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# *CIS*-FATTY ACIDS, WHICH ACTIVATE PROTEIN KINASE C, ATTENUATE $Na^+$ AND $Ca^{2+}$ CURRENTS IN MOUSE NEUROBLASTOMA CELLS

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SUMMARY

1. Activation of protein kinase C (PKC) by phorbol esters or diacylglycerols has been shown to modulate a number of ionic currents carried by $Ca^{2+}$, $K^+$ and $Cl^-$. Recently, it has been demonstrated that PKC may be activated by *cis*-fatty acids in the absence of either phospholipid or $Ca^{2+}$. We wished to determine if this new class of PKC-activating compound would also modulate ionic currents. To this end we applied the whole-cell voltage-clamp technique to N1E-115 neuroblastoma cells.

2. Analysis of families of currents evoked under voltage clamp by depolarizing steps from a holding potential of −85 mV during external application of 5 $\mu$M-oleate (a *cis*-fatty acid) showed a 36% reduction of the peak inward current with no shift in either the peak or the reversal potential of the current–voltage relation and no alteration of outward current.

3. External application of the *cis*-fatty acids oleate, linoleate and linolenate reversibly attenuated voltage-dependent $Na^+$ current with approximate half-maximal dose values of 2, 3, and 10 $\mu$M respectively. Oleate was approximately 2 times more potent when applied internally ($ED_{50} = 1$ $\mu$M). Externally applied elaidate (a *trans*-isomer of oleate) and stearate (a saturated fatty acid) which do not activate PKC, had no effect. Since *cis*-fatty acids are known to fluidize membranes, as well as to activate PKC, we sought to dissociate these functions by applying compounds that fluidize membranes but do not activate PKC: methyloleate and lysophosphatidylcholine. Neither compound affected $Na^+$ current when applied externally at concentrations of 1–50 $\mu$M.

4. In contrast to *cis*-fatty acids, three classical PKC activators, phorbol-12,13-dibutyrate (PDB), phorbol-12,13-diacetate (PDA), and 1,2-oleoylacetylglycerol (OAG) were found to have no effect on the voltage-dependent $Na^+$ current when applied externally at 10 nM–1 $\mu$M (phorbol esters) or 1–150 $\mu$M (OAG) for incubation periods up to 1 h.

5. External application of the PKC inhibitors polymyxin B, H-7, sphingosine and staurosporine blocked the attenuation of the $Na^+$ current by *cis*-fatty acid in a dose-dependent manner, with maximal inhibition occurring at doses of 50, 10, 200 and 0·1 $\mu$M, respectively. The cyclic nucleotide-dependent protein kinase inhibitor H-8

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was much less effective in blocking the *cis*-fatty acid effect. Polymyxin B and staurosporine were more potent when applied internally.

6. Chronic (24 h) exposure to 1 $\mu$M phorbol-12-myristate-13-acetate (TPA) was employed to down-regulate PKC. This treatment did not alter the baseline characteristics of the isolated $Na^+$ current, but was effective in blocking the attenuation of $Na^+$ current produced by subsequent external application of *cis*-fatty acid.

7. To determine whether the distinction between *cis*-fatty acids and classical PKC activators was specific to the $Na^+$ current, these compounds were applied externally to isolated $Ca^{2+}$ currents. Both classes of compound attenuated both transient and sustained $Ca^{2+}$ currents without altering their kinetics of activation. *Cis*-fatty acids, but not the classical activators, were significantly more potent with internal application. Inhibitors of PKC blocked the effect of externally applied *cis*-fatty acid, and were more potent with internal application.

8. These data suggest two broad classes of explanation. First, *cis*-fatty acid attenuation of the $Na^+$ current could be mediated in part through a non-PKC mechanism. The second explanation, which we favour, is that activation of the PKC family of enzymes by *cis*-fatty acids and the classical PKC activators (phorbol esters, diacylglycerols) could result in different patterns of substrate phosphorylation such that *cis*-fatty acid activation of PKC produces attenuation of the $Na^+$ current in N1E-115 cells, while stimulation of PKC by classical activators does not. In contrast, phosphorylation of proteins necessary for $Ca^{2+}$ current attenuation would be produced by either class of activator.

## INTRODUCTION

Protein kinase C (PKC) is a family of enzymes (Coussens, Parker, Rhee, Yang-Feng, Chen, Waterfield, Francke & Ullrich, 1986) which have been implicated in a vast array of transmembrane signalling processes (see Nishizuka, 1986, 1988, for reviews). These enzymes have been described as $Ca^{2+}$ and phospholipid dependent, with diacylglycerol, a breakdown product of phosphoinositide hydrolysis, serving as a second messenger for activation (Kikkawa, Takai, Minakuchi, Inohara & Nishizuka, 1982). More recently, it has been demonstrated that PKC may be activated by *cis*-fatty acids in the absence of $Ca^{2+}$ and phospholipid (Murakami & Routtenberg, 1985; Murakami, Chan & Routtenberg, 1986). Furthermore, specific subtypes of the PKC family have been suggested to be selectively activated by *cis*-fatty acids (Nishizuka, 1988).

Protein kinase C is found in high concentration in neural tissues, and has been implicated in the modulation of several neuronal functions including process outgrowth, neurotransmitter release, long-term potentiation, and the modulation of ionic channels. The involvement of PKC in the latter process is evidenced by changes in the electrical properties of neurones following application of phorbol esters, diterpine compounds which directly activate PKC by substituting for diacylglycerol (Castagna, Takai, Kaibuchi, Sano, Kikkawa & Nishizuka, 1982), 1,2-oleoylacetyl-glycerol (OAG) or purified PKC itself. Activation of PKC has been shown to modulate ionic currents carried by $Ca^{2+}$ (DeRiemer, Strong, Albert, Greengard &

Kaczmarek, 1985; Di Virgilio, Pozzan, Wollheim, Vincentini & Meldolesi, 1986; Farley & Auerbach, 1986; Rane & Dunlap, 1986; Werz & Macdonald, 1987; Doerner, Pitler & Alger, 1988; Marchetti & Brown, 1988), $K^+$ (Baraban, Snyder & Alger, 1985; Farley & Auerbach, 1986; Higashida & Brown, 1986; Doerner *et al.* 1988) and $Cl^-$ (Madison, Malenka & Nicoll, 1986).

We wished to determine if another class of PKC-activating compounds, *cis*-fatty acids, would also modulate ionic currents in a neuronal cell, and if so, in what manner. To this end we applied *cis*-fatty acids to N1E-115 neuroblastoma cells using the whole-cell configuration of the patch-clamp recording technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and found that *cis*-fatty acid produced a reversible attenuation of inward current and, with addition of the appropriate channel blockers, of isolated $Na^+$ and $Ca^{2+}$ currents. The present report describes the electrophysiological characteristics of this attenuation and a series of experiments conducted to determine whether the observed effect of *cis*-fatty acids on these currents is mediated by PKC activation.

## METHODS

### *Cell culture*

Cells of the murine neuroblastoma clone N1E-115 were obtained from Dr M. Nirenberg (National Institutes of Health, Bethesda, MD) and were grown at 37 °C in a humidified atmosphere containing 10% $CO_2$. The proliferating cultures were grown in T-75 culture flasks (Costar; Cambridge, MA) and were fed on alternate days during logarithmic growth with Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% newborn bovine serum (GIBCO) and gentamycin (100 $\mu$M/ml). Six days before their use, confluent cells were suspended by mechanical agitation and replated at a density of $3 \times 10^5$ cells per 35 mm culture dish (Costar) in the medium described above, supplemented with 4% dimethylsulphoxide (DMSO). The cells were fed again, three days after replating. Treatment of N1E-115 cells with 4% DMSO caused the cells to develop highly excitable membranes and large (40 $\mu$M), rounded cell bodies with no process outgrowth (Kimhi, Palfrey, Spector, Barak & Littauer, 1976) which (unlike cells treated with 2% DMSO, which grow elaborate processes) are ideal for voltage-clamp experiments because they eliminate the problem of space-clamp failure.

### *Recording*

The whole-cell patch-clamp technique was used to record ionic currents from the neuroblastoma cells (Hamill *et al.* 1981). Patch electrodes were pulled from borosilicate microfilament glass (1·50 mm o.d., 1·12 mm i.d., obtained from A–M Systems Inc., Everett, WA) and were polished on a microforge to yield a tip diameter of 2–3 $\mu$M and a resistance of 0·8–2·0 MΩ when recorded with normal internal and external solutions (see below). Ground potential was measured with a Ag–AgCl wire inserted into a 3 M-KCl–agar electrode in the bath. The patch electrode was placed onto the cell surface and suction was applied to yield a seal resistance of greater than 2 GΩ. The underlying membrane was then disrupted by further suction, after which capacitance compensation was introduced. Only cells that exhibited resting potentials greater than −30 mV and input resistances greater than 50 MΩ were used in this study. Cells were clamped at the selected holding potential by the SEVC circuit of the Axoclamp 2A voltage-clamp amplifier (Axon Instruments, Burlingame, CA) operating at a switching frequency of 9 kHz. Membrane currents were filtered at 1 kHz, digitized with a 12-bit analog-to-digital converter (LabMaster, Scientific Solutions Inc., Solon, OH) OH) operating at a sampling frequency of 10 kHz, and stored on the hard disc of an IMB PC-XT microcomputer for further analysis with the pCLAMP software package (Axon Instruments). Linear leakage current was determined by hyperpolarizing command pulses and was digitally subtracted from current–voltage ($I$–$V$) plots. Current traces and $I$–$V$ plots represent single

observations. All other electrophysiological measures are reported as the mean ± standard error of the mean (S.E.M.) of four independent observations unless otherwise noted. All experiments were performed at 20–24 °C.

*Solutions*

Solutions were delivered to the recording chamber (volume = 1·5 ml) by gravity at a rate of 2 ml/min. Solutions for the recording of total and isolated $Na^+$ current were slightly modified versions of those used by Ikeda, Schofield & Weight (1986). The standard external solution contained (mM): NaCl, 140·0; KCl, 5·4; $CaCl_2$, 2·0; $MgCl_2$, 0·8; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10·0; dextrose, 25·0. The external solution used for $Na^+$ current isolation contained: NaCl, 70·0; $MgCl_2$, 10·0; HEPES, 10·0; dextrose, 25·0. The external solution used for $Ca^{2+}$ current isolation contained: $BaCl_2$, 20·0; $MgCl_2$, 1·0; HEPES, 10·0; tetraethylammonium chloride (TEA-Cl); 120·0. The standard internal solution contained: KCl, 140·0; $CaCl_2$, 1·0; $MgCl_2$, 2·0; HEPES, 10·0; ethylene-bis-(oxyethylenenitrile)tetraacetic acid (EGTA), 11·0. The internal solution used for $Na^+$ current isolation contained: NaF, 25·0; *N*-methylglucamine chloride (NMG-Cl), 140·0; TEA-Cl, 2·0; $CaCl_2$, 1·0; $MgCl_2$, 2·0; HEPES, 10·0; EGTA, 11·0. The internal solution used for $Ca^{2+}$ current isolation contained: NMG-Cl, 140·0; HEPES, 10·0; EGTA, 10·0; TEA-Cl, 2·0; Mg-ATP, 1·0. All solutions were adjusted to pH 7·3. Internal and external solutions were adjusted to 310 and 330 mosM respectively by the addition of sucrose.

All test compounds added to the external solutions were applied in sub-millimolar concentrations and hence were not substituted for any ionic species, with the exception of TEA-Cl (15 mM) and choline chloride (45 mM) which were substituted for NaCl in an equimolar fashion. Test compounds applied internally were present in the patch electrode. Protonated forms of the fatty acids oleate (*cis*-9-octadecenoate), linoleate (all-*cis*-9,12-octadecadienoate), linolenate (all-*cis*-9,12,15-octadecatrienoate), elaidate (*trans*-9-octadecenoate), and stearate (*n*-octadecanoate), as well as the methyl ester of oleate, were directly dispersed in 20 mM-Tris-HCl by 1 min vigorous vortex mixing, $N_2$ gas bubbling and 10 min sonication at 4 °C (Murakami *et al.* 1986). The phorbol esters, phorbol-12,13-diacetate (PDA), phorbol-12,13-dibutyrate (PDB), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were prepared as stock solutions in 10% ethanol. Lysophosphatidylcholine (LPC) and OAG were dissolved in chloroform, which was evaporated under $N_2$ gas prior to their use. Neither the ethanol carrier solution nor a solution used to flush tubes containing evaporated chloroform was seen to have any effect on baseline $Na^+$ current ($n = 4$ per case). Protein kinase inhibitors were prepared as stock solutions in 20 mM-Tris-HCl. Aliquots of all the stock solutions were stored under $N_2$ at −20 °C.

All reagents were obtained from Sigma Chemical Company (St Louis, MO) with the exception of the phorbol esters, obtained from LC Services Corporation (Woburn, MA), the protein kinase inhibitors 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8), and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7), obtained from Seikagaku America, Inc. (St Petersburg, FL), and staurosporine, obtained from Boehringer Mannheim (Indianapolis, IN).

*Protein kinase C distribution assay*

Cells were washed three times with 10 ml serum-free DMEM, and then with 10 ml ice-cold homogenizing buffer (5 mM-HEPES; 2 mM-dithiothreitol (DTT); leupeptin, 10 μM/ml). Cells were collected in 2 ml homogenizing buffer by scraping with a rubber policeman, and were then homogenized with a Teflon–glass homogenizer (15 up-and-down strokes). The homogenate was spun at 100000 *g* for 1 h at 4 °C and the supernatant was saved as the cytosolic fraction. The pellet was resuspended in homogenizing buffer supplemented with 0·5% Triton X-100 for 1 h. This suspension was spun at 100000 *g* for 1 h at 4 °C, and the supernatant was collected as the membrane fraction. Separated cytosolic and membrane factions were applied to DEAE-cellulose minicolumns (bed volume = 0·2 ml) pre-equilibrated with column buffer (1 mM-EGTA; 1 mM-EDTA; 50 mM-Tris-HCl; leupeptin, 10 μg/ml; pH = 7·5). Columns were washed twice with 1 ml washing buffer (1 mM-EGTA, 2 mM-DTT, 50 mM-Tris-HCl, pH = 7·5) and eluted with washing buffer supplemented with 0·25 M-KCl. The activity of PKC was measured using an exogenous histone substrate as previously described (Murakami *et al.* 1986).

## RESULTS

### *Observations under normal conditions*

The mean resting membrane potential and input resistance recorded under current-clamp conditions using the standard internal and external solutions were

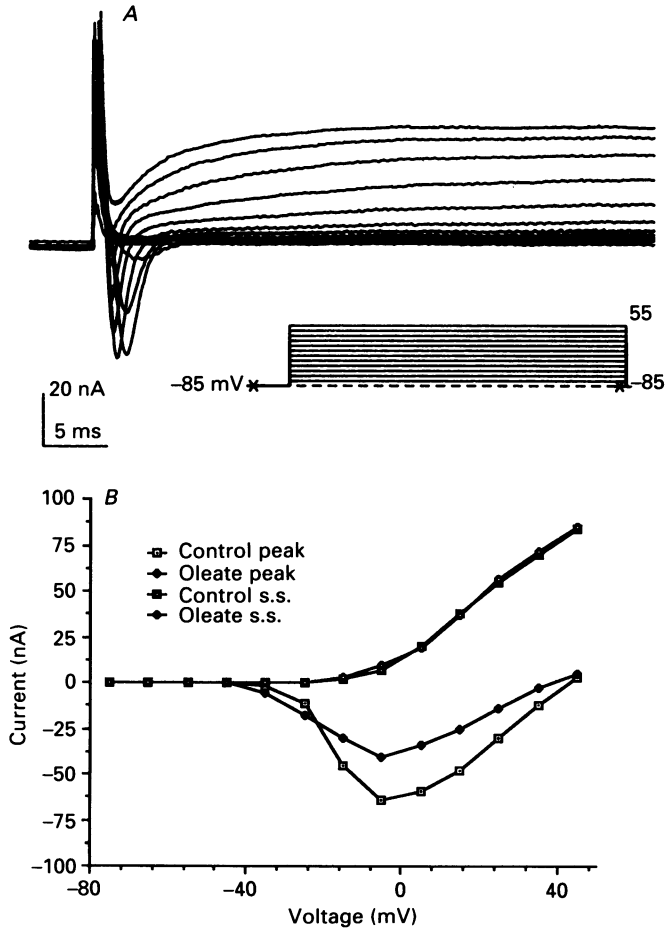


Fig. 1. Current records from a N1E-115 neuroblastoma cell evoked by step depolarizations from a holding potential of −85 mV. A family of currents was evoked from a single cell bathed in the normal external solution (*A*). The *I–V* relation of currents evoked by this protocol indicates that 5 μM-oleate reduces the peak inward current without altering its kinetics of activation, or the sustained outward current (*B*). s.s. = steady state.

−42·4 ± 4·7 mV (mean ± S.E.M., $n = 8$) and 126·0 ± 11·8 mΩ respectively. Neither of these measurements was significantly altered by the application of either oleate, a *cis*-fatty acid, or stearate, a saturated fatty acid, at concentrations of 1–50 μM.

Figure 1*A* shows a series of currents evoked under voltage-clamp conditions by depolarizing steps superimposed upon a holding potential of −85 mV. A large, transient, voltage-dependent inward current was initiated at −40 mV, peaked at −5 mV,

and reversed direction at +45 mV. A large, delayed, sustained outward current was initiated at −20 mV and exhibited an S-shaped voltage dependence with a linear, ohmic region situated between 0 and +35 mV. The *I–V* relation of these currents is shown in Fig. 1*B*.

Application of 5 μM-oleate produced a 36·2±2·1% reduction of the peak inward current with no shift in either the peak or the reversal potential of the *I–V* relation (Fig. 1*B*). In addition, application of oleate appeared to produce a small enhancement of the inward currents evoked by depolarizing steps to −35 and −25 mV. No effects on the outward current were observed.

### *Observations on the isolated $Na^+$ current*

#### *Characterization of the cis-fatty acid effect*

Previous work using this cell type has suggested that the inward current recorded using the above parameters is largely carried by the $Na^+$ ion (Moolenaar & Spector, 1978, 1979). Thus, we sought to isolate the $Na^+$ current to determine if the action of oleate on the inward current could be attributed, at least in part, to an alteration of $Na^+$ conductance. The protocol for the isolation of the $Na^+$ current used application of external $Mg^{2+}$, and internal $F^-$ and TEA, to block currents carried by $Ca^{2+}$ and $K^+$. Experiments to determine the ionic basis of the currents isolated by this protocol are illustrated in Fig. 2. Currents evoked by depolarizing steps from −85 to −5 mV were seen to have no outward component other than a very small amount of linear leakage. First, the inward current evoked under these conditions was unaffected by the external application of the $K^+$ channel blocker TEA-Cl (15 mM). Secondly, this inward current was completely and reversibly blocked by the application of the classical $Na^+$ channel blocker, tetrodotoxin. Thirdly, this inward current was completely and reversibly blocked by the substitution of 45 mM-external $Na^+$ with choline to reduce the driving force for the $Na^+$ current to zero.

Application of 5 μM-oleate reduced the peak isolated $Na^+$ current by 36·5±1·9% (see Fig. 2*D*). In agreement with the previous observations on total inward current (Fig. 1), this reduction was not accompanied by a shift in either the peak or the reversal potential of the *I–V* relation, but was accompanied by an enhancement of those small $Na^+$ currents produced by steps to command potentials near −40 mV (see Fig. 3*A*). Application of a tenfold higher dose of oleate produced a slightly larger reduction of the peak $Na^+$ current, 49·0±3·4%, and a similar alteration of the *I–V* relation (Fig. 3*B*). To determine if the action of oleate could be duplicated by other *cis*-fatty acids, we applied 5 μM-linolenate and 20 μM-linoleate, and then observed the *I–V* relation (Fig. 3*C* and *D*). These treatments produced mean reductions of the peak $Na^+$ current of 35·0±3·6 and 30·7±2·8%, respectively, and otherwise duplicated the characteristics of the oleate effect detailed above. The steady-state inactivation function of the voltage-dependent $Na^+$ current was determined by stepping from various holding potentials to −5 mV, and was not altered by the application of 5 μM-oleate (Fig. 3*E*).

We suspected that the small shift in activation kinetics seen with application of *cis*-fatty acids (see Figs 1 and 3) was actually a time-dependent recovery of the $Na^+$ current that was merely superimposed upon the larger attenuation effect produced by the *cis*-fatty acid. To test this notion, evoked $Na^+$ currents were recorded from a

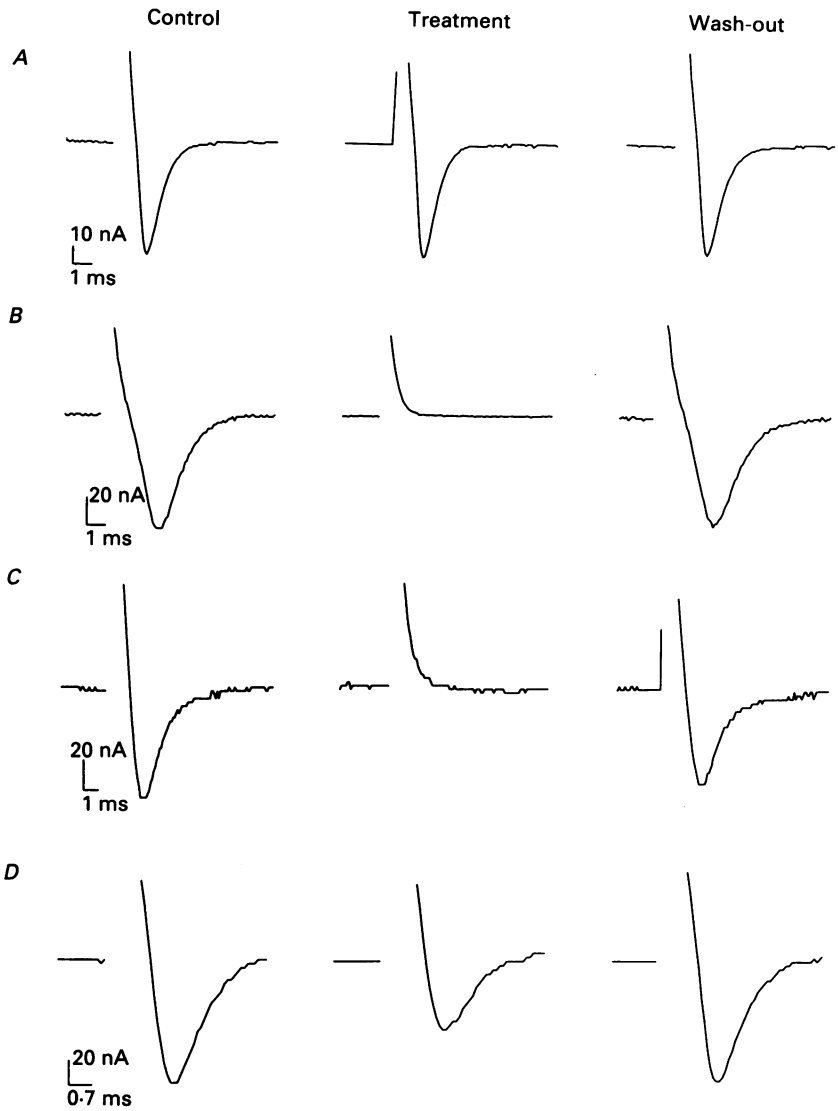


Fig. 2. Experiments to determine the ionic basis of current isolated by the $Na^+$ current isolation protocol. Inward current evoked by a step depolarization from a holding potential of −85 mV to a command potential of −5 mV was recorded in the presence of 15 mM-TEA-Cl (*A*). 1 μM-tetrodotoxin (*B*). and in a medium in which 45 mM-choline chloride was substituted for NaCl in an equimolar fashion (*C*). The pharmacological profile of these treatments indicates that the current isolated in this manner is indeed carried by $Na^+$. Externally applied 5 μM-oleate attenuates this current in a reversible manner (*D*).

cell before and after a 10 min settling period in which no compounds were added to the bath. The *I–V* relation as an effect of this treatment is shown in Fig. 3*F*. It illustrates that a small shift in activation kinetics is indeed a result of time-dependent settling, and not a result of the application of *cis*-fatty acid.

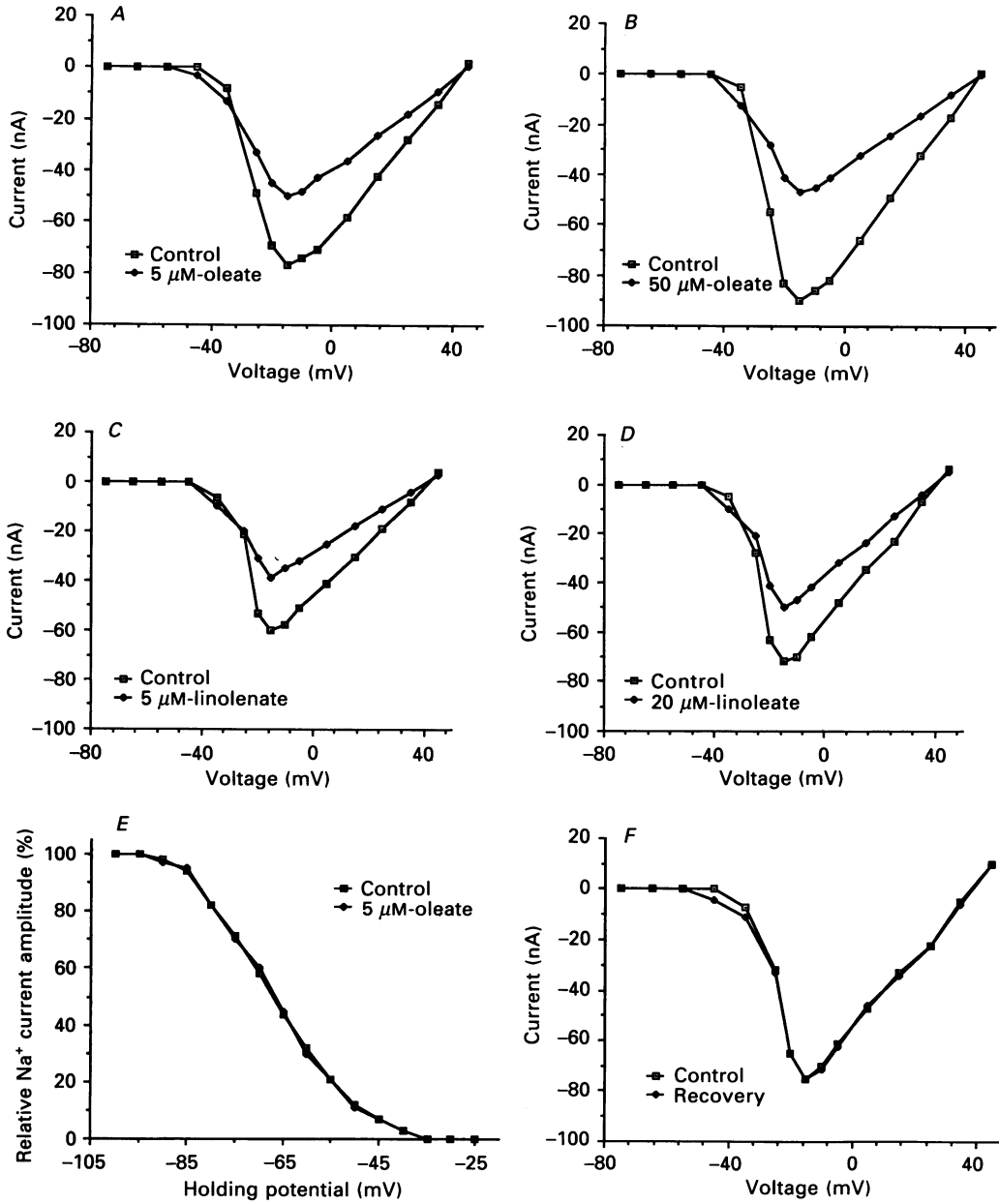


Fig. 3. The *I–V* relations determined from peak isolated $Na^+$ currents recorded in the presence of externally applied 5 μM-oleate (*A*), 50 μM-oleate (*B*), 5 μM-linolenate (*C*), and 20 μM-linoleate (*D*), and the steady-state inactivation function of the isolated $Na^+$ current in the presence of 5 μM-oleate (*E*). In *F*, the time-dependent recovery of isolated $Na^+$ currents is indicated in this *I–V* relation determined from currents evoked before (⊡) and after (◆) a 10 min settling period.

Figure 4 illustrates the time course of the actions of oleate and stearate on the peak $Na^+$ current. First, it may be observed that application of 5 or 50 $\mu$M-stearate, a saturated fatty acid, had no effect on the peak $Na^+$ current. In addition, there was no effect of this compound on any other aspect of the *I–V* relation (data not shown).

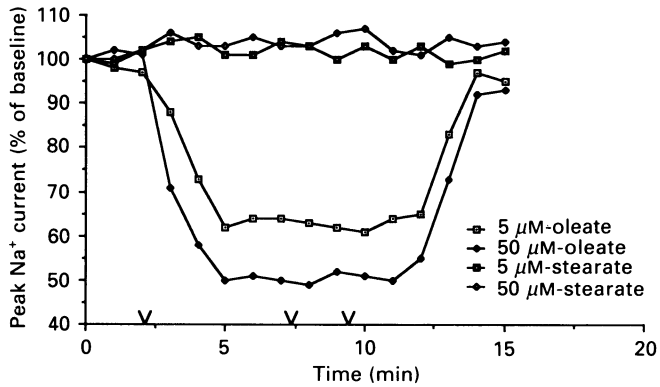


Fig. 4. The time course of the oleate and stearate action on the normalized peak $Na^+$ current. The test lipid is applied to the bath at $t = 2$ min, the stable effect of the lipid is assessed at $t = 7$ min, and wash-out begins at $t = 9$ min (time points are indicated by arrowheads).

Secondly, the reduction of peak $Na^+$ current produced by either 5 or 50 $\mu$M-oleate was evident within 1 min, but required approximately 4 min to reach a stable level. Thirdly, wash-out of oleate did not begin to reverse the $Na^+$ current attenuation until 3 min after it was started. However, within 6 min, the effect of oleate was almost completely eliminated.

*Mediation of the effect of cis-fatty acid: correlational experiments*

There are several known effects of *cis*-fatty acids on cellular processes. We wished to determine which of these effects might mediate the observed attenuation of $Na^+$ current by these compounds. First, *cis*-fatty acids have been demonstrated to activate PKC in the absence of $Ca^{2+}$ and phospholipid (Murakami & Routtenberg, 1985; Murakami *et al.* 1986). Secondly, *cis*-fatty acids in monomeric form have been demonstrated to fluidize cellular membranes (Ahkong, Fisher, Tampion & Lucy, 1973; Takenaka, Horie & Kawasaki, 1983; Kitagawa, Endo & Kametani, 1985). Thirdly, *cis*-fatty acids, like all fatty acids, have the capacity to form micelles, which by fusing with the cellular membrane may disrupt membrane and ion channel function.

It is difficult to dissociate the PKC activation effect of *cis*-fatty acids from their membrane fluidization effects as there appears to be an excellent correlation between the two functions (see Table 1). Fortunately, there do exist compounds which are as effective as *cis*-fatty acids in membrane fluidization, but which do not activate PKC. Thus, we chose to examine the dose response of three classes of lipid compounds: *cis*-fatty acids (oleate, linoleate and linolenate) which both activate PKC and are effective fluidizers of cellular membranes, saturated (stearate) or *trans*-unsaturated (elaidate) fatty acids which are not effective either as PKC activators or as

membrane fluidizers, and a third class of compounds (methyloleate and LPC) which are effective membrane fluidizers (Poole, Howell & Lucy, 1970; Ahkong *et al.* 1973) but do not activate PKC (Murakami & Routtenberg, 1985; K. Murakami, personal communication).

TABLE 1. The potency of lipids for attenuation of $Na^+$ current is predicted by their capacity for PKC activation, but not by their capacity for membrane fluidization

| Fatty acid | $V_{max}$ for PKC activation (pmol/min)* | Membrane fluidization† | % Inhibition of $I_{Na}$ at 20 $\mu$M‡ |
|---|---|---|---|
| Oleate | 112 | + | 45·2 ± 3·7 |
| Linoleate | 106 | + | 30·7 ± 2·8 |
| Linolenate | 64 | + | 41·2 ± 3·6 |
| Methyloleate | 6 | + | −3·5 ± 1·1 |
| LPC | 11 | + | −9·8 ± 2·0 |
| Elaidate | 14 | ○ | −8·5 ± 4·2 |
| Stearate | 14 | ○ | −7·3 ± 3·9 |

* Murakami & Routtenberg (1985); Murakami *et al.* (1986).
† Poole *et al.* (1970); Ahkong *et al.* (1973); Kitagawa *et al.* (1985).
‡ Mean ± S.E.M., $n = 4$ per group.

Attenuation of the $Na^+$ current was produced only by those compounds which have been previously shown to activate PKC, the *cis*-fatty acids, and not by compounds which fluidize membranes but do not activate PKC (see Fig. 5*A*). Among the *cis*-fatty acids, oleate and linolenate were equipotent, the approximate values for the half-maximal dose ($ED_{50}$) being 2 and 3 $\mu$M, respectively. Linoleate, on the other hand, was less potent, with an approximate $ED_{50}$ of 10 $\mu$M. We have measured the maximum potential ($V_{max}$) for *cis*-fatty acid activation of PKC using both exogenous histone H1 protein (see Table 1) and purified protein F1 (S. Chan, R. Nelson, K. Murakami & A. Routtenberg, unpublished observations). Using histone H1 as a substrate, linolenate and linoleate activate PKC at 57 and 95% of the oleate $V_{max}$, respectively. When purified protein F1 is used as a substrate, linolenate and linoleate activate PKC at 98 and 38% of the oleate $V_{max}$ respectively. Thus, the relative potency of these *cis*-fatty acids for $Na^+$ current attenuation is reflected in their potency for PKC activation when purified protein F1 is used as a substrate, but not when purified H1 histone protein is used as a substrate.

The critical micelle concentrations of selected fatty acids used in this experiment were determined by light scattering at 400 nm using the method of Blomquist, Lindemann & Hakanson (1985). The critical micelle concentrations of oleate, linoleate, linolenate, elaidate and stearate, as measured in the primary external medium used for recording isolated $Na^+$ current, were 195, 240, 290, 255 and 175 $\mu$M, respectively. Thus is appears unlikely that the attenuation of the $Na^+$ current produced by *cis*-fatty acids applied at 1–50 $\mu$M is a result of micelle formation and consequent disruption of the cellular membrane.

If the attenuation of $Na^+$ current by *cis*-fatty acids were mediated by PKC activation, then *cis*-fatty acids should be more potent when applied internally. To assess this prediction, the *cis*-fatty acid, oleate, and the non-PKC activating control compound, methyloleate, were applied both internally and externally over a broad

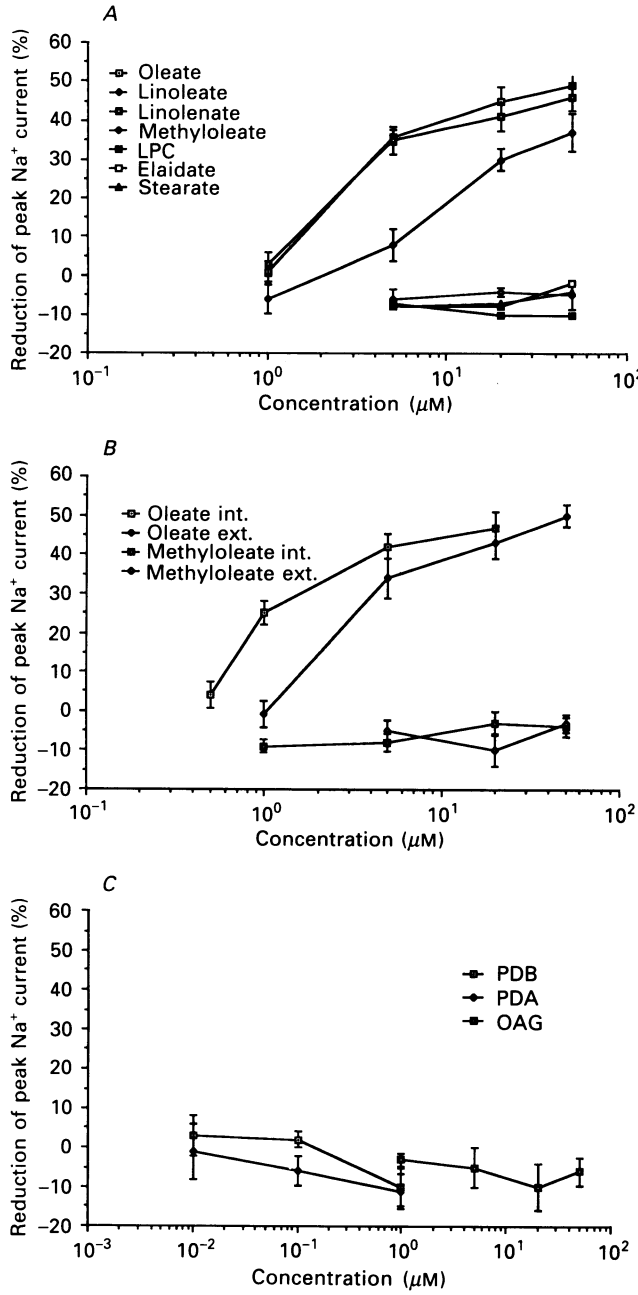


Fig. 5. Effects of PKC activators on the peak $Na^+$ current. *A*, dose response of the peak $Na^+$ current to external application of three classes of lipid (see text) illustrates that, of the seven compounds tested, only the three *cis*-fatty acids (oleate, linoleate and linolenate) attenuated the $Na^+$ current. *B*, oleate is more potent with internal as opposed to external application. The non-PKC activating control compound, methyloleate, is ineffective in either case. *C*, Dose response of the peak $Na^+$ current following a 30 min bath application of three classical PKC activators, the phorbol esters PDB and PDA, and the diacylglycerol OAG, indicates that these compounds have no effect on the $Na^+$ current in N1E-115 neuroblastoma cells. Bars represent the S.E.M., and $n = 4$ per point in this and following figures, except where otherwise indicated.

range of doses (Fig. 5*B*). The $ED_{50}$ for oleate attenuation of $Na^+$ current was reduced approximately twofold with internal application. The maximal level of attenuation was unchanged by the method of oleate administration, and methyloleate produced no significant alteration in either case.

If attenuation of the $Na^+$ current produced by *cis*-fatty acids were mediated by activation of PKC, then one might predict that other compounds known to activate PKC would also attenuate the $Na^+$ current. This prediction was tested by applying two phorbol esters, PDB and PDA, as well as the synthetic diacylglycerol, OAG. All three of these compounds are known to activate PKC both *in vivo* and *in vitro*, with the phorbol esters being effective in the nanomolar range (Castagna *et al.* 1982; Werz & MacDonald, 1987) and OAG being effective in the micromolar range (Nishizuka, 1986; Rane & Dunlap, 1986). Neither the phorbol esters at 10 nM-1 $\mu$M, nor OAG applied at 1–150 $\mu$M had a significant effect on the amplitude of the peak $Na^+$ current (see Fig. 5*C*), or any other aspect of the *I–V* relation (data not shown). These compounds were without significant effect when applied to the cells for periods up to 1 h. Application of the highest dose of phorbol esters, 1 $\mu$M, was seen to have a slight facilitory effect on peak $Na^+$ current. In addition, external application of the phorbol ester, TPA, at 1 $\mu$M concentration was ineffective in altering $Na^+$ current ($2{\cdot}4 \pm 1{\cdot}9$% increase). This last test was undertaken because TPA was the phorbol ester used to down-regulate PKC with chronic application in a subsequent portion of this study. Aliquots of all these compounds were tested for their ability to activate purified PKC *in vitro*, by the method of Murakami *et al.* (1986), and were found to be potent PKC activators.

*Mediation of the effect of cis-fatty acids: interventive experiments*

If the attenuation of $Na^+$ current produced by application of *cis*-fatty acids is mediated by PKC activation, then one would predict that inhibitors of PKC activation would block their effect. There exist several compounds which are inhibitors of PKC activation. Unfortunately, none of these compounds are completely specific, as they all inhibit $Ca^{2+}$–calmodulin-dependent protein kinase or the cyclic nucleotide-dependent protein kinases to some degree. Polymyxin B and sphingosine are compounds which strongly inhibit PKC, weakly inhibit $Ca^{2+}$–calmodulin-dependent protein kinase, and do not inhibit cyclic nucleotide-dependent protein kinases (Mazzei, Katoh & Kuo, 1982; Hannun, Loomis, Merrill & Bell, 1986). Staurosporine and H-7 strongly inhibit both PKC and cyclic nucleotide-dependent kinases, but only weakly inhibit $Ca^{2+}$–calmodulin-dependent protein kinase (Kawamoto & Hidaka, 1984; Tamaoki, Nomoto, Takahashi, Kato, Morimoto & Tomita, 1986). The compound H-8 strongly inhibits cyclic nucleotide-dependent kinases, but only weakly inhibits PKC and $Ca^{2+}$–calmodulin-dependent protein kinase (Hidaka, Inagaki, Kawamoto & Sasaki, 1984). The compound W-7 inhibits $Ca^{2+}$–calmodulin-dependent protein kinase more strongly than PKC or cyclic nucleotide-dependent protein kinase (Asano & Hidaka, 1984). Thus, if the effects of *cis*-fatty acids were mediated by PKC activation, one would predict that staurosporine, polymyxin B, H-7 and sphingosine would be more potent inhibitors of this effect than either H-8 or W-7.

In order to evaluate the effectiveness of these six inhibitor compounds on the blockade of the *cis*-fatty acid effect, it first became necessary to evaluate their effects

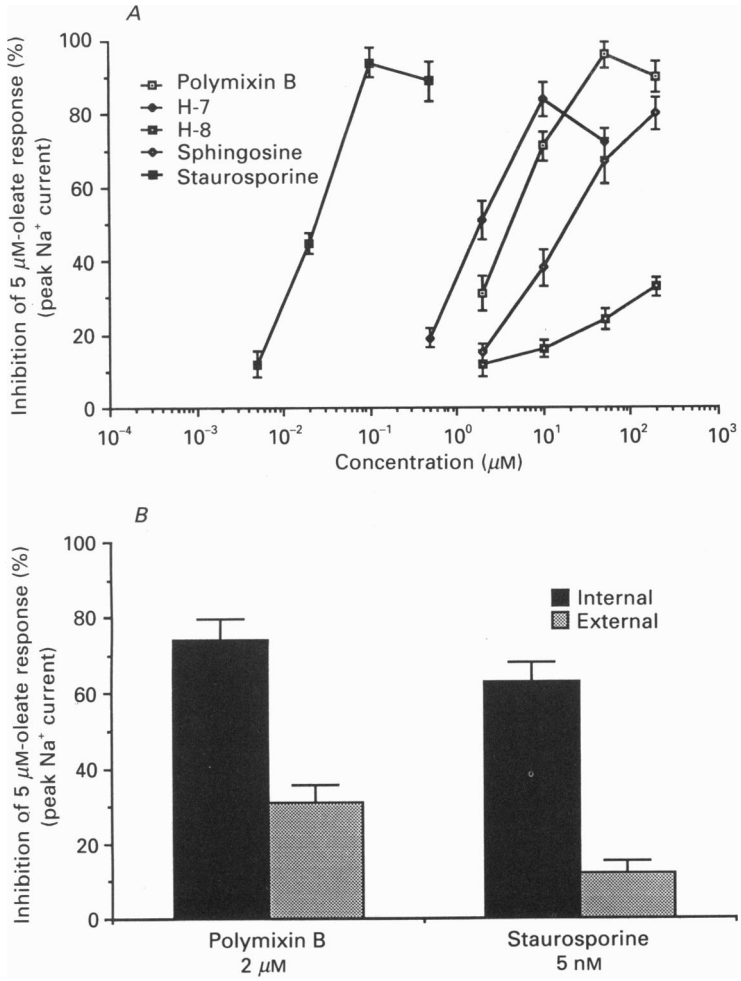


Fig. 6. Effects of PKC inhibitors on *cis*-fatty acid attenuation of the peak $Na^+$ current. *A*, external application of the PKC inhibitors polymyxin B, H-7, sphingosine and staurosporine block the attenuation of the peak $Na^+$ current produced by external application of 5 $\mu$M-oleate in a dose-dependent manner, while H-8, an inhibitor of cyclic nucleotide-dependent protein kinase is much less effective. The potency of a given compound for PKC inhibition *in vitro* predicts its effectiveness for blocking the *cis*-fatty acid effect (see text). *B*, both polymyxin B and staurosporine are most effective in blocking the *cis*-fatty acid effect when applied internally. Doses of these two inhibitors were chosen to produce a small but measurable effect with external application (see text for a discussion of this issue). $n = 6$ per point.

when administered alone. None of these compounds, except W-7, had an effect on the $Na^+$ current *I–V* relation when applied in doses ranging from 0·5–200 $\mu$M. The compound W-7 produced a drastic (90%) reduction of the peak $Na^+$ current amplitude that was only partially reversible over a 20 min wash-out period, and thus it was excluded from further study.

To assess the effects of the five remaining inhibitors on the attenuation of the $Na^+$

current by *cis*-fatty acids, cells were bathed in a solution containing the inhibitor minus oleate for 10 min prior to the application of medium containing both 5 $\mu$M-oleate and inhibitor. The medium used to wash out the oleate also contained the inhibitor. Consistent with the proposed PKC mediation of the *cis*-fatty acid effect,

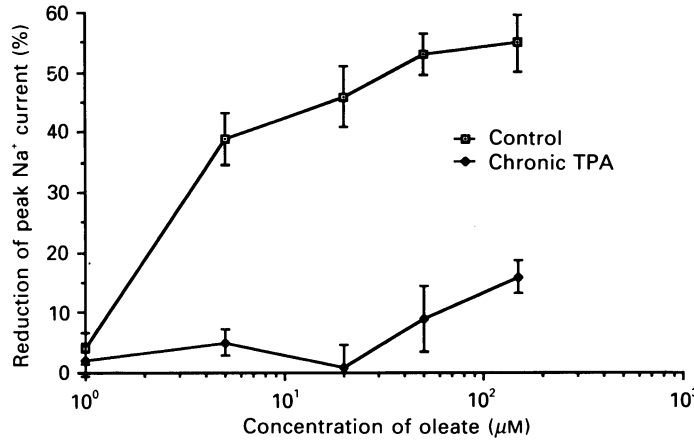


Fig. 7. N1E-115 neuroblastoma cells were pre-treated with either 1 $\mu$M-phorbol-12-myristate-13-acetate (chronic TPA) or the carrier solution (control) for 24 h prior to recording a dose response of the $Na^+$ current to application of oleate. Control cells showed a dose response very similar to other cells which received no pre-treatment (see Fig. 6), while TPA-pre-treated cells showed a near-total blockade of the oleate attenuation of $Na^+$ current which was partially overcome at the highest doses of oleate.

polymyxin B, H-7, sphingosine and staurosporine all blocked the attenuation of the $Na^+$ current produced by *cis*-fatty acid in a dose-dependent manner (see Fig. 6*A*). The most effective doses were 50 $\mu$M for polymyxin B (96·2 $\pm$ 3·5% inhibition), 10 $\mu$M for H-7 (83·8 $\pm$ 4·6%), 200 $\mu$M for sphingosine (80·3 $\pm$ 4·3%), and 0·1 $\mu$M for staurosporine (94·0 $\pm$ 4·1%). The compound H-8 showed a much smaller dose-dependent blockade of the *cis*-fatty acid effect, achieving a 37·1 $\pm$ 2·6% inhibition at a dose of 200 $\mu$M. Significantly, the relative potency of these inhibitors for PKC inhibition *in vitro* predicts their relative potency for inhibition of the *cis*-fatty acid effect on $Na^+$ current: staurosporine $\gg$ H-7 > polymyxin B > sphingosine > H-8.

In addition, polymyxin B (2 $\mu$M) and staurosporine (10 nM) were approximately 2·5 and 5 times more effective with internal application, respectively. The doses of these compounds were chosen to provide a small but measurable effect with external application. Consequently, the internal/external efficacy ratio of these single doses should not be taken as an indication that a constant ratio exists across a broad range of doses (see Fig. 5*B* for an illustration of this problem with oleate application).

Another way to block PKC application in neuronal cells is to expose them to high concentrations of a phorbol ester, such as TPA, for 24 h or longer, in order to induce down-regulation of both cytosolic and membrane-bound PKC (Matthies, Palfrey, Hirning & Miller, 1987). We found that this treatment reduced total (C+M) PKC activity from 318·8 pmol/min in control cells (incubated in the TPA carrier solution) to 75·0 pmol/min in TPA-treated cells, a reduction of 76·5%. Chronic exposure to 1 $\mu$M-TPA did not significantly alter the baseline *I–V* relation of the isolated $Na^+$

current. However, this treatment was effective in blocking the attenuation of the $Na^+$ current produced by oleate (see Fig. 7). Oleate (5 $\mu$M) produced a $5{\cdot}4 \pm 2{\cdot}1\%$ reduction of peak $Na^+$ current in TPA-pre-treated cells, and a $38{\cdot}7 \pm 4{\cdot}4\%$ reduction in control cells pre-treated with the TPA carrier solution. This blockade was partially overcome with high doses of oleate: 150 $\mu$M-oleate produced a $16{\cdot}0 \pm 2{\cdot}8\%$ reduction in TPA-pre-treated cells compared to a $55{\cdot}5 \pm 4{\cdot}8\%$ reduction in the control.

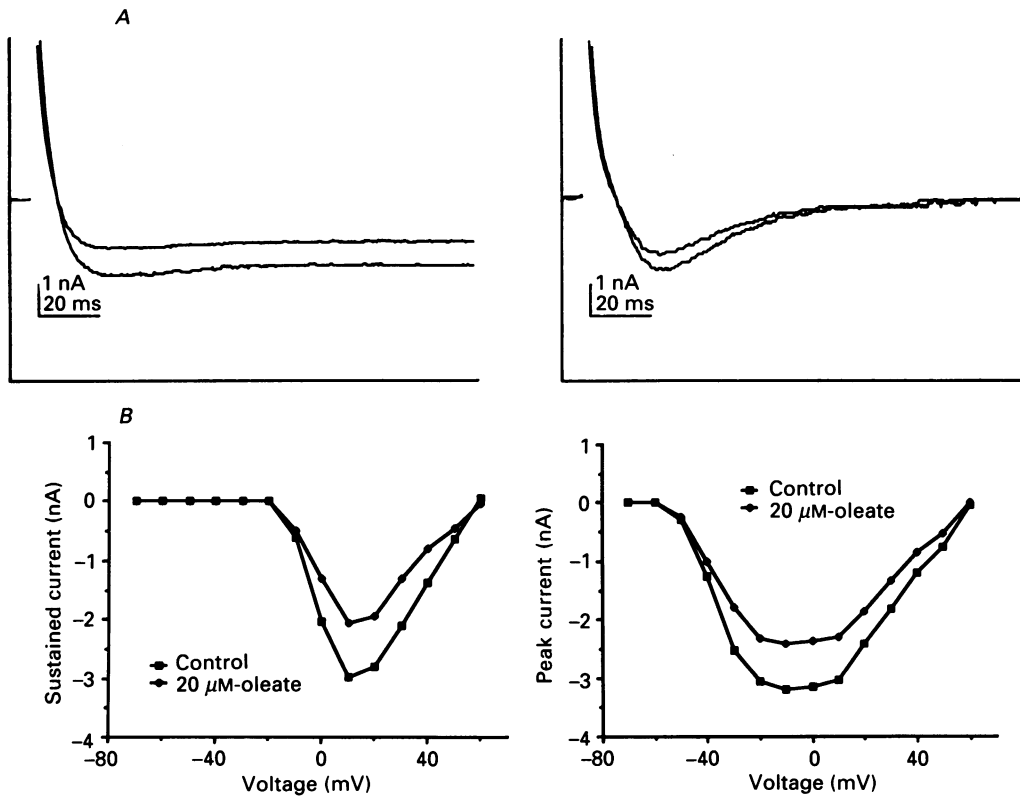


Fig. 8. Externally applied oleate, a *cis*-fatty acid, attenuates both transient and sustained components of $Ca^{2+}$ current. *A*, step depolarizations from a holding potential of $-85$ mV to command potentials of $-20$ and $+10$ mV produced the current traces used for measuring transient and sustained $Ca^{2+}$ currents, respectively. *B*, *I–V* relations of peak and sustained currents show a reduction in conductance with no shift in activation kinetics or reversal potential. The *I–V* plots have been corrected for linear leakage.

### *Observations on the isolated* $Ca^{2+}$ *current*

#### *Effects of PKC activators and inhibitors*

As *cis*-fatty acids were seen to attenuate $Na^+$ current while classical PKC activators did not, we wished to determine whether this distinction would be present in another ionic current. We chose to examine voltage-dependent $Ca^{2+}$ currents, which have been widely reported to be modulated by classical PKC activators. This modulation has generally taken the form of attenuation in vertebrate- or vertebrate-derived cells (Di Virgilio *et al.* 1986; Rane & Dunlap, 1986; Werz & Macdonald, 1987;

Doerner *et al.* 1988; Marchetti & Brown, 1988) and facilitation in invertebrate cells (DeRiemer *et al.* 1985; Farley & Auerbach, 1986). The $Ca^{2+}$ current in N1E-115 neuroblastoma cells is composed of two separable types, a low-voltage activated, transient current, and a high-voltage activated, long-lasting current. These currents

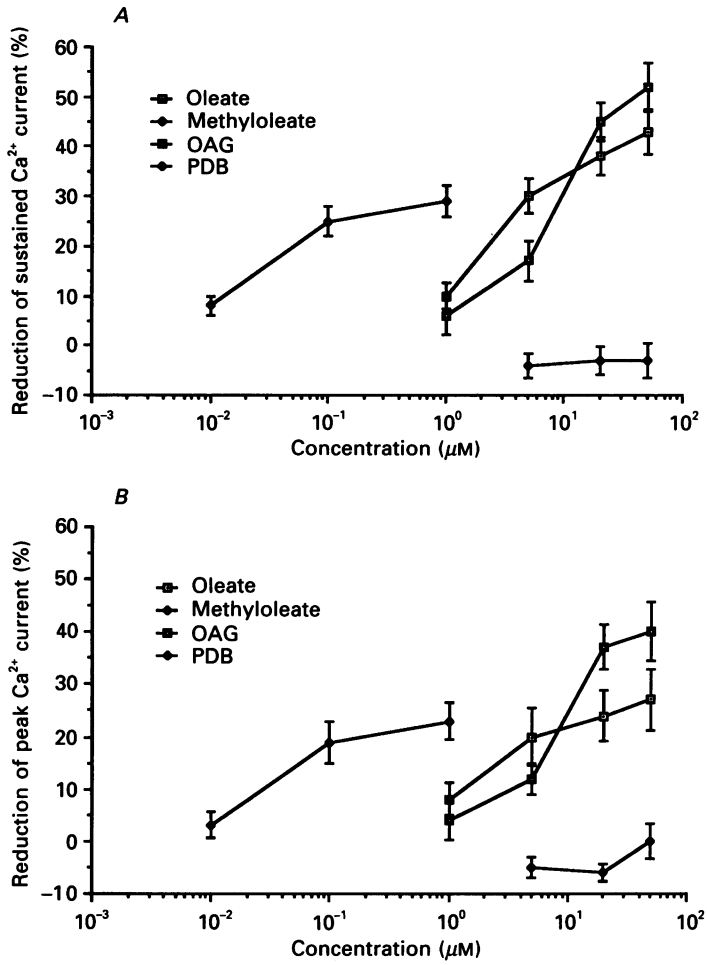


Fig. 9. The dose–response functions of sustained (*A*) and peak (*B*) $Ca^{2+}$ current to externally applied PKC activators illustrate that both classical PKC activators (OAG, PDB) and *cis*-fatty acid (oleate) attenuate both types of current. Methyloleate, which does not activate PKC, was ineffective.

have been called types I and II, respectively (Narahashi, Tsunoo & Yoshii, 1987) and are similar to currents called 'T' and 'L' in chick dorsal root ganglion (DRG) neurones (Nowycky, Fox & Tsien, 1985; for review, see Tsien, Lipscombe, Madison, Bley & Fox, 1988).

Figure 8*A* illustrates $Ca^{2+}$ current (in this case, $Ba^{2+}$ current flowing through $Ca^{2+}$ channels) evoked by step depolarizations from a holding potential of −85 mV to command potentials of −20 and +10 mV. In agreement with a previous report (Narahashi *et al.* 1987), this current was completely blocked by the addition

of 5 $\mu$M-$La^{3+}$ to the external medium, and the transient component of this current was blocked by a 150 ms pre-pulse to 0 mV (data not shown). Step depolarization to −20 mV produced a current with a near-maximal transient component and no measurable sustained component. Therefore, the peak current amplitude at −20 mV

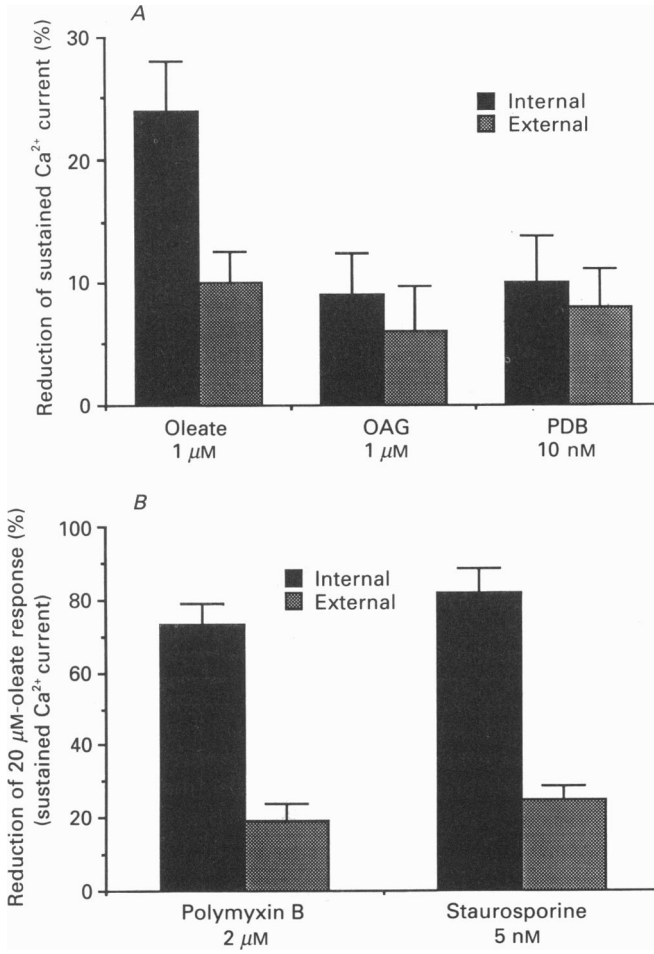


Fig. 10. Effects of internally and externally applied PKC activators and inhibitors on sustained $Ca^{2+}$ current. Doses were chosen so that external application would produce a small but measurable effect. *A*, the *cis*-fatty acid oleate is significantly more potent when applied internally, while the classical PKC activators OAG and PDB are not. *B*, two PKC inhibitors, polymyxin B and staurosporine, are more potent in blocking the effect of externally applied 20 $\mu$M-oleate, when applied internally. $n = 6$ per point.

may be taken as a measure of type I $Ca^{2+}$ current. Conversely, step depolarization to +10 mV produced a current with a near-maximal sustained component. As the transient component of $Ca^{2+}$ current was completely inactivated prior to 150 ms (the duration of the depolarizing step) the amplitude of the sustained current at this time point may be taken as a measure of type II $Ca^{2+}$ current.

External application of either *cis*-fatty acid (oleate, 1–50 $\mu$M) or classical PKC

activators (PDB, 10 nM-1 $\mu$M; OAG, 1–50 $\mu$M) reduced both peak and sustained $Ca^{2+}$ currents without altering their kinetics of activation (Fig. 8*B*). Methyloleate was ineffective in altering $Ca^{2+}$ currents. A dose–response study using these four compounds produced several notable results (Fig. 9). First, all three PKC activators attenuated the sustained $Ca^{2+}$ current to a greater degree than the peak (transient) $Ca^{2+}$ current. This finding is consistent with previous studies which have examined the effects of classical PKC activators on chick DRG neurones (Marchetti & Brown, 1988) and guinea-pig hippocampal pyramidal neurones (Doerner *et al.* 1988). Secondly, the PKC activators attenuated both types of $Ca^{2+}$ at $ED_{50}$ values consistent with their known potency for PKC activation *in vitro*. Thirdly, OAG produced a larger maximal attenuation of both types of $Ca^{2+}$ current than either PDB or oleate.

If the attenuation of $Ca^{2+}$ current produced by these two classes of PKC activator is indeed mediated by PKC activation, then these compounds should be more potent when applied internally. Doses of PKC activators were chosen to produce a small but measurable attenuation of the sustained $Ca^{2+}$ current when applied externally. Internal application of this dose of oleate produced a significantly larger effect ($P < 0{\cdot}01$, Student's $t$ test) while internal application of OAG or PDB produced a slightly, but not significantly, larger effect ($P < 0{\cdot}10$; see Fig. 10*A*). This same distinction was reflected in measures of the peak $Ca^{2+}$ current (data not shown). To examine further if the effects of oleate on $Ca^{2+}$ current were mediated by PKC activation, we applied the PKC inhibitors polymyxin B (2 $\mu$M) and staurosporine (10 nM) either internally or externally, in conjunction with 20 $\mu$M (externally applied) oleate. Figure 10*B* illustrates that both PKC inhibitors blocked the oleate effect on $Ca^{2+}$ current, and were significantly more potent with internal application. Neither inhibitor altered $Ca^{2+}$ current when applied alone, either internally or externally, at the doses stated above.

## DISCUSSION

There were five major findings of this study. First, *cis*-fatty acids reversibly attenuated the voltage-dependent $Na^+$ current by reducing the peak current amplitude without altering voltage-dependent activation or inactivation (Fig. 3). Secondly, when three classes of lipids were applied, one which was effective in both PKC activation and membrane fluidization (*cis*-fatty acids), a second which was effective in membrane fluidization but not PKC activation (LPC, methyloleate), and a third which was not effective in either function (stearate, elaidate), only the *cis*-fatty acids were seen to attenuate the $Na^+$ current (Fig. 5*A*). Thirdly, phorbol esters and OAG, two classical activators of PKC, had no effect on the $Na^+$ current when applied over a wide range of doses (Fig. 5*B*). Fourthly, reduction of PKC activity either by acute application of inhibitory drugs (Fig. 6), or by down-regulation using chronic exposure to phorbol ester (Fig. 7) was effective in blocking the attenuation of the $Na^+$ current produced by *cis*-fatty acids. Fifthly, in contrast to the $Na^+$ current, $Ca^{2+}$ currents were attenuated both by classical PKC activators and *cis*-fatty acids. However, *cis*-fatty acids, but not the classical activators, were significantly more potent with internal application.

When oleate was applied to a cell bathed in normal medium, no effects on outward current were observed (Fig. 1). However, the lack of oleate effect on net outward current does not preclude the possibility that oleate may have effects on outward currents recorded in isolation. Phorbol esters which activate PKC have been demonstrated to inhibit the $Ca^{2+}$-dependent $K^+$ current in *Hermissenda* type B photoreceptors (Farley & Auerbach, 1986) but not in NG105-15 neuroblastoma–glioma hybrid cells, where they appear to block the M-current (Higashida & Brown, 1986). Phorbol esters also reduce two outward currents in rat hippocampal pyramidal cells: the $Ca^{2+}$-dependent $K^+$ current, which is presumed to mediate the slow component of the after-hyperpolarization (Baraban *et al.* 1985), and a voltage-dependent $Cl^-$ current (Madison *et al.* 1986).

Is it likely that the observed attenuation of the voltage-dependent $Na^+$ current is a consequence of a modulation of the driving force acting on the $Na^+$ as opposed to an alteration of $Na^+$ conductance? There are two possibilities to be considered in this regard. The first is an alteration of the resting membrane potential and the second is an alteration of the $Na^+$ equilibrium potential achieved by altering the concentration of internal free $Na^+$. This second possibility may be particularly important in the light of two ion pumping mechanisms, an electrogenic pump that transports $Na^+$ out of and $K^+$ into the cell, and an electrically silent pump that transports $Na^+$ into, and protons out of, the cell. The latter pump has been demonstrated to be activated by phorbol esters and OAG, and blocked by the PKC inhibitor H-7 (Ober & Pardee, 1987). It appears unlikely, however, that any of these mechanisms underlies the attenuation of the voltage-dependent $Na^+$ current by *cis*-fatty acids as application of the latter does not appear to alter either the resting membrane potential or the reversal potential of the isolated $Na^+$ current (Fig. 3).

We have demonstrated that over the range of lipids tested, the ability of fatty acids to attenuate $Na^+$ current correlates with PKC activation, and not with membrane fluidization or the formation of micelles (Fig. 5*A* and Table 1). Takenaka, Horie & Hori (1987) have reported that application of both saturated and unsaturated fatty acids to the squid giant axon results in a reversible attenuation of voltage-dependent $Na^+$ current. However, they used different lipids (medium chain length: C8–C14) and much higher doses (16 mM-laurate in their study, as opposed to 5 $\mu$M-oleate in the present study). The reduction in peak inward current produced by their treatment was different from that observed herein as it was accompanied by a rightward shift in the *I–V* relation and was also accompanied by a small facilitation of outward current. Takenaka *et al.* (1987) saw no difference between saturated and unsaturated fatty acids in the attenuation of $Na^+$ current, and attributed their effect to a disruption of the $Na^+$ channel m gate produced by fluidization of the membrane. This attribution is supported by measurements which show increases in membrane fluidity as measured by fluorescence photobleaching recovery (Takenaka *et al.* 1983), as well as cell membrane expansion (Horie, Kawasaki & Takenaka, 1987), following application of millimolar quantities of 2-decenoate (C11:1), a compound shown to reduce $Na^+$ current. When comparing the present results with those of Takenaka *et al.* (1987) it is clear that they differ significantly in terms of effect (attenuation of $Na^+$ current with a shift in activation kinetics) and in the means of their production (millimolar quantities of lipid). We take these differences as indications that the

attenuation of $Na^+$ current produced by micromolar quantities of *cis*-fatty acid is not mediated by membrane fluidization.

To our knowledge, there have been three previous reports of PKC activators altering voltage-dependent $Na^+$ current, two of which are difficult to interpret. First, chronic (48 h) but not acute (30 min) exposure to 10 nM-phorbol ester caused an irreversible elimination of voltage-gated $Na^+$ current in approximately one-third of a population of RINm5f cells (Rorsman, Arkhammar & Berggren, 1986). Acute exposure of the cells to 10 nM-phorbol ester did produce a consistent increase in late $Ca^{2+}$ current, a result which is at odds with a reduction in voltage-dependent $Ca^{2+}$ current produced by 30 nM-phorbol ester in the same cell type (Di Virgilio *et al.* 1986). The effect of chronic phorbol ester application on the $Na^+$ current is difficult to interpret because it is unclear if this treatment ultimately results in a greater degree of PKC activation, or a lesser degree of activation as a result of PKC down-regulation (Matthies *et al.* 1987).

Secondly, ionophoretic application of PKC and phorbol ester to cat spinal motoneurones *in vivo* has been demonstrated to increase the maximal rate of spike rise and the maximal spike height measured under constant-current conditions, results which the authors suggest indicate a PKC-mediated facilitation of voltage-dependent $Na^+$ current (Zhang & Krnjević, 1987). However, since this mode of recording does not allow for the resolution of ionic currents, this suggestion remains unproven.

Thirdly, application of 10 nM-TPA or 50 $\mu$M-OAG produces an attenuation of voltage-dependent $Na^+$ current recorded in *Xenopus* oocytes injected with mRNA from chick forebrain (Sigel & Baur, 1988). As in the present study, the attenuation of $Na^+$ current by PKC activators was blocked by co-application of a PKC inhibitor (tamoxifen), and was not accompanied by an alteration of voltage-dependent activation or inactivation. The attenuation produced by OAG was reversible upon wash-out, while that produced by TPA was not.

The finding in the present study, that the classical activators of PKC, phorbol esters and OAG, do not alter voltage-dependent $Na^+$ current in N1E-115 neuroblastoma cells, differs with the above result of Sigel & Baur (1988). The basis of this difference is likely to lie in the different preparations used: the native $Na^+$ channel and its associated regulatory machinery of the differentiated N1E-115 neuroblastoma cell may have different properties than their counterparts in the *Xenopus* oocytes which require injection of exogenous mRNA for their expression. This distinction is supported by the finding that application of OAG to chick DRG neurones produces an attenuation of voltage-dependent $Ca^{2+}$ current (Rane & Dunlap, 1986), while application of OAG to *Xenopus* oocytes injected with chick brain mRNA produces an enhancement of this current (Sigel & Baur, 1988).

Consistent with previous reports using vertebrate- or vertebrate-derived cells (Di Virgilio *et al.* 1986; Rane & Dunlap, 1986; Werz & Macdonald, 1987; Doerner *et al.* 1988; Marchetti & Brown, 1988), we have found that both classical PKC activators and *cis*-fatty acids attenuate voltage-dependent $Ca^{2+}$ current (Fig. 9). This result is significant because it argues that the failure of classical activators to attenuate $Na^+$ current is unlikely to result from a failure of these compounds to activate PKC in N1E-115 cells. Thus, the distinction between the *cis*-fatty acid and classical activator effects appears to be specific to the $Na^+$ current.

A recent report has called into question the notion that the attenuation of $Ca^{2+}$ current by classical PKC activators is mediated by PKC activation. Hockberger, Toselli, Swandulla & Lux (1989) have shown that classical activators attenuate $Ca^{2+}$ current in a manner independent of PKC activation, possibly through a direct interaction with the $Ca^{2+}$ channel. They report that external, but not internal, application of OAG was effective in attenuating $Ca^{2+}$ current in chick DRG neurones, and that internal application of PKC inhibitors (sphingosine, staurosporine, H-7) did not affect the response to externally applied OAG or phorbol ester. In addition, they report that externally applied OAG or phorbol ester produces near-maximal attenuation of the $Ca^{2+}$ within 500 ms of application, a time course which they interpret to be more suggestive of a channel-blocking action than of an action mediated by a membrane-bound kinase. Two reports which are inconsistent with the findings of Hockberger *et al.* (1989) have recently appeared. In cultured guinea-pig hippocampal pyramidal neurones, application of the PKC inhibitor, H-7, either internally (Doerner & Alger, 1988) or externally (Doerner *et al.* 1988) blocked the attenuation of voltage-dependent $Ca^{2+}$ current produced by externally applied phorbol ester. The observation that internally applied H-7 can block the effect of externally applied phorbol ester is particularly important in that it suggests that H-7 is not merely blocking an action of phorbol ester on the external surface of the $Ca^{2+}$ channel.

In the present study, we have found *cis*-fatty acid to be more potent in attenuating both $Na^+$ and $Ca^{2+}$ currents when applied internally (Figs 5*B* and 10*A*). Likewise, we have found PKC inhibitors to be more potent in blocking the effects of externally applied *cis*-fatty acid on these currents when applied internally (Figs 6*B* and 10*B*). Thus, our results are consistent with those of Alger and co-workers (Doerner & Alger, 1988; Doerner *et al.* 1988), but not with those of Hockberger *et al.* (1989). The results herein suggest that the action of *cis*-fatty acid on $Na^+$ and $Ca^{2+}$ currents is mediated by PKC activation, and not by a direct interaction of *cis*-fatty acid with ion channel proteins.

While oleate, a *cis*-fatty acid, was more potent in its action on $Ca^{2+}$ current when applied internally, OAG and PDB were approximately equipotent with internal and external application (Fig. 10*A*). We interpret this distinction to mean that PDB and OAG gain access to membrane-bound PKC more effectively than *cis*-fatty acid. Consequently, externally applied OAG or PDB produce the maximal PKC activation possible for that dose, while externally applied *cis*-fatty acid does not.

The lack of a classical activator effect on voltage-dependent $Na^+$ current in the N1E-115 cell is not consistent with a simple model of PKC mediation of the *cis*-fatty acid effect. It is possible that the effect of *cis*-fatty acids on the $Na^+$ current may be dependent upon coactivation of a PKC mechanism and a non-PKC mechanism (such as membrane fluidization). However, we favour one of the following two models which do not require invoking a non-PKC mechanism. In model A, *cis*-fatty acids and classical activators have different effects on the $Na^+$ current because they activate different PKC subtypes (or sets of PKC subtypes). This model is consistent with observations that PKC is a family of enzymes (Coussens *et al.* 1986) which show a heterogenous response to *cis*-fatty acid activation (Sekiguchi, Tsukuda, Ase, Kikkawa & Nishizuka, 1988; Nishizuka, 1988). Alternatively, model B states that the substrate specificity of a single PKC subtype could be dependent upon its mode

of activation such that activation by *cis*-fatty acid could result in phosphorylation of the $Na^+$ channel protein, or of a protein that interacts with it, while activation of the same PKC subtype by a classical activator does not.

The observation that acutely applied phorbol esters are ineffective in altering the $Na^+$ current, but that chronically applied phorbol esters are effective in blocking the attenuation of this current by *cis*-fatty acids, would appear to argue against model A. However, this argument is dependent upon the assumption that the specificity of activation of PKC subtypes and the specificity of down-regulation of PKC subtypes is the same for a given activator. This assumption may not be correct. In RBL-2H3 cells, chronic exposure to TPA resulted in greater down-regulation of type II PKC than type III PKC as assayed by immunoblot (Huang, Yoshida, Cunha-Melo, Beaven & Huang, 1989). However, when activation of purified type II and type III PKC by TPA was measured *in vitro* (in the presence of phosphatidylserine and $Ca^{2+}$) using H1 histone as a substrate, no discernible differences in the kinetics of activation could be seen (Sekiguchi *et al.* 1988). Thus, subtype specificity for down-regulation and activation may not be identical for a given PKC activator. It should be noted that model B is consistent with either case of the down-regulation/activation issue detailed above.

Either model is supported by evidence that addition of *cis*-fatty acid or OAG to N1E-115 cell homogenate results in different patterns of protein phosphorylation as resolved by polyacrylamide gel electrophoresis: addition of exogenous *cis*-fatty acid, but not OAG, resulted in an increase in the phosphorylation of proteins with molecular weights 40 and 58 kDa (K. Murakami, S. G. Chen & A. Routtenberg, unpublished observation). This finding is consistent with previous observations that addition of exogenous phorbol esters and diacylglycerols can result in different patterns of substrate protein phosphorylation in isolated rat liver plasma membranes (Kiss & Luo, 1986), or different patterns of physiological response (including such measures as prolactin release, prolactin synthesis, and cell stretching) in $GH_4$ pituitary cells (Ramsdell, Pettit & Tashjian, 1986).

Purified PKC phosphorylates the $\alpha$-subunit of the $Na^+$ channel *in vitro* using either purified $Na^+$ channel from rat brain or native $Na^+$ channel in rat brain synaptosomes (Costa & Catterall, 1984). It is not known if this phosphorylation is a function of a particular PKC subtype, or if the phosphorylation of the $\alpha$ subunit by PKC has any particular functional consequence. If activation of PKC by *cis*-fatty acids does lead to attenuation of $Na^+$ current in neuronal cells, what function might this serve? A route for speculation is provided by a recent finding that $Na^+$ influx regulates the turnover of phosphatidylinositol (Gusovsky, Hollingsworth & Daly, 1986), one consequence of which is diacylglycerol production. Diacylglycerol serves as a second messenger for PKC activation (Nishizuka, 1986), and also serves as a substrate for diacylglycerol lipase, which functions to liberate free fatty acids. Thus, it is tempting to speculate that a PKC-mediated attenuation of voltage-dependent $Na^+$ current might function as a negative feedback regulator of PKC activation and/or voltage-dependent $Na^+$ current.

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