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Voltage-Dependent Neuromodulation of Na^+ Channels by D1-Like Dopamine Receptors in Rat Hippocampal Neurons

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Activation of D1-like dopamine (DA) receptors reduces peak Na^+ current in acutely isolated hippocampal neurons through phosphorylation of the α subunit of the Na^+ channel by cAMP-dependent protein kinase (PKA). Here we report that neuromodulation of Na^+ currents by DA receptors via PKA is voltage-dependent in the range of -110 to -70 mV and is also sensitive to concurrent activation of protein kinase C (PKC). Depolarization enhanced the ability of D1-like DA receptors to reduce peak Na^+ currents via the PKA pathway. Similar voltage-dependent modulation was observed when PKA was activated directly with the membrane-permeant PKA activator DCl-cBIMPS (cBIMPS; 20 μM), indicating that the membrane potential dependence occurs downstream of PKA. PKA activation caused only a small (-2.9 mV) shift in the voltage dependence of steady-state inactivation and had no effect on slow inactivation or on the rates of entry into the fast or slow inactivated states, suggesting that another mechanism is responsible for coupling of membrane potential changes to PKA modulation. Activation of PKC with a low concentration of the membrane-permeant diacylglycerol analog oleylacetyl glycerol also potentiated modulation by SKF 81297 or cBIMPS, and these effects were most striking at hyperpolarized membrane potentials where PKA modulation was not stimulated by membrane depolarization. Thus, activation of D1-like DA receptors causes a strong reduction in Na^+ current via the PKA pathway, but it is effective primarily when it is combined with depolarization or activation of PKC. The convergence of these three distinct signaling modalities on the Na^+ channel provides an intriguing mechanism for integration of information from multiple signaling pathways in the hippocampus and CNS.

Key words: Na^+ current; neuromodulation; cAMP-dependent protein kinase; protein kinase C; hippocampus; dopamine receptors; phosphorylation

Voltage-gated Na^+ current is the primary inward current underlying excitability in the hippocampus and throughout the CNS. The α subunit of the neuronal voltage-gated Na^+ channel is a primary target for phosphorylation by both cAMP-dependent protein kinase (PKA) (Costa et al., 1982; Costa and Catterall, 1984; Rossie and Catterall, 1987; Murphy et al., 1993) and protein kinase C (PKC) (Costa and Catterall, 1984; Murphy et al., 1993). The neurotransmitters dopamine (DA) and acetylcholine modulate Na^+ currents via these signaling mechanisms in hippocampal neurons (Cantrell et al., 1996, 1997). Application of oleylacetyl glycerol (OAG), a membrane-permeant activator of PKC, reduces peak Na^+ current and slows the rate of inactivation of Na^+ channels expressed in *Xenopus* oocytes or cultured mammalian cells (Sigel and Baur, 1988; Dascal and Lotan, 1991; Numann et al., 1991; West et al., 1991). Activation of muscarinic acetylcholine receptors coupled to phospholipase C exerts a similar PKC-dependent inhibitory effect on Na^+ current in acutely isolated hippocampal pyramidal neurons (Cantrell et al., 1996).

The α subunit of brain Na^+ channels is phosphorylated by PKA at multiple consensus sites on the intracellular loop between domains I and II (Rossie et al., 1987; Rossie and Catterall, 1989; Murphy et al., 1993). PKA reduces peak Na^+ current amplitude in cultured rat brain neurons (Li et al., 1992) and in mammalian cells (Li et al., 1992) or *Xenopus* oocytes (Gershon et al., 1992; Smith and Goldin, 1996, 1997) expressing brain Na^+ channels. Similarly, activation of D1-like DA receptors, which couple to activation of adenylyl cyclase, decreases endogenous Na^+ current in acutely isolated striatonigral and hippocampal neurons (Surmeier et al., 1992; Schiffmann et al., 1995; Cantrell et al., 1997). This modulatory effect requires direct phosphorylation of the Na^+ channel α subunit by PKA at Ser 573 (Cantrell et al., 1997; Smith and Goldin, 1997).

Small changes in resting membrane potential can have dramatic effects on the integrative properties and on the threshold and frequency of action potential firing in central neurons (Jahnsen and Llinas, 1984; Hultborn and Kiehn, 1992; McCormick and Von Krosigk, 1992; Gorelova and Reiner, 1996; Kiehn et al., 1996; McCormick and Bal, 1997; Surmeier and Kitai, 1997). In the experiments reported here, we have examined the role of membrane potential and PKC activation in modulation of whole-cell Na^+ current by D1-like DA receptors in acutely isolated rat hippocampal pyramidal neurons. Our results show that membrane potential is a crucial determinant of the neuromodulation of Na^+ channels via the PKA pathway. Depolarization strongly enhances the effect of dopaminergic agonists and PKA activation. This voltage dependence does not reflect a simple shift in the steady-state inactivation of Na^+ channels after phosphorylation. Concurrent activation of PKC also enhances the ability of D1-like DA receptor activation to modulate the functional properties of the neuronal voltage-gated sodium current via a parallel pathway. These results provide the first evidence, to our knowledge, for voltage-dependent neuromodulation and raise the possibility that membrane voltage and PKC phosphorylation alter the extent or

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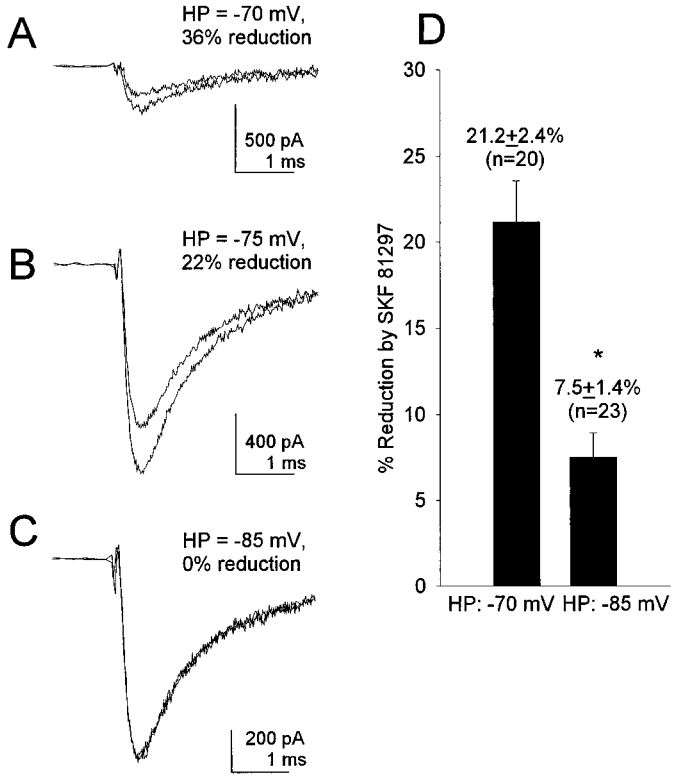

Figure 1. Modulation of whole-cell Na^+ current by D1-like dopamine receptor activation is enhanced at depolarized holding potentials in rat hippocampal neurons. *A–C*, Representative current traces elicited by a test pulse to −20 mV from the indicated holding potential in control and in the presence of 5 μM SKF 81297 (*smaller trace*). *D*, Bar graph summarizing the effect of membrane depolarization on the magnitude of SKF 81297 modulation in a population of neurons ($n \geq 20$ for each group). The mean of each population is indicated on the graph. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test).

pattern of phosphorylation of Na^+ channels by PKA and thereby alter neuromodulation of channel activity.

MATERIALS AND METHODS

Acute dissociation of hippocampal neurons

Hippocampal neurons from adult (>25 d postnatal) male rats were acutely isolated using procedures described previously (Bargas et al., 1994; Howe and Surmeier, 1995; Cantrell et al., 1996). In brief, rats were decapitated under metofane anesthesia. Brains were quickly removed, iced, and blocked before slicing. Slices (400–500 μm) were cut and transferred to a low-calcium (100 μM), HEPES-buffered saline solution containing (in mM): 140 Na isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES, pH 7.4, 300–305 mOsm/l. All solutions were bubbled with 100% O_2 before slicing. Slices were then incubated for 1–6 hr in $NaHCO_3$-buffered Earle's balanced salt solution (Sigma, St. Louis, MO) bubbled with 95% O_2, 5% CO_2, pH 7.4, 300–305 mOsm/l. Single slices were then removed into the low-calcium buffer, and with the aid of a dissecting microscope, regions of hippocampus were removed and placed in a treatment chamber containing protease type XIV (Sigma) (0.7 mg/ml) in HEPES-buffered HBSS (Sigma) at 35°C, pH 7.4, 300–305 mOsm/l. After 5–10 min of the enzyme treatment, the tissue was rinsed several times in the low-calcium buffer and mechanically dissociated using a series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm tissue culture dish (Corning, Corning, NY) mounted on the stage of an inverted microscope containing 1 ml of HEPES-buffered phosphate-free HBSS. After the cells were allowed to settle (~5 min), the solution bathing the cells was changed to normal external recording solution.

Mammalian cell transfection

tsA-201 cells, an embryonic kidney cell line stably transfected with simian virus 40 large tumor antigen (Robert Dubridge, Cell Genesis, Foster City, CA), were used for transfection experiments. tsA-201 cells were maintained in DMEM/F12 medium (Life Technologies/RBL) supplemented with 10% fetal calf serum (Hyclone), 25 U/ml penicillin, and 25 μg/ml streptomycin (Sigma). They were cotransfected with cDNA encoding the human CD8 marker protein (EBO-pCD-leu2; American Type Culture Collection) and a plasmid encoding wild-type or mutant rat brain type IIa Na^+ channel α subunit. Cells were transfected using the calcium phosphate precipitation method as described previously (Margolskee et al., 1993). Successfully transfected cells were then identified by labeling with magnetic polystyrene microspheres coated with anti-CD8 antibody (Jurman et al., 1994) (Dynabeads M-450 CD8, Dynal, Great Neck, NY).

Whole-cell recording

Hippocampal neurons. Whole-cell currents were recorded from pyramidally shaped hippocampal neurons that had at most one to two short processes (Hamill et al., 1981; Bargas et al., 1994; Howe and Surmeier, 1995). Electrodes were pulled from 75 μl micropipette glass (VWR Scientific, West Chester, PA) and fire-polished before use. The external recording solution consisted of (in mM): 20 NaCl, 10 HEPES, 1 $MgCl_2$, 0.4 $CdCl_2$, 55 CsCl, 5 $BaCl_2$, 80 glucose, pH 7.3 with NaOH, 300–305 mOsm/l. The internal recording solution consisted of (in mM): 188.9 *N*-methyl D-glucamine, 40 HEPES, 4 $MgCl_2$, 1 NaCl, 0.1 BAPTA, 25 phosphocreatine, 2–4 Na_2ATP, 0.2 Na_3GTP, 0.1 leupeptin, pH 7.2 with H_2SO_4, 270–275 mOsm/l. SKF 81297 (RBI, Natick, MA) and $PKCI_{19-36}$ (Peninsula Labs, Belmont, CA) were prepared as fresh concentrated stocks in water and frozen in aliquots before use. Sp-5,6-DCl-cBIMPS (cBIMPS; BioLog, LaJolla, CA) and OAG (Alexis Biochemicals, San Diego, CA) were prepared as concentrated stocks in DMSO and diluted before use. Appropriate vehicle controls were performed where necessary.

Electrode resistances were typically 3–6 MΩ in the bath. Final series resistance values averaged 6–8 MΩ, of which 80% was compensated electronically. The series resistance compensation did not change significantly during a typical experiment. Recordings were obtained using an Axon Instruments 1C patch clamp (Axon Instruments, Foster City, CA). Voltage-pulses were delivered and currents were recorded using a personal computer running Basic-FASTLAB software to control an AD/DA interface (IDA, Indec Systems, Sunnyvale, CA).

Pharmacological compounds were applied using a gravity-fed sewer pipe system. The array of application capillaries (~150 mm inner diameter) was positioned a few hundred micrometers away from the cell under study. Solution changes were made by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Irvine, CA). Complete solution changes were achieved within <1 sec as judged by the rate of TTX block of Na^+ current and changes in reversal potential in response to a change in Na^+ concentration.

tsA-201 cells. Whole-cell currents were recorded from successfully transfected cells identified using DynaBeads. The external recording solution consisted of (in mM): 140 NaCl, 10 HEPES, 1 $MgCl_2$, 0.4 $CdCl_2$, 25 CsCl, 5 $BaCl_2$, pH 7.3 with NaOH, 300–305 mOsm/l. The internal recording solution was identical to that described for hippocampal neurons. Other recording parameters were as described above.

Data analysis

Data were collected using standard voltage step protocols. Least-squares curve fitting and statistical analysis were performed using Sigma Plot (Jandel Scientific). Statistics are presented as means ± SEM.

RESULTS

Modulation of Na^+ current by the D1 dopamine receptor agonist SKF 81297 is enhanced by membrane depolarization

Activation of D1-like DA receptors, which are coupled to the stimulation of adenylyl cyclase, decreases endogenous Na^+ cur-

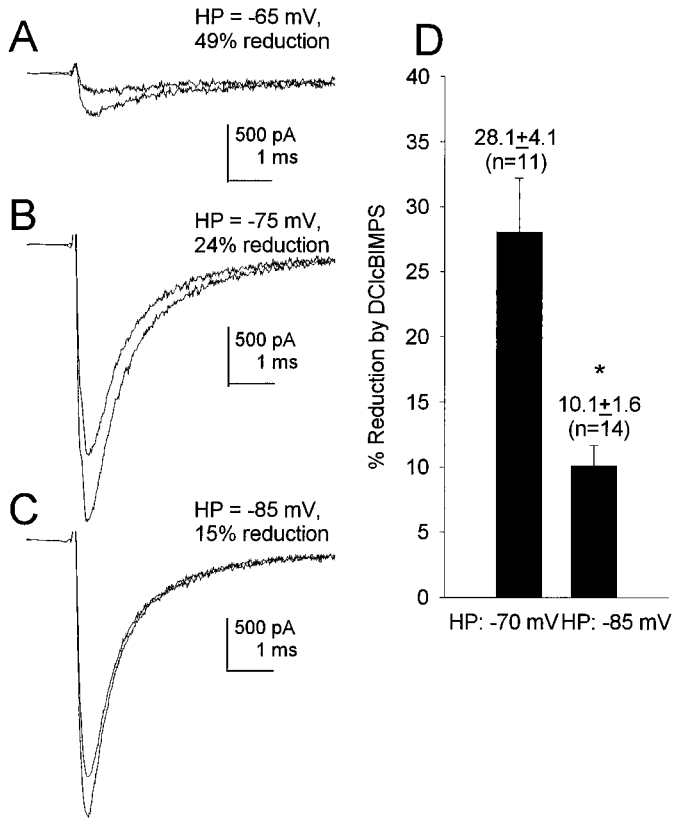

Figure 2. Membrane potential-dependent modulation of Na^+ current by cBIMPS in rat hippocampal neurons. *A–C*, Representative current traces elicited by a test pulse to −20 mV from the indicated holding potential in control and in the presence of 50 μM cBIMPS (*smaller trace*). *D*, Bar graph summarizing the effect of membrane depolarization on the magnitude of cBIMPS modulation in a population of neurons. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test).

rent in acutely isolated hippocampal neurons via phosphorylation by PKA (Cantrell et al., 1997). To determine whether membrane potential could affect physiological modulation of Na^+ currents by DA receptor activation, hippocampal neurons were studied under whole-cell voltage clamp using a holding potential of either −70 or −85 mV. From a holding potential of −70 mV, a 40 msec test pulse to −20 mV was applied once every 2 sec for 1–2 min in control solution until the Na^+ current magnitude stabilized. The D1 agonist, SKF 81297 (5 μM), was then applied. Reduction in peak Na^+ current was measured as the difference in the magnitude of the current elicited by the test pulse in control conditions and in the presence of the agonist. The agonist was then washed out of the bath and the current amplitude was allowed to recover. This control–drug–wash sequence was then repeated at the hyperpolarized potential. At a holding potential of −70 mV, application of SKF 81297 reduced peak Na^+ current by 21.2 ± 2.4% ($n = 20$) (Fig. 1*A,D*), but only by 7.5 ± 1.4% when the holding potential was −85 mV ($n = 23$) (Fig. 1*C,D*). Similar results were obtained when the control–drug–wash sequence was performed at the hyperpolarized potential and then repeated at the depolarized potential. Together, these results indicate that the effect of DA receptor activation on Na^+ current is voltage dependent.

Direct activation of PKA with the membrane-permeant activator cBIMPS (20 μM) revealed similar dependence on membrane potential (Fig. 2). Depolarization from −85 to −75 mV or −65 mV caused an increase in the percentage reduction of sodium current by treatment with cBIMPS from 15 to 49% (Fig. 2*A–C*). Application of cBIMPS at a holding potential of −85 mV reduced peak Na^+ current only 10.1 ± 1.6% ($n = 14$), but the reduction was 28.1 ± 4.1% ($n = 11$) at a holding potential of −70 mV (Fig. 2*D*). This experiment activates PKA downstream of adenylyl cyclase, placing the membrane potential-sensitive component of this regulatory pathway downstream of cAMP production. The most likely voltage-sensitive component in the pathway is the Na^+ channel itself. In contrast, PKC-dependent modulation of the Na^+ channel via muscarinic receptor activation as described previously (Cantrell et al., 1996) was not sensitive to membrane potential (data not shown).

Similar effects of membrane potential on PKA modulation of brain Na^+ channel α subunits expressed in tsA-201 cells

PKA modulation of Na^+ channels via PKA-dependent phosphorylation at Ser 573 is reproduced when type IIA Na^+ channel α subunits are heterologously expressed in human embryonic kidney tsA-201 cells (Cantrell et al., 1997). To determine whether depolarization of the membrane exerts similar effects on PKA-dependent regulation of Na^+ current in this heterologous expression system, we compared the reduction of peak Na^+ current in response to PKA stimulation by cBIMPS at holding potentials of −110 mV, −85 mV, and −70 mV. As shown in Figure 3, the magnitude of modulation was greatly increased by membrane depolarization, with an average reduction in peak current of 0.2 ± 0.2% ($n = 16$) at −110 mV and 19.6 ± 1.2% ($n = 64$) at −70 mV. Thus, voltage-dependent enhancement of Na^+ channel modulation by PKA is reconstituted when the Na^+ channel α subunit is expressed alone in a non-neuronal mammalian cell expression system.

We repeated the experiments shown in Figures 1 and 2 with 20 mM BAPTA in the intracellular solution to determine whether elevations in the intracellular calcium concentration might be responsible for the observed voltage dependence of the modulation. This concentration of BAPTA should be sufficient to rapidly buffer intracellular calcium to the low nanomole range. We found that the voltage-dependence of D1/PKA modulation was similar for cells dialyzed with 0.1 mM or 20 mM BAPTA in the intracellular solution, indicating that the intracellular calcium concentration is not a determining factor for this aspect of Na^+ channel modulation (data not shown).

The voltage dependence of D1/PKA modulation is not caused by a shift in voltage dependence of steady-state inactivation

The effects of membrane potential on D1/PKA modulation could result from a simple hyperpolarizing shift in the steady-state inactivation curve after phosphorylation, because this would cause a net increase in the inhibitory effect of phosphorylation as the cell is depolarized. We therefore tested whether PKA-dependent phosphorylation of the sodium channel α subunit had any effects on the voltage dependence of inactivation.

We first examined steady-state fast inactivation with a standard prepulse protocol. Na^+ currents were measured during test pulses to 0 mV after a series of 50 msec depolarizing prepulses between −110 and 50 mV. The current amplitude was plotted as a function of prepulse voltage and fit with a Boltzmann function to determine the half-inactivation voltage and slope factor. Inac-

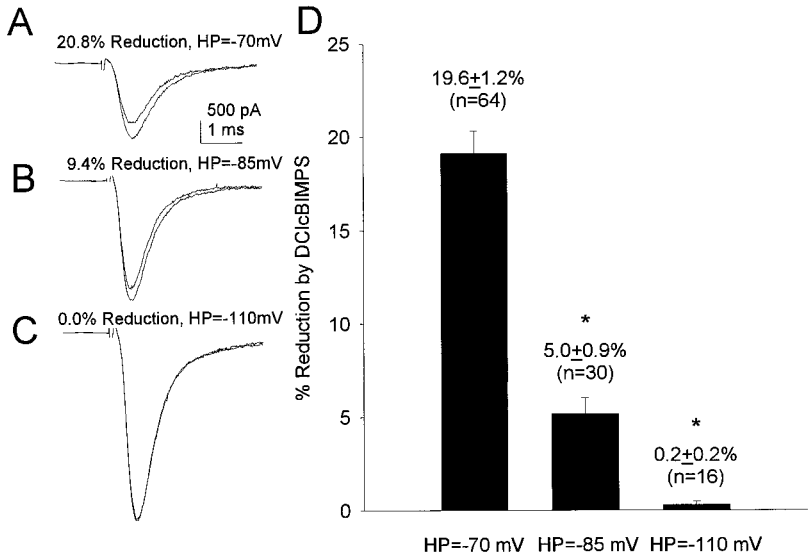

Figure 3. Membrane potential-dependent modulation of Na^+ current by cBIMPS in tsA-201 cells transiently transfected with type IIA Na^+ channel α subunits. *A–C*, Representative current traces elicited by a test pulse to 0 mV from the indicated holding potential in control and in the presence of 50 μM cBIMPS (*smaller trace*). *D*, Bar graph summarizing the effect of membrane depolarization on the magnitude of cBIMPS modulation in a population of tsA-201 cells expressing type IIA Na^+ channel α subunits. An *asterisk* indicates statistical significance ($p < 0.05$; Student's *t* test).

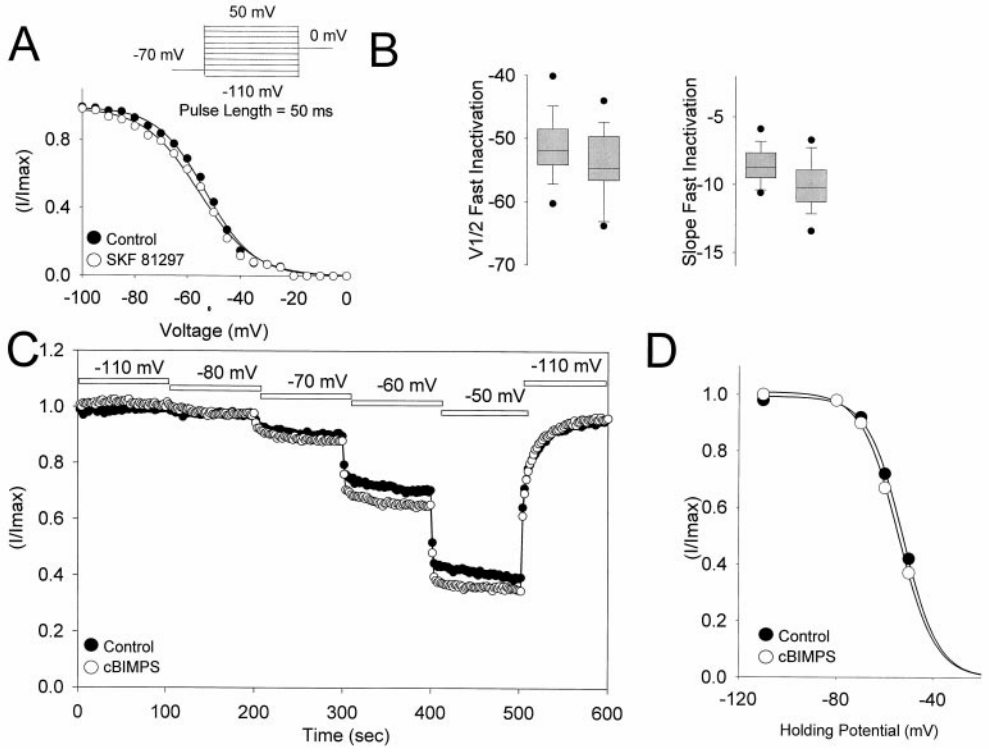

Figure 4. Effects of PKA phosphorylation on the voltage dependence of steady-state inactivation. *A*, From a holding potential of −70 mV, hippocampal neurons were depolarized to the indicated membrane potentials for 50 msec and then further depolarized to 0 mV to record peak Na^+ currents. Steady-state fast inactivation curves are presented for control conditions (●) and in the presence of 10 μM SKF 81297 (○). *B*, Box plot summarizing the effects of PKA phosphorylation on the half-inactivation voltage and the slope factor. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test). *C*, The peak Na^+ current in response to test pulses to OmV is plotted as a function of time in control (●) or 50 μM cBIMPS (○) in response to depolarization of the membrane to the indicated holding potential. *D*, Voltage dependence of the sum of fast and slow inactivation of control and in the presence of cBIMPS derived from the data in *C*.

tivation curves were compared in control solution and in the presence of the D1 agonist SKF 81297 (10 μM). As shown in Figure 4*A*, there was only a small negative shift in the half-inactivation voltage in the presence of the D1 agonist. The mean values for half-inactivation voltage and slope factor in control conditions were −51.8 ± 1.5 mV ($n = 13$) and 9.2 ± 0.7 mV ($n = 13$), respectively, and −54.7 ± 1.6 mV ($n = 13$) and 10.8 ± 0.9 mV ($n = 13$) in the presence of SKF 81297 (Fig. 4*B*). This small shift (−2.9 mV, $p < 0.05$), although statistically significant, would cause only a 4.7% reduction in peak current at −85 mV and a 9.2% reduction at −70 mV. Thus, the effects of D1 receptor activation and subsequent PKA phosphorylation are not caused primarily by a shift in steady-state fast inactivation.

Na^+ channels are also inactivated by a mechanistically distinct slow inactivation process (Narahashi, 1964; Adelman and Palti, 1968; Rudy, 1978; Ogata and Tatebayashi, 1992). Therefore, we next examined the effects of PKA phosphorylation on steady-state slow inactivation because a hyperpolarizing shift in slow inactivation could also account for our results. To test slow inactivation, cells were held at −110 mV for 100 sec. Current amplitude was determined by applying a 40 msec test pulse to 0 mV once every 2 sec. After 100 sec at −110 mV, the holding potential was changed to −80 mV for 100 sec, −70 mV for 100 sec, −60 mV for 100 sec, and −50 mV for 100 sec (Fig. 4*C*). This protocol allowed time for the channels to enter both the fast and slow inactivated states. The peak current elicited by the test pulse at each membrane potential was then plotted as a function of voltage and fit with a Boltzmann equation to determine the half-inactivation

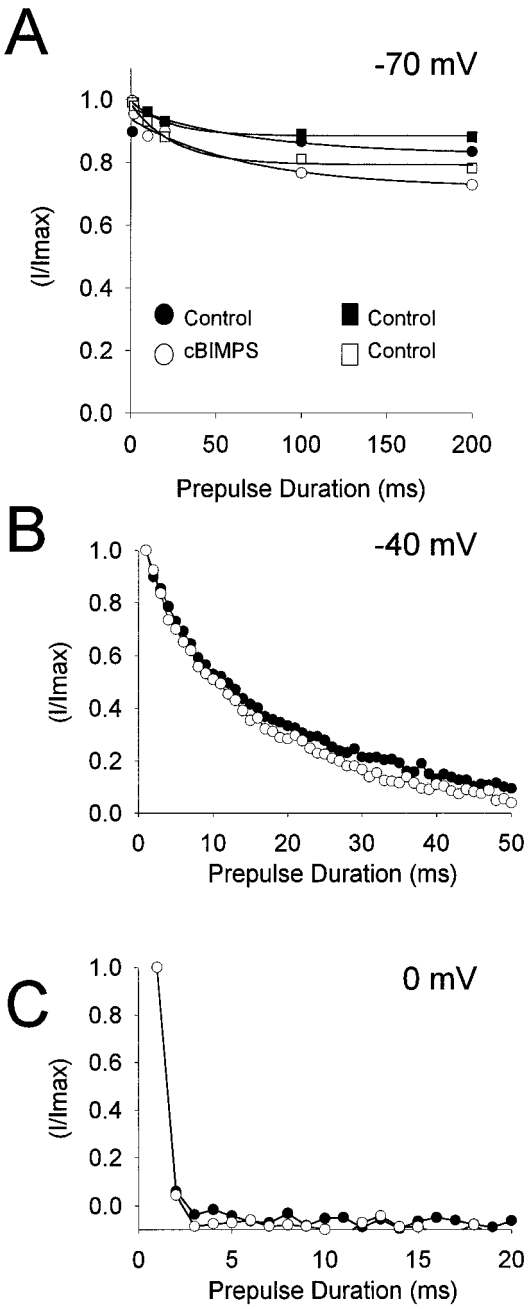

Figure 5. Effects of PKA phosphorylation on the rate of inactivation. *A*, Hippocampal neurons were depolarized to −70 mV for the indicated times and then peak currents were recorded at 0 mV. A plot of normalized peak current versus prepulse duration at −70 mV in control conditions (●) and cBIMPS (○). The *solid lines* are single exponential fits of the data to determine the inactivation rate constant. Note that the small increase in inactivation in the presence of cBIMPS was also observed when the pulse protocol was repeated twice in the absence of cBIMPS (■ vs □). *B*, Plot of normalized peak current versus prepulse duration at −40 mV in control and in the presence of 50 μM cBIMPS demonstrating the rate inactivation from the final closed states. *C*, Plot of normalized peak current versus prepulse duration at 0 mV in control and in the presence of 50 μM cBIMPS demonstrating the rate of inactivation from the open state.

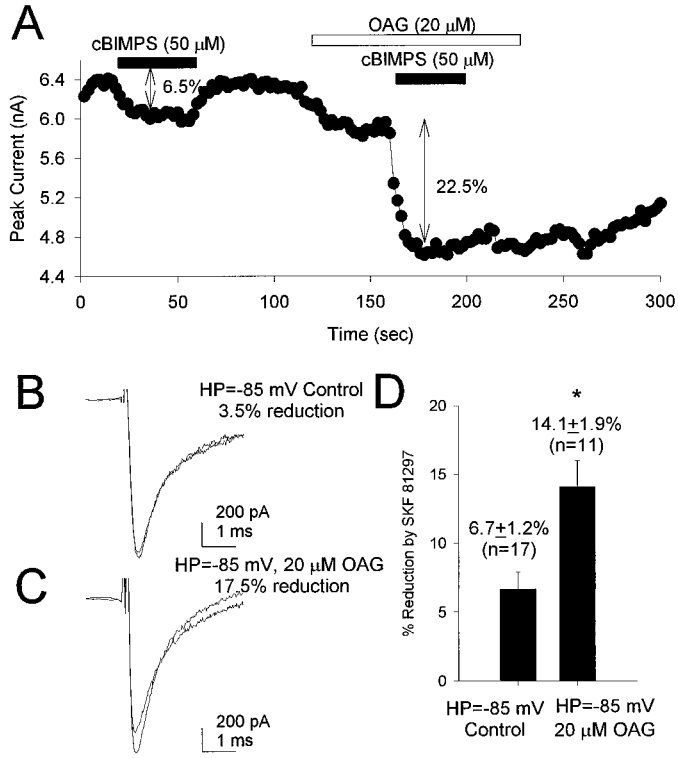

Figure 6. Modulation by SKF 81297 and cBIMPS is enhanced by activation of PKC at a hyperpolarized holding potential in rat hippocampal neurons. *A*, Time course of modulation by cBIMPS in the presence and absence of OAG. The peak current is plotted as a function of time in the presence of various neuromodulators as indicated by the *bars*. *B, C*, Representative current traces elicited by a test pulse to −20 mV from a holding potential of −85 mV demonstrating modulation by SKF 81297 with or without previous activation of PKC by OAG. *D*, Bar graph summarizing the facilitatory effect of previous exposure to a low dose (20 μM) of OAG on the magnitude of SKF 81297 modulation in a population of neurons. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test).

voltage and slope factor (Fig. 4*D*). The protocol was then repeated in the same cell in the presence of 50 μM cBIMPS. As shown in Figure 4*C,D*, there was little shift in the half-inactivation voltage or slope factor in the presence of the PKA activator cBIMPS (50 μM). The half-inactivation voltage and slope factors in control conditions were −60.0 ± 1.8 mV (n = 12) and 6.4 ± 0.6 mV (n = 12), respectively, and −62.6 ± 1.8 mV (n = 12) and 7.1 ± 0.2 mV (n = 12) in the presence of cBIMPS (Fig. 4*D*). This small shift (−2.6 mV, $p < 0.05$), although statistically significant, is comparable to the effect on fast inactivation alone and would account for only a 8.6% reduction at −70 mV. Thus, we can conclude that the effects of D1 receptor activation and subsequent PKA phosphorylation of the channel are not caused primarily by changes in the voltage dependence of steady-state slow or fast inactivation.

We next examined the effects of PKA phosphorylation on the rate of inactivation from the closed state, because an increase in the rate of closed-state inactivation would cause more channels to inactivate before opening and thereby reduce peak Na^+ current. Cells were held at −100 mV and depolarized to −70 mV for varying intervals of time from 1 to 200 msec to induce channel inactivation without channel opening. The extent of inactivation was then assessed by depolarization to 0 mV for 5 msec and recording the remaining current. This protocol was performed for cells in control conditions and in the presence of cBIMPS. Comparison of the results gave a direct measure of the effects of PKA phosphorylation on closed-state inactivation. We found that cells treated with cBIMPS inactivated more during the second appli-

cation of the long pulse protocol than under control conditions during the first application of the pulse protocol (Fig. 5*A*, *circles*) ($n = 6$). However, this was an effect of the pulse protocol or time of the experiment because the same small increase in inactivation was observed when the pulse protocol was repeated twice in the absence of cBIMPS (Fig. 5*A*, *squares*) ($n = 6$). Furthermore, fitting these data to an exponential function gave rate constants of 71.7 and 67.6 msec for control and cBIMPS, respectively. This change (4.1 msec) was not significantly different from that observed in control solution during a similar recording protocol. Thus, we conclude that the effects of D1 receptor activation and subsequent PKA phosphorylation of the channel occur without alterations in the rate of steady-state inactivation at −70 mV.

Finally, we compared the effects of PKA phosphorylation on the rate of onset of inactivation from closed state(s) further along the activation pathway and from the open state. The current amplitude in response to a test pulse to 0 mV was measured after a prepulse to −40 mV (to study inactivation from the final closed states) or 0 mV (to study open-state inactivation). Prepulse duration was increased in 1 msec increments from 1 to 50 msec to study closed-state inactivation and from 1 to 20 msec to study open-state inactivation. These experiments were conducted in control solution and then repeated in the presence of 50 μM cBIMPS. As shown in Figure 5*B,C*, no difference in the onset of inactivation was observed in the presence of cBIMPS from either the closed state or the open state. Thus, we can conclude that the effects of D1 receptor activation and subsequent PKA phosphorylation of the channel occur without substantial alterations in the rate of onset of inactivation from either the closed states or the open state. Altogether, our results indicate that the observed effects of membrane potential on D1/PKA modulation are not caused primarily by changes in the rate or voltage dependence of inactivation from closed or open states. This rules out a simple negative shift in gating parameters as the mechanism of interaction between membrane potential and PKA regulation. The possibility that membrane potential changes alter the extent of phosphorylation or the pattern of phosphorylation of the PKA sites in the Na^+ channel is considered in Discussion.

Modulation of Na^+ current by PKA is also enhanced by the activation of PKC

Previous work in this laboratory showed that concurrent activation of PKC potentiated the effects of PKA activation on Na^+ currents in cultures of embryonic neurons and transfected cells studied at a negative holding potential of −110 mV (Li et al., 1992, 1993). To determine whether PKC potentiates D1/PKA regulation of Na^+ current at physiologically relevant resting membrane potentials (−70 to −85 mV) in hippocampal pyramidal neurons, we used the membrane-permeant activator of PKC, OAG. The magnitude of the modulation was assessed as the difference in the magnitude of the Na^+ current elicited by a test pulse to −20 mV in control and in the presence of 50 μM cBIMPS before and after the application of OAG (20 μM) (Fig. 6*A*). At a holding potential of −85 mV, a 40 msec test pulse to −20 mV was applied once every 2 sec for 1–2 min in control solution until the current magnitude stabilized, and 50 μM cBIMPS was then applied. A reduction in peak Na^+ current of 6.5% was observed in this experiment in the presence of cBIMPS. The cBIMPS was then washed out of the bath, and the current amplitude was allowed to recover. A low concentration of OAG was applied, which itself caused a small reduction in peak Na^+ current, and the control–drug–wash sequence was then repeated in the continued presence of the PKC activator. The magnitude of the cBIMPS effect was significantly increased to 22.5% reduction of Na^+ current in this experiment after activation of PKC (Fig. 6*A*). In 13 similar experiments at −85 mV, activation of PKC increased modulation by cBIMPS from 8.9 ± 2.0% ($n = 13$) in control to 16.8 ± 2.9% ($n = 13$) in the presence of OAG.

We performed similar experiments with the D1-like agonist SKF 81297 (Fig. 6*B–D*). As for cBIMPS, concurrent activation of PKC with OAG significantly increased the extent of neuromodulation by SKF 81297 (Fig. 6*B,C*). At a holding potential of −85 mV, the mean peak Na^+ current was decreased 6.7 ± 1.2% ($n = 17$) by SKF 81297 in control and 14.1 ± 1.9% ($n = 11$) in the presence of OAG. These results show that PKA-dependent modulation of Na^+ currents is enhanced by both membrane depolarization and PKC activation.

Similar effects of OAG were observed on PKA modulation of Na^+ channel α subunits expressed in tsA-201 cells (Fig. 7*A–C*). At −110 mV, essentially no modulation was observed under control conditions, but significant modulation was recovered after application of OAG. Larger effects of cBIMPS were observed at −85 and −70 mV, as expected from the voltage dependence of PKA modulation, but these effects were significantly increased by concurrent activation of PKC at holding potentials of −85 and −70 mV as well. The relative effect of PKC activation is greatest at −110 mV, where activation of PKA has no detectable effect alone.

We repeated the experiments shown in Figures 6 and 7 in the presence of 20 mM BAPTA in the intracellular solution to rapidly buffer intracellular calcium to the low nanomole range. The increase in D1/PKA modulation in response to concurrent PKC activation was similar for cells dialyzed with 0.1 mM or 20 mM BAPTA (data not shown). These results indicate that the intracellular calcium concentration is not a determining factor for enhancement of modulation of Na^+ channels by PKC.

Membrane potential-dependent enhancement of PKA modulation is independent of phosphorylation by PKC

To determine whether basal activation of endogenous PKC was responsible for the effect of membrane depolarization, we tested the magnitude of the PKA-dependent modulation of Na^+ currents at a holding potential of −70 mV in hippocampal neurons dialyzed with the specific PKC inhibitor $PKCI_{(19–36)}$ (House and Kemp, 1987). When $PKCI_{(19–36)}$ was added to the recording pipette at a concentration of 20 μM and 5–10 min were allowed for dialysis of the peptide, no significant differences in the magnitude of the modulation by cBIMPS were observed between the peptide-containing neurons and control neurons (Fig. 8*A–C*). In comparable control experiments, this treatment is sufficient to completely prevent the modulation of currents by OAG or by activation of muscarinic acetylcholine receptors in hippocampal neurons (Cantrell et al., 1996), consistent with the conclusion that membrane potential-dependent enhancement of PKA modulation does not require phosphorylation by PKC.

To further address this question, we used a mutant Na^+ channel in which the conserved serine residue 1506 in the loop connecting domains III and IV of the Na^+ channel α subunit was mutated to alanine (S1506A). Previous studies have demonstrated that phosphorylation of this serine residue is required to observe PKC-dependent modulation of the Na^+ current (West et al., 1991) and PKC-dependent enhancement of PKA modulation of the Na^+ current (Li et al., 1993). If phosphorylation of the

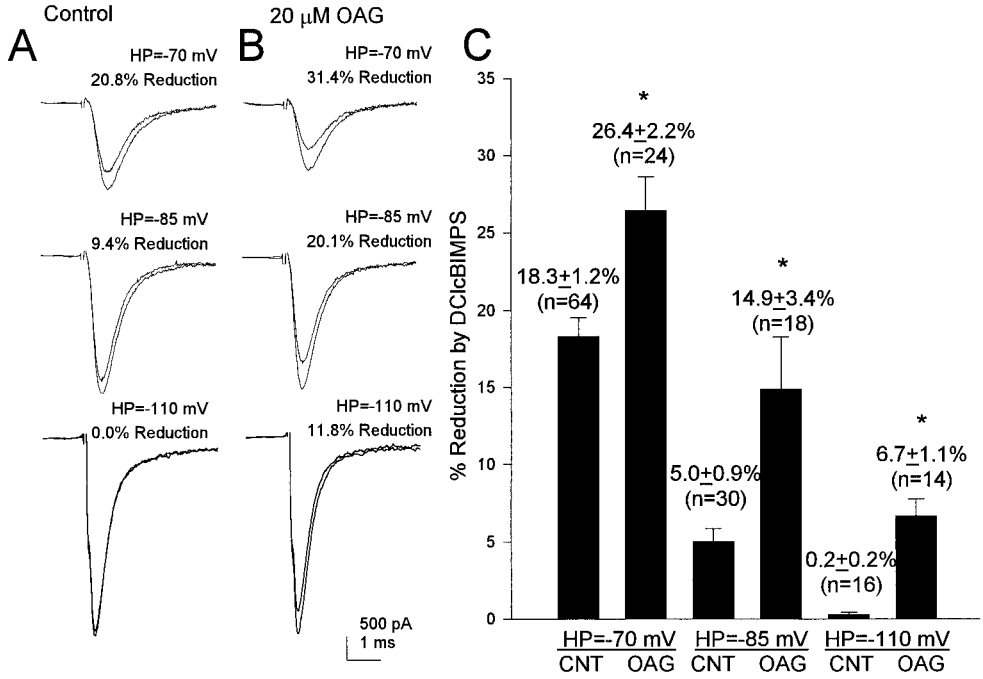

Figure 7. Modulation by cBIMPS is also potentiated by PKC activation in transiently transfected tsA-201 cells expressing type IIA Na^+ channel α subunits. *A, B*, Representative current traces elicited by a test pulse to 0 mV from the indicated holding potential demonstrating modulation by cBIMPS without (*A*) or with (*B*) previous activation of PKC by OAG. *C*, Bar graph summarizing the facilitatory effect of previous exposure to a low dose (20 μM) of OAG on the magnitude of cBIMPS modulation in a population of tsA-201 cells. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test).

channel by PKC is necessary for the voltage dependence of the D1/PKA effect, mutation of serine 1506 should prevent voltage-dependent PKA modulation. As illustrated in Figure 9, we observed similar voltage-dependent PKA modulation of this mutant channel. Application of cBIMPS at a holding potential of −110 mV reduced peak sodium current only 2.8 ± 1.2% (Fig. 9*B,C*) ($n = 4$), but the reduction was 17.8 + 5.6% (Fig. 9*A,C*) ($n = 4$) at a holding potential of −70 mV. The magnitude of the voltage-dependent increase in PKA modulation is similar to that observed for wild-type Na^+ channels, confirming that phosphorylation of serine 1506 by PKC is not needed for voltage-dependent enhancement of PKA modulation.

DISCUSSION

Membrane depolarization enhances the modulation of Na^+ channels by PKA

Phosphorylation of the α subunit of neuronal Na^+ channels serves an important regulatory function in the CNS. Na^+ current in striatonigral and hippocampal neurons is modulated by activation of D1-like DA receptors, which act through PKA to reduce peak Na^+ current (Surmeier et al., 1992; Schiffmann et al., 1995; Cantrell et al., 1997). Our results provide the first evidence for an effect of membrane potential on modulation of sodium channels

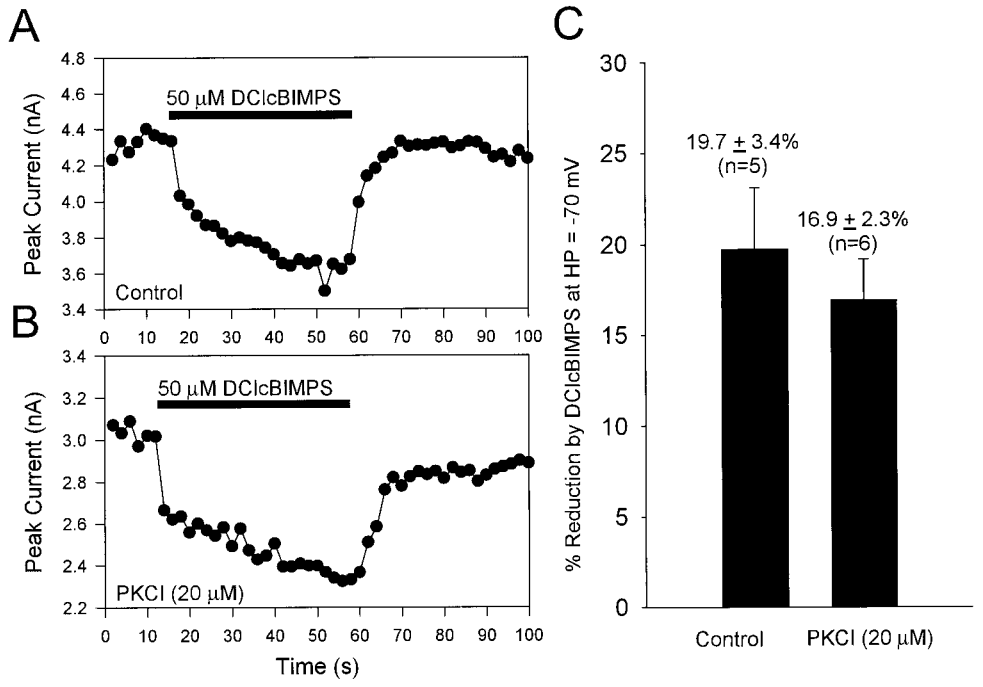

Figure 8. Membrane potential-dependent enhancement of PKA modulation does not require phosphorylation by PKC. *A, B*, Peak current elicited by a test pulse to −20 mV from a holding potential of −70 mV plotted as a function of time in control solution and in the presence of 50 μM cBIMPS for a control cell and for a cell dialyzed with 20 μM PKCI. *C*, Bar graph summarizing the magnitude of PKA modulation in control and in the presence of 20 μM PKCI for a population of neurons. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test).

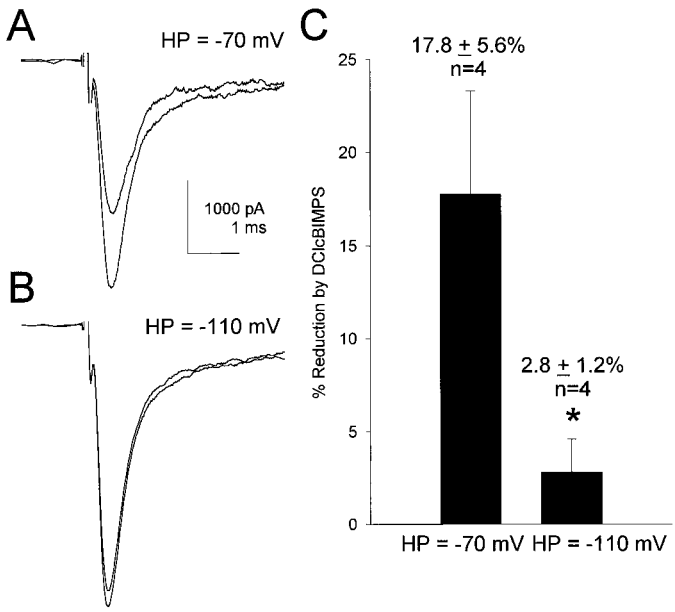

Figure 9. D1/PKA modulation is voltage dependent in tsA-201 cells expressing mutant S1506A Na^+ channel α subunits. *A, B*, Representative current traces elicited by a test pulse to 0 mV from the indicated holding potential in control and in the presence of 5 μM SKF 81297 (*smaller trace*). *C*, Bar graph summarizing the effect of membrane depolarization on the magnitude of SKF 81297 modulation in a population of tsA-201 cells ($n \geq 20$ for each group). The mean of each population is indicated on the graph. An *asterisk* indicates statistical significance ($p \leq 0.05$; Student's *t* test). These results indicate that PKC phosphorylation and membrane depolarization affect PKA modulation of Na^+ current by parallel mechanisms that both enhance the PKA-dependent reduction in peak Na^+ current.

by the D1/PKA pathway and, to our knowledge, the first evidence for voltage-dependent neuromodulation of any ion channel. Depolarization in the range from −110 to −70 mV substantially enhances the reduction of peak Na^+ current by PKA activation. At −110 mV, no detectable reduction in peak Na^+ current is caused by activation of PKA. In contrast, the reduction in peak Na^+ current grows progressively with depolarization, approaching a 30% reduction in peak current at −70 mV. Thus, over the range of voltages near the normal resting membrane potential, small depolarizations caused by synaptic input or by subthreshold inward current through voltage-gated channels would enhance the effects of DA on Na^+ channels, thereby reducing peak Na^+ current and increasing the threshold for action potential generation.

Voltage-dependent neuromodulation may be particularly important in light of recent reports that the response properties of some populations of neurons are state-dependent (Jahnsen and Llinas, 1984; Hultborn and Kiehn, 1992; Gorelova and Reiner, 1996; Kiehn et al., 1996; McCormick and Bal, 1997; Surmeier and Kitai, 1997). For example, neostriatal neurons can exist in either an "up-state," in which the membrane is depolarized, or a "down-state," in which the membrane is hyperpolarized (Surmeier and Kitai, 1997). This voltage-dependent shift in the function of striatonigral neurons has profound effects on their integrative and action potential firing properties. In light of our observations, Na^+ channels in a neuron residing in the up-state would be expected to be responsive to an incoming DA signal, whereas neurons in the down-state would not. The integration of PKA-dependent and membrane potential-dependent regulation of Na^+ channels may contribute in an important way to the regulation of neuronal spiking activity by activation of dopaminergic neurotransmitter pathways and other regulatory pathways using cAMP as second messenger.

Mechanism of voltage-dependent neuromodulation of Na^+ channels

Previous studies of modulation of single Na^+ channels in cultured brain neurons have shown that the reduction of peak Na^+ current caused by phosphorylation by PKA results from a shift of channels to a null gating mode in which channels do not open in response to depolarization (Li et al., 1992). This effect of PKA might acquire voltage dependence in two ways. First, changes in membrane potential may alter the extent of PKA modulation via an effect of the voltage-dependent gating processes of Na^+ channels on phosphorylation or dephosphorylation of the channel. Second, phosphorylation might cause a shift in steady-state inactivation of channels, which would impart a voltage dependence to the functional effects of phosphorylation. We tested the possibility that PKA may alter steady-state inactivation using protocols designed to measure the rate and voltage dependence of both fast and slow inactivation. We observed only a small shift in the voltage dependence of steady-state fast inactivation and no shift in the voltage dependence of slow inactivation or in the rates of fast or slow inactivation in the presence of a PKA activator. Because phosphorylation does not strongly affect the gating parameters of Na^+ channels, the alternative hypothesis that voltage directly alters the extent or pattern of PKA phosphorylation in response to dopaminergic stimulation deserves further study. In this case, the prolonged changes in membrane potential in the range of −70 to −110 mV imposed in our experiments may cause slow conformational changes in the sodium channel phosphorylation sites that lead to differences in their rates of phosphorylation or dephosphorylation. Development of new methods will be required to test this idea because the extent of Na^+ channel phosphorylation must be measured in single cells whose membrane potential is altered in the range of −110 to −70 mV under voltage-clamp conditions.

Synergistic interaction between membrane depolarization and PKC in enhancing regulation of Na^+ channels by PKA

Previous work on brain Na^+ channels expressed in transfected cells showed that PKA and PKC act in a convergent manner to regulate Na^+ channels and cause a reduction in peak Na^+ current (Li et al., 1993). In hippocampal pyramidal neurons, activation of muscarinic acetylcholine receptors modulates Na^+ channels, causing a slowing of inactivation and a reduction of peak Na^+ current (Cantrell et al., 1996). Those effects required activation of PKC but not activation of PKA. In transfected cells at a holding potential of −110 mV, little effect of PKA on Na^+ current is observed unless PKC is also activated, as reported previously (Li et al., 1992). We were interested in determining whether PKC-dependent enhancement of the effects of PKA could be demonstrated in hippocampal neurons under physiological conditions. In the experiments described here, we observed significant modulation of Na^+ currents by the PKA pathway in the absence of PKC activation at −85 or −70 mV, but that modulation was enhanced by concomitant activation of PKC at both membrane potentials. Evidently, membrane potential and PKC act in a parallel manner to enhance PKA modulation of

peak Na^+ current in brain neurons. A recent report (Kondratyuk and Rossie, 1997) has suggested that phosphorylation of purified sodium channels by PKC decreases dephosphorylation of cAMP-dependent phosphorylation sites by calcineurin or protein phosphatase 2A. This may be the mechanism by which PKC enhances the ability of PKA to regulate channel function. In striatonigral neurons residing in the hyperpolarized down-state (Surmeier and Kitai, 1997), this mechanism would enhance responsiveness to an incoming DA signal in the presence of an additional signal that activates PKC, such as activation of muscarinic receptors coupled to PKC stimulation as described previously (Cantrell et al., 1996).

Functional implications of convergent regulation of Na^+ channels in hippocampal neurons by PKA, PKC, and membrane depolarization

The hippocampus receives both cholinergic innervation from neurons of the basal forebrain (Frotscher and Leranth, 1985; Price et al., 1993; Wainer et al., 1993) and dopaminergic innervation from the mesocorticolimbic dopamine system (Civelli et al., 1993). PKC-dependent slowing of inactivation of Na^+ currents would increase the duration of the action potential and possibly alter the pattern of action potential firing. Reduction of neuronal Na^+ currents attributable to phosphorylation by PKC and/or PKA would be expected to strongly influence the functional properties of the target neurons, and these effects would be strengthened by small depolarizations of the resting membrane potential. Reduction of peak Na^+ current would be expected to shift the voltage threshold for action potential generation toward more depolarized potentials. Thus, a stronger depolarization would be required to elicit a response. The frequency at which the cell is capable of generating action potentials might also be reduced. The convergent regulation of Na^+ current by membrane potential, PKC, and PKA allows signals mediated by voltage-gated ion channels, neurotransmitter receptors that directly affect membrane conductance, and neurotransmitter receptors that activate either PKA or PKC to be integrated at the level of the Na^+ channel, which is the final common pathway for signal output from the cell body in the form of action potentials.

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