Multisite Phosphorylation Mechanism for Protein Kinase A Activation of the Smooth Muscle ATP-Sensitive K⁺ Channel

Kathryn V. Quinn, Jonathan P. Giblin, Andrew Tinker

Abstract—The activation of ATP-sensitive K⁺ channels by protein kinase A in vascular smooth muscle is an important component of the action of vasodilators. In this study, we examine the molecular mechanisms of regulation of the cloned equivalent of this current sulfonylurea receptor 2B/inward rectifier K⁺ (SUR2B/Kir6.1). Specifically, we focus on whether the channel is directly phosphorylated and the sites at which this occurs in the protein complex. We identify one site in Kir6.1 (S385) and two sites in SUR2B (T633 and S1465) using a combination of biochemical and functional assays. Our work supports a model in which multiple sites in the channel complex have to be phosphorylated before activation occurs. (Circ Res. 2004;94:000-000.)

Key Words: Kir6.1 ▪ ATP-sensitive potassium channel ▪ ion channel regulation ▪ protein kinase A ▪ protein phosphorylation

A TP-sensitive potassium (K<sub>ATP</sub>) channels are found in a variety of tissues, and one of their roles is to allow the metabolic state of the cell to be reported at the plasma membrane.1–4 The channels exist in the cell membrane as an octamer of 4 pore-forming inward rectifier K⁺ (Kir6.X) subunits with 4 sulfonylurea receptor (SUR) subunits.5,6 The channels are closed by raised intracellular ATP, stimulated by MgADP, and are sensitive to K⁺ channel openers such as pinacidil and diazoxide. In addition, the channels are blocked by antidiabetic sulfonylureas.7–9 The differences in physiological and pharmacological properties in specific tissues are accounted for by differential subunit expression.7–9

The phosphorylation of ion channels by protein kinases is an important mechanism by which membrane excitability is regulated by cell signaling pathways.10 This is of particular physiological importance for K<sub>ATP</sub> channels in vascular smooth muscle, where they control smooth muscle tone in response to hormones and neurotransmitters.11–13 In particular, endogenous vasodilators such as calcitonin gene-related peptide and adenosine have been shown to act via protein kinase A (PKA) to stimulate glibenclamide-sensitive currents.14–16 What is the molecular counterpart of the channel in smooth muscle? The pharmacological properties of the native channel are compatible with the known properties of SUR2B-containing channel complexes.9 Furthermore, channel activity in many native smooth muscles is absolutely dependent on nucleotide diphosphates, and this is characteristic of the behavior of Kir6.1.17–19 Finally, more direct studies of this issue with the Kir6.1 and SUR2 knockout mice and our own work in a human primary pulmonary artery smooth muscle cell line are also consistent with the native vascular channel being composed of Kir6.1/SUR2B.20–22

However, little is known of the molecular mechanisms by which protein kinases and more specifically PKA modulate the vascular K<sub>ATP</sub> channel. We investigate this problem here using biochemical and electrophysiological approaches. We ask whether channel modulation occurs with the cloned channel isoforms, whether this is mediated by direct phosphorylation of the protein complex, and which residues are involved.

Materials and Methods

Molecular Biology
Stable lines were generated in human embryonic kidney 293 (HEK293) cells containing either Kir6.1 with SUR2B or Kir6.2 with SUR2B, as described previously.23 The consensus site recognition program in Prositie ([R/K]-[R/K]-x-[S/T] for PKA; http://ca.expasy.org/tools/scnpsite.html) was used to identify 2 PKA consensus sites (T234, S385) in Kir6.1 and 2 PKA consensus sites in SUR2B (T633, S1465). Single point mutations were introduced into Kir6.1 and SUR2B using the QuikChange kit (Stratagene) according to the instructions of the manufacturer. Transient transfections were performed using lipofectamine (Invitrogen) or Polyfect (Qiagen), with enhanced green fluorescent protein-N1 (Clontech) as a marker for transfected cells. Cystic fibrosis transmembrane conductance regulator (CFTR) was used as previously described.24

Generation of Maltose-Binding Protein Fusions for SUR2B NBD1 and NBD2
Polymerase chain reaction (PCR) products corresponding to NBD1 (amino acids 623–957) and NBD2 (amino acids 1322–1546) of mouse SUR2B were subcloned in frame into the pMALc2x expression vector (New England Biolabs). A shortened version of the NBD1 fusion protein with and without a point mutation (amino acids
623–734, SUR2BΔ1s, T633As) was also made in a similar fashion. A fusion of maltose-binding protein (MBP) with the C terminus of Kir6.1 (amino acids 179–424) was generated in a previous study. MBP fusions containing mutated PKA sites were generated in an identical manner using the mutant cDNA as the PCR template.

### Production and Purification of MBP Fusion Proteins

Transformation, expression, and purification of fusion proteins was done as described previously. A small aliquot of bound fusion protein was eluted from the resin with 10 mmol/L maltose in column buffer, and the protein content was measured using a protein assay kit (Bio-Rad).

**In Vitro Phosphorylation Assays**

The fusion proteins bound to the amylose resin were washed 5×1 mL HEPES buffer, and the protein was eluted with 10 mmol/L sodium fluoride, 1 mmol/L dithiothreitol, EDTA-free complete protease inhibitor cocktail (Roche). A total of 5 U of the catalytic subunit of PKA (PKAcat), 12 μL of 5X phosphorylation buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L EDTA, 10% glycerol, 5 mmol/L sodium pyrophosphate, 50 mmol/L sodium chloride, 10 EGTA, 1.2 MgCl2, 2.6 CaCl2) contained the following (in mmol/L): 100 K aspartate, 40 NaCl, 5 HEPES, 1.2 MgCl2, and 10 EGTA. Pipette solutions (pH 7.2) contained the following (in mmol/L): 160 NaCl, 6 KCl, 5 HEPES, 1 MgCl2, and 1.5 CaCl2. Pipette solutions (pH 7.2) contained the following (in mmol/L): 100 K aspartate, 40 NaCl, 6 KCl, 5 HEPES, 1.2 MgCl2, and 2.6 CaCl2. For experiments using CFTR, bath solutions (pH 7.4) contained the following (in mmol/L): 160 NaCl, 6 KCl, 10 HEPES, 1 MgCl2, and 1.5 CaCl2. Pipette solutions (pH 7.2) contained the following (in mmol/L): 100 K aspartate, 40 NaCl, 10 HEPES, 1.2 MgCl2, 1.9 CaCl2, 5 EGTA (free Ca2+=100 nM), and 3 (Na)ATP. Forskolin was dissolved in dimethyl sulfoxide (DMSO) and diluted so that bath concentration of DMSO did not exceed 0.1% and so control experiments with DMSO alone had no effect on currents. Currents were measured at −60 mV unless stated otherwise. Drugs were applied either using a gravity-driven perfusion system or a pipette manifold solution changer (MSC-200; Biologic).

**The cAMP Assay**

For the measurement of forskolin-mediated accumulation of cAMP, HEK293 cells were grown to 20% confluence in 6-well dishes. Cells were prelabeled with 5 μCi of [3H]adenine per well (in minimum essential medium) overnight at 37°C and then incubated with the phosphodiesterase inhibitor Ro20–1724 (100 μmol/L) in serum-free medium for 30 minutes at 37°C. Forskolin (10 μmol/L) was then added and incubated for 15 minutes at 37°C. Medium was then aspirated and cells washed with serum-free medium. Reactions were terminated by the addition of 2 5% perchloric acid and 0.1 mmol/L Na2EDTA at 4°C. [3H]cAMP was isolated by sequential chromatography using Dowex 50-alumina columns. Each fraction was collected in a vial containing 5 mL of Ultima Gold MV scintillant (Packard) and counted in a liquid scintillation counter. Reactions were done in triplicate, and data are expressed as percentage conversion of [3H]ATP to [3H]cAMP.

**Data Analysis**

Data are expressed as mean±SEM. Either Student paired t test or 1-way ANOVA with a Bonferroni post hoc test was used as appropriate to calculate statistical significance. All data presentation and statistical analysis were performed using Origin 6.0 (Microcal) or Prism v3.0 (GraphPad).

**Reagents**

All compounds were from VWR International, with the exception of forskolin, 1,9-dideoxy forskolin (Calbiochem), PKAcat, (Mg)ATP, (Na)UDP, glibenclamide, diazoxide (Sigma), levocromakalim, Rp-cAMPs, and Ro20–1724 (Tocris Cookson).

**Results**

We first established that HEK293 cells possessed the relevant cellular signaling machinery for PKA-mediated channel modulation. We have previously observed that forskolin, a direct activator of adenylyl cyclase, was able to elevate cAMP in HEK293 cells. We confirmed that it was able to do so in the Kir6.1/SUR2B stable line (Figure 1A). The CFTR is a chloride channel, the activity of which is characteristically dependent on direct PKA phosphorylation of the channel protein. Application of forskolin to HEK293 cells transiently expressing CFTR led to pronounced activation of a chloride current (Figure 1B).

We then addressed whether cloned KATP channels could be activated by PKA in the whole-cell configuration. We have described and characterized stable HEK293 cell lines expressing Kir6.1/SUR2B or Kir6.2/SUR2B. These cells con-
One possibility is that forskolin may block the Kir6.1/SUR2B current still occurred and was reversible (data not shown). A cAMP assay, comparing percent conversion [3H]ATP with [3H]cAMP in HEK293 cells (Kir6.1/SUR2B stable line) under basal conditions and in those treated with forskolin. **P<0.001. B, Currents evoked during voltage steps from -100 to +120 mV in WT HEK293 cells transiently transfected with CFTR in control conditions (n) and after perfusion with 10 μmol/L forskolin (C; n=9). Currents shown are mean of 300 ms of recording taken from the middle of each 1-second voltage step. *P<0.05.

Figure 1. HEK293 cells contain the necessary machinery for studying PKA-mediated signaling. A, A cAMP assay, comparing percent conversion [3H]ATP with [3H]cAMP in HEK293 cells (Kir6.1/SUR2B stable line) under basal conditions and in those treated with forskolin. **P<0.001. B, Currents evoked during voltage steps from -100 to +120 mV in WT HEK293 cells transiently transfected with CFTR in control conditions (n) and after perfusion with 10 μmol/L forskolin (C; n=9). Currents shown are mean of 300 ms of recording taken from the middle of each 1-second voltage step. *P<0.05.

Thus, it is apparent that Kir6.1/SUR2B currents can be regulated in a PKA-dependent fashion in the whole-cell patch clamp configuration. Kir6.1/SUR2B channel pore, probably from the cytoplasmic side, in addition to its action on adenylate cyclase. This accounts for the slow inhibition observed during the application of forskolin and derivatives. Finally, we investigated the role that particulate PKA may have in channel modulation.
configuration. However, such experiments do not discriminate between direct channel phosphorylation and indirect mechanisms in which PKA is an intermediary component of a signaling cascade. We used 2 assays to address this. First, we synthesized bacterial fusion proteins of domains of the channel-containing consensus sites for PKA phosphorylation. We expressed and purified the C terminus of Kir6.1 and the nucleotide binding domains of SUR2B fused to MBP. We did not examine the N terminus of Kir6.1 because it does not contain a consensus site. Figure 3A shows coomassie-stained SDS-PAGE gels loaded with the indicated proteins. We then subjected such purified protein to in vitro phosphorylation assays with PKA<sub>cat</sub>. It is clear from the autoradiograms shown in Figure 3B that MBP-Kir6.1C, MBP-SUR2B-NBD1, and MBP-SUR2B-NBD2 are substrates for PKA-mediated phosphorylation. In contrast, MBP control protein is not phosphorylated, and thus, the radioactive phosphate is likely to be incorporated into serine/threonine residues present in the fused domain. Therefore, domains of the channel complex can act as direct substrates for PKA-mediated phosphorylation. As a second assay, we performed inside-out patch experiments in which we examined single-channel NPo before and after exposure to PKA<sub>cat</sub>. In the absence of ATP and nucleotide diphosphates, there was little Kir6.1/SUR2B channel activity; however, upon application of 1 mmol/L (Mg)ATP and 0.5 mmol/L (Na)UDP, there was an immediate increase. The Kir6.1/SUR2B channel activity did not run down with time under the indicated conditions. The subsequent addition of PKA<sub>cat</sub> together with the nucleotides resulted in an additional increase in activity (Figure 4). Although it is formally possible that a membrane-bound intermediary might still exist taken together with the biochemistry, the data are highly suggestive that channel activation occurs by direct phosphorylation of the protein complex.

Given the results shown in Figures 3 and 4, we attempted to establish which serine and threonine residues are phosphorylated in the cytoplasmic domains of Kir6.1 and SUR2B. The consensus site recognition program in Prosite ([R/K]-[R/K]-x-[S/T] for PKA; http://ca.expasy.org/tools/scnpsite.html) was used to identify 2 PKA consensus sites (T234, S385) in Kir6.1 and 2 PKA consensus sites in SUR2B (T633, S1465).

Figure 3. Domains of Kir6.1 and SUR2B can act as substrates for direct phosphorylation by the PKA<sub>cat</sub>. A shows coomassie-stained SDS-PAGE gels loaded with 10 μg of the MBP fusion proteins indicated after purification on amylose resin and in vitro phosphorylation with the PKA<sub>cat</sub> (see Materials and Methods). The lane labeled L corresponds to the molecular weight standards. The arrows denote the positions of the fusion proteins. The higher molecular weight band observed with the purified MBP-Kir61C fusion protein is likely to represent an SDS-resistant oligomer. B shows autoradiographs of the gels in 3A after 24 hours of exposure to film. Control experiments in which PKA<sub>cat</sub> was omitted from the phosphorylation reaction did not yield any phosphorylated species (data not shown).

Figure 4. The PKA<sub>cat</sub> activates Kir6.1/SUR2B in inside-out patches. A, Example of Kir6.1/SUR2B channel activity recorded at −60mV from an inside-out patch; patches were initially excised into a low-Ca<sup>2+</sup> solution, and 1mmol/L (Mg)ATP and 0.5 mmol/L (Na)UDP (and later, 10 U/mL PKA<sub>cat</sub>) were applied to the intracellular face of the channel using a manifold perfusion system. B, Expanded traces from sections labeled in 4A. C, Bar chart showing mean NPo with and without ATP and UDP, and with PKA<sub>cat</sub>. Application of 10 U/mL of PKA<sub>cat</sub> to the channel led to a significant increase in channel activity. Dotted lines indicate closed levels and arrows indicate open levels. *P<0.05.
Initially, in vitro phosphorylation assays were performed on point mutations MBP-Kir6.1C-T234A, MBP-Kir6.1C-S385A, MBP-Kir6.1-T234A-S385A, MBP-SUR2B-NBD1-T633A, and MBP-SUR2B-NBD2-S1465A. All point mutations expressed comparable levels of protein compared with wild-type and could be purified in an analogous fashion. Representative autoradiograms are shown in Figure 5A, and quantitation from a number of experiments is shown in Figure 5B. Phosphorylation by PKA was abolished in MBP-Kir6.1C-T234A, MBP-Kir6.1C-S385A, MBP-Kir6.1-T234A-S385A, and MBP-SUR2B-NBD2-S1465A. The abolition of MBP-SUR2B-NBD2-S1465A is not quite significant from the quantitation, although it is clearly present from the gels. This results from the relatively lower levels of incorporation in this particular fusion compared with the others. We further investigated the ability of T633 in NBD1 to act as a substrate for PKA phosphorylation. We synthesized a shorter fusion protein (SUR2NBD1s; see Materials and Methods) and found that it was a substrate for PKA phosphorylation. The mutant T633As were not phosphorylated (Figure 5C). In addition, we synthesized short peptides (see Materials and Methods) corresponding to this region with and without the T633A mutation. The nonmutant peptide was phosphorylated to a significantly higher degree (Figure 5D). Thus, T633 does act as substrate for phosphorylation by PKA, and its phosphorylation in full-length NBD1 is probably masked by phosphorylation at other sites.

We next analyzed the potential functional significance of these consensus residues. We generated 2 point mutations in Kir6.1 (T234A and S385A) and 2 in SUR2B (T633A and S1465A). These mutants were transiently transfected along with SUR2B (in the case of the Kir6.1 mutants) or with Kir6.1 (in the case of the SUR2B mutants) into HEK293 cells so that each channel complex contained 1 point mutation. In whole-cell recordings, all point mutations removed the stim-
ulation in steady-state current seen with forskolin using wild-type Kir6.1 and SUR2B cells. Figure 6A shows some representative recordings, and Figure 6B shows the mean data together with statistical analysis. All the mutants expressed robust basal whole-cell currents. Furthermore, we were able to activate these mutants using a potassium channel opener (Figure 6C shows an example). The effect of inclusion of a phosphodiesterase inhibitor is shown below.

These point mutations were further investigated by applying the PKA cat to the intracellular face of the channel during inside-out patch recordings of single-channel activity. In such studies, 3 of these point mutants had attenuated activation (S385A in Kir6.1 and T633A and S1465A in SUR2B), whereas T234A was still significantly activated (Figure 7A and 7B). However, a K-channel opener was still able to activate the currents (Figure 7A).

There is a potential discrepancy between the data for the T234A mutation with the different experimental configurations (ie, whole-cell with forskolin and inside-out patch recording with PKAcat). We investigated this further and used forskolin in combination with a phosphodiesterase inhibitor. Under these circumstances, there was significant activation of T234A, but S385A was not activated (Figure 8).

**Discussion**

We have studied the regulation of the Kir6.1/SUR2B complex by PKA, and we can recapitulate many aspects of native channel regulation in a HEK293 heterologous expression system. Our results strongly suggest that regulation occurs because of direct channel phosphorylation. What kind of model then can we propose for the link between the phosphorylation of individual sites and channel activity? It is important to appreciate that a single alanine substitution actually removes 4 potential sites because the channel com-
plex is an octomer of 4 Kir6.1 subunits and 4 SUR2B subunits. Our hypothesis is that channel activation is largely mediated via phosphorylation at S385A in Kir6.1 and T633 and S1465 residues in SUR2B. Furthermore, significantly enhanced channel activity only occurs at high levels of phosphorylation (ie, when multiple sites are phosphorylated). Such a mechanism is appealing because it would be of value in stopping channel activation from occurring because of fluctuations in basal PKA activity. In vivo, this would prevent precipitous changes in blood pressure. How well does our data support such a scheme?

We have examined the role of 4 consensus sites. Alanine substitution of S385 in Kir6.1 and S1465 in SUR2B clearly abolishes biochemical and functional evidence of regulation. T633A is convincingly implicated from the functional studies; however, the fusion protein MBP-NBD1-T633A is still significantly phosphorylated. It seems likely that other sites are phosphorylated in NBD1, and this masks phosphate incorporation at T633. Biochemical studies on a shorter fusion of this domain support this contention. It is known that PKA may potentially phosphorylate at sites such as R-X-[S/T].29 T234 in Kir6.1 seems to be of relatively less functional importance. It can be activated by PKA$_{cat}$ applied in inside-out patches in a quantitatively comparable fashion to wild-type Kir6.1. Furthermore, the mutant channel can be activated by forskolin in the whole-cell configuration in the presence of a phosphodiesterase inhibitor, likely to lead to a higher and more sustained rise in intracellular cAMP than forskolin alone, whereas Kir6.1 S385A is not. A further interesting biochemical feature is that phosphorylation of the Kir6.1 residues may be intrinsically cooperative as alanine substitution of either one of the residues results in a substantial reduction of signal. Thus, the functional effects arising from the T234A mutation may well occur from diminished ability to phosphorylate the S385 residue at a given level of PKA activity.

Other investigators have examined PKA modulation of the Kir6.2/SUR1 complex.30,31 The exact details of the mechanism varied between the reports and are also different from our proposed model for the Kir6.1/SUR2B complex. Gonoi et al did find evidence for a phosphorylated site on both SUR1 and Kir6.2.30 The residue identified in SUR1 was responsible for basal activity, and the residue on the channel (equivalent to our S385 residue) was responsible for activation. The residues identified by us on SUR2B are clearly involved in PKA-mediated activation, and it is not clear whether they have an effect on basal activity as well. Lin et al identified another distinct single site on the channel responsible for

Figure 8. Stimulatory effects of forskolin can be restored in Kir6.1T234A by incubation with a phosphodiesterase inhibitor. Ai, Example of Kir6.1T234A/SUR2B current after 15 minutes of preincubation with 50 µmol/L Ro-20 to 1724. Short voltage steps (25-ms duration) were applied from a holding potential of 0 to −60 mV every 3 s, and the evoked current at −60 mV is plotted against time. Ai, Example of Kir6.1S385A/SUR2B current recorded using the same protocol as 8A, also following incubation with Ro-20 to 1724. B, Column graphs summarizing mean currents after 2 minutes of perfusion of forskolin for Kir6.1T234A/SUR2B and Kir6.1S385A/SUR2B after pre-incubation with Ro-20 to 1724. *P<0.05.
activity (homologous to our T234 residue). It is possible that mechanisms similar to those suggested by us to account for the functional effects of the T234A mutant in the Kir6.1/SUR2B complex may pertain to the Kir6.2/SUR1 complex as well. In our hands, there seems to be a fundamental difference in the mechanism of regulation of Kir6.2/SUR2B compared with Kir6.1/SUR2B. Also in this study, we found that activation of Kir6.2/SUR2B by forskolin only occurred in perforated patch recordings. Our data are generally comparable with that obtained in native tissues; however, 1 point on which they differ is the role of AKAPs. In native smooth muscle cells, it has been intriguing question is how mechanistically PKA acts to increase NPo. For example, in CFTR, phosphorylation of residues in the R domain are thought to be important for channel activation. However, activation appears to be more graded in relation to the phosphorylation status. One interesting issues to pursue in further studies.

Are there any precedents for multisite PKA phosphorylation in the regulation of other ion channels? One particularly well studied example is CFTR, in which a number of residues in the R domain are thought to be important for channel activation. However, activation appears to be more graded in relation to the phosphorylation status. PKA may not be critical for functional activation. The observations may reflect the predominant distribution of PKA in the 2 different cell systems. It is known that the majority of endogenous PKA in HEK293 cells is present in the soluble fraction.

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References


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