

Potentialiation of rat brain sodium channel currents by PKA in *Xenopus* oocytes involves the I-II linker

RAYMOND D. SMITH AND ALAN L. GOLDIN

Department of Microbiology and Molecular Genetics, University of California, Irvine, California 92697-4025

Smith, Raymond D., and Alan L. Goldin. Potentialiation of rat brain sodium channel currents by PKA in *Xenopus* oocytes involves the I-II linker. *Am J Physiol Cell Physiol* 278: C638–C645, 2000.—Functional modulation of voltage-gated sodium channels affects the electrical excitability of neurons. Protein kinase A (PKA) can decrease sodium currents by phosphorylation at consensus sites in the cytoplasmic I-II linker. Once the sites are phosphorylated, however, additional PKA activity can increase sodium currents by an unknown mechanism. When the PKA sites were eliminated by substitutions of alanine for serine, peak sodium current amplitudes were increased by 20–80% when PKA was activated in *Xenopus* oocytes either by stimulation of a coexpressed β_2 -adrenergic receptor or by perfusion with reagents that increase cAMP. Potentiation required the I-II linker of the brain channel, in that a chimeric channel in which the brain linker was replaced with the comparable linker from the skeletal muscle channel did not demonstrate potentiation. Using a series of chimeric and deleted channels, we demonstrate that potentiation is not dependent on any single region of the linker and that the extent of potentiation varies depending on the total length and the residues throughout the linker. These data are consistent with the hypothesis that potentiation by PKA is an indirect process involving phosphorylation of an accessory protein that interacts with the I-II linker of the sodium channel.

modulation; ion channel; phosphorylation; protein kinase A; site-directed mutagenesis

VOLTAGE-GATED SODIUM channels in electrically excitable cells initiate and propagate action potentials in response to depolarizing inputs that achieve threshold level. Therefore, modulation of the functional properties of sodium channels can significantly affect the likelihood that an action potential is initiated. The rat brain IIA sodium channel is functionally modulated by protein kinase A (PKA) activity in two distinct ways, resulting in either a decrease or an increase in sodium current amplitudes (6, 8, 9, 16, 19, 20). The brain sodium channel contains five PKA consensus sites located within the cytoplasmic linker that connects domains I and II of the channel, and the channel is phosphorylated by PKA at four of these sites (11, 14, 15). PKA-mediated attenuation of current amplitude

involves phosphorylation at specific consensus site(s) within the I-II linker, with the serine at position 573 being the most important (2, 20, 21). When the I-II linker PKA sites were collectively mutated or deleted or when the serine at position 573 was selectively replaced with alanine, sodium currents were increased rather than decreased by activation of PKA activity (20, 21).

In contrast to the brain sodium channel, currents through the rat skeletal muscle sodium channel (SkM1) are not affected by PKA activation. The I-II linker in the muscle channel is substantially shorter than the linker in the brain channel. We previously reported that substitution of the entire brain I-II linker with the linker from the muscle channel resulted in a chimeric channel that did not show either current attenuation or current potentiation (20). These results demonstrated that the I-II linker of the brain channel was required for potentiation of current amplitudes by PKA activation. However, the fact that currents through brain sodium channel mutants that lacked the PKA sites in the I-II linker were still increased by PKA activation indicates that potentiation does not involve phosphorylation at any of the consensus PKA sites in the linker. Sodium currents through the rat cardiac sodium channel (SkM2) are similarly potentiated by PKA activation (17). Eight PKA consensus sites in that channel were eliminated by site-directed mutagenesis in an effort to correlate current potentiation with phosphorylation of particular site(s), but none of the mutations eliminated the potentiation by PKA (4). By constructing a series of chimeric channels between the cardiac and muscle sodium channels, it was shown that the I-II linker region of the cardiac channel was required for potentiation (4).

To investigate the importance of specific regions of the brain sodium channel I-II linker, we constructed a series of chimeric channels in which brain channel sequences were replaced by sequences from comparable regions of the skeletal muscle channel. In parallel experiments, we constructed deletions within the I-II linker to determine whether those regions are required for current potentiation. To examine the effects of PKA activation in *Xenopus* oocytes, PKA was activated either by stimulating an exogenous β_2 -adrenergic receptor (β_2 -AR) with isoproterenol or by perfusion with a cocktail that contained forskolin, cAMP analogs, and IBMX. These results demonstrate that potentiation of sodium current amplitudes by PKA does not require

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any one specific region of the I-II linker but rather depends on the total length and residues throughout the linker.

MATERIALS AND METHODS

Construction of deletion mutants and chimeric sodium channels. The Δ PKA mutant was constructed by subcloning a portion of the sodium channel containing the I-II linker into M13 and looping out of a region that encodes the PKA consensus sites as previously described (20). Other deletion mutants were made by PCR mutagenesis. The following anti-sense oligonucleotides were used to loop out targeted regions: Δ PKA, [CAAGTCCATAGAGACGTGGTA]-[CTCGTAGGTCAATCTACTTCC]; Δ PKABIG, [CCAGCAGGGTGGGCA]-[CTCCTGCTGCTTCTTTCAGTTGC]; Δ PKA-L, [AGCGGCAAATCTCTT]-[GCCTGCCCGCTGAA]; Δ PKA-R, [CCAGCAGGGTGGGCA]-[CAACAAGTCCATAGAGAC]. Nucleotide sequences that flank the regions looped out are indicated in brackets. PCR mutagenesis was done by amplifying two overlapping fragments that together spanned the region between two unique restriction sites. The fragments were gel purified, joined together, and amplified as one large fragment by PCR. Primers upstream and downstream of unique restriction sites on either side of the deletion were used, in addition to a primer that was complementary to the mutagenic oligonucleotide. PCR amplification was done with 2.5 units of *Pfu* DNA polymerase (Stratagene) in a reaction mixture that contained 20 μ M dNTPs, and 2 μ M primers. Thermal cycling was done by denaturing at 95°C for 30 s, annealing at 5°C below the melting temperatures for oligonucleotide pairs for 1 min, and extending at 72°C for 4 min for 30 cycles.

The BMB chimera was constructed as described previously (20). BMB-Left and BMB-Right chimeras were constructed to contain muscle sodium channel sequence either upstream or downstream of mutated PKA sites, respectively. This was done by merging the first portion of the linker from the BMB chimera with the PKACOMP-A mutant or by merging the downstream region of the BMB chimera with the PKACOMP-A mutant, as diagrammed in Fig. 1. To merge these regions together, PCR mutagenesis was done with overlapping fragments amplified from the BMB chimera and PKACOMP-A in a manner similar to the way in which the deletion mutations were made. The following oligonucleotide primers containing muscle and brain sodium channel sequence just upstream or downstream of the mutant PKA sites were used: BMB-Left (antisense, brain muscle), [GCGGAGCGGCAAATCTCTT]-[GTCCCCATCTGCCTCCTCTCC]; BMB-Right (sense, brain muscle), [GTCTCTATGGACTTGTG]-[GAGAAGGGGCCCAAGGCC].

Transcription of RNA and expression in *Xenopus* oocytes. Sodium channel RNA transcripts were synthesized from *Not I* linearized DNA templates using a T7 RNA polymerase transcription kit (Ambion, Austin, TX). *BamH I* was used to linearize the pBART plasmid containing the β_2 -AR coding region downstream from an SP6 polymerase promoter (23), which was generously provided by Dr. Mike White (Allegheny Univ. of Health Sciences). Stage V oocytes were removed from adult female *Xenopus laevis* frogs and prepared as previously described (7), and incubated in ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5) supplemented with 0.1 mg/ml gentamicin, 0.55 mg/ml pyruvate, and 0.5 mM theophylline. Sodium channel RNA was injected at ~100 pg/oocyte and β_2 -AR RNA was injected at 50 pg/oocyte. After a 40-h incubation at 20°C in ND-96,

sodium currents were recorded using a two-electrode voltage clamp at room temperature as previously described (12).

Activation of PKA in oocytes. The bath solution consisted of ND-96 for all electrophysiological recordings. PKA was activated by two different methods. The first method involved perfusing oocytes that had been coinjected with β_2 -AR RNA with 4 μ M isoproterenol in bath solution for 10 min. The second method involved perfusing oocytes with a cocktail consisting of 25 μ M forskolin, 10 μ M chlorophenylthio-cAMP (CPT-cAMP), 10 μ M dibutyryl-cAMP (DBcAMP), and 10 μ M IBMX for 10 min (21). Each of these reagents increases cytoplasmic cAMP levels and therefore activates PKA. Forskolin activates adenylyl cyclase (3), CPT-cAMP and DBcAMP are membrane permeable, stable analogs of cAMP (10, 13), and IBMX is an inhibitor of phosphodiesterases that convert cAMP to AMP (1). Forskolin was prepared at a stock concentration of 50 mM in DMSO, CPT-cAMP and DBcAMP were prepared at stock concentrations of 10 mM in water, and IBMX was prepared at a stock concentration of 10 mM in ethanol. All of these reagents were obtained from Sigma (St. Louis, MO). Stock solutions were stored at -20°C. Stock solutions of isoproterenol (Sigma) were made at a concentration of 100 mM in water and stored at -20°C. The rate of perfusion with bath solution was carefully adjusted to 0.1 drop per second to minimize fluctuations in current amplitude resulting from changes in flow rate.

Data analysis. Sequence comparison of the RIIA and SkM1 I-II linker sequences were made using Clustal V multiple sequence alignment software. Electrophysiological data were recorded and analyzed using pCLAMP software (Axon Instruments; Foster City, CA) and further analysis utilized Excel (Microsoft; Redmond, WA) and SigmaPlot and SigmaStat (Jandel; San Rafael, CA). In some cases drift in the peak current amplitude was observed even after allowing for recovery from slow inactivation. In those cases the peak current measurements were adjusted by subtracting out a linear relationship that was fit to data acquired during the first 10 min before PKA stimulation as previously described (21). The maximum subtraction represented a change of less than 25% over the entire 50-min-recording interval.

RESULTS

Sodium channel deletion mutants and chimeras. To test the involvement of specific regions of the I-II linker in potentiation of sodium current amplitudes, we constructed a series of deletions within the I-II linker of the brain sodium channel and we created chimeric channels by substituting regions of the muscle I-II linker into the brain sodium channel. The I-II linkers of these mutant channels are shown schematically in Fig. 1A, in which the presence of each of five consensus PKA sites is indicated by the letter "S," and the presence of a mutation in which the consensus serine is substituted with an alanine is indicated by the letter "A." The complete sequence of the linker regions, indicating the specific regions that are deleted or substituted in the chimeras, is shown in Fig. 1B. These wild-type and mutant sodium channels were used to test the role of the I-II linker in PKA-mediated potentiation of sodium current.

Sodium current potentiation by PKA activation. The functional impact of sodium channel phosphorylation was tested by expression of each of the channels in *Xenopus* oocytes, followed by activation of PKA activity

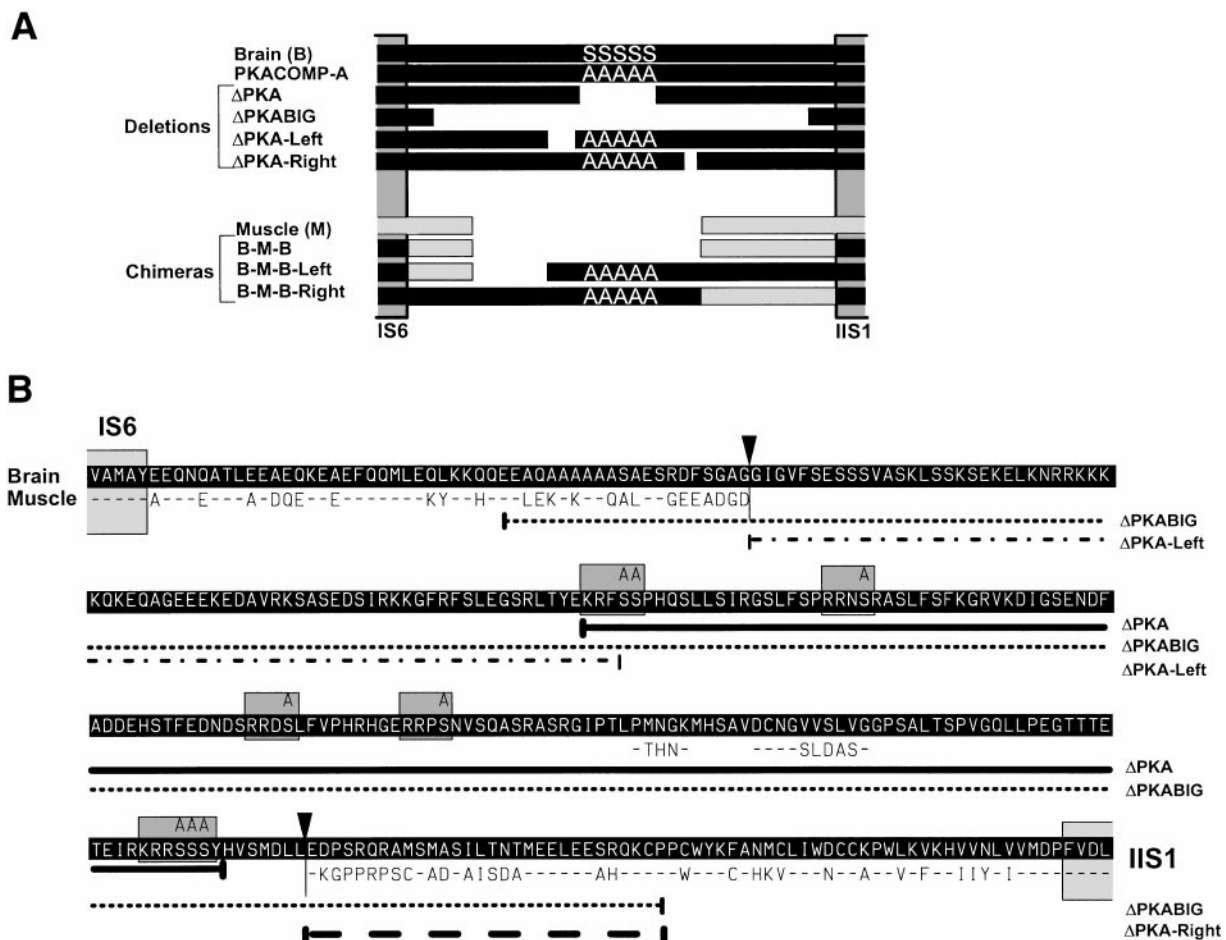


Fig. 1. Diagram of sodium channel I-II linker showing deletion and chimeric constructs. *A*: cytoplasmic linker that connects domains I and II of the sodium channel is shown schematically for wild-type rat IIA brain sodium channel (Brain), a mutant of the brain sodium channel in which 5 protein kinase A (PKA) consensus sites were eliminated by site-directed mutagenesis by converting serines to alanines (PKACOMP-A) and deletions of different regions within the brain channel linker. Each of the 5 consensus PKA sites is indicated by the letter "S," and elimination of each site by substitution of an alanine residue is indicated by the letter "A." Δ PKA and Δ PKABIG have varying amounts of linker deleted. Δ PKA-Left and Δ PKA-Right have deletions on either side of mutated, but otherwise intact, PKA consensus sites. Chimeric channels were constructed by combining various regions of the brain and muscle channels. BMB chimera consists of brain channel with entire I-II linker replaced by muscle linker. BMB-Left chimera has muscle sequence extending from end of domain I to mutated PKA sites in I-II linker. BMB-Right chimera contains muscle sequence extending from just downstream of mutated PKA sites to start of domain II. *B*: amino acid sequence alignment for I-II linker of brain and muscle sodium channels. Brain linker sequence is shown in white lettering against black background, and muscle sequence is shown underneath in black lettering, with identical amino acids indicated by dashes. Serine-to-alanine mutations in PKACOMP-A that eliminate the 5 PKA consensus sites are indicated by "A" in shaded boxes containing PKA consensus sites. Specific boundaries of Δ PKA, Δ PKABIG, Δ PKA-Left, and Δ PKA-Right deletions in brain channel are indicated by solid and dashed lines with accompanying labels to right. Specific boundaries for chimeric channels are indicated above brain sequence by downward arrowheads.

by two independent means. The first method involved stimulation of a coexpressed β_2 -AR for 10 min with isoproterenol. The β_2 -AR is coupled to adenylyl cyclase through G_{α_s} , so that stimulation of the receptor with isoproterenol activates PKA by triggering a transient increase in cytoplasmic cAMP. For the wild-type rat brain sodium channel, the peak sodium current amplitude was reduced by 20% 10 min after PKA activation (Fig. 2*A*, thick line with asterisk). The second method of inducing PKA involved perfusion with a cocktail containing forskolin, DBcAMP, CPT-cAMP, and IBMX, all of which activate PKA by increasing cytoplasmic cAMP levels (20). After cocktail perfusion for 10 min, the

sodium current amplitude was reduced by 10% (Fig. 2*B*, thick line with asterisk). The reduction of peak sodium current amplitude by either of these manipulations is consistent with previous reports for the effects of PKA on the wild-type rat brain sodium channel (8, 20, 21).

We previously showed that sodium current attenuation by PKA requires phosphorylation at PKA consensus site(s) located in the linker between domains I and II of the sodium channel (20, 21). Mutants in which the five PKA sites were collectively mutated by replacing serine residues with alanines (PKACOMP-A, Fig. 1) or in which the central portion of the I-II linker containing

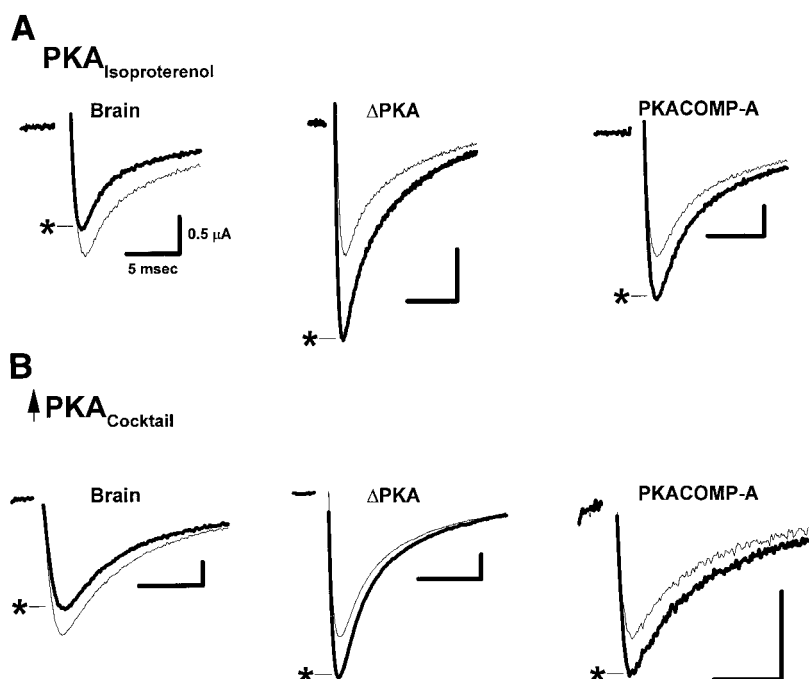


Fig. 2. Current traces showing modulation of sodium current by PKA activation. *A*: currents were elicited by membrane depolarization from holding potential of -100 to -10 mV before and 20 min after activation of PKA by isoproterenol stimulation of coexpressed β_2 -adrenergic receptor (β_2 -AR). PKA activation consisted of perfusing $100 \mu\text{M}$ isoproterenol in ND-96 bath solution (96 mM NaCl , 2 mM KCl , 1.8 mM CaCl_2 , 1 mM MgCl_2 , and 5 mM HEPES , pH 7.5) during 10-min interval followed by washout with ND-96. Currents that were recorded before PKA activation are indicated by thin lines, and currents measured after PKA activation are indicated by thick lines that are marked with asterisks. Currents were recorded for wild-type brain sodium channel and ΔPKA and PKACOMP-A mutants. Calibration bars for current amplitude and time are $0.5 \mu\text{A}$ and 5 ms , respectively. *B*: sodium currents for same 3 channels were recorded as indicated in *A*, except that PKA was activated by perfusion with cocktail containing $25 \mu\text{M}$ forskolin, $10 \mu\text{M}$ dibutyryl-cAMP, $10 \mu\text{M}$ chlorophenylthio-cAMP, and $10 \mu\text{M}$ IBMX. Current traces are shown before (thin lines) and 20 min after PKA activation (thick lines marked with asterisks). Calibration bars for current amplitude and time are $0.5 \mu\text{A}$ and 5 ms , respectively.

the five PKA sites was deleted (ΔPKA , Fig. 1) did not show current reduction. Instead, the sodium current amplitude was potentiated by PKA activation for these mutant sodium channels. When PKA was activated by isoproterenol stimulation of the β_2 -AR, peak sodium current amplitude through the ΔPKA channel was increased by 50% and currents through the PKACOMP-A channel were increased by $\sim 20\%$ (Fig. 2*A*, thick lines with asterisks). When PKA was alternatively activated by perfusion with the PKA activation cocktail, current amplitudes were similarly increased by $\sim 20\%$ for both ΔPKA and PKACOMP-A (Fig. 2*B*, thick lines with asterisks). Therefore, in the absence of the PKA sites in the I-II linker, PKA activation potentiates sodium current amplitude.

Biphasic modulation of sodium current by PKA. The time courses for the changes in peak current amplitude for the wild-type, ΔPKA , and PKACOMP-A channels following stimulation of the β_2 -AR receptor are shown in Fig. 3. The time course of current reduction for the wild-type channel is somewhat faster than that of potentiation, with the maximal effect occurring ~ 10 min after isoproterenol stimulation. In comparison, maximal potentiation for the ΔPKA and PKACOMP-A channels is relatively delayed, with maximal current observed 15–20 min after PKA activation. Both attenuation and potentiation of current are transient, with current levels returning to baseline following washout of isoproterenol. Similar time courses were obtained when PKA was activated by PKA activation cocktail (data not shown). The difference in time of onset for current attenuation and potentiation was consistently observed in this and previous studies (20, 21). These data demonstrate that PKA activation potentiates sodium current amplitude in a reversible manner when the I-II linker PKA sites are absent. In addition, the temporal separation of attenuation and potentiation

indicates that the effects of PKA are biphasic, with the onset of attenuation preceding that of potentiation.

Substitution of muscle sequence into brain channel I-II linker reduces potentiation. We previously demonstrated that neither current attenuation nor potentiation was observed for the wild-type rat muscle sodium channel in response to PKA activation (19, 20). The I-II linker of the muscle channel is substantially shorter than that of the brain I-II linker, and it does not contain

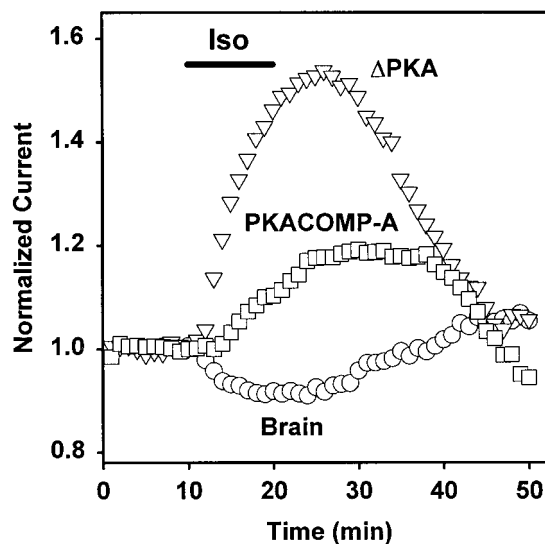


Fig. 3. Time course of peak sodium current amplitude during PKA activation. Peak sodium current amplitudes recorded from wild-type brain channel (\circ) and ΔPKA (∇) and PKACOMP-A (\square) mutant channels are shown during 50-min time course. Baseline current level was established during initial 10-min interval. PKA was activated during following 10 min by perfusion with $100 \mu\text{M}$ isoproterenol in ND-96, as indicated by bar denoted "Iso", followed by washout with ND-96. Peak current amplitudes were adjusted and normalized to initial peak amplitude measured during first 10 min before PKA activation.

any consensus PKA phosphorylation sites (Fig. 1). When isoproterenol was perfused on oocytes expressing both the muscle channel and the β_2 -AR, no change in peak current amplitude was observed (Fig. 4A). The attenuation response of the brain sodium channel following PKA activation is shown for comparison. Average and standard deviation values for the percent change in current amplitude for multiple oocytes are summarized in Fig. 4C. To determine whether differences in the sequences of the I-II linkers between the two channels were responsible for the different responses to PKA activation, we constructed a chimeric channel in which the entire I-II linker of the brain channel was substituted with corresponding muscle channel sequence (BMB, Fig. 1). The effects of PKA activation by isoproterenol stimulation of a coexpressed β_2 -AR are shown in Fig. 4A. Replacement of the brain I-II linker with muscle sequence completely eliminated the attenuation of sodium current amplitude and eliminated the majority of sodium current potentiation as well. These data demonstrate that sequences unique to the brain channel I-II linker compared with the muscle channel are required for both sodium current attenuation and potentiation by PKA.

We previously demonstrated that the consensus PKA phosphorylation sites are necessary and sufficient for the attenuation of sodium current amplitudes (20, 21), and the muscle channel does not contain any of these consensus sites. However, the results with the Δ PKA and PKACOMP-A mutants demonstrate that the consensus PKA sites are not necessary for the potentiation of sodium current amplitude (Fig. 2). To determine

which region(s) of the brain channel I-II linker are involved in potentiation, we constructed two additional chimeras in which smaller regions within the brain I-II linker were replaced with corresponding muscle sequence (Fig. 1). For each of these chimeras, the PKA sites in the brain channel sequence were eliminated by replacement of the serines with alanines as in PKACOMP-A to eliminate the effect of current attenuation. These chimeras were designed to contain muscle sequence upstream of the PKA consensus sites (BMB-Left) or downstream of the PKA sites (BMB-Right). Both BMB-Left and BMB-Right expressed sodium currents that were potentiated by PKA activation (Fig. 4, B and C). The BMB-Right chimera demonstrated a level of potentiation that was similar to the PKACOMP-A channel. However, the amount of potentiation for the BMB-L chimera was significantly reduced relative to the PKACOMP-A mutant. Currents through the PKACOMP-A channel were potentiated by $26 \pm 4\%$, whereas currents through the BMB-Left chimera were potentiated by only $15 \pm 9\%$. Therefore, current potentiation was disrupted by a region-specific replacement with muscle sequence. Substitution of muscle sequence upstream and downstream of the PKA sites changed two aspects of the brain sodium channel linker at the primary amino acid level. First, the amino acid sequence was altered due to differences between the muscle and brain isoforms, as shown in Fig. 1B. Second, the overall length of the I-II linker was shortened in the case of the BMB-Left chimera due to the fact that the region of the muscle channel upstream of the PKA sites is shorter (Fig. 1). The length of the I-II

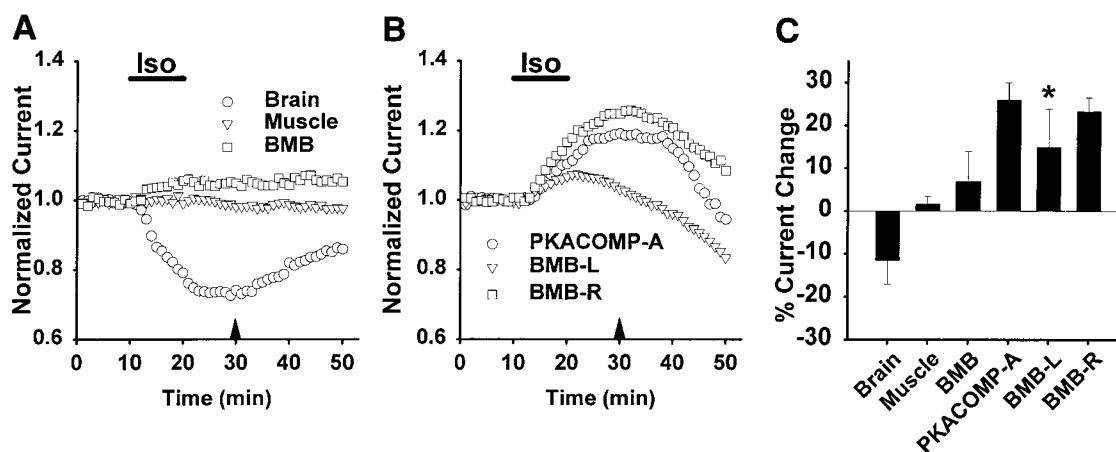


Fig. 4. Potentiation of chimeric brain channels containing different extents of muscle channel sequence. *A*: normalized peak current amplitudes are plotted as function of time for brain (\circ) and muscle (∇) channels and BMB chimera (\square) during 50-min time course. Peak amplitudes were measured from current traces elicited by depolarizations from holding potential of -100 to -10 mV. Representative values are shown. Baseline current level was established during initial 10-min interval, after which PKA was activated for 10 min by perfusion with $100 \mu\text{M}$ isoproterenol in ND-96, as indicated by bar denoted "Iso", followed by washout with ND-96. Peak current amplitudes were adjusted and normalized to initial amplitude measured during first 10 min before PKA activation. Average values from multiple oocytes for peak current value at 30-min time point (indicated by arrowheads in *A* and *B*) are summarized in *C*. *B*: normalized peak current amplitudes were measured as in *A* for PKACOMP-A mutant (\circ), and BMB-Left (L) (∇) and BMB-Right (R) (\square) chimeric channels. *C*: average percentage changes in peak current amplitude measured 20 min after PKA activation (at 30-min time point indicated by arrowheads in *A* and *B*) are shown. SD values are indicated by error bars. Asterisk above bar for BMB-L chimera indicates that percentage change after PKA activation for that chimera was significantly different from that for PKACOMP-A mutant (t -test $P < 0.05$). Percentage change for BMB-R chimera was not significantly different from that for PKACOMP-A mutant. Number of oocytes tested: 5 brain, 5 muscle, 4 BMB, 6 PKACOMP-A, 3 BMB-L, and 3 BMB-R.

linker in the BMB-Right chimera was preserved relative to that of the brain sodium channel linker (Fig. 1).

Deletions of small regions of I-II linker disrupt current potentiation. To determine if the disruption of potentiation by the BMB-Left chimera resulted from a shortening of the linker rather than a change in the amino acid sequence, three deletions in the I-II linker were constructed. A large deletion termed Δ PKABIG removed 267 amino acids, including the majority of the I-II linker and the consensus PKA sites (Fig. 1). Two smaller deletions were constructed in the PKACOMP-A mutant on either side of the mutated PKA sites to examine the effects of deleting different regions of the I-II linker. These smaller deletion mutants were termed Δ PKA-Left (71 amino acids deleted) and Δ PKA-Right (30 amino acids deleted) (Fig. 1). The channels all expressed currents that were similar to the wild-type channel with respect to current amplitude and inactivation kinetics (data not shown). The effects of PKA activation on each of these deletion mutants were then examined by stimulation of a coexpressed β_2 -AR with isoproterenol (Fig. 5). Data are included for the original Δ PKA mutant in which the central region of the linker (138 amino acids) containing the five PKA consensus sites was deleted. Representative responses observed in individual oocytes are shown in Fig. 5A, and the average percentages of current change in multiple oocytes are summarized in Fig. 5B. The deletion mutants all showed some potentiation, but the magnitude of the response was consistently smaller for the Δ PKABIG, Δ PKA-Left, and Δ PKA-Right mutants when compared with the Δ PKA mutant (Fig. 5B). The reduction in potentiation for each of these mutants was similar, demonstrating that the decrease in potentiation resulted from a shortening of the linker, rather than from removal of specific sequences in any one region of the I-II linker.

DISCUSSION

We have demonstrated that PKA activation in *Xenopus* oocytes potentiates currents through mutant rat brain sodium channels lacking PKA sites in the I-II linker, consistent with our previous results (19–21). It also has been shown previously that the wild-type brain sodium channel is attenuated by PKA phosphorylation (2, 8, 9, 20, 21). We propose that both forms of modulation occur for the wild-type channel, but that the net effect is a reduction in current amplitude because attenuation normally dominates. In addition, both forms of modulation require the I-II linker of the brain sodium channel.

Shortening of I-II linker reduces potentiation. By constructing deletions of various regions of the I-II linker, we have determined that mutations that shorten the linker on either side of the centrally located PKA sites reduce the level of potentiation. Changing the amino acid sequence downstream of the central PKA sites by a substitution of muscle sequence (BMB-Right) maintained the length of the linker and had no effect on the potentiation response. In contrast, a deletion of 30 amino acids within the same region significantly re-

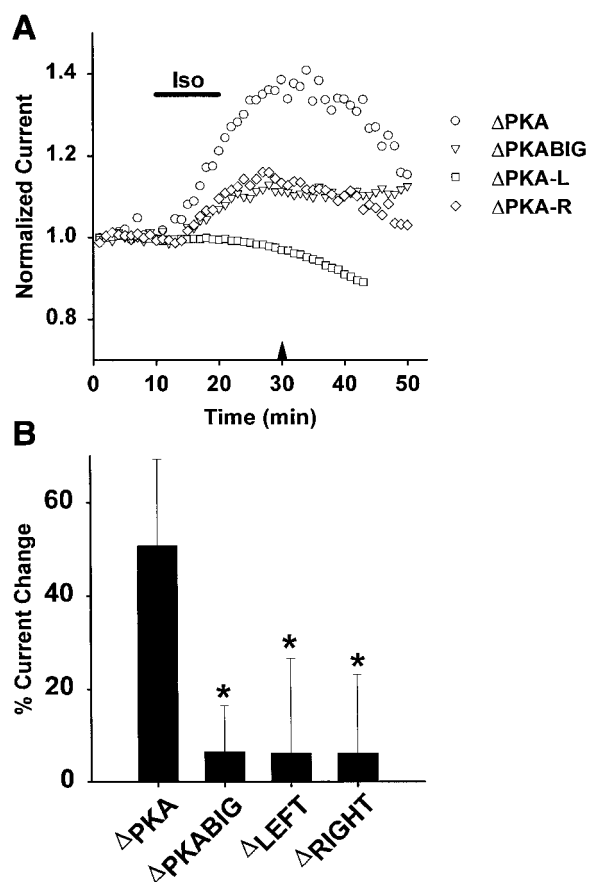


Fig. 5. Deletions within I-II linker reduce potentiation by PKA. **A:** normalized peak current amplitudes are plotted as function of time for Δ PKA (\circ), Δ PKABIG (∇), Δ PKA-L (\square), and Δ PKA-R (\diamond) deletion mutants during 50-min time course. Sodium current amplitudes were measured from current traces elicited by depolarizations from holding potential of -100 to -10 mV. Representative values are shown. Baseline current level was established during initial 10-min interval, after which PKA was activated for 10 min by perfusion with $100 \mu\text{M}$ isoproterenol in ND-96, as indicated by bar denoted "Iso", followed by washout with ND-96. Peak current amplitudes were adjusted and normalized to initial amplitude measured during first 10 min before PKA activation. Average values from multiple oocytes for peak current value at 30-min time point (indicated by arrowhead) are summarized in **B**. **B:** average percentage changes in peak current amplitude measured 20 min after PKA activation (at 30-min time point indicated by arrowhead in **A**) are shown. SD values are indicated by error bars. Asterisk indicates that percentage change after PKA activation for that deletion mutant was significantly different from that for Δ PKA mutant (t -test $P < 0.05$). Number of oocytes tested: 3 Δ PKA, 5 Δ PKABIG, 4 Δ PKA-L, and 4 Δ PKA-R.

duced potentiation. When the region of the brain channel I-II linker upstream of the PKA sites was replaced with corresponding muscle channel sequence (BMB-Left), the length was reduced by 71 amino acids and the level of potentiation was significantly reduced. The central portion of the linker that was deleted in the Δ PKA mutant is apparently not required for potentiation, as the level of potentiation observed for the Δ PKA mutant is at least as large as that observed for the PKACOMP-A mutant. There are at least two possible explanations for this result. First, it is possible that adjacent regions of the linker are important for potentiation because they are in closer proximity to the

putative transmembrane regions, so that deletions in these regions are more likely to affect the structure of the channel. Alternatively, it is possible that the central region of the I-II linker that was deleted in the Δ PKA mutant has a compact secondary structure, so that deletion of this region does not alter the overall structure of the remaining linker. The potentiation of current that was observed in all cases was not secondary to alterations in the electrophysiological properties of the channels, as neither the deletion mutations nor the chimeric constructs significantly affected any of these properties (data not shown).

Biphasic modulation of brain sodium channel by PKA. Our data suggest that PKA-mediated potentiation of sodium current underlies the normally observed attenuation of sodium current expressed from the wild-type channel. It is possible that each type of modulation might occur under different physiological conditions. For example, activation of PKA to a low-to-moderate level could be sufficient to phosphorylate the PKA sites in the I-II linker and attenuate current. However, if the basal level of phosphorylation was already sufficient to phosphorylate the I-II linker sites, additional activation of PKA might trigger the secondary mechanism that potentiates current. Consistent with this hypothesis, we previously observed potentiation of the wild-type sodium channel under conditions that were predicted to result in an elevated basal level of PKA activity (19). In that study, we coexpressed the β_2 -AR at a high level (about 1,000 \times higher than in this study), so that unstimulated β_2 -AR activity would activate PKA above the normal baseline level. Under those conditions, sodium current amplitude already would have been attenuated to a new basal level. We propose that when PKA was activated to even higher levels by isoproterenol stimulation, we observed the secondary effect of current potentiation.

Further support for the hypothesis that potentiation by PKA occurs even when the consensus sites are phosphorylated is provided by results with a mutant channel in which the serine residues in the consensus PKA sites have been replaced with aspartates. The negative charge of the aspartate residues should mimic the negative charge of the phosphorylated PKA sites and resulted in smaller current amplitudes compared with the wild-type channel (20). Currents through this mutant channel were also potentiated when PKA was activated by stimulation of a coexpressed β_2 -AR (20).

Potential mechanisms for potentiation. Although these data demonstrate that the I-II linker of the brain sodium channel is required for potentiation, we do not know the molecular mechanism involved in the process. Sodium current potentiation was observed using two independent means of PKA activation, which confirms that PKA phosphorylation plays a central role. Our results indicate that potentiation of current does not involve direct phosphorylation of the I-II linker, because it was possible to disrupt the response by nonoverlapping deletions (compare the Δ PKA-Left and Δ PKA-Right mutants). In addition, there are no consensus PKA sites in the I-II linker other than the five sites

that were mutated in the PKACOMP-A mutant. One possible mechanism is that a cellular protein in the oocyte is phosphorylated by PKA, and this protein then interacts with the I-II linker of the sodium channel by binding to region(s) upstream or downstream from the centrally located PKA sites. Candidate proteins include cytoskeletal proteins such as ankyrin, spectrin, syntrophin, and tenascin, all of which have been shown to interact with the sodium channel (18, 22, 24). Consistent with the hypothesis that a secondary protein is involved in current potentiation, the onset for current attenuation by phosphorylation of the I-II linker sites is faster than the appearance of current potentiation observed for mutants that lack the I-II linker PKA sites. The delay in response suggests that potentiation occurs through a separate and perhaps indirect mechanism.

Potentiation of brain and cardiac sodium channels is similar. Our results are similar to previous data for the cardiac sodium channels, which also has been shown to be potentiated by PKA activation in *Xenopus* oocytes (4, 5, 17). In that case, potentiation was abolished when the I-II linker of the cardiac channel was substituted with corresponding muscle sodium channel sequence. Elimination of eight PKA sites throughout the cardiac sodium channel did not affect current potentiation, prompting the authors to speculate that potentiation of the cardiac channel occurred either by phosphorylation of a nonoptimal PKA site or by phosphorylation of an oocyte protein that acts as an accessory protein. Assuming that the same mechanism is responsible for potentiation of brain and cardiac sodium channel currents, our data and those of Frohneser et al. (4, 5) suggest that potentiation is an indirect process.

We thank Drs. Marianne Smith, Michael Pugsley, Ted Shih, and Daniel Allen for helpful discussions during the course of this work and Mimi Reyes for excellent technical assistance.

This work was supported by the National Institute of Neurological Disorders and Stroke Grant NS-26729. A. L. Goldin is an Established Investigator of the American Heart Association.

Present address of R. D. Smith: Dept. of Biology, Univ. of California San Diego, La Jolla, CA 92093-0357.

Address for reprint requests and other correspondence: A. L. Goldin, Dept. of Microbiology and Molecular Genetics, Univ. of California, Irvine, CA 92697-4025 (E-mail: AGoldin@uci.edu).

Received 28 July 1999; accepted in final form 21 October 1999.

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