Identification and Localization of an Actin-binding Motif That Is Unique to the Epsilon Isoform of Protein Kinase C and Participates in the Regulation of Synaptic Function

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Abstract. Individual isoforms of the protein kinase C (PKC) family of kinases may have assumed distinct responsibilities for the control of complex and diverse cellular functions. In this study, we show that an isoform specific interaction between PKCe and filamentous actin may serve as a necessary prelude to the enhancement of glutamate exocytosis from nerve terminals. Using a combination of cosedimentation, overlay, and direct binding assays, we demonstrate that filamentous actin is a principal anchoring protein for PKCe within intact nerve endings. The unusual stability and direct nature of this physical interaction indicate that actin filaments represent a new class of PKC-binding protein. The binding of PKCe to actin required that the kinase be activated, presumably to expose a cryptic binding site that we have identified and shown to be located between the first and second cysteine-rich regions within the regulatory domain of only this individual isoform of PKC. Arachidonic acid (AA) synergistically interacted with diacylglycerol to stimulate actin binding to PKCe. Once established, this protein–protein interaction securely anchored PKCe to the cytoskeletal matrix while also serving as a chaperone that maintained the kinase in a catalytically active conformation. Thus, actin appears to be a bifunctional anchoring protein that is specific for the PKCe isoform. The assembly of this isoform-specific signaling complex appears to play a primary role in the PKC-dependent facilitation of glutamate exocytosis.

Evolution has conserved a fundamental mechanism that ensures the sorting and fusion of secretory vesicles upon their delivery to appropriate destinations along the secretory pathway. In many eukaryotes, however, alternative pathways have emerged in which vesicle delivery and fusion have become rigorously regulated events that are capable of manifesting use-dependent variations in the probability of their occurrence. Glutamate exocytosis is a particularly striking example of a secretory event that, in presynaptic nerve terminals, has acquired a highly evolved mechanism for regulating the efficiency of excitatory synaptic transmission. Moreover, it has been established that use-dependent changes in the reliability (Stevens and Wang, 1994) and extent (Schulz et al., 1994) of glutamate exocytosis directly participate in certain forms of synaptic plasticity.

Studies from many laboratories suggest that a conditional relationship may exist between the efficiency of glutamate release and the activity of presynaptic protein kinase C (PKC). These studies have shown that the Ca2+-dependent release of glutamate is significantly enhanced in the presence of phorbol esters, which activate most isoforms of PKC, and that this effect can be blocked by inhibitors of serine/threonine kinase activity (Yamamoto et al., 1987; Madison et al., 1991; Huang et al., 1992). Confirmation of these results in preparations of isolated nerve endings (synaptosomes) established a presynaptic locus for the PKC-dependent enhancement of sustained glutamate release and provided a simple system in which to identify the molecular components of the PKC signaling pathway that are capable of facilitating synaptic vesicle recruitment during bouts of intense synaptic activity (Terrian et al., 1993). The PKC family includes at least 11 different isoforms that possess different requirements for activation and are widely believed to have assumed distinctive cellular responsibilities (for reviews see Nishizuka, 1992; Hug

1. Abbreviations used in this paper: AA, arachidonic acid; GAP-43, growth-associated protein; GS1-406, truncated recombinant gelsolin; OAG, oleyl-acetyl-glycerol; P3, hippocampal mossy fiber-enriched synaptosomal fraction; PDBu, phorbol 12,13-dibutyrate; PS, phosphatidylserine; PKC, protein kinase C; RACK, receptors for activated C kinase; TX100, Triton X-100.
The coexistence of various PKC isoforms within an assort-
gene family is divided into three subfamilies; the
PKC-binding protein known to us has been shown to be
preferentially interact with certain isoforms of PKC
PKC-binding proteins have now been cloned and shown to
anchor proteins that are expressed by the cell. These intracellular
peptides (Kiley et al., 1992; Disatnik et at., 1994; Staudinger
et al., 1995). Cell specific patterns of redistribution may
also be observed for an individual PKC isoform, depend-
ing on the complement of isoform specific PKC-binding
Proteins that are expressed by the cell. These intracellular
PKC-binding proteins, sometimes referred to as "recep-
tors for activated C kinase" (RACKs; Mochly-Rosen et al., 1991) or as proteins that interact with C-kinase (PKCs;
Staudinger et al., 1995), are believed to determine the to-
pogenic fate of the PKC isoforms by differentially anchor-
ing the PKCs to specific cellular compartments. Several
PKC-binding proteins have now been cloned and shown to
preferentially interact with certain isoforms of PKC
(Chapline et al., 1993; Ron et al., 1994). However, no
PKC-binding protein known to us has been shown to be
specifically paired with an individual isoform of PKC.
Such absolute targeting specificity would require that the
anchoring protein contain a binding site that is uniquely
matched to an isoform-specific localization signal.

In the present study, we have examined the potential in-
volvement of individual PKC isoforms in the enhancement of sustained glutamate exocytosis, the pattern of their sub-
cellular redistribution, the determinants of isoform spe-
cific targeting within presynaptic terminals, and the condi-
tions required for their constitutive activation. The results
revealed that at least one requirement for the enhance-
ment of sustained glutamate exocytosis may be the posi-
tioning of activated PKC within the cytoskeletal matrix of
presynaptic terminals. Four independent lines of evidence indicated that the PKCe isoform was selectively anchored to
this complex aggregate of insoluble proteins through an
unusually secure and direct protein–protein interaction
with filamentous actin. Using purified actin and recombi-
nant PKCe, it was confirmed that complementary domains
within these proteins physically interacted with one an-
other through the formation of a salt-bridge that was sta-
ble at physiological ionic strength. In addition, an actin-

binding motif was identified in the regulatory domain of
PKCe that appeared to be conformationally hidden when the kinase was inactive and that is not conserved in any
other known members of the PKC family. These observa-
tions support the notion that PKCe contains an actin-bind-
ing sequence that assigns a distinct responsibility to this
individual kinase in mediating the PKC-dependent replen-
ishment of synaptic vesicles during repetitive bouts of pre-
synaptic depolarization.

Materials and Methods

In Vivo Destruction of Dentate Granule Cells
Male Sprague-Dawley rats were anesthetized and positioned in a Kopf
stereotaxic apparatus. Two unilateral intradentate injections of colchicine
(3.5 μg) were delivered, using the coordinates described previously (Con-
ner-Kerr et al., 1993). Contralateral sham injections were also delivered to
each animal. After a postinjection period of 10 d, animals were sacrificed,
hippocampi were removed, and synaptosomes were prepared (see below)
using either the sham- or colchicine-injected hippocampi as starting tissue.
Colchicine selectively destroyed the rat, but not guinea pig, dentate gran-
ule cell-mossy fiber pathway under these conditions (Conner-Kerr et al.,
1993).

Synaptosomal Preparation
Male Hartley guinea pigs were used in all experiments that did not require
the use of colchicine-induced lesions. Animals were decapitated, the hip-
ncampi removed and manually disrupted in a 0.3 M sucrose medium con-
taining 1 mM MgSO 4 and 15 mM sodium N-tris(hydroxymethyl) methyl-2-aminoethane-sulfonic acid (pH 7.4) at 0-5°C, using custom-made
Dounce type homogenizers (Terrian et al., 1988). The differential centrif-
geration procedures used to prepare hippocampal mossy fiber–enriched
fractions (P3) have been described in detail (Terrian et al., 1988). An iden-
tical protocol was used to isolate P3 fractions from sham- or colchicine-
injected rat hippocampi. Synaptosomal pellets were resuspended in an oxy-
genated, Ca2+-free, Krebs-bicarbonate medium (Terrian, 1995).

Phorbol Ester Preincubation Conditions
Synaptosomes were incubated for 5 min at 30°C before introducing CaCl2
(1.4 mM) to the medium and incubating for an additional 15 min. Aliquots
of the synaptosomal suspension were then transferred to separate tubes
and incubated at 30°C for 10 min in the presence of the PKC activator, 4β-
phorbol 12,13-dibutyrate (4β-PDBu; 1 μM), or an equimolar concentra-
tion of the inactive phorbol ester, 4α-PDBu. Where specified, intact syn-
aptosomes were exposed to 1 μM cytochalasin D, 1 μM staurosporine, or
100 μM sphingosine 20 min before the introduction of phorbol esters.

Superfusion Procedures
Aliquots of the synaptosomal suspension were layered onto parallel su-
perfusion columns (Terrian, 1995) and superfused (0.5 ml/min) at room
temperature with an oxygenated Krebs-bicarbonate medium containing
1.4 mM CaCl2. The relatively hydrophilic phorbol ester, PDBu, is com-
pletely washed out of intact synaptosomes within a 28-min exposure to
these superfusion conditions (Terrian et al., 1993). Beginning at 27 min,
four sequential 5-min superfusate fractions were collected and prepared for
fluorometric assay of glutamate by measuring the production of
NADH by glutamic acid dehydrogenase (Terrian et al., 1988). Glutamate
release was evoked by the introduction of 35 mM KCl to the superfusion
medium, in equimolar substitution for NaCl (Terrian and Ways, 1995).

Cellular Fractionation
Synaptosomes were disrupted by sonication in 20 mM Tris-HCl (pH 7.4)
containing 10 mM EDTA, 2 mM EGTA, 100 mM β-glycerophosphate,
0.05 mg/ml phenylmethylsulfonyl fluoride, 0.1 mg/ml aproatin, 1 mg/ml
leupeptin, 2 μM peptatin, and 0.1 mg/ml ovomucin. Cytosolic fractions
were obtained by centrifugation (78,000 g for 20 min at 4°C). Using a re-
combiant PKCe standard, it was determined that the resulting superna-
tant contained a weight ratio of 7 mg PKCe/1 μg cytosolic protein. The


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particulate fractions (0.5 mg protein) were extracted with 250 µl of a 1% (vol/vol) Triton X-100 (TX100) solution at 4°C for 20 min, unless specified otherwise. The TX100-soluble and -insoluble fractions were then separated by sedimentation (20 min at 78,000 g). In some experiments, the TX100-insoluble material was reextracted using a variety of additional solubilization protocols for 30 min at 4°C (see Table I). All TX100-insoluble fractions were finally resuspended in a cytoskeletal buffer that consisted of 125 mM Tris-HCl (pH 6.8), 1.25% SDS, 3 mM EDTA, 0.25 M sucrose, 10% glycerol, 1% 2-mercaptoethanol, and protease inhibitors (see above).

**Cosedimentation Assays**

The Ca²⁺-independent, in vitro, binding of native PKCs to the 1% TX100-insoluble fraction was analyzed using a modification of the cosedimentation assay described by Mochly-Rosen et al. (1991). In brief, 100 µg of 1% TX100-insoluble synaptosomal protein was resuspended in 50 µl of 20 mM Tris-Cl (pH 7.4) containing 10 mM EDTA, 2 mM EGTA, 100 mM β-glycerophosphate, and 0.05 mg/ml PMSF, and preincubated for 30 min at 30°C. Where indicated, the 1% TX100-insoluble fraction was pre-treated with trypsin (Mochly-Rosen et al., 1991) or 25 µg of recombinant gelsolin GS-406 (Sun et al., 1994). Sonicated phosphatidylserine (PS) liposomes (20 µg/ml) and CaCl₂ (1 mM) were also added to the reaction medium, with specified. Native synaptosomal cytosol (150 µg protein) was then incubated in the presence of the indicated amount of either 4α-PDBu or 4β-PDBu, to a final volume of 120 µl incubation medium. TX100-insoluble aggregates were sedimented and subjected to immunoblot analysis as previously described (Terrian et al., 1991), using antipeptide antisera specific for the α, β₁, δ, and ε isoforms of PKC. These antisera recognize proteins of the predicted molecular mass for their respective isoforms. The specificity of the detected proteins was confirmed by the ability of the immunizing peptide to abolish detection of these bands (Ways et al., 1991; Terrian et al., 1993). Antibody-reactive bands were visualized with either peroxidase-labeled secondary antibodies and enhanced chemiluminescence or 125I-protein A. Densitometric analysis of the autoradiograms was performed to quantify changes in the relative content of PKC.

**Actin-binding Assays**

Varying amounts of purified rabbit skeletal muscle actin were preincubated for 30 min at room temperature, in 150 µl of 4 mM Tris-HCl (pH 7.5) containing 10 µM CaCl₂, 30 mM KCl, 120 mM potassium phosphate, 1 mM MgCl₂, 150 µM ATP, and 100 mM ZnCl₂, before the addition of 50 µg synaptosomal cytosol (350 ng PKC). The volume of the incubation mixture was adjusted to 220 µl and samples were incubated for 30 min at 30°C, in the presence of either 1 µM 4α-PDBu or 4β-PDBu. Where indicated, varying amounts of synthetic peptides (LKKQET, PKCα19-22, or annexin I139-151) were added to the incubation medium at the same time as PDBu. Mixtures of purified actin and native cytosol were also incubated in the presence of 1 µM oleyl-acyetyl-glycerol (OAG), with or without varying concentrations of arachidonic acid (AA). Filamentous actin was sedimented (78,000 g for 5 min) and resuspended in cytoskeletal buffer for immunoblot analysis. The final actin concentration was determined by SDS-PAGE and transferred to nitrocellulose membranes that were incubated for 3 h at 4°C with a Tris-buffered saline (TBS) that contained 5% milk powder. The TBS consisted of 10 mM Tris-Cl (pH 7.4) and 150 mM NaCl. Nitrocellulose filter-bound proteins were then washed twice with TBS and incubated for 1 h at room temperature in TBS containing 10 µg/ml cytosolic protein, 10 mg/ml bovine serum albumin, 1 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotonin, and 1 µM 4β-PDBu. After two more washes with phosphate-buffered saline, the membranes were blotted using either anti-PKCα, ε, ζ, or anti-β-actin antisera.

**In Vitro Protein Phosphorylation**

Synaptosomal 1% TX100-insoluble protein (150 µg) was resuspended in 200 µl of Tris-HCl (pH 7.4) that contained native cytosolic protein (80 µg), 0.1 mM EGTA, and 1 µM 4α-PDBu or 4β-PDBu. After a 5-min incubation at room temperature, insoluble aggregates were sedimented (15,500 g for 5 min). Where specified, these pellets were washed three times with 200 µl of 20 mM Tris-HCl (pH 7.4). The final pellets were all resuspended in 185 µl of 20 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA. The assay of in vitro phosphotransferase activity was initiated by adding 15 µl of 20 mM Tris-HCl containing 180 mM MgCl₂, 12 mM ATP, and [γ-³²P]ATP (0.26 mCi/ml). Samples were incubated for 5 min at 30°C and sedimented as before. Pellets were washed twice with 200 µl of 20 mM Tris-HCl (pH 7.4) and resuspended in cytoskeletal buffer before SDS-PAGE using 5–15% linear gradient gels and autoradiographic analysis.

**Miscellaneous**

Treatment effects were evaluated by determining the significance of the difference between means using a paired Student’s t test. SDS-PAGE using 5–15% linear gradient gels and immunoblot analysis of the autoradiograms was performed to quantify changes in the relative content of PKC.

**Overlay Assays**

Denatured, 1% TX100-insoluble, synaptosomal proteins that are capable of binding to native PKC under in vitro conditions were analyzed using a modification of the overlay assay described by Jaken and colleagues (Liao et al., 1994). TX100-insoluble proteins (150 µg) were denatured and separated by SDS-PAGE and transferred to nitrocellulose membranes that were incubated for 3 h at 4°C with a Tris-buffered saline (TBS) that contained 5% milk powder. The TBS consisted of 10 mM Tris-Cl (pH 7.4) and 150 mM NaCl. Nitrocellulose filter-bound proteins were then washed twice with TBS and incubated for 1 h at room temperature in TBS containing 10 µg/ml cytosolic protein, 10 mg/ml bovine serum albumin, 1 mM EGTA, 10 µg/ml leupeptin, 10 µg/ml aprotonin, and 1 µM 4β-PDBu. After two more washes with phosphate-buffered saline, the membranes were blotted using either anti-PKCα, ε, ζ, or anti-β-actin antisera.

**Results**

Presynaptic Localization of PKCα, PKCβ, and PKCζ

We have taken advantage of the fact that colchicine selectively destroys the rat dentate granule cell - mossy fiber pathway in vivo to identify proteins that are endogenous to hippocampal mossy fiber nerve endings. Comparisons of the P3 fractions that were prepared from ipsilateral (lesioned) and contralateral (sham-injected) hippocampi indicated that destruction of the mossy fibers reduced the content of synaptophysin and GAP-43; proteins that are localized to the presynaptic terminals of all hippocampal neurons, by 93% and 63%, respectively (Fig. 1 A). In contrast, the synaptosomal content of PKCβII and γ was unaltered by colchicine (Fig. 1 B), indicating that mossy fiber nerve endings are unlikely to be the source of these PKC isoforms. The antipeptide antisera against PKCβII did not detect any proteins of the predicted molecular mass in the P3 fraction. Although a ~116-kD immunoreactive protein that may represent a high molecular size isoform of PKCβII (Sublette et al., 1993) was observed, colchicine appeared to increase rather than reduce the content of this unidentified protein (Fig. 1 B). Colchicine-induced lesions also increased the content of PKCβ in the P3 fraction. An antipeptide antisera against PKCα detected no proteins in
Figure 1. Presynaptic localization of PKC isoforms by deletion analysis. (A) Effects of selectively removing mossy fiber nerve endings from hippocampal P3 subcellular fractions, using in vivo intradentate injections of colchicine, on the relative content of two presynaptic marker proteins, synaptophysin, and GAP-43. Hippocampal homogenates (H) are shown for reference. (B) Effects of colchicine-induced lesions on the recovery of various PKC isoforms (PKCa, PKCβI, PKCβII, PKCγ, PKCe, PKCζ, and PKCη) in the rat hippocampal P3 subcellular fraction. “ND” means not detected. Equal amounts of protein were applied to the adjacent lanes of each gel shown in A and B, above. Representative blots are shown from two (PKCe) or three independent experiments and data are reported as the means ±SEM.

either the P3 fraction or whole rat brain standard (data not shown). Only PKCe, PKCζ, and PKCη were significantly decreased as a result of this lesion (Fig. 1B). These observations indicate that hippocampal mossy fiber nerve endings contain at least one representative from each of the three subfamilies of PKC.

4β-PDBu Selectively Promotes the Chelator-Stable Association of PKCe with a TX100-resistant Fraction That Is Maintained, In Situ, in the Absence of 4β-PDBu

Once established, the 4β-PDBu–induced enhancement of glutamate exocytosis has been shown to become stabilized in intact synaptosomes and independent of phorbol esters, while remaining sensitive to PKC inhibitors (Terrian et al., 1993; Terrian and Ways, 1995). In an attempt to determine whether this persistent facilitation of release was mediated by either reversible or irreversible hydrophobic interactions of PKC with membrane lipids (Bazzi and Nelsestuen, 1988; Kazanietz et al., 1992), translocation assays were performed using intact synaptosomes. Both mechanisms are thought to contribute to the constitutive activation of PKC. The principal criteria used to distinguish between the reversibly bound and “membrane-inserted” forms of PKC are that, in the later case, the solubility of the enzyme is reduced when intact cells are treated with phorbol esters and that the association of PKC with the particulate fraction becomes independent of phorbol esters and resists extraction with EGTA, but not the detergent TX100 (Bazzi and Nelsestuen, 1988; Kazanietz et al., 1992). In addition, the enhancement of glutamate release does not stabilize during an acute (1 min) exposure to 4β-PDBu, but persists when synaptosomes are pretreated for at least 10 min with 4β-PDBu, and then washed to remove this phorbol ester (Terrian et al., 1993). Using these criteria, synaptosomes were incubated for 45 min in the presence of either the in-
active phorbol ester, 4α-PDBu, or 4β-PDBu for 1, 10, or 45 min and sonicated in the presence of a buffer that contained 2.4 mM EGTA. The solubility of PKC isoforms, following their 4β-PDBu-dependent redistribution, was initially analyzed by extracting the particulate protein with 0.1% TX100, at a detergent to protein ratio of 5:1. Cytosolic, 0.1% TX100-soluble, and 0.1% TX100-resistant fractions were prepared from whole synaptosomal lysates. Where specified, PDBu was washed out of the intact synaptosomes before cellular fractionation. No effect of 4β-PDBu on the distribution of PKCζ was detected under these conditions (Fig. 2 A), indicating that the disruption of intact synaptosomes itself was not responsible for an artifactual redistribution of the other, phorbol ester-responsive, isoforms of PKC. Like PKCζ, PKCe also did not respond to 4β-PDBu treatment (data not shown). The content of cytosolic PKCa, βII, γ, and ε decreased within 1 min of treatment with 4β-PDBu. However, this decrease in PKCa, βII, and γ solubility was completely reversed by removal of 4β-PDBu (Fig. 2 and data not shown). PKCe was the sole member of the PKC family that could be localized to mossy fiber terminals (Fig. 1), maintained a persistent association with a particulate fraction in the absence of 4β-PDBu in situ, and resisted extraction with 2.4 mM EGTA in vitro (Fig. 2C). In contrast to the membrane-inserted form of PKC, however, PKCe could not be extracted from the particulate fraction using 0.1% TX100 and 45 min (condition 10W). Amounts of protein loaded into adjacent lanes of a given immunoblot were equivalent, but varied between fractions and isoforms. These immunoblots are representative of the results obtained in three independent experiments using 0.1% TX100. At a final concentration of 1.0% TX100, the same results were obtained in two additional experiments.

Figure 2. In situ translocation of endogenous PKCa, ε, and ζ. Effects of 4β-PDBu on the subcellular distribution of PKCζ (A), PKCa (B), and PKCe (C). Intact synaptosomes were incubated for 45 min and exposed to 1 μM 4β-PDBu during the final period of incubation, for the time indicated. Fractions obtained from synaptosomes that were incubated for 45 min with the inactive phorbol ester, 4α-PDBu, are indicated as a 0-min exposure. Synaptosomes were then disrupted and separated into cytosolic and particulate fractions. Particulate fractions were extracted using 0.1% TX100 and further separated into TX100-soluble and TX100-resistant fractions. Immunoblot analysis was used to determine the effect of 4β-PDBu on the solubility of PKCa, ε, and ζ. The stability of in situ PKC interactions were examined by removing 4β-PDBu from intact synaptosomes after a 10-min exposure (condition 10W). Amounts of protein loaded into adjacent lanes of a given immunoblot were equivalent, but varied between fractions and isoforms. These immunoblots are representative of the results obtained in three independent experiments using 0.1% TX100. At a final concentration of 1.0% TX100, the same results were obtained in two additional experiments.

Activated PKCe Becomes Insoluble Due to Saturable, Ionic, Protein–Protein Interactions That Are Independent of Calcium or Phosphatidylserine

To determine the nature of PKCe binding that is established under in situ conditions, intact synaptosomes were
The solubilization of PKCe after in situ targeting to the synaptic 1% TX100-insoluble matrix by 4β-PDBu

| Reextraction         | Insoluble protein* (Total) | Insoluble PKCe1 (4α-PDBu) |
|----------------------|---------------------------|----------------------------|
|                      | %                         | %                          |
| None                 | 44                        | 174                        |
| Octyl Glucoside      | 7                         | 179                        |
| (NH4)2SO4            | 14                        | 108                        |
| NaCl                 | 13                        | 86                         |
| pH 12                | 6                         | 109                        |

Table I. Solubilization of PKCe after In Situ Targeting to the Synaptosomal 1% TX100-Insoluble Matrix by 4β-PDBu

Intact synaptosomes were treated with either 1 μM 4α-PDBu or 4β-PDBu. Particulate fractions were obtained by centrifugation of synaptosomal lysates and initially extracted with 1% TX100. All subsequent extractions from this 1% TX100-insoluble aggregate were performed at 4°C for 30 min. Octyl glucoside was used at a final concentration of 100 mM, (NH4)2SO4 at 250 mM, and NaCl at 3 M concentrations. Alkaline conditions were established using a 0.1 M Na2CO3 buffer. 4α-PDBu-treated synaptosomes were used as a paired control.

*Insoluble protein is expressed as the percent of total synaptosomal protein that resisted extraction using 1% TX100 alone (None) or with an additional reextraction by the methods specified.

1Insoluble PKCe represents the relative amount of PKCe that resisted extraction, using the method specified. Aliquots from the same synaptosomal suspension were exposed to either 4α- or 4β-PDBu, extracted with 1% TX100, and reextracted in an identical manner. Values are expressed as the percent of insoluble PKCe recovered in the paired 4α-PDBu control for each treatment condition.

The ability of PKCe to resist solubilization by non-ionic detergents, samples of the TX100-insoluble material were reextracted using octyl glucoside. Octyl glucoside is a considerably more stringent detergent than TX100 and displaces even detergent-resistant glycosylphosphatidylinositol anchored proteins from cell membranes (Brown and Rose, 1992). After reextracting the 1% TX100-insoluble material with 100 mM octyl glucoside, at a detergent to protein ratio of ~6:1, only 7% of the total synaptosomal protein remained insoluble (Table I). Western blots confirmed that the presynaptic integral membrane protein, synaptophysin, was completely solubilized by this combination of detergent extractions (data not shown). However, no PKCe was recovered in the additional protein that had been solubilized by octyl glucoside (Table I). Thus, the 4β-PDBu-stabilized binding of PKCe was clearly not dependent on the direct interaction of this kinase with membrane lipids. In contrast, PKCe was completely displaced from the 1% TX100-insoluble matrix when the ionic strength (3 M NaCl) or alkalinity (pH 12) of the extraction buffer was increased (Table I), indicating that the in situ binding of PKCe may be mediated by electrostatic interactions between two or more proteins. The fact that Na2CO3 buffers titrated to a pH of 11 failed to interfere with the binding of PKCe suggested that these interactions were unusually stable (data not shown).

Specific protein interactions are both saturable and concentration dependent. After the coincubation of native cytosolic and 1% TX100-insoluble fractions in the presence of 4β-PDBu, neither PKCe nor PKCa cosedimented with the insoluble fraction (data not shown). As in the previous experiment, however, 4β-PDBu significantly increased the association of PKCe with the 1% TX100-insoluble fraction (Fig. 3 A). PKCe binding was saturable and strictly dependent on the concentration of soluble PKCe that was included in the incubation mixture (Fig. 3 B). To evaluate whether PS and Ca2+ mediate the interaction of PKCe with an unidentified PS-binding protein (Wolf and Sahyoun, 1986; Liao et al., 1994), sonicated PS liposomes and

A)

Figure 3. In vitro binding of native, cytosolic, PKCs to a 1% TX100-insoluble aggregate of synaptosomal proteins. (A) Conditions required for in vitro binding of PKCe. Cytosol (150 μg) and 1% TX100-insoluble fractions (100 μg) were coincubated in the absence (lanes 1 and 2) or presence of the PKC activators, 1.4 mM CaCl2 (lanes 4, 6, and 7), 20 mg/ml PS (lanes 3, 4, 6, and 7) and 1 μM 4β-PDBu (lanes 5, 6, and 7). In lane 7, the 1% TX100-insoluble fraction was exposed to mild trypsinization before the addition of a trypsin inhibitor and cytosol, according to the method of Mochly-Rosen et al. (1991). The same results were obtained in three independent experiments. (B) Cytosolic PKCe binding to the 1% TX100-insoluble fraction (50 μg protein) at different concentrations of cytosol. CaCl2, PS, and 4β-PDBu were present at all points, in the concentrations specified above. Data are the means ± SEM of the values obtained in three independent experiments.
Ca\(^{2+}\) were added to the in vitro cosedimentation assay mixture. As shown in Fig. 3 A, PKCe binding was increased by 4β-PDBu (lane 5) and abolished by pretreating the 1% TX100-insoluble fraction with trypsin (lane 7). However, PS had no effect on the solubility of PKCe, in either the absence or presence of Ca\(^{2+}\) (Fig. 3 A, lanes 3 and 4), and the combination of PS and Ca\(^{2+}\) did not augment the 4β-PDBu-induced binding of PKCe (Fig. 3 A, lane 6). These data demonstrate that PKCe binds to the detergent-insoluble fraction via direct protein–protein interactions that are not dependent on the formation of a "PS-Ca\(^{2+}\) bridge" (Wolf and Sahyoun, 1986; Liao et al., 1994).

It has previously been demonstrated that the 4β-PDBu-dependent enhancement of glutamate exocytosis is dependent on the presence of an intact actin-based cytoskeleton (Terrian and Ways, 1995). Therefore, we tested the ability of 250 mM \((\text{NH}_4)_2\text{SO}_4\) to solubilize PKCe because of its reported efficiency in extracting the filamentous, actin-based, cytoskeleton from cellular lysates (Fey et al., 1984). \((\text{NH}_4)_2\text{SO}_4\) almost completely solubilized the PKCe (Table I).

**PKCe Remains Insoluble in the Absence of 4β-PDBu and Native Cytosol**

To determine how tightly PKCe associates with the detergent-insoluble components of nerve endings, we examined the kinetics for the dissociation of this complex. Synaptosomal cytosolic and 1% TX100-insoluble fractions were isolated and then reintroduced to one another, under in vitro conditions, in the presence of either 1 μM 4α-PDBu or 4β-PDBu, 10 mM EDTA, and 2 mM EGTA. Essentially all of the soluble PDBu, and >90% of the bound PDBu, was then removed by washing the TX100-insoluble aggregate three times (Fig. 4, inset). The washed pellets were next resuspended in a Tris buffer and aliquots of this suspension were removed at specified times to determine the amount of PKCe that remained bound to the TX100-insoluble fraction. Data were analyzed using the following equation: B(t) = B0 × e\(^{(−kt)}\); where B(t) was the amount of PKCe bound at any given time point, B0 was the maximal amount of bound PKCe, k was the turnover constant (1/τ), and τ was time. It was observed that, in the absence of 4β-PDBu and in the presence of chelators, PKCe and the TX100-insoluble fraction remained tightly associated with one another and exhibited a turnover constant of 0.0134 (τ = 75 min; Fig. 4).

**PKCe Remains Catalytically Active within the Presynaptic Cytoskeletal Matrix after the Removal of Phorbol Ester**

Phosphotransferase activity is not required for PKCe to bind the detergent-insoluble proteins. Indeed, all in vitro cosedimentation assays were performed in the absence of ATP and under conditions which do not support this activity. Thus, an alternative mechanism must account for the ability of 4β-PDBu to stabilize the repositioning of PKCe. It is well established that when phorbol esters bind to the cytosine-rich regions of PKCs the cryptic pseudosubstrate and substrate-binding domains of these enzymes dissociate and become exposed (for review see Hug and Sarre, 1993). This observation raised the possibility that PKCe could only interact with anchoring proteins when the enzyme assumed an active conformation. An important functional consequence of such a requirement would be that PKCe remained constitutively active while it was anchored to native PKCe-binding proteins. The fact that PKCe was the only endogenous isoform that bound to the detergent-

**Figure 4.** Rate of dissociation for PKCe binding in the absence of 4β-PDBu. PKCe binding to a 1% TX100 insoluble aggregate of synaptosomal proteins was established under in vitro conditions by coincubation with the native cytosol in the presence of 1 μM 4β-PDBu. Three washes were performed to remove >90% of the bound 4β-PDBu, this was confirmed using \[^{3}P\]4β-PDBu (inset). After incubation in the absence of phorbol ester for the time specified, soluble and insoluble forms of PKCe were separated and immunoblot analyses were performed to determine the relative amount of PKCe that remained insoluble. Data are the means ± SEM of the values from three independent experiments. The turnover constant for bound PKCe was estimated by performing a curve fit on the mean values using Sigma Plot Scientific Graphing Software 2.0 (Jandel Scientific, Inc.).

**Figure 5.** In vitro phosphorylation of native TX100-insoluble proteins by bound PKCe. Soluble PKCe was co-incubated with 1% TX100-insoluble protein in the presence of either the inactive phorbol ester, 1 μM 4α-PDBu (lanes 1 and 3), or 1 μM 4β-PDBu (lanes 2 and 4). Insoluble proteins were then sedimented and the absence of bound PKCα and ζ was confirmed by immunoblot analysis of the unwashed pellets (data not shown). The phosphotransferase reaction was initiated by adding \[^{32}P\]ATP in the absence (lanes 3 and 4) or presence (lanes 1 and 2) of 1 μM PDBu. Insoluble pellets were washed three times to remove PDBu (lanes 3 and 4). PKCe-dependent phosphotransferase activity was estimated by measuring changes in the phosphorylation of an unidentified PKC substrate, having a molecular size of 80 kD.
insoluble fraction of P3 synaptosomes (see above) provided the opportunity to directly test this hypothesis. Synaptosomal cytosolic and 1% TX100-insoluble fractions were co-incubated and the native PKCe was induced to translocate to the insoluble fraction by adding 1 μM 4β-PDBu, in the absence of ATP, to the incubation medium. Insoluble proteins were then sedimented and either assayed directly or first washed three times to remove PDBu (Fig. 4, inset). Thus, all subsequent 4β-PDBu-induced phosphotransferase activity in the washed TX100-insoluble fraction could be attributed to the insoluble form of PKCe. PKCe-dependent phosphotransferase activity was examined, in the presence of 0.1 mM EGTA, by measuring the autoradiographic densitometric intensity of an endogenous 80-kD phosphoprotein (p80; Fig. 5). Consistent with our working hypothesis, it was observed that the selective targeting of PKCe to the TX100-insoluble fraction was associated with increased catalytic activity (Fig. 5, lanes 1 and 2). Moreover, this increase in PKCe phosphotransferase activity was maintained (Fig. 5, lanes 3 and 4) after removing >90% of the bound [3H]4β-PDBu (Fig. 4, inset).

4β-PDBu Stimulates PKCe Translocation, PKCe-dependent Phosphorylation, and Glutamate Exocytosis with Equivalent Potency

Unless the 4β-PDBu-induced translocation and activation of PKCe are independent events, 4β-PDBu should stimulate both responses with equal potency. In vitro cosedimentation and phosphorylation assays were performed in the presence of increasing concentrations of 4β-PDBu to compare the EC50 values for these potentially interdependent responses. The data were analyzed using the equation: V = (Vmax × [S])/((EC50 + [S]); where V was PKCe binding, phosphorylation or glutamate exocytosis, Vmax was the maximal V, n was the Hill coefficient, [S] was the 4β-PDBu concentration employed, and EC50 was the 4β-PDBu concentration required to achieve Vmax/2. Estimates of the EC50 values for the 4β-PDBu-dependent translocation of PKCe in situ (EC50 = 193 nM) and the in vitro PKCe-dependent phosphorylation of p80 (EC50 = 402 nM) did not markedly differ. Moreover, the EC50 values for these responses were found to be nearly equivalent to that which was calculated from the results of our earlier release experiments (EC50 = 159 nM; Terrian, 1995). Regression analysis revealed a high degree of correlation between the amount of PKCe bound and both the PKCe-dependent phosphorylation of p80 (r² = 0.737) and Ca²⁺-dependent release of glutamate (r² = 0.988). These data provide circumstantial evidence that the 4β-PDBu-induced translocation and activation of PKCe are interdependent events that may represent a necessary prelude to the enhancement of glutamate exocytosis.

Conditions which Prevent the Interaction of PKCe with the Presynaptic Cytoskeletal Matrix Prevent the 4β-PDBu-dependent Enhancement of Glutamate Exocytosis

Two independent methods for preventing the 4β-PDBu-dependent enhancement of glutamate exocytosis have been established (Terrian and Ways, 1995). These treatments were used to perform a preliminary analysis of whether the cytoskeletal localization of PKCe was essential for the 4β-PDBu-dependent enhancement of glutamate exocytosis. First, intact synaptosomes were pretreated with the PKC inhibitors, sphingosine and staurosporine, before the introduction of either 4α-PDBu or 4β-PDBu. Synaptosomal suspensions were then used to conduct release and translocation assays in parallel. The presence of inhibitors was maintained throughout all phases of the release assay, since it has been shown previously that continuous inhibition was required to fully prevent the enhancement of glutamate release (Terrian and Ways, 1995). The ability of this treatment to completely abolish the 4β-PDBu-dependent facilitation of glutamate release was confirmed in this experiment (Fig. 6 A). In agreement with predictions that the recruitment of PKCe to the cytoskeletal matrix was required for the enhancement of glutamate release, sphingosine and staurosporine also blocked the in situ translocation of PKCe (Fig. 6 A). In a second series of experiments, intact synaptosomes were incubated with cytochalasin D before the addition of PDBu. This treatment has been shown to completely abolish the 4β-PDBu-dependent enhancement of glutamate exocytosis (Terrian and Ways, 1995). Here, it is reported that cytochalasin D also prevented the in situ accumulation of insoluble PKCe (Fig. 6 B).

4β-PDBu–dependent Interactions of PKCe with Actin

The above observations suggest that ionic protein–protein interactions mediate the binding of PKCe to either filamentous actin itself or to the actin-based skeletal framework of isolated nerve endings. To determine whether PKCe was anchored in the cytoskeletal matrix by either direct, or indirect, interactions with actin, we used recombinant gelsolin (GS1-406), a highly specific actin filament severing protein (Sun et al., 1994). Based on the known properties of GS1-406, it could be predicted that GS1-406 activity would substantially decrease the average size of actin filaments in synaptosomal TX100-insoluble fractions and, thereby, solubilize proteins that were anchored to this aggregate by the insoluble form of filamentous actin. A portion of the 1% TX100-insoluble fraction was pre-treated with GS1-406 and the in vitro binding of PKCe was stimulated by 4β-PDBu. GS1-406 decreased the amount of insoluble β-actin (data not shown) and PKCe (Fig. 7 A), providing independent confirmation of the results obtained using cytochalasin D in situ (Fig. 6 B). Overlay assays were next performed in our attempt to identify the PKCe-binding protein(s). These assays revealed that the synaptosomal 1% TX100-insoluble fraction contained a 43-kD protein that, after denaturation, was capable of binding the native PKCe in vitro. Interestingly, the mobility of this protein on SDS-PAGE gels precisely matched that of the endogenous β-actin that was present in the same TX100-insoluble fraction (Fig. 7 B). The simplest interpretation of this finding was that PKCe binds directly to actin. To test this hypothesis, in vitro cosedimentation assays were performed using synaptosomal cytosolic fractions and purified filamentous actin. The data reported that native PKCe was capable of binding to purified actin filaments, under physiological ionic strength conditions, and that this binding was both saturable and significantly
enhanced by the active isoform of PDBu (Fig. 7 C). Neither PKCα nor PKCζ were detected by immunoblot analysis of these same actin pellets (data not shown). Although 4β-PDBu did not alter the affinity of native PKCε for filamentous actin, the $V_{\text{max}}$ was increased almost fourfold (Fig. 7 C). ATP was required to obtain consistent, 4β-PDBu-dependent, binding of PKCε and Zn$^{2+}$ enhanced this effect (data not shown). At a fixed concentration of purified actin (1 μM) and cytosolic PKCε (41 nM), 4β-PDBu proved to be a potent stimulus for the in vitro association of these native-binding partners (Fig. 7 D; EC$_{50}$ = 212 nM).

**Identification of a Putative Actin-binding Motif in PKCε**

Sequence motifs that function as recognition signals for protein–protein interactions have previously been identified in competition experiments by coincubating the binding proteins with synthetic peptides that are homologous to the putative binding sites. A series of such experiments indicated that none of the known PKC-binding domains were directly involved in actin binding. Indeed, neither the pseudosubstrate peptide, PKCα$_{19-31}$ (10–55 μM) nor annexin I$_{338-345}$ (5–20 μM) had any effect on the in vitro binding of native PKCε to actin in the absence or presence of 1 μM 4β-PDBu (data not shown). However, analysis of the PKCε sequence revealed the presence of a motif that had previously been identified as a putative actin-binding motif in several other actin-binding proteins (Fig. 8 A; Vancompernolle et al., 1991). While this actin-binding motif was found to be conserved in the human, mouse, rat, and rabbit PKCε, it was not present in any other known isoform of PKC, suggesting a mechanism that could account for the selective redistribution of cellular PKCε. Therefore, we examined the ability of a synthetic hexapeptide that corresponded to the putative actin-binding domain of PKCε, PKCε$_{223-228}$ (Fig. 8 A), to compete with native PKCε for binding to purified actin and, in a separate experiment, 1% TX100-insoluble synaptosomal fractions. Consistent with the involvement of PKCε$_{223-228}$ in the recognition and binding of actin, this hexapeptide (LKKQET) competed, in a dose-dependent manner, with native PKCε for binding to either substrate (Fig. 8 B). Thus, PKCε contains a unique, actin-binding motif that appears to play an important part in the binding of this isoform to actin.

What are the structural determinants for actin binding to PKCε? The fact that PKCε-actin interactions were stimulated by 4β-PDBu indicated that the actin-binding domains in PKCε may normally be hidden when this protein is in an inactive conformation. These contact sites for actin could be integrated into the secondary structure of native PKCε or consist of linear sequences that are complementary to actin (Vandekerckhove and Vancompernolle, 1992). To better understand the requirements for the formation of this complex, equimolar concentrations of denatured, and presumably unfolded, recombinant PKCα, δ, and ε were individually tested for their ability to bind puri-
fied actin. Significant binding of purified actin to PKCe was observed at a physiological ionic strength and in the absence of PDBu (Fig. 8 C). Actin binding to PKCe or δ was not detected (data not shown), suggesting that PKCe selectively interacted with actin under these conditions. The ability of the synthetic peptide, LKKQET, to effectively compete with recombinant PKCe for actin binding (Fig. 8 C) provided independent support for our previous observations in which cytosol had been used as an alternative source of PKCe (Fig. 8 B). These findings indicated that actin was a PKCe binding protein and that small sequence motifs in the primary structure of PKCe could contain the information that was required for actin recognition and binding.

**AA and Diacylglycerol Synergistically Interact to Promote the Binding of PKCe to Actin Filaments**

While phorbol esters have greatly facilitated investigations into the presynaptic role of PKCe, physiological agonists will ultimately be required for evaluating the potential functional significance of our observations. The triggering events that expose the putative actin-binding domain in PKCe in vivo are not known. However, the diacylglycerol and AA that are liberated from membrane phospholipids are widely regarded as putative endogenous signals for PKC activation (Nishizuka, 1992). To determine whether these putative endogenous activators of PKC stimulate the binding of PKCe to purified actin, actin cosedimentation assays were performed using AA as the PKC activator, in the presence and absence of the diacylglycerol analog, OAG (1 μM). The data were analyzed using the equation:

\[
V = \frac{V_{\text{max}} \times [S]^n}{EC_{50} + [S]^n}
\]

where \(V\) was PKCe binding, \(V_{\text{max}}\) was the maximal \(V\), \(n\) was the Hill coefficient, \([S]\) was the AA concentration employed, and \(EC_{50}\) was the AA concentration required to achieve \(V_{\text{max}}/2\). Fig. 9 indicates that AA was a potent stimulus for the binding of PKCe to actin (\(EC_{50} = 10.9 \mu M\)). The potency of AA was substantially increased (\(EC_{50} = 0.6 \mu M\)) when OAG, was added to the incubation medium (Fig. 9).

**Discussion**

We have identified a binding site for filamentous actin on PKCe that is unique to this individual isoform of PKC. This actin-binding site was located between the first and second cysteine-rich regions (cys1 and cys2) of the regulatory C1 domain of PKCe, residues 223–228 (Fig. 8 A), in a variety of different species. Although it remains to be determined whether other PKC isoforms have also conserved such unique binding motifs, the existence of PKCe223-228 makes it clear that a mechanism has been established for the pairing of individual PKC isoforms with specific intracellular PKC-binding proteins. This complexity in the control of PKC targeting raises the question of what role these isoform-specific pathways may play in the regulation of cell function. In the case of PKCe, the remarkably stable interaction between PKCe223-228 and filamentous actin anchors this kinase to the cytoskeletal matrix of presynaptic terminals, where the holoenzyme remains constitutively active and appears to play a primary role in sustaining the...
Figure 9. Effect of AA and OAG on PKCe binding to purified actin. Actin-binding assays were performed as described in Materials and Methods by coincubating polymerized actin (100 μg) and native PKCe (50 μg cytosol) in the absence or presence of OAG (1 μM) and in the presence of increasing concentrations of AA. Curve fitting was performed on the mean of two (AA alone) or three (AA with OAG) independent determinations, using Sigma Plot Scientific Graphing Software 2.0 (Jandel Scientific, Inc.).

Figure 8. A synthetic peptide that is homologous to PKC\textsubscript{e223-228} competes with PKCe for actin binding. (A) Sequence and location of the putative actin-binding domain in PKCe and comparison with the homoogous motif in other known actin-binding proteins. (B) Effect of PKC\textsubscript{e223-228} (LKKQET) on the in vitro binding of native PKCe to purified actin and detergent-insoluble synaptosomal components. Polymerized actin (100 μg) or 1% TX100-insoluble fractions (100 μg) were preincubated with 50 μg of synaptosomal cytosol (∼350 ng of native PKCe), 1 μM 4β-PDBu, and the specified concentration of PKC\textsubscript{e223-228} for 30 min before cosedimenting filamentous actin and insoluble PKCe. The efficacy of 4β-PDBu was confirmed in parallel controls that were simultaneously incubated, without PKC\textsubscript{e223-228}, in the presence of 1 μM 4β-PDBu or 4β-PDBu (see inset). (C) Actin binding to recombinant, denatured, PKCs measured by cosedimentation. Human recombinant PKC\textsubscript{e} (100 ng) was preincubated with purified actin (5 μM) in the absence (lane 2) or presence (lane 3) of the synthetic hexapeptide, LKKQET (80 μM). Lane 1 was a control that contained only polymerized actin. Actin pellets were washed, fractionated by SDS-PAGE, and visualized by immunoblotting with anti-PKC\textsubscript{e}. The same results were obtained in two independent experiments.

slow phase of glutamate exocytosis. While a surplus of mature synaptic vesicles are typically docked and primed for fusion, the supply of these vesicles must be actively replenished during bouts of high frequency stimulation (Lim et al., 1990). Many details regarding the mechanism that controls membrane recycling and the recruitment of fresh synaptic vesicles remain obscure. A step towards understanding how this mechanism operates is the identification of PKCe as one of the signaling molecules in this complex pathway.

Do other members of the PKC family also participate in the recruitment of endogenous glutamate and long lasting facilitation of exocytosis? This possibility cannot be formally excluded without further investigation. However, the present study provides several observations of direct significance. Endogenous PKCe, βII, γ, δ, ε, and ζ were contained in the hippocampal P3 subcellular fraction. However, the results of our colchicine lesion experiments indicated that only PKCe, ε, and ζ were localized to the hippocampal mossy fiber nerve endings in this synaptosomal preparation. This finding was consistent with electron microscopic observations that PKCe immunoreactivity is located in the hippocampal mossy fiber terminals (Saito et al., 1993), while both PKC\textsubscript{βII} and γ appear to be excluded from hippocampal nerve endings (Kose et al., 1990). PKCa is principally associated with the Golgi complex of hippocampal neurons (Ito et al., 1990) and little PKCe immunoreactivity is evident in this brain region (Merckenthaler et al., 1993). Although PKC\textsubscript{e} appeared to be an abundant PKC isoform of mossy fiber origin, translocation assays confirmed that this enzyme does not respond to 4β-PDBu. 4β-PDBu stimulated the in situ translocation of PKC\textsubscript{a}, βII, and γ. However, the association of these PKC isoforms with the particulate fraction was reversed by ei-
of PKC~II and γ) or by removing 4β-PDBu (PKCe). PKCe was the only endogenous isoform of PKC to maintain a chelator-resistant interaction with an intracellular protein that could not be dissociated by removing 4β-PDBu. These properties distinguished PKCe binding from either the Ca²⁺-induced membrane binding, or membrane insertion, of PKC (Bazzi and Nelsestuen, 1988). Thus, multiple isoforms of PKC may contribute to the transient effects of 4β-PDBu on synaptic function, but the long lasting presynaptic effects of 4β-PDBu appeared to be mediated by an isoform-specific pathway. In this regard, it is of interest that the transfection-induced overexpression of PKCe, but not other PKC isoforms, dramatically increases the secretory activity of pituitary GH₂C₁ cells (Akita et al., 1994).

Actin filaments are a principal target for activated PKCe in presynaptic terminals. This conclusion is supported by four independent lines of evidence. First, activated PKCe remained soluble when intact synaptosomes were pretreated with cytochalasin D and when an aggregate of native, detergent-insoluble, synaptosomal proteins were pretreated with recombinant gelsolin. Second, overlay assays demonstrated that activated PKCe, but not other isoforms, bound to a detergent-insoluble protein that comigrated with β-actin on SDS-PAGE gels. Third, both native and purified PKCe bound directly to purified actin. Using cytosolic PKCe, it was demonstrated that in the absence of PS this binding was dose dependent, saturable, and specific for the PKCe isoform. Fourth, the binding of either native or recombinant PKCe to purified actin was effectively inhibited by a synthetic hexapeptide with the sequence of the constitutive phosphotransferase activity (Schaap et al., 1990). While the carboxyl terminal catalytic fragment of PKCa that is generated by limited proteolysis has been shown to bind nonneuronal intermediate filaments, it does not bind to actin (Omari et al., 1992). As actin filaments were the principal binding protein for PKCe in the synaptosomal preparation, this catalytic fragment would not be expected to cosediment with the washed detergent-insoluble aggregate to which the full-length PKCe was bound. Moreover, it may be concluded that the constitutive activation of PKCe did not result from hydrophobic interactions of this kinase with a lipid bilayer. This would be consistent with the observations that bound PKCe could not be solubilized by high concentrations of either TX100 or octyl glucoside and that the in vitro PKCe binding and phosphotransferase activity were unaffected by phospholipids, such as PS. Whether autophosphorylation was responsible for the persistent activation of PKCe is unknown. The available evidence indicates that the activity of PKCe may be influenced by an intramolecular or intermolecular form of kinase activity (Koide et al., 1992; Saïdo et al., 1992). Studies are in progress to determine whether dephosphorylation alters the constitutive phosphotransferase activity of actin-bound PKCe. However, the simplest possibility is that activators of PKCe simultaneously produce an allosteric activation of the enzyme and expose a cryptic binding sequence for actin filaments (Fig. 10). This implies that a transient increase in PKCe activity could be maintained, in the absence of stimulatory cofactors and indifferent to changes in PKCe phosphorylation, for several hours by the ability of actin filaments to tightly bind and activate PKCe. The model in Fig. 10 provides a basis for our findings and identifies a PKCe binding and activating protein, whose
Activator

PKC-ε

Actin filaments

PKC substrate(s)

Enhancement of glutamate release

Figure 10. Model for the assembly and constitutive activation of a PKCe-specific signaling complex. Binding of an activator, such as arachidonic acid/diacylglycerol, would be required to simultaneously promote the allosteric activation of PKCe and to expose a cryptic binding site for the cytoskeletal protein, actin (step 1). The anchoring of PKCe to actin (step 2) does not require, but is associated with, increased phosphotransferase activity. Metabolism of the endogenous activator for PKCe would result in the reversal of some actin binding and the resolubilization of inactive PKCe (step 3). However, a pool of the PKCe that remains tightly bound to actin continues to be catalytically active in the absence of the agonist that initially targeted this PKC isoform into the cytoskeletal matrix (step 4). Reversible and stable binding of PKCe to this protein may be preferentially induced by partial and complete agonists, respectively. This model suggests that actin represents a bifunctional, chaperone and anchoring protein that is specific for the PKCε isoform. The assembly and stabilization of this actin-PKCε complex appears to play a primary role in the PKC-dependent enhancement of sustained glutamate exocytosis (step 5).

existence was first suggested by Parker and colleagues (Pears et al., 1991). Such a mechanism would assign an additional level of functional significance to bifunctional PKC-anchoring proteins that are capable of maintaining PKCs in a catalytically active conformation.

PKCe binding to purified actin was synergistically stimulated by AA and the diacylglycerol analog, OAG. The presence of PKCe in the TX100-insoluble fraction of freshly isolated synaptosomes indicated that the enzyme had been physiologically activated in these terminals. Candidate signals for PKCe activation included AA and diacylglycerol, because these two lipid metabolites had previously been shown to increase PKCe-dependent phosphotransferase activity (Koide et al., 1992; Kasahara and Kikkawa, 1995) and the evoked release of endogenous glutamate (Zhang and Dorman, 1993). The present results are in agreement with the hypothesis that AA is a potent endogenous activator of PKCe, while PS effectively activates PKCa, β, γ, and δ, but not PKCe (Kasahara and Kikkawa, 1995). In the absence of either Ca2+ or PS, AA significantly increased the binding of PKCe to filamentous actin. Although OAG by itself had no effect on the binding of actin by PKCe, the addition of OAG when AA was present caused a synergistic enhancement of PKCe binding. A similar interaction results in the potentiation of glutamate release from synaptic preparations (Zhang and Dorman, 1993). In this preparation, the stimulation of metabotropic glutamate receptors effectively mimics the permissive effect of OAG on AA-induced release (Herrero et al., 1992). Thus, phospholipid metabolism may well participate in the physiological activation of PKCe and this mechanism seems to be closely regulated. Optimal activation appears to be contingent upon the coactivation of presynaptic metabotropic glutamate receptors, for the production of diacylglycerol and phospholipase A2, for the liberation of AA (Nishizuka, 1992). Both conditions are likely to be satisfied during the bouts of intense presynaptic activity that produce a long lasting increase in neurotransmitter release (Schulz et al., 1994).

Formal and direct proof that a causal relationship exists between the actin binding of PKCe, the constitutive activation of this kinase, and the enhancement of glutamate release will require further investigation. However, it is possible to estimate the likelihood that these response data sets represent interdependent events by comparing their individual EC50 values for 4β-PDBu. As the ratio of these EC50 values approach unity there is a corresponding increase in the confidence that such a relationship exists. The result of such an analysis estimated that there is a reasonably high (>95%) probability that the translocation of PKCe, PKCe binding to actin, and the enhancement of glutamate exocytosis were functionally related. These results, together with the demonstration that treatments which prevent PKCe binding and activation also abolish the enhancement of glutamate release by 4β-PDBu, are in agreement with the suggestion that the extent to which phorbol esters facilitate the slow phase of glutamate exocytosis may be influenced by the binding of PKCe to actin.

The present results suggest that the individual isoforms of PKC may have assumed distinct responsibilities for the control of regulated secretion. In the case of PKCe, we have discovered that this specificity could have been achieved through the conservation of an actin-binding motif in this, but not in other PKC isoforms. The existence of this conserved binding site in PKCe explains how this isoform becomes specifically paired with, and tightly bound to, actin filaments in the presynaptic cytoskeletal matrix. Use-dependent changes in lipid metabolism have been shown to be capable of generating the endogenous signals that are required to expose this actin-binding site in native PKCe. Once bound to actin, PKCe remained constitutively active in the absence of lipid metabolites or membrane phospholipids. Thus, actin not only appears to be a novel and specific PKCe-binding protein, but may also act as an

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agonist that is capable of maintaining this kinase in a cata-
lactically active conformation for several hours (Fig. 10).
The assembly of this isoform-specific signaling complex may have multiple and diverse influences on synaptic function.
The present results, together with our previous studies, indicate that one outcome of functional significance may be the enhanced availability of synaptic vesicles for exocytosis during intense bouts of presynaptic activity.

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