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Multiple Effects of Protein Kinase C Activators on Na^+ Currents in Mouse Neuroblastoma Cells

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Abstract. The effects of externally applied different protein kinase C (PKC) activators on Na^+ currents in mouse neuroblastoma cells were studied using the perforated-patch (nystatin-based) whole cell voltage clamp technique. Two diacylglycerol-like compounds, OAG (1-oleoyl-2-acetyl-*sn*-glycerol), and DOG (1-2-dioctanoyl-*rac*-glycerol) attenuated Na^+ currents without affecting the time course of activation or inactivation. The reduction in Na^+ current amplitude caused by OAG or DOG was dependent on membrane potential, being more intense at positive voltages. The steady-state activation curve was also unaffected by these substances. However, both OAG and DOG shifted the steady-state inactivation curve of Na^+ currents to more hyperpolarized voltages. Surprisingly, phorbol esters did not affect Na^+ currents. *Cis*-unsaturated fatty acids (linoleic, linolenic, and arachidonic) attenuated Na^+ currents without modifying the steady-state activation. As with DOG and OAG, *cis*-unsaturated fatty acids also shifted the steady-state inactivation curve to more negative voltages. Interestingly, inward currents were more effectively attenuated by *cis*-fatty acids than outward currents. Oleic acid, also a *cis*-unsaturated fatty acid, enhanced Na^+ currents. This enhancement was not accompanied by changes in kinetic or steady-state properties of currents. Enhancement of Na^+ currents caused by oleate was voltage dependent, being stronger at negative voltages. The inhibitory or stimulatory effects caused by all PKC activators on Na^+ currents were completely prevented by pretreating cells with PKC inhibitors (calphostin C, H7, staurosporine or polymyxin B). By themselves, PKC inhibitors did not affect membrane currents. *Trans*-unsaturated or saturated fatty acids, which do not activate PKC's, did not modify Na^+ currents. Taken together, the experimental results suggest that PKC activation modulates the behavior of Na^+ channels by at least three distinct mechanisms. Because qualitatively different results were obtained with different PKC activators, it is not clear how Na^+ currents would respond to activation of PKC under physiological conditions.

Key words: Protein kinase — Membrane currents — Modulation

Introduction

The activity of different ion channels can be modulated by phosphorylation (Kaczmarek & Levitan, 1987; Hille, 1992). Na^+ channels, which are transmembrane proteins responsible for the generation and propagation of electrical activity in different excitable cells, are good substrates for both protein kinases A (PKA) and C (PKC) (Rossie & Catterall, 1987; Emerick & Agnew, 1989; Yang & Barchi, 1990; Murphy & Catterall, 1992; *see review by* Catterall, 1992). Costa and Catterall (1984) demonstrated that purified brain Na^+ channels can be phosphorylated by PKC in detergent solutions. Following this observation, a few studies attempted to evaluate the significance of PKC phosphorylation for Na^+ channel function. While Sigel and Baur (1988) showed that PKC depressed Na^+ currents in *Xenopus* oocytes injected with chick brain RNA (*see also* Lotan et al., 1990), Moorman, Kirsch and Brown (1989) reported that phorbol esters increased Na^+ currents in heart cells (*see* Benz, Herzig & Kohlhardt, 1992, for a different result in cardiac myocytes). Using mouse neuroblastoma cells (N1E-115), Linden and Routtenberg (1989) demonstrated that *cis*-unsaturated fatty acids decreased Na^+ currents, while the more classical activators of PKC (diacylglycerol

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(DAG)-like compounds) had no effect. The α-subunit of type IIA rat brain Na^+ channel can be readily phosphorylated by PKC (Murphy & Catterall, 1991; Li et. al., 1993) decreasing the amplitude of Na^+ currents (Dascal & Lotan, 1991; Numann, Caterall & Scheuer, 1991; West et al., 1991), and slowing inactivation (Numann et al., 1991; West et al., 1991; Li et al., 1993).

In the course of our investigations on the effects of PKC activators on Na^+ currents in neuroblastoma (N1E-115) cells, we noticed that different PKC activators have different effects on Na^+ currents. Because suggestions for the involvement of PKC on the modulation of ion channels have, in most cases, relied on the effects of its activators and/or inhibitors, it is important to compare the effects of these different substances on ionic currents. In this study, the effects of various PKC activators and/or inhibitors on Na^+ currents were evaluated. The perforated (nystatin-based) patch whole cell clamp technique was used in neuroblastoma cells. This method preserves the intracellular milieu (Horn & Marty, 1988; Korn et al., 1991) allowing a normal or close-to-normal physiological characterization of different PKC-related substances on Na^+ currents. We demonstrate that different PKC activators, namely, DAG-like compounds, *cis*-unsaturated fatty acids (linoleic, linolenic, and arachidonic acids), and oleic (another *cis*-fatty acid) acid all modify Na^+ currents. The first two groups of substances attenuated, while oleic acid increased Na^+ currents. These alterations in Na^+ currents seem to be linked to PKC activation, and are specific for each group of substances.

Materials and Methods

Tissue Culture

N1E-115 mouse neuroblastoma cells (kindly provided to us by Dr. F.N. Quandt, Department of Physiology, Rush Medical School, and Dr. M.W. Niremberg, NIH) were grown in flasks (70 ml), and on small plastic coverslips in culture dishes at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn bovine serum (GIBCO, Grand Island, NY), and 0.1% penicillin and streptomycin. Confluent cells were split once a week, and replated at a density of 2.10^4 cells/ml. Culture medium was replaced every 2–3 days.

Solutions

The bath (extracellular) solution contained (in mM): 75 NaCl, 75 choline-Cl, 2 $CaCl_2$ 10 *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.2 $CdCl_2$ (pH = 7.4, adjusted with NaOH). The pipette solution contained (in mM): 10 NaF, 90 CsF, 30 NaCl, 10 ethyleneglycol-bis *N,N,N',N'*-tetraacetic acid (EGTA) and 10 HEPES (pH = 7.4, adjusted with CsOH). In several experiments with *cis*-unsaturated fatty acids, the transmembrane electrochemical equilibrium potential for Na^+ (V_{Na}) was modified by altering the concentrations of external and/or internal Na^+. These specific changes in Na^+ concentrations will be properly identified in the text. The following substances were purchased from Sigma (St. Louis, MO), and Calbiochem (San Diego, CA): phorbol 12,13-didecanoate, phorbol 12,13-diacetate, phorbol 12-myristate 13-acetate, oleic acid, linoleic acid, linolenic acid, elaidic acid, DOG, OAG, arachidonic acid, stearic acid, staurosporine, calphostin C, polymixin B, and H7. PKC-related substances were dissolved in dimethylsulfoxide (DMSO) and added to the extracellular solution upon sonication. Final DMSO concentration was less than 0.1%, and at this concentration DMSO had no effects on Na^+ currents.

Electrophysiology

Cell-attached coverslips were transferred to the experimental chamber (volume = 200 µl) mounted on an inverted microscope. Membrane currents were recorded using the perforated-patch whole cell method using nystatin (Horn & Marty, 1988; Korn et al., 1991). We were confident that our cells were under the perforated voltage-clamp method, and not under the conventional whole cell condition, because the experimental results described in this study are not the same when the conventional whole cell recording is used (M. Renganathan and S. Cukierman, *in preparation; see also* Linden & Routtenberg, 1989 who applied the conventional whole cell recording method to N1E-115 cells and obtained different experimental results for the effects of PKC activators on Na^+ currents). All experiments were performed at room temperature (21–23°C). Patch electrodes were pulled from borosilicate capillaries (W.P. Instruments, FL) using a programmable puller (P-87, Sutter Instrument, CA). Electrode tip diameters were typically 2 µm and had resistances of 0.8–1.2 mΩ. To reduce the pipette capacitance, the tip of the patch electrode was covered with a hydrophobic paint (Cukierman, 1992). Nystatin stock solutions in DMSO were prepared daily. The final concentration of nystatin in the pipette was 120 µg/ml. In our experiments, $t = 0$ is defined as the time when the membrane patch was functionally perforated with nystatin yielding an access resistance equal or less than 2 MΩ. Control current recordings were obtained at $t = 15$ min. Membrane currents were recorded and analyzed using an Axopatch 1D amplifier, and pCLAMP software, respectively (Axon Instruments, Foster City, CA).

Data Analysis

Two different voltage clamp pulse protocols were applied to cells from a −100 mV holding potential. The first protocol, used to construct activation curves, consisted of 5 msec pulses from the holding voltage to +60 mV in 10 mV steps at a frequency of 1 Hz. The second protocol, used to determine steady-state inactivation curves, consisted of 5 msec test pulses to −20 mV preceded by 500 msec prepulses of variable voltages (−90 to 60 mV). Membrane currents were filtered at 5 kHz and digitized at a sample frequency of 33 kHz. Peak sodium conductances were calculated at each test voltage by

$$G_{Na} = I_{Na,peak}/(V - V_{Na}), \quad (1)$$

where V_{Na} is the Na^+ reversal potential. Activation curves were determined by plotting $G_{Na}/G_{Na,max}$ (m) *vs.* membrane potential. Experimental points were fitted to the expression

$$m = [1 + \exp(V - V_{1/2})/k]^{-1}, \quad (2)$$

where $V_{1/2}$ is the midpoint of activation curve, and k is the curve steepness. The steady-state inactivation curves ($I_{Na\ peak}/I_{Na\ peak,\ max} = h$ *vs.*

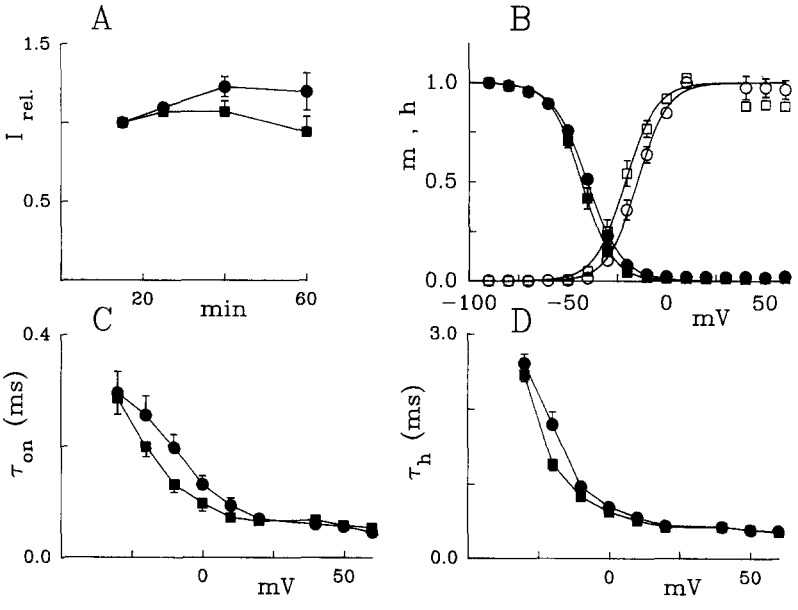

Fig. 1. Time-dependent alterations in the properties of Na^+ currents in N1E-115 cells. Results are expressed as mean ± SEM for five different cells. (*A*) Time-dependent increase in peak Na^+ currents elicited by voltage clamp pulses from −100 to −10 mV or 60 mV. Voltage clamp pulse protocols were delivered 15–60 min after the membrane patch was perforated with nystatin in the "whole" cell configuration (t = 0). I_{rel} is the peak current at time t divided by the peak at t = 15 min; (*B*) voltage dependence of steady-state inactivation (h, filled symbols), and activation (m, open symbols) of Na^+ currents. Circles and squares were obtained 15 and 40 min after the beginning of recordings, respectively. Curves were drawn according to nonlinear regression analysis with the following parameters for the fittings: h, circles, $V_{1/2}$ = −40.89 ± 0.37 mV, and k = 8.84 ± 0.32 mV; squares $V_{1/2}$ = −42.01 ± 0.24 mV, and k = 7.97 ± 0.20 mV; m, circles, $V_{1/2}$ = −14.68 ± 0.43 mV, and k = −7.70 ± 0.38 mV; squares, $V_{1/2}$ = −20.74 ± 0.50 mV, and k = −7.91 ± 0.42 mV. *C* and *D* show the voltage dependence of the activation and inactivation time constants of Na^+ currents at 15 (circles) and 40 min (squares) after the beginning of recordings, respectively.

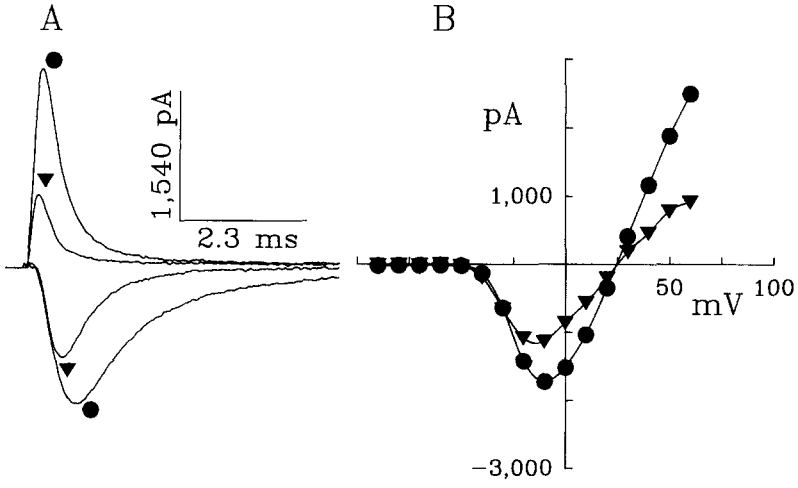

Fig. 2. (*A*) Na^+ currents in response to voltage clamp pulses to +60 (outward currents) and −10 mV (inward currents) from a holding membrane potential of −100 mV. Circles identify recordings obtained in control conditions (t = 15 min), and triangles after exposure to 10 μM OAG for 25 min. (*B*) Peak Na^+ current voltage relationships in control (circles) and in the presence of 10 μM OAG (triangles).

holding potential) were determined by a similar expression. The time constant of Na^+ current activation (τ_{on}) was determined by the best fit of the onset of Na^+ currents to:

$$I_{Na} = I_{Na,\,peak} \cdot [1 - \exp(t/\tau_{on})]^3 \quad (3)$$

The time constant of inactivation of Na^+ currents (τ_h) was determined by fitting the decay of Na^+ currents with a single exponential. Fitting of experimental points to equations described above was done with Sigmaplot (Jandel Scientific, Corte Madera, CA). Paired or unpaired Student's t-tests were used to evaluate the statistical significance of changes in Na^+ current parameters caused by different experimental conditions.

Results

Time-dependent Alterations in Na^+ Currents in Control Cells

Na^+ currents in N1E-115 cells display a significant time-dependent increase in their peak amplitudes. In order to quantify the effects of different PKC activators and/or inhibitors, it is necessary to evaluate the time course of changes in different parameters of Na^+ currents in control conditions. Figure 1 summarizes the re-

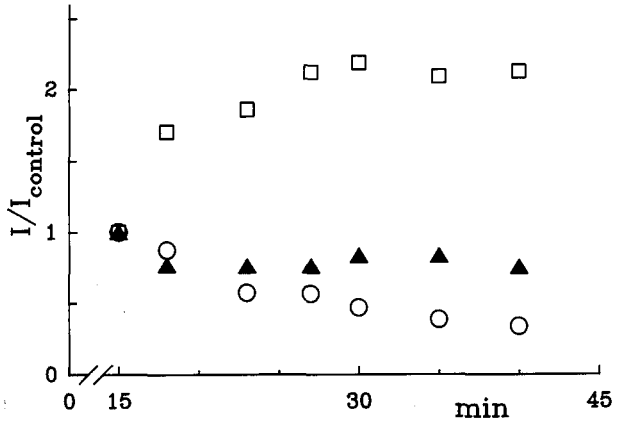

Fig. 3. Time course of effects of PKC activators on peak Na^+ currents in response to voltage clamp pulses from -100 to -10 mV. PKC activator was applied and washed-out at $t = 15$ and 25 min, respectively. $I/I_{control}$ is the peak Na^+ current normalized to the peak Na^+ current at $t = 15$ min. Circles, 75 μM DOG; triangles, 4 μM linolenic acid; squares, 4 μM oleic acid.

sults obtained in a control group of five different cells. In panel *A*, relative peak currents in response to depolarizing voltage steps from -100 to -10 mV (circles) and to 60 mV (squares), are plotted as a function of time. For voltage steps to -10 mV, peak Na^+ currents increased during the first 15–40 min after nystatin perforated the membrane patch, and remained constant thereafter. This increase is statistically significant ($P < 0.0001$) and was observed for all voltage steps between -40 and 0 mV. For test pulses larger than 0 mV, there were relatively small increases in peak Na^+ current amplitudes which were not significant ($0.45 < P < 0.68$, *see* squares in panel *A*). These observations suggest that the activation curve of Na^+ currents shifts to more negative potentials during the initial 15–40 min. Figure 1*B* shows that indeed the activation curve (m) of Na^+ currents shifted by approximately 6 mV during the first 15–40 min in the hyperpolarizing direction. In the same panel, it is demonstrated that the steady-state inactivation curve (h) is essentially unaltered during that period. The shift in the activation curve of Na^+ currents to more negative voltages could explain the increase in Na^+ currents observed in the voltage range of -40 to 0 mV, and the absence of changes in peak currents for voltages > 0 mV. Panel *C* shows that τ_{on} decreased significantly at voltages more negative than 0 mV during the first 15–40 min. This acceleration of Na^+ current activation can be seen in several figures in this report (*see* Figs. 2, 6, 7, 10, 11, and 12). It is likely that the negative shift of the activation curve of Na^+ currents (panel *B*, open symbols) is a consequence of this decrease in τ_{on}. On the other hand, panel *D* shows that the inactivation time course of Na^+ currents (τ_h) did not change appreciably during these control experiments.

Table 1. Effects of PKC activators and related compounds on Na^+ currents[a]

Substances	Changes in Peak Na^+ currents
Control[b]	1.23 ± 0.06 (5)
DOG (75 μM)	0.43 ± 0.01 (5)
OAG (10 μM)	0.40 ± 0.01 (5)
Phorbol Esters (1 μM)	1.15 ± 0.05 (9)
Oleate (4 μM)	2.00 ± 0.01 (9)
Linolate (4 μM)	0.52 (2)
Linolanate (4 μM)	0.82 ± 0.07 (5)
Arachidonate (4 μM)	0.41 ± 0.02 (4)
Elaidate	1.25 (3)
Stearate	1.30 (2)

[a] Relative changes in peak current amplitude (mean ± sem, n) caused by different PKC activators or similar compounds in response to voltage clamp pulses from -100 to -10 mV. Alterations in Na^+ currents were assessed by measuring peak currents 15 min after the perforated-patch, whole cell clamp condition was attained (control condition), and 25 min after addition of substance.

[b] A 23% increase in peak Na^+ currents occurs during the first 15–40 min after the perforated-patch whole cell clamp condition is attained (*see* Fig. 1). This normal increase must be taken into consideration in evaluating the effects of PKC-related substances. The effects of phorbol esters, elaidate, or stearate, were not significantly different from control conditions ($0.90 < P < 0.57$). Other substances shown in the table changed the peak amplitude of Na^+ currents significantly ($0.001 < P < 0.00003$).

Effects of DAG-like Compounds on Na^+ Currents

Figure 2*A* shows the effects of 10 μM OAG on Na^+ currents elicited by voltage clamp pulses from -100 to -10 mV (inward currents) and $+60$ mV (outward currents). At each membrane voltage, the larger current recordings were obtained in control conditions while the smaller current traces were obtained 25 min after addition of OAG. OAG caused a clear reduction in current amplitudes at all voltages. Another noticeable alteration in this figure is an acceleration in τ_{on}. However, this effect was not significantly different from what normally occurred in control conditions (*see* Fig. 1*C*). In Fig. 2*B*, the relationships between membrane voltage and peak Na^+ currents are depicted in control conditions (circles) and 25 min after exposure to 10 μM OAG (triangles). The effects of DAG-like compounds on Na^+ currents were not reversible even after prolonged (15–30 min) cell superfusion with control solution. The time course of effects of 75 μM DOG on peak Na^+ currents in response to voltage steps from -100 to -10 mV is shown in Fig. 3 as circles. In this figure, DOG was applied and withdrawn at $t = 15$ and 25 min, respectively. Notice that while the onset of attenuation of Na^+ currents was relatively rapid and complete within ≈10–15 min, the attenuation did not reverse within the duration of the experiment.

Table 2. Effects of different PKC-related substances on Na^+ current activation and inactivation parameters

Experimental Condition	Activation			Inactivation		
	$\Delta V_{1/2}$(mV)	k (mV)	τ_{on} (msec)	$\Delta V_{1/2}$(mV)	k (mV)	τ_{inact} (msec)
Control (5)	−6.06 ± 2.64	9.58 ± 2.82	0.18 ± 0.01	−1.12 ± 0.69	−8.2 ± 0.63	0.83 ± 0.04
DOG (5)	−3.91 ± 2.20	8.47 ± 0.87	0.16 ± 0.03	−22.68 ± 1.67*	−9.12 ± 0.53	0.64 ± 0.02
OAG (5)	−10.04 ± 2.71	9.70 ± 0.92	0.14 ± 0.01	−8.4 ± 1.16**	−7.53 ± 0.43	0.64 ± 0.04
Phorbol (9)	−5.95 ± 0.76	9.25 ± 0.88	0.15 ± 0.01	−1.38 ± 0.37	−7.82 ± 0.77	0.64 ± 0.09
Oleate (9)	−5.53 ± 2.24	8.17 ± 0.74	0.17 ± 0.02	−2.28 ± 0.42	−9.13 ± 0.36	1.19 ± 0.15
Linolate (2)	−4.75	8.3	0.14	−26.97*	−10.62	0.68
Linolanate (5)	−8.86 ± 4.19	7.96 ± 1.13	0.15 ± 0.02	−11.30 ± 2.83***	−9.22 ± 0.26	0.88 ± 0.06
Arachidonic (2)	−8.77	7.85	0.17	−19.81*	−9.79	0.91
Elaidate (2)	−4.90	6.85	0.24	−1.6	−8.35	1.17
Stearate (2)	−3.99	8.58	0.21	−2.3	−7.67	0.92

The effects of different PKC-related substances on $\Delta V_{1/2}$ (half point of steady-state activation or inactivation curves), k (steepness of activation or inactivation curves) and τ_{on} and τ_{inact} (activation and inactivation time constants, respectively) measured at −10 mV, were evaluated at t = 15 min (control) and 25 min after exposure of cell to substance (t = 40 min). Control shows the mean variation of parameters (at t = 15 and 40 min) in cells (*see* Fig. 1) that were not treated with PKC-related substances. Because cells normally present a temporal variation in some (but not all) parameters (Fig. 1), the statistical significance of alterations caused by different PKC-related substances must be related to that control group of cells. Asterisks indicate statistically significant differences found between measurements in control group of cells and cells treated with PKC-related compound: $*P < 6.10^{-6}$; $**P < 0.005$; $***P < 0.02$. Values are expressed as mean ± SEM. Concentrations of different substances are the same as indicated in Table 1. Even though the displayed values for τ's were measured at −10 mV, PKC-related substances did not affect these time constants at other test voltages (*results not shown*).

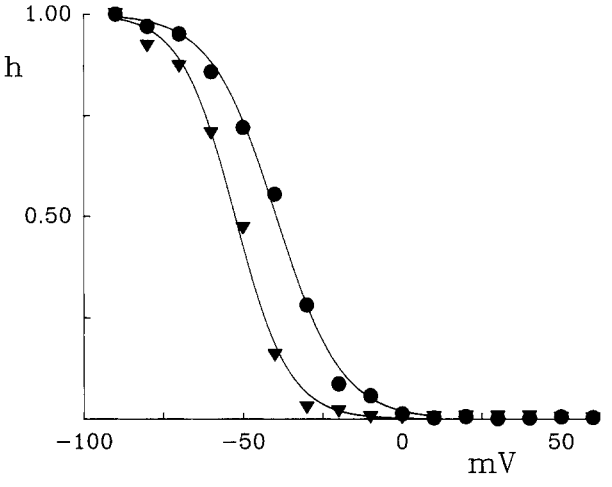

Fig. 4. Steady-state inactivation curves (h) in control (circles) and 25 min after addition of 10 μM OAG. Nonlinear regression curves were drawn through the experimental points with the following values (*see* Materials and Methods for equation): $V_{1/2}$ = −39.51 (circles), and −52.46 mV (triangles); k = 10.18 (circles), and 8.41 mV (triangles).

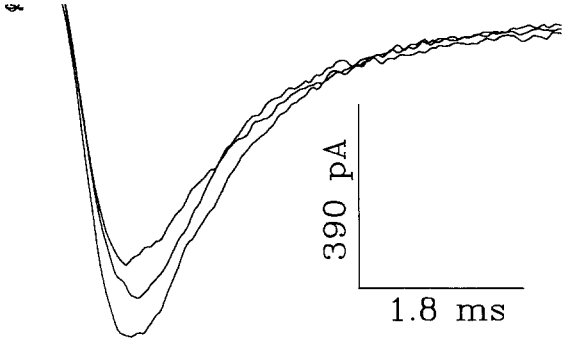

Fig. 5. Effects of calphostin C on the modulation of Na^+ currents by OAG. Three Na^+ current traces are shown in response to voltage clamp pulses from −100 to −10 mV. The smaller trace was obtained in control (15 min after the beginning of the experiment) conditions. The middle and larger current recordings were obtained after 10 min of perfusion with 0.1 μM calphostin C (t = 25 min), and 20 min after addition of 10 μM OAG (t = 45 min), respectively.

Essentially similar results to the one depicted in Fig. 2 were observed in four other experiments with 10 μM OAG and in five experiments with 75 μM DOG (*see* Table 1). Reduction in peak Na^+ currents caused either by OAG or DOG were highly significant in relation to control conditions (OAG: $P < 0.0005$; DOG: $P < 0.00003$). The effects of either OAG or DOG on τ_{on} and steady-state activation curves ($V_{1/2}$ and k) were not significant (*see* Table 2 and Fig. 1). On the other hand, DAG-like substances significantly shifted the steady-state inactivation curve (h) to more negative voltages. The result of an experiment illustrating this shift is shown in Fig. 4 (*see* Table 2 for the average values).

The effects of OAG or DOG on Na^+ currents were fully prevented by pre-exposing cells with different PKC inhibitors. In four different experiments with 1 μM staurosporine, one experiment with 0.1 μM calphostin C, and two other experiments with H7 (10 μM) or polymyxin-B (200 μM), the decrease in Na^+ currents that would

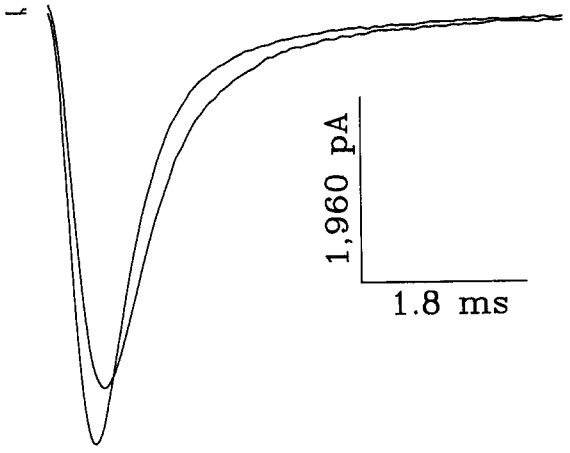

Fig. 6. Effects of 1 μM phorbol 12, 13 didecanoate on Na^+ currents. Two current traces in response to voltage clamp pulses from −100 to −10 mV in control conditions (smaller trace, t = 15 min), and 25 min after addition of phorbol ester.

have been caused by DAG-like components did not occur. A typical experiment of this group is shown in Fig. 5. In this figure, current traces in response to voltage clamp steps from −100 to −10 mV are illustrated. The smaller current trace was recorded in control conditions, while the middle and larger recordings were obtained in the presence of 0.1 μM calphostin C, and 0.1 μM calphostin C plus 10 μM OAG, respectively. It can be seen that calphostin C not only prevented the inhibitory effect of OAG on Na^+ currents, but also did not prevent the increase in Na^+ current amplitude that does normally occur in control conditions (*see* Fig. 1).

Phorbol esters Do Not Modify Na^+ Currents in N1E-115 Neuroblastoma Cells

Phorbol ester compounds are extremely effective in activating PKC (Stryer, 1988). Surprisingly, in nine different experiments, phorbol esters did not modify Na^+ currents. Figure 6 shows two current traces in control conditions, and after addition of a relatively high concentration (1 μM) of phorbol 12, 13 didecanoate. Phorbol esters did not prevent the increase in Na^+ current nor the decrease of its activation time course that normally occurred within 15–40 min after beginning of recordings (*see* Table 1 and Fig. 1). As indicated in Table 2, phorbol esters neither affected the steady-state properties of the activation (m) and inactivation (h) curves, nor the activation and inactivation time constants of Na^+ currents.

Cis-unsaturated Fatty Acids (Linolate, Linolenate, and Arachidonate) Attenuate Na^+ Currents

Some *cis*-unsaturated fatty acids can activate PKC's (Asaoka et al., 1992; Azzi, Boscoboinik & Hensey, 1992). At least one PKC isoform (PKCζ) can be specifically activated by *cis*-fatty acids (Asaoka et al., 1992). Na^+currents were attenuated by linolate (*cis*-9,12-octadecadienoic acid), linolenate (*cis*-9,12,15-octadecatrienoic acid), and arachidonate (*cis*-5,8,11,14,-eicosatetraenoic acid).

Figure 7*A* shows Na^+ currents in response to −10 and +60 mV voltage clamp pulses in control (larger traces) and in the presence of 4 μM linolenic acid. Linolenate depressed Na^+ currents. As with DAG-like compounds, this inhibition was not accompanied by alterations in τ_{on}, τ_h, or steady-state activation curves. The time course of effects of 4 μM linolenic acid on peak Na^+ currents are shown in Fig. 3 as triangles. As with DAG-like compounds, the attenuation of Na^+ currents caused by *cis*-unsaturated fatty acids developed rapidly but did not show significant signs of reversibility during experiments.

Different CUFA's (*cis*-unsaturated fatty acids) shifted considerably the steady-state inactivation curve of Na^+ currents to more negative voltages (*see* Table 2). An extremely important observation in Fig. 7*B* is that, for membrane potentials more negative than +20 mV, the inhibition of Na^+ currents was significantly more pronounced than at more positive voltages. This is a very reproducible and clear distinction between Na^+ current inhibition induced by DAG-like compounds (*see* Fig. 2) and the one caused by this group of *cis*-fatty acids. In Fig. 8, this point is better illustrated by plotting the relative peak Na^+ currents ($I/I_{control}$) *vs.* membrane potential for the experiments illustrated in Figs. 2 (circles) and 7 (filled triangles). While the blocking effect caused by DAG-like compounds was stronger for voltages > 20 mV, the attenuation caused by *cis*-unsaturated fatty acids was almost completely relieved at these voltages.

In the experiment of Fig. 7, the calculated V_{Na} was 16 mV. Because the attenuation of Na^+ currents by *cis*-unsaturated fatty acids was considerably relieved at voltages > 20 mV, it is possible that these PKC activators preferentially attenuate *inward* Na^+ currents. To examine this possibility, experiments were performed with *cis*-unsaturated fatty acids under different V_{Na}'s. This is illustrated in Fig. 9. In this figure, the relative attenuation of peak Na^+ currents was plotted against membrane potential for three different experiments. Circles were obtained from an experiment in which the calculated V_{Na} was 51 mV, and squares were obtained from a different experiment where V_{Na} was 0 mV. (Triangles in this figure are from the same experiment shown in Fig. 7.) Interestingly, for test pulses above V_{Na} there was a significant relief of attenuation of peak Na^+ currents caused by *cis*-fatty acids.

Na^+ current attenuation caused by *cis*-fatty acids could also be prevented by PKC inhibitors. This is illustrated in Fig. 10 which shows Na^+ currents in response to depolarizing voltage steps from −100 to −10 mV. The smallest current trace was obtained in control

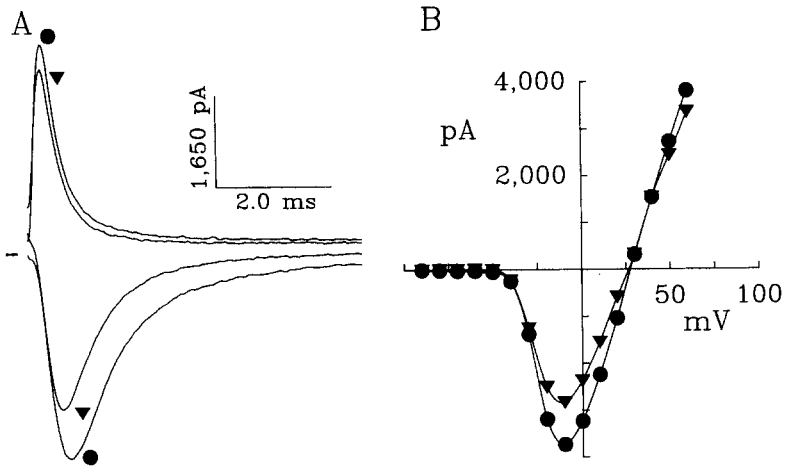

Fig. 7. Effects of 4 μM linolenic acid on Na^+ current amplitude. (*A*) Membrane currents in response to −10 (inward currents) and +60 (outward currents) mV from a holding membrane potential of −100 mV. Triangles indicate recordings obtained 25 min after exposure to linolenate, and circles were obtained in control (t = 15 min after beginning of experiment) conditions. (*B*) Current-voltage relationships of the same experiment illustrated in *A*.

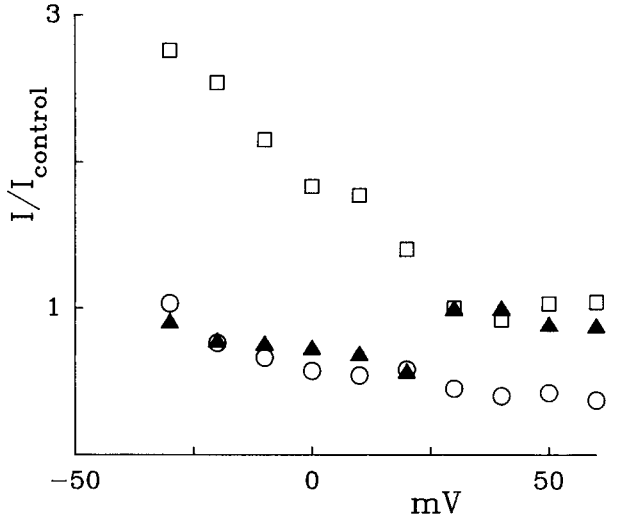

Fig. 8. Relative peak Na^+ currents ($I/I_{control}$) in the presence of OAG (circles), linolenate (triangles), or oleate (squares). Experimental points were extracted from experiments illustrated in Figs. 2, 5, and 7. Notice the difference between Na^+ current inhibition caused by OAG or linolenate.

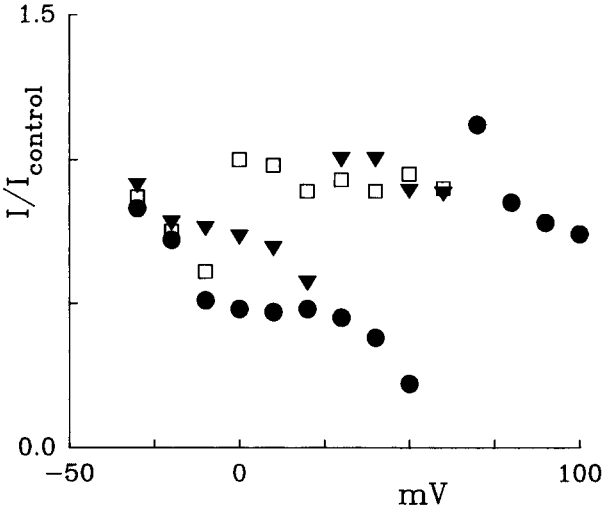

Fig. 9. Relative peak Na^+ currents ($I/I_{control}$) and voltage clamp test pulses. In these three different experiments, 4 μM linolenic acid was used to attenuate Na^+ currents. Triangles were obtained from experiment shown in Fig. 7. Squares were obtained from an experiment with 75 mM Na^+ in the pipette and bath solutions (V_{Na} = 0 mV). In this experiment 35 mM intracellular CsCl was replaced with the same concentration of NaCl. Circles were obtained from a different experiment where calculated V_{Na} is 51 mV (20 mM NaCl in the pipette solution was replaced by the same concentration of CsF, while 75 mM CholineCl in th external bath was replaced with the same concentration of NaCl). Notice that for the three different experiments, Na^+ outward currents are substantially less attenuated than the inward currents.

conditions, the middle trace after exposure to 0.1 μM calphostin C, and the larger trace after addition of 4 μM linolenate to the calphostin C containing solution. Notice that neither calphostin C nor linolenate prevented the time-dependent increase in Na^+ currents that are normally observed in these cells. In nine other experiments, the inhibitory effects of *cis*-fatty acids on Na^+ currents were prevented by pretreating cells with 0.1 μM calphostin C (n = 3), 10 μM H7 (n = 2) or 1 μM staurosporine (n = 4).

Oleate (Another *cis*-unsaturated Fatty Acid) Enhances Na^+ Currents

Oleate (*cis*-9-octadecenoic acid) enhanced Na^+ currents, and said enhancement could be abolished by pretreating N1E-115 cells with different PKC inhibitors. This is shown in the next two figures. Figure 11*A* shows the original current traces in response to voltage clamp pulses from −100 to −10 (inward currents) and to +60 mV (outward currents) in control conditions (circles), and after addition of 4 μM oleate to the perfusion solution (triangles). In panel *B* of the same figure, the complete *I-V* relationship is shown. The voltage-dependent enhancement of peak Na^+ currents caused by oleic acid is also illustrated in Fig. 8 as squares.

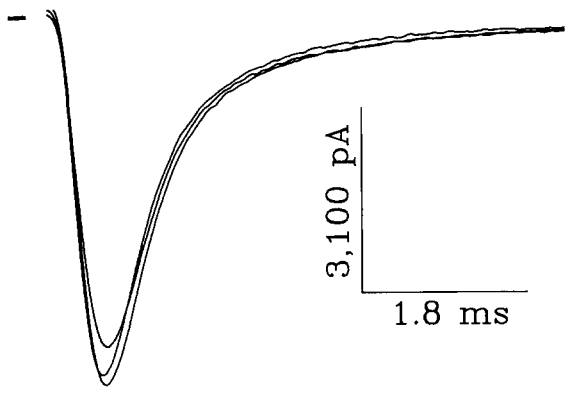

Fig. 10. Depression of Na^+ currents caused by linolenate is prevented by PKC inhibitors. Na^+ current recordings in response to voltage steps from −100 to −10 mV in control (t = 15 min after beginning of experiment, smaller current trace), 10 min after adding 0.1 μM calphostin C (t = 25, middle trace), and after adding 4 μM linolenate to the calphostin C containing solution (t = 45 min, larger current trace).

As with other PKC activators, the effects of oleate were rapidly induced but were not reversible even after extensive perfusion of drug from external solution (*see squares* in Fig. 3). Figure 12 shows different Na^+ current recordings in response to voltage pulses from −100 to −10 mV. The smaller current trace was obtained in control conditions (t = 15 min), while the middle and the larger traces were obtained 10 min after perfusion with 1 μM staurosporine, and 25 min after adding oleate to the perfusing system, respectively. Similar results were also obtained in one experiment with 0.1 μM calphostin C, and in two experiments with H7 (10 μM). The effects of oleate were limited to an increase in Na^+ current amplitude (*see* Tables 1 and 2).

Saturated and *trans*-unsaturated Fatty Acids Do Not Change Na^+ Currents in N1E-115 Cells

Saturated and *trans*-unsaturated fatty acids do not activate PKC's (Asaoka et al., 1992). Elaidic (*trans*-9-octadecenoic, three experiments), stearic (octadecanoic, two experiments), and myristic (tetradecanoic) acids (one experiment) did not affect Na^+ currents (*see* Tables 1 and 2). These results support the conclusion that the *cis*-unsaturated fatty acids used in this study are modulating Na^+ channel function via activation of PKC's.

Discussion

The most important experimental conclusion of this study is that different PKC activators modulate Na^+ currents in three distinct ways. DAG-like substances and *cis*-unsaturated fatty acids (but not oleate) attenuated, while oleate enhanced Na^+ currents. The attenuation of Na^+ currents by DAG-like substances or *cis*-fatty acids was accompanied by a significant shift of the steady-stage inactivation curves to more hyperpolarized potentials, while the activation curve and the time constants of inactivation or activation at different test voltages remained essentially unchanged. The enhancement of Na^+ currents by oleate was not accompanied by other significant alterations of these currents. The effects of PKC activators could be completely prevented by pretreating neuroblastoma cells for 10–15 min with PKC inhibitors. PKC inhibitors, by themselves, did not modify Na^+ currents. Moreover, saturated or *trans*-unsaturated fatty acids did not affect Na^+ currents. The alterations caused by PKC activators on Na^+ currents were irreversible within the duration of experiments. It is possible that such "irreversibility" is due to the hydrophobic nature of PKC activators. They probably bind to different intracellular components, lengthening the time required for these compounds to leave the intracellular environment and interrupt the activation of PKC. Surprisingly, phorbol esters did not affect Na^+ currents. Taken together, these results are suggestive that alterations in Na^+ currents caused by PKC activators are being mediated by PKC-induced phosphorylation of Na^+ channels and/or an intracellular component capable of interacting with and modulating Na^+ channels.

Na^+ current inhibition caused by DAG-like substances had different characteristics from the one caused by *cis*-fatty acids. While the inhibition caused by DAG-like substances was continuously dependent on membrane voltage, being stronger at positive voltages (*see* Figs. 2 and 8, and also Fig. 1*B* in Numann et al., 1991), *cis*-fatty acids preferentially attenuated inward Na^+ currents with little or no effects on outward currents (*see* Figs. 7–9, and compare *I-V* plots between Figs. 2 and 7). *Cis*-fatty acids modulated (enhanced with oleic acid, or attenuated with other fatty acids) preferentially inward Na^+ currents. This is a novel and puzzling observation for which, at present, we are unable to provide a reasonable explanation.

PKC activation has been linked with attenuation of Na^+ currents in different cells (*cf.* Catterall, 1992; *see references* in Introduction). Of particular interest to our results is the work by Linden and Routtenberg (1989). Those authors have investigated the effects of different PKC activators on Na^+ currents in the same cell line used in the present investigation. Significant differences were noticed between their experimental findings and ours: (i) Linden and Routtenberg did not observe effects of OAG on Na^+ currents. In our experiments, OAG and DOG clearly inhibited Na^+ currents; (ii) *cis*-unsaturated fatty acids (*including oleate*) attenuated Na^+ currents without affecting steady-state inactivation. In our experiments, the inhibition caused by *cis*-unsaturated fatty acids (excluding oleic acid)

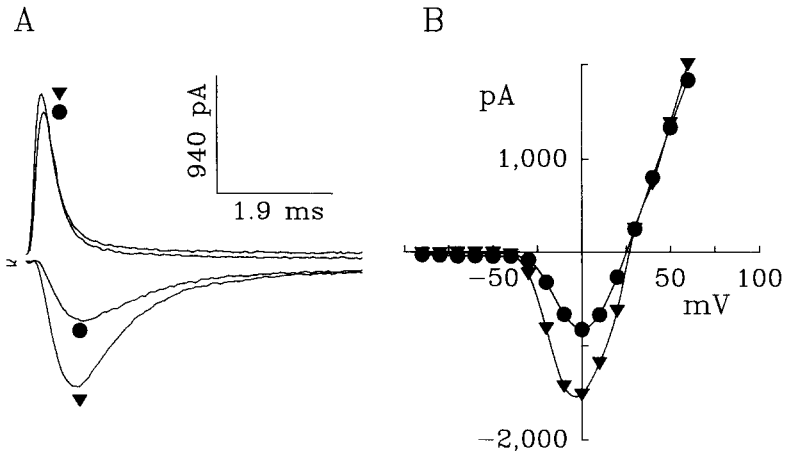

Fig. 11. Enhancement of Na^+ currents caused by oleic acid (4 μM). (*A*) Current recordings in response to voltage pulses from −100 to −10 (inward currents) and +60 mV (outward currents). Control (t = 15 min), circles, and 25 min after perfusing the cell with oleic acid. (*B*) Peak Na^+ current-voltage relationship from the same experiment in *A*.

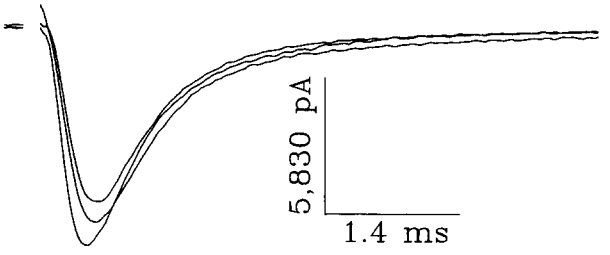

Fig. 12. Effects of oleic acid are prevented by PKC inhibitors. Three Na^+ current recordings obtained in control (t = 15 min), 10 min after perfusion with μM staurosporine (t = 25 min), and with staurosporine and 4 μM oleate (t = 45 min). Notice that staurosporine as well as oleate did not prevent the Na^+ current from having the normal increase in amplitude and decrease in activation time.

was accompanied by an appreciable hyperpolarizing voltage shift in the steady-state inactivation curves (*see* Table 2); (iii) another important difference concerns the reversibility of effects of PKC activators: Linden and Routtenberg (1989) reported that the effects of PKC activators were reversible within several minutes; (iv) In our experiments oleic acid caused a dramatic increase in Na^+ current amplitude. Therefore, the only common observation shared by us and Linden and Routtenberg (1989) is the absence of effects of phorbol esters on Na^+ currents in N1E-115 neuroblastoma cells. The effects of PKC activators on Na^+ currents described by Linden and Routtenberg (1989) were also prevented with different PKC inhibitors. A significant methodological difference exists between our experimental approach and the one followed by Linden and Routtenberg. While they used the whole cell voltage clamp technique to record Na^+ currents, we have been using the perforated patch clamp method which seems to provide a close-to-normal intracellular milieu by avoiding the washout of macromolecules from the cytoplasm (Horn & Marty, 1988). It is thus possible that the full physiological effects of PKC activators on Na^+ currents might require the presence of a normal or close-to-normal intracellular environment to modify Na^+ currents in the manner shown in the present study. In a first pass, the discrepancies observed between our experimental results and those previously reported (Linden & Routtenberg, 1989) might be a consequence of the use of different patch clamp methodologies. If this is correct (experiments in progress in our laboratory are addressing this possibility), then the modulation of Na^+ currents by different PKC activators can be a very complex phenomenon under physiological conditions.

Different nonmutually exclusive hypotheses could explain the diversity of experimental results obtained in this study. One of them is related to the existence of different PKC isoforms (Asaoka et al., 1992; Azzi et al., 1992), and to the presence of several phosphorylation sites on Na^+ channels (Murphy & Catterall, 1992). It is plausible that different PKC activators could mobilize different sets of PKC isoforms (Khan, Blobe & Hannum, 1992). These, in turn, could phosphorylate Na^+ channels with different patterns leading to different modulations of Na^+ channel function. In this respect, it is important to mention that PKCζ lacks the binding domain for phorbol esters (Asaoka et al., 1992). Because phorbol esters did not modify Na^+ currents in N1E-115 cells, it is possible that PKCζ needs to be necessarily activated in order for Na^+ currents to be modulated. It is thus possible that a common isoform present among different sets of PKC isoforms mobilized by different PKC activators is PKCζ.

Another possibility that must be seriously considered is that an unknown intracellular component after being phosphorylated by PKC might interact with and modulate Na^+ channels. This is an interesting possibility because the effects of PKC activation on Na^+ currents seem to depend on whether the normal intracellular environment is or is not maintained (*see above*).

Also, it has been suggested (*cf.* Ordway et al., 1991) that fatty acids can modulate some ion channels by directly interacting with them. Consequently, it is possi-

ble that some of the effects seen in this study might arise as a consequence of such direct interaction with Na^+ channels. Some evidence for this was published (Takenaka, Horie & Hiro, 1987; Wieland, Fletcher & Gong, 1992). Because PKC inhibitors prevented the effects of *cis*-fatty acids on Na^+ currents, it is important to note that this hypothetical direct effect may depend on PKC activation of the channel and/or an unknown intracellular component. However, the implicit assumption in this latter possibility that needs to be investigated is that PKC inhibitors do not prevent the direct effects of fatty acids on Na^+ channels.

The multiple effects of different PKC activators on Na^+ currents raise a word of caution in attempting to infer the physiological consequences of PKC activation for a given biological phenomenon. In this sense, an important and general signaling mechanism in physiology is the activation of phospholipases leading to the simultaneous production of DAG's and *cis*-unsaturated fatty acids. Because these substances might have completely different effects on Na^+ currents, it is not clear how Na^+ currents and action potential generation or propagation in excitable cells will react to a physiological activation of phospholipases.

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