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Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells

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1 The effects of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on membrane currents and on single channel K currents in smooth muscle cells isolated from canine trachea were examined by use of tight seal whole cell- and patch-clamp techniques.

2 Depolarizing current applied through a recording pipette did not elicit an action potential under current clamp. A strong outward rectification was observed.

3 In most cells under voltage-clamp, only an outward current was observed upon depolarization from −60 mV when a pipette solution contained mainly KCl. The outward current consisted of three components; a large initial transient, a following sustained component and an additional component of irregular small transients on the sustained one. The two transient components were almost abolished when extracellular and pipette solutions contained 2.2 mM Cd^{2+} (0 mM Ca^{2+}) and 10 mM EGTA, respectively. The sustained component was well maintained under these conditions.

4 TEA at low concentrations (< 1 mM) effectively decreased the transient components and made the outward current smooth; it also suppressed the sustained component at higher concentrations. In outside-out patches, external 1 mM TEA reduced the single channel conductance of Ca-activated K channels by about 87% whereas 3 mM 4-AP did not. 4-AP at low concentrations (< 3 mM) selectively reduced the sustained component of the outward current.

5 A Ca current recorded after the suppression of outward current by internal Cs^+ had a peak of approximately 200 pA at +10 mV (holding potential: −60 mV). The half inactivation voltage in the steady-state was approximately −30 mV.

6 Simultaneous application of 1 mM TEA and 4-AP reduced the outward current and unmasked a Ca current. Under these conditions, an action potential with overshoot was easily elicited under current clamp.

7 It is concluded that the low excitability of canine tracheal smooth muscle cell upon depolarization is due to a large outward K current which consists of Ca-dependent and Ca-independent components. The peak amplitude of the Ca current is similar to that in highly excitable smooth muscle cells such as those of the ureter.

Introduction

The smooth muscle of canine (Stephens & Kroeger, 1970; Suzuki *et al.*, 1976) and bovine trachealis (Kirkpatrick, 1975) shows low excitability. No action potential is elicited even when the cell is depolarized by stimulation with electrical pulse(s) or agonists such as acetylcholine, histamine, 5-hydroxytryptamine etc. (see Kirkpatrick, 1980). Therefore a significant contribution of pharmaco-mechanical coupling to the agonist-induced contraction has been suggested in these preparations (Farley & Miles, 1977; Coburn & Yamaguchi, 1977). In guinea-pig trachea, the smooth muscle cells often exhibit spontaneous electrical slow waves (Small, 1982).

It has been found that a K channel blocker, tetraethylammonium (TEA), increases the membrane excitability in canine and bovine tracheal smooth muscle cells and even elicits repetitive action potentials and concomitant rhythmic contractions similar to the spontaneous contractions of intestinal smooth muscle (Stephens *et al.*, 1975; Kroeger & Stephens, 1975; Kirkpatrick, 1975). Subsequently, it was reported that other K channel blockers such as 4-aminopyridine (4-AP) (Imaizumi & Watanabe, 1983; Kannan *et al.*, 1983) and procaine (Imaizumi & Watanabe, 1982) also have similar but not identical effects to those of TEA on the muscle. The decrease in outward rectification upon depolarization by these K channel blockers was observed in quiescent smooth muscles by use of conventional microelectrodes and the partition method (Casteels *et al.*, 1977; Kirkpatrick, 1975; Suzuki *et al.*, 1976; Hara *et al.*, 1980), and appeared to be responsible for the increase in excitability. Moreover, ^{42}K- and ^{86}Rb-efflux experiments suggest that these compounds decrease K^+ permeability perhaps, by suppressing different K channels (Imaizumi & Watanabe, 1981; 1982; 1983).

The application of the patch-clamp technique to single smooth muscle cells from canine trachea reveals the existence of a Ca-activated K channel on the cell membrane (McCann & Welsh, 1986) as well as in other smooth muscle cells (Inoue *et al.*, 1985; Benham *et al.*, 1985; 1986; Singer & Walsh, 1987; Bregestovski *et al.*, 1988). Recently, a small voltage-dependent Ca channel current has been recorded in single canine tracheal smooth muscle cells (Kotlikoff, 1988).

The present study using whole cell- and patch-clamp techniques was undertaken to elucidate the mechanisms involved in the increase of membrane excitability of canine tracheal smooth muscle cells by TEA and 4-AP. The objective was to determine whether TEA and 4-AP block different K currents and whether the Ca current in tracheal smooth muscle really is much smaller than in other highly excitable smooth muscle cells.

Methods

Cell isolation

Circular muscle strips were obtained from the cervical trachea of dogs weighing 8–14 kg. The mucosa, adventitial, areolar

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and connective tissues were carefully removed from the smooth muscle layer in normal Krebs solution under a binocular microscope (× 30). The procedure of cell isolation was the same as that described by Imaizumi *et al.* (1989a). The tissue was immersed in Ca-free Krebs solution (Ca omitted, no EGTA) for 40–60 min in a test-tube at 37°C. Thereafter, the solution was replaced with Ca-free Krebs solution containing 0.1% albumin and 0.1% collagenase (490 u ml^{-1}, YAKULT, Tokyo) for 30 min. The solution was then replaced with Ca-free and collagenase-free Krebs solution. The tissue was gently agitated with a glass pipette with a short shank and a fire-polished tip. After approximately 10 min agitation, the solution became turbid due to the presence of isolated smooth muscle cells. A few drops of cell suspension were placed in a recording bath (0.3 ml) mounted on the stage of a phase-contrast microscope (Nikon TMD) for electrical measurements.

Solutions

The normal Krebs solution contained (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 123.5, HCO_3^- 25, $H_2PO_4^-$ 1.2, glucose 14 and was gassed with a mixture of 95% O_2 and 5% CO_2. For electrical recordings, a HEPES buffered solution of the following composition was used (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 149.7, glucose 14, HEPES 10. The solution was gassed with 100% O_2 and the pH was adjusted to 7.2 with NaOH. The pipette solution contained (mM): K^+ 140.0, Na^+ 10.0, Mg^{2+} 4.0, Cl^- 148.0, adenosine-triphosphate (ATP) 5.0, ethyleneglycol-bis-(β' amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA) 0.5, the pH of which was adjusted to 7.2 with KOH. When measuring Ca currents, the 140 mM KCl in the pipette solution was replaced with equimolar CsCl and the concentration of EGTA was increased to 10 mM. The pCa of the solutions for measuring Ca activated K channel current activity in excised patches was adjusted with a Ca-EGTA buffer (Benham *et al.*, 1986).

Electrical recordings

Whole cell voltage and current clamp and patch clamp commands were applied to single cells or patches by the method introduced by Hamill *et al.* (1981). The details were similar to those described previously (Imaizumi *et al.*, 1989a,b). The resistance of the pipette was between 2 and 5 MΩ for the whole cell-clamp and between 7 and 15 MΩ for the patch-clamp. A hydraulic micromanipulator (MO-102 or MO-203 Narishige Scientific Instruments, Tokyo, Japan) was used to position the microelectrode near the cell. All procedures for approaching the cell, making a GΩ seal and rupturing the cell membrane were done under × 800 magnification. Inside- or outside-out patch-clamp procedures (Hamill *et al.*, 1981) were performed to examine the effects of TEA and 4-AP on single K channel currents by use of a List EPC-7 amplifier. Single channel current recordings were filtered at 1 kHz by a 4 pole Bessel filter and sampled at 250 μs (see below). The seal resistance formed between cell membrane and tip before impalement was between 10 and 50 GΩ. All measurements were performed at 25 ± 1°C.

Data storage and analysis

Membrane potential and current were monitored on a storage oscilloscope (VC-10, Nihon-koden, Tokyo, Japan or VC-6041, Hitachi, Tokyo, Japan) and stored on video-tape after digitizing by use of a PCM-recording system (PCM 501ES, SONY, Tokyo, Japan; modified to obtain a frequency-response from d.c. to 20 kHz). The data on the tape were replayed later and loaded into a computer (IBM-AT) through an A-D converter (Datatranslation; DT2801A) using data-acquisition software (AQ). Data analysis was performed on an IBM-AT using software, DAS, developed in Dr Wayne Giles' laboratory (University of Calgary, Canada; also AQ). Part of the analysis of single channel current was performed by use of a programme developed by Dr John Dempster (University of Strathclyde, Great Britain). Selected records were printed out with a laser printer (HP Laser Jet Series II) or an X-Y plotter (Roland, DXY-1300).

Statistics and drugs

Summarized data are shown as mean values with s.e.mean. Statistical differences were tested by Student's *t* test. The following drugs were used; tetraethylammonium chloride, 4-aminopyridine, EGTA (Wako Pure Chemical Industries, Ltd., Osaka). Nicardipine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were kindly supplied by Yamanouchi Pharmaceutical Comp. and Bayer (Japan), respectively.

Results

Only relaxed cells having lengths of between 100 and 180 μm were used for electrical recordings (approximately 150 cells). These cells showed contractile responses to acetylcholine, 10 μM, and also to KCl leaked from recording pipettes. Figure 1a shows typical recordings of membrane potential changes elicited by depolarizing or hyperpolarizing current applied through the recording pipette to a single smooth muscle cell under the current clamp mode. The resting membrane potential of this cell was −44 mV and the average of 19 cells

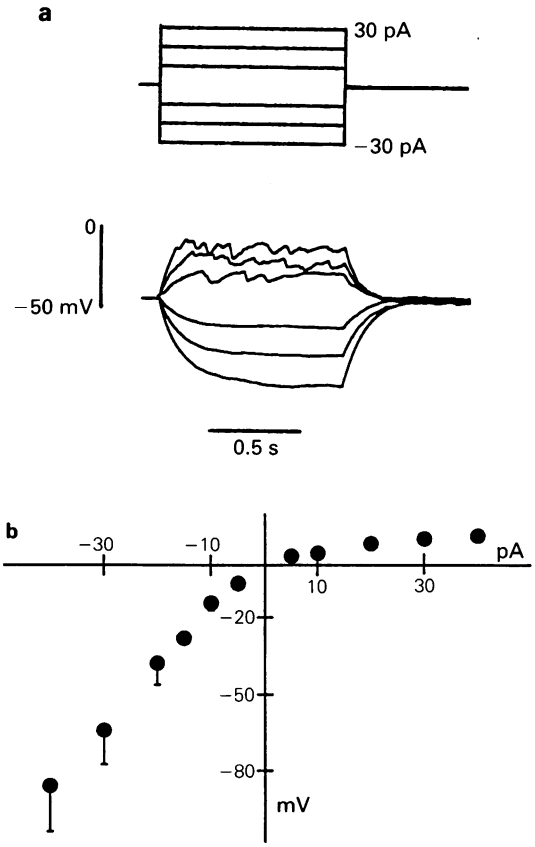

Figure 1 (a) Membrane potential changes (lower traces) recorded under the current clamp mode from a single smooth muscle cell isolated from canine trachea. Six traces were superimposed. The resting membrane potential of this cell was −44 mV. Depolarizing or hyperpolarizing current (upper traces) was applied through the recording pipette. (b) Summarized data showing the relationship between the applied current amplitude (abscissa scale) and resulting changes in membrane potential (ordinate scale) ($n = 6$). Note the strong outward rectification.

derived from 8 trachealis was -41.8 ± 1.5 mV. Neither action potentials nor active depolarizing responses were observed upon depolarization in any cell examined ($n = 19$), while hyperpolarizing humps were often observed when a strong depolarizing current was applied. As illustrated in Figure 1b, the cell membrane showed quite strong outward rectification. The membrane resistance determined between approximately -60 and -100 mV assuming a linear current-voltage relationship was 2.6 ± 0.5 GΩ ($n = 6$). The total membrane capacitance calculated from the membrane time constant (121.8 ± 12 ms, $n = 8$) and the membrane resistance was 47 pF.

Figure 2a shows the outward current evoked upon depolarization from a holding potential of -60 mV under voltage clamp conditions. Without subtraction of leakage current, no inward current was observed in 25 cells out of 30. In five cells, a small and very transient inward current was detected between the capacitive transient and the activation of outward current (not shown). The outward current upon depolarization was very noisy in all cells examined. At potentials positive to 0 mV, a large transient outward current was activated during the initial part of a depolarizing pulse. The current then decreased to a sustained level (sustained component) on which small transient outward currents were superimposed. This differentiation of the depolarization-evoked outward current has previously been observed in the longitudinal smooth muscle cells from the rabbit small intestine (Ohya *et al.*, 1987).

To examine whether the occurrence of transient outward currents was random or not, five recordings obtained at the same potential were averaged (Figure 2a(ii)). It is clear that they only occurred irregularly except at the initial part of the depolarizing pulse. Figure 2b shows the outward currents recorded after extracellular Ca^{2+} was replaced with equimolar Cd^{2+}. The transient components disappeared and the current was activated more slowly and smoothly at all potentials. The peak outward current (Figure 2c) was decreased by about 55% at +10 mV. Figure 2d compares the minimum outward current in normal solution with the current evoked at the end of a 500 ms pulse in Cd^{2+} solution. The steady component was decreased by about 25% at +10 mV. These observations suggest that the transient components of outward current depend quite strongly upon Ca influx during depolarization whereas the sustained component is relatively independent of extracellular Ca.

Exposure to 4-AP (0.3 and 3 mM) had little effect on the transient component of outward current but significantly decreased the sustained phase (Figure 3a(i) and (ii)). After Ca^{2+} was replaced with Cd^{2+}, 4-AP (1 mM) markedly reduced the smooth outward current (Figure 3b). The effect of 4-AP was more apparent in the initial part of depolarization under these conditions. Such results mean that the activation of the outward current was slowed down by 4-AP or, alternatively, the blocking effect of 4-AP was reduced during a long depolarizing pulse (500 ms). The decrease in the sustained component produced by 3 mM 4-AP was about 60% at +10 mV whereas the initial transient current was reduced by about 25% (Figure 3c; see also Figure 6). If the effect of 1 mM 4-AP on the outward current was measured at 50 ms from the start of the depolarization in Cd^{2+} solution, there was a greater reduction at 0 mV (by about 60%) than at more positive potentials (by about 40% at +30 mV, Figure 3d).

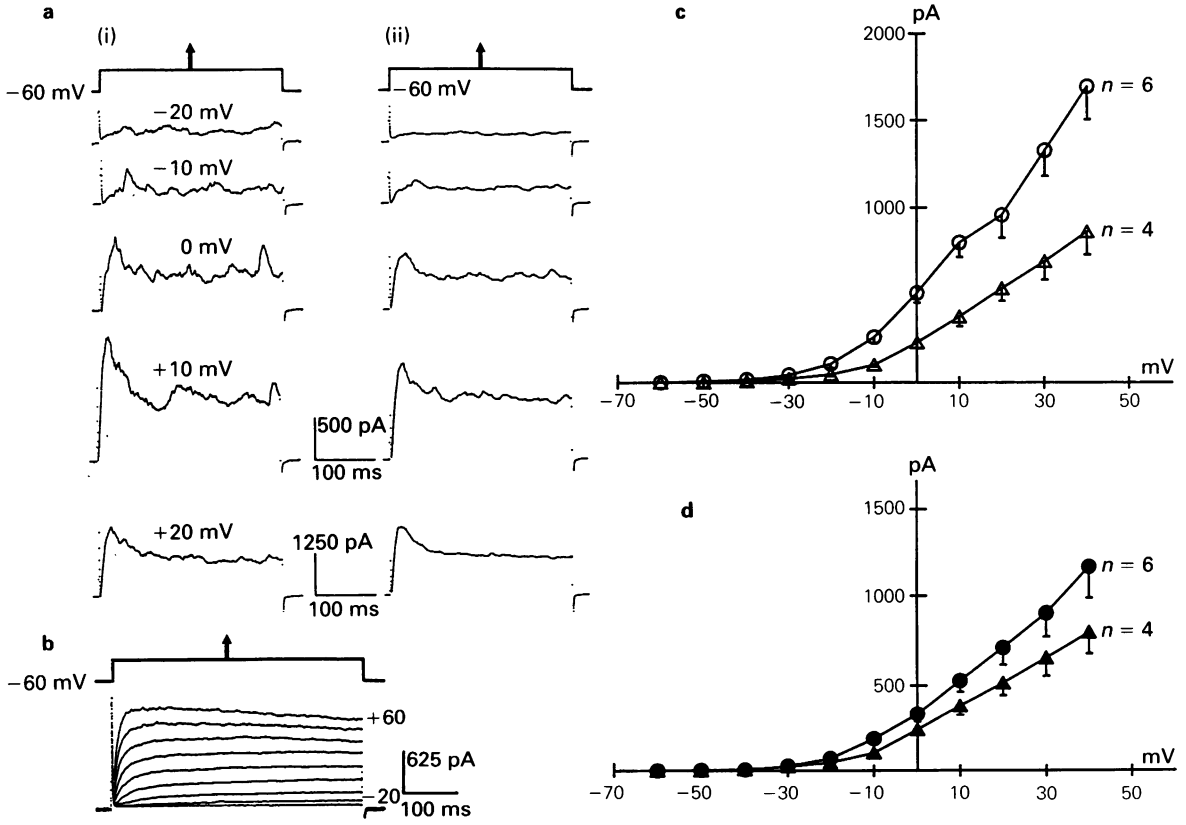

Figure 2 (a) (i) Membrane current elicited by depolarization from a holding potential of -60 mV to the potentials indicated. (ii) Average of five traces at potentials shown in corresponding traces in a(i). Note that the calibration for the trace at +20 mV is different from that for less positive potentials. Leakage current was not subtracted. (b) Outward currents elicited by depolarization from -60 to the potentials between -20 and +60 mV by 10 mV steps in a solution containing 2.2 mM Cd^{2+} instead of 2.2 mM Ca^{2+}. Nine current traces were superimposed. Note that the outward currents were rather smooth. (c) Summarized data of peak outward currents activated at potentials shown on abscissa scale. Circles (○) and triangles (△) indicate the amplitude of peak outward current in normal and Ca-free Cd solutions, respectively. (d) Circles (●) denote the summarized data of least outward current amplitude after the initial large transient outward current in normal solution. These approximate to the amplitude of the steady component: Triangles (▲) illustrate the outward current amplitude measured at the end of a 500 ms pulse in Ca-free Cd solution.

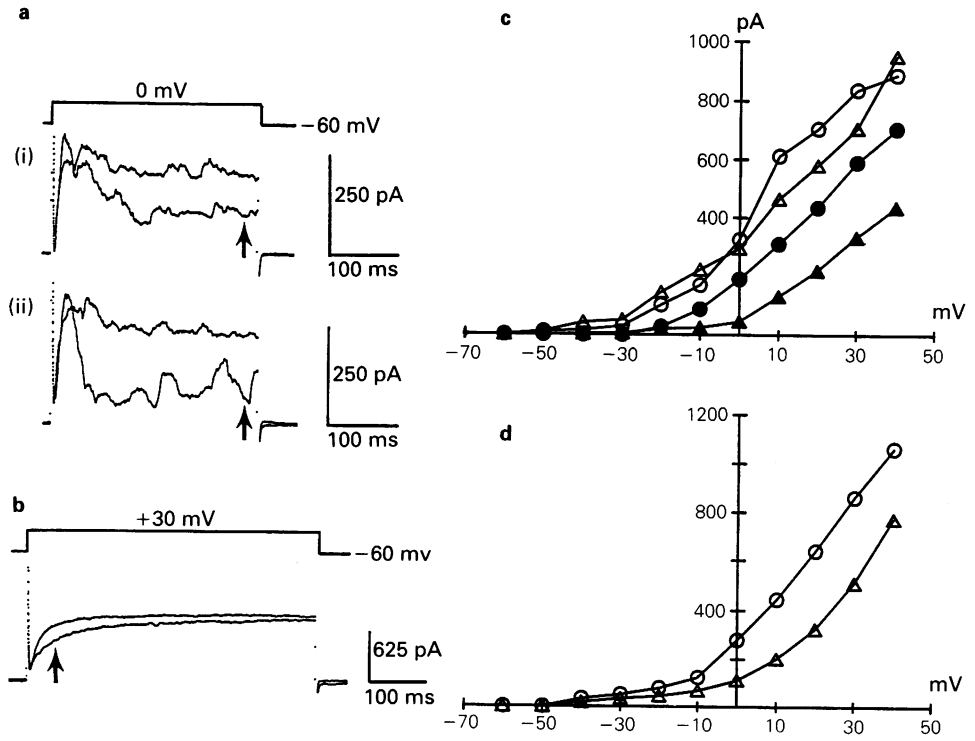

Figure 3 (a and b) Effects of 4-aminopyridine (4-AP) on the outward current elicited by depolarization from −60 to 0 and +30 mV in normal (a(i), (ii)) and Ca-free Cd solutions (b), respectively. Note that the initial transient outward current was not affected by 0.3 (a(i)) and 3 mM (a(ii)) 4-AP, while later part of current was markedly decreased. In the Cd solution (b), the outward current was apparently decreased by 1 mM 4-AP at the initial but not the later part of depolarization. (c) Open and closed symbols indicate the maximum and minimum amplitude of outward currents, respectively, at potentials on the abscissa scale. Circles and triangles denote the amplitude in the absence and presence of 3 mM 4-AP, respectively. (d) Effects of 1 mM 4-AP on the outward current measured 50 ms after the start of depolarization in Ca-free Cd solution. Circles and triangles indicate the current in the absence and presence of 1 mM 4-AP, respectively.

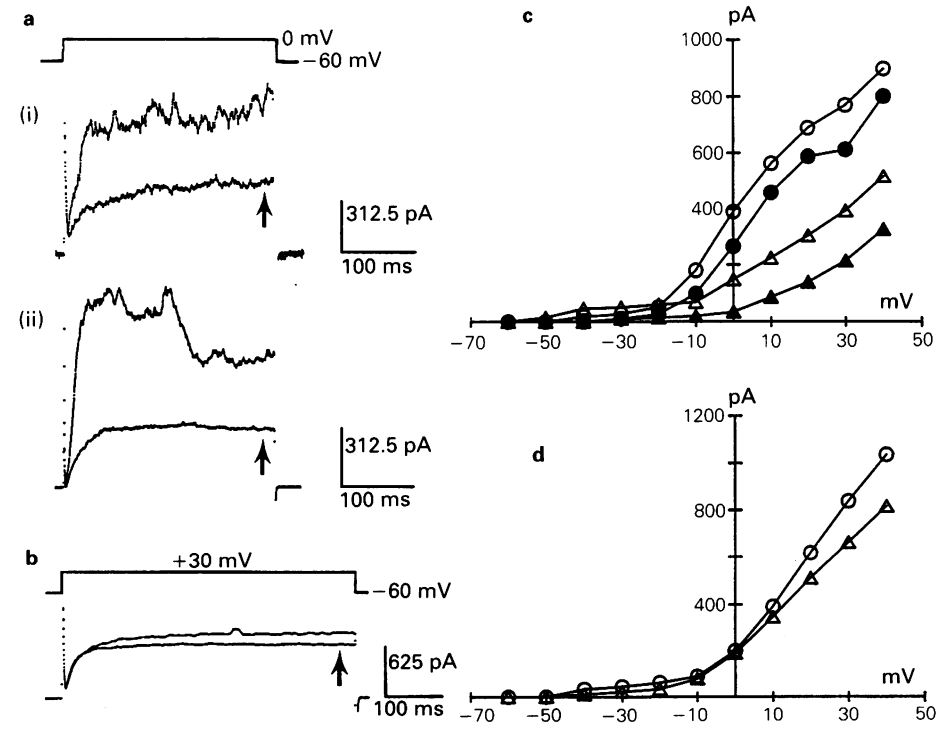

Figure 4 (a and b) Effects of tetraethylammonium (TEA) on the outward currents in normal (a(i), (ii)) and Ca-free Cd solutions (b), respectively. Both transient and sustained components were decreased by 0.3 (i) and 3 mM (ii) TEA. Note that the TEA-resistant current is smooth as that in the Ca-free Cd solution. In the Ca-free Cd solution, 1 mM TEA decreased the later part of outward current (b). (c) Summarized data of outward current amplitude in the absence (circles) or presence (triangles) of 3 mM TEA in the normal solution. Open and closed symbols denote the maximum and minimum amplitude, respectively. (d) The outward currents measured at the end of a 500 ms pulse before (circles) and after (triangles) the application of 1 mM TEA in the Ca-free Cd solution. Note that the effect of TEA is more apparent at positive potentials.

In contrast to 4-AP, TEA (0.3 and 3 mM) affected the transient components of evoked outward current (Figure 4a(i) and (ii)). The current traces became smooth and similar to those observed when extracellular Ca^{2+} was replaced with Cd^{2+}. In a Cd^{2+} solution (Figure 4b), the inhibitory effect of 1 mM TEA on the smooth current appeared gradually during a long depolarizing pulse. The data obtained in normal solutions are summarized in Figure 4c. It is clear that 3 mM TEA inhibits both transient and sustained components. In Cd^{2+} solution, the block by 1 mM TEA is more apparent at positive potentials (Figure 4d).

Data on the effects of TEA and 4-AP on the transient and steady components of outward current elicited by depolarization from −60 to 0 mV at 0.05 Hz are plotted as concentration-response relationships in Figure 5. The amplitude of both components in each cell was determined by averaging the values obtained from three or four traces (not from averaged traces) after the effect of the drug had reached a steady level. Current amplitude was normalized as the value relative to the peak or the minimum outward current in each cell before drug application. The approximate IC_{50} values of TEA for the inhibition of transient and sustained components were 0.3 and 3 mM, respectively. The IC_{50} values of 4-AP were >10 mM and about 0.3 mM, respectively. The results strongly indicate that 4-AP selectively decreases the steady component whereas TEA inhibits both the transient and steady components, with the former more susceptible to TEA than the latter.

The experiments in which Cd^{2+} solution was used suggested that the initial and subsequent repetitive transient components were due to the activation of Ca^{2+}-dependent K^+ current. In order to confirm the implication that TEA, in contrast to 4-AP blocks Ca^{2+}-activated K^+ channels, single channel recordings were performed in which an outside-out patch configuration was used. In preliminary experiments, the relationship between the channel open probability and the pCa of the internal solution was determined from inside-out patches (data not shown). The number of channels included in each patch was quantified at pCa 3.7. There was an average of 3.5 channels per patch ($n = 10$; pipette resistance: ≃10 MΩ). The open probability of the channel was calculated at a certain pCa and potential using current amplitude histograms similar to those shown in Figure 6a(iii) and b(iii). At 0 mV, the approximate open probabilities at pCa 7.0 and 6.5 were 0.1

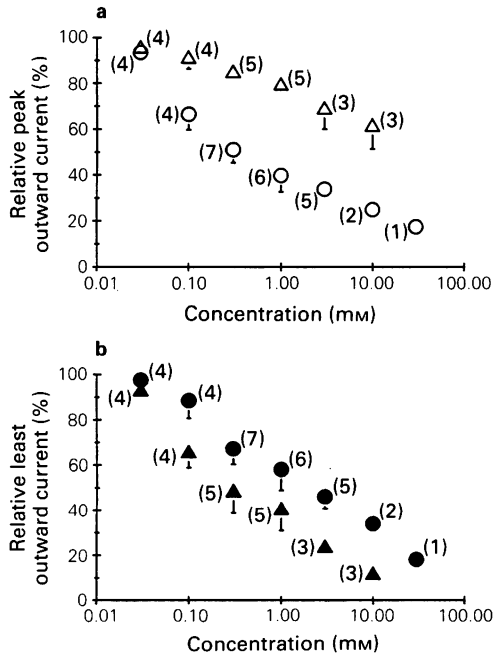

Figure 5 The relationships between the concentration of tetraethylammonium (TEA) (circles) or 4-aminopyridine (4-AP) (triangles) and the effects on the outward current elicited by depolarization from −60 to 0 mV in normal solution. The maximum (a) and the minimum (b) amplitudes of outward current were normalized against those before the application of drugs, respectively, and plotted on the ordinate scale. The numbers in the parentheses are the numbers of cells used.

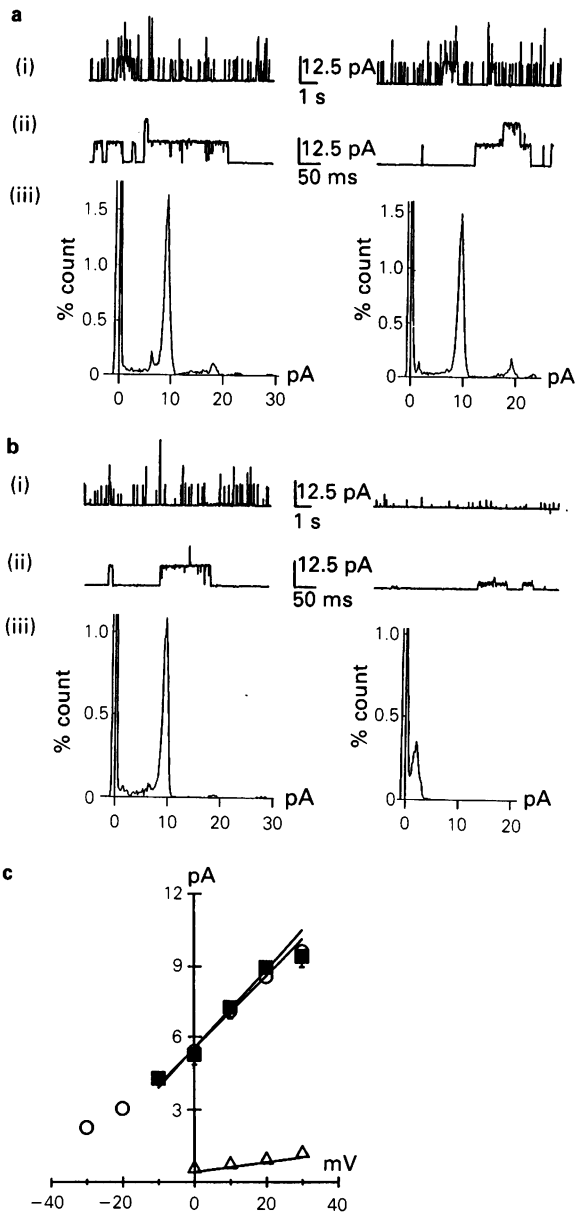

Figure 6 (a) Effect of 1 mM 4-aminopyridine (4-AP) on the single channel current of Ca-activated K channel under outside-out configuration. The pCa of the pipette solution was set at 7.5 to keep the open probability low. The bathing and pipette solutions include 5.9 and 140 mM K^+ respectively. The patch was held at +20 mV. The unitary current of 9.4 pA was little affected by 1 mM 4-AP (a(i) and (ii)). The histograms (iii) indicate that the open probability was also unaffected by 1 mM 4-AP (left: control; right: 1 mM 4-AP). The ordinate scale of the histogram is the percentage of total sample count in each bin (0.2 pA step) relative to the count during the recording period (8.2 s). The ratio of total open time (integration of bins distributed around the peaks at about 9.4) vs. recording period was 0.135 and 0.121 in the control and in the presence of 1 mM 4-AP, respectively. (b) Both the unitary current and the open probability of the K-channel at +20 mV were decreased markedly by the presence of 1 mM tetraethylammonium (TEA) ((i), (ii) and (iii)). Note that the current at open state is very noisy (ii). (c) The effects of 3 mM 4-AP (closed squares) and 2 mM TEA (triangles) on the conductance of the channel. The channel conductance in the control was 154 ± 10 pS (open circle, $n = 5$). The conductance was markedly decreased by 2 mM TEA (19.9 ± 2.3 pA, $n = 3$, $P < 0.001$ vs. control) but not by 3 mM 4-AP (166 ± 5.5 pA, $n = 3$, $P > 0.05$ vs. control). The solid lines were fitted by eye.

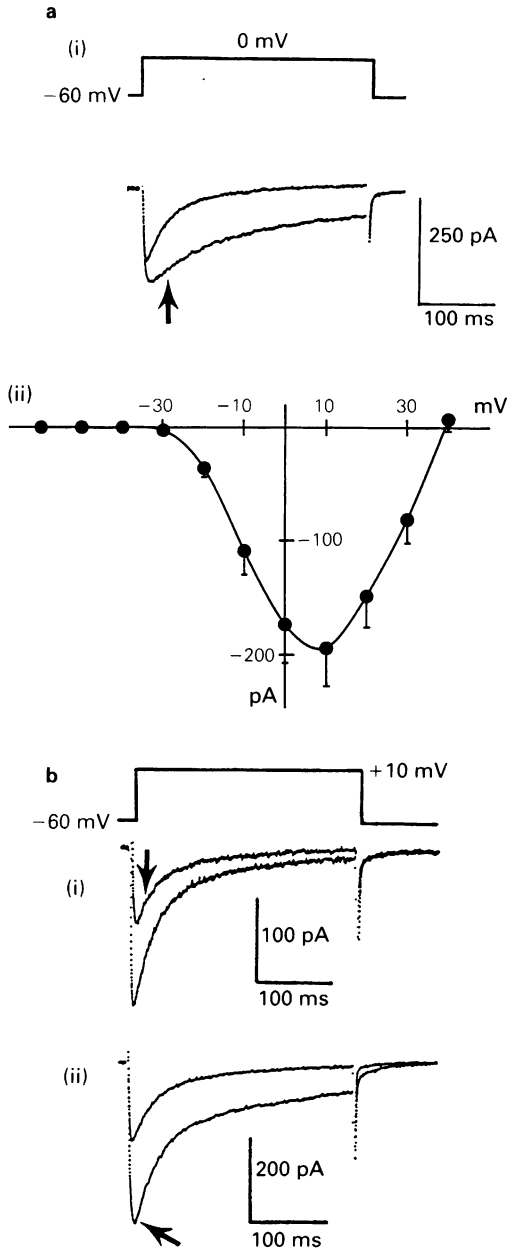

Figure 7 (a) Ca current activated by depolarization from −60 to 0 mV (i) and the summarized data of current-voltage relationship of I_{Ca} ((ii), $n = 6$). Outward currents were blocked by 140 mM Cs^+ in the pipette solution. When extracellular 2.2 mM Ca^{2+} was replaced with equimolar Ba^{2+}, the inward current increased and became sustained (a(i), indicated by an arrow). (b) Ca current elicited by depolarization from −60 to +10 mV. I_{Ca} was decreased by the exposure to 0.1 μM nicardipine ((i), arrow). In the presence of 1 μM Bay K 8644, the inward current was markedly enhanced ((ii), arrow).

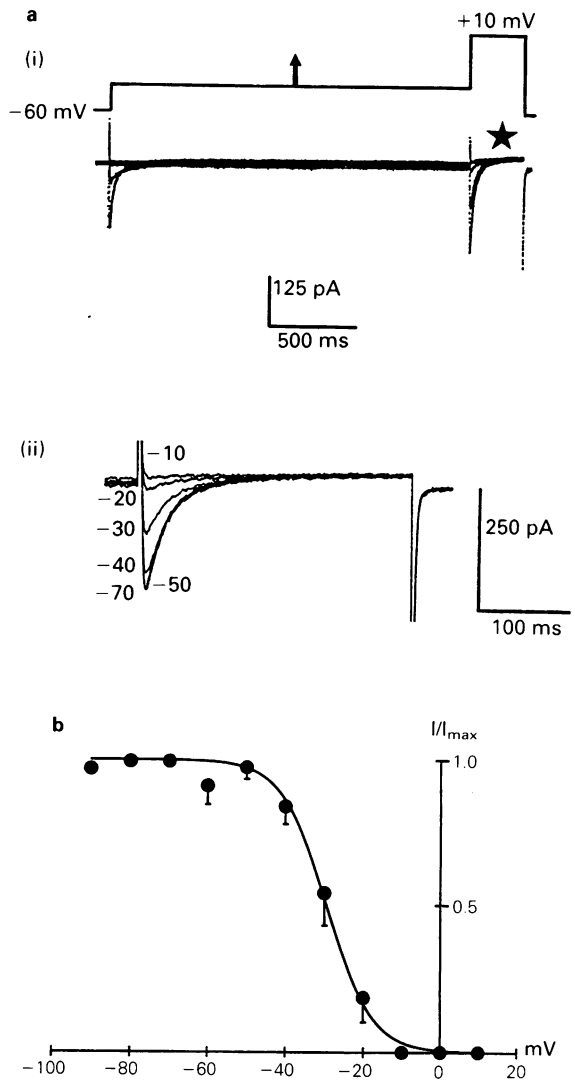

Figure 8 The voltage-dependence of steady-state inactivation of Ca channel current in canine tracheal smooth muscle cells. (a) The conventional double pulse protocol (upper trace in (i)). The duration of P_1 and P_2 were 2 s and 300 ms, respectively. The P_2 potential was +10 mV. The P_1 potential was changed by 10 mV steps at 0.05 Hz. In the lower panel in a(i), five traces at different P_1 potentials are superimposed. The Ca currents at P_2 pulse (indicated by a star) are shown on a faster time scale in a(ii). (b) The summarized data of the steady-state inactivation obtained in the manner shown in (a) ($n = 5$). The data for each cell were fitted by the Boltzmann function (see Text). The values for the half inactivation voltage and the slope factor are −29.9 ± 3.8 mV and −5.8 ± 0.9 mV ($n = 5$), respectively and were used to plot the line shown.

and 0.8, respectively and, therefore, highly dependent upon the pCa ($n = 3$, data not shown). In these preliminary experiments, the pipette and bath solutions contained 140 and 5.9 mM K^+, respectively. Figure 6a shows the effect of 1 mM 4-AP on the single channel events of a large conductance Ca^{2+}-activated K^+ channel (see also Figure 6c) using an outside-out configuration. The concentrations of Ca^{2+} in the pipette and bath solutions were pCa 7.5, and 2.2 mM, respectively. Under these conditions, neither the unit current size nor the open probability was changed by 1 mM 4-AP (Figure 6(a)). Similar results were obtained in a further four cells. In contrast to 4-AP, 1 mM TEA decreased the unitary current from 8.5 to 2.5 pA and increased the noise at the open state (Figure 6b(i) and (ii)). The open probability also appeared to be decreased by 1 mM TEA (Figure 6b(iii)). Data on the effects of 2 mM TEA and 3 mM 4-AP on the single channel conductance are summarized in Figure 6(c). The conductance was significantly decreased by 2 mM TEA from 154 ± 10 pS ($n = 5$) to 19.9 ± 2.3 pS ($n = 3$) but not by 3 mM 4-AP (166 ± 5.5 pS, $n = 3$).

Although the large outward current observed upon depolarization must be at least partially responsible for the strong rectification and for the lack of excitability under current clamp conditions, it is also possible that the low excitability is attributable to a small Ca inward current. Figure 7a(i) shows recordings of the inward current activated by depolarization from −60 to 0 mV. The pipette solution contained 140 mM Cs^+ instead of K^+ (Klöckner & Isenberg, 1985b). In almost all cells examined (>30 cells), a substantial inward current (>100 pA at the peak) was recorded in the region of +10 mV. The inactivation of this current was fast but was markedly slowed by the substitution of extracellular 2.2 mM Ca^{2+} with equimolar Ba^{2+} (Figure 7a(i)). The peak current amplitude

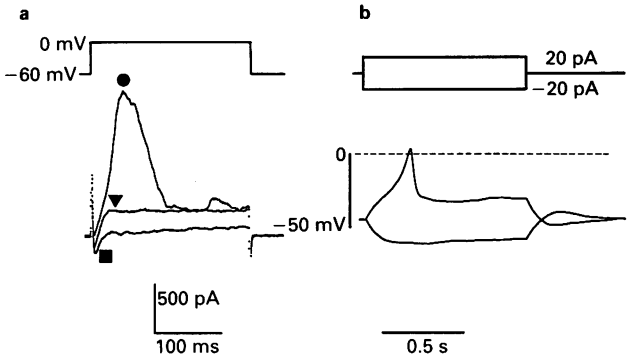

Figure 9 The effects of simultaneous application of 1 mM tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on membrane currents (a) and membrane excitability (b). The initial transient outward current elicited by depolarization from −60 to 0 mV (a, indicated by a circle) was effectively suppressed by 1 mM TEA (a, indicated by a triangle). Additional application of 1 mM 4-AP decreased the sustained component (a, indicated by a square) and unmasked the Ca current. In the presence of 1 mM TEA and 4-AP, a clear action potential with overshoot (5 mV) was elicited (b) in the same cell. The resting membrane potential of this cell in the presence of 1 mM TEA and 4-AP was −45 mV.

was increased by about 40% ($n = 3$) after the substitution. A summary of data on I_{Ca} is illustrated in Figure 7a(ii). Peak current (194.7 ± 33.2 pA, $n = 6$) was obtained near +10 mV in normal solution containing 2.2 mM Ca^{2+}. The current was markedly decreased by 0.1 μM nicardipine to about 40% of the control (Figure 7b(i)) and blocked completely by the addition of 500 μM Cd^{2+} (data not shown, $n = 3$). Exposure to 1 μM Bay K 8644 doubled the peak amplitude of I_{Ca} (Figure 7b(ii); $n = 3$). The voltage-dependence of I_{Ca} inactivation was examined by use of a conventional double pulse protocol (Figure 8a) in normal solution. The inactivation occurred at potentials positive to −60 mV and was complete in the region of −10 mV (Figure 8b). The data obtained from each cell were well described by the Boltzmann function.

$$I/I_{max} = 1/[1 + \exp\{(V_{1/2} - V_m)/S\}]$$

where $V_{1/2}$ is the membrane potential at which one-half inactivation occurs and S is the slope factor at $V_m = V_{1/2}$. The averaged $V_{1/2}$ and S were −29.9 ± 3.8 mV and −5.8 ± 0.9 mV ($n = 5$), respectively.

From the approximate IC_{50} values of TEA and 4-AP for the inhibition of the transient and sustained components of outward current, respectively, the simultaneous application of TEA and 4-AP (each 1 mM) should inhibit a large part of the outward current and increase membrane excitability. The results of such an experiment are shown in Figure 9. The application of 1 mM TEA significantly reduced the transient outward current upon depolarization from −60 to 0 mV under voltage clamp. The addition of 1 mM 4-AP produced a further decrease in the remaining sustained component of the outward current, and revealed a greater inward current. Under these conditions, a clear action potential was elicited by depolarizing current (Figure 9b; the same cell). A single application of 1 mM TEA or 4-AP did not allow the cell to elicit an action potential in response to depolarizing stimulation, whereas small active depolarizing responses of about 10 mV were occasionally observed. Similar observations were obtained from 5 separate cells.

Discussion

The pharmacological approach using TEA and 4-AP in the present study clearly shows that at least two distinct K^+ currents are activated upon depolarization in canine tracheal smooth muscle cells. Such a result was expected from a previous study in canine trachea in which the inhibitory effects of TEA and 4-AP on ^{86}Rb-efflux were additive (Imaizumi & Watanabe, 1983). The existence of two distinct K currents has also been identified by use of TEA and 4-AP as pharmacological tools in rabbit portal vein (Beech & Bolton, 1987). The transient component of outward current is apparently a K current which flows through a Ca activated K channel and which can be blocked by extracellular TEA at relatively low concentrations (see Inoue *et al.*, 1985). Although three kinds of Ca activated K channel have been reported in smooth muscle cells (Inoue *et al.*, 1985; 1986), the K channel with a large conductance of approximately 200 pS (140 mM K^+ symmetry; $K_{L\text{-}Ca}$ channel) and with a high sensitivity to extracellular TEA appears to be the major K^+ channel responsible for the outward current in several types of smooth muscle cell (urinary bladder: Klöckner & Isenberg, 1985a; stomach: Mitra & Morad, 1985; Walsh & Singer, 1987; ureter: Imaizumi *et al.*, 1989a; taenia caeci: Yamamoto & Kao, 1989). The IC_{50} of TEA for the inhibition of the transient outward current was 0.3 mM in the present study. Although the measurement of inhibition might be an underestimate due to the lack of inward current subtraction, the value appears to be reasonable since 1 mM TEA reduced the charge movement per unit time through a single $K_{L\text{-}Ca}$ channel by over 70% (see Figure 6b(iii)). It is, therefore, clear that the transient outward current is more susceptible to TEA ($IC_{50} = 0.3$ mM) than the sustained component ($IC_{50} = 3$ mM). The present results also showed that low concentrations of 4-AP (<3 mM) did not block the $K_{L\text{-}Ca}$ channel. At higher concentrations, however, 4-AP (>3 mM) appeared to decrease the open probability of the $K_{L\text{-}Ca}$ channel. This contrasts with the data obtained from rabbit portal vein (Beech & Bolton, 1987) and suggests that the action of 4-AP on $K_{L\text{-}Ca}$ could vary depending on the type of smooth muscle cell.

In tracheal smooth muscle, the outward current at 0 mV was decreased only by about 50% in Ca-free Cd solution or on exposure to 3 mM TEA which probably suppresses the $K_{L\text{-}Ca}$ channel almost completely. The remaining sustained component of outward current was a delayed rectifier K current (I_K) which was activated voltage-dependently and was quite sensitive to 4-AP ($IC_{50} = 0.3$ mM). TEA also inhibited this component ($IC_{50} = 3$ mM). These two IC_{50} values are rather accurate since the inactivation of I_{Ca} is fast enough to disregard its negative contribution to the sustained component of outward current. The possibility that I_K is modulated by intra- and/or extracellular Ca^{2+} was not examined in the present study. Moreover, it is also quite possible that I_K may have been partly suppressed by the replacement of Ca^{2+} with Cd^{2+}, since some voltage- and time-dependent K currents are sensitive to divalent cations such as Mn^{2+}, Cd^{2+} and Co^{2+} (Mayer & Sugiyama, 1988; Beech & Bolton, 1989; Yamamoto & Kao, 1989). This type of K current which is voltage-dependent, Ca-independent, highly sensitive to 4-AP and exhibits relatively slow activation and inactivation, has been observed in nerves (Meeves & Pichon, 1977; Hermann & Gorman, 1981; as single channel current: Quandt, 1988) and muscles (Fink & Wettwer, 1978) including the smooth muscle cells from rabbit pulmonary (Okabe *et al.*, 1987), rabbit femoral, bovine coronary arteries (Muraki & Takeda, unpublished observation), rat aorta (Toro & Stefani, 1987), rabbit portal vein (Beech & Bolton, 1987) and rabbit iris sphincter (Furuichi, unpublished observation). However, cells from the taenia caeci (Imaizumi *et al.*, 1989a), urinary bladder (Klöckner & Isenberg, 1985a), and ureter (Imaizumi *et al.*, 1989a) of the guinea-pig do not possess a delayed rectifier K current which is highly sensitive to 4-AP. This might suggest that in general, smooth muscle cells which show low excitability possess a substantial 4-AP-sensitive I_K, although a similar current has been described in rabbit portal vein which is spontaneously excitable (Beech & Bolton, 1989).

The I_K in canine tracheal smooth muscle can be easily distinguished from the Ca^{2+}-independent transient outward current, the so called A-current, I_A, which is also highly sensitive to 4-AP (Thompson, 1982; Rogawski, 1985). The I_A has a

much faster inactivation, is less sensitive to TEA than I_K, and has been found in smooth muscle cells from portal vein (Beech & Bolton, 1989), ureter (Imaizumi *et al.*, 1989a; Lang, 1989) and vas deferens (Muraki & Takeda, unpublished observation). In the present study, the single channel currents underlying the I_K were not identified and are the subject of further study.

The blockade of I_K by TEA and 4-AP exhibited different characteristics (see Figure 3c and Figure 4c). In Cd^{2+} solution the effect of TEA developed gradually during a long depolarizing pulse, whereas those of 4-AP were apparent as a slow-down of I_K activation. TEA may act as an open channel blocker from the inner surface in a manner similar to that described for squid axon (reviewed by Armstrong, 1975) or alternatively it may act from the outside in other neurones (reviewed by Stanfield, 1983). It has been reported that the affinity of 4-AP for K channels is decreased by depolarization (Ulbricht & Wagner, 1976; Yeh *et al.*, 1976). This may explain one observed feature of 4-AP-induced blockade of I_K in the tracheal cells; a decrease in blockade during depolarization rather than a slow-down of I_K activation.

Under the conditions of the present study, it was confirmed that the inward current upon depolarization in tracheal smooth muscle was an almost pure Ca current as has already been reported in several smooth muscle cells (Klöckner & Isenberg, 1985b; Mitra & Morad, 1985; Ganitkevich *et al.*, 1986; Ohya *et al.*, 1986; Nakazawa *et al.*, 1987; Kotlikoff, 1988). Although a small I_{Ca} was previously reported in the same preparation (< 50 pA; Kotlikoff, 1988), we found a relatively large I_{Ca} (> 100 pA, $n > 30$) in most well-shaped cells in the normal solution containing 2.2 mM Ca^{2+}. The amplitude of the I_{Ca} (average 200 pA in 2.2 mM Ca solution) corresponds to that in cells from guinea-pig ureter (Imaizumi *et al.*, 1989a) and is greater than that in aorta (Toro & Stefani, 1987) and pulmonary (Okabe *et al.*, 1987), ear (Arronson, *et al.*, 1989), coronary and femoral arteries (Muraki & Imaizumi, unpublished observation). The reason for the difference in the amplitude of I_{Ca} in canine tracheal smooth muscle cells in the two studies (Kotlikoff, 1988; present investigation) is not clear. However, it may be related to the different types of dog employed and to modification of channel properties during cell isolation.

In the present study, the types of Ca channel were not examined carefully (Benham *et al.*, 1987; Yatani *et al.*, 1987; Yoshino *et al.*, 1988; Loirand *et al.*, 1989; Arronson *et al.*, 1989). The I_{Ca} in our preparation showed high sensitivity to nicardipine and also to Bay K 8644, whereas the latter was not effective in a previous study (Kotlikoff, 1988). The present observations favour the presence of an L type channel (Yatani *et al.*, 1987). The half inactivation voltage of −30 mV obtained in normal solution (2.2 mM Ca^{2+}) is not really consistent with the presence of a T type channel as has been suggested by Kotlikoff (1988). The relatively fast inactivation of I_{Ca} in the present study could be explained by a large degree of Ca^{2+}-dependent inactivation of I_{Ca} since Ba^{2+} currents exhibit much slower inactivation rates (Ganitkevich *et al.*, 1987; Nakazawa *et al.*, 1987; Ohya *et al.*, 1988). The contribution of high threshold (L-) type Ca channel current may be significant in canine tracheal smooth muscle as in other several smooth muscle cells (ureter: Imaizumi *et al.*, 1989a; vas deferens: Nakazawa *et al.*, 1988; portal vein: Loirand *et al.*, 1989; saphenous vein: Yatani *et al.*, 1987; taenia caecum: Yoshino *et al.*, 1988).

The simultaneous application of relatively low concentrations of TEA and 4-AP markedly increased membrane excitability (Figure 9). This was due to the additive inhibitory effects of these drugs on outward current. In vascular smooth muscle it has been suggested that TEA may also directly increase the Ca permeability (Haeusler *et al.*, 1980). However, our preliminary experiments with canine tracheal cells suggest that TEA and 4-AP (3 mM) do not markedly affect the amplitude of I_{Ca} under conditions in which K currents were blocked by internal 140 mM and external 10 mM Cs^+ (Muraki, unpublished observation). In guinea-pig ureter, spike potentials are observed under physiological conditions and I_{Ca} is very similar in amplitude to I_{Ca} in dog trachea (Imaizumi *et al.*, 1989a; present study). Although the inactivation of I_{Ca} in trachea is much faster than in ureter, it is similar to that observed in other spike-generating smooth muscles such as taenia caeci (Ganitkevich *et al.*, 1986), portal vein (Arronson *et al.*, 1988) and urinary bladder (Klöckner & Isenberg, 1985b). Thus the presence of 4-AP-sensitive delayed rectifier K current (I_K) in dog trachea and its absence from guinea-pig ureter may be a critical feature in determining the excitability of these two types of smooth muscle.

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