The regulation of synaptic reactivity by protein kinase C and its substrate proteins has been studied using both behavioral and long-term potentiation (LTP) paradigms. We have studied the effects of protein kinase C activators and inhibitors on the durability of synaptic reactivity. The main conclusion to be drawn is that protein kinase C plays a pivotal and indeed necessary role for enhanced durability. In combination with a neural signal, PKC demonstrates a profound synergism enhancing synaptic efficacy.
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Final Technical Report

1 October 1989 - 28 February 1993

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

Final Technical Report for AFOSR 90-0240

Submitted to:
Directorate of Life Sciences
Air Force Office of Scientific Research

Submitted by:
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Final Technical/Scientific Report

0. Project Period  The project period includes October 1, 1989 to February 29, 1993. The present report is being filed in July 1993.

1. Summary

We have recently proposed a testable model of the mechanics of synaptic plasticity (Colley and Routtenberg, 1993). This model summarizes the results of experiments carried out during the tenure of this grant and will be discussed here in relation to this recently proposed model.

The regulation of synaptic reactivity by protein kinase C and its substrate proteins has been studied using both behavioral and long-term potentiation (LTP) paradigms. We have studied the effects of protein kinase C activators and inhibitors on the durability of synaptic reactivity. The main conclusion to be drawn is that protein kinase C plays a pivotal and indeed necessary role for enhanced durability. In combination with a neural signal PKC demonstrates a profound synergism enhancing synaptic efficacy.

Three separate inhibitors of protein kinase C activity, polymyxin B, mellitin and H-7, each with a different mechanism of action block LTP persistence. Application was made by micro-pressure ejection into the molecular layer of the dentate gyrus before or after LTP. The major result of this study was that inhibitors had no effect on the initiation of LTP but completely eliminated the enhanced response 10-15 min after its initiation. This provides strong support for our view that PKC plays a critical role in the persistence but not the initiation of LTP. As indicated at the bottom of Figure 1 from Colley & Routtenberg there is a "PKC independent phase" which is not blocked by inhibitors.

Both PDBu, a diacylglycerol (DAG) analogue, and the cis unsaturated fatty acid (CUFA) olate facilitate LTP and do so, according to the model in Figure 1, in a synergistic fashion on the beta PKC in the presynaptic terminal. A crucial question in the analysis of the role of PKC in synaptic reactivity is the site of action of each compound. Indirect evidence suggested a synaptic site since PKC is found in high concentration there. To assess this view directly we compared application dosages required to facilitate synaptic reactivity duration in the dentate hilus, a nearby site, and the molecular layer of the dentate, 100 micra from the granule cells, precisely the point where perforant path terminals synapse. We have found that only 10-16% of the dosage is required when the application, iontophoretic or micro-pressure, is made at the synaptic zone. This provides strong support for the synaptic site of action of these protein kinase C activators. As shown in Figure 1 the site within the synapse where such interaction can take place is in the presynaptic terminal where both act upon the beta isoform of PKC.
A major contribution from our laboratory has been the discovery of a novel protein kinase C (PKC) activator, the cis unsaturated fatty acids (CUFAs), which we have proposed represent an alternative route of activation of PKC to DAG since full activation can occur in the absence of calcium or phospholipid. We have zeroed in on this enzyme because there is a growing body of evidence that PKC regulates synaptic reactivity and learning and memory. To establish the cellular basis for this regulation we have studied ionic currents in cell lines and dissociated cells of the hippocampus where long-term potentiation paradigm (LTP) is demonstrated. As shown in Figure 1 the CUFa PKC activator can exert its influence both on presynaptic and postsynaptic processes.

Since CUFAs are "physiological drugs" they offer the promise of a potentially safe methods for facilitating performance by enhancement of enzymes of the brain linked to memory and learning.

2. Statement of Work

We wished to know the answers to the following questions:

1. Is PKC activity important presynaptically, postsynaptically or both?
2. What are the precise time frames in which PKC is active? Is this different for presynaptic and postsynaptic mechanisms?
3. Is PKC expressed uniformly in the nervous system? Are there differential regional expression of isoforms of PKC?

3. Status of research

Significant accomplishments made during this period were:

a. Presynaptic vs postsynaptic specific effects of PKC inhibitors.

The locus of the synaptic mechanism for persistence of long-term potentiation (LTP) is a focus of current debate: presynaptic or postsynaptic or an ongoing dialogue between the two. One specific molecule relevant to this debate is protein kinase C (PKC) which this and other laboratories have found is necessary for persistence of LTP1. The relative importance of presynaptic and postsynaptic PKC in LTP and the time when participation occurs has not been established since prior studies used intracellular leakage of PKC inhibitors. To address this problem in the hippocampal slice preparation, low concentrations of PKC inhibitor were injected by iontophoresis into CA1 somata either before or after LTP. Two different PKC inhibitors, polymyxin B (PMXB) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) injected 10
min before LTP induction caused potentiated responses to return to baseline 15-35 min after LTP induction without affecting the initial magnitude of potentiation. There was no effect on LTP persistence when H-7 or PMXB was injected intracellularly 5 min after LTP induction. In contrast, 15 or 30 min, but not 60 min after LTP induction, focal extracellular micro-pressure ejection of PKC inhibitor in the stratum radiatum caused decay of LTP to baseline. This is likely acting presynaptically since intracellular inhibitors injected postsynaptically are ineffective 5 min after LTP induction. Focal application to stratum pyramidale produced a weaker delayed decay relative to application to stratum radiatum. Our data suggest that activation of postsynaptic PKC activity is necessary for LTP persistence but is time-limited to about 5 min. Presynaptic PKC activity is also necessary for persistence and is time-limited to less than 60 min. It is attractive to think that these two events are sequentially activated and that they use PKC subtypes differentially localized to pre- and postsynaptic elements.

b. Synapse-specific effects of PKC activators

Protein kinase C (PKC) stimulators, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) or cis-unsaturated fatty acid (UFA), have been shown to prolong synaptic enhancement induced by long-term potentiation (LTP). This observation suggests a role for PKC in the biochemical mechanisms underlying maintained enhancement.

To determine if PKC stimulators prolong LTP by acting selectively at synapses given high frequency stimulation or by actions that are not synapse specific (e.g. increased postsynaptic excitability) we examined the effect of TPA or UFA on input-selective enhancement. Population EPSPs evoked in the same granule cell population by either the medial (MPP) or lateral (LPP) perforant path can be selectively enhanced leaving the other perforant path input which receives only low frequency stimulation as an internal control for PKC stimulator effects not specific to enhanced synapses.

Synapse specific effects were in fact observed as UFA or TPA selectively prolonged MPP enhancement following two trains of high frequency MPP stimulation, without affecting responses evoked by the LPP. A similar synapse selectivity of PKC stimulator action was seen following high frequency LPP stimulation.

These findings suggest that PKC stimulators prolong enhancement by acting specifically at high frequency stimulated synapses. PKC stimulators do not appear to affect either postsynaptic neuron excitability or synapses given only low frequency stimulation. This provides further evidence that PKC acts synergistically with the consequences of repetitive synaptic activation to maintain enhancement.

c. Ionic currents regulated by CUFA mediated PKC activation

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 (c-FAs) 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, and if so, to determine the nature of that modulation. To this end, we applied the whole-cell patch clamp recording technique to N1E-115 neuroblastoma cells differentiated in 4% dimethylsulphoxide (DMSO).

Analysis of families of currents evoked under voltage clamp by depolarizing steps from a holding potential of -85 mV during application of 5 uM oleate (a c-FA) showed a 36% reduction of the peak inward current with no shift in either the peak or the reversal potential of the current-voltage (I-V) relation and no alteration of outward current.

Inward current of this cell is largely carried by Na^+, we sought to record the isolated Na^+ current by application of external Mg^{2+}, internal F^- and tetraethylammonium (TEA), and the replacement of internal K^+ with N-methylglucamine. The isolated Na^+ current recorded in this manner was completely and reversibly abolished by tetrodotoxin or removal of external Na^+, and was unaffected by application of external TEA.

Application of the c-FAs oleate, linoleate, and linolenate reversibly attenuated voltage-dependent Na^+ current with approximate ED50's of 2, 3, and 10 uM respectively. 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 at concentrations of 1-50 uM.

Three classical PKC activators, phorbol-12,13-dibutyrate, phorbol-12,13-diacetate, and 1,2-oleoylacetlylglycerol (OAG) were found to have no effect on the voltage-dependent Na^+ current when applied at 10nM-1uM (phorbol esters) or 1-150uM (OAG) for incubation periods up to 1 h.

The PKC inhibitors polymyxin B and H-7 were seen to block the attenuation of the Na^+ current by c-FA in a dose-dependent manner, with maximal inhibition occurring at doses of 50 and 10 uM, respectively. The cyclic nucleotide-dependent protein kinase inhibitor H-8 was much less effective in blocking the c-FA effect.

Using a different approach to determine whether the c-FA effect was mediated by PKC, chronic (24 h) exposure to 1 uM phorbol ester was employed to down-regulate this enzyme. 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 application of c-FA.

These data suggest two broad classes of explanation. First, c-FA attenuation of the Na^+ current could be mediated in part through a non-PKC mechanism. The second explanation, which we favor, is that activation of the PKC family of enzymes by c-FAs and the classical PKC activators
(phorbol esters, diacylglycerols) could result in different patterns of substrate phosphorylation such that c-FA activation of PKC produces attenuation of the Na+ current in N1E-115 cells, while stimulation of PKC by classical PKC activators does not.

d. Glial-neuronal interaction

A unique glial-synapse interaction suggested by recent studies showing an interaction between the protein kinase C substrate, F1/GAP43, and the glial protein, S100. (Sheu et al. 1993) We determined whether the phosphorylation of a neuronal protein F1/GAP43 could be regulated by glial factors. The results demonstrated that either the S100b or a mixture of S100a and S100b, both from a brain glia source, could inhibit phosphorylation of F1/GAP43 by PKC in vitro in a dose-dependent manner. Purified S100b differentially inhibited the F1 phosphorylation by four recombinant PKC subtypes: the IC50 of S100b for betaI and betaII PKC was 8 uM while for alpha and gamma PKC was 64 uM. Since the pure S100b did not inhibit histone III-S phosphorylation by the four PKC subtypes, the inhibition of S100b on F1/GAP43 phosphorylation could not be explained by a direct inhibition of kinase activity. S100b inhibition is thus both a substrate-specific and subtype-specific effect. Along with earlier studies implicating S100 in synaptic plasticity and neurite outgrowth, the present results suggest that S100 may regulate these functions through its inhibition of neuron-specific PKC substrate (F1/GAP43) phosphorylation.

4. Articles published, accepted for publication and submitted.

Published


6. Collier, T.J., Quirk, G.S., and Routtenberg, A.
Separable roles of hippocampal granule cells in forgetting and pyramidal cells in remembering spatial information. *Brain Research*, 987, 409, 316-328.


17. Routtenberg, A. Protein kinase C and protein F1: Potential molecular mediators of lesion-induced synaptic plasticity.


5. Personnel

<table>
<thead>
<tr>
<th>Name</th>
<th>Title</th>
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<tr>
<td>A. Routtenberg</td>
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* - Assistant Professor, Vanderbilt
** - Assistant Professor, University of Buffalo
*** - Assistant Professor, Johns Hopkins University

6. Coupling Activities

A. Spoken papers at meetings

6. Nelson, R.B., Linden, D.J., and Routtenberg, A. Two growth cone-enriched C-kinase substrates and two vesicle-associated phosphoproteins are directly...

7. Sheu, F.-S., Murakami, K., and Routtenberg, A. Subcellular distribution of protein kinase C (PKC) in rat brain regions suggests that hippocampus has the highest capacity for kinase translocation, and that this capacity may be diurnally regulated. *Soc. Neurosci.*, 1987, 13, 1009.


In an initial study we have found that protein synthesis inhibitors block LTP only when given 4 hr, but not 30 min. before induction. Does the maintenance of LTP require new synthesis or the presence of existing proteins? We attempted to resolve this controversy (e.g. Neurosci., 28:519, 1989 vs Synapse, 1:90, 1987) by studying LTP in the anesthetized male albino mouse injecting cycloheximide (CXM) or anisomycin (ANI) subcutaneously 30 min or 4 hr prior to LTP. Population responses of dentate gyrus cell layer were obtained by perforant path stimulation from entorhinal cortex. Population spike amplitudes, before and after high frequency stimulation (HFS), were compared among saline, CXM and ANI injected-mice. Initial potentiated responses were similar for all 3 groups, i.e., 200% of baseline spike amplitude 20 min after HFS. This implies that CXM or ANI does not affect the induction stage of LTP in contrast to prior reports (J. Neurosci., 4:3080, 1984 & Synapse, 1:90,
Injection of CXM 4 hr before HFS resulted in a decay of LTP that appeared at 90 min. The decreased responses remained at a lower level (60%) for 4 hr after HFS (P<0.01). ANI when injected 4 hr before HFS did not change the potentiated responses. CXM or ANI injected 30 min before HFS had no effect on the response at 4 hr after HFS though the response was temporarily lowered after HFS in the 40-90 min period for both inhibitors.

These results demonstrate that the inhibition of protein synthesis by CXM but not by ANI blocks the maintenance of LTP. In contrast, another study using rats (Neurosci., 28:519, 1989) found ANI to impair LTP maintenance. This inconsistency may reflect different methods or species used. Since CXM injected 4 hr before HFS but not 30 min before blocked LTP persistence, this implies that protein(s) constitutively present at the time of HFS with a half-life of more than 30 min and less than 4 hr is necessary for LTP maintenance. Moreover, the absence of an effect at 30 min suggest that new protein synthesis is not required.