*Proc. Natl. Acad. Sci. USA*
Vol. 77, No. 10, pp. 5812–5816, October 1980
Biochemistry

# Calcium-regulated phosphorylation in synaptosomal cytosol: Dependence on calmodulin

(protein kinase/antipsychotics)

JAMES P. O'CALLAGHAN, LAWRENCE A. DUNN, AND WALTER LOVENBERG

Section on Biochemical Pharmacology, Hypertension–Endocrine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

*Communicated by C. B. Anfinsen, July 14, 1980*

ABSTRACT **Calcium stimulated the phosphorylation of several specific synaptosomal cytosolic proteins. The effects of calcium were both concentration and time dependent and were most apparent for proteins with molecular weights of 50,000, 55,000, and 60,000. Exogenous calcium (1.0–100 μM) enhanced the net incorporation of phosphate into protein by as much as 23-fold. In the absence of added calcium, the calcium chelator [ethylenebis(oxyethylenenitrilo)]tetraacetic acid did not lower the phosphorylation of any protein below control levels. The antipsychotic, fluphenazine (1.0–100 μM), caused a concentration-dependent decrease in calcium-stimulated protein phosphorylation. When the heat-stable calcium-binding protein, calmodulin, was removed from synaptosomal cytosol by affinity chromatography on fluphenazine-Sepharose, calcium-stimulated protein phosphorylation was abolished. Responsiveness to calcium could be restored by the addition of calmodulin to the phosphorylation assay. These results indicate that calcium-dependent protein kinases are of major importance in regulating the phosphorylation of specific cytosolic proteins in neuronal tissue. Furthermore, it would appear that one of the three substrates under investigation is specific to synaptosomal cytosol whereas the other two are present in both the cytosol and membrane fractions.**

Calcium ion regulates the endogenous phosphorylation of specific proteins found in several particulate fractions of neuronal tissue, including synaptic plasma membranes (1, 2), synaptic vesicles (3), cortical slices (4), synaptosomes (5), and synaptosomal lysates (6). Evidence indicates that the calcium-dependent phosphorylation of synaptic plasma membranes (2) and synaptic vesicles (3) requires the heat-stable calcium-binding protein, calmodulin. Thus, the effect of calcium on the phosphorylation of specific proteins present in these fractions may be mediated through one or more protein kinases that require calmodulin as a coenzyme. The calcium- and calmodulin-activated kinases present in these particulate fractions have not been purified. Specific calcium-regulated protein kinases have been identified in neuronal cytosol (7, 8). However, the endogenous cytosolic substrates for these kinases have not been characterized and a role for calmodulin in the regulation of these cytosolic enzymes has yet to be established. In the present report, we present evidence that calcium regulates the phosphorylation of several specific synaptosomal cytosolic proteins, an effect that was found to be dependent on the presence of calmodulin.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

## MATERIALS AND METHODS

**Materials.** The following chemicals, obtained from commercial sources, were used: dithiothreitol, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), and cyclic AMP (cAMP) from Sigma; Hepes from Calbiochem; and Tris from Schwarz/Mann. Sepharose 4B was purchased from Pharmacia. Fluphenazine·HCl was generously provided by S. J. Lucania (Squibb Institute of Medical Research, Princeton, NJ). The materials used in the preparation of the polyacrylamide gels and associated buffers were of the highest purity commercially available. All other chemicals were of analytical reagent grade. Adenosine 5′-[$\gamma$-$^{32}$P]triphosphate (10–40 Ci/mmol); 1 Ci = 3.7 × $10^{10}$ becquerels) was purchased from New England Nuclear.

The subjects were male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 150–200 g. Because the rat striatum is a rich source of synaptosomes and contains a high concentration of soluble calmodulin (9), it was used as the sole source of synaptic membranes and synaptosomal cytosol.

Calmodulin was prepared from rat striatum or whole rat brain (lacking the cerebellum) by affinity chromatography on a fluphenazine-Sepharose matrix. Fluphenazine was covalently coupled to Sepharose 4B by the bisoxirane technique (10) as described (11). Brain extracts were prepared by homogenization in 3 vol of 50 mM Hepes, pH 7.0/0.5 M NaCl/5 mM $CaCl_2$. A supernatant fraction was obtained from the homogenate by centrifugation at 100,000 × *g* for 30 min. This supernatant was heated for 5 min in a boiling water bath. A second supernatant was then prepared by centrifugation (100,000 × *g*, 30 min) to remove the precipitated proteins. For chromatography, the heated supernatant fraction was placed onto a 1.0 × 20 cm fluphenazine-Sepharose column that had been equilibrated with a column buffer (10 mM Hepes, pH 7.0/0.5 mM $CaCl_2$). The column was washed with column buffer containing 300 mM NaCl, and calmodulin was subsequently eluted with 10 mM Tris·HCl, pH 8.0/10 mM EGTA. EGTA was removed from the calmodulin-rich eluate by exhaustive dialysis against 5 mM $CaCl_2$. Calmodulin obtained in this manner appeared as a single band in $NaDodSO_4$/polyacrylamide gels and comigrated with purified calmodulin prepared by the method of Klee (12). The presence of calmodulin in the EGTA eluate was also verified by the assay procedure described by Schulman and Greengard (2).

Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; p50, p55, and p60 bands, bands containing proteins of $M_r$ 50,000, 55,000, and 60,000, respectively; cAMP, cyclic AMP.

**Tissue Preparation.** Rats were killed by decapitation, the brains were removed, and the striata were dissected within 30 sec. A crude synaptosomal lysate was prepared from a striatal homogenate by the method of Jones and Matus (13). The lysate was centrifuged at 140,000 × *g* for 90 min. The resulting supernatant fraction was used as the source of synaptosomal cytosol. The pelleted membranes were used to prepare a synaptic plasma membrane fraction by a discontinuous sucrose gradient technique (13). Proteins were assayed by the method of Bradford (14), with bovine plasma gamma globulin as a standard.

**Phosphorylation Assays.** The net incorporation of phosphate into striatal synaptosomal cytosolic proteins (100 μg of protein), or in some cases into synaptic plasma membrane proteins (100 μg of protein), was assayed at 30°C in a standard assay mixture containing (200 μl final volume) 50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 1.0 mM dithiothreitol, and 5 μM [$\gamma$-$^{32}$P]ATP ($1 \times 10^7$ dpm/nmol). Where indicated, EGTA, $CaCl_2$, fluphenazine, and calmodulin were included in the assay at the concentrations listed. After a 1-min preincubation of the assay mixture, the phosphorylation reaction was initiated by the addition of the cytosolic or membrane proteins. After 1 min (except where indicated otherwise), the reaction was terminated by solubilizing the cytosol or membrane proteins in 100 μl of a sample buffer containing 9% (wt/vol) $NaDodSO_4$, 30 mM Tris·HCl (pH 8.0), 6% (vol/vol) 2-mercaptoethanol, 3 mM EDTA, and 27% (wt/vol) sucrose.

**Removal of Calmodulin from Synaptosomal Cytosol.** In the experiments in which calmodulin was extracted from synaptosomal cytosol, fluphenazine-Sepharose columns (0.5 × 5.0 cm) were first equilibrated in the assay buffer containing 50 μM $CaCl_2$. Prior to chromatography on fluphenazine-Sepharose, the cytosol samples were suspended in the assay buffer at a final calcium concentration of 50 μM. The amount of brain extract chromatographed never exceeded 50% of the column bed volume to avoid overloading the gel. After chromatography, the eluates were divided into aliquots and the protein values were adjusted to 100 μg per sample. Calmodulin was then added to half of the samples and all tubes were assayed for phosphorylation as described above.

**Gel Electrophoresis, Autoradiography, and Microdensitometry.** The solubilized proteins were resolved on $NaDodSO_4$/polyacrylamide slab gels (10 × 14 × 0.15 cm) (Hoefer Scientific Instruments, San Francisco, CA). The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained $NaDodSO_4$ at a final concentration of 0.1%. The running and gel buffers were as described by O'Farrell (15) and were prepared in deionized water with conductivity values of 15 MΩ or greater. Electrophoresis was performed at constant power (7.5 W/gel). After electrophoresis, the gels were fixed and stained with 0.1% Coomassie brilliant blue R250 and 50% (vol/vol) methanol/10% (vol/vol) acetic acid. The gels were destained by diffusion in 30% methanol/10% acetic acid before they were dried under heat and reduced pressure. Autoradiography was carried out for 1–2 days with Kodak RP x-ray film. The molecular weights of the protein bands were estimated from molecular weight standards (Bio-Rad). Incorporation of phosphate into specific protein bands were determined by microdensitometry of the autoradiographs with an EC 920 transmission microdensitometer (E-C Apparatus, St. Petersburg, FL). Protein phosphorylation was quantified from the densitometric scans by integration of the areas under the peaks corresponding to specific protein bands. The background darkness of the autoradiographs was taken as the baseline value for these determinations. All microdensitometry values obtained were linear with respect to the range of exposures observed after autoradiography.

## RESULTS

**Effects of Calcium on Phosphorylation of Synaptosomal Cytosolic Proteins.** Autoradiographs were obtained for the synaptosomal cytosolic proteins that were resolved on $NaDodSO_4$/polyacrylamide gels after phosphorylation in the presence and absence of calcium. These data are presented in Fig. 1. Of the more than 50 proteins resolved by electrophoresis, only 2 or 3 major bands were phosphorylated in the absence of calcium. Endogenous calcium did not appear to play a role in this basal level of phosphorylation because the addition of EGTA to the assay did not affect the incorporation of phosphate into any proteins. When 10 μM cAMP was added to the assay mixture, it resulted in an 2-fold stimulation of the phosphorylation of a protein with an apparent $M_r$ of 80,000 (data not shown). The addition of calcium resulted in a concentration-dependent increase in the incorporation of phosphate into at least a dozen cytosolic proteins. The effects were observed at a concentration of calcium as low as 1.0 μM. The most highly phosphorylated proteins were those with apparent $M_r$ of 50,000, 55,000, and 60,000 which will hereafter be referred to as the p50, p55, and p60 bands. In this and all following experiments, the presentation of quantitative data concerning the net incorporation of phosphate into specific proteins will be limited to the values obtained for the p50, p55, and p60 bands. Quantitative data for the effects of calcium on the phosphorylation of these proteins are presented in Fig. 2. The results show that calcium caused a linear increase in the phosphorylation of all three protein bands within a range of 1–50 μM. Maximal effects were observed at a calcium concentration of 100 μM and resulted in a 15-, 19-, and 23-fold stimulation above control levels of phosphorylation for the p50, p55, and p60 bands, respectively. These effects were completely reversed by the inclusion of EGTA in the assay mixture (data not shown).

The effect of calcium on the phosphorylation of the synaptosomal cytosolic proteins was time dependent. From the autoradiographs (Fig. 3) it is apparent that the net incorporation of phosphate into protein in the presence of calcium (50 μM) followed a gradual increase over a period of minutes with a gradual decline to nearly control levels within 2 hr. However, the net increase and decrease in the phosphorylation of the p50, p55, and p60 protein bands did not follow an identical time course. This is apparent from the autoradiographs (Fig. 3) and was confirmed by microdensitometry (Fig. 4). The patterns of phosphorylation and dephosphorylation for the p50, p55, and p60 bands could be distinguished from each other based on differences in both the rate of incorporation of phosphate and

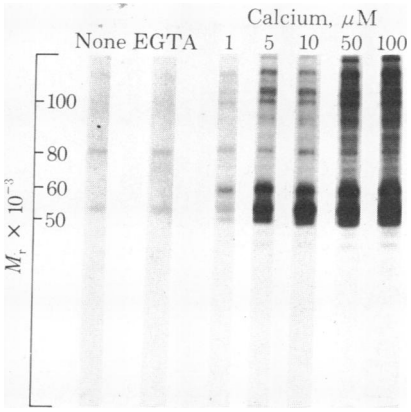


FIG. 1 Autoradiographs showing the effects of EGTA (100 μM) and $CaCl_2$ on the phosphorylation of synaptosomal cytosolic proteins. Synaptosomal cytosol was incubated under the standard conditions.

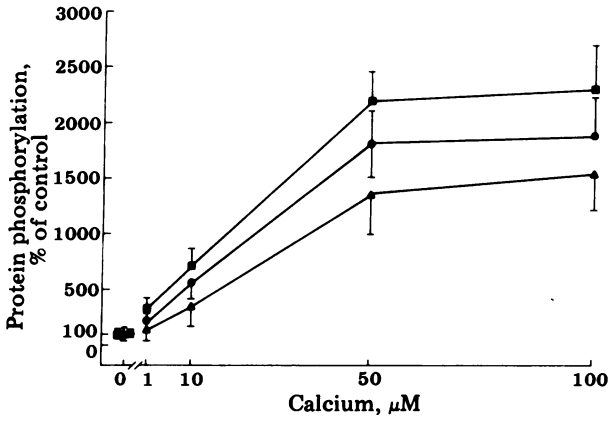


FIG. 2. Phosphorylation of specific synaptosomal cytosolic proteins as a function of calcium concentration. The net incorporation of phosphate into specific proteins was quantified from the densitometric scans of the autoradiographs and is expressed as a percent (±SEM) of control phosphorylation. ■, p60 band; ▲, p55 band; ●, p50 band.

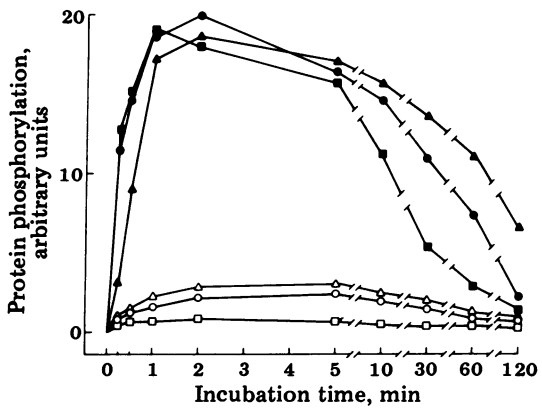


FIG. 4. Phosphorylation of specific synaptosomal cytosolic proteins as a function of incubation time. ○, △, and □, Specific protein phosphorylation observed in the absence of calcium; ●, ▲, and ■, phosphorylation of the same proteins in the presence of 50 μM $CaCl_2$. The net incorporation of phosphate into specific proteins was quantified from the densitometric scans of the autoradiographs and is expressed in arbitrary units of density. ■ and □, p60 band; ▲ and △, p55 band; ● and ○, p50 band.

the rate of its removal. When the data were expressed as a percentage of the phosphorylation observed in the presence of calcium, the maximal incorporation of phosphate into protein was at an incubation time of 1 min (Fig. 4). In the absence of calcium the net incorporation of phosphate reached a peak for the three proteins between 2 and 5 min of incubation. This pattern of phosphorylation differs from that observed in the presence of calcium and was found to be insensitive to EGTA at all time points. Additionally, the maximal incorporation of phosphate in the presence of EGTA amounted to no more than 20% of the maximal phosphorylation observed in the presence of calcium.

**Effect of Fluphenazine *in Vitro* on Calcium-Regulated Phosphorylation.** A variety of neuroleptic agents bind to calmodulin *in vitro* (16). Of these agents, the phenothiazine antipsychotics are among those that show the highest binding affinities (16). Therefore, in order to examine the role of calmodulin in the calcium-regulated phosphorylation of synaptosomal cytosolic proteins, we added a phenothiazine antipsychotic, fluphenazine, to the standard assay. Autoradiographs from these experiments are shown in Fig. 5, lanes 1–7; the microdensitometry values obtained from these autoradiographs are shown in Fig. 6. The qualitative and quantitative results presented in these figures indicate that, in the presence of calcium (50 μM), fluphenazine caused a concentration-dependent decrease in the phosphorylation of the cytosol proteins. In the presence of as little as 25 μM fluphenazine, the calcium-stimulated phosphorylation of the p50, p55, and p60 bands was reduced to levels equal to those observed in the absence of calcium. When fluphenazine (100 μM) was added to the standard assay in the absence of calcium, it had no effect on the phosphorylation of any protein (data not shown).

**Fluphenazine-Sepharose Chromatography.** The calmodulin dependence for calcium-regulated phosphorylation of synaptosomal cytosol was further substantiated by chromatography of the cytosol over an affinity column of fluphenazine-Sepharose 4B. This technique was shown to effectively remove endogenous calmodulin from the synaptosomal cytosol based on the following lines of evidence: (*i*) when synaptosomal cytosol proteins were subjected to gel electrophoresis both before and after affinity chromatography, a comparison of the resulting electrophoretic profiles revealed that a protein corresponding to the electrophoretic mobility of authentic calmodulin was

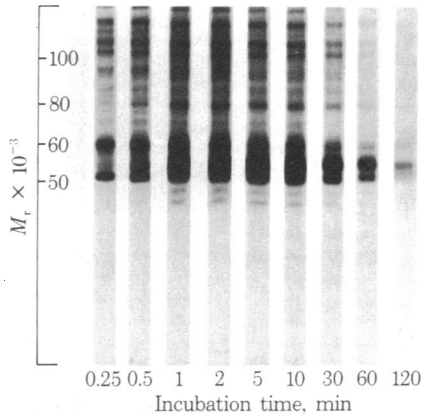


FIG. 3. Autoradiographs showing the effects of $CaCl_2$ (50 μM) on the phosphorylation of synaptosomal cytosolic proteins as a function of incubation time.

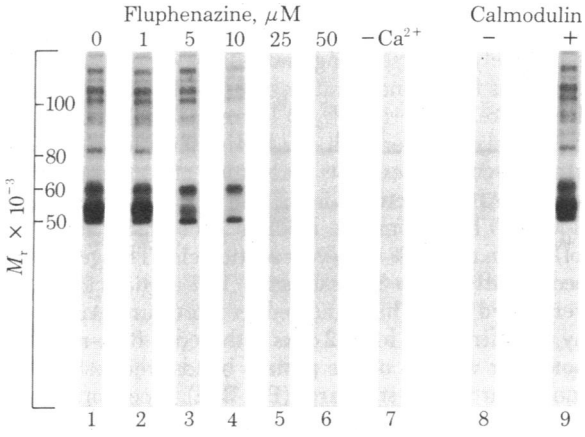


FIG. 5. Autoradiographs showing the effects of fluphenazine on the phosphorylation of synaptosomal cytosolic proteins in the presence of 50 μM $CaCl_2$ (lanes 1–6). Lane 7, incorporation of phosphate into synaptosomal cytosolic proteins in the absence of calcium. Lanes 8 and 9, phosphorylation of these same proteins in the presence of $CaCl_2$ (50 μM) after removal of calmodulin (lane 8) and removal of calmodulin followed by addition of exogenous calmodulin (0.5 μg) (lane 9). Calmodulin was removed from synaptosomal cytosol and prepared from whole brain by affinity chromatography on fluphenazine-Sepharose.

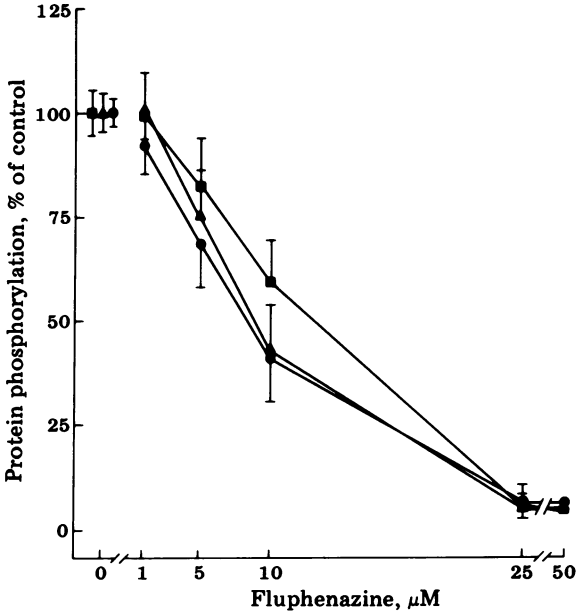


FIG. 6. Phosphorylation of specific synaptosomal cytosolic proteins as a function of fluphenazine concentration. Calcium was included in the standard incubation mixture at a final concentration of 50 μM. ■, p60 band; ▲, p55 band; ●, p50 band.

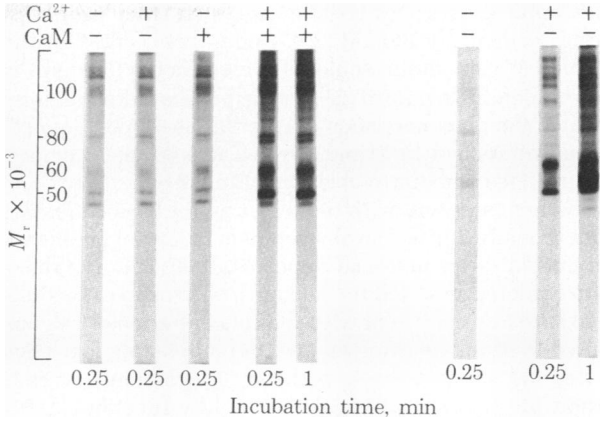


FIG. 7. Autoradiographs showing the phosphorylation of synaptic plasma membrane proteins (*Left*) and synaptosomal cytosolic proteins (*Right*) under various conditions. Where indicated, synaptic plasma membranes were incubated in the presence of 100 μM $CaCl_2$ with or without 0.5 μg of calmodulin (CaM). Synaptosomal cytosol was incubated in the absence or presence of 50 μM $CaCl_2$.

missing from the chromatographed cytosol; (*ii*) subsequent washing of the column with EGTA eluted a protein whose electrophoretic mobility and biological activity (assayed as described) (2) were identical to those of native calmodulin; and (*iii*) when the chromatographed cytosol was treated with heat and assayed for the presence of calmodulin, no activity was observed. That calmodulin was required for the calcium-stimulated phosphorylation of synaptosomal cytosol is evident from the autoradiographs in Fig. 5. When the chromatographed cytosol was assayed for phosphorylation in the presence of calcium, the levels of phosphorylation could not be distinguished from those observed in the absence of calcium (Fig. 5, compare lanes 7 and 8). However, phosphorylation could be restored to control levels by the addition of calmodulin (0.5 μg) to the chromatographed cytosol (Fig. 5, compare lanes 1 and 9).

**Calcium-Regulated Phosphorylation: Comparison Between Phosphoprotein Substrates in Synaptic Plasma Membranes and Synaptosomal Cytosol.** Synaptic plasma membranes were phosphorylated in the presence and absence of calcium and calmodulin (Fig. 7). As has been reported (2), the addition of calcium to the membranes did not alter the pattern of phosphorylation. When calmodulin was added to the assay, it resulted in a calcium-dependent phosphorylation of several membrane proteins as previously described (2). Several calcium- and calmodulin-regulated phosphoproteins from synaptic plasma membranes had electrophoretic mobilities that matched those observed for synaptosomal cytosol. The phosphoproteins common to both membrane and cytosol fractions included bands with apparent $M_r$ of 50,000 and 60,000; however, the p55 band observed in synaptosomal cytosol was not observed in synaptic plasma membranes at incubation times of up to 2 hr. In the presence of calcium and calmodulin, maximal phosphorylation of the membrane proteins was reached within 15 sec instead of the 60 sec required for maximal incorporation of phosphate into cytosol proteins.

## DISCUSSION

We have demonstrated that calcium regulates the phosphorylation of specific synaptosomal cytosolic proteins from rat striatum and that this effect has an absolute requirement for the heat-stable calcium-binding protein, calmodulin. The effects of calcium on phosphorylation were large in magnitude and both time and concentration dependent. Findings similar to these have been reported previously for the regulation of the phosphorylation of synaptic plasma membranes (2) and synaptic vesicles (3). Additionally, while the present work was in progress, Yamauchi and Fujisawa (16) reported that the phosphorylation of whole-brain cytosol was calcium dependent and could be influenced by the addition of calmodulin. Thus, our observations serve as one more example of the expanding number of biochemical reactions that are regulated by calmodulin.

Levin and Weiss (17) have demonstrated that a number of antipsychotic drugs bind to calmodulin with high affinity in a calcium-dependent fashion. In the present investigation, we used the phenothiazine antipsychotic, fluphenazine, in an attempt to demonstrate that calmodulin was required for the calcium-regulated phosphorylation of synaptosomal cytosolic proteins. Two approaches were chosen. In the first, fluphenazine was added to the phosphorylation assay to serve as a calmodulin-specific antagonist of the phosphorylation reaction. In the second, fluphenazine was linked to a Sepharose matrix in order to extract calmodulin from synaptosomal cytosol. We have previously shown this technique to be effective in removing calmodulin from brain extracts (18). Fluphenazine *in vitro* proved to be completely effective in antagonizing the effects of calcium on phosphorylation, and fluphenazine-Sepharose, which removed calmodulin from synaptosomal cytosol, resulted in a loss of calcium-stimulated phosphorylation. Exogenous calmodulin added to synaptosomal cytosol after fluphenazine-Sepharose chromatography restored the effects of calcium on phosphorylation, suggesting that fluphenazine does indeed block calcium-regulated phosphorylation by binding to endogenous calmodulin. The results of the studies using fluphenazine, combined with the ability of EGTA to block calcium-stimulated phosphorylation, satisfy most of the criteria for calmodulin-regulated reactions that have been proposed by Cheung (19).

The approximate steady-state levels of calcium in the "resting" cell are 10–100 nM (20). Under these conditions, all intracellular calmodulin would be present in the biologically inactive calcium-free form (21). In the present study, calcium stimulated the phosphorylation of specific cytosol proteins only at a concentration of 1 $\mu$M and above. These values encompass those associated with the soluble compartment of "excited" cells (20) and are consistent with those that might be encountered intraneuronally during the development of a presynaptic calcium current after neuronal depolarization (22). The dissociation constants reported for calcium's binding to calmodulin (20, 21) are also consistent with the concentration of calcium required to stimulate cytosolic protein phosphorylation. Thus, our findings support the hypothesis that calcium serves to transmit information within the cytosol by functioning as a second messenger when bound to specific soluble proteins such as calmodulin (20). That a calcium-regulated phosphorylation system in neuronal cytosol may play an important role in synaptic function is indicated by the findings that enzymes that control the biosynthesis of neurotransmitters (e.g., tyrosine hydroxylase and tryptophan hydroxylase) are activated under phosphorylating conditions (23, 24) and can be regulated in a calcium-dependent (24, 25) or even a calmodulin-dependent (18, 26) manner.

Of the several synaptosomal cytosolic proteins that were phosphorylated in the presence of calcium, three proteins, designated the p50, p55, and p60 bands, were the most highly phosphorylated. The magnitude of the incorporation of phosphate into these proteins in response to the addition of calcium reached levels between 15 and 23 times those of control. These values were in sharp contrast to the small (2-fold) cAMP-induced increases in the phosphorylation of a specific cytosolic protein (data not shown) and were much greater than the 3- to 6-fold increases in the phosphorylation of specific synaptic membrane substrates that have been observed in the presence of cAMP (27). The gradual return to control levels of phosphorylation for the p50, p55, and p60 bands was indicative of the presence of endogenous phosphatases, which *in vivo* would be expected to limit the extent and duration of the phosphorylation reactions. The distinct time-effect relationships for the phosphorylation of the p50, p55, and p60 bands may reflect differences in substrate specificity for either protein kinases or phosphatases. However, the net incorporation of phosphate into any of the soluble substrates that we have detected may be a function not only of the activities of particular kinases or phosphatases (or both) but also of the endogenous inhibitors (28, 29) that affect these enzyme systems. Furthermore, because the determinants of substrate specificity for protein kinases may reside in elements of both primary structure (30) and conformational flexibility (31), the isolation and chemical and physical characterization of each phosphoprotein substrate would seem to be prerequisite for the clarification of the role of these proteins in neuronal function.

With respect to only the p50 and p60 bands, phosphoproteins with similar electrophoretic mobilities have been observed in preparations of synaptic vesicles (3) and synaptic plasma membranes (2) (see Fig. 4). Calcium and calmodulin also appear to be required for the phosphorylation of these proteins (2). These observations raise the possibility that substrates of a specific calmodulin-regulated protein kinase(s) may be common to synaptic vesicles, synaptic plasma membranes, and synaptosomal cytosol. If the p50 and p60 proteins are common to both particulate and cytosolic fractions, it will be necessary to establish their relative distribution and respective degree of phosphorylation in these two compartments in order to further understand their role in synaptic function. In contrast, the p55 band present in synaptosomal cytosol was not observed in the synaptic plasma membrane fraction, even at an incubation time that corresponded to the maximal incorporation of phosphate into this protein. Identification of these phosphoproteins and their corresponding protein kinase(s) will be necessary in order to correlate the degree of phosphorylation with the functional state of these specific proteins.

J.P.O'C. is a Staff Fellow, Pharmacology Research Associate Program, National Institute of General Medical Sciences, National Institutes of Health.

1. DeLorenzo, R. J., Emple, G. P. & Glaser, G. H. (1977) *J. Neurochem.* **28,** 21–30.
2. Schulman, H. & Greengard, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5432–5436.
3. DeLorenzo, R. J., Freedman, S. D., Yohe, W. B. & Maurer, S. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1838–1842.
4. Forn, J. & Greengard, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5195–5199.
5. Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.* **252,** 2764–2773.
6. Sieghart, W., Forn, J. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2475–2479.
7. Browning, M., Bennett, W. & Lynch, G. (1979) *Nature (London)* **278,** 273–275.
8. Takai, Y., Kishimoto, A., Inoue, M. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252,** 7603–7609.
9. Egrie, J. C., Campbell, J. A., Flangas, A. L. & Siegel, F. L. (1977) *J. Neurochem.* **28,** 1207–1213.
10. Sundberg, L. & Porath, J. (1974) *J. Chromatogr.* **90,** 87–98.
11. Charbonneau, H. & Cormier, M. J. (1979) *Biochem. Biophys. Res. Commun.* **90,** 1039–1047.
12. Klee, C. B. (1977) *Biochemistry* **16,** 1017–1024.
13. Jones, D. H. & Matus, A. I. (1974) *Biochim. Biophys. Acta* **356,** 276–287.
14. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
15. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
16. Yamauchi, T. & Fujisawa, H. (1979) *Biochem. Biophys. Res. Commun.* **90,** 1172–1178.
17. Levin, R. M. & Weiss, B. (1979) *J. Pharmacol. Exp. Ther.* **208,** 454–459.
18. Kuhn, D. M., O'Callaghan, J. P., Juskevich, J. C. & Lovenberg, W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4688–4691.
19. Cheung, W. Y. (1980) *Science* **207,** 19–27.
20. Kretsinger, R. H. (1979) *Adv. Cyclic Nucleotide Res.* **11,** 1–26.
21. Wolff, D. J., Poirier, P. G., Brostrom, C. O. & Brostrom, M. A. (1977) *J. Biol. Chem.* **252,** 4108–4117.
22. Blaustein, M. P., Ratzlaff, R. W. & Kendrick, N. K. (1978) *Ann. N.Y. Acad. Sci.* **307,** 195–212.
23. Ames, M. W., Lerner, P. & Lovenberg, W. (1978) *J. Biol. Chem.* **253,** 27–31.
24. Kuhn, D. M., Vogel, R. L. & Lovenberg, W. (1978) *Biochem. Biophys. Res. Commun.* **82,** 759–766.
25. Simon, J. R. & Roth, R. H. (1979) *Mol. Pharmacol.* **16,** 224–233.
26. Yamauchi, T. & Fujisawa, H. (1979) *Biochem. Biophys. Res. Commun.* **90,** 28–35.
27. Ueda, T., Maeno, H. & Greengard, P. (1973) *J. Biol. Chem.* **248,** 8295–8305.
28. Szmigielski, A., Guidotti, A. & Costa, E. (1977) *J. Biol. Chem.* **252,** 3848–3853.
29. Khandelwal, R. L. & Zinman, S. M. (1978) *J. Biol. Chem.* **253,** 560–565.
30. Kemp, B. E., Bylund, D. B., Huang, T.-S. & Krebs, E. G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3448–3452.
31. Bylund, D. B. & Krebs, E. G. (1975) *J. Biol. Chem.* **250,** 6355–6361.