Role of nitric oxide in \( \beta_3 \)-adrenoceptor activation on basal tone of internal anal sphincter

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Banwait, Kuldip S. and Satish Rattan. Role of nitric oxide in \( \beta_3 \)-adrenoceptor activation on basal tone of internal anal sphincter. Am J Physiol Gastrointest Liver Physiol 285: G547–G555, 2003. First published May 7, 2003; 10.1152/ajpgi.00545.2002.—Effects of activation of \( \beta_3 \)-adrenoceptor (\( \beta_3 \)-AR) have not been determined in the spontaneously tonic smooth muscle of the internal anal sphincter (IAS). The effects of disodium (R,R)-5-[2-[2–3-chlorophenyl]-2-hydroxyethyl]-amino[propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316243), a selective \( \beta_3 \)-AR agonist, on the basal smooth muscle tone and direct release of nitric oxide (NO) by circular smooth muscle strips of the opossum IAS were determined. We also examined the presence of endothelial nitric oxide synthase (eNOS) protein by Western blot studies. CL 316243 produced a concentration-dependent relaxation of the smooth muscle that remained unmodified by different neurohumoral antagonists. The smooth muscle relaxation by CL 316243 was selectively antagonized by L 748337, a \( \beta_3 \)-selective antagonist. The effect of CL 316243 was accompanied by an increased release of NO; this was partly transduced via NOS, possibly smooth muscle nitric oxide synthase (NOS) activation may explain the characteristic IAS relaxation in response to activation of the \( \beta_3 \)-AR. It is well known that stimulation of \( \beta \)-ARs (\( \beta_1 \), \( \beta_2 \), or \( \beta_3 \)) causes release of \( G_{\alpha} \), which then activates adenylate cyclase to produce cAMP (34, 41). Elevated levels of cAMP produce relaxation of smooth muscle via activation of cAMP-dependent protein kinase (PKA). There are significant differences in the intracellular signaling pathways used by \( \beta_1 \) and \( \beta_2 \) vs. \( \beta_3 \)-AR subsequent to activation of PKA. Fully activated PKA may cause phosphorylation of \( \beta_1 \)- and \( \beta_2 \)-ARs, leading to their inactivation (7, 26). \( \beta_3 \)-ARs lack PKA phosphorylation sites; desensitization or internalization of \( \beta_3 \)-receptors is relatively less prevalent compared with \( \beta_1 \) and \( \beta_2 \)-receivers (15, 33). In addition, it has been shown that endothelial nitric oxide synthase (eNOS) may partly modulate relaxation of vascular smooth muscles in response to activation of the \( \beta_3 \)-AR (5, 40).

We determined the effect of selective \( \beta_3 \)- vs. nonselective \( \beta \)-AR agonists in the basal tone of the internal anal sphincter (IAS) and its mechanism of action. Effects of such agents on the basal IAS tone and understanding the basic mechanisms by which they induce relaxation of the smooth muscle may provide a basis for development of potential therapeutics for various conditions such as constipation with megacurema, anal fissures, and Hirschsprung’s disease. The purpose of the present investigation is twofold: 1) to examine effect of \( \beta_3 \)-AR activation in the basal IAS tone and 2) to examine the role of NOS in \( \beta_3 \)-AR-induced relaxation of the spontaneously tonic smooth muscle of the IAS.

MATERIALS AND METHODS

Preparation of smooth muscle strips. Smooth muscle strips from the IAS of opossums were prepared for recording of isometric tension as described previously (32). Briefly, the animals were anesthetized with pentobarbital sodium (40–50 mg/kg ip), and the entire anal canal was isolated and transferred to oxygenated (95% O\(_2\)-5% CO\(_2\)) Krebs physiological solution (in mM: 118.07 NaCl, 4.69 KCl, 2.52 CaCl\(_2\), 1.16 MgCl\(_2\), 1.2 H\(_2\)PO\(_4\), 25.2 H\(_2\)CO\(_3\), 15.5 glucose, 0.50 each of NaHCO\(_3\) and Ca-EDTA). The temperature was maintained at 37°C.

THE \( \beta_3 \)-ADRENOCEPTOR (\( \beta_3 \)-AR), discovered in 1989 (11), has been identified in different regions of gastrointestinal (GI) tract, ureter, urinary bladder, uterus, prostate, tate, white fat, and in the cardiovascular system in humans (1–3, 8, 12, 28, 31, 36). We have shown (10) that the characteristics of relaxation of the lower esophageal sphincter (LES) smooth muscle in response to activation of the \( \beta_3 \)-AR are very different from relaxation in response to activation of the \( \beta_1 \) or \( \beta_2 \)-AR: \( \beta_3 \)-AR activation by the selective agonist is characteristically antagonized by the \( \beta_3 \)-selective antagonist.
MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose. The IAS was carefully freed of striated muscle fibers belonging to the external anal sphincter as well as other extraneous tissues such as large blood vessels. The anal canal was opened and pinned flat onto a dissecting tray containing oxygenated Krebs solution; the mucosal side was bathed directly by the Krebs solution. The mucosal and submucosal layers were removed by dissection, and circular smooth muscle strips of the IAS circular smooth muscle (~1 × 10 mm) were prepared.

All protocols for use of animals were approved by Thomas Jefferson University’s Animal Care and Use Committee.

**Measurement of isometric tension.** The smooth muscle strips were secured at both ends with silk sutures and transferred to muscle baths that contained 2 ml oxygenated Krebs solution (35°C). One end of each smooth muscle strip was anchored at the bottom of the muscle bath, and the other end was attached to a force transducer (model FTO3; Grass Instruments, Quincy, MA) for the measurement of isometric tension on a computerized PowerLab/800. The smooth muscle tension in the basal state and following different stimuli was amplified by ETH-400 Bridge Amplifiers (CB Sciences, Dorset, VT). Baseline and optimal length (L₀) of the smooth muscle strips were determined as described previously (32). Only smooth muscle strips that developed steady-state tone. Baseline and optimal length (L₀) of the smooth muscle strips were determined as described previously (32). Only smooth muscle strips that developed steady-state tone and relaxed in response to electrical field stimulation (EFS) were included in the study. The studies employed 32 adult opossums of either sex. Each animal usually yielded three to four usable smooth muscle strips, and only one antagonist was used in each smooth muscle strip. n Refers to different smooth muscle strips from different animals.

Nonadrenergic noncholinergic nerve stimulation with EFS. EFS was delivered with a Grass stimulator (model S88; Grass Instruments) connected in series to a Med-Lab Stimu-Splitter II (Med-Lab Instruments, Loveland, CO). We used optimal stimulus parameters for the neural stimulation (10–15 V, 0.5-ms pulse duration, 4-s train) at frequencies that varied from 0.5 to 20 Hz. The electrodes used for the EFS consisted of a pair of platinum wires (model MA-6400; Grass Instruments) connected in series to a Med-Lab Stimu-Splitter II (Med-Lab Instruments, Quincy, MA). The signals were then processed through computer-based acquisition and data-analysis systems by use of a PowerLab/800 analog-to-digital converter (AD Instruments, Mountain View, CA) and recorded on a PC-based Hewlett-Packard computer (model HP Vectra VE, Hewlett-Packard, Palo Alto, CA) equipped with Windows 98. The data were analyzed with PowerLab software. The smooth muscle strips were stretched at 10 mN tension initially and allowed to equilibrate for at least 1 h with intermittent washings. During this period, the muscle strips developed steady-state tone. Baseline and optimal length (L₀) of the smooth muscle strips were determined as described previously (32). Only smooth muscle strips that developed steady tone and relaxed in response to electrical field stimulation (EFS) were included in the study. The studies employed 32 adult opossums of either sex. Each animal usually yielded three to four usable smooth muscle strips, and only one antagonist was used in each smooth muscle strip. n Refers to different smooth muscle strips from different animals.

Western blot studies. Western blot analysis of eNOS in the IAS smooth muscle of opossums was determined according to the protocol of BD Transduction Laboratories (San Diego, CA). Briefly, the circular smooth muscle of the IAS (after the removal of the mucosa, muscular mucosa, serosal, and longitudinal muscle layer) was carefully prepared. The IAS tissue thus prepared was cut into small pieces (1- to 2-mm cubes) and rapidly homogenized in boiling lysis buffer (1% SDS, 1.0 mM sodium orthovandate, 10 mM Tris, pH 7.4; 250 mg of tissue/3.5 ml of the buffer), then put into the microwave for 10 s. The homogenate was centrifuged (16,000 g at 15°C) for 5 min. The pellet was discarded, and the protein contents of the supernatants were determined by the method of Lowry et al. (27) using BSA as the standard. All of the samples were mixed with 2× sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) and heated by placing samples in a boiling water bath for 4 min. Total protein samples (0–40 μg) were loaded to commercially available 7.5% polyacrylamide PAGEr Gold Gel (BioWhitaker Molecular Applications, Rockland, ME) and applied to 7.5% SDS-PAGE by the method of Laemmli (25). The separated proteins were electrophoretically transferred to nitrocellulose membrane (NCM) at 4°C. To block nonspecific antibody binding, the NCM were immersed overnight at 4°C in Super Block Tris-buffered saline (TBS) blocking buffer in TBS (Pierce Biotechnology, Rockford, IL). The NCM were incubated with 1:1,000 diluted primary eNOS antibody (Ab) (BD Transduction Laboratories) for 1 h at room temperature. After being washed with the blocking buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary Ab (1:1,500) (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. A separate NCM was incubated with primary Ab for platelet endothelial marker (platelet endothelial cell adhesion molecule (PECAM-1), Santa Cruz, CA) at a dilution of 1:1,000 and subsequently, after being washed, incubated at 1:1,500 dilution of HRP-conjugated donkey anti-rabbit IgG secondary Ab (Santa Cruz) (1:1,500). The membranes were blotted semidry by placing them in between two filter papers, and bands were identified by chemiluminescence using the enhanced chemiluminescence (ECL) detection system and Hyperfilm ECL (Amersham Pharmacia Biotech, Little Chalfont, UK). The bands were then scanned (Astra 2420, UMAX, Dallas, TX), and their relative densities were determined using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

**Determination of NO.** Measurement of total NO (NO + NO₂ + NO₃⁻) was used as a measure of release of NO by the tissues. Once released, NO is rapidly oxidized to NO₂ + NO₃⁻. Therefore, it is necessary to measure all forms of NO (17) to determine accurately the amount of NO released by tissues. Total NO was determined with a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) specifically designed for this purpose. Per fusates were collected from the muscle baths before treatment of the smooth muscle strips with any agents (basal condition) and after treatment with either disodium (R,R)-5-[2-[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316243; 3 × 10⁻⁶ M) or isoproterenol (1 × 10⁻⁶ M). Such perfusates were collected before and after selective NO donors. After treatment with CL 316243 or isoproterenol, perfusates (100 μl) were collected at the onset of a fall in the basal IAS tone and transferred immediately to 1.5-ml vials at −20°C before analysis for content of NO₃⁻.

A commercially available (Cayman Chemical) nitrate/nitrite colorimetric assay kit was used to measure the total nitrate/nitrite concentration in a simple two-step process in which 1) nitrate in the sample is converted to nitrite by nitrate reductase and 2) nitrite is converted to a deep purple azo compound on the addition of Griess Reagent (17, 35). Eighty microliters of each sample were pipetted into a 96-well plate, and absorbance at 540 nm was determined using a spectrophotometer. Nitrite concentration in each sample was calculated by comparison with a standard curve generated immediately before analysis of the test samples. The standards and the test samples were analyzed in duplicate. Drugs and chemicals. The following chemicals were used in the study: N°-nitro-l-arginine (l-NNA); l-N°-l-(1-iminoethyl)ornithine hydrochloride (l-NIO; eNOS inhibitor) (9); 1-[2-(trifluoromethylphenyl)imidazole (TRIM; nNOS inhibitor) (19); CL 316243; isoproterenol hydrochloride, atropine sulfate, guanethidine, pyrilamine, cimetidine, indomethacin, and guanosine 5′-triphosphate (GTP) solution.
sodium nitroprusside, sodium nitrate (Sigma, St. Louis, MO); TTX (Calbiochem, San Diego, CA); VIP-(10–28) (Peninsula Laboratories, Belmont, CA); N-(3-aminomethyl)benzyl acetamide 2HCl (1400D; INOS inhibitor; Alexis Biochemicals, San Diego, CA) (14); NO (Matheson Gas, Bridgeport, NJ); and EGTA tetrasodium (Fisher Scientific, Pittsburgh, PA).

When possible, all chemicals were dissolved and diluted in Krebs solution on the day of the experiment. Vials and pipette tips were siliconized, and the muscle baths were treated with 2.5% bovine serum albumin to reduce binding of peptides to the surface of the glass or plastic.

Drug responses. Pretreatment with different concentrations of NOS inhibitors (1 × 10^{-6} to 1 × 10^{-4} M) was used to examine their effects on decrease in the basal IAS tone by CL 316243, isoproterenol, or an NO donor [sodium nitroprusside (SNP)]. All agonists were given in a cumulative fashion. Once the concentration response curve (CRC) for an agent was determined, the smooth muscle strips were washed at least six times and resting tone was allowed to recover to the original level. The effects of the various agonists, administered at their EC_{max}, on NO release were determined in the presence or absence of selective inhibitors (1 × 10^{-6} to 1 × 10^{-4} M) of NOS.

Data analysis. The fall in the basal IAS tone is expressed as the percent maximal fall (100%) induced by a supramaximal concentration (5 mM) of EGTA (4). Statistical significance between different groups was determined by using paired or unpaired t-tests. ANOVA was performed to compare the entire frequency-response curve or CRC before and after administration of NOS inhibitors. The results are expressed as means ± SE of different experiments. A P value <0.05 was considered statistically significant.

RESULTS

Effect of β3-AR agonist CL 316243: influence of β3-AR selective antagonist propranolol on IAS smooth muscle relaxation by CL 316243. β3-AR selective agonist CL 316243 and nonselective agonist isoproterenol caused a concentration-dependent fall in the basal tone of the IAS (Figs. 1 and 2). Maximal relaxation with CL 316243 ranged from 74 to 85% with 3 × 10^{-6} M. EC_{50} for the β3-AR agonist in these experiments was 1.5 × 10^{-7} M. The agonist was found to be several times more potent in the IAS than in the LES, where EC_{50} was 6 × 10^{-7} M and EC_{max} was 1 × 10^{-4} M (unpublished data).

The IAS smooth muscle relaxation caused by CL 316243 but not that by isoproterenol was selectively antagonized by L 748337 in a concentration-dependent manner (*P < 0.05; n = 5–8; Fig. 1). The basal IAS tone in CL 316243 and isoproterenol experiments was 1.48 ± 0.18 and 1.52 ± 0.12 g, respectively. The smooth muscle tone was not significantly modified by the β3-antagonist. The corresponding values of basal IAS tone in these experiments in the presence of L 748337 was 1.35 ± 0.23 and 1.38 ± 0.18 g, respectively (*P > 0.05; n = 5–8). Conversely, the IAS smooth muscle relaxation by isoproterenol but not by CL 316243 was significantly and concentration dependently antagonized by propranolol (*P < 0.05; n = 5–8; Fig. 2). The lower concentration of propranolol (that causes a significant rightward shift in the CRC of isoproterenol; Fig. 2B)

Fig. 1. Influence of the selective β3-adrenoceptor (β3-AR) antagonist L 748337 (1 × 10^{-6} and 3 × 10^{-8} M) on internal anal sphincter (IAS) smooth muscle relaxation induced by CL 316243 (A) and isoproterenol (ISO; B). Note that L 748337 produces a concentration-dependent and significant antagonism of CL 316243-induced relaxation of the IAS (*P < 0.05; n = 5–8), whereas it has no significant effect on the effects of ISO (*P > 0.05; n = 5–8). The data points in this and the subsequent figures represent means ± SE.

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had no significant effect on CL 316243-induced fall in the basal IAS tone \((P > 0.05; n = 5–8; \text{Fig. 2A})\). Only a higher concentration of propranolol caused a moderate antagonism of CL 316243 effect \((*P < 0.05; n = 5–8)\).

Relaxation of the IAS smooth muscle induced by CL 316243 was not modified by neither neurohumoral inhibitors nor the neurotoxin TTX \((1 \times 10^{-6} \text{ M}; P > 0.05; n = 5–8)\). Different neurohumoral inhibitors examined were atropine (muscarinic antagonist; \(1 \times 10^{-6} \text{ M}\)), guanethidine (adrenergic blocking agent; \(3 \times 10^{-6} \text{ M}\)), pyrilamine and cimetidine (H\(_1\) and H\(_2\) histamine receptor antagonists, respectively; \(1 \times 10^{-5} \text{ M}\)), indomethacin (prostaglandin synthetase inhibitor; \(1 \times 10^{-5} \text{ M}\)), and VIP-(10–28) (VIP antagonist; \(1 \times 10^{-6} \text{ M}\)). These data suggest that CL 316243 directly induces relaxation of the IAS smooth muscle via selective activation of \(\beta_3\)-ARs. Further support for the notion that CL 316243 acts directly on smooth muscle cells (SMC) of the IAS is the observation that it induced relaxation of the isolated SMCs (data not shown).

Time course for the effects of CL 316243 and isoproterenol on the basal tone of the IAS smooth muscle. Relaxation of the IAS smooth muscle induced by CL 316243 was significantly longer in duration than that induced by isoproterenol (Fig. 3). The IAS relaxation by isoproterenol recovered to near original baseline within 20 min. By contrast, the total duration of IAS relaxation with CL 316243 was \(240 \pm 20 \text{ min}\). In these series of experiments with CL 316243, the basal tone recovered to \(~70\%\) of the original level at the end of 3 h.

Effects of L-NNA, a general inhibitor of NOS, on IAS smooth muscle relaxation by either CL 316243 or isoproterenol. IAS smooth muscle was treated with L-NNA, a general inhibitor of NOS, followed by either CL 316243 or isoproterenol. As shown, the smooth muscle relaxation by CL 316243 is of significantly longer duration compared with ISO. The IAS relaxation by ISO recovers to near original baseline within 20 min, whereas with CL 316243 it takes \(>3 \text{ h}\) for plateau to occur. At the end of 3 h, the basal IAS tone following CL 316243 recovers to \(~70\%\) of original tone.
316243 or isoproterenol to determine whether NOS had a role in agonist-induced relaxation of this muscle. L-NNA caused significant attenuation of the CL 316243-induced fall in basal IAS tone (\( *P < 0.05; n = 5–8 \)) (Fig. 4A) but had no significant effect on the isoproterenol-induced fall in IAS tone (Fig. 4B). In these experiments, CL 316243 (\( 3 \times 10^{-6} \) M) caused a 75.03 ± 1.7% fall in the basal IAS tone that was attenuated to 35.6 ± 2% following the pretreatment with L-NNA.

**Effects of different NOS inhibitors on IAS smooth muscle relaxation induced by either CL 316243 or isoproterenol.** The decrease in IAS smooth muscle tone induced by CL 316243 was not significantly affected by a selective inhibitor of either nNOS (TRIM) or of iNOS (1400DW; \( 1 \times 10^{-6}; 1 \times 10^{-5} \), and \( 1 \times 10^{-4} \) M; Figs. 5, A and B; \( P > 0.05; n = 5–8 \)).

In contrast, L-NIO (1 \( \times 10^{-6} \) and 1 \( \times 10^{-5} \) M) significantly attenuated the CL 316243-induced fall in IAS tone in a concentration-dependent fashion (\( *P < 0.05; n = 5–8 \); Fig. 6). L-NIO caused no significant effect on IAS smooth muscle relaxation by SNP (\( P > 0.05; n = 5–8 \); Fig. 7). The IAS smooth muscle relaxation caused by EFS was, however, partially but significantly attenuated by L-NIO (\( *P < 0.05; n = 5–8 \)).

In these series of experiments, the overall CRC for the fall in the basal IAS tone by different concentrations of isoproterenol was not affected significantly by any of the inhibitors of NOS (\( P > 0.05; n = 5–8 \); Fig. 8).

**Presence of eNOS protein in the IAS smooth muscle.** Western blot studies revealed the presence of eNOS-specific protein in the circular smooth muscle of the IAS (Fig. 9). The expression of eNOS in the IAS smooth muscle was found to be significant and specific, because PECAM-1 (an endothelial cell marker) was poorly expressed under the identical experimental conditions.

**Effect of CL 316243 on NO release in the IAS smooth muscle before and after different inhibitors: comparison with isoproterenol and EFS.** To ascertain the direct release of NO and the nature of NOS involved, NO release was determined by measuring the amount of total NOX as explained in MATERIALS AND METHODS. The standard curves for NOX generated by the addition of sodium nitrate (from 1 to 100 nmol) were linear with a coefficient of correlation (\( r \)) of 0.993. CL 316243 (\( 3 \times 10^{-6} \) M) caused a significant increase in the basal levels of NO in the IAS smooth muscle. The eNOS inhibitor L-NIO selectively and significantly blocked this increase in NO release (Fig. 10). The basal release from the IAS smooth muscle tissues was 19 ± 4 nmol, and CL 316243 increased these levels to 38 ± 9 nmol. L-NIO (1 \( \times 10^{-5} \) M) attenuated CL 316243-induced increased NO release to 20 ± 3 nmol (\( *P < 0.05; n = 4 \); Fig. 10). Conversely, neuronal and inducible NOS inhibitors TRIM and 1400DW, respectively, had no significant effect on CL 316243-induced increase in the levels of NO release (Fig. 10; \( P < 0.05; n = 4 \)).

We also monitored the release of NO following isoproterenol (1 \( \times 10^{-6} \) M) and EFS (10–15 V, 0.5-ms pulse duration, 4-s train, and 5 Hz). EFS caused a significant increase in the NO release; this was partly attenuated by TRIM but not by 1400DW and L-NIO. Isoproterenol did not produce a significant increase in NO release in the IAS smooth muscle.
DISCUSSION

This is the first report on the effects and mechanism of action of β3-AR agonist in the spontaneously tonic smooth muscle of the IAS. The studies suggest that β3-AR agonist causes potent and prolonged relaxation of the IAS via β3-AR distinct from the conventional β-ARs known to produce relaxation of the gastrointestinal smooth muscles. In addition, the relaxation of the IAS smooth muscle following β3-AR stimulation uses a novel mechanism involving activation of NOS.

**Fig. 5.** Influence of the neuronal NOS (nNOS) inhibitor 1-[2-(trifluoromethylphenyl)imidazole (TRIM; 1 \times 10^{-6}, 1 \times 10^{-5}, and 1 \times 10^{-4} M) (A) and inducible NOS (iNOS) inhibitor N-(3-aminomethyl)benzyl acetamide 2HCl (1400DW; 1 \times 10^{-6}, 1 \times 10^{-5}, and 1 \times 10^{-4} M) (B) on IAS smooth muscle relaxation induced by CL 316243. Note that the % maximal fall in IAS tone induced by CL 316243 is not significantly modified by inhibitors of n- or iNOS (P > 0.05; n = 5–8).

**Fig. 6.** Influence of putative eNOS inhibitor L-N5-(1-iminoethyl)ornithine dihydrochloride (l-NIO; 1 \times 10^{-6} and 1 \times 10^{-5} M) on IAS smooth muscle relaxation induced by CL 316243. l-NIO produces a concentration-dependent and significant attenuation of IAS smooth muscle relaxation induced by CL 316243 (\*P < 0.05; n = 5–8).

**Fig. 7.** Comparison of the influence of L-NIO on IAS smooth muscle relaxation induced by different agonists and nonadrenergic, noncholinergic nerve stimulation by electrical field stimulation (EFS). As shown, L-NIO produces selective and significant attenuation of IAS smooth muscle relaxation induced by CL 316243 and EFS (\**P < 0.01; \*P < 0.05; n = 5–8) but not that induced by ISO or sodium nitroprusside (SNP).
Data suggest that the IAS relaxation by CL 316243 is caused by the activation of the $\beta_3$-AR in the smooth muscle. Relaxation of the IAS smooth muscle induced by CL 316243 was not antagonized by propranolol in a concentration-dependent manner known to primarily block the effects of $\beta_1$- and $\beta_2$-AR activation. This finding supports the suggestion that CL 316243 is an agonist for $\beta_3$-AR and induces relaxation of the IAS smooth muscle via those specific receptors. This suggestion is supported further by our finding that $\beta_3$-AR antagonist L 748337 (6) inhibits CL 316243-induced relaxation of the IAS smooth muscle in a concentration-dependent manner. Conversely, the smooth muscle relaxation by isoproterenol is antagonized by propranolol but not by L 748337. Different neurohumoral inhibitors and neurotoxin TTX do not modify the smooth muscle relaxation caused by CL 316243. The direct action of $\beta_3$-agonist at the smooth muscle is further demonstrated by its inhibitory action at the SMC.

We further investigated the intracellular mechanism of IAS smooth muscle relaxation by $\beta_3$-AR activation.

Fig. 8. Influence of n-, i-, and eNOS inhibitors, TRIM, 1400DW, and L-NIO (1 × 10^{-5} M), respectively, on ISO-induced relaxation of the IAS smooth muscle. In contrast to the effect of CL 316243, the IAS smooth muscle relaxation induced by ISO is not significantly affected ($P > 0.05; n = 5–8$) by L-NIO.

Fig. 9. Western blot of eNOS protein in the circular smooth muscle of the IAS (IAS-CSM) using specific eNOS antibody (top) and the corresponding densitometric analysis (bottom). Different amounts of sample proteins (5, 10, 20, and 40 µg) of IAS-CSM are run on SDS-PAGE, blotted onto nitrocellulose membrane, and incubated with anti-eNOS (1:1,000). The intensity of the eNOS signal is dependent on the amount of protein sample used. Not shown, under the identical experimental conditions and the exposure time, the levels of platelet endothelial cell adhesion model were virtually undetectable.

Fig. 10. Release of NO in the IAS smooth muscle in response to CL 316243 (3 × 10^{-6} M) or ISO (1 × 10^{-6} M) before and after treatment with L-NIO. CL 316243 causes a significant increase in release of NO (*$P < 0.05; n = 4–5$). Among different NOS inhibitors examined, the increase in NO release is selectively attenuated by L-NNA and L-NIO (**$P < 0.05; n = 4–5$). In contrast, ISO does not stimulate a significant increase in the release of NO before or after treatment of the IAS smooth muscle with L-NIO ($P > 0.05; n = 4$).

Data suggest that the IAS relaxation by CL 316243 is caused by the activation of the $\beta_3$-AR in the smooth muscle. Relaxation of the IAS smooth muscle induced by CL 316243 was not antagonized by propranolol in a concentration-dependent manner known to primarily block the effects of $\beta_1$- and $\beta_2$-AR activation. This finding supports the suggestion that CL 316243 is an agonist for $\beta_3$-AR and induces relaxation of the IAS smooth muscle via those specific receptors. This suggestion is supported further by our finding that $\beta_3$-AR antagonist L 748337 (6) inhibits CL 316243-induced relaxation of the IAS smooth muscle in a concentration-dependent manner. Conversely, the smooth muscle relaxation by isoproterenol is antagonized by propranolol but not by L 748337. Different neurohumoral inhibitors and neurotoxin TTX do not modify the smooth muscle relaxation caused by CL 316243. The direct action of $\beta_3$-agonist at the smooth muscle is further demonstrated by its inhibitory action at the SMC.

We further investigated the intracellular mechanism of IAS smooth muscle relaxation by $\beta_3$-AR activation.
In our preliminary studies, the smooth muscle relaxation by CL 316243 was inhibited by the general NOS inhibitor L-NNA, suggesting the involvement of NOS. To investigate the nature of NOS involved, we examined the influence of selective NOS inhibitors. Detailed experimentation with selective inhibitors of different NOS isozymes revealed that only L-NIO causes a substantial and concentration-dependent attenuation of the smooth muscle relaxation by CL 316243. Inhibitors of inducible and neuronal NOS have no significant effect. Our Western blot studies using specific eNOS antibody further show the presence of eNOS in the circular smooth muscle of the IAS. The conclusion is supported by recent findings in a different system, where it was shown that activation of β3-AR causes vascular smooth muscle relaxation by activation of eNOS (5, 40).

It is quite clear that the enzymatic activity of eNOS in IAS smooth muscle is increased on stimulation of the β3-adrenoceptor and is associated with relaxation of that smooth muscle. That is, inhibitor of eNOS attenuates the production of NO and the relaxation of the IAS smooth muscle in response to CL 316243. Furthermore, increased production of NO is unique to the β3-AR, because treatment of IAS smooth muscle with isoproterenol (primarily β1/β2-AR) does not stimulate production of NO. eNOS responsible for β3-AR-mediated relaxation of IAS smooth muscle may be present in the SMC. The role of eNOS from endothelial cells in our smooth muscle preparations is expected to be minimal because of the limited presence of endothelial cells. In addition, the inhibitory effect of CL 316243 is preserved in the presence of different neurohumoral inhibitors and is demonstrated directly in the isolated SMC. Therefore, we speculate that the β3-AR are present in the SMC and that relaxation of the IAS smooth muscle is partly mediated by direct interaction of agonists with those receptors. Western blot studies further delineate the presence of eNOS in the IAS smooth muscle. PECAM-1, on the other hand, was not found in significant amounts. Other investigators (13) have also found eNOS to be present in SMCs of the GI tract and to be involved in smooth muscle relaxation by a number of neurohumoral agonists. However, the role for NOS pathway in β-AR agonist-induced relaxation of the GI smooth muscle has not been examined before.

Recent studies by König et al. (24) in the GI tract are in general agreement with the concept that eNOS is not restricted to endothelial cells and that it also occurs in other cells such as interstitial cells of Cajal (ICC) (42) and SMC (39). The proposed role of SMC eNOS in the present studies is different from previous studies in the vascular smooth muscle where β3-agonist-induced relaxation is mediated primarily by eNOS in the endothelium (5, 40). Interestingly, in heart, eNOS present in cardiac myocytes has been shown to be involved in the negative inotropic effect of β3-AR activation (16). Additional studies are needed to ascertain the definitive and relative roles of eNOS in the SMC vs. the endothelial cells and ICC in the IAS smooth muscle relaxation by β3-AR activation.

β3-agonist-induced prolonged relaxation of the IAS smooth muscle may have important therapeutic implications in conditions such as anal fissures, Hirschsprung’s disease, irritable bowel syndrome, severe constipation due to spastic gut, and achalasia. Presently, such conditions are being treated with topical glyceryl trinitrate (GTN), cardizem ointment, botulinum toxin injection, and surgical intervention (21, 22, 29, 37, 38). One of the deleterious effects of botulinum toxin injection includes fecal incontinence, whereas topical nitrites cause headaches in 20–100% of cases. Surgical intervention may lead to long-term complications (notably incontinence) (30). It is of interest that both GTN and β3-agonist act via the release of NO, but β3-agonist has fewer cardiovascular side effects (10).

β-ARs, especially β3-AR activation-mediated smooth muscle, involve a number of intracellular mechanisms. In the present investigation, we did not examine the relative role of other intracellular mechanisms such as cAMP/PKA (34, 41), decrease in free intracellular Ca2+ levels (18, 20, 23), and lack of PKA phosphorylation sites in β3- vs. β1- and β2-receptors (7, 26). We speculate that a combination of the NOS activation and other intracellular events may be responsible for the prolonged IAS smooth muscle relaxation by β3-agonists.

In summary, β3-AR agonists possess unique characteristics as prolonged IAS smooth muscle relaxation and a novel mechanism of action via the activation of NOS. This combined with other intracellular pathways may be responsible for the prolonged IAS smooth muscle relaxation. These attributes and minimal cardiovascular side effects suggest that selective β3-agonists may have clinical relevance for treating anorectal motility disorders.

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DISCLOSURES

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