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9. Carbonate, by LECO carbon determinator, and x-ray fluorescence, courtesy Dr. R. H. McNutt, McMaster University, Ontario. X-ray diffraction courtesy Dr. L. Evans, Guelph University, Ontario.
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Regulation of a Heart Potassium Channel by Protein Kinase A and C

KENNETH B. WALSH AND ROBERT S. KASS

The enzymes adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (protein kinase A) and protein kinase C regulate the activity of a diverse group of cellular proteins including membrane ion channel proteins. When protein kinase A was stimulated in cardiac ventricular myocytes with the membrane-soluble cAMP analog 8-chlorphenylthio cAMP (8-CPT cAMP), the amplitude of the delayed-rectifier potassium current (I_K) doubled when recorded at 32°C but was not affected at 22°C. In contrast, modulation of the calcium current (I_{Ca}) by 8-CPT cAMP was independent of temperature with similar increases in I_{Ca} occurring at both temperatures. Stimulation of protein kinase C by phorbol 12,13-dibutyrate also enhanced I_K in a temperature-dependent manner but failed to increase I_{Ca} at either temperature. Thus, cardiac delayed-rectifier potassium but not calcium channels are regulated by two distinct protein kinases in a similar temperature-dependent fashion.

PROTEIN KINASES MODULATE THE electrical activity of various cells through their actions on membrane ion channels (*1–3*). In cardiac muscle, enhancement of the voltage-gated L-type calcium current (I_{Ca}) by β-adrenergic agonists, such as isoproterenol, may result from a direct phosphorylation of Ca^{2+} channel protein by cAMP-dependent protein kinase (protein kinase A) (*4–6*). In smooth muscle and neuronal cells, another protein kinase (protein kinase C), activated by diacylglycerol (or synthetic phorbol ester compounds) and intracellular Ca^{2+} (*7*), regulates a number of K^+ and Ca^{2+} currents (*8–12*).

Isolated ventricular cells were obtained from adult guinea pig hearts by a procedure similar to that of Mitra and Morad (*13*). Recordings of membrane currents were made with the whole-cell patch clamp technique (*14*) in a chamber containing normal external solution consisting of 138 m*M* NaCl, 4.8 m*M* KCl, 1.2 m*M* $MgCl_2$, 1.0 m*M* $CaCl_2$, 5 m*M* dextrose, 5 m*M* Hepes, and 5 μ*M* tetrodotoxin (TTX), *p*H 7.4. Electrodes (2 to 5 megohms) contained 50 m*M* KCl, 60 m*M* potassium glutamate, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 11 m*M* EGTA, 5 m*M* adenosine triphosphate, and 10 m*M* Hepes. The ratio of EGTA to $CaCl_2$ sets the free intracellular Ca^{2+} concentration at 10 n*M* (*15*). The *p*H of this solution was adjusted to 7.3 with KOH, bringing the total K^+ concentration to 140 m*M*. The temperature of the recording chamber was set at either 22° or 32°C. In order to study I_{Ca} and I_K, cell membrane potential was held at −30 mV to inactivate the fast Na^+ current. The I_{Ca} was elicited by test pulses at 40-ms duration to potentials of −10 to +10 mV, and I_K deactivating tails were recorded on return to the holding potential after a 1.5-s prepulse to +20 through +40 mV. Protein kinase A and C were stimulated by external application of 8-CPT cAMP (Boehringer Mannheim) and phorbol 12,13-dibutyrate (PDB) (Sigma), respectively, to the recording chamber.

Addition of 150 μ*M* 8-CPT cAMP to a cell at 22°C produced little change in the I_K tails but caused over a threefold increase in the amplitude of I_{Ca} (Fig. 1A and Table 1). The modulation of I_{Ca} provides evidence that exposure to 8-CPT cAMP stimulates protein kinase A in these cells. Enhancement of I_{Ca} by 8-CPT cAMP occurred in a voltage-dependent manner, with the largest change in I_{Ca} occurring at more negative potentials (Table 1). A similar voltage-dependent increase in I_{Ca} has been reported during stimulation of the β-receptor with isoproterenol (*16*).

At 32°C, I_K doubled in size in the presence of 8-CPT cAMP, whereas I_{Ca} was enhanced to an extent similar to that at 22°C (Fig. 1B and Table 1). As was the case with I_{Ca}, the enhancement of I_K mediated by protein kinase A occurred in a voltage-dependent manner. The increase in tempera-

Table 1. Summary of changes in amplitude of I_K and I_{Ca} during stimulation by protein kinase A and C. All values represent the mean ± SEM (n = 4 for all experiments except 8-CPT cAMP at 22°C, where n = 3). I_K tails were recorded on return to the holding potential (−30 mV) after a 1.5-s prepulse to the indicated potential.

Condition	I_{Ca}			I_K		
	Potential (mV)	Change in amplitude (%) at		Potential (mV)	Change in amplitude (%) at	
		22°C	32°C		22°C	32°C
150 μ*M* 8-CPT cAMP	−10	549 ± 130	607 ± 156	20	16 ± 2	154 ± 21
	0	220 ± 55	237 ± 55	30	12 ± 6	124 ± 11
	10	111 ± 19	97 ± 33	40	3 ± 2	92 ± 16
	20	84 ± 10	80 ± 29			
10 n*M* PDB	−10	1 ± 16	7 ± 9	20	−2 ± 4	46 ± 4
	0	−10 ± 10	−6 ± 3	30	−3 ± 4	49 ± 5
	10	−9 ± 10	3 ± 6	40	−1 ± 4	45 ± 5
	20	−5 ± 3	9 ± 6			

Department of Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642.

ture did not alter the voltage dependence of I_{Ca} modulation.

Single exponential fits to the I_K tails revealed that enhancement of I_K at 32°C by 8-CPT cAMP was accompanied by a slight slowing in the rate of decay of the tail. For the tails shown in Fig. 1B, the time constant of decay (τ) increased from 665 to 692 ms. Overall in three experiments τ increased by 7.2 ± 1.2% (mean ± SEM) after return from a voltage step to +40 mV.

Our results indicated that I_K but not I_{Ca} was regulated by protein kinase A in a temperature-dependent manner. We were interested in determining if enhancement of I_K by protein kinase C, another prominent kinase present in cardiac ventricular cells (*17, 18*), might also be temperature-dependent, and what effect stimulation of this kinase might have on I_{Ca}. At concentrations of up to 50 n*M*, PDB, a potent activator of protein kinase C (*19*), produced no change in the amplitude of I_K recorded at 22°C (Fig. 2A and Table 1) or in I_{Ca} recorded at 22° and 32°C (Fig. 2, A and B, and Table 1). However, addition of 10 n*M* PDB at 32°C resulted in a modest but consistent enhancement of I_K (Fig. 2B and Table 1). Increasing the PDB concentration to 50 n*M* resulted in no further increase in I_K. The I_K could also be enhanced at 32°C by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*20, 21*).

Although phorbol ester compounds increased I_K, this regulation differed from that mediated by protein kinase A in two important ways. Unlike the results with 8-CPT cAMP, increases in I_K resulting from stimulation of protein kinase C were voltage-independent with similar changes produced over the voltage range studied (Table 1). In addition, PDB was found to increase the rate of decay of the I_K tails with τ decreasing by 10.7 ± 2.3% (n = 4).

We also examined the effects of protein kinase A and C on I_K under conditions in which I_{Ca} was blocked and determined whether these two kinases regulated I_K in an additive manner. Stimulation of protein kinase C by PDB resulted in an increase of the amplitude of I_K that was activated during a voltage step to +50 mV and was deactivated upon return to the holding potential of −30 mV (Fig. 3). During the peak of the PDB effect, addition of 8-CPT cAMP produced a further enhancement in the size of I_K.

Our observation that enhancement of I_K occurs during stimulation of both protein kinase A and C, lends support to the view that these two protein kinases can react with and modify the activity of the same ion channel protein in a single cell. However, the different kinetic effects produced on I_K by PDB and 8-CPT cAMP, the voltage-dependent (8-CPT cAMP) versus voltage-independent (PDB) modes of action, and the additive effects of the two compounds suggest that these two enzymes regulate the I_K channel in different ways, possibly by phosphorylating distinct amino acid residues on the channel protein.

Regardless of the site of action of protein kinase A and C, stimulation of both of these kinases increased I_K in a temperature-dependent manner, indicating that a similar temperature-dependent step exists in these two regulatory pathways for the I_K channel. This temperature-dependent step does not ap-

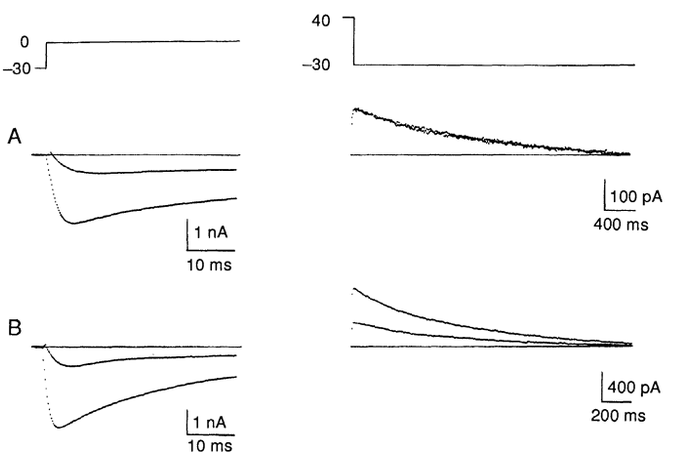

Fig. 1. Stimulation of protein kinase A by 8-CPT cAMP enhances I_K but not I_{Ca} in a temperature-dependent manner. I_{Ca} was recorded during a 40-ms voltage step to 0 mV (left panel), and I_K was recorded after a 1.5-s voltage step to +40 mV (right panel). The holding potential was −30 mV. Each pair of records shows currents obtained before and after addition of 150 μ*M* 8-CPT cAMP. (**A**) Currents recorded at 22°C from cell DL. (**B**) Currents recorded at 32°C from cell DM. I_K and I_{Ca} were sampled at 167 Hz and 5 kHz, respectively, and filtered at 50 Hz and 2 kHz, respectively. Single exponentials were fit to the I_K tails by means of a nonlinear fitting algorithm called "simplex" (*23*). Capacity transients were removed with an analog blanking circuit. Time-independent currents have been subtracted in these and other records.

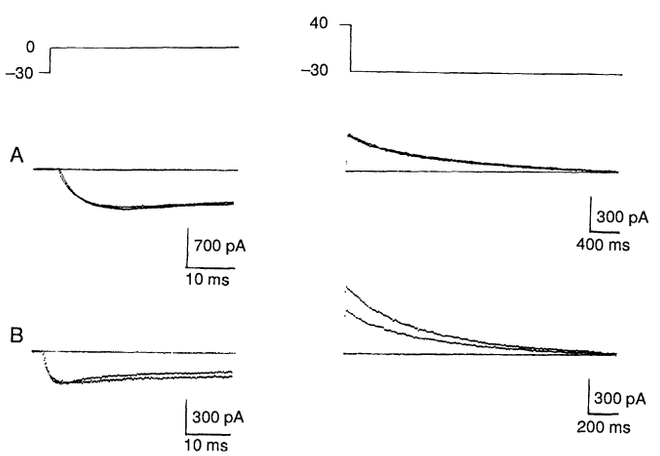

Fig. 2. Stimulation of protein kinase C enhances I_K in a temperature-dependent manner. I_{Ca} recorded during a 40-ms voltage step to 0 mV (left panel) and I_K recorded after a 1.5-s voltage step to +40 mV (right panel). The holding potential was −30 mV. (**A**) Currents recorded at 22°C from cell DQ in the presence and absence of 50 n*M* PDB. Similar results were obtained with 10 n*M* PDB at 22°C (Table 1). (**B**) Currents recorded at 32°C from cell DC in the presence and absence of 10 n*M* PDB. Increasing the concentration of PDB to 50 n*M* in this experiment produced no further increase in the amplitude of I_K, indicating that maximal enhancement of I_K can be obtained with 10 n*M* PDB.

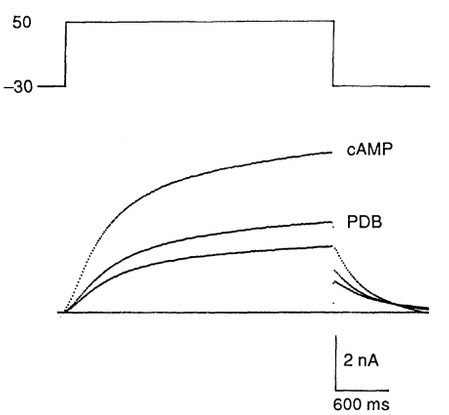

Fig. 3. Enhancement of I_K by protein kinase A and C is additive. I_K recorded during a 3-s voltage step to +50 mV and on return to the holding potential of −30 mV. Currents were recorded at 32°C before and after addition of 10 m*M* PDB in an external solution containing 160 m*M* tris base–HCl, 3 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 5 m*M* dextrose, and 200 μ*M* of the dihydropyridine Ca^{2+} channel blocker nisoldipine. During the peak enhancement of I_K, 150 μ*M* 8-CPT cAMP (cAMP) was added to the recording chamber. Cell DG. Addition of PDB or 8-CPT cAMP to another cell at 22°C caused no change in I_K (not shown).

pear to be involved in the modulation of the Ca^{2+} channel by protein kinase A. Along with our finding that stimulation of protein kinase C did not alter I_{Ca}, these results provide further evidence that cardiac K^+ and Ca^{2+} channels are regulated differently (*22*).

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Distinct Cloned Class II MHC DNA Binding Proteins Recognize the X Box Transcription Element

HSIOU-CHI LIOU, MARK R. BOOTHBY, LAURIE H. GLIMCHER

The class II (Ia) major histocompatibility complex (MHC) antigens are a family of integral membrane proteins whose expression is limited to certain cell types. A pair of consensus sequences, X and Y, is found upstream of all class II genes, and deletion of each of these sequences eliminates expression of transfected genes. Furthermore, the absence of a specific X box binding protein in patients with severe combined immunodeficiency disease whose cells lack class II suggests an important role for these proteins in class II regulation. Here, the cloning of two λgt11 complementary DNAs encoding DNA binding proteins (murine X box binding proteins λmXBP and λmXBP-2) is reported. Both phage-encoded fusion proteins bind specifically to the X box of the Aα, but not to Eα or Eβ class II genes. These two independent isolates do not cross-hybridize. The λmXBP complementary DNA hybridizes to two RNA species, 6.2 and 3.0 kilobases in mouse, that are expressed in both Ia positive and Ia negative cells. By means of DNA blot analysis with the λmXBP complementary DNA insert and probes generated from each end of this complementary DNA insert, λmXBP was found to arise from a multigene family. These data illustrate the high degree of complexity in the transcriptional control of this coordinately regulated gene family.

THE MURINE MAJOR HISTOCOMPATIBILITY complex (MHC) locus encodes four coexpressed class II genes: Aα, Aβ, Eα, and Eβ whose expression is coordinately regulated in response to a number of stimuli. Constitutive class II expression in B cells is under the control of at least one dominant locus located on mouse chromosome 16 that encodes a cross-species transacting factor (*1*). Control regions for eukaryotic genes are subject to trans-regulation by sequence-specific DNA binding proteins (*2*). Several DNA binding activities specific for sequences upstream of class II genes, including the X and Y boxes, have been identified (*3–7*). DNA binding proteins have been identified that recognize the consensus X box transcription element of human (*5, 6*) and murine (*3, 7*) class II genes; the proteins that bind class II Aα X boxes differ from those that bind Eα and Eβ X boxes (*7*). Patients with severe combined immunodeficiency disease, whose cells do not express class II genes, lack an X box binding protein as indicated by gel retardation assays (*8*). We have now identified cDNA clones that direct the synthesis of X(Aα) binding proteins in order to better characterize the proteins that bind these transcription elements.

A method developed by Singh *et al.* (*9*) enabled these investigators to molecularly clone a DNA binding protein whose recognition properties overlap those of transcriptional factors H2TF1 and NF-κB. We, therefore, screened a λgt11 mouse spleen cDNA expression library with ^{32}P-labeled X(Aα)44, a 44 bp oligonucleotide containing the Aα X box as well as the interspace sequence separating the X and Y boxes (Fig. 1B). From 750,000 plaques examined, two clones remained positive after three rounds of screening and were negative with a control probe BRE(Aα)39. The frequency of the message is therefore at least 1/400,000, which is consistent with the predicted frequency for rare messages encoding other transcription factors [1/100,000 in (*9*)].

Protein lysates made from the first of the two positive clones (λmXBP) were tested for DNA binding activity in a gel retardation assay. A λcOVA (chicken ovalbumin λgt11 cDNA clone) protein lysate and a B cell nuclear extract that contained X box binding activity were included as negative and positive controls, respectively. A DNA binding activity was detected specifically in the lysate of the λmXBP lysogen (Fig. 1A). This activity was sequence-specific since competition for retarded band was provided by unlabeled X(Aα)44 but not by Y(Aα)37, an oligonucleotide containing the Y box plus eight bases of the interspace-element (Fig. 1A, lanes 3 to 6). Furthermore, no retarded bands unique to λmXBP were detected when labeled Y(Aα)37 and BRE(Aα)39 oligonucleotides were used as probes (Fig. 1A, lanes 9 to 18). The faint bands present in all lanes with both the Y(Aα)37 and BRE(Aα)39 probes probably represent nonspecific binding. These complexes were eliminated with amounts of poly(dI-dC) that did not affect the formation of the X(Aα) complexes. The activity was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), indicating that it was linked to the lac Z fusion gene (Fig. 1A, lanes 3, 4, 19, and 20). Neither the nonspecific λcOVA (with or without IPTG induction) nor Y1089 host lysates showed any corresponding retarded bands (Fig. 1A, lanes 2, 7, and 8). To obtain direct evidence that the β-galactosidase fusion protein en-

H.-C. Liou, Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts, 02115. M. R. Boothby and L. H. Glimcher, Department of Cancer Biology, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA 02115.