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Buprenorphine inhibits bradykinin-induced release of calcitonin gene-related peptide from rat trigeminal neurons *via* both μ-opioid and nociceptin/orphanin peptide receptors

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ABSTRACT

In this study we used the dual opioid and nociceptin/orphanin peptide (NOP) agonist buprenorphine to investigate the relative contributions of opioid and NOP systems in regulating bradykinin-stimulated calcitonin-gene related peptide (CGRP) release from primary cultures of neonatal rat trigeminal neurons. We found that: bradykinin stimulates CGRP secretion either by a direct effect or after applying so-called "bradykinin -priming" protocol. In both cases, buprenorphine was able to inhibit bradykinin-stimulated CGRP secretion; however, inhibition was mediated by NOP receptors when buprenorphine was added to the incubation medium along with bradykinin, whereas it appeared to be mediated by μ-opioid receptors in bradykinin priming experiments. Bradykinin treatments also caused an increase in neuronal prostaglandin production; prostanoids appeared to be involved in the stimulatory effects of bradykinin as well as in buprenorphine inhibition, through apparently unrelated mechanisms.

1. Introduction

In spite of important clinical issues such as physical dependence, cognitive disorders and the development of tolerance, opioids are used in malignant and non-malignant chronic pain conditions, including trigeminal neuropathy (Attal et al., 2006). The lack of recent, well-designed clinical trials limits the use of opioids in the treatment of migraine (Evers et al., 2006), although evidence of their efficacy in this condition is reported (Tornabene et al., in press).

It is well established that peripheral, spinal and supra-spinal opioid receptors act together in mediating opioid-induced analgesia; in the trigemino-cervical complex, opioids regulate nociceptive neurotransmission through an action on trigeminal nucleus caudalis neurons (Storer et al., 2003). However, the relative contribution of each site to a given analgesic effect is still matter of dispute. A large body of evidence shows that the activation of opioid receptors on primary afferent neurons in the peripheral nervous system plays a crucial role in opioid analgesia (Gupta et al., 2001; Dionne et al., 2001; Stein et al., 2003). Opioid receptors have been described on cell bodies of primary afferent neurons as well as on peripheral processes (Fields et al., 1980); co-localisation studies confirmed their presence on C- and A-fibres and in neurons expressing substance P and calcitonin gene-related peptide (CGRP) (Minami et al., 1995). Recent studies have pointed out that systemic opioids act predominantly through the activation of peripheral receptors (Shannon and Lutz, 2002). Moreover, opioid peripheral analgesia is increased by local inflammatory conditions, as demonstrated in different animal models of inflammatory and neuropathic pain, as well as in patients with chronic inflammation (Obara et al., 2004; Pertovaara and Wei, 2001; Stein et al., 2001; Truong et al., 2003; Whiteside et al., 2005).

Buprenorphine is a synthetic opiate with broad clinical applications, ranging from pain to opioid drug addiction (Cowan, 1995). As far as analgesia is concerned, buprenorphine shows a unique and complex mode of action. In fact, it acts as partial agonist at μ-opioid receptors; partial agonism might explain buprenorphine safety and–at least in part–such features such as the ceiling effect and the long duration of action, which have been observed in *in vivo* studies (Dum and Herz, 1981; Walsh et al., 1994). In addition, buprenorphine also exerts agonistic activity on nociceptin/orphanin peptide (NOP) receptors (Wnendt et al., 1999; Bloms-Funke et al., 2000; Huang et al., 2001). NOP receptor and its endogenous ligand nociceptin are structurally related to the opioid system, but exhibit a distinct pharmacological profile; in fact, the opioid peptides dynorphin A, enkephalin and β-endorphin are not able to cross-activate NOP receptors, and naloxone shows no antagonistic effect (Meunier et al., 1995; Mollereau et al., 1994; Hawkinson et al., 2000). NOP activation has been reported to produce anti-analgesic and analgesic and/or hyperalgesic effects, depending on the supra-spinal or spinal site of action, respectively (Mogil and Pasternak, 2001; Wang et al., 1999).

In *in vivo* animal models, the analgesic effects of buprenorphine seem to be mediated mostly by the activation of μ-opioid receptors (Lutfy et al., 2003); however, at variance with pure μ-agonists, buprenorphine also displays anti-hyperalgesic activity (Koppert et al.,

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2005). After systemic buprenorphine administration, the activation of supra-spinal NOP receptors induced by the drug caused a reduction in buprenorphine analgesic effects; moreover, in NOP knock-out mice buprenorphine showed enhanced μ-opioid-receptor-mediated antinociception (Lutfy et al., 2003). Thus, the activation of supra-spinal NOP receptors by buprenorphine appears to oppose the effects of μ-receptor stimulation; however, the existence of one such drug-receptor interplay at peripheral level remains to be demonstrated.

Rat primary trigeminal neuron cultures maintain several features of *in vivo* sensory nerve endings; the degree of functional activation of these cells can be evaluated through the measurement of CGRP release (Malin et al., 2007). In fact, trigeminal neurons express both μ-opioid and NOP receptors (Li et al., 1998; Xie et al., 1999; Hou et al., 2003) and release CGRP, a key neuropeptide of the trigeminal-vascular system involved in different pain syndromes such as migraine (Goadsby et al., 1988). Using trigeminal neuron primary cultures, we have recently shown that a NOP full agonist inhibits basal and stimulated CGRP release, suggesting a modulatory role for nociceptin in trigeminal nociceptive transmission (Capuano et al., 2007). In this paper, we used the same experimental paradigm to investigate the effect of buprenorphine on CGRP release and the relative contribution of NOP and μ-opioid in mediating such effect.

2. Methods

2.1. Trigeminal neuronal cultures

Trigeminal neuronal cultures were prepared from 6- to 7-day-old Wistar rats as previously described (Capuano et al., 2007). The use of animals for this experimental work has been approved by the Italian Ministry of Health (licensed authorization to P. Navarra). Briefly, ganglia from both sides were aseptically removed. Tissues were collected in a Petri dish containing 3–5 ml of ice-cold phosphate buffer saline without Ca^{++} and Mg^{++} (PBS w/o; Sigma), supplemented with antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin; Sigma) and D-glucose (6 g/l). Tissues were then digested in 5 mg/ml collagenase (Biospa, Milan, Italy) for 20 min at 37 °C, followed by 5 min incubation in 0.125% trypsin (EuroClone, Pero, Milan, Italy). 10 μl of DNAse I 1 mg/ml (2320 Kunitz/ml) was added in the last 5 min of incubation. Digested tissues were resuspended in 5 ml Ham's F12 medium (Biospa, Milan, Italy), containing 10% heat-inactivated endotoxin-free fetal calf serum (Gibco, Milan, Italy) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin), and cells were mechanically dissociated using a Pasteur pipette. Cell suspension was plated on a 25 cm^2 flask (Corning, Turin, Italy) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2–3 h (pre-plating). This additional step allowed us to separate neurons from non-neuronal cells on the basis of differential adhesion. At the end of pre-plating incubation, neurons were harvested from the flask and plated in 24-well tissue culture plates, previously coated with poly-D-lysine (40 mg/ml; MW 70000–130000, Sigma) at a density of 100,000 cells/well. The incubation volume was 1 ml/well of complete culture medium (see above), enriched with 50 ng/ml of 2.5 S murine Nerve Growth Factor (Alexis-Vinci Biochem, Vinci, Florence, Italy). The culture medium was changed within 24 h from seeding and 10 mM cytosine arabinoside (ARA-C) was added to further reduce non-neuronal cell growth. All experiments were carried out 6–7 days after dissection, when cells reached complete *in vitro* maturation.

2.2. CGRP release studies

In these studies, complete Ham's F12 medium (containing 10% fetal calf serum, 1 μM bacitracin, 10 mg/ml aprotinin, without Nerve Growth Factor) was used. Basal and stimulated CGRP release was assessed incubating cultures with medium alone or medium containing test drugs. The incubation volume was 300 μl; all experiments were performed at 37 °C and 5% CO_2 and lasted 10 min. In the experiments with receptor antagonist drugs, a 5-min pre-incubation in the presence of the receptor antagonist was performed. Thereafter, the medium was replaced with fresh release-medium containing test drugs. At the end of the experiments, media were collected and stored at −35 °C until the assays were performed.

2.3. Radioimmunoassays (RIAs)

CGRP release was measured by a radioimmunoassay technique using an antiserum produced and validated in our laboratory (Tringali et al., 2005). Each sample was assayed in duplicate. The release findings were expressed as pg/well. The mean IC_{50} of the standard curve was 50.8 ± 2.0 pg/tube. The intra-assay and inter-assay coefficients of variation were1.27% and ±0.95% at the lowest (1.95 pg/tube) and 13.7% and ±12.6% at the highest (1000 pg/tube) level of standard, respectively. The lowest concentration that could be measured with 95% confidence (i.e. 2 S.D. at zero) was 1.95 pg/tube. Thus, the detection limit was 19.5 pg/ml of the original sample (as 100 μl of each sample were used in the RIA system).

Prostaglandin E_2 (PGE_2) was measured as previously described (36). In brief, 100 μl of incubation medium were assayed; sample volume was diluted to 250 μl with 0.025 M phosphate buffer (pH 7.5). 2500–3000 cpm of [^{3}H]PGE_2 and antiserum at a final dilution of 1:115,000 were added together. A duplicate standard curve (ranging from 1 to 400 pg/tube, with an EC_{50} of 28 pg/tube) was run with each assay. The intra-assay and inter-assay coefficients of variation were 5% and 10%, respectively. Separation of free from antibody-bound PGE_2 was obtained with charcoal, which absorbs 95–98% of free PGE_2. After centrifugation for 10 min at 4 °C, supernatants were decanted directly into 10 ml of liquid scintillation fluid and radioactivity measured by liquid scintillation counting.

2.4. Drugs

Buprenorphine was kindly provided by Formenti–Grünenthal (Italy) as hydrochloride salt; the drug was dissolved in distilled water and 100 mM-aliquots were stored at −35 °C. Bradykinin was purchased by Vinci-Biochem (Florence, Italy) and dissolved in a 5% acetic acid solution at 100 mM. [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO), N(Phe) Nociceptin(1–13) (NPheNOC), the selective NOP receptor antagonist and naloxone hydrochloride were purchased from Tocris (Cookson, UK), dissolved in distilled water at 100 mM and aliquots stored at −35 °C. Indomethacin meglumine salt from commercial sources (Chiesi Farmaceutici, Italy) was used; the drug was first dissolved in sterile water and then diluted at final concentration of 1 μg/ml. All working dilutions were made using the releasing-medium. Study drugs were assayed as blanks in RIAs, and showed no interference with the assay up to the highest concentrations tested.

2.5. Statistical analysis

All data are presented as mean ± S.E.M., unless otherwise noted. Significant differences between groups were assessed by one-way analysis of variance (ANOVA) with Newman–Keuls' multiple comparison *post-hoc* test, unless otherwise stated. A comparison analysis between two linear regressions was performed. All data were analysed using a PrismTM computer program (GraphPad, San Diego, CA, USA).

3. Results

3.1. Bradykinin induces CGRP and PGE2 release from rat trigeminal neurons

A peptide endowed with potent pro-inflammatory activity, bradykinin induced a concentration-dependent increase in CGRP release from 0.1 nM onward, reaching the maximal stimulatory effect at 1 μM (Fig. 1). The latter concentration was used in all subsequent

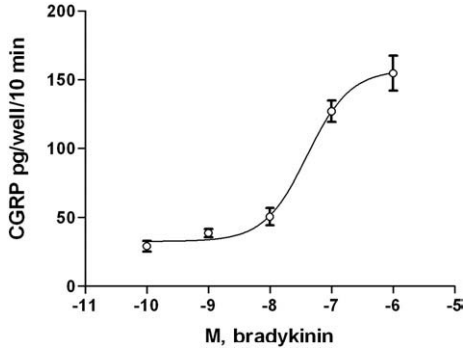

Fig. 1. CGRP release stimulated by bradykinin. Trigeminal neurons were exposed for 10 min to medium containing graded doses of bradykinin. Media were collected and CGRP was measured. Data are expressed as pg of CGRP/well/10 min, the means ± S.E.M. of $n = 6$ replicates for each experimental group. ***$P < 0.001$ *vs* controls.

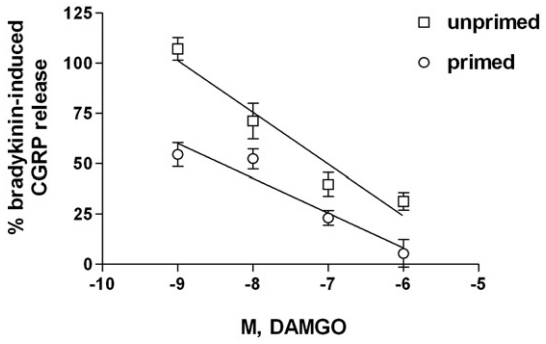

Fig. 3. Effects of DAMGO on bradykinin-induced CGRP release in unprimed and primed cultures. In unpriming protocol, trigeminal neurons were incubated with medium containing graded doses of full μ-opioid agonist DAMGO along with 1 μM bradykinin, and CGRP release was measured after 10 min. In priming experiments, neurons were exposed to 1 μM bradykinin for 15 min and further incubated with plain medium or medium containing graded doses of DAMGO, and CGRP release was measured for 10 min. Regression curves were constrained to parallelism (see Results for details) and a relative potency was calculated as indirect measure of receptorial arrangement induced by bradykinin priming. Data are expressed as percentage of bradykinin-induced CGRP release, the mean ± S.E.M. of 8 replicates of two independent experiments.

experiments. The effects of bradykinin were also tested on PGE_2 secretion in this paradigm; 1 μM bradykinin induced a marked ($P < 0.01$) increase in PGE_2 release from cultured neurons after 10 min of incubation, and this effect persisted up to 40 min (Fig. 2).

3.2. Bradykinin challenge: unpriming vs priming experimental paradigms

The actions of buprenorphine on the modulation of CGRP release from rat trigeminal neurons were assessed using two experimental protocols. In fact, the prolonged *in vitro* exposure of trigeminal neurons to bradykinin has been reported to induce functional competence and trafficking of opioid-receptors (Berg et al., 2007). Since buprenorphine is known to act, at least in part, through the activation of these receptors, we sought to investigate whether a prolonged exposure to bradykinin might unmask responses to buprenorphine that are mediated by μ-opioid receptors as well. In the unpriming protocol we treated neuron cultures with medium containing testing doses of buprenorphine, given along with bradykinin, and CGRP release was measured after 10 min. Conversely, in the bradykinin priming protocol we first exposed neuron cultures to 1 μM bradykinin for 15 min, then we replaced medium with plain medium or medium containing buprenorphine and measured CGRP released after 10 min; under these conditions, a marked increase in CGRP secretion caused by previous bradykinin exposure is observed.

3.3. DAMGO displays different inhibitory profile in unprimed and primed neuron cultures

The modulatory effect of bradykinin-priming protocol on the receptorial arrangement of μ-opioid receptors on neuronal surface was first investigated in control experiments with the full μ-opioid receptor, DAMGO. The latter, given in the range 1 nM–1 μM, induced a dose-dependent inhibition of bradykinin-evoked CGRP release in both experimental paradigms (Fig. 3). A linear regression analysis was performed to compare the two dose-response curves from unpriming and priming protocols. EC_{50} values were −6.934 M ± 0.025 M and −8,413 M ± 0.040 M for unprimed and primed neurons, respectively. The parallelism test performed on the two regression equations showed a not significant difference between the slopes (t calculated = 1.772; t tabular = 2.060; n.s.); thus, the relative potency ($R = 27.8$) provided a quantitative estimate of the effect of priming over the degree of receptor expression in this paradigm.

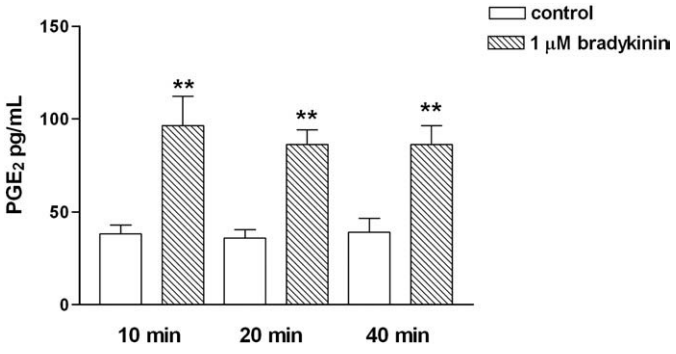

Fig. 2. Time-dependent increase of PGE2 release from primary culture of rat trigeminal neurons. Cells were incubated with plain medium (controls) or medium containing 1 μM bradykinin. PGE2 secretion was evaluated at different time points (up to 40 min). Data are expressed as pg/ml of PGE_2, the means ± S.E.M. of $n = 4$ replicates for each experimental group. **$P < 0.01$ *vs* controls.

3.4. Buprenorphine inhibits bradykinin-evoked CGRP release via NOP receptor in unprimed cultures

Buprenorphine, given in the range 0.1 nM–10 μM, elicited a concentration-dependent decrease in bradykinin-stimulated CGRP release, reaching a maximum at 0.1 μM (−37% *vs* bradykinin alone; $P < 0.001$); higher buprenorphine concentrations were associated to lower, albeit significant reductions (Fig. 4). The inhibitory effect of

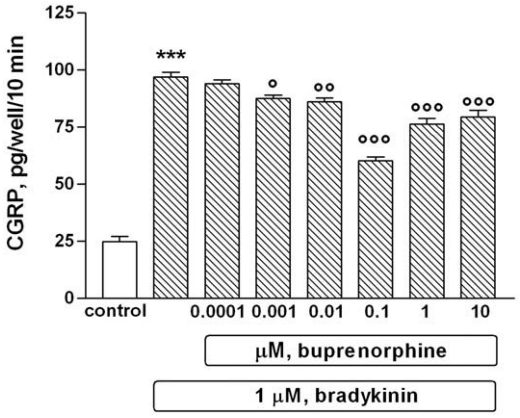

Fig. 4. Effects of buprenorphine on bradykinin-induced CGRP release. Trigeminal neurons were incubated with medium containing 1 μM bradykinin alone or bradykinin plus graded doses of buprenorphine (unpriming protocol). Buprenorphine inhibited bradykinin evoked CGRP release showing a dose-dependent U-shaped profile. Data are expressed as pg of CGRP/well/10 min, the means ± S.E.M. of $n = 8$ replicates for each experimental group. ***$P < 0.001$ *vs* controls; °, °° and °°°$P < 0.05$, $P < 0.01$ and $P < 0.001$ *vs* bradykinin given alone, respectively.

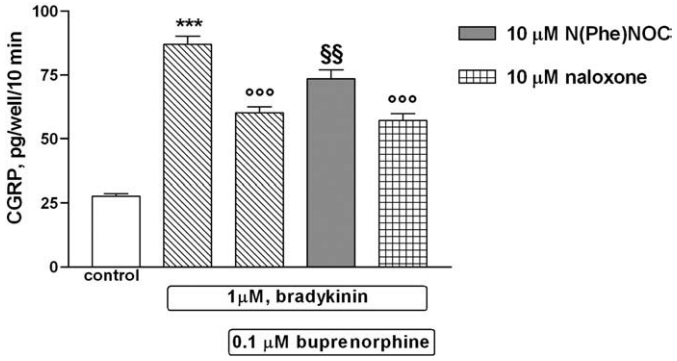

Fig. 5. Primary cultures of trigeminal neurons were incubated with medium containing 1 μM bradykinin alone or in combination with 0.1 μM buprenorphine (unpriming protocol). Where indicated, neurons were pre-exposed for 5 min to 10 μM NPheNOC, a NOP receptor antagonist, or to10 μM naloxone. NPheNOC antagonized the effects of 0.1 μM buprenorphine on bradykinin-stimulated CGRP release, while naloxone had no effects. Data are expressed as pg of CGRP/well/10 min, the means ± S.E.M. of $n = 4–5$ replicates for each experimental group. ***$P<0.001$ *vs* controls; °°°$P<0.001$ *vs* bradykinin given alone; §§$P<0.01$ *vs* bradykinin + buprenorphine.

0.1 μM buprenorphine was significantly counteracted by 10 μM of the selective NOP receptor antagonist NPheNOC ($P<0.01$ *vs* buprenorphine alone), whereas 10 μM naloxone had no effect whatsoever, indicating that the inhibitory effect of buprenorphine over bradykinin stimulation of CGRP release is mediated *via* the activation of NOP receptors (Fig. 5).

3.5. Buprenorphine inhibits bradykinin-evoked CGRP release via μ-receptor in primed neurons

Buprenorphine, given in the range 0.1 nM–10 μM, displayed a concentration-dependent inhibitory profile, with maximal effect at 10 μM (Fig. 6)(−37% *vs* bradykinin alone, $P<0.001$). As a consequence of priming, the shape of buprenorphine dose-response curve changed from U-shaped (Fig. 4) into linear; therefore, a statistical analysis was carried out using the linear regression method to compare the two dose-range responses of buprenorphine obtained in unprimed *vs* primed neurons. The parallelism test performed on the two regression equations revealed a non significant difference between slopes, and buprenorphine displayed similar relative potencies under unprimed and primed conditions (t calculated = 1.634; t tabular = 2.011; n.s.).

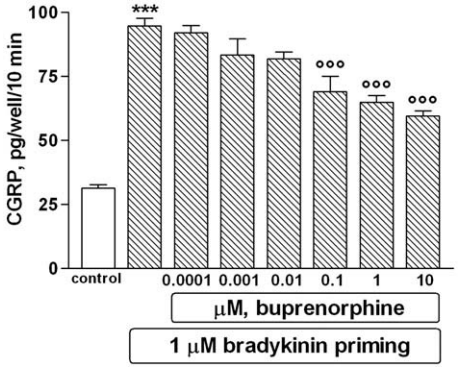

Fig. 6. Effects of buprenorphine on CGRP release in bradykinin-primed trigeminal neurons. Cultures of rat trigeminal neurons were incubated with medium containing 1 μM bradykinin (priming). After 15 min, medium was removed, and neurons were incubated with plain medium or medium containing tested doses of buprenorphine for additional 10 min. Bradykinin-priming significantly increased CGRP release, which was inhibited by buprenorphine in a dose-dependent manner, reaching the maximal effect at 10 μM. Data are expressed as pg of CGRP-LI/well/10 min, the means ± S.E.M. of $n = 6$ replicates for each experimental group. ***$P<0.001$ *vs* controls; °°°$P<0.001$ *vs* bradykinin given alone.

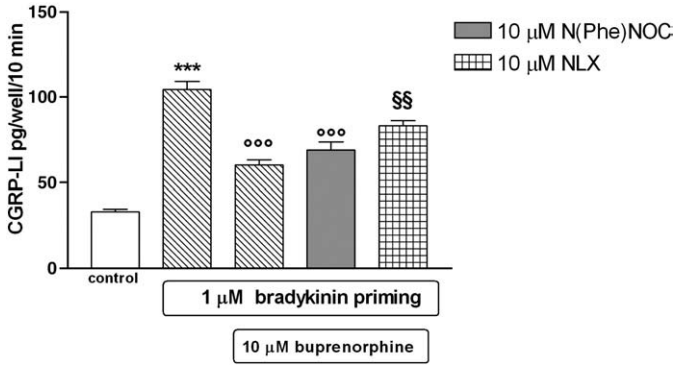

Fig. 7. Effects of NPheNOC and naloxone on buprenorphine effects on CGRP release in bradykinin-primed cultures. Trigeminal neurons were exposed to 1 μM bradykinin for 15 min (priming) and then, incubated with plain medium or medium containing 10 μM buprenorphine. When indicated, neurons were treated with 10 μM NPheNOC or naloxone. Naloxone reverted the effects of 10 μM buprenorphine, while NPheNOC showed no effects. Data are expressed as pg of CGRP/well/10 min, the means ± S.E.M. of $n = 4–5$ replicates for each experimental group. ***$P<0.001$ *vs* controls; °°°$P<0.001$ *vs* bradykinin given alone; §§$P<0.01$ *vs* bradykinin + buprenorphine.

However, the priming procedure appeared to modify the pattern of buprenorphine-receptor interaction; in fact, after priming NPheNOC loose its capability to antagonize buprenorphine inhibition of bradykinin effect, while on the contrary such effect was significantly counteracted by naloxone (Fig. 7).

3.6. Indomethacin partially counteracts the effects of buprenorphine on primed cultures

A pre-treatment with the non selective cyclooxygenase inhibitor indomethacin, given at 1 μg/ml concentration, caused a weak, albeit significant reduction in bradykinin-priming-stimulated CGRP release (Fig. 8). As expected, 10 μM buprenorphine given alone also significantly reduced bradykinin stimulation of CGRP release; however, buprenorphine inhibition was partially counteracted by indomethacin pre-treatment (Fig. 8).

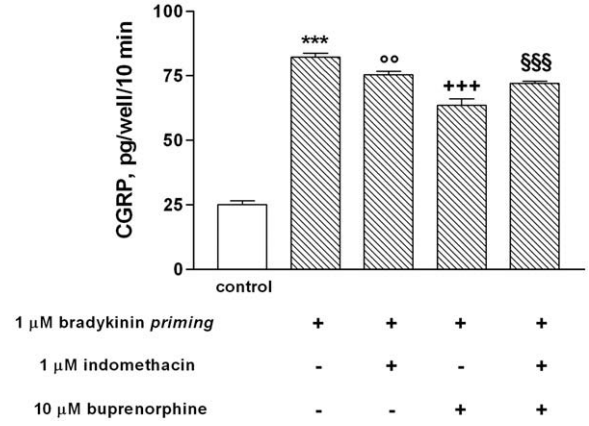

Fig. 8. Effects of indomethacin on CGRP release from bradykinin-primed cultures. Bradykinin-primed neurons (15 min exposure to 1 μM bradykinin), were incubated with plain medium or medium containing 10 μM buprenorphine with or without a pre-treatment with 1 μg /ml indomethacin. Indomethacin weakly counteracted the effects of buprenorphine on CGRP release from bradykinin-primed neurons. Data are expressed as pg of CGRP/well/10 min, the means ± S.E.M. of $n = 6$ replicates for each experimental group. ***$P<0.001$ *vs* controls; °°$P<0.01$ *vs* bradykinin given alone; +++$P<0.001$ *vs* bradykinin given alone; §§§$P<0.001$ *vs* buprenorphine without indomethacin.

4. Discussion

4.1. Bradykinin is a useful pharmacological tool to investigate in vitro effects of inflammation on CGRP release from rat trigeminal neurons

In this study we used bradykinin, a known activator of sensory neurons endowed with potent inflammatory activity (Dray and Perkins, 1993; Calixto et al., 2000), as a tool to stimulate CGRP release. Bradykinin was shown to enhance CGRP release from cultured dorsal root ganglia as well as from trigeminal ganglion neurons (Vasko et al., 1994; Jenkins et al., 2001). These effects are mediated by G-protein coupled receptor B1 and B2 receptor subtypes, which are both expressed on trigeminal neurons (Calixto et al., 2004; Ma et al., 2000).

In our model, we characterized the effects of bradykinin on CGRP release from rat trigeminal neurons, confirming that bradykinin exerts a potent and dose-dependent stimulatory effect on CGRP. Indeed, the peptide stimulates CGRP secretion within 10 min of incubation either by a direct effect or after applying the bradykinin-priming protocol (see Section 3.2 for details). In addition, bradykinin was able to increase PGE_2 production and release from trigeminal neurons, providing further evidence for the involvement of prostanoids in generating and maintaining inflammatory pain process (Jenkins et al., 2003).

4.2. Bradykinin priming induces a functional expression of μ-opioid receptors on trigeminal neurons in vitro: the DAMGO paradigm

Bradykinin was used with two different experimental designs, either as a constant (unpriming protocol) or a pulse (bradykinin priming) stimulus. Indeed, in trigeminal neurons, bradykinin priming was shown to induce functional competence and trafficking of delta-opioid receptors, and a rapid modulation of μ-opioid receptor signaling (Berg et al., 2007; Patwardhan et al., 2005). The changes in receptor arrangement induced by bradykinin priming seem to be mediated by B_2 receptor, with the subsequent activation of both PKC-dependent intracellular signals and cyclooxygenase (COX)-dependent release of arachidonic acid products (Berg et al., 2007).

In this context, we tested the effects of the full μ-opioid receptor agonist DAMGO in primed and unprimed culture. In both paradigms, DAMGO exerted a dose-dependent inhibitory effect on bradykinin-stimulated CGRP release. Moreover, the priming protocol was able to left-shift the regression curve of DAMGO (as demonstrated by statistical analysis), providing indirect evidence that bradykinin priming modulates the number, and possibly the function, of μ-opioid receptors expressed on the cell surface.

4.3. The effects of buprenorphine on bradykinin-evoked CGRP release is inhibitory but the profile of drug-receptor interaction is complex

Some evidence supports the notion that local opioid treatments are more effective in the presence of local inflammatory states. In fact, under such conditions as tissue injury, inflammation and neuropathic pain, an increased expression of opioid receptors as well as facilitated drug-receptor interaction and coupling efficiency have been described (Zollner et al., 2003). Such phenomena are commonly observed in animal models of inflammatory and neuropathic pain, although local opiod application in clinical trials and human pain models did not show clear advantages compared to systemic treatments (Stein et al., 2003) In our model, in both unpriming and priming protocols buprenorphine exhibited an inhibitory profile on bradykinin-induced CGRP release, which was different in maximal efficacy but similar in potency. Indeed, in the presence of increased CGRP secretion as induced by bradykinin, the effects of buprenorphine were consistently inhibitory, but inhibition was mediated by NOP receptors when buprenorphine was added to the incubation medium along with bradykinin (unpriming paradigm), whereas it appeared to be mediated by in bradykinin priming experiments. These two different experimental conditions allowed us to differentiate between NOP and μ-opioid receptorial actions of buprenorphine: in unprimed cultures, buprenorphine inhibition was antagonized by NPheNOC, whereas naloxone was able to counteract buprenorphine inhibitory effect in primed cultures. These findings are in keeping with the notion that opioid efficacy is enhanced under local inflammatory conditions.

The activation of classical opioid receptors, either at central or peripheral level, produces antinociception (Stein et al., 1989, 2003; Truong et al., 2003), whereas NOP activation induces different or even opposite effects, depending on whether the activation occurs at supraspinal, spinal or peripheral level (Mogil and Pasternak, 2001; Wang et al., 1999). In this framework, buprenorphine represents a unique pharmacological tool to investigate the effects of simultaneous activation of the two receptor systems. Co-activation of NOP and μ-opioid receptors has been extensively studied in *in vivo* models of antinociception; evidence indicates that central NOP activation tends to compromise the overall antinociceptive activity of buprenorphine, and to override the antinociceptive effects of buprenorphine induced by the activation of spinal μ-opioid receptors (Lutfy et al., 2003;Yamamoto et al., 2006). Our experimental paradigm allows us to investigate the relative contribution of opioid and NOP in mediating the effects of buprenorphine, devoid of the influence of central receptor activation.

4.4. Role of prostanoids in mediating the effect of bradykinin priming

In our paradigm, we showed that the blockade of COX activity by indomethacin caused a significant reduction, but not a complete reversal of bradykinin stimulatory effects on CGRP release, suggesting that prostanoids acts as positive modulators–rather than mediators–of bradykinin activity. The reduced levels of prostaglandins following indomethacin treatments affected the inhibitory effects of buprenorphine in bradykinin-priming experiments. Such phenomenon can be explained by the fact that prostaglandins play a role in exposing μ-opioids receptors on cell surface (Berg et al., 2007), and the reduced availability of these receptors ensuing COX blockade impairs buprenorphine inhibitory capability.

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