Role of Adenylate and Guanylate Cyclases in $\beta_1$-, $\beta_2$-, and $\beta_3$-Adrenoceptor-Mediated Relaxation of Internal Anal Sphincter Smooth Muscle

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ABSTRACT

The purpose of the present study was to ascertain the role of adenylate (AC) versus guanylate cyclase (GC) signaling pathways in the internal anal sphincter (IAS) smooth muscle relaxation by $\beta_1$, $\beta_2$, and $\beta_3$-adrenoceptor (AR) activation by xamoterol, procaterol, and disodium 5-[(2R)-2-(3-chlorophenyl)-2-hydroxy-ethyl]amino)piperyl]1,3-benzodioxole-2,2-dicarboxylate (CL 316243), respectively. The above-mentioned agonists produced concentration-dependent relaxation of the smooth muscle strips. Both the selective $G_{\alpha}\alpha$ and $G_\alpha$ antagonists, 8,8'-[carbonylbis(imino-3,1-phenylene)]bis-(1,3,5-naphthalene trisulfonic acid) (NF 023) and 4,4',4''-[carbonylbis(imino-5,1,3-benzenetetrabenzyl(dimine)])tetrakis-benzene-1,3-disulfonic acid (NF 449), respectively, inhibited the relaxation induced by procaterol. However, only NF 023 inhibited the relaxation induced by xamoterol and CL 316243. 1H[1,2,4]Oxadiazol[4,3-a]quinolin-1-one, a soluble GC inhibitor, significantly inhibited the relaxation induced by different agonists. In contrast, the selective AC inhibitor [9-(tetrahydro-2'-furyl)adenine] (SQ 22536) inhibited only the relaxation induced by procaterol. (9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-I][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720), a CAMP-dependent protein kinase inhibitor, attenuated the relaxation by procaterol, whereas (9S,10R,12R)-2,3,9,10,11,12, hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-I][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT 5823), a selective cGMP-dependent protein kinase (PKG) inhibitor, attenuated the relaxation induced by xamoterol and CL 316243. Xamoterol produced significant increase in cGMP levels, whereas only procaterol enhanced the cAMP levels. Western blot analysis confirmed the presence of $\beta_1$, $\beta_2$, and $\beta_3$-AR subtypes in the IAS. In summary, $\beta_3$-AR activates both $G_{\alpha}G_{\alpha}$ and $G_{\alpha}\alpha$-protein subunits and induces relaxation in the rat IAS via both CAMP/cGMP pathways. In contrast, the $\beta_1$/$\beta_2$-ARs activation causes the smooth muscle relaxation via $G_{\alpha}G_{\alpha}$-protein subunit/GC/GMP/PKG pathway. These studies are important for the understanding of intracellular mechanisms underlying IAS smooth muscle relaxation and in turn the pathophysiology of certain anorectal motility disorders.
prolonged relaxation without untoward effects (Cook et al., 2001; DiMarino et al., 2002). Some reports have well described that the gastrointestinal smooth muscles relax in response to postjunctional β-adrenoceptors (β-ARs) activation (Manara et al., 1995; Gauthier et al., 2000), making these receptors a target for therapeutic potential.

One of the problems in delineating the pharmacology of β-ARs in the gastrointestinal tract has been the lack of subtype-selective ligands. This problem was partially solved with the introduction of the β1- and β2-selective agonists xamoterol (Malta et al., 1985) and procaterol (Kotsonis and Majewski, 1994), respectively. Because of significant cardiovascular effects associated with β1- and β2-AR activation (Brodde, 1991), there is less interest in the pursuit of β-AR agonists for their therapeutic use in gastrointestinal motility disorders. β1-AR agonists have emerged as potential therapeutic agents for several gastrointestinal motility disorders. Recent studies have shown that CL 316243, a potent and selective β2-AR agonist (Takeda et al., 2000), produces prolonged relaxation of the IAS without the undesirable systemic effects of the β1 and β2 types. The same has been shown by in vivo studies in the lower esophageal sphincter of opossums (DiMarino et al., 2002). Despite the information on β-ARs in different mammalian tissues (Manara et al., 1995; Gauthier et al., 2000), little is known about the signal transduction mechanisms underlying the relaxation of gastrointestinal tract smooth muscles, especially the IAS.

It has been suggested that the activation of β-ARs produces smooth muscle relaxation via increase in the intracellular levels of cyclic nucleotides, such as cyclic adenosine 3′,5′-cyclic monophosphate (cAMP) and cyclic guanosine 3′,5′-cyclic monophosphate (cGMP), the products of adenylyl (AC) and guanylate (GC) cyclase activation as shown in the rabbit urinary bladder (Morita et al., 1992) and in the rat corpus cavernosum (Andersson, 2001). These data are in line with previous works showing that cAMP and cGMP constitute major pathways for the IAS and lower esophageal sphincter smooth muscle relaxation (Rattan and Moumni, 1989; Baird and Muir, 1990; Joslyn et al., 1990; Szewczak et al., 1990; Murray et al., 1992).

Rathi et al. (2003) reported the functional and molecular characterization of β-ARs in the opossum IAS. The authors identified three different types of β-ARs mediating relaxation: the β1, β2, and β3. However, intracellular messengers activated in response to β-AR were not analyzed. Because the IAS has significant importance in the pathophysiology of a number of motility disorders (Lennard-Jones, 2000; Schiller, 2000), patients could benefit from agents that cause selective and prolonged relaxation of the IAS. Selective and prolonged relaxation of the IAS smooth muscle has been reported in our recent studies with β2-AR activation (Banwait and Rattan, 2003). The present study was designed to characterize the signaling pathways underlying the activation of β-ARs in the IAS smooth muscle. The studies were carried out in rat tissues because recent studies have shown that for the IAS investigations, rat is an equally good animal model compared with other species (Fan et al., 2002). The routing of intracellular pathways related to AC and GC were followed by using selective inhibitors of the sequential events. Direct measurements of tissue cAMP/cGMP levels after selective β-ARs activation was measured by enzyme immunoassay. In addition, the presence of membrane bound β-AR was determined through Western blot analysis.

Materials and Methods

Tissue Preparation. Male Sprague-Dawley rats (300–350 g) were humanely killed by decapitation, and the anal canal with an adjacent region of the rectum was quickly removed and transferred to oxygenated (95% O2 + 5% CO2) Krebs’ physiological solution of the following composition: 118.07 mM NaCl, 4.69 mM KCl, 2.52 mM CaCl2, 1.16 mM MgSO4, 1.01 mM NaH2PO4, 25 mM NaHCO3, and 11.10 mM glucose at 37°C. Extrinsic tissue and the external anal sphincter connected to the IAS were carefully removed by sharp dissection. The anorectal region was then opened and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs’ physiological solution. After the removal of the mucosa, the IAS was identified as the thickened circular smooth muscle of the lowermost part of the anal canal. The fine dissection to prepare the smooth muscle strips (∼1 mm in width and 1 cm in length) was performed under the dissecting microscope. The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and was 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 containing oxygenated Krebs’ solution at 37°C. One end of the muscle strip was anchored at the bottom of the muscle bath, whereas the other was connected to a force transducer (model FT03; Grass Instruments, Quincy, MA). Isometric tension was measured by the PowerLab/SPS data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia) and recorded using Chart 4.1.2 (ADInstruments Pty Ltd.). Each smooth muscle strip was initially stretched to a tension of 0.7 g. The muscle strips were then given at least 1 h to equilibrate during which they were washed with Krebs’ solution every 20 min. Only smooth muscle strips that developed spontaneously tone and responded to electrical field stimulation were used in this study. The changes in tension from various drugs were expressed as the percentage of maximal relaxation achieved by 50 mM EGTA at the end of the experiment.

The procedure to describe details of tissue preparation and isometric tension recording in the IAS smooth muscle has been published in a number of our earlier publications (Moumni and Rattan, 1998; Banwait and Rattan, 2003; Rathi et al., 2003; Sarma et al., 2003).

Drug Response. To determine the effect of β1-, β2-, and β3-AR agonists on cAMP/cGMP levels, IAS strips were exposed to 30 μM xamoterol, procaterol, and CL 316243, respectively. Those experiments were performed in the presence and absence of NF 023 (100 nM), NF 449 (100 nM), ODQ (1 μM), SQ 22536 (1 μM), KT 5720 (1 μM), and KT 5823 (1 μM) and forskolin (10 μM) were used as positive controls. 3-isobutyl-1-methylxanthine (IBMX, 100 μM), a nonspecific phosphodiesterase inhibitor, was added to the organ bath 10 min before the other agents to avoid cyclic nucleotides degradation. In certain experiments, the effect of ODQ (1 μM) or SQ 22536 (1 μM), by themselves added before IBMX, was also determined. At the point of the agonist-induced maximal relaxation
of IAS, the tissues were instantaneously frozen in liquid nitrogen as described previously (Torphy et al., 1986; Rattan et al., 1991) and stored at –72°C for further analysis. Briefly, the smooth muscle strips after their full exposure from the muscle baths were flash-frozen with modified Wollenberger clamps (precooled in liquid N2).

Extraction and Determination of Cyclic Nucleotides. The individual frozen tissues were transferred to a cold 6% trichloroacetic acid solution to give a 5% (w/v) homogenate. The tissue samples were homogenized on ice using an Ultraturrax T8 tissue homogenizer (Tekmar tissuemizer; Tekmar, Cincinnati, OH). The homogenized samples were centrifuged at 3000 g for 15 min at 4°C to separate the precipitated protein. The supernatant was separated from the precipitate, and the excess trichloroacetic acid was removed from the supernatant after four times extraction with 5 volumes of water-saturated diethyl ether. The aqueous extracts remaining were concentrated in an Eppendorf Vacufuge concentrator (Westbury, NY).

The cAMP and cGMP contents were measured in duplicate after acetylation using BIORACK RPN225 and RPN226 (Ohbayashi et al., 1998) assay kits, respectively, obtained from Amersham Biosciences Inc. (Piscataway, NJ). The protein content of the homogenate was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The values of cyclic nucleotide content were expressed as picomoles per milligram of protein.

β-AR Analysis by Western Blot. Western blot analysis of β1-, β2-, and β3-agonists xamoterol, procaterol, and CL 316243, respectively, on the basal tone of the rat IAS (shown as percentage of maximal fall in IAS tension) in the absence (control) and presence of the selective Gia and Gα antagonists NF 023 and NF 449, respectively. A, NF 023 significantly inhibit the relaxation induced by xamoterol (*, p < 0.05; n = 4–6) but not NF 449. B, both NF 023 and NF 449 inhibit the relaxation induced by procaterol (**, p < 0.05). C, NF 023 inhibits the relaxation by CL 316243 (+, p < 0.05; n = 4–6), whereas it has no significant effect on the Emax of the agonist. In all panels, the values represent the mean ± S.E. of four to six independent determinations (* denotes significance on comparison of entire curve with respect to control).
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% bromphenol blue, and 2% β-mercaptoethanol) and placed in boiling water bath for 3 min. An aliquot of (20 μl containing 40 μg of protein) of each sample was separated by 12% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (NCM) at 4°C. To block nonspecific antibody binding, the NCM was soaked overnight at 4°C in Tris-buffered saline/Tween (composed of 20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween 20) containing 1% BSA. The NCM was then incubated with the specific primary antibodies (rabbit polyclonal IgG, 1:5000) with the corresponding secondary antibody (donkey anti-rabbit IgG, 1:2500) for 1 h at room temperature. After washing with Tris-buffered saline/Tween, the membranes were incubated with horseradish peroxidase labeled-secondary antibody (1:10000) for 1 h at room temperature. The corresponding bands were visualized with enhanced chemiluminescence substance using Western blot detection kit and Hyperfilm MP (Amersham Biosciences Inc.). Bands corresponding to different proteins on X-ray films were scanned (SnapScan 310; Agfa, Ridgefield Park, NJ, and the respective integrated optical densities (IODs) determined by using Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Drugs and Chemicals. IBMX, dimethyl sulfoxide (DMSO), and EGTA were purchased from Sigma-Aldrich (St. Louis, MO). Xamoterol and procateterol were purchased from Tocris Cookson Inc. (Ellisville, MO). CL 316243, NF 023, NF 449, KT 5720, and KT 5823 were from Calbiochem (La Jolla, CA). Drugs and Chemicals. All agents except KT 5720 and KT 5823 were dissolved and diluted in Kreb’s buffer. Initial stock solutions (10 mM) of KT 5720 and KT 5823 were prepared using DMSO, which were then diluted with Kreb’s buffer. The final concentration of DMSO in the organ bath did not exceed 0.01%.

Data Analysis. Results are expressed as means ± S.E. Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (GraphPad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies and maximal responses were expressed as negative logarithm of the molar concentration of agonist producing 50% of the maximum response (pEC50) and maximum effect elicited by the agonist (Emax), respectively. Statistical significance was determined by using the one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. In all cases, probability levels of less than 0.05 (p < 0.05) were considered statistically significant.

Results

Effects of β1-, β2-, and β3-AR Agonists Xamoterol, Procateterol, and CL 316243, Respectively, on the IAS Tone. Selective agonists for β1-, β2-, and β3-AR xamoterol, procateterol, and CL 316243, respectively, produced relaxation of the IAS smooth muscle. However, significant differences were observed in the Emax values causing smooth muscle relaxation. Xamoterol was the least effective in causing IAS smooth muscle relaxation (Emax = 45.97 ± 9.09%; ANOVA, *p < 0.05), followed by procateterol (Emax = 72.34 ± 2.76%) and CL 316243 (Emax = 86.26 ± 1.96%). No significant difference among the pEC50 values was observed.

Effect of Gβγα and Gα Inhibitors NF 023 and NF 449, Respectively, on IAS Relaxation by the Selective β-AR Agonists. To identify the type of G protein involved in the response induced by each β-AR type, CRs for the selective agonists were obtained in the absence and presence of NF 449 (Gβγα inhibitor) and NF 023 (Gβγα inhibitor). Results show that these inhibitors significantly attenuated the relaxation induced by procateterol. In contrast, the responses of xamoterol and CL 316243 were inhibited only by NF 023 (Table 1). Consequently, the data suggest that the β2-AR activates both G protein subunits, whereas β1/β3-ARs activate primarily the Gβγα subunit.

Effect of GC and AC Cyclase Inhibitors ODQ and SQ 22536, Respectively, on the IAS Smooth Muscle Relaxation by the Selective β-AR Agonists. ODQ, a selective inhibitor of guanylate cyclase, significantly inhibited the relaxation induced by different β-AR agonists. On the other hand, SQ 22536, a selective inhibitor of adenylate cyclase, only inhibited the relaxation induced by procateterol (Fig. 2, A–C; Table 1). Results suggest that β3-AR-activated response is dependent upon both cAMP/cGMP syntheses, whereas β1/β2-AR activation induces responses dependent primarily upon the cGMP production.

Effect of cAMP-Dependent Protein Kinase (PKA) Inhibitor KT 5720 and a cGMP-Dependent Protein Kinase (PKG) Inhibitor KT 5823 on the Relaxation by Selective β-AR Agonists. KT 5720 and KT 5823 were used to identify the nature of protein kinase involved in the IAS smooth muscle relaxation caused by the selective agonists of each subtype of β-AR. KT 5720 significantly inhibited the relaxation induced by procateterol but not by xamoterol and CL 316243. Conversely, KT 5823 inhibited the relaxation by xamoterol and CL 316243 but not by procateterol (Fig. 3, A–C; Table 1), suggesting that the signaling mechanism activated by the β2-AR is dependent primarily upon PKA phosphorylation, whereas the β1 and β3-activated pathways involve PKG activation.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Emaxa</th>
<th>pEC50b</th>
</tr>
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<tbody>
<tr>
<td>Xamoterol</td>
<td>45.97 ± 9.09</td>
<td>8.06 ± 0.12</td>
</tr>
<tr>
<td>+ 1 μM ODQ</td>
<td>2.22 ± 4.16</td>
<td>—</td>
</tr>
<tr>
<td>+ 1 μM SQ 22536</td>
<td>34.43 ± 11.58</td>
<td>8.19 ± 0.21</td>
</tr>
<tr>
<td>+ 100 nM NF 023</td>
<td>44.16 ± 9.75</td>
<td>7.89 ± 0.22</td>
</tr>
<tr>
<td>+ 100 nM NF 449</td>
<td>31.15 ± 8.85</td>
<td>7.89 ± 0.10</td>
</tr>
<tr>
<td>+ 1 μM KT 5823</td>
<td>12.86 ± 3.57</td>
<td>7.21 ± 0.11</td>
</tr>
<tr>
<td>Procaterol</td>
<td>72.34 ± 2.76</td>
<td>7.45 ± 0.17</td>
</tr>
<tr>
<td>+ 1 μM ODQ</td>
<td>24.53 ± 3.10</td>
<td>7.18 ± 0.17</td>
</tr>
<tr>
<td>+ 1 μM SQ 22536</td>
<td>41.01 ± 5.34</td>
<td>6.43 ± 0.12</td>
</tr>
<tr>
<td>+ 100 nM NF 023</td>
<td>22.09 ± 2.03</td>
<td>6.51 ± 0.28</td>
</tr>
<tr>
<td>+ 100 nM NF 449</td>
<td>55.78 ± 1.49</td>
<td>5.79 ± 0.14</td>
</tr>
<tr>
<td>+ 1 μM KT 5720</td>
<td>59.52 ± 3.85</td>
<td>7.17 ± 0.03</td>
</tr>
<tr>
<td>+ 1 μM KT 5823</td>
<td>71.59 ± 3.18</td>
<td>7.65 ± 0.25</td>
</tr>
<tr>
<td>CL 316243</td>
<td>86.26 ± 1.96</td>
<td>7.05 ± 0.14</td>
</tr>
<tr>
<td>+ 1 μM ODQ</td>
<td>34.24 ± 2.62</td>
<td>7.01 ± 0.15</td>
</tr>
<tr>
<td>+ 1 μM SQ 22536</td>
<td>74.51 ± 2.98</td>
<td>7.31 ± 0.17</td>
</tr>
<tr>
<td>+ 100 nM NF 023</td>
<td>32.27 ± 4.88</td>
<td>6.75 ± 0.11</td>
</tr>
<tr>
<td>+ 100 nM NF 449</td>
<td>70.24 ± 7.88</td>
<td>8.46 ± 0.13</td>
</tr>
<tr>
<td>+ 1 μM KT 5720</td>
<td>82.44 ± 4.50</td>
<td>7.05 ± 0.19</td>
</tr>
<tr>
<td>+ 1 μM KT 5823</td>
<td>33.87 ± 4.64</td>
<td>7.78 ± 0.13</td>
</tr>
</tbody>
</table>

One-way ANOVA, *p < 0.05.

a Emax is defined as maximal relaxation of basal tone of IAS smooth muscle elicited by the agonist compared that by 50 mM EGTA. Data are expressed as mean ± S.E. of four to six independent determinations.

b pEC50 = −log EC50 (concentration of the agonist that produces 50% relaxation of the IAS smooth muscle).

—, unable to calculate.
1.77 pmol/mg, respectively. Forskolin (10 μM) significantly enhanced the cAMP level while causing decrease in the levels of cGMP (*, p < 0.05; n = 4). Conversely, SNP (10 μM) significantly decreased cAMP while causing increases in the tissue levels of cGMP. Data with the measurements of cAMP and cGMP levels are shown in Fig. 4.

Guanylate and adenylate cyclase inhibitors by themselves also had different effects on tissue levels of cyclic nucleotides. Incubation of the IAS strips with ODQ produced no significant effect on cAMP levels but caused significant decrease in the cGMP levels. On the other hand, SQ 22536 produced a significant decrease in cAMP levels without affecting cGMP (p > 0.05). Xamoterol and CL 316243 significantly decreased (*, p < 0.05) the cAMP level, whereas they enhanced the cGMP level. In contrast, procaterol caused significant increase in the levels of cAMP (*, p < 0.05) without any significant change on cGMP levels (p > 0.05). Results suggest that the β2-AR primarily activates cAMP production, whereas β1- and β3-ARs activate cGMP. In addition, the data suggest the existence of a cross talk between the cAMP and the cGMP systems. Data are summarized in Fig. 4, A and B.

Western Blot for β1, β2, and β3 Receptors. To detect the presence of membrane-bound β-ARs in the rat IAS, Western blot analyses for all of the β-ARs was performed. The immunooblots on Fig. 5A demonstrate the presence of the expected molecular size for β1-AR (63 kDa), β2-AR (68 kDa), and β3-AR (65 kDa). Figure 4B shows significant differences (p < 0.05) in the relative distribution of different β-AR. β-actin was used as standard for the normalization of calculations. The relative expressions of β-ARs were calculated in relation to

Fig. 2. Effect of the selective β1-, β2-, and β3-agonists xamoterol, procaterol, and CL 316243 on the basal IAS tone (percentage of maximal fall in IAS tension) in the absence (control) and presence of AC and GC inhibitors SQ 22536 and ODQ, respectively. A, SQ 22536 does not produce any significant effect on procaterol CRC; however, ODQ significantly inhibited the relaxation (*, p < 0.05). B, SQ 22536 does not produce any significant effect on the CRC of CL 316243; however, ODQ significantly inhibits the relaxation (*, p < 0.05). C, both SQ 22536 and ODQ cause significant inhibition of the relaxation by procaterol (*, p < 0.05). The values represent the mean ± S.E. of four to six independent determinations.
Results show that the highest relative expression of $\beta_2$-AR (1.93) was followed by that of $\beta_1$-AR (2.51) and $\beta_3$-AR (1.16). Discussion

The studies for the first time show the relative role of AC and GC pathways in the IAS smooth muscle relaxation after selective activation of $\beta$-AR. The effects of different inhibitors on the IAS relaxation by different agonists are summarized in Table 2. The studies demonstrate 1) the presence of three subtypes of $\beta$-AR ($\beta_1$, $\beta_2$, and $\beta_3$); 2) relaxation of the smooth muscle by the selective agonism of different $\beta$-ARs, leading to distinct and in some cases overlapping AC and GC pathways; and 3) $\beta_3$-AR activation clearly following the course of GC activation.

The functional and molecular characterization of $\beta_1$, $\beta_2$, and $\beta_3$-ARs responsible for the opossum IAS relaxation has been reported recently (Rathi et al., 2003). The pharmacological characterization of the $\beta$-ARs in rat IAS was performed using the selective $\beta_1$, $\beta_2$, and $\beta_3$-AR agonists xamoterol, procaterol, and CL 316243, respectively. Results show that the selective agonists induce concentration-dependent relaxation as reported previously in different systems (Goldberg and Frishman, 1995; De Ponti et al., 1996; Roberts et al., 1997; Rathi et al., 2003). However, important differences among the calculated $E_{\text{max}}$ values were found in the rat IAS smooth muscle. Xamoterol was the least efficacious in causing relaxation ($E_{\text{max}} = 35.15 \pm 5.99\%$; ANOVA, $p < 0.05$), followed by procaterol ($E_{\text{max}} = 72.89 \pm 5.09\%$) and CL 316243 ($E_{\text{max}} = 86.24 \pm 1.96\%$). The possible explanation may be differences in the signal transduction mechanisms, partial agonism by the agonist, and cross talk between dif-
ferent pathways, and finally the animal species differences. It is worth noting that in the case of opossums IAS, xamoterol was found to be a full agonist (Rathi et al., 2003).

SQ 22536 (a selective AC inhibitor) attenuates the relaxation induced by procaterol, but not by CL 316243. On the other hand, ODQ (a selective GC inhibitor) attenuates the relaxant effect of CL 316243. The data suggest the role of GC pathway in 2-AR activation and that of AC in 3-AR-mediated relaxation of the IAS. These results are in agreement with previous findings showing that the 3-AR stimulates the cAMP-dependent signaling pathway (Gilman, 1987; Scott et al., 2003), whereas the 2-AR stimulates the cGMP-dependent pathway (Trochu et al., 1999; Cirino et al., 2003). The studies further show higher efficacy with 3-AR activation ($E_{\text{max}} = 86.24 \pm 1.96\%$) compared with 2-AR activation ($E_{\text{max}} = 72.89 \pm 5.09\%$). Whether this is related to the higher potency of cGMP versus cAMP is not known. It is of interest that the cyclic nucleotides measurements show that the tissue levels of cAMP (48.42 ± 3.96 pmol/mg) are ~4 times higher than the cGMP (10.73 ± 1.77 pmol/mg) in the basal state. The observations are similar to those in ovine pulmonary arteries where ~4-fold higher concentration of cAMP versus cGMP was required to achieve EC50 in causing the smooth muscle relaxation (Dhanakoti et al., 2000).

ODQ, but not SQ 22536, inhibited the relaxation induced by xamoterol. This suggests that 1-AR activates the cGMP pathway. Because cGMP is more potent than cAMP in the IAS (Moummi and Rattan, 1988), one could predict that xamoterol would induce relaxation with $E_{\text{max}}$ values higher than obtained with procaterol and close to that with CL 316243 because the 2 agonist activates the cAMP pathway and the 3 agonist activates the cGMP. However, on actual experimentation, xamoterol did not produce any significant effect ($p > 0.05; n = 4$). One-way ANOVA compared with the control: *, $p < 0.05$.

SNP causes increase in cGMP, but decrease in cAMP levels. Conversely, forskolin decreases cGMP levels, while caus-
3-AR. As an additional support to the functional data, Western blot analysis for the β1-AR, β2-AR, and β3-AR present on the smooth muscle cells membranes of the rat IAS. A, immunoblots with the expected molecular size for β1-AR (63 kDa), β2-AR (68 kDa), and β3-AR (65 kDa). B, relative distribution of β-AR. Data show significant differences in the receptor relative distributions of different subtypes of β-AR, β1 being the lowest followed by β2 and β3. β-Actin was used as standard for the calculations, which are expressed as β-AR IOD in relation to β-actin IOD (β-AR IOD/β-actin IOD). A 12% SDS-polyacrylamide gel was used for electrophoresis for 60 min, transferred to NCM, and probed by isofrom antibodies for all proteins. The values are the mean ± S.E. of three independent determinations. One-way ANOVA: * , p < 0.05.

**TABLE 2**

Summary of effect of inhibitors of sequential events pertaining to the pathways examined, on β1-AR, β2-AR, and β3-AR activation

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Inhibitor</th>
<th>Effect on β1-AR (Agonist) (Xamoterol)</th>
<th>Effect on β2-AR (Agonist) (Procaterol)</th>
<th>Effect on β3-AR (Agonist) (CL 316243)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate cyclase</td>
<td>G protein (Gα)</td>
<td>NF 449a NA</td>
<td>↓</td>
<td>↑ (?)</td>
</tr>
<tr>
<td>AC activation</td>
<td>SQ 22536</td>
<td>NA</td>
<td>↓</td>
<td>NA</td>
</tr>
<tr>
<td>PKA activation</td>
<td>KT 5720</td>
<td>NA</td>
<td>↓</td>
<td>NA</td>
</tr>
<tr>
<td>Guanylate cyclase</td>
<td>G protein (Gα)</td>
<td>NF 023</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>GC activation</td>
<td>ODQ</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PKG activation</td>
<td>KT 5823</td>
<td>NA (?)</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

NA, not affected; ↓, inhibition; ↑, augmentation; (?) explanations for these unanticipated observations have been given in the text.

All β-ARs belong to the large family of G protein-coupled receptors, characterized by seven-transmembrane helices (Emorine et al., 1989). It is well known that activation of β-ARs causes release of Gα or Gα/βγ subunit, which then activate cyclic nucleotides production by the activation of AC and GC (Nantel et al., 1995). In the present study, we show that selective antagonism of the subunits Gα and Gβ of the G proteins coupled to β-ARs by NF 023 and NF 449, respectively, causes selective antagonism of β-AR action. Both antagonists attenuate the relaxations induced by procaterol, implicating the role of these G protein subunits in β-AR-activated relaxant response. This is in agreement with the findings that ODQ and SQ 22536 attenuate the relaxation induced by procaterol and suggests the involvement of cGMP and cAMP in response to β2-AR activation.

Although, in general, β2-AR are known to activate Gα/βγ-activated cAMP pathway (Gilman, 1987; Scott et al., 2003), it has been described in several animal species (Kuschel et al., 1999; Wang et al., 2003), including humans (Kilts et al., 2003), that the β2-AR couples to different signaling pathways via both Gα and Gβγ proteins. An additional evidence has been presented that the signal from the β2-AR may involve a switch in receptor coupling from Gα to Gβγ proteins due to a feedback phosphorylation of the β2-AR itself by PKA after stimulation by β agonists (Daaka et al., 1997). In contrast, only NF 023 attenuates the relaxation induced by CL 316243 and xamoterol, suggesting that β1 and β3 activate signaling pathways primarily via Gβγ subunits. These observations are in agreement with the previous reports in other systems (Trochu et al., 1999; Cirino et al., 2003). It is noteworthy that NF 449, rather than inhibiting the IAS relaxation by CL 316243, either causes an increase in the relaxation (considering Emax) or no effect (considering Emax). This suggests the possibility that the Gα coupling plays a functional break for the Gβγ/α pathway in the signaling transduction process by β2-AR.

PKG-selective antagonist KT 5823 attenuates the relaxation induced by xamoterol and CL 316243 but not by procaterol. Conversely, PKA-selective inhibitor KT 5720 attenuates the relaxation by procaterol but not by xamoterol and CL 316243. These results are in agreement with previous works showing that β2-AR and β3-AR activation pathways mainly go through PKA (Gilman, 1987; Scott et al., 2003) and PKG (Trochu et al., 1999; Cirino et al., 2003), respectively. In general, the observed effects with PKA and PKG inhibitors flow well with the effects of AC and GC inhibitors. Unexpectedly however, KT 5823 does not produce any significant effect on the smooth muscle relaxation by β2-AR activation. A likely explanation for this is the involvement of mechanisms regulated by cGMP yet independent of PKG activation as proposed in other systems (Dhanakoti et al., 2000; Shimoda et al., 2003).

As an additional support to the functional data, Western blot studies demonstrate the presence of β1, β2, and β3-AR with the expected sizes of 63, 68, and 65 kDa, respectively. Interestingly, the relative distribution analysis reveals that the β2-AR, in contrast with the observed potencies in causing relaxation, is present in lower levels. A plausible explanation for the disparity between the potency of the selective agonists versus the level of receptor expression is the lack of uniformly potent and selective agonists, partial agonism of different β-AR agonists (in certain species), and the nature of antibo-
ies used that may not uniformly and fully recognize different types of β-ARs.

In summary, the present studies demonstrate β1-, β2-, and β3-AR subtypes in the rat IAS smooth muscle and provide valuable information on the role of AC and GC signaling pathways in the IAS relaxation. β1-AR activation produces smooth muscle relaxation via activation of Gi/o protein subunit, leading to cGMP synthesis and PKG activation, whereas β2-AR primarily activates the Gs protein subunit, causing increase in cAMP levels with consequent activation of PKA. β3-AR activation clearly follows the course of GC pathway (Gs, Gi/o/cGMP/PGK). This hypothesis is further corroborated by the actual determinations of cGMP levels. Biochemical pathway for β3-AR activation-mediated IAS smooth muscle relaxation is similar to that by neuronal nitric-oxide synthase and endothelial nitric-oxide synthase activation in the IAS (Rattan and Chakder, 1992; Chakder and Rattan, 1993; Banwait and Rattan, 2003). Data with β3-AR activation in the IAS in view of its prolonged, selective, systemic effects is still to be explored (DiMarino et al., 2002; Banwait and Rattan, 2003) combined with the utilization of biochemical pathway common to nonadrenergic noncholinergic nerve stimulation are important from the standpoint of anorectal and other gastrointestinal motility disorders.

References


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