and Wolfe (5) showed that [^{14}C]methyl coenzyme M is formed from [^{14}C]methanol, indicating a common methyl transfer route. A comparative study of the mechanisms of the nonenzymatic reaction and that of this B_{12} enzyme can now be formulated (scheme 1). This reaction mechanism explains how methyl coenzyme M is synthesized. Each of the proposed intermediates in the catalytic cycle occurs at the slower rate of the nonenzymatic reaction (4). During the last 10 years of research on the mechanism of methane biosynthesis, a role for a B_{12} cofactor has been sought, without much success. This research shows that a B_{12}-dependent methyltransferase is important in the biosynthesis of methane by *Methanosarcina barkeri*, although no similar B_{12} protein can be found in extracts of *Methanobacterium thermoautotrophicum* (6). Our results indicate that B_{12}-dependent pathways are operative in the biological formation of methane in addition to other pathways that are B_{12}-independent.

J. M. WOOD
Department of Biochemistry, Gray Freshwater Biological Institute, University of Minnesota, Navarre 55392
ISABEL MOURA
J. J. G. MOURA
M. H. SANTOS
A. V. XAVIER
Departmento de Biologia e Bioengenharia, Universidade Nova de Lisboa, Centro de Quimica Estrutural, Complexo Interdisciplinar, 1000 Lisboa, Portugal
J. LEGALL
Department of Biochemistry University of Georgia, Athens 30602
M. SCANDELLARI
Laboratoire de Chimie Bacterienne, Centre National de la Recherche Scientifique 13274 Marseille, Cedex 2 France

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Interaction of Brain Synaptic Vesicles Induced by Endogenous Ca^{2+}-Dependent Phospholipase A_2

Abstract. *Endogenous phospholipase A_2 activity of brain synaptic vesicles was Ca^{2+}-dependent and was increased by prostaglandin $F_{2\alpha}$, calmodulin, adenosine 3′,5′-monophosphate, and adenosine triphosphate, whereas the activity was inhibited by prostaglandin E_2 in the absence or presence of calmodulin. Light-scattering measurements demonstrated that stimulation of the enzyme's activity correlated with the induction of vesicle-vesicle aggregation. The effects of these compounds on endogenous synaptic vesicle phospholipase A_2 activity may imply a common end point of their purported neuromodulatory actions, and indicate that synaptic vesicle phospholipase A_2 may play a central role in presynaptic neurotransmission.*

According to the vesicle hypothesis (1), an influx of Ca^{2+} into the presynaptic axon terminal leads to exocytosis of neurotransmitter from synaptic vesicles into the synaptic cleft. The mechanism of this Ca^{2+}-induced event is unresolved. Calcium is postulated to mediate this process directly by cross-bridging membranes (2), neutralizing membranal surface charges (3), or inducing membrane phase transitions (4). Other hypotheses of Ca^{2+}-mediated exocytosis invoke activation of brain actomyosin (5) or membrane protein phosphorylation (6).

In this study we assayed purified synaptic vesicles from bovine brain (6, 7) for phospholipase A_2 (PLA_2) (E.C. 3.1.1.4) activity using [2-^{14}C]arachidonyl phosphatidylcholine as substrate (8). When synaptic vesicles were incubated with 2.8 nmole of substrate in the presence of 1 m*M* EGTA and increasing concentrations of $CaCl_2$ (10^{-7} *M* to 10^{-1} *M*) at *p*H 9, PLA_2 activity increased with increasing Ca^{2+} concentration reaching maximum activity at 10 μ*M* $CaCl_2$. In the presence of EGTA, the amount of arachidonic acid released was 0.07 ± 0.009 nmole/mg-hour. Calcium (2 m*M*) increased the activity approximately sixfold, the amount of arachidonic acid released reaching 0.40 ± 0.13 nmole/mg-hour.

When synaptic vesicles were incubated at 37°C with increasing substrate concentrations (0.2 to 4.0 nmole) in the presence of 2 m*M* $CaCl_2$, *p*H 9.0, for 60 minutes (9), analysis of the substrate concentration curve by transformation into a Lineweaver-Burk plot revealed the Michaelis constant (K_m) to be 60 μ*M* and the maximum velocity (V_{max}), 2.0 nmole/mg-hour. Further experiments were performed under the same conditions in the presence of a variety of compounds known to be present in neurons and to modulate neurotransmission (Table 1). In no case was there a significant effect on the K_m. Calmodulin, a multifunctional Ca^{2+}-binding protein (10) reported to stimulate the release of neurotransmitter (6), caused about a fivefold increase in the enyzme's V_{max}. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is synthesized in the brain (11) and stimulates autonomic neurotransmission (12), caused an eightfold increase in PLA_2 activity. Prostaglandin E_2 (PGE_2), which is synthesized in the brain (11), inhibits autonomic neurotransmission (12) and acts as a sedative (13), not only inhibited PLA_2 activity in the presence of Ca^{2+} alone, but also inhibited the calmodulin stimulating effect.

Individually, adenosine triphosphate (ATP) inhibited by 50 percent and adenosine 3′,5′-monophosphate (cyclic AMP), a nucleotide reported to stimulate PLA_2 activity (14), activated by 50 percent the synaptic vesicle PLA_2. However, cyclic AMP in conjunction with ATP activated PLA_2 by 200 percent. The difference in individual versus combined effects of these compounds on PLA_2 activity led us to speculate that the mechanism of the

Table 1. Effects of various conditions on the V_{max} of synaptic vesicle PLA_2. Reaction mixtures were incubated at 37°C for 60 minutes with 100 μg of synaptic vesicle protein in increasing substrate concentrations (0.2 to 4.0 nmole), brought to a final volume of 200 μl with tris buffer, *p*H 9.0, in the presence of 2 m*M* $CaCl_2$.

Conditions and concentration	V_{max} (nmole/mg-hour)	Percentage change*
Cyclic AMP (1 m*M*)	3.0	50 (+)
ATP (1 mm)	1.0	50 (−)
Cyclic AMP (1 m*M*) plus ATP (1 m*M*)	6.0	200 (+)
Calmodulin (1 μmole)	9.0	350 (+)
PGE_2 (4.0 nmole)	0.6	70 (−)
PGE_2 (4.0 nmole) plus calmodulin (1 μmole)	1.0	50 (−)
$PGF_{2\alpha}$ (4.0 nmole)	16.0	700 (+)

*Plus signs indicate stimulation and minus signs indicate inhibition.

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combined cyclic AMP and ATP potentiation of PLA_2 may involve a protein kinase.

We also conducted phosphorylation experiments with synaptic vesicles, using 1.6 μM [γ-^{32}P]ATP and standard conditions of incubation, sodium dodecyl sulfate gel electrophoresis, and autoradiography (*15*). We found that 100 μM cyclic AMP stimulated phosphorylation of a protein with a molecular weight of 40,000 (40K), as well as proteins of molecular weights 175K, 100K, 80K, 57K, 55K, 53K, and 30K (Fig. 1), confirming the presence of an endogenous synaptic vesicle cyclic AMP–dependent protein kinase. Hirata (*16*) reported that lipomodulin, a 40K protein found in neutrophils and synaptic plasma membrane (*17*), inhibits PLA_2. Upon phosphorylation by Ca^{2+} or cyclic AMP, however, lipomodulin's inhibitory effect is suppressed. It is possible that the phosphorylated 40K protein in our synaptic vesicle preparation is lipomodulin and that the stimulation induced by cyclic AMP and ATP on synaptic vesicle PLA_2 is affected by way of phosphorylation of this protein. Although Ca^{2+} inhibits cyclic AMP–dependent protein kinases (*18*), it is possible that this protein kinase is dependent on both cyclic AMP and Ca^{2+}, as appears to be the implication in another brain protein kinase system (*19*).

To correlate the modulation of synaptic vesicle PLA_2 with function we assayed synaptic vesicle aggregation with a Behring laser nephelometer which measured the amount of light scattered by synaptic vesicles. When synaptic vesicles were incubated with 2 mM $CaCl_2$, only a 40 percent increase in light scattering was observed (Table 2). However, when vesicles were incubated with PLA_2 (*Vipera russelli*) or lysolecithin in the presence of $CaCl_2$, within 10 seconds the increase in light scattering was 250 and 220 percent, respectively. Phase-contrast and electron microscopy of PLA_2-treated synaptic vesicles confirmed that the increase in light scattering was associated with aggregation, lysis, and fusion of synaptic vesicles (data not shown). The similar effects of these two compounds implied that exogenous PLA_2 hydrolyzed synaptic vesicle phosphoglycerides with subsequent production of lysolecithin which in turn induced aggregation and fusion of synaptic vesicles. Lysolecithin induces fusion in a variety of membranes (*20*) and has been implicated in the secretion of adrenal chromaffin granules (*21*).

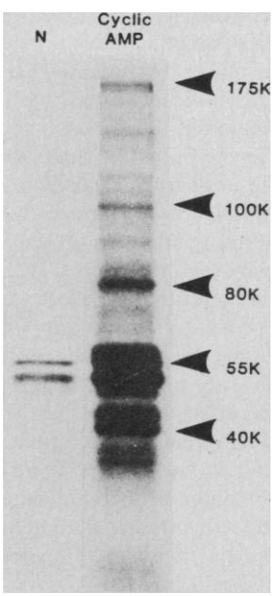

Fig. 1. Autoradiograms of synaptic vesicles incubated with (*N*) no reagents added, or with $MgCl_2$ and 100 μM cyclic AMP in the presence of [γ-^{32}P]ATP and resolved by electrophoresis, on 5 to 15 percent sodium dodecyl sulfate polyacrylamide gel.

Calmodulin induced a 250 percent increase in light scattering within 10 seconds of being added to the vesicles. This effect was attributed to the stimulation of endogenous synaptic vesicle PLA_2, since preincubation of the vesicles with either 80 mM mepacrine or 80 mM parabromophenacylbromide, both inhibitors of PLA_2 (*22, 23*), diminished this effect (Table 2). Calmodulin stimulation was diminished 150 percent by 4.0 nmole of PGE_2. In contrast, $PGF_{2\alpha}$ (4.0 nmole) enhanced the effect of Ca^{2+} by 275 percent and the effect of calmodulin by 50 percent.

Adenosine triphosphate and cyclic AMP individually induced 30 and 50 percent increases in light scattering, respectively. The addition of both compounds led to a 150 percent increase in light scattering. The effects of these compounds on synaptic vesicle behavior were attributed to their interaction with endogenous synaptic vesicle PLA_2, since preincubation of the synaptic vesicles with parabromophenacylbromide diminished these effects.

Thus compounds that stimulated or inhibited synaptic vesicle PLA_2 also stimulated or inhibited, respectively, synaptic vesicle light scattering, and these effects in turn were correlated with vesicle-vesicle aggregation and possible fusion (*24, 25*).

The addition of exogenous PLA_2 to brain slices causes depletion in the number of synaptic vesicles (*26*). Moreover, application of β-bungarotoxin, a component of which is a Ca^{2+}-dependent PLA_2, causes an initial enhancement of neurotransmitter release from axon terminals (*27*). It is of interest, therefore, to find an endogenous PLA_2 in synaptic vesicles that is Ca^{2+}-dependent. On the basis of these data we postulate that

Table 2. Effects of various conditions on synaptic vesicle light scattering. Baseline nephelometer recordings of synaptic vesicles (30 μg/ml) prior to the addition of the ion or compounds was 0.9 to 1.10. The nephelometer units are directly proportional to the intensity of scattered light detected by a photodiode-generated signal. All incubations took place at 25°C in a total volume of 300 to 600 μl, *p*H 6.5. When more than one compound was added, they were added in 1-minute sequences. The presence of quinacrine or parabromophenylacylbromide (*p*BPB) indicates that the synaptic vesicles were incubated with these compounds for 60 minutes before other compounds were added. The percentage change in nephelometry units indicates the change that occurred from baseline to the peak of light scattering prior to synaptic vesicle sedimentation. Values shown represent the average of three to five separate determinations.

Conditions and concentration	Percentage increase in light scattering (nephelometer units)
$CaCl_2$ (2 mM)	40
Ca^{2+} (2 mM) and PLA_2 (*Vipera russelli*) (25 μg)	250
Ca^{2+} (2 mM) and lysolecithin (egg yolk) (20 μg)	220
$CaCl_2$ (2 mM) and calmodulin (1.0 μmole)	250
$CaCl_2$ (2 mM), calmodulin (1.0 μmole), and quinacrine (80 mM)	10
$CaCl_2$ (2 mM), calmodulin (1.0 μmole), and *p*BPB (80 mM)	50
$CaCl_2$ (2 mM), calmodulin (1.0 μmole), and PGE_2 (4.0 nmole)	100
$CaCl_2$ (2 mM) and $PGF_{2\alpha}$ (4.0 nmole)	150
$CaCl_2$ (2 mM), $PGF_{2\alpha}$ (4.0 nmole), and *p*BPB (80 mM)	50
$CaCl_2$ (2 mM), $PGF_{2\alpha}$ (4.0 nmole), and calmodulin (1 μmole)	350
$CaCl_2$ (2 mM), $PGF_{2\alpha}$ (4.0 nmole), calmodulin (1 μmole), and *p*BPB (80 mM)	50
$CaCl_2$ (2 mM) and ATP (1 mM)	30
$CaCl_2$ (2 mM), $MgCl_2$ (10 mM), and cyclic AMP (1 mM)	90
$CaCl_2$ (2 mM), $MgCl_2$ (10 mM), cyclic AMP (1 mM), and *p*BPB (80 mM)	30
$CaCl_2$ (2 mM), $MgCl_2$ (10 mM), cyclic AMP (1 mM), and ATP (1 mM)	150
$CaCl_2$ (2 mM), $MgCl_2$ (10 mM), cyclic AMP (1 mM), ATP (1 mM), and *p*BPB (80 mM)	50

activation of synaptic vesicle PLA_2 may be one of the mechanisms involved in Ca^{2+}-mediated stimulus-secretion coupling in axon terminals. The fact that this enzyme can be modulated by a variety of compounds present in the brain and known to modify neuronal activity suggests that it may have an important role in presynaptic neuronal events.

NATHAN MOSKOWITZ
WILLIAM SCHOOK
SAUL PUSZKIN

Division of Molecular Pathology, Department of Pathology, Mount Sinai School of Medicine of the City University of New York, New York 10029

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24. When light-scattering experiments were performed at *p*H 9.0, the results were similar to those in Table 2. However, at *p*H 9.00 parabromophenacylbromide induced a 24.3 percent (±8.4) greater inhibition of light scattering than at *p*H 6.5. This was in line with the observation that this compound exerts its maximum inhibitory effect on PLA_2 at basic *p*H values (*23*).
25. The physiological importance of synaptic vesicle PLA_2 in function is underlined by the effects of these compounds on vesicle-vesicle interaction at physiological cellular *p*H (6.5). Although maximum PLA_2 activity as determined by the enzymatic assay was achieved at *p*H 9.0, we emphasize that this assay measured the amount of exogenously added phosphatidylcholine capable of being hydrolyzed by endogenous synaptic vesicle PLA_2. This high *p*H may be necessary for the maximum exposure of synaptic membrane PLA_2 to come into contact with and subsequently hydrolyze exogenous substrate. The nephelometer experiments, however, represent a functional manifestation of synaptic vesicle PLA_2 hydrolysis of endogenous synaptic vesicle phosphatidylcholine. At *p*H 6.5 there probably is adequate interaction between the substrate and enzyme.
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X-ray Induction of Persistent Hypersensitivity to Mutation

Abstract. *The progeny of x-irradiated V79 cells are hypersensitive to PUVA-(8-methoxypsoralen plus longwave ultraviolet light) induced mutation at the locus for hypoxanthine-guanine phosphoribosyl transferase. This hypersensitivity is most evident at low doses of PUVA that do not induce mutation in non–x-irradiated cells. The hypersensitivity is evoked by x-irradiation delivered as a single dose or as multiple fractions over a long period and persists for at least 108 days of exponential growth. This radiation-induced hypersensitivity to subsequent mutation is a new phenomenon that may be relevant to multistage carcinogenesis.*

The reaction of a human population to various agents may depend in part on the population's previous exposure to radiation and chemicals. For example, psoriatic patients show an increased risk for cutaneous carcinoma after PUVA therapy (8-methoxypsoralen plus longwave ultraviolet light) if they have previously been treated with ionizing radiation (*1*). The incidence of cutaneous carcinoma in psoriatic patients is 15 times higher than expected for an age, sex, and geographically matched population (*2*). The present investigation was designed to determine whether the progeny of cells treated in vitro with x-radiation display a subsequent hypersensitivity to the mutagenic effects of PUVA.

Preliminary studies indicated that progeny of V79 cells irradiated with a single, high dose of x-radiation (600 to 900 rads) were sensitive to mutation induced by low doses of PUVA. These studies also indicated that the dose-response pattern of the induced hypersensitive state could best be studied by selecting from the progeny of x-irradiated cells a number of cells (10^4) that would make the probability of including x-ray–induced mutants in the population rather small. The maximum number of mutants observed after x-irradiation in these cells was 25×10^{-6}. From each of ten populations of x-irradiated cells we cultured 10^4 survivors; the progeny of these survivors exhibited background levels of mutation that were similar to those of non–x-irradiated cells ($13.4 \pm 1.0 \times 10^{-6}$ mutants per survivor). All ten populations, however, exhibited increased sensitivity to PUVA-induced mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus when compared to non–x-irradiated cells. Although increased sensitivity to PUVA-induced mutation was always observed when larger numbers of cells were selected from the x-irradiated populations, the increased number of x-ray–induced mutants partially obscured the pattern of increased mutation induced by low doses of PUVA.

From four populations of x-irradiated V79 cells we cultured 10^4 survivors. Twelve days later we exposed the progeny of these cells, and of control (non–x-irradiated) cells, to PUVA, and examined their cytotoxic and mutational responses. The survival pattern, which was characteristic of cells exposed to PUVA (*3*, *4*), was essentially the same for the x-irradiated and control populations (Fig. 1A). In contrast, the rate of mutation at the HGPRT locus (indicated by 6-thioguanine resistance) was significantly greater in the x-irradiated cells than the control populations. The frequency of PUVA-induced mutation in the progeny of x-irradiated cells is the difference between the frequency after PUVA treatment and the background frequency in replicate cultures of x-irradiated cells not exposed to PUVA. Similarly, the frequency of PUVA-induced mutations in non–x-irradiated control cells is the difference between the frequency in non–x-irradiated cells treated with PUVA and the background frequency in replicate cultures exposed neither to x-rays nor PUVA. Historical or laboratory control levels are not used for any calculation; each data point in Fig. 1B has its individual non–PUVA-treated control.

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