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Nociceptin Inhibits Uterine Contractions in Term-Pregnant Rats by Signaling Through Multiple Pathways[1]

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ABSTRACT

The actions of the endogenous peptide nociceptin (PNOC; previously abbreviated as N/OFQ) on the myometrium have not been investigated previously. Our aim was to study the presence and functional role of PNOC in the modulation of uterine contractility in pregnant rats at term. The presence of PNOC and its receptors (OPRL1; previously called NOP) in the uterus were detected by radioimmunoassay and radioligand-binding experiments. The PNOC-stimulated G protein activation was assessed by a [^{35}S]GTPgammaS-binding technique. The effects of PNOC in uterine rings precontracted with KCl or oxytocin were also tested in vitro. Uterine levels of cAMP were measured by enzyme immunoassay. The K^+ channel blockers tetraethylammonium and paxilline were used to study the role of K^+ channels in mediating the uterine effects of PNOC. Both PNOC and OPRL1 were present in the uterus. PNOC revealed a maximum contraction inhibition of approximately 30%, which was increased to 40% by naloxone. Naloxone and pertussis toxin significantly attenuated the G protein-stimulating effect of PNOC. The uterine cAMP levels were elevated by PNOC and naloxone and after preincubation with pertussis toxin. Tetraethylammonium and paxilline reduced the contraction-inhibiting effect of PNOC and naloxone to approximately 10% and 15%, respectively. We presume that PNOC plays a role in regulating uterine contractility at term. Its effect is mediated partly by stimulatory heterotrimeric G (G_s) proteins coupled to OPRL1 receptors and elevated cAMP levels, and also by Ca^{2+}-dependent K^+ channels. Our results demonstrate a novel action and signaling pathway for PNOC that might be a potential drug target.

cyclic adenosine monophosphate, neuropeptides, pregnancy, signal transduction, uterus

INTRODUCTION

Nociceptin (also known as orphanin FQ, or OFQ; hereafter referred to as PNOC [molecular weight = 1809.06]) is the endogenous ligand for orphan opioid receptor-like 1 (ORL-1 or NOP; hereafter referred to as OPRL1) [1, 2]. PNOC and its receptor comprise a relatively novel peptide-receptor system that is widely expressed in the central nervous system and in peripheral tissues. The amino acid sequences for both PNOC and OPRL1 are highly homologous to those of opioid ligands and receptors, respectively. Classical opioid ligands exhibit little affinity for OPRL1, and their actions cannot be antagonized on OPRL1 by naloxone, which is a hallmark of classical opioid receptors [3]. The central actions of PNOC are various, possibly because of its widespread localization in the brain, involving the central control of nociception, and its participation in locomotor activity and in the processes of reward, learning and memory, anxiety, fear, stress, and feeding behavior [4].

Previous results have shown that in the female rat, PNOC has a role in the neuroendocrine control of reproductive functions [5]. However, the peripheral actions of PNOC have not been fully described. Outside the brain, OPRL1 has been detected in the rat peripheral ganglia, airways, and intestines; the mice heart, vas deferens, and colon; the guinea pig retina; and in the human airways and mononuclear cells [6–11]. As regards the visceral smooth muscles, PNOC has been found to inhibit nonadrenergic-noncholinergic bronchoconstriction in the guinea pig airways via the prejunctional modulation of tachykinin release [12] and to inhibit distal colon motility in the rat [13]. However, the possible actions of PNOC on the smooth muscle of the female reproductive organs have not been discussed.

The nociceptive peptide PNOC is considered to be a pronociceptive peptide. Several previous findings have confirmed that such endogenous oligo- and polypeptides (e.g., oxytocin [14]; tachykinin, previously called substance P [15, 16]; and CALCA, previously called calcitonin gene-related peptide [17–19]) have pronociceptive and also potent modulatory effects on the uterine contractility. Investigation of the actions of these neuropeptides can provide substantial new information relating to the mediation of uterine contractions at term, either physiological or pathological. The onset of preterm uterine contractions, which can lead to preterm labor and birth, is one of the most common pathological conditions in obstetrics. Because its underlying causes and molecular pathways have not been fully elucidated, a need exists for investigations of endogenous factors that might control uterine activity, with the perspective of improving tocolytic therapy.

Our aim was to investigate the possible role of the endogenous nociceptive peptide PNOC in the modulation of uterine contractions in pregnant rats at term (Day 22 of pregnancy). We studied the expressions of PNOC and OPRL1 in the pregnant as well as the nonpregnant rat uterus. The intracellular signaling pathways of PNOC were studied by measuring PNOC-stimulated uterine G protein activation and local cAMP accumulation and by investigating the role of large

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conductance voltage and Ca^{2+}-dependent (outward rectifying) K^+ (KCNMA1; previously known as BK_{Ca}) channels. The present study provides experimental evidence regarding the presence and regulatory role of PNOC in the pregnant rat uterus at term, mediated partly by G_s proteins coupled to OPRL1 receptors and elevated cAMP levels, and also by KCNMA1 channels.

MATERIALS AND METHODS

Animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration no. IV./01758–2/2008) and under the control of the ISO-9001:2008 Quality Management System.

Sexually mature, female Sprague-Dawley rats (body mass, 140–160 g; age, 50–60 days) were mated in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of copulation was considered to be the first day of pregnancy. Nonpregnant females used in the experiments were cyclic, virgin rats at the estrous phase. The animals were housed in rooms with regulated temperature (20–23°C), humidity (40%–60%), and light (12L:12D), with water and food intake ad libitum.

PNOC Tissue Levels

The uterine levels of PNOC were measured in nonpregnant and 22-day pregnant rats. The extraction of PNOC was carried out by a validated method [20]. During the validation of the extraction procedure, the stability of PNOC as well as the reproducibility, recovery, and linearity of recovery in the concentration range of 1–100 pg/100 mg tissue were determined. The recovery was 87% ± 0.68% (mean ± SEM), and all other parameters were in the normal ranges.

The tissue extracts were subjected to radioimmunoassay (RIA) for PNOC using a commercially available ^{125}I-PNOC RIA Kit (Nociceptin/Orphanin FQ [rat] - RIA Kit; Phoenix Pharmaceuticals Inc.) with a minimum sensitivity of 1 pg/ml. Data were evaluated with Isodata 20/20 software. Five rats per group (nonpregnant group and 22-day pregnant group, respectively) were used for each point. Significance was calculated by Student *t*-test.

Radioligand-Binding Experiments

Membrane preparation. Radioligand-binding experiments were carried out on nonpregnant or 22-day pregnant rat uterus membrane preparations. The uterine tissues were cut and homogenized in ice-cold buffer (0.05 M Tris-HCl [pH 7.4]) using a Braun Teflon-glass homogenizer (10–15 strokes) and filtered through four layers of gauze to remove large aggregates. The homogenate was centrifuged (Sorvall RC5C centrifuge, SS34 rotor) at $40\,000 \times g$ for 20 min at 4°C, and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37°C. The centrifugation step was repeated, and the final pellet was resuspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at −70°C until use [21]. Protein concentration was determined by the method of Bradford [22].

Radioligand-binding assay. For the homologue displacement experiments, the reaction mixture contained 100 μl of membrane preparation (~0.3–0.4 mg protein/ml), 100 μl of [^{3}H]PNOC-NH_2 with a specific activity of 25 Ci/mM, and 100 μl of unlabeled PNOC (10^{-12}–10^{-5} M) or 100 μl of incubation buffer (consisting of 0.05 M Tris-HCl, 0.01 M $MgCl_2$, and 2.5% ethanol [total buffer = pH 7.42]) for total binding. Incubation was started by addition of the membrane suspension and continued in a shaking water bath until a steady state was achieved (24°C, 60 min). At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandel M24R Cell harvester (Semat) through Whatman GF/C filters (Semat Technical Ltd.) presoaked in polyethyleneimine (0.3%, pH 10) for 30 min and washed with three times with 10 ml of ice-cold buffer (0.05 M Tris-HCl [pH 7.42]). The radioactivity of the dried filters was detected in UltimaGold F scintillation cocktail (Packard) with a Packard Tricarb 2300TR liquid scintillation counter [21].

Specific binding was determined by subtracting the nonspecific binding value from the total binding value. All assays were carried out at least three times in duplicate, and values are given as the mean ± SEM. The experiments were individually analyzed, and the maximum numbers of binding sites (B_{max}) and the equilibrium dissociation constants (K_d) were calculated with the Prism 4.0 computer program (GraphPad, Inc.).

[^{35}S]GTPγS-binding assay. The uterine tissue samples obtained from 22-day pregnant rats were homogenized [9] and diluted in 50 mM Tris-HCl buffer (pH 7.4) to obtain appropriate protein content for the assays (~10 μg protein/sample). The membrane fractions were incubated at 30°C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, and 100 mM NaCl and containing 20 MBq per 0.05 ml of [^{35}S]GTPγS (0.05 nM) and increasing concentrations of PNOC tested in the presence of excess GDP (30 μM) in a final volume of 1 ml, according to Sim et al. [23] and Traynor and Nahorski [24], with slight modifications. The effect of PNOC was investigated together with 10^{-6} M naloxone. The G_i protein-activating effect of PNOC and naloxone was also measured in the presence of 500 ng pertussis toxin (PTX)/ml. Total binding was measured in the absence of test compound; nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding. The difference (total binding − nonspecific binding) indicates the basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters (Whatman) with a Brandel M24R Cell harvester. Filters were washed three times with 5 ml of ice-cold buffer (pH 7.4), and the radioactivity of the dried filters was detected in UltimaGold F scintillation cocktail (Packard) with a Packard Tricarb 2300TR liquid scintillation counter. Stimulation is given as a percentage of the specific [^{35}S]GTPγS-binding observed in the absence of receptor ligands (basal activity). The [^{35}S]GTPγS-binding experiments were performed in triplicate and repeated at least three times. Data were analyzed with the sigmoid dose-response curve fit option. The maximal effect and pD_2 (the negative logarithm of the half-maximum effective concentration; −log median effective concentration) values were also determined with Prism 4.0.

In Vitro Contractility Studies

On day 22 of pregnancy (term), the rats were killed by carbon dioxide inhalation, and the uteri were removed and prepared for the in vitro contractility assay as reported previously [19]. Briefly, the isolated uterine horns were immediately placed in an organ bath (de Jongh solution; 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4, and 6 mM glucose [pH 7.4]) perfused with a mixture of 95% oxygen and 5% carbon dioxide, after which fat was trimmed and the fetoplacental units were removed. The temperature was maintained at 37°C. Four rings (length, 1 cm) were sliced from the middle part of each horn, including implantation sites, and tested in parallel; they were mounted vertically in the above-mentioned organ bath under the same conditions. After mounting, the initial tension was set at 1.5 g, and the rings were equilibrated for 60 min. Rhythmic contractions were then elicited with 25 mM KCl. The effects of PNOC and/or naloxone on the uterine rings were measured in the concentration range of 10^{-12}–10^{-7} M in a noncumulative manner. After each concentration of PNOC or naloxone, the rings were washed three times, allowed to recover for 5 min, and then contracted again with KCl.

In another set of experiments, uterine contractions were elicited with 10^{-6} M oxytocin, and the contraction-inhibiting effects of PNOC alone and in combination with naloxone were tested in a noncumulative manner. These experiments were carried out in the presence of the nonselective K^+ channel blocker tetraethylammonium (TEA; 10^{-3} M) and the KCNMA1 selective blocker paxilline (10^{-3} M).

The tension of the myometrial rings was measured with a strain gauge transducer (SG-02; Experimetria Ltd.) and recorded and analyzed with the SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd.). The areas under the curves were evaluated, and the effects of PNOC or naloxone were expressed as a percentage of the KCl- or oxytocin-induced contractions. The pD_2 values and maximum contraction-inhibiting values were calculated and analyzed (ANOVA Newman-Keul test) with Prism 4.0.

Measurement of Uterine cAMP Accumulation

Uterine tissue samples were incubated in de Jongh solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4, and 6 mM glucose [pH 7.4]) at 37°C, perfused with a mixture of 95% oxygen and 5% carbon dioxide. Cyclic AMP accumulation was detected in the presence of the nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (10^{-3} M; IBMX), PNOC (10^{-8} M), and/or naloxone (10^{-8} M) and forskolin (10^{-5} M). The effect of the inhibitory heterotrimeric G (G_i/G_o) protein inhibitor PTX (400 ng/ml) on uterine cAMP accumulation was also tested in the presence of IBMX, PNOC, naloxone, and forskolin. The samples were then immediately frozen in liquid nitrogen and stored until the extraction of cAMP [25]. Next, frozen tissue samples were ground, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid, and centrifuged at $1000 \times g$ for 10

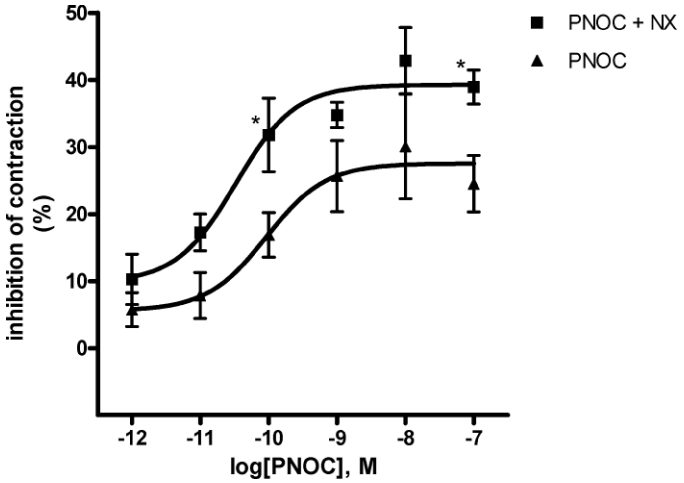

FIG. 1. Inhibitory effect of nociceptin on pregnant uterine contractions in vitro. The contractions were elicited with 25 mM KCl in 22-day pregnant uterine rings from the rat. The concentration-dependent relaxing effect of nociceptin (PNOC) was potentiated by the presence of 10^{-8} M naloxone (PNOC + NX). Naloxone alone caused a nonsignificant inhibition of KCl-evoked contractions (data not shown). n = 8; $^{*}P < 0.05$.

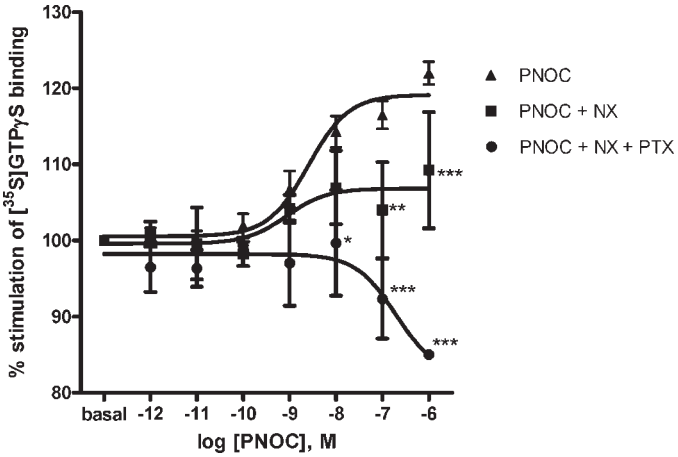

FIG. 2. Effect of nociceptin on G protein activation in the pregnant rat myometrium in vitro. Nociceptin increased the concentration of activated G protein in a dose-dependent manner (PNOC). The presence of 10^{-6} M naloxone significantly decreased the nociceptin-induced G protein activation (PNOC + NX), while the G_i protein inhibitor pertussis toxin elicited a strong decline in activated G protein level (PNOC + NX +PTX). n = 6; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

min. The supernatants were extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70°C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP enzyme immunoassay kit, and tissue cAMP levels were expressed as pmol/mg tissue. The tissue cAMP levels were calculated and analyzed (ANOVA Newman-Keul test) with Prism 4.0.

Materials

The radioligand ([^{3}H]PNOC) was radiolabeled by Drs. Judit Farkas and Geza Toth (Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary). The ^{125}I-PNOC RIA Kit was obtained from Phoenix Pharmaceuticals, Inc. Guanosine-5′-[γ-^{35}S]triphosphate ([^{35}S]GTPγS) was purchased from Amersham. PNOC was from Bachem and Sigma-Aldrich Ltd. Naloxone, IBMX, the cAMP enzyme immunoassay kit, tetraethylammonium chloride (i.e., TEA), paxilline, guanosine 5′-diphosphate (GDP), guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS), and polyethyleneimine were from Sigma-Aldrich Ltd. PTX was from Tocris Cookson Ltd. Oxytocin was from Richter Gedeon Ltd.

RESULTS

PNOC Tissue Levels

The tissue levels of PNOC were measured by RIA in the uteri of nonpregnant and 22-day pregnant rats, respectively (n = 5 for each group). In the nonpregnant rats, PNOC was present at a concentration of 2.11 ± 0.21 pg per 100 mg wet tissue. In the 22-day pregnant rats, uterine PNOC concentration was 3.65 ± 0.12 pg per 100 mg wet tissue, which is significantly higher ($P < 0.05$) than in the nonpregnant rats.

Radioligand-Binding Studies

The presence of OPRL1 receptors in the uterus was detected by radioligand-binding experiments (n = 4 for each group). In the uteri of nonpregnant females, the B_{max} of the OPRL1 receptors was 87.3 ± 5.2 fmol protein/mg membrane, with a K_d of 2.19 ± 0.14 × 10^{-8} M. In the membrane fractions of the 22-day pregnant uteri, the corresponding B_{max} and K_d values were 99.6 ± 2.31 fmol protein/mg membrane and 1.95 ± 0.09 × 10^{-8} M, respectively. No significant difference ($P > 0.05$) was found between the B_{max} or K_d values in the nonpregnant versus the 22-day pregnant rat uteri.

In Vitro Contractility Studies

Figure 1 illustrates the effects of PNOC in uterine rings precontracted with KCl or oxytocin. The KCl-evoked rhythmic contractions in the pregnant rat uterus were inhibited in a concentration-dependent manner by PNOC. The maximum inhibition of contractions was 27.5% ± 3.1% (pD_2 = 10.03 ± 0.40). In the presence of the opioid antagonist naloxone, the maximum contraction-inhibiting effect of PNOC was increased to 39.3% ± 3.2% (pD_2 = 10.48 ± 0.30). Naloxone alone caused a nonsignificant inhibition of the KCl-evoked contractions (n = 8 for each set of experiments; data not shown).

[^{35}S]GTPγS-Binding Assay

Because the central effects of PNOC are mediated via the activation of GNAI proteins coupled to the OPRL1 receptors and also the outward rectifying K^+ channels, the PNOC-stimulated G protein activation was additionally tested on the membrane fractions of the 22-day pregnant rat uteri (Fig. 2). PNOC stimulated the [^{35}S]GTPγS binding through the OPRL1 receptors by 119.1% ± 1.2% (pD_2 = 8.59 ± 0.17). In the presence of naloxone, the PNOC-stimulated G protein activation was decreased to 106.8% ± 1.2% (pD_2 = 8.35 ± 0.50). In the presence of PTX and naloxone, however, the maximum G protein activation decreased to 82.3% ± 3.0% (pD_2 = 6.71 ± 0.29), with the activation declining to below the basal level (n = 6 for each set of experiments).

Measurement of Uterine cAMP Accumulation

To investigate the mechanism by which PNOC inhibits uterine contractions, the effect of PNOC on uterine cAMP accumulation was also measured (Fig. 3). The PNOC (10^{-8} M) and naloxone (10^{-8} M)-stimulated uterine cAMP accumulations were detected in the presence of the nonspecific phosphodiesterase inhibitor IBMX (10^{-3} M) and the adenylyl cyclase activator forskolin (10^{-5} M). PNOC alone did not evoke a significant increase ($P > 0.05$) in the uterine cAMP accumulation (14.0 ± 0.6 pmol/mg tissue) as compared with the nontreated control samples (13.2 ± 0.2 pmol/mg tissue). However, its combination with naloxone caused a significant

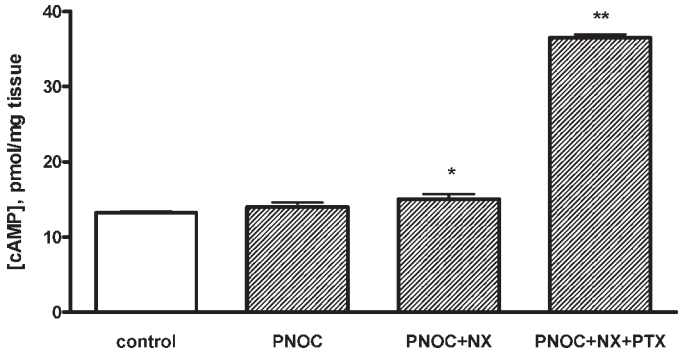

FIG. 3. Effect of nociceptin on the intracellular cAMP level in the pregnant rat myometrium. Nociceptin (PNOC) itself did not increase the cAMP level as compared with the control (white column). The presence of naloxone (PNOC + NX) increased the nociceptin-induced intracellular cAMP level, while the addition of pertussis toxin (PNOC + NX + PTX) resulted in a robust elevation in cAMP level. n = 6; *$P < 0.05$, **$P < 0.01$.

elevation ($P < 0.05$) in the uterine cAMP level (15.0 ± 0.7 pmol/mg tissue). Moreover, if the uterine tissue samples were preincubated with the G_i protein inhibitor PTX (400 ng/ml), PNOC with naloxone elevated the uterine cAMP level far higher ($P < 0.01$) than that without preincubation (36.5 ± 0.5 pmol/mg tissue), which points to the involvement of G_s proteins in the intracellular signaling pathways of PNOC and naloxone in the pregnant rat uterus (n = 6 for each set of experiments).

Investigation of KCNMA1 Channels in Isolated Organ Bath

Because the central actions of PNOC and naloxone are mediated via the activation of the KCNMA1 channels and hyperpolarization of the neurons, the effects of PNOC and naloxone on the uterine samples were also tested in the presence of the nonselective K^+ channel inhibitor TEA and the KCNMA1 channel-selective paxilline (both at 10^{-3} M) (Fig. 4). In these experiments, the rhythmic uterine contractions were evoked by oxytocin instead of KCl. TEA and paxilline themselves did not change the pattern of oxytocin-induced contractions. However, the uterus-relaxing effect of PNOC on oxytocin-evoked rhythmic contractions (30.9% ± 3.6%; pD_2 = 9.58 ± 0.40) was significantly attenuated by TEA (10.6% ± 1.7%; pD_2 = 9.14 ± 0.34) and paxilline (15.1% ± 2.7%; pD_2 = 9.60 ± 0.83). Similarly, when both PNOC and naloxone were present, their common contraction-inhibiting effect (38.1% ± 3.9%; pD_2 = 9.89 ± 0.34) was significantly decreased by TEA (16.5% ± 2.1%; pD_2 = 8.99 ± 0.42) and paxilline (17.7% ± 2.3%; pD_2 = 9.12 ± 0.90) (n = 6 for each set of experiments).

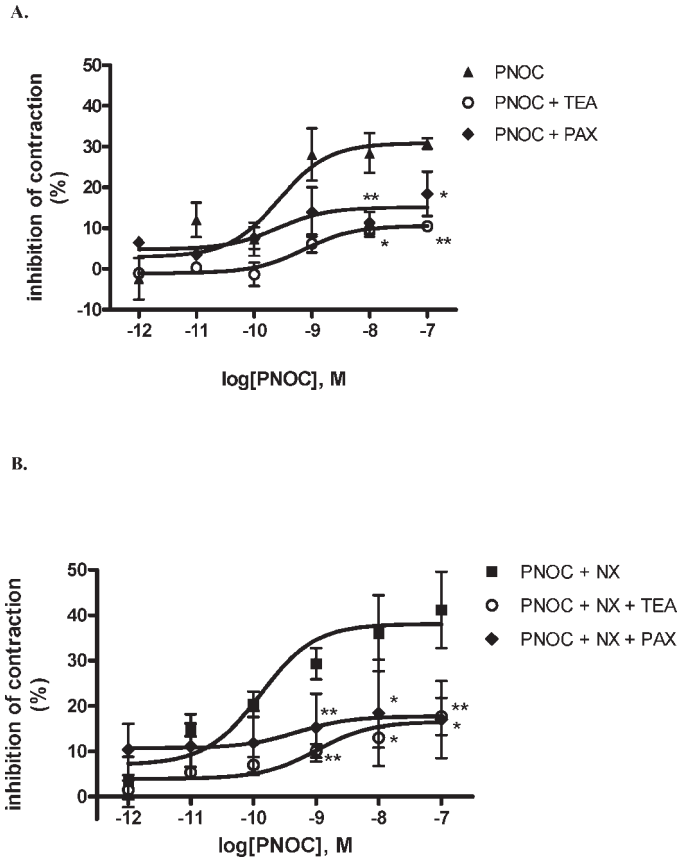

FIG. 4. Effects of potassium channel inhibitors on the pregnant uterine-relaxing effect of nociceptin in vitro. The contractions were elicited with 10^{-6} M oxytocin in 22-day pregnant uterine rings from the rat. **A**) The concentration-dependent relaxing effect of nociceptin (PNOC) was decreased both by the nonselective K^+ channel inhibitor tetraethylammonium (10^{-3} M; PNOC + TEA) and by the outward rectifying K^+ channel inhibitor paxilline (10^{-3} M; PNOC + PAX). No significant difference was found between the effects of the two K^+ channel blockers (n = 6). **B**) The relaxing effect of nociceptin was slightly increased by naloxone (10^{-8} M; PNOC + NX). Their joint effect was decreased both by the nonselective K^+ channel inhibitor tetraethylammonium (10^{-3} M; PNOC + NX + TEA) and by the outward rectifying K^+ channel inhibitor paxilline (10^{-3} M; PNOC + NX + PAX). No significant difference was found between the effects of the two K^+ channel blockers. n = 6; *$P < 0.05$, **$P < 0.01$.

DISCUSSION

Regarding its effects on the central nervous system, PNOC is considered to act as an important neuroendocrine regulator of female reproductive functions. Foradori et al. [26] found that in the human brain, gonadotropin-releasing hormone (GNRH) immunoreactive nerve cells colocalize PNOC in a high percentage, without any regional restriction. It was also demonstrated that the pulsatile GNRH secretion is under the control of ovarian steroids and PNOC. Furthermore, in the limbic system and hypothalamus, the expressions of *Pnoc* and *Oprl1* mRNA are site-specifically regulated by estrogens and progesterone [27]. These findings strongly indicate that the actions of GNRH are modulated, in part, by PNOC, with this mechanism controlling the reproductive functions by regulating release of the gonadotropic hormones follicle-stimulating hormone and luteinizing hormone from the pituitary.

The direct actions of opioid peptides, but not of PNOC, in the reproductive system of rats were studied by Vértes et al. [28], who observed direct cross-talk between opioid and estrogenic signaling in the control of uterine cell proliferation. Potential involvement of the opioid system in the female reproductive functions is shown by the fact that endogenous opioid peptides (among which dynorphin A exhibits some interesting homologies to PNOC) and the classical opioid receptors mu, delta, and kappa have been detected in the female rat reproductive tract [29], but to our knowledge, their exact roles have not been clarified. The presence and effects of PNOC in the female rat uterus have not been investigated previously.

The idea that endogenous pronociceptive substances might have a modulating effect on the uterine smooth muscle contractility stems from the ancient experience that labor contractions are extremely painful. Thus, labor pain and powerful contractions are usually related. However, previous

findings demonstrated that when administered directly into the periaqueductal gray of rats, oxytocin exerts an antinociceptive effect [30]. This effect of centrally administered oxytocin is attenuated by the opioid antagonist naloxone, suggesting that by stimulating the opioid neurons in the periaqueductal gray, oxytocin may contribute to the activation of descending antinociceptive pathways [31]. Sensory neuropeptides (e.g., tachykinin, calcitonin gene-related peptide, and galanin), released in an efferent fashion from sensory nerve endings, regulate uterine contractility and also play important roles in the mediation of peripheral nociception [15, 18, 19]. Previous results on the role of PNOC in central and peripheral pain processing [4, 32] aroused our interest regarding its participation in the modulation of uterine contractions of the late-pregnant rat.

First, we investigated the expression of PNOC in the uterus of nonpregnant and pregnant rats. The RIA results confirmed the presence of PNOC in both the nonpregnant and the pregnant rat uterus, with a moderate increase with gestation. This may suggest that PNOC likely is involved in local mechanisms regulating the uterine functions at term. Previously, the ovarian expression of prepronociceptin (*Pnoc*), the precursor for PNOC, was also confirmed [33]; its level was 10-fold higher after hCG-induced ovulation. In situ hybridization studies showed a similar up-regulation of the mRNA for *Pnoc* in the theca cell layer of preovulatory follicles, implicating PNOC as a possible paracrine factor in the periovulatory ovary.

We also detected specific binding sites for OPRL1 in both the nonpregnant and the term-pregnant rat uterus, with their concentration being similar to that in most brain regions [34]. In concordance with studies dealing with OPRL1 receptor characterization [35], we identified a single, high-affinity, saturable binding site in the nonpregnant and the 22-day pregnant rat uteri, with the same B_{max} or K_d values. The expression of specific OPRL1 receptors in the rat uterus further supports the hypothesis that PNOC exerts a local regulatory role.

Regarding its effects on the uterus, PNOC was found to inhibit both KCl- and oxytocin-evoked rhythmic contractions. To exclude the possible involvement of classical opioid receptors in the mediation of this effect, naloxone was coadministered with PNOC onto the isolated uterine tissue samples. Our results revealed that naloxone significantly increased the uterus-relaxing effect of PNOC, suggesting that naloxone has a relaxing effect on its own. The effect of naloxone alone on the uterus appeared to be insignificant. The potentiating effect of naloxone may seem unexpected, but a recent publication reported an elevated uterine tone in humans after intrathecal administration of opioids during labor [36], which suggests that endogenous opioids might have a contraction-enhancing effect on the uterus that can be attenuated by naloxone.

The high homology between the OPRL1 receptor and the three opioid receptor subtypes raised the question of whether the OPRL1 receptor is a G_i coupled receptor that regulates adenylyl cyclase activity, K^+ channels, and voltage-gated Ca^{2+} channels. The cellular actions of PNOC were found to be similar to those of opioids—namely, inhibition of the formation of cAMP, closure of voltage-sensitive Ca^{2+} channels, and enhancement of outward K^+ conductance [37]. Hence, we also tested the G protein-activating effect of PNOC on the term-pregnant rat uterus and found a significant elevation of [^{35}S]GTPγS binding through the OPRL1 receptors. Thus, the actions of PNOC on the uterus are, at least in part, mediated by G proteins. Furthermore, the presence of naloxone decreased the maximum [^{35}S]GTPγS binding and, at the same time, increased the uterus-relaxing effect of PNOC. A possible explanation for this phenomenon might be that naloxone interferes with the G_i protein-activating potency of PNOC. In the presence of naloxone and PTX, which uncouples the receptor from the G_i protein and prevents the inhibition of adenylyl cyclase activity, we detected a dramatic fall in PNOC-induced G protein production. This inhibition of the G_i proteins resulted in activation below the basal level.

To investigate further the mechanism by which PNOC inhibits uterine contractions, the effect of PNOC on uterine cAMP accumulation was also measured. Coadministration of naloxone with PNOC increased the uterine cAMP level. To test the participation of G_s proteins in the actions of PNOC, the changes in cAMP level in the uterus were assessed in the presence of PTX. In these experiments, the cAMP level rose markedly, which suggests that OPRL1 receptors are probably coupled to both G_i and G_s proteins in the late-pregnant rat uterus, and if naloxone is present, the net effect is an elevation in cAMP concentration. We also presume that PNOC and naloxone compete for intracellular G_i protein activation.

The phenomenon that individual receptors are able to activate multiple pathways by switching between different G proteins is well established [38, 39]. The coupling of beta-adrenergic receptors to G_s proteins leads to the activation of adenylate cyclase and the consequent phosphorylation of protein kinase A, which phosphorylates the receptor and diminishes its coupling to G_s but increases the coupling to G_i. Thus, the G_i-mediated stimulation of the mitogen-activated protein kinase and cellular actions require prior phosphorylation of the receptor by protein kinase A. We presume that naloxone interferes with the intracellular pathways activated by PNOC and promotes its coupling to G_s. This mechanism results in an elevation of the myometrial cAMP level and the inhibition of myosin light-chain kinase phosphorylation and uterus relaxation.

The changes in local cAMP levels, however, do not seem to be the only intracellular pathway regulated by PNOC via the OPRL1 receptors. PNOC has been reported to enhance an outward K^+ conductance, which reduces neuronal excitability and transmitter release in the brain [37]. In the uterus, KCNMA1 channels are abundant and play an important role in limiting depolarization, thereby relaxing the uterine smooth muscle. Moreover, these KCNMA1 channels are activated by a cAMP-dependent phosphorylation cascade in cultured human myometrial cells [40]. We found that blockade of the K^+ channels with TEA or paxilline diminishes the uterus-relaxing effect of PNOC, applied either alone or in combination with naloxone, which again suggests that this effect of PNOC on the pregnant rat uterus is, in part, mediated by the activation of KCNMA1 channels, resulting in a more negative electric potential inside the myometrial cells. No difference was found between the PNOC-inhibiting effects of the nonselective K^+ channel blocker TEA and the KCNMA1-selective blocker paxilline. This suggests that KCNMA1 channels may have a crucial role in the uterus-relaxing effect of PNOC. In the presence of TEA or paxilline, PNOC, either alone or in combination with naloxone, still produced a limited uterus-relaxing effect, which is suspected to be mediated by the elevated cAMP level.

These results indicate that PNOC has a direct muscle-relaxant effect in the pregnant rat uterus, mediated by the OPRL1 receptors, and that it can be potentiated by naloxone. We have provided evidence that the OPRL1 receptors are coupled to multiple G proteins and that their stimulation leads to the activation of KCNMA1 channels in the term-pregnant rat uterus. Since the identification of PNOC in 1995 and the

deorphanization of OPRL1 receptors, several nonpeptide agonists have been synthesized and evaluated in animal or human studies [41]. OPRL1 receptors comprise a potential new drug target. We assume that after a systematic evaluation of their effect on the human myometrium, PNOC or OPRL1-related (preferably nonpeptide) agonists might be considered as prospective candidates for tocolytic therapy.

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