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 μM) block of Kir6.2/SUR2A currents gradually declined with time, whereas inhibition of Kir6.2/SUR1 or Kir6.2ΔC36 currents did not change. The decline in Kir6.2/SUR2A ATP sensitivity was not observed in Mg$^{2+}$-free solution and was blocked by the phosphatidylinositol (PI) 3-kinase inhibitors LY 294002 (10 μM) and wortmannin (100 μM). 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 μM 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.

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¹ The abbreviations used are: K$_{ATP}$, ATP-sensitive potassium; NBD, nucleotide-binding domain; PIP$_2$, PI(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PIP$_3$, phosphatidylinositol 3,4,5-trisphosphate; PIP$_{3,4}$, phosphatidylinositol 3,4-bisphosphate; PIP$_{4,5}$, phosphatidylinositol 4-phosphate; TM, transmembrane domain; PIP$_{3,4,5}$, phosphatidylinositol 3-phosphate.

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tion. This ATP-dependent activation of Kir6.2/SUR2A currents by MgATP can be prevented by 10 μM LY 294002, a specific inhibitor of PI 3-kinase (28), suggesting that it results from MgATP-dependent production of PKC or PI(3,4)P2 rather than PI(4,5)P2 (PI(4,5)P2). Both PKC and PI(4,5)P2, 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 (GenBank accession number D50581; Refs. 3 and 4), rat SUR1 (GenBank accession number L40624; Ref. 5), and SUR2A (GenBank accession number D83598; Ref. 6) cDNAs were cloned in the pEF vector. A truncated form of Kir6.2 (Kir6.2C), 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 Kir6.2C 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 kΩ 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 °C using an EPC7 patch-clamp amplifier (List Electronic, Darmstadt, Germany; Ref. 30). The pipette (external) solution contained 140 mM KCl, 2.6 mM NaCl, 10 mM HEPES (pH 7.4 with KOH). The intracellular (bath) solution contained 107 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 10 mM EGTA, 10 mM HEPES (pH 7.2 with KOH; final [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), phallloidin, and cytochalasin (CalBiochem) were dissolved in Me2SO to make 10 mM stock solutions. Stock solutions (1 mM) of PIP2 and PIP3 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 ± 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 KATP channel.

Time Course of ATP Inhibition—Fig. 1A shows that application of 100 μM ATP to the intracellular membrane surface initially inhibited both Kir6.2/SUR1 and Kir6.2/SUR2A currents by ~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 KATP current by 100 μM 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 (Ki) of Kir6.2/SUR1 and Kir6.2/SUR2A channels is produced by 28 and 29 μM ATP, respectively (31). The Ki value for Kir6.2/SUR1 is in good agreement with that found when the channel is expressed in mammalian cells (8–47 μM; Refs. 21 and 32) and with what is found for the native β-cell KATP channel (26 μM; Ref. 33). Reported values for half-maximal inhibition of cloned Kir6.2/SUR2A channels and for native cardiac KATP channels vary widely, from 17 to 100 μM (6, 31, 32).
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 μM ATP was 97.7 ± 0.6% (n = 6) compared with 87.6 ± 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 μM 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 KATP 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 μM (27, 36). Thus, at a concentration of 10 μM, PI 3-kinase is totally inhibited, but there is little effect on PI 4-kinase, whereas at a concentration of 100 μM, PI 4-kinase is also completely blocked (36). As shown in Fig. 3, 10 μM 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 PIP3, PIP2, or PI4P is required for the response. 100 μM LY 294002 also blocked the ATP-dependent activation of Kir6.2/SUR2A currents and further increased the extent of inhibition by 100 μM ATP. Wortmannin (100 μM, n = 3), which also blocks PI 3-kinase (37), and neomycin (100 μM, n = 3), which chelates phospholipids, produced an immediate decline in Kir6.2/SUR2A currents containing this mutation (Kir6.2/SUR2A-K2A).
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 mM LY 294002, this activation cannot be mediated by PIP(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 after 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 $\beta$-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$_3$ 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 $\mu$m) increased the time-dependent current activation produced by 100 $\mu$m MgATP, whereas phalloidin (20 $\mu$m) 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 $\mu$m 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) and 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 containing 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$_3$, or PIP$_3$, or PIP$_3$, on Kir6.2/SUR1 and Kir6.2/SUR2A currents in the presence of 100 $\mu$m 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/SUR1 (Fig. 5B), providing additional support for the idea that...
Activation of Kir6.2/SUR2A and Kir6.2/SUR1

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 \(K_{\text{ATP}}\) conductance recorded for Kir6.2/SUR2A (\(\bullet\), \(n = 3\)) or Kir6.2/SUR2A (\(\bullet\), \(n = 3\)) at different times after the addition of 5 \(\mu M\) PIP3 and 100 \(\mu M\) 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 \(\mu M\) PIP3 and 100 \(\mu M\) ATP to the intracellular solution in the presence of 20 \(\mu M\) phalloidin (\(\bullet\), \(n = 3\)) or 10 \(\mu M\) LY 294002 (\(\square\), \(n = 3\)). 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.

**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,
PPI3 (P(4,5)P2). All three phospholipids are also phosphorylated by PI 3-kinase to produce P(3,4)P2 and P(3,4,5)P3 respectively (Fig. 3C). Biochemical studies have shown that 10 μM LY 294002 specifically inhibits PI 3-kinase and is without effect on PI 4-kinase but that at a concentration of 100 μM, LY 294002 also totally blocks PI 4-kinase (36). Because 100 μM LY 294002 abolished the magnesium-dependent loss of ATP sensitivity, we conclude that either PI(3,4,5)P3 and/or PIP3 is involved in the response. At this concentration, LY 294002 does not prevent 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 μM 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 KATP channel activity simply by sequestering the amount of PIPs available for interaction with the channel.

Mechanism of Action of PIP2—Our results suggest that the effect of PIP2 on the ATP sensitivity of the KATP channel is not mediated via the cell cytoskeleton, because it was not blocked by phallolidin. 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 phallolidin 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 μM LY 294002. This might suggest that disruption of the cytoskeleton enhances PIP2 levels (or targeting to the KATP channel), whereas, conversely, stabilization of the cytoskeleton by phallolidin reduces PIP2 levels. Furthermore, the ability of phallolidin 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 phallolidin did not prevent the loss of ATP sensitivity produced by direct application of PIP2 to the intracellular membrane surface. In contrast, the stimulatory action of PIP2 on native β-cell KATP channels is prevented by phallolidin (24, 45). Presumably, this difference reflects the different cell types, which may metabolize PIP2 differently.

Interestingly, cytochalasin reduced the activity of native cardiac KATP 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 PIP2 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 KATP 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 μM MgATP. Moreover, when PIP2 was added directly to the patch in the presence of 100 μM 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 Kir6.2ΔC 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 KATP channel and its Kd for ATP inhibition, and it has been suggested that PIP2 mediates its effect on the KATP channel ATP sensitivity, at least in part, via changes in the channel open probability (49). Whether or not a given increase in open probability (Po) results in a change in ATP sensitivity will therefore depend on the initial Po. Because this is lower for Kir6.2/SUR1 and Kir6.2ΔC 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 Po of Kir6.2/SUR2A channels means that a further increase in Po 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 PIP2 is able to interact directly with the SUR2A subunit of the KATP 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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