Neurokinin A and Ca^{2+} current induce Ca^{2+}-activated Cl^- currents in guinea-pig tracheal myocytes

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1. Membrane currents were recorded by a patch clamp technique in guinea-pig tracheal myocytes, using the whole cell mode with Cs^+ internal solution.

2. Both neurokinin A (NKA, 1 μM) and caffeine (10 mM) evoked Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) transiently. In Ca^{2+}-free bathing solution, the first application of NKA or caffeine elicited $I_{Cl(Ca)}$ but the second application of these substances failed to activate it. In addition, pretreatment with ryanodine in the presence of caffeine abolished the response to both NKA and caffeine whilst heparin (200 μg ml^{-1}) only blocked the NKA-induced response. $I_{Cl(Ca)}$ was also elicited by inositol 1,4,5-trisphosphate (IP_3).

3. Command voltage pulses positive to 0 mV from a holding potential of −60 mV activated the voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) and late outward current. Upon repolarization to the holding potential, slowly decaying inward tail currents were recorded. The outward current during the depolarizing pulses and the inward tail current were enhanced by Bay K 8644, but completely blocked by Cd^{2+} or nifedipine. Replacement of external Ca^{2+} with Ba^{2+}, removal of Ca^{2+} from the bath solution, or inclusion of EGTA (5 mM) in the patch pipette, also led to abolition of these currents, indicating that they were Ca^{2+} dependent, and that Ca^{2+} influx due to $I_{Ca,L}$ activated the currents.

4. When $[Cl^-]_o$ or $[Cl^-]_i$ was changed, the reversal potential (E_{rev}) of the Ca^{2+}-activated currents shifted, thus behaving like a Cl^--selective ion channel as predicted by the Nernst equation. DIDS (1 mM) completely abolished the currents, also suggesting that they were $I_{Cl(Ca)}$.

5. NKA (1 μM) and caffeine (30 mM) transiently activated $I_{Cl(Ca)}$, and after that both agents markedly reduced $I_{Cl(Ca)}$ induced by $I_{Ca,L}$. This is probably due to sarcoplasmic reticulum (SR) Ca^{2+} release induced by NKA or caffeine, followed by inhibition of the Ca^{2+}-induced Ca^{2+} release from the SR.

6. The present results indicate that $I_{Cl(Ca)}$ can be activated by SR Ca^{2+} release due to NKA or caffeine (through IP_3 or ryanodine receptors) as well as by Ca^{2+} influx due to $I_{Ca,L}$. It also suggests that activation of $I_{Cl(Ca)}$ by NKA may be mediated by the production of IP_3, which releases Ca^{2+} from the SR.

Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) are widely recognized in a variety of cells such as cardiac myocytes and smooth muscle cells (Byrne & Large, 1988; Pacaud, Loirand, Lavie, Mironneau & Mironneau, 1989; Amédée, Large & Wang, 1990; Loirand, Pacaud, Mironneau & Mironneau, 1990; Zygmunt & Gibbons, 1991; Akbarali & Giles, 1993). The activation of Cl^- currents can: (1) lead to membrane depolarization since the Cl^- equilibrium potential is more positive than the resting membrane potential (Aickin, 1990), (2) induce an after-polarization following an action potential (Nishimura, Akasu & Tokimasa, 1991) or (3) change the action potential duration and consequently alter

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Ca^{2+} influx (Korn, Bolden & Horn, 1991). In tracheal smooth muscle cells (TSMCs) from the guinea-pig (Small, 1982), slow waves in the membrane potential, as found in other smooth muscle cells (Langton, Burke & Sanders, 1989), had been recorded and were thought to induce spontaneous fluctuations of muscle tone (Christensen, Caprilli & Lund, 1969; Sanders & Smith, 1986). Since slow-wave discharge in airway smooth muscle is potential dependent (Kirkpatrick, 1981), it is likely that the alterations in electrical activities of airway smooth muscle elicited by activation of Cl^- currents may play important roles in regulating the tracheal smooth muscle tone.

Tachykinins, such as neurokinin A (NKA) and substance P have been identified in non-cholinergic sensory nerves in the airways and are recognized as neurotransmitters which control airway resistance (Barnes, Chung & Page, 1988). These neuropeptides contract tracheal smooth muscle, and increase microvascular permeabilities (Lundberg, Saria, Brodin, Rosell & Folkers, 1983). Tachykinins have been reported to affect several ionic currents in smooth muscle cells. In gastric smooth muscle cells, substance P enhances the voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) (Clapp, Vivaudou, Singer & Walsh, 1989). In colonic smooth muscle cells, it has been shown that the neurokinin-1 receptor agonist, substance P methylester, activates a Cl^- current via GTP-binding proteins probably in a membrane-delimited manner (Sun, Supplisson, Torses, Sachs & Mayer, 1992). In guinea-pig TSMCs, NKA and substance P activate $I_{Cl(Ca)}$ (Janssen & Sims, 1994; Nakajima, Hazama, Hamada, Omata & Kurachi, 1995), but the characteristics of activation of Cl^- currents have not been fully investigated. In addition, in cardiac myocytes, vascular and oesophageal smooth muscle cells, a rise in $[Ca^{2+}]_i$ due to $I_{Ca,L}$ can activate $I_{Cl(Ca)}$ (Pacaud *et al.* 1989; Zygmunt & Gibbons, 1991; Akbarali & Giles, 1993). In the former, a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism is involved in activating the Cl^- current (Zygmunt & Gibbons, 1991). However, it has not been examined whether the Ca^{2+} influx due to $I_{Ca,L}$ can activate $I_{Cl(Ca)}$ and whether the CICR mechanism is involved in activating $I_{Cl(Ca)}$ in TSMCs.

The main purpose of this study is to clarify the characteristics and physiological significance of $I_{Cl(Ca)}$ in single TSMCs, using the whole cell clamp techniques. The present study shows that $I_{Cl(Ca)}$ can be activated by sarcoplasmic reticulum (SR) Ca^{2+} release due to NKA or caffeine (through inositol 1,4,5-trisphosphate (IP_3) or ryanodine receptors) as well as Ca^{2+} influx due to the Ca^{2+} current. It also indicates: (1) that the activation of Cl^- currents by NKA may be due to IP_3 production through pertussis toxin (PTX)-insensitive GTP-binding proteins, which release SR Ca^{2+}, and (2) that the CICR mechanism may be partly involved in the activation of $I_{Cl(Ca)}$ by Ca^{2+} influx due to $I_{Ca,L}$. Since $I_{Cl(Ca)}$ substantially contributed to the action potential shapes, it appears to play important roles in regulating the muscle tone in tracheal smooth muscle.

METHODS

Preparations

Single smooth muscle cells were obtained from the guinea-pig trachea using an isolation method described previously (Hisada, Kurachi & Sugimoto, 1990). Briefly, adult guinea-pigs were killed by cervical dislocation following sodium pentobarbitone anaesthesia and the trachea was removed. Then, after removing the surrounding connective tissue, the cartilaginous portion of the trachea was cut open longitudinally and the mucosa removed with a scalpel. The membranous portion of the trachea was cut into small pieces and incubated in the dissociation medium containing 0·5 mg ml^{-1} papain (Sigma) and 0·05% bovine serum albumin at 4 °C for 14–18 h. The tissue was then incubated with an enzymatic solution containing 3 mg ml^{-1} collagenase (Worthington CLS II, Freehold, NJ, USA), 0·5 mg ml^{-1} trypsin inhibitor (Sigma Type I-S) and 0·15 mM dithiothreitol (Sigma) at 37 °C for 45 min. Subsequently, the tissue was transferred to the enzyme-free dissociation medium and kept at 4 °C. Single smooth muscle cells were dispersed by trituration in enzyme-free dissociation medium just prior to the experiments. This yielded an acceptable number of viable single smooth muscle cells. All experiments were performed at 35–37 °C. The dissociation medium contained (mM): NaCl, 110; $NaHCO_3$, 10; KCl, 5; $MgCl_2$, 0·5; NaH_2PO_4, 0·5; $CaCl_2$, 0·16; EDTA, 0·49; taurine, 10; Phenol Red, 0·02; Hepes-NaOH buffer, 10; glucose, 11 (pH 8·0). In experiments using PTX-treated cells, isolated smooth muscle cells were incubated at 35 °C in normal Tyrode solution for 6 h with and without PTX (5 μg ml^{-1}). Also, in experiments using ryanodine-treated cells, single cells were incubated for approximately 5 min under perfusing ryanodine (50 μM) in the presence of caffeine (10 mM).

Solutions and drugs

The composition of the normal Tyrode solution was as follows (mM): NaCl, 136·5; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·53; glucose, 5·5; Hepes-NaOH buffer, 5 (pH 7·4). The Ca^{2+}-free Tyrode solution was the same as the normal Tyrode solution with the exception that $CaCl_2$ was omitted. When the external Cl^- ($[Cl^-]_o$) or internal Cl^- concentration ($[Cl^-]_i$) was changed, Cl^- was exchanged for aspartic acid. When the external Na^+ concentration ($[Na^+]_o$) was altered, Na^+ was replaced with equimolar $Tris^+$ or tetraethylammonium (TEA^+) while maintaining the Cl^- concentration constant. The patch pipette solution contained (mM): CsCl, 140; EGTA, 0·15; $MgCl_2$, 2; Na_2ATP, 1; guanosine 5′-triphosphate (sodium salt, Sigma) 0·1 and Hepes-CsOH buffer, 5 (pH 7·2). To record the $I_{Ca,L}$, the composition of the bath solution was as follows (mM): NaCl, 126·5; $CaCl_2$, 5·0; $MgCl_2$, 0·53; TEA, 10; glucose, 5·5; Hepes-CsOH buffer, 5 (pH 7·4). The patch pipette contained (mM): CsCl, 140; $MgCl_2$, 2; Na_2ATP, 1; guanosine 5′-triphosphate (sodium salt, Sigma) 0·1 and Hepes-KOH buffer 5 (pH 7·2). In some experiments, 5 mM EGTA was included in the patch pipette to chelate intracellular Ca^{2+}. In addition, in some experiments, guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS, Boehringer, Mannheim, Germany) or GDPβS was added to the internal solution instead of GTP. NKA was purchased from Peptide Ins (Osaka, Japan). Caffeine, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), heparin and IP_3 were purchased from Sigma. Heparin or IP_3 was added to the pipette solution, and NKA, caffeine, Bay K 8644 or DIDS was applied to the bath solution. The chamber was continuously perfused by the bathing solution. The recording chamber (2 mm in width and 15 mm in length) was used, and the depth of the perfusion solution was about 0·7–1 mm. The solution was perfused at a rate of about 5–7 ml min^{-1} with gravity, and 90% changes of the bath solution occurred within 1 s.

Recording technique and data analysis

Membrane currents were recorded with glass pipettes using the whole cell voltage clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) employing a patch clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany). The heat-polished patch pipettes, filled with the artificial internal solution (composition described above), had a tip resistance of 3–6 MΩ. Membrane currents were monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikusui Electronics, Tokyo, Japan). The series resistance was compensated at the start of the experiments. The data were stored on video tape using the PCM converter system (RP-880, NF electronic circuit design, Tokyo, Japan). Later, the data were reproduced, low-pass filtered at 2 kHz (−3 dB) with a Bessel filter (FV-625, NF, 48 dB/octave slope attenuation), sampled at 5 kHz, and analysed off-line with a computer using pCLAMP software (Axon Instruments). Voltage-ramp command pulses were used to generate current–voltage (I–V) relationships. Statistical data are expressed as means ± S.D. Student's t test was used for statistical analysis and $P < 0.05$ was considered to be significant.

RESULTS

NKA and caffeine activate Cl^- currents in single TSMCs

The effect of NKA or caffeine on membrane currents was investigated in single TSMCs from the guinea-pig with Cs^+ internal solution. NKA (1 μM) transiently evoked a prominent inward current at the holding potential of −40 mV (Fig. 1*A*). The peak amplitude of the inward current was 13·5 ± 7·9 pA pF^{-1} (mean ± S.D., $n = 15$). The current

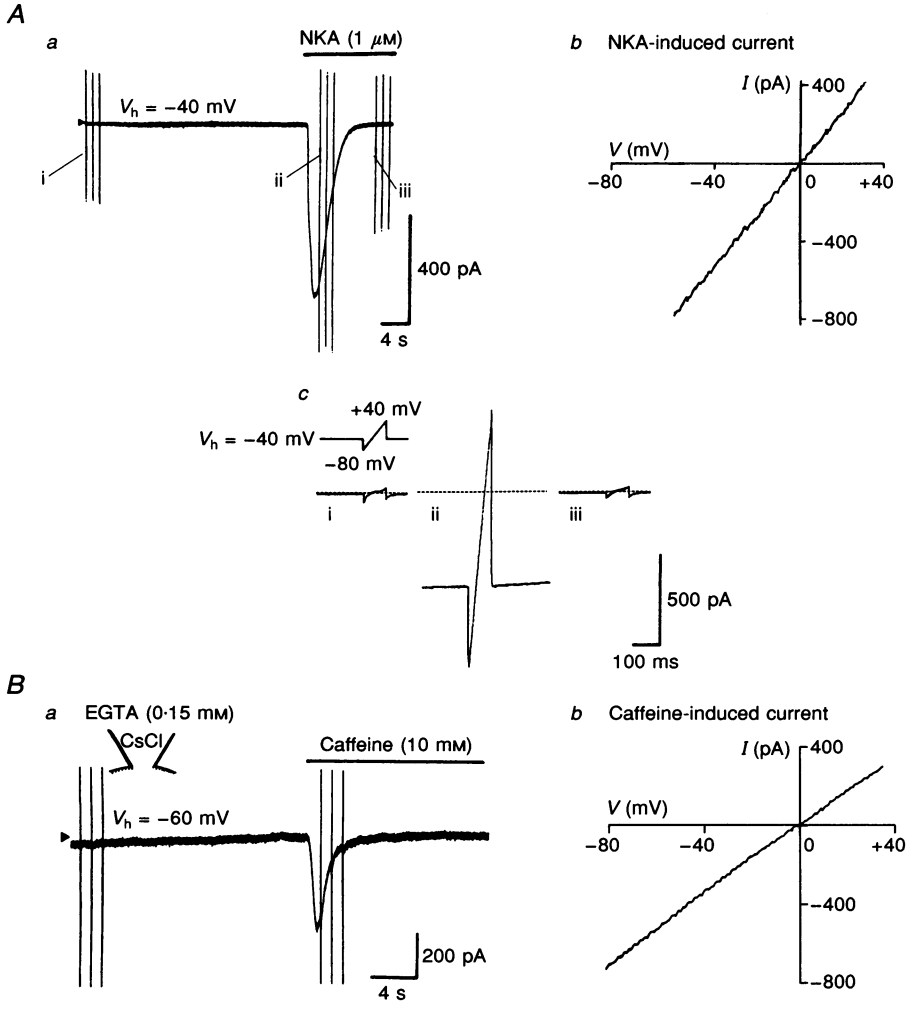

Figure 1. Activation of Cl^- currents by NKA and caffeine in isolated TSMCs

A, activation of Cl^- currents by NKA in isolated TSMCs. The patch pipette contained the Cs^+ internal solution. The cell was held at −40 mV, and the continuous monitoring of the holding current is illustrated in *a*. In controls (i) and during NKA application (1 μM) (ii), the ramp voltage pulses (from −80 to +40 mV for 100 ms) were applied. The current traces (i–iii) during the ramp pulse are illustrated in *c*. The zero current level is indicated by the dashed line. The NKA-induced current is taken as the difference between the current in the control (i) and that in the presence of NKA (ii). The I–V relationships of the NKA-induced current are plotted in *b*. *B*, effects of caffeine on membrane currents of TSMCs. *a*, the holding potential was −60 mV and voltage ramps from −80 to +40 mV were applied in the control conditions and during caffeine application (10 mM). The I–V relationship (*b*) of caffeine-induced current was obtained by subtracting the control current during the ramp pulse from that in the presence of caffeine.

faded within 10–20 s in the continued presence of NKA. The *I–V* relationships of the NKA-induced current obtained by subtracting the control current from that in the presence of NKA (Fig. 1*A a*) were roughly linear between −80 and +40 mV, and crossed the zero current level at about 0 mV, suggesting that the current flows mainly through either non-selective cation channels or Cl^- currents. Caffeine (10 mM), which is known to release Ca^{2+} from intracellular storage sites, also evoked a transient inward current (Fig. 1*B*). The caffeine-induced current also showed a linear *I–V* relationship, which reversed at about 0 mV as with NKA. The changes in the extracellular and intra-pipette Cl^- concentration ($[Cl^-]_o$ and $[Cl^-]_{pip}$, respectively) shifted the E_{rev} of the NKA- or caffeine-induced current according to the expected alterations of the E_{rev} for a Cl^--selective ion channel. In addition the current was abolished by a Cl^--channel blocker, DIDS, (1 mM) (not shown), suggesting that both NKA and caffeine activate Cl^- currents in single TSMCs as reported previously (Nakajima *et al.* 1995).

To confirm the mechanisms of activation of Cl^- currents, we examined the effects of EGTA in the patch pipettes as shown in Fig. 2*A*. The concentration of EGTA was increased from 0·15 to 5 mM in the pipette solution. With 5 mM EGTA in the pipette, neither NKA (1 μM) nor caffeine (10 mM) evoked Cl^- currents. Therefore, an increase in $[Ca^{2+}]_i$ may mediate the NKA induction of Cl^-

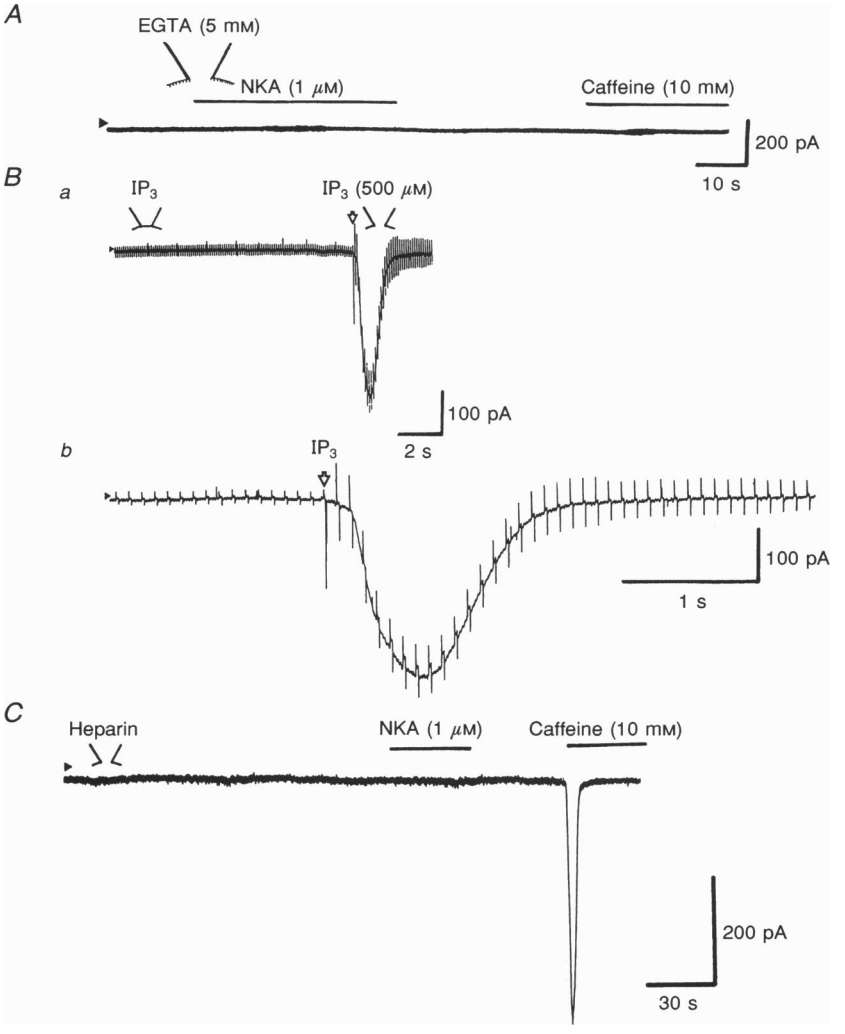

Figure 2. Effects of EGTA, IP_3 and heparin on caffeine- and NKA-induced currents

A, effect of high EGTA on caffeine- and NKA-induced currents. The Cs^+ internal solution contained 5 mM EGTA. The holding potential was −60 mV and caffeine (10 mM) or NKA (1 μM) was added to the bathing solution. *B*, IP_3-activated inward current. The patch pipette contained 500 μM IP_3. The cell was held at −60 mV. Immediately after the rupture of the membrane, a transient inward current was elicited by IP_3. Slight depolarizing command steps from a holding potential of −60 mV were applied every 100 ms. Data with an expanded time scale are illustrated in *b*. *C*, effect of heparin on the activation of inward current by NKA and caffeine. The cell was held at −60 mV and the holding current was continuously recorded. The patch pipette was filled with Cs^+ internal solution containing heparin (200 μg ml^{-1}).

currents in guinea-pig TSMCs. Figure 2*B* and *C* illustrate the effects of intracellular IP_3 on membrane currents and those of heparin, an IP_3-receptor antagonist, on NKA- and caffeine-evoked Cl^- currents. As shown in Fig. 2*B*, the application of IP_3 (500 μM) activated an inward current transiently. DIDS (1 mM) also abolished the IP_3-evoked current, suggesting that IP_3 activated the Cl^- current. In addition, the effects of heparin were also investigated to clarify the involvement of the IP_3 receptor in the activation of Cl^- currents by NKA (Fig. 2*C*). When heparin (200 μg ml^{-1}) was present in the pipette, NKA could not activate Cl^- currents. However, the subsequent application of caffeine (10 mM) elicited huge Cl^- currents. These results suggest that IP_3, which releases Ca^{2+} from intracellular Ca^{2+} storage sites, plays the role of an intracellular second messenger in the activation of Cl^- currents by NKA.

The underlying mechanisms of activation of Cl^- currents were further investigated (Fig. 3). With 2 mM GDPβS in the pipette solution (Fig. 3*A*), NKA (1 μM) did not induce a noticeable response when the cells were held at −60 mV ($n = 5$). In contrast, caffeine (10 mM) induced Cl^- currents. As caffeine was still effective in the presence of intracellular GDPβS, these results suggest that the blockade of the NKA-induced response by GDPβS is not due to depletion of the internal calcium store or direct inhibition of $I_{Cl(Ca)}$. Figure 3*Ba* and *b* illustrate the comparative effects of the inclusion of GTP and GTPγS in the pipette solution on the NKA-induced response. In GTP (200 μM)-loaded cells, low doses of NKA (0·1 μM) did not elicit the Cl^- current, while NKA (1 μM) brought about marked activation. On the other hand, when GTPγS (200 μM, a non-hydrolysable GTP analogue) was added to the pipette, NKA evoked the Cl^-

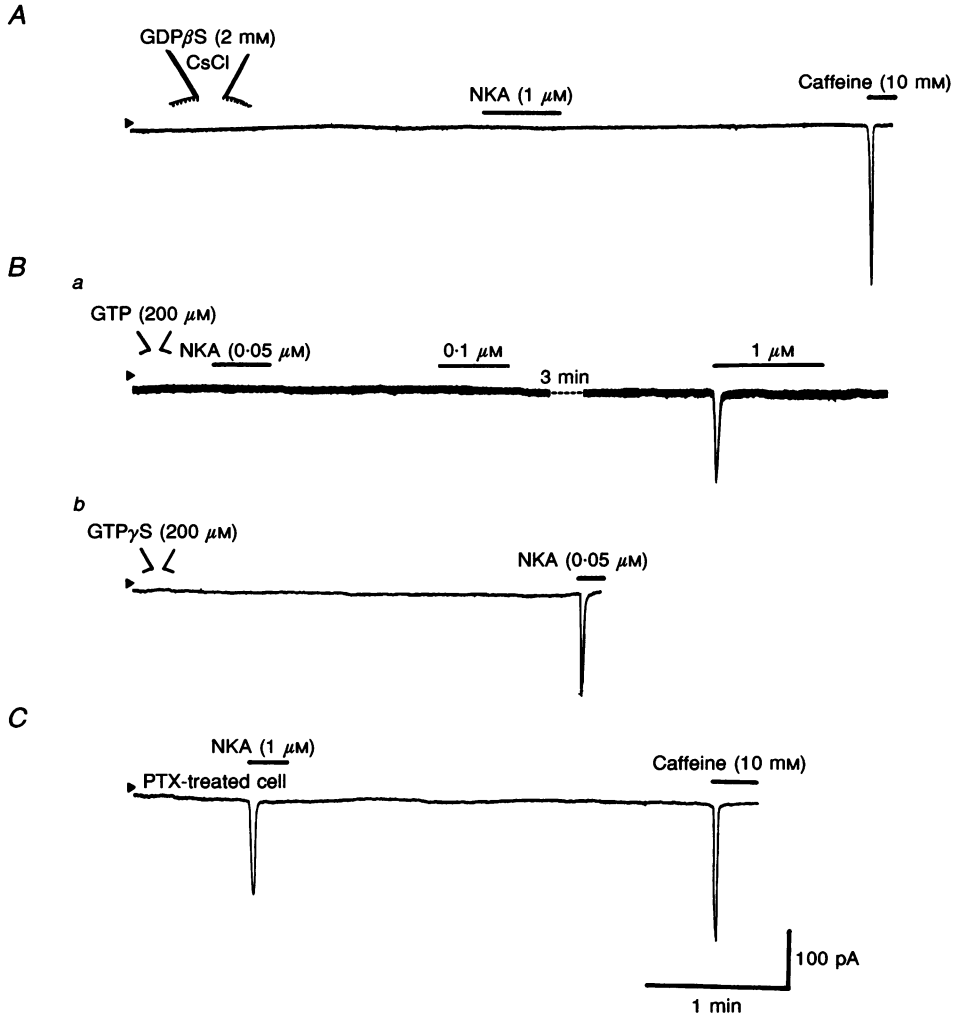

Figure 3. Effects of GDPβS, GTPγS and pertussis toxin (PTX) on the activation of an inward current by NKA

In *A*, *Ba* and *b*, the patch pipette contained GDPβS (2 mM), GTP (200 μM) and GTPγS (200 μM), respectively. In *C*, the cell was pretreated with PTX (5 μg ml^{-1}). In each case, the cell was held at −60 mV, and the holding current was monitored. The drug sequence is indicated in the upper part of each current trace.

current, even at a concentration of 0·05 μM. Incubation of the cells in the presence of 5 μg ml^{-1} PTX for 6 h did not affect the amplitude of the NKA-induced Cl^- current (Fig. 3*C*). NKA (1 μM) induced Cl^- currents of 11·3 ± 4·3 and 10·3 ± 3·8 pA pF^{-1} in control and PTX-treated cells, respectively ($n = 5$, P = n.s).

Involvement of intracellular calcium stores on NKA- and caffeine-activated Cl^- currents in single TSMCs

To elucidate the involvement of intracellular calcium stores on NKA-activated Cl^- currents, the effects of extracellular Ca^{2+} were investigated in Fig. 4. The effects of extracellular Ca^{2+} on the caffeine-activated Cl^- currents are shown in Fig. 4*A*. In normal Tyrode solution containing Ca^{2+} (Fig. 4*A*, upper trace), caffeine (10 mM) elicited a Cl^- current. After washout, the second application of caffeine also elicited the same amplitude for the inward current. When caffeine was applied soon after the cell was exposed to Ca^{2+}-free Tyrode solution (Fig. 4*A*, lower trace), the response to caffeine was not altered. However, the second application of caffeine was not able to activate the Cl^- current at all, suggesting that

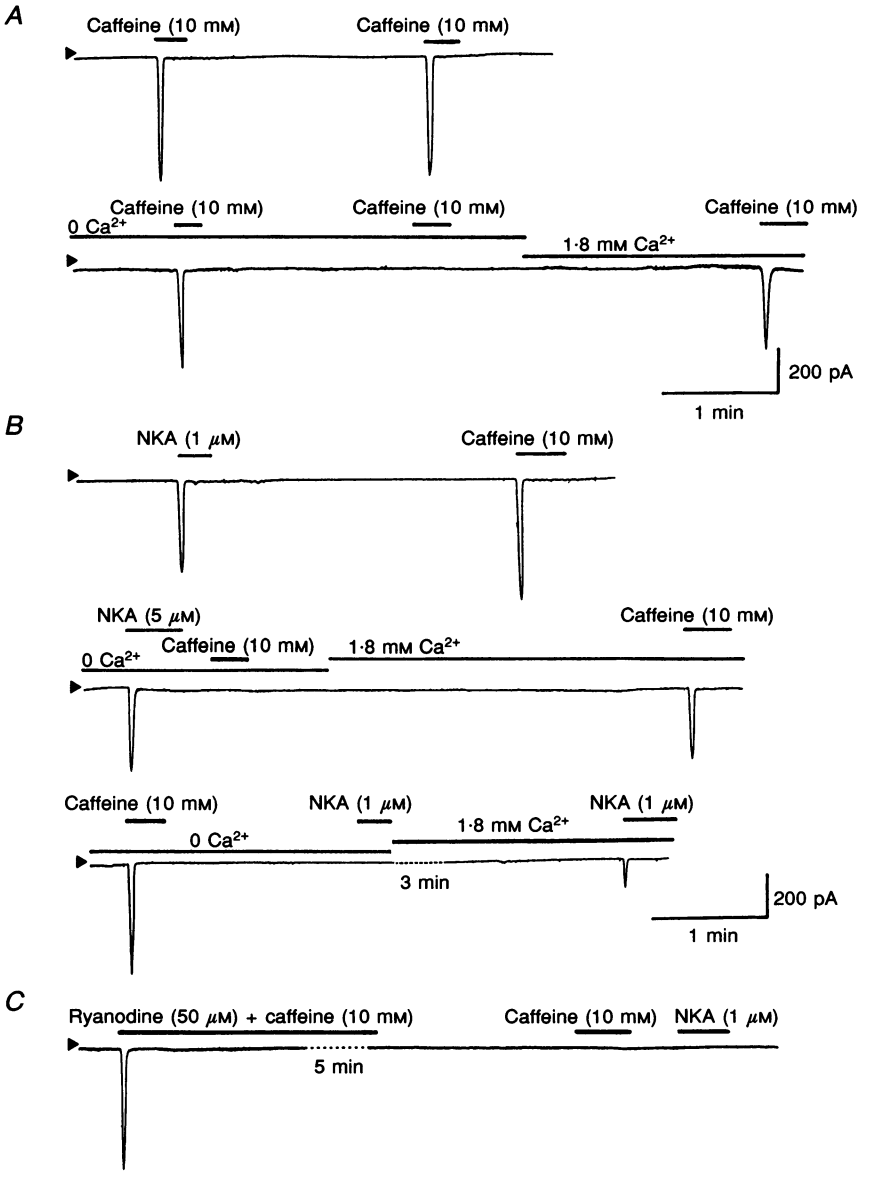

Figure 4. Effects of extracellular Ca^{2+} and ryanodine on NKA- and caffeine-activated inward current

A and *B*, effects of extracellular Ca^{2+} on NKA- and caffeine-activated inward current. The cells were held at −60 mV in each case. The drug sequence is shown in the upper part of the original current traces. In each case, the holding current was continuously monitored (see text for further details). *C*, effects of ryanodine on the NKA- or caffeine-induced Cl^- current. Cells were treated with ryanodine (50 μM) in the presence of caffeine (10 mM) for approximately 5 min. Note that immediately after the application of caffeine and ryanodine, $I_{Cl(Ca)}$ was activated transiently.

intracellular Ca^{2+} from the SR had been depleted during the first application of caffeine (10 mM). When the cell was again bathed in normal Tyrode solution, caffeine activated the Cl^- current. These results are in accordance with the notion that caffeine activates the Cl^- current by releasing Ca^{2+} from the SR. The effects of extracellular Ca^{2+} on the NKA-activated Cl^- current are presented in Fig. 4*B*. When NKA was applied soon after the cells were exposed to Ca^{2+}-free Tyrode solution (Fig. 4*B*, middle trace), the response to NKA was unaltered. These data suggest that external Ca^{2+} does not contribute in any major way towards the generation of the Cl^- current evoked by both NKA and caffeine. In Ca^{2+}-free Tyrode solution, after NKA (5 μM) had elicited a huge Cl^- current, the application of caffeine (10 mM) failed to evoke the current significantly (Fig. 4*B*, middle trace). However, when cells were bathed in normal Tyrode solution, caffeine did evoke a response. Alternatively, Cl^- currents were induced by 10 mM caffeine in Ca^{2+}-free Tyrode solution, but subsequently the application of NKA (1 μM) failed to evoke the current (Fig. 4*B*, lower trace). These results suggest that both NKA and caffeine-sensitive SR Ca^{2+} stores may overlap in TSMCs.

Ryanodine is known to inhibit caffeine-induced Ca^{2+} release in smooth muscle cells (Meissner, 1986; Ito, Takakura, Sato & Sutko, 1986; Iino *et al.* 1989; Kanmura, Missiaen, Raeymaekers & Casteels, 1988; Katsuyama, Ito & Kuriyama, 1991). Therefore, the effects of ryanodine on activation of the Cl^- currents by NKA were investigated (Fig. 4*C*). Following the application of ryanodine (50 μM) in the presence of caffeine (10 mM) for approximately 5 min, subsequent application of caffeine (10 mM) and NKA (1 μM) were unable to activate Cl^- currents (Fig. 4*C*). The same results were obtained from five cells. These findings suggest that ryanodine inhibits the activation of Cl^- currents by NKA as well as caffeine in guinea-pig TSMCs.

Ca^{2+} influx due to $I_{Ca,L}$ also induces $I_{Cl(Ca)}$ in single TSMCs

In single airway smooth muscle cells isolated from guinea-pig and human bronchial smooth muscle cells (Hisada *et al.* 1990), it has been shown that the voltage-dependent Ca^{2+} current mainly consists of a dihydropyridine-sensitive high-threshold Ca^{2+} channel, which can be classified as L-type. Figure 5 shows the effects of Bay K 8644 on membrane currents in single TSMCs. Without EGTA in the patch pipette, the cell was held at −60 mV, and command voltage pulses to +20 mV were applied at 0·1 Hz. In the controls, the $I_{Ca,L}$ was recorded, followed by the outward current. A slowly decaying inward tail current was elicited upon repolarization (Fig. 5*A*). After application of Bay K 8644 (500 nM), $I_{Ca,L}$ increased. The outward current during the command pulse and the inward tail current were also

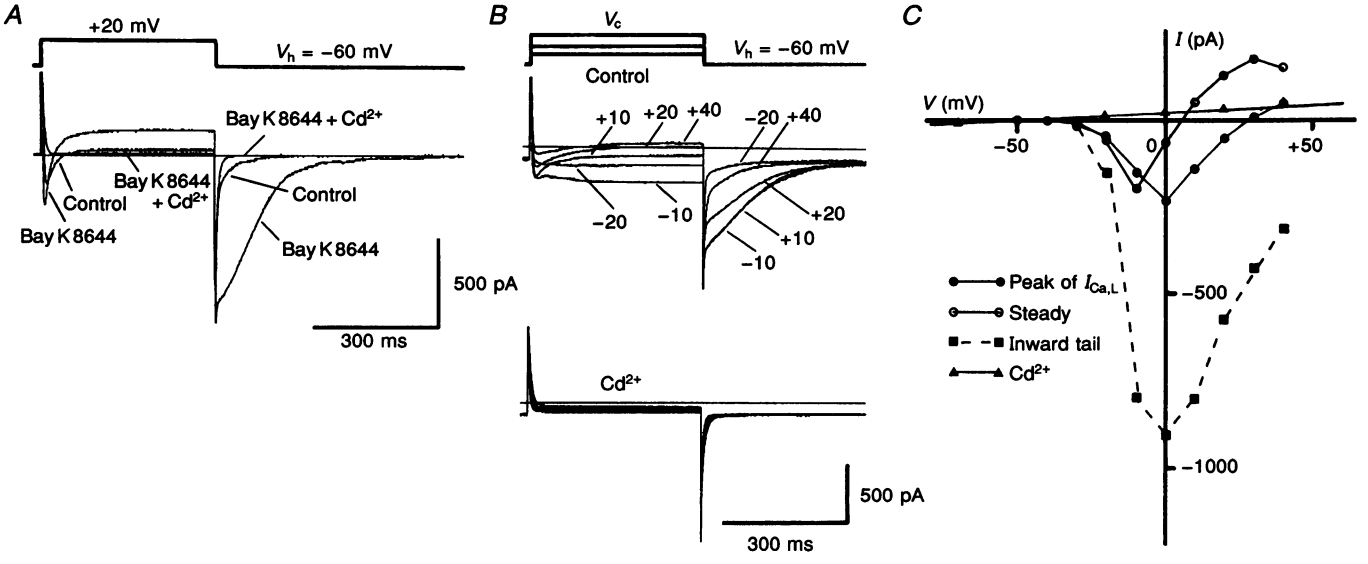

Figure 5. Effects of Bay K 8644 and Cd^{2+} on membrane currents in TSMCs

Without EGTA in the patch pipette, the cell was held at −60 mV and command voltage steps to +20 mV were applied at 0·1 Hz. The original current traces in *A* are shown for controls, Bay K 8644 (500 nM) and additional application of Cd^{2+} (0·3 mM). Note that Bay K 8644 increased the outward current during the depolarizing pulses and the inward tail current. The zero current level is indicated by the horizontal lines. *B*, original current traces elicited during various command voltage steps shown in controls and in the presence of Cd^{2+} (0·3 mM). The *I–V* relationships at the initial peak of Ca^{2+} current and measured at the end of the pulses are illustrated for controls and in the presence of Cd^{2+} in *C*. In the presence of Cd^{2+}, the *I–V* relationship was linear and superimposed on the leakage current which was obtained by extrapolating the currents that corresponded to small hyperpolarizing and depolarizing potentials. ■, amplitude of the inward tail current plotted against each command voltage potential.

dramatically enhanced. The amplitude of $I_{Ca,L}$ and the inward tail current increased from −180 to −310 pA and −210 to −870 pA, respectively. Cd^{2+} (0·3 mM) or nifedipine (1 μM, not shown) completely abolished the activation of these currents, even in the presence of Bay K 8644. Figure 5*B* illustrates typical current traces obtained from a cell at each command voltage step in controls and after application of Cd^{2+} (0·3 mM), and Fig. 5*C* presents its *I–V* relationships. In controls (Fig. 5*B*), $I_{Ca,L}$ was followed by a steady inward current at potentials negative to 0 mV, and the outward current was elicited during the command pulses at potentials positive to 0 mV. Upon repolarization to the holding potential, an inward tail current was observed. The *I–V* relationship at the steady state in controls and after Cd^{2+} crossed at approximately +6 mV in this cell. As shown in Fig. 5*C*, the amplitude of the inward tail current plotted against each command pulse reached a peak value at 0 mV, where $I_{Ca,L}$ was maximal. The relationship was U-shaped. These findings suggest that $I_{Ca,L}$ might be involved in the activation of the current during command pulses and the inward tail current.

To investigate the involvement of a rise in intracellular Ca^{2+} due to $I_{Ca,L}$ in these currents, the effects of Ca^{2+} in the bath solution were examined (Fig. 6). In controls (Fig. 6*A a*), $I_{Ca,L}$ was followed by an outward current, and the inward tail current was evoked. However, after extracellular Ca^{2+} was removed from the bath solution, $I_{Ca,L}$ was abolished. The outward current and inward tail current then also disappeared (Fig. 6*A b*). When Ca^{2+} was again added to the Ca^{2+}-free Tyrode solution, these currents reappeared (Fig. 6*A c*). The time courses of amplitude of $I_{Ca,L}$ and inward tail current in the control and after depletion of Ca^{2+} from the bath are presented in Fig. 6*B*. Immediately after removal of Ca^{2+} from the bath, both the inward tail current and $I_{Ca,L}$ were abolished. Figure 6*C* shows the effects of EGTA (5 mM) in the patch pipette. Only $I_{Ca,L}$ was observed in the controls and in the presence of Bay K 8644 (500 nM) under these conditions. The outward current and the inward tail current were totally abolished. These results indicate that the current elicited by $I_{Ca,L}$ was Ca^{2+}-activated and that the rise in $[Ca^{2+}]_i$ from the Ca^{2+} influx due to $I_{Ca,L}$ was involved in evoking the current.

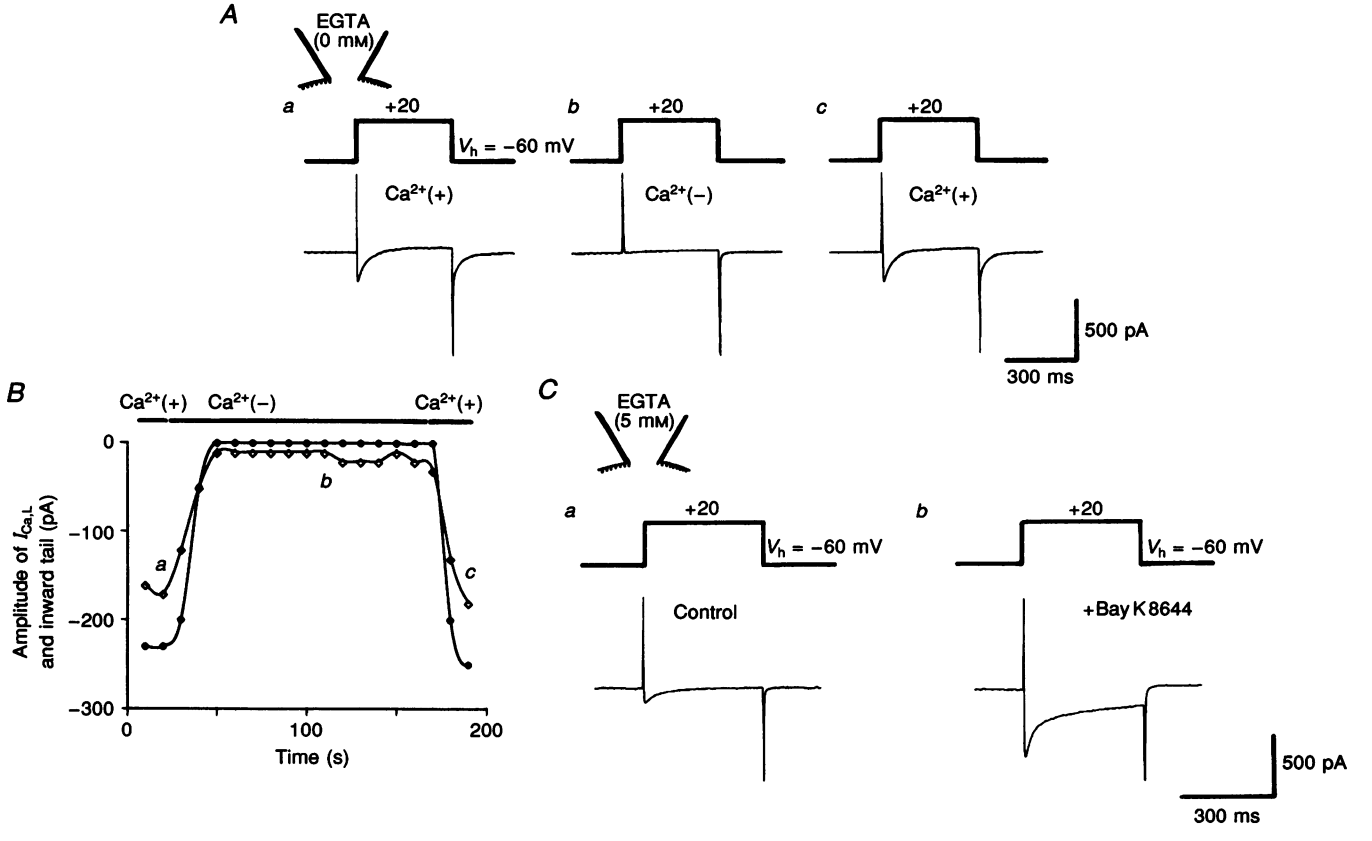

Figure 6. Effect of extracellular Ca^{2+} or EGTA in the pipette solution on the Ca^{2+}-activated current

A, effects of extracellular Ca^{2+}. The cell was held at −60 mV and command voltage steps to +20 mV were applied at 0·1 Hz (*a* and *c*). When Ca^{2+} was absent from the bathing solution (*b*), the voltage-dependent L-type Ca^{2+} current ($I_{Ca,L}$) was abolished. The time courses of amplitude of $I_{Ca,L}$ (●) and inward tail current (◇) are shown in the presence or absence of extracellular Ca^{2+} (*B*). *C*, with 5 mM EGTA in the patch pipette, Bay K (500 nM) markedly enhanced $I_{Ca,L}$ but did not induce Ca^{2+}-activated currents at all.

Since Ba^{2+} is known to pass through the $I_{Ca,L}$ channel, an experiment was conducted in which Ba^{2+} was substituted for Ca^{2+} (Fig. 7). In Ca^{2+}-Tyrode solution, Bay K 8644 (500 nM) significantly increased $I_{Ca,L}$ and Ca^{2+}-activated currents (Fig. 7*A b*). When Ba^{2+} (5 mM) was substituted for Ca^{2+}, the amplitude of $I_{Ca,L}$ was increased and its inactivation was slowed (Fig. 7*A c*), while stimulation of the Ca^{2+}-activated current was completely abolished (Fig. 7*A c*). The *I–V* relationships of $I_{Ca,L}$ in both Ca^{2+}- and Ba^{2+}-Tyrode solution are presented in Fig. 7*B*. In Ba^{2+}-Tyrode solution, the components of the Ca^{2+}-activated currents activated during the command pulse disappeared completely (Fig. 7*B*). Thus, it is likely that Ba^{2+} was unable to induce the Ca^{2+}-activated currents. The dependence of the Ca^{2+}-activated inward tail currents on $[Ca^{2+}]_o$ was further examined as shown in Fig. 7*C*. It would be expected that increasing the duration of the pulse would result in an increase in the amount of Ca^{2+} entering the cell through the Ca^{2+} channels, resulting in an

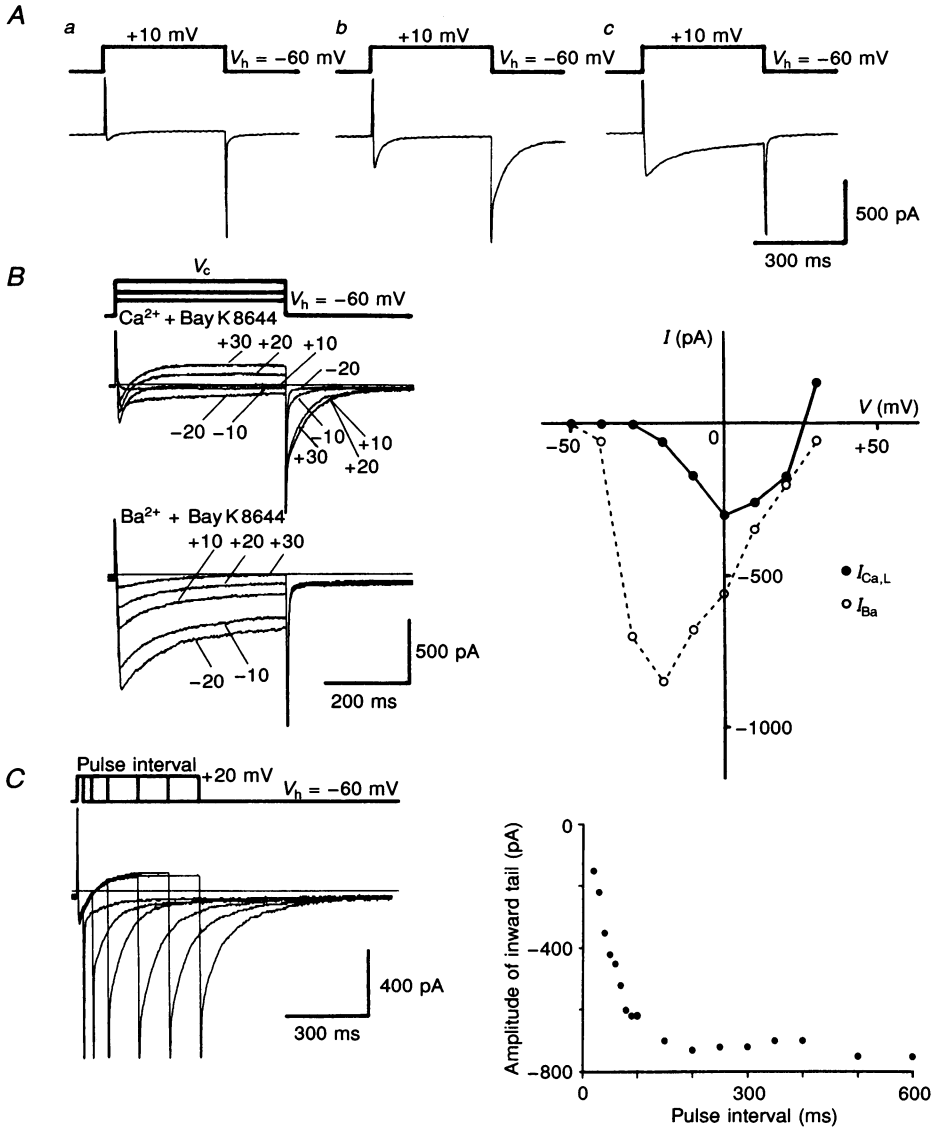

Figure 7. Effects of substitution of Ba^{2+} for Ca^{2+} and increasing the duration of the depolarizing voltage steps on Ca^{2+}-activated currents in TSMCs

A, effect of substitution of Ba^{2+} for Ca^{2+} on Ca^{2+}-activated currents in TSMCs. *A*, cell was held at −60 mV and command voltage pulses to +20 mV were applied. The original current traces are presented for the control Ca^{2+}-containing solution (*a*), the addition of Bay K 8644 (500 nM) (*b*) and in Ba^{2+}-containing solution in the presence of Bay K 8644 (*c*). Note that when Ba^{2+} was substituted for Ca^{2+}, the inactivation of $I_{Ca,L}$ was slowed and the inward tail current was completely abolished. *B*, original current traces in the presence of Bay K 8644 (500 nM) in both Ca^{2+}- and Ba^{2+}-containing solutions are indicated at various command pulses. The *I–V* relationships measured at the initial peak are shown for each solution. *C*, effects of increasing the duration of the depolarizing voltage step. The cell was held at −60 mV and command pulses with different pulse intervals (20–600 ms) were applied.

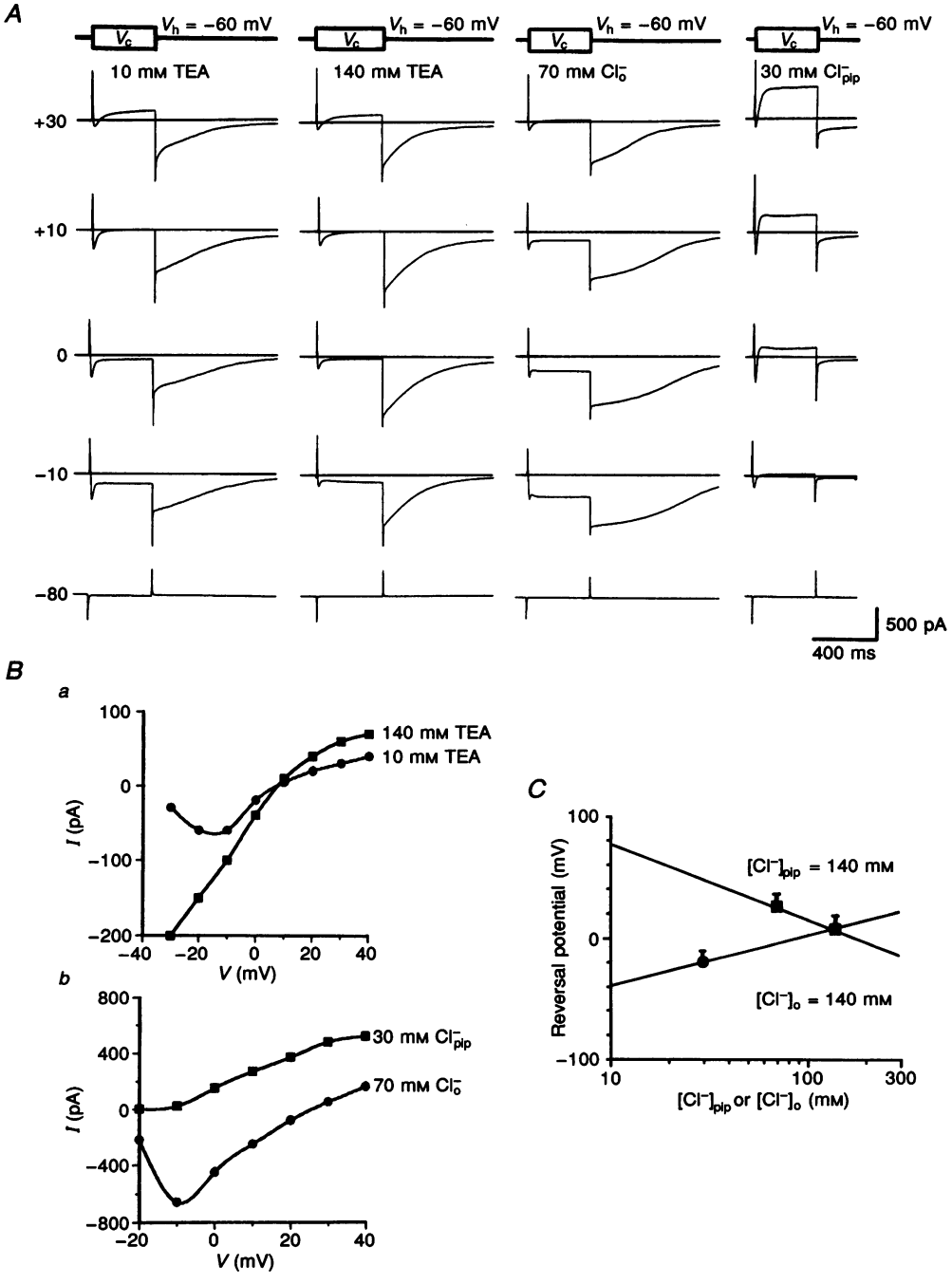

Figure 8. Effects of substituting TEA^+ for extracellular Na^+ and various external or internal $[Cl^-]$ on the E_{rev} of Ca^{2+}-activated currents

A, effects of substituting TEA^+ for extracellular Na^+ and various external or internal $[Cl^-]$ on Ca^{2+}-activated currents. The cell was held at −60 mV and command steps were applied to various membrane potentials. *B*, effects of substituting TEA^+ for extracellular Na^+ and various external or internal $[Cl^-]$ on Ca^{2+}-activated currents. The *I–V* relationships measured at the end of the pulse after the leakage current was subtracted are shown. The reversal potential in control and TEA-containing solutions was approximately +5 mV in this case (*a*). Also, the reversal potential was −20 mV in 30 mM Cl^-_{pip} and +24 mV in 70 mM Cl^-_o in *b*. *C*, relationship between the reversal potential of Ca^{2+}-activated currents and extracellular (■) or pipette (●) concentration of Cl^-.

increase in $[Ca^{2+}]_i$. Therefore, the results from an experiment in which the duration of the depolarizing pulse was increased from 20 to 600 ms are shown in Fig. 7*C*. The envelope of the tail currents indicates a progressive increase in the amplitude of the inward tail currents as the pulse interval is increased. The maximum inward tail current was observed at about 100–200 ms (Fig. 7*C*, right panel).

To elucidate the ionic selectivity of these Ca^{2+}-activated currents, the reversal potential (E_{rev}) was measured. Figure 8 presents the results of an experiment in which extracellular Na^+ was totally replaced with TEA^+. The original current traces in the controls (10 mM TEA) and TEA solution (140 mM TEA) and its corresponding *I–V* relationship at the steady state are presented in Fig. 8*A* and *Ba*. The E_{rev} was estimated to be +4 mV ± 6 mV ($n = 6$) in the control solution and +5 ± 6 mV ($n = 5$) in the TEA solution. Thus, the replacement of Na^+ with TEA^+ did not significantly alter E_{rev}, indicating that Na^+ was not a main charge carrier, and that the Na^+–Ca^{2+} exchange current was also not involved. Figure 8*A* and *Bb* show the current records and the corresponding *I–V* relationship in experiments where the $[Cl^-]_o/[Cl^-]_{pip}$ were 70 mM/140 mM (Fig. 8*A a*) and 140 mM/30 mM (Fig. 8*B b*), respectively. The E_{rev} in 70 mM $[Cl^-]_o$/140 mM $[Cl^-]_{pip}$ was +21 ± 8 mV ($n = 5$), while that in 140 mM $[Cl^-]_o$/30 mM $[Cl^-]_{pip}$ was −23 ± 8 mV ($n = 4$). Figure 8*C* illustrates the relationship between E_{rev} and $[Cl^-]_o$ or $[Cl^-]_{pip}$. The E_{rev} of the current changed by about −56 mV with a 10-fold change in $[Cl^-]_o$, and by about 44 mV with a 10-fold change in $[Cl^-]_{pip}$. These values may be sufficient to bring about the expected alterations in E_{rev} for the Cl^--selective ion channel. To confirm this, the actions of the chloride channel blocker, DIDS, on Ca^{2+}-activated currents were investigated (Fig. 9). The *I–V* relationships in controls and in the presence of DIDS are illustrated in Fig. 9*B*. DIDS (1 mM) only slightly decreased $I_{Ca,L}$ (from −400 to −300 pA), but it dramatically suppressed the Ca^{2+}-activated currents. Niflumic acid (10 μM) also suppressed the currents. These results are compatible with the notion that the Ca^{2+}-activated currents were also Cl^- currents.

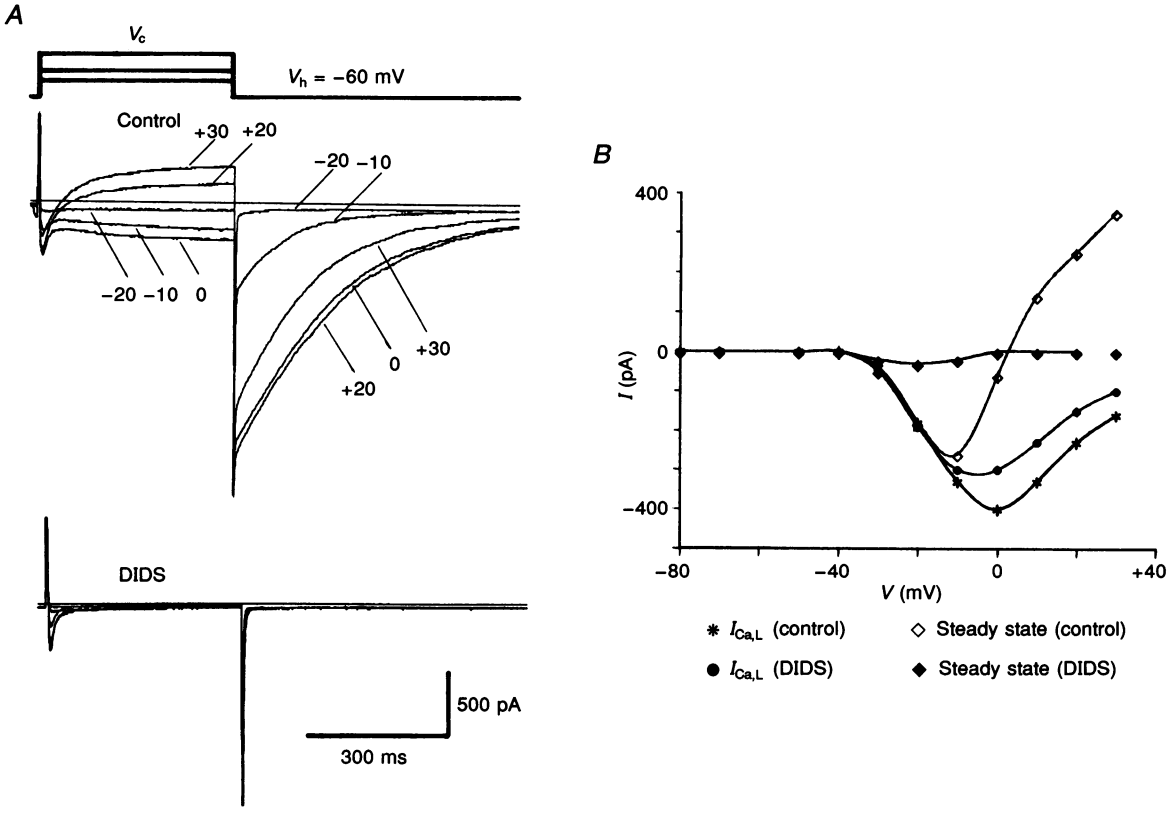

Figure 9. Effects of DIDS on the $I_{Cl(Ca)}$ activated by Ca^{2+} currents in TSMCs

The cell was held at −60 mV and various membrane potentials were applied at 0·1 Hz. *A*, original current traces for controls and in the presence of DIDS (1 mM). *B*, *I–V* relationships measured at the initial peak and at the end of the pulse.

NKA inhibits $I_{Cl(Ca)}$ evoked by Ca^{2+} influx due to $I_{Ca,L}$

The above results indicate that Cl^- currents in TSMCs can be activated by $I_{Ca,L}$, as has been reported for other smooth muscle cell types (Pacaud *et al.* 1989; Akbarali & Giles, 1993). Subsequently, we investigated the effects of NKA on $I_{Cl(Ca)}$ evoked by Ca^{2+} currents ($I_{Ca,L}$). Figure 10 illustrates a typical experiment. Without EGTA in the pipette solution, the cell was held at −60 mV, and command voltage pulses to +20 mV were applied at 0·1 Hz. When NKA (1 μM) was added to the bath, the transient activation of Cl^- currents reflecting Ca^{2+} release from the storage sites was recorded. Following this, NKA markedly inhibited both the inward tail current and outward Cl^- currents elicited during depolarizing pulses. NKA (1 μM) also decreased $I_{Ca,L}$ from −720 to −600 pA in this cell. The *I–V* relationships revealed that $I_{Ca,L}$ was decreased slightly at any given voltage pulse without any change in voltage threshold. The $I_{Ca,L}$ current maximum was decreased by −15 ± 6% in the presence of NKA (1 μM), a finding which also supports the observation that the transient activation of Cl^- currents by NKA was not due to the enhancement of $I_{Ca,L}$. In addition, although NKA inhibited $I_{Ca,L}$, the inhibitory effect of NKA on $I_{Ca,L}$

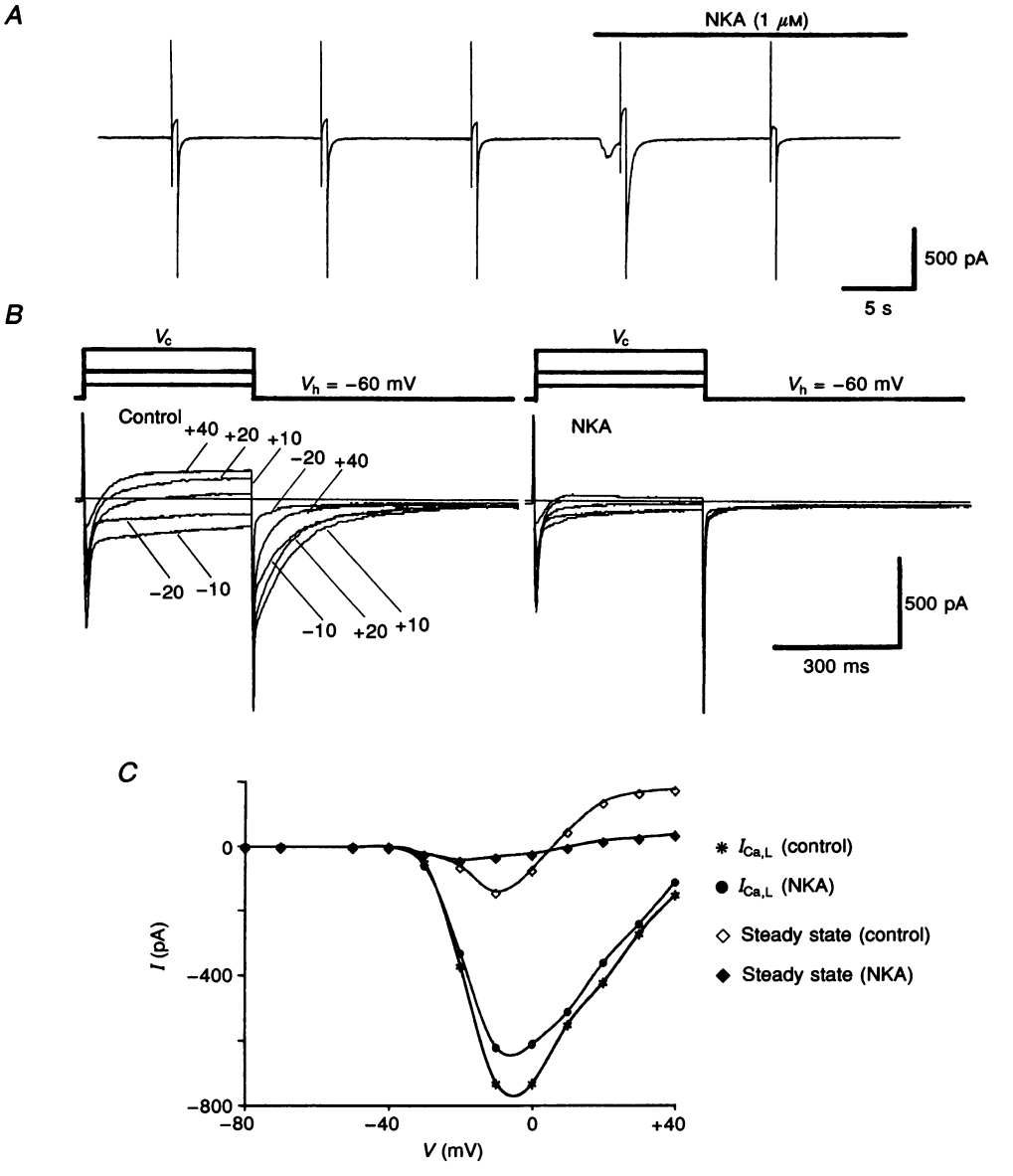

Figure 10. Effects of NKA on the $I_{Cl(Ca)}$ evoked by Ca^{2+} currents in single TSMCs

The cell was held at −60 mV and command steps to +20 mV were applied at 0·1 Hz. *A*, original current traces. Note that NKA activated a transient inward current. *B*, currents evoked by various depolarizing pulses for control and after the application of NKA (1 μM). *C*, *I–V* relationships measured at the initial peak and at the end of the pulse for controls and in the presence of NKA.

was relatively small, compared with that on Cl^- currents, suggesting that NKA may inhibit $I_{Cl(Ca)}$ activated by $I_{Ca,L}$, possibly due to the Ca^{2+} release from storage sites, followed by inhibiting the CICR from Ca^{2+} storage sites.

The effects of caffeine (30 mM) on $I_{Cl(Ca)}$ elicited by $I_{Ca,L}$ were also investigated (Fig. 11). Caffeine (10 mM) transiently activated $I_{Cl(Ca)}$ at a holding potential of −60 mV. Caffeine then markedly inhibited $I_{Cl(Ca)}$ activated during a command pulse of +20 mV, and the inward tail Cl^- current in a similar manner to NKA. Caffeine (30 mM) also reduced the $I_{Ca,L}$ current by $-30 \pm 5\%$ ($n = 5$).

Effects of intracellular Cl^- concentration on action potentials in a Cs^+-loaded cell

The above results indicate that $I_{Cl(Ca)}$ was activated by Ca^{2+} influx due to the voltage-dependent L-type Ca^{2+} channels. Therefore, to clarify whether the Cl^- current actually affects the action potential configurations in TSMCs, the effects of intracellular Cl^- concentration and DIDS on the action potentials were investigated as shown in Fig. 12. The patch pipette contained the Cs^+ internal solution, and action potentials were elicited from −60 mV at a stimulation of 0·1 Hz. With 40 mM Cl^- in the patch pipette (Fig. 12*A a*), the spike was followed by a long-lasting positive after-

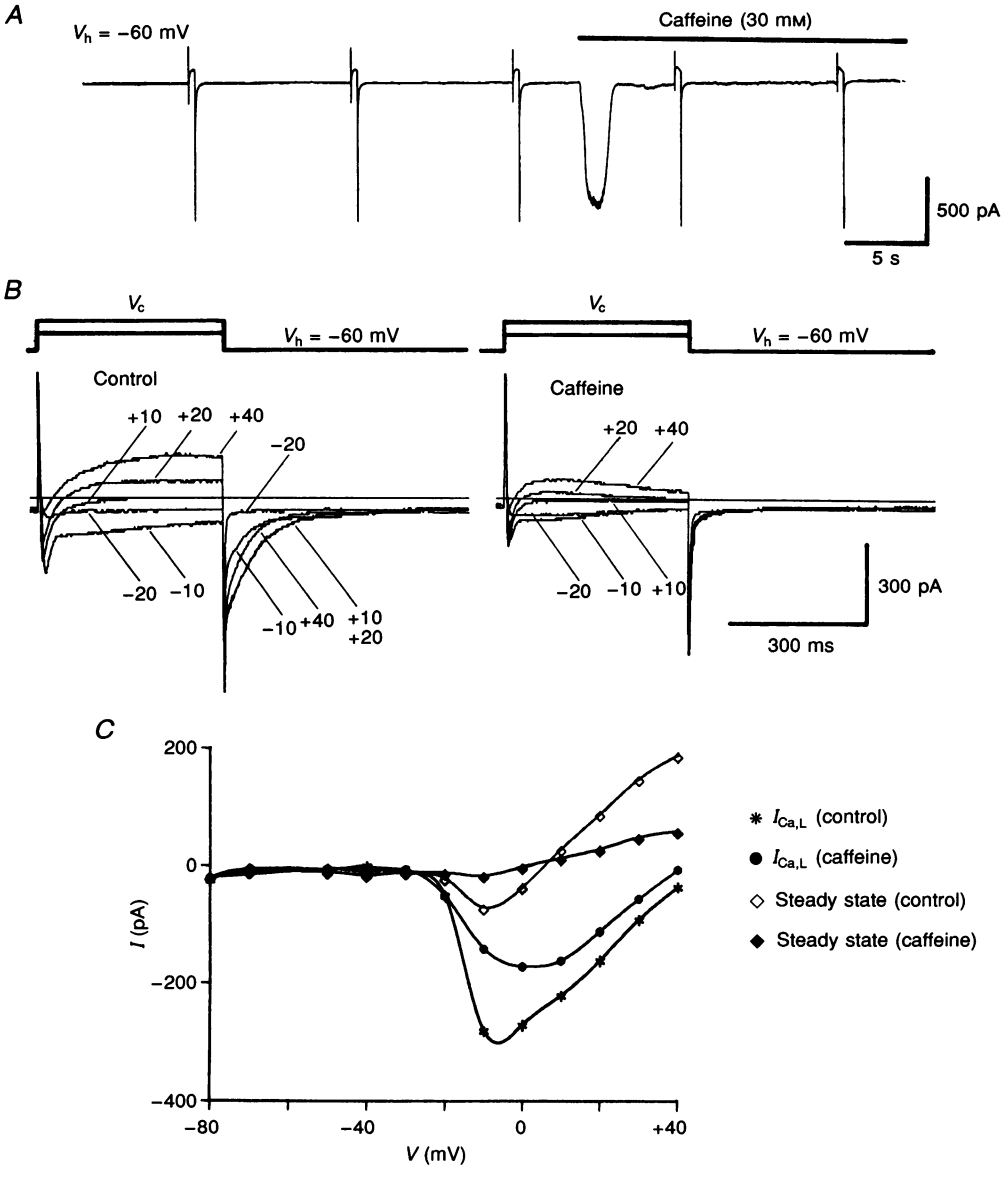

Figure 11. Effects of caffeine on the $I_{Cl(Ca)}$ evoked by Ca^{2+} current in single TSMCs

The cell was held at −60 mV and command steps to +20 mV were applied at 0·1 Hz. *A*, original current traces. *B*, currents evoked by various depolarizing pulses for control and after the application of caffeine (30 mM). *C*, *I–V* relationship measured at the initial peak and at the end of the pulse.

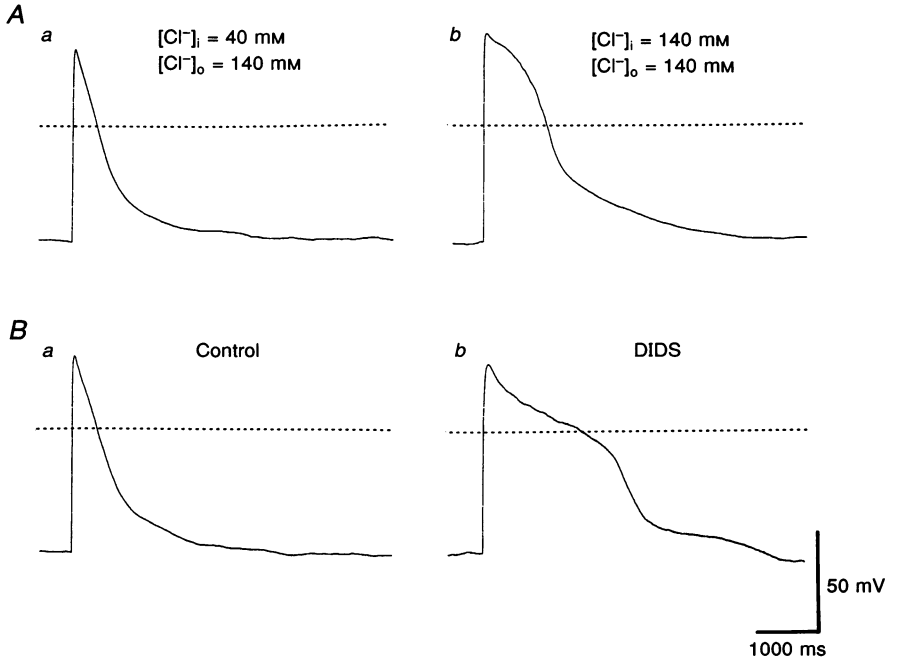

Figure 12. Effects of $[Cl^-]_i$ and DIDS on the action potentials in single TSMCs

A, influence of intracellular Cl^- concentration on the action potentials in single TSMCs. The action potentials were elicited at a stimulation of 0·1 Hz from a membrane potential of −60 mV. The patch pipette contained 40 mM CsCl (*a*) or 140 mM CsCl (*b*) in the patch pipette. *B*, effect of DIDS (1 mM) on the action potential. The patch pipette contained 40 mM CsCl.

potential and the action potential duration measured at 50% repolarization (APD_{50}) was 520 ± 110 ms ($n = 4$). When the Cl^- concentration in the patch pipette was increased to 140 mM, the action potential duration was markedly prolonged (1500 ± 600 ms in APD_{50}) (Fig. 12*A b*). In addition, with 40 mM Cl^- in the patch pipette, DIDS (1 mM) dramatically prolonged the action potential duration (470 ± 50 ms ($n = 3$) in control and 2200 ± 300 ms ($n = 3$) in the presence of DIDS as shown in Fig. 12*B*. These results suggest that $I_{Cl(Ca)}$ may contribute to the formation of the after-potential and plateau of the action potentials in TSMCs.

DISCUSSION

The major findings of the present study are as follows. (1) $I_{Cl(Ca)}$ was activated by SR Ca^{2+} release due to NKA or caffeine (through IP_3 or ryanodine receptors) as well as by Ca^{2+} influx due to the voltage-dependent L-type Ca^{2+} currents in single TSMCs. (2) The activation of $I_{Cl(Ca)}$ by NKA may be due to the production of IP_3 through PTX-insensitive GTP-binding proteins, which releases Ca^{2+} from the SR. (3) Since ryanodine inhibited the activation of $I_{Cl(Ca)}$ induced by both caffeine and NKA, IP_3-sensitive Ca^{2+} storage sites may be the same as, or closely correlated to the ryanodine-sensitive sites in single TSMCs. (4) The CICR mechanism may be partly involved in the activation of $I_{Cl(Ca)}$ by Ca^{2+} influx due to $I_{Ca,L}$. (5) The activation of Cl^- currents may play an essential role in regulating action potentials and thus muscle tone in single TSMCs.

NKA and caffeine activate Cl^- currents in single TSMCs

Many contractile agonists cause rapid depolarization of the membrane, usually through the activation of either non-selective cation channels or Cl^- channels in a variety of smooth muscle cells (Benham, Bolton & Lang, 1985; Byrne & Large, 1988; Amédée *et al.* 1990; Loirand *et al.* 1990; Inoue & Isenberg, 1990; Janssen & Sims, 1992; 1993; 1994). With the Cs^+ pipette solution, the NKA-induced current reversed in accordance with the expected alteration of the E_{rev} for a Cl^--sensitive ion channel and was completely inhibited by a Cl^- channel blocker, DIDS. Therefore, NKA may activate a Cl^- channel current in TSMCs, which is compatible with the previous paper (Nakajima *et al.* 1995). Similarly, Janssen & Sims (1992, 1993, 1994) have reported that ACh, histamine and substance P activated Cl^- currents in guinea-pig TSMCs.

An increase in $[Ca^{2+}]_i$ appears to be responsible for the NKA induction of the Cl^- current since an increase in EGTA in the pipette solution from 0·15 to 5 mM abolished the current and caffeine evoked a current similar to NKA. Thus, NKA may cause the release of Ca^{2+} from intracellular storage sites as in the cases for ACh and histamine

in TSMCs (Janssen & Sims, 1992, 1993). Since the NKA induction of Cl^- currents was inhibited by GDPβS and potentiated by GTPγS added to the pipette solution, GTP-binding proteins may be involved in the NKA induction of $[Ca^{2+}]_i$-activated Cl^- currents in TSMCs. In colonic smooth muscle cells using the single channel recordings, it has been shown that the neurokinin 1 receptor agonist, substance P methylester, activates Cl^- channels via GTP-binding proteins, probably in a membrane-delimited manner (Sun *et al.* 1992). This mechanism may not be involved in the NKA action in TSMCs since 5 mM EGTA or heparin (IP_3 receptor antagonist) in the pipette abolished the response. Since tachykinins, such as substance P, induce inositol phosphoinositide hydrolysis in various kinds of smooth muscle cells including trachea (Bristow, Suman-Chauhan & Watling, 1987; Grandordy, Frossard, Rhoden & Barnes, 1988), these results suggest that the production of IP_3 through NKA receptors, which releases Ca^{2+} from storage sites, may be involved in the activation of Cl^- currents by NKA. PTX, which ADP-ribosylates GTP-binding proteins (G_i or G_o), could not inhibit the activation of Cl^- currents by NKA, suggesting that PTX-insensitive G proteins are involved in the activation of Cl^- currents by NKA.

Relationship between NKA (IP_3 receptor)- and caffeine (ryanodine receptor)-sensitive Ca^{2+} stores in TSMCs

It is known that caffeine releases ryanodine-sensitive intracellular Ca^{2+} from smooth muscle cells (Iino, 1989); however, uncertainty remains as to the degree of overlap of the intracellular Ca^{2+} sources mobilized by neurotransmitters in TSMCs. In rabbit mesenteric artery, noradrenaline only partially depletes the caffeine-sensitive store (Haeusler, Richards & Thorens, 1981). In porcine coronary artery, and guinea-pig taenia coli (Casteels & Raeymaekers, 1979; Itoh, Kahiwara, Kitamura & Kuriyama, 1982), ACh appears to release Ca^{2+} from an additional caffeine-insensitive store. On the other hand, in rabbit aorta (Leijten & van Breemen, 1984), a complete overlap exists between the caffeine and noradrenaline-sensitive Ca^{2+} stores. The present study provides evidence that there is a relationship between NKA- (IP_3) and caffeine-sensitive Ca^{2+} stores in TSMCs. In Ca^{2+}-free bathing solution, caffeine and NKA elicited Cl^- currents, but the second application of caffeine or NKA could not activate Cl^- currents, suggesting that these agents release Ca^{2+} from the SR and that Ca^{2+} stores had been depleted in Ca^{2+}-free solution during the first application of these agents. In addition, after Ca^{2+} had been depleted by caffeine in Ca^{2+}-free bathing solution, the application of NKA did not show any response, suggesting that an overlap may exist between the caffeine- and NKA- (IP_3 receptor) sensitive Ca^{2+} stores in guinea-pig TSMCs. Similar findings were also reported for noradrenaline- and caffeine-activated Cl^- currents in rabbit ear artery (Amedee *et al.* 1990).

Ryanodine has been shown to activate Ca^{2+}-release channels and deplete Ca^{2+} from SR membranes, thereby inhibiting caffeine-induced Ca^{2+}-release from the SR (Ito *et al.* 1986; Kanmura *et al.* 1988). Actually, following the application of ryanodine (50 μM) in the presence of caffeine (10 mM) for approximately 5 min, the effects of caffeine on Cl^- currents were completely abolished. Ryanodine also abolished the activation of Cl^- currents by NKA. These results provide an additional evidence that the NKA-sensitive (IP_3-sensitive) Ca^{2+} storage sites may be identical to or closely correlated with the caffeine-sensitive (ryanodine-sensitive) sites in guinea-pig TSMCs.

$I_{Cl(Ca)}$ is activated by an increase in $[Ca^{2+}]_i$ due to the $I_{Ca,L}$ in single TSMCs

In addition to the transient activation of $I_{Cl(Ca)}$ by NKA or caffeine, the present studies show that $I_{Cl(Ca)}$ was also activated by an increase in intracellular Ca^{2+} due to $I_{Ca,L}$ as reported in various kinds of cells, including vascular or oesophageal smooth muscle cells (Pacaud *et al.* 1989; Akasu, Nishimura & Tokimasa, 1990; Zygmunt & Gibbons, 1991; Akbarali & Giles, 1993). The following results support this notion. (1) The Ca^{2+}-activated current was activated without EGTA in the pipette and enhanced by Bay K 8644. In contrast, it was abolished when Ca^{2+} was absent in the bath solution, or blocked by Cd^{2+} or nifedipine. (2) The reversal potential of the currents changed with the external or internal Cl^- concentration as predicted by the Nernst equations, but was not altered when Na^+ was replaced by TEA^+. Thus, these Ca^{2+}-activated currents are neither Na^+–Ca^{2+} exchange currents nor non-selective cation currents, because TEA^+ cannot be the charge carrier in both currents (Giles & Shimoni, 1989). (3) Cl^- channel blockers such as DIDS or niflumic acid inhibited the Ca^{2+}-activated currents.

In other smooth muscle cells, contractile agonists such as noradrenaline and substance P have been reported to enhance $I_{Ca,L}$ (Clapp *et al.* 1989; Loirand *et al.* 1990). In gastric smooth muscle cells, Clapp *et al.* (1989) reported that substance P, as well as ACh, enhances $I_{Ca,L}$. The present study indicates that NKA inhibits $I_{Ca,L}$ only slightly in TSMCs. As caffeine also inhibited Ca^{2+} currents, the inhibitory effect of NKA on Ca^{2+} current may be due to a Ca^{2+}-dependent inactivation process (Pacaud, Loirand, Mironneau & Mironneau, 1987). Thus, since NKA inhibited Ca^{2+} currents in TSMCs, it is unlikely that the voltage-dependent Ca^{2+} channels are involved in NKA-activated Cl^- currents.

The Ca^{2+} influx due to the Ca^{2+} channels can release Ca^{2+} from ryanodine-sensitive storage sites (CICR mechanism) in cardiac myocytes (Endo, 1977; Zygmunt & Gibbons, 1991). Similarly, the CICR mechanism has been reported in smooth muscle cells (Iino, 1989). Though NKA and caffeine inhibit Ca^{2+} currents in TSMCs, the inhibitory actions of

these agents on $I_{Cl(Ca)}$ activated by Ca^{2+} currents may be due to Ca^{2+}-release and Ca^{2+}-depletion from storage sites, then followed by the inhibition of the CICR from Ca^{2+}-storage sites. As indicated in Figs 10 and 11, after NKA or caffeine transiently activated Cl^- currents by releasing Ca^{2+} from IP_3- or ryanodine-sensitive Ca^{2+} storage sites, $I_{Cl(Ca)}$ was markedly suppressed, but not completely abolished. Thus, these findings provide the evidence that CICR from Ca^{2+} storage sites may be partly involved in the activation of Cl^- currents evoked by Ca^{2+} current in TSMCs.

Physiological significance of the $I_{Cl(Ca)}$ in TSMCs

The present studies indicate that $I_{Cl(Ca)}$ is activated by Ca^{2+} influx through the Ca^{2+} channels as well as NKA or caffeine. Guinea-pig trachealis muscle shows slow waves in the membrane potential and generates spontaneous fluctuations of muscle tone (Small *et al.* 1982). Since slow-wave discharge in airway smooth muscle is potential dependent (Kirkpatrick, 1981), it is likely that the membrane depolarization due to the activation of $I_{Cl(Ca)}$ may affect spontaneous fluctuations of tracheal smooth muscle tones. Also, in Cs^+-loaded TSMCs, where K^+ conductances were inhibited, action potentials were easily elicited as shown in Fig. 12. The action potential shapes were: (1) dramatically influenced by the concentration of Cl^- ions in the patch pipettes, and (2) affected by DIDS, suggesting that the activation of Cl^- currents may alter the action potential duration and consequently alter Ca^{2+} influx in TSMCs. Though further investigation will be required to elucidate the physiological role of the $I_{Cl(Ca)}$ in TSMCs, the alterations in electrical activities of airway smooth muscle elicited by activation of Cl^- currents may play a role in regulating tracheal smooth tone, especially under the conditions where K^+ conductances are blocked by neurotransmitter such as NKA and ACh (Janssen & Sims, 1992; Nakajima *et al.* 1995).

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