Depolarization of rat brain synaptosomes increases phosphorylation of voltage-sensitive sodium channels*

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Depolarization of rat brain synaptosomes causes an increase in phosphorylation of serine residues 573, 610, 623, and 687 on voltage-sensitive sodium channels. Although these sites have been shown to be phosphorylated by cAMP-dependent protein kinase in vitro and in situ, the depolarization-induced increase in their state of phosphorylation is not due to increased cAMP-dependent protein kinase activity, but requires calcium influx and protein kinase C. Since phosphorylation at this cluster of sites inhibits sodium current and would decrease neuronal excitability, this may be an important negative feedback mechanism whereby calcium influx during prolonged or repetitive depolarization can attenuate neuronal excitability and prevent further calcium accumulation. Phosphorylation of purified channels by protein kinase C decreases dephosphorylation of cAMP-dependent phosphorylation sites by purified calcineurin or protein phosphatase 2A. This suggests that one mechanism by which protein kinase C may increase phosphorylation of cAMP-dependent phosphorylation sites in sodium channels is to inhibit their dephosphorylation. This represents an important new mechanism for convergent regulation of an ion channel by two distinct signal transduction pathways.

Sodium influx through voltage-sensitive sodium channels is responsible for the initiation and propagation of action potentials in most neurons, and ultimately promotes the opening of voltage-sensitive calcium channels at the synapse, leading to calcium influx and exocytosis. Because of their central role in these processes, the response properties of sodium channels can control neuronal excitation and secretion. Phosphorylation of brain sodium channels by PKA† and PKC dramatically alters their electrophysiological responses. Cyclic AMP-dependent phosphorylation decreases sodium current in response to membrane depolarization (1, 2) due to a decrease in the probability of channel opening (1). Phosphorylation by PKC slows channel inactivation (3), decreases sodium current (3–5), and is required for the electrophysiological effect of cAMP-dependent phosphorylation to be observed (6). Two electrophysiological studies have demonstrated that neurotransmitters modulate sodium channel function via second messenger-induced phosphorylation. Stimulation of D1 dopamine receptors in dissociated rat nigrostriatal neurons reduces sodium current in a cAMP-dependent manner (7). Stimulation of muscarinic receptors in cultured hippocampal neurons, via activation of PKC, also reduces sodium current (8).

The α subunit of brain sodium channels is phosphorylated by PKA on multiple serine residues clustered in a single intracellular loop between homologous domains I and II of the channel protein (9–11). Replacement of this loop with the corresponding loop from a skeletal muscle sodium channel lacking these phosphorylation sites abrogates the cAMP-mediated inhibition of channels expressed in Xenopus oocytes (12). Thus, this intracellular segment contains all phosphorylation sites necessary for cAMP-dependent inhibition of brain sodium channels.

In the present study, we observed that depolarization of rat brain synaptosomes caused a calcium-dependent increase in phosphorylation of cAMP-dependent phosphorylation sites on sodium channels. This response was not due to increased PKA activity, but required activation of PKC. These results suggest that accumulation of calcium and activation of PKC in nerve terminals may lead to increased phosphorylation and consequent decreased responsiveness of sodium channels. We also found that purified reconstituted sodium channels phosphorylated by both PKC and PKA were poorly dephosphorylated by purified calcineurin or PP2A, in contrast to sodium channels phosphorylated by PKA alone. This observation suggests one potential mechanism by which PKC activation during synaptic depolarization could lead to an increase in phosphorylation of cAMP-dependent phosphorylation sites on sodium channels. The ability of one phosphorylation site on a given substrate to control dephosphorylation of a separate site represents a potentially important molecular mechanism for conditional or convergent regulation of the responses of ion chan-

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¶ The abbreviations used are: PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2A; (8)-cAMPs, adenosine 3′,5′-cyclic monophosphorothioate-(R); BIM, bisindolylmaleimide; PMA, phorbol 12-myristate 13-acetate; MPMA, 4-O-methylphorbol 12-myristate 13-acetate.
nals and other phosphoproteins by distinct signal transduction pathways.

**EXPERIMENTAL PROCEDURES**

**Materials, Purified Proteins, and Antibodies**—Sodium channels were purified through the wheat germ-agglutinin chromatography step according to Hartshorne and Catterall (13). Heterotrimeric PP2A containing a mixture of PP2Aβ and PP2Aγ was purified from rat brain as described previously (14). The catalytic subunit of PKA was purified from bovine heart (15). Monoclonal antibody 1G11 generated toward cAMPS, okadaic acid, sodium channels were immuno-

phosphopeptide maps were generated from SDS-polyacrylamide gel
described previously (14). The catalytic subunit of PKA was purified
from bovine heart (15). Okadaic acid was included during treatment with calcineurin to inhibit low levels of okadaic acid-sensitive phosphatase activity present in wheat germ-agglutinin purified sodium channel preparations.2 Samples were incubated at 37 °C for varying amounts of time, then the reaction was terminated with 100 μl of 10% trichloroacetic acid and 10 μl of 10 mg/ml bovine serum albumin. Protein was then sedimented, and acid-soluble [32P]P, was quantified.

**RESULTS**

Depolarization of Synaptosomes Increases the Phosphorylation of cAMP-dependent Phosphorylation Sites in Sodium Channels—Back phosphorylation (23) was used to measure changes in phosphorylation of cAMP-dependent phosphorylation sites on sodium channels. In this technique, endogenous phosphate is incorporated into channels in synaptosomes during experimental treatments, then channels are isolated under conditions preventing dephosphorylation and treated with the catalytic subunit of PKA and [γ-32P]ATP to incorporate radioactive phosphate into sites that were not phosphorylated in situ. Thus, a decrease in back phosphorylation reflects an increase in endogenous phosphorylation. Back phosphorylation of immunopurified sodium channels with the catalytic subunit of PKA specifically labels serine residues 573, 610, 623, and 687 of rat brain type IIA sodium channels (11). Treatments that elevate CAMP in synaptosomes (24) or cultured rat brain cells (19) block subsequent back phosphorylation of these sites, indicating that these sites are phosphorylated by PKA in situ. Serine residues 573, 610, 623, and 687 will be collectively referred to as cAMP-dependent phosphorylation sites in this study. It is not known whether other kinases also phosphorylate these sites in vivo.

Depolarization of synaptosomes by high potassium in the presence of calcium decreased back phosphorylation of channel cAMP-dependent phosphorylation sites, indicating an increase in endogenous phosphorylation (Fig. 1). The magnitude of this response varied from experiment to experiment, but ranged from 25 to 50% in 30 independent experiments. In the absence of calcium no change in phosphorylation occurred, suggesting that the increase in phosphorylation during depolarization requires calcium influx.

The Depolarization-induced Increase in Channel Phosphorylation Is Not Due to an Increased PKA Activity—The role of PKA in this response was examined. Depolarization of synaptosomes did not increase PKA activity (Fig. 2). In addition, the increase in channel phosphorylation caused by depolarization was not blocked by pretreatment with the inhibitory CAMP derivative, (R)-cAMPS (25) (Fig. 3). In contrast, forskolin increased PKA activity in synaptosomes and decreased back phosphorylation of sodium channels; both of these responses were blocked by (R)-cAMPS (Figs. 2 and 3). Treatment with (R)-cAMPS alone had no effect on control kinase activity or channel phosphorylation. These results indicate that the depolarization-induced increase in phosphorylation of cAMP-dependent phosphorylation sites on sodium channels is not

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2 B. Law and S. Rossie, unpublished observation.
caused by increased PKA activity. However, since (R<P>)cAMPS may not completely block basal PKA activity (26), basal PKA activity may still be responsible for the accumulation of phosphate in cAMP-dependent phosphorylation sites that occurs during depolarization.

The Response to Depolarization Requires PKC and Calcium—Because depolarization of synaptosomes increases PKC-mediated phosphorylation (27, 28) and sodium channels are a potential substrate for PKC phosphorylation in synaptosomes (29), the involvement of PKC in this response was examined. Treatment of synaptosomes with PMA (100 ng/ml) mimicked the effect of depolarization on back phosphorylation of sodium channels (Fig. 4A). The response to depolarization or PMA treatment was blocked by BIM (1 μM), a specific inhibitor of PKC (30, 31), suggesting that the effect of depolarization on sodium channel phosphorylation is mediated by PKC. The response to PMA, like that to depolarization, was prevented by removal of buffer calcium. Treatment of synaptosomes with BIM alone (Fig. 4A) or with the inactive phorbol ester MPMA (Fig. 4B) had no effect on channel phosphorylation.

Protein kinase C and PKA both phosphorylate Ser-610 in vitro, but other phosphorylation sites are not shared (20). To determine whether the increase in endogenous phosphorylation at cAMP-dependent phosphorylation sites was due solely to phosphorylation of Ser-610, tryptic phosphopeptide maps of back phosphorylated sodium channel α subunits were prepared and the changes in phosphorylation for each of the four cAMP-dependent phosphorylation sites were quantified. As shown in Fig. 5, depolarization affected all four cAMP-dependent phosphorylation sites to a similar extent. This demonstrates that the depolarization-induced increase in phosphorylation of cAMP-dependent phosphorylation sites is not solely due to the direct phosphorylation of Ser-610 by PKC. However, we cannot rule out the possibility that PKC phosphorylates additional cAMP-dependent phosphorylation sites in vivo, since each of these sites...
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### DISCUSSION

This study suggests that an important physiologic stimulus, neuronal depolarization, influences the responsiveness of voltage-dependent brain sodium channels by controlling the state of phosphorylation of cAMP-dependent phosphorylation sites. In addition, two novel aspects of the role of PKC in controlling sodium channel phosphorylation are revealed; PKC is required for the effect of synaptosomal depolarization on sodium channel phosphorylation, and the direct phosphorylation of purified sodium channels by PKC reduces the ability of phosphatases to attack cAMP-dependent phosphorylation sites.

### FIG. 5
Analysis of individual cAMP-dependent phosphorylation sites of sodium channels after depolarization of synaptosomes. Sodium channels from control or depolarized synaptosomes were isolated and back phosphorylated, then trypsin phosphopeptide maps were prepared from \(^{32}\)P-labeled sodium channel α subunits. The \(^{32}\)P content of phosphopeptides corresponding to each cAMP-dependent phosphorylation site was quantified. Results are expressed as the percent decrease in back phosphorylation compared with control samples and represent the average ± S.E. of three experiments.

### FIG. 6
The effect of phosphatase inhibitors and depolarization on sodium channel phosphorylation. Synaptosomes were treated with 300 nM okadaic acid (O.A.) for 10 min or 1 μM cyclosporin A for 30 min, and depolarized (depol) or treated with control buffer during the last minute of incubation. After treatment, channels were isolated and back phosphorylated. Results are expressed as the percent decrease in back phosphorylation compared with control samples and represent the average ± S.E. of three experiments.

### FIG. 7
Dephosphorylation of reconstituted purified sodium channels after phosphorylation with PKA alone, PKC alone, or PKA and PKC. Purified sodium channels were reconstituted and phosphorylated with the catalytic subunit of PKA (●), with PKC (○), or with both kinases (▲), then treated with calcineurin (A) or PP2A (B), and phosphate release was assessed as described under "Experimental Procedures." Results are expressed as moles of P\(_i\) released per mol of phosphoserine present in each reaction and represent the average ± S.E. of seven experiments.
be investigated in synaptosomes. Although it is not clear whether the role of PKC in the depolarization-induced elevation of sodium channel phosphorylation is due to a direct phosphorylation of sodium channels or is indirect, it is unlikely that PKC acts solely by promoting calcium influx or membrane depolarization. Although PKC inhibition during synaptosomal depolarization decreases calcium influx and membrane depolarization to a moderate extent (37), treatment of synaptosomes with phorbol ester has little or no effect on calcium levels (38, 39) or membrane depolarization (39) and does not enhance either of these responses to treatment with high potassium (37). In the present study, PMA increased sodium channel phosphorylation in the absence of a depolarizing stimulus, conditions under which calcium elevation is not expected (38, 39). Although the effect of PMA was prevented by incubating synaptosomes in a calcium-free medium, the loss of responsiveness may be explained by a depletion of calcium in synaptosomes during treatment with calcium-free buffer containing EGTA. Others have demonstrated that PKC can phosphorylate different synaptosome substrates depending on the means of activation (40). To determine whether PKC directly phosphorylates sodium channels during depolarization, the phosphorylation of Ser-1506 should be measured. Although phosphorylation of this site in vivo has not yet been examined, it is phosphorylated by PKC but not PKA in vitro (20) and this site plays a key role in the electrophysiologic effects of PKC on sodium channels (6).

In addition to its essential role in initiating exocytosis, depolarization-induced calcium influx can modulate the subsequent excitability of the synaptic terminal by activating a host of calcium-dependent enzymes which can alter ion channel function (41, 42). Because phosphorylation at cAMP-dependent phosphorylation sites inhibits brain sodium channels, the enhanced state of channel phosphorylation after depolarization would be expected to inhibit subsequent synaptic excitation, decreasing further calcium influx and secretion. However, a number of studies suggest that activation of PKC enhances neurosecretion (reviewed in Refs. 39 and 43). One possible explanation reconciling these two views may be that rapid or early PKC-mediated phosphorylation events enhance secretion, whereas the increase in channel phosphorylation at cAMP-dependent sites that we observe may require periods of repetitive or sustained depolarization. Early PKC-mediated events may include the phosphorylation of sodium channels at Ser-1506. Phosphorylation at this site slows channel inactivation and would be expected to prolong depolarization and promote calcium influx. Thus, one possible scenario may be that depolarization and calcium influx activates PKC, which phosphorylates a number of target proteins, including sodium channels on Ser-1506, enhancing neurosecretion. During repetitive or prolonged depolarization, the phosphorylation of cAMP-dependent phosphorylation sites is gradually enhanced due to increased phosphorylation, decreased dephosphorylation, or both. This leads to inhibition of sodium channels during subsequent depolarizing stimuli. This may represent a negative feedback mechanism by which neurons are protected from accumulating dangerously high levels of calcium during periods of prolonged depolarization or hyperexcitation. Several cases have been described in which phosphorylation of one site on a given substrate controls the phosphorylation of another site (44). Although fewer examples of one phosphorylation site controlling the dephosphorylation of a separate phosphorylation site have been reported, several important cases have recently been described. These include an enhanced rate of phosphotyrosine dephosphorylation in the presence of a nearby phosphothreonine residue on mitogen-activated protein kinase by dual-specificity phosphatases (45, 46), the inhibition of calcineurin-mediated dephosphorylation of neuronal DARPP-32 at Thr-34 by casein kinase I-mediated phosphorylation at Ser-137 (47), and the resistance to dephosphorylation of PKCs induced by phosphorylation of Thr-638 (48). Such interaction between phosphorylation sites represents an important molecular mechanism for the conditional regulation of a given response. Our results with purified sodium channels suggest that the ability of phosphatases to dephosphorylate cAMP-dependent phosphorylation sites on sodium channels is modulated by PKC phosphorylation of the channel itself. This effect may contribute to the convergent regulation of sodium channel function by PKC and PKA, observed in electrophysiological studies (6), and to the increased phosphorylation of cAMP-dependent phosphorylation sites during synaptosomal depolarization. Since PKC phosphorylation of Ser-1506 is critical for cAMP-dependent inhibition of sodium channel function (6), it will be important to learn if phosphorylation of Ser-1506 is responsible for the effect on dephosphorylation of cAMP-dependent phosphorylation sites observed in vitro.

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