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REVIEW

Phorbol esters and neurotransmitter release: more than just protein kinase C?

*,[1]Eugene M. Silinsky & [1]Timothy J. Searl

[1]Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611, U.S.A.

This review focuses on the effects of phorbol esters and the role of phorbol ester receptors in the secretion of neurotransmitter substances. We begin with a brief background on the historical use of phorbol esters as tools to decipher the role of the enzyme protein kinase C in signal transduction cascades. Next, we illustrate the structural differences between active and inactive phorbol esters and the mechanism by which the binding of phorbol to its recognition sites (C1 domains) on a particular protein acts to translocate that protein to the membrane. We then discuss the evidence that the most important nerve terminal receptor for phorbol esters (and their endogenous counterpart diacylglycerol) is likely to be Munc13. Indeed, Munc13 and its invertebrate homologues are the main players in priming the secretory apparatus for its critical function in the exocytosis process.
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Keywords: Protein kinase C; Munc13; neurotransmitter release; phorbol esters

Abbreviations: DAG, diacylglycerol; DOC2α, double C2 domain protein that interacts with Munc13; EPPs, end-plate potentials; EPSCs, excitatory postsynaptic currents; HR, hinge region of protein kinase C; LTX, α-latrotoxin; MHD, munc homology domain; NSF, *N*-ethylmaleimide-sensitive factor (an ATPase, not the National Science Foundation), PLC, phospholipase C; PDBu, 4β-phorbol-12,13-dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; PS, pseudosubstrate domain; RIM, rab-interacting molecule; *RRP*, readily releasable pool of neurotransmitter; SNAPs, soluble NSF attachment proteins; SNAREs, SNAP receptors (syntaxin, SNAP-25 and synaptobrevin); SNAP-25, synaptosomal-associated protein of 25 kDa, TPA, 4β-12-*O*-tetradecanoylphorbol-13-acetate

Background: phorbol esters and protein kinase C (PKC)

Members of the plant family *Euphorbiaceae* have been prominent players in the studies of both the genesis of mammalian tumors and the enzymological basis of signal transduction. Specifically, selected plants in this family, which include decorative houseplants (*Poinsettia*) and historical sources of purgatives (*Croton*), have been used as sources of the compounds known as the phorbol esters. Phorbol esters have been found to produce a myriad of physiological effects on cells in a variety of tissues (Nishizuka, 1988; Kikkawa *et al*., 1989; Newton, 1995), with one of the most commonly observed effects being the promotion of tumor growth (see e.g. Van Duuren *et al*., 1979). The importance of phorbol esters to models of human carcinogenesis thus led to investigations to discover the target moieties for which phorbol esters possess high affinity. The search for such targets initially led to one of the major players in mammalian signal transduction cascades, the enzyme protein kinase C (PKC) (Nishizuka, 1988; Kikkawa *et al*., 1989; Newton, 1995). Indeed, phorbol esters can both activate PKC and after more extended incubation periods, cause downregulation of the enzyme. Such observations, reproduced in a multitude of tissues, led to the widespread use of phorbol esters as experimental research tools to evaluate the importance of PKC in biological processes. Phorbol esters are effective in this regard as they act as stable substitutes for the natural activator of PKC, diacylglycerol (DAG).

The generic structure of active phorbol esters (β phorbols) is shown in Figure 1 (left, see also Van Duuren *et al*., 1979). The phorbols are polyclic (four-ringed) compounds, with the rings being depicted in Figure 1 as A, B, C and D. The three most widely used active phorbols, TPA (4β-12-*O*-tetradecanoyl-phorbol-13-acetate), PDBu (4β-phorbol-12,13-dibutyrate) and PMA (phorbol 12-myristate-13-acetate), differ only by their substitutions at positions 12 and 13 of ring B (see R1 and R2, Figure 1). Inactive α phorbols (Figure 1, right) are often used as controls for nonspecific effects of phorbol esters in experiments in which a β phorbol produces a biological effect. The structural differences between the epimeric α and β phorbols are apparent when one considers the OH group at the 4 position on ring C (red). In the active β phorbols, the OH group is in the β position (projecting towards the reader in Figure 1, left). In the inactive phorbols, the OH group is in the α position (Figure 1, right); this in turn changes the spatial arrangement of the cyclopentenone group (ring D) and precludes the activation of PKC or other structurally similar phorbol ester receptors. The inactive α phorbols thus have the same lipophilicity and physicochemical properties as the active β phorbols, but because of these conformational shifts, are unable to activate a biological response in most instances.

*Author for correspondence; E-mail: e-silinsky@northwestern.edu

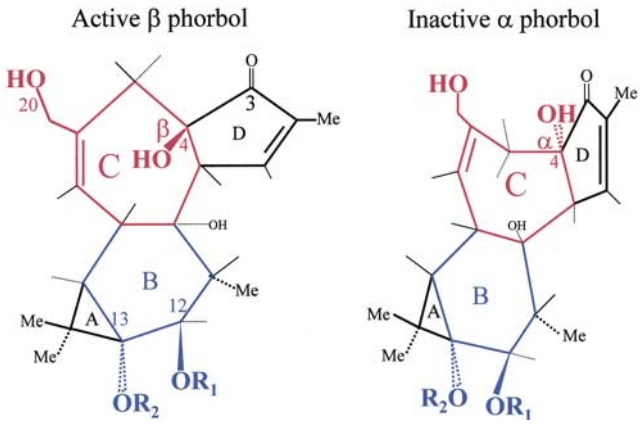

Figure 1 Structure of phorbol esters with respect to differences between active and inactive compounds. The structure on the left depicts the active (β) conformation of the generic 4 ringed (A – D) phorbol ester structure. This β conformation is defined by the 4-OH group that is projecting towards the reader. Positions 12 and 13 are the sites where substituents are added, hence differentiating PDBu from PMA and other phorbols (see text). In the inactive (α) conformation (right), the 4-OH is in the alpha position projecting away from the reader. Other sites important for the binding of phorbols to its receptors (termed C1 domains – see Figure 2) are depicted (positions 3, 4 and 20 are important in this regard). See text for further details.

The commonly accepted targets for phorbol esters, the PKCs, fall into three distinct families of isozymes: (1) the *conventional* PKCs (α,β,γ), (2) the *novel PKCs* (δ,ε,η, and μ) and (3) the *atypical PKCs*. For the sake of convenience, we will focus on the conventional PKC families. For further details of the other families, see Newton (1995), Jaken (1996) and the legend to Figure 2.

A general schematic for the different regions of the classical PKCs is shown in Figure 2a. The regulatory domain of PKC contains the binding sites for phorbol esters, the C1 domains. These domains are the natural receptors for the endogenous ligand DAG, which is formed from membrane phospholipids (predominantly phosphatidylinositol bisphosphate) after the activation of phospholipase C (PLC) by a wide variety of biological modulators and neurotransmitters. As mentioned above, active phorbol esters were found to provide a stable substitute for DAG. The C1 region is a zinc-finger receptor, a common term for these regions because of their richness in cysteine residues, even though the domain is not structurally related to the zinc-finger proteins that bind nucleic acids (Zhang *et al*., 1995). The zinc-finger receptors maintain the active conformation of the C1 domain.

The purpose of the C1 domain is to help recruit proteins to membranes. A sketch based upon the crystal structure of this domain is shown in more detail in Figure 2b (see Hommel *et al*., 1994; Zhang *et al*., 1995). The upper part of this domain, which serves as the binding region for phorbol esters, possesses a strategic region containing two β sheets (Figure 2b, 1 and 2) which separate a water-filled cavity. After displacing a molecule of water, the phorbol esters fit into this cavity, replacing lost hydrogens by oxygens at the C3, C4 and C20 positions. The binding of phorbols in the C1 domain provides a hydrophobic cover allowing the complex to be inserted and anchored into membranes with little conformational change. After insertion into the membrane, the catalytic domain of PKC is activated (see Figure 2a, C3) and, with the appropriate substrates bound to the C4 domain, phosphorylation ensues.

Another intriguing aspect of the regulatory domain of the classical PKCs is the presence of calcium binding domains (Figure 2a, C2). These domains were first discovered in PKC and named C2 domains (Kaibuchi *et al*., 1989); a C2 domain consists of five conserved aspartate residues situated in two loops (see Sutton *et al*., 1995; Sudhof & Rizo, 1996). C2 domains, rather than undergoing conformational changes like calmodulin and other proteins with EF calcium binding hands, can serve as electrostatic switches that rapidly transduce calcium (and phospholipid) binding into a biological response. This binding of calcium and phospholipid assists in translocating PKC to membranes and thereby activating the enzyme, a process generally initiated by DAG binding to the C1 domain (although the C1 and C2 domains can act as independent membrane targeting domains). In addition to the PKCs, C2 domains have also been found in a significant array of presynaptic proteins, where they are thought to provide calcium sensors for the release of neurotransmitter substances (e.g. synaptotagmin I – see Sudhof, 1995; Sudhof & Rizo, 1996; Augustine, 2001).

The catalytic domain of PKC possesses both an ATP binding domain, which is responsible for the protein kinase activity (Figure 2, C3), and a substrate binding domain (Figure 2, C4). This paper will focus on the C1 and C2 domains of the PKCs and other phorbol ester receptors. For further details of the other parts of Figure 2a and how they relate to the enzymatic activity of the various PKC isoforms – see figure legend.

Other targets for phorbol esters: Unc13 and its homologues

For a number of years, the effects of phorbol esters on biological processes (often when coupled to the absence of effects of inactive α phorbols) has been interpreted as demonstrating a role for PKC in that process. In some studies, PKC antagonists were used to confirm a role for PKC. Furthermore, the binding of tritiated PDBu has been used to determine the cellular localization of PKC in a variety of tissues. In spite of this well-entrenched tautology between phorbol ester effects and PKC, there are other important receptors for phorbol esters. One such receptor, Unc13 (uncoordinated mutant #13), was first described over a quarter century ago in *Caenorhabditis elegans* (Brenner, 1974). Mutants devoid of the *Unc13* gene were known to have uncoordinated movements and inappropriately sluggish pharyngeal muscle contractions as well as disturbances of cholinergic function. Subsequently, Maruyama & Brenner (1991), who cloned this gene and sequenced the cDNA, found that a central core region of the 1734 amino-acid protein bears a striking similarity to PKC. This domain was expressed in *E. coli* and found to be a phorbol ester/DAG receptor (Maruyama & Brenner, 1991). Moreover, treatment of *C. Elegans* with phorbol esters produced uncoordinated movements.

Further detailed studies of the binding of phorbols to Unc13 revealed a cysteine-rich region (the zinc-finger domain), that is the high-affinity binding site for phorbol esters (Kazanietz *et al*., 1995; Kazanietz, 2002). When the binding properties of this region were compared to the C1 domain of PKC, the

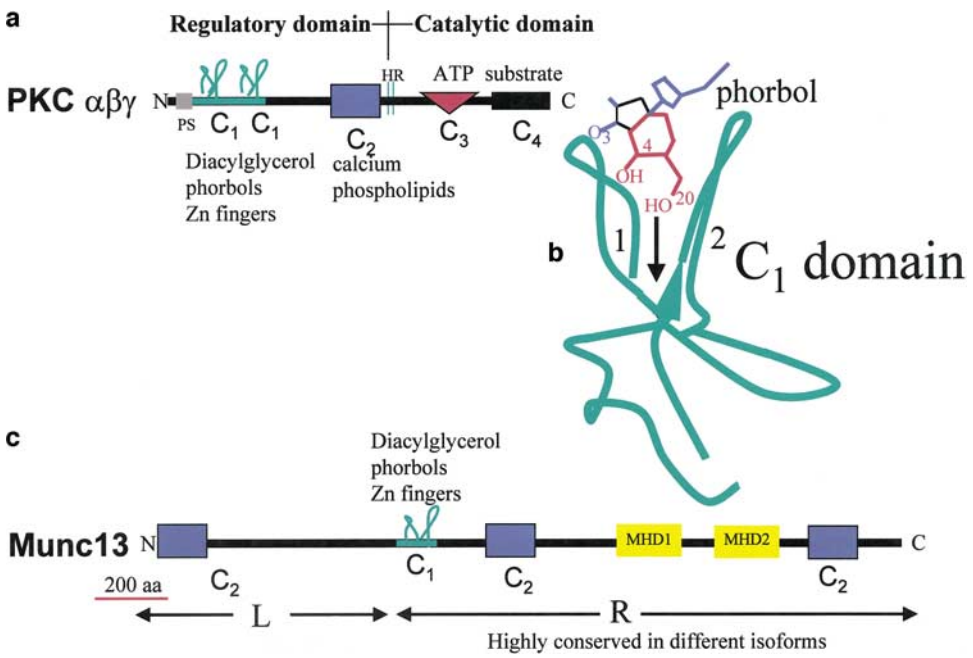

Figure 2 Phorbol ester receptors; protein kinase C (PKC) and Munc13. (a) The important domains of the classical PKCs. Note the N terminal regulatory region, of 20 – 40 kDa and the C term catalytic region (about 45 kDa). The pseudosubstrate (PS) domain, a domain that also binds acidic lipids such as phosphatidylserine, is near the C1 domain and serves an autoinhibitory function. The PS domain actually occupies the active site in the inactive enzyme and is not subject to proteolysis. Hence, the conventional linear diagram for PKC (as depicted in the figure for convenience) is somewhat unrepresentative as the PS domain actually curves around to bind to the active site prior to enzyme activation (for an excellent representation, see Newton, 1995). When PKC is activated by DAG (or phobol esters), calcium ions and phosphatidylserine, the PS domain is removed from the active site. Indeed, PKCs devoid of a PS domain demonstrate constitutive activity. The hinge region (HR) separates the regulatory and catalytic domains. The HR becomes sensitive to proteolysis when PKC interacts with its target membranes. In contrast to the classical PKCs, the novel PKCs, while possessing a C2-like domain, do not bind calcium ions. The atypical PKCs only have one C1 domain and may not bind phorbol esters. In addition, C2 domains of atypical PKCs lack key residues that maintain its active conformation. (b) The structure of the typical C1 domain in more detail (see text). (c) The generic Munc13 structure. At the C terminus, the R region is highly conserved in the various isoforms of Munc13. The R regions contains two C2 domains, two Munc homology domains (MHDs – for specific details see Koch *et al*., 2000), and the phorbol ester binding site (C1 domain). At the N terminus, the L domain is the region that exhibits variability between isoforms. Most interacting proteins (e.g. syntaxin) bind to the R region. RIM1 and calmodulin bind only to the L region however. With regard to the expression of the various Munc isoforms, Munc13-1 is ubiquitously expressed in all neurons in the rat central nervous system while Munc13-2 and Munc13-3 have more restricted locations. Munc13-2 is only detectable in rostral regions of the rat central nervous system (including the cerebral cortex and hippocampus), and Munc13-3 is found mostly in the cerebellum. Another variant, Munc13-4, is only distantly related to the other munc-13 isoforms and has no C1 domain. Munc 13-4, is expressed in secretory cells in the lung (goblet cells and secretory epithelium).

similarities were remarkable. A thorough comparison of the structure – activity relation of a series of phorbol receptor agonists revealed small statistically significant (but perhaps physiologically irrelevant) differences between Unc13 and PKC δ. A modest difference between PKCδ and Unc13 was also observed as to the phospholipid dependence of phorbol binding. These studies on worms have been extended to *Drosophila* (Dunc13) and mammals (Munc13). Mammalian species possess at least three different *Munc13* genes that act as phorbol ester receptors: *Munc13-1*, *Munc13-2* and *Munc13-3* (Brose *et al*., 1995; Koch *et al*., 2000). Munc13-1 is the most ubiquitous mammalian brain isoform and it has been suggested that this protein plays an essential role in the process of neurotransmitter release (Brose *et al*., 1995).

The generic structure of the Munc13 proteins is depicted schematically in Figure 2c. In addition to the C1 domain, Munc13 (depending on the isoform) has two to three C2 domains, As alluded to above, these domains are generally characteristic of calcium binding sites. Beginning at the C terminus is the R domain (so named because of the similarity to one of the splice variants of Unc13); the R domain is conserved in all species studied. This conserved region contains C2 domains, two Munc homology domains (Figure 2, MHD1 and MHD2 – for details see Koch *et al*., 2000) and the phorbol ester binding site (C1). Variability occurs in the remaining part of the N terminus delineated as the L region (Figure 2c, see also Brose *et al*., 2000; Betz *et al*., 2001). For further details of the localization of the various Munc13 isoforms, see legend to Figure 2.

Electrophysiological studies of neurotransmitter release: PKC or an Unc13 homologue as the targets for phorbol esters

The skeletal neuromuscular junction appears to be an appropriate synapse to use as a 'case study', as seminal studies of phorbol esters and neurotransmitter release were performed on this synapse. In the classical paper of Shapira *et al*. (1987), it was found that active phorbol esters were potent stimulators of the evoked and spontaneous release of the neurotransmitter acetylcholine (ACh) from amphibian motor nerve endings, while inactive phorbols had no effect on synaptic function. The conclusion, which was reasonable at the time because of our

lack of knowledge of other phorbol ester receptors, was that PKC is an important modulator of ACh secretion.

As more studies were published on phorbol ester effects on neuronal cell bodies and synaptic loci, we became puzzled by the apparent dichotomy between the effects of phorbols on calcium currents and neurotransmitter release (see Introduction to Redman *et al*., 1997). Specifically, more often than not, Ca^{2+} currents recorded from cell bodies were decreased by phorbol esters, yet neurotransmitter release was increased. The phenomenon was investigated further at the skeletal neuromuscular junction, where we have been able to make simultaneous measurements of the nerve terminal Ca^{2+} currents that initiate transmitter release ('Perineural Ca currents' – see Redman & Silinsky, 1995) and the electrophysiological correlates of evoked ACh release (end-plate potentials, EPPs). Figure 3a shows that the Ca^{2+} currents were decreased by phorbol esters, while evoked neurotransmitter release was enhanced (see figure legend for an explanation of the currents).

Based upon the upward shift by PDBu of the dose–response curve for extracellular [Ca^{2+}] and transmitter release (Figure 3b), and the data depicted in Figure 3a, it was concluded that the stimulatory effect of phorbol esters occurred downstream of membrane ionic channels. It was suggested that this specific effect on neurotransmitter release could be due to an increase in the number of activatable release sites or synaptic vesicles, that is, an increase in the readily releasable pool (*RRP*) of neurotransmitter (Redman *et al*., 1997). An alternative view is that the efficiency by which Ca^{2+} activates the secretory process is increased by phorbol esters (Redman *et al*., 1997).

A related issue concerned the numerous anecdotal reports to the authors that a number of protein kinase inhibitors failed to affect the actions of phorbol esters, results attributed more often than not to inactive antagonists rather than to a non-PKC phorbol receptor. Somewhat to our dismay, we found that the enhancement of secretion by active phorbols was not blocked by a number of PKC inhibitors (Figure 3c), even at heroic concentrations. We thus capitulated and suggested that Munc13 or a related protein mediates the stimulatory effects of phorbol esters on neurotransmitter secretion (Redman *et al*., 1997; Searl & Silinsky, 1998).

Since the publication of this work, several authors using electrophysiological techniques have implicated a non-PKC mechanism in the effects of phorbol esters on neurotransmitter secretion. In a study of mossy fiber terminals in the mouse hippocampus, phorbol esters were found to potentiate a component of neurosecretion independent of changes in free presynaptic calcium concentrations and of PKC (Honda *et al*., 2000). Hori *et al*. (1999) studied the effects of phorbol esters at the giant presynaptic glutamatergic terminal of the rat calyx of Held, an experimentally accessible system in the auditory brain stem in which the giant presynaptic element can be patch clamped. In this study, it was found that phorbol esters increased neurotransmitter release without a change in membrane ionic currents. In addition, a component of the phorbol ester effect was independent of PKC. This component was likely to be because of Munc13, as a synthetic peptide mimicking a protein that interacts with Munc13 reduced the effects of phorbol esters, presumably by reducing the availability of Munc13. This peptide corresponds to the N terminus of DOC2α, a calcium binding protein, so named because of a double C2 domain. Both of these studies thus support the conclusions of Redman *et al*. (1997) that the target sites for the action of phorbol esters to stimulate neurotransmitter release are downstream of Ca^{2+} entry. Finally, in a study of *Xenopus* nerve-muscle cultures (Betz *et al*., 1998), overexpression of Munc13 increased both evoked and spontaneous neurotransmitter release and increased the phorbol ester sensitivity of neurotransmitter release at the amphibian neuromuscular junction. The essential equipotency and similar selectivity sequences of various phorbol esters on PKC and on Munc13 were also confirmed in this vertebrate study. In addition, inactive 4α phorbols were found to have no effect on Munc13.

This relatively recent spate of publications supports the results of Scholfield & Smith (1989), who found that two nonselective PKC inhibitors, H7 and acridine orange did not alter the stimulatory actions of PDBu and other phorbol esters in slices of guinea-pig olfactory cortex. At the time, these authors were lost voices in the crowd when they suggested the possibility that 'phorbol esters were working in some way not involving protein kinase C'. In this study on olfactory cortex, Ca^{2+} appeared to antagonize the action of phorbol esters, an effect not observed in other studies (see e.g. Figure 3b above).

Further details of the mechanisms of action of the Unc13 isoforms in the process of neurotransmitter secretion require a brief discussion of the secretory apparatus. For the convenience of the reader, an overview of the strategic components of the neurotransmitter release machinery follows.

Proposed subcellular mechanisms for the stimulatory effects of phorbol esters on neurotransmitter release

A discussion of the constituents of the secretory apparatus generally begins with the core complex of three presynaptic proteins (termed the SNARES - see legend to Figure 4). The three SNARES, which are depicted in the unprimed state in Figure 4a are: (1) *syntaxin* (an intrinsic presynaptic membrane protein that is linked to voltage-gated calcium channels), (2) *SNAP-25* (synaptosomal-associated protein of 25 kDa, loosely affiliated with the presynaptic plasma membrane by palmitolyation through cysteine residues) and (3) *synaptobrevin* (an intrinsic synaptic vesicle protein also termed vesicle-associated membrane protein or VAMP). In contrast to the unprimed state, when the secretory apparatus is in the *primed* state (Figure 4e), the α helices of these three proteins are arranged as four intertwined tails, with SNAP-25 contributing two helices, the other two SNAREs contributing one helix each (see inset from Sutton *et al*., 1998). Vesicles in this fusion-competent, primed state represent what is termed the readily releasable pool (*RRP*) of neurotransmitter quanta. Intimately associated with the SNARES is the synaptic vesicle protein synaptotagmin (omitted from Figure 4 for the sake of clarity). Synaptotagmin I contains two C2 domains and is generally believed to be the calcium sensor for synchronous evoked exocytosis (Fernandez-Chacon *et al*., 2002; Mackler *et al*., 2002), that is, release detected electrophysiologically as EPPs (see Figure 3) or excitatory postsynaptic currents (EPSCs).

This structural arrangement depicted schematically in Figure 4e for the primed complex is crucial for the rapid coupling of Ca^{2+} entry to the release of neurotransmitter substances. Indeed, priming might even involve a partial fusion reaction of the vesicular membrane with the nerve terminal membrane (Jahn & Sudhof, 1999). Vesicles filled with neurotransmitter and docked

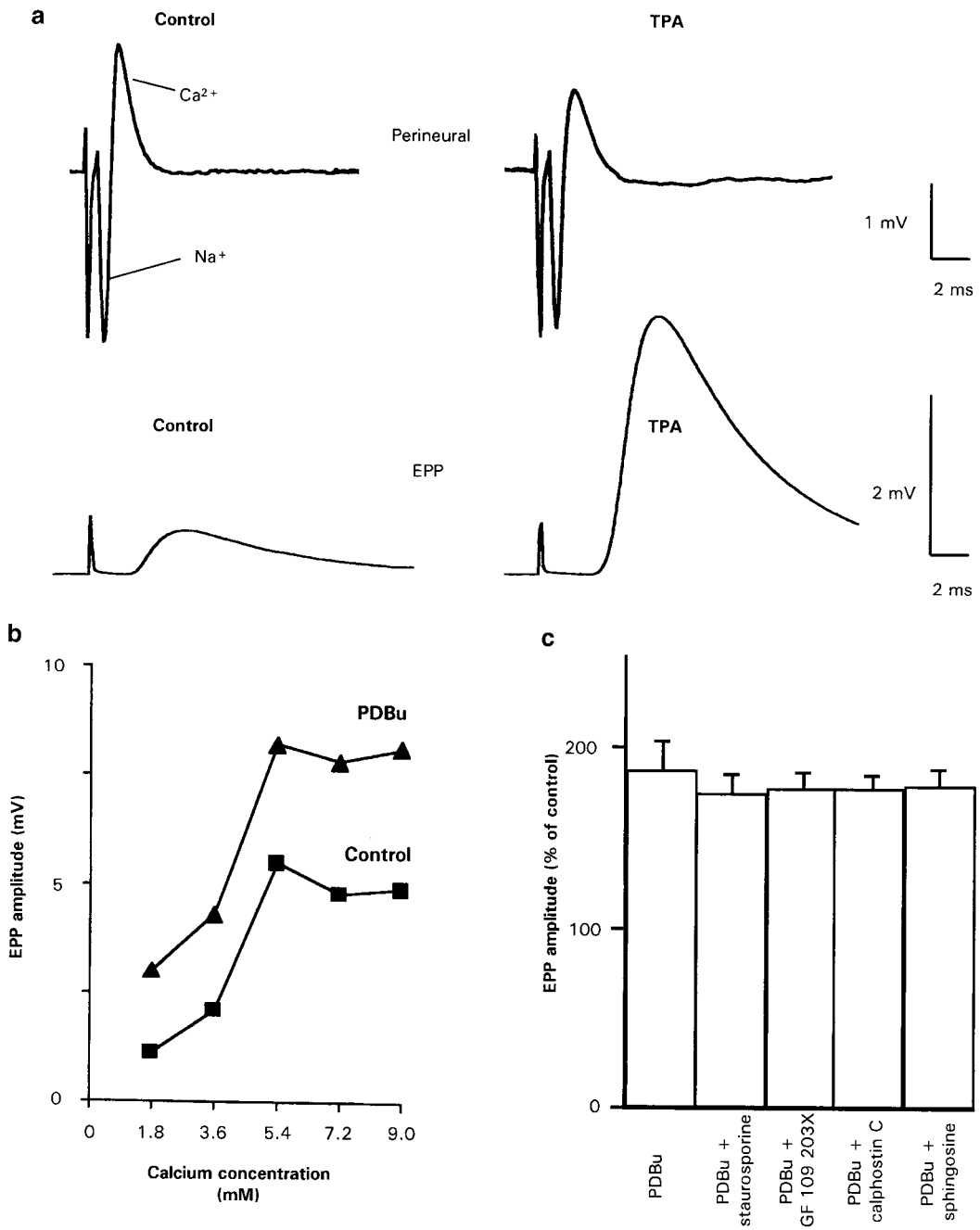

Figure 3 Effects of phorbol esters on evoked ACh release and calcium currents and the lack of effect of different calcium concentrations (b) or PKC antagonists (c) on the actions of phorbols on ACh release from motor nerve endings. (a) The effects of the β phorbol TPA on perineural waveforms resulting from sodium and calcium currents in the nerve endings ('perineural currents' – upper traces) and physiologically functional ACh release (EPPs, lower traces). Perineural currents were measured before and after application of TPA. Note that the Ca^{2+}-related signal is upward (outward) since the highest density of Ca^{2+} channels is at the nerve terminal and the inward Ca^{2+} current at the terminal is registered as a voltage change reflective of an outward current back at the recording electrode (which records the extracellular signal near the junction of the myelinated and nonmyelinated axons). Modest concentrations of potassium channel blockers were employed to prevent contamination of the peak of calcium signal with activation of voltage-gated potassium channels (see Redman & Silinsky (1995) and Redman *et al.* (1997) for specific details). A stimulation frequency of 0.05 Hz was used to minimize synaptic depression. Each trace is the averaged response to 20 stimuli. (b) Concentration – response curves for extracellular Ca^{2+} and evoked ACh release (EPPs) in the presence and absence of PDBu (200 nM). Each individual data point represents the mean amplitude of eight individual EPPs. (a) and (b) were reprinted with permission from Redman *et al.* (1997). (c) Shows that a variety of protein kinase inhibitors have no effect on the stimulatory actions of phorbol esters (reprinted with permission from Searl & Silinsky, 1998). Data depict mean $\pm$ 1 s.e.m. where the percent of control reflects the ratio of the EPP amplitudes produced by PDBu to the EPP amplitudes in the presence of the respective inhibitors ($n = 11$ experiments for PDBu and $n = 4$ experiments for the PKC antagonists). The concentrations of the antagonists were: staurosporine (1 μM), GP 109203X (10 μM), calphostin C (25 μM) and sphingosine (0.5 mM). No significant differences were observed between the groups (one-way ANOVA). As discussed in Searl & Silinsky (1998), the absence of effects of calphostin C may indicate differences in the binding characteristics of invertebrate and vertebrate unc with respect to C1 domain antagonists. Alternatively, the access of calphostin C to its targets in the nerve ending may be impaired in the intact preparation (see Searl & Silinsky, 1998 for further discussion). All experiments in this figure were made using isolated cutaneous pectoris neuromuscular preparations from the frog.

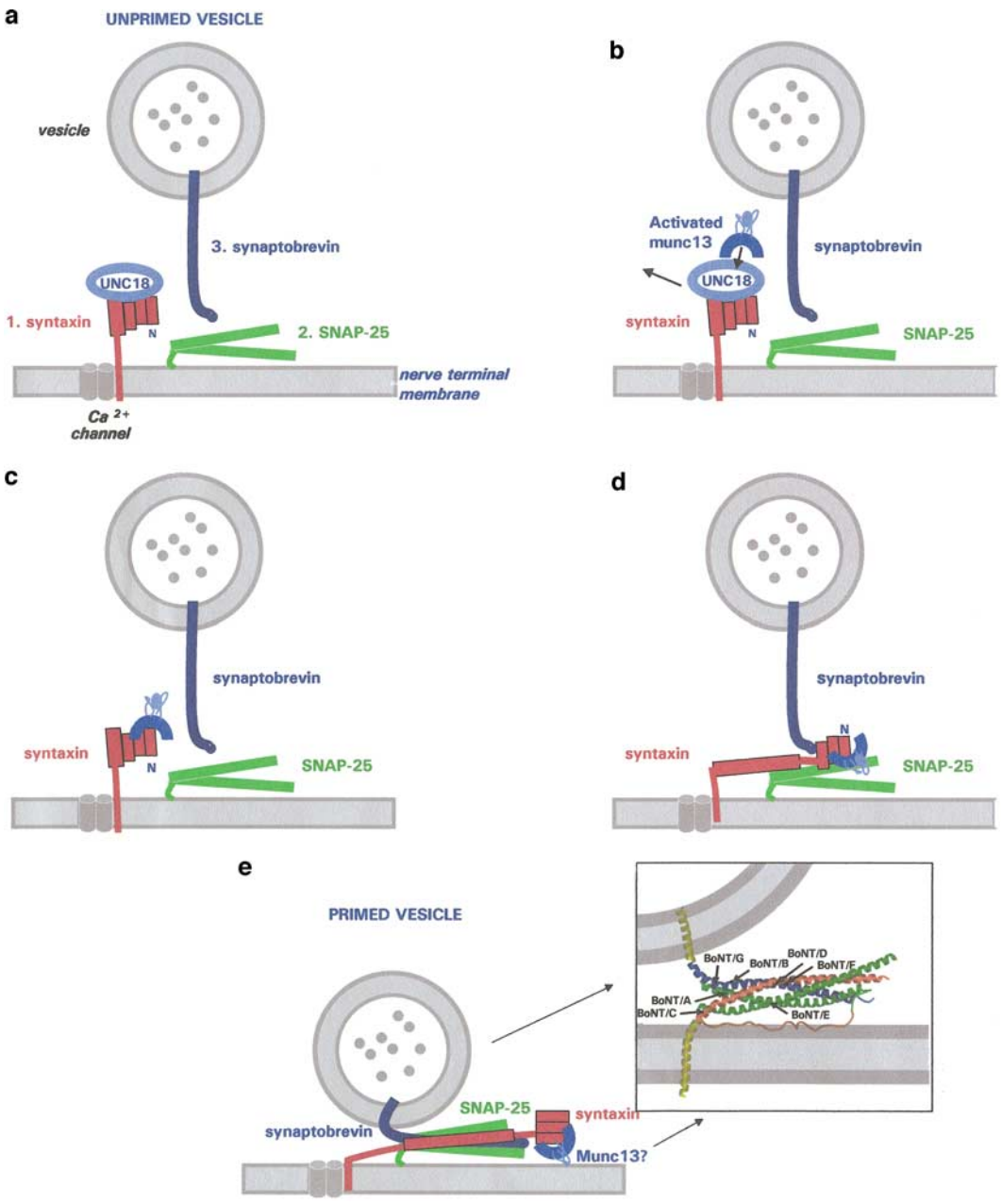

Figure 4 The secretory apparatus and the involvement of unc13 isoforms in the priming process. (a) The unprimed vesicle docked near the active zone. The three SNARE proteins syntaxin (red, in association with calcium channels), SNAP-25 (green) and synaptobrevin (dark blue) are also depicted in the nonprimed state. Prior to priming, syntaxin is held in an inactive, four-helix bundle by Munc18 (unc18, light blue); (b) The arrival of an activated Munc13 (dark blue) and the removal of the Munc18 (most likely by displacement). The figure depicts an activated C1 domain, which is required for priming by exogenous phorbols or endogenous DAG (see text for a discussion on the possibility of different pools of Munc13). (c) Munc13 bound near the N terminus of syntaxin and the initiation of the priming of the core complex by syntaxin. During the priming by the active state of syntaxin (d), the helical core domain nearest the membrane (the Hcore domain also called the H3 domain) participates in the 4-helix bundle with SNAP-25 and synaptobrevin, while the other three N terminal helices (HA-HC) do not (e – see also Fiebig *et al*., 1999). (e) A primed vesicle, comparing our cartoon and the depiction of Sutton *et al.* (1998) gleaned from their elegant X-ray crystallographic data (inset). For an additional review of articles on the topic of neurotransmitter secretion not cited in the text (see Zucker, 1996; Robinson & Martin, 1998; Gerst, 1999; Lin & Scheller, 2000). The inset in (e) also demonstrates that the botulinum toxins cleave specific SNARES at very specific residues. For example, a nine-amino-acid segment at the C terminus of SNAP-25 is cleaved by the clinically employed fraction of botulinum toxin (type A or BOTOX). In neurons, the regions of munc-13 that bind both RIM and syntaxin are essential for neuronal function (this stringent requirement is not true in adrenal chromaffin cells). In addition, priming of chromaffin granules is considerably more complex, with both a readily releasable pool and a slowly releasable pool being hypothesized for exocytosis (see Brose *et al*., 2000). The use of the SNARE nomenclature for members of the core complex continues, often to the great confusion of nonsynaptophiles (see Rothman & Warren, 1994; Gerst, 1999). This excursion into terminology begins with *N*-ethylmaleimide sensitive factor or *NSF*, an ATPase that acts a chaperone for *dissociating* the critically important presynaptic proteins that constitute the core complex (i.e. a chaperone for dissociating syntaxin, SNAP-25 and synaptobrevin). To perform its chaperone function, NSF requires the assistance of soluble *N*SF attachment proteins or ***SNAPs*** (in α,β, and even historically γ flavors) to dissociate the three members of the core complex. These Hellenic SNAPs (which are not related to SNAP-25, a member of the core complex), need to bind to the core complex before NSF can bind. As syntaxin, SNAP-25 and synaptobrevin acts as *receptors for the SNAPS*, these three proteins are termed *SNA*p receptors or ***SNAREs***.

at active zones of secretion (e.g. Figure 4a) are not necessarily competent to fuse. These vesicles must mature in some manner to be fusion competent, that is must receive the appropriate priming stimulus to be associated with the plasma membrane SNAREs at the nerve terminal active zone. The plasma membrane protein syntaxin is the coordinator of the priming process, but syntaxin needs to be in the active conformation to perform this priming function. The results to be summarized below gleaned from studies in mouse (Munc13), *Drosophila* (Dunc13) and *C. elegans* (Unc13) demonstrate that Munc13 is essential for the priming process by activating syntaxin. For concise reviews of the comparative Unc13 studies, see Tokumaru & Augustine (1999) and Brose *et al*. (2000).

As shown in Figure 4a, prior to priming, syntaxin is maintained in an inactive conformation by its association with another protein termed Munc18 (also called nsec-1 and depicted in Figure 4 as unc18). Munc13 then binds to the N terminus of syntaxin causing Munc18 to dissociate (Figure 4b, c – see Sassa *et al*., 1999). The Munc13 – syntaxin interaction serves to initiate the assembly of the primed core complex as depicted in Figure 4d and e. Evidence in favor of the importance of Munc13 for priming is that *C. elegans* mutants that produce constitutively active open states of syntaxin no longer require Unc13 for vesicle priming (Richmond *et al*., 2001). The preceding discussion of the priming process is also applicable to the enhancement of priming by exogenous phorbol esters or increases in endogenous DAG (see legend to Figure 4).

The review thus far has focused on the effects of phorbol esters, with the evidence mounting suggesting that a major target for the actions of these exogenous activators of C1 domains is Munc13. What is the importance of endogenous Munc13 in the control of neurotransmitter release?

Molecular genetic studies on the importance of Munc13 in the process of neurotransmitter release; relation to PKC

In the studies on invertebrates (Unc13 and Dunc13), the importance of these moieties is incontrovertible; neurotransmitter release is completely eliminated in the deletion mutants of *C. elegans* (Richmond *et al*., 1999) and *Drosophila* (Aravamudin *et al*.,1999), and this effect is independent of the nature of the neurotransmitter (Richmond *et al*., 1999, for a review see Brose *et al*., 2000). The failure of neurotransmitter secretion is not due to the sparsity of neurotransmitter as the vesicle numbers are actually *increased* in *Dunc13* and *Unc13* mutants (Aravamudin *et al*., 1999; Richmond *et al*., 1999). As alluded to above, *C. elegans* and *Drosophila* each possess only a single *Unc13*/*Dunc13* gene, while mammalian neurons have three independent genes for the phorbol ester-sensitive Muncs, *Munc13-1*, *Munc 13-2 and Munc13-3*. The effects in mice thus depend on the nature of the neurotransmitter and the specific munc13 isoform. A discussion of the relative importance of Munc13-1 and Munc13-2 in the processes of neurotransmitter release and as physiologically relevant phorbol ester receptors follows (for further details of the Munc13 isoforms, see legend to Figure 2).

In the seminal study on Munc13 in mouse hippocampal neurons (Augustin *et al*., 1999), the brain-specific isoform *Munc13-1* was deleted and it was found that glutamate release from the mutant was decreased by 90%. This decrease in secretion was associated with a decrease in the *RRP* (and thus primed vesicles) as assessed by applying sucrose and quantifying the massive avalanche of asynchronous release that ensues upon sucrose addition (a common method for assessing the size of the *RRP*). The fusion process was unaffected in the deletion mutant as determined by the application of α-latrotoxin (LTX), which produced an increase in neurotransmitter output in *Munc13-1*-deficient mice. This effect of LTX may result from the presence of Munc13-2 in these cells as deletion of both *Munc13-1* and *Munc13-2* renders neurons essentially unresponsive to LTX (see Varoqueaux *et al*., 2002 and below). (The mode of action of LTX is controversial. However, current evidence suggests that, the principal action of LTX, after binding to either of its two different presynaptic receptors, is to produce pores in the cell membrane; these pores in turn promote the influx of extracellular calcium – see e.g. Hlubek *et al*., 2000; Ushkaryov, 2002.) Vesicles were analyzed from *Munc13-1* deletion mutants morphometrically and no changes in the overall active zone structure or the number or distribution of synaptic vesicles were observed. These results, when coupled to the evidence that Unc13 does not act on either the storage of neurotransmitter or the endocytotic recycling pathways (see Tokumaru & Augustine, 1999 for review), led to the suggestion that the deficit in *Munc13-1* mutants is associated with the priming process (Augustin *et al*., 1999).

What about the observation that 10% of the glutamatergic neurons and all of the GABAergic neurons were unaffected in the *Munc13-1* deletion mutants? Both knockout and knockin (ie rescue) studies suggest that those glutamatergic synapses that were unaffected by the deletion of *Munc13-1* and the GABAergic synapses use Munc13-2 to perform their priming function (Rosenmund *et al*., 2002). In addition, when both the *Munc13-1* and *Munc13-2* genes were deleted, the morphology of nerve endings was normal in the double mutant, but evoked and spontaneous neurotransmitter release were completely absent. In these double mutants, application of LTX produced infrequent spontaneous events – less than one every 10 s in contrast to the frequency of $23.4\,s^{-1}$ in neurons in which *Munc13-2* alone was deleted (Varoqueaux *et al*., 2002). These rare spontaneous events demonstrated, however, that the postsynaptic apparatus was normally responsive to neurotransmitter. Rescue of the double mutant by overexpressing either Munc13-1 or Munc13-2 restored synaptic transmission. It was thus of interest to determine if there were differences in the pattern of synaptic activity in neurons that use the different munc-13 isoforms as priming factors. The results suggest that Munc13-2 is responsible for a facilitatory process known as augmentation (Rosenmund *et al*., 2002). Augmentation is a Ca^{2+}-dependent process of enhanced release that decays with a time constant of approximately 8 s (for a review of augmentation and other facilitatory processes, see Silinsky, 1985). The source of the calcium dependence of Munc13-2-mediated augmentation appears to come from endogenous PLC activation as the specific PLC inhibitor U72122 prevents augmentation (the inactive congener, U73343 did not affect augmentation).

How do these molecular genetic studies on Munc13 relate to the action of phorbol esters in neurotransmitter release? In the most dramatic example, Rhee *et al*. (2002) used a knockin strategy based upon the *Munc13-1* locus to create mice that expressed a mutation in the phorbol ester binding site of Munc13-1; the mutant neurons were completely insensitive to phorbol esters. Under these conditions, there was no change in the expression of PKC or in the ability of PKC to phosphorylate its targets at the secretory apparatus (e.g.

SNAP-25). These authors strongly suggested that many previous examples of PKC-mediated effects of phorbol esters on neurotransmitter release might actually be because of the effects on Munc13. They also found that the Munc13 C1 domain is an absolute requirement for neurotransmitter release to be maintained at substantial levels during repetitive high-frequency presynaptic stimulation, as a mutation in the phorbol ester binding site produces a marked increase in depression under these conditions (Rhee *et al*., 2002). These results suggest that the activation of the C1 domain of Munc13 by DAG (because of endogenously active signal transduction pathways) or by exogenous phorbol esters is an important physiological mechanism that increases the size of the *RRP*.

Phorbol ester receptors in general and their interactions with other presynaptic proteins and trans-synaptic processes

The focus of this review on Munc-13 by no means excludes other phorbol esters or even PKC (Stevens & Sullivan, 1998) as important mediators of secretory function. Indeed, the majority of cells contain several proteins other than Munc-13 and PKC with C1 domains that serve as phorbol ester/DAG receptors and thus act to translocate the protein to strategic sites in the cell (see e.g. Fabbri *et al*., 1994). These other C1-domain proteins include the chimaerins (which are implicated in a plethora of cell processes – see Ahmed *et al*., 1993, Kazanietz, 2002), and DAG kinase γ as well as two other C1 domain proteins that are important players in signal transduction cascades in eukaryotes (Brose & Rosenmund, 2002; Kazanietz, 2002). For example, T lymphocytes, via the activation of G-protein coupled receptors or tyrosine kinase coupled receptors, mediate their biological effects through the Ras/Raf/MEK/ERK pathway. Activation of this pathway is dependent upon the C1 domain of RasGRP (which activates Ras and similar small G-proteins) rather than upon PKC. Finally, protein kinase D1, an enzyme essential for targeting proteins to the cell surface, is translocated to strategic regions of the Golgi apparatus after binding of DAG or phorbols esters to its C1 domain. As this review focuses on neurotransmitter release, Munc13 and PKC, the reader interested in these alternative global pathways of DAG/phorbol signalling is referred to the excellent review by Brose & Rosenmund (2002) for the original citations.

With respect to PKC, Yawo (1999) found that PKC increases the sensitivity of the release mechanism to calcium entering via calcium channels at the giant synapse of the chick ciliary ganglion (as is evidenced by a leftward shift in the dose–response curve for extracellular calcium and acetylcholine release – but see Figure 3b). This effect was blocked by bisindolylmaleimeide (BIS), an inhibitor of ATP binding to the C3 domain (see Figure 2). Furthermore, studies with BIS suggest that PKC mediates increases in glutamate release in the rat cochlear nucleus (Oleskevich & Walmsley, 2000). While it is true that presynaptic proteins SNAP-25 (see above), Munc18, and synaptotagmin are substrates for phosphorylation by PKC (Hilfiker & Augustine, 1999), it is important to note that the bisindolylmaleimeide derivatives used in both of these studies may be toxic to excitable cells (see Brose & Rosenmund, 2002, for discussion).

Most of the interactions mediated by phorbol ester receptors and presynaptic proteins do not require phosphorylation by PKC. As alluded to above, Munc13 interacts with syntaxin and promotes its active state. In addition, the interaction between Munc13 with the vesicle Ca^{2+} binding protein DOC2α (see above) could alter the calcium sensitivity of this double C2 domain protein (see Hilfiker & Augustine, 1999). Munc13 might also interact with a GTP/GDP exchange factor protein for small G-proteins of the Arf family (Neeb *et al*., 1999) and with a brain isoform of β spectrin (Sakaguchi *et al*., 1998). One intriguing transynaptic interaction between a nonphorbol binding unc-13 homologue and unc-13 has been described by Doi & Iwasaki (2002) and merits discussion. Specifically, a novel protein homologous to Munc13-4 (a distantly related isoform found in secretory cells in lung that does not bind phorbol esters – see legend to Figure 2) has been found at neuromuscular junctions in *C. elegans* (Doi & Iwasaki, 2002). This protein, which is termed aex-1, couples a Munc13 homology domain (see Figure 2, MHD) with a potential C2 domain and is found postsynaptically in the muscle. Despite being postsynaptic, mutations of the *aex-1* gene causes abnormal presynaptic localization of Unc13; this aberrant localization is caused by the absence of an important retrograde messenger normally released from the muscle when aex-1 is active.

Perhaps, the most significant interaction between a phorbol ester receptor and a presynaptic protein occurs between Munc13-1 and rab interacting molecule-1 (RIM1) (Betz *et al*., 2001). RIM1 is so named because it is a presynaptic membrane effector of rab3a, a small GTP-binding protein that is found associated with the synaptic vesicles in the GTP-bound state (see e.g. Geppert & Sudhof, 1998). Indeed, RIM1 is one of the rare synaptic proteins that is specifically localized to the active zone. Its presence in the region has suggested its important role in tethering the vesicle near the active zone. Structurally, RIM1 contains a zinc-finger domain, a PDZ domain (characteristic of a select group of membrane proteins that helps bring multiprotein transduction process together) and two C2 domains. It was found that RIM1 binding region is conserved between Munc13-1 and Munc 13-2, and hence RIM 1 binds to the N terminus of both Munc13-1 and Munc 13-2 (see Figure 2c, L), competing with Rab3A for the same zinc-finger region of RIM1 (Betz *et al*., 2001). The part of Munc13 that binds syntaxin (and is thus responsible for priming) differs from that which binds RIM1. The interaction of Munc13 with RIM1 appears to occur at the variable L region that precedes the C1 domain at the N terminus of Munc13 (Figure 2), while the priming interaction with syntaxin is in the R region (Figure 2) and includes the C1 domain.

Of great interest are the results from studies in which the interaction between Munc13-1 and RIM1 is disrupted. This disruption was accomplished by overexpressing a Munc13-1 variant that possesses the RIM1 binding region of Munc13-1 without the ability to prime the secretory apparatus (Betz *et al*., 2001). Indeed, the observed presynaptic deficiency that occurs with overexpression of this RIM binding, but priming incompetent variant of Munc13-1 looks strikingly like that of Munc13-1-deficient neurons, with a decrease in the *RRP* and EPSCs being observed. From these results, it thus appears that the regions of Munc13 that bind RIM and syntaxin are both essential for neuronal function. The most intellectually alluring hypothesis based on these data can be summarized as follows. Once a synaptic vesicle with its attached rab3a arrives at the active zone, it attaches to RIM and the vesicle is now docked at the active zone (but see Koushika *et al*., 2001). RIM

can then bind to Munc13, switching its initial function in the docking process to one of assisting Munc13 in promoting the syntaxin-dependent priming of the secretory apparatus (Schoch *et al*., 2002). RIM thus appears to subserve the function of a *Renaissance* molecule at the synapse, docking the synaptic vesicle (via an interaction with rab3a), and then enhancing the activation of syntaxin and the priming process via an interaction with Munc13-1. Indeed, an open form of syntaxin can rescue the RIM mutant (Koushika *et al*., 2001), further supporting the role of RIM in promoting the syntaxin-dependent priming process.

From these results, it appears that the activity of the Munc13 pool at the active zone can be regulated both by C1 domain activators (DAG and phorbol esters) and by protein – protein interactions with molecules such as RIM1, in turn regulating the *RRP* of synaptic vesicles.

Unanswered questions on non-PKC phorbol ester receptors and neurotransmitter release

Based upon the effects at the C1 domain of Munc13, what electrophysiological correlates might be observed after treatment of nerve endings with phorbol esters? Using the standard nomenclature in which the mean number of packets (vesicles) of neurotransmitter released (m) is equal to the product of the number of primed vesicles in the *RRP* (n) multipled by the average probability of release (p) (i.e. $m = np$), it would be expected that the *RRP* would be increased. Two approaches support this conclusion. First, a train of stimuli (100 Hz) delivered to the mouse motor nerve ending shows an increase in release during the first few stimuli but no changes later in the train (Figure 5). This suggests that the mobilization of vesicles from a reserve pool to the docked state is not affected by phorbols, rather the number of primed vesicles (i.e., the size of the *RRP*) is increased. Second, preliminary experiments using binomial analysis of neurotransmitter release demonstrate an increase in the binomial parameter n without a change in the probability of release (p) after phorbol ester treatment at the amphibian neuromuscular junction (Searl & Silinsky, unpublished data in preparation). For a complete discussion of how the binomial parameters n and p might relate to specific parts of the secretory apparatus, see Searl & Silinsky (2002).

In the studies of Rosenmund *et al*. (2002), where the *RRP* is assumed to be measurable by examing the effects of sucrose on asynchronous neurotransmitter release, only a 30% increase in the *RRP* was observed compared with a 500% increase in EPSC amplitudes. Given the current model for phorbol ester actions on Munc13, which appear to be exclusively involved in priming the secretory apparatus, it is not clear why there should be such a large difference in the potentiation of the single evoked EPSC and the more modest increase in the *RRP*. One possibility is that the probability of release of the individual vesicle is increased as suggested by Rhee *et al*. (2002) and Rosenmund *et al*. (2002). While this effect cannot be ruled out, for example phorbol esters could act on the ultimate fusion reaction itself (Tokumaru & Augustine, 1999), this implies a further unknown involvement of phorbol esters in the release process (in addition to simply increasing the numbers of primed vesicles). Another possibility is that the degree of correlation between sucrose-evoked release and the *RRP* is not as great as previously thought. In the studies of Rhee *et al*. (2002), the sucrose-delineated pool is decreased

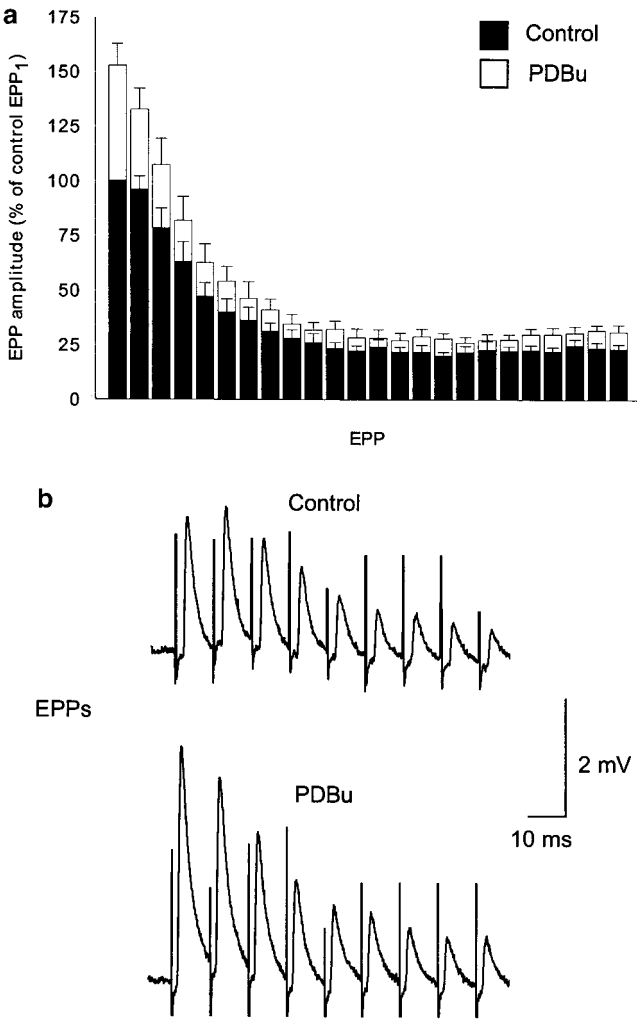

Figure 5 The effects of phorbol dibutyrate (PDBu) on evoked acetylcholine release (EPP amplitudes) during high-frequency trains in the mouse. EPPs were measured from the end plates of wild-type mouse phrenic nerve hemidiaphragm preparations in the presence of the protein kinase C inhibitor RO-318220 (1 μM). High-frequency trains (24 stimuli at 100 Hz, 2 mM Ca^{2+}) were applied at 60 s intervals. Under these stimulation conditions, the rapid rundown in EPP amplitudes at the beginning of the train presumably represents the depletion of the 'readily releasable pool' (*RRP*) of the transmitter. The plateau at the end of the train presumably represents a balance between the release of transmitter and the 'mobilization' of transmitter for release. Amplitudes of the EPP trains were averaged (between six and nine trains) in control and then following application of 100 nM PDBu (recorded from the same end plate). In the upper panel (a), averaged results are shown for $n = 5$ preparations. In (b), the first nine EPPs (averages of trains from the same end plate in control and in PDBu) from a typical experiments in (a) are shown. As shown here, the first 2 – 3 EPPs recorded at the beginning of the trains of stimuli are potentiated strongly by PDBu, whereas the EPPs at the end of the train are less affected compared with the control EPPs. This result is consistent with PDBu increasing the size of the readily releasable pool while having little effect on the probability of release. For details of the experimental conditions in the mouse preparation, see Hirsh *et al*. (2002).

when the phorbol binding site is mutated (i.e. a decrease in *RRP*) with no change in the mean EPSC (m). These results led to the conclusion that the probability of release is increased when the phorbol binding site on Munc13 is mutated (as $m = np$, then a decrease in n with no change in m requires an increase in p). This interpretation must be taken with caution

as the rate of refilling of the sucrose pool was not affected by deletion of the phorbol binding site while the rate of refilling of the action-potential-evoked pool was decreased by this mutation. This suggests that the pool of synaptic vesicles might differ for release evoked by action potentiates and by sucrose.

One of the confounding issues in the scheme presented in Figure 3 relates to the surprising result that the EPSC in response to discrete nerve impulses was not affected by the elimination of a crucial amino acid required for phorbol ester binding in Munc13-1. This is in contrast to the effects of repetitive stimulation discussed above, whereby pronounced depression occurs with higher frequency of stimulation in the mutant because the phorbol binding site is unavailable to mediate increases in the *RRP*. A further complication arises if it can be confirmed that Munc13-1 binding to syntaxin is not affected by phorbol esters (see Rhee *et al*., 2002, p. 131). These observations have led to the suggestions that evoked release at low stimulation frequencies occurs from a pool of vesicles primed by a form of Munc13 that is attached to the cytoskeleton and is functionally independent of the C1 domain (Rhee *et al*., 2002); this pool is only slowly replenished. If this is true, then, speculatively, this could be the pool attached to spectrin (Sakaguchi *et al*., 1998). The other pool, as discussed above, is dependent upon the C1 domain and contributes to an increase in the *RRP* when phorbol esters are applied.

The final issue relates to the C2 domains in Munc13, domains normally important in binding calcium. What is the importance of the three C2 domains in Munc13-1? In worms and flies, some data suggest that the C2 domains of these unc-13 isoforms might be involved in the calcium sensing process (Aravamudin *et al*., 1999; but see Richmond *et al*., 1999). This may not be the case with Munc13 isoforms as the calcium sensitivity of release is unchanged in chromaffin cells in which munc13-1 is overexpressed (but see caveat in legend to Figure 4) nor do the C2 domains apparently bind Ca^{2+} (Brose *et al*., 1995 – see also Augustin *et al*., 1999, p. 359). Further experiments are indeed necessary to determine the importance of the multiple C2 domains in Munc13 (see also Tokumaru & Augustine, 1999). In particular direct measurements of the binding properties of Ca^{2+} and other divalent cations to these C_2 domains may provide further insights into the possible role of domains.

In conclusion, while the data summarized in this review suggest a possible role for PKC in the control of neurotransmitter secretion, the critical role that non-PKC phorbol ester receptors such as Munc13 play in the process of neurotransmitter release demonstrates that phorbol esters and neurotransmitter secretion are indeed more than just PKC.

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