

# The Antitussive Activity of $\delta$ -Opioid Receptor Stimulation in Guinea Pigs

CHARLES J. KOTZER, DOUGLAS W. P. HAY, GIULIO DONDIO, GIUSEPPE GIARDINA, PAOLA PETRILLO, and DAVID C. UNDERWOOD

Departments of Pulmonary Pharmacology (C.J.K., D.W.P.H., D.C.U.), Medicinal Chemistry (G.D., G.G.), and Biology (P.P.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania

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## ABSTRACT

In this study, the activity of the  $\delta$ -opioid receptor subtype-selective agonist, SB 227122, was investigated in a guinea pig model of citric acid-induced cough. Parenteral administration of selective agonists of the  $\delta$ -opioid receptor (SB 227122),  $\mu$ -opioid receptor (codeine and hydrocodone), and  $\kappa$ -opioid receptor (BRL 52974) produced dose-related inhibition of citric acid-induced cough with ED<sub>50</sub> values of 7.3, 5.2, 5.1, and 5.3 mg/kg, respectively. The nonselective opioid receptor antagonist, naloxone (3 mg/kg, i.m.), attenuated the antitussive effects of codeine or SB 227122, indicating that the antitussive activity of both compounds is opioid receptor-mediated. The  $\delta$ -receptor antagonist, SB 244525 (10 mg/kg, i.p.), inhibited the anti-

tussive effect of SB 227122 (20 mg/kg, i.p.). In contrast, combined pretreatment with  $\beta$ -funaltrexamine ( $\mu$ -receptor antagonist; 20 mg/kg, s.c.) and norbinaltorphimine ( $\kappa$ -receptor antagonist; 20 mg/kg, s.c.), at doses that inhibited the antitussive activity of  $\mu$ - and  $\kappa$ -receptor agonists, respectively, was without effect on the antitussive response of SB 227122 (20 mg/kg, i.p.). The  $\sigma$ -receptor antagonist rimcazole (3 mg/kg, i.p.) inhibited the antitussive effect of dextromethorphan (30 mg/kg, i.p.), a  $\sigma$ -receptor agonist, but not that of SB 227122. These studies provide compelling evidence that the antitussive effects of SB 227122 in this guinea pig cough model are mediated by agonist activity at the  $\delta$ -opioid receptor.

Opioid receptor agonists are classified by their activity at three opioid receptor subtypes known as  $\mu$  (Wang et al., 1994),  $\kappa$  (Mansson et al., 1994), and  $\delta$  (Knapp et al., 1994). Members of this class of compounds have been demonstrated to have antitussive effects. For example, compounds such as morphine and codeine, considered to be the most potent and effective antitussive drugs currently on the market, are categorized generally as agonists at the  $\mu$ -opioid receptor (Eddy et al., 1969; Karlsson et al., 1990). In addition,  $\kappa$ -opioid-selective agonists have been shown to inhibit cough in laboratory animals (Kamei et al., 1990).

Although the involvement of  $\delta$ -opioid receptors in cough has been described (Kamei et al., 1992; Dondio et al., 1997), some of the data are conflicting. Some reports indicate that  $\delta$ -receptor antagonists produce an antitussive effect against capsaicin-induced cough in rats and mice (Kamei et al., 1993c, 1994b). Furthermore, evidence has been presented that  $\delta$ -opioid receptor agonists can either reduce (Kamei et al., 1991) or enhance (Kamei et al., 1993d) the antitussive effect of  $\mu$ -receptor agonists [e.g., D-Ala<sup>2</sup>-Me-Phe<sup>4</sup>-Gly-ol<sup>5</sup>-

enkephalin (DAMGO), morphine]. The reason(s) for these discrepancies is not known but may be linked to the limited selectivity of the compounds that were investigated.

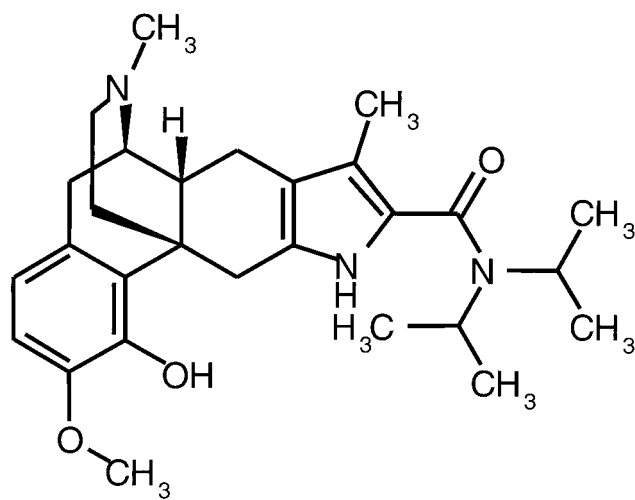
The goal of this study was to clarify the influence of  $\delta$ -receptors in a cough model. Specifically, we investigated the ability of the novel  $\delta$ -opioid receptor agonist, SB 227122 ([10*R*, 4*bS*-(4*b* $\beta$ ,9*a* $\beta$ )]-7-diisopropylaminocarbonyl-8,14-dimethyl-4-hydroxy-3-methoxy-4*b*,5,9,9*a*,10,11-hexahydro-(6*H*)-[2,3-*h*]-pyrrolo[10,4-*b*]iminoethenophenanthrene; Fig. 1), to inhibit citric acid-induced cough in the guinea pig. SB 227122 is a nonpeptide agonist with high affinity for the human  $\delta$ -receptor ( $K_i = 6.9$  nM from binding studies) and  $K_i > 2$   $\mu$ M versus  $\mu$ - or  $\kappa$ -receptors (Petrillo et al., 1998). In addition, we attempted to confirm the mechanism of the antitussive activity of SB 227122 by using subtype-selective opioid receptor antagonists.

## Materials and Methods

**Cell Lines.** Stable expression of human  $\delta$ -opioid receptors (h-DORs) and  $\mu$ -opioid receptors (h-MORs) in Chinese hamster ovary (CHO) cells and human  $\kappa$ -opioid receptors (h-KORs) in human embryonic kidney 293 (HEK-293) cells were prepared in-house. The h-DOR was cloned by screening primers based on the public se-

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**ABBREVIATIONS:** DAMGO, D-Ala<sup>2</sup>-Me-Phe<sup>4</sup>-Gly-ol<sup>5</sup>-enkephalin; h-DOR, human  $\delta$ -opioid receptor; h-MOR, human  $\mu$ -opioid receptor; h-KOR, human  $\kappa$ -opioid receptor;  $\beta$ -FNA,  $\beta$ -funaltrexamine; Nor-BNI, norbinaltorphimine; CHO, Chinese hamster ovary; HEK-293, human embryonic kidney 293; RT-PCR, reverse-transcription polymerase chain reaction; DADLE, D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin; S.A., specific activity; PLSD, protected least-squares difference.



## SB 227122

**Fig. 1.** Structure of SB 227122 ([10*R*,4*bS*-(4*b* $\beta$ ,9*a* $\beta$ )]-7-diisopropylamino-carbonyl-8,14-dimethyl-4-hydroxy-3-methoxy-4*b*,5,9,9*a*,10,11-hexahydro-(6*H*)-[2,3-*b*]-pyrrolo[10,4-*b*]iminoethenophenanthrene).

quence (GenBank accession no. U10504); reverse-transcription polymerase chain reaction (RT-PCR) was done using whole human brain poly(A)<sup>+</sup> RNA as the template. The h-MOR was cloned by screening a whole brain human cDNA library with a coding probe from a truncated h-KOR cDNA. A truncated version of the h-KOR was cloned by screening a whole brain cDNA library using the ORL-1 receptor as the probe. The missing 5'-end was cloned by RT-PCR using whole brain poly(A)<sup>+</sup> RNA as the template. The full-length cDNA for the three opioid receptors was inserted into *Asp718* and *HindIII* sites of pCDN mammalian expression vector (Aiyar et al., 1994). CHO or HEK-293 cells were transfected by electroporation then plated for isolation of single clones. CHO-transfected cells were grown in suspension culture in serum-free medium (1017 S03; Proprietary-SmithKline Beecham Pharmaceuticals, King of Prussia, PA) in the presence of 0.05% pluronic acid (F68; GIBCO BRL, Paisley, Scotland), maintained at 37°C, and gassed with 5% CO<sub>2</sub>. Selection for CHO transfectants was performed by growth in the absence of nucleotides. The maximum cell density for these cell lines was 4 × 10<sup>6</sup> cells/ml. From binding studies it was determined that transfected CHO cell lines express h-DOR and h-MOR at a density of 11.5 and 6.9 pmol/mg of protein, respectively.

HEK-293 cells were grown attached to T-150 flasks in Earle's minimum essential medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine; G418 was included for selection. The stable cell line expresses h-KOR at a density of 3.6 pmol/mg of protein from binding studies.

**Cells Membrane Preparation.** Membranes were prepared by hypotonic lysis according to previously described methods with minor modification (Scheideler and Zukin, 1990). Briefly, cells were harvested in PBS (1 × 10<sup>6</sup> cells/ml) and collected by centrifugation (800*g* for 5 min). The pellets were resuspended in the same volume of ice-cold 10 mM potassium phosphate buffer, pH 7.2 (buffer A), and centrifuged at 40,000*g* for 10 min. The cells were hypo-osmotically lysed by resuspension in the same volume of buffer A for 20 min in ice, centrifuged at 800*g* (5 min), and the supernatant saved. The resulting pellets were resuspended in buffer A, and the last step was repeated two additional times, saving the supernatants each time. Supernatants derived from the three low-speed centrifugations were pooled and centrifuged at the high speed. Pellets were resuspended in buffer A containing 0.32 M sucrose and 5 mM EDTA (buffer B) to wash and concentrate the membranes. The final pellets were resuspended in buffer B at a final concentration of 1 to 2 mg of protein/ml

(ca. 40 × 10<sup>6</sup> cells/ml) and stored at -80°C. Protein was determined with the protein assay kit from Sigma Chemical Co. (Milan, Italy).

**Radioligand Binding Assays.** [<sup>3</sup>H]D-Ala<sup>2</sup>-D-Leu<sup>5</sup>-enkephalin ([<sup>3</sup>H]DADLE; *K*<sub>d</sub> = 1.2 nM), [<sup>3</sup>H]DAMGO (*K*<sub>d</sub> = 1.2 nM), and [<sup>3</sup>H]U69593 (*K*<sub>d</sub> = 1.6 nM) were used to label  $\delta$ -,  $\mu$ -, and  $\kappa$ -binding sites, respectively. Binding experiments were performed in triplicate at a final protein concentration of 10  $\mu$ g/ml in buffer A and a radiolabeled ligand concentration of 0.4 to 0.5 nM. The nonspecific binding was determined in the presence of 10  $\mu$ M naloxone, and was less than 1% of the radioligand added under these assay conditions. Samples (final volume of 2 ml) were incubated for 60 min at 37, 30, and 25°C for  $\delta$ -,  $\mu$ -, and  $\kappa$ -binding assays, respectively. The reaction was terminated by rapid filtration through Whatman GF/B filters and two washes with cold assay buffer A (4 ml) using a M48 Brandel Cell Harvester (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). Filters used for [<sup>3</sup>H]U69593 binding were presoaked in buffer A containing 0.05% polyethylenimine. Radioactivity on the filters was measured by liquid scintillation counting with a Canberra Packard 2500TR beta counter (Milan, Italy).

The data obtained from competition binding experiments were analyzed with nonlinear fit analysis using the RS/1 software (BBN Software Products Corp., Cambridge, MA) (Benfenati and Guardabasso, 1984; Baron et al., 1991). *K*<sub>i</sub> values were determined from IC<sub>50</sub> using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

**cAMP Accumulation.** Whole CHO cells, expressing the human  $\delta$ -receptor, were incubated with vehicle or test compounds in 200 mM Krebs-Ringer, buffered with HEPES, containing 125 mM NaCl, 5 mM KCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 12 mM glucose, and 1 mM isobutylmethylxanthine. Cells were treated with 10 mM forskolin to stimulate cAMP synthesis, and cAMP content was determined after 10 min using a double antibody <sup>125</sup>I-cAMP radioimmunoassay (RPA 509; Amersham, Milan, Italy).

**Cough Model.** All animal studies conform to Animal Care and Use Committee protocols filed at SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

Male Hartley guinea pigs (550–750 g; Charles River, Portage, MI) were used in all experiments. Animals were placed in a clear plastic exposure chamber with an internal volume of 6 liters. A bias airflow was applied to the chamber at a rate of 2 l/min for the duration of the experiment. The changes in airflow inside the chamber were measured by a pressure transducer (MP 45-14; Validyne Engineering Corp., Northridge, CA) with a range of  $\pm 2$  cm of H<sub>2</sub>O and a pneumotachograph (consisting of nine 325-mesh screens) mounted on the top of the exposure chamber. Flow signals were output to a preamplifier bank (Buxco Electronics Inc., Sharon, CT) and routed to a chart recorder (Linearcorder WR 3320; Western Graphtech, Irvine, CA) for analyses. Using this system, an incidence of cough was denoted by a larger than normal inspiration (at least twice the normal tidal deflection) followed immediately by a rapid, forceful expiration (more than three times the normal excursion from baseline) (Piiirila and Sovijarvi, 1995). A cough was easily distinguished from animal movement, augmented breaths, and gasps by close inspection of the flow tracing. Because a sneeze and cough have similar flow patterns, they were differentiated by visual observation of the animals (Laude et al., 1993).

Cough was induced by inhalation of an aerosol of 0.4 M citric acid. This has been shown previously to induce the cough reflex in guinea pigs (Forsberg et al., 1988, 1992). The aerosol was administered to the animals via a small-volume ultrasonic nebulizer (AeroSonic model 5000D; DeVilbiss, Somerset, PA) connected to the bias flow port immediately before the exposure chamber inlet. A volume of 2 ml of citric acid solution was placed into the ultrasonic nebulizer. During the 1-min aerosolization period, approximately 0.5 ml of the solution was nebulized. The incidences of cough in 13 min (aerosolization time + observation time) were recorded. Animals were used for only one citric acid challenge due to tachyphylaxis of the cough response (Laloo et al., 1995).

Drugs were administered i.p., i.m., or s.c. before cough challenge. Pretreatment times and doses varied for each compound based on experiments conducted in this study and published reports. Several different studies were performed: 1) comparison of the antitussive activity of the  $\mu$ -receptor agonists, codeine and hydrocodone (i.p.; 30-min pretreatment), with the  $\kappa$ -receptor agonist BRL 52974 (s.c.; 30-min pretreatment), and the  $\delta$ -receptor agonist SB 227122 (i.p.; 20-min pretreatment) in doses ranging from 0.5 to 20 mg/kg; 2) examination of the effects of the nonselective opioid receptor antagonist, naloxone (3 mg/kg, i.m.; 10-min pretreatment) against the antitussive activity of codeine (10 mg/kg, i.p.; 30-min pretreatment) and SB 227122 (5 or 10 mg/kg, i.p.; 20-min pretreatment); 3) comparison of the effects of opioid subtype-selective receptor antagonists,  $\beta$ -funaltrexamine ( $\beta$ -FNA) ( $\mu$ -selective; 20 mg/kg, s.c.; 24-h pretreatment), norbinaltorphimine (Nor-BNI) ( $\kappa$ -selective; 20 mg/kg, s.c.; 4-h pretreatment), and SB 244525 ( $\delta$ -selective; 10 mg/kg, i.p.; 25-min pretreatment), to inhibit the antitussive activities of hydrocodone, BRL 52974, and SB 227122, respectively; and examination of the effect of a combination of  $\beta$ -FNA and Nor-BNI against the  $\delta$ -selective opioid receptor agonist SB 227122; and 4) exploration of the effects of rimcazole (3 mg/kg, i.p.; 45-min pretreatment), a  $\sigma$ -receptor antagonist, on the antitussive activity of the  $\sigma$ -receptor agonist, dextromethorphan (i.p.; 30-min pretreatment), and SB 227122.

**Drugs.** [ $^3$ H]DADLE [specific activity (S.A.) 55.3  $\mu$ Ci/nmol] and [ $^3$ H]DAMGO (S.A. 54.5  $\mu$ Ci/nmol) were obtained from New England Nuclear (Brussels, Belgium). [ $^3$ H]U-69593 (55  $\mu$ Ci/nmol) was supplied by Amersham (Milan, Italy). Codeine and hydrocodone were purchased from Mallinckrodt, Inc. (St. Louis, MO) and Salars (Como, Italy), respectively. BRL 52974 [4-(pyrrolidin-1-yl)methyl-5- $\beta$ -(3,4-dichlorophenyl)acetyl-4,5,6,7-tetrahydroimidazo[4,5-c]pyridine]; SB 227122 [(10*R*,4*bS*-(4*b* $\beta$ ,9*a* $\beta$ ))-7-diisopropylaminocarbonyl-8,14-dimethyl-4-hydroxy-3-methoxy-4*b*,5,9,9*a*,10,11-hexahydro-(6*H*)-[2,3-*h*]-pyrrolo[10,4-*b*]iminoethenophenanthrene);  $\beta$ -FNA [(*E*)-4-[[5*a*,6*b*]-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6yl]amino]-4-oxo-2-butenic acid methyl ester hydrochloride]; Nor-BNI dihydrochloride tetrahydrate; naltrindole hydrochloride (8*R*-(4*bS*\*,8*a*,8*a* $\beta$ ,14*b* $\beta$ ))-7-(cyclopropylmethyl)-5,6,7,8,14,14*b*-hexahydro-4,8-methanobenzofuro[2,3-*a*]pyrido[4,3-*b*]carbazole-1,8*a*(9*H*)-diol hydrochloride); and SB 244525[(8*R*-(4*bS*\*,8*a*,8*a* $\beta$ ,12*b* $\beta$ ))-7-allyl-11-isobutylcarbonyl-1-methoxy-10-methyl-5,6,7,8,12,12*b*-hexahydro-(9*H*)-4,8-methano-benzofuro[3,2-*e*]pyrrolo[2,3-*g*]isoquinolin-8-*ol* hydrochloride) were synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals. Naloxone (4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)morphinan-6-one hydrochloride); dextromethorphan (*d*-3-methoxy-*N*-methylmorphinan); and rimcazole (*cis*-9-[3-(3,5-dimethyl-1-piperazinyl)propyl]-9*H*-carbazole dihydrochloride) were purchased from Sigma (St. Louis, MO). Citric acid monohydrate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All chemicals were dissolved in 0.9% saline for i.m., s.c., i.p., and aerosol drug administration except for dextromethorphan, which was solubi-

lized in 4% acetic acid, Nor-BNI, which was dissolved in 0.1 N HCl, and SB 227122 and BRL 52974, which were dissolved in 0.1 N CH<sub>3</sub>SO<sub>3</sub>H for parenteral administration.

**Statistics.** Data were analyzed using StatView statistical analysis software (Abacus Concepts, Inc., Berkeley, CA). ANOVA was performed for each experiment, followed by a Fisher's protected least-squares difference (PLSD) test. *P* values are provided under *Results*, with a value less than .05 regarded as significant. Results are presented as means or means  $\pm$  S.E.

## Results

### In Vitro Studies

**Receptor Binding.** The binding affinity of agonists and antagonists used in cough studies were determined in assays using the h-DOR, h-MOR, and h-KOR cell lines (Table 1). The  $\delta$ -receptor agonist SB 227122 demonstrated a 294- and >725-fold greater affinity for the  $\delta$ -receptor than the  $\mu$ - or  $\kappa$ -receptor, respectively. The  $\delta$ -receptor antagonist SB 244525 also proved to be highly selective for the  $\delta$ -receptor with a  $\mu/\delta$ - and  $\kappa/\delta$ -ratio from binding studies (*K<sub>i</sub>* values) of 294 and 170, respectively. The selectivities of the other opioid receptor agonists (codeine, hydrocodone, and BRL 52974) and antagonists ( $\beta$ -FNA, Nor-BNI, and naloxone) as well as the poor affinities of  $\sigma$ -receptor agonist (dextromethorphan) and antagonist (rimcazole) for the opioid receptors were also confirmed (Table 1).

**Functional Experiments.** The functional in vitro activities of SB 227122 and SB 244525 were assessed by determining their effects on cAMP levels in CHO cells expressing the human cloned  $\delta$ -opioid receptor. SB 227122 (0.1–1000 nM) displayed full agonist activity for decreasing cAMP (*IC*<sub>50</sub> = 12 nM); this effect was completely antagonized by naltrindole (100 nM). SB 244525 alone, at concentrations up to 1  $\mu$ M, lacked any significant effect on cAMP levels. However, SB 244525 (0.1–1000 nM) potentially antagonized the inhibitory effects of SB 227122 or (–)TAN-67, a nonpeptide  $\delta$ -opioid receptor agonist, on cAMP levels (data not shown).

### In Vivo Studies

**Antitussive Activity of Receptor Subtype-Selective Agonists.** The antitussive activities of four subtype-selective opioid receptor agonists, codeine, hydrocodone, BRL 52974, and SB 227122, were determined in the guinea pig citric acid-induced cough model. Cough incidence in vehicle-treated animals ranged from 10 to 18 coughs, averaging 15  $\pm$

TABLE 1  
Agonist and antagonist binding to human  $\delta$ -,  $\kappa$ -, and  $\mu$ -opioid receptors

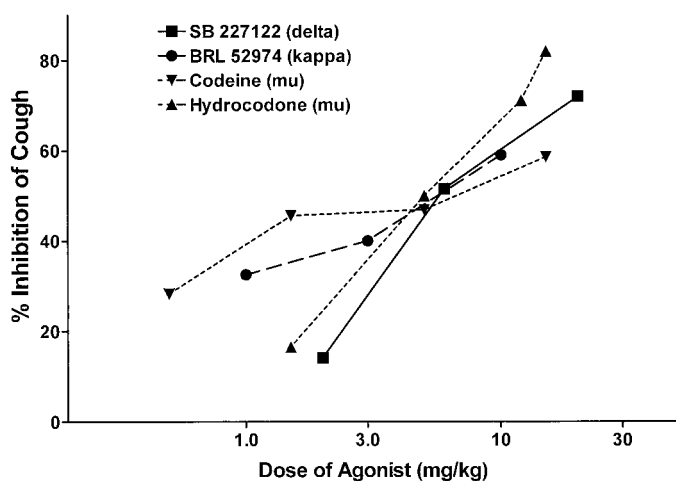
Compound	Class	Receptor <i>K<sub>i</sub></i>		
		$\delta$ h-DOR/CHO	$\mu$ h-MOR/CHO	$\kappa$ h-KOR/HEK-293
		<i>nM</i>		
SB 227122	$\delta$ -agonist	6.90 $\pm$ 1.07	2030 $\pm$ 519	>5000
SB 244525	$\delta$ -antagonist	4.76 $\pm$ 1.04	1223 $\pm$ 206	810 $\pm$ 327
Naltrindole	$\delta$ -antagonist	0.24 $\pm$ 0.02	7.23 $\pm$ 1.71	4.36 $\pm$ 0.49
Codeine	$\mu$ -agonist	>1000	161.3 $\pm$ 32.4	>2500
Hydrocodone	$\mu$ -agonist	1021 $\pm$ 238	36.4 $\pm$ 13.15	717 $\pm$ 177
$\beta$ -FNA	$\mu$ -antagonist	10.9 $\pm$ 1.7	0.62 $\pm$ 0.20	0.82 $\pm$ 0.15
BRL 52974	$\kappa$ -agonist	2904 $\pm$ 341	899 $\pm$ 199	0.48 $\pm$ 0.06
Nor-BNI	$\kappa$ -antagonist	2.9 $\pm$ 0.5	2.7 $\pm$ 0.6	0.79 $\pm$ 0.26
Naloxone	opioid antagonist	33.8 $\pm$ 6.53	2.38 $\pm$ 0.41	2.52 $\pm$ 0.51
Dextromethorphan	$\sigma$ -agonist	>2500	1277 $\pm$ 247	2007 $\pm$ 342
Rimcazole	$\sigma$ -antagonist	>2500	>2500	896.3 $\pm$ 218

Results are given as means  $\pm$  S.E., *n* = 3–4.

1 coughs in the 13-min monitoring period ( $n = 8$ ). All four opioid agonists produced dose-related inhibition of the cough response resulting in at least 60% maximum inhibition (Fig. 2). The  $ED_{50}$  values of the  $\mu$ -receptor agonists, codeine and hydrocodone, were determined to be 5.2 mg/kg ( $n = 5$ ) and 5.1 mg/kg ( $n = 5$ ), respectively, when administered i.p. 30 min before citric acid challenge. The  $\kappa$ -receptor agonist BRL 52974 and the  $\delta$ -receptor agonist SB 227122 had  $ED_{50}$  values of 5.3 mg/kg (s.c.; 30-min pretreatment) and 7.3 mg/kg (i.p.; 20-min pretreatment), respectively ( $n = 4-8$ ; Fig. 2).

**Effect of Naloxone on Opioid Antitussive Activity.** Naloxone, a nonselective opioid receptor antagonist, was administered (3 mg/kg, i.m.) after vehicle; codeine or the  $\delta$ -receptor agonist SB 227122 were administered 10 min before cough challenge. Higher doses of naloxone produced central nervous system effects, such as ataxia and sedation, as well as antitussive activity (data not shown). Naloxone (3 mg/kg, i.m.) alone was without influence on the incidence of cough after citric acid challenge: control =  $17 \pm 2.5$  coughs ( $n = 4$ ); +naloxone =  $17 \pm 1.2$  coughs ( $n = 4$ ). Guinea pigs treated with codeine (10 mg/kg, i.p.) coughed an average of  $8.6 \pm 1.4$  times ( $n = 5$ ), which represented 49% inhibition of the cough reflex ( $P < .05$ , ANOVA). In the group of codeine-treated animals that received naloxone (3 mg/kg, i.m.), the incidence of cough was  $14 \pm 2.8$  ( $P < .05$  compared with the group receiving codeine alone, ANOVA;  $n = 4$ ; Fig. 3a), demonstrating an attenuation of the antitussive effect of codeine by naloxone.

In an identical experimental protocol, the selective  $\delta$ -opioid receptor agonist SB 227122 (5 or 10 mg/kg, i.p.) inhibited cough by 49 and 71%, respectively, as demonstrated by a decrease from  $15.8 \pm 2.1$  coughs in the vehicle-treated animals to  $8.0 \pm 2.2$  coughs at 5 mg/kg or  $4.6 \pm 1.3$  coughs at 10 mg/kg ( $P < .05$ , ANOVA;  $n = 4-5$ ; Fig. 3b). Naloxone (3 mg/kg, i.m.) significantly reduced antitussive activity of the SB 227122 (5 or 10 mg/kg, i.p.) to 4% ( $15.2 \pm 1.2$  coughs) and 35% ( $10.4 \pm 1.9$  coughs) inhibition, respectively; the incidence of cough with SB 227122 (5 mg/kg, i.p.) in the presence



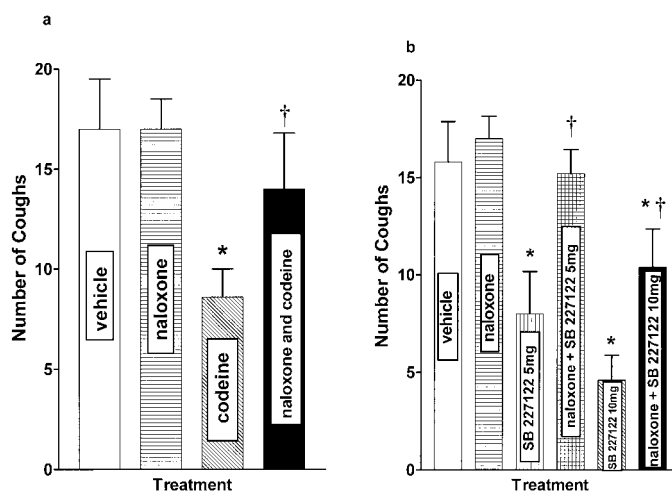
**Fig. 2.** Inhibition of citric acid-induced cough in guinea pigs after the administration of selective  $\mu$ - (codeine and hydrocodone),  $\kappa$ - (BRL 52974), and  $\delta$ - (SB 227122) opioid receptor agonists. Codeine and hydrocodone were administered i.p. 30 min before cough challenge, whereas SB 227122 and BRL 52974 were given i.p. 20 min and s.c. 30 min before treatment, respectively. The results are expressed as percentage of inhibition of the citric acid-induced cough response as compared with vehicle-treated animals;  $n = 4-8$ . Standard error bars are omitted for clarity.

of naloxone was not different from vehicle-treated, citric acid-exposed animals ( $P > .05$ , ANOVA;  $n = 4-5$ ; Fig. 3b).

**Effect of Selective Opioid Receptor Antagonists on the Antitussive Activity of Opioid Agonists.** To test the activity of a selective  $\mu$ -receptor antagonist,  $\beta$ -FNA (20 mg/kg, s.c.) or vehicle was administered 24 h before the selective  $\mu$ -receptor agonist hydrocodone (12 mg/kg, i.p.; 30-min pretreatment) (Kamei et al., 1993b).  $\beta$ -FNA alone had no effect on citric acid-induced cough occurrences (control =  $16.3 \pm 0.9$  coughs,  $n = 8$ ; + $\beta$ -FNA =  $15 \pm 1.9$  coughs,  $n = 4$ ). However,  $\beta$ -FNA substantially reduced the ability of hydrocodone (12 mg/kg, i.p.) to inhibit the citric acid-induced cough reflex (hydrocodone =  $4.8 \pm 1.4$  coughs,  $n = 8$ ; hydrocodone +  $\beta$ -FNA =  $12.8 \pm 1.8$  coughs,  $n = 8$ ;  $P < .05$ , ANOVA; Fig. 4a). The cough incidence in animals treated with both hydrocodone and  $\beta$ -FNA was not significantly different from vehicle-treated animals ( $P > .05$ , ANOVA; Fig. 4a).

In a similar experiment the selective  $\kappa$ -receptor antagonist Nor-BNI (20 mg/kg, s.c.; 4-h pretreatment) (Kamei et al., 1994a) was administered to animals that were later treated with BRL 52974 (10 mg/kg, s.c.), a selective  $\kappa$ -receptor agonist, 30 min before challenge with citric acid. BRL 52974 significantly inhibited the citric acid-induced cough response as compared with vehicle-treated animals: control =  $16.5 \pm 2.0$  coughs,  $n = 8$ ; +BRL 52974 =  $6.4 \pm 1.4$  coughs,  $n = 7$  ( $P < .05$ , ANOVA; Fig. 4b). Nor-BNI alone ( $12.8 \pm 2.7$  coughs,  $n = 4$ ) did not significantly influence citric acid-induced cough compared with vehicle-treated animals ( $P > .05$ , ANOVA). Nor-BNI essentially abolished the inhibitory effects of BRL 52974 against citric acid-induced cough: +Nor-BNI and BRL 52974 =  $12.6 \pm 2.3$  coughs; +Nor-BNI and vehicle =  $12.8 \pm 2.6$  coughs;  $n = 4$  ( $P > .05$ , ANOVA; Fig. 4b).

The effects of a combination of the selective  $\mu$ - and  $\kappa$ -receptor antagonists  $\beta$ -FNA (20 mg/kg, s.c.) and Nor-BNI (20 mg/kg, s.c.), respectively, against the antitussive effect of SB 227122 were examined. The combined antagonist pretreatment alone did not significantly alter the cough response:  $12.7 \pm 2.6$  coughs ( $n = 4$ ) as compared with  $16.3 \pm 0.9$  coughs



**Fig. 3.** The effect of the opioid antagonist naloxone (3 mg/kg, i.m.; 10-min pretreatment) on the antitussive activity of codeine (10 mg/kg, i.p.; 30-min pretreatment; panel a) or SB 227122 (5 or 10 mg/kg, i.p.; 20-min pretreatment; panel b). The results are expressed as the total number of coughs induced by a 1-min 0.4 M citric acid aerosol during the 13-min observation period;  $n = 4-5$ . \* $P < .05$ , ANOVA, Fisher's PLSD, as compared with vehicle-treated animals; and † $P < .05$ , ANOVA, Fisher's PLSD, as compared with opioid-agonist-treated animals.

( $n = 8$ ) in vehicle-treated animals ( $P > .05$ , ANOVA). In addition,  $\beta$ -FNA and Nor-BNI did not affect the antitussive activity of SB 227122 (20 mg/kg, i.p.; 20-min pretreatment; Fig. 4c). Thus, the group treated with SB 227122 exhibited  $4.3 \pm 1.3$  coughs, which represented a 72% inhibition compared with vehicle-treated animals, whereas the group pretreated with both antagonists and SB 227122 produced  $4.0 \pm 1.1$  coughs (74% inhibition;  $P < .05$  compared with vehicle-treated animals, ANOVA;  $n = 4-8$ ; Fig. 4c).

When administered alone, the  $\delta$ -selective receptor antagonist SB 244525 (10 mg/kg, i.p.; 25-min pretreatment) did not affect citric acid-induced cough: control =  $16.3 \pm 1.0$  coughs,  $n = 8$ ; +SB 244525 =  $15 \pm 1.4$  coughs,  $n = 6$  (Fig. 4d). When the  $\delta$ -receptor agonist SB 227122 was administered to animals treated with the  $\delta$ -receptor antagonist SB 244525, an average of  $10 \pm 2.5$  coughs was produced by citric acid expo-

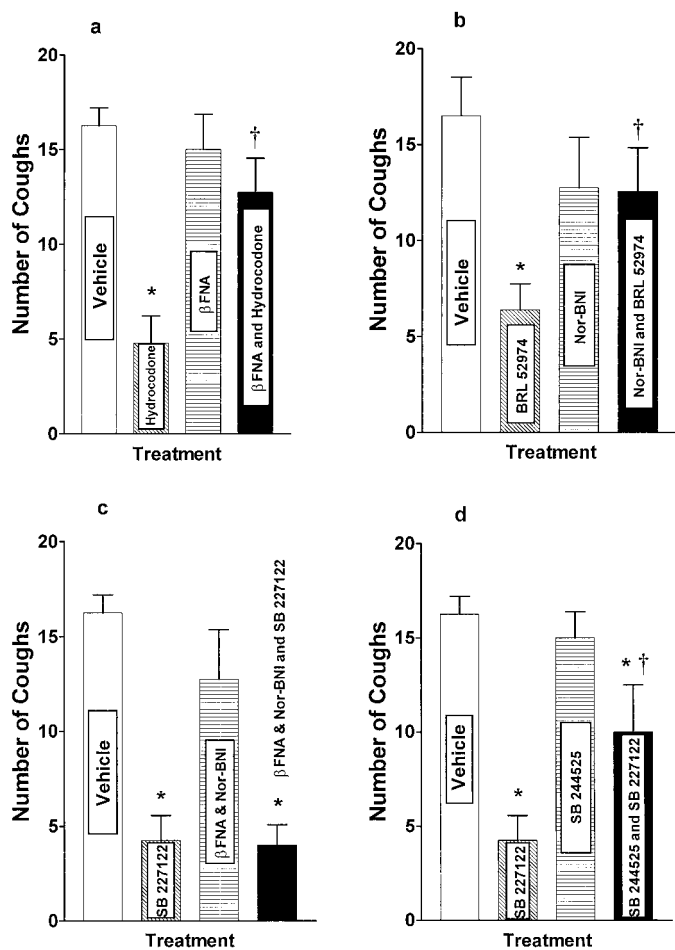
sure. This represented a significant reduction in the antitussive activity of SB 227122 (from 72 to 34% inhibition;  $P < .05$ , ANOVA, Fisher's PLSD;  $n = 6-8$ ; Fig. 4d).

**Effect of Selective  $\sigma$ -Antagonist Rimcazole on Dextromethorphan or SB 227122.** The  $\sigma$ -receptor antagonist rimcazole (3 mg/kg, i.p.) alone had no effect on citric acid-induced cough: control =  $16 \pm 1.0$  coughs,  $n = 4$ ; +rimcazole =  $16.3 \pm 1.1$ ,  $n = 4$ ;  $P > .05$ , ANOVA (Table 2). The  $\sigma$ -receptor agonist dextromethorphan inhibited citric acid-induced cough at doses of 10 and 30 mg/kg, i.p. ( $12 \pm 2.4$  coughs,  $n = 5$  and  $6 \pm 1.8$  coughs,  $n = 7$ , respectively;  $P < .05$ , ANOVA at the 30 mg/kg dose only). The antitussive activity of dextromethorphan (30 mg/kg, i.p.) was significantly inhibited by rimcazole (3 mg/kg, i.p.): cough incidence =  $6 \pm 1.8$  coughs ( $n = 7$ ) in dextromethorphan-treated animals versus  $15 \pm 2.2$  coughs ( $n = 5$ ) in the animals treated with rimcazole and dextromethorphan ( $P < .05$ , ANOVA). In contrast, rimcazole was without effect on SB 227122 (20 mg/kg, i.p.)-induced inhibition of coughs: +SB 227122 =  $4.6 \pm 1.2$  coughs,  $n = 8$ ; +SB 227122 and rimcazole =  $6.2 \pm 3.0$  coughs,  $n = 4$ ;  $P > .05$ , ANOVA; Table 2).

## Discussion

The major findings of this study, using the citric acid-induced cough model in guinea pigs, are: 1) the four subtype-selective opioid receptor agonists, codeine ( $\mu$ ), hydrocodone ( $\mu$ ), BRL 52974 ( $\kappa$ ), and SB 227122 ( $\delta$ ), demonstrated dose-dependent antitussive activity; 2) the opioid antagonist naloxone inhibited the antitussive activity of codeine ( $\mu$ -selective receptor agonist) or SB 227122 ( $\delta$ -selective receptor agonist); and 3) the antitussive activity of the  $\delta$ -opioid receptor agonist SB 227122 was not affected by treatment with a combination of  $\mu$ -receptor- and  $\kappa$ -receptor-selective antagonists, or rimcazole, a  $\sigma$ -receptor-selective antagonist but was inhibited by SB 244525, a  $\delta$ -receptor-selective antagonist (Petrillo et al., 1998). Collectively, these data indicate that the antitussive activity of the  $\delta$ -receptor agonist SB 227122 is due to stimulation of this specific opioid receptor.

Selective agonists for each of three opioid receptors, i.e., codeine and hydrocodone for the  $\mu$ -receptor, BRL 52974 for the  $\kappa$ -receptor, and SB 227122 for the  $\delta$ -receptor (Giardina et al., 1995), demonstrated dose-dependent antitussive activity with a range of ED<sub>50</sub> values from 5.1 to 7.3 mg/kg (i.p.) across the group of agonists. In addition to similar ED<sub>50</sub> values, all



**Fig. 4.** a, the effects of selective opioid  $\mu$ -antagonist ( $\beta$ -FNA; 20 mg/kg, s.c.; 24-h pretreatment) on the antitussive effects of hydrocodone (12 mg/kg, i.p.; 30-min pretreatment), a selective  $\mu$ -receptor agonist; b, the effects of Nor-BNI, a selective  $\kappa$ -antagonist (20 mg/kg, s.c.; 4-h pretreatment), on the antitussive effects of BRL 52974, a selective  $\kappa$ -agonist (10 mg/kg, s.c.; 30-min pretreatment); c, the effects of a combination of  $\beta$ -FNA ( $\mu$ ) and Nor-BNI ( $\kappa$ ) on the antitussive effects of SB 227122 (20 mg/kg, i.p.; 20-min pretreatment); d, the effects of selective  $\delta$ -opioid receptor antagonist SB 244525 (10 mg/kg, i.p.; 25-min pretreatment) on the antitussive effects of SB 227122 (20 mg/kg, i.p.; 20-min pretreatment). The results are expressed as the total number of coughs induced by a 1-min 0.4 M citric acid aerosol during the 13-min observation period;  $n = 4-5$ . \* $P < .05$ , ANOVA, Fisher's PLSD, as compared with vehicle-treated animals; and † $P < .05$ , ANOVA, Fisher's PLSD, as compared with opioid agonist-treated animals.

**TABLE 2**  
Effects of rimcazole on the antitussive activity of dextromethorphan and SB 227122

Treatment	Incidence of Cough	Percentage of Inhibition	<i>n</i>
Vehicle	$16.6 \pm 0.9$		12
Rimcazole (3 mg/kg)	$16.3 \pm 1.1$	$4.4 \pm 6.5$	4
Dextromethorphan (10 mg/kg)	$12.2 \pm 2.4$	$26.8 \pm 14.4$	5
Dextromethorphan (30 mg/kg)	$5.9 \pm 1.8^*$	$64.9 \pm 10.9$	7
Rimcazole (3 mg/kg) and dextromethorphan (30 mg/kg)	$14.8 \pm 2.2^\dagger$	$12.9 \pm 12.7$	5
SB 227122 (20 mg/kg)	$4.6 \pm 1.2^*$	$72.0 \pm 7.4$	8
Rimcazole (3 mg/kg) and SB 227122 (20 mg/kg)	$6.2 \pm 3.0^*$	$63.2 \pm 18.19$	4

Results are given as means  $\pm$  S.E.

\* $P < .05$  versus vehicle group, ANOVA.

† $P < .05$  versus dextromethorphan 30 mg/kg, Fisher's PLSD test.

four opioid agonists produced similar degrees of inhibition ( $\geq 60\%$ ) at the maximum dose tested.

SB 227122 has been identified previously as a selective agonist at the  $\delta$ -opioid receptor with a  $K_i$  of 6.9 nM in a binding assay using human  $\delta$ -receptor; selectivity is indicated by the  $\mu/\delta$ - and  $\mu/\kappa$ -ratio from binding studies of 294 and  $>725$ , respectively (Petrillo et al., 1998; Table 1). This study represents the initial comprehensive description of the binding and functional activity profiles of SB 227122 and SB 244525. The results clearly indicate that SB 227122 is a potent and selective  $\delta$ -opioid receptor agonist, whereas SB 244525 is a potent and selective  $\delta$ -opioid receptor antagonist.

To determine whether the antitussive activity of SB 227122 was attributable to activity at an opioid receptor, it was tested in the presence of naloxone, a nonselective opioid receptor antagonist. Codeine was used as a standard opioid receptor agonist, whose antitussive activity has been shown previously to be inhibited by naloxone (Karlsson et al., 1990). Naloxone has a  $K_i$  of 33.8, 2.4, and 2.5 nM for the  $\delta$ -,  $\mu$ -, and  $\kappa$ -human opioid receptors, respectively (Table 1). The inhibition by naloxone of the antitussive properties of both codeine and SB 227122 strongly suggests that the latter exerts its antitussive activity via activation of opioid receptors.

Receptor subtype-selective ligands were used to further explore the mechanism of action of SB 227122.  $\beta$ -FNA has demonstrated  $\mu$ -receptor-selective antagonist activity when administered 24 h before challenge with a  $\mu$ -receptor agonist (Hayes et al., 1985).  $\beta$ -FNA alone had no effect on citric acid-induced cough, but it substantially reduced the antitussive activity of hydrocodone, a  $\mu$ -receptor selective agonist. Hydrocodone has an affinity ( $K_i$ ) for the human  $\mu$ -receptor of 36.4 nM, which is 28 and 20 times greater than its affinity for the  $\delta$ -opioid and  $\kappa$ -opioid receptors, respectively (Maguire et al., 1993). BRL 52974 has a  $K_i$  of 0.48 nM against h-KOR and  $K_i$  values of greater than 18,000 nM for both the  $\mu$ - and  $\delta$ -opioid receptors (Table 1). Nor-BNI has been identified as a  $\kappa$ -opioid-selective receptor antagonist and this was confirmed in our study, in which Nor-BNI inhibited the antitussive effects of BRL 52974, the  $\kappa$ -receptor selective agonist (Portoghese et al., 1992). Pretreatment with a combination of  $\beta$ -FNA ( $\mu$ ) and Nor-BNI ( $\kappa$ ), at doses that inhibited their corresponding selective agonists, had no effect on the antitussive activity of SB 227122. This finding suggests that the antitussive effect of the compound in this model is independent of activity at the  $\mu$ - or  $\kappa$ -opioid receptors.

SB 244525 is a selective  $\delta$ -receptor antagonist, which has a  $K_i$  of 4.8 nM for the human  $\delta$ -receptor, a  $K_i$  of 1223 nM for the human  $\mu$ -receptor, and a  $K_i$  of 810 nM for the human  $\kappa$ -receptor, and is without activity in the cAMP functional assay, suggesting that it has no  $\delta$ -agonist activity (Petrillo et al., 1998). SB 244525 significantly inhibited the antitussive effect of SB 227122. This finding provides further evidence that the inhibition of cough associated with SB 227122 is mediated through the  $\delta$ -receptor. The antagonist SB 244525 alone did not inhibit cough, which correlates with its lack of functional agonist activity at the  $\delta$ -receptor. Moreover, SB 244525 (10 mg/kg) did not inhibit the antitussive effects of either the  $\mu$ -opioid agonist hydrocodone (12 mg/kg) or the  $\kappa$ -opioid agonist BRL 52974 (10 mg/kg) (data not shown). It has previously been reported that a purported  $\delta$ -receptor antagonist, naltrindole, demonstrated dose-dependent antitussive activity in mice and rats (Kamei et al., 1993c). This action was

attributed to both direct  $\delta$ -receptor antagonism and activation of  $\kappa$ -opioid receptors (Kamei et al., 1993c). In this study, we show significant binding of naltrindole to the  $\mu$ - and  $\kappa$ -receptors (Table 1), albeit at least 20-fold less potent than to  $\delta$ -receptors; these data are consistent with the results of a previous study (Rogers et al., 1990). Antitussive activity of naltrindole was confirmed in our laboratory ( $ED_{50} = 8.2$  mg/kg, i.p.), and was found to be inhibited by pretreatment with the combination or  $\mu$ - ( $\beta$ -FNA) and  $\kappa$ - (Nor-BNI) receptor antagonists (data not shown). This suggests that the antitussive influence of naltrindole is not due to  $\delta$ -receptor antagonism per se but may be mediated via activation of  $\mu$ - and  $\kappa$ -receptors.

In addition to opioid receptor-related antitussive activity of SB 227122, we investigated the possible involvement of the  $\sigma$ -receptors. Dextromethorphan is a commonly used nonnarcotic antitussive, which has a binding  $K_i$  of 15 nM at the  $\sigma$ -receptor (Chen et al., 1991). We confirmed dose-related antitussive activity of dextromethorphan in this guinea pig model of cough (Callaway et al., 1991; Braga et al., 1994) and its reduction by treatment with rimcazole, a  $\sigma$ -receptor antagonist (Kamei et al., 1993a). Rimcazole, at a dose which significantly inhibited the antitussive activity of dextromethorphan, did not affect the activity of SB 227122. Thus, although structurally both SB 227122 and dextromethorphan are classified as morphinans (although with opposite optical sign rotation), we have excluded the participation of the  $\sigma$ -receptor in the antitussive activity of SB 227122.

The selectivities of the compounds in this study were determined in human cloned receptors, whereas the antitussive studies were conducted in guinea pigs. It is possible that species differences exist in the affinities of compounds for guinea pig and human opioid receptors. To the best of our knowledge, the guinea pig  $\delta$ - and  $\mu$ -opioid receptors have not been cloned. However, it has been determined that homology among the human, rat, and mouse  $\delta$ -opioid receptors is 93%, with 100% homology within the transmembrane regions (Quock et al., 1999). In addition, no binding discrepancy was detected in a direct comparison of human and mouse  $\delta$ -opioid receptors (Simonin et al., 1994). In binding studies with several opioid ligands in guinea pig brain tissue and human cloned receptor, we have determined that the correlation coefficients for the affinities of the compounds for the guinea pig and human receptors were 0.9861, 0.9926, and 0.9274 for  $\delta$ -,  $\mu$ -, and  $\kappa$ -receptors, respectively (data not shown). Collectively, the data would suggest that no significant species differences are likely to exist in the affinities of SB 227122 and SB 244525 for human and guinea pig opioid receptors.

$\mu$ -receptor agonists have been recognized for some time to have significant side effects including respiratory depression, constipation, and physical dependence (Zenz and Willweber-Strumpf, 1993). Undesirable side effects with  $\kappa$ -agonists include diuresis, dysphoria, and sedation (Dionne et al., 1991; Coltro and Clarke, 1995). There is some evidence that the  $\delta$ -opioid agonists may be less likely to produce some of the untoward effects associated with either the  $\mu$ - or  $\kappa$ -agonists, suggesting that members of the former class of compounds may have a greater therapeutic index as potential antitussives (Rapaka and Porreca, 1991). We have evaluated several other  $\delta$ -opioid agonists in the same structural class as SB 227122 and demonstrated antitussive activity of these com-

pounds in the guinea pig model with ED<sub>50</sub> values ranging from 5 to 15 mg/kg, i.p. (data not shown).

In summary, we have demonstrated that the selective  $\delta$ -opioid receptor agonist SB 227122 inhibits, in a dose-dependent manner, citric acid-induced cough in the guinea pig. The antitussive activity of SB 227122 is due to selective activation of the  $\delta$ -subtype opioid receptor. Members of this class of compounds may have potential as novel antitussive drugs.

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**Send reprint requests to:** Dr. David C. Underwood, Department of Pulmonary Pharmacology, UW2532, SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd., King of Prussia, PA 19406. E-mail: david\_c\_underwood@sbphrd.com