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Effect of KC399, a newly synthesized K^+ channel opener, on acetylcholine-induced electrical and mechanical activities in rabbit tracheal smooth muscle

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1 Effects of KC399, an opener of ATP-sensitive K^+ channels were investigated on membrane potential, isometric force and intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization induced by acetylcholine (ACh) in smooth muscle from the rabbit trachea.

2 In these smooth muscle cells, ACh (0.1 and 1 μM) depolarized the membrane in a concentration-dependent manner. KC399 (1–100 nM) hyperpolarized the membrane whether in the presence or absence of ACh. When the concentration of ACh was increased, the absolute values of the membrane potential induced by the maximum concentration of KC399 were less negative.

3 ACh (0.1 to 10 μM) concentration-dependently produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. KC399 (above 3 nM) lowered the resting $[Ca^{2+}]_i$ and attenuated the ACh-induced phasic and tonic increases in $[Ca^{2+}]_i$ and force, in a concentration-dependent manner. The magnitude of the inhibition was greater for the ACh-induced tonic responses than for the phasic ones. Nicardipine (0.3 μM), a blocker of the L-type Ca^{2+} channel, attenuated the ACh-induced tonic, but not phasic, increases in $[Ca^{2+}]_i$ and force. KC399 further attenuated the ACh-induced tonic responses in the presence of nicardipine.

4 In β-escin-skinned strips, Ca^{2+} (0.3–10 μM) produced a contraction in a concentration-dependent manner. KC399 (0.1 μM) had no effect on the Ca^{2+}-force relationship in the presence or absence of ATP with GTP. However, at a very high concentration (1 μM), this agent slightly shifted the relationship to the right and attenuated the maximum Ca^{2+}-induced contraction.

5 We conclude that, in rabbit tracheal smooth muscle, the membrane hyperpolarization induced by KC399 attenuates the ACh-induced tonic increase in $[Ca^{2+}]_i$ through an inhibition of nicardipine-sensitive and -insensitive Ca^{2+}-influxes, thus causing an inhibition of the ACh-induced tonic contraction. The ACh-induced phasic increase in $[Ca^{2+}]_i$ and force are also inhibited, but less effectively than the tonic ones, suggesting that the action of such K^+ channel openers on agonist-induced responses may be slightly different in tracheal from vascular smooth muscle.

Keywords: Tracheal smooth muscle; K^+ channel opener, acetylcholine; myofilament Ca^{2+} sensitivity; membrane hyperpolarization; Ca^{2+} release; Ca^{2+} influx

Introduction

In recent years, many K^+ channel openers that activate the glyburide- and ATP-sensitive K^+ channel have been synthesized for possible use as anti-spasmogenic agents in vascular and tracheal smooth muscle (for reviews, see Cook, 1988; Quast, 1992; Edwards & Weston, 1993). These K^+ channel openers hyperpolarize the membrane of smooth muscle cells and their action was inhibited by a sulphonylurea, glyburide. Following work on vascular smooth muscle, it was originally thought that such K^+ channel openers might inhibit the activation of the L-type Ca^{2+} channel as a result of the membrane hyperpolarization they evoke and that this was responsible for their inhibition of agonist-induced contraction (Standen *et al.*, 1989). Later, it was found, in vascular smooth muscle from various vessels, that the membrane hyperpolarization induced by such K^+ channel openers also inhibits agonist-induced production of inositol 1,4,5-trisphosphate ($InsP_3$) (Ito *et al.*, 1991; Itoh *et al.*, 1992) and/or lowers the sensitivity of the contractile machinery to Ca^{2+} (Okada *et al.*, 1993). Thus, it seems likely that these three mechanisms may all contribute to the K^+channel opener-induced muscle relaxation in vascular smooth muscle.

In smooth muscle cells, Ca^{2+} influx can be increased by agonists through activation of both the L-type Ca^{2+} channel and the receptor-operated non-selective cation channel (Benham & Tsien, 1987; Inoue & Isenberg, 1990; Pacaud & Bolton, 1990). These channels are voltage-sensitive, but the latter channel is insensitive to organic Ca^{2+} channel blockers in longitudinal smooth muscle of the guinea-pig ileum (Inoue & Isenberg, 1990). We recently found that, in the rabbit mesenteric artery, the membrane hyperpolarization induced by Y-26763 (a K^+ channel opener) abolished the tonic increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA), while nicardipine only partly inhibits the NA-response (Itoh *et al.*, 1994a). These results suggest that an agent that hyperpolarizes the smooth muscle membrane would be likely to inhibit the NA-induced contraction more than an organic Ca^{2+} channel blocker in rabbit mesenteric artery. In airway smooth muscle, many ATP-sensitive K^+ channel openers also inhibit agonist-induced contraction (Black *et al.*, 1990; Taylor *et al.*, 1992; Small *et al.*, 1992). However, the mechanisms underlying this action of K^+ channel openers in tracheal smooth muscle have yet to be fully clarified.

KC399 (*N*-(2-cyanoethyl)-2,2-bisfluoromethyl-6-nitro-2*H*-1 benzopyran-4-carbothioamide) has recently been synthesized in our laboratory. Its synthesis was based on a computer-assisted analysis of the pharmacophore model of K^+ channel openers (Koga *et al.*, 1993) and it was found to be a quite potent, glyburide-sensitive muscle relaxant of the smooth muscle in dog and guinea-pig airways (Imagawa *et al.*, 1993; Kamei *et al.*, 1994).

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To help clarify the mechanisms underlying the actions of K^+ channel openers in tracheal smooth muscle, the effects were studied of KC399 on the increases in membrane potential, $[Ca^{2+}]_i$ and force induced by various concentrations of ACh in rabbit tracheal smooth muscle. The effects on the increase in $[Ca^{2+}]_i$ and force were compared with those of nicardipine. Further, the effect of KC399 on ACh-induced Ca^{2+} release was studied by assessing its effect on the ACh-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. In addition, the direct action of KC399 on myofilament Ca^{2+}-sensitivity in the presence and absence of ACh was studied in β-escin-skinned smooth muscle.

Methods

Male albino rabbits, weighing 1.9–2.5 kg, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1},i.v.) and then exsanguinated. A cervical portion of the tracheal tissues was dissected away from both ends of the cartilage in Krebs solution, then carefully cleaned by removal of connective tissues under a binocular microscope at room temperature.

Electrophysiological experiments

A glass microelectrode filled with 3M KCl was made from borosilicate glass tube (o.d., 1.0 mm with a core inside, Glass Filament, 6010, A-M System, Inc., Everett, WA, U.S.A.). The resistance of the electrodes was 30–60 MΩ. The microelectrode was held in an electrode-holder (MEH-1S10, World Precision Instruments, Inc., New Haven, CT, U.S.A.) connected to a high input impedance preamplifier (MEZ-7200, Nihon Kohden, Tokyo, Japan). Signals from the preamplifier were recorded on a chart recorder (model VP-6538A, Panasonic, Yokohama, Japan). The tissue was cut into segments, 2 to 3 mm wide and 5 mm long, in the transverse direction and pinned onto a rubber plate in a chamber (2 ml bath volume) and perfused at room temperature with Krebs solution at a flow rate of 3 ml min^{-1}. The electrode was inserted into smooth muscle cells from the inner surface of the tissue.

Ca^{2+} and force measurement

To enable recording of isometric force, fine circularly cut smooth muscle preparations (0.3–0.5 mm in length, 0.05–0.07 mm in width, 0.05–0.07 mm thick) were prepared. The smooth muscle preparation was transferred to a chamber of 0.3 ml volume and mounted horizontally on an inverted microscope (Diaphoto TMD with special optics for epifluorescence, Nikon). The preparation was moved to the centre of the field and a mask (0.04 mm square) placed in an intermediate image plane of the microscope.

To load Fura 2 into the smooth muscle cells, 2 μM-acetoxy methyl ester of Fura 2 (Fura 2AM) was applied for 2 h in Krebs solution at room temperature (25–26°C). After this period, the solution containing Fura 2AM was washed with Krebs solution for 0.5 h to ensure sufficient esterification of Fura 2AM in the cells. The Fura 2 fluorescence emission at 510 nm (passed through an interference filter centred at 510 nm with a full width at half-transmission of 40 nm) was passed through the objective lens (20 × fluor, Nikon) and collected with a photomultiplier tube (R928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphoto-TMD microscope. Two alternative excitation wavelengths, 340 and 380 nm (each slit 5 nm), were applied by a spectrofluorimeter (CA 200DP, Japan Spectroscopic Co. Ltd, Tokyo, Japan) and the data analyzed with software developed in our laboratory.

The ratio of Fura 2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of background fluorescence. Background fluorescence (including the autofluorescence of the preparation) excited by 340 and 380 nm u.v. light was measured before the application of Fura 2AM. The background fluorescence intensity was 10–15% of the Fura 2 signal in smooth muscle preparations at either excitation wavelength. Cytosolic Ca^{2+} concentrations were calculated with the formula described by Grynkiewicz *et al.* (1985) and *in vitro* calibration, as described previously (Itoh *et al.*, 1992). The K_d value for Fura 2 was estimated to be 200 nM (Becker *et al.*, 1989).

Experiments on chemically skinned smooth muscle

To study the direct effect of KC399 on the contractile proteins in rabbit tracheal smooth muscles, β-escin-treated skinned smooth muscle preparations were used. β-Escin-skinned smooth muscles retain the receptor-GTP binding protein-phospholipase C coupling mechanism, i.e., in the skinned smooth muscle, agonists can release Ca^{2+} from the intracellular storage sites and enhance the myofilament Ca^{2+}-sensitivity in the presence of GTP (Itoh *et al.*, 1992; 1994a,b).

The methods used to skin the muscle strips and the compositions of the solutions used have been described elsewhere (Itoh *et al.*, 1986; 1992; 1994b). Briefly, skinned muscle preparations were obtained by treatment with 25 μM β-escin in Ca^{2+}-free solution containing EGTA and ATP ('relaxing solution') for 25 min, after the constant amplitude of contraction induced by 128 mM K^+ had been recorded. When the Ca^{2+}-force relationship was to be determined, the concentration of EGTA in the solution was 4 mM and 1 μM ionomycin was applied to avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle. To prevent deterioration of the Ca^{2+}-induced contraction, 0.1 μM calmodulin was applied throughout the experiments, as described previously (Itoh *et al.*, 1986). Various concentrations of Ca^{2+} were applied cumulatively from low to high concentration. The amplitude of contraction induced by each of the various concentrations of Ca^{2+} was normalized with respect to that induced by 10 μM Ca^{2+} in the same strip.

To observe the effect of ACh with GTP on the Ca^{2+}-force relationships, a series of experiments was done in each muscle preparation. After skinning the strip, Ca^{2+}-force relationship was first obtained in the absence of ACh with GTP (control Ca^{2+}-force relationship). Following washout of the preparation with relaxing solution (to relax the muscle strip), the 2nd Ca^{2+}-force relationship was then obtained in the presence of 10 μM ACh with 30 μM GTP in the same preparation.

To observe the effect of KC399 (0.1 and 1 μM), a series of experiments was also done in each muscle preparation, in which Ca^{2+}-force relationship was first obtained (control Ca^{2+}-force relationship). After washing of the preparation with relaxing solution containing 0.1 μM KC399, the 2nd Ca^{2+}-force relationship was then obtained in the presence of 0.1 μM KC399, followed by a washout with relaxing solution containing 1 μM KC399. The third Ca^{2+}-force relationship was subsequently obtained in the presence of 1 μM KC399. A similar protocol was used to observe the effect of KC399 on the Ca^{2+}-force relationship in the presence of ACh with GTP except that the control Ca^{2+}-force relationship was obtained in the presence of ACh with GTP. The reproducibility of the Ca^{2+}-force relationships under these experimental conditions was reported previously (Itoh *et al.*, 1986; 1992).

The slope of the concentration-response for the effect of Ca^{2+} on force is shown by the Hill coefficient (n) and midpoint position ($pK = (-\log K)$, where K is the dissociation constant). These parameters were obtained by fitting the data points for each curve to eqn. (1) by a non-linear least-squares method.

$$F/F_0 = (C/K)^n/[1 + (C/K)^n] \quad (1)$$

Where C represents the concentration of Ca^{2+}, F is the amplitude of contraction at any given concentration of Ca^{2+} and F_0 is the maximum response evoked by 10 μM Ca^{2+} expressed as a relative force of 1.0.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The concentration of K^+ was modified by replacing NaCl with KCl, isosmotically. To make Ca^{2+}-free solution, 2.6 mM $CaCl_2$ was replaced with $MgCl_2$ and 2 mM EGTA was then added. The solutions were bubbled with 95% O_2–5% CO_2 and their pH maintained at 7.3–7.4.

For experiments on skinned muscle, the composition of the relaxing solution was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N*-*N*'-*bis*-(2-ethanesulphonic acid; PIPES), 5.1 mM $Mg(MS)_2$, 5.2 mM ATP, 5 mM phosphocreatine and 4 mM ethylenglycol-*bis*-(β-aminoethyl) *N*,*N*,*N*',*N*'-tetraacetic acid (EGTA). Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(MS)_2$ to 4 mM EGTA, based on the calculation given previously (Itoh *et al.*, 1986). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 M by changing the amount of KMS added.

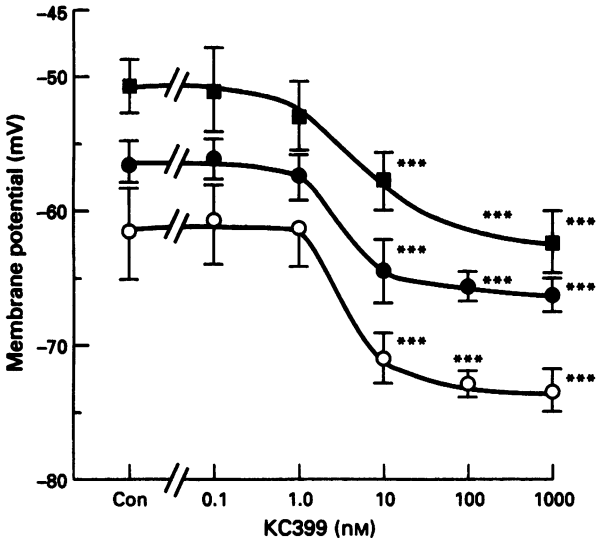

Figure 1 Effect of KC399 on membrane potential in rabbit tracheal smooth muscle cells in the absence (○) and presence of acetylcholine (ACh, ●, 0.1 μM; ■, 1 μM). Results shown are each the mean of data from 5–6 cells with s.d. ***$P<0.001$ for comparison with values before application of KC399 (control).

Drugs

Drugs used were Fura 2, Fura 2AM, EGTA and PIPES (Dojin, Japan), GTP (Boehringer Mannheim GMBH, Germany), β-escin and glyburide (Sigma) and acetylcholine (ACh, Ovisot, Daiichi Seiyaku Co. Ltd., Japan). KC399 (*N*-(2-cyanoethyl)-2,2-bisfluoromethyl-6-nitro-2*H*-1-benzopyran-4-carbothioamide) and levcromakalim were synthesized in our laboratory. KC399 and levcromakalim were dissolved to 10–20 mM in DMSO (dimethylsulphoxide).

Statistics

The values recorded were expressed as means ± s.d. and statistical significance determined by Student's paired or unpaired

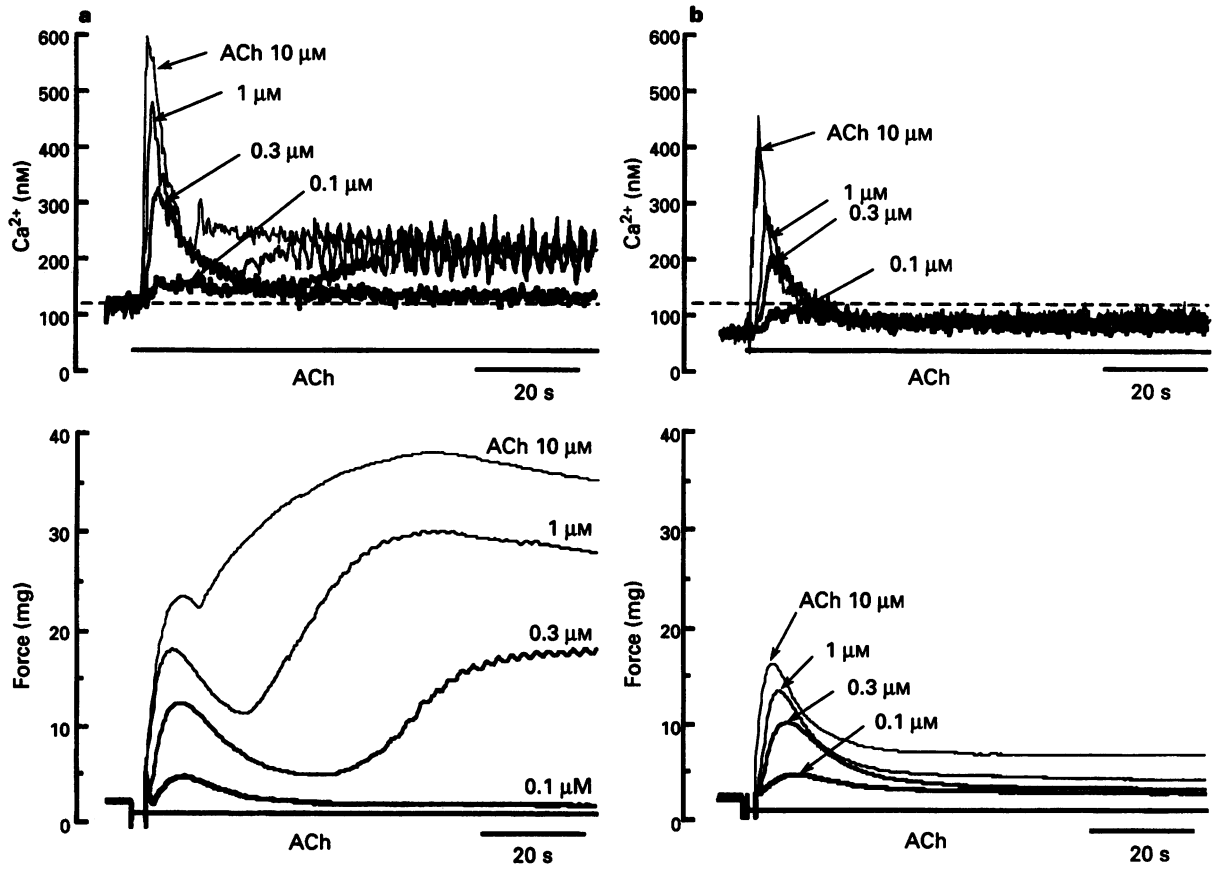

Figure 2 Effect of KC399 (100 nM) on increase in $[Ca^{2+}]_i$ and force induced by various concentrations of acetylcholine (ACh) in a smooth muscle strip of the rabbit trachea. Upper panels, $[Ca^{2+}]_i$; lower panels, force. Various concentrations of ACh were applied for 1.5 min at 10 min intervals from low to high concentration (a), and then the strip was pretreated with 100 nM KC399 15 min before various concentrations of ACh were applied in the presence of 100 nM KC399 using the same protocol (b). Broken lines indicate the resting level of $[Ca^{2+}]_i$. The experiment was done on a single smooth muscle strip.

t test and one factor ANOVA with repeated measures. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effects of KC399 on membrane potential

Smooth muscle cells of the rabbit trachea were electrically quiescent and the resting membrane potential was -61.5 ± 3.7 mV ($n=6$). ACh (0.1 and 1 μM) depolarized the membrane in a concentration-dependent manner. The membrane potential was -56.5 ± 1.8 mV ($n=6$, $P<0.05$) in the presence of 0.1 μM ACh and -50.7 ± 2.0 mV ($n=6$, $P<0.05$) in 1 μM ACh. Under these conditions, oscillatory changes in the membrane potential were not observed.

In the absence of ACh, KC399 (over 1 nM) concentration-dependently hyperpolarized the membrane, the maximum hyperpolarization (to -73.2 ± 1.6 mV, $n=5$) being obtained at 0.1 μM (Figure 1). KC399 also hyperpolarized the membrane in the presence of 0.1 μM and 1 μM ACh, again in a concentration-dependent manner. With an increase in the concentration of ACh from 0.1 μM to 1 μM, the absolute values of the membrane potential induced by the maximum concentration of KC399 were less. However, Δ values (difference in membrane potential before and after application of the maximum concentration of KC399) were not significantly different. They were 11.4 ± 3.0 mV ($n=6$) in the absence of ACh, 9.8 ± 2.7 mV ($n=6$) in the presence of 0.1 μM ACh and 12.6 ± 1.7 mV ($n=6$) in 1 μM ACh.

Effects of KC399 on ACh-induced increases in $[Ca^{2+}]_i$ and force

In thin smooth muscle strips from the rabbit trachea, the resting $[Ca^{2+}]_i$ was 126 ± 18 nM ($n=8$). ACh was applied for 1.5 min at 10 min intervals to obtain reproducible responses. KC399 (100 nM) was applied for 15 min before and during the application of ACh. Under these conditions, ACh (0.1–10 μM) concentration-dependently produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force (Figures 2 and 3). In 2 out of 5 preparations, oscillatory responses were imposed on the ACh-induced tonic increase in $[Ca^{2+}]_i$ and force (Figure 2). KC399 (100 nM) reduced the resting $[Ca^{2+}]_i$ to 106 ± 24 nM (range from 76 nM to 125 nM, $n=4$, $P<0.05$) and attenuated both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by any given concentration of ACh (0.1–10 μM).

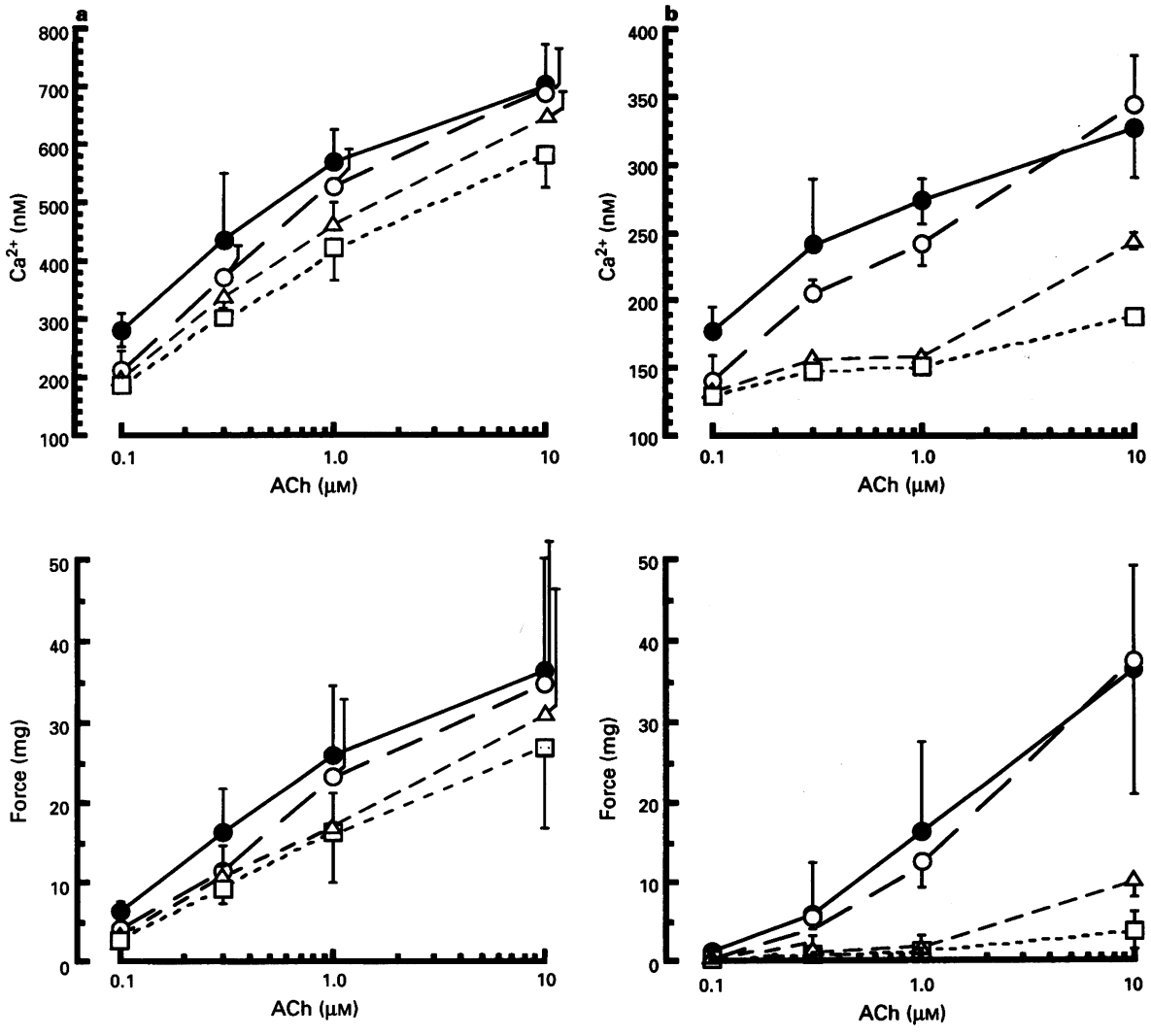

Figure 3 Concentration-dependent effects of KC399 on the phasic (a) and tonic (b) increases in $[Ca^{2+}]_i$ and force induced by various concentrations of acetylcholine (ACh) in rabbit tracheal smooth muscle. Upper panels, $[Ca^{2+}]_i$; lower panels, force. (●) Control; in the presence of KC399 (○, 3 nM; Δ, 10 nM; □, 100 nM). ACh was applied for 1.5 min and the tonic responses were obtained 1.5 min after the application. Results shown are each the mean of data from 4 preparations with s.d.

The concentration-dependent effect of KC399 on the increases in $[Ca^{2+}]_i$ and force induced by various concentrations of ACh (0.1–10 μM) is illustrated in Figure 3. KC399 (3 nM) consistently attenuated both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by 0.1 μM ACh in all preparations ($n=4$, $P<0.05$ by one way repeated ANOVA) but had no significant effect on those induced by 0.3–10 μM ACh. Before application of 3 nM KC399, the phasic increases in $[Ca^{2+}]_i$ induced by 0.1 μM ACh were 281±33 nM and 212±36 nM in the absence and presence of 3 nM KC399, respectively. The tonic increases in $[Ca^{2+}]_i$ induced by 0.1 μM ACh were 178±18 nM and 140±21 nM in the absence and presence of 3 nM KC399, respectively (Figure 3).

KC399 (10 nM) significantly inhibited the tonic increase in $[Ca^{2+}]_i$ and force induced by any concentration of ACh (0.1–10 μM) (Figure 3, $n=4$, $P<0.05$ by one way repeated ANOVA). At 100 nM, KC399 inhibited both the phasic and tonic increase in $[Ca^{2+}]_i$ and force induced by ACh (0.1–10 μM). Glyburide (10 μM, a blocker of the ATP-sensitive K^+ channel) did not modify the resting $[Ca^{2+}]_i$ and abolished all these inhibitory actions of KC399 (not shown).

High K^+ (128 mM) produced a large phasic, followed by a small tonic increase in $[Ca^{2+}]_i$ and force (Figure 4). The tonic increase in $[Ca^{2+}]_i$ induced by 128 mM K^+ (355±67 nM, $n=4$) was almost the same as that induced by 10 μM ACh (328±21 nM, $n=4$), but the tonic force induced by high K^+ (7.1±4.6 mg, $n=4$) was only one sixth of that induced by 10 μM ACh (36.6±15.5 mg, $n=4$). These results indicate that the efficacy with which $[Ca^{2+}]_i$ produces contraction in the presence of ACh is higher than that in high K^+. KC399 (100 nM) had no effect on the increase in $[Ca^{2+}]_i$ and force induced by 128 mM K^+, but greatly attenuated the ACh-induced responses (Figure 4).

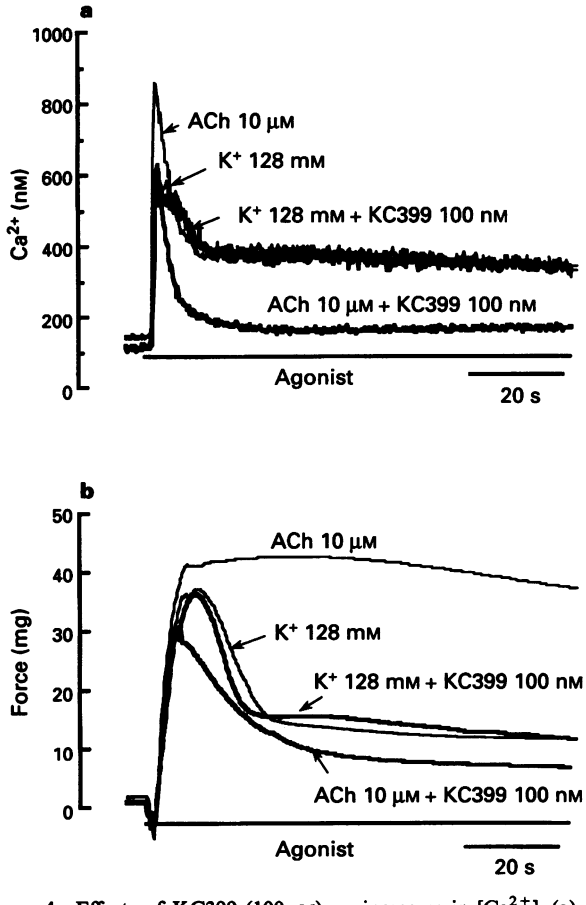

Figure 4 Effects of KC399 (100 nM) on increases in $[Ca^{2+}]_i$ (a) and force (b) induced by 128 mM K^+ and by 10 μM acetylcholine (ACh) in a smooth muscle strip of the rabbit trachea. Stimulants were applied where indicated by the bars. When used, KC399 was given as pretreatment for 15 min and was present during the application of ACh and high K^+. Thinner lines, in the absence of KC399; thicker lines, in the presence of KC399. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

Effects of nicardipine on ACh-induced increases in $[Ca^{2+}]_i$ and force

Figure 5a shows the effect of 0.3 μM nicardipine on the increases in $[Ca^{2+}]_i$ and force induced by 128 mM K^+ in smooth muscle from the rabbit trachea. Nicardipine potently attenuated both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by high K^+, the tonic contraction being completely abolished. Nicardipine (0.3 μM) slightly attenuated the phasic increases in $[Ca^{2+}]_i$ and force induced by 1 μM ACh, but strongly inhibited the ACh-induced tonic responses (Figure 5b). In the absence of nicardipine, the phasic and tonic increases in $[Ca^{2+}]_i$ induced by 1 μM ACh were 648±75 nM and 325±15 nM, respectively, and these were 585±86 and 251±34 nM in the presence of 0.3 μM nicardipine, respectively ($n=4$). Nicardipine (1 μM) did not further attenuate the ACh-induced tonic increase in $[Ca^{2+}]_i$.

In the presence of 0.3 μM nicardipine, KC399 (0.1 μM) lowered the resting $[Ca^{2+}]_i$ (from 103±21 nM to 78±15 nM, $n=4$) and further attenuated both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by 1 μM ACh (Figure 5b). In the presence of 0.1 μM KC399 with 0.3 μM nicardipine, the ACh-induced phasic and tonic increase in $[Ca^{2+}]_i$ were 450±120 nM and 187±25 nM, respectively ($n=4$).

Effects of KC399 on ACh-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution

To study the effect of KC399 on agonist-induced release of Ca^{2+} from its storage sites, we observed its effect on the ACh-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution containing 2 mM EGTA. After a 2 min application of Ca^{2+}-free solution, 1 μM ACh was applied for 1.5 min, followed by a 1 min washout; then the preparation was left for 10 min in Krebs solution. KC399 was applied for 8 min in Krebs solution and 3.5 min in Ca^{2+}-free solution so that it was present for 10 min before and for 1.5 min during the application of ACh.

Following the application of Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ decreased from 100±12 nM to 71±5 nM ($n=3$) and the subsequent application of ACh transiently increased $[Ca^{2+}]_i$ and force. In Ca^{2+}-free solution, KC399 (10 and 100 nM) slightly attenuated the ACh-induced transient increases in $[Ca^{2+}]_i$ (Figure 6a) and force (Figure 6b) in a concentration-dependent manner. The Δ changes in $[Ca^{2+}]_i$ (peak minus baseline) induced by 1 μM ACh in Ca^{2+}-free solution were 200±33 nM and 161±31 nM, respectively in the presence and absence of 0.1 μM KC399 ($n=3$, $P<0.05$ by paired t test). Glyburide (10 μM) did not modify the resting $[Ca^{2+}]_i$ and greatly attenuated the inhibitory action of KC399 on these ACh-induced responses (not shown).

To study the effect of KC399 on myofilament Ca^{2+} sensitivity in the presence of ACh, the relationship between $[Ca^{2+}]_i$ and force was plotted as a function of time after the application of 1 μM ACh in the presence and absence of KC399 (Figure 6c). Since ACh was injected over 1 s in the present experiments, the initial phase of the force response (within 1–2 s) was impossible to measure and so the measurement of force started 2 s after the injection. The plotted points indicate that the relationship between $[Ca^{2+}]_i$ and force was similar whether in the presence of 10 nM (○) or 100 nM KC399 (□) or in the absence (△) of KC399.

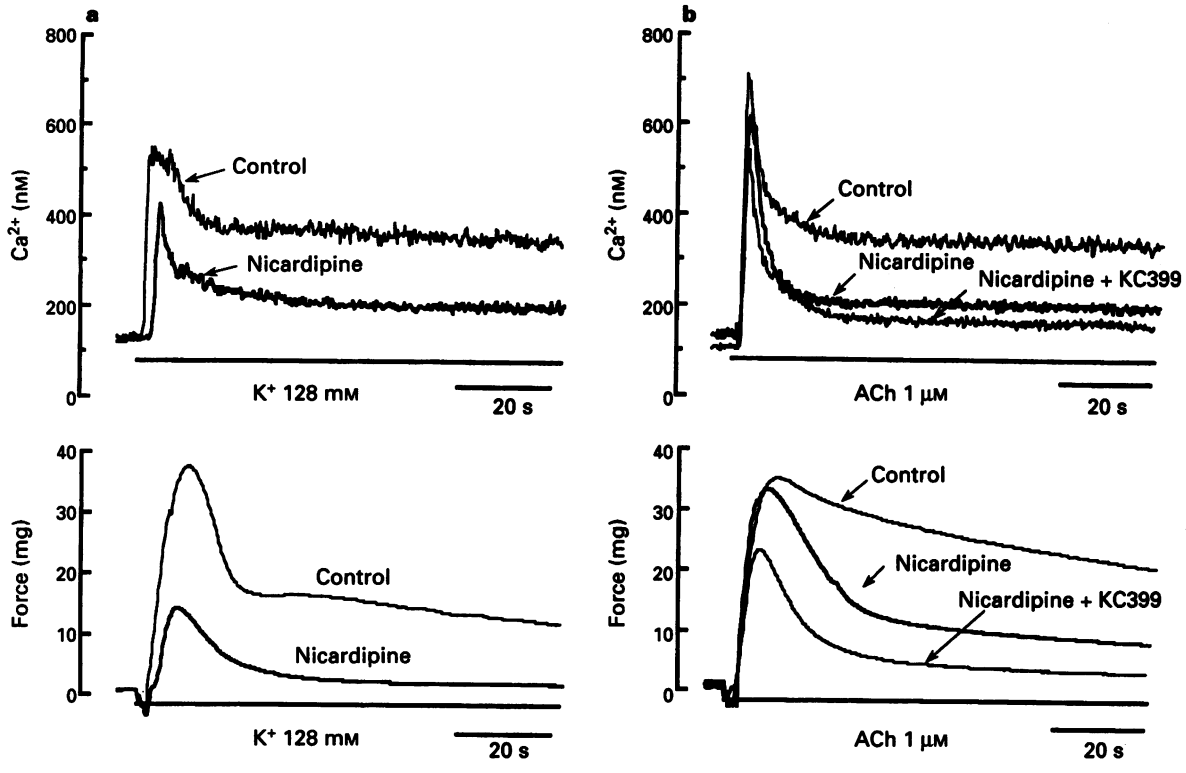

Figure 5 Effect of nicardipine (0.3 μM) with and without KC399 (100 nM) on increases in $[Ca^{2+}]_i$ (upper panels) and force (lower panels) induced by 128 mM K^+ (a) and 1 μM acetylcholine (ACh, b) in a smooth muscle strip of the rabbit trachea. Stimulants were applied where indicated by the bars. Nicardipine, with or without KC399, was given as pretreatment for 15 min and was present during the application of ACh and high K^+. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

Effects of KC399 on Ca^{2+}-induced contraction in β-escin-skinned smooth muscle

To study the direct effect of KC399 on the contractile apparatus in tracheal smooth muscle, the effect of KC399 was observed on the contractions induced by various concentrations of Ca^{2+} in the presence and absence of ACh with GTP in β-escin-skinned smooth muscle strips. In β-escin-skinned muscles, application of Ca^{2+} (0.3–10 μM) produced contraction in a concentration-dependent manner. ACh (10 μM) with GTP (30 μM) always shifted the Ca^{2+}-force relationship to the left without a change in the maximum Ca^{2+}-induced contraction ($n=4$). The half maximum contraction was obtained at 1.4 ± 0.3 μM Ca^{2+} in control (in the absence of ACh with GTP) and 1.1 ± 0.2 μM in the presence of ACh with GTP ($n=4$). These were significantly different. These results suggest that the sensitivity of the contractile apparatus to Ca^{2+} was enhanced by the application of ACh with GTP.

KC399 (0.1 μM) did not modify the Ca^{2+}-force relationship whether in the presence or absence of 10 μM ACh with 30 μM GTP. In the absence of ACh with GTP, the ED_{50} value for Ca^{2+} in the presence of KC399 was 1.5 ± 0.2 μM ($n=4$, $P>0.05$, compared with that in the absence of KC399 using a paired t test). In the presence of ACh + GTP together with KC399, the ED_{50} value for Ca^{2+} was 1.3 ± 0.2 μM ($n=4$, $P>0.05$). However, at a higher concentration (1 μM), KC399 shifted the Ca^{2+}-force relationship to the right and attenuated the maximum contraction induced by 10 μM Ca^{2+} in the presence and absence of ACh with GTP (Figure 7). The ED_{50} values for Ca^{2+} in the absence and presence of ACh with GTP were, respectively, 1.6 ± 0.1 μM ($n=4$, $P<0.05$, compared with that in the absence of KC399 using a paired t test) and 1.6 ± 0.2 μM ($n=4$, $P<0.05$). When the concentration of Ca^{2+} was increased to 30 μM in the presence of 1 μM KC399, the amplitude of the Ca^{2+}- induced contraction was similar to that induced by 10 μM Ca^{2+} in 1 μM KC399 (1.0 ± 0.1 times, $n=3$).

Discussion

Effect of KC399 on membrane activities in the presence of ACh

In our preparations, rabbit tracheal smooth muscle cells were electrically quiescent and the resting membrane potential was -61.5 ± 3.7 mV. These electrical properties are similar to those observed in canine tracheal smooth muscle cells (Kamei *et al.*, 1994). In canine tracheal smooth muscle, ACh (>30 nM) produces a membrane depolarization with a concomitant increase in membrane oscillation (Janssen & Daniel, 1991; Kamei *et al.*, 1994). In the present experiments, in rabbit tracheal smooth muscle cells, ACh (0.1 and 1 μM) produced a sustaining membrane depolarization without electrical oscillations. Thus, the ACh-induced electrical events may be slightly different in rabbit tracheal smooth muscle cells from those in the dog.

In the present experiments, KC399 concentration-dependently hyperpolarized the membrane in the presence or absence of ACh (0.1 or 1 μM). The Δ membrane potential change (difference in the membrane potential before and after application of KC399) induced by the maximum concentration of KC399 was about 10 mV whether in the presence or absence of ACh. However, the absolute membrane potential level attained with the maximum concentration of KC399 became less negative when ACh was added or when the concentration of ACh was increased, suggesting that the complex relationship between membrane potential and K^+ conductance may be present in the presence of a K^+ channel opener with ACh in

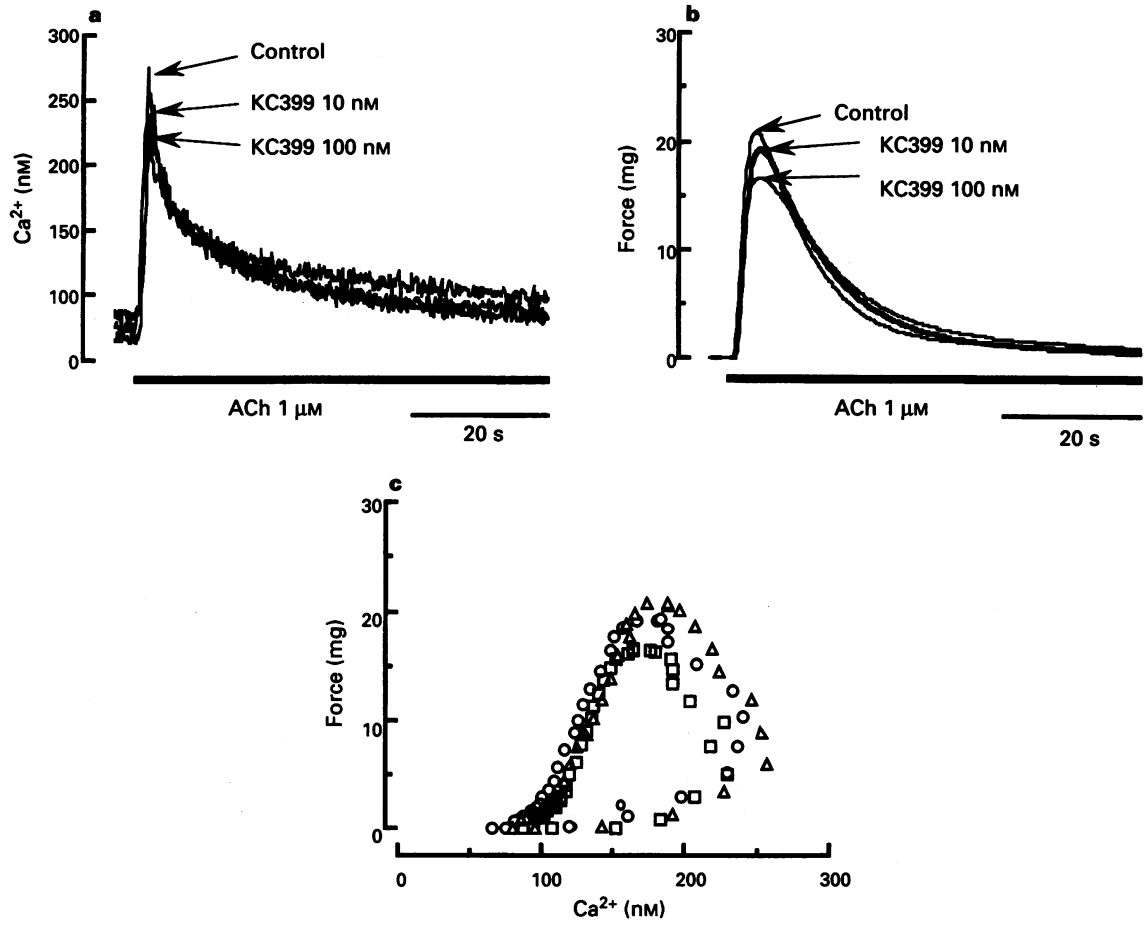

Figure 6 Effect of KC399 on increase in $[Ca^{2+}]_i$ (a) and force (b) induced by 1 μM acetylcholine in Ca^{2+}-free solution containing 2 mM EGTA in rabbit tracheal smooth muscle. The preparation was pretreated for 8 min with KC399 in Krebs solution, for 2 min in Ca^{2+}- free solution and KC399 was present during the application of ACh. Ca^{2+}-free solution was applied 2 min before application of ACh. (c) The relationship between $[Ca^{2+}]_i$ and force as a function of time in the presence of 1 μM ACh in the presence (○, 10 nM; □, 100 nM) and absence (Δ) of KC399 in Ca^{2+}-free solution. After application of ACh, $[Ca^{2+}]_i$ immediately increased with a slight increase in force, and $[Ca^{2+}]_i$ then decayed as force developed. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 2 strips.

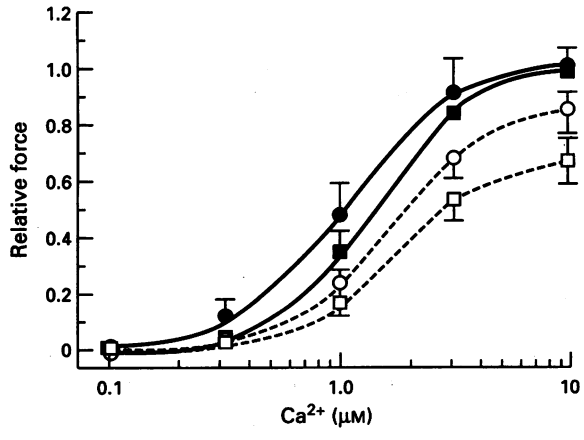

Figure 7 Effect of 1 μM KC399 on Ca^{2+}-force relationship in the presence (○,●) and absence (□,■) of 10 μM acetylcholine with 30 μM GTP in β-escin-skinned smooth muscle strips of rabbit tracheal smooth muscle. After strips had been skinned by application of 25 μM β-escin for 25 min, various concentrations of Ca^{2+} were applied in the absence (filled symbols) and presence (open symbols) of 1 μM KC399. KC399 was given as pretreatment for 5 min and was present during the application of Ca^{2+}. Results shown are each the mean of data from 4–8 preparations with s.d.

rabbit tracheal smooth muscle cells. In swine tracheal smooth muscle, ACh produces an inward cation current and causes a membrane depolarization (Saunders & Farley, 1991). Further, it was recently suggested that, in the smooth muscle of the guinea-pig urinary bladder, muscarinic receptor stimulation inhibits the ATP-sensitive K^+ channel (Bonev & Nelson, 1993). The contribution of these to KC399-induced membrane hyperpolarization in the presence of ACh remains to be clarified in tracheal smooth muscle cells.

Effects of KC399 on ACh-induced Ca^{2+}-influxes

In rabbit tracheal smooth muscle, ACh produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. In Ca^{2+}-free solution containing 2 mM EGTA, the phasic increase in $[Ca^{2+}]_i$ and force induced by ACh remained, but the tonic responses disappeared, indicating that the phasic response is mainly due to ACh-induced Ca^{2+} release from the intracellular storage sites, whereas the tonic response is caused by Ca^{2+} influxes activated by ACh.

In the present experiments, KC399 concentration-dependently attenuated both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by ACh, the effect being more potent on the tonic responses than on the phasic ones. The concentration-dependency of the inhibition of ACh-induced tonic responses

by KC399 was similar to that seen when the same agent induced membrane hyperpolarization in the presence of ACh. K^+ channel openers hyperpolarize the membrane through an activation of ATP-sensitive K^+ channel and the action is abolished in the presence of concentrations over 50 mM K^+ (Edward & Weston, 1993). In canine tracheal smooth muscle cells, we previously found that KC399 did not hyperpolarize the membrane in the presence of high K^+ (> 20 mM) (Kamei *et al.*, 1994). Glyburide is well known to be an inhibitor of the ATP-sensitive K^+ channel which is activated by various K^+ channel openers (Edward & Weston, 1993). In the present experiments, KC399 (100 nM) failed to modify either the phasic or tonic increases in $[Ca^{2+}]_i$ and force induced by 128 mM K^+ (Figure 4). Significantly, the inhibitory action of KC399 on the ACh-induced tonic responses was blocked by 10 μM glyburide. Furthermore, KC399 (3–100 nM) had no effect on the Ca^{2+}-force relationship whether in the presence or absence of ACh with GTP in β-escin-skinned smooth muscle. These results suggest that KC399 hyperpolarizes the membrane and then inhibits ACh-activated Ca^{2+}-influxes, thus causing the observed inhibition of the ACh-induced tonic contraction.

Nicardipine (a blocker of the L-type Ca^{2+} channel, 0.3–1 μM) has been shown to abolish completely the tonic increases in $[Ca^{2+}]_i$ and force induced by 128 mM K^+ in rabbit arterial smooth muscle (Itoh *et al.*, 1994a). In the present experiments, nicardipine (0.3 μM) greatly attenuated the tonic increase in $[Ca^{2+}]_i$ induced by 128 mM K^+ and abolished the tonic contraction in rabbit tracheal smooth muscle (Figure 5a). Similar results were observed on the high K^+-induced tonic contraction in rabbit coronary artery using nisoldipine, another L-type of Ca^{2+} channel blocker (Itoh *et al.*, 1984). These results suggest that nicardipine, at this concentration, can effectively inhibit an L-type Ca^{2+} channel activity in rabbit tracheal smooth muscle.

Nicardipine (0.3 μM) attenuated the tonic increase in both $[Ca^{2+}]_i$ and force induced by 1 μM ACh, although this agent had a very minor effect on the ACh-induced phasic responses (about 10% inhibition) (Figure 5b). The magnitude of the inhibition induced by 0.1 μM KC399 on the ACh-induced tonic response was bigger than that induced by nicardipine (Figure 2b for KC399 and Figure 5 for nicardipine). Moreover, in the presence of nicardipine, 0.1 μM KC399 further attenuated the tonic increase in $[Ca^{2+}]_i$ and force induced by 1 μM ACh (Figure 5b). In smooth muscle cells, Ca^{2+} influx can be increased by agonists through activation of both the L-type Ca^{2+} channel and the receptor-operated non-selective cation channel (Benham & Tsien, 1987; Inoue & Isenberg, 1990; Pacaud & Bolton, 1990). These channels are voltage-sensitive, but the latter channel is insensitive to organic Ca^{2+} channel antagonists in longitudinal smooth muscle of the guinea-pig ileum (Inoue & Isenberg, 1990). These results suggest that the membrane hyperpolarization induced by KC399 may inhibit the ACh-induced tonic increase in $[Ca^{2+}]_i$ through an inhibition of L-type Ca^{2+} channel-sensitive and -insensitive Ca^{2+} influxes in rabbit tracheal smooth muscle.

Effect of KC399 on ACh-induced Ca^{2+} release from intracellular storage sites

It is believed that $InsP_3$ plays an essential role in ACh-induced Ca^{2+} release in tracheal smooth muscle (Chilvers *et al.*, 1990; Katsuyama *et al.*, 1990). In smooth muscle of the rabbit mesenteric artery, the membrane hyperpolarization induced by pinacidil or levcromakalim (another type of K^+ channel opener) inhibits noradrenaline (NA)-induced $InsP_3$ production and attenuates the NA-induced release of Ca^{2+} from its storage sites (Ito *et al.*, 1991; Itoh *et al.*, 1992). In the present experiments, on rabbit tracheal smooth muscle, KC399 slightly attenuated the ACh-induced phasic increase in $[Ca^{2+}]_i$ and force whether in the presence or absence of extracellular Ca^{2+}. A similarly weak effect on ACh-induced Ca^{2+} release is exerted by 0.1 μM levcromakalim, suggesting that this is not a specific characteristic of KC399. These results suggest that, in contrast to the situation in vascular smooth muscle, the membrane hyperpolarization induced by K^+ channel openers may not play a significant role in ACh-induced Ca^{2+} release in tracheal smooth muscle.

In summary, KC399 hyperpolarizes the membrane and inhibits ACh-activated voltage-sensitive Ca^{2+} influxes and thus attenuates the ACh-induced tonic contraction. KC399 also inhibits ACh-induced Ca^{2+} release, but in this action it is less potent than on ACh-induced Ca^{2+} influxes. These results suggest that the actions of K^+ channel openers on agonist-induced responses may differ slightly in tracheal and in vascular smooth muscle.

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References

BECKER, P.L., SINGER, J.J., WALSH, J.V. & FAY, F.S. (1989). Regulation of calcium concentration in voltage-clamped smooth muscle cells. *Science*, **244**, 211–214.

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**, 275–278.

BLACK, J.L., ARMOUR, C.J., JOHNSON, P.R.A., ALOUAN, L.A. & BARNS, P.J. (1990). The action of a potassium channel activator, BRL 38227 (lemakalim), on human airway smooth muscle. *Am. Rev. Respir. Dis.*, **142**, 1384–1389.

BONEV, A.D. & NELSON, M.T. (1993). Muscarinic inhibition of ATP-sensitive K^+ channels by protein kinase C in urinary bladder smooth muscle. *Am. J. Physiol.*, **265**, C1723–C1728.

COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.*, **9**, 21–28.

CHILVERS, E.R., BATTY, I.H., BARNES, P.J. & NAHORSKI, S.R. (1990). Formation of inositol polyphosphates in airway smooth muscle after muscarinic receptor stimulation. *J. Pharmacol. Exp. Ther.*, **252**, 786–791.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

IMAGAWA, J., YOSHIDA, S., KOGA, T., KAMEI, K. & NABATA, H. (1993). The effect of a novel benzopyrane derivative, KC399, on the isolated guinea-pig trachealis and human bronchi. *Gen. Pharmacol.*, **24**, 1505–1512.

INOUE, R. & ISENBERG, G. (1990). Effects of membrane potential on acetylcholine-induced inward current in guinea-pig ileum. *J. Physiol.*, **424**, 57–71.

ITO, S., KAJIKURI, J., ITOH, T. & KURIYAMA, H. (1991). Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. *Br. J. Pharmacol.*, **104**, 227–233.

ITOH, T., ITO, S., SHAFIQ, J. & SUZUKI, H. (1994a). Effects of a newly synthesized K^+ channel opener, Y-26763, on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of the rabbit mesenteric artery. *Br. J. Pharmacol.*, **111**, 165–172.

ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inorganic phosphate regulates the concentration-relaxation cycle in skinned muscles of the rabbit mesenteric artery. *J. Physiol.*, **376**, 231–252.

ITOH, T., KANMURA, Y., KURIYAMA, H. & SUZUKI, S. (1984). Nisoldipine-induced relaxation in intact and skinned smooth muscles of rabbit coronary arteries. *Br. J. Pharmacol.*, **83**, 243–258.

ITOH, T., SEKI, N., SUZUKI, S., ITO, S., KAJIKURI, J. & KURIYAMA, H. (1992). Membrane hyperpolarization inhibits agonist-induced synthesis of inositol 1,4,5-trisphosphate in rabbit mesenteric artery. *J. Physiol.*, **451**, 307–328.

ITOH, T., SUZUKI, S., SUZUKI, A., NAKAMURA, F., NAKA, M. & TANAKA, T. (1994b). Effects of exogenously applied calponin on Ca^{2+}-regulated force in skinned smooth muscle of the rabbit mesenteric artery. *Pflügers Arch.*, **427**, 301–308.

JANSSEN, L.J. & DANIEL, E.E. (1991). Depolarizing agents induce oscillations in canine bronchial smooth muscle membrane potential: possible mechanisms. *J. Pharmacol. Exp. Ther.*, **259**, 110–117.

KAMEI, K., NABATA, H. & KURIYAMA, H. (1994). Effects of KC399, a novel ATP-sensitive K^+ channel opener, on electrical and mechanical responses in dog tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **268**, 319–327.

KATSUYAMA, H., SUZUKI, S. & NISHIE, E. (1990). Actions of second messengers synthesized by various spasmogenic agents and their relation to mechanical responses in dog tracheal smooth muscle. *Br. J. Pharmacol.*, **100**, 41–48.

KOGA, H., SATO, H., ISHIZAWA, T., KUROMARU, K., MAKINO, T., TAKA, N., TAKAHASHI, T., SATO, T. & NABATA, H. (1993). Benzopyran-4-carboxamide K^+ channel openers. *Bioorg. Med. Chem. Lett.*, **3**, 1115–1118.

OKADA, Y., YANAGISAWA, T. & TAIRA, N. (1993). BRL 38227 (levcromakalim)-induced hyperpolarization reduces the sensitivity to Ca^{2+} of contractile elements in canine coronary artery. *Naunyn Schmied. Arch. Pharmacol.*, **347**, 438–444.

PACAUD, P. & BOLTON, T.B. (1990). Relation between muscarinic cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *J. Physiol.*, **441**, 477–499.

QUAST, U. (1992). Potassium channel openers: Pharmacological and clinical aspects. *Fundam. Clin. Pharmacol.*, **6**, 279–293.

SAUNDERS, H.H. & FARLEY, J. (1991). Pharmacological properties of potassium currents in swine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **260**, 1038–1044.

SMALL, R.C., BERRY, J.L., BURKA, J.F., COOK, S.J., FOSTER, R.W., GREEN, K.A. & MURRAY, M.A. (1992). Potassium channel activators and bronchial asthma. *Clin. Exp. Allergy*, **22**, 11–18.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

TAYLOR, S.G., ARCH, J.R.S., BOND, J., BUCKLE, D.R., SHAW, D.J., TAYLOR, J.F. & WARD, J.S. (1992). The inhibitory effects of cromakalim and its active enantiomer BRL 38227 against various agonists in guinea pig and human airways: comparison with pinacidil and verapamil. *J. Pharmacol. Exp. Ther.*, **261**, 429–437.

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