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# The Regulation of Neurotransmitter Secretion by Protein Kinase C

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## Abstract

The effect of protein kinase C (PKC) on the release of neurotransmitters from a number preparations, including sympathetic nerve endings, brain slices, synaptosomes, and neuronally derived cell lines, is considered. A comparison is drawn between effects of activation of PKC on neurotransmitter release from small synaptic vesicles and large dense-cored vesicles. The enhancement of neurotransmitter release is discussed in relation to the effect of PKC on:

1. Rearrangement of the F-actin-based cytoskeleton, including the possible role of MARCKS in this process, to allow access of large dense-cored vesicles to release sites on the plasma membrane.
2. Phosphorylation of key components in the SNAP/SNARE complex associated with the docking and fusion of vesicles at site of secretion.
3. Ion channel activity, particularly $Ca^{2+}$ channels.

**Index Entries:** Protein kinse C; secretion; SH-SY5Y; cytoskeleton; vesicles; $Ca^{2+}$ channels.

## Introduction

Regulated exocytosis is the process by which vesicular and plasma membranes fuse as a consequence of a sudden rise in intracellular calcium ($[Ca^{2+}]_i$). Several stages occur during the secretory process of which exocytosis is the final step. These include movement of vesicles to release sites, and docking of vesicles at the plasma membrane prior to fusion and retrieval of membrane components by endocytosis (Rothman, 1994; Sudhof, 1995; Martin, 1997). The exocytotic step is dependent on an increase in $[Ca^{2+}]_i$ following activation of voltage-gated $Ca^{2+}$ channels, which can be regulated by receptors situated on nerve endings.

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Although several studies have shown that secretion is highly regulated by protein kinases, including calcium/calmodulin kinase II, protein kinase A, and protein kinase C (PKC; Pang et al., 1988; De Camilli et al., 1990; Coffey et al., 1994; Liu et al., 1994; Vitale et al., 1995; Majewski and Musgrave, 1995; Turner et al., 1996; Goodall et al., 1997b), little is known about the detailed molecular mechanisms underlying regulation by kinases.

This article will focus on recent studies on the regulation of neurosecretion by PKC. In particular, the possible mechanisms at the molecular level by which PKC enhances neurosecretion will be discussed. Several possibilities exist to account for the regulation of neurotransmitter release by PKC. These include:

1. Activation of ion channels (particularly $Ca^{2+}$ channels).
2. Removal of the cytoskeletal F-actin barrier to vesicle movement (in particular, the extent to which activation of PKC leads to the accumulation of vesicles at release sites.
3. Modification of vesicle docking/fusion steps.

PKC has also been reported to evoke secretion of mucin from colonic cells (Hong et al., 1997), histamine from mast cells (Ludowyke et al., 1996), and insulin from pancreatic β-cells (Deeney et al., 1996; Efanov et al., 1997). These examples fall outside the nervous system, so they will not be considered further in this article.

## Preparations Used to Study the Effect of PKC on Secretion

In order to understand the molecular mechanisms underlying the regulation of exocytosis by PKC, it is necessary to have an exocytotic model that lends itself to molecular manipulations. Mammalian brain slices and synaptosomes provide convenient preparations with which to demonstrate receptor regulation of exocytosis (Chesselet, 1984; Middlemiss, 1988; Sanchez-Prieto et al., 1996). However, owing to the complexity of the tissue, the heterogeneity of nerve endings and the predominance of glial and other nonneuronal cell membranes, these preparations are limited in their usefulness for studies on the molecular mechanisms by which receptors regulate exocytosis. For example, receptors for a number of neurotransmitters and neuromodulators are located on astrocytes as well as neurons (*see*, e.g., Hamprecht, 1986), thus making receptor-mediated changes in second messengers difficult to interpret. It is however important to establish that phenomena observed in cells in culture are also observed in more intact preparations and to use these latter to test hypotheses based on data obtained from cells in culture.

### *Central Nervous System Preparations*

#### *Brain Slices*

Early studies (Malenka et al., 1986) showed that the phorbol esters 4-β-phorbol-12,13-dibutyrate (PDBu) and phorbol-12,13-diacetate (PDBAc), which activate PKC, enhanced synaptic transmission in rat hippocampal brain slices. In a later study, these authors found that PDBu and PDAc enhanced both spontaneous postsynaptic potentials and $K^+$-evoked glutamate release, the main excitatory neurotransmitter in the hippocampus (Malenka et al., 1987). In contrast, application of 4-α phorbol-12,13-didecanonate, which does not activate PKC, did not enhance $K^+$ evoked release of glutamate.

Subsequent studies confirmed these initial observations, since it was found that the PKC activators, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and PDBu enhanced NA release from mouse brain cortical slices evoked by electrical stimulation. This effect was attenuated by the PKC inhibitor polymyxin B and was not observed with 4-α-phorbol 12,13-dibutyrate, which does not activate PKC. Downregulation of PKC (by exposure of slices to 1 μ*M* PDBu, for 10 h) abolished the facilitatory effect of acutely applied PDBu on NA release (Schroeder et al., 1995). Furthermore, Parfitt and Madison (1993) reported a potentiation of transmission (detected electrophysiologically) owing to activation of PKC by phorbol esters in hippocampal slice prepara-

tions by a presynaptic, $Ca^{2+}$-dependent mechanism. The potentiation of synaptic transmission is also observed by coapplication of arachidonic acid and either diacylglycerol (DAG; Bramham et al., 1994) or the metabotropic glutamate receptor agonist 1-aminocyclopentane-1$_S$,3$_R$-dicarboxylic acid (ACPD; Collins and Davies, 1993). These observations imply that activation of glutamate receptors gives rise to elevated DAG levels leading to activation of PKC. Thus, activation of PKC appears to enhance neurotransmitter secretion from multicellular central nervous system preparations.

*Synaptosomes*

Synaptosomes are preparations of nerve endings obtained when brain is homogenized under isotonic conditions. They represent an enriched source of nerve endings and are the simplest neuronal preparation with which to study presynaptic mechanisms, including receptor regulation of release (*see*, e.g., Sanchez-Prieto et al., 1996). Several studies have shown that neurotransmitter release from synaptosomes is modified by activation of PKC, usually with phorbol esters.

In a recent detailed study, Terrian and Ways (1995) reported that PDBu increases $Ca^{2+}$-dependent glutamate release during continuous depolarization of synaptosomes by elevated extracellular $K^+$. The same study also provided evidence that sustained activation of PKC increases synaptic vesicle recycling in the presence of saturating concentrations of $Ca^{2+}$. In addition, it appears that PDBu did not increase the availability of cytosolic free calmodulin or the activity of $Ca^{2+}$/calmodulin-dependent protein kinase. Since cytochalasin D totally occluded the effect of PDBu, the authors suggest that there is a direct interaction between PKC isoforms and cytoskeletal PKC binding proteins. The same group (Terrian, 1995) also found that the persistent effects on $Ca^{2+}$-dependent glutamate release were directly related to the dose of PDBu used as well as being an independent of method of depolarization. These persistent effects of PDBu were not observed with glutamate release evoked by the calcium ionophore ionomycin and were prevented by VSCC blockers. Thus, the persistent effects of PKC activation on depolarization-evoked glutamate release were dependent on the route of $Ca^{2+}$ entry. They conclude that PKC affects the slow phase of glutamate release by increasing the efficiency of vesicle mobilization and recycling. More recently, an interesting report from the same group (Prekeris et al., 1996) suggests that there is an isoform-specific interaction between PKC-ε and F-actin, and this serves as a prelude to the enhancement of glutamate release from synaptosomes. These authors found that F-actin is a principal anchoring protein for PKC-ε within the nerve endings The binding of PKC-ε to actin requires that the kinase be activated. They also report that arachidonic acid interacted, synergistically with diacylglycerol to stimulate F-actin binding to PKC-ε. Once established, PKC-ε remained securely fixed to the cytoskeleton, which also acted as a chaperone maintaining the kinase in a catalytically active conformation. Thus, F-actin appears to be a bifunctional anchoring protein specific for the PKC-ε isoform. The authors suggest that the assembly of this isoform-specific signaling complex appears to play a primary role in the PKC-—dependent facilitation of glutamate exocytosis. In a parallel study (Zhang et al., 1996) found that arachidonic acid and oleoylacetylglycerol facilitate, synergistically, $Ca^{2+}$-dependent glutamate release from hippocampal mossy fiber nerve endings. Interestingly, disruption of cytoskeletal organization with cytochalasin D prevented the lipid-dependent facilitation of both KCl and ionomycin-evoked glutamate release. In addition, arachidonic acid plus glutamatergic or cholinergic agonists enhanced glutamate release, and a role for PKC was suggested by using specific inhibitors. The overall conclusion from the study was that lipid-dependent facilitation of glutamate release from mossy fiber nerve endings requires $Ca^{2+}$ and involves multiple presynaptic effects, some of which depend on PKC.

A separate study Nicholls and his group reports that phorbol esters enhance 4-aminopyridine-evoked glutamate release from cortical synaptosomes, whereas activation (or inhibition) of $K^+$-elicited glutamate release is not affected. The authors conclude that PKC does not play an obligatory role in a single cycle of exocytosis (as elicited by "clamped" depolarization with KCl). In contrast, glutamate release evoked by spontaneous action potentials (which inhibits $K^+$ channels) in the presence of 4-aminopyridine is enhanced by PKC (Barrie et al., 1991; Coffey et al., 1993). These authors also found that phorbol esters had little effect on the resting membrane potential, but greatly enhanced the 4-aminopyridine-evoked depolarization. This suggested an enhancement of spontaneous action potentials owing to inhibition of $K^+$ channels, (possibly "delayed rectifier" or A-type) based on inhibition of PKC facilitation of glutamate release by 1 m*M* $Ba^{2+}$ and 0.50 μ*M* clofilium (Sanchez-Prieto et al., 1996). Of particular interest is the observation by Nicholls and his group that the metabotropic glutamate receptor agonist ACPD greatly enhances glutamate release evoked by 4-AP, but not by KCl. They also only find this enhancement in the presence of exogenous arachidonic acid (AA), which is also required to detect ACPD-dependent phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS). The authors interpret their data in terms of a model in which AA sensitizes PKC to the transient increase in DAG resulting from activation of metabotropic glutamate receptors by ACPD (Coffey et al., 1994; *see* Sanchez-Prieto et al., 1996 for review).

In another series of experiments, Nicholls and colleagues (Barrie and Nicholls, 1993; Budd and Nicholls, 1995) reported that 4-aminopyridine- and $K^+$-evoked glutamate release from cerebrocortical synaptosomes could be inhibited by the adenosine $A_1$ receptor agonist cyclohexyl adenosine (CHA). This inhibition was associated with a decreased 4-aminopyridine and $K^+$-evoked increase in $[Ca^{2+}]_i$. Futhermore, the CHA-induced inhibition is suppressed by 3 n*M* PBDu or by activation of the PKC-coupled metabotropic glutamate receptor by ACPD. In addition, a tonic inhibition owing to leaked exogenous adenosine can be reversed by adenosine deaminase or by PDBu.

The overall conclusion from studies on synaptosomes is that multiple mechanisms occur to account for the enhancement of glutamate release following activation of PKC. One mechanism that is of particular interest, in view of recent studies on the effect of PKC activation on release of catecholamines from chromaffin cell and the human neuroblastoma SH-SY5Y (*see later*), is that PKC regulates the movement of vesicles from the reserve pool, which are attached to F-actin to release sites on the plasma membrane.

Further examples of the use of brain slices and synaptosomes to study the role of PKC in the regulation of NA release will be provided later in this article when the role of the PKC substrate, GAP-43, is discussed.

### *Peripheral Nervous System Preparations*

Several studies have suggested that PKC regulates NA release from cardiac nerve endings. Thus, bradykinin (acting at $B_2$ receptors) enhances electrically induced NA release from rat atria preincubated with [$^3$H] NA. In addition, the PKC inhibitor, bisindolymaleimide reduced significantly the facilitatory effect of BK, AII, and PDBu on NA release from rat atria (Chulak et al., 1995). Thus, BK appears to enhance NA release via prejunctional $B_2$ receptors in rat atria by a mechanism involving PKC. A more recent study (Kurz et al., 1997) found that although BK enhanced electrically evoked NA release from isolated perfused rat heart, this effect was not inhibited by inhibitors of PKC, cyclooxygenase, blockage of NO synthesis, or tyrosine kinase. BK enhancement of NA release was, however, inhibited by the phospholipase $A_2$ inhibitor AACOCF3. The authors concluded, therefore, that presynaptic $B_2$ receptors located on cardiac sympathetic neurons enhanced exocytoytic release of NA

via activation of phospholipase $A_2$. In addition, the muscarinic $M_1$ agonist, McNeil A 343, has been suggested to enhance electrically evoked NA release from postganglionic sympathetic nerves in mouse atria by a mechanism involving PKC (Costa et al., 1993). Further support for suggestions that PKC regulates NA release from cardiac nerves is provided by the observation that TPA enhances electrically evoked NA release from guinea pig atria (Brasch, 1993). In contrast, PDBu has been found to attenuate the facilitatory effect of nicotine on electrically evoked NA release from bovine cerebral vessels (Sanchez-Merino et al., 1995). Thus it appears that activation of PKC can decrease the facilitatory effect of nicotine possibly by increasing the desensitization of the nicotinic receptor. In this context, it is of interest that inhibition of PKC either by downregulation with phorbol esters or using the inhibitor GF 109203X has been reported to decrease nicotine-evoked release of catecholamines from bovine chromaffin cells (Cox and Parsons, 1997). Thus, depending on conditions, nicotine-evoked secretion of catecholamines appears to depend on the activation of PKC.

## Cell Culture

Cultures of neurons overcome some of the complexities and ambiguities associated with studies on molecular mechanisms using brain slices and synaptosomes. Although primary cultures of central nervous system (CNS) neurons from rat cerebellum and cortex have been used to study the release of glutamate and GABA, respectively (Pocock et al., 1995; Takei et al., 1997), less success has been achieved with primary cultures of adrenergic neurons. This is mainly because of the difficulty of obtaining a sufficient number of cells and the heterogeneity of cell type.

One approach that offers considerable promise for future studies is the development of catecholaminergic cell lines by the use of oncogenes driven by cell-specific promotors that direct tumorigenesis to brainstem neurons of transgenic mice. Of particular interest are the studies of Chikaraishi and her group, who have isolated a catecholaminergic cell line, CATH.a from tyrosine hydroxylase-positive tumours in transgenic mice carrying the SV40 T-antigen oncogene under the the transcriptional control of 773 bp of 5′-flanking sequences from the rat TH gene (Suri et al., 1993). More recently it has been shown that CATH.a cells deprived of serum differentiate to put out processes that contain vesicles in their endings (Qi et al., 1997). It remains to be established whether the differentiated cell line (CAD) will exhibit depolarization-evoked, $Ca^{2+}$-dependent release of catecholamines.

More success has been achieved with primary cultures of sympathetic neurons (*see later*). For example, primary cultures of superior cervical ganglia, which coexpress high levels of catecholamines and neuropeptide Y (NPY), were used as a model to examine whether sympathetic neuronal peptide and transmitter content or secretion is differentially regulated (May et al., 1995). Interestingly, depolarization stimulated NPY secretion 18-fold, whereas catecholamine secretion increased only threefold. Addition of forskolin or dibutyryl cAMP led to a concentration-dependent, sustained stimulation of NPY secretion and a decrease in cellular NPY content. Stimulation of catecholamine release was also observed, but total catecholamine content was also increased. The phorbol ester TPA stimulated NPY secretion to a lesser extent than activation of PKA and had a minimal stimulatory effect on catecholamine release. The magnitude of the secretory responses of sympathetic neurons to these regulators was far greater than changes in NPY or catecholamine content, biosynthesis, or mRNA levels. Thus, release is the primary site of regulation. These studies imply that release from large electron-dense core vesicles (LDCVs) predominates in these cultures and should be compared with recent studies on sheep spleen (De Potter et al., 1995, 1997), which also suggest that NA release occurs from LDCVs. The work of May et al. (1995)

also suggests that there are fundamental differences in the biosynthetic pathways, vesicular compartmentalization, uptake, and metabolism of neuropeptides and neurotransmitters.

### *Neuroendocrine Cells*

Important increases in our understanding of the mechanisms underlying neurotransmitter/hormone release have come from studies using chromaffin cells. These correspond to primary cultures and have been used extensively to study the release of NA and adrenaline, and will be referred to in more detail in later sections of this article. An alternative approach is to use continuous cell lines derived from neoplastic tissues that express $Ca^{2+}$-dependent release of hormones or neurotransmitters similar to the parent tissue. One cell line that has been used extensively to study the release of catecholamines is the PC12 cell line isolated from a rat pheochromocytoma. This clone occurs in culture in two forms, $PC12^+$, which expresses morphological and some functional properties of a chromaffin cell, and $PC12^+$ isolated when PC12 cells are differentiated with nerve growth factor, which exhibits morphological and some functional properties of sympathetic neurons. PC12 cells have been used by several groups to study the depolarization and cholinergic-evoked release of either dopamine (DA; Ritchie, 1979; Baizer and Weiner, 1985; Meldolesi et al., 1988; Martin, 1997) or NA (Rabe and McGee, 1983; Rabe et al., 1987). Early work provided limited pharmacological data showing that cholinergic agonists evoke release of catecholamines by acting at nictotinic receptors. More recent work, however, suggests that muscarinic receptors are also involved, although the amounts of catecholamine released were very small and required a sensitive perfusion assay to detect them (Meldolesi, et al., 1988). Thus, catecholamine release from PC12 cells appears to be regulated mainly by depolarization following activation of nicotinic receptors with muscarinic receptors playing a minor role. However, the cholinergic regulation of catecholamine release from PC12 cells could be more complex and depends on the differentiation state of the cells. The major catecholamine in PC12 cells is DA, which is present at 5–10 times the NA content (Ritchie, 1979). This decreases the value of PC12 cells as a model for studying the regulation of NA release, since represents a discrepancy with the parent adrenal gland, which secretes adrenaline and NA as the major catecholamines rather than DA. Nethertheless, PC12 is an important cell line for studying the effect of PKC on release of catecholamines from LDCVs (*see* e.g., Oda et al., 1997) as will be evident later in the article.

### *The Human Neuroblastoma SH-SY5Y*

One of the aims of this article is to consider the merits of an alternative cell line, the neuroblastoma clone SH-SY5Y derived from a human sympathetic ganglion (Ross and Biedler, 1995), which is becoming of increasing importance. Previous studies have shown that SH-SY5Y can be differentiated to express many properties of a mature sympathetic neuron (Pahlman et al., 1990). In particular, following treatment for several days with low concentrations (16 n*M*) of TPA, SH-SY5Y synthesizes NA, has a higher content of NA compared to DA, and in preliminary studies, was found to express high-affinity uptake and depolarization-evoked $Ca^{2+}$-dependent release of NA (Scott et al., 1986). During the past 10 years, several groups have established the value of SH-SY5Y as a model for studying the regulation of differentiation by PKC (*see* e.g., Heikkila et al., 1989; Leli et al., 1992; Parrow et al., 1995). These studies suggest that downregulation of PKC-α and PKC-ε was incompatible with differentiation. During the past 7 years, it has been found that SH-SY5Y is an excellent cell line for studying the molecular mechanisms underlying NA release. Thus, we have demonstrated high-affinity uptake of NA and glutamate (O'Neil et al., 1994). In addition, we have demonstrated depolarization (induced by raised $K^+$, veratridine, nicotinic agonists, and $Ba^{2+}$) and $Ca^{2+}$-ionophore (A23187) evoked release of [$^3$H]NA, which is dependent on extracellular $Ca^{2+}$. Furthermore, we have

shown that activation of muscarinic ($M_3$), bradykinin ($B_2$), and (in cells transfected with the rat $AT_{1A}$ receptor) angiotensin II receptors also evokes NA release by a mechanism that largely uses intracellular $Ca^{2+}$ (Murphy et al., 1991; Vaughan et al., 1993; 1995a; McDonald et al., 1994; 1995). An important difference between our studies and previous work is that NA release is measured in cultures of SH-SY5Y cells, which have not been differentiated by exposure to phorbol esters for several days (Vaughan et al., 1995b). In this connection, pilot studies Vaughan, unpublished observations) found no difference in the extent of NA release or the proportion of release inhibited by L-type (nifedipine) and *N*-type (ω-conotoxin) VSCC antagonists in cultures of SH-SY5Y cells treated with TPA, retinoic acid, or nerve growth factor.

SH-SY5Y cells have been found to express muscarinic $M_1$,$M_2$ (Adam et al., 1987) and $M_3$ (Lambert et al., 1989) receptor subtypes, as well as μ- and δ-opioid receptors (Kazmi and Mishra, 1987) and $\alpha_2$-adrenoceptors (Kazmi and Mishra, 1989). More recently, our group and others (Purkiss et al., 1995) have provided electrophysiological evidence (*see later*) and NA release data which suggest that SH-SY5Y expresses bradykinin $B_2$ (McDonald et al., 1994, Purkiss et al., 1995), muscarinic $M_1$ (McDonald et al., 1994) $Y_2$ receptors (McDonald et al., 1994, 1995). SH-SY5Y also expresses the G-proteins $G_s\alpha$, $G_i\alpha1$, $G_i\alpha2$, $G_o\alpha$, $G_z\alpha$, and Gβ. $G_o\alpha$ is the major G-protein found in the CNS, and $G_z\alpha$ appears to be closely associated with neuronal tissue (Ammer and Schultz, 1994). Thus SH-SY5Y cells express several G-proteins found in the mammalian nervous system (Meneray and Bennett, 1995). In our studies so far, we have detected $G_s\alpha$, $G_i\alpha$ and and $G_o\alpha$ and the small GTP binding protein rab 3A by Western blot analysis of SH-SY5Y. In addition, the observations that activation of muscarinic $M_3$ and bradykinin $B_2$ receptors is accompanied by changes in inositol phosphates (Lambert and Nahorski, 1990; Purkiss et al., 1995) $[Ca^{2+}]_i$ (Lambert and Nahorski, 1990; Murphy et al., 1991; McDonald et al., 1994; Purkiss et al., 1995) suggest that G-proteins are important in the regulation of $[Ca^{2+}]_i$ in SH-SY5Y. Additional support for this view is provided by our observations that muscarinic ($M_3$) and NPY ($Y_2$) inhibition of $Ca^{2+}$ currents is inhibited by pertussis toxin (*see later*).

Release of NA is maximally enhanced by 8 min of pretreatment with TPA (100 n*M*) which activates PKC (Murphy et al., 1992; Turner et al., 1994; 1996). Our current studies (Turner et al., 1994, 1996) using Western blots have detected PKC subtypes α, ε, and ζ in SH-SY5Y. Furthermore pretreatment of SH-SY5Y cell layers with phorbol 12,13-dibutyrate for 48 h results in the differential downregulation of PKC-α by over 90% compared with PKC-ε (approx 50%) and little effect on expression of PKC-ζ as detected by Western blots. These differences have been refined by comparing the effect of PDBu and bryostatin-1 (also shown to downregulate PKCs in SH-SY5Y [Parrow et al., 1998]) on the expression of PKC subtypes. Treatment with PDBu and bryostatin has proven particularly useful, since PKC-α is decreased by 40 and 60%, respectively, after 2 h, whereas PKC-ε and PKC-ζ are not affected. Since acute exposure to TPA no longer enhances NA release in cells exposed to bryostatin-1 for 2 h (Turner et al., 1996) it is probable that PKC-α is the important subtype in mediating the enhancement of NA release in SH-SY5Y cells. These findings have enabled us to establish conditions under which activation of PKC will enhance release and to identify PKC substrates important in this process in terms of:

1. Their phosphorylation time.
2. Their intracellular relocalization.
3. How these events are affected by selective downregulation of PKC-α (Goodall et al., 1997b).

A more detailed account of these changes will be presented later. The observation that TPA enhances NA release in SH-SY5Y is in agreement with other workers using synaptosomes, brain slices, and primary cultures of striatal neurons (Jalava et al., 1993) who have also reported enhancement of neurotransmit-

ter release following activation of PKC by phorbol esters. The human neuroblastoma SH-SY5Y thus provides us with an ideal cell line with which to study the role of PKC substrates associated with the cytoskeleton, vesicles, and plasma membranes in the regulation of NA release.

## Synaptic Vesicles and Large Dense Core Vesicles

Two main types of secretory vesicles are found in the nervous system. Small synaptic vesicles (SSV; 40–60 nm diameter) that occur mainly in nerve endings in the CNS and (LDCV >75 nm diameter) that are present mainly in the sympathetic nervous system and are similar to the much larger (250–300 nm diameter) chromaffin granules found in chromaffin cells. SSVs contain only the "classical" neurotransmitters, such as acetylcholine, catecholamines, glutamate, and GABA. They are located in two pools. The first is an active pool docked at the plasma membrane adjacent to voltage-sensitive $Ca^{2+}$ channels (particularly N-type), which are poised for very rapid release of transmitter in response to an influx of $Ca^{2+}$ (McMahon and Nicholls, 1991). In addition, a second reserve pool of SSVs is found to be associated with the F-actin cytoskeleton (*see later*). In contrast, LDCVs in addition to "classical" neurotransmitters, such as catecholamines, also contain peptide neurotransmitters, such as enkephalins and NPY. LDCVs are found in sympathetic neurons (where they occur at between 5 and 30% of the SSVs), but are relatively sparse in the CNS. In resting chromaffin cells, they are located at a distance from release sites and excluded from the plasma membrane by a barrier of actin filaments. LDCVs resemble very closely the adrenal chromaffin granule. Thus, they store as secretory products NA, ATP, chromagranins, enkephalins, and NPY. However, although much important information on the regulation of secretion has come from studies on chromaffin cells, a word of caution is necessary. Not only are the storage granules considerably larger than LDCVs, but also their exceptionally large numbers could lead to differences in the storage and movement of granules compared with LDCVs in sympathetic neurons, which are more sparsely located. In addition, up to 30% of secretory vesicles in PC12 cells can be found associated with the plasma membrane under resting conditions. This implies that caution should be taken in extrapolating results obtained using PC12 cells to study release from LDCVs to sympathetic neurons, since details between storage of LDCVs appear to differ.

Some sympathetic neurons contain small dense-cored vesicles (SDCVs) in addition to LDCVs. SDCVs have electron dense cores under certain fixation conditions and a diameter of 40–60 nm. SDCVs differ from LDCVs mainly because they do not contain soluble proteins or peptides, e.g., chromagranins, enkephalins, and NPY. Another important difference between SDCVs and LDCVs is that the former express the vesicle protein synaptophysin, whereas the latter do not. However, they can take up catecholamines and nucleotides as well as synthesize noradrenaline. Recent studies using isolated, perfused sheep spleen have suggested that NA is released only from LDCVs and not from SDCVs inspite of the numerical superiority of the latter vesicles (De Potter et al., 1995, 1997). In addition, PC12 cells, in common with other endocrine cells, possess a poulation of "synaptic-like microvesicles" (SLMVs), which contain many of the integral proteins of SSVs (such as synaptotagmin, synaptobrevin, and synaptophysin). They do not store catecholamines and appear to release acetylcholine (Bauerfeind et al., 1993, 1995). Recent studies, however, suggest that release of acetlycholine from SLMVs and of catecholamines from LDCVs occur by the same basic mechanism (Nishiki et al., 1997). Pathways for the synthesis of these vesicles differ. LDCVs, since they contain peptides and soluble proteins, must be synthesized in the cell body Golgi apparatus, whereas the small vesicles could be early endosomes located within the varicosities (*see*, e.g., Bauerfeind et al., 1994). Recent studies suggest

that synaptophysin is delivered to SLMVs via the plasma membrane and an internal compartment (Schmidt et al., 1997). This provides additional support for the view that SLMVs represent a different pool of vesicles and are not precursors of LDCVs in neuroendocrine cells.

Recent advances in the identification of vesicle-specific proteins have led to the development of antibodies against vesicle-specific proteins, such as synapsin, synaptotagmin, synaptophysin, synaptobrevin, and rab 3A (Sudhof and Jahn, 1991; Jahn and Sudhof, 1994). Using these antibodies together with methods for isolating the different types of vesicles, it appears that SSVs express all the above proteins whereas LDCVs do not appear to express synaptophysin (*see*, e.g., Walch-Solimena et al., 1993; Goodall et al., 1997a). Our electron microscopic and subcellular fractionation data indicate that SH-SY5Y cells contain dense-cored secretory vesicles of 120-nm average diameter, which costore NA and NPY as well as secretogranins-I and II and dopamine-β hydroxylase corresponding to LDCVs (Goodall et al., 1997a; Ou et al., 1998). In addition, our immunocytochemical and Western blot studies suggest that SH-SY5Y cells express two types of vesicles. LDCVs, which express synaptotagmin-I, synaptobrevin, synapsin-I, and rab 3A, and a population of smaller vesicles (possibly corresponding to the SLMVs of PC12 cells), which express synaptophysin as well, but do not contain NA, NPY, or secretogranin-II (Goodall et al., 1997a).

## Cytoskeletal and Vesicular Proteins and Exocytosis

Current hypotheses (Sudhof and Jahn, 1991; Trifaro and Vitale, 1993; Jahn and Sudhof, 1994) propose that SSVs accumulate in distinct regions of the cytoplasm (active zones) and that only those vesicles apposed to the inner layer of the plasma membrane are able to release their contents in response to the rise in $[Ca^{2+}]_i$. The bulk of the vesicles are prevented from diffusion to the plasma membrane by interaction with cytoskeletal proteins, such as F-actin. For example, synapsin I is a neuron-specific vesicle-associated protein that is phosphorylated in response to depolarization. In the dephosphorylated state, synapsin I binds SSVs to F-actin and, thus, traps the vesicles in a cytoskeletal mesh. One consequence of depolarization is activation of the $Ca^{2+}$/calmodulin-dependent protein kinase (CAM kinase II), resulting in the phosphorylation of synapsin I. This leads to the liberation of vesicles from their links with F-actin and increased migration of vesicles to the plasma membrane (Dc Camilli et al., 1990; Robinson, 1991; Trifaro and Vitale, 1993).

A different situation occurs in chromaffin cells, which are used as a convenient model for studying exocytosis from LDCVs. Current hypotheses (Cheek and Burgoyne, 1986; Trifaro and Vitale, 1993; Vitale et al., 1995) suggest that under resting conditions, the majority of secretory granules are located at least 150 nm from the plasma membrane in chromaffin cells. This suggests that there is a barrier preventing the movement of vesicles to the plasma membrane. Immunocytochemical studies show that chromaffin cells have a continuous ring of F-actin underneath the plasma membrane. Furthermore, quantitative electron microscopic studies show that in any given section of a resting chromaffin cell, < 10% of granules are seen within 50 nm of the plasma membrane. Following treatment with 100 n*M* TPA, this number increases to 12–15% (Vitale et al., 1995). Chromaffin granules are retained within the cortical actin network, where they are associated with F-actin by α-actinin (Trifaro et al., 1992). The F-actin filaments are linked to the plasma membrane by fodrin (a member of the spectrin family of proteins that links F-actin to plasma membranes in most cells). Additional support for the general hypothesis is provided by the recent studies of Gillis et al. (1996), who used membrane capacitance measurements to assay $Ca^{2+}$-triggered exocytosis in single bovine adrenal chromaffin cells. Treatment with TPA increased the size of the readily releasable pool of secretory granules. Thus, TPA increased the

amplitude, but not the time-course of the exocytotic burst that resulted from rapid elevation of $[Ca^{2+}]_i$ owing to photolysis of DMI-nitrophen ("caged" $Ca^{2+}$). The authors conclude that PKC affects a late step in secretion, but not the $Ca^{2+}$ sensitivity of the final step.

An alternative mechanism proposes a role for the CAM binding, F-actin regulatory protein caldesmon as well as the $Ca^{2+}$-dependent F-actin-severing enzymes scinderin and gelsolin. At low $[Ca^{2+}]_i$, caldesmon stimulates actin assembly and crosslinks F-actin to form an actin "cage", which provides a barrier to exocytosis (Aunis and Bader, 1988). Entry of $Ca^{2+}$ following depolarization activates calmodulin, which binds to caldesmon leading to a disruption of the actin crosslinks. Thus, increasing $[Ca^{2+}]_i$ not only disrupts links between vesicles and F-actin, but also dissolves the cytoskeletal gel. There is good evidence showing that following $K^+$-evoked or nicotinic receptor stimulation-induced depolarization, the subplasmalemmal F-actin disassembles, allowing the secretory granules in chromaffin cells to reach the plasma membrane (Trifaro and Vitale, 1993). Studies by Trifaro et al. have suggested that the major cause of F-actin disassembly following depolarization is activation of the $Ca^{2+}$-dependent F-actin-severing enzyme, scinderin, similar to gelsolin and villin (Trifaro et al., 1992; Marcu et al., 1994). In support of this view are the observatiions that scinderin, but not gelsolin, diffuses out of permeabilized chromaffin cells and that loss of scinderin is acompanied by a loss of chromaffin cell secretory response (Vitale et al., 1992). Furthermore Vitale et al. (1991) reported that following depolarization of chromaffin cells owing to activation of nicotinic receptors or elevated $K^+$, subcortical scinderin, but not gelsolin redistributes to foci on the plasma membrane and this redistribution precedes catecholamine secretion. More recently, Zhang et al. (1996). Have shown that recombinant scinderin potentiates F-actin disassembly and exocytosis in permeabilized chromaffin cells, and that this action is blocked by two peptides with sequences corresponding to two F-actin binding sites on scinderin. In addition, studies on tissue distribution suggest that scinderin is restricted to tissues with high secretory activity (Tachakarov et al., 1990).

Treatment of chromaffin cells with phorbol esters causes a partial disruption of the cortical actin cytoskeleton, increases the number of LDCVs in the 0 to 50-nm cortical zone, and potentiates nicotine, potassium, and electrically evoked catecholamine release (Vitale et al., 1995). These observations together with the inhibitory effect of cyclic AMP on nicotine-induced F-actin disassembly and secretion suggest that cortical actin networks are also a site for second messenger modulation of exocytosis. Aditional support for a role for calmodulin in release from LDCVs was provided by the study of Schweitzer et al. (1995) on NA release from PC12 cells. In this study it was reported that the $Ca^{2+}$/CAM kinase II inhibitor, KN-42, inhibited approx 50% of NA release evoked by either carbachol or direct depolarization. KN-42 did not inhibit the rise in $[Ca^{2+}]_i$ observed following depolarization or application of carbachol. The authors conclude that two pools of LDCVs occur in PC12 cells, one of which is able to release NA in response to a rise in $[Ca^{2+}]_i$. Release from the other pool requires a $Ca^{2+}$/CAM kinase II-dependent step in addition. Clearly this pool of LDCVs has analogies to the requirement for phosphorylation of synapsin-I in the liberation of the reserve pool of SSVs from F-actin in central synapses.

Additional support for the hypothesis that F-actin acts as a barrier to secretion from LDCVs is provided by the recent study of Chen and Wagner (1997), which found that $K^+$ and ATP-evoked release of NA from PC12 cells is accompanied by a $Ca^{2+}$-dependent decrease in F-actin.

## Candidate Target Proteins for PKC Substrates

### *MARCKS*

The mechanism by which PKC promotes cytoskeletal rearrangements has not yet been

established, although several studies have suggested that the PKC substrates MARCKS and GAP-43 are important proteins in regulating F-actin plasma membrane interactions and possibly exocytosis (Dekker et al., 1991; Coffey et al., 1994; Lui et al., 1994). Similar changes in the actin-based cytoskeleton and phosphorylation of MARCKS underlie the phorbol ester-activated release of prolactin from $GH_4C_1$ cells (Kiley et al., 1992), the secretion of pepsinogen from gastric chief cells (Raufman et al., 1997), glucose-induced insulin secretion (Calle et al., 1992), and of glutamate from hippocampal synaptosomes (Terrian and Ways, 1995). In the nonphosphorylated state, MARCKS binds to F-actin and is associated with the plasma membrane (*see* 109, Blackshear, 1993; Aderem, 1995 for reviews). Thus, MARCKS is a candidate protein for a role in maintaining the tight F-actin barrier associated with the plasma membrane in chromaffin cells (Trifaro and Vitale, 1993) and SH-SY5Y cells (Goodall et al., 1997b). MARCKS migrates anomalously on SDS gels with $M_r$ ranging from 60,000 to 87,000, although the primary sequence indicates an $M_r$ value of 31 k Dalton for the human protein (Harlan et al., 1991). It has a highly conserved N-terminal region and an effector domain of 25 amino acids, referred to as the phosphorylation site domain (PSD), which also contains calmodulin and F-actin binding sites (Graff et al., 1989; Hartwig et al., 1992). Another interesting property of MARCKS that it shares with a second PKC substrate, GAP-43, is its stability to boiling in the absence of sodium dodecyl sulfate (SDS), which helps to identify and isolate these proteins.

MARCKS can be phosphorylated both in whole cells and cell-free extracts with a stoichiometry of three or four phosphates (depending on species) on serines, all of which are located in the PSD (Manenti et al., 1992). Recent studies using recombinant $His_6$-MARCKS and PKC subtypes expressed and purified from baculovirus-infected insect cells found that the serine residues in the PSD were phosphorylated in a definite order: $S_{156}$=> $S_{163}$>>$S_{152}$, although there was little difference in the overall phosphorylation of MARCKS by PKC-$\beta_1$, PKC-$\delta$ and PKC-$\varepsilon$ (Herget et al., 1995). In addition, although PKC-$\zeta$ phosphorylated a peptide corresponding to the PSD in MARCKS, no phosphorylation of histone IIIS, $His_6$-MARCKS, or GST-MARCKS could be observed (Herget et al., 1995). Phosphorylation results in the dissociation of MARCKS from the plasma membrane and in the loss of its ability to crosslink F-actin (Thelen et al., 1991). For example, recent studies in fibroblasts have shown that PKC-dependent phosphorylation regulates the movement of MARCKS between the plasma membrane and Lamp-1-positive lysosomes (Allen and Aderem, 1995). A similar phosphorylation and movement of MARCKS could lead to a selective, partial dissassembly of the F-actin cytoskeleton, and therefore, the migration of secretory vesicles to release sites on the plasma membrane in chromaffin and SH-SY5Y cells. Furthermore, it has been shown that the related protein MacMARCKS is associated with secretory vesicles in the PC12 cells and SSVs in cerebrocortical synaptosomes (Chang et al., 1996). Further support for a role for MARCKS in the regulation of secretion by PKC is provided by studies on the release of ACTH release from pituitary cells by AVP (Lui et al., 1994). Exposure of cultured ovine anterior pituitary cells to 1 $\mu M$ AVP resulted in the rapid phosphorylation of MARCKS reaching a maximum plateau by 30 s. In the same cells AVP stimulated ACTH release biphasically. During the first 30 s there was a rapid burst of ACTH release followed by a slower sustained secretion. The authors suggest from the correlation between MARCKS phosphorylation and ACTH secretion, that MARCKS phosphorylation may be involved in the initial events associated with secretion of this hormone. An alternative possibility is that MARCKS phosphorylation represents a priming step for the slower sustained phase of secretion. Thus, there are several observations that provide additional support for the suggestion that the MARCKS family of proteins play a role in integrating $Ca^{2+}$ and PKC-

dependent signals in the regulation of neurosecretion.

## GAP-43

Recent studies on GAP-43 (B-50, F1, pp46 or P-57) have clarified its role in regulating the growth state of axonal terminals. Phosphorylation of GAP-43 by PKC appears to be involved in transducing intra- and extracellular signals to regulate cytoskeletal organization in the nerve ending both in relation to nerve terminal sprouting and long-term potentiation (*see* Benowitz and Routtenberg, 1997 for a recent review). GAP-43 has also been implicated in exocytosis. This protein, which is located on the inner leaflet of the plasma membrane, has been found to become phosphorylated during release of NA from rat hippocampal slices and rat brain cortical synaptosomes (Dekker et al., 1991; Hens et al., 1993a). Hens et al. (1993b), have also found that inhibition of PKC activity using the synthetic peptide $PKC_{19-36}$, and downregulation of PKC by TPA did not affect $Ca^{2+}$-evoked NA release in streptolysin *O*-permeabilized synaptosomes, although these treatments inhibited phosphorylation of GAP-43. These observations suggest that GAP-43 is involved in the processing of vesicles prior to the $Ca^{2+}$ trigger rather than in exocytotic events subsequent to the increase in $[Ca^{2+}]_i$. One possibility is that GAP-43 acts as a CAM store, since phosphorylation of GAP-43 has been found to decrease its binding to calmodulin. Furthermore (Hens et al., 1995), report that monoclonal antibody (MAb) NM2, directed toward the N-terminal residues 39–43 of rat GAP-43, dose-dependently inhibited $Ca^{2+}$-induced NA release from streptolysin *O*-permeabilized synaptosomes. In contrast, antibody NM6, directed against the C-terminal residues 132–213, was without effect. In addition, NM2 inhibited PKC-dependent phosphorylation of Ser41 and the binding of CAM in permeated synaptosomes. These observations suggest that the N-terminal residues 39–43 of rat GAP-43 play an important role in $Ca^{2+}$-dependent NA release, presumably by serving as a local CAM store. Thus, a consequence of phosphorylation of GAP-43 is that intracellular levels of CAM would increase, leading to an activation of CAM kinase II and the subsequent phosphorylation of synapsin I (Vitale et al., 1991). This could lead to an increase in the number of SSVs at release sites and, hence, an enhancement of secretion. Support for the involvement of GAP-43 in catecholamine release is also provided by the study of Ivins et al. (1993), who reported that isolation of stably transfected clones of PC12 cells expressing GAP-43 cDNA in the antisense orientation had decreased depolarization-evoked dopamine release. However, in similar clones prepared by R. Neve (Mailman Research Center, Harvard Medical School) of SH-SY5Y cells in which GAP-43 was either overexpressed or synthesis decreased (as detected by Western blots) no effect on depolarization or muscarinic $M_3$-evoked release of NA could be observed (Vaughan, unpublished observations). Thus, the phosphorylation of GAP-43 does not appear to act as a regulator of NA release in SH-SY5Y cells.

## Role of MARCKS in PKC Enhancement of Secretion in SH-SY5Y

A possible insight into the mechanism by which PKC could enhance exocytosis has been provided by recent studies using SH-SY5Y. Pretreatment of SH-SY5Y cell layers with TPA for 10 min increased the incorporation of $^{32}P_i$ into two proteins with apparent molecular weights (76 and 46 kDa) and isoelectric points (p*I* = 4.0 and 3.7) corresponding to MARCKS and GAP-43, respectively. The identity of these phosphorylated proteins was confirmed using Western blots. Thus, both MARCKS and GAP-43 became phosphorylated during the time-course of TPA enhancement of NA release. In addition, using a combination of electron microscopy, immunocytochemistry, and cell fractionation, the following changes were observed (Goodall et al., 1997b; Danks, 1999).

1. PKC-α migrated from the cytosol to the plasma membrane.

2. The number of LDCVs in a 100 nm zone adjacent to the plasma membrane increased three fold.
3. MARCKS became phosphorylated, and approx 50% of MARCKS was relocated from membrane to cytosolic fractions. Under these conditions, although GAP-43 became phosphorylated, no redistribution between cytosol and membrane fractions was observed.
4. Confocal microscopy using fluroscein isothiocyanate-labeled phalloidin showed that the F-actin cytoskeleton, which forms a tight barrier at the plasma membrane, partially disassembled.

Furthermore, translocation of MARCKS from the plasma membrane to the cytosol is correlated with the presence of PKC-α (Goodall et al., 1997b). Thus, pretreatment of SH-SY5Y cells with the phorbol ester PDBu for 2 h (conditions that led to the loss of 40% immunostaining for PKC-α, but no decrease in PKC-ε immunostaining (Turner et al., 1996) resulted in a 40% decrease in the translocation of MARCKS to the cytosol induced by TPA (Goodall et al., 1997b). Also, treatment with PDBu for 48 h, which leads to the downregulation of PKC-α and PKC-ε by 90% and 50%, respectively (Turner et al., 1996), completely inhibited the translocation of MARCKS. Interestingly, phosphorylation of MARCKS was only decreased by about 40% following TPA treatment of cells exposed to PDBu for 48 h (Goodall et al., 1997b). Thus our recent studies suggest that in intact SH-SY5Y cells, PKC-ε and PKC-ζ are unable to phosphorylate all the serines in the PSD of MARCKS. In addition, our studies are in agreement with the previous report that all the serine residues in the PSD of MARCKS need to be phophorylated before translocation can take place (Thelen et al., 1991).

These observations have suggested the following working hypothesis to account for the enhancement of NA release by phorbol esters in SH-SY5Y cells. The initial step is migration of the PKC-α subtype to the plasma membrane. Our previous studies (Turner et al., 1996) have found that NA release is enhanced by PKC-α rather than either of the other two PKC subtypes (PKC-ε and PKC-ζ) present in SH-SY5Y cells (Leli et al., 1993; Turner et al., 1994; Parrow et al., 1995). A consequence of migration of PKC-α to the plasma membrane is that serines in the PSD of MARCKS are phosphorylated, resulting in a dissociation of the F-actin filaments crosslinked to the PSD (*see* e.g., Thelen et al., 1991). This leads to dissociation of a pool of MARCKS from the plasma membrane and disruption of the links between MARCKS and F-actin filaments, resulting in the partial breakdown of the actin cytoskeletal barrier. The loss of this barrier leads to an increase in the number of LDCVs adjacent to release sites, so that following elevation of $[Ca^{2+}]_i$, NA release is enhanced. Our proposed mechanism could also account for the action of phorbol esters on chromaffin cell F-actin disassembly (Vitale et al., 1992). Our studies also suggest that although all of the serine residues in the PSD of MARCKS need to be phosphorylated for translocation to occur, some of these serine residues are selectively phosphorylated by PKC-α in intact cells. Thus, under the conditions used to enhance NA release, PKC-ε is unable to phosphorylate sufficient serines for translocation of MARCKS to occur.

Our current views on the mechanisms by which activation of PKC-α could lead to the redistribution of LDCVs in SH-SY5Y cells are summarized in Fig. 1.

## Other Candidate Proteins as Targets for PKC

### *Vesicle-Associated Proteins and Proteins Involved in the Docking/Fusion Process*

Several proteins associated with secretory vesicles and the plasma membrane that have been implicated in the mechanism by which the secretory pathway is regulated have been identified and characterized over the past few years (Sudhof, 1995; Calakos, and Scheller, 1996; Hanson et al., 1997; Morgan and Burgoyne, 1997) These include synaptotagmin (Sudhof and Rizo, 1997), the vesicle-associated

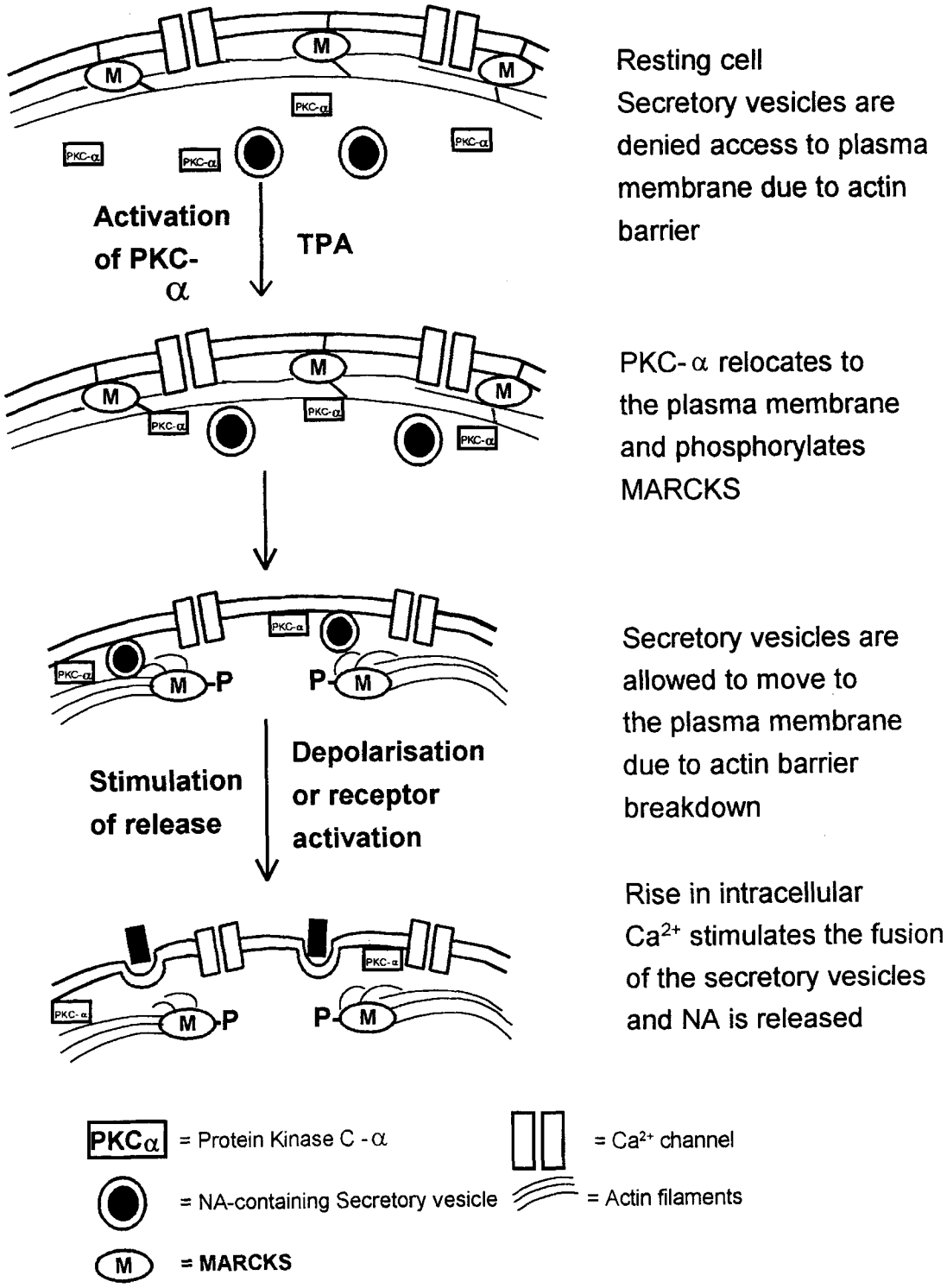


Fig. 1. Scheme summarizing the effect of activation of PKC-α by TPA on the F-actin cytoskeleton and distribution of LDCVs in SH-SY5Y cells. *See text* for details.

membrane protein (VAMP)/synaptobrevin (Trimble et al., 1988; Baumart et al., 1989), and the plasma membrane proteins syntaxin (Bennett et al., 1992) and 25-kDa synaptosomal protein (SNAP-25; Oyler et al., 1989). One of the strongest lines of support for the involvement of these proteins in secretion has come from the use of clostridial neurotoxins that act as specific (zinc-dependent) neurotoxins to cleave synaptobrevin (Schiavo et al., 1992), syntaxin and SNAP-25 (Blasi et al., 1993; Montecucco and Schiavo, 1994; Neimian et al., 1994). In addition, it has been found that synaptobrevin, syntaxin, and SNAP-25 can be isolated as a complex and act as receptors for the soluble protein α-soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (α-SNAP), which in turn recruits NSF (Sollner et al., 1993 a,b). In addition, the *Drosophila* mutant *comatose* has defective neurotransmission owing to mutations in the gene coding for NSF (Pallanck et al., 1995). Thus, recent and previous studies (see, e.g., Rothman, 1994) have suggested that these proteins, all which have homologs essential for consititutive exocytosis in yeast (Ferro-Noric and John, 1994) were part of a general, conserved mechanism for the docking and fusion of vesicles in both constitutive and regulated secretory pathways.

### *Synaptotagmin*

Synaptotagmin is an integral membrane protein localized to both SSVs and LDCVs, which has been implicated in the docking and fusion steps of $Ca^{2+}$-dependent exocytosis. Thus, microinjection of antibodies against synaptotagmin, but not against the vesicle proteins synaptobrevin, rab 3a, synaptophysin, and $SV_2$, decreased $K^+$-evoked $Ca^{2+}$-dependent increases in dopamine β hydroxylase staining on the surface of PC12 cells (Elferink et al., 1993). Synaptotagmin is a major vesicle protein comprising 7–8% of total protein in synaptic vesicles. The large cytoplasmic domain contains two $C_2$ motifs, each similar to the $Ca^{2+}$ and phospholipid binding domain of PKC. Studies with recombinant protein suggest that both cytoplasmic $C_2$ domains are necessary to bind to and aggregate chromaffin granules in a $Ca^{2+}$-dependent manner (Damer and Creutz, 1994). Similar studies showed the recombinant synaptotagmin binds to artificial liposomes in a $Ca^{2+}$-dependent manner (Chapman and Jahn, 1994). Further studies have shown that there is an interaction between synaptotagmin and synapsin I which is mediated by the C-terminal region of the latter protein. This interaction appears to be regulated by a novel $Ca^{2+}$-binding site, which does not require phospholipids (Chapman et al., 1995). Synaptotagmin contains many potential phosphorylation sites that could regulate binding to membranes. In one study (Popli, 1993), immunoprecipitation of synaptotagmin from endogenously phosphorylated synaptic vesicles suggested that phosphorylation is mediated by $Ca^{2+}$/CAM kinase II. A possible note of caution that synaptotagmin might not be involved in secretion from all cell types is provided by the study of Wendland and Scheller (1994). These workers found that stable expression of fragments of rat synaptotagmin I, comprising the first C2 repeat, the second C2 repeat, or the entire cytoplasmic domain (in the mouse anterior pituitary cell line AtT-20), had no effect on the release of [$^3$H]choline or ACTH. The rationale behind these experiments is that the fragments may compete with endogenous synaptotagmin for membrane binding sites. The fragments were expressed and found to be localized to both soluble and membrane fractions, and in the case of the second C2 repeat, to coimmunoprecipitate with synaptotagmin. Constitutive secretion and endocytosis were also unaffected. The authors conclude that either a redundant pathway exists or that synaptotagmin is not involved in membrane trafficking in endocrine cells. However, the possibility remains that the fragments fail to compete successfully with synaptotagmin for its membrane binding sites.

An interesting study was carried out by Bauerfeind et al. (1995), who used synaptotagmin I- and II- defficient PC12 cells to compare the role of this protein in $Ca^{2+}$-dependent

release from LDCVs and SLMVs. They found that although catecholamine and protein secretion from LDCVs was not affected, depolarization-evoked release of acetylcholine from SLMVs was no longer $Ca^{2+}$-dependent (Bauerfeind et al., 1995). The authors proposed that depolarization-evoked secretion from SLMVs occurs by both $Ca^{2+}$ dependent and independent pathways. Furthermore, only the $Ca^{2+}$-dependent pathway requires synaptotagmin.

*Munc-18*

Munc-18 is the mammalian equivalent of a family of ubiquitous proteins highly expressed in neurons (also known as Sec-1 in yeast [Halachmi and Lev, 1996], unc-18 in *Caenorhabditis elegans* [Gengyo-Ando et al., 1996], and Rop protein in *Drosophila* [Harrison et al., 1994]). Both unc-18 (Gengyo-Ando et al., 1996) and Rop (Harrison et al., 1994) have been implicated in neurotransmission, which suggests that munc-18 and its homologs might be essential components of the secretory pathway. In vitro studies have shown that munc-18 binds to syntaxin with high affinity (Hata et al., 1993; Garcia et al., 1994; Hodel et al., 1994; Pevsner et al., 1994), and inhibits binding of syntaxin to SNAP-25 and synaptobrevin (Pevsner, 1996). Recently, it has been shown that munc-18 is a substrate for PKC and that phosphorylation decreases its affinity for syntaxin: Thus, Fujita et al. (1996) found that recombinant munc-18 is phosphorylated by PKC in a $Ca^{2+}$- and phospholipid-dependent manner on serines 306 and 313. Furthermore, they reported that munc-18 complexed with syntaxin is not phosphorylated, and that PKC-catalyzed phosphorylation of munc-18 inhibits its interaction with syntaxin. It has been suggested therefore that munc-18 plays a role in the docking of vesicles by controlling the formation of the complex between syntaxin, SNAP-25 (t-SNAREs), and synaptobrevin (a v-SNARE). Furthermore, the regulation of this interaction by the phosphorylation state of munc-18 suggests a possible site for the control of secretion by PKC.

*14–3–3 Proteins*

These proteins are found in a broad range of organisms and tissues with particularly high concentrations in mammalian brain. They have also been implicated in catecholamine release from bovine chromaffin cells, since they leach out of permeabilized cells and have been shown to enhance exocytosis on reconstitution (Morgan and Burgoyne, 1992). These proteins have been implicated in many diverse functions, including signal transduction pathways, exocytosis, and cell-cycle regulation. Intact 14–3–3 proteins inhibit PKC in vitro, and contain conserved sequences that ressemble the pseudosubstrate domain of PKC and the C-terminus of annexins (Xiao et al., 1995; Wheeler et al., 1996). 14–3–3 Proteins are found at high concentrations on synaptic plasma membranes where they bind through the N-terminal 12-kDa region (Jones et al., 1995). There is some confusion in the literature concerning whether these proteins act as inhibitors or activators of PKC. Thus, although several studies found that 14–3–3 proteins are potent inhibitors of PKC (Jones et al., 1995), 14–3–3 proteins, isolated from bovine forebrain, have been reported to activate PKC (Tanji et al., 1994). This confusion was partly resolved when it was realized that several isoforms of 14–3–3 proteins exist. Thus, Dellambra et al. (1995) report that the $\sigma$ and $\zeta$ isoforms of 14–3–3 proteins stimulate PKC in keratinocytes, whereas Aitken et al. (1995) report that posttranslationally modified forms of $\beta$ and $\delta$ enhance the inhibitory action of these proteins on PKC twofold. In addition, site-specific mutagenesis of several regions in the $\tau$ isoform of 14–3–3, including mutation of a putative pseudosubstrate site to a potential substrate sequence, did not alter its inhibitory activity. Part of the confusion may also arise from the differing effect of the various isoforms of 14–3–3 proteins on the several PKC subtypes. Thus Acs et al. (1995) found that the 14–3–3 $\zeta$ isoform activated the classical PKC isozymes approximately twofold, had no effect on PKC-$\delta$, but greatly activated PKC-$\varepsilon$. Recently, 14–3–3 proteins have been implicated

in the rearrangement of the F-actin cytoskeleton associated with the movement of secretory granules to the plasma membrane in chromaffin cells (Chamberlain et al., 1995; Rorh and Burgoyne, 1995). It remains to be determined whether this represents a direct action of 14–3–3 proteins or a secondary effect consequent to their effect on PKC.

*Dephosphin/Dynamin*

This protein is a 94,000/96,000 mol-wt neuronal-specific PKC substrate that is located in nervous tissue. Using a specific polyclonal antibody, Powell and Robinson (1995) studied the distribution of dephosphin by immunoblotting and immunocytochemistry. From this study, they concluded that dephosphin was found only in the brain, and not in the testes, lung, kidney, adrenals, heart, liver, or skeletal muscle. A more detailed analysis showed that dephosphin occurred in high levels in the perikarya, axons, and nerve terminals of cerebellar neurons, but not in radial Bergman glial cells. Thus, dephosphin appears to be neuron-specific, distributed widely throughout the brain and concentrated in nerve endings, where it is regulated by PKC-mediated phosphorylation (Powell and Robinson, 1995). Phosphorylation converts dephosphin to a tetramer and activates an intrinsic GTPase activity (Liu et al., 1996). An important observation providing insight into its mechanism of action is that dephosphin becomes dephosphorylated following depolarization owing to $Ca^{2+}$-dependent activation of the $Ca^{2+}$/CAM-dependent phosphatase calcineurin (Nichols et al., 1994). This cycle of depolarization-regulated phosphorylation/dephosphorylation has led to suggestions that dephosphin may play a role in endocytosis (Robinson et al., 1993, 1994; Liu et al., 1994). Support for this hypothesis has come from the observation that dephosphin, labeled with $^{32}P_i$, was dephosphorylated following depolarization with 40 m*M* $K^+$ in synaptosomes prepared from hippocampus, cerebellum, and cerebral cortex. The evidence available to date supports the current hypotheses, suggesting a role for dephosphin in synaptic vesicle recycling in nerve terminals. Recent studies (*see* e.g., McClure and Robinson, 1996; Wigge et al., 1997) suggest that dynamin is assembled into a collar or fusion ring that surrounds the "neck" of recycling synaptic vesicles by binding to the Scr homology 3 (SH3) domain of amphiphysin.

*Annexins*

Annexins are a family of $Ca^{2+}$-binding proteins that relocate to cell membranes on elevation of $[Ca^{2+}]_i$ (Raynal and Pollard, 1994; Mohti et al., 1995; Barwise and Walker, 1996; Blanchard et al., 1996).

Many roles have been proposed for annexins, including $Ca^{2+}$-dependent mediators of exocytosis and endocytosis (Sarafian et al., 1991; Creutz, 1992; Harder and Gerke, 1993; Chasserotgolaz et al., 1996). Annexins assocaite with both phospholipids and with the cytoskeleton in a $Ca^{2+}$-dependent manner. The phospholipid binding is mediated by $Ca^{2+}$-binding loops that project from highly conserved domains of 70 amino acids that are repeated four times in all annexins, except annexin VI, which contains 8 repeats. The N-termini of annexins are not conserved between mammalian annexins, which vary considerably in length and sequence. For certain annexins, the N-termini interact with members of another family of $Ca^{2+}$-binding proteins, S-100 proteins.

The N-termini of certain annexins contain sites for phosphorylation by receptor-linked tyrosine kinases and PKC. In particular, annexin II is phosphorylated by the tyrosine kinase of pp60src, whereas annexin II (Waisman, 1995) and annexin IV (Raynal and Pollard, 1994) have been reported to be substrates for PKC. Interestingly, annexin V, which is not a substrate for PKC, has been found to inhibit PKC (Schlaepfer et al., 1992; Raynal et al., 1993; Hyatt et al., 1994; Rothhut et al., 1995). It is not clear, however, whether the inhibition is owing to annexin V, competing with PKC for binding to phospholipid or to a more direct action of annexin V on PKC. An additional link between annexins and PKC is the presence of a sequence in annexins similar to the one found

in RACK-1 and 14–3–3 (Ron et al., 1994). RACK-1 is thought to be an anchoring protein that helps the targeting of activated PKC to specific intracellular locations (Mochly-Rosen et al., 1991). 14–3–3 Proteins bind to and inhibit PKC (Aiken et al., 1995) and are thought to help reorganize the cortical actin cytoskeleton of cells. In permeabilized chromaffin cells, addition of 14–3–3 stimulates catecholamine release (Rothand Burgoyne, 1995).

We have demonstrated that SH-SY5Y cells contain annexins I, II, IV, V, VI, and possibly annexin III (Blanchard et al., 1996). Annexin II was predominantly associated with the plasma membrane, but annexin V showed a cytosolic and nuclear location. On elevation of $[Ca^{2+}]_i$ with the ionophore A23187 or depolarization with 100 m*M* KCl annexins II and V relocate and attach to membranes in a way that is not extractable by EGTA, but requires Triton-X-100 for solubilization. The resistance to extraction of annexins from membranes following an increase in $[Ca^{2+}]_i$ has been observed in several cell types (Mohti et al., 1995; Trotter et al., 1995; Barwise and Walker, 1996). In mammalian brain, annexin V is enriched in glial cells (Woolgar et al., 1990), but it has also been found in axon terminals associated with synaptic vesicles (Gotow et al., 1996). Early work on cholinergic synapses (Fielder and Walker, 1985; Walker, 1982) also demonstrated an association of calelectrin (annexin II) with cholinergic synaptic vesicles at sites of contact with the nerve terminal plasma membrane.

The first annexin implicated in mediating membrane–membrane interactions was annexin VII (synexin), which was isolated using an assay based on its ability to aggregate chromaffin granule membranes in the presence of $Ca^{2+}$ (Raynal and Polland, 1994). Subsequently, it was shown that membrane fusion could be induced by addition of free fatty acids to membranes aggregated by annexin VII (Raynal and Pollard, 1994). Annexin II has also been shown to be able to aggregate chromaffin granules (Regnouf et al., 1995). Interestingly, in this case, membrane fusion was found to result from subsequent activation of PKC.

Annexins may be purified owing to their $Ca^{2+}$-dependent affinity for cytoskeletal fractions, and several have been shown to bind to F-actin in a $Ca^{2+}$-dependent manner (Raynal and Pollard, 1994). These data suggest that annexins may play an important role at the interface between F-actin and the plasma membrane in SH-SY5Y cells. A recent report of relevance to this suggestion is that annexin VI binds to the calb domain of p120GAP (Davis et al., 1996). The calb domain of this protein is located in the $Ca^{2+}$ and phospholipid binding C2 domain of p120GAP. C2 domains are also present in other proteins that bind to annexins. These include PKC (Mochly-Rosen et al., 1991, Hyatt et al., 1994) and cytosolic phospholipase $A_2$ (Kin et al., 1994; Mira et al., 1997). C2 domains are also present in phospholipase C, phosphoinositol 3-kinase, and the vesicle-associated protein synaptotagmin. It would be particularly interesting if annexins mediated interactions between proteins containing C2 domains and the cytoskeleton/phospholipid interface of the mammalian cell.

## Neuronal Ion Channels and PKC

Neuronal excitability dictates synaptic transmitter release and is determined by the activity of plasma membrane ion channels. The temporally coordinated activity of voltage-gated $Na^+$, $K^+$, and $Ca^{2+}$ channels underlies the spreading of action potentials along axonal and dendritic pathways, and also controls $Ca^{2+}$ influx at synapses, thereby regulating exocytosis. Such a fundamental role for voltage-gated ion channels implies that fine-tuning their activity will have important influences on synaptic transmission. This is indeed the case: ion channels are important regulatory targets for hormones and indeed for the transmitters whose release is controlled by channel activity. Numerous second messenger pathways exist, which couple a vast array of receptors to ion channels, and this section considers the various ways in which PKC activation can alter channel activity. We have confined our com-

ments to the regulation of neuronal voltage-gated $Ca^{2+}$ channels, since the regulation of neuronal $Na^+$ and $K^+$ channels by PKC and other kinases represents a vast and rapidly expanding field, and has recently been reviewed in detail elsewhere (Catterall, 1993, 1996a,b; Levitan, 1994; Cantrell et al., 1996; Cukierman, 1996; Aidley and Stanfield, 1996).

Neuronal voltage-gated $Ca^{2+}$ channels represent a large family of heteromultimeric proteins. Each consists of a pore-forming $\alpha_1$-subunit, which possesses the voltage sensor as well as being the binding site for numerous therapeutically important drugs (e.g., dihydropyridines). Although when expressed alone in recombinant systems the $\alpha_1$-subunit can function as a $Ca^{2+}$ channel, its properties are normalized (i.e., modulated such that they resemble more closely native $Ca^{2+}$ channels) when coexpressed with the auxiliary $\beta$- and $\alpha_2$-—subunits. The classification of neuronal voltage-gated $Ca^{2+}$ channel has developed from the early low- and high-voltage-activated subtypes (representing T-type and all others, respectively) such that high-voltage-activated $Ca^{2+}$ channels have been subdivided into five separate types (L-, N-, P-, Q-, and R-types), distinguishable by their biophysical and pharmacological properties (Dolphin, 1995). Further diversity is provided by splice variants of both the $\alpha_1$- and $\beta$ subunits (Dunlap et al., 1995). The majority of evidence suggests that N-type channels are of primary importance in mediating transmitter release in peripheral neurons, whereas in the central nervous system, there is good evidence for both P- and N-type channels mediating synaptic transmission, although the relative importance of these and other subtypes depends on the synaptic pathway under study (Dunlap et al., 1995).

As discussed earlier, investigations into the possible modulatory roles of PKC on $Ca^{2+}$ channel activity have been heavily reliant on the use of phorbol esters. Awareness that phorbol esters could modulate voltage-gated $Ca^{2+}$ channels emerged from early studies that indicating that $Ca^{2+}$-dependent exocytosis in various secretory models, including chromaffin cells (Knight and Baker, 1983; Burgoyne et al., 1988), PC-12 cells (Harris et al., 1985), and brain synaptosomes (Nichols et al., 1987), could be enhanced by phorbol esters through PKC activation. This field of research expanded enormously with the application of the patch-clamp technique (Hamill et al., 1981): with whole-cell and single-channel recording techniques, the specific enhancement of subtypes of channel could be directly investigated. An earlier review has summarized the extensive reports of modulation of $Ca^{2+}$ channels by phorbol esters (Shearman et al., 1989), and a striking feature of these studies is the diversity of reported actions of phorbol esters. Both enhancement and inhibition of $Ca^{2+}$ currents have been reported, and in other cases, lack of discernible effects has been noted (Shearman et al., 1989). There are many possible reasons for such apparently diverse responses, not the least being the fact that such roles for PKC have been sought in a wide range of tissues, and the numerous subtypes of $Ca^{2+}$ channels are likely to vary considerably in the number and accessibility of phosphorylation sites available for PKC.

Among the first reports of the effects of phorbol esters on $Ca^{2+}$ channel activity, there was a measure of agreement that activation of PKC enhanced $Ca^{2+}$ channel opening. This was demonstrated originally in invertebrate neurons, where both TPA and the purified enzyme produced similar enhancements of $Ca^{2+}$ currents (DcReimer et al., 1985). Similar effects were also reported at the single-channel level in cardiac cells and in sympathetic neurons (Lacerde et al., 1988; Lipscombe et al., 1988), More recently, evidence has emerged that PKC can enhance activity of recombinant $Ca^{2+}$ channels (Bourinet et al., 1992; Singer-Lahat et al., 1992). Interestingly, an isolated report using *Aplysia* neurons indicated that PKC activation could induce activity of $Ca^{2+}$ channels that were previously inactive (Strong et al., 1987). However, this recruitment of channels by PKC activation has yet to be demonstrated in mammalian neurons. Instead, a more recent report has indicated that PKC activation may enhance

$Ca^{2+}$ currents in central and peripheral neurons by disrupting the tonic inhibition of channels by G-proteins (Swartz, 1993). Thus, in cortical and pyramidal neurons, phorbol esters enhanced $Ca^{2+}$ currents and reduced the ability of baclofen and 2-chloro-adenosine to inhibit these currents. Similar results were obtained in peripheral neurons (from superior cervical and dorsal root ganglia). These observations could also account for the effects of PKC on recombinant $Ca^{2+}$ channels, since recombinant channels are capable of coupling to endogenous G-proteins in various expression systems.

In contrast to the above reports, there are several accounts of phorbol esters causing inhibition of neuronal $Ca^{2+}$ channels. For example, Schroeder et al. (1990) demonstrated that phorbol esters selectively inhibited T-type $Ca^{2+}$ channels in sensory neurons. Furthermore, evidence has been provided that PKC activity mediates the inhibitory action of certain transmitters or hormones on neuronal $Ca^{2+}$ channels; for example, the inhibitory actions of cholecystokinin in *Helix aspersa* neurons were enhanced by intracellular injection of concentrations of purified PKC, which by themselves were too low to cause a discernible effect (Hammond et al., 1987). Furthermore, pretreatment of cells with levels of diacylglycerol analogs, which themselves inhibited $Ca^{2+}$ currents in rat sensory neurons, prevented further inhibition by noradrenaline (Rane and Dunlap, 1986). More recently it has been reported that TPA strongly inhibits $K^+$-evoked $[Ca^{2+}]_i$ transients and $Mn^{2+}$ quenching of fura-2 fluoresence in bovine adrenal chromaffin cells. In contrast 4α-PDBu had no effect (Sena et al., 1995). Thus, it appears that under certain conditions, activation of PKC can lead to an inhibition of VSCCs.

Thus, contrasting effects of PKC activation on $Ca^{2+}$ channel activity are apparent. One possible reason for this emerged when phorbol esters were shown to inhibit $Ca^{2+}$ currents without activating PKC. This effect was originally reported in neurons (Hockberger et al., 1989), and subsequently also in cardiac tissue (Asai et al., 1991). Indeed, within the same preparation, both PKC-dependent and PKC-independent effects of phorbol esters have been reported (Doerner et al., 1990; Reeve et al., 1995). In the study by Reeve et al., (1995), phorbol esters were shown to enhance L-type $Ca^{2+}$ currents selectively, independently of PKC activation, in a manner that was at least superficially similar to the actions of Bay K 8644, the dihydropyridine L-type $Ca^{2+}$ channel agonist (i.e., caused a voltage-dependent enhancement and prolonged tail currents). However, in these same cells (SH-SY5Y), PKC-dependent enhancements of L- and N-type currents were also demonstrated, a finding that agreed with studies in frog sympathetic neurons (Yang and Tsien, 1993). Phorbol esters have clearly been extremely useful tools in probing the effects of PKC on $Ca^{2+}$ channel activity, but the fact that they can have other (possibly direct) effects indicates that they should be used in conjunction with other agents known to act selectively on PKC, one of the most selective of all being the PKC pseudosubstrate, PKC 19–36 (House and Kemp, 1987). With the continuing emergence of increasingly selective agents acting on PKC, many of the above-described studies are worthy of re-examination.

## Summary and Conclusions

This article has described the current understanding of the mechanisms by which PKC regulates secretion in neuroendocrine systems. The main effect is an enhancement of $Ca^{2+}$-dependent release rather than the ability to evoke release in the absence of additional secretagogs. This is particularly apparent for depolarization-evoked release, since PKC inhibitors do not affect this process. Although the enhancement of release by PKC is well documented, little clear understanding of mechanism appears. The major exception relates to secretion from LDCVs based on studies using secretory cell lines, such as chromaffin cells and SH-SY5Y cells. In this situation, a plausible

hypothesis relating to the movement of LDCVs to release sites following rearrangement of the F-actin cytoskeleton is emerging. It will be a future challenge to see if this mechanism, derived from cells in culture, also occurs for the release of catcholamines and neuropeptides from LDCVs in sympathetic nerves.

A further weakness in establishing the importance of PKC in the regulation of secretion is the extent to which the evidence for a role for PKC relies on studies with phorbol esters. A very important requirement is to establish that activation of PKC via receptors coupled to phospholipase C will also modify the secretion of neurotransmitters and hormones. The studies suggesting a role for PKC in overcoming the inhibition of glutamate release by adenosine $A_1$ receptor activation in synaptosomes and the work suggesting the bradykinin-enhanced release of NA from rat atria are particularly important in helping to establish a role for PKC in the regulation of secretion.

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