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Relaxing actions of procaterol, a β_2-adrenoceptor stimulant, on smooth muscle cells of the dog trachea

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1 The effects of procaterol, a β_2-adrenoceptor agonist, on smooth muscle cells of the dog trachea were investigated by use of microelectrode and isometric tension recording methods, and by measurement of Ca transients as estimated fromthe fura-2 fluorescence, adenosine 3′: 5′-cyclic monophosphate (cyclic AMP) and breakdown of phosphatidylinositols.

2 Procaterol hyperpolarized the membrane and increased the ionic conductance (above 10 nM) in a dose-dependent manner. These actions were inhibited by propranolol.

3 Procaterol inhibited the mechanical responses evoked by acetylcholine (ACh), histamine or 5-hydroxytryptamine (5-HT), in the presence or absence of Ca^{2+} in the bath solution, but not that evoked by high concentrations of ACh (1 μM). The ID_{50} value of procaterol for the peak amplitude of the ACh-induced contraction (30 nM) was 0.3 nM. The equivalent values for the histamine-induced phasic and tonic responses (10μM) were 0.15 and 0.01 nM), respectively.

4 Procaterol (over 1 nM) increased the amount of cyclic AMP in a dose-dependent manner which was blocked by prior application of propranolol.

5 Procaterol did not alter the changes in the amounts of phosphatidylinositol 4,5-bisphosphate ($PI-P_2$) and phosphatidic acid (PA) induced by ACh, histamine or 5-HT. Thus, the synthesis of inositol 1,4,5-trisphosphate is not affected by stimulation of the β_2-adrenoceptor.

6 ACh increased the free Ca^{2+} concentration to a greater extent than that produced by histamine or 5-HT. These changes were reduced by procaterol, except for those induced by high concentrations of ACh (over 1μM).

7 It is concluded that procaterol relaxes tissues precontracted by various agonists due to a reduction in the free Ca^{2+}. This inhibitory action may be due to an increase in the amount of cyclic AMP but does not result from an inhibition of the hydrolysis of phosphatidyl inositols. The hyperpolarization induced by procaterol may partly contribute to the observed relaxation.

Introduction

Yabuuchi *et al.* (1977) found that in anaesthetized dogs, procaterol, 5-(1-hydroxy-2-isopropylaminobutyl) -8-hydroxycarbostyril hydrochloride was a β-adrenoceptor agonist with bronchodilator selectivity. Its actions were blocked by propranolol or pindolol. Procaterol was as active as isoprenaline and trimetoquinol but was approximately 3 times more active than salbutamol in inhibiting histamine-induced increase in bronchial resistance. In anaesthetized cats, procaterol and salbutamol showed similar β_2-adrenoceptor agonist activity (Yamashita *et al.*, 1978). Using blood perfusing procedures in tracheal and cardiac muscles, Himori & Taira (1977) reported that procaterol was about 6000 times more selective than isoprenaline on tracheal smooth muscle compared with ventricular muscle, as estimated from relative potency calculations.

Irie *et al.* (1979) classified procaterol as a β_2-adrenoceptor agonist from its metabolic products in the beagle dog. Takayanagi *et al.* (1977) found that procaterol increased tissue levels of cyclic AMP and that this action was greater in the guinea-pig trachea than in the atria and was prevented by prior application of propranolol. The β_2-adrenoceptor agonist action of procaterol was confirmed in various tissues (vascular and cardiac tissues; Borkowski & Quinn, 1984; lung; Delhaye *et al.*, 1983, parotid gland; Hata *et al.*, 1985).

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Although procaterol has a high potency and selectivity for tracheal smooth muscle and has shown a long duration of action in the treatment of bronchial asthma, the mechanism underlying β_2-adrenoceptor agonist activity is not well understood. The present experiments were an attempt to clarify this aspect of the action of procaterol on dog tracheal smooth muscle by use of microelectrode and isometric tension recording methods. The effects of procaterol on changes in myoplasmic free Ca^{2+} concentration and the hydrolysis of inositol phospholipids were also measured.

Methods

Mongrel dogs of either sex, weighing 10–15 kg, were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.v.). Segments of the cervical trachea were excised, and dorsal strips of transversely running smooth muscle fibres were separated from the cartilage. The mucosa and adventitial areolar tissues were carefully removed, under microscopic observation.

Membrane potential recording

The membrane potential and resistance of strips of smooth muscle (1 mm in width and 15 mm in length) were measured by the partition stimulating method. Microelectrodes (40–80 MΩ resistances, filled with 3 M KCl) were inserted from the mucosal side superfused with warmed (32°C) Krebs solution, at a flow rate of 2–3 ml min^{-1}. Electrical responses were displayed on a pen-writing recorder (Nihonkohden Recticorder RJG 4024).

Tension recording

The muscle strips were cut to a diameter of 0.05–0.08 mm and a length of about 0.3 mm, under a binocular microscope. The preparation was set up in a small chamber with a capacity of 0.9 ml through which the test solution was rapidly superfused (Itoh *et al.*, 1981). Both ends of the preparation were fixed between pieces of Scotch double tape (3M Co., St Paul, Minn, U.S.A) with thin silk thread. The tension evoked by various stimulants was recorded with a strain gauge transducer (U-gauge, Shinko). Tension development in the absence of Ca^{2+} was measured in a Ca^{2+}-free solution containing 2 mM EGTA (ethylene glycol bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; Dozin) (Itoh *et al.*, 1981; 1985).

Measurements of cytosolic free Ca^{2+}

Dispersed single smooth muscle cells were prepared by collagenase dispersion, as described by Sumimoto & Kuriyama (1986) and dispersed cells were collected by centrifugation at 30 *g* for 5 min. They were then loaded with fura-2 (50–100 μM) in the presence of 1 μM fura-2 acetoxymethyl ester (fura-2/AM) for 60 min at 37°C as described for quin2 by Sumimoto & Kuriyama (1986). After loading with fura-2, cell viability assessed by the trypan blue exclusion test was over 90%. Before measurement of the fluorescence, the cell suspension was equilibrated in HEPES buffer solution containing 1 mM Ca^{2+} for 30 min and then preincubated at 37°C for 10 min.

Fluorescence of fura-2 loaded cells was measured at 37°C in a Hitachi 650-40 fluorescence spectrophotometer with a thermostatically-equipped cell holder, using an excitation wavelength 340 nm (slit 4 nm) and emission 490 nm (slit 10 nm). The cells were suspended in 2 ml of HEPES buffer solution containing 1 mM $CaCl_2$ at concentrations of $0.5–1 \times 10^6$ cells ml^{-1} in 1 cm square quartz cuvette and the preparation was stirred continuously.

Following completion of the fluorescence measurement, the cells were lysed with 0.2% Triton X 100 and the dye-saturated 1.0 mM Ca^{2+} gave the maximum fluorescence of the sample, F_{max}. F_{min} was the fluorescence obtained by adjusting the pH of the lysed cells to 8.5 with Tris base followed by the addition of 10 mM EGTA.

The concentration of intracellular free Ca^{2+}, $[Ca^{2+}]_i$, was calculated from the equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d, an effective dissociation constant for Ca^{2+}-fura-2 is 224 nM (Grynkiewicz *et al.*, 1985).

Assays of inositol phospholipids, phosphatidic acid and inositol phosphate

Concentrations of phosphatidylinositol 4,5-bisphosphate, PI-P_2, and phosphatidic acid (PA) in muscle cells of the trachea were measured before and after application of agonists, as described by Hashimoto *et al.* (1985). Briefly, muscle strips were labelled in a phosphate-free, HEPES-buffered Krebs solution containing $^{32}P_i$ 40 μCi ml^{-1} (specific radioactivity, 30–40 Ci $mmol^{-1}$; Japan Atomic Energy Research Institute) at 37°C for 6 h. The strips were then washed three times with the above solution without $^{32}P_i$ and were incubated with procaterol or isoprenaline for 10–240 s. The reaction was halted by adding a solvent containing chloroform, methanol and concentrated HCl (100:200:2, v/v), and strips were homogenized in a glass homogenizer. Crude phospholipid extracts in the solvent were chromatographed on Silica Gel 60 plates (Merck). The plates were then autoradiographed for 12–15 h using Sakura X-ray film. The fractions corresponding to PI-P_2 and PA on the plate were excised, and counted for radioactivity in a liquid scintillation counter.

Assays of cyclic AMP

Dissected tissues weighing 50–100 mg wet weight were mounted in an organ bath under zero load. Tissues kept at 37°C for about 60 min in bubbled Krebs solution served as the control. To observe the effects of isoprenaline or procaterol on the content of cyclic AMP, various concentrations of these agents were applied for the final 3 min of the 60 min incubation period. After 60 min incubation, the tissue was frozen with liquid nitrogen and homogenized with 6% trichloroacetic acid. The level of cyclic AMP of the extracts was measured with a radioimmunoassay kit (Yamasa Shoyu, Tokyo). Further details of the procedures have been given elsewhere (Hirata & Kuriyama; 1980).

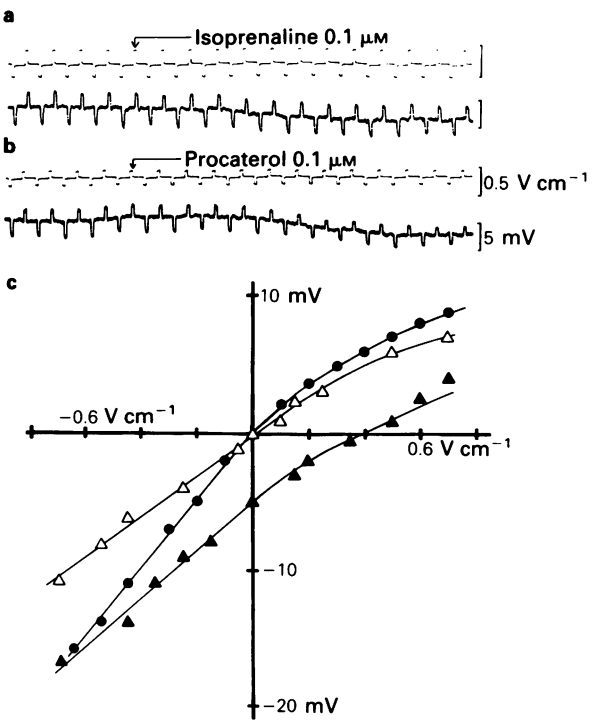

Figure 1 Effects of isoprenaline (a) or procaterol (b and c) on the membrane resistance measured from single smooth muscle cells. The microelectrode was inserted into the cell within 100 μM of the stimulating electrode. (a and b) To record the electrotonic potential before and during application of procaterol or isoprenaline, inward and outward currents of constant intensity were applied alternately. (c) Various intensities of inward and outward current were applied (pulse duration; 2 s). To measure the membrane resistance at the resting membrane potential level in the presence of procaterol, an outward d.c. current was applied to return the membrane potential to the previous resting level: (●) control; (▲) procaterol 0.1 μM; (△) procaterol plus current injection.

Solutions and drugs

The ionic composition of the Krebs solution was as follows (mM) Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+}2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.4, glucose 11.5; 97% O_2:3% CO_2 was bubbled into the solution, the pH of which was adjusted to 7.4. High K^+ solution was prepared by replacing NaCl with KCl, isotonically.

Drugs used in the experiments were procaterol ((±)-procaterol, Otsuka Pharmac.), isoprenaline ((−)-isoprenaline (+)-bitartrate, Nakarai Chemicals), propranolol ((±)-propranolol HCl, ICI Pharmac.), acetylcholine (acetylcholine Cl, ACh) and tetrodotoxin (TTX; Sigma), histamine (histamine HCl, Ishizu Pharmac.), 5-hydroxytryptamine (serotonin creatine sulphate, 5-HT) and atropine (atropine sulphate; Merck), guanethidine sulphate (Tokyo Kasei) and fura-2/AM (Dozin). All solutions were freshly prepared before each experiment. The water used in this study was glass-twice distilled water and all other chemicals were of the highest reagent grade available.

Statistics

The measured values are expressed as the mean ± s.d. (standard deviation) and the number of observations or as the mean ± s.e. (standard error) in case of the measurements of the Ca transient, cyclic AMP or phosphatidylinositols. The statistical significance was assessed by Student's *t* test for paired or unpaired values. *P* values of less than 0.05 were considered to be statistically significant.

Results

Effects of procaterol on the membrane potential and resistance

The cells of resting muscle strips were electrically quiescent and had a mean membrane potential of -61 ± 1.2 mV ($n = 50$ cells, 5 strips). With application of various concentrations of procaterol (>10 nM), the membrane was hyperpolarized in a concentration-dependent manner. Isoprenaline hyperpolarized the membrane to -70.2 ± 2.4 mV, $n = 30$. This was greater than that produced by procaterol (-65.4 ± 1.8 mV, $n = 30$) at concentrations above 1 μM ($P < 0.01$). Propranolol (0.1 μM) inhibited the hyperpolarization induced by concentrations of procaterol or isoprenaline up to 10 μM. Thus,

the hyperpolarization induced by these drugs is mediated through activation of β-adrenoceptors. To clarify the nature of the hyperpolarization induced by these agents, the current-voltage relation was observed in the presence or absence of the two agonists (0.1 μM). As shown in Figure 1a and b, alternately applied, constant intensity, inward and outward currents provoked electrotonic potentials. With application of 0.1 μM isoprenaline or procaterol, the membrane was hyperpolarized and the amplitude of the electrotonic potential was reduced. When the current-voltage relationship was plotted with various intensities of inward and outward currents before and after application of procaterol, membrane resistance was shown to be reduced by application of 0.1 μM procaterol. When the above current-voltage relationship was observed after displacement of the membrane potential to the resting level by application of outward direct current, the membrane resistance was still reduced.

General features of the contraction evoked by various stimulants

When slightly stretched smooth muscle strips of dog trachea were superfused with Krebs solution (1.1 times slack length; at which length, the isometric active force was maximum, and the resting tension was about 1–2 mg), tone resistant to tetrodotoxin (1 μM) or atropine (1 μM) gradually developed during 2–3 h and was sustained for 2–3 h (Figure 2). During the development of tone, the application of 10 μM ACh produced a contraction of minute amplitude (Figure 2a). After 5–6 h, tone gradually declined to a basal level and 10 μM ACh then produced a large contraction (Figure 2b). In Figure 2c, the contractions evoked by various stimulants (128 mM K^+, 10 μM ACh, 10 μM histamine and 10 μM 5-HT) are illustrated.

To clarify whether the exogenously applied stimulants exerted direct (Foster *et al.*, 1983) and/or indirect (Kuriyama *et al.*, 1982) actions, tissues were superfused with 3 μM atropine plus 5 μM guanethidine. The amplitude of the tonic component of contraction evoked by 10 μM ACh in control conditions was normalized as a relative amplitude (RA) of 1.0. In the presence of atropine and guanethidine (test amplitude, $n = 5$), the RA of the phasic component of contraction was consistently reduced (128 mM K^+: RA = 0.32 ± 0.02 (control), 0.26 ± 0.01 (test), $P < 0.05$; 10 μM 5-HT: RA = 0.65 ± 0.08 (control), 0.42 ± 0.01 (test), $P < 0.05$; 10 μM histamine: RA = 0.64 ± 0.18 (control), 0.32 ± 0.06 (test), $P < 0.01$; 10 μM ACh: RA = 0.92 ± 0.09 (control), 0.01 ± 0.01 (test), $P < 0.01$). The tonic response was also markedly inhibited in the case of histamine and ACh ($P < 0.01$, $n = 5$).

To estimate the sources of Ca^{2+} contributing to the

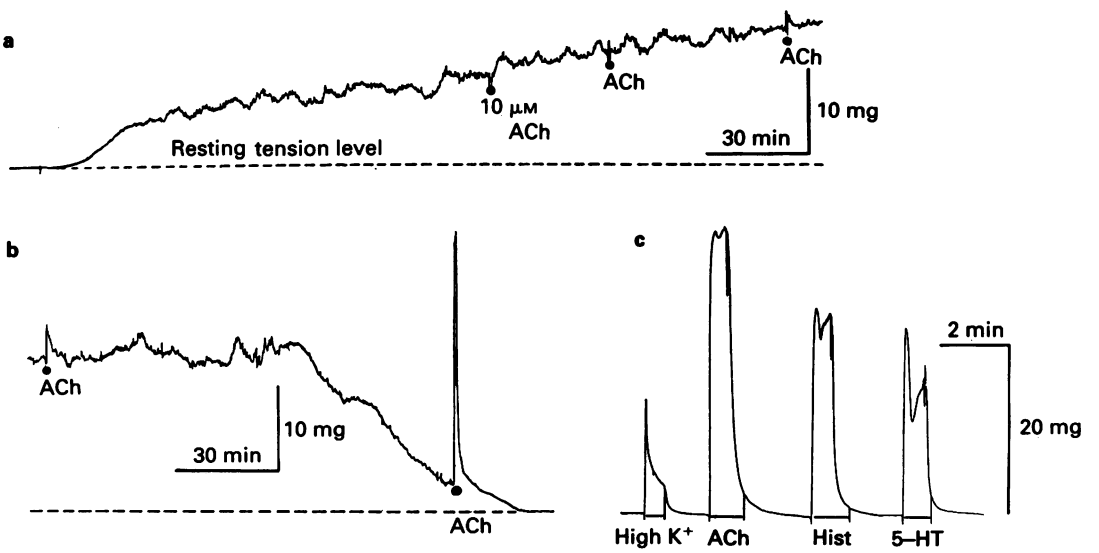

Figure 2 Effects of various stimulants, 128 mM K^+ (high K^+), 10 μM acetylcholine (ACh), histamine (Hist) or 5-hydroxytryptamine (5-HT) on the contraction evoked in small muscle strips excised from the dog trachea. (a and b) Continuous records. During generation of the contraction, ACh (10 μM) was repetitively applied as indicated by dots; (c) after the tissue relaxed completely to the resting level, the four stimulants were successively applied. Thereafter, the resting tension level was kept at about 1 mg (1.1 times the slack length).

generation of contraction evoked by various stimulants, contractions were evoked after 2 min pre-application of Ca^{2+}-free solution containing 2 mM EGTA. High K^+ in Ca^{2+}-free solution evoked neither a phasic nor a tonic response, while 10 μM histamine, 1.0 μM ACh or 10 μM 5-HT evoked phasic but not tonic responses. The amplitude of the phasic response evoked by 5-HT was markedly inhibited, while that evoked by histamine or ACh was inhibited to a lesser extent.

Effects of procaterol on agonist-induced contractions

The maximum contractions produced by histamine and 5-HT (each 10 μM) were much smaller than the ACh maximum (also 10 μM) and corresponded to the effects of 50 nM ACh. Thus the effects of two different concentrations of ACh were studied. After 3 min pretreatment with procaterol, both phasic and tonic components of the contraction evoked by 50 nM ACh, 10 μM histamine and 10 μM 5-HT were inhibited (Figure 3A). The contraction evoked by 1.0 μM ACh was only slightly inhibited while the rising phase of the contraction was markedly slowed. Such inhibition of ACh-induced responses was also observed in the absence of Ca^{2+}. As shown in Figure 3B, the 30 nM ACh-induced contraction was markedly inhibited in Ca^{2+}-free solution containing 2 mM EGTA. Addition of 2.6 mM Ca^{2+} restored the mechanical response (Figures 3Ba and b). Following application of 0.1 μM procaterol, the ACh-induced contraction ceased (Figure 3Bc). When the same experimental protocols were adopted using 1.0 μM ACh, only a phasic response appeared in the presence of Ca^{2+}-free solution and the re-addition of Ca^{2+} produced the same amplitudes of phasic and tonic responses as were observed in the control (Figures 3Ba′ and b′). When 0.1 μM procaterol was applied beforehand, the amplitude of the phasic contraction remained the same, but the rate of rise of the contraction was reduced (Figure 3 Bc′).

Figure 4 shows the effects of procaterol or isoprenaline on the 30 nM and the 1 μM ACh-induced contractions. In concentrations over 0.1 nM, procaterol or isoprenaline consistently inhibited the 30 nM ACh-induced contraction, in a dose-dependent manner (Figure 4a). The tonic responses rapidly declined and ACh produced only a transient phasic contraction. Procaterol and isoprenaline had much the same actions on the ACh-induced contraction. On the other hand, up to 0.1 μM procaterol or isoprenaline had no effect on the amplitude of the tonic component of the 1.0 μM ACh-induced contraction. The ID_{50} values of procaterol or isoprenaline observed on the 30 nM

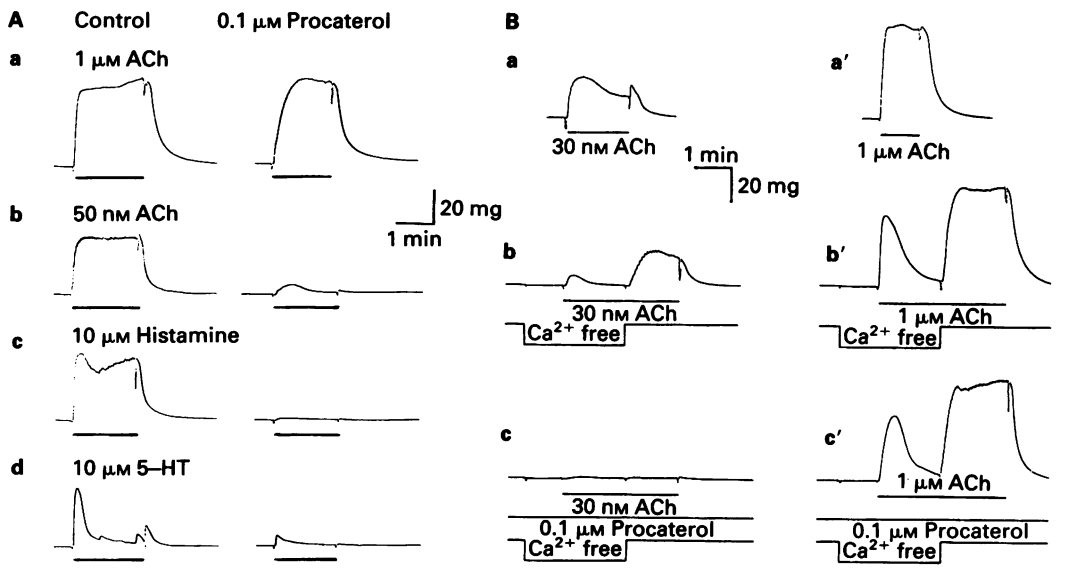

Figure 3 (A) Effects of 0.1 μM procaterol on the agonist-induced contraction: (a) 1 μM acetylcholine (ACh), (b) 50 nM ACh, (c) 10 μM histamine, (d) 10 μM 5-hydroxytryptamine (5-HT). Procaterol was applied 3 min before application of the agonists. (B) Effects of 0.1 μM procaterol on the ACh-induced contraction in the presence (a and a′) or absence of Ca^{2+} (b, b′, c and c′); 30 nM (a – c) or 1 μM (a′ – c′) was applied. Tissues were pretreated with Ca^{2+}-free solution containing 2 mM EGTA for 1 min before application of ACh.

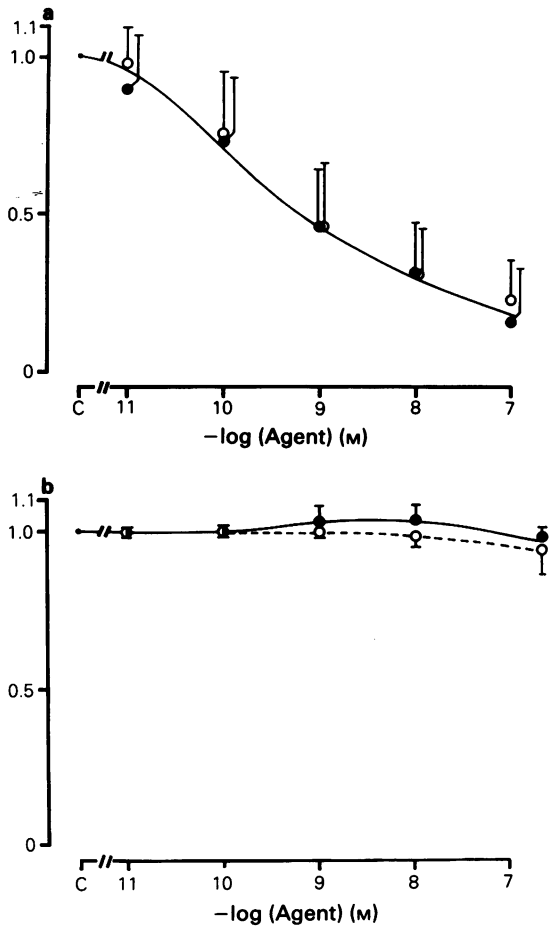

Figure 4 Effects of various concentrations of procaterol (○) or isoprenaline (●) on the maximum amplitude of the 30 nM acetylcholine (ACh)-induced contraction (a) or on the tonic component of a 1 μM ACh-induced contraction (b). The peak amplitude of the contraction evoked by 30 nM ACh (a) or 1 μM ACh (b) was normalized as a relative tension of 1.0, respectively ($n = 5$). Procaterol or isoprenaline was applied 3 min before application of ACh. Vertical lines indicate s.d., $n = 5$-6 different muscle strips.

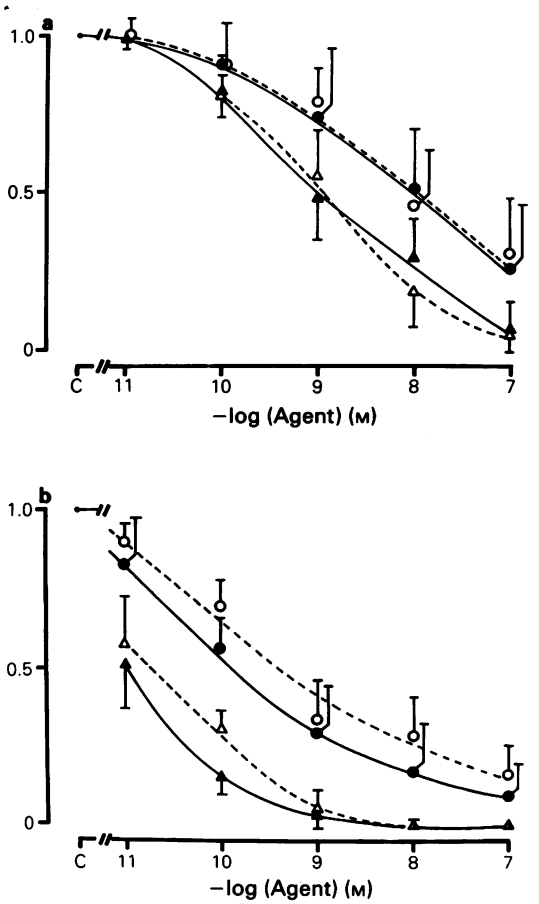

Figure 5 Effects of various concentrations of procaterol (●, ▲) or isoprenaline (○, △) on the 10 μM 5-hydroxytryptamine (a) or 10 μM histamine-induced contraction (b). The experimental procedures were the same as described for Figure 4. $n = 5$ different muscle strips.

ACh-induced contraction were each 0.3 nM ($n = 5$).

Similar experiments were carried out on 10 μM 5-HT (Figure 5a) or 10 μM histamine-induced contractions (Figure 5b) in the presence of 3 μM atropine with 5 μM guanethidine. Both the phasic and tonic responses to 5-HT were consistently inhibited at concentrations over 10 nM, with a greater effect on tonic responses. The ID_{50} value of procaterol against 5-HT-induced contractions was 10 nM for phasic or 1 nM for tonic responses ($n = 5$). No difference between the inhibitory actions of procaterol and isoprenaline on agonist-induced contractions was observed. Both agents inhibited the phasic and tonic responses induced by 10 μM histamine. The ID_{50} values of isoprenaline and procaterol against phasic responses were 0.4 nM and 0.15 nM, respectively, and those against the tonic responses were 0.02 nM and 0.01 nM, respectively ($n = 5$).

The effects of 1 nM or 0.1 μM procaterol on the histamine-induced phasic or tonic response of the contraction were observed. Contractions evoked by histamine (up to 10 μM) were blocked by procaterol (0.1 μM), and 1 nM procaterol produced selective inhibition of tonic responses. However, the mode of inhibition of the phasic and tonic responses induced by procaterol was not by a parallel shift to the right.

Effects of procaterol and isoprenaline on production of cyclic AMP

Figure 6 shows the effects of procaterol or isoprenaline on the amount of cyclic AMP synthesized in the smooth muscle cells of the dog trachea. With application of procaterol or isoprenaline (0.1 nM–1 μM), the cyclic AMP concentration was significantly increased. Further increase in the concentration of both agents no longer produced a further increase in the amount of cyclic AMP. These changes were antagonized by propranolol.

Effects of procaterol or isoprenaline on hydrolysis of phosphatidyl inositols induced by spasmogens

When 1 μM ACh was added to the bath, the amount of PI-P_2 was reduced within 10 s to 0.8 times the control. Although a slight inhibition of the hydrolysis of PI-P_2 occurred 10 s after application of ACh, the hydrolysis of PI-P_2 induced by ACh was not subsequently modified by either procaterol or isoprenaline (Figure 7a). When the amounts of synthesized PA were measured, there was a consistent increase 10 s after the application of ACh (the amount of PA measured before application of ACh was 9574 ± 728 c.p.m. mg^{-1} protein, $n = 15$, and this value was normalised as the control of 1.0). After 60 s, no steady value had been reached (Figure 7b). On exposure to procaterol, the amount of PA synthesized exceeded that seen with application of isoprenaline ($P < 0.05$, $n = 4–7$).

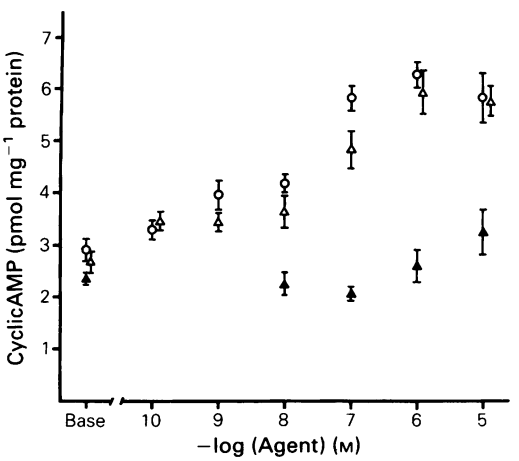

Figure 6 Effects of various concentrations of procaterol (○) or isoprenaline (△) on the amount of cyclic AMP. Experimental procedures were as described in Methods. The effects of procaterol were also observed under conditions of pretreatment with 1 μM propranolol (▲). Vertical bars indicate s.e., $n = 6–7$ samples.

Similar experiments were carried out by applying 10 μM 5-HT in the presence of 3 μM atropine and 5μM-guanethidine, to reduce possible indirect effects. The amount of PI-P_2 was reduced and that of PA was increased by application of 10 μM 5-HT, as expected from the previous observations with ACh (after 30 s, PI-P_2 reduced to 0.78 times the control and PA increased to 2.1 times over the control, $n = 5$; after 60 s, it was reduced to 0.64 times and was increased to 3.6 times the control, respectively, $n = 5$). These actions of 5-HT were not modified in the presence of isoprenaline or procaterol. When the effects of 10 μM histamine on the hydrolysis of PI-P_2 were observed in the presence of atropine with guanethidine, a reduc-

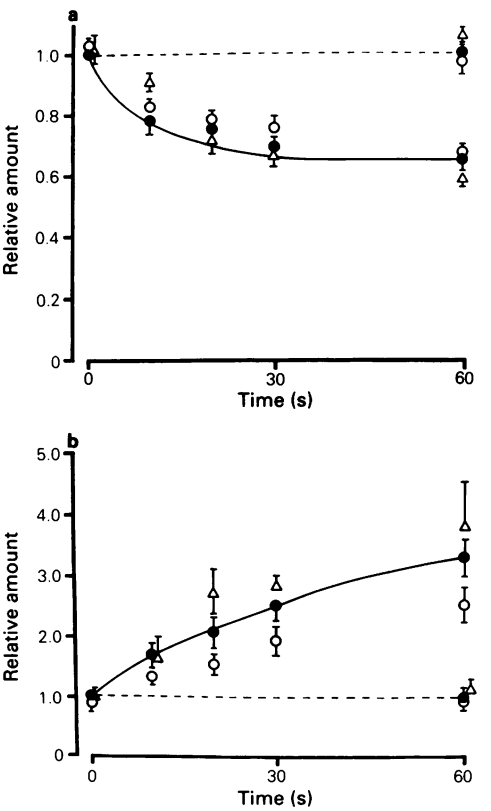

Figure 7 Effects of 1 μM procaterol (△) or isoprenaline (○) on the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-P_2; a) and phosphatidic acid (PA; b) induced by 1 μM acetylcholine (ACh) against time (up to 60 s). Vertical bars indicate s.e., $n = 4–10$. The amounts of PI-P_2 and PA were also measured after 60 s without the application of ACh (dotted lines). The amounts of PI-P_2 and PA measured in the absence of ACh were 79506 ± 7516 c.p.m. mg^{-1} protein, $n = 15$ and 15603 ± 2986 c.p.m. mg^{-1} protein, $n = 10$, respectively, and these values were taken as 1.0, respectively.

tion in the amount of PI-P_2 and an increase in that of PA similar to that observed on exposure to ACh (1 μM) were detected. Neither procaterol (1 μM) nor isoprenaline had any effect on the histamine-induced changes in the amounts of PI-P_2 and PA.

Effects of procaterol on cytosolic Ca^{2+} as measured with fura-2

Figure 8A shows the effects of procaterol on the basal $[Ca^{2+}]_i$ and on the Ca transient observed in the presence of histamine or ACh (each 10 μM). The resting (basal) free Ca^{2+} concentration was 121 ± 9 nM ($n = 19$), and 1 μM procaterol transiently reduced the amount of free Ca^{2+} to just below the basal level (Fgiure 8Aa). When histamine or ACh was applied, the Ca transient developed rapidly and declined with a short duration to a sustained level just above the basal value. Neither 1 μM atropine nor 3 μM guanethidine had any effect on the histamine-induced Ca transient. ACh (10 μM) increased the free Ca^{2+} to a greater extent than that seen with the application of 10 μM histamine. During the sustained increase provoked by application of 10 μM histamine, 1 μM procaterol caused a transient decrease in the $[Ca^{2+}]_i$ and there was a gradual reversion to the resting level (Figure 8Ab). In the case of 10 μM ACh, the reduction in the Ca transient induced by procaterol was much smaller than that seen with histamine (Figure 8Ac). After 2 min pretreatment with 1 μM procaterol, the Ca transient obtained by application of 0.1 μM ACh, 10 μM histamine or 10 μM 5-HT was inhibited, while that evoked by 10 μM ACh was unaffected (Figure 8B).

Figure 9a shows the relationship between the Ca transient and various concentrations of ACh. In concentrations over 1 nM, ACh increased the free Ca^{2+} concentration. When 1 μM procaterol was applied for 2 min, the amplitude of the subsequently provoked Ca transient was reduced when concentrations of ACh were below 0.1 μM. Procaterol had no effect on the Ca transient evoked by a further increase in the concentrations of ACh (1–10 μM). Figure 9b shows the effects of various concentrations of procaterol on the histamine-induced Ca transient. In concentrations over

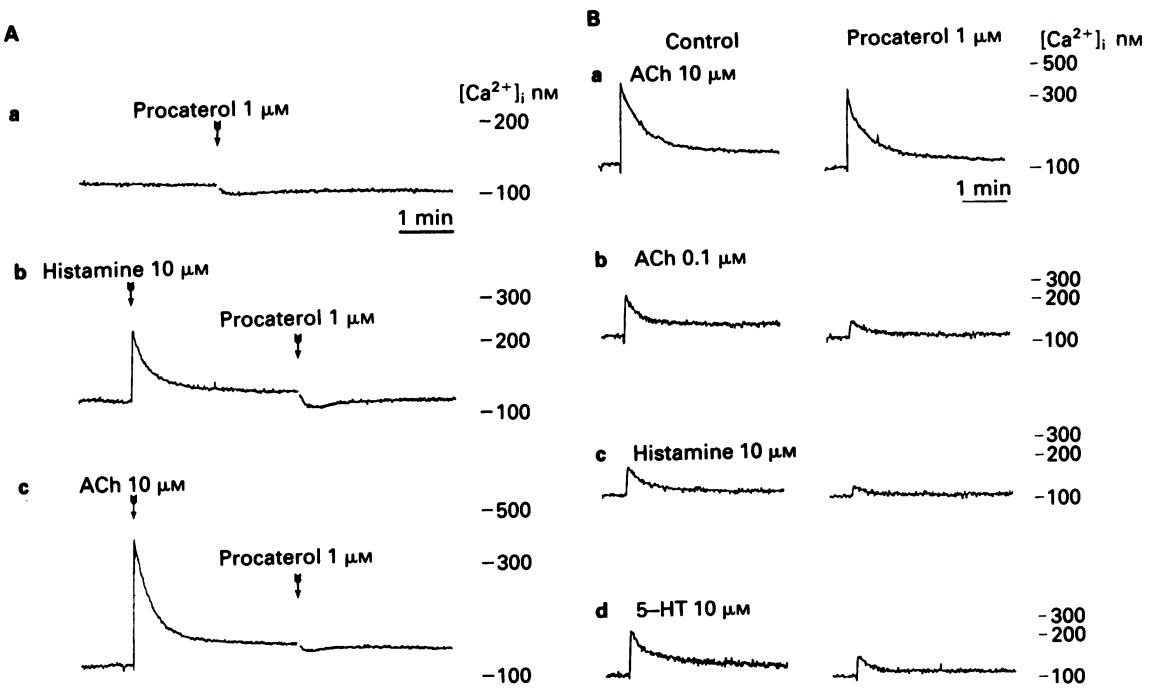

Figure 8 (A) Effects of 1 μM procaterol on the resting (a), 10 μM histamine (b) or 10 μM acetylcholine (ACh)-induced (c) Ca transient as estimated from the fura-2 fluorescence. Arrows indicate application of various agonists, including procaterol. The amount of free Ca^{2+} was calculated from the intensity of fura-2 fluorescence, as described in Methods. (B) Effects of 1 μM procaterol on Ca transients provoked by various agonists: (a) 10 μM ACh, (b) 0.1 μM ACh, (c) 10 μM histamine, (d) 10 μM 5-hydroxytryptamine (5-HT). Tissues were pretreated with procaterol for 2 min before the application of agonists.

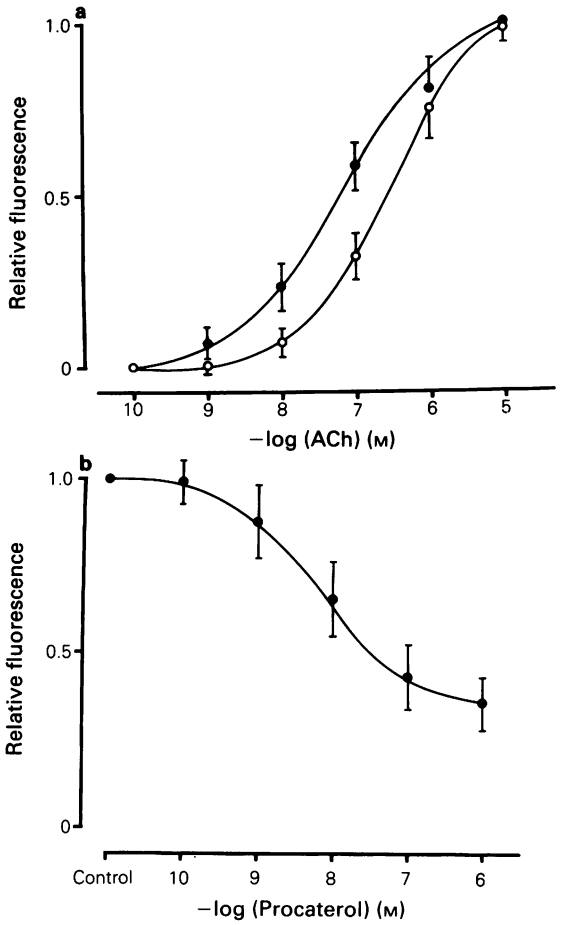

Figure 9 (a) Effects of procaterol on various concentrations of the acetylcholine (ACh)-induced Ca transient. The peak Ca transient recorded by application of 10 μM ACh was normalised at 1.0 (the mean value of 410 nM, $n = 5-6$). Vertical bars indicate s.e. ($n = 5-6$ pellets): (●) control; (○) in the presence of procaterol. (b) Effects of various concentrations of procaterol on the histamine-induced Ca transient under conditions of pretreatment with 1 μM atropine or 3 μM guanethidine for 3 min before the application of histamine. Vertical bars indicate s.e., $n = 4-6$ pellets. The amplitude of the Ca transient, as estimated from the fura-2 fluorescence, provoked by 10 μM histamine was normalised as a relative value of 1.0 (the absolute value was 198 nM, $n = 5$ pellets).

1 nM, procaterol consistently inhibited the Ca transient, in a concentration-dependent manner ($n = 3-6$), while 1 μM procaterol did not completely block the response. The ID_{50} value for procaterol on the 10 μM histamine-induced Ca transient was calculated to be 20 nM ($n = 5$).

Discussion

In the initial stages of the study, the mechanisms associated with the contractile agents themselves were investigated. The 128 mM K^+-induced contraction was smaller than that produced by ACh, histamine or 5-HT. This suggests that the voltage-dependent Ca^{2+} channel may be poorly developed in comparison to receptor-operated mechanisms. In a previous study with saponin-skinned tracheal muscle, the contraction evoked by 10 μM Ca^{2+} was much larger than that evoked by 128 mM K^+ and was similar in amplitude to that evoked in intact tissues by 10 μM ACh (Ito & Itoh, 1984). In the present study, some agonists, in particular histamine, stimulated not only the muscle directly but also the release of transmitters (mainly ACh) from the nerve terminals. Such a prominent indirect component was not found in an earlier investigation in guinea-pig trachea (Foster *et al.*, 1983) and is presumably a reflection of differences between the dog and guinea-pig.

The phasic response evoked by agonists is mainly evoked by Ca^{2+} released from cellular stores and in Ca^{2+} free solution, only the phasic component of agonist-induced contractions persists (Kuriyama *et al.*, 1982). In the dog trachea, this Ca^{2+} release is thought to be mediated by inositol 1,4,5-trisphosphate ($InsP_3$) liberated following hydrolysis of PI-P_2 (Hashimoto *et al.*, 1985). In the present experiments, application of ACh, histamine and 5-HT indeed increased the breakdown of PI-P_2 and the amount of PA.

In resting conditions, the amount of free Ca^{2+} estimated with fura-2 in the smooth muscle cells of dog trachea was similar to that observed in other smooth muscles using either aequorin, fura-2 or quin2 (100–180 nM) (Morgan & Morgan, 1984; Williams *et al.*, 1985; Rembold & Murphy, 1986; Sumimoto & Kuriyama, 1986).

In the present study, procaterol inhibited the tonic mechanical component of agonist-induced responses more than the phasic component. These effects were associated with a reduction in the fura-2 transient and in the sustained phase of the fura-2 response. A relatively small reduction in the sustained phase may have a critical effect on the magnitude of agonist-induced responses. These procaterol-induced changes in intracellular Ca^{2+} concentration may be due to an inhibition in the release of Ca^{2+} from the sarcoplasmic reticulum, an acceleration of Ca^{2+} re-uptake into the sarcoplasmic reticulum, an extrusion of Ca^{2+} to the extracellular space or inhibition of Ca^{2+} influx. Neither procaterol nor isoprenaline had much effect on $InsP_3$ synthesis and thus, a reduction in Ca^{2+} release from the sarcoplasmic reticulum is not responsible for the observed inhibitory effects of procaterol.

The amount of cyclic AMP was increased by

procaterol in the dog trachea and this synthesis ceased with the pre-application of propranolol. These results confirmed findings obtained in guinea-pig trachea (Takayanagi *et al.*, 1977) and the beagle dog (Irie *et al.*, 1979). In the smooth muscle cells of pig aorta, cyclic AMP accelerated the Ca pump as estimated from the accumulation of Ca^{2+} into the sarcolemma-rich fraction, and also accelerated Ca^{2+} re-uptake into the sarcoplasmic reticulum (Suematsu *et al.*, 1984). Cyclic AMP phosphorylates myosin light chain kinase and inhibits the phosphorylation of the myosin light chain activated by the Ca-calmodulin complex (Conti & Adelstein, 1980; Kerrick & Hoar, 1981). Thus, a reduction in free Ca^{2+} and in the amplitude of contraction would result from an increase of cyclic AMP concentrations, following application of procaterol. It is clear from the present study that if the intracellular trigger is of sufficient magnitude (as in the case of high ACh concentrations), the ability of procaterol to modify this signal is limited and relatively little reduction in mechanical response occurs.

The ID_{50} value of procaterol for the histamine-induced contraction was much smaller than that for the histamine-induced Ca transient. This discrepancy could be explained by the threshold Ca^{2+} concentration required to generate a contraction, i.e. the basal level of free Ca^{2+} concentration was about 120 nM and the minimum concentration of Ca^{2+} required to provoke a contraction was about 150–200 nM. In this way, a slight reduction in free Ca^{2+} may induce a more marked reduction in the mechanical response.

In dog trachea, isoprenaline and procaterol hyperpolarized the membrane, an effect associated with a decrease in membrane resistance. The importance of hyperpolarization in relaxant processes in the trachea (and other smooth muscles) is not clear. For example, Allen *et al.* (1985) showed that hyperpolarization was relatively unimportant in isoprenaline-induced relaxation in guinea-pig trachea. It seems reasonable that an increase in membrane potential will be important when the action of the spasmogen is associated with membrane depolarization and the opening of voltage-dependent ion channels. Furthermore, it may serve to close any voltage-dependent Ca^{2+} channels which are open under any given conditions. In the case of agonists like ACh, stored Ca plays an important role in the ensuing contraction (Ito & Itoh, 1984) and hyperpolarization is probably of limited importance in this situation.

Isoprenaline and procaterol had similar effects on the smooth muscles of dog trachea as estimated from increased cyclic AMP concentrations and the ID_{50} values for the contraction evoked by these agonists were also similar. However, isoprenaline is also a β_1-adrenoceptor stimulant on cardiac muscles (Himori & Taira, 1977).

In conclusion, procaterol relaxes pre-contracted tracheal tissue, with a reduction in the free Ca^{2+} in the cytosol. This inhibitory action may be closely linked with an increase in the amount of cyclic AMP but is apparently not associated with the hydrolysis of phospholipids. Procaterol also hyperpolarizes the membrane but this action probably plays only a minor role in its inhibitory effects.

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