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G protein activation and cyclic AMP modulation by naloxone benzoylhydrazone in distinct layers of rat olfactory bulb

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1 Naloxone benzoylhydrazone (NalBzoH) has initially been developed as an agonist of the pharmacologically defined κ_3-opioid receptor and has recently been employed as an antagonist at the opioid receptor-like (ORL1) receptor. In the present study, we investigated the ability of NalBzoH to elicit agonist-like effects on receptor signalling in distinct layers of rat olfactory bulb, a brain region where we have demonstrated the presence of opioid and ORL1 receptors coupled to both stimulation and inhibition of cyclic AMP formation.

2 In membranes of the olfactory nerve-glomerular layer (ON-GL), external plexiform layer (EPL) and granule cell layer (GRL), NalBzoH elicited a concentration-dependent stimulation of guanosine-5′-*O*-(3-[^{35}S]-thio)triphosphate ([^{35}S]GTPγS) binding with pEC_{50} values ranging from 7.36 to 7.86, whereas the κ_1-opioid receptor agonists (−)-U-50,488 and U-69,593 were inactive.

3 In membranes of GRL, but not ON-GL and EPL, NalBzoH stimulated basal adenylyl cyclase activity by 40% with a pEC_{50} of 8.14, and significantly potentiated the net enzyme stimulation elicited by corticotropin-releasing hormone and pituitary adenylate cyclase-activating peptide 38. Pertussis toxin prevented the NalBzoH stimulations of [^{35}S]GTPγS binding and adenylyl cyclase activity.

4 In membranes of EPL and GRL, but not ON-GL, NalBzoH elicited a concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity with pEC_{50} values of 8.07 and 8.08, respectively.

5 At concentrations that completely blocked the actions of nociceptin/orphanin FQ (N/OFQ), the ORL1 receptor antagonists CompB and [Nphe^1]N/OFQ(1–13)NH_2 failed to antagonize either the stimulatory or the inhibitory effect of NalBzoH on cyclic AMP formation. Similarly, the κ_1-opioid receptor antagonist nor-binaltorphimine counteracted the NalBzoH effects with relatively low potencies (pK_i values = 7.67–8.09).

6 Conversely, the selective δ-opioid receptor antagonist TIPP ($pK_i = 9.10$) and the selective μ-opioid receptor antagonist CTAP ($pK_i = 8.27$) reduced the inhibitory effect of NalBzoH by 70 and 30%, respectively. Moreover, TIPP and CTAP potently inhibited the NalBzoH stimulation of cyclic AMP, each antagonist maximally causing 50% blockade of the agonist response.

7 These data demonstrate that in the olfactory bulb NalBzoH activates receptor signalling by acting through δ- and μ-opioid receptors and independently of ORL1 and κ_1-opioid receptors.
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Keywords: Opioid receptors; ORL1 receptor; naloxone benzoylhydrazone; [^{35}S]GTPγS binding; adenylyl cyclase; olfactory bulb

Abbreviations: α_{TGDP}, GDP-bound form of the α subunit of transducin; BSA, bovine serum albumin; CRH, corticotropin-releasing hormone; DTT, dithiothreitol; EPL, external plexiform layer of the main olfactory bulb; FSK, forskolin; GRL, granule cell layer of the main olfactory bulb; NalBzoH, naloxone benzoylhydrazone; N/OFQ, nociceptin/orphanin FQ; nor-BNI, nor-binaltorphimine; Nphe, [Nphe^1]N/OFQ(1–13)NH_2; ON-GL, olfactory nerve-glomerular layer of the main olfactory bulb; ORL1, opioid receptor-like; PACAP 38, pituitary adenylate cyclase-activating polypeptide 38; PL017, (*N*-Me-Phe^3,D-Pro^4)-casomorphin (1–4) amide; PTX, pertussis toxin

Introduction

Naloxone benzoylhydrazone (NalBzoH) is a naloxone derivative that acts on opioid receptors with a complex pharmacological profile. Early studies *in vivo* showed that NalBzoH potently antagonized morphine-induced analgesia and, at higher doses, caused analgesia independently of μ-, δ-, and κ_1-opioid receptors and through the activation of a novel subtype of κ-opioid receptor, termed κ_3 (Paul *et al*., 1990). Accordingly, radioligand-binding studies identified the occurrence of three distinct κ-opioid receptor subtypes, termed κ_1, κ_2 and κ_3, and showed that [^{3}H]NalBzoH labeled the κ_3 subtype with high affinity (Clark *et al*., 1989; Cheng *et al*., 1992; Berzetei-Gurske *et al*., 1995). The κ_3-opioid receptor subtype was characterized as being relatively insensitive to both κ_1-opioid receptor agonists, such as U-50,488 and U-69,593, and the κ_1-opioid receptor antagonist nor-binaltorphimine (nor-BNI) (Clark *et al*., 1989; Cheng *et al*., 1992;

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Berzetei-Gurske *et al*., 1995). Moreover, functional studies demonstrated that NalBzoH behaved as a potent antagonist at the μ-opioid receptor (Dunnill *et al*., 1996; Brown & Pasternak, 1998), whereas it acted as an agonist at κ_3-opioid receptors expressed in BE(2)-C and SH-SY5Y neuroblastoma cell lines (Standifer *et al*., 1994; Cheng *et al*., 1995). However, so far a single gene for each opioid receptor subtype has been identified and the pharmacological profile of the cloned κ receptor corresponds to that of the putative κ_1 receptor subtype (Dhawan *et al*., 1996). Thus, the molecular nature of the putative κ_3-opioid receptor subtype is still obscure. In the attempt to isolate the gene for the κ_3-opioid receptor subtype, Pan *et al*. (1995) reported the identification of a cDNA clone, termed KOR3, which was found to correspond to the mouse homologue of the human opioid receptor-like (ORL1) receptor for the endogenous peptide nociceptin/orphanin FQ (N/OFQ) (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Mogil & Pasternak, 2001). When transfected in host cells, the ORL1 receptor displayed pharmacological properties distinct from that of the cloned opioid receptor subtypes and from the pharmacologically defined κ_3-opioid subtype (Calo' *et al*., 2000b; Mogil & Pasternak, 2001). Nonetheless, a number of studies have shown that NalBzoH is a ligand of the ORL1 receptor, behaving either as a potent N/OFQ antagonist or partial agonist (Dunnill *et al*., 1996; Abdulla & Smith, 1997; Nabeshima *et al*., 1999; Chiou, 2001; Bigoni *et al*., 2002). Moreover, behavioural studies in mice have shown that NalBzoH antagonized the hyperalgesic effect of N/OFQ, and that the antinociceptive effect of NalBzoH was lost in mice made deficient of ORL1 receptor, suggesting that blockade of ORL1 receptor, rather than stimulation of κ_3 opioid receptor, mediates the action of NalBzoH (Noda *et al*., 1998). On the other hand, in neuroblastoma BE(2)-C cell line, pharmacological and antisense mapping data showed that NalBzoH can inhibit cyclic AMP formation independently of ORL1 receptor activation, thus providing support to the idea that in this cell system the receptor mechanism of NalBzoH and N/OFQ are distinct (Mathis *et al*., 2001).

We have previously shown that in rat olfactory bulb, activation of μ and δ opioid and ORL1 receptors exerts a bimodal control of cyclic AMP formation, causing stimulation of basal and neurotransmitter-stimulated adenylyl cyclase activities and inhibition of forskolin (FSK)- and Ca^{2+}/calmodulin-stimulated enzyme activities (Olianas & Onali, 1992; 1994; Onali *et al*., 2001). Evidence has been provided indicating that these opposite effects on cyclic AMP are mediated through the $\beta\gamma$ subunits of G proteins of the G_i/G_o type (Olianas & Onali, 1999), which differentially affect the activity of Ca^{2+}-insensitive and Ca^{2+}-sensitive isoforms of adenylyl cyclase (Sunahara *et al*., 1996). In the course of these studies, we noticed that NalBzoH was able to exert agonist effects on receptor signalling, which were similar to those elicited by N/OFQ and μ- and δ-opioid receptor agonists.

In the present study, we show that NalBzoH causes G protein activation and modulates adenylyl cyclase activity in tissue membranes of specific layers of the rat main olfactory bulb. Moreover, by using selective receptor agonists and antagonists, we demonstrate that NalBzoH acts independently of ORL1 and κ_1-opioid receptors and exerts its agonist activity through the stimulation of δ- and μ-opioid receptors.

Methods

Tissue dissection and membrane preparation

Male Sprague–Dawley rats (200–300 g) were used. Animals were maintained in a 12 h light/dark cycle with food and water *ad libitum*. Experiments were performed according to the principles of laboratory animal care (Law on animal experiments in Italy, D.L. 116/92). Rats were killed by decapitation and tissue dissections were performed as previously described (Onali *et al*., 2001). Briefly, with the use of a tissue slicer, the olfactory bulbs were cut in coronal sections (300 μm thick), which were kept in an ice-cold phosphate- buffered saline (PBS) solution. With the aid of a stereoscopic microscope equipped with a diascopic illuminator base, the bulb layers were clearly identified on the basis of their characteristic cytoarchitecture and each slice was free-hand dissected into three portions: the olfactory nerve-glomerular layer (ON-GL); the external plexiform layer including the mitral cell layer (EPL); the granule cell layer (GRL). ON-GL was microdissected by making a cut along the border between this layer and EPL, whereas EPL was separated from GRL by cutting along the deep part of the mitral cell layer. The tissue layers from individual slices were pooled and homogenized in an ice-cold buffer containing 10 mM HEPES–NaOH, 1 mM EGTA, 1 mM $MgCl_2$ (pH 7.40) using a Teflon-glass tissue grinder. The homogenate was centrifuged at 27,000 $\times g$ for 20 min at 4°C. The pellet was resuspended in the same buffer at a protein concentration of 0.8–1.0 mg ml^{-1} and either stored at −80°C for binding assays or used immediately for adenylyl cyclase assays. Striatal tissue (dorsal striatum) was microdissected from 300 μm thick coronal slices of a brain tissue block obtained by performing a first transverse cut just anterior to the olfactory tubercle and a second transverse cut at the level of the median eminence. Tissue from five to six sections was collected and membranes were prepared as described for the olfactory bulb. For each experiment, a fresh tissue preparation was used.

Assay of [^{35}S]GTPγS binding

Tissue membranes were diluted in 10 mM HEPES/NaOH (pH 7.4) and 1 mM EDTA, centrifuged at 27,000 $\times g$ for 20 min at 4°C and resuspended in the same buffer. The binding of [^{35}S]GTPγS was assayed in a reaction mixture (final volume 100 μl) containing 25 mM HEPES/NaOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 50 μM GDP and 1.0 nM [^{35}S]GTPγS. KCl was used instead of NaCl as preliminary experiments indicated that, while equally effective in reducing basal [^{35}S]GTPγS binding, KCl allowed a higher receptor-mediated stimulation than NaCl. The incubation was started by the addition of the membrane suspension (5–8 μg of protein) and was carried out at 30°C for 60 min. The incubation was terminated by adding 5 ml of ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.4) and 1 mM $MgCl_2$, immediately followed by rapid filtration on glass fibre filters presoaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 μM GTPγS. Assays were performed in duplicate.

To investigate the reversibility of NalBzoH stimulation of [^{35}S]GTPγS binding, time-course experiments were performed. Tissue membranes were preincubated for 10 min at 30°C with vehicle or 100 nM NalBzoH. At time 0, reaction was started by the addition of [^{35}S]GTPγS (final concentration 1 nM) and stopped at 2 min intervals for the following 20 min. Naloxone (final concentration 10 μM) was added to either vehicle- or NalBzoH-treated samples 6 min after the beginning of the reaction.

Protein content was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Adenylyl cyclase assay

Adenylyl cyclase activity was assayed in a 100 μl reaction mixture containing 50 mM HEPES/NaOH (pH 7.4), 2.3 mM $MgCl_2$, 0.3 mM EGTA, 0.2 mM [α-^{32}P]ATP (50–70 c.p.m. pmol^{-1}), 0.5 mM [^{3}H]cyclic AMP (80 c.p.m. nmol^{-1}), 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U ml^{-1} creatine phosphokinase, 100 μM GTP, 50 μg of BSA, 10 μg of bacitracin, 10 μM bestatin and 10 kallikrein inhibitor units of aprotinin. When forskolin (FSK) was used, it was added to the reaction mixture at the final concentration of 10 μM. The reaction was started by adding the tissue preparation (30–40 μg of protein) and was carried out at 30°C for 10 min. When the effect of the GDP-bound form of the α subunit of transducin (α_{TGDP}) was investigated, 10 μl of tissue membranes (10–15 μg of protein) were preincubated with an equal volume of a solution containing either 2 μg of α_{TGDP} (kindly donated by Dr H.E. Hamm, Northwestern University, Chicago, IL, U.S.A.) or vehicle at 4°C for 60–80 min. Thereafter, NalBzoH or vehicle was added (10 μl) immediately followed by the addition of the reaction mixture (20 μl). The incubation was carried out for 10 min at 30°C. Cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns as described by Salomon *et al*. (1974). The recovery of [^{32}P]cyclic AMP from each sample was calculated on the basis of the recovery of [^{3}H]cyclic AMP. Assays were carried out in duplicate.

Intracerebral injection of pertussis toxin (PTX)

PTX (Sigma RBI) dissolved in a vehicle containing 50 mM sodium phosphate buffer and 250 mM NaCl (pH 7.0) was stereotaxically injected into the right olfactory bulb at two positions (total amount of 3.5 μg in 7 μl), as previously described (Olianas & Onali, 1994). The control animals were injected with an equal volume of vehicle containing 3.5 μg of BSA. The rats were killed 60–68 h after surgery and olfactory bulb from vehicle- and toxin-injected animals were microdissected and tissue membranes were prepared as described above. Three tissue preparations were tested.

Materials

[α-^{32}P]ATP (30–40 Ci mmol^{-1}), [2,8-^{3}H]cyclic AMP (25 Ci mmol^{-1}) and [^{35}S]GTPγS (1306 Ci mmol^{-1}) were from Perkin-Elmer-New England Nuclear (Boston, MA, U.S.A.). N/OFQ and corticotropin-releasing hormone (CRH) (human, rat) were from Neosystem (Strasbourg, France). [Nphe^1]N/OFQ(1–13)NH_2 (Nphe) and CompB (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzamidazol-2-one) were kindly provided by Drs. G. Calo' (University of Ferrara, Italy) and S. Ozaki (Banyu Tsukuba Research Institute, Tsukuba, Japan), respectively. Deltorphin I, (N-Me-Phe^3,D-Pro^4)-casomorphin (1–4) amide (PL017), TIPP and pituitary adenylate cyclase-activating polypeptide 38 (PACAP 38) were obtained from Bachem AG (Bubendorf, Switzerland). FSK and GTPγS were from Calbiochem, (San Diego, CA, U.S.A.) and Boehringer (Mannheim, Germany), respectively. (−)-U-50,488 hydrochloride (trans-(−)3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide hydrochloride), nor-BNI dihydrochloride and CTAP were from Tocris Cookson Ltd (U.K.). NalBzoH, U-69,593 ((5α, 7α, 8β)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro (4-5)dec-8-yl)benzeneacetamide), aprotinin, bestatin, bacitracin and the other reagents were from Sigma RBI (St Louis, MO, U.S.A.). Naloxone hydrochloride was obtained from Salars (Como, Italy).

Statistical analysis

Results are reported as means ± s.e.m. Data from agonist concentration–response curves were analysed by a least-squares curve fitting computer programme (Graph Pad Prism, San Diego, CA, U.S.A.). Agonist concentration producing half-maximal effect (EC_{50} values) were converted to the logarithmic form (pEC_{50} = negative logarithm of EC_{50}) as these values are log-normally distributed (Fleming *et al*., 1972). Antagonist potencies were determined in experiments where the compounds were examined for their ability to reverse the agonist effect in a concentration-dependent manner. The data were analysed as competition curves by nonlinear regression analysis, and the antagonist inhibitory constant (K_i) was calculated according to the Cheng–Prusoff equation (Cheng & Prusoff, 1973). In a limited number of experiments involving the antagonism of (−)-U-50,488 by nor-BNI in rat striatum, the effect of a fixed concentration of antagonist on the agonist concentration response curves was examined and the K_i value was determined according to the equation $EC_{50}b = EC_{50}a\,(1 + I/K_i)$, where $EC_{50}a$ and $EC_{50}b$ are the agonist concentrations producing half-maximal effects in the absence and in the presence of the antagonist and I is the antagonist concentration. K_i values were converted to the logarithmic form (pK_i). Statistically significant differences between concentration–response curves were determined by two-way analysis of variance with repeated measures. Statistical significance of the difference between means was determined by Student's *t*-test.

Results

Effects on [^{35}S]GTPγS binding

In ON-GL, EPL and GRL membranes, NalBzoH elicited a concentration-dependent stimulation of [^{35}S]GTPγS binding to membrane G proteins with pEC_{50} values of 7.36 ± 0.08, 7.80 ± 0.04 and 7.86 ± 0.05, respectively, and maximal effects corresponding to 19.4 ± 2.0% ($P<0.05$, $n=3$), 99.8 ± 3.2% ($P<0.01$, $n=3$) and 59.5 ± 2.1% ($P<0.01$, $n=3$) increase of basal values, respectively (Figure 1). For comparison, we examined the effects of other opioid receptor agonists under the same experimental conditions used for NalBzoH. In ON-GL membranes, the ORL1 agonist N/OFQ

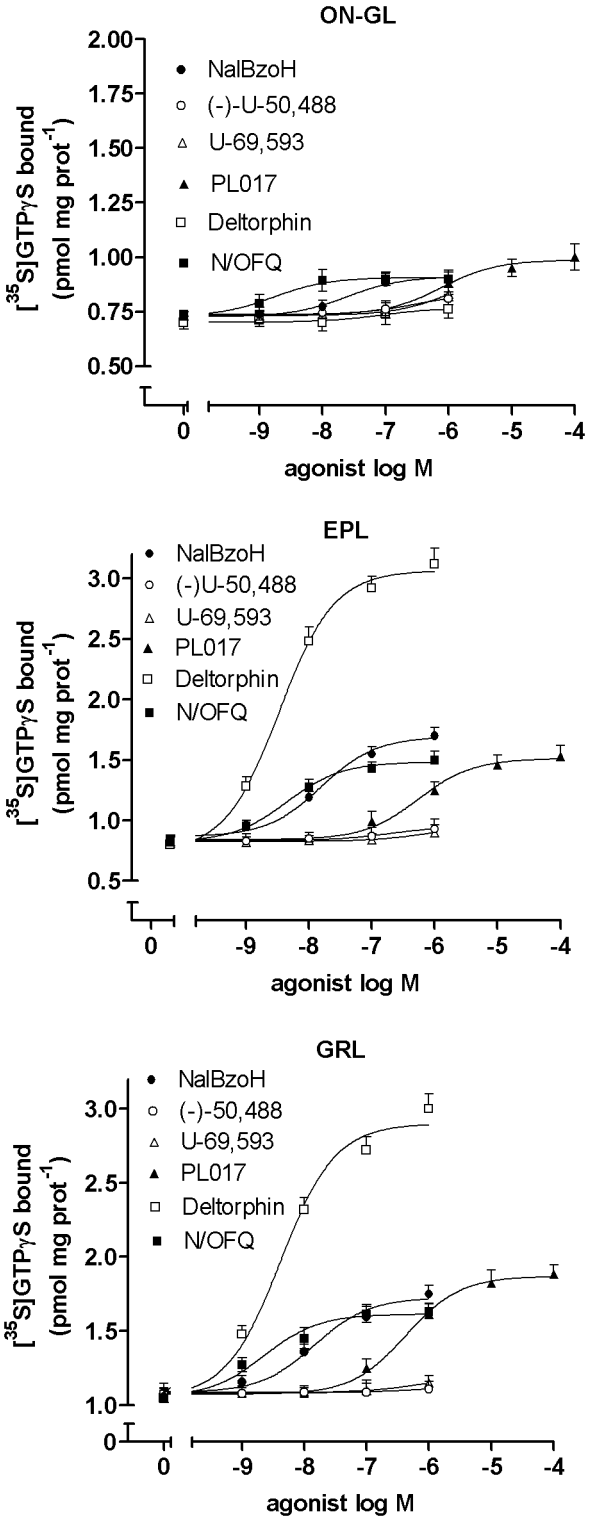

Figure 1 Concentration-dependent effects of NalBzoH and other opioid receptor agonists on [^{35}S]GTPγS binding in ON-GL, EPL and GRL membranes. Values are the mean$\pm$s.e.m. of three experiments.

($pEC_{50}=8.78\pm0.06$) and the selective μ-opioid receptor agonist PL017 (Chang *et al*., 1983) ($pEC_{50}=6.18\pm0.07$) maximally stimulated [^{35}S]GTPγS binding by 25.0 ± 3 and 37.9 ± 4%, respectively ($P<0.01$) (Figure 1). The selective δ-opioid receptor agonist deltorphin I ***(Erspamer *et al*., 1989)

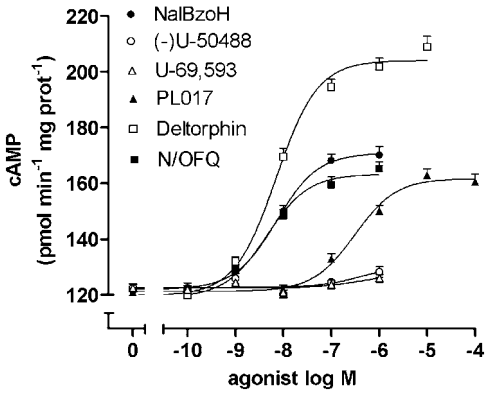

Figure 2 Concentration-dependent effects of NalBzoH and other opioid receptor agonists on basal adenylyl cyclase activity in GRL membranes. Values are the mean$\pm$s.e.m. of three to eight experiments.

and the κ_1-opioid receptor agonists (−)-U-50,488 and U-69,593 (Clark *et al*., 1989; Remmers *et al*., 1999), tested at the same concentrations of NalBzoH, failed to significantly affect [^{35}S]GTPγS binding. In EPL membranes, N/OFQ ($pEC_{50}=8.35\pm0.03$), PL017 ($pEC_{50}=6.28\pm0.05$) and deltorphin I ($pEC_{50}=8.47\pm0.04$) increased the binding of [^{35}S]GTPγS by 80.1 ± 3.0, 81.9 ± 2.8 and 219.8 ± 8.2%, respectively. Similar results were obtained in GRL membranes, where deltorphin I was the most effective (175.1 ± 5.7% increase, $pEC_{50}=8.37\pm0.05$), followed by PL017 (74.0 ± 3.0% increase, $pEC_{50}=6.36\pm0.04$) and N/OFQ (52.0 ± 4.0% increase, $pEC_{50}=8.64\pm0.05$). As in ON-GL membranes, (−)-U-50,488 and U-69,593 were found to be inactive in these tissue layer preparations (Figure 1).

Time-course experiments indicated that in EPL membranes preincubated for 10 min with 100 nN NalBzoH, there was a rapid stimulation of [^{35}S]GTPγS binding which was evident 2 min after the addition of the radioligand (the earliest time point investigated) and linear with time for at least 20 min. Under these conditions, NalBzoH increased the rate of [^{35}S]GTPγS binding by about two-fold when compared to basal rate. The addition of the nonselective opioid receptor antagonist naloxone (final concentration 10 μM) 6 min after the beginning of the reaction caused a rapid reversal of the NalBzoH stimulation, decreasing the binding rate to basal value (results not shown).

Effects on basal adenylyl cyclase activity

NalBzoH failed to affect basal adenylyl cyclase activity of ON-GL and EPL membranes (results not shown), whereas it caused a concentration-dependent increase of cyclic AMP formation in GRL membranes (Figure 2). The pEC_{50} value was 8.14 ± 0.05 and the maximal stimulation corresponded to 40.6 ± 3.6% ($P<0.01$, $n=8$) increase of basal activity. In the same tissue layer, N/OFQ ($pEC_{50}=8.34\pm0.06$, $n=6$), deltorphin I ($pEC_{50}=8.10\pm0.04$, $n=3$) and PL017 ($pEC_{50}=6.50\pm0.10$, $n=3$) increased cyclic AMP formation by 36.0 ± 4.0, 71.5 ± 5.0 and 34.4 ± 4.3%, respectively ($P<0.01$) (Figure 2). In EPL membranes, deltorphin I, but not PL017 and N/OFQ, stimulated basal adenylyl cyclase activity by 42.5 ± 3.5% ($n=5$, $P<0.01$). In ON-GL mem-

branes, both deltorphin I and PL017 were without effect, whereas N/OFQ inhibited the cyclic AMP formation by 19.6±3% ($n=3$, $P<0.05$) (results not shown). (−)-U-50,488 and U-69,593 failed to affect basal adenylyl cyclase activity in GRL (Figure 2) and the other tissue layers (results not shown).

Effects of PTX and α_{TGDP}

In GRL membranes prepared from olfactory bulbs injected with PTX *in vivo*, NalBzoH (1 μM) failed to stimulate either [^{35}S]GTPγS binding or adenylyl cyclase activity, while it elicited significant responses in membranes obtained from vehicle-treated bulbs (Figure 3).

Preincubation. of GRL membranes with α_{TGDP}, a scavenger of βγ subunits (Federman *et al*., 1992), completely blocked the stimulatory effect of NalBzoH and reduced basal adenylyl cyclase activity by 18%. Enzyme activity values (expressed as pmol of cyclic AMP min^{-1} mg protein±s.e.m., $n=3$) were: basal 98.2±4.1, NalBzoH (1 μM) 133.8±5.0 ($P<0.01$ vs basal), α_{TGDP} (2 μg) 80.8±3.4 ($P<0.05$ vs basal), α_{TGDP} + NalBzoH 85.2±5.6 ($P>0.05$ vs α_{TGDP} alone).

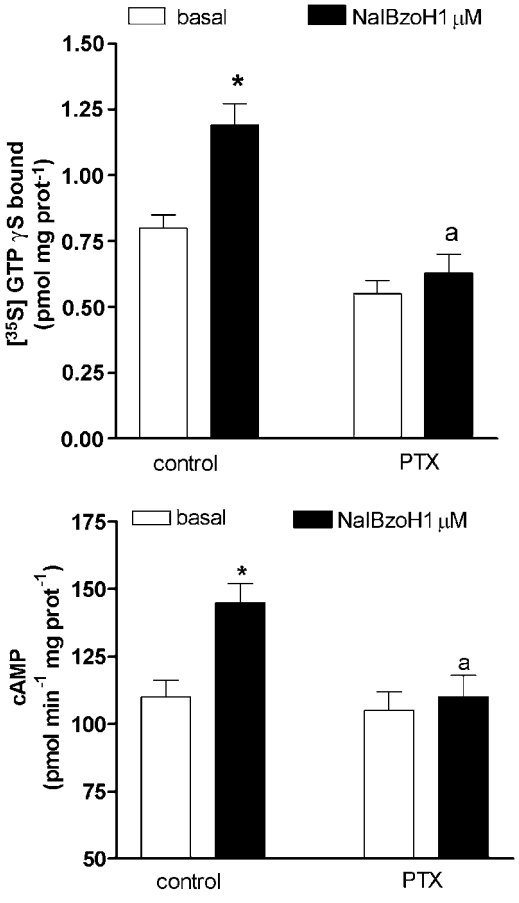

Figure 3 Effects of NalBzoH (1 μM) on [^{35}S]GTPγS binding (upper panel) and basal adenylyl cyclase activity (lower panel) in GRL membranes of olfactory bulb treated *in vivo* with either pertussis toxin (PTX) or vehicle (control). Values are the mean±s.e.m. of three experiments carried out on three distinct tissue preparations. *$P<0.05$; [a]$P>0.05$ vs basal.

Effects of NalBzoH on neurotransmitter-stimulated adenylyl cyclase activity

In GRL membranes, the neuropeptides CRH ($pEC_{50}=7.51\pm0.08$) and PACAP 38 ($pEC_{50}=9.52\pm0.06$) maximally stimulated adenylyl cyclase activity by 35.2±3 and 265±10%, respectively. The coaddition of NalBzoH (1 μM) significantly enhanced the net enzyme stimulations elicited by CRH and PACAP 38 by 45.4±3.0 and 31.0±3.0% ($P<0.05$) without significantly changing the potencies of the neuropeptides (pEC_{50} values were 7.46±0.06 and 9.62±0.08, respectively) (Figure 4).

Effects on FSK-stimulated adenylyl cyclase activity

NalBzoH failed to affect FSK-stimulated adenylyl cyclase activity in ON-GL membranes (results not shown), but significantly inhibited the enzyme activity in EPL ($pEC_{50}=8.07.\pm0.05$, maximal inhibition = 22.1±1.4%, $P<0.05$) and in GRL membranes ($pEC_{50}=8.08\pm0.08$,

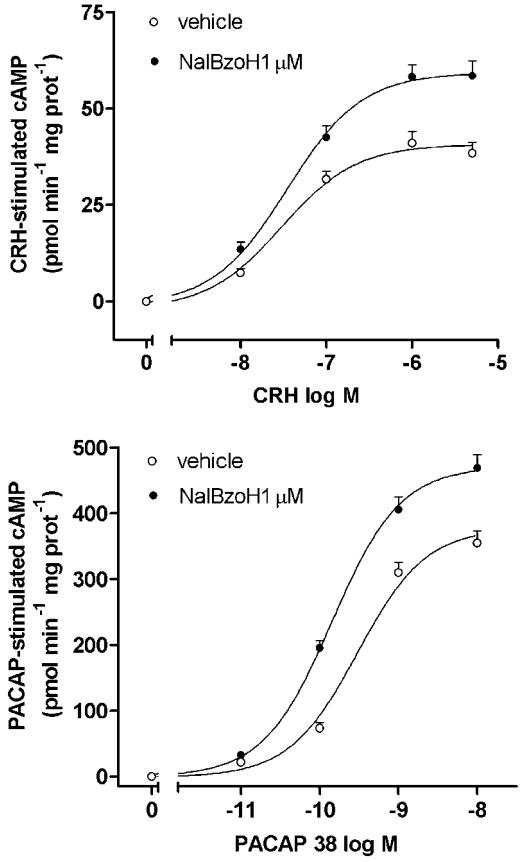

Figure 4 Effects of NalBzoH (1 μM) on CRH (upper panel)- and PACAP 38 (lower panel)-stimulated adenylyl cyclase activities in GRL membranes. The enzyme activity was assayed at the indicated concentrations of the neuropeptides in the presence of either vehicle or NalBzoH. Values indicate the net enzyme activities stimulated by either CRH or PACAP 38 above control activities and are the mean±s.e.m. of three experiments. Control activities (expressed as pmol of cyclic AMP min^{-1} mg protein $^{-1}$) were: vehicle 124±5, 1 μM NalBzoH 168±7. The CRH and PACAP 38 concentration–response curves in the presence of NalBzoH were significantly different from those in the presence of vehicle ($P<0.05$ by analysis of variance).

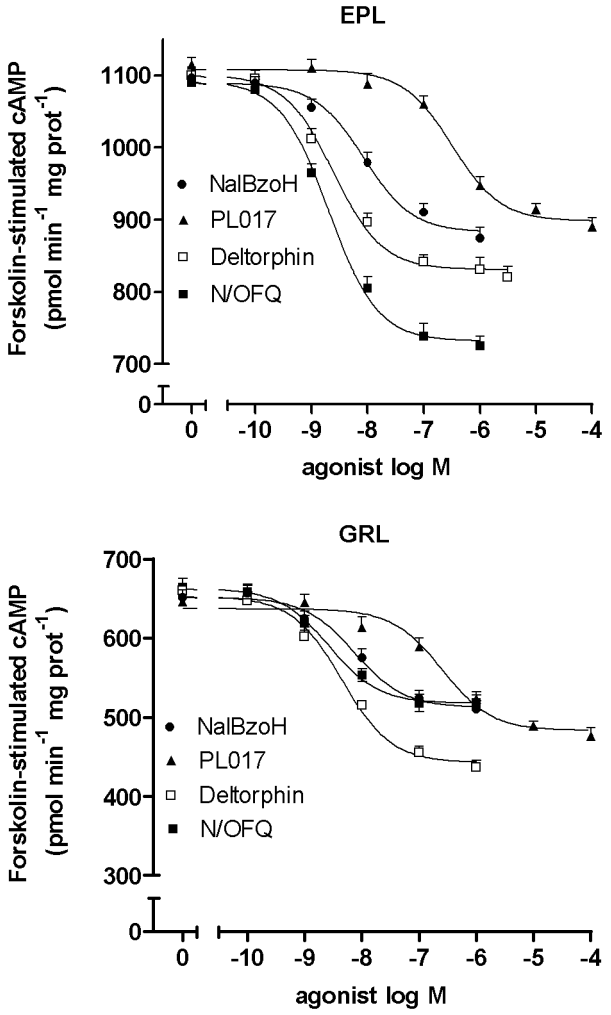

Figure 5 Concentration-dependent inhibition of FSK-stimulated adenylyl cyclase activities in EPL and GRL membranes by NalBzoH and other opioid receptor agonists. Values are the mean±s.e.m. of three experiments.

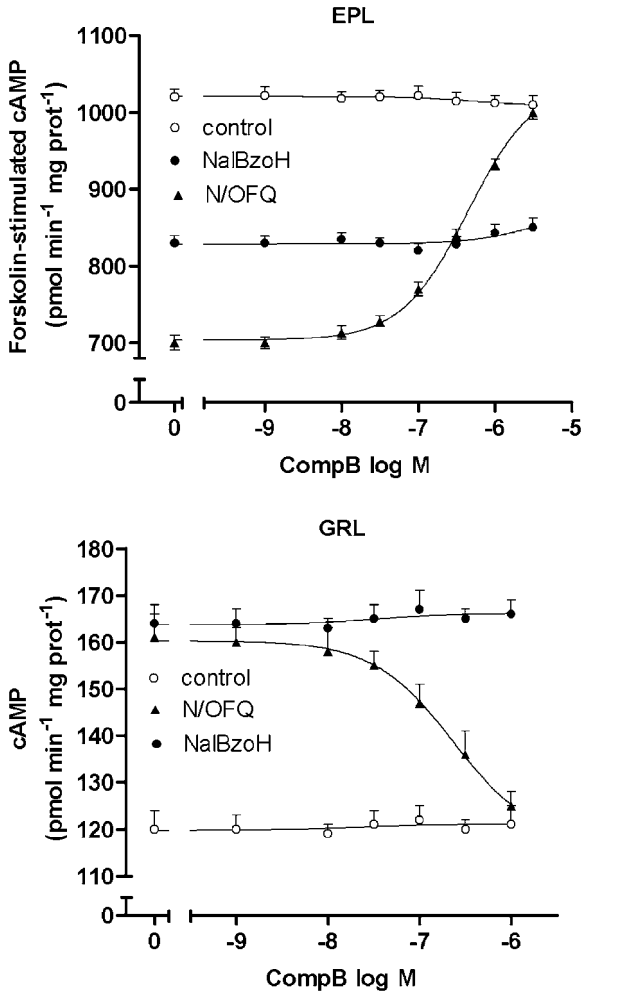

Figure 6 Effects of increasing concentrations of CompB on NalBzoH (1 μM)- and N/OFQ (1 μM)-induced inhibition of FSK-stimulated adenylyl cyclase activity in EPL membranes and on NalBzoH (1 μM)- and N/OFQ (1 μM)-induced stimulation of basal enzyme activity in GRL membranes. Control samples were incubated with vehicle. Values are the mean±s.e.m. of three experiments.

maximal inhibition = 23.3±3.0%, $P<0.05$) (Figure 5). With regard to the effects of other opioid receptor agonists, in ON-GL membranes, deltorphin I (0.1 nM–1.0 μM), PL017 (10 nM–100 μM) failed to affect the FSK-stimulated cyclic AMP formation , whereas N/OFQ caused a significant inhibitory effect (16.4±2.0% reduction, $P<0.05$, $n=3$) with a pEC_{50} of 8.80±0.05 (results not shown). In EPL and GRL membranes, a concentration-dependent inhibition of FSK-stimulated adenylyl cyclase activity was elicited by deltorphin I (27±3 and 32±4% reduction, $P<0.01$, $n=3$; pEC_{50} values = 8.67±0.04 and 8.40±0.03, respectively), PL017 (20±2 and 26±4% reduction, $P<0.05$, $n=3$; pEC_{50} values = 6.42±0.07 and 6.47±0.05, respectively) and N/OFQ (33±4 and 22±2% reduction, $P<0.01$, $n=3$; pEC_{50} values = 8.69±0.04 and 8.50±0.06, respectively) (Figure 5). In contrast, (−)-U-50,488 (1 μM) failed to significantly affect FSK-stimulated enzyme activity in each layer (results not shown). On the other hand, in striatal membranes, (−)-U-50,488 caused a concentration-dependent inhibition of FSK-stimulated adenylyl cyclase activity with an pEC_{50} of 7.79±0.05 and a maximal effect corresponding to 20.2±1.9% decrease of control activity ($P<0.05$, $n=3$) (results not shown).

Effects of receptor antagonists

In EPL membranes, addition of increasing concentrations of the selective ORL1 receptor antagonist CompB (Ozaki *et al.*, 2000) failed to affect the NalBzoH inhibition of FSK-stimulated adenylyl cyclase activity, while it completely antagonized the inhibitory effect of N/OFQ with a pK_i of 9.05 (Figure 6, upper panel and Table 1). Similarly, in GRL membranes, CompB was without effect on the cyclase stimulation by NalBzoH, but potently blocked the action of N/OFQ (Figure 6, lower panel and Table 1). Nphe (1 nM–1 μM), another selective ORL1 antagonist (Calo' *et al.*, 2000a), behaved similarly to CompB (Table 1).

The selective δ-opioid receptor antagonist TIPP (Schiller *et al.*, 1992) maximally reduced the adenylyl cyclase inhibitory effect of 1 μM NalBzoH by 70±3% with a pK_i value of 9.27 (Figure 7, upper panel). TIPP completely antagonized the inhibition by deltorphin I with a similar potency (Table 1), while it was inactive on the PL017-induced inhibition (Figure 7, upper panel). In GRL membranes (Figure 7, lower panel),

Table 1 Potencies of opioid receptor antagonists (pK_i values) in counteracting agonist modulation of adenylyl cyclase activity in distinct layers of rat olfactory bulb

	NalBzoH	*Deltorphin I*	*PL017*	*N/OFQ*
		Inhibiton of FSK-stimulated adenylyl cyclase in EPL		
CompB	>5.5	N.T.	N.T.	9.05±0.06
Nphe	>6.0	N.T.	N.T.	8.81±0.05
nor-BNI	7.67±0.05	7.65±0.04	7.58±0.06	>6.0
CTAP	8.27±0.09	>5.0	8.54±0.07	N.T.
TIPP	9.27±0.04	9.07±0.03	>5.0	N.T.
		Stimulation of basal adenylyl cyclase in GRL		
CompB	>6.0	N.T.	N.T.	8.98±0.05
Nphe	>6.0	N.T.	N.T.	8.74±0.06
nor-BNI	8.09±0.07	7.81±0.05	8.07±0.03	>6.0
CTAP	8.37±0.10	>5.0	8.72±0.09	N.T.
TIPP	9.10±0.09	8.85±0.08	>5.0	N.T.

N.T., not tested; pK_i values were calculated from antagonist competition curves according to Cheng & Prusoff (1973). Data are the mean±s.e.m. of three determinations.

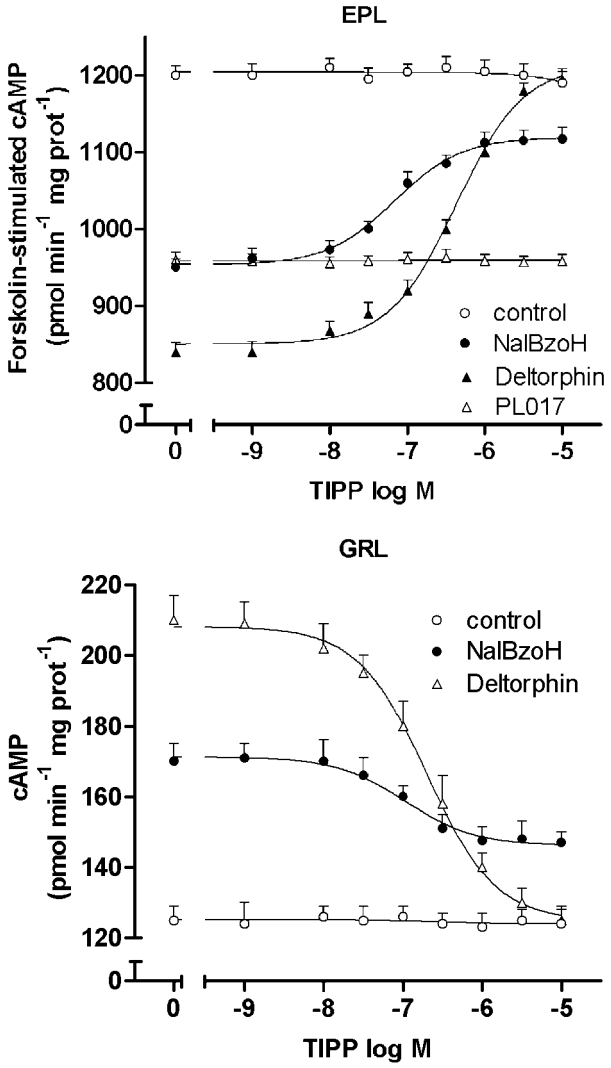

Figure 7 Effects of increasing concentrations of TIPP on NalBzoH (1 μM)-, deltorphin I (1 μM)- and PL017 (50 μM)-induced inhibition of FSK-stimulated adenylyl cyclase activity in EPL membranes and on NalBzoH (1 μM)- and deltorphin I (1 μM)-induced stimulation of basal enzyme activity in GRL membranes. Control samples were incubated with vehicle. Values are the mean±s.e.m. of three experiments.

TIPP reduced the NalBzoH stimulation by 50±2% with a pK_i of 9.10 and completely blocked the deltorphin I stimulation, while having no effect on PL017 stimulation (Table 1).

The μ-opioid receptor antagonist CTAP (Pelton *et al*., 1986) yielded a 30±3 and 47±4% blockade of the NalBzoH inhibitory and stimulatory effect, respectively, with pK_i values similar to those displayed in antagonizing the PL017 effects (Figure 8 and Table 1). At the concentrations used, CTAP had no effect on deltorphin I.

The κ_1-opioid receptor antagonist nor-BNI (Takemori *et al*., 1988; Remmers *et al*., 1999) completely reversed the NalBzoH-induced adenylyl cyclase inhibition in EPL and stimulation in GRL membranes with pK_i values of 7.67 and 8.09, respectively (Figure 9 and Table 1). Nor-BNI displayed similar potencies in antagonizing the PL017 and deltorphin I effects in both layers (Figure 9 and Table 1). On the other hand, in striatal membranes nor-BNI potently antagonized the (−)-U-50,488-induced inhibition of FSK-stimulated adenylyl cyclase activity with a pK_i of 9.49±0.05 ($n=3$) (result not shown).

Discussion

In the present study, we show that in homogenates of the rat main olfactory bulb, NalBzoH is capable of exerting agonist effects on receptor signalling, as demonstrated by the stimulation of [^{35}S]GTPγS binding and the dual regulation of adenylyl cyclase activity. We also show that these responses occur in a layer-specific manner. Thus, NalBzoH stimulated [^{35}S]GTPγS binding with a greater potency and efficacy in EPL and GRL than in ON-GL membranes. Moreover, NalBzoH had no effect on adenylyl cyclase activity in ON-GL, but significantly affected cyclic AMP formation in EPL and GRL membranes. The layer distribution of the NalBzoH effects was compared with that displayed by the ORL1 receptor agonist N/OFQ, the δ-opioid receptor agonist deltorphin I, the μ-opioid receptor agonist PL017 and the κ_1-opioid receptor agonists (−)-U-50,488 and U-69,593. The pattern of activity of NalBzoH was different from that of N/OFQ, which was effective in modulating cyclic AMP formation in each olfactory bulb layer, but appeared similar to that of deltorphin I and PL017, which were inactive in ON-GL and effective in the other layers. On the other hand, the κ_1-opioid receptor agonists (−)-U-50,488 and U-69,593 failed to significantly

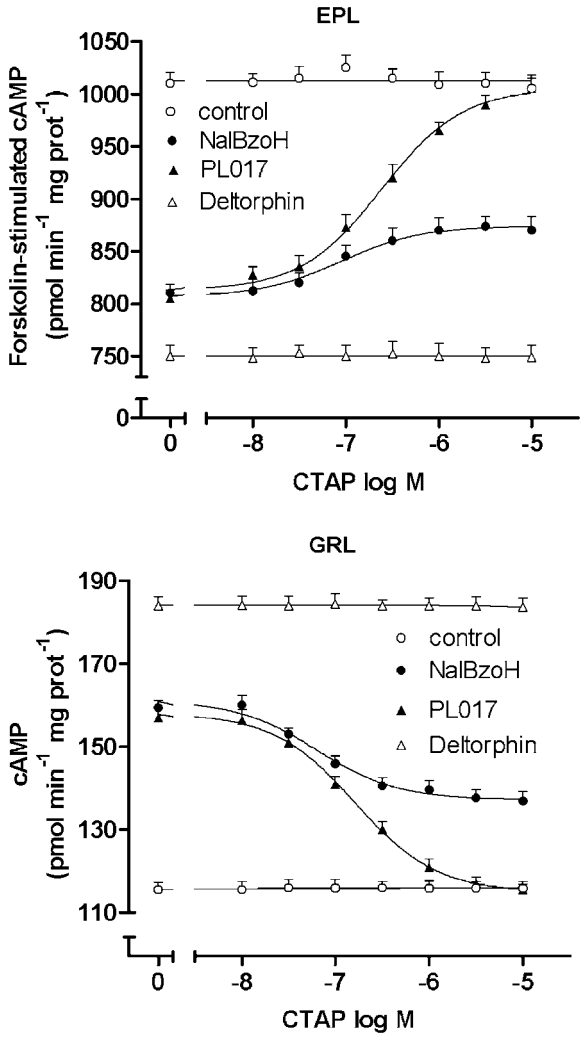

Figure 8 Effects of increasing concentrations of CTAP on NalBzoH (1 μM)-, deltorphin I (0.1 μM)- and PL017 (30 μM)-induced inhibition of FSK-stimulated adenylyl cyclase activity in EPL membranes and on NalBzoH (0.1 μM)-, deltorphin I (0.1 μM)- and PL017 (30 μM)-induced stimulation of basal enzyme activity in GRL membranes. Control samples were incubated with vehicle. Values are the mean $\pm$ s.e.m. of three experiments.

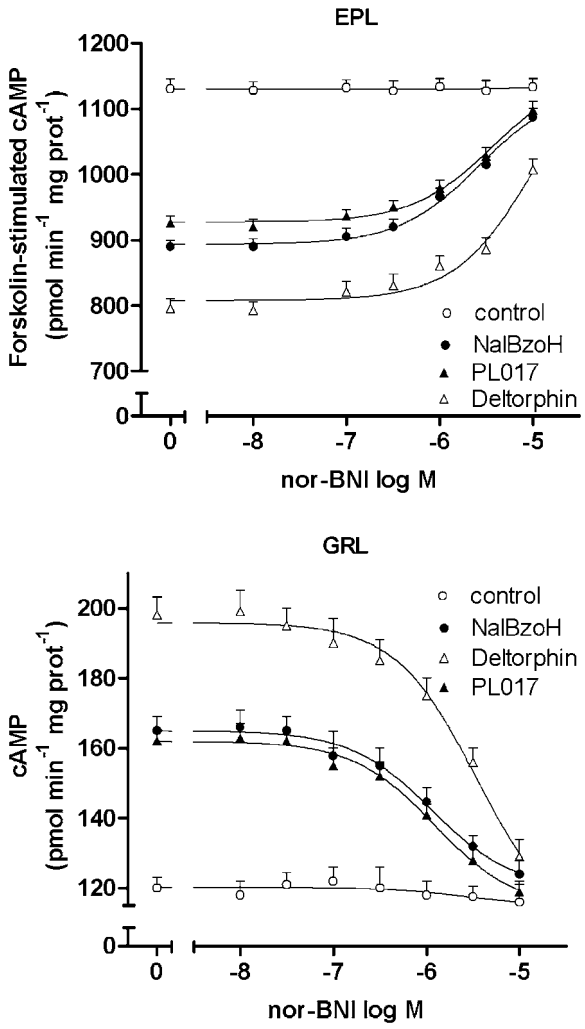

Figure 9 Effects of increasing concentrations of nor-BNI on NalBzoH (1 μM)-, deltorphin I (1 μM)- and PL017 (50 μM)-induced inhibition of FSK-stimulated adenylyl cyclase activity in EPL membranes and on NalBzoH (1 μM)-, deltorphin I (1 μM)- and PL017 (50 μM)-induced stimulation of basal enzyme activity in GRL membranes. Control samples were incubated with vehicle. Values are the mean $\pm$ s.e.m. of three experiments.

affect either [^{35}S]GTPγS binding or adenylyl cyclase activity in each layer of the olfactory bulb. The failure to detect κ_1-opioid receptor-mediated effects was not attributable to the assay conditions, as in membranes of rat striatum, an area enriched of κ_1-opioid receptors (Mansour *et al*., 1995), (−)-U-50,488, caused a significant cyclase inhibition with a potency ($pEC_{50} = 7.79$) that well correlated with that found in similar functional assays of the cloned κ_1-opioid receptor ($pEC_{50} = 8.11$; Prather *et al*., 1995). In general, the layer localization of the responses elicited by the different opioid receptor agonists correlates with the reported localization of the respective receptors (Mansour *et al*., 1995). Moreover, the lack of effects of (−)-U-50,488 and U-69,593 agrees with the poor expression of κ_1-opioid receptors in all layers of the rat olfactory bulb (Mansour *et al*., 1995).

Radioligand binding studies using bovine brain membranes indicated that [^{3}H]NalBzoH bound to μ-opioid receptors in a pseudoirreversible manner (Price *et al*., 1989). It was therefore important to investigate whether the NalBzoH stimulation of [^{35}S]GTPγS binding was reversible. Time-course experiments indicated that the addition of naloxone to membranes preincubated with NalBzoH caused a rapid and complete reversal of the NalBzoH stimulation, indicating that the compound freely dissociated from the receptors. It is noteworthy that guanine nucleotides, which were present in the receptor functional assays, have been reported to promote the dissociation of the pseudoirreversible [^{3}H]NalBzoH binding (Price *et al*., 1989).

As observed for agonists acting on μ- and δ-opioid and ORL1 receptors, NalBzoH stimulated basal adenylyl cyclase activity in the rat main olfactory bulb. The NalBzoH stimulatory effect was restricted to the GRL and was elicited with a potency ($pEC_{50} = 8.14$) close to that displayed in stimulating [^{35}S]GTPγS binding ($pEC_{50} = 7.86$). These values are consistent with the reported receptor binding affinity of [^{3}H]NalBzoH ($pK_i \geqslant 9.0$) in brain membranes (Clark *et al*., 1989; Cheng *et al*., 1992; Berzetei-Gurske *et al*., 1995). Both the stimulatory effects of NalBzoH on [^{35}S]GTPγS binding and

adenylyl cyclase activity were prevented by *in vivo* injection of olfactory bulbs with PTX, indicating the involvement of G proteins of the G_i/G_o family in these responses. Moreover, the cyclase stimulation was significantly inhibited by membrane preincubation with α_{TGDP}, a scavenger of G protein $\beta\gamma$ subunits. These findings indicate that the NalBzoH stimulation occurred independently of activation of the G protein G_s, which has been proposed to mediate cyclic AMP stimulation by opioids in other cell systems (Cruciani *et al*., 1993; Wang & Gintzler, 1997), but was likely mediated by G_i/G_o $\beta\gamma$ subunits activating type II/IV adenylyl cyclases expressed in GRL membranes (Onali *et al*., 2001). This possibility is further supported by the finding that in GRL NalBzoH potentiated the stimulation of adenylyl cyclase activity by G_s-coupled CRH and PACAP 38 receptors. Indeed, the $\beta\gamma$-induced stimulation of type II/IV adenylyl cyclases is known to be markedly enhanced when the enzymes are concurrently activated by the α subunit of G_s (Sunahara *et al*., 1996). Previous studies have demonstrated that G_i/G_o $\beta\gamma$ subunits may also mediate the stimulatory effects on cyclic AMP formation elicited by activation of δ- and μ-opioid and ORL1 receptors in rat olfactory bulb (Olianas & Onali, 1999; Onali *et al*., 2001).

Besides enhancing basal and neurotransmitter-stimulated adenylyl cyclase activity, NalBzoH induced a concentration-dependent inhibition of the enzyme activity stimulated by FSK. The inhibitory effect was evident in EPL and GRL membranes and was absent in ON-GL. The inhibitory effects correlated with the stimulation of [^{35}S]GTPγS binding in the same layers and can be explained by postulating that in specific anatomical sites G protein activation elicited by NalBzoH decreases the activity of some cyclase isoforms, such as types I and V/VI, which are inhibited by G protein α and $\beta\gamma$ subunits (Sunahara *et al*., 1996) and are expressed in EPL and GRL (Onali *et al*., 2001). The lack of detecting the inhibitory effect in the absence of FSK is possibly due to the low basal activity of these cyclase isoforms and/or to the concomitant stimulatory effects on other cyclase isoforms.

The specific expression of the dual regulation of cyclic AMP formation by NalBzoH and selective δ- and μ-opioid receptor agonists in EPL and GRL, which are the sites of intrinsic and centrifugal regulation of olfactory processing (Mori *et al*., 1999), suggests that this mechanism may be relevant for the opioid modulation of some forms of olfactory learning (Kinsley *et al*., 1995; Roth & Sullivan, 2003). By sensitizing type II/IV adenylyl cyclases, opioids may enhance the responses of mitral cells and interneurons to CRH and PACAP peptides acting through G_s-coupled receptors, whereas, by inhibiting type I/VIII adenylyl cyclases they may curtail the cyclic AMP elevation elicited by Ca^{2+}-mobilizing neurotransmitters, thus selectively affecting the efficiency of synaptic transmission. Whether chronic administration of NalBzoH or other opioid agonists can alter the dual modulation of cyclic AMP in the olfactory bulb is a matter of future investigation.

As the agonist-like effects of NalBzoH were qualitatively similar to those elicited by activation of ORL1 and opioid receptors, it was important to investigate whether these receptors mediated the NalBzoH effects. Therefore, competition experiments using a full range of antagonist concentrations were performed to assess whether the effects of NalBzoH persisted when the activity of other opioid receptor agonists was completely suppressed. We found that the selective ORL1 antagonists CompB and Nphe, which potently blocked the cyclase regulation by N/OFQ, had no effect on the agonist actions of NalbzoH, thus ruling out the participation of this receptor subtype. On the other hand, 70% of the cyclase inhibition and about 50% of the stimulation elicited by NalBzoH were blocked by the selective δ-opioid receptor antagonist TIPP with potencies similar to those shown in antagonizing deltorphin I. As expected by its high receptor subtype selectivity, TIPP had no effect on the responses elicited by PL017. These data indicate that in the olfactory bulb a large fraction of the NalBzoH agonist activity was mediated by stimulation of δ-opioid receptors. Following the same experimental paradigm, we examined the effects of the selective μ-opioid receptor antagonist CTAP. Increasing concentrations of this compound reversed the inhibitory and stimulatory effects of NalBzoH by 30 and 50%, respectively, with the same potencies displayed in blocking PL017. The selectivity of the CTAP antagonism was confirmed by the lack of effects on deltorphin I even at concentrations as high as 10 μM. These data indicate that a portion of the NalBzoH responses, corresponding to the fraction not blocked by TIPP, was mediated by activation of μ-opioid receptors. To complete the picture, we investigate the effects of the κ_1-opioid receptor antagonist nor-BNI. Although this compound was capable of completely blocking the dual action of NalBzoH, the antagonism occurred with potencies similar to those displayed in blocking deltorphin I and PL017 and several-fold lower than that shown in counteracting (−)-U-50,488 in striatum. These data indicate that nor-BNI antagonized the action of NalBzoH by blocking δ- and μ-opioid receptors.

The combined agonist action of NalBzoH on different opioid receptors in the olfactory bulb was likely responsible for the atypical sensitivity to opioid antagonists with lower subtype selectivity, which in a preliminary study was interpreted as due to the involvement of the putative κ_3-opioid receptor (Olianas & Onali, 2002). It will be of interest to investigate whether also in other cell systems the use of highly subtype-selective antagonists can disclose agonist effects of NalBzoH on δ- and μ-opioid receptor activity.

The maximal stimulatory and inhibitory effects of NalBzoH were significantly smaller than those elicited by deltorphin I, a highly efficacious δ-opioid receptor agonist and comparable to those elicited by the full μ-opioid receptor agonist PL017. As the antagonist data indicated that each NalBzoH effect resulted from the sum of δ- and μ-opioid receptor activations, it is possible that the compound acted as a partial agonist at both receptors. Further studies on cell systems expressing a single opioid receptor subtype are required for the definite assessment of the NalBzoH intrinsic activity.

The finding that NalBzoH activates μ-opioid receptors is in contrast with a previous study reporting that the compound behaved as a pure antagonist with no effect, *per se*, on cyclic AMP accumulation in cells transfected with the cloned μ-opioid receptor (Brown & Pasternak, 1998). The reason for this discrepancy is not clear, as cell lines overexpressing the receptors are generally an optimal system to detect agonist effects even by compounds with low intrinsic activity. It is possible that differences in the assay conditions (i.e. intact cells vs cell membranes) and/or sensitivity may be responsible for the divergent results.

In conclusion, the present study shows for the first time that in the brain NalBzoH is capable of triggering signalling mechanisms by acting on δ- and μ-opioid receptors. These properties do not support the assumption that NalBzoH is a selective agonist of the pharmacologically defined κ_3-opioid receptor and should be taken into consideration when the compound is employed in cell systems expressing multiple opioid receptor subtypes.

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