A HIGHLY ION-SENSITIVE ATP-PHOSPHORYLATION SYSTEM IN LOBSTER NERVE

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SUMMARY The transfer of -phosphate from ^{32}P labeled adenosine-triphosphate (ATP) at low concentrations (10^{-10} to 10^{-7}M) into the peripheral nerve of the lobster was found to be highly sensitive to external ionic environments. The phosphorylation process is inhibited at conditions similar to extracellular environments (high Na^+, Ca^{++} and pH) and stimulated by those close to intracellular medium (high K^+, Mg^{++} and low pH). This system is not related to Na-K ATPase (pump ATPase) which is highly sensitive to ouabain and is active only at higher ATP concentrations ($> 10^{-6}$M). The system is membrane bound and sensitive to a variety of neuro-active agents which are known to interfere with ionic conductance changes in axons.

In studying the mechanism of nerve excitation electrophysiologists have been using the nerve cords of the walking legs of large crustacean species, since they are rich with axonic material including giant axons without synaptic process. Only a few researchers have made attempts to study the biochemical properties of these nerve materials. The presence of Na and K ATPase (EC 3.6.1.3) in nerve material has been well documented (1). The function of this enzyme has been correlated with the active transport, but not with the membrane conductance changes (2, 3).

There could be other ATP involving systems present in these nerve materials that might be logically related to the mechanisms of membrane conductance changes. For instance, it has been reported that there is a large titer of ATPase activity associated with contractile protein (4).

By using γ-labeled ^{32}P ATP Skou and Hilberg (5) were able to demonstrate that patterns of ^{32}P incorporation into a peripheral nerve component largely depends upon the concentrations of Na, K, and Mg ions. Their system, however, is likely to be related to a Na and K ATPase that is similar to

Table I

Ionic Dependence of ^{32}P Incorporation at high and low Concentrations of ATP

Data are expressed in picomoles of ^{32}P incorporated in 10 minutes at 37°C into crude supernatant, containing 8 mg wet weight equivalent of lobster nerve matter. Buffer used was prepared in 30 mM tris-HCl, pH 7.0, containing .6 mM of EGTA. The mM quantities of Na^+, K^+, and Mg^{++} present in the buffer were as indicated in the table.

Ionic composition of buffer used (mM)	Concentrations of ATP	
	2.25×10^{-5}M	7×10^{-8}M
60 Na^+/60 K^+/10 Mg^{++}	30.70	.113
10^{-4}M Ouabain	36.22	.116
10^{-5}M DDT	30.26	.159
60 Na^+/10 Mg^{++}	53.5	.051
10^{-4}M Ouabain	71.5	.054
10^{-5}M DDT	33.1	.062
60 K^+/10 Mg^{++}	90.0	.483
10 Mg^{++}	103.4	.528

Table II

Effects of Neuroactive Agents on ^{32}P Incorporation into 149,000 g Precipitate Fraction at 37°C in 10 min. (Buffer containing 60 mM Na^+, 60 mM K^+, and 10 mM Mg^{++} in 30 mM tris-HCl at pH 7.0, ATP concentration: 7×10^{-8}M).

Compound	Final concentration (M)	Picomoles ^{32}P incorporated
Control	-	.126
DDT	10^{-5}	.164
Ouabain	10^{-4}	.125
Tetrodotoxin[a] (TTX)	10^{-6}	.120
Hemicholinium-3	10^{-4}	.094
Procaine-HCl	5×10^{-4}	.093
Acetylcholine (I)	10^{-4}	.084
N-acetylimidazole	10^{-3}	.073
Sparteine	5×10^{-4}	.071
2,4-Dinitrophenol	5×10^{-4}	.062
Diphenylhydantoin	5×10^{-4}	.053
$BeCl_3$	10^{-4}	.000

[a] Unlike other agents Tetrodotoxin is known to block only the external Na gate which explains why this compound has no effect on this phosphorylation system which is activated under intracellular environments.

the one found in the lobster (6). The key to the above conclusion is that the system is sensitive to ouabain, an acknowledged Na and K ATPase inhibitor, and shows ion sensitivities that are expected by the Na and K ATPase actions.

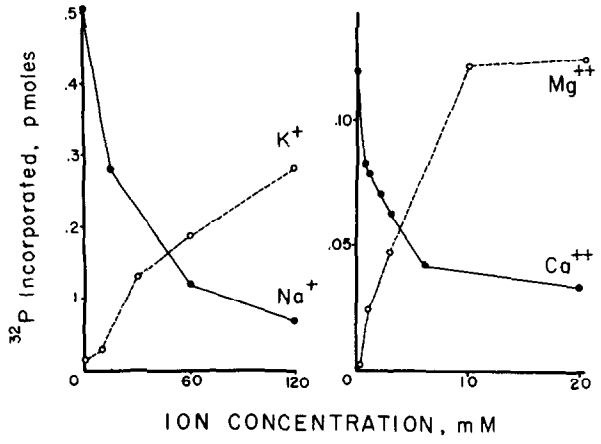

Fig. 1. ^{32}P incorporation from γ-^{32}P labeled ATP into proteins in the crude supernatant as a function of Na^+, K^+, Mg^{++}, and Ca^{++} at 7×10^{-8}M ATP. Data are expressed in picomoles of ^{32}P incorporated in ten minutes into 8 mg equivalent of wet weight lobster nerve. Left figure (Na) variation of Na^+ at 60 mM K^+, 10 mM Mg^{++}; (K) variation of K^+ at 40 mM Na^+, 10 mM Mg^{++}. Right figure (Ca) variation of Ca^{++} at 60 mM Na^+, 60 mM K^+, 10 mM Mg^{++}; (Mg) variation of Mg^{++} at 60 mM Na^+, 60 mM Na^+, 60 mM K^+. All in 30 mM tris-HCl, pH 7.0 containing .6 mM EGTA (ethyleneglycol-bis(β-aminoethyl ether) N, N'-tetraacetic acid).

We report here the discovery of another ATP related system in the lobster nerve. The system, basing upon studies of ^{32}P incorporation, is strongly influenced by mono- and divalent cations but not by ouabain. It exhbits the characteristics of a system responsible for conductance changes.

MATERIALS AND METHODS

Peripheral nerve cords were excised from the walking legs and pincers of the lobster Homerus americanus and homogenized at 80 mg/ml in 725 mM sucrose at 0°C. To determine the extent of ^{32}P incorporation, 0.1 ml of a crude supernatant obtained by centrifuging the homogenized axons 1000 g for ten min was mixed with 0.9 ml of incubation medium containing defined but variable amounts of Na, K, Mg, and Ca (i.e. see Table I and Figure I). The reaction was initiated by adding 1.0 µl of (γ-^{32}P)-ATP to make a final ATP concentration of 7×10^{-8}M or similarly a mixture of labeled and unlabeled ATP to give a higher concentration of ATP (see Table I).

After 10 min of incubation at 37°C, the reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA). The labeled nerve components were co-precipitated with bovine albumin as described by DeLange (7). Blank values, obtained by adding TCA prior to the addition of labeled ATP, were subtracted from each set of data to assure that only enzymatic incorporation was being assayed.

RESULTS

In the experiments shown in Table I the effects of ions and inhibitors on ^{32}P incorporation at two different ATP concentrations were compared. At the high level of ATP (2.25×10^{-5}M) the ionic effects were very similar to the ones obtained by Skou and Hilberg (5) on Na and K ATPase, while a completely different set of results were observed at the low ATP concentration. The chief differences were in the divergent effects of K at each level and the lack of ouabain effects at low ATP concentrations. This discovery is confirmed and clarified in Figure 1 (left) showing the dependence of ^{32}P incorporation on changes in monovalent cation concentration. Specifically, this system, operating at low ATP concentrations, accepts decreased amounts of phosphates at higher Na concentrations; whereas increasing the K concentration in the presence of at least a trace of Na, increases the amount of ^{32}P incorporation. The same system is stimulated by Mg and inhibited by Ca (Figure 1, right). The pH of the medium also influences the extent of phosphorylation: there was a steady decrease obtained by raising the pH from 6.6 to 7.9 (compare .185 picomoles to .100 picomoles).

To study the localization of this system, the crude supernatant was centrifuged at 20,000 g for 10 min, the resulting supernatant was diluted to a sucrose concentration of 250 mM and further centrifuged at 149,000 g for 120 min at 0°C to collect the membraneous material. The results of the ionic effects relative to variation of Na and K indicated the same pattern of influence obtained in Figure 1 when the collected membraneous

material was assayed after being re-suspended in 725 mM sucrose. Table II shows that various other neuroactive agents cause inhibition of the amount of ^{32}P transferred from the γ-labeled ATP. All of these chemicals are known to affect the Na and/or K permeability.

The possibility that the observed incorporation being an artifact of uptake of orthophosphate can be ruled out because under these experimental conditions, the maximum of ^{32}P incorporation was observed in one minute and the level declined thereafter (to be published). There was sufficient ATP and hence liberated orthophosphate so that the incorporation should have increased with time as noted by previous workers (8), if an uptake of orthophosphate was being studied. Also since ouabain is known to inhibit the production of orthophosphate from ATP, it should have inhibited the ^{32}P incorporation. The enzymatic nature of this phosphorylation is further established by the observation that mersalyl at $10^{-4}M$ (an -SH inhibitor) caused 50% inhibition into the crude supernatant.

DISCUSSION

There are a number of indications to support the view that the novel phosphorylation system presented here could play a role in regulating ionic flow across the axonic membrane. For instance, (a) it is highly sensitive to changes in Na, K, Mg and Ca concentrations as well as pH in the manner that is compatible with the theory of nerve excitation, (b) its centrifugal behavior indicates that it is membrane bound, (c) it is operative at extremely low ATP concentrations (important from the energy consideration) and (d) it is sensitive to a variety of neuroactive agents that are known to affect ion permeability changes. Also in agreement with our observation is the reports by other workers (9,10) that there are changes in the state of radio-labeled high energy triphosphates associated with the lobster nerve activity during evoked nerve excitation.

It is known that isolated axons, even when they are internally perfused with artificial medium, can maintain the state of excitability for a

number of hours in the presence of CN^- which inhibits ATP synthesis (3). Thus the energy required for conductance changes must be stored in a large energy pool, or it is self regenerating (11). Assuming that phosphorylated protein complex can act as an energy pool, the former possibility appears to be more compatible with our observation.

Examples of allosteric changes in conformation of phosphorylated proteins are numerous. Moreover several protein kinase systems have been already found to play important roles in nerve function (12). Thus there is a likely possibility that a phosphorylation system such as the one reported here participate in some way in the process of conductance changes during action potentials. However, more direct biochemical and electrophysiological evidence are needed to confirm such a possibility, since our current knowledge is not complete enough to permit accurate assessment for the energy requirement and the speed of such phosphorylation and dephosphorylation reactions.

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