Nociceptin/orphanin FQ inhibits ischaemia-induced glutamate efflux from rat cerebrocortical slices

R. M. Nelson,[1,4,CA] G. Calo',[2] R. Guerrini,[3] A. H. Hainsworth,[1] A.R. Green[1,5] and D. G. Lambert[4]

[1]School of Pharmacy and Pharmaceutical Sciences, DeMontfort University, Leicester, UK; Departments of [2]Pharmacology and [3]Pharmaceutical Sciences, University of Ferrara, Italy; [4]Department of Anaesthesia LRI, Leicester LE1 5WW; [5]AstraZeneca, Charnwood, Loughborough, UK

[CA,4]Corresponding Author and Address

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Nociceptin/orphanin FQ (NC), the endogenous ligand for the G-protein coupled nociceptin receptor (NCR), has a modulatory role in various physiological processes including neurotransmitter release. We have examined the effects of NC, the analogues NC(1-13)NH_2 and [F/G]NC(1-13)NH_2 and the competitive antagonist [Nphe^1]NC(1-13)NH_2 (Nphe^1) on glutamate efflux during an acute simulated ischaemic challenge in rat cerebrocortical slices. The increase in glutamate efflux seen with ischaemia was inhibited by NC (EC_{50} 250 nM). At micromolar concentrations, the analogues were found to have a similar effect on glutamate efflux compared to NC. In all cases, inhibition of glutamate efflux was abolished by Nphe^1. These results suggest a neuroprotective action for NC. *NeuroReport* 11:1–4 © 2000 Lippincott Williams & Wilkins.

Key words: Glutamate efflux; Ischaemia; Neuroprotection; Nociceptin/orphanin FQ; Stroke

INTRODUCTION

Nociceptin/orphanin FQ (NC), is a 17 amino acid peptide which acts as the endogenous agonist of the $G\alpha_{i/o}$ G-protein coupled nociceptin receptor (NCR). We have been involved in the identification and characterization of novel peptide ligands with activity at the NCR [1,2]. NC(1-13)NH_2 is the shortest NC fragment to retain full biological activity. With this template we have identified [F/G]NC(1-13)NH_2 which displays a range of pharmacological activities from pure antagonist to full agonist, although its true pharmacological profile is likely to be as a partial agonist [1]. In addition, shifting the benzyl side chain of Phe^1 from the carbon to the nitrogen atom to produce [Nphe^1]NC(1-13)NH_2 yields a pure, selective and competitive (pA_{2-6}) NCR antagonist [3].

NCR is widely expressed throughout the CNS, mainly on nerve fibres and neuronal cell bodies, suggesting a presynaptic action for NC [1], although the gene encoding for the NCR has been found at other sites, including glia [4]. The activation of NCR is involved in a variety of central and peripheral functions including the impairment of spatial learning, reduction of gastrointestinal motility and the production of analgesia or hyperalgesia depending on the site of administration [1]. Of particular interest is the ability of NCR activation to inhibit the release of a variety of neurotransmitters including serotonin [5] and glutamate [6], suggesting both anxiolytic and anti-epileptic actions attributable to NC. As other compounds with anxiolytic and anti-epileptic actions have been shown to inhibit ischaemia-induced glutamate efflux *in vitro* [7] and to be neuroprotective *in vivo* [8,9] in several models of cerebral ischaemia, we have investigated the *in vitro* effects of NC and the analogues NC(1-13)NH_2 and [F/G]NC(1-13)NH_2 on glutamate efflux in response to an acute ischaemic challenge. In addition, the effects of the antagonist [Nphe^1]NC(1-13)NH_2 have been studied.

MATERIALS AND METHODS

Materials: NC, NC(1-13)NH_2, [F/G]NC(1-13)NH_2 and [Nphe^1]NC(1-13)NH_2 were synthesized at one of our institutes as described previously [2,10]. Glutamate dehydrogenase was obtained from Calbiochem-Novabiochem Corp., Nottingham, UK and cell microsieve (100 μm) mesh was purchased from BDH, Dorset, UK. All other reagents were purchased from Sigma Chemical Co., Dorset, UK.

Preparation of slices: Female Wistar rats (200–250 g) were killed by cervical dislocation, the cerebral cortex rapidly dissected and placed in ice-cold Krebs bicarbonate buffer containing (in mM) NaCl 115, KCl 4.6, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, glucose 8.8, pH 7.4.

Prisms of cerebral cortex (cross-sectional area 350 μm^2) were prepared and agitated in a shaking water bath at 37°C for 40 min. Equivalent volumes of slice suspension were aliquoted into mesh baskets and subjected to a 30 min preincubation period at 37°C in aerated HEPES buffered saline (control HBS) containing (in mM) NaCl 140, KCl 2.5, $MgCl_2$ 0.5, $CaCl_2$ 2, HEPES acid 10, glucose 10, pH 7.4.

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Experimental design: Baskets were incubated for 30 min in either 2 ml control HBS or hypoxic/hypoglycaemic (no added glucose, bubbled with nitrogen: ischaemia) HBS with the appropriate gas gently bubbled over the surface throughout the experiment (control: air, ischaemia: nitrogen). To generate a concentration-response curve, the ischaemic medium contained nociceptin (NC, 1 nM–30 μM) with peptidase inhibitors (capotril, amastatin, bestatin and phosphoramidon, 30 μM each). In a separate set of experiments, slices were incubated with NC, NC(1-13)NH_2 or [F/G]NC(1-13)NH_2 (1 μM) with or without the competitive antagonist Nphe^1 (30 μM). At the end of the incubation period, baskets were transferred to 1% Triton X-100 for 5 min to lyse cell membranes and release any remaining glutamate. The supernatants were spun at 12 000 r.p.m. at 4°C, for 5 min. Glutamate was measured fluorimetrically (excitation 366 nm, emission 450 nm), using the conversion of $NADP^+$ to NADPH by glutamate dehydrogenase using minor modifications of published methods [10].

Data analysis: All data are presented as mean ± s.e. mean unless stated otherwise. Glutamate efflux data is expressed as a percentage of the total glutamate present (i.e. $[Glut_L/(Glut_L + Glut_T)] \times 100$, where $Glut_L$ is glutamate released during the 30 min incubation and $Glut_T$ is glutamate released into the Triton X-100). EC_{50} values were calculated where appropriate by fitting Langmuir–Hill curves to glutamate efflux data after subtraction of the percentage control efflux. Curves were fitted by least squares non-linear regression (Graphpad Prism). Statistical analysis was performed using repeated measures ANOVA with Tukey's multiple comparisons (Graphpad Prism). Results were considered significant at a value of $p \leq 0.05$.

RESULTS

Effect of nociceptin on ischaemia-induced glutamate efflux: There was a marked increase in glutamate efflux under ischaemic conditions which was inhibited in a concentration-dependent manner by nociceptin (NC, Fig. 1). The EC_{50} was 0.25 μM (95% confidence intervals 0.1–0.6 μM) with an E_{max} of 51.6 ± 2.8% ($n = 5$). The inhibition of glutamate efflux under ischaemic conditions produced by 1 μM NC was abolished in the presence of 30 μM Nphe^1 ($n = 6$, Fig. 2). In control or ischaemic conditions, peptidase inhibitors and Nphe^1 alone were found to have no effect on glutamate efflux whilst NC had no significant effect on control values (data not shown).

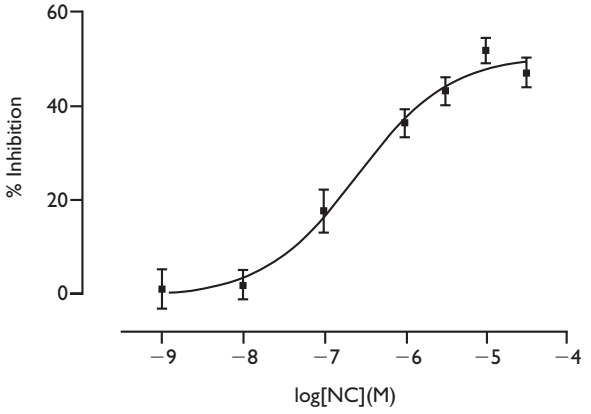

Fig. 1. Effect of increasing concentrations of nociceptin on ischaemia-induced glutamate efflux from rat cortical prisms over 30 min. Results reported as the percentage inhibition of the ischaemia-induced efflux are shown as mean ± s.e.m. ($n = 5$). The calculated EC_{50} for nociceptin (NC) was 0.25 μM; Hill coefficient = 0.75.

Effect of NC(1-13)NH_2 and [F/G]NC(1-13)NH_2 on ischaemia-induced glutamate efflux : NC(1-13)NH_2 ((1-13)) and [F/G]NC(1-13)NH_2 ([F/G]), at a concentration of 1 μM, both produced an inhibition of glutamate efflux which, in each case, was abolished by 30 μM Nphe^1 ($n = 5$, Fig. 3). The inhibition produced by [F/G] and (1-13) was

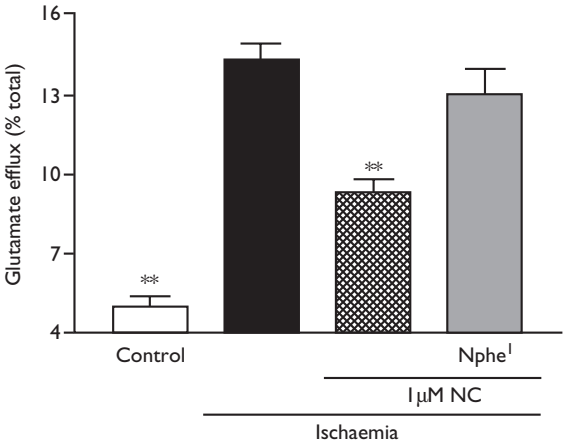

Fig. 2. Effect of control conditions or simulated ischaemia on glutamate efflux from rat cortical prisms over 30 min and the effect of nociceptin (NC, 1 μM). The effect of [Nphe^1]NC(1-13)NH_2 (Nphe^1, 30 μM) on the inhibitory effect of nociceptin is also shown. Results shown as mean ± s.e.m. ($n = 6$). **Different from ischaemia ($p \leq 0.01$). Addition of Nphe^1 abolished the inhibitory effect of nociceptin on ischaemia-induced glutamate efflux.

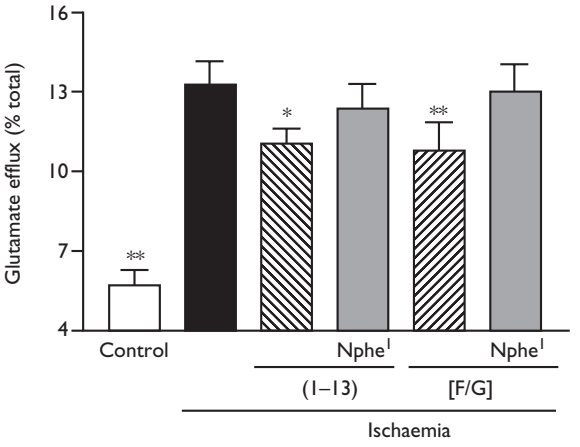

Fig. 3. Effect of NC(1-13)NH_2 and [F/G]NC(1-13)NH_2 on ischaemia-induced glutamate efflux from rat cortical prisms over 30 min. The effect of Nphe^1 on the inhibitory action of NC(1-13)NH_2 ((1-13)) and [F/G]NC(1-13)NH_2 ([F/G]) is also shown. Results shown as mean ± s.e.m. ($n = 5$). Different from ischaemia ** $p \leq 0.01$, * $p \leq 0.05$. Both (1-13) and [F/G] (1 μM) reduced the glutamate efflux seen under simulated ischaemic conditions. Addition of Nphe^1 (30 μM) abolished the inhibitory effect of both (1-13) and [F/G].

$33.5 \pm 4.8\%$ and $29.01 \pm 9.1\%$ respectively, slightly lower than that seen for 1 μM NC ($36.2 \pm 3\%$). Under control conditions, (1-13) and [F/G] had no significant effect on glutamate efflux (data not shown).

DISCUSSION

This study is the first to show that nociceptin, the endogenous ligand for the opioid-like receptor NCR, inhibits glutamate efflux from rat cerebrocortical slices in response to an acute ischaemic challenge. This inhibition occurs in a concentration dependent manner with an EC_{50} of 250 nM. Furthermore, this study demonstrates the NC analogues $NC(1\text{-}13)NH_2$ and $[F/G]NC(1\text{-}13)NH_2$, at a concentration of 1 μM, also inhibit ischaemia-induced glutamate efflux, producing levels of inhibition similar to those seen with 1 μM NC. K^+-evoked glutamate release from rat cerebrocortical slices has been shown to be inhibited by nociceptin with an EC_{50} of 51 nM [1], having a greater potency than observed in the present study. This disparity probably reflects the different contributions of synaptic release to the glutamate efflux measured by the two different methods. All of the inhibitory effects seen with NC and the analogues investigated were abolished by the selective antagonist $[Nphe^1]NC(1\text{-}13)NH_2$. The observation that $[Nphe^1]NC(1\text{-}13)NH_2$ alone did not increase ischaemia-induced glutamate levels in the extracellular medium suggests that endogenous nociceptin released during ischaemia does not control glutamate efflux under ischaemic conditions.

It is interesting to note that in the present study $[F/G]NC(1\text{-}13)NH_2$ acts as an agonist of the NCR as this analogue has previously been shown to range in function from an antagonist to full agonist depending on the tissue preparation studied [1]. The differing pharmacological actions of $[F/G]NC(1\text{-}13)NH_2$ may be due to differences in receptor expression levels in the different tissues. In transfected cell lines, $[F/G]NC(1\text{-}13)NH_2$ acts as an antagonist if low expression levels of the NCR occur, whilst it acts as a full or partial agonist in cells with high expression levels [11]. As high levels of receptor expression have been shown in the rat cortex [12], this would account for the agonist activity of $[F/G]NC(1\text{-}13)NH_2$ reported here.

Previous studies have demonstrated various cellular effects due to NCR activation, including decreased cAMP accumulation [13,14], increased potassium conductance [15,16] and inhibition of high voltage activated calcium channels [17,18]. It appears probable that NC exerts its effect on ischaemia-induced glutamate efflux either by increasing potassium conductance or inhibiting calcium channels.

It is generally accepted that one of the initiating factors for neuronal death in cerebral ischaemia is the disruption of ionic gradients and neuronal depolarization, leading to cytotoxic levels of calcium accumulating within the cell [19,20]. In animal models, voltage operated calcium channel antagonists are neuroprotective, reducing post-ischaemic brain oedema and infarct size [21,22]. Furthermore, P/Q and N type calcium channel antagonists attenuate the massive increases in extracellular glutamate that occur during ischaemia [23]. As nociceptin has been shown to inhibit all of these channels [17,18], it is possible that the effects seen with nociceptin in this study contribute to voltage-operated calcium channel inhibition. However, in previous work, we have shown that removal of extracellular calcium has no effect on ischaemia-evoked glutamate efflux in our system [7], suggesting that the inhibitory action of nociceptin is not due to calcium channel antagonism.

We have previously shown that the neuroprotective GABA-mimetic chlomethiazole reduces glutamate efflux, probably due to an increase in chloride conductance causing decreased neuronal excitability [7]. Increasing potassium conductance would also reduce neuronal excitability, thus limiting cytotoxic calcium levels [19,24]. Nociceptin receptors have been shown to activate potassium currents in neurons within rat locus coeruleus [15] and ventromedial hypothalamic slices [16], both being of the inward rectifier type. Reductions in inwardly rectifying potassium currents have been shown in glial cells surrounding ischaemic brain lesions [25]. As NCR gene expression has been shown in glial cultures [4], it is possible that NCR activation, through its ability to increase potassium currents, helps to maintain local ion homeostasis, thereby limiting the hyperexcitability associated with ischaemia. These findings suggest that the inhibition of ischaemia-induced glutamate efflux due to NCR activation seen in this study may be due to increasing potassium conductance, leading to a reduction in cell excitability.

CONCLUSION

The present study demonstrates the ability of nociceptin and its analogues $NC(1\text{-}13)NH_2$ and $[F/G]NC(1\text{-}13)NH_2$ to inhibit ischaemia-induced glutamate efflux *in vitro*. We hypothesize that this effect is due to the ability of NCR activation to increase potassium conductance, producing a reduction in neuronal excitability. The data presented here indicate that NCR activation may have neuroprotective properties.

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