Block by Calcium of ATP-activated Channels in Pheochromocytoma Cells

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ABSTRACT We have investigated the effects of Ca^{2+} on Na^+ influx through ATP-activated channels in pheochromocytoma PC12 cells using single channel current recordings. Under cell-attached patch-clamp conditions with 150 mM Na^+ and 2 mM Ca^{2+} in the pipette, the unitary current activity showed an open level of about −4.3 pA at −150 mV. The channel opening was interrupted by flickery noise as well as occasional transition to a subconducting state of about −1.7 pA at −150 mV. The open level was decreased with increased external Ca^{2+}, suggesting that external Ca^{2+} blocks Na^+ permeation. We assessed the block by Ca^{2+} as the mean amplitude obtained with heavy filtration according to Pietrobon et al. (Pietrobon, D., B. Prod'hom, and P. Hess, 1989. *J. Gen. Physiol.* 94:1–21). The block was concentration dependent with a Hill coefficient of 1 and a half-maximal concentration of ~6 mM. A similar block was observed with other divalent cations, and the order of potency was $Cd^{2+} > Mn^{2+} > Mg^{2+} \fallingdotseq Ca^{2+} > Ba^{2+}$. High Ca^{2+}, Mg^{2+}, and Ba^{2+} did not block completely, probably because they can carry current in the channel. The block by external Ca^{2+} did not exhibit voltage dependence between −100 and −210 mV. In the inside-out patch-clamp configuration, the amplitude of inward channel current obtained with 150 mM external Na^+ was reduced by increased internal Ca^{2+}. The reduction was observed at lower concentrations than that by external Ca^{2+}. Internal Ba^{2+} and Cd^{2+} induced similar reduction in current amplitude. This inhibitory effect of internal Ca^{2+} was voltage dependent; the inhibition was relieved with hyperpolarization. The results suggest that both external and internal Ca^{2+} can block Na^+ influx through the ATP-activated channel. A simple one–binding site model with symmetric energy barriers is not sufficient to explain the Ca^{2+} block from both sides.

INTRODUCTION

Ion channels activated by extracellular ATP were first described by Kolb and Wakelam (1983) in cultured chick skeletal muscle cells and Krishtal, Marchenko, and Pidoplichko (1983) in mammalian sensory neurons. Since then, similar ATP-activated channels have been reported in a variety of cells such as smooth muscle cells (Benham, Bolton, Byrne, and Large, 1987; Benham and Tsien, 1987; Nakazawa and Matsuki, 1987; Friel, 1988), cardiac cells (Friel and Bean, 1988; Bean and Friel,

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1990), sensory neurons (Bean, 1990; Bean, Williams, and Ceelen, 1990), and pheochromocytoma cells (Inoue, Nakazawa, Fujimori, and Takanaka, 1989; Nakazawa, Fujimori, Takanaka, and Inoue, 1990*a*, 1991; Neuhaus, Reber, and Reuter, 1991). The ATP-activated channels are cation selective but do not discriminate strongly among cation species (e.g., Benham and Tsien, 1987; Friel, 1988; Bean, 1990; Nakagawa, Akaike, Kimitsuki, Komune, and Arima, 1990; Nakazawa et al., 1990*a*). Divalent cations as well as monovalent cations are permeable through these channels, and permeability of Ca^{2+}, estimated from reversal potentials of macroscopic currents under the whole-cell recording conditions, is several times larger than that of Na^+ (Benham and Tsien, 1987; Nakagawa et al., 1990; Nakazawa et al., 1990*a*; Thomas and Hume, 1990).

Ca^{2+} is not only a permeable cation but also reduces ATP-activated macroscopic currents (Honoré, Martin, Mironneau, and Mironneau, 1989; Nakazawa et al., 1990*a*). Honoré et al. (1989) speculated that the decrease in macroscopic currents at higher concentrations of Ca^{2+} may arise from a decrease in the concentration of the free form of ATP. However, external Ca^{2+} reduces ATP-activated single channel currents (Benham and Tsien, 1987; Krishtal, Marchenko, Obukhov, and Volkova, 1988; Nakazawa, Inoue, Fujimori, and Takanaka, 1990*b*; Neuhaus et al., 1991), which suggests that block occurs at least in part at the single channel level. The Ca^{2+} concentration necessary to block Na^+ influx through the channel is different among different cell types. Millimolar Ca^{2+} is necessary to block Na^+ influx through ATP-activated channels in smooth muscle from rabbit ear artery (Benham and Tsien, 1987) or pheochromocytoma cells (Nakazawa et al., 1990*b*; Neuhaus et al., 1991), whereas micromolar Ca^{2+} is enough to reduce the Na^+ influx in rat sensory neurons (Krishtal et al., 1988). These results suggest that Ca^{2+} might compete with Na^+ at a binding site in the channel pore. However, detailed analyses of the block by Ca^{2+} have been lacking. These analyses are important because they may also provide information to clarify the mechanisms underlying ion permeation through the ATP-activated channel.

In this study we characterized the reduction of the current amplitude associated with Ca^{2+} in ATP-activated channels of rat pheochromocytoma PC12 cells. The reduction of the current was observed with both external Ca^{2+} and internal Ca^{2+}. We analyzed and discussed the results based mainly upon an idea of block by Ca^{2+} of Na^+ permeation through the channel.

Some of the results in this report have already been presented in abstract form (Nakazawa and Hess, 1992).

METHODS

Cell Culture

Pheochromocytoma (PC12) cells were grown in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum, 7.5% calf serum, 4 mM glutamine, 1% penicillin-streptomycin, and 2 μg/ml nerve growth factor (NGF) prepared from mouse salivary glands. The cells were plated on glass coverslips coated with poly-L-lysine, and were used for experiments 7–10 d after plating. We used NGF-treated cells to record the single channel

activity because desensitization to ATP in these cells was reduced compared with untreated cells (Nakazawa et al., 1991).

Patch-Clamp Recordings

Single channel recordings were made with standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Cells were placed in an experimental chamber mounted on an inverted microscope (model IMT-2; Olympus, Tokyo, Japan). Under cell-attached conditions, the membrane potential outside the patch was nulled (Hess, Lansman, and Tsien, 1986) by superfusing the cells with a bathing solution of the following composition (mM): 140 K-aspartate, 5 EGTA, and 10 HEPES, titrated to pH 7.4 with KOH. In the case of recordings from inside-out patches, a bathing solution was selected so that the inside of the membrane patch was exposed to a desired ion composition. Patch pipettes were pulled from Boralex hematocrit micropipettes (P5251; USA Scientific Plastics, Ocala, FL), coated with Sylgard (Dow Corning Corp., Midland, MI), and fire-polished. The pipette solution contained 150 mM NaCl, 5 mM HEPES, and a desired concentration of Cl-salt of a divalent cation. The solutions were titrated to pH 7.5 with NaOH.

Current signals were recorded with an EPC-7 patch-clamp amplifier (LIST-Electronic, Darmstadt, FRG). The recordings were performed at room temperature (~25°C). The currents were filtered at 1 kHz with an eight-pole Bessel filter and sampled at 5 kHz. The signals were stored and analyzed on a PDP 11-73 laboratory computer.

Identification of ATP-activated Channels

ATP (adenosine 5'-triphosphate disodium salt; Sigma Immunochemicals, St. Louis, MO) was added to the pipette solution. After achievement of tight seals, the patch potential was routinely set at −150 mV to obtain large amplitude currents. With an appropriate concentration of ATP (e.g., 10 μM with 2 mM $CaCl_2$), single ATP-activated channels similar to those obtained with an outside-out patch from PC12 cells (Nakazawa et al., 1990*b*) could be recorded in almost all the patches (>90%; e.g., 26 of 28 patches for 10 μM ATP with 2 mM $CaCl_2$) with little simultaneous opening of multiple channels. Channel activity was not observed with ATP-free pipette solutions (n = 12). The appropriate concentration of ATP varied with the concentration of divalent cations included in the pipette solution: for example, 10 μM ATP was enough to activate the channels at 2 mM $CaCl_2$ but 1 mM ATP was required at 60 mM $CaCl_2$. This shift is consistent with a decrease in the free ATP concentration as suggested by Honoré et al. (1989). At higher concentrations (e.g., 1 mM ATP with 2 mM $CaCl_2$), simultaneous openings of a number of channels (usually more than five) were observed and this channel activity disappeared within 2 min, presumably due to desensitization to ATP (Bean and Friel, 1990; Nakazawa et al., 1990*a*). We usually used 10 or 100 μM ATP at lower divalent cation concentrations (<3 mM), 300 μM ATP at moderate divalent cation concentrations (3–10 mM), and 1 mM ATP at higher divalent cation concentrations (>10 mM).

Data Analysis

Amplitude histograms were obtained from the data directly, with the bin size equal to the maximal resolution (204.8 points/pA at the highest gain). To assess the blocking effects of divalent cations, the currents were refiltered heavily (usually 30 Hz), according to Pietrobon, Prod'hom, and Hess (1989). This method is based on an assumption that filtering will produce a mean current corresponding to the average proportion of time the channel spends at each conductance level (Pietrobon et al., 1989). The open level of the heavily filtered current was determined by manual fitting with a cursor on a PDP 11-73 computer. All the data shown in

this paper are representative of results obtained in at least three experiments under identical conditions.

RESULTS

General Features of ATP-activated Channel Currents in PC12 Cells

Fig. 1 *A* shows an ATP-activated channel current recorded from a cell-attached patch with 150 mM NaCl and 2 mM $CaCl_2$ in the pipette. The current included an open level (about −4.3 pA at −150 mV; O) and a closed state (C). The open level was concomitant with a flickery noise, which indicates an unresolved rapid kinetic behavior of the channel (see below). The opening of the channel contained periodic transitions to a subconducting state (about −1.7 pA at −150 mV; S), as is the case

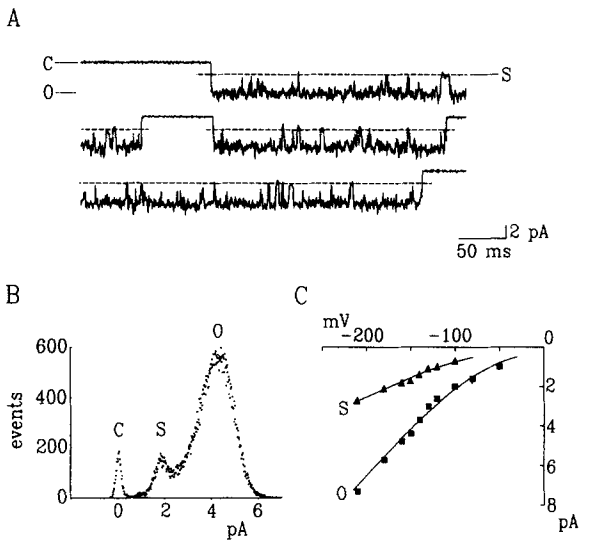

FIGURE 1. ATP-activated single channel currents with 150 mM Na^+ and 2 mM Ca^{2+} obtained under the cell-attached conditions. The pipettes contained 10 μM ATP. (*A*) Typical single channel activity recorded from a cell-attached patch. C and O are a closed and an open level, respectively. During openings, the channel exhibited periodical transitions to a subconducting level (*S*; indicated also by dashed lines). Holding potential was −150 mV. (*B*) Amplitude histogram for openings of a single channel. The data were sampled at 5 kHz from the same patch as in *A* and shown with a bin size of 400 ms. (*C*) Current–voltage relationship for the open level (*O*) and the subconducting level (*S*). The smooth curves were fitted to the data by eye.

with ATP-activated channels in rat sensory neuron (Krishtal et al., 1988). The existence of the subconducting state was further demonstrated by an amplitude histogram (Fig. 1 *B*). The amplitude of the open level (O) was increased with hyperpolarization and showed an inward rectification (Fig. 1 *C*) as seen in the ATP-activated macroscopic currents with the whole-cell recordings from PC12 cells (Nakazawa et al., 1990*a*) and sensory neurons (Bean et al., 1990), and single channel currents from rat sensory neurons (Krishtal et al., 1988; Bean et al., 1990). The amplitude of the subconducting level also increased with hyperpolarization (Fig. 1 *C*).

The flickery noise at the open level was observed over a range of Ca^{2+} concentrations tested (2–60 mM; Fig. 3 *A*). This noise was also observed when Ca^{2+} was removed and 10 mM EDTA or EGTA was added to the pipette solution (e.g., Fig.

3 *A*, top trace). A similar flickery noise was reported in rat sensory neurons (Krishtal et al., 1988; Bean et al., 1990).

We estimated the kinetics of the "unresolved" flickery noise using the approximation methods with beta distributions (FitzHugh, 1983; Yellen, 1984; Pietrobon et al., 1989). We assume here the following simple kinetic model:

$$\text{full open level} \underset{k_o}{\overset{k_s}{\rightleftharpoons}} \text{reduced level} \qquad \text{(Scheme 1)}$$

where k_s and k_o are rate constants for transitions to a full open level and a reduced level, respectively. We analyzed the data in two separate runs, assuming that the reduced level was either the subconducting state or the closed state. The amplitude histogram in the absence of Ca^{2+} could be fitted by a k_o of 42,000/s and a k_s of 55,000/s (Fig. 2 *A*) if the subconducting state was adopted as the reduced level, and it could be fitted by a k_o of 90,000/s and a k_s of 70,000/s if the closed state was adopted as the reduced level (Fig. 2 *B*). The k_s values suggest that the transitions to the

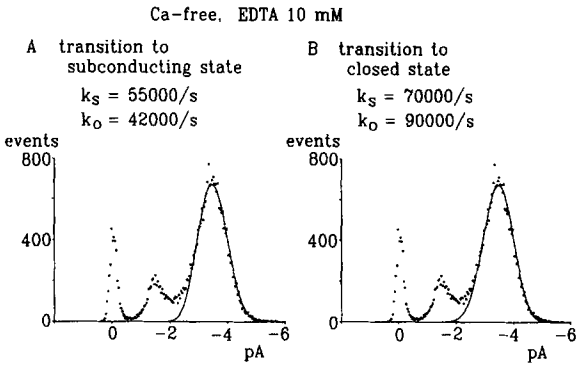

FIGURE 2. (*A* and *B*) Estimation of rate constants for Ca^{2+}-independent fast transitions by fitting with beta distributions. The peak for the open level in the absence of Ca^{2+} (with 10 mM EDTA) was fitted with beta distributions (*smooth curves*) assuming a full open level of 7.5 pA and a reduced current level of 1.8 (*A*) or 0 pA (*B*). Rate constants used for the fitting are indicated in each panel.

reduced level are more rapid than the conformational change in L-type Ca^{2+} channels associated with protons during Na^+ permeation (Pietrobon et al., 1989; Prod'hom, Pietrobon, and Hess, 1989).

Reduction of Current Amplitude by Extracellular Ca^{2+}

Fig. 3 shows single channel currents recorded at different Ca^{2+} concentrations. The open level became smaller with increased external Ca^{2+}, suggesting that Ca^{2+} blocks Na^+ permeation through the channel. The reduction of the current amplitude was indicated by a leftward shift of a peak corresponding the open level in the amplitude histogram (Fig. 3 *B*). With external $Ca^{2+} > 10$ mM, the peak corresponding to the open level overlapped with a peak corresponding to the subconducting level, and these two peaks could no longer be discriminated at 20 mM Ca^{2+} (Fig. 3 *B*).

The open level of the channel current cannot readily be determined from raw traces, due to the flickery noise (Fig. 3 *A*), or from amplitude histograms, due to overlapping of the subconducting level (Fig. 3 *B*). Therefore, to assess the concentration dependence of the channel block by external Ca^{2+}, we estimated mean channel

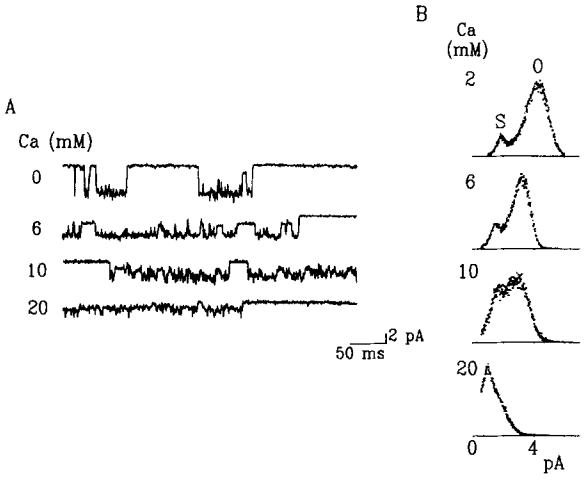

FIGURE 3. (*A*) Changes in single channel currents with increasing external Ca^{2+} (top to bottom). The traces were obtained from four different cell-attached patches held at −150 mV. The pipette solutions contained 150 mM Na^+ and various concentrations of Ca^{2+} (indicated on the left). Ca^{2+}-free solution (*top trace*) also contained 10 mM EDTA. The concentrations of ATP in the pipette solution were 100 μM (Ca^{2+}-free and 6 mM Ca^{2+}), 300 μM (10 mM Ca^{2+}), or 1 mM (20 mM Ca^{2+}). (*B*) Shift of the open level (*O*) toward smaller amplitude with increasing external Ca^{2+}. The amplitude histograms for the single channel current measured at −150 mV in cell-attached conditions with 150 mM Na^+ and various concentrations of Ca^{2+} (indicated on the left). The peak corresponding to the closed level was not plotted for clarity.

currents by heavy filtration (Fig. 4, *A* and *B*) according to Pietrobon et al. (1989). The decrease in the mean channel currents could be fitted by a curve with a Hill coefficient of 1 and an EC_{50} of 6 mM if we assume a minimal current level of 0.9 pA (Fig. 4 *C*). This minimal current level may be related to the permeation of Ca^{2+} through the channel (Benham and Tsien, 1987; Bean et al., 1990; Nakagawa et al.,

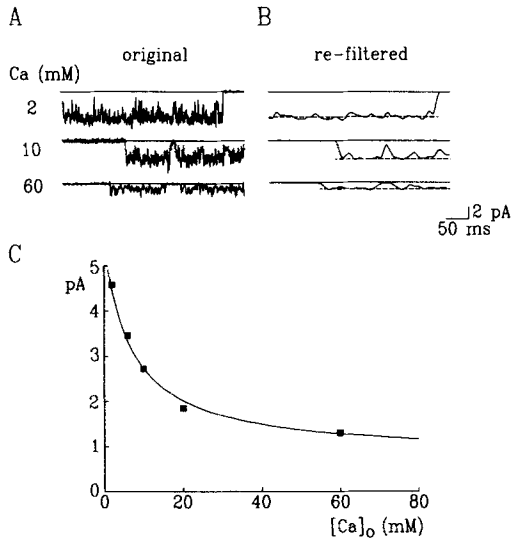

FIGURE 4. The estimation by heavy filtration (30 Hz) of the effects of external Ca^{2+} on single channel currents. The current was obtained from cell-attached patches held at −150 mV. (*A* and *B*) Single channel currents obtained at various external Ca^{2+} concentrations with 150 mM Na^+ (*A*), and their refiltered features (*B*). Straight and dashed lines indicate the zero current level and estimated mean open level, respectively. (*C*) Concentration dependence of the Ca^{2+}-induced decrease in mean single channel current obtained with the heavy filtration. The smooth curve was fitted to the data according to the formula $i = (i_{max} - i_{min}) \cdot [K_d/([Ca]_o + K_d)] + i_{min}$, using values of i_{max} = 5.5 pA, i_{min} = 0.9 pA, and K_d = 6 mM.

1990; Nakazawa et al., 1990*a*). We did not explain the significance of the minimal current level further, but subtracted this level in estimation of the concentration dependence for convenience.

The subconducting level also reduced with increasing external Ca^{2+}. In the case of data shown in Fig. 3 *A*, the amplitude directly determined from row traces was −1.8 pA without Ca^{2+} and −1.5 pA with 6 mM Ca^{2+}. The subconducting state was reduced less (to ~83% of control) than was the fully open level (to ~73% of control; Fig. 6 *C*). This may suggest that these two conducting states have a different affinity for Ca^{2+}. However, we did not pursue this problem further because the determination of the subconducting level was difficult with high concentrations of Ca^{2+}.

Effects of Other Divalent Cations

We examined whether other divalent cations (Mg^{2+}, Ba^{2+}, Cd^{2+}, and Mn^{2+}) exert a similar inhibitory effect on the ATP-activated channel. When Mg^{2+} (2 mM) or Ba^{2+} (10 mM) was added to the pipette solution instead of Ca^{2+}, the channel currents (Fig. 5 *A*) exhibited similar characteristics to those obtained with Ca^{2+} (Fig. 1). We could

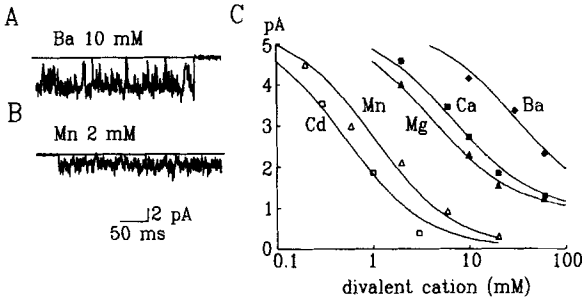

FIGURE 5. Block by various divalent cations of the ATP-activated single channel currents with 150 mM Na^+. Holding potential was −150 mV. Cell-attached conditions. (*A* and *B*) Single channel currents in the presence of 10 mM Ba^{2+} (*A*) or 2 mM Mn^{2+} (*B*). The Mn^{2+} solution also contained 0.6 mM Ca^{2+} to maintain the channel activity (see text). (*C*) The concentration–response relationships. The mean current was estimated with the heavy filtration as in Fig. 4. Smooth curves were fitted to the data assuming binding of divalent cations with a Hill coefficient of 1 and a maximal current of 5.5 pA. As for Mg^{2+}, Ca^{2+}, and Ba^{2+}, a minimal current of 0.9 pA was assumed.

not resolve channel currents when adding Cd^{2+} or Mn^{2+} to the pipette solution instead of Ca^{2+}; in most cases, the current from the patches with these cations exhibited a brief (<100 ms) "ATP-activated channel-like" activity followed by a continuous and inconsistent fluctuation of inward currents. We do not know whether this fluctuation is related to the ATP-activated channels or not. We tried recording channel currents by including 0.6 mM Ca^{2+} with these divalent cations to the pipette solution. This concentration of Ca^{2+} itself has only negligible effects, judging from the concentration dependence of block (Fig. 4) and the comparison of actual traces of the channel unitary current with 0.6 mM Ca^{2+} and those with 10 mM EDTA (not shown). With these conditions, we could analyze the effects of Cd^{2+} or Mn^{2+} (Fig. 5 *B*). We estimated the concentration dependence with the heavy filtration as we did for Ca^{2+} (Fig. 5 *C*). The order of potency was $Cd^{2+} > Mn^{2+} > Mg^{2+} \fallingdotseq Ca^{2+} > Ba^{2+}$. The channel current with Mg^{2+} and Ba^{2+} was not completely abolished at the

highest concentrations tested. Unlike Mg^{2+} and Ba^{2+}, the currents with Cd^{2+} and Mn^{2+} approached the zero-current level at higher concentrations (Fig. 5 *C*).

Lack of Voltage Dependence of Block by Ca^{2+}

Fig. 6 shows a comparison of the inhibitory effect of Ca^{2+} at three different potentials, −100, −150, and −210 mV. The concentration–response relationships at these three potentials were almost identical, suggesting that the Ca^{2+}-induced inhibition is not voltage dependent. We also compared the inhibitory effect of 10 mM Ba^{2+} and 0.6 mM Mn^{2+} at these three potentials; there was also no voltage dependence with these cations.

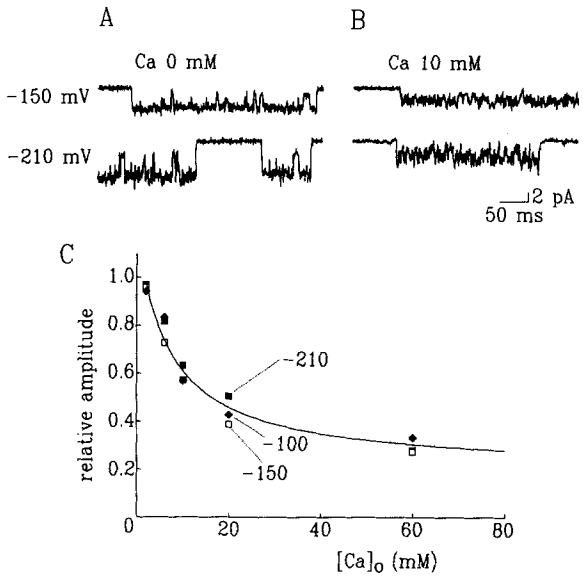

FIGURE 6. Lack of voltage dependence of inhibition induced by external Ca^{2+}. Data were obtained from cell-attached patches. (*A* and *B*) Single channel current recorded at −150 mV (*upper traces*) or −210 mV (*lower traces*) with Ca^{2+}-free (*A*) or 10 mM Ca^{2+}-containing external solution (*B*). The Ca^{2+}-free solution contained 10 mM EDTA. (*C*) Concentration–response relationship for Ca^{2+}-induced inhibition at −100 (*diamonds*), −150 (*open squares*), and −210 mV (*filled squares*). The mean current amplitude was estimated as in Fig. 4 and normalized to maximal current obtained with the Ca^{2+}-free solution. The smooth curve was drawn as in Fig. 4, assuming relative amplitude of a minimal current of 0.2.

Block of Na^+ Influx through the Channel by Divalent Cations from the Inside

Fig. 7 *A* shows the Na^+ influx through the channel measured at −150 mV in an inside-out patch. When the current was measured in a bathing solution (inside) without divalent cations, the channel current was quite similar to that in the cell-attached condition (see Fig. 1). The current was significantly inhibited by addition of low concentrations of Ca^{2+} (0.6 and 2 mM) to the bath solution (Fig. 7 *A*). Similar inhibition was observed with internal Ba^{2+} and Cd^{2+}. As the block by the internal divalent cations was also accompanied by a flickery noise, we estimated the mean channel currents with heavy filtration (Fig. 7 *B*) as in the case of the current block by external divalent cations (Fig. 5). Both Ca^{2+} and Ba^{2+} blocked the channel

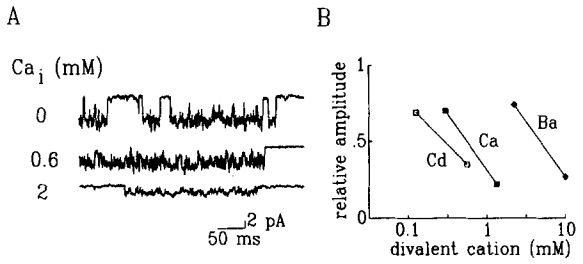

FIGURE 7. Block by internal divalent cations of Na^+ influx through ATP-activated channels recorded from inside-out patches. Both internal and external solutions contained 150 mM Na^+. The patches were held at −150 mV. (*A*) Decrease in single channel current amplitude by internal Ca^{2+}. The current trace obtained with nominally Ca^{2+}-free or 0.6 or 2 mM Ca^{2+}-containing internal solution (top to bottom). (*B*) Concentration–response for internal Ca^{2+}-, Ba^{2+}-, and Cd^{2+}-induced inhibition. The mean channel current was obtained with the heavy filtrations as in Fig. 4 and normalized to maximal current with divalent cation–free internal solution.

current from the inside more potently than from the outside. Cd^{2+} blocked from the inside as potently as from the outside.

Unlike block by external divalent cations, the block by internal divalent cations exhibited clear voltage dependence (Fig. 8). The block by 0.6 mM internal Ca^{2+} and by 3 or 10 mM internal Ba^{2+} (Fig. 8, *A* and *B*) was reduced at strongly hyperpolarized potentials. Fig. 8 *A* shows an example with 10 mM internal Ba^{2+}; the channel current at −150 mV was reduced to 28% of control, but the current at −210 mV was reduced only to 96% of control if comparing the mean amplitude after heavy filtration.

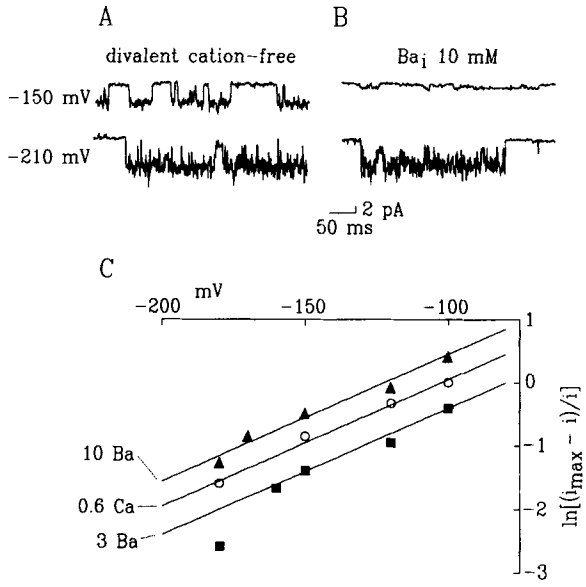

FIGURE 8. Voltage dependence of internal divalent cation–induced inhibition of the Na^+ influx through the ATP-activated channel. Inside-out patches with 150 mM Na^+ in both sides. (*A* and *B*) Removal of internal Ba^{2+}-induced inhibition by hyperpolarization. The single channel current at −150 mV (*upper traces*) or −210 mV (*lower traces*) in the absence (*A*) or presence (*B*) of 10 mM Ba^{2+} inside was shown. (*C*) Voltage dependence of inhibition induced by 0.6 mM Ca^{2+} (*open circles*) and 3 (*filled squares*) or 10 mM (*filled triangles*) Ba^{2+}. The mean current amplitude was estimated by heavy filtration and normalized to maximal current with divalent cation–free internal solution. Inhibition induced by these divalent cations was plotted semilogarithmically against holding potentials (see Discussion). Straight lines with a single slope were fitted to the data.

DISCUSSION

We have characterized the reduction of the ATP-activated channel by external and internal divalent cations, including Ca^{2+}. Ca^{2+} is permeant in ATP-activated channels (Benham and Tsien, 1987; Bean et al., 1990; Nakagawa et al., 1990; Nakazawa et al., 1990*a*), as also supported by the existence of a minimal conductance level remaining after the block of single channel currents by high concentrations of external Ca^{2+} (Fig. 4). Thus, our results can probably be explained by the blocking effects of Ca^{2+} on Na^+ permeation occurring in the channel pore. Several reports have already referred to the relationship between single ATP-activated channel currents and external divalent cations, including Ca^{2+}. The ATP-activated channels in sensory neurons are highly sensitive to Ca^{2+}. Krishtal et al. (1988) reported that the channel current is halved at Ca^{2+} concentrations as low as 100 μM, and the amplitude is monotonically reduced up to 5 mM Ca^{2+}. On the other hand, the channels in smooth muscle cells from rabbit ear artery (Benham and Tsien, 1987), like those in pheochromocytoma cells (Nakazawa et al., 1990*b*; Neuhaus et al., 1991), were less sensitive to Ca^{2+}: only a small reduction of the channel current was observed with millimolar concentrations of Ca^{2+}. Detailed properties of these Ca^{2+}-induced inhibitory effects such as voltage dependence have not been previously reported.

The Na^+ influx through the ATP-activated channel was blocked by various divalent cations from both the outside (Fig. 5) and the inside (Fig. 7). The block by these divalent cations may be mediated through the same binding site as that for the block by Ca^{2+}. Like Ca^{2+}, Mg^{2+} and Ba^{2+} did not block completely even at high concentrations (Figs. 4 and 5). This is consistent with their permeability to the ATP-activated channels (Benham and Tsien, 1987; Honoré et al., 1989). On the other hand, the currents disappeared with Cd^{2+} and Mn^{2+} at the highest concentrations (Fig. 5 *C*). Thomas and Hume (1990) reported a similar abolishment of the ATP-activated macroscopic current in chick skeletal muscle. The disappearance of the channel current may suggest that these divalent cations are impermeable to the ATP-activated channels.

Estimation of Position of the Binding Site for the Block by Ca^{2+}

Ca^{2+} blocked Na^+ influx through the ATP-activated channel from both the outside and the inside of the channel. In this section, we estimate the position of the Ca^{2+} binding site for the channel block. For simplicity, we assume that the reduction in the current amplitude with increased Ca^{2+} is solely due to block of Na^+ permeation in the channel pore, and that the appearance of the subconducting level (Fig. 1) and the unresolved rapid transitions observed as a flickery noise (Fig. 2) are not influenced by an increase in Ca^{2+} concentrations, although we have no evidence for this assumption.

Our hypothesis is that the block occurs when the binding site, which normally mediates Na^+ permeation, is occupied by Ca^{2+}. We assume a simple one–binding site model with symmetrical energy barriers for the permeation of Ca^{2+}:

$$Ca^{2+}_{out} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Ca^{2+}\cdot X \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} Ca^{2+}_{in} \qquad \text{(Scheme 2)}$$

where X is the binding site, and k's are rate constants (Hille, 1992*b*). The rate constant for the association of external Ca^{2+} (k_{assoc}) equals k_{+1}, and that for dissociation (k_{dissoc}) is given by $k_{dissoc} = k_{-1} + k_{+2}$. If k_{-1} is negligible, i.e., Ca^{2+} occupies the binding site and then mostly proceeds toward the inner mouth, the requirement for the lack of voltage dependence (Fig. 6) is that the binding site for Ca^{2+} is located at the midpoint of the electrical distance of the channel pore (Hille, 1992*a*). If k_{+2} is negligible, i.e., external Ca^{2+} occupies the binding site and then is completely "repelled" toward the outer mouth, the location is estimated to be very close to the outer mouth of the channel pore. In a general case where neither k_{+2} nor k_{-1} is negligible, the location can be regarded as somewhere in the half of the electrical distance closer to the outer mouth.

The block by internal Ca^{2+} exhibited voltage dependence (Fig. 8). If scheme 2 is applied to this block, $k_{assoc} = k_{-2}$, and $k_{dissoc} = k_{+2} + k_{-1}$. We assume that k_{-1} is negligible because internal Ca^{2+} or other divalent cations may not permeate through the channel pore at the holding potentials we used (more negative than −100 mV). With this assumption, this block can be regarded as a Boltzmann equilibrium distribution under a potential difference (Woodhull, 1973; Hille, 1992*c*). Current remaining after block by divalent cations (i) is given by

$$i = i_{max} \cdot \{K_B/([B]_{in} + K_B)\} \quad (1)$$

where i_{max} is current without divalent cations, $[B]_{in}$ is the concentration of internal divalent cations, and K_B is a dissociation constant. An equation that is available for estimation of voltage dependence of the block is (Ravindran, Schild, and Moczydlowski, 1991):

$$\ln \{(i_{max} - i)/i\} = \ln \{[B]_{in}/K_B(0)\} - 2 \cdot \theta EF/RT \quad (2)$$

where $K_B(0)$ is the value of K_B at zero membrane potential, θ is the electrical distance measured from the inner mouth of the channel, and E, F, R, and T are their general meanings. We plotted results of linear regression analyses of the block by internal divalent cations based on Eq. 2 in Fig. 8 *C*. The block by 0.6 mM Ca^{2+} or 3 or 10 mM Ba^{2+} could be fitted by lines assuming a single value of θ (= 0.25). Thus, the binding site may exist around 25% of the total electrical distance from the inner mouth of the pore.

The location of the binding site estimated from the block by external Ca^{2+} (between 0 and 50% from the outside) does not agree with the value estimated from the block by internal Ca^{2+} (25% from the inside). The discrepancy suggests that a simple one–binding site model cannot account for the channel block by Ca^{2+}. This may indicate that more than one binding site exists in the channel pore. A model consisting of a single binding site with asymmetrical energy barriers can also account for the discrepancy of electrical distance.

Comparison of the Block by Ca^{2+} with That in Other Channels

Na^+ permeation through *N*-methyl-D-aspartic acid (NMDA) receptor channels is blocked by millimolar Ca^{2+} (Mayer and Westbrook, 1987; Ascher and Nowark, 1988). In nicotinic acetylcholine receptor channels expressed from complementary DNA, millimolar external Ca^{2+} or Mg^{2+} blocks inward currents carried by monovalent

cations (Imoto, Methfessel, Sakmann, Mishina, Mori, Konno, Fukuda, Kurasaki, Bujo, Fujita, and Numa, 1986; Imoto, Busch, Sakmann, Mishina, Konno, Nakai, Bujo, Mori, Fukuda, and Numa, 1988). Block by millimolar Ca^{2+} of monovalent cation permeation was also recently reported for a non-NMDA type of glutamate receptor channels (Gu and Huang, 1991) and neuronal nicotinic receptor channels (Vernino, Amador, Luetje, Patrick, and Dani, 1992). Perhaps ATP-activated channels have a binding site in the channel pore qualitatively similar to those in other ligand-gated channels. The block of Na^+ permeation by millimolar Ca^{2+} was also reported for the voltage-gated Na^+ channels (Ravindran et al., 1991). In contrast, Na^+ permeation through voltage-gated Ca^{2+} channels is blocked by micromolar Ca^{2+} (Almers and McCleskey, 1984; Hess and Tsien, 1984; Hess et al., 1986; Lansman, Hess, and Tsien, 1986).

Contribution of the Block by Ca^{2+} to ATP-activated Macroscopic Current

Extracellular Ca^{2+} reduces the ATP-activated macroscopic current in a concentration-dependent manner (Honoré et al., 1989; Nakazawa et al., 1990*a*). In addition to the block by Ca^{2+} in the channel pore described in this report, another mechanism that may account for the inhibition of the macroscopic current has been reported: namely, a decrease in the free form of ATP (Honoré et al., 1989). The necessity of higher ATP concentrations to record channel activity with higher Ca^{2+} concentrations (see Methods) is indicative of decrease of free ATP concentration. It is difficult to determine the contribution of each mechanism to the inhibition of the macroscopic current and cellular responses initiated by this current (for reviews see Bean and Friel, 1990; Bean, 1992; Inoue and Nakazawa, 1992). The block by competitive permeation at least can account for the inhibition of the macroscopic current because the K_d of 6 mM for the Ca^{2+}-induced single channel inhibition (Fig. 4) is almost the same as that obtained for the inhibition of the macroscopic current in PC12 cells (Nakazawa et al., 1990*a*). The complete disappearance of the macroscopic current at the highest concentrations of Ca^{2+} (Nakazawa et al., 1990*a*) or Ba^{2+} (Honoré et al., 1989) suggests that a decrease in the free ATP concentration does occur because the inhibition by Ca^{2+} in the channel pore leaves a conducting level at high external Ca^{2+}.

Flickery Noise at the Open Level

The flickery noise at the open level of the ATP-activated channel was observed in the absence of Ca^{2+} (Fig. 3 *A*). However, this result does not exclude the possibility that Ca^{2+} modifies the transitions observed as this flickery noise. In addition, the noise at the open level in the presence of Ca^{2+} may also contain a component that arises from the block of Na^+ by external Ca^{2+} in the channel pore. This is speculated from the following consideration. If association of Ca^{2+} to the binding site responsible for the block of the channel pore is $\sim 10^8/(M \cdot s)$ as in the case of various kinds of Ca^{2+}-binding molecules (see Discussion section of Lansman et al., 1986), the association rate constant with 2 mM Ca^{2+}, for example, will be 200,000/s. This value is only three to four times larger than the estimated k_s value for the Ca^{2+}-independent flickery noise (55,000 or 70,000/s in Fig. 2). The flickery noise at the open levels in the presence of external Ca^{2+} in this report (Figs. 1 and 3) and those in

ATP-activated channels in other types of cells (e.g., Nakazawa and Matsuki, 1987; Krishtal et al., 1988; Bean et al., 1990) may consist of both the Ca^{2+}-independent transitions and the block by Ca^{2+} of the channel pore.

Other Interpretations of the Present Results

The block by internal Ca^{2+} can probably be interpreted as a phenomenon in the channel pore because of its voltage dependence (Fig. 8). However, the lack of voltage dependence of the block by external Ca^{2+} can also be explained by other mechanisms. One possible mechanism is an allosteric effect: external Ca^{2+} may bind to a site outside of the channel pore and induce conformational changes so that the channel current reduces to the subconducting level (Fig. 1) or some other smaller conductance level.

Another mechanism that can contribute to reduction of the channel current is changes in surface potentials: neutralization by external Ca^{2+} of negative surface potential may reduce effective concentration of Na^+ and in turn decrease the current. In the models proposed for various types of channels, the surface charge density ranges from one negative charge per 100 A^2 to one per 400 A^2 (Hille, 1992*d*). The relation between surface potential (ψ) and charge density (σ) in a solution containing Na^+ and Ca^{2+} is given by an equation (Grahame, 1947; Hille, 1992*d*):

$$\sigma^2 = 2\epsilon\epsilon_0 RT[[Na^+](\exp(-F\psi/RT) - 1) + [Ca^{2+}](\exp(-2F\psi/RT) - 1)] \quad (3)$$

where ϵ_0 is the dielectric constant ($\sim$80 in water; Hille, 1992*d*), and ϵ, F, R, and T have their general meanings. If a density of one negative charge per 200 A^2 is assumed, surface potential calculated using Eq. 3 is -66 mV with 150 mM Na^+, or -55 mV with 150 mM Na^+ and 6 mM Ca^{2+}. Na^+ concentration at the surface ($[Na^+]_s$) is given by an equation (Hille, 1992*d*):

$$[Na^+]_s = [Na^+]\exp(-F\psi/RT) \quad (4)$$

With Eq. 4, the calculated reduction by Ca^{2+} of surface potential (-66 to -55 mV) results in a 46% decrease in Na^+ concentration at the surface. Thus, the neutralization of surface potential can also explain the observed reduction of the channel current. However, if we also apply this idea to the effects of other divalent cations (Fig. 5), a simple scheme where neutralization depends only on charges but not on ion species is not sufficient, and we must postulate a binding site that exhibits ion selectivity. In conclusion, the block by external divalent cations of ATP-activated channels suggests the existence of a specific binding site whether the phenomena are explained by block of channel pore, allosteric inhibition, or change in surface potentials.

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