Biochimica et Biophysica Acta, 1070 (1991) 332–342

BBAMEM 75439

ATP as a positive effector of the sodium efflux in single barnacle muscle fibers

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(Received 12 February 1991)
(Revised manuscript received 21 May 1991)

Key words: Sodium ion efflux; Barnacle fiber; Microinjection; ATP

A study has been made of the mechanism by which the injection of $ATPNa_2$ stimulates the ouabain-insensitive Na efflux in fibers from the barnacle, *Balanus nubilus*. The results of this study are as follows: $ATPNa_2$ is found to be a more potent effector of the Na efflux in unpoisoned fibers than ATPMg on an equimolar basis, but not more potent than $ADPNa_2$. In ouabain-poisoned fibers $ATPNa_2$ and ATPMg are equipotent but the former is more potent than $ADPNa_2$. The magnitude of the response to $ATPNa_2$ injection into ouabain-poisoned fibers depends on: (i) the ouabain concentration used; (ii) the concentration of $ATPNa_2$ injected, and (iii) the external Ca^{2+} concentration. Ouabain is without effect when it is applied at the time of $ATPNa_2$ injection. Responsiveness to ouabain, however, is found to return if the glycoside is applied after complete decay of the response to ATP. Under these conditions, the effect of ouabain in fibers injected with $ATPNa_2$ is significantly less than in fibers injected with ATPMg. Preinjection of EGTA in high concentrations fails to reduce the size of the response to $ATPNa_2$ injection. Injection of Mg^{2+} following peak stimulation by ATP almost completely reverses the response. The response to Mg^{2+} is concentration-dependent. Ryanodine but not neomycin reduces the response to ATP. ATPγS is not as effective as $ATPNa_2$. Nor is AMP-PNP consistently as effective as $ATPNa_2$. Collectively, these results support the hypothesis that the response of the Na efflux to $ATPNa_2$ injection involves the operation of the putative Na^+-Ca^{2+} exchanger in the reverse mode and that a raised Ca_i^{2+} is not an absolute requirement. They also strongly suggest that two other governing factors are the Na^+ gradient across the sarcolemma and the myoplasmic pMg. Mg^{2+} seems to act as an inhibitor.

Introduction

In 1975, Bittar and Tong [1] demonstrated the occurrence of transitory stimulation of the resting Na efflux following the injection of $ATPNa_2$ into barnacle muscle fibers, and that this response involves the ouabain-insensitive component of the Na efflux. They raised the possibility that the observed effect might be due to chelation of internal free Mg^{2+} by ATP. This seemed reasonable particularly in the light of evidence that the concentration of free Mg^{2+} in these fibers is about 5 mM [2,3]. Since current thinking holds that ATP in skeletal muscle promotes opening of the sarcoplasmic reticulum (SR) Ca^{2+} release channel, whilst Mg^{2+} promotes closure (see, for example, Ref. 4), the possibility arises that the natriferic action of ATP may be partly or wholly due to a rise in free Ca_i^{2+} brought about by the action of ATP as an allosteric effector, as well as by the attending decrease in internal free Mg^{2+} brought about by the removal of some free Mg^{2+} by ATP. This line of reasoning is based on abundant and solid evidence that the injection of Ca^{2+} into unpoisoned and ouabain-poisoned fibers stimulates the Na efflux, the minimal effective concentration being about 1 μM [5,6], and the fact that the stability constant of ATP and Mg^{2+} is 4.24 [7,8]. Furthermore, current thinking holds that the operation of the Na^+-Ca^{2+} exchanger in the sarcolemma requires both Ca_i and

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; AMP-PNP, 5′-adenylyl imidodiphosphate.

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ATP and that a raised internal Na concentration promotes Na^+-Ca^{2+} exchange in the reverse mode (see, for example, Ref. 9). Thus, a new working hypothesis can be framed which takes into account not only the behavior of the SR release channel towards ATP but also the behavior of the Na^+-Ca^{2+} exchanger towards ATP and Mg^{2+}. The following communication describes experiments the results of which support the hypothesis that ATP is a powerful activator of reverse Na^+-Ca^{2+} exchange, whilst a raised Ca_i^{2+} is not a strict requirement and that a sudden rise in Mg_i^{2+} almost completely reverses the response of the Na efflux to ATP injection.

Materials and Methods

The species of barnacles, the method of dissection, cannulation, microinjection and counting of ^{22}Na activity in the effluent and the fiber were essentially as those described by Bittar [10]. The artificial seawater (ASW) used had the following composition (mM): NaCl 465, KCl 10, $MgCl_2$ 10, $CaCl_2$ 10, $NaHCO_3$ 10 and pH 7.8. Solutions of nominally Ca^{2+}-free ASW were prepared by omitting calcium chloride and raising NaCl in an osmotically equivalent amount. Solutions for injection were prepared using 3 mM Hepes (pH 7.2). The volume of test solution or a 3 mM Hepes solution injected into these fibers was 0.3–0.4 μl. This is diluted by the myoplasm by a factor of roughly 100. All experiments were carried out at a room temperature of 22 °C to 24°C.

The results given in this paper are expressed as the mean ± standard error. Student's *t*-test was employed to compare the data statistically. Values for $P < 0.05$ were considered as being significant. Estimates of the size of the observed effects on the ^{22}Na efflux were calculated on the basis of the rate constant plots. For the case where two stimulatory or inhibitory phases were present in succession, the size of the second response was arrived at by taking the difference between the two combined phases and the first phase. Some of the figures shown are composites of several semilog efflux plots. This is done in preference to showing a single typical experiment solely because the behavior of fibers isolated from the same muscle bundle in such experiments is quite uniform.

All reagents used were analytical grade. Ouabain, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes), ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), $ATPNa_2$, ATPMg, adenosine 5'-*O*-(3-thiotriphosphate (ATPγS) (tetralithium salt), and 5'-adenylyl imidodiphosphate (AMP-PNP) (tetralithium salt) were purchased from Sigma Chemical Co., St. Louis, Missouri. Ryanodine was supplied by S.B. Penick and Company, 100 Church Street, New York City.

Results

Unpoisoned fibers

Injection of $ATPNa_2$

Injection of 0.5 M-$ATPNa_2$ into unpoisoned fibers causes a rise in the resting Na efflux, the magnitude of which averages 62 ± 10% (n = 8). The response is not always prompt in onset, as it may take 5–10 mins to occur, and it is usually transitory in nature.

Comparison of $ATPNa_2$ with ATPMg injection

Knowing that the stability constant (log *K*) of ATP and Mg^{2+} is 4.24, and of ATP and Na^+ is 0.83 (at 37 °C and 0.15 M-Na^+) [8], it seemed of special interest to find out if $ATPNa_2$ is more potent than ATPMg as the result of its ability to remove internal free Mg^{2+}. Injection of 0.5 M $ATPNa_2$ and 0.5 M ATPMg into fibers isolated from the same muscle bundle causes stimulatory responses of the order of 48 ± 8% ($n = 8$) and 17 ± 3% ($n = 8$), respectively. The difference is significant (P being < 0.05). This is illustrated in Fig. 1.

Effect of $ADPNa_2$ injection

Because ADP is considerably weaker than ATP as an effector of the SR Ca^{2+} release channel (Meissner, K., private communication) and has only one-tenth the ability of ATP to bind Mg^{2+} [7,8], and because ADP in contrast to ATP is completely without effect on Na^+-Ca^{2+} exchange e.g. in the squid axon [11], it was thought likely that the response of the resting Na efflux to ATP injection would be found to be appreciably larger than the response to ADP. However, the results of experiments do not bear this out, viz. injection of 0.5 M $ADPNa_2$ causes a stimulatory response of the order of 33 ± 5% ($n = 8$) (Fig. 2), a value significantly greater

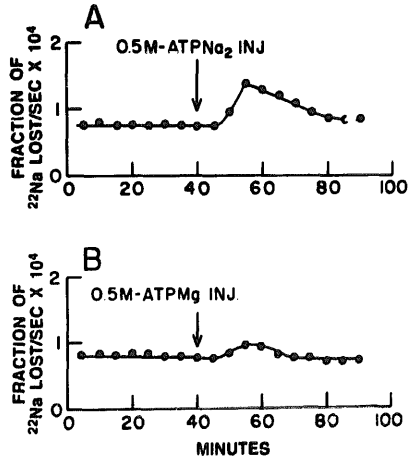


Fig. 1. The effect on the Na efflux from fibers injected with $ATPNa_2$ and ATPMg in equimolar concentrations (rate constant plots for ^{22}Na loss).

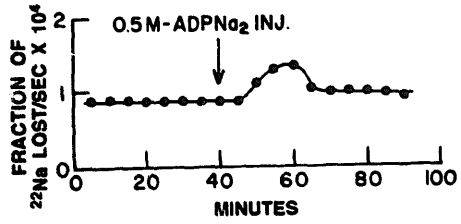


Fig. 2. The effect of injecting 0.5 M $ADPNa_2$ on the resting Na efflux.

than $9 \pm 4\%$ obtained by injecting 3 mM Hepes into companion controls (P being < 0.01) but not significantly different from $48 \pm 8\%$ stimulation ($n = 8$) obtained with $ATPNa_2$. Such a comparison is important partly because the fibers used were from the same muscle bundle. Such data are consistent with the hypothesis that ATP and ADP act as the result of releasing Ca^{2+} from the SR and/or removal of internal free Mg^{2+}. However, they do not exclude the possibility that the response to ATP or ADP is the result of newly formed cAMP. In the latter case, several possibilities suggest themselves. First, a sharp rise in myoplasmic ADP may elicit ATP release by mitochondria via the adenylate translocase system [12]. Second, ADP is an effector of the adenylate kinase reaction, thus giving rise to ATP and AMP formation [13]. And third, both ADP and AMP are known activators of the phosphofructokinase reaction, a principal site of control in the glycolytic pathway [14].

Ouabain poisoned fibers

Injection of $ATPNa_2$ into poisoned fibers

As illustrated in Fig. 3, injection of 0.5 M $ATPNa_2$ into a fiber poisoned with 10^{-4} M ouabain beforehand leads to a prompt and sharp rise in the remaining Na efflux (the magnitude of which averages $463 \pm 88\%$, $n = 5$). Characteristically, the response reaches a peak within 20 min of ATP injection, and then decays rather rapidly.

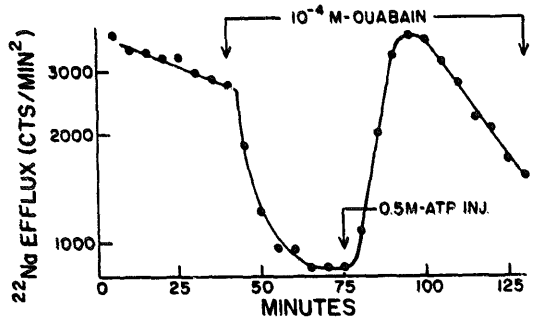


Fig. 3. The kinetics of the response of the ouabain-insensitive Na efflux to the injection of 0.5 M $ATPNa_2$ (a composite of five semilog efflux plots). Ordinate: rate at which ^{22}Na leaves the fiber in counts/min per min.

Comparison of $ATPNa_2$ with ATPMg

Injection of 0.5 M $ATPNa_2$ and 0.5 M ATPMg into two separate groups of fibers isolated from the same bundle and pretreated with ouabain causes a response the magnitude of which averages $463 \pm 88\%$ ($n = 5$) and $347 \pm 44\%$ ($n = 5$), respectively. The observed difference is not significant. Such a result is consistent with the concept that $ATPNa_2$ and ATPMg are equipotent as activators of the Na^+-Ca^{2+} exchanger in the reverse mode, provided the prevailing Na^+ gradient is not steep, and provided basal myoplasmic pMg in these fibers is not very low.

Comparison of $ATPNa_2$ with $ADPNa_2$

As in the preceding protocol, solutions of 0.5 M $ATPNa_2$ and 0.5 M $ADPNa_2$ were injected into ouabain-poisoned fibers. The results show a stimulatory response to $ATPNa_2$ of the order of $214 \pm 18\%$ ($n = 5$), a value which is significantly larger than $119 \pm 9\%$ ($n = 5$) obtained by injecting $ADPNa_2$. This is in keeping with the view that ADP is not only without effect on the Na^+-Ca^{2+} exchanger (see, for example, Ref. 11) but also much weaker than ATP as a positive effector of the SR Ca^{2+} release channel [15]. Further, it is in keeping with the view that ATP is a more powerful Mg^{2+} chelator than ADP.

Dependence of the response to $ATPNa_2$ on the external ouabain concentration

The activation of reverse Na^+-Ca^{2+} exchange is known to be a function of Na_i, the apparent K_m for Na^+ being ≈ 30 mM in internally perfused barnacle fibers (see, for example, Ref. 16) and 21 mM in guinea pig ventricular muscle fibers [17]. It also is recognized that the Na^+ gradient is the most effective monovalent gradient in driving Ca^{2+} influx (see, for example, Ref. 18). These values are close to what Na_i would be expected to be when the membrane Na^+/K^+ ATPase system is fully inhibited by ouabain. As illustrated in Fig. 4, the magnitude of the response of the Na efflux to the injection of 0.5 M $ATPNa_2$ is a function of the external ouabain concentration. Notice that the minimal effective concentration of ouabain lies in the low μmolar range. Ouabain in a concentration exceeding 10^{-3} M was not employed because such concentrations cause a diphasic effect viz. a fall in Na efflux, followed 20 min later by a steady increase in ^{22}Na loss [19]. In fact, concentrations exceeding 10^{-3} M of ouabain are not used usually because ethanol as the vehicle reduces the resting efflux. These results are in accord with the view that inhibition of the Na pump by ouabain increases Na_i, and that a raised Na_i in turn, leads to an increase in internal free Ca^{2+} resulting not only from reduced Na^+-Ca^{2+} exchange in the forward mode but also from increased Na^+-Ca^{2+} exchange in the reverse mode.

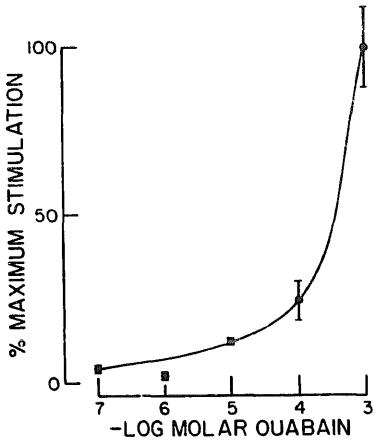


Fig. 4. The dependence of the response of the Na efflux to the injection of 0.5 M $ATPNa_2$ on the external ouabain concentration. Each plotted point is the mean value of three measurements. Vertical bars span ± S.E. The fibers used were isolated from the same barnacle specimen.

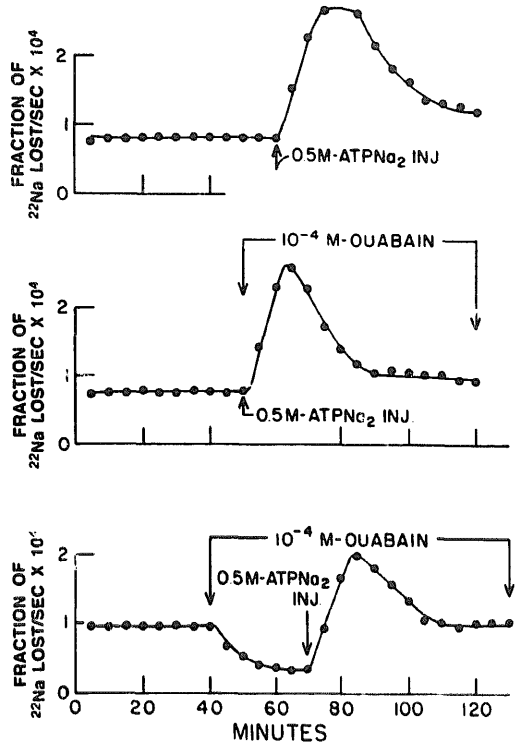


Fig. 5. The response to the injection of 0.5 M $ATPNa_2$ in an (i) unpoisoned fiber (upper panel), (ii) fiber poisoned with 10^{-4} M ouabain simultaneously (middle panel), and (iii) fiber poisoned with 10^{-4} M ouabain beforehand (lower panel).

Response to simultaneous ouabain application and injection of ATP

Information as to whether ouabain is effective in reducing the Na efflux in fibers suddenly injected with ATP was next sought, particularly since next to nothing is yet known about this problem. Three representative experiments are given in Fig. 5 where it can be seen (upper panel) that the injection of 0.5 M $ATPNa_2$ causes, as expected, a transitory rise in the resting Na efflux (168 ± 27%, $n = 5$), and that simultaneous 10^{-4} M ouabain application externally and injection of $ATPNa_2$ (middle panel) also causes a transitory rise (181 ± 19%, $n = 5$) but no subsequent fall below the original resting level of the Na efflux. Rather, as in the preceding experiment, complete decay of the response fails to occur. Also illustrated is that injection of 0.5 M $ATPNa_2$ into poisoned fibers results in a response which decays only partially (lower panel). Notice however that the efflux in this instance returns to its original pre-ouabain resting level. A comparison of the size of the response (arrived at by taking the difference between the rate constant value found prior to ouabain application and peak response to ATP) with that obtained by injecting ATP into unpoisoned fibers reveals that they are not significantly different, P being < 0.1.

Having found that ouabain does not potentiate the response to $ATPNa_2$ following its application at the time of injection of the nucleotide, it became desirable to determine whether ouabain sensitivity is present after complete disappearance of the response to ATP. To this end, fibers isolated from the same muscle bundle were injected with 0.5 M $ATPNa_2$ and with 0.5 M ATPMg 40 min prior to treatment with ouabain. These experiments with ATPMg were included as a control since an appreciable rise in myoplasmic pMg resulting from the injection of $ATPNa_2$ would be ex-

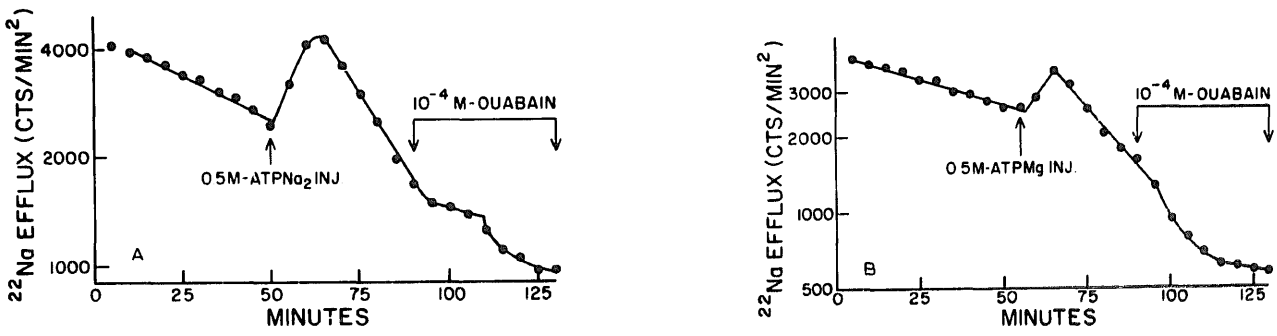


Fig. 6. (A) Marked inhibition of the Na efflux by external application of 10^{-4} M ouabain subsequent to complete decay of the response to the injection of 0.5 M $ATPNa_2$ (semilog plot). (B) Less inhibition of the Na efflux by external application of 10^{-4} M ouabain subsequent to complete decay of the response to the injection of 0.5 M ATPMg (semilog plot).

pected to impair the ability of ouabain to bind to the membrane Na^+/K^+-ATPase, e.g. ATP is bound to the enzyme via a Mg^{2+} ion, and/or to reduce the activity of the enzyme, e.g. optimal activity requires a Mg/ATP ratio of 1 : 1 (see Ref. 20). As illustrated in Fig. 6A and b which is a composite of four efflux semilog plots the injection of 0.5 M $ATPNa_2$ and 0.5 M ATPMg prior to ouabain application fails to produce a significant difference in the behavior of the effluxes. Notice that the resulting slopes are practically the same. However, the application of 10^{-4} M ouabain at $t = 90$ min (i.e. after disappearance of the response to $ATPNa_2$ injection as judged on the basis of the rate constant plots) leads to a delayed step-down in the Na efflux. The magnitude of this inhibition averages $25 \pm 5\%$ ($n = 4$). Further, as illustrated in Fig. 6B, inhibition by ouabain in the fiber preinjected with ATPMg is more rapid in onset and averages $56 \pm 3\%$ ($n = 4$) in size. This value is significantly larger than the preceding value of $26 \pm 5\%$, P being < 0.05.

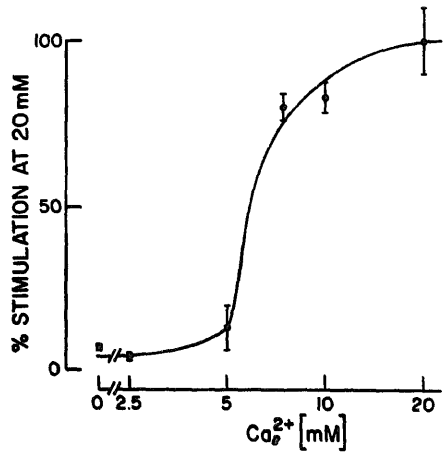


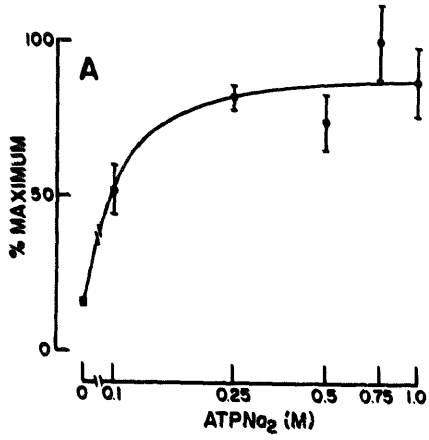
Fig. 8. The response of the ouabain-insensitive Na efflux to the injection of 0.5 M $ATPNa_2$ as a function of the external Ca^{2+} concentration. Each plotted point is the mean value-of three measurements. Vertical bars span ± S.E. The fibers used were isolated from the same barnacle specimen.

Concentration-response curve for $ATPNa_2$

Summarized in Fig. 7A are the results obtained by injecting $ATPNa_2$ in varying concentration into fibers treated with 10^{-4} M ouabain. Notice that the EC_{50} is 0.1 M and that saturation kinetics are reached when the concentration of injected ATP exceeds 0.25 M. More experiments of this type were done using fibers from another barnacle. The reason was simple: it seemed necessary to show that a response is obtainable with $ATPNa_2$ concentrations less than 0.1 M. The results obtained are given in Fig. 7B where it can be seen that an identical EC_{50} in the range of 50–100 mM is obtained.

Dependence of the ATP effect on external Ca^{2+}

Shown in Fig. 8 is that the magnitude of the response to ATP injection depends on the external Ca^{2+} concentration, and that saturation kinetics fail to take place over the 15–20 mM Ca^{2+} concentration range. These results were confirmed by repeating this type of experiment. The absence of absolute dependency of the response to ATP on external Ca^{2+} is most reasonably accounted for by assuming the occurrence of Na^+-Na^+ exchange [10] and/or the presence of some residual Ca^{2+} deep in the region of the invaginations of the sarcolemma, thereby permitting a low degree of Na^+-Ca^{2+} exchange.



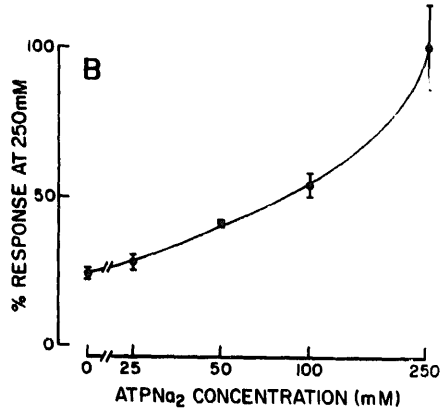


Fig. 7. Concentration-response curve for the stimulatory action of injected $ATPNa_2$ on the ouabain-insensitive Na efflux. Ordinate: % of maximum response; abscissa: ATP concentration of solution before injection plotted on a logarithmic scale. Each plotted point is the mean value of three measurements. Vertical bars span ± S.E. (A) the results obtained with fibers that were isolated from a single barnacle specimen are shown. (B) The results shown are based on fibers isolated from a second barnacle specimen. Notice that the companion controls when injected with 3 mM Hepes show a response which is not very different from that seen after injecting a solution of 25 mM $ATPNa_2$.

The ATP effect in unpoisoned and ouabain-poisoned fibers preinjected with EGTA

Rasgado-Flores, Santiago and Blaustein [21] have shown that the Ca_e-dependent Na efflux in dialyzed barnacle muscle fibers is increased when Ca_i^{2+} is raised, say from 10^{-7} M to 10^{-6} M. Similarly, in the squid axon, Ca_i^{2+} acts as an activator of Na^+-Ca^{2+} exchange, the apparent K_m for Ca^{2+} being 10 μM or 0.6 μM [22,23]. Further, ATP without Ca_i is known to activate reverse Na^+-Ca^{2+} exchange [11]. It therefore seemed well worthwhile to check the validity of this idea by stabilizing myoplasmic pCa e.g. by injecting EGTA, and then injecting $ATPNa_2$. The results obtained by injecting 0.25 M EGTA (pH 7.2) into unpoisoned and ouabain-poisoned fibers 1 h prior to 0.5 M $ATPNa_2$ are as follows: (i) Injection of 0.5 M $ATPNa_2$ into unpoisoned fibers preinjected with 0.25 M EGTA causes stimulation of the order of $105 \pm 25\%$ ($n = 4$), a value significantly larger than $38 \pm 7\%$ ($n = 4$) obtained with companion control fibers preinjected with 3 mM-Hepes. And (ii) Injection of 0.5 M $ATPNa_2$ into ouabain-poisoned fibers preinjected with 0.25 M EGTA causes stimulation of the order of $336 \pm 25\%$ ($n = 4$), a value significantly larger than $170 \pm 27\%$ ($n = 4$) obtained with companion control fibers preinjected with 3 mM Hepes. These experiments were repeated but this time 0.25 M EGTA was injected 2 h prior to $ATPNa_2$, thereby allowing more time for complete equilibration of the chelator. The results thus obtained indicate that (i) injection of 0.5 M $ATPNa_2$ into unpoisoned fibers injected 2 h beforehand with 0.25 M EGTA causes stimulation of the order of $78 \pm 11\%$ ($n = 4$), a value which is the same as that found in companion controls viz. $79 \pm 11\%$ ($n = 4$). And (ii) injection of 0.5 M $ATPNa_2$ into *ouabain-poisoned* fibers injected 2 h beforehand with 0.25 M EGTA causes stimulation of the order of $477 \pm 59\%$ ($n = 4$), a value which is not significantly different from $308 \pm 52\%$ ($n = 4$) obtained in companion controls, P being > 0.05. These results were confirmed by repeating this type of experiment, viz. unpoisoned fibers: $209 \pm 27\%$ ($n = 4$) vs. $208 \pm 34\%$ ($n = 4$) and poisoned fibers: $349 \pm 56\%$ ($n = 4$) vs. $313 \pm 17\%$ ($n = 4$). Thus, the only conclusion possible seems to be that a raised Ca_i is not an absolute requirement for the occurrence of a full response to ATP.

Reversal by Mg^{2+} injection of the ATP effect

Mg^{2+} is known to inhibit the operation of the Na^+-Ca^{2+} exchanger in both the forward and reverse modes, e.g. in the squid axon [11]. Illustrated in Fig. 9 (upper panel) is that the injection of 0.5 M $MgCl_2$ following the onset of peak stimulation by $ATPNa_2$ almost completely abolishes the response to ATP ($85 \pm 5\%$ reversal, $n = 4$). By contrast, injection of 3 mM Hepes fails to alter the course of the stimulated efflux

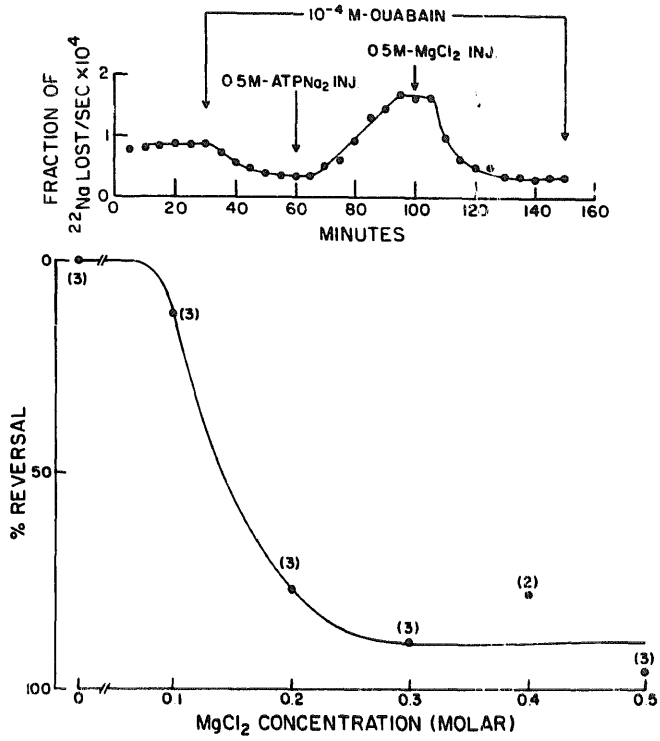


Fig. 9. (Upper panel) Complete reversal of the response of the ouabain-insensitive Na efflux to the injection of 0.5 M $ATPNa_2$ by injecting 0.5 M $MgCl_2$. (Lower panel) Dose-response curve for the effect of Mg^{2+} injection after $ATPNa_2$.

in companion controls ($n = 4$). Repetition of this type of experiment confirms this finding ($71 \pm 4\%$, reversal, $n = 4$). In order to be more certain of this result, namely that Mg^{2+} injection reverses the response to $ATPNa_2$ injection, experiments were undertaken to determine whether this effect of Mg^{2+} injection is concentration-dependent. Shown in Fig. 9 (lower panel) is that this is the case and that the effects obtained with Mg^{2+} in the 0.3–0.5 M range are considerable. Injection of Hepes (3 mM), as expected, is ineffective. However, it may be ojected that these results fail to take into account the fact that the response to the injection of 0.5 M $ATPNa_2$ is frequently found to decay. Which means that in situations where reversal by Mg^{2+} injection is incomplete, the decay phase of the remaining response reflects a Mg^{2+}-insensitive mechanism. In order to check this point more closely, experiments were designed in which various controls were done. Test fibers were injected with 0.5 M $MgCl_2$ following peak stimulation by injected 0.5 M $ATPNa_2$. The results show complete reversal in three fibers, and 56% reversal in the third. The control fibers were divided into two groups. In the first group, the microinjector was inserted and withdrawn, showing little or no effect on the behavior of the decay phase of the response to $ATPNa_2$ injection. Estimates of the decay recorded at $t = 140$ min show a value of $38 \pm 5\%$

($n = 3$). In the second group, 3 mM Hepes was injected. At $t = 140$ min the decay amounts to $36 \pm 3\%$ ($n = 3$). The fact that there are fibers showing no decay of the response to $ATPNa_2$ injection or almost complete reversal to the injection of 0.5 M $MgCl_2$ in combination with observations of situations where slow decay does occur but is never complete (at $t = 140$ min) but responses which are also almost fully reversed by injecting solutions of 0.5, 0.4, and 0.3 M $MgCl_2$ represent prima facia evidence that a sustained response to $ATPNa_2$ is fully Mg-sensitive, whilst a response that decays rather slowly and is incompletely reversed by Mg^{2+} may represent the presence of a Mg^{2+}-insensitive component. Such fibers may well have a low myoplasmic pMg despite Mg removal by injecting $ATPNa_2$.

Injection of ryanodine before ATP

To clarify the problem of whether ATP injection results in the release of Ca^{2+} by the SR, ryanodine was used. This plant alkaloid is known to act as a specific inhibitor of the SR Ca^{2+} release channel (see, for example, Ref. 24), and to produce a transient rise in the ouabain-insensitive Na efflux following its internal or external application (Bittar, E.E. and Huang, Y.-P., unpublished data). The latter data are in line with the observation that skeletal muscle SR Ca^{2+} release channels incorporated into lipid bilayers open and close when ryanodine is added (see, for example, Ref. 25). The representative experiment given in Fig. 10 (upper panel) shows quite clearly that the injection of 10^{-5} M ryanodine (upper panel) causes a rise in the ouabain-

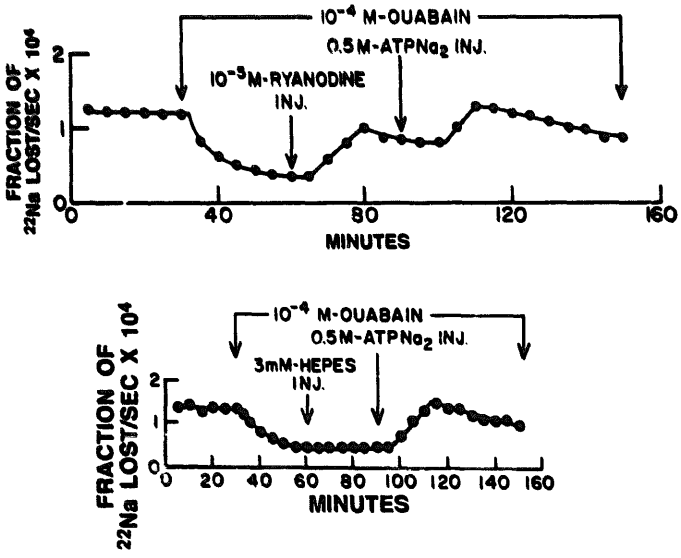


Fig. 10. (Upper panel) Blunting of the response of the ouabain-insensitive Na eflux to the injection of 0.5 M $ATPNa_2$ by prior injection of 10^{-5} M ryanodine. (Lower panel) Lack of effect of injection of 3 mM Hepes, followed by full effect of 0.5 M $ATPNa_2$ injection.

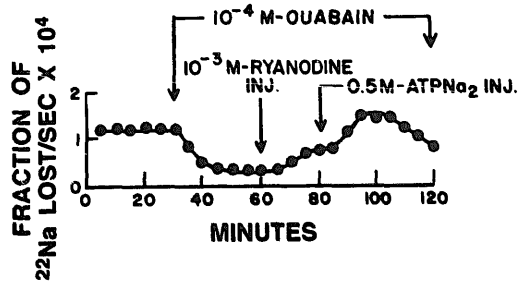


Fig. 11. Failure of 10^{-3} M ryanodine injection to stop the response to 0.5 M $ATPNa_2$ from occurring.

insensitive Na efflux, whereas this is not the case when 3 mM Hepes is injected into a companion control fiber (Fig. 10, lower panel), and that-the response of the test fiber to 0.5 M $ATPNa_2$ is reduced by ryanodine (viz. $142 \pm 41\%$ ($n = 7$) in test fibers, vs. $326 \pm 32\%$ ($n = 8$) in companion controls the difference being significant). In contrast, a sharp rise in efflux occurs following ATP injection into the control fiber. In view of this finding, the next series of experiments involved the injection of a higher concentration of ryanodine, e.g. 10^{-3} M to see if it can abolish the response to ATP. The results of these experiments show that ryanodine is able to reduce but not completely abolish the response to ATP (viz. $188 \pm 40\%$ ($n = 4$) stimulation in test fibers, vs. $345 \pm 47\%$ ($n = 4$) in companion controls, the difference being significant) (Fig. 11). The evidence therefore suggests the possibility that ryanodine may have a second point of action, namely the Na^+-Ca^{2+} exchanger itself. This is more apparent from the data obtained with neomycin (vide infra).

Lack of effect of neomycin

Palade [26] and Meissner and Henderson [27] found that neomycin inhibits the SR Ca^{2+} release channel. The results obtained show that preinjection of 10^{-3} M neomycin does not reduce the size of the response to 0.5 M $ATPNa_2$ injection (viz. $344 \pm 45\%$ ($n = 4$) in test fibers vs. $342 \pm 36\%$ ($n = 8$) in companion controls. Such a result is consistent with the hypothesis that the requirement for a raised Ca_i is not absolute, as found in the experiments with EGTA.

Comparison of ATPγS with $ATPNa_2$

The ATP analogue, ATPγS, is a poor substrate for most ATPases but is a good substrate for protein kinases [28]. Further, transfer of the phosphorothioate residue to the kinase renders the protein fairly resistant to the action of phosphatases [28]. The solution of ATPγS used for injection was a Li salt. This did not seem objectionable for two valid reasons: first, the stability constants of ATP (data on ATPγS being unavailable) and Na^+, Li^+ and Mg^{2+} are 1.31, 1.8 and 4.55, respectively, at 25°C and 0.1 M Me_4N^+ [7]. And second, injection of Li^+ in a concentration less than 1

M into unpoisoned fibers is without effect on the Na efflux [29]. The results of experiments show that injection of 0.5 M $ATP\gamma SLi_2$ into ouabain-poisoned fibers produces a small response, viz. $78 \pm 21\%$ ($n = 5$), a value that is significantly less than $312 \pm 63\%$ obtained by injecting 0.5 M $ATPNa_2$ ($n = 5$) and less than $218 \pm 53\%$ obtained by injecting 0.5 M $ATPNa_2$ in a solution containing 0.5 M LiCl, pH 7.2 ($n = 5$). The difference between the two latter values is not significant.

Comparison of AMP-PNP with ATP and ATPγS

Meissner and Henderson [27] who tested the effect of the non-hydrolyzable ATP analogue AMP-PCP by adding it to skeletal and cardiac muscle SR vesicles found a 5 mM concentration to cause a large release in Ca^{2+}. Since non-hydrolyzable ATP analogues are known to be without effect on Na^+-Ca^{2+} exchange in the squid axon, e.g AMP-PNP [22], it seemed worthwhile to see if the response of ouabain-poisoned fibers to this analogue is less than that obtainable with $ATPNa_2$. The results of experiments show that the injection of 0.5 M $AMP\text{-}PNPLi_2$ (pH 7.2) causes a stimulation of the order of $291 \pm 47\%$ ($n = 5$), a value which is not very different from $392 \pm 36\%$ ($n = 5$) obtained by injecting 0.5 M $ATPNa_2$ but significantly greater than $139 \pm 37\%$ ($n = 5$) obtained by injecting 0.5 M $ATPNa_2$ in a solution containing 0.5 M LiCl (pH 7.2). The latter result is not totally unexpected since Li^+ is known to interrupt Na-Na exchange e.g. red cells [30]. In view of this data, and the fact that the effect obtained with ATPγS in the earlier experiments was small, the next experiments involved a comparison of the effects of AMP-PNP and ATPγS using fibers from the same muscle bundle. The results obtained are as follows: Injection of 0.5 M $AMP\text{-}PNPLi_2$ and 0.5 M $ATP\gamma SLi_2$ into ouabain-poisoned fibers causes stimulation of the order of $86 \pm 12\%$ ($n = 4$) and $70 \pm 6\%$ ($n = 4$), respectively. Both are significantly less than the values of $303 \pm 49\%$ ($n = 4$) and $337 \pm 35\%$ ($n = 4$), obtained by injecting 0.5 M $ATPNa_2$ in a solution containing 0.5 M LiCl, and 0.5 M $ATPNa_2$, respectively.

Comparison of AMP-PNP and ATPγS in fibers preloaded with Na^+

Merely from these results with ATPγS one could not infer that a kinase reaction is not involved in the response of the Na efflux to ATP. For example, it could be argued that these analogues are rapidly hydrolyzed in barnacle myoplasm or that wide variation is a problem. Moreover, both analogues were injected in the form of a Li salt rather than a Na salt. Hence experiments with ATPγS and AMP-PNP were repeated but fibers preloaded with Na^+ 30 min prior to injecting the analogue were included this time. The

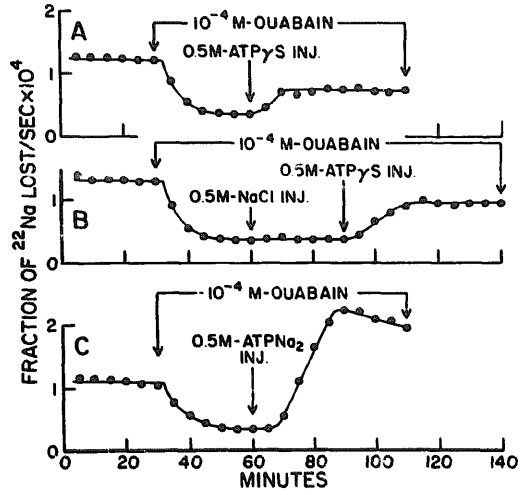


Fig. 12. A comparison between the response of the ouabain-insensitive Na efflux to the injection of (i) 0.5 M ATPγS (A, upper panel), (ii) 0.5 M ATPγS following 0.5 M NaCl (B, middle panel) and (iii) 0.5 M $ATPNa_2$ (C, lower panel).

results of these experiments are as follows: (i) Injection of 0.5 M ATPγS and 0.5 M AMP-PNP into fibers pretreated with 10^{-4} M ouabain causes stimulation of the order of $151 \pm 23\%$ ($n = 3$) and $189 \pm 20\%$ ($n = 3$), respectively. Both are significantly less than the value of $430 \pm 42\%$ ($n = 5$) obtained by injecting $ATPNa_2$. And (ii) injection of 0.5 M AMP-PNP and 0.5 M ATPγS into ouabain-poisoned fibers loaded 30 mins earlier with 0.5 M NaCl (in 3 mM Hepes, (pH 7.2)) cause stimulation of the order of $219 \pm 81\%$ ($n = 3$) and $128 \pm 27\%$ ($n = 3$), respectively. These are not significantly different from each other. Nor are they significantly different from those obtained in the preceding experiments involving 'unloaded' fibers. Representative experiments carried out by injecting ATPγS are given in Fig. 12. As will be seen, the magnitude of the effect obtained with poisoned fibers (upper panel) and Na^+ loaded, ouabain-poisoned fibers (middle panel) following the injection of ATPγS are in both instances strikingly smaller than the response observed upon the injection of $ATPNa_2$. If anything, such results indicate that these analogues are mimetics but they are not as effective as ATP on an equimolar basis.

Response to $ATPNa_2$ following NaCl, LiCl and Hepes injection

This last series of experiments were specifically designed to further substantiate the conclusion that the injection of NaCl or LiCl into ouabain-poisoned fibers is ineffective and that injecting them before $ATPNa_2$ does not modify the size of the response to the nucleotide. The results obtained are as follows: Injection of 0.5 M NaCl prior to 0.5 M $ATPNa_2$ produces a $25 \pm 9\%$ ($n = 5$) rise in ouabain-insensitive Na efflux, whilst injection of the nucleotide 30 min later produces

a rise the magnitude of which averages $161 \pm 20\%$ ($n = 5$). These results are to be compared with those obtained by injecting 0.5 M LiCl, viz. $7 \pm 7\%$ ($n = 5$) rise in the ouabain-insensitive Na efflux (P being > 0.2) and stimulation by the nucleotide of the order of $210 \pm 42\%$ ($n = 5$). In parallel control experiments, the injection of 3 mM Hepes causes a $36 \pm 8\%$ ($n = 5$) rise in the ouabain-insensitive Na efflux, whilst the nucleotide causes stimulation of the order of $177 \pm 32\%$ ($n = 5$). The finding that the effects of NaCl and Hepes injection are alike lends support to the idea that the effect observed with NaCl can be dismissed. But what is noteworthy is that prior injection of Li^+ fails to reduce the size of the response to $ATPNa_2$ injection (see p. 339, $ATPNa_2$ solution containing LiCl).

Discussion

One of the most notable features emerging from these studies is that the mechanism underlying stimulation of the Na efflux by ATP does not necessarily require a rise in internal free Ca^{2+}. This is indicated by the fact that the response to ATP of unpoisoned and ouabain-poisoned fibers preinjected with EGTA is the same as the response to ATP of companion controls. Such a finding is most simply explained by assuming that fibers injected with EGTA have not only a high pCa, but also a high pMg. As will be remembered, the stability constants for EGTA and Ca^{2+} and EGTA and Mg^{2+} are 11 and 5.2, respectively [31]. That de-inhibition caused by a raised pMg might play a key role in this response is suggested by the observation that Mg^{2+} injection practically reverses the response to ATP. This behavior parallels that of the Na^+-Ca^{2+} exchanger in the squid axon where Mg^{2+} is found to be a negative effector [11]. Thus, the new picture which emerges is that EGTA enhances the response of these fibers to ATP, presumably by raising myoplasmic pMg and that this compensates for the absence of a raised Ca_i. However, the objection may be raised that Mg^{2+} reverses the ATP effect not by acting as a negative effector but rather as an inhibitor of a kinase reaction, e.g. cAMP-protein kinase reaction [32], and that this might conceivably be the case if the injection of ATP leads to newly formed cAMP. Such a possibility is real, particularly if the resulting ATP concentration is not in excess of the free myoplasmic Mg^{2+} and/or the free Mg^{2+} concentration is not rendered too low. This is because both an excess of free ATP^{4-} and a very low internal Mg^{2+} are conditions that bring about inhibition of adenylate cyclase activity (see, for example, Refs. 33, 34). Although data concerning the time-course of internal cAMP levels before and after ATP injection are not yet available, it will be recalled that Granot et al. [35] and Armstrong et al. [36] were able to demonstrate with the aid of NMR that excess Mg^{2+} is strongly inhibitory to cAMP-protein kinase activity. This argument is reinforced by evidence that the response of the Na efflux to injected cAMP or catalytic subunit of cyclic AMP is virtually abolished by injecting Mg^{2+} (see, for example, Ref. 37). The fact that it is necessary to use a high concentration of Mg^{2+} to reverse the response to catalytic subunit or ATP is not an unexpected finding in the light of the work of Stralfors and Belfrage [38] showing that a concentration of Mg^{2+} in excess of 10 mM is required for inhibition of the catalytic reaction.

The experiments carried out with ouabain have yielded useful information. First, convincing evidence has been produced that the size of the response to ATP increases as the concentration of ouabain is raised and that the glycoside is effective in the low μmolar concentration range. Specific evidence indicating a rise in internal Na_i^+ in barnacle muscle fibers following ouabain application is provided by the studies of White and Hinke [39] who employed the sodium microelectrode. Thus, the inference can safely be drawn that the response of *unpoisoned* fibers to ATP injection is not large primarily because the internal free Na_i is low. If this is the correct view, it follows then that variability in the size of the response to ATP in unpoisoned fibers may well be due to the wide range of internal Na^+, e.g. 15–34 mmol/kg fiber water obtaining in these fibers [40]. Together, then, these observations are significant because they uphold the generally accepted view that the Na^+ gradient across the fiber membrane plays a governing role in the regulation of Na^+-Ca^{2+} exchange in the reverse mode [9]. Another reason for attaching importance to reverse Na^+-Ca^{2+} exchange in fibers poisoned with ouabain is that the Na pump of barnacle fibers is electrogenic [10] and if so, one would then expect the resting membrane potential following ouabain application to be reduced [41]. Assuming then that this is indeed the case, and since the E_m in cannulated fibers is lower than the E_m of uncannulated, i.e., intact fibers [10], the possibility arises that the Na efflux also changes on account of depolarization of the fiber membrane by ouabain. This follows from the fact that the reverse potential of Na^+-Ca^{2+} exchange is a function of the membrane potential in addition to being a function of the Na^+ and Ca^{2+} gradients. Expressed in another way, the energy from the Ca^{2+} electrochemical gradient drives the Na efflux (viz. $\Delta\tilde{\mu}_{Ca} > 3\Delta\tilde{\mu}_{Na}$). As for the observation that on occasion fibers preinjected with EGTA show augmentation of the response to ATP injection, this is of some significance and in accord with the concept that the $Na^+ : Ca^{2+}$ stoichiometry of the exchanger may vary between 2.5 : 1 and 3 : 1 [42] or may be more variable, and that a raised myoplasmic pMg induced by EGTA in mMolar concentration amplifies the response to ATP injection.

The inability of ouabain to augment the response to ATP upon its application at the time of $ATPNa_2$ injection is a piece of evidence relevant to the question of what happens to the transport enzyme. Such failure is reasonably accounted for by assuming that a rise in myoplasmic pMg impairs the binding of ouabain to membrane Na^+/K^+-ATPase. Why inhibition is not seen following full decay of the response to $ATPNa_2$ may be taken as suggesting that restoration of myoplasmic pMg to its original value fails to occur or that it may occur somewhat slowly. This is a situation which closely parallels that following the application of ouabain to fibers injected with GTP or Gpp(HH)p, where ouabain is found not to inhibit the Na efflux [43]. However, the loss of ouabain sensitivity here is only temporary since it is restored following full decay of the response to ATP. A striking feature that cannot be overlooked is that the fibers injected with $ATPNa_2$ show not only a more delayed onset of the ouabain effect but also reduced sensitivity to the glycoside. These observations may carry several implications for clinical medicine.

Other features arising from the present data are interesting but somewhat puzzling. One is that the injection of the ATP analogue AMP-PNP does not always produce an effect comparable to that obtainable with $ATPNa_2$ or $ATPNa_2$ with LiCl. Although the analogue like ATP acts as an allosteric effector of the SR Ca^{2+} release channel (see, for example, Ref. 44), it cannot be assumed that the level of cooperative interaction between nucleotide and the available free Mg^{2+} is always the same. For example, the internal free Mg^{2+} may be high rather than low in certain fibers. Another possible explanation for such a discrepancy is that the injection of $ATPNa_2$ results in the addition of 5 mM Na to the existing myoplasmic Na which in the case of fibers already loaded with Na^+ by having pretreated them with ouabain, further stimulates reverse Na^+-Ca^{2+} exchange. However, this does not seem likely since the injection of 0.5 M NaCl prior to ATPγS fails to modify the rate constant for ^{22}Na efflux. In other words, a fall in internal specific activity of ^{22}Na is, for all practical purposes, matched by a rise in Na efflux. The other puzzling result is the failure of injected ATPγS to fully mimic the effect of $ATPNa_2$. One reason for the failure of ATγS to produce a large effect is that it is rapidly broken down by ATPases. For example, Gratecos and Fischer [45] found that the SR ATPase of dogfish muscle hydrolyzes ATPγS 10-times more rapidly than ATP within only one minute of addition. That kinases do not play a role is strongly suggested by the fact that the response to ATPγS is small, more particularly since ATPγS is known to render phosphorylated substrate(s) relatively resistant to dephosphorylation by phosphatases. In this connexion, however, it is important perhaps to keep in mind the observation of DiPolo and Beaugé [11] that ATPγS in the absence of internal Mg^{2+} is ineffective in the dialyzed squid axon. Again, this underscores the role of internal pMg.

The existence of a second SR Ca^{2+} release channel, namely that activated by the second messenger *myo*-inositol 1,4,5-trisphosphate (IP_3) in skeletal muscle (see, for example, Ref. 46) raises the question as to whether the response to ATP injection may additionally involve IP_3. This possibility however seems rather remote in view of two lines of evidence: first, Lea, Griffiths, Tregear and Ashley [47] who found IP_3 ineffective in releasing Ca^{2+} from the SR of skinned frog and barnacle muscle fibers also reported that the addition of D-2,3-diphosphoglycerate does not modify this irresponsiveness. And second, work in progress in this laboratory shows that the injection of IP_3 or the non-hydrolyzable analogue, D-*myo*-inositol 1,4,5-trisphosphorothioate (D-IP_3S) into unpoisoned and ouabain-poisoned fibers fails to stimulate the Na efflux (Bittar, E.E., Huang, Y.-P. and Potter, B.V.L., unpublished data).

Further information bearing on the problem of a fall in myoplasmic pCa is the observation that, as a rule, injection of $ATPNa_2$ into unpoisoned fibers often elicits a mild contraction which is followed by relaxation or fails to elicit a contraction, whilst ouabain-poisoned fibers show a prompt and powerful contraction upon injection of the nucleotide. Only partial relaxation follows. It is therefore entirely reasonable to draw the provisional conclusion that myoplasmic pCa in unpoisoned fibers reaches the threshold value for excitation-contraction coupling (i.e. a pCa of approx. 6) following the injection of $ATPNa_2$. On this view, then, it would seem that myoplasmic pCa in poisoned fibers falls below 6 following $ATPNa_2$ injection. This favors the hypothesis that the response to ATP involves the entry of trigger Ca^{2+} via the putative Na^+-Ca^{2+} exchanger and/or increased release of Ca^{2+} via the SR Ca^{2+} release channel. This interpretation gains further credence if a rise in free Ca_i causes so-called Ca^{2+}-induced release of Ca^{2+} by the SR.

Lastly, the case for activation of Na^+-Ca^{2+} exchange in the reverse mode in ouabain-poisoned fibers by injecting ATP into them leaves unanswered the question whether a phosphorylation reaction regulates the behavior of the exchanger. In heart muscle, for example, Caroni and Carafoli [48] produced evidence that phosphorylation-dephosphorlyation reactions are involved in the mechansim of Na^+-Ca^{2+} exchange across the sarcolemmal membrane. However, Philipson [49] does not hold this view. Whether the barnacle fiber preparation will prove to be a useful system for studying this particular problem remains to be seen.

Acknowledgement

This work was supported by NIH grant 5142.

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