Pharmacological Characterization of the Nociceptin/Oriphanin FQ Receptor Antagonist SB-612111 \([(–)-cis-1-Methyl-7-\[4-(2,6-dichlorophenyl)piperidin-1-yl\]methyl\]-6,7,8,9-tetrahydro-5\(H\)-benzocyclohepten-5-ol]: In Vitro Studies

Barbara Spagnolo, Giacomo Carrà, Martina Fantin, Carmela Fischetti, Chris Hebbes, John McDonald, Timothy A. Barnes, Anna Rizzi, Claudio Trapella, Giulia Fanton, Michele Morari, Dave G. Lambert, Domenico Regoli, and Girolamo Calò

Department of Experimental and Clinical Medicine, Section of Pharmacology and Istituto Nazionale di Neuroscienze (B.S., G.C., M.F., A.R., M.M., D.R., G.C.) and Department of Pharmaceutical Science and Biotechnology Center (C.T., G.F.) University of Ferrara, Ferrara, Italy; and Department of Cardiovascular Sciences, University of Leicester, Leicester, United Kingdom (C.H., J.M., T.A.B., D.G.L.)

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ABSTRACT

The compound SB-612111 \([(–)-cis-1-methyl-7-\[4-(2,6-dichlorophenyl)piperidin-1-yl\]methyl\]-6,7,8,9-tetrahydro-5\(H\)-benzocyclohepten-5-ol] was recently identified as a selective antagonist for the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP). In the present study, the in vitro pharmacological profile of SB-612111 at human recombinant NOP receptors expressed in Chinese hamster ovary (CHO) cells [receptor binding, guanosine 5\(\text{'}\)-O-(3-[\(\text{35S}\)]thio)triphosphate (GTP\(\text{'}\)) binding, and cAMP level experiments] as well as at native NOP receptors expressed in peripheral (mouse and rat vas deferens, guinea pig ileum) and central (mouse cerebral cortex synaptosomes releasing \([\text{3H}]\text{cAMP}\)) preparations was evaluated and compared with that of the standard nonpeptide antagonist \((\pm)\)-J-113397 \([(\pm)\text{-trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one}]. SB-612111 produced a concentration-dependent displacement of \([\text{3H}]\text{N/OFQ}\) binding to CHO\(_{\text{NOP}}\) cell membranes, showing higher affinity and NOP selectivity over classical opioid receptors than \((\pm)\)-J-113397. SB-612111 and \((\pm)\)-J-113397 competitively antagonized the effects of N/OFQ on GTP\(\text{'}\)\([\text{35S}]\) binding in CHO\(_{\text{NOP}}\) cell membranes \(K_{\text{d}}\) values in the range of 8.20 to 8.50. In parallel experiments, \((\pm)\)-J-113397 was found to be 2- to 9-fold less potent than SB-612111. In the electrically stimulated tissues, 1 \(\mu\)M SB-612111 did not modify the effects of classical opioid receptor agonists. In conclusion, the results of the present study demonstrated that SB-612111 is among the most potent and NOP-selective nonpeptide antagonists identified to date.

Nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995; Reinscheid et al., 1995) is a neuropeptide that selectively interacts with the NOP receptor, a novel member of the opioid receptor family (Cox et al., 2000). The N/OFQ-NOP receptor system has been shown to be involved in the regulation of a variety of central and peripheral functions (Calò et al., 2000). For deeply understanding the biological roles of the N/OFQ-NOP receptor system, potent and selective antagonists, possibly of nonpeptide nature, are required. The first reported molecule displaying such features was J-113397 (Ozaki et al., 2000), which was shown to bind with nanomolar affinity to NOP receptors and to display 100- to 300-fold selectivity over classical opioid receptors (Ozaki et al., 2000).

ABBREVIATIONS: N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ peptide; \((\pm)\)-J-113397, \((\pm)\text{-trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one} \); GTP\(\text{'}\)\([\text{35S}]\), 5\(\text{'-O-(3-[\text{35S}]\text{thio})triphosphate; SB-612111, (\pm)\text{-cis-1-methyl-7-} [[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5\(H\)-benzocyclohepten-5-ol; CHO, Chinese hamster ovary; MOP, \(\mu\)-opioid peptide; DOP, \(\delta\)-opioid peptide; KOP, \(\kappa\)-opioid peptide; BSA, bovine serum albumin.
J-113397 antagonized N/OFQ effects at human NOP in a competitive manner with $\mu_A$ values in the range of 7.50 to 8.90 in cAMP and guanosine 5'-O-(3-[35S]thiophosphoryl) (GTP[y35S]) assays. The selective antagonist properties of J-113397 were confirmed at native NOP receptors expressed in isolated tissues and in brain preparations evaluated with biochemical, neurochemical, and electrophysiological techniques (Calo et al., 2002). J-113397 actions were also investigated in vivo against a variety of N/OFQ effects, confirming its NOP antagonist features (Calo et al., 2002). Recently, Zarrutin et al. (2004) synthesized a new molecule, SB-612111, as a member of a novel class of nonpeptide antagonists structurally unrelated to the known nonpeptide NOP ligands (Zarrutin et al., 2004). In competition radioligand binding experiments, SB-612111 displayed subnanomolar affinity for the NOP receptor and high selectivity over classical opioid receptors. Functional studies in HEK293 cells expressing the human NOP receptor demonstrated pure, competitive, and high-potency antagonism exerted by this molecule against N/OFQ (Zarrutin et al., 2004). Finally, these findings were confirmed in vivo in the mouse, where SB-612111 antagonized the pronociceptive action of N/OFQ given supraspinally and potentiated the action of morphine in animals made tolerant to morphine (Zarrutin et al., 2004). In the present study, we further investigated the in vitro pharmacological profile of SB-612111 and made a systematic comparison with the standard nonpeptide NOP antagonist J-113397.

Materials and Methods

Cell Culture and Membrane Preparation. For these studies, CHO cells stably expressing individual human NOP, MOP, DOP, or KOP receptors were used. These cells typically express 0.80, 3.05, and 0.84 pmol of receptor/mg protein, respectively (Kitayama et al., 2003).

CHO_hNOP cells were maintained in Dulbecco’s modified Eagle’s medium/Nutrient F12 (50:50) supplemented with 5% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml), and fungizone (2.5 $\mu$g/ml). Stock cultures were further supplemented with genetin (G418, 200 $\mu$g/ml) and hygromycin B (200 $\mu$g/ml) as described previously (McDonald et al., 2003). CHO_hMOP, CHO_hDOP, and CHO_hKOP cells were maintained in Nutrient F12 supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 $\mu$g/ml), and fungizone (2.5 $\mu$g/ml). Stock cultures were further supplemented with G418 (200 $\mu$g/ml). Cells were cultured at 37°C in 5% carbon dioxide humidified air, subcultured as required, and used when confluent. For binding experiments, membranes were prepared from freshly harvested cell suspensions in Tris (50 mM) and MgCl2 (5 mM), pH 7.4 for hNOP and Tris (50 mM), pH 7.4 for hMOP, hDOP, and hKOP. For GTP[y35S] assays, membranes were prepared from freshly harvested cell suspensions in Tris (50 mM) and EGTA (0.2 mM) pH 7.4. All tissues were homogenized, then centrifuged at 10,000 rpm for 30 min. The supernatant was assayed according to Lowry et al. (1951).

[Leucyl-$\text{[3H]}$]N/OFQ Binding. Fifteen micrograms of CHO_hNOP homogenate protein was incubated in 0.5-ml volumes of buffer supplemented with 10 $\mu$M peptidease inhibitors (amastatin, bestatin, captorplir, and phosphoramidon), 0.5% bovine serum albumin (BSA), increasing concentrations of SB-612111 and (±)-J-113397, and approximately 200 pM [Leucyl-$\text{[3H]}$]N/OFQ. Total radiolabel bound was much less than 10%. Nonspecific binding was determined in the presence of 1 $\mu$M unlabeled N/OFQ. In some experiments, N/OFQ was included as a reference. Reactions were incubated for 1 h at room temperature and terminated by vacuum filtration (Brandel Harvester; Brandel Inc., Gaithersburg, MD) through Whatman GF/B filters (Whatman, Clifton, NJ) soaked in 0.5% polyethyleneimine. Bound radioactivity was determined after 8-h extraction in Optiphase Safe (Wallac, Loughborough, UK) using liquid scintillation spectroscopy (McDonald et al., 2003).

$\text{[3H]}$Diprenorphine Binding. Thirty micrograms of CHO_hMOP/hDOP/hKOP membrane protein was incubated in 0.5-ml volumes of buffer supplemented with peptidease inhibitors (as above), 0.5% BSA, increasing concentrations of SB-612111 and (±)-J-113397, and 1 nM $\text{[3H]}$DPN. Total radiolabel bound was much less than 10%. Nonspecific binding was determined in the presence of 10 $\mu$M naloxone. In some experiments, MOP/DOP/KOP-selective reference compounds were included. Reactions were incubated for 1 h at room temperature and terminated by vacuum filtration (Brandel Harvester) through Whatman GF/B filters soaked in 0.5% polyethyleneimine (McDonald et al., 2003). Bound radioactivity was determined as above.

GTP[y35S] Binding. Twenty micrograms of CHO_hNOP membranes was incubated in 0.5 ml of buffer containing Tris (50 mM), EGTA (0.2 mM), MgCl2 (1 mM), NaCl (100 mM), bacitracin (0.15 mM), peptidease inhibitors (as above), GDP (100 $\mu$M), and approximately 150 pM GTP[y35S] (McDonald et al., 2003). N/OFQ, SB-612111, and (±)-J-113397 were included in different combinations and various concentrations. SB-612111 and (±)-J-113397 were preincubated for 15 min at 30°C. Nonspecific binding was obtained in the presence of unlabeled 10 $\mu$M GTPyS. The reaction was incubated for 1 h at 30°C with gentle shaking and terminated by filtration through Whatman GF/B filters using a Brandel Harvester.

cAMP Accumulation Experiments. Whole cells were suspended in Krebs/HEPES buffer containing isobutylmethylxanthine (1 mM) and forskolin (1 $\mu$M) as described by Kitayama et al. (2003). N/OFQ, SB-612111, and (±)-J-113397 were preincubated for 15 min at 37°C. cAMP was extracted and assayed using a specific protein binding assay (Brown et al., 1971).

Electrically Stimulated Isolated Tissues. Tissues were taken from male Swiss mice (30–35 g), albino guinea pigs (300–350 g), and Sprague-Dawley rats (300–350 g). The mouse vas deferens, the guinea pig ileum, and the rat vas deferens were prepared as described previously (Bigoni et al., 1999). These were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1-ms duration and 0.05-Hz frequency. The electrically evoked contractions (twitches) were measured isotoniaically with a strain gauge transducer (Basile 7006; UgoBasile s.r.l., Varese, Italy) and recorded with the personal computer-based acquisition system Power Lab (ADInstruments Pty Ltd., Castle Hill, Australia). After an equilibration period of approximately 60 min, the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration-response curves to N/OFQ were performed (0.5-log unit step) in the absence or in the presence of increasing concentrations of SB-612111. In the same series of experiments, the effects of SB-612111 were compared with that of (±)-J-113397 (0.1 $\mu$M) with 15-min preincubation time. In a separate series of experiments performed in the mouse vas deferens, concentration-response curves to N/OFQ were performed 1, 2, or 3 h after washing out SB-612111 (100 nM) or (±)-J-113397 (300 nM). The selectivity of action of SB-612111 was assessed by challenging the compound at 1 $\mu$M versus opioid receptor agonists such as deltorphin (DOP selective) in the mouse vas deferens (Hughes et al., 1975), dermorphin (MOP selective) in the guinea pig ileum (Paton, 1957), and etorphine (nonselective opioid agonist) in the rat vas deferens (Schulz et al., 1979).

Mouse Cerebral Cortex Synaptosomes. Male Swiss mice (25–30 g) (Morini, Reggio Emilia, Italy) were used for these studies. In the morning of the experiment, mice were decapitated under light ether anesthesia, and the frontoparietal cortex was isolated. Synaptosomes were prepared as described previously (Shrenna et al., 2000). Synaptosomes were preloaded with [3H]HT for incubation for 25 min in medium containing 50 nM [3H]HT (specific activity, 27.8 Ci/mmol; NEN DuPont, Boston, MA). Sample collection (every 3
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Fig. 1. Effects of N/OFQ, SB-612111, and (±)-J-113397 on [3H]N/OFQ binding to membranes prepared from CHO<sub>NOP</sub> cells. Points represent means, and vertical lines represent S.E.M. of three separate experiments.

min) was initiated after a 20-min period of filter washout. K<sup>+</sup> stimulation (1-min pulse) was applied at the 38th min (Mela et al., 2004). N/OFQ was added to the superfusion medium 9 min before the K<sup>+</sup> pulse and maintained until the end of the experiment. SB-612111 (10 nM) and (±)-J-113397 (100 nM) were added 3 min before N/OFQ. Radioactivity in the superfusate samples and filters (dissolved with 1 ml of 1 M NaOH followed by 1 ml of 1 M HCl) was determined by liquid scintillation spectrophotometry using a Beckman LS 1800 β-spectrometer (Beckman Coulter, Fullerton, CA) and Ultima Gold XR scintillation fluid (Packard Instruments B.V., Groningen, The Netherlands). Spontaneous 5-HT release from synaptosomes was calculated by subtracting the estimated spontaneous efflux (obtained by interpolation between the samples preceding and following the stimulation) from the total efflux observed in the stimulated sample. The ability of SB-612111 to bind to NOP as well as classical opioid receptors has been evaluated using membranes of CHO cells expressing the human recombinant proteins. The binding profile of SB-612111 has been compared with that of the NOP antagonist (±)-J-113397. Both SB-612111 and (±)-J-113397 produced a concentration-dependent displacement of [3H]N/OFQ binding to membranes prepared from CHO<sub>NOP</sub> cells (Fig. 1), with p<sub>KD</sub> values for (±)-J-113397 being approximately 6-fold higher affinity than (±)-J-113397 and only approximately 3-fold lower affinities than the NOP endogenous ligand N/OFQ. At all classical opioid receptors (MOP/DOP/KOP), SB-612111 was essentially inactive up to 1 μM, showing high NOP selectivity over classical opioid receptors. (±)-J-113397 displayed activity at MOP, and based on an assumed maximal displacement of 100%, selectivity over NOP was crudely estimated at around 1 order of magnitude. The results of this series of experiments are summarized in Table 1.

GTPγ<sup>[35S]</sup> Binding. In CHO<sub>NOP</sub> membranes, N/OFQ stimulated the GTPγ<sup>[35S]</sup> binding in a concentration-depen-

**TABLE 1**

Receptor binding profile of SB-612111 and (±)-J-113397 to human recombinant NOP, MOP, DOP, and KOP receptors expressed in CHO cells

<table>
<thead>
<tr>
<th></th>
<th>CHO&lt;sub&gt;NOP&lt;/sub&gt;</th>
<th>CHO&lt;sub&gt;MOP&lt;/sub&gt;</th>
<th>CHO&lt;sub&gt;DOP&lt;/sub&gt;</th>
<th>CHO&lt;sub&gt;KOP&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB-612111</td>
<td>9.18 ± 0.08</td>
<td>&lt;6.0</td>
<td>&lt;5.3</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>(±)-J-113397</td>
<td>8.42 ± 0.06</td>
<td>7.48 ± 0.03</td>
<td>&lt;5.3</td>
<td>&lt;6.0</td>
</tr>
</tbody>
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Drugs. The peptides used in this study were prepared and purified as described previously (Guerrini et al., 1997). J-113397 was prepared as a racemic mixture, according to De Risi et al. (2001); to underlie this, the compound is referred to as (±)-J-113397. The compound SB-612111 was synthesized following the experimental conditions and protocols described in details in the patent (WO 03/040099 A1) by Palombi and Ronzoni (2003). Captorplir, amastatin, bestatin, naloxone, cAMP, 3-isobutyl-1-methyloxanthine, HEPEIS, Tris, BSA, EGTA, GTPγS, and forskolin were from Sigma (Poole, UK), and phosphoramidon was from the Peptide Institute (Osaka, Japan). All tissue culture media and supplements were from Gibco (Paisley, UK). [2,3,8-3H]cAMP (28.4 Ci/mmol), GTPγ<sup>35S</sup> (1250 Ci/mmol), and [leucyl-3,4,5-3H]-N/OFQ ([3H]N/OFQ, 75–133 Ci/mmol) were from NEN DuPont. GDP was obtained from Sigma (Deisenhofen, Germany). Bacitracin, obtained from Merck (Darmstadt, Germany), was heated for 1 h at 70°C in water to inactivate any enzymatic activity before use. SB-612111 was solubilized in dimethyl sulfoxide at the final concentration of 10 mM, and the successive dilutions were performed in saline, whereas the other compounds were solubilized in saline solution.

Data Analysis and Terminology. The pharmacological terminology adopted in this article is consistent with the International Union of Basic and Clinical Pharmacology recommendations (Neubig et al., 2003). All data are expressed as means ± S.E.M. of n experiments. In competition binding studies, the log concentration of competitor producing 50% inhibition of specific binding (pIC<sub>50</sub>) was corrected for the competing mass of radiolabel according to Cheng and Prusoff (1973) to yield p<sub>Ki</sub> values. p<sub>D</sub> values for [3H]N/OFQ in CHONOP and [3H]DPN in CHONOP and CHOSOP and CHOKOP were 0.091, 0.182, 0.313, and 0.137 nM, respectively (in-house laboratory values). In GTPγ<sup>[35S]</sup> assays, agonist-stimulated binding is expressed as stimulation factor using the following formula: stimulation factor = (agonist stimulated binding – nonspecific binding)/(basal binding – nonspecific binding). cAMP data are presented as an inhibition of forskolin-stimulated cAMP formation. pIC<sub>50</sub>, pEC<sub>50</sub>, and E<sub>max</sub> values are obtained by computer-assisted curve fitting of individual curves using Prism version 5.0 (GraphPad Software Inc., San Diego, CA). For in vitro studies on electrically stimulated tissues, the potency of SB-612111 was evaluated by Schild analysis, whereas p<sub>EC50</sub> values for (±)-J-113397 were calculated using the Gaddum Schild equation, p<sub>EC50</sub> = −log(CR − 1)/(agonist) + 1, assuming a slope equal to unity, where C<sub>R</sub> is the ratio of the EC<sub>50</sub> of the agonist in the presence and in the absence of the antagonist. Data have been analyzed statistically using the Student’s t test for unpaired data or the one-way analysis of variance followed by the Dunnett’s test, as specified in table and figure legends; p values less than 0.05 were considered significant.

Results

Receptor Binding. The ability of SB-612111 to bind to NOP as well as classical opioid receptors has been evaluated using membranes of CHO cells expressing the human recombinant proteins. The binding profile of SB-612111 has been compared with that of the NOP antagonist (±)-J-113397. Both SB-612111 and (±)-J-113397 produced a concentration-dependent displacement of [3H]N/OFQ binding to membranes prepared from CHO<sub>NOP</sub> cells (Fig. 1), with p<sub>Ki</sub> values of 9.18 ± 0.08 and 8.42 ± 0.06, respectively. SB-612111 displayed approximately 6-fold higher affinity than (±)-J-113397 and only approximately 3-fold lower affinities than the NOP endogenous ligand N/OFQ. At all classical opioid receptors (MOP/DOP/KOP), SB-612111 was essentially inactive up to 1 μM, showing high NOP selectivity over classical opioid receptors. (±)-J-113397 displayed activity at MOP, and based on an assumed maximal displacement of 100%, selectivity over NOP was crudely estimated at around 1 order of magnitude. The results of this series of experiments are summarized in Table 1.

GTPγ<sup>[35S]</sup> Binding. In CHO<sub>NOP</sub> membranes, N/OFQ stimulated the GTPγ<sup>[35S]</sup> binding in a concentration-depen-

Data are mean ± S.E.M. of three separate experiments.
dent manner, with pEC₅₀ and Eₘₐₓ of 9.20 ± 0.11 and 8.69 ± 0.21, respectively (Fig. 2). Both SB-612111 and (±)-J-113397 at 300 nM preincubated for 15 min were inactive per se but produced a parallel rightward shift of the concentration-response curve to N/OFQ, yielding pKᵦ values of 9.70 and 8.71, respectively (Fig. 2; Table 2). Thus, SB-612111 was approximately 10-fold more potent than (±)-J-113397 in this assay.

cAMP Accumulation. We also investigated the effects of SB-612111 and (±)-J-113397 on forskolin-stimulated cAMP formation in whole CHOₜNOP cells. N/OFQ produced a concentration-dependent inhibition of forskolin-stimulated cAMP formation, with mean pEC₅₀ and Eₘₐₓ values of 10.32 ± 0.14 and 100 ± 2%, respectively (Fig. 3). Both SB-612111 and (±)-J-113397 at 300 nM preincubated for 15 min were inactive per se but produced a parallel rightward shift of the concentration-response curve to N/OFQ, yielding pKᵦ values of 8.63 and 7.95, respectively. SB-612111 was approximately 5-fold more potent than (±)-J-113397 in this assay.

Electrically Stimulated Isolated Tissues. SB-612111 was assessed against N/OFQ in the electrically stimulated mouse and rat vas deferens and guinea pig ileum. At 1 μM (but not at lower concentrations), SB-612111 induced per se inhibitory effects in some mouse vas deferens (−44 ± 4% in 17 of 25 tissues) and guinea pig ileum (−46 ± 4% in 9 of 14) tissues while being completely inactive in the rat vas deferens. In the 10 to 1000 nM range, SB-612111 produced a concentration-dependent, parallel rightward shift of the concentration-response curve to N/OFQ, without significantly affecting the maximal agonist response. Schild analysis was compatible with competitive antagonism in all the preparations (slope values not significantly different from unity), and the calculated pA₂ values were in the range 8.20 to 8.50 (Fig. 4; Table 2). In parallel experiments (±)-J-113397 was found to be 2- to 9-fold less potent than SB-612111 (Table 2).

In a separate series of experiments performed in the electrically stimulated mouse vas deferens, the reversibility of SB-612111 (100 nM) and (±)-J-113397 (300 nM) action was evaluated. The antagonist effect of (±)-J-113397 can be easily, although not completely, reversed by washing the tissues for 1 h (Fig. 5, left). On the contrary, the antagonist effect exerted by SB-612111 could not be reversed even after 3 h of washing; as a matter of fact, the concentration-response curves to N/OFQ obtained in the presence of SB-612111 and after 3 h of washing were superimposable (Fig. 5, right).

Finally, the selectivity of action of SB-612111 was investigated in isolated tissues by challenging the compound against opioid receptor agonists. One micromolar SB-612111 did not affect the concentration-response curve to the DOP-selective agonist deltorphin I in the mouse vas deferens (con-
trol, pEC\(_{50}\) (95% confidence limit), 12.64 (12.45–12.83); \(E_{\text{max}}\), 82 ± 2%; 1 μM SB-612111, pEC\(_{50}\) 12.73 (12.56–12.90); \(E_{\text{max}}\) 83 ± 3%), to the MOP-selective agonist dermorphin in the guinea pig ileum [control, pEC\(_{50}\) 9.63 (9.43–9.83); \(E_{\text{max}}\) 84 ± 2%; 1 μM SB-612111, pEC\(_{50}\) 9.87 (9.47–10.27); \(E_{\text{max}}\) 79 ± 2%], and to the universal opioid receptor agonist etorphine in the rat vas deferens [control pEC\(_{50}\) 7.77 (7.56–7.98); \(E_{\text{max}}\) 84 ± 4%; 1 μM SB-612111, pEC\(_{50}\) 7.92 (7.74–8.40); \(E_{\text{max}}\) 83 ± 3%].

**Mouse Cortical Synaptosomes.** According to Mela et al. (2004), a 1-min pulse of 10 mM KCl evoked a robust [\(^3\)H]5-HT overflow, amounting to 2.0 ± 0.4% of the tritium synaptosome content, that was inhibited in a concentration-dependent manner by N/OFQ. Analysis of the N/OFQ concentration-response curve yielded a pEC\(_{50}\) value of 7.85 and an \(E_{\text{max}}\) of 69 ± 5% of control values. Both SB-612111 (10 nM) and (\(±\))J-113397 (100 nM) did not modify K\(^+\)-stimulated [\(^3\)H]5-HT overflow per se but antagonized N/OFQ inhibitory effects without modifying the maximal effects elicited by the agonist (Fig. 6). pK\(_{B}\) values of 8.45 and 8.20 were derived from these experiments for SB-612111 and (\(±\))J-113397, respectively. Table 2 summarizes the values of antagonist po-

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**Fig. 4.** Electrically stimulated mouse vas deferens (top), guinea pig ileum (middle), and rat vas deferens (bottom). Left, concentration-response curve to N/OFQ obtained in the absence and in the presence of increasing concentrations of SB-612111 (10–1000 nM); the corresponding Schild plot is shown on the right. Points represent means, and vertical lines represent S.E.M. of six experiments.
113397 displaced [3H]N/OFQ in a concentration-dependent four experiments. Points represent means, and vertical lines represent S.E.M. of 113397. In CHOhNOP cell membranes, SB-612111 and (●) and selectivity than the nonpeptide antagonist (○) binant NOP receptors displaying higher potency (by 2–10-fold) and selectivity (by 2004) demonstrating that SB-612111 behaves as a pure, competitive, and selective antagonist at both native and recombinant peripheral receptors (Zaratin et al., 2004). However, based on the 300-fold difference between the concentration active at NOP receptor (~3 nM) and that needed to exert inhibitory effects in the electrically stimulated tissues (approximately 1000 nM), it is unlikely that interaction with adrenergic receptors will confound the use of SB-612111. Thus, interactions between SB-612111 and adrenergic receptors were not further investigated.

The competitive nature of SB-612111-NOP receptor interaction was demonstrated by classical Schild analysis performed in isolated tissues. Very similar values of potency were obtained for SB-612111 in all these preparations (range, 8.20–8.63), with only the exception of the GTPγ[S] binding assay, where a statistically significant higher value of potency was calculated (pK_B, 9.7). Higher potency in this particular assay compared with the other tests was not a peculiar feature of SB-612111 since we obtained similar results with (±)J-113397 (pK_B value in the GTPγ[S] binding assay, 8.71; range, 7.53–8.15 in the other tests) and with NOP-selective peptide antagonists such as [Nphe1]N/OFQ(1–13)[NH2] (pK_B value in the GTPγ[S] binding assay, 7.0; range, 6.0–6.4 in the other tests) (Caló et al., 2002) and UFP-101 (pK_B value in the GTPγ[S] binding assay, 9.1; range, 7.1–7.7 in the other tests) (Caló et al., 2005). This might be due to the higher receptor accessibility in membranes (where the GTPγ[S] binding assay is performed) than in whole cells or tissue preparations (where the other...
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Address correspondence to: Dr. Girolamo Calo, Department of Experimental and Clinical Medicine, Section of Pharmacology, via Fossato di Mortara 19, 44100 Ferrara, Italy. E-mail: g.calo@unife.it

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