cAMP-dependent Phosphorylation of Two Sites in the \( \alpha \) Subunit of the Cardiac Sodium Channel*

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The voltage-sensitive \( \text{Na}^+ \) channel is responsible for generating action potentials in the heart which are critical for coordinated cardiac muscle contraction. Cardiac \( \text{Na}^+ \) channels are regulated by cAMP-dependent phosphorylation, but the sites of phosphorylation are not known. Using mammalian cells expressing the rat cardiac \( \text{Na}^+ \) channel (rH1) \( \alpha \) subunit and site-specific antibodies, we have shown that the \( \alpha \) subunit of rat heart \( \text{Na}^+ \) channel is phosphorylated selectively by cAMP-dependent protein kinase (PKA) \textit{in vitro} and in intact cells. Analysis of the sites of phosphorylation by two-dimensional phosphopeptide mapping and site-directed mutagenesis of fusion proteins revealed that the cardiac \( \alpha \) subunit is phosphorylated selectively \textit{in vitro} by PKA on Ser\(^{526}\) and Ser\(^{529}\) in the intracellular loop connecting homologous domains I and II (LI-II). These two residues were phosphorylated in intact cells expressing the rH1 \( \alpha \) subunit when PKA was activated. Our results define a different pattern of phosphorylation of LI-II of cardiac and brain \( \text{Na}^+ \) channels and implicate phosphorylation of Ser\(^{526}\) and Ser\(^{529}\) in the differential regulation of cardiac and brain \( \text{Na}^+ \) channels by PKA.

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‡ This abbreviation is used: LI-II, intracellular loop between homologous domains I and II of the \( \alpha \) subunit; PKA, cAMP-dependent protein kinase (protein kinase A); PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GST, glutathione S-transferase; DCl-cBIMPS, dichloro-1-\( \beta \)-D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphate.

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**EXPERIMENTAL PROCEDURES**

Materials—Protein A-Sepharose was purchased from Sigma. \([\gamma-\text{\(^{32}\)P}]\text{ATP} \) (3,000 Ci/mmol) was obtained from DuPont NEN. Sequencing grade modified porcine trypsin was purchased from Promega Corp. (Madison, WI). Chromogram sheets and x-ray film were purchased from Eastman Kodak. Molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE) were purchased from Novex (San Diego).

Phosphorylation of Cardiac \( \text{Na}^+ \) Channel by PKA—Immunoprecipitated rat \( \text{Na}^+ \) channel or Na\(^{+}\) channel fusion proteins were phosphorylated by incubation at 37 °C in 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10 mM MgCl\(_2\), 1 mM EGTA, 0.15 μM \([\gamma-\text{\(^{32}\)P}]\text{ATP} \) (3,000 Ci/mmol), or 100 μM unlabeled ATP, in the presence of 0.25 μM purified catalytic subunit of PKA for the times indicated. Phosphorylation reactions were terminated by heating at 65 °C for 3 min in 80 mM Tris-HCl (pH 6.8), 10% glycerol, 10 μM dithiothreitol, and 2% SDS.

SDS-PAGE and Immunoblotting—Denatured \([\gamma-\text{\(^{32}\)P}]\text{P}-\text{labeled Na}^+\) channel protein was analyzed by SDS-PAGE in 6% Laemmli gels (23 using a U.S.C. Section 1734 solely to indicate this fact.
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RESULTS

Phosphorylation of Cardiac Na" Channel Isolated from Cultured Cells Expressing the α Subunit of Rat Heart Na" Channel—To determine if the rat heart Na" channel is a substrate for PKA in vitro, Chinese hamster lung cells (line 1610) expressing the α subunit of rat heart Na" channel (SNα-rH1, 5) were solubilized with 1% Triton X-100, and the α subunit was immunoprecipitated with either anti-SP19 or nonimmune IgG. Isolated channels were then phosphorylated in the presence of PKA and [γ-32P]ATP and analyzed by SDS-PAGE on a 6% Laemmli gel. 32P-Labeled α subunits were visualized by autoradiography. Molecular weight markers are represented as M = 10^-5. Panel B, 32P-labeled α subunits were excised from the wet gel and processed for phosphoamino acid analysis as described under “Experimental Procedures.” Acid-hydrolyzed samples were subjected to two-dimensional electrophoresis on thin layer cellulose plates at pH 1.9 (first dimension), and at pH 3.5 (second dimension). The migration fragments of phosphothreonine (T), phosphoserine (S), and phosphotyrosine (Y) standards are designated by the broken circles and arrows.

by SDS-PAGE, and 32P-labeled phosphoproteins were visualized by autoradiography as described under “Experimental Procedures.” Fig. 1A shows that anti-SP19 specifically immunoprecipitated a single protein with an apparent molecular mass of approximately 240 kDa which is a substrate for PKA. Preimmune IgG failed to immunoprecipitate this phosphoprotein, confirming its specificity (Fig. 1A).

Two-dimensional phosphoamino acid analysis (Fig. 1B) showed that PKA phosphorylated the α subunit in vitro solely on serine residues. Phosphorylation of the α subunit was examined further by two-dimensional tryptic phosphopeptide mapping as described under “Experimental Procedures.” Following autoradiography, two major phosphopeptides (I and II) were visualized (Fig. 2A). Overexposure of these phosphopeptide maps indicates the presence of three reproducible minor phosphopeptides (III–V) whose migration positions are illustrated in Fig. 2B. These phosphopeptides are not clearly visible in this photograph but are visible in the original overexposed autoradiographs (also see Figs. 5 and 7). A minor phosphopeptide near phosphopeptide III was also visualized in overexposed maps. The presence of this peptide varied from experiment to experiment and was distinct from phosphopeptide III.

Together these results suggest that the cardiac Na" channel α subunit is phosphorylated by PKA in vitro on multiple serine residues.

Phosphorylation of GST Fusion Proteins Containing L1,11—The rat brain Na" channel α subunit is phosphorylated on four residues clustered in L1,11 both in vitro and in intact cells in response to activation of PKA (12, 13). Although these residues are not conserved in the rat heart isoform of the channel (3), several potential consensus sequences for PKA recognition are found in this intracellular loop. Lack of an efficient purification procedure for cardiac Na" channels precludes the identification of phosphorylation sites by conventional protein microsequencing techniques. We therefore constructed and expressed GST fusion proteins containing portions of L1,11 of the rat heart Na" channel α subunit (Fig. 3A). NaFH1 corresponds in sequence to amino acids 436–645 of the full-length cardiac α subunit predicted by cDNA cloning (3). NaFH1 was purified by chromatography on glutathione-Sepharose, incubated under phosphorylating conditions in the presence of [γ-32P]ATP and PKA.
[γ-32P]ATP, and analyzed by SDS-PAGE and autoradiography as described under "Experimental Procedures." Fig. 4A shows that the NaFH1 was a good substrate for PKA. A faster migrating phosphoprotein is present in this preparation and likely represents a proteolytically cleaved fragment of full-length NaFH1. GST alone is not a substrate for PKA (data not shown). 32P-Labeled NaFH1 was then subjected to two-dimensional tryptic phosphopeptide mapping as described under "Experimental Procedures" (Fig. 4B). Two major and one minor phosphopeptide were visualized whose position in two-dimensional maps corresponded with phosphopeptides I, II, and III derived from tryptic digestion of 32P-labeled α subunit isolated from intact cells (Fig. 2A). Overexposed autoradiographs also contain two other minor phosphopeptide spots whose migration positions correspond to phosphopeptides IV and V (data not shown). These results suggest that the rat heart Na+ channel α subunit isolated from intact cells is phosphorylated by PKA in vitro solely on residues contained in Iα-II α subunit, as observed previously for the brain Na+ channel α subunit. Lα-II of the cardiac Na+ channel α subunit contains eight candidate Ser residues within the PKA consensus sequence KRXXS, RXXS, or RXS (Fig. 3B), which are all distinct from phosphorylation sites in the brain Na+ channel. To locate the region that is phosphorylated by PKA, two new fusion proteins were constructed which divide NaFH1 into two parts. NaFH2 and NaFH3 correspond in sequence to amino acids 436–511 and 491–645, respectively. Equal amounts of purified NaFH2 and NaFH3 were incubated under phosphorylating conditions in the presence of PKA and [γ-32P]ATP and analyzed by SDS-PAGE and autoradiography as described above. NaFH2 was not a substrate for PKA (data not shown). NaFH3, however, was a good substrate in vitro for PKA (Fig. 5A). A faster migrating phosphoprotein is present in this lane and likely represents a proteolytically cleaved fragment of full-length NaFH3. When 32P-labeled NaFH3 was subjected to two-dimensional tryptic phosphopeptide mapping, two major and one minor phosphopeptide were visualized (Fig. 5B) whose migration positions corresponded to phosphopeptides I, II, and III, respectively, in the peptide map derived from the full-length α subunit isolated from intact cells (Fig. 2A) and NaFH1 (Fig. 4B). Overexposed maps of 32P-labeled NaFH3 also contained the minor phosphopeptides IV and V (data not shown). These results suggest that the rat heart Na+ channel α subunit isolated from intact cells is phosphorylated by PKA on serine residues located between amino acids 512 and 645 in Lα-II.

To narrow further the region phosphorylated by PKA, NaFH3 was split into two fusion proteins (NaFH4 and NaFH5) which correspond in sequence to amino acids 491–569 and 530–645, respectively (Fig. 3A). Fig. 6 shows that when equivalent amounts of the fusion proteins NaFH4 and NaFH5 were incubated under phosphorylating conditions in the presence of PKA and [γ-32P]ATP, only NaFH4 was phosphorylated. When 32P-labeled NaFH4 was subjected to two-dimensional tryptic phosphopeptide mapping, two major and one minor phosphopeptide were visualized whose migration positions corresponded to phosphopeptides I, II, and III in the peptide map derived from the full-length α subunit (data not shown). Overexposed maps of 32P-labeled NaFH4 also contained the minor phosphopeptides IV and V (data not shown). These results further narrow the region of the heart Na+ channel phosphorylated by PKA to residues 511–530 in Lα-II. This region contains five serine residues; residues 520, 526, and 529 conform to the RXS consensus motif for PKA recognition (Fig. 3B).

Individual Serine Residues Phosphorylated by PKA—To determine which of these serine residues is phosphorylated by PKA, three additional fusion proteins were generated, corresponding in sequence to NaFH3 but with the serines at positions 520 (NaFH-S520A), 526 (NaFH-S526A), and 529 (NaFH-S529A) mutated to alanine residues. Each fusion protein was
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Fig. 4. Phosphorylation and tryptic phosphopeptide mapping of the fusion protein NaFH1. Panel A, purified NaFH1 was phosphorylated in the presence of PKA and [γ-32P]ATP, analyzed by SDS-PAGE on a Novex 10–20% Tricine gel, and 32P-labeled fusion protein was located by autoradiography. Molecular weight markers are represented as M, × 10^-3. Panel B, phosphorylated NaFH1 was analyzed on a 7.5% Laemmli SDS gel and located by autoradiography. 32P-Labeled NaFH1 was excised from the wet gel and processed for tryptic phosphopeptide analysis as described under “Experimental Procedures.” Tryptic phosphopeptides were separated in two dimensions by high voltage electrophoresis (pH 8.9) followed by thin layer chromatography. Arrows designate the direction of electrophoresis (+) and chromatography (C).

Fig. 5. Phosphorylation and tryptic phosphopeptide mapping of the fusion protein NaFH3. Panel A, purified NaFH3 was phosphorylated in the presence of PKA and [γ-32P]ATP, analyzed by SDS-PAGE on a Novex 10–20% Tricine gel, and 32P-labeled fusion protein was located by autoradiography. Molecular weight markers are represented as M, × 10^-3. Panel B, phosphorylated NaFH3 was analyzed on a 7.5% Laemmli SDS gel and located by autoradiography. 32P-Labeled NaFH3 was excised from the wet gel and processed for tryptic phosphopeptide analysis as described under “Experimental Procedures.” Tryptic phosphopeptides were separated in two dimensions by high voltage electrophoresis (pH 8.9) followed by thin layer chromatography. Arrows designate the direction of electrophoresis (+) and chromatography (C).

Fig. 6. Phosphorylation of the fusion proteins NaFH4 and NaFH5 by PKA. Equal amounts (0.1 μg) of purified NaFH4 and NaFH5 were incubated under phosphorylating conditions in the presence of PKA and [γ-32P]ATP and analyzed by SDS-PAGE on a Novex 10–20% Tricine gel. 32P-Labeled fusion proteins were located by autoradiography. Molecular weight markers are represented as M, × 10^-3.

Phosphorylation and tryptic phosphopeptide mapping of phosphorylated fusion proteins S525A and S529A. Purified fusion proteins were phosphorylated in the presence of PKA and [γ-32P]ATP, analyzed by SDS-PAGE on 7.5% Laemmli gels, located by autoradiography, and subjected to two-dimensional mapping as described under “Experimental Procedures.” Tryptic phosphopeptides were separated in two dimensions by high voltage electrophoresis (pH 8.9) followed by thin layer chromatography. Arrows designate the direction of electrophoresis (+) and chromatography (C). Broken circles designate positions of absent phosphopeptides.

Fig. 7. Two-dimensional tryptic phosphopeptide mapping of phosphorylated fusion proteins S525A and S529A. Purified fusion proteins were phosphorylated in the presence of PKA and [γ-32P]ATP, analyzed by SDS-PAGE and autoradiography, and subjected to two-dimensional mapping as described under “Experimental Procedures.” Arrows designate the direction of electrophoresis (+) and chromatography (C). Broken circles designate positions of absent phosphopeptides.

Purified NaFH3 which contains Ser526 and Ser529 (Fig. 8). NaFH-S560/1A, like NaFH5 by PKA. Equal amounts (0.1 μg) of purified NaFH4 and NaFH5 were incubated under phosphorylating conditions in the presence of PKA and [γ-32P]ATP and analyzed by SDS-PAGE on a Novex 10–20% Tricine gel. 32P-Labeled fusion proteins were located by autoradiography. Molecular weight markers are represented as M, × 10^-3.

To confirm this conclusion, an additional fusion protein was generated, corresponding in sequence to NaFH3 but with the serines corresponding to residue 525, 526, and 529 mutated to alanine residues (NaFH-S525/6/9A). The ability of PKA to phosphorylate NaFH-S525/6/9A was then compared with its ability to phosphorylate the fusion protein NaFH-S560/1A, which contains Ser526 and Ser529 (Fig. 8). NaFH-S560/1A, like NaFH5, is a good substrate for PKA (data not shown). Equi-
Fig. 8. Phosphorylation of fusion proteins S560/1A and S525/6/9A. Purified S560/1A and S525/6/9A were phosphorylated for 1 min (panel A) or 10 min (panel B) in the presence of PKA and [γ-32P]ATP and analyzed by SDS-PAGE on a Novex 10–20% Tricine gel. 32P-Labeled fusion proteins were visualized by autoradiography. Molecular weight markers are represented as Mw × 10−3. Panel C, to ensure that equal amounts of fusion proteins were phosphorylated, they were analyzed by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-GST antibodies, and visualized on x-ray film using chemiluminescent detection.

Phosphospecific Anti-peptide Antibodies Directed against Phosphorylated Ser526 and Ser529—To examine directly the PKA-dependent phosphorylation of Ser526 and Ser529 in the fusion proteins encompassing the intracellular loop connecting domains I and II, phosphospecific sequence-directed antibodies were generated against the phosphopeptides SH-(521–531)-Ser526-P and SH-(526–535)-Ser529-P as described under “Experimental Procedures.” Fig. 9A shows that anti-(SH-(521–531)-Ser526-P) did not react with unphosphorylated NaFH3 but reacted with NaFH3 previously phosphorylated by PKA. Anti-(SH-(521–531)-Ser526-P) also reacted strongly with the mutant fusion protein NaFH-S529A after it was phosphorylated by PKA. Anti-(SH-(521–531)-Ser526-P), however, did not react with either phosphorylated NaFH-S525/6A or NaFH-S526A. These results are consistent with data presented above which indicate that PKA phosphorylates Ser526 in the fusion protein NaFH3.

Fig. 9B shows that anti-(SH-(526–535)-Ser529-P) did not react with unphosphorylated NaFH3, whereas it reacted with NaFH3 previously phosphorylated by PKA. Anti-(SH-(526–535)-Ser529-P) reacted strongly with the mutant fusion proteins NaFH-S525/6A and NaFH-S526A after phosphorylation by PKA but not in the unphosphorylated state (Fig. 9B). Anti-(SH-(526–535)-Ser529-P), however, did not react with phosphorylated NaFH-S529A. These results are consistent with our finding that PKA phosphorylates Ser529 in the fusion protein NaFH3.

Phosphorylation of Ser526 and Ser529 of the Cardiac Na+ Channel a Subunit in Intact Cells in Response to Activation of PKA—We next determined whether Ser526 and Ser529 were phosphorylated in intact cells by PKA in response to changes in the concentration of intracellular cAMP. To address this question we utilized the technique of “back-phosphorylation.” Cells expressing the cardiac Na+ channel a subunit were incubated in the absence or presence of the cell permeant cAMP analog DCl-eBIMPS to activate intracellular PKA. Cells were then solubilized, and the Na+ channel a subunit was isolated by immunoprecipitation and incubated under phosphorylating conditions in the presence of PKA and [γ-32P]ATP as described under “Experimental Procedures.” In this experiment, a decrease in the in vitro PKA-mediated incorporation of 32P into the a subunit from treated cells is proportional to cAMP-stimulated phosphorylation of the a subunit in situ in intact cells. PKA-mediated in vitro incorporation of 32P into the a subunit was reduced by 65% ± 2% (n = 3) in cells pretreated for 10 min with 100 μM DCl-eBIMPS (Fig. 10, A and B). To assess the effect of stimulation with DCl-eBIMPS on the PKA-mediated 32P incorporation into specific Ser residues, we generated tryptic phosphopeptide maps of the a subunit isolated from treated and untreated cells. Fig. 11A shows the phosphopeptide map of the a subunit derived from untreated cells which contains the major phosphopeptides I and II seen in previous maps (Fig. 2A). Diminished back-phosphorylation of phosphopeptides I and II was observed in the phosphopeptide map derived from DCl-eBIMPS-treated cells (Fig. 11B). Fig. 11A also shows that the minor variable phosphopeptide near the position of phosphopeptide III was present in this experiment and was not diminished by the treatment of cells by DCl-eBIMPS. These results indicate that this phosphopeptide is not phosphorylated in intact cells by PKA in response to treatment with a membrane-permeant derivative of cAMP. Together these results indicate that both Ser526 and Ser529 are phosphorylated by PKA in intact cells in response to increased intracellular levels...
subunit but did not abolish the observed effect of the kinase. Of the five potential PKA sites mutated, one was located in the amino terminus, two were located in the intracellular loop connecting homologous domains II and III, and two were located in the intracellular loop connecting homologous domains I and II. Neither of these sites in L_{4-11} corresponded to Ser^{256} or Ser^{269}. Thus, previous experiments have not succeeded in identifying sites that are required for the physiological effect of phosphorylation by PKA, possibly because the consensus sites that have been examined by mutagenesis are not actually phosphorylated in vivo.

Phosphorylation of Cardiac Na⁺ Channel a Subunit Isolated from Mammalian Cells—Our results show that cardiac Na⁺ channels immunoprecipitated from mammalian cells expressing the rat cardiac Na⁺ channel a subunit are phosphorylated by PKA in vitro. These results are in agreement with those of Gordon et al. (1) and Cohen and Levitt (2), which showed that Na⁺ channel immunoprecipitated from solubilized rat cardiac membranes is a substrate for PKA in vitro. PKA phosphorylation of rat cardiac a subunit occurs exclusively on serine residues, and the a subunit is phosphorylated by PKA in intact cells in response to increases in intracellular levels of cAMP.

Principal Sites of Phosphorylation of the Cardiac Na⁺ Channel a Subunit—Since brain Na⁺ channel activity is modulated by phosphorylation of the a subunit in L_{4-11} (12, 13, 15, 16), we focused on this region as a possible target for PKA phosphorylation in the rat cardiac Na⁺ channel. This loop contains 11 consensus sequences for PKA with the motifs of KRXXS, RXXS, and RXS but does not contain any serine residues within the canonical RXXS motif. A fusion protein containing most of L_{4-11} was a substrate for PKA in vitro, and the tryptic phosphopeptide map of ^32P-labeled NaFH1 was similar to the map obtained from ^32P-labeled a subunit isolated from intact cells, indicating that the same residues were being phosphorylated in NaFH1 as in the full-length channel. These results demonstrate that in vitro, PKA phosphorylates the cardiac Na⁺ channel a subunit solely on residues contained in the intracellular loop connecting domains I and II.

Using a series of truncated fusion proteins within L_{4-11}, we narrowed the phosphorylation sites to the sequence between residues 511 and 530. This region contains eight Ser residues including three (Ser^{256}, Ser^{256}, and Ser^{269}) in RXS consensus motifs for PKA recognition. Comparison of tryptic phosphopeptide maps of wild type and mutant fusion proteins showed that Ser^{256} was required for the generation of phosphopeptides I, II, and IV, whereas Ser^{256} was required for the generation of phosphopeptides I, III, and V. A mutant fusion protein (NaFH-S925/69/8A) in which Ser^{256}, Ser^{256}, and Ser^{256} were all mutated to alanine residues was a very poor substrate for PKA, consistent with the conclusion that Ser^{256} and Ser^{256} are the major sites of phosphorylation. The low level of residual phosphorylation of NaFH-S925/69/8A is not surprising since this fusion protein still contains 17 other Ser residues including four in RXS consensus motifs for PKA recognition. It is likely, therefore, that in the absence of the preferential phosphorylation sites (Ser^{256} and Ser^{256}), PKA phosphorylates these other Ser residues to a very low extent. Consistent with this conclusion, the two-dimensional tryptic phosphopeptide map of the low level of phosphopeptides derived from phosphorylation of NaFH-S925/6/8A does not contain spots that coincide with maps of NaFH1 and full-length a subunit (data not shown). Thus, all of our results support the conclusion that Ser^{256} and Ser^{256} are the principal sites of phosphorylation in the a subunit of the cardiac Na⁺ channel.

Origin of Multiple Phosphopeptides—We detected two major and three minor phosphopeptides in our tryptic two-dimen-

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**FIG. 10.** cAMP-dependent phosphorylation of rat cardiac Na⁺ channel a subunit in intact cells. Panel A, SNa-rH1 cells expressing the rat cardiac Na⁺ channel a subunit were incubated in the absence (control, CONT) or presence of the PKA activator DCI-cBIMPS, solubilized in 1% Triton X-100, and the a subunit was isolated by immunoprecipitation as described under “Experimental Procedures.” Isolated a subunit was then back-phosphorylated in the presence of PKA and [γ-^32P]ATP and analyzed by SDS-PAGE on a 6% Laemmli gel. ^32P- Labeled a subunit was visualized by autoradiography. Molecular weight markers are represented as M̅, 10^6. Panel B, ^32P incorporated into the cardiac Na⁺ channel a subunit was quantitated by liquid scintillation counting; 100% equals ^32P incorporated into the a subunit of untreated cells. Results are the average of three separate experiments ± S.E.

**FIG. 11.** Two-dimensional tryptic mapping of back-phosphorylated Na⁺ channel a subunit isolated from control and DCI-cBIMPS-treated cells. ^32P-Labeled back-phosphorylated cardiac Na⁺ channel a subunits isolated from control (panel A) and DCI-cBIMPS-treated SNa-rH1 cells (panel B) were analyzed by SDS-PAGE on a 6% Laemmli gel, located by autoradiography, and processed for tryptic phosphopeptide mapping as described under “Experimental Procedures.” Tryptic phosphopeptides were separated in two dimensions by high voltage electrophoresis (pH 8.9) followed by thin layer chromatography. Arrows designate the direction of electrophoresis (+) and chromatography (C).

**DISCUSSION**

Cardiac Na⁺ Channels Are Regulated by cAMP-dependent Phosphorylation—Cardiac voltage-sensitive Na⁺ channels are modulated by activation of β-adrenergic receptors acting through both direct and indirect pathways (17–22). The effect of adrenergic agents on cardiac Na⁺ channel activity is significant, suggesting that it may be an important physiological mechanism in the heart. Recent mutagenesis work by Schreibmayer et al. (8) has shown that activation of β2 receptors in Xenopus oocytes expressing the a subunit of the rat cardiac Na⁺ channel modulates Na⁺ channel activity. These authors mutated five potential PKA phosphorylation sites in the a
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Sional maps which apparently are generated from phosphorylation of only two Ser residues, Ser526 and Ser529. Trypsin cleaves at the carboxyl side of Lys and Arg residues. The amino acid sequence surrounding Ser526 and Ser529 contains Arg at positions 533, 544, and 545. Tryptic cleavage may occur at one or more of these positions generating multiple peptides. In addition, the Arg at position 522 is followed by a Pro residue at position 523. Since trypsin does not cleave Arg-Pro bonds efficiently (30), multiple peptides could be generated by incomplete cleavage at this site.

Our data show that mutation of either Ser525 or Ser529 caused the loss of phosphopeptide I. This can be explained based on the specificity of trypsin cleavage. Trypsin does not cleave efficiently after Arg residues in the sequence RXS when the serine is phosphorylated (30). If Ser526 and Ser529 were both phosphorylated, trypsin would not cleave after Arg254 or Arg257 efficiently (Fig. 3B), so it is plausible that phosphopeptide I represents a doubly phosphorylated peptide including Ser526 and Ser529. When Ser526 is mutated to Ala to prevent phosphorylation, trypsin can cleave after Arg254 more efficiently. Therefore, phosphopeptides I, III, and IV are absent from tryptic maps of NaFH-S526A. When Ser529 is mutated to Ala to prevent phosphorylation, trypsin can cleave after Arg257 more efficiently. Therefore phosphopeptides I, II, and IV are absent from tryptic maps of NaFH-S529A. When Ser529 is mutated to Ala to prevent phosphorylation, trypsin can cleave after Arg257 more efficiently. Therefore phosphopeptides I, II, and IV are the major tryptic fragments in the maps of phosphorylated α subunit isolated from mammalian cells and are present in roughly equal intensity. The fact that phosphopeptide V, which is only generated when Ser526 but not Ser529 is phosphorylated, is only a minor component in maps of the full-length α subunit suggests that Ser529 is phosphorylated extensively and therefore the preferential site of phosphorylation of the α subunit by PKA. Studies using synthetic peptides have shown that a hydrophobic residue immediately following the phosphorylated residue is an important determinant of high reactivity of PKA (31, 32). Ser529 is followed immediately by Ile, whereas Ser526 is followed by an Arg residue. This is consistent with the conclusion that Ser529 is the preferred site for PKA phosphorylation.

Comparison with Physiological Studies—As mentioned above, Schreibmayer et al. (8) mutated two Ser residues in L1-L4, as well as several residues elsewhere, and found no effect on regulation of Na⁺ channel function by protein phosphorylation. The sites in L1-L4 correspond to Ser485 and Ser594 in our studies (Fig. 3B). Thus, their results are consistent with ours in that we do not detect cAMP-dependent phosphorylation of either Ser485 or Ser594 in vitro or in intact cells in response to cAMP stimulation. In contrast, our results show that the rat cardiac Na⁺ channel α subunit is phosphorylated in vitro and in intact cells in a cAMP-dependent manner on Ser526 and Ser529 and therefore suggest that Ser526 and/or Ser529 may play a role in the cAMP-dependent regulation of cardiac Na⁺ channel activity. Identification of these phosphorylation sites will allow the design of mutagenesis experiments to determine the precise role of phosphorylation of these amino acid residues in the modulation of cardiac Na⁺ channel activity.

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REFERENCES

1. Gordon, D., Merrick, D., Wellner, D. A., and Catterall, W. A. (1988) Biochemistry 27, 7032–7038
2. Cohen, S. A., and Levitt, L. K. (1993) Circ. Res. 73, 735–742
3. Rogart, R. B., Cristis, L. K., Maglia, D. D., Kephart, D. L., and Kaiser, M. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8170–8174
4. Kallen, R. G., Sheng, Z. H., Yang, J., Chen, L. Q., Rogart, R. B., and Barchi, R. L. (1990) Neuron 4, 233–242
5. Wu, Y., Rogers, J., Tanada, T., Scheuer, T., and Catterall, W. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3283–3289
6. Cristis, L. L., Satin, J., Fozzard, H. A., and Catterall, W. A. (1990) FERS Lett. 275, 195–200
7. Gellens, M. E., George, A. L., Jr., Chen, L. Q., Chahine, M., Horn, R., Barchi, R. L., and Kallen, R. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 554–558
8. Schreibmayer, W., Frohnwieser, W., Dascal, N., Platter, D., Spreitzer, B., Zechner, R., Kallen, R. G., and Lester, H. A. (1994) Receptors Channels 2, 339–350
9. White, M. M., Chen, L. Q., Kleinfield, R., Kallen, R. G., and Barchi, R. L. (1991) Mol. Pharmacol. 39, 604–608
10. Costa, M. R. C., Casnellie, J. E., and Catterall, W. A. (1982) J. Biol. Chem. 257, 7918–7921
11. Costa, M. R. C., and Catterall, W. A. (1984) J. Biol. Chem. 259, 8210–8218
12. Murphy, B. J., Rossie, S., De Jongh, K. S., and Catterall, W. A. (1993) J. Biol. Chem. 268, 27355–27362
13. Rossie, S., and Catterall, W. A. (1987) J. Biol. Chem. 262, 12735–12744
14. Gershon, E., Weigl, L., Lotan, I., Schreibmayer, W., and Dascal, N. (1992) J. Neurosci. 12, 3743–3752
15. Li, M., West, J. W., Lai, Y., Scheuer, T., and Catterall, W. A. (1992) Neuron 8, 1151–1159
16. Smith, R. A., and Goldin, A. L. (1996) J. Neurosci. 16, 1965–1974
17. Schubert, B., VanDongen, A. M., Kirsch, G. E., and Brown, A. M. (1989) Science 245, 516–519
18. Ono, K., Kiyosue, T., and Arita, M. (1989) Am. J. Physiol. 256, C1313–C1317
19. Sunami, A., Fan, Z., Nakamura, F., Naka, M., Tanaka, T., Sawanobori, T., and Hirase, M. (1991) J. Pflugers Arch. 419, 415–417
20. Matsuda, J. J., Lee, H., and Shibata, E. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 59–70
21. Ono, K., Fozzard, H. A., and Hanek, D. A. (1993) Circ. Res. 72, 807–815
22. Frohnwieser, B., Weigl, L., and Schreibmayer, W. (1993) J. Pflugers Arch. 400, 751–753
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Murphy, B. J., and Catterall, W. A. (1992) J. Biol. Chem. 267, 16129–16134
25. De Jongh, K. S., Murphy, B. J., Colvin, A. A., Hell, J. W., Takahashi, M., and Catterall, W. A. (1996) Biochemistry 35, 10392–10402
26. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 85, 51–59
27. West, J. W., Scheuer, T., Maechler, L., and Catterall, W. A. (1992) Neuron 8, 59–70
28. Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2154–2159
29. Gordon, D., Merrick, D., Auld, V., Dunn, R., Goldin, A. L., Davidson, N., and Catterall, W. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8862–8866
30. Hardie, D. G., Campbell, D. G., Caudwell, F. B., and Haystead, T. A. J. (1993) in Protein Phosphorylation: A Practical Approach (Hardie, D. G., ed) pp. 61–85, IRL Press, Oxford
31. Meggio, F., Chessa, G., Borin, G., Pinna, L. A., and Marchiori, F. (1980) Biochem. Biophys. Acts 662, 94–101
32. Songyang, Z., Blechner, S., Hoagland, N., Hockstra, M. F., Pfwna-Worms, H., and Cantley, L. C. (1994) Curr. Biol. 4, 973–982