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Caffeine induces periodic oscillations of Ca^{2+}-activated K^+ current in pulmonary arterial smooth muscle cells

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Abstract. The periodic oscillations of outward currents were studied in smooth muscle cells of the rabbit pulmonary artery. The combined stimuli of superfusion with 1 mM caffeine and depolarization of the membrane potential to 0 mV evoked periodic oscillations of outward currents with fairly uniform amplitudes and intervals. The oscillating outward currents induced by caffeine were dependent on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and had a reversal potential near to the equilibrium potential for K^+. So the oscillating outward currents are carried by K^+ through Ca^{2+}-dependent K^+ channels ($I_{K(Ca)}$), and may reflect the oscillations of $[Ca^{2+}]_i$. The oscillating outward currents were abolished, or their frequency reduced, by lowering external $[Ca^{2+}]$, Ca^{2+} channel blockers, or by 1 μM ryanodine, indicating that: (1) there is a continuous influx of Ca^{2+} through the plasma membrane at a holding potential of 0 mV; (2) the periodic transient increases of $[Ca^{2+}]_i$ are ascribed to the rhythmic release of Ca^{2+} from ryanodine-sensitive intracellular store by the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). On the basis of the above results, we simulated the oscillation of $[Ca^{2+}]_i$ induced by caffeine, which is known to lower the threshold of CICR. The patterns of peak amplitude histograms of spontaneous transient outward currents (STOC) in the oscillating cells were different from those in non-oscillating cells. The amplitudes of STOC in the latter were more variable than those in the former. The oscillating outward currents were modulated by 1 μM forskolin and 1 μM sodium nitroprusside, but STOC were little affected. The above differences between STOC and oscillating outward currents suggest that the two currents are activated by the Ca^{2+} originating from different intracellular Ca^{2+} stores which are functionally heterogeneous.

Key words: Caffeine – Calcium-dependent potassium current – Calcium oscillation – Vascular smooth muscle

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Introduction

The oscillation of intracellular free Ca^{2+} level ($[Ca^{2+}]_i$) has been observed in many cell types. Such oscillations were studied first in excitable cells such as cardiac myocytes [7] and sympathetic ganglion cells [17], then in non-excitable cells such as hepatocytes [28], HeLa cells [26], endothelial cells [15] and fibroblasts [10]. In excitable cells, intracellular Ca^{2+} overload or a low concentration of caffeine which lowers the threshold of Ca^{2+}-induced Ca^{2+} release (CICR), could induce the spontaneous oscillation of $[Ca^{2+}]_i$. Studies of the oscillation mechanism and of the effects of caffeine and ryanodine have contributed to the understanding of intracellular Ca^{2+} regulation by the sarcoplasmic reticulum (SR) [19].

In non-excitable cells, on the other hand, hormones or neurotransmitters which raise the level of intracellular inositol 1,4,5-trisphosphate (IP_3) are necessary to induce oscillations of $[Ca^{2+}]_i$. According to the model proposed by Goldbeter et al. [8], IP_3 plays a priming role in the generation of $[Ca^{2+}]_i$ oscillations in the following way. IP_3 not only releases Ca^{2+} from the IP_3-sensitive intracellular store, but also creates a constant influx of external primer Ca^{2+} through its specific receptor. The subsequent rise in $[Ca^{2+}]_i$ in turn triggers the release of Ca^{2+} from internal stores by the mechanism of CICR. Although the initiating agents vary with cell types, the CICR is proposed as a common mechanism maintaining the oscillation.

In pulmonary arterial smooth muscle cells, which are electrically stable compared with neurons or cardiac myocytes, we observed that a combined stimulus of a low concentration of caffeine and sustained depolarization could induce periodic spikes of outward current. The train of outward currents we observed appeared to be different from the spontaneous transient outward currents (STOC) described by Benham and Bolton [1], in uniformity of amplitudes of spikes and intervals between them. We assumed that the oscillating outward current

reflects the oscillation of $[Ca^{2+}]_i$. In this study, we investigated the underlying mechanism of the oscillating outward currents.

Materials and methods

After induction of anaesthesia in rabbits (1−1.5 kg) by intravenous injection of sodium pentobarbital (50 mg/kg) and heparin (1000 IU/kg) via the ear vein, the heart and lung were removed and immersed in Normal Tyrode solution which had been aerated with 100% O_2. The primary branch of the main pulmonary artery was dissected free of the surrounding adventitia and connective tissues. The dissected tissue was cut into small strips and bathed in Ca^{2+}-free Normal Tyrode solution for 15 min, and then incubated at 37°C in Ca^{2+}-free Normal Tyrode solution containing elastase (Sigma, type II-A, 0.3 mg/ml), collagenase (Worthington, 120 IU/ml), taurine (20 mM) and dithiothreitol (1 mg/ml) for 40−50 min. Single cells were isolated by gentle agitation of the digested arterial strips with a wide-bored pipette in Kraft-Brühe (KB) solution.

The normal tyrode solution used to superfuse cells contained (in mM): NaCl, 143; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 5; adjusted to pH 7.4 with NaOH. Potassium aspartate internal solution inside the whole-cell patch electrode contained (in mM): potassium aspartate, 110; KCl, 20; $MgCl_2$, 1; MgATP, 5; di-TRIS-creatine phosphate, 5; ethyleneglycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA), 0.1; HEPES, 5; adjusted to pH 7.4 with KOH. Potassium aspartate and KCl were replaced with equimolar caesium aspartate and tetraethylammonium chloride (TEA), respectively, in the patch electrode containing caesium aspartate for blocking of K^+ currents.

For recording in the cell-attached patch configuration, the solution inside the patch electrode contained (in mM): KCl, 150; $CaCl_2$, 1; $MgCl_2$, 0.5; HEPES, 5; adjusted to pH 7.4 with TRIS base.

Currents from single cells were recorded using the whole-cell voltage-clamp technique [9] with an Axopatch-1C amplifier (Axon Instruments). Data were displayed on an oscilloscope (Philips PM 3350) and a pen recorder (Gould Recorder 220), and were pulse-code modulated (Medical System) before being recorded on a video cassette recorder for later analysis.

For quantitative analysis of STOC, the amplitudes of individual STOC were measured and the peak amplitude histograms of STOC were drawn from the digitized records sampled for 52 s using an ADC interface (TL-1, Axon Instrument) and an IBM-compatible PC.

Single-channel currents recorded using cell-attached patch-clamp technique were filtered at 2 kHz through a 4-pole Bessel filter and digitized at 5 kHz. Data analysis was performed with pClamp 5.5.1 software. Values for channel open probability (P_o) were obtained from 30-s recordings of data. Most experiments were performed at 30−35°C, unless indicated separately.

Data were calculated as mean ± SEM (n = number of experiments). Statistical significance was determined using Student's t-test.

Results

Induction of rhythmic oscillation of outward current by depolarization and caffeine

When a pulmonary arterial smooth muscle cell was superfused with 1 mM caffeine, with its membrane potential being held at 0 mV, a large sinusoidal outward current was induced initially, and this was followed by a train of spikes of outward current with rather uniform intervals and an amplitude of about 3.5 nA (Fig. 1A). As shown in Fig. 1A, the outward currents during the superfusion with 1 mM caffeine could be classified into two groups on the basis of amplitude and duration. One had short durations of about 100−200 ms and small amplitudes below 1 nA, and the other had longer durations of about 4 s and larger amplitudes above 3 nA. While the former appeared at irregular intervals, the latter had a fairly uniform frequency. The properties of the small currents indicate that they might correspond to STOC which are evoked by sporadic release of Ca^{2+} from internal stores [1]. We therefore refer to these currents as STOC. However, the uniform amplitude and frequency of the latter suggest that it is a K^+ current activated by Ca^{2+} released synchronously from internal stores, and will be referred to as oscillating outward currents. After the caffeine had been washed out with Normal Tyrode solution, the train of oscillating outward currents was interrupted and the STOC which were observed in the control state before the superfusion of caffeine were resumed (Fig. 1A). About 15 min after washing out the caffeine, large outward currents of fairly long duration, which had also been seen long after the sustained depolarization in control (not shown), were observed. On resuperfusion of caffeine the rhythmic oscillation of the outward current resumed.

In another cell, in which little activity of STOC was shown at a holding potential of −40 mV in spite of the superfusion with 1 mM caffeine, depolarization to 0 mV evoked oscillating outward currents after a latent period of 50 s without the initial sinusoidal oscillation of outward current (Fig. 1B). Also during the appearance of the large spikes of outward currents, STOC were scarcely observed. The above findings indicate that combining the stimuli of caffeine and depolarization induces the oscillating outward currents, irrespective of the order of stimuli. We induced the periodic oscillation of outward currents by the above method in the following experiments. The mean amplitude and frequency of the oscillating outward currents which were measured during the first 5 min of induction were 2.44 ± 0.43 nA and 2.86 ± 0.68 min^{-1}, respectively (n = 13).

Not all cells we tested, however, showed the oscillating outward currents. In 5 of 18 cells, we failed to induce the oscillation. Characteristically, STOC were rapidly inactivated in most cells which had shown the oscillation, while they remained active in cells which failed to generate the oscillation. The chart records of representative examples for the non-oscillating cells are shown in Fig. 9A and C. In non-oscillating cells, the mean amplitude of STOC was 163.3 ± 25.6 pA (n = 3) which was measured within 5 min after caffeine treatment. The above mean value obtained from non-oscillating cells was significantly larger than 68.5 ± 3.1 pA (n = 5), the mean amplitude of STOC which was measured in five cells which showed a caffeine-induced oscillation of outward currents ($P < 0.05$). The mean capacitance of cells used in this study was 24.2 ± 2.2 pF (n = 5).

There was a difference in the amplitude variability of STOC as well as the mean value between oscillating

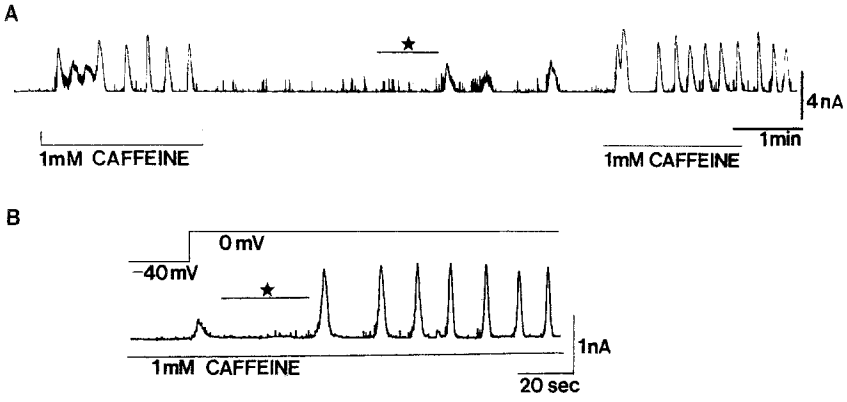


Fig. 1A, B. Periodic oscillation of outward currents induced by the combined stimuli of 1 mM caffeine and depolarization to 0 mV. **A** Oscillating outward currents were induced by superfusion with 1 mM caffeine and a holding potential of 0 mV. **B** In another cell, depolarization from −40 mV to 0 mV, during superfusion with 1 mM caffeine, also induced the similar oscillating outward currents with fairly uniform amplitudes and intervals

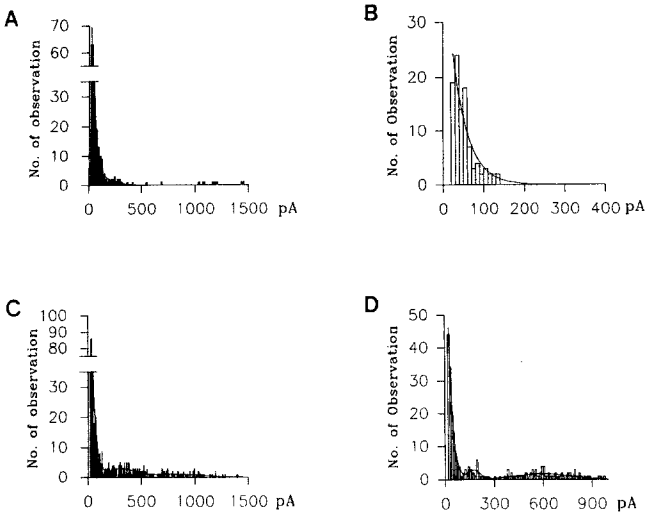


Fig. 2. Amplitude histograms of spontaneous transient outward currents (STOC) sampled from the data displayed in Fig. 1A (**A**), Fig. 1B (**B**), Fig. 9A (**C**) and Fig. 9C (**D**). The sampling period was marked with an *asterisk* in each paper record. The amplitude histograms of STOC regression lines were fitted with the following equation:

$$a = a_1 \cdot \exp(-x/m_1) + a_2 \cdot \exp[-(x - m_2)^2/2s_2^2] + a_3 \cdot \exp[-(x - m_3)^2/2s_3^2]$$

where x, a_i, m_i and s_i are the amplitude of STOC, the maximum number of observations, mean amplitude and variance of the ith component of distribution function, respectively. The following parameters are used in the fitting: **A** $a_1 = 149.88$, $m_1 = 31.64$, $a_2 = 2.02$, $m_2 = 131.75$, $s_2 = 124.40$; **B** $a_1 = 47.57$, $m_1 = 37.37$; **C** $a_1 = 152.36$, $m_1 = 31.79$, $a_2 = 2.70$, $m_2 = 308.54$, $s_2 = 122.69$, $a_3 = 1.11$, $m_3 = 769.73$, $s_3 = 284.47$; **D** $a_1 = 135.45$, $m_1 = 23.26$, $a_2 = 2.82$, $m_2 = 177.16$, $s_2 = 36.09$, $a_3 = 1.72$, $m_3 = 601.82$, $s_3 = 175.72$. The total numbers of observations were 312 (**A**), 100 (**B**), 417 (**C**) and 231 (**D**). Amplitudes of STOC less than 20 pA were not taken into consideration in the evaluation

cells and non-oscillating cells. A comparison of STOC peak amplitude histogram from oscillating and non-oscillating cells demonstrates this difference. Peak amplitude histograms of STOC obtained from non-oscillating cells have typical distribution patterns which could be fitted by a combination of one exponential and several normal distributions [16] (Fig. 2C, D). In representative cells which generated oscillating outward current, however, the peak amplitudes of STOC, sampled at the latent period between the depolarization and the first oscillatory outward current, have only one exponential distribution (Fig. 2B). Generally, a cell that generates a more regular and uniform oscillating outward current shows less variation in the amplitude of its STOC.

Characterization of oscillating outward currents induced by caffeine

In order to confirm whether channels other than the Ca^{2+}-activated K^+ channel, which is known to be responsible for STOC [1], are involved in the outward currents induced by caffeine, the following experiments were performed. With internal potassium aspartate solution, the train of outward currents induced by caffeine was blocked by external 10 mM TEA (Fig. 3A). Voltage ramps (0.8 V/s) were applied from a holding potential of −40 mV before injecting caffeine (inset of Fig. 3C, a) and at the peak of the outward current induced by injection of caffeine (Fig. 3C, b). The relationship to voltage of the two currents was obtained from the negative slope of the ramp (from + 40 mV to −120 mV for 200 ms). The *I/V* plots for these two currents are displayed in Fig. 3C. The outward current induced by caffeine was reversed at −69 mV, which is close to the calculated equilibrium potential for $K^+(E_K)$, −84.8 mV. The discrepancy between E_K and the reversal potential might be due to the imperfect selectivity of the current for K^+, since the constant field equation gives the reversal potential of $I_{K(Ca)}$ as −69 mV, assuming the channel has permeability of Na^+ with respect to K^+ (P_{Na}/P_K) as 0.03, based on Benham et al. [2]. To see whether the caffeine-induced current is carried by cations other than K^+, the following experiment was performed. Since the ratio of [K]/[Na] in Normal Tyrode solution is about 0.036, it would be unclear in the presence of external Normal Tyrode solution, assuming $P_{Na}/P_K = 0.03$, whether the charge carrier of inward current induced by caffeine was Na^+ or K^+. So we replaced external K^+ with equimolar Cs^+ in the experiment shown in Fig. 3B. When K^+ inside the patch electrode and in Normal Tyrode solution was replaced with Cs^+, very small oscillating inward currents were observed at −40 mV (Fig. 3B). The *I/V* curve plotted in a similar manner to the voltage ramp showed that caffeine induced only in-

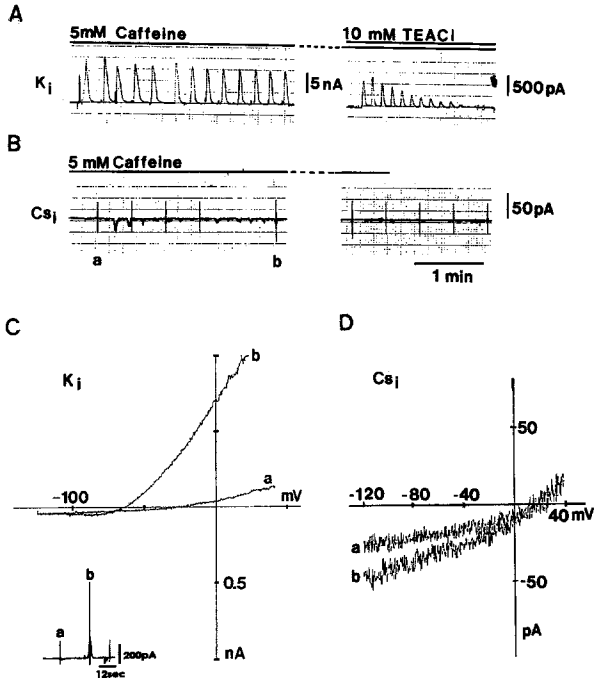


Fig. 3A–D. Characterization of oscillating outward currents induced by caffeine. **A** The oscillating outward current elicited by 5 mM caffeine at a holding potential of 0 mV was abolished by the addition of 10 mM tetraethylammonium chloride (TEACl). **B** With caesium aspartate internal solution, 5 mM caffeine induced a train of transient inward currents at a holding potential of −40 mV. **C** With potassium aspartate internal solution, a transient outward current was induced by the injection of 5 mM caffeine from a large-bore pipette (internal tip diameter; about 500 μm) placed beside the cell. The current responses to the descending limb of a voltage ramp illustrated in Fig. 5B under control conditions (*a*) and at the peak of the transient outward current (*b*) are superimposed on this *I/V* plot. **D** In the same cell as **B**, the *I/V* relationships were obtained at *a* and *b* with the same method as in **C**. Only the inward current was elicited by caffeine within the most of the voltage range tested

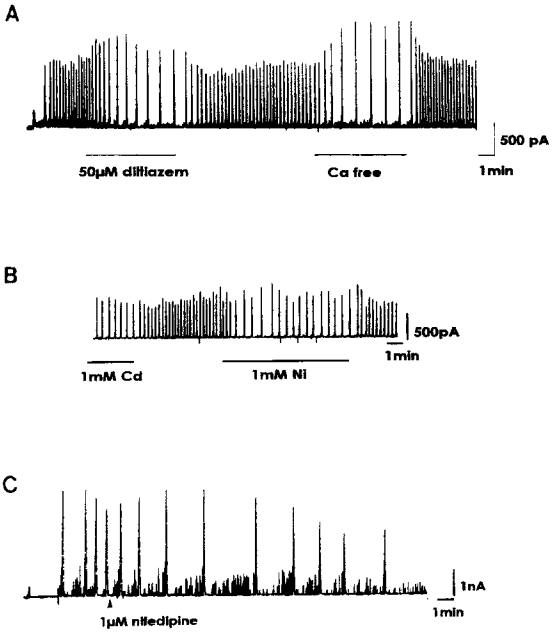


Fig. 4. The effects of Ca^{2+} channel blockers, 50 μM diltiazem (**A**), 1 mM Cd^{2+}, 1 mM Ni^{2+} (**B**), 1 μM nifedipine (**C**) and nominally Ca^{2+}-free solution (**A**) on the oscillating outward currents induced by 1 mM caffeine at a holding potential of 0 mV. Such conditions, which are expected to inhibit the influx of Ca^{2+}, reduced the frequency of the oscillation (**A, B**) or abolished it (**C**). The effect of nifedipine was not recovered by washing out with normal tyrode solution

ward current over most of the voltage range we tested (Fig. 3D), indicating that the caffeine-induced inward current is carried mostly by Na^+. However, it could not be determined in this experiment whether the inward current was ascribed to Ca^{2+}-activated K^+ channels or Ca^{2+}-activated non-selective cation channels. Nevertheless outward K^+ current would be predominant at 0 mV, since the inward current was reversed at near 0 mV. The oscillating outward currents were not observed in the presence of 5 mM EGTA in the internal solution. These findings indicate that the oscillating outward current induced by caffeine is $I_{K(Ca)}$, and that it is substantially the same as STOC. Since the P_o of the $I_{K(Ca)}$ channel is dependent on $[Ca^{2+}]_i$ at a constant membrane potential [20], the oscillating outward current and STOC can reflect the change in $[Ca^{2+}]_i$. The discrete nature of these two currents (the rhythmicity and uniformity of the oscillating outward currents vs irregularity of STOC) would implicate the presence of two different levels of intracellular Ca^{2+} handling mechanisms.

Effect of external Ca^{2+} concentration and Ca^{2+} channel blockers

The oscillation frequency seemed to be very sensitive to the Ca^{2+} influx rate. The lowering of external Ca^{2+}, or the external application of Ca^{2+} blockers, reduced the frequency of the oscillation or abolished it ($n = 4$). Representative data are shown in Fig. 4. The removal of extracellular Ca^{2+}, or the external application of 50 μM diltiazem, reduced the frequency from about 1/10 s^{-1} to 1/50 s^{-1}, while the amplitudes of Ca^{2+} spikes became larger than in control (Fig. 4A). The large magnitude of Ca^{2+} spikes in Ca^{2+}-free bathing solution, however, could be partly ascribed to the removal of the screening effect of external Ca^{2+} [11]. The effects of other organic and inorganic Ca^{2+} blockers such as nifedipine, Cd^{2+} and Ni^{2+} were similar to that of diltiazem (Fig. 4B, C). These findings are mostly consistent with the model proposed by Kuba and Takeshita [18]. The fact that external Ca^{2+} is necessary to generate the oscillation led us to assume that non-inactivating channels are responsible for the continuous Ca^{2+} influx, which is blocked by organic and inorganic Ca^{2+} channel blockers. Possible routes in the cellular membrane responsible for the continuous Ca^{2+} influx could be as follows: (1) non-inactivating voltage-dependent L-type Ca^{2+} channel [13]; (2) Na-Ca exchange; (3) Ca-dependent or agonist-induced non-specific cation channels.

When caesium aspartate solution containing 5 mM EGTA was used in the patch electrode, we recorded a downward deflection of the current around 0 mV on the

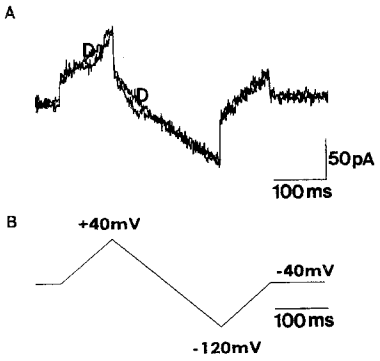


Fig. 5. Current recordings (**A**) responding to command voltage ramp (**B**), using caesium aspartate as the internal solution. The inward deflection at about 0 mV is considered to be a window Ca^{2+} current because it was abolished by diltiazem (marked by *D*) or the removal of extracellular Ca^{2+} (not shown)

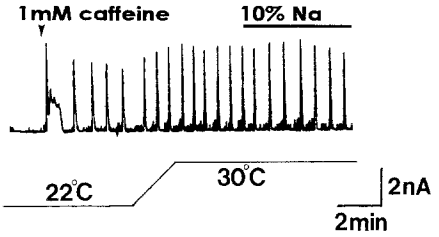


Fig. 7. Effects of temperature and reduction of external Na^+ concentration on the oscillation. With the holding membrane potential at 0 mV, superfusion of 1 mM caffeine induced periodic oscillation of the outward current. Raising temperature from 22°C to 30°C increased the frequency and amplitudes of the oscillating outward current, from 1/40 s^{-1} to 1/30 s^{-1} and from 3.40 ± 0.06 nA to 4.34 ± 0.05 nA, respectively. Replacement of 90% of external Na^+ with equimolar *N*-methyl-D-glucamine (indicated by *10% Na*) had no appreciable effect on the oscillation

voltage ramp as shown in Fig. 5B. The inward deflection was abolished by the removal of extracellular Ca^{2+} or application of 50 μM diltiazem (Fig. 5A). These findings suggest that the window-type Ca^{2+} current might be the route of continuous Ca^{2+} entry.

Since this current was blocked by organic Ca^{2+} channel blockers, the current might flow through non-inactivating L-type Ca^{2+} channels. However, contradictory results were also observed: hyperpolarization of the membrane potential which is expected to deactivate L-type Ca^{2+} channels, increased the oscillation frequency (n = 3) (Fig. 6). An explanation for the contradiction between (1) the sensitivity of oscillator frequency to organic Ca^{2+} channel blockers and (2) the effect of membrane potential remains to be elucidated. Two possible mechanisms underlying the effect of membrane potential are presented in the Discussion.

To rule out the possibility of Ca^{2+} influx via Na-Ca exchange, 90% of extracellular Na^+ was replaced with *N*-methyl-D-glucamine, but the reduction of extracellular Na^+ concentration had little effect on the oscillation (Fig. 7).

Figure 7 also shows that the oscillation is affected by bath temperature. A rise in temperature was associated with an increase in the frequency and amplitude of oscillating outward currents as well as basal STOC activities (n = 3). The shortened duration of the Ca^{2+} spike suggests that cytosolic Ca^{2+} is pumped out or taken up more rapidly at higher temperatures.

Effects of a higher concentration of caffeine and ryanodine on Ca^{2+} transients

To investigate the role of internal Ca^{2+} stores, we tested the effects of a higher concentration of caffeine and of ryanodine on the oscillation. Caffeine has been reported to shift the CICR rate/pCa relation towards lower Ca^{2+} concentrations in smooth muscle [12]. When the concentration of caffeine was increased from 1 mM to 5 mM, the oscillation was transformed from a transient type

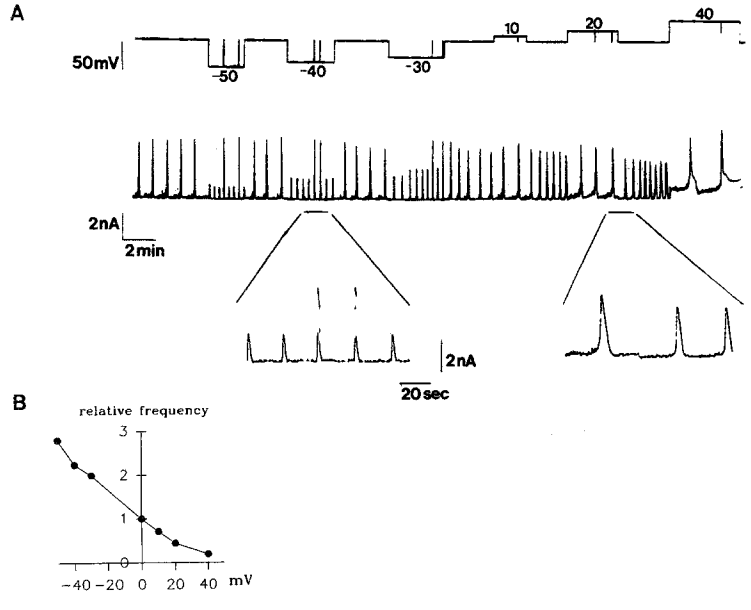


Fig. 6A, B. Voltage dependence of the frequency of the oscillating outward current. **A** The change of holding potential is recorded on the *top trace* and the corresponding oscillating outward current is shown on the *lower trace*. In order to exclude the effect of the electrochemical driving force for K^+, the holding potentials were changed to 0 mV instantaneously from each voltage (*lowest traces* show selected regions on an expanded time scale). The magnitudes of outward currents at 0 mV from all holding potentials were similar to those at 0 mV. This result suggests that the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) at the peaks of the oscillating outward current are not affected by holding potential. Some spontaneous increase in amplitudes of oscillating outward current at a holding potential of −30 mV could be due to the recovery to 32°C from a short drop in temperature to 27°C. **B** The relationship between the holding membrane potentials and the relative frequencies of the oscillating outward current. The relative frequencies were calculated from the ratio of the frequency at each voltage to that in the immediately preceding period at 0 mV

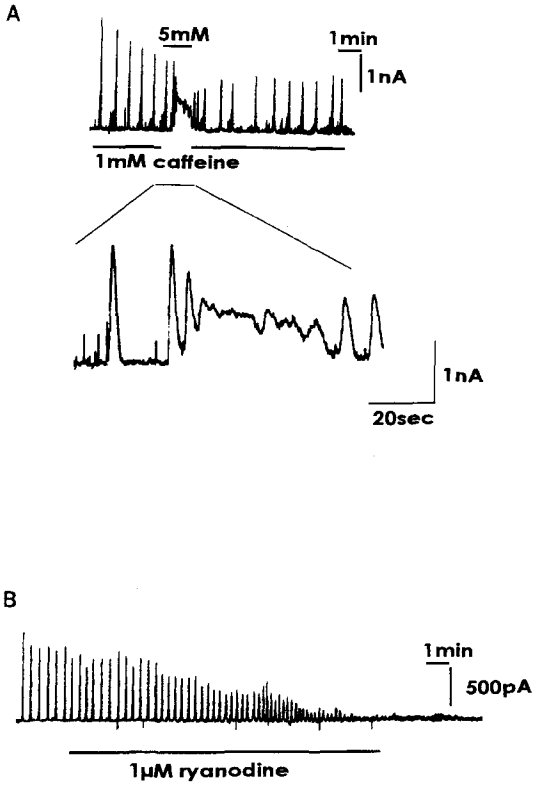


Fig. 8. Effects of 5 mM caffeine (**A**) and 1 μM ryanodine (**B**) on the oscillating outward current induced by 1 mM caffeine. Caffeine (5 mM) induced sustained outward current with sinusoidal oscillation which is shown in the *lower trace* of (**A**) on an expanded time scale. On the other hand, 1 μM ryanodine irreversibly decreased the amplitude of the oscillating outward current without transforming the oscillation type

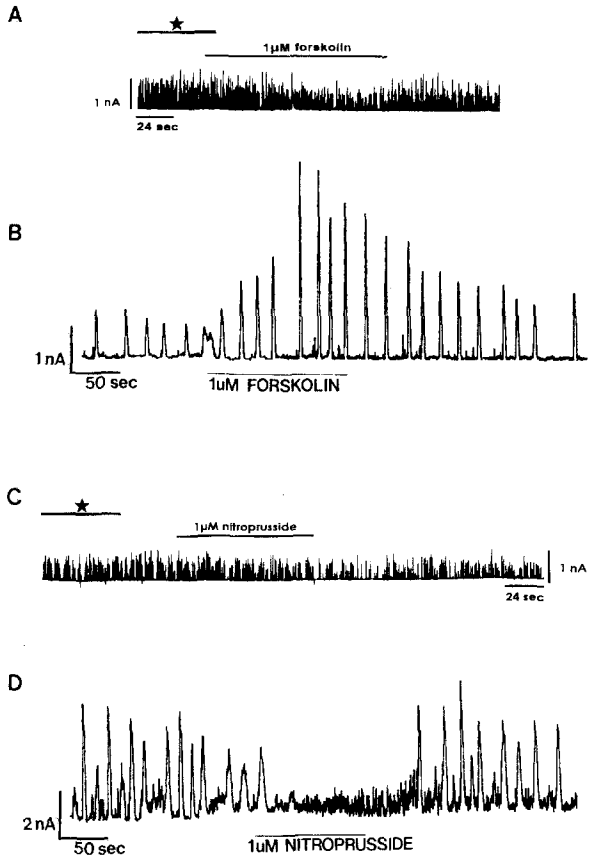


Fig. 9. Effect of 1 μM forskolin (**A, B**) and 1 μM sodium nitroprusside (**C, D**) on the oscillating outward currents and STOC. While 1 μM forskolin had little effect on the STOC activity recorded from a cell which did not show the oscillating outward currents (**A**), it enhanced the amplitudes of the oscillating outward currents induced by 1 mM caffeine (**B**). Sodium nitroprusside (1 μM) abolished the oscillating outward currents, but not basal STOC (**D**). It had no effect on the STOC in another non-oscillating cell (**C**). Holding potential was 0 mV and 1 mM caffeine was superfused in all experimental conditions

into a sinusoidal type, which reflects the sustained elevation of $[Ca^{2+}]_i$ (Fig. 8A, see also [3]). The transient type of oscillation was restored by lowering the caffeine concentration to 1 mM. The above findings suggest that during superfusion of 1 mM caffeine $[Ca^{2+}]_i$ is kept fairly low by the balancing role of Ca^{2+} pumps in the sarcolemma and SR against Ca^{2+} release from the SR and the influx from outside of cell, but the higher concentration of caffeine breaks the balance by sensitizing the Ca^{2+} release channel of the SR to Ca^{2+}, resulting in more release of Ca^{2+} from the SR. Therefore the maximal concentration which induces the transient type of oscillation should depend on the functional capacity of cellular Ca^{2+} extrusion mechanisms. In fact the caffeine concentration which elicits the transient type of oscillation varied from cell to cell. In one case, the transient type of oscillations could be induced even with 5 mM caffeine, as shown in Fig. 3.

Micromolar ryanodine is known to bind to CICR channels and lock the channel into its open state with half-maximal conductance, thus depleting the store which releases Ca^{2+} by CICR in skeletal muscle [24]. The superfusion of 1 μM ryanodine shortened the intervals between current spikes, decreased the amplitudes of spikes gradually over 14 min, and finally abolished the oscillation completely (Fig. 8B). The slow reduction of the amplitude of oscillatory outward current caused by ryanodine is consistent with the gradual depletion of the Ca^{2+} pool which is known to occur in the presence of this drug.

These findings indicate that the slow build-up of $[Ca^{2+}]_i$ by influx from outside the cell triggers the release of Ca^{2+} from ryanodine-sensitive internal Ca^{2+} stores by CICR.

Differential effect of forskolin and sodium nitroprusside (SNP) on STOC and oscillating outward currents

The K^+ current oscillation was also modulated by drugs which are known to affect the level of intracellular second messengers such as forskolin and SNP (Fig. 9).

Superfusion with 1 μM forskolin increased amplitudes of $I_{K(Ca)}$ spikes and shortened the spike interval (Fig. 9B), suggesting that cyclic adenosine monophosphate (cAMP) potentiates the activity of Ca^{2+} pumps in the SR to take up Ca^{2+} more rapidly. However, forskolin had little effect on the STOC in cells in which caffeine failed to induce the oscillating outward currents

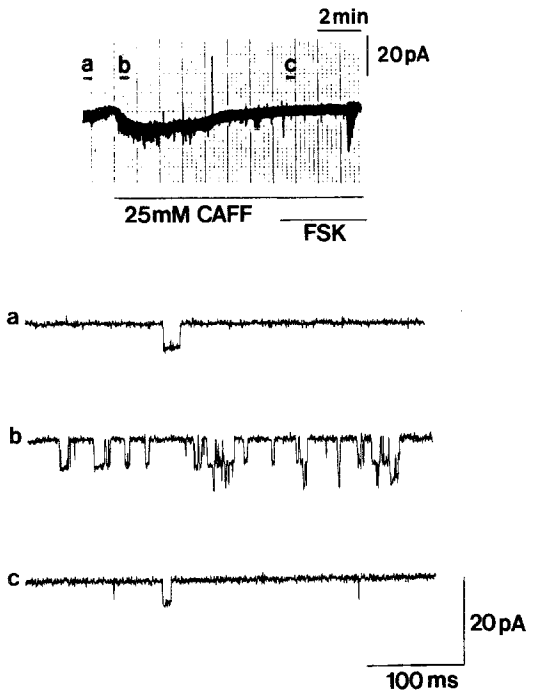


Fig. 10. Effect of 1 μM forskolin on Ca^{2+}-activated K^+ current [$I_{K(Ca)}$] activity. The chart record is of current flow through a cell-attached membrane patch while voltage inside the patch electrode was held at −100 mV. Outward current was recorded as a downward deflection. The presence of $I_{K(Ca)}$ channels inside the patch was tested by superfusion with Normal Tyrode solution containing 25 mM caffeine. This significantly increased NP_o (number of channels×open probability) of the channels from 0.014 to 0.14. The addition of 1 μM forskolin (*FSK*) to the external solution had no significant effect on the channel activities recorded from this cell-attached patch (NP_o = 0.011)

(Fig. 9A). It is unlikely that the K^+ channel activity is directly modulated by cAMP, because forskolin slightly reduced the outward current in the whole-cell configuration (not shown) and had little effect on $I_{K(Ca)}$ channel activity in the cell-attached patch mode (Fig. 10).

SNP is known to elevate intracellular cyclic guanosine monophosphate (cGMP) levels by releasing nitric oxide which activates soluble guanylate cyclase [23]. Whereas STOC were not affected in non-oscillating cells (n = 3, Fig. 9C), the oscillating outward currents were quickly abolished by the extracellular application of 1 μM SNP (Fig. 9D). In contrast to the effect of SNP on the oscillating outward currents, the STOC activity was not suppressed (Fig. 9D). During wash out of SNP, the STOC activity was more enhanced and the Ca^{2+} oscillation was eventually resumed (Fig. 9D). The effect of SNP on STOC is consistent with that described by Clapp and Gurney [6]. The underlying mechanism of the effect of forskolin and SNP remains to be elucidated.

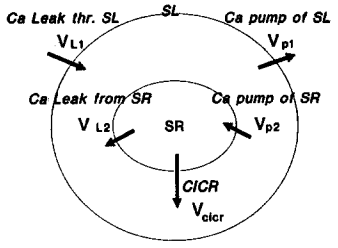
Discussion

In this study, we have demonstrated that a low concentration of caffeine and simultaneous depolarization of membrane potential could induce periodic oscillation of $I_{K(Ca)}$ which reflects $[Ca^{2+}]_i$ and that the oscillation is modulated by extracellular Ca^{2+} concentration, ryanodine and chemicals which are known to modulate the level of intracellular second messengers. Since membrane hyperpolarization has been reported to inhibit noradrenaline-induced synthesis of IP_3 in the mesenteric artery of the rabbit [14], we studied the effect of heparin to see whether IP_3 synthesis is activated in our experimental conditions. The oscillation of outward current could be induced by inclusion of heparin (0.57 mg/ml) in the electrode (in three out of nine cells). Although further studies are necessary to elucidate whether properties of the oscillation are similar to those in the absence of heparin, our results indicate that IP_3 is not essential for the oscillation of outward current. However, in the presence of heparin the oscillation was unstable to interfering factors such as the removal of external Ca^{2+}, and the success rate of induction of the oscillation was reasonably lower than that in the absence of heparin. So it is our impression that IP_3 is not essential in the $[Ca^{2+}]_i$ oscillation, but plays an enhancing role in it. Similar results have been reported also in the heart [30]. The underlying cause of the effect of heparin on the oscillation is unclear. A recent paper [4], which demonstrated that IP_3 and Ca^{2+} act as co-agonists for the release of Ca^{2+} from internal stores in cerebellar Purkinje cells, indicates that this might be a plausible mechanism of the effect of heparin.

According to the model proposed by Berridge [3], our experimental findings suggest that the depolarization stimulus which leads to constant influx of external Ca^{2+} plays a role similar to IP_3 in the Ca^{2+} oscillation in non-excitable cells, while caffeine facilitates CICR by lowering its threshold. This combination of depolarization and caffeine induces repetitive release and uptake of Ca^{2+} by ryanodine-sensitive internal Ca^{2+} stores in vascular smooth muscle cells. The simulation programmed on the basis of the model proposed by Goldbeter et al. [8] accounts for the frequency response to lowering the external Ca^{2+} level. Figure 11 shows our model and simulation result, which explain the frequency response of the oscillation to lowering the level of external Ca^{2+}. In the simulation result, reducing Ca^{2+} influx rate by 30% of the control decreased frequency, while it increased the peak amplitudes of Ca^{2+} spikes. Our simulation result demonstrated that the mechanism of the oscillation was very similar to that of the rhythmic hyperpolarization of sympathetic ganglion cells induced by caffeine [17], and particularly similar to the $[Ca^{2+}]_i$ oscillation induced by K^+ depolarization combined with caffeine [21].

It was unexpected that hyperpolarization increased the oscillation frequency, since Ca^{2+} influx through L-type Ca^{2+} channel is supposed to decrease. There are two possible mechanisms underlying our result. First, the increase in the oscillation frequency could result not only from increase in Ca^{2+} influx, but also from the following factors: (1) an increase in the rate of Ca^{2+} release from internal store, (2) a decrease in the activity of the Ca^{2+} pump, and (3) a decrease in the threshold for CICR of the Ca^{2+} release channel in SR. So the decrease in oscillation frequency could be interpreted as a decrease in Ca^{2+} influx, only if none of the above factors

A



B

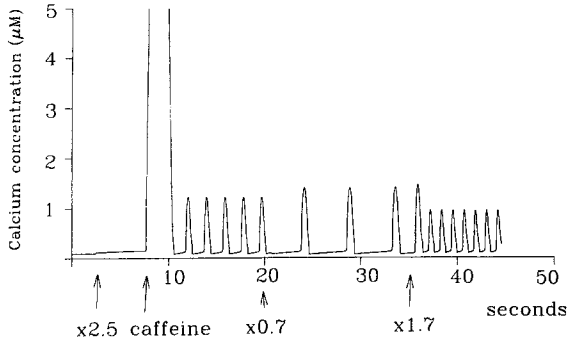


Fig. 11. A A schematic representation of the model for Ca^{2+} oscillation induced by caffeine. The following initial values were used for simulation: $X = 0.1$ μM, $Y = 180$ μM, $Ca_o = 1.8$ mM, $M_{cicr} = 0.7$ μM/ms, $K_{c1} = 0.9$, $K_{c2} = 10$ μM, $K_{p1} = 0.06$ μM, $K_{p2} = 0.02$ μM, $K_{L1} = 0.000002$/ms, $K_{L2} = 0.0006$/ms, $R = 10$. The following formulations were used for simulation, based on Kuba and Takeshita (1981):

$V_{cicr} = M_{cicr} \cdot X^4 \cdot Y^2 / (X^4 + K_{c1}^4)(Y^2 + K_{c2}^2)$

$V_{p1} = M_{p1} \cdot X^2 / (X^2 + K_{p1}^2)$, $V_{p2} = M_{p2} \cdot X^2 / (X^2 + K_{p2}^2)$

$V_{L1} = K_{L1}(Ca_o - X)$, $V_{L2} = K_{L2}(Y - X)$

$dX/dt = (V_{cicr} + V_{L1})/R + V_{L2} - V_{p1} - V_{p2}$,

$dY/dt = R \cdot V_{p2} - V_{cicr} - V_{L2}$

B Result of the simulation of $[Ca^{2+}]_i$ oscillation induced by caffeine based on the above formulations. At 2.5 s, K_{L1} was increased from control value, 2×10^{-6} to 5×10^{-6} to simulate the depolarization of the membrane potential. At 7.5 s, K_{c1} was decreased from 0.9 to 0.2 to simulate the effect of caffeine. At 20 s, K_{L1} was lowered by 1.5×10^{-6}/ms, which reduced the frequency of the oscillation while increasing the amplitudes of the $[Ca^{2+}]_i$ spikes. At 35 s, K_{L1} was increased from 3.5×10^{-6}/ms to 6×10^{-6}/ms, which affected the frequency and the amplitude of the oscillation oppositely. SL, Sarcolemma; subscription "1" and "2" in following abbreviations indicate "pertaining to SL" and "pertaining to SR", respectively; X, Y, Ca_o, the concentration of Ca^{2+} in cytosol, in Ca^{2+}-induced Ca^{2+} release (CICR) pool and in extracellular space, respectively; M_{p1}, M_{p2}, maximal rate of Ca^{2+} pump in SL_1 and SR_2; M_{cicr}, maximal rate of CICR; K_{p1}, K_{p2}, threshold constants for Ca^{2+} pumping in SL_1 and SR_2; K_{c1}, K_{c2}, threshold constants for CICR; K_{L1}, K_{L2}, rate constants for passive leak of Ca^{2+} into the cytosol through SL_1 and SR_2; V_{p1}, V_{p2}, pumping rates of Ca^{2+} pump in SL_1 and SR_2; V_{cicr}, rate of CICR; V_{L1}, V_{L2}, rate of Ca^{2+} leak through SL_1 and SR_2; R, volume ratio of cytosol to SR

other than Ca^{2+} influx rate is influenced by depolarization of the membrane potential. Specifically, Lydrup et al. [22] suggested the $[Ca^{2+}]_i$-lowering effect of high K^+ solution in mesotubarium smooth muscle might be considered to be due to stimulation of the SR Ca^{2+} pump, based on the observation that decline in $[Ca^{2+}]_i$ was diminished by ryanodine. The second possibility is that Ca^{2+} influx pathways other than via the L-type Ca^{2+} channel are involved. Most of the Ca^{2+} oscillations we observed were affected by organic Ca^{2+} channel blockers such as diltiazem and nifedipine. In one case, however, nifedipine (2 μM) had no effect although the oscillation disappeared during superfusion of Ca^{2+}-free solution containing 0.1 mM EGTA. The above finding indicates the possibility of Ca^{2+} influx through a pathway other than the L-type Ca^{2+} channel. The R-type Ca^{2+} channel [5] or Ca^{2+}-activated non-selective cation channel might be possible candidates for the entry pathways. Nevertheless the fact that effects of diltiazem and nifedipine are similar to the removal of external calcium suggests that non-inactivating L-type Ca^{2+} channel is a major Ca^{2+} influx pathway.

Most pulmonary arterial cells which generated periodic Ca^{2+} oscillation showed very scarce and small STOC activity, compared with cells which failed to generate Ca^{2+} oscillation. The STOC activity, if any, faded out in spite of sustained depolarization. The differences between the amplitude histograms of oscillating cells and non-oscillating cells provide evidence that the subsarcolemmal free Ca^{2+} level is more variable in the latter than in the former. Why are STOC and oscillating outward currents incompatible in a cell? The answer to this question is uncertain at this moment. We used $I_{K(Ca)}$ as an indicator of $[Ca^{2+}]_i$. However, $I_{K(Ca)}$ is a poor indicator of $[Ca^{2+}]_i$, because the dependence of the magnitude of $I_{K(Ca)}$ on $[Ca^{2+}]_i$ is not linear and $I_{K(Ca)}$ reflects only the Ca^{2+} concentration of the inner surface of the sarcolemma. Recently, the superficial buffer barrier hypothesis has indicated that the SR in smooth muscle cells is composed of superficial and deep SR which are functionally heterogeneous; superficial SR takes up Ca^{2+} entering through the sarcolemma by its Ca^{2+}-ATPase before it can activate myofilaments; it is then preferentially released toward the plasmalemma to be extruded into the extracellular space [27]. The higher Ca^{2+} concentration near the inner surface of the plasmalemma generated by the superficial buffer barrier would activate not only $I_{K(Ca)}$ but also Ca^{2+} extrusion mechanisms such as Na-Ca exchange and Ca-ATPase. The fact that $I_{K(Ca)}$ activity reflects the Ca^{2+} concentration of the inner surface rather than the overall $[Ca^{2+}]_i$ suggests that STOC activity could also reflect the Ca^{2+} efflux rate by the Ca^{2+} pump or by other Ca^{2+} extrusion mechanisms in the sarcolemma. If the hypothesis is correct, high STOC activity in a cell could be interpreted as the effective extrusion of Ca^{2+} released by superficial SR. The maintenance of high subsarcolemmal Ca^{2+} makes it possible that the amount of Ca^{2+} stored in the intracellular CICR pool is kept low, because the Ca^{2+} which entered through the sarcolemma would be trapped by superficial SR and released towards the sarcolemma and pumped out. The interesting paper by Xiong et al. [29] provided evidence of the existence of subsarcolemmal Ca^{2+} stores.

The $[Ca^{2+}]_i$ oscillation might not be peculiar to pulmonary arterial smooth muscle, because the induction of oscillations is also possible in smooth muscle cells of the rabbit ear artery under the same conditions (data not shown). However, we failed to elicit oscillations in intestinal smooth muscle cells which showed large and sustained STOC activities compared with vascular smooth

muscle cells. We have the impression that vascular smooth muscle cells which have relatively little STOC activity could charge their internal Ca^{2+} stores with entered Ca^{2+} which then leads the store to a readily releasable state.

There would be two possible mechanisms underlying the dissociative effects of forskolin and SNP on STOC and Ca^{2+} oscillation. One is the possibility that $I_{K(Ca)}$ responsible for STOC differs from $I_{K(Ca)}$ for oscillating outward current. The other possibility is that two different Ca^{2+} stores contribute to STOC and oscillating outward current. Saunders and Farley [25] suggested that small STOC arise from smaller conductance Ca^{2+}-activated K^+ (SK) channels than large-conductance K^+ (BK) channels which are responsible for large STOC. However, we have some evidence against this possibility. We also found two types of Ca^{2+}-activated K^+ channels whose conductances differ, but BK channels are most prevalent (about 10 000/cell), especially when the membrane potential is depolarized around 0 mV. The contribution of SK channels in STOC might be rather small when the membrane potential is depolarized as in our experimental conditions, since SK channels have little dependence on membrane potential. In addition, 1 μM forskolin had no significant effect on the outward current in patch-clamp experiments in the whole-cell configuration with KCl internal solution the free Ca^{2+} concentration of which was fixed at pCa 6.75 using 5 mM EGTA (data not shown). In order to see whether SK channels are more involved in either STOC and oscillating outward current, we examined the effects of 0.1 μM apamin on STOC and oscillating outward current, recorded from a non-oscillating cell and an oscillating cell, respectively. However, no appreciable effect of apamin was found in either case (data not shown). So the same type of channels, large-conductance BK channels, would be responsible for both of the oscillating outward currents and STOC. Therefore the dissociative effects of forskolin and SNP on them suggest that the oscillating outward currents we observed involve an internal Ca^{2+} store of large capacity which is more regulated by intracellular second messengers, compared with the Ca^{2+} store responsible for STOC. Hence the induction of periodic $[Ca^{2+}]_i$ oscillation by caffeine and depolarization would be useful to elucidate the effects of intracellular second messengers on SR function.

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