(2)

# AD-A183 309 REPORT DOCUMENTATION PAGE



| | |
|---|---|
| Unclassified | 1b. RESTRICTIVE MARKINGS: None |
| 2a. SECURITY CLASSIFICATION AUTHORITY | 3. DISTRIBUTION/AVAILABILITY OF REPORT: Unlimited |
| 2b. DECLASSIFICATION/DOWNGRADING SCHEDULE | |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S): F49620-83-K-0035 | 5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 87-0912 |
| 6a. NAME OF PERFORMING ORGANIZATION: Yale University (Sch. Med.) / 6b. OFFICE SYMBOL (If applicable) | 7a. NAME OF MONITORING ORGANIZATION: AFOSR/NL |
| 6c. ADDRESS (City, State and ZIP Code): New Haven, CT 06510 | 7b. ADDRESS (City, State and ZIP Code): Building 410, Bolling AFB, D.C. 20332-6448 |
| 8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR-Life Sciences Contract / 8b. OFFICE SYMBOL (If applicable) | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: F49620-83-K-0035 |
| 8c. ADDRESS (City, State and ZIP Code): AFOSR-Life Sciences Directorate, Department of the Air Force, AFSC, Bolling Air Force Base, DC 20332-6448 | 10. SOURCE OF FUNDING NOS. |

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO. | WORK UNIT NO. |
|---|---|---|---|
| 61102F | 2312 | A5 | |

11. TITLE (Include Security Classification): The Effects of Hydrazine and Related Compounds on Calcium-Calmodulin Regulated Synaptic Processes

12. PERSONAL AUTHOR(S): Robert J. DeLorenzo, M. D., Ph. D., M. P. H.

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Yr., Mo., Day) | 15. PAGE COUNT |
|---|---|---|---|
| Final | FROM 11/83 TO 85/8/14 | July 10, 1986 | |

16. SUPPLEMENTARY NOTATION

| 17. COSATI CODES | | |
|---|---|---|
| FIELD | GROUP | SUB. GR. |
| | | |
| | | |

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): Neuronal Excitability, Hydrazine, Calcium, Calmodulin Kinase ~~II~~, Cytoskeleton.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Results from this research effort have provided insights into the effects of hydrazines on calcium calmodulin regulated synaptic processes. The effects of hydrazines on the polarization-dependent calcium uptake into synaptazomes was studied. Initial studies performed by others suggested that hydrazines alter the depolarization-dependent calcium uptake by synaptozomes. After a detailed study to confirm these results and to look at the molecular mechanisms involved, we could not reproduce these reported effects of hydrazine on calcium fluxes. Hydrazine in concentrations as high as 100 micromolar did not have any significant effect on potassium stimulated calcium uptake into synaptazomes. Thus, our conclusion was that under physiological conditions used to study transmitter release, calcium uptake and protein phosphorylation in our laboratory, we did not see hydrazine effecting the calcium entry cross the membrane.

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| 20. DISTRIBUTION/AVAILABILITY OF ABSTRACT | 21. ABSTRACT SECURITY CLASSIFICATION |
|---|---|
| UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS ☐ | Unclassified |

| 22a. NAME OF RESPONSIBLE INDIVIDUAL | 22b TELEPHONE NUMBER (Include Area Code) | 22c. OFFICE SYMBOL |
|---|---|---|
| Lorris G. Cockerham, Lt Col | (202)767-5021 | NL |

DD FORM 1473, 83 APR EDITION OF 1 JAN 73 IS OBSOLETE.

SECURITY CLASSIFICATION OF THIS PAGE

28 MAY 1987

Hydrazine, however, had a dramatic effect on calcium calmodulin-dependent protein phosphorylation in neuronal preparations. As hydrazine concentrations were increased, hydrazine was found to initially stimulate calcium calmodulin-dependent protein phosphorylation in membrane preparations. As hydrazine was increased to high levels beyond 500 micromolar, hydrazine then inhibited CaM Kinase II activity. These effects were studied and the concentration curve was calculated. Hydrazine seemed to have a direct effect on the kinase and was not indirectly effecting phosphatase activity in these preparations. Further studies were planned to study these effects on purified calmodulin-dependent protein kinase from brain. These results provide an important insight into the potential neurotoxic effect of hydrazines. Calmodulin-dependent protein phosphorylation has been implicated in mediating many of the effects of calcium on neuronal function. The fact that hydrazine can alter the activity of this kinase may provide a molecular insight into some of its toxic effects on neuronal function. This is an important area for further investigation and may have clinical implications in preventing some of the toxic effects of hydrazines on the nervous system.

**Final Scientific Report**

**Title:** **THE EFFECTS OF HYDRAZINE AND RELATED COMPOUNDS ON CALCIUM-CALMODULIN REGULATED SYNAPTIC PROCESSES**

**Robert J. DeLorenzo, M.D., Ph.D., M.P.H. - Principal Investigator**

**Period:** 8/1/83 - 6/30/85

**DoD Number:** AFOSR-F4920-83-K-0035
Department of the Air Force

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**Final Report: AFOSR-F4920-83-K-0035**

**Period: 8/1/83 - 6/30/85**

# THE EFFECTS OF HYDRAZINE AND RELATED COMPOUNDS ON CALCIUM-CALMODULIN REGULATED SYNAPTIC PROCESSES

## PROGRESS REPORT

### A. Objectives

The overall objectives of this study were to determine the role of calcium and calmodulin-stimulated protein phosphorylation in mediating neuronal excitability and plasticity and how hydrazine may regulate these effects of calcium. Previous results from our laboratory have suggested that this important phosphorylation system in brain may modulate the effects of calcium and phenytoin on neurotransmitter release, and thus may play an important role in the regulation of the seizure threshold. Thus, if hydrazine regulated this important enzyme or other calcium regulated processes, it would provide an important insight into the biochemical effects of hydrazine on neuronal tissue.

To test our hypothesis, we studied calcium-dependent protein phosphorylation in several in vitro and in vivo model systems that have been developed in this laboratory. We correlated the effects of calcium and hydrazine on protein phosphorylation with their effects on neurotransmitter release and morphological changes in several preparations.

## B. Research Progress During This Grant Period

Research during this grant was directed at understanding the molecular action of hydrazine and how it related to calcium-dependent protein phosphorylation and neurotransmitter release in presynaptic nerve terminal and synaptic vesicle preparations. Identification of a and b tubulin as major protein components of DPH-L and DPH-M was accomplished and the correlation of tubulin phosphorylation with neurotransmitter release was investigated.

We accomplished the major goals of our initial project application. We were successful in studying the role of calcium and calmodulin processes in mediating synaptic excitability. We also determined the effects of hydrazine on specific phosphorylation systems. We did not confirm previous studies from Bern's group that hydrazines inhibited voltage dependent calcium uptake in intact nerve terminal preparations. These results provided a major insight into the effects of hydrazine on calcium regulated processes. The results from this research are summarized below.

### 1. The Calmodulin Hypothesis of Neurotransmission

Results obtained in this research effort over the last two years have provided significant evidence that calmodulin mediates some of calcium's effects on synaptic function. The effects

of calcium on synaptic protein phosphorylation, synaptic vesicle neurotransmitter release, and vesicle-membrane interactions were shown to require the calcium binding protein, calmodulin. The calmodulin hypothesis for neurotransmission developed from these studies states that as $Ca^{2+}$ enters the presynaptic nerve terminal during depolarization, it binds to a high affinity $Ca^{2+}$ receptor protein, calmodulin, and initiates several $Ca^{2+}$-calmodulin dependent biochemical processes that modulate synaptic activity. The anticonvulsants phenytoin, carbamazepine, and the benzodiazepines have been shown to inhibit the target sites of several $Ca^{2+}$-calmodulin regulated synaptic processes.

To implicate calmodulin in synaptic function, it is necessary to demonstrate that calmodulin is present at the synapse. A vesicle-bound heat stable protein was isolated from highly enriched preparations of synaptic vesicles from rat cortex that had the same molecular weight as calmodulin in this project. This vesicle bound protein could be removed from the vesicles in the presence of EGTA and was found to bind $Ca^{2+}$ at micromolar concentrations. When compared to calmodulin isolated from whole rat brain, the vesicle-$Ca^{2+}$ binding protein was found to be identical to calmodulin in molecular weight, amino acid composition, isoelectric point, and in its ability to stimulate vesicle protein kinase, adenylate cyclase and phosphodiesterase activity. Vesicle calmodulin represented 0.92% of the total protein in synaptic vesicle fraction.

A heat-stable, $Ca^{2+}$-binding protein, was also isolated from nerve terminal synaptoplasm prepared by standard procedures. This synaptic protein was found to be identical to whole brain calmodulin in molecular weight, isoelectric point, amino acid composition and ability to activate adenylate cyclase and phosphodiesterase. Synaptic CaM comprised 0.71% of the total protein in the synaptoplasm preparation. Because the concentration of CaM in whole brain fractions is approximately 1% of the total brain protein, the high percentage of CaM in

synaptoplasm and synaptic vesicle fractions strongly indicates the presence of this $Ca^{2+}$-receptor protein in the presynaptic nerve terminal. Calmodulin was isolated and characterized from postsynaptic density preparations. The ability to isolate CaM from highly enriched preparations of pre- and postsynaptic fractions strongly indicates that CaM is a trans-synaptic protein. Therefore, CaM is very well suited to mediate the effects of $Ca^{2+}$ on both the pre- and postsynaptic sides of the synapse.

## 2. Calmodulin and $Ca^{2+}$-Dependent Neurotransmitter Release

Since the evidence indicates that CaM is present at the synapse, this $Ca^{2+}$-receptor protein is a possible presynaptic protein for modulating the effects of $Ca^{2+}$ on neurotransmitter release and turnover. As $Ca^{2+}$ enters the presynaptic ending, it can bind to this high-affinity receptor and initiate several biochemical processes involved in synaptic function. Initial evidence for a role of calmodulin involvement in neurotransmitter release was developed employing preparations of intact synaptosomes and isolated synaptic vesicles.

### a. Synaptic Vesicle Studies

A more physiological procedure for isolating synaptic vesicles has been developed in this research effort. Vesicles from this isolation procedure were shown to be much more responsive to $Ca^{2+}$ than vesicles prepared under the standard hypotonic isolation methods. $Ca^{2+}$ in the presence of ATP and $Mg^{2+}$ simultaneously initiated the release of vesicle neurotransmitter substances vesicle protein phosphorylation, and vesicle and membrane interactions. The $Ca^{2+}$ responsive synaptic vesicle preparation was then studied to determine if calmodulin mediated the effects of $Ca^{2+}$ on vesicle neurotransmitter release.

The vesicles prepared under more physiological procedures that simulated the intracellular environment also contained calmodulin. The calmodulin in the vesicle preparation was tightly bound to the vesicle surface and could be selectively removed by washing the vesicles with the $Ca^{2+}$ chelating agent, EGTA. Thus, it was possible to obtain preparations of calmodulin containing (plain vesicles) and calmodulin depleted (treated vesicles) vesicles. The calmodulin depleted vesicle fractions were then studies for neurotransmitter release (Table 1).

$Ca^{2+}$ in the presence of calmodulin stimulates the release of norepinephrine and acetylcholine from calmodulin depleted vesicles ( Table 1). $Ca^{2+}$ or calmodulin alone, however, had no significant effect on neurotransmitter release ( Table 1). Trifluoperazine, a phenothiazine that inactivates calmodulin, also inhibited $Ca^{2+}$-calmodulin stimulated vesicle neurotransmitter release (Table 1). The calmodulin kinase inhibitors, phenytoin and diazepam, were found to inhibit $Ca^{2+}$-calmodulin stimulated vesicle neurotransmitter release (Table 1). The $Ca^{2+}$-calmodulin stimulation of release was also shown to be dependent on $Mg^{2+}$ and ATP and vesicles prepared under hypotonic conditions did not show significant $Ca^{2+}$-calmodulin stimulated release of neurotransmitter substances.

b. Intact Synaptosome Studies

Although vesicle preparations offer several advantages for studying the effects of $Ca^{2+}$ on vesicular transmitter release, it is important to correlate the results from the isolated vesicle fractions with data obtained from neurotransmitter release studies of intact nerve terminal preparations. Isolated intact nerve terminals (synaptosomes) are excellent preparations for studying the effects of $Ca^{2+}$ and membrane depolarization on neurotransmitter release.

In this study we have employed the intact synaptosome system to study the role of CaM in neurotransmitter release as summarized in Table 2. The disadvantage of the synaptosome system for studying the effects of CaM on neurotransmitter release is that is not yet possible to remove CaM from the synaptosome without destroying the viability of the preparation. However, various pharmacological inhibitors of CaM (e.g., trifluoperazine) and $Ca^{2+}$-CaM protein kinase activity (e.g., phenytoin and diazepam), which inhibit $Ca^{2+}$-CaM release in vesicle preparations, have been used to probe the possible involvement of CaM in neurotransmitter release from intact nerve terminals.

Conditions that induce $Ca^{2+}$ entry by the depolarization of the synaptosome membrane (e.g., high K+ or veratridine or by producing $Ca^{2+}$ channels (e.g., $Ca^{2+}$ ionophore A23187) caused significant synaptosomal release of norepinephrine and acetylcholine (Table 2). This increased release of neurotransmitter substances produced by both elevated K+ and A23187 was significantly inhibited by trifluoperazine (Table 2). these results suggest that inhibition of CaM by trifluoperazine blocks the release process. However, it is not possible from these experiments to determine if trifluoperazine is inhibiting release by blocking $Ca^{2+}$ uptake or by inhibiting a specific $Ca^{2+}$-regulated process within the nerve terminal.

To test these possibilities, the effects of trifluoperazine on $Ca^{2+}$ uptake (Table 2) was investigated. Trifluoperazine inhibits the depolarization-dependent uptake of $Ca^{2+}$ into intact synaptosomes induced by both elevated K+ and veratridine. However, the $Ca^{2+}$ uptake produced by A23187 was not inhibited by trifluoperazine. This data indicated that trifluoperazine inhibits release in two ways: (1) by inhibiting depolarization-dependent $Ca^{2+}$ uptake, and (2) by blocking a $Ca^{2+}$-regulated process that modulates release even when $Ca^{2+}$ is entering the nerve terminal in the presence of A23187.

The anticonvulsant, phenytoin, and the benzodiazepine, diazepam, also blocked norepinephrine and acetylcholine release from intact synaptosomes produced by A23187 under conditions where they do not block $Ca^{2+}$ uptake, (Table 2). These inhibitors of CaM kinase activity also blocked the $Ca^{2+}$-CaM-dependent release of neurotransmitter substances from isolated vesicles, further suggesting that CaM is involved in neurotransmission. Studies from both isolated vesicles and intact synaptosome preparations indicate that CaM may act as a $Ca^{2+}$ receptor mediating some of the effects of $Ca^{2+}$ on neurotransmission.

3. Effects of Hydrazine on Depolarization Dependent Calcium Uptake

To study the effects of hydrazine on calcium uptake into nerve terminals, intact nerve terminal preparations (synaptosomes) were prepared and employed in this study. Previous reports by Bern's group indicated that hydrazine blocked voltage dependent calcium uptake. Over twenty experiments were conducted with intact synaptosomes employing $Ca^{45}$. The synaptosomes were depolarized with elevated potassium. Under these conditions, depolarization caused significant uptake of $Ca^{45}$ (Table 2).

In the presence of hydrazine, we studied $Ca^{45}$ uptake. Hydrazine in concentrations from $10^{-1}$ to $10^{-2}$ had no significant effect on calcium uptake (Table 2). Some inhibition modification was seen at the higher concentrations, but it was not reproducible. Thus, it was our conclusion from these results, that hydrazine did not significantly alter calcium fluxes in synaptosome preparations. However, further experimentation with other neuronal systems should be completed before any firm conclusions are drawn.

## 4. A Molecular Approach to the Actions of Calcium and Calmodulin on Neurosecretion

The evidence presented previously suggest that $Ca^{2+}$-CaM regulated synaptic biochemical processes may regulate the effect of $Ca^{2+}$ on synaptic activity. Therefore, it is important to determine which CaM-regulated enzyme systems are involved in specific aspects of synaptic function. Evidence from several laboratories has suggested that $Ca^{2+}$-calmodulin regulated protein phosphorylation may play a role in regulating the release of neurotransmitter substances. Studies from Puszkin's laboratory have indicated that $Ca^{2+}$-calmodulin may regulate vesicle membrane interactions through alteration of membrane lipid environments. Although the precise biochemical mechanisms mediating the effects of $Ca^{2+}$ on release are not known, research is now being focused on several promising areas.

## 5. $Ca^{2+}$-Stimulated Protein Phosphorylation

Calcium-stimulated protein phosphorylation in brain was initially described in whole brain homogenates and highly enriched preparations of synaptosomes. These results demonstrated that $Ca^{2+}$-stimulated the endogenous phosphorylation of many brain proteins, but particularly proteins in the 10,000-20,000, 50,000-45,000, 60,000-64,000, and 150,000-300,000 molecular weight ranges. Two protein bands with molecular weights of 52,000-54,000 and 60,000-64,000 (proteins DPH-M and DPH-L, respectively) were of particular interest because they were most dramatically stimulated by $Ca^{2+}$ and inhibited by phenytoin, an anticonvulsant that blocks several $Ca^{2+}$-dependent processes, including neurotransmitter release. The phosphorylation of synaptosomal proteins DPH-L and DPH-M was also enriched in synaptic vesicle fractions prepared from intact synaptosomes. These results demonstrated that the $Ca^{2+}$-stimulated phosphorylation observed in synaptosome fractions was occurring within the synaptosomes and at in some other membrane contaminations in the preparation.

An hypothesis was developed from these findings, suggesting that $Ca^{2+}$-dependent protein phosphorylation (a new phosphorylation system distinct from cyclic AMP kinases) may regulate the effects of $Ca^{2+}$ on synaptic function and neurotransmitter release. The $Ca^{2+}$-dependent pattern of endogenous protein phosphorylation has been observed by other researchers in several isolated brain fractions and in preparations of other tissues such as the adrenal medulla and the electric organ of Torpedo.

Depolarization-dependent $Ca^{2+}$ uptake in intact synaptosomes was shown to stimulate the phosphorylation of an 80,000-dalton protein (protein I) in intact synaptosomes. The levels of phosphorylation of proteins with identical molecular weights to proteins DPH-L and DPH-M seen in isolated synaptosomes were shown to be stimulated in intact synaptosomes by depolarizing conditions that stimulated $Ca^{2+}$ entry and simultaneously initiated neurotransmitter release from intact synaptosomes. The phosphorylation of these proteins in intact preparations was also shown to be occurring in the synaptic vesicle, synaptic membrane, and postsynaptic density preparations from these intact fractions. These results provided the first evidence that the depolarization-dependent phosphorylation of specific proteins in intact synaptosome preparations was actually occurring within the synaptosomes and not associated with other contaminants in the synaptosome preparations. The level of phosphorylation of MW 50,000-60,000 proteins also correlate with norepinephrine release in intact adrenal medulla cells.

6. Effects of Hydrazine on Protein Phosphorylation

Since hydrazine did not effect voltage gated calcium-uptake in intact nerve terminal preparations, we directed our efforts at studying other calcium-regulated processes that might be effected by hydrazine. Initially in this grant period, we studied the effects of hydrazine on rat brain protein phosphorylation. The results of these experiments are provided below.

The effects of hydrazine was similar to trifluoperizine or dilantin in its ability to inhibit protein phosphorylation.

7. Effect of Hydrazine on the Endogenous Phosphorylation of Rat Brain Proteins

When homogenate preparations were incubated under standard assay conditions in the presence and absence of hydrazine (HDZ) and subjected to acrylamide gel electrophoresis and autoradiography, it was found that HDZ significantly reduced the net level of phosphorylation of two specific bands on the autoradiograph (Fig. 1). The radioactive bands affected by DPH were demonstrated to be associated with ($^{32}$P) phosphoprotein by the following methodology. Treatment of phosphorylated brain protein with protease completely abolished both the protein staining pattern and the associated radioactivity seen on the autoradiograph of the DPH-affected bands. Conversely, treatment with ribonuclease A had no effect on the protein staining pattern, the autoradiograph, or the effect of HDZ. Triple lipid extraction with ethanol-ether (3:1) of the labelled homogenate protein and hydrolysis of DNA by boiling acidified (10%TCA) labelled homogenate, had no significant effect on the protein staining pattern, the radioactivity of the bands or the effect of HDZ. The results of these experiments indicate that both of the HDZ specific radioactive bands were associated with protein. These bands are clearly designated in Fig. 1. The percent inhibition by HDZ under standard conditions of the endogenous phosphorylation of protein DPH-L was consistently greater than that of protein DPH-M. The terms endogenous phosphorylation and dephosphorylation are employed to indicate the increase or decrease in ($^{32}$P) phosphate content of individual protein bands, and not to describe the activity of protein kinase or protein phosphatase. The net level of ($^{32}$P) phosphate incorporated into each protein represents a balance between protein kinase and protein phosphatase activity.

Proteins DPH-L and DPH-M were the only two proteins with phosphorylation consistently affected by HDZ under standard reaction conditions. However, increasing the HDZ concentration produced inhibition of other phosphoproteins. Results qualitatively similar to those shown in Fig. 1 were observed in 30 experiments with rat brain homogenates. The pattern of endogenous phosphorylation and the effect of HDZ were the same when either male or female rats were employed as the source of brain protein. Freezing of the homogenate preparations at -20°C for less than one week caused approximately a 10 per cent reduction in phosphorylation of proteins DPH-L and DPH-M. Protein DPH-L seemed more sensitive to storage at -20°C. Prolonged storage for more than two weeks caused a substantial decrease in the effect of HDZ on the phosphorylation of these proteins. Some variation was observed in the percent decrease in phosphorylation of proteins DPH-L and DPH-M in different homogenate preparations, but the effect of HDZ was always consistently observed.

8. Effect of Varying the Concentration of DPH

The effects of varying the concentration of hydrazine on the endogenous phosphorylation of proteins DPH-L and DPH-M is shown in Fig. 2. The concentration of HDZ required to produce a half-maximal decrease in the level of phosphorylation of proteins DPH-L and DPH-M were estimated from the curves in fig. 2 to be $4x10^{-3}$M and $6x10^{-3}$, respectively. The phosphorylation of both proteins could be almost completely inhibited in the presence of concentrations of HDZ greater $1x10^{-2}$M. The minimum HDZ concentration for decreasing the level of phosphorylation of proteins DPH-L and DPH-M greater than 80 per cent were $8x10^{-3}$M and $1x10^{-2}$ respectively.

Effect of varying the concentration of ATP. The effects of HDZ on the amount of ($^{32}$P) phosphate incorporated into proteins DPH-L and DPH-M were independent of ATP

concentration over the range from 0.11 uM to 50uM (Fig. 3). These results demonstrate that the net decrease in phosphorylation of these two proteins was not due to a competition between HDZ and ATP. The results further suggest that the effect of HDZ on the endogenous phosphorylation of these two proteins is not attributable to an indirect action of HDZ, causing a decrease in the concentration of ATP.

9. Time Course of Endogenous Phosphorylation of Proteins DPH-L and DPH-M

Hydrazine decreased both the initial rate and net level of phosphorylation of proteins DPH-L and DPH-M under standard reaction conditions (Fig. 4). The maximal levels of phosphorylation of proteins DPH-L and DPH-M were reached in 60 and 40 seconds, respectively. The level of phosphorylation of both proteins decreased very gradually after reaching maximal levels. Figure 4 also demonstrates the effect of toxic levels of HDZ on the time course of phosphorylation of proteins DPH-L and DPH-M. Both the initial rates and net levels of phosphorylation were significantly reduced at this higher concentration ($1x10^{-2}$M).

10. The Effect of Calcium and Magnesium on the Phosphorylation of Proteins DPH-L and DPH-M

In the absence of both magnesium and calcium there was no significant incorporation of ($^{32}$P) phosphate into proteins DPH-L and DPH-M. In the presence of magnesium alone, protein DPH-M was phosphorylated to near maximal levels, while protein DPH-L was only phosphorylated approximately 50 per cent of standard conditions. When EGTA (2mM) was employed to chelate any endogenous calcium in the homogenate preparation, magnesium alone was not sufficient to support the phosphorylation of proteins DPH-L and DPH-M. Both calcium and magnesium were required to maximally incorporate ($^{32}$P) phosphate into proteins

DPH-L and DPH-M. HDZ had no effect on the phosphorylation of proteins DPH-L and DPH-M in the absence of calcium ions. The effect of HDZ on the phosphorylation of these proteins was dependent upon the presence of calcium ions (see Table 2).

11. Antagonistic Actions of Hydrazine and Calcium

To further correlate the action of HDZ on protein phosphorylation with its effects on neuronal tissue, the relationship of calcium concentration to the HDZ induced changes in protein phosphorylation was studied in rat brain homogenates. The opposing actions of HDZ and calcium on the level of ($^{32}P$) phosphate incorporation into specific brain proteins has been demonstrated in this laboratory, by employing the techniques of acrylamide ge electrophoresis and autoradiography.

The effects of HDZ in the presence and absence of calcium on the pattern of endogenous phosphorylation of proteins in homogenates of rat cerebrum are shown in Fig. 5. When homogenate-(EGTA preparations were incubated under standard conditions in the presence and absence of HDZ, it was observed that HDZ significantly reduced the net level of phosphorylation of two specific bands on the autoradiograph in the presence of calcium ions (10mM). DPH had no apparent effect on the endogenous phosphorylation of brain proteins in the absence of calcium. The two bands affected by HDZ have been shown to be phosphoprotein in nature.

The effect of calcium on the pattern of endogenous phosphorylation of proteins from rat brain homogenates is also shown quantitatively in Fig. 5. In the presence of calcium ions, the incorporation of ($^{32}P$) phosphate into proteins DPH-L and DPH-M was markedly stimulated. It can be clearly seen from Fig. 5 that in the absence of calcium the endogenous phosphorylation

of proteins DPH-L and DPH-M was almost completely reduced to zero. Calcium also stimulated the phosphorylation of other proteins, but the most dramatic effect of calcium was observed in the DPH-specific proteins. These effects of calcium on protein phosphorylation were observed over a side range of ATP concentrations, and thus also were independent of ATP concentration.

## 12. Calmodulin Mediation of the Effects of Ca2+ on Protein Kinase Activation

$Ca^{2+}$-stimulated endogenous membrane protein phosphorylation was shown to be dependent on CaM in crude preparations of brain membrane and in several other tissues. Calmodulin was subsequently demonstrated to mediate the effect of $Ca^{2+}$ on the phosphorylation of specific synaptic vesicle proteins. Calmodulin was also shown to modulate the effects of $Ca^{2+}$ on the endogenous phosphorylation of highly enriched synaptic membrane, synaptic junctional complex, and postsynaptic density preparations. Furthermore, depolarization-dependent $Ca^{2+}$ uptake by intact synaptosomes was shown to simultaneously stimulate the release of neurotransmitter substances and the phosphorylation of specific (especially proteins DPH-L and DPH-M) synaptic vesicle, synaptic membrane, synaptic junctional and postsynaptic density proteins isolated from the intact synaptosomes following depolarization-dependent $Ca^{2+}$ uptake.

## 13. Summary

In summary, evidence from this research has confirmed with both intact and broken synaptosome fractions the initial observations in isolated synaptosome fractions that $Ca^{2+}$ stimulates the phosphorylation of synaptic proteins. Phosphorylation of DPH-L and DPH-M and possibly other MW 50,000-60,000 proteins appears to be consistently observed and

correlates with neurotransmitter release; however, protein I, low-molecular-weight proteins, and several high-molecular-weight proteins have also been described as serving as endogenous substrates for endogenous the $Ca^{2+}$-CaM kinase activity. Hydrazines effect the calcium stimulated phosphorylation of proteins DPH-L and DPH-M. Preliminary results at the end of this grant period indicated that the effects of toxic concentrations of hydrazine inhibit calmodulin dependent protein phosphorylation.

**Table 1. Effects of Calmodulin and $Ca^{2+}$-Calmodulin Kinase Inhibitors on $Ca^{2+}$-Calmodulin Stimulated Protein Phosphorylation and Neurotransmitter Release in Isolated Synaptic Vesicles**

| Condition | Neurotransmitter Release (%) | | Protein DPH-M |
|---|---|---|---|
| | Acetylcholine | Norepinephrine | Phosphorylation (%) |
| Control | 34 | 38 | 21 |
| $Ca^{2+}$ | 41 | 44 | 25 |
| Calmodulin | 36 | 39 | 22 |
| $Ca^{2+}$ + Calmodulin | 100 | 100 | 100 |
| $Ca^{2+}$ + Calmodulin | | | |
| + Trifluoperazine | 62 | 68 | 55 |
| + Phenytoin | 69 | 72 | 49 |
| + Diazepam | 61 | 73 | 47 |

Calmodulin depleted synaptic vesicles were isolated and studied for neurotransmitter release and protein DPH-M phosphorylation as described previously (DeLorenzo, et al. 1979). The data give the means of 10 determinations and are expressed as percent of the maximally stimulated condition (100%). The largest ± S.E.M. was 5.7. The effects of trifluoperazine (15 μM), phenytoin (80 μM), and diazepam (75 μM) were found to be statistically significant in comparison to maximally stimulated values. $P < 0.001$.

Table 2. Effects of calmodulin and $Ca^{2+}$-calmodulin kinase inhibitors on $Ca^{2+}$ uptake, neurotransmitter release and protein phosphorylation in intact nerve terminal preparations*

| Conditon | $Ca^{2+}$ Uptake,% | Neurotransmitter release,% | | Protein phosphorylation, | |
|---|---|---|---|---|---|
| | | Acetylcholine | Norepinephrine | Whole synaptosomes | Synaptic vesicles |
| Control | - | 45 | 52 | 58 | 39 |
| $Ca^{2+}$ | 40 | 51 | 56 | 61 | 44 |
| $Ca^{2+}$, K | 100 | 100 | 100 | 100 | 100 |
| $Ca^{2+}$, K | | | | | |
| + trifluoperazine | 61 | 63 | 68 | 69 | 61 |
| + phenytoin | 65 | 68 | 70 | 68 | 67 |
| + diazepam | 66 | 64 | 59 | 72 | 67 |
| $Ca^{2+}$, A23187 | 95 | 94 | 98 | 91 | 96 |
| $Ca^{2+}$, A23187 | | | | | |
| + trifluoperazine | 68 | 69 | 73 | 72 | 76 |
| + phenytoin | 73 | 74 | 76 | 73 | 70 |
| + diazepam | 77 | 78 | 75 | 75 | 72 |
| + hydrazine | 98 | 73 | 72 | 80 | 75 |

* Intack synaptosomes were incubated under various conditions after preincubation with $^{32}P$ followed by quantitation of $Ca^{2+}$ uptake, neurotransmitter release and protein DPH-M phosphorylation as described (DeLorenzo, 1981a, 1982). Concentrations of trifluoperazine, phenytoin, and diazepam were 15, 80 and 20 μM, respectively. The data give the means of eight determinations and are expressed as percentage of the maximally stimulated condition (100%). The largest ±SEM was 6.3. The effects of changes produced by all three drugs in comparison to the maximally stimulated condition were statistically significant. $P < 0.001$.

Table 3

Effects of therapeutic and toxic concentrations of DPH on the endogenous phosphorylation of brain protein from rat brain homogenates*

| Band | Approximate Mol. Wt. ( $\times 10^{-3}$) | Toxic Level A | | Toxic Level B | |
|---|---|---|---|---|---|
| | | HDZ ($5 \times 10^{-3}$M) | % Inhibition | HDZ ($5 \times 10^{-2}$M) | % Inhibition |
| Protein DPH-L | 60-63 | 41+ | 59 | 2 | 98 |
| Protein DPH-M | 49-53 | 59 | 41 | 11 | 89 |
| A | 250-300 | 94 | 6 | 62 | 38 |
| B | 180-220 | 92 | 8 | 58 | 42 |
| C | 87-92 | 106 | 6 | 107 | 7 |
| D | 8-12 | 97 | 3 | 67 | 33 |

*Reactions were conducted under standard conditions. Incorporation of [$^{32}$P] phosphate is expressed as percent of control value in the absence of HDZ. Each value represents the mean of results obtained from 3 experiments. The largest range about the mean was 5 and the ranges were omitted for clarity

+percentage of control

Table 4

Effect of Calcium and Magnesium on
the Phosphorylation of Proteins DPH-L and DPH-M*

| | | Protein DPH-L | | Protein DPH-M | |
|---|---|---|---|---|---|
| Preparation | Additions | - HDZ | + HDZ | - HDZ | + HDZ |
| Homogenate | none | 1 + | 1 | 2 | 3 |
| | $MgCl_2$ | 11 | 12 | 33 | 35 |
| | $CaCl_2$ | 0 | 0 | 2 | 2 |
| | $MgCl_2$+$CaCl_2$ | 26 | 11 | 98 | 22 |
| Homogenate + EGTA (2 mm) | none | 0 | 0 | 0 | 0 |
| | $MgCl_2$ | 0 | 0 | 0 | 0 |
| | $CaCl_2$ | 0 | 0 | 0 | 0 |
| | $MgCl_2$+$CaCl_2$ | 28 | 14 | 47 | 19 |

*Reactions were conducted under standard conditions except for the variation of metal ions ($MgCl_2$, 10 mM and $CaCl_2$, 10 mM) and homogenate preparations as indicated. Each value represents the mean from 4 experiments. The largest range about the mean was $\pm$ 4 arbitrary units and thus the ranges were omitted for clarity.

+arbitrary units of $[^{32}P]$ phosphate incorporation.

## Figure 1

Effect of HDZ on the endogenous phosphorylation of rat brain homogenates. The reactions were initiated by the addition of [$\gamma$-$^{32}$P] ATP and conducted under standard conditions for 20 seconds. The concentration of HDZ was $5 \times 10^{-3}$M. The autoradiograph demonstrates the incorporation of [$^{32}$P]phosphate into specific bands in the dried acrylamide gel. HDZ-specific proteins, DPH-L and DPH-M, are labelled for clarity with arrows. The autoradiograph was scanned with a microdensitometer and the density of each band in the presence and absence of HDZ is plotted as a function of distance from the origin and expressed in arbitrary units. The resultant desitogram demonstrates the quantitative effect of HDZ on proteins DPH-L and DPH-M. The molecular weight scale was estimated with known molecular weight markers (See later: Materials and Methods). The results of the experiment illustrated are representative of 15 individual experiments.

Figure 1 on Next Page

**Figure 1**

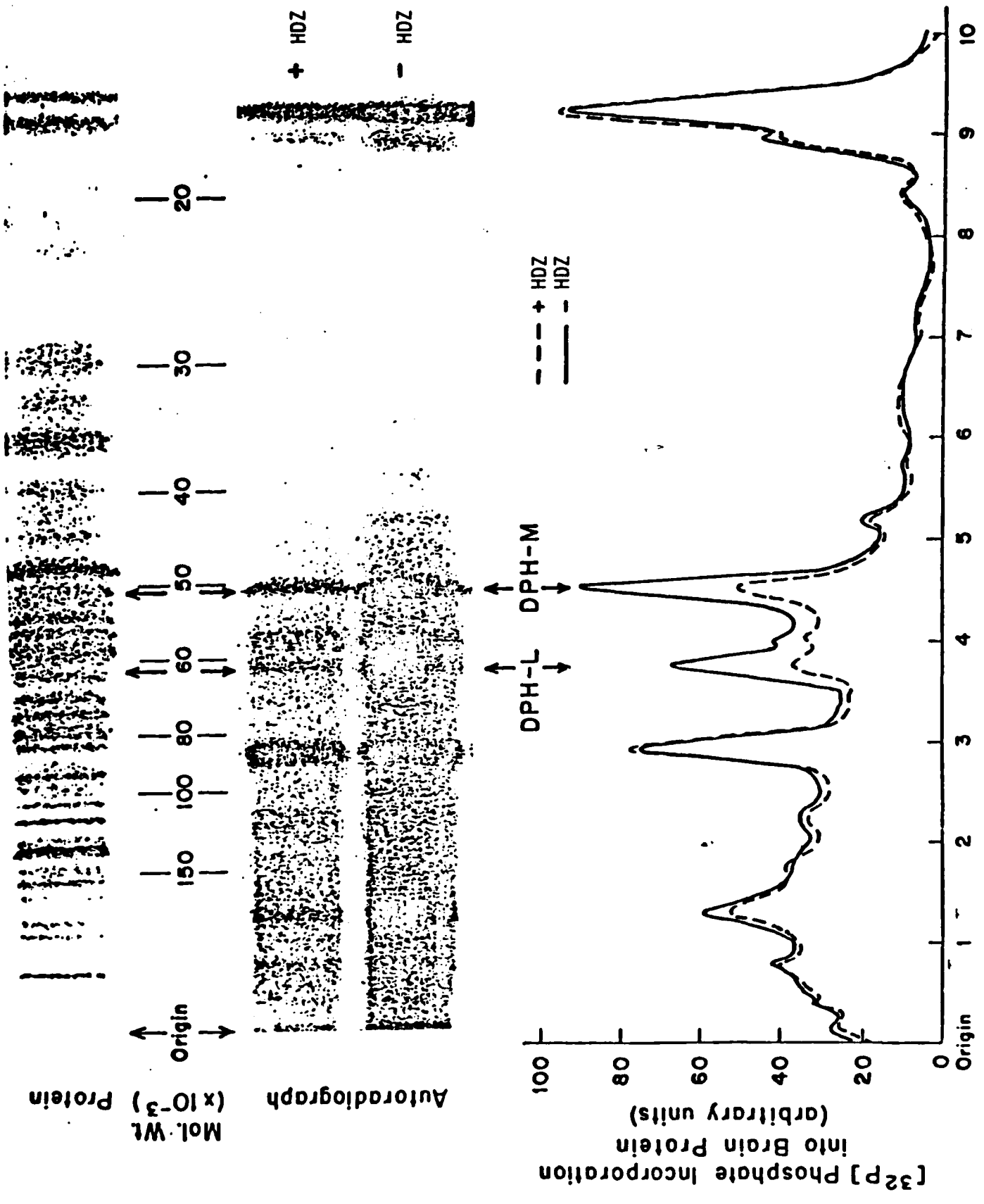

+ HDZ
- HDZ
Origin
20
30
40
50
60
80
100
150
Mol. Wt. (x 10⁻³)
Protein
Autoradiograph
DPH-L
DPH-M
+ HDZ
- HDZ
[32P] Phosphate Incorporation into Brain Protein (arbitrary units)
100
80
60
40
20
0
Origin
1
2
3
4
5
6
7
8
9
10

**Figure 2**

**Effect of HDZ concentration on the level of phosphorylation of proteins DPH-L (A) and DPH-M (B). Reactions were performed under standard conditions except for the variations in DPH concentration. The data give the mean values and ranges for 6 experiments.**

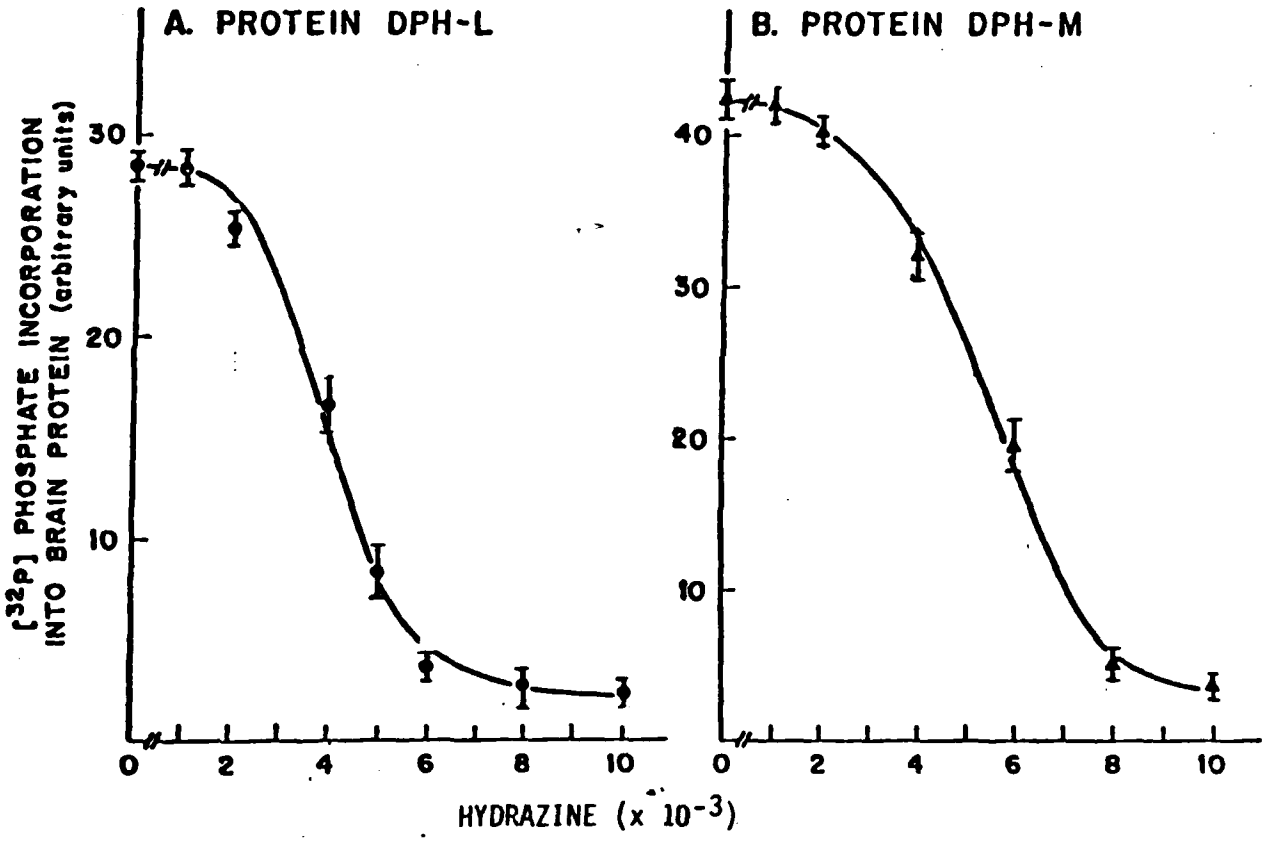


**Figure 3**

**Effect of ATP concentration on the phosphorylation of proteins DPH-L (A) and DPH-M (B) in the absence ( o—o, ——) or presence ( o—o, ——) of $5 \times 10^{-3}$M HDZ. Reactions were conducted under standard conditions except for the variation of ATP concentrations. Data give the mean values and ranges for 4 experiments.**

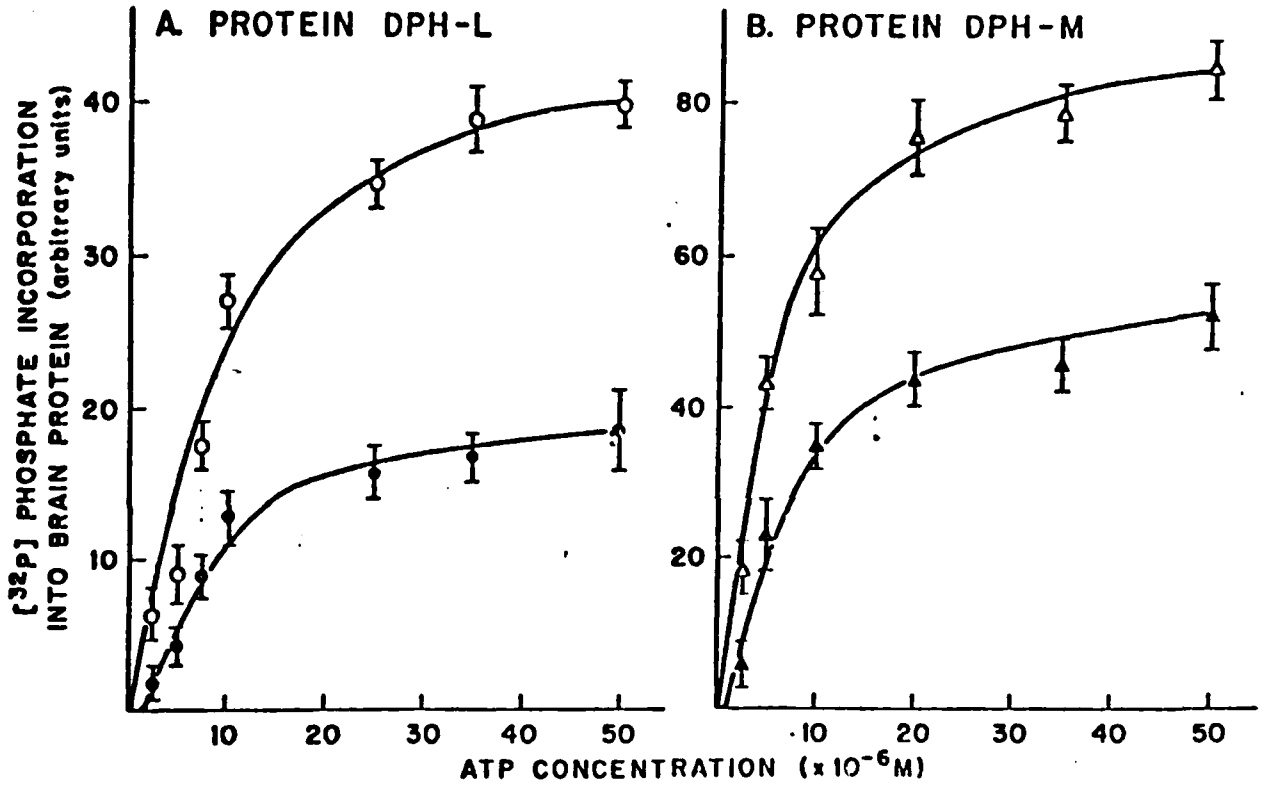


**Figure 4**

**Time course of $^{32}P$ phosphate incorporation into proteins DPH-L (A) and DPH-M (B) in the presence and absence of HDZ. Reactions were conducted under standard conditions except for the variations in incubation times and with the following additions: HDZ, $5 \times 10^{-3}$M ( o—o, —— ); HDZ, $1 \times 10^{-2}$M (——, ——); and no additions ( o—o, —— ). Data give the mean values and ranges for 6 experiments.**

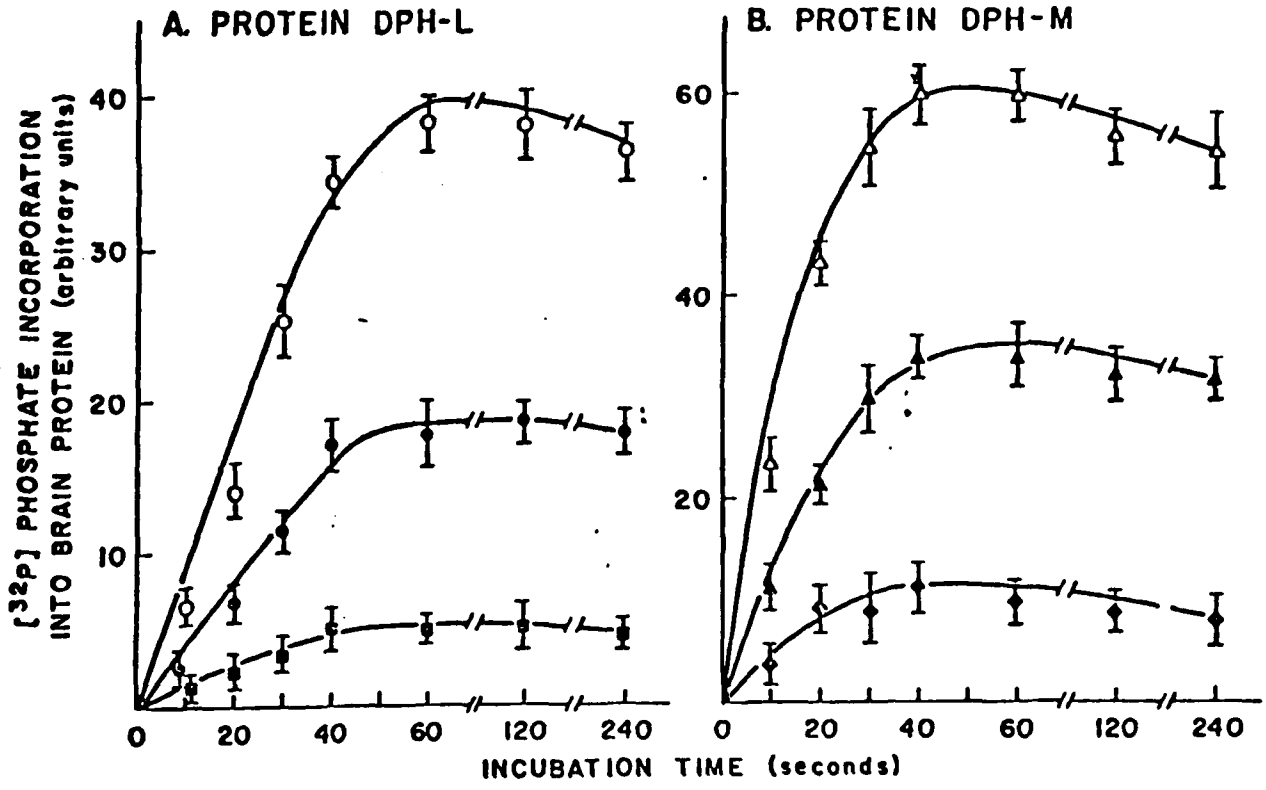
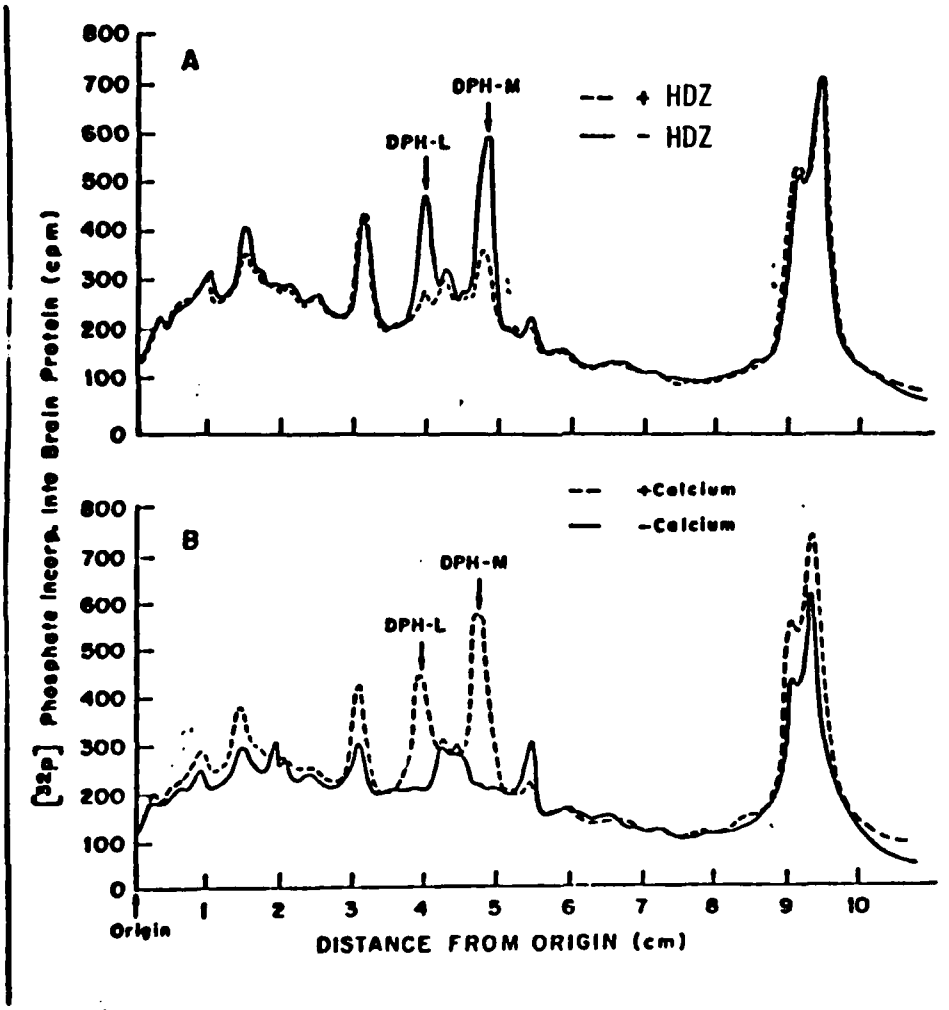


**Figure 5**

Quantitation of the effects of HDZ and/or calcium on the endogenous phosphorylation of rat brain homogenates. The dried gel used to produce the autoradiographs was cut into 2-mm pieces starting from the origin. The radioactivity in each piece of gel was determined by liquid scintillation counting, and the counts per minute (cpm) for each piece of gel was plotted as a function of distance from the origin. The points were connected in a smooth curve for clarity. The effect of HDZ ($4 \times 10^{-2}$ M) in the presence of 10 mM calcium chloride (A) and the effect of 10 mM calcium chloride (B) on the pattern of endogenous phosphorylation of specific bands are presented. The peaks corresponding to proteins DPH-L and DPH-M are marked for clarity.



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