Block of Cardiac ATP-sensitive K⁺ Channels by External Divalent Cations Is Modulated by Intracellular ATP

Evidence for Allosteric Regulation of the Channel Protein

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ABSTRACT   We have investigated the interactions between extracellular divalent cations and the ATP-sensitive potassium channel in single guinea pig ventricular cells and found that, under whole-cell patch clamp recording conditions, extracellularly applied Co^{2+}, Cd^{2+}, and Zn^{2+} block current through the ATP-sensitive K channel (I_{KATP}). The respective K_d's for block of I_{KATP} by Cd^{2+} and Zn^{2+} are 28 and 0.46 µM. The K_d for Co^{2+} is >200 µM. Extracellular Ca^{2+} and Mg^{2+} appear to have no effect at concentrations up to 1 and 2 mM, respectively. Block of I_{KATP} by extracellular cations is not voltage dependent, and both onset and recovery from block occur within seconds. Single-channel experiments using the inside-out patch configuration show that internally applied Cd^{2+} and Zn^{2+} are not effective blockers of I_{KATP}. Experiments in the outside-out patch configuration confirm that the divalent cations interact directly with I_{KATP} channel activity. Our study also shows that this block of I_{KATP} is dependent on intracellular ATP concentrations. Under whole-cell conditions, when cells are dialyzed with $[ATP]_{pipette} = 0$, the degree of cation block is reduced. This dependence on intracellular ATP was confirmed at the single-channel level by experiments in excised, inside-out patch configurations. Our results show that some, but not all, divalent cations inhibit current through I_{KATP} channels by binding to sites that are not within the transmembrane electric field, but are on the extracellular membrane surface. The interdependence of internal ATP and external divalent cation binding is consistent with an allosteric interaction between two binding sites and is highly suggestive of a modulatory mechanism involving conformational change of the channel protein.

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INTRODUCTION

Modulation of ionic currents by cations can be studied to probe the functional architecture of ion channels and their membrane environment. Divalent and monovalent ion-induced gating shifts of most voltage-dependent ion channels have been analyzed within the framework of Guoy-Chapman surface potential theory to provide insights into the location and density of surface charges on or near channel proteins (Hille, Woodhull, and Shapiro, 1975; Gilly and Armstrong, 1982a, b; Krafte and Kass, 1988; Armstrong and Cota, 1990; Zalman, Dukes, and Morad, 1991; see also Hille, 1992). Investigation of voltage-dependent block of ionic current has also provided information about intra-pore cation binding sites that define the functional properties of the open ion channel (Woodhull, 1973; Vandenberg, 1987; Matsuda, Saigusa, and Irisawa, 1987; Matsuda, 1988). Divalent ions may also contribute to the biochemical regulation of channel activity. For example, the slow cardiac delayed rectifier potassium channel is inhibited by intracellular magnesium (Mg_i^{2+}) in a manner that is consistent, not with occlusion of the channel pore, but rather with the regulation of channel dephosphorylation (Duchatelle-Gourdon, Hartzell, and Lagrutta, 1989; Tarr, Trank, and Goertz, 1989; Duchatelle-Gourdon, Lagrutta, and Hartzell, 1991).

This study focuses on interactions between adenosine-5′-triphosphate (ATP)–sensitive potassium channels and extracellular divalent cations. ATP-sensitive potassium channels, first discovered in cardiac cells in 1983 (Noma, 1983), are regulated by the metabolic state of the cell. Channel activity is controlled by intracellular ATP and by changes in the ATP/ADP ratio (Findlay, 1988; Lederer and Nichols, 1989). Current through ATP-sensitive K channels (I_{KATP}) is sensitive to internal divalent cations: intracellular Ca^{2+}, Mg^{2+}, Ba^{2+}, and Sr^{2+} block I_{KATP} in a voltage-dependent manner, suggesting binding sites within the membrane electric field or channel pore (Findlay, 1987; Horie, Irisawa, and Noma, 1987). Studies of interactions of external divalent ions and I_{KATP} have been more limited. Extracellular Ba^{2+} and Cs^+, well-known potassium channel blockers, block I_{KATP} in a voltage-dependent manner, consistent with an intra-pore binding site (Quayle, Standen, and Stanfield, 1988), but extracellular Ca^{2+} and Mg^{2+} have no effect (Horie et al., 1987). In this study, we find unexpectedly that I_{KATP} is inhibited by other extracellularly applied divalent ions, but in a voltage-independent manner. This inhibition is observed for channel activity induced by low levels of both intracellular ATP and pinacidil, a drug that has been shown to increase I_{KATP} in cardiac cells (Arena and Kass, 1989a, b). Our results suggest that these divalent cations inhibit current through I_{KATP} channels by binding to sites that are not within the transmembrane electric field, but are on the extracellular membrane surface, and are distinct from previously described intra-pore cation binding sites. Binding of external divalent ions to these sites may have important regulatory consequences for the I_{KATP} channel. We also find that the ability of the externally applied divalent cations to inhibit I_{KATP} is dependent on intracellular ATP concentrations. This result suggests an allosteric interaction between an external divalent cation binding site with an intracellular nucleotide binding site.

These results have been reported in preliminary form (Kwok and Kass, 1992, 1993).

METHODS

Cell Isolation and Recording Procedures

Single myocytes were isolated from either ventricule of adult male or female guinea pigs (Charles River Laboratories, Wilmington, MA) weighing ~200–350 g. The isolation procedure is a modification of that of Mitra and Morad (1985), which has been previously described (Arena and Kass, 1988).

Current measurements were obtained in the inside-out and outside-out single-channel and whole-cell configurations of the patch clamp procedure as described by Hamill, Marty, Neher, Sakmann, and Sigworth (1981). Pipettes were pulled from glass (Clay Adams, Inc., Parsippany, NJ). The resistances of the pipettes were typically 2–3 MΩ for the whole-cell experiments and 5–15 MΩ for the single-channel experiments. Series resistance compensation was adjusted to give the fastest possible capacity transients without producing ringing. Recordings were made at room temperature (20–25°C) from a Plexiglas chamber mounted on the stage of an inverted Olympus microscope. Solution change was accomplished either by exchanging the entire bath volume or by using a multibarreled ejection pipette which allows for fast local solution exchange. Current was measured with a Yale IV patch clamp amplifier and analyzed with the pCLAMP (Axon Instruments, Inc., Foster City, CA) software package. For experiments carried out in the inside-out and outside-out patch configurations, currents were low-pass filtered at 500 Hz and sampled at 1 kHz. An opening was interpreted as a crossing of a 50% threshold level from the baseline to the first open channel amplitude. Because of multiple channels in a patch, open probability, P_o, was calculated in two ways. In most experiments, P_o was calculated as a cumulative open probability, i.e., as a fraction of the total length of time the channels were in an open state over the total recording duration. This provided us with a qualitative, but comparative, method in monitoring effects of the divalent cations on I_{KATP} at the single-channel level.

In one set of single-channel experiments where changes in P_o were monitored at different ATP concentrations, we carried out a more quantitative approach in calculating P_o. The number of channels in a patch was determined from overlapping channel activity at zero ATP, where we assumed that the channels were maximally activated. We then calculated P_o in the following manner. For a one-channel patch, the probability that the patch shows no channel activity is given by $P_c = 1 - P_o$. Thus, for n channels in the patch, $P_c = (1 - P_o)^n$. The probability that the patch shows no channel activity, P_c, is calculated as a fraction of the total length of time where no channel activity was detected over the total recording duration. Solving for P_o gives $P_o = 1 - P_c^{1/n}$.

Ionic Conditions

Isolated cells were initially placed in a standard Tyrode solution consisting of (mM): 132 NaCl, 4.8 KCl, 3 MgCl₂, 1 CaCl₂, 5 dextrose, and HEPES, pH 7.4. In the whole-cell configuration, after establishment of whole-cell voltage clamp, the external bath solution was changed to one that isolates potassium channel current (mM): 132 N-methyl-D-glucamine, 1 CaCl₂, 10 HEPES, 2 MgCl₂, 5 dextrose, 5 KCl, and 200 nM nisoldipine, pH 7.4 with HCl. Divalent cations (Co²⁺, Cd²⁺, and Zn²⁺) were added as needed. The standard pipette solution for whole-cell experiments contained (mM): 110 K-aspartate, 10 HEPES, 1 MgCl₂, 1 CaCl₂, 11 EGTA, and 0.5–5 K₂ATP, pH 7.4 with KOH.

For experiments done in the inside-out patch configuration, cells were initially placed in standard Tyrode's solution. After establishing a gigaohm seal, the bath solution was changed to one containing (mM): 110 K-aspartate, 5 EGTA, 5 HEPES, 1 MgCl₂, and 1 K₂ATP, pH 7.4 with KOH. The patch was then excised, exposing the intracellular side to this solution, and the ATP

concentration was changed to activate or inhibit channel activity. The standard pipette solution (extracellular side) contained (mM): 110 K-aspartate, 1 $CaCl_2$, 5 HEPES, and 1 $MgCl_2$, pH 7.4 with KOH.

Seals were established in a similar manner for outside-out patch experiments, but in these cases patches were excised into normal whole-cell extracellular solutions. The pipette solution was identical to that used in whole-cell experiments except that the ATP concentration was 100 μM.

Pinacidil, a gift of Lilly Research Laboratories (Indianapolis, IN), was dissolved as a 10-mM stock solution in 3% 1N HCl and diluted appropriately before use. Glibenclamide (Sigma Chemical Co., St. Louis, MO) was dissolved as a 1-mM stock solution in 0.1 N NaOH and also diluted appropriately before use. Nisoldipine, a gift from Miles Laboratories Inc. (New Haven, CT), was dissolved in polyethylene glycol as a 1-mM stock solution and diluted to the appropriate concentrations before use.

Voltage Protocols

Whole-cell currents were measured at the end of 50-ms voltage steps applied from a −40-mV holding potential to minimize overlap with the delayed rectifier components (Arena and Kass, 1988; Sanguinetti and Jurkiewicz, 1990). Sodium channel currents were inactivated at the −40-mV holding potential and L-type calcium channel activity was eliminated by nisoldipine (200 nM) (Kass, 1982).

In single-channel experiments, the criteria used to identify I_{KATP} channels were: (a) unitary current amplitude; (b) sensitivity to intracellular ATP; and, in some cases, (c) inhibition by glibenclamide (Arena and Kass, 1989b). Experiments in the inside-out patch configuration were conducted under either symmetrical (140 mM) or physiological (extracellular [K] = 5 mM; intracellular [K] = 140 mM]) potassium concentrations. Experiments in the outside-out patch configuration were conducted at physiological potassium concentrations.

Cell Dialysis

In whole-cell experiments, the cytosolic ATP concentration is controlled by the recording pipette ATP concentrations. To allow for sufficient diffusional exchange between the cytosol and the reservoir of the pipette, control traces were typically recorded after 10–15 min. The extracellular (bath) solution was then changed to one containing pinacidil and currents were recorded 5–15 min later, allowing a total dialysis time of 15–30 min. After changing the bath solution to one containing pinacidil and the test divalent cation, another 5–10 min elapsed before currents were measured. Hence, ∼25–40 min have elapsed by the time the pinacidil-activated current has reached steady state. Given the variations in the sizes of the cells (as estimated by cell capacitance to be ∼100 pF) and the access resistance of the pipettes, this elapsed time would have allowed ∼90–95% of complete diffusional exchange between the cytosol and the pipette reservoir (Pusch and Neher, 1988).

RESULTS

Divalent Cation Block of Pinacidil-induced I_{KATP}

To test for interactions between I_{KATP} channels and externally applied divalent cations, we initially induced I_{KATP} under whole-cell recording conditions with pinacidil, a drug that has previously been shown to enhance the activity of ATP-sensitive K^+ channels in cardiac ventricular cells (Arena and Kass, 1989a, b). Fig. 1 illustrates the effects of externally applied Zn^{2+} on pinacidil-induced currents in a ventricular cell

dialyzed with 2 mM ATP. The current traces illustrate the effects of both pinacidil and Zn^{2+} at voltages positive and negative to V_K. In the absence of Zn^{2+}, pinacidil (100 μM) induced a time-independent current at the positive voltage, but had little effect on currents measured negative to V_K as has previously been described for this drug under the same experimental conditions (Arena and Kass, 1989a). Addition of
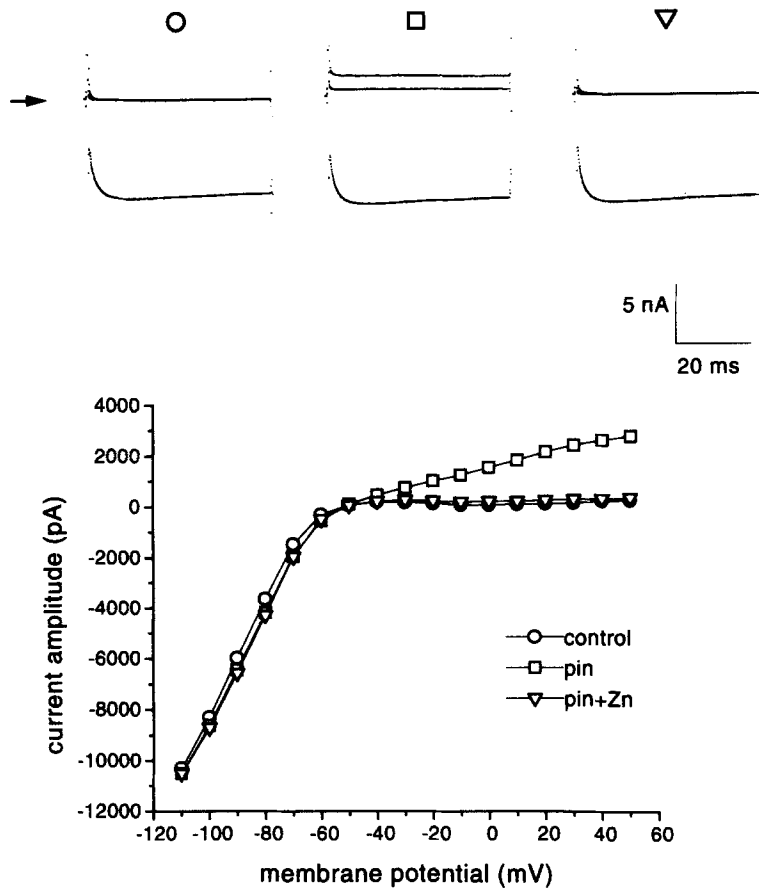

FIGURE 1. External Zn^{2+} inhibits pinacidil-induced current: whole-cell conditions. Whole-cell currents were measured during 50-ms test pulses applied from a -40-mV holding potential in control (O), in the presence of 100 μM pinacidil (□), and in the presence of 5 μM Zn^{2+} and pinacidil (∇). Current traces shown were recorded at membrane potentials of -100, -20, and $+20$ mV. The arrow indicates the zero current level. Plotted in the graph are currents measured at the end of the test pulse vs. pulse voltage for each condition.

Zn^{2+} (5 μM) to the external solution markedly decreased the current induced by pinacidil, but had no effect on currents negative to V_K (inward rectifier channel currents), suggesting Zn^{2+} block of I_{KATP} but not inward rectifier currents. The effect of Zn^{2+} on the current–voltage relationship measured at the end of 50-ms test pulses, summarized in the lower half of the figure, confirms that the effects of Zn^{2+} are most

pronounced over the voltage range previously reported to be dominated by I_{KATP} in whole-cell recordings (Arena and Kass, 1989a). Block of the pinacidil-induced current by external Zn^{2+} was reversible by washout (data not presented). Because we found that the inhibitory effects of Zn^{2+} were much less pronounced in cells dialyzed with very low ATP concentrations (see below), we maintained $[ATP]_i$ between 0.5 and 5 mM in our pipette solutions unless otherwise specified in the experiments that follow.

Zn^{2+} was not the only divalent ion that inhibited I_{KATP} during extracellular application. We also found that external Cd^{2+} and Co^{2+} reduced I_{KATP} in a manner consistent with the effects of Zn^{2+}. However, I_{KATP} did not appear to be sensitive to the presence of Ca^{2+} or Mg^{2+} (millimolar concentration range) in the extracellular solution, consistent with the results of Horie et al. (1987).
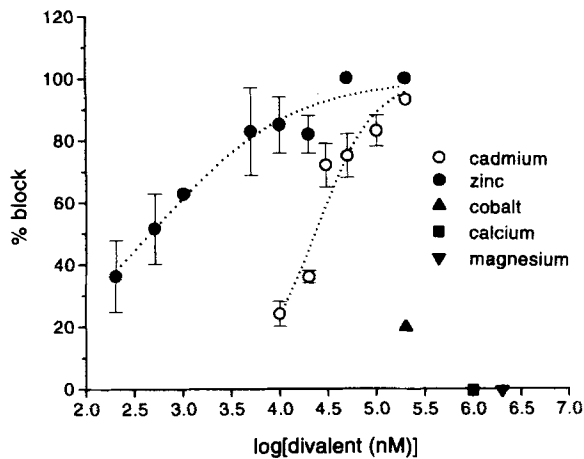

FIGURE 2. Concentration–response curves for divalent cation block of pinacidil-activated I_{KATP}. Pinacidil-sensitive current at $+10$ mV was determined by subtracting the control currents (pinacidil-free), or in some cases, the glibenclamide-sensitive currents from the pinacidil-activated currents. Current amplitude was measured at the end of a 50-ms pulse from a holding potential of -40 mV. Percent block was measured as percent red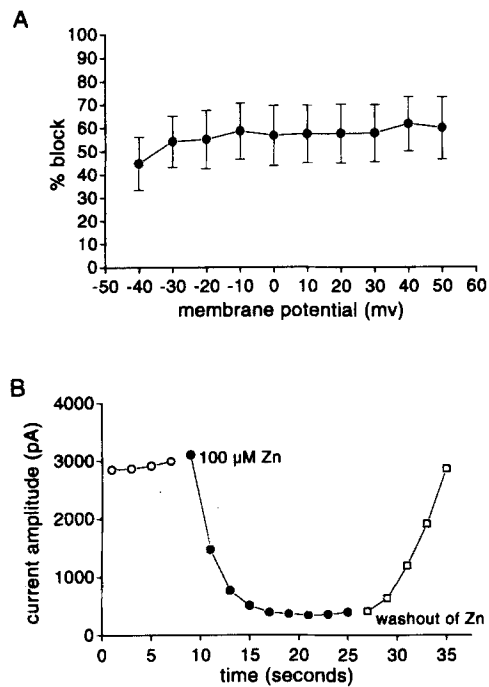uction in the pinacidil-sensitive current in the presence of divalent cations. The curves were fit by nonlinear regression to a simple sigmoidal function: %block = $1/[1 + (K_d/[C])^n]$, where [C] is the divalent cation concentration, K_d is the cation concentration for half-maximal effect, and n is the Hill coefficient. Each point represents an average of three or more experiments and the error bars indicate standard error. The Hill coefficient and K_d for Zn^{2+} were 0.7 and 460 nM, respectively, and for Cd^{2+} were 1.4 and 28 µM.

Concentration Dependence of Divalent Cation Block

The differences between the inhibitory activity of pinacidil-induced current by the divalent cations we studied are summarized in Fig. 2, which shows the percentage of pinacidil-induced current blocked as a function of the concentration of divalent cation tested. The order of blocking potency that we found is: Zn^{2+} ($K_d = 0.46$ µM) > Cd^{2+} ($K_d = 28$ µM) > Co^{2+} (20% block at 200 µM) > Ca^{2+}, Mg^{2+}. The Hill coefficients obtained by fitting the data with a simple sigmoidal function (%block = $1/[1 + (K_d/[divalent])^n]$), were ~1 for both Zn^{2+} and Cd^{2+}, indicative of noncooperativity.

Evidence for an External Divalent Ion Regulatory Binding Site

Voltage-independent block. Because it is known that I_{KATP} is blocked by intracellular divalent cations (Findlay, 1987; Horie et al., 1987), it was important to determine experimentally whether the inhibition of pincidal-induced current by extracellular Cd^{2+} and Zn^{2+} which we have measured is due to binding of these cations to intracellular sites. To test for this possibility, we first tested for an effect of membrane potential on block by these divalent ions, because intracellular divalent ion block of I_{KATP} has been shown to be voltage dependent (Quayle et al., 1988). If block is due to the binding of the blocking ion to a site within the membrane electric field and/or

FIGURE 3. Evidence that the divalent ion binding site is extracellular. (*A*) Block of I_{KATP} is not voltage dependent. Block of I_{KATP} was determined by measuring the decrease in current amplitude of the pinacidil-sensitive current caused by 500 nM externally applied Zn^{2+} relative to control (Zn^{2+} free). This %block was measured at a series of voltages and plotted against test membrane potential in the figure. No statistical difference was observed for percent block of pinacidil-sensitive current at −40 and +50 mV (Student's t test, 95% confidence level). Each point represents an average of three experiments and the error bars indicate standard error. (*B*) Onset of and recovery from block of I_{KATP} by external Zn^{2+} is rapid. Whole-cell I_{KATP} was recorded at +10 mV in the continued presence of pinacidil and monitored every 2 s. Control records were obtained (O) and the cell was then exposed to 100 μM Zn^{2+} (●), followed by washout of this divalent cation (□). The plot shows current amplitude measured at the end of a 200-ms pulse as a function of time during the experiment.

channel pore, block should be enhanced at negative membrane potentials for extracellularly applied cations. Fig. 3 *A* shows that block of pinacidil-induced current by extracellularly applied Zn^{2+} is not voltage dependent. Plotted in the figure is the percent block of current measured at the end of test voltage pulses vs. test pulse voltage. There was no significant difference between the inhibition of I_{KATP} by Zn^{2+} at −40 and +50 mV, providing evidence against an intra-pore/membrane field binding site. Similar results (not shown) were found for Cd^{2+} block of pinacidil-induced currents. The observed lack of voltage-dependent block by Zn^{2+} and Cd^{2+} supports the view that these divalent cations do not block I_{KATP} by binding to intra-pore sites.

Time Course of Onset of Block of I_{KATP} by Zn^{2+} and Cd^{2+}. The experiments summarized in Fig. 3 *A* rule out the possibility of an intra-pore or trans-membrane field binding site location, but they do not rule out the possibility of an intracellular binding site location that is not within the pore/field and thus not subject to modulation by membrane potential. If this were the case, then the blocking ions would have to cross the membrane via hydrophilic pathways (channels) in order to access these binding sites. We measured the time course of the onset of and recovery from block by Zn^{2+} and Cd^{2+} in order to test for a possible intracellular location. Here we changed solutions surrounding the test cells with a local solution changer (see Methods) and monitored membrane current every 2 s at +10 mV after exposing the cell to pinacidil. Application of Zn^{2+} (Fig. 3 *B*) or Cd^{2+} (data not shown) resulted in rapid block and recovery from block after return to cation-free solution. In Fig. 3 *B*, block of I_{KATP} reaches steady state in ~5 s and washout is complete in ~10 s. Similar results were obtained in three experiments each for Zn^{2+} and Cd^{2+}.

The rapid onset of and recovery from block by Zn^{2+} and Cd^{2+} do not support the hypothesis of ions traversing a membrane barrier. This result and the lack of voltage-dependent block, however, are consistent with external cation binding sites. A more stringent and direct test of this hypothesis is to determine whether internally applied Zn^{2+} and Cd^{2+} can block I_{KATP} with similar potency as when applied externally.

Effects of Internal Zn^{2+} and Cd^{2+}

To investigate the effects of internally applied Zn^{2+} and Cd^{2+}, experiments were carried out in the inside-out, excised patch configuration of patch clamp, which provided direct access to the intracellular side of the membrane. In this approach, ATP-sensitive potassium channels could be activated either by lowering intracellular ATP concentrations or by addition of pinacidil.

Fig. 4 shows that I_{KATP} channels are not inhibited by intracellularly applied Zn^{2+}. Current traces, recorded in 15-s segments under symmetrical K (135 mM) conditions, revealed I_{KATP} channel activity when cytoplasmic ATP was reduced from 1 to 0.1 mM (Fig. 4, *A* and *B*), as has been previously reported (Arena and Kass, 1989*b*). Under these conditions, ATP-sensitive K channels can be identified as the dominant 1.5-pA (corresponding to 50 pS) events measured at +30 mV, and it is clear that subsequent application of Zn^{2+} (100 μM) did not affect the amplitude of these events (Fig. 4 *C*). The insensitivity to intracellular Zn^{2+} indicated in the current traces of this figure is confirmed by the corresponding cumulative amplitude histograms (Fig. 4, *A–C*). The histograms also suggest that the cumulative probability of opening, P_o, is not affected by the internal application of Zn^{2+}, despite the fact that this Zn^{2+} concentration is ~200-fold greater than the K_d obtained under whole-cell conditions (Fig. 2). This was verified by computing P_o (see Methods): $P_{o,100 \mu M ATP} = 0.96$ and $P_{o,ATP+Zn} = 0.83$ for the data presented in the figure. Under similar experimental conditions, intracellular Cd^{2+} also had no effect on I_{KATP} channel activity (data not shown).

Because pinacidil was used to activate I_{KATP} in the whole-cell experiments, the effects of intracellular Zn^{2+} and Cd^{2+} on I_{KATP} channels activated by pinacidil in the inside-out patch configuration were also investigated. Fig. 5 illustrates one of these experiments and shows that single-channel activity induced by pinacidil is not
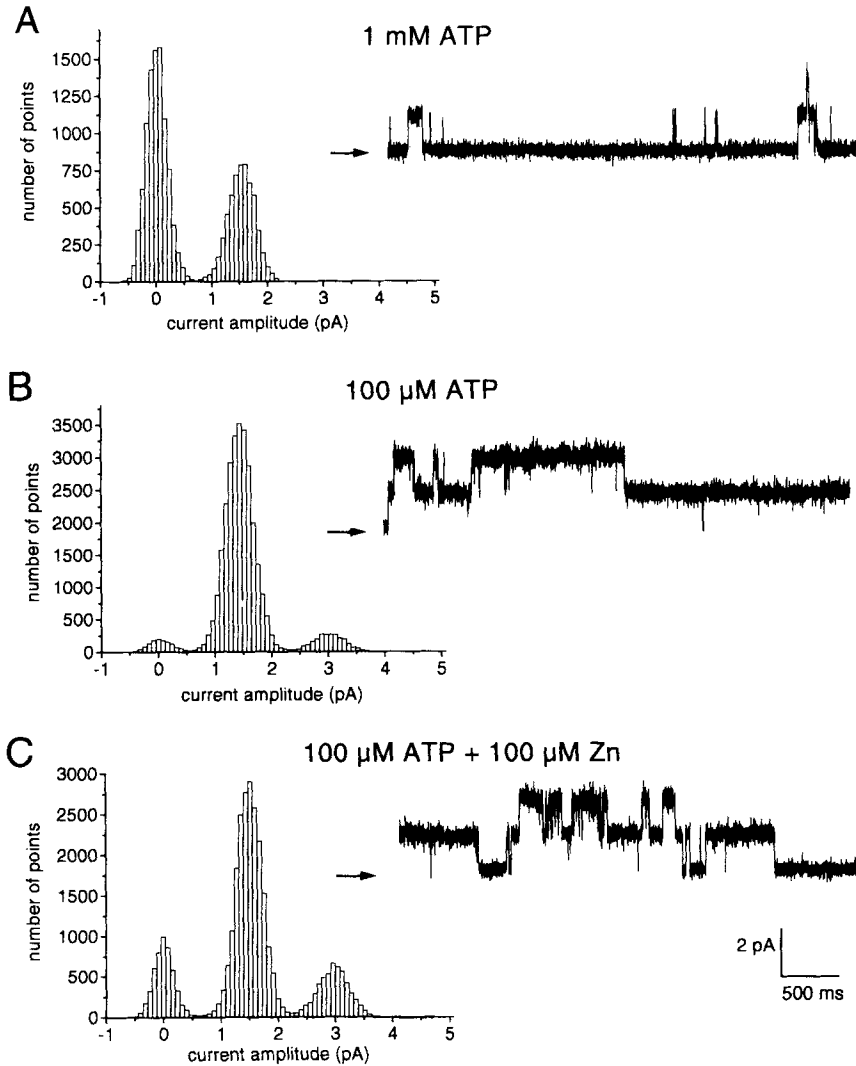
FIGURE 4. I_{KATP} channel activity is not affected by Zn^{2+} applied to the cytosolic membrane face in excised patch recordings. Single-channel current traces and accompanying all-points histograms obtained from an inside-out membrane patch are shown in control (1 mM ATP, A), in the presence of 100 μM ATP (B), and in 100 μM Zn^{2+} in the continued presence of 100 μM ATP (C). These solutions correspond to cytosolic conditions. Membrane potential was set at +30 mV and single-channel currents are indicated by upward deflections. Zero current levels are indicated by arrows. Histogram shown in A is obtained from a current recording duration of 15 s and those in B and C are obtained from a recording duration of 30 s.

inhibited by intracellular Cd^{2+}. I_{KATP} channel activity was stimulated by 200 μM pinacidil in the presence of 1 mM ATP (Fig. 5, A and B). Cd^{2+} affected neither the single-channel amplitude nor the P_o's of the pinacidil-induced I_{KATP} channel activity (Fig. 5 C). The computed cumulative P_o's were: $P_{o,pin} = 0.29$ and $P_{o,pin+Cd} = 0.36$. Similar results were obtained with Zn^{2+} on pinacidil-induced single-channel activity.
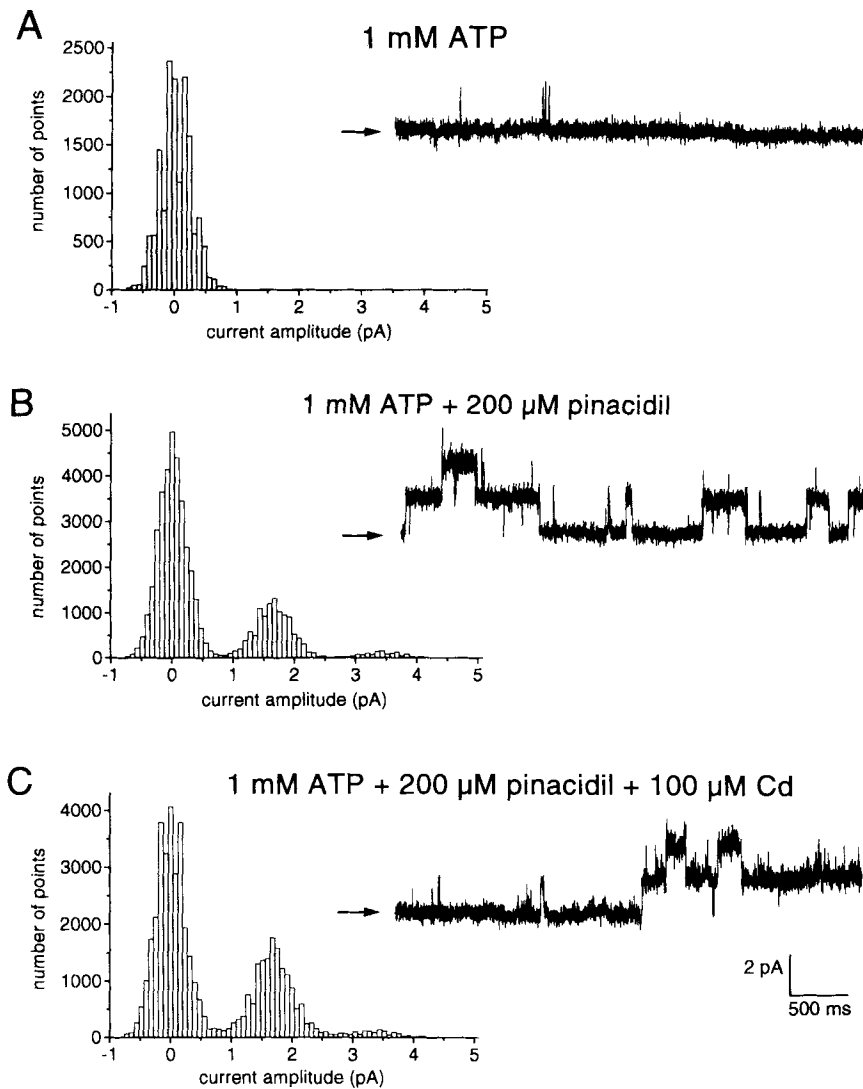
FIGURE 5. Pinacidil-induced single-channel activity is not blocked by cytosolic Cd^{2+}. Single-channel current traces and accompanying all-points histograms obtained from an inside-out membrane patch in symmetrical (140 mM) potassium are shown during control (1 mM ATP, A), activation by 200 μM pinacidil in the presence of 1 mM ATP (B), and exposure to 100 μM Cd^{2+} in the presence of both ATP and pinacidil (C). Membrane potential was set at +30 mV, and single-channel currents are indicated by upward deflections. Zero current levels are indicated by arrows. Histogram in A is obtained from a current recording duration of 15 s and those in B and C are from a recording duration of 45 s.

In a total of 12 inside-out patch experiments, we failed to measure reversible inhibition of channel activity by either Zn^{2+} (100 μM) or Cd^{2+} (100 μM) during cytosolic application, regardless of whether channel activity was activated by low ATP or pinacidil.

Zn^{2+} and Cd^{2+} Block I_{KATP} Channels Induced by Reduction of ATP

The insensitivity to intracellularly applied Zn^{2+} and Cd^{2+}, the rapid onset of block by extracellular divalent cations, and the apparent lack of voltage-dependent block all support the hypothesis that Zn^{2+} and Cd^{2+} bind to external sites. However, the question remains whether external divalent cation block of I_{KATP} channels is somehow pinacidil dependent, since our whole-cell data were obtained only after pinacidil-induced activation of I_{KATP}. To test this possibility directly, outside-out patch experiments were conducted so that I_{KATP} could be activated by lowering cytoplasmic ATP without the use of drug. Fig. 6 shows the effect of 100 µM Cd^{2+} on I_{KATP} channel activity in an outside-out patch recorded under conditions that resemble those for our previous whole-cell recordings. Physiological potassium concentrations ($[K]_{out} = 5$ mM, $[K]_{in} = 140$ mM) were used, cytoplasmic ATP was set to 100 µM in order to reveal I_{KATP} channels, and currents were recorded at 0 mV, a voltage at which pronounced whole-cell I_{KATP} can be measured. Under control conditions multiple channel openings can be seen in the current traces and in the corresponding events histogram. In the presence of Cd^{2+}, the number of open channels decreased, with the corresponding cumulative P_o decreasing from 0.64 to 0.21, but the apparent single-channel conductance was not affected. Upon washout, multiple channel openings were detected again with the cumulative P_o increasing to 0.75. The corresponding current–voltage relationship for the single-channel current is presented in Fig. 7 in both the presence and absence of Cd^{2+}. The channel conductance, both in control and in the presence of Cd^{2+}, is 14 pS, in agreement with previously reported I_{KATP} conductances recorded under physiological conditions (Kakei, Noma, and Shibasaki, 1985; Spruce, Standen, and Stanfield, 1987). These data indicate that the effect of divalent cation block of I_{KATP} is to decrease the number of channel openings, and that the block of I_{KATP} by Zn^{2+} and Cd^{2+} is not linked to activation of this channel by pinacidil.

Effectiveness of Zn^{2+} and Cd^{2+} Block of I_{KATP} Is Dependent on Intracellular ATP Concentrations

As described above, in our initial whole-cell experiments we found that if cells were dialyzed with ATP concentrations <0.5 mM, the effectiveness of block by the external divalent cations decreased. We investigated this phenomenon systematically by monitoring external divalent cation block of I_{KATP} on cells dialyzed with an intracellular ATP concentration of nominally 0 mM. We confirmed our preliminary observations and found that, in contrast with results obtained from cells dialyzed with [ATP] $\geq$ 0.5 mM, the inhibitory effects of Cd^{2+} and Zn^{2+} were greatly reduced for cells dialyzed with 0 mM ATP. The results are summarized in Fig. 8. It is not likely that this marked change in the effectiveness of divalent cation block of I_{KATP} is due to leak current or activation of other types of channels, because low concentrations of glibenclamide, a potent inhibitor of I_{KATP} (Arena and Kass, 1989a), is not affected by intracellular [ATP] (Fig. 8). This result suggested a possible interaction between externally applied divalent cations and intracellular ATP.

Since under whole-cell conditions pinacidil was used to activate I_{KATP}, we further investigated this ATP-dependent effect at the single-channel level under pinacidil-free conditions, excluding any pinacidil-mediated pathways. Experiments in the
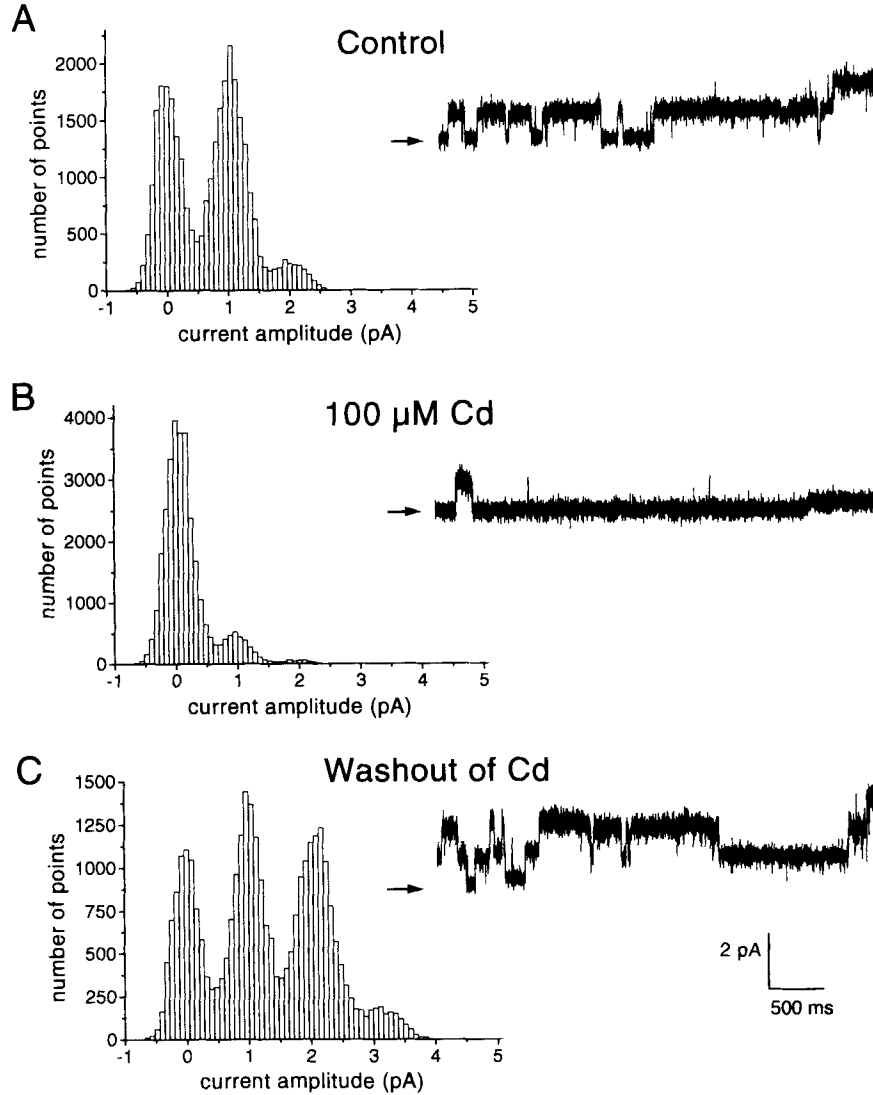
FIGURE 6. Block of I_{KATP} single-channel activity by externally applied Cd^{2+}: evidence that block is not dependent on pinacidil stimulation of the channel. Single-channel current traces and accompanying all-points histograms obtained from an outside-out membrane patch are shown before (A), during (B), and after (C) exposure to externally applied 100 μM Cd^{2+}. ATP concentration of cytosolic membrane (pipette) solution was 100 μM. Current traces were recorded in 5 mM extracellular and 140 mM intracellular potassium concentrations. The membrane potential was set at 0 mV, and single-channel currents are indicated by upward deflections. Zero current levels are indicated by arrows. Histograms were obtained from recording durations of 30 s.

inside-out patch configuration were designed to monitor changes in divalent cation block of I_{KATP} at two intracellular ATP concentrations, 200 μM ATP and ATP free. Physiological potassium concentrations of 5 mM on the extracellular side and 140 mM on the cytosolic side were used. As shown in Fig. 9 A, in the presence of external Cd^{2+}, channel activity is limited ($P_o = 0.05$) in 200 μM ATP, suggestive of Cd^{2+} block of I_{KATP}. This is demonstrated by the sample single-channel current trace and the corresponding cumulative amplitude histogram. (Single-channel P_o was calculated by first determining the number of channels in a patch as described in Methods.) As intracellular ATP concentration is changed to ATP free, an increase in channel activity is evident ($P_o = 0.20$; Fig. 9 B). This indicated a decrease in the blocking ability of Cd^{2+} to inhibit I_{KATP} in the absence of intracellular ATP.

To more quantitatively compare channel P_o's in the presence and absence of external divalent cation at the two ATP concentrations, and to account for patch
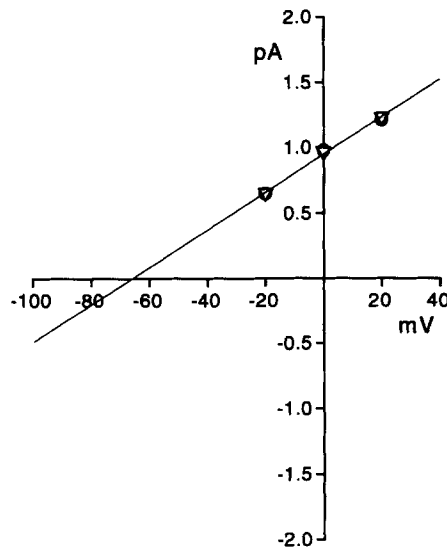

FIGURE 7. Divalent cation block of I_{KATP} does not alter channel conductance. The current–voltage relationship obtained from an outside-out membrane patch in 5 mM extracellular and 140 mM intracellular potassium concentrations is shown in the absence (O) and presence (∇) of Cd^{2+}. I_{KATP} was revealed by setting cytosolic ATP equal to 100 μM. Lines were fit by linear regression with a slope (conductance) of 14 pS. $E_{rev} = -67$ mV.

variability, we carried out several parallel experiments to statistically determine any differences. The results are summarized in Fig. 10. In the presence of intracellular ATP (200 μM), the P_o of the I_{KATP} channel in control (Cd^{2+} free) was significantly different from that obtained in the presence of Cd^{2+}, with $P_{o,Cd-free} = 0.16 \pm 0.05$ ($n = 6$) and $P_{o,Cd} = 0.04 \pm 0.02$ ($n = 7$) (average $\pm$ SEM), respectively. On the other hand, in the absence of intracellular ATP, the channel P_o's were not significantly different in Cd^{2+}-free and in 100 μM Cd^{2+}, with $P_{o,Cd-free} = 0.43 \pm 0.20$ ($n = 6$) and $P_{o,Cd} = 0.31 \pm 0.11$ ($n = 7$), respectively. The result demonstrates that in the absence of intracellular ATP, external Cd^{2+} does not inhibit channel activity. Yet, in the presence of ATP, external Cd^{2+} inhibits channel activity. The decrease in P_o observed at the single-channel level in the presence of 100 μM Cd^{2+} is equivalent to an inhibition of 75%. This is in agreement with block of I_{KATP} by 100 μM Cd^{2+} at the whole-cell level where block was ~80%.

DISCUSSION

In this study, we report that the ATP-regulated potassium channel is inhibited by very low concentrations of the divalent cations Cd^{2+} and Zn^{2+}. Cadmium and zinc ions are well-documented modulators of voltage-gated channels that either cause shifts in gating due to titration or screening of negative surface charges or, additionally, induce changes in channel protein configurations (Hille et al., 1975; Gilly and Armstrong, 1982a, b; Lansman, Hess, and Tsien, 1986; Zalman et al., 1991). The I_{KATP} channel is not a voltage-gated channel. It is controlled by intracellular ATP and is thus a ligand-gated channel. The effects of Cd^{2+} and Zn^{2+} on I_{KATP} cannot therefore be due to changes in membrane surface potential and associated shifts in voltage-dependent gating. These ions appear to bind to an extracellularly located site based on our experimental tests for voltage dependence, kinetics of onset and recovery, and excised patch experiments. However, the binding site does not appear
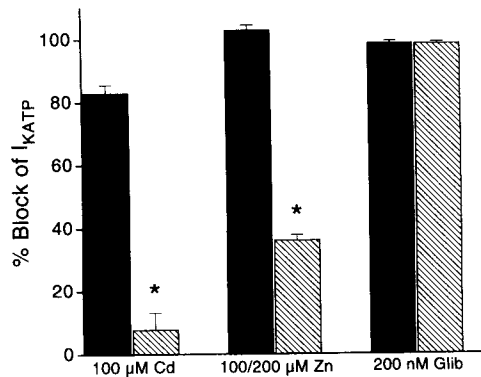

FIGURE 8. Divalent cation block of pinacidil-activated I_{KATP} is dependent on intracellular ATP concentrations. Percent block of I_{KATP} by external divalent cation was measured as percent reduction in the pinacidil-sensitive current at +10 mV under whole-cell conditions. Intracellular (pipette) ATP concentrations were either nominally zero (▨) or ≥0.5 mM (■). The data for external Zn^{2+} were pooled from experiments done in 100 and 200 μM Zn^{2+}. For both Cd^{2+} and Zn^{2+}, there were statistical differences (indicated by *) for block of I_{KATP} obtained with an intracellular ATP concentration of >0.5 mM (Cd^{2+}, $n = 5$; Zn^{2+}, $n = 4$) and a concentration of nominally 0 mM (Cd^{2+}, $n = 4$; Zn^{2+}, $n = 6$: Student's t test, 95% confidence level). No statistical difference was observed for the effect of glibenclamide ([ATP] > 0.5 mM, $n = 7$; [ATP] = 0 mM, $n = 4$). Error bars indicate SEM.

to be within the channel pore. Thus the inhibition of I_{KATP} by these divalent ions appears more consistent with regulation of channel activity than with a physical block of the permeation pathway.

A recent study showed that external Cd^{2+} may interact directly with a voltage-gated potassium channel in cat ventricular myocytes by stabilizing the channel in a high conductance state (Follmer, Lodge, Cullinan, and Colatsky, 1992). This mode of interaction is not unlike a previously proposed model for the regulation of some voltage-gated potassium channels by external divalent cations (Gilly and Armstrong, 1982b; see also Begenisich, 1988). Gilly and Armstrong (1982b) found that extracellular divalent cations affect gating kinetics of the neuronal delayed rectifier potassium channel in a manner inconsistent with simple surface charge theory and proposed a model in which binding of divalent ions to an extracellular region of the channel protein induced conformational changes that affected channel gating. In the model,
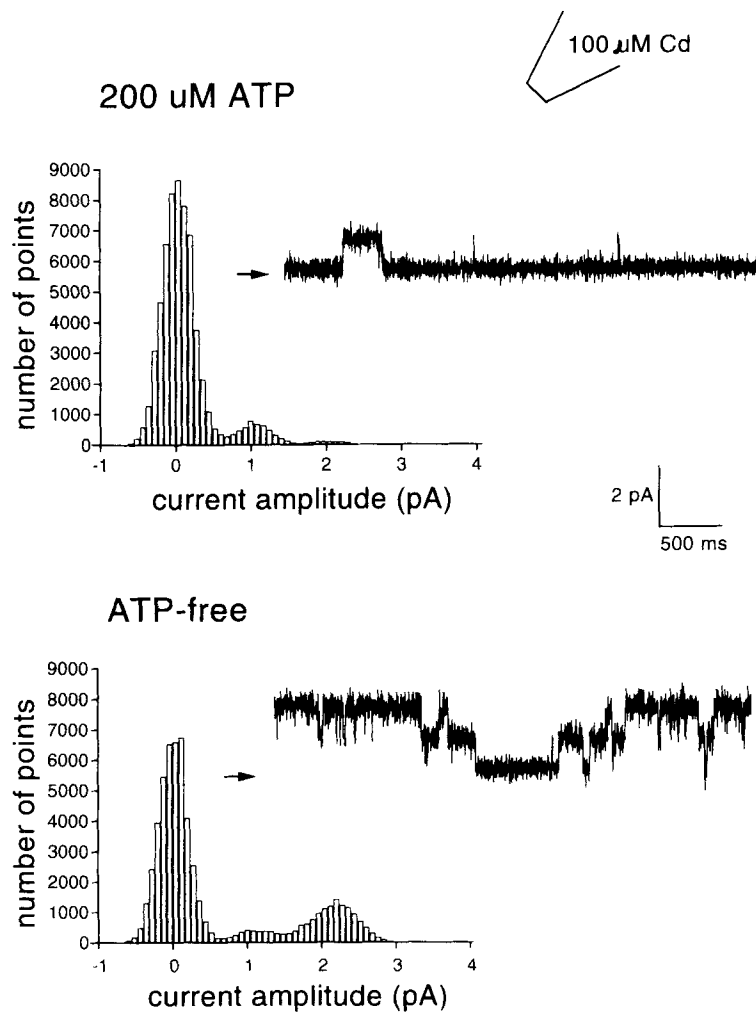
FIGURE 9. Block of I$_{KATP}$ single-channel activity by externally applied Cd is dependent on intracellular ATP concentrations. Sample current traces and corresponding all-points histogram obtained from an inside-out membrane patch at intracellular ATP concentrations of 200 μM (*A*) and ATP-free (*B*) are shown in the presence of extracellular Cd (100 μM). Current traces were recorded in 5 mM extracellular and 140 mM intracellular potassium concentrations. The membrane potential was set at 0 mV, and single-channel currents are indicated by upward deflections. Zero current levels are indicated by arrows. Histograms were obtained from current recording durations of 60 s.

the gating apparatus of the channel consists of several subunits, each consisting of interdigitating fingers of negatively and positively charged amino acid residues. As the set of negative charges moves relative to the set of positive charges, the channel makes a transition from the closed to the open state. The channel in the closed conformational state consists of an unpaired negative charge on the extracellular

side. When a counterion is attracted to the immediate vicinity of the unpaired charge and binds to an external binding site, the closed state of the channel is stabilized.

How can such a model explain the results of this study? Because the activity of I_{KATP} channels is regulated by the binding and unbinding of ATP to intracellular sites on or associated with the channel protein, it is reasonable to postulate that the binding of ATP replaces the voltage-dependent opening step in the above model in changing channel conformation from open to closed states. Then, binding of divalent ions to an extracellular site might stabilize the channel in a conformation that favors the binding of internal ATP and thus the closed state of the channel. This type of allosteric model predicts that internal ATP-dependent changes in channel conformation can alter the interactions of externally applied divalent ions.
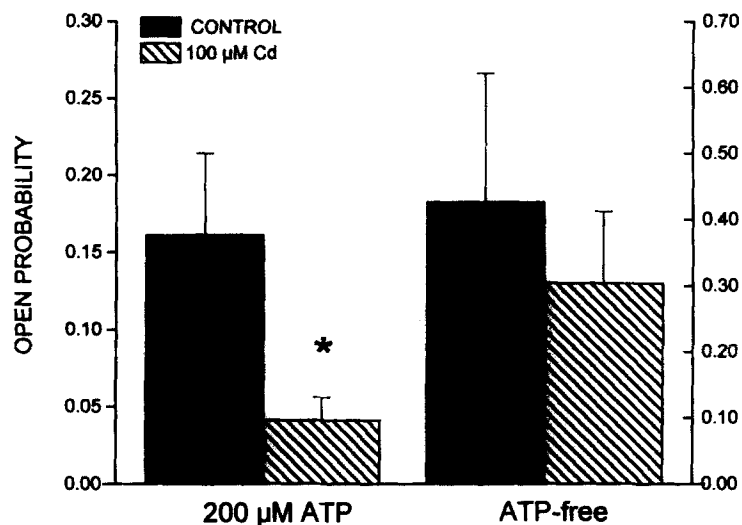

FIGURE 10.   Effect on single-channel P_o by externally applied Cd is dependent on intracellular ATP. P_o is calculated from $P_o = 1 - P_c^{1/n}$, as described in Methods. The graph is a summary of experiments as described in Fig. 9. Statistical significance is indicated by * (Student's t test, 95% confidence level). Control (external Cd-free) conditions are indicated by the filled bars, and external Cd-present conditions are indicated by the hatched bars. ATP concentrations shown are intracellular concentrations. Error bars indicate SEM.
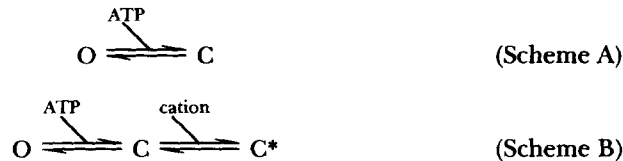
Our observation that, under whole-cell conditions, dialyzing the cell with very low ATP changes the effectiveness of block of I_{KATP} by Cd^{2+} and Zn^{2+}, is consistent with the predictions discussed above. This dependence on intracellular ATP was also confirmed at the single-channel level, where in zero ATP concentrations external divalent cations had no effect on channel activity. This may be due to the possibility that when ATP is not bound to the I_{KATP} channel protein, a shift in the charged residues results in a conformational change such that the extracellular cation binding site for Cd^{2+} and Zn^{2+} may be inaccessible. Recent studies have shown that the phosphate from ATP may interact with the voltage sensor of a phosphorylated axonal delayed rectifier potassium channel by electrostatic interactions (Perozo and Beza-

nilla, 1990). In addition, Gilly and Armstrong (1982a, b) have also proposed the idea of a "disappearing" receptor accompanying activation in their model for sodium and potassium channels. In any event, the most likely explanation of change in internal ATP affecting the interactions of external divalent ions with the channel is that both actions lead to interrelated changes in the channel protein conformation.

Recently, Treherne and Ashford (1992) reported that extracellular monovalent cations, K^+ or Na^+ ions, affect an I_{KATP} channel's sensitivity to intracellular ATP in hypothalamic neurons. They observed that an increase in the extracellular K^+ concentration (or a concurrent decrease in the extracellular Na^+ concentration) further sensitized the channel to ATP. The modulatory mechanism involved in their observation may be similar to the one discussed here involving conformational change of the channel protein. This allosteric interaction between an external cation binding site and an intracellular nucleotide binding site, however, is not a common property of I_{KATP} found in various tissue types. Teherne and Ashford (1992) also reported that no similar changes in ATP sensitivity were observed in I_{KATP} channels from an insulin-secreting cell line, CR1-G1.

A possible scheme involving the conformational change in the I_{KATP} channel protein is a simple, three-state model as shown below.

$$O \underset{}{\overset{ATP}{\rightleftharpoons}} C \qquad \text{(Scheme A)}$$

$$O \underset{}{\overset{ATP}{\rightleftharpoons}} C \underset{}{\overset{cation}{\rightleftharpoons}} C^* \qquad \text{(Scheme B)}$$

When ATP is bound, the channel makes a transition from the open state, O, to a closed state, C, as depicted in Scheme A. This closed state is further stabilized when an external divalent cation binds, as depicted in Scheme B. The further stabilization of the closed state with both ATP (internally) and cation (externally) bound to the channel protein is denoted by C^*. The dependence on intracellular ATP for external divalent cation block prevents the channel from making a direct transition from O to C^*. This is a simplified model and we did not attempt to take into account the predicted multiple binding sites for ATP (Noma and Shibasaki, 1985; Lederer and Nichols, 1989). Other schemes are certainly possible and future experiments should provide more information on the modulatory mechanism and the rate constants between states.

The results from the experiments of Gilly and Armstrong (1982a, b) also showed that the group IIB metal ions, Zn^{2+}, Cd^{2+}, and Hg^{2+}, were most effective in affecting gating kinetics on the voltage-gated potassium channel in nerve membrane. For the various ions studied, the degree of potency was $Hg^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+} > Mn^{2+} > Cu^{2+} > Ca^{2+}$. This is similar to the results of the present study, where the effectiveness of block of I_{KATP} was $Zn^{2+} > Cd^{2+} > Co^{2+} > Mg^{2+}$, Ca^{2+}. The ion specificity is probably not due to the size of the ion or its free energy of hydration. For example, Zn^{2+} and Co^{2+} have similar Pauling radii and hydration energies (Hille, 1992), but their ability to block I_{KATP} is considerably different. Rather, as was hypothesized for the potassium channel in nerve, the high specificity of Zn^{2+} and Cd^{2+} for I_{KATP} may be due to the electronic structure of the group IIB metal ions.

These ions are highly polarizable and can covalently bind to uncharged molecular entities, while Ca^{2+}, for example, binds strongly only to charged entities. The similar ion selectivity for the results reported by Gilly and Armstrong (1982b) and this study also support the view of a common underlying mechanism.

In the guinea pig ventricular cells used in this study, we found that Zn^{2+} and Cd^{2+} blocked neither the inward rectifier (Fig. 1) nor delayed rectifier potassium channel currents, I_{KS} (data not shown), at concentrations sufficiently high to completely block I_{KATP}, suggesting that the site at which these divalent ions bind is unique to the I_{KATP} channel in these cells. This possibility and the interdependence of external divalent ion binding and internal [ATP] will be the subjects of future investigations.

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