Homeostatic Regulation of Kv1.2 Potassium Channel Trafficking by Cyclic AMP*

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The Shaker family potassium channel, Kv1.2, is a key determinant of membrane excitability in neurons and cardiovascular tissue. Kv1.2 is subject to multiple forms of regulation and therefore integrates cellular signals involved in the homeostasis of excitability. The cyclic AMP/protein kinase A (PKA) pathway enhances Kv1.2 ionic current; however, the mechanisms for this are not fully known. Here we show that cAMP maintains Kv1.2 homeostasis through opposing effects on channel trafficking. We found that Kv1.2 is regulated by two distinct cAMP pathways, one PKA-dependent and the other PKA-independent. PKA inhibitors elevate Kv1.2 surface levels, suggesting that basal levels of cAMP control steady-state turnover of the channel. Elevation of cAMP above basal levels also increases the amount of Kv1.2 at the cell surface. This effect is not blocked by PKA inhibitors, but is blocked by inhibition of Kv1.2 endocytosis. We conclude that Kv1.2 levels at the cell surface are kept in dynamic balance by opposing effects of cAMP.

A key modulator of Kv1.2 function is the cyclic AMP/protein kinase A (PKA)2 signaling pathway. Stimulation of β2-adrenergic receptors causes a potent and reversible enhancement of Kv1.2 ionic current. Part of the mechanism for this increase is alteration of the channel’s biophysical properties by PKA (9); activation of PKA increases the time that single channels spend in higher conductance states. Mutational analysis revealed that an N-terminal threonine (Thr–46) within a PKA consensus sequence is required for the response to PKA, suggesting direct phosphorylation of the channel protein at this site (9). In addition to positive regulation by PKA, Kv1.2 can be negatively regulated by tyrosine phosphorylation (11). Activation of muscarinic acetylcholine receptors, for example, elicits tyrosine phosphorylation of Kv1.2 and the resultant suppression of its ionic current (11). Tyrosine phosphorylation of Kv1.2 also elicits endocytosis of the channel, and this endocytosis is a mechanism for current suppression (10). This process is dependent upon the proteins dynamin and cortactin, both of which are well known for their roles in endocytosis (12–14). Therefore, tyrosine kinase-dependent trafficking is an important mechanism for regulation of Kv1.2 activity. It is not known whether trafficking plays an equally important role in Kv1.2 regulation by cAMP/PKA.

Trafficking of a variety of membrane proteins is modulated by the cAMP/PKA pathway. Elevation of cAMP inhibits the rapid endocytosis of the cystic fibrosis transmembrane conductance regulator, thus increasing the amount of channel at the cell surface (15). The mechanisms by which cAMP affects the cystic fibrosis transmembrane conductance regulator are varied and may involve both PKA-dependent and PKA-independent mechanisms (16–18). Phosphorylation of aquaporin-2 by PKA causes channel insertion into the plasma membrane (19); however, this can also occur via a PKA-independent mechanism (20, 21). Furthermore, increasing CAMP enhances trafficking of the amiloride-sensitive sodium channel (ENaC) to the plasma membrane, and defects in this trafficking are associated with Liddle syndrome (22). Therefore, the cAMP/PKA pathway is an important regulator of plasma membrane protein trafficking. Furthermore, the mechanisms involved are varied; in some cases, cAMP modulates trafficking via PKA, but in other cases, cAMP works independently of PKA.

Here, we show that the cAMP/PKA pathway influences Kv1.2 trafficking. Our experiments reveal distinct PKA-

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2 The abbreviations used are: PKA, protein kinase A; BFA, brefeldin A; GFP, green fluorescent protein; WT, wild type; FA, formic acid; HPLC, high pressure liquid chromatography; HEK, human embryonic kidney; MS/MS, tandem mass spectrometry; PDE, phosphodiesterase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; siRNA, small interfering RNA.
dependent and PKA-independent effects on Kv1.2 surface levels. Our findings suggest this occurs through CAMP-dependent modulation of channel endocytosis. Low CAMP levels maintain basal PKA activity, which enhances constitutive Kv1.2 endocytosis, and high CAMP levels inhibit constitutive endocytosis of the channel independently of PKA. We conclude that homeostasis of Kv1.2 expression in the plasma membrane is dependent on a critical balance of CAMP levels within the cell.

**Experimental Procedures**

**Materials**—Antibody directed against the first extracellular loop of Kv1.2 (anti-Kv1.2e) was developed with assistance from BIOSOURCE International (Camarillo, CA). Anti-phosphorylated RX(S/T) antibody was purchased from Cell Signaling (Danvers, MA), and anti-Kv1.2 and anti-Kvβ2 monoclonal antibodies from the University of California (Davis, CA). A rabbit anti-Kv1.2 antibody raised against a peptide region within the carboxyl terminus of Kv1.2 (anti-Kv1.2p) was developed with assistance from Cocalico (Reamstown, PA). Alexa Fluor-conjugated secondary antibodies and phalloidin (Invitrogen) were used for immunofluorescence. Non-targeting siRNA (D-0012106) and cortactin siRNA (M-010508) were purchased from Dharmacon (Lafayette, CO). Isoproterenol, propranolol, and isobutylmethylxanthine were purchased from Sigma; myristoylated PKA inhibitor peptide was from BIOMOL International (Plymouth Meeting, PA); and all other reagents were from EMD Biosciences (San Diego, CA) unless noted otherwise.

**Tissue Culture**—Two different stable cell lines were used: clonal human embryonic kidney 293 cells stably expressing M1 muscarinic acetylcholine receptors (HEK-M) and the HEK-M clonal cell line also stably expressing Kv1.2α and Kvβ2 (HEK-K). Cells were cultured as reported previously (10). Confluent cultures were plated to a low density (25,000 cells/cm²) onto poly-d-lysine-coated tissue culture plates (Corning Glass Works, Corning, NY) and subsequently placed in serum-free medium overnight. Following stimulus treatment where appropriate, cells were metabolically poisoned with 0.5% sodium azide for 30 min at 37 °C to block endocytosis and then processed for flow cytometry or Western blot analysis as described previously (10).

**Transient Transfection of HEK Cells**—Dynamin was expressed using pEGFP-N1. Dynamin K44A was generously provided by Dr. Mark A. McNiven. GFP was expressed using pEGFP-N1 (Clontech). Kv1.2α and Kvβ2 were expressed using the pRK5 mammalian expression vector. Mutagenesis primers were purchased from Operon Biotechnologies (Huntsville, AL), and mutagenesis was performed with the Stratagene QuikChange site-directed mutagenesis kit (La Jolla, CA). HEK-M or HEK-K cells were transiently transfected with calcium phosphate. HEK-M or HEK-K cells were transiently transfected with calcium phosphate.

**Detection of Kv1.2 Surface Levels by Flow Cytometry**—Surface Kv1.2 was labeled with a rabbit polyclonal antibody (anti-Kv1.2e) directed against an extracellular epitope within the channel. Secondary labeling was done with a fluorescein-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL). Kv1.2 surface levels were quantified as the number of cells emitting at 667 nm with fluorescence intensity above a threshold value determined using control IgG-labeled cells. For experiments requiring transient transfection, GFP was used as a marker, and emission at 667 nm was taken only from cells with green fluorescence. Flow cytometry was done with the EasyCyte single laser flow cytometer (Guava Technologies, Hayward, CA). Analysis of cell populations and histograms was done with FCS Express and WinMDI softwares, respectively.

**Alkaline Phosphatase Treatment**—Cell lysates were centrifuged at 20,000 × g for 4 min at 4 °C, and the supernatant was ultracentrifuged at 95,000 × g for 10 min at 4 °C. The supernatant was incubated with calf intestinal alkaline phosphatase (20 units) for 60 min at 37 °C in a buffer designed to support alkaline phosphatase activity (100 mM NaCl, 10 mM Tris, 10 mM MgCl₂, pH 8.2) or a buffer designed to inhibit alkaline phosphatase activity (100 mM NaCl, 10 mM Tris, 10 mM EDTA, pH 4.3).

**Immunoprecipitation and Immunoblot**—Cells were washed with ice-cold phosphate-buffered saline and lysed in radioimmuno precipitation assay buffer (50 mM Tris, 150 mM NaCl, 11 mM EDTA, 0.25% deoxycholate, 1% Nonidet P-40, 10% glycerol, 1 mM NaF, 1 mM NaVO₃, 1 mM NaN₃, BAPTA, 1 mM dithiothreitol, protease inhibitors (Sigma, catalog no. P8340), phosphatase inhibitors (Calbiochem, catalog nos. 524624 and 524625), pH 8.0). The lysate was centrifuged at 20,000 × g for 4 min, and the resulting supernatant was ultracentrifuged at 95,000 × g for 10 min. Kv1.2 was immunoprecipitated from the ultracentrifugate supernatant using anti-Kv1.2p antibody. Bound proteins were eluted from the beads and then separated by SDS-PAGE. Western blotting detection of Kv1.2 was done with anti-Kv1.2 monoclonal antibody. Blots were imaged and quantified with the Odyssey infrared imaging system (Li-Cor, Lincoln NE).

**Electrophysiology**—HEK-K cells were grown on poly-D-lysine-coated 35-mm tissue culture dishes as described above, but were not treated with sodium azide. Kv1.2 ionic current was measured using a whole-cell voltage clamp. The pipette solution contained 120 mM KCl, 3.69 mM CaCl₂ (60 nM free Ca²⁺), 94 mM MgCl₂, (100 nM free Mg²⁺), 5 mM BAPTA, 5 mM NaHEPES, and 5 mM glucose, pH 7.1; the bath solution contained 100 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 23 mM glucose, and 5 mM NaHEPES, pH 7.4. The experimental chamber was maintained at 35 °C. Maximum series resistance error was calculated to be <4% and was not compensated. Currents were evoked with a family of 75-ms voltage steps in 10-mV increments from −70 to +50 mV. Recordings were made immediately after break-in to limit spontaneous current rundown. Data collection was done with an Axopatch 200D amplifier interfaced via a Digi-Data 1322A D/A converter to a Windows-based computer running the pCLAMP data acquisition program Clampex (Molecular Devices, Sunnyvale, CA). Data analysis was performed with the pCLAMP data analysis program Clampfit (Molecular Devices) and with Origin (Microcal, Northampton, MA).

**Immunofluorescence**—Immunofluorescence imaging was performed as described previously (10). Images were acquired with the DeltaVision deconvolution restoration microscopy system (Applied Precision, Issaquah, WA).
Mass Spectrometry—A Coomassie-stained band corresponding to Kv1.2 was excised, diced, and subjected to in-gel digestion with sequencing grade modified trypsin (6 ng/μl; Promega, Madison, WI) in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides were extracted with 50% acetonitrile (MeCN), 2.5% formic acid (FA) and then dried. Peptides were then resuspended in 2.5% MeCN, 2.5% FA and loaded using a Micro-AS autosampler (Thermo Electron, Waltham, MA) onto a 100-μm inner diameter fused silica microcapillary column prepared in-house and packed with 12 cm of reverse phase MagicC18 material (5 μm, 200 Å; Michrom Bioresources, Inc., Auburn, CA). Using a Surveyor MS HPLC Pump Plus (Thermo Electron), peptides were eluted using a 5–35% MeCN (0.15% FA) gradient over 50 min, after a 14-min isocratic loading at 2.5% MeCN, 0.15% FA. The HPLC solvents used to achieve separation were 2.5% MeCN, 0.15% FA (solvent A) and 97.5% MeCN, 0.15% FA (solvent B). Mass spectra were acquired in an LTQ-XL linear ion trap mass spectrometer (Thermo Electron) over the entire run using 10 MS/MS scans following each survey scan. Instrument control was performed using the Xcalibur software package (version 2.0, SR2, Thermo Electron). Raw data were searched against a Kv1.2 sequence data base using Sequest software (version 27, revision 12, Thermo Electron) with no enzyme specificity and a 2-Da mass tolerance. Cysteine residues were required to have a static increase in 71 Da for acrylamide addition. Differential modifications of 80 Da for serine, threonine, and tyrosine residues, as well as 16 Da for methionine residues, were permitted, and phospho-specific neutral loss increases to XCorr scoring were turned off. After initially identifying each phospho-tryptic peptide corresponding to serines 440 and 449, targeted MS/MS analyses on the corresponding m/z values were performed, confirming the initial results.

Statistical Analysis—Descriptive statistics are provided in figures as bar graphs indicating the sample mean, with error bars indicating S.E. Detection of statistical difference between two independent measurements was by a one-way t test. Comparison of percent changes between pairs of independent measurements was by a two-way analysis of variance. Sample populations were considered to be significant at p ≤ 0.05.

RESULTS
cAMP Enhances Surface Expression of Kv1.2—Stimulation of β2-adrenergic receptors with isoproterenol in Xenopus oocytes augments the ionic current of Kv1.2 (9). We hypothesized that trafficking exists as a possible mechanism for this process. Flow cytometry was used to analyze changes in Kv1.2 surface levels. The basal levels of the channel were measured with cells treated with an antibody directed against an external loop of Kv1.2 (anti-Kv1.2e), as compared with a control group of cells treated with IgG. A representative histogram shift depicting increased fluorescence between the IgG control and the cells labeled with anti-Kv1.2e antibody is shown in Fig. 1A. The inset shows the population of intact cells selected to generate the histogram. Endogenous β2-adrenergic receptors activated with the norepinephrine analog isoproterenol (10 μM, 10 min) caused a significant increase in channel density at the cell surface (n = 12, p ≤ 0.001) (Fig. 1B). This response was blocked by pretreatment with propranolol (10 μM, 30 min; p = 0.1), a β-adrenergic receptor antagonist. Thus, not only does β2-adrenergic stimulation cause an increase in Kv1.2 ionic current as reported previously, it also causes a significant increase in the quantity of channels present at the surface of the cell.

Isoproterenol stimulates a signaling cascade that includes the activation of adenylyl cyclase and cAMP, and therefore, we wanted to confirm that this pathway was involved. Increasing concentrations of forskolin, an activator of adenylate cyclase, Kv1.2 surface levels are shown as a function of forskolin concentration. Data points at each concentration were taken 10 min after the application of forskolin. B, Kv1.2 surface levels are shown as a function of time after the application of 10 μM forskolin. C, mean Kv1.2 surface levels are shown after treatment with 10 μM forskolin (FSK) for 10 min or 250 μM CPT-cAMP for 30 min, revealing that such treatments significantly increase surface Kv1.2 relative to the control (CTR). D and E, the PDE inhibitors isobutylmethylxanthine (IBMX; 100 μM) and rolipram (ROL; 10 μM) significantly increased the surface levels of Kv1.2. **, p < 0.01 versus control.
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Application of the cell-permeable cAMP analog chlorophenylthio-cAMP (250 μM, 30 min) displayed a similar response to forskolin (10 μM, 10 min), suggesting that cAMP is the main downstream effector (n = 12, p < 0.0001) (Fig. 2C). To confirm this idea, phosphodiesterase (PDE) inhibitors were used to inhibit the degradation of cAMP, thus increasing the intracellular concentration of the second messenger. The general PDE inhibitor, isobutylmethylxanthine (100 μM, 30 min), caused a significant increase in Kv1.2 surface levels (n = 18, p < 0.0001) (Fig. 2D). Because isobutylmethylxanthine can inhibit both cAMP- and cGMP-specific PDEs, we decided to test a cAMP-specific PDE inhibitor. Rolipram (10 μM, 3 min), which inhibits PDE4, also increased Kv1.2 surface levels (n = 6, p < 0.0001). Therefore, elevating cAMP increases Kv1.2 expression on the cell surface.

CAMP Affects Kv1.2 Surface Levels through PKA-independent and PKA-dependent Mechanisms—Because increasing cAMP levels enhanced the amount of Kv1.2 on the cell surface, we hypothesized that these effects were mediated by the main cAMP downstream effector, PKA. To investigate this, we treated cells with a panel of PKA inhibitors, including myristoylated PKA inhibitor peptide (10 μM, 10 min), KT5720 (5 μM, 30 min), and H89 (10 μM, 30 min). These inhibitors did not prevent either forskolin or chlorophenylthio-cAMP from increasing Kv1.2 surface levels (n = 12, p ≤ 0.01) (Fig. 3, A–C). This suggests that cAMP can enhance surface levels independent of PKA. Interestingly, PKA does appear to have a role because application of the PKA inhibitors alone caused a significant increase in Kv1.2 surface levels (p ≤ 0.01). Therefore, we sought to confirm the existence of basal PKA activity in unstimulated cells. To that end, we inhibited adenylate cyclase with SQ22536 (500 μM, 30 min), which resulted in a similar increase in Kv1.2 surface levels (n = 25, p ≤ 0.0001). Furthermore, we evaluated the level of substrates phosphorylated by PKA in cell lysates with an antibody directed against a phosphorylated PKA consensus sequence (RXX(S/T)). Forskolin cause a significant increase in the amount of phosphorylated protein (n = 2, p ≤ 0.01). Conversely, both the adenylate cyclase inhibitor, SQ22536, and the PKA inhibitor, H89, decreased phosphorylation (n = 3, p ≤ 0.05) (Fig. 3E). This suggests that the mechanism for cAMP regulation of Kv1.2 surface levels can occur in both PKA-dependent and PKA-independent manners.

CAMP and PKA Modulate Kv1.2 Function—We next asked whether the increased surface expression of Kv1.2 caused by forskolin or inhibitors of PKA was correlated with an increase in Kv1.2 ionic current. Forskolin induced a significant increase in Kv1.2 ionic current relative to the control; 6.4 ± 1.0 nA versus 3.3 ± 1.2 nA (n = 9, p < 0.01) (Fig. 4A). KT5720 also caused a similar increase in Kv1.2 ionic current relative to the control; 12.6 ± 0.9 nA versus 5.8 ± 3.5 nA (n = 5, p < 0.01) (Fig. 4B). Half-activation voltage was determined from tail current activation curves fitted by a Boltzmann function. The half-activation voltage was 10.3 ± 1.4 mV in control cells and −11.7 ± 3.3 mV in forskolin-treated cells. These values are not significantly different (p = 0.28). Therefore, the increase in ionic current caused by forskolin is not attributable to a shift in voltage dependence of Kv1.2 activation. Interestingly, KT5720 did produce a significant depolarizing shift in Kv1.2 voltage dependence (−6.6 ± 0.7 mV, p < 0.01). However, because it is a positive shift, it cannot underlie the KT5720-induced increase in Kv1.2 steady-state ionic current. These findings indicate that the additional Kv1.2 channels in the plasma membrane upon treatment with forskolin or with PKA inhibitors are functional and thus capable of affecting membrane excitability.

**FIGURE 3. Effects of cAMP and PKA on Kv1.2.** Treatment with the PKA inhibitors 1 μM myristoylated PKA inhibitor peptide (mPKI; A), 10 μM H89 (B), or 5 μM KT5720 (KT; C) caused a significant elevation of Kv1.2 surface levels. These inhibitors did not block the increase of surface Kv1.2 caused by the addition of 250 μM chlorophenylthio-cAMP (A) or 10 μM forskolin (FSK; B and C). D, the adenylate cyclase inhibitor SQ22536 (SQ; 500 μM) also significantly increased Kv1.2 surface levels. E, an immunoblot of control (CTR) cell lysates or lysates from cells treated with forskolin, SQ22536, or H89 was probed with an antibody directed against a phosphorylated PKA consensus sequence (left). Equal loading was confirmed by probing with anti-GAPDH antibody. Phosphorylated RXX(S/T) substrate band densities were normalized to GAPDH, averaged over at least two experiments, and are depicted as bar graphs (right). **, p < 0.01, and *, p ≤ 0.05 relative to control; /, p < 0.05 relative to inhibitor alone.
Serines 440 and 449 Are Phosphorylated in Forskolin-treated Cells, but Are Not Responsible for the Forskolin-mediated Increase in Surface Kv1.2—In the course of the preceding experiments, we observed that forskolin produced a shift in the electrophoretic mobility of Kv1.2. Loss of the higher molecular mass band after treatment of cell lysates with alkaline phosphatase indicates that this shift is caused by channel phosphorylation \((n = 2)\) (Fig. 5A). Because forskolin caused a PKA-independent increase in Kv1.2 surface levels, we hypothesized that this shift was caused by a PKA-independent phosphorylation of Kv1.2. Moreover, the PKA inhibitor, H89 \((10 \mu M, 30 \text{ min})\) (Fig. 5B; \(n = 2\)), had no effect on the forskolin-induced band shift. Interestingly, this suggests that PKA is not the kinase involved and indicates that forskolin signals through a different kinase/phosphatase pathway to elicit phosphorylation of Kv1.2.

We next used mass spectrometry to identify the phosphorylation sites responsible for the shift in Kv1.2 mobility. Kv1.2 was immunoprecipitated from forskolin-stimulated HEK-K cells and then resolved by SDS-PAGE. Coomassie-stained bands of Kv1.2 were excised, diced, and digested in-gel with trypsin. Extracted peptides were subjected to liquid chromatography MS/MS and data base search analysis identified two unique phospho-tryptic peptides harboring phosphoserine 440 and phosphoserine 449 (Fig. 6A). To determine whether phosphorylation at these sites causes the mobility shift seen in Fig. 5A, we introduced the serine-to-alanine mutations S440A, S449A, and S440/449A. The forskolin-induced doublet was not observed in any of these mutant channels, confirming that these sites are critical for forskolin-induced phosphorylation of Kv1.2 (Fig. 6B).

Given that Ser-440 and Ser-449 are vital to the forskolin-induced band shift, we evaluated their roles in channel surface expression. To our surprise, these point mutations had no effect on the forskolin-mediated increase in Kv1.2 surface levels \((n = 18, p = 0.01)\) (Fig. 6C). The inset to Fig. 6C depicts the percent change induced by forskolin, normalized to saline control. Therefore, the forskolin-induced shift in electrophoretic mobility and the increase in surface channel levels are not causally related. Interestingly, the serine-to-alanine mutations caused a significant decrease in the unstimulated Kv1.2 surface levels \((p = 0.01)\).

Thr-46 Is Necessary for the cAMP-mediated Effects on Kv1.2 Surface Levels—Previous work has shown that mutation of the Kv1.2 N-terminal threonine 46 to valine \((T46V)\) produces channels that are resistant to PKA-mediated current increases \((9)\). This suggests that phosphorylation of this site by PKA underlies the biophysical changes associated with current enhancement. Our findings indicate that the cAMP-mediated increase in surface Kv1.2 is PKA-independent. We therefore sought to clarify the role of Thr-46 in Kv1.2 regulation. Introduction of the T46V mutation significantly decreased Kv1.2 surface levels \((n = 31, p < 0.0001)\) (Fig. 7A). Furthermore, T46V blocked the forskolin-mediated increase \((p = 0.36)\); thus, channels harboring the T46V mutation were refractory to cAMP-dependent effects on
To investigate the role of phosphorylation at Thr-46, we performed mass spectrometry on WT Kv1.2 derived from forskolin-treated cells. Whereas we cannot definitively conclude that Thr-46 is not phosphorylated, we did not find any peptide where Thr-46 was phosphorylated, even when we performed targeted MS/MS analysis on masses consistent with Thr-46 phospho-tryptic peptides in multiple charge states. Conversely, we did detect tryptic peptides that included Thr-46 in its unphosphorylated state. This suggests that channel modulation does not involve phosphorylation of Kv1.2 at Thr-46.

To determine whether decreased surface expression caused by the T46V mutation results from a change in Kv1.2 protein expression or stability, we evaluated protein levels by immunoblotting. The levels of T46V protein were not lower than those of WT protein (Fig. 7B), suggesting that the T46V mutation alters surface channel levels by a mechanism that does not involve changes in channel protein levels. We also note that T46V did not alter the forskolin-induced mobility shift, consistent with the idea that the band shift is not involved in stimulus-induced changes in surface levels of Kv1.2.

**Forskolin-mediated Effects on Kv1.2 Are Independent of Kvβ2**—The auxiliary Kvβ2 subunit binds to Kv1.2 and can serve as a chaperone protein, guiding newly synthesized channels to the cell surface (23). This interaction requires an intact T1 domain within Kv1.2, and mutation of Thr-46, which lies within the T1 domain of Kv1.2, disrupts Kvβ2 binding (24). Our finding that the T46V mutation also blocks forskolin effects on surface
cAMP Modulates Kv1.2 Trafficking at the Plasma Membrane—Elevation of Kv1.2 surface levels can be achieved by increased outward trafficking or by decreased inward trafficking of channels. Possibly, cAMP exerts its effects by regulating trafficking of newly synthesized channels from the Golgi apparatus to the plasma membrane. To test this idea, brefeldin A (BFA) was used to compromise the integrity of the Golgi apparatus (25). Although BFA treatment (5 μM, 1 h) disrupted the Golgi apparatus (Fig. 9A), it had no effect on the ability of forskolin to increase surface levels of Kv1.2 (n = 18, p < 0.0001) (Fig. 9B). This indicates that the cAMP-mediated increase in Kv1.2 surface levels does not involve changes in Golgi trafficking of the channel.

Alternatively, cAMP may decrease inward trafficking of Kv1.2 channels from the cell surface. Endocytosis is an important mechanism for the regulation of Kv1.2 (10). We hypothesized that cAMP inhibits the steady-state endocytotic trafficking of Kv1.2, thereby increasing surface channel levels. Dynamin affects endocytotic trafficking in multiple ways (13) and has a key role in Kv1.2 endocytosis (10). We therefore targeted this protein to block Kv1.2 inward trafficking. Overexpression of a dominant negative form of dynamin, dynamin K44A, completely blocked the forskolin-mediated increase in Kv1.2 surface levels (n = 12, p = 0.19) (Fig. 9C). To further explore this idea, we targeted cortactin, another protein with a central role in Kv1.2 trafficking (26). We have shown previously that depletion of cortactin with siRNA blocks Kv1.2 inward trafficking (12). Cortactin knockdown completely inhibited the ability of forskolin to modulate surface levels of Kv1.2 (n = 8, p = 0.08) (Fig. 9, D and E). Thus, both cortactin and dynamin are essential for cAMP-mediated effects on Kv1.2 surface levels. We therefore conclude that cAMP exerts its effects by modulating the inward trafficking of Kv1.2.

**DISCUSSION**

Altered cAMP levels can result in changes in neuronal plasticity (27), hormonal secretion (28), and gene expression (29); divergent feedback loops ensure a regulated balance of cAMP (30). In this study, we have shown dual mechanisms for cAMP modulation of Kv1.2 trafficking. First, we demonstrated that elevation of cAMP caused a significant increase in Kv1.2 surface levels and that this effect is independent of PKA. Next, we showed that decreasing cAMP also caused a significant increase in Kv1.2 surface levels, but that this effect is PKA-dependent. Thus, basal cAMP levels form a homeostatic set point for Kv1.2 surface expression. We propose that through its effects on Kv1.2 function, cAMP homeostasis may act as a buffer for cellular excitability (Fig. 10).

Previous studies in Xenopus oocytes show that application of PKA alters the biophysical properties of Kv1.2 to increase the ionic current (9). Here we showed in HEK293 cells that inhibiting PKA increases Kv1.2 at the cell surface (Fig. 3) and that this increase corresponds to an increase in ionic current (Fig. 4). Thus, it appears that in Xenopus oocytes PKA increases Kv1.2 activity, whereas in HEK293 cells PKA decreases its activity. Therefore, depending on the cellular environment, PKA can affect Kv1.2 function by directly impacting the channel’s biophysical properties or by affecting channel trafficking. Indeed,
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Although PKA does have a role in Kv1.2 trafficking, the effect of forskolin on Kv1.2 is independent of PKA. Despite this, we found that mutation of a potential PKA phosphorylation site (T46V) completely eliminates the effect of forskolin on Kv1.2 surface levels. This result may be explained by a phosphorylation-independent role for Thr-46. Minor et al. (31) have shown that Thr-46 forms a hydrogen bond involved in maintaining T1 domain structure and that mutation of Thr-46 disrupts the T1 domain. This prevents interaction of the isolated T1 domain of Kv1.2 with the Kvβ2 subunit (24). These findings added a new facet to previous research that shows a chaperone-like role for Kvβ2 in promoting cell surface expression (23) and axonal targeting of Kv1.2 (32). We confirmed that the entire α subunit harboring the T46V mutation cannot bind Kvβ2 (Fig. 8). This is consistent with our finding that Kv1.2 T46V had significantly reduced surface expression relative to the wild-type channel. However, the mechanism by which T46V blocks the forskolin-induced trafficking of the channel (Fig. 7) does not involve the loss of Kvβ2 binding (Fig. 8). Therefore, correct positioning of the T1 domain appears to be required for multiple but distinct types of channel trafficking, including Kvβ2-mediated and, as we have reported here, Kvβ2-independent and cAMP-dependent trafficking of Kv1.2.

We found no evidence for forskolin-induced phosphorylation of Thr-46; however, forskolin does induce phosphorylation at other sites. Biochemical and mass spectrometry studies suggest that forskolin induces a PKA-independent phosphorylation of Kv1.2 at Ser-440 and Ser-449 (Fig. 6). We found that these sites are critical for Kv1.2 surface expression, but surprisingly they do not appear to be involved in the forskolin-induced increase in surface channel. Nevertheless, phosphorylation at one or both of these sites may be necessary for the channel to reach the cell surface or to prevent channel degradation. In fact, Ser-440 seems to be critical for protein stability, as the S440A mutation visibly decreases protein levels (Fig. 6B). Furthermore, we observed a notable change in the pattern of higher molecular mass Kv1.2 bands in the S449A mutant channel. This may represent altered channel glycosylation, indicating disruption of normal channel maturation. Therefore, these sites are important for channel surface expression, but do not appear to play a direct role in the acute response to forskolin.

Further evidence that altered biosynthetic trafficking is not involved comes from our finding that disruption of the Golgi these mechanisms are not necessarily mutually exclusive and may act in concert with PKA-independent effects of cAMP to precisely modulate Kv1.2 function.
apparatus with brefeldin A has no effect on the cAMP-dependent, PKA-independent increase in Kv1.2 surface levels. Instead, this effect appears to involve modulation of constitutive channel endocytosis. Previous reports have shown that Kv1.2 surface expression is modulated by endocytosis of the channel protein. This process is dependent on both dynamin (10) and cortactin (12). We showed that disruption of either dynamin or cortactin blocks the forskolin-induced increase in surface levels (Fig. 9). We conclude that forskolin modulates Kv1.2 surface levels by modulating the constitutive endocytosis of the channel. Therefore, both positive and negative regulation of Kv1.2 surface expression are governed by modulated channel endocytosis. In this way, Kv1.2 serves to link membrane excitability with a complex array of trafficking signals.

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