The Journal of Neuroscience, January 1988, 8(1): 281–288

Protein Phosphorylation in Nerve Terminals: Comparison of Calcium/Calmodulin-Dependent and Calcium/Diacylglycerol-Dependent Systems

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Rat cerebral cortical synaptosomes that had been prelabeled with ^{32}P-orthophosphate were exposed to either (1) K^+ depolarization which causes Ca^{2+} influx and hence would be expected to activate Ca^{2+}-dependent enzymes, including Ca^{2+}/calmodulin-dependent and Ca^{2+}/diacylglycerol-dependent protein kinases (Ca/CaM kinases and protein kinase C, respectively); or (2) phorbol esters or 1-oleoyl-2-acetylglycerol (OAG), which selectively activate protein kinase C. Proteins whose state of phosphorylation was affected by these treatments could be divided into 3 classes. *Class A* includes 5 phosphoproteins that showed rapidly increased phosphorylation by synaptosomal depolarization but not by OAG or phorbol ester. Four of these proteins, synapsins Ia and Ib and proteins IIIa and IIIb, are neuron-specific, synaptic vesicle-associated proteins known to be substrates for Ca/CaM kinases I and II. These phosphoproteins were rapidly dephosphorylated upon synaptosomal repolarization. *Class B* is composed of 2 phosphoproteins that showed rapidly increased phosphorylation by either synaptosomal depolarization or treatment with phorbol ester or OAG. These 2 acidic proteins of *M*$_r$ 87 and 49 kDa are known from *in vitro* studies to be specific substrates for protein kinase C. Thermolytic peptide mapping indicated that the 87 kDa protein in synaptosomes was phosphorylated by protein kinase C *in situ*. These 2 phosphoproteins were slowly dephosphorylated upon synaptosomal repolarization. *Class C* comprises 4 phosphoproteins that were rapidly dephosphorylated upon synaptosomal depolarization and may be substrates for Ca^{2+}-activated protein phosphatase(s).

These data suggest that Ca^{2+} influx into nerve terminals activates Ca/CaM kinases I and II, protein kinase C, and unidentified protein phosphatase(s). It seems likely that the activation of these multiple Ca^{2+}-regulated protein phosphorylation and dephosphorylation pathways underlies pleiotropic physiological actions of Ca^{2+} at the nerve terminal.

Two distinct Ca^{2+}-dependent protein kinases have recently been implicated in the regulation of neurotransmitter release. One of these, Ca^{2+}/calmodulin-dependent protein kinase II (Ca/CaM kinase II), increases neurotransmitter release when injected into the squid giant synapse (Llinás et al., 1985). The other is protein kinase C, which has a similar effect when activated in several non-neuronal preparations (Knight and Baker, 1983; Tanaka et al., 1984; Wakade et al., 1985), in cultured fetal brain neurons (Zurgil and Zisapel, 1985), and in rat brain synaptosomes (Nichols et al., 1987). These enzymes presumably exert their effects on neurotransmitter release by phosphorylating specific protein substrates in the nerve terminal (Nichols et al., 1987). Previous studies have shown that Ca^{2+} influx into nerve terminals leads to altered phosphorylation of a variety of proteins (Krueger et al., 1977; Wu et al., 1982; Robinson and Dunkley, 1983; Robinson and Dunkley, 1985; Dunkley et al., 1986a). It is therefore important to characterize the Ca^{2+}-dependent protein phosphorylation systems present in nerve terminals, in particular, the protein kinase C and Ca/CaM kinase systems.

Our experimental approach to these studies was first to examine the overall patterns of Ca^{2+}-dependent protein phosphorylation in nerve terminals and then to analyze the activity of particular protein kinases by monitoring the state of phosphorylation of their specific substrate proteins. We used prelabeled synaptosomes, since the radioactive ATP generated in this preparation is made only in intact nerve ending particles that contain metabolically active mitochondria (Krueger et al., 1977), thus precluding significant labeling of extrasynaptosomal proteins. The stimulation conditions used were (1) K^+ depolarization to induce Ca^{2+} influx, which would be expected to activate Ca^{2+}-dependent enzymes, including Ca/CaM kinases and protein kinase C; and (2) treatment with selective activators of protein kinase C, namely, tumor-promoting phorbol esters (Castagna et al., 1982) and the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) (Kaibuchi et al., 1983), to identify specific synaptosomal substrates for protein kinase C. To monitor the activity of the Ca/CaM kinases, we measured the state of phosphorylation of synapsin I, a neuron-specific, synaptic vesicle-associated protein (De Camilli et al., 1983a, b; Huttner et al., 1983), which is phosphorylated on distinct sites by Ca/CaM kinases I and II (Huttner et al., 1981; Kennedy and Greengard, 1981). To monitor the activity of protein kinase C, we measured the state of phosphorylation of the 87 kDa protein, previously shown to be a specific substrate for this kinase in lysed synaptosomes (Walaas, 1982; Wu et al., 1982) and brain homogenates (Walaas et al., 1983a, b). Our results demonstrate the Ca/CaM kinase I, Ca/CaM kinase II, and protein kinase C are all activated by Ca^{2+} influx into synaptosomes, each with distinct substrate specificities and temporal characteristics. A preliminary report of this study has been presented in abstract form (Wang et al., 1985).

Received Mar. 17, 1987; revised June 3, 1987; accepted June 18, 1987.

This work was supported by USPHS Grant MH 39237.

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Materials and Methods

Phorbol esters were obtained from LC Services Corp. (Woburn, MA), A23187 and OAG from Sigma (St. Louis), *Staphylococcus aureus* V8 protease from Miles Laboratories (New Haven, CT), thermolysin from Calbiochem (San Diego, CA), and ^{32}P-orthophosphate from New England Nuclear (Boston). All other reagents were of analytical grade and were obtained from standard commercial sources.

Preparation and prelabeling of synaptosomes. Male Sprague-Dawley rats were decapitated and the cerebral cortex was homogenized in 0.32 M sucrose. A crude synaptosomal fraction (P_2) was prepared as described (Krueger et al., 1977). The final pellet was resuspended in an oxygenated Krebs-Ringer buffer containing (in mM) NaCl, 132.0; $MgSO_4$, 2.4; KCl, 4.8; $CaCl_2$, 1.0; glucose, 10.0; and HEPES-NaOH, 20.0, pH 7.4. In some experiments the P_2 fraction was purified over Percoll gradients as described (Dunkley et al., 1986b). Synaptosomes were prelabeled at 37°C for 45 min with 1 mCi/ml of ^{32}P-orthophosphate, centrifuged at 1000 × *g* for 5 min, and resuspended at a protein concentration of 2 mg/ml. Reactions were initiated by addition of 100 μl of synaptosomes to tubes containing the appropriate drug solutions and terminated by addition of "Stop Solution" [final concentration, 3% (wt/vol) SDS, 5% glycerol, 68 mM Tris HCl, and 2% β-mercaptoethanol] and heating in a boiling water bath for 2 min. When synaptosomes were depolarized by high KCl concentrations, tonicity was maintained by decreasing the NaCl concentration correspondingly. For the repolarization experiments, prelabeled synaptosomes were resuspended at a protein concentration of 16 mg/ml. After depolarization by 40 mM KCl, the synaptosomes were diluted 8-fold into KCl-free buffer so that the final KCl concentration was 5 mM. Aliquots were then added to "Stop Solution" at the appropriate time points.

Phorbol esters were dissolved in 100% dimethylsulfoxide (DMSO) and diluted into buffer with a final solvent concentration of no more than 0.1%, which by itself had no effect on protein phosphorylation (data not shown). OAG was sonicated in DMSO before use as described by Kaibuchi et al. (1983).

Polyacrylamide gel electrophoresis. One-dimensional SDS/PAGE was performed by standard techniques (Laemmli, 1970), and gels were processed as described (Walaas et al., 1983a). The 2-dimensional detergent/urea-based gel system was used as described (Imada and Sueoka, 1980), with the first dimensional gel containing 3% acrylamide, 0.1% SDS, 0.3% Triton CF-10, and 9 M urea, and the second dimension being a conventional SDS/PAGE slab gradient gel of 7–14% acrylamide. Two-dimensional analysis by isoelectric focusing and SDS/PAGE was performed as described (O'Farrell, 1975; O'Farrell et al., 1977).

Peptide mapping of partially proteolysed phosphoproteins. SDS/PAGE was carried out as described above except that the gels were not fixed in acid prior to drying. An autoradiogram was used as a guide to cut out the protein bands of interest. These were then subjected to limited proteolysis by 10 μg/sample of *S. aureus* protease, the proteolytic fragments were separated on a 15% gel as described (Cleveland et al., 1977; Huttner and Greengard, 1979) and quantitated by liquid scintillation spectroscopy. This method was quantitative and eluted more than 90% of the radioactivity in the original gel pieces. Thermolysin digestion for 2-dimensional phosphopeptide fingerprinting was carried out as described (Albert et al., 1984).

Immunoprecipitation of phosphoproteins. Synaptosomes were treated as described above except that the incubation was stopped by 1% SDS followed by 2 min of heating in a boiling water bath. Immunoprecipitation of synapsin I and protein III, by an antiserum that recognizes both proteins (gift of J. H. Haycock and M. Browning, The Rockefeller University), and of the 87 kDa protein, by an antiserum raised against purified rat brain 87 kDa protein (J. K. T. Wang, K. A. Albert, and P. Greengard, unpublished results), were carried out as described (Goelz et al., 1981). The immunoprecipitated proteins were separated and analyzed by SDS/PAGE as described above.

Results

Two-dimensional gel electrophoretic analysis

In preliminary experiments, a prelabeled P_2 fraction from rat cerebral cortex was exposed to phorbol esters or 50 mM KCl, followed by conventional 1-dimensional SDS/PAGE. The resolution of this technique was insufficient for detailed analysis of the complicated protein phosphorylation patterns observed (not shown). We therefore utilized a 2-dimensional system developed by Imada and Sueoka (1980) to improve the resolution. This system separates SDS-solubilized proteins in a detergent/urea-containing first-dimensional tube gel mainly according to their charge properties and hydrophobicity, followed in the second dimension by a conventional separation based on molecular mass. Examples of phosphorylation patterns so obtained with a crude synaptosomal fraction are shown in Figure 1. Purified synaptosomes prepared over Percoll gradients (fractions 4 and 5 of Dunkley et al., 1986b) provided similar results (not shown).

Effect of depolarization-induced Ca^{2+} influx on the protein phosphorylation pattern

K^+-depolarized synaptosomes showed a complex pattern of changes in protein phosphorylation (Fig. 1). Phosphorylation of synapsin Ia and Ib (86 and 80 kDa, labeled as I in Fig. 1), proteins IIIa (74 kDa) and IIIb (57 kDa), and substrates at 87, 49, and 64 kDa, was increased. The highly basic synapsin I and the acidic 87 kDa protein (which migrated at 83 kDa in this gel system) were well-separated from each other. Their identities were confirmed by limited proteolysis by *S. aureus* protease, which yielded the appropriate phosphopeptide fragments of 13 and 9 kDa for the 87 kDa protein and 35 and 10 kDa for synapsin I (Fig. 2) (Huttner and Greengard, 1979; Wu et al., 1982). The 49 kDa protein, upon limited proteolysis, yielded a peptide (not shown) similar to that observed for a 47 kDa substrate for protein kinase C in lysed synaptosomes (Wu et al., 1982) and is likely to be the same protein. In contrast to these increases in phosphorylation, 4 distinct proteins with higher apparent molecular masses (99, 99, 135, and 170 kDa) were consistently dephoshorylated by this treatment. All of the changes induced by depolarization were dependent on the presence of Ca^{2+} in the incubation buffer, and were mimicked by the Ca^{2+} ionophore A23187 (data not shown).

Effects of phorbol ester and OAG on the protein phosphorylation pattern

To identify possible physiological substrates for protein kinase C, ^{32}P-prelabeled synaptosomes were treated with 100 nM phorbol 12,13-dibutyrate (PDBu) for 1 min or 100 μg/ml OAG for 10 min. This resulted in detectable increases in phosphorylation of only the 87 and 49 kDa proteins (Fig. 1), i.e., the same 2 major acidic proteins that responded to K^+ depolarization.

When the concentration of PDBu was increased to 1 μM and the incubation time extended to 20 min, there was increased phosphorylation of additional substrates at 55, 67, and 64 kDa (Fig. 3). One of these unidentified proteins, the weakly responsive 64 kDa protein, was prominently phosphorylated upon K^+ depolarization (see Fig. 1). Phosphorylation of these additional substrates required high PDBu concentrations and long incubation times. Quantitative analysis by peptide mapping (as in Fig. 2) also revealed that phosphorylation of the 10 kDa peptide of synapsin I increased 28 ± 4% (mean ± SE; $n = 5$) in synaptosomes treated with the high (1 μM) concentration of PDBu. Small increases in phosphorylation were also observed for protein III separated on 2-dimensional gels or immunoprecipitated with specific antiserum (data not shown). In contrast, the 35 kDa peptide of synapsin I showed no significant increase in phosphorylation (10 ± 8%) in response to PDBu.

The phosphoproteins that were affected by the various treatments, except for those that were dephosphorylated by depolarization, are summarized in Table 1. The 2 substrates that

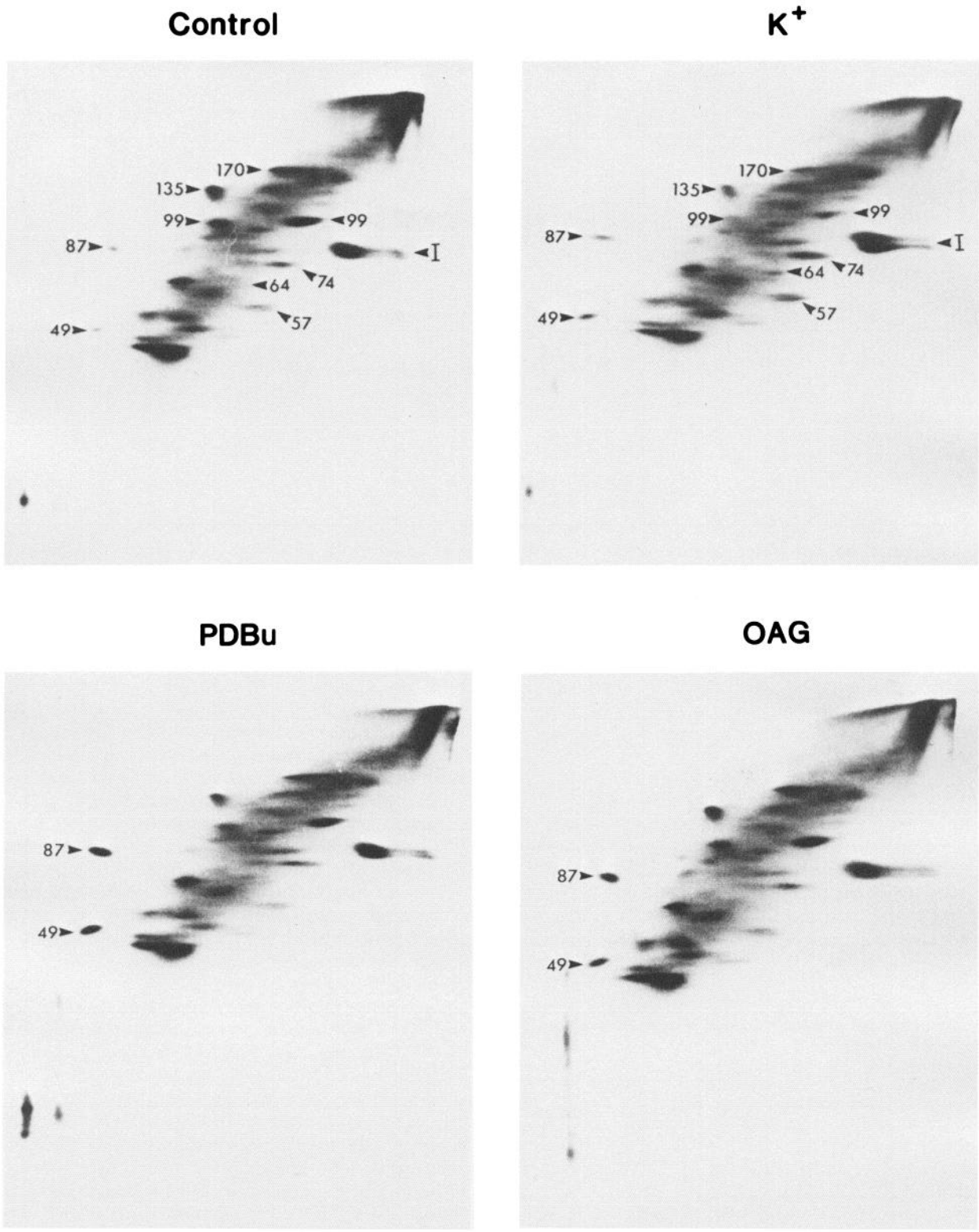

Figure 1. Two-dimensional electophoretic analysis of proteins from ^{32}P-prelabeled synaptosomes. Detergent/urea-based 2-dimensional gel electrophoresis was performed as described in Materials and Methods. *Arrows* point to proteins that responded to the indicated treatment (KCl 50 mM, 30 sec; PDBu 100 nM, 1 min; OAG 100 μg/ml, 10 min), with the numbers denoting the M_r in kDa. *I*, synapsin I.

responded to OAG and to low concentrations of PDBu, namely, the 87 and 49 kDa proteins, have previously been shown *in vitro* to be specific substrates for protein kinase C (Wu et al., 1982; Aloyo et al., 1983; Albert et al., 1986). Two-dimensional analysis by isoelectric focusing and SDS/PAGE (not shown) further confirmed their identity, with the 87 kDa protein yielding multiple isoelectric spots between pI 4.0–4.6, and the 49 kDa protein yielding spots of pI 4.8–5.0, in agreement with

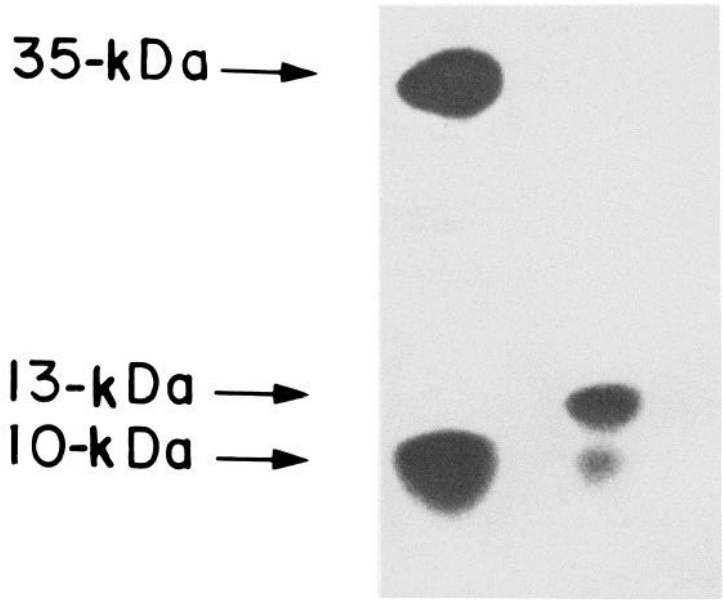

Figure 2. Limited proteolysis by *S. aureus* protease. Synapsin I (*left*) and the 87 kDa protein (*right*) were excised from a detergent/urea-based 2-dimensional gel and subjected to limited proteolysis by *S. aureus* protease as described in Materials and Methods. An autoradiogram of the final 15% polyacrylamide gel on which the peptides were resolved is shown.

previous reports (Aloyo et al., 1983; Albert et al., 1986). Since both K^+ depolarization and active phorbol esters (or OAG) increased phosphorylation of these proteins, it appears that Ca^{2+} influx into nerve terminals activated protein kinase C. This interpretation was further supported by thermolytic peptide fingerprinting (Albert et al., 1984) of the 87 kDa protein from synaptosomes treated with high KCl, PDBu, or OAG. In all cases similar phosphopeptide patterns were observed (not shown), indicating that the same sites were being phosphorylated under all 3 conditions, consistent with an activation of protein kinase C.

Quantitative analysis by limited proteolytic peptide mapping and by immunoprecipitation

To further characterize the 2 Ca^{2+}-dependent protein phosphorylation systems, we studied the activity of protein kinase C and the Ca/CaM kinases in a quantitative manner. Phosphorylation of the 13 kDa peptide derived from the 87 kDa protein was used as a marker of protein kinase C activity. Phosphorylation of the 10 and 35 kDa peptides derived from synapsin I was used as a marker of the activity of Ca/CaM kinases I and II, respectively. Following limited proteolysis of the 87 kDa protein and synapsin I by *S. aureus* protease, the resultant phosphopeptides were quantitated (Huttner and Greengard, 1979; Wu et al., 1982). When an antiserum against the 87 kDa protein became available during the course of these studies (J. K. T. Wang, K. A. Albert, and P. Greengard, unpublished observations), phosphorylation of this protein was also quantitated by immunoprecipitation. The 2 procedures gave similar results (not shown).

Treatment of prelabeled synaptosomes with 50 mM KCl for 30 sec increased the radioactivity incorporated into the phosphopeptides derived from synapsin I and the 87 kDa protein. The percentage increases over control (mean ± SE, $n = 7$) were as follows: synapsin I 35 kDa peptide, 91 ± 15%; synapsin I 10 kDa peptide, 35 ± 6%; 87 kDa protein 13 kDa peptide, 65 ± 11%. In contrast, treatment with 100 μg/ml OAG specifically increased ^{32}P-incorporation into the 13 kDa peptide from the 87 kDa protein (99 ± 18%) without any effect on the 10 and 35 kDa peptides from synapsin I, consistent with a selective stimulation of protein kinase C.

A number of phorbol ester analogs stimulated phosphorylation of the 87 kDa protein in a concentration-dependent manner (Fig. 4). PDBu was an effective activator and was more potent than phorbol 12,13-diacetate. Phorbol 13-acetate, a very weak tumor promoter, had only a small effect. 4α-PDBu, and phorbol was inactive (data not shown). This rank order of potencies is consistent with the potency series for phorbol ester binding to brain membranes (Dunphy et al., 1980). The dose–response curves for the active phorbol esters were not affected by the absence of Ca^{2+} in the incubation medium (Fig. 4). Likewise, the effect of OAG was also independent of extracellular Ca^{2+} (data not shown). In contrast, KCl-induced phosphorylation of both the 87 kDa protein and synapsin I required extracellular Ca^{2+} (data not shown), in agreement with previous reports (Krueger et al., 1977; Wu et al., 1982; Robinson and Dunkley, 1983). The increase in phosphorylation of the 87 kDa protein was significant within 1 min of PDBu treatment and reached a maximum by 10 min, with the plateau maintained to at least 20 min (Fig. 5).

The time course of phosphorylation of substrates induced by

Table 1. Ca^{2+}-regulated phosphorylation of proteins in synaptosomes from rat cerebral cortex

Phosphoprotein		Treatment			
kDa	Name	K^+	OAG	PDBu (low)	PDBu (high)
Identified proteins					
83	87 kDa	⇈	⇈	⇈	⇈⇈
49	(B50?)	⇈	⇈	⇈	⇈⇈
86	Synapsin Ia	⇈	NC	NC	↑
80	Synapsin Ib	⇈	NC	NC	↑
74	Protein IIIa	⇈	NC	NC	↑
57	Protein IIIb	⇈	NC	NC	↑
Unidentified proteins					
55	?	NC	NC	NC	↑
64	?	⇈	NC	NC	↑
67	?	NC	NC	NC	↑

PDBu (low), 100 nM, 1 min; PDBu (high), 1 μM, 20 min. NC, no change in the state of phosphorylation. Arrows, increased state of phosphorylation, ⇈⇈ > ⇈ > ↑.

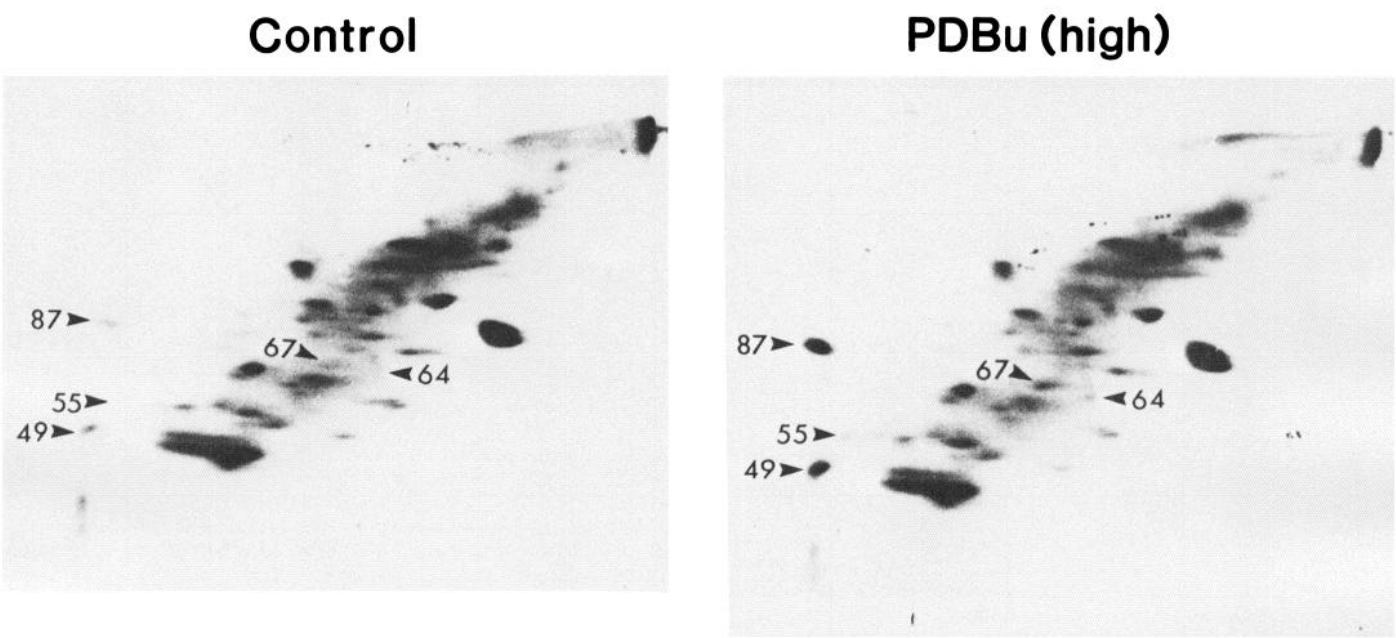

Figure 3. Effect of high concentration of PDBu on pattern of protein phosphorylation. Prelabeled synaptosomes were treated with 1 μM PDBu for 20 min. Detergent/urea-based 2-dimensional electrophoresis was then performed as described in Materials and Methods.

K^+ depolarization was also examined. Consistent with previous results (Krueger et al., 1977; Robinson et al., 1983), we found that the phosphorylation of synapsin I (both the 35 and the 10 kDa peptides, which showed similar time courses) increased rapidly, reaching a maximum within 10 sec. This was followed by a steady decline in phosphorylation, despite continued depolarization, such that control levels were reached within 2 min (Fig. 6). Declines in the phosphorylation of proteins IIIa and IIIb and the 64 kDa protein were also observed in the presence of continued depolarization (data not shown). In contrast, phosphorylation of the 87 kDa protein was rapid in onset, reaching a maximum by 15 sec, and remained elevated for at least 10 min (Fig. 6).

Repolarization of synaptosomes after a brief period of depolarization resulted in rapid reversal of the phosphorylation of synapsin I and a slow reversal of the phosphorylation of the 87 kDa protein. Thus, when prelabeled synaptosomes were depolarized for 2 sec (similar results were obtained after 15 sec of depolarization), and then repolarized by dilution, the phosphorylation of synapsin I (both the 35 and 10 kDa peptides) declined rapidly and reached basal levels by 1 min (Fig. 7). Repolarization also reversed the phosphorylation of the 87 kDa protein, although it occurred at a slower rate than that of synapsin I. Phosphorylation of the 87 kDa protein remained elevated for at least 1 min before a gradual decline to basal level between 3 and 5 min.

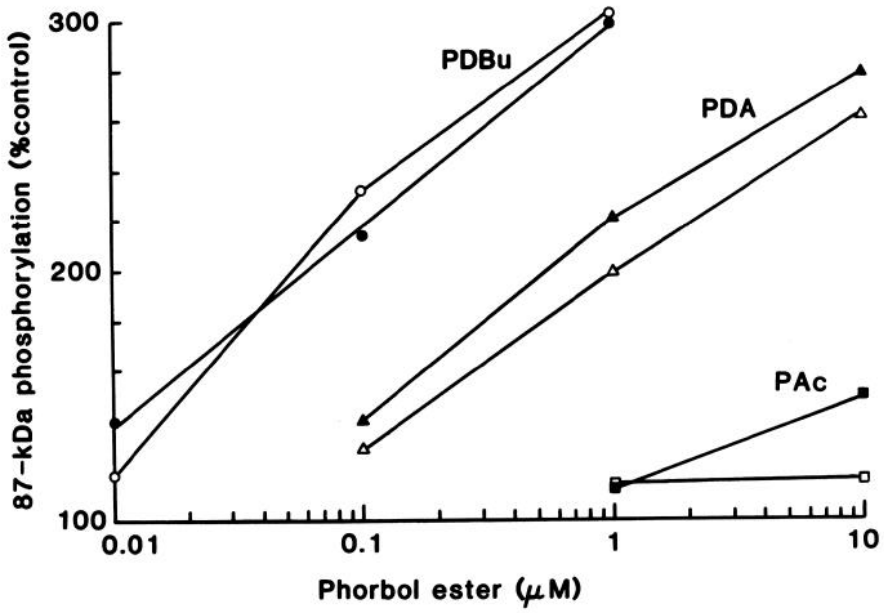

Figure 4. Concentration and Ca^{2+} dependency of phorbol ester-stimulated phosphorylation of the 87 kDa protein. Prelabeled synaptosomes were treated for 20 min with the indicated concentrations of phorbol esters, and ^{32}P-incorporation into the 13 kDa peptide derived from the 87 kDa protein was quantitated by limited proteolysis by *S. aureus* protease as described in Materials and Methods. *Open symbols,* 1 mM $CaCl_2$ in the buffer; *closed symbols,* 0.1 mM EGTA and no $CaCl_2$ in the buffer. *PDBu,* phorbol 12,13-dibutyrate; *PDA,* phorbol 12,13-diacetate; *PAc,* phorbol 13-acetate.

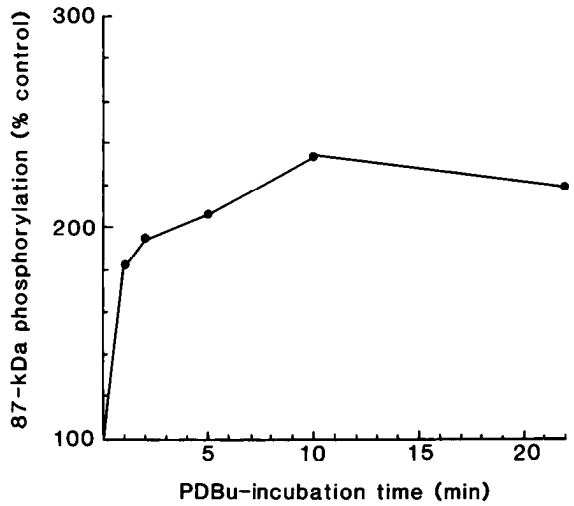

Figure 5. Time course of PDBu-stimulated phosphorylation of the 87 kDa protein. Prelabeled synaptosomes were exposed to 100 nm PDBu for various periods of time. ^{32}P-incorporation into the 13 kDa peptide derived from the 87 kDa protein was quantitated by limited proteolysis by *S. aureus* protease as described in Materials and Methods.

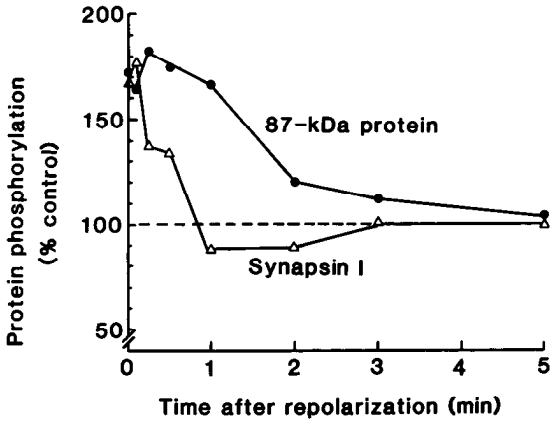

Figure 7. Time course of reversal of the phosphorylation of synapsin I and the 87 kDa protein. Prelabeled synaptosomes were depolarized by 40 mM KCl for 2 sec and then repolarized by dilution as described in Materials and Methods. Aliquots were removed at various intervals. Phosphorylation of synapsin I and the 87 kDa protein was quantitated as described in the legend to Figure 6.

Discussion

In this study we have characterized 2 major Ca^{2+}-dependent protein phosphorylation systems in nerve terminals from rat cerebral cortex. The 2 systems are independently regulated by protein kinase C and Ca/CaM kinases and display distinct activation mechanisms, temporal characteristics, and subcellular localization. The phosphorylation system regulated by protein kinase C appears to contain a limited number of major substrates since selective activation of the kinase by PDBu (or OAG) detectably increased the phosphorylation of only the 87 and 49 kDa proteins. The effect of phorbol ester was time and dose dependent, with a potency series similar to that for protein kinase C activation. It was independent of Ca^{2+} in the incubation buffer, consistent with the finding that, *in vitro,* protein kinase C can be fully activated at low Ca^{2+} concentrations if diglycerides and phospholipids are both present (Kishimoto et al., 1980). The lesser potency of OAG, compared with phorbol esters, might be due to the presence of an active diacylglycerol kinase to synaptosomes (Lapetina and Hawthorne, 1971) that would rapidly metabolize OAG (Kaibuchi et al., 1983). Very high concentrations of PDBu at longer incubation times increased the state of phosphorylation of several additional proteins, including synapsin I and protein III, both of which are substrates for Ca/CaM kinase I and cAMP-dependent protein kinase. However, these proteins are not good substrates for protein kinase C *in vitro* (A. C. Nairn, personal communication). Hence these effects of PDBu may be mediated by secondary activation of other protein kinases or phosphatases. Whether these effects are physiologically significant or are mere artifacts of the high concentrations of phorbol esters used is presently unclear.

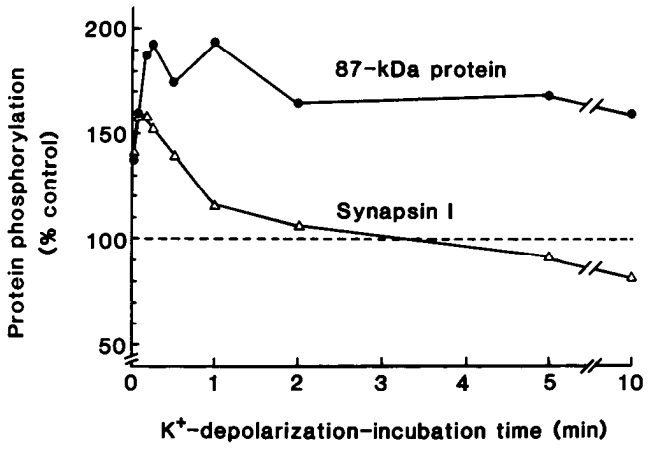

Figure 6. Time course of depolarization-induced phosphorylation of synapsin I and the 87 kDa protein. Prelabeled synaptosomes were exposed to 40 mM KCl for the indicated period of time. Synapsin I phosphorylation was analyzed by limited proteolysis by *S. aureus* protease as described in Materials and Methods. Radioactivity in the 35 and 10 kDa peptides was quantitated and found to follow very similar time courses; their counts were hence combined and shown here as the time course for synapsin I. The 87 kDa protein was immunoprecipitated, and its state of phosphorylation was quantitated as described in Materials and Methods.

K^+ depolarization of synaptosomes altered the phosphorylation of a number of proteins, some of which have been described previously (Krueger et al., 1977; Wu et al., 1982; Robinson and Dunkley, 1983; Dunkley et al., 1986a). In particular, there was increased phosphorylation of synapsin I, protein III, and the 87 and 49 kDa proteins. The increased phosphorylation of synapsin I occurred in both the 35 kDa peptide, a substrate for Ca/CaM kinase II, and the 10 kDa peptide, a substrate for both Ca/CaM kinase I and cAMP-dependent protein kinase (Huttner et al., 1981; Kennedy and Greengard, 1981). Protein III is likewise a substrate for Ca/CaM kinase I and cAMP-dependent protein kinase (see Nestler and Greengard, 1984; Nairn et al., 1985; Nairn and Greengard, 1987). It has been suggested that the Ca^{2+}-induced phosphorylation of the 10 kDa peptide of synapsin I and of protein III in intact nerve terminals is mediated by activation of cAMP-dependent protein kinase (Dunkley et al., 1986a). However, nerve terminals contain appreciable activity of Ca/CaM kinase I (Nairn and Greengard, 1987), which can label the 10 kDa synapsin I peptide in lysed synaptosomes *in vitro* in the presence of the specific inhibitor of cAMP-dependent protein kinase (unpublished data). Hence, one could account for the changes observed in synapsin I and protein III by Ca^{2+} influx causing activation of Ca/CaM kinases I and II. Upon K^+ depolarization, increased phosphorylation of the 87 kDa protein occurred in the same proteolytic peptides

that were affected by treatment with phorbol ester or OAG. Hence, Ca^{2+} influx also appears to activate protein kinase C in the nerve terminal. The 4 proteins that were dephosphorylated upon depolarization may be substrates for one or more Ca^{2+}-dependent protein phosphatases (Shields et al., 1985). However, we cannot rule out the possibility that these 4 proteins are substrates for a highly active protein kinase that is inactivated by Ca^{2+} influx. In conclusion, Ca^{2+} influx into nerve terminals appears to activate, at the minimum, CaM kinases I and II, protein kinase C, and possibly one or more unidentified protein phosphatases.

The 87 and 49 kDa proteins appear to be the major substrates for protein kinase C in nerve terminals. Both proteins have also been identified as prominent substrates for protein kinase C in nerve growth cone particles (Katz et al., 1985). The 87 kDa protein is a widely distributed brain-enriched phosphoprotein concentrated in synaptosomal membranes (Albert et al., 1986; Blackshear et al., 1986). The 49 kDa protein is likely to be the protein called B-50 or F-1, which has been described as a neuron-specific, acidic substrate for protein kinase C localized to presynaptic membranes (Sorensen et al., 1981; Aloyo et al., 1983; Gispen et al., 1985; Nelson and Routtenberg, 1985). Protein kinase C has also been shown to be localized mainly to the particulate fraction in a brain crude mitochondrial preparation (Kikkawa et al., 1982). Thus, the kinase and its 2 major substrates in nerve terminals are enriched in the membrane fraction, suggesting that the protein kinase C-regulated system may be particularly active at synaptic membranes. This is in contrast to the Ca/CaM kinase system, which appears to phosphorylate mainly the synaptic vesicle-associated proteins synapsins Ia and Ib and proteins IIIa and IIIb in presynaptic terminals.

The rapid onset and decline of synapsin I phosphorylation, whether during continued depolarization or after repolarization, is consistent with a short-term, transient role for the protein in regulating presynaptic functions. It is interesting that Ca/CaM kinase II, when activated by Ca^{2+} influx, does not appear to have long-lasting effects on synapsin I phosphorylation in nerve terminals. This is in contrast to what might have been expected from the *in vitro* demonstration that the kinase undergoes a Ca^{2+}/calmodulin-dependent autophosphorylation, resulting in an active enzyme that is no longer dependent on Ca^{2+} or calmodulin (Saitoh and Schwartz, 1985; Lai et al., 1986; Miller and Kennedy, 1986).

In summary, our results indicate that depolarization of the nerve terminal results in the activation of at least 2 highly regulated Ca^{2+}-dependent protein phosphorylation systems, each with distinct substrate specificity, temporal characteristics, and subcellular localization. Ca/CaM protein kinases, through a rapid and transient phosphorylation of synaptic vesicle-associated substrates such as synapsin I, may be involved in short-term regulation of neurotransmitter release. Protein kinase C, on the other hand, through a rapid but longer-lasting phosphorylation of membrane-associated proteins, may play a modulatory role at the level of the plasma membrane. Further studies of the Ca^{2+}-dependent protein phosphorylation systems described in this report should facilitate our understanding of the mechanism of action and the physiological role of Ca^{2+}-dependent protein kinases and their substrate proteins in nerve terminals.

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