J. Membrane Biol. 144, 59–69 (1995)

The Journal of
Membrane
Biology

Direct Modulation of Na^+ Currents by Protein Kinase C Activators in Mouse Neuroblastoma Cells

M. Renganathan, C.M.G. Godoy, S. Cukierman
Department of Physiology, Loyola Medical School, 2160 South First Avenue, Maywood, Illinois 60153

Received: 31 May 1994/Revised: 20 October 1994

Abstract. We investigated the effects of different protein kinase C (PKC) activators on Na^+ currents using the conventional whole-cell and the inside-out macropatch voltage-clamp techniques in mouse neuroblastoma cells (N1E-115). Two different categories of PKC activators were investigated: the cis-unsaturated fatty acids (CUFAs): oleic (*cis*-9-octadecenoic), linoleic (*cis*-9-12-octadecadienoic), and linolenic acid (*cis*-9-12-15-octadecatrienoic), and, the diacylglycerol (DAG) derivative 1-2-dioctanoyl-sn-glycerol (DOG). These substances caused the following alterations on Na^+ currents: (i) Na^+ currents were attenuated as a function of voltage. While DOG attenuated both inward and outward Na^+ currents in a monotonic and continuous voltage-dependent manner, CUFAs preferentially attenuated inward currents; (ii) the steady-state activation curve of Na^+ currents shifted to more depolarized voltages; (iii) opposite to the activation curve, the steady-state inactivation curve of Na^+ channels (h curve) shifted to more hyperpolarized voltages; (iv) the time course of inactivation development was accelerated by PKC activators, while the recovery from inactivation was not affected; (v) substances that inhibit different metabolic pathways (PKC activation, cyclooxygenase, lipooxygenase, and P-450 pathways) did not prevent the effects of PKC activators on Na^+ currents. One fully saturated fatty acid (octadecanoic acid), a trans-unsaturated fatty acid (*trans*-9-octadecenoic), and different phorbol esters did not affect Na^+ currents; (vi) effects of different PKC activators on Na^+ currents were completely reversible. These observations suggest that PKC activators might interact with Na^+ channels directly. These direct effects must be taken into consideration in evaluating the overall effect of PKC activation on Na^+ channels. Moreover, it is likely that this direct interaction could account, at least in part, for the diversity of effects of PKC activators on Na^+ channels.

Key words: Na channels — Modulation — Protein kinase C — Activators — Membrane currents

Introduction

Ion channels are transmembrane proteins responsible for the electrical properties of cells. As with proteins in general, ion channels can be modulated by intracellular messengers via activation of protein kinases (Kaczmarek & Levitan, 1987; Hille, 1992). Activators of protein kinase C (PKC) are an important category of intracellular messengers. Cis-unsaturated fatty acids (CUFAs) and diacylglycerol compounds (DAGs), which activate PKC, can be released from the intracellular side of the membrane following receptor mediated activation of phospholipases (*see* Asaoka et al. 1992 for review).

Considerable attention has been directed to the effects of protein kinase C activators on Na^+ channels (*cf.* Catterall, 1992). Activation of protein kinase C by CUFAs (Linden & Routtenberg, 1989), or DAG-like compounds, depressed Na^+ currents in different cells (*see* Catterall, 1992 for review). However, direct (independent of metabolic pathways) effects of CUFAs or DAGs on K^+ channels (Kim & Clapham, 1989; Ordway et al. 1989; Bowlby & Levitan, 1994), Ca^{2+} channels (Hockberger et al. 1989; Shimada & Somlyo, 1992), and many other ionic channels (*see* review by Meves, 1994) have been reported recently. Therefore, the possibility that some PKC activators modulate Na^+ channels independently or in addition to PKC activation exists. Indeed, Fraser et al. (1993) have shown that arachidonic acid attenuated Na^+ currents in striatal neurons. Their results suggested a direct interaction between arachi-

Correspondence to: S. Cukierman

donic acid and Na^+ channels. On the other hand, Linden & Routtenberg (1989) proposed that cis-unsaturated fatty acids (oleic, linoleic, and linolenic acid) depressed Na^+ currents on N1E-115 neuroblastoma cells via PKC activation.

Our previous study on the effects of protein kinase C activators in neuroblastoma N1E-115 cells using the perforated patch method (Godoy & Cukierman, 1994) revealed that these compounds modulate Na^+ currents via activation of PKC. In that study however, Na^+ currents did not respond in the same way to different CUFAs and DAGs (Godoy & Cukierman, 1994; *see* Discussion). We reasoned that differences in responses of Na^+ currents to different PKC activators could be a consequence of a combination of different direct (independent of metabolic pathways) effects of these substances on Na^+ channels, and phosphorylation of channels (and/or unknown intracellular component capable of modulating Na^+ channels) via PKC activation (Godoy & Cukierman, 1994). The main purpose of this study was to address the possibility of direct (independent of metabolic pathways) effects of PKC activators (CUFAs and DAG-like substances) on Na^+ currents in neuroblastoma N1E-115 cells. Are there direct effects of these substances on Na^+ channels (Fraser et al. 1993), or do PKC activators modulate Na^+ currents via PKC activation only (Linden & Routtenberg, 1989; Godoy & Cukierman, 1994)? Using two different electrophysiological techniques (conventional whole-cell voltage-clamp method, and inside-out macropatches), we have demonstrated that different PKC activators shifted the activation curve of Na^+ currents to more *depolarized* voltages, and attenuated Na^+ currents. As to Na^+ current inactivation, PKC activators shifted the steady-state inactivation curve to more *hyperpolarized* voltages, and accelerated its development. These effects were rapidly reversible, and did not depend on PKC activation or on lipoxygenase, cyclooxygenase, or P-450 metabolic pathways.

Material and Methods

Tissue Culture

N1E-115 mouse neuroblastoma cells (kindly provided to us by Dr. M.W. Niremberg, NIH) were grown in 70 ml flasks, 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 10^4 cells/ml. Culture medium was replaced every day after 48 hr of cell splitting.

Solutions

In whole-cell voltage-clamp experiments, 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 (intracellular) solution contained (in mM): 10 NaF, 90 CsF, 30 NaCl, 10 ethyleneglycol-bis N,N,N′N′Tetraacetic (EGTA) and 10 HEPES (pH = 7.4, adjusted with CsOH). These solutions were used to study inward and outward Na^+ currents while blocking K^+ and Ca^{2+} currents. In a few experiments (*see* Fig. 4), 75 mM extracellular choline-Cl was replaced by an equimolar concentration of NaCl, and on the intracellular side, 20 mM NaCl was replaced with the same concentration of CsCl (total concentration of CsF = 110 mM). In experiments using inside-out macropatches, the pipette and bath solutions were the extracellular and intracellular solutions (described above), respectively.

Drugs

The following substances were purchased either from Sigma (St. Louis, MO), or Calbiochem (San Diego, CA): phorbol 12,13-didecanoate, phorbol 12,13 diacetate, phorbol 12-myristate 13-acetate, oleic, linoleic, linolenic, elaidic acids, DOG (1-2-dioctanoyl-sn-glycerol), indomethacin, nordihydroguaiaretic, and stearic acid, staurosporine, calphostin C, and H7. The acetylenic analog of arachidonic acid (ETYA) was obtained from Boehringer-Mannheim (New York, NY). Hydrophobic compounds were dissolved in dimethylsulfoxide (DMSO) and added to solutions upon sonication. Final DMSO concentration was less than 0.1% and at this concentration did not affect Na^+ currents.

Electrophysiology

Cell attached plastic coverslips were transferred to the experimental chamber (volume = 200 µl) mounted on an inverted microscope, and perfused at a rate of 1 ml/min. Membrane currents were recorded using the conventional whole-cell voltage-clamp method or excised inside-out macropatches (Hamill et al., 1981). Experiments were performed at room temperature (21–23°C). Patch electrodes were pulled from borosilicate capillaries (W.P. Instruments, Miami, FL) using a programmable puller (P-87, Sutter Instrument, Novato, CA). Electrode tip diameters were typically 2 µm and had resistances of ≈0.6 MΩ when filled with pipette solutions. Typical access resistance values were between 0.8 and 1.4 MΩ, and, in most experiments, series compensation was used at a level of 50 to 75%. To reduce pipette capacitance, the tip of the patch electrode was covered with hydrophobic paint (Pap Pen, Mt. Prospect, IL; *see* Cukierman, 1992). In our experiments, t = 0 is defined as the time when the whole-cell voltage-clamp (or inside-out macropatch) condition was obtained. Control current recordings were obtained at t = 15 min. Membrane currents were recorded and analyzed with an Axopatch 1D amplifier, and pClamp software, respectively (Axon Instruments, Foster City, CA).

Experimental Protocol

After attaining the whole-cell voltage-clamp condition (t = 0), control Na^+ current recordings were obtained at t = 15 min. Immediately after these recordings, a PKC activator was added to extracellular solution, and its effect on Na^+ currents were evaluated at t = 40 min. Following these recordings, the cell was superfused with a solution without PKC activator, and another set of Na^+ currents were recorded at t = 60 min. The exact same experimental protocol was used in a control group of cells which were not exposed to PKC activators (control experiments). In other experiments with different metabolic inhibitors, these substances were added to the extracellular solution prior to PKC activators

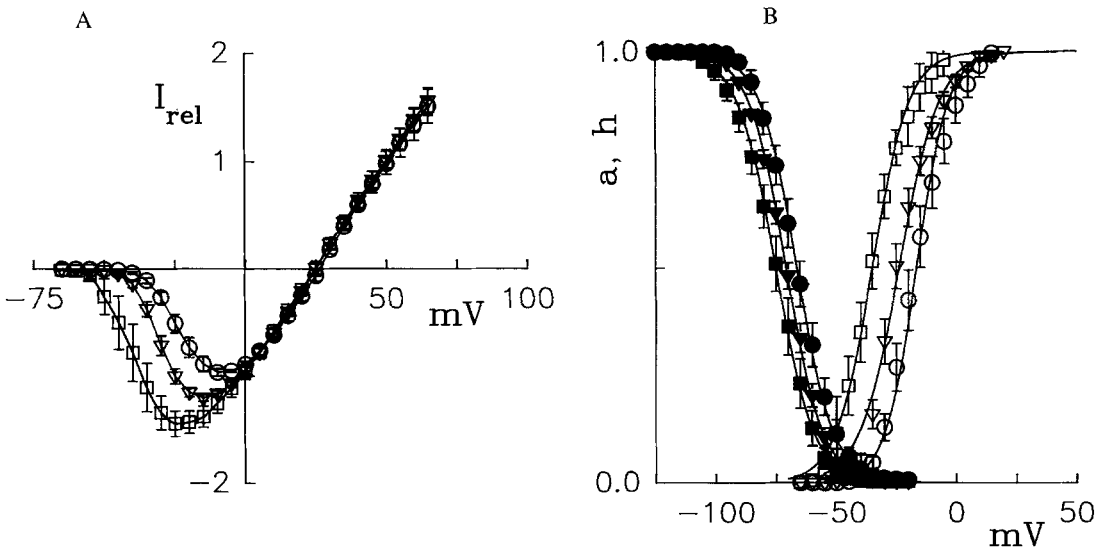

Fig. 1. Time dependent alterations of Na^+ currents following the establishment of the whole-cell voltage-clamp condition (t = 0 min). (*A*) shows normalized peak Na^+ current-voltage relationship for a group of seven different cells at t = 15 min (circles), 40 min (triangles), and 60 min (squares). For each cell, peak currents were normalized to peak Na^+ current in response to a voltage step from −130 to −10 mV at t = 15 min. (*B*) shows the steady-state activation (*a*, open symbols) and inactivation (*h*, filled symbols) curves of Na^+ currents at t = 15 min (circles), 40 min (triangles), and 60 min (squares). Experimental points are mean ± SEM (n = 7 cells). Curve-fitting parameters for the a and h curves were: a curves, $V_{0.5}$ s = −16.7 mV (circles), −23.7 mV (triangles), and −35.2 mV (squares); ks = 7.21 ± 0.46 mV (circles), 7.34 ± 0.28 mV (triangles), and 7.59 ± 0.14 (squares); h curves (filled symbols), $V_{0.5}$ s = −66.2 mV (circles), −70.7 mV (triangles), and −75.1 mV (squares); ks = −7.46 ± 0.23 mV (circles), −7.49 ± 0.22 mV (triangles), and −8.11 ± 0.21 (squares).

(*see* Results), and the same experimental protocol described above was followed.

Data Analysis

Different voltage-clamp pulse protocols were applied to cells from a −130 mV holding potential. The first protocol, used to construct activation curves, consisted of 5 msec pulses from a holding voltage to +60 mV in 5 mV steps at a frequency of 0.5 Hz. The second protocol, used to determine steady-state inactivation curves, consisted of 5 msec test pulses to 50 mV preceded by 100 msec prepulses of variable voltages (−120 to 0 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 the equation,

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

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

$$a = [1 + \exp(V_{0.5} - V)/k]^{-1}, \quad (2)$$

where $V_{0.5}$ is the midpoint of activation curve, and k is the curve steepness. Steady-state inactivation curves ($I_{Na\ peak}/I_{Na\ peak,\ max}$ = h *vs.* holding potential) were determined by a similar expression. Two other pulse protocols were used to determine the time course of inactivation, and recovery from inactivation of Na^+ currents. One of them, consisted in applying a test pulse to +50 mV following variable prepulse durations to voltages between −60 to −30 mV. Another pulse protocol consisted in applying twin voltage clamp pulses from −130 to +50 mV with variable interpulse intervals.

Fitting of experimental points to equations described above was done with Sigmaplot (Jandel Scientific, Corte Madera, CA). Paired or unpaired Student's *t*-test was used to evaluate the statistical significance of changes in Na^+ current parameters caused by different experimental conditions.

Results

Time-dependent Alterations of Na^+ Currents in Control Experiments

It has been shown in different cells that significant time-dependent alterations in Na^+ current gating occur during experiments in whole-cell voltage-clamp (Fernandez, Fox & Krasne, 1984; Hanck & Sheets, 1992). To properly evaluate the effects of different PKC activators on Na^+ currents, it is essential to characterize the time-dependent alterations that these currents might have during control conditions. Figure 1 shows time-dependent changes in peak Na^+ currents (*A*), and in steady-state activation and inactivation curves (*B*) in control conditions. Na^+ currents were measured at t = 15 min (circles), t = 40 min (triangles), and t = 60 min (squares) following the establishment of whole-cell voltage-clamp condition (t = 0). (*A*) demonstrates that there is an appreciable increase in peak Na^+ current amplitude within 15–60 min after start of experiment. The increase in peak current

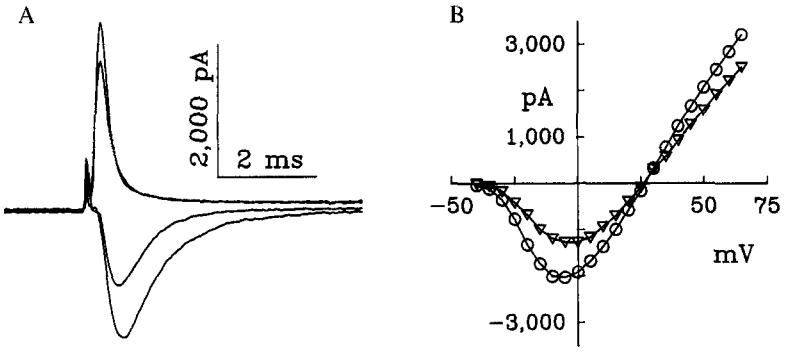

Fig. 2. Effects of 6 μM linolenic acid on Na^+ currents. (*A*) Na^+ currents in response to voltage clamp pulses from −130 to −10 mV (inward [negatively deflected] currents) and to +60 mV (outward [positively deflected] currents). For each pair of recordings, the largest trace was obtained in control conditions (t = 15 min), and the lower trace, 25 min after application of linolenate (t = 40 min). (*B*) peak Na^+ current-voltage relationships from the same experiment illustrated in *A*.

amplitude was limited to voltage steps between −60 and −5 mV. For voltage steps > −5 mV, there was no alteration in peak Na^+ currents with time. These observations suggest that the increase in peak Na^+ current is a consequence of changes in activation and inactivation gating properties. In seven different cells, there was an average shift from −16.7 ± 0.31 mV (t = 15 min) to −23.7 ± 1.4 (t = 40 min), and to −35.2 ± 1.0 (t = 60 min) for the activation curve of Na^+ currents (*B*, open symbols). The steady-state inactivation curve (h, filled symbols in *B*) also shifted in the hyperpolarizing direction: from −66.2 ± 1.8 mV (t = 15 min) to −70.7 ± 1.2 mV (t = 40 min), and to −75.1 ± 1.5 mV (t = 60 min). The steepness of the a and h curves were not significantly altered (*see* Fig. 1 legend for mean ± SEM).

Effects of PKC Activators on Na^+ Currents

Activation of Na^+ Currents

Figure 2*A* shows Na^+ current recordings in response to voltage steps from −130 to −10 mV (inward currents) and to 60 mV (outward currents). In each group of traces, the larger trace was obtained in control conditions (t = 15 min) and the smaller trace recorded 25 min after application of 6 μM linolenic acid to the extracellular solution (t = 40 min). This fatty acid caused a substantial attenuation of Na^+ currents as also illustrated in the *I-V* plot of *B*. Different from control experiments (Fig. 1), peak Na^+ current-voltage relationships in *B* indicate that attenuation is not accompanied by significant hyperpolarizing voltage shifts of the activation curve. This is better illustrated in Fig. 3*A* which shows activation curves for the same experiment. Notice that in control experiments (no treatment with PKC activator), and between t = 15 and 40 min, an average shift of −7.0 mV in the activation curve occurred (*see* Fig. 1; Table 1, column 2). Similar observations described in Figs. 2 and 3*A* with linolenate, were also seen with other PKC activators. Table 1 shows that all PKC activators tested (CUFAs and DOG) attenuated Na^+ currents (column 2), and prevented the significant hyperpolarizing shift of the activation curve that occurred in control conditions within 15–40 min of experiment (column 3). It is interesting to notice that phorbol esters, one C18 saturated fatty acid (stearate), and the trans-isomer of oleic acid (elaidate), which do not modulate Na^+ currents via PKC activation in these cells (Godoy & Cukierman, 1994), neither attenuated Na^+ currents nor induced voltage shifts in the activation curves in relation to control experiments (*see* Table 1, columns 2 and 3).

Attenuation of Na^+ currents caused by DAGs and CUFAs did not share the same features. While DOG blocked Na^+ currents in a continuously voltage-dependent manner (positive voltages enhancing attenuation), CUFAs attenuated *inward* Na^+ currents more effectively than outward currents. This is demonstrated in Fig. 4*A* which shows the relative attenuation of peak Na^+ currents ($I_r = I_{peak}$ in presence of DOG $/I_{peak}$ control) plotted as a function of different test potentials.[1] In 4*B*, relative peak Na^+ currents were plotted as a function of voltage following 25 min of application of external 6 μM linolenic acid. Circles were obtained with standard solutions (reversal potential ≈20 mV) while squares were obtained with extracellular and intracellular solutions modified to give a reversal potential of ≈50 mV for Na^+ currents. Notice that a continuous voltage-dependent attenuation of Na^+ currents occurs for inward Na^+ currents, and there is a clear decrease of attenuation of outward Na^+ currents in presence of linolenic acid. While a complete characterization of the different blocking effects of PKC activators on Na^+ currents will be the object of future studies, it is interesting to note that unidirectional block of Na^+ currents has been reported with paragracine (Seyama, Wu & Naharashi, 1980), and strychnine (Cahalan & Shapiro, 1976)

Inactivation of Na^+ Currents

In addition to attenuating Na^+ currents and shifting the activation curve in the depolarizing direction, PKC acti-

[1] In some (*see* Figs. 4 and 8*B*), but not in all experiments, and at voltages close to the activation threshold of Na^+ currents (−40 to −30 mV), enhancement of peak Na^+ currents occurred. While the reasons for this behavior are not clearly understood, it should be pointed out that for voltages > −30 mV, PKC activators inhibited Na^+ currents.

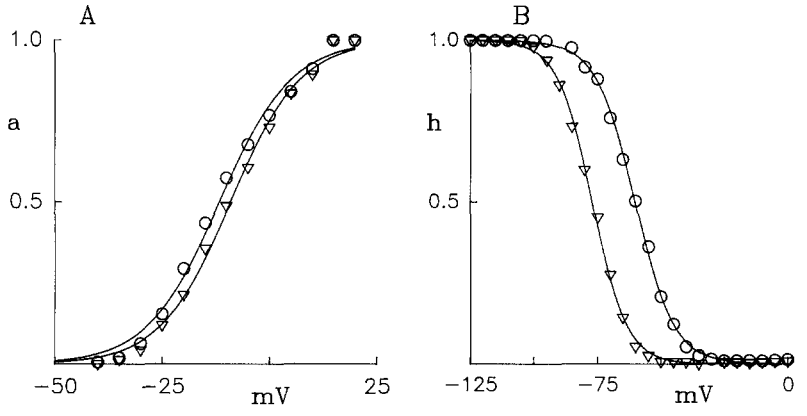

Fig. 3. (*A*) Steady state activation curves of Na^+ currents in control (t = 15 min, circles), and 25 min following exposure to 6 μM linolenic acid (t = 40 min, triangles). Same experiment as in Fig. 2. Curves were drawn according to equations described in methods with $V_{0.5}$ s = −11.8 mV (circles), and −8.2 mV (triangles), and ks = 9.15 mV (circles), and 7.93 mV (triangles). Notice that, unlike control a curves (*see* Fig. 1*B*), linolenate prevented the hyperpolarizing voltage shift that normally occurs between t = 15 and t = 40 min; (*B*) steady-state inactivation curves of Na^+ currents in control (t = 15 min, circles), and 25 min after extracellular application of 6 μM linolenic acid (t = 40 min, triangles). Curves drawn according to the following fitting parameters: $V_{0.5}$ s = −60.2 mV (circles), and −77.0 mV (triangles); ks = −7.74 (circles), and −6.83 (triangles). CUFAs enhanced the hyperpolarizing shift normally seen in control conditions between t = 15 and 40 min (*see* Fig. 1*B* and Table 1).

Table 1. Effects of different PKC-related compounds on Na^+ current properties[a]

	I_{rel}[b]	I_{rel}[c]	$\Delta V_{0.5}$[d], mV	$\Delta V_{0.5}$[e], mV	$\Delta V_{0.5}$[f], mV	$\Delta V_{0.5}$[g], mV
Control (n = 7)	1.21 ± 0.03	1.25 ± 0.09	−7.0 ± 1.1	−18.5 ± 1.0	−4.5 ± 1.5	−8.9 ± 2.4
Oleate (n = 8)	0.82 ± 0.04[h]	1.19 ± 0.12	−1.4 ± 0.6[h]	−16.3 ± 0.7	−10.4 ± 3.1[h]	−12.9 ± 4.2
Linolate (n = 6)	0.45 ± 0.04[h]	1.07 ± 0.39	−3.0 ± 1.1[h]	−21.4 ± 1.3	−27.3 ± 2.4[h]	−16.3 ± 4.7
Linolenate (n = 6)	0.52 ± 0.04[h]	1.15 ± 0.12	−0.4 ± 1.1[h]	−14.3 ± 1.4	−16.9 ± 1.2[h]	−9.9 ± 1.8
DOG (n = 6)	0.43 ± 0.03[h]	1.41 ± 0.03	−2.2 ± 1.8[h]	−18.5 ± 1.6	−14.1 ± 1.8[h]	−12.6 ± 4.3
Phorbol esters (n = 3)	1.23 ± 0.08		−5.3 ± 3.8		−5.0 ± 0.9	
Stearate (n = 3)	1.06 ± 0.07		−5.9 ± 0.7		−3.6 ± 0.1	
Elaidate (n = 2)	1.13		−5.4		−6.0	

[a] Different parameters shown in this Table were measured at t = 15 min (control conditions), at t = 40 min (25 min after exposure to drug), and at t = 60 min (20 min after drug wash out). In control experiments (line 2), no drug was applied to cells and data with different substances must be evaluated in relation to this control group of cells (*see* Materials and Methods). Except for DOG which was used at a concentration of 25 μM, all other substances were applied at a concentration of 6 μM. Data expressed as mean ± SEM.

[b,c] Relative changes in peak Na^+ currents in response to voltage steps from −130 to −10 mV. Currents were measured at t = 15 min, (control), t = 40 min (25 min after exposure to drug), and 20 min after recovery from effects of drug (t = 60 min). $I_{rel} = I_{40min}/I_{15min}$ (column 2), or I_{60min}/I_{15min} (column 3).

[d,e] Voltage shifts of the midpoint of activation curves of Na^+ currents at t = 40 min and t = 60 min, respectively. These differences were calculated in relation to $V_{0.5}$ at t = 15 min.

[f,g] Voltage shifts of the midpoint of inactivation curves (h) of Na^+ currents at t = 40 min and t = 60 min, respectively. These differences were calculated in relation to $V_{0.5}$ at t = 15 min.

[h] Statistically significant difference in relation to control group of cells ($P < 0.001$).

vators caused a significant hyperpolarizing shift of the steady-state inactivation curve (h curve). In the experiment illustrated in Fig. 3*B*, the h curve shifted by approximately −17 mV after application of 6 μM linolenic acid. Notice that in control experiments (cells not treated with PKC activators; Fig. 1 and Table 1, column 5), there is an approximately −4.5 shift of the h curve between 15 and 40 min. However, all PKC activators tested shifted the inactivation curve of Na^+ currents by significantly larger voltages, ≈2–6-fold in relation to control conditions (*see* Table 1, column 5). As with activation of Na^+ currents, phorbol esters, stearic and elaidic acid did not shift the h curve of Na^+ currents in relation to control conditions.

In control conditions, inactivation of Na^+ currents developed with an exponential time course with a time constant of 44.1 ± 4.2 msec ($n = 5$) for a voltage prepulse of −60 mV, and 5.8 ± 0.9 msec ($n = 7$) at −30 mV. Development of inactivation of Na^+ currents was speeded by different PKC activators. This is shown in Fig. 5 for an experiment with 25 μM DOG. When a conditioning prepulse of −60 mV (*A*) was applied, the time constant of inactivation development decreased from 44.5 to 19.6 msec (2.3-fold decrease). With a prepulse of −30 mV

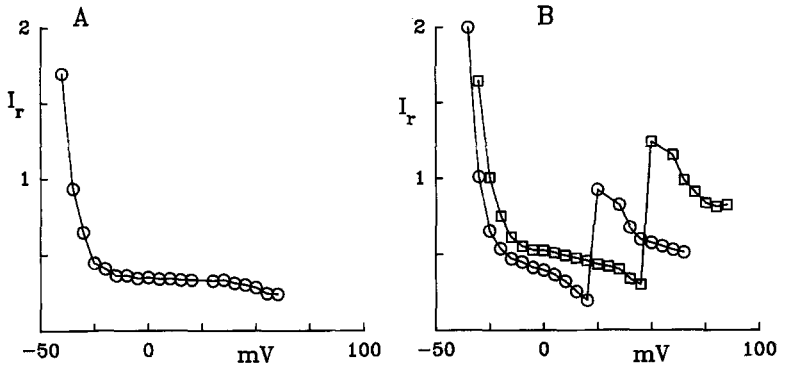

Fig. 4. Voltage-dependent attenuation of peak Na^+ currents in the presence of 25 μM DOG (*A*) or 6 μM linolenic acid (*B*). Peak Na^+ currents were normalized to control recordings (I_r). In *B*, circles were obtained using standard solutions while squares were obtained with solutions designed to change the electrochemical potential of Na^+ to ≈50 mV.

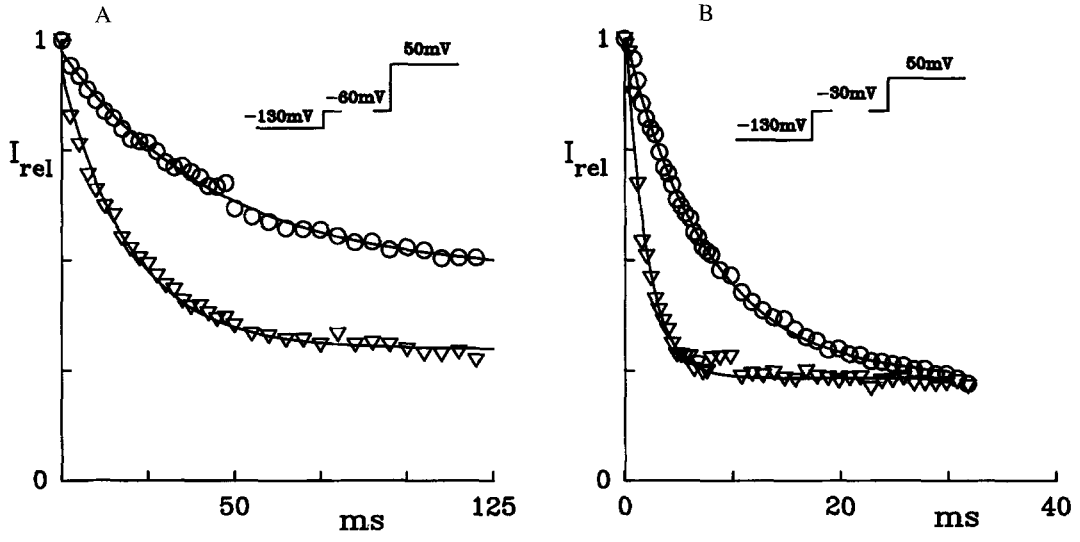

Fig. 5. Time course of development of inactivation in control conditions (circles) and 25 min after exposure of cell to 25 μM DOG. Voltage clamp pulse protocols are illustrated in the inset of each panel. Curves were fitted to the following expression $I_{rel} = a \cdot exp\ (-t/b) + c$, with the following values for *a*, *b*, and *c*, respectively: (*A*) 0.5, 44.5 msec 0.47 (circles); 0.63, 19.6 msec, 0.3 (triangles); (*B*) 0.78, 7.7 msec, 0.23 (circles); 0.84, 1.9 msec, 0.23 (triangles).

(*B*), the time constant decreased 4.1-fold (from 7.7 to 1.9 msec). Comparable quantitative results were obtained in two other experiments with DOG (*not shown*). CUFAs also accelerated the development of inactivation in relation to control conditions. In two different experiments, linolenic and linoleic acid decreased the time constant of inactivation development by 2.2-fold (with a −60 mV prepulse, average of two experiments), and 4.9-fold (with a −30 mV prepulse).

While inactivation in the presence of different intracellular activators proceeded with a faster time course in relation to control conditions, the recovery from inactivation was not changed. For example, at −130 mV recovery from inactivation under the effects of linoleic acid had a single exponential time course with a time constant of 4.1 ± 0.79 msec (mean ± SEM, n = 3). This value was not significantly different from control conditions (4.5 ± 0.85 msec, n = 3, paired observations). Quantitatively similar results were also observed in two experiments with DOG, two experiments with oleic, and one experiment with linolenic acid (*results not shown*).

Reversibility of Effects of PKC Activators on Na^+ Currents

The effects of PKC activators on Na^+ currents were completely reversible. The time courses of DOG-induced Na^+ current attenuation, and recovery from attenuation are illustrated in Fig. 6. Peak Na^+ currents were measured for voltage steps from −130 to −10 mV and plotted as filled circles in this figure. As with other PKC activators, the effects of DOG stabilized within 10–15 min. Notice that during exposure to DOG (indicated by vertical dashed lines), there was no appreciable shift in the midpoint of activation curves (open squares in Fig. 6; *see also* Figs. 2*B* and 3*A*). Following perfusion with a DOG-

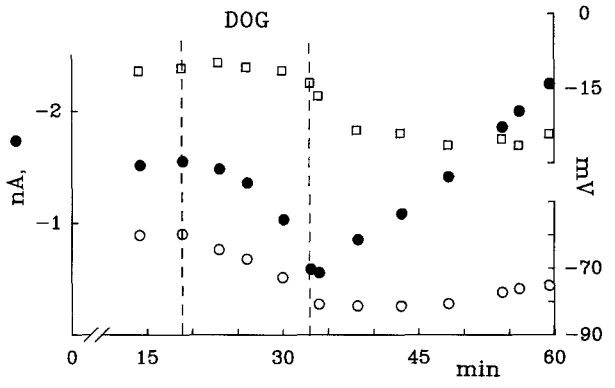

Fig. 6. Time course of effects of 25 μM DOG on peak Na^+ currents (filled circles), and on the midpoint of activation (squares), and inactivation (open circles) curves of currents. DOG was present in the extracellular solution during interval indicated by dashed lines. Peak currents were measured following voltage clamp steps from −130 to −10 mV. Solution exchange in the experimental chamber was complete in less than 10 sec.

free solution, there was an increase in peak current amplitude, which by the end of the experiment at t = 60 min, was considerably larger than at t = 15 min. This increase did normally occur in control experiments (*see* Fig. 1; column 2 in Table I), and is a consequence of hyperpolarizing voltage shifts in the activation curve of Na^+ channels. In Fig. 6, the overall shift in $V_{.05}$ between t = 15 and 60 min was ≈−17 mV, which was comparable to the average shift observed in the control group of cells (−18.5 mV, column 3 in Table 1).

The effects of PKC activators on the inactivation of Na^+ currents were also reversible. In Fig. 6, the midpoints of h curves were plotted as open circles. In this experiment, DOG induced a −21 mV shift in the h curve. After wash out of DOG, the h curve shifted by 6 mV in the depolarizing direction. The overall shift of the h curve following application and wash out of DOG was −15 mV, which was not significantly different from the average shift of control cells (column 6; Table 1).

The Effects of PKC Activators on Na^+ Currents Are Not Mediated by PKC Activation

In different experiments, N1E-115 cells were superfused with different PKC inhibitors (0.1–1 μM calphostin C, 1 μM staurosporine, or 10 μM H-7) for 15–20 min prior and throughout addition of PKC activators. PKC inhibitors did not prevent the effects of PKC activators on Na^+ currents. One such experiment is illustrated in Fig. 7. In this figure, the cell was superfused for 15 min with 1 μM calphostin-C before and during the experiment. *A* of this figure shows current recordings in response to voltage steps from −130 to −10 mV (inward currents) and to 60 mV (outward currents). For each group of recordings, the middle traces were obtained after the cell attained the whole-cell voltage-clamp condition (t = 0 min), the larger trace at t = 15 min (in absence of PKC activator, but still in presence of calphostin-C), and the smaller current trace 25 min after addition of 25 μM DOG. Notice that preexposure of cells to calphostin-C neither prevented the normal increase in Na^+ current amplitude that occurs after the beginning of the recordings, nor the current attenuation by DOG. In *B*, it is also shown that the hyperpolarizing shift in the h curve caused by DOG was not prevented by calphostin-C.

The type of experiment shown in Fig. 7 with DOG was also performed with different combinations of PKC inhibitors and activators. The addition of linoleic acid to cells previously treated with either 1 μM calphostin-C or 1 μM staurosporine attenuated peak Na^+ currents in response to voltage steps to −10 mV by 0.47 ± 0.04 (n = 5) or 0.45 ± 0.09 (n = 3), respectively, and also shifted the h curves by −25.2 ± 2.1 mV (n = 5) or −29.5 ± 3.9 mV (n = 3), respectively. These figures are not significantly different from data with cells that were not treated with PKC inhibitor before addition of PKC activator (*see* Table 1). In two different experiments, addition of oleate or linolenate to cells previously exposed to 1 μM staurosporin or calphostin-C attenuated peak Na^+ currents at −10 mV, by 0.67- or 0.52-fold. At the same time, the steady-state inactivation curve shifted by −13.0 or −19.4 mV, respectively. Finally, in two additional experiments with DOG, calphostin C (1 μM) and staurosporine (1 μM) were unable to prevent attenuation of Na^+ currents and changes in inactivation (*results not shown*).

In two different experiments, 1 μM staurosporine was added to the solution inside the pipette, and did not prevent the attenuation of peak Na^+ currents (average decrease of 0.47 for voltage steps to −10mV) caused by 6 μM linolenic acid. Taking into consideration that the pipette solution contained no ATP, and that extracellular as well as intracellular PKC inhibitors did not prevent the effects of PKC activators, it is possible to conclude that such effects are not being mediated by PKC activation.

Inhibitors of Cyclooxygenase, Lipoxygenase, and P-450 Metabolic Pathways Did Not Prevent the Effects of PKC Activators on Na^+ Currents

Cis-unsaturated fatty acids containing a pentadiene structure (–C = C – C – C = C–) are substrates for lipoxygenase, cyclooxygenase, and cytochrome P450 metabolic pathways. Even though oleic acid, which does not contain the pentadiene structure, attenuated Na^+ currents in qualitatively the same way as other CUFAs (*see* Table 1), the possibility that these metabolic pathways might be involved in the modulation of Na^+ currents was experimentally addressed. To this end, cells were superfused for 15–20 min before and during the experiments either

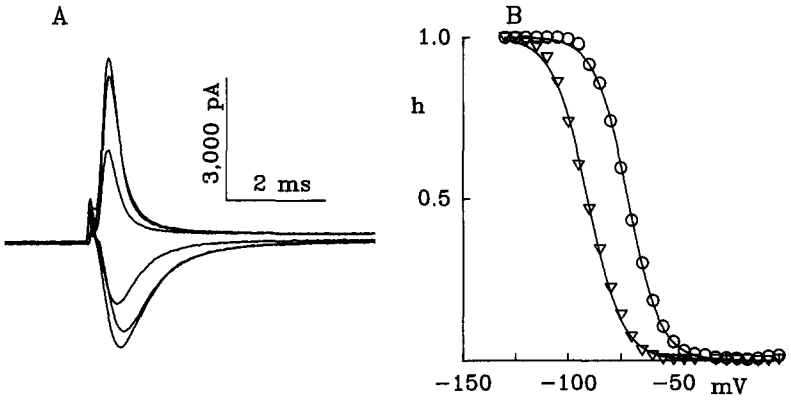

Fig. 7. PKC inhibitors did not prevent effects of PKC activators on Na^+ currents. (*A*) Na^+ currents in response to voltage clamp steps from −130 to −10 mV (inward currents) and to +60 mV (outward currents). In each group of traces, the middle and larger traces were recorded at t = 0 and 15 min after addition of 1 μM calphostin-C (t = 15), respectively. The smaller trace was obtained after 25 min in presence of calphostin-C and 25 μM DOG (t = 40 min). (*B*) h curves at t = 15 min (circles) and 40 min (triangles). These curves were fitted with the following parameters: $V_{0.5}$ = −71.7 mV (circles) and −90.8 mV (triangles); k = −7.62 (circles), and −8.1 (squares).

with external 10 μM indomethacin (a cyclooxygenase inhibitor, Egan & Gale, 1984), or external 50 μM nordihydroguaiaretic acid (NDGA, a lipoxygenase inhibitor, Shen & Winter, 1979), or 50 μM ETYA (acetylenic analogue of arachidonic acid that inhibits these metabolic pathways) loaded in the patch pipette. Figure 8 shows the effects of 6 μM linolenic acid on Na^+ currents in cells that were superfused with 10 μM indomethacin. *A* shows current recordings in response to voltage-clamp steps from −130 to −10 and +60 mV, in control and in presence of linolenate. *B* shows the complete *I-V* curves for this experiment in control (circles) and after linolenic acid (triangles). The h curve in this experiment shifted by −18 mV (*results not shown*). In two other experiments, 50 μM intracellular ETYA (loaded in the pipette solution) did not prevent current attenuation by 6 μM linolenic acid (an average reduction of peak Na^+ currents by 0.43 in response to test voltages of −10 mV was observed), nor the hyperpolarizing voltage shifts in the h curves (average shift of −21.9 mV). Essentially similar results occurred with different cells pretreated with NDGA (n = 2), and in one additional cell pretreated with indomethacin and exposed to DOG.

Attenuation of Na^+ Currents by PKC Activators Were Also Observed in Inside-out Macropatches

Figure 9 shows the effects of 0.6 μM oleic acid on Na^+ currents in an excised inside-out macropatch. In *A*, recordings were obtained in control conditions, and in *B*, 5 min after addition of oleate to bath ("intracellular" side). *C* shows that, as also demonstrated in whole cell recordings, oleate induced a −17 mV shift in the steady-state inactivation curve of Na^+ channels. Qualitatively similar observations to those reported in Fig. 9 with an excised patch were also observed in two additional experiments with 0.6 μM oleic acid, in five experiments with 6 μM linoleic acid, and in one experiment with DOG (*results not shown*).

Discussion

The novel results presented in this study are: (i) different categories of PKC activators (CUFAs and DOG) attenuated Na^+ currents and shifted the a and h curves in the depolarizing and hyperpolarizing directions, respectively; (ii) while DOG attenuated both peak inward and outward Na^+ currents in a continuously voltage-dependent manner, attenuation of peak outward currents by CUFAs was considerably less pronounced than inward currents, (iii) PKC activators accelerated the development of inactivation of Na^+ currents without changing the time course of recovery from inactivation; (iv) effects i–iii are independent of different metabolic pathways: (a) different PKC inhibitors did not prevent the effects of PKC activators; (b) inhibitors of cyclooxygenase, lipoxygenase, and metabolic P450 pathways did not prevent modulation of Na^+ currents by PKC activators, and, (c) PKC activators were still able to modulate Na^+ currents in inside-out macropatches, which is an experimental situation that presumably disrupts different metabolic pathways; (v) interestingly, various phorbol esters, one trans-unsaturated fatty acid, and a fully saturated fatty acid did not modify Na^+ currents. Although the presented results do not rigorously demonstrate the presence of a direct interaction between PKC activators and Na^+ channels, they support such a conclusion.

Even though a quantitative account for the inhibition of Na^+ currents by different PKC activators was not attempted, it is still possible to gain some insights into the basic mechanisms responsible for such modulation. The finding that the time course of inactivation development is accelerated by different PKC activators in a voltage-dependent manner (Fig. 5), could account for the voltage-dependent attenuation of Na^+ currents: as test voltages are made increasingly positive, channels will inactivate faster than at more negative voltages, leading to a voltage-dependent attenuation of peak Na^+ currents. Also, as the holding potential is made more positive (Fig. 3*B*), considerably more channels will inactivate at these potentials in relation to more negative potentials, de-

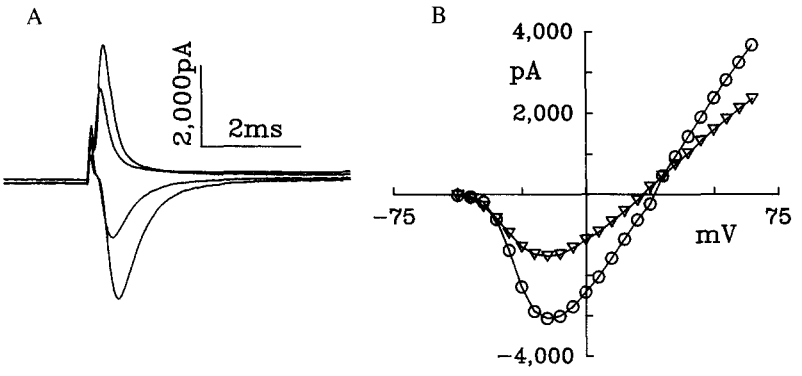

Fig. 8. Effects of 6 μM linolenic acid on Na^+ currents in cells pretreated with 10 μM indomethacin. (*A*) Na^+ currents in response to voltage steps from −130 to −10 or +60 mV in control conditions (cells superfused 15 min before and during voltage clamp), and after linolenate (smaller current traces). (*B*) Peak current-voltage relationships for experiment in *A*.

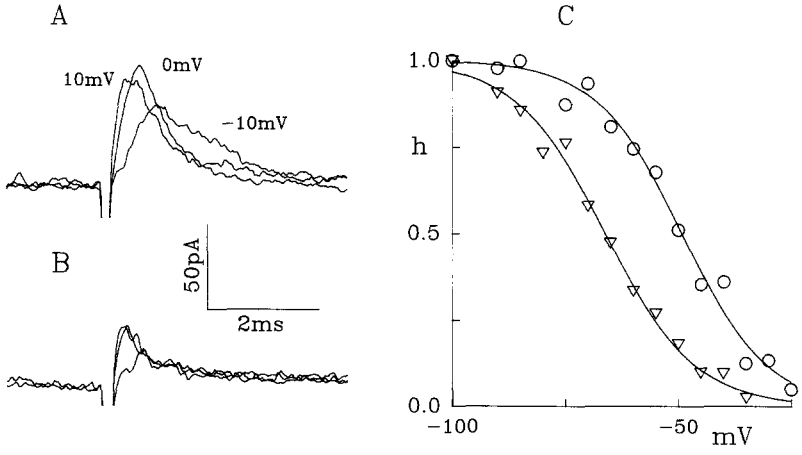

Fig. 9. Na^+ current recordings from an inside-out macropatch in response to different voltage steps from a holding voltage of −100 mV; (*A*) control; (*B*) 5 min after addition of 0.6 μM oleic acid to bath. (*C*) Steady-state inactivation curves in control (circles), and after 0.6 μM oleic acid (triangles). Continuous curves were fit to experimental points with following parameters: circles, −49.2 mV ($V_{0.5}$), −9.44 mV (k); triangles, −66.2 mV ($V_{0.5}$), k = −9.91 mV (k).

creasing the number of channels available to open in response to a test pulse. This would have the effect of decreasing peak Na^+ currents by a relatively larger proportion at more positive holding voltages, contributing to a shift of the h curve to more negative voltages. However, alteration of Na^+ channel gating (acceleration of inactivation) does not seem to be the sole effect of PKC activators. Our results with CUFAs clearly demonstrated that outward Na^+ currents were considerably less attenuated than inward currents.

It is instructive to compare different results obtained in our laboratory, concerning PKC activation and Na^+ currents, using the same cell line, tissue culture procedures, and solutions. The only difference was that in a previous study (Godoy & Cukierman, 1994), the perforated patch variation of the whole-cell voltage-clamp method was used with the implicit assumption that Na^+ channels were being exposed to a normal, or close-to-normal, intracellular physiological environment. Table 2 compares the effects of PKC activators on Na^+ currents using the conventional and the perforated whole-cell voltage-clamp methods. Using the perforated-patch method, we have previously demonstrated that if conventional PKC inhibitors were applied to cells prior to and during exposure to PKC activators, the effects of PKC activators on Na^+ currents were totally prevented (Godoy & Cukierman, 1994). On the other hand, the present study clearly demonstrated that PKC activators modulated Na^+ currents independent of PKC activation. The obvious question is why do external PKC activators attenuate Na^+ currents independent of PKC activation only when the conventional whole-cell voltage-clamp method is being used. We do not have a clear answer to this question. One set of possibilities is that dialysis of intracellular medium with salt solution leads to allosteric changes in Na^+ channels, or washes away an unknown intracellular modulator of Na^+ channels, making the channels responsive to direct modulation by external PKC activators. Another possibility is that PKC inhibitors under the perforated-patch method somehow prevent the direct effects of PKC activators on Na^+ channels. Our observations that Na^+ channel behavior is quite different under different recording methods is not unique. It has been shown that attenuation of Na^+ currents by protein kinase A also depends on the recording method: in cell-attached patches, PKA activation does not attenuate rat brain IIA Na^+ currents, while in excised inside-out patches it does (*see* Li et al., 1993).

Table 2 shows that linolate, linolenate, and DOG attenuated Na^+ currents and shifted the h curves in the hyperpolarizing direction under conventional whole-cell and perforated-patch methods. These results indicate

Table 2. Comparison between effects of PKC activators on Na^+ currents in N1E-115 cells under perforated patch and conventional whole-cell recording

	Perforated patch[b]	Whole cell
Oleic acid	Enhancement	Attenuation
Linoleic, linolenic, arachidonic acids	Attenuation	Attenuation
DAG-like compounds	Attenuation	Attenuation
Phorbol esters[a]	No effects	No effects
	Irreversible[c]	Reversible
	No shift of activation curve	Positive shift of activation curve
	Negative shift of h curve (except for oleate which did not cause shifts)	Negative shift of h curve
Other characteristics	PKC inhibitors prevent effects	PKC inhibitors do not prevent effects
	Acceleration of inactivation	Acceleration of inactivation
	Recovery from inactivation unaffected	Recovery from inactivation unaffected

[a] Different phorbol esters tested are mentioned in Materials and Methods.
[b] Data from Godoy and Cukierman (1994).
[c] Reversibility is defined in the context of the duration of a typical experiment (≈60 min, of which ≈20 min of wash out of cells with a PKC activator-free solution).

that PKC activators are capable of interfering with inactivation independent of PKC activation. The preferential attenuation of inward Na^+ currents by fatty acids is also observed with both recording methods. This indicates that the main difference between attenuation of Na^+ currents by DAG-like compounds and CUFAs is probably related to different interactions of these substances with the permeation pore, and not due to phosphorylation of channels or proteins interacting with them.

On the other hand, oleic acid had dramatically different effects on Na^+ currents with different recording methods. At −10 mV, oleate enhanced peak Na^+ currents by approximately 100% with the perforated-patch method (Godoy & Cukierman, 1994), and attenuated currents by approximately 30% with the conventional whole-cell recording method. One possibility that cannot be presently discarded is that with the perforated-patch technique, oleate is activating or recruiting a normally inactive population of Na^+ channels via phosphorylation, in addition to blocking Na^+ channels via direct interaction, with the overall effect of enhancing Na^+ currents. Another possibility is that PKC phosphorylated Na^+ channels respond to oleate differently from non-phosphorylated channels.

The effects of PKC activators on Na^+ currents under the perforated-patch method were not reversible (Godoy & Cukierman, 1994). In the experiments reported in this study, they were reversible within ≈10 min (*also see* Linden & Routtenberg, 1989). A slower reversibility (or no reversibility at all) is expected to occur during modulation of Na^+ currents by PKC activation with the perforated-patch method. Recovery from PKC activation requires not only the wash out of PKC activators, as recovery from a simple direct effect does, but also, subsequent return of PKC activity to basal levels and activation of phosphatases, leading to removal of phosphate groups from Na^+ channels (and/or phosphorylated protein modulating the channel). Indeed, Numann, Catterall and Scheuer (1991) showed that the time course of reversibility of DAG-induced activation of PKC on Na^+ currents in intact cells is at least five times slower than reported in the present study.

Using the whole-cell technique in N1E-115 cells, it was previously found that CUFAs attenuated Na^+ currents without shifting the a and h curves along the voltage axis (Linden & Routtenberg, 1989). In that study, Na^+ current attenuation caused by PKC activators was prevented by different PKC inhibitors, most of which were also used in our experiments with completely different results. Contrary to our conclusions, those authors proposed that Na^+ current attenuation by CUFAs is a consequence of PKC activation. Another serious experimental discrepancy between Linden and Routtenberg's results and ours was that DAGs did not affect Na^+ currents in their studies, while it clearly inhibited Na^+ currents in our experiments. We cannot explain the conceptually important discrepancies between our results and those of Linden and Routtenberg (1989).

On the other hand, Fraser et al. (1993) showed that arachidonic acid attenuated Na^+ currents and shifted h curves in the hyperpolarizing direction in striatal neurons under the conventional whole-cell voltage-clamp method. These effects were reversible, and were independent of PKC activation, and cyclooxygenase, lipoxygenase, and P-450 pathways. Such observations are compatible with arachidonate attenuating Na^+ currents via direct interaction with channels. Our experimental results extend their observations with arachidonic acid to different PKC activators, and demonstrated, in addition, that these substances also attenuated Na^+ currents in in-

side-out patches and accelerated the development of inactivation. Moreover, we demonstrated that the two different categories of PKC activators have different mechanisms of attenuation of Na^+ currents.

Wieland, Fletcher and Gong (1992) showed that external oleate or arachidonate attenuated Na^+ currents in cultured skeletal muscle, while stearate, a saturated fatty acid, did not. Takenaka, Horie and Hori (1987) demonstrated that external CUFAs (in concentration range of mM) attenuated Na^+ currents and shifted the activation curve in the depolarizing direction in squid giant axons. However, in both studies (Takenaka et al., 1987; Wieland et al., 1992) it was not possible to eliminate the possibility that some of these effects were being mediated by metabolic pathways.

Results similar to ours were obtained on different ionic currents. Shimada and Somlyo (1992) demonstrated in isolated smooth muscle cells that CUFAs (but not trans-unsaturated or saturated fatty acids) attenuated Ca^+ currents, and shifted the inactivation curve to negative voltages independently of metabolic pathways. Also in relation to Ca^+ currents, it was demonstrated that OAG and phorbol esters attenuated these currents via extracellular effects and not via PKC activation (Hockberger et al., 1989). Direct effects of PKC activators have also been proposed for different K^+ channels. CUFAs enhanced outwardly rectifying K^+ currents in smooth and heart muscle cells (Kim & Clapham, 1989; Ordway et al., 1989). A recent report from Bowlby & Levitan (1994) indicated that DAG interacts directly with voltage-dependent K^+ channels (Kv1.3) causing a decrease in peak currents and faster current decay.

In summary, our experimental results suggest that PKC activators can directly interact with Na^+ channels in a complex manner causing current attenuation. Similar direct interactions were also reported for Na^+ (Fraser et al., 1993), Ca^+ (Hockberger et al., 1989; Shimada & Somlyo, 1992), and K^+ channels (Bowlby & Levitan, 1994; *see* Meves, 1994), suggesting that these different channels (and/or different modulatory proteins interacting with channels) might have binding sites to PKC activators. Moreover, the response of Na^+ currents to different PKC activators clearly depends on the presence of a normal intracellular environment.

This work was supported in part by a grant-in-aid from the American Heart Association (National Center).

References

Asaoka, Y., Nakamura, S., Yoshida, K., Nishizuka, Y. 1992. Protein kinase C, calcium and phospholipid degradation. *Trends Biochem. Sci.* **17:**414–417

Bowlby, M.R., Levitan, I.B. 1994. Diacylglycerol induces fast inactivation in slowly inactivating voltage-gated potassium channels. *Biophys. J.* **66:**A208 (*Abstr.*)

Cahalan, M.D., Shapiro, B.I. 1976. Current and frequency dependent block of sodium channels by strychnine. *Biophys. J.* **16:**76*a* (*Abstr.*)

Catterall, W.A. 1992. Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* **72:**S15–S48

Cukierman, S. 1992. Characterization of K^+ currents in rat malignant lymphocytes (Nb2 cells). *J. Membrane Biol.* **126:**147–157

Egan, R.W., Gale, P.H. 1984. Comparative biochemistry of lipoxygenase inhibitors. *In:* Prostaglandins, Leukotrienes, and Lipoxins. J.M. Bailey, editor. pp. 593–607. Plenum Press, New York

Fernandez, J.M., Fox, A.P., Krasne, S. 1984. Membrane patches and whole-cell membranes: a comparison of electrical properties in rat clonal (GH3) pituitary cells. *J. Physiol.* **356:**565–585

Fraser, D.D., Hoehn, K., Weiss, S., MacVicar, B.A. 1993. Arachidonic acid inhibits sodium currents and synaptic transmission in cultured striatal neurons. *Neuron* **11:**633–640

Godoy, C.M., Cukierman, S. 1994. Multiple effects of protein kinase C activators on Na^+ channels in mouse neuroblastoma cells. *J. Membrane Biol.* **140:**101–110

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hanck, D.A., Sheets, M.F. 1992. Time-dependent changes in kinetics of Na^+ current in single canine cardiac Purkinje cells. *Am. J. Physiol.* **262:**H1197–H1207

Hille, B. 1992. Ionic channels of excitable membranes. 2nd. edition, Sinauer Associates Inc., Sunderland, MA

Hockberger, P., Toselli, Swandulla, D., Lux, H.D. 1989. A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature* **338:**340–342

Kaczmarek, L.K., Levitan, I.B. 1987. Neuromodulation. The biochemical control of neuronal excitability. Oxford University Press, New York

Kim, D., Clapham, D. 1989. Potassium channels in cardiac cells activated by arachidonic acid and phospholipids. *Science* **244:**1174–1176

Li, M., West, J.W., Numann, R., Murphy, B.J., Scheuer, T., Catterall, W.A. 1993. Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. *Science* **261:**1439–1442

Linden, D.J., Routtenberg, A. 1989. Cis-fatty acids, which activate protein kinase C, attenuate Na^+ and Ca^{2+} currents in mouse neuroblastoma cells. *J. Physiol.* **419:**95–119

Meves, H. 1994. Modulation of ion channels by arachidonic acid. *Prog. Neurobiol.* **43:**175–186

Numann, R., Catterall, W.A., Scheuer, T. 1991. Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science* **254:**115–118

Ordway, R.W., Walsh, J.V., Singer, J.J. 1989. Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells. *Science* **244:**1176–1179

Seyama, I., Wu, C.H., Naharashi, T. 1980. Current-dependent block of nerve membrane sodium channels by paragracine. *Biophys. J.* **29:**531–538

Shen, T.W., Winter, C.A. 1979. Chemical and biological studies on indomethacin, sulindac and analogs. *Adv Drug Res* **12:**89–245

Shimada, T., Somlyo, A.P. 1992. Modulation of voltage dependent Ca channel current by arachidonic acid and other long-chain fatty acids in rabbit intestinal smooth muscle. *J. Gen. Physiol.* **100:**27–44

Takenaka, T., Horie, H., Hori, H. 1987. Effects of fatty acids on membrane currents in the squid axon. *J. Membrane Biol.* **95:**113–120

Wieland, S.J., Fletcher, J.E., Gong, Q.H. 1992. Differential modulation of a sodium conductance in skeletal muscle by intracellular and extracellular fatty acids. *Am. J. Physiol.* **263:**C308–C312