Surface Charge and Properties of Cardiac ATP-sensitive K+ Channels

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ABSTRACT   ATP-sensitive K+ (K_{ATP}) channels are present in a wide variety of tissues. The sensitivity of these channels to closure by cytosolic ATP (ATP_i) varies significantly among different tissues and even within the same tissue. The purpose of this study was to test the hypothesis that negative surface charges modulate the sensitivity of the K_{ATP} channels to ATP_i by influencing surface potential in the vicinity of the ATP-binding site(s) of the channel. Unitary currents through K_{ATP} channels were measured in inside-out membrane patches excised from rabbit ventricular myocytes using the patch-clamp technique. Agents known to be effective at screening negative surface charges were applied to the cytosolic surface of the patches, and their effects on ATP sensitivity were examined. These agents included Mg^{2+} (2–15 mM), Ba^{2+} (2–10 mM), and the polycations protamine (0.01–10 μM), poly-L-lysine (500 μM), and poly-L-arginine (0.5 μM). The divalent cations and the various polycations all dramatically reduced the concentration of ATP_i required to half-maximally suppress current through K_{ATP} channels (K_d), from ~100 μM in the absence of these agents to 1.6–8 μM in their presence. The effects were dose dependent. Protamine also reduced the sensitivity of K_{ATP} channels to block by cytosolic ADP. The sensitivity of K_{ATP} channels to block by ATP was independent of membrane potential, suggesting that the ATP-binding site is not located within the transmembrane voltage field. The effects of the polycation poly-L-lysine on ATP sensitivity were also independent of membrane potential or the direction (inward or outward) of current through K_{ATP} channels. In addition to increasing ATP sensitivity, Mg^{2+}, Ba^{2+}, and the polycations all caused dose-dependent block of inward and outward currents through K_{ATP} channels over similar concentration ranges as their effects on ATP sensitivity. The block of inward current by polycations was not associated with reduction of single-channel conductance or evidence of fast open channel block. However, the polycations did cause a modest reduction in single-channel conductance of outward current. These results are consistent with the presence of negative surface charges that reduce the local ATP concentration at the ATP-binding site(s) on the channel, relative to the bulk cytosolic ATP concentration. Screening these negative surface charges with divalent cations or polycations

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decreases the local ATP gradient, resulting in a decrease in the apparent K_d for ATP. Applying surface potential theory to the Mg^{2+} data, a rough calculation of the surface charge density necessary to account for these effects yielded a value of 1 negative charge per 199 $Å^2$, corresponding to surface potentials in the range of -50 to -70 mV. The putative negative surface charges are most likely located on the K_{ATP} channel protein itself rather than in the lipid bilayer because the negatively charged amphiphile SDS, which inserts into the lipid bilayer and increases negative surface charge sensed by voltage-dependent ion channels, had no effect on the ATP sensitivity of K_{ATP} channels when applied to the cytoplasmic surface. Our results suggest that variability in negative surface charge density near the ATP-binding site(s) of K_{ATP} channels may in part explain the large differences in the ATP sensitivity of K_{ATP} channels in various tissues.

INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels are a class of K^+ channels that link the membrane potential to the bioenergetic state of the cell. K_{ATP} channels have been shown to be important in the regulation of such diverse functions as the cardiac action potential duration during metabolic impairment (Noma, 1983; Nichols, Ripoll, and Lederer, 1991; Deutsch, Klitzner, Lamp, and Weiss, 1991), ischemic preconditioning and stunning in the heart (Auchampach, Maruyama, Cavero, and Gross, 1992; Gross and Auchampach, 1992), vasomotor tone (Standen, Quayle, Davies, Brayden Huang, and Nelson, 1989), insulin secretion (Petersen and Findlay, 1987), skeletal muscle excitability (Spruce, Standen, and Stanfield, 1987), neurotransmitter release (Amoroso, Schmid-Antomarchi, Fosset, and Lazdunski, 1990), renal tubular function (Tsuchiya, Wang, Giebisch, and Welling, 1992), and oocyte maturation (Wilbrand, Honore, and Lazdunski, 1992). The defining attribute of K_{ATP} channels is that intracellular ATP (ATP_i) closes the channel. However, the intracellular ATP concentration ($[ATP]_i$) required for half-maximal suppression of K_{ATP} channels (K_d) in excised membrane patches varies significantly, >100-fold, among different tissue types (Ashcroft and Ashcroft, 1990), and it also varies between different membrane patches in the same tissue (e.g., ≤ 60-fold variability in cardiac K_{ATP} channels) (Findlay and Faivre, 1991; Weiss, Venkatesh, and Lamp, 1992). The ATP sensitivity of K_{ATP} channels can also be modified by various factors, including metabolic inhibition (Deutsch and Weiss, 1993), rundown (Escande, Thuringer, Le Greun, and Cavero, 1988; Deutsch and Weiss, 1993), and treatment with trypsin (Deutsch and Weiss, 1993, 1994; Proks and Ashcroft, 1993). The mechanisms underlying this variability in ATP sensitivity, however, are poorly understood.

Ion channels function in an environment with many local surface charges. The source of these charges may be acidic and basic amino acid residues of membrane proteins (Kyte and Doolittle, 1982; Catterall, 1988), negatively charged phosphate groups added to amino acid residues during protein phosphorylation (Perozo, Bezanilla, and Dipolo, 1989), and charged phospholipids in the plasma membrane, which exist as charged zwitterions at physiologic pH (Storch and Kleinfeld, 1985). Surface potential theory has been successful in explaining a number of phenomena affecting the function of proteins, including ion channels. A surface potential is created when charged residues on or near the aqueous face of a protein or lipid

polarize the surrounding aqueous environment. For a membrane-spanning protein such as an ion channel, this surface potential can shift the effective transmembrane potential difference sensed by voltage sensors of the channel, adding an electrical bias that alters voltage-dependent gating (Green and Andersen, 1991). The ability of extracellular cations to cause a hyperpolarizing shift in the gating of voltage-dependent ion channels has been attributed to their effectiveness at screening negative extracellular surface charges present on biological membranes (Hille, 1992). Surface charge also influences the local concentration of ions, increasing the concentration of oppositely charged ions and decreasing the concentration of like-charged ions in the immediate vicinity of the membrane. The magnitude of these effects can be large; bilayers of negatively charged phospholipids bathed in a Ringer's solution have surface potentials ranging from -40 to -120 mV, and the surface concentration of Ca^{2+} ions is predicted to be 20 to 1,000 times higher at the bilayer surface than in the bulk solution (McLauglin, Szabo, and Eisenman, 1971). Surface charges may, in addition, be an important mechanism for concentrating permeant ions in the vestibules of ion channels to increase permeation rates through the pore (Green and Andersen, 1991) and to increase the affinity of toxins at their binding sites (Green, Weiss, and Andersen, 1987). Certain enzymes, such as superoxide dismutase, also appear to use surface charges located near substrate-binding sites to increase the local concentration of the substrate, thereby increasing enzyme efficiency (Cudd and Fridovich, 1982).

In view of these precedents, we speculated that negative surface charges in the region of the ATP-binding site(s) on the K_{ATP} channel may, by altering local ATP concentration, be a determinant of the sensitivity of K_{ATP} channels to closure by ATP. We were intrigued by the observation that the ATP sensitivity of K_{ATP} channels is altered by cytosolic Mg^{2+} because divalent cations are effective surface charge screening agents. In pancreatic beta cells and skeletal muscle, cytosolic Mg^{2+} decreased the apparent sensitivity of K_{ATP} channels to ATP, which was attributed to a higher affinity of the ATP-binding site to ATP^{4-} than $MgATP^{2-}$ (Ashcroft and Kakei, 1989; Vivaudou, Arnoult and Villaz, 1991). For cardiac K_{ATP} channels, however, Mg^{2+} increased their sensitivity to ATP (Findlay, 1988; Koyano, Kakei, Nakashima, Yoshinaga, Matsuoka, and Tanaka, 1993). As will be shown in this paper, the latter effect cannot be fully explained by a higher affinity of cardiac K_{ATP} channels for $MgATP^{2-}$ than for ATP^{4-}. This raises the possibility that Mg^{2+} exerts its effects by screening negative surface charges near the ATP-binding site(s) on the channel, thereby increasing the local ATP concentration and lowering the apparent K_d for block by cytosolic ATP. We have evaluated this hypothesis by examining the effects of agents known to screen negative surface charge on the sensitivity of K_{ATP} channels to block by cytosolic ATP. We find that these agents have a dual effect: they markedly increase the sensitivity of K_{ATP} channels to block by ATP, and, in the absence of ATP, they decrease channel open probability. These observations can explained by a model of the K_{ATP} channel where negative surface charges are present near the ATP-binding site(s) on the K_{ATP} channel. The postulated charges determine ATP sensitivity by influencing local ATP concentration near the ATP-binding site(s), and they modulate channel open probability through electrostatic interactions with the

gating mechanism. Our evidence also suggests that these negative surface charges are located on the channel protein rather than in the surrounding lipid environment.

MATERIALS AND METHODS

Cell Isolation

New Zealand White rabbits of either sex (2–3 kg) were anesthetized intravenously with a lethal dose of sodium pentobarbital. The heart was rapidly excised through a thoracotomy incision
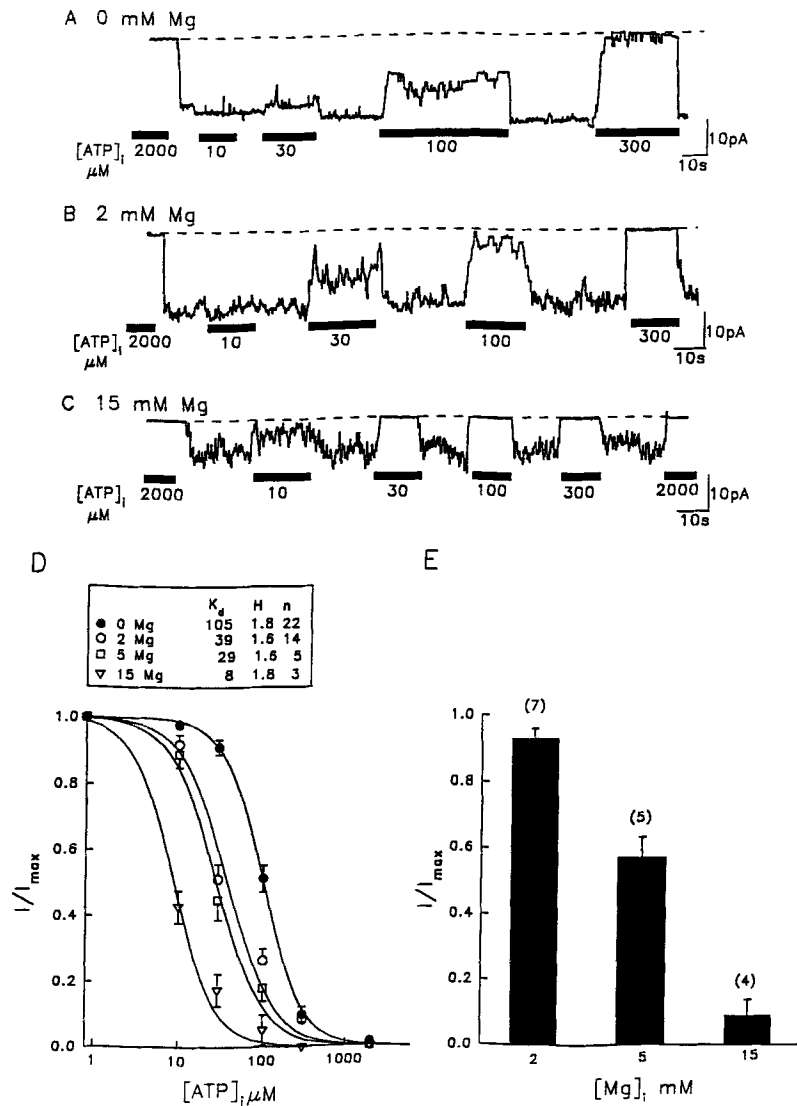

FIGURE 1.

and rapidly mounted on a Langendorff perfusion apparatus. Single ventricular myocytes were isolated by enzymatic digestion with collagenase and protease (Mitra and Morad, 1986). Myocytes were prepared for the giant patch technique using the method of Hilgemann (Collins, Somlyo, and Hilgemann, 1992) to allow sarcolemmal blebbing by incubating them overnight at room temperature in a high K^+, low Ca^{2+} solution.

Patch Clamp Methods

Cells were placed in a 0.5-ml capacity experimental chamber mounted on the stage of an inverted microscope, and they were continuously perfused at a rate of 1–4 ml/min. Patch electrodes were mounted on the headstage of an amplifier (Axopatch 200; Axon Instruments, Burlingame, CA). Membrane current and voltage signals were recorded on a chart recorder and a hard disk using Axotape software (Axon Instruments) for later computer analysis with customized software. For measuring single K_{ATP} channel currents, patch electrodes were fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL) and had a tip resistance of 2–4 $M\Omega$ when filled with the standard electrode solution. A 10-channel multibarreled rapid perfusion device with a common opening was used to facilitate rapid solution changes at the cytoplasmic surface (facing the bath) of the inside-out patches. The 90% exchange time of the bath solution was typically <200 ms (Weiss et al., 1992). For measuring Na^+-K^+ pump currents, giant membrane patches were formed with electrodes of 18–25 μm inner diameter pulled from borosilicate glass. To improve the stability of the seals, the electrode tips were passed through a viscous mixture of parafilm and light mineral oil (Collins et al., 1992). Solution changes were made using a device similar to that described by Collins et al. (1992). All patch clamp experiments were performed at room temperature (21–25°C).

Figure 1 (*opposite*).   Effect of $[Mg^{2+}]_i$ on the ATP sensitivity and inward current in cardiac K_{ATP} channels. (*A*) An inside-out patch was excised from a rabbit myocyte and exposed to different ATP concentrations (as indicated above the bars) in the presence of an Mg^{2+}-free bath solution. The space between the bars represent exposure to ATP-free solution. (*B*) The same membrane patch was reexposed to various ATP concentrations in the presence of a free $[Mg^{2+}]_i$ of 2 mM, showing increased sensitivity to block by $[ATP]_i$ compared to *A*. (*C*) A different membrane patch was exposed to various $[ATP]_i$ in the presence of 15 mM free $[Mg^{2+}]_i$. The sensitivity of K_{ATP} channels to closure by $[ATP]_i$ was further increased. In *A–C*, zero current levels are indicated by the dashed lines. Both the patch electrode and bath solutions contained 150 mM KCl. The membrane potential was held at −40 mV, and traces were filtered at 200 Hz. (*D*) Dose-response curves of K_{ATP} channels to $[ATP]_i$ in inside-out membrane patches in the presence of free $[Mg^{2+}]_i$ concentrations of 0, 2, 5, and 15 mM. For each patch, current (*I*) at each $[ATP]_i$ was normalized to the current in the absence of ATP_i (I_{MAX}). Data points are the mean ± SEM. Smooth curves represent best fits to a Hill equation (Eq. 1). The K_d, Hill coefficient (*H*), and mean number of patches for each treatment is indicated in the inset. Statistical comparison of the I/I_{MAX} values over the $[ATP]_i$ range of 10–300 μM was as follows: 0 Mg^{2+} vs 2 Mg^{2+}, $P <$ 0.05; 0 Mg^{2+} vs 5 Mg^{2+}, $P <$ 0.05; 0 Mg^{2+} vs 15 Mg^{2+}, $P <$ 0.05; 2 Mg^{2+} vs 15 Mg^{2+}, $P <$ 0.05; 2 Mg^{2+} vs 5 Mg^{2+}, $P >$ 0.05; 5 Mg^{2+} vs 15 Mg^{2+}, $P >$ 0.05. (*E*) Effect of a 10–20-s exposure to 2, 5, and 15 mM free Mg^{2+} on inward current through K_{ATP} channels in the absence of ATP. The number of patches is indicated above each bar. For each patch, current (*I*) at each $[Mg^{2+}]$ was normalized to the current in the absence of Mg^{2+} (I_{MAX}). Only patches in which the measurement of current in the presence of Mg^{2+} was bracketed by current measurements in the absence of Mg^{2+} have been included.

Solutions and Experimental Procedures

For measuring single-channel K_{ATP} channel currents, the standard patch electrode solution contained (in mM): 4 KCl, 145 NaCl + NaOH, and 5 Hepes, pH 7.35. In some experiments, Na^+ was replaced by equimolar K^+. The standard bath solution consisted of (in mM) 150 KCl + KOH, 0.5 $CaCl_2$, 2 EDTA, 0–2 K_2ATP, and 5 Hepes, pH 7.25. In some experiments, $MgCl_2$ was added to the bath solution and MgATP and EGTA were used in place of K_2ATP and EDTA to maintain a free $[Mg^{2+}]$ of ~2, 5, or 15 mM. In other experiments, $BaCl_2$ or $LaCl_3$ were added to the standard bath solution to maintain a free $[Ba^{2+}]$ of ~2, 4, or 10 mM and a free $[La^{2+}]$ of ~10 mM. Other reagents added to the bath solution in different experiments included (in μM): 100–300 ADP, 0.01–10 protamine (mol wt ~7,000, Upjohn, Kalamazoo, MI), 5 U/ml heparin (Upjohn), 0.5 poly-L-arginine (mol wt 50,000–150,000, Sigma P8137), 500 poly-L-lysine (mol wt 1,000–4,000, Sigma P0879), 10 dodecyltrimethylammonium (DDTMA), and 10 SDS. The sensitivity of the K_{ATP} channel to closure by $[ATP]_i$ was tested by varying the [ATP] of these solutions from 0 to 2 mM. The free Ca^{2+} concentration was estimated at ~50–100 nM. The concentration of free and bound ligands was calculated using MaxC software (Bers, Patton, and Nuccitelli, 1993).

With 4 mM K^+ in the patch electrode, the patch electrode potential was held at 0 mV to record outward currents through K_{ATP}^+ channels. In experiments with 150 mM K^+ in the patch electrode, the patch electrode was generally held at either +40 or −40 mV to examine inward or outward currents through K_{ATP} channels. Because of the large number of channels (> 10) in most patches, recording membrane current for 10–12 s was adequate to assess the effects of a given $[ATP]_i$ or a given cation on channel activity while minimizing channel rundown. Each $[ATP]_i$ or cation tested was bracketed by exposure to ATP-free or cation-free bath solution, and data were accepted for analysis only if the current in the ATP-free or cation-free solution returned to >80% of the pretest value.

The Na^+-K^+ pump current was isolated in giant membrane patch experiments. The patch electrode solution contained (in mM): 100 N-methyl-D-glutamine (NMG), 100 MES, 20 Hepes, 20 TEA-OH, 5 EGTA, 20 CsCl, 5 KCl, 1 $MgCl_2$, and 1 $BaCl_2$, pH 7.0 (adjusted with MES). The bath solution contained either 100 NaOH or 100 CsOH, 20 CsCl, 100 MES, 20 TEA-OH, 20 Hepes, 10 EGTA, 0–1 MgATP, and 0 or 10 μM protamine, pH 7.0 (adjusted with MES). The Na^+-K^+ pump current was activated by switching from the Cs^+ to the Na^+ bath solution containing various concentrations of ATP_i.

Drugs and Chemicals

All chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise indicated.

Data Analysis

Statistical analysis was performed by ANOVA or Student's t test with the appropriate Bonferroni correction. $P < 0.05$ was considered significant. All results are presented as mean ± SE.

RESULTS

Effects of Mg^{2+} on ATP Sensitivity of K_{ATP} Channel

Fig. 1 illustrates the effect of cytosolic Mg^{2+} on the ATP sensitivity of K_{ATP} channels in an excised inside-out membrane patch from a guinea pig ventricular myocyte. With symmetrical 150 mM K^+ on each side of the membrane and a membrane potential of −40 mV, inward current through multiple K_{ATP} channels was observed

when 2,000 μM ATP was removed from the bath (cytosolic surface). In an EDTA-buffered Mg^{2+}-free bath solution, the K_d for block by ATP was ~100 μM (Fig. 1 A). In the presence of a free Mg^{2+} concentration of 2 mM, however, the K_d for block by ATP was reduced to ~30 μM in the same patch (Fig. 1 B). Increasing free Mg^{2+} to 15 mM further reduced the K_d to <10 μM (Fig. 1 C). Fig. 1 D summarizes the effects
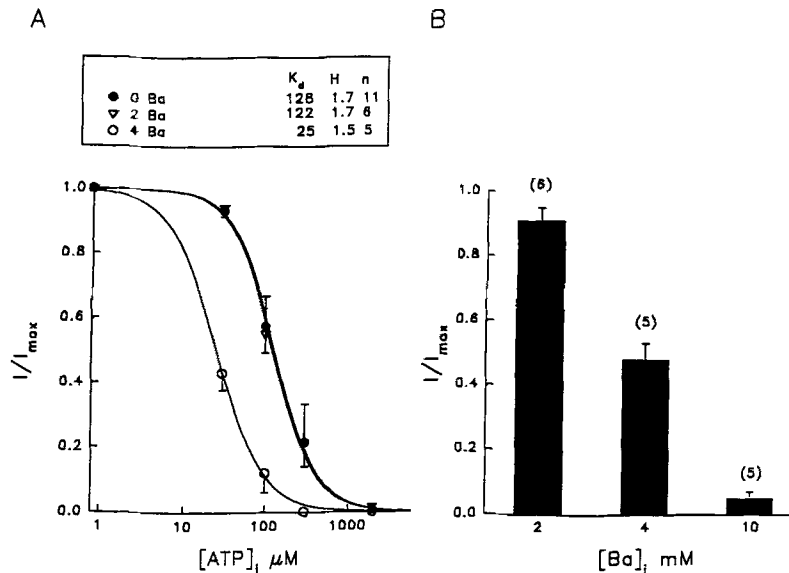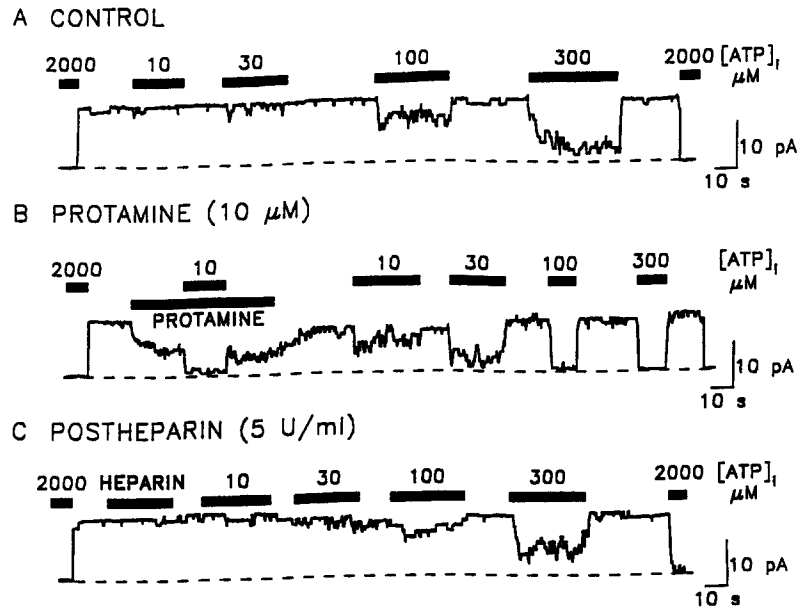

FIGURE 2. Effect of $[Ba^{2+}]_i$ on the ATP sensitivity and inward current in K_{ATP} channels. (A) Dose-response curve of K_{ATP} channels to $[ATP]_i$ in inside-out membrane patches in the presence of free $[Ba^{2+}]_i$ concentrations of 0, 2, and 4 mM. For each patch, current (I) at each $[ATP]_i$ was normalized to the current in the absence of ATP, (I_{MAX}). Data points are the mean ± SEM. Smooth curves represent best fits to a Hill equation (Eq. 1). The K_d, Hill coefficient (H), and mean number of patches for each treatment is indicated in the inset. Statistical comparison of the I/I_{MAX} values over the $[ATP]_i$ range of 30–300 μM were as follows: 0 Ba^{2+} vs 2 Ba^{2+}, P > 0.05; 0 Ba^{2+} vs 4 Ba^{2+}, P < 0.05; 2 Ba^{2+} vs 4 Ba^{2+}, P < 0.05. (B) Effect of a 10–20-s exposure to 2, 4, and 10 mM $[Ba^{2+}]_i$ on inward current through K_{ATP} channels in the absence of ATP. The number of patches is indicated above each bar. For each patch, current (I) at each $[Ba^{2+}]$ was normalized to the current in the absence of Ba^{2+} (I_{MAX}).

of Mg^{2+} on the K_d for block by ATP. Data from multiple patches were fit to the Hill equation:

$$I/I_{MAX} = 1/\{1 + ([ATP]_i/K_d)^H\} \tag{1}$$

In the presence of 0, 2, 5, and 15 mM Mg^{2+}, the best fits to this equation yielded K_d's for block by ATP of 105 μM (n = 22 patches), 39 μM (n = 14 patches), 29 μM (n = 5 patches), and 8 μM (n = 3 of 11 patches tested where sufficient current remained in the presence of 15 mM Mg^{2+} to test ATP sensitivity), respectively. The Hill coefficients (H) were 1.8, 1.6, 1.6, and 1.8, respectively. In nine patches, the ATP sensitivity was tested again in the absence of Mg^{2+} after exposure to 2 (n = 6 patches)
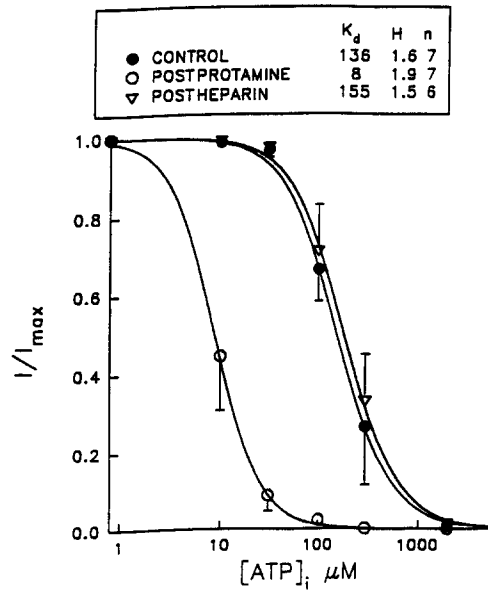
A CONTROL

B PROTAMINE (10 μM)

C POSTHEPARIN (5 U/ml)

D

		K_d	H	n
●	CONTROL	136	1.6	7
○	POSTPROTAMINE	8	1.9	7
▽	POSTHEPARIN	155	1.5	6

FIGURE 3.

and 5 mM free Mg^{2+} ($n = 3$ patches). Although the current through the K_{ATP} channels had rundown by 8 ± 2% and 14 ± 5%, respectively, the K_d's returned to the original values, (101 and 113 μM, respectively). Thus, it seems unlikely that rundown caused the increased sensitivity to ATP_i during exposure to cytosolic Mg^{2+}.

Millimolar Mg^{2+} also caused a marked reduction in inward current through K_{ATP} channels (Fig. 1 E), as has been noted previously in cardiac myocytes and pancreatic beta cells (Findlay, 1987a and 1987b; Aschroft and Kakei, 1989). Unfortunately, detailed investigation of the kinetic features of Mg^{2+} block of inward current was not possible because we were unable to obtain membrane patches with only a single active channel. However, it has previously been shown in both guinea pig ventricular myocytes and pancreatic beta cells that 5–10 mM $[Mg^{2+}]_i$ had no effect on the single-channel conductance of inward currents through K_{ATP} channels (Horie, Irisawa, and Noma, 1987; Ashcroft and Kakei, 1989), suggesting that the reduction of inward current by internal Mg^{2+} was not caused by fast open channel block.

The finding that removal of internal Mg^{2+} led to a decrease in the sensitivity of K_{ATP} channels to block by ATP is consistent with the possibility that the ATP-binding site(s) of the cardiac K_{ATP} channel has a higher affinity for $MgATP^{2-}$. However, the observation that the sensitivity to block by ATP increased further when Mg^{2+} was increased from 2 to 15 mM cannot be explained on this basis. With 30 μM ATP, the calculated proportion of ATP bound to Mg^{2+} was nearly identical in the presence of either 2, 5, or 15 mM Mg^{2+} (28.9, 29.5, and 29.8 μM, respectively). Yet in the presence of 30 μM ATP, current through K_{ATP} channels was suppressed by 49 ± 5% with 2 mM free Mg^{2+} present, 55 ± 6% with 5 mM free Mg^{2+} present, and by 83 ± 5% with 15 mM free Mg^{2+} present. In view of the ability of divalent cations to screen negative charges, an alternative explanation is that Mg^{2+} screens negative charges in

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Figure 3 (*opposite*). Effect of protamine on the ATP sensitivity of the K_{ATP} channel. (*A*) An inside-out patch excised from a rabbit ventricular myocyte was exposed to different ATP concentrations as indicated above the bars. The spaces between the bars represent exposure to ATP-free solution. (*B*) The same membrane patch was exposed to protamine (10 μM) in the absence of ATP_i, which resulted in partial blockade of the current. The patch was then exposed to 10 μM $[ATP]_i$ in the continued presence of protamine and showed a dramatically increased sensitivity to closure by ATP_i. Protamine was washed out, and the current returned towards baseline. After washout of protamine, the patch was reexposed to the various $[ATP]_i$ indicated, and it still demonstrated a markedly increased sensitivity to closure by ATP. (*C*) The same membrane patch was then exposed briefly to heparin (5 U/ml), and its sensitivity to closure by $[ATP]_i$ returned to what it was before protamine treatment. The zero current levels are indicated by the dashed lines. The patch electrode solution contained 4 mM KCl, and the bath solution contained 150 mM KCl and was Mg^{2+}-free. The membrane potential was 0 mV, and current traces were filtered at 200 Hz. (*D*) Dose response of K_{ATP} channels to $[ATP]_i$ in inside-out membrane patches before and after exposure to 10 μM protamine and after exposure to heparin (5 U/ml). For each patch, current (*I*) at each $[ATP]_i$ was normalized with respect to the current in the absence of ATP_i (I_{MAX}). Data points are the mean ± SEM. Smooth curves represent best fits to a Hill equation. The K_d, Hill coefficient (*H*), and mean number of patches for each treatment is indicated in the inset. Statistical comparison of the I/I_{MAX} values over the $[ATP]_i$ range of 10–300 μM were as follows: control vs postprotamine, $P < 0.01$; postprotamine vs postheparin, $P < 0.01$; control vs postheparin, $P > 0.05$.

the vicinity of the ATP-binding site(s) of the channel, leading to a local increase in [ATP] and reducing the apparent K_d for block by ATP. Since the ability of ATP to block K_{ATP} is insensitive to transmembrane voltage (see below; Takano and Noma, 1993), it is likely that these putative negative charges are surface charges.

Effects of Other Divalent Cations on the ATP Sensitivity of the K_{ATP} Channels

To test this hypothesis further, we tested whether other divalent and trivalent cations had similar effects as Mg^{2+}. Ca^{2+} and La^{3+} were not useful because they induced irreversible complete block of inward and outward currents through K_{ATP} channels when applied in millimolar concentrations. Sr^{2+} has also been reported to cause irreversible block (Findlay, 1987b), and so it was not tested. Ba^{2+} also caused nearly complete block at 10 mM, but lower concentrations could be tested. Fig. 2 A shows the effects of 2 and 4 mM free Ba^{2+} on the ATP sensitivity of K_{ATP} channels in excised inside-out patches. Whereas 2 mM free Ba^{2+} did not significantly alter ATP sensitivity, 4 mM free Ba^{2+} significantly reduced the K_d from 128 to 25 μM. In four patches, the ATP_i sensitivity was retested after exposure to 4 mM free Ba^{2+}. In the absence of Ba^{2+}, the current through K_{ATP} channels returned to 95 ± 3% of the original current, and the K_d was similar (121 μM). Similar to Mg^{2+}, Ba^{2+} also suppressed inward current through K_{ATP} channels (Fig. 2 B).

Effects of Protamine on the ATP Sensitivity of the K_{ATP} Channels

Most divalent cations bind to ATP. To avoid the potential complication of different binding affinities of the K_{ATP} channel for ATP^{4-} vs divalent cation-bound forms of ATP, such as $MgATP^{2-}$, we examined the effects of other agents that are known to be effective at screening negative surface charge. A variety of large polycations have been shown to screen negative surface charge very effectively at low concentrations (Singh, Kasinath, and Lewis, 1992). Fig. 3 illustrates the effects of the polycation protamine in the absence of cytosolic Mg^{2+}. When applied to the cytoplasmic surface of an excised inside-out membrane patch, 10 μM protamine had two effects: it suppressed current through K_{ATP} channels in the absence of ATP, and it dramatically increased the sensitivity of the channel to closure by $[ATP]_i$. After washout of protamine, the current returned towards the control level in the absence of ATP, but the K_{ATP} channels remained more sensitive to closure by ATP, with the K_d decreasing from ~ 100 μM before exposure to protamine to < 30 μM after protamine (Fig. 3 B). Subsequent brief exposure of the patch to heparin (5 U/ml), a potent polyanion that binds strongly to protamine, restored the ATP sensitivity to the pre-protamine level (Fig. 3 C). Fig. 3 D summarizes the results in seven patches. Fitting the data to the Hill equation (Eq. 1), the K_d for ATP in the absence of cytosolic Mg^{2+} averaged 136 μM under control conditions, decreased to 8 μM after exposure to 10 μM protamine, and returned to 155 μM after exposure to heparin. Exposure of excised membrane patches to heparin (5 U/ml) before protamine treatment had no significant effect on the ATP sensitivity of K_{ATP} channels ($n = 5$, data not shown).

Fig. 4 A illustrates the effects of different protamine concentrations on the

sensitivity of K_{ATP} channels to closure by ATP_i in the absence of Mg^{2+}_i. In all of these patches, protamine remained present during the determination of ATP sensitivity, unlike in Fig. 3. At 0.01 μM, protamine had no significant effect on the K_d (71 vs 86 μM) or Hill coefficient. At 0.1 μM, the dose response to ATP was shifted to the left, indicating increased sensitivity to ATP (K_d 8 μM), but the shape was not well fit by the
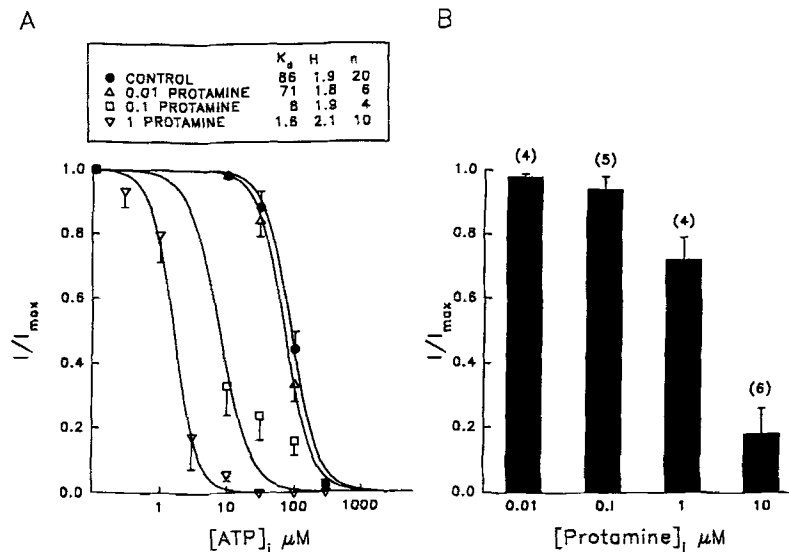

FIGURE 4. Effect of various concentrations of protamine (0.01–10 μM) on the dose-response curve of K_{ATP} channels to $[ATP]_i$ in inside-out membrane patches. For graphic illustration, the control $[ATP]_i$ dose-response curves were pooled. For each patch, current (I) at each $[ATP]_i$ was normalized to the current in the absence of ATP_i (I_{MAX}). Data points are the mean ± SEM. Smooth curves represent best fits to a Hill equation. The K_d, Hill coefficient (H), and mean number of patches for each treatment are indicated in the inset. The data for 0.1 μM protamine was arbitrarily fit to a Hill coefficient of 1.9 because of the paucity of data points on the upper arm of the curve. Statistical comparison of the I/I_{MAX} values over the $[ATP]_i$ range of 10–300 μM were as follows: control vs 1 μM protamine, $P < 0.01$; control vs 0.1 μM protamine, $P < 0.01$; control vs 0.01 μM protamine, $P > 0.05$. Experimental conditions were the same as described in Fig. 3. (B) Effect of a 10–20-s exposure to 0.01–10 μM protamine on inward current through K_{ATP} channels in the absence of ATP. Number of patches is indicated above each bar. Only patches in which the current in the absence of protamine was measured both before and after the current in the presence of protamine were used. The data includes additional patches in which the ATP sensitivity was not tested. For each patch, current (I) at each [protamine] was normalized to the current in the absence of protamine (I_{MAX}).

Hill equation, suggesting a mixed population of channels with different ATP sensitivities. At 1 μM protamine, all of the K_{ATP} channels showed increased sensitivity to ATP, with a K_d of 1.6 μM. As shown in Fig. 4 B, increasing protamine concentrations also progressively decreased the open probability of K_{ATP} channels. At 10 μM, protamine completely blocked inward and outward current through K_{ATP} channels in

A   CONTROL, +40 mV

B   CONTROL, −40 mV

C   POLY−L−LYSINE, +40 mV

D   POLY−L−LYSINE, −40 mV

E

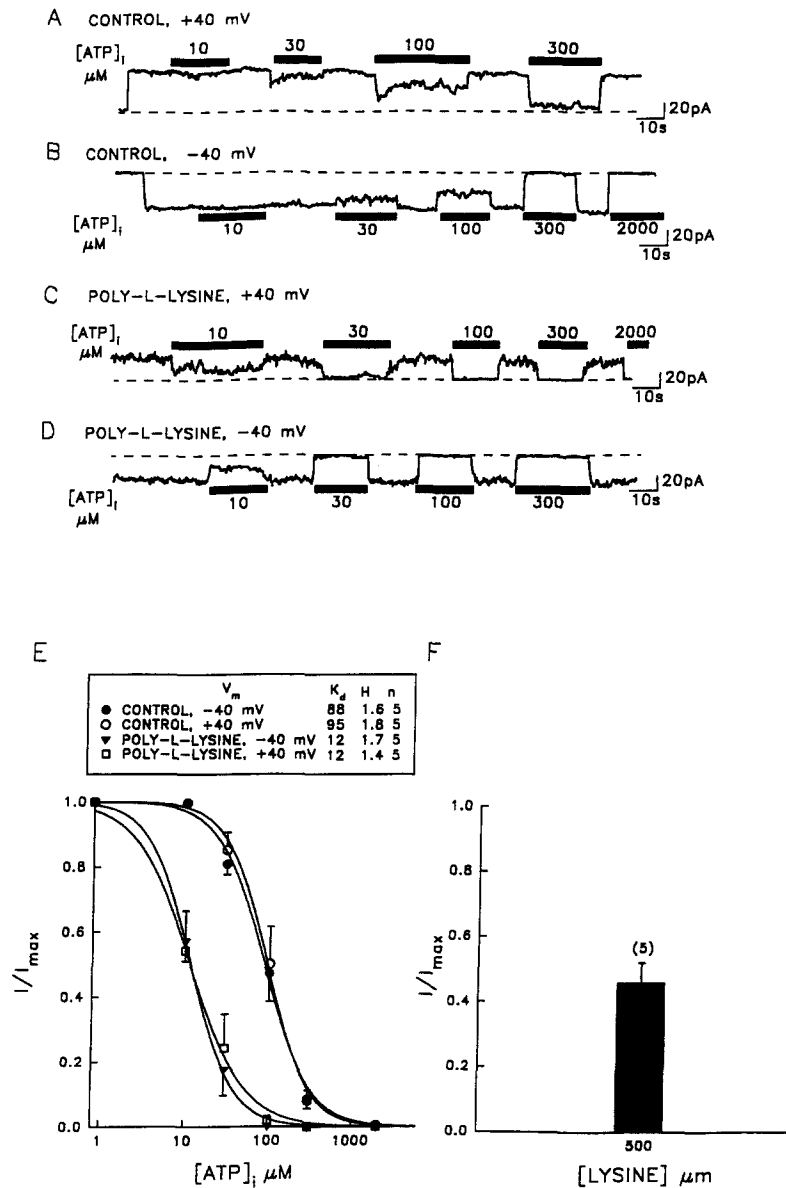| V_m	K_d	H	n
● CONTROL, −40 mV	88	1.6	5
○ CONTROL, +40 mV	95	1.8	5
▼ POLY−L−LYSINE, −40 mV	12	1.7	5
□ POLY−L−LYSINE, +40 mV	12	1.4	5

F

FIGURE 5.

most patches (the patch in Fig. 3 is an exception), and the ATP sensitivity could not be reliably determined until after removal of protamine. Prolonged exposure to protamine at this concentration also appeared to accelerate channel "rundown." The potency of protamine in shifting the $[ATP]_i$ dose response curve was similar to the half-neutralization value of 1 μM for protamine in screening surface charge of glomerular epithelial cells (Singh et al., 1992).

*Other Polycations also Increase the ATP Sensitivity and Decrease the Open
Probability of K_{ATP} Channels*

In addition to protamine, we also tested the effects of other polycations known to be
effective surface charge screening agents (Singh et al., 1992). Fig. 5 illustrates the
effects of poly-L-lysine (mol wt 1,000–4,000). With symmetrical 150 mM KCl on both
sides of the excised inside-out patch membrane and Mg^{2+}-free conditions, the K_d for
ATP was similar (∼ 100 μM), whether the membrane potential was held at −40 or
+40 mV to induce inward or outward current, respectively, through K_{ATP} channels,
confirming that the ATP sensitivity of K_{ATP} channels is not voltage dependent. This
finding indicates that the ATP-binding site(s) is not located within the transmem-
brane voltage field. In the presence of 500 μM poly-L -lysine, the K_d was reduced to
∼ 10 μM at both membrane potentials. Fig. 5 *E* summarizes the results in five
patches. Similar to protamine, poly-L-lysine reduced the K_d for suppression of K_{ATP}
channels by ATP dramatically from ∼ 90 to 12 μM. Furthermore, the effect was
identical for both inward and outward current through K_{ATP} channels, indicating that
the effect of poly-L-lysine was not voltage dependent. The latter finding is consistent
with the hypothesis that the effects of poly-L-lysine are mediated by screening surface
charges and not charges within the membrane voltage field. In addition to increasing
ATP sensitivity, poly-L-lysine also decreased the open probability of K_{ATP} channels,
both for inward (Fig. 5 *F*) and outward current (not shown), similar to protamine,
Mg^{2+}, and Ba^{2+}. Also similar to protamine, the effects of poly-L-lysine on ATP
sensitivity and open probability in the absence of ATP persisted after washout, but
could be fully reversed by a brief exposure to heparin (not shown).

Fig. 6 illustrates the effects of 0.5 μM poly-L-arginine (mol wt 50,000–150,000). At
this concentration, poly-L-arginine completely suppressed current through K_{ATP}

Figure 5 (*opposite*).   Effect of poly-L-lysine on the ATP sensitivity and inward current through
K_{ATP} channels. (*A–D*) An excised inside-out membrane patch was exposed to the different ATP
concentrations as indicated above the bars. The space between the bars indicates exposure to
ATP-free solution. The patch electrode and bath solutions both contained 150 mM KCl, and
the bath solution was free of Mg^{2+} throughout. The membrane potential was held at +40 mV in
A and *C*, and −40 mV in *B* and *D*. The zero current line is indicated by the dashed line. In *C*
and *D*, the bath solution contained 500 μM poly-L-lysine. (*E*) Dose response of K_{ATP} channels to
[ATP]ᵢ in inside-out membrane patches before and during exposure to 500 μM poly-L-lysine at
membrane potentials of +40 mV and −40 mV. For each patch, current (*I*) at each [ATP]ᵢ was
normalized to the current in the absence of ATP (I_{MAX}). Data points are the mean ± SEM.
Smooth curves represent best fits to a Hill equation. The K_d, Hill coefficient (*H*), and mean
number of patches for each treatment is indicated in the inset. Statistical comparison of the
I/I_{MAX} values over the [ATP]ᵢ range of 10–300 μM were as follows: control (+40 mV) vs
poly-L-lysine (+40 mV), $P < 0.05$; control (−40 mV) vs poly-L-lysine (+40 mV), $P < 0.05$;
control (+40 mV) vs control (−40 mV), $P > 0.05$; poly-L-lysine (+40 mV) vs poly-L-lysine (−40
mV), $P > 0.05$. Experimental conditions were the same as described in Fig. 6. (*F*) Effect of a
10–20 s exposure to 500 μM poly-L-lysine on inward current through K_{ATP} channels in the
absence of ATP in five patches. For each patch, current (*I*) in the presence of poly-L-lysine was
normalized to the current in the absence of poly-L-lysine (I_{MAX}).
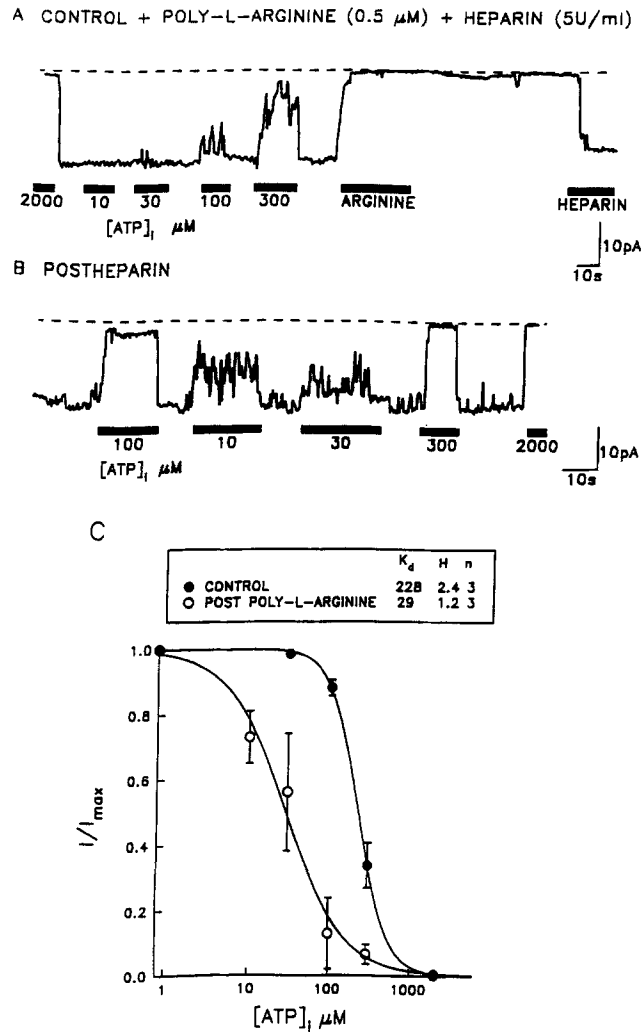
FIGURE 6. Effect of poly-L-arginine on the ATP sensitivity of the K_{ATP} channel. (A) An inside-out patch was exposed to different ATP concentrations as indicated above the bars. The space between the bars represent exposure to ATP-free solution. The patch was then exposed to poly-L-arginine (0.5 μM) in the absence of ATP_i, which resulted in complete blockade of the current. Washout of the poly-L-arginine did not restore the current, but after a brief exposure to heparin (5 U/ml), the current returned towards baseline levels. (B) The same membrane patch after exposure to heparin (5 U/ml), demonstrated increased sensitivity to closure by $[ATP]_i$. The zero current levels are indicated by the dashed lines. The patch electrode and bath solution contained 150 mM KCl, and the bath solution was free of Mg^{2+}. The membrane potential was -40 mV, and traces were filtered at 200 Hz. (C) Dose-response curve of K_{ATP} channels to $[ATP]_i$ in inside-out membrane patches under control conditions and after treatment with (and washout of) poly-L-arginine (0.5 [67od] mM) and heparin (5 U/ml). For each patch, current (I) at each $[ATP]_i$ was normalized to the current in the absence of ATP_i (I_{MAX}). Data points are the mean ± SEM. Smooth curves represent best fits to a Hill equation. The K_d, Hill coefficient (H), and mean number of patches for each treatment are indicated in the inset. Statistical comparison of the I/I_{MAX} values over the $[ATP]_i$ range of 10–300 μM for control vs post poly-L-arginine and heparin was $P < 0.05$.

channels in the inside-out patch, and the effect persisted after washout of poly-L-arginine. Subsequent exposure to heparin (5 U/ml) restored the current to the control level. However, the ATP sensitivity of the channels in the patch was increased, with the K_d decreasing from >100 μM to ~10 μM (Fig. 6 *B*). Similar findings were obtained in two additional patches, and the results are summarized in Fig. 6 *C*. In all three patches, 0.5 μM poly-L-arginine completely blocked the current through the K_{ATP} channel (Fig. 6 *A*). After washout of poly-L-arginine and treatment with heparin, the K_d was 29 μM, compared to 228 μM under control conditions in these patches (Fig. 6 *C*). We did not test whether treatment of the patch with heparin at a higher dose or for a longer period of time fully reversed the effects of poly-L-arginine on ATP sensitivity of the K_{ATP} channels. Of the three polycations tested, poly-L-arginine is the most potent at screening negative surface charge (Singh et al., 1992).

Polycations Do Not Affect Single-Channel Conductance or Induce Rapid Open Channel Block of K_{ATP} Channels

As illustrated in Figs. 1 *E*, 2 *B*, 4 *B*, 5 *E*, and 6 *A*, all of the negative surface charge screening agents partially blocked inward and outward current through K_{ATP} channels in the absence of ATP. Unfortunately, it was not possible to obtain membrane patches with a single K_{ATP} channel to analyze the kinetic features of channel block by polycations in detail. However, in several patches, there were few enough K_{ATP} channels to assess the effects on single-channel conductance and to draw some qualitative conclusions about the nature of channel block by the polycations in the absence of ATP. As shown in Fig. 7, neither protamine nor poly-L-lysine significantly altered the single-channel slope conductance for inward current, which averaged 67 ± 1.8 pS under control conditions, 63 ± 2.1 pS after exposure to 10 μM protamine, and 59 ± 2.3 pS during exposure to 500 μM poly-L-lysine (*n* = 5). Thus, the block of inward current by polycations cannot be attributed to a reduction in the single-channel conductance caused by fast open channel block. Qualitative inspection of the inward single channel currents in Fig. 7 does not reveal any obvious changes in the kinetics of gating. We did not characterize whether the block of inward current was voltage dependent.

Effect of Charged Amphiphiles on the ATP Sensitivity of the K_{ATP} Channel

The location of the negative surface charges influencing the ATP sensitivity of K_{ATP} channels could be on the lipid bilayer or on the channel protein itself. To distinguish between these two possibilities, we examined the effects of the positively and negatively charged amphiphiles, DDTMA and SDS. These amphiphiles insert into the lipid bilayer and alter the lipid-borne surface charge. It has been shown that at a concentration of 20 μM, these agents cause significant shifts in gating of voltage-gated Na^+, Ca^{2+}, and K^+ channels in a manner consistent with altered membrane surface charge density (Ji, Weiss, and Langer, 1993). If lipid-borne surface charges were important determinants of the ATP sensitivity of K_{ATP} channels, then DDTMA would be expected to increase ATP sensitivity by increasing positive lipid-borne surface charge density, while SDS should decrease ATP sensitivity by adding negative surface charge. At 20 μM, DDTMA caused block of both inward and outward current through K_{ATP} channels, so that its effects on ATP sensitivity could not be evaluated.
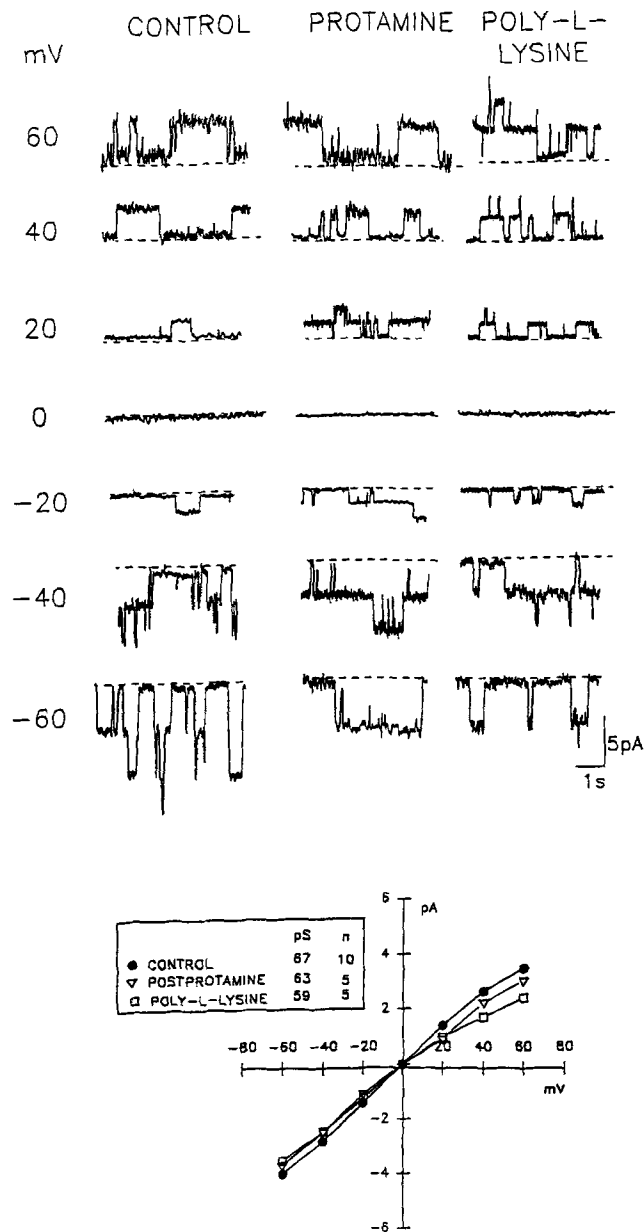
FIGURE 7. Effects of protamine and poly-L-lysine on unitary conductances of K_{ATP} channels. (A) Recordings of unitary currents were made with 150 mM KCl in both the patch electrode and bath solutions, and the bath solution was free of Mg^{2+}. Left, middle, and right traces are control, after washout of 10 μM protamine, and in the presence of 500 μM poly-L-lysine, respectively. Zero current line is indicated by the dashed line, and the membrane potential (in millivolts) is indicated to the left of traces. Traces were filtered at 200 Hz. (B) Single-channel I-V relationship for excised inside-out patches under the three conditions. Each point is the average of measurements in five patches at various membrane potentials. The single-channel conductance in the inward direction is shown in the inset for the three conditions. For graphic illustration, the data for the control patches were pooled. SE bars are smaller than the symbols.
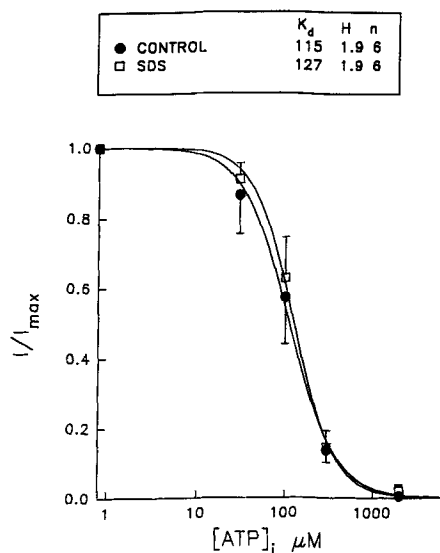
	K_d	H	n
● CONTROL	115	1.9	6
□ SDS	127	1.9	6

FIGURE 8. Dose-response curve of K_{ATP} channels to [ATP]$_i$ in inside-out membrane patches before and during exposure to 20 μM SDS. For graphic illustration, the data for the control patches were pooled. For each patch, current (I) at each [ATP]$_i$ was normalized to the current in the absence of ATP$_i$ (I_{MAX}). Data points are the mean ± SEM for six patches. Smooth curves represent best fits to a Hill equation. The K_d and Hill coefficient (H) before and during SDS exposure are indicated in the inset. There was no significant difference between the I/I_{MAX} values over the [ATP]$_i$ range of 10–300 μM between control and SDS ($P > 0.05$).

After removal of DDTMA, however, the ATP sensitivity was the same as under control conditions ($n = 6$ patches). At 20 μM, SDS did not block current through K_{ATP} channels, and it did not significantly alter the K_d for block by ATP (Fig. 8). These results suggest that the surface charges modulating ATP sensitivity are located on the K_{ATP} channel protein rather than in the adjacent lipid bilayer, and that the surface charge screening agents do not act by screening surface charges on the lipid bilayer.
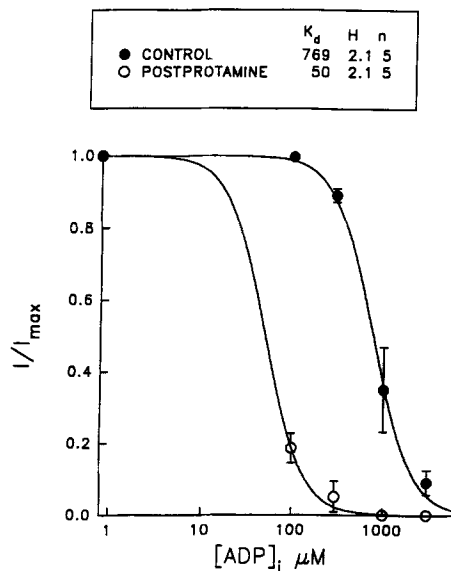

	K_d	H	n
● CONTROL	769	2.1	5
O POSTPROTAMINE	50	2.1	5

FIGURE 9. Effect of 10 μM protamine on the sensitivity of K_{ATP} channels to closure by cytosolic ADP ([ADP]$_i$) in the absence of Mg^{2+}]$_i$. Each data point represents the average from five patches fitted to a Hill equation (smooth curves). The K_d, Hill coefficient (H), and number of patches are indicated in the inset. I_{max} is average current through K_{ATP} channels in absence of cytosolic ATP or ADP. Compared to control, the sensitivity of K_{ATP} channels to block by ADP after washout of 10 μM protamine was markedly increased ($P < 0.05$ for data range of 100–1000 μM [ADP]$_i$).
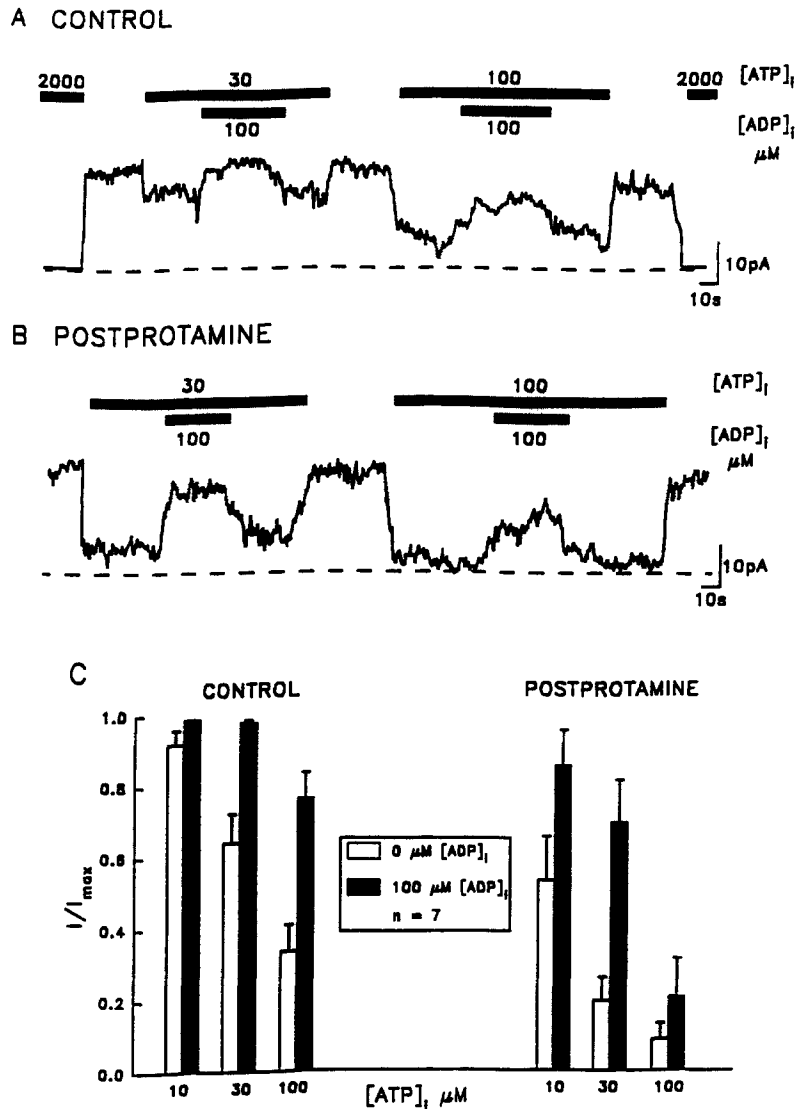
FIGURE 10. Effect of 10 μM protamine treatment on the stimulatory effect of cytosolic ADP on K_{ATP} channel activity in the presence of 2 mM free $[Mg^{2+}]_i$. (A) In an excised inside-out membrane patch, K_{ATP} channels were maximally activated by removing $[ATP]_i$ (between bars) and then partially suppressed by 30 or 100 μM $[ATP]_i$, as indicated. In the presence of either $[ATP]_i$, 100 μM $[ADP]_i$ caused an increase in current. (B) The same membrane patch after exposure to 10 μM protamine for 20 s. The patch was more sensitive to closure by ATP_i, but in the presence of either 30 or 100 μM $[ATP]_i$, 100 μM $[ADP]_i$ reversibly increased $I_{K_{ATP}}$ as before protamine. Zero current levels are indicated by dashed lines. All solutions contained 2 mM free $[Mg^{2+}]_i$. Current traces were filtered at 200 Hz. (C) Summary of the effect of protamine on ADP/ATP interaction, in data averaged from seven excised inside-out patches before and after exposure to 10 μM protamine. ADP (100 μM, with 2 mM free intracellular Mg^{2+} present) was applied to patches after $I_{K_{ATP}}$ had been partially suppressed by 10, 30, or 100 μM ATP_i, both before and after exposure to 10 μM protamine. I_{MAX} represents average current in absence of both ATP_i or ADP_i. After exposure of the membrane patch to protamine, the stimulatory effect of ADP_i remained present when comparing the current levels in the presence and absence of ADP_i ($P < 0.05$ at both 30 and 100 μM ATP_i before protamine treatment and at 10, 30, and 100 μM ATP_i after protamine treatment).

Effect of Screening Surface Charge on ADP Block of K_{ATP} Channel and on the ATP-ADP Interaction

In the absence of cytosolic Mg^{2+}, millimolar cytosolic ADP blocks K_{ATP} channels (Noma, 1983). To investigate whether surface charge screening agents alter the sensitivity of K_{ATP} channels to block by ADP, we exposed excised inside-out membrane patches to various [ADP]$_i$ in a Mg^{2+}-free bath solution before and after treatment of the patch with 10 μM protamine. Fig. 9 shows that after exposure to protamine, the K_d for block of K_{ATP} channels by [ADP]$_i$ decreased from 769 to 50 μM ($n = 5$), similar to the effects of protamine on the ATP sensitivity of the channels.
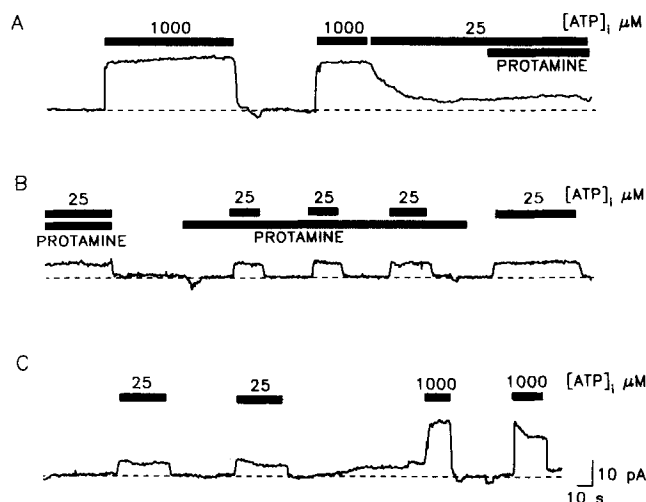

FIGURE 11.   Effect of protamine on outward Na^+-K^+ pump current in an excised inside-out giant membrane patch. The patch electrode solution contained 5 mM K^+, and the bath solution contained 100 mM Na^+. *A–C* are continuous tracings. The Na^+-K^+ pump current was reversibly and maximally activated by exposing to the cytoplasmic membrane surface to 1 mM $MgATP^{2-}$. Reducing cytosolic $MgATP^{2-}$ to 25 μM caused steady state current to decline by ~80%, and 10 μM protamine had no effect in repeated challenges. At the end of the tracing, the current was again maximally activated by 1,000 μM ATP$_i$, showing only a modest rundown. Zero current level is indicated by dashed lines. Current traces were acquired at a digitization rate of 30 Hz.

In the presence of intracellular Mg^{2+}, cytosolic ADP interferes with the ability of ATP to close K_{ATP} channels. Fig. 10 shows the effect of protamine on this stimulatory effect of ADP. In this excised inside-out patch, outward current through multiple K_{ATP} channels was partially suppressed by 30 or 100 μM [ATP]$_i$ with 2 mM free Mg^{2+} present, and it was significantly potentiated when 100 μM [ATP]$_i$ was added to the bath (Fig. 10 *A*). After exposure to 10 μM protamine in the same patch, the ATP sensitivity was increased (Fig. 10 *B*). However, the ability of 100 μM [ADP]$_i$ to potentiate current through K_{ATP} channels remained. Fig. 10 *C* summarizes the results from seven patches exposed to the same protocol and demonstrates that ADP

remained effective at relieving the inhibition of K_{ATP} channels by ATP after screening of surface charge with protamine.

Effect of Protamine on the ATP Sensitivity of the Na^+-K^+ Pump

To determine whether the ATP sensitivity of other ATP-binding proteins might be affected by surface charge screening agents in a similar manner to the K_{ATP} channel, we examined the effects of protamine on the Na^+-K^+ pump, using the giant inside-out membrane patch technique developed by Hilgemann (Collins et al., 1992). In Fig. 11, outward Na^+-K^+ pump current was maximally activated by adding 1 mM $MgATP^{2-}$ to the cytosolic surface with 100 mM Na^+ in the bath and 5 mM K^+ in the patch electrode. Reducing cytosolic $MgATP^{2-}$ to 25 μM decreased the Na^+-K^+ pump current by ~80%, and subsequent application of 10 μM protamine had no significant effect on the magnitude of the current during multiple applications of $MgATP^{2-}$. Similar results were obtained in two additional giant patches. The outward current elicited by application of $MgATP^{2-}$ required the presence of Na^+ in the bath. Similar to previous published results, half-maximal activation of the outward current occurred at a $[Na^+]_i$ of 2–3 mM, and half-maximal activation by $MgATP^{2-}$ at ~100 μM. The outward current induced by $MgATP^{2-}$ + Na^+ was not affected by 10 or 100 μM glibenclamide, a blocker of K_{ATP} channels.

DISCUSSION

Surface Charge and the ATP Sensitivity of K_{ATP} Channels

This study demonstrates that exposure of the cytosolic region of excised inside-out patches to Mg^{2+}, Ba^{2+}, and various polycations (protamine, poly-L-lysine, and poly-L-arginine) dramatically increased the sensitivity of the K_{ATP} channel to closure by its negatively charged ligand, ATP. Despite their marked differences in molecular weight, total charge, and effective concentration range (differing by over four orders of magnitude), the common ability of this diverse group of cations to increase the sensitivity of K_{ATP} channels to ATP is most consistent with the hypothesis that the effect of the cations is caused by screening and/or binding to negative surface charges in the region of the ATP-binding site(s) on the K_{ATP} channel protein. We consider these putative negative charges to be surface charges because neither the ability of ATP to block K_{ATP} channels nor the effects of surface charge screening agents was sensitive to the transmembrane voltage gradient (Fig. 5). All of the agents used in this study have been shown to be effective at screening negative surface charge in the concentration ranges tested in this study, and their relative potencies are comparable to those reported from surface charge neutralization experiments in other assays (Singh et al., 1992; Hille, 1992). Several of the polycations (protamine and poly-L-arginine) had high affinity, requiring only submicromolar concentrations to exert their effects. The observation that their effect on ATP sensitivity persisted after they were washed out from the bath solution suggests that they bind to the negative surface charges and dissociate slowly. However, their effect was either completely or partially reversed by exposure to heparin, a highly charged polyanion, which has been shown to readily reverse the surface charge screening effects of these polycations in other assays (Singh et al., 1992). The divalent cations may also bind to

the negative surface charges (Hille, 1992); in fact, this is one possible explanation for the observation that the effects of Mg^{2+} and Ba^{2+} were not quantitatively identical.

We envision the mechanism by which surface charges modulate the ATP sensitivity of K_{ATP} channels to be the following (Fig. 12): negative charges are present in a region of K_{ATP} channel protein through which the anionic ATP molecule must pass to reach the actual ATP-binding site(s) on the protein. The effect of the surface potential generated by these negative surface charges is to cause a reduction in the local concentration of ATP near the ATP-binding site. By screening these negative charges and reducing this surface potential, the difference between the bulk ATP concentration and that in the region of the ATP-binding site(s) is reduced, resulting in an increase in the apparent affinity of the K_{ATP} channel for ATP. This is analogous to the effects of surface charges near the active sites of enzymes, such as superoxide
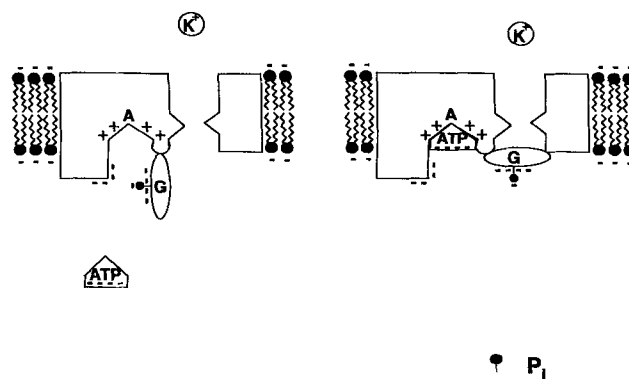

FIGURE 12.   Hypothetical model of the K_{ATP} channel illustrating how negative surface charges near the positively charged ATP-binding site (*A*) may alter the apparent sensitivity of the K_{ATP} channel to closure by ATP and modulate open-close transitions of the gate (*G*). Left and right panels show open and closed states of the channel, respectively. Negative phosphate groups (P_i) are indicated by the symbol. Only one ATP-binding site is shown for simplicity (although the Hill coefficient typically near 2 indicates that two ATP-binding sites are more likely). See text for details.

dismutase, in which the apparent affinity of the enzyme for an oppositely charged substrate (e.g., the superoxide anion) is increased by locally concentrating the substrate in that region (Cudd and Fridovich, 1982); and that of tetrodotoxin- and saxitoxin-binding sites in Na^+ channels, in which negative charges increase the apparent binding affinity by increasing local toxin concentration (Green et al., 1987). Similarly, negative charges at the entrance to the pores of a number of ion channels, including AChR (Dani and Eisenman, 1987), voltage-dependent Na^+ channels (Green et al., 1987), inwardly rectifying K^+ channels (Kell and DeFelice, 1988), L-type Ca^{2+} channels (Prod'hom, Pietrobon, and Hess, 1989), and Ca^{2+}-activated K^+ channels (MacKinnon, Latorre, and Miller, 1989), enhance permeation rates by increasing the local concentration of oppositely charged permeant ions. A novel

feature of surface charge effect as it relates to the K_{ATP} channel, however, is that it acts to decrease rather than increase the apparent affinity of ATP for its binding site.

Unlike the voltage sensors of voltage-gated ion channels, which appear to sense the surface potential produced by negative charges in the lipid bilayer (Ji et al., 1993), we found no evidence that increasing negative surface charge on the inner (cytosolic) surface of the membrane, by exposing the patch to the negatively charged amphiphile SDS, had any effect on the ATP sensitivity of K_{ATP} channels (Fig. 8). This concentration of SDS produced up to a 22-mV shift in the gating parameters of the sodium current (Ji et al., 1993). A change in surface potential of this magnitude would be sufficient to decrease the local concentration of $MgATP^{2-}$ and ATP^{4-} at the membrane surface by factors of 5.7 and 32.4, respectively (see Eq. 2 below), yet SDS had no effect on the ATP sensitivity of K_{ATP} channels. This effect of SDS suggests that the negative surface charges modulating the ATP sensitivity of K_{ATP} channels are located on the K_{ATP} channel protein itself, rather than on the adjacent lipid bilayer. We could not test the effects of the positively charged amphiphile DDMTA because at the concentration that shifted gating parameters of voltage-gated ion channels (Ji et al., 1993), DDTMA completely blocked current through K_{ATP} channels.

The density of surface charges that would be necessary to produce the observed effects on ATP sensitivity can be roughly estimated using surface potential theory (McLaughlin et al., 1971). The relationship between surface potential (Ψ_0) and local ion concentration $[C_i(0)]$ can be expressed by the relationship:

$$C_i(0) = C_i^* \exp\left(-z_i F \Psi_0 / RT\right), \qquad (2)$$

where C_i is the bulk concentration of the ionic species, z_i is the valence of the ion, and R, T, and F have their usual meanings. The K_d for suppression of K_{ATP} channels by ATP was 39 μM in the presence of 2 mM $[Mg^{2+}]_i$ and 29 μM in the presence of 5 mM free $Mg[^{2+}]_i$. We assume that (a) the predominant species blocking the K_{ATP} channels was $MgATP^{2-}$ because the concentration of other ATP species would be very low with such a large excess of free Mg^{2+}; (b) the local $MgATP^{2-}$ concentration at the ATP-binding site(s) required to produce half-maximal suppression of channel activity was the same at both free Mg^{2+} concentrations; and (c) the difference in apparent K_d's resulted from a greater unscreened surface potential in the presence of 2 mM free Mg^{2+}, requiring a greater bulk $MgATP^{2-}$ concentration to achieve the same local $MgATP^{2-}$ concentration at the ATP-binding site(s) on the channel, by the ratio of 39/29. Using Eq. 2, the surface potentials in the presence of 2 mM free Mg^{2+} $[\Psi_0(2)]$ and 5 mM free Mg^{2+} $[\Psi_0(5)]$ are therefore related by the expression:

$$\exp\left(+2F\Psi_0(2)/RT\right) = (39/29)^*\exp(+2F\Psi_0(5)/RT)$$

or

$$\Psi_0(2) = \ln(39/29) + \Psi_0(5) \qquad (3)$$

Making the simplifying assumptions that the surface charge near the ATP-binding site is uniformly distributed, that the dielectric constant in the aqueous phase is constant and equal to its bulk value, and that the ions act as point charges and screen but do not bind to surface charges, the relationship between surface charge density

and surface potential is described by the Graham equation:

$$\sigma^2 = 2\epsilon\epsilon_0 RT\Sigma\{C_i^*\exp(-z_i\Psi_0/RT) - C_i\}$$

or

$$(272\sigma)^2 = \Sigma\{C_i^*\exp(-z_i\Psi_0/RT) - C_i\} \qquad (4)$$

where C_i is the molar concentration of the ith ion for all the ionic species in the solution, ϵ is the dielectric constant of aqueous solution, and ϵ_0 is the permittivity of free space. Considering K^+, Mg^{2+}, and Cl^- as the major ions in solution, Eq. 4 can be written for the two cases corresponding to 2 and 5 mM free Mg^{2+}. Since the left hand side of both equations are the same, the right-hand sides can be set equal to each other and then solved for $\Psi_0(2)$ by substituting Eq. 3. The solution of this cubic polynomial gives a value of -63.5 mV for $\Psi_0(2)$, and it yields values of -59.8 mV for $\Psi_0(5)$, and 1 charge per 199 Å^2 for σ when substituted back into Eq. 4. These are reasonable values of surface charge density and surface potentials for biological membranes (McLaughlin et al., 1971). The value of $\Psi_0(2)$ or $\Psi_0(5)$ can also be used in Eq. 3 to calculate $C_i(0)$, the local concentration of $MgATP^{2-}$ at the ATP-binding site that causes half-maximal suppression of $I_{K,ATP}$. The value of $C_i(0)$ is 0.26 μM, which is equivalent to the "intrinsic" K_d of the ATP-binding site in the absence of a surface potential. The above calculations are very rough estimates and are subject to considerable error from the simplifying assumptions used in applying the Graham equation to ligand binding in a protein, where only a few charged residues in a complex geometrical arrangement are likely to be important. Also, binding of cations to surface charges and the different affinities of various ATP species for the ATP-binding site have not been considered, and it is difficult to estimate what the distribution of ATP species with different valences would be near the ATP-binding site(s) under influence of the local surface potential. In the absence of divalent cations, this latter factor would greatly complicate any attempt to analyze quantitatively the effects of screening surface potential by altering monovalent cation concentration.

Major differences in the affinity of the ATP-binding site(s) for $MgATP^{2-}$ and ATP^{4-} or the trivalent ATP species may be the explanation for the different effects of Mg^{2+} on cardiac vs pancreatic and skeletal muscle K_{ATP} channels. Unlike cardiac K_{ATP} channels, Ashcroft and Kakei (1987) have shown that K_{ATP} channels in pancreatic beta cells are blocked with much higher affinity by ATP^{4-} ($K_d \sim 4 \mu$M) than $MgATP^{2-}$. Similar findings have been obtained in skeletal muscle K_{ATP} channels (Vivadou et al., 1991). It is perhaps noteworthy that the K_d for block by ATP^{4-} in K_{ATP} channels from these tissues is similar to that of cardiac K_{ATP} channels in the presence of the surface charge screening polycations (e.g., Figs. 4 and 5). This could be accounted for if the putative negative surface charges modulating the ATP sensitivity of cardiac K_{ATP} channels were reduced or absent in pancreatic and skeletal muscle K_{ATP} channels. It should be possible to test this idea by determining whether polycations have any effect on the ATP sensitivity of pancreatic and skeletal muscle K_{ATP} channels. Unlike pancreatic and skeletal muscle K_{ATP} channels, however, cardiac K_{ATP} channels appear to be readily blocked by $MgATP^{2-}$ as well as by ATP^{4-}. A compelling argument against ATP^{4-} as the sole high affinity blocking species in

cardiac K_{ATP} channels is the following: in the presence of 2 mM free Mg^{2+} and a total ATP concentration of 30 μM, current through cardiac K_{ATP} channels was suppressed by 49 $\pm$ 5% at a calculated free ATP^{4-} concentration of 1.1 μM. However, in the presence of 15 mM free Mg^{2+} and the same total ATP concentration, current was suppressed to a greater extent (by 83 $\pm$ 5%) by a much lower free ATP^{4-} concentration of 0.2 μM (Fig. 1 D). The ability of a lower free ATP^{4-} concentration to produce a greater degree of block cannot be attributed to the additional surface charge screening effect of 15 vs 2 mM Mg^{2+} because, in the presence of polycationic surface charge screening agents, significantly higher concentrations of free ATP^{4-} (>1.6 μM) were required to produce this degree of block. Thus, our results support the hypothesis that $MgATP^{2-}$ blocks the cardiac K_{ATP} channel.

Blocking Effects of Surface Charge Screening Agents on K_{ATP} Channels

In addition to their effect on ATP sensitivity, Mg^{2+}, Ba^{2+} and all of the polycations tested caused block of inward current through K_{ATP} channels in the absence of cytosolic ATP. The ability of Mg^{2+} to block inward current through K_{ATP} channels has been described previously in cardiac and pancreatic cells (Findlay, 1987a, 1987b; Ashcroft and Kakei, 1989), although not in skeletal muscle (Forestier and Vivaudou, 1993). Unlike block of outward current by Mg^{2+}, which is voltage-dependent and associated with a reduction in apparent single-channel conductance consistent with fast open-channel block (Findlay, 1987a; Horie et al., 1987; Ashcroft and Kakei, 1989), block of the inward current by Mg^{2+} does not show features of fast open-channel block (Horie et al., 1987; Aschroft and Kakei, 1989). Mg^{2+} block of the inward current appears to be qualitatively similar, from a kinetic standpoint, to the block produced by the various polycations, which also did not cause a change in single-channel conductance or flickery block (Fig. 6). Unfortunately, the effects on channel gating kinetics could not be rigorously quantitated because of our inability to obtain patches with a single K_{ATP} channel.

It is notable that the potency of these agents in blocking inward current generally paralleled their potency in altering ATP sensitivity (Figs. 1, D and $E2$, A and B; and 5, E and F). An intriguing possibility is that the block of inward current by polycations is related to their surface charge screening effect. For example, the relevant negative surface charges might be located in the region of the ATP-dependent gate (G), as illustrated schematically in Fig. 12, such that their electrostatic interaction with positive charges at the ATP-binding region (A) promote the open state of the gate. When ATP binds and adds negative charge to the A site, this electrostatic interaction promoting the open state of the gate is broken, and the gate closes. Screening the negative charges with surface charge screening agents would also inhibit the electrostatic interaction and promote gate closure, and would raise the local ATP concentration in the immediate vicinity of the A site, decreasing the apparent K_d for ATP. This hypothetical model provides a unifying scheme to explain the dual effects on ATP sensitivity and decreased open probability. The model is analogous in some respects to calmodulin-binding proteins, in which a highly charged calmodulin-binding domain interacts electrostatically with another region of the protein producing autoinhibition (Soderling, 1990; Chapman, Au, Alexander, Nicolson, and Storm,

1991). By binding to this site, calmodulin prevents the electrostatic interaction and relieves autoinhibition, activating the enzymatic function of the protein.

There are several attractive aspects to this K_{ATP} channel model. The dual effects of an increase in ATP sensitivity accompanied by a decrease in channel open probability are not unique to surface-charge screening agents, but they constitute a general pattern common to a number of interventions that alter K_{ATP} channel function. K_{ATP} channel rundown is associated simultaneously with a reduction in open probability and an increased sensitivity to closure by ATP (Deutsch and Weiss, 1993). One or several of the key negative surface charges on the gate of the K_{ATP} channel might be negatively charged phosphate groups (Fig. 12). Reduction of this negative charge as a result of dephosphorylation would inhibit the electrostatic interaction with the unoccupied A site, which keeps the gate open in the absence of ATP, promoting "rundown." At the same time, the loss of the negatively charged phosphate group(s) would result in an increase in local ATP concentration at the A site relative to bulk cytosolic ATP concentration, increasing the apparent affinity for ATP. A similar explanation has been invoked to explain the role of phosphorylation in the regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel; namely, that channel phosphorylation adds negative charge to specific domains of the channel and causes electrostatic interactions within the protein that promote the open state (Rich, Berger, Cheng, Travis, Saxena, Smith, and Welsh, 1993). The converse scenario might explain the dual effects of ADP in the presence of Mg^{2+} (Lederer and Nichols, 1989; Weiss et al., 1992) and of trypsinization of the cytosolic membrane surface (Proks and Ashcroft, 1993; Deutsch and Weiss, 1994), which simultaneously increase channel open probability and decrease ATP sensitivity.

Physiological Significance

K_{ATP} channels are found in a variety of tissues including cardiac muscle, skeletal muscle, vascular and nonvascular smooth muscle, pancreatic beta cells, neural tissue, renal epithelium, and follicular cells. It is remarkable how widely the sensitivity of K_{ATP} channels to block by ATP varies among different tissues and even within the same tissue. For instance, the K_d for closure by $[ATP]_i$ is in the 1–100 μM range for cardiac and pancreatic cells (Noma, 1983; Findlay, 1987a; Ashcroft and Kakei, 1989; Lederer and Nichols, 1989), but in the millimolar range for ventral medial hypothalamic neurons and renal epithelial cells (Ashcroft and Ashcroft, 1990; Tsuchiya et al., 1992). For cardiac K_{ATP} channels, the K_d in different membrane patches varied from 9 to 580 μM ATP_i in rat ventricle (Findlay and Faivre, 1991) and from 5 to 79 μM in guinea pig ventricle (Weiss et al., 1992). Differences in ATP sensitivity between endocardial and epicardial K_{ATP} channels have also been described (Furukawa, Kimura, Furukawa, Bassett, and Myerburg, 1991), and ATP sensitivity of cardiac K_{ATP} channels is markedly altered by metabolic inhibition, channel rundown, and trypsinization (Thuringer and Escande, 1990; Deutsch and Weiss, 1993; Proks and Ashcroft, 1993; Deutsch and Weiss, 1994). By providing evidence that negative surface charge density in the region of the ATP-binding site(s) has dramatic effects on the ATP sensitivity of K_{ATP} channels, the present study suggests a potential explanation for this variability; that the density of negative surface charges located at the access to the ATP-binding site(s) varies in a tissue-specific manner, providing a means for

regulating the sensitivity of K_{ATP} channels to cytosolic ATP concentrations in that tissue.

We also considered the possibility that variation in negative surface charge density may be a general mechanism for regulating ATP sensitivity of ATP-binding proteins. In this context, we studied the effects of protamine on the ATP sensitivity of the Na^+-K^+ pump. However, we found that protamine had no significant effect, suggesting that this mechanism may be specific for K_{ATP} channels.

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