PHOSPHOPROTEIN REGULATION OF SYNAPTIC REACTIVITY: ENHANCEMENT AND CONTROL. (U) NORTHWESTERN UNIV EVANSTON IL CRESAP NEUROSCIENCE LAB A ROUTTENBERG MAR 85
PHOSPHOPROTEIN REGULATION OF
SYNAPTIC REACTIVITY:
ENHANCEMENT AND CONTROL OF A
MOLECULAR GATING MECHANISM

Progress Report for AFOSR 83-0335
Submitted to:
Directorate of Life Sciences
Air Force Office of Scientific Research

Submitted by:
Dr. Aryeh Routtenberg
Cresap Neuroscience Laboratory
Northwestern University
Evanston, Illinois 60201
**11. TITLE (Include Security Classification)**

Phosphoprotein Regulation of Synaptic Reactivity: Enhancement and Control of a Molecular Gating Mechanism

**12. PERSONAL AUTHOR(S)**

Dr. Arveh Routtenberg

**13a. TYPE OF REPORT**

Annual

**13b. TIME COVERED**

FROM 9/30/83 TO 9/29/84

**14. DATE OF REPORT (Yr., Mo., Day)**

March 1985

**15. PAGE COUNT**

35

**17. COBOL CODES**

<table>
<thead>
<tr>
<th>FIELD</th>
<th>GROUP</th>
<th>SUB. GR.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)**

Protein Fl, Kinase, Phosphorylation

**19. ABSTRACT (Continue on reverse if necessary and identify by block number)**

Protein Fl, a brain specific synaptically located protein, is phosphorylated in direct relation to the enhancement of synaptic communication. Studies demonstrated that the kinase involved in phosphorylation of protein Fl is protein kinase C. Results suggest that the mode of activation of the kinase C by synaptic enhancement may involve translocation of the enzyme from the cytosol to the cell membrane.
I. Summary

It was previously shown that protein F1, a brain specific synaptically localized protein, was phosphorylated in direct relation to the enhancement of synaptic communication. It was proposed that the increased phosphorylation resulted from activation of F1 kinase.

Evidence obtained in the first 12 months of the grant supported this proposal: (1) F1 kinase is protein kinase C; (2) protein kinase C was activated by synaptic enhancement; (3) the mode of activation by synaptic enhancement of the C kinase involved enzyme translocation.

Using two-dimensional gel electrophoresis protein F1 was shown to be an acidic (pI=4.5), 47 kDa protein with a characteristic "tilted lozenge" microheterogeneity. A phosphoprotein with identical characteristics has been identified in primate cerebral cortex. Neither of two calmodulin kinases (PBK or Type II) increased F1 phosphorylation. Nor was cyclic AMP effective. Thus, kinase C has been the only kinase identified to date as the F1 kinase.

In parallel experiments protein kinase C and protein F1 were isolated and purified to near homogeneity. When the purified substrate (F1) and the purified kinase were incubated in the presence of AT(32)P, phosphorylation of F1 was observed, but only in the presence of calcium and phosphatidylserine(PS).
Thus, as expected, protein F1 phosphorylation was absolutely dependent upon a calcium/phospholipid kinase, protein kinase C.

To determine whether protein kinase C was activated during synaptic enhancement, histone H1 was used as the substrate and hippocampal tissue homogenate from "enhanced" preparations was used as the crude enzyme preparation. Following enhancement, significant activation of PS stimulated kinase C activity was observed. Recent evidence indicates that this enhancement occurs as a result of translocation of the kinase from the cytosol to the membrane. This mechanism invites the following considerations: a. the metabolic energy requirement for effecting a change in synaptic reactivity may be minimal; b. the role of calcium in regulating synaptic plasticity may be to increase the hydrophobicity of the kinase and promote its translocation to the membrane; c. regional differences in kinase C activity may be accompanied by differences in membrane/cytosol kinase C ratios which may determine the capacity for synaptic reactivity among brain regions. Figure 1 summarizes the proposal that the phosphorylation of protein F1 by protein kinase C is a critical step in the regulation of synaptic reactivity.
II. Introduction

A direct link has been established between the phosphorylation state of an identified protein (Protein F1) and the state of synaptic strength measured electrophysiologically (see refs. 1, 2 and 8 in List of References (section V.).

To achieve this we have studied enhancement of synaptic reactivity in the hippocampus. Using high frequency stimulation of the perforant path, long-term enhancement (LTE) of the dentate gyrus synaptic response can persist for up to 4 hr in acute preparations, and several days or even weeks in chronic preparations. It was shown in both cases that the increase in Protein F1 phosphorylation was selective: in acute preparations studied for minutes or hours and in animals with chronic electrodes 3 days after LTP (ref. 4). That is, of the phosphoproteins studied in the 30-90 kDa range only protein F1 phosphorylation was significantly increased.

If the change in synaptic strength depended on the state of protein F1 phosphorylation, then one would predict that the extent of synaptic enhancement would be directly related to protein F1 phosphate content. In support of this prediction, it was found (ref. 8) that a direct relation existed between F1 phosphate content and change in spike amplitude.
II. Research Objectives

These findings have prompted the following questions which we have addressed during the first year of the research grant.

(1) Is the increase in Protein F1 phosphorylation related to an increase in kinase activity?

(2) Is the increase in kinase activity related to a subcellular redistribution of the kinase?

In order to answer these questions it was first necessary to determine the identity of the kinase that phosphorylates Protein F1 and establish that it alone is capable of phosphorylating F1.

In the first year of the grant we have begun a program of purification, isolation and characterization of F1 and its kinase.

Thus, the research objectives for the first 12 month period of the grant were:

a. Identification of protein F1 kinase

b. Isolation of protein F1 kinase

c. Identification and isolation of protein F1

d. Identify the regulators –second messengers – of protein F1 phosphorylation

e. Determine F1 kinase activity following synaptic enhancement

f. Describe protein F1 phosphorylation in the 150,000xg
particulate to synaptic enhancement

  g. Determine whether subcellular redistribution of the kinase occurs after enhancement.

  h. Establish the brain regional distribution of protein kinase C and protein F1.

IV. Status of the Research

The electrophysiological methods have been described in detail (see refs. 1,2 and 8 in List of Publications in Section V). Briefly, anesthetized and chronically prepared subjects had stimulating electrodes placed in the perforant path to evoke synaptic responses recorded with a micropipette placed in the dentate gyrus (see Figure 2). Eight trains of eight 0.4 msec cathodal pulses were delivered (1 train every 30 sec) to produce an enhancement of the synaptic response. The change from baseline was monitored for selected durations, at which time, the brain was rapidly frozen (< 5 sec to elimination of brain activity) in situ.

Procedures for preparation of the tissue, in vitro phosphorylation and densitometric analysis of phosphate content of electrophoretically separated phosphoproteins have been described in detail elsewhere (see refs. 2,4,5 and 8).

In the first year of the grant, the following experiments have been carried out.

  a. Identification of protein F1 kinase

-6-
We have recently demonstrated that the increase in Protein F1 phosphorylation in vitro following enhancement can be observed with extremely short reaction durations and micromolar concentration of ATP (in ref. 8 see Fig 1). Such reaction conditions almost wholly depend on the level of kinase activity, since less than 1% of Protein F1 substrate available would be phosphorylated. It was shown that the selective alteration in Protein F1 is also observed under these conditions. This means that the enhancement procedure is likely to activate Protein F1 kinase. In addition, the most clearcut direct relation between F1 phosphorylation and synaptic reactivity was observed with the 10 sec reaction.

We have recently shown that Protein F1 kinase is protein kinase C (see ref. 3). Protein F1 was stimulated both by calcium (5 uM) and by a phospholipid dependent mechanism. Partially purified exogenous kinase C stimulated the phosphorylation of protein F1. In addition, the tumour promoting phorbol ester, TPA, which stimulates protein kinase C activity, stimulated the phosphorylation of protein F1. We have recently shown (ref. 5) that protein F1 observed in two-dimensional gels is increased in its phosphorylation consequent to incubation with exogenous purified protein kinase C and by phosphatidylserine. In the latter case we have directly assessed the amount of radioactivity in protein F1 by
cutting out the protein spot from the 2-dimensional gel.

Evidence from studies with isolated enzyme and substrate, to be discussed in the next section, confirms that protein F1 is a brain substrate protein for protein kinase C.

b. Isolation of protein F1 kinase (protein kinase C)

Because of the evidence indicating that protein F1 kinase was, in fact, protein kinase C, the enzyme was isolated using a unique sequence of column chromatographic techniques developed by Dr. Murakami in this laboratory. As shown in Table 1, which provides the details of the separation and purification, the enzyme has now been purified nearly 1000-fold. We had not anticipated such rapid progress in this area. We are now in a position to study the effect of intracellular injection of this enzyme preparation on the biophysical parameters and synaptic reactivity of the hippocampal neuron.

c. Identification and isolation of protein F1

We have sought to identify the protein substrate for protein kinase C. To do so we developed both equilibrium and nonequilibrium pH gel electrophoresis in the first dimension followed by SDS-gel electrophoresis in the second dimension. This "protein fingerprinting" has proven a powerful tool for identification of the Protein F1 substrate. We have found that the F1 protein whose phosphorylation is increased by enhancing synaptic reactivity has a molecular weight of 47 kD and an
isoelectric point of 4.5, properties similar to the B-50 protein of Gispen and co-workers. This finding is important because Protein F1 is then a brain specific protein, enriched in the synaptic membrane fraction and concentrated in those brain regions likely to be involved in the regulation of synaptic reactivity. The development of monoclonal antibodies to protein F1 should provide direct immunocytochemical assessment of this hypothesis.

A program of protein F1 purification has been undertaken by Dr. Shew Chan in this laboratory (see Table 2). The crucial observation to date is that a highly purified preparation, probably greater than 500-fold, is phosphorylated by a highly purified protein kinase C in the presence of certain regulators, to be discussed in the next section (see Figures 3, 4, 5, and 6).

During the course of purification certain observations were made that may be important for understanding the function and identity of protein F1. First, during the extraction from the P100 column F1 is eluted in the void volume suggesting that in its native form it is composed of at least another monomer with a mol wt. greater than 100 kDa. This may be the 68 kDa band (see Figure 5) that co-purifies with protein F1 and is the only other band that is detected in the P-100 fraction. Second, in several of the fractions a potent inhibitor of F1 phosphorylation was found. In the next grant period we plan to
isolate and purify the inhibitor, which appears to be a protein, and then use this purified fraction in iontophoretic studies to be discussed in the Research Progress and Forecast Report.

d. **Identity of regulators —second messengers— of protein F1 phosphorylation: absolute dependence of Protein F1 phosphorylation on protein kinase C and phospholipid**

As shown in Figure 3 and 4, the phosphorylation of protein F1 only occurs in the presence of phosphatidylserine. Thus, there is an absolute dependence on PS, indicating that protein F1 is a substrate for the multifunctional protein kinase C enzyme. As shown in Figure 1, other regulators of protein kinase C include growth factors, peptides, neurotransmitters and tumour promoters. We have shown, in the case of insulin, ACTH, phorbol ester and opioids that these regulators influence protein F1 phosphorylation in the predicted direction: inhibitors of kinase C activity decrease and activators increase F1 phosphorylation.

e. **Activation of C kinase activity following synaptic enhancement**

By using the hippocampal tissue homogenate as the enzyme preparation we have observed that LTP significantly increases protein kinase C activity (see ref. 8). In this enzyme assay, phospholipid dependent kinase activity is measured with histone H1, a preferential substrate for Kinase C, as phosphate.
In the proposed research it was predicted that "F1 phosphorylation will be increased in osmotically-shocked membranes following long-term enhancement." In a recently completed study (ref. 9) this prediction was confirmed by a high positive correlation ($r=0.86$, $df=6$, $p<.05$) between the level of synaptic enhancement and the extent of F1 phosphorylation present in osmotically-shocked membranes pelleted at 150,000xg. These results suggest the hypothesis that enhancement translocates protein kinase C to the membrane. In addition, once translocated, the enzyme must be intercalated within the membrane to an extent sufficient to resist removal by high g-force. In the next section a system modeling translocation and a direct test of this hypothesis are described.

In the next section a system modeling translocation and a direct test of this hypothesis are described.
Fig. 3: Autoradiograph showing the dependency of F1 phosphorylation on kinase C (K-C) and phosphatidylyserine (PS) in ACE fractions. Two different ACE fractions were used here, each was treated with and without K-C or PS. Only when K-C and PS (100 ug/ml) were both present, phosphorylation of F1 were observed. Other experimental conditions are explained in text. Arrow indicates the position of F1. It is evident that other kinase C substrates are still present in the ACE fraction.
TABLE 2

12 rats (~12gm) in ice cold 0.32M sucrose + 2mM DTT (60ml) ↓
Homogenization. Then dilute with another 60ml 0.32M sucrose solution ↓
1,200xg for 10 min ↓
collect supernatant ↓
10,000xg for 20 min ↓
Discard supernatant. Pellet (P2) resuspended in 60ml of lysing solution (10mM EGTA + 10mM Tris + 2mM DTT, pH 7.2) ↓
Lyse for 1/2 hr at 4°C, slow stirring and then spin at 13,000xg ↓
Pellet (P2') resuspended in 10ml of 1mM Mg acetate. ↓
Adjust pH to 11.5 with 1N NaOH ↓
80,000xg for 20 min ↓
supernatant acidified to pH 5.5 by 1M Na-acetate-acetic acid at pH 5.0 ↓
50,000xg for 20 min ↓
supernatant (ACE) ↓
40-80% (NH₄)₂SO₄ precipitation ↓
Hydroxyloapaptate (HP) column (1x2m) ↓
30 to 75mM KPi elution ↓
Phenyl-sepharose CL-4B column (1x2m) ↓
Biogel P-100 gel filtration column (1.5 x 50cm) ↓
Purified F1

Flow chart for steps leading to isolation of protein F1.
Dr. Aryeh Rcuttenberg  
Annual Report  
AFOSR 83-0355

**TABLE 1**

Purification of Protein Kinase C

In two separate experiments, a single band between 68K and 94K was obtained. Apparent Mr is 84K which is similar to the value reported by Nishizuka. Autophosphorylation of this polypeptide has been detected.

The purification data is summarized below.

**Isolation A**

<table>
<thead>
<tr>
<th></th>
<th>protein, mg</th>
<th>spec. act. (µ/mg)</th>
<th>purification</th>
<th>yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1960</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>CX</td>
<td>526</td>
<td>0.654</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE</td>
<td>72.2</td>
<td>3.03</td>
<td>4.6</td>
<td>64</td>
</tr>
<tr>
<td>AcA34</td>
<td>4.25</td>
<td>24.0</td>
<td>36.7</td>
<td>30</td>
</tr>
<tr>
<td>Hydroxylapatite(HA)</td>
<td>0.456</td>
<td>198</td>
<td>303</td>
<td>26</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>0.011</td>
<td>624</td>
<td>954</td>
<td>2</td>
</tr>
</tbody>
</table>

**Isolation B**

<table>
<thead>
<tr>
<th></th>
<th>protein, mg</th>
<th>spec. act. (µ/mg)</th>
<th>purification</th>
<th>yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1920</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>CX</td>
<td>540</td>
<td>0.916</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE</td>
<td>122</td>
<td>2.12</td>
<td>2.3</td>
<td>53</td>
</tr>
<tr>
<td>AcA34</td>
<td>2.73</td>
<td>72.7</td>
<td>79</td>
<td>40</td>
</tr>
<tr>
<td>HA</td>
<td>0.928</td>
<td>201</td>
<td>220</td>
<td>38</td>
</tr>
<tr>
<td>Phenyl-S</td>
<td>0.044</td>
<td>609</td>
<td>664</td>
<td>6</td>
</tr>
</tbody>
</table>

As shown in the table, purification fold is 650-1000 with yield of 2-6%. Although there may be some room to improve the yield of the enzyme, the basic method of isolation is now established. We test the direct interaction of C-kinase with phospholipids, fatty acids, and divalent cation without the interference of impurities. Below is shown sharp PS-dependent peak even with 40-80 fold purification.

**PARTIAL PURIFICATION OF PROTEIN KINASE C**

![Partial Purification of Protein Kinase C](image-url)
Figure Captions

Fig. 2. Schematic of stimulating and recording electrode locations. Note that bottleneck of homo stimulation leads to broader invasion of dentate gyrus.
Regulators: Calcium, Diacylglycerol, Phorbol, Phosphatidylserine, mACH, Insulin, Opioids, ACTH

Novel, Phospholipid Dependent Enzyme: Protein Kinase C

Multi-Functional Actions: Protein, Phospho-Protein F1, Enhanced Synaptic Reactivity

Functional Consequence:

Fig. 1
Figure Captions

Fig. 1. Flow chart provides event sequence overview of regulators of protein kinase C leading to enhanced synaptic reactivity.
VII. Interactions (Coupling Activities)


Invited speaker, April, 1984, University of Illinois, Champaign-Urbana. "Protein kinase C regulation of synaptic growth and plasticity."


Invited speaker, Friedrich-Miesche Institute, Basle, Switzerland. September 3, 1984, "Protein kinase C and synaptic plasticity."

Invited speaker, FIDIA Research Laboratories, Abano Terme, Italy. September 6, 1984, "Protein kinase C regulation of synaptic plasticity."

VI. Professional Personnel

Research Associates
Shew Chan, Ph.D.
Kentaro Murakami, Ph.D.

Graduate Assistants
Ray Akers, M.A.
Robert Nelson, M.A.
David Lovinger, M.A.
Patricia Colley, B.Sc.
David Linden, B.A.


5. Nelson, R., and Routtenberg, A.
Characterization of protein F1 (47kD, 4.5 pI): a kinase C substrate directly related to neural plasticity.


In preparation:


V. List of Publications: Published, in press and in preparation

Published:


In press:

4. Lovinger, D., Barnes, C.A., McNaughton, B.L. and Routtenberg, A. A selective increase in hippocampal protein F1 phosphorylation directly related to three day growth of long term synaptic enhancement. Brain Research, 1985, in press.
protein kinase C activity have recently been observed in an analysis of monkey cortex and in a regional analysis of rat brain (ref. 10; see Figure 9). In both primate and rodent brain there is a non-uniform distribution of this phosphorylation system. One attractive hypothesis is that regions with increased activity of the kinase have an increased regulatory function in synaptic reactivity. This view is quite consistent with the finding that the highest level of protein F1 phosphorylation in primate temporal cortex is the anterior temporal tip regions, a cortical locus associated with storage of visual representations of the environment.
phosphorylation reaction using calcium buffered at 50 uM in all three reaction conditions. The prediction was that F1 phosphorylation in osmotically - shocked synaptosomes will be greatest in those preparations previously treated with calcium.

We have evidence that supports this prediction though we have found that 10 micromolar calcium is sufficient to hold the kinase to the membrane. Moreover, the addition of calcium during the initial osmotic shock appears to have a persistent effect, since a subsequent osmotic shock does not remove the kinase from the substrate.

If this system were to model the alterations in kinase C subcellular distribution observed during synaptic enhancement then we should observe translocation of the kinase following procedures related to the enhanced physiological response itself. In a recently completed study (Akers and Routtenberg, 1985; ref. 9) we have shown that protein kinase C activity is increased in the membrane while, at the same time, it is reduced in the cytoplasm (see Figures 7 and 8). This suggests that the translocation of the kinase has occurred. We plan to directly visualize this event by tracking kinase C movement using immunocytochemical methods with a monospecific antibody to protein kinase C.

h. **Brain regional distribution of protein kinase C**

Regional variations in protein F1 phosphorylation and
Fig. 4: Autoradiograph showing the phosphorylation of F1 by kinase C (K-C) in the 30 to 75mM KPi eluant (HP column). Again, the phosphorylation of F1 was observed only when PS and K-C were both present. Adjacent lanes using the 0-30mM KPi eluant did not contain F1. Arrow indicates the expected position of protein F1.
Fig. 5. An electrophoresis SDS gel showing protein profiles in various purification steps. Arrows indicate the two protein bands visualized in the purified P-100 fraction. HOMO = homogenate; $P_2^*$ = an osmotically shocked membrane fraction; AcE = pH extracted fraction; 40-80% ASP = 40-80% ammonium sulfate precipitated AcE fraction; HP = a 30-75mM KPi eluted fraction of hydroxylapatite column; P-100 = Bio-gel P-100 gel filtration purified fraction. Various purification steps are discussed in "Preliminary Studies" and "Methods: Section a".
Fig. 6. Autoradiographs of two-dimensional gels (2-D gel) depicting the molecular weight (MW), isoelectric point (pI) and phosphatidylserine (PS) dependency of the 47K protein (assumed to be protein F1) in the P-100 gel filtration purified fraction. (A) represents a 2-D autoradiograph of the P-100 purified fraction when PS was not added for phosphorylation reaction. In the absence of PS, no F1 phosphorylation by exogenous kinase C (K-C) was found. (B) represents the autoradiograph when 50 μg/ml PS was added. A prominent phosphorylated protein was found at Mr = 47K and pI = 4.5 position. This phospho-protein matched the phosphorylated protein F1 in a HP fraction (C) under the same phosphorylation conditions. The microheterogeneity of the two phosphorylated bands also appeared to be similar.
Figure 7. Protein kinase C activity is increased in membranes one hour after LTP stimulation.

Perforant path innervation into the dentate gyrus of male albino rats was determined. High frequency repetitive stimulation (LTP) (8 trains of 8 pulses at 100 Hz), or low frequency control stimulation (LFC) (64 pulses at 0.1 Hz) was then delivered. One minute or one hour later, the animals were sacrificed by total today immersion into liquid nitrogen. The innervated regions of dorsal hippocampi were dissected on a block of dry ice, and homogenized 50 mM Tris (pH 7.2) and 10 μg/ml leupeptin. Homogenates were spun at 100,000 xg for one hour. Resulting supernatants were run through a 0.4x1 cm DEAE-cellulose minicolumn. The column was washed, and protein kinase C activity eluted with 0.3M NaCl. 1000,000 xg pellets were resuspended in 50mM Tris (pH 7.2) 0.1% Triton X-100, 2mM EDTA, 2mM EGTA, and 10 μg/ml leupeptin, and left for one hour at 4°C. Following centrifugation at 100,000xg for one hour, the supernatants containing extracted protein kinase C were passed through DEAE-cellulose minicolumns, and protein kinase C was eluted as before.

Protein kinase C activity was determined in the following assay mix: 50mM Tris (pH 7.2), 5mM MgCl, 0.5mM EGTA, 0.5mM EDTA, 100 μg histone H1, 3-5 μg enzyme preparation, and 0.5mm [γ-32P]-ATP (sp. act. = 50 c.p.m./pmol), + 100 μg/ml phosphatidylserine. The reaction was run for 10 min. at 30°C, quenched with the addition of a saturated EDTA solution, and spotted onto phosphocellulose paper. The papers were washed, and counted by liquid scintillation spectrometry. Protein kinase C activity was taken
as the difference between activity seen in the presence or absence of phosphatidylserine. All enzyme assays were linear with respect to time and enzyme concentration. All values are mean ± s.e.m.; n=6 for all groups except naive controls, where n=7.
Membrane C Kinase Activity

(nmol Pi/min · mg Protein)

Unop Control 1 min. 60 mins.

CON LTP

p<0.05

Fig. 7
Figure 8. LTP stimulation shifts the subcellular distribution of protein kinase C activity.

Methods as described in Fig. 8 legend. All animals were sacrificed one hour after the delivery of LTP stimulation. All values mean ± s.e.m.; all n=6.
Translocation of Protein Kinase C Activity from Cytosol to Membrane

**Fig. 8**

- Protein Kinase C Activity (% of total kinase C activity)
- Con LTP Supernatant
- Con LTP Membrane
- N.S.
- Con LTP Total Activity

- Protein Kinase C Activity (nmol Pi/min/mg protein)
Figure Captions

Fig. 9. Kinase C activity in selected brain regions.
Regional Analysis of Protein Kinase C Activity

Protein Kinase C Activity (nmol P/h/mg of protein)

Fig. 9