Multi-site 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 channel comprising the sulfonylurea receptor 2B and the inward rectifier 6.1 subunit (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;94:1359-1366.)

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

The phosphorylation of ion channels by protein kinases is an important mechanism by which membrane excitability is regulated by cell signaling pathways. This is of particular physiological importance for K_{ATP} channels in vascular smooth muscle, where they control smooth muscle tone in response to hormones and neurotransmitters. 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. The differences in physiological and pharmacological properties in specific tissues are accounted for by differential subunit expression.

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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. 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). 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. The cystic fibrosis transmembrane conductance regulator (CFTR) was used as previously described.

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 H11001—The activation of ATP-sensitive K_{ATP} 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 channel comprising the sulfonylurea receptor 2B and the inward rectifier 6.1 subunit (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;94:1359-1366.)
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 amyllose resin were washed 5×1 mL HEPES buffer, and the protein content was measured using a protein assay kit (Bio-Rad). A total of 5 U of the catalytic subunit of PKA (PKAcat), 12 μL of 5× phosphorylation buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L EDTA, 10% glycerol, 5 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L dithiothreitol, EDTA-free protease inhibitor cocktail (Roche)). A total of 5 U of the catalytic subunit of PKA (PKAcat), 12 μL of 5× phosphorylation buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L ATP, 50 mmol/L MgCl2), 26 μL of HEPES buffer, 13 μL of 50 mmol/L HEPES, pH 7.4+1.5% (v/v), Triton X-100, 1 μL of 5′-γ-ATP (370 MBq/mL, 110 TBq/mmol; Amberson Biosciences) was added to 10 μg of protein. The sample was incubated at 37°C for 2 hours and then washed 5 times with 1 mL HEPES buffer. The protein was eluted with 6× Laemmli gel-loading buffer and run on an SDS-PAGE gel, dried, and subjected to autoradiography. Quantitation was performed using Scion Image (Scion).

Peptide Phosphorylation Experiments

Peptides corresponding to amino acids 629–640 of SUR2B were synthesized and high-performance liquid chromatography-purified to 85% purity (Eurogentec). Two peptides were synthesized: 1 corresponding to the wild-type sequence-designated 633T (CKKHT-GVQSKP) and the other (designated 633A) containing an alanine mutation of the putative phosphorylation site (CKKHAGVQSKPI). A total of 1 mg of each peptide was coupled at a concentration of 1 mg/mL in 90 mmol/L HEPES, pH 7.2, to 500 μL of Affi-gel 10 (Bio-Rad) according to the instructions of the manufacturer. The efficiency of coupling was observed to be 100% as assessed by protein assay (Bio-Rad) according to the instructions of the manufacturer. The protein concentration of a 1:1 slurry of coupled peptide was 1 mg/mL in 90 mmol/L HEPES, pH 7.2, to 500 μL of Affi-gel 10 (Bio-Rad) according to the instructions of the manufacturer. The efficiency of coupling was observed to be 100% as assessed by protein assay (Bio-Rad), and the protein concentration of a 1:1 slurry of coupled peptide was 1 mg/mL. The protein concentration of a 1:1 slurry of coupled peptide was 1 mg/mL. Phosphorylation experiments were done in the manner described earlier in this section until the final wash step using 100 μg of coupled peptide per reaction. After the final wash, the coupled peptide was resuspended in 1 mL of distilled water, and the radioactivity in three 250-μL aliquots was determined by counting in 10 mL of distilled water using a Tri-Carb 2100TR liquid scintillation analyzer (Packard Biosciences).

Electrophysiology

Membrane currents were studied with the whole-cell and inside-out patch-clamp technique using an Axopatch 200B amplifier (Axon Instruments). Currents were filtered at 1 kHz and sampled at 2 kHz via a Digidata 1200 interface. Data were analyzed using Clampfit, Fetchan, and Pstat software (Axon Instruments). Single channel open probability (Npo) was calculated from 1 minute of continuous recording, generally with multiple channels present in the patch. Whole-cell patch pipettes were manufactured from borosilicate glass (OD 1.5 mm, ID 1.2 mm) using a PP-830 puller and fire polished in a similar way, using glass with thicker walls (OD 1.5 mm, ID 0.86 mm), with final pipette resistances of 5 to 10 mol/L. Pipette capacitance was reduced by coating tips with a paraffin/mineral oil suspension and was compensated for electronically, whereas series resistance was compensated to 70% using the amplifier. Whole-cell bath solutions (pH 7.4) contained the following (in mmol/L): 140 KCl, 5 HEPES, 1.2 MgCl2, and 2.6 CaCl2. Pipette solutions (pH 7.2) contained the following (in mmol/L): 140 KCl, 5 HEPES, 1.2 MgCl2, 10 EGTA, 1 CaCl2, 1 (Mg)ATP, and 0.5 (Na)UDP. In the whole-cell configuration, currents generally took 10 minutes after breakthrough to fully stabilize at a steady state. For excised patch recordings, bath solutions (pH 7.2) contained the following (in mmol/L): 140 KCl, 5 HEPES, 1.2 MgCl2, 10 EGTA, 1 CaCl2 [supplemented with 1 mmol/L (Mg)ATP and 0.5 (Na)UDP where indicated]. Pipette solutions (pH 7.4) contained the following (in mmol/L): 140 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 KCl, 10 HEPES, 1 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 cAMP 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), levromakalim, 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,25 was able to elevate cAMP in HEK293 cells.26 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.23 These cells con-
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 (A) 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 2. Kir6.1/SUR2B currents (but not Kir6.2/SUR2B currents) are activated by forskolin. All examples show currents recorded from HEK293 cell stable lines, and bars indicate application of various compounds. A, Example of Kir6.1/SUR2B current. 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. Aii, Also shown are examples of current evoked during voltage steps from −100 to +100 mV during a 250-ms period recorded before,1 at the peak response to forskolin,2 and with glibenclamide.3 B, Forskolin does not activate Kir6.2/SUR2B currents. C, Example of Kir6.1/SUR2B current recorded using the same protocol as in 2A during application of 8-bromo-cAMP (8-br-cAMP). D, Example of Kir6.1/SUR2B current recorded using the same protocol as in Figure 1A, with the inclusion of 200 μmol/L Rp-cAMPS in the patch pipette. E, Column graph showing mean current density for Kir6.1/SUR2B control currents compared with currents recorded after a 120-s application of forskolin, forskolin with Ro−20 1724, 8-Br-cAMP, forskolin with Rp-cAMPs, 1,9-dideoxyforskolin, and forskolin with Ht31. *P<0.05; **P<0.01; ***P<0.001.

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. RII-containing PKA subunits are scaffolded to the plasma membrane (and intracellular sites) through A kinaseanchoring proteins (AKAPs). 27,28 This interaction can be disrupted using a peptide (Ht31). We included this peptide in the patch pipette, but it did not abrogate forskolin activation of Kir6.1/SUR2B currents (Figure 2E).

Thus, it is apparent that Kir6.1/SUR2B currents can be regulated in a PKA-dependent fashion in the whole-cell...
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 PKAcat. 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 PKAcat. 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 PKAcat 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 PKAcat. 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 PKAcat (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. The arrows denote the positions of the fusion proteins. Control experiments in which PKAcat was omitted from the phosphorylation reaction did not yield any phosphorylated species (data not shown).

Figure 4. PKAcat activates Kir6.1/SUR2B in inside-out patches. A, Example of Kir6.1/SUR2B channel activity recorded at −60 mV from an inside-out patch; patches were initially excised into a low-Ca2+ solution, and 1 mmol/L (Mg)ATP and 0.5 mmol/L (Na)UDP (and later, 10 U/mL PKAcat) 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 PKAcat. Application of 10 U/mL of PKAcat 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-
pore-blocking action of forskolin. Similarly, both point mutations in SUR2B removed the significant stimulation by forskolin.

*P*<0.05; **P**<0.001. C, Response of Kir6.1S385A/SUR2B to levocromakalim.

Figure 6. Mutation of single PKA phosphorylation sites in Kir6.1 or SUR2B removes stimulatory effect of forskolin on whole-cell currents. HEK293 cells were transiently transfected with Kir6.1, along with either SUR2BT633A or SUR2BS1465A, or SUR2B along with Kir6.1T234A or Kir6.1S385A, and whole-cell currents were recorded. A, Time-series examples are shown of currents recorded during 25-ms steps to −60 mV (from a holding potential of 0 mV) every 3 seconds for Kir6.1T234A/SUR2B, Kir6.1S385A/SUR2B, Kir6.1/SUR2BT633A, and Kir6.1/SUR2BS1465A. Mean data for these mutations are compared with stable Kir6.1/SUR2B channel currents in the bar chart in 6B. There was a nonsignificant increase in Kir6.1T234A/SUR2B current after 2-minute applications of 10 μmol/L forskolin, and in Kir6.1S385A/SUR2B, current significantly decreased after forskolin application. This reduction in current amplitude can be accounted for by the

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 PKAcat 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 complex 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 fluctuations in basal PKA activity. In vivo, this would prevent precipitous changes in blood pressure. How well does our data support such a scheme?

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Other investigators have examined PKA modulation of the Kir6.2/SUR1 complex. 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. 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 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 convincingly demonstrated that the Ht31 peptide abolishes forskolin and receptor-mediated activation of the native current. However, in HEK293 cells, it was still possible to activate the current by including PKA cat in the pipette. Thus, it is plausible that the particular cellular location of 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.

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. One intriguing question is how mechanistically PKA acts to increase NPo. For example, in CFTR, phosphorylation of the R domain by PKA is essential before ATP can activate the channel by binding at the nucleotide-binding domains. The situation is different with the Kir6.1/SUR2B complex, in which channel opening is critically dependent on the provision of nucleotide diphosphates and PKA fine tunes that activity. A second mechanistic point is the potential for protein kinases to act by modulating anionic phospholipid sensitivity. It is plausible that for Kir6.2, PKA might increase the phosphatidyl inositol bisphosphate (PIP2) affinity resulting in channel activation. This is an unlikely mechanism with Kir6.1 because it already has a very high affinity for anionic phospholipids, and addition of further PIP2 does not lead to activation. These are interesting issues to pursue in further studies.
In summary, we have begun to unravel the molecular mechanisms of PKA regulation of Kir6.1/SUR2B. Our data implicate at least 3 sites in playing an important role in this modulation and support a model in which a large number of these sites need to be phosphorylated before substantial channel activation occurs.

Acknowledgments
This work was supported by the British Heart Foundation (BHF) and the Wellcome Trust. J.P.G. is a BHF junior fellow.

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Circ Res. 2004;94:1359-1366; originally published online April 15, 2004;
doi: 10.1161/01.RES.0000128513.34817.c4

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