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Effects of sigma ligands on the cloned μ-, δ- and κ-opioid receptors co-expressed with G-protein-activated K^+ (GIRK) channel in *Xenopus* oocytes

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1 Taking advantage of the functional coupling of the opioid receptors with the G-protein-activated K^+ (GIRK) channel, we investigated the effects of sigma (σ) ligands of various structural and pharmacological classes, (+)-N-allylnormetazocine ((+)-SKF10047) and (+)-cyclazocine, (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3PPP), 1,3-di-(2-tolyl)guanidine (DTG), carbetapentane and haloperidol, on the inward K^+ current responses in *Xenopus* oocytes co-injected with each of the cloned μ-, δ- and κ-opioid receptor mRNAs and the GIRK1 mRNA.

2 (+)-SKF10047 acted as a δ- and κ-agonist (EC_{50} values (μM) = 0.618 and 0.652, respectively) and μ-antagonist (IC_{50} value (μM) = 8.51). (+)-Cyclazocine acted as a κ-agonist and μ-antagonist (IC_{50} = 33.2). (+)-3PPP acted as a κ-agonist (EC_{50} = 18.08) and a μ-antagonist. DTG acted as a μ- and κ-agonist (EC_{50} = more than 30 and 14.88, respectively). Carbetapentane acted as a κ-agonist and μ-antagonist (IC_{50} = 11.2). Haloperidol acted as a μ- and δ-agonist (EC_{50} = 5.683 and 7.389, respectively).

3 All currents induced by σ ligands were reduced by 1 μM naloxone, an opioid receptor antagonist, and blocked by 300 μM Ba^{2+}, a GIRK channel blocker. It was also indicated that the antagonism by naloxone at the δ- and κ-opioid receptors was weaker than that of naloxone at the μ-opioid receptor. The σ ligands tested had no effect on the current responses in the oocytes injected with each of the opioid receptor mRNAs alone or with the GIRK1 mRNA alone.

4 We conclude that various σ ligands directly interact with the cloned μ-, δ- and κ-opioid receptors in *Xenopus* oocytes. Our results suggest that the effects of the σ ligands may be partly mediated by the opioid receptors.

Keywords: σ Ligand; (+)-N-allylnormetazocine ((+)-SKF10047); (+)-cyclazocine; 1,3-di-(2-tolyl)guanidine (DTG); (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3PPP); carbetapentane; haloperidol; opioid receptor; G-protein-activated K^+ (GIRK) channel; *Xenopus* oocytes

Introduction

N-allylnormetazocine (SKF10047), a racemic benzomorphan opiate, causes psychotomimetic effects in human subjects (Keats & Telford, 1964) and canine excitation which is related to the psychotomimetic effects in man (Martin *et al.*, 1976). Martin and colleagues (1976) postulated that the psychotomimetic effects were mediated by sigma (σ) receptors, subtypes of the opioid receptors. Subsequently, σ receptors were found to differ from the other opioid receptors in that they are insensitive to naloxone and enantioselective for the (+)-isomers of opiates, whereas the opioid receptors are antagonized by naloxone and enantioselective for the (−)-isomers of opiates (Tam, 1983; 1985; Largent *et al.*, 1984; Tam & Cook, 1984; Weber *et al.*, 1986). The benzomorphan opiates and phencyclidine (PCP), psychotomimetics, were thought to act at a common recognition site termed the σ/PCP receptor (Zukin & Zukin, 1981). However, σ receptors are highly sensitive to antipsychotics, such as haloperidol and perphenazine (Tam, 1983; Largent *et al.*, 1984; Tam & Cook, 1984; Weber *et al.*, 1986), while the PCP site is insensitive to them (Tam, 1983; Largent *et al.*, 1986) and is present on the N-methyl-D-aspartate (NMDA) receptor channels (Lodge & Johnson, 1990). In addition, autoradiographic studies have revealed that the localization of σ receptors in the brain (Largent *et al.*, 1986; Walker *et al.*, 1992) differs from those of the opioid receptors (Mansour *et al.*, 1995) and the PCP site (Largent *et al.*, 1986). Therefore, σ receptors have been considered as unique sites.

Compounds which bind with high affinity to σ receptors are termed σ ligands. σ Ligands are composed of diverse chemical classes including benzomorphans, butyrophenones, phenothiazines, guanidines, 3-phenylpiperidines, peptides and steroids, such as (+)-SKF10047, haloperidol, perphenazine, 1,3-di-(2-tolyl)guanidine (DTG), (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3PPP), neuropeptide Y and progesterone, respectively (Walker *et al.*, 1990).

Since σ receptors are widely distributed in distinct brain areas, such as limbic structures, cerebellum, motor nuclei in the brainstem and hypothalamus at high density (Largent *et al.*, 1986; Walker *et al.*, 1990; Jansen *et al.*, 1991), the effects of σ ligands in some psychiatric disorders (Debonnel, 1993), movement and posture (Walker *et al.*, 1988; 1993), and neuroendocrine regulation (Iyengar *et al.*, 1990) have been investigated. The psychotomimetic effects of (+)-SKF10047 and antipsychotic effects of haloperidol suggest that σ receptors play a role in schizophrenia and drug abuse. Several typical antipsychotic drugs, such as haloperidol and perphenazine, exhibit high affinity for the D_2-dopamine receptor and σ receptors (Tam & Cook, 1984), and blockade of the D_2-dopamine receptor has been thought to play an important role in the clinical efficacy of antipsychotic drugs (Seeman & Van Tol, 1994). However, novel atypical antipsychotic drugs which have

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moderate to high affinity for σ receptors and only low affinity for the D_2-dopamine receptor have recently been shown to be useful for treating schizophrenia, with minimal extrapyramidal side effects (Den Boer *et al.*, 1990; Lewander *et al.*, 1990). The antipsychotic effects of σ ligands have elicited interest in their therapeutic application to schizophrenia and drug abuse (Debonnel, 1993). It has also been suggested that σ ligands may be useful for the treatment of pain (Chien & Pasternak, 1994; Kest *et al.*, 1995), dystonia (Walker *et al.*, 1988; 1993), cerebral ischaemia (O'Neill *et al.*, 1995), amnesia (Earley *et al.*, 1991), epilepsy (Tortella & Musacchio, 1986; Tortella *et al.*, 1989), ulcer (Pascaud *et al.*, 1990; Harada *et al.*, 1994), and tumours (Brent & Pang, 1995).

σ Ligands modulate the dopaminergic (Steinfels & Tam, 1989), noradrenergic (Gonzalez-Alvear & Werling, 1995) and acetylcholinergic (Junien *et al.*, 1991; Matsuno *et al.*, 1993) systems, the NMDA receptor-mediated response (Monnet *et al.*, 1992), carbachol-induced phosphatidylinositol turnover (Candura *et al.*, 1990), and blockade of tonic potassium channels (Wu *et al.*, 1991; Morio *et al.*, 1994). However, functional properties of σ ligands at various receptors have not yet been identified.

σ Receptors also exist in various peripheral organs and tissues including adrenal gland, testis, ovary, spleen, peripheral blood leukocytes, vas deferens, liver (Walker *et al.*, 1990), kidney (Hellewell *et al.*, 1994), gastrointestinal tract (Roman *et al.*, 1988) and heart (Dumont & Lemaire, 1991). σ Ligands modulate the endocrine and immune systems (Wolfe & De Souza, 1994; Liu *et al.*, 1995).

Many benzomorphan opiates elicit psychotomimetic effects via the κ-opioid receptor (Pfeiffer *et al.*, 1986) as well as σ receptors. The (−)-isomers, but not the (+)-isomers, of the benzomorphans have been shown to elicit psychotomimetic effects via the κ-opioid receptor (Pfeiffer *et al.*, 1986). It has also been shown that σ ligands modulate the μ-, δ- and κ-opioid analgesia in an analgesic assay (Chien & Pasternak, 1994) and the hyperpolarization induced by [Met^5]enkephalin, a nonselective opioid agonist, in a neuronal slice preparation (Bobker *et al.*, 1989). However, whether σ ligands directly interact with opioid receptors is largely unknown.

Recently, the μ-, δ- and κ-opioid receptors have been cloned, and their structure, functions and distributions have been investigated (Knapp *et al.*, 1995; Minami & Satoh, 1995). Using a *Xenopus* oocyte expression system, activation of each of the μ, δ- and κ-opioid receptors has been revealed to gate the G-protein-activated K^+ (GIRK) channel via G-protein (Chen & Yu, 1994; Henry *et al.*, 1995; Kovoor *et al.*, 1995; Ma *et al.*, 1995; Ikeda *et al.*, 1995; 1996). This functional assay system has made it possible to characterize the functional properties of various ligands at the opioid receptors. To investigate the effects of σ ligands of various structural and pharmacological classes on the cloned μ-, δ- and κ-opioid receptors, we performed this functional assay in oocytes co-injected with each of the cloned opioid receptor mRNAs and the GIRK1 mRNA. The results of the present study demonstrate that various σ ligands interact directly with the cloned μ-, δ- and κ-opioid receptors.

Methods

Specific mRNA preparation

Plasmids containing the entire coding sequences for the mouse κ- and δ-opioid receptors and for the mouse GIRK1 channel were obtained using the polymerase chain reaction (PCR) method with the mouse whole brain cDNA as a template, and designated pSPORκ, pSPORδ and pSPGIRK1, respectively (Ikeda *et al.*, 1995; Kobayashi *et al.*, 1995). A plasmid containing the entire coding sequence for the mouse μ-opioid receptor, pSPORμ (Ikeda *et al.*, 1996), was also obtained using the PCR method (Kobayashi *et al.*, 1995) with the mouse whole brain cDNA as a template and with a pair of specific primers, 5′-AACCATGGACAGCAGCGCCG-3′ and 5′-GCTCTAGATTAGGGCAATGGAGCAGTTTCT-3′, which were synthesized on the basis of the nucleotide sequence for the mouse μ-opioid receptor. pSPORμ, pSPORδ and pSPGIRK1 were linearized by digestion with EcoRI and pSPORκ by digestion with SacI. The specific mRNAs were synthesized *in vitro* from the linearized plasmids with SP RNA polymerase in the presence of cap dinucleotide ^{7}mGpppG (Ambion mMESSAGE mMACHINE In Vitro Transcription Kit).

Expression in Xenopus *oocytes and electrophysiological analyses*

Xenopus laevis oocytes were injected with each opioid-receptor mRNA (~10 ng 100 nl^{-1} per oocyte) together with the GIRK1 mRNA (~12 ng 100 nl^{-1} per oocyte). The oocytes were incubated at 19°C in Barth's solution (composition, mM: NaCl 88, KCl 1, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, Tris-HCl (pH 7.4) 7.5, gentamicin sulphate 0.1 mg ml^{-1}. Oocytes were defolliculated by manual dissection after 1 mg ml^{-1} collagenase (Wako) treatment for 1 h. The oocytes were superfused with a high-potassium solution (KCl 96 mM, NaCl 2 mM, $MgCl_2$ 1 mM and $CaCl_2$ 1.5 mM) at 19°C. Whole-cell currents of the oocytes were recorded from 3 to 10 days after injection with a conventional two-micropipette voltage clamp (Sakimura *et al.*, 1992). The membrane potential was held at −70 mV. Membrane resistances of oocytes were approximately 0.2 MΩ at −70 mV. Data were fitted to a standard logistic equation using SigmaPlot (Jandel Scientific) to compute the EC_{50} and the IC_{50} in analysis of concentration-response relationships. The values obtained are expressed as mean ± s.e.mean and *n* is the number of oocytes tested.

Compounds

σ Ligands, (+)-N-allylnormetazocine hydrochloride ((+)-SKF10047), (+)-cyclazocine, 1,3-di-(2-tolyl)guanidine (DTG), (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine hydrochloride ((+)-3PPP), carbetapentane citrate and haloperidol were purchased from Research Biochemicals Inc. A selective μ-opioid-receptor agonist, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), a selective δ-opioid-receptor agonist, [D-$Pen^{2,5}$]enkephalin (DPDPE), a selective κ-opioid-receptor agonist, *trans*-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide (U50488H), and an opioid-receptor antagonist, naloxone, were purchased from Sigma Chemical Co. (+)-Cyclazocine, DTG and haloperidol were dissolved in ethanol, methanol and dimethyl sulphoxide (DMSO), respectively. Other compounds were dissolved in distilled water. The stock solutions of all compounds were stored at −20°C until use. They were added to the high-potassium solution in appropriate amounts immediately before the experiment.

Results

Opioid receptor activation by σ ligands

To investigate the effects of σ ligands on the cloned μ-, δ- and κ-opioid receptors, we performed the *Xenopus* oocyte functional assay with each of the three opioid receptor mRNAs co-injected with the GIRK1 mRNA. In the oocytes co-injected with the μ-opioid receptor and GIRK1 mRNAs, application of haloperidol or DTG produced an inward current (Figure 1a). Application of (+)-SKF10047, (+)-cyclazocine, (+)-3PPP or carbetapentane, even at 30 μM, induced no response in the same oocytes (data not shown). In the oocytes co-injected with the δ-opioid receptor and GIRK1 mRNAs, application of (+)-SKF10047 or haloperidol produced an inward current (Figure 1b). Application of (+)-cyclazocine, DTG, (+)-3PPP or carbetapentane, at 10 μM, induced no response in the same oocytes

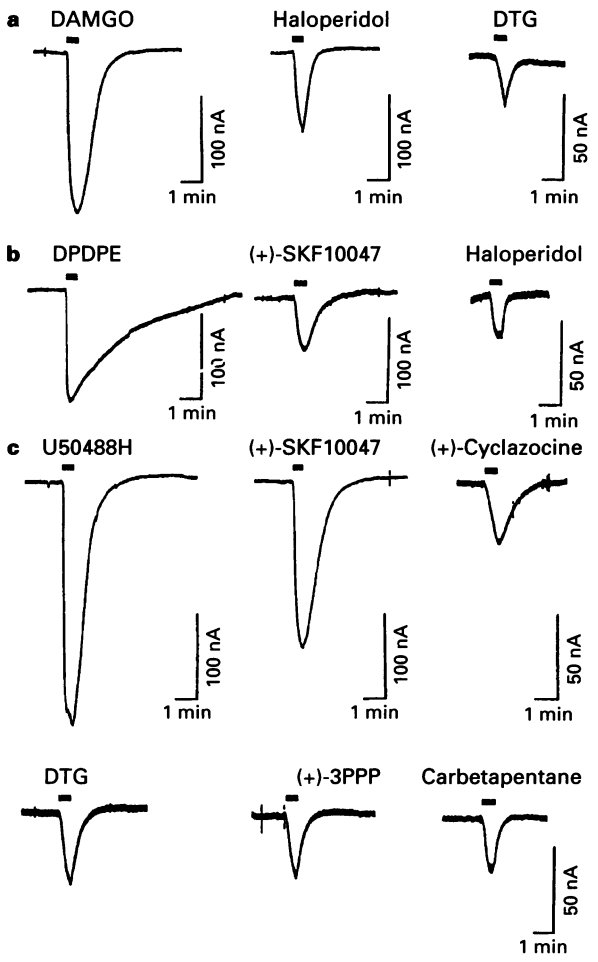

Figure 1 Current responses to σ ligands and selective opioid agonists in *Xenopus* oocytes co-injected with opioid receptor mRNA and GIRK1 mRNA. (a) Current responses in the oocytes co-injected with μ-opioid receptor and GIRK1 mRNAs. Responses to 100 nM DAMGO and 10 μM haloperidol in one oocyte, and response to 30 μM DTG in another oocyte. (b) Current responses in an oocyte co-injected with δ-opioid receptor and GIRK1 mRNAs to 100 nM DPDPE, 10 μM (+)-SKF10047 and 10 μM haloperidol. (c) Current responses in an oocyte co-injected with κ-opioid receptor and GIRK1 mRNAs to 100 nM U50488H, 10 μM (+)-SKF10047, 10 μM (+)-cyclazocine, 10 μM DTG, 10 μM (+)-3PPP and 10 μM carbetapentane. The time intervals between the applications of ligands were approximately 10 min. Current responses were measured at a −70 mV membrane potential in a high-potassium solution. Bars above the traces show the duration of application. Inward current is downward.

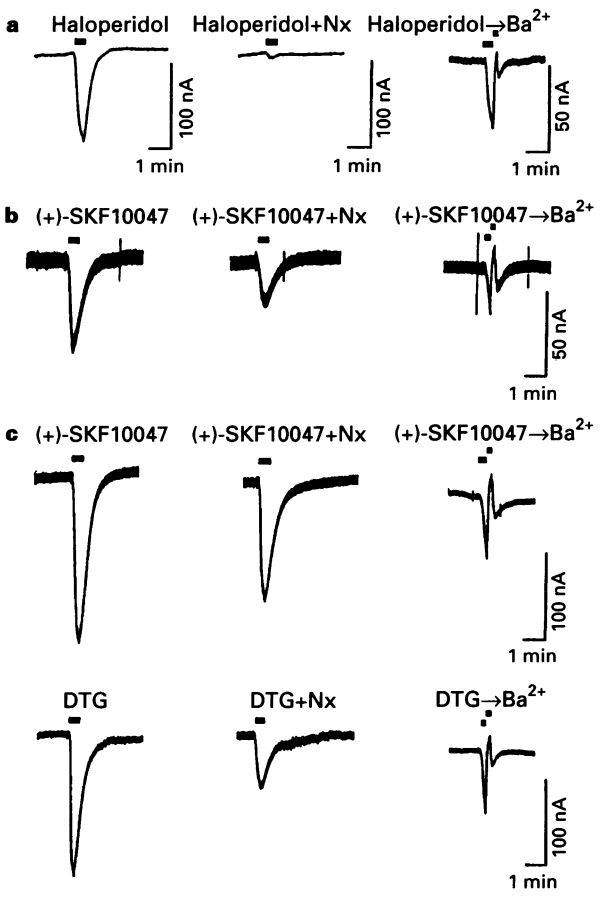

Figure 2 Inhibition of σ ligand-induced current responses by naloxone and Ba^{2+}. (a) Current responses in the oocyte co-injected with μ-opioid receptor and GIRK1 mRNAs to 50 μM haloperidol, 50 μM haloperidol plus 1 μM naloxone (Nx), and 300 μM Ba^{2+} after 50 μM haloperidol. (b) Current responses in the oocyte co-injected with δ-opioid receptor and GIRK1 mRNAs to 6 μM (+)-SKF10047, 6 μM (+)-SKF10047 plus 1 μM Nx, and 300 μM Ba^{2+} after 6 μM (+)-SKF10047. (c) Current responses in the oocyte co-injected with κ-opioid receptor and GIRK1 mRNAs to 6 μM (+)-SKF10047, 6 μM (+)-SKF10047 plus 1 μM Nx, and 300 μM Ba^{2+} after 6 μM (+)-SKF10047 (upper row) and 100 μM DTG, 100 μM DTG plus 1 μM Nx, and 300 μM Ba^{2+} after 100 μM DTG (lower row). Current responses were measured at a −70 mV membrane potential in a high-potassium solution. Bars above the traces show the duration of application, and lower and upper bars in the right column show the duration of application of a σ ligand and Ba^{2+}, respectively. Inward current is downward.

(data not shown). In the oocytes co-injected with the κ-opioid receptor and GIRK1 mRNAs, application of (+)-SKF10047, (+)-cyclazocine, DTG, (+)-3PPP or carbetapentane produced an inward current (Figure 1c). Application of haloperidol (30 μM) induced no response in the same oocytes (data not shown). Application of any one of the σ ligands tested induced no response in the oocytes injected with each of the opioid receptor mRNAs alone or with the GIRK1 mRNA alone (data not shown). Application of the solvent vehicles at the highest concentration (0.1%) in this experiment had no effect on the current responses in the oocytes injected with each opioid receptor mRNA and/or the GIRK1 mRNA.

In the oocytes co-injected with each opioid receptor mRNA and the GIRK1 mRNA, all of the current responses induced by the σ ligands were reduced by 1 μM naloxone, an opioid-receptor antagonist (Figure 2, middle). Interestingly, the current responses in the oocytes co-injected with the μ-opioid receptor and GIRK1 mRNAs were almost completely abolished by 1 μM naloxone, while the current responses in the oocytes co-injected with either the δ- or the κ-opioid receptor mRNA and the GIRK1 mRNA were not completely abolished by 1 μM naloxone, suggesting that the σ ligands cause the responses through activation of the δ- and κ-opioid receptors even in the presence of naloxone. Furthermore, all of the current responses induced by the σ ligands were rapidly blocked by 300 μM Ba^{2+}, which blocks a family of inward-rectifier K^+ channels including the GIRK channel (Kovoor *et al.*, 1995), and recovered immediately after its washout (Figure 2, right). Ba^{2+} (300 μM) alone caused an upward shift of membrane current traces in the oocytes co-injected with each opioid receptor mRNA and the GIRK1 mRNA as well as in the oocytes injected with the GIRK1 mRNA alone, but the shifts were too small to elucidate the blocking of the agonist-induced currents (data not shown). Also Ba^{2+} (300 μM) induced no responses in the oocytes injected with each opioid receptor mRNA alone or in uninjected oocytes (data not shown). These results suggest that the σ ligands which produced the inward currents directly activate the opioid receptors, and that the responses are mainly mediated by the GIRK1 channel.

Concentration-dependence of σ ligand-induced current responses

To investigate the concentration-dependence of the effects of the σ ligands on the opioid receptors, we compared the current responses induced by σ ligands with the full response induced by each selective opioid agonist. Since the degree of desensitization of the current responses became negligible after a few applications of a selective opioid agonist, quantitative experiments were carried out after the applications. The selective μ-, δ- and κ-opioid agonists, DAMGO, DPDPE and U50488H, were used at 1 μM, 1 μM and 500 nM, respectively (Ikeda *et al.*, 1995; 1996). The maximum current responses induced by the selective μ-, δ- and κ-opioid agonists were 52.5–950 nA (415.2±74.1 nA; $n=12$), 51.25–267.5 nA (116.5±11.5 nA; $n=21$) and 81.25–315 nA (172.3±10.1 nA; $n=30$), respectively. The magnitudes of the inward current responses induced by the σ ligands were concentration-dependent (Figue 3). The EC_{50} values and Hill coefficient (n_H) values of these σ ligands obtained from the concentration-response relationships are shown in Table 1. In the oocytes co-injected with the μ-opioid receptor and GIRK1 mRNAs, haloperidol and DTG produced current responses at micromolar concentrations, and haloperidol was more potent than DTG at concentrations of up to ~43 μM (Figure 3a). At 100 μM, haloperidol and DTG produced 25.8±2.5% ($n=5$) and 39.5±6% ($n=7$) of the control current response to DAMGO, respectively. In the oocytes co-injected with the δ-opioid receptor and GIRK1 mRNAs, (+)-SKF10047 produced current responses even at nanomolar concentrations. Haloperidol was less efficacious and potent than (+)-SKF10047, but produced low current responses at micromolar concentrations. (+)-SKF10047 (3 μM) and haloperidol (100 μM), at the highest concentrations tested, produced 54.4±5.6% ($n=5$) and 26.8±2.8% ($n=7$) of the control current response to DPDPE, respectively. In the oocytes co-injected with the κ-opioid receptor and GIRK1 mRNAs, (+)-SKF10047 produced current responses even at nanomolar concentrations. Both DTG and (+)-3PPP produced current responses at micromolar concentrations. (+)-SKF10047 (3 μM), DTG (100 μM) and (+)-3PPP (100 μM), at the highest concentrations tested, produced 63.0±3.8% ($n=7$), 46.2±4% ($n=9$) and 20.6±1.7% ($n=7$) of the control current response to U50488H, respectively.

Inhibitory effects of σ ligands on the opioid receptors

To investigate the inhibitory effects of the σ ligands on the μ-, δ- and κ-opioid receptors, we applied a σ ligand together with a selective opioid agonist. The selective opioid agonists were used at the concentrations of 10 fold the respective EC_{50} values at which each current response was near the peak response in the concentration-response curve (Ikeda *et al.*, 1995; 1996).

In the oocytes co-injected with the μ-opioid receptor and GIRK1 mRNAs, the control current responses to DAMGO were reversibly suppressed by (+)-SKF10047, (+)-cyclazocine, carbetapentane and (+)-3PPP which induced no responses themselves at 30 μM (Figure 4a). As shown in Figure 4b, these σ ligands dose-dependently suppressed the current responses to DAMGO. At the highest concentration tested (30 μM), (+)-SKF10047, (+)-cyclazocine, carbetapentane and (+)-3PPP reduced the current response to 19.9±3.9% ($n=7$), 48.5±3.2% ($n=6$), 22.2±6.0% ($n=5$) and 77.2±3.4% ($n=5$) of the control current response, respectively. The IC_{50} and Hill coefficient values obtained from concentration-response relationships are shown in Table 2. The rank order of potency was (+)-SKF10047, carbetapentane > (+)-cyclazocine > (+)-3PPP (Figure 4b). These results suggest that (+)-SKF10047, (+)-cyclazocine, carbetapentane and (+)-3PPP act as antagonists at the μ-opioid receptor.

The current response via the δ-opioid receptor activated by DPDPE was not affected by 10 μM (+)-cyclazocine, DTG,

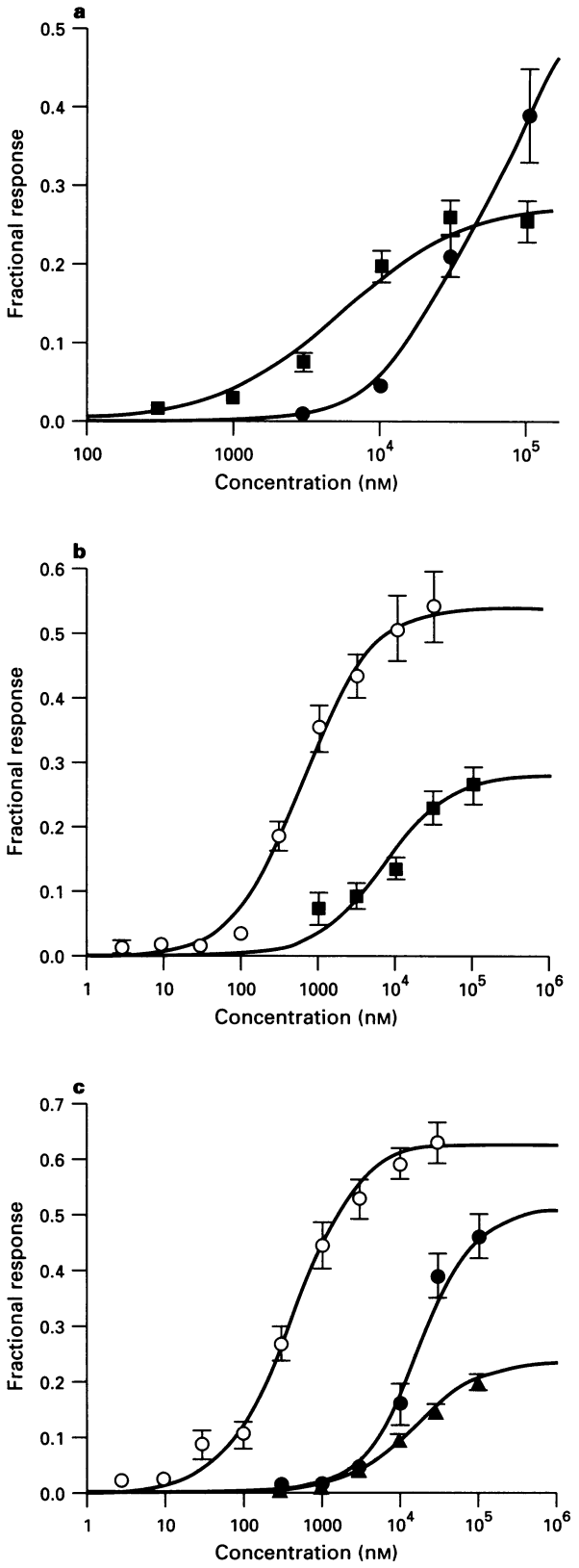

Figure 3 Concentration-response relationships for (+)-SKF10047 (○), DTG (●), (+)-3PPP (▲) and haloperidol (■). (a) The concentration-response relationships for DTG and haloperidol in the oocytes co-injected with μ-opioid receptor and GIRK1 mRNAs. (b) The concentration-response relationships for (+)-SKF10047 and haloperidol in the oocytes co-injected with δ-opioid receptor and GIRK1 mRNAs. (c) The concentration-response relationships for (+)-SKF10047, DTG and (+)-3PPP in the oocytes co-injected with κ-opioid receptor and GIRK1 mRNAs. The fractional responses are the ratios of the σ ligand-induced responses to the control response to the selective opioid agonist. Each point represents the mean and s.e.mean of the fractional responses obtained from 5 to 9 oocytes. Data points of each σ ligand are fitted using a logistic equation.

Table 1 The effects of σ ligands on the cloned μ-, δ- and κ- opioid receptors

	μ		δ		κ	
Compound	EC_{50}	n_H	EC_{50}	n_H	EC_{50}	n_H
(+)-SKF10047	–		0.618±0.167	1.54±0.21	0.652±0.223	0.98±0.15
DTG	>30		–		14.88±1.407	2.17±0.30
(+)-3PPP	–		–		18.08±4.688	0.96±0.05
Haloperidol	5.683±0.758	1.69±0.22	7.389±1.270	0.98±0.13	–	

The mean±s.e.mean of the EC_{50} (μM) and Hill coefficient (n_H) values are shown. Dash (–) indicates that an agonist effect was not observed.

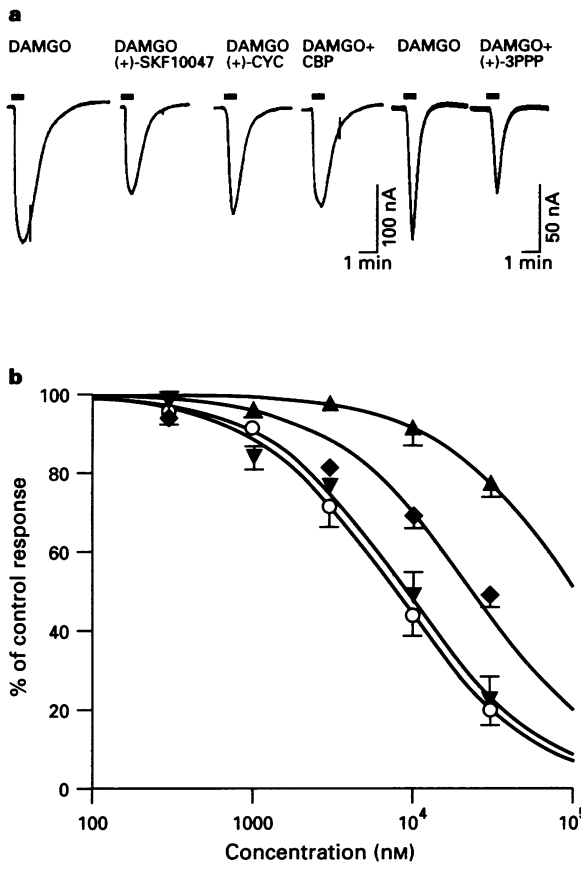

Figure 4 Inhibition of DAMGO-induced current responses by σ ligands in the oocytes co-injected with μ-opioid receptor and GIRK1 mRNAs. (a) Current responses to 180 nM DAMGO, 180 nM DAMGO plus 10 μM (+)-SKF10047, 180 nM DAMGO plus 10 μM (+)-cyclazocine ((+)-CYC) and 180 nM DAMGO plus 10 μM carbetapentane (CBP) in one oocyte, and responses to 180 nM DAMGO and 180 nM DAMGO plus 30 μM (+)-3PPP in another oocyte. Current reponses were measured at a −70 mV membrane potential in a high-potassium solution. Bars above the traces show the duration of application. Inward current is downward. (b) Concentration-dependent inhibition of the responses by (+)-SKF10047 (○), (+)-cyclazocine (◆), (+)-3PPP (▲) and carbetapentane (▼). Each point represents the mean and s.e.mean % of the control responses obtained from 5 to 7 oocytes. Data points of each σ ligand are fitted using a logistic equation.

(+)-3PPP or carbetapentane (data not shown). The current response via the κ-opioid receptor activated by U50488H was not affected by 10 μM haloperidol (data not shown).

Discussion

In the present study, we demonstrated the effects of σ ligands of various structural and pharmacological classes, (+)-SKF10047, (+)-cyclazocine, DTG, (+)-3PPP, carbetapentane and haloperidol, on the cloned μ-, δ- and κ-opioid receptors co-expressed with the GIRK1 channel in *Xenopus* oocytes. (+)-SKF10047 acted as an agonist at the δ- and κ-opioid receptors and as an antagonist at the μ-opioid receptor. (+)-Cyclazocine, (+)-3PPP and carbetapentane acted as agonists at the κ-opioid receptor and as antagonists at the μ-opioid receptor. DTG acted as an agonist at the μ- and κ-opioid receptors. Haloperidol acted as an agonist at the μ- and δ-opioid receptors.

Table 2 Potency of σ ligands as antagonists for the cloned μ-opioid receptor

Compound	IC_{50} (μM)	n_H	n
(+)-SKF10047	8.51±1.60	1.08±0.07	7
(+)-Cyclazocine	33.2±8.4	0.69±0.15	6
Carbetapentane	11.2±3.4	1.04±0.08	5

The mean±s.e.mean of the IC_{50} (μM) and Hill coefficient (n_H) values are shown. *n* is the number of oocytes tested.

SKF10047 is a prototypic σ ligand of benzomorphans, and (+)-SKF10047 has been used experimentally as a σ_1 agonist (Beart *et al.*, 1989; Itzhak, 1989; Quirion *et al.*, 1992). It has been known that (+)-SKF10047 has moderate affinity for the PCP site labelled with [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine (TCP) (Largent *et al.*, 1986) and that (±)-SKF10047 and PCP block the same site on NMDA receptor channels to similar extents in a *Xenopus* oocyte expression system (Yamakura *et al.*, 1993). Since the K_i values of (+)-SKF10047 for the μ-, δ- and κ-opioid receptors are above micromolar concentrations (Tam, 1985) and the behavioural effects of (+)-SKF10047 are not blocked by naloxone (Walker *et al.*, 1990), (+)-SKF10047 has not been considered to interact with opioid receptors. In the present study, (+)-SKF10047 activated the δ- and κ-opioid receptors even at nanomolar concentrations and antagonized the μ-opioid receptor at micromolar concentrations, and the activation of the δ- and κ-opioid receptors by (+)-SKF10047 was not completely antagonized by naloxone. Some of the naloxone-insensitive effects of (+)-SKF10047 reported previously may be caused by the activation of the δ- and κ-opioid receptors. It has been reported that the naloxone-sensitive psychotomimetic and aversive effects mediated via the κ-opioid receptor are elicited by (−)-isomers of benzomorphans, but not (+)-isomers which elicit naloxone-insensitive psychotomimetic effects via σ receptors (Pfeiffer *et al.*, 1986). However, since the (+)-isomers of benzomorphans, such as (+)-SKF10047 and (+)-cyclazocine, activated the cloned κ-opioid receptor, the psychotomimetic effects of (+)-isomers of benzomorphans may be partly mediated by the κ-opioid receptor.

Carbetapentane, which exhibits high affinity for σ_1 binding sites (Quirion *et al.*, 1992) and the M_1-muscarinic receptor (Hudkins & DeHaven-Hudkins, 1991), has been used clinically as a non-opioid antitussive agent with low dependence liability and has been found to have anticonvulsant properties (Tortella & Musacchio, 1986; Tortella *et al.*, 1989). Since carbetapentane acted as a κ-agonist in the present study and κ-agonists exhibit aversive effects (Millan, 1990) and anticonvulsant

activity (Tortella *et al.*, 1986), the low dependence liability and the anticonvulsant properties of carbetapentane may be partly mediated by the κ-opioid receptor.

Haloperidol, a typical antipsychotic drug of butyrophenones, exhibits high affinity not only for the D_2-dopamine receptor (Seeman & Van Tol, 1994) but also for σ receptors (Largent *et al.*, 1984; Tam & Cook, 1984; Weber *et al.*, 1986). The effects of haloperidol on the opioid receptors have not been investigated since Clay & Brougham (1975) reported that the IC_{50} value of haloperidol for the binding of [^{3}H]-(−)-naloxone to rat brain homogenates was 880 nM. The present findings firstly demonstrate that haloperidol acts as a μ- and δ-agonist. Since the concentration of haloperidol in the brain is considered to reach low micromolar concentrations soon after the administration of high dosages of haloperidol in clinical practice (Öhman *et al.*, 1977; Wurzburger *et al.*, 1981; Korpi *et al.*, 1984), the μ- and δ-opioid receptors in the brain may be activated in haloperidol-treated schizophrenic patients and drug abusers. Di Chiara & Imperato (1988) showed that haloperidol increased the extracellular dopamine concentration in the nucleus accumbens (NAc), and suggested that the increase was caused by a feedback response induced by blockade of the dopamine receptors. However, since both μ-agonists and δ-agonists have been shown to increase the dopamine concentration in the NAc (Spanagel *et al.*, 1990), the increase in the dopamine concentration may be partly caused by the activation of the μ- and δ-opioid receptors by haloperidol.

Both DTG and (+)-3PPP, which are selective σ ligands, have been widely used as σ ligands for investigating the pharmacological and biochemical properties, distributions and functional roles of σ receptors. The effects of DTG and (+)-3PPP in micromolar concentrations have been analyzed using many *in vivo* response assays and using a variety of bioassays in preparations including neuronal slice, ileum longitudinal muscle/myenteric plexus (LMMP) and vas deferens. The effects of DTG and (+)-3PPP on the μ- and κ-opioid receptors observed in the present study may be part of the molecular mechanism of the functional roles of the σ ligands in these assays.

Electrophysiological studies have revealed that DAMGO and [Met^5]enkephalin (M-Enk), a nonselective opioid agonist, increase potassium conductance via the μ-opioid receptor in neurones of the locus coeruleus (LC) in neuronal slice preparations and hyperpolarize the cell membrane (North *et al.*, 1987). Bobker and colleagues (1989) reported that both DTG and (+)-3PPP (1 − 100 μM) inhibited the M-Enk-induced hyperpolarization in neurones of the LC, but did not block it completely, and that haloperidol (1 − 10 μM) weakly inhibited the M-Enk-induced hyperpolarization. Since the κ-opioid receptor is moderately expressed in the LC (Mansour *et al.*, 1995), M-Enk activates the κ-opioid receptor as well as the μ-opioid receptor. The present findings demonstrate that DTG, (+)-3PPP and haloperidol interact with the μ- and/or κ-opioid receptors. The inhibitory effects of the σ ligands may be partly mediated by the opioid receptors.

Guinea-pig ileum LMMP preparations have been used in bioassays for characterizing the activation of the μ- or κ-opioid receptors, and the activation of the opioid receptors has been found to inhibit electrically stimulated smooth muscle contraction (Leslie, 1987). Campbell *et al.* (1989) have demonstrated that various σ ligands at micromolar concentrations inhibit the electrically stimulated contractions of the LMMP in the presence of naloxone and in the preparation treated with an irreversible opiate antagonist and that both (+)-SKF10047 and (+)-cyclazocine potentiate the stimulated contraction in the same preparation, suggesting that the effects may be mediated by σ receptors, not by opioid receptors. Since then, the effects of σ ligands in LMMP preparations have been investigated without consideration of the effects of σ ligands on opioid receptors (Campbell *et al.*, 1991; Coccini *et al.*, 1991). However, the present findings indicate that the interaction of various σ ligands with the μ- and/or κ-opioid receptors should be considered in evaluating the effects of σ ligands at micromolar concentrations in LMMP preparations.

The activation of the μ-, δ- or κ-opioid receptors in the mouse and rat vasa deferentia inhibits electrically stimulated twitch contractions (Leslie, 1987). DeHaven-Hudkins *et al.* (1991) have demonstrated that various σ ligands including DTG (10 nM − 100 μM) and haloperidol (10 nM − 30 μM) inhibit electrically stimulated twitch contractions in the mouse vas deferens even at nanomolar concentrations and that the inhibitory effects of DTG are not antagonized by 0.3 μM naloxone. Also, Kennedy & Henderson (1989) have demonstrated that both DTG and haloperidol at high concentrations (30 and 100 μM) inhibit the twitch contractions. In the present study, DTG and haloperidol at micromolar concentrations acted as a μ- and κ-agonist and a μ- and δ-agonist, respectively, and the activation of the δ- or κ-opioid receptors by the σ ligands tested was not completely antagonized by 1 μM naloxone. Our results suggest that the inhibitory effect of haloperidol and DTG at high concentrations in the vas deferens preparations is partly mediated via the direct activation of the opioid receptors by the σ ligands.

It has been shown that σ ligands modulate the μ-, δ- and κ-opioid analgesia in the tail flick assay (Chien & Pasternak, 1994) and that DTG and haloperidol exhibit antinociceptive effects in the tail flick assay, although the antinociceptive effect of DTG is blocked by rimcazole, a σ ligand, but not by naloxone (Kest *et al.*, 1995). The modulation of the opioid analgesia and antinociceptive effect by σ ligands may be also related to the interaction of the σ ligands with the opioid receptors.

In the present study, (+)-SKF10047 activated the δ- and κ-opioid receptors at nanomolar concentrations and the σ ligands of various chemical classes interacted with the opioid receptors at micromolar concentrations. These results indicate that the effects of σ ligands at these concentrations may reflect the interaction of the σ ligands not only with σ receptors but also with the opioid receptors *in vivo* and *in vitro* and that the σ ligands tested in this study cannot be used as selective probes in investigating the functional effects of the σ ligands at micromolar concentrations. To investigate further the functional properties of σ ligands and σ receptors, studies are required to develop specific and potent σ ligands and to identify σ receptors and endogenous σ ligands.

Xenopus oocyte membranes contain an intrinsic σ_2-like binding site (Patterson *et al.*, 1994). In the present study, the oocytes injected with the GIRK1 mRNA alone did not respond to all of the σ ligands tested. The σ_2-like binding site in *Xenopus* oocytes may not functionally couple with the GIRK1 channel in the signal transduction, although all of the σ ligands tested may not act as agonists at the σ_2-like binding site.

Binding assays can be used to investigate the affinity of a ligand, but not its functional properties, such as whether it is a full agonist, partial agonist or antagonist. The *Xenopus* oocyte co-expression system with the synthesized opioid receptor and GIRK1 mRNAs can be used to characterize the functional properties of known opioid ligands as well as novel ligands at each opioid receptor, although this system will not replace many *in vivo* response assays and a variety of bioassays with isolated tissues, such as the guinea pig ileum and mouse vas deferens. Furthermore, this system may be very useful for screening novel ligands for each opioid receptor subtype and developing specific opioid ligands. Since the heteromultimeric GIRK channels in *Xenopus* oocytes have recently been reported to produce larger inward currents than those in the oocytes injected with the GIRK1 mRNA alone (Kofuji *et al.*, 1995; Krapivinsky *et al.*, 1995; Nichols *et al.*, 1995), the *Xenopus* oocyte expression system may be improved by co-injection of GIRK subunit mRNAs.

In conclusion, we have demonstrated that σ ligands of various chemical classes directly interact with the cloned μ-, δ- and κ-opioid receptors co-expressed with the GIRK1 channel in *Xenopus* oocytes. Our results suggest that the functional roles of the σ ligands may be partly mediated by the opioid receptors.

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