

ATP Modulation of ATP-sensitive Potassium Channel ATP Sensitivity Varies with the Type of SUR Subunit*

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ATP-sensitive potassium (K_{ATP}) channels comprise Kir and SUR subunits. Using recombinant K_{ATP} channels expressed in *Xenopus* oocytes, we observed that MgATP (100 μ M) block of Kir6.2/SUR2A currents gradually declined with time, whereas inhibition of Kir6.2/SUR1 or Kir6.2 Δ C36 currents did not change. The decline in Kir6.2/SUR2A ATP sensitivity was not observed in Mg²⁺ free solution and was blocked by the phosphatidylinositol (PI) 3-kinase inhibitors LY 294002 (10 μ M) and wortmannin (100 μ M), and by neomycin (100 μ M). These results suggest that a MgATP-dependent synthesis of membrane phospholipids produces a secondary decrease in the ATP sensitivity of Kir6.2/SUR2A. Direct application of the phospholipids PI 4,5-bisphosphate and PI 3,4,5-trisphosphate in the presence of 100 μ M MgATP activated all three types of channel, but the response was faster for Kir6.2/SUR2A. Chimeric studies indicate that the different responses of Kir6.2/SUR2A and Kir6.2/SUR1 are mediated by the first six transmembrane domains of SUR. The MgATP-dependent loss of ATP sensitivity of Kir6.2/SUR2A was enhanced by the actin filament disrupter cytochalasin and blocked by phalloidin (which stabilizes the cytoskeleton). Phalloidin did not block the effect of PI 3,4,5-trisphosphate. This suggests that MgATP may cause disruption of the cytoskeleton, leading to enhanced membrane phospholipid levels (or better targeting to the K_{ATP} channel) and thus to decreased channel ATP sensitivity.

ATP-sensitive potassium (K_{ATP})¹ channels are widely distributed, being found in pancreatic B-cells, cardiac, smooth, and skeletal muscles, and neurones (1). They play important functional roles in all these tissues by linking cellular metabolism to electrical activity. Opening of the K_{ATP} channel produces a voltage-independent K⁺ current that hyperpolarizes the cell and reduces its electrical excitability; conversely, K_{ATP} channel closure usually decreases membrane excitability.

The K_{ATP} channel is an octamer, formed by the physical association of four inwardly rectifying potassium channel sub-

units (Kir6.2) and four regulatory sulfonylurea receptor subunits (2). K_{ATP} channels in different tissues are composed of different Kir and SUR subunits. In most tissues, Kir6.2 acts as the pore-forming subunit (3, 4). Two different sulfonylurea receptor genes (SUR1 and SUR2) have been identified, and further diversity is created by alternative splicing of SUR2 (5–9). In this paper, the major isoform of SUR2 (6) is called SUR2A. It shares 67% sequence identity with SUR1. There is evidence that SUR1 serves as the regulatory subunit of the K_{ATP} channel in pancreatic B-cells and some types of neurone (5, 10), whereas a similar role is played by SUR2A in cardiac and skeletal muscle (6).

Binding sites for drugs and modulatory agents are found on both Kir6.2 and SUR subunits. The nucleotides ATP and ADP bind to an intracellular site on Kir6.2 and bring about K_{ATP} channel closure (11, 12), whereas a range of magnesium nucleotides (including ATP and ADP) interact with the NBDs of SUR and thereby increase channel activity (13–16). The balance between these inhibitory and stimulatory effects results in channel inhibition by most concentrations of MgATP and high concentrations of MgADP (10 mM) and in channel activation by low micromolar concentrations of MgADP. It is thought that metabolic regulation of K_{ATP} channel activity is mediated, in part, by changes in the intracellular concentrations of these nucleotides.

Recent studies indicate that membrane phospholipids also play an important role in modulating K_{ATP} channel activity. Thus, phosphatidylinositol 4,5-bisphosphate (PIP₂) increases the activity and reduces the ATP sensitivity of both native and cloned K_{ATP} channels (17–21). Overexpression of PI5-kinase, which enhances PIP₂ levels, reduces the ATP sensitivity of the channel in membrane patches (21), whereas breakdown of PIP₂ by phospholipase C enhances the K_{ATP} channel ATP sensitivity (22). This is in agreement with data indicating that PIP₂ binds directly to Kir channels (23). Interestingly, application of PIP₃ and PI 3-P also enhances K_{ATP} channel activity (19, 20, 24).

The rundown of both native and cloned K_{ATP} channels that occurs in excised membrane patches is reversed by application of MgATP (25–27), and PIP₂ has also been implicated in this effect (25). It is postulated that PIP₂ is produced in plasma membrane by serial phosphorylation of PI and that this process is sequentially catalyzed by PI 4-kinase and PI 5-kinase. MgATP generation of phospholipids, therefore, has multiple but related effects on K_{ATP} channel activity. It decreases the rate of channel rundown, it causes reactivation of the channel after removal of ATP, and it reduces the channel ATP sensitivity.

In this paper, we demonstrate that the time course of K_{ATP} channel inhibition by ATP is markedly different for Kir6.2/SUR1 and Kir6.2/SUR2A. Whereas inhibition of Kir6.2/SUR1 currents by 100 μ M ATP does not change with time, that of Kir6.2/SUR2A decreases during prolonged nucleotide applica-

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¹ The abbreviations used are: K_{ATP} , ATP-sensitive potassium; NBD, nucleotide-binding domain; PIP₂, and PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PI, phosphatidylinositol; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; TM, transmembrane domain; PI(3)P, phosphatidylinositol 3-phosphate.

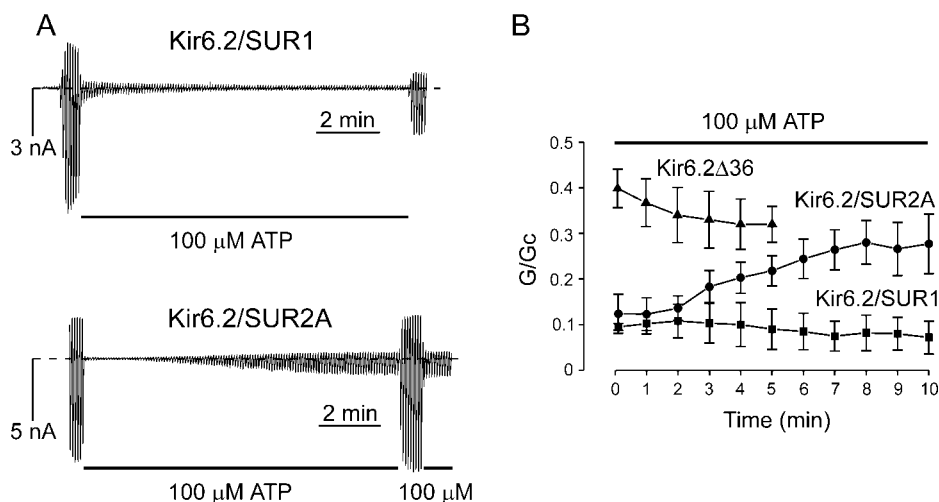


FIG. 1. Time course of current response in the presence of 100 μ M MgATP. A, macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR1 or SUR2A in response to a series of voltage ramps from -110 to $+100$ mV. ATP was added to the intracellular solution as indicated by the bars. All solutions contained Mg^{2+} . B, mean K_{ATP} conductance recorded for Kir6.2 Δ C36 (\blacktriangle , $n = 3$), Kir6.2/SUR1 (\blacksquare , $n = 6$), or Kir6.2/SUR2A (\bullet , $n = 7$) currents at different times after the addition of 100 μ M MgATP to the intracellular solution. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP.

tion. This ATP-dependent activation of Kir6.2/SUR2A currents by MgATP can be prevented by 10 μ M LY 294002, a specific inhibitor of PI 3-kinase (28), suggesting that it results from MgATP-dependent production of PIP_3 or $\text{PI}(3,4)\text{P}_2$ rather than $\text{PI}(4,5)\text{P}_2$ (PIP_2). Both PIP_3 and PIP_2 , however, are able to promote channel activity in excised patches. Studies with chimeric SUR further suggest that the different responses of Kir6.2/SUR1 and Kir6.2/SUR2A channels are conferred by the first set of transmembrane domains of the sulfonylurea receptor.

EXPERIMENTAL PROCEDURES

Molecular Biology—Mouse Kir6.2 (GenBankTM accession number D50581; Refs. 3 and 4), rat SUR1 (GenBankTM accession number L40624; Ref. 5), and SUR2A (GenBankTM accession number D83598; Ref. 6) cDNAs were cloned in the pBFP vector. A truncated form of Kir6.2 (Kir6.2 Δ C), which lacks the C-terminal 36 amino acids and forms functional channels in the absence of SUR, was prepared as described previously (11). Chimeras between SUR1 and SUR2A were constructed as described previously (29). Capped mRNA was prepared using the mMESSAGE mMACHINE large scale *in vitro* transcription kit (Ambion, Austin, TX), as previously described (30).

Oocyte Collection—Female *Xenopus laevis* were anesthetized with MS222 (2 g/liter added to the water). One ovary was removed via a mini-laparotomy, the incision was sutured, and the animal was allowed to recover. Immature stage V and VI oocytes were incubated for 60 min with 1.0 mg/ml collagenase (Sigma, type V) and manually defolliculated. Oocytes were either injected with ~ 1 ng of Kir6.2 Δ C mRNA or coinjected with ~ 0.1 ng of Kir6.2 mRNA and ~ 2 ng of mRNA encoding either SUR1 or SUR2A. The final injection volume was 50 nl/oocyte. Isolated oocytes were maintained in Barth's solution and studied 1–4 days after injection (30).

Electrophysiology—Patch pipettes were pulled from borosilicate glass and had resistances of 250–500 k Ω when filled with pipette solution. Macroscopic currents were recorded from giant excised inside-out patches at a holding potential of 0 mV and at 20–24 $^{\circ}\text{C}$ using an EPC7 patch-clamp amplifier (List Elektronik, Darmstadt, Germany; Ref. 30). The pipette (external) solution contained 140 mM KCl, 1.2 mM MgCl_2 , 2.6 mM CaCl_2 , 10 mM HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained 107 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM EGTA, 10 mM HEPES (pH 7.2 with KOH; final $[\text{K}^+]$, ~ 140 mM). The magnesium-free intracellular solution contained 140 mM KCl, 1 mM EGTA, 10 mM HEPES (pH 7.2 with KOH). LY 294002 (CalBiochem), phalloidin, and cytochalasin (CalBiochem) were dissolved in Me_2SO to make 10 mM stock solutions. Stock solutions (1 mM) of PIP_2 and PIP_3 were made in magnesium-free intracellular solution and diluted to the desired concentration and sonicated (30 min on ice) immediately before use. Rapid exchange of solutions was achieved by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the

bath. Test solutions were applied in random order unless otherwise stated.

In most experiments, currents were recorded in response to repetitive 3-s voltage ramps from -110 mV to $+100$ mV. They were filtered at 10 kHz, digitized at 0.4 kHz using a Digidata 1200 Interface, and analyzed using pClamp software (Axon Instruments, Foster City, CA). Records were stored on videotape and resampled at 20 Hz for presentation in the figures. The slope conductance was measured by fitting a straight line to the current-voltage relation between -20 and -100 mV; the average response to five consecutive ramps was calculated in each solution. In other experiments, macroscopic currents were recorded at a fixed holding potential of -50 mV in response to nucleotide or drug applications. Currents were sampled at 20 Hz and analyzed using Microcal Origin software (Microcal Software, Northampton, MA). Data are presented as the means \pm S.E.

RESULTS

Macroscopic currents were recorded in inside-out patches from *Xenopus* oocytes expressing either Kir6.2/SUR1 or Kir6.2/SUR2A. Current amplitudes were similar for both types of K_{ATP} channel.

Time Course of ATP Inhibition—Fig. 1A shows that application of 100 μ M ATP to the intracellular membrane surface initially inhibited both Kir6.2/SUR1 and Kir6.2/SUR2A currents by $\sim 90\%$. Inhibition of Kir6.2/SUR1 currents did not change, or even slightly increased, over the course of a 10-min exposure to ATP. In contrast, there was a gradual decline in the ATP sensitivity of Kir6.2/SUR2A currents with time; despite the continued presence of nucleotide, a slow increase in current was observed that began about 2 min after the onset of ATP application and stabilized 8–10 min later at around 75% block. This decrease in ATP sensitivity was not reversed by a 1-min exposure to nucleotide-free solution (Fig. 1A). Mean data are shown in Fig. 1B.

The extent of initial block of both types of K_{ATP} current by 100 μ M ATP is in agreement with that previously published for Kir6.2/SUR1 and Kir6.2/SUR2A channels. When expressed in oocytes and measured immediately after patch excision, half-maximal inhibition (K_i) of Kir6.2/SUR1 and Kir6.2/SUR2A channels is produced by 28 and 29 μ M ATP, respectively (31). The K_i value for Kir6.2/SUR1 is in good agreement with that found when the channel is expressed in mammalian cells (8–47 μ M; Refs. 21 and 32) and with what is found for the native β -cell K_{ATP} channel (26 μ M; Ref. 33). Reported values for half-maximal inhibition of cloned Kir6.2/SUR2A channels and for native cardiac K_{ATP} channels vary widely, from 17 to 100 μ M (6, 31,

34). It seems possible that this may reflect, at least in part, the time at which the measurements were made during exposure to ATP. It may also reflect differences in the concentration of endogenous membrane phospholipids.

To confirm that the difference in nucleotide response between Kir6.2/SUR1 and Kir6.2/SUR2A currents was conferred by the SUR subunit, we also measured the effect of continued exposure to ATP on a truncated form of Kir6.2 (Kir6.2ΔC), expressed in absence of SUR. Fig. 1B shows that Kir6.2ΔC currents resemble Kir6.2/SUR1 currents and show a slow decline with time in the presence of ATP, despite the lower ATP sensitivity. These data argue that the time-dependent activation of Kir6.2/SUR2A currents is conferred by the SUR2A subunit.

Effect of Walker A Mutation on the ATP Sensitivity of SUR 2A—It is possible that the time-dependent stimulatory effect of MgATP is mediated via the NBDs of SUR2A, either directly or via hydrolysis of MgATP to MgADP. It is known that MgADP is able to stimulate both Kir6.2/SUR1 (13–16, 31) and Kir6.2/SUR2A (31). Mutation of the lysine residue in the Walker A motif of NBD2 (but not NBD1) of SUR2A abolishes this stimulatory effect (35). We therefore examined the effect of MgATP

on channels containing this mutation (Kir6.2/SUR2A-K2A).

Fig. 2 shows that initial ATP sensitivity of Kir6.2/SUR2A-K2A currents is slightly greater than that found for the wild type channel; the mean block by 100 μ M ATP was $97.7 \pm 0.6\%$ ($n = 6$) compared with $87.6 \pm 4.3\%$ ($n = 7$) for Kir6.2/SUR2A-K2A and Kir6.2/SUR2A, respectively. This is not unexpected because a similar increase in ATP sensitivity is found when the equivalent residue is mutated in SUR1 (14). As in the case of SUR1, therefore, it may result from loss of MgATP activation mediated via the NBDs of SUR. Despite the enhanced ATP sensitivity, however, Kir6.2/SUR2A-K2A currents showed a time-dependent activation in the presence of MgATP that resembled that found for wild type channels; it began 1–2 min after exposure to ATP, and the current amplitude increased 3-fold within 5 min. This decrease in ATP sensitivity did not occur in the presence of 100 μ M LY 294002, an inhibitor of PI 3-kinase (see below); rather the currents declined with time (as was also observed for Kir6.2/SUR2A; Fig. 3B). These results therefore suggest that the MgATP-dependent decline in ATP sensitivity is not mediated via nucleotide interaction with the NBDs of SUR.

Mechanism of ATP-dependent Activation—Another mechanism by which the ATP sensitivity of the K_{ATP} channel might be reduced is by the ATP-dependent generation of PIP₂ in the patch membrane caused by the action of endogenous lipid kinases (21). To test this hypothesis, we examined the effect of the lipid kinase inhibitor LY 294002 on the ATP sensitivity of Kir6.2/SUR2A currents (Fig. 3). LY 294002 is a potent and relatively specific inhibitor of PI 3-kinase with an IC₅₀ of 1.5–4 μ M (27, 36). Thus, at a concentration of 10 μ M, PI 3-kinase is totally inhibited, but there is little effect on PI 4-kinase, whereas at a concentration of 100 μ M, PI 4-kinase is also completely blocked (36). As shown in Fig. 3, 10 μ M LY 294002 blocked the time-dependent decline in ATP sensitivity observed for Kir6.2/SUR2A currents and prevented the reduction in ATP sensitivity produced by preincubation with ATP (compare Figs. 1 and 3). It also slightly enhanced the ATP sensitivity of the channel. This suggests that production of PI(3)P, PI(3,4)P₂, or PIP₃ is required for the response. 100 μ M LY 294002 also blocked the ATP-dependent activation of Kir6.2/SUR2A currents and further increased the extent of inhibition by 100 μ M ATP. Wortmannin (100 μ M, $n = 3$), which also blocks PI 3-kinase (37), and neomycin (100 μ M, $n = 3$), which chelates phospholipids, produced an immediate decline in Kir6.2/SUR2A

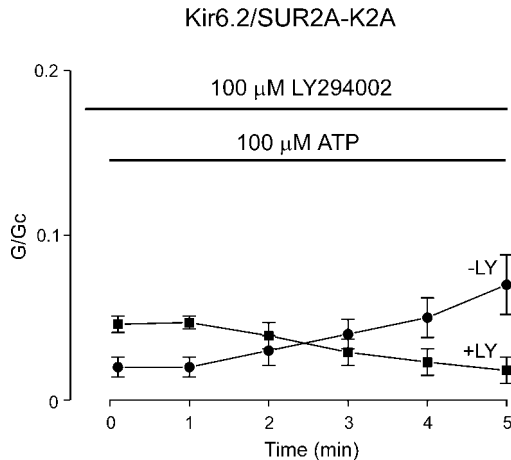


FIG. 2. Effects of an NBD mutation on the response to MgATP. Mean conductance recorded for Kir6.2/SUR2A-K2A currents at different times after the addition of 100 μ M MgATP to the intracellular solution in the absence (●, $n = 6$) or presence (■, $n = 3$) of 100 μ M LY 294002. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP.

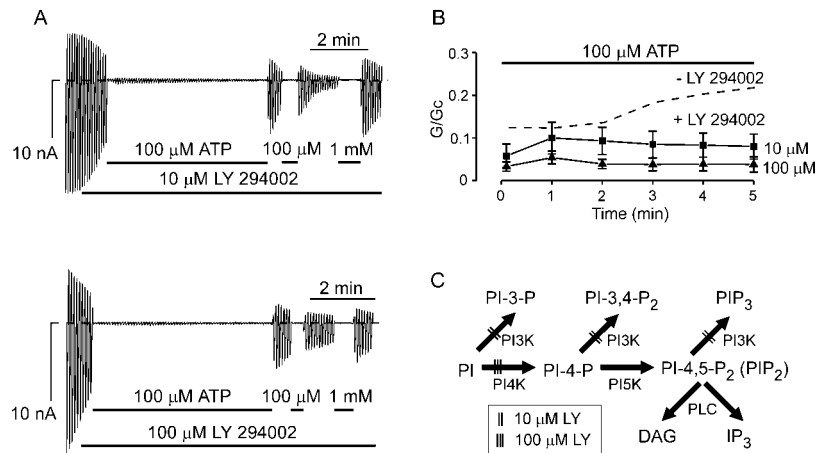


FIG. 3. Effects of LY 294002 on Kir6.2/SUR2A currents. A, macroscopic Kir6.2/SUR2A currents recorded in response to a series of voltage ramps from -110 to $+100$ mV. ATP and LY 294002 (10 μ M, above; 100 μ M, below) were added to the intracellular solution as indicated by the bars. All solutions contained Mg²⁺. B, mean conductance recorded for Kir6.2/SUR2A currents at different times after the addition of 100 μ M MgATP to the intracellular solution in the presence of 10 μ M (■, $n = 3$) or 100 μ M (▲, $n = 3$) LY 294002. The dashed line indicates the data obtained in control solution (as shown in Fig. 1B). The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP. C, schematic illustrating the metabolism of phospholipids. The pathways inhibited by 10 and 100 μ M LY 294002 are indicated.

channel activity and prevented the MgATP-dependent loss of ATP sensitivity (data not shown). As expected if a lipid kinase is involved, ATP was not effective at stimulating Kir6.2/SUR2A currents in the absence of Mg^{2+} (data not shown).

Taken together, these results suggest that MgATP is used as a substrate by PI 3-kinase in the oocyte membrane to generate PIP_3 or other membrane lipids and that the gradual accumulation of PIP_3 causes a slow decline in the ATP sensitivity of Kir6.2/SUR2A currents. Because PI 4-kinase is not blocked by 10 μM LY 294002, this activation cannot be mediated by $PI(4,5)P_2$ (Fig. 3C). Earlier studies have shown that K_{ATP} channel is very sensitive to the level of PI 5-kinase activity (21), which favors the possibility that the effects of MgATP are mediated via production of PIP_3 rather than $PI(3)P$ or $PI(3,4)P_2$. Although we cannot formally exclude a role for the latter two phospholipids, for simplicity, we will simply refer to PIP_3 (rather than PIP_3 , $PI(3)P$, or $PI(3,4)P_2$) in the rest of this paper.

The time lag observed before ATP induces activation of Kir6.2/SUR2A currents may therefore reflect the time taken for sufficient PIP_3 to accumulate within the membrane to cause a measurable reduction in ATP sensitivity. Accumulation of PIP_3 within the membrane may also explain why the ATP sensitivity of Kir6.2/SUR2A currents is not immediately restored on return to control solution. Clearly, if the lipid is not rapidly removed from the membrane, then subsequent ATP application will produce a smaller inhibitory response.

Mechanism of Action of PIP_3 —Although exogenously applied PIP_3 is known to modulate β -cell K_{ATP} channel activity (20, 38), it need not necessarily interact directly with the channel. It might also exert its effect indirectly, by modulating a protein that regulates the K_{ATP} channel. It is well established that PIP_2 and other membrane lipids influence the cell cytoskeleton by inhibiting the activity of actin-binding proteins that sever and cap actin filaments (39). Likewise, PIP_3 associates with the Rho family of GTPases (40), which are key regulators of actin filament structure (41). Agents that modulate the cytoskeleton also influence the activity of native K_{ATP} channels in cardiac membranes in both the presence and absence of ATP (42, 43). We therefore examined the effects of phalloidin, which stabilizes the cytoskeleton, and of cytochalasin, which disrupts the cytoskeleton, on the ATP-dependent activation of Kir6.2/SUR2A currents. Cytochalasin (10 μM) increased the time-dependent current activation produced by 100 μM ATP, whereas phalloidin (20 μM) diminished the extent of activation (Fig. 4A). These results argue that the ATP-dependent activation of Kir6.2/SUR2A currents involves the cell cytoskeleton and that disruption of the cytoskeleton enhances the ATP-dependent decline in the channel ATP sensitivity.

The effect of cytochalasin might be mediated subsequent to PIP_3 production, or it might affect generation of the phospholipid. To distinguish between these possibilities, we examined whether LY 294002 was able to inhibit the response to cytochalasin; if cytochalasin influences PIP_3 production, then LY 294002 should block the response whereas, conversely, if cytochalasin has an effect downstream of the phospholipid it should still be effective in the presence of LY 294002. As shown in Fig. 4 (A and B), both 10 and 100 μM LY 294002 completely abolished the response to cytochalasin. The simplest explanation of this result is that changes in the cell cytoskeleton do not modify the ATP sensitivity of Kir6.2/SUR2A currents directly. Rather, disruption of the cytoskeleton enhances MgATP-dependent production of PIP_3 (or its metabolites) or facilitates PIP_3 targeting to the K_{ATP} channel and thereby enhances the loss of ATP sensitivity.

In contrast to Kir6.2/SUR2A channels, K_{ATP} channels con-

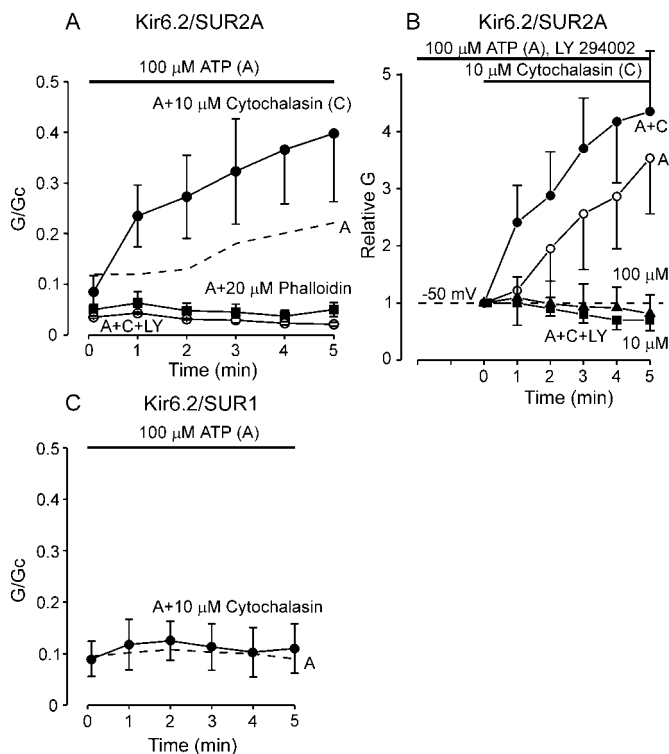


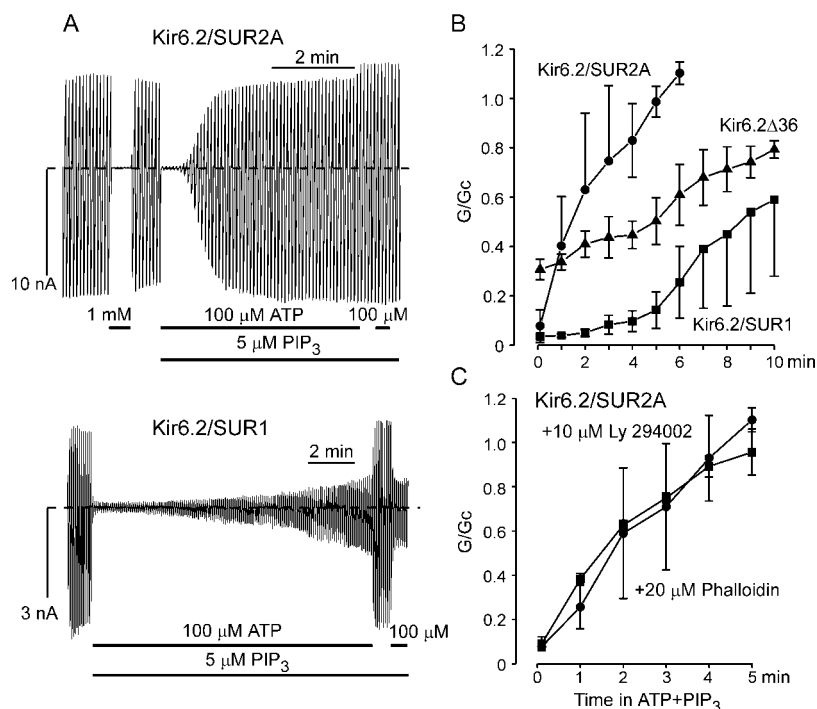
FIG. 4. Effects of cytoskeletal agents. A, mean K_{ATP} conductance recorded for Kir6.2/SUR2A currents at different times after the addition of 100 μM MgATP to the intracellular solution in the presence of 10 μM cytochalasin (\bullet , $n = 4$) or 20 μM phalloidin (\blacksquare , $n = 3$) or of 10 μM cytochalasin plus 10 μM LY 294002 (\circ , $n = 3$). The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP. The dashed line indicates the control response in the absence of either agent. B, mean conductance recorded for Kir6.2/SUR2A currents at -50 mV at different times after the addition of 100 μM MgATP (\circ , $n = 5$), 100 μM MgATP plus 10 μM cytochalasin (\bullet , $n = 4$), 100 μM MgATP and 10 μM cytochalasin plus 10 μM LY 294002 (\blacktriangle , $n = 5$). The dashed line indicates the initial current level. The conductance (G) is expressed as a fraction of the mean (G_c) of that obtained at -50 mV in control solution before exposure to ATP. The patch was held at -50 mV throughout the experiment. C, mean conductance recorded for Kir6.2/SUR1 currents at different times after the addition of 100 μM MgATP to the intracellular solution in presence of 10 μM cytochalasin ($n = 3$). The dashed line indicates the response in the absence of cytochalasin. The slope conductance (G) was measured using a ramp protocol and is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP.

taining the SUR1 subunit were not activated by cytochalasin in the presence of ATP (Fig. 4C). Thus, like the ATP-dependent activation itself, this response is specific to SUR2A.

Differential Sensitivity of Kir6.2/SUR1 and Kir6.2/SUR2A Currents to PIP_3 —It is striking that whereas Kir6.2/SUR2A currents show an ATP-dependent activation that appears to be mediated by PIP_3 production, this is not the case for Kir6.2/SUR1 currents. In some patches, however, we observed a slow, time-dependent activation of Kir6.2/SUR1 currents when exposed to 1 mM ATP (data not shown). This suggests that Kir6.2/SUR1 channels may simply be less sensitive to PIP_3 than Kir6.2/SUR2A channels.

We therefore tested the effect of direct application of PIP_2 or PIP_3 on Kir6.2/SUR1 and Kir6.2/SUR2A currents in the presence of 100 μM ATP. Fig. 5 (A and B) shows that (in the absence of Mg^{2+}) PIP_3 produces a time-dependent decline in the ATP sensitivity of both types of channel but that this effect was more rapid and pronounced in the case of Kir6.2/SUR2A than Kir6.2/SUR1. The magnitude and time course of the PIP_3 activation of Kir6.2 $\Delta C36$ was similar to that observed for Kir6.2/SUR1 (Fig. 5B), providing additional support for the idea that

FIG. 5. Time course of current response in the presence of 5 μ M PIP₃ and 100 μ M MgATP. *A*, macroscopic currents recorded from oocytes coexpressing Kir6.2 and either SUR2A (*above*) or SUR1 (*below*) in response to a series of voltage ramps from -110 to $+100$ mV. ATP and PIP₃ were added to the intracellular solution as indicated by the bars. All solutions were Mg²⁺-free. *B*, mean K_{ATP} conductance recorded for Kir6.2 Δ C36 (\blacktriangle , $n = 3$); Kir6.2/SUR1 (\blacksquare , $n = 3$) or Kir6.2/SUR2A (\bullet , $n = 3$) currents at different times after the addition of 5 μ M PIP₃ and 100 μ M ATP to the intracellular solution. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP. All solutions were Mg²⁺-free. *C*, mean conductance recorded for Kir6.2/SUR2A currents at different times after the addition of 5 μ M PIP₃ and 100 μ M ATP to the intracellular solution in the presence of 20 μ M phalloidin (\bullet , $n = 3$) or 10 μ M LY 294002 (\blacksquare , $n = 3$). The dashed line indicates the response in the absence of either agent. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP.



the differential action of the phospholipid on Kir6.2/SUR1 and Kir6.2/SUR2A is mediated by the SUR2A subunit. Similar results were found with PIP₂ ($n = 2-3$ in each case, data not shown). Because the effects of PIP₃ were tested in the absence of Mg²⁺, we can be certain that current activation is the result of the phospholipid rather than ATP itself. It has previously been reported that the ATP sensitivity of both types of channel is reduced by PIP₂ in excised patches (18–20, 22, 44), but the difference in sensitivity has not been remarked.

Fig. 5C shows that neither LY 294002 nor phalloidin is able to block the effect of PIP₃ on Kir6.2/SUR2A currents. Similar results were found with PIP₂ (data not shown). The lack of effect of phalloidin is consistent with the idea that stabilization of the cell cytoskeleton is involved in the MgATP-dependent generation of phospholipids rather than directly affecting the K_{ATP} channel. The data also indicate that PIP₂ does not have to be metabolized to PIP₃ to mediate its effect, because its effect was not blocked by LY 294002.

Molecular Basis of the Differential Sensitivity of Kir6.2/SUR1 and Kir6.2/SUR2A Currents to Phospholipids—To determine the molecular basis of the differential sensitivity of Kir6.2/SUR1 and Kir6.2/SUR2A currents to phospholipids, we made chimeras between SUR1 and SUR2A and investigated the ability of 100 μ M MgATP to produce a time-dependent activation of K_{ATP} channels containing chimeric SUR. These results are summarized in Fig. 6. SUR1 containing both NBDs, or just the last 42 amino acids of SUR2A, behaved like SUR1. Likewise, SUR2A containing the last 42 amino acids, or NBD2, of SUR1 behaved like the parent channel. When the first six TMs of SUR1 were transferred into SUR2A, however, the ability of MgATP to stimulate K_{ATP} channel activity was abolished (Fig. 6A). Thus, it appears that this region of SUR is critical for the different response of Kir6.2/SUR1 and Kir6.2/SUR2A channels to MgATP.

DISCUSSION

Our results suggest that the gradual loss of ATP sensitivity of Kir6.2/SUR2A currents is due to a MgATP-dependent synthesis of membrane phospholipids, which causes a secondary decrease in the channel ATP sensitivity. This hypothesis is supported by the facts that LY 294002, wortmannin, neomycin,

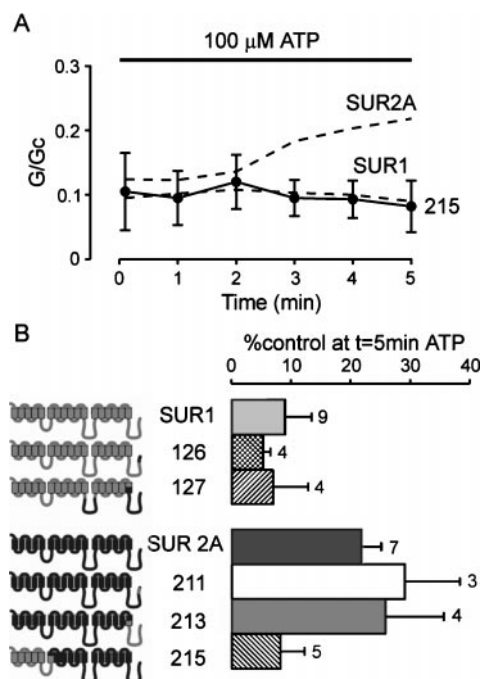


FIG. 6. Effects of chimeras. *A*, mean conductance recorded from an inside-out patch excised from an oocyte coexpressing Kir6.2 and SUR-215, at different times after the addition of 100 μ M MgATP to the intracellular solution. The slope conductance (G) is expressed as a fraction of the mean (G_c) of that obtained in control solution before exposure to ATP. The dashed lines indicate the response of Kir6.2/SUR1 or Kir6.2/SUR2A currents. *B*, macroscopic conductance recorded from patches excised from oocytes coexpressing Kir6.2 and either SUR1, SUR2A, or the SUR chimera indicated, after 5 min of exposure to MgATP. Mean conductance in the presence of the test solution (G) is expressed relative to the mean conductance in the control (nucleotide-free) solution before nucleotide addition (G_c). The number of patches is given by each bar.

and Mg²⁺-free solutions prevent this effect and that the effect of MgATP is mimicked by the phospholipids PIP₂ and PIP₃.

In the cell membrane, PI 4-kinase phosphorylates PI to give PI(4)P, which is subsequently phosphorylated by PI 5-kinase to

PIP₂ (PI(4,5)P₂). All three phospholipids are also phosphorylated by PI 3-kinase to produce PI(3)P, PI(3,4)P₂, and PIP₃ respectively (Fig. 3C). Biochemical studies have shown that 10 μ M LY 294002 specifically inhibits PI 3-kinase and is without effect on PI 4-kinase but that at a concentration of 100 μ M, LY 294002 also totally blocks PI 4-kinase (36). Because 10 μ M LY 294002 abolished the magnesium-dependent loss of ATP sensitivity, we conclude that either PI(3)P, PI(3,4)P₂, and/or PIP₃ is involved in the response. At this concentration, LY 294002 does not prevent PI(4)P or PI(4,5)P₂ formation, suggesting that neither of these lipids is responsible for the magnesium-dependent decrease in ATP sensitivity.

When applied directly to the membrane patch, both PIP₂ and PIP₃ were able to reduce the ATP sensitivity of Kir6.2/SUR2A, although no MgATP-dependent activation of the channel was observed in the presence of 10 μ M LY 294002. This suggests that PIP₃ is a more potent regulator of the channel and that, in the presence of LY 294002, PIP₂ generated by addition of MgATP does not accumulate to a concentration sufficient to reduce the channel ATP sensitivity. This may be due to the action of endogenous phospholipase C or lipid phosphatases. It is also worth pointing out that because many proteins bind PIPs, they may influence K_{ATP} channel activity simply by sequestering the amount of PIPs available for interaction with the channel.

Mechanism of Action of PIP₃—Our results suggest that the effect of PIP₃ on the ATP sensitivity of the K_{ATP} channel is not mediated via the cell cytoskeleton, because it was not blocked by phalloidin. We did observe, however, that agents that perturb the cytoskeleton have a marked effect on the MgATP-dependent decline in ATP sensitivity. Notably, disruption of actin microfilaments by cytochalasin enhanced the response, whereas stabilization of microfilaments by phalloidin decreased the response. It is possible that the effect of cytochalasin may be mediated upstream of PI 3-kinase because it can be blocked by 10 μ M LY 294002. This might suggest that disruption of the cytoskeleton enhances PIP₃ levels (or targeting to the K_{ATP} channel), whereas, conversely, stabilization of the cytoskeleton by phalloidin reduces PIP₃ levels. Furthermore, the ability of phalloidin to inhibit the ATP-dependent loss of ATP sensitivity suggests that MgATP may mediate its effect by inducing cytoskeletal disruption. This idea is supported by the fact that phalloidin did not prevent the loss of ATP sensitivity produced by direct application of PIP₃ to the intracellular membrane surface. In contrast, the stimulatory action of PIP₃ on native β -cell K_{ATP} channels is prevented by phalloidin (24, 45). Presumably, this difference reflects the different cell types, which may metabolize PIP₃ differently.

Interestingly, cytochalasin reduced the activity of native cardiac K_{ATP} channels in the absence of ATP (42) but enhanced it when ATP was present (43). It seems plausible that the latter effect is due to an increase in MgATP-mediated PIP₃ generation, as in the case for the cloned channel Kir6.2/SUR2A. A different mechanism may underlie the action of cytochalasin in the absence of ATP, which promotes K_{ATP} channel rundown (42).

Differential Sensitivity of Kir6.2/SUR1 and Kir6.2/SUR2A Channels—In contrast to Kir6.2/SUR2A currents, Kir6.2/SUR1 currents were not activated by 100 μ M MgATP. Moreover, when PIP₃ was added directly to the patch in the presence of 100 μ M MgATP, the phospholipid produced a much faster and more marked activation of Kir6.2/SUR2A than of Kir6.2/SUR1. Our results indicate that these different responses are mediated by the first set of transmembrane domains of SUR (TMs 1–6). It is noteworthy that this region of SUR confers the different gating kinetics of Kir6.2/SUR1 and Kir6.2/SUR2A channels (46, 47).

This region of SUR may therefore be involved in transducing conformational changes in SUR into gating of the channel pore.

Both Kir6.2/SUR1 and Kir6.2 Δ C channels have shorter bursts of openings and a lower open probability than Kir6.2/SUR2A channels. Loussouarn and colleagues (48) have shown that there is an exponential correlation between the burst duration (or open probability) of the K_{ATP} channel and its K_i for ATP inhibition, and it has been suggested that PIP₂ mediates its effect on K_{ATP} channel ATP sensitivity, at least in part, via changes in the channel open probability (49). Whether or not a given increase in open probability (P_o) results in a change in ATP sensitivity will therefore depend on the initial P_o . Because this is lower for Kir6.2/SUR1 and Kir6.2 Δ C currents, the likelihood of observing a change in ATP sensitivity will be less.

It is therefore possible that the different responses of Kir6.2/SUR2A and Kir6.2/SUR1 are due to their different single-channel kinetics; the higher P_o of Kir6.2/SUR2A channels means that a further increase in P_o produced by PIP₂ is more likely to reduce the channel ATP sensitivity than in the case of Kir6.2/SUR1 channels. An alternative interpretation of our results, however, is that PIP₃ is able to interact directly with the SUR2A subunit of the K_{ATP} channel to decrease the channel ATP sensitivity (in addition to its effect on Kir6.2) and that the functional effect of this interaction involves the first six TMs of SUR.

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**ATP Modulation of ATP-sensitive Potassium Channel ATP Sensitivity Varies with
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