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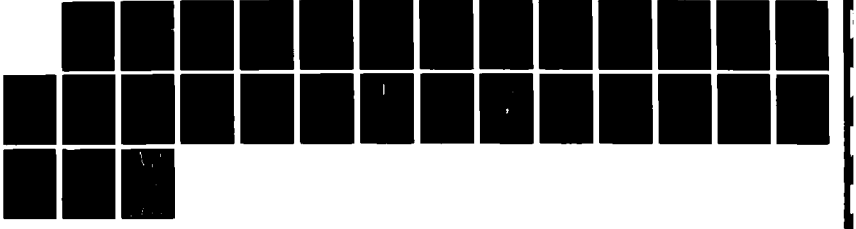

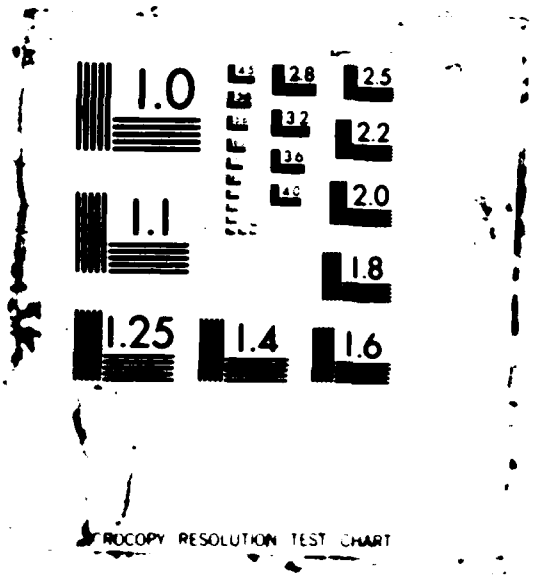
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Final Scientific/Technical Report
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PHOSPHOPROTEIN REGULATION OF SYNAPTIC REACTIVITY: ENHANCEMENT AND CONTROL OF A MOLECULAR GATING MECHANISM

Progress Report for AFOSR 83-0335
Submitted to:
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Submitted by:
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This research is focussed on the role of molecular switches in brain which regulate synaptic reactivity. We have identified a 47,000 mol wt phosphoprotein and its kinase (C) which play a pivotal role in this regulation. We have enhanced communication among nerve cells studied electrophysiologically by activation of this kinase. In the proposed research we specifically manipulate this brain phosphoprotein with novel kinase activating agents. In a new initiative, both the kinase and the F1 substrate will be cloned with the goal of enhancing synaptic reactivity by regulating the expression of proteins that play a pivotal role in this process.

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Final Technical/Scientific Report

1. Project Period The project period includes September 30, 1983 to September 29, 1986. The present report is being filed in February, 1987.

2. Personnel

Name	Title	Dates of Service	% Effort
A. Routtenberg	Professor/PI	2/83-present	25%
S. Chan	Res. Neurobiologist	2/84-present	25%
K. Murakami	Res. Neurobiologist	4/84-present	25%
R. Akers	Grad. Res. Asst.	7/83-present	50%
P. Colley	Grad. Res. Asst.	7/83-5/85	50%
D. Linden	Grad. Res. Asst.	9/84-present	50%
D. Lovinger	Grad. Res. Asst.	7/83-present	50%
R. Nelson	Grad. Res. Asst.	7/83-present	50%
F. Sheu	Grad. Res. Asst.	9/85-present	50%
K. Wong	Grad. Res. Asst.	4/86-present	50%

3. Achievement of Goals

In the past project period the initial goal was to analyze post-translational modification of phosphoproteins using in vitro phosphorylation following long-term potentiation in the intact hippocampal formation. At that time (6/83) only scanty evidence existed on the participation of particular brain phosphoproteins. One note was sounded which turned out to be predictive, i.e., because of the important role that calcium plays in LTP, those phosphoproteins specifically related to calcium function were suggested to be involved.

4. Major research findings

a. LTP increases protein F1 phosphorylation

Initial evidence first reported at the Federation meetings in April 1983 (Routtenberg et al., 1983), discussed in relation to protein kinase C (PKC) in Routtenberg (1984) and reported in detail in Routtenberg, Lovinger and Steward (1985); Lovinger, Barnes, McNaughton and Routtenberg (1985) and Lovinger, Akers, Nelson and Routtenberg (1986) pointed to protein F1, a 47 kD, acidic (4.5) phosphoprotein (Nelson and Routtenberg, 1985) as closely associated wtih LTP production. Thus, LTP significantly increased its phosphorylation and the extent of phosphorylation in vitro was related to the extent of enhancement measured in vivo.

These results focussed on the participation of protein F1 in synaptic plasticity and called attention to the phosphorylation mechanism regulating its activity. To understand the significance of these results it was necessary to determine the F1 kinase and characterize its activity after LTP. In the next few sections evidence on one major issue, i.e., the identity of the kinase for protein F1 is reviewed; in section h recent evidence concerning the identity and function of the F1 substrate protein is presented.

b. Purification of protein kinase C and protein F1

As a first step to understanding the significance of

the increase in in vitro phosphorylation of protein F1 then, it was essential to identify the kinase and then characterize its activity after LTP. We knew that F1 was unlikely to be a cAMP kinase substrate since our first studies on protein phosphorylation (Ehrlich and Routtenberg, 1974; Routtenberg and Ehrlich, 1975) demonstrated that neither cyclic AMP nor cyclic GMP stimulate the phosphorylation of protein F1. The role of calcium in protein phosphorylation (Cheung, 1982; Nishizuka, 1986) and in LTP (e.g., Bainbridge et al., 1983; Lynch et al., 1985) suggested that a calcium dependent kinase might be important. We therefore investigated both calmodulin and phospholipid dependent kinases. In our first study (Akers and Routtenberg, 1985) we demonstrated that protein F1 was stimulated by phospholipid (phosphatidyl-serine) in a dose-dependent fashion and that the calcium/phospholipid dependent kinase activator, phorbol ester, stimulated the endogenous phosphorylation of protein F1. Moreover, calmodulin did not stimulate protein F1 phosphorylation.

To investigate this proposal, it was necessary to demonstrate that purified F1 could be phosphorylated by purified protein kinase C and that such phosphorylation was regulated by calcium and phospholipid. Using histone H1 as substrate, protein kinase C was purified 1000-fold to near homogeneity by Murakami et al. (1986).

[We have discovered that in addition to the calcium phospholipid dependent activation, there exists another separate mechanism for protein kinase C activation that involves activation by cis fatty acids such as oleate (Murakami and Routtenberg, 1985; see Section f).

Protein F1 purification has been achieved by Chan et al. (1986). Addition of purified protein kinase C in the presence of calcium and phospholipid led to the incorporation of approximately 1 mole of phosphate per mole protein. Since purification was about 50% we suggested the possibility that each F1 molecule may have two phosphorylatable sites. Using Edman degradation and high voltage paper electrophoresis, greater than 97% of the phosphate was covalently bound to serine residues. The stability of that covalent linkage may be of some importance in long-term regulation of synaptic plasticity. No detectable phosphorylation was observed on tyrosine or threonine. Three different calmodulin kinases including calmodulin kinase II (Kennedy et al.) did not produce any detectable phosphoryalation of protein F1. Moreover, calmodulin inhibited the kinase C dependent phosphorylation in a dose-dependent fashion (Chan et al., 1986).

We have concluded that protein F1 is phosphorylated by protein kinase C. The exclusivity of this reaction has yet to be determined, though at this stage protein kinase C is the only kinase known to phosphorylate protein F1.

c. Translocation of protein kinase C by LTP

Since protein kinase C phosphorylates protein F1 and F1 phosphorylation is altered by LTP, the next step in our investigations focussed on the question of the impact of LTP

on protein kinase C activity. This study revealed a novel mechanism for the regulation of synaptic plasticity translocation of the kinase from the cytosol to the membrane (Akers et al., 1986). Additionally, this activation took time: it was not observed 1 min after LTP but was observed 1 hr after its induction. This is consistent with our observation that protein F1 phosphorylation is not increased 1 min after LTP (Routtenberg et al., 1985). We suppose that the increase in membrane kinase C, via translocation of its activity from the cytosol to membrane could lead to the increase in protein F1 phosphorylation.

1'. Calcium-mediated translocation of protein kinase C in synaptosomes: A "model LTP"?

The translocation of protein kinase C represents a novel mechanism for regulating synaptic plasticity, raising certain intriguing issues. For example, what regulates translocation? Is there a specific signal? Once translocated, what is its persistence? To approach this issue Akers and Routtenberg (manuscript in Appendix) studied the role of calcium in translocating protein kinase C activity in a hippocampal synaptosomes. Evidence in this and other laboratories indicated that calcium increases the hydrophobicity of protein kinase C (Walsh, et al., 1984; Murakami, unpublished observations). Thus the influx of calcium following activation could expose the hydrophobic domain of protein kinase C so that protein kinase C more readily attaches to the plasma membrane.

Akers showed that at 5 X 10-6 M, calcium causes translocation of protein kinase C activity to the membrane and a correlated ($r = +0.86$, $p < .01$) increase in protein F1 phosphorylation. Interestingly, when now a subsequent lower concentration of calcium was used (10-7) that normally decreases membrane protein kinase C, a persistence of membrane protein kinase C activity was observed. This suggests the possibility that protein kinase C translocation could serve as a "latching" mechanism persisting in its membrane attachment for a period of time that could conceivably extend for several days based on the half-life of protein kinase C. Such a mechanism could be important for the persistent increase in F1 phosphorylation observed three days after LTP (Lovinger, et al., 1985).

2'. Regional differences in protein kinase C distribution

If protein kinase C translocation were critically involved in regulating synaptic function one might predict that its endogenous distribution within different brain regions would be different. Fwu-Shan Sheu in our laboratory has, in fact, discovered that the intracellular distribution of protein kinase C is differentially distributed, for example, in hypothalamus cerebellum and frontal cortex as opposed to hippocampus (Table 1). Surprisingly, the ratio of cytosol/membrane protein kinase C in hippocampus is greater than this ratio in cerebellum. Moreover, the total protein kinase C activity is greater in hippocampus.

Table 1
Regional Differences in the
Cytosol and Membrane Distribution of protein kinase C activity
(pmol/min/mg-protein)

Brain Region	Cytosol	Membrane	C/M
Hippocampus	1560	2380	0.65
Frontal Cortex	1220	2590	0.47
Hypothalamus	1233	2400	0.51
Cerebellum	510	1950	0.26

It appears that the translocation mechanism may be regulated to a particular mean level both throughout the life of the organism and also shift in relation to inputs; the long-term life-span regulation may provide, in fact, the potential for input-dependent shifts in distribution necessary to regulate synaptic strength.

d. Phorbol ester "DAG-type" activation of protein kinase C promotes synaptic plasticity

Since we had shown that LTP activates/translocates protein kinase C, we wished to manipulate protein kinase C and observe the effects on LTP. Specifically, if protein kinase C translocation were important for synaptic plasticity then induction of translocation should regulate that plasticity. Fortunately a rather specific compound exists that translocates protein kinase C. Castagna et al. (1982) discovered that phorbol esters from Croton oil stimulated protein kinase C and Kraft and Anderson (1983) found that phorbol esters translocated the kinase from the cytosol to the membrane. It is now known that protein kinase C co-purifies with the phorbol ester receptor (Niedel et al., 1983; Kikkawa et al., 1983) suggesting that protein kinase C is indeed a phorbol receptor.

Nishizuka (1984) has suggested that phorbol esters act by substituting for DAG reducing the calcuim requirement for protein kinase C activation. I shall refer to this as a "DAG-type" of protein kinase C activation (this contrasts with the "Oleate-type" protein kinase C activation to be described shortly). Diacylglycerol (DAG) is therefore a second messenger for protein kinase C activation (see Figure 1 in appendix). DAG is the metabolic product of phospholipase C hydrolysis of phosphoinositide diphosphate (PIP2). Because DAG is rapidly degraded it was proposed to act as a signal. The persistent activation of protein kinase C observed following enhanced synaptic plasticity, however, would not likely be related to persistent elevation of this second messenger.

It was predicted on the basis of findings just reviewed that the influence on synaptic plasticity would be one of enhancement. Using the recording and iontophoresis set-up shown in Figure 2 we have found indeed that intrahippocampal ejections of the phorbol ester, 12-O-tetradecanoylphorbol

13-acetate(TPA), a potent protein kinase C activator, prolongs the enhanced response of LTP (Lovinger et al., 1985; Routtenberg et al., 1986) in the intact in vivo hippocampus. Using an in vitro hippocampal slice preparation, Malenka et al. (1986) have confirmed this growth promoting influence of TPA on LTP. It may be noted that in this and other studies perfusing the hippocampal slice with TPA (Baraban et al., 1985; Malenka et al., 1986) a difficulty exists in identifying the site of action of the drug. Since the drug will influence protein kinase C present in terminals (Girard et al., 1985), cell bodies and dendrites (Nishizuka, 1986) phorbol ester would likely influence many different functions.

e. Iontophoresis of phorbol ester into the synaptic zone induces plasticity of the response

To influence the synaptic zone directly we plan in the proposed research, and have initial evidence to be discussed, to record/eject in the molecular layer, specifically in the synaptic terminal zone of the perforant path fibers. It now appears that protein F1 and protein kinase C are co-localized to the presynaptic terminal (Gispen et al., 1985; Girard et al., 1985; Worley et al., 1986). It is therefore reasonable to eject the compounds of interest into the zone where the synaptic terminals are present. Because of the rigidly laminated structure of the hippocampus precise placement into the region of synaptic termination is readily accomplished. There is evidence that protein kinase C is present in high concentration within the hippocampus (Nishizuka, unpublished observations; F.S. Sheu and Routtenberg, unpublished observations).

Multi-barreled pipettes are placed in the molecular layer following laminar profile analysis. The hilar response, the granule cell layer and the null zone provide suitable landmarks as the pipette is raised into the molecular layer (dorsal leaf). The selected site of recording/ejection is the region with the maximum negative slope. We apply the phorbol ester PDBu (Phorbol 12, 13 dibutyrate) directly to the locus of synaptic termination of perforant path fibers, in the molecular layer.

Without the use of high-frequency stimulation PDBu in the synaptic zone potentiated the synaptic response (slope of EPSP) generated by low frequency (0.1 Hz) stimulation (see Figure 3A). After 2 hrs, high-frequency stimulation now failed to produce a further increase in synaptic response in contrast to controls receiving the tris vehicle (Figure 3B). Therefore, the process engendered by phorbol ester occludes the response to LTP. This is consistent with a role for protein kinase C in the synaptic plasticity of LTP. The amount of phorbol ester ejected and the use of phorbol esters with different potencies will be carefully evaluated in the proposed research. In addition, inactive phorbol esters of the 4-alpha type represent important controls for the non-specific effects of the phorbol moiety.

f. A novel "oleate-type" mechanism for protein kinase C activation and regulation of synaptic plasticity

Murakami and Routtenberg (1985) have proposed that another mechanism exists for activation of protein kinase C in addition to that by calcium and phospholipid. This is by cis fatty acids such as oleate which can fully activate protein kinase C in the absence of calcium or phospholipid (Murakami and Routtenberg, 1985; Murakami, Chan and Routtenberg, 1986).

We have proposed that protein kinase C is dually regulated: one limb is mediated by phospholipase C (PLC) activation leading to DAG elevation (Figure 1), the other by phospholipase A2 (PLA2) activation leading to elevation of oleic acid (Figure 4). That a second distinct activation mechanism for protein kinase C may exist is suggested by the discovery that oleic acid can activate protein kinase C in the absence of calcium and phospholipid and that DAG but not oleate requires micelle formation to stimulate protein kinase C activity (Murakami, Chan and Routtenberg, 1985; 1986). We proposed that this mechanism may be separate from the DAG-mediated protein kinase C activation (Nishizuka, 1984).

If TPA prolongs the durability of enhanced synaptic reactivity by activating protein kinase C then one would predict that protein kinase C activators other than TPA such as oleic acid or arachidonic acid should possess the same ability. Since cis fatty acid iontophoresis had not been attempted previously we wished to determine, before testing this prediction, the reliability of ejection and the possibility of performing dose-response studies. To determine the dose/current relation with oleate and arachidonate iontophoresis, we have used 3H-oleate and measured the amount ejected from two different pipettes using the same batch of radioactive oleate or arachidonate (see Figure 5). There appears to be little variation in the amount ejected from different pipettes at a given ejection current.

The predicted enhancing effects of oleate have recently been confirmed (Linden, Murakami and Routtenberg, 1986). Thus, the cis fatty acid oleate iontophoresed into the hippocampal hilus promoted growth of synaptic plasticity. The trans-isomer, elaidic acid, had little influence, and was no different than vehicle controls. Because oleic acid, unlike TPA, is capable of activating protein kinase C in the absence of calcium, its iontophoresis into hippopocampus might not produce effects identical to those observed with TPA or DAG. Such oleate-specific effects distinct from phorbol ester have recently been observed.

g. Enhanced effectiveness of oleate iontophoretically-applied to the synaptic zone

To determine whether the action of oleate on promoting growth of the potentiated response that we have recently described (Linden et al., 1986) is related to a synaptic

site of action, we plan to determine whether iontophoretically-applied oleate to the molecular layer is, in fact, more potent in enhancing synaptic plasticity. Initial results suggest that the enhancing effects of oleate can be observed with a lower dosage than hilar application consistent with the idea of a perforant path/dentate granule cell synaptic site of action.

These initial results also suggest that, in contrast to phorbol esters, oleate by itself may not induce a potentiated response but rather appears synergistic with high-frequency stimulation to produce growth of the response once it is engendered by high-frequency stimulation. This model is based on the proposal by Nishizuka (1984). A calcium-mediated event independent of kinase C and protein kinase C activation act synergistically to produce a physiological response, release of thrombin from platelets. We have proposed that a similar mechanism may be operating in the generation of the neuroplastic response (1986). A synergism may thus exist between the activation of protein kinase C by oleate and the enhancing consequences of the high-frequency trains.

h. Identification and function of protein F1

An exciting development during 1986 was the realization that protein F1 was probably identical to brain proteins studied in a different functional context by several other laboratories. One of the challenges facing these laboratories and our own is to discover the functional commonality among our research findings. To this end a meeting was held in May, 1986 at the Neuroscience Institute at Rockefeller University, chaired by Bernice Grafstein, Larry Benowitz and the PI. We learned that an acidic phosphoprotein of molecular weight (tentative) 46-48 kD, primarily regulated by protein kinase C activity, is related to at least four functions: phospholipid metabolism (protein B-50), axonal regeneration (GAP-43: Growth Associated Protein initially estimated to be 43 kD); developmental growth of axons (pp46); synaptic plasticity (protein F1). In the past year we have collaborated with three laboratories to show that protein F1 is likely the same as protein B-50 (Gispen, DeGraan, Chan and Routtenberg, 1986), GAP-43 (Snipes, Chan, Costello, Norden, Freeman and Routtenberg, 1986) and pp46 (Nelson, Hyman, Pfenninger and Routtenberg, 1985).

I have suggested (1985a) that these converging lines of evidence point to a role of protein F1 in axonal growth, and that during the strengthening of the synapse a physical growth of the presynaptic terminal occurs, possibly coordinated with post-synaptic growth (as in the synaptic spinule, Tarrant and Routtenberg, 1977)..

1. Brief summary of progress and recent developments

In the past project period we have identified an enzyme, protein kinase C, and its substrate, protein F1, that are closely associated with the enhancement of synaptic reactivity. In addition to identification and

characterization of the kinase and substrate in this past project period, we have discovered two new mechanisms that may regulate protein kinase C activity: enzyme translocation and cis fatty acid activation of protein kinase C. The specific role of these new mechanisms in regulating synaptic plasticity represents a major thrust of our present studies. mechanism in the regulation of synaptic In the past year there has appeared convergent evidence from other laboratories, mostly unpublished at this time, indicating that protein kinase C plays an important role in the regulation of synaptic activity.

4. Publications
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1. Figures

Figure 1: Schematic depiction of "DAG-type" activation of protein kinase C

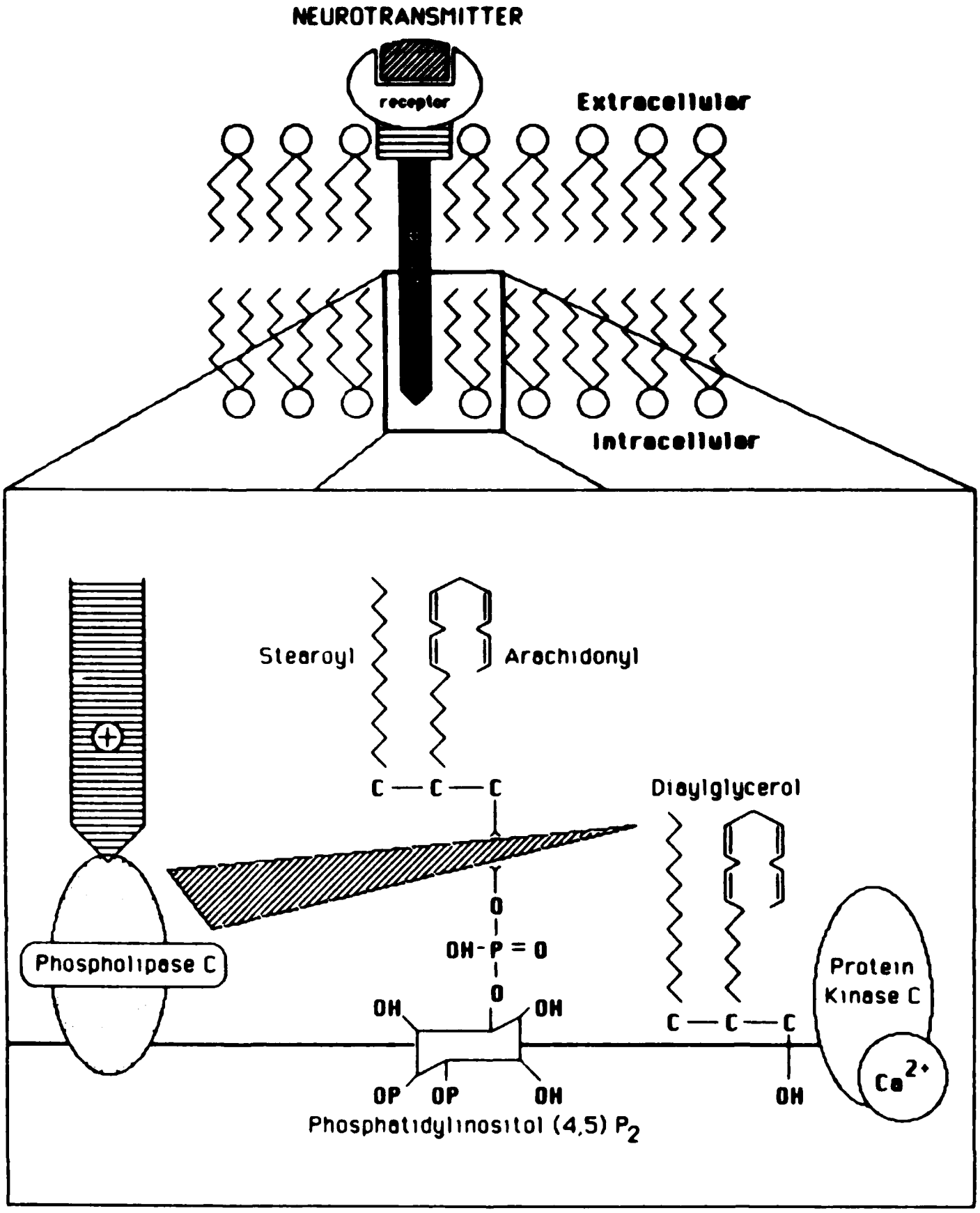

NEUROTRANSMITTER
receptor
Extracellular
Intracellular
Stearoyl
Arachidonyl
C — C — C
Diacylglycerol
O
OH-P = O
O
OH
OH
OP
OP
OH
Phosphatidylinositol (4,5) P2
Phospholipase C
Protein Kinase C
Ca2+
C — C — C
OH

Figure 2: Set-up for iontophoresis of protein kinase C regulators into intact hippocampal dentate gyrus. Stimulating electrode activates perforant path (PP), the cells of origin are in entorhinal cortex (EC) projecting to granule cells of dentate gyrus (DG) where a multi-barreled pipette is positioned. CA1, CA3-pyramidal cell zones of hippocampal gyrus.

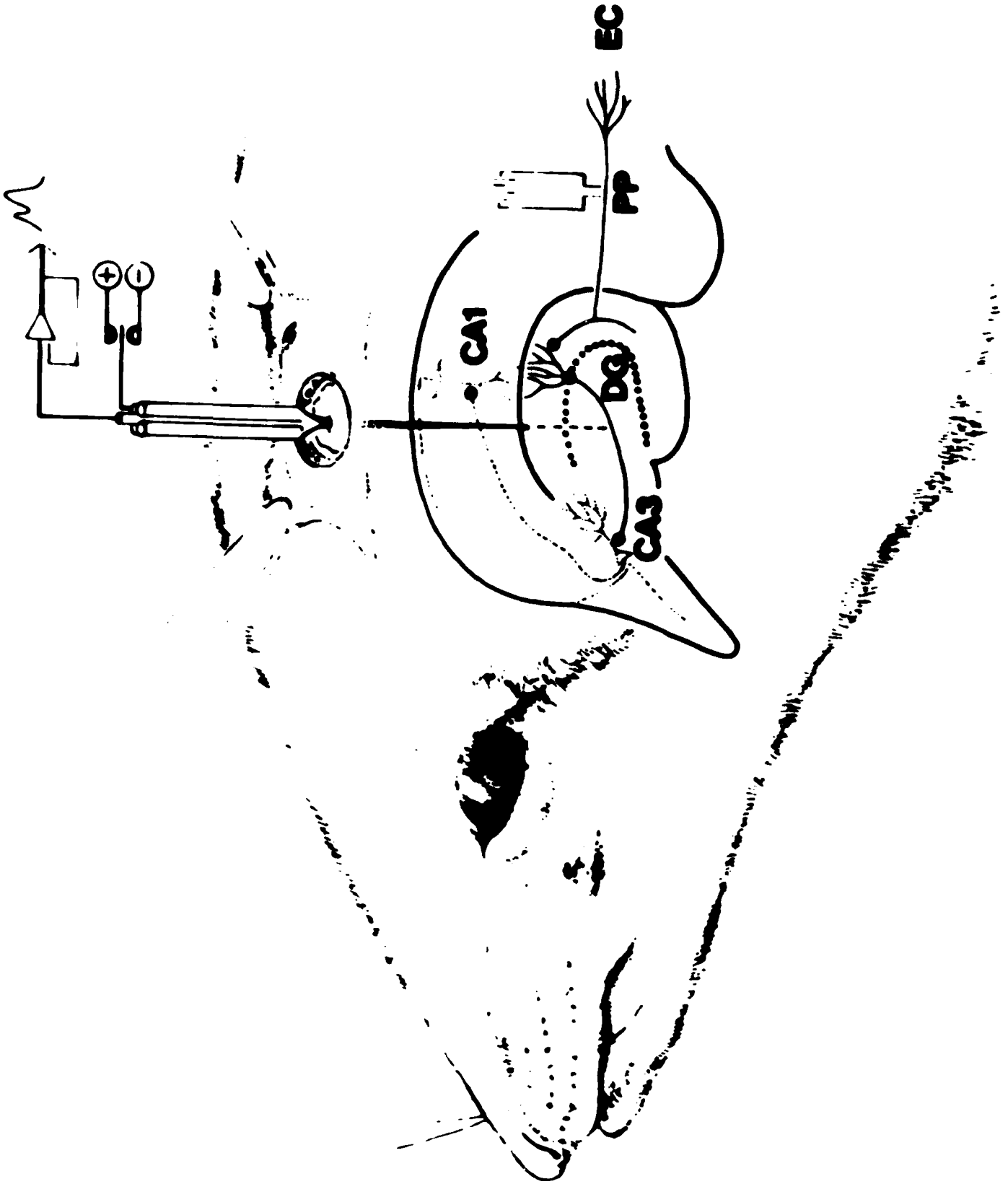

EC
PP
CA1
DG
CA3

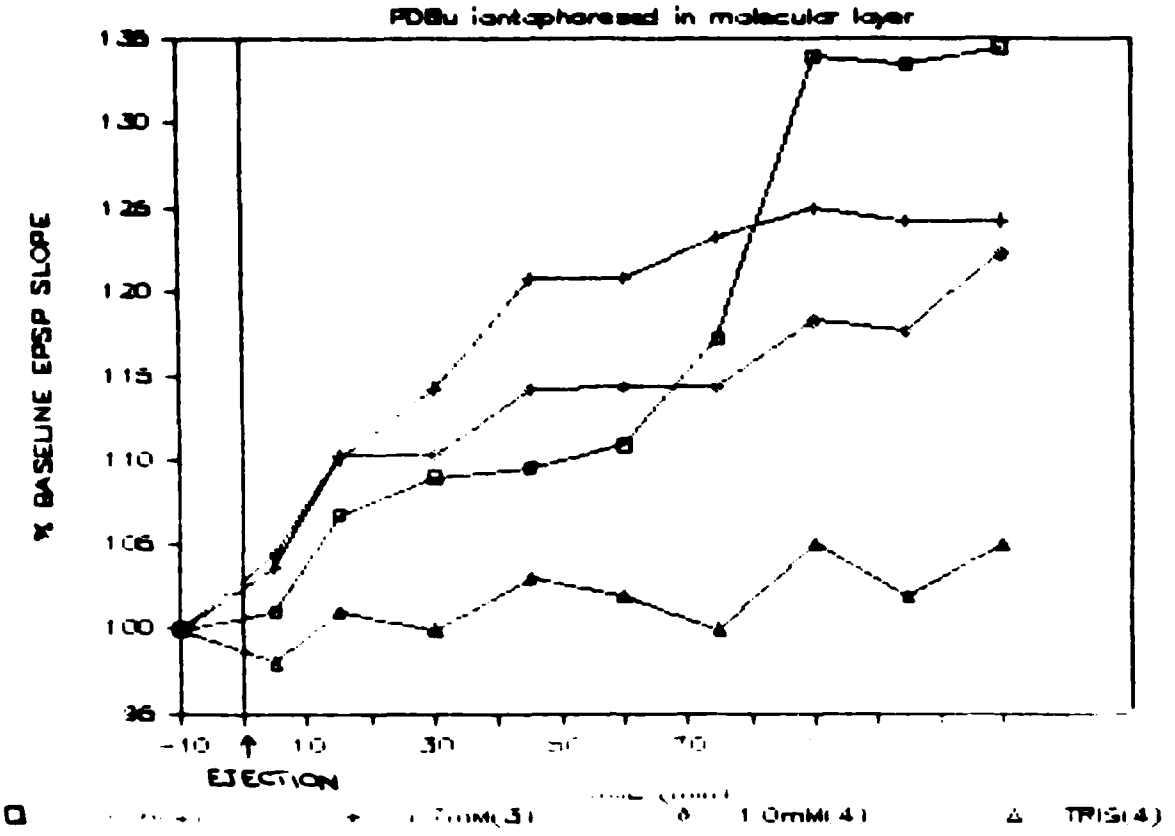
Figure 3

Figure 3a: Phorbol dibutyrate (PDBu) iontophoretically applied to the molecular layer of the intact rat dentate gyrus induces growth of baseline synaptic activation. Once baseline was determined, PDBu or TRIS vehicle was ejected (25 nA anodal, 5 min). Amount of PDBu ejected was from 30 pM to 60 pM. A dose-related effect on baseline synaptic activation monitored for two hours after the ejection was observed No effects of TRIS vehicle were detected.

Figure 3b: PDBu induced growth of EPSP blocks long-term potentiation two hours. 30 pM of PDBu was iontophoretically applied after PDBu ejection to the intact molecular layer of the dentate gyrus with a 25 nA anodal current for 5 minutes. Baseline low-frequency synaptic activation was monitored for two hours after which eight trains of 400 Hz stimulation (10V, 0.4 msec) were delivered. PDBu animals showed increase in baseline EPSP slope with the ejection alone and a failure to potentiate. Vehicle control animals, however, showed no change following the ejection, and EPSP slope potentiation to the level of growth seen with PDBu ejection alone.

2A.

PDBu INDUCES SYNAPTIC PLASTICITY



2B.

PDBu OCCLUDES LTP 2Hr LATER

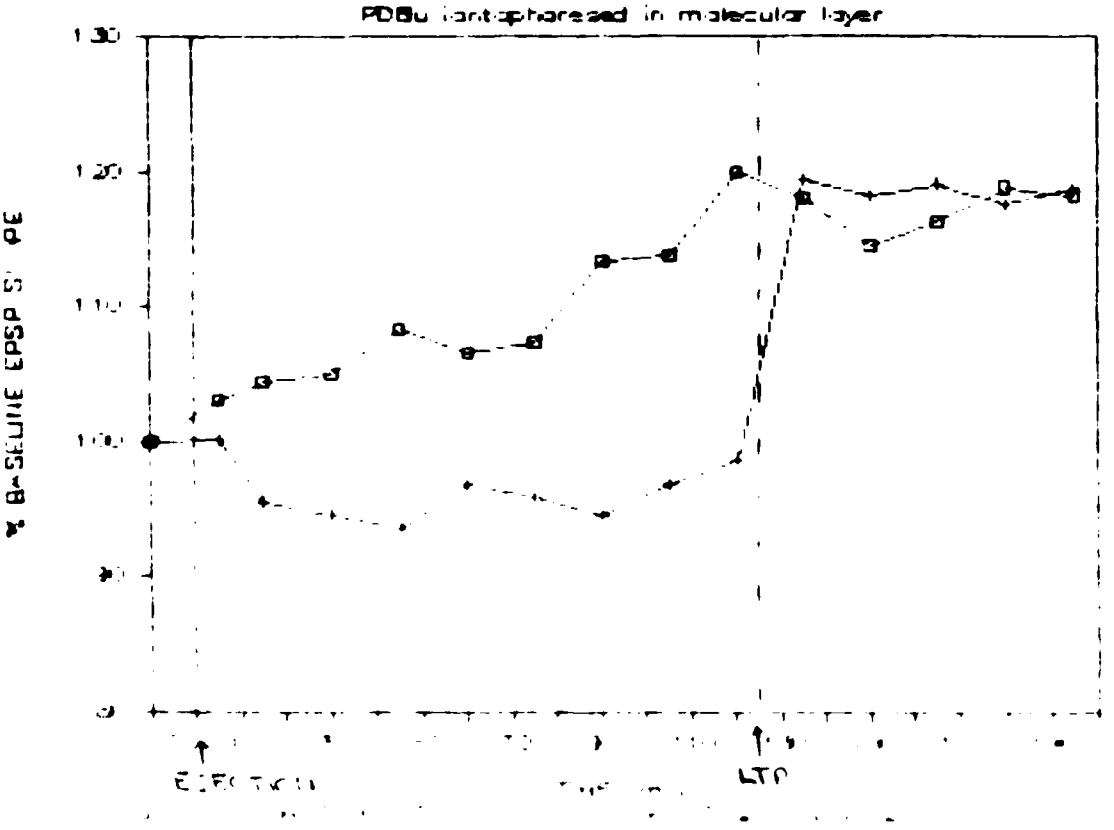


Figure 4: Schematic depiction of "oleate-type" activation of protein kinase C.

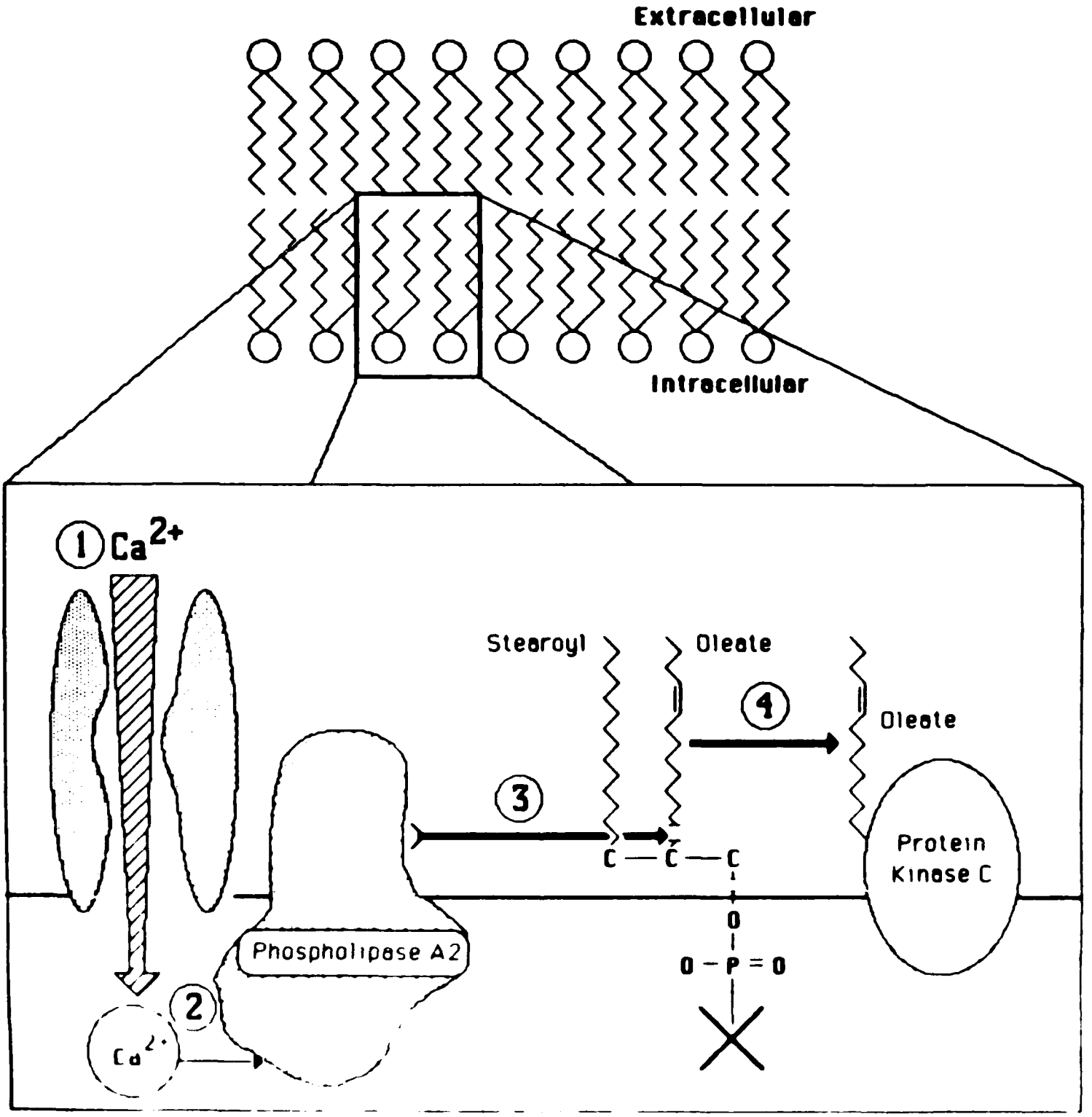
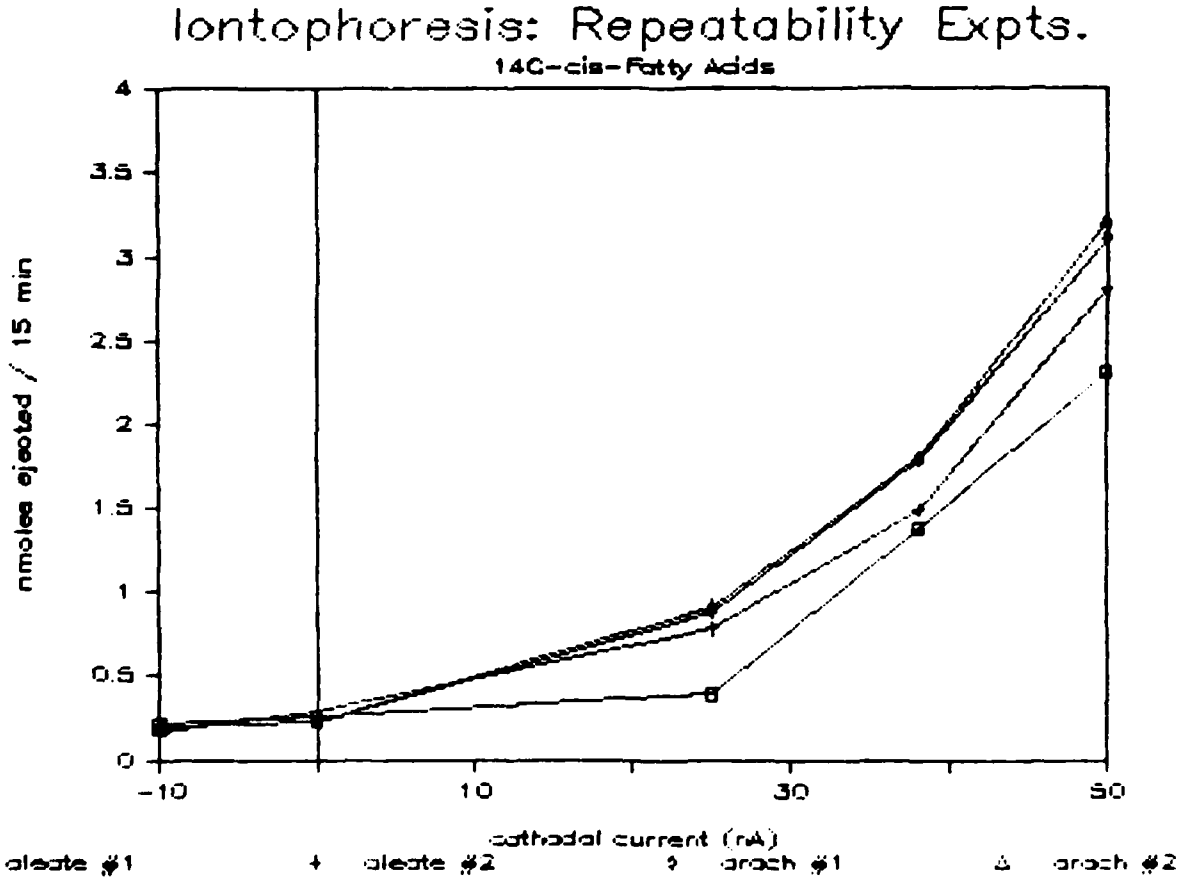

Extracellular
Intracellular
1 Ca2+
Stearoyl
Oleate
4
Oleate
3
Protein Kinase C
Phospholipase A2
2
Ca2+
O
O — P = O

Figure 5: C14-labelled oleate and arachidonate were directly dispersed into 20 mM tris buffer, vortexed, nitrogen bubbled and sonicated. The concentration of these solutions was 5.2 mM. The fatty-acids were ejected with cathodal current from a single barrel of a five-barreled micropipette (~20 Mohm impedence) into 100 ul of normal saline; and this saline was subsequently assayed for radioactivity using liquid scintillation counting. Data are shown for ejections using two different pipettes for each fatty acid. These data show that 1) oleate and arachidonate have similar kinetics of ejections; and 2) there is not a large variability between pipettes for ejection of a given fatty acid.


Iontophoresis: Repeatability Expts.
14C-cis-Fatty Acids
nmoles ejected / 15 min
4
3.5
3
2.5
2
1.5
1
0.5
0
-10
10
30
50
cathodal current (nA)
oleate #1
oleate #2
arach #1
arach #2

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This research is focussed on the role of molecular switches in brain which regulate synaptic reactivity. We have identified a 47,000 mol wt phosphoprotein and its kinase (C) which play a pivotal role in this regulation. We have enhanced communication among nerve cells studied electrophysiologically by activation of this kinase. In the proposed research we specifically manipulate this brain phosphoprotein with novel kinase activating agents. In a new initiative, both the kinase and the F1 substrate will be cloned with the goal of enhancing synaptic reactivity by regulating the expression of proteins that play a pivotal role in this process.

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