Phosphorylation of a conserved protein kinase C site is required for modulation of Na^+ currents in transfected Chinese hamster ovary cells

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INTRODUCTION

Voltage-gated Na^+ channels mediate the generation and propagation of action potentials in excitable cells. Phosphorylation of the α subunit of the Na^+ channel by protein kinase C slows inactivation and reduces peak Na^+ currents without reducing the number of Na^+ channels in the cell surface membrane. Phosphorylation of serine 1506, located in a conserved intracellular loop between domains III and IV involved in inactivation of the Na^+ channel, is required for both modulatory effects. Mutant Na^+ channels lacking this phosphorylation site have normal functional properties in unstimulated cells, but do not respond to phosphorylation by protein kinase C. This phosphorylation site is conserved among different Na^+ channel α subunits and may regulate electrical activity in a wide range of excitable tissues.

RESULTS AND DISCUSSION

Na^+ currents recorded in dissociated rat brain neurons can be reduced by >90% and their macroscopic inactivation slowed significantly by addition of the protein kinase C (PKC) activator OAG (1-oleoyl-2-acetyl-sn-glycerol) to the bathing solution (1). We studied the modulation of Na^+ channels by PKC in more detail in the Chinese hamster ovary (CHO) cell line CNaIIA-1 expressing Type IIA Na^+ channel α subunits (2). Whole cell Na^+ currents of 4–25 nA are typically observed in these cells, and the high density of Na^+ channels allows recording of macroscopic currents in cell-attached or excised membrane patches. Within 5 min, following addition of 25 μM OAG to the cell bathing solution, Na^+ currents in a typical cell-attached patch were decreased and there was a significant slowing of inactivation. Maximal slowing was observed at 40–50 μM OAG, whereas maximal reduction of peak Na^+ current required greater than 65 μM OAG, without further slowing of the Na^+ current.

We measured ^{3}H-saxitoxin (STX) binding (3) to CNaIIA-1 cells following incubation for 5–60 min in the presence of 50 μM OAG (Fig. 1), and showed that the number of ^{3}H-STX binding sites is unchanged within the time frame of the observed electrophysiological response to PKC activation and is not significantly reduced even after 60 min OAG treatment. Internalization of Na^+ channels is therefore unlikely to be responsible for the reduction in peak Na^+ current in response to PKC phosphorylation.

The presence of a consensus PKC phosphorylation site (4) centered at ser^{1506} (Fig. 2 *A* and *B*), within a domain of the channel involved in inactivation (5, 6), led to the hypothesis that phosphorylation at ser^{1506} might be involved in modulation of Na^+ channel activity. The synthetic peptide SP19 (Fig. 2 *B*, *boxed*) was rapidly phosphorylated in vitro by PKC but not by cAMP-dependent protein kinase (Fig. 2 *C*), consistent with a modulatory role for PKC phosphorylation of ser^{1506}.

To test this hypothesis we developed a transfected CHO cell line expressing a mutant Na^+ channel α

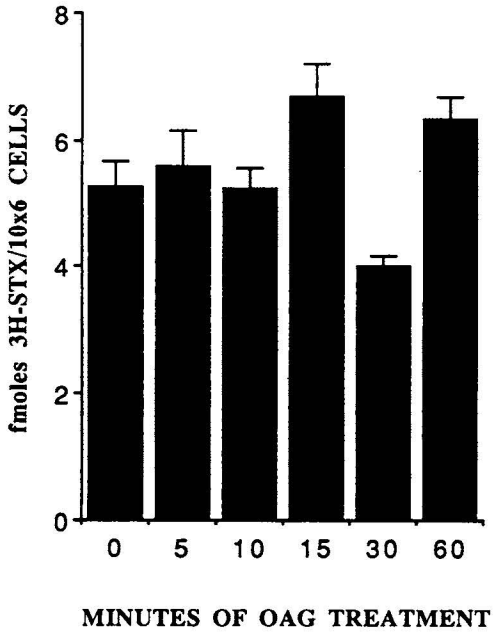

FIGURE 1 Effects of OAG on saxitoxin binding to CNaIIA-1 cells. CNaIIA-1 cells were cultured in the wells of a 24 well Linbro plate. 36 wells were incubated for 0, 5, 10, 15, 30, or 60 min in the presence of 50 μM OAG. ^{3}H-STX binding was measured as previously described (3).

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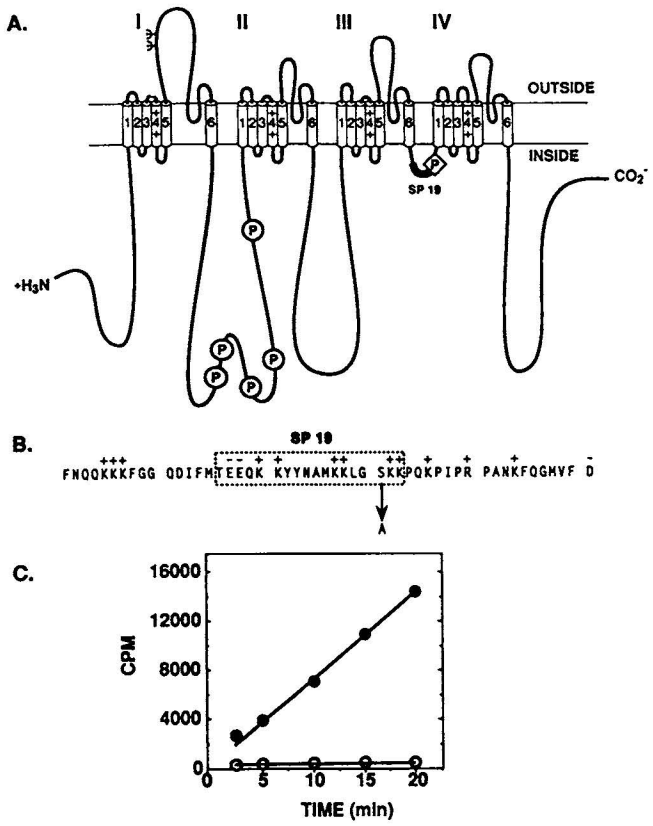

FIGURE 2 (*A*) The transmembrane folding model of the Na^+ channel α subunit is shown with experimentally determined cAMP-dependent protein kinase phosphorylation sites Ⓟ, and the putative PKC phosphorylation site ◈. (*B*) The single letter amino acid code of the primary sequence of the peptide loop between homologous domains III and IV. The arrow indicates the position of theserine 1506 to alanine mutation. (*C*) The synthetic peptide SP19, delineated by the dotted box, was rapidly phosphorylated by PKC in the presence of phosphatidyl serine (PS) and diolein (●). Purified catalytic subunit of cAMP-dependent protein kinase does not phosphorylate SP19 (○).

subunit with alanine substituted for ser^{1506} (CNaS1506A-9). Na^+ currents were recorded in every cell examined with a mean Na^+ current of 4.63 nA. The voltage dependence of Na^+ channel activation in CNaS1506A-9 cells ($V_{½} = -24$ mV, $k = -6.62$, $n = 9$) is unaltered with respect to wild type IIA Na^+ channels as expressed in CNaIIA-1 cells ($v_{½} = -22$ mV, $k = -7.21$, $n = 22$). The voltage dependence of steady-state inactivation is slightly shifted in the depolarizing direction, but the time course is unchanged (CNaIIA-1, $V_{½} = -59$ mV, $k = 5.42$, $n = 12$; CNaS1506A-9, $V_{½} = -54$ mV, $k = 5.27$, $n = 6$).

The effects of PKC phosphorylation of ser^{1506} in the Na^+ channel was analyzed in both CNaIIA-1 and CNaS1506A-9 cells. In cell-attached patches, addition of 20 μM OAG to the bathing solution caused a rapid decrease in peak current and a slowing of inactivation in CNaIIA-1 cells (Fig. 3 *A*), but 75 μM OAG had no effect on Na^+ currents in CNaS1506A-9 cells (Fig. 3 *C*, $n = 17$). In excised membrane patches, addition of PKC to the bathing solution caused a rapid decrease in peak current and a slowing of inactivation for CNaIIA-1 cells (Fig. 3 *B*), but had no effect on Na^+ currents in CNaS1506A-9 cells (Fig. 3 *D*, $n = 5$).

Our results demonstrate that phosphorylation of $serine^{1506}$ in Type IIA Na^+ channel α subunit leads to both slowing of inactivation and reduction of the peak Na^+ current. Preliminary evidence indicating differential sensitivity of the two effects to OAG suggests a more

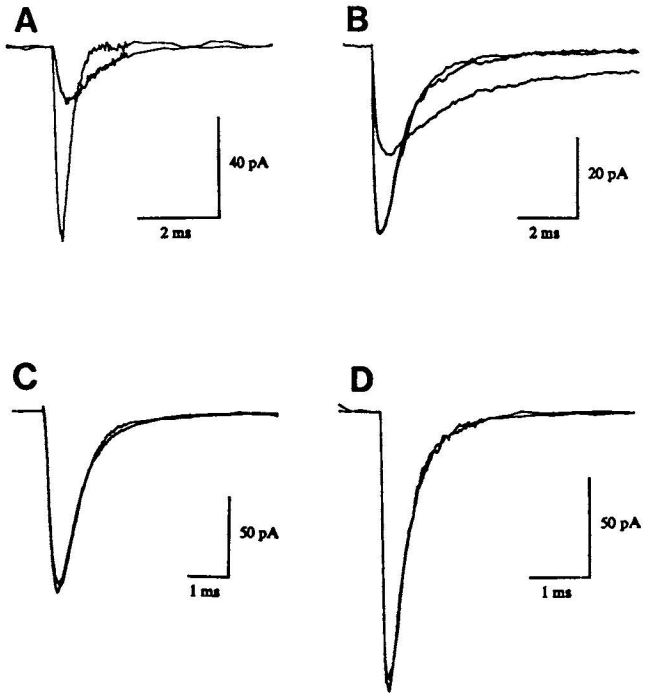

FIGURE 3 PKC was activated in cell attached patches by addition of 25–75 μM OAG to the recording chamber. Inside-out membrane patches were treated with OAG, PS, and ATP alone or in the presence of PKC by addition to the recording chamber. (*A*) Ensemble currents evoked in a cell-attached patch from CNaIIA-1 cells by depolarization to −30 mV from a holding potential of −100 mV. The smaller current was recorded from the same patch after 30 min exposure to 20 μM OAG. (*B*) Ensemble current average of 50 current traces recorded from a single-membrane patch excised from a CNaIIA-1 cell. Currents measured in the same patch following treatment of the cytoplasmic surface with OAG, PS, and ATP are superimposed. The smallest current is recorded from the same patch following addition of PKC. (*C*) Ensemble currents recorded from a CNaS1521A-9 cell attached patch in the presence or absence of OAG are superimposable. (*D*) Ensemble current average of 50 current traces recorded from a membrane patch excised from a CNaS1521A-9 cell. Currents measured in the same patch following treatment of the cytoplasmic surface with OAG, PS, ATP, and PKC are superimposable with current measured in the untreated patch.

complex mechanism perhaps involving additional phosphorylation sites. Alternatively, phosphorylation of ser^{1506} may mediate both effects in a time dependent manner, immediate slowing of inactivation followed by a slowly developing reduction in Na^+ current. Additionally, we show that the reduction of Na^+ current in response to activation of PKC is not mediated by removal of Na^+ channels from the cell surface.

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