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PKA-dependent Regulation of mKv1.1, a Mouse *Shaker*-like Potassium Channel Gene, When Stably Expressed in CHO Cells

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Potassium (K) channels are important regulators of cellular physiology and can themselves be modulated by phosphorylation. We have investigated the potential protein kinase A (PKA) regulation of mKv1.1, a mouse *Shaker*-like K channel gene, when it is expressed in stably transfected Chinese hamster ovary (CHO) cell lines. Whole-cell patch-clamp records show that expression of mKv1.1 gives rise to a rapidly activating, sustained K^+ current, referred to classically as a delayed rectifier–type current. In order to study the effects of PKA, we compared cell lines transfected with mKv1.1 alone with lines cotransfected with both mKv1.1 and a plasmid encoding a dominant negative mutation in the regulatory subunit of PKA. These mutant regulatory subunits bind to endogenous catalytic subunits of PKA but do not respond to cAMP, thereby causing a chronic reduction in the basal PKA activity in these cells. We found that mKv1.1 current kinetics are unaltered but current density is 3.4-fold higher in the cell lines expressing mutant regulatory subunit than in lines expressing only mKv1.1. RNase protection assays indicate that levels of the specific RNA for mKv1.1 are increased almost twofold in the lines expressing mutant regulatory subunit over the lines expressing mKv1.1 only. Further, the levels of mKv1.1 protein, assayed using an mKv1.1 channel–specific antibody, are increased by almost a factor of 3 between the two types of cell lines. These results suggest that PKA can regulate mKv1.1 channel expression by changing steady-state levels of RNA and by other posttranscriptional mechanisms.

[Key words: potassium channel, delayed rectifier, phosphorylation, protein kinase A, channel regulation, gene expression]

Voltage-gated potassium channels are a diverse family of structurally related proteins, each with unique kinetics, pharmacology, and tissue distribution. Molecular cloning and expression of a number of K channel genes have provided new insight into channel structure and the functions of various domains of the channel (Miller, 1991). Relatively little is known, however, about the mechanisms regulating K channel gene expression or those regulating assembly, distribution, and stability of the channel proteins. These processes are likely to be important in determining the contribution of each channel type to the various K^+ currents measured in cells where each gene is expressed.

The first mammalian K channel genes were isolated from mouse brain (Tempel et al., 1988) and from rat brain (Baumann et al., 1988) based on the similarity of these genes to the *Drosophila Shaker* K channel genes (Jan and Jan, 1989; Salkoff et al., 1992). Recently, these genes have been designated Kv1.1 to indicate their ionic selectivity, voltage sensitivity, subfamily membership, and order of isolation (Chandy et al., 1991). Functional expression of the rat brain clone in *Xenopus* oocytes and in stably transfected mammalian Sol8 cells demonstrated that rKv1.1 (previously RCK1) currents were rapidly activating, with inactivation over tens of seconds (Stuehmer et al., 1988; Christie et al., 1989; Koren et al., 1990). Similar kinetics are observed when mKv1.1 (previously referred to as MBK1) is expressed in *Xenopus* oocytes (Hopkins and Tempel, 1992). The Kv1.1 gene sequences from all mammalian species studied to date predict a consensus PKA phosphorylation site in the carboxyl end of the channel subunit. Similar sites are observed in other related K channels (Chandy et al., 1990; Drewe et al., 1992).

A large body of work has demonstrated that kinase activity, in particular protein kinase A (PKA) activity, is important in gene regulation, development, learning, and at times when the nervous system undergoes plastic remodeling (Montminy et al., 1989; Mayford et al., 1992). However, fewer studies have reported on the regulation of expression of ion channels. Modulation of Ca channels by hormone action in cardiac tissue has been shown to upregulate current by direct PKA-mediated phosphorylation of the channel (Trautwein and Hescheler, 1990). In stably transfected CHO cells expressing Na channel cDNA, acute stimulation of PKA activity suppresses Na^+ current. Removal of basal PKA activity in those cells upregulates channel expression by a posttranscriptional mechanism (Li et al., 1992). In contrast, during NGF-mediated PC12 cell differentiation, the endogenous Na^+ current is upregulated by cAMP stimulation via a pathway that requires a functional PKA catalytic subunit (Kalman et al., 1990). Reduction of basal PKA activity in this system blocks functional Na channel expression although Na channel transcript is upregulated (Ginty et al., 1992).

Physiological evidence suggests that voltage-gated K channels are also modulated by hormones in many tissues. Indeed, PKA modulation of delayed rectifier–type K^+ currents has been shown clearly in heart and in lymphocytes (Giles et al., 1989; Soliven and Nelson, 1990). In cardiac tissue, however, the molecular species of K channel(s) modulated by PKA is not known since several different K channel genes with potential PKA phos-

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phorylation sites are expressed in cardiac tissue (Roberds and Tamkun, 1991). In addition, a unique, small K channel gene that is expressed in heart and involved in the establishment of a slowly activating K^+ current, I_{sK}, has been shown to be modulated by PKA-induced insertion of channels into the membrane of the *Xenopus* oocyte expression system (Blumenthal and Kaczmariek, 1992).

Here we address the question of PKA regulation of the expression of a cloned K channel cDNA, mKv1.1, by using a molecular tool to block endogenous PKA activity. We have stably transfected mKv1.1 into CHO cells, and also into CHO cells that contain a plasmid encoding a dominant mutant regulatory subunit of PKA (Clegg et al., 1987). This PKA regulatory subunit has been mutated in both the A and B cAMP binding sites (AB^-), and therefore remains constitutively bound to the catalytic subunit of PKA, inactivating the endogenous PKA. We have used this model to study the effects of PKA on expression of mKv1.1 RNA, protein, and current. Our results suggest that reducing basal PKA activity leads to an increase in mKv1.1 K^+ current via an increase in RNA as well as other posttranscriptional mechanisms.

Materials and Methods

Plasmids. For establishment of the stable cell lines, a fragment of the mKv1.1 potassium channel cDNA clone MK9.6 (Tempel et al., 1988) was inserted into the BamHI restriction endonuclease site of pZEM 228 (kindly provided by E. Mulvihill, Zymogenetics Inc., Seattle, WA). The subcloned region of mKv1.1 extends from the SmaI site 40 base pairs (bp) upstream of the AUG start codon through the HindIII site downstream from the TAA stop codon. Thus, in this pZEM–mKv1.1 construct, the coding region of mKv1.1 is placed downstream of a mouse metallothionine promoter with high constitutive activity and upstream of SV40 polyadenylation and termination sequences. In order to generate riboprobes that protect the 5′ end of the mKv1.1 sequence, a 252 bp fragment from the SmaI site to the SphI site, encompassing the AUG start codon, was cloned into pBluescript SK^+ (Strategene, La Jolla, CA). For riboprobes that protect the 3′ end of the mKv1.1 sequence, a 197 bp fragment from BclI to HindIII, including the TAA stop codon, was cloned into pBluescript SK^+ (see Fig. 5*B*).

Cell culture and transfection. Wild-type CHO cells were maintained using standard tissue culture techniques in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 50 U of penicillin/ml, and 50 μg of streptomycin/ml. CHO/AB^- cells containing the mutant regulatory subunit of PKA (kindly provided by the laboratory of Dr. William Catterall, University of Washington) were maintained with the same medium supplemented with 200 μg/ml hygromycin. Both lines of CHO cells were transfected with 15 μg of pZEM–mKv1.1 plasmid DNA by electroporation using a Bio-Rad Gene Pulser Transfection Apparatus (Richmond, CA). Two days after the transfection procedure, G418 (Geneticin, GIBCO–Bethesda Research Labs, Gaithersburg, MD) was added to 500 μg/ml. Established lines were thereafter maintained with G418 at 250 μg/ml. Two days prior to harvest, cells were seeded simultaneously for electrophysiology, RNA preparation, and protein analysis. On the day that the electrophysiology was done, parallel cultures were harvested for RNA and protein analyses.

RNA analysis. In order to isolate total RNA, cells were removed from two T-25 flasks with versene and pelleted in a 50 ml conical centrifuge tube. The cell pellet was rapidly vortexed in 1 ml of 4 M guanidinium thiocyanate and processed under acidic conditions to yield total RNA free from DNA (Chomczynski and Sacchi, 1987). RNA concentrations were determined by A260 optical density measurements done in duplicate. RNase protection assays were performed on 2.5 or 10 μg of total RNA according to methods in Ausubel et al. (1991). Riboprobes were synthesized in the presence of ^{32}P-UTP to a specific activity of 4×10^7 cpm/μg cRNA; 2.5×10^5 cpm was added to each sample for hybridization. The hybridization mix was incubated at 50°C overnight, digested with RNase A and RNase T1, phenol extracted, concentrated, and separated on a denaturing 6% acrylamide sequencing gel. The amount of RNA protected in each sample was quantified by PhosphorImager analysis using the Molecular Dynamics scanner at the Fred Hutchinson Cancer Research Center, Image Analysis Laboratory, Seattle, WA.

Protein analysis. For both total protein (Western) blot analysis and immunoprecipitation experiments, cells were incubated in equal volumes of culture medium for 20 hr, one without radioactivity for Westerns and one containing 0.1 mCi/ml ^{35}S-methionine for immunoprecipitation. Cells were then rinsed in culture medium several times, removed by versene treatment, and pelleted in a 50 ml conical. The pellet was resuspended in 1 ml of Tris-saline (TBS; 20 mM Tris-HCl, 100 mM NaCl, pH 7.5) and disrupted in a glass–glass homogenizer. The membrane fraction was separated from the soluble fraction by centrifuging at $15,000 \times g$ for 10 min, and the pellet was resuspended in 1 ml of 1% Triton-X in TBS on ice for 1 hr. After duplicate resuspensions and centrifugations at $15,000 \times g$ for 30 min (4°C), the supernatant was saved as the membrane protein fraction. Total protein concentration was determined in duplicate using the DC Protein Assay kit supplied by Bio-Rad Laboratories (Hercules, CA).

For Western blots, 8–10 μg of protein was separated by SDS-PAGE and electrotransferred to either PVDF or nitrocellulose membranes using Protean mini-gel apparati (Bio-Rad). The protein blots were incubated first with polyclonal antibodies developed against a C-terminal peptide of mKv1.1 (α-Kv1.1; residues 427–495; Wang et al., 1993). The antibody had been adsorption purified twice against an mKv1.2–glutathionine-S-transferase fusion protein–coupled sepharose column. (The mKv1.2 clone is a closely related *Shaker*-like channel with high sequence homology; this step was used to make the antibody more specific.) The secondary antibody for chemiluminescence was alkaline phosphatase–conjugated goat anti-rabbit IgG, and for color reactions was horseradish peroxidase–conjugated goat anti-rabbit IgG. The substrate for the chemiluminescent reaction was Lumi-Phos 530 (Boehringer Mannheim Corporation, Indianapolis, IN), and for the color reaction was 3,3′-diaminobenzidine. The Western blot images were densitized and analyzed at the Fred Hutchinson Cancer Research Center, Image Analysis Laboratory.

For immunoprecipitation experiments, either 20 μg protein/cell line or 7×10^6 cpm/cell line was precleared by incubation with 3 μl of preimmune serum for 1 hr at 4°C followed by addition of 30 μl of Protein A–sepharose (2 mg Protein A/ml sepharose) in dilution buffer (0.1% bovine hemoglobin and 0.1% Triton-X in TBS), incubation for 30 min at 4°C, and then centrifugation at $5000 \times g$ for 5 min. Protein was immunoprecipitated by replacing preimmune serum with 3 μl of adsorption-purified α-Kv1.1 antibody in the same protocol, with the exception that 10 μl of Protein A–sepharose was used. Precipitated protein was washed with dilution buffer three times, twice with TBS, and once with 0.5 M Tris, pH 6.8; 25 μl of 5× sample buffer was added, and the samples were boiled for 10 min and separated using 10% SDS-PAGE. The gel was soaked in fluorography enhancing solution (Entensify, Amersham Corporation, Arlington Heights, IL) and dried on 3 mm filter paper. Blots were placed in a phosphorimager cassette for 5 d, and the image was collected and analyzed at the Fred Hutchinson Cancer Research Center.

Electrophysiology. CHO cells were grown in adherent culture at low density (5–10% confluency) on 35 mm plastic tissue culture dishes. For recording, a Sylgard (Dow Chemical, Midland, MI) chamber was constructed inside the dish, such that the total bath volume was 0.2 ml. Perfusion of the bath was achieved by continuous gravity flow, and changes in the perfusion solution were made by switching a four-way valve. The approximate dead volume between solutions was 0.3 ml, and the bath flow was approximately 0.5 ml/min. Pipette solution was (mM) K-aspartate, 100; KCl, 20; $MgCl_2$, 3; K_2EGTA, 10; K_2HEPES, 25; ATP, 0.3; GTP, 0.1; pH 7.4 with KOH. Standard bath Ringer solution was (mM) NaCl, 140; KCl, 2.5; $CaCl_2$, 3; $MgCl_2$, 1; Na_2HEPES, 10; pH 7.4 with NaOH. For 0 Na experiments the NaCl was replaced by equimolar *N*-methyl-D-glucamine (NMDG), and HCl was used for titration. For experiments with increased K, NaCl or NMDG was replaced by equimolar KCl. All experiments were corrected for junction potentials, which were −18 mV for the NMDG solution and −10 mV for the Ringer solution. For most experiments with standard bath Ringer solution, 1 μM tetrodotoxin was added to the solution to block the endogenous Na^+ current in the CHO cells. Most reagent salts and pharmacological blockers were obtained from Sigma. Fraction I of dendrotoxin (DTX-I) was purified by HPLC as in Newitt et al. (1991).

The whole-cell variation of the patch-clamp technique was used to record mKv1.1 K^+ currents (Hamill et al., 1981). Recording pipettes were constructed using a two-pull procedure from VWR micropipette

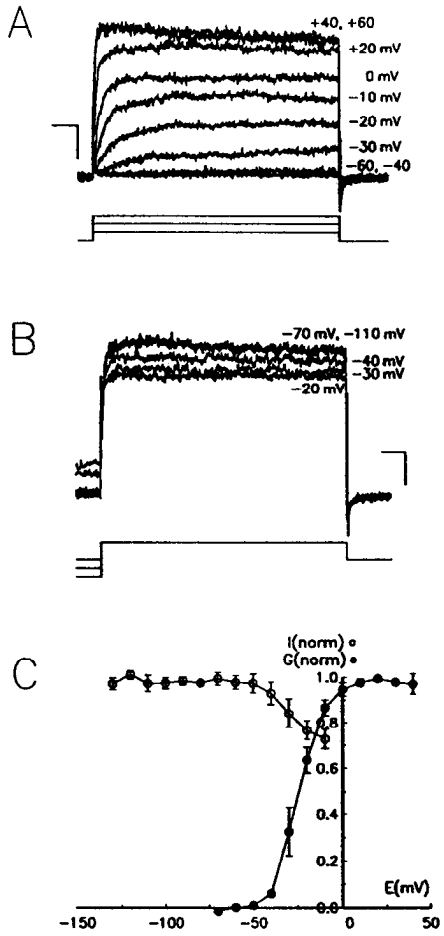

Figure 1. Activation and inactivation of mKv1.1 currents expressed in CHO/MK1 cells. *A*, Currents recorded from a holding potential of −90 mV, during potential steps to −60 through +60 mV, as indicated next to the traces. *B*, Steady-state inactivation of currents from same cell. The holding potential was changed from −110 mV to −20 mV for 7.5 sec before a step to +30 mV to elicit the currents shown here. Calibration: 50 msec, 50 pA (for *A* and *B*). *C*, Resultant conductance–voltage curves from data in *A* (*solid circles*) and steady-state inactivation curve from data in *B* (*open circles*).

glass (75 μl). Dishes were mounted on the stage of a Nikon Diaphot microscope, and pipette manipulation was achieved using a Newport (Fountain Valley, CA) XYZ translator (with one motorized dimension) mounted on a holder for the Orbital Stage (Meridian Instruments, Kent, WA). Currents and voltages were recorded using an Axopatch 200 (Axon Instruments, Burlingame, CA), and currents were sampled on line and voltages delivered using an Atari STE computer, ITC16 interface board, and M2Lab software (Instrutech, Elmont, NY). Current traces were not leak subtracted.

Results

Electrophysiological and pharmacological characteristics of mKv1.1

Outward potassium currents in CHO/MK1 cells were recorded using a paradigm of stepping from a potential of −90 mV to between −60 and +60 mV for 500 msec. Current began to activate between −40 and −30 mV, and reached a maximum at +40 mV (Fig. 1*A*). The activation time constant decreased with depolarization and ranged from 36 msec at −20 mV to 6 msec at +20 mV (mean of four cells). The mKv1.1 current inactivated little, although at the most positive potentials there was 5–8% inactivation at the end of the 500 msec pulse. The

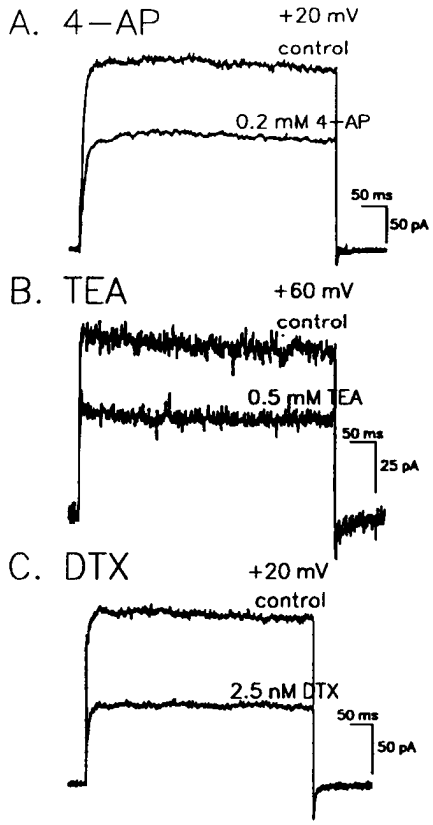

Figure 2. Pharmacology of block of mKv1.1 currents recorded from different cells. *A*, Block by 0.2 mM 4-AP during a step to +20 mV from a holding potential of −70 mV. *B*, Block by 0.5 mM TEA during a step to +60 mV from a holding potential of −90 mV. *C*, Block by 2.5 nM DTX during a step to +20 mV from a holding potential of −70 mV.

steady-state conductance was derived by dividing the average current over the last 30 msec of the pulse by the driving force ($E - E_K$), and plotted in a conductance–voltage curve (Fig. 1*C*; $n = 5$). The curve can be fit by a Boltzmann function with a half-maximal voltage of −24.9 (±2.6) mV; the conductance changes *e*-fold in 6.3 (±0.5) mV.

The steady-state inactivation curve was constructed by varying the holding potential of the cell for 7.5 sec and stepping from that potential to +30 mV to activate the remaining current (Fig. 1*B*). A plot of this data (Fig. 1*C*; $n = 5$) shows that negative to −50 mV, no steady-state inactivation is seen. The maximal inactivation measured was 26% of the total current, from a holding potential of −10 mV, where considerable current is activated. This is consistent with the minimal degree of inactivation seen during 500 msec test pulses with mKv1.1 (Fig. 1*A*).

We have studied the sensitivity of mKv1.1 expressed in CHO cells to several K channel blockers (Fig. 2). The block by 4-aminopyridine (4-AP) was half-maximal at 0.35 mM ($n = 15$). At higher concentrations of 4-AP (2–10 mM) an increase in leak was seen, and the effects of the 4-AP were difficult to reverse. Block by tetraethylammonium (TEA) was half-maximal at 0.5 mM (fit of $n = 7$ cells). DTX-I, a snake toxin, has been used as a biochemical probe to identify K channels from a variety of tissues as well as to purify K channels from mammalian brain (Rehm et al., 1989; Newitt et al., 1991). DTX-I was tested for its blocking effects on mKv1.1 channels and was shown to block

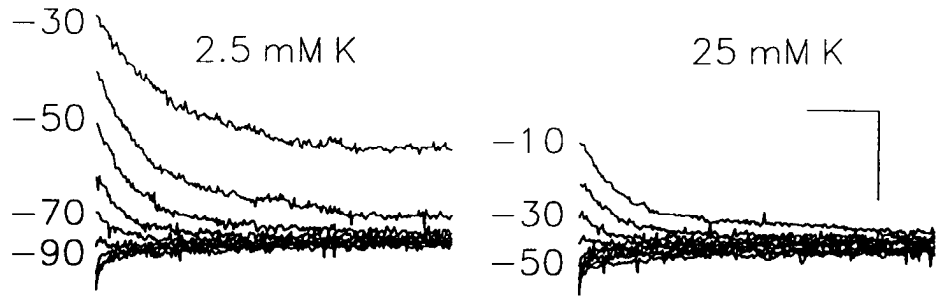

Figure 3. The reversal potential of the tail currents changes with changes in external K^+. At the *left* are tail currents recorded during a potential step to the voltages indicated after a step to +30 mV to elicit current. External solution contained 2.5 mM K^+; current reverses from inward to outward between −80 and −90 mV. At the *right* are tail currents from the same cell, after external solution was changed to one containing 25 mM K^+. Tail current reverses from outward to inward between −30 and −40 mV. Calibration: 10 pA, 10 msec.

reversibly, with a half-maximal value of about 2.5 nM (fit of n = 10 cells). At higher concentrations (25–50 nM), washing for approximately 30 min did not reverse the block by DTX-I.

The ionic selectivity of mKv1.1 was tested. Figure 3 shows tail current traces obtained after a pulse to +30 mV to elicit current, returning to potentials ranging from −110 to −20 mV. The left panel shows the tail currents in 2.5 mM external K, under which conditions the current reverses at −85 mV. In the same cell, the external K was raised to 25 mM, causing the currents to reverse at −35 mV. Three cells were tested in a similar manner, and the mean change was 49 mV, which is close to a shift of 57 mV that would be expected for an ideal K^+ ion-selective conductance.

Comparison of CHO/MK1 and CHO/AB⁻/MK1 cells

The CHO/AB⁻ cell line was transfected with a plasmid encoding a mutant regulatory subunit of PKA that does not bind cAMP, and remains bound to the catalytic subunit (Clegg et al., 1987). The cAMP-stimulated PKA activity in this CHO/AB⁻ cell line was less than 10% of that observed in parental CHO cells without the mutant subunit (Li et al., 1992). A similar reduction of endogenous PKA activity has been observed in other cell lines expressing the same mutant regulatory subunit construct (Ginty et al., 1991; Schecterson and McKnight, 1991).

Cells containing both the mutant regulatory subunit and mKv1.1 plasmids (CHO/AB⁻/MK1) expressed greater mKv1.1 K^+ current density than cells without the mutant regulatory subunit (CHO/MK1). However, the electrophysiological characteristics of the current were the same between the two groups of cells. Figure 4*A* shows the conductance–voltage curves for the two types of cells, with the CHO/MK1 values shown as circles and the CHO/AB⁻/MK1 values as triangles. The two curves were not significantly different; similar or greater shifts were observed within the same experimental group of cell lines as between different groups.

Figure 4*B* shows the amount of current density present in each of three CHO/MK1 or CHO/AB⁻/MK1 cell lines, and for cell lines containing pZEM plasmid alone (B2, B4). The CHO/AB⁻/MK1 cells express on average 3.4 times the current density (in pA/pF) that is expressed in CHO/MK1 cell lines. All three CHO/AB⁻/MK1 current densities (C1, C9, C10) were significantly greater than the three CHO/MK1 cells (E2, E3, E4; $p < 0.001$). Recordings from control cells [CHO (B2) or CHO/AB⁻ (B4) cells transfected with control plasmid] showed no outward currents. It is interesting to note that all CHO/AB⁻/MK1 cell lines had a smaller cell size, averaging 11.5 ± 3.0 pF (n = 34) as compared to 17.0 ± 4.6 pF (n = 35) in the CHO/MK1 lines. Furthermore, all cell lines did express a TTX-sensitive sodium current, which was not changed in density between any of the cell lines (data not shown). An additional CHO/AB⁻/MK1 cell line was recorded under the same conditions and found to express 30.1 pA/pF, increasing the average current density to 26 pA/pF, a 3.6-fold increase over the CHO/MK1 lines. This cell line (C4) was not included in other experiments.

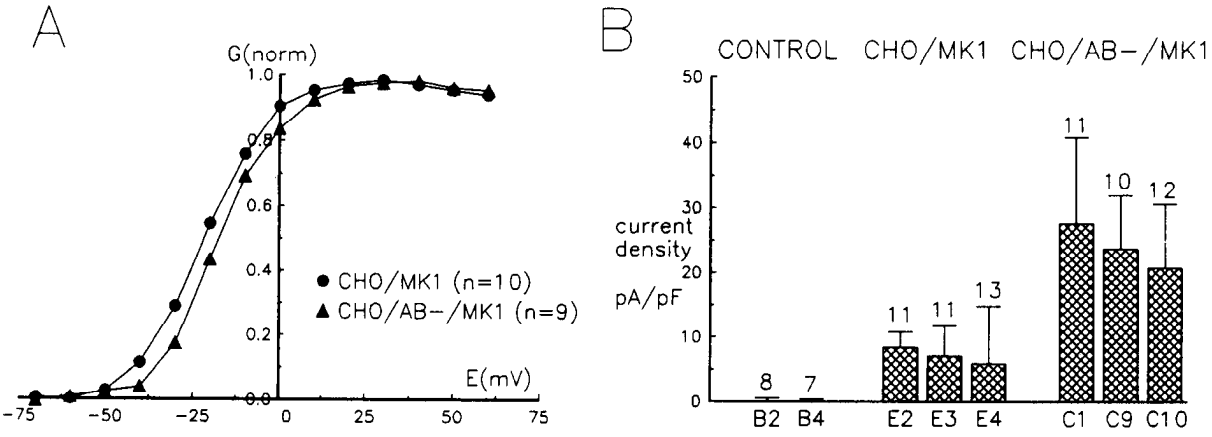

Figure 4. Comparison of currents in CHO/MK1 cells and CHO/AB⁻/MK1 cells. *A*, Conductance–voltage curves for CHO/MK1 cells (*circles*; n = 10) and CHO/AB⁻/MK1 cells (*triangles*; n = 9). *B*, Histogram of current densities (pA/pF) from three CHO/MK1 cell lines (E2, E3, E4) and three CHO/AB⁻/MK1 cell lines (C1, C9, C10). Error bars indicate SD, and *numbers above error bars* are number of cells from each cell line. The average current density for the CHO/MK1 lines was 7.1 pA/pF, and for the CHO/AB⁻/MK1 lines was 24.0 pA/pF.

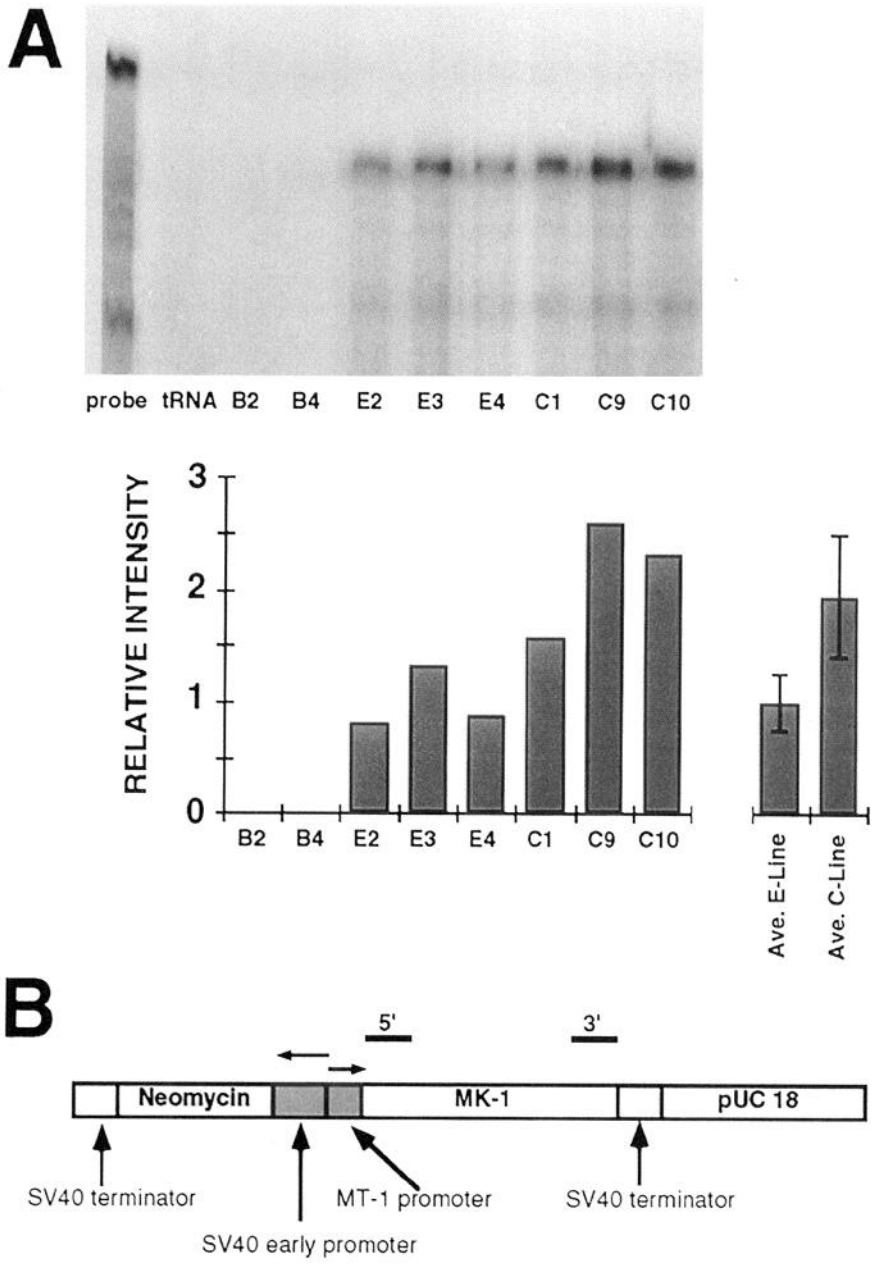

Figure 5. RNase protection assays. *A,* Digitized phosphorimager scan of the gel of an RNase protection assay run with the 5′ riboprobe is shown in the *upper panel.* The *lower panel* is a histogram of the scan. *B2* is a control cell line in which the pZEM vector without the mKv1.1 insert was transfected into wild-type CHO cell lines; *B4* is the CHO/AB⁻ cell line with the same vector construct. *E2, E3,* and *E4* are CHO/MK1 cell lines. *C1, C9,* and *C10* are CHO/AB⁻/MK1 cell lines. The undigested probe is shown (*far left lane*), as well as a tRNA control. The image is quantified as shown on the histogram below. The two values on the *far right* indicate the average normalized intensities of the signal for the five RNase protections assays that were quantified. Averaged CHO/MK1 (E lines) was 1.00 ± 0.22 (n = 14 lanes) and averaged CHO/AB⁻/MK1 (C lines) was 1.94 ± 0.57 (n = 13 lanes). *B,* Diagram of the pZEM–mKv1.1 plasmid construct used for transfection. The positions of the 5′ and 3′ riboprobes are indicated by *bars above. MT-1* indicates the mouse metallothionine promoter.

RNase protection assays

Seven sets of RNase protection assays were performed. Results from an experiment using the 5′ probe (see Fig. 5*B* for plasmid structure) are shown in Figure 5. An assay using the 3′ probe gave similar results (data not shown). Figure 5*A* shows that no mKv1.1-specific protected fragment was detected using the 5′ probe to examine the control cell lines B2 (CHO with pZEM alone) or B4 (CHO/AB⁻ with pZEM alone). Additional control cell lines transfected with pZEM containing mKv1.1 in the antisense orientation showed no mKv1.1 mRNA expression (data not shown). In contrast, an mKv1.1-specific protected fragment was detected using either the 5′ or the 3′ probe to examine all six cell lines expressing mKv1.1 (Fig. 5*A*). The three CHO/MK1 lines (E2, E3, and E4) have a lower level of specific mRNA expression, as determined by relative intensity on a phosphorimager scan, than the corresponding CHO/AB⁻/MK1 cell lines C1, C9, and C10. Five of the seven RNase protection assays were quantitated by phosphorimaging analysis. In three of these assays we also quantified the levels of 18S RNA, expressing mKv1.1 RNA relative to 18S RNA levels. The average increase in mKv1.1-specific RNA in the CHO/AB⁻/MK1 lines relative to the CHO/MK1 lines was 1.94 ± 0.57 ($p < 0.001$). These results indicate that, at steady state, there is an increased level of mKv1.1 RNA in the cells containing the mutant regulatory subunit. This does not appear to be a general feature of RNA expression in these cells, as the RNA levels for 18S are not significantly different between the two cell types (average 18S density of C lines relative to E lines was 1.045, n = 2). We do not know if this increase in steady-state mKv1.1 transcript levels is the result of changes in transcription rates or stability of the RNA (see Discussion).

mKv1.1 protein levels

The 3.4-fold increase in mKv1.1 current levels in the CHO/AB⁻/MK1 cells (C lines) over the CHO/MK1 cells (E lines) cannot be accounted for by taking only the twofold increase in mKv1.1 RNA transcript into consideration. In order to explore further the mechanisms involved in upregulation of mKv1.1 current in cells expressing the mutant regulatory subunit of PKA, protein levels of mKv1.1 were assessed using a polyclonal antisera specific to mKv1.1 (α-Kv1.1; Wang et al., 1993). Figure 6*A* shows an immunoblot (Western) in which total membrane protein from the indicated cell lines was probed with α-Kv1.1. A single major band of 55–65 kDa is detected first in the CHO/MK1 (E2, E3) and more strongly in the CHO/AB⁻/MK1 cell lines (C1, C9). The size of the recognized protein is somewhat larger than the predicted molecular weight of the unglycosylated mKv1.1 protein (56.4 kDa; Tempel et al., 1988), suggesting that some glycosylation has occurred in the CHO cell lines. The band was not detected by preimmune sera or when α-Kv1.1 sera were preincubated with mKv1.1 peptide (data not shown), indicating that α-Kv1.1 specifically detects the expressed mKv1.1 protein. Staining intensity of the 60 kDa region in each lane was measured by chemiluminescent detection and densitometric scanning; the results are graphed below the gel. Six such Western blots were analyzed. On average, the CHO/AB⁻/MK1 cell lines (C lines) have a 2.8-fold increase in protein compared to the CHO/MK1 cell lines (E lines; $p < 0.001$). The control cell lines, which are transfected with the pZEM plasmid only, showed no detectable mKv1.1 protein expression, consistent with their lack of mKv1.1 current.

Immunoprecipitation experiments on metabolically labeled cells confirmed the finding that mKv1.1 protein levels were increased in CHO/AB⁻/MK1 cell lines (Fig. 6*B*). Control and MK1-expressing cell lines were metabolically labeled with ^{35}S-methionine for 20 hr, harvested, and immunoprecipitated with α-Kv1.1. Precipitated proteins were separated by SDS-PAGE and analyzed by phosphorimaging. Consistent with the Western immunoblot data, the presumed mKv1.1 band appeared in all lines transfected with mKv1.1, was increased an average of 3.0-fold in the CHO/AB⁻/MK1 lines ($p < 0.001$, $n = 6$), but was not detectable in cell lines transfected with control plasmid only. Quantitatively comparable data was obtained when the metabolically labeled samples were independently analyzed by Western blot, indicating that steady-state levels of mKv1.1 protein were reached during the labeling period (data not shown). Thus, chronic inhibition of PKA activity leads to a significant increase in mKv1.1 protein assayed either by immunoblot or by immunoprecipitation.

Discussion

Using whole-cell recording techniques, we have observed that expression of the mKv1.1 K channel gene in transfected CHO cells gives rise to a rapidly activating, sustained K^+ current that activates at −40 mV and is sensitive to block by 4-AP, TEA, and DTX. These physiological and pharmacological characteristics are similar to those observed for rKv1.1, the rat homolog of mKv1.1, when it is expressed transiently in *Xenopus* oocytes (Stuehmer et al., 1988). We also found that, in CHO cells, coexpression of mKv1.1 with a plasmid encoding a dominant negative mutation of the regulatory subunit of PKA (AB⁻) causes an increase in mKv1.1 K^+ current density. Significant increases are seen in both mKv1.1 RNA levels and protein levels. These results suggest that the increased K^+ current seen in the CHO/AB⁻/MK1 cell lines relative to the CHO/MK1 cell lines occurs by mechanisms that are posttranscriptional and may include regulation of RNA levels. These findings are distinct from the results of comparable studies on PKA regulation of Na channel expression (Ginty et al., 1992; Li et al., 1992).

Several mechanisms might give rise to an increased current in the PKA-deficient cell lines. It is unlikely that PKA is affecting mKv1.1 gene transcription in our system. While PKA has been shown to regulate transcription of various genes through the transcription factor CREB (Montminy et al., 1989; Habener, 1990), in our transformed CHO cell system, mKv1.1 expression is driven by the PKA-insensitive metallothionine-1 promoter in the ZEM plasmid. The mKv1.1 gene endogenous to the CHO cell genome is apparently also insensitive to changes in PKA activity, as there was no induction of mKv1.1 current in CHO cells where PKA activity was decreased in cells containing the plasmid control vector without the mKv1.1 insert (CHO/AB⁻ line B4). Thus, the upregulation of mKv1.1 RNA is more likely to occur by modification of PKA-sensitive gene products that may be involved in the processing, modification, or stabilization of functional mKv1.1 RNA. Several examples of second messenger modulation of mRNA have been reported. In a rat hepatoma cell line, dibutyryl-cAMP increases the half-life of mRNA for phosphoenolpyruvate carboxykinase by 10-fold, via a mechanism that does not require protein synthesis and that enhances the stability of the polysome-free mRNA (Hua and Hod, 1992). In a cell line expressing corticotropin-releasing hormone, stimulation of protein kinase C by phorbol esters increases mRNA levels for this hormone, and also increases the length of the polyA tail of the mRNA by threefold (Adler et al., 1992), thus leading to a possible increase in mRNA stability due to an increased ability to bind to the polyA binding protein and protection from exonucleolytic digestion (Bernstein and Ross, 1989; Bernstein et al., 1989). Although we do not know the specific mechanism by which mKv1.1 RNA is increased in our system, it is unlikely that this increase alone (1.9-fold) could entirely account for the increase seen in current densities (3.4-fold) or protein levels (2.8-fold).

It remains possible that some portion of the increase in mKv1.1 current density is determined by PKA during translation, assembly, insertion, or function of the mKv1.1 channels. Phosphorylation of Na channels in excised patches using cAMP and PKA catalytic subunit causes a decrease in the Na^+ current (Li et al., 1992). In addition, using a strategy similar to the one used in the present study, coexpression of the mutant regulatory subunit of PKA in CHO cells causes upregulation of Na^+ current, suggesting that the endogenous level of PKA activity in CHO cells is sufficient to modulate the level of Na channel expression. Unlike the situation with mKv1.1 K channels, Na channel mRNA levels are unchanged while Na channel–specific saxitoxin (STX) binding sites on the cell surface increase when PKA activity is reduced. These data suggest that inhibition of PKA causes Na channel number and current to increase via an unknown posttranscriptional mechanism (Li et al., 1992). In contrast, hormonally induced PKA activity in differentiating PC12 cells causes an increase in Na^+ current (Kalman et al., 1990). Recent studies (Ginty et al., 1992) indicate that this upregulation requires PKA activity at the posttranslational level: PC12 cells transfected with a mutant regulatory subunit of PKA respond to hormone by relatively normal morphological differentiation, by increasing Na channel mRNA, and by increasing the number

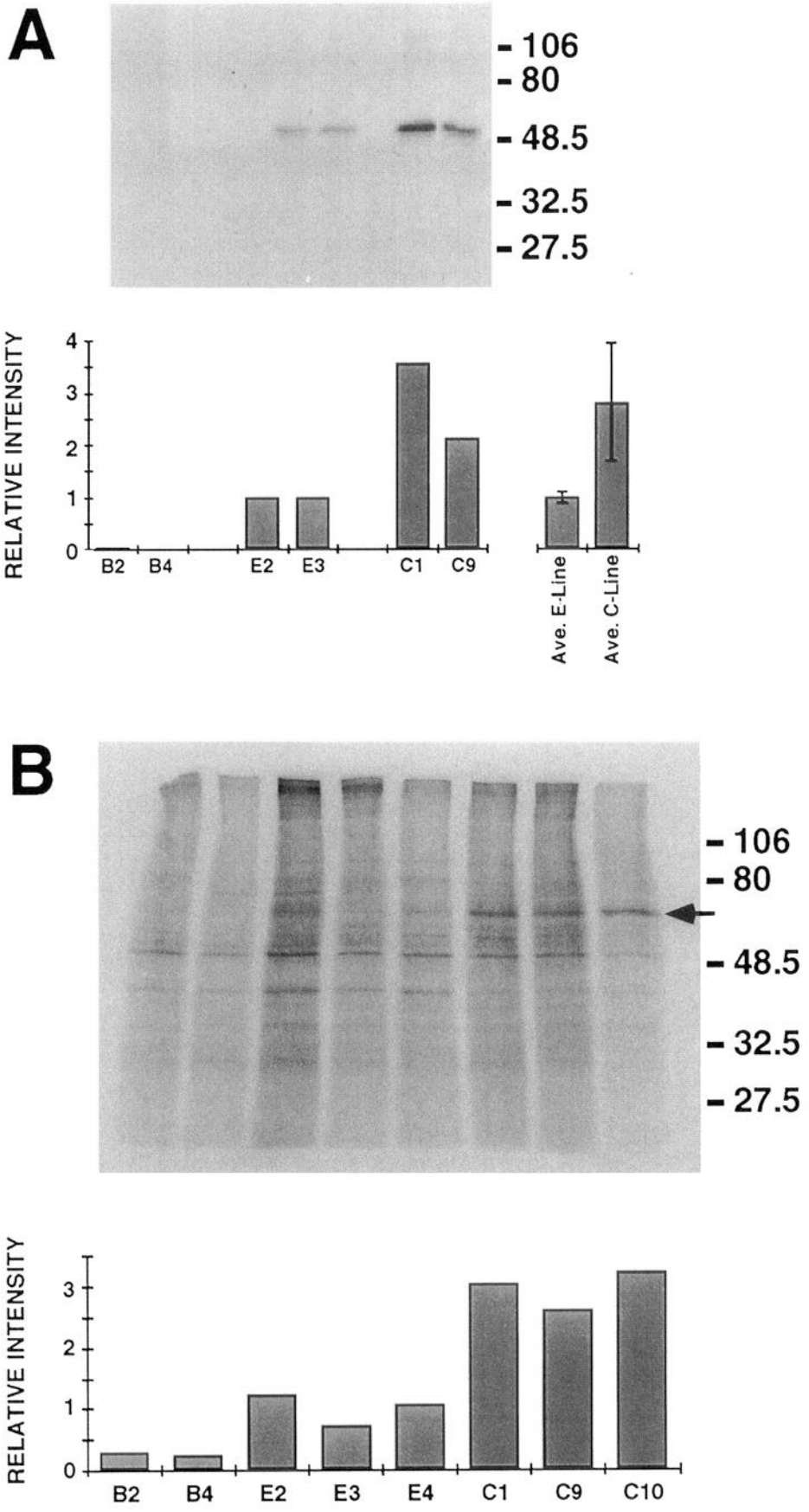

Figure 6. Analysis of mKv1.1 protein levels. *A*, Total membrane protein (Western) gel demonstrating that the CHO/AB$^-$/MK1 lines (*C1, C9*) express more mKv1.1-specific antibody–labeled protein than does the CHO/MK1 line (*E2, E3*). *B2* and *B4* are control cell lines (as in Fig. 5). Averaged normalized intensities for the six Western blots of CHO/MK1 (E lines) were 1.00 ± 0.09 (n = 12 lanes), and for the CHO/AB$^-$/MK1 (C lines), were 2.78 ± 1.20 (n = 12 lanes). *B*, Immunoprecipitation of ^{35}S-methionine–labeled mKv1.1 protein after a 20 hr incubation. *E2, E3,* and *E4* are CHO/MK1 cell lines; *C1, C9,* and *C10* are CHO/AB$^-$/MK1 cell lines. All lanes were loaded with 7 × 10^6 cpm/μg total radioactivity. The *arrow* indicates the protein labeled at approximately 70 kDa.

of STX-binding Na channels on the cell surface. However, no significant Na^+ current is observed in any of the PC12 cells expressing the mutant regulatory subunit of PKA, suggesting that PKA activity is required posttranslationally to establish functional Na channels. Thus, chronic reduction of PKA results in the reduction of Na^+ current in PC12 cells, an increase in Na^+ current in transfected CHO cells, and a comparable but mechanistically unique increase in K^+ current in our studies.

In a few cell types, endogenously expressed delayed rectifier–type K^+ currents have been shown to be functionally modulated by PKA. In human T lymphocytes, stimulation with β-adrenergic agonists or cAMP directly modulate a K^+ current, causing a decrease in peak current, an increase in inactivation during the pulse, a decrease in the voltage dependence of steady-state inactivation, and a negative shift in the current–voltage relation (Soliven and Nelson, 1990). This K^+ current inactivates during a maintained pulse, has a K_i for block of TEA of 8–16 mM, and resembles the *n*-type K channel found in lymphocytes (DeCoursey et al., 1987a). This channel has been identified as MK3 or mKv1.3 (Grissmer et al., 1990; also called RGK5, Douglass et al., 1990), a channel closely related to mKv1.1. On a longer time scale, the *n*-type channel has also been shown to be upregulated by mitogenic stimulation in mouse T lymphocytes (DeCoursey et al., 1987b) due, at least in part, to synthesis of new protein. Thus, this mKv1.1-related channel can be upregulated developmentally or downregulated when PKA activity is increased acutely. In our experiments, PKA activity was inhibited chronically, resulting in an increase in mKv1.1 current.

In cardiac tissue, the effect of cAMP and of the catalytic subunit of PKA is to upregulate K^+ current (Giles et al., 1989; Yakawa and Kameyama, 1990). The molecular species of K channel modulated by adrenergic input, cAMP, and the catalytic subunit of PKA is not known in heart, and thus the molecular structure of the phosphorylation site is also unknown. It is possible that at least one of the K channels regulated by PKA in heart is not closely related to *Shaker* or to mKv1.1. Specifically, a small (130 amino acid–encoding) cDNA, min-K, is expressed in cardiac tissue and induces a very slowly activating current, I_{sK}, when expressed in *Xenopus* oocytes (Folander et al., 1990). The kinetics of activation and deactivation of the min-K–induced current resemble closely those recorded in several types of cardiac cells. Furthermore, the K^+ current induced by min-K in oocytes is upregulated by PKA stimulation. This PKA stimulation, however, is not affected by a site-directed mutation of the putative PKA phosphorylation site in the expressed min-K cDNA (Blumenthal and Kaczmariek, 1992). Instead, the upregulation seen in oocytes expressing min-K appears to be caused by insertion of new channels into the cell membrane.

We conclude from the literature that, depending on cell type being studied, changing cellular PKA activity can result in either increased or decreased channel expression and that this change can be brought about either directly or indirectly via transcriptional, posttranscriptional, or posttranslational mechanisms. Given the ubiquity of predicted PKA phosphorylation sites on cloned ionic channel proteins, the elucidation of various cell-specific mechanisms involved in PKA regulation of these currents may provide important insights into the observed complexity of cellular signaling. Our results suggest that chronic inhibition of PKA activity in CHO cells expressing mKv1.1 results in increased K channel expression at the levels of RNA, protein, and current density. At present we do not know the molecular nature of this regulation. It will be interesting to know whether channel regulation requires direct phosphorylation of the channel protein.

References

Adler GK, Rosen LB, Fiandaca MJ, Majzoub JA (1992) Protein kinase-C activation increases the quantity and poly(A) tail length of corticotropin-releasing hormone messenger RNA in NPLC cells. Mol Endocrinol 6:476–484.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1991) Current protocols in molecular biology. New York: Wiley.

Baumann A, Grupe A, Ackermann A, Pongs O (1988) Structure of the voltage dependent potassium channel is highly conserved from *Drosophila* to vertebrate central nervous system. EMBO J 7:2457–2463.

Bernstein P, Ross J (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability. Trends Biochem Sci 14:373–377.

Bernstein P, Peltz SW, Ross J (1989) The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability *in vitro*. Mol Cell Biol 9:659–670.

Blumenthal EM, Kaczmeriek LK (1992) Modulation by cAMP of a slowly activating potassium channel expressed in *Xenopus* oocytes. J Neurosci 12:290–296.

Chandy KG, Williams CB, Spencer RH, Aguilar BA, Ghanshani S, Tempel BL, Gutman GA (1990) A family of three mouse potassium channel genes with intronless coding regions. Science 247:973–975.

Chandy KG, Douglass J, Gutman GA, Jan L, Joho R, Kaczmarek L, MacKinnon D, North RA, Numa S, Ribera A, Rudy B, Salkoff L, Swanson R, Tanouye M, Tempel BL (1991) A simplified nomenclature for vertebrate voltage-gated potassium channel genes. Nature 352:26.

Christie MJ, Adelman JP, Douglass J, North RA (1989) Expression of a cloned rat brain potassium channel in *Xenopus* oocytes. Science 244:221–224.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Clegg CH, Correll LA, Cadd GG, McKnight GS (1987) Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. J Biol Chem 262:13111–13119.

DeCoursey TE, Chandy KG, Gupta S, Cahalan MD (1987a) Two types of potassium channels in murine T lymphocytes. J Gen Physiol 89: 379–404.

DeCoursey TE, Chandy KG, Gupta S, Cahalan MD (1987b) Mitogen induction of ion channels in murine T lymphocytes. J Gen Physiol 89:405–420.

Douglass J, Osborne PB, Cai Y-C, Wilkinson M, Christie MJ, Adelman JP (1990) Characterization and functional expression of a rat genomic DNA clone encoding a lymphocyte K^+ channel. J Immunol 144:4841–4850.

Drewe JA, Verma S, Frech G, Joho RH (1992) Distinct spatial and temporal expression patterns of K^+ channel mRNAs from different subfamilies. J Neurosci 12:538–548.

Folander K, Smith JS, Antanavage J, Bennett C, Stein RB, Swanson R (1990) Cloning and expression of the delayed-rectifier I_{sK} channel from neonatal rat heart and diethylstilbestrol-primed rat uterus. Proc Natl Acad Sci USA 87:2975–2979.

Giles W, Nakajima T, Ono K, Shibata EF (1989) Modulation of a delayed rectifier K^+ current by isoprenaline in bull-frog atrial myocytes. J Physiol (Lond) 415:233–249.

Ginty DD, Glowacka D, DeFranco C, Wagner JA (1991) Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities. J Biol Chem 266:15325–15333.

Ginty DD, Fanger GR, Wagner JA, Maue RA (1992) The activity of cAMP-dependent protein kinase is required at a posttranslational level for induction of voltage-dependent sodium channels by peptide growth factors in PC12 cells. J Cell Biol 116:1465–1473.

Grissmer S, Dethlefs B, Wasmuth J, Goldin AL, Gutman GA, Cahalan MD, Chandy KG (1990) Expression and chromosomal localization of a lymphocyte K^+ channel gene. Proc Natl Acad Sci USA 87:9411–9415.

Habener JF (1990) Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol Endocrinol 4:1087–1094.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp technique for high resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–100.

Hopkins WF, Tempel BL (1992) Members of a mouse subfamily of genes encoding voltage-gated potassium channel subunits form heteromultimers when co-expressed in *Xenopus* oocytes. Soc Neurosci Abstr 18:1093.

Hua J, Hod Y (1992) The role of protein synthesis in the decay of phosphoenolpyruvate carboxykinase messenger RNA. Mol Endocrinol 6:1418–1424.

Jan LY, Jan YN (1989) Voltage-sensitive ion channels. Cell 56:13–25.

Kalman D, Wong B, Horvai AE, Cline MJ, O'Lague PH (1990) Nerve growth factor acts through cAMP-dependent protein kinase to increase the number of sodium channels in PC12 cells. Neuron 4:355–366.

Koren G, Liman ER, Logothetis DE, Nadal-Ginard B, Hess P (1990) Gating mechanism of a cloned potassium channel expressed in frog oocytes and mammalian cells. Neuron 2:39–51.

Li M, West JW, Lai Y, Scheuer T, Catterall WA (1992) Functional modulation of brain sodium channels by cAMP-dependent phosphorylation. Neuron 8:1–20.

Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER (1992) Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*. Science 256:638–644.

Miller C (1991) 1990: annus mirabilis of potassium channels. Science 252:1092–1096.

Montminy MR, Gonzalez GA, Yamamoto KK (1990) Regulation of cAMP-inducible genes by CREB. Trends Neurosci 13:184–188.

Newitt RA, Houamed KM, Rehm H, Tempel BL (1991) Potassium channels and epilepsy: evidence that the epileptogenic toxin, dendrotoxin, binds to potassium channel proteins. Epilepsy Res [Suppl] 4:263–273.

Rehm H, Newitt RA, Tempel BL (1989) Immunological evidence for a relationship between the dendrotoxin-binding protein and the mammalian homologue of the *Drosophila Shaker* K^+ channel. FEBS Lett 249:224–228.

Roberds SL, Tamkun MM (1991) Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart. Proc Natl Acad Sci USA 88:1798–1802.

Salkoff L, Baker K, Butler A, Covarrubias M, Pak MD, Wei A (1992) An essential 'set' of K^+ channels conserved in flies, mice and humans. Trends Neurosci 15:161–166.

Schecterson LC, McKnight GS (1991) Role of cyclic adenosine 3′,5′-monophosphate-dependent protein kinase in hormone-stimulated beta-endorphin secretion in AtT20 cells. Mol Endocrinol 5:170–178.

Soliven B, Nelson DJ (1990) Beta adrenergic modulation of K^+ current in human T lymphocytes. J Membr Biol 117:263–274.

Stuehmer W, Stocker M, Sakmann B, Seeburg P, Baumann A, Grupe A, Pongs O (1988) Potassium channels expressed from rat brain cDNA have delayed rectifier properties. FEBS Lett 242:199–206.

Tempel BL, Jan YN, Jan LY (1988) Cloning of a probable potassium channel gene from mouse brain. Nature 332:837–839.

Trautwein W, Hescheler J (1990) Regulation of cardiac L-type calcium current by phosphorylation and G proteins. Annu Rev Physiol 52: 257–274.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric K^+ channels are located in axonal nodes of Ranvier and in synaptic terminals. Nature, in press.

Yakawa K, Kameyama M (1990) Mechanism of receptor-induced modulation of the delayed outward potassium current in guinea-pig ventricular myocytes. J Physiol (Lond) 421:135–150.