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Characteristics of caffeine-induced and spontaneous inward currents and related intracellular Ca^{2+} storage sites in guinea-pig tracheal smooth muscle cells

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Abstract

Characteristics of caffeine-induced inward current (I_{caf}) and spontaneous transient inward current were examined in single smooth muscle cells isolated from guinea-pig trachea. When a pipette solution contained mainly CsCl, an application of 10 mM caffeine elicited transient I_{caf} at a holding potential of −60 mV. Spontaneous transient inward currents were recorded in about 15% of cells examined and were abolished by caffeine. Both were Cl^- current activated by an increase in intracellular Ca^{2+} concentration ($I_{Cl\text{-}Ca}$). When 10 mM caffeine was puff-applied twice with various intervals, the amplitude of the second I_{caf} depended upon the period of the interval. The relationship between the amplitude and the interval represents the recovery time course of I_{caf}, which was significantly slowed by 30 μM cyclopiazonic acid. The I_{caf} was not significantly affected by addition of Cd^{2+}. Removal of external Ca^{2+} did not affect the first I_{caf} but markedly reduced the second one, regardless of the presence of Cd^{2+}. In conclusion, I_{caf} is evoked by activation of $I_{Cl\text{-}Ca}$ via Ca^{2+} release. The recovery time course of I_{caf} indicates the refilling of Ca^{2+} storage sites by the cyclopiazonic acid-sensitive Ca^{2+} pump. The refilling at −60 mV depends strongly upon Ca^{2+} influx through the Cd^{2+}-insensitive pathway. Spontaneous transient inward currents may be also due to $I_{Cl\text{-}Ca}$ activated by spontaneous Ca^{2+} release from local storage sites.

Keywords: Smooth muscle cell; Trachea; Caffeine; Ca^{2+}-dependent Cl^- channel; Sarcoplasmic reticulum; Ca^{2+} pump

1. Introduction

Caffeine increases Ca^{2+} sensitivity of Ca^{2+}-releasing channels for Ca^{2+}-induced Ca^{2+} release in endoplasmic and sarcoplasmic reticulum and, therefore, has been widely used as a convenient pharmacological tool to investigate Ca^{2+}-induced Ca^{2+} release mechanisms and the characteristics of the Ca^{2+} storage sites in various types of cells including smooth muscle cells (see as a review, Sawynok and Yaksh, 1993). A great advantage of using caffeine as a Ca^{2+} releaser is that the compound is membrane permeable, can be removed easily by washout and, therefore, is far easier to handle than inositol 1,4,5-trisphosphate (IP_3) as a compound inducing Ca^{2+} release directly. Moreover, the results obtained using caffeine are more simply interpreted than those obtained from receptor-operated Ca^{2+} mobilization, even though caffeine has some additional effects on ionic channels (Hughes et al., 1990; Ganitkevich and Isenberg, 1992) and cAMP phosphodiesterase (Butcher and Sutherland, 1962).

It has been reported that application of caffeine elicits a transient outward current which is due to activation of the Ca^{2+}-dependent K^+ current by Ca^{2+} release from intracellular Ca^{2+} stores at a holding potential near the resting membrane potential in several types of smooth muscle cells (Benham and Bolton, 1986; Suzuki et al., 1992; Ohta et al., 1993). Stimulation by an agonist via receptor activation has been demonstrated to elicit inward currents, in addition to outward currents, in smooth muscle cells of rabbit ear artery (Amédée et al., 1990), rabbit and rat portal vein (Byrne and Large, 1988; Pacaud et al., 1989), guinea-pig trachea (Janssen and Sims, 1992, 1993b). These inward currents are due to activation of Ca^{2+}-dependent Cl^-

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and/or nonspecific cationic channels resulting from formation of IP_3 via receptor-linked phospholipase C activation and subsequent Ca^{2+} release from intracellular stores.

In portal vein (Wang et al., 1992) and pulmonary artery (Wang and Large, 1993) of the rabbit, spontaneous transient inward currents have been recorded. It has been suggested that spontaneous transient inward currents are generated by the activation of Ca^{2+}-dependent Cl^- channels resulting from cyclical spontaneous Ca^{2+} release from Ca^{2+} storage sites in a similar way to spontaneous transient outward currents (Benham and Bolton, 1986). Characteristics of spontaneous transient inward currents in tracheal smooth muscle cells have not been examined yet.

The present study was undertaken to determine the characteristics of the caffeine-induced inward current and also spontaneous transient inward currents, if observed, in tracheal smooth muscle cells. The relationship between intracellular Ca^{2+} storage sites and Ca^{2+}-dependent inward currents at near the resting membrane potential was examined. Moreover, the refilling process of caffeine-sensitive Ca^{2+} storage sites by Ca^{2+} influx and the Ca^{2+} pump, which may be strongly correlated with the activation of spontaneous transient inward currents, was examined by measuring the caffeine-induced inward current as a monitor of the caffeine-releasable amount of Ca^{2+} in storage sites.

2. Materials and methods

2.1. Cell isolation

Male Hartley guinea-pigs, weighing about 200 g, were killed by a blow to the head. The trachea was isolated. Single cells were dispersed using collagenase and papain as described previously (Imaizumi et al., 1989). Only relaxed, spindle-shaped cells which had a length over 100 μm were used for electrical recordings. These cells showed a good contractile response to 10 μM acetylcholine or histamine. The size of the cells was similar to that reported for isolated cells from guinea-pig trachea (Janssen and Sims, 1992).

2.2. Electrophysiological measurements

Standard whole-cell voltage clamping (Hamill et al., 1981) was performed using an EPC7 (List, Germany) or a CEZ-2300 (Nihon-koden, Japan) amplifier. In some experiments, the nystatin-perforated patch method was used (Horn and Marty, 1988). Current and voltage signals were stored on videotape using a PCM system and were later captured on an IBM computer using DA2801A as analog-digital converter and an acquisition program for precise analysis as described previously (Imaizumi et al., 1990). All experiments were carried out at 23 ± 2°C.

Caffeine was applied by an exchange of bathing solution or micro-application. Micro-application of caffeine was performed by a puff with pressure ejection from a pipette filled with the extracellular medium plus caffeine. The tip diameter of the pipette for the puff application was about 50 μm. The pipette was always placed downstream of the medium flow and at < 100 μm from a target cell. Since the tip diameter of puff pipettes was large, the concentration of caffeine around the cell reached very close to that in the pipette during a puff for 5 s. The change in holding current induced by puff application of 40 mM K^+ occurred within 100 ms and was exactly the same as that induced by rapidly changing the external solution.

2.3. Solutions

The physiological salt solution for electrophysiological experiments contained (mM): NaCl 137, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.2, glucose 10, Hepes 10. The pH was adjusted to 7.2 using NaOH. A solution for dispersing cells was prepared by omitting Ca^{2+} and Mg^{2+} from the physiological salt solution. The pipette solution for electrical recordings contained (mM): CsCl 140, $MgCl_2$ 4, ATP-2Na 5, EGTA 0.05, Hepes 10. To reduced the Cl^- concentration in the pipette solution, 50% of 140 mM CsCl was replaced with Cs aspartate. The pipette solution for the nystatin-perforated patch method contained (mM): CsCl 140, $MgCl_2$ 4, nystatin (200 ~ 600 μg/ml). Nystatin was dissolved in dimethyl sulfoxide (DMSO) before use (Horn and Marty, 1988). To record total membrane currents, including caffeine-induced outward current, cesium ions were replaced by equimolar potassium ions. The pH in these solutions was adjusted to 7.2 using KOH.

2.4. Chemicals

Caffeine, acetylcholine, histamine, forskolin, CsCl, $CdCl_2$, $CoCl_2$, ryanodine, choline chloride, dithiothreitol, dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical (Osaka, Japan). Cyclopiazonic acid, thapsigargin, adenosine trisphosphate disodium salt (ATP), nystatin, niflumic acid, CsOH, aspartic acid, papain, 4,4,-diisothiocyanatostilbene-2,2,-disulfonic acid (DIDS), 8-bromo-cAMP were obtained from Sigma (St. Louis, MO, USA). Bovine serum albumin (Miles, fraction V), collagenase (500 unit/mg Yakult, Tokyo, Japan), EGTA (Dojin Kagaku, Kumamoto, Japan), tetraethylammonium chloride (Tokyo Kasei, Tokyo, Japan), atropine sulfate (Nakalai Tesque, Kyoto, Japan) were from the companies shown in parentheses.

2.5. Statistics

Results are given as means ± S.E.M. and were compared using Student's *t*-test for paired or unpaired experiments. $P < 0.05$ was considered significant, indicated in the figures by an asterisk; P values are presented after adjustment for multiple comparisons with the Bonferroni correction.

3. Results

3.1. Responses to caffeine in tracheal smooth muscle cells

When a pipette solution contained 140 mM KCl (see Materials and methods), application of 10 mM caffeine by an exchange of the external solution produced several types of responses depending upon holding potentials under whole-cell voltage clamping (Fig. 1). At a holding potential of −60 mV, mixed transient outward and inward currents, which occasionally recorded as oscillatory currents were observed in response to caffeine. The currents decayed during the presence of caffeine (Fig. 1A,a). Application of caffeine always caused cell contraction and its withdrawal induced relaxation. At a holding potential of 0 mV, however, caffeine evoked only an outward current which was also transient. Sometimes a transient outward current was followed by oscillating outward currents (Fig. 1A,b). When most of the K^+ in the pipette solution was replaced by cesium (see Materials and methods), only an inward current was recorded in response to 10 mM caffeine at holding potentials of −90, −60, and −30 mV (I_{caf}) (Fig. 1B). The current disappeared at around 0 mV and reversed to outward at +30 mV. The amplitude of the peak inward current recorded at −60 mV in 50 cells ranged from 50 to 2400 pA, with a mean amplitude of 585 ± 51 pA. In some cells, oscillating inward currents were also observed at −60 mV. The amplitude of these 'oscillations' decreased progressively with time. These caffeine-induced membrane currents were also observed in a similar manner when nystatin-perforated patch clamping was performed ($n = 8$; I_{caf} with a mean amplitude of 625 ± 81 pA).

Fig. 1C summarizes the relationship between concentrations of caffeine and the relative amplitude of I_{caf} elicited at a holding potential of −60 mV. The pipette solution contained mainly CsCl. The amplitude of I_{caf} elicited by 10 mM caffeine was taken as 1.0. The broken line indicates the mean amplitude of I_{caf} in 5 cells. It is notable, however, that the activation of I_{caf} in 5 individual cells did not occur in a graded manner with the increase in caffeine concentration but did so in an all-or-none manner. This result suggests that activation of I_{caf} may be a type of all-or-none response.

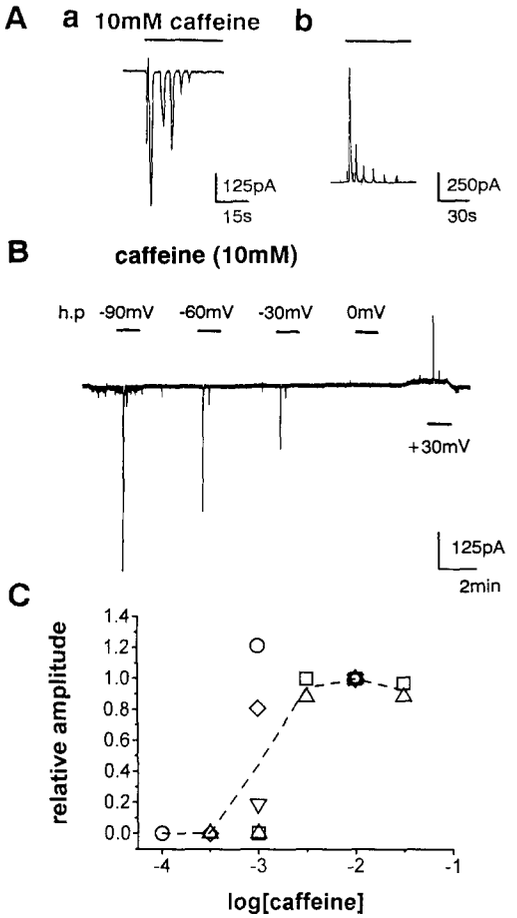

Fig. 1. Caffeine-induced outward and inward currents in single smooth muscle cells isolated from guinea-pig trachea. A: When a recording pipette was filled with KCl-rich solution, application of 10 mM caffeine elicited mainly an inward current at holding potential of −60 mV (A,a) and an outward current at 0 mV (A,b). Caffeine was applied by rapid exchange of bathing solution (< 2 s). Both inward and outward currents were transient and/or oscillatory. B: Current-voltage relationship of caffeine-induced current. The pipette solution contained mainly CsCl in order to block K^+ currents. Caffeine (10 mM) was applied for 60 s at five different holding potentials. Note that the current reversed at near 0 mV. C: Concentration-response relationship between caffeine and inward current at a holding potential of −60 mV. Symbols (○ □ △ ▽ ◇) indicate the peak amplitude of the inward current induced by caffeine at selected concentrations in five separate cells. The data were normalized, taking the peak amplitude induced by 10 mM caffeine as 1.0 in each cell. The broken line indicates mean relative amplitude of inward current in five cells tested. Note that the inward current was activated by caffeine in an all-or-none manner in each cell.

3.2. Ionic basis of I_{caf}

In order to investigate the contribution of Cl^- current to I_{caf}, reversal potential of I_{caf} was measured under conditions where the transmembrane Cl^- gradient was altered by replacing internal Cl^- with aspartate$^-$. Fig. 2A,a shows the protocol to measure the E_r of the I_{caf}. Cells were depolarized by paired pulses from a holding potential of −60 mV to 0 and +30 mV

with 150 ms duration, respectively, with an interpulse duration of 500 ms, once every 1.5 s. The reduction of internal Cl^- from 150 to 70 mM caused a change in E_r from -0.3 ± 0.3 ($n = 15$) to -23.1 ± 1.3 mV ($n = 4$) as shown in the current-voltage (I-V) relationship (Fig. 2A,b). Based upon internal Cl^- concentrations of 150 and 70 mM, the reversal potentials of Cl^- (E_{Cl}) are calculated to be 0 and -26 mV, respectively. These results indicate that I_{caf} appears to be mainly due to an increase in membrane Cl^- conductance.

In the next series of experiments, the contribution of Na^+ to I_{caf} was examined since the inward current may also have been due to an increase in conductance through a non-selective cationic channel. In these experiments, the extracellular Na^+ was replaced with equimolar tetraethylammonium$^+$ or choline$^+$. The E_r of I_{caf} was measured as shown in Fig. 2A. Fig. 2B,a and B,b show the I-V relationships of I_{caf} in the extracellular solution containing 126 mM tetraethylammonium$^+$ ($E_r = +2.6 \pm 0.9$ mV, $P > 0.05$ vs. control: $+0.7 \pm 0.4$ mV, $n = 5$) or choline$^+$ ($E_r = +0.1 \pm 0.2$ mV, $P > 0.05$, vs. control: -1.0 ± 1.1 mV, $n = 4$) in the presence of 3 μM atropine, respectively. Replacement of 126 mM Na^+ with equimolar tetraethylammonium$^+$ or choline$^+$ did not alter the amplitude and the E_r of I_{caf}, indicating that monovalent cations, especially Na^+, did not contribute to I_{caf}.

Moreover, to confirm that I_{caf} is mainly carried by Cl^-, the effects of niflumic acid and DIDS, chloride channel blockers, on I_{caf} were examined. The amplitude of I_{caf} induced by 10 mM caffeine in the presence of 10 μM niflumic acid or 1 mM DIDS in the external solution was 26.4 ± 5.4 and 27.7 ± 8.5% of the control, respectively ($n = 4$ for each, $P < 0.05$ vs. control). I_{caf} was abolished by 100 μM niflumic acid or 10 mM DIDS ($n = 2$ for each). These results also strongly suggest that I_{caf} is due to an increase in chloride conductance.

3.3. Dependence of I_{caf} on $[Ca^{2+}]_i$ and intracellular Ca^{2+} storage sites

Since caffeine releases Ca^{2+} from intracellular Ca^{2+} storage sites, I_{caf} may be due to activation of Ca^{2+}-dependent Cl^- channels by released Ca^{2+}. When the concentration of the calcium chelator, EGTA, in the pipette solution was raised from 0.05 mM to 10 mM, application of 10 mM caffeine failed to evoke the inward current ($n = 5$, not shown), indicating that elevation of $[Ca^{2+}]_i$ is required to elicit I_{caf}. The relation between I_{caf} and intracellular Ca^{2+} storage sites was examined further.

Ryanodine is a substance which keeps Ca^{2+}-releasing channels on the sarcoplasmic reticulum membrane

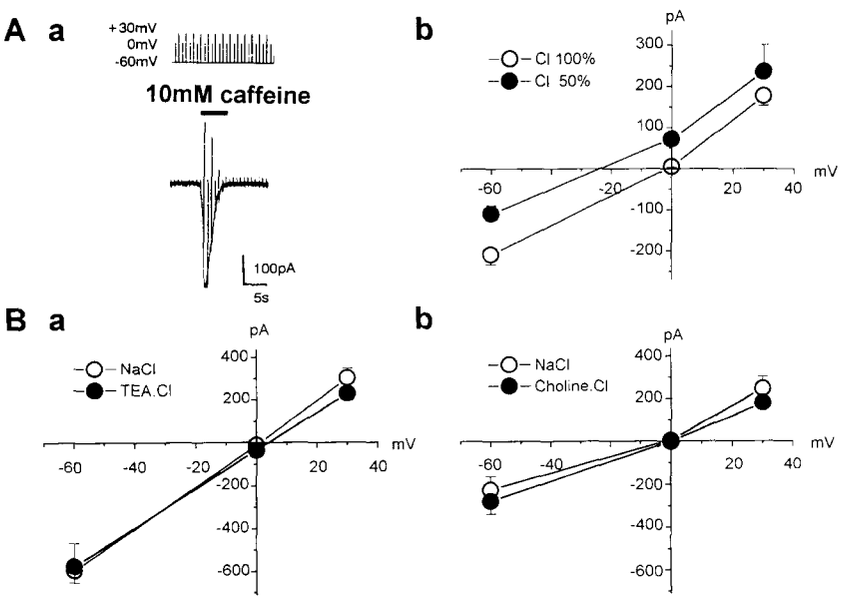

Fig. 2. Reversal potential and ion selectivity of caffeine-induced current. The pipette solution contained mainly CsCl. A: Reversal potential of caffeine-induced current was measured with a paired pulse protocol. A cell was depolarized from -60 to 0 and $+30$ mV for 150 ms, respectively, with interpulse duration of 500 ms, once every 1.5 s. The peak amplitude of membrane current was measured during application of caffeine at -60, 0, and $+30$ mV as shown in (a) and was plotted against the potentials in (b). ○: Averaged amplitude of currents recorded when the pipette solution contained 150 mM CsCl ($n = 15$). ●: Averaged amplitude of currents recorded when half of CsCl in the pipette solution was replaced with Cs aspartate ($n = 4$). Under these conditions, the calculated Cl^- equilibrium potentials were 0 and -26 mV, respectively, and the reversal potentials of the caffeine-induced current were 0 mV and approximately -23 mV, respectively. B: Reversal potentials were measured in the same manner as shown in (A,a). When NaCl in the bathing solution was completely replaced with TEA · Cl (a) or choline · Cl (b), the reversal potential was not significantly affected.

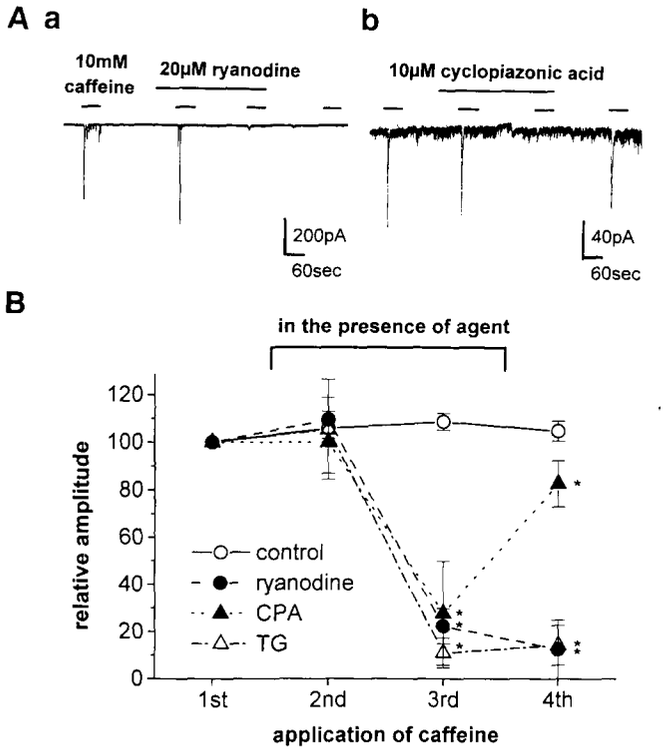

Fig. 3. Effects of several agents on caffeine-induced inward current. A: Application of 10 mM caffeine was repeated 4 times with intervals of 3–4 min. The 2nd and 3rd applications were carried out in the presence of 20 μM ryanodine (a) and 10 μM cyclopiazonic acid (CPA, b), respectively. The pipette solution contained mainly CsCl. The holding potential was −60 mV. B: Effects of 20 μM ryanodine (●), 10 μM cyclopiazonic acid (▲) and 100 nM thapsigargin (TG, △) on caffeine-induced inward current were examined as shown in (A) for ryanodine and cyclopiazonic acid. The peak amplitude of the inward current obtained with the 1st application of caffeine was taken as the control (1.0) in each cell. In the presence of ryanodine, cyclopiazonic acid or thapsigargin, the inward current for the 2nd application of caffeine had an amplitude similar to that of the control. The inward current for the 3rd application of caffeine was, however, much smaller than the control. The inhibitory effect of cyclopiazonic acid was removed by washout, whereas those of ryanodine and thapsigargin were not. $^{*}P < 0.05$ vs. control.

in the half-open state and consequently depletes stored Ca^{2+} in sarcoplasmic reticulum (Rios et al., 1992). Fig. 3A,a shows the effects of 20 μM ryanodine on I_{caf}. Caffeine (10 mM) was applied 4 times at intervals of about 4 min. The second and third applications of caffeine were performed in the presence of ryanodine. Ryanodine did not affect the second I_{caf} but greatly reduced the third one. Summarized data are shown in Fig. 3B. The amplitude of first I_{caf} was taken as 1.0. In four trials, the amplitude of I_{caf} declined slightly but not significantly in the absence of additional agents. The relative amplitude of the third I_{caf} which was performed in the presence of ryanodine was significantly smaller than the control ($n = 3$, $22.3 \pm 7.5\%$, $P < 0.05$ vs. control). These results suggest that I_{caf} is activated by Ca^{2+} release from ryanodine-sensitive Ca^{2+} store sites. The effect of ryanodine was not removed by washout.

It has been reported that cyclopiazonic acid and thapsigargin are highly selective inhibitors of Ca^{2+}-ATPase on the endoplasmic and sarcoplasmic reticulum membrane in various types of cell, including smooth muscle cells (Seidler et al., 1989; Thastrup et al., 1990; Suzuki et al., 1992; Uyama et al., 1992). Therefore, effects of cyclopiazonic acid (Fig. 3A,b, Fig. 3B) and thapsigargin (Fig. 3B) on I_{caf} were investigated. Experiments were performed with the same procedure used to examine the effects of ryanodine. In the presence of 10 μM cyclopiazonic acid, although the amplitude of the second I_{caf} was not affected significantly, the amplitude of the third I_{caf} was markedly reduced. Summarized data (Fig. 3B) show that both 10 μM cyclopiazonic acid and 100 nM thapsigargin inhibited the third I_{caf} significantly (cyclopiazonic acid: $27.8 \pm 22.1\%$, $n = 4$, thapsigargin: $10.9 \pm 6.3\%$, $n = 4$, $P < 0.05$ vs. control). The effect of cyclopiazonic acid was mostly removed by washout, whereas that of thapsigargin was irreversible.

The effects of Cd^{2+}, a divalent cation which blocks Ca^{2+} influx through voltage-dependent Ca^{2+} channels and also non-selective cation channels (Inoue, 1991), on I_{caf} were examined. Experiments were performed with the same procedure as used to examine the effects of ryanodine. Of four applications of caffeine, the second and third were performed in the presence of 1 mM Cd^{2+} (closed triangles, Fig. 4). The amplitude of the second I_{caf} was not affected by the presence of Cd^{2+}; $100.4 \pm 4.6\%$ of that of the control ($P > 0.05$, $n = 4$). The amplitude of the third I_{caf} in the continuous presence of Cd^{2+} was slightly but significantly smaller ($80.1 \pm 10.0\%$, $P < 0.05$ vs. control) than the control. When 1 mM Co^{2+} was used to block Ca^{2+} influx, the results were similar to those for Cd^{2+}; the amplitudes of the second and third I_{caf} were $107.3 \pm 7.8\%$ and $93.8 \pm 10.5\%$, respectively ($n = 4$, $P > 0.05$ vs. control).

Dependence of I_{caf} activation on extracellular Ca^{2+} was tested in the same manner as shown above. In the absence of extracellular Ca^{2+} and the presence of 1 mM EGTA, the amplitude of the second I_{caf} was similar to that of the control (Fig. 4, closed squares, $96.5 \pm 11.7\%$, $n = 3$, $P > 0.05$). The third I_{caf} in the continuous absence of Ca^{2+} was significantly smaller than the control ($n = 3$; $9.0 \pm 3.3\%$, $P < 0.05$). Re-admission of extracellular Ca^{2+} mostly restored I_{caf} (closed square, fourth). When external 2.2 mM Ca^{2+} was replaced with equimolar Cd^{2+}, the amplitude of the second I_{caf} was not significantly smaller than the control (Fig. 4; open triangles, $111.5 \pm 8.8\%$, $n = 5$, $P > 0.05$ vs. control) but that of the third I_{caf} was markedly reduced ($31.4 \pm 12.3\%$, $n = 5$, $P < 0.05$ vs. control). The fourth I_{caf}, which was recorded in the

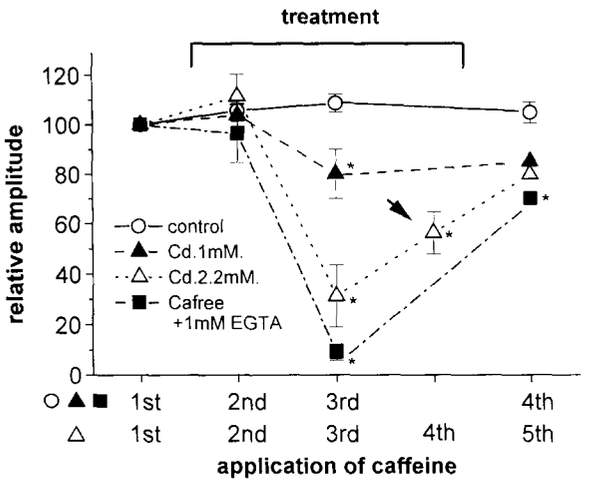

Fig. 4. Ca^{2+} dependence of caffeine-induced inward current. Effects of cadmium (Cd^{2+}) and Ca^{2+}-free solution on caffeine-induced inward current were examined at −60 mV as shown in Fig. 3A for ryanodine and cyclopiazonic acid (CPA). The pipette solution contained mainly CsCl. The peak amplitude of the inward current obtained with the 1st application of caffeine was taken as the control (1.0) in each cell. ○: Control ($n = 4$). ▲: The 2nd and 3rd applications of 10 mM caffeine were performed in the presence of 1 mM Cd^{2+} in a standard bathing solution ($n = 4$). △: The 2nd and 3rd applications were performed in a bathing solution where 2.2 mM Ca^{2+} was replaced with equimolar Cd^{2+}. The 4th application (indicated by an arrow) was done after the addition of 2.2 mM Ca^{2+} in the presence of 2.2 mM Cd^{2+} ($n = 4$). ■: The 2nd and 3rd application were done in a Ca^{2+}-'free' solution containing 1 mM EGTA ($n = 3$). The amplitude of I_{caf} induced by the 2nd application of caffeine was almost identical under these four different conditions. Note that the absence of external Ca^{2+} markedly reduced the 3rd I_{caf} regardless of the presence of Cd^{2+}. * $P < 0.05$ vs. control.

presence of both 2.2 mM Ca^{2+} and Cd^{2+} and indicated by an arrow, recovered partially (56.3 ± 8.4%, $n = 4$). The fifth I_{caf} recorded in the presence of 2.2 mM Ca^{2+} and the absence of Cd^{2+} recovered further (79.9%, $n = 2$). Thus, the refilling of intracellular Ca^{2+} storage sites after caffeine-induced Ca^{2+} release requires extracellular Ca^{2+}, regardless of the presence of Cd^{2+}, suggesting that the influx of Ca^{2+} for refilling was only partly blocked by Cd^{2+}. The extrusion of released Ca^{2+} to outside of the cell membrane may not be significantly blocked by Cd^{2+} either.

3.4. The recovery time course of I_{caf}

Since Ca^{2+}-ATPase inhibitors reduced I_{caf}, the refilling process of Ca^{2+} storage sites with Ca^{2+} after caffeine-induced release may strongly depend upon Ca^{2+}-ATPase activity. The recovery time course of I_{caf} in the presence or absence of cyclopiazonic acid (30 μM) was examined by paired puff applications of 10 mM caffeine with various intervals at the holding potential of −60 mV. The protocol for these experiments is shown in Fig. 5A. I_{caf} evoked by puff application for 5 s reached a peak within 2 s and declined to the resting level during the application. There was a clear tendency for I_{caf} induced by puff application to have faster activation and decay time courses and a larger peak amplitude than that induced by bath application as pointed out for other smooth muscle cells (Ganitkevich and Isenberg, 1992). The amplitude of the first I_{caf} in the absence of cyclopiazonic acid was taken as 1.0. Fig. 5B shows the recovery time course of I_{caf} which was well described by a single exponential function with a time constant of 24.6 ± 1.1 s in 7 cells out of 8 cells. Similar experiments were carried out in the presence of 30 μM cyclopiazonic acid (Fig. 5C). The recovery of I_{caf} in the presence of cyclopiazonic acid was significantly delayed and was not fitted well by a single or sum of double exponential functions. Fig. 5D shows re-normalized data, in which the amplitude

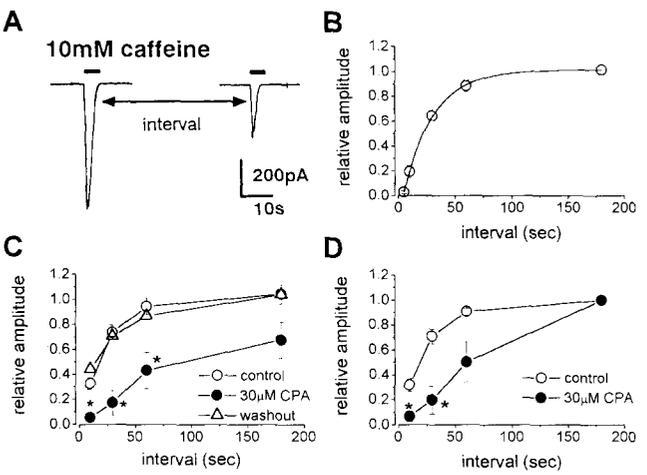

Fig. 5. Recovery time course of caffeine-induced inward current. A: Caffeine (10 mM) was applied twice with a certain interval (paired application). The holding potential was −60 mV. The pipette solution contained mainly CsCl. Caffeine was puff-applied to a cell by pressure ejection for 5 s from a pipette filled with the bathing solution containing 10 mM caffeine. The amplitude of the inward current induced by the latter application in a pair depended strongly on the interval. B: Pooled data for the peak amplitude obtained in experiments such as shown in (A) were plotted against the interval ($n = 8$). The peak amplitude measured when the interval was 175 s was taken as the control (1.0) in each cell. The recovery time course was well fitted by a curve described by a single exponential function with time constant of 24.6 ± 1.1 s in most cells examined (7 out of 8). C: Effects of 30 μM cyclopiazonic acid (CPA) on the recovery time course of I_{caf} were examined as shown in (A) ($n = 3$). The recovery time course was measured after the effect of cyclopiazonic acid on I_{caf} had reached a steady level. In the presence of 30 μM cyclopiazonic acid, the amplitude of the latter I_{caf} in a pair with an interval shorter than 60 s was markedly reduced. The effect of cyclopiazonic acid was removed by washout. Note that the effect of cyclopiazonic acid on I_{caf} at a longer interval was smaller than that observed when caffeine was applied by solution exchange (cf. Fig. 3). D: The effect of 30 μM cyclopiazonic acid shown in (C) was replotted taking the amplitude at the interval of 175 s as 1.0 in each cell. Note that the recovery was significantly slower in the presence of cyclopiazonic acid.

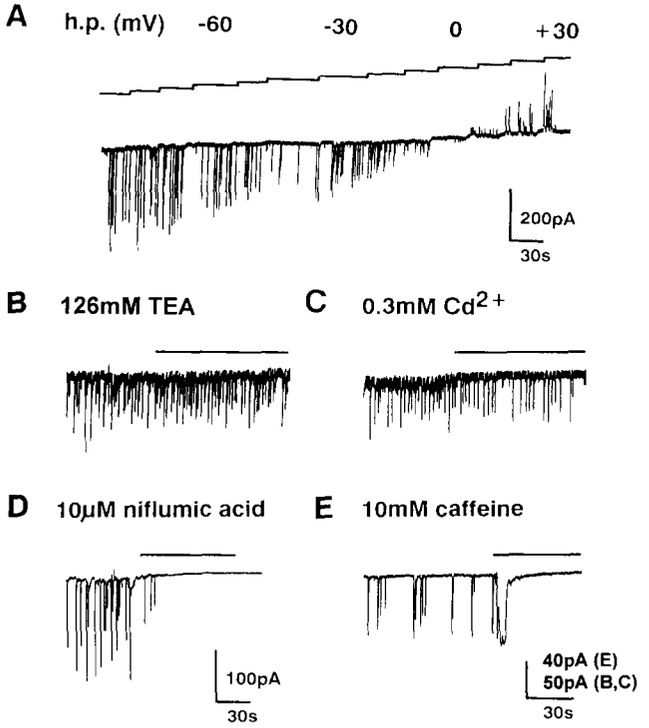

Fig. 6. Pharmacological characteristics of spontaneous transient inward currents in tracheal smooth muscle cells. The pipette solution contained mainly CsCl. The current-voltage relationship of spontaneous transient inward currents was recorded in (A) by changing the holding potential from −90 to +30 mV in 10 mV steps. The current reversed at near 0 mV. The replacement of external 126 mM Na^+ with equimolar tetraethylammonium$^+$ (TEA, B) and addition of 0.3 mM Cd^{2+} (C) did not affect spontaneous transient inward currents. Spontaneous transient inward currents were blocked by application of 10 μM niflumic acid (D) or 10 mM caffeine (E). Each treatment (B–E) is indicated by a bar.

of I_{caf} at the interval of 175 s in each preparation in Fig. 5C in the absence or presence of cyclopiazonic acid was taken as 1.0. These results indicate that the recovery of I_{caf} was relatively slow at 23°C and was significantly delayed by cyclopiazonic acid. It can be suggested that the amplitude of I_{caf} depends on the Ca^{2+} content in intracellular Ca^{2+} storage sites which is mainly regulated by Ca^{2+}-ATPase activity.

3.5. Spontaneous transient currents

In about 15% of cells examined, spontaneous transient inward currents were recorded at a holding potential of −60 mV, when the CsCl-rich pipette solution was used under both the conventional whole-cell mode and the nystatin-perforated patch mode. The E_r of spontaneous transient inward currents was about 0 mV as shown in Fig. 6A. The replacement of extracellular Na^+ with tetraethylammonium$^+$ (Fig. 6B) and addition of 0.3 mM Cd^{2+} to the extracellular solution did not affect spontaneous transient inward currents (Fig. 6C). Addition of 10 μM niflumic acid inhibited spontaneous transient inward currents (Fig. 6D). In these cells, puff- or bath-applied 10 mM caffeine (Fig. 6E) evoked inward currents which were followed by a period of inhibition of spontaneous transient inward currents. Application of 10 μM cyclopiazonic acid, 100 nM thapsigargin or 20 μM ryanodine also inhibited spontaneous transient inward currents (not shown). These results were observed in at least 3 cells for each procedure and suggest that pharmacological characteristics of spontaneous transient inward currents are identical to those of I_{caf}, implying that spontaneous transient inward currents are Ca^{2+}-dependent Cl^- currents stimulated by the cyclical release of Ca^{2+} from internal Ca^{2+} storage sites. The application of 8-bromo-cAMP (1 mM) or forskolin (20 μM) did not elicit the inward currents (not shown, $n = 3$ or 5 for each).

4. Discussion

The present study clearly showed that the application of caffeine activates the Ca^{2+}-dependent Cl^- current in addition to the Ca^{2+}-dependent K^+ current by Ca^{2+}-release from Ca^{2+} storage sites in tracheal smooth muscle cells of the guinea-pig. It is also shown that spontaneous transient inward currents are occasionally observed in these cells and may also be due to a Ca^{2+}-dependent Cl^- current activated by cyclical spontaneous Ca^{2+} release from local storage sites (Wang et al., 1992; Wang and Large, 1993) as has been suggested for spontaneous transient outward currents (Benham and Bolton, 1986).

Based upon the reversal potential measurement in several different ionic environments, it can be concluded that I_{caf} occurs as a result of the increase in membrane Cl^- conductance. The shift in E_r on replacement of Cl^- with aspartate$^-$ is very close to the predicted E_{Cl} and consistent with that reported in the rat portal vein (Pacaud et al., 1989). The possibility that a non-selective cationic current also contributes to I_{caf} can be discarded, whereas simultaneous activation of these two kinds of inward currents has been shown in response to acetylcholine in guinea-pig tracheal smooth muscle cells (Janssen and Sims, 1992) and to noradrenaline in ear artery of the rabbit (Amédée et al., 1990). Activation of large conductance non-selective cationic channels by caffeine has been reported in smooth muscle cells of rat portal vein (Loirand et al., 1991) and toad stomach (Guerrero et al., 1994). An increase in only non-selective cation conductance by acetylcholine or noradrenaline has been found in intestinal smooth muscle cells of the guinea-pig (acetylcholine: Inoue and Isenberg, 1990; Pacaud and Bolton, 1991), and portal vein of the rabbit (noradrenaline: Wang and Large, 1991). The present results are, therefore, rather similar to those observed for responses to

noradrenaline in portal vein (Byrne and Large, 1988; Pacaud et al., 1989). It is also unlikely that spontaneous transient inward currents in guinea-pig tracheal smooth muscle cells include a non-specific cationic current in addition to Cl^- current.

In airway epithelial cells and cardiac cells, a cAMP-dependent Cl^- current ($I_{Cl\text{-}cAMP}$) is elicited by β-adrenoceptor agonists, cAMP nonhydrolytic analogs or cAMP-dependent protein kinase catalytic subunits (Bahinski et al., 1989; McCann and Welsh, 1990). Although caffeine inhibits phosphodiesterase (Butcher and Sutherland, 1962), the contribution of $I_{Cl\text{-}cAMP}$ to I_{caf} can be ruled out in the present study, since the application of cAMP nonhydrolytic analogs (8-bromo-cAMP) or forskolin neither elicited inward currents nor affected I_{caf} markedly. Moreover, I_{caf} occurred transiently and in all-or-none manner in contrast to sustained $I_{Cl\text{-}cAMP}$. $I_{Cl\text{-}cAMP}$ does not appear to be elicited by caffeine in guinea-pig tracheal smooth muscle cells.

Application of caffeine abolished spontaneous transient inward currents after the activation of I_{caf}, indicating that spontaneous transient inward currents are due to Ca^{2+} release from caffeine-sensitive Ca^{2+} storage sites. Both I_{caf} and spontaneous transient inward currents were inhibited or markedly reduced by the treatment with ryanodine, cyclopiazonic acid or thapsigargin. Ryanodine keeps Ca^{2+} releasing channels on the sarcoplasmic reticulum membrane in a half open-state and consequently depletes stored Ca^{2+} in sarcoplasmic reticulum (Rios et al., 1992). Cyclopiazonic acid and thapsigargin selectively inhibit Ca^{2+}-ATPase on endoplasmic and sarcoplasmic reticulum membrane in various types of cells including smooth muscle cells (Seidler et al., 1989; Thastrup et al., 1990). The decrease in Ca^{2+} uptake into sarcoplasmic reticulum caused by treatment with cyclopiazonic acid or thapsigargin in smooth muscle cells results in a decrease in Ca^{2+}-induced Ca^{2+} release which is triggered by Ca^{2+} entering through voltage-dependent Ca^{2+} channels upon depolarization (Suzuki et al., 1992). The present results indicate that both I_{caf} and spontaneous transient inward currents are due to Ca^{2+} release from ryanodine-sensitive Ca^{2+} storage sites which are filled by Ca^{2+} uptake sensitive to cyclopiazonic acid and thapsigargin. The reason for the lower probability of finding spontaneous transient inward currents than spontaneous transient outward currents in tracheal cells is not clear from the present study. It has been reported that the Ca^{2+} sensitivity of Ca^{2+}-activated Cl^- channels was lower than that of Ca^{2+}-activated K^+ channels (Pacaud et al., 1992; Hogg et al., 1993). In tracheal cells where spontaneous transient outward currents but not spontaneous transient inward currents were recorded, the Ca^{2+} transients might not reach a level high enough to activate the Cl^- currents.

Neither I_{caf} nor spontaneous transient inward currents were changed by the addition of Cd^{2+} or Co^{2+}, indicating that Cl^- channel activity is not directly affected by external Cd^{2+} or Co^{2+}. Refilling of storage sites with Ca^{2+} after caffeine-induced release strongly depended on external Ca^{2+} and was not affected significantly by the presence of external Cd^{2+}. It was a rather unexpected observation that neither the extrusion of cytoplasmic Ca^{2+} nor the entry of Ca^{2+} for the refilling was significantly affected by external Cd^{2+}. It has been reported that Ca^{2+} entry after depletion of intracellular storage sites is, at least in part, sensitive to 1,4-dihydropyridine, a Ca^{2+} channel antagonist (Janssen and Sims, 1993a). It is, therefore, of interest that the major pathway for the refilling of caffeine-sensitive Ca^{2+} storage sites in guinea-pig tracheal smooth muscle cells appeared to be insensitive to external Cd^{2+}.

If the amplitude of I_{caf} induced by 10 mM caffeine reflects the amount of releasable Ca^{2+} in storage sites, the recovery time course of I_{caf}, which was measured as the interval between paired applications of caffeine, may indicate the refilling process, which probably consists of Ca^{2+} extrusion from and entry to the cell and Ca^{2+} uptake into the storage sites, as has been suggested for rat portal vein (Baron et al., 1991). The recovery time course of I_{caf} in tracheal smooth muscle of the guinea-pig was comparable to that in the rat portal vein (half recovery time of 29 s at 22°C) (Baron et al., 1991). The finding that the recovery time course was slowed in the presence of cyclopiazonic acid gave evidence supporting the interpretation that the recovery is due to the refilling of intracellular Ca stores. The inactivation of Ca^{2+}-releasing channels, however, has been speculated upon by Fabiato (1985). In addition, Missiaen et al. (1992) have reported that Ca^{2+} release by IP_3 may occur, not through an all-or-none release mechanism but through a steady-state release mechanism, in which the decreasing Ca^{2+} content of the intracellular Ca^{2+} storage sites slows down further release in permeabilized A7r5 smooth muscle cells. Therefore, we cannot rule out completely the possibility that the small amplitude of I_{caf} on the latter application of caffeine in a pair does not reflect directly a smaller amount of stored Ca^{2+}.

Since single channel currents of Ca^{2+}-dependent Cl^- channels were not recorded in the present study, characteristics of the channels which were responsible for I_{caf} and/or spontaneous transient inward currents were not identified. So far, very few single channel recordings of Cl^- channels have been reported in smooth muscle cells. Recently, Klöckner (1993) has reported that intracellular Ca^{2+} ions activate a low-conductance Cl^- channel in smooth muscle cells isolated from human mesenteric artery. In rabbit proximal colon, a neurokinin A-evoked large conductance Cl^- channel current has been recorded (Sun et al., 1992),

whereas the Cl^- current is not dependent on $[Ca^{2+}]_i$. The contribution of Cl^- channel currents induced by cell swelling observed in cardiac cells (Sorota, 1992) to I_{caf} was not investigated in the present study.

I_{caf} and spontaneous transient inward currents in the present study may have been greater than those under physiological conditions since E_r of Cl^- was about 0 mV under our experimental conditions whereas it is more negative under physiological conditions from −11 to −47 mV (Lamb et al., 1994). A Ca^{2+}-dependent Cl^- current is considered to be responsible for the membrane depolarization induced by agonists such as acetylcholine, histamine and substance P which activate phospholipase C and enhance IP_3 formation in tracheal smooth muscle cells (Janssen and Sims, 1992,1993b). On the other hand, spontaneous transient inward currents may play a role under pathological conditions where cells are overloaded with Ca^{2+} (Wang et al., 1992).

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References

Amédée, T., C.D. Benham, T.B. Bolton, G. Byrne and W.A. Large, 1990, Potassium, chloride and non-selective cation conductances opened by noradrenaline in rabbit ear artery cells, J. Physiol. (London) 423, 551.

Bahinski, A., A.C. Narin, P. Greengard and D.C. Gatsby, 1989, Chloride conductance regulated by cyclic-AMP dependent protein kinase in cardiac myocytes, Nature 340, 718.

Baron, A., P. Pacaud, G. Loirand, C. Mironneau and J. Mironneau, 1991, Pharmacological block of Ca^{2+}-activated Cl^- current in rat vascular smooth muscle cells in short-term primary culture, Pflüg. Arch. 419, 553.

Benham, C.D. and T.B. Bolton, 1986, Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit, J. Physiol. (London) 281, 385.

Butcher, R.W. and E.W. Sutherland, 1962, Adenosine 3′,5′-trisphosphate in biological materials. I. Purification and properties of cyclic 3′,5′-phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in humane urine, J. Biol. Chem. 237, 1244.

Byrne, N.G. and W.A. Large, 1988, Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein, J. Physiol. (London) 404, 557.

Fabiato, A., 1985, Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell, J. Gen. Physiol. 85, 247.

Ganitkevich, V.YA. and G. Isenberg, 1992, Caffeine-induced release and reuptake of Ca^{2+} by Ca^{2+} stores in myocytes from guinea-pig urinary bladder, J. Physiol. (London) 458, 99.

Guerrero, A., F.S. Fay and J.J. Singer, 1994, Caffeine activates a Ca^{2+}-permeable, nonselective cation channel in smooth muscle cells, J. Gen. Physiol. 104, 375.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann and F.J. Sigworth, 1981, Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches, Pflüg. Arch. 391, 85.

Hogg, R.C., Q. Wang and W.A. Large, 1993, Time course of spontaneous calcium-activated chloride currents in smooth muscle cells from the rabbit portal vein, J. Physiol. (London) 464, 15.

Horn, R. and A. Marty, 1988, Muscarinic activation of ionic currents measured by a new whole-cell recording method, J. Gen. Physiol. 92, 145.

Hughes, A.D., S. Hering and T.B. Bolton, 1990, The action of caffeine on inward barium current through voltage-dependent calcium channels in single rabbit ear artery cells, Pflüg. Arch. 416, 462.

Imaizumi, Y., K. Muraki and W. Watanabe, 1989, Ionic currents in single smooth muscle cells from the ureter of the guinea-pig, J. Physiol. (London) 411, 131.

Imaizumi, Y., K. Muraki and M. Watanabe, 1990, Characteristics of transient outward currents in smooth muscle cells from the ureter of the guinea-pig, J. Physiol. (London) 427, 301.

Inoue, R., 1991, Effect of external Cd^{2+} and other divalent cations on carbachol-activated non-selective cation channels in guinea-pig ileum, J. Physiol. (London) 442, 447.

Inoue, R. and G. Isenberg, 1990, Effect of membrane potential on acetylcholine-induced inward current in guinea-pig ileum, J. Physiol. (London) 424, 57.

Janssen, L.J. and S.M. Sims, 1992, Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes, J. Physiol. (London) 453, 197.

Janssen, L.J. and S.M. Sims, 1993a, Emptying and refilling of Ca^{2+} store in tracheal myocytes as indicated by ACh-evoked currents and contraction, Am. J. Physiol. 265, C877.

Janssen, L.J. and S.M. Sims, 1993b, Substance P activates Cl^- and K^+ conductances in guinea-pig tracheal smooth muscle cells, Can. J. Physiol. Pharmacol. 72, 705.

Klöckner, U., 1993, Intracellular calcium ions activate a low-conductance chloride channel in smooth muscle cells isolated from human mesenteric artery, Pflüg. Arch. 424, 231.

Lamb, F.S., K.A. Volk and E.F. Shibata, 1994, Calcium-activated chloride current in rabbit coronary artery myocytes, Circ Res. 75, 742.

Loirand, G., P. Pacaud, A. Baron, C. Mironneau and J. Mironneau, 1991, Large conductance calcium-activated non-selective cation channel in smooth muscle cells isolated from rat portal vein, J. Physiol. 437, 461.

McCann, J.D. and M.J. Welsh, 1990, Regulation of Cl^- and K^+ channels in airway epithelium, Annu. Rev. Physiol. 52, 115.

Missiaen, L., H.D. Smedt, G. Droogmans and R. Casteels, 1992, Ca^{2+} release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca^{2+} in permeabilized cells, Nature 357, 599.

Ohta, T., S. Ito and Y. Nakazato, 1993, Chloride currents activated by caffeine in rat intestinal smooth muscle cells, J. Physiol. (London) 465, 149.

Pacaud, P. and T.B. Bolton, 1991, Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells, J. Physiol. (London) 441, 477.

Pacaud, P., G. Loirand, C. Mironneau and J. Mironneau, 1989, Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein, Br. J. Pharmacol. 97, 139.

Pacaud, P., G. Loirand, G. Gregoire, C. Mironneau and J. Mir-

roneau, 1992, Calcium-dependence of the calcium-activated chloride current in smooth muscle cells of rat portal vein, Pflüg. Arch. 421, 125.

Rios, E., G. Pizarro and E. Stefani, 1992, Charge movement and the nature of signal transduction in skeletal muscle excitation-contraction coupling, Annu. Rev. Physiol. 54, 109.

Sawynok, J. and T.L. Yaksh, 1993, Caffeine as an analgesic adjuvant: a review of pharmacology and mechanisms of action, Pharmacol. Rev. 45, 43.

Seidler, N.W., I. Jona, M. Vegh and A. Martonosi, 1989, Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum, J. Biol. Chem. 264, 17816.

Sorota, S., 1992, Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell patch-clamp method, Circ. Res 70, 679.

Sun, X.P., S. Supplisson, R. Torres, G. Sachs and E. Mayer, 1992, Characterization of large-conductance chloride channels in rabbit colonic smooth muscle, J. Physiol. (London) 448, 355.

Suzuki, M., K. Muraki, Y. Imaizumi and M. Watanabe, 1992, Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells, Br. J. Pharmacol. 107, 134.

Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley and A.P. Dawson, 1990, Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} store by specific inhibition of the endoplasmic reticulum, Proc. Natl. Acad. Sci. USA 87, 2466.

Uyama, Y., Y. Imaizumi and M. Watanabe, 1992, Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile responses in skinned ileal smooth muscle, Br. J. Pharmacol. 106, 208.

Wang, Q. and W.A. Large, 1991, Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells, J. Physiol. (London) 435, 21.

Wang, Q. and W.A. Large, 1993, Action of histamine on single smooth muscle cells dispersed from the rabbit pulmonary artery, J. Physiol. (London) 468, 125.

Wang, Q., R.C. Hogg and W.A. Large, 1992, Properties of spontaneous transient inward currents recorded in smooth muscle cells isolated from the rabbit portal vein, J. Physiol. (London) 451, 525.