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CICLO XXII

Cyclic nucleotide signalling interplay in rat neonatal cardiac myocytes: FRET-based targeted biosensors reveal compartment-specific modulation of cAMP levels by cGMP

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1 RIASSUNTO DELL'ATTIVITÀ SVOLTA

L'adenosina monofosfato ciclico (AMP ciclico o cAMP) e la guanosina monofosfato ciclico (GMP ciclico o cGMP) sono importanti modulatori della funzione cardiaca. L'AMP ciclico è generato dalle adenilato ciclasi (AC) in seguito all'attivazione di recettori accoppiati a proteine G (GPCR). L'AMP ciclico è un importante mediatore dello stimolo adrenergico e tramite l'attivazione del suo principale effettore, la protein chinasi A (PKA), regola la forza e la frequenza cardiaca. Il GMP ciclico, generato dalle guanilato ciclasi (GC) in risposta a ossido nitrico (NO) e peptidi natriuretici, attiva la proteina chinasi G (PKG) e modula l'inotropia e la vasodilatazione.

Recenti studi hanno dimostrato che le vie di segnale mediate da cAMP sono compartimentalizzate e che l'attivazione selettiva di differenti GPCR porta alla generazione di distinti pool di cAMP ognuno dei quali attiva un diverso set di PKA. Una volta attivata PKA fosforila una serie di substrati che si trovano in sua prossimità. Ogni singolo pool di cAMP è controllato da un diversa combinazione di fosfodiesterasi, gli enzimi che degradano i nucleotide ciclici. Le PDE sono elementi essenziali nella trasduzione del segnale; infatti, da un lato limitano la diffusione del secondo messaggero prevenendo l'inappropriata attivazione di bersagli aspecifici, dall'altro, potendo regolare i livelli intracellulari di cAMP, modulano l'entità e la durata dei segnali che generano cAMP.

Nella prima parte del mio progetto di dottorato ho dimostrato che anche le vie di segnale mediate da cGMP sono compartimentalizzate. Anche cGMP infatti viene generato in singoli compartimenti ciascuno regolato da diverse PDE. In particolare, la stimolazione della forma particolata della GC da parte del peptide natriuretico atriale (ANP) genera un pool di cGMP controllato esclusivamente dalla PDE2 mentre l'attivazione della guanilato ciclasi solubile ad opera di NO genera un pool di cGMP che è sotto stretto controllo di PDE2 e PDE5. Inoltre i miei risultati mostrano che il cGMP generato dalla stimolazione dei recettori β_3 è

controllato da PDE2 e PDE5. Come per l'AMP ciclico, dunque anche il controllo spaziale del GMP ciclico sembra giocare un ruolo importante nella fisiologia della cellula cardiaca.
Negli ultimi anni, nuovi e interessanti avanzamenti nella biochimica delle PDE e nelle cascate di segnale dei nucleotidi ciclici hanno dimostrato che le vie di segnale mediate da cAMP e cGMP sono altamente interconnesse e che l'integrazione di queste vie è mediata dalle PDE. Infatti, la capacità del cGMP di modulare l'attività enzimatica di alcune PDE che degradano l'cAMP costituisce un importante punto di contatto tra le due cascate. In particolare cGMP può attivare la PDE2, agendo su siti di legame allosterici, nominati domini GAF; al contrario cGMP può inibire la PDE3 competendo con cAMP per il legame al sito catalitico. È dunque presumibile che il cGMP possa alterare i livelli intracellulari di cAMP modulando questi importanti enzimi. Inoltre è possibile predire l'esistenza di domini di trasduzione del segnale in cui il cGMP agendo su PDE2 o PDE3 può alterare e modulare in modo selettivo i livelli di cAMP.

Studi effettuati in precedenza nel nostro laboratorio hanno dimostrato che in cardiomiociti di ratto neonato, pool diversi di cAMP, generati da differenti stimoli, attivano in modo selettivo le due isoforme di PKA. La PKA di tipo I e la PKA di tipo II sono localizzate in distinti compartimenti sub-cellulari. Tale localizzazione è resa possibile dai domini di dimerizzazione e di ancoraggio presenti nelle subunità regolatorie che permettono a queste due isoforme di ancorarsi a determinate proteine denominate AKAP (A kinase anchoring proteins). Questa diversa distribuzione spaziale permette il generarsi di microdomini in cui l'AMP ciclico vene generato selettivamente in seguito all'attivazione di specifici GPCR. I vari pool di cAMP sono regolati da diverse famiglie di PDE. In questo modo, stimoli specifici generano cAMP in domini localizzati della cellula e attivano in modo selettivo solo richiesti pool di PKA con conseguente fosforilazione di specifiche proteine.

Nella seconda parte del mio progetto, ho cercato di capire se pool diversi di cGMP, agendo su PDE2 o PDE3, possono regolare in modo differenziale il segnale mediato da cAMP nei compartimenti che ospitano PKA di tipo I e PKA di tipo II. In particolare mi sono focalizzata

sul l'impatto di cGMP sulla risposta β-adrenergica. Per investigare meglio l'interazione tra cAMP e cGMP, ho modificato due sensori molecolari basati sul FRET in modo da direzionarli nei due compartimenti che ospitano PKAI e PKA II (compartimento RI e compartimento RII). Per ottenere questa localizzazione ho generato delle proteine chimeriche in cui ho fuso ad Epac1-camps (sensore per cAMP) e a cygnet-2.1 (sensore per cGMP) i domini di dimerizzazione e ancoraggio di PKAI e PKAII.

In cardiomiociti stimolati con isoproterenolo (ISO) l'aumento di cAMP si è dimostrato più elevato nel compartimento RII rispetto al compartimento RI, confermando risultati ottenuti in precedenza. Al contrario, quando i cardiomiociti sono stati trattati con il donatore di NO SNAP (S-nitroso-N-acetylpenicillamine) si è verificata un'inversione dei gradienti di cAMP in risposta ad ISO. La risposta a ISO, infatti, si è dimostrata più elevata nel compartimento RI rispetto al compartimento RII. Successivi studi hanno rivelato che questa inversione è dovuta alla generazione di cGMP. Il pretrattamento con un inibitore della guanilato ciclasi solubile, ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one) ha completamente abolito l'effetto di SNAP sull'inversione dei gradienti. Inoltre studi di FRET-imaging per cGMP hanno dimostrato che la somministrazione di SNAP genera lo stesso aumento di cGMP in entrambi i compartimenti, escludendo come possibile causa dell'inversione una non omogenea generazione di cGMP tra i compartimenti RI e RII. E' stato quindi ipotizzato che l'inversione dei gradienti di cAMP in risposta ad ISO fosse determinata dalla regolazione compartimento-specifica delle PDE da parte di cGMP; nello specifico, abbiamo ipotizzato che cGMP inibisse PDE3 nel compartimento RI e che attivasse PDE2 nel compartimento RII.

Esperimenti di imaging effettuati con inibitori selettivi di PDE2 e PDE3 hanno confermato questa ipotesi. L'inibitore di PDE2, BAY 60-7550 (BAY), infatti, previene l'effetto di SNAP nel compartimento RII (ma non nel compartimento RI), suggerendo che l'attivazione cGMP-mediata di PDE2 occorre specificamente nel compartimento RII. Al contrario, in presenza dell'inibitore di PDE3 cilostamide (CILO) l'aumento di cAMP nel compartimento RII è ancora basso rispetto alle cellule di controllo, ma nel compartimento RI non si verifica nessun

ulteriore aumento di cAMP in seguito a stimolazione con ISO, indicando che PDE3 è già stata inibita dal cGMP generato da SNAP.

La sovra-espressione di una PDE2 cataliticamente inattiva, in grado di scalzare la PDE2 endogena dai corrispettivi siti di ancoraggio, ha rivelato che la localizzazione di PDE2 è essenziale per l'osservata inversione dei gradienti. In presenza del mutante, infatti, l'inversione dei gradienti di cAMP in risposta ad ISO indotta da SNAP è completamente abolita.

L'organizzazione topografica di PDE2 e PDE3 è stata ulteriormente confermata da esperimenti in cui cardiomiociti sono stati stimolati con il donatore di NO SNAP in presenza di inibitori selettivi di PDE2 e PDE3. Questi dati confermano che nel compartimento RII l'aumento di cGMP che deriva dall'attivazione della guanilato ciclasi solubile, risulta nell'attivazione di PDE2 e di conseguenza nella riduzione della concentrazione di cAMP in questo compartimento. Al contrario, un aumento di cGMP nel compartimento RI implica principalmente l'inibizione di PDE3 e di conseguenza in un aumento locale della concentrazione di cAMP.

Infine ho voluto investigare se l'alterazione dei livelli di cAMP indotta da cGMP potesse compromettere l'attivazione selettiva delle isoforme di PKA. A questo scopo ho eseguito degli esperimenti di imaging utilizzando un reporter dell'attività enzimatica di PKA basato sul FRET (AKAR3). Anche questo sensore è stato ingegnerizzato per indirizzarlo ai compartimenti RI e RII. La stimolazione con ISO ha generato una robusta attivazione di PKAII rispetto a PKA I, confermando risultati ottenuti in precedenza; al contrario, in presenza di SNAP, ISO ha generato un massiccia attivazione di PKAI rispetto a PKA II. Questi dati indicano che l'alterazione dei livelli di cAMP da parte di cGMP si riflette in una alterata attivazione delle isoforme di PKA.

In modo interessante, cardiomiociti trattati con ISO e SNAP hanno dimostrato un aumento dei livelli di fosforilazione di troponina I, prevenuto da l'inibitore della guanilato ciclasi solubile ODQ. Questi risultati confermano che la modulazione di cAMP da parte di cGMP può influenzare il livello di fosforilazione di bersagli a valle di PKA.

In conclusione, i dati raccolti durante la mia tesi di dottorato dimostrato che in cardiomiociti il cGMP può modulare la via di segnale mediata dall'cAMP modo compartimento-specifico modulando l'attività enzimatica di PDE2 e PDE3. Questa modulazione, inoltre, dipende dalla sorgente di cGMP dal momento che l'attivazione della guanilato ciclasi particolata o solubile ha effetti diversi sulla modulazione di pool di cAMP localizzati. La modulazione compartimento-specifica di cAMP ad opera di cGMP potrebbe essere molto rilevante nella fisiologia della cellula dal momento che altera l'attivazione selettiva delle isoforme di PKA e il livello di fosforilazione di alcune proteine.

2 SUMMARY

The second messengers cAMP and cGMP are important regulators of cardiac function. cAMP is generated by adenylyl cyclases (AC) upon G protein-coupled receptor (GPCR) stimulation and activates PKA as its main downstream effector. The cAMP signal generated in response to catecholamines regulates the strength and the frequency of cardiac contraction. cGMP is generated by guanylyl cyclases (GC) in response to nitric oxide (NO) and natriuretic peptides, activates PKG and modulates inotropy and vasorelaxation.

Recent studies have shown that cAMP signalling is compartmentalized and that the activation of different GPCRs leads to the generation of discrete pools of cAMP that activate specific subset of PKA. Activated PKA in turn phosphorylates specific downstream targets that are in close proximity. Each cAMP pool is shaped by a combination of different phosphodiesterases (PDEs) the only cAMP degrading enzymes, thus PDEs are essential components in the spatial and temporal control of cAMP response. They restrict the diffusion of cAMP, preventing unspecific activation of PKA and phosphorylation of downstream targets, and regulate the duration of the signal.

In the first part of my project I demonstrated that, similar to cAMP, also cGMP shows restricted diffusion in rat neonatal cardiac myocytes (RNCMs), suggesting that spatial control may be as important for cGMP-mediated signalling as it is for cAMP-mediated signalling. My studies revealed that intracellular cGMP generated by stimulation of the particulate GC (pGC) by atrial natriuretic peptide (ANP) or stimulation of the soluble GC (sGC) by the NO donor SNP is compartmentalized into separate microdomains. These cGMP microdomains are regulated by different families of PDEs. In particular, PDE2 appears to control the pool of cGMP generated by the pGC whereas the pool of cGMP generated by the sGC is shaped by both PDE2 and PDE5. In addition, I found that both PDE2 and PDE5 control the cGMP response to β_3-adrenergic receptors stimulation.

Recent advances in our understanding of cyclic nucleotide signalling, in particular of the activity and regulation of PDEs, have shown that cAMP and cGMP signalling pathways are highly interconnected. Integration between these two pathways is arbitrated by the modulation of cAMP-degrading PDEs by cGMP. cGMP acts as a competitive inhibitor for PDE3 whereas it activates PDE2 by binding to allosteric binding sites called GAF domains. PDE2 and PDE3 therefore may act to coordinate crosstalk between these two signalling cascades and it is possible to predict the existence of distinct signalling units in which cGMP, by acting on PDE2 or PDE3, can selectively modulate cAMP levels.

Previous work conducted in our laboratory in RNCMs has demonstrated that confined pools of cAMP generated in response to specific extracellular stimuli selectively activate individual PKA isoforms. PKA type I and PKA type II, by binding to AKAPs (A kinase binding proteins), are tethered to different subcellular locales. This defines exclusive signalling domains within which the cAMP signal is uniquely generated via activation of specific GPCRs and their associated G proteins. The dynamics of cAMP within the compartments defined by the localization of PKA type I and PKA type II are uniquely modulated by the activity of different subsets of PDEs. The generation of distinct pools of cAMP and the consequent activation of selected PKA isoforms results in a stimulus-specific phosphorylation of downstream protein targets (Di Benedetto et al 2008).

In the second part of my project I set out to understand whether discrete pools of cGMP, by acting either on PDE2 or PDE3, can differently regulate cAMP signalling in the two compartments defined by PKA type I and PKA type II. In particular I focused on the impact of cGMP signals on the cAMP response to β-adrenergic stimulation. To fully characterize the interplay between cGMP and cAMP in these two compartments I took advantage of targeted versions of the FRET-based biosensors for cAMP and cGMP. Epac1-camps and cygnet-2.1 were fused to the unique dimerization/docking domain sequences that mediate anchoring of PKA-RI and PKA-RII subunits to AKAPs (Honda et al 2001; Nikolaev et al 2004). In myocytes stimulated with isoproterenol (ISO) the cAMP response was significantly higher

in the PKA type II compartment (RII compartment) than in the PKA type I compartment (RI compartment), confirming previous results. In striking contrast, when myocytes were pre-treated with the NO donor SNAP (S-nitroso-N-acetylpenicillamine) and stimulated with ISO, an inversion of these gradients was observed where the cAMP response was higher in the RI compartment than in the RII compartment. Such inversion was dependent on cGMP, as it was completely abolished by the guanylyl cyclase inhibitor ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one). In addition, cGMP FRET-imaging showed that SNAP generated the same amount of cGMP in the RI and RII compartments excluding the possibility that the observed effect was due to an uneven generation of cGMP in the two compartments. I therefore hypothesized that this inversion of the gradients was the dependent on a compartment-specific modulation of PDEs by cGMP and, specifically, on the cGMP-dependent inhibition of PDE3 in the RI compartment and on the cGMP-dependent activation of PDE2 in the RII compartment. Imaging experiments performed with selective inhibitors of PDE2 and PDE3 confirmed this hypothesis. The PDE2 inhibitor BAY 60-7550 (BAY) prevented the effect of SNAP in the RII compartment (but not in the RI compartment), suggesting that a cGMP-activation of PDE2 activity occurs specifically in the RII compartment. On the contrary, in the presence of the PDE3 inhibitor cilostamide (CILO) the cAMP increase in the RII compartment was still smaller than control cells, but in the RI compartment no further increase in cAMP occurred upon ISO stimulation, indicating that PDE3 had already been inhibited by the cGMP generated by SNAP.

Overexpression of a catalytically dead mutant of PDE2 that displaced endogenous PDE2 from its cognate anchor sites inside the cell, revealed that compartmentalization of the PDE2 is essential for the observed inversion of the cAMP gradients. In the presence of the mutant PDE2, indeed, the SNAP-induced inversion of the cAMP gradients upon ISO stimulation is completely abolished.

The topographical organization of PDE2 and PDE3 was further confirmed by experiments in which RNCMs were stimulated with the NO donor SNAP in the presence of PDE2 or PDE3 inhibitors. These data confirm that in the RII compartment the rise of cGMP secondary to sGC activation results in the activation of PDE2 and, consequently, in a reduction of the

cAMP concentration in this compartment. On the contrary, increasing cGMP concentration in the RI compartment leads mainly to PDE3 inhibition and therefore in a local increase in cAMP concentration.

Finally I wanted to assess whether alteration of cAMP levels induced by cGMP also affects activation of PKA isoforms. To this aim I performed imaging experiments using FRET-based A kinase activity reporters (AKAR3) (Zhang et al 2001) engineered to be targeted to the RI and RII compartments. ISO stimulation led to a much robust activation of PKA type II over PKA type I, in agreement with previous results. In contrast, in the presence of SNAP, ISO stimulation activated more PKA type I than PKA type II. These data indicate that the alteration of cAMP levels in the RI and RII compartments translates in a different activation of the two PKA isoforms. Interestingly, RNCMs, treated with SNAP and ISO showed an increased phosphorylation level of Troponin I that was prevented by the sGC inhibitor ODQ, confirming that cGMP-mediated control of cAMP levels can affect PKA-mediated phosphorylation of downstream targets.

In conclusion, the data collected during my thesis project show that cGMP can modulate cAMP signalling in cardiac myocytes in a compartment specific manner by acting on cGMP-regulated cAMP-hydrolyzing PDEs. In addition, this effect depends on the source of cGMP as activation of the pGC or the sGC has different effects on the modulation of local cAMP pools. Finally, the compartment-specific modulation of cAMP signals by cGMP is functionally relevant as it affects the selective activity of PKA isoforms and the phosphorylation level of PKA targets.

3 INTRODUCTION

3.1 Cyclic nucleotide signal transduction

3'-5'-Cyclic adenosine monophosphate (cAMP) and 3'-5'-Cyclic guanosine monophosphate (cGMP) are important intracellular signalling mediators that regulate a bewildering number of cellular processes. cAMP is the second messenger mediating the “fight of flight” response, our body's ancestral, automatic, inborn response to perceived attack or risk to our survival. It also regulates cell growth and differentiation, hormone secretion, metabolism, gene transcription and many other functions. In the heart, it modulates excitation contraction coupling (ECC) thereby controlling the force and frequency of cardiac contraction. cGMP is critical to the maintenance of vascular tone, cardiac contractility, cardioprotective response to ischemia and cellular proliferation (Baxter 2004; Costa et al 2005; D'Souza et al 2004; Ignarro et al 1999; Waldman & Murad 1988).

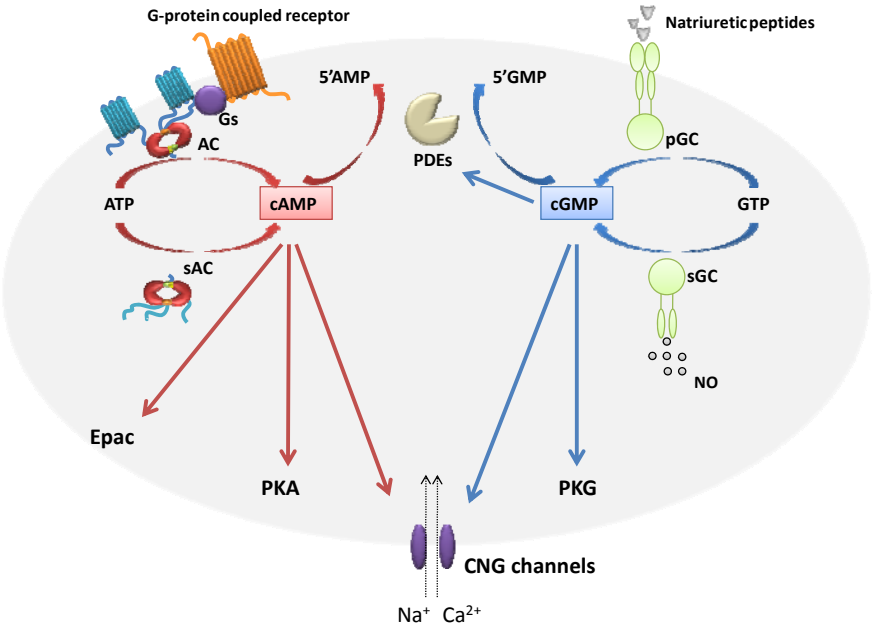


Figure 3-1 Schematic representation of cAMP and cGMP signalling pathways. AC, adenylyl cyclase; sAC, soluble adenyly cyclase; pGC, particulate guanylyl cyclase; sGC, soluble guanylyl cyclase; ATP, adenosine triphosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; 5'-AMP, adenosine monophosphate, 5'-GMP, guanosine monophosphate; PDEs, phosphodiesterases; Epac, Exchange protein directly activated by cyclic AMP; PKA, protein kinase A; PKG, protein kinase G; CNG, cyclic nucleotide gated ion channels; NO, nitric oxide.

The signalling pathways using cAMP and cGMP as second messengers involve a complex system of proteins, many or all of which are independently regulated. Intracellular levels of these second messengers are determined by the balance between generation by cyclases, and degradation by phoshodiesterases (PDEs). cAMP is synthesized by adenylyl cyclases (ACs) in response to G-protein coupled receptor activation and its main effectors are protein kinase A (PKA), the guanine nucleotide exchange factors (GEFs), Epac1 and Epac2, and cyclic nucleotide gated (CNG) ion channels. cGMP is generated by guanylyl cyclases (GCs) in response to NO or natriuretic peptides, it activates protein kinase G (PKG) and regulates the activity of some PDE isoforms. cGMP also regulates CNG ion channels.

3.2 From phosphorylase to cAMP-PKA signalling: an historical overview of the key steps in the discovery of hormones signalling cascades

3.2.1 *Hormones and glycogen breakdown*

Figure 3-2 Earl Wilbur Sutherland (1915-1974).

The study of cAMP signal transduction begun about fifty years ago with studies by Earl Wilbur Sutherland (1915-1974) (Figure 3-2) on the glycogenolytic effects of epinephrine and glucagon. Back in 1958, while at Western Reserve Academy (Hudson, Ohio) Sutherland performed the experiments that would have been worth the Nobel Prize in Physiology or Medicine in 1971 “for his discoveries concerning the mechanisms of the action of hormones” (Rall & Sutherland 1958; Sutherland & Rall 1958). The stimulating and vibrant scientific environment of the early 50s contributed to the establishment of the basic biochemistry of glycogen breakdown with phosphorylase, phosphoglucomutase, and glucose 6-phosphatase being the basic enzymes involved. At that time, Sutherland was carrying out studies on a phosphorylase, the enzyme responsible for the conversion of glycogen to glucose, whose activity was increased by hormones like

glucagon and norepinephrine (Sutherland & Cori 1951). While purifying phosphorylase from dog liver extracts, Sutherland found another enzyme which catalyzes the inactivation of phosphorylase. Since in the inactivation reaction the molecular weight did not vary, he suspected that a minor change might be responsible for the loss of activity, with the loss of a phosphate group being only one of many possibilities. Later on, he realized that the inactivating enzyme was indeed a phosphatase (Wosilait & Sutherland 1956) (Figure 3-3).

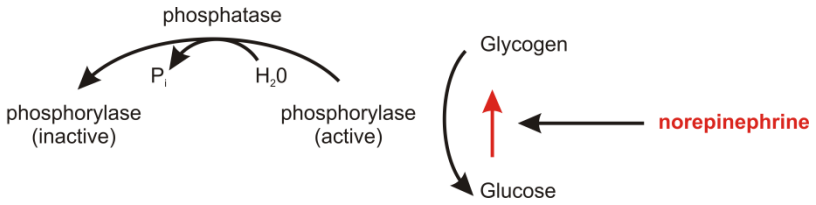


Figure 3-3 Effect of norepinephrine on glycogen breakdown. Norephinephrine enhances glycogen breakdown by stimulating phosphorylase activity. Phosphorylase is converted into the inactive form through a phophatase.

When Ted Rall joined Sutherland at Western Reserve Academy, they started a fruitful period of collaboration. They demonstrated that the concentration of active phosphorylase in liver resulted from a balance between inactivation by a phosphatase and reactivation by a process in which a phosphate was donated to the protein, a reaction still not well characterized (Rall et al 1956a; Rall et al 1956b; Sutherland & Wosilait 1956; Wosilait & Sutherland 1956). When Cori and colleagues crystallized the phosphorylase, they found that it exists in two forms, phosphorylase *a*, which is active without the addition of AMP, and phosphorylase *b*, which is inactive without AMP (Cowgill & Cori 1955; Taylor et al 1948). In the same years, Edwin G. Krebs joined the Cori's laboratory as a post doctoral fellow and together with Fischer decided to determine the mechanism by which the inactive phosphorylase *b* was converted into the active form. They soon found that the interconversion of phosphorylase was the result of an enzyme-catalyzed phosphorylation-dephosphorylation reaction that required ATP and Mg^{2+}. The way in which AMP promoted phosphorylase activation was eventually elucidated when Krebs and Fisher discovered phosphorylase kinase, an enzyme that phosphorylates phosphorylase (Krebs & Fischer 1956). In addition, they

observed that phosphorylase kinase itself exists in a highly activated phosphorylated form and in a less active unphosphorylated form (Figure 3-4).

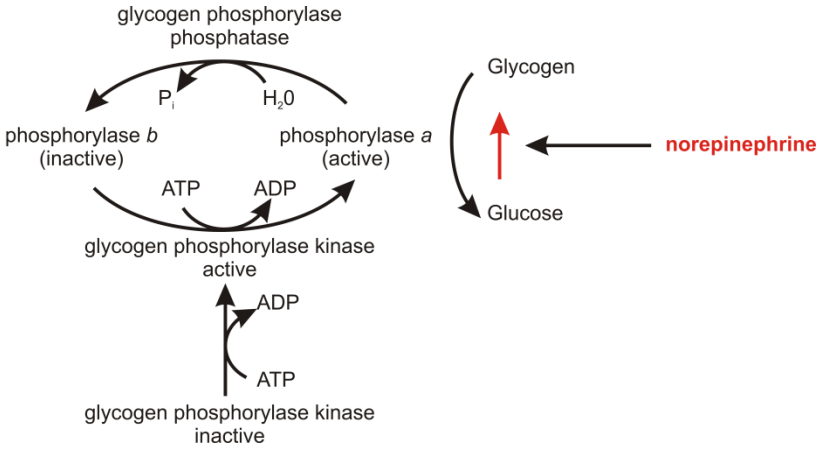


Figure 3-4 Glycogen phosphorylase kinase is the enzyme that converts phosphorylase into its active state. Glycogen phosphorylase kinase exists in active or inactive forms.

3.2.2 *The discovery of cyclic AMP and Adenylyl Cyclases*

Rall and Sutherland continued their studies on the effect of hormones on glycogen breakdown and found, interestingly, that if they centrifuged their homogenates, to remove "cellular debris", the hormonal response was lost. Conversely, if they recombined the particulate fraction with the original supernatant, the response could be restored. In addition, when they incubated the particulate fraction with hormones, a heat stable factor was produced that could activate phosphorylase when added to the supernatant fraction (Berthet et al 1957).

They set out to identify this heat stable factor and they showed that it was an adenine ribonucleotide that could be produced from ATP by the particulate fraction from different tissues: liver, heart, skeletal muscle and brain (Rall & Sutherland 1958). In the same years, Lipkin and colleagues isolated the same compound and established its structure as adenosine 3'-5'-monophosphate, now commonly referred to as cyclic AMP or cAMP (Lipkin et al 1959). The enzyme present in the particulate fractions that converted ATP to cAMP was originally named "adenyl cyclase". Furthermore, they found that in animal tissue cAMP was rapidly inactivated by a yet uncharacterized

enzyme with adenosine 5'-phosphate being the product of the reaction. It begun to appear clear that the concentration of cAMP was determined by a balance between formation from ATP and degradation to 5'-AMP and following studies were focused on the enzymes responsible for this reactions. In 1963, Ashman started to elucidate adenyl cyclase activation mechanism. Adenyl cyclase had been shown to be held at the membrane in a inhibited form and he suggested that hormones may act by relieving this inhibition (Ashman et al 1963). In 1967, Robinson proposed that the membrane adenylyl cyclase was composed of two types of subunits: a regulatory subunit facing the extracellular space and a catalytic subunit with its active domain in contact with the cytoplasm (Robison et al 1967) (Figure 3-5). Shortly thereafter, the enzymes that degrade cAMP, phosphodiesterase, were described (Beavo et al 1970a; 1971; Beavo et al 1970b; Butcher & Sutherland 1962; Sutherland 1972).

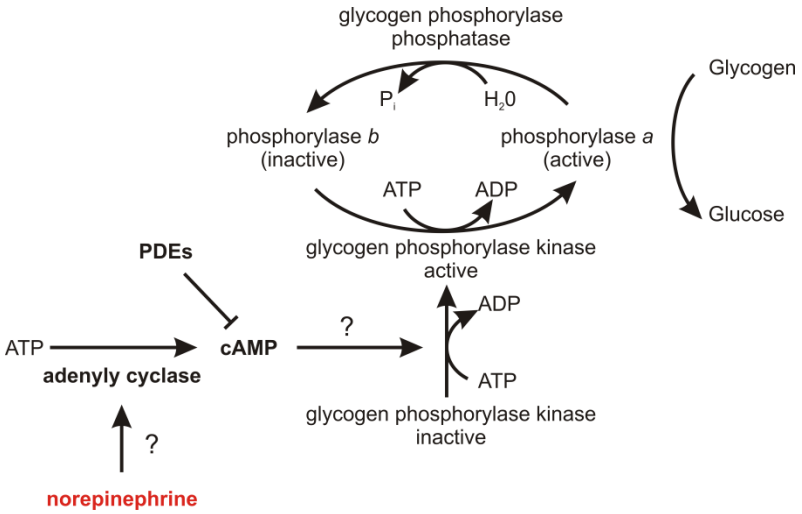


Figure 3-5 Adenylyl cyclase generates cAMP that in turn activates glycogen phosphorylase.

3.2.3 *PKA and the kinase cascade*

One of Krebs' projects was focused on the mechanism through which cAMP promoted phosphorylase activation via phosphorylase kinase. This was determined by Krebs' postdoctoral fellow Donald A. Walsh that, in 1968, discovered in rabbit skeletal muscle the cAMP-dependent protein kinase A (PKA) (Walsh et al 1968). With these last findings

the cascade of glycogen break down was fully elucidated (Figure 3-6). The discovery of PKA established the existence of the first protein kinase cascade in which one kinase activates another kinase and led to a growing interest on the field of protein phosphorylation. Later on, Krebs described for the first time what it is now known as the MAP kinase cascade (Ahn et al 1991; Seger & Krebs 1995).

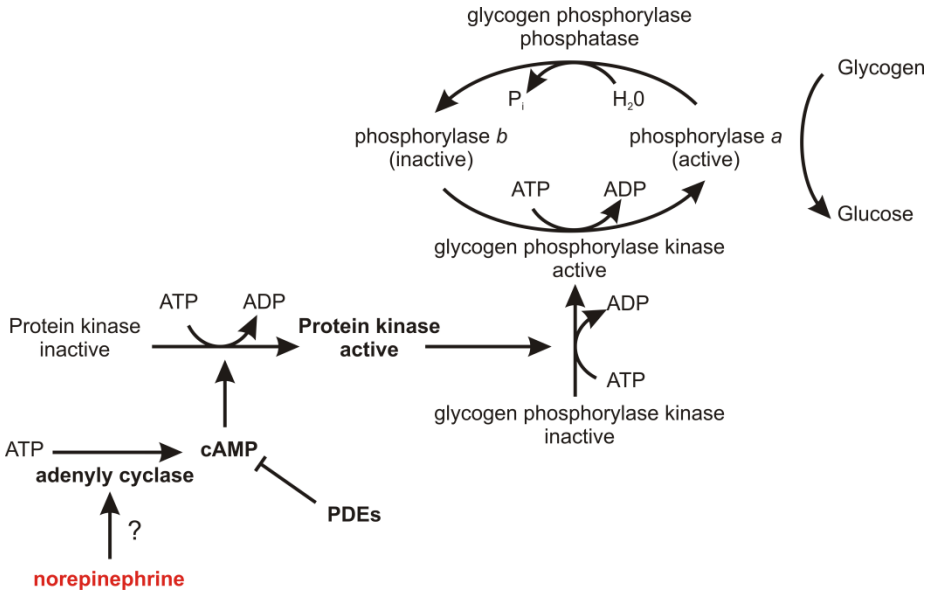


Figure 3-6 cAMP activates glycogen phosphorylase kinase thruogh PKA activation.

A few years later, Gill and Garren showed that PKA is composed of two regulatory subunits that bind cAMP, and two catalytic subunits which are inhibited by the regulatory subunits (Gill & Garren 1971). When cAMP binds to the regulatory subunit, the catalytic subunit dissociates and becomes active. Protein kinase A was found to be widely distributed and to mediate the many known effects of cAMP (Kuo & Greengard 1969; Wosilait & Sutherland 1956).

3.2.4 *G proteins*

By this time, it had become clear that epinephrine and glucagon increased the accumulation of cAMP by stimulating adenylyl cylcase; however, how the cell surface receptors interacted with adenylyl cylase was not clear yet. In 1977-1978 two papers

from Gilman and colleagues provided new data to clarify this mechanism. They noticed that two proteins were necessary for adenylyl cyclase activity, one of which was a regulatory protein, made of two functional components. They soon discovered that the protein was a guanine nucleotide binding protein capable of binding activated AC. This protein was then called G-protein. Eventually, they determined that binding of a hormone to a specific receptor triggers the exchange of GTP to GDP in the G-protein, causing a conformational change which induces the dissociation of its α subunit bearing GTP from its $\beta\gamma$ subunits with the Gα-GTP subunit then activating AC. The GTP bound to the α subunit is eventually hydrolyzed to GDP and the subunits reassociate (Figure 3-7).

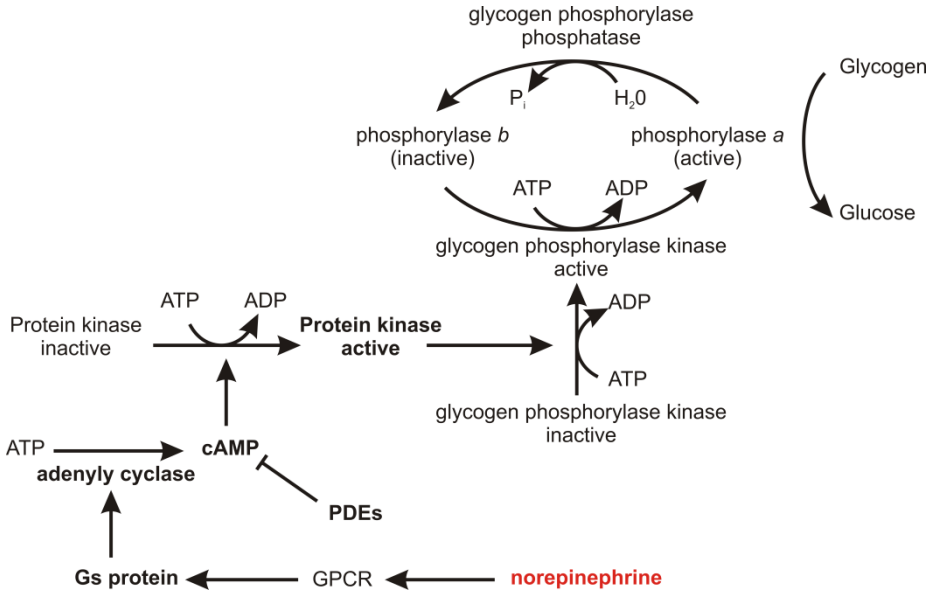


Figure 3-7 Signalling cascade through which norepinephrine stimulates glycogen break down.

3.2.5 *The concept of "second messenger"*

Evidence began to accumulate to suggest that some of the effects of other hormones might be mediated by this same mechanism (Sutherland et al 1965; Sutherland & Robison 1966). These and other developments resulted in the definition of a new and original role for cAMP as an intermediary in many hormonal functions. Figure 3-8 shows how Sutherland illustrated this concept. In his model, different cells contain receptors for different hormones and the interaction between hormone and the

receptor stimulates adenyl cyclase leading to an increase of cAMP. cAMP in turn acts intracellularly to generate appropriate physiological responses. Since different cells contain different enzymes, the end result of the change in cAMP levels will differ from one type of cell to the other, for example phosphorylase activation in hepatic cells, steroidogenesis in the adrenal cortex, and so on.

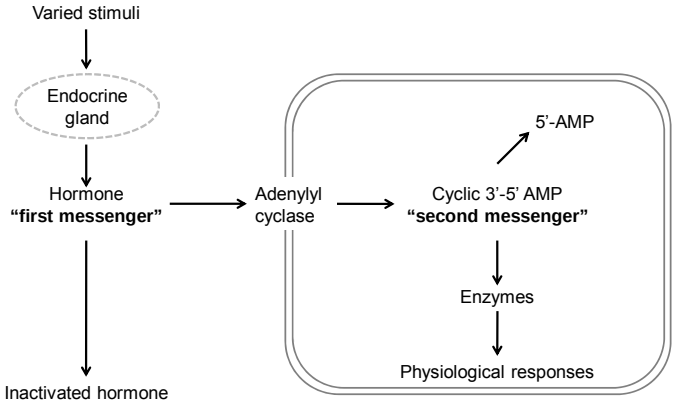


Figure 3-8 Sutherland's concept of the "Second messenger", adapted from (Sutherland & Robison 1966).

Although this model represents a true key milestone in our understanding of cell physiology, it is now clear that this is an oversemplification of what really happens inside the cell. We now know that the system is much more complex and less linear than initially envisaged. Research conducted in the last few decades has shown that parallel and spatially segregated signalling pathways coexist within the cell. Indeed, each individual cell can express many different G protein-coupled receptors and a huge number of enzymes and proteins that are potential targets for PKA, the main effector of cAMP. In the heart for example, cAMP mediates the catecholaminergic control on heart rate and contractility but at the same time it transduces the signal of many others hormones and neurotransmitters. Understanding how the myocyte can decode such a large number of intracellular stimuli and deliver the appropriate downstream responses, has recently been a fascinating and debated topic among the scientific community and the focus of intense investigation. However, the molecular details of how appropriate signal propagation is achieved are still not fully elucidated.

3.2.6 *cGMP railed behind a bit…*

3'-5'-Cyclic guanosine monophosphate (cGMP) was first isolated from rat urine in 1963 by Ashman and colleagues. cAMP research started from an effect - epinephrine and glucagone were known to stimulate glycogen breakdown - and eventually led to the isolation of a nucleotide; for cGMP, in contrast, all begun with the discovery of a nucleotide but nothing was known about its effects on cell physiology. Until the 1980s, in fact, it was unknown which hormone stimulated the synthesis of cGMP or which metabolic pathway it regulated. After its identification, several laboratories started to look for enzymes responsible for its synthesis and degradation, for effectors and biological outcomes. At the turn of the 70s guanyly cyclases, cGMP phosphodiesterases and cGMP-dependent protein kinase (PKG) were described.

Ferid Murad and colleagues begun to examine guanylyl cyclase activity in different tissue homogenates. Surprisingly, they found activity in both high-speed supernatant and particulate fractions. However, while the enzyme activity present in the particulate fraction showed cooperative catalytic kinetics, the soluble guanylyl cylcase activity demonstrated typical Michaelis-Menten kinetics. These findings suggested that the enzyme could exist in different isoforms. This hypothesis was difficult to demonstrate though, due to lack of proper purification techniques. The crude preparations used in these experiments, in fact, were more likely contaminated by nucleotidase, phosphatases and phosphodiesterases that were competing for substrate and products. To overcome this problem and to avoid purification, cloning, expression, and characterization of the enzyme, the authors started to add to the crude lysates a large variety of substances in order to inhibit these contaminants. These shortcuts allowed them to better illustrate the kinetic behaviour of the enzyme and at the same time led them to a serendipitous observation. They noticed that some of these substances, for example sodium azide, sodium nitrite and hydroxylamine, activated the enzyme. Azide activation was tissue specific, required oxygen, was enhanced by thiols and took several minute to reach the maximum effect. This suggested that during incubation it was converted in a sGC activator. They also found that azide effect was inhibited by a

substance present in heart extracts. After intensive investigation they realized that hemoglobin and myoglobin were indeed inhibitors of sodium azide. By testing further substances, they build up a list of sGC activators that also showed relaxant effect in tracheal, gastrointestinal and vascular smooth muscle (nitrite, sodium nitroprusside, nitrolycerin). Since chemically generated NO was able to induce cGMP synthesis in their preparation, Murad and collegues hypothesised that these prodrugs were converted to nitric oxide and coined the term “nitrovasodialtors”. In addition, the high affinity of NO for heme, explained the inhibitory effect of haemoglobin and myoglobin (Murad & Pak 1972).

Murad *et al.* proposed that NO could act like a second messenger for hormones and drugs (Katsuki et al 1977; Murad 1994). NO was mainly considered a pollutant released from cigarette smoke and automobile exhaust and the idea that a free radical could activate an enzyme and mediate the action of hormones was not well accepted by the scientific community. This view of nitric oxide completely changed in the 1980s, thanks to the development of proper essays to measure NO. Robert Furchgott identified a labile factor realised from endothelial cells when they were stimulated with acetylcholine, bradykinin and histamine. This factor induced vascular relaxation and was termed “endothelial derived relaxant factor” (EDRF). In 1983, Murad and colleagues demonstrated that EDRF was able to stimulate sGC and generate cGMP, it activated the cGMP dependent protein kinase and led to the phosphorylation of the same substrates as nitrovasodilators did. In 1988, Furchgott and Ignarro proposed that EDRF was nitric oxide. Robert Furchgott, Louis J. Ignarro and Ferid Murad in 1998 were awarded by the Nobel Prize “for their discoveries concerning nitric oxide and cGMP as signalling molecules in the cardiovascular system”.

Natriuretic peptide-mediated cGMP signalling was discovered only in the mid 80s when a polipeptide, the atrial natriuretic peptide (ANP), was isolated from atrial muscle tissue. This peptide was found to have potent diuretic and hypotensive properties and it was able to increase cGMP levels also in smooth muscle, kidney and adrenal gland (Ackermann et al 1984; Atlas et al 1984; Bloch et al 1985; de Bold 1982; 1985) .

Evidence for the existence of cGMP-specific PDEs came from the work of Brooker and colleagues where they found in rat brain a phosphodiesterase isoform activated by cyclic GMP (Brooker et al 1968). The first studies on cGMP dependent protein kinase (PKG), instead, have been carried out by Greengart and colleagues. In 1970, they partially purified from lobster muscle a kinase activated by 3'-5'-guanosine monophosphate (Kuo & Greengard 1970).

3.3 Role of cyclic nucleotides in the heart

Both cAMP and cGMP play important roles in heart function. The cAMP/PKA signalling system controls cardiac excitation contraction coupling. Inotropic and lusitropic responses to β-adrenergic stimulation are finely tuned by PKA mediated phosphorylation of specific proteins. Phosphorylation of L-type Ca^{2+} (LTCC) channels and the ryanodine receptor (RyR) increases the amount of Ca^{2+} available for contraction (positive inotropic effect). Phosphorylation of troponin I (TnI) accelerates troponin C-Ca^{2+} off-rate and accelerates force development and shortening during systole and force relaxation and re-lengthening during diastole. In addition, phosphorylation of PLB, releases the inhibitory effect this protein exerts on the Ca^{2+}-ATPase pump (SERCA), enhancing the Ca^{2+} re-uptake in the sarcoplasmic reticulum and myofilament relaxation (lusitropic effect). Regulation of the cAMP-PKA signalling is crucial for the appropriate catecholamine-mediated modulation of cardiac contractility (Bers 2002; 2008). This signalling system, however, is responsible for the functional response of a variety of other hormones and neurotransmitter. For example, the transcription of many genes is regulated by PKA direct phosphorylation of transcription factors. One of the best characterized transcription factor is the cyclic AMP response element (CRE)-binding protein (CREB) (Gonzalez & Montminy 1989; Gonzalez et al 1989; Sands & Palmer 2008; Shaywitz & Greenberg 1999; Wadzinski et al 1993).

Not only cAMP is essential for proper functioning of the heart but this signalling pathway is also involved in pathological processes. Heart failure and dilated cardiomyopathy are carachterized by reduced responsiveness to β-adrenergic

stimulation (Movsesian & Bristow 2005) and chronic activation of the cAMP-PKA pathway leads to cadiac hypertrophy (Osadchii 2007; Port & Bristow 2001).

The second messenger cGMP is implicated in a vast array of functions in multiple cell types within the cardiovascular system. It mediates the effect of NO and natriuretic peptides and is an important modulator of vascular tone, endothelial permeability, and vascular homeostasis and proliferation. In the heart the cGMP-PKG singnaling regulates contractile function (Hofmann et al 2006), and serves to caunteract both acute and chronic stress responses (Lincoln et al 2001). In addition the cGMP-PKG pathway seems to play a crucial role in the protective effect of ischemic pre and post-conditioning (Yellon & Downey 2003) and in cardiac remodelling. Recently, it has been published that enhancement of cGMP-PKG signalling, through PDE5 or PDE1 inhibition, prevents and reduces cardiac hypertrophy (Hofmann et al 2006; Lincoln et al 2001; Miller et al 2009; Takimoto et al 2005a; Takimoto et al 2005b).

3.4 Cyclic nucleotides synthesis: adenylyl cyclases and guanylyl cyclases

3.4.1 *Adenylyl cyclases*

Adenylyl cyclases (ACs) are ATP-pyrophosphate lyases that convert ATP to cAMP and pyrophosphate. In mammals, nine membrane-bound isoforms (AC1-AC9) and one soluble form (sAC) of adenylyl cyclase have been identified. Membrane-bound AC isoforms are large proteins of approximately 120–140 kDa that share a common secondary structure comprising a short and variable amino-terminus, two repeats of a module composed of six transmembrane spans (M1 and M2) and two cytoplasmic domains (C1 and C2) (Figure 3-9). The ATP-binding C1 and C2 domains are the most conserved regions between AC isoforms and their interaction forms the catalytic active site. The catalytic domain can be expressed independently of the rest of the molecule to form $G_{\alpha s}$- and forskolin-stimulatable enzymes (Sunahara et al 1996). Within this motive two aspartic acid residues coordinate two Mg^{2+} metal ions necessary for catalytic activity. Nucleotide binding and catalysis are determined by C1 and C2. As a consequence, any factor that modifies the relative orientation of these two domains

can alter the structure of the active site thereby affecting substrate affinity and catalytic velocity. The catalytic core has been crystallized in two different conformations: a forskolin-bound eterodimer and and a $G_{s\alpha 2}$-bound eterodimer. The two structures differ by a rotation of 7 degrees that brings key catalytic elements from the two domains close to each other. These data indicate that both forskolin and G_s are capable of driving the observed structural changes. The mechanism by which $G_{s\alpha}$ and $G_{i\alpha}$ modulate AC activity has been largely explored; briefly, while $G_{s\alpha}$ plays the role of helping the catalytic domain to collapse around the substrate, $G_{i\alpha}$ maintains the catalytic domain in an open conformation thus inhibiting its activity (Hurley 1999; Sunahara et al 1996; Sunahara et al 1997; Tesmer et al 1997).

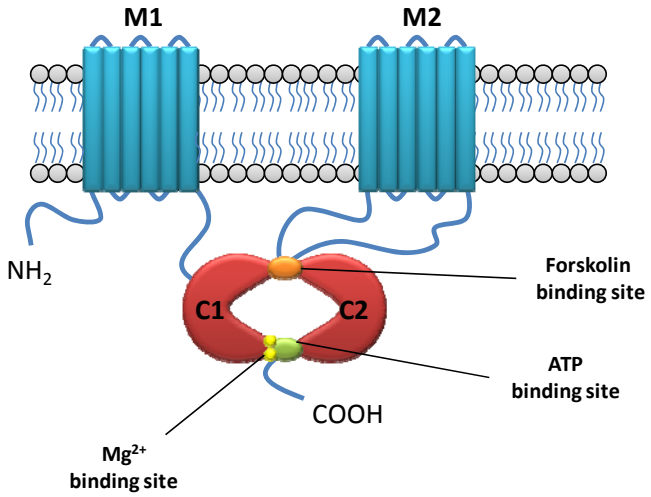


Figure 3-9 Schematic representation of adenylyl cyclase.

The various family members are all stimulated by forskolin and by $G_{\alpha s}$, with the only exception of sAC. In addition, each of the ten known AC isoforms is uniquely regulated via a combination of different mechanisms that comprise $G_{\beta\gamma}$-stimulation, $G_{i\alpha}/Ca^{2+}$ inhibition, and Ca^{2+} stimulation (Table 1).

G-protein subunits exert type specific effects on ACs. AC2, AC4 and AC7 isoforms are stimulated by the $G_{\beta\gamma}$ subunits of the heterotrimeric G proteins, and by PKC phosphorylation, whereas they are insensitive to $G_{\alpha i}$ and to Ca^{2+}. AC1 and AC8, are

inhibited by $G_{\alpha i}$ and by $G_{\beta\gamma}$ subunits, and are stimulated by Ca^{2+} in a calmodulin (CaM)-dependent manner. AC5 and AC6 are both inhibited by $G_{\alpha i}$, by Ca^{2+} and by PKA phosphorylation. AC3, which is inhibited by G_{α}, is also negatively regulated by CaM-kinase. AC9 is inhibited by $G_{\alpha i}$, as well as by PKC ad calcineurin. Indications exist that also tyrosine kinase receptors can modulate ACs (Nair et al 1995).

The only soluble mammalian adenyl cyclase described so far, sAC (Chen et al 2000) completely differs from all the other AC isoforms. sAC is regulated by calcium (Jaiswal & Conti 2003), but it is not stimulated by $G_{\alpha s}$ or forskolin, it is not inhibited by $G_{\alpha i}$ and it is not affected by $G_{\beta\gamma}$. Uniquely, this enzyme is directly stimulated by bicarbonate, in a pH-independent manner (Cooper 2003; Willoughby & Cooper 2007).

Ac Isoform	G protein regulators		Protein kinases		Calcium
	stimulatory	inhibitory	stimulatory	inhibitory	
Group I					
AC1	Gsα	Gα, i, z, o, Gβγ	PKCα (weak)	CaMK IV	↑CaM
AC8	Gsα	Gβγ			↑CaM
AC3	Gsα	Gβγ	PKCα (weak)	CaMK II	↑CaM
Group II					
AC2	Gsα, Gβγ		PKCα		
AC4	Gsα, Gβγ			PKCa	
AC7	Gsα, Gβγ		PKCα		
Group III					
AC5	Gsα, Gβγ	Gα, i, z	PKC (a, ζ)	PKA	↓Free Ca^{2+}
AC6	Gsα, Gβγ	Gα, i, z		PKA, PKC (δ,ε)	↓Free Ca^{2+}
Group IV					
AC9	Gsα			PKC	↓ via calcineurin

Table 1 ACs regulation mechanisms adapted from (Sadana & Dessauer 2009).

3.4.2 *Guanylyl cyclases*

Guanylyl cyclases are a family of enzymes that catalyze the conversion of GTP to 3'-5-guanine monophosphate. Although cGMP-forming activity was first described in 1969 it took until the mid-1970s to find out that there are two different types of GC which were subsequently found to differ not only in their cellular localization (cytosolic versus

membrane-bound) but also in their structure and regulation (Chrisman et al 1975; Garbers & Gray 1974; Kimura & Murad 1974).

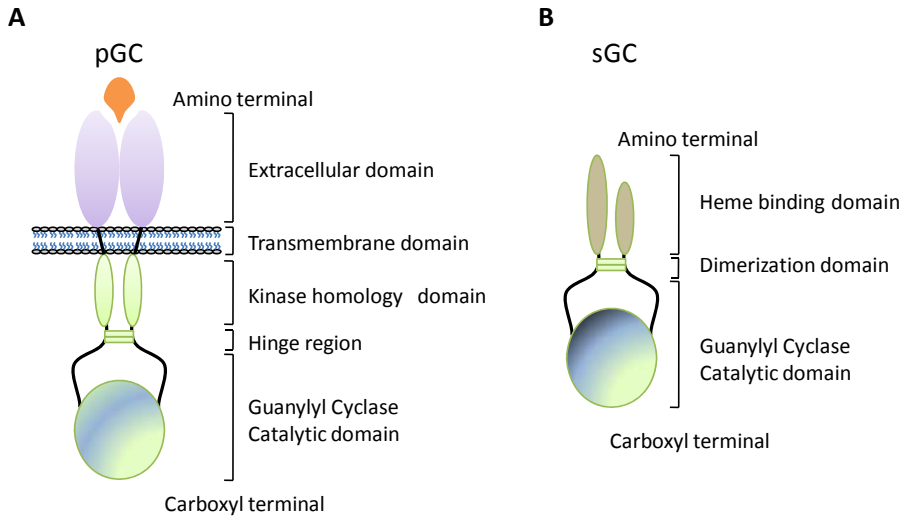


Figure 3-10 Structure of particulate (A) and soluble (B) guanylyl cyclases. Adaped from (Lucas et al 2000).

3.4.2.1.1 Particulate guanylyl cyclases

Seven membrane-bound isoforms of guanylyl cyclases (GC-A to GC-G) have been identified. They are structurally related to the cytosolic enzymes, but they are not stimulated by NO. These GCs belong to the group of receptor-linked enzymes containing one membrane-spanning region. They exhibit highly conserved structure including an extracellular peptide receptor domain at the N-terminus, a short transmembrane domain, a regulatory kinase-homology domain (KHD), a hinge domain and a C-terminal catalytic domain (Figure 3-10-A). On the basis of their ligand preference, pGC have been classified in natriuretic peptide receptor, intestinal peptide-binding receptors and orphan receptor. GC-A and GC-B bind and are activated by ANP, BNP and C-type natriuretic peptides. GC-C is activated by guanylyl, uroguanyly and lymphoguanylyl. Ligands for GC-D, -E, -F and G remain to be identified. The natriuretic peptide receptor-like GCs, including GC-A, -B and –G are broadly expressed in many tissue. On the contrary, GCs-C, -D, -E, and F are expressed in sensory organs and possess a characteristic C-terminal tail. The function of this additional domain is still

unknown, however different hypothesis have been formulated. Wada and colleagues proposed a role in the regulation of catalytic activity (Wada et al 1996), other works suggested that it could be responsible for the association with cytoskeleton (Hakki et al 1993), or that it could mediate internalization of guanylyl cyclase receptors (Urbanski et al 1995). The regulation of pGC activity by natriuretic peptides occurs in different steps. In the absence of ligand, pGCs exist like homodimers complexes in which each monomer is phosphorylated at the level of KHD. Oligomerization and phosphorylation confer to the dimer high affinity towards ligands and allow the cytoplasmic domain to respond to ligand-receptor interactions. When peptides bind to the pGC they cause a conformational change in the KHD. As a consequence, ATP binds to the same domain and releases the repression that the KHD exerts on catalytic activity of pGC. This, in turn, increases the V_{max} of cGMP production. Association of ATP with the KHD reduces the affinity of the extracellular binding domain for the substrate and induces dephosphorylation of the KHD. This results in dissociation of ATP and ligand leading to desensitization. Regeneration of a responsive receptor is probably gained through re-phosphorylation of the KHD (Lucas et al 2000).

3.4.2.1.2 Soluble guanylyl cyclases

The NO-sensitive GC is a heterodimer consisting of two different subunits termed α and β; expression of both subunits is required for catalytic activity. Both α and β subunits exist in two isoforms $\alpha1$, $\alpha2$ and $\beta1$, $\beta2$ respectively. The most prevalent sGC isotype is the $\alpha1\beta1$ eterodimer. Each etrodimer is carachterized by an amino terminal regulatory domain that contains heme prosthetic group. The prosthetic group contains a ferrous (Fe^{3+}) core that forms an imidazole axial bond with the hystidine 105 of the β subunit. In addition, sGCs possess a dimerization domain and a carboxyl terminal catalytic domain (Figure 3-10-B).

NO activates sGC in a two-step activation process. NO, indeed, can bind to both heme and non heme sites of sGC. Initially, NO binds to the ferrous, transforming the five-coordinate heme moiety of sGC into a NO-responsive six-coordinate nitrosyl

intermediate. Upon further NO-binding, and in the presence of magnesium, cGMP, and pyrophosphate, the nitrosyl intermediate immediately converts to a five-coordinate nitroxyl complex (Russwurm & Koesling 2004a; 2004b; Tsoukias et al 2004; Zhao et al 1999). As a consequence, the bond between the heme iron and the protein histidine axial ligand breaks, triggering a conformational change in the catalytic domain of the enzyme and accelerating the basal rate of conversion of GTP to cGMP by several hundred folds. The six-coordinate nitrosyl intermediate sGC cannot be activated by NO in the absence of magnesium, cGMP, or pyrophosphate. Thus, at low levels of NO, sGC remains in a low-activity state, whereas at high levels of NO and substrates/products, the low-activity state sGC can be converted to the highly active state.

3.4.2.1.3 NO synthases

Nitroxic synthase enzymes (NOS) catalyse the reaction of L-arginine, NADPH, and oxygen to the free radical NO, citrulline and NADP. They contain relatively tightly-bound cofactors (6R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (heme). Three isoforms of NOS have been identified; they are generated by different genes and show different localization, regulation, catalytic properties and inhibitor sensitivity. Neuronal NOS (nNOS) (also known as Type I, NOS-I and NOS-1) is the isoform first found (and predominating) in neuronal tissue, inducible NOS (iNOS) (also known as Type II, NOS-II and NOS-2) is the isoform which is inducible in a wide range of cells and tissues and endothelial NOS (eNOS) (also known as Type III, NOS-III and NOS-3) is the isoform first found in vascular endothelial cells.

3.5 Degradation of cyclic nucleotides: phosphodiesterases (PDEs)

PDEs are a superfamily of metallophosphohydrolases that specifically cleave the 3', 5'-cyclic phosphate moiety of cAMP and/or cGMP to produce the corresponding 5'-nucleotide, 5'-AMP and 5'-GMP.

Twenty-one genes have been indentified in human, rat and mouse. They are categorized into 11 different families (PDE1-PDE11) on the basis of their amino acid

sequences, substrate specificities, endogenous and exogenous regulators and pharmacological properties. These genes encode multiple protein products generated by alternative splicing and transcription from multiple promoters; as a consequence more than 50 different PDE proteins are expressed in mammalian cells.

Individual PDEs exert specific functional roles as a consequence of the unique combination of regulatory mechanisms, intracellular localization and enzyme kinetics (Baillie et al 2005; Conti & Beavo 2007).

3.5.1 *General structure*

The mammalian PDEs share a common structural organization, with a conserved catalytic domain located near the C-terminus, and regulatory domains both at the C- and at the N-terminus of the protein (Figure 3-11).

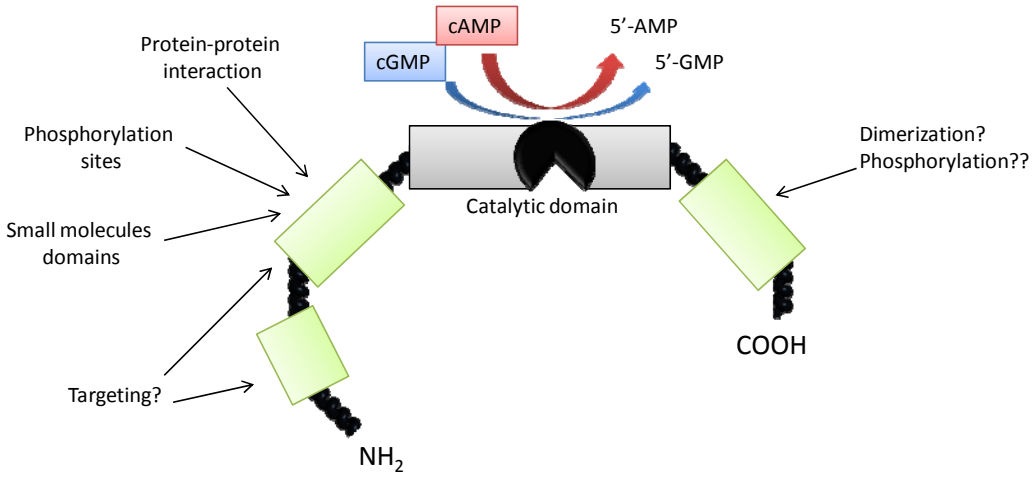


Figure 3-11 Schematic representation of PDE structure adapted from (Mehats et al 2002).

The catalytic domain possesses a particular signature motif $HD(X_2)H(X_4)N$, conserved in all mammalian PDEs, that include consensus metal-binding domains (Zn^{2+} and Mg^{2+}) related to those of metal-ion phopshohydrolases (Francis et al 2001). The N-terminus is the most variable domain but it includes sequences that are quite conserved within each family and correspond to functional motifs (Conti & Jin 1999). These domains are

involved in the regulation of the catalytic domain and determine the subcellular localization of these enzymes. They include binding sites for small molecules, such as cyclic nucleotides, protein-protein interaction domains, and phosphorylation sites for kinases. The function of some conserved domains located at the C-termini is still unknown; nevertheless, it has been proposed that this region might serve for dimerization or it can contain further sites of regulation, for example additional phosphorylation sites (Hoffmann et al 1999; Lenhard et al 1996).

3.5.2 *PDEs substrate specificity*

In mammals, 3 of the 11 PDE families selectively hydrolyze cAMP (PDEs 4, 7, and 8), 3 families are selective for cGMP (PDEs 5, 6, and 9), and 5 families hydrolyze both cyclic nucleotide with varying efficiency (PDE 1, 2, 3, 10, and 11) (Figure 3-12). Several structural studies on PDEs suggest that the selectivity is determined by one particular invariant glutamine in the catalytic site (Zhang et al 2004). Zhang and colleagues solved the structures of PDE4B and PDE4D in complex with AMP, the structure of chimeric PDE5A in complex with GMP, and the apo-structure of the dual-specific enzyme PDE1B. They proposed a glutamine switch mechanism for the control of PDE selectivity toward cyclic nucleotides. They found that an invariant glutamine residue can adapt two different orientations in space: in one orientation the hydrogen bond (H-bond) network supports guanine binding leading to cGMP selectivity and in the other orientation the network supports adenine binding resulting in selectivity toward cAMP. For both nucleotides to bind well, this glutamine must be able to rotate freely. They thus proposed that in dual-specific PDEs the orientation of the key glutamine residue switches between the two orientations resulting in dual specificity towards cyclic nucleotides.

3.5.3 *Regulatory domains*

The unique regulatory characteristics of each PDE family are defined by protein domains located at the N-terminus. These domains are essential for ligand binding,

oligomerization and kinase recognition/phosphorylation. As shown in Figure 3-12 PDE families (PDE2, 5, 6, 10 and 11) contain two tandem protein domains named GAF (GAFA and GAFB). The GAF acronym comes from the name of the first classes of proteins in which they were identified: cGMP-specific and –regulated cyclic nucleotide phosphodiesterases, Adenylyl cylase, and *E.coli* transcription factor Fh1A. GAF domains are known to function as regulatory elements that bind nucleotides or other small molecules; they are involved in cGMP-mediated allosteric regulation and dimerization of PDEs, although some GAF domains have also been shown to bind cAMP (Aravind & Ponting 1997; Gross-Langenhoff et al 2006). PDE1 contains a Ca^{2+}/calmodulin binding (CaM) site; PDE3 has a transmembrane domain that includes six hydrophobic helices; PDE4 has upstream conserved regions (UCRs), that may function as a binding domain for acidic phospholipids such as phosphatidic acid and phopshatidylserine (Grange et al 2000; Houslay & Adams 2003; Houslay et al 1998). PDE8 contains a response regulator receiver (REC) domain and a per-arnt-sim (PAS) domain, named after the three eukaryotic proteins [period, aryl-hydrocarbon receptor nuclear translocator (ARNT), and single minded] in which they were first described. Like GAF domains, PAS domains are involved in ligand binding and protein-protein interactions, and they share some structural similarities with the GAF domains (Fukunaga et al 1995; Huang et al 1993). The functional consequences of PDEs dimerization are not completely understood; however, loss of oligomerization in general is associated with loss of regulation of catalytic activity (Richter & Conti 2004).

The third conserved feature of the N-terminus of several PDEs is the presence of phosphorylation sites in the vicinity of the dimerization domain. Phosphorylation of these domains has been shown to modify the catalytic activity of PDEs. For PDE5, for example, allosteric binding of cGMP to GAF domain increases the accessibility of the Ser 92/102 residue and promotes PKG-mediated phosphorylation. This phosphorylation then activates the catalytic domain and decreases the K_m for the substrate (Bessay et al 2007). Phosphorylation sites are also present in PDE1, PDE3 and in the long forms of PDE4. PDE1A1, 1B and 1C can be phosphorylated at the N-terminus by PKA and CaM-

kinase II (CaMKII) and phosphorylation decreases the binding and activation by calmodulin *in vitro* (Florio et al 1994; Sharma & Wang 1985). PDE2A can also be phosphorylated, apparently by tirosin kinase (Bentley 2005). PDE3A and 3B can be phosphorylated and activated by PKA and PKB; PKB-mediated phosphorylation of PDE3B increases the catalytic activity of the enzyme (Kitamura et al 1999; Shakur et al 2001; Zmuda-Trzebiatowska et al 2006). At the N-terminus of UCR1 of all the PDE4 long forms there is a highly conserved motif (RRESF) that can be phosphorylated by PKA thereby leading to an increased catalytic activity of the enzymes (MacKenzie et al 2002; Sette & Conti 1996; Sette et al 1994). PDE4 subfamilies, except for PDE4A, contain an ERK consensus motif (Pro-Xaa-Ser-Pro) whose serine can be phosphorylated by ERK (extracellular signal-regulated kinase). ERK phosphorylation can exert a positive or negative effect on PDE4 activity depending on the presence or absence of UCR1/UCR2 domains. UCR1 and UCR2 mediate dimerization of PDE4 long forms, they interact through ionic interactions and they form a regulatory module that directs the functional outcomes of PKA and ERK phosphorylation. ERK phosphorylation leads to inhibition of long isoforms (they contain both UCR1 and UCR2), activation of short isoforms, and weak inhibition of super-short isoforms. Therefore cAMP signalling can be enhanced or decreased by ERK depending on the isoform expressed. Interestingly ERK inhibition of PDE4 long isoforms can be abolished by PKA phosphorylation at the UCR1 domain. Phosphorylation of PDE4 by ERK blocks the enzyme activity thereby increasing cAMP levels. As a consequence, activated PKA phosphorylates a serine residues in the UCR1, releasing PDE4 inhibition. This constitutes a feedback regulatory mechanism and allow transient modulation of cAMP levels by ERK signalling (Baillie et al 2000; Hoffmann et al 1999; MacKenzie et al 2000).

Some PDEs possess also auto inhibitory domains, PDE6 for example has an inhibitory subunit (Pγ) whose affinity for the enzyme is enhanced by the binding of cGMP to the GAF domains (Cote 2004). PDE7 and PDE9 have no identified specific protein domains. Several properties of the C-terminal domains have also been described for PDEs,

including phosphorylation sites or prenylation sites; however, there are not confirmed concepts about the function of the C-terminal region of PDEs.

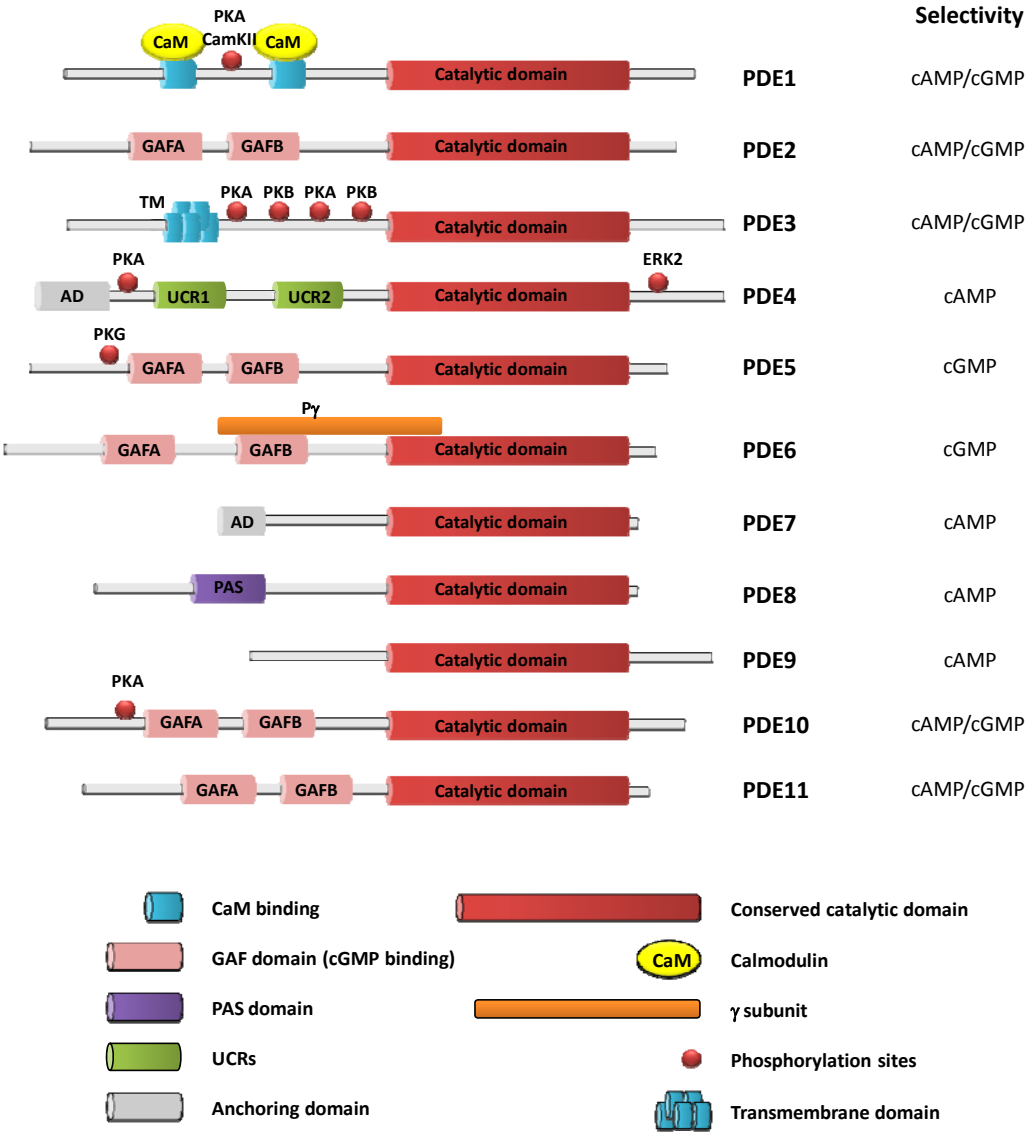


Figure 3-12 PDEs families adaped from (Conti & Beavo 2007).

3.6 Effectors of cyclic nucleotides

Four major cyclic nucleotide binding protein families have been described: protein kinase A and G, phosphodiesterases (PDEs), cyclic nucleotide-gated channels (CNG) and exchange proteins directly activated by cyclic AMP (Epacs).

3.6.1 *Protein kinases A and G*

3.6.1.1.1 cAMP dependent protein kinase (PKA)

PKA is a heterotetramer composed of two catalytic (C) and two regulatory (R) subunits Each R subunit owns two cAMP binding sites (A and B) to which cAMP binds cooperatively. In the inactive holoenzyme the two catalytic subunits are bound noncovalently to a regulatory dimer and only the B site is exposed and available for binding. When a molecule of cAMP occupies the B site it induces an intramolecular conformational change that enhances the binding of a cAMP molecule to the A site. When four molecules are bound to the PKA enzyme the catalytic subunits dissociate and become catalitically active. Active catalytic subunits are then free to phosphorylate Serine and Threonine residues in target proteins (Gill & Garren 1971; Kopperud et al 2002; Krebs & Beavo 1979; Taylor et al 1990);(Taylor et al 1999).

PKA is formed by a combination of several isoforms of R and C subunits. Each of the three C subunit isoforms (α, β and γ) can associate with any of the two RI (RIα, RIβ) and RII (RIIα and RIIβ) subunits. While all C subunits show common kinetic features and substrate specificity (Taylor et al 1992), R subunits possess different physical and biological properties and establish the characteristics of the PKA holoenzyme (Skalhegg & Tasken 2000). PKA holoenzymes are classified as either type I or type II, depending on the subtype of regulatory subunit present. PKA isoforms show different biochemical properties and distinct localization. PKA type I is more readily dissociated by cAMP than PKA type II (Cummings et al 1996; Dostmann et al 1990). Intracellular localization of PKA isoenzymes is mediated by the N-terminal dimerisation/docking (D/D) domain of the R-subunits (Figure 3-13). PKA type II is associated with the particulate fraction of cells; it binds to scaffolding proteins, A kinase anchoring proteins (AKAPs) (Wong & Scott 2004). PKA type I is found mainly in the soluble fraction of cell lysates. However, it has been shown that also PKAI binds to AKAPs although with a lower affinity (Burton et al 1997; Colledge & Scott 1999) and dual specificity AKAPs have been described as well as RI-selective AKAPs (Angelo & Rubin 1998; Gronholm et al 2003; Li et al 2001).

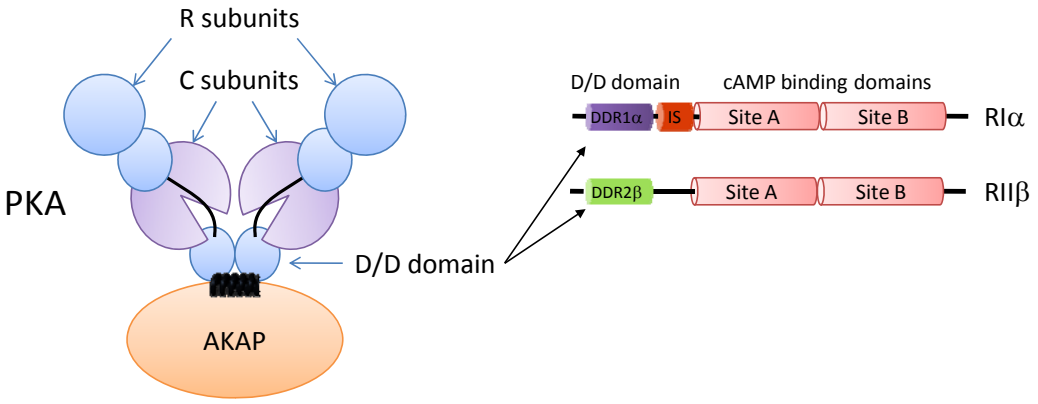


Figure 3-13 Binding to AKAPs is mediated by the dimerization docking domains of regulatory subunits.

3.6.1.1.2 cGMP dependent protein kinase (PKG)

cGMP-dependent protein kinase (PKG) belongs to the family of Serine/Threonine kinases. Three isotypes of PKGs have been identified: PKGIα, PKGIβ, and PKGII. PKGI (76 kDa) is the prominent isotype in the cardiovascular system, it is expressed at very high levels in vascular smooth muscle cells (both α and β splice variants) and endothelial cells (PKGIβ) and at lower levels in cardiac myocytes (PKGIα). PKGII (86 kDa) is mainly expressed in the kidney, brain, and intestine. PKGs are dimers comprised of two identical monomers. Each monomer consists of three functional domains: an N-terminal domain, a regulatory domain, and a kinase domain (or catalytic domain). Each regulatory domain contains two tandem cGMP binding sites that bind cGMP with high and low affinity and interact allosterically. The catalytic domain contains the Mg^{2+}/ATP and peptide binding pockets. The N-terminal domain mediates PKG homodimerization, suppresses kinase domain activity in the absence of cGMP, and interacts with target substrate proteins. Binding of cGMP to both regulatory domains releases the inhibition of the catalytic centre by the NH_2-terminal autoinhibitory/pseudosubstrate site and allows the phosphorylation of Serine/Threonine residues in target proteins and in the amino-terminal autophosphorylation site. Autophosphorylation has been shown to increase the basal activity and PKGI and PKGII (Smith et al 1996; Vaandrager et al 2003). Amino-terminal modifications appear to control intracellular localization and

hence function of PKG. In the intestine, for example, PKGII is involved in the regulation of ion and water transport. It acts as a key regulator of ion transport systems, including the cystic fibrosis transmembrane conductance regulator (CFTR)-chloride channel. Myristoylation has been shown to be a prerequisite for the positioning of PKGII in the apical membrane of the enterocyte and to explicate its functions (Vaandrager et al 1996).

3.6.2 *CNG channels*

Cyclic nucleotide-gated (CNG) ion channels are non selective cation channels that are important part of the signal transduction pathway in the visual and olfactory systems. The function of CNG channels in photoreceptors and olfactory sensory neurons has been established beyond reasonable doubt, whereas for other cell types either their expression is uncertain or their function is not fully understood. They have been reported to be expressed also in airway epithelial cells, endothelial cells of pulmonary artery and kidney. CNG channel activity shows very limited voltage dependence, however, they are classified as voltage dependent ion channels. Cyclic nucleotides directly bind and activate CNG channels in a concentration dependent way; probably four molecules of cyclic nucleotide are necessary to fully open the channel. All CNG channels subunits contain a COOH-terminal region that exhibits significant sequence similarity to the cyclic nucleotide binding domain of PKA and PKG (for references see review (Kaupp & Seifert 2002)).

Different models for channels activation have been proposed (Li et al 1997) but this is still matter of debate. All known CNG channels respond to both cAMP and cGMP, but lower concentration of cAMP then cGMP are sufficient to open the channels. CNG channels of cones and rods are able to distinguish between cAMP and cGMP, on the contrary, channels in chemiosensitive cilia of olfactory sensory neurons respond equally to both ligands. They form eterotetrameric complexes that can be constituted by two or three different types of subunits. CNG channels' subunits are encoded by six

different genes (CNGA1-4 and CNGB1-2). The subunit composition of each channel determines functional features like sensitivity, selectivity, ion permeation and gating.

3.6.3 *Epac proteins*

Epac1 (Exchange protein directly activated by cyclic AMP) and Epac2 proteins were discovered about 10 years ago as a new class of effectors for the second messenger cyclic AMP. In 1998 two independent papers described for the first time these two proteins and their function. De Rooij and colleagues performed a database search looking for proteins that could explain the insensitivity of cAMP-induced activation of Rap1 to PKA inhibitors. They found two proteins that contained a cAMP-binding site and a domain homologous to domains of known GEFs (guanine nucleotide exchange factors) for Ras and Rap1 (de Rooij et al 1998).

The same proteins, named cAMP-GEFI and cAMP-GEFII, were discovered independently by Kawasaki and colleagues in a screen for brain-specific genes with second messenger-binding motifs (Kawasaki et al 1998). Epac proteins are guanine nucleotide exchange factors for the Ras-like small GTPases Rap1 and Rap2 and function independently of protein kinase A. Small G proteins, including Rap, act as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state. cAMP-dependent activation of Epac promotes the exchange of GDP for GTP, hence switching on the Rap GTPases (Bos et al 2007).

Epac1 and Epac2, are encoded by two distinct genes, RAPGEF3 and RAPGEF4 (de Rooij et al 1998; Kawasaki et al 1998). RAPGEF3 codes for Epac1, whereas RAPGEF4 generates a long and a short variant named Epac2A and Epac2B, respectively (Niimura et al 2009).

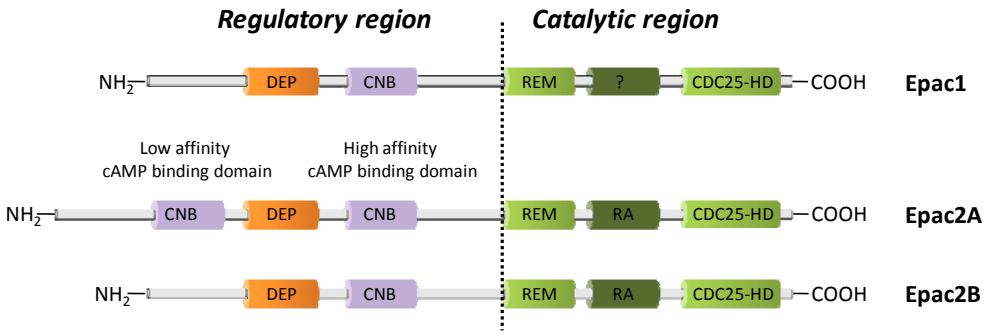


Figure 3-14 Structure of Epac proteins adapted from (Metrich et al 2009). DEP, (Disevelled, Egl-10, Pleckstrin) domain; CNB, cyclic nucleotide binding domain; REM, Ras exchange motif; HD, homology domain; RA, Ras-association domain.

Epac proteins consist of an N-terminal regulatory region and a C-terminal catalytic region (Figure 3-14). The C-terminal catalytic region activates Rap1 but not Ras, Ral, or R-ras. This region comprises the enzymatic GEF domain CDC25-homology domain (HD) and the Ras exchange motif (REM), which is needed for stability of the GEF domain. Between REM and CDC25-HD there is a domain with ubiquitin fold. For Epac2 it has a clear feature of Ras –association (RA) domains, found in many Ras-interacting proteins. The N-terminal part of the protein contains a DEP (Disevelled, Egl-10, Pleckstrin) domain and a CNB (cyclic nucleotide binding) domain that binds cAMP. Epac2A shares a similar organization, but possesses an additional cyclic nucleotide-binding domain, of uncertain biological function. This domain does bind cAMP, but with low affinity compared with the second CNB domain. Furthermore, its deletion affects neither auto-inhibition nor the binding of cAMP to the second CNB domain, suggesting that this first CNB domain might not be directly involved in the regulation of Epac2 (de Rooij et al 2000). The DEP domain's function is not clear yet; however, it has been shown that deletion of this domain results in a more cytosolic localization of the protein, suggesting that DEP domain, rather than being involved in the regulation of Epac by cAMP, is involved in the localization of the protein, probably through interaction with a lipid or a membrane protein (de Rooij et al 2000; Ponsioen et al 2004). The cAMP binding domain (CNB) is similar to the cAMP binding domains in the regulatory subunit of PKA. cAMP is required for the activation of full-length Epac in vitro, and deletion of

the regulatory N-terminal part containing the cAMP binding domain results in the constitutive activation of Epac, indicating that it serves as an auto-inhibitory domain.

In the closed conformation of the protein, the binding site for Rap is completely covered by the regulatory region; this closed conformation is achieved thanks to the 'switchboard' (a pseudo β-sheet between the regulatory and catalytic region) and the 'ionic latch' [an ionic interaction between the second CNB domain and the CDC25-homology domain (HD)]. Binding of cAMP to Epac induces large conformational changes that disrupt the ionic latch followed by a reorientation of the second CNB domain towards the switchboard region and releasing of the auto-inhibitory effect of the N-terminal region, leading to Rap activation (Figure 3-15) (Rehmann et al 2006).

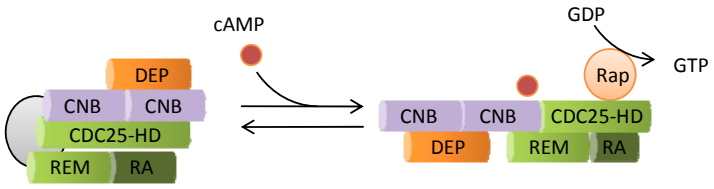


Figure 3-15 Activation of Epac (indicated for Epac2) by cAMP results in the opening of the protein to enable interaction with Rap and, consequently, the conversion of RapGDP to RapGTP (Bos 2006).

3.6.4 *PDEs as integrators of cAMP and cGMP signalling pathways*

Dual specificity PDEs and PDEs that contain allosteric binding sites are capable of integrating cAMP and cGMP signalling pathways. cGMP can indeed act as a regulator of the activity of cAMP-degranding PDEs, such as that the intracellular concentration of cGMP can influence the intracellular concentration of cAMP. The modulatory effect of cGMP on cAMP-hydrolizing PDEs occurs at nanomolar to micromolar concentrations of cGMP. This concentration is compatible with that at which cGMP activates targets such as PKG (K_a~100 nmol/L) (Ruth et al 1991) and cyclic nucleotide-gated channels (K_a~20 µmol/L) (Biel et al 1994). Cardiac myocytes express several cGMP regulated PDEs, which will be discussed in the next paragraphs.

3.6.4.1.1 cGMP activated PDEs: PDE2 and PDE5

PDE2

The PDE2A gene encodes 3 N-terminal splice variants: PDE2A1, PDE2A2 and PDE2A3 (Rosman et al 1997). The isoform PDE2A2 contains an unique hydrophobic N-terminal sequence that could explain why its activity has been found in both membrane-associated and soluble fractions of the cell (Yang et al 1994). In cardiac myocytes PDE2 has been shown to be cytosolic as well as associated with the sarcolemma, the sarcoplasmic reticulum membrane (Lugnier & Komas 1993), the Golgi apparatus and the nuclear envelope (Geoffroy et al 1999). PDE2 hydrolizes both cAMP and cGMP, with K_m values of 30 µmol/L for cAMP and 10 µmol/L for cGMP (Osadchii 2007).

All PDE2 isoforms contain two GAF domains located at the N-terminus and cGMP binds with high affinity to GAFB. This binding causes an allosteric modification that lowers the apparent K_m of PDE2 for cAMP and results in higher catalytic activity of the enzyme. This allosteric modulation by cGMP is more important than its competitive inhibition of cAMP hydrolysis, although cAMP hydrolysis can be inhibited, *in vitro*, by high cGMP concentrations (>50 µmol/L). This dual substrate specificity allows PDE2 to mediate negative crosstalk between the cGMP and cAMP singalling pathways. Very recently, the X-ray crystal structure of human PDE2A has been reported. The crystal included both catalytic and regulatory domains. Analysis of these structures led to a general mechanism of activation of PDE2. The model proposed involves a mechanism whereby access to the substrate pocket is blocked by the dimer partner, through an H-loop. Allosteric binding of cGMP to one of the GAFB domains causes a relative movement of the catalytic domains, relieving this blockage (Pandit et al 2009).

PDE5

PDE5 is a homodimer of 93 KDa subunits that specifically hydrolyzes cGMP. Like PDE2, it contains two GAF domains but the high affinity cGMP-binding occurs only to the GAFA (k_D 40 nmol/L). Cyclic nucleotide binding to this domain is ≈100-fold selective for cGMP over cAMP (Zoraghi et al 2005). Interestingly, PDE2 GAFB, PDE5 GAFA and PDE6

GAFA, all of which bind cGMP, show substantial structural homology. As with cGMP binding to the GAFB domain, it has been shown that the binding of cGMP to the PDE5 GAFA domain stimulates enzyme catalytic activity about 10-fold and that blockade of this binding inhibits activity (Rybalkin et al 2003). There is also evidence that cGMP binding is stabilized by phosphorylation of a nearby Serine that enhances cGMP affinity for GAFA domain (Francis et al 2002). The kinase responsible for this phosphorylation has been shown to be PKG (Shimizu-Albergine et al 2003); however, when the GAFA is already occupied by cGMP, PKA can also phosphorylate this site (Corbin et al 2000). This two mechanisms, allosteric cGMP binding and PKG mediated phosphorylation, constitute a way for the cell to prolong the activation of PDE5 in a feedback loop initiated by the synthesis of cGMP.

3.6.4.1.2 cGMP inhibited PDEs: PDE1 and PDE3

PDE1

The PDE1 family of phosphodiesterases includes three genes (PDE1A, PDE1B and PDE1C); in rat myocardial tissue PDE1C but not PDE1A or PDE1B expression was found using RT-PCR analysis (Kostic et al 1997). For a long time PDE1 was considered to be expressed only in non-myocyte cardiac cells such as endothelial and vascular smooth muscle cells, or fibroblast, rather than cardiac myocytes. A recent study has reported that PDE1C is highly expressed in the human myocardium and present in human cardiomyocytes (Vandeput et al 2007); however, the physiological and pathological roles of PDE1 in the heart are still unknown. PDE1 is a Ca^{2+}/Calmodulin-activated isoenzyme that hydrolyzes both cAMP and cGMP with similar substrate specificity in human (Reeves et al 1987), dog (Weishaar et al 1987), rabbit (Shahid & Nicholson 1990) and guinea-pig (Weishaar et al 1987) myocardial tissue. However, PDE1 preferentially hydrolyzes cGMP in rat and bovine myocardium (Harrison et al 1986; Weishaar et al 1987). Ca^{2+}/Calmodulin increases PDE1 V_{max} values for both cAMP and cGMP without any effect on K_m values. The three gene products exhibit different affinity for cAMP. PDE1A has the lowest affinity (K_m=50 to 100 μmol/L), PDE1B has an intermediate

affinity (K_m=7 to 24 µmol/L) and PDE1C has the highest affinity (K_m<1 µmol/L). For all these isoforms, therefore, cAMP hydrolyzing activity can be inhibited by cGMP. However, cGMP-mediated inhibition of the cAMP-hydrolyzing activity of PDE1 has been demonstrated only *in vitro*. PDE1 activity is also regulated by protein-kinase mediated phosphorylation. In particular, PDE1A1 and PDE1A2 isoenzyme can be phosphorylated by PKA and Ca^{2+}/Calmodulin-activated kinase II, an effect that reduces PDE1 affinity for calmodulin and calcium. Moreover PDE1 has been shown to be phosphorylated by protein kinase C in hamster heart providing 2-fold increase in PDE1 V_{max} for cAMP without changing K_m value (Lee et al 1994; Yu et al 1996).

PDE3

The PDE3 family of phosphodisterases includes two genes, PDE3A and PDE3B. In human myocardium three PDE3 isoforms have been identified: PDE3A1, PDE3A2 and PDE3A3. They are generated by alternative transcription and alternative translation from the PDE3A gene (Choi et al 2001; Wechsler et al 2002). The amino acid sequence of these isoforms is almost identical; the catalytic regions are identical with respect to catalytic activity and sensitivity to cGMP and other inhibitors of cAMP hydrolysis, they mainly differ at the N-terminus were different domains responsible for intracellular localization and phosphorylation sites are present. These isoforms localize to different intracellular compartments. PDE3A1 has been found only in the microsomal fraction of human myocardium; it is characterized by two domains, NHR1 and NHR2 that determine its intracellular localization. NHR1 is a hydrophobic loop that inserts into intracellular membranes whereas NHR2 localizes the enzyme via protein-protein interactions (Kenan et al 2000; Shakur et al 2000). PDE3A2 contains only the NHR2 domain and is recovered both in microsomal and cytosolic fractions; PDE3A3 lacks both NHR1 and NHR2 domains and its distribution in primarily cytosolic.

PDE3 is the most abundant PDE isoenzyme in myocardial tissue of most mammalian species (in rats only the PDE3A gene is expressed, and PDE4 is more abundant than PDE3 (Mongillo et al 2004; Rochais *et al* 2006)). Immunocytochemical studies have

shown that PDE3 is associated mainly with sarcolemma and intracellular membranes in rat cardiac myoytes (Mongillo *et al* 2004; Okruhlicova et al 1996). PDE3 enzymes bind with high affinity both cAMP (K_m≈80 nmol/L) and cGMP (K_m≈20 nmol/L), which are mutually competitive substrates. Because of a much higher catalytic rate (Kcat's) for cAMP that for cGMP, PDE3 phosphodiesterases appear to function mainly as cGMP-inhibited cAMP hydrolyzing enzymes (Shakur *et al* 2001).

3.7 Spatial control in cyclic nucleotide signal transduction

Each individual cell expresses a relative large number of different GPCRs that signal trough the generation of cAMP and PKA activation. PKA, in turn, can phosphorylate many intracellular targets. Nevertheless, the activation of each individual receptor results in a specific functional outcome raising the question of how signal specificity is accomplished.

In the past ten years a number of studies have tried to unravel the complex mechanisms that allow cAMP signals to retain signal specificity inside the cell (Fischmeister et al 2006; Scott & Pawson 2009; Zaccolo 2009; Zaccolo et al 2006). We now know that compartmentalization of cAMP and of all the components of the cAMP pathway is pivotal in ensuring the necessary fidelity of signal transduction.

The first evidence that cAMP is not uniformly distributed inside the cell and that not all cAMP signals provoke the same downstream response was initially provided by Brunton and colleagues over 30 years ago in a series of biochemical studies performed on the rat heart (Brunton et al 1979) or in isolated ventricular myocytes (Buxton & Brunton 1983). When comparing the effects of isoproterenol (ISO) and prostaglandin E1 (PGE1) on cAMP accumulation and PKA activation in the particulate and soluble fractions, these authors found that ISO increases cAMP and activates PKA in both fractions, whereas PGE1 increases cAMP and activates PKA only in the soluble fraction (Brunton et al. 1979; Buxton & Brunton, 1983). Further studies demonstrated that ISO causes phosphorylation of phosphorylase kinase (Keely 1977), TnI (Brunton et al 1979) and other PKA targets (Hayes et al 1979), whereas PGE1 had no effect on these

substrates. These results suggested that not only PKA is targeted to specific locations within the cell but also that different pools of cAMP must coexist within the cell.

3.7.1 *cAMP-PKA compartmentalization*

Several studies demonstrated that tight control in space and in time of the cAMP-PKA signalling pathway is crucial to maintain specificity of the downstream response. It is well appreciated that subsets of PKA are sequestered into discrete subcellular domains in vicinity of specific targets. In this way, PKA is accessed by cAMP generated by specific activators and in turn it can phosphorylate and modulate particular substrates that are in close proximity. Compartmentalization of PKA is accomplished by AKAPs (A kinase anchoring proteins) a large family of functionally related multiscaffolding proteins that bind PKA and allow focal organization of signalling proteins within the cell. All members of this family share a conserved PKA anchoring domain that consists of a conserved amphipatic helix of 14-18 residues. This helix interacts with an hydrophobic groove in the dimerisation/docking domain (D/D) located at the N-terminus of the regulatory subunit dimer (Gold et al 2006; Kinderman et al 2006). The functional relevance of such spatial regulation has been demonstrated by using peptides able to disrupt AKAP-anchored PKA thereby ablating the regulation of coupled proteins (Colledge & Scott 1999; Diviani & Scott 2001; Diviani et al 2001).

An example of the importance of PKA anchoring comes from studies in cardiac myocytes. β-adrenergic regulation of cardiac contractility is largely mediated by PKA phosphorylation of target proteins, such as L-type Ca^{2+} channels, phospholamban (PLB), ryanodine receptor (RyR) and contractile proteins. Fink and colleagues have shown that in cardiac myocytes anchoring of PKA by AKAPs is indispensable for appropriate response to β-adrenergic stimulation. Disruption of the PKA-AKAP complex with the ht31 peptide, in fact, led to a reduced PKA-dependent phosphorylation of troponin I and myosin binding protein C. As a consequence, rate and amplitude of cell shortening as well as relaxation were increased (Fink et al 2001). In the last years, many AKAPs

have been shown to control calcium handling and cardiac contractility, among others AKAP18α, AKAP18δ and mAKAP (Hulme et al 2006; Kapiloff et al 1999).

AKAP18δ for example regulates the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) and mediates the lusitropic effect of β-adrenergic stimulation. AKAP18δ has been shown to interact with PLB, a transmembrane protein that in its dephosphorylated state exerts an inhibitory effect on SERCA2. When catecholamines impinge on β adrenergic receptors they lead to the generation of cAMP and PKA activation. PKA in turn phosphorylates PLB on Ser16, PLB dissociates from SERCA and relieves its inhibitory effect thereby allowing calcium influx in the sarcoplasmic reticulum. Lyngren and colleagues generated a short peptide from PLB covering the AKAP18δ-binding domain to compete with and displace the AKAP18δ–PLB interaction. Rat neonatal cardiac myocytes containing the disrupting peptide lost the localization of AKAP18δ at the z-lines, and upon isoproterenol stimulation, PLB phosphorylation was reduced by almost 50%. Additionally, these myocytes showed significantly reduced Ca^{2+} re-uptake into the sarcoplasmic reticulum, both at the basal level and after treatment with norepinephrine (Lygren et al 2007).

AKAPs do not only anchor PKA but also coordinate other signalling enzymes such as other kinases, phosphatases, GTPases, PDEs and other regulatory proteins. This fundamental characteristic permits control over the amplitude and duration of the signal and at the same time ensures integration and processing of multiple signals within discrete locales (Beene & Scott 2007).

3.7.2 *GPCR and AC compartmentalization*

Compartmentalization of PKA, though, implies also compartmentalization of the upstream components of the signalling pathway. If GPCRs and ACs were free to fluctuate within the plasma membrane, every pool of PKA could be non-specifically activated in response to each individual stimulus, thereby overcoming the advantage of having PKA anchored in proximity to specific substrates. Interestingly, β-adrenergic receptors, serotonin receptors and other GPCRs have been shown to localize in specific

membrane microdomains, lipid rafts and caveolae (Patel et al 2008). Lipid rafts are regions of the membrane rich in cholesterol and other lipids and caveolae are a subset of lipid rafts observed in invaginations of the plasma membrane that are stabilized by one or more isoforms of caveolin. Caveoleae are thought to be important in the organization of signal transduction events, particularly in insulin and cAMP signalling (Cohen et al 2003). In addition, it has been shown that also regulatory molecules involved in the synthesis of cAMP (ACs and G proteins) concentrate in these particular structures of the plasma membrane (Ostrom & Insel 2004; Patel et al 2008; Penela et al 2003; Willoughby & Cooper 2007).

ACs have also been found in complexes with AKAPs. Since AKAPs target PKA to specific downstream targets it is not surprising that macromolecular complexes include ACs to facilitate the generation of local pools of cAMP. AKAP79/150 is known to anchor different proteins, for example calcineurin (CaN), protein kinase C (PKC), protein kinase A (PKA), β_1 and β_2 –adrenergic receptors (Dodge & Scott 2000). Recently, in rat brain extracts, it has been shown AKAP79/150 can also bind AC5. AKAP79/150 by assembling AC5 and PKA allows the generation of a feedback control mechanism on cAMP synthesis. PKA-mediated phosphorylation inhibits AC5. When cells are treated with β_2-agonists cAMP is synthetized and PKA is activated. PKA phosphorylates AC5 contributing to a burst of cAMP synthesis (Bauman et al 2006). Other few examples are present in the literature, in which a physiological relevance has been proved for the interaction of AKAPs-ACs. The mAKAPβ-AC5 complex, for example, seems to be involved in the transduction of sympathetic hypertrophic cAMP signalling in cardiac myocytes (Kapiloff et al 2009). Also the plasma membrane targeted AKAP Yotiao associates with adenylyl cyclases. In the brain it serves as a scaffold protein for AC1, AC3, and AC9 to bring PKA in proximity to the cAMP synthesis machinery and to enhance the phosphorylation dependent termination of cAMP synthesis. In contrast, binding of Yotiao to AC2 directly inhibits the cyclase activity. The mechanism for such inhibition is not clear yet but it could be due to the recruitment of an inhibitory protein to the complex (Piggott et al 2008).

3.7.3 *Compartmentalization of cAMP*

cAMP is a small and hydrophilic molecule that shows a diffusion constant in the range of 270-780 $\mu m^2/s$ (Kasai & Petersen 1994). Accordingly, cAMP should diffuse rapidly through the cell and have equal access to all cellular compartments. This is how cAMP is thought to relay information to sites that are remote from the site of synthesis at the plasma membrane. However, if this were what happens, this would also imply that cAMP could fill almost instantaneously the entire cell, leading to the non-selective activation of all PKA subsets regardless of their localization near specific targets. As a consequence, any advantage deriving from spatial organization of macromolecular complexes would be abolished. Different mechanism to explain spatial restriction of cAMP have been proposed: these include a physical barrier, in which elements of the endoplasmic reticulum underneath the plasma membrane limit cAMP diffusion inside the cytosol (Rich et al 2000), and electrostatic tunnelling, in which cAMP directly shifts from the AC to PKA (Karpen & Rich 2001).

Definitely though, an important mechanism contributing to cAMP compartmentalization relays in PDEs activity. In cardiac myocyes, cAMP microdomains generated by catecholamines were completely abolished by the non-selective inhibitor of PDEs, suggesting that indeed PDE degrading activity contributes to cAMP restriction (Zaccolo et al 2002; Zaccolo & Pozzan 2002). Several studies have shown that there is a functional and physical coupling of individually localized PDEs with selected pools of ACs that are activated in response to different hormones. In neonatal cardiac myocytes the cAMP generated by the β adrenergic stimulation is controlled mainly by PDE4 whereas PDE3, although representing about 30% of the total PDE activity, exerts only a marginal effect. This result has been explained by the different localization of the two PDEs; indeed, immunostaining of PDE3 and PDE4 revealed a different intracellular localization (Mongillo et al 2004). Also PDE2, that contributes only 1% of the total PDE activity in rat cardiac myocytes, was shown to degrade a large proportion of the cAMP generated by β-AR stimulation in these cells. Again, PDE2 was shown to localize at specific subcellular sites (Mongillo et al 2006). Interestingly, displacement of

endogenous PDEs has been shown to disrupt their control over specific cAMP pools (Terrin et al 2006). In cardiac myocytes, overexpression of a catalytically dead mutant of PDE4 abolished the control mediated by the β-arrestin-recruited PDE4 over the β_2-AR switch from G_s to G_i, thereby enhancing ERK pathway activation (Baillie et al 2003).

The concepts of physical or enzymatic barrier, however, do not totally explain specific activation of compartmentalized PKA. In fact, they imply the generation of a cAMP gradient from the site of synthesis at the plasma membrane to the deep cytosol. As a result, PKA pools at the plasma membrane would be preferentially activated compared to the PKA pools localized inside the cytosol. In addition, activation of the PKA localized deep inside the cell would necessarily imply concomitant activation of PKA at the plasma membrane. A new mechanism has been proposed to explain specific activation of PKA subset irrespectively of their subcellular localization and distance from the site of cAMP synthesis. It has been shown that PDEs by degrading cAMP act as sinks to locally drain cAMP (Terrin et al 2006). Simultaneous intracellular gradients can therefore be generated irrespective of their distance from the AC that generated the cAMP. PDEs regulate cAMP intracellular levels in a compartment specific manner allowing activation of certain PKA subsets and keeping cAMP levels under the threshold of PKA activation in the proximity of those targets that need to be protected from inappropriate phosphorylation (Terrin et al 2006).

3.8 Compartimentalization of cGMP

The existence of distinct generators of cGMP (pGC and sGC) that localize in different intracellular locations suggests that cGMP production may not be distributed uniformly within the cell. Several studies have shown differential effects of cGMP produced by sGC and pGC on various cell functions. Stimulation of HEK-293 cells with atrial natriuretic peptide (ANP) but not exposure to NO donors triggers redistribution of PKG to the plasma membrane (Airhart et al 2003). In cultured vascular smooth cells (VSMCs), stimulation with ANP activated CNG channels more readily than the NO-donor S-nitroso-N-acetylpenicellamine (SNAP) (Piggott et al 2006). In a human

epithelial cell line, Zolle and colleagues found that the pGC and the sGC modulated $[Ca^{2+}]_c$ through different Ca^{2+} homeostatic mechanisms. Indeed, stimulation of the particulate but not the soluble GC leads to the inhibition of Ca^{2+} extrusion (Zolle et al 2000). Differences between pGC and sGC activation have also been reported in cardiac preparations. In mouse ventricular myocytes the cGMP generated by the CNP-pGC pathway mainly decreased Ca^{2+} levels, whereas cGMP produced by the NO-sGC mainly decreases Ca^{2+} sensitivity (Su et al 2005). In frog ventricular myocytes, sGC activation causes a pronounced inhibition of the L-type Ca^{2+} current ($I_{Ca,L}$) on stimulation by intracellular cAMP, whereas pGC activation has little effect (Gisbert & Fischmeister 1988; Mery et al 1993). These data suggest that cGMP signals are functionally localized into different subcellular compartments.

The first direct evidence for intracellular cGMP compartmentation came from the study of Castro *et al.* performed in intact adult cardiac myocytes (Castro et al 2006). Subsarcolemmal cGMP signals were monitored in real time by expressing the rat olfactory cyclic nucleotide–gated (CNG) channel α-subunit and by recording the associated cGMP-gated current (I_{CNG}). These studies demonstrated that PDEs restrict cGMP diffusion and are therefore important players in the compartmentalization mechanism. Different isoforms of PDEs shape the two distinct cGMP pools generated by natiruretic pepetides and NO donors. In particular, PDE2 controls both particulate and soluble pools of cGMP, whereas PDE5 specifically controls the soluble pool of cGMP. Differential spatiotemporal distributions of cGMP may therefore contribute to the specific effects of natriuretic peptides and NO donors on cardiac function.

3.9 Compartimentalization of PKG

The first cGMP-dependent protein kinase anchoring proteins (GKAPs) have been described by Vo and colleagues in 1998. By using a protein overlay approach, they identified numerous proteins able to bind PKG type II in rat aorta, brain and intestine. Among these, myosin has been shown to be a ubiquitous GKAP. Deletion mutagenesis studies identified the regions of PKG that were required for GKAP interaction. The

anchoring domain has been shown to localize at the N-terminus of PKG type II between residues 27 and 60 (Vo et al 1998). The dimerization docking domain of PKG has also been mapped near this region, suggesting a structural organization very similar to that of PKA (Atkinson et al 1991; Takio et al 1984).

In another study, a genetic yeast-2-hybrid screen identified troponin T as a PKG binding protein and determined that the interaction was depended on a leucine zipper motif (Yuasa et al 1999). Troponin T is not a substrate of PKG, however, its interaction with PKG allows phosphorylation of troponin I. Troponin I, troponin T and troponin C constitute the thin filaments of the sarcomere. These findings suggest that PKG anchoring to the contractile machinery may be important for the regulation of contraction. Another important cardiac target of PKG is the natriuretic peptide A receptor (Airhart et al 2003). Activation of the receptor by ANP recruits PKG from the cytosol; binding of PKG and phosphorylation of the receptor enhances the intrinsic cyclase activity thereby enhancing cGMP production. The NPRA-PKG association may represent a novel mechanism for compartmentalization of cGMP-mediated signalling and regulation of receptor sensitivity. Finally, in rat aorta endothelium a complex consisting of sGC, PKA, PKG and Caveolin-1 has been isolated. Disruption of the complex impaired relaxation stimulated by NO (Linder et al 2005).

3.10 cAMP and cGMP signalling crosstalk

The cAMP and cGMP signalling pathways are tightly interconnected. A first level of crosstalk lays in the opposing effect of PKA and PKG –mediated phosphorylation of target proteins. For example, phosphorylation of L-type Ca^{2+} channels by PKA has a stimulatory effect; on the contrary, phosphorylation by PKG seems to have and inhibitory effect on I_{Ca} (Ruiz-Velasco et al 1998). At a second level, interaction between these two pathways occurs via cGMP-mediated regulation of cAMP-degrading PDEs. In the case of PDE2, this regulation involves the allosteric stimulation of cAMP hydrolysis by cGMP. For PDE3 cGMP acts as a competitive inhibitor of cAMP hydrolysis.

The cGMP binding affinities for PDE2 and PDE3 vary by two orders of magnitude. Therefore, it is possible to expect that the impact of cGMP on cAMP-hydrolizing PDEs depends on the intracellular concentration of cGMP. At concentrations <50 nmol/L cGMP will exclusively inhibit PDE3, whereas at concentrations between 200 and 500 nmol/L cGMP will also activate PDE2. This mechanism could explain the biphasic behavior of cGMP in several cellular processes. For example, in human atrial myocytes cGMP was found to regulate I_{ca} by modulating cAMP levels. While perfusion with low concentrations of cGMP stimulated I_{Ca} presumably by inhibiting PDE3, perfusion with high cGMP concentrations led to inhibition of I_{Ca} probably through PDE2 stimulation (Mery et al 1993). In another study, dose-dependent modulation of cAMP levels resulted in an opposite effect on thrombin-induced endothelial permeability. Low concentrations of cGMP inhibited endothelial permeability via PDE3 inhibition. On the contrary, high cGMP concentrations increased endothelial permeability via PDE2 activation (Surapisitchat et al 2007).

As a consequence of this cross-talk mechanism, cAMP hydrolyzing PDEs can serve as targets for cGMP mediated signalling and some of the effects of cGMP may result from effects on the cAMP pathway. Increasing evidence indicates that the modulation of PDE2 and PDE3 by cGMP is of physiological and pathophysiological importance in a variety of cell types. In endothelial cells the effect of cGMP on endothelial permeability is dependent on the cGMP concentration present and on the relative expression levels of PDE2 and PDE3 (Surapisitchat et al 2007). In glomerulosa cells of bovine adrenal cortex ANP reduces aldosteron secretion through PDE2 activation (MacFarland et al 1991). In frog ventricular cells, PDE2 has been suggested to mediate the inhibitory effect of cGMP on cAMP-stimulated Ca^{2+}current (Mery et al 1995). In human platelets, activation of PDE2 by sodium niroprusside has been shown to limit cAMP accumulation, thus inhibiting thrombin-induced aggregation (Dickinson et al 1997). In adult rat cardiac fibroblasts, NO attenuates cAMP accumulation via sGC–cGMP-PDE2 stimulation (Gustafsson & Brunton 2002). cGMP has been shown to be involved in the trafficking of NKCC2 ($Na^+/K^+/2Cl^-$ contransporter) at the apical membrane of the thick

ascending limb (THALs) cells (cells extracted from the outer medulla of rat kidneys). The mechanism has not be fully elucidated yet, but it seems that increases in cGMP and activation of PDE2 reduce cAMP-mediated exocytosis of the contrasporter (Ares et al 2008). cGMP has also been shown to regulate cAMP and meiosis in the mouse oocytes trough PDE3 inhibition (Norris et al 2009).

An example of how intricated cAMP and cGMP signalling can be is the multiple and sometimes contradictory actions reported for NO in the heart. NO modulates several aspects of physiological myocardial function, excitation-contraction coupling, myocardial relaxation, heart rate and β-adrenergic inotropic response. However, the intracellular signalling mechanisms responsible for these effects remain poorly understood. NO can exert its functions both trough cGMP-indipendent and cGMP-dependent mechanisms (Vila-Petroff et al 1999). cGMP-indipendent mechanisms include stimulation of adenylyl cyclases and alteration of Ca^{2+} fluxes by nytrosilation of L-type Ca^{2+} channels (Vila-Petroff et al 1999), ryanodine receptor (Ziolo et al 2001) or phospholamban (Kohr et al 2008). cGMP-mediated effects comprise either activation of PKG or modulation of PDEs activity. For example, it has been reported that while low concentrations of NO donors increase myocardial contractility, high concentrations of NO exert a negative inotropic effect. It has been proposed that low levels of NO lead to cGMP-mediated inhibition of PDE3. This results in an enhancement of cAMP levels, activation of PKA and achievement of positive inotropic effect, via stimulation of Ca^{2+} current (Mery et al 1993). On the contrary, the negative inotropic effect of high NO donors concentrations has been largely attributed to a cGMP-mediated reduction in myofilament Ca^{2+} responsiveness, possibly via activation of PKG (Shah 1996; Vila-Petroff et al 1999; Wegener et al 2002). However, the mechanism responsible for reduced myofilaments Ca^{2+} is still poorly understood. PKG phosphorylation of troponin I may mediate the same offect of PKA phosphorylation, leading to an increase of the troponin C-Ca^{2+} off rate (Robertson et al 1982). There is also some evidence that PKG can suppress basal and PKA-stimulated Ca^{2+} currents (Matsumoto et al 2000; Mery et al

1991). Finally, it has been recently reported that in adult rat cardiac myocytes isolated from failing hearts CNP (C-type natriuretic peptide) stimulation enhances β_1-AR mediated contractile response though inhibition of PDE3 (Qvigstad et al 2009).

As shown by the above examples, a large body of evidence supports the existence and the physiological relevance of a cAMP/cGMP signalling crosstalk. However, much still remains to be elucidated of the intricacy of such interaction. A better understanding of how cGMP and cAMP signalling pathways are interconnected appears to be particularly critical in the heart where these pathways are the target of drugs that are commonly used for the treatment of heart failure and ischemic heart disease, two of the most frequent heart pathologies. For instance, patients with heart failure may be treated simultaneously with β-blockers, to reduce cAMP levels, and nitrates that increase cGMP levels and thereby, through the cGMP-mediated modulation of PDEs, may in turn have an effect on cAMP levels. A detailed understanding of how cGMP impacts on cAMP signalling could be critical to reduce side effects and improve pharmacological treatments.

3.11 Real time detection of cyclic nucleotides

cAMP signalling is one of the most important and highly studied signal transduction pathway. Soon after its discovery in 1953, it has become clear that cAMP was involved in many other cellular processes than glycogen metabolism. Alterations in cAMP levels in fact, are responsible not only for metabolic reactions, but also for electrical, cytoskeletal, transcriptional and other responses. cAMP is generated by different hormones and neurotransmitters that impinge on the plasma membrane. How stimulation of different receptors that act via the same second messenger can elicit the appropriate cellular response has been an exciting area of study. However, methodologies to measure cAMP levels in single living cells have been lacking for a long time. The determination of cAMP levels has been mainly based on indirect measurements, for example recording of the activation of reactions dependent on cAMP concentration, or on radio-immuno assays (RIA). RIA have been introduced in the

late 60s and are based on detection of intracellular changes in cAMP using the competition between cAMP in the sample and exogenous labelled cAMP for binding to an anti-cAMP antibody (Steiner et al 1969). This approach is being gradually replaced by non-radioactive methods such as Fluorescence Polarisation (FP) (Prystay et al 2001), Homogeneous Time Resolved Fluorescence (HTRF) or AlphaScreen (Amplified Luminescence Proximity Homogeneous Assay) (Gabriel et al 2003). In these essays, like in the original RIA, cAMP present in the sample competes with a cAMP tracer (fluorescein labelled in the case of FP, biotinylated in AlphaScreen), resulting in a dose-dependent signal decrease. Thanks to their reliability and high sensitivity, these methods are still used as valid research tools. However, they show a big limitation that derives from the necessity of fixing and/or lysing the samples. As a consequence any information on the spatial organization of the cAMP signal is lost. Also the time resolution of these assays is rather poor. Antibody-based methods can only detect cAMP concentration in steady state conditions or changes within a time scale of minutes, but can not resolve faster dynamics of cAMP signalling. In addition, these technologies provide average information on total cAMP changes in a cell population whereas a study of cAMP fluctuations in single cells could be more physiologically relevant. Therefore, these methods are inappropriate to investigate highly dynamic or localized intracellular events mediated by cAMP.

A great step forward in the study of the temporal and spatial components of cAMP signal transduction came from the development of real time detection approaches to measure cAMP in intact living cells. We can basically single out two principal ways of measuring cAMP in intact living cells: biosensors based on cyclic nucleotide gated ion channels or biosensors based on Fluorescence Resonance Energy Transfer (FRET). These approaches have been optimized and constitute a valid tool for the study of signal transduction. The characteristics, advantages and limitations of these sensors will be discusses in the next paragraphs.

3.11.1 *Detection of cAMP in living cells*

3.11.1.1.1 Channel-based biosensors

One technique to monitor cAMP dynamics in intact living cells relays on the use of genetically modified cyclic nucleotides gated channels (CNG) as indirect cAMP biosensors. This method, developed by Rich and colleagues (Rich et al 2000; Rich et al 2001a; Rich et al 2001b), is based on transfection or adenoviral infection of wild type (wt) or mutants of the α subunit of rat olfactory CNG channel (CNGA2). CNGs are directly opened by cyclic nucleotides and their activation can be measured by electrophysiological recordings of Ca^{2+} currents or Ca^{2+} imaging with a Ca^{2+}-indicator (e.g.Fura-2). Wild-type CNG channels show several limitations. They exhibit a lower affinity for cAMP than for cGMP (Dhallan et al 1990; Rich et al 2000; Rich et al 2001a; Rich et al 2001b), they can be directly opened by nitric oxide (Broillet 2000) and the opening of these channels is strongly inhibited upon binding of the Ca^{2+}-calmodulin complex (Liu et al 1994) .

To overcome some of these negative aspects, the wt CNG channels have been mutagenized (C460W and E583M) to obtain a new sensor "channel Δ61–90/C460W/E583M" virtually insensitive to cGMP and sensitive to low cAMP concentrations. In addition, the regulation of the channel by Ca^{2+}-calmodulin has been removed by deletion of residues 61–90 (Rich et al 2001b).

Notwistanding these improvements this detection method still presents some limitations. Changes in cAMP concentrations can be detected only at the plasma membrane and it is not possible to compare changes at the plasma membrane with changes in other cellular districts. In addition, overexpression of the channel and consequent influx of Ca^{2+} may affect the intracellular concentrations of cAMP by acting on Ca^{2+}-sensitive ACs or PDEs. As a consequence, the cAMP concentration that is detected by the sensor may not be physiological.

3.11.1.1.2 FRET-based biosensors

A more direct approach to monitor cAMP in single living cells is based on fluorescent dyes or proteins, Fluorescence resonance energy transfer (FRET) (Box1), and imaging microscopy. Fluorescence resonance energy transfer is a technique half a century old. However, thanks to the development of new fluorescent dyes and also new optical methods and instrumentation with higher spatial resolution, it has found novel biological applications. A strong contribution to the development of FRET based approaches has come from the molecular cloning (Prasher et al 1992) and subsequent

BOX1. Fluorescence resonance energy transfer (FRET)

FRET relies on a non radiative, distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore. The donor fluorophore is excited by incident light, and if an acceptor is in close proximity, the excited state energy from the donor can be transferred. As a consequence, donor's fluorescence intensity and excited state lifetime are reduced, and the acceptor's emission intensity is increased. For FRET to occur, the donor-acceptor distance must be comprised between 1 and 10nm, the two fluorophores must be appropriately oriented in space, and there must be a substantial overlap (at least 30%) between the donor's emission spectrum and the acceptor's excitation spectrum (Clegg 1996; Lakowicz 1999). The efficiency of this process (E) depends on the donor-to-acceptor separation distance r with an inverse 6th power law: $E=R_0^6/(R_0^6+r^6)$ where R_0 is the distance at which half of the energy is transferred, and depends on the spectral characteristics of the fluorophores and their relative orientation (Förster 1948). R_0 can be calculated using $R_0=9.78x10^3\ [Q_d\ K^2 n^{-4}\ J]^{1/6}$ Å, where Q_d is the quantum yield of the donor, n is the refractive index of the medium and is generally assumed to be 1.4 (range 1.33-1.6) for proteins, K^2 is the orientation factor and J is the overlap integral *(Van Der Meer et al 1994)*. The overlap integral J represents the degree of overlap between the donor fluorescence spectrum and the acceptor absorption spectrum and is given by $J=_0\int^{\infty} f_D(\lambda)\ \varepsilon_A(\lambda)\ \lambda^4\ d\lambda$, where λ is the wavelength of the light, $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor at that wavelength, and $f_D(\lambda)$ is the fluorescence spectrum of the donor normalised on the wavelength scale. The orientation factor is defined as $K^2=(\cos\theta_T-3\cos\theta_d\ \cos\ \theta_a)^2$ where θ_T is the angle between the donor emission transition moment and the acceptor absorption transition moment, θ_d and and θ_a are the angles between the donor-acceptor connection line and the donor emmission and the acceptor absorption transition moments, respectively. K^2 varies between 0 and 4. In the Förster equation K^2 assumes a numerical value of 2/3 provided that both probes can undergo unrestricted isotropic motion. According to the Förster's equation, doubling of the distance between the two fluorophores, for example, from R_0 to $2R_0$, decreases the efficiency of transfer from E=50% to E=1.5%. This makes FRET an exquisitely sensitive approach to measure conformational changes and protein-protein interactions.

engineering of the green fluorescence protein (GFP) from the bioluminescent jellyfish *Aequorea Victoria* (Tsien 1998).

GFP possesses several features that make it suitable for *in vivo* imaging. It can be expressed in many cell types and it does not need cofactors to become fluorescent, it can be fused to peptides or proteins and therefore targeted to distinct organelles or subcellular locales. In addition, mutagenesis of GFP has generated a lot of mutants with different spectral and physico-chemical characteristics, like photostability, resistance to pH and Cl^- ions (Griesbeck et al 2001; Nagai et al 2002; Tsien 1998).

One of the classic applications of FRET relies on the study of protein-protein interaction or interaction between protein domains. Recently, it has been exploited to generate cellular biosensors for second messengers like Ca^{2+} (Miyawaki 2003; Miyawaki et al 1997), IP_3 (Tanimura et al 2004), cAMP and cGMP (see below) or to monitor the activation of proteins, such G protein coupled receptor (Janetopoulos et al 2001) or PKA activation (Allen & Zhang 2006; Ni et al 2006; Zhang et al 2001).

The first FRET-based method to detect cAMP was developed by Adams and co-workers (Adams et al 1991). They generated a sensor based on PKA, named FlCRhR[1]. The regulatory (R) and catalytic (C) subunits of PKA were labelled with rhodamine and fluoresceine, respectively. In the absence of cAMP, R and C subunits are associated to form a tetramer (R_2C_2) and the two dyes are close enough for FRET to occur. When cAMP binds to the R subunits, the C subunits dissociate and FRET is impaired. Although this methodology allowed, for the first time, to image cAMP fluctuations in a living sample, it has not found a wide application owing to some limitations and technical difficulties. It requires the microinjection of a large amount of probe (μM concentrations) and the labelled subunits generate aggregates and interact with sub cellular structures (Goaillard et al 2001).

[1] pronounced “flicker”(Fl) Fluoresceine, (C) catalytic subunit, (Rh) rhodamine, (R) regulatory subunit

A great advance in the field of cAMP imaging came from the development of the first genetically encoded, FRET-based cAMP indicator, R-CFP/C-YFP (Zaccolo et al 2000). The α-catalytic subunit (Cα) and the RIIβ regulatory subunit (RIIβ) of PKA were fused to the yellow and cyan mutants of the green fluorescent protein, respectively (Figure 3-16). Also this sensor exploits the conformational change that occurs upon cAMP binding to the R subunits and FRET variations are used as a read out for the intracellular cAMP fluctuations. Being this sensor based on PKA it shows high selectivity and sensitivity for cAMP with EC_{50} = 0.3 μM (Mongillo et al 2004). In addition, the RIIβ subunit, via its dimerisation and docking domain, binds to endogenous AKAPs thereby localizing the sensor in specific compartments. By using the R-CFP/C-YFP sensor it has been possible to visualize, for the first time, microdomains of cAMP in cardiac myocytes and unravel the role of different PDEs in shaping intracellular cAMP gradients (Mongillo et al 2004; Zaccolo et al 2000).

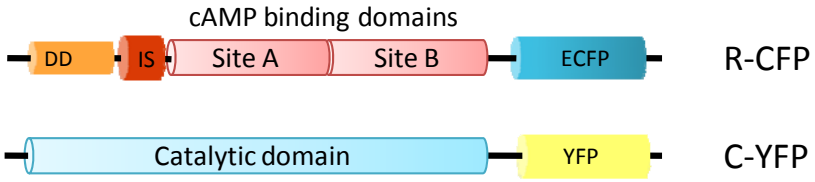


Figure 3-16 Schematic representation of PKA-based sensor.

Through the years, this sensor has been modified to achieve different characteristics, like different dynamic range, cAMP sensitivity or targeting to specific intracellular locales. In spite of these improvements, the R-CFP/C-YFP sensor still presents some limitations. First, since C and R are expressed from two distinct plasmids, it is difficult to predict if equimolar concentrations of C and R are present in transfected cells. Second, the cAMP-dependent dissociation of R and C subunits occurs trough a cooperative mechanism (Taylor et al 2005) and therefore the kinetics reported by the sensors may be slower than the actual kinetics of cAMP. Finally, the catalytic subunit is active and can phosphorylate downstream targets like PDEs or ACs thereby affecting cAMP levels.

To overcome some of the drawbacks of PKA-based biosensors, a new class of uni-molecular indicators based on Epac proteins have been developed. The first sensor of this class was generated by fusing the amino terminus of the Epac 1 protein to CFP and the C-terminus to YFP (DiPilato et al 2004; Nikolaev et al 2004; Ponsioen et al 2004). Since this sensor showed membrane localization, in particular to the nuclear envelope and to perinuclear compartments, the DEP domain (amino acids 1–148) was deleted in order to obtain a cytosolic probe. Additional mutations (T781A, F782A) were introduced to generate the sensor CFP–Epac (δDEP-CD)–YFP (also termed H30), which is catalytically inactive (Figure 3-17). This probe displays faster activation kinetics and an extended dynamic range but a lower affinity for cAMP than PKA (EC_{50} = 12.5 µM) limiting its application to systems characterized by large changes in cAMP concentrations. To better investigate subcellular dynamics of cAMP, Epac-based indicators have been genetically engineered to target them to selective subcellular compartments. Two new versions of the H30 probe have been developed to target the sensor to the plasma membrane (mp-H30) and to the nucleus (nls-H30) (Figure 3-17) (Terrin et al 2006). Imaging microscopy performed with these differently targeted probes has shown that contiguous intracellular domains with different cAMP concentration exist inside the cell.

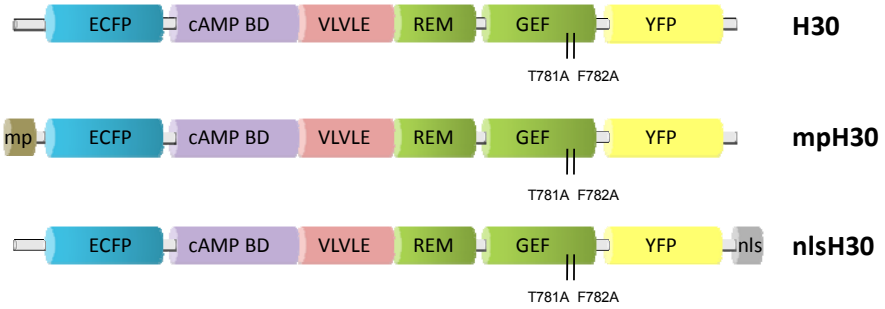


Figure 3-17 Schematic representation of epac-based sensors.

A few years ago, Nikolaev and colleagues generated a class of sensor with a simplified structure. These sensors are constituted by a single cAMP binding domain from either Epac 1 (Epac1-camps) (Figure 3-18), Epac 2 (Epac2-camps) or PKA (PKA-camps) that

have been sandwiched between CFP and YFP (Nikolaev et al 2004). Containing only one cAMP-binding domain, these probes lack cooperativity and display higher temporal resolution; in addition, they do not contain any catalytic or targeting domains that might interfere with intracellular regulatory processes.

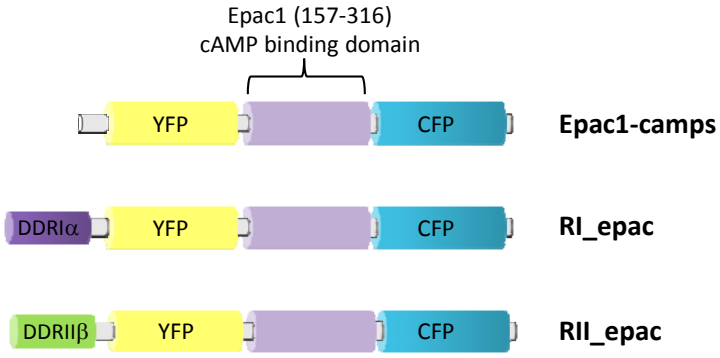


Figure 3-18 Structure of cAMP sensors based on the cAMP binding domain of Epac1.

Recentely, in our lab we have generated two new targeted sensors based on Epac1-camps, RI_epac and RII_epac by fusing at the N-terminus of Epac1-camps the dimerisation and docking domains of the PKA regulatory subunit RIα and RIIβ, respectively (Figure 3-18). In this way we have targeted the cAMP sensor to those subcellular compartments where PKA type I and PKA type II normally reside inside the cell. When expressed in cardiac myocytes these probes show a different distribution pattern, RI_epac shows a tight striated pattern overlaying with both the Z and the M sarcomeric lines whereas RII_epac shows a very strong localization that corresponds to the M line and a much weaker localization overlaying the Z line (Figure 3-19). The different localisation of the probes is due to the binding to specific endogenous AKAPs, mediated by the respective D/D domains (Di Benedetto et al 2008).

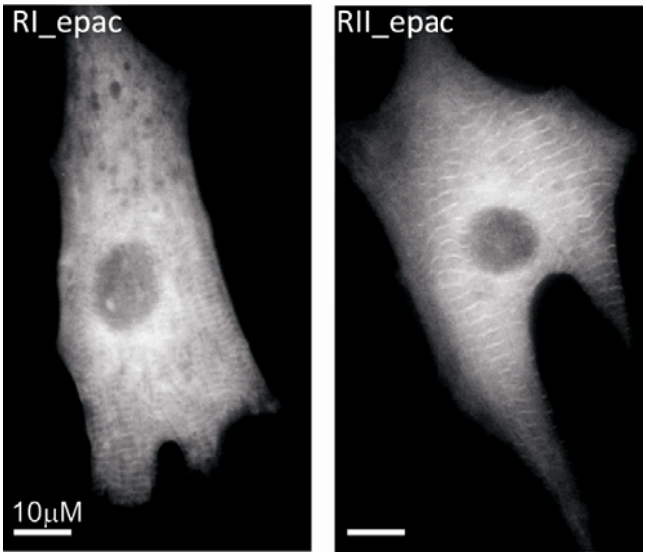


Figure 3-19 RNCMs expressing RI_epac and RII_epac.

Real time imaging preformed with these sensors allowed the identification of two independent subcellular compartments within which PKA type I and PKA type II are activated by discrete cAMP pools generated in a stimulus dependent manner. Isoproterenol (ISO) was found to generate a cAMP pool that selectively activates PKA type II whereas prostaglandin1 (PGE1) generated a cAMP signal that selectively activates PKA type I, revealing the existence of independent compartments where cAMP levels are uniquely regulated (Figure 3-20) (Di Benedetto et al 2008). Such cAMP pools are shaped by different subsets of PDEs that, by limiting diffusion of cAMP outside the boundaries of these compartments prevent cross activation of PKA isoforms. The generation of these distinct pools of cAMP also results in distinct downstream functional outcomes with ISO leading to the phosphorylation of PLB, TnI and β-AR, whereas PGE1 does not affect the phosphorylation level of these substrates.

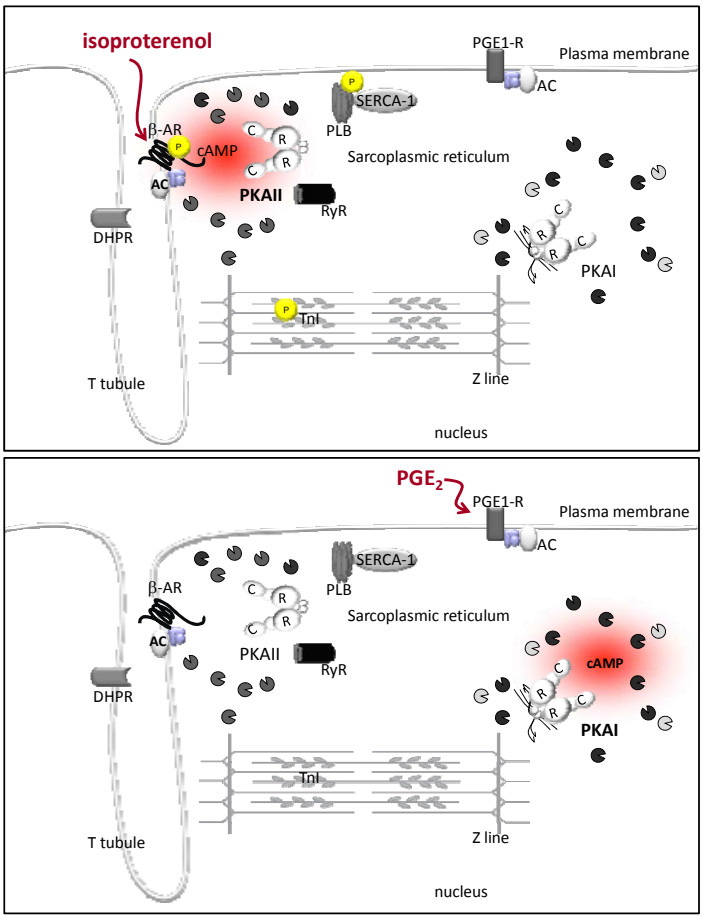


Figure 3-20 PKA type I and PKA type II define exclusive, stimulus coupled signalling domains.

3.11.2 *Detection of cGMP in living cells*

As for cAMP, the first methods to measure cGMP levels were based on biochemical essays, such as radio- or enzyme-immunoassay. These destructive techniques allow for quantitation of total cellular cGMP on a cell population, without providing any information on the concentration of free cytoplasmic cyclic nucleotide and ignoring any spatial localization (Leitman & Murad 1986). Barsony and colleagues tried to detect cGMP in fixed cells combining a microwave irradiation method to fix the samples and immune detection (Barsony & Marx 1990). Microwave fixation prevented some disadvantages of classical fixation methods (freezing or formaldehyde) like nucleotide loss from cells, nucleotide diffusion within cells, and chemical modification of

immunologic epitopes. Nonetheless, this method does not allow time-lapse experiments and precise quantification of intracellular cGMP levels.

3.11.2.1.1 Non FRET-based cGMPsensors

A possible way to measure cGMP levels in intact, living cells is to use genetically encoded cyclic nucleotide gate channels. The first real time detection of cGMP in living cells was reported by Trivedi and co-workers, in 1998 (Trivedi & Kramer 1998). They performed "patchcramming" experiments in neuroblastoma cells to monitor the effect of muscarinic agonists and NO on cGMP intracellular levels. As a detector for cGMP they used an exogenously expressed CNG channel engineered to be especially sensitive and selective for cGMP (Goulding et al., 1994). In "patchcramming" experiments (Kramer, 1990), an excised, inside-out membrane patch containing these channels is "crammed" into a receiver cell. The activity of these channels is monitored and reflects the concentration of free cGMP in the recipient cell. In 2000, Rich and colleagues developed the new technique for subsarcolemmal cAMP detection described above and based on patch clamp measurements of a CNG channel. In this case cells were transfected or infected with the wild-type (wtT) α-subunit of the rat olfactory cyclic nucleotide–channel (CNGA2) (Rich et al 2000). This channel binds cGMP with a 10-fold higher affinity than cAMP. However, while it is not appropriate for cAMP detection it can still be used as a real time sensor for cGMP. Others non-FRET sensors have been developed in 2008 by Nausch and colleagues. These sensors, named FlincGs (*fl*uorescent *in*dicators of *cG*MP), contain a single GFP-based fluorescent unit, the circular permutated GFP, fused at the N-terminus of regulatory domain fragments of PKG type I (Figure 3-21) (Nausch et al 2008). When cGMP binds to the cGMP binding domains, the sensor undergoes a conformational change that modifies the conformation of the GFP fluorophore. As a consequence when excited at 480 nm, an increase of the emission intensity at 510 nm is observed and the changes in fluorescence emission at 510 nm can be used as a read out of cGMP levels. Three variants of FlincGs have been engineered from PGK type I. α-FlincG contains the entire

regulatory domain of PKGIα, β–FlincG holds the entire regulatory domain of PKGIβ, whereas δ-FlincG consists of the regulatory domain of PKG Iα deleted of the entire N-terminal domain (Δ1-77). These sensors possess different K_D (35 nM, 1.1 µM, and 170 nM, respectively) and different cGMP/cAMP selectivity (1.140, 30, 280, respectively). The α and β -FlincG isoforms display a decrease in the maximal intensity change when expressed in vascular smooth cells (VSM), due probably to interactions the endogenous PKG through the N-terminal dimerization domain. δ-FlincG lacking this domain, in fact, did not show any reduction. In addition, the δ-FlincG sensor displayed a second, blue-shifted excitation peak at 410 nm that resulted in an emission peak at 508 nM. These two excitation wavelengths can be combined and analyzed ratiometrically (480/410 nm excitation), similar to the Ca^{2+} indicator Fura (Grynkiewicz et al 1985).

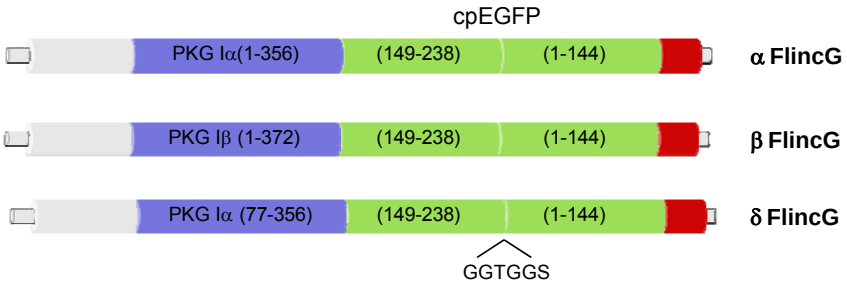


Figure 3-21 Structure of FlincG sensors.

3.11.2.1.2 FRET-based cGMP sensors

A first set of FRET-based sensors for cGMP was developed in 2000 by Sato and colleagues. In these sensors, called CGY, cGMP-dependent protein kinase Iα (PKG Iα) and different deletion mutants were fused with blue- and red-shifted green fluorescent proteins (GFPs) to their N- and C-termini, respectively. Among all the variants, only the PKG Iα-Δ1-47 construct (CGY-Del 1), in which the dimerization domain was deleted, showed an increase in FRET upon cGMP generation (Figure 3-22).

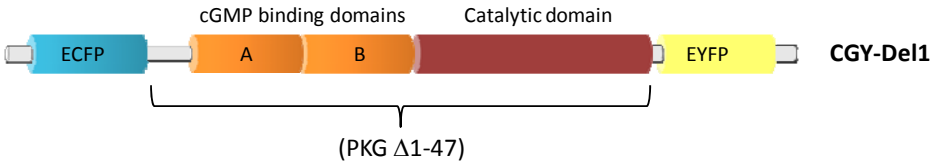


Figure 3-22 CGY-Del1 structure.

In the same periods, another group was developing cGMP sensor based on PKG. Honda and co-workers inserted the full length PKGIα and several deletion mutants between ECFP and EYFP or Citrine, a pH-insensitive variant of YFP (Honda et al 2001). The full length version did not display any FRET change upon cGMP generation. The C-terminus deletion mutants dispayed cGMP selective and dependent changes in FRET but the cGMP-induced ratio changes were irreversible, making them unsuitable for intracellular studies. Among the N-terminus deletion mutants, a Δ1-77 construct, yielded a functioning protein that responded to cGMP. This sensor was named cygnet-1 (*cy*clic *G*MP i*n*dicator using *e*nergy *t*ransfer) (Figure 3-23). This sensor has been further modified to silence the catalytic activity by the introduction of the T516A, leding to the generation of cygnet-2 (Figure 3-23). Later, the substitution of EYFP with Citrine generated the sensor cygnet-2.1 (Figure 3-23). The use of CGY and Cygnet sensors, however, is limited by low specificity (in the case of CGY), a relatively small dynamic range and relatively low temporal resolution (Honda et al 2001).

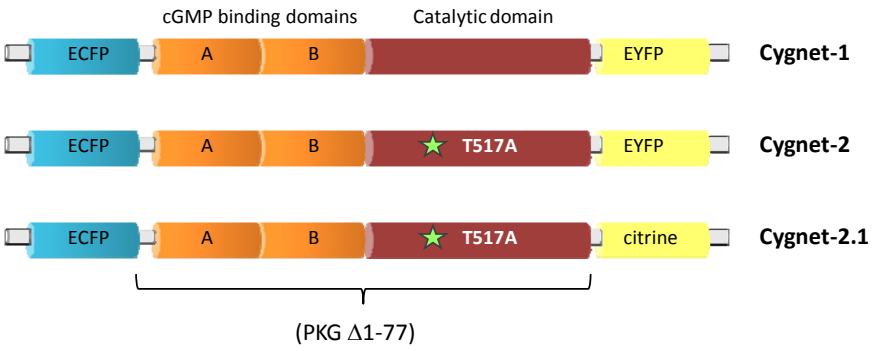


Figure 3-23 Structure of Cygnet sensors.

Simpler sensors were engineered by Nikolaev and colleagues. Rather than using entire proteins with all functional domains, they sandwiched only cGMP binding domains between CFP an YFP. They used the cGMP binding domain B from PKG (cGES-GKIB), the regulatory GAFB domain from PDE2A (cGES-DE2) and the regulatory GAFA domain from PDE5A (cGES-DE5) (Figure 3-24) (Nikolaev et al 2006).

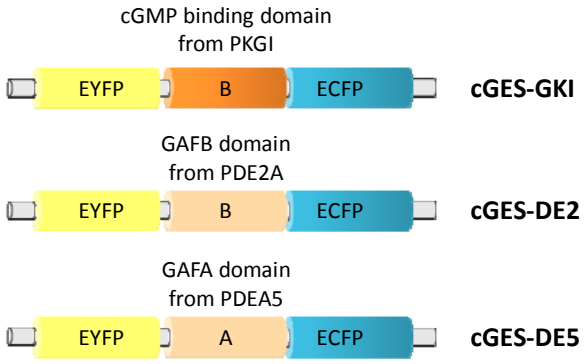


Figure 3-24 Structure of cGES sensors.

They compared these new sensors with previous CGY and cygnet-2.1 in terms of selectivity, sensitivity and kinetic properties (Table 2). cGES-GKIB responded to cGMP with a decrease in FRET whereas cGES-DE2 and cGES-DE5 responded with an increase in FRET signal. In addition, the response of the PDE based constructs was twofold greater than the one obtained with CGY-Del 1 and cygnet-2.1 sensors. The CGY sensors displayed high affinity for cGMP (20 nM), all the others sensors showed affinities in the micromolar range. The most selective sensor resulted cygnet-2.1 followed by cGES-DE2 and c-GES-DE5, cGES-GKIB and CGY-Del1 instead have moderate selectivity. Finally they compared the kinetic properties of these sensors in response to isoproterenol in HEK293 transfected cells. FRET changes were rather slow in cGES-GKIB, CGY and cygnet-2.1 whereas they were fairly rapid in PDE based sensors. Finally, they compared kinetic properties of cGES-DE5 and cygnet-2.1. They reasoned that only these two probes had reasonable selectivity for faithfully reporting cGMP signals. They expressed these two sensors in HEK293 cells and provided SNP and showed that cygnet-2.1 responded slowly, with a delay of about 10 seconds. In addition, its final response was

twofold smaller than the one registered with cGES-DE5. Furthermore, when cells were stimulated with pulses of SNP at subsaturating concentration only the cGES-DE5 sensor detected very rapid fluctuations of cGMP. From this study they concluded that cGES-DE5 is the best performing probe for measuring intracellular dynamics of cGMP.

Sensor	Backbone	EC_{50} cGMP	EC_{50} cAMP	Selectivity (cGMP/cAMP)	FRET response (CFP/YFP)
CGY-Del1	Truncated GKI	20.0 ±1.8nM	152.3 ±16.8 nM	7.6	↓
cygnet-2.1	Truncated GKI	1.7 ±0.2 mM	>1000 mM	>600	↑
cGES-GKIB	GKI-B domain	5.0 ±1.2 mM	485 ±82 mM	97	↑
cGES-DE2	PDE2A GAF-B domain	0.9 ±0.1 mM	115 ±17 mM	128	↓
cGES-DE5	PDE5A GAF-A domain	1.5 ±0.2 mM	630 ±100 mM	420	↓

Table 2 Comparison of cAMP versus cGMP selectivity of different cGMP sensors *in vitro*, adapted from (Nikolaev et al 2006).

3.12 Aims of the thesis

A large body of evidence indicates that both cAMP and cGMP are generated in a compartmentalized manner, with distinct pools of cyclic nucleotides coexisting inside the cell. There is also convincing evidence that an extensive crosstalk between these two signalling pathways exists inside the cell and that a mechanism responsible for such interplay may be the cGMP-mediated modulation of cAMP degrading PDEs. It is therefore possible to predict the existence of multiple independent signalling units in which distinct pools of cGMP can selectively affect specific cAMP hydrolyzing PDEs and the associated, discrete cAMP pools.

The aim of my project was to study the interplay between cAMP and cGMP signalling pathways in neonatal cardiac myocytes and to establish if such interplay occurs in a compartment-selective manner. I used a FRET-based, real time imaging approach to determine the dynamics cAMP and cGMP in intact, living myocytes and I have investigated the role that PDEs play in such crosstalk, focusing in particular on the β-adrenergic mediated response.

4 EXPERIMENTAL PROCEDURES

4.1 CELLULAR BIOLOGY

4.1.1 *Cardiac myocytes' culture: isolation protocol and transfection*

Isolation Protocol

Primary cultured cardiac ventricular myocytes were isolated from hearts of 1- to 3-days old Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) with the following protocol (this protocol has been optimized for 12-13 hearts).

Day 1

Before starting the isolation protocol prepare:

ADS 1X from 10X stock (500 mL)

Digestion mix

Medium 1st day (M1) (200 mL needed)

Scissor for cervical dislocation

Small straight scissor (to cut sternum)

Small curved scissors (to mince hearts)

Two small Petri dishes (pre-cooled at -20°C)

Put some ADS into each Petri dish and keep ice cold.

Thaw aliquots of laminin on ice.

Laminin coating

Sterilize and clean high quality 24 mm cover glasses (VWR International, Cat No 6310161) with an ether-ethanol solution (1:1), mount them on 6 multi-well TC plates and let them dry.

Prepare a solution of laminin in medium without serum (20 µg/mL) (see 4.2). Coat each cover slip with 250 µL of laminin, leave in the incubator at 37°C for at least 90 minutes. Discard the laminin solution by aspiration before seeding the cells.

Step 1: hearts extraction and processing

Sacrifice the rats by cervical dislocation. Remove rapidly the hearts and collect them in a 50 mL tube with 5 mL ADS.

Aspirate the ADS and transfer all the hearts in one small Petri dish supplied with ice-cold buffer ADS. Wash out blood from hearts by pushing gently on surface, aspirate ADS and add fresh one.

With straight scissors cut and discard atrium and aorta and place ventricles in the second Petri dish. Mince hearts in small pieces (about 1 mm^3) with curved scissors[2].

Collect the pieces in 1 mL portions with a 5 ml pipette into a 50 ml sterile tube. Aspirate the heart debris and the supernatant.

Add 9 ml of the Digestion mix. Put a tiny magnetic stirrer in the tube.

Incubate at 37°C for 5 min, constantly but gently stirring.

Discard the supernatant[3].

Step 2: digestion

Add 9 ml of the Digestion mix.

Incubate at 37°C for 20 minutes, with gently stirring[4].

Collect the supernatant[5] and transfer it in a 50 mL tube.

Add 1 ml NCS.

---

[2] Make sure that all the former operations are performed at 4°C as fast as possible to avoid hearts undergo ischemia

[3] This first digestion step serves to get rid of most of the pericardial collagen. This is the only time in which the supernatant is discarded.

[4] During the first 20-min digestion step, prepare the laminin-coated coverslips (see protocol).

[5] Cells are in the supernatant, the pellet contains undigested tissue.

The remaining pellet is processed again starting from step 1.

Spin at 1250 rpm, 5 minutes, without centrifuge's brake.

Discard the supernatant. Cells are now in the pellet.

Resuspend the pellet in 2 ml NCS and put the tube in the incubator. Leave cap loosen to consent O_2/CO_2 exchange.

All the digestions will be then collected in a 50 mL tube for the subsequent preplating[6].

Step 3: preplating

Gather up all cells collected in the previous steps and spin at 1250 rpm for 5 minute without centrifuge's brake.

Resuspend the pellet in Medium 1st day (M1) (10 ml every 5 hearts).

Plate the suspension onto 10 cm-cell culture dishes (one dish every 5 hearts).

Incubate at 37°C for about 2 hours[7].

Collect the supernatant from the dishes.

Spin at 1250 rpm for 5 minutes without centrifuge's brake.

Resuspend the pellet in 20 mL of medium M1.

Dilute the suspension 1:10 in M1.

Count the cells on a Thoma hemocytometer. Score only well-rounded bigger cells, with a well-defined dark membrane and a clear cytoplasm at the phase contrast.

Plate 300000 cells/ml on the laminin-coated coverslips.

Incubate at 37°C.

Day 2

Wash cells with ADS three times. Shake gently to remove dead cells.

Change medium to Medium 2nd day (M2).

[6] Steps 1 to 6 have to be repeated until all the tissue is digested (usually 4 times).

[7] This step induces fibroblasts and endothelial cells adhere to the dish while myocytes will be retained in suspension.

Incubate the cells for at least 2 hours in the incubator before using them for physiology or transfection.

From this moment onwards, change medium every second day.

Transfection protocol

Isolated cardiomyocytes were transfected with TransFectin™ Lipid Reagent (BIO-RAD 170-3351); the protocol has been optimized as follow:

Make two mixes (scale up quantities as required):

Solution A: 250 μL of MEM 199 and 2.5 μg DNA

Solution B: 250 μL of MEM 199 and 7 μl TransFectin™

Keep the two solutions at room temperature for 5 minutes.

The two solutions are then mixed and incubated at room temperature for 20 minutes. Add 500 μl of the mixed solution drop wise to the wells.

4.1.2 *CHO culture and transfection*

Chinese Hamster Ovary cells were grown in HAM F-12 supplemented with 10% FBS, and 50 U/mL Penicillin, and 50 μg/mL streptomicin, and 2mM glutamine.

CHO thawing

Thaw the cryovial in a water bath at 37°C. Transfer the contents of the thawed vial into a 10 ml centrifuge tube and then add dropwise, 9 ml of warm medium under continuous swirling. Centrifuge the cells' suspension at 1200 rpm for 5 minutes. Resuspend the pellet in 5 mL of medium and transfer it into a T25 flask. Incubate at 37°C, 5% CO_2.

CHO freezing

Change the medium of the cells the day before freezing the cells and freeze them at confluency of 80%. Wash the cells 3 times with PBS. Trypsinize the cells with 1 mL of 0.05% trypsin-EDTA and spin them down at 1250 rpm for 5 minutes. Resuspend them

with 0.75 mL of ice-cold FBS. Transfer the suspension into cryovials which have been pre-chilled in ice. Add dropwise 0.25 mL of HAM F-12 supplemented with 120 g/L glucose and 40% DMSO. Place the vials in the -70°C freezer overnight, for slow cooling, and then transfer them into liquid nitrogen.

CHO transfection

CHO were transfected with TransIT®Transfection Reagent, MIRUS (MIR 2300) as follow (scale up quantities as required):

Prepare a mix of medium w/o serum (100 μL) and transfection reagent (6 μL).
Incubate at room temperature for 5 minutes.
Add DNA (2-2.5 μg).
Incubate at room temperature for 20 minute.
Add the mix dropwise to the wells (~110 μl).

4.2 MEDIA, BUFFERS AND SUBSTANCES FOR CELLULAR BIOLOGY

ADS 10X		(Doses for 250 ml)
NaCl	1060 mM	15.4 g
Hepes	200 mM	11.9 g
NaH_2PO_4	8 mM	0.284 g
KCl	53 mM	0.988 g
$MgSO_4$	4 mM	0.247 g
Glucose	50 mM	2.253 g

pH 7.4

Digestion mix

The digestion mix, 0.45 g/L Collagenase and 1.25 g/L of Pancreatin, should be made fresh before the culture.

For 50 mL of digestion mix weigh 22.5 mg of Collagenase and dissolve in ADS, add 162.5 mg of Pancreatin and mix; filter through 0.22 μm filter. Keep ice-cold.

Medium 1st Day (M1)[8] doses for 100 mL

D-Mem 25mM Hepes	67.1%	67.1 mL
M-199	16.8%	16.8 mL
Horse serum (HS)	10%	10 mL
Newborn Calf Serum (NCS)	5%	5 mL
Glutamine 200 mM	1%	1 mL
Pen/strep	0.1%	0.1 mL

Medium 2nd Day (M2) doses for 100 mL

D-Mem 25mM Hepes	74.7%	74.7 mL
M-199	18.7%	18.7 mL
Horse serum (HS)	5%	5 mL
Newborn Calf Serum (NCS)	0.5%	0.5 mL
Glutamine 200 mM	1%	1 mL
Pen/strep	0.1%	0.1 mL

Preparation of laminin-coated coverslips:

Laminin is diluted 1:10 in sterile water and aliquots (100 μg/mL) are kept at -80°C.

Let laminin aliquots thaw slowly on ice.

[8] It is preferable if media are prepared fresh

Dilute 1:5 in serum-free medium (e.g. M199).

Coat each coverslip with 250-300 µl of laminin (final concentration 20 µg/mL).

Incubate at 37°C for at least 1 hour.

Remove excess laminin.

Media and enzymes

Phosphate-Buffered Saline (PBS, Cat.n° 10010031), Ham F12 (Cat.n°31765092), DMEM High Glucose (Cat.n°42430025), MEM199 (Cat.n°31150022), Horse Serum (Cat.n°26050088), New Calf born Serum (Cat.n°26170043), Penicilline/Streptomicine (10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)/ml, Cat.n°15140122) and Glutamine (Cat.n°25030024) are from Invitrogen.

Collagenase (Cat.n°10103586001) is from Roche, Pancreatin (Cat. n°1750) is from Sigma

Laminin (mouse) is from BD Biosciences (Cat.n°354232).

4.3 MOLECULAR BIOLOGY

4.3.1 *Generation of competent cells*

With the following protocol it is possible to obtain competent bacteria with transformation efficiency of $5x10^6$ - $2x10^7$ transformants/µg DNA.

Pick a single colony from a freshly streaked selective plate of One Shot TOP10®Competent Cells (Invitrogen) and inoculate a starter culture of 3 ml of sterile LB medium. Do not add antibiotic, since these cells do not have a resistance plasmid in them.

Grow the cells on a shaker at 37°C until they reach an OD (600 nm) of 0.3 to 0.4 (1 cm path length). Divide the bacterial culture in pre-cooled 50 mL tube and keep on ice for 10 minutes. Pellet the bacteria at 4.000 rpm for 10 minutes at 4°C. Cool 1 mM $CaCl_2$ and 1 mM $CaCl_2$ in glycerol 10% w/v solutions.

Discard supernatant. Gently resuspend each bacteria pellet in 10 mL of ice-cold $CaCl_2$ 0.1 M, work on ice, preferably in the cold room. Centrifuge the cell suspension at 4,000 rpm for 10 minutes. Discard the supernatant.

Resuspend the pellet with 2mL of ice-cold $CaCl_2$ 0.1M in glycerol 10% w/v.

Dispense in 100 μL aliquots and snap freeze cells in liquid nitrogen.

Store at -80°C.

4.3.2 *Bacteria transformation*

Competent cells were transformed with the following protocol:

Take an aliquot of competent cells and thaw it on ice.

Add 2-2.5 μg DNA to competent cells without pipetting.

Incubate on ice for 20 minutes.

Heat shock at 42°C for 30 seconds and incubate on ice for 1-2 minutes.

Add 250 μL of LB and incubate the suspension at 37°C for 45 minutes.

Spread 100 –150 μl of the bacterial suspension onto a pre-warmed LB plate with the required antibiotic for selection.

Allow the plate to dry and then incubate it overnight at 37°C.

4.3.3 *Cloning*

DNA purification

The DNA has been purified from bacteria culture using Hispeed Plasmid Maxi Kit from QIAgen (Cat. No. 12163), following the manufacturer's instructions.

DNA quantification

DNA concentration has been determined using the Biorad Spectrophotometer, measuring the absorbance of the sample at 260 nm; the amount of DNA has been quantified using the formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{OD_{260} \times \text{ dilution factor } \times 50\ \mu g/ml}{1000}$$

The ratio of OD_{260}/OD_{280} has been used to assess the purity of the sample[9].

PCR and DNA purification

PCR products and digested DNA were purified using QIAquick PCR Purification Kit (Cat. No. 28106, QIAgen) and QIAquick Gel Extraction Kit (Cat. No. 28706, QIAgen) following manufacterure's instructions.

Restriction enzymes and Ligation

All restriction enzyme were purchased by New England Biolabs, for ligation reactions, Rapid DNA Ligation Kit Cat. No. 11 635 379 001, from Roche, was used.

4.3.3.1.1 Generation of FRET-Based constructs

Epac1-camps, RI_epac and RII epac

The Epac1-camps sensor for cAMP (Nikolaev et al 2004) was kindly provided by M.Lohse (Institute of Pharmacology and Toxicology, University of Wurzburg, Germany). RI_epac and RII_epac were generated by fusion to the N-terminus of Epac-1 of the sequence encoding for the dimerization-docking (DD) domain of RIα (64 aa) or RIIβ (49 aa), respectively. Between the DD domain and Epac-1, the 27 aa linker $A(EAAAK)_5A$ was inserted. Such a linker was chosen because it folds as a monomeric hydrophilic α-helix and is effective in separating heterofunctional domains in a fusion protein, avoiding inadequate interactions between the domains and allowing them to work independently

[9]A ratio between 1.8 and 2.0 denotes that the absorption in the UV range is due to nucleic acids; a ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers; a ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) it is advisable to re-precipitate the DNA.

Generation of RI_cGES-DE2 and RII_cGES-DE2

The construct cGES-DE2 (pcDNA3-EYFP-PDE2A2(murine)-ECFP) was a kind gift of Martin Lohse, Institute of Pharmacology and Toxicology, Wurzburg, Germany. RIα and RIIβ dimerization and docking domains (D/D) were amplified from RI_epac and RII_epac, respectively, and inserted it the HindIII site of the MCS of cGES-DE2 construct.

Primers to amplify RI

RI_NheI_for: CTAGCTAGCATGGAGTCTGGCCAGTACCGC

RI_DD_linker_rev: GAGAAGGAGGAGGCAAAACAGGAGGCCGCGGCGAA

Primers to amplify RII

CMV forward: CGCAAATGGGCGGTAGGCGTG

RIIb_DD_linker_rev: CCCAAGCTTGCTAGCATGAGCATCGAGATCCCCGC

Generation of RI_cygnet-2.1 and RII_cygnet-2.1

Cygnet-2.1 (pcDNA3.1-Cygnet-2.1) was gently supplied by Wolfgang Dostmann, Departments of Pharmacology and Molecular Physiology and Biophysics, University of Vermont, Burlington. RIα and RIIβ dimerization and docking domain (D/D) were amplified from RI_epac and RII_epac, respectively, and inserted between NheI and XhoI sites of the MCS of pcDNA3 cygnet-2.1 construct.

Primers to amplify RI:

RI_NheI_fwr: AAG CTT GCT AGC ATG GAG TCT

RI_HindII_XhoI_rev: CCC TCG AGC ATA AGC TTG GCG GC

Primers to amplify RII

RII_Hind_NheI_fwr: AAG CTT GCT AGC ATG AGC ATC

RII_HindII_XhoI_rev: CC CTC GAG AAG CTT GGC GGC TGC CTT

Generation of RI_AKAR3 and RII_AKAR3

The construct AKAR3 (pcDNA3 AKAR3) was kindly provided by Jin Zhang, Department of Pharmacology and Molecular Sciences, the Johns Hopkins University School of Medicine, Baltimore. RI_AKAR3 and RII_AKAR3 were generated subcloning RIα and RIIβ D/D in the HindIII site of the multiple cloning site of the original AKAR3.

4.3.3.1.2 Generation of PDE2A2wt-mRFPI and PDE2A2mut-mRFPI

Site diected mutagnesis of PDE2A2

PDE2A2 construct (pcDNA3.1-PDE2A2(mouse)) was a kind gift of Joseph Beavo, Department of Pharmacology, University of Washington Medical School, Seattle. The catalitycally dead mutant of PDE2A2 (PDE2A2mut-mRFPI) was generated introducing the D685A and D796A mutations in pcDNA3.1-PDE2A2 (mouse)

Primers to for D685A mutation

D685A_PDE2A2_fwd: CCTGCATGTGTCATGcCCTGGACCACAGAGGCGTTTATTTCCTG
CATGTGTCATGcCCTGGACCACAGAGGCACAAACAA

D685A_PDE2A2_rev: GCCTCTGTGGTCCAGGgCATGACACATGCAGGTTGTTTGTGCCTC
TGTGGTCCAGGgCATGACACATGCAGGAAATAAAC

Primers to for D796A mutation

D796A_PDE2A2_fwd: CCTCATGACCTCCTGTGcCCTCTCTGACCAGACGTGCCTCCTC
ATGACCTCCTGTGcCCTCTCTGACCAGACAAAGGG

D796A_PDE2A2_rev: GTCTGGTCAGAGAGGgCACAGGAGGTCATGAGGCCCTTTGTC
TGGTCAGAGAGGgCACAGGAGGTCATGAGGAGGCAC

Generation of PDE2A2wt-mRFPI

In the lab wild type and catalytically dead PDE2A2 were subcloned NheI at the N-terminus of mRFPI (pEYFP-C1-mRFPI).

Primers to amplify PDE2A2

NheI_PDE2A2_FRW: CGATCGGCTAGCATGGTCCTGGTGTTGC

NheI_PDE2A2_REV: TGATCGGCTAGCGCCCTCGAGGCTGCAG

4.4 WESTERN BLOTTING

Cell lysis

Cardiac myocytes were treated as indicated and washed twice with ice cold PBS (phosphate buffered saline) and cellular lysates were prepared with lysis buffer containing 25 mM Hepes, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 10% (v/v) glycerol and 1% (v/v) Triton X-100 and Complete TM EDTA-free protease inhibitor cocktail tablets (Roche).

Quantification

Protein concentration of lysates was quantified using the Bradford assay and all samples were equalized for protein concentration.

Preparation of samples

Samples were boiled at 75°C for 10 minutes in lithium dodecyl sulphate sample buffer (LDS, NuPAGE LDS Cat.n° NP00084) and 1,4-Dithiothreitol (DTT, 100 mM).

Type of gels and membranes

Proteins were separated by gel electrophoresis with NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm (NP0321BOX, Invitrogen), and transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore).

Blocking

Membranes were bloked with Protein-Free T20 (TBS) Blocking Buffer (37571, Thermo Scientific) for 1 hour, room temperature.

Primary incubation

The following antibodies were used to detect phospho-proteins and native proteins in lysates from control and treated cells: Phospho-Troponin I (cardiac) Ser23/24 (4004, Cell Singalling), Troponin I (4002, Cell Singalling).

Primary antibodies were diluted 1:1000 in TBS-Tween and incubated for 1 hour.

Secondary incubation

The secondary fluorescently labeled antibodies were rabbit anti-mouse conjugated with Alexa Fluor680 (A2157, Invitrogen) and goat anti-rabbit IgG conjugated with IRDye800CW (611732127, Rockland Immunochemicals).

Antibodies were diluted 1:10,000 in TBS-Tween and incubated for 1hour.

Detection

Western blot were detected using The Odyssey® Infrared Imaging System (LI-COR).

Analysis

Quantification was done using Image J software. Arbitrary phosphorylation units were calculated and results plotted against controls.

4.5 MEDIA, BUFFERS AND SUBSTANCES FOR WESTERN BLOTTING

Tris-Buffered Saline Tween-20 (TBST): 20 mM Tris pH 7.4, 500 mM NaCl, 0.5% tween 20

Running buffer: NuPAGE® MES SDS Running Buffer (NP0002, Invitrogen)

Transfer buffer: NuPAGE® Transfer Buffer with 10% methanol (NP0006, Invitrogen)

4.6 PDE ACTIVITY ASSAY PROTOCOL

Procedure

1. Label tubes in triplicate for each sample to be assayed - keep on ice (include blanks).
2. Thaw all stock solutions and prepare working solutions in appropriate buffers.
3. Switch on water baths (30°C and boiling).

4. Prepare sample(s) to be assayed in a total of 50µl Buffer A (20 mM Tris-Cl pH7.4). If doing inhibitor studies (i.e. dose-response) then make sample up to 40 µl and add 10 µl of appropriately diluted inhibitor. For blanks add 50 µl of Buffer A.

5. Add 50 µl oflabelled cAMP substrate mix to each tube (including to blanks) and vortex.

6. Incubate samples for 10 minutes at 30°C.

7. Boil for 2 minutes to terminate reaction.

8. Cool samples on ice for at least 15 minutes.

9. Add 25 µl of 1 mg/ml snake venom (i.e. 0.2 mg/ml final).

10. Incubate for 10 minutes at 30°C

11. Add 400 µl Dowex and vortex (keep dowex stirring! ensure thoroughly resuspended before adding).

12. Cool samples on ice for minimum 15 minutes.

13. Vortex tubes thoroughly then centrifuge at 13,000 rpm for 3 minutes.

14. Meanwhile add 1 ml scintillant to freshly labelled tubes.

15. Remove 150 µl of supernatant and add to appropriate tube with scintillant (at this stage prepare totals. For totals add 50 µl of cAMP mix to 1 ml scintillant - prepare 2 x totals tubes).

16. Vortex all tubes thoroughly.

17. Count on scintillation counter.

18. Analyse data.

4.7 REAGENTS FOR PDE ACTIVITY ASSAY

Buffer A : 20 mM Tris-Cl pH7.4.

Buffer B : 20 mM Tris-Cl pH7.4, 10 mM $MgCl_2$.

Dowex : stock is 1:1 mix of dowex:H_20. Prior to use add 2 parts of this mix to 1 part Ethanol (100%) (e.g. 2 ml dowex/ H_20 + 1 ml Ethanol).

Snake venom: 10 mg/ml stock in buffer A. Dilute to 1 mg/ml (in buffer A) prior to use.

cAMP mix: 2 µl of 'cold' + 3 µl of 'hot' 8-[3H]-labelled cAMP per 1ml of buffer B (i.e. 2 µl + 3 µl + 995 µl buffer).
'cold' unlabelled cAMP stock solution = 1 mM (will be 2 µM final)
'hot' 8-[3H]-labelled cAMP = 1 µCi/µl (will be 3 µCi/ml final)

Work out total volume(s) of cAMP mix, Snake venom, Dowex that will be required for whole assay (including pilot) and prepare in advance.

4.8 FRET IMAGING

Generally, a FRET-based cAMP indicator is composed by a cAMP sensor, which may consist of either two separate interacting protein domains or a single protein domain undergoing a conformational change upon cAMP binding, and a donor and an acceptor chromophore fused to the cAMP sensor. Changes in conformation or in the distance of interacting protein partners that occurs upon cAMP binding affect the distance or the orientation of the two fluorophores, and thus the efficiency of energy transfer. Variations in energy transfer efficiency, accordingly, correlate with changes in cAMP concentration.

During a FRET experiment cells expressing the FRET-sensor are excited at a wavelength of 430 nm and the CFP (480 nm) and YFP (545 nm) emissions are collected by the camera on the two adjacent halves of the camera chip.
Figure 4-1 illustrates the mechanism of action of Epac1-camps. In the unbound conformation of the probe, the two fluorophores are in close proximity and in proper orientation and FRET occurs. When CFP is excited at its proper wavelength (430 nm), part of its excited-state energy is transferred to YFP, which can emit at its own wavelength (545 nm). Both CFP and YFP emission wavelength can be detected. When cAMP binds to the probe a conformational change occurs, the two fluorophores move apart and FRET is reduced. Therefore, only CFP emission (480 nm) can be detected upon excitation of CFP whereas YFP emission (545 nm) decreases. FRET changes can be

measured as changes in the emission spectrum of the probe (emission CFP/emission YFP) upon illumination at a wavelength that excites selectively the donor CFP (430 nm).

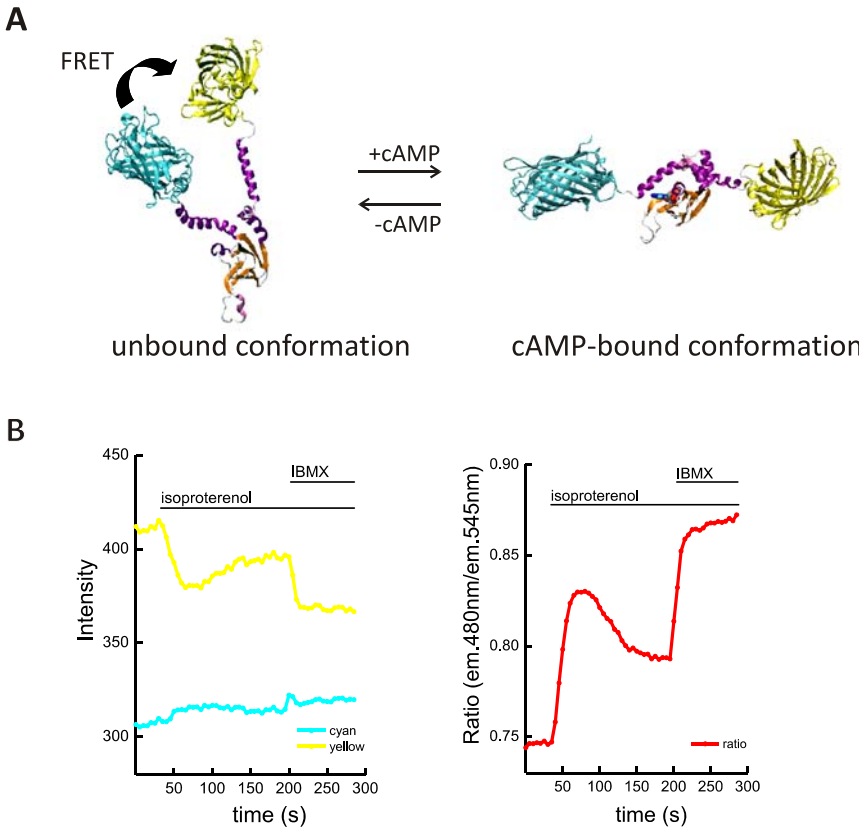


Figure 4-1 (A) FRET-based sensor for cAMP; (B) representative intensity plots of CFP and YFP emission (left) and CFP/YFP Ratio (right).

4.8.1 *Data processing*

Once the FRET experiment is completed the raw data stored in the computer memory will consist of two series of images, or stacks, of the analyzed cell along time. These two stacks represent the fluorescence intensity of CFP and YFP emission at each time point. Data are processed offline to check for alignment, drift of the focus or change in the position of the cell[10]. The analysis consists in alignment of CFP and YFP stacks in order to

[10] If the cell changes position during the experiment it is necessary to calculate the intensities and ratio values frame by frame, appropriately repositioning the ROI at every time point

optimally superimpose the images[11] and drawing of regions of interest (ROIs) on the cell and on the background for both CFP and YFP channels. At this point mean intensities values are calculated from the stacks and background is subtracted from both CFP and YFP channels. For each frame, the ratio (R) between CFP and YFP mean intensity is calculated.

The estimated change in cAMP concentration after a stimulus is expressed as $\Delta R/R_0\%$ where $\Delta R = Rt_2 - Rt_1$. Rt_1 corresponds to the average of at least 5 ratio values before the addition of the stimulus; Rt_2 corresponds to the average of at least 5 ratio values calculated at the plateau reached after the correspondent stimulus. R_0 corresponds to emission 480 nm/emission 545 nm at time t_0.

$$\frac{\Delta R}{R_0}\% = \frac{Rt_2 - Rt_1}{R_0} * 100$$

Ratiometric measurements correct for unequal probe distribution and, within certain limits, for bleaching occurring during the experiment and changes in focus. However, care must be taken when aligning the two channels (to avoid artefacts) and when interpreting ratio values. To assess whether the increases in ratio correspond to actual increases in cAMP plot CFP and YFP intensities over time and accurately analyze how fluorescence intensity changes in the two channels over time. Ratio changes can reliably be considered as FRET changes only when determined by a change in donor emission which is paralleled by an opposite change in acceptor emission (e.g. an increase in CFP emission must be paralleled by a decrease in YFP emission).

[11] A perfect alignment is very important in order to avoid artifacts in the ratio calculation

4.9 IMAGING SET UP

The basic setup for FRET imaging consists of a wide-field fluorescence microscope, a light source for excitation of CFP, a device to separate YFP and CFP emission signals, a digital camera to collect them, and a computer to store the images and analyze the data.

Exemplified below is the FRET imaging system we use in our laboratory:

1. **Microscope:** Olympus IX71 inverted microscope
2. **Illumination source:** Lambda LS Stand Alone Xenon Arc Lamp and Power Supply; Sutter Instrument (0661301)
3. **Neutral Density filters:** filters series 22000, filter set 22000A 25mm (N.D.: 0.2, 0.4, 0.6, 1.0, 1.5), Chroma Technology
4. **CFP illumination setting:** excitation filter ET436/20x, dichroic mirror T455LP, emission filter ET480/40m (Chroma Technology)
5. **YFP illumination setting:** excitation filter ET500/30x, dichroic mirror T515LP, emission filter ET535/30m (Chroma Technology)
6. **FRET illumination setting.** Filter cube: CFP excitation filter ET436/20x, dichroic mirror 455DCLP (all from Chroma Technology). This dichroic reflects the wavelengths below 455 nm to the sample therefore exciting CFP at 430 nm. It also allows the wavelengths above 455 nm to pass through, therefore permitting emission of CFP at 480 nm and YFP at 545 nm to reach the beam splitter. Beam Splitter: dichroic mirror 505DCLP, YFP emission 545 nm, CFP emission 480 nm (all from Chroma Technology). Light emitted by the sample comprises both CFP and YFP emission wavelengths. The dichroic mirror splits the emitted light in two beams. Wavelengths below 505 nm, which include CFP emission at 480 nm, are reflected and directed through a series of mirrors towards to the CFP emission filter. Wavelengths over 505 nm, which include YFP emission at 545 nm, pass through the dichoric and are directed to the YFP filter

7. **Objective:** Olympus PlanApoN, 60X, NA 1.42 oil, 0.17/FN 26.5
8. **Immersion oil:** Immersion oil "IMMERSOL" 518F , Carl Zeiss
9. **Beam splitter:** Dual-view simultaneous-imaging system (DV2 mag biosystem, Photometrics, ET-04-EM)
10. **Detector:** coolSNAP HQ monochrome camera system, Photometrics
11. **Shutter:** Lambda 10-3 optical filter changer with smartshutter, Sutter Instrument
12. **Computer:** Dell DE6700, 2.66 GHz Intel Core 2 Duo CPU, 3.50 GB RAM, 400 GB hard drive, Windows XP Professional version 2002
13. **Image acquisition and analysis software:** Meta imaging series 7.1, MetaFluor, Molecular Devices (www.moleculardevices.com)

4.10 MEDIA, BUFFERS AND SUBSTANCES FOR FRET

Cell bathing solution

NaCl	125 mM
KCl	5 mM
Na_3PO_4	1 mM
$MgSO_4$	1 mM
Hepes	20 mM
Glucose	5.5 mM
$CaCl_2$	1 mM

Adjust to pH 7.4

Stimuli

Atrial natriuretic peptide (A6791), isoproterenol (I2760), norepinephrine (N5785), cilostamide (C7971), rolipram (R6520), IBMX (I5879), and forskolin (F6886) are from Sigma-Aldrich. SNAP (82250), ODQ (81410) and L-NAME (80210) are from Cayman. Bay 60-7550 is from Alexis.

4.11 STATISTICS

Data are presented as average ± Standard Error. Non-parametric t-test was used to compare groups of experiments. Number of replicates is indicated in the text.

* $p<0.05$

** $0.05<p<0.01$

*** $p<0.001$

5 RESULTS

5.1 Intracellular dynamics of cGMP in cardiac myocytes

5.1.1 *Introduction*

The second messenger cyclic guanosine monophosphate (cGMP) is an important mediator of cardiac function. It is synthesised by two isoforms of guanylyl cyclase: the particulate GC (pGC), a membrane bound enzyme activated by natriuretic peptides (ANP, BNP and CNP) and the soluble GC (sGC) that is cytosolic and is stimulated by nitric oxide (NO). Both natriuretic peptides and NO donors stimulate the production of cGMP, nonetheless, they exert different effects on cardiac and vascular smooth muscle function. For instance, in mouse ventricular myocytes activation of the pGC by CNP decreases Ca^{2+} levels, whereas activation of the sGC by NO decreases Ca^{2+} sensitivity (Su et al 2005). In frog ventricular myocytes, sGC activation causes a pronounced inhibition of the L-type Ca^{2+} current ($I_{Ca,L}$) induced by isoprenaline, whereas pGC activation has little effect (Gisbert & Fischmeister 1988; Mery et al 1993).

Similar to what has recently emerged for cAMP, it has been hypothesized that intracellular compartmentation of cGMP could explain these differences. However, very little is known about cGMP dynamics in intact cardiac myocytes. The first evidence of compartmentalization of cGMP singalling pathway was published in 2006 by Rodolphe Fischmeister's group. They found that cGMP generated by the soluble and the particulate isoforms of GC are differently regulated by PDE2 and PDE5. According to their studies PDE5 controls the cGMP generated by the sGC but not by the particulate GC, whereas PDE2 exclusively controls the cGMP generated by the pGC. In these experiments, cGMP signals were monitored in adult rat cardiomyocytes by expression of the rat olfactory cyclic nucleotide–gated (CNG) channel α-subunit and by recordings of the associated cGMP-gated current (I_{CNG}). This technique allows detection only of subsarcolemmal cGMP and no information can be collected on the cGMP dynamics that occur deep inside the cell. In my first set of experiments, therefore, I set out to

investigate the role of phosphodiesterase (PDE) subtypes in the control of cGMP levels in the bulk cytosol of primary cultured rat neonatal cardiac myocytes (RNCMs). For these experiments I performed real time imaging of cGMP by using cytosolic FRET-based sensors. In addition, I analyzed the role of individual PDEs in shaping the cGMP response elicited by the β-adrenergic stimulation. Our laboratory previously published that catecholamine stimulation of RNCMs leads not only to the activation of β_1 and β_2 adrenoceptors but also to the activation of β_3-adrenergic receptors which are functionally coupled to an endothelial nitric oxide synthase (eNOS). In the presence of norepinephrine or isoproterenol, activated eNOS generates NO, which in turn stimulates cGMP synthesis by soluble GC (Mongillo et al 2006). It was therefore relevant to study the contribution of individual PDEs to the control of cGMP signals generated by catecholamines.

AIMS:

- study the intracellular cGMP dynamics upon activation of sGC or pGC in RNCMs
- establish the role of PDE2 and PDE5 in controlling and shaping such cGMP signals in the bulk cytosol

5.1.2 *Results*

5.1.3 *PDEs involved in the control of intracellular basal levels of cGMP*

I first asked whether in cardiac myocytes there is a basal generation of cGMP that is counteracted by a basal PDE activity. To answer this question primary cultured RNCMs were transfected with the FRET-based sensor for cGMP cGES-DE2 (Figure 5-1C) (Nikolaev et al 2006). Figure 5-1A shows a RNCM expressing the sensor cGES-DE2 with its typical cytosolic localization. When RNCMs expressing cGES-DE2 were treated with the non selective PDE inhibitor IBMX (isobutyl-methyl-xantine, 100 μM) a clear increase of cGMP could be detected (Figure 5-1B). In the absence of other stimuli, IBMX generated an avarage FRET response of $\Delta R/R_0$=3.84±0.70% (n=7) (Figure 5-2). This result indicates that in RNCMs guanylyl cyclases are constitutively active. Next, I investigated

the role of individual cGMP-degrading PDEs in controlling basal levels of cGMP. RNCMs express four families of cGMP degrading enzymes, PDE1, PDE2, PDE3, and PDE5. No PDE1 selective inhibitors are presently available. In addition, previous studies have reported that PDE2 and PDE5 are the enzyme mainly responsible for cGMP degradation in the subsarcolemmal compartment in these cells. I therefore focused on these PDE subtypes. To test the relative contribution of PDE2 and PDE5 in controlling basal levels of cGMP, RNCMs expressing the cGMP sensor cGES-DE2 were challenged with the selective PDE2 inhibitor BAY 60-7550 (BAY, 10 μM) or with the PDE5 selective inhibitor sildenafil (SILD, 1 nM) (Figure 5-2).

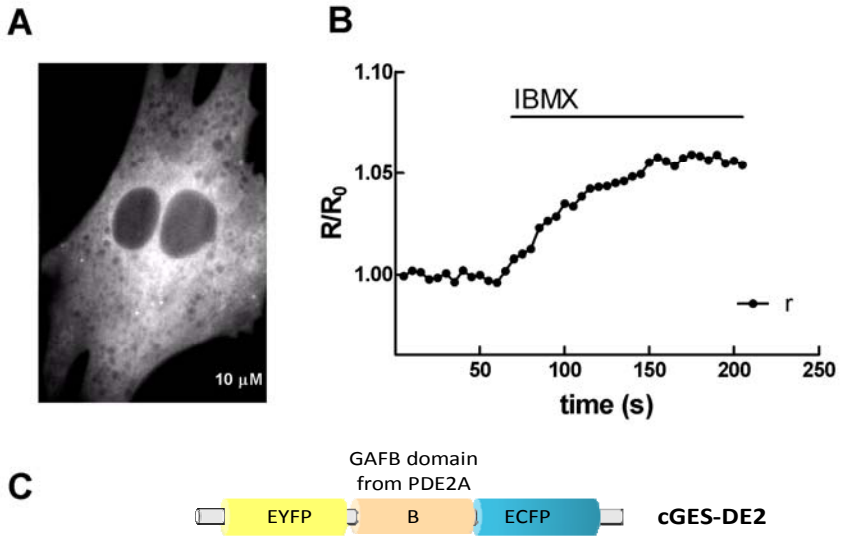


Figure 5-1 (A) RNCM expressing the cGES-DE2 sensor; (B) representative kinetic of cGMP changes obtained with this sensor upon IBMX treatment; schematic representation of cGES-DE2.

The analysis of FRET changes showed that cells treated with BAY generated a response of $\Delta R/R_0$=1.4±0.31% (n=11), whereas cells stimulated with SILD generated a response of $\Delta R/R_0$=0.77±0.14% (n=25). The simultaneous application of BAY and SILD generated an increase in cGMP corresponding to a FRET change of $\Delta R/R_0$=2.38±0.44% (n=23). Thus, inhibition of PDE2 led to an increase in cGMP significantly higher than the increase in cGMP generated by PDE5 inhibition ($p<0.05$), indicating that PDE2 plays a more prominent role than PDE5 in degrading intracellular basal levels of cGMP in these cells. In addition, inhibition of both PDE2 and PDE5 showed an additive effect, with a FRET

change not statistically different from the FRET change generated by IBMX, indicating that PDE2 and PDE5 account for the total cGMP degrading activity in these cells.

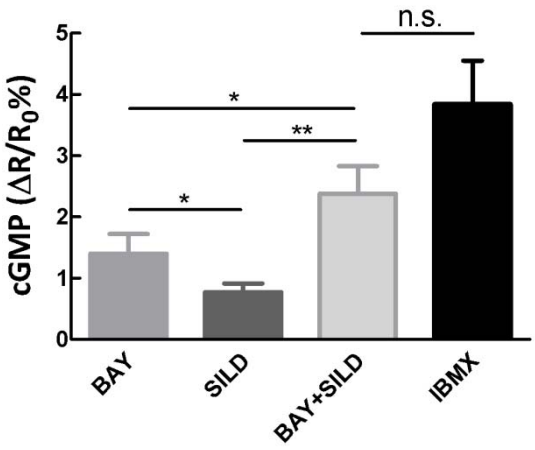


Figure 5-2 Effect of PDEs inhibition on cGMP basal levels.

5.1.3.1.1 PDEs involved in the control of cGMP generated by the pGC

The aim of this set of experiments was to understand the relative role of PDE2 and PDE5 in shaping the cGMP response generated by the pGC. RNCMs transfected with the cytosolic probe cGES-DE2 were treated with atrial natriretic peptide (ANP, 100 μM) alone or in combination with a selective PDE inhibitor (Figure 5-3). ANP generated a FRET response $\Delta R/R_0$=0.55±0.08% (n=30). ANP plus the PDE2 inhibitor BAY generated a response that was significantly higher than ANP alone $\Delta R/R_0$=1.84±0.35% (n=10), $p<0.05$. Conversely, ANP plus the PDE5 inhibitor SILD generated the same amount of cGMP as ANP alone $\Delta R/R_0$=0.75±0.23% (n=10). ANP plus the combination of BAY and SILD generated a FRET response $\Delta R/R_0$=2.87±0.46% (n=7), comparable to FRET change detected upon treatment with ANP+BAY, suggesting that PDE2 is the only PDE that controls the levels of cGMP generated by the pGC whereas PDE5 does not appear to participate in this control.

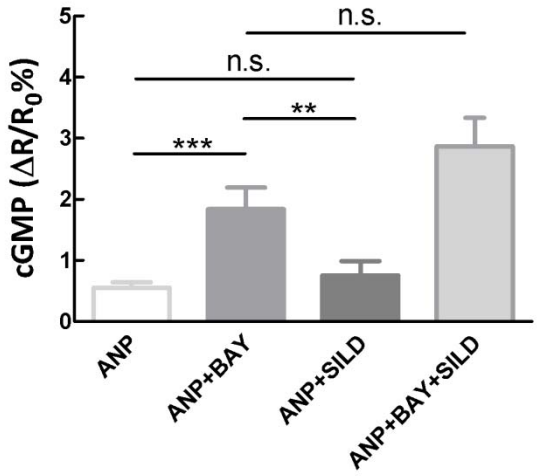


Figure 5-3 PDEs involved in the control of the cGMP generated by the pGC.

5.1.3.1.2 PDEs involved in the control of cGMP generated by sGC

I next explored the relative role of PDE2 and PDE5 in shaping the cGMP response upon activation of the sGC. RNCMs expressing cGES-DE2 were challenged with the NO donor sodium nitroprusside (SNP, 1.5 μM) alone or in combination with PDE2 and PDE5 selective inhibitors (Figure 5-4). SNP generated a FRET response of $\Delta R/R_0$=0.75±0.13% (n=22). SNP plus BAY (10 μM) generated a FRET change of $\Delta R/R_0$=2.01±0.86% (n=13) whereas SNP plus SILD (1 nM) generated a FRET response of $\Delta R/R_0$=0.85±0.86% (n=15). In both cases, the response in the presence of the PDE inhibitor was significantly higher than in the presence of SNP alone (for both $p<0.05$), suggesting that both PDE2 and PDE5 contribute to limit the cGMP levels generated by sGC. Treatment with SNP in the presence of BAY and SILD generated a response of $\Delta R/R_0$=3.71±0.80% (n=7) that was approximately equal to the sum of the response generated by inhibition of the sole PDE2 and the sole PDE5. These data suggest that PDE2 and PDE5 may not control the same pool of sGC-generated cGMP. Indeed, if PDE2 and PDE5 were acting on the same pool of cGMP one would expect that, unless both PDE were working at saturation, upon inhibition of one PDE the increase of cGMP may be compensated by the activity of the other PDE. If this were the case, the amount of cGMP released upon inhibition of both

PDE would be expected to be larger than the sum of the responses detected upon inhibition of each PDE alone.

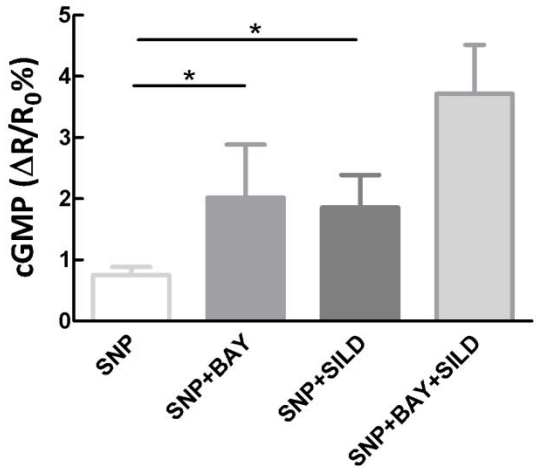


Figure 5-4 PDEs involved in the control of the cGMP generated by sGC.

5.1.3.1.3 PDEs involved in the control of cGMP generated by β-AR stimulation

To monitor cGMP dynamics upon β-adrenergic stimulation I choose to use a different sensor, cygnet-2.1 (Figure 5-5C) (Honda et al 2001) because of its higher selectivity for cGMP versus cAMP (cGMP/cAMP selectivity: >600 for cygnet-2.1 and =128 for cGES-DE2 (Nikolaev et al., 2006)). Given the large cAMP response generated by β-adrenergic stimulation the results obtained with a sensor with low selectivity for cGMP versus cAMP may have been difficult to interpret. Cygnet-2.1 expression and performance were tested in cardiac myocytes. The probe showed a cytosolic localization (Figure 5-5A) and gave the expected FRET change to cGMP raising stimuli (Figure 5-5B).

RNCMs expressing cygnet-2.1 were treated with selective PDEs inhibitors and IBMX (Figure 5-6A). BAY generated a FRET response of $\Delta R/R_0$=1.29±0.31% (n=14) whereas SILD generated a FRET response of $\Delta R/R_0$=0.30±0.15% (n=12). Simultaneous treatment with BAY and SILD produced a response of $\Delta R/R_0$=2.97±0.01% (n=5). IBMX generated a $\Delta R/R_0$=4.62±1.61% (n=6).

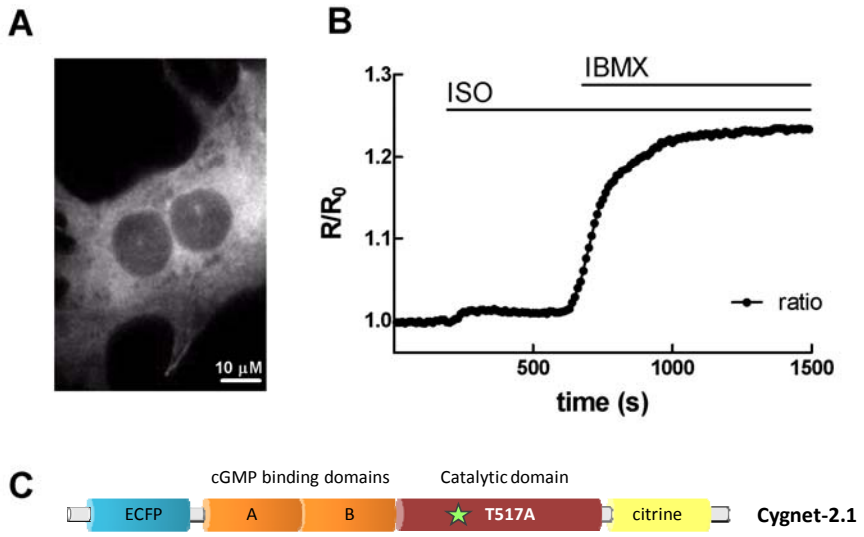


Figure 5-5 Cygnet-2.1 localization in RNCMs (A); representative kinetic of cGMP changes obtained with this sensor upon isoproterenol (ISO) and IBMX treatment; schematic of cygnet-2.1 (C).

This set of data is in agreement with previous results described in 5.1.2.1 with the cGES-DE2 sensor, where the basal levels of cGMP were shown to be entirely controlled by PDE2 and PDE5, with a major role played by PDE2. Cells were then stimulated with the β agonist isoproterenol (ISO 10 μM) alone or in combination with PDE2 and PDE5 inhibitors (Figure 5-6B). Upon stimulation with ISO 10 μM the sensor detected a FRET change of $\Delta R/R_0$=1.32±0.16% (n=39). ISO plus BAY generated a $\Delta R/R_0$=3.45±0.58% (n=13). ISO plus SILD caused a FRET change of $\Delta R/R_0$=3.61±0.77% (n=12). ISO plus the combination of BAY and SILD resulted in a FRET change of $\Delta R/R_0$=7.02±1.35% (n=14). These data indicate that β-adrenergic stimulation results in cGMP generation and that this cGMP pool is controlled by both PDE2 and PDE5. To better dissect the contribution of each PDE in the control of cGMP generated by ISO the data Figure 5-6C shows the contribution to cGMP levels of ISO alone, of the PDE inhibitor alone and of the PDE inhibitors in the presence of ISO. As shown, on ISO stimulation, the amount of cGMP detected in the presence of SILD is larger than the amount of cGMP detected in the presence of BAY. This suggests that cGMP generated by β-adrenoceptor stimulation is controlled predominantly by PDE5 and to a lesser extent by PDE2.

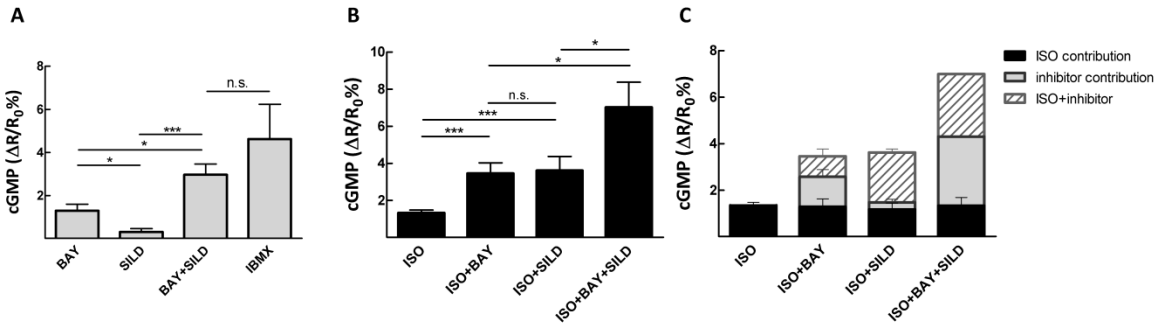


Figure 5-6 Contribution of PDE2 and PDE5 in shaping the cGMP pool generated by the β-adrenergic stimulation.

5.1.4 *Discussion*

cGMP is a ubiquitous intracellular second messenger that can be generated by both the soluble and the particulate guanylyl cyclases (GCs) in response to nitric oxide and natriuretic peptides, respectively. It modulates different aspects of myocardial function: excitation-contraction coupling, myocardial relaxation, diastolic function, heart rate, β-adrenergic inotropic response, and myocardial energetic and substrate metabolism (Semigran 2005; Shah & MacCarthy 2000). Chronic elevation of cGMP levels induced by natriuretic peptides or PDE5 inhibition has been shown to prevent and reverse cardiac hypertrophy (Rosenkranz et al 2003; Takimoto et al 2005b). Activation of the pGC or the sGC produces distinctive downstream effects suggesting that segregated cGMP pools transduce specific signals. In this chapter I analyzed the dynamics of cGMP in intact RNCMs using FRET-based, real-time imaging of cGMP and I showed that, not all cGMP is indifferently accessible to all cGMP-degrading PDEs, suggesting that, similar to cAMP, intracellular cGMP is not evenly distributed within cardiac myocytes but it is compartmentalized into distinct microdomains. Such cGMP microdomains are selectively regulated by distinct PDEs. In particular, PDE2 appears to control the pool of cGMP generated by the pGC whereas the pool of cGMP generated by the sGC is shaped equally by PDE2 and PDE5. In a previous study Castro and colleagues reported that the cGMP generated by sGC is shaped exclusively by PDE5. A possible reason for these contrasting results is that different approaches were used to monitor cGMP in the two

studies. Castro *et al.* used a CNG ion channel as a sensor for cGMP. This sensor, being localized at the plasma membrane, allows monitoring of cGMP exclusively in the subsarcolemmal compartment, whereas in this study a cytosolic cGMP sensor was used. It is possible to speculate that PDE5 acts mainly in the sub-plasma membrane region, thereby showing a larger contribution when cGMP levels are detected selectively in this compartment. In addition, an enrichment of PDE5 underneath the plasma membrane may act as a barrier that prevents cGMP released upon PDE2 inhibition in compartments deeper in the cytosol to reach, and be detected by, a sensor that is targeted to the plasma membrane.

Another conclusion that can be drawn from the experiments described above is that PDE2 and PDE5 contribute differently to the control of cGMP generated in response to β_3-adrenergic receptors stimulation (β_3-AR), with PDE5 showing a predominant role. As *in vitro* studies indicate that both PKA and PKG can phosphorylate and activate PDE5, it is possible to envisage a negative feed-back loop mechanism whereby β-adrenoceptor stimulation, either via cAMP-PKA or via cGMP-PKG, can lead to the activation of PDE5 and to the consequent reduction of the cGMP response. However, it is presently unknown if PDE5 can be activated *in vivo* and to confirm this hypothesis additional experiments would be required.

Our laboratory recently demonstrated that PDE2 integrates cGMP and cAMP signals in a feedback control loop in which activation of β_3-AR counteracts cAMP generation obtained via stimulation of β_1/β_2-adrenoceptor (Mongillo et al., 2006). This mechanism would contrast excessive β-adrenergic drive in normal conditions but would underlie the progressive nature of functional cardiac impairment in heart failure by further reducing the already blunted cAMP response. The mechanism proposed for this feedback loop involves activation of β_3-adrenergic receptors leading to the activation of endothelial nitric oxide synthase (eNOS). Activated eNOS generates NO, which stimulates cGMP synthesis by soluble GC; cGMP in turn activates the cAMP-hydrolyzing activity of PDE2. Interestingly, data from our laboratory also show that both PDE2 and PDE5 can localize at Z-band structures in cardiac myocytes (Mongillo et al., 2006; Takimoto et al., 2005). It

is therefore interesting to note that PDE5 can very likely play a role in this feedback loop mechanism by reducing cGMP levels and therefore, through reduced PDE2 activity, increase cAMP levels. This is particularly relevant if considering that PDE5 inhibitors such as Viagra® are currently widely used not only for the treatment of erectile dysfunctions but are also indicated for the treatment of pulmonary arterial hypertension. PDE5 inhibition may increase cGMP levels and, in turn, reduce local cAMP via activation of PDE2, thereby further reducing the contractile reserve in a condition where cardiac contractility may already be defective.

5.2 cAMP/cGMP interplay

5.2.1 *Introduction*

In the past few years, compartmentalization of the cAMP signal transduction pathway has emerged as an important mechanism to ensure the necessary specificity of response. It is now well accepted that cAMP-PKA singalling is compartmentalized in discrete subcellular domains. PKA is anchored to A kinase–anchoring proteins (AKAPs) in close proximity to specific targets and is activated by restricted pools of cAMP. An important contribution to cAMP compartmentalization derives from the activity of PDEs, that act as sinks to locally drain cAMP diffusion and to switch off the signal in specific locations (Terrin et al 2006).

Recently, our laboratory has published evidence that the two PKA isoforms, PKA type I and PKA type II, define distinct subcellular compartments within which cAMP is selectively generated by different hormonal stimuli (Di Benedetto et al 2008). PKA type I and PKA type II possess different structural features and distinct biochemical characteristics, resulting in non redundant function (Cummings et al 1996; Dostmann et al 1990; Skalhegg & Tasken 2000). PKA type I and type II also have a different subcellular localization. Classically, it is assumed that PKA type II is mainly associated with the particulate fraction of cell lysates whereas PKA type I has been found preferentially in the cytosolic fraction (Corbin et al 1977). Several studies have shown that the intracellular localization of PKA isoforms is determined by the unique dimerization/docking domain of the regulatory subunits (R) that has been shown to mediate anchoring of PKA-RI and PKA-RII to AKAPs (Carr et al 1991). With the aim of studying the selectivity of PKA isoforms activation, two new cAMP sensors were developed in our laboratory that target to the subcompartments in which the two isoforms of PKA normally reside. To achieve this, the dimerization/docking domain of the regulatory subunit of either PKA type I (RI) or PKA type II (RII) were fused to the cytosolic FRET-based cAMP sensor Epac1-camps (Nikolaev et al 2004), to generate the two targeted sensors RI_epac and RII_epac, respectively. FRAP experiments and confocal microscopy studies confirmed that, by binding to specific endogenous AKAPs,

these two sensors localize to distinct intracellular locales (Di Benedetto et al 2008). RII_epac shows a strong localization at the M lines and a weaker localization at the Z lines, whereas RI_epac shows a striated pattern that overlaps with both with Z and M lines. Real time imaging experiments performed with these sensors showed that PKA type I and PKA type II define exclusive singalling domains within which cAMP is generated in a stimulus-specific manner, leading to selective activation of PKA isoforms and to the phosphorylation of a distinct pattern of downstream targets. Specifically, ISO was found to generate a restricted cAMP pool that selectively activates PKA type II over PKA type I and to lead to the phosphorylation of TnI, PLB, and β_2-AR. In contrast, prostaglandin 1 (PGE1) selectively activates PKA type I and does not increase the phosphorylation level of these targets. These studies demonstrated that different hormones generate spatially distinct pools of cAMP that selectively activate different PKA isoforms which in turn mediate different functional effects. A reasonable hypothesis is that distinct subsets of PDEs contribute to the restriction of these cAMP pools preventing cross activation of PKA isoenzymes. However, the exact contribution of individual PDEs to such compartmentalization has not been determined yet.

Recently, the concept of compartmentalized signal transduction has been expanded also to cGMP and emerging data suggest a tight interconnection between the cAMP and the cGMP singalling pathways involving the activity of the cyclic nucleotide-degrading enzymes PDEs. Several cGMP-mediated responses in cardiac cells, including the potentiation of Ca^{2+} currents and the reduction of the responsiveness to β-adrenergic receptor agonists, have been suggested to result from the effects of cGMP on PDE-mediated cAMP hydrolysis (Layland et al 2002; Vila-Petroff et al 1999). Cardiac myocytes express two families of cGMP-regulated cAMP-hydrolyzing PDEs, PDE2 and PDE3. They differ with respect to subcellular localization, affinity, and specificity for cAMP and cGMP (Conti and Beavo, 2007; Zaccolo and Movsesian, 2007). In the case of PDE2, this regulation involves the allosteric stimulation of cAMP hydrolysis by cGMP (Martinez et al., 2002; Michie et al., 1996). For PDE3, on the contrary, cGMP acts as a competitive inhibitor of cAMP hydrolysis (Shakur et al., 2001).

Therefore, cGMP acts as a regulator of the activity of cAMP-hydrolyzing PDEs. Modulation of PDE activity can potentially affect intracellular cAMP gradients and, consequently, the functional outcome of cAMP signals. In addition, given that both cGMP and cAMP appear to be compartmentalized, it is interesting to explore if cGMP-mediated regulation of cAMP occurs in a compartment-specific manner.

AIMS:

- investigate whether cAMP/cGMP crosstalk occurs in neonatal rat ventricular cardiac myocytes
- determine how changes in cGMP affect cAMP levels
- assess if cGMP-mediated modulation of cAMP is compartment-specific

5.2.2 *Results*

5.2.2.1.1 Effects of sGC activation and PDE5 inhibition on cAMP levels

My first question was whether alteration of cGMP levels could affect global cAMP levels. For these experiments I used the sensor Epac1-camps, a cytosolic probe for cAMP (Nikolaev et al 2004)(Figure 5-7C). Epac-1 expressed in RNCMs showed the expected uniform cytosolic distribution (Figure 5-7A). When cardiac myocytes expressing Epac1-camps, were treated with the NO donor SNAP (S-nitroso-N-acetylpenicillamine) or the PDE5 selective inhibitor Sildenafil (SILD) a clear increase in intracellular cGMP concentration could be detected (Figure 5-7B).

RNCMs expressing the cAMP probe Epac1-camps and treated with SNAP (100 μM) generated a FRET response of $\Delta R/R_0$=3.02±1.03% (n=3) and further addition of IBMX (100 μM) led to a total FRET change of $\Delta R/R_0$=17.04±1.40% (n=3) (Figure 5-8).

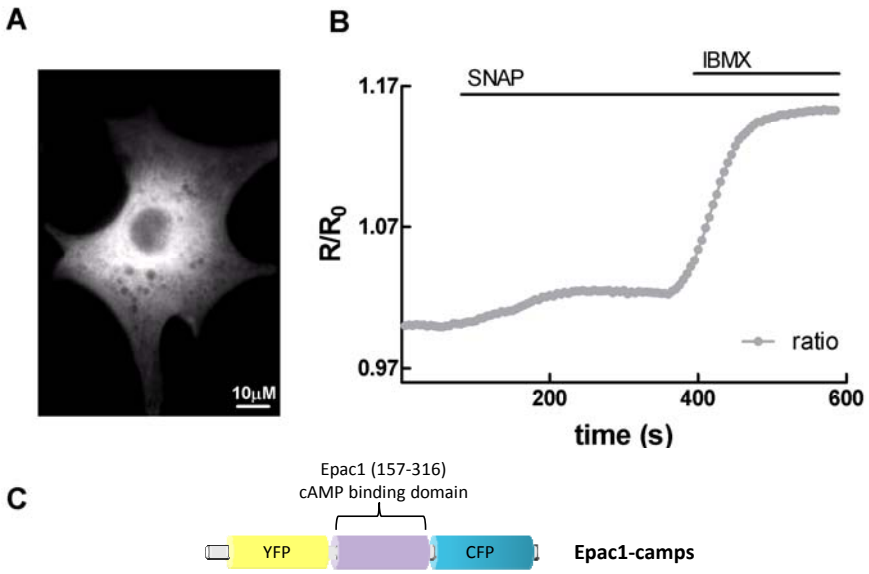


Figure 5-7 Epac1-camps localization in RNCMs (A), representative kinetic of FRET changes (R/R_0) in experiments in which cardiac myocytes were treated with SNAP and IBMX (B); structure of Epac1-camps (C).

Myocytes challenged with the PDE5 inhibitor Sidenafil (SILD, 1 nM) generated a FRET response of $\Delta R/R_0$=2.42±0.93% (n=14) and SILD and IBMX 100 μM generated a FRET increase of $\Delta R/R_0$=11.97±1.87% (n=14) (Figure 5-8). These preliminary experiments provided the first evidence that stimuli that increase cGMP levels may also affect cAMP levels.

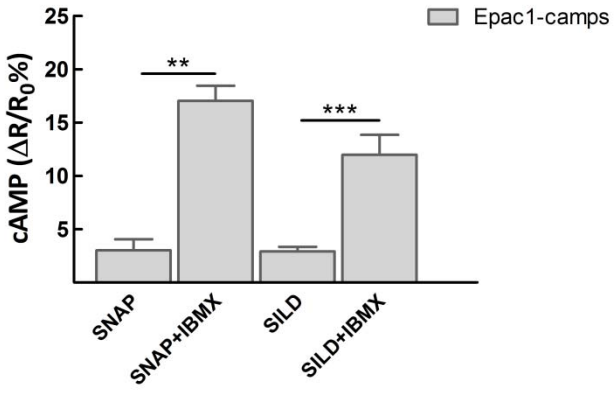


Figure 5-8 Effect of SNAP and SILD on cAMP levels.

5.2.2.1.2 cGMP reshapes the cAMP response to ISO in a compartment-specific manner

I next asked whether the generation of cGMP could affect the cAMP response to isoproterenol (ISO). RNCMs expressing the cytolsolic sensor Epac1-camps were challenged with ISO 10 nM and SNAP 100 μM. In these conditions the majority of analyzed cells showed no changes in cAMP concentration upon SNAP application (Figure 5-9A); however, in a limited number of cells a small change could be detected (Figure 5-9B). We reasoned that, being Epac1-camps a cytosolic sensor that detects bulk changes in cAMP occurring globally in the cytosol we may be underestimating changes that occur in specific compartments, particularly if an increase in cAMP concentration occurs concomitantly with a decrease in cAMP concentration in different locales.

Previous work in the laboratory has established that PKA type I and type II isoforms define distinct subcellular compartments in which cAMP is uniquely regulated. Therefore, to explore the possibility that the NO donor SNAP may induce different changes in cAMP in individual compartments I focused on the compartment defined by PKA type I and PKA type II by using the targeted sensors RI_epac or RII_epac.

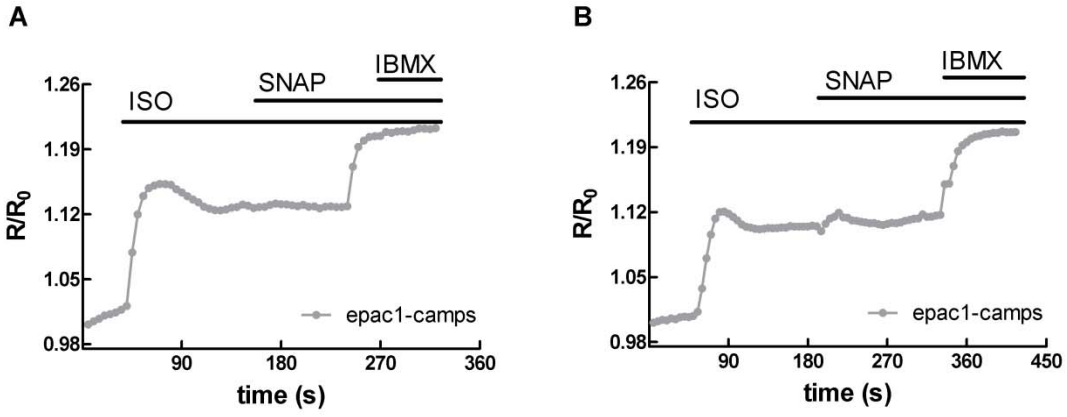


Figure 5-9 Effect of SNAP on cAMP response to ISO.

Cardiomyocytes expressing either RI_epac or RII_epac were treated with ISO 10 nM. The cAMP response was higher in the RII compartment as compared to the RI compartment with FRET changes of $\Delta R/R_0$=6.39±0.51% (n=18) detected by RII_epac and

$\Delta R/R_0$=4.36±0.32% (n=13) by RI_epac ($p<0.01$) (Figure 5-10), confirming previous results obtained in the laboratory (Di Benedetto et al 2008). Pretreatment of the cells with the NO donor SNAP (100 μM) dramatically affected this response, inducing a higher FRET change in the RI compartment as compared to the RII compartment ($\Delta R/R_0$=7.20±0.76% (n=13) for RI_epac and $\Delta R/R_0$=3.19±0.35% (n=11) for RII_epac, $p<0.001$ (Figure 5-10). To test whether this inversion of the cAMP gradients observed in the presence of SNAP was dependent on cGMP generation, cells were pretreated with ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 10 μM), an inhibitor of the sGC. In cells pretreated with ODQ, the effect of SNAP was completely abolished and the response to ISO in the presence of SNAP in the two compartments was comparable to the response in control conditions (ISO alone) ($\Delta R/R_0$=3.54±0.27%, n=15 for RI_epac and $\Delta R/R_0$=5.10±0.47%, n=13 for RII_epac, $p<0.05$) (Figure 5-10). These experiments indicate that cGMP can affect the response to ISO in a compartment specific manner and specifically, that cGMP can significantly increase the cAMP response to ISO in the RI compartment ($p<0.001$) whereas it significantly decreases the cAMP response to ISO in the RII compartment ($p<0.001$). This alteration in cAMP levels is due to cGMP generation since the inhibition of the sGC with ODQ totally prevents the effect of the NO donor SNAP on the cAMP response.

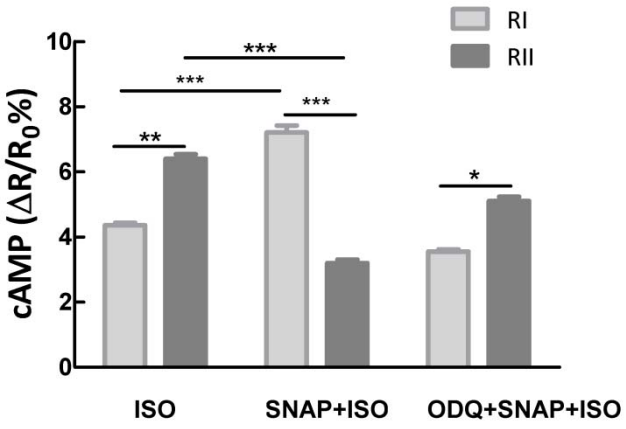


Figure 5-10 Effect of SNAP in the response to ISO in RI and RII compartments.

5.2.2.1.3 Generation of cGES-DE2 sensors for cGMP selectively targeted to the PKA-RI and PKA-RII subcompartments

One possible explanation for the observed cGMP-dependent inversion of the cAMP gradients generated in response to ISO in the presence of SNAP is that the effect of the NO donor is not homogeneous within the cell and that SNAP pretreatment induces different cGMP changes in the RI and RII compartments. To investigate if this is the mechanism involved I set out to monitor cGMP changes selectively in the two compartments defined by PKA type I and PKA type II. To this aim, I modified the cGMP probe cGES-DE2 in order to target it to these subcompartments. I took advantage of the unique dimerization/docking domain sequences that had been previously shown to mediate anchoring of PKA-RI and PKA-RII subunits to AKAPS. Thus, by fusing the dimerization/docking domain from either RIα (amino acids 1 to 64) or RIIβ (amino acids 1 to 49) to the N-terminus of the soluble cGES-DE2 I generated two new sensors named RI_cGES-DE2 and RII_cGES-DE2 (Figure 5-11B). RI_cGES-DE2 AND RII_cGES-DE2 localize as the endogenous PKAI and PKAII (Figure 5-11A)

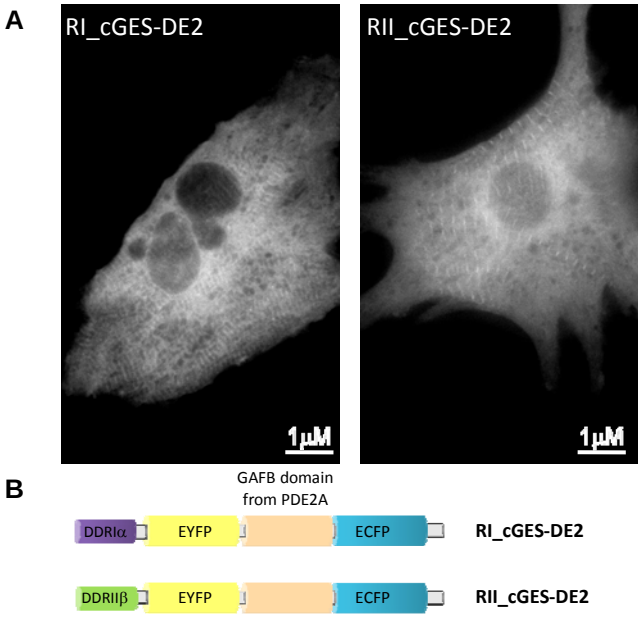


Figure 5-11 (A) Localization of RI_cGES-DE2 and RII_cGES-DE2 in RNCMs, (B) structure of the two sensors.

5.2.2.1.4 Modification of the cGMP sensor cGES-DE2 failed to generate functioning sensors

Preliminary experiments performed in cardiac myocytes using the sensors RI_cGES-DE2 and RII_cGES-DE2 indicated that the probes were not working properly. The expression level resulted to be extremely low (Figure 5-10A), making detection of FRET changes very problematic. In addition, the FRET changes detected in response to IBMX resulted to be low and in some instances negative responses were detected as well (Figure 5-10B).

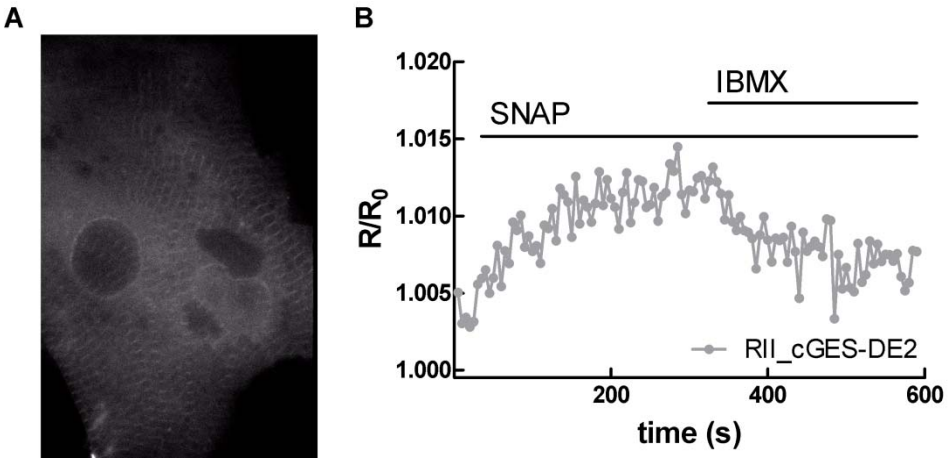


Figure 5-12 Low expression level of RII_cGES-DE2 in RNCMs (A); representative trace obtained with RII_cGES-DE2.

In the attempt to further characterize the FRET response elicited by these modified sensors I performed a number of experiments in CHO cells, a simpler cellular model than cardiac myocytes. CHO cells expressing either RI_cGES-DE2 or RII_cGES-DE2, were challenged with a saturating concentration of SNAP 100 μM and IBMX 100 μM to generate maximal FRET responses. Cygnet-2.1 and the original cytosolic probe cGES-DE2 were transfected as controls (Figure 5-13). The maximal FRET changes recorded for these sensors resulted to be extremely low: $\Delta R/R_0$=0.37±0.37% (n=4) for RI_cGES-DE2 and $\Delta R/R_0$=0.34±0.31% (n=4) for RII_cGES-DE2, $\Delta R/R_0$=4.41±0.86% (n=17) for Cygnet-2.1 and $\Delta R/R_0$=1.35±1.14% (n=8) for cGES-DE2.

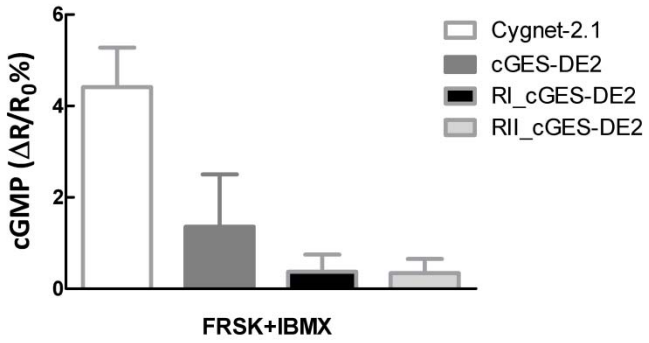


Figure 5-13 Performance of cGMP sensors.

These experiments show not only that, contrary to what previously published (Nikolaev et al 2006) the sensor cygnet-2.1 appears to give larger FRET changes to saturating stimuli then the untargeted cGES-DE2 but also that the fusion with the D/D has interfered with the performance of the targeted probes, making these new constructs not suitable for FRET experiments. Fusion with the dimerisation/docking domain is known to influence FRET efficiency. Previous experiments performed in our laboratory had shown that fusion of Epac1-camps with RI and RII dimerization and docking domains reduced maximal FRET response by half when compared to the parent sensors (Di Benedetto et al 2008).

5.2.2.1.5 Targeting of the cGMP sensor cygnet-2.1 to the PKA-RI and PKA-RII subcompartments

Given its better performance compared to cGES-DE2, I decided to modify the cGMP sensor cygnet-2.1 to target it to the PKA type I and type II compartments by fusing to its N-terminus the dimerization/docking domain of RIα and RIIβ (Figure 5-14C). As shown in Figure 5-14A when expressed in RNCM these probes showed the expected intracellular localization.

In addition, both targeted sensors generated a robust FRET change to increased levels of cGMP (Figure 5-14B).

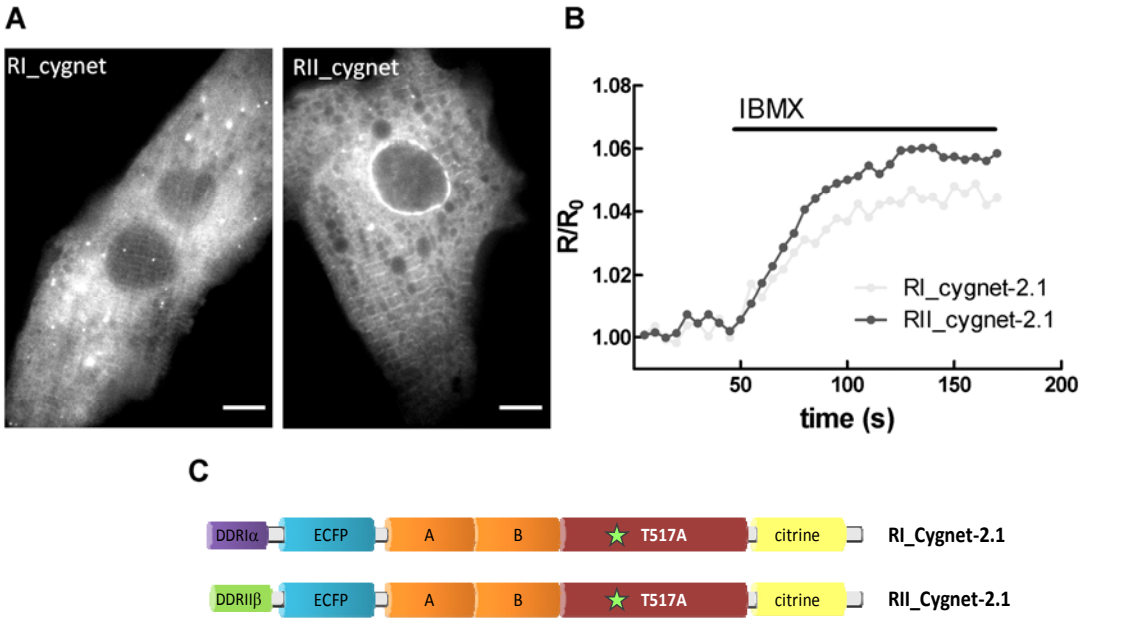


Figure 5-14 (A) Intracellular localization of RI_cygnet-2.1 and RII_cygnet-2.1 in RNCMs; (B) representative kinetics obtained with these two sensors upon IBMX stimulation; (C) schematic representation of the sensors.

5.2.2.1.6 SNAP generates a comparable increase in cGMP levels in both RI and RII compartments

I next investigated whether the effect of the NO donor SNAP on the generation of cGMP was homogeneous or whether SNAP pretreatment was inducing different cGMP changes in the RI and RII compartments. A number of experiments were performed in which cardiac myocytes expressing either RI_cygnet-2.1 or RII_cygnet-2.1 were treated with SNAP 100 μM (Figure 5-15A). The two differently targeted sensors detected a comparable rise in cGMP upon SNAP stimulation with $\Delta R/R_0$=5.45±0.98% (n=14) for RI_cygnet-2.1 and $\Delta R/R_0$=6.67±0.98% (n=10) for RII_cygnet-2.1. These results indicate that the same amount of cGMP is generated in the two compartments upon stimulation of the sGC and suggest that the sGC is equally associated with both RI and RII compartments.

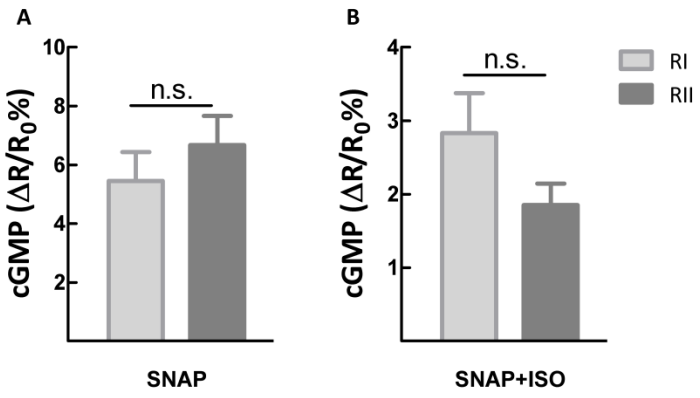


Figure 5-15 Effect of SNAP and ISO+SNAP on cGMP levels.

In a second series of experiments NRVMs transfected with RI_cygnet-2.1 and RII_cygnet-2.1 were pretreated with SNAP and stimulated with ISO 10 nM (Figure 5-15B). ISO generated the same cGMP response in both RI and RII compartments with $\Delta R/R_0$=2.82±0.29% (n=7) for RI_cygnet-2.1 and $\Delta R/R_0$=1.85±0.54% (n=8) for RII_cygnet-2.1.

5.2.2.1.7 Are PDEs involved in the inversion of the cGMP-dependent cAMP gradients?

The experiments presented in the previous paragraph show that the NO donor SNAP induces an increase of cGMP that is uniform in the RI and RII compartments. In addition, in cells pretreated with SNAP, ISO stimulation generates the same amount of cGMP in both compartments. We can therefore exclude that the cGMP dependent inversion of the ISO-induced cAMP gradients is dependent on an uneven generation of cGMP in the RI and RII compartments. Another possible explanation for the observed effect is a cGMP-mediated modulation of PDEs activity. The hypothesis here would be that, in the presence of SNAP a cGMP-inhibition of PDE3 occurs in the RI compartment leading to an increased cAMP response to ISO whereas a reduced increase in cAMP is detected in the RII compartment due to cGMP-mediated activation of PDE2 in that locale. To test this hypothesis NRCMs transfected with either RI_epac or RII_epac were stimulated with ISO in the presence of SNAP plus selective pharmacological inhibitors of either PDE2 or PDE3

(Figure 5-16). In control experiments, as expected, cardiomyocytes treated with ISO (10 nM) alone showed a higher cAMP response in the RII compartment than in the RI compartment ($\Delta R/R_0$=6.35±0.32% for RII_epac, n=18 and $\Delta R/R_0$=4.36±0.32%, n=18, for RI_epac, p<0.05). NRCM pre-incubated with SNAP (100 μM) and stimulated with ISO showed an higher response in the RI compartment as compared to the RII compartment with FRET changes of $\Delta R/R_0$=7.20±0.76% (n=13) for RI_epac and $\Delta R/R_0$=3.19±0.35% (n=11) for RII_epac. This set of experiments confirms the results presented in 5.2.2.2 in which pretreatment with SNAP induced an inversion of the cAMP gradients typically observed in RI and RII compartments in response to ISO.

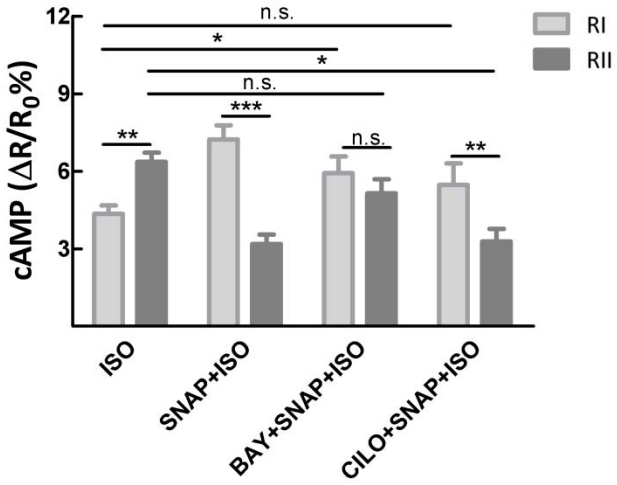


Figure 5-16 Effect of PDE2 and PDE3 inhibition on the cGMP-induced inversion of the gradients upon ISO stimulation.

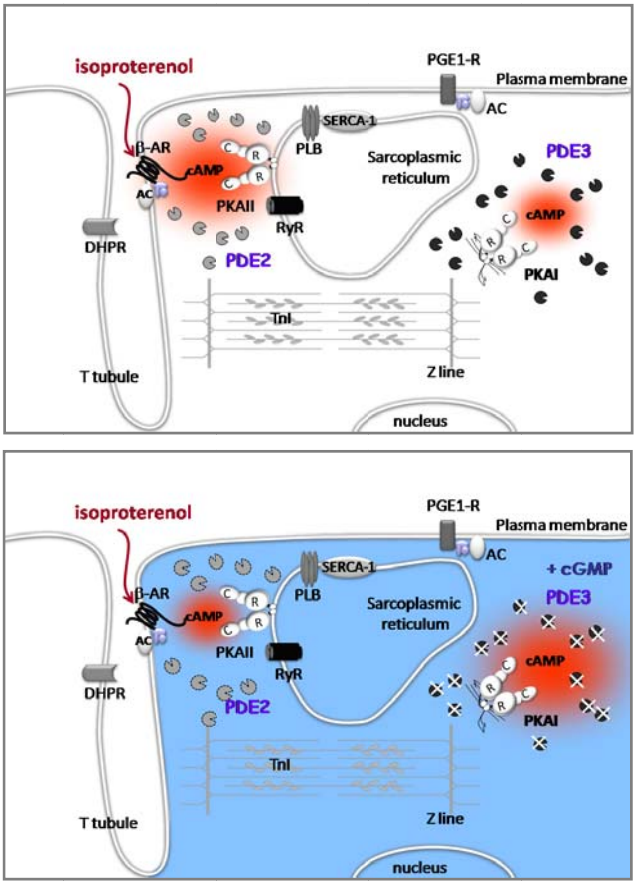
When cardiomyocytes were pretreated with SNAP and the PDE2 inhibitor BAY (10 μM), ISO stimulation generated a comparable FRET change in the RI and RII compartments ($\Delta R/R_0$=5.39±0.63%, n=16 and $\Delta R/R_0$=5.15±0.54%, n=15, respectively). In RNCMs pretreated with SNAP and the PDE3 inhibitor CILO (10 μM), ISO stimulation generated a higher increase in the FRET change in the RI compartment as compared to the RII compartment (with a FRET change of $\Delta R/R_0$=5.48±0.82%, n=7 and $\Delta R/R_0$=3.28±0.48%, n=8, respectively, p<0.001).

In the RII compartment, cells pretreated with SNAP and BAY generated the same response to ISO as control cells (ISO alone). On the contrary, cells pretreated with SNAP and CILO generated a smaller response to ISO as compared to control cells ($p<0.05$). A possible mechanism to explain these results is that PDE2 is functionally associated with the RII compartment and that the reduced increase in cAMP upon ISO stimulation in the presence of SNAP can be attributable to a cGMP-mediated activation of PDE2.

In the RI compartment, cells pretreated with SNAP and BAY still showed a higher response to ISO than control cells ($p<0.05$). Conversely, when cells were pretreated with SNAP and CILO, no further increase in cAMP was observed upon ISO stimulation. These data are compatible with a functional association of PDE3 with the RI compartment where the SNAP-induced cGMP inhibits PDE3 thereby increasing the amount of cAMP detectable in response to ISO stimulation. In contrast, when myocytes are pre-incubated with CILO, the cGMP generated upon SNAP treatment acts on a PDE3 that is already inhibited by the pharmacologic inhibitor and therefore the effect of SNAP on PDE3 activity is not detectable.

5.2.2.1.8 A spatially confined PDE2 appears to be necessary for the cGMP-mediated control of local cAMP signals

Based on the data presented so far, the following working hypothesis can be formulated: PDE2 is functionally coupled with the PKAII compartment whereas PDE3 is functionally coupled with the PKAI compartment (Figure 5-18). Thus, SNAP leads to a reduced response to ISO in the PKAII compartment as a consequence of cGMP-mediated activation of PDE2, and to an increased cAMP response in the PKAI compartment as a result of cGMP-mediated PDE3 inhibition in that locale.



5-17 Figure 5-18 Schematic representation of the working hypothesis.

In the following set of experiments I wanted to investigate whether the cGMP-mediated activation of PDE2 in the RII compartment was involved in the inversion of the cAMP gradients in the response to ISO. Cardiomyocytes expressing either RI_epac or RII_epac were treated with ISO 10 nM (Figure 5-19). In agreement with previous results, the sensors detected a higher increase in the RII compartment as compare to the RI compartment with FRET changes of $\Delta R/R_0$=6.45±0.97% (n=11) for RII_epac and $\Delta R/R_0$=4.43±0.41% (n=13) for RI_epac ($p<0.05$). Cells pretreated with SNAP (100 μM), ISO generated a higher response in the RI compartment as compared to the RII compartment with FRET change $\Delta R/R_0$=6.32±0.60% (n=19) detected by RI_epac and $\Delta R/R_0$=3.21±0.44% (n=19) detected by RII_epac ($p<0.001$), confirming the results presented in 5.2.2.2. Next, RNCMs were pretreated with SNAP and stimulated with ISO

and BAY (10 μM) (Figure 5-19). The response detected by the cAMP sensors was comparable in the two compartments, with $\Delta R/R_0$=6.10±0.64% (n=7) for RI_epac and $\Delta R/R_0$=6.13±0.82% (n=8) for RII_epac. These data show that the gradient generated by ISO stimulation between the RI and the RII compartments is completely abolished by the PDE2 inhibitor BAY. Thus these experiments confirm the results presented in 5.2.2.4 in which inhibition of PDE2 preceded the treatment with SNAP and ISO and further support a mechanisms whereby SNAP-induced cGMP activates a PDE2 pool that is functionally coupled with the PKA type II compartment.

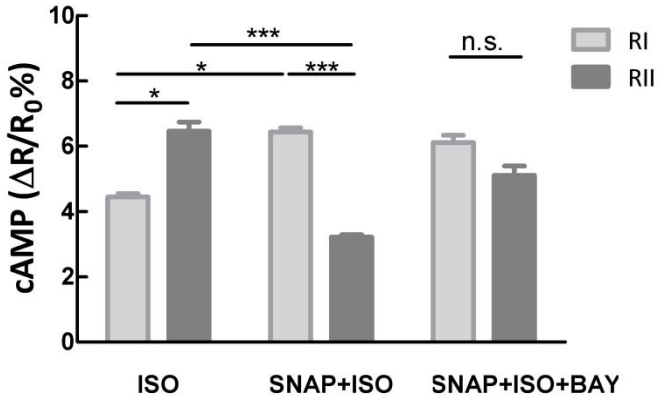


Figure 5-19 Effect of PDE2 inhibition on the SNAP-induced inversion of the gradients.

5.2.2.1.9 Effects of displacing the endogenous PDE2 on the SNAP-dependent inversion of the cAMP gradients

The hypothesis presented above implies a physical coupling of PDE2 with the PKA type II compartment, as a PDE2 enzyme free to diffuse in the cytosol and activated by a homogeneous SNAP-induced cGMP increase (as shown in 5.2.2.2) would not be expected to exert its effects selectively in the PKA type II compartment and not in the PKA type I compartment.

To test the hypothesis that spatial confinement of PDE2 is necessary for PDE2 to exert its effect upon SNAP treatment, a possible approach is to displace the endogenous PDE2 in RNCMs from its cognate intracellular anchor sites and to assess the effect of SNAP on cAMP levels in the RI and RII compartments in these conditions.

For these experiments we applied the same strategy developed in M. Houslay's laboratory for PDE4. In this approach, to gain insight into the functional role of an anchored PDE, a dominant negative mutant of the PDE is generated (Baillie et al 2003). This involves engineering specific mutations in the enzyme catalytic site such that they rendered catalytically inactive. The mutant (catalitically dead) PDE is then overexpressed in cells to such a level that it will serve to displace the corresponding endogenous active PDE isoform from its functionally relevant anchor site. As a consequence, the catalytically dead PDE will localize at those sites where normally the endogenous active enzyme resides and the active displaced enzyme will be free to diffuse in the cell.

To apply this approach to PDE2 I introduced two mutations in the catalytic site of the wild type PDE2A2 enzyme. These mutations, D685A and D796A, convert two aspartic acid residues into two alanine residues (Figure 5-20). These two Asp residues are located in the substrate binding pocket and coordinate two divalent metals, Zn^{2+} or Mg^{2}, they are absolutely conserved among all the PDEs and are necessary for the catalytic activity.

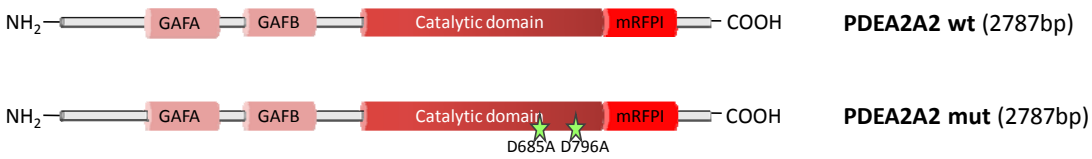


Figure 5-20 Stucture of PDE2A2 wt and PDE2A2 mut.

To verify that the introduced mutations were sufficient to abolish the activity of the enzyme, the activity of the PDE2A2mut was tested in the laboratory using an enzymatic essay. As shown in Figure 5-21 the catalytic activity of the mutant resulted completely abolished.

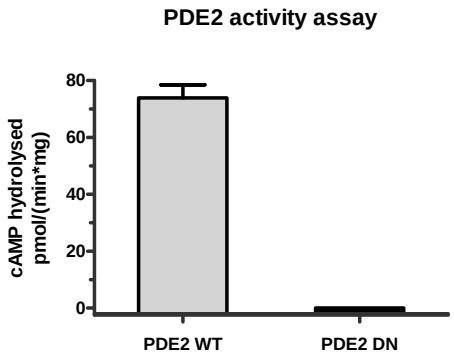


Figure 5-21 Activity essay of PDE2A2wt and PDE2A2mut (PDE2DN).

Subsequently, both the wild type PDE2A2 and the catalytically dead mutant PDE2A2 were fused to a monomeric RFP, so that for FRET experiments it was then possible to select cells expressing both the FRET-sensors and the PDE2A2 constructs (Figure 5-22).

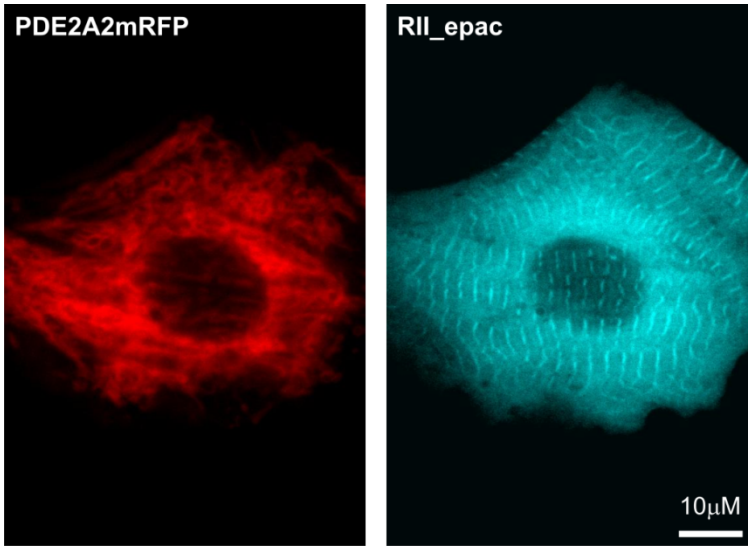


Figure 5-22 RNCM coexpressing PDE2A2mRFPI and RII_epac.

Control experiments were performed in myocytes transfected with the FRET-based sensors only. In agreement with previous findings, cardiomyocytes expressing either RI_epac or RII_epac showed a response to ISO that was higher in the RII compartment and SNAP generated the inversion of the cAMP gradients (Figure 5-23).

In striking contrast, in myocytes expressing PDE2A2mut isoform, the effect of SNAP on the inversion of the cAMP gradients in the RI and RII compartments was completely abolished and the response to ISO resulted to be higher in the RII compartment than in the RI compartment ($\Delta R/R_0$=4.52±0.44%, n=16, and $\Delta R/R_0$=3.42±0.42%, n=14, respectively, p<0.05) (Figure 5-23).

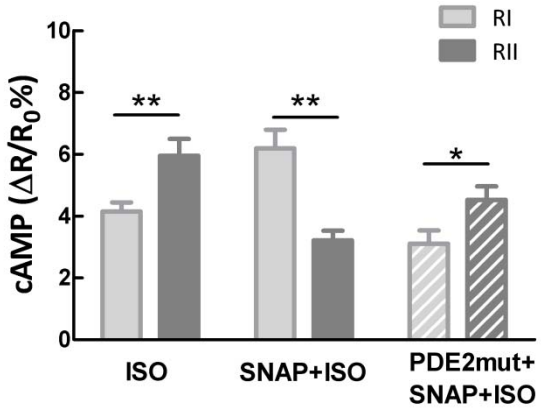


Figure 5-23 Effect of the displacement of PDE2 on the SNAP-induced inversion of the gradients.

A number of FRET experiments were carried out in RNCMs transfected with either RI_epac or RII_epac and the wt form of PDE2A2.

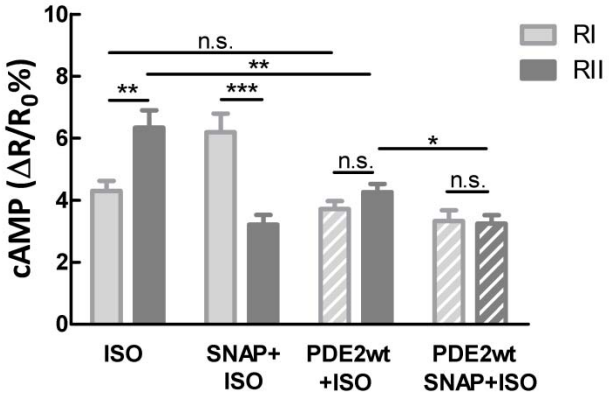


Figure 5-24 Effect of the overexpression of PDE2A2wt.

When PDE2A2wt was overexpressed no difference between the two compartments was detected in response to ISO or ISO in the presence of SNAP (Figure 5-24).

These results could be interpreted as follows: overexpression of the catalytically active PDE2 results in an accumulation of the enzyme in the RII compartment. This would be responsible to the reduced cAMP response to ISO in this compartment. In the presence of SNAP, the active PDE2 enzyme that has accumulated in the RII compartment is further activated by NO-induced cGMP, leading to a further reduction of cAMP levels as compared to cells that do not overexpress PDE2wt and are treated with SNAP+ISO. In addition, in the presence of SNAP, the increased cAMP response to ISO normally detected in the RI compartment is blunted due to the presence of excess PDE2 that, perhaps due to saturation of anchor sites in the RII compartment, accumulates also in the RI compartment.

Overall these data show that a physical coupling of PDE2 with the RII compartment is necessary both for the different response to ISO in RI and RII compartments and for determining the inversion of the cAMP gradients in the presence of SNAP.

5.2.2.1.10 Effect of nitric oxide synthase inhibition on basal cAMP levels

In section 5.2.2.1 data were presented that show that cAMP levels can be affected by cGMP fluctuations even in the absence of a GPCR agonist. The aim of this set of experiments was to assess the effect of reducing/abolishing cGMP production on cAMP basal levels. To this aim, cardiomyocytes were treated with L-NAME (L-Nitroarginine methyl ester) a nitric oxide synthase inhibitor. Nitric oxide synthases are enzymes that catalyze the production of nitric oxide (NO) from L-Arginine; NO, in turn, stimulates sGC to produce cGMP. As a read out for cAMP basal levels the basal FRET ratio was measured. The basal ratio is calculated as $Intensity_{CFP}/Intensity_{YFP}$ upon excitation at 430 nm at time zero in the absence of stimulus (both CFP and YFP intensities are subtracted of background). When binding to the FRET sensors cAMP causes a conformational change that increases the distance between CFP and YFP fluorophores, with consequent reduction of FRET and decrease in the emission intensity of YFP. This leads to an increase of the ratio (I_{CFP}/I_{YFP}) value. Accordingly, the higher the cAMP levels, the higher the ratio.

In the absence of L-NAME, the basal levels of cAMP detected in the two compartments were comparable: R=0.58±0.008 (n=24) in RI and R=0.57±0.017 (n=20) in RII; in cells pre-treated with L-NAME, basal levels of cAMP were higher in the RII compartment (R=0.62±0.008 (n=17)) as compared to the RI compartment (R=0.57±0.01 (n=15)), $p<0.05$ (Figure 5-25). In addition, in the RII compartment cells treated with L-NAME showed higher cAMP levels as compared to control cells. The increase in cAMP levels in RII can be explained as the consequence of L-NAME inhibition of NO synthase, reduced activation of sGC and reduced cGMP-mediated activation of PDE2 hydrolytic activity.

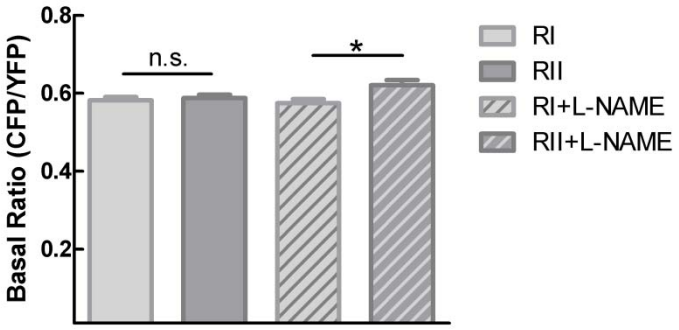


Figure 5-25 Effect of inhibition of nitric oxide synthetase on cAMP basal levels.

5.2.2.1.11 Activation of the pGC by ANP does not lead to an inversion of the ISO-induced cAMP gradients

Natriuretic peptides and NO donors activate the pGC and the sGC respectively and mediate different and sometime opposite effects on cardiac and vascular function. Experimental evidence (data presented in 5.1.2 and in (Castro et al 2006)) suggests that cGMP is not uniformly generated inside the cell but is spatially segregated and that different cGMP pools generated by the pGC and the sGC are shaped by different PDEs.

I therefore set out to investigate whether the generation of cGMP through the activation of the pGC by the atrial natriuretic peptide (ANP) would elicit on the cAMP response to ISO the same effect as SNAP. To this aim I pretreated RNCMs with ANP and I studied the reponse to ISO (Figure 5-26).

Control cardiomycytes expressing either RI_epac or RII_epac were treated with ISO 10 nM. In this set of experiments the two sensors detected a higher increase in the RII compartment as compared to the RI compartment with FRET changes of $\Delta R/R_0$=6.39±0.51% (n=18) for RII_epac and $\Delta R/R_0$=4.36±0.32% (n=13) for RI_epac, p<0.01. When cells were pretreated with ANP (100 μM), ISO generated the same response in RI and RII compartments with a FRET change of $\Delta R/R_0$=4.05±0.44% (n=21) for RI_epac and $\Delta R/R_0$=4.27±0.52% (n=20) for RII_epac. In the presence of ANP the response to ISO in the RII compartment was significantly lower than the response detected in control cells (p<0.001). Conversely, no difference could be appreciated in the RI compartment with or without ANP.

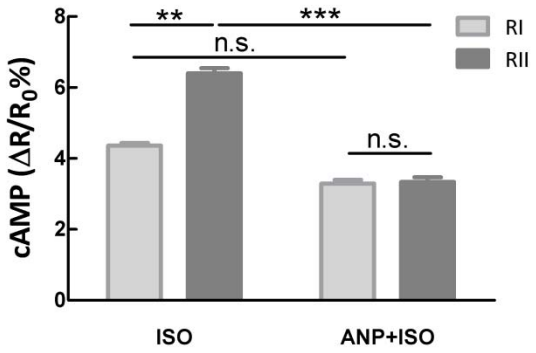


Figure 5-26 Effect of activation of the pGC on the cAMP response to ISO.

To better understand the mechanism responsible for the effect of ANP on the cAMP response to ISO, the effect of atrial natriuretic peptide (ANP, 100 μM) on cGMP levels in the RI and the RII compartments was assessed (Figure 5-27). When cardiomycytes expressing either RI_cygnet-2.1 or RII_cygnet-2.1 were treated with ANP 10 μM a higher increase in the RII compartment as compared to the RI compartment with FRET changes of $\Delta R/R_0$=2.73±0.51% (n=15) for RII_cygnet-2.1 and $\Delta R/R_0$=1.59±0.20% (n=18) for RI_cygnet-2.1, p<0.05.

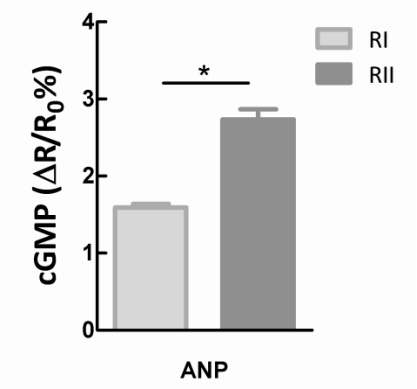


Figure 5-27 Effect of ANP on cGMP levels in the RI and RII compartments.

These data indicate that the pGC generates a larger pool of cGMP in the RII compartment. Based on these data, we can hypothesize that a pGC-PDE2 signalling domain is active in the PKA type II compartment and that activation of the pGC with ANP generates a cGMP pool that activates PDE2. PDE2 in turn controls and degrades the cAMP pool generated by the β-adrenergic stimulation. The lower amount of cGMP is generated in the RI compartment may be not sufficient to inhibit PDE3 activity in this compartment. To confirm this hypothesis, additional experiments in which RNCM are treated with ANP plus ISO in the presence of PDE2 and PDE3 inhibitors will be necessary.

5.2.2.1.12 Effect of SNAP on the PDEs basal activity in the RI and RII compartments

The data presented so far indicate that there is a functional association between PDE2 and the RII compartment and between PDE3 and the RI compartment. This functional coupling is responsible for the compartment-specific modulation of the cAMP response to ISO operated by cGMP. Our laboratory has previously demonstrated that in RNCMs there is a clearly detectable basal activity of the adenylyl cyclases that is counterbalanced by the activity of PDEs (Mongillo et al 2004). If a differential coupling of PDE2 and PDE3 with RII and RI, respectively, exists, it should be operative also in the control of basal levels of cAMP. Therefore, seeking further evidence for such differential coupling, I investigated the effect of SNAP on basal cAMP levels, in the absence of other

stimuli. For this, cardiomyocytes expressing either RI_epac or RII_epac were treated with SNAP alone (100 μM) (Figure 5-28).

Contrary to what expected, RI_epac and RII_epac in these conditions detected a comparable FRET change in the two compartments, with $\Delta R/R_0$=5.34±0.73% (n=16) for RI_epac and $\Delta R/R_0$=5.88±0.80% (n=19) for RII_epac. In fact, according to the proposed hypothesis, one would expect a higher cAMP increase in the RI compartment than in the RII compartment upon SNAP stimulation, as a result of PDE3 inhibition and PDE2 activation, respectively.

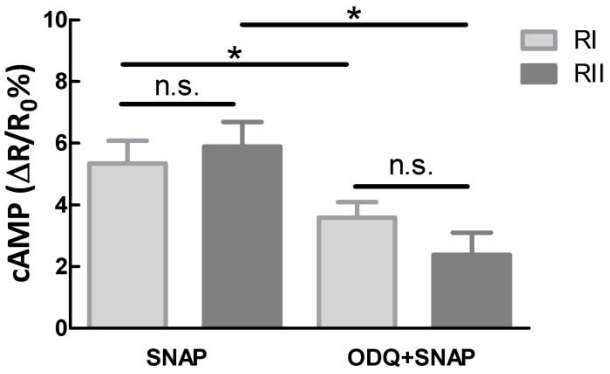


Figure 5-28 Effect of the inhibition of sGC on the response to SNAP.

Interestingly, though, when RNCMs were pretreated with the sGC inhibitor ODQ (10 μM) in combination with SNAP a FRET change of $\Delta R/R_0$=3.59±0.49% (n=8) for RI_epac and $\Delta R/R_0$=2.38±0.71% (n=14) for RII_epac was detected, suggesting that the effect of SNAP on cAMP levels in these conditions could not be attributable solely to the cGMP-mediated modulation of PDEs activity (Figure 5-28). It has been recently published that AC5 and AC6 can be activated by nytrosilation (Vila-Petroff et al 1999). To test whether SNAP-induced nitrosylation of ACs may be a contributing mechanism in these experimental conditions, cAMP changes were recorded in RNCMs that were pretreated with IBMX and subsequently challenged with SNAP (Figure 5-29). The aim of these experiments was to test the effect of SNAP in a condition in which all the PDEs were inhibited, to exclude any contribution of cGMP-mediated modulation of PDEs on cAMP

levels. When RNCMs expressing either RI_epac or RII_epac were challenged with IBMX the RI_epac sensor detected an increase in cAMP of $\Delta R/R_0$=4.67±0.87% (n=5) that was comparable with the increase detected by RII_epac, $\Delta R/R_0$=7.81±1.70% (n=6), confirming that in RNCMs there is a substantial basal activity of the ACs that is counterbalanced by the activity of PDEs. The subsequent addition of SNAP led to a further increase in cAMP in both compartments with RI_epac detecting a FRET change of $\Delta R/R_0$=7.68±2.35% (n=5) and RI_epac detecting a FRET change of $\Delta R/R_0$=9.45±2.30% (n=6). These data suggest that there might be a possible activation mechanism of the cyclases by SNAP. To confirm this mechanism additional experiments would be required; however, this effect seems to affect in a similar way both the RI and the RII compartments.

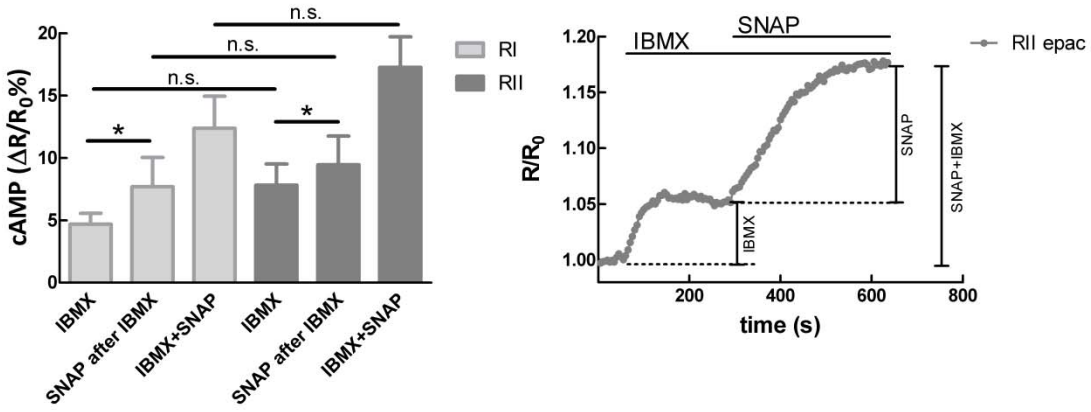


Figure 5-29 Effect of SNAP on cAMP levels.

It appears therefore that SNAP stimulation leads to an increase in cAMP levels, to which both ACs activation and PDEs modulation contribute, resulting in a comparable cAMP increase in both compartments.

A different way to establish if PDE2 and PDE3 differential coupling with RII and RI compartments, respectively, contributes to the maintenance of cAMP basal levels is to assess the effect of SNAP when PDE2 or PDE3 have been previously selectively inhibited. If such differential coupling is in place, one would expect that in the presence of inhibited PDE2, SNAP leads to a larger increase in the RII compartment than in the RI

compartment whereas in the presence of selectively inhibited PDE3 a larger increase in the RI compartment is expected. As shown in Figure 5-30 NRVMs expressing either RI_epac or RII_epac and treated with SNAP 100 μM alone showed a similar increase in cAMP in both compartments ($\Delta R/R_0$=5.68±1.17% for RI_epac (n=12) and $\Delta R/R_0$=3.65±0.34% (n=9) for RI epac). When cells were pretreated with the PDE2 inhibitor BAY, RII_epac detected a higher increase in cAMP than the RI_epac sensor ($\Delta R/R_0$=9.05±1.10% for RII_epac (n=8) and $\Delta R/R_0$=6.16±0.66% (n=6) for RI epac). This increase in cAMP was significantly higher as compared to the increase generated by SNAP alone ($p<0.05$). This result is in line with the hypothesis of an association of PDE2 with the RII compartment. The higher increase in cAMP levels generated by SNAP in the presence of BAY, indeed, can be attributable to the lack of the contribution of PDE2 hydrolytic activity in this compartment. Notably, PDE2 inhibition has no effect on the cAMP levels in the RI compartment.

When myocytes expressing either RI_epac or RII_epac were pretreated with CILO and challenged with SNAP the RI_epac sensor registered a higher increase in cAMP than the RII_epac sensor ($\Delta R/R_0$=7.98±1.18% for RI_epac (n=8) and $\Delta R/R_0$=4.62±0.86% (n=7) for RII epac). This increase was significantly higher as compared to the increase in cAMP generated by SNAP alone ($p<0.05$) and can be attributable to the cGMP-mediated inhibition of PDE3. In agreement with the original hypothesis, PDE3 inhibition has no effect on the cAMP levels in the RII compartment.

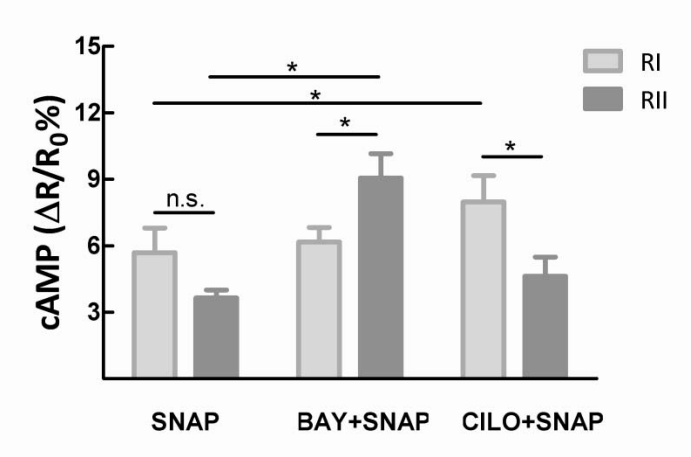


Figure 5-30 Effect of PDE2 and PDE3 inhibition on SNAP stimulation.

5.2.2.1.13 Effect of PDE5 inhibition on cAMP-mediated response to ISO

PDE5 is a cGMP-selective PDEs that is expressed at high levels in rat heart. Selective inhibition of PDE5 would, therefore, increase intracellular cGMP levels in these cells. In a preliminary set of experiments I have investigated if PDE5 inhibition with sildenafil may affect the cAMP response to ISO. In these experiments cardiac myocytes expressing either the RI_epac sensor or the RII_epac sensor were challenged with ISO (10 nM) and the PDE5 inhibitor SILD (1 µM). In Figure 5-31 two representative kinetics of the FRET change detected by the two sensors are shown. As expected, ISO stimulation generated a higher response in the RII compartment as compared to the RI compartment. Upon SILD application, a drop in the FRET ratio was detected by the RII_epac sensor, whereas a small increase was detected by the RI_epac sensor. These results can be interpreted as follows: SILD inhibition of PDE5 leads to an increase of cGMP that in the RII compartment leads to the activation of PDE2 that in turn degrades cAMP, hence the observed reduction in the FRET ratio. On the contrary, the SILD-induced cGMP increase inhibits PDE3 in the RI compartment, leading to a cAMP increase in this locale.

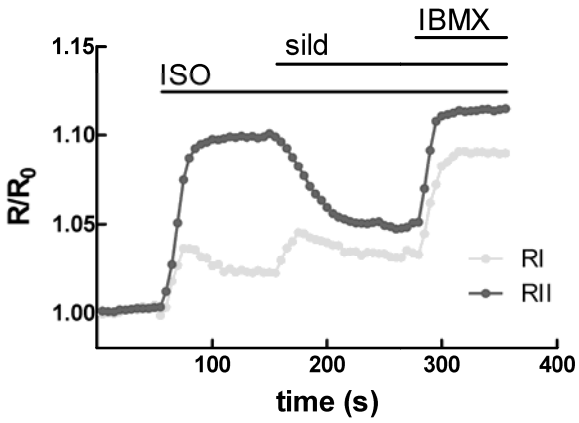


Figure 5-31 Representative kinetics of FRET changes detected by RI_epac and RII_epac upon sequential stimulation with ISO, SILD and IBMX.

5.2.3 *Discussion*

We have previously shown that the cAMP effectors PKAI and PKAII define exclusive signalling domains within which phosphodiesterases (PDEs) selectively contribute to cAMP spatial control thus preventing cross activation of PKA isoenzymes (Di Benedetto et al 2008). Several studies have also shown that cGMP can modulate PDE2 and PDE3 activity and can therefore potentially affect intracellular cAMP gradients and cAMP mediated responses.

In this study I provide evidence of an interplay occurring between cAMP and cGMP signalling pathways in RNCMs. FRET-based sensors targeted to the PKAI and PKAII compartments were generated in order to detect localized levels of cGMP and cAMP in these specific locales.

As previously published, myocytes stimulated with 10 nM isoproterenol generated a cAMP response that was significantly higher in the PKAII compartment than in the PKAI compartment. In order to assess the effect of cGMP signals on cAMP response to ISO in these two compartments, myocytes were pre-treated with the NO donor SNAP. NO released by SNAP activates the soluble guanylyl cyclase (sGC) leading to an increase in cGMP. Stimulation with isoproterenol in the presence of SNAP resulted in an inversion of the cAMP gradients in the PKAI and PKAII compartments. Specifically, we detected a significantly higher cAMP response in the PKAI compartment and a smaller cAMP response it the PKAII compartment as compared to cells treated with isoproterenol alone. The gradients inversion was completely abolished by the guanylyl cyclase inhibitor ODQ, suggesting that this mechanism is cGMP-dependent. In addition, cGMP-FRET imaging showed that this inversion is not due to a different level of cGMP generated upon SNAP in the two compartments. We therefore hypothesize that a functional association between PDE2 and the RII compartment and PDE3 and the RI compartment could underpin a compartment specific modulation of cAMP levels by cGMP. Thus, SNAP leads to a reduced level of cAMP in the PKAII compartment as a consequence of cGMP-mediated activation of PDE2, whereas the level of cAMP increases in the PKAI compartment as a result of cGMP-mediated PDE3 inhibition. This

hypothesis is further supported by experiments performed with selective inhibitors of PDE2 and PDE3. In fact, selective inhibition of PDE2 blocks the effect of SNAP in the RII compartment but does not affect the response to ISO in the RI compartment. In contrast, selective inhibition of PDE3 has no effect on cAMP levels in the RII compartment, confirming a selective coupling of PDE3 with the RI compartment.

Displacement of the endogenous PDE2 with a catalytically dead mutant of PDE2 completely abolished the effect of SNAP on the inversion of the gradients upon ISO stimulation, suggesting that a compartmentalized PDE2 in the RII compartment is necessary to achieve this compartment specific modulation.

A further confirmation of this topographical organization of PDE2 and PDE3 comes from experiments in which I investigated the effect of SNAP on the basal levels of cAMP. In the presence of the PDE2 selective inhibitor BAY 60-7550 the cAMP response to SNAP stimulation was higher in the RII compartment as compared to the response detected in the RI compartment, confirming a functional coupling between PDE2 and the RII compartment. On the contrary, selective inhibition of PDE3 with cilostamide led to a higher increase in cAMP in the RI compartment as compared to the RII compartment, demonstrating a functional association of PDE3 with the RI compartment.

Preliminary experiments have shown that inhibition of PDE5 led to distinct effect on cAMP levels in the RI and RII compartments. While in the RII compartment SILD generated a decrease in cAMP possibly via PDE2 activation, in the RI compartment it led to a small increase in cAMP, due to PDE3 inhibition. These data suggest that PDE5 may contribute to regulate cAMP-cGMP interplay. PDE5 have been shown to be localized in specific sub compartments in cardiac myocytes and to have access only to a fraction of cGMP produced in these cells, suggesting that PDE5 has a spatially specific role (Takimoto et al 2005a). These data shed light on possible new therapies in which local control on cAMP levels and associated responses can be achieved by modulation of cGMP levels. It has been documented that PDE5 inhibition has beneficial effects on cardiac hypertrophy, that it can reduce infarct size after coronary obstruction and that it

has a protective effect against necrosis and apoptosis of myocardial cells. However, which are the mechanisms responsible for these effects are not clear yet and it is interesting to speculate that at least in part, they maybe the consequences of cGMP modulation of cAMP levels.

Finally, activation of the particulate guanylyl cyclase (pGC) by ANP does not lead to an inversion of the cAMP gradients by ISO stimulation as observed with SNAP. The data indicate that ANP reduces the cAMP response to ISO in the RII compartment but does not affect the cAMP response in the RI compartment. Based on these results it can be envisaged a pGC-PDE2 signalling domain active in the RII compartment where the cGMP generated by the pGC blunts the cAMP response to β-AR stimulation via activation of PDE2. These findings further support the hypothesis of a compartmentalization of cGMP signalling in which not all the stimuli that rise cGMP generate the same downstream effect.

5.3 Further studies to define the topography of PDE2 and PDE3 in NRVM

5.3.1 *Introduction*

In cardiac myocytes, β-adrenergic signalling mediates the positive inotropic effect of cathecolamines, mainly through cAMP generation and PKA activation. By performing real time imaging in intact cardiac myocytes, our laboratory has previously established in NRCMs the contribution of individual PDEs isoforms in shaping and controlling cAMP levels in basal conditions and upon β-adrenergic stimulation (Mongillo et al 2004). 90% of the total PDE activity is provided by PDE4 and PDE3, whereas PDE2 represents only a minor fraction of this activity (less than 1%). PDE4 isoforms contribute for 60% of total basal activity with PDE4D subfamily contributing for 40% of the total PDE4 activity. In the presence of norepinephrine, inhibiting only 10% of the total PDE4 activity produces a dramatic effect on cAMP levels, whereas PDE3 inhibition shows little effect (Mongillo et al 2004). These data suggested a functional coupling between PDE4 and a subset of AC that are activated upon β-adrenergic stimulation. Conversely, when the entire pool of ACs is stimulated by forskolin, PDE3 inhibition shows an effect that is markedly greater than partial inhibition of PDE4. This suggests that PDE3 is mainly involved in regulating basal cAMP levels. Analysis of the kinetic of response of PDE3 and PDE4 inhibition during catecholamine stimulation indicates that PDE4 rather than PDE3 modulates the amplitude and duration of the cAMP response to β-agonists. These studies and work from other laboratories have also established that PDEs contribute to the compartmentation of cAMP singalling by modulating cAMP levels in selected functional compartments. Confocal studies have shown that different PDE families, including PDE4, PDE3 and PDE2, but also isoforms within the same family, such as PDE4B and PDE4D, possess a distinct intracellular localization (Mongillo et al 2004; Mongillo et al 2006). Spatial confinement of different PDEs isoforms to discrete compartments and their functional coupling to individual receptors can therefore provide an efficient mechanism to control local cAMP level in a stimulus specific manner.

More recently, work performed in our laboratory established that PKA isoforms constitute another level of compartmentalization of the cAMP signals. PKA type I and PKA type II, by binding to AKAPs, define distinct intracellular signalling compartments. They respond to distinct cAMP pools generated by different GPCR and phosphorylate specific downstream targets. The PKA type II isoform is selectively activated by β-adrenergic stimulation and mediates the phosphorylation of specific downstream targets involved in the ECC (excitation-contraction coupling), whereas PKA type I is selectively activated by different hormones (e.g. glucagone or PGE1). The cAMP pools associated with the PKAI or PKAII compartments are shaped by distinct subsets of PDEs as the selectivity of PKA isoforms activation is lost in the presence of the PDE inhibitor IBMX. However, which PDEs are engaged in the control of the cAMP response to β-AR stimulation in each single compartment has not been established.

AIMS:

- further define a topographical map of PDEs isoforms associated with the PKA type I and type II compartments
- dissect the role of individual PDEs in the control of the cAMP response to catecholamines in the PKA type I and type II compartments

5.3.2 *Results*

In a series of preliminary experiments cardiac myocytes expressing either RI_epac or RII_epac were challenged with ISO 10 nM (Figure 5-32). The two sensors detected a higher increase in the RII compartment as compare to the RI compartment with FRET changes of $\Delta R/R_0$=6.45±0.97% (n=11) for RII_epac and $\Delta R/R_0$=4.43±0.41% (n=13) for RI_epac ($p<0.05$), confirming previous results. Cells stimulated with ISO in combination with the non selective PDEs inhibitor IBMX generated the same cAMP response in both RI and RII compartments (Figure 5-32). These data confirmed previous data showing that IBMX dissipates the gradients between the two compartments suggesting that PDEs play a pivotal role in generating different intracellular gradients of cAMP.

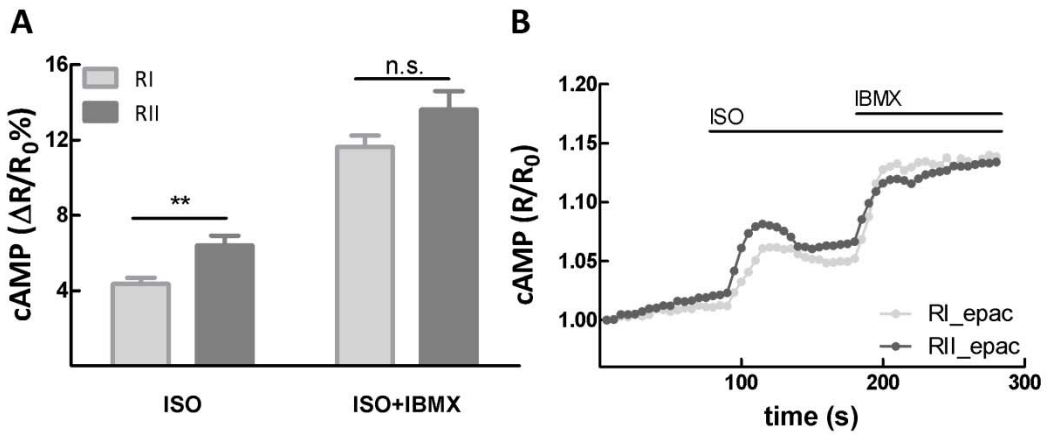


Figure 5-32 (A) Effect of ISO and IBMX on cAMP levels; (B) representative traces obtained with RI_epac and RII_epac on stimulation with ISO and IBMX.

In a second series of experiments, I set out to explore the role of individual cAMP-degrading enzymes in defining the cAMP response to ISO. To determine the contribution of each PDE family, cardiomyocytes expressing either RI_epac or RII_epac were treated with ISO 10 nM followed by selective inhibitors of PDE2, PDE3 or PDE4. In all the experimental groups, internal controls showed that ISO generated a higher response in the RII compartment as compared to the RI compartment, as expected. In the presence of ISO inhibition of PDE2 with BAY (Figure 5-33A and B), showed an increase in cAMP that was higher in the RII compartment as compared to the RI compartment (RII_epac detected a change of $\Delta R/R_0$=4.28±0.92% (n=10) whereas RI_epac detected a FRET change of $\Delta R/R_0$=2.44±0.38% (n=14), ($p<0.05$)). These data indicate that PDE2 is functionally coupled with the RII compartment and selectively degrades cAMP generated by ISO in this locale.

In contrast, in RNVMs challenged with ISO inhibition of PDE3 with CILO showed a significantly higher response in the RI compartment than in the RII compartment (RI_epac detected a FRET change of $\Delta R/R_0$=1.80±0.46% (n=11) whereas RII_epac detected a change of $\Delta R/R_0$=0.52±0.36% (n=9), ($p<0.05$)) (Figure 5-33C and D).

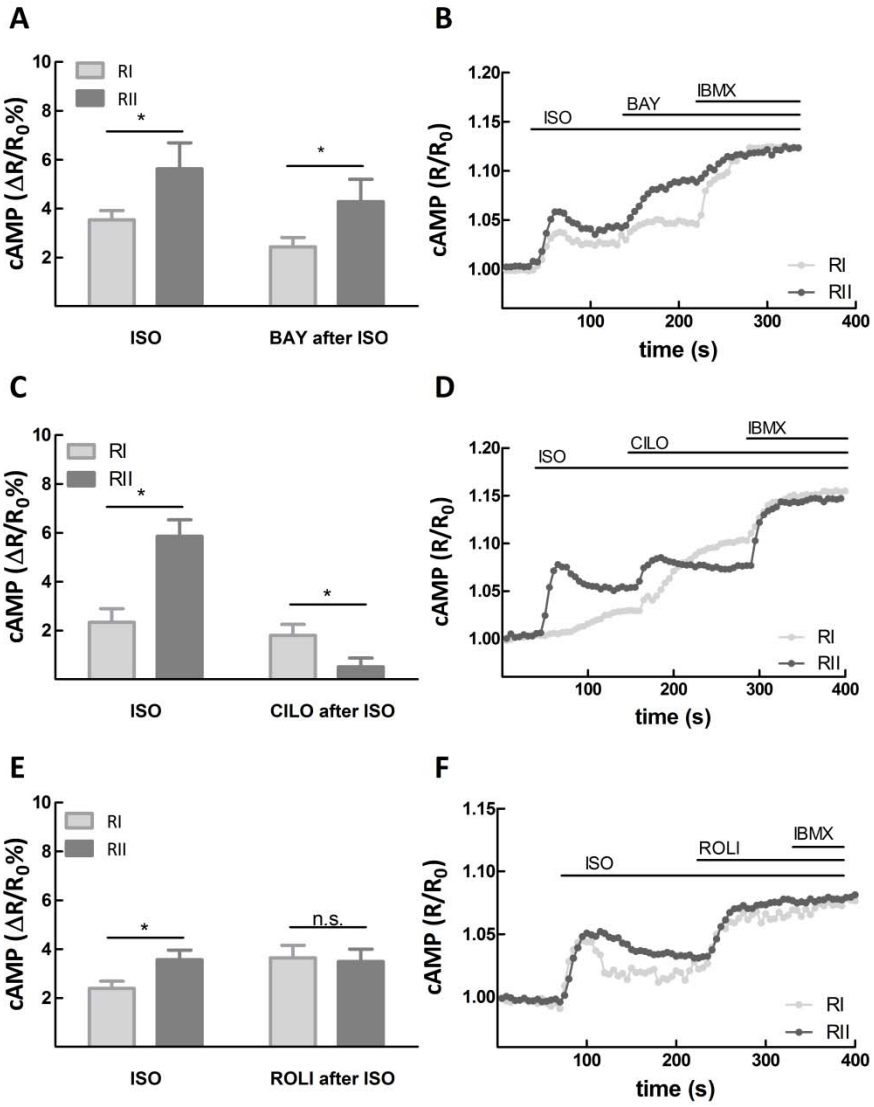


Figure 5-33 Effect of selective inhibition of PDE2 (A), PDE3 (C) and PDE4 (E) after ISO stimulation; in B, D and F representative kinetics obtained with RI_epac and RII_epac.

These data indicate that the main role of PDE3 is to degrade the ISO-generated cAMP within the RI compartment. Finally, myocytes treated with ISO and the PDE4 inhibitor rolipram (10 µM, ROLI), showed a comparable response to PDE4 inhibition in both compartments with FRET changes of $\Delta R/R_0$=3.65±0.51% (n=22) for RI_epac and $\Delta R/R_0$=3.49±0.51% (n=19)) (Figure 5-33E and F). These data suggest that PDE4 may be equally associated with both compartments.

5.3.3 *Discussion*

Overall, the data presented above are compatible with a functional association of PDE2 with the RII compartment and PDE3 with the RI compartment. This different association underpins the compartment specific modulation of cAMP levels by cGMP signals described in 5.2.2. In addition, the effects of selective inhibition of cAMP-PDEs points out a different role of the different PDE families. Based on the data presented above are compatible with a model whereby PDE4 operates equally in both compartments to modulate the cAMP response to β-adrenergic stimulation. In this respect, PDE4 may constitute the primary mechanism to control and contain the cAMP generated by this stimulus and act as a "master regulator" of cAMP pools boundaries. The fact that PDE4 is the most abundant PDE expressed in rat cardiomyocytes and responsible for the 60% of total PDE basal activity is compatible with such predominant role of PDE4. On the contrary, the control on cAMP levels exerted by PDE2 and PDE3 appears to be restricted to selective compartments. In particular PDE2 appears to control a pool of cAMP that is confined to the PKA-II compartment whereas PDE3 appears to control a cAMP pool confined to the PKA-I compartment. Notably, previous localization studies have shown that PDE2 and PDE3 possess a different pattern of localization in cardiac myocytes with PDE2 localizing at the sarcomeric Z-lines whereas PDE3 displays non homogeneous trabecular localization, compatible with localization on internal membranes (Mongillo et al 2004; Mongillo et al 2006). Consistent with the model proposed, PDE4 shows a wider subcellular distribution, with localization at both the Z and the M line as well as at the intersarcomeric space (Mongillo et al 2004).

5.4 Functional outcomes of the cGMP-mediated inversion of the cAMP gradients

5.4.1 *Introduction*

In the heart cAMP is a key regulator of excitation–contraction coupling. β-adrenergic stimulation represents the main physiological mechanism to face increases in circulatory demand, mediating positive inotropic and positive lusitropic effects. These effects are exerted by PKA mediated phosphorylation of pivotal target proteins. During systole PKA phosphorylates L-type Ca^{2+} channels (DHPRs, dihydropyridine receptors) and the ryanodine receptor (RyR), thereby increasing the amount of Ca^{2+} ions available for the sarcomere to contract. These events are thought to be largely responsible for the positive inotropic effect of β-adrenoceptor stimulation. At diastole, PKA controls the reuptake of Ca^{2+} in the sarcoplasmic reticulum by phosphorylating phospholamban (PLB) and thus blocking the inhibitory effect that PLB exerts on the sarcoplasmic reticulum Ca^{2+} ATPase pump (SERCA). Furthermore, PKA phosphorylates sarcomeric proteins, such as the myosin binding Protein C and troponin I (TnI), exerting control also on their sensitivity to Ca^{2+} and significantly contributing to the lusitropic effects of β-adrenergic stimulation.

Catecholamine-mediated control of the contractile performance of the heart is a vital mechanism that allows the necessary adjustments to confront imminent danger or to prevent abrupt drops in blood pressure. Enhanced and sustained cardiac adrenergic signalling, however, may contribute to disease states such as heart failure (HF) (Floras, 2003). It is therefore imperative that the cAMP signals are delivered correctly to appropriately tune the contractile response to catecholamines. Previous results in our laboratory have shown that key proteins involved in the catecholamine-mediated regulation of cardiac contractility are under control of restricted cAMP pools that selectively activate subsets of PKA isoenzymes. ISO has been shown to preferentially activate PKA type II whereas PGE1, glucagon, and glucagon-like peptide activate PKA type I. Activation of PKAII by ISO but not by PGE1 leads to an increase in phosphorylation of PLB, TnI and β_2–adrenergic receptors. PGE1 stimulation on the

contrary, decreases phosphorylation of PLB and TnI through a G_i-mediated mechanism. In the previous chapter I have shown that cGMP signals can alter cAMP levels in a compartment dependent manner, by modulating the activity of PDE2 and PDE3. In particular, I have presented data showing that the activation of the sGC leads to an inversion of the cAMP gradients generated in response to ISO, leading to a higher increase of cAMP in the PKA type I compartment and to a comparably lower increase in cAMP in the PKA type II compartment. I next investigated if such inversion translates in an inversion of the PKA activation levels in the two compartments and if this, in turn, affects the phosphorylation level of critical PKA substrates.

AIMS:

- investigate the effect of SNAP stimulation on PKA isoforms activation
- study the effect of SNAP on the catecholamine-induced PKA-mediated phosphorylation of downstream targets (PLB, TnI, β-AR)

5.4.2 *Results*

5.4.2.1.1 SNAP alters the PKA isoforms selective activation induced by ISO

My aim here was to assess whether alteration of cAMP levels induced by cGMP has an effect on the activation of different PKA isoforms. To detect PKA activity I took advantage of the FRET-based A kinase activity reporter AKAR3 developed by Zhang and colleagues (Allen & Zhang 2006; Ni et al 2006; Zhang et al 2001). AKAR3 is a four-part chimeric protein consisting of ECFP, a phosphoamino acid binding domain FHA1 (14–3-3τ), a PKA-specific phosphorylatable peptide sequence (LRRASLP) and CpV E172 (circular permutated version of the YFP named venus (Nagai et al 2004)) (Figure 5-34A). In the unphosphorylated state of the probe, the two fluorophores are too far away for FRET to occur. When PKA phoshorylates the substrate domain, the intramolecular binding of the substrate to the FHA1 domain drives a conformational change, the two fluorophores move closer and FRET increases (Figure 5-34B).

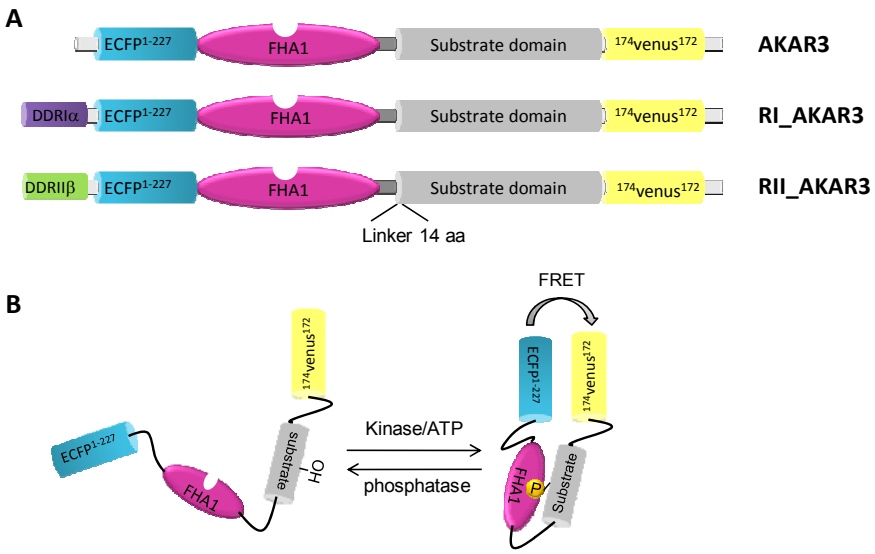


Figure 5-34 (A) Schematic representation of AKAR3 sensors; (B) schematic representation of the mechanism of action of AKAR3 sensor.

In the laboratory this sensor has been engineered in order to monitor PKA activation in the RI and RII compartments by fusion of the dimerization/docking domain from either RIα (amino acids 1 to 64) or RIIβ (amino acids 1 to 49) to the N-terminus of the soluble AKAR3 (Figure 5-34A). Both RI_AKAR3 and RII_AKAR3 showed the typical intracellular localization of the other RI/RII targeted sensors in cardiac myocytes (Figure 5-35).

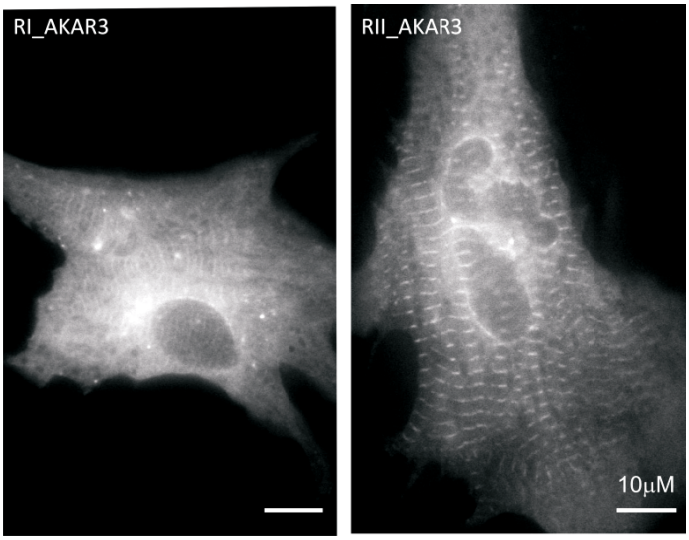


Figure 5-35 RNCMs expressing RI_AKAR3 and RII_AKAR3.

Using these targeted sensors of PKA-mediated phosphorylation I set out to investigate the effect of SNAP on PKA isoforms activation (Figure 5-36). Cardiac myocytes expressing either RI_AKAR3 or RII_AKAR3 were challenged with ISO 1 nM. Upon ISO stimulation a higher FRET change was detected by RII_AKAR ($\Delta R/R_0$=7.10±1.32% (n=6)) than RI_AKAR ($\Delta R/R_0$=0.43±0.52% (n=7)), p<0.001, suggesting a prominent activation of PKA in the RII compartment. In striking contrast, when cells were pretreated with SNAP, ISO generated a higher response in the RI compartment ($\Delta R/R_0$=8.60±1.86% (n=6)), as compare to the RII compartment ($\Delta R/R_0$=3.17±1.18% (n=7), p<0.05. These data indicate that alteration of cAMP levels in the RI and RII compartments translates in an inversion of the PKA-mediated phosphorylation activity in the two compartments resulting in a prominent activation of PKA type I over PKA type II.

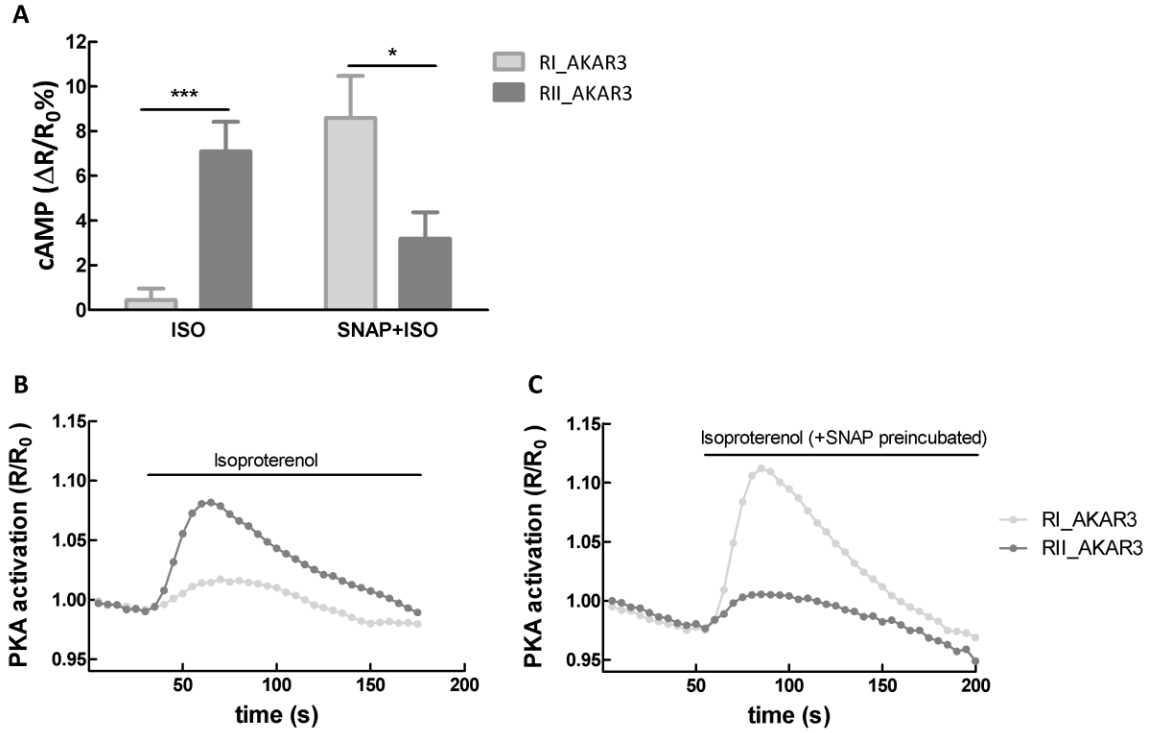


Figure 5-36 (A) Effect of ISO and SNAP+ISO on PKA activation; (B) representative traces obtained with RI_AKAR3 and RII_AKAR3 in RNCMs stmulated with ISO; (C) representative traces obtained wih RI_AKAR3 and RII_AKAR3 in RNCMs pretreated with SNAP and stimulated with ISO.

5.4.2.1.2 Effect of SNAP on the catecholamine-induced phosphorylation of PKA downstream targets

The aim of the following set of experiments was to investigate whether the effect of SNAP on the activation of the PKA type I and type II has an effect on the phosphorylation of known PKA type II downstream targets. Here I set out to study the phosphorylation level of the PKA target TnI. Western blot analysis showed that, as expected, stimulation with ISO (10 nM) leads to an increase in the phosphorylation level of TnI (Figure 5-37). Interestingly, RNVMs treated with ISO (10 nM) and SNAP (100 μM) show a further increase in TnI phosphorylation and this effect is prevented by the soluble guanylyl cyclase inhibitor ODQ.

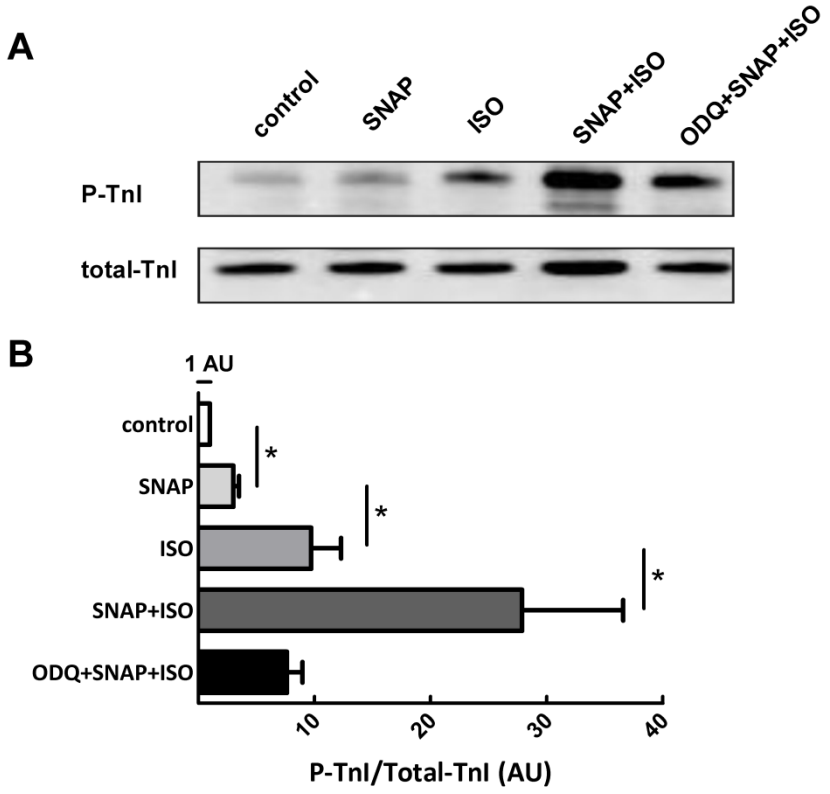


Figure 5-37 (A) Representative western blot of TnI and Phospho-TnI in RNCMs lysates treated as indicated; (B) WB quantification (mean of 5 separate experiments).

These data indicate that SNAP can affect TnI phosphorylation levels. These results should however be interpreted with caution as it has been shown that TnI, in addition to being a substrate for PKA, can also be phosphorylated by PKG at Ser23/24. Therefore further experiments in the presence of PKG inhibitors will be necessary in order to establish the contribution of a cGMP-PKG activity on the observed increase in TnI phosphorylation upon SNAP+ISO stimulation. However, in support of a contribution of cAMP-PKA to the observed increased phosphorylation of TnI, it should be observed that the effect of SNAP alone does only marginally, although significantly, increase TnI phosphorylation, suggesting that the large increase in phosphorylation observed in the presence of SNAP+ISO may largely depend on cAMP and PKA activity.

5.4.3 *Discussion*

In this set of experiments I investigated the effect of NO on the activation of PKA isoforms upon β-AR stimulation. To monitor PKA activity in RI and RII compartments I used two FRET-based A Kinase activity reporters (AKAR) specifically targeted in these compartments. By performing real time FRET-experiments I found that the NO donor SNAP inverts the preferential activation of PKA isoforms upon ISO stimulation yielding a higher activation of PKA type I than PKA type II. These results are in agreement with my previous finding that SNAP leads to an inversion of the cAMP gradients in the PKA type I and type II compartments upon catecholamine stimulation. In addition, these results show that this alteration in PKA activation affects the phosphorylation level of the downstream PKA target TnI as western blot analysis of phospho-TnI shows increased phosphorylation after stimulation with SNAP and ISO. Interestingly, such increase is prevented by the sGC inhibitor ODQ. Future experiments will extend this analysis to other PKA targets such as β-AR and PLB.

6 CONCLUSION AND PERSPECTIVES

In this thesis I investigated the intracellular dynamics of cAMP and cGMP in cardiac myocytes by performing FRET-based, real time imaging in intact living cells.

In the first part of my project I focused on cGMP signalling. My studies revealed that intracellular cGMP generated by different stimuli is not uniformly distributed within the cell but rather it is compartmentalized into distinct microdomains. Phosphodiesterases (PDEs), the only cGMP degrading enzymes, play a key role in shaping and organizing these cGMP pools. I found that cGMP domains are regulated by different families of PDEs. In particular, PDE2 controls the cGMP pool generated by the particulate guanylyl cyclase whereas the pool of cGMP generated by the soluble guanylyl cyclase is shaped by both PDE2 and PDE5. In addition I found that cGMP generated via β_3-AR activation is controlled exclusively by PDE5, revealing a further compartmentalization mechanism.

In the second part of my PhD I investigated whether interplay between cAMP and cGMP signalling pathways occurs in RNCMs. In particular, I focused on the impact of cGMP signals on the cAMP response to ISO in the compartments defined by the two isoforms of PKA, PKA type I and PKA type II. My results indicate that in RNCMs cAMP and cGMP pathways are interconnected and that cGMP can alter cAMP levels and cAMP mediated responses through the modulation of PDE2 and PDE3 activity. I found that the NO donor SNAP leads to an inversion of the cAMP gradients upon ISO stimulation. This inversion is dependent on cGMP generation and on the compartment specific modulation of PDE2 and PDE3. In particular my data indicate that there is a functional association between PDE2 and the RII compartment and PDE3 and the RI compartment.

In addition, I showed that this inversion is dependent on the physical coupling between PDE2 and the RII compartment as the overexpression of a catalytically dead PDE2 completely abolished the effect of SNAP. Future experiments are planned to displace the endogenous PDE3 to investigate the relevance of its association with the RI compartment in this mechanism.

I investigated also the role of different families of PDEs in shaping the cAMP response to ISO in the RI and RII compartment. With this study we gain more information on the topographical organization of PDEs in cardiac myocytes. We propose a model in which PDE4 act as a master regulator of boundaries between compartments, whereas PDE2 and PDE3 control on cAMP levels is more restricted to selective compartments. These results confirm previous data on the role of spatially confined PDEs in the regulation of specific associated cAMP pool and support the hypothesis of functional association of PDE2 and PDE3 with the RII and RI compartment, respectively.

Finally I showed that the cGMP-induced inversion of the cAMP levels affects the selectivity of PKA isoforms activation. I found than ISO stimulation in the presence of SNAP shifted the selectivity of ISO-induced activation of PKA isoforms from PKA type II to PKA type I. I set out to investigate whether alteration of cAMP levels and PKA activation had any consequences on the phosphorylation pattern of selective downstream targets of PKA. Stimulation of RNCMs with SNAP and ISO led to an increase in TnI phosphorylation as compared to the stimulation with ISO alone. This increase in TnI phosphorylation is prevented by the treatment with the sGC inhibitor ODQ suggesting that the large increase in phosphorylation observed in the presence of SNAP plus ISO may depend on cGMP-modulated cAMP signals and PKA activity. Future experiments will extend this analysis to other PKA targets such as β-AR and PLB.

Precisely time-regulated and space-confined activity of the cAMP/PKA transduction pathway is essential for an accurate control of the ECC system and is necessary for proper catecholamine response. In the light of the new findings reported here it is possible to envisage that the negative inotropic effect exerted by NO donors, so far largely attributed to PKG-activation, could be due, at least in part, to an alteration of PKA isoforms activation and consequent change in targets phosphorylation, with important consequences on how the cAMP and cGMP pathways may be targeted for the treatment of heart disease.

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