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Neuropeptide FF receptors couple to a cholera toxin-sensitive G-protein in rat dorsal raphe neurones

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Abstract

In rat dorsal raphe neurones, nociceptin (300 nM) reduced the peak $[Ca^{2+}]_i$ transient, triggered by depolarization, by 36.7 ± 1.8% ($n = 46$). This effect of nociceptin decreased to 16.7 ± 2.9% ($n = 18$) after pre-treatment of the neurones with pertussis toxin (5 μg/ml, 2–6 h) but was unchanged (37.4 ± 2.1%, $n = 44$) after pre-incubation with cholera toxin (5 μg/ml, 2–6 h). This suggests that, in dorsal raphe neurones, the ORL1 receptor couples to inhibitory ($G_{i/o}$) G-proteins. The neuropeptide FF analogue, [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (10, 100, 1000 nM), acted as an anti-opioid and reduced the effect of nociceptin (300 nM, 30 s) by 62.0 ± 3.3% ($n = 28$). Following pre-incubation with cholera toxin (5 μg/ml, 2–6 h) [D-Tyr^1, (N-Me)Phe^3] neuropeptide FF was unable, at the three concentrations tested, to block nociceptin activity. We conclude that, in rat dorsal raphe neurones, neuropeptide FF receptors couple to stimulatory G-proteins (G_s). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Neuropeptide FF; Nociceptin; Ca^{2+}, intracellular; Pertussis toxin; Cholera toxin; Dorsal raphe

1. Introduction

Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2), first isolated from bovine brain (Yang et al., 1985), acts through specific receptors that are not recognized by opiates and which are located in spinal cord and brain areas related either to the processing of nociceptive messages or to sensory input and visceral functions (Roumy and Zajac, 1998). The cDNAs encoding human, bovine, rat and mouse peptides have been reported (Perry et al., 1997; Vilim et al., 1999), and recently the genes coding for two neuropeptide FF receptors (neuropeptide FF_1 and neuropeptide FF_2) have been cloned from humans and rats (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000). Neuropeptide FF modulates opioid functions: in the rat, for example, it antagonizes morphine analgesia after intracerebroventricular injection but induces long-lasting analgesia after intrathecal administration (Roumy and Zajac, 1998).

The binding of radiolabelled analogues of neuropeptide FF to rat spinal cord and brain membranes is reduced by non-hydrolysable analogues of guanosine triphosphate (GTP) (Payza and Yang, 1993). Similarly, non-hydrolysable analogues of GTP decrease neuropeptide FF binding in membranes of mouse olfactory bulb, where neuropeptide FF stimulates adenylate cyclase (Gherardi and Zajac, 1997). These results suggest that neuropeptide FF receptors are coupled to G-proteins, possibly a stimulatory G-protein (G_s). However, the recently cloned neuropeptide FF_1 and neuropeptide FF_2 receptors have been described as $G_{i/o}$-protein coupled when expressed in Chinese hamster ovary cells (Hinuma et al., 2000) or human embryonic kidney 293 cells (Elshourbagy et al., 2000), respectively.

In rat dorsal raphe neurones, neuropeptide FF dose dependently ($EC_{50} = 1.8$ nM) reduces the inhibitory effect of nociceptin/orphanin FQ on the transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggered by depolarization (Roumy and Zajac, 1999). In the present study, we investigated the effects of pertussis and cholera toxins on this cellular activity induced by neuropeptide FF.

2. Materials and methods

In all, 15 young (8 to 20 days) Sprague–Dawley rats were used in these experiments. The isolation of dorsal raphe neurones has been already described (Roumy and Zajac, 1999). In brief, the dorsal raphe was dissected from

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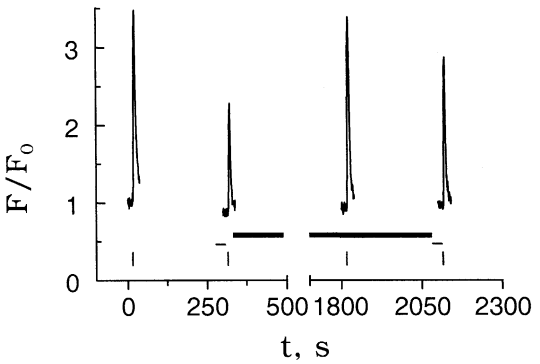

Fig. 1. Recordings of $[Ca^{2+}]_i$ transient triggered by depolarization. Neurones were depolarized by short (5 s) perfusions with 20 mM K^+ (thin vertical lines). Nociceptin (300 nM 30 s, thin horizontal lines) applied immediately before depolarization reduced the amplitude of the Ca^{2+} transient. This effect of nociceptin was larger before (left part of the figure) than after (right part) an 18-min perfusion with 100 nM [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (thick line). In this example, [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF reduced the nociceptin effect by 56%. Note the interruption of the time axis.

four to five 200-μm brain slices and suspended in 1 ml buffer (in mM: NaCl, 150; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; HEPES, 10; pH = 7.30) containing 1 mg trypsin (type XIII) and incubated for 45 min at 25°C. The pieces of tissue were further incubated (23–24°C) for 3 min in the presence of soybean trypsin inhibitor (1 mg/ml) and bovine serum albumin (0.1%) and washed three times in 1 ml of buffer plus 0.1% bovine serum albumin. Neurones were dispersed by trituration with Pasteur pipettes of decreasing tip diameter.

The neurone suspension (350 μl) was incubated 90 min at 23–24°C in the presence of 1.5 μM of the cell-permeant Fluo4 acetoxymethyl ester (Fluo4-AM). After centrifugation at $200 \times g$ for 3 min at 4°C, the neurones were re-suspended in 350 μl buffer plus bovine serum albumin (0.1%) and further incubated for 30 min to allow for complete intracellular de-esterification of Fluo4-AM. An 80-μl aliquot was deposited on a microscope slide and 5 min was allowed for neurone attachment before the start of perfusion (1.8 ml/min). Cholera and pertussis toxins (5 μg/ml) were added at the same time as Fluo4-AM and were present until the neurones were used for recordings.

Neurones were viewed with a 40/0.65 objective, illuminated at 488 nm (10 nm bandwidth interference filter) and imaged with a cooled CCD camera (MicroMax 782-Y, Princeton Instruments) driven by MetaView software (Universal Imaging). The average pixel intensity within user-defined regions of interest was measured and saved on a computer hard drive. The fluorescence intensity is expressed as F/F_0, where F and F_0 are the fluorescence intensity at any time and the mean resting fluorescence intensity preceding the first depolarization, respectively.

Neurones were perfused at 1.8 ml/min with a medium containing (in mM) NaCl, 150; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; HEPES, 10. The pH was adjusted to 7.30 and bovine serum albumin (0.1%) was added. The neurones were depolarized during 5 s with a perfusion medium in which KCl was raised to 20 mM and NaCl reduced accordingly to maintain osmotic pressure. An electrically operated valve effected a rapid change of solution that was complete within 8 s. The depolarization was repeated every 5 min. For each neurone, after the response to nociceptin had been recorded, the peptidase-resistant analogue [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (1DMe, 10, 100, 1000 nM) was perfused for 18 min before the effect of nociceptin was measured again. In control experiments, the effect of [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (10, 100, 1000 nM) upon the inhibitory effect of nociceptin was measured in neurones not treated with toxins.

Data are presented as means ± S.E.M. The effects of [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF before and after cholera toxin were analysed by a one-way analysis of variance followed by Bonferroni's Multiple Comparison Test (Prism software). The level of significance was chosen as 0.05 in all analyses.

3. Results

Nociceptin (300 nM, 30 s) reduced the magnitude of the Ca^{2+} transient triggered by depolarization (Fig. 1). In control neurones, nociceptin reduced the peak fluorescence intensity by 36.7 ± 1.8% ($n = 46$). In neurones pre-in-

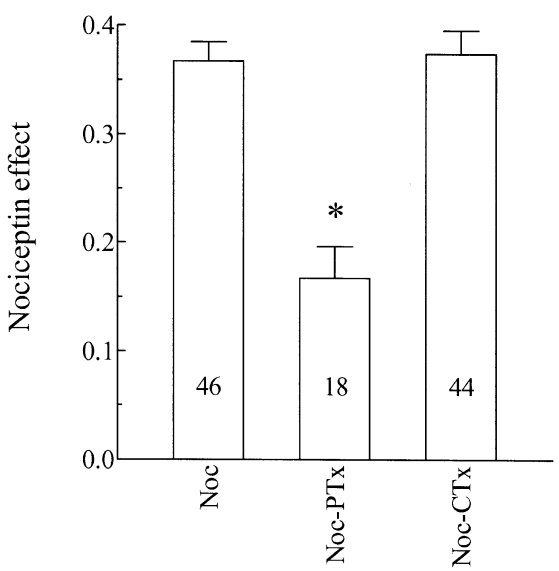

Fig. 2. Pertussis toxin, but not cholera toxin, reduced the effect of nociceptin on Ca^{2+} transients. The activity of nociceptin (300 nM, 30 s) was measured in control neurones (Noc) and in neurones pre-incubated with either pertussis toxin (Noc-PTx) or cholera toxin (Noc-CTx). Nociceptin effect was measured as $E = 1 - [(Fp)_{Noc}/(Fp)_c]$, where $(Fp)_{Noc}$ and $(Fp)_c$ are the peak fluorescence intensity, during K^+-induced depolarization, following 30 s nociceptin and in the control state, respectively. Numbers of neurones are indicated in each column. * Significantly ($P < 0.05$) different from controls.

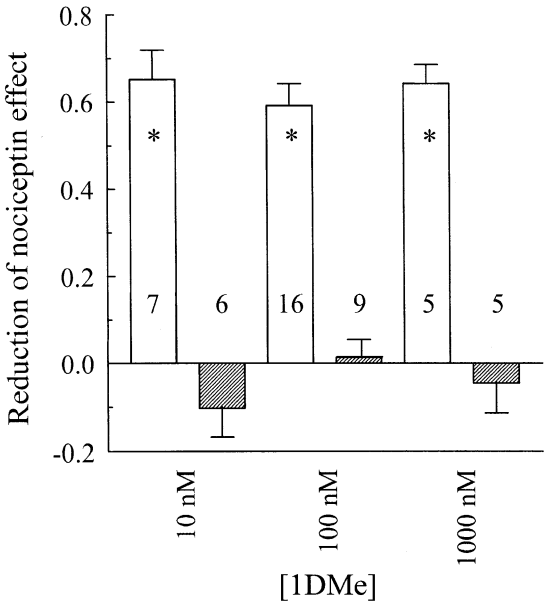

Fig. 3. [D-Tyr^1, (N-Me)Phe^3]Neuropeptide FF no longer reduced the nociceptin effect in neurones pre-treated with cholera toxin. The effect of nociceptin (300 nM, 30 s) was measured before and after an 18-min perfusion with [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (10, 100, 1000 nM) in control neurones (empty columns) and in neurones pre-treated with cholera toxin (hatched columns). The reduction of the nociceptin effect was calculated as: $1-(E_{1DMe}/E_c)$, where E_c and E_{1DMe} were the nociceptin effects (see legend to Fig. 2) before and after [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF superfusion, respectively. Numbers of neurones are indicated for each situation. * Significant ($P < 0.05$) difference in the effect of each concentration of [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF between cholera toxin-treated and control neurones.

cubated with pertussis toxin (5 μg/ml, 2–6 h), this effect of nociceptin was significantly reduced (Fig. 2), amounting to only 16.7 ± 2.9% ($n = 18$). After pre-incubation with cholera toxin (5 μg/ml, 2–6 h), the magnitude of the nociceptin-induced reduction of the Ca^{2+} transient was not affected (Fig. 2), being 37.4 ± 2.1% ($n = 44$).

[D-Tyr^1, (N-Me)Phe^3]Neuropeptide FF dose dependently reduces the inhibitory effect of nociceptin upon the Ca^{2+} transient triggered by depolarization (Roumy and Zajac, 1999). In control neurones, the 62% maximum reduction was already reached at 10 nM [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF (Fig. 3). Following pre-incubation with cholera toxin (5 μg/ml, 2–6 h) this effect of [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF was lost at the three concentrations tested (Fig. 3).

4. Discussion

Nociceptin either increases an inwardly rectifying K^+ conductance and/or reduces Ca^{2+} channel conductances in various types of neurones (Amano et al., 2000; Connor et al., 1996a; Connor et al., 1996b; Connor and Christie, 1998; Emmerson and Miller, 1999; Ikeda et al., 1997; Knoflach et al., 1996; Madamba et al., 1999; Meis and Pape, 1998; Slugg et al., 1999; Vaughan and Christie, 1996; Vaughan et al., 1997). In rat dorsal raphe neurones, nociceptin enhances an inwardly rectifying K^+ conductance (Vaughan and Christie, 1996) and reduces Ca^{2+} channel conductances (Roumy and Zajac, 1999). The modulated Ca^{2+} channels are very likely the same channels modulated by activation of 5-HT_{1A} receptors (Penington et al., 1991), since the effects of saturating concentrations of serotonin and nociceptin are not additive (Roumy, unpublished observations). In the present study, we demonstrated that pre-treatment of the neurones with pertussis toxin, but not with cholera toxin, reduced the inhibitory effect of nociceptin on the $[Ca^{2+}]_i$ transient triggered by depolarization by 55%, suggesting that the ORL1 receptor is coupled to $G_{i/o}$ inhibitory G- proteins. This is in agreement with previous studies demonstrating that the modulation of K^+ and Ca^{2+} conductances by nociceptin is dependent upon $G_{i/o}$ proteins (Connor et al., 1996b; Knoflach et al., 1996; Meis and Pape, 1998).

We previously demonstrated (Roumy and Zajac, 1999) that, in rat dorsal raphe neurones, the neuropeptide FF analogue [D-Tyr^1, (N-Me)Phe^3]neuropeptide FF dose dependently reduced the inhibitory effect of nociceptin upon $[Ca^{2+}]_i$ transients with an EC_{50} of 1.8 nM. We now report that this effect is abolished by pre-treatment of the neurones with cholera toxin. This toxin, which catalyses the ADP ribosylation of the α-subunit of G_s, leads first to a persistent activation and then to a loss of G_s from the membrane (Boehm et al., 1996). The initial activation of G_s could lead to accumulation of cAMP, with perhaps an effect on the response of dorsal raphe neurones to nociceptin. This is not likely, however, since the response to nociceptin was not altered by the pre-treatment with cholera toxin. The long-lasting effect of cholera toxin corresponds to a reduction of G_s in the plasma membrane of chick sympathetic neurones, detected after a 3-h treatment with cholera toxin (Boehm et al., 1996). Our results therefore suggest that, in the dorsal raphe, the neuropeptide FF receptor couples to a cholera toxin-sensitive G-protein (G_s) to activate a signalling pathway leading to a reduction of the effect of nociceptin on Ca^{2+} conductances.

This conclusion is consistent with the stimulation of adenylate cyclase activity in mouse olfactory bulb membranes by neuropeptide FF (Gherardi and Zajac, 1997), but is at variance with the coupling of both neuropeptide FF_1 and neuropeptide FF_2 receptors to a $G_{i/o}$ protein in Chinese hamster ovary cells (Hinuma et al., 2000) and human embryonic kidney 293 cells (Elshourbagy et al., 2000), respectively. However, Bonini et al. (2000) also observed that neuropeptide FF_1 receptors couple to a G_s-protein, though less efficiently than to G_i. Thus, in the dorsal raphe, the action of cholera toxin could be due to the blockade of the activation of neuropeptide FF_1 receptors. However, only neuropeptide FF_2 receptor mRNA has been detected in this nucleus (Gerald et al., 1999). It is now, however, widely accepted that receptors can couple to

several G-proteins, including G-proteins that transduce divergent signalling pathways such as G_s and G_i (Sidhu and Niznik, 2000). Such multiple couplings have now been reported for many receptors (Chakraborty et al., 1991; Crain and Shen, 1998; Cruciani et al., 1993; Daaka et al., 1997; Eason et al., 1992; Fan et al., 1992; Herrlich et al., 1996; Kuhn and Gudermann, 1999; Luo et al., 1999; Negishi et al., 1993; Schnefel et al., 1990; Schwindinger et al., 1998; Shreeve et al., 2000; Sidhu et al., 1991, 1998; Stanislaus et al., 1998; Wenzel-Seifert and Seifert, 2000). These results may therefore indicate that neuropeptide FF receptors transduce different cellular effects through coupling to various G-proteins.

We conclude that, in rat dorsal raphe neurones, the activation of neuropeptide FF receptors reduces the nociceptin-induced inhibition of Ca^{2+} channels through coupling to a stimulatory (G_s) G-protein.

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