

The Characterization of the Increase of Membrane Conductance after Depolarization in Single Rat Adrenal Chromaffin Cells

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The conductance change evoked by step depolarization was studied in primarily cultured rat adrenal chromaffin cells using patch-clamp and capacitance measurement techniques. When we applied a depolarizing pulse to a chromaffin cell, the inward calcium current was followed by an outward current and depolarization-induced exocytosis was accompanied by an increase in conductance trace. The slow inward tail current which has the same time course as the conductance change was observed in current recording. The activation of slow tail current was calcium-dependent. Reversal potentials agreed with Nernst equation assuming relative permeability of Cs^+ to K^+ is 0.095. The outward current and tail current were blocked by apamin (200 nM) and d-tubocurarine (2 mM). The conductance change was blocked by apamin and did not affect membrane capacitance recording. We confirmed that conductance change after depolarization comes from the activation of the SK channel and can be blocked by application of the SK channel blockers. Consequently, it is necessary to consider blocking of the SK channel during membrane capacitance recording.

Key Words: Chromaffin cell, Small conductance calcium-activated potassium channel, Patch-clamp techniques, Membrane conductance, Membrane capacitance

INTRODUCTION

Chromaffin cells have been widely used for studying exocytotic mechanisms, as they have many characteristics of both neuronal and endocrine cells. A method of measuring vesicle fusion by detecting capacitance changes in voltage clamp has enabled neuroendocrine release to be assayed with time resolution in the order of milliseconds (Neher & Marty, 1982). Elevation of intracellular calcium is essential for the release of secretory vesicle (Augustine & Neher, 1992; von Rüden & Neher, 1993; Thomas et al, 1993; Heidelberger et al, 1994) and calcium entry through the voltage dependent calcium channel evoked by depolarization is the main source of cal-

cium (Douglas, 1968; Boarder et al, 1987). However, the entry of calcium seemed to induce not only exocytosis but also a calcium-dependent increase in conductance in the perforated patch or a low Ca buffering condition. This conductance change was known in bovine chromaffin cells, but it was simply attributed to an increase in access resistance (Engisch & Nowycky, 1996).

Integration of the inward calcium current, which is often used as a parameter of calcium entry, cannot be properly evaluated in the presence of a large outward current. When conductance changes become large, capacitance changes can be contaminated by the changes of conductance.

In the present experiment we identified the underlying mechanism of a calcium-dependent increase in membrane conductance (G_m) and revealed the small conductance calcium-activated potassium currents are the main cause of the conductance change.

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METHODS

Cell preparation

Cells were prepared as described in the earlier paper with some modification (Kim et al, 1995). In short, both adrenal glands were dissected from an adult Sprague-Dawley rat (150~200 g) of either sex. In a physiological buffer solution (PBS) containing (mM): 145 NaCl, 5.4 KCl, 1 NaH₂PO₄, 11.2 glucose, 15 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH adjusted to 7.4, the capsule and cortex were removed and the isolated medullae were minced into small pieces with scalpels. Cells were digested for 30 min at 37°C in a PBS containing: 2 mg/ml collagenase type II (Worthington Biomedical Co., Freehold, NJ, USA), 1 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA), and 0.1 mg/ml DNase type I (Sigma) and then for another 10 min in a PBS containing 1.25 mg/ml trypsin (Boeringer Mannheim, Mannheim, Germany). The digested tissues were rinsed 3 times with PBS and triturated gently 20 times with yellow tipped pipette. The dissociated cells were centrifuged down at 900 r.p.m. for 10 min and suspended in a media (Dulbecco's modified Eagle's medium with supplements: 10% fetal bovine serum, 50 unit/ml penicillin, 50 mg/ml streptomycin) and centrifuged again. The cell suspensions in the final amount of media (0.35 ml) were divided onto dry, polyethylenimine-coated, glass coverslips. After waiting for about an hour until cells were settled on the surface, the coverslips were flooded with media and kept for up to 5 days in a CO₂ incubator at 37°C.

Electrophysiological recording

The whole-cell (Hamill et al, 1981) and perforated whole-cell configuration (Horn & Marty, 1988) of patch clamp methods were employed to measure ionic currents and membrane capacitance (C_m) using an EPC-7 amplifier (List Electronic, Darmstadt, Germany). Experiments were performed on cells in culture for 1~3 days at room temperature (22~25°C). Borosilicate glass electrodes with tip resistance of 2~3 M Ω were used. Bath solution contained (mM): 140 NMG, 10 CaCl₂, 0.5 MgCl₂, 5.4 KCl, 10 HEPES and 10 glucose, pH adjusted to 7.4 by HCl. The internal solution for whole-cell recording (Fig. 4) contained (mM): 120 CsCl, 20 TEACl, 2 MgCl₂, 0.5 or 0.05 EGTA, 10 HEPES, pH adjusted to 7.4 by CsOH. An

experiment was started when the access resistance reached 20 M Ω in the case of the perforated patch configuration. The tip of the patch pipette was filled with an internal solution containing (mM): 90 CsSO₄, 50 CsCl, 2 MgCl₂, 20 HEPES, pH adjusted to 7.4 by CsOH and then the pipette was backfilled with the same internal solution containing 200 μ g/ml nystatin in dimethyl sulfoxide. All chemicals were purchased from Sigma. To monitor exocytosis, we used a software-based phase-sensitive detector (PSD) (O'Shaughnessy & Kim, 1995). In short, after neutralization of the capacitive transient using the EPC-7 amplifier's cancellation circuitry, a 10~15 mV r.m.s., 1 kHz sinusoidal stimulus was added to a holding potential of -80 mV. From the resulting currents, resistive and capacitive component were separated at two orthogonal phase angles, using the PSD. The correct angle for the PSD was located with changes of 100 fF in the potentiometer of the C_{slow} compensation circuit. At right angle, the deflection of a 100 fF change in the C_m trace was not projected on the conductance trace and the deflection also served as a calibration of the 100 fF changes in C_m .

RESULTS

Activation of the calcium current and the ensuing outward and inward current

Fig. 1A shows a representative recording of capacitance and conductance from rat chromaffin cells in the perforated patch configuration of a patch clamp technique. When we applied a 1 sec depolarizing pulse to a chromaffin cell, inward I_{Ca} was followed by the outward current and the depolarization-induced exocytosis was accompanied by an increase in G_m . Activation of an outward current and an increase of G_m was observed in most cells. The increase of the G_m made us suspect that exocytosis monitored by C_m might be contaminated with an artifact from a conductance change because a large change in cell conductance results in a shift of phase angle and might be a potential source of error. Fig. 1B shows a current recording from the same cell. Slow inward tail current was activated when the test potential was returned to -80 mV. The time course of the tail current was qualitatively the same as that of G_m . So, it is quite reasonable that the increase of G_m reflects the activation of certain channels in the membrane and

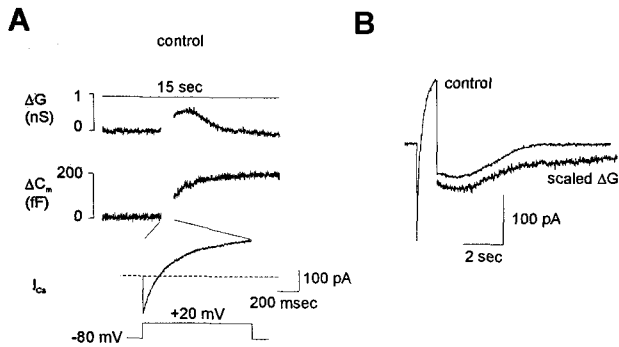


Fig. 1. Outward current and conductance change after depolarization in rat chromaffin cells in perforated patch configuration. A: Conductance, capacitance and current recording were obtained by a 1 sec depolarization from holding potential of -80 to $+20$ mV. B: The currents were evoked by a 1 sec depolarization from a holding potential of -80 to $+20$ mV. Arbitrarily scaled conductance trace was paralleled to compare the time course.

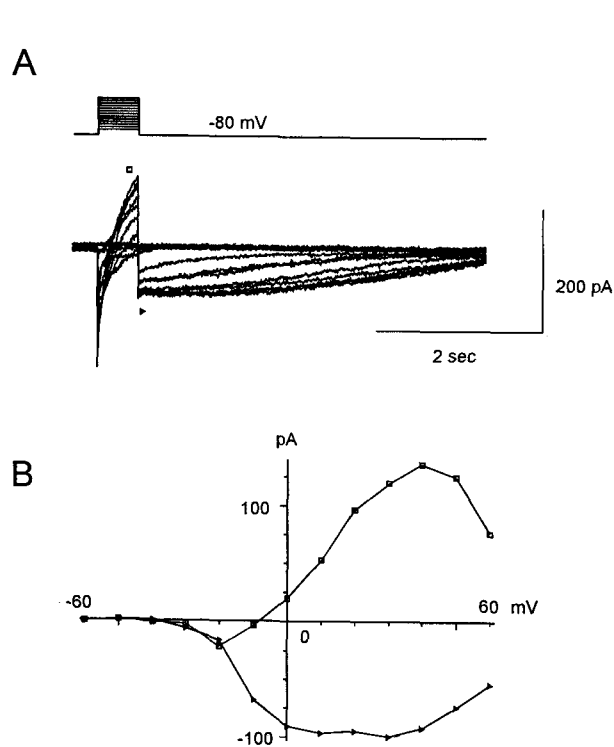


Fig. 2. Current-voltage relationship of outward and inward tail current. A: A series of 750 msec voltage pulses between -60 to $+60$ mV were applied from a holding potential of -80 mV. B: Plot of amplitude of outward currents (square) and tail currents (triangle) measured at 200 msec after depolarization.

not the gating charge movements of the Na^+ channel (Horrigan & Bookman, 1994).

Calcium dependency of the outward and inward current

The current-voltage relationship (Fig. 2A, B) shows the dependency of the slow tail current amplitude on the depolarizing potential. Activation of the tail currents required a depolarization positive to -30 mV, reaching a peak in a voltage range between $+10$ to $+30$ mV and decreasing with higher depolarization. In general the shape of current voltage relationship for the tail current suggests that the activation of the slow tail currents depends on calcium entries during depolarization.

As the duration of the conditioning pulse increased, so did the amplitude of the tail currents. As seen the cell shown in Fig. 3A, the amplitude of the tail current sharply increased after a 1 sec conditioning

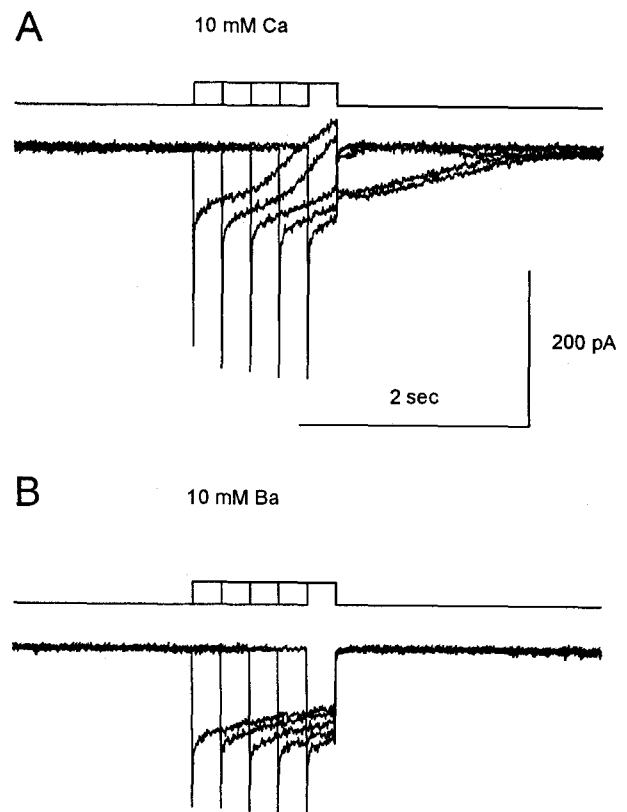


Fig. 3. Ca^{2+} dependence of tail current. A: The duration of depolarizing pulses increased by 250 msec. Bath solution contained Na^+ instead of NMG. B: External Ca^{2+} was changed to Ba^{2+} in the same cell.

pulse. Generally the activation of the tail current was evident in most cells in the perforated patch configuration when duration of conditioning pulses were larger than a second.

In the same cell, substitution of extracellular Ca^{2+} by Ba^{2+} resulted in an abolition of the tail current. Taken together, the above results show that the activation of the outward and inward tail currents are calcium-dependent.

K^+ permeability

To test which ion is responsible for the slow tail current, we measured the reversal potential with different $[\text{K}^+]_o$ in a whole-cell configuration. Return

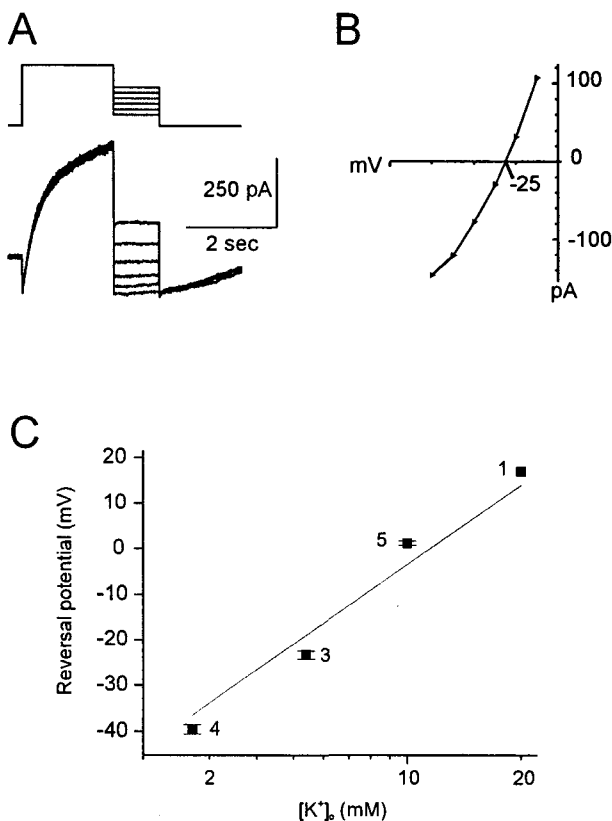


Fig. 4. K^+ permeability of tail currents. A: The cell was stepped to 20 mV for 2 sec from a holding potential of -80 mV, then returned to different potentials for 1 sec. Recovery of 30 s or more was allowed between pulses. $[\text{K}^+]_o=5.4$ mM. B: Current-voltage relationship from A. C: Plot of reversal potential against $[\text{K}^+]_o$. The numbers of cells tested are indicated. The data point were fitted to the modified Nernst equation $(-58\log(P_K[\text{K}^+]_o/P_{\text{Cs}}[\text{Cs}^+]_i))$, where $P_{\text{Cs}}/P_K=0.095$, $[\text{Cs}^+]_i=120$ mM.

voltage was changed to measure reversal potential. Fig. 4A shows the tail current recorded with 5.4 mM $[\text{K}^+]_o$. Fig. 4B shows the current-voltage relationship from the experiments in A. The measured reversal potentials from four values of $[\text{K}^+]_o$ were plotted in Fig. 4C. The values could be fitted by the modified Nernst equation $(-58\log(P_K[\text{K}^+]_o/P_{\text{Cs}}[\text{Cs}^+]_i))$ assuming that the relative permeability of Cs^+ to K^+ is 0.095. The slow tail current did not have Cl^- permeability. When internal or external $[\text{Cl}^-]$ were varied there was no change in the reversal potential (data not shown).

Therefore, it is concluded that the tail current at negative holding potentials following depolarization steps consists of K^+ currents through Ca^{2+} -activated K^+ channels ($I_{\text{K}(\text{Ca})}$) which has relatively high Cs^+ permeability. In chromaffin cells, there are at least two types of $I_{\text{K}(\text{Ca})}$ including large conductance (BK) and small conductance $I_{\text{K}(\text{Ca})}$ (SK) (Neely & Lingle, 1992). However, it is unlikely that the BK channel is responsible for the tail current since the BK channel is voltage-dependent and thus deactivated within 30 msec after returning to holding potential of -80 mV (Neely & Lingle, 1992) also it does not have a high Cs^+ permeability (Hille, 1992).

Blockade of the tail current and conductance change by the SK channel blocker

An SK channel blocker was applied to confirm whether the slow tail currents results from the activation of the SK channel (Park, 1994). (+)-tubocurarine (dTC), a diquaternary neuromuscular blocking agents, showed reversible block of the tail current at the concentration of 2 mM (Fig. 5A). The blocking

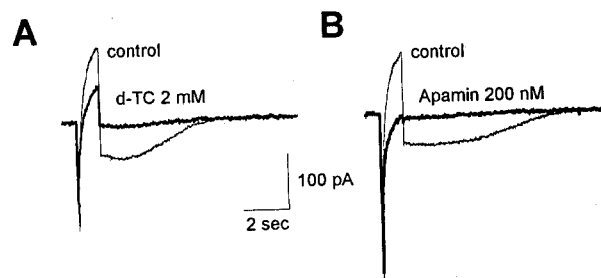


Fig. 5. Blocking of tail currents by d-tubocurarine, and apamin. A: Effect of external 2 mM d-tubocurarine on tail current. A one second depolarization was applied from a holding potential of -80 mV to 20 mV. B: Effect of external 200 nM apamin on tail current.

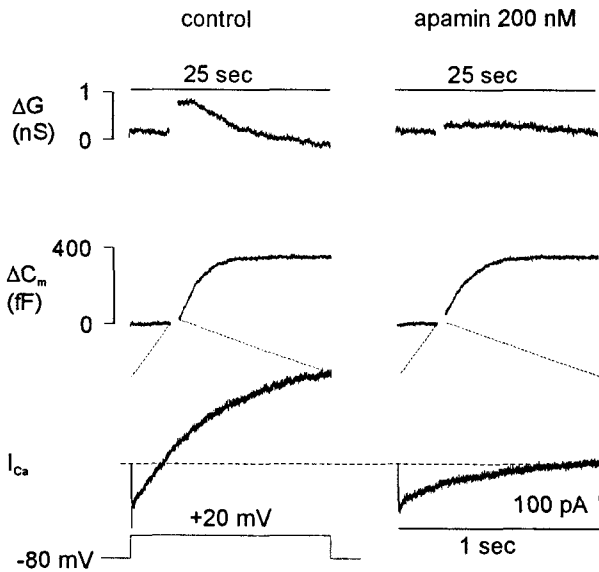


Fig. 6. Effect of apamin on membrane conductance. The pulse protocol was the same as in Fig 1A. Each pulse was spaced at interval of 150 sec. Apamin was applied 100 sec before the second pulse.

effect of 2 mM dTC shows a large cell to cell variation. Apamin (200~400 nM), an octadecapeptide from honey-bee venom, almost completely reduced the outward current as well as the inward tail current (Fig. 5B). The blocking was fast but washout was slow and incomplete. Fig. 6 shows that 200 nM of apamin effectively blocks conductance change as well as the outward current activated by step depolarization ($n=12$). Conductance change did not affect the membrane capacitance.

DISCUSSION

The inward tail current was not activated when the internal solution was buffered by a high EGTA, which is generally used to record I_{Ca} . The increase in the duration of the conditioning pulse resulted in the increase of the tail current amplitude. The current-voltage relationship of the tail current shows the dependency of slow tail current amplitude on the conditioning potential. Replacement of the charge carrier with barium resulted in the abolition of the tail current. From these results it is evident that the activation of the tail current is dependent on calcium entry through the voltage-dependent calcium channel and the following elevation of intracellular calcium

concentration. The tail current was not always observed, when we used the internal solution which contained 0.5 mM EGTA in whole-cell recording condition. By using the internal solution containing 0.05 mM EGTA, we activated the tail current. This suggests that the natural calcium buffer power in rat chromaffin is equivalent to a EGTA between 0.05 and 0.5 mM.

Ca^{2+} -activated conductances are potential obstacles in monitoring exocytosis by C_m measurements because large changes of G_m result in a shift of phase angle and might be a source of error (Neher & Marty, 1982). There are several calcium-activated channels which can be responsible for the change of membrane conductance after depolarization (Marty, 1989). Calcium-activated potassium channels, calcium-activated chloride channels, calcium-activated cation channels are the possible candidates for the change. Although as yet only two types of Ca^{2+} -activated K^+ channels have been reported in rat chromaffin cells, we had to rule out involvement of all of the above calcium activated conductances. The involvement of chloride was excluded by changing of the internal or external chloride. The involvement of non-selective cation channels were also excluded by replacement of internal Cs^+ to Li^+ (data not shown).

The measurements of reversal potential in different external K^+ concentrations proved K^+ selectivity of the channel. Both BK and SK channels have been reported in rat chromaffin cells (Neely & Lingle, 1992). Of the two, the SK channel was more probably involved in the tail current. Remaining BK currents after being inactivated were shown to deactivate within 30 msec following repolarization (Neely & Lingle, 1992). Conclusively, blocking of the tail current by apamin and dTC provided the concrete evidence that the tail currents are from the activation of SK channels.

The ion selectivity of the SK channel was described in rat chromaffin cells (Park, 1994). The SK channel has a permeability sequences similar to those of other K^+ channels except that Cs^+ was quite permeable. The relative permeability of Cs^+ to K^+ was reported to be 0.16. In this paper this value was somewhat smaller (0.095) but still high. Since our primary purpose was not to examine the ion selectivity of SK channels, we did not go further to elucidate the difference. In the presence of SK channels large outward currents are inevitable especially due to its high Cs^+ permeability.

Another important characteristics of the SK channel is that it is not voltage-dependent (Park, 1994). This enables SK to produce long hyperpolarization pauses after action potentials (after-hyperpolarization) as well as spike frequency adaptation during depolarizing influences (Yarom et al, 1985; Lang & Ritchie, 1990).

In conclusion we confirmed that conductance change after depolarization comes from the activation of the SK channel and that it can be blocked by an application of a SK channel blocker. Consequently, it is necessary to consider blocking the SK channel during membrane capacitance recording.

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