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Increased Presynaptic ATP Levels Coupled to Synaptic Activity at the Crayfish Neuromuscular Junction

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Levels of ATP and related adenylates were measured in the terminal region of efferent nerves in the crayfish opener muscle using the luciferin-luciferase method. Following 1 min of stimulation at 50 Hz, the average (±SE) ATP content rose from 13.4 (±1.5) to 19.0 (±2.1) nmol/mg dry weight. The amounts of ADP, AMP, and the phosphagen phosphoarginine did not change significantly. Thus, the increased ATP was not derived from any of these potential sources. The increase was found to depend on synaptic activation, however, for its magnitude was directly related to the concentration of extracellular Ca^{2+}, and it was blocked when $CoCl_2$, verapamil, ruthenium red, or γ-methylglutamate and picrotoxin were added to the bath. Addition of ATP to the bath solution also increased nerve ATP levels. Based upon measurements of sucrose distribution, only 50% of this increase was in the extracellular water space. The remainder of the ATP had either entered the nerve, become adsorbed extracellularly, or both. Addition of 2-deoxy-D-glucose and γ-^{32}P-ATP to the bath resulted in the formation of ^{32}P-2-deoxy-D-glucose-6-P by the nerve. This suggests that a fraction of the extracellular ATP does enter the neuron chemically intact. To determine whether exogenous ATP is the source of the increased ATP measured in the nerve following stimulation, the bath was assayed for ATP. Stimulation did cause ATP levels to increase significantly; however, the maximum concentration was 3 orders of magnitude lower than that required to increase ATP levels in resting nerve. Thus, the ATP released during stimulation, presumably from the muscle, may account for the increased nerve ATP; however, the local concentration of ATP immediately surrounding the nerve must either be higher than that assayed in the bathing medium or ATP must be taken up more readily during nerve activity.

Free energy from ATP hydrolysis is required to maintain long-term excitability of nerve cells. Specifically, ionic gradients across the membrane are maintained by the Na^+-K^+ ATPase (Brinley and Mullins, 1967). Thus, when axons are exposed to metabolic inhibitors, such as cyanide, they become depolarized and inexcitable (Hodgkin and Keynes, 1955). There is also active transport of Ca^{2+} out of the axon, driven at the expense of ATP (DiPolo and Beauge, 1979).

During repetitive action potential discharge, there is an elevated requirement for ATP due to the increased active transport of these ions (Baker and Connelly, 1966; Ritchie, 1967). In isolated nerves, this is manifest as an increased metabolic rate (Gerard, 1932). Indeed, there is a general relationship between discharge rate and oxygen consumption (Ritchie, 1967). The ability of a nerve cell to compensate for this greater demand for ATP may largely determine its capacity to sustain repetitive activity. In several preparations, axonal ATP and phosphocreatine (PCr) content reportedly decreased during high-frequency (>50 Hz) stimulation (Greengard and Straub, 1959; Montant and Chmouliovsky, 1968; *cf.* Okada and McDougal, 1971; Smith, 1980b).

To assess the relationship between changes in ATP levels and nerve activity, we measured high-energy phosphates in the terminal region of motor axons in the crayfish. In contrast to results obtained in other preparations, ATP was found to increase significantly following high-frequency stimulation. Moreover, this increase was dependent on synaptic activation, most probably of the postsynaptic cell. Stimulation also causes the level of ATP in the bath to increase, suggesting this as a possible source of the increased presynaptic ATP content.

Materials and Methods

Experimental preparation

All experiments were performed on the efferent nerve innervating the opener muscle of the first walking leg of the crayfish (*Procambarus clarkii*). In each preparation, the closer muscle and the blood vessels were removed, exposing the inner surface of the opener muscle. The nerve, consisting of one excitor and one inhibitor axon, could then be seen clearly as it projected along the surface of the muscle.

After exposing the nerve, the tissue was placed in a 23°C circulating bath of well-oxygenated saline solution (Smith, 1983) for 25 min. The entire preparation—including the opener muscle, its associated exoskeleton, and the nerve—was then quick-frozen in liquid nitrogen either immediately (*nonstimulated*) or after repetitive stimulation (*stimulated*) of the nerve for 1 min at 50 Hz. Transferring the preparation from the bath to the liquid nitrogen takes about 5 sec, during which time there is a negligible change in ATP levels in crayfish (Smith, 1980b).

Electrophysiology

The nerve was stimulated in the meropodite region using a suction electrode. The efficacy of stimulation was monitored by recording action potentials from the nerve along the proximal surface of the opener muscle using microelectrodes filled with 4 M NaCl. Synaptic activity was monitored by recording excitatory postsynaptic potentials intracellularly in the opener muscle using microelectrodes filled with 3 M KCl. In addition, activity at single synaptic release sites was recorded using focal extracellular microelectrodes. The criteria for selecting recording sites and specific technical details are presented in Niles and Smith (1982).

Assay procedures

ATP

To determine the ATP levels in a small sample of the nerve, it was necessary to dissect the appropriate tissue without allowing any signif-

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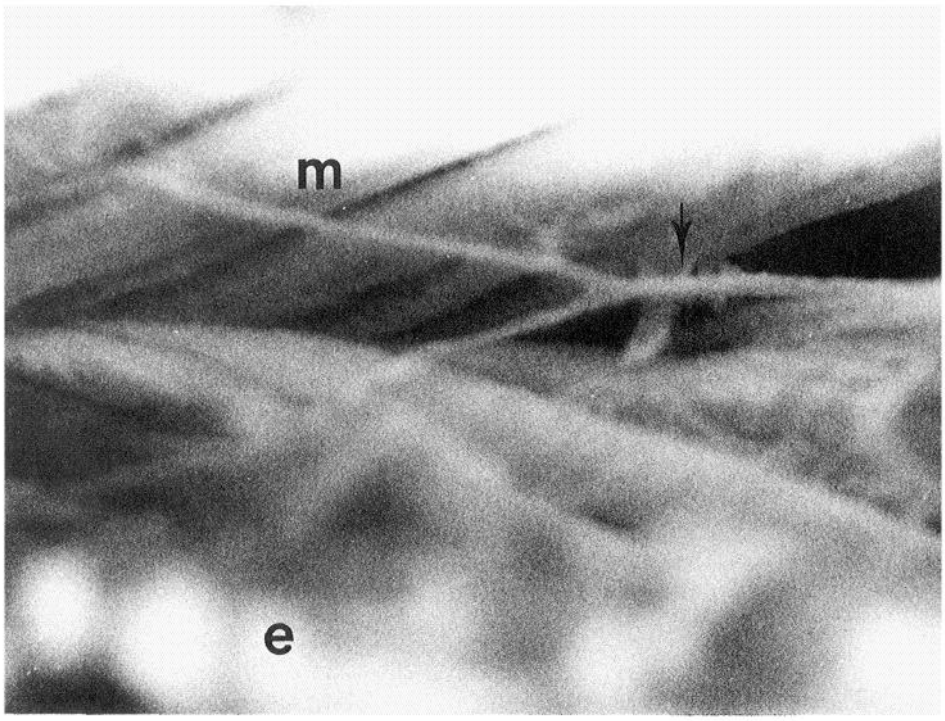

Figure 1. Opener muscle innervation following quick-freezing and lyophilization. The nerve (*arrow*) in this region is dissected free, weighed, and prepared for the subsequent assays. Underlying opener muscle fibers (*m*) and surrounding exoskeleton (*e*) are also visible. ×160.

icant metabolic activity to occur. Lyophilization of the tissue has been shown to prevent hydrolysis of high-energy phosphates, permitting the nerve to be dissected at room temperature without a decrease in ATP levels (Lowry and Passonneau, 1972). Thus, the frozen specimens were placed in a lyophilizer and brought to a vacuum (≤0.005 mm Hg) before they could thaw. They were then freeze-dried overnight.

Following this freeze-drying, a small nerve sample was dissected from the terminal region (Fig. 1). Numerous small branches came off with the larger parent axons. These presumably included synaptic release sites, since they are located in varicosities along these finer processes (Florey and Cahill, 1982). The dissected nerve was then weighed. In early experiments, a Cahn Electrobalance (model 26) was used; in the latter part of this study, quartz-fiber "fishpole" balances were used (Lowry and Passonneau, 1972) to improve resolution. The dry weight of the samples was usually less than 1 μg.

ATP was assayed using the luciferin–luciferase ("Firefly") method (Lust et al., 1981). After weighing the tissue, it was placed in 50 μl of 0.1 N NaOH and vortexed. A 10 μl aliquot was then added to 100 μl of buffer solution containing 50 mM glycylglycine (pH 8.1), 7.5 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM EGTA, and 0.04% BSA. Luciferin–luciferase (50 μl; DuPont) was added. After swirling the preparation for 5 sec, the reaction cuvette was placed in a photometer (Aminco), and the light intensity at 5 sec was recorded. The amount of ATP represented by this peak was then calculated from standard curves according to the method described by Lust et al. (1981). With this assay, quantities of ATP as small as 10^{-14} mol could be detected.

In some experiments, ATP levels in the bath solution were assayed. A small aliquot from the bath was added directly to the 50 μl of luciferin–luciferase in these cases.

Total adenylates

Levels of total adenylates (ATP, ADP, and AMP) were measured in a 3-stage modification of the basic ATP assay. The lyophilized tissue was placed in 50 μl of 0.1 N HCl and vortexed. A 10 μl portion of this tissue extract was placed into 200 μl of imidazole buffer solution containing 100 mM imidazole, 2 mM $MgCl_2$, 75 mM KCl, and 3 mM phosphoenolpyruvate. The ATP levels in a 50 μl sample were then measured using the luciferin–luciferase procedure. To measure ATP + ADP, another 10 μl aliquot of the tissue extract was added to 200 μl of the imidazole buffer solution plus pyruvate kinase (25 units/ml of buffer solution), which catalyzes phosphorylation of ADP to ATP. This was allowed to incubate for 30 min at room temperature. The ATP content, representing tissue ATP + ADP in this case, was then assayed in a 50 μl sample. Total adenylates (ATP + ADP + AMP) were measured in a third 10 μl aliquot of tissue extract. Myokinase (7.5 units/ml imidazole buffer solution), which catalyzes formation of ADP from AMP and ATP, and pyruvate kinase were added to the incubation medium, and the tissue was allowed to incubate for 60 min at room temperature. ATP levels measured in these samples represent total adenylates in the tissue. The corresponding values for ADP and AMP content were calculated from these data. Conversion of AMP and ADP to ATP by these reactions was >95% (see Lust et al., 1981).

Phosphoarginine

The phosphagen phosphoarginine (PArg) was measured by converting it to arginine via arginine kinase and then measuring the increase in ATP concentration. Specifically, lyophilized tissue was placed in 50 μl of 0.1 N NaOH. A 20 μl aliquot was added to 200 μl of an imidazole buffer containing 50 mM imidazole-HCl (pH 7.0), 1 mM $MgCl_2$, 0.6 μM purified ADP, and 0.02% BSA. Following a 30 min incubation, tissue ATP levels were than assayed in this preparation. A second 20 μl aliquot of the tissue extract was added to 200 μl of this imidazole buffer plus arginine kinase (20 units/ml of buffer solution). After the 30 min incubation, ATP levels, representing tissue ATP + PArg, were assayed. The PArg values were calculated from these data. Conversion to ATP was >95% (Lust et al., 1981).

The success of this PArg assay depends upon adequate purification of the ADP added to the reaction mixture. We used the enzymatic procedure specified by Lowry and Passonneau (1972, p. 153) without modification.

Chromatography

Adenosine, AMP, and ADP

Adenosine, AMP, and ADP were detected by UV absorbance (254 nm) following chromatographic separation on a reverse-phase high-pressure liquid chromatography (HPLC) column (Ultrasphere ODS) using the ion-pairing reagent tetrabutylammonium dihydrogen phosphate (TBA). A 2-solvent system was used. Solvent A consisted of 4% acetonitrile in 0.03 M KH_2PO_4 with 5 mM TBA at pH 6.0; solvent B was 100% acetonitrile. The following gradient was found to give optimal separation of the desired compounds: 4 min with 5% solvent B and then 2 min with 15% solvent B, followed by a linear gradient from 15 to 25% solvent B over a period of 4 min. Flow rate was 1.5 ml/min.

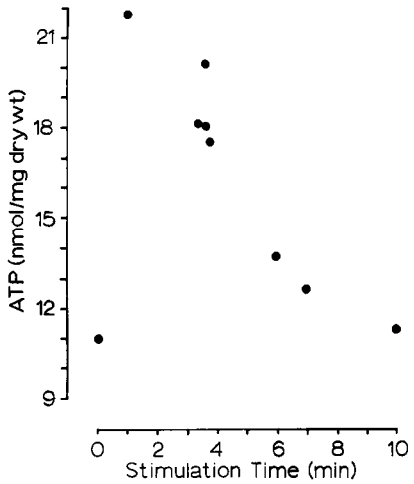

Figure 2. Increased ATP levels following axon stimulation until conduction failure occurred. Tissue was bathed in normal saline for 20 min and then stimulated at 50 Hz for the indicated time until conduction block occurred in nerve terminals included in the region assayed. Each point represents values measured in a single nerve.

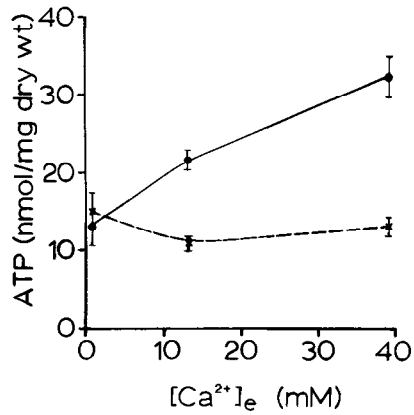

Figure 3. Calcium dependence of the ATP increase. Values of ATP were determined in nerves that had been stimulated for 1 min at 50 Hz (*circles*) and in nonstimulated controls (*crosses*). Extracellular Ca^{2+} concentration varied from 0.01 to 39 mM. Each point is the average (±SE) of 8 measurements from different animals.

Deoxyglucose-6-P

Deoxyglucose-6-P was isolated by a method described by Gatley et al. (1984). Briefly, the preparation was incubated in a saline solution containing ^{3}H-deoxyglucose (10 mM, 25 mCi/mmol) and ^{32}P-ATP (5 mM, 50 mCi/mmol) for a specified time, then washed for 5 min in cold saline, and frozen immediately in liquid nitrogen. The frozen specimens were lyophilized, and the nerve was dissected from the muscle. The dissected nerve was subsequently weighed on the quartz-fiber "fishpole" balance and homogenized in 50% acetonitrile. The mixture was centrifuged to separate the protein and 20 μl aliquots of the supernatant were analyzed isocratically by HPLC on an anion-exchange column (Altex Ultrasil-AX) with 20 mM K_2PO_4 (pH 6.7) as the mobile phase at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and assayed for ^{3}H and ^{32}P by liquid scintillation counting.

Electron microscopy

The methods were identical to those referenced in Smith (1983).

Results

Tissue levels of high-energy phosphates

Tissue levels of ATP, ADP, AMP, and PArg obtained in a typical assay are presented in Table 1. The excitor and inhibitor axons could not be separately dissected in the terminal regions, so the assay measured their combined ATP levels. We were able to calculate concentrations (as mol/liter of cell water) from the measured values of nmol/mg dry weight using the following relationships for crayfish nerve (Smith, 1980b): wet wt/dry wt = 6.8, and mg wet wt/μl water = 1.2. Following stimulation of the excitor axon for 1 min at 50 Hz, the ATP content of the nerve increased from 13.4 to 19.0 nmol/mg dry weight (Table 1). This difference (42%) is statistically significant at the 0.05 level. The ADP and AMP levels also rose (Table 1). These changes represent a significant (0.05 level) increase in total adenylates.

The energy charge, (½[ADP] + [ATP])/([AMP] + [ADP] + [ATP]), which quantifies the extent to which the adenylate system is "filled" with high-energy phosphates did not differ significantly. The values were 0.87 and 0.86 in the nonstimulated and the stimulated tissue, respectively. This indicates that there were no major changes in the responses of the ATP regulatory enzymes to the relative concentrations of ATP, ADP, or AMP. Similar results were observed in 4 other series of experiments.

In this preparation, high-frequency (50 Hz) stimulation is associated with conduction block in the excitor axon (Smith, 1980a). The ATP content was assayed in axons stimulated at 50 Hz until extracellularly recorded action potentials observed at a proximal recording site failed to propagate to a more distal recording site within the region to be analyzed (Smith, 1980a). These times ranged from 1 to 10 min. As shown in Figure 2, smaller changes in ATP were observed following longer stimulation times, but the levels were always higher than in nonstimulated cells. After 10 min of repetitive action potentials, the ATP measurements were not significantly different from those of nonstimulated cells. Thus, blockage was not readily associated with reduced ATP; on the contrary, ATP levels were generally elevated at the time of failure, although very localized deficiencies cannot be ruled out.

Table 1. Levels of high-energy phosphates in crayfish axons

	Nonstimulated			Stimulated	
Compound	nmol/mg	mM		nmol/mg	mM
ATP	13.4 ± 1.5	2.4 ± 0.3	*	19.0 ± 2.1	3.4 ± 0.4
ADP	4.7 ± 1.1	0.8 ± 0.2		6.4 ± 0.8	1.1 ± 0.1
AMP	0.1 ± 0.1	0.02 ± 0.02		0.3 ± 0.2	0.1 ± 0.04
Total adenylates	18.2 ± 1.8	3.2 ± 0.3	*	25.7 ± 2.4	4.5 ± 0.4
PArg	12.2 ± 0.5	2.2 ± 0.1		14.6 ± 3.9	2.6 ± 0.7

Average (±SE) values of data obtained from 8 preparations. The nerve was stimulated for 1 min at 50 Hz. Asterisks indicate that the corresponding values for nonstimulated and stimulated tissue differ statistically at the 0.05 level (2-tailed *t* test).

Table 2. Effects of agents that block synaptic transmission on ATP levels in crayfish axons

Compound (mM)	Nonstimulated		Stimulated	
	nmol/mg	mM	nmol/mg	mM
$CoCl_2$ (10)	10.5 ± 1.0	2.2 ± 0.2	10.6 ± 0.6	1.9 ± 0.1
Verapamil (0.1)	14.2 ± 2.8	2.5 ± 0.5	19.4 ± 2.4	3.4 ± 0.4
Ruthenium red (0.01)	13.9 ± 3.6	2.5 ± 0.6	15.5 ± 1.5	2.7 ± 0.3
γ-Methylglutamate (2) and picrotoxin (2)	11.8 ± 2.0	2.1 ± 0.4	12.5 ± 1.1	2.2 ± 0.2

Average (±SE) values of ATP levels obtained from at least 6 preparations. The nerve was stimulated for 1 min at 50 Hz. The concentrations (mM) of the compounds added are indicated in parentheses.

An immediate source of the increased ATP might be the phosphagen, PArg. Thus, its levels were also assayed. The results, presented in Table 1, indicated that the PArg content did not change significantly during the 1 min stimulation. Indeed, the lack of any decrease in ADP indicates that the increased ATP is not derived from endogenous neuronal adenylates.

Control measurements of ATP levels in stimulated mammalian nerve

For comparison, we measured the effects of stimulation on ATP levels of rat phrenic nerve. In this mammalian preparation, however, nerve terminal regions could not be dissected free from the surrounding muscle. Thus, samples were taken from a region about 1 cm proximal to the diaphragm muscle. Small nerve bundles, with an average (±SD) dry weight of 123 (±88) μg, were dissected from 4 different animals. The ATP content was then measured in nonstimulated and stimulated tissue; stimulation was delivered at 20 Hz for 1 min. The average (±SE) values were 11.5 (±2.3) and 6.0 (±0.5) nmol/mg dry weight, respectively. In contrast to the crayfish results, stimulation resulted in a 48% reduction of the ATP levels in rat nerve. This difference is significant at the 0.05 level.

The observed drop in ATP levels during high-frequency repetitive stimulation of the rat nerve is consistent with data reported by previous investigators (Greengard and Straub, 1959; Montant and Chmouliovsky, 1968). However, the rise in ATP and total adenylates seen in single crayfish axons is unique, and further experiments were performed to elucidate the underlying mechanisms.

Dependence on synaptic transmission

Further investigation in crayfish indicated that the increase in ATP associated with nerve stimulation required synaptic transmission.

The magnitude of the ATP increase during stimulation became larger as $[Ca^{2+}]_e$ was increased. In Figure 3, this relationship is shown for $[Ca^{2+}]_e$ ranging from 0.01 to 39 mM. Furthermore, addition of 10 mM Co^{2+} to the extracellular bath, which effectively blocks Ca^{2+} currents and transmitter release in this system, blocks the stimulation-induced rise in ATP levels. Verapamil (10^{-4} M) and ruthenium red (10^{-5} M) also reduced the amount by which ATP rose during stimulation, although the effect was less pronounced. However, these 2 compounds were found in this study to decrease average quantal release by 31 and 35%, respectively, after 25 min. These results are summarized in Table 2.

Thus, the stimulation-induced rise in ATP depends on the influx of Ca^{2+} into the presynaptic nerve, suggesting that transmitter release is required. This requirement was further characterized in a separate series of experiments in which antagonists of glutamate and GABA, the putative excitatory and inhibitory transmitters, were added to the bath. Picrotoxin (2 mM) was added to the bath, for it effectively blocks the inhibitory responses. It has proved more difficult to block the excitatory junctional potential completely. However, γ-methylglutamate (2–20 mM) was found to reduce postsynaptic potential amplitudes by about 50% (Lowagie and Gerschenfeld, 1970; Zucker, 1974). Thus, it was added to the bath. As shown in Table 2, axonal ATP did not increase during stimulation under these conditions. Similar results were also obtained when γ-methylglutamate but not picrotoxin was added to the bath.

Presynaptic accumulation of exogenous ATP

Studies were then performed to determine whether ATP levels in the bath affect axonal ATP content. ATP, in concentrations ranging from 1 to 10 mM, was added to the bath solution, and the entire preparation was allowed to incubate for 3 min. The tissue was then rinsed in distilled water and axonal ATP was measured. As shown in Figure 4, there was a significant increase in ATP associated with the axon in the presence of 5 and 10 mM ATP. At 1 mM, there was no measurable increase. To determine the apportionment of exogenous ATP between intra- and extracellular spaces of the isolated nerve, the distribution of added ATP was compared to that of sucrose, an extracellular marker. Tissue samples from 8 different preparations were incubated for 3 min in bath solutions containing ^{14}C-sucrose (673 mCi/mmol) and ^{3}H-ATP (26 Ci/mmol). The concentrations of sucrose and ATP added to the bath were 0.2 and 5 mM, respectively. The tissue ATP and the activities of ^{3}H-ATP and ^{14}C-sucrose in the dissected nerve were then assayed using the

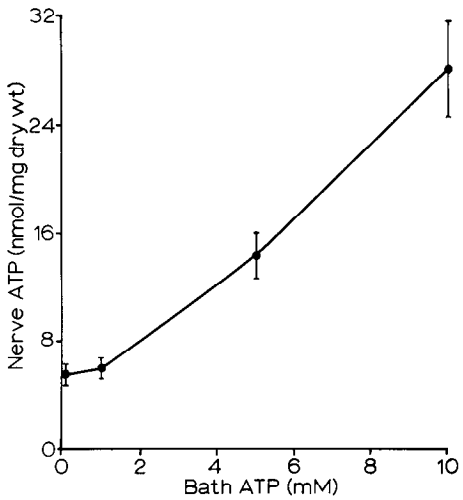

Figure 4. Incorporation of bath-applied ATP into the nerve. ATP was added to the bath, and the entire preparation was allowed to incubate for 3 min. The ATP content of the nerve was then assayed. Each point represents the average (±SE) of at least 7 measurements from different preparations.

Table 3. Sucrose space, ATP space, and nerve ATP levels

^{14}C-sucrose space (nl)	^{3}H-ATP space (nl)	Tissue ATP increase (nmol/mg dry wt)		
		Total increase	Increase in sucrose space	Net increase
0.67 ± 0.09	1.30 ± 0.27	11.4 ± 2.2	6.7 ± 0.9	4.7 ± 2.4

The average (±SE) values of data obtained from 8 different preparations. The concentrations of sucrose and ATP added to the bath were 0.2 and 5 mM, respectively. The incubation time was 3 min. The amount of ATP in the sucrose space was calculated using the formula

$$\left(\frac{\text{ATP in space}}{\text{(nmol/mg dry wt)}}\right) = \left(\frac{\text{tissue } ^{14}\text{C DPM/mg dry wt}}{\text{bath } ^{14}\text{C DPM/liter}}\right) \times \text{(nmol bath ATP/liter)}$$

luciferin–luciferase method and a scintillation counter, respectively. The sucrose space, the ATP space and the total ATP within these spaces were calculated from the ^{14}C, ^{3}H, and ATP contents of the tissue sample and the bath saline. Similarly, 8 more preparations were dissected and incubated for 3 min in normal saline, and tissue ATP was measured. The increase in tissue ATP was calculated by subtracting the average ATP level in the control tissue from the average ATP level in the preparations incubated with the sucrose and ATP.

The results of these experiments are shown in Table 3. They indicate that after correcting for the ATP in the sucrose space, at least 40% of the total increase in ATP remains. Another way to analyze these results is to calculate the apparent ATP space. Assuming that the added ATP became distributed uniformly throughout the extracellular compartment, the ATP space was calculated to be 50% larger than the sucrose space. These observations suggest that a significant (approximately 50%) portion of the exogenous ATP is either entering the nerve, becoming adsorbed extracellularly, or both.

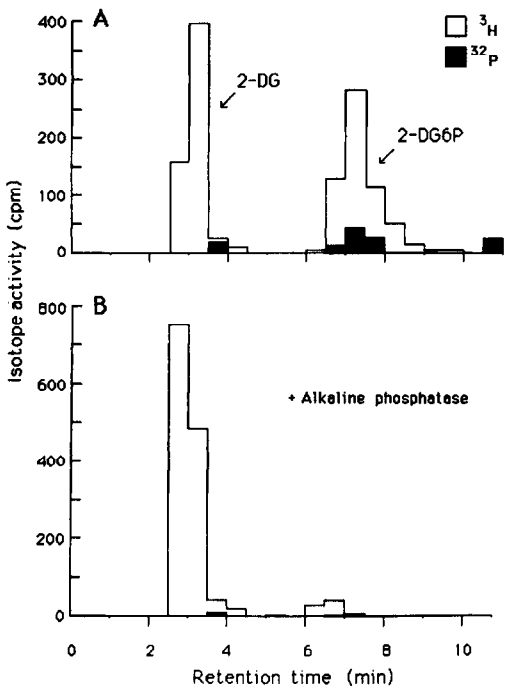

Figure 5. Chromatographic analysis of nerve. The preparation was incubated with ^{3}H-deoxyglucose and ^{32}P-ATP for 15 min and then washed for 5 min in cold saline. *Open bars,* amount of ^{3}H found in each fraction; *closed bars,* ^{32}P. In *B* the nerve extract was treated with alkaline phosphatase for 60 min immediately prior to analysis.

The possibility that exogenous adenosine (1 mM) might also lead to increased axonal ATP levels was tested in similar experiments and ruled out. Indeed, ATP content decreased by 35%. Thus, the increase in tissue ATP elicited by exogenous ATP is not due to the formation of adenosine, which then entered the nerve and induced ATP synthesis.

Metabolism of bath-applied ATP

Although the above observations suggest that exogenous ATP is being taken up by the nerve, they do not exclude the possibility that the ATP is merely becoming adsorbed extracellularly to the nerve tissue. Therefore, the following experiments were performed to determine whether extracellular ATP can be metabolized by the nerve, for this would indicate whether ATP is being taken up into the axoplasm. The first step in the metabolism of glucose, the phosphorylation of glucose to glucose-6-P, was examined. This reaction, catalyzed by the enzyme hexokinase, was chosen because the reaction is essentially irreversible; moreover, an analog of glucose exists, 2-deoxy-D-glucose, that becomes phosphorylated in the cell but is not metabolized further (Wick et al., 1957). Thus, the product 2-deoxy-D-glucose-6-P accumulates in the cell once it is formed, increasing its ease of detection. If it could be demonstrated that the extracellular application of ^{32}P-labeled ATP causes deoxyglucose to acquire a ^{32}P label during its conversion to deoxyglucose-6-P, this would indicate that ATP was being taken up by the neuron. Experiments were performed to test this possibility.

The preparation was incubated in buffer containing ^{3}H-deoxyglucose and γ-^{32}P-ATP, and the nerve metabolites were then separated by HPLC (see Materials and Methods) and assayed for both ^{3}H and ^{32}P. The ^{3}H label was used to identify the metabolites related to 2-deoxy-D-glucose. Since 2-deoxy-D-glucose undergoes only one reaction in the cell, the ^{3}H label should only be associated with the neutral sugar, 2-deoxy-D-glucose, and its phosphate ester, 2-deoxy-D-glucose-6-P. The ^{32}P label, on the other hand, may become associated with many different metabolites in the cell if it does, in fact, cross the cell membrane. The correspondence of ^{32}P label with ^{3}H, however, would suggest that the ^{32}P label had been incorporated into 2-deoxyglucose-6-P.

In Figure 5*A* the results of such an experiment are shown. Of the total ^{3}H label, 49% eluted at the void volume, which is expected for neutral sugars, while 51% was retained as expected for sugar phosphates (Gatley et al., 1984). In contrast, most of the ^{32}P label could not be eluted from the column with 20 mM K_2PO_4, but required raising the concentration of K_2PO_4 to at least 200 mM. Nevertheless, in 20 mM K_2PO_4 some ^{32}P did elute in the same fractions as the deoxyglucose-6-P. The total ^{32}P counts (±SE) measured in this peak were 1280 ± 98, which is significantly different from measurements of background (390 ± 32; 0.05 level). Similar results were obtained in 18 trials.

This suggested that the 2-deoxy-D-glucose had acquired some

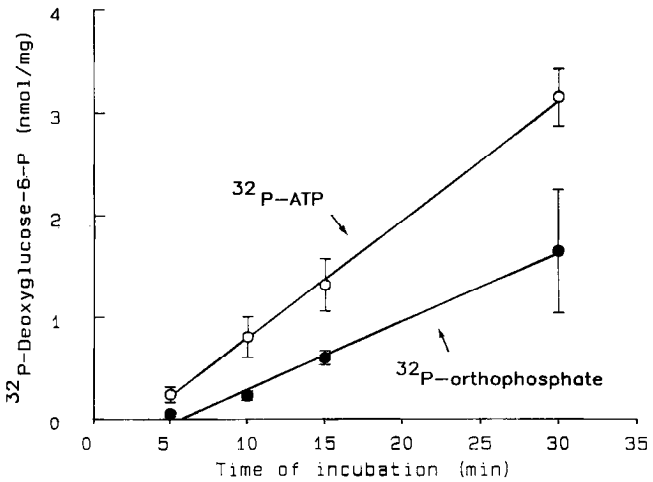

Figure 6. Time course of the incorporation of ^{32}P into deoxyglucose. The amount of extracellularly derived ^{32}P label found in the same fractions as deoxyglucose-6-P is plotted as a function of time of incubation with ^{3}H-deoxyglucose and either ^{32}P-ATP (*open circles*) or ^{32}P-orthophosphate (*closed circles*). The amount of ^{32}P-labeled deoxyglucose-6-P was calculated using the formula:

$$\left(\begin{matrix}\text{^{32}P-deoxyglucose-6-P}\\ \text{(nmol/mg dry wt)}\end{matrix}\right) = \left(\frac{\text{tissue ^{32}P DPM/mg dry wt}}{\text{bath ^{32}P DPM/liter}}\right) \times \left(\begin{matrix}\text{mmol}\\ \text{bath}\\ \text{ATP/liter}\end{matrix}\right)$$

where tissue ^{32}P DPM refers to the total isotope activity in the fractions associated with the 2-deoxyglucose-6-P. Each point represents the average (±SE) of at least 3 measurements from different preparations.

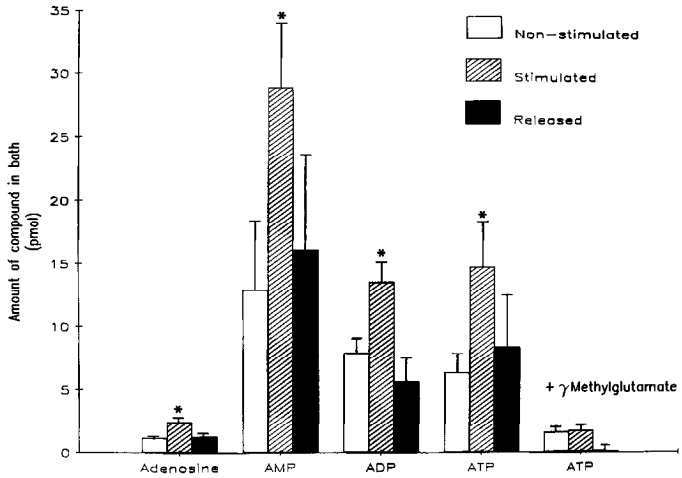

Figure 7. Release of adenine nucleotides and adenosine upon stimulation of the excitor axon. Stimulation-induced release was calculated by subtracting the amounts measured in the bath in the nonstimulated cases from the amounts measured following stimulation (20 min, 10 Hz). Bath volume was 5 μl; this was attained by sealing the exoskeleton surrounding the opener muscle with petroleum jelly to form a small chamber. Values represent the averages (±SE) of 5 or 6 measurements taken from different preparations. *Asterisks* indicate the stimulated levels are statistically different from the nonstimulated levels (0.05 level).

of the ^{32}P label. To determine whether the label was truly bound covalently to the deoxyglucose, alkaline phosphatase was added to the nerve extract to hydrolyze any phosphate esters that might be present. After treatment of the nerve extract with alkaline phophatase, the retained ^{3}H peak and its corresponding ^{32}P peak were reduced by 87 and 84%, respectively (Fig. 5*B*), showing that it indeed represents the phosphate ester, 2-deoxyglucose-6-P. Thus, this experiment demonstrated that ^{32}P label provided extracellularly on ATP becomes covalently linked to a cellular metabolite, 2-deoxy-D-glucose-6-P.

The most direct way for this to occur would involve the entry of ^{32}P-ATP into the nerve, followed by the transfer of the ^{32}P label to 2-deoxy-D-glucose via the hexokinase reaction. However, it is also possible that the ^{32}P-ATP became hydrolyzed outside the cell to ADP and ^{32}P-orthophosphate. The ^{32}P label could then have entered the cell as ^{32}P-orthophosphate, recombined with ADP inside the cell, and then transferred to deoxyglucose via hexokinase.

To test whether a significant amount of the ^{32}P label might be entering the nerve as orthophosphate following the extracellular hydrolysis of ^{32}P-ATP, 28 preparations were incubated with ^{3}H-deoxyglucose and either 5 mM ^{32}P-ATP or 5 mM ^{32}P-orthophosphate for periods of time ranging from 5 to 30 min. For each preparation the amount of ^{32}P label found in the same fractions as ^{3}H-deoxyglucose-6-P was determined. The results are shown in Figure 6, where the average amount of ^{32}P label originating from the extracellular space and incorporated into deoxyglucose is plotted as a function of time of incubation with either ^{32}P-ATP or ^{32}P-orthophosphate. The rate of incorporation produced by ^{32}P-orthophosphate was half the rate measured in the presence of ^{32}P-ATP. Thus, if the latter mechanism does contribute, it would be too slow to account completely for the observed incorporation of ^{32}P label into deoxyglucose even if the highly unrealistic assumption is made that all of the exogenous ATP is hydrolyzed to ADP and orthophosphate.

Nevertheless, a further experiment was performed to eliminate this possibility. The contribution of extracellular ^{32}P-orthophosphate was made even less probable by the inclusion of a large amount of unlabeled orthophosphate (10 mM) in the bath so that any ^{32}P-orthophosphate produced extracellularly from the hydrolysis of ^{32}P-ATP would be "diluted" by the unlabeled orthophosphate. If a considerable amount of the ^{32}P label enters the nerve cell as orthophosphate, then the incorporation of ^{32}P into deoxyglucose should be significantly reduced under these conditions. If, on the other hand, ^{32}P label enters the nerve primarily as ^{32}P-ATP, the incorporation of ^{32}P label into deoxyglucose should not be altered significantly.

Six preparations were incubated with 10 mM ^{3}H-deoxyglucose and 5 mM ^{32}P-ATP for 15 min along with the additional 10 mM nonlabeled orthophosphate. The average (±SE) amounts of total deoxyglucose-6-P and ^{32}P-labeled deoxyglucose-6-P were 49.4 (±15.3) and 0.89 (±0.06) nmol/mg dry wt, respectively. The corresponding average amounts measured in 5 control preparations incubated 15 min with 10 mM ^{3}H-deoxyglucose and 5 mM ^{32}P-ATP are 51.5 (±6.9) and 1.11 (±0.10) nmol/mg dry wt. Neither the total nor the ^{32}P-labeled deoxyglucose-6-P levels are significantly different between the 2 groups. Thus, the ^{32}P enters the cell primarily on ATP and not orthophosphate, supporting the suggestion that exogenous ATP can enter nerve cells chemically intact.

Release of ATP into the extracellular space

The demonstration that exogenous ATP can elevate presynaptic ATP levels suggested that exogenous ATP might be the source of the increased nerve ATP observed during stimulation. To test this possibility, the bath was assayed for ATP using the luciferin–luciferase assay. Its immediate breakdown products, ADP, AMP, and adenosine were also measured, using their UV absorbance following separation on an HPLC column.

The results, presented in Figure 7, indicate that all of these compounds exist in the bathing solution and their levels increase significantly during stimulation of the excitor axon (0.05 level).

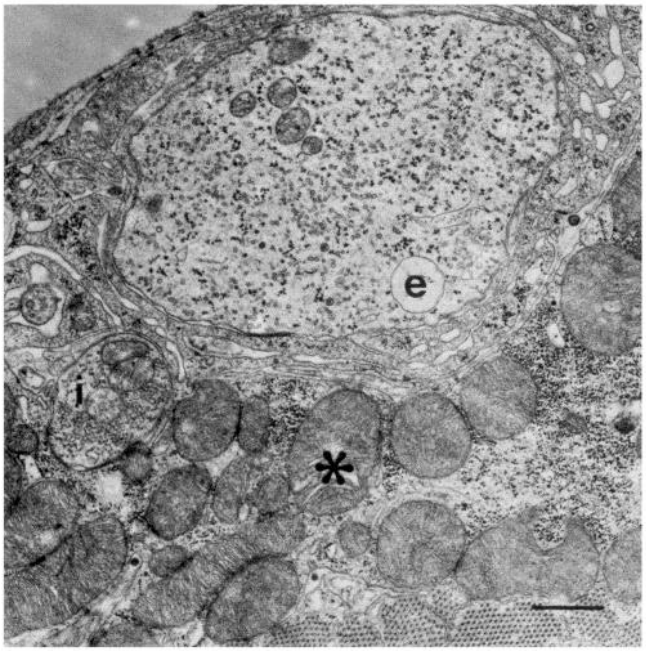

Figure 8. Muscle mitochondria lie in close proximity to associated motor nerve. Numerous large muscle mitochondria, one of which is denoted by an *asterisk*, are tightly aligned against the postsynaptic membrane near the excitor (*e*) and inhibitor (*i*) axons. Similar close proximity of large muscle mitochondria and motor nerves can be seen in rodents (unpublished observations). Calibration bar, 1 μm.

Total adenylate levels (not including adenosine) in a 5 μl bath sample increased by 30 ± 8 (SE) pmol; this corresponds to a 6 μM increase to a total level of 12 μM. The presence of ADP, AMP, and adenosine indicates that either these compounds were released into the bath along with ATP or were generated from the hydrolysis of ATP following its release.

Bath levels of adenosine and adenine nucleotides were measured in 5 more preparations in which the bathing solution contained 20 mM γ-methylglutamate to block the postsynaptic effects of the glutamate released during stimulation. In this case, the resting level of each substance was reduced dramatically. Adenosine, AMP, and ADP levels were reduced below their detection limits (1 pmol). ATP, which was measured separately by the luciferin–luciferase method, could still be detected, but its resting level was reduced, and it did not increase following nerve stimulation. This finding suggests that during stimulation, the normal activation of the muscle by glutamate leads to the release of ATP into the extracellular space. Furthermore, the fact that addition of γ-methylglutamate resulted in a decrease in the background levels of each of the adenylates in the bath suggests that resting levels of glutamate leakage may lead to a sizable release of ATP and also possibly some of its products of hydrolysis in the absence of stimulation.

Discussion

This study demonstrates that ATP levels in the terminal region of a nerve increase during stimulation. The observed adenylate levels in nonstimulated tissue, which generally ranged from 3 to 4 mM, are comparable to those reported in other studies. Following stimulation, the ATP and total adenylate values were increased by 1.0 and 1.3 mM, respectively. Since our technique measures total ATP associated with the nerve, we do not know whether the entire increase occurred within the neuron or whether some fraction of it was due to extracellular adsorption.

Slightly increased levels of ATP in nervous tissue during electrical stimulation have been reported in other preparations. At moderate stimulation rates (≤30 Hz), ATP content has been seen to rise by 8 and 17% in mixed nerve bundles from rabbit (Greengard and Straub, 1959) and crayfish (Smith, 1980b), respectively. However, these changes were not significant statistically in either case, nor were they as large as those observed in this study. Furthermore, in the rabbit, stimulation at 50 Hz for 15 sec led to a drop in both ATP and PCr; this is similar to the results observed in rat phrenic nerve in this study.

The results obtained from crayfish indicate that the nerve is capable of taking up exogenous ATP, and the amount of ATP in the bath is increased following stimulation. It thus seemed possible that the increased nerve ATP measured following stimulation was derived from an extracellular source. Moreover, since γ-methylglutamate blocked both the neuronal increase and the increase in the bath, the muscle was thought to be the source of the extracellular ATP. The nerve terminals cannot be ruled out as a source of the ATP since a high concentration of γ-methylglutamate (20 mM) was required to inhibit the postsynaptic response and therefore might have caused presynaptic effects as well. However, the muscle is the most likely source, for it is improbable that the terminals contain sufficient ATP to account for the observed increase. Specifically, the total ATP content of the approximately 6000 terminals per muscle—60 terminals per muscle fiber, 100 fibers per muscle (Dudel and Kuffler, 1961)—is about 0.6 pmol; this assumes that the terminals are 2 μm spheres (Onodera and Takeuchi, 1980) and that the terminal ATP concentrations are 2.4 mM, as in the rest of the nerve. This is not enough ATP to account for the release into the bath of at least 6 pmol.

In contrast, the surrounding muscle is a rich source of ATP. In both vertebrates and invertebrates, large muscle mitochondria are often densely clustered in the noncontractile regions in very close proximity to the associated motor nerves. An example from this crayfish preparation is shown in Figure 8. Moreover, postsynaptic release of ATP has been reported in other preparations, such as frog (Abood et al., 1962), *Torpedo* (Israel et al., 1976), and human (Forrester, 1972).

In crayfish, quantities of the ATP metabolites ADP, AMP, and adenosine were also increased in the bath following stimulation. It is not known whether these compounds were actually released from the source, which is presumably muscle, or whether they were generated from the hydrolysis of ATP following its release. However, the concentration of extracellular ATP required to increase nerve ATP levels significantly is still much greater than the maximum concentration of total adenylates measured in the bath. Namely, 5 mM exogenous ATP was required to increase neuronal ATP by the same amount as observed following 1 min of 50 Hz stimulation (5.6 pmol; compare Table 1 with Fig. 4). During stimulation, though, total adenylates measured in the bath rose only to 12 μM.

However, local ATP concentrations may be much higher. If the 5.6 pmol increase in neuronal ATP is derived from an extracellular source, such as the muscle, it must have distributed into an extracellular water (or sucrose) space of 0.67 nl (see Table 3). If 60% of the total ATP increase is in this space, then the extracellular ATP within this region would have risen by 3.4/0.67 pmol/nl, or about 5 mM. Thus, the effects of nerve stimulation on neuronal ATP are consistent with a distribution of 5 mM increased extracellular ATP in the region of the nerve and either an intracellular accumulation or the extracellular binding of 2.2 pmol/μg dry wt. This is, in fact, quite similar to the observed accumulation of ATP when the tissue was exposed to 5 mM exogenous ATP.

The technique used to measure bath levels of ATP may have grossly underestimated the concentration of ATP in the extracellular space immediately surrounding the nerve. The terminal portion of the nerve, which was analyzed in this study, lies in

very close proximity to the muscle, with its connective tissue sheath actually confluent with that of the muscle (Fig. 8). This may result in a highly sequestered extracellular environment. In fact, the slow diffusion of bath-applied compounds to the neuromuscular junction has suggested the existence of a diffusion barrier between the extracellular space and the bathing medium (Smith, 1983; Zucker, 1974). It is possible that close physical association of the nerve and muscle is necessary for significant transfer of ATP to occur, and it may explain why such a process has not been observed before.

Regardless of whether the increased ATP has an extracellular source, it is noteworthy that exogenous ATP enters the nerve. Cells are generally thought to be impermeant to highly charged anions, such as ATP, although there is evidence that certain cells do incorporate exogenous ATP. Exposure of various lines of transformed cells to external ATP causes them to become permeable to several normally impermeant molecules, including nucleotides (Rozengurt and Heppel, 1979). In rat soleus muscle, addition of ^{14}C-ATP (5 mM) to the incubation medium leads to an apparent accumulation of ATP within the muscle fibers (Chaudry and Gould, 1970). Similarly, incubation of rat neonatal heart cells with 5 mM ATP increased their ATP content by 75% with a Q_{10} of approximately 2.5 (Williams et al., 1979). Very low concentrations of ATP (8.6 μM) specifically induce a permeability in rat mast cells to nucleotides and sugar phosphates (Cockcroft and Gomperts, 1979). Furthermore, in squid axons, extracellularly applied γ-^{32}P-ATP effectively phosphorylates 2 axoplasmic proteins (Pant et al., 1979). In this preparation, it was demonstrated that exogenous γ-^{32}P-ATP is capable of phosphorylating 2-deoxy-D-glucose. Since this could not be explained by the extracellular hydrolysis of the ^{32}P-ATP to ADP and ^{32}P-orthophosphate, extracellular ATP must be able to enter the nerve chemically intact. All of these observations are consistent with the cellular uptake of ATP.

Thus, if the local ATP concentration within the extracellular space surrounding the nerve does increase more than measurements of bath aliquots would suggest, some of it could enter the ATP pool of the nerve and provide a source of energy during periods of intense activity. Alternatively, the presynaptic nerve may take up ATP more readily when the nerve is active. Lower concentrations of bath ATP may be sufficient to increase nerve ATP levels during synaptic activity since a portion of the uptake may occur during the recycling of synaptic vesicles (*cf.* Heuser and Reese, 1973).

Another role for the released ATP is in the regulation of axon terminal excitability and synaptic transmission. At the time of conduction failure, nerve ATP levels are elevated above those measured in nonstimulated "resting" tissue, suggesting that extracellular ATP and/or adenosine may also be correspondingly high. Both of these compounds have been shown to depress presynaptic activity (Ribeiro and Walker, 1975; Schubert et al., 1982; Silinsky and Ginsborg, 1983), and adenosine, in particular, inhibits calcium spikes (Proctor and Dunwiddie, 1983). These congruent observations indicate that ATP or adenosine emanating from the muscle may decrease axonal excitability and/or synaptic transmission through a negative-feedback mechanism. This feedback role was tested, and, indeed, exogenous ATP was found to inhibit transmitter release in this preparation by altering stimulation-induced Ca^{2+} uptake (Lindgren and Smith, 1984).

In summary, the results are consistent with the following mechanism. During synaptic activation, ATP is released into the extracellular space due to the action of glutamate on its receptors, increasing the local ATP concentration within the extracellular space surrounding the muscle. Some of this ATP may be taken up by the nerve, contributing to the increase in nerve ATP levels observed during stimulation. ATP also inhibits the release of glutamate from the presynaptic terminals (Lindgren and Smith, 1984). Thus, exogenous ATP may serve a dual role by both moderating synaptic activity and providing a source of energy during periods of intense activity.

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