It is well known that postjunctional β-adrenoceptors (β-ARs) mediate the inhibitory effects of sympathetic nerve stimulation in different smooth muscles including those of the gastrointestinal tract (Manara et al., 1995b; Gauthier et al., 2000). The intestinal β-AR was originally described as a β1- and β2-AR (Lands et al., 1967). Further studies with gastrointestinal preparations from several species established the relaxed effect of classical β-AR (β1 and β2) agonists (Bennett, 1965; Hedges and Turner, 1969; De Ponti et al., 1996a). Subsequently, studies investigating β-ARs in gastrointestinal smooth muscle from several species demonstrated relaxation responses that were resistant to propranolol and displayed lower affinity to other conventional β-AR antagonists (Arch and Kaumann, 1993; Goldberg and Frishman, 1995; Strosberg, 1997; Manara et al., 2000). This finding, along with the emergence of a new class of β-AR agonists described first in adipocytes (Feve et al., 1991), suggested the presence of an “atypical” class of β-ARs. In 1989, Emorine et al. (1989) cloned and sequenced β3-AR and found that it shared the pharmacological characteristics of the atypical β-AR.

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Recent studies have demonstrated the selective, potent, and prolonged relaxant effect of CL 316,243, a selective β3-AR agonist, on the sphincteric smooth muscles of the opossum lower esophageal sphincter (DiMarino et al., 2002), without the significant systemic cardiovascular side effects that are associated with β1- and β2-AR agonists.

In the past few years, β3-agonists have emerged as potential therapeutic agents for several gastrointestinal motility disorders including irritable bowel syndrome (Scarpignato and Pelosini, 1999). Anorectal dysfunctions such as Hirschprung’s disease, constipation, anal fissures, and hemorrhoids may also be associated with either hypertensive IAS or failure of sphincteric relaxation in response to the recto-anal inhibitory reflex (Azpiroz and Whitehead, 2002). Characterization of neurohumoral receptors that mediate selective, potent, and prolonged relaxation of IAS and other GI smooth muscles without untoward systemic effects will be of considerable interest in the treatment of anorectal and other GI motility disorders.

The present investigation was carried out to characterize β-AR in the gastrointestinal tonic smooth muscle of the IAS by comprehensive studies using a combination of classical pharmacology, receptor binding, and molecular biology approaches.

The aim of the present study is to determine the presence of and characterize the β-AR subtypes involved in mediating relaxation of the IAS smooth muscle. We used selective agonists and antagonists to determine the receptor binding profiles of each β-AR subtype. The presence of membrane bound β-AR and mRNA encoding for the three β-AR subtypes was determined through Western blot studies and reverse transcription-polymerase chain reaction (RT-PCR) analysis, respectively.

Materials and Methods

Preparation of Smooth Muscle Strips. Adult male opossums (Didelphis virginiana) weighing 2.5 to 3.5 kg were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Laparotomy was performed, and a part of the rectum along with the anal canal was removed using sharp dissection. The IAS was identified by manometry as high-pressure zone and marked by means of sutures in situ. The animals were sacrificed by exsanguinations; the anorectal region was then dissected out and transferred to 2-ml muscle baths (Radnoti Glass Technology, Monrovia, CA) containing oxygenated Krebs’ solution every 15 min. Only smooth muscle strips that developed spontaneous tone and responded to electrical field stimulation were used in this study. The changes in tension from various drugs were expressed as the percent maximal relaxation achieved by 50 mM EGTA, at the end of each experiment. Each smooth muscle served as its own control.

Drug Responses. To determine the concentration-response curves (CRCs) with β1-, β2-, and β3-AR agonists on the basal tone of the IAS smooth muscles, xamoterol, procaterol, and ZD 7114 [5S]-[2-hydroxy-3-phenoxypropylaminoethoxy]-N-(2-methoxyethyl)phenoxyacetamide], respectively, were added to the muscle bath in cumulative concentrations (Rattan and Moumni, 1989). Successive concentrations of the agonists were not added until the response of the previous concentration stabilized. When no effect was observed, 10 minutes were allowed between additions of different concentrations. In preliminary studies, when a single concentration was used, we noted that this was an appropriate time to gauge the maximal effect of a given concentration of the agonist. No difference in the results occurred with longer exposures.

To determine the effects of β1-, β2-, and β3-AR antagonists, CGP 20712A [1,2,3,4-tetrahydro-1-naphthalenyl[amino]- (2-methoxyethyl)phenoxy]-1H-imidazol-2-ylphenoxy][propyl]amino[ethoxy]-benzamide methanesulfonate salt], ICI 118551, and SR 59230A [1-(2-ethylphenoxy)-3-[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino][(2S)-2-propanol hydrochloride], respectively (in concentrations ranging from 1 × 10^-6 to 1 × 10^-6 M), were added 30 min before obtaining the CRC of the test agonist.

Adrenoceptor (β-AR) Analysis by Western Blot. Western blot analysis of β1-, β2-, and β3-AR in the IAS and rectum of the opossums was performed according to the protocol of Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Circular smooth muscle tissues of the IAS and rectum were cut into small pieces (2 × 2 mm cubes) and rapidly homogenized in 3 ml of boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris, pH 7.4) and then put into the microwave for 10 s. The homogenates were centrifuged (16,000g, 4°C) for 15 min. The pellet obtained was dissolved in Krebs’ buffer (composition already described) containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (combined pH of 7.6). The protein contents were determined by the method described by Lowry et al. (1951) using bovine serum albumin as the standard.

All of the samples were mixed with 2X sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% β-mercaptoethanol) and boiled for 4 min. A total of 20 µl (40 µg total protein) of each sample was applied to commercially available 7.5% SDS polycrylamide gel PAGEEr Gold Gel (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) applied to a 7.5% SDS-polyacrylamide gel apparatus by the method of Laemmli (1970), using 150 V for 1 h. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (NCM) at 4°C for 90 min at 100 V. To block nonspecific antibody binding, the NCMs were immersed overnight at 4°C in Super Block Tris-buffered saline/Tween blocking removed. Circular smooth muscle strips of the IAS (approximately 1 × 10 mm) were prepared and tied on either end using 3-0 silk suture in preparation for measurement of isometric tension.

The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Measurement of Isometric Tension. The smooth muscle strips were transferred to 2-ml muscle baths (Radnoti Glass Technology, Monrovia, CA) containing oxygenated Krebs’ solution at 35°C. One end of the muscle strip was anchored at the bottom of the muscle bath while the other end was connected to a force transducer (model PTO3; Grass Instruments, Quincy, MA). Isometric tension was recorded by the PowerLab/8SP data acquisition system using Chart 4.1.2 (ADInstruments, Grand Junction, CO). Each smooth muscle strip was initially stretched to a tension of 0.7 g. The muscle strips were then given at least an hour to equilibrate, during which time they were washed with Krebs’ solution every 15 min. Only smooth muscle strips that developed spontaneous tone and responded to electrical field stimulation were used in this study. The changes in tension from various drugs were expressed as the percent maximal relaxation achieved by 50 mM EGTA, at the end of each experiment.
buffer (Pierce Biotechnology, Rockford, IL). The NCM was divided into three smaller sections labeled as β1, β2, and β3. The NCMs were then incubated with the respective diluted isoform specific primary (1°) antibodies corresponding to the specific β-AR subtype. The NCMs were incubated with rabbit β1, β2, and goat β3 polyclonal antibodies, respectively (Santa Cruz Biotechnology Inc.) at a dilution of 1:500. All membranes were incubated with 1° antibody for 1 h at room temperature. The membranes were then washed with Tris-buffered saline/Tween three times. Afterward, the membranes corresponding to β1 and β2 were incubated separately in 1:1000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences Inc., Piscataway, NJ) in 2° antibody buffer for 1 h at room temperature. The remaining membrane was incubated in 1:5000 diluted horseradish peroxidase-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology Inc.) in 2° antibody buffer. The bands were identified by chemiluminescence using the ECL detection system and Hyperfilm MP (Amersham Biosciences). Densitometric analysis of the bands was performed using Image Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Membrane Preparation for Receptor Binding Studies. The circular smooth muscle of the IAS was dissected free by the aforementioned procedure and placed immediately in ice-cold Krebs' buffer (composition already described above) containing 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (combined pH of 7.6). The IAS was minced with scissors and homogenized in 5 volumes of ice-cold TED buffer (20 mM Tris Cl, 1 mM EDTA, 1 mM dithiothreitol, pH 8) by the use of a Tekmar Tissuemizer (Tekmar-Doehrmann, Mason, OH) for 15 s. The homogenates were centrifuged at 100,000g for 1 h at 4°C. The supernatant was filtered through a 500-mesh nitex mesh. The membranes were resuspended in cold Krebs' buffer (pH 7.6) and stored at -80°C until used. Protein content was determined by the method of Lowry et al. (1951).

Radioligand Binding Studies. The radioligand (1-3-[125I]iodocyanopindolol ([125I]CYP; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) was used for identifying β-AR. For equilibrium determination, membranes at a protein concentration of 40 μg per tube were incubated with [125I]CYP (specific activity 2000 Ci/mmol) for 0, 15, 30, 45, 60, 90, 120, and 180 min. The experiments were carried out in the presence or absence of 100 μM propranolol (a nonselective β-AR antagonist). The incubation mixture was composed of 50 mM Tris HCl buffer, pH 7.4, containing 10 mM MgCl2, and 1 mM EDTA in a final volume of 250 μl. A time course using the above-mentioned time points was carried out in duplicate at 35°C to determine the optimal time needed for equilibrium. The incubation was terminated by rapid filtration through Whatman GF/C glass-fiber filters (24-mm circles) (Whatman, Clifton, NJ) using a 1225 sampling manifold (Millipore Corp., Bedford, MA), followed by washing three times with 5 ml of ice-cold 25 mM Tris HCl buffer, pH 7.4. The filters were counted in the Auto-Gamma Counting System (model 5550; PerkinElmer Life Sciences, Boston, MA) at an efficiency of 80%. Specific binding was calculated by subtracting nonspecific binding from total binding.

For saturation assays, membranes were incubated at 37°C for 120 min with increasing concentrations of [125I]CYP (5–3000 pM). All values in binding experiments are the average of duplicates. Specific binding was defined as binding inhibited by 100 μM propranolol. The equilibrium dissociation constant (KD) and the maximum binding capacity (Bmax) were determined by nonlinear regression analysis by GraphPad Prism software (GraphPad Software Inc., San Diego, CA). KD is the concentration of the radioligand required to occupy 50% of the binding sites. Bmax is defined as the maximal specific binding obtained with increasing concentration of radioligand, and it is a measure of receptor density in the tissue under investigation. Displacement experiments were performed with varying amounts of [125I]CYP, depending on the appropriate KD of the high- and low-affinity sites of the IAS. In the IAS, 66 pM and 1.61 nM [125I]CYP were used in the high- and low-affinity sites, respectively. The KD values were calculated by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as: KD = IC50/(1 + L/KD), where IC50 represents the concentration of competitor causing 50% inhibition and L signifies the concentration of radioligand.

**Isolation and Quantification of Total RNA.** Tissue specimens from the circular smooth muscle of the IAS were carefully dissected and homogenized as described under Membrane Preparation for Receptor Binding Studies. Total RNA was extracted from the tissue homogenate using the TRI reagent (Molecular Research Center, Cincinnati, OH) protocol based on the method of Chomczynski and Sacchi (1987). RNA samples were then dissolved in diethylpyrocarbonate (DEPC)-treated water (pH 7.5). The optical density (OD) of each sample was determined by a UV-visible spectrophotometer (Amersham Biosciences) at a wavelength of 260 nm (λ260). The yield and quality of the RNA were assessed by measuring the OD λ260/OD λ280 ratio.

**Preparation and Amplification of cDNA Encoding β1-, β2-, and β3-ARs (RT-PCR Analysis).** RNA samples of 2 μl (1 μg) that were of acceptable quality were used as templates for the synthesis of cDNA. Primers for β1-, β2-, and β3-AR, and β-actin (internal standard), based on the previous report (Dincer, 2002), were synthesized by Thomas Jefferson University facilities (Kimmel Cancer Institute, Nucleic Acid Facility). The sequence and accession numbers listed in Table 1 are based on published sequences in the National Center for Biotechnology Information GenBank database (http://www3.ncbi.nlm.nih.gov/entrez). cDNAs were synthesized by reverse transcription of 1.0 μg of each total RNA. The reaction mixture consisted of 10× reverse transcription buffer, deoxyadenosyl-5′-triphosphates (20 mM), MgCl2 (25 mM), 18 U of RNasin ribonuclease inhibitor, and 20 U of AMV reverse transcriptase in a final volume of 20 μl. The contents of reaction mixture were purchased from Promega (Madison, WI), and a reverse transcription mixture was incubated at 42°C for 45 min and then at 95°C for 5 min. PCR amplification was done on segments of cDNA encoding each of the three subtypes of β-AR using gene-specific primers as a way of determining the amount of transcripts present. The PCR reaction mixture was added directly to RT tubes and consisted of 10× reaction buffer, 25 mM MgCl2, 3.5 μM of recombinant TaqDNA polymerase (Takara Shuzo Co., Shiga, Japan), and 20 μM concentrations of the respective sense and antisense primers. DEPC water was added for a final volume of 50 μl. PCR amplification was carried out in a Mark cycle gradient thermal sequencer (Eppendorf, Inc., Westbury, NY). After initial heating of samples at 95°C, each cycle of amplification consisted of 45 s at 94°C, followed by 45 s at 60°C, and 2 min of extension at 72°C; this sequence was repeated for a total of 38

<table>
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<th>Primer</th>
<th>Strand</th>
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<th>Location</th>
<th>Accession Number</th>
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<td>3203–3222</td>
<td>VO-1217-J0691</td>
</tr>
</tbody>
</table>

**TABLE 1**

Primer used in RT-PCR for amplification of mRNA encoding β-AR and β-actin in IAS smooth muscle.
cycles. At the end of the reactions, 15 μl of samples was mixed with 5 μl of 6× green/purple loading dye. The samples were loaded onto a 2% agarose gel containing ethidium bromide and electrophoresed for approximately 1 h at 100 V. The gels were visualized with an ultraviolet transilluminator (312-nm variable intensity; Fisher Scientific, Pittsburgh, PA) and photographed using a UV gel electrophoresis camera (Polaroid GH 10; Polaroid, Hertfordshire, UK). Densitometric analysis of the gel bands was carried out using Kodak Image Analysis software (Eastman Kodak, Rochester, NY).

**Drugs and Chemicals.** SR 59230A hydrochloride, propranolol hydrochloride ([±]-1-isopropylamino-3-(1-naphthoxy)-2-propanol hydrochloride), CGP 20712A (methanesulfonate salt), dimethyl sulfoxide (DMSO), and EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Xamoterol hemifumarate [1-(4-hydroxyphenoxy)-3-[2-(4-morpholino-carboxamido)ethylamino]-2-propanol], ICI 118,551 hydrochloride ([±]-1-[2,3-dihydro-7-methyl-1H-inden-4-yl]oxy)-3-[1-methylthethyl]-amino)-2-butanol), procaterol hydrochloride ([±]-erythro-8-hydroxy-5-[1-hydroxy-2-isopropylamino]butylcarbostyril), and ZD 7114 hydrochloride were purchased from Tocris Cookson (Ballwin, MO).

[125I]CYP was purchased from Amersham Biosciences UK Ltd. All agents except SR 59230A and ZD 7114 were dissolved and diluted in Krebs’ buffer. Initial stock solutions (10−2 M) of SR 59230A and ZD 7114 were prepared using DMSO and were then diluted accordingly with Krebs’ buffer to arrive at the desired final concentrations in the muscle baths. The amounts and concentrations of DMSO used for any of the final concentrations had no effect on the basal tone of the IAS smooth muscle.

**Data Analysis.** The fall in basal tension of the IAS smooth muscle following administration of agonists was expressed as the percentage of maximal relaxation as explained above. The results were expressed as means ± S.E. of different experiments. The statistical significance between different groups was determined by analysis of variance and by paired or unpaired t test. A p value smaller than 0.05 was considered significant. Agonist potencies, pA2 of antagonists, and receptor binding data (Bmax, Kd, and KI) were calculated using GraphPad Prism software. pA2 values were calculated based on the earlier method (Arunlakshana and Schild, 1959).

**Results**

**Effect of ZD 7114 on the Basal Tone of IAS Smooth Muscle.** The β3-AR agonist ZD 7114 (formerly ICI D7114) (Growcott et al., 1993b) produced a concentration-dependent fall in the basal tension of the IAS smooth muscle (Fig. 1A) with an EC50 value of 5.30 × 10−8 M (n = 8–10). The concentration causing maximal relaxation (ECmax) was 1 × 10−6 M. The maximal relaxation in different experiments ranged from 80.7 to 88.5%. The selective β3-AR antagonist SR 59230A (De Ponti et al., 1996b) significantly attenuated the relaxant response to ZD 7114 in a concentration-dependent manner (p < 0.05; n = 5–8; Fig. 1A). A Schild plot produced a line with a slope of 0.90 ± 0.15 (Fig. 1B) and a corresponding pA2 value of 7.8 ± 0.24.

Both the selective β3-AR antagonist CGP 20712A (Dooley et al., 1986) (1 × 10−7 M) and the selective β2-AR antagonist ICI 118551 (Bilski et al., 1983) (1 × 10−7 M) failed to produce any significant shifts in the CRCs of ZD 7114 (p > 0.05; n = 5–8; Fig. 1C). The EC50 and pA2 values of β3- and other β-AR agonists and antagonists are given in Table 2.

**Effect of Procaterol on the Basal Tone of IAS Smooth Muscle.** Procaterol, a β2-AR selective agonist (Kotsonis and Majewski, 1994) produced a concentration-dependent fall in basal tension of the IAS smooth muscle with an EC50 value of 2.51 × 10−5 M (n = 5–8) (Fig. 2A). The concentration causing maximal relaxation (ECmax) was 3 × 10−6 M. The maximal relaxation in different experiments ranged from 79.1 to 83.7%. The selective β2-AR antagonist ICI 118551 (Bilski et
al., 1983) significantly attenuated the relaxant response to ZD 7114 in a concentration-dependent manner (p < 0.05; n = 5–8; Fig. 2A). A Schild plot produced a line with a slope of 0.88 ± 0.07 (Fig. 2B) and a corresponding pA2 value of 7.70 ± 0.31.

The selective β1-AR antagonist CGP 20712A (1 × 10⁻⁷ M) and the selective β2-AR antagonist SR 59230A (1 × 10⁻⁷ M) did not produce any significant shifts in the CRC of procatel (p < 0.05; n = 5–8; Fig. 2C).

**Effect of Xamoterol on the Basal Tone of IAS Smooth Muscle.** The β1-AR agonist xamoterol (Malta et al., 1985) produced a concentration-dependent fall in the basal tension of the IAS smooth muscle (Fig. 3A) with an EC50 value of 1.02 × 10⁻⁷ M (n = 5–8). The concentration causing maximal relaxation (ECmax) was 3 × 10⁻⁶ M. The maximal relaxation in different experiments ranged from 71.5 to 78.7%. The selective β2-AR antagonist CGP 20712A (Dooley et al., 1986) caused a significant shift in the CRC of xamoterol in a concentration-dependent manner (p < 0.05; n = 5–8; Fig. 3A). A Schild plot produced a line with a slope of 0.82 ± 0.08 (Fig. 3B) and a corresponding pA2 value of 7.12 ± 0.18.

The selective β3-AR antagonist ICI 118551 (1 × 10⁻⁷ M) did not inhibit relaxation by xamoterol at concentrations below 3 × 10⁻⁷ M. However, ICI 118551 significantly reduced the xamoterol-mediated relaxation at higher concentrations (p < 0.05; n = 5–8). The selective β3-AR antagonist SR 59230A (1 × 10⁻⁷ M) did not produce any significant shifts in the CRC of xamoterol (p < 0.05; n = 4; Fig. 3B).

**Receptor Binding Studies on β-ARs in IAS Smooth Muscle.** To characterize and determine the levels of β-ARs in the IAS, we conducted radioligand binding studies with [¹²⁵I]CYP. Based on reports that [¹²⁵I]CYP has a significantly lower affinity for β1/β2-AR than for β3-AR (Donigan et al., 2000; Kohout et al., 2001), we investigated the binding profiles of the three β-AR subtypes in the IAS. Initially, to determine the appropriate time need for the equilibrium, a time course was plotted. [¹²⁵I]CYP specifically bound to membrane preparations of the IAS in a time-dependent fashion, with equilibrium achieved at 90 min (35°C), and remained constant for 180 min (data not shown).

When membrane preparations derived from the circular smooth muscle layer of the IAS were incubated with increasing concentrations of radioiodin (5–3000 pM) and 100 μM propranolol, the nonselective β-antagonist, the specific binding of [¹²⁵I]CYP was found to be saturable with a plateau of saturation between 750 and 1200 pM radioligand (Fig. 4A). Sigmoid representation of the data illustrates the binding of [¹²⁵I]CYP over large concentration ranges from the high affinity site (picomolar) to the low affinity site (nanomolar) (Fig. 4B). The two populations of β-ARs were also evident by the curvilinear Scatchard plot of the data (Fig. 4C). Nonlinear regression analysis revealed that the saturation binding isotherm was best fit by a double hyperbolic plot, indicating the presence of two distinct binding sites with high (R1) and low (R2) affinities for [¹²⁵I]CYP.

The respective Kd and Bmax (Kd1 and Bmax1) values at the high affinity site (R1) were 96.4 ± 8.7 pM and 12.5 ± 0.6 fmol/mg protein, whereas the Kd and Bmax (Kd2 and Bmax2) at the low affinity site (R2) were 1.96 ± 0.17 nM and 58.7 ± 4.3 fmol/mg protein, respectively.

The presence of two populations of β-AR binding sites in the IAS smooth muscle was assessed by performing competition experiments against [¹²⁵I]CYP binding with β-subtype-specific ligands used in functional studies. To focus on the ligand binding properties of the low- or high-affinity sites, experiments were performed at both RH (96.4 pM) and RL (1.96 nM) radioligand concentrations. In the presence of a low concentration of radioligand (66 pM), the rank order potency for the selective β-AR antagonist causing 50% displacement of [¹²⁵I]CYP (IC50) was as follows: ICI 118551 > CGP 20712A > SR 59230A (Fig. 5). By contrast, at concentrations of [¹²⁵I]CYP indicative of the R1 (1.60 nM), there was an inversion of the ligand binding profile where SR 59230A > ICI 118551 > CGP 20712A (Fig. 6). Similar trends were seen with the respective selective β1-, β2-, and β3-AR agonists (data not shown). The Kc value was calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973), and the resultant values at both the high- and low-affinity sites are listed in Table 3.

Additional calculations based on specific binding revealed the predominant presence of low-affinity β3-ARs. From the entire population of β-ARs, high-affinity (β1/β2-AR) constituted 21.3% and low affinity (β3-ARs) comprised 78.7%.
Determination of $\beta_1$, $\beta_2$, and $\beta_3$-AR Membrane Protein in the IAS and Rectum. To identify and quantify $\beta$-AR protein expression in the rectum and IAS, the membrane preparations were fractionated by SDS-polyacrylamide gel electrophoresis and subjected to Western blotting by primary antibodies specific to each $\beta$-AR subtype (see Materials and

Fig. 2. A, CRC showing IAS smooth muscle relaxation by proclerol ($\beta_2$-agonist) before and after a selective $\beta_2$-antagonist, ICI 118551. As shown, ICI 118551 causes a significant and concentration-dependent attenuation in the CRC of proclerol (*, $p < 0.05; n = 5–8$). B, Schild plot of different concentrations of ICI 118551 versus log($r - 1$) of proclerol. The $pA_2$ value for ICI 118551 in antagonizing proclerol-induced relaxation of the IAS smooth muscle is 7.7. C, $\beta_1$-AR (CGP 20712A) and $\beta_3$-AR (SR 59230A) antagonists, in contrast, have no significant effect on the IAS smooth muscle relaxation caused by proclerol ($p > 0.05; n = 5–8$).

Fig. 3. CRC showing percent maximal fall in basal IAS tone with xamoterol ($\beta_1$-agonist) before and after different concentrations of CGP 20712A ($\beta_2$-antagonist). Data show that CGP 20712A (with the exception of $1 \times 10^{-8} \text{M}$) causes a significant and concentration-dependent inhibition of the IAS smooth muscle relaxation by xamoterol (*, $p < 0.05; n = 5–8$). B, Schild plot of different concentrations of CGP 20712A versus log($r - 1$) of xamoterol. The $pA_2$ value for SR 59230A in antagonizing xamoterol-induced relaxation of the IAS smooth muscle is 7.12. C, influence of $\beta_2$- and $\beta_3$-AR antagonists ICI 118,551 and SR 59230A, respectively, on percent maximal fall in basal IAS tone by xamoterol. Data show that the $\beta_1$-AR agonist-mediated fall in the basal tone of the IAS smooth muscle was not significantly modified by SR 59230A ($p > 0.05; n = 5–8$) but was modestly antagonized by ICI 118551 (*, $p < 0.05; n = 5–8$).
Methods). All three subtypes of β-AR were found to be present in the rectum and IAS membranes as shown by the representative blots in Fig. 7. The blots demonstrate the relative distribution of membrane receptor proteins for β1-AR (63 kDa), β2-AR (68 kDa), and β3-AR (65 kDa) in these tissues. Data suggest that the distribution of the three subtypes of membrane β-AR in these tissues was similar (p > 0.05; Fig. 7).

Detection of β-AR mRNA in the IAS Using RT-PCR. RT-PCR amplification was used to detect β1-, β2-, and β3-AR, and β-actin mRNA in the circular smooth muscle layer of the IAS. To ensure that the PCR products were exclusively derived from mRNA, total RNA samples were treated with DNase to eliminate genomic DNA. As shown in Fig. 8, the resultant PCR products demonstrated the expected sizes of 608 (β1-AR), 194 (β2-AR), and 444 bp (β3-AR). The PCR product for β-actin, an internal standard, was also detected in each preparation at its expected size of 387 bp.

Discussion

The studies demonstrate a systematic and comprehensive characterization of β-adrenoceptors (β-ARs) in the tonic smooth muscle of the gastrointestinal tract. The IAS smooth muscle served as the prototype using functional, classical pharmacology, molecular, and receptor binding approaches. The studies demonstrate: 1) the presence of membrane-bound β-AR through Western blotting and β-AR mRNA through RT-PCR; 2) the role of a heterogeneous population of β-ARs (β1, β2, and β3) in mediating potent relaxation of the
TABLE 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>High-Affinity β-AR Sites</th>
<th>Low-Affinity β-AR Sites</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_i$ ($nM$)</td>
<td>Hill Slope $b$</td>
</tr>
<tr>
<td>CGP 20712A</td>
<td>114.1 ± 16.5</td>
<td>−0.88 ± 0.20</td>
</tr>
<tr>
<td>ICI 118551</td>
<td>30.4 ± 9.7</td>
<td>−0.55 ± 0.18</td>
</tr>
<tr>
<td>SR 59230A</td>
<td>853.3 ± 120</td>
<td>−0.63 ± 0.15</td>
</tr>
</tbody>
</table>

*a* $K_i$ values were determined by the Cheng-Prusoff equation as described under Materials and Methods.

*b* Hill slope was determined using GraphPad Prism 3.0. The 95% confidence intervals of the Hill slope included the value of −1.0 for each ligand.

affinity values for antagonism by SR 59230A (pA$_2$ of 7.8) are consistent with previous studies in guinea pig ileum (pA$_2$, 7.7) (Roberts et al., 1999) and human colon (pA$_2$, 8.3) (De Ponti et al., 1996b). Proacaterol, a β$_2$-selective agonist (Kotsonis and Majewski, 1994), also causes a concentration-dependent relaxation of the smooth muscle strips with a pEC$_{50}$ of 7.6, whereas ICI 118,551 (β$_2$-selective antagonist) (Bilski et al., 1983) antagonizes this relaxation with a pA$_2$ value of 7.7. This is consistent with pA$_2$ values reported by Strosberg (1997). Likewise, xamoterol (β$_1$ agonist) (Malta et al., 1985), causes a concentration-dependent relaxation of the IAS smooth muscle that is selectively antagonized by CGP 20712A (β$_1$-AR antagonist).

In the rat (Roberts et al., 1999; Brown and Summers, 2001) and mouse (Hutchinson et al., 2001) ileum, it has been shown that β$_3$-ARs play a predominant role, whereas β$_1$-ARs have only a small role in smooth muscle relaxation. The presence of atypical or β$_3$-ARs was established in rat ileum by [125I]CYP binding studies (Roberts et al., 1995) and by β$_3$-mRNA on RT-PCR analysis (Roberts et al., 1999). Roberts et al. (1995, 1999) were able to show the presence of β$_3$-AR but not those of β$_1$- and β$_2$-AR binding sites, even under classical binding conditions. Roberts et al. (1995, 1999) did, however, find an abundance of β$_3$-AR mRNA, in addition to β$_3$. Nevertheless, the role of β$_2$-AR was discounted because smooth muscle relaxation caused by zinterol (β$_2$-AR-selective agonist) was antagonized by the β$_2$ antagonist SR 58894A and not by ICI 118551. The exact reason for the differences in the functional, binding, and molecular findings in these studies has not been fully delineated.

In contrast, the results of our studies in the IAS are in agreement with those in human colonic smooth muscle (De Ponti et al., 1996b) showing that the respective β$_1$- and β$_2$-selective antagonists CGP 20712A and ICI 118551 inhibit isoprenaline-mediated relaxation, which is further inhibited by SR 59230A. Differences between various studies may be reconciled on the basis of variations in species and tissues. The present studies, like those in human colon (De Ponti et al., 1996b), were conducted in spontaneously tonic smooth muscle, compared with others in which contraction was elicited by different contractile agonists. Whether such contractile agonists have attenuating effects in the functional expression of different β-ARs remains to be determined.

Receptor binding, Western blot, and RT-PCR studies provide additional support in favor of the functional data. The receptor binding studies demonstrate, for the first time in the GI tract, the presence of two binding sites. These binding sites correspond to high affinity (R$_{HI}$) β$_1$/β$_2$ and low affinity (R$_L$) β$_3$ sites. We identified these binding sites with $K_i$ values of 96 nM and 1.96 nM, respectively. The $K_a$ values of the respective binding sites are similar to those described in...
adipocytes and Chinese hamster ovary (CHO) cells (Feve et al., 1991).

Two classes of binding sites were identified using competition studies with β-AR subtype-selective antagonists. The rank order potency of the antagonists at the high-affinity site is CGP 20712A > SR 59230A with Kᵢ values of 3.04 x 10⁻⁸, 1.14 x 10⁻⁷, and 8.53 x 10⁻⁷ M, respectively. When radioligand concentrations were employed in the low-affinity (1.61 nM), the potency was reversed, with SR 59230 > ICI 118551 > CGP 20712A. The corresponding Kᵢ values with these antagonists were 4.81 x 10⁻⁸, 8.80 x 10⁻⁷, and 1.78 x 10⁻⁶ M, respectively. The Kᵢ values of CGP 20712A and ICI 118551 at the RH are consistent with those reported at β₁- and β₂-ARs in CHO cells (Mejean et al., 1995). The Kᵢ value for SR 59230A at the RH is similar to that of the β₂-AR found in rat colon (Manara et al., 1995a). The Kᵢ values of CGP 20712A and ICI 118551 are similar to those reported in guinea pig ileum and vascular smooth muscles (Kohout et al., 2001).

ZD 7114 was first described as a selective β₃-AR agonist in brown fat and guinea pig ileum (Holloway et al., 1991). Some subsequent studies have described ZD 7114 as having atypical β₂-AR antagonistic effects in certain tissues (Growcott et al., 1993a). In the IAS smooth muscle, ZD 7114 behaves as a full β₂-AR-selective agonist causing relaxation that is potently inhibited by SR 59230A. Therefore, the actions of ZD 7114 may be tissue- and species-specific.

SR 59230A was developed as the first β₂-AR-selective antagonist for the gut (Manara et al., 1995a). Recently, Horinouchi and Koike (2001) have raised the possibility that the effects of SR 59230A are tissue-specific. In the guinea pig gastric fundus and duodenum, SR 59230A may possess atypical β₂-AR-agonistic activity by recognizing an aminotetralin moiety in the β-AR. In our study, however, SR 59230A was found to be a selective β₂-AR antagonist with a Pₐₛ value of 7.8. It causes a concentration-dependent rightward shift in the CRC of ZD 7114 without modifying the effects of β₁- and β₂-AR agonists. In addition, SR 59230A alone does not cause a fall in IAS basal tone at concentrations up to 1 x 10⁻⁴ M. It is possible that the presence of a bulky group on the arylenethanolamine or aryloxypropanolamine side chain on both ZD 7114 and SR 59230A (Horinouchi and Koike, 2001) may render the receptor tissue- and species-specific. However, the opposing actions of ZD 7114 and SR 59230A in the IAS may not support that concept.

Receptor binding analysis reveals a higher receptor density of β₂-AR in the IAS smooth muscle. This is supported by the severalfold higher Bₐₜₙₙ in the case of low-affinity β-AR (β₂-AR) compared with high-affinity β-AR (β₁/β₂-AR). With this information, one would have expected higher potencies of β₂- versus β₁- and β₂-AR agonists in causing IAS smooth muscle relaxation. The functional studies, however, show that in this respect, β₁-, β₂-, and β₃-agonists are nearly equipotent. We speculate three possible explanations for this disparity. The first and simplest explanation is the lack of effective β₂-AR agonists as compared with β₁- and β₂-AR agonists for the IAS smooth muscle at the present time. Second, β₂-AR in the IAS smooth muscle may have a large number of spare receptors. Third, β₂-AR may represent a heterogeneous population such as β₃a- and β₃b-ARs, as suggested by the recent studies in CHO (Hutchinson et al., 2002). Furthermore, the activation and signal transduction of such a β₃a- and β₃b-AR complex may prevent the full potency of the β₂-AR agonist. Therefore, it is no surprise that the β₂-AR agonist ZD 7114 has variable effects in different GI smooth muscle preparations (Growcott et al., 1993a,b). The involvement of β₃a- and β₃b-AR complex and the exact signal transduction involved in β₂-AR-mediated IAS relaxation by agonists such as ZD 7114 remains to be determined.

In addition to receptor binding studies, the presence of β-AR in the IAS smooth muscle is further demonstrated by Western blot and RT-PCR studies. Western blot studies using primary antibodies specific to each β-AR subtype reveal the presence of all three subtypes of β-AR (β₁-AR, 63 kDa; β₂-AR, 68 kDa; and β₃-AR, 65 kDa) in the rectum and IAS membranes. RT-PCR amplification was used to detect β₁-, β₂-, and β₃-AR in the circular smooth muscle layer of the IAS. The PCR products demonstrated the expected sizes of 608 (β₁-AR), 194 (β₂-AR), and 444 bp (β₃-AR).

The present studies, therefore, provide comprehensive evidence for the presence and actions of β₁-, β₂-, and β₃-AR in IAS smooth muscle. In light of these findings, combined with the previously described actions of β₂-AR activation in the lower esophageal sphincter (DiMarino et al., 2002) with limited side effects and prolonged smooth muscle relaxation, we suggest that β₂-AR agonists in particular may have considerable physiological and therapeutic implications in anorectal and other spastic gastrointestinal motility disorders.

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