Calcium-Promoted Translocation of Protein Kinase C to Synaptic Membranes: Relation to the Phosphorylation of an Endogenous Substrate (Protein F1) Involved in Synaptic Plasticity

Raymond F. Akers and Aryeh Routtenberg

Cresap Neuroscience Laboratory, Northwestern University, Evanston, Illinois 60201

The translocation of protein kinase C between membrane and cytosol has been implicated in several cellular processes (Kraft and Anderson, 1983; Wooten and Wrenn, 1984; Akers et al., 1985, 1986; Hirota et al., 1985; Wolf et al., 1986). We desired to identify potential trigger mechanisms underlying the translocation of protein kinase C activity to neural membranes following the synaptic plasticity observed after long-term potentiation (LTP; Akers et al., 1986). Takai et al. (1979) have suggested an important role for calcium in protein kinase C translocation; we have therefore studied the effects of Ca^{2+} on both the translocation of protein kinase C activity and the *in vitro* phosphorylation of its endogenous substrate, protein F1, in rat hippocampal synaptosomes. Since identical free Ca^{2+} levels were maintained in subsequent assays of synaptosomal membranes (SPM) and cytosol preparations, alterations in endogenous enzyme activity and in *in vitro* phosphorylation were due to the Ca^{2+} present during treatment of synaptosomes, and not to the Ca^{2+} present during assays of enzymatic activity. This afforded the opportunity to relate directly such enzyme translocation to endogenous substrate phosphorylation. The major findings were as follows:

1. Following treatment of synaptosomes with Ca^{2+} , protein kinase C activity in synaptic membrane and protein F1 *in vitro* phosphorylation were elevated in a dose-dependent manner.

2. The greatest increment in membrane protein kinase C activity and protein F1 *in vitro* phosphorylation occurred when Ca^{2+} was increased from 0.1 to 1.0 μM . Maximal levels of enzyme activity were seen following treatment with 10 μM Ca^{2+} , and minimum levels were observed following treatment with EGTA.

3. Protein F1 phosphorylation was positively correlated with the protein kinase C activity found in membranes ($r = 0.869$; $P < 0.001$).

4. Treatment with Ca^{2+} increased the amount of protein kinase C activity in membranes and decreased the amount of kinase C activity in the soluble fraction. The sum total of synaptic membrane and soluble protein kinase C activities

was similar in different preparations lysed with different Ca^{2+} levels, suggesting that a redistribution, or translocation, of protein kinase C had occurred.

These results indicate that Ca^{2+} regulates the amount of protein kinase C activity associated with synaptic membranes. This association is directly correlated with protein F1 *in vitro* phosphorylation. Since after LTP, protein kinase C activity is translocated to membranes (Akers et al., 1986) and membrane-bound protein F1 *in vitro* phosphorylation is elevated (Routtenberg et al., 1985), the translocation of protein kinase C to membranes, triggered by Ca^{2+} , may account in part for the observed changes in protein kinase C and protein F1 following LTP.

Ca^{2+} /phospholipid-sensitive protein kinase C activity is most enriched in brain (Kikkawa et al., 1982), suggesting that the enzyme plays an important role in neuronal processes (Nishizuka, 1984). We have proposed a specific neural function for protein kinase C in adult synaptic plasticity (Routtenberg, 1985; Routtenberg, 1986). This suggestion was based on evidence that membrane protein kinase C activity was directly related to the persistence of the synaptic enhancement following long-term potentiation (LTP) in rat hippocampus (Akers et al., 1986). Related studies showed that protein F1 (M_r 47 kDa; IEP, 4.5) is an endogenous protein kinase C substrate (Akers and Routtenberg, 1985; Nelson and Routtenberg, 1985; Chan et al., 1986). Protein F1 phosphorylation *in vitro* has been related to the persistence of LTP (Routtenberg et al., 1983, 1985; Lovinger et al., 1985, 1986).

The increase in membrane protein kinase C activity following LTP appears to result from a translocation of kinase activity from the cytosol to the membrane (Akers et al., 1986).¹ The increase in protein F1 *in vitro* phosphorylation following LTP could thus result from increased membrane protein kinase C activity. Mechanisms controlling this enzyme translocation, and the relation of enzyme translocation to endogenous substrate phosphorylation, however, remain unclear.

Protein kinase C can be activated by Ca^{2+} and phospholipid *in vitro*. It has been proposed that Ca^{2+} may increase the affinity of the enzyme for membranes *in vivo* (Takai et al., 1979), thus activating the enzyme. In essence, then, Ca^{2+} would regulate the amount of enzyme associated with membranes by redistributing enzyme between cytosol and membrane. Such a mechanism could operate to control protein kinase C activity and protein F1 phosphorylation during LTP in the hippocampus, where changes in Ca^{2+} flux have been observed following high-frequency

Received Dec. 17, 1986; revised May 8, 1987; accepted June 11, 1987.

Special thanks to Bob Nelson for skillful assistance in the preparation of 2-dimensional gels, and for encouragement and advice throughout this study. Gratitude is expressed to Drs. Shew Chan and Kentaro Murakami for comments on the manuscript. Peter Naumann provided valuable assistance with computer related issues. This work was supported by Grants MH25281 and AFOSR 83-0335.

Correspondence should be addressed to Dr. Aryeh Routtenberg, Northwestern University, Cresap Neuroscience Laboratory, Rm. 311, 2021 Sheridan Road, Evanston, IL 60201.

Copyright © 1987 Society for Neuroscience 0270-6474/87/123976-08\$02.00/0

¹ We use the phrase "translocation of protein kinase activity" to include the possibility that translocation includes a shift from loosely membrane-attached to tightly membrane-bound.

quency repetitive stimulation (Morris et al., 1983). To examine enzyme-membrane interactions and their relation to the phosphorylation of endogenous substrates, we developed an *in vitro* model system in which Ca^{2+} levels in hippocampal synaptosomes were controlled by Ca^{2+} /EGTA buffers and osmotic lysis. Enzyme translocation during Ca^{2+} treatment of synaptosomes was determined by subsequent assay of protein kinase C activity and protein F1 *in vitro* phosphorylation in synaptic membranes (SPM).

Materials and Methods

Subcellular fractionation. Hippocampi from male albino rats (Charles River) were homogenized in 0.32 M sucrose, and crude synaptosomes (P2) were prepared as described previously (Akers and Routtenberg, 1985). P2 material was resuspended in 0.32 M sucrose, and then lysed for 30 min at 4°C. Lysis media contained 10 mM Tris (pH 7.2), 10 $\mu\text{g}/\text{ml}$ leupeptin, and a free Ca^{2+} buffered with 1 mM EGTA. Free Ca^{2+} ranged from 10^{-8} to 10^{-4} M. Free Ca^{2+} was calculated by the equation,

$$K = \text{Ca EGTA} / \text{Ca}^{2+} \text{ EGTA}.$$

The apparent affinity constant for EGTA at pH 7.2 and 4°C was calculated with the program described by Fabiato and Fabiato (1979). Absolute stability constants for Ca/EGTA binding used were

$$\log K_1 = 10.716; \quad \log K_2 = 5.33.$$

Lysis was also performed in a minimal-calcium lysing medium containing 10 mM Tris (pH 7.2) and 5 mM EGTA.

Following lysis, the suspension was centrifuged at $20,000 \times g$ for 30 min. Supernatants containing synaptosomal cytosol were either collected, concentrated, and dialyzed with a Millipore CX concentrating unit (for *in vitro* phosphorylation studies) or passed through a DEAE-cellulose column (for assay of protein kinase C activity). The pellets, containing synaptosomal membranes (SPM), were washed in 100 mM Tris (pH 7.2), centrifuged at $20,000 \times g$ for 30 min, and resuspended in 50 mM Tris (pH 7.2) and 1 mM EDTA. Membranes were stored at -70°C ; all assays were performed within 24 hr of sample preparation.

Assay of membrane and cytosol protein kinase C activity. Protein kinase activity was partially purified prior to assay. For the determination of membrane protein kinase C activity, SPM preparations were solubilized in 0.1% Triton X-100, 10 mM Tris (pH 7.2), 2 mM EGTA, and 2 mM EDTA for 1 hr at 4°C. Solubilized membranes were then centrifuged at $100,000 \times g$ for 1 hr to eliminate debris. Resulting supernatants, containing solubilized protein kinase C activity, were then passed through DEAE-cellulose columns (0.5×0.5 cm). Following washing of the columns with 50 mM Tris (pH 7.2), 2 mM EGTA, and 2 mM EDTA, membrane protein kinase C activity was eluted with a single step of 0.15 M NaCl. Of the total recoverable phospholipid-dependent kinase activity, >95% was eluted in this fraction. These procedures minimized the presence of interfering substances during enzyme assay (Kraft and Anderson, 1983).

To determine cytosolic protein kinase C activity in DEAE eluates, synaptosomal cytosol was obtained by first collecting the supernatants resulting from lysed synaptosomes (described above). These supernatants were then passed through DEAE-cellulose columns, and protein kinase C was then partially purified as described herein.

Protein kinase C activity in both membrane and cytosol was measured as the difference in kinase activity observed in the presence and absence of phosphatidylserine (PS). Protein kinase C fractions were incubated for 10 min at 30°C in 50 mM Tris (pH 7.2), 5 mM MgCl_2 , 0.5 mM EGTA, 0.5 mM EDTA, 1.5 mM CaCl_2 , 100 μg histone H1, 0.5 mM gamma- ^{32}P -ATP (sp act, 100 cpm/pmol), $\pm$ 100 $\mu\text{g}/\text{ml}$ PS. The reaction was stopped with a saturated solution of EDTA and immediately spotted onto phosphocellulose paper. The papers were dried and counted in a Beckman LS-150 liquid-scintillation counter. All enzyme assays were linear with respect to time and protein concentration. These assay conditions, identical to those used by Akers et al. (1986), give a free calcium concentration of 500 μM . Protein concentration was determined by a modification of the Lowry method (Lowry et al., 1951).

In vitro phosphorylation assay. Protein F1 *in vitro* phosphorylation was determined essentially as described previously (Akers and Routtenberg, 1984). Briefly, SPM were reacted for 10 sec at 30°C in a medium containing 50 mM Tris (pH 7.2), 1 mM free Mg^{2+} , 0.1 μM free Ca^{2+} (buffered with 1 mM EDTA), and 50 μM gamma- ^{32}P -ATP. The reaction was quenched with an SDS-stop solution. Reacted protein sample was

separated by SDS-PAGE. Dried 1-dimensional gels were exposed to Kodak AR x-ray film. Resultant autoradiograms were microdensitometrically scanned and analyzed with a mouse-driven IBM PC XT equipped with an IBM Data Acquisition Board. ^{32}P -phosphate incorporation into phosphoprotein bands was machine-computed as the area under the densitometric curve minus background.

Two-dimensional PAGE by isoelectric focusing and SDS molecular mass separation was performed according to Nelson and Routtenberg (1985).

Results

Effect of Ca^{2+} on protein kinase C activity and protein F1 phosphorylation in synaptosomes

Increasing levels of Ca^{2+} present during lysis of synaptosomes increased membrane protein kinase C activity (as assayed into histone H1) and increased endogenous protein F1 *in vitro* phosphorylation in synaptic membranes (Fig. 1A). Maximal SPM protein kinase C activity and protein F1 *in vitro* phosphorylation were observed after lysis in 10 μM free Ca^{2+} while lysis in EGTA produced the SPM lowest in kinase C activity and F1 phosphorylation (see Fig. 2A). In fact, SPM protein kinase C activity was increased 2–3-fold, and protein F1 phosphorylation 5–6-fold, by lysis in 10 μM Ca^{2+} , as compared to lysis in EGTA. Lysis in 100 μM free Ca^{2+} decreased both kinase C activity and protein F1 phosphorylation in SPM relative to lysis in 10 μM free Ca^{2+} , perhaps because of activation of a Ca^{2+} -sensitive protease (Kishimoto et al., 1983).

Protein kinase C activity was directly related to protein F1 phosphorylation in synaptic membranes (Fig. 1B; $r = 0.869$, $n = 16$; $p < 0.001$). Further significant correlations were observed between free calcium concentration at lysis and protein kinase C activity ($r = 0.809$, $n = 16$; $p < 0.001$) and protein F1 *in vitro* phosphorylation ($r = 0.656$, $n = 16$; $p < 0.01$).

Two-dimensional gel-electrophoretic profiles (Fig. 2B) of SPM *in vitro* phosphorylated products shown in 1 dimension in Figure 2A confirmed that the 47 kDa phosphoprotein altered by lysis in Ca^{2+} was indeed protein F1. A 47 kDa, 4.5 IEP spot corresponding to protein F1 (Nelson and Routtenberg, 1985) was heavily phosphorylated following lysis in 10 μM free Ca^{2+} , as compared to lysis in EGTA.

Subcellular redistribution of protein kinase C activity and protein F1 phosphorylation

The subcellular distribution of protein kinase C activity in SPM and cytosol was determined following lysis in either EGTA or 10 μM Ca^{2+} . SPM protein kinase C activity, as expected, was increased by lysis in 10 μM free Ca^{2+} relative to EGTA-lysed SPM (Fig. 3). Approximately 38.5% of protein kinase C activity in synaptosomes was associated with membranes lysed in EGTA, while 86.8% of kinase C activity was associated with membranes lysed in 10 μM Ca^{2+} .

A corresponding decrease was observed in cytosolic kinase C activity pretreated with 10 μM free Ca^{2+} relative to EGTA-pretreated cytosol (see Fig. 3). Moreover, the sum total of SPM and cytosol protein kinase C activities was similar in EGTA- and 10 μM Ca^{2+} -lysed groups. Differences between the EGTA- and 10 μM Ca^{2+} -lysed fractions, then, most likely resulted from a subcellular redistribution of kinase activity, rather than from changes in kinase activity due to proteolysis of enzyme or proenzyme.

Subcellular analysis of protein F1 *in vitro* phosphorylation revealed a distinct enrichment in SPM, while protein F1 phosphorylation was not observed in cytosol under any conditions studied (data not shown). Moreover, analysis with 2-dimen-

Figure 1. Effect of free Ca^{2+} during lysis of synaptosomes on protein kinase C activity and protein F1 *in vitro* phosphorylation in SPM. P2 were lysed as described in Materials and Methods to yield 8 different SPM preparations. Free Ca^{2+} used during lysis ranged from pCa 8 to pCa 4; the EGTA condition contained 5 mM EGTA with no added Ca^{2+} . Each free Ca^{2+} used during lysis thus yielded a distinct SPM preparation. **A**, Protein kinase C activity was determined in SPM as described in Materials and Methods and measured as the difference in protein kinase activity seen in the presence and absence of 100 $\mu\text{g}/\text{ml}$ PS. Protein kinase C activity (nmol P_i/min mg protein) is plotted here as a function of Ca^{2+} present during lysis. Protein F1 *in vitro* phosphorylation was performed as described in Materials and Methods. Briefly, reacted SPM protein was separated by SDS-PAGE. The gels were dried, exposed to x-ray film, and resultant autoradiograms were scanned with a microdensitometer. ^{32}P -phosphate incorporation into protein F1 was taken as the curve densitometric area minus background. Endogenous protein F1 *in vitro* phosphorylation (densitometric area, cm^2) in SPM is plotted vs free Ca^{2+} present during lysis. Both protein kinase C activity and protein F1 *in vitro* phosphorylation were expressed as the percentage of the enzyme activity measured following 10 μM Ca^{2+} lysis. SPM protein kinase C activity at 10 μM Ca^{2+} was 15.83 nmol P_i/min mg protein; SPM protein F1 *in vitro* phosphorylation at 10 μM Ca^{2+} was 63.18 cm^2 (densitometric area under the curve). These data are representative of 4 experiments. **B**, SPM were prepared from P2 lysed in 8 different free Ca^{2+} as in **A**. Protein kinase C activity and protein F1 *in vitro* phosphorylation were then determined as described in Materials and Methods. Protein kinase C activity (nmol P_i/min mg protein) and ^{32}P -phosphate incorporation into protein F1 (densitometric area, cm^2) from the same preparations were compared and are graphed here. Data from 2 separate experiments were combined. $r = 0.869$, $n = 16$, $t = 6.47$; $p < 0.001$.

Figure 2. Analysis of SPM *in vitro* phosphorylation by gel electrophoresis. P2 were lysed in media containing either 5 mM EGTA or 10 μM free Ca^{2+} , and SPM were prepared as described previously. SPM material was then phosphorylated *in vitro*, as described in Materials and Methods. **A**, Separation of SPM protein by single-dimension SDS-PAGE. **B**, Separation of SPM protein by 2-dimensional gel electrophoresis. A gradient of pH 3–10 was maintained in the first dimension, and proteins were separated by SDS-PAGE in the second dimension, as in Materials and Methods. These autoradiograms were exposed and developed for identical amounts of time.

sional gel-electrophoretic techniques revealed an absence of protein F1 staining in cytosol, and a clearly observable protein F1 stain in SPM (Fig. 4). This is especially dramatic since 24% of the recovered cytosolic protein was loaded onto the 2-dimensional gel (25 μg protein loaded; 104 μg protein recovered), whereas just 3% of the recovered SPM protein (25 μg protein loaded; 911 μg protein recovered) was analyzed by 2-dimensional gel electrophoresis.

Analysis of 2-dimensional gels further revealed qualitatively similar protein F1 stains from SPM lysed with EGTA or 10 μM free Ca^{2+} (Fig. 4). Thus, the difference in protein F1 phosphorylation between EGTA- and Ca^{2+} -treated groups could not be explained by a significant alteration in the amount of protein F1 substrate in these preparations.

Effects of phosphatidylserine on protein F1 phosphorylation

Protein F1 *in vitro* phosphorylation was marginally detectable in SPM following lysis in EGTA (see Fig. 2A). This could have occurred because EGTA pretreatment removed protein kinase

B

Figure 2. Continued.

C from membrane, or, alternatively, because EGTA pretreatment prevented the activation of protein kinase C by its cofactors. To distinguish between these 2 possibilities, we incubated SPM lysed in EGTA with the protein kinase C cofactor, phosphatidylserine. PS had no effect on protein F1 phosphorylation in SPM prepared with EGTA (Fig. 5) under conditions optimal for protein F1 *in vitro* phosphorylation. Interestingly, phosphoproteins of 82 and 78 kDa were stimulated by PS under these conditions, indicating the presence of a phospholipid-sensitive kinase in this preparation. PS did, however, stimulate SPM protein F1 phosphorylation under identical conditions in SPM prepared with 10 μM Ca^{2+} .

Effects of further Ca^{2+} treatment on membrane protein kinase C activity in SPM

To assess the strength of the Ca^{2+} -promoted attachment of protein kinase C with membranes, we next attempted to dissociate protein kinase C from SPM. After the elevated Ca^{2+} levels present during lysis promoted a strong association between kinase and membranes, it was difficult to dissolve that association with a second treatment of membranes in minimal Ca^{2+} . Membrane kinase C activity in SPM initially lysed in 10 μM Ca^{2+} , then subsequently treated with 0.1 or 0.01 μM Ca^{2+} , was not decreased to control levels, but remained elevated (Table 1). In fact, the second treatment with 0.1 μM Ca^{2+} had

Figure 3. Subcellular distribution of protein kinase C activity following lysis. SPM and synaptosomal cytosol were prepared as in Materials and Methods from P2 lysed in either 5 mM EGTA or 10 μM free Ca^{2+} . Protein kinase C activity (nmol P_i /min/mg protein) was determined as described in Materials and Methods. TOTAL refers to the sum of SPM and synaptosomal cytosol activities. These data are representative of 5 experiments.

MEMBRANE

SOLUBLE

Figure 4. Subcellular distribution of protein F1 as analyzed by 2-dimensional gel electrophoresis. SPM and synaptosomal cytosol were prepared as described in Materials and Methods, with lysis of P2 in either 5 mM EGTA or 10 μ M free Ca^{2+} . Synaptosomal cytosol protein was concentrated by lyophilization and then dialyzed with a Millipore CX concentrating unit prior to electrophoresis. Twenty-five micrograms of protein were loaded onto each isoelectrofocusing tube. Two-dimensional electrophoresis was carried out as described in Figure 3 and Materials and Methods. Proteins were separated by a pH gradient in the first dimension, and then by SDS-PAGE. Protein was stained by the silver method of Merrill et al. (1981). Arrows point to the 4.5 IEP, 47 kDa spot corresponding to protein F1.

virtually no effect on membrane kinase C activity as compared to the effect on the group that was initially lysed in 10 μ M Ca^{2+} , and subsequently treated again with 10 μ M Ca^{2+} .

Discussion

This study assessed protein kinase C activity and the phosphorylation of one of its substrates, protein F1, in synaptosomes ex-

posed to fluctuations in free Ca^{2+} levels. We reasoned that if Ca^{2+} regulated the amount of protein kinase C activity associated with membranes, changes in intracellular free Ca^{2+} would shift protein kinase C activity between membranes and cytosol without altering the total amount of protein kinase C activity. Synaptosomes were thus treated in a system designed to allow maximal Ca^{2+} buffering of cytosol and membrane components,

Figure 5. Effects of phosphatidylserine (PS) on SPM protein phosphorylation *in vitro*. SPM was prepared by lysis of P2 in media containing 5 mM EGTA or 10 μ M free Ca^{2+} , as described in Materials and Methods. *In vitro* phosphorylation was then performed by incubating SPM (1 mg protein/ml) for 1 min at 30°C in 50 mM Tris (pH 7.2), 0.025% Triton X-100, 1 mM free Mg^{2+} , and 0.1 μ M free Ca^{2+} (buffered with 1 mM EDTA), $\pm$ 100 μ g/ml PS. Gamma- 32 P-ATP was then added for 10 sec, and the reaction quenched as before. SDS-PAGE was performed as previously described. The resulting autoradiograms are shown here. Arrows point to phosphoprotein bands stimulated by PS.

while at the same time providing a means to separate the 2 phases. Since identical free Ca^{2+} levels were maintained in subsequent assays of SPM and cytosol preparations, alterations in endogenous enzyme activity and *in vitro* phosphorylation were due to the Ca^{2+} present during treatment of synaptosomes, and not the Ca^{2+} present during assays of enzymatic activity. Thus, these experiments were designed to study the effects of Ca^{2+} on enzyme translocation only, and not on enzyme activation (Wolf et al., 1986).

We report here that Ca^{2+} regulates the association of protein kinase C to synaptic membranes, and in so doing controls the endogenous level of protein F1 *in vitro* phosphorylation. The presence of protein kinase C activity in membranes was directly related to the endogenous *in vitro* phosphorylation of its membrane-bound substrate, protein F1. The changes in protein F1 phosphorylation did not appear to be due to any fluctuations in the endogenous levels of protein kinase cofactors, or to alterations in the levels of the protein F1 substrate, as detected by silver-staining of protein. The Ca^{2+} -induced translocation of protein kinase C activity to membranes, then, determined protein F1 *in vitro* phosphorylation.

Table 1. Effects of Ca^{2+} treatment on protein kinase C activity in SPM

pCa			Protein kinase C activity (% of maximum activity)
Initial lysis	Second treatment		
8	8		36.9
7	7		54.3
5	8		77.6
5	7		96.8
5	5		100.0

Synaptosomes were lysed in media containing free Ca^{2+} ranging from pCa 8 to pCa 5. Following lysis, SPM material was obtained by centrifugation and washed, as described in Materials and Methods. Resulting SPM pellets were then resuspended in lysis media and incubated at 4°C for 30 min (second treatment). The suspensions were then centrifuged at $20,000 \times g$ for 30 min and the pellets examined for protein kinase C activity, as in Materials and Methods. Protein kinase C activity is given as a percentage of the activity measured in DEAE eluates of membranes first lysed with pCa 5 and treated again with pCa 5.

While a close relationship exists between membrane protein kinase C activity and protein F1 *in vitro* phosphorylation, the mechanisms regulating each process are likely to be similar but not identical. For example, protein F1 *in vitro* phosphorylation was marginally detectable in SPM treated with EGTA, yet 39% of the total protein kinase C activity in synaptosomes was present in these membranes. The addition of PS to these EGTA-treated membranes did not stimulate protein F1 *in vitro* phosphorylation, but was able to stimulate the phosphorylation of other phosphoprotein bands. Thus, the phospholipid-sensitive protein kinase present in these membranes was able to phosphorylate several endogenous substrates, but not protein F1. This observation could be explained by (1) a lower affinity of the phospholipid-sensitive kinase present in these membranes for protein F1 than for other substrates, or (2) the specific removal or inactivation with the EGTA treatment of a subtype of phospholipid-sensitive protein kinase selective for protein F1. This latter possibility is consistent with polyacrylamide gel and cDNA evidence that several distinct phospholipid-dependent protein kinases with different molecular properties may exist in brain (Kikkawa et al., 1986; Nishizuka, 1986; Parker et al., 1986). In addition, one or more of these subtypes may be activated by a mechanism distinct from the Ca^{2+} -phospholipid system, i.e., by cis fatty acids (Murakami et al., 1986).

The attachment of enzyme to membrane, promoted by Ca^{2+} , may be partly attributed to changes in the hydrophobic nature of protein kinase C. Ca^{2+} increases the affinity of protein kinase C to hydrophobic phenylsepharose columns (Walsh et al., 1984; K. Murakami, unpublished observations); Ca^{2+} may thus enhance the hydrophobicity of protein kinase C, allowing association of kinase with synaptic membranes.

The evidence to date suggests that this translocation mechanism may be involved in neuronal responses requiring a more prolonged enzyme activation. For example, the translocation of protein kinase C to membranes persists for at least 1 hr after the induction of LTP in hippocampus; moreover, membrane kinase C activity is directly related to the persistence of synaptic enhancement (Akers et al., 1986). The involvement of this kinase C translocation in the 3-d persistence of LTP and protein F1 phosphorylation (Lovinger et al., 1985) has yet to be studied.

The present study indicates that the protein kinase C activity associated with synaptic membranes in the presence of $10\ \mu\text{M}$ Ca^{2+} is not readily dissociable in minimal Ca^{2+} . Elevated Ca^{2+} levels may therefore promote a strong attachment of enzyme to synaptic membranes. This contrasts with effects seen with inside-out ghosts derived from non-neural cells, where a strong attachment is only seen when synergistically promoted by Ca^{2+} and phorbol esters (Wolf et al., 1985).

The translocation of protein kinase C to membranes, regulated by Ca^{2+} , may play a pivotal role in neuronal activation. For example, protein kinase C activity has been associated with the regulation of ionic currents in *Aplysia* (DeRiemer et al., 1985) and *Hermisenda* (Alkon et al., 1986), as well as in rat hippocampal pyramidal cells (Baraban et al., 1985). Protein kinase C activity has also been implicated in processes of neural plasticity in both vertebrate (Akers et al., 1986) and invertebrate (Farley and Auerbach, 1986) systems. Since large changes in Ca^{2+} flux are observed following high-frequency repetitive stimulation in hippocampus (Morris et al., 1983), it may be that Ca^{2+} -induced enzyme translocation increases membrane protein kinase C activity (Akers et al., 1986) and leads to elevation of protein F1 *in vitro* phosphorylation (Lovinger, et al., 1985; Routtenberg et al., 1985) following the neural plasticity of LTP. In view of the synaptic localization of protein kinase C (Girard et al., 1985) and protein F1 (as B-50 protein; Gispén et al., 1985), this translocation process may occur at the synaptic terminal.

References

- Akers, R. F., and A. Routtenberg (1984) Brain protein phosphorylation: Selective substrate action of insulin. *Life Sci.* 35: 809–813.
- Akers, R. F., and A. Routtenberg (1985) Protein kinase C phosphorylates a 47 Mr protein (F1) directly related to synaptic plasticity. *Brain Res.* 334: 147–151.
- Akers, R. F., P. Colley, D. Linden, D. M. Lovinger, K. Murakami, and A. Routtenberg (1985) Protein kinase C (PKC) activity is translocated to the membrane following hippocampal synaptic plasticity. *Fed. Proc.* 44: 1421.
- Akers, R. F., D. M. Lovinger, P. A. Colley, D. J. Linden, and A. Routtenberg (1986) Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* 231: 587–589.
- Alkon, D. L., M. Kubota, J. T. Neary, S. Naito, D. Coulter, and H. Rasmussen (1986) C-Kinase activation prolongs Ca^{++} -dependent inactivation of K^{+} currents. *Biochem. Biophys. Res. Commun.* 134: 1245–1255.
- Baraban, J. M., S. H. Snyder, and B. E. Alger (1985) Protein kinase C regulates ionic conductance in hippocampal pyramidal neurons: Electrophysiological effects of phorbol esters. *Proc. Natl. Acad. Sci. USA* 82: 2538–2542.
- Chan, S. Y., K. Murakami, and A. Routtenberg (1986) Phosphoprotein F1: Purification and characterization of a brain kinase C substrate related to plasticity. *J. Neurosci.* 6: 3618–3627.
- DeRiemer, S. A., J. A. Strong, K. A. Albert, P. Greengard, and L. K. Kaczmarek (1985) Enhancement of calcium current in *Aplysia* neurons by phorbol ester and protein kinase C. *Nature* 313: 313–316.
- Fabiato, A., and F. Fabiato (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experimentation in skinned muscle cells. *J. Physiol. (Paris)* 75: 463–505.
- Farley, J., and S. Auerbach (1986) Protein kinase C activation induces conductance changes in *Hermisenda* photoreceptors like those seen in associative learning. *Nature* 319: 220–223.
- Girard, P. R., G. J. Mazzei, J. G. Wood, and J. F. Kuo (1985) Polyclonal antibodies to phospholipid/ Ca^{++} -dependent protein kinase and immunocytochemical localization of the enzyme in rat brain. *Proc. Natl. Acad. Sci. USA* 82: 3030–3034.
- Gispén, W. H., J. L. Leunissen, A. B. Oestreicher, A. J. Verkleij, and H. Zwiers (1985) Presynaptic localization of B-50 phosphoprotein: The (ACTH)-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. *Brain Res.* 328: 381–385.
- Hirota, K., T. Hirota, G. Aguilera, and K. J. Catt (1985) Hormone-induced redistribution of calcium-activated phospholipid-dependent protein kinase in pituitary gonadotrophs. *J. Biol. Chem.* 260: 3243–3246.
- Kikkawa, U., Y. Takai, R. Minakuchi, S. Inohara, and Y. Nishizuka (1982) Calcium-activated, phospholipid-dependent protein kinase from rat brain. *J. Biol. Chem.* 257: 13341–13348.
- Kikkawa, U., M. Go, J. Koumoto, and Y. Nishizuka (1986) Rapid purification of protein kinase C by high performance liquid chromatography. *Biochem. Biophys. Res. Commun.* 135: 636–644.
- Kishimoto, A., N. Kajikawa, M. Shiota, and Y. Nishizuka (1983) Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. *J. Biol. Chem.* 258: 1156–1164.
- Kraft, A. S., and W. B. Anderson (1983) Phorbol esters increase the amount of Ca^{++} , phospholipid dependent protein kinase associated with plasma membrane. *Nature* 301: 621–625.
- Lovinger, D. M., R. F. Akers, R. B. Nelson, C. A. Barnes, B. L. McNaughton, and A. Routtenberg (1985) A selective increase in hippocampal protein F1 phosphorylation directly related to three day growth of long term synaptic enhancement. *Brain Res.* 343: 137–143.
- Lovinger, D., R. Akers, P. Colley, D. Linden, and A. Routtenberg (1986) Direct relation of synaptic plasticity to phosphorylation of membrane protein F1: Potential association with protein kinase C translocation. *Brain Res.* 399: 205–211.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
- Merrill, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211: 1437–1438.
- Morris, M. E., K. Krnjevic, and N. Ropert (1983) Changes in free Ca^{++} recorded inside hippocampal neurons in response to fimbrial stimulation. *Soc. Neurosci. Abstr.* 9: 395.
- Murakami, K., S. Y. Chan, and A. Routtenberg (1986) Protein kinase C activation by *cis*-fatty acid in the absence of Ca^{++} and phospholipids. *J. Biol. Chem.* 263: 15424–15429.
- Nelson, R. B., and A. Routtenberg (1985) Characterization of protein F1 (47 kDa, 4.5 pl): A kinase C substrate directly related to neural plasticity. *Exp. Neurol.* 89: 213–224.
- Nishizuka, Y. (1984) Turnover of inositol phospholipids and signal transduction. *Science* 225: 1365–1370.
- Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* 233: 305–312.
- Parker, P. J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield, and A. Ullrich (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* 233: 853–859.
- Routtenberg, A. (1985) Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. *Behav. Neural Biol.* 44: 186–200.
- Routtenberg, A. (1986) Synaptic plasticity and protein kinase C. In *Phosphoproteins in the Nervous System. Progress in Brain Research*, vol. 69, W. H. Gispén and A. Routtenberg, eds., pp. 211–234, Elsevier/North Holland, Amsterdam.
- Routtenberg, A., D. Lovinger, S. Cain, R. Akers, and O. Steward (1983) Effects of long-term potentiation of perforant path synapses in the intact hippocampus on *in vitro* phosphorylation of a 47 kD protein (F1). *Fed. Proc.* 42: 755.
- Routtenberg, A., D. Lovinger, and O. Steward (1985) Selective increase in the phosphorylation of a 47 kD protein (F1) directly related to long-term potentiation. *Behav. Neural Biol.* 43: 3–11.
- Takai, Y., A. Kishimoto, Y. Iwasa, Y. Kawahara, T. Mori, and Y. Nishizuka (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.* 254: 3692–3695.
- Walsh, M. P., K. A. Valentine, P. K. Ngai, C. A. Carruthers, and M. D. Hollenberg (1984) Ca^{++} -dependent hydrophobic interaction chromatography. *Biochem. J.* 224: 117–127.
- Wolf, M., P. Cuatrecasas, and N. Sahyoun (1985) Interaction of protein kinase C with membranes is regulated by Ca^{++} , phorbol esters, and ATP. *J. Biol. Chem.* 260: 15718–15722.

Wolf, M., H. LeVine, W. S. May, P. Cuatrecasas, and N. Sahyoun (1986) A model for intracellular translocation of protein kinase C involving synergism between Ca^{++} and phorbol esters. *Nature* 317: 546–549.

Wooten, M. W., and R. W. Wrenn (1984) Phorbol ester induces intracellular translocation of phospholipid/ Ca^{2+} -dependent protein kinase and stimulates amylase secretion in isolated pancreatic acini. *FEBS Lett.* 171: 183–186.