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Relaxant actions of isoprenaline on guinea-pig isolated tracheal smooth muscle

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1 The effects of isoprenaline on membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in guinea-pig isolated tracheal muscle were studied by use of intracellular micro-electrodes and fura-2 signals respectively. Measurements of membrane potential were carried out in the presence of spontaneously-generated muscle tone, whereas fura-2 signals were measured during contraction produced by exogenous prostaglandin E_2 (100 nM). The potency of isoprenaline in causing relaxation was the same in these two different situations.

2 Isoprenaline (0.01 μM) produced relaxation accompanied by 5 mV hyperpolarization. A combination of tetraethylammonium (TEA, 10 mM) and verapamil (3 μM) did not alter the effects of isoprenaline. Removal of external K^+ did not increase the degree of hyperpolarization produced by isoprenaline.

3 In the presence of TEA (10 mM) and verapamil (3 μM), isoprenaline (0.03-1 μM) reduced $[Ca^{2+}]_i$ concentration-dependently. A similar degree of inhibition was observed when isoprenaline was applied during the maintained contraction induced by prostaglandin E_2 and against the contraction evoked by the addition of Ca^{2+} to tissues bathed in a Ca^{2+}-free medium and pretreated with both isoprenaline and prostaglandin E_2.

4 It is concluded that activation of TEA-sensitive Ca^{2+}-dependent K^+channels does not play a significant role in isoprenaline-induced relaxation. We propose that, in the guinea-pig tracheal muscle, isoprenaline may produce relaxation mainly by inhibiting a receptor-operated pathway for Ca^{2+} influx across the plasma membrane which is normally activated by prostaglandins.

Keywords: Airway muscle; isoprenaline; relaxation; TEA; β-adrenoceptor

Introduction

Isoprenaline, an agonist of β-adrenoceptors, produces relaxation in most smooth muscles. There are many different mechanisms involved in this relaxation (Bülbring & Tomita, 1987). An increase of adenosine 3':5'-cyclic monophosphate (cyclic AMP) resulting from β-adrenoceptor activation may reduce the affinity of Ca^{2+}-calmodulin for myosin light chain kinase. Alternatively, cyclic AMP may reduce $[Ca^{2+}]_i$ by facilitating Ca^{2+} transport into intracellular stores and/or across the plasma membrane. Also, membrane hyperpolarization caused by K^+-channels activated by cyclic AMP may inhibit Ca^{2+} influx through voltage-dependent Ca^{2+} channels. In addition, there is also the possibility that isoprenaline may inhibit a receptor-operated pathway for Ca^{2+} influx which is resistant to organic Ca^{2+} channel blockers. In guinea-pig tracheal muscle, for example, contractions induced by prostaglandins are not inhibited by Ca^{2+} channel blockers such as verapamil or nifedipine but are inhibited by isoprenaline (Foster *et al.*, 1984; Ito *et al.*, 1985).

By use of the patch clamp technique it has been shown that isoprenaline increases the open probability of Ca^{2+}-activated K^+ channels through phosphorylation mediated by cyclic AMP-dependent kinase in rabbit (Kume *et al.*, 1989) and porcine tracheal muscle (Muraki *et al.*, 1992). Therefore, the hyperpolarization accompanying isoprenaline-induced relaxation (Allen *et al.*, 1985; Honda *et al.*, 1986) may be the result of an increase in outward currents through Ca^{2+}-activated K^+ channels. Involvement of the activation of these K^+ channels in β-adrenoceptor-mediated relaxation has already been suggested by Jones *et al.* (1990). This suggestion was based on their observations of the inhibitory action of charybdotoxin (a Ca^{2+}-activated K^+ channel blocker) on isoprenaline-induced relaxation of guinea-pig tracheal muscle precontracted by carbachol. On the other hand, only a poor correlation between membrane hyperpolarization and relaxation has been noted in relaxation caused by β-adrenoceptor activation (Cook *et al.*, 1993). Furthermore, there is evidence that the inhibitory action of charybdotoxin on isoprenaline-induced relaxation is due to an increase in Ca^{2+} influx through voltage-gated Ca^{2+} channels after membrane depolarization caused by the toxin (Huang *et al.*, 1993 ; Cook *et al.*, 1995). Thus the mechanisms underlying isoprenaline-induced relaxation of airways smooth muscle are still not well understood. In the present experiments, we aimed to study further the effects of isoprenaline in producing relaxation. In most experiments tetraethylammonium (TEA, 10 mM) and verapamil (3 μM) were added to the perfusing solution to minimize the contributions of Ca^{2+}-activated K^+ channels and voltage-dependent L-type Ca^{2+} channels. Some of the results have been briefly reported (Tomita *et al.*, 1994).

Methods

Guinea-pigs (300-350 g) of either sex were killed by stunning and bleeding. Segments of cervical trachea were excised and the tracheal ring was opened by cutting longitudinally at the cartilaginous region opposite the membranous portion. Mucosal and connective tissues were carefully removed with fine forceps under microscopic observation. Segments containing one cartilaginous ring (for Ca^{2+} measurements) or three cartilaginous rings (for membrane potential measurements) were prepared.

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Tension and membrane potential recordings

The tracheal preparation was fixed in a small chamber (0.2 ml) with small pins at the cartilaginous regions. The outermost cartilage ring was connected to a strain gauge with a fine silk thread for tension recording. The preparation was superfused with normal solution (see below) prewarmed to 35°C at a constant flow of 2 ml min^{-1}. Membrane potential was measured by a conventional micro-electrode technique. Micro-electrodes (resistance of 30–40 MΩ, filled with 3 M KCl) were inserted from the mucosal side.

Fura-2 loading and cytosolic Ca^{2+} measurements

The method of measuring $[Ca^{2+}]_i$ was essentially as previously described (Iino *et al.*, 1994). A preparation containing a single cartilaginous ring was mounted in a small chamber. One end of the preparation was anchored and the other end was connected to a strain gauge with a fine thread. The chamber was fixed on a stage of an inverted microscope (IMT-2, Olympus Optical Co., Tokyo, Japan). After equilibrating in normal solution at 35°C for about 1 h, indomethacin (1 μM) and atropine (1 μM) were added to the perfusate throughout the experiments to block contributions of endogenous prostaglandins and acetylcholine. Fura-2 was loaded by incubating with 10 μM fura-2/AM for 2 h at room temperature (20–22°C). During the loading, a cholinesterase inhibitor (physostigmine 10 μM or diisopropyl phosphorofluoridate, DFP, 10 μM) was also added to facilitate fura-2 loading (Maruyama *et al.*, 1989) and probenecid (10 μM) to prevent a possible sequestration and secretion of fura-2 (Di Virgilio *et al.*, 1988; Watanabe *et al.*, 1992). When used together with atropine, the anticholinesterase had little effect on mechanical responses to prostaglandin E_2 (PGE_2) and isoprenaline. Before starting measurements the chamber was perfused with normal solution prewarmed to 35°C at least for 20 min to remove extracellular fura-2-AM and to improve the condition of the tissue. $[Ca^{2+}]_i$ was estimated by monitoring the ratio of fluorescence intensities of fura-2 excited at 340 and 380 nm ($R_{340/380}$). Fluorescence signals were collected at 3 s intervals with an image processor (ARGUS-50, Hamamatsu Photonics, Japan). The size of the spot where the fluorescence signals were detected was about 60 μm square. To study effects of isoprenaline, PGE_2 (100 nM) was used to raise muscle tone.

Solutions, drugs, and statistical analysis of results

The normal solution had the following composition (mM): NaCl 117, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 11.8, Tris-HCl 10 (pH 7.4 at 35°C).

Drugs used were: (±)-isoprenaline hydrochloride, indomethacin, tetraethylammonium chloride (TEA), atropine sulphate, PGE_2, verapamil hydrochloride, probenecid, physostigmine, DFP (all from Sigma, U.S.A.), fura-2-AM, and ethyleneglycol-*bis*-(β-aminoethylester)-N,N'-tetraacetic acid (EGTA) (Dojin, Japan). Indomethacin and fura-2/AM were dissolved in ethanol and dimethylsulphoxide, respectively. EGTA (500 mM) was dissolved by neutralizing with 1 N NaOH as a stock solution. Isoprenaline stock solution (1 mM) was prepared in 0.1 M HCl and a fresh dilution was made just before application. Data are expressed as means ± s.e.mean with number of preparations used (n). Students unpaired t test was used to evaluate the statistical significance of differences between means. Values of $P<0.05$ were considered to be significant.

Results

Effects of isoprenaline on mechanical and electrical activities

In the guinea-pig isolated tracheal muscle the tonic contraction which develops spontaneously is probably due to production of endogenous prostaglandins (Orehek *et al.*, 1975; Baba *et al.*, 1985). After equilibration for more than 1 h, muscle tone was maintained with only small fluctuations and the membrane potential was relatively stable (−48.5 ± 5.3 mV, $n=32$). Small rhythmic electrical activity was often observed (22 out of 32 preparations), as reported previously (Small, 1982; Allen *et al.*, 1985; Honda *et al.*, 1986). Under these conditions, 0.01 μM isoprenaline produced nearly complete relaxation, membrane hyperpolarization (5.7 ± 2.3 mV, $n=12$) and suppression of electrical activity (Figure 1a), as also previously observed (Allen *et al.*, 1985; Honda *et al.*, 1986). When K^+ was removed from the external solution there was a decrease in muscle tone and inhibition of spontaneous electrical activity accompanied by a small depolarization (2–3 mV). As shown in Figure 1b, the absolute value of membrane potential reached during isoprenaline-induced hyperpolarization was not significantly affected by K^+ removal. However, because of the depolarization produced by K^+ removal, the absolute degree of isoprenaline-induced hyperpolarization was increased (7.6 ± 3.1 mV, $n=4$). Reapplication of K^+ (6 mM) transiently hyperpolarized the membrane before the membrane potential returned towards the control level. The degree of this hyperpolarization was similar in the presence and absence of 0.01 μM isoprenaline (6.3 ± 2.6 mV with, and 7.1 ± 1.9 mV without, $n=4$).

TEA (10 μM) depolarized the membrane, and induced, or increased, rhythmic electrical activity, confirming previous results (Allen *et al.*, 1985; Tomita, 1989). As the amplitude of rhythmic activity was increased, the most negative level of membrane potential tended to return to the value observed before TEA application. In the presence of 10 mM TEA, isoprenaline (0.01 μM) still produced clear hyperpolarization, blocked electrical slow wave activity and caused relaxation (as is shown for 0.1 μM isoprenaline in the presence of 6 mM TEA: Tomita, 1989). Due to dislodgement of intracellular micro-electrodes it was difficult to examine the effects of isoprenaline on the same cell before and after TEA application. When examined in different cells, hyperpolarization induced by isoprenaline (0.01 μM) was slightly larger in the presence of 10 mM TEA (8.4 ± 3.5 mV, $n=5$).

In the presence of 10 mM TEA, verapamil (3 μM) reduced muscle tone, depolarized the membrane, and converted regular rhythmic electrical activity to irregular activity (Figure 2a). In the presence of both 10 mM TEA and 3 μM verapamil, isoprenaline (0.01 μM) produced further relaxation accompanied by membrane hyperpolarization (Figure 2b). The degree of hyperpolarization was difficult to measure due to irregular fluctuation in membrane potential before isoprenaline application, but a similar magnitude of hyperpo-

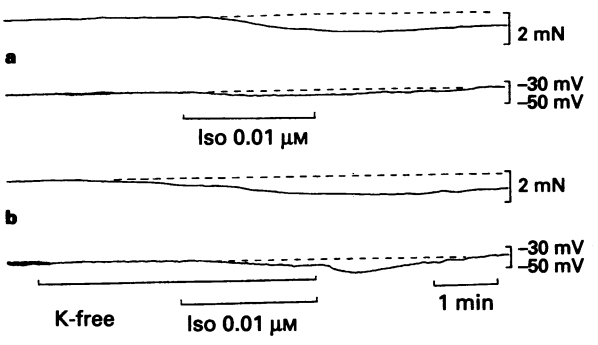

Figure 1 Guinea-pig isolated trachealis: effects of isoprenaline on tension (upper trace) and membrane potential recorded intracellularly (lower trace) in normal Krebs solution (a) and in K^+-free solution (b). The membrane potential recordings are taken from the same cell. Isoprenaline (Iso, 0.01 μM) and K^+-free solution were applied as indicated below the records. Isoprenaline was applied for 2 min at an interval of 20 min. See text for further explanation.

larization was observed in the presence and absence of verapamil.

Effects of isoprenaline on $[Ca^{2+}]_i$

Effects of isoprenaline on $[Ca^{2+}]_i$ were studied when muscle tone was increased by PGE_2 (100 nM) and spontaneous tone was inhibited by indomethacin (1 μM). These conditions were chosen because the muscle tone generated within 10–20 min varied to a great extent after fura-2 loading at room temperature. The solution also contained 10 mM TEA and 3 μM verapamil and preparations were first exposed to Ca^{2+}-free solution before addition of 2.4 mM Ca^{2+}. Two different approaches were used in these experiments. In the first, isoprenaline was applied during the sustained contraction induced by 2.4 mM Ca^{2+}. In the second, 2.4 mM Ca^{2+} was readmitted in the presence of isoprenaline. These two different types of experiments yielded very similar results. Figure 3 shows a typical result from the first type of experiment. Isoprenaline-induced relaxation was concentration-dependent and accompanied by a concentration-dependent decrease in fura-2 signal ($R_{340/380}$). There was a reasonable correlation in the degree and the rate of recovery between relaxation and fura-2 signal. When applied for 2 min, 0.3 μM isoprenaline produced almost maximum relaxation (96.8 ± 1.5%, $n=4$), but the decrease in fura-2 signal was slightly less (84.5 ± 4.1%), assuming that the fura-2 signal in Ca^{2+}-free solution (with 0.1 mM EGTA) is 100%.

In the second type of experiment Ca^{2+} (2.4 mM, 4 min) was readmitted 2 min after isoprenaline application (Figure 4). At concentrations greater than 0.3 μM, the inhibitory effects of isoprenaline were similar to those seen in the first type of experiment. When the concentration was lower than 0.3 μM, however, the effects of isoprenaline were stronger than those seen in the first type of experiment, probably due to the 2 min pre-exposure to isoprenaline. For example, when 0.03 μM isoprenaline was used, tension decreased to 33.1 ± 7.6% and the fura-2 signal decreased to 70.6 ± 12.2% in the first type of experiment ($n=5$) whereas tension decreased to 8.1 ± 3.6% and the fura-2 signal to 46.0 ± 7.9% in the second type ($n=6$).

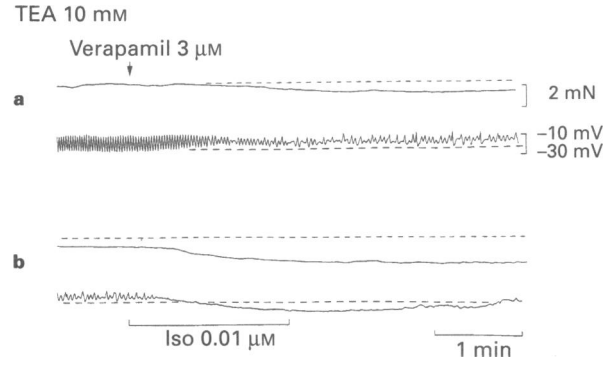

Figure 2 Guinea-pig isolated trachealis: effects of isoprenaline on tension (upper trace) and membrane potential (lower trace) in the presence of 10 mM TEA and 3 μM verapamil. (a) and (b) are continuous recordings. Note that isoprenaline (0.01 μM) was still effective in producing relaxation and hyperpolarization.

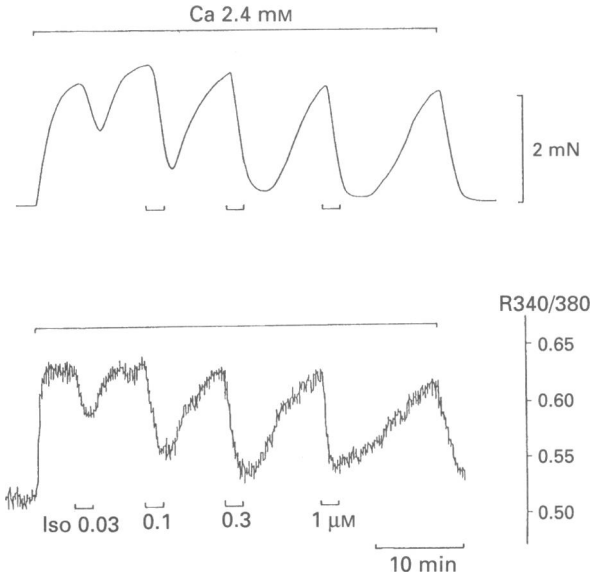

Figure 3 Guinea-pig isolated trachealis: effects of isoprenaline on tension (upper trace) and ratio of fura-2 signals ($R_{340/380}$) recorded simultaneously. Ca^{2+} (2.4 mM) was applied in the presence of PGE_2 (100 nM) to produce tonic contraction as shown above each trace. Isoprenaline at different concentrations (0.03–1 μM) was applied for 2 min as indicated below each trace. The preparation was pretreated with indomethacin (1 μM), physostigmine (10 μM), atropine (1 μM), TEA (10 mM) and verapamil (3 μM).

Effects of cyclopiazonic acid

Figure 5 shows a typical effect of cyclopiazonic acid, an inhibitor of the Ca^{2+} pump in sarcoplasmic reticulum in a similar type of experiment to Figure 3. After observing a control response to 0.1 μM isoprenaline, 10 μM cyclopiazonic acid (CPA) was applied. CPA increased both tension and the fura-2 signal, but did not significantly affect relaxation and the decrease in the fura-2 signal produced by isoprenaline. This was confirmed in two other preparations. In other experiments in which only the mechanical response was recorded under the same experimental conditions, relaxation produced by 0.01, 0.03 and 0.1 μM isoprenaline was 29.4 ± 3.2, 65.2 ± 5.1 and 93.0 ± 2.0% in the absence and 27.6 ± 4.3, 58.7 ± 5.2 and 86.3 ± 3.0% in the presence of 10 μM CPA, respectively ($n=7$). There was no statistical difference between these two groups.

Effects of isoprenaline on muscle tone

Since membrane potentials were measured in the presence of spontaneously generated muscle tone whereas changes in $[Ca^{2+}]_i$ were estimated during contractions produced by

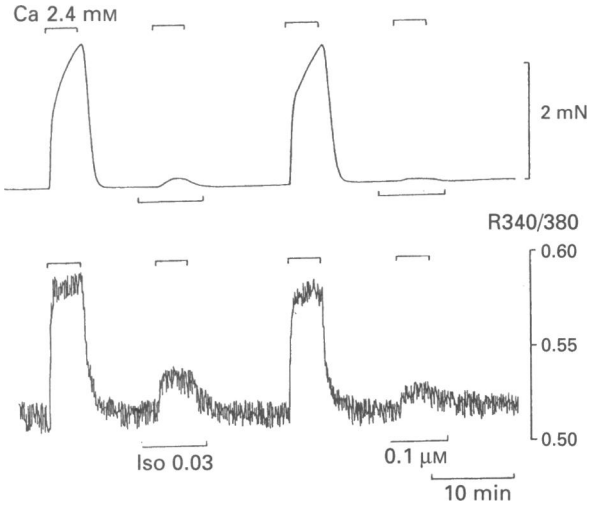

Figure 4 Guinea-pig isolated trachealis: similar experiment to that shown in Figure 3, but 2.4 mM Ca^{2+} was applied for 4 min as indicated above the trace. Isoprenaline (0.03 and 0.1 μM) was applied for 8 min as indicated below each trace. Isoprenaline application was started 2 min before addition of Ca^{2+}.

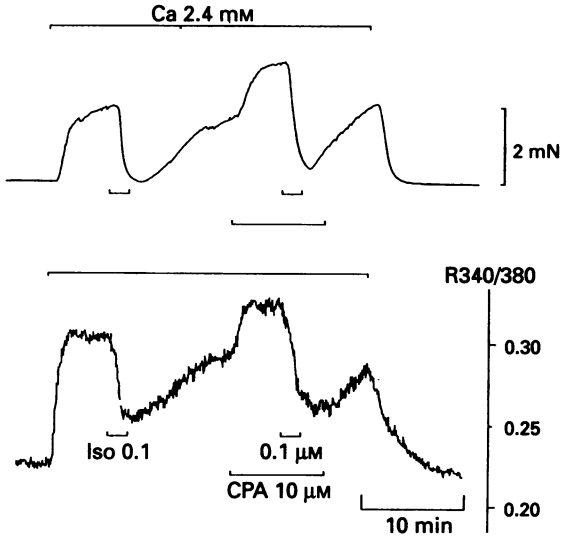

Figure 5 Guinea-pig isolated trachealis: effect of cyclopiazonic acid (CPA) on tension (upper trace) and fura-2 signal (lower trace). In the presence of prostaglandin E_2 (100 nM) and indomethacin (1 μM), 2.4 mM Ca^{2+} was applied as indicated above the recordings. Isoprenaline (Iso, 0.1 μM) was applied in the absence and presence of CPA (10 μM). Tension and fura-2 signal were simultaneously recorded.

100 nM PGE_2 in the presence of indomethacin, we compared relaxant effects of isoprenaline under these two different conditions using the same preparations. The results are summarized in Table 1. The muscle tone induced by 100 nM PGE_2 was nearly the same as the spontaneously generated tone. The effects of isoprenaline (0.01 μM) appeared slightly stronger in K-free solution, but this was not statistically significant. TEA (10 mM), which increased muscle tone by 50–70%, reduced the isoprenaline-induced relaxation. Verapamil (3 μM) not only reversed the increased muscle tone caused by TEA as previously reported (Foster *et al.*, 1984) but also removed TEA-induced antagonism of isoprenaline, as found for nifedipine (Huang *et al.*, 1993). No significant difference in the effects of isoprenaline was found between spontaneously generated muscle tone and contraction produced by PGE_2.

We also examined the effect of probenecid (10 μM) and physostigmine (10 μM) or DFP (10 μM) used for fura-2 loading. Although physostigmine and DFP increased the muscle tone, this was completely abolished by atropine (1 μM). The presence of atropine, probenecid and physostigmine or DFP had no clear effect on PGE_2-induced contractions and the relaxant effects of isoprenaline.

Discussion

At concentrations higher than 0.01 μM, isoprenaline produces clear membrane hyperpolarization in the guinea-pig tracheal muscle. A possible mechanism may be an increase in K^+ conductance mediated by activation of Ca^{2+}-dependent K^+ channels (Kume *et al.*, 1989; Muraki *et al.*, 1992). Hyperpolarization may reduce Ca^{2+} influx through voltage-gated Ca^{2+} channels and thereby result in relaxation. This possibility has been proposed as an explanation of the antagonistic effects of charybdotoxin, a Ca^{2+}-dependent K^+ channel blocker, on isoprenaline-induced relaxation (Jones *et al.*, 1990; Murray *et al.*, 1991). There are, however, several lines of evidence against this idea. (1) A poor correlation has been observed between membrane hyperpolarization and relaxation during activation of β-adrenoceptors (Cook *et al.*, 1993). (2) TEA, a non-selective K^+ channel blocker, has little, or only a weak, inhibitory action on isoprenaline-induced relaxation (Allen *et al.*, 1985 and the present experiments), although Ca^{2+}-dependent K^+ channels are highly sensitive to TEA (Tomita & Kume, 1994). In canine tracheal muscle, for example, 1 mM TEA reduces the activity of the Ca^{2+}-dependent K^+ channel by 87% (Muraki *et al.*, 1990). (3) In guinea-pig trachealis, verapamil is not effective (Duncan & Douglas, 1984; Foster *et al.*, 1984; Baba *et al.*, 1985), but isoprenaline is very effective in inhibiting spontaneously generated muscle tone and PGE_2-induced muscle tone. In the present experiments, no clear difference in isoprenaline-induced relaxation was found in the absence and in the presence of 10 mM TEA and 3 μM verapamil both for spontaneously generated muscle tone and for PGE_2-induced contraction. These results strongly suggest that K^+ channel activation, membrane hyperpolarization and inhibition of voltage-gated Ca^{2+} channels are not playing a major role in isoprenaline-induced relaxation. The inhibitory effect of charybdotoxin found by Jones *et al.* (1990) might be due to activation of voltage-gated Ca^{2+} channels by the toxin-induced depolarization and hence functional antagonism of β-adrenoceptor-mediated relaxation, as suggested by Huang *et al.* (1993). In the experiments of Jones *et al.* (1990) carbachol (0.34 μM) was used to raise tissue tone. A further possible explanation of the antagonism of isoprenaline by charybdotoxin is that the toxin modifies the spasm induced by carbachol in such a manner that the spasm is less susceptible to suppression by isoprenaline.

The spontaneous generation of muscle tone in the guinea-pig tracheal muscle is inhibited by Ca^{2+} removal and indomethacin. Therefore, the tone is likely to be due to Ca^{2+} influx through a verapamil-insensitive pathway in the plasma membrane which is activated by prostaglandins (Orehek *et al.*, 1975; Baba *et al.*, 1985; Ito *et al.*, 1985). It is thus possible that isoprenaline inhibits Ca^{2+} influx through this pathway. Measurements of $[Ca^{2+}]_i$ with fura-2 fluorescence signals strongly suggest that this is the main mechanism underlying the re-

Table 1 Relaxation produced by isoprenaline (0.01 μM) in isolated tracheal smooth muscle

Spontaneously generated muscle tone				
	Normal solution	*K-free*	*TEA* (10 mM)	*TEA* (10 mM) + *verapamil* (3 μM)
Tension before isoprenaline	100%	75.3 ± 2.9%	168.4 ± 9.3%	105.4 ± 9.6%
Relaxation by isoprenaline	77.1 ± 3.0% (n = 12)	72.6 ± 6.3% (n = 6)	63.8 ± 5.8% (n = 6)	81.0 ± 3.4% (n = 6)
Contraction produced by PGE_2 (100 nM)				
	Normal solution	*K-free*	*TEA* (10 mM)	*TEA* (10 mM) + *verapamil* (3 μM)
Tension before isoprenaline	100%	75.6 ± 3.5%	154 ± 3.9%	112.5 ± 7.5%
Relaxation by isoprenaline	76.2 ± 5.1% (n = 12)	84.4 ± 7.4% (n = 6)	58.7 ± 9.0% (n = 6)	83.5 ± 2.0% (n = 6)

Tension before isoprenaline was expressed by taking the value in normal solution as 100% and the level in Ca^{2+}-free solution to be 0%. Relaxation by isoprenaline was measured from the tension just before isoprenaline application taking the value in Ca^{2+}-free solution as 100%.

laxation caused by isoprenaline. In the presence of TEA (10 mM) and verapamil (3 μM), relaxation produced by isoprenaline applied during PGE_2-induced contraction is accompanied by a clear decrease in fura-2 signals indicating a decrease in $[Ca^{2+}]_i$. Since isoprenaline is also very effective in inhibiting contractions and increases in $[Ca^{2+}]_i$ produced by an external application of Ca^{2+}, in the presence of PGE_2, it is more likely that these effects are due to inhibition of Ca^{2+} influx rather than to facilitation of Ca^{2+} sequestration or Ca^{2+} efflux. The observation that cyclopiazonic acid failed to inhibit relaxation and a decrease in $[Ca^{2+}]_i$ produced by isoprenaline supports this idea. Isoprenaline-induced hyperpolarization observed in the presence of 10 mM TEA could be due either to activation of TEA-insensitive K^+ channels or to inhibition of inward currents activated by PGE_2. The failure to observe an increase in the hyperpolarization by removal of external K^+ suggests the latter possibility, but further experiments are necessary to substantiate this hypothesis.

If a decreased sensitivity of contractile protein to Ca^{2+}-calmodulin was mainly responsible for the relaxation, one would expect to see a poor correlation between the degree of relaxation and a decrease in $[Ca^{2+}]_i$, but in fact a relatively good correlation was found. Therefore, a decrease in $[Ca^{2+}]_i$ is considered to be mainly responsible for isoprenaline-induced relaxation. At a concentration higher than 0.03 μM, isoprenaline produced almost complete relaxation but some fura-2 signal remained, assuming the signal in Ca^{2+}-free medium is the control level. This result might indicate that $[Ca^{2+}]_i$ must increase above a certain level to allow tension development or, alternatively, a decrease in the sensitivity of myosin light chain kinase to Ca^{2+}-calmodulin pathway uncouples the increase in $[Ca^{2+}]_i$ and tension. Although these problems were not investigated in the present experiments, these factors may play some role in tension development. The relaxation and the decrease in $[Ca^{2+}]_i$ (or inhibition of a rise of $[Ca^{2+}]_i$) can be most easily explained by assuming that isoprenaline inhibits a pathway for Ca^{2+} influx which is activated by prostaglandins and is insensitive to verapamil.

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