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The Role of Protein Kinase C and Its Neuronal Substrates* Dephosphin, B-50, and MARCKS in Neurotransmitter Release

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Contents

* While this manuscript was under review, another comprehensive review of the role of PKC in neurotransmitter release was published by Gispen's group (Dekker et al., 1991a).

Abstract

This article focuses on the role of protein phosphorylation, especially that mediated by protein kinase C (PKC), in neurotransmitter release. In the first part of the article, the evidence linking PKC activation to neurotransmitter release is evaluated. Neurotransmitter release can be elicited in at least two manners that may involve distinct mechanisms: *Evoked* release is stimulated by calcium influx following chemical or electrical depolarization, whereas *enhanced* release is stimulated by direct application of phorbol ester or fatty acid activators of PKC. A markedly distinct sensitivity of the two pathways to PKC inhibitors or to PKC downregulation suggests that only *enhanced* release is directly PKC-mediated. In the second part of the article, a framework is provided for understanding the complex and apparently contrasting effects of PKC inhibitors. A model is proposed whereby the site of interaction of a PKC inhibitor with the enzyme dictates the apparent potency of the inhibitor, since the multiple activators also interact with these distinct sites on the enzyme. Appropriate PKC inhibitors can now be selected on the basis of both the PKC activator used and the site of inhibitor interaction with PKC. In the third part of the article, the known nerve terminal substrates of PKC are examined. Only four have been identified, tyrosine hydroxylase, MARCKS, B-50, and dephosphin, and the latter two may be associated with neurotransmitter release. Phosphorylation of the first three of these proteins by PKC accompanies release. B-50 may be associated with *evoked* release since antibodies delivered into permeabilized synaptosomes block *evoked*, but not enhanced release. Dephosphin and its PKC phosphorylation may also be associated with *evoked* release, but in a unique manner. Dephosphin is a phosphoprotein concentrated in nerve terminals, which, upon stimulation of release, is rapidly dephosphorylated by a calcium-stimulated phosphatase (possibly calcineurin [CN]). Upon termination of the rise in intracellular calcium, dephosphin is phosphorylated by PKC. A priming model of neurotransmitter release is proposed where PKC-mediated phosphorylation of such a protein is an obligatory step that *primes* the release apparatus, in preparation for a calcium influx signal. Protein dephosphorylation may therefore be as important as protein phosphorylation in neurotransmitter release.

Index Entries: Dephosphin; protein kinase C; isozymes; phorbol esters; protein phosphorylation; synaptosomes; neurotransmitter release; protein kinase C inhibitors; calcineurin.

Abbreviations: AA, arachidonic acid; CaM, calmodulin; CaM-PK, calmodulin-dependent protein kinase; CN, calcineurin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLA_2, phospholipase A2; PMA, phorbol 12-myristate 13-acetate; PS, L-phosphatidyl-L-serine.

Neurotransmitter Release

Neurotransmitter release occurs when depolarization of the membrane of presynaptic nerve terminals opens voltage-sensitive calcium channels, and stimulates calcium influx and diffusion into the cell. It is initiated by the increase in intracellular calcium, which binds to an intracellular receptor protein that subsequently mediates fusion of synaptic vesicles with the plasma membrane resulting in release of vesicle contents. Since there are many effects of calcium, mediated by many proteins, the nature of the intracellular receptor for calcium that mediates release is unknown and remains one of the fundamental questions in neurobiology. Much attention is focused on protein kinase C (PKC) as the possible calcium trigger, but even if its involvement is proven, it would still remain to be determined which PKC substrate mediated the effect. In the last six years, a wide body of research has directly associated PKC activation in nerve terminals with neurotransmitter release or PKC activation in secretory cells to exocytosis. However, there are many possible roles PKC might play in neurotransmitter release.

There are four broad stages to the neurotransmitter release cycle. Calcium-stimulated processes commence at stage 2, and each stage overlaps.

1. *Priming* of the nerve terminal in preparation for release. This is a new concept whereby phosphorylation of a specific protein prepares the nerve terminal for subsequent calcium influx.
2. *Mediation* (or initiation of the fusion event) of release. This is initiated by calcium entry, involves synaptic vesicle fusion with the plasma membrane, and has been proposed to be regulated by a single protein complex.
3. *Reactivation* of the nerve terminal by initiation of events that prepare the terminal for the next stimulus (some aspects of this role overlap with those of priming). For example, movement and docking of synaptic vesicles, cytoskeletal rearrangement, and maintenance of energy and ion homeostasis.
4. *Modulation* of the amount or rate of release. This can occur by regulation of the availability of neurotransmitter or synaptic vesicles for secretion, by altering calcium homeostasis, or by modification of proteins involved in other stages.

Priming, mediation, and reactivation are cyclic events that underpin the stimulation and dynamically prepare the nerve terminal for multiple action potentials. Because of the complexity of neurotransmitter release and the multiple stages involved, it may not be sufficient to "associate" PKC with neurotransmitter release, but it is more important to determine at which stage of release PKC might be involved. In this article, the potential role of PKC in all of these broad stages is examined. Limitations in our understanding of the molecular events underlying each step also limit our ability to place PKC in the pathway accurately.

Neurotransmitter release is a controlled fusion of synaptic vesicles with the presynaptic plasma membrane and subsequent release of the vesicle contents. The released neurotransmitters then activate postsynaptic receptors in the next neuron or target tissue, and trigger a cascade of intracellular signals resulting in generation of a biological response. The basic mechanisms are presumed to be analogous to (or identical with) controlled secretory release from endocrine or exocrine gland cells, mast cells, platelets, and neutrophils; these nonneuronal models of exocytosis are also drawn upon in this article. There are many recent and comprehensive reviews encompassing the biochemistry of, or proteins involved in neurotransmitter release (Augustine et al., 1987; Kelly, 1988; Jahn et al., 1990; Valtorta et al., 1990; De Camilli and Jahn, 1990; Zimmermann, 1990; Thureson-Klein and Klein, 1990; Almers and Tse, 1990; Trimble et al., 1991) or exocytotic release (Knight and Baker, 1987; Harper, 1988; Knight et al., 1989; Plattner, 1989; Burgoyne, 1990; Almers, 1990). The final cascade of biochemical events that underlie release is unknown, and although many major processes have been implicated, the PKC system would appear to be among the strongest candidates. There are, however, few reviews concerning the possible roles of PKC in neurotransmitter release or exocytosis (Harper, 1988; Kaczmarek, 1987; Zimmermann, 1990), and new trends in the literature suggest that PKC primes, reactivates, and modulates, but is not required for mediation of release.

Elevation of intracellular calcium is the first event of the cascade (Augustine et al., 1987; Zimmermann, 1990) (Fig. 1). Intracellular calcium presumably binds to a receptor molecule within the nerve terminal, the binding of which somehow promotes fusion of synaptic vesicles with the presynaptic membrane at the active zone. The key to defining the subsequent events lies in determining the immediate target for calcium binding in the nerve terminal. A short cascade of molecular steps might be involved, such as calcium binding to PKC, which phosphorylates a protein that then mediates fusion, or to CN, which dephosphorylates a protein that mediates fusion, and evidence exists for both models.

The mechanism triggering fusion of the synaptic vesicle and plasma membranes is unknown. Fusion could be very rapid if the synaptic vesicle were initially docked at the plasma membrane and a fusion or trigger protein were already associated with the vesicles. Binding of calcium

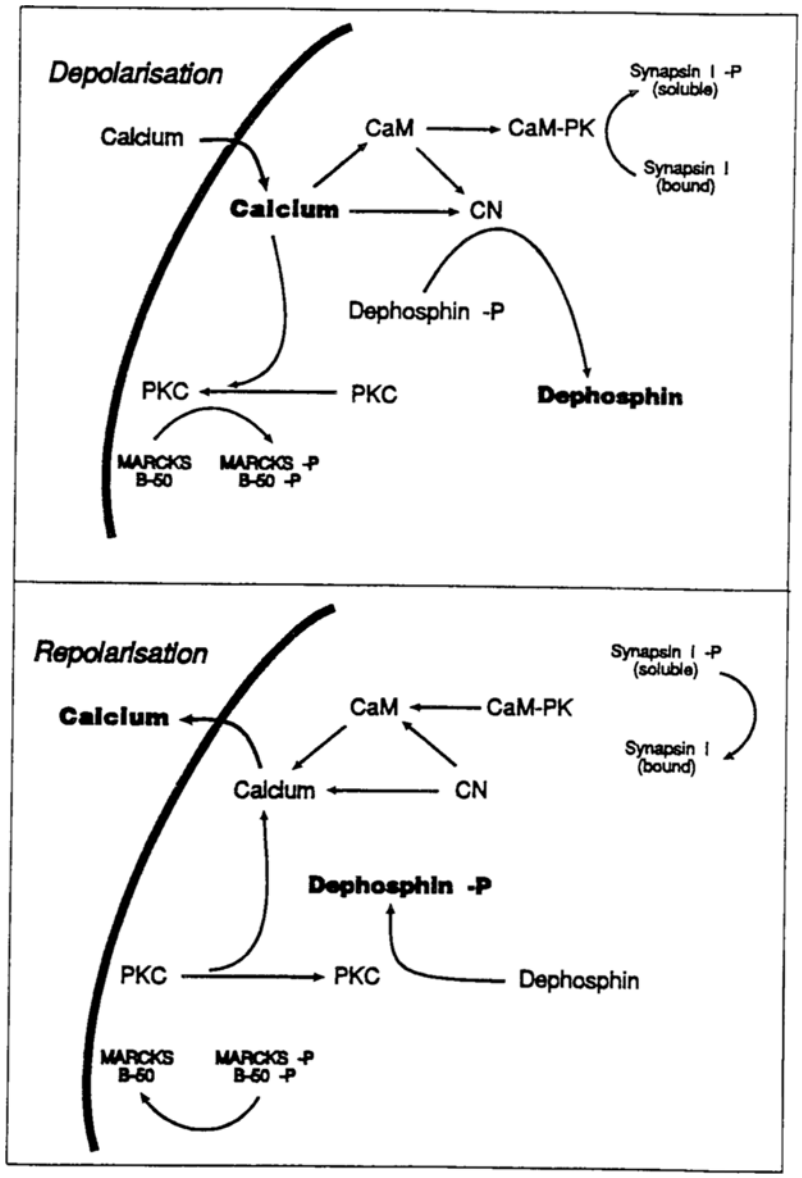

Fig. 1. Model for the mechanism of phosphorylation and dephosphorylation of dephosphin in nerve terminals. *Depolarization:* Under depolarizing conditions, calcium entry into nerve terminals activates a multitude of calcium-dependent enzymes. Shown here are activation of PKC, which phosphorylates MARCKS and B-50, activation of calmodulin (CaM) and calmodulin-dependent protein kinase II (CaM-PK-II), which phosphorylates synapsin I and releases it from synaptic vesicles, and proposed activation of calcineurin (CN), which dephosphorylates dephosphin and accompanies neurotransmitter release. Note that the specific identity of this phosphatase as CN is only speculative at this stage. *Repolarization:* Under repolarizing conditions, calcium is rapidly extruded from the nerve terminal, and resting intracellular calcium levels are restored. This results in removal of the stimulus for CaM-PK-II, CN, and PKC, and thereby returns them to their basal activities. This allows basal activities of protein phosphatases to dephosphorylate synapsin I, B-50, and MARCKS. However, some PKC is active or activated, and phosphorylates dephosphin to restore it to its resting phosphorylated state. This serves to "prime" dephosphin to be ready to respond to a second or subsequent stimulus. The mechanism of how PKC could be active towards dephosphin, but not B-50 or MARCKS, under these conditions is not known.

may trigger a conformational change in this protein that allows a fusion pore to be formed through which the membrane lipids diffuse and merge. Therefore, the molecule most likely responsible for neurotransmitter release could be expected to interact with synaptic vesicles at some point (Augustine et al., 1987; Zimmermann, 1990; Trimble et al., 1991). Identification of vesicle-associated proteins or cytosolic proteins that reversibly interact with vesicles may provide a clue to molecules responsible for vesicle fusion and/or translocation. Candidates for this putative calcium trigger include:

1. PKC;
2. Calmodulin (CaM)—which further activates a variety of enzymes, such as CaM-dependent protein kinase-II (CaM-PK-II), CaM-dependent adenylate cyclase, CaM-dependent phosphodiesterase, or CN;
3. Vesicle-associated calcium binding proteins (e.g., calelectrin, synexin, or other annexins, a family of calcium-dependent membrane-binding proteins (Burgoyne, 1990);
4. Membrane proteins, such as "mediatophore," which mediates calcium-dependent acetylcholine release (Israel et al., 1988), or dephosphin (Dunkley and Robinson, 1986);
5. Cytosolic phosphoproteins, such as parafusin (Plattner, 1989; Satir et al., 1989); or
6. G proteins that can translocate from synaptic vesicles to the cytosol during exocytosis (Fischer von Mollard et al., 1991).

However, other possibilities are likely to exist. The mechanics of fusion of the two membranes is also not understood, but interested readers are referred to a detailed hypothetical process (Almers, 1990).

In determining the potential intracellular targets for calcium, it is also necessary to be aware of the interactions among any or all of the pathways outlined below. For example, CaM stimulates apparently conflicting pathways of phosphorylation via CaM-PK-II and dephosphorylation via CN, as well as inhibiting PKC phosphorylation of certain substrates. Therefore, drugs or inhibitory CaM antibodies may affect multiple pathways. Similarly, fatty acids or phospholipids that stimulate PKC may also stimulate PKA while inhibiting CaM-PK-II. Many such apparently opposing biochemical events are known to occur in intact cells simultaneously. It is also important to be aware that few biochemical tools are highly specific, and this may partly expound the difficulty in elucidating the mechanisms underlying neurotransmitter release.

In this article, a model is proposed whereby there are three roles of PKC in neurotransmitter release. The first role is in *priming* the nerve terminal to be ready to respond to a stimulus. The increase in intracellular calcium is proposed to activate a phosphatase (such as CN) and the dephosphorylation of dephosphin or other PKC substrate, which, in turn, directly mediates neurotransmitter release. PKC may therefore phosphorylate and *prime* other proteins, such as dephosphin, to mediate release. The model links PKC directly to neurotransmitter release in a preparative role. This phosphatase or its substrate protein(s) would then be the calcium trigger that mediates secretion. The second role of PKC is in *modulation* of neurotransmitter release. In this situation, PKC activation can enhance neurotransmitter release by mechanisms distinct from those mediating calcium- or depolarization-evoked release. The third role of PKC is in *reactivation* of the nerve terminal from release, and includes phosphorylation and activation of tyrosine hydroxylase to restore depleted neurotransmitter levels. Therefore, PKC may play direct roles in neurotransmission that include most stages of the process, but not *mediation*. The evidence for these roles is developed in the following sections.

Role of Protein Phosphorylation

Many studies have demonstrated a broad correlation between phosphorylation and neurotransmitter release (Dunkley and Robinson, 1986; Dekker et al., 1989b,1990a,b) or exocytosis (*see* references in Cote et al., 1986). Such correlations

would be better viewed as broad associations, rather than as evidence of actual involvement in neurotransmitter release. However, depolarization of intact rat brain synaptosomes stimulates calcium influx and the phosphorylation of a variety of synaptosomal proteins (Robinson, 1987) (Fig. 2). Phosphorylation is owing to the activation of CaM-PK-II, which phosphorylates synapsin I (marked 75 kDa in Fig. 2A) and other proteins (Huttner and Greengard, 1979; Kennedy and Greengard, 1981; Dunkley et al., 1986; Dunkley and Robinson, 1986), and of PKC. PKC phosphorylates an 83-kDa protein termed MARCKS (Dunkley et al., 1986; Rodnight and Perrett, 1986; Nichols et al., 1987; Wang et al., 1988,1989) and a 45-kDa protein called B-50 (Dunkley et al., 1986; Dunkley and Robinson, 1986; Robinson et al., 1987; Rodnight and Perrett, 1986; Wang et al., 1988; Dekker et al., 1989b; De Graan et al., 1989). Depolarization is also accompanied by the activation of phosphatase(s), and the rapid dephosphorylation of at least two prominent synaptosomal phosphoproteins termed P139 and dephosphin (the latter is a doublet that was formerly called P96 and P93) (Robinson et al., 1987; Robinson, 1987,1991; Yip and Kelly, 1989; Wang et al., 1988; Guillemette et al., 1990) (Fig. 2). These events are summarized in Fig. 1.

Other evidence for a role of protein phosphorylation in release comes from studies of release or exocytosis in permeabilized cells. Exocytosis may have an absolute Mg-ATP requirement in both synaptosomes and nonneuronal cells (Knight, 1987; Knight et al., 1988; Plattner, 1989; Deldcer et al., 1991b), although the requirement may not be essential for the final stage of vesicle fusion (Howell et al., 1989). In permeabilized chromaffin or mast cells, intracellular Mg-ATP primes the cells so that the initial component of fusion is independent of exogenous ATP and the ATP partially maintains the primed state by acting before calcium in the secretory pathway (Holz et al., 1989; Howell et al., 1989). The simplest way to link the role of calcium and Mg-ATP to release may be by supporting the phosphorylation of proteins prior to or during release (Dekker et al., 1991b). Alternatively, the Mg-ATP may be required to maintain phosphorylation of a protein that is dephosphorylated upon activation of secretion (Howell et al., 1989; Plattner, 1989). The latter possibility is also supported by findings that okadaic acid (an inhibitor of phosphatases 1 and 2A) enhances neurotransmitter release at neuromuscular junctions, which would increase phosphorylation of proteins prior to release activation (Abdul-Ghani et al., 1991).

Protein phosphorylation could be involved in any or all stages of neurotransmitter release. Although phosphorylation plays clear roles in *reactivation* and *modulation* of neurotransmitter release (reviewed in Dunkley and Robinson, 1986), in contrast, there is little compelling evidence linking phosphorylation to *mediation* of neurotransmitter release (Augustine et al., 1987; Almers, 1990; Trimble et al., 1991). However, there are a number of possible ways in which protein kinases or phosphatases could mediate neurotransmitter release. The relevant protein kinase or phosphatase and substrate phosphoprotein may have a specialized location at the active zone or on the synaptic vesicle, which allows for much greater enzyme reaction rates than achievable in steady-state reaction assays in vitro. Furthermore, the initial rate of enzyme activity is likely to be considerably greater than derived steady-state reaction velocities. This could account for the submillisecond time available for initiation of release to occur (Almers, 1990). There are also alternate possibilities, such as kinase–substrate or phosphatase–substrate complexes that are "preprimed" and ready for a calcium signal, or that only a few molecules of substrate may be required to be phosphorylated or dephosphorylated in order to bring about movement or fusion of a single docked synaptic vesicle. These possibilities argue against time constraints as the factor that limits mediation to a single molecular conformational change (Augustine et al., 1987; Almers, 1990; Trimble et al., 1991). In fact, a small cascade of steps is possible, especially if all of the elements are primed and correctly localized prior to the influx of calcium.

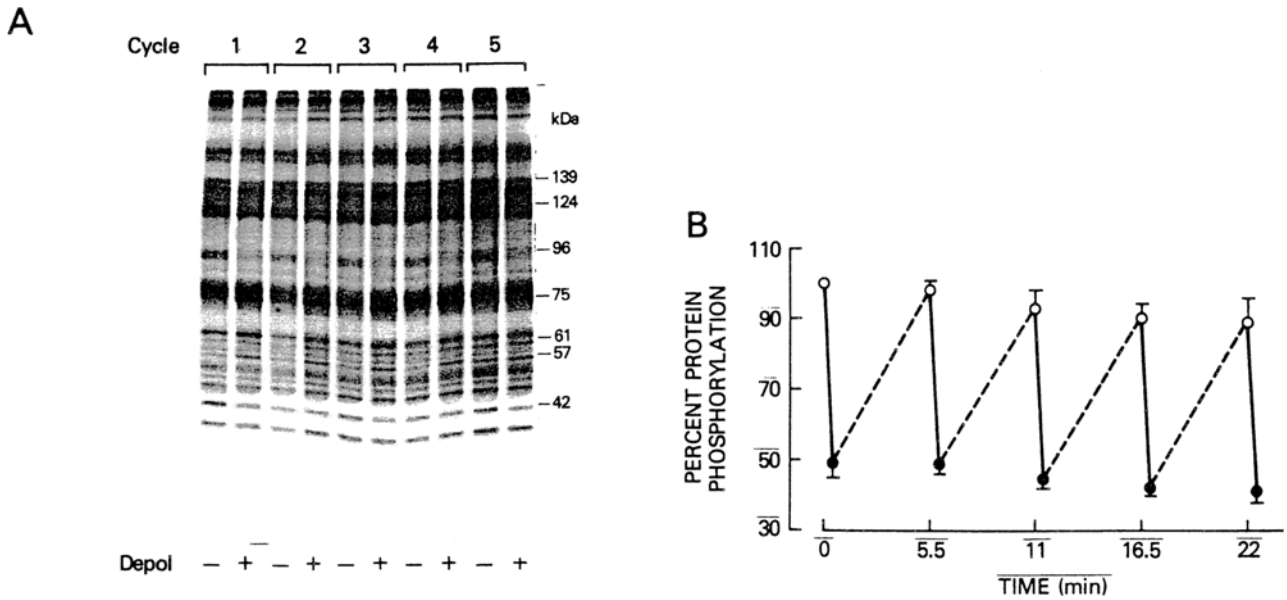

Fig. 2. Reversibility of dephosphin's phosphorylation and dephosphorylation in intact synaptosomes is shown in the autoradiograph in A and quantified in B. Dephosphin and P139 are normally phosphorylated after 45 min of prelabeling of intact synaptosomes with $^{32}P_i$ (Robinson et al., 1987) and are dephosphorylated after 15 s of depolarization with 41 m*M* K^+. After depolarization, an aliquot is removed for the polyacrylamide gel, and the synaptosomes were pelleted in a microfuge and resuspended in low K^+ buffer for 5 min. After 5 min of this repolarization, dephosphin phosphorylation was essentially completely restored, and a second depolarization elicited the same extent of dephosphorylation. Phosphorylation and dephosphorylation proceeded through five cycles of depolarization and repolarization, indicating the reversible nature of the phenomenon and that dephosphorylation is not owing to proteolysis. This indicates that regulation of dephosphin phosphorylation in intact cells is both dynamic and robust. Results in panel B represent quantitative analysis of dephosphin phosphorylation and dephosphorylation during depolarization and repolarization (Robinson et al., 1987). Similar results were obtained with P139, but were not quantified because of the migration on gels of P139 in a region of high background.

Protein Phosphatases

Protein dephosphorylation has also been linked to neurotransmitter release from synaptosomes (Sim, 1991). The only known neuronal examples of this are dephosphin (or P96), P124, and P139 (Robinson et al., 1987) (Figs. 2 and 3). These phosphoproteins are phosphorylated in resting intact synaptosomes, and are rapidly dephosphorylated upon depolarization-dependent calcium influx and neurotransmitter release. Phosphorylation of dephosphin occurs via PKC in intact synaptosomes and in vitro (Figs. 4 and 5), and preliminary evidence suggests that the dephosphin phosphatase may be CN (Sim et al., 1991; Sim, 1991). The role of the protein in neurotransmitter release is unknown, but its properties are reviewed in detail below (*see* "Dephosphin" *below*). The role of dephosphorylation in neurotransmitter release is further supported by studies with okadaic acid, the potent inhibitor of phosphatases 1 and 2A. In one study, okadaic acid doubled the quanta of neurotransmitter released, supporting the idea that phosphorylation/dephosphorylation of proteins constantly modulates release (Abdul-Ghani et al., 1991). In another study, okadaic acid increased basal release of excitatory amino acids from synaptosomes (Sim, 1991). These studies support phosphatases as at least modulators of release. However, the use of okadaic acid to study neurotransmitter release must be considered with caution, since

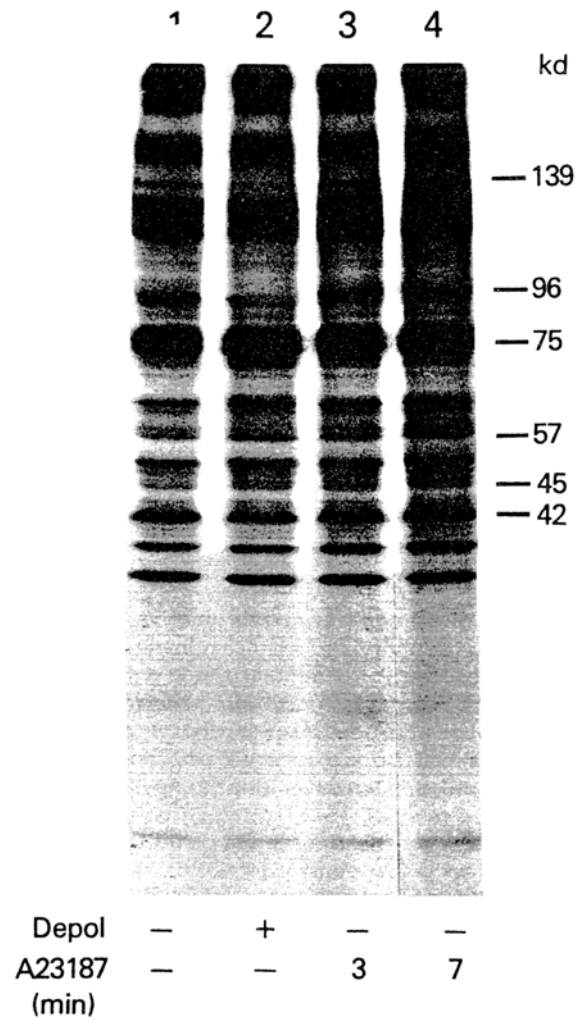

Fig. 3. Dephosphin dephosphorylation in intact synaptosomes is dependent upon the method of Ca^{2+} influx as well as a rise in intracellular Ca^{2+}. Autoradiograph shows phosphorylation of dephosphin (96 kDa) or P139 in resting intact synaptosomes (lane 1) labeled with $^{32}P_i$; and 41 m*M* K^+ depolarization-stimulated dephosphorylation (lane 2). The calcium ionophore A23187 (1 μ*M*), at concentrations that do not depolarize the synaptosomes (Robinson et al., 1987), stimulates an increase in intracellular Ca^{2+} and phosphorylation of synapsin I (75 and 80 kDa) and synapsin II (72 and 57 kDa) by CaM-PK-II, but does not stimulate dephosphin or P139 dephosphorylation (lanes 3 and 4). Thus, dephosphin dephosphorylation requires more than a simple rise in intracellular Ca^{2+}, and is sensitive to the method of Ca^{2+} entry through voltage-sensitive calcium channels.

its effects may be mediated by a wide variety of intracellular phosphorylation/dephosphorylation events. For example, okadaic acid inhibits catecholamine release from chromaffin cells by an effect on calcium influx rather than an effect on a potential calcium trigger for release (Yanagihara et al., 1991), but in contrast, okadaic acid mimics the effect of decreased intracellular calcium in synaptosomes (Sim et al., 1991).

Dephosphorylation of proteins accompanies exocytotic release in a number of nonneuronal systems, such as islet cells (Jones et al., 1988), chromaffin cells (Cote et al., 1986), and *Paramecium* (Plattner, 1989; Strecher et al., 1987). In chromaffin cells, many proteins are dephosphorylated upon stimulation, but only dephosphorylation of a 20.4-kDa protein precedes the onset of neurotransmitter release (Cote et al., 1986). In *Paramecium*, one protein, parafusin (formerly PP65), is rapidly (<1 s) dephosphorylated on exocytosis and then rephosphorylated within 5–20 s (Satir et al., 1988; Plattner, 1989). A parafusin-like molecule has been found in the cytosol of a variety of species, including bovine chromaffin cells (Fournier et al., 1989) and humans, where it is proposed to play a role in exocytosis and membrane fusion (Satir et al., 1989). It is possible that intact rat brain synaptosomes also contain a parafusin-like protein (P65) (Gomez-Puertas et al., 1991). Parafusin has been proposed to be related (Plattner, 1989) to the synaptic vesicle-associated 65-kDa protein described by a number of groups (Fournier et al., 1989; Floor and Feist, 1989; Perin et al., 1990). Dephosphorylation of parafusin may be mediated by CN, since microinjection of anti-CN antibodies into *Paramecium* specifically inhibits exocytosis and parafusin dephosphorylation (Momayezi et al., 1987; Plattner, 1989). Parafusin is also a substrate for alkaline phosphatases, microinjection of which stimulates exocytotic release from *Paramecium* (Momayezi et al., 1987). These studies make a strong case for a role of dephosphorylation in exocytotic release and implicate CN as the mediator of calcium-induced release.

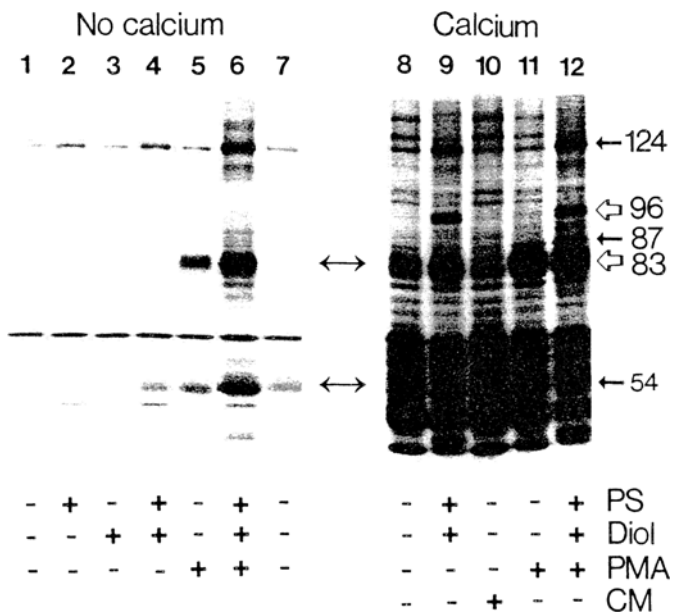

Fig. 4. Autoradiograph showing in vitro phosphorylation of dephosphin (96 kDa) and MARCKS (83 kDa) by PKC in synaptosomal cytosol. PMA alone increased the phosphorylation of MARCKS (lane 5), an effect that was enhanced by PS (lane 6). Ca^{2+} stimulated phosphorylation of a variety of proteins owing mostly to CaM-PK-II activity (lane 8), since this effect was completely prevented by calmodulin antibodies (not shown). However, Ca^{2+}/PS stimulated the phosphorylation of both MARCKS and dephosphin (lane 9). This shows that PS/PMA (without Ca^{2+}) stimulates phosphorylation of only a subset of all synaptosomal PKC substrates, and illustrates that phosphorylation of some proteins is unaffected by phorbol esters. However, P124 is an excellent example of a protein phosphorylated in the presence of either PS/PMA or Ca^{2+}/PS (lanes 6, 9, and 12). Activation of protein kinase C by PS/PMA (without Ca^{2+}) has been reported by other groups (Vandenbark et al., 1984; Couturier et al., 1984; Ashendal, 1985; Arcoleo and Weinstein, 1985; Bazzi and Nelsestuen, 1988,1989). PS/PMA therefore alters the substrate specificity of PKC such that it phosphorylates a distinct group of proteins to those phosphorylated in the presence of Ca/PS as observed by others (Kreutter et al., 1985; Kiss and Luo, 1986; Yamamoto et al., 1988; Kumar et al., 1987). Synaptosomes were prepared from rat brain, lysed, and a high-speed supernatant prepared (Robinson, 1991). Endogenous proteins were phosphorylated for 60 s by endogenous PKC in the presence of the activators shown (Robinson, 1991; Robinson and Lovenberg, 1988). The concentrations of various additions were 1 m*M* EGTA (present in all samples), 40 μg/mL phosphatidylserine (PS), 4 μg/mL diolein (DAG), 200 n*M* PMA, or 100 μ*M* free Ca^{2+}. After terminating the reactions with SDS, the proteins were processed by gel electrophoresis and autoradiography (Robinson and Dunkley, 1983a). Essentially the same results were obtained with addition of exogenous purified rat brain PKC.

Calcineurin (CN)

CN is a calcium-dependent phosphatase, with calcium sensitivity conferred at two points. First, the small subunit of CN (CN-B, 19 kDa) is a calcium binding protein that confers calcium dependence to the enzyme, and CaM provides a second stimulation that is additive with the first. The CaM binding site of CN is found in the large subunit (CN-A, 61 kDa) in residues 391–414, which also contains a phosphorylation site for PKC or CaM-PK-II (Hashimoto and Soderling, 1989; Calalb et al., 1990). CaM binding blocks phosphorylation or dephosphorylation of this site, but phosphorylation does not block CaM binding (Calalb et al., 1990). Phosphorylation only partly inactivates the enzyme, and the major CN phosphatase is likely to be phosphatase 2A, although autodephosphorylation can occur slowly (Hashimoto and Soderling, 1989). CN can

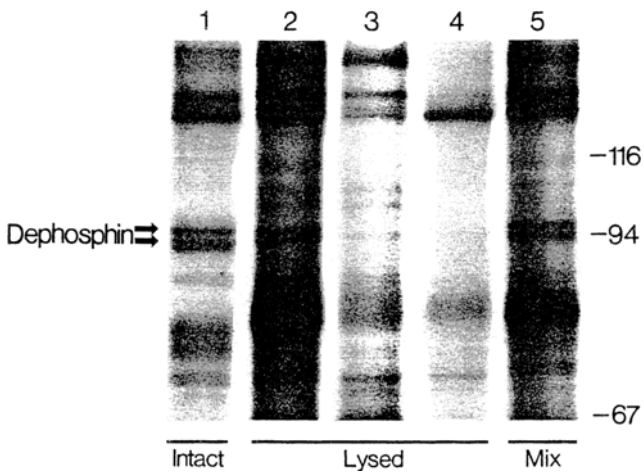

Fig. 5. Dephosphin is phosphorylated in intact and lysed synaptosomes. The autoradiograph shows phosphorylation of dephosphin in $^{32}P_i$-labeled intact synaptosomes, [γ-^{32}P]-ATP-labeled synaptosomal cytosol, or a mixture of both. Intact synaptosomes were labeled with $^{32}P_i$ for 60 min (Robinson and Dunkley, 1983b), washed twice in a microfuge to remove $^{32}P_i$, lysed with lysis buffer, and centrifuged at 100,000*g* for 30 min to prepare cytosol from labeled synaptosomes (lane 1). Phosphorylation of synaptosomal cytosol for 60 s with [γ-^{32}P]-ATP was performed as described (Robinson and Lovenberg, 1988) in the presence of EGTA (lane 4), Ca^{2+} (lane 3) or Ca^{2+}/PS to activate PKC (lane 2). Equal total counts of sample in lanes 1 and 2 were mixed for running in lane 5. The results show that dephosphin from intact synaptosomes is a 96,000- and 93,000 doublet, and that the doublet comigrates with PKC-phosphorylated dephosphin in lysed synaptosomal cytosol. The mixing experiment reinforces the equivalent migration of the proteins from the in vivo and in vitro conditions. This suggests that PKC is the dephosphin kinase in intact synaptosomes. Dephosphin from intact or lysed synaptosomes also had the same isoelectric points, phosphopeptide maps, and presence only of phosphoserine (Robinson, 1991).

be specifically inhibited by synthetic peptides based on sequences near the COOH-terminal of CN that may represent an autoinhibitory domain (Hashimoto et al., 1990). These peptides do not inhibit CaM-PK-II or phosphatases I or IIA. CN can also be inhibited by micromolar zinc, vanadate (Kocher and Clemetson, 1991), and phenothiazines (Orgad et al., 1987). Other divalent cations, strontium and barium, can replace calcium in CN activation; however, a second group of metal divalent cations comprising nickel, manganese, and cobalt can produce much greater activation than the former group of cations by acting directly on the enzyme rather than on CaM (King and Huang, 1983). CN shares with PKC the ability to undergo reversible calcium-dependent translocation from the cytosol to the membrane (Gopalakrishna et al., 1986; Niedel and Blackshear, 1986; Akers and Routtenberg, 1987). Binding is initially rapid, reversible, calcium-dependent, that leads to stimulation of phosphatase activity, and is followed by a slower phase of binding that strengthens the interaction, but that may inhibit enzyme activity toward certain substrates. The subunit containing the phospholipid binding domain is CN-B (Politino and King, 1990).

Two recent studies suggest that CN may be activated in intact cells after calcium entry into rat brain slices stimulated by the NMDA receptor. In the first, NMDA stimulates calcium influx and DARPP-32 dephosphorylation in striatal slices (Halpain et al., 1990). CN was proposed to mediate dephosphorylation of DARPP-32, an in vitro substrate of cAMP-dependent protein kinase and CN, and lead to inactivation of DARPP-32's ability to inhibit phosptase 1. This would result in activation of phosphatase 1 in nerve cells (Halpain et al., 1990). The same teleological arguments were applied to NMDA receptor activation of MAP-2 phosphorylation in hippocampal slices (Halpain and Greengard, 1990). However, there has been no direct demonstration of CN activation in either of these studies. A role for CN in neurotransmitter release has not been previously considered, since few CN substrates have yet been identified, apart from the regulatory subunit (R-II) of PKA, DARPP-32, MAP-2, and B-50 (Klee and Cohen, 1988). However, CN has been suggested to be the major dephosphin phosphatase in synaptosomes (*see below*) (Robinson et al., 1987; Robinson, 1987; Wang et al., 1988; Sim et al., 1991; Sim, 1991) and the parafusin phosphatase in *Paramecium* (Momayezi et al., 1987). Given that CN is concentrated in nerve terminals and, like PKC, is regulated in activity and subcellular localization by calcium, it should be considered as a strong candidate for mediation of neurotransmitter release.

Role of Calmodulin (CaM)

CaM is one protein that could be involved in *mediation* of neurotransmitter release, but the evidence in support of this is sparse. There is excellent evidence, however, that CaM is involved in *reactivation* and *modulation* of release. CaM is a small protein that binds up to four calcium ions cooperatively and is found in virtually all eukaryotic cells. Because of its diverse roles in cellular functions, it has been viewed by many as the possible calcium trigger for neurotransmitter release. However, the same diversity of function has led to the difficult task of determining which CaM-activated enzyme mediates its effects. CaM is present in nerve terminals and associated with synaptic vesicles (DeLorenzo, 1981). Inhibitors, such as phenothiazines, block neurotransmitter release or protein phosphorylation in synaptosomes and many other cells, often under conditions where calcium influx is not also blocked (Robinson et al., 1984; Ronning and Martin, 1986; Robinson and Dunkley, 1987). However phenothiazines also block many other proteins that have been associated with neurotransmitter release, including PKC (Wise and Kuo, 1983), annexins (Pollard et al., 1982), and CN (Orgad et al., 1987). CaM inhibitors, however, do not inhibit calcium-evoked catecholamine release from PC12 cells, providing one argument against a role for CaM (Matthies et al., 1988). More direct evidence for a link between CaM and release came with nonneuronal models. Microinjection of anti-CaM antibodies using preloaded red blood cell ghosts inhibits exocytosis from chromaffin cells (Kenigsberg and Trifaro, 1985). However, even this result is clouded by interpretation of which CaM-mediated events might be inhibited by this approach. Since CaM itself may not cause vesicle fusion (Augustine et al., 1987, but *see also* DeLorenzo, 1981), its possible effects on neurotransmitter release are more likely to be mediated by one of numerous CaM binding proteins, such as CaM-PK, CN, P65, B-50, or MARCKS (*see below*). Many of the effects of CaM occur by activation of CaM-PK-II.

Role of Calmodulin-Dependent Protein Kinase II (CaM-PK-II)

CaM-PK is particularly abundant in brain, where it may have a multitude of roles (Dunkley, 1991; Kelly, 1991), such as *reactivation* and *modulation* of neurotransmitter release. CaM-PK-II is activated after depolarization-dependent calcium influx into synaptosomes (Dunkley et al., 1986; Dunkley and Robinson, 1986) and phosphorylates a range of nerve terminal proteins (Robinson and Dunkley, 1983a,1985; Dunkley and Robinson, 1986), including tyrosine hydroxylase and synapsin I (75 kDa in Fig. 2). These phosphorylation events play important roles in *reactivation* and *modulation* of neurotransmitter release (respectively). Phosphorylation of synapsin I by CaM-PK-II results in its dissociation from the synaptic vesicles that it normally coats, to the cytosol (Sihra et al., 1989), and thereby increases the probability that the decoated vesicles will fuse with the plasma membrane on stimulation (Llinas et al., 1985) (Fig. 1). These experiments were performed by microinjection of CaM-PK-II into nerve terminals, or by injection of phospho- or dephosphosynapsin I (Nichols et al., 1989; Llinas et al., 1985). Similarly, introduction of thiophosphorylated CaM-PK-II into synaptosomes augments release by relieving a constraint on release (Nichols et al., 1990). These microinjection experiments dramatically illustrate the modulatory role of CaM-PK-II in neurotransmitter release. Synapsin I and other cytoskeletal-associated proteins therefore modulate exocytosis by regulating the availability of synaptic vesicles at the synaptic junction. Other experimental evidence also indicates that CaM-PK-II may not mediate release. For example, manganese, a CaM activator, fails to support release from PC12 cells (Matthies et al., 1988) and barium, which does not activate CaM (Chao et al., 1984) or CaM-PK-II (Robinson and Dunkley, 1983b), but stimulates neurotransmitter release from synaptosomes (Crosland et al., 1983; Sanchez-Prieto et al., 1987). Caution is required in the final interpretation of such studies, since it is by no means certain

that barium-mediated neurotransmitter release occurs via the same biochemical steps as calcium-evoked release.

Role of Protein Kinase C (PKC)

PKC is a family of phospholipid-dependent protein kinases, at least four of which are stimulated by calcium, whereas at least four others (sometimes termed the nPKC family) are calcium-independent (for reviews, *see* Nishizuka, 1988; Nelsestuen and Bazzi, 1991). The calcium requirement, in turn, is regulated by 1,2-diacylglycerol and possibly by other factors (Nishizuka, 1988; Oishi et al., 1988). The requirement of PKC for calcium and Mg-ATP, along with its ability to associate with acidic phospholipids reversibly in a calcium-dependent manner, makes the possibility that PKC is the primary target for intracellular calcium in triggering release attractive. There is now strong evidence that the activation of PKC is associated with calcium-dependent neurotransmitter release from a variety of neuronal tissues or secretory cells, but there is no evidence that PKC might also *mediate* release (*see below*). Universally, activation of PKC is associated with an enhanced rate of neurotransmitter release or exocytosis. The activation of PKC that accompanies neurotransmitter release may be elicited separately by a rise in intracellular calcium (by depolarization or a calcium ionophore) or by addition of exogenous calcium-independent PKC activators (either phorbol esters or arachidomic acid [AA]) (*see below*). These activators work synergistically to stimulate release (Tanaka et al., 1984; Zurgil and Zisapel, 1985; Taniyama et al., 1990), but appear to operate by distinct biochemical pathways.

The role of PKC in neurotransmitter release has been investigated by various combinations of three basic approaches.

1. Activation of intracellular PKC by addition of one of the following classes of compounds to neuronal cell models: phorbol esters, synthetic cell-permeable diacylglycerols, or fatty acids (such as AA);
2. Inhibition of PKC in intact cells by one of two approaches: cell-permeable PKC inhibitors or downregulation of PKC by chronic phorbol ester treatment; or
3. The most direct approach, microinjection into intact nerve cells of either PKC or antibodies to its specific substrates.

All have inherent advantages, disadvantages, and strict limitations. The use of these approaches to correlate PKC activation with neurotransmitter release is discussed in the following sections.

Phorbol Esters

Many studies have now shown an enhanced release elicited by phorbol esters from synaptosomes (Nichols et al., 1987; Shuntoh et al., 1988; Shu and Selmanoff, 1988; Diaz-Guerra et al., 1988; Davis and Patrick, 1990; Guitart et al., 1990), brain slices (Allgaier and Hertting, 1986; Tanaka et al., 1986; Wang and Friedman, 1987; Versteeg and Ulenkate, 1987; Allgaier et al., 1988; Huang et al., 1988; Dekker et al., 1989b), cultured neurons (Zurgil et al., 1986; Shuntoh et al., 1989; Weiss et al., 1989, Taniyama et al., 1990; Finch and Jackson, 1990) and sympathetic neurons (Tanaka et al., 1984; Wakade et al., 1985; Malhotra et al., 1988), but no effects are observed in the absence of extracellular calcium. This effect of phorbol esters is not mediated by a rise in intracellular calcium (Tanaka et al., 1986; Chandler and Leslie, 1989). Phorbol ester enhancement is often greater at lower concentraions of extracellular calcium (Zurgil and Zisapel, 1985; Zurgil et al., 1986; Nichols et al., 1987; Shuntoh et al., 1988,1989). In many studies, phorbol ester activation of neurotransmitter release from synaptosomes is potentiated by calcium ionophores (Bradford et al., 1983; Tanaka et al., 1984,1986; Nichols et al., 1987; Israel et al., 1987; Shu and Selmanoff, 1988; Taniyama et al., 1990). This has led to a model whereby activation of PKC and hence phosphorylation of specific proteins result in an increased sensitivity of the neurotransmitter release pro-

cess to intracellular calcium levels (Tanaka et al., 1984; Zurgil and Zisapel, 1985; Nichols et al., 1987; Shu and Selmanoff, 1988). Since phorbol esters are likely to be specific for activation of PKC, these studies show that PKC can modulate release.

The studies are limited in that phorbol ester-enhanced release may be unrelated to calcium-evoked release (*see below*). It is also important to note that recent studies have shown that phorbol esters do not activate the isozyme PKC-α (in the absence of a rise in calcium) and will not stimulate phosphorylation of a variety of substrates, such as dephosphin (*see* Dephosphin *below*). Therefore, many PKC substrates, such as dephosphin, are unlikely to be involved directly in phorbol ester-enhanced release. Furthermore, the effects of phorbol esters may not always be directly mediated by PKC, since for example, PKC might activate other kinases or phosphatases in a cascade process. Specific examples of this are the findings that tyrosine kinase phosphorylation of a 40-kDa protein in hippocampal slices is stimulated by phorbol ester and muscarinic agonists, an effect that is blocked by the PKC inhibitor H-7 (Stratton et al., 1989), and that PMA stimulates phosphatase 2A in mouse skin (Gschwendt et al., 1989). Such cascades could underlie any of the observed effects of phorbol esters on neurotransmitter release.

Synthetic DAG

Another approach to demonstrating an intracellular role for PKC in a biological process is the use of cell-permeable analogs of diacylglycerol, such as l-oleoyl-2-acetylglycerol (OAG) or 1,2-dioctanoylglycerol (DiC8), which activate intracellular PKC. Note, however, that such agents may not mimic all instances of PKC activation unless accompanied by a simultaneous increase in intracellular calcium. Application of DAG in concert with calcium ionophores is not done in all studies and may partly account for the lack of success of the approach common to many studies. There are few studies examining the effects of OAG or DAG on neurotransmitter release in neuronal models. In one, dopamine and serotonin release from striatal synaptosomes was stimulated by PMA or OAG, and this was paralleled by activation of cytosolic PKC (Davis and Patrick, 1990). Similarly, OAG enhances synaptosomal glutamate release (Lynch and Bliss, 1986). Dop-amine release from retinal neurons or from PC12 cells is also increased by OAG, the former effect being blocked by H-7 or staurosporine (Kato et al., 1990; Pozzan et al., 1984). One of the specific neuronal effects of OAG or DOG is inhibition of voltage-gated potassium channels, an effect blocked by PKC inhibitors (Colby and Blaustein, 1988; Doerner et al., 1988). These agents can also recruit previously covert calcium channels in Aplysia neurons (Strong et al., 1987). In nonneuronal models of exocytosis, a variety of studies have also found an enhancement of exocytosis by OAG that is potentiated by calcium ionophores (Gerrard et al., 1989; Roldan and Harrison, 1990; Chakravarty et al., 1990; Chakravarty, 1990).

Arachidonic Acid

In addition to triggering neurotransmitter release, calcium also increases intracellular AA in neuronal and many other secretory cells, predominantly through the action of calcium-dependent phospholipases, such as PLA_2 (reviewed by Shimizu and Wolfe, 1990). (Alternatively, AA is generated by the PLC pathway by phosphorylation of diacylglycerol to phosphatidic acid by diacylglycerol kinase.) AA is metabolized either by cyclooxygenase (aspirin or indomethacin inhibited) to prostaglandins and thromboxanes or by lipoxygenases (nordihydroguaiaretic acid [NDGA] inhibited) to leukotrienes and lipoxins, or it is autooxidized. Fatty acids are normally present in low concentrations in the brain, but accumulate after a number of pathological conditions, such as hypoxia, hypoglycemia, and electroconvulsive seizures. AA is capable of interacting with other intracellular second messenger systems by stimulating phospholipase C, guanylate cyclase and PKC, and by inhibition of calcium-dependent neutral proteases.

The generation of AA within the nerve terminals by activation of PLA_2 is associated with neurotransmitter release from synaptosomes (Bradford et al., 1983; Asakura and Matsuda, 1984). Application of AA to synaptosomes, brain slices, or chromaffin cells elicits release, in the presence or absence of calcium (Asakura and Matsuda, 1984; Rhoads et al., 1983; Lynch and Voss, 1990; Koda et al., 1989; Taniyama et al., 1990). Linoleic, Linolenic, and oleic acids elicit the same responses, but less potently (Rhoads et al., 1983; Asakura and Matsuda, 1984). Often the AA signal persists, but in synaptosomes, it may be transient since AA is rapidly metabolized (Asakura and Matsuda, 1984). The lipoxygenase metabolites of AA, HETE, and HPETE, also elicit release when applied to synaptosomes (Lynch and Voss, 1990). AA also stimulates calcium influx into synaptosomes, an effect that is indomethacin-inhibited and mimicked by addition of prostaglandins (Kandasamy and Hunt, 1990). In synaptosomes, application of indomethacin to block metabolism of AA has no effect on depolarization-dependent release, indicating that prostaglandins and thromboxanes are not obligatory for this mode of release (Shu and Selmanoff, 1988). AA also promotes exocytosis from a variety of other secretory cells in the presence or absence of calcium: PC12 cells (Matthies et al., 1987), chromaffin cells (Koda et al., 1989; Tachikawa et al., 1990), islet cells (Metz, 1988), pituitary cells (Chang et al., 1986), and pancreatic acinar cells (Wooten and Wrenn, 1988). In permeabilized chromaffin cells, AA-stimulated release is dependent on MgATP (Koda et al., 1989), supporting a role for protein kinases in mediating the effect. As suggested for phorbol esters above, AA-enhanced release may be a distinct biochemical pathway to calcium-evoked release, since it is abolished by PKC inhibitors or PKC downregulation, whereas calcium-evoked release is not (Morgan and Burgoyne, 1990).

The major intracellular receptor for AA may be PKC, and the role fatty acids play in neurotransmitter release may therefore be mediated by PKC. Arachidonic acid and other *cis*-unsaturated fatty acids (Murakami et al., 1986; O'Brian and Weinstein, 1987; Sekiguchi et al., 1987; El Touny et al., 1990) or some of their oxygenation products (Hansson et al., 1986; O'Brian et al., 1988; Shearman et al., 1989) activate PKC by directly binding to the enzyme. This activation occurs in the presence or absence of calcium. Like the other activators, fatty acid activation resuts in translocation of protein kinase C from the cytosol to the membrane (Boscá et al., 1989), but unlike other activators, Ca/AA does not appear to induce autophosphorylation (El Touny et al., 1990). Although fatty acids do not interact with the PS/diacylglycerol binding site of protein kinase C, they may activate via either a distinct site or one that overlaps with the PS site (O'Brian and Weinstein, 1987; Murakami et al., 1986; El Touny et al., 1990). Fatty acid activation of PKC is synergistic with diacylglycerol activation in vitro or in intact cells (Shinomura et al., 1991). It is likely that fatty acids activate only the soluble form of PKC, since they do not interact with membrane-bound forms (Dell and Severson, 1989; El Touny et al., 1990).

All isozymes of PKC so far examined are activated by fatty acids (Wooten and Wrenn, 1988; Naor et al., 1988; Sekiguchi et al., 1987; Leibersperger et al., 1990), but PKC-γ is the most sensitive (Naor et al., 1988; Shearman et al., 1989), whereas PKC-α (which is the most widespread isozyme) responds with by far the greatest degree of stimulation (Sekiguchi et al., 1987). Therefore, selective activation of PKC-γ may be possible in the low micromolar range of AA, whereas higher concentrations may give a powerful stimulation of PKC-α. However, in hippocampal synaptosomes, which lack PKC-γ, AA and other fatty acids still activate PKC-α and PKC-β, suggesting that all isozymes may be stimulated (Shearman et al., 1991). AA or oleic acid also stimulates phosphorylation by PKC of a variety of cellular proteins in vitro (Hansson and Ingelman-Sundberg, 1987; Wooten and Wrenn, 1988), including dephosphin (Robinson and Lovenberg, 1986), or in intact cells (Halenda et al., 1989; Fan et al., 1990). For example, basal phosphorylation of MARCKS by PKC is stimulated by addition of AA to intact synapto-

somes, but depolarization-dependent phosphorylation is unaltered (Piomelli et al., 1989). This supports the idea that PKC may play a role in priming the nerve terminal to respond to calcium influx with enhanced release.

Other biochemical actions of AA could also play a role in intact cells. One such action is inhibition of CaM-PK-II, and not CaM-PK-I or III (Piomelli et al., 1989). This specific inhibition is exerted on the regulatory region of the kinase. Inhibition also occurs on application of AA to intact synaptosomes, where phosphorylation of synapsin I by CaM-PK-II is inhibited.

PKC Inhibitors

Studies with PKC inhibitor effects on neurotransmitter release reveal clear biochemical distinctions between evoked and enhanced release, with only the latter usually being sensitive to inhibitors. The PMA enhancement of neurotransmitter release is always prevented by PKC inhibitors H-7, polymyxin B, staursporine, or sphingosine, providing support that PKC is involved (Allgaier and Hertting, 1986; Tanaka et al., 1986; Wang and Friedman, 1987; Versteeg and Ulenkate, 1987; Feuerstein et al., 1987; Allgaier et al., 1987,1988; Daschmann et al., 1988; Shuntoh et al., 1988; Shu and Selmanoff, 1988; Dekker et al., 1989b,1990b). Similarly, AA activation of neurotransmitter release from Purkinje cells is also inhibited by the PKC inhibitors sphingosine and polymyxin B, suggesting that PKC may also be the intracellular target for the effects of AA on neurotransmitter release (Taniyama et al., 1990). It is not surprising that all effective PKC inhibitors should block the enhancement of release produced by direct PKC activators.

However, calcium- or depolarization-evoked release is insensitive or only partially sensitive to most PKC inhibitors, such as H-7 or the pseudosubstrate peptide inhibitor PKC_{19-31} (Matthies et al., 1987; Wang and Friedman, 1987; Daschmann et al., 1988; Tachikawa et al., 1990; Guitart et al., 1990; Dekker et al., 1991b). These inhibitors are known to be potent and moderately selective for PKC in intact cells (Hidaka and Hagiwara, 1987; Ohta et al., 1988; Schachtele et al., 1988) and always inhibit the PMA- or AA-enhanced component of neurotransmitter release. Further evidence that enhanced and evoked release involve different biochemical pathways came from studies with acetylcholine release from *Torpedo* synaptosomes (Guitart et al., 1990). In this study, PMA-enhanced release was abolished by H-7, whereas depolarization-evoked release was unaffected, as found for most such studies. Conversely, botulinum toxin, a strong blocker of evoked release, had no effect on enhanced release (Guitart et al., 1990), strongly supporting distinct underlying mechanisms. Similarly, with the use of permeabilized chromaffin cells and the peptide pseudosubstrate inhibitor of PKC (PKC_{19-31}), inhibition of the PMA-enhanced component of release can occur without effect on calcium-evoked secretion (TerBush and Holz, 1990). Complementary results were obtained with AA-enhanced release using staurosporine (Morgan and Burgoyne, 1990). This suggests that activation of PKC is not required for evoked release, but is a modulator, and that PKC only plays a direct role in enhanced release.

There appears to be one major exception to the above inhibitor results. In neuronal cells, polymyxin B, which is a widely used PKC inhibitor common to most of these studies, effectively inhibits both enhanced and evoked release (Allgaier and Hertting, 1986; Tanaka et al., 1986; Versteeg and Ulenkate, 1987; Feuerstein et al., 1987; Dekker et al., 1989b,1990b,1991b; Tachikawa et al., 1990). However, polymyxin B is a potent inhibitor of other enzymes apart from PKC, such as CaM-PK-II (equipotent with PKC; P. J. Robinson, unpublished), myosin light chain kinase (Mazzei et al., 1982), calcium channels, PLA_2, and calcium-stimulated potassium channels (Qi et al., 1983; Chang et al., 1987; Greenberg et al., 1987; Varecka et al., 1987). Most importantly, polymyxin B has been shown to inhibit CaM potently (Mazzei et al., 1982; Hegemann et al., 1991). Thus, its effects on evoked release may equally be mediated by CaM and could even be used to argue in favor of a role for CaM in mediation of release (e.g., Tachikawa et al., 1990).

Activator-Dependence of PKC Inhibitors

Caution is always required in interpretation of PKC inhibitor studies. There are numerous inconsistencies in the literature regarding the apparent effectiveness of PKC inhibitors to inhibit PMA- or AA-stimulated responses when compared to hormonal or calcium-mediated responses. This concept has led to the suggestion that PKC inhibitors may show "activator—dependence," i.e., the effectiveness of an inhibitor is related to the nature of the activator used—PMA, AA, or Ca/PS (calcium plus phosphatidylserine). This has also been described for in vitro assays of protein kinase C activity, but the underlying mechanisms are not understood (O'Brian and Weinstein, 1987; Ronning and Martin, 1986; Roghani et al., 1987; Schachtele et al., 1988; Seifert et al., 1988; Seifert and Schachtele, 1988; El Touny et al., 1990). For example, rhodamine 6G is 10 times more potent an inhibitor of Ca/AA-activated PKC than of Ca/PS or phosphatidylserine plus PMA- (PS/PMA)-activation (O'Brian and Weinstein, 1987). Chlorpromazine was less potent when PMA was used to activate PKC rather than Ca/PS (Roghani et al., 1987) and sphingosine was more potent in inhibiting PS/DAG activation than fatty acid-activated PKC (El Touny et al., 1990).

Distinct inhibitors of protein kinase C do not always inhibit cellular responses stimulated by the different activators calcium, hormone, PMA, or AA (Ronning and Martin, 1986; Schachtele et al., 1988; Seifert and Schachtele, 1988; Grove and Mastro, 1988; Stevens et al., 1989; Lowe et al., 1990). In exocytotic cells, retinal inhibited hormone- or PMA-induced LH release, but not AA-induced release (Chang et al., 1986), trifluoperazine and polymyxin B were less potent inhibitors of PMA-stimulated prolactin release than calcium-stimulated release (Ronning and Martin, 1986), and the potencies of PKC inhibitors on NADPH oxidase activation in neutrophils appeared to vary with different activators used (Seifert and Schachtele, 1988). There are many further examples where the PKC activator determines the effectiveness or potency of the PKC inhibitor (Grove and Mastro, 1988; Stevens et al., 1989; Lowe et al., 1990; Wright and Hoffman, 1986; Schachtele et al., 1988). Disparate effects of different PKC inhibitors may reflect either distinct PKC-dependent and PKC-independent pathways or the distinct mode of PKC activation employed in each case using either:

1. Depolarization or hormonally induced increases in intracellular calcium (and DAG); or
2. Phorbol ester-induced activation; or
3. AA activation.

There are two underlying, possibly concomitant explanations for this. First, in the case of neurotransmitter release, PKC plays a role only in phorbol ester- and AA-enhanced release, and is not involved in calcium-evoked release. However, the second explanation is that the mechanism of action of each PKC inhibitor differs, and the site of interaction of inhibitor with PKC must be considered in concert with the activator being used. This concept is summarized in Table 1 and Fig. 6. Accordingly, PKC inhibitors can be broadly divided into four classes according to their site of action with the enzyme (operationally defined as competitive inhibition) at:

1. The active sites: the ATP binding site, e.g., H-7 and staurosporine (Hidaka et al., 1984; Tamaoki et al., 1986) or the substrate binding site, e.g., $PKC_{19\text{-}31}$ (House and Kemp, 1987);
2. The DAG or PMA binding site, e.g., sphingosine (Hannun et al., 1986);
3. The phospholipid binding site e.g., palmitoylcarnitine or polymyxin B (Wise and Kuo, 1983; Kiss et al., 1987); or
4. The AA binding site, e.g., rhodamine 6G (O'Brian and Weinstein, 1987).

Disparate effects of inhibitors may reside in the distinct biochemical site of action of each class of protein kinase C inhibitor and would therefore be related to the nature of the activator used to stimulate PKC. A further complication of the use of such inhibitors is their possible effects on other enzymes, as illustrated above for polymyxin B.

Table 1
Grouping of PKC Inhibitors According to Their Site of Interaction with the Enzyme

Inhibitor Class	Inhibitor	Reference
A		
Substrate		
A1-Mg/ATP	H-7	Hidaka et al., 1984
	Staurosporine	Tamaoki et al., 1986
	MDL 27,032	Robinson et al., 1990
	Quercetin	Nakadate et al., 1988
	Erbstatin	Bishop et al., 1990
A2 - Protein	PKC_{19-31}	House and Kemp, 1987
	Chelerythrine	Herbert et al., 1990 B
BPMA	Lipophospho-glycan	McNeely and Turco, 1987
	Sphingosine	Hannun et al., 1986
	Acridine orange	Hannun and Bell, 1988
	Gossypol	Nakadate et al., 1988
	Calphostin	Tamaoki et al., 1990
C		
Phospholipid	Palmitoyl-carnitine	Wise and Kuo, 1983; Nakadate and Blumberg, 1987
	Retinal	Isakov, 1988
	Polymyxin B	Kiss et al., 1987
	Trifluoperazine	Wise and Kuo, 1983
	Tamoxifen	Nakadate et al., 1988
	Adriamycin	Wise and Kuo, 1983; Nakadate et al., 1988
D		
Fatty acid	Rhodamine 6G	O'Brian and Weinstein, 1987

*PKC inhibitors can be divided into the same A–D categories as described in the legend to Fig. 6 (other categories are possible) according to their mode of kinetic inhibition of the enzyme (shown in the reference cited). It is proposed that each inhibitor that interacts in the regulatory domain of the enzyme will more potently inhibit activity when the activator used is also targeted to the same site (however, multiple sites of drug interaction can occur for some inhibitors). Judicious use of PKC inhibitors would first take into consideration which activators were employed and then select inhibitors from group A, as well from groups B–D (as appropriate for the activator—PMA, AA, or calcium/phospholipid). Inhibitors from the remaining groups B–D may have particularly weak effects. Other inhibitors may be added to this list as their mechanisms of action are determined.

Downregulation of PKC

Downregulation of PKC by long-term treatment with phorbol ester prevents the phorbol ester enhancement of release, but only partly affects depolarization-evoked neurotransmitter release from cultured neuronal cells (Matthies et al., 1987) or PC12 cells (Matthies et al., 1988). These studies complement the PKC inhibitor studies above and support the conclusion that PKC is not essential for evoked release, i.e., PKC does not *mediate* release. Downregulation of PKC was also used to demonstrate that the PKC-γ isozyme is probably not involved in PMA-enhanced neurotransmitter release from synaptosomes (Oda et al., 1991). Brief incubation of synaptosomes with PMA produced selective downregulation of PKC-α and PKC-β, but not PKC-γ. In these PKC-γ-enriched synaptosomes, PMA enhancement of release was abolished. Depletion of cellular PKC by downregulation also decreases the effectiveness of PMA or AA to enhance exocytosis, but only partly inhibits evoked exocytosis in nonneuronal cells (Burgoyne et al., 1988; Metz, 1988; Morgan and Burgoyne, 1990; Tachikawa et al., 1990). In chromaffin cells, downregulation of PKC with PMA for 24 h abolishes enhanced secretion, but calcium-evoked secretion is maintained (Burgoyne et al., 1988). In permeabilized chromaffin cells, only AA-enhanced release, rather than calcium-evoked release is abolished after PKC downregulation (Morgan and Burgoyne, 1990). This complements the PKC inhibitor studies described above, and suggests that activation of PKC is obligatory for enhanced, but not evoked neurotransmitter release.

Intracellular Injection of PKC

Microinjection of PKC into *Aplysia* bag cell neurons enhances the calcium action potential by effects on calcium channels (DeRiemer et al., 1985). This effect is distinct from the enhancement produced by PKA in that the latter involves decreases in potassium currents. Modulation of calcium channels is a common feature of PKC activity (Hammond et al., 1987; Rane et al., 1989).

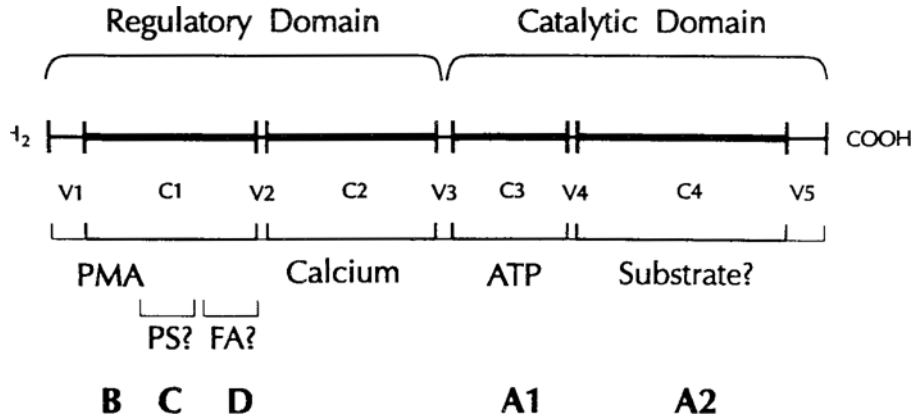

Fig. 6. Model of possible sites of interaction of inhibitors with PKC. The PKC amino acid sequence can be divided into four constant (C1–C4) and five variable domains (V1–V5), based on sequence homology between the isozymes (adapted from Nishizuka, 1988). Isozymes α, βI, βII, and γ can be cleaved into two major fragments, comprising the regulatory or catalytic domains, by trypsin or calpain in the V3 domain. Several sites of interaction of substrates, cofactors, and activators are indicated: **A1**: The ATP binding sequence, Gly–X–Gly–X–X–Gly....Lys, lies within the C3 domain. **A2**: The precise location of the protein substrate binding site is unknown, but may lie in an acidic region of C4 (House et al., 1989). **B**: Phorbol esters, such as PMA, and DAG interact with the zinc fingers of the C1 domain (Ono et al., 1989). **C**: The location of the binding site for phosphatidylserine (PS) and other phospholipids has not been precisely determined, but may lie within C1. **D**: The binding site for unsaturated fatty acids (FA) is also unknown and is designated as lying within the C1 domain in this diagram, since it may overlap with the PS binding site (El Touny et al., 1990). A binding site for calcium is not listed, since it has yet to be shown to interact directly with the kinase, rather than with the acidic phospholipid head group, and no specific competitive inhibitors of calcium have yet been described. Parker originally proposed it to be in the V3 domain (Parker et al., 1986), but since the δ, ε, ζ, and η isozymes (collectively termed nPKC) lack the C2 domain and are calcium-independent, it is also possible that calcium interacts with the C2 domain.

Microinjection of PKC modulates synaptic activity in several different neurons (Zhang and Krnjezic, 1987; Hu et al., 1987; Hammond et al., 1987). These methods were also used to show that PKC also plays a role in synaptic plasticity. In hippocampal slices, microinjection of PKC elicits long-lasting enhancement of synaptic transmission resembling LTP (Hu et al., 1987). Antibodies against B-50 introduced into streptolysin O permeabilized synaptosomes inhibit neurotransmitter release, implicating this protein in neurotransmitter release (Dekker et al., 1989a). These antiB-50 antibodies inhibit only calcium-evoked release, and not PMA-enhanced release (Dekker et al., 1991b), once again suggesting two independent release mechanisms.

Summary of PKC Role in Neurotransmitter Release

The above studies indicate that, in either neuronal or nonneuronal cells, secretion of neurotransmitters can be divided into at least two components, each of which could involve more than one distinct biochemical pathways: *Evoked* release (calcium- or depolarization-stimulated), which can also be called *mediation*, and *enhanced* release (PMA- or AA-stimulated), a form of *modulation* of release. Other components are also known, but may be unrelated to PKC, such as cAMP-enhanced release or barium-evoked release. Enhanced release is regulated by PKC, particularly since direct PKC activators were used to elicit

the response; it is sensitive to all PKC inhibitor classes, prevented by PKC downregulation, and not inhibited by introducing antiB-50 antibodies, which block evoked release. In synaptosomes, enhanced release would involve isozymes PKC-α and/or PKC-β, since PKC-γ plays no role in enhanced release, nor is its presence required for evoked release (Oda et al., 1991). Evoked release, however, does not directly involve PKC. Evoked release is insensitive or poorly sensitive to otherwise highly effective PKC inhibitors, such as H-7, staurosporine, or PKC_{19-31}. Furthermore, downregulation of PKC does not abolish neurotransmitter release, and often has little effect on evoked release (Matthies et al., 1987; Morgan and Burgoyne, 1990). Conversely, botulinum toxin prevents evoked release without blocking enhanced release (Guitart et al., 1990). Therefore, PKC enhancement of neurotransmitter release occurs by a mechanism that is distinct from that which regulates evoked release. However, PKC may still play an indirect role in evoked release by *priming*—or phosphorylating—a key protein that itself mediates release. This PKC substrate would be distinct from that which controls enhanced release.

The Priming Model of Neurotransmitter Release

Protein phosphorylation, and in particular PKC, appears to "prime" the exocytotic or neurotransmitter release process (Howell et al., 1989; Plattner, 1989; Holz et al., 1989; TerBush and Holz, 1990) by phosphorylation of an unspecified nerve terminal phosphoprotein. This phosphoprotein would then directly *mediate* release, or indirectly mediate release via its dephosphorylation or its phosphorylation-dependent interaction with calcium. Thus, phosphorylation of a specific protein may be required for release to occur. Enhanced phosphorylation of such a protein could enhance the subsequent release, whereas decreased phosphorylation may reduce subsequent release. The priming model for neurotransmitter release accounts for the apparent requirement for calcium and Mg-ATP, the correlation of release with phosphorylation and dephosphorylation, and the role of PKC and its modulators (phorbol esters, fatty acids) in regulation of release. However, it leaves open the identity of the specific target for calcium, which could be the phosphoprotein itself, its phosphatase, or other intermediary protein, such as CaM.

Support for the *priming* model comes from a variety of studies in neuronal or nonneuronal systems. In cultured neuronal cells, downregulation of PKC by chronic phorbol esters abolishes phorbol ester-enhanced neurotransmitter release, but only partly inhibits depolarization-evoked release (Matthies et al., 1987). Similarly, PKC inhibitors completely prevent phorbol ester-enhanced release from brain cortical slices, but only weakly inhibit depolarization-stimulated release (Wang and Friedman, 1987). Additional support for the priming model of release comes from studies with nonneuronal models. In permeabilized chromaffin cells, calcium or PMA enhance noradrenaline release; however, in PKC downregulated cells, calcium-induced release is maintained and is unaffected by a variety of PKC inhibitors (Tachikawa et al., 1990). In these cells, intracellular Mg-ATP primes the cells so that the initial component of secretion is independent of exogenous ATP and the ATP partially maintains the primed state by acting before calcium in the secretory pathway (Holz et al., 1989). Priming of chromaffin cells for release can also occur by pretreatment with calcium-dependent phospholipase A_2 (PLA_2) and fusion can then still occur in the absence of calcium (Karli et al., 1990). This process was thought to be mediated by an AA metabolite, which may in turn activate PKC. Priming has also been linked to exocytotic secretion from chromaffin cells by thiophosphorylation experiments. Thiophosphorylation of permeabilized cells retards protein dephosphorylation and blocks secretion (Brooks et al., 1984; Brooks and Brooks, 1985; Plattner, 1989). Similar experiments have not been performed in nerve terminals, although thiophosphorylation of

extracellular proteins had no effect on release of GABA or dopamine (Hauptmann et al., 1985). There are few known candidates for a synaptic phosphoprotein that might prime release. In intact synaptosomes, three proteins, dephosphin, P124, and P139, are rapidly dephosphorylated upon calcium influx and neurotransmitter release that could be candidates for the priming protein. Of these three, only dephosphin is presently known to be a PKC substrate, and the scope of this article is restricted to PKC substrates.

Neuronal Substrates of PKC

Given the strong association between PKC activity and neurotransmitter release, it is essential to determine what the nerve terminal targets of PKC might be. Only four synaptosomal substrates for PKC have been recognized in intact synaptosomes or nerve cells, although multiple substrates have been detected in synaptosomal cytosolic fractions (Wrenn et al. 1980; Kuo et al., 1984; Robinson and Lovenberg, 1988). The first three are MARCKS, B-50, and tyrosine hydroxylase (Dunkley et al., 1986; Rodnight and Perrett, 1986; Rodnight et al., 1986; Yip and Kelly, 1989), all of which are phosphorylated in intact synaptosomes after depolarization-dependent calcium entry (Dunkley et al., 1986; Rodnight and Perrett, 1986; Yip and Kelly, 1989). B-50 (but not its PKC phosphorylation) has been associated with a mediatory role in neurotransmitter release. The fourth protein is a novel PKC substrate, dephosphin, that is dephosphorylated on depolarization and is subsequently phosphorylated by PKC after the stimulus is removed (repolarization) (Robinson, 1991). These proteins will be reviewed in detail below.

Dephosphin

Dephosphin is a synaptic phosphoprotein in intact nerve terminals that is rapidly dephosphorylated upon depolarization and calcium influx (Robinson and Dunkley, 1983,1985; Robinson et al., 1987; Robinson, 1987). The rapid dephosphorylation of dephosphin precedes the phosphorylation of any other protein in the nerve terminal. Although dephosphorylation is rapid in response to depolarization (<2 s), dephosphin is more slowly rephosphorylated upon removal of the depolarization stimulus (<2 min) (Robinson et al., 1987). Dephosphorylation is fully reversible for at least five cycles of depolarization and repolarization, indicating a physiological role for the process (Fig. 2). Phosphorylation of dephosphin is intimately linked to the membrane potential, and dephosphorylation requires both calcium influx into the nerve terminals and depolarization of the membrane (Robinson et al., 1984; Robinson and Dunkley, 1985; Robinson et al., 1987) (Fig. 3). These properties suggest a dynamic role for the protein in nerve terminal function.

A 96,000-dalton phosphoprotein (called P96) was also described in synaptosomal cytosol and is a substrate of PKC (Robinson et al., 1987; Robinson and Lovenberg, 1988) (Fig. 4). This is illustrated in Fig. 4, where various activators of PKC are used to stimulate phosphorylation of endogenous substrates in nerve terminal cytosol. Phosphorylation of MARCKS is stimulated by PS/PMA (without calcium), whereas phosphorylation of P96 and many other substrates was insensitive to PS/PMA, but stimulated by Ca/PS (Fig. 4). This in vitro PKC substrate runs on longer polyacrylamide gels as a doublet of 96,000- and 93,000-dalton subunits, both of which are indistinguishable from dephosphin (also a doublet) in intact synaptosomes (Fig. 5). Dephosphin labeled in vitro or in intact synaptosomes also had identical isoelectric points and phosphopeptide maps, and were all phosphorylated on serine residues (Robinson, 1991). Therefore, dephosphin is an in vitro substrate of PKC and *in situ* phosphorylated protein.

Dephosphin is also a PKC substrate in intact synaptosomes. To demonstrate this, a novel approach was required since the protein appeared to be already highly phosphorylated in the resting nerve terminal (Fig. 7). Therefore, intact synaptosomes were briefly depolarized to stimulate

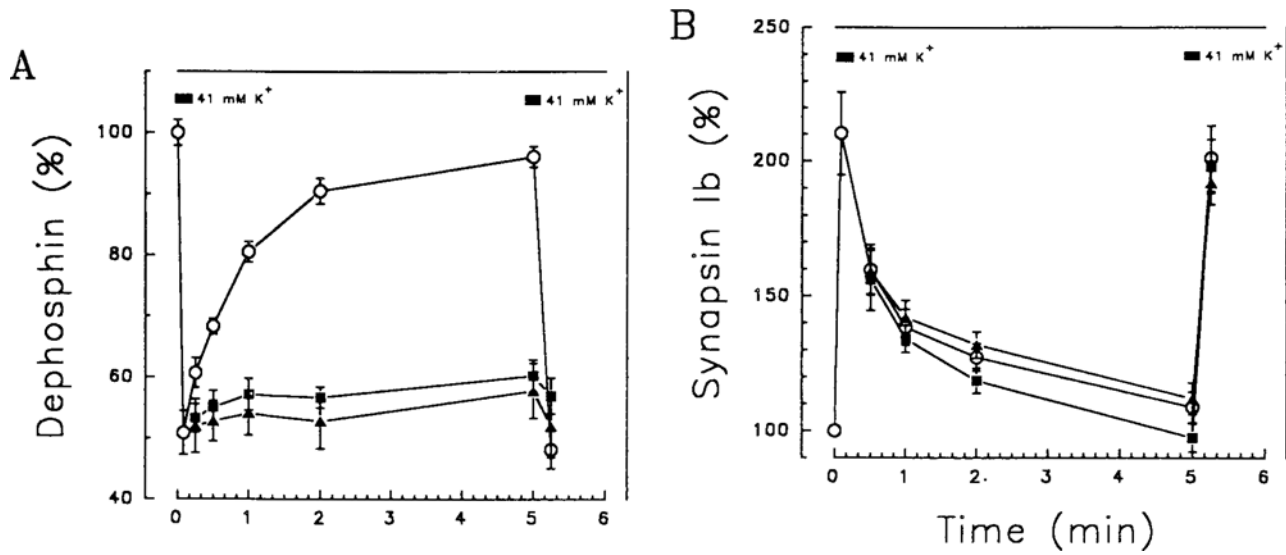

Fig. 7. Dephosphin is phosphorylated by PKC in intact synaptosomes. The figure shows the effect of protein kinase C inhibitors on dephosphin rephosphorylation (**A**) or on synapsin Ib phosphorylation (**B**) after 41 m*M* K^+ depolarization. Synaptosomes were prelabeled with $^{32}P_i$ for 60 min, washed, and depolarized with 41 m*M* K^+ for 10 s (indicated by the solid bars at the top). H-7 (75 μ*M*, ▲) or palmitoylcarnitine (200 μ*M*, ■) was added to controls (○) 5 min prior to the first depolarizing stimulus and was present for all subsequent events. After depolarization, the synaptosomes were repolarized by resuspension in low K^+ buffer for the indicated times. At the end of the repolarization period, the synaptosomes were subjected to a second depolarization stimulus. Samples were run on a polyacrylamide gel and autoradiographed. Dephosphin was almost fully rephosphorylated within 2 min after repolarization. The inhibition of rephosphorylation by PKC inhibitors shows that PKC is the dephosphin kinase. The specificity of the inhibitors was checked using phosphorylation of synapsin Ib by CaM-PK-II as a control, which was completely unaffected by either inhibitor. Dephosphin and synapsin Ib phosphorylation were determined by densitometry of the autoradiographs, and results are presented as a percent of phosphorylation in unstimulated synaptosomes for 11 (control curves) or 6 (in the presence of inhibitors) experiments.

dephosphorylation, and the stimulus was then removed (repolarization) to allow rephosphorylation of dephosphin. Rephosphorylation was essentially complete within 2 min. The effect of PKC inhibitors on rephosphorylation was examined. Preincubation of the synaptosomes with the PKC inhibitors H-7, palmitoylcarnitine (Fig. 7A), polymyxin B, or MDL 27,032 completely prevented dephosphin rephosphorylation. The inhibitors also blocked phosphorylation of MARCKS, a known synaptosomal substrate of PKC (not shown), but were without effect on depolarization-dependent synapsin Ib phosphorylation by CaM-PK-II (Fig. 7B), indicating a relative specificity of the inhibitors for PKC. In parallel experiments, the intrasynaptosomal calcium concentration was determined with fura-2 fluorescence (Fig. 8). Depolarization produced an immediate increase in intracellular calcium that was restored to basal levels almost immediately on repolarization. Therefore, the increase in dephosphin phosphorylation during rephosphorylation occurs in the absence of a rise in intracellular calcium. It is not known how PKC might stimulate dephosphin phosphorylation in the absence of such a rise in intraterminal calcium.

Other Properties and Purification

Other properties of dephosphin suggest that it may be nervous system-specific. It is concentrated in nerve endings, and not found in kidney, skeletal muscle, heart, liver, lung, testes, adrenal gland, or salivary glands, although its presence in low concentrations in other tissues cannot yet

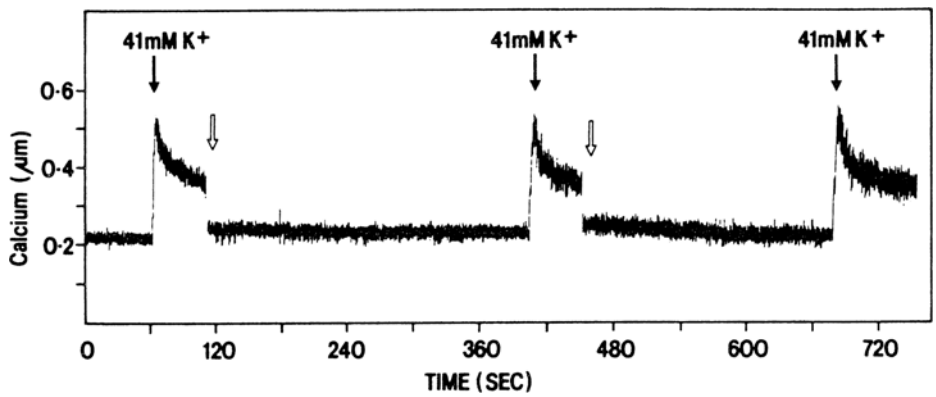

Fig. 8. Repolarization of synaptosomes rapidly restores resting intracellular Ca^{2+} levels. Synaptosomes were incubated under the same conditions as described in Fig. 5, except for the absence of $^{32}P_i$ and the presence of fura-2-am for 20 min. After washing, intracellular free Ca^{2+} was determined (Stauderman and Pruss, 1989) during depolarization (filled arrows) and repolarization (open arrows), using the same times described above. Depolarization produced a very rapid rise in intracellular free Ca^{2+} that decreased, but remained elevated as long as the stimulus was maintained. Repolarization produced an apparently equally rapid return of intracellular levels to baseline. Three cycles of depolarization and repolarization of the same synaptosomes produced the same effect each time. Particularly, note that during the first 2 min of repolarization intracellular Ca^{2+} is unchanged from basal levels; however, dephosphin phosphorylation is occurring over the same time frame (Fig. 5).

be ruled out owing to the sensitivity limit of the assay (Fig. 9). Dephosphin is also present in fresh human brain synaptosomal cytosol, where its molecular weight and phosphopeptide maps are identical to that of the rat (not shown). This may indicate little evolutionary modification of the protein in mammals, but also indicates a potential role for the protein and PKC in human brain.

Although originally shown to be a cytosolic substrate of PKC (Robinson, 1991), dephosphin is also associated with membranes. Subcellular fractionation of rat brain synaptosomes reveals that its in vitro phosphorylation can readily be detected in brain synaptic vesicles (Fig. 9A), but not in synaptic membranes, suggesting that it may associate with the synaptic vesicle population. In homogenized whole brain, < 5% of the dephosphin is soluble; the remainder can be dissociated from total brain membranes by modest increases in ionic strength (Fig. 10). Thus, it is released by low concentrations of Mg^{2+}, EGTA, NaCl, or KCl. However, Ca^{2+} does not elute dephosphin from membranes (Fig. 10). Rather, Ca^{2+} stabilizes the membrane-bound form so that NaCl is less effective in releasing bound dephosphin (not shown). This suggests that calcium modulates the subcellular location of dephosphin. Therefore, the protein is predominantly a peripheral membrane protein that is also associated with synaptic vesicles, and Ca^{2+} stabilizes this association.

A scheme has recently been developed for the purification of dephosphin from rat brain. Initially, rat brain is homogenized in the presence EGTA and EDTA, and membranes prepared. Dephosphin is eluted from the membranes by homogenization in 100 m*M* NaCl, resulting in an extract that is highly enriched in dephosphin. Further purification is achieved by Q-sepharose, S-sepharose, and hydroxyapatite chromatographies, to about 99 % homogeneity. The purified protein is also a substrate for purified rat brain PKC, but, as described above in synaptosomal cytosol, it is not or is poorly phosphorylated when PS/PMA is the activator (Fig. 11).

Lack of Effect of Phorbol Esters

Surprisingly, when intact synaptosomes were treated with 200 n*M* PMA to activate intracellular PKC, there was no effect on dephosphin phos-

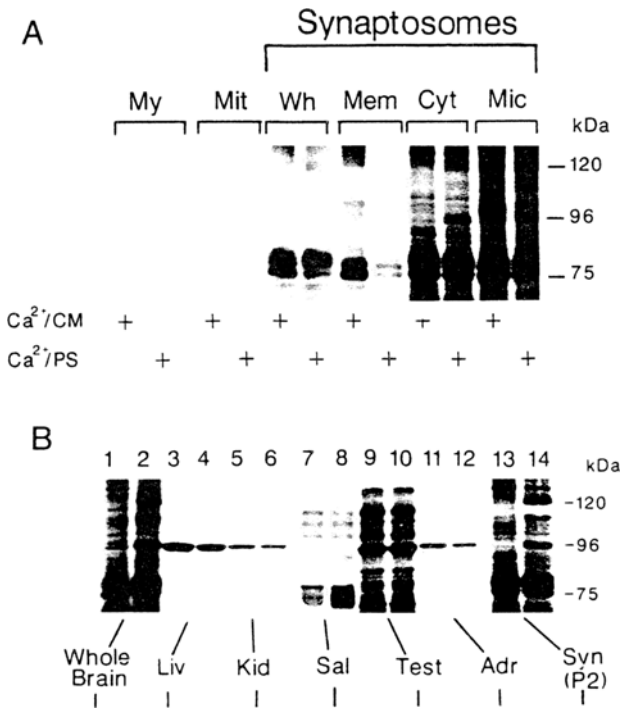

Fig. 9. Localization of dephosphin phosphorylation to neuronal tissue and to the cytosolic and synaptic vesicle fractions. **A**: Synaptosomes were prepared by sucrose density gradient to produce myelin (My), mitochondrial (Mit), and synaptosomal (Wh) fractions. The purified synaptosomes were further subfractionated to synaptic plasma membranes (Mem), cytosol (Cyt), and synaptic vesicles (includes other microsomes, Mic). Each fraction was phosphorylated for 30 s with [γ-^{32}P]-ATP and endogenous kinases in the presence of Ca^{2+}/PS or Ca^{2+}/CaM as described above. The autoradiograph shows that phosphorylation of dephosphin could be detected only in the cytosolic and synaptic vesicle fractions. The identity of dephosphin was verified by phosphopeptide mapping using V8 protease (not shown). **B**: Cytosolic fractions were prepared from 100,000*g* supernatants of homogenized whole brain, liver (Liv), kidney (Kid), salivary glands (Sal), testes (test), adrenals (Adr), or crude P2 synaptosomes (Syn P2). Each fraction was phosphorylated for 60 s in the presence of exogenous purified rat brain PKC and either Ca^{2+}/CaM (odd lanes) or Ca^{2+}/PS (even lanes). PKC stimulated phosphorylation of dephosphin only in brain or synaptosomes. The 94-kDa phosphoprotein detected in most other tissues correlates with the size, phosphorylation characteristics, and tissue distribution of phosphorylase b, and was not dephosphin, since it had a distinctly different peptide map (not shown).

phorylation, although phosphorylation of B-50 (not shown) and MARCKS (Fig. 12) were stimulated, indicating that PKC was indeed activated. The lack of effect of PMA on dephosphin in intact cells was also found in vitro, where PKC, stimulated by phosphatidylserine plus PMA (PS/PMA), did not support dephosphin phosphorylation in synaptosomal cytosol, despite stimulation of MARCKS and B-50 (Fig. 4). Purified rat brain dephosphin is also an extremely poor substrate for purified rat brain PKC, which is stimulated by PS/PMA (Fig. 11). Of other commercially available substrates tested, PS/PMA did not support casein phosphorylation either (Fig. 11). Myelin basic protein was phosphorylated to a greater extent by PS/PMA, histone to a lesser extent, and protamine phosphorylation was activator-independent. Therefore, the lack of effect of phorbol esters on dephosphin phosphorylation is not unique. This suggests that PS/PMA alters the apparent substrate specificity of PKC such that it phosphorylates a distinct group of proteins to those phosphorylated in the presence of Ca/PS. Similar observations have been made by others, but could not be explained (Kreutter et al., 1985; Kiss and Luo, 1986; Yamamoto et al., 1988; Kumar et al., 1987). Studies on the effect of PS/PMA on the autophosphorylation of PKC showed that PS/PMA induces autophosphorylation of all major calcium-dependent PKC isozymes, but autophosphorylation of PKC-α results in a retarded mobility in polyacrylamide gels (Fig. 13) and suggests that PS/PMA has unique effects on this isozyme.

The biochemical basis of the lack of phorbol ester activation lies partly in the finding that at least one isozyme of PKC differs in substrate specificity and in its ability to be activated by phorbol esters. PKC from rat brain was separated into the four major isozymic forms by hydroxyapatite chromatography (House et al., 1989), and only three (βI, βII, and γ) isozymes were activated by PS/PMA (Fig. 14). Recent independent findings on the comparatively rare isozymes δ and ε, indicate that they also are activated by PS/PMA. Activation of PKC by PS/PMA in the absene of

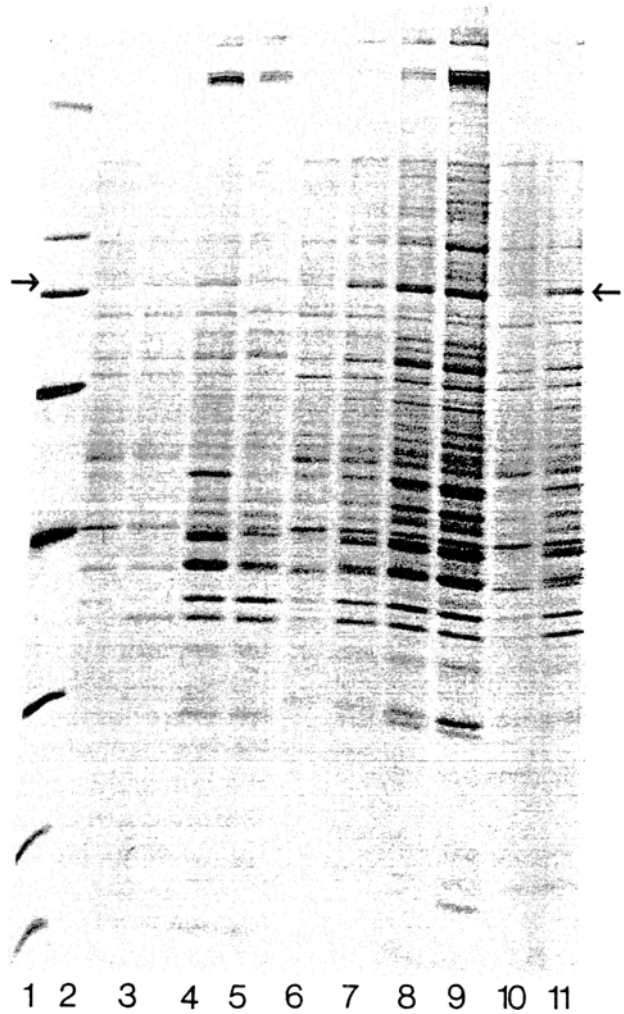

Fig. 10. Dephosphin is a peripheral membrane protein. A Coomassie blue-stained polyacrylamide gel is shown, indicating the extraction of dephosphin from homogenized brain membranes by small to moderate increases in ionic strength. Brain was homogenized in the presence of 1 m*M* EGTA and 1 mM EDTA and centrifuged at 48,000*g* for 30 min. The pellet was rehomogenized twice more in the same buffer to remove contaminating soluble proteins. The membranes were then divided into 10 aliquots and homogenized in the presence of various agents of differing ionic strengths, incubated 30 min on ice, and recentrifuged. An equal aliquot of each supernatant was run on the gel. The extraction buffers were based on the original homogenization buffer (lane 2) and included either 10 m*M* EGTA (lane 3), 10 m*M* Mg^{2+} (lane 4), 10 m*M* Ca^{2+} (lane 5), NaCl at 10, 50, 100, or 250 m*M* (lanes 6–9), or KCl at 10 or 50 m*M* (lanes 10,11). Dephosphin was fully extracted by 100 m*M* NaCl, and higher concentrations simply extracted other proteins as well. Small amounts of dephosphin (about 5%) were extracted by 10 m*M* EGTA, NaCl, or KCl. Significantly more dephosphin was extracted with 10 m*M* Mg^{2+}, although Mg^{2+} more selectively extracted other proteins. These results suggest that modest changes in ionic strength release membrane-bound dephosphin. Note, however, that 10 m*M* Ca^{2+} did not extract any dephosphin (a faint band in lane 5 ran with a higher molecular weight and could not subsequently be phosphorylated by PKC), despite the fact that it extracted most of the same proteins as were extracted by Mg^{2+}. In contrast, Ca^{2+} apparently stabilized the dephosphin association with membranes, since in other experiments, its addition greatly decreased the extraction of dephosphin normally achieved by 100 m*M* NaCl (not shown). This raises the possibility that dephosphin is a Ca^{2+} binding protein. Molecular-weight standards were run in lane 1 (in kDa from the top, 200, 116, 94, 67, 43, 30, 20.4, and 14.4).

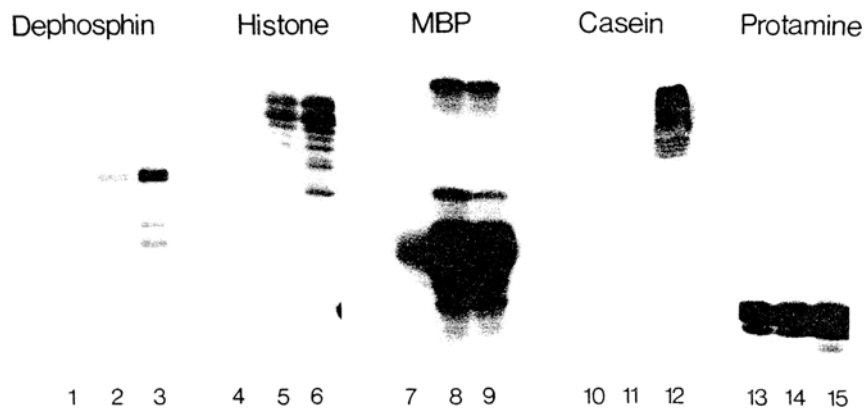

Fig. 11. Autoradiograph showing the phosphorylation of purified dephosphin by PKC, but not in the presence of phorbol esters. Purified rat brain PKC (mixture of isozymes a, b, and g) was used to phosphorylate purified dephosphin (200 ng/lane) or a variety of commercially available substrates (2 μg/lane). Phosphorylation was for 5 min in the presence of PS (40 μg/mL, lanes 1, 3, 7, 10, and 13), PS/PMA (200 n*M*, lanes 2, 5, 8, 11, and 14), or Ca^{2+}/PS (100 μ*M*, lanes 3, 6, 9, 12, and 15). Note that Ca^{2+}/PS stimulated the phosphorylation of each substrate, but the response of each substrate to the activators PS/PMA varied markedly. Only dephosphin and casein were not phosphorylated in the presence of PS/PMA. Histone was a poorer substrate for PS/PMA activation, whereas myelin basic protein was a better substrate for PS/PMA activation than Ca^{2+}/PS activation. Therefore, the apparent substrate specificity of PKC is determined by the activators used, and dephosphin is not a substrate for phorbol ester-activated PKC. Note that for the dephosphin panel the high molecular weight part of an autoradiograph is presented, whereas for all other lanes, the low-molecular-weight region of the autoradiograph is shown.

calcium has been oserved by others, but remained unexplained (Vandenbark et al., 1984; Coutuier et al., 1984; Ashendal, 1985; Arcoleo and Weinstein, 1985; Bazzi and Nelsestuen, 1989). As little as 50 n*M* PMA activates PKC and causes an irreversible translocation of the kinase to the membranes, an effect not seen with DAG (Bazzi and Nelsestuen, 1989), and results in calcium-independent kinase activity (Bazzi and Nelsestuen, 1988). Therefore, only one form, PKC-α, does not respond to phorbol ester activation. Since this isozyme is the most widely distributed form, this result modifies the widely held notion that phorbol esters are the best tool for demonstrating PKC involvement in signal transduction. Furthermore, the results predict that dephosphin could be a substrate for only PKC-α. This is a particularly interesting possibility, since few substrate specificity differences have been detected for PKC isozymes.

Is Calcineurin the Dephosphin Phosphatase?

CN coud be the dephosphin phosphatase (Robinson et al., 1987; Robinson, 1987; Wang et al., 1988; Sim, 1991). Dephosphin dephosphorylation is calcium-dependent (Robinson and Dunkley, 1983a; Robinson et al., 1987), inhibited by phenothiazine inhibitors of PKC and CN (Robinson et al., 1984), and only partially sensitive to low concentrations of okadaic acid, the potent inhibitor of phosphatases 1 and 2A (Haystead et al., 1989), but fully inhibited by higher concentrations of okadaic acid, which are known to inhibit CN (Sim et al., 1991; Sim, 1991). The okadaic acid studies show that phosphatases 1 and 2A probably do not dephosphorylate dephosphin and implicate CN as the major dephosphin phosphatase, but more specific experiments are still required.

The mechanism of phosphorylation of dephosphin in intact synaptosomes has been partly elucidated, and has revealed new principles of modes of activation of PKC in living cells (summarized in Fig. 1). Dephosphin is phosphorylated by PKC in resting synaptosomes. Depolarization activates calcium influx and PKC, but does not increase dephosphin phosphorylation because calcium also activates a dephosphin

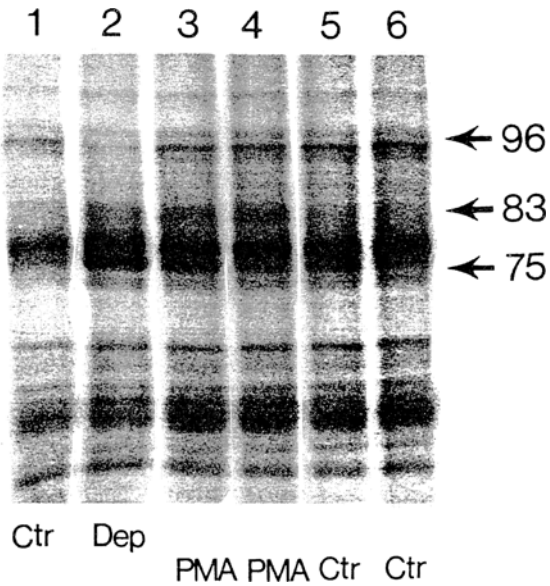

Fig. 12. Autoradiograph showing phorbol ester stimulation of phosphorylation of MARCKS, but not dephosphin in intact synaptosomes. Synaptosomes were prelabeled with $^{32}P_i$; for 60 min, and washed to remove free $^{32}P_i$. Synaptosomes were then incubated for 15 s in control (Ctr, lane 1) or 41 m*M* K^+ buffer (Dep, lane 2), or for 5 min in the presence (lane 3 and 4) or absence (lane 5 and 6) of 200 n*M* PMA, and the proteins separated on a 7.5% acrylamide gel to resolve the MARCKS protein (83,000 daltons) from the synapsin I doublet (80,000 and 75,000 daltons). Phorbol esters clearly stimulated the phosphoryl-ation of MARCKS and of B-50 (not shown in this gel), but never had any effect on dephosphin phosphorylation in at least nine experiments.

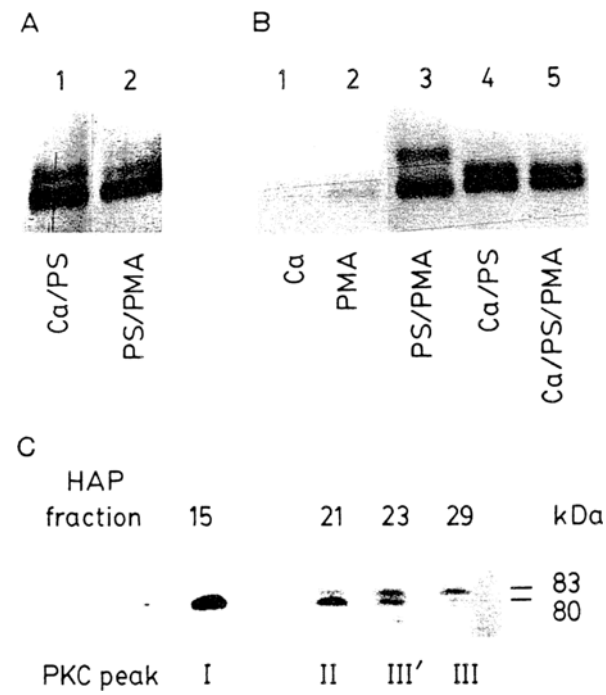

Fig. 13. Effect of phorbol ester on PKC autophosphorylation. Purified rat brain PKC was autophosphorylated in the presence of either Ca/PS or PS/PMA (as described above), and the proteins separated on a gel. A silver-stained gel is shown in panel A. Note that autophosphorylation in the presence of PS/PMA results in a mobility shift of the 81-kDa form of PKC to 83 kDa (the 83-kDa form did not reproduce well in the photographic processes). The autoradiograph in panel B shows that PS/PMA produces a distinct pattern of autophosphorylation of the higher molecular-weight PKC species, causing a slower migration in SDS gels. The autoradiograph in panel C shows autophosphorylation in the presence of PS/PMA after separation of the PKC isozymes by hydroxyapatite chromatography into isozymes I (or γ), II (or β), or III (or α). The lane marked III' is the leading edge of the third peak. The results indicate that PKC-α is markedly altered by autophosphorylation in the presence of PS/PMA.

phosphatase. Upon termination of calcium influx, phosphorylation of dephosphin occurs to prepare the protein for the next depolarization stimulus. Such properties make dephosphin an ideal candidate for the protein that *primes* neurotransmitter release, but its identity as such still remains to be determined. It will also be important to determine the effects of calcium and of phosphorylation on the subcellular location of dephosphin and its association with synaptic vesicles.

The B-50 protein (synonyms: GAP-43, F1, neuromodulin, P-57) is specific to neurons (Karns et al., 1987) and a limited group of astrocytes (Vitkovic et al., 1988). Its properties have been comprehensively reviewed (Gispen et al., 1991; Coggins and Zwiers, 1991). It is initially synthesized as a soluble protein, much of which is subsquently located on the inner surface of the plasma membrane (Gispen, 1986; Skene, 1989). It is further enriched in the menbranes of growth cones and nerve terminals (Van Hooff et al., 1988,1989), but only slightly lower levels are found in cytosol (Cimler et al., 1985). Its concentration in brain is about 10 μ*M*, which is similar to that of CaM (Cimler et al., 1987). Sequencing

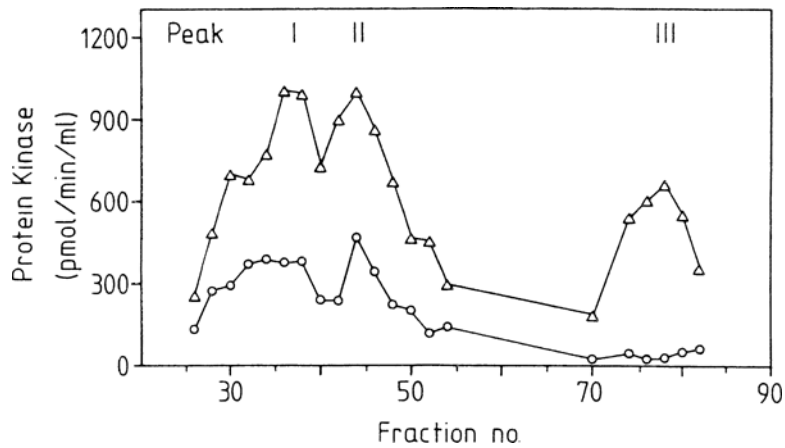

Fig. 14. Phorbol esters do not activate PKC-α. Rat brain PKC was separated by hydroxyapatite chromatography into three peaks, I, II, and III, previously shown to contain PKC-γ, β, and α respectively. Ca^{2+}/PS stimulated the histone phosphorylation activity of all three isozymes (Δ), whereas PS/PMA stimulated only PKC-γ and -β, (O). This shows that PKC-α does not respond to phorbol ester activation. Note that in the presence of Ca^{2+} PMA probably acts in a similar manner to diacylglycerol and stimulates all of these isozymes. The result presented here refers to the special situation of little or no free Ca^{2+}.

shows that it has an unusually hydrophilic and polar composition, and is predicted to be highly elongated with a high percent of random coil (Masure et al., 1986; Wakim et al., 1987; Cimler et al., 1987; Karns et al., 1987). It is composed of 70% Glu/Gln, Ala, Lys, Asp/Asn and Pro, with only a single aromatic amino acid (Phe-42) (Masure et al., 1986). It has a predicted size of 24 kDa, but this compares with an anomalous size of 45 kDa on polyacrylamide gels (dependent upon the acrylamide concentration and crosslinker), and the difference is apparently not the result of posttranslational modifications (Wakim et al., 1987; Cimler et al., 1987; Karns et al., 1987). It shows no transmembrane domains or N-linked glycosylation sites. It can be posttranslationally modified by fatty acylation of Cys near the N-terminus (Skene and Virag, 1989). The effect of this modification may be to target the protein to the membrane, but fatty acylation has a high turnover rate, suggesting a reversible interaction of B-50 with the membrane. The N-terminal sequence of B-50 also appears to target the protein to the plasma membrane (Zuber et al., 1989).

It shares with MARCKS and neurogranin the property of binding to CaM in the absence of calcium and releasing CaM in the presence of calcium (Andreasen et al., 1983; Alexander et al., 1987; Baudier et al., 1991), and is known to associate with CaM in native synaptic membranes (De Graan et al., 1990). The CaM binding peptide sequence is RGHITRKKL, and CaM binding occurs with a K_d of only 2 μ*M*. CaM is released in the presence of calcium, a property that has been utilized in purification (Andreasen et al., 1983; Alexander et al., 1987). It is phosphorylated by PKC, preferentially by the PKC-β isozyme (Sheu et al., 1990). The stoichiometry of phosphorylation is 0.5–1.3 and suggests only a single phosphorylation site (Alexander et al., 1987; Apel et al., 1990). The K_m for PKC is 1.0 μ*M* (Apel et al., 1990), which is comparable to the best-known substrates, such as histone (K_m 0.6 μ*M*) or GS peptide (0.5 μ*M*) (House and Kemp, 1987). The phosphorylation site is Ser-41, which is adjacent to or within the CaM binding site ($B\text{-}50_{43\text{–}51}$) (Apel et al., 1990; Nielander et al., 1990; Alexander et al., 1988). PKC phosphorylation of B-50 or a synthetic peptide based on the phosphorylation site is only partially dependent upon calcium, but completely dependent on phospholipids (Apel et al., 1990). Mutagenesis of Ser-41 to Thr or Ala com-

pletely prevents PKC phosphorylation, suggesting that it is the sole phosphorylation site (Nielander et al., 1990). PKC phosphorylation of this site inhibits CaM binding (Alexander et al., 1987). B-50 is also phosphorylated by casein kinase II, and on a serine (possibly Ser-192) with a stoichiometry of 1.2 and a K_m of 4 μM (Pisano et al., 1988). B-50 is not phosphorylated by PKA or CaM-PK-I or II, but does contain consensus sequences consistent with a possible phosphorylation site for proline-directed protein kinase (Mitchell et al., 1990). B-50 is dephosphorylated by calcineurin with a K_m of 2.6 μM (Liu and Storm, 1989; Schrama et al., 1989) and another cellular phosphatase that is magnesium-stimulated (Dokas et al., 1990). It shares sequence homology with a 17-kDa brain protein termed neurogranin or BICKS, but only within the CaM binding and PKC phosphorylation site sequences, suggesting there may be a family of proteins sharing these binding and regulatory motifs (Baudier et al., 1991).

Phosphorylation of B-50 occurs in intact synaptosomes (Dunkley et al., 1986, Dunkley and Robinson 1986; Wang et al., 1988; Van Hooff et al., 1989; Dekker et al., 1990a; Heemskerk et al., 1990) or brain slices (Dekker et al., 1989b; Yip and Kelly, 1989). Phosphorylation is stimulated by depolarization-dependent calcium influx, phorbol esters, or receptor activation, and is blocked by PKC inhibitors H-7 or polymyxin B (Dekker et al., 1990a). B-50 phosphorylation has recently been correlated with neurotransmitter release from synaptosomes (Dekker et al., 1990b) and hippocampal slices (Dekker et al., 1989b), since phorbol esters enhance B-50 phosphorylation and neurotransmitter release, and both were inhibited by polymyxin B. Antibodies against B-50 introduced into permeabilized synaptosomes inhibit noradrenaline release, implicating this protein in neurotransmitter release (Dekker et al., 1989a,1991b). In these studies, synaptosomes were permeabilized with streptolysin-O and noradrenaline release regulated by changes in the buffer calcium. Anti-B-50 antibodies, which completely inhibited B-50 phosphorylation by PKC, also inhibited evoked neurotransmitter release, but not PMA-enhanced release (Dekker et al., 1991b). Caution is required in final interpretation of such studies, since B-50's interaction with CaM or its regulation of phosphatidylinositol 4-phosphate kinase may also play a role (Kenigsberg and Trifaro, 1985; Dekker et al., 1991b).

Other B-50 functions have also been proposed. One is to bind and concentrate CaM at specific sites in neuronal growth cones or nerve terminals, where CaM may be locally released after PKC phosphorylation (Alexander et al., 1987). Another may be regulation of G proteins, such as G_o, since the N-terminal 21 amino acids of B-50 mimic the cytoplasmic tails of most receptors from the seven-transmembrane domain family and activate G_o (Strittmatter et al., 1990). Its phosphorylation has also been correlated with neurite outgrowth (Karns et al., 1987; Van Hooff et al., 1989). B-50 may also play a role in synaptic plasticity. Phosphorylation of B-50 has been associated with LTP (Routtenberg, 1985; Lovinger et al., 1987; Akers and Routtenberg, 1987) and neurons expressing the highest levels of B-50 mRNA are in the associative regions of the human brain, rather than the sensory or initial input regions (Neve et al., 1987). B-50 is also an inhibitor of phosphatidylinositol-4-phosphate kinase and may thereby act as a feedback inhibitor of the phosphatidyl inositol response (Gispen, 1986; Jork et al., 1984).

MARCKS

The MARCKS protein has been widely studied, and recently cloned and sequenced (Graff et al., 1989a; Stumpo et al., 1989a,b; Sakai et al., 1989). It is found in virtually all cells, where it is a substrate for only PKC both in vitro and in intact cells. It is associated with both the membranes and cytosol (Patel and Kligman, 1987). It occurs in quite high concentrations in the brain, where it represents 0.2% of cellular protein (or about 12 μM) (Graff et al., 1989a). Although associated with a majority of cell types in the central nervous system, MARCKS' immunoreactivity was particularly prominent in association with microtu-

bules (Ouimet et al., 1990), and its mRNA is particularly enriched in specific cell types in the brain (Graff et al., 1989d). MARCKS has the unusual property that it is soluble in 2% trichloroacetic or perchloric acids (Witters and Blackshear, 1987; Baudier et al., 1989), or in 40% acetic acid (P. J. Robinson, manuscript in preparation). This property has been utilized to extract the phosphorylated protein selectively from $^{32}P_i$-labeled intact cells (De la Escalera et al., 1989, P. J. Robinson, manuscript in preparation). This property can also be utilized in rapid purification of the protein (Baudier et al., 1989). The protein has been purified by a number of laboratories and appears to be an extremely elongated monomer (Patel and Kligman, 1987; Albert et al., 1987; Baudier et al., 1989; Morris and Rozengurt, 1988). It has an unusually high content of Ala and Glu/Gln. The protein has been cloned and the cDNA sequenced from a variety of species (Graff et al., 1989a; Sakai et al., 1989; Stumpo et al., 1989a,1989b). The bovine brain form contains 335 amino acids, with a predicted mol wt of 32,000 (which contrasts with its mobility on polyacrylamide gels at 80–87 kDa). The NH terminus contains a consensus sequence for myristoylation, which may target the protein to the membrane (Aderem et al., 1988; Graff et al., 1989c).

MARCKS is phosphorylated by PKC (Fig. 4) with a stoichiometry of 3–4 mol/mol and is phosphorylated exclusively on serine (Patel and Kligman, 1987; Albert et al., 1987). Four phosphopeptides were generated by tryptic digestion and a 25-amino acid domain within the center of the molecule (residues 151–175 contained all of these phosphorylation sites) (Graff et al., 1989b). This sequence is highly conserved between species. The phosphorylation of the MARCKS protein is stimulated on depolarization or phorbol ester stimulation of intact synaptosomes (Fig. 12) (Dunkley et al., 1986; Dunkley and Robinson, 1986) or brain slices (Rodnight and Leal, 1990). This also demonstrates that PKC is active after stimulation of intact nerve endings (Fig. 1). Stimulation of phosphorylation by either depolarization or phorbol esters results in its translocation from the synaptosomal membrane to the cytosol, suggesting that PKC phosphorylation usually occurs on the membrane (Wang et al., 1989).

Like B-50, MARCKS is a CaM binding protein, but only in the absence of calcium (Graff et al., 1989d). CaM binding is inhibited by MARCKS phosphorylation by PKC, which occurs, adjacent to, or within the CaM binding region (Graff et al., 1989d). Conversely, CaM binding inhibits MARCKS' phosphorylation (Albert et al., 1984b). Therefore, calcium influx into nerve terminals leads to MARCKS' phosphorylation, release of CaM, and translocation of MARCKS from the nerve terminal membrane to the cytosol (Wang et al., 1989). Although functions for MARCKS are unknown, these properties suggest that MARCKS could regulate the local cellular concentration of free CaM. Its near ubiquitous cellular distribution and association with cell growth, differentiation, and neurotransmitter release suggests a fundamental cellular function in a process, such as signal transduction, but not necessarily with neurotransmitter release. All these events are associated with profound cytoskeletal rearrangement, and MARCKS is known to be associated with the cytoskeleton of neurons or other cells. Therefore, MARCKS may play a general role in the shape of the cell or nerve terminal (Sato et al., 1985; Ouimet et al., 1990; Thelen et al., 1990), and thereby in *reactivation* or *modulation* of release.

Tyrosine Hydroxylase

Tyrosine hydroxylase (TH) is a mixed-function monooxygenase catalyzing hydroxylation of tyrosine to dopa using molecular oxygen and the cofactor BH_4 (reviewed in Zigmond et al., 1989). TH exists as a homotetramer of 60-kDa subunits, which have been cloned and sequenced. The enzyme exists as both cytosolic and membrane-bound forms, the latter being both peripheral and integral membrane forms (Zigmond et al., 1989; Kuhn et al., 1990). TH is stimulated by neurotransmitter receptor activation or by depolarization in a variety of secretory systems, including synap-

tosomes (Zigmond et al., 1989; Haycock, 1987; Chowdhury and Fillenz, 1988; Colby et al., 1989; Koda et al., 1989; Mitchell et al., 1990). Activation is mediated by phosphorylation by one of at least four classes of protein kinase (Zigmond et al., 1989) correlating with phosphorylation of distinct sites (Mitchell et al., 1990; Haycock, 1990). PKC-mediated phosphorylation was demonstrated with the use of phorbol esters, which activate dopa synthesis. In addition, OAG also activates TH in PC12 cells. Evidence for a role for PKC stimulation of TH in permeabilized chromaffin cells comes from studies correlating AA activation of TH and exocytosis from these cells (Koda et al. 1989). It has also been shown that TH is a direct substrate for PKC (Albert et al., 1984a). It remains to be fully resolved which protein kinase phosphorylates precisely which site in TH in different cells (Zigmond et al., 1989; Haycock, 1990). Currently, it is known that PKC, PKA, and PKG phosphorylate Ser-40, CaM-PK-II phosphorylates Ser-19 (and slowly Ser-40), and a novel proline-directed protein kinase may phosphorylate Ser-31 (Mitchell et al., 1990; Haycock, 1990). Phosphorylation of tyrosine hydroxylase at Ser-40 or Ser-31 results in activation and the increased synthesis of dopamine, presumably to replenish stores depleted by exocytosis, to allow the terminal to recover from a stimulus (Haycock, 1987; Colby et al., 1989; Haycock, 1990; Mitchell et al., 1990). Phosphorylation at Ser-19 makes the enzyme sensitive to the effects of a regulatory protein. The role of PKC is not particularly specific in this system, since many other kinases may achieve the same result. However, the system serves to illustrate one of the *reactivation* roles that PKC plays in neurotransmitter release (Chowdhury and Fillenz, 1988).

Summary of Role of PKC Substrates

There are extremely few currently known and studied PKC substrates in nerve terminals, despite there being the potential for the existence of many (for example, *see* Fig. 4). Much of the restriction of research to these few substrates lies in the reliance on phorbol esters to demonstrate a PKC-mediated event, whereas phorbol esters may reveal only a subset of PKC-mediated phosphoproteins. The substrates that are under current investigation are likely to play distinct roles in different phases of the neurotransmitter release process. TH is likely to be involved in the *reactivation* phase by replenishing depleted neurotransmitter stores. PMA-enhanced release (i.e., one form of release *modulation*) is associated with phosphorylation of MARCKS, and may be regulated by this or other substrates that are yet to be identified. A role for dephosphin in the PMA enhancement can be excluded, since it is unaffected by phorbol esters (although it is affected by AA), and a role for B-50 appears unlikely, since its antibodies do not inhibit PMA-enhanced release. Calcium-evoked release (i.e., *mediation* of release) appears unlikely to be caused by direct increases in phosphorylation of any of these PKC substrates, but B-50 and dephosphin may still be involved. B-50's involvement may be at the level of its CaM binding or other biochemical property. Evoked release may also require *priming*, by the phosphorylation of proteins by PKC. Dephosphin is an excellent candidate for such a protein. The dephosphorylation of, or calcium interaction directly with, such substrates is associated with and might underlie evoked release.

Multiple Roles of PKC in Neurotransmitter Release

It is likely that the steps involved in the molecular cascade of neurotransmitter release include liberation of vesicles from their association with the cytoskeleton and the phosphorylation by PKC of proteins that have the ability to alter location between membrane-bound and cytoplasmic forms, and thus, facilitate or initiate the fusion between synaptic vesicles and the plasma membrane (Zimmermann, 1990). From the preceding discussion, it is apparent that the intracellular target of calcium important in mediating transmitter release is unknown. PKC activation is

strongly associated with neurotransmitter release in *priming*, *reactivation*, and *modulatory* roles, but appears unlikely to be directly mediatory. In other words, PKC itself is unlikely to be the calcium trigger for release. The only specific protein(s) that have been clearly associated with mediation of release are CaM and B-50, but the latter appears not to involve PKC phosphorylation, and these two proteins reversibly interact with each other.

This article has highlighted the current body of literature that overwhelmingly links PKC activation to neurotransmitter release or exocytosis. However, evaluation of the work indicates that PKC is not directly involved in all stages of release. PKC activators, PMA or fatty acids, enhance release by a mechanism that directly involves PKC activation. This *modulation* of release is exquisitely sensitive to PKC inhibitors or to PKC downregulation. Depolarization- or calcium-evoked release does not convincingly require PKC activation and is poorly sensitive or insensitive to inhibitors and downregulation. Although PKC does not directly *mediate* evoked release, a role for PKC is still possible in *priming* another protein by phosphorylation in preparation for release. A review of the currently known neuronal substrates for PKC that are also phosphorylated in intact systems reveals only four substrates, and these are unlikely to represent all of the major neuronal PKC substrates. Two of the major tasks ahead are to identify other possible nerve terminal or synaptic vesicle-associated PKC substrates, and to determine which of these proteins underlie the effects of PKC on three of the four stages of neurotransmitter release.

A New Model for the Mechanism of Release?

The work regarding the role of PKC in neurotransmission and the properties of its unique substrate dephosphin allow construction of a speculative mechanistic model that is consistent with the data reported here. The model requires that the underlying mechanism of neurotransmitter release be considered in two stages—*priming* and *mediation*. Multiple steps probably underlie each stage, but the following initial steps are proposed: *Priming*—phosphorylation of a protein(s) by PKC *primes* the nerve terminal prior to the depolarizing stimulus (or as a consequence of the previous stimulus). This explains both the apparent requirement of exocytosis for calcium and Mg-ATP and the *modulation* of the process by PKC. *Mediation*—depolarization stimulates calcium influx and CaM release from its binding proteins (such as B-50 and/or MARCKS) in the nerve terminal. This could account for the demonstrated ability of anti-CaM and antiB-50 antibodies to inhibit neurotransmitter release or exocytosis. Activated and released CaM binds to and activates the calcium trigger. CN is an ideal candidate for that trigger, and dephosphin or mammalian parafusin-like proteins are good candidates for substrates that could mediate this response. Dephosphorylation of one such substrate may initiate vesicle fusion and neurotransmitter release. Alternatively, direct calcium association with such a substrate may mediate release.

This model, although speculative, provides a stimulating framework for future investigation into the properties of such proteins and the biochemical basis of neurotransmitter release. It attempts to break away from previous concepts of a single entity mediating release, but allows for biological complexity and involvement of multiple calcium signals to play critical roles in neurotransmitter release. It does not yet identify the calcium trigger protein or the mechanism of fusion itself, but highlights a novel area for future research by the proposal that CN dephosphorylation of dephosphin may be part of the calcium trigger that mediates neurotransmitter release.

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