Biochimica et Biophysica Acta, 1103 (1992) 77–84

BBAMEM 75481

The ability and inability of ATP to stop aluminum from reducing the sodium efflux in unpoisoned barnacle muscle fibers

Yong-Ping Huang and E. Edward Bittar

Department of Physiology, University of Wisconsin, Madison, WI (USA)

(Received 14 March 1991)
(Revised manuscript 24 July 1991)

Key words: Sodium ion efflux; Aluminum; ATP; (Barnacle fiber)

A study has been made in single barnacle muscle fibers with the object of determining whether ATP is able to protect the resting Na efflux from the effects of injected aluminum (Al) and whether Al is able to reduce or abolish the stimulatory action of ATP on the efflux. The results of the experiments show that neither ATPMg nor $ATPNa_2$ preinjection stops Al from reducing the basal Na efflux in unpoisoned fibers which undergo a large fall (hypersensitive fibers). Preinjection of Al into such fibers reduces or abolishes the stimulatory response of the Na efflux to ATP injection. In less hypersensitive fibers, however, ATPMg is protective. This is also true of $ATPNa_2$ preinjection in both classes of fibers showing stimulation. Injection of a mixture of $AlCl_3$-$ATPNa_2$ into unpoisoned fibers causes less inhibition than $AlCl_3$ injection. The hypothesis that both ATPMg and $ATPNa_2$ are protective is also supported by the results obtained with ouabain-poisoned fibers: (i) Al injection after ATP fails to reverse the stimulatory response to ATP, while ATP injection after Al exerts only a small or no effect. (ii) Mg^{2+} injection fails to reverse the stimulatory response to Al injection in poisoned fibers. And (iii) Anti-proteolysis agents e.g. leupeptin and pepstatin, upon preinjection, do not alter the kinetic results obtained by injecting Al into unpoisoned and ouabain-poisoned fibers.

Introduction

The work of Bittar and Huang [1] provides evidence in support of the hypothesis that stimulation of the ouabain-insensitive Na efflux by injecting $ATPNa_2$ into barnacle muscle fibers involves operation of the Na^+-Ca^{2+} exchanger in the reverse mode and that a raised internal free Ca^{2+} is not a prerequisite for the occurrence of this response. It also provides evidence that Mg^{2+} is able to reverse this response to ATP injection, thus confirming the current view that internal free Mg^{2+} is an inhibitor of this exchanger. The study of this exchanger has more recently become of particular interest for at least two reasons. One is the lack of information on the question whether it is a primary target of aluminum (Al) and the other whether ATP is able to protect the Na efflux from the untoward effects of Al. Thus, the following communication represents in some measure an examination of both questions. As will be recalled, GTP is protective against the inhibitory effects of Al but not in hypersensitive fibers [2], i.e. fibers which show a great fall in Na efflux when Al is injected. Considering that the stability constants of ATP and Al, and GTP and Al are the same, that is, the log K_s value is 10.9 [3,4], this being seven units larger than the log K_s of ATP and Mg^{2+} (e.g. see Ref. 5), and considering that the ATP/GTP ratio in these fibers is quite high (e.g. ATP 4.4 mM and GTP 0.27 mM [6]), it seemed worthwhile to verify the idea that ATP which is readily plentiful may be more protective than GTP as a chelator and hence stop Al from inhibiting the resting Na efflux. However, as will be shown, this is not the case. The evidence presented also supports the idea that the ATPAl complex formed as the result of injecting ATP after Al probably behaves as a 'dead-end' substrate. This is also true of the GTPAl complex [9].

Abbreviation: Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: E.E. Bittar, Department of Physiology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA.

Materials and Methods

The species of barnacles, the methods of dissection, cannulation, microinjection and counting of ^{22}Na activ-

ity in the effluent and in the fiber were essentially as described by Bittar [7]. 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. The solutions of $ATPNa_2$, ATPMg and $MgCl_2$ used for injection were prepared using 3 mM-Hepes (pH 7.2). Solutions containing $AlCl_3$ were prepared using double-distilled, de-ionized water at a low pH e.g. a 0.5 M $AlCl_3$ solution, pH 1.9. Information about speciation of Al in solutions with a varying pH is given by Huang and Bittar [2]. The volume of test fluid, water or a 3 mM-Hepes solution injected into a fiber was 0.3–0.4 μl. This is diluted by the myoplasm by a factor of roughly 100. All experiments were carried out at an environmental temperature of 22°–24°C.

The results are given as the mean ± S.E. Student's *t*-test for unpaired values was used to determine significance levels. Differences between means were considered significant at $P < 0.05$. Estimates of the size of the observed effects on the ^{22}Na efflux were calculated on the basis of the rate constant plots (i.e., fraction of ^{22}Na lost/s vs. time). For the case where two stimulatory or inhibitory phases were present in succession, the size of the second response was computed by taking the difference between the two combined phases and the first phase. Moreover, two rules based on experience acquired with the technique of microinjection are applicable here. The first is that the injection of a 3 mM Hepes solution or water alone in approximately 0.4 μl volumes into control unpoisoned or ouabain-poisoned fibers is often without effect on the Na efflux. However, a transitory rise of the order of 10–20% is sometimes seen. This is attributed to a slight and temporary rise in internal free Ca^{2+} resulting from injury caused by the insertion of the microinjector down the axis of the fiber. Such results obtained with companion control fibers are not dismissed. For the case where the injection of a test solution elicits a transitory rise in the Na efflux of less than 20%, the result is regarded as significant, providing companion control fibers show a lack of effect with Hepes or water. As for the second rule, the injection of a 3 mM Hepes solution or only water never causes a decline in the Na efflux in both unpoisoned and ouabain-poisoned fibers. Thus, results showing a decline in the Na efflux following the injection of a test solution are considered significant.

Some of the figures shown in this paper are composites of several efflux plots. These are based on experiments carried out with fibers isolated from the same barnacle specimen. This is done in preference to showing a representative experiment solely because of the observed uniformity in behaviour of the Na efflux in these experiments.

All reagents used were analytical grade. Ouabain, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), $ATPNa_2$, ATPMg, leupeptin (hemisulfate salt) and pepstatin A were purchased from Sigma Chemical Company, St. Louis, MO. $AlCl_3$ was purchased from Fisher Scientific Company, Fair Lawn, New Jersey. Deferoxamine was a gift from Ciba-Geigy Corporation, Summit, NJ.

Results

Al injection before and after ATPMg into unpoisoned fibers

Keeping in mind that the effect of $AlCl_3$ injection into unpoisoned fibers is either biphasic with inhibition of the resting Na efflux following transitory stimulation, or more commonly, monophasic with inhibition occurring promptly and taking at least an hour to reach a maximum [8], experiments were first undertaken in which the ATPMg complex was used. The results obtained show: (i) (a) Injection of 0.5 M $AlCl_3$, as illustrated in Fig. 1A, exerts a biphasic effect, viz. stimulation is followed by inhibition. This stimulatory response is of the order of 49 ± 4% ($n = 5$), while the inhibition is of the order of 47 ± 19% ($n = 5$). The latter value is not different from 42 ± 8% ($n = 4$) inhibition obtained by injecting Al into ATPMg-enriched fibers (Fig. 1B). Notice that the inhibitory effect is not yet complete (Fig. 1A). Also shown is that the injection of ATPMg 70 mins after Al exerts a transitory and negligible rise in the efflux and that it fails to stop the decline in the rate constant for ^{22}Na efflux. (b) As illustrated in Fig. 1B, injection of 0.5 M ATPMg produces a stimulatory response of the resting Na efflux (of the order of 62 ± 12%, $n = 4$) and that the subsequent injection of 0.5 M $AlCl_3$ (at $t = 70$ mins) produces a biphasic

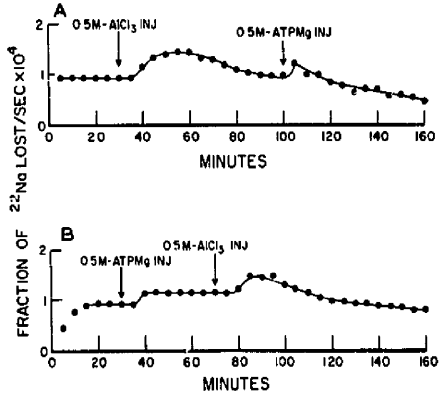

Fig. 1. (A) The biphasic response of the basal Na efflux to the injection of 0.5 M $AlCl_3$, followed by a negligible transient response to 0.5 M ATPMg injection. (B) The biphasic response of the Na efflux to the injection of 0.5 M $AlCl_3$ in a fiber preinjected with 0.5 M ATPMg.

response, viz. stimulation followed by inhibition. The reason for the stimulation has been tentatively elucidated by Bittar and Huang [9]. The delayed inhibition averages $42 \pm 8\%$ ($n = 4$) in magnitude, as already mentioned. It is thus quite clear that in the case of hypersensitive fibers, the injection of ATPMg after Al is rather ineffective. This is also the case with the preinjection of ATPMg. (c) In additional parallel experiments, 0.5 M deferoxamine was injected 30 mins before 0.5 M $AlCl_3$. This completely stops Al from exerting an inhibitory effect on the resting Na efflux ($n = 4$). In sharp contrast, companion controls injected with 3 mM-Hepes, followed by 0.5 M $AlCl_3$ show $40 \pm 25\%$ inhibition ($n = 4$). (ii) In less sensitive fibers, however, prior injection of ATPMg is found to prevent the inhibitory effect of Al from occurring. For example, fibers isolated from the same bundle of fibers exhibiting a fall of $20 \pm 6\%$ ($n = 4$) in the resting Na efflux following the injection of 0.5 M $AlCl_3$ fail to show a fall when Al is injected 60 mins after 0.5 M ATPMg ($n = 4$). By 'less sensitive' is meant a reduction in the resting Na efflux by Al injection of about 20% or less.

Al injection before and after $ATPNa_2$ into unpoisoned fibers

It is known that the injection of $ATPNa_2$ into unpoisoned fibers produces a larger response than ATPMg [1]. This difference is attributed to the ability of the $ATPNa_2$ complex to raise myoplasmic pMg as the result of chelating internal free Mg^{2+}. It therefore seemed of special interest to see if the prior addition of Al to the myoplasm would reduce the response to the injection of $ATPNa_2$. The results obtained are as follows: (a) Injection of 0.5 M $ATPNa_2$ after 0.5 M $AlCl_3$ produces stimulation of the order of $32 \pm 11\%$ ($n = 4$), a value significantly less than $114 \pm 21\%$ ($n = 4$) obtained by injecting 0.5 M $ATPNa_2$ (prior to $AlCl_3$) and $91 \pm 15\%$ ($n = 4$) obtained by injecting 0.5 M $ATPNa_2$ after water (pH 1.9). (b) Injection of 0.5 M $AlCl_3$ after $ATPNa_2$ produces stimulation of the order of $28 \pm 9\%$ ($n = 4$). However, this value is the same as that obtained by injecting water (pH 1.9), viz. $28 \pm 10\%$ ($n = 4$). Thus, when stimulation in unpoisoned fibers occurs following the injection of Al, preinjection of $ATPNa_2$ is protective. Further, it is noteworthy that Al injection after ATP fails to produce any inhibition ($n = 4$). (c) In parallel experiments, injection of 0.5 M $AlCl_3$ into companion controls produces a biphasic effect, viz. stimulation of the order of $41 \pm 4\%$ ($n = 4$), followed by inhibition of the order of $17 \pm 9\%$ ($n = 4$). However, the significance of the observed stimulatory phase is dismissed, since the injection of water (pH 1.9) into companion controls causes a transitory rise of the order of $42 \pm 6\%$ ($n = 4$). Together, then, these results indicate that the preinjection of ATP prevents Al from

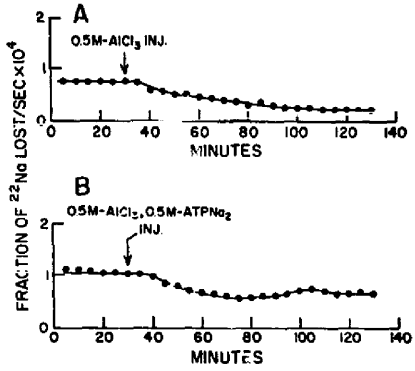

Fig. 2. (A) The monophasic inhibitory action of 0.5 M $AlCl_3$ injection on the basal Na efflux. (B) The monophasic inhibitory action of injecting a solution of 0.5 M $AlCl_3$, 0.5 M $ATPNa_2$ in a companion test fiber.

reducing the basal Na efflux and that Al preinjection stops ATP from exerting its full stimulatory effect.

Injection into unpoisoned fibers of a solution of 0.5 M $AlCl_3$–0.5 M $ATPNa_2$ (pH 1.9)

The idea that ATP does act as a chelator of free Al^{3+} was put to the test in a rather direct way, namely by injecting a solution of a mixture of 0.5 M $AlCl_3$–0.5 M $ATPNa_2$ (pH 1.9) into unpoisoned fibers to see whether it reduces the Na efflux and whether the size of such an effect is smaller than that of injected $AlCl_3$ alone. The results of experiments reveal that injection of the mixture causes a fall in the resting Na efflux, the magnitude of which averages $32 \pm 11\%$ ($n = 4$). This is to be compared with a $70 \pm 3\%$ fall ($n = 4$) caused by injecting 0.5 M $AlCl_3$ into companion controls. The difference is significant. A typical experiment of each type is given in Fig. 2A and B. Sensitivity to Al of this magnitude is unusual. Hence it seemed important to include less hypersensitive fibers, and further experiments were done, the results of which show that the mixture causes $13 \pm 6\%$ inhibition ($n = 12$), whilst injection of 0.5 M $AlCl_3$ causes $35 \pm 8\%$ ($n = 12$) inhibition. The difference is significant, P being < 0.05. Such results are interpreted as strongly suggesting that ATP does act as a powerful chelator of Al when both are introduced into the myoplasm simultaneously and that the phenomenon of hypersensitivity is rather unlikely to be the result of the direct addition of the ATPAl complex to the myoplasm.

Al injection into poisoned fibers before and after ATPMg

Bittar and Huang [1] found that ATPMg and $ATPNa_2$ are equipotent following injection into fibers prepoisoned with 10^{-4} M-ouabain. They had also found that as a rule Al injection into poisoned fibers leads to

stimulation of the remaining Na efflux [9]. The results of experiments show: (i) Injection of 0.5 M $AlCl_3$ into fibers pretreated with 10^{-4} M-ouabain causes a rise in the remaining Na efflux of the order of $69 \pm 6\%$ ($n = 4$), which is not significantly different from $102 \pm 20\%$ ($n = 4$) obtained by injecting Al after 0.5 M ATPMg or $106 \pm 20\%$ ($n = 4$) obtained by injecting Al after 3 mM Hepes. (ii) However, injection of 0.5 M ATPMg causes a rise in the Na efflux of the order of $174 \pm 17\%$ ($n = 4$), a value which is significantly larger than $37 \pm 27\%$ ($n = 4$) obtained by injecting ATPMg after 0.5 M $AlCl_3$. Repetition of this type of experiment confirmed these results. It is thus quite clear that Al preinjection reduces the size of the response to ATPMg. Such a result suggests that Al stops the Na^+–Ca^{2+} exchanger from responding or, as is more likely newly formed ATPAl is relatively ineffective as an effector or substrate. Alternatively, Al is able to stop the membrane adenylate cyclase system from responding to the sudden addition of ATPMg as the result of the formation of ATPAl which then acts as a competitive inhibitor (e.g. Refs. 10 and 11).

Al injection into poisoned fibers before and after $ATPNa_2$

The results of experiments carried out with fibers preexposed to 10^{-4} M-ouabain show: (i) Whereas Al injection prior to $ATPNa_2$ causes a rise in the ouabain-insensitive Na efflux which averages $74 \pm 25\%$ ($n = 4$), its injection after $ATPNa_2$ is completely without effect ($n = 4$). (ii) Whereas injection of $ATPNa_2$ causes a $422 \pm 106\%$ rise in the ouabain-insensitive Na efflux ($n = 4$), its injection after Al causes only an $86 \pm 24\%$ rise ($n = 4$). The difference is significant, P being < 0.02. (iii) Injection of 3 mM Hepes before or after Al is without effect ($n = 4$ in each case). However, Al injection before Hepes is found to lead to a delayed decline in the ouabain-insensitive Na efflux, the magnitude of which averages $41 \pm 2\%$ ($n = 4$). No prompt inhibition is seen when Al is injected prior to ATP ($n = 4$). Such results are taken to mean that prior injection of $ATPNa_2$ is protective against the biphasic effect of Al on the ouabain-insensitive Na efflux but that prior injection of Al reduces the response to $ATPNa_2$. The latter finding is not unexpected in the light of the preceding results with ATPMg.

(i) Shown in Fig. 3A is a composite of three semilog efflux plots illustrating that the injection of 0.5 M $ATPNa_2$ causes a prompt and sharp rise in the remaining Na efflux, and that the injection of 0.5 M $AlCl_3$ following the onset of peak stimulation by ATP produces a negligible transient rise in this efflux. In contrast, the composite of four plots shown in Fig. 3B illustrates that the injection of 0.5 M $AlCl_3$ after 3 mM Hepes into companion controls causes a transitory but sharp rise in the efflux. Such results constitute clear evidence in favor of the suggestion that if the injection

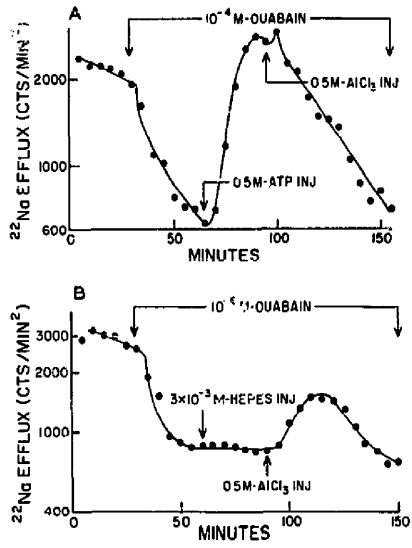

Fig. 3. (A) The injection of 0.5 M $AlCl_3$ into fibers poisoned with 10^{-4} M ouabain shortly after the onset of peak stimulation of the ouabain-insensitive Na efflux by injecting 0.5 M ATP (composite of three semilog efflux plots). (B) The injection of $3 \cdot 10^{-3}$ M Hepes into fibers poisoned with 10^{-4} ouabain, followed by the injection of 0.5 M $AlCl_3$ (composite of four semilog efflux plots).

of $ATPNa_2$ leads to stimulation of reverse Na^+–Ca^{2+} exchange as suggested by Bittar and Huang [1], then the sudden introduction of Al into the myoplasm does not seem to disturb the operation of the exchanger in a significant way or the added Al is rapidly chelated and little or no free Al^{3+} is allowed to occur.

Illustrated in Fig. 4 is that the injection of 0.5 M $ATPNa_2$ following peak stimulation by Al causes a rather small transitory rise in the efflux. Injection of 3 mM Hepes after Al, as illustrated in Fig. 4B is without effect.

The question now asked was this: Does the sudden addition of ATP to the myoplasm result in significant splitting of this nucleotide, for example, to cAMP and PPi, and if so, is it then possible to explain why prior injection of ATP into unpoisoned fibers fails to protect the resting Na efflux from the inhibitory action of Al? A similar question was asked in connexion with the study of the protective action of GTP [2]. However, because of the lack of data on the time-course of injected ATP, as well as internal cAMP, this question was examined in another way, namely by halving the concentration of the $AlCl_3$ solution used for injection. The results of these experiments show that the injection of 0.25 M $AlCl_3$ following 0.5 M $ATPNa_2$ into unpoisoned fibers produces $40 \pm 5\%$ inhibition in the resting Na efflux ($n = 8$), a value not significantly different from $49 \pm 5\%$ inhibition obtained by injecting

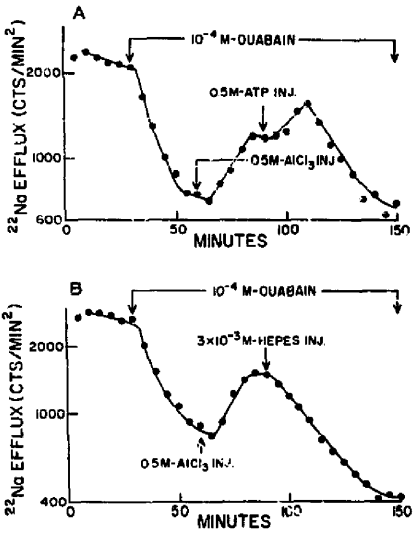

Fig. 4. (A) The injection of 0.5 M ATP into ouabain-poisoned fibers shortly after peak stimulation by injecting 0.5 M $AlCl_3$ (composite of four semilog efflux plots). (B) The injection of $3\cdot10^{-3}$ M Hepes shortly after peak stimulation by injecting 0.5 M $AlCl_3$ (composite of three semilog efflux plots).

0.25 M $AlCl_3$ into companion controls ($n = 7$), or $44 \pm 5\%$ inhibition obtained by injecting 0.25 M $AlCl_3$ after 3 mM Hepes. Thus, the only conclusion possible is that $ATPNa_2$ injection into unpoisoned fibers, as in the case of GTP, is not protective in fibers hypersensitive to the inhibitory effect of Al.

Comparison of ATPMg with $ATPNa_2$ using poisoned fibers

To test the possibility of competition between Al^{3+} and Mg^{2+}, experiments were done in which 0.5 M $AlCl_3$ was injected into ouabain-poisoned fibers before and after 0.5 M ATPMg and 0.5 M $ATPNa_2$. The results are as follows: (i) Al injection into fibers subsequently injected with ATPMg produces a rather delayed small inhibition in one of the four fibers tested (of the order of 27%), whereas Al injection into fibers subsequently injected with $ATPNa_2$ produces delayed inhibition in the four fibers, the magnitude of which averages $20 \pm 6\%$. However, the stimulatory responses observed after the injection of ATPMg and $ATPNa_2$ into both groups of fibers are not significantly different from each other (viz. $20 \pm 12\%$, $n = 4$ vs. $41 \pm 17\%$, $n = 4$). (ii) ATPMg injection before Al causes stimulation of the order of $123 \pm 26\%$ ($n = 4$), whereas $ATPNa_2$ injection causes stimulation of the order of $362 \pm 63\%$ ($n = 4$). The difference is significant. Both values are significantly larger than those obtained by injecting ATPMg and $ATPNa_2$ after Al, viz. $20 \pm 12\%$ and $41 \pm 17\%$, respectively. Further, injection of Al after ATPMg produces inhibition in one of the four fibers tested (of the order of 33%), whereas Al after $ATPNa_2$ produces $24 \pm 8\%$ inhibition in the four fibers tested. Such data suggests the possibility that fibers injected with $ATPNa_2$ are more vulnerable to inhibition by Al than fibers injected with ATPMg.

Repetition of the above experiments led to the following data: (i) Al injection (before ATPMg) into poisoned fibers produces only a monophasic response, viz. stimulation of the order of $197 \pm 38\%$, ($n = 4$). This is the same as that obtained by injecting Al before $ATPNa_2$ (viz. $167 \pm 19\%$, $n = 4$). Little or no response to the injection of ATPMg or $ATPNa_2$ after Al is seen in both groups of fibers, whereas the injection of ATPMg and $ATPNa_2$ (before Al) produces stimulation of the order of $132 \pm 11\%$ ($n = 4$) and $308 \pm 27\%$ ($n = 4$), respectively. And (ii) injection of 0.5 M $AlCl_3$ long after peak stimulation by ATPMg injection produces a prompt rise in the Na efflux averaging $115 \pm 23\%$ ($n = 4$). However, injection of Al long after peak stimulation by $ATPNa_2$ produces a rise in the Na efflux in only two of the four fibers tested. This effect averages $208 \pm 49\%$ in magnitude. A representative experiment from each group is shown in Fig. 5. Notice the occurrence of a slight delay in the onset of the response to Al injection in both experiments.

Lack of effect of Mg^{2+} injection after Al in poisoned fibers

If it be true that Al does not act as an inhibitor of the Na^+–Ca^{2+} exchanger, then the question arises as to whether or not the stimulatory response resulting from the injection of Al into ouabain-poisoned fibers is unaffected by Mg^{2+}. As shown in Fig. 6 (a composite of four plots), injection of 0.5 M $MgCl_2$ fails to inter-

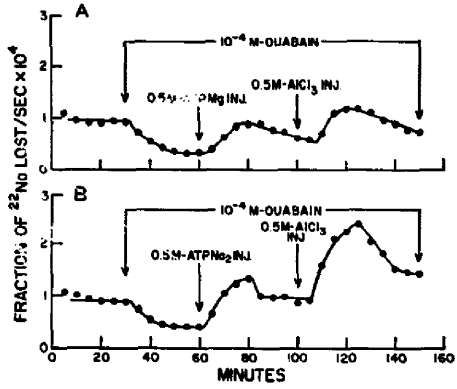

Fig. 5. (A) Stimulation following the injection of Al into a poisoned fiber preinjected with ATPMg. (B) Stimulation following the injection of Al after $ATPNa_2$.

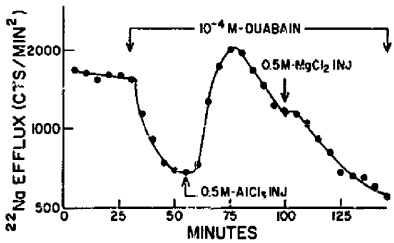

Fig. 6. The failure of 0.5 M $MgCl_2$ injection to reverse the stimulatory response of the ouabain-insensitive Na efflux to the injection of 0.5 M $AlCl_3$ (composite of four semilog efflux plots).

rupt the response of the ouabain-insensitive Na efflux to the injection of 0.5 M $AlCl_3$.

Leupeptin and pepstatin before Al

It is now widely recognized that the activation of Ca^{2+}-dependent proteases by Ca^{2+} leads to cell injury e.g. hepatocytes (e.g. Ref. 12). Since barnacle fibers usually contract following the injection of Al, the objection may be raised that the observed alterations in the response to ATP in fibers pre-injected with Al reflect the existence of cell injury rather than the ability of ATP to form a 'dead-end' effector in the presence of free Al^{3+}. For this reason experiments were carried out in which two known inhibitors of Ca^{2+}-activated proteins, leupeptin and pepstatin [12,13] were tested by injecting them prior to Al. Briefly, the results obtained indicate that both protease inhibitors are unable to influence the behavior of the resting Na efflux toward Al injection. Notice the lack of effect of subsequent application of 10^{-4} M-ouabain, a result confirming the observation of Huang and Bittar [2] that Al renders these fibers less sensitive to the glycoside. Fig. 7B shows that the preinjection of 10^{-3} M PP fails to interfere with the inhibitory action of 10^{-4} M-ouabain and that subsequent injection of 0.5 M $AlCl_3$ leads to a monophasic stimulatory response of the remaining Na efflux (viz. 207 ± 34% stimulation, $n = 4$). Fig. 7C shows an identical response to Al injection into a ouabain-poisoned fiber preinjected with 10^{-2} M LP (viz. 183 ± 59% stimulation, $n = 4$). And Fig. 7D confirms these kinetic results in that the preinjection of a mixture of LP and PP fails to stop the stimulatory response to Al from taking place. Though the size of the response to Al appears somewhat small, viz. 87 ± 31% ($n = 3$), it is not statistically different from the two preceding values, P being > 0.05 and > 0.2, respectively. Experiments involving the injection of LP or PP (or both) followed by Al, and then ATP were considered but not carried out since this would mean more damage to these fibers by having to inject them thrice (counting the procedure of loading with ^{22}Na).

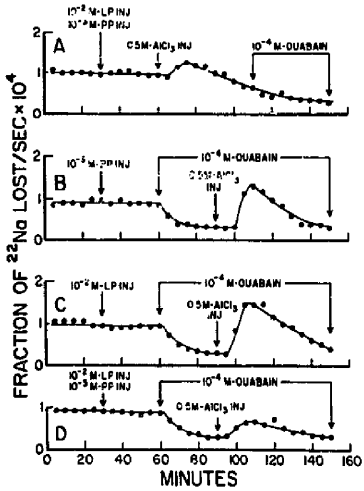

Fig. 7. (A) The biphasic response of the basal Na efflux to the injection of 0.5 M $AlCl_3$ in a fiber preinjected with 10^{-2} M LP and 10^{-3} M PP. Notice the relative lack of effect of 10^{-4} M ouabain when applied after the onset of the inhibitory phase of Al action. (B) The injection of 0.5 M $AlCl_3$ into a fiber injected with 10^{-3} M PP, and then exposed to 10^{-4} M ouabain. (C) The injection of 0.5 M $AlCl_3$ into a fiber injected with 10^{-2} M LP, and then exposed to 10^{-4} M ouabain. (D) The injection of 0.5 M $AlCl_3$ into a fiber injected with 10^{-2} M LP and 10^{-3} M PP, and then exposed to 10^{-4} M ouabain. In experiments of this type the fibers are isolated from the same muscle bundle.

Discussion

The present results provide support for the view that neither ATPMg nor $ATPNa_2$ protects hypersensitive unpoisoned fibers from the inhibitory effect of Al injection. As will be remembered, this is also true of GTP and Gpp(NH)p [2] whose mechanisms of action are largely different from those of ATP. In an effort to gain some insight into the significance of this mechanism, the powerful Al chelator, deferoxamine, was injected prior to Al and found to prevent the occurrence of inhibition in such fibers. This was not unexpected since deferoxamine is known to bind Al 10^{11} times more strongly than does ATP [14]. If for the moment one accepts that the data obtained with LP and PP suggest that the inhibitory action of Al is not the result of a Ca^{2+}-mediated proteolytic reaction, then the simplest way of explaining Al hypersensitivity in unpoisoned fibers is to suppose that it is largely due to the binding by Al^{3+} of not only ATP but also internal phosphates e.g. P_i. In this connexion, it is perhaps worth mentioning that the ^{27}Al-NMR evidence obtained by Jackson [15] reveals that in the presence of 1 mM H_2PO_4 and 100 μM Al the predominant species is $[Al(PO_4)_2H]^{2-}$ over almost the entire pH range. As it happens, the possibility of phosphate precipitation

was considered by Huang and Bittar [2] when in a recent pilot study they preinjected phosphate into hypersensitive fibers and found it to almost completely abolish inhibition of Na efflux by Al. This work is now in progress.

In the light of the comparison drawn between the protectiveness of ATPMg and $ATPNa_2$, it is well to keep in mind that Al seems to act by competing with Mg^{2+}. That is, removal of internal free Mg^{2+} by injecting $ATPNa_2$ renders these fibers more sensitive to the untoward effect of Al. This is clearly illustrated by poisoned fibers injected with $ATPNa_2$ or ATPMg, followed by Al. However, a less satisfactory solution of this problem presents itself when ouabain-poisoned fibers are examined. This is perhaps mainly because the inhibitory phase of Al injection is seen rather seldom within the time-frame of experiments of this type. Nonetheless, a salient finding to emerge is that ATPMg and $ATPNa_2$ are protective insofar as the stimulatory phase of Al injection is concerned. Both reduce or eliminate the stimulatory response of the ouabain-insensitive Na efflux elicited by Al but this depends inter alia on how long after ATP injection Al injection is carried out (vidé Fig. 5). That the response itself is not likely to be related to a proteolytic reaction is suggested by the fact that LP or PP or both together fail to modify the behavior of the efflux toward Al (Fig. 7).

It is significant that the injection of Al shortly after peak stimulation of the ouabain-insensitive Na efflux by ATP injection fails to reverse this response to ATP. Such a result offers a powerful argument that little or no free Al^{3+} is left in the myoplasm to inhibit the Na^+–Ca^{2+} exchanger. This is of course based on the assumption that ATP injection into poisoned fibers stimulates reverse Na^+–Ca^{2+} exchange, as suggested by Bittar and Huang [1]. Alternatively, this result can be simply explained by assuming that any free Al^{3+} occurring in the myoplasm does not act as an inhibitor of the exchanger. Such an interpretation can readily be reconciled with the observation that Mg^{2+} injection fails to reverse the response to Al injection, if both Al^{3+} and Mg^{2+} are genuine inhibitors of the exchanger. The question then is: Which is the preferred explanation? Clearly, the former, for it is not only straightforward but also supported by experiments which show that the injection of a mixture of $AlCl_3$–$ATPNa_2$ is not as inhibitory as $AlCl_3$ injection. Additionally, it is attractive conceptually because ATPAl may be regarded as a dead-end complex for a wide range of enzymes, notably the Na^+-K^+-ATPase system. This is reminescent of the work of Womack and Colowick [10] and Viola et al. [11] who investigated the actions of Al on yeast and brain hexokinase. They concluded that Al causes slow-binding inhibition as the result of competition between ATPAl and ATPMg—a situation strikingly similar to that created by injecting a mixture of $AlCl_3$–ATP and finding that the Na efflux is, in fact, reduced by such a maneuver. However, the problem might not be this simple, since allowance must be made for the possibility that myoplasmic ATPMg in some barnacle fibers might be low, e.g. 1.4 mM [16] and that such estimates carried out with the aid of firefly reflect an overall value of ATP occurring in micro-domains. Moreover, too much should not be made of ATPAl formation to the exclusion of several other considerations. One is that the Brönsted theory dictates that there is a close relationship between a reaction rate and ionic strength but this problem is not amenable to independent study in this type of work. The other concerns systems having particularly high conditional log K values e.g. 2,3-diphosphoglycerate which has a value of 12 (Martin, B. and MacDonald, T., private communication). In other words, situations where free Mg^{2+} is replaced by Al^{3+} are bound to involve inhibition of Mg^{2+}-dependent processes including many key reactions. A prime example is the slowing down of GTP hydrolysis by Al^{3+} e.g. bovine brain microtubules which involves GTPAl formation [17]. Another but less striking example is that provided by bovine retinal rod GTPase which appears to be a poor target of Al^{3+} [18]. This is also the case with liver fluke adenylate cyclase: though very active, the cyclase is not very sensitive to Al in high concentrations [19]. For example, 13–38% inhibition of basal cyclase requires 25 μM $AlCl_3$. The fact that ATP was present in the preincubation reaction mixture immediately suggests that inhibition of the enzyme by Al might well be the result of competition between dead-end ATPAl and ATPMg. Whether the observed inhibition of the resting Na efflux by injecting Al into barnacle fibers is due to inhibition of basal adenylate cyclase by a mechanism involving competition beween ATPAl and ATPMg is not yet clear. Such a possibility remains real, despite evidence that the injection of PKI into these fibers fails to reduce basal Na efflux but stops injected cAMP from stimulating the Na efflux [20]. Altogether, if Al acts as an inhibitor by precipitating internal free phosphates, then the intriguing question is to understand how P_i precipitation in myoplasm having a high ArP (this is the phosphagen in invertebrates in lieu of CrP, e.g. 24 mM [21]) leads to a fall in the active extrusion of Na in these fibers. In order to understand this it will be necessary to focus on the Mg^{2+}-dependent arginine kinase reaction [22] which if inhibited by Al^{3+} might be unable to buffer myoplasmic ATPMg sufficiently. And more importantly, in *hypersensitive* unpoisoned fibers with a low myoplasmic ATPMg such a disturbance could turn out to be a key event leading to a fall in resting Na efflux.

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