β_1 -Adrenoceptor-mediated relaxation with isoprenaline and the role of MaxiK channels in guinea-pig esophageal smooth muscle

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Abstract

The possible functional coupling between β_1 -adrenoceptor and MaxiK channels which results in smooth muscle relaxation was examined in the guinea-pig esophageal muscularis mucosae. Isoprenaline-elicited relaxation of esophageal smooth muscle was confirmed to be mediated through β_1 -adrenoceptors as the response was competitively antagonized by a β_1 -selective antagonist atenolol with a pA_2 value of 7.01. Iberitoxin (IbTx, 10^{-7} M), a selective MaxiK channel inhibitor, substantially diminished the relaxant response to isoprenaline. The extent of the MaxiK channel contribution to the relaxant response was 15–40% of the control response when estimated as the $E_{50\%}$ – $E_{\max}$ responses to isoprenaline. The relaxation to isoprenaline was also attenuated by high-KCl (80 mM) to the same degree as the relaxant response generated in the presence of IbTx, and thus the estimated extent of the K^+ channel contribution was 10–40%. These findings indicate that β_1 -adrenoceptors are substantially coupled with MaxiK channels to produce relaxation of esophageal smooth muscle in the guinea-pig. Although MaxiK channels account for the contribution of K^+ channels to the β_1 -adrenoceptor-mediated relaxation in this smooth muscle preparation, their contribution seems to be less when compared to the β_2 -adrenoceptor-mediated relaxation of tracheal smooth muscle.

Key words: β_1 -adrenoceptor, esophageal smooth muscle, iberitoxin, isoprenaline, large-conductance Ca^{2+} -activated K^+ (MaxiK) channels

Introduction

Smooth muscle relaxes in response to β -adrenoceptor stimulation. The mechanisms by which such stimulation generates smooth muscle relaxation are still to be established. However, recent physiological and pharmacological studies have shown evidence for a significant role for K^+ channels in β -adrenoceptor-mediated smooth muscle relaxation (Kume *et*

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et al., 1989; Jones *et al.*, 1990; Huang *et al.*, 1993; Jones *et al.*, 1993; Scornik *et al.*, 1993; Kume *et al.*, 1994; Torphy, 1994; Johnson, 1998; Nara *et al.*, 1998; Horinouchi *et al.*, 2003; Tanaka *et al.*, 2003). Particular attention has been given to the roles of large-conductance, Ca^{2+} -activated K^+ (MaxiK) channels (Nelson *et al.*, 1995; Toro *et al.*, 1998; Orio *et al.*, 2002; Tanaka *et al.*, 2004) in the relaxation mediated through β -adrenoceptors. This is both because the conductance of this type of K^+ channel is extremely high and this channel type is highly expressed in smooth muscle cells (Wallner *et al.*, 1995; Toro *et al.*, 1998; Jiang *et al.*, 1999). In this regard, we have recently shown that in the β_2 -adrenoceptor-mediated relaxation of tracheal smooth muscle by isoprenaline, the contribution by MaxiK channels is substantial. The relaxant response can almost entirely be ascribed to the activation of this type of K^+ channel when isoprenaline concentrations are $\leq \text{EC}_{50}$ value (Tanaka *et al.*, 2003). On the other hand, in gastrointestinal smooth muscle, the contribution by MaxiK channels is practically negligible to the relaxation mediated through β_3 -adrenoceptors (Horinouchi and Koike, 2002; Horinouchi *et al.*, 2003). These findings suggest that the extent of functional coupling between β -adrenoceptors and MaxiK channels in generating smooth muscle relaxation depends on the receptor subtype, with the present tentative rank order for the coupling being β_2 -subtype $\gg \beta_3$ -subtype. However, little information is available concerning the response mediated through β_1 -subtype adrenoceptors.

The present study was thus carried out to determine whether β_1 -adrenoceptor-mediated relaxation of smooth muscle involves MaxiK channel activation or not. For this purpose, in the present study, we employed guinea-pig esophageal smooth muscle (esophageal muscularis mucosae) (Kamikawa and Shimo, 1987) since the expression of the mRNA for β_1 -adrenoceptors is abundant in this smooth muscle tissue (Horinouchi *et al.*, 2003), and the relaxation to isoprenaline is mediated entirely by β_1 -receptors (Uchida, 1983; Horinouchi *et al.*, 2003).

Materials and Methods

Male and female Hartley guinea-pigs weighing 300–600 g (Saitama Experimental Animals Co., Ltd, Saitama, Japan) were used in the present study. Guinea-pigs were housed in rooms in which the temperature (20–22°C) and relative air humidity ($50 \pm 5\%$) were strictly regulated. Food and water were available *ad libitum* to all animals. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (accredited by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan).

Preparation of longitudinal smooth muscle of esophageal muscularis mucosae

Longitudinal smooth muscle of the esophageal muscularis mucosae was prepared as reported previously (Kamikawa and Shimo, 1987; Horinouchi *et al.*, 2003). Briefly, esophageal tissues were carefully isolated from the guinea-pig and immersed in Krebs solution (mM: NaCl, 118.0; KCl, 4.75; CaCl_2 , 2.54; MgSO_4 , 1.20; KH_2PO_4 , 1.19; NaHCO_3 , 25.0; and glucose, 11.0) bubbled with a mixture of 95% O_2 and 5% CO_2 . Subsequently, the outer striated muscle coat of the tissue was carefully dissected away and the longitudinal tube containing the muscularis

mucosae was cut very cautiously into about 10 mm lengths under a dissecting microscope so that the smooth muscle layer was not damaged.

Measurement of tension changes

Preparations were suspended vertically in a 20-ml organ bath containing Krebs solution which was maintained at $32 \pm 1^\circ\text{C}$ and bubbled with the $\text{O}_2\text{--CO}_2$ mixture. Tension changes of the esophageal smooth muscle preparations were isometrically recorded with a force-displacement transducer (TB-612; Nihon Kohden, Tokyo, Japan) connected to an amplifier (high-gain DC amplifier: model, AD 632J; Nihon Kohden, Tokyo, Japan).

Assessment of the action of isoprenaline on muscle tone and the inhibitory effects of atenolol and iberiotoxin

The effects of isoprenaline were examined as follows. Each smooth muscle preparation was preloaded with an initial tension of 0.5 g, since at this tension, the contractile response to acetylcholine (ACh) at 3×10^{-7} M was maximal, but declined with further preloads of up to 2.5 g ($n=2$). Preparations were then incubated for 60 min with renewal of the bath medium each 20 min, following which they were contracted with 3×10^{-6} M ACh to confirm that normal contractile responses could be obtained. ACh was subsequently washed out. After this procedure, each preparation was contracted with methacholine (10^{-6} M). When the active tension generated with methacholine reached a steady-state level 30–40 min after its application, isoprenaline was applied cumulatively to the bath medium until the maximum relaxant response was obtained. When examining the effects of atenolol and iberiotoxin (IbTx), they were added to the bath medium simultaneously with methacholine. At the end of each experiment, papaverine (10^{-4} M) was applied to the bath medium to determine the potentially maximum relaxant level.

In some experiments, contraction was generated with isotonic high-KCl (80 mM) Krebs solution (mM): NaCl, 42.75; KCl, 80.0; CaCl_2 , 2.54; MgSO_4 , 1.20; KH_2PO_4 , 1.19; NaHCO_3 , 25.0; and glucose, 11.0.

Indomethacin (3×10^{-6} M) was present in the bath medium throughout the experiments to prevent the possible release of endogenous prostaglandins.

Drugs

Drugs used in the present study were as follows: methacholine (*O*-acetyl- β -methylcholine) chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan); (–)-isoprenaline hydrochloride, CGP-20,712A [(±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide] methanesulfonate, indomethacin, papaverine hydrochloride (Sigma-Aldrich Co., St. Louis, Mo., USA); iberiotoxin (IbTx) (Peptide Institute, Minoh-City, Osaka, Japan); (±)-atenolol (Sigma-RBI, Natick, Mass., USA). All other chemicals used were of analytical grade. Indomethacin was dissolved in pure ethanol to make a stock solution of 10^{-2} M just prior to its usage. Distilled water was used to dissolve and dilute all other drugs. All drugs are expressed as molar concentrations in the bathing solution.

Data analysis and statistics

To construct concentration-response relationships for the relaxation to isoprenaline, the percentage of the relaxant response was calculated considering the tension level before application of isoprenaline as 0% relaxation, and the tension level just before application of methacholine or high-KCl as 100% relaxation. Data were plotted as a function of isoprenaline concentration and fitted to the equation:

$$E = E_{\max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is the percentage relaxation at a given isoprenaline concentration, $E_{\max}$ is the maximum relaxation, A is the concentration of isoprenaline, n_H is the slope function and EC_{50} is the effective isoprenaline concentration that produce a 50% response. The curve fitting was carried out using GraphPad Prism™ (Version 3.00) (GraphPad Software, Inc., San Diego, Calif., USA). The EC_{50} values were converted to logarithmic values (pD_2 , $-\log EC_{50}$) for statistical analysis. The competitive antagonistic potency of atenolol was expressed as pA_2 value, which was calculated according to the method originally reported by Arunlakshana and Schild (1959).

Data are presented as means $\pm$ S.E.M. or mean values with 95% confidence intervals in parentheses while n refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad Prism™ by paired *t*-test, unpaired *t*-test, and unpaired *t*-test with Welch's correction if necessary. A *P* value of less than 0.05 was considered to be statistically significant.

Results

β_1 -Type of adrenoceptor mediates relaxation of guinea-pig esophageal smooth muscle in response to isoprenaline

Isoprenaline relaxed esophageal smooth muscle preparations contracted with methacholine (10^{-6} M) in a concentration-dependent fashion (Fig. 1A, open circles) with a pD_2 value of 8.34 (8.29–8.39, $n=12$). Concentration-response curves for isoprenaline were shifted rightwards in a parallel fashion by atenolol (a β_1 -selective adrenoceptor antagonist) (Fig. 1A, filled symbols). Schild plot analysis gave a straight line with a slope of 1.01 (0.83–1.19) and pA_2 value of 7.01 (6.85–7.25) ($n=12$ for each) (Fig. 1B). The relaxant response to isoprenaline was also antagonized competitively by another β_1 -adrenoceptor antagonist, CGP-20,712A, with its β_1 -corresponding pA_2 value (data not shown).

MaxiK channels contribute substantially towards isoprenaline-elicited relaxation

Figure 2 shows the effect of a MaxiK channel selective inhibitor, IbTx, on the relaxant response of esophageal smooth muscle preparations to isoprenaline. The response to isoprenaline was inhibited by IbTx (10^{-7} M), which was reflected by the significant decrease in its pD_2 value: control, 8.26 (8.22–8.30); IbTx, 7.98 (7.92–8.04) ($n=4$, $P<0.05$). The maximum response ($E_{\max}$) attained with 10^{-6} M isoprenaline was also reduced by IbTx: control, $91.8 \pm 1.5\%$; IbTx, $76.9 \pm 1.4\%$ ($n=4$, $P<0.01$). Therefore, when estimated at $E_{\max}$, the extent of the MaxiK channel contribution to isoprenaline-elicited relaxation was 16.2%. Similarly, the extent of the MaxiK channel contribution was calculated as 38.2% at the $\approx E_{50\%}$ (50% response) elicited by 10^{-8} M isoprenaline.

Fig. 1. Isoprenaline-elicited relaxation is mediated mainly through β_1 -adrenoceptors in guinea-pig esophageal smooth muscle. **A:** Concentration-response relationships for isoprenaline-elicited relaxation in the absence and presence of different concentrations of ($\pm$)-atenolol. Muscle relaxation is calculated with respect to the tension level before administration of isoprenaline (0% relaxation) and the tension level before application of methacholine (10^{-6} M) (100% relaxation). Data are plotted as means $\pm$ S.E.M. of four experiments in each case. **B:** Schild plot analysis carried out for atenolol against isoprenaline. The data analysed ($n=12$ points) are from the experiments shown in A. Dotted lines show the 95% confidence intervals of the regression line.

Evidence for the role of K^+ channels in isoprenaline-elicited relaxation

Figure 3 shows the effects of high-KCl on the relaxant response to isoprenaline. Basically, high-KCl (80 mM) suppressed the relaxation produced by isoprenaline to the same amount of that obtained with IbTx (Fig. 2). The pD_2 and E_{max} values were both significantly reduced in high-KCl-contracted tissue: pD_2 , 8.43 (8.37–8.49) (control) *vs.* 7.97 (7.93–8.01) (high-KCl) ($n=4$, $P<0.05$); E_{max} , $95.2 \pm 0.3\%$ (control) *vs.* $87.8 \pm 0.7\%$ (high-KCl) ($n=4$, $P<0.01$). Therefore, the extent of K^+ channel contribution at E_{max} was calculated as 7.7% and it was 39.0% at $\approx E_{50\%}$ (10^{-8} M isoprenaline).

Fig. 2. The MaxiK channel blocker, iberiotoxin, prevents esophageal smooth muscle relaxation to isoprenaline. Relaxant responses (%) to isoprenaline in the absence (○, control) and presence (●) of iberiotoxin (10^{-7} M) are calculated with respect to basal tension (100% relaxation) and steady-state contractile responses to methacholine (10^{-6} M) or methacholine plus iberiotoxin (0% relaxation). Muscle tension levels before administration of isoprenaline are: methacholine only, 1.82 ± 0.25 g; methacholine plus iberiotoxin, 2.10 ± 0.27 g ($n=4$ for each, $P<0.05$). Data are plotted as means $\pm$ S.E.M. of four experiments in each case. * $P<0.05$; ** $P<0.01$; *** $P<0.001$: significant differences between two groups.

Fig. 3. High-KCl suggests a significant role for K^+ channels in esophageal smooth muscle relaxation to isoprenaline. The relaxant effects of isoprenaline were examined in preparations precontracted with methacholine (10^{-6} M, ○) or high-KCl (80 mM, ●). The percentage of esophageal relaxation is calculated with respect to basal tension (100% relaxation), and methacholine (10^{-6} M) or high-KCl (80 mM) steady-state contractile responses (0% relaxation). Muscle tension levels before administration of isoprenaline are: methacholine, 1.66 ± 0.12 g; high-KCl, 1.03 ± 0.11 g ($n=4$ for each, $P<0.001$). Data are means $\pm$ S.E.M. of four experiments for each group. * $P<0.05$; ** $P<0.01$; *** $P<0.001$: significant differences between two groups.

Discussion

The present findings clearly show that MaxiK channels are involved in β_1 -adrenoceptor-mediated relaxation of esophageal smooth muscle (longitudinal muscularis mucosae). This is the first evidence to show a functional coupling between β_1 -adrenoceptors and MaxiK channels in the mechanical response of smooth muscle. Although the functional coupling with MaxiK channels seems to be much greater for β_1 -adrenoceptors than for β_2 -adrenoceptors, it is likely to be less than that with β_2 -adrenoceptors.

The role of β_1 -adrenoceptors as the principal subtype of β -adrenoceptor to mediate the relaxation of guinea-pig esophageal smooth muscle to isoprenaline is evidenced from the observation that the relaxant response was competitively antagonized by atenolol, a β_1 -selective antagonist (Fig. 1). Its pA_2 value was 7.01 and this was consistent with the β_1 -receptor corresponding value of ≈ 7.00 : (O'Donnell and Wanstall, 1983; Henry and Goldie, 1990). A competitive antagonism of another β_1 -selective antagonist, CGP-20,712A, against isoprenaline (data not shown) also supports this concept. The present findings using atenolol are consistent with our previous report to show that isoprenaline-elicited relaxation of guinea-pig esophageal muscularis mucosae is antagonized by CGP-20,712A and ICI-118,551 with their pA_2 values against β_1 -adrenoceptor (Horinouchi *et al.*, 2003). The primary role for this type of β_1 -adrenoceptor in the mediation of the relaxant response to isoprenaline can also be supported by the abundant expression of its mRNA in this smooth muscle (Horinouchi *et al.*, 2003).

As for other smooth muscle preparations, MaxiK channels have been identified in esophageal smooth muscle in other species such as human (Wade *et al.*, 1999), opossum (Zhang and Paterson, 2001) and cat (Salapatek *et al.*, 2002). In addition, other types of K^+ channels have also been identified with techniques of electrophysiology, pharmacology and molecular biology. They are: ATP-sensitive K^+ channel in rabbit (Hatakeyama *et al.*, 1995); delayed rectifier K^+ channels in human ($K_v1.2$, $K_v1.5$) (Wade *et al.*, 1999), opossum (Zhang and Paterson, 2001) and cat ($K_v1.2$) (Salapatek *et al.*, 2002); inwardly rectifying K^+ channel in cat (Ji *et al.*, 2000); and human ether-à-go-go related gene (HERG)-like channel in opossum (Arbarali *et al.*, 1999). However, possible functional coupling of these K^+ channels, including MaxiK channels, with β -adrenoceptors (β_1 -subtype) has not been elucidated so far. The present study has shown that MaxiK channels are substantially involved in the β_1 -adrenoceptor-mediated relaxation of esophageal smooth muscle and for the first time, indicated a functional coupling between β_1 -adrenoceptors and MaxiK channels in the smooth muscle mechanical response. This conclusion is clearly drawn based on the observation that isoprenaline-elicited relaxation was suppressed by a specific inhibitor of this ion channel, IbTx (Fig. 2). Diminution of the relaxant response to isoprenaline by IbTx through inhibition of this K^+ channel was also supported by the significant attenuation of the relaxant response by high-KCl (Fig. 3).

The functional coupling between β_1 -adrenoceptors and MaxiK channels to produce smooth muscle relaxation as shown in the present study implies an obligatory role for this K^+ channel as a downstream effector of classical β -adrenoceptors (β_1 -, β_2 -receptors) since the contribution of MaxiK channels is also substantial in β_2 -adrenoceptor-mediated relaxation (Tanaka *et al.*, 2003). Furthermore, this ion channel might also function as a universal membrane protein which

translates the chemical signals received on G_s-protein-coupled receptors into membrane potential changes, since the channel role is also significant in the relaxations triggered through G_s-protein-coupled receptors other than β -adrenoceptors (Tanaka *et al.*, 1999; Turcato and Clapp, 1999; Yamaki *et al.*, 2001). The only exception as far as we know is the gastrointestinal smooth muscle relaxation mediated through β_3 -adrenoceptors which was not affected by IbTx or 2 mM TEA in the guinea-pig stomach and duodenum (Horinouchi and Koike, 2002; Horinouchi *et al.*, 2003). Thus involvement of MaxiK channels can be ruled out in β_3 -receptor-mediated responses. By contrast, in the β_2 -adrenoceptor-mediated relaxation to isoprenaline in guinea-pig tracheal smooth muscle, the contribution of MaxiK channels is $\approx 80\%$ at $E_{50\%}$ (10^{-8} M isoprenaline) and $\approx 40\%$ at $E_{\max}$ (10^{-7} M isoprenaline) (Tanaka *et al.*, 2003). Therefore, the tentative rank order for the extent of the role of MaxiK channels in β -adrenoceptor-mediated relaxation is: β_2 -subtype $>$ β_1 -subtype $\gg$ β_3 -subtype.

On the other hand, the extent of the dependence on any K⁺ channel activation is given as a different rank order. As judged from the attenuation of isoprenaline-elicited relaxation in high-KCl-contracted muscle (Fig. 3), the extent of K⁺ channel contribution to β_1 -adrenoceptor-mediated relaxation is 10–40% at $\approx E_{50\%}$ – $E_{\max}$, and the rest of the component of more than 50% is produced through a K⁺ channel-independent pathway. By contrast, β_2 -adrenoceptor-mediated relaxation generated in tracheal smooth muscle of the guinea-pig is completely abolished in high-KCl-contracted muscle (our unpublished observation) though the subtypes of non-MaxiK K⁺ channels remain to be established. Similarly, in gastrointestinal smooth muscle cells, although MaxiK channels are not involved, β_3 -adrenoceptor-mediated relaxation largely depends on the activation of K⁺ channels ($> \approx 60$ –70%), and the most prevailing candidate is the 4-aminopyridine-sensitive voltage-gated K⁺ channel (Horinouchi *et al.*, 2003). Therefore, the tentative rank order for the extent of K⁺ channel roles in β -adrenoceptor-mediated relaxation is: β_2 -subtype $>$ β_3 -subtype $>$ β_1 -subtype. As for the β_3 -subtype, possible endogenous agonists for this receptor subtype are still controversial. By contrast, judging from the differences in receptor affinity between noradrenaline and adrenaline, the most dominant endogenous agonist for the β_1 -subtype would be noradrenaline and the one for the β_2 -subtype would be adrenaline. Both subtypes (β_1 , β_2) are classified into the seven transmembrane receptor superfamily and coupled with the G_s-protein-adenylyl cyclase system (Johnson, 1998). However, downstream to the receptor, the molecular mechanisms by which smooth muscle relaxation is generated are not identical for the β_1 - and β_2 -receptor systems and this difference might be associated with the kinds of agonist. The present findings which show the possible presence of subtype-specific mechanisms including K⁺ channels for smooth muscle relaxation in β -adrenoceptor-mediated systems denote the significance of being able to reveal this fundamental but ubiquitous drug receptor system at a closer molecular level.

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