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Mark M. Rasenick, Ph.D.

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333 Cedar Street
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This research effort studied the effects of hydrazines and organophosphates on various synaptic processes. The cyclic nucleotide related portions of this study included an examination of the effects of hydrazines upon synaptic membrane adenylate cyclase, cyclic nucleotides and any dynamic membrane events which might be related to the above processes. We were able to show distinct effects of hydrazines upon both catalytic moiety and catalytic moiety-G unit regulated adenylate cyclase. The calcium calmodulin kinase studies in this project investigated the effects of hydrazines and organophosphates on isolated

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20. ABSTRACT continued:

membrane fractions and intact synaptosome preparations. We found that hydrazines and organophosphates have effects on the incorporation of ^{32}P-phosphate into several specific synaptic proteins and attempted to probe the molecular mechanism mediating the effects of these compounds. These studies provide an insight into the toxic effects of hydrazines and organophosphates on neuronal tissue and may help develop effective methods to prevent and treat these effects in man.

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Final Scientific Report

Title: The Effects of Hydrazines and Related Compounds on Calcium Calmodulin in Regulated Synaptic Processes

Robert J. DeLorenzo, Principal Investigator

Mark M. Rasenick, Co-Investigator

Period: 6/1/82 - 7/1/83

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Technical Monitor: Dr. William Berry
Program Manager
Life Science Directorate
(203) 767-5021

Institution: Yale University School of Medicine
Department of Neurology
333 Cedar Street
New Haven, Connecticut 06510

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Final Report: AFOSR-82-0284

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The Effects of Hydrozines and Related Compounds on Calcium Calmodulin Related Synaptic Processes

The specific aims of this proposal included plans to study the effects of hydrazines and organophosphates on various synaptic processes. The cyclic nucleotide related portions of this study included an examination of the effects of hydrazines upon synaptic membrane adenylate cyclase, cyclic nucleotides and any dynamic membrane events which might be related to the above processes. In this regard, we investigated the effects of hydrazines upon calmodulin sensitive and calmodulin insensitive neuronaladenylate cyclase. We also investigated the effects of hydrazines upon the ability of synaptic membrane adenylate cyclase to be activated by various compounds which work either directly through the catalytic moiety, or through the catalytic moiety regulatory subunit (G unit) complex. In the past year, despite our failure to distinquish the efect of hydrazines on calcium calmodulin versus non calcium calmodulin activated adenylate cyclase, we were able to show distinct effects of hydrazines upon both catalytic moiety and catalytic moiety-G unit regulated adenylate cyclase. The calcium calmodulin kinase studies in this project investigated the effects of hydrazines and organophosphates on isolated membrane fractions and intact synaptosome preparations. We studied the effects of hydrazines and organophosphates on the incorporation of ^{32}P-phosphate

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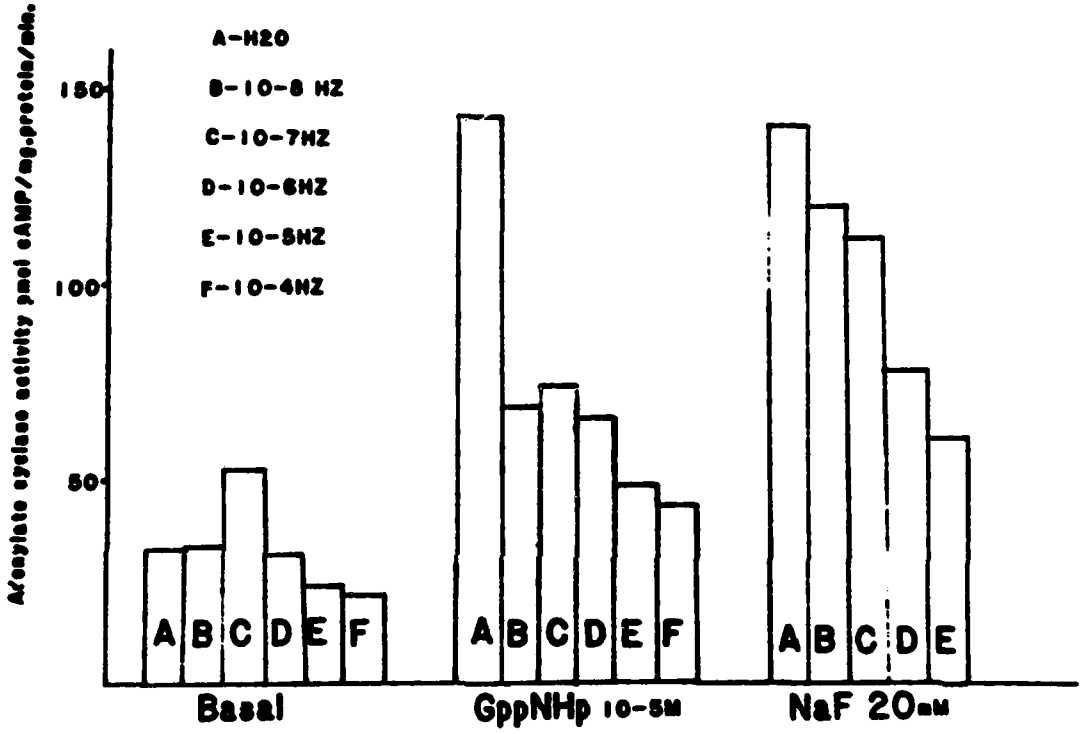
Figure 1. Effects of Hydrazine Preincubation upon Adenylate Cyclase.



Hydrazine (concentrations as indicated) is incubated with synaptic membranes 30°C 10 min. H_2O (Basal) GPPNHP or NaF are added as indicated and samples are assayed for adenylate cyclase 10 min 30°C. (Note misprint: 10^{-5} GPP if actually 10^{-6} GPPNHP.)

into several specific synaptic proteins and attempted to probe how these compounds affected this calcium regulated process.

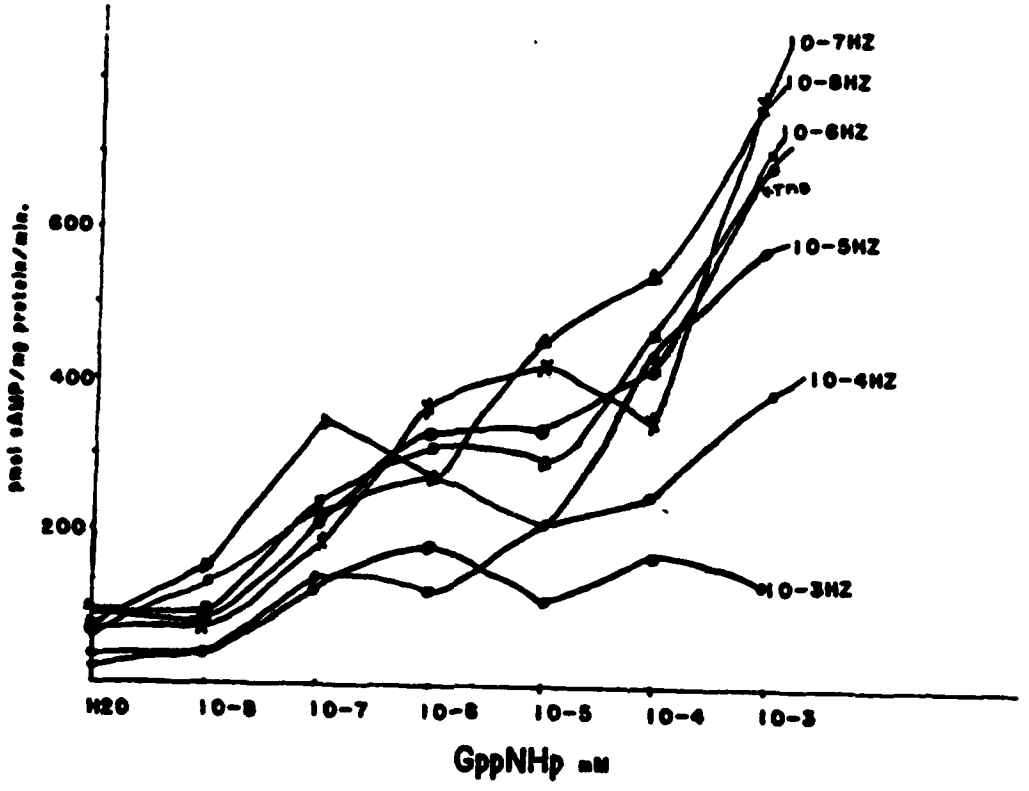
Cyclic Nucleotide Studies

Initial experiments (shown in Figue 1) demonstrate that hydrazines, in a dose dependent fashion, will inhibit adenylate cyclase which has been assayed in the presence of the non-hydrolysable GTP analogue (GPPNHP) or sodium fluoride. No effects of hydrazines are noted on basal adenylate cyclase activity.

In Figure 2, we demonstrate the ability of hydrazines to both augment and inhibit adenylate cyclase which has been pre-activiated by GPPNHP. In these experiments, both hydrazines and then GPPNHP are pre-incubated with synaptic membranes. This results in a considerably more efficatious augmentation of adenylate cyclase by GPPNHP, and a more profound inhibition by higher dose hydrazines at very high levels of GPPNHP. Intriguingly however, at low doses of GPPNHP (10^{-8} to 10^{-7}), there is a noticable effect of low dose hydrazines on an augmentation of adenylate cyclase.

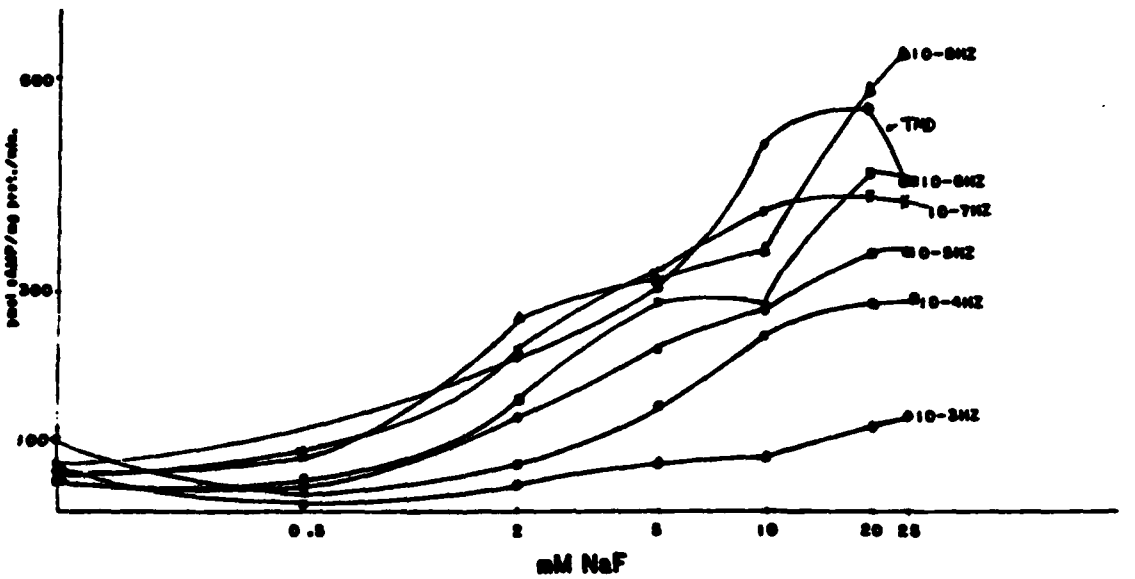
A somewhat different picture is seen when the ability of sodium fluoride to activate adenylate cyclase in the presence of hydrazine is examined. In these studies (Figure 3), it is noted that there is a simple dose dependent inhibition by hydrazines among a variety of fluoride concentrations all the way up to 25 mM. However, very low dose hydrazines (10^{-8}) will mitagate the inhibition of adenylate cyclase seen at high (25 mM) sodium fluoride concentrations. Furthermore, the commonly noted decrease in adenylate cyclase activity at 25 mM sodium fluoride is not seen with any of the

Figure 2. Effects of Hydrazine upon Adenylate Cyclase Activated by Varying Concentrations of GPPNHP.



Membranes are preincubated with hydrazine as indicated (10 min 30°C) and then preincubated with GPPNHP at the indicated concentrations. Adenylate cyclase is then assayed as in Figure 1.

Figure 3. Effects of Hydrazine upon Adenylate Cyclase Activated by Varying Concentrations of NaF.



Membranes are treated as in Figure 2 except NaF is substituted for GPPNHP.

concentrations of hydrazines despite the net adenylate cyclase activity being lower when in the presence of these compounds.

The preceding three figures have described the effects of hydrazines when these compounds are pre-incubated with synaptic membranes and adenylate cyclase is subsequently assayed. Under all these conditions hydrazines are present during the assay of adenylate cyclase as well as the pre-incubation. When hydrazines are pre-incubated with synaptic membranes and these membranes are subsequently washed, a very different picture emerges. Table 1 illustrates these observations that is, there is a dose dependent increase in adenylate cyclase observed when hydrazines are pre-incubated with synaptic membranes and subsequently washed, and hydrazines do not appear in the assay. If hydrazines are present in the assay step as well, however, then the augmentation of adenylate cyclase observed by hydrazine presence and subsequent washing is tempered. Curiously however, the ability of manganese to activate adenylate cyclase (a test of catalytic moiety activation) is augmented when hydrazines are present in the pre-incubation step and subsequently washed. Furthermore, the ability of hydrazines to augument manganese activated adenylate cyclase is further augmented by the continued presence of hydrazines during the assay step despite the blunting of GPPNHP and NaF stimulated activities. It is also noteworthy, that hydrazines did not effect manganese activated adenylate cyclase when present during the assay step yet not initially washed. The reasons for this are thus far inexplicable. However it should be noted, that manganese is as strong an oxidant as hydrazine is a reducing agent and it is possible that these redox properties are involved under these conditions.

Table I. Reversibility of Hydrazine Effects of Washing after Hydrazine Incubation.

Additions to Assay:	Membranes preincubated (prior to assay) with: H_2O/H_2O	10^{-4} HZ/H_2O	10^{-4} HZ/10^{-4} HZ
	Adenylate cyclase activity*		
None	2.4	13.1	0
10^{-7} Gpp(NH)p	4.8	58.8	10.6
10^{-4} Gpp(NH)p	24.4	70.0	46.4
NaF (20mM)	62.8	84.4	58.1
$MnSO_4$ (10mM)	150	230	314.

* pmol cAMP /mg protein /min

Membranes are incubated with the indicated compound (before slash) 10 min 30°C and washed with 20 volumes of buffer. Membranes are then resuspended and assayed for adenylate cyclase with the indicated activation and H_2O or hydrazine (indicated--/hydrazine after slash).

Considerable work will be necessary to understand the complexities of hydrazine effects upon synaptic membranes and adenylate cyclase. Several intriguing aspects of this study which bear directly on basic projects involving adenylate cyclase activation include the differential affects of hydrazines when present during the assay or when pre-incubated and washed. Another very important parameter that has been noted in these studies is the apparent ability of hydrazines to distinquish between a fluoride and GPPNHP activation of adenylate cyclase. In 1980, this investigator had discovered a protein which was able to distinquish between guanylnucleotide and fluoride activation of synaptic membrane adenylate cyclase. It would be intriguing if hydrazines display similar effects. This will be investigated along with the previous phenomenon. Furthermore, we hope to be able to be in a better position to examine the effects of calmodulin upon adenylate cyclase through the use of GPPNHP ligand binding.

Calmodulin Kinase Activity

Our initial studies during this first year were directed at studying the effects of hydrazine on the endogenous phosphorylation of brain membrane proteins in the presense or absence of calcium and/or calmodulin. Hydrazines, over a broad concentration range (10^{-9}-10^{-2} M), were incubated with calmodulin depleted rat brain synaptosome membrane for 1 min to 1 hr intervals. The endogenous kinase activity in the presence of γ-^{32}P-ATP with or without calcium and/or calmodulin.

Hydrazine at concentrations above 10^{-4} M had a significant inhibitory effect on kinase activity when incubated with brain

membrane for greater than 30 min. The effects of various hydrazine concentrations as a function of incubation time with membrane are shown in Table 2. Concentrations below 10^{-4} M had no effect on kinase activity. In addition, hydrazines had to be in contact with the membrane fractions for longer than 30 min (Table 3) to demonstrate significant membrane effects.

At a concentration of 10^{-3} M hydrazine, the effects of this compound on kinase activity was studied as a function of the time of incubation with the membrane preparation (Table 3). These studies demonstrated a clear time dependent effect on this membrane enzyme system. At a concentration of 10^{-3} M hydrazines, the inhibitory effect on brain membrane became significantly apparent at times greater than 30 min. These results indicate that hydrazines take time to interact with hidden membrane sites or that they may be gradually affecting the properties of the membrane: i.e., altering its stability or fluidity.

To explore these effects further, we determined the effects of hydrazine on highly enriched preparations of calcium calmodulin kinase activity isolated from brain cytosol. Hydrazine did not significantly inhibit the isolated kinase system up to concentrations of 10^{-2} M with up to 2 hrs of equilibration with the enzyme protein. These results suggest that the effect of hydrazine on kinase activity is not the result of a direct effect on the kinase itself, but an affect of hydrazine on the membrane structure or fluidity which then can indirectly affect kinase activity.

Our results with kinase activity during this one year of study parallel our findings with with adenylate cyclase regulation. It appears that the hydrazines play a major role in interacting with

Table 2. Concentration Effects of Hydrazines on Brain Membrane Calmodulin Kinase Activity.

Condition	Hydrazine Concentrations (M)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Control	15	12	16	14	14
Calcium	31	30	28	27	29
Calmodulin	16	15	18	12	14
Calcium & Calmodulin	100	101	97	85	68

Hydazine at several concentrations was incubated with brain membrane for 1 hr at 0°C. The treated membrane was then incubated to study endogenous membrane calmodulin kinase activity. The untreated membrane demonstrated marked calciumm calmodulin stimulated kinase activity. Data are expressed as precent of maximal stimulation which was designated as 100%. Incorporation of ^{32}P-phosphate into the major 54,000 dalton peptide is shown. Effects on the phosphorylation of this protein were representative of the effects on the level of phosphorylateion of other brain proteins.

Table 3. Effects of Various Times of Exposure to Hydrazine on Membrane Kinase Activity.

	Calcium Calmodulin Phosphorylation	
Time of Exposure Min.	Control	Hydrazine
1	100	100
10	99	98
20	97	95
30	98	89
45	101	81
60	97	73

Data give the mean of 8 determinations and are representative of 6 experiments. See legend for Table 2 for conditions. Data are expressed as percent of maximal condition in the presence of calcium and calmodulin.

neuronal membrane in a time dependent manner, possibly altering membrane fluidity. We will more directly investigate this hypothesis in subsequent studies utilizing fluorescent membrane probes.

During this year of study, we also examined the effects of several organophosphates on calcium calmodulin kinase activity. Calcium calmodulin tubulin kinase activity is very unstable post mortem and this instability was found to be primarily the result of protease activity. We employed the organophosphates diisopropyl phosphorofluoridate (DFP) and (PMSF) to attempt to inhibit this protease activity. Both DFP and PMSF produced a significant enhancement in enzyme activity. Thus, we routinely employ these organophosphates in our enzyme purification buffers.

These results suggested that organophosphates administered acutely or chronically could alter the endogenous calcium calmodulin kinase activity in neuronal tissue. Since our kinase system is present at the synapse and associated with microtubules, this affect of the organophosphates might account for some of their toxic action on neuronal function. Thus, several studies were initiated to study and probe this possibility.

Chronic treatment of rats with organophosphates produced significant elevation of calcium calmodulin tubulin kinase activity when compared to appropriate control animals. In addition, acute treatment with DFP just prior to sacrifice also produced elevated endogenous calmodulin kinase activity in brain membrane and cytosol fractions. Direct addition of DFP to the reaction tube, however, did not significntly alter endogenous tubulin phosphorylation by the

kinase. Thus, DFP was not directly stimulating the kinase. Presumably, it acts by indirectly inhibiting the proteases that regulate the level of the kinase in brain tissue.

Preliminary studies with intact synaptosomes, from control and chronically treated animals, demonstrated that organophosphates increased depolarization dependent protein phosphorylation in synaptosomes. These results (Table 4) suggested that the affects of organophosphates on neuronal tissue may regulate the expression of calcium enzyme systems. In addition, these findings with intact synaptosomes indicate that organophosphates may have a major impact on synaptic function that could occur at sublethal concentrations of these agents. Thus, the synaptosome and calcium system models employed in these studies may provide important insight into our understanding of some of the potentially serious toxic effects of these compounds on the central nervous system when humans are exposed to sublethal concentrations of the agents. We plan to actively investigate these issues over the next three years.

In addition to the results obtained concerning the effects of hydrazines and organophasphates on calcium systems in brain, we have also gained insights into the basic mechanism involved in mediating the effects of calcium on neuronal activity. Hydrazine is clearly a useful pharmacological tool for altering membrane fluidity. Thus, we plan to utilize this information to study the effect of alterations in membrane fluidity on synaptic modulation. The use of organophosphates to inhibit enodgenous brain protease activity has allowed us to stabilize the calcium calmodulin tubulin kinase system. This result was a major breakthrough for our research

Table 4. Effects of Chronic DFP Exposure on Depolarization Sensitive Protein Phosphorylation and Neurotransmitter Release in Intact Synaptosomes

Conditions	Protein Phosphorylation	Norepinephrine Release
Control Animals		
Calcium	41	50
Calcium, Potasium	100	100
DFP Animals		
Calcium	65	71
Calcium, Potasium	183	166

Data are expressed as the mean of 10 determinations and are representative of 6 separate experiments. The largest standard error of the means was ±7.1. Control animals received placebo and DFP treated animals received sub-lethal doses of DFP for 4 days prior to sacrifice. Data are expressed as percent of maximal control condition in the presence of calcium, potasium.

effort and has resulted in the purification of this important enzyme system in brain.

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