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Functional and Molecular Characterization of Beta Adrenoceptors in the Internal Anal Sphincter

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Running Title: **β-adrenoceptors in IAS smooth muscle**

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ABBREVIATIONS: β-AR, β–adrenergic receptor; IAS, internal anal sphincter; CRC, concentration response curve; EC_{max}, concentration causing maximal relaxation; EC_{50}, concentration causing 50% of maximal relaxation; ICYP, iodocyanopindolol; ZD 7114 hydrochloride,(S)-4-[2-hydroxy-3-phenoxypropylaminoethoxy]-N-(2-methoxyethyl)phenoxyacetamide); SR 59230A hydrochloride, (1-(2-ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride); ICI 118,551 hydrochloride, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CGP 20712A methanesulfonate salt, (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt)

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ABSTRACT

The purpose of the present study was to characterize different β-adrenoceptors (β-ARs) and determine their role in the spontaneously tonic smooth muscle of the internal anal sphincter (IAS). The β-AR subtypes in the opossum IAS were investigated by functional *in vitro*, radioligand binding, western blot, and reverse transcription polymerase chain reaction (RT-PCR) studies. ZD 7114, a selective β_3-AR agonist, caused a potent and concentration-dependent relaxation of the IAS smooth muscle that was antagonized by the β_3-AR antagonist SR 59230A. Conversely, the IAS smooth muscle relaxation caused by β_1 and β_2-AR agonists (xamoterol and procaterol, respectively) was selectively antagonized by their respective antagonists CGP 20712 and ICI 118551. Saturation binding of [^{125}I]iodocyanopindolol ([^{125}I]CYP) to β-AR subtypes revealed the presence of a high affinity site (K_{d1} = 96.4 ± 8.7 pM; B_{max1} = 12.5 ± 0.6 fmol/mg protein) and a low affinity site (K_{d2} = 1.96 ± 1.7 nM; B_{max2} = 58.7 ± 4.3 fmol/mg protein). Competition binding with selective β-AR antagonists revealed that the high affinity site correspond to β_1/β_2-AR and the low affinity site to β_3-AR. Receptor binding data suggest the predominant presence of β_3-AR over β_1/β_2-AR. Western blot studies identified β_1, β_2, and β_3-AR subtypes. The presence of β_1, β_2, and β_3-AR was further demonstrated by mRNA analysis using RT-PCR. The studies demonstrate a comprehensive functional and molecular characterization of β_1, β_2, and β_3-ARs in IAS smooth muscle. These studies may have important implications in anorectal and other gastrointestinal motility disorders.

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It is well known that post-junctional β-adrenoceptors (β-ARs) mediate the inhibitory effects of sympathetic nerve stimulation in different smooth muscles including those of the gastrointestinal tract (Gauthier et al., 2000; Manara et al., 1995b). The intestinal β-AR was originally described as a β_1-and β_2-AR (Lands et al., 1967). Further studies with gastrointestinal preparations from several species established the relaxant effect of classical β-AR (β_1 and β_2) 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; Strosberg, 1997; Manara et al., 2000; Goldberg and Frishman, 1995). 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 and colleagues (Emorine et al., 1989) cloned and sequenced the β_3-AR and found that it shared the pharmacological characteristics of the “atypical” β-AR.

The β_3-AR has been found in a variety of mammalian tissues (Berkowitz et al., 1995) including white and brown adipocytes (Muzzin et al., 1991), trachea (Webber and Stock, 1992), heart (Kaumann and Molenaar, 1996; Gauthier et al., 2000), gastrointestinal tract (Bardou et al., 1998; De Ponti et al., 1995), and the urinary tract (Tomiyama et al., 1998). In the GI tract, recent studies have focused on the ability of β_3-AR specific agonists to cause relaxation in a number of different smooth muscle tissues including rat ileum, jejunum, colon, guinea-pig ileum, and duodenum (Manara et al., 1995b). One of the problems in delineating the pharmacology of β-ARs in the gastrointestinal tract has been the lack of subtype-selective agonists and antagonists,

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especially those for β_3-AR. Recent *in vivo* studies have demonstrated the selective, potent, and prolonged relaxant effect of CL 316,243, a selective β_3-AR agonist, on the sphincteric smooth muscles of the opossum LES (DiMarino et al., 2002), without the significant systemic cardiovascular side effects that are associated with β_1- and β_2-AR agonists.

In the past few years, β_3-agonists have emerged as potential therapeutic agents for several gastrointestinal motility disorders including irritable bowel syndrome (Scarpignato and Pelosini, 1999). Anorectal dysfunctions such as Hirschsprung's disease, constipation, anal fissures, and hemorrhoids may also be associated with either hypertensive IAS or failure of sphincteric relaxation in response to the rectoanal 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.

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 to 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

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through western blot studies and reverse transcription polymerase chain reaction (RT-PCR) analysis, respectively.

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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 exsanguination, the anorectal region was then dissected out and transferred immediately to oxygenated (95% O_2 + 5% CO_2) Krebs' physiological solution of the following composition: 118.07 mM NaCl, 4.69 mM KCl, 2.52 mM $CaCl_2$, 1.16 mM $MgSO_4$, 1.01 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11.10 mM glucose. A longitudinal incision along the length of isolated anorectal region was made and the tissue was pinned flat in a Sylgard (Dow Corning Corp., Midland, MI) coated petri dish. Once the lumen was fully exposed, the mucosa and submucosa were removed carefully by sharp dissection. The tissue was then turned on the serosal side and all extraneous tissue including the outer longitudinal muscle was removed. Circular smooth muscle strips of the IAS (approximately 1x10 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, Inc., 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 FT03; Grass Instruments, Quincy, MA). Isometric tension was recorded by the PowerLab/8SP data acquisition system using Chart 4.1.2 (ADInstruements, 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 they were washed with Krebs' solution every 15 min. Only smooth muscle strips that developed spontaneous tone and responded to electrical field stimulation (EFS) 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 (CRC) with β_1-, β_2-, and β_3-AR agonists on the basal tone of the IAS smooth muscles, xamoterol, procaterol, and ZD 7114, respectively were added to the muscle bath in cumulative concentrations (Rattan and Moummi, 1989). Successive concentrations of the agonists were not added till the response of the previous concentration stabilized. Ten minutes between additions of different concentrations were allowed when no effect was observed. In preliminary studies, when a single concentration was used, we noted that this was an appropriate time needed to gauge the maximal effect of a given concentration of the agonist. No difference in the results occurred with longer exposures. In order to determine the effects of β_1-, β_2-, and β_3-AR antagonists, CGP 20712A, ICI 118551, and SR 59230A, respectively (in concentrations ranging from $1x10^{-8}$ to $1x10^{-6}$ M) were added 30 min before obtaining the CRC of the test agonist.

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Beta-Adrenoceptor (β-AR) Analysis by Western Blot. Western blot analysis of β_1-, β_2-, and β_3- in the IAS and rectum of the opossum was performed according to the protocol of Santa Cruz Biotechnology (Santa Cruz, CA). Circular smooth muscles tissues of the IAS and rectum were cut into small pieces (2 x 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), then put into the microwave for 10 seconds. The homogenates were centrifuged (16,000g, 4^{o}C) for 15 min. The pellet obtained was dissolved in Krebs' buffer (composition already described above) containing 1 mM EDTA, 1mM DTT, and 1 mM PMSF (combined pH of 7.6). The protein contents were determined by the method described by Lowry et al (Lowry et al., 1951) using BSA 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% bromophenol blue, 2% β-mercaptoethanol) and boiled for 4 minutes. 20 µl (40 µg total protein) of each sample was applied to commercially available 7.5% SDS polyacrylamide gel PAGEr Gold Gel (BioWhitaker Molecular Applications, Rockland, ME) applied to 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS Page) apparatus by the method of Laemmli (Laemmli, 1970) using 150 V for 1 hr. The separated proteins were electrophoretically transferred to nitrocellulose membrane (NCM) at 4^{o}C for 90 min at 100 V. To block nonspecific antibody binding, the NCM were immersed overnight at 4^{o}C in Super Block Tris-buffered saline Tween (TBS) blocking buffer (Pierce Biotechnology, Rockford, IL). The NCM was divided into three smaller sections labeled as β_1, β_2, and β_3. The NCM were then incubated with the respective diluted isoform specific primary (1^o) antibodies corresponding to the specific β-AR subtype. The NCM were incubated with rabbit β_1, β_2, and goat β_3 polyclonal antibodies, respectively (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1:500. All membranes were incubated with 1^o antibody for 1 hr at room temperature. The membranes

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were then washed with TBS-T three times. Afterwards, the membranes corresponding to β_1 and β_2 were incubated separately in 1:1000 diluted horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) in 2^o antibody buffer for 1 hr at room temperature. The remaining membrane was incubated in 1: 5,000 diluted horseradish peroxidase conjugated bovine anti-goat IgG (Santa Cruz Biotechnology) in 2^o 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, 1mM DTT, and 1 mM PMSF (combined pH of 7.6). The IAS was minced with scissors and homogenized in 5 volumes of ice-cold TED buffer by the use of Tekmar Tissuemizer (Tekmar & Co., Cincinnati, OH) for 15 s. The homogenates were centrifuged at 100,000 g for 1 hr at 4°C. The supernatant was filtered through a 500 μ Nitex mesh. The pellets were resuspended in cold Krebs' buffer and stored at -80°C until used. Protein content was determined by the method of Lowry et al (Lowry et al., 1951).

Radioligand Binding Studies. The radioligand (-)-3-[^{125}I]Iodocyanopindolol ([^{125}I]CYP); Amersham Pharmacia Biotech UK limited, Buckinghamshire, UK) was used for identifying β-AR. For equilibrium determination, membranes at a protein concentration of 40 μg per tube were incubated with [^{125}I]CYP (specific activity: 2,000 Ci/mmol) for 0, 15, 30, 45, 60, 90, 120,

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150 and 180 min. The experiments were carried out in the presence or absence of 100 μM propranolol (a non-selective β−AR antagonist). The incubation mixture was composed of 50 mM Tris HCl buffer pH 7.4, containing 10 mM $MgCl_2$ and 1 mM EDTA in a final volume of 250 μl. A time course (using above mentioned time points) was carried out in duplicate at 35^{o}C in order to determine optimal time needed for equilibrium. The incubation was terminated by rapid filtration through Whatman GF/C glass-fiber filters (24 mm circles) (Whatman Inc., Clifton, NJ) using 1225 Sampling Manifold (Millipore Corp., Bedford, MA), followed by washing three times with 5 ml ice-cold 25 mM Tris HCl buffer, pH 7.4. The filters were counted in the Auto-Gamma Counting System (model 5550; Packard Instrument Co., Downers Grove, IL) 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 [^{125}I]CYP (5 to 3,000 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 (K_d) and the maximum binding capacity (B_{max}) were determined by non-linear regression analysis by GraphPad Prism software (San Diego, CA). K_d is the concentration of ligand required to occupy 50% of the binding sites. B_{max} is defined as the maximal specific binding obtained with increase in concentration of radioligand, and it is a measure of receptor binding in the tissue under investigation. Displacement experiments were performed with varying amount of [^{125}I]CYP depending on the appropriate K_d of the high and low affinity sites of the IAS. In the IAS, 66 pM and 1.61 nM [^{125}I]CYP were used in the high and low affinity sites respectively. The K_i value was calculated by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as follows:

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$K_i = IC_{50}/(1+L/K_d)$ where IC_{50} 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 above in the section of 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 (Chomczynski and Sacchi, 1987). RNA samples were then dissolved in dethylpyrocarbonate (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 (λ_{260}). The yield and quality of the RNA were assessed by measuring the OD λ_{260}/OD λ_{280} ratio.

Preparation and Amplification of cDNA encoding β_1, β_2, β_3-ARs (RT-PCR analysis). RNA samples 2 µl (1 µg) of acceptable quality were used as templates for the synthesis of cDNA. Primers for β_1, β_2, β_3-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 10X reverse transcription buffer, dNTPs (20 mM), $MgCl_2$ (25 mM), 18 U RNasin ribonuclease inhibitor, and 20 U AMV Reverse Transcriptase in a total volume of 20 µl. The contents of reaction mixture were purchased from

Promega (Madison, WI). Following brief centrifugation, the reactions mixtures were incubated at 42^{o}C for 45 min, then at 95^{o}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 10x reaction buffer, 25 mM $MgCl_2$, 3.5 µl of recombinant Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan) and 20 mM of 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, Westbury, NY). Following initial heating of samples at 95^{o}C, each cycle of amplification consisted of 45s at 94^{o}C, followed by 45s at 60^{o}C, and 2 min extension at 72^{o}C; this sequence was repeated for a total of 38 cycles. At the end of the reactions, 15 µl of samples was mixed with 5 µl of 6x green/purple loading dye. The samples were loaded onto a 2% agarose gel containing ethidium bromide and electrophoressed for approximately 1 hr at 100 V. The gels were visualized with an ultraviolet transluminator (312 nm variable intensity, Fisher Biotech, Fisher Scientific, Pittsburgh, PA) and photographed using UV gel electrophoresis camera (Polaroid GH 10, UK). Densitometric analysis of the gel bands was carried out using Kodak Image Analysis software (Rochester, NY).

Drugs and Chemicals. SR 59230A hydrochloride (1-(2-Ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride), propranolol hydrochloride (±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride, CGP 20712A (methanesulfonate salt (±)-2-Hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt), DMSO

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(dimethyl sulfoxide) and EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Xamoterol hemifumarate **(**1-(4-Hydroxyphenoxy)-3-[2-(4-morpholinocarboxamido) ethylamino]-2-propanol), ICI 118,551 hydrochloride (±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol), procaterol hydrochloride **(**(±)-*erythro*-8-Hydroxy-5-[1-hydroxy-2-(isopropylamino) butyl]carbostyril), ZD 7114 hydrochloride (S)-4-[2-Hydroxy-3-phenoxypropylaminoethoxy]-N-(2-methoxyethyl)phenoxyacetamide) were purchased from Tocris Cookson Inc. (Ellisville, MO). [^{125}I]CYP (-)3-[^{125}I]Iodocyanopindolol was purchased from Amersham Pharmacia Biotech (UK).

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, which 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 agonists was expressed as the percent of maximal relaxation as explained above. The results were expressed as means ± SE of different experiments. The statistical significance between different groups was determined by analysis of variance (ANOVA) and by paired or unpaired *t*-test. A p value smaller than 0.05 was considered significant. Agonist potencies, pA_2 of antagonists, and receptor binding data (B_{max}, K_d, and K_i) were calculated using GraphPad Prism software (San Diego, CA). pA_2 values were calculated based on the earlier method (Arunlakshana and Schild, 1959).

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Results

Effect of ZD 7114 on the Basal Tone of IAS Smooth Muscle. The β_3-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 EC_{50} value of 5.30 x 10^{-8} M (n = 8-10). The concentration causing maximal relaxation (EC_{max}) was 1x10^{-6} M. The maximal relaxation in different experiments ranged from 80.7 to 88.5%. The selective β_3-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 pA_2 value of 7.8 ± 0.24.

Both the selective β_1-AR antagonist CGP 20712A (Dooley et al., 1986) (1x10^{-7} M) and the selective β_2-AR antagonist ICI 118551 (Bilski et al., 1983) (1x10^{-7} M) failed to produce any significant shifts in the concentration response curves (CRC) of ZD 7114 ($p > 0.05$; n = 5-8; Fig. 1C). The EC_{50} and pA_2 values of β_3-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 β_2-AR selective agonist (Kotsonis and Majewski, 1994) produced a concentration-dependent fall in basal tension of the IAS smooth muscle with a EC_{50} value of 2.51 x 10^{-8} M (n = 5-8) (Fig. 2A). The concentration causing maximal relaxation (EC_{max}) was 3x10^{-6} M. The maximal relaxation in different experiments ranged from 79.1 to 83.7%. The selective β_2-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 pA_2 value of 7.70 ± 0.31.

Both the selective β_1- CGP 20712A (1x10^{-7} M) and β_3-AR antagonist SR 59230A (1x10^{-7} M) did not produce any significant shifts in the CRC of procaterol ($p<0.05$; n = 5-8; Fig. 2C).

Effect of Xamoterol on the Basal Tone of IAS Smooth Muscle. The β_1-AR agonist

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xamoterol (Malta et al., 1985) produced a concentration-dependent fall in the basal tension of the IAS smooth muscle (Fig. 3A) with an EC_{50} value of 1.02 x 10^{-7} M (n = 5-8). The concentration causing maximal relaxation (EC_{max}) was 3x10^{-6} M. The maximal relaxation in different experiments ranged from 71.5 to 78.7 %. The selective β_1-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. 1A). A Schild plot produced a line with a slope of 0.82 ± 0.08 (Fig. 3B) and a corresponding pA_2 value of 7.12 ± 0.18.

The selective β_2-AR antagonist ICI 118551 (1x10^{-7} M) did not inhibit relaxation at concentrations below 3x 10^{-7} M. However, at higher concentrations ICI 118551 significantly reduced the xamoterol-mediated relaxation with an EC_{50} of 4.80 x 10^{-6} M ($p < 0.05$; n = 5-8). The selective β_3-AR antagonist SR 59230A (1x10^{-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. In order to characterize and determine the levels of β-ARs in the IAS, we conducted radioligand binding studies with [^{125}I]CYP. Based on reports that [^{125}I]CYP has a significantly lower affinity for β_1/β_2-AR than β_3 (Kohout et al., 2001; Dunigan et al., 2000), 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. [^{125}I]CYP specifically bound to membrane preparations of the IAS in a time-dependent fashion with equilibrium achieved at 90 min (35^{o}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 radioligand (5 to 3000 pM) and 100 µM of the non-selective β-antagonist propranolol, the specific binding of [^{125}I]CYP was found to be saturable with a plateau of saturation between 750 and 1200 pM of the radioligand (Fig. 4A). Sigmoid

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representation of the data illustrates the binding of [^{125}I]CYP over large concentration ranges from the high affinity site (pM) to the low affinity site (nM) (Fig. 4B). The two populations of β-ARs were also evident by the curvilinear Scatchard plot of the data (Fig. 4C). Non-linear 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 (R_H) and low (R_L) affinities for [^{125}I]CYP.

The respective K_d and B_{max} (K_{d1} and B_{max1}) at the high affinity site(R_H) were 96.4 ± 8.7 pM and 12.5 ± 0.6 fmol/mg protein while the K_d and B_{max} (K_{d2} and B_{max2}) at the low affinity site (R_L) 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 [^{125}I]CYP binding with β-subtype specific ligands used in functional studies. In order to focus on the ligand binding properties of the low or high affinity sites, experiments were performed at both R_H (96.4 pM) and R_L (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 [^{125}I]CYP (IC_{50}) was as follows: ICI 118551 > CGP 20712A > SR 59230A (Fig. 5). By contrast, at concentrations of [^{125}I]CYP indicative of the R_L (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 β_1-, β_2-, and β_3-AR agonists (data not shown). The K_i 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.

Further calculations based on specific binding, revealed the predominant presence of low affinity β_3-AR. From the entire population of β-AR, high affinity (β_1/ β_2-AR) constituted 21.3% and low affinity (β_3-ARs) were 78.7%.

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Determination of β_1, β_2, β_3-AR Membrane Protein in the IAS and Rectum. In order to identify and quantify β-AR protein expression in the rectum and IAS, the membrane preparations were fractionated by SDS-PAGE and subjected to Western blotting by primary antibodies specific to each β-AR subtype (see Materials and 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 Figure 7. The blots demonstrate the relative distribution of membrane receptor proteins for β_1-AR, (63 kDa), β_2-AR (68 kDa), and β_3-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 β_1, β_2, and β_3-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 sample were treated with DNAse to eliminate genomic DNA. As shown in Figure 8, the resultant PCR products demonstrated the expected sizes of 608 (β_1-AR), 194 (β_2-AR), and 444 bp (β_3-AR). The PCR product for β–actin, an internal standard, was also detected in each preparation at its expected size of 387 bp.

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Discussion

The studies demonstrate a systematic and comprehensive characterization of β-adrenoceptors (β-AR) 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 (β_1, β_2, and β_3) in mediating potent relaxation of the IAS smooth muscle; and 3) the presence of both high (β_1/β_2) and low (β_3) affinity-binding sites, with a significantly higher population of β_3-AR as compared to β_1/β_2.

The contribution of the three β-AR subtypes in mediating IAS smooth muscle relaxation is in general agreement with previous reports in different smooth muscles including the GI tract (Goldberg and Frishman, 1995; Roberts et al., 1997; De Ponti et al., 1996a; Strosberg, 1997). The conclusions are based on the ability of β_1-, β_2-, and β_3-agonists to cause a full relaxation that is selectively antagonized by their respective antagonists. ZD 7114, a β_3 selective agonist (Growcott et al., 1993b), produces a concentration-dependent relaxation of the IAS smooth muscle that is antagonized by the β_3 antagonist SR 59230A (De Ponti et al., 1996b) but not by CGP 20712A or ICI 118551 (β_1-, and β_2-AR antagonists, respectively). The 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). Procaterol, 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, while 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 (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 (Brown and Summers, 2001; Roberts et al., 1999) and mouse (Hutchinson et al., 2001) ileum, it has been shown that β_3-AR play a predominant role while β_1-AR have only a small role in smooth muscle relaxation. The presence of atypical or β_3-AR was established in rat ileum by [^{125}I]CYP binding studies (Roberts et al., 1995) and by β_3-mRNA on RT-PCR analysis (Roberts et al., 1999). The authors 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. In addition to β_3, the authors did however, find an abundance of β_2-AR mRNA. The role of β_2-AR, however, was discounted because smooth muscle relaxation caused by zinterol (β_2-AR selective agonist) was antagonized by β_3-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 β_1 and β_2 selective antagonists CGP 20712A and ICI 118551, respectively 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 as compared to others where

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contraction was elicited by different contractile agonists. Whether such contractile agonists have attenuating effects in the functional expression of different β-AR 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_H) β_1/β_2 and low affinity (R_L) β_3 sites. We identified these binding sites with K_d values of 96 pM and 1.96 nM, respectively. The K_d 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 ICI 118551 > CGP 20712A > SR 59230A with K_i 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 range (1.61 nM), the potency was reversed with SR 59230 > ICI 118551 > CGP 20712A. The corresponding K_i values with these antagonists were 4.81 x 10^{-8}, 6.80 x 10^{-7}, and 1.78 x 10^{-6} M, respectively. The K_i values of CGP 201712A and ICI 118551 at the R_H are consistent with those reported at β_1 and β_2-ARs in CHO cells (Mejean et al., 1995). The K_i value for SR 59230A at the R_L is similar to that of the β_3-AR found in rat colon (Manara et al., 1995a). The K_i 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 β_3-AR agonist in brown fat and guinea-pig ileum

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(Holloway et al., 1991). Some subsequent studies have described ZD 7114 as having atypical β_3-AR antagonistic effects in certain tissues (Growcott et al., 1993a). In the IAS smooth muscle, ZD 7114 behaves as a full β_3-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 β_3-AR selective antagonist for the gut (Manara et al., 1995a). Recently, Horinouchi and Koike have raised the possibility that the effects of SR 59230A are tissue-specific (Horinouchi and Koike, 2001). In the guinea-pig gastric fundus and duodenum, SR 59230A may possess atypical β-AR agonistic activity by recognizing a aminotetralin moiety in the β-AR. In our study, however, SR 59230A was found to be a selective β_3-AR antagonist with a pA_2 value of 7.8. It causes a concentration-dependent rightward shift in the CRC of ZD 7114 without modifying the effects of β_1-and β_2-AR agonists. In addition, SR 59230A alone does not cause a fall in IAS basal tone at concentrations up to $1x10^{-4}$ M. It is possible that the presence of a bulky group on the arylethanolamine or aryloxypropanololamine 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 β_3-AR in the IAS smooth muscle. This is supported by the several-fold higher B_{max} in the case of low affinity-β–AR (β_3-AR) as compared with high affinity-β–AR (β_1-/β_2-AR). With this information, one would have expected higher potencies of β_3- vs. β_1- and β_2-AR agonists in causing IAS smooth muscle relaxation. The functional studies however, show that in this respect, β_1, β_2, and β_3-agonists are

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nearly equipotent. We speculate three possibilities to explain this disparity. First and simplest explanation is the lack of effective β_3-AR agonists as compared to β_1 and β_2-AR agonists for the IAS smooth muscle at the present time. Second, β_3-AR in the IAS smooth muscle may have a large number of spare receptors. Third, β_3-AR may represent a heterogeneous population such as β_{3a}- and β_{3b}-ARs as suggested by the recent studies in CHO (Hutchinson et al., 2002). Furthermore, the activation and signal transduction of such a β_{3a}- and β_{3b}-AR complex may prevent the full potency of the β_3-AR agonist. Therefore, it is no surprise that the β_3-AR agonist ZD 7114 has variable effects in different GI smooth muscle preparations (Growcott et al., 1993b; Growcott et al., 1993a). The involvement of β_{3a}- and β_{3b}-AR complex and the exact signal transduction involved in β_3-AR mediated 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 (β_1-AR, 63 kDa; β_2-AR, 68 kDa, and β_3-AR, 65 kDa) in the rectum and IAS membranes. RT-PCR amplification was used to detect β_1, β_2, and β_3-AR, in the circular smooth muscle layer of the IAS. The PCR products demonstrated the expected sizes of 608 (β_1-AR), 194 (β_2-AR), and 444 bp (β_3-AR). The present studies, therefore, provide comprehensive evidence for the presence and actions of β_1, β_2-, and β_3-AR in IAS smooth muscle. In the light of these findings, combined with the previously described actions of β_3-AR activation in the LES (DiMarino et al., 2002) with limited side effects and prolonged smooth muscle relaxation, we suggest that β_3-AR

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agonists in particular may have considerable physiological and therapeutic implications in anorectal and other spastic gastrointestinal motility disorders.

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Footnotes

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TABLE 1

Primers used in RT-PCR reactions for amplifications of mRNA encoding β-AR and β-actin in IAS smooth muscle

Primer	Strand	Primer Sequence (5′-3′)	Location	Accession No
β_1-AR	Forward	GCCGATCTGGTCATGGGA	307-324	NM-012701.1
	Reverse	GTTGTAGCAGCGGCGCG	617-635	
β_2-AR	Forward	ACCTCCTCCTTGCCTATCCA	591-610	NM-012492.1
	Reverse	TAGGTTTTCGAAGAAGACCG	1131-1150	
β_3-AR	Forward	AGTGGGACTCCTCGTAATG	465-483	NM-013108.1
	Reverse	CGCTTAGCTACGACGAAC	891-908	
β-actin	Forward	CGCTTAGCTACGACGAAC	2750-2768	VO-1217-J00691
	Reverse	AGCCATGCCAAATGTGTCAT	3203-3222	

TABLE 2

Comparison of potencies of β_1, β_2, and β_3-AR agonists and their respective antagonists SR 59230A, ICI 118551, and CGP 201712 in the IAS smooth muscle

Agonist	**Antagonist** (nM)	E_{max}[a] (%)	EC_{50}[b] (nM)	pEC_{50}[c]	pA_2[d]
ZD 7114	-	84.6 ± 3.9	53.0	7.28 ± 0.13	
	SR 59230A (1)	79.5 ± 4.4	72.1	7.14 ± 0.10	7.80 ± 0.24
	SR 59230A (10)	78.3 ± 3.1	193.6	6.71 ± 0.12	
	SR 59230A (100)	73.3 ± 2.9	528.4	6.28 ± 0.03	
Procaterol	-	81.4 ± 2.3	25.1	7.60 ± 0.14	
	ICI 118551 (1)	81.4 ± 2.9	87.9	7.06 ± 0.12	7.70 ± 0.31
	ICI 118551 (10)	80.5 ± 1.8	140.9	6.85 ± 0.09	
	ICI 118551 (100)	75.1 ± 3.9	220.8	6.66 ± 0.06	
Xamoterol	-	75.1 ± 3.6	101.9	6.99 ± 0.08	
	CGP 201712 (1)	72.8 ± 3.9	153.5	6.81 ± 0.11	7.12 ± 0.18
	CGP 201712 (10)	71.1 ± 2.5	301.3	6.52 ± 0.11	
	CGP 201712 (100)	69.3 ± 2.6	580.8	6.24 ± 0.12	

[a]E_{max} was defined as maximal relaxation of basal tone of the IAS smooth muscle elicited by the agonists in comparison to that by 50 mM EGTA. Data are expressed as the mean ± SE of n = 6-8 experiments.

[b]EC_{50} is defined as the concentration of the agonist that produces 50% relaxation of the IAS smooth muscle.

[c]$pEC_{50} = -\log EC_{50}$.

[d]pA2 is the negative log of the concentration of the antagonist that causes a two-fold increase in EC_{50}. This was calculated by extrapolation of respective Schild plots by Graphpad Prism 3.0.

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TABLE 3
K_i values of β-subtype selective antagonists from competition binding experiments with [^{125}I]CYP

Ligand	**High Affinity β-AR sites**		**Low Affinity β-AR sites**	
	[a]K_i (nM)	Hill Slope[b]	K_i (nM)	Hill Slope
CGP 20712A	114.1 ± 16.5	-0.88 ± 0.20	1783.0 ± 293.0	-0.73 ± 0.17
ICI 118551	30.4 ± 9.7	-0.55 ± 0.18	680.0 ± 220.0	-0.56 ± 0.10
SR 59230A	853.3 ± 120	-0.63 ± 0.15	48.1 ± 14.3	-0.86 ± 0.14

[a]K_i, values determined by Cheng-Prussoff equation as described in 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.

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Figure Legends

Fig. 1. A. Effect of β_3-agonist ZD 7114 on the basal tone of the IAS (shown as percent maximal fall in basal IAS tone) in the absence (control) and in the presence of the β_3-selective antagonist SR 59230A. SR 592330A causes significant and concentration-dependent rightward shift in the control concentration-response curve (CRC) of ZD 7114 (*; $p < 0.05$; n = 5 to 8). The values represent mean ± SE. **B.** Schild plot of different concentrations of SR 59230A vs. log(r-1) of ZD 7114. The pA_2 value of SR 59230A in antagonizing ZD 7114-induced relaxation of the IAS smooth muscle is 7.8. **C.** Influence of β_2- and β_1-AR antagonists ICI 118,551 and CGP 20712A respectively, on percent maximal fall in basal IAS tone. Data show that β_3-AR agonist-mediated fall in the basal tone of the IAS smooth muscle was not significantly modified by β_2- and β_1-AR antagonists ($p > 0.05$; n 5 to 8).

Fig. 2. A. CRC showing IAS smooth muscle relaxation by procaterol (β_2-agonist) before and after a selective β_2-antagonist ICI 118551. As shown, ICI 118551 causes a significant and concentration-dependent attenuation in the CRC of procaterol (*; $p < 0.05$; n 5 to 8). **B.** Schild plot of different concentrations of ICI 118551 vs. log(r-1) of procaterol. The pA_2 value ICI 118551 in antagonizing procaterol-induced relaxation of the IAS smooth muscle is 7.7. **C.** β_1- (CGP 20712A) and β_3-AR (SR 59230A) antagonists on the other hand have no significant effect on the IAS smooth muscle relaxation caused by procaterol ($p > 0.05$; n 5 to 8).

Fig. 3. CRC showing percent maximal fall in basal IAS tone with xamoterol (β_1-agonist) before and after different concentrations of CGP 20712A (β_1-antagonist). Data show CGP 20712A (with the exception of $1x10^{-8}$ M) causes a significant and concentration-dependent inhibition of

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the IAS smooth muscle relaxation by xamoterol (*; $p < 0.05$; n 5 to 8). **B.** Schild plot of different concentrations of CGP 20712A vs. log(r-1) of xamoterol. The pA_2 value SR in antagonizing xamoterol-induced relaxation of the IAS smooth muscle is 7.12. **C.** Influence of β_2- and β_3-AR antagonists ICI 118,551 and SR 59230A, respectively on percent maximal fall in basal IAS tone by xamoterol. Data show that β_1-AR agonist-mediated fall in the basal tone of the IAS smooth muscle was not significantly modified by SR 59230 ($p > 0.05$; n 5 to 8), but was modestly antagonized by ICI 118551 (*; $p < 0.05$; n 5 to 8).

Fig. 4. A. Binding of [^{125}I]Iodocyanopindolol to β-AR in IAS membrane preparations. Representative equilibrium binding curves show saturable and specific binding to β-AR. Membrane preparations of opossum IAS (40 μg/tube) were incubated with increasing concentrations of radioligand. Specific binding was quantified as described in Materials and Methods.

B. Sigmoid representation of the data illustrates the binding of [^{125}I]CYP over large concentration ranges from the high affinity site (pM) to the low affinity site (nM).

C. Scatchard transformation of the data reveals a curvilinear plot demonstrating the presence of two binding sites. Linear regression analysis resulted in a two-site binding model characterized by a high (K_d = 96 pM [^{125}I]CYP; B_{max} = 12.5 fmol/mg protein) and low (K_d = 1.96 pM [^{125}I]CYP; B_{max} = 58.7 fmol/mg protein) affinity site.

Fig. 5. Competition curves of β-AR selective antagonists for [^{125}I]CYP binding to opossum IAS membrane preparations at a high affinity site. Membrane preparations of opossum IAS (40 μg/tube) were incubated with 66 pM [^{125}I]CYP and increasing concentrations of subtype

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selective antagonists. The figure represents means ± SE from four displacement experiments. The corresponding inhibition constants were obtained by the Cheng-Prussof equation and are listed in Table 3.

Fig. 6. Competition curves of selective β-AR antagonists for [^{125}I]CYP binding to opossum IAS membrane preparations at a low affinity site. Membrane preparations of opossum IAS (40 μg/tube) were incubated with 1.61 nM [^{125}I]CYP and increasing concentrations of subtype selective antagonists. The figure represents means ± SE from four displacement experiments.

Fig. 7. A. Western blot analysis of β_1-AR, β_2-AR, and β_3-AR expression in the plasma membrane of rectum and IAS demonstrating relative distribution of expected size protein for β_1-AR, (63 kDa), β_2-AR (68 kDa), β_3-AR (65 kDa). The membrane protein (40 μg/well) was run on a 7.5% SDS-PAGE polyacrylimide gel, electrophoresed for 60 min, transferred to nitrocellulose membranes, and probed by isoform-specific antibodies for each β -AR protein. **B.** Densitometric analysis shows equal distribution of different β-AR in the IAS and rectum. Data are means ± SE of four experiments.

Fig. 8. A. Detection of β_1-AR, β_2-AR, and β_3-AR mRNAs (run in triplicate) in the circular smooth muscle layer of the opossum IAS. Expected sizes of PCR products were 608, 194, 444, and 387 bp for β_1-AR, β_2-AR, β_3-AR, and β-actin, respectively. PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide.

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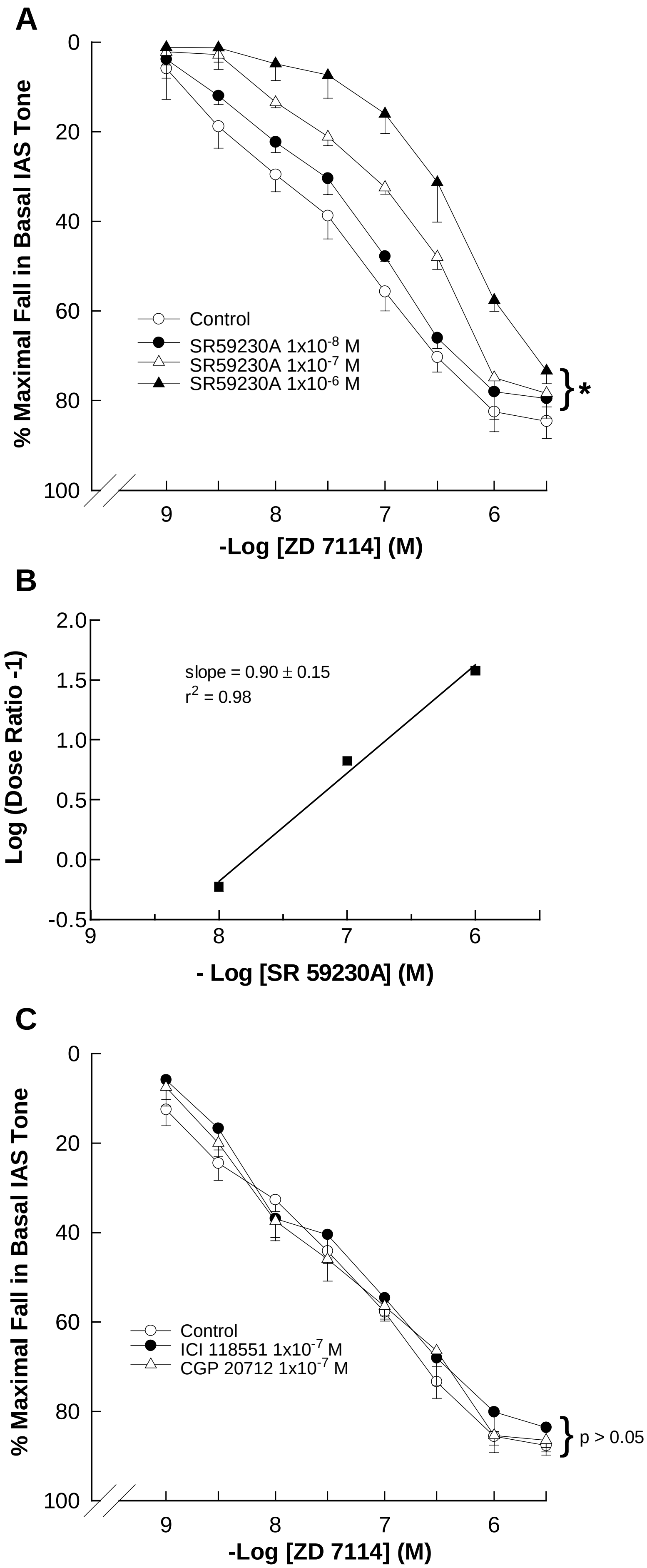

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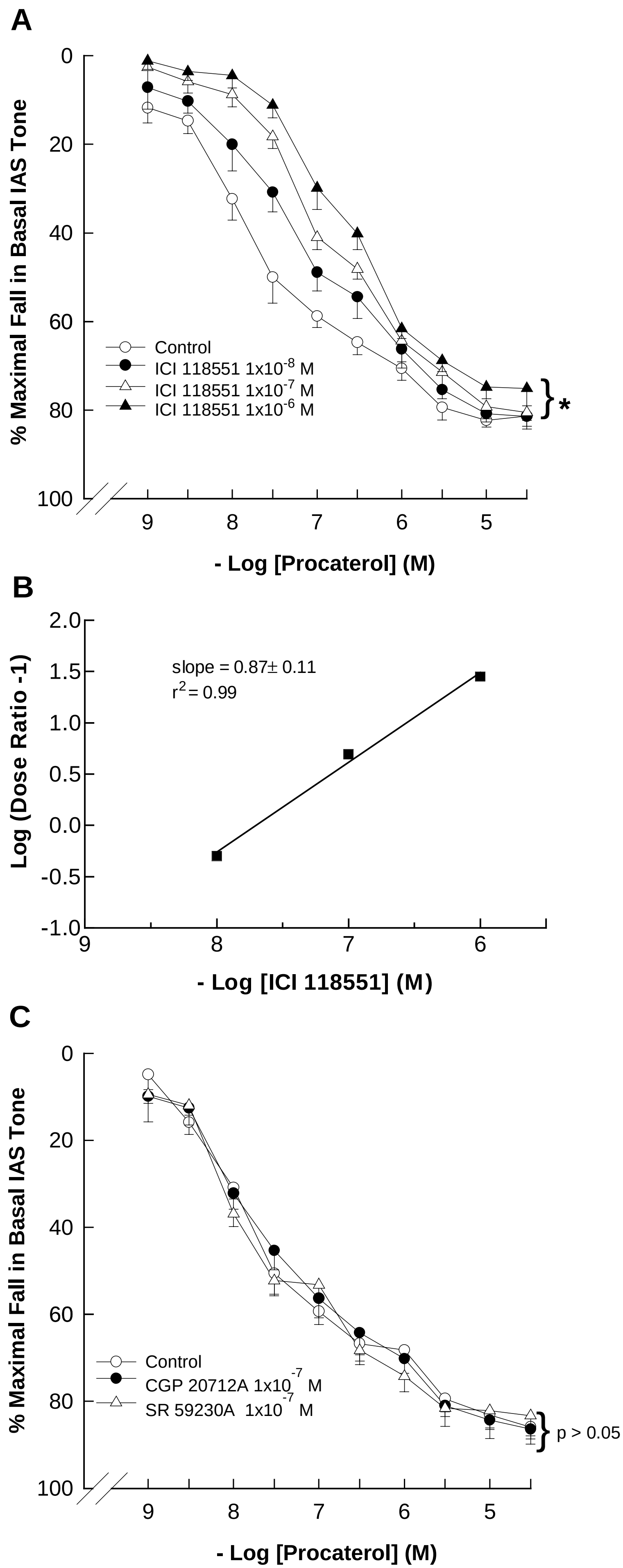

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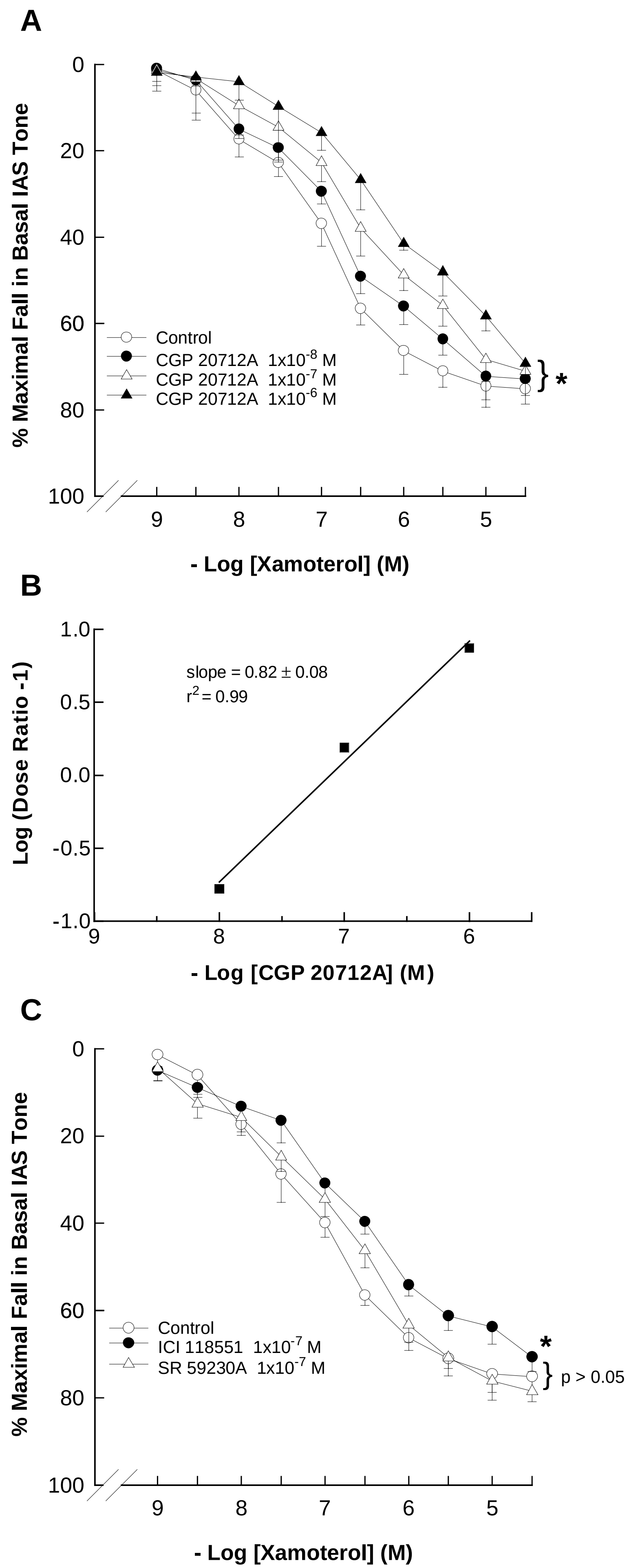

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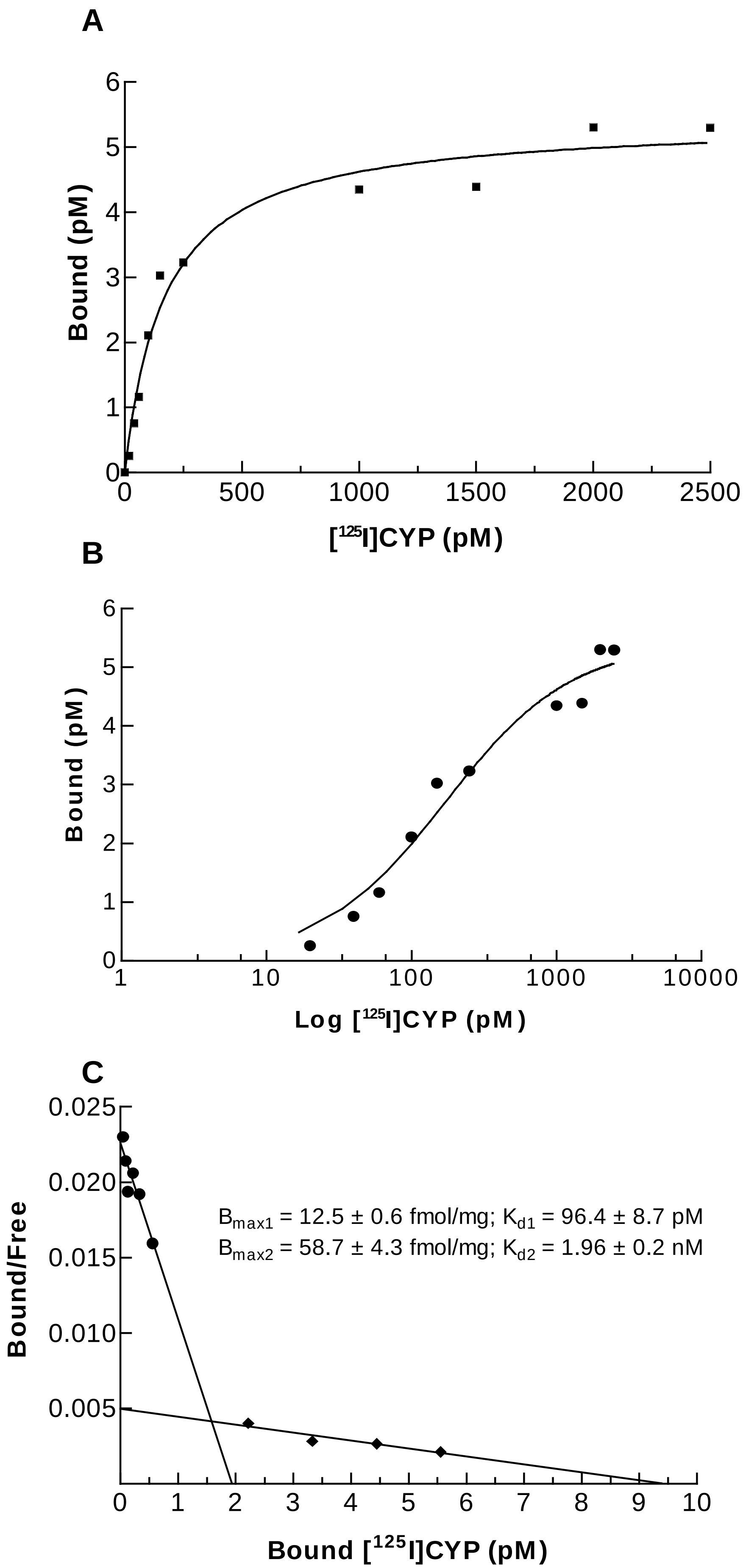

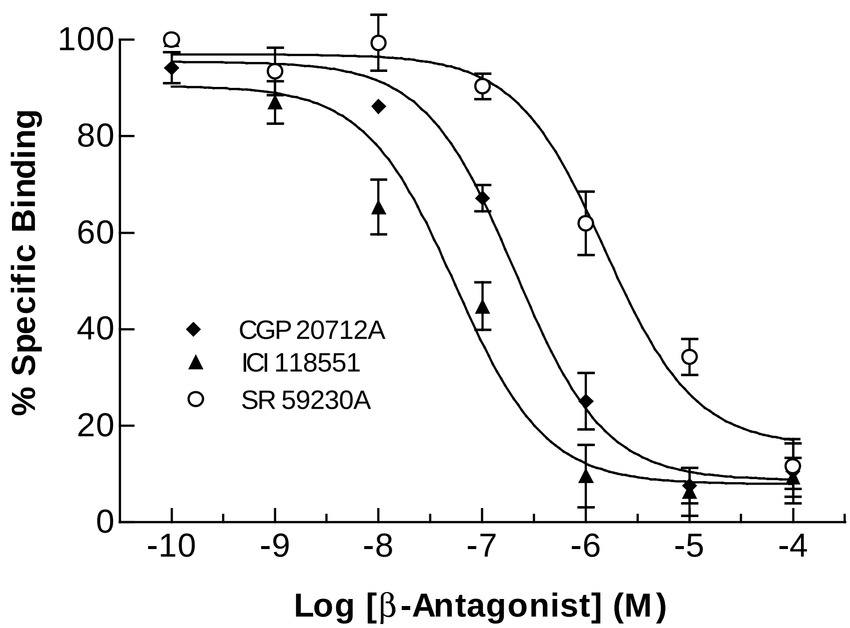
100
80
60
40
20
0
% Specific Binding
CGP 20712A
ICI 118551
SR 59230A
-10
-9
-8
-7
-6
-5
-4
Log [β-Antagonist] (M)

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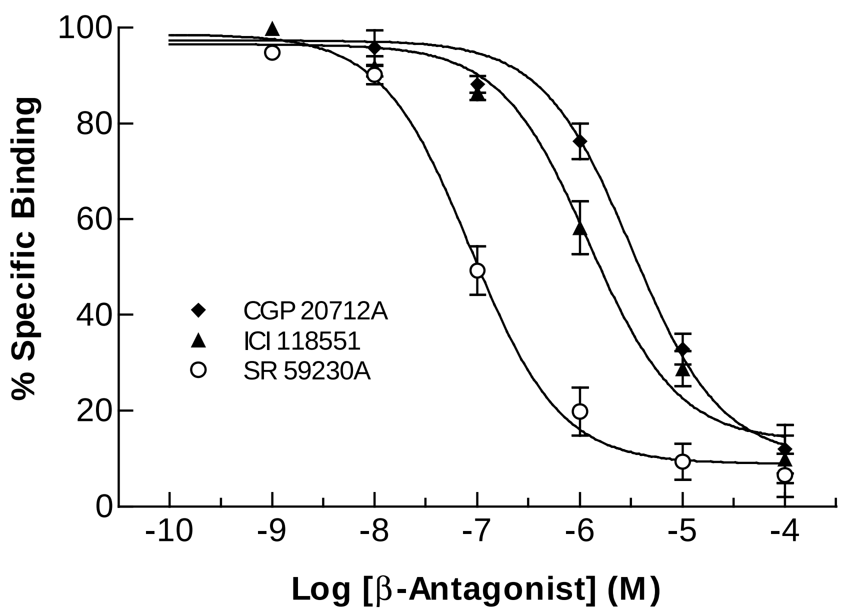

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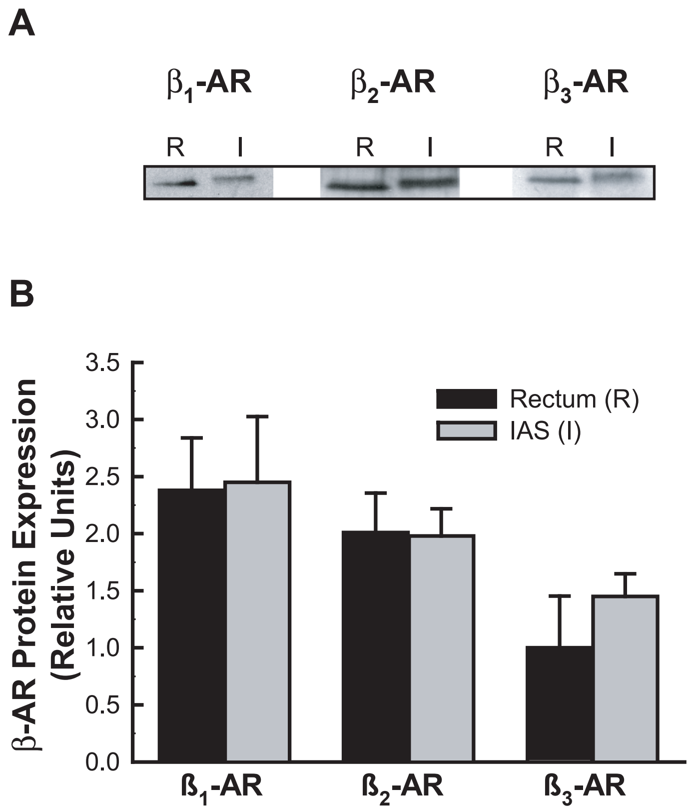

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