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EFFECTS OF ATPγS IN ISOLATED RAT BRAIN SYNAPTOSOMES*

MIROSLAWA HAUPTMANN†, DAVID F. WILSON‡ and MARIA ERECIŃSKA
Department of Biochemistry and Biophysics and Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

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Abstract—The effects of ATPγS, a slowly hydrolyzable analogue of ATP, were investigated in the preparation of synaptosomes isolated from rat cerebral cortex. It was found that addition of [^{35}S]ATPγS resulted in substantial magnesium-dependent incorporation of ^{35}S into synaptosomal proteins which was prevented completely by ATP. The most prominently labeled polypeptides were those with apparent molecular weights of 100,000; 84,000; 74,000; 62,000; 55,000; 48,000; and 41,000. The rate and extent of thiophosphorylation were unaffected by addition of cAMP, veratridine or sodium fluoride. ATPγS at 50–100 μM had no effect on either uptake or release of γ-aminobutyric acid (GABA) and dopamine; at a concentration of 1 mM it inhibited incorporation of dopamine by about 20%. This inhibition was also seen with 1 mM GTP, β, γ-methylene-adenosine 5′-triphosphate and adenylylimidodiphosphate, which suggests that the nucleotide triphosphates themselves, and not membrane protein phosphorylation, were responsible for the effect observed. It is concluded that ATPγS is an effective tool for studying the possible role of ATP released in synaptic transmission. The results obtained thus far suggest that neither extrasynaptosomal ATP nor phosphorylation of external proteins of the presynaptic membrane is sufficient for modulation of neurotransmitter uptake or release. They may, however, play a role in combination with other conditions.

The role of adenosine triphosphate (ATP) at synaptic junctions in both peripheral and central nervous systems has been the subject of considerable debate ever since Holton and Holton [1] put forward a suggestion that ATP may be a neurotransmitter. The experimental evidence accumulated over the past 15 years suggests that this nucleotide may be released from nervous tissue by two mechanisms (for review see Refs. 2–6). In the first, ATP is viewed as a neurohormonal agent which is secreted from one cell upon excitation to modulate activity of either the same or another cell but not by depolarization of the postsynaptic membrane. This situation is exemplified by release of ATP in conjunction with either catecholamines [7] or acetylcholine [8, 9] from specific adrenergic or cholinergic terminals. In the second mechanism, ATP is postulated to act as neurotransmitter in a specific purinergic system [2, 10].

There have been a number of studies which showed that ATP is released from nervous tissue during activity [11–15] and subsequently hydrolyzed in the extracellular environment [3, 16–18]. Release of ATP has been reported after exposure of nervous tissue to elevated potassium concentrations or veratrum alkaloids [14, 19]. It is not clear, however, whether the effects of ATP are presynaptic, postsynaptic or both. It seems that in peripheral tissues the major presynaptic actions of ATP are mediated by an adenosine receptor [20–24] since the inhibitory effect of this nucleotide is sensitive to theophylline [25]. On the other hand, there is some evidence that ATP itself can act on postsynaptic sites [26, 27]. In the central nervous system, purines are reported to exert primarily depressant effects on neuronal firing although occasional excitatory responses have been noted [28, 29]. Detailed evaluation of the depression of spontaneous activity of neurons in cerebral and cerebellar cortices by iontophoretically applied ATP and various adenine nucleotide derivatives and analogues showed, moreover, that dephosphorylation of ATP to adenosine was required for expression of depressant activity [29]. On the other hand, Brennan and Cantrill [30] postulated that membrane protein phosphorylation caused by ATP may be a mechanism for the presynaptic control of transmitter release.

In this paper we have addressed the question of whether ATP *per se* or ATP-dependent phosphorylation of the external surface of the presynaptic terminal modulates the release and/or uptake of neurotransmitters. To do this we have utilized preparations of rat brain synaptosomes which respond to electrical stimulation and depolarization with increased release of adenine nucleotides [3, 12, 14] and exhibit a number of neuronal properties such as uptake and secretion of neurotransmitters (see Ref. 31 for review). Thus, these preparations provide a convenient model system in which the effects of adenine nucleotides on these properties can be studied. Our preliminary unpublished results showed, however, that externally added ATP was hydrolyzed very rapidly by synaptosomes, hence it was not possible to differentiate between the effects of ATP itself and those of its metabolites (such as adenosine).

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† On leave of absence from: Psychoneurological Institute, 1/9 Sobieskiego St., 02-957 Warsaw, Poland.

‡ Direct all correspondence to: Dr. David F. Wilson, University of Pennsylvania, Department of Biochemistry and Biophysics, Philadelphia, PA 19104.

We have used, therefore, a slowly hydrolyzable analogue of ATP, ATPγS, which is a substrate for many kinases, and the resulting thiophosphate esters are resistant to hydrolysis by phosphatases [32–34]. Our results show that addition of ATPγS results in substantial incorporation of ^{35}S into synaptosomal proteins and suggests that this is a promising approach to determining the possible role of phosphorylation of the external surfaces of nerve ending in modulating synaptic function.

MATERIALS AND METHODS

Synaptosomal preparation. Synaptosomes were isolated from the forebrains of male Sprague–Dawley rats according to the method of Booth and Clark [35]. The synaptosomal pellet was washed once in a modified Krebs–Henseleit medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1 mM sodium-phosphate, 10 mM Tris–HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid), pH 7.4, and suspended in the same medium at a final concentration of 5–10 mg protein/ml.

In all experiments, synaptosomal suspensions were subjected to 5-min preincubation with 10 mM glucose and 2.5 mM $CaCl_2$ at 27° and then diluted to the protein concentration required by the experimental protocol. Protein concentration was determined by the method of Lowry *et al.* [36] using bovine serum albumin as a standard.

Measurements of ATP, ATPγS and ADP. ATP, ATPγS and ADP were determined in the neutralized trichloroacetic acid (TCA) extracts of synaptosomal preparations (see legend to Fig. 1) by high performance liquid chromatography (HPLC, Perkin–Elmer, series 2 liquid chromatograph). Chromatographic separations were carried out on an EM Regents Hibar EC-RT 125 mm × 4.0 mm column packed with 5 micron Lichrosorb NH_2 particles. Nucleotide concentrations were determined by relative peak areas, using a Perkin–Elmer LC-75 spectrophotometric detector (254 nm) that was coupled to a Hewlett–Packard 3390A reporting integrator. An isocratic elution at 2 ml/min using a 0.4 M $NH_4H_2PO_4$ (J. T. Baker) buffer with a pH of 3.1 (NaOH) gave a separation of ADP, ATP and ATPγS with retention times of 1.65, 2.8 and 4.4 min respectively.

Thiophosphorylation of synaptic membranes and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Synaptosomes (about 1.5 mg protein/ml) were incubated with 10 μM [^{35}S]ATPγS (20 μCi/ml incubation mixture) at 27°. Samples (200 μl) were withdrawn after 1-min and 15-min incubations and quenched by the addition of 50% TCA to a final concentration of 5%. The precipitated protein was washed four times with cold 5% TCA, dissolved in 150 μl of a "stop" solution containing 3% sodium dodecyl sulfate (SDS), 2% mercaptoethanol, 5% glycerol, 100 mM sodium-phosphate buffer, pH 7.0, and a small amount of bromophenol blue dye and heated at 100° for 1 min. Electrophoresis of 50-μl samples (about 100 μg protein/gel) was carried out for about 7 hr according to the method of Weber and Osborn [37]. The gels contained 7.5% acrylamide and 0.27% bisacrylamide in 0.1 M sodium-phosphate buffer, pH 7.0, with 0.2% SDS and were chemically polymerized by addition of *N,N,N',N'*-tetramethylethylenediamine (TEMED) (0.1%) and freshly made ammonium persulfate solution (0.15%). The gels were run at a constant current of 8 mA/gel.

Molecular weights were estimated using the following proteins as molecular weight standards: bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400) and lysozyme (14,300).

Following electrophoresis, the gels were cut into 0.11 cm slices and incubated overnight with 250 μl of a 95:5 mixture of 30% hydrogen peroxide and 58% ammonium hydroxide at 45°. Their radioactivity was measured using an aqueous scintillant (ACS-II, Amersham, Arlington Heights, IL). Gels with standard proteins were first stained with Coomassie blue solution and then destained in a mixture of acetic acid–methanol–water in a model 18-1 Quick Gel destainer (Ames Co., Division Miles Laboratories, Inc.).

Incorporation of [^{35}S]ATPγS into synaptosomal membranes. Synaptosomes (about 2.0 mg/ml) were incubated with 10 or 25 μM [^{35}S]ATPγS (1–3 μCi/ml incubation mixture); samples were withdrawn at the intervals specified in the figures, filtered through glass fiber filters (Schleicher & Schuell, Inc., Keene, NH) and washed twice with 5-ml aliquots of Krebs–Henseleit medium. The filters were air-dried and their radioactivity was measured in a Searle Delta 300 liquid scintillation counter, using aqueous scintillant (ACS II, Amersham).

Release of neurotransmitter. At the end of the initial 5-min preincubation, 0.2 μM [^{3}H]dopamine (sp. act. 28.4 Ci/mmole) with 0.1 mM pargyline or 2 μM [^{14}C]-γ-aminobutyric acid ([^{14}C]GABA) (sp. act. 208.0 mCi/mmole; New England Nuclear, Boston, MA) with 2 mM aminooxyacetic acid was added and the suspension further incubated for 10 min. Aliquots of the incubation mixture were then diluted 10-fold in the same medium (but without added GABA or dopamine), and 200-μl samples were immediately withdrawn and rapidly centrifuged (Beckman microfuge) through a layer of silicone oil (sp. g. 1.03, General Electric, Waterford, NY). Approximately 10 sec later, 60 μM veratridine and 40 mM KCl, or 15 mM KCl (see figure legends for details) were added to achieve depolarization. Samples were withdrawn and centrifuged through silicone oil, and radioactivity was measured in both the pellet and the supernatant fraction.

Uptake of neurotransmitters. Synaptosomal suspensions were diluted in the appropriate media to a protein concentration of about 2 mg/ml, and uptake measurements were started by the addition of one of the radioactive neurotransmitters. Samples (200 μl) of the incubation mixture were withdrawn at the time intervals indicated in the figures and centrifuged through a layer of silicone oil. Radioactivity was measured both in the pellet and the supernatant fraction.

[^{35}S]Adenosine 5'-[γ-thio]triphosphate, sp. act. 65.7 to 80.4 Ci/mmole; 3,4-[7-^{3}H(N)]dihydroxyphenylethylamine (dopamine), sp. act. 24.9 Ci/mmole; and [^{14}C(U)]gamma-aminobutyric acid, sp. act.

208.0 mCi/mmole were purchased from the New England Nuclear Corp. ATPγS was obtained from Boehringer Mannheim (Indianapolis, IN). Acrylamide and *N,N'*-methylenebisacrylamide were obtained from the Eastman Kodak Co. (Rochester, NY).

RESULTS

Hydrolysis of ATP and ATPγS by synaptosomal preparation. Figure 1 compares the time-courses of hydrolysis of ATP and ATPγS by preparation of synaptosomes incubated in Krebs–Henseleit buffer containing 2.5 mM Ca^{2+}. It can be seen that the concentration of ATP in the quenched samples declined very rapidly, and at 60 sec after the addition of 50 μM triphosphate its concentration was almost undetectable. The concentration of ADP initially increased as a mirror image of the decrease in ATP concentration, reached a maximum at 15–30 sec, and then declined at a somewhat slower rate. AMP plus adenosine showed a lag phase followed by a continuous rise.

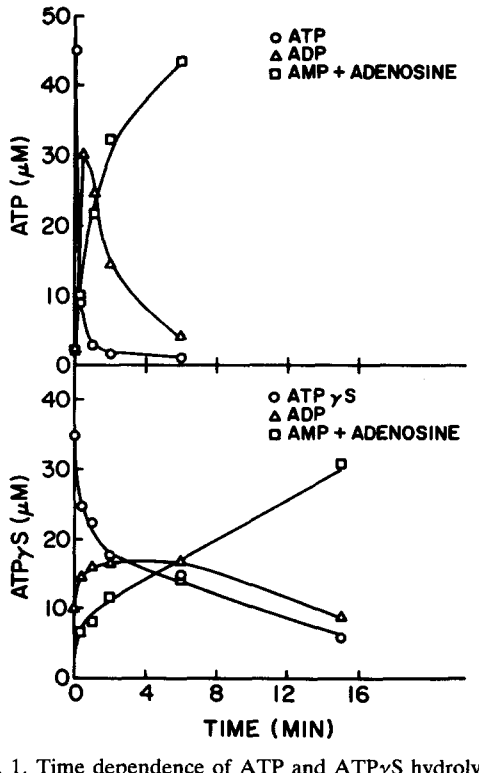

Fig. 1. Time dependence of ATP and ATPγS hydrolysis. Synaptosomes (about 1.5 mg protein/ml) were suspended in Krebs–Henseleit buffer, pH 7.4, containing 10 mM glucose and 2.5 mM $CaCl_2$ and incubated for 5 min at 27° with constant shaking. ATP (50 μM) or ATPγS (50 μM) was then added and the incubation continued. Samples (200 μl) were withdrawn at the times indicated and quenched by the addition of 50% trichloroacetic acid (final concentration of 3%). The precipitated protein was sedimented by centrifugation, and the supernatant fraction removed and neutralized with 5 M K_2CO_3. The concentrations of ATP, ATPγS and ADP were determined by high pressure liquid chromatography (Perkin–Elmer, series-2 liquid chromatograph). The concentrations of AMP plus adenosine were estimated by subtraction of the sum of ATP and ADP measured at each time point from the total ATP + ADP added to the blank (no synaptosomes present).

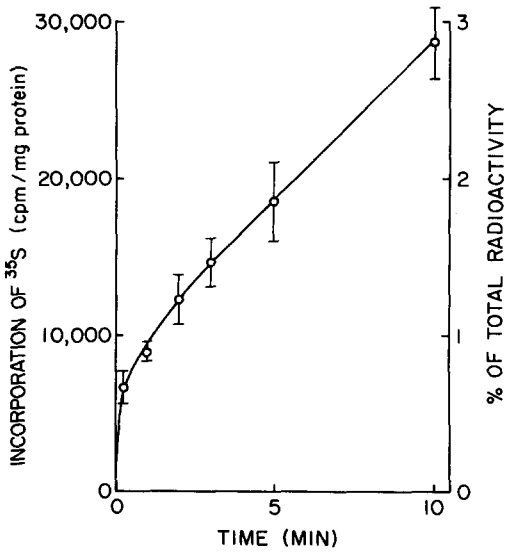

Fig. 2. Time dependence of the incorporation of ^{35}S into synaptic membranes. The synaptosomal suspensions (1.5 to 2.0 mg protein/ml) were incubated with 10 μM [^{35}S]ATPγS (1 μCi/ml of incubation mixture) as described in the legend to Fig. 1. Duplicate samples (200 μl) were removed at the times indicated, diluted to 6 ml with cold Krebs–Henseleit solution, and rapidly filtered through glass fiber filters. The filters were washed twice with 5 ml of cold Krebs–Henseleit buffer and dried in air, and their radioactivity was measured. Samples of an incubation mixture without synaptosomes were filtered and used as a blank. Incorporation of ^{35}S is shown as counts per minute per mg protein or as percent of total radioactivity added. Values are means ± S.D. (N = 4).

By contrast, ATPγS was hydrolyzed at a much slower rate: after 5 min about 40% of the starting concentration was still present in solution. Consequently ADP, and AMP + adenosine increased much more slowly.

Incorporation of ^{35}S into synaptosomes. Figure 2 shows the rate of incorporation of radioactive sulfur into synaptosomes suspended in the presence of 1.3 mM magnesium and 2.5 mM calcium. With 10 μM [^{35}S]ATPγS, the rate of radioactivity increase was biphasic; the initial "jump" completed in about 20 sec was followed by a slower phase whose rate was almost linear in time; at 1 min about 0.7% of the total radioactivity was found in the synaptosomal fraction whereas at 10 min this value was about 3%. Incorporation of radioactive sulfur was dependent on the concentration of magnesium in the suspending medium and increased with the rise in $[Mg^{2+}]_{ext}$ (Fig. 3). By contrast, removal of calcium, addition of veratridine in combination with increase in extracellular potassium concentration, and addition of adenosine 3':5'-monophosphate (cAMP) (in the presence of theophylline) did not affect either the rate or the extent of incorporation of ^{35}S into the synaptosomes (data not shown).

Incorporation of radioactive sulfur was also measured by precipitating the proteins with TCA (5%

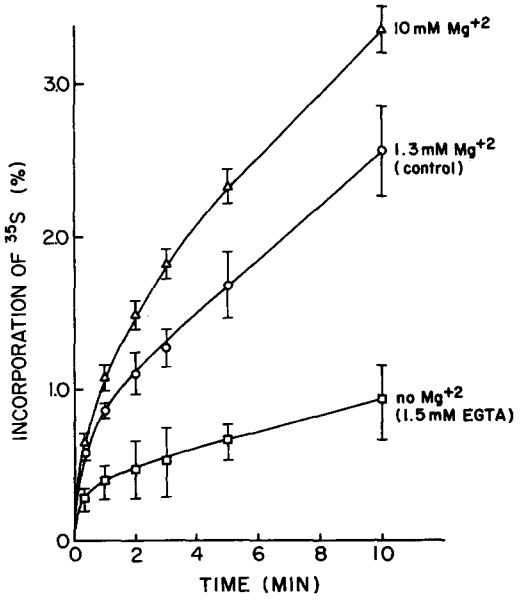

Fig. 3. Dependence of the incorporation of [^{35}S]ATPγS on magnesium concentration. Synaptosomes were suspended and treated as described in the legend of Fig. 2. Values are means of three experiments ± S.D.

final concentration) at various times after the addition of [^{35}S]ATPγS. (The precipitated proteins were washed four times with 5% TCA and finally suspended in 50 μl of "stop" solution (see Materials and Methods for details) before counting with Amersham ACS II aqueous counting scintillant.) The results of such experiments showed that the rate and amount of incorporation of the radioactivity were, within the limits of experimental error, the same as those obtained with filtration techniques.

The stability of thiophosphorylated membrane components was determined by measuring the release of radioactivity from synaptosomes prelabeled with radioactive ATPγS. Synaptosomes were preincubated for 10 min with 10 μM [^{35}S]ATPγS at 27° and centrifuged in the cold (2 min, Beckman microfuge); the pellet was resuspended in the original volume of Krebs–Henseleit buffer containing calcium and glucose. At various times (15 sec to 5 min), aliquots were withdrawn and rapidly filtered (see Materials and Methods). There was no decrease of radioactivity in the pellet over the time interval investigated.

Preincubation of synaptosomes for 1 min with 1 mM ATP before the addition of [^{35}S]ATPγS caused nearly complete inhibition of incorporation of the radioactivity into the sample incubated with the radioactive nucleotide triphosphate for 1 min, whereas after a 15-min incubation a substantial amount of radioactivity was found in the synaptosomal protein (Table 1) consistent with the hydrolysis of ATP in less than 15 min (Fig. 1). Analysis of the radioactivity pattern on SDS-polyacrylamide gel electrophoresis showed that predominant labeling occurred in polypeptides with apparent mol. wt. of 100,000; 84,000; 74,000; 62,000; 55,000; 48,000; and 41,000 (Fig. 4). Some polypeptides with lower molecular weights were also labeled. The presence of protein bands in the respective places on the gels was confirmed by parallel staining with Coomassie blue. The incorporation of ^{35}S was time dependent in that the amount of radioactivity found in the individual peaks was higher at 15 min than in 1 min. The lower panel of Fig. 4 shows, in agreement with the results in Table 1, that 1 mM ATP inhibited incorporation of radioactive sulfur in samples incubated for 1 min with [^{35}S]ATPγS, but the effect was much smaller in the sample incubated for 15 min. The labeling pattern observed in the presence of ATP was not substantially different from that in its absence.

Addition of 10–50 mM NaF to the incubation medium prior to [^{35}S]ATPγS had no measurable effect on either the amount of label incorporated into the synaptosomal peptides or the pattern of their labeling after gel electrophoresis (data not shown).

Figure 5 shows that the rate and extent of incorporation of ^{35}S increased with increasing concentration of ATPγS in the range of 10–500 μM. There was no evidence for saturation even at 500 μM adenine nucleotide.

Effects of hypotonicity and detergent treatment on thiophosphorylation. Figure 6 shows that 7-fold dilu-

Table 1. Effect of ATP on the incorporation of [^{35}S]ATPγS*

Incubation time with [^{35}S]ATPγS (min)	Control		+ ATP	
	cpm (total)	Incorp. (%)	cpm (total)	Incorp. (%)
1	31,875	0.62	1,140	0.02
15	71,190	1.38	44,630	0.88

* The synaptosomal suspension (1.95 mg protein/ml) was preincubated for 2 min with 1 mM ATP, and then 10 μM [^{35}S]ATPγS was added at time zero. Duplicate samples (200 μl) were taken out after 1-min and 15-min incubations, and the reaction was terminated by the addition of TCA to a final concentration of 5%. The precipitated protein was washed four times with 1-ml aliquots of cold 5% TCA, and the pellet was dissolved in 150 μl of a "stop" solution containing 3% SDS, 2% mercaptoethanol, 5% glycerol, 100 mM phosphate buffer, pH 7.0, and a small amount of bromophenol blue dye and heated at 100° for 1 min. The incorporation of ^{35}S was expressed as percent of total radioactivity added.

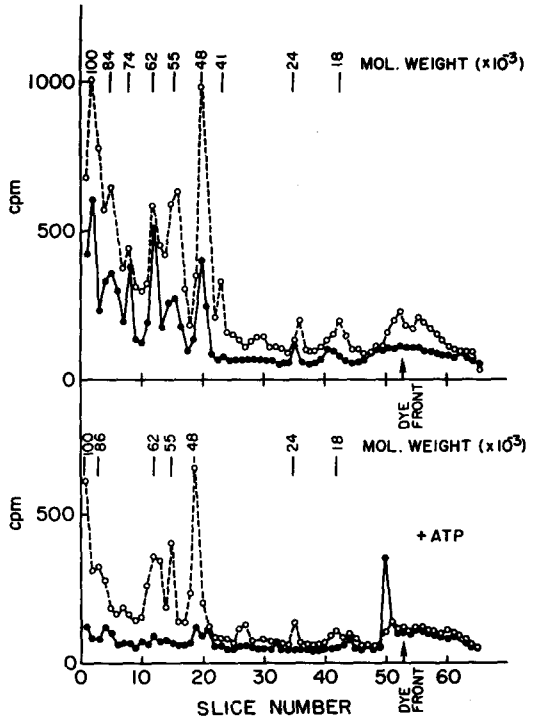

Fig. 4. Molecular weight distribution of thiophosphorylated proteins on SDS-polyacrylamide (7.5%) gel electrophoresis. Samples for gel electrophoresis, taken after 1-min (●—●) and 15-min (○—○) incubations, were prepared as described in the legend to Table 1. Electrophoresis of 50-μl samples was carried out for about 7 hr according to the procedure of Weber and Osborn [37] described in Materials and Methods. Following electrophoresis, the gels were cut into 0.11 cm slices and incubated overnight at 45° with 250 μl of a 30% hydrogen peroxide, 58% ammonium hydroxide (95:5) mixture; the radioactivity was counted after addition of aqueous counting scintillant (Amersham ACS II).

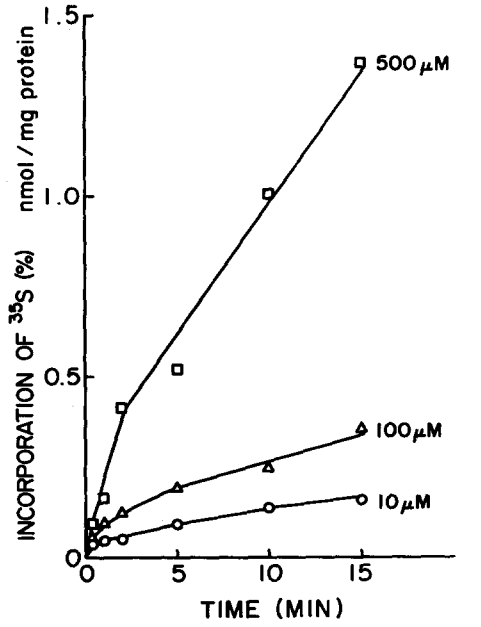

Fig. 5. Dependence of the incorporation of ^{35}S on the concentration of ATPγS. Synaptosomes (2.8 mg protein/ml) were incubated in the presence of 10 μM (510 μCi/mmole) (○), 100 μM (51 μCi/mmole) (△) or 500 μM (10 μCi/mmole) (□) [^{35}S]ATPγS, and filtration of the duplicate samples was carried out as described in the legend to Fig. 2.

tion of synaptosomes in distilled water containing 1.3 mM magnesium and 2.5 mM calcium caused a 2-fold stimulation of incorporation of ^{35}S into the synaptosomes, whereas treatment with Triton X-100 (0.1% final concentration) had a much bigger effect (9-fold stimulation). Raising the magnesium concentration from 1.3 to 10 mM caused a further enhancement in the incorporation of radioactive sulfur although the effect was much more pronounced in hypotonically-treated synaptosomes.

The incorporation of radioactive sulfur into the TCA-precipitable fraction of hypotonically- or detergent-treated synaptosomes was analyzed on polyacrylamide gels in the presence of sodium dodecyl sulfate. Figure 7 shows that the amount of radioactivity recovered in various peaks was increased by both types of treatments but especially by the addition of detergents. Although the overall pattern of labeling does not seem to show striking differences among the three samples, the increases in radioactivities of the various peaks were not identical. In detergent-treated synaptosomes, the greatest changes were seen in peaks with apparent mol. wt. of 100,000; 80,000; 64,000; and 34,000–40,000,

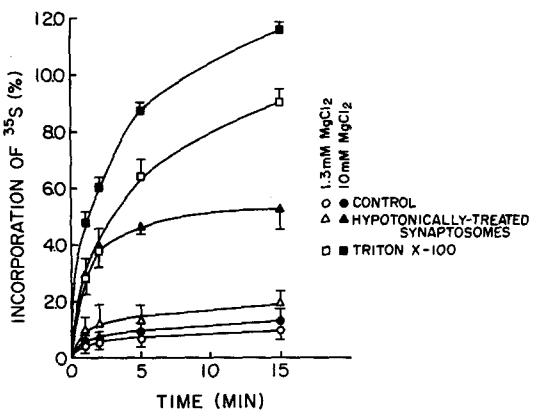

Fig. 6. Incorporation of ^{35}S after hypotonic and detergent lysis. Synaptosomes (about 10–12 mg protein/ml) were incubated for 5 min at 27° and then diluted 7-fold, either in Krebs–Henseleit buffer (control, ○, ●) or in Krebs–Henseleit buffer containing 0.1% Triton X-100 (□, ■) or in distilled water (hypotonically-treated synaptosomes, △, ▲). Incubations were carried out in the presence of either 1.3 mM Mg^{2+} (○, △, □) or 10 mM Mg^{2+} (●, ▲, ■) and with 10 μM [^{35}S]ATPγS added at time zero. Duplicate samples (200 μl) were taken out at the times indicated and rapidly quenched by the addition of TCA to a final concentration of 5%. The precipitated protein was washed three times with 5% TCA and finally dissolved in 50 μl of "stop" solution (see Materials and Methods). The radioactivity of the sample was counted in a Searle Delta 300 counter. Values are means ± S.D. for two experiments.

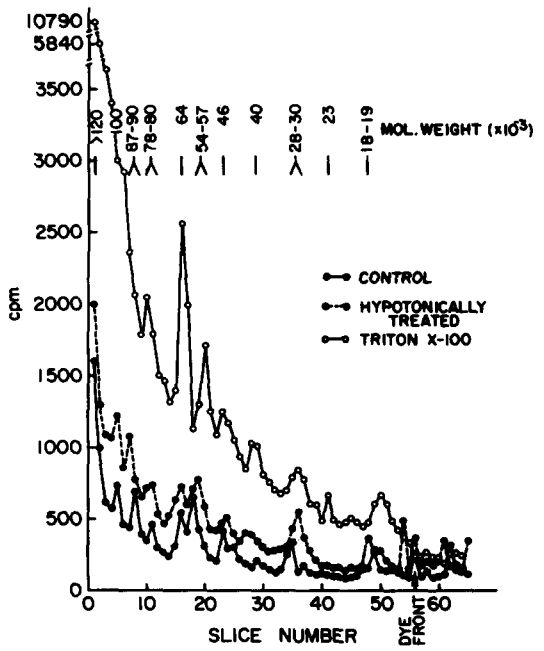

Fig. 7. Effect of hypotonicity and detergent lysis on the labeling pattern of synaptosomal proteins. Conditions and procedures are described in the legend of Fig. 6.

whereas in hypotonically-treated synaptosomes the largest increases in radioactivity were found in peaks with apparent mol. wt. of 100,000; 78,000; and 39,000–40,000.

Interaction of ^{35}S incorporation with neurotransmitters and neurotransmitter transport. Addition of dopamine (10^{-5} M), norepinephrine (10^{-5} M) or carbachol (10^{-4} M) did not affect the incorporation of the radioactive sulfur. In some experiments, a slight inhibition (10–20%) was observed, which was larger with carbachol than with the catecholamines.

ATPγS at 50 μM had no measurable effect on either the uptake or release of GABA, dopamine norepinephrine and 5-hydroxytryptamine (data not shown). A higher concentration of ATPγS (1 mM) did not affect either the uptake or release of GABA but it decreased the transport of dopamine (Fig. 8). Although this inhibitory effect was small (16 ± 1.0%, N = 6), the difference between the control and ATPγS-inhibited samples was statistically significant at the level of $P < 0.025$. Slowly hydrolyzable ATP analogues, methylene adenosine 5′-triphosphate and adenylyl imidodiphosphate (AMP-PNP) and GTP caused, at concentrations of 1 mM, a similar inhibition of dopamine uptake.

Release of dopamine induced by veratridine and potassium was not influenced by 1 mM ATPγS.

* W. R. Bettz and K. J. O'Brien, "End Labelling of DNA Restriction Fragments Using [S³⁵]Adenosine 5′-(γ-ther)Triphosphate followed by Mercury Affinity Chromatography", Meeting of the American Society of Biological Chemistry, St. Louis, MO, 1–4 June, 1981, Abstr. 1787 as expanded in the *New England Nuclear Bulletin*, January 1982.

DISCUSSION

The goal of this investigation was to evaluate the role of ATP in regulating presynaptic function in the central nervous system. Advantage was taken of the availability of an ATP analogue, ATPγS, which is known to serve as a substrate for many phosphorylases and kinases (see e.g. Refs. 32–34) while being a poor substrate for phosphatases. Moreover, the thiophosphate esters that are formed are more resistant to hydrolysis by phosphatases than are their respective phosphate esters. It should be kept in mind, however, that the kinetic parameters for reactions with ATPγS are not the same as those with ATP. In the situations thus far investigated, the K_m values for ATPγS have been found to be higher and the V_{max} values lower [33,*]. Hence, quantitative comparisons in terms of effective concentrations of ATPγS as compared to those of ATP are not always possible, especially when mixtures of phosphatases, kinases and phosphorylases are present in the same tissue preparation. Nevertheless, an evaulation of the effects of ATPγS in an experimental system can provide a qualitative measure of the role of ATP itself.

Thiophosphorylation of synaptosomal proteins. The results presented in this paper show that addition of ATPγS to a preparation of rat brain synaptosomes caused magnesium-dependent thiophosphorylation of synaptosomal proteins which was unaffected by calcium, cyclic AMP and veratridine. The incorporation of radioactive sulfur was biphasic, with the initial rapid phase being completed in less than 15–20 sec. This rapid phase would be expected to be much faster with ATP and may represent phosphorylation on the time scale of the events of synaptic transmission. The incorporation of ^{35}S was completely prevented by ATP which indicates that ATPγS and ATP compete for the same "substrate" sites on synaptosomal proteins. The most prominantly labeled polypeptide chains were those of 100,000; 84,000; 74,000; 62,000; 55,000; 48,000; and 41,000 daltons. It is interesting that the incorporation of ^{32}P into synaptosomes incubated with either ^{32}P-labeled inorganic phosphate or [^{32}P]ATP reported by other authors [38–42] occurs into proteins with similar molecular weights.

Demonstration of thiophosphorylation of synaptosomal proteins raises the question of the nature of sites that are labeled by radioactive sulfur. Preparations of synaptosomes are heterogenous in that they contain presynaptic "pinched-off" vesicles, some postsynaptic elements still attached to the vesicles, and possibly membranes derived from glial cells [31]. Although electron microscopic studies showed that the preparation we use [43] consists of over 80% presynaptic elements, labeling of either the postsynaptic nerve fragments or glia-derived vesicles nevertheless remains a real possibility.

The next problem concerns the site of thiophosphorylation on each of the individual vesicles. It is known that nucleotide triphosphates, including ATP, cross biological membranes extremely slowly [44, 45]. Moreover, [^{35}S]thiophosphate formed by hydrolysis of the ATP-γ-thiophosphate is not readily transported into synaptosomes and, even if it enters,

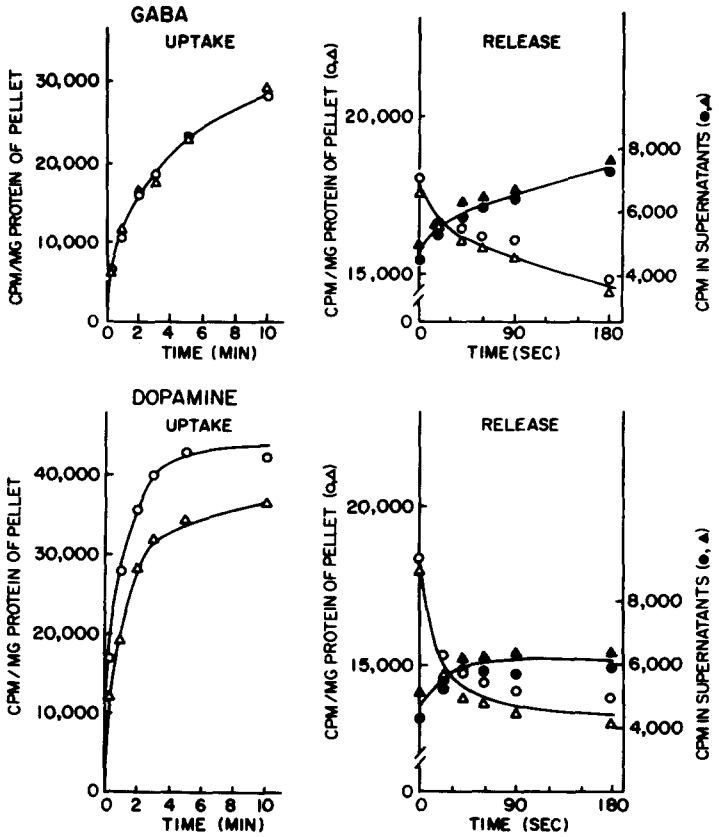

Fig. 8. Influence of 1 mM ATPγS on uptake and release of GABA from total brain synaptosomes and of dopamine from striatal synaptosomes. Brain or striatal synaptosomes (about 9.0 mg protein/ml) were suspended in Krebs–Henseleit buffer, pH 7.4, and preincubated with 10 mM glucose and 2.5 mM $CaCl_2$ for 5 min at 27°. At the end of the preincubation period, in release experiments, 2.0 μM [^{14}C]GABA (with 2.0 mM aminooxyacetic acid) or 0.2 μM [^{3}H]dopamine (with 0.1 mM pargyline) was added, and the samples were further incubated for 10 min. After the 10-min incubation, aliquots were taken out and diluted 10-fold into media either with (△) or without (○) 50 μM ATPγS and incubated for 1 min before adding 40 mM KCl. The first sample (300 μl) (zero time) was taken 5 sec before the addition of KCl. Further samples were withdrawn at the intervals indicated and rapidly centrifuged through a layer of silicone oil. Radioactivity was measured in the pellets (○, △) and the supernatant fractions (●, ▲). In the case of uptake experiments, striatal or brain synaptosomes were diluted in Krebs–Henseleit buffer, pH 7.4, to the final protein concentration of 1 to 2.0 mg/ml and incubated with 10 mM glucose and 2.5 mM $CaCl_2$ in the absence (○) and presence (△) of 50 μM ATPγS for 5 min at 27°. The uptake was started by the addition of 2.0 μM [^{14}C]GABA or 0.4 μM [^{3}H]dopamine. Duplicate samples (200 μl) were withdrawn, centrifuged through a layer of silicone oil, and treated as above. Each point represents a mean of three to five independent experiments where individual values were within 5% of each other.

no significant ATP-γ-thiophosphate will be formed [46]. Thus, incorporation of radioactive sulfur occurs almost exclusively at the surfaces exposed to the suspending medium. This is in contrast to experiments using ^{32}P-labeled inorganic phosphate and ATP where the label is incorporated exclusively (inorganic phosphate) or predominantly (ATP, through hydrolysis to ^{32}P-labeled inorganic phosphate) into internal proteins from metabolically synthesized ATP. In our experiments, labeling includes the inside of a small number of "leaky" vesicles although the functional integrity of the preparation we use [47, 48] and the several-fold stimulation of thiophosphorylation by Triton X-100 seem to argue against this being the major cause of labeling in our system. Although all currently known protein kinases are intracellular [49], it is not unreasonable that the external surface of synaptic membranes, which is unusual in being exposed to ATP as part of its physiological function, may possess an ecto protein kinase (or kinases) responsible for phosphorylation of specific membrane proteins.

It should be pointed out that F^-, an inhibitor of non-specific phosphatases [50], caused no change in either the amount of label incorporated into the peptides or the pattern of labeling observed following SDS gel electrophoresis. It is unlikely, therefore, that incorporation of label into phosphatases played a significant role in the phenomena described in this paper.

Effect of thiophosphorylation and ATPγS on neurotransmitter uptake and release. ATPγS at concentrations of 50–100 μM had no effect on either uptake or release of GABA or dopamine. This

implies that neither an ATP receptor nor phosphorylation of external surface of presynaptic membrane proteins affects these functions. This interpretation is in contrast with the suggestion of Brennan and Cantrill [30] that phosphorylation of synaptosomal proteins which occurred during a 15-min incubation with 1 mM ATP was responsible for inhibition of GABA release induced by high concentration of potassium. Since a prolonged incubation with ATP must have led to a complete hydrolysis of this nucleotide to adenosine, it is possible that what was observed was the inhibitory effect of adenosine [5].

Our results show that high concentrations of ATPγS (about 1 mM) inhibited the uptake of dopamine by about 20%. This inhibition was caused by the nucleoside triphosphate itself and not by thiophosphorylation of synaptosomal proteins because the slowly- or non-hydrolyzable analogues of ATP exhibited the same inhibitory property. The effect may be caused either by nonspecific interactions between dopamine, a positively charged amine, and the negatively charged phosphate residues on the nucleoside triphosphates or by interactions of ATP with membrane binding sites which affect directly or indirectly dopamine uptake. It is difficult to evaluate the physiological significance of this observation because of the small size of the inhibitory effect. However, as long as the actual concentrations of ATP and dopamine during synaptic transmission remain unknown, it should be kept in mind that formation of complexes between ATP and the biogenic amines may cause transient changes in the concentrations of free amines in the synaptic cleft and hence affect the various pre- and postsynaptic events.

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