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ATP-Sensitive Potassium Channel Traffic Regulation by Adenosine and Protein Kinase C

Keli Hu, Cindy Shen Huang, Yuh Nung Jan, and Lily Yeh Jan*
Howard Hughes Medical Institute
Department of Physiology
Department of Biochemistry
University of California, San Francisco
San Francisco, California 94143

Summary

ATP-sensitive potassium (K_{ATP}) channels activate under metabolic stress to protect neurons and cardiac myocytes. However, excessive channel activation may cause arrhythmia in the heart and silence neurons in the brain. Here, we report that PKC-mediated downregulation of K_{ATP} channel number, via dynamin-dependent channel internalization, can act as a brake mechanism to control K_{ATP} activation. A dileucine motif in the pore-lining Kir6.2 subunit of K_{ATP}, but not the site of PKC phosphorylation for channel activation, is essential for PKC downregulation. Whereas K_{ATP} activation results in a rapid shortening of the action potential duration (APD) in metabolically inhibited ventricular myocytes, adenosine receptor stimulation and consequent PKC-mediated K_{ATP} channel internalization can act as a brake to lessen this APD shortening. Likewise, in hippocampal CA1 neurons under metabolic stress, PKC-mediated, dynamin-dependent K_{ATP} channel internalization can also act as a brake to dampen the rapid decline of excitability due to K_{ATP} activation.

Introduction

ATP-sensitive potassium (K_{ATP}) channels control insulin release and blood flow and may protect muscle, heart, kidney, and brain under metabolic stress (Noma, 1983; Seino, 1999; Ashcroft, 1988; Schwanstecher et al., 1999; Terzic et al., 1995; Nelson and Quayle, 1995). K_{ATP} channel inhibition by ATP and stimulation by nucleotide diphosphates allows the membrane potential and excitability to be regulated by the metabolic state of the cell. The K_{ATP} channel contains four pore-lining subunits of the Kir6 subfamily of inwardly rectifying potassium channels (Tucker et al., 1997) and four regulatory subunits of the ATP binding cassette (ABC) superfamily. The regulatory subunits, the sulphonylurea receptors (SUR), mediate channel inhibition by sulphonylurea drugs such as glibenclamide (Aguilar-Bryan and Bryan, 1999; Babenko et al., 1998; Seino, 1999; Fujita and Kurachi, 2000; Loussouarn et al., 2002; Tucker and Ashcroft, 1998). Differences in endogenous K_{ATP} channel properties and pharmacology (Edwards and Weston, 1993) may be accounted for by differences in subunit composition: Kir6.2 and SUR1 (pancreatic β cells), Kir6.2 and SUR2A (cardiac myocytes), Kir6.2/Kir6.1 and SUR2B (vascular smooth muscles), or Kir6.2 and SUR1/2B (central neurons) (Aguilar-Bryan et al., 1998).

K_{ATP} channels activate under metabolic stress such as hypoxia, ischemia, and metabolic inhibition and protect heart, brain, and muscle cells against ischemic injury (Nichols and Lederer, 1991; Pang et al., 1997; Yamada and Inagaki, 2002). For example, ischemia causes adenosine to accumulate in the heart, brain, and kidney (Mubagwa et al., 1996; Mubagwa and Flameng, 2001; Nieber et al., 1999; Reshef et al., 1998; Shryock and Belardinelli, 1997), leading to tissue protection likely due to PKC activation (Simkhovich et al., 1998; Schulz et al., 2001). PKC stimulates K_{ATP} channels in cardiac myocytes (Hu et al., 1996; Light et al., 1996; Liu et al., 1996) and recombinant K_{ATP} channels, most likely by phosphorylating Kir6.2 at threonine 180 (Light et al., 2000). The protection afforded by K_{ATP} activation to central neurons and cardiac myocytes during ischemia is largely due to energy preservation (Yamada et al., 2001; Wann, 1993) and reduced Ca^{2+} influx (Edwards and Weston, 1993; Hearse, 1995).

While activation of K_{ATP} channels during metabolic stress is protective against injury, excessive K_{ATP} channel opening may have deleterious consequences. In the heart, active K_{ATP} channels cause the action potential duration (APD) to shorten and contribute to cellular K^+ loss, both risk factors for arrhythmia (Janse and Wit, 1989; Wilde, 1993). In the brain, opening of K_{ATP} channels in neurons results in hyperpolarization and reduced excitability, raising the risk of silencing the neurons (Yamada et al., 2001). It is therefore important to understand how K_{ATP} channels might be regulated under metabolic stress to confer protection without incurring serious damage.

In this study, we ask whether adenosine and PKC might modulate K_{ATP} channels in ways other than their known involvement in channel activation. To explore the effects of PKC activation, we first employed the phorbol ester 4-β-phorbol 12-myristate 13-acetate (PMA) and found that 15–30 min PMA treatments reduced K_{ATP} channel activity by promoting channel internalization via dynamin-dependent endocytosis. Having determined that the pore-lining Kir6.2 subunit is the primary target for PKC-mediated downregulation, we mutated PKC consensus sites in Kir6.2 including T180 and found no effect on channel downregulation by PKC. Instead, we found a dileucine motif in Kir6.2 to be important for constitutive as well as PKC-induced channel internalization, in cultured cardiac myocytes as well as COS-7 cells. The physiological significance for such a brake against excessive K_{ATP} activation is underscored by its ability to retard not only APD shortening in metabolically inhibited ventricular myocytes but also loss of excitability of hippocampal CA1 neurons under metabolic inhibition.

*Correspondence: gkw@itsa.ucsf.edu

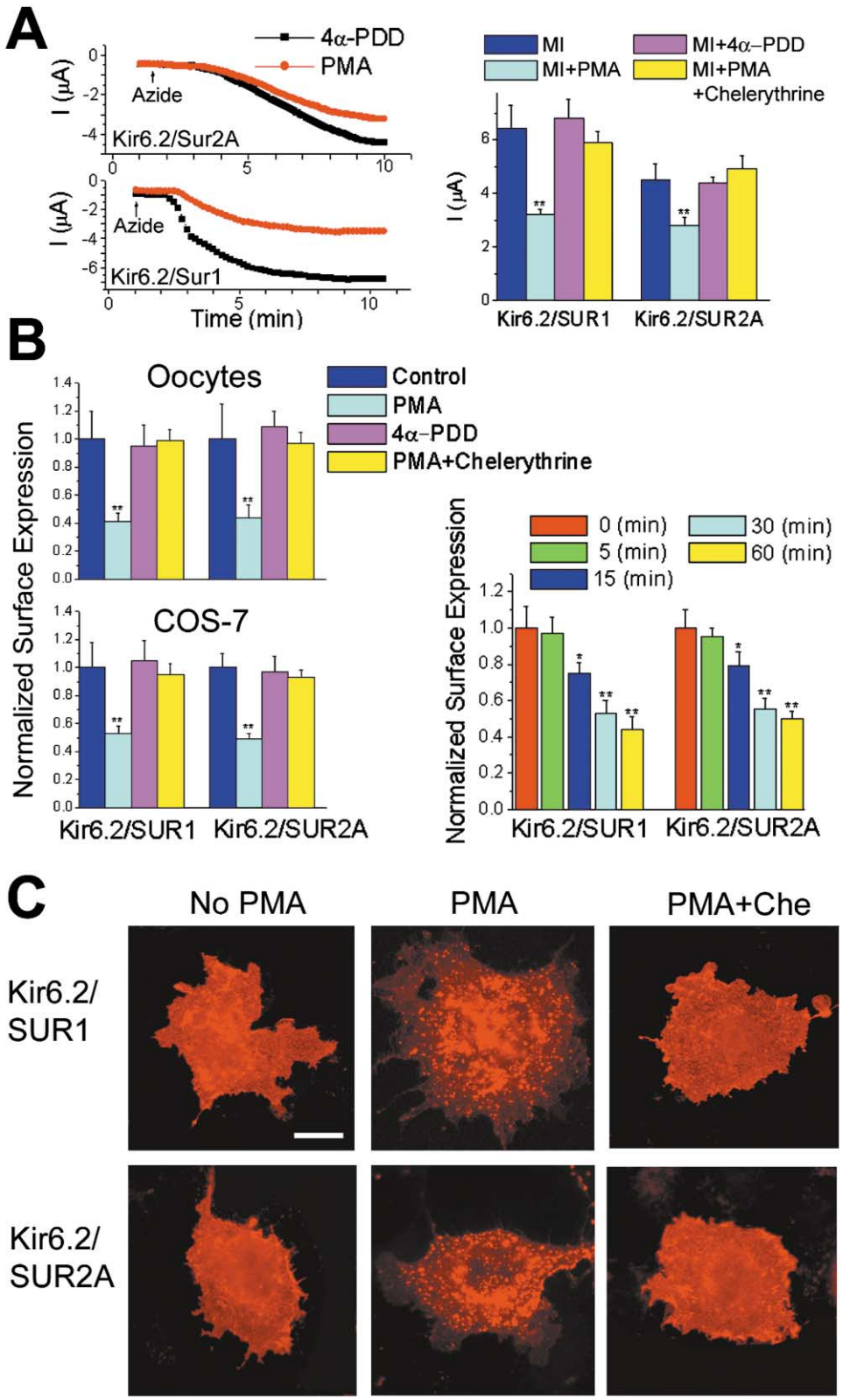


Figure 1. PKC Activation by PMA Pretreatment Downregulates ATP-Sensitive K^+ Channels via Channel Internalization

(A) PMA pretreatment for 30 min decreases azide-induced K^+ current measured at −60 mV in *Xenopus* oocytes expressing Kir6.2 and SUR1/2A. Left panel with representative traces shows current reduction after pretreatment with PMA (100 nM) but not the inactive congener 4α-PDD (100 nM). Recordings were made using a ramp protocol between −120 and 40 mV every 5 s. Right panel shows averaged current amplitudes measured after 10 min superfusion with azide (3 mM) (MI, metabolic inhibition) from oocytes pretreated with PMA, 4α-PDD, or PMA plus chelerythrine (10 μM). **$p < 0.01$.

Results

PMA Causes a Reduction of Surface K_{ATP} Channels in *Xenopus* Oocytes and COS-7 Cells

Exposing oocytes expressing Kir6.2 and SUR1/2A to the metabolic inhibitor sodium azide (3 mM) for 10 min induced the K_{ATP} current, with a somewhat variable time course of onset. This azide-induced current was significantly reduced by 100 nM PMA pretreatment (Figure 1A). This K_{ATP} downregulation was evident when cells were exposed to PMA (100 nM) for 15 min (Figure 1B, right panel; 75.5% $\pm$ 6.2% for Kir6.2 and SUR1 and 78.6% $\pm$ 8.1% for Kir6.2 and SUR2A, $p < 0.05$ versus control [MI], $n = 7$); stronger effects were observed after 30 min of PMA treatment (Figures 1A and 1B, right panel; 53.3% $\pm$ 7.4% for Kir6.2 and SUR1 and 55.8% $\pm$ 6.7% for Kir6.2 and SUR2A, $p < 0.01$ versus control [MI], $n = 7$). We have therefore carried out all the quantitative analyses following 30 min of PMA treatment. No reduction resulted from treatment with the inactive congener 4-α-phorbol 12,13-didecanoate (4α-PDD, 100 nM) (Figure 1A; $n = 7$). The PKC inhibitor chelerythrine (10 μM) prevented the downregulation by PMA (Figure 1A, right panel; $n = 7$), consistent with the notion that PKC activation by PMA decreases K_{ATP} currents.

A decrease in K_{ATP} current may result from alteration of either channel function or channel number. To examine whether PMA affects the K_{ATP} channel number, we quantified surface K_{ATP} channel proteins with the hemagglutinin (HA) epitope inserted in an extracellular loop of Kir6.2, via a chemiluminescence assay (Zerangue et al., 1999). PMA treatment caused a significant, 2-fold reduction in surface channel number in oocytes (Figure 1B, top left panel) (41.5% $\pm$ 6.2% for Kir6.2 and SUR1 and 44.3% $\pm$ 9.1% for Kir6.2 and SUR2A, $n = 8$, $p < 0.01$ versus control). Similar reduction was observed for channels expressed in COS-7 cells (Figure 1B, lower left panel) (53.2% $\pm$ 5.4% for Kir6.2 and SUR1 and 49.1% $\pm$ 4.3% for Kir6.2 and SUR2A, $n = 7$, $p < 0.01$ versus control). No reduction was observed when cells were exposed to PMA together with the PKC inhibitor chelerythrine (10 μM) (Figure 1B, lower left panel; $n = 6$). The inactive congener 4α-PDD (100 nM) also had no effect (Figure 1B, lower left panel; $n = 7$). These results indicate that the PKC downregulation of channel activity could be accounted for by a 2-fold reduction in the number of channels on the cell membrane.

PMA Facilitates Endocytosis of K_{ATP} Channels in COS-7 Cells

A plausible mechanism for the observed reduction in surface channel number is that PKC stimulates channel internalization. To test this possibility, we transiently transfected COS-7 cells with HA-tagged Kir6.2 and SUR1/2A, then treated live cells with primary antibodies against the extracellular HA epitope. These cells were subsequently treated with PMA or PMA plus chelerythrine for 30 min at 37°C, then fixed, permeabilized, and labeled with fluorescent secondary antibodies at room temperature. Most of the labeled channels appeared to have remained on the plasma membrane if cells were not exposed to PMA. In contrast, significant endocytosis of K_{ATP} channels followed the PMA treatment, as indicated by the shift of antibody-labeled channels from the plasma membrane to numerous endocytic vesicles inside the cell (Figure 1C). The PKC inhibitor chelerythrine prevented PMA from causing channel internalization (Figure 1C), indicating that endocytosis of K_{ATP} channels is induced by PKC activation.

Distribution of Internalized K_{ATP} Channels Following PKC Activation

Different endocytic markers were used in double labeling experiments to characterize the vesicles containing endocytosed K_{ATP} channels marked with antibodies applied to live cells. First, live cells were exposed to rhodamine-conjugated WGA (wheat germ agglutinin) to label surface glycoproteins, some of which were subsequently internalized. Following PMA treatment, endocytosed K_{ATP} channels colocalized with WGA (Figure 2A). There was also partial overlap between endocytosed channels and GFP-Rab7 (Figure 2B), a marker for late endosomes and lysosomes (Bucci et al., 2000), indicating that some of the internalized channels may be targeted for degradation.

Many housekeeping receptors such as the transferrin receptor rapidly recycle to the cell surface via recycling endosomes (Mukherjee et al., 1997) in a Rme1-dependent process (Grant et al., 2001; Lin et al., 2001). Expression of the dominant-negative Rme1 (G429R) mutant significantly slowed recycling of transferrin receptors, causing their accumulation in the Rme1-associated recycling compartment (Figure 3A). By contrast, there was relatively minor overlap between internalized channels and the default recycling pathway marked by Rme1 (G429R) (Figure 3A).

To further explore the mechanism for PKC-induced channel endocytosis, we tested the effect of expressing the dominant-negative mutant dynamin 1 (K44E). Disruption of dynamin function blocked PKC downregulation of K_{ATP} channels on the COS-7 cell surface (Figure 3B, left panel; $p < 0.01$ versus PMA alone, $n = 6$) and prevented PKC from reducing K_{ATP} current in metabolically inhibited oocytes (Figure 3B, right panel; $p < 0.01$ versus PMA alone, $n = 8$). These findings suggest that PKC promotes K_{ATP} channel internalization by dynamin-dependent endocytosis.

A Dileucine Motif in Kir6.2 Is Essential for PKC to Promote Internalization of Kir6.2 and SUR1/2A

The K_{ATP} channel is composed of two different types of subunit, Kir6.2 and SUR1/2A (Figure 4A, top panel). To

(B) Chemiluminescence measurement of channels containing HA-tagged Kir6.2 and SUR1/2A on the surface of nonpermeabilized oocytes (top left), COS-7 cells (lower left), and time course of PMA-induced reduction of channel proteins on the COS-7 cell surface (right). PMA (100 nM) treatment for 15, 30, or 60 min significantly reduces cell surface expression. **$p < 0.01$, *$p < 0.05$.
(C) PMA (100 nM) facilitates endocytosis of channels containing HA-tagged Kir6.2 and SUR1/2A in transiently transfected COS-7 cells. The PKC inhibitor chelerythrine (Che, 10 μM) blocks the PMA effect. Scale bar equals 15 μm.

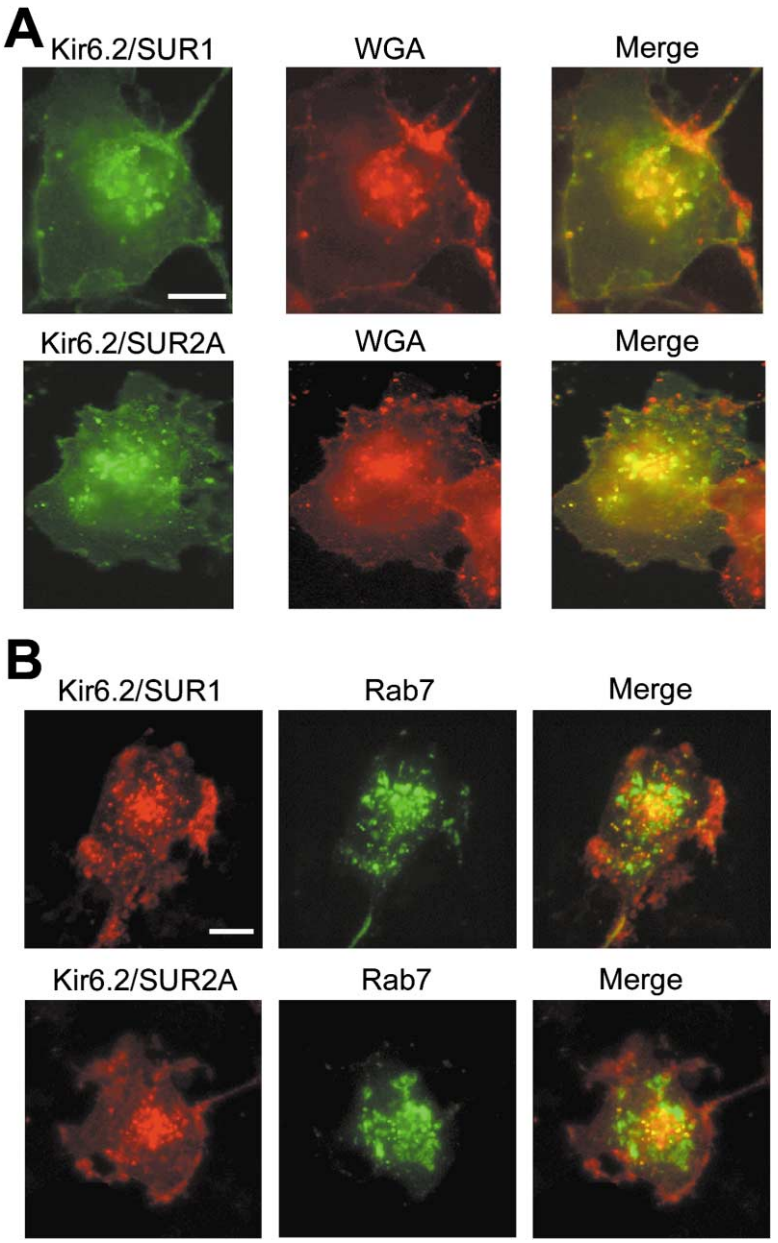


Figure 2. Partial Overlap of Endocytosed K_{ATP} Channels with Endocytosed Wheat Germ Agglutinin or the Late Endosome Marker GFP-Rab-7

(A) Application of HA antibody to live cells label surface K_{ATP} channels with an extracellular HA epitope, revealing that most channels that are internalized following PMA (100 nM) treatment accumulate in intracellular vesicles that also contain endocytosed rhodamine-WGA. Scale bar equals 15 μm; WGA, wheat germ agglutinin.

(B) A small portion of these endocytosed channels colocalizes with the late endosome/lysosome marker GFP-Rab-7. Scale bar equals 15 μm.

test whether PKC can downregulate channels containing only Kir6.2 subunits, we removed the RKR signal for ER retention/retrieval (Schwappach et al., 2000; Zerangue et al., 1999), either by alanine substitutions or by deleting the last 36 residues of Kir6.2 (Kir6.2Δ36) (Tucker et al., 1997; Figure 4A, lower panel), and expressed tetrameric Kir6.2 channels without SUR. Cell surface expression of HA-tagged Kir6.2Δ36 was not altered by PMA treatment (Figure 4B, left panel; 104.5% ± 6.3%, n = 7). In contrast, alanine substitution of LRKR in Kir6.2 (Kir6.2C4A) yielded tetrameric channels that were downregulated by the PMA treatment (Figure 4B, left panel; 69.7% ± 4.7%, n = 7, $p < 0.05$ versus Kir6.2C4A without PMA). Thus, PKC downregulates tetrameric Kir6.2 channels as well as octameric K_{ATP} channels, and the last 36 amino acids of Kir6.2 harbor sequences crucial for channel downregulation by PKC.

A dileucine motif is located within the last 36 amino acids of Kir6.2. Dileucine motifs mediate endosomal targeting by interacting with the AP2 adaptor protein complex (Sandoval et al., 1994; Trowbridge et al., 1993). Indeed, mutating this dileucine sequence of Kir6.2 (355-356LL-AA) caused an increase of surface octameric channels (Figure 4B, right panel; 126.5% ± 9.2% for mutant Kir6.2 and SUR1 and 130.2% ± 10.9% for mutant Kir6.2 and SUR2A, n = 8, $p < 0.05$ versus wild-type), suggesting that the dileucine motif plays a role in constitutive channel endocytosis. Interestingly, these mutant channels no longer responded to PMA treatment with a decrease in cell surface expression (Figure 4B, right panel; 121.1% ± 12.3% for mutant Kir6.2 and SUR1 and 132.7% ± 10.9% for mutant Kir6.2 and SUR2A, n = 8). These mutant channels also yielded similar azide-induced K_{ATP} currents with or without PMA treatment (Figure 4C). Moreover, PMA treatment of COS-7 cells failed to induce internalization of K_{ATP} channels lacking

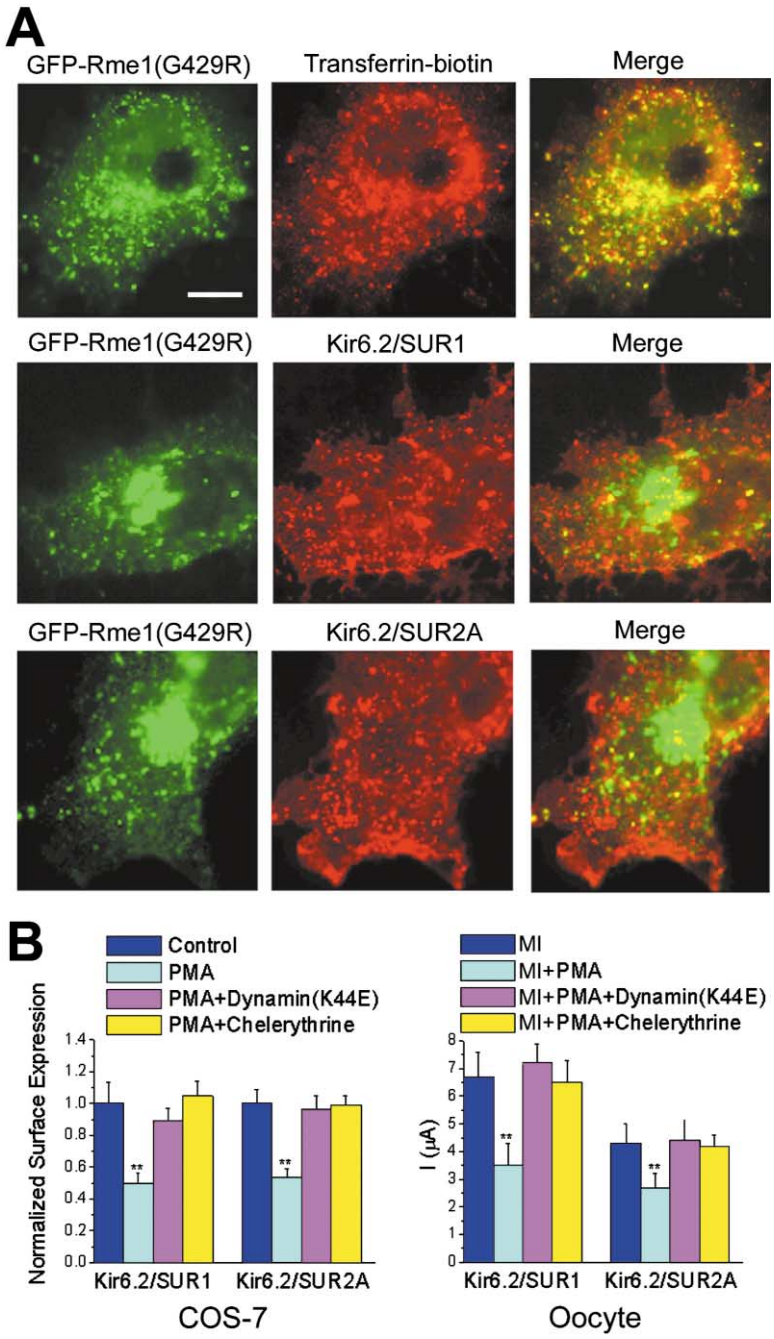


Figure 3. Dynamin-Dependent PKC-Induced Channel Endocytosis with Partial Overlap of Internalized Channels and the Recycling Endosomal Marker GFP-Rme1 (G429)

(A) Expression of a dominant-negative Rme1 mutant, Rme1 (G429R), inhibits recycling of transferrin receptors but does not cause significant accumulation of channels in the compartment that contains transferrin receptors and GFP-Rme1 (G429R). Scale bar equals 15 μm.

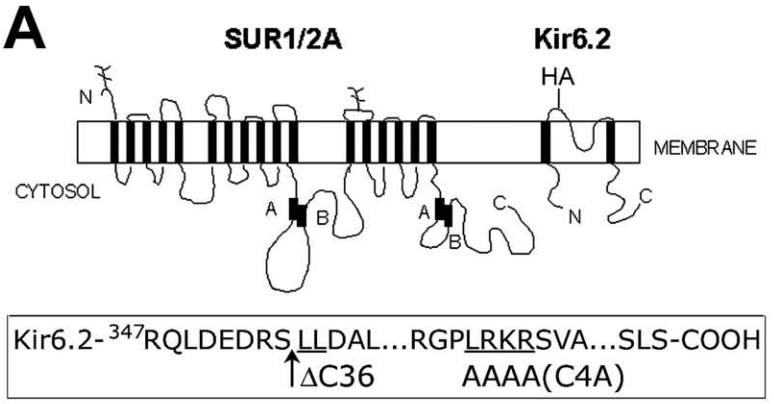
(B) Dominant-negative dynamin mutant (K44E) blocks PMA-induced reduction of surface K_{ATP} channels in COS-7 cells (left) and similar reduction of K_{ATP} current at −60 mV after 10 min metabolic inhibition of oocytes coexpressing Kir6.2 and SUR1/2A (right). $^{**}p < 0.01$.

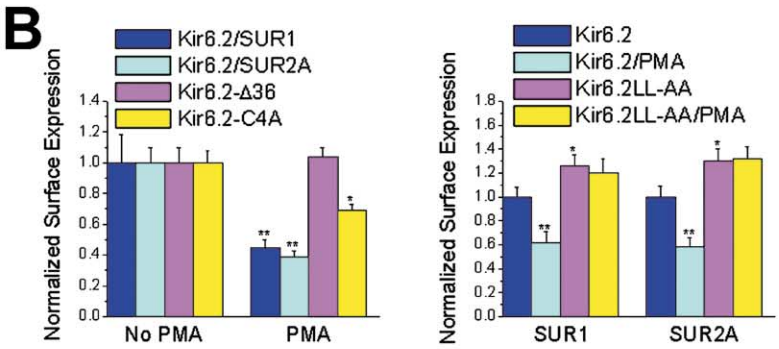
the dileucine motif (Figure 4D). These results strongly suggest that the dileucine motif in Kir6.2 functions in constitutive endocytosis as well as PKC-induced internalization of K_{ATP} channels.

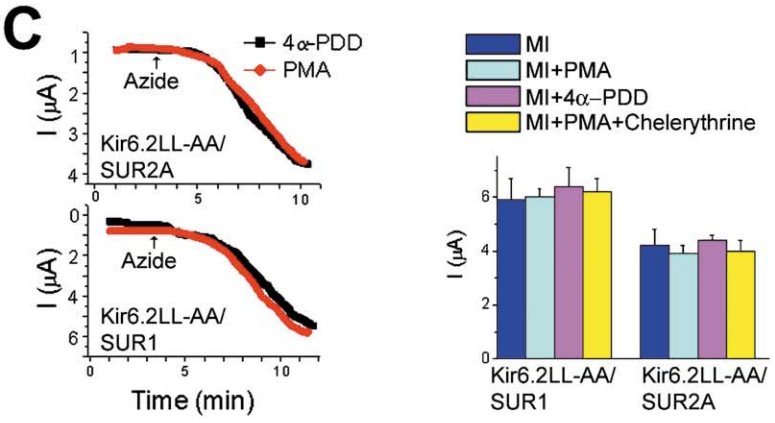
To test whether Kir6.2 phosphorylation and activation by PKC is a prerequisite for channel internalization, we examined the effect of PMA on COS-7 cells expressing K_{ATP} channels with mutated PKC consensus sites in Kir6.2 (Figure 5A; T180A, T224A, T336A, or T345A) (Kemp and Pearson, 1990). PMA reduced the surface expression of these mutant K_{ATP} channels by half (Figure 5B), as in the case of wild-type K_{ATP} channels. Moreover, K_{ATP} channels lacking any one or all four PKC consensus sites were still internalized following PMA treatment (Figure 5C and data not shown). PKC phosphorylates primarily T180, causing channel activation (Light et al., 2000). These findings present interesting contrast with desensitization of G protein-coupled receptors (GPCR); whereas GPCR activation and ensuing phosphorylation of the activated receptors is a prerequisite for their internalization (Claing et al., 2002), K_{ATP} channel activation via PKC phosphorylation is not required for PKC-induced channel internalization.

PKC Stimulates Internalization of K_{ATP} Channels in Cardiac Myocytes

K_{ATP} channels in cardiac myocytes are stimulated within 5 min by acute PKC activation by PMA (Hu et al., 1996);







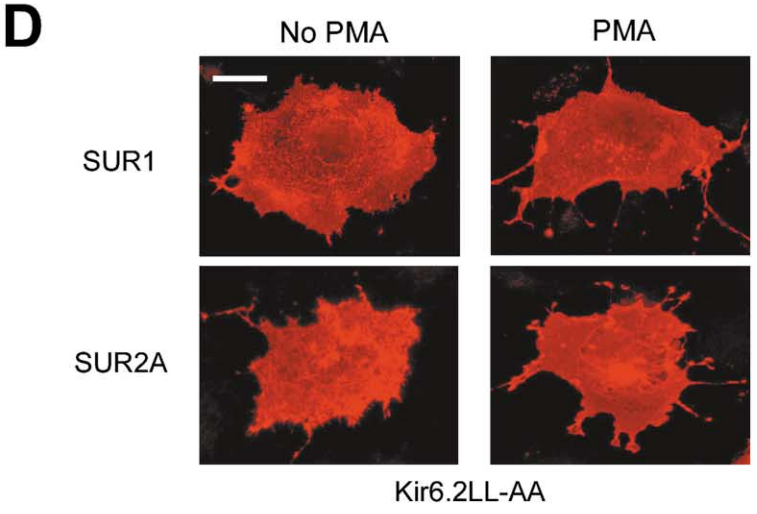


Figure 4. A Dileucine Motif in Kir6.2 Is Required for PMA-Induced Endocytosis of K_{ATP} Channels

(A) Schematic depiction of Kir6.2 and SUR1/2A membrane topology (top) and Kir6.2 residues mutated in this study (underlined) (bottom).

(B) Chemiluminescence measurements of surface protein level in COS-7 cells expressing HA-tagged Kir6.2ΔC36 or Kir6.2C4A alone (left) or HA-tagged Kir6.2 with its dileucine replaced with alanines (Kir6.2LL-AA) together with SUR1/2A (right) reveal that PMA (100 nM) fails to stimulate endocytosis of channels without the dileucine motif in Kir6.2. *$p < 0.05$, **$p < 0.01$.

(C) Replacing dileucine with alanines prevents PMA from reducing azide-induced K^+ current recorded at −60 mV in *Xenopus* oocytes. Left panel shows representative traces. Right panel shows average inward current measured at −60 mV after 10 min metabolic inhibition, following 30 min pretreatment with PMA (100 nM), 4α-PDD (100 nM), or PMA plus chelerythrine (10 μM). Recordings in oocytes were made using a ramp protocol between −120 and 40 mV every 5 s.

(D) PMA treatment does not result in significant endocytosis of channels containing Kir6.2LL-AA and SUR1/2A. Scale bar equals 15 μm.

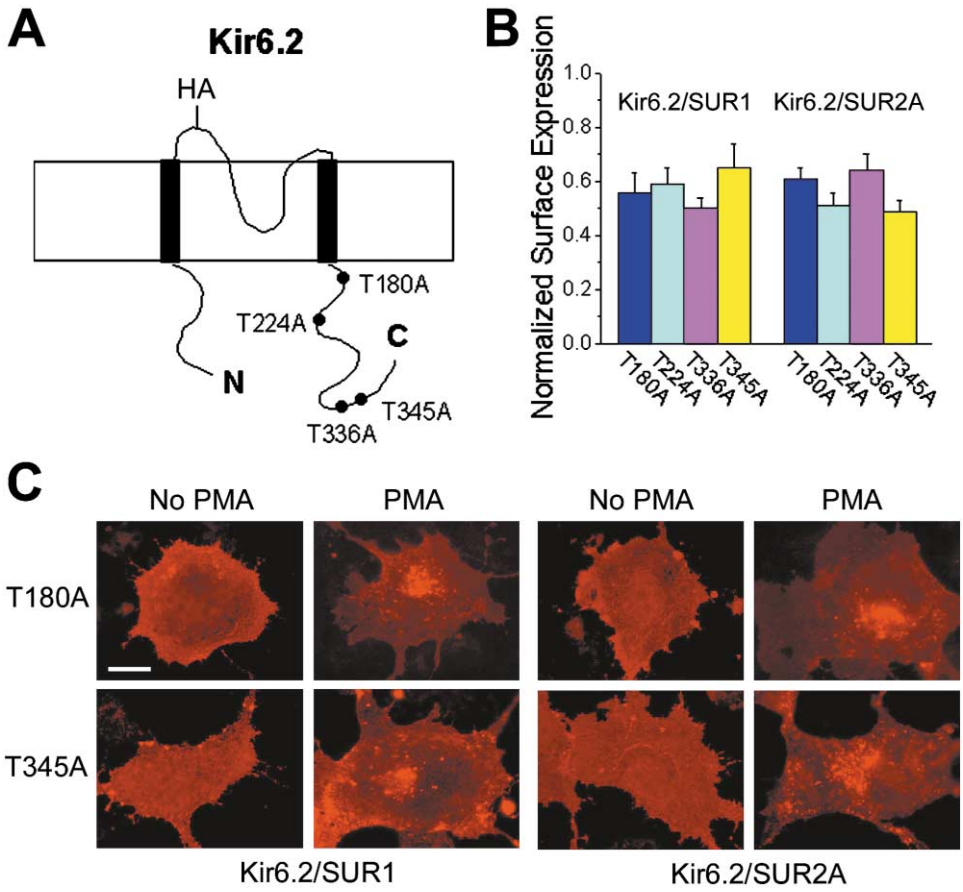


Figure 5. Potential PKC Phosphorylation Site(s) of Kir6.2 Are Not Required for PKC-Mediated Endocytosis

(A) Consensus sites for PKC phosphorylation in Kir6.2.

(B) Chemiluminescence measurement reveals a 2-fold reduction of surface protein level by PMA (100 nM) in COS-7 cells expressing HA-tagged Kir6.2 mutants (T180A, T224A, T336A, and T345A) and SUR1/2A.

(C) Representative immunofluorescence staining showing that mutating potential phosphorylation sites (T180A, T345A) in Kir6.2 does not prevent PMA-induced endocytosis of channels containing HA-tagged Kir6.2 and SUR1/2A. Scale bar equals 15 μm.

20 min after establishing whole-cell patch-clamp recording with 400 μM ATP in pipette solution to reduce the cytosolic ATP level, PMA (100 nM) was added to the bath, causing activation of K_{ATP} current within 5 min (Figure 6A). In contrast, pretreatment of ventricular myocytes with PMA for 30 min reduced the K_{ATP} current induced by metabolic inhibition (Figure 6B). Consequently, whereas cardiac K_{ATP} channel activation causes action potentials to shorten during ischemia or metabolic inhibition (Nichols and Lederer, 1991; Wilde, 1993), PMA pretreatment reduced action potential shortening of myocytes exposed to glucose-free solutions containing the metabolic inhibitors cyanide (2 mM) and 2-deoxy-glucose (10 mM) (Figure 6C; $n = 7$). The 30 min PMA pretreatment did not affect the resting membrane potential (-83.5 ± 5.3 mV, $n = 21$) nor the shape or duration of action potentials prior to metabolic inhibition (MI) (data not shown). Thus, whereas PKC pretreatment does not appear to affect channels that are active in healthy myocytes, it reduces the extent of endogenous K_{ATP} channel activation during metabolic stress.

To test whether PKC downregulates endogenous K_{ATP} channels by promoting internalization, we examined the effects of a dominant-negative dynamin peptide (D10) on action potentials in adult rat ventricular myocytes. D10 is a 10 amino acid peptide that interferes with the binding of amphiphysin with dynamin, thereby disrupting dynamin-dependent endocytosis (Kittler et al., 2000). For control, we used a scrambled peptide of the same amino acid composition, S10. The D10 or S10 peptide was added to the solution in the patch clamp electrode and allowed to diffuse into the cardiac myocyte under whole-cell patch clamp for 20 min before these myocytes were treated with PMA for 30 min. There were no significant alterations in APD_{90} after dialyzing peptides into the myocytes or after the subsequent PMA treatment for 30 min (Figure 6D, top panel). Thus, these treatments had no detectable effects on cardiac channels other than K_{ATP} channels, which remained quiescent in healthy myocytes. Eight minutes of metabolic inhibition caused more severe reduction of APD_{90} in PMA-treated cells dialyzed with D10, compared to PMA-treated cells dialyzed with S10 (Figure 6D; 13.8 ± 1.4 ms for D10 group versus 32.8 ± 2.4 ms for S10 group, $p < 0.01$, $n = 8$). Therefore, the effect of PKC on APD shortening is dynamin dependent.

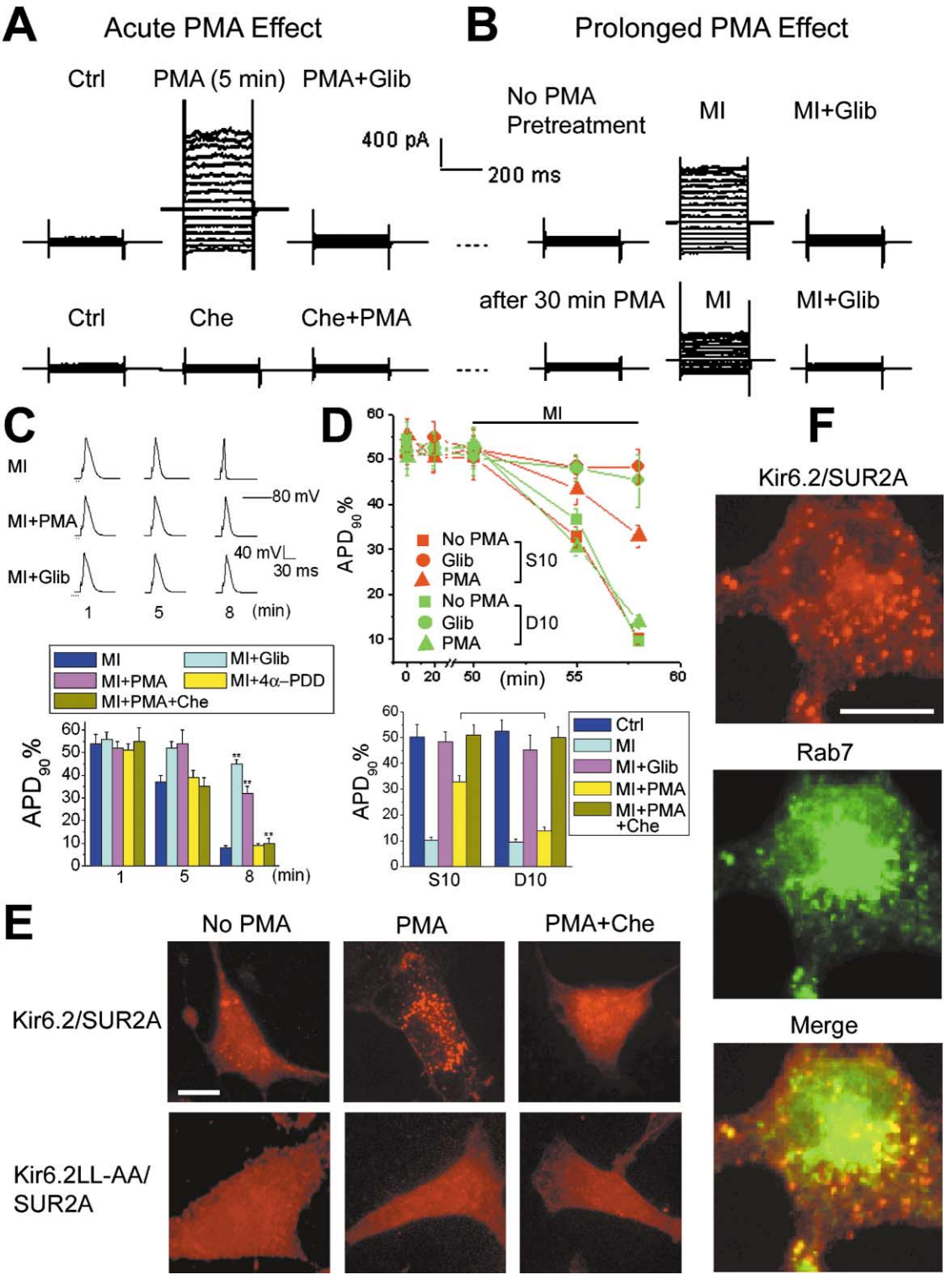


Figure 6. Channel Activation due to Brief PKC Activation by PMA in Contrast to Dynamin- and Dileucine-Dependent Channel Downregulation due to Prolonged PKC Activation in Rat Ventricular Myocytes

(A) Acute PMA (100 nM) treatment for 5 min greatly increases K_{ATP} current recorded in ventricular myocytes with 400 μM ATP in the whole-cell patch-clamp pipette solution. The K_{ATP} channel blocker glibenclamide (Glib, 10 μM) blocks this current, and the PKC inhibitor chelerythrine (Che, 10 μM) blocks the PMA effect. Representative whole-cell currents are shown, from a holding potential of −40 mV to 300 ms voltage pulses between −100 mV and 50 mV. Dotted lines indicate zero current levels.

(B) PKC activation by PMA (100 nM) pretreatment for 30 min reduces metabolic inhibition (cyanide plus 2-deoxyglucose)-induced K_{ATP} current in rat ventricular myocytes. Subsequent addition of glibenclamide (Glib) blocks this current. Same voltage-clamp protocol as in (A).

(C) Shortening of action potential duration in rat ventricular myocytes exposed to metabolic inhibitors cyanide and 2-deoxy glucose is due

Further support for a role of PKC in channel traffic regulation was obtained in neonatal ventricular myocytes transfected with HA-tagged Kir6.2 and SUR2A. The PMA pretreatment for 30 min induced endocytosis of the HA-tagged channels (Figure 6E), causing part of these internalized channels to reside in Rab7-positive late endosomes (Figure 6F). Moreover, PKC-induced channel internalization in these neonatal ventricular myocytes required the dileucine endocytic motif of Kir6.2 (Figure 6E).

PKC can be activated by adenosine in vivo. Adenosine accumulates in tissues under metabolic stress (Mubagwa and Flameng, 2001). Adenosine released into the interstitial fluid in metabolically stressed heart could activate various adenosine receptor subtypes that are coupled to multiple effectors including enzymes, channels, and transporters. Indeed, PKC activation has been implicated in adenosine-mediated events such as ischemic preconditioning (Downey and Cohen, 1997). It is thus plausible that adenosine receptor activation may stimulate PKC in cardiac myocytes and cause K_{ATP} channel internalization. We tested this scenario by adding adenosine to isolated ventricular myocytes.

First, we found that PMA or adenosine pretreatment for 30 min reduced endogenous K_{ATP} currents in metabolically stressed adult rat ventricular myocytes (Figure 7A). Metabolic inhibition with cyanide activated K_{ATP} current that was significantly blocked by 10 μM glibenclamide (1.02 $\pm$ 0.13 nA in MI versus 0.21 $\pm$ 0.03 nA in MI plus glibenclamide, $p < 0.01$, $n = 5$). PMA pretreatment significantly reduced this K_{ATP} current (0.47 $\pm$ 0.06 nA in PMA followed with MI versus MI group, $p < 0.01$, $n = 7$). Adenosine pretreatment also reduced K_{ATP} current activated by metabolic inhibition (Figure 7A, left panel; 0.56 $\pm$ 0.08 nA in adenosine followed with MI versus MI group, $p < 0.01$, $n = 7$). The PKC inhibitor chelerythrine blocked this adenosine-induced K_{ATP} current reduction (Figure 7A, right panel). Thus, adenosine can activate endogenous PKC to downregulate cardiac K_{ATP} channels.

Next, we found that adenosine significantly reduced APD shortening after 8 min exposure of adult ventricular myocytes to metabolic inhibitors (35.7 $\pm$ 4.1 ms for adenosine followed with MI [adenosine group] and 9.3 $\pm$ 1.2 ms for MI, $n = 6$, $p < 0.01$; Figure 7B). This adenosine effect was abolished by the adenosine receptor antagonist 8-phenyltheophyline (8-PT) (8.9 $\pm$ 1.1 ms, $n = 6$, $p < 0.01$ versus adenosine group) and by the PKC inhibitor chelerythrine (14.2 $\pm$ 1.8 ms, $n = 6$, $p < 0.01$ versus adenosine group).

Adenosine (100 μM)-induced endocytosis of K_{ATP} channels was readily detected in neonatal rat ventricular myocytes expressing channels with extracellular HA tag. This adenosine effect was also sensitive to the PKC inhibitor chelerythrine (Figure 7C). Furthermore, K_{ATP} channels internalized following adenosine treatment showed partial overlap with the late endosome/lysosome marker GFP-Rab7 (Figure 7D), as expected for PKC downregulation of the channel. Taken together, these findings indicate that adenosine induces K_{ATP} channel internalization in cardiac myocytes by activating adenosine receptors and the downstream PKC, a process that could partially counter runaway K_{ATP} channel activation during metabolic inhibition and hence reduce the detrimental effects due to APD shortening.

PKC Stimulates Internalization of Endogenous K_{ATP} Channels in Hippocampal CA1 Neurons

We next tested whether adenosine causes endogenous K_{ATP} channel downregulation in a PKC-dependent manner in central neurons and, if so, whether PKC-mediated downregulation of neuronal K_{ATP} channels is dynamin dependent. To approach these questions, we recorded from cultured hippocampal slices. Healthy CA1 neurons had a resting potential of -55.3 ± 2.1 mV ($n = 25$; Figure 8A) and, when depolarized, fired action potentials repeatedly (Figure 8B), both in neurons dialyzed with the scrambled control peptide S10 and in neurons dialyzed with the dominant-negative dynamin peptide D10 via the whole-cell patch-clamp electrode. PKC pretreatment for 30 min did not affect these properties, indicating little effect on channels that are active in these healthy neurons.

Metabolic inhibition with 0.5 mM cyanide caused hyperpolarization of CA1 neurons; 10 μM glibenclamide blocked this hyperpolarization due to endogenous K_{ATP} channel activation (Figure 8A; -69.5 ± 3.2 mV in MI+S10 versus -57.4 ± 2.9 mV in MI+Glib+S10 group, $p < 0.01$, $n = 8$). It also caused a gradual decrease in firing rate of action potentials in the CA1 neurons dialyzed with S10, resulting in action potential failure within 5 min of metabolic inhibition (Figure 8B, left panel). Glibenclamide (10 μM) prevented cyanide-induced decrease in firing rate, indicating that the loss of excitability results from K_{ATP} channel activation during metabolic stress. PMA pretreatment for 30 min allowed metabolically in-

to activation of K_{ATP} channels, as indicated by the effect of the K_{ATP} channel blocker glibenclamide (Glib, 10 μM). Pretreatment with PMA (100 nM) for 30 min significantly reduces action potential shortening induced by metabolic inhibition. Action potentials were elicited by 2 ms twice-threshold pulses every 5 s. Top panel shows representative traces and bottom panel shows the averaged data. Che indicates chelerythrine (10 μM). **$p < 0.01$.

(D) The dominant-negative dynamin peptide D10 (100 μM in patch-clamp pipette solution) diffuses into cardiac myocyte after establishment of whole-cell recording (time 0) and prevents the PMA (100 nM) pretreatment (time 20–50 min) from reducing the shortening of APD_{90}% due to metabolic inhibitors cyanide (2 mM) and 2-deoxy glucose (10 mM) (MI, top panel). The average APD_{90}% before (control, Ctrl) and 8 min into MI (bottom panel) reveals that D10 but not scrambled peptide S10 (100 μM) prevents PMA from reducing the shortening of APD_{90}% caused by MI. **$p < 0.01$ for MI + PMA, D10 versus S10 (bracket).

(E) PMA (100 nM) treatment for 30 min induces endocytosis of channels containing SUR2A and HA-tagged Kir6.2 but not Kir6.2LL-AA in primary culture of rat neonatal ventricular myocytes. The PKC inhibitor chelerythrine (Che, 10 μM) blocks the PMA effect. Scale bar equals 20 μm.

(F) Endocytosed HA-tagged Kir6.2 and SUR2A induced by PMA (100 nM) treatment for 30 min shows partial overlap with late endosome/lysosome marker GFP-Rab7 in cardiac myocytes. Scale bar equals 20 μm.

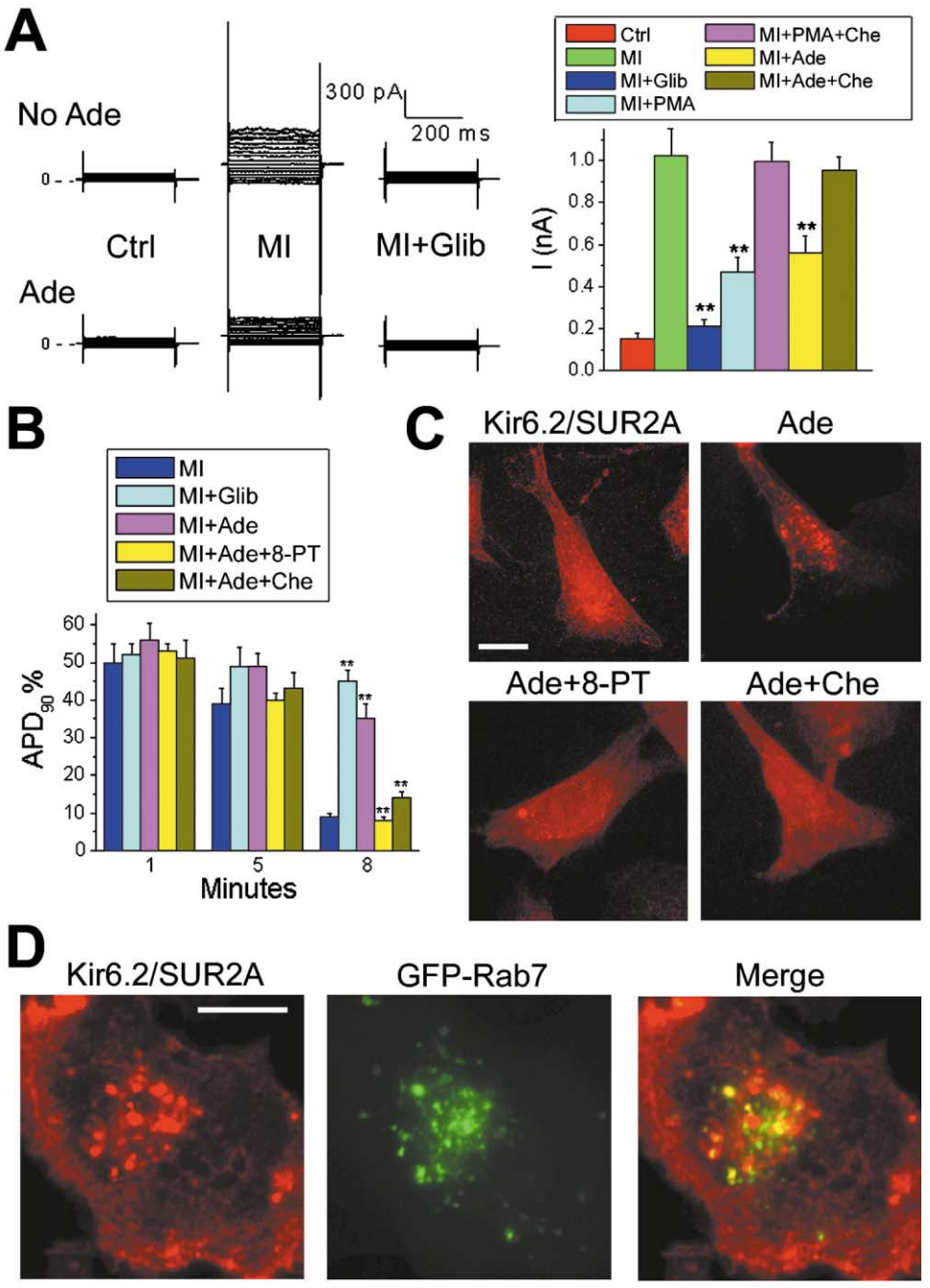


Figure 7. Adenosine Causes PKC-Dependent Endocytosis of Both Transfected and Endogenous K_{ATP} Channels in Rat Ventricular Myocytes

(A) PKC activation by adenosine significantly reduces MI-induced activation of K_{ATP} current. Pretreatment for 30 min with adenosine (Ade) significantly reduces MI-induced activation of K_{ATP} current in rat ventricular myocytes (holding potential, −40 mV; 300 ms voltage pulses between −100 mV and 50 mV) (left). Average K_{ATP} current amplitude at 50 mV before (control, Ctrl) or 8 min into MI in the absence or presence of Glib (10 μM) or pretreatment with PMA (100 nM), PMA plus Che (10 μM), ade (100 μM), or ade plus Che (right). **$p < 0.01$.

(B) Average action potential duration at 90% repolarization in rat ventricular myocytes. Adenosine significantly reduces action potential shortening 8 min after the onset of metabolic inhibition. Action potentials were elicited by 2 ms twice-threshold pulses. **$p < 0.01$.

(C) Adenosine (Ade, 100 μM)-induced endocytosis of HA-tagged Kir6.2 and SUR2A expressed in neonatal rat ventricular myocytes is blocked by the adenosine receptor antagonist 8-PT (10 μM) and the PKC inhibitor chelerythrine (Che). Scale bar equals 20 μm.

(D) Endocytosis of HA-tagged Kir6.2 and SUR2A induced by adenosine shows partial overlap with late endosome/lysosome marker GFP-Rab7 in cardiac myocytes. Scale bar equals 20 μm.

hibited neurons dialyzed with S10 to continue firing action potentials (Figure 8B, left panel), consistent with the notion of PKC downregulation of K_{ATP} channels. In cells dialyzed with D10, PMA treatment no longer had any effect on action potentials (Figure 8B, right panel). PKC activation by PMA also reduced cyanide-induced

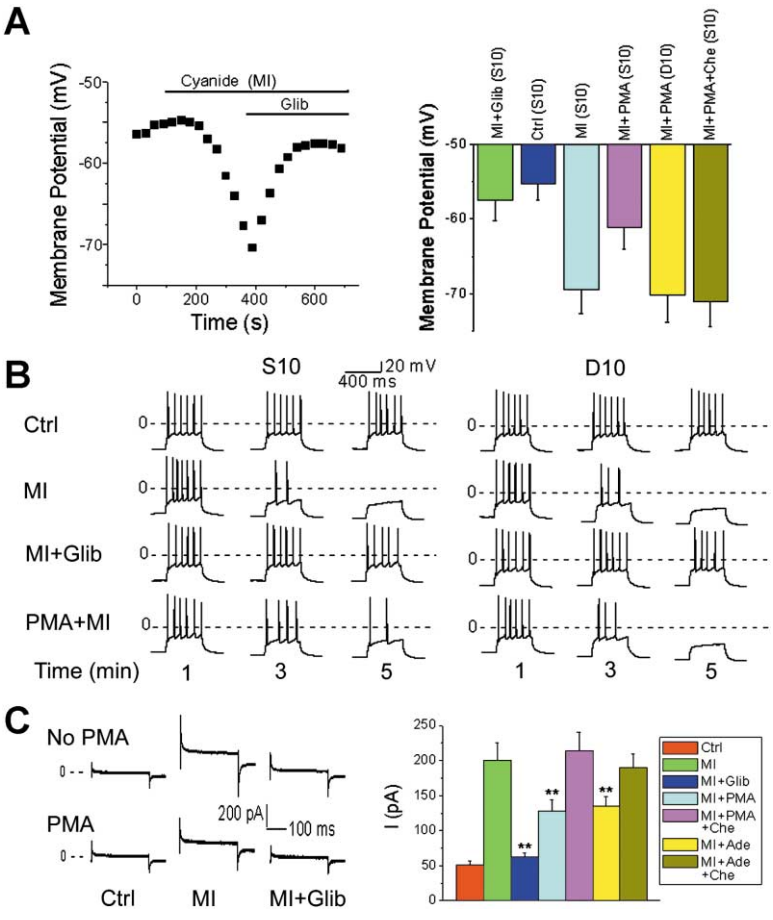


Figure 8. PKC-Induced, Dynamin-Dependent, Downregulation of K_{ATP} Channels in Hippocampal Neurons May Be Triggered by Adenosine

(A) Hyperpolarization of the resting membrane potential (RMP) due to metabolic inhibition (MI)-induced K_{ATP} activation is reduced by PMA in a dynamin-dependent manner. Left panel shows a representative time course of changes in RMP. Right panel shows mean data before (control, Ctrl) and 5 min into MI in cells dialyzed with either S10 (scrambled peptide, 100 μM) or D10 (dominant-negative dynamin peptide, 100 μM) under whole-cell patch clamp, and pretreated with PMA (100 nM) or PMA plus chelerythrine (Che, 10 μM). The dominant-negative dynamin peptide completely blocks the PMA effect.

(B) The number of action potentials evoked by 400 ms current pulses of 200 pA is reduced to zero following 5 min of metabolic inhibition (MI) in hippocampal neurons dialyzed with S10 (100 μM, left) under whole-cell patch-clamp. This reduction in excitability is due to K_{ATP} activation and can be prevented by application of glibenclamide (Glib, 10 μM). PMA (100 nM) pretreatment partially blocks the reduction of AP firing frequency in metabolically inhibited neurons containing S10 (left) but not in metabolically inhibited neurons containing the dominant-negative dynamin peptide D10 (100 μM, right).

(C) Metabolic inhibition (MI)-induced whole-cell currents recorded at −60 mV for 300 ms from a holding potential of −70 mV in hippocampal neurons. The MI-induced current is blocked by K_{ATP} inhibitor glibenclamide (10 μM) and reduced by PMA pretreatment (100 nM, left). Statistically significant reduction of neuronal K_{ATP} is induced by PMA or adenosine (100 μM). The adenosine as well as the PMA effects are blocked by the PKC inhibitor Che (10 μM). **$p < 0.01$.

hyperpolarization (−61.1 ± 3.0 mV in PMA+MI+S10 versus MI+S10 group, $p < 0.05$, $n = 8$), an effect that was eliminated by D10 (−70.2 ± 3.7 mV in PMA+MI+D10 versus PMA+MI+S10 group, $p < 0.05$, $n = 8$) (Figure 8A, right panel). These findings suggest that the PMA effect is due to dynamin-dependent internalization of K_{ATP} channels by PKC.

To further verify that PKC treatment of CA1 neurons downregulates endogenous K_{ATP} channels, we recorded whole-cell K_{ATP} currents during a 300 ms step to −60 mV from a holding potential of −70 mV (Figure 8C). Metabolic inhibition with cyanide (0.5 mM) induced K_{ATP} current (200.5 ± 25.4 pA in MI versus 51.2 ± 5.3 pA in control, $p < 0.01$, $n = 7$) that was blocked by glibenclamide (62.1 ± 6.3 pA, $n = 7$). PMA or adenosine treatment significantly decreased the endogenous K_{ATP} currents (127.7 ± 16.5 pA in PMA+MI versus MI group, $p < 0.01$, $n = 7$; 134.8 ± 13.2 pA in Ade+MI versus MI group, $p < 0.01$, $n = 7$). Downregulation by adenosine or PMA was blocked by the PKC inhibitor chelerythrine (213.5 ± 26.7 pA in PMA+Che+MI and 189.3 ± 20.1 pA in Ade+Che+MI group, $n = 7$). Thus, metabolic inhibition with cyanide causes hyperpolarization of CA1 neurons and depression of neuronal activity by activating K_{ATP} channels. Activation of endogenous PKC reduces neuronal K_{ATP} channel activity most likely by promoting K_{ATP} channel internalization in a dynamin-dependent manner, thereby rescuing metabolically stressed neurons from an imminent loss of excitability.

Discussion

PKC Regulates K_{ATP} Channel Trafficking

K_{ATP} channels mediate protection against ischemic injury in the heart and brain (Miki et al., 2002; Suzuki et al., 2002; Yamada et al., 2001; Hiraoka and Furukawa, 1998; Rejdak et al., 2001). Therapeutic agents such as sulphonylurea drugs also target these channels (Edwards and Weston, 1993; Fujita and Kurachi, 2000). Given that channel activity depends critically on the number of functional channels, traffic regulation could be an important, though still poorly understood, mechanism of K_{ATP} channel modulation. Our study reveals that PKC activation downregulates K_{ATP} channels in cardiac myocytes and central neurons, most likely by promoting channel internalization. Importantly, K_{ATP} channels endogenous to the cardiac myocytes and hippocampal neurons may be downregulated by adenosine and its downstream effector PKC. Our findings, therefore, identify a brake against excess channel activation by removing some of the K_{ATP} channels from the plasma membrane. This traffic regulation provides a negative feedback in controlling

K^+ efflux and APD shortening in cardiac myocytes, and in maintaining neuronal excitability in the brain. It may afford the heart cells with a means to delay or avoid ventricular arrhythmia, the major cause of mortality from coronary artery disease (Goldman and Cook, 1984). It may also prevent or reduce a rapid descent of central neurons into dormancy (Alzheimer and ten Bruggencate, 1988).

K_{ATP} channels may be modulated by many factors including ATP, ADP, protons, and G protein-coupled receptors such as adenosine receptors (Mubagwa et al., 1996; Shryock and Belardinelli, 1997). PKC can either inhibit or activate K_{ATP} channels in several noncardiac tissues, depending on the time course of experiment and signaling pathway involved (Bonev and Nelson, 1993; de Weille et al., 1989; Dunne, 1994; Honore and Lazdunski, 1993; Wang and Giebisch, 1991). In cardiac myocytes, several studies have consistently shown that brief (5 to 10 min) PKC activation stimulates K_{ATP} channel function, due to phosphorylation of T180 in Kir6.2 (Wang and Lipsius, 1995; Hu et al., 1996; Liu et al., 1996; Light et al., 1996, 2000; Hu et al., 1999). We have found that prolonged PKC activation (15 to 30 min) downregulates K_{ATP} channel function via channel internalization. Thus, PKC has dual effects on K_{ATP} channel modulation: activation of surface channels via channel phosphorylation and reduction of surface channel number by promoting channel internalization. It is generally accepted that PKC may serve as a central mediator in ischemic preconditioning, which causes prolonged elevation of PKC activity during subsequent ischemia (Nakano et al., 2000). Our findings suggest a potential role for the regulation of K_{ATP} channel trafficking as a mechanism for providing cytoprotection against ischemia, possibly by optimizing the number of active K_{ATP} channels on the cell surface. Although the data presented are consistent with a role for this mechanisms in vivo, further experiments will be required to demonstrate directly the contribution of this mechanism to a physiological context.

PKC-Induced K_{ATP} Channel Internalization Is Dynamin Dependent and Requires a Dileucine Motif in Kir6.2, but not Channel Activation by PKC Phosphorylation

We have shown that PKC-mediated K_{ATP} channel internalization is dynamin dependent but appears to be independent of Kir6.2 phosphorylation. Moreover, we have identified a dileucine internalization motif that is required for PKC-induced K_{ATP} internalization. Mutation of dileucine in Kir6.2, when coexpressed with SUR1/2A, significantly increased the cell surface channel number in the absence of PKC activation. It also prevented PKC from causing K_{ATP} channel endocytosis. Thus, the dileucine motif is involved in constitutive endocytosis as well as PKC-mediated internalization of K_{ATP} channels. Interestingly, PKC-induced channel internalization does not depend on phosphorylation in Kir6.2. This may allow PKC to remove a fraction of K_{ATP} channels from the cell membrane regardless of their state of activation by PKC, in a process that runs in parallel to but is temporally and mechanistically independent of K_{ATP} channel activation via PKC phosphorylation.

Dileucine signals may target membrane proteins from TGN to endosomes or the basolateral surface of polarized epithelial cells (Pond et al., 1995). Dileucine motifs may also target proteins from the plasma membrane to endosomes (Bremnes et al., 1994; Letourneur and Klausner, 1992; Sandoval et al., 1994). This motif is recognized by AP2 (Heilker et al., 1996). Phosphorylation of threonine 156 of the μ2 subunit of the AP2 complex is essential for endocytosis (Olusanya et al., 2001). The endogenous μ2 kinase is most likely the adaptor-associated kinase 1 (AAK1), although cyclin G-associated kinase (GAK) has also been implicated (Smythe, 2002). Whether PKC might act upstream of these kinases is not known. Interestingly, myocardial PKCϵ is associated with adaptin-β, which is at much elevated levels in transgenic mice with enhanced cardioprotection due to the expression of constitutively active PKCϵ (Ping et al., 2001).

Physiological Implications in the Brain and the Heart

The physiological relevance of our findings is suggested from the ability of adenosine and the downstream PKC to downregulate endogenous K_{ATP} in both cardiac myocytes and central neurons, thereby reducing cardiac APD shortening and sustaining neuronal activity. Adenosine accumulates during ischemia and locally mediates the protection due to K_{ATP} channel activation (Mubagwa and Flameng, 2001; Nieber et al., 1999; Reshef et al., 1998). Adenosine A1- and A3-receptors, identified as part of the mechanism for preconditioning, are coupled to PKC through the activation of G protein and phospholipase C (PLC) or D (PLD) (Schulz et al., 2001). PLC activation is typically very rapid in onset, and the resulting DAG production is short-lived, peaking at 30 s. In contrast, PLD activation is delayed but accounts for prolonged production of DAG and prolonged activation of PKC. It is therefore likely that PLD rather than PLC is involved in adenosine-mediated K_{ATP} channel internalization.

K_{ATP} activation by PKC has been associated with adenosine-mediated protection both in the heart and brain (Blondeau et al., 2000; Perez-Pinzon and Born, 1999; Schulz et al., 2001). Activation of K_{ATP} channels causes APD shortening in the heart (Nichols et al., 1991). APD shortening has been proposed to be one of the mechanisms for the protective effects of K_{ATP} channel opening during metabolic stress (Edwards and Weston, 1993; Hearse, 1995). The beneficial effect of K_{ATP} channel opening may also involve processes other than APD shortening (Grover et al., 1995), such as hyperpolarization (Suzuki et al., 2002). Notwithstanding the rapid action of adenosine and PKC in activating K_{ATP} channels, chronic elevation of PKC activity confers cardiac protection in transgenic mice (Dorn et al., 1999; Ping et al., 2001). Similarly, chronic treatment with adenosine uptake inhibitors causes sustained cardioprotection against ischemia/reperfusion injury (Mubagwa and Flameng, 2001). It would be of interest to examine in these cases the possible contribution of channel traffic regulation.

K_{ATP} channels are present in many brain regions (Mourre et al., 1991; Schwanstecher and Panten, 1993; Ashford et al., 1990; Guatteo et al., 1998). These channels may exert protection against neurodegeneration

or neuronal death during ischemia (Liss et al., 1999a, 1999b). Loss of K_{ATP} channel function in Kir6.2 knockout mice results in hypoxia-induced generalized seizure (Yamada et al., 2001). The protective effect of K_{ATP} channels has been associated with hyperpolarization and reduced excitability in neurons (Hansen et al., 1982; Alzheimer and ten Bruggencate, 1988; Ashcroft and Ashcroft, 1990; Perez-Pinzon et al., 1996; Reshef et al., 1998; Yamada et al., 2001). On the other hand, excessive K_{ATP} channel activation may contribute to depression of higher brain functions by hypoxia or cerebral ischemia, resulting in disorientation and loss of consciousness (Rossen et al., 1943). K_{ATP} channel activation causes hyperpolarization and complete cessation of spontaneous electrical activity in dopaminergic SN neurons (Roper and Ashcroft, 1995; Yamada et al., 2001). In brain slice preparations, hypoxia or metabolic inhibition causes hyperpolarization and reduced excitability in hippocampal neurons (Theodore et al., 1991; Englund et al., 2001), due to an increase in K_{ATP} conductance (Godfraind and Krnjevic, 1993; Hyllienmark and Brismar, 1996). Similar to our observations in ventricular myocytes, we found that adenosine likely activates PKC in hippocampal neurons to cause K_{ATP} channel internalization, so as to provide a brake against rapid loss of neuronal activity.

Our observations uncover an important adaptive mechanism allowing K_{ATP} channels to be downregulated by prolonged PKC activation via traffic regulation. This braking mechanism may play a critical role in regulating K_{ATP} channel function to overcome metabolic stress, though its implication in heart and brain physiology and pathology requires careful scrutiny given the complexity of cardiac and neuronal protection during ischemia. Given that Kir6.2 is the subunit common to K_{ATP} channels in the pancreas, heart, and brain, the regulation of K_{ATP} channel trafficking by PKC characterized in this study is likely to be of general relevance. Our studies provide evidence that, in parallel with K_{ATP} channel activation under metabolic stress, K_{ATP} channels in cardiac myocytes and central neurons are regulated at the level of trafficking to control the extent of channel activation.

Experimental Procedures

Molecular Biology

The HA epitope was introduced into mouse Kir6.2 cDNA by sequential overlap extension polymerase chain reaction (PCR) as described by Zerangue et al. (1999). The epitope was inserted at position 102 of Kir6.2. The 11 amino acids (^{98}GDLYAYMEKGIT^{99}) were inserted into Kir6.2 before the HA epitope. The Kir6.2Δ36, Kir6.2C4A (LRKR-AAAA, 368–371), and Kir6.2LL-AA (355–356) mutant constructs were generated by PCR. All PCR-derived sequences were entirely sequenced. Mammalian expression constructs were all in pcDNA3 (Invitrogen). For oocyte expression, constructs were either in pGEMHE (Liman et al., 1992) or in pGEMHEm (Margeta-Mitrovic et al., 2000). cRNA was transcribed using T7 RNA polymerase.

Cell Isolation and Cell Culture

COS7 cells were cultured in Dulbecco's modified Eagle's medium DMEM/F12 (Mediatech) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin-streptomycin (cell culture facility, UCSF). Cells were grown on coverslips for immunofluorescence and in 35 mm tissue culture dishes for chemiluminescence assay of surface protein. Cells were transfected with FuGENE6 (Roche) and stained 2–3 days later.

Primary culture of ~90% pure cardiac ventricular myocytes was prepared from the heart of 1-day-old rats according to the method of Simpson et al. (Simpson and Savion, 1982). The cell suspension was finally plated onto 3.5 cm collagen-coated coverslips at a density of 2×10^5 per 35 mm dishes. Adherent viable myocytes contracted spontaneously 24 hr after plating. Neonatal ventricular myocytes were transfected using FuGENE6 3 days after cell preparation. Cells were stained 2 days after transfection. Rhodamine-labeled phalloidin (1:200, Sigma), which binds to the myofibrils with a characteristic cross-striated pattern, was used to distinguish myocyte from nonmyocyte cells in the culture. Nonmyocyte cells were less than 10% of total cells.

Rat ventricular myocytes were isolated from adult SD rats (200 to 300 g, Charles River) by enzymatic dissociation, with methods similar to those previously described (Hu et al., 1996). Hippocampal slices (400 μm) were cut from postnatal 6- to 8-day-old rats with tissue chopper and cultured in medium as described (Shi et al., 1999).

Quantitative Chemiluminescence Detection of Proteins on the Surface of Oocytes and COS7 Cells

Chemiluminescence measurement of HA-tagged surface proteins has been previously described (Zerangue et al., 1999). Treatment with PMA or other interventions was done by incubation at 37°C prior to fixation. For *Xenopus* oocytes, cells were prepared and maintained as previously described (Collins et al., 1997). Two to three days after oocytes were injected with 1 ng cRNA for Kir6.2 constructs and 5 ng for SUR1/2A constructs, oocytes were blocked, labeled with primary antibody, and then washed and incubated with HRP-coupled secondary antibody. Individual oocytes were placed in 50 μl Power Signal Elisa (Pierce) and incubated at room temperature for 1 min before the measurement. For COS7 cells, cells plated in 35 mm tissue culture dishes were transfected with FuGENE and surface expression of all constructs was assayed 2–3 days after transfection as previously described (Margeta-Mitrovic et al., 2000). Cells were fixed, blocked, and then labeled with primary antibody (3F10) for 1 hr and with an appropriate HRP-coupled secondary antibody for 20 min. Chemiluminescence for cells in the whole 35 mm dish was quantified after 15 s incubation in Power Signal Elisa solution. Chemiluminescence for both oocytes and COS7 cells was quantified using a TD-20/20 luminometer (Turner Designs). For the detection of HA-tagged proteins, we used anti-HA rat monoclonal antibody (3F10, Roche, 0.2 μg/ml) and goat anti-rat HRP-conjugated F(ab')2 fragments (Jackson, 1:1000 dilution).

Immunofluorescence

COS7 cells were blocked with 5% goat serum in PBS (30 min at 4°C) labeled with primary antibody (2 hr at 4°C). Cells were then treated with PMA or other interventions for 30 min at 37°C, fixed with 4% formaldehyde in PBS (30 min), blocked in 5% goat serum in PBS with 0.1% Triton X-100 (30 min), and labeled with secondary antibody (1 hr); after cells were fixed, all steps were done at room temperature. We used anti-HA monoclonal rat primary antibody (3F10, Roche, 1 μg/ml) and Cy3- or Cy2-conjugated donkey anti-rat secondary antibody (Jackson, 1:500). Immunofluorescence was visualized with a Nikon inverted microscope.

Electrophysiology

For *Xenopus* oocytes, recordings were made 3–5 days after cRNA injection using standard two-electrode voltage clamp technique. K_{ATP} currents were recorded in the solution containing (in mM) 90 KCl, 10 Na HEPES, 1.8 $CaCl_2$, 1.0 $MgCl_2$ (pH 7.4) and determined by subtracting control current (−60 mV) from currents recorded after 10 min application of 3 mM sodium azide. Oocytes were treated with PMA or other reagents prior to recordings. All recordings in oocytes were made using a ramp protocol between −120 and 40 mV every 5 s for time course measurement.

For cardiac ventricular myocytes, all cells for electrophysiological measurements were rod-shaped and showed clear cross striations. The bath solution contains (mM) 135 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 0.33 NaH_2PO_4, 10 HEPES, and 10 glucose at pH 7.4 (pH adjusted with NaOH). The pipette solution contained (mM) 120 K glutamate, 25 KCl, 1.0 $MgCl_2$, 10 HEPES, 5 EGTA, 0.1 GTP, and 1

K_2ATP at pH 7.3 (pH adjusted with KOH) except that 0.4 mM K_2ATP was added to the pipette solution for the studies on the effect of acute PKC activation. Whole-cell K_{ATP} currents were recorded using voltage-clamp technique. $CdCl_2$ (0.2 mM) and 4-aminopyridine (2 mM) were added to the bath solution to inhibit Ca^{2+} current and transient outward current, respectively. In current-clamp mode, action potentials were elicited by 2 ms twice-threshold pulses and were recorded every 5 s. APD_{90} were compared in various groups. All recordings were conducted at room temperature (21°C to 25°C).

For hippocampal CA1 neurons, slices were transferred to the recording chamber perfused with physiological solution (21°C–25°C) containing (mM) 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose at pH 7.4 and gassed with 5% CO_2 and 95% O_2. Patch recording pipettes were filled with internal solutions containing (mM) 140 K gluconate, 5 HEPES, 2 $MgCl_2$, 1.1 EGTA, 1 Na_2ATP, 3 Na_3GTP, at pH 7.25. Cells were held in either voltage-clamp mode for recording whole-cell K_{ATP} currents or current-clamp for action potentials. A depolarizing current pulse (400 ms, 200 pA) was applied to elicit action potentials in the current-clamp mode.

Chemicals and Statistical Analysis

Group data are expressed as mean ± SE. Unpaired t test or ANOVA with a range test (the least significant difference test) compare the data between groups. Differences with a two-tailed value of $p < 0.05$ were considered statistically significant.

Collagenase (Type II) was purchased from Worthington. Other chemicals were purchased from Sigma. PMA and 4α-PDD were stored in ethanol at −20°C. The K_{ATP} channel blocker glibenclamide were stored in DMSO. Other chemicals were stored in distilled H_2O. Dominant-negative dynamin peptide D10 (QVPSRPNRAP) and scrambled peptides S10 (PRAPNSRQPV) were dissolved at 100 μM in the internal solution (pipette solution).

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