Activation of Protein Kinase C Increases Neuronal Excitability by Regulating Persistent Na\textsuperscript{+} Current in Mouse Neocortical Slices

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Rapid Communication

Introduction

The dynamics of neocortical circuit function depend on the excitability of individual neurons as well as on the synaptic interactions between them. Neocortical neuronal excitability may be modulated by a variety of ligands that regulate voltage-gated channels through activation of protein kinase C (PKC). Although most attention in this regard has focused on K\textsuperscript{+} channel regulation (Krnjevic et al. 1971; McCormick et al. 1993), there is considerable evidence that PKC-mediated phosphorylation of the Na\textsuperscript{+} channel may also be of considerable functional significance. Studies at the channel level have shown that this regulation results in a decrease in Na\textsuperscript{+} channel availability (Cantrell et al. 1996; Lotan et al. 1990; Numann et al. 1991); this would be expected to reduce neuronal excitability. Recent reports, however, indicate that in hippocampus, PKC activation increases dendritic excitability and thereby reverses the activity-dependent reduction in dendritic action-potential propagation (Colbert and Johnston 1998; Tsubokawa and Ross 1997).

In the present study, we sought to determine the effect of PKC activation on neocortical neurons. We found that, although exposure to phorbol esters causes a decrease in persistent Na\textsuperscript{+} current (I_{\text{NaP}}) at depolarized voltages, it also leads to a marked decrease in threshold for action-potential generation. This paradoxical effect is related to a hyperpolarizing shift in the voltage dependence of I_{\text{NaP}} activation. Portions of this work were previously presented in abstract form (Fleidervish et al. 1997).

Methods

CD1 mice of either sex, postnatal day 7 to postnatal day 28, were anesthetized with pentobarbital sodium (60 mg/kg) and decapitated. Brains were removed, and 400-μm-thick coronal slices of somatosensory cortex were prepared as previously described (Fleidervish et al. 1996; Fleidervish and Gutnick 1996). Recordings were made in a small (300 μl) interface-type recording chamber, where slices were continuously perfused with artificial cerebrospinal fluid whose composition was (in mM) 124 NaCl, 3 KCl, 2 CaCl\textsubscript{2}, 2 MgSO\textsubscript{4}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 10 glucose (pH 7.3 at 32 ± 0.5°C when bubbled with a 95% O\textsubscript{2}-5% CO\textsubscript{2} gas mixture).

Whole cell recordings from 108 layer V neurons were made using the patch-clamp technique (Hamill et al. 1981) as modified for \textquotedblleft blind\textquotedblright recording in slices (Blanton et al. 1989). Patch pipettes, pulled from borosilicate glass capillaries (Hilgenberg, Germany) on a Narishige PP83 puller, had resistances of 1.5 ±3.5 MΩ. The pipette solution for persistent sodium current recording consisted of (in mM) 135 CsCl, 4 NaCl, 2 MgCl\textsubscript{2}, and 10 N-2-hydroxyethylpiperazine-N’-2-ethansulfonic acid (HEPES, cesium salt), pH 7.25. Care was taken to maintain membrane access resistance as low as possible (usually 3–4 MΩ and always <10 MΩ); series resistance was 80% compensated using the built-in circuitry of an Axopatch-1D amplifier (Axon Instruments). Command voltage protocols were generated, and data were acquired on-line with an Axolab 1100 A/D interface. Data were low-pass filtered at 2 kHz (~3 dB, 4-pole Bessel filter) and sampled at 5–10 kHz digitalization frequency. Before data acquisition, linear leak current was subtracted using the built-in circuits of the amplifier.

An Axoclamp-2A amplifier in a Bridge mode was used for current-clamp experiments. The pipette solution contained (in mM) 135 K gluconate, 2 MgCl\textsubscript{2}, and 10 HEPES (potassium salt), pH 7.25. Data were low-pass filtered at 10 kHz (~3 dB, 4-pole Bessel filter) and sampled at 20 kHz digitalization frequency. Voltages in Fig. 1 have not been corrected for liquid junction potential.

Data were analyzed using PClamp 5.5 and Microcal Origin 3.78 software. Spike amplitudes were measured from threshold to peak. Apparent input resistance (R_{\text{in}}) was determined as the slope of...
neuronal voltage-current curve measured in the hyperpolarizing the (linear) range. Membrane time constant ($\tau_m$) was determined by an exponential fit to the decay of the voltage response following a small hyperpolarizing current step.

The protein kinase C activator, phorbol 12-myristate 13-acetate (PMA; Sigma) was applied intracellularly through the patch pipette to limit its action to the neuron under study. 4-α-phorbol 12-myristate 13-acetate (Sigma) was used as a negative control. The specific PKC inhibitor chelerythrine (Biomol) was applied to the bath solution. The specific PKC inhibitor calphostin C (Biomol) was added to the pipette solution. Bath applied tetrodotoxin (TTX; Sigma; 1 μM) was used to block Na$^+$ currents, and CdCl$_2$ (200 μM) was used to block Ca$^{2+}$ currents.

RESULTS

Figure 1 shows a current-clamp recording from a representative layer V neuron with PMA (100 nM) included in the pipette solution. As the phorbol ester diffused into the cell, neuronal excitability increased (Fig. 1A), such that a given depolarizing current step evoked progressively longer and longer trains of spikes. This was closely associated with a hyperpolarizing shift in the voltage threshold for action-potential generation (Fig. 1B). Immediately upon establishment of whole cell recording, spike threshold of this cell was $-39$ mV. As PMA continued to diffuse into the neuron, however, spike threshold gradually decreased until it finally reached a new steady value between $-55$ and $-60$ mV (Fig. 1B), which is close to the activation threshold of fast Na$^+$ current in neocortical neurons (Fleidervish et al. 1996). As is illustrated in Fig. 1C, a similar effect of PMA on voltage threshold was observed in six of the seven layer 5 neurons tested, although the time course and extent of the effect varied from neuron to neuron. These effects were caused by the phorbol ester, because threshold voltage was stable for

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**FIG. 1.** Phorbol 12-myristate 13-acetate (PMA) causes an increase in neuronal excitability. A: in a current-clamp whole cell recording from layer V neuron, PMA (100 nM) increases neuronal excitability. Note, that as PMA diffuses into the cell, an initially just threshold depolarizing current step of 165 pA elicits progressively longer trains of spikes. Top traces: membrane voltage. Middle traces: intracellularly applied current pulse. Bottom traces: time differential of the voltage trace. Note that the increase in excitability is associated with a small decrease in spike overshoot potential and in maximal upstroke velocity. B: in the same neuron, PMA induces a shift of the voltage threshold for action-potential generation from $-39$ to $-58$ mV over 50 min. Note, that the current necessary to elicit a spike decreases from 165 to 65 pA. Spikes have been truncated at $-30$ mV. C: time course of the effect of 100 nM PMA on the voltage of spike threshold in 7 neurons. Note that the time course of the effect varied somewhat from cell to cell, and that in one neuron, threshold was not changed. D: effect of 23-min exposure to 100 nM intracellular PMA on spike amplitude and rate of depolarization, and on apparent input resistance and membrane time constant. Shown are means ± SE, n = 7. The changes in amplitude, $dV/dt_{\text{max}}$ and $R_m$, were statistically significant (paired t-test, P < 0.05).
hours in recordings under the same experimental conditions but without PMA in the pipette (n = 10 cells). Figure 1D shows that the PMA-induced hyperpolarizing shift in threshold was not accompanied by an increase in action-potential amplitude or maximal depolarization rate, as might be expected if the availability of Na⁺ current underlying the action potential itself were to increase. Indeed, in most neurons, including that shown in Fig. 1, PMA caused a slight progressive decrease in spike overshoot and a slight reduction in maximal rate of rise. These observations indicate that the threshold decrease occurred despite a parallel reduction in the overall availability of Na⁺ channels, which is consistent with previous reports that PKC-mediated Na⁺ channel phosphorylation causes a decrease in Na⁺ current (Cantrell et al. 1996; Lotan et al. 1990; Numann et al. 1991).

In previous reports, increased neocortical neuronal excitability due to the actions of various neuromodulators has generally been ascribed to decreased K⁺ conductance (Krtrjevic et al. 1971; McCormick et al. 1993). Figure 1D shows, however, that the PMA-induced effect on voltage threshold we report was not associated with the changes in apparent input resistance and membrane time constant that would be expected if K⁺ conductances were reduced. Indeed, enhanced neuronal excitability caused by a decrease in K⁺ conductance would be expected to be associated with an increase in apparent input resistance, and to entail a reduction in the current required to reach spike threshold (McCormick et al. 1993); it would not, however, be expected to alter the threshold voltage itself, which, in neocortical neurons, depends primarily on the availability of I_{NaP} (Crill 1996; see, for review, Taylor 1993).

Results of the current-clamp experiments strongly suggested that PKC activation by PMA affects neuronal excitability via modulation of I_{NaP}, whose prominence in neocortical neurons is well documented (Alzheimer et al. 1993a; Connors et al. 1982; Stafstrom et al. 1985). We therefore examined the effect of intracellularly applied PMA on I_{NaP} in voltage clamp mode by applying 2-s depolarizing voltage ramps from −70 to 0 mV; a rising rate slow enough to maximize the current-voltage (I-V) relationship. These observations indicate that the PMA-induced reduction in I_{NaP} was not accompanied by an increase in action-potential amplitude or maximal depolarization rate, as might be expected if the availability of Na⁺ current underlying the action potential itself were to increase. Indeed, in most neurons, including that shown in Fig. 1, PMA caused a slight progressive decrease in spike overshoot and a slight reduction in maximal rate of rise. These observations indicate that the threshold decrease occurred despite a parallel reduction in the overall availability of Na⁺ channels, which is consistent with previous reports that PKC-mediated Na⁺ channel phosphorylation causes a decrease in Na⁺ current (Cantrell et al. 1996; Lotan et al. 1990; Numann et al. 1991).

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Intracellular exposure to PMA (100 nM to 1 μM) had a dual effect on I_{NaP}. Starting from the first minutes of breaking into the cell, there was a decrease in I_{NaP} amplitude at very depolarized membrane potentials. Also, the activation of I_{NaP} shifted in the hyperpolarizing direction, such that there was a significant increase in I_{NaP} at potentials more negative than −45 mV (Fig. 2A).

The PMA effect on I_{NaP} was via PKC activation, because the inactive PMA analogue, 4α-PMA, had no apparent effect

**FIG. 2.** Protein kinase C (PKC) activation by PMA decreases I_{NaP} at depolarizing potentials and shifts its activation to more negative potentials. A: in a voltage-clamp recording with PMA (100 nM) included into the pipette solution, instantaneous current-voltage (I-V) curve during a slow (35 mV/s) depolarizing ramp was checked every minute. Note that while PMA diffuses into the cell, the inward currents decrease at depolarizing voltages and increase at potentials more hyperpolarizing than −45 mV. K⁺ currents were blocked by using Cs⁺ as the main intracellular monovalent cation, and Ca²⁺ currents were blocked by adding 200 μM Cd²⁺ to the bath. Access resistance 4 MΩ; leakage subtracted. B: when tetrodotoxin (TTX; 1 μM) was added to the bath solution, PMA did not affect the instantaneous I-V curve. C: PKC inhibitor, chelerythrine (20 μM), when added to the bathing solution, prevented PMA effect on I_{NaP}.
on $I_{\text{NaP}}$ ($n = 6$ neurons; data not shown). Furthermore, in slices that were preincubated for $>1$ h with the specific PKC inhibitor chelerythrine (20 $\mu$M; $n = 6$), intracellularly applied PMA did not affect $I_{\text{NaP}}$ (Fig. 2C). In slices bathed in TTX (1 $\mu$M) $I_{\text{NaP}}$ was completely blocked (Fig. 2B). Under these conditions, the intracellular PMA had no evident effect on the surviving responses to voltage ramps (Fig. 2B), indicating that PKC activation did not affect $I_{\text{NaP}}$, and that its effect on the instantaneous $I-V$ curve was not due to induction of an additional current.

Figure 3A shows the time course of the PMA effect on $I_{\text{NaP}}$ as determined in 11 neurons. Current amplitudes at $-55$ mV and at $-35$ mV were used as measures of $I_{\text{NaP}}$ availability at these potentials (Fig. 3, inset). Values have been normalized to the initial amplitude at $-35$ mV, because, at this potential, $I_{\text{NaP}}$ is near maximal activation, yet it does not overlap with $I_{\text{Na}}$ (Fig. 2B). At $-35$ mV, after a short delay, PMA caused a steady decrease in $I_{\text{NaP}}$ such that, after 10 min, only 45 $\pm$ 11% (mean $\pm$ SE) of the initial current amplitude remained. By contrast, at $-55$ mV, $I_{\text{NaP}}$ gradually increased from near zero to $\sim$25% of the initial amplitude at $-35$ mV. These effects of intracellular PMA were not observed in neurons exposed to the specific PKC inhibitors chelerythrine (preincubation as above, $n = 7$, Fig. 3B), and calphostin C (10 $\mu$M in the pipette solution, $n = 7$, data not shown).

**DISCUSSION**

Our data demonstrate that PKC activation by intracellular PMA lowers spike threshold and thereby increases the excitability of layer V neocortical neurons. This finding was unexpected because several studies have shown that PKC activation causes a decrease in the availability of fast-inactivating Na$^+$ channels (Cantrell et al. 1996; Lotan et al. 1990; Numann et al. 1991). Indeed, we find that $I_{\text{NaP}}$, which reflects a distinct kinetic mode of these same Na$^+$ channels (Alzheimer et al. 1993a; Patlak and Ortiz 1985), is also decreased by exposure to PMA. However, $I_{\text{NaP}}$ is only reduced at membrane potentials more depolarized than spike threshold; that is, voltages where the persistent fraction of the Na$^+$ current has virtually no influence on the neuron’s behavior because it is so much smaller than the fast-inactivating $I_{\text{Na}}$. The functionally significant effect of PKC activation was a leftward shift in the $I_{\text{NaP}}$ activation curve, which in any event is $\sim$10 mV more negative than that of the transient $I_{\text{Na}}$ (Brown et al. 1994; French et al. 1990). We propose that the consequent increase in persistent inward current at subthreshold voltages was responsible for the hyperpolarizing shift in spike threshold and the change in voltage trajectory toward threshold. It is important to note that the Na$^+$ channel kinetics in neocortical neurons is such that at threshold potential, only a small fraction of the total transient $I_{\text{NaP}}$ is available for activation (Fleidervish et al. 1996). The voltage dependence of $I_{\text{NaP}}$ inactivation is very steep, however. Thus a PKC-mediated shift in $I_{\text{NaP}}$ activation serves to compensate for the overall reduction in Na$^+$ channel availability and can account for the observed enhancement of neuronal excitability.

In direct measurements of $I_{\text{NaP}}$, the effect of PMA appeared within 4–5 min, whereas the effect on spike threshold in current-clamp recordings evolved over a longer time period. This discrepancy may reflect the time necessary for PMA to diffuse from the somatic pipette to the spike initiation zone, which is likely to be quite distant in the axons of pyramidal neurons (Colbert and Johnston 1996; Stuart et al. 1997).

Various modulators may regulate neocortical excitability by activating one or another isozyme of PKC, and the molecular details underlying the novel effect on $I_{\text{NaP}}$ we report here have yet to be elucidated. The extreme diversity within the broad family of PKC (Dekker and Parker 1994; Nishizuka 1988), as well as differences in experimental conditions, may explain the difference between our findings with phorbol ester and the recent report by Mittmann and Alzheimer (1998) that...
in isolated neocortical neurons at room temperature, activation of muscarinic receptors depressed $I_{\text{Na}}$ without a voltage shift in activation.

The functional consequences of regulating $I_{\text{Na}}$ are likely to be very significant, because of the role this persistent inward current plays not only in determining spike threshold and firing properties, but also in synaptic integration and regulation of dendritic excitability. In this last regard, it is interesting that in hippocampal pyramidal cells, activity-dependent reduction in dendritic spike amplitude, which has been attributed to a reduction in $\text{Na}^+$ channel availability (Colbert et al. 1997; Jung et al. 1997), is reversed both by carbachol (Tsukakawa and Ross 1997) and by phorbol esters that activate PKC (Colbert and Johnston 1998).

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REFERENCES


Colbert, C. M., Magee, J. C., Hoffman, D. A., and Johnston, D. Slow recovery from inactivation of $\text{Na}^+$ channels underlies the activity-depen-
dent attenuation of dendritic action potentials in hippocampal CA1 pyra-


