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The reduction of the inhibitory effect of aluminum on Na^+ efflux in barnacle muscles fibers by preinjecting phosphate

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The object of the present study was to test the hypothesis that the pre-enrichment of single muscle fibers from the barnacle *Balanus nubilus* with inorganic phosphate may protect the basal Na efflux from the inhibitory effect of Al injection. This approach was adopted in the light of evidence that the preinjection of ATP fails to stop the Na efflux in unpoisoned fibers from falling following the injection of Al. The results of the experiments are as follows: (i) Preinjection of K_2HPO_4 into unpoisoned fibers reduces the magnitude of the inhibitory effect on the basal Na efflux of injected Al in a dose-dependent manner but fails to completely stop it from occurring. (ii) Injection of K_2HPO_4 following Al into unpoisoned fibers fails to arrest or reverse the inhibitory effect of injected Al. (iii) Injection of K_2HPO_4 in a concentration as high as 0.5 M is without effect on the course of the basal Na efflux. (iv) Injection of K_2HPO_4 into ouabain-poisoned fibers fails to stop Al from stimulating the ouabain-insensitive Na efflux. Injection of K_2HPO_4 following peak stimulation by injecting Al is also without effect. (v) Injection of K_2HPO_4 in a concentration as high as 0.5 M is without effect on the course of the ouabain-insensitive Na efflux. Collectively, the results obtained with unpoisoned 'hypersensitive' fibers are consistent with the view that a significant fraction of the injected inorganic phosphate binds Al^{3+}, and hence protects the basal Na efflux from the untoward action of Al^{3+}.

Introduction

Huang and Bittar [1] put forward evidence supporting the view that neither ATPMg nor $ATPNa_2$ protects hypersensitive unpoisoned fibers from the barnacle *Balanus nubilus* from undergoing a fall in the basal Na efflux following the injection of aluminum (Al). This is in sharp contrast to the ability of the chelator deferoxamine to totally stop the Na efflux from falling [1]. Calcium-dependent proteolysis was considered as a possible reasonable explanation of the inhibitory action of injected Al but experiments involving the preinjection of both leupeptin and pepstatin failed to substantiate this idea. Thus, one important question which remains unanswered is whether the mechanism underlying the inhibitory action of Al involves the complexing of internal inorganic phosphate (P_i) and whether pre-enrichment of these fibers would protect the basal Na efflux from the untoward effect of Al. The possibility of internal P_i playing a key role is suggested by the work of Jackson [2], based on ^{27}Al-NMR, indicating that in the presence of coordinating phosphate, e.g., at pH 7.0, the species $[Al(PO_4)_2H]^{2-}$ predominates. Keeping in mind that the ^{31}P-NMR spectroscopic studies of Hansen et al. [3] show that single barnacle fibers possess a P_i content of 2.7 mmol/kg fiber water but that chemical analysis provides a higher value, e.g., 7.6 mmol/kg fiber water (Bárány, M., private communication) and hence, posing the problem of an NMR-invisible pool (see p. 126), it seemed desirable to determine whether this hypersensitivity of the basal Na efflux to Al injection can be reduced or eliminated by simply preloading these fibers with K_2HPO_4. The purpose of the following communication is to bring forward evidence which indicates that experiments with K_2HPO_4 go some way toward strengthening the hypothesis that an elevated internal P_i partly protects the basal Na efflux against the toxic effect of Al and that the basal Na efflux in both unpoisoned and ouabain-poisoned fibers is unaffected by a sudden rise in myoplasmic P_i.

Materials and Methods

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

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

ity in the effluent and in the fiber were the same as those described by Bittar [4]. 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 for injecting $AlCl_3$ and K_2HPO_4 were prepared using double-distilled, de-ionized water and the pH was adjusted as necessary to 1.9 and 7.3, respectively. The volume of test fluid injected into a fiber was 0.3–0.4 μl. Dilution by the myoplasm may be taken as being 100-fold. All experiments were carried out at an environmental temperature of 22–24°C.

The data are expressed as the mean value ± S.E. Student's t-test was used 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 based on the rate constant plots. Since ^{22}Na efflux from these fibers follows simple exponential kinetics, the rate constants were calculated using the approximation:

$$\text{rate constant (in s}^{-1}) \simeq \frac{\text{average } ^{22}\text{Na lost per s}}{\text{average } ^{22}\text{Na present in fiber}}$$

The figures presented in this paper are rate constant plots. All reagents used were analytical grade. Ouabain, Hepes and dipotassium phosphate (K_2HPO_4) were supplied by Sigma, St. Louis, MO. $AlCl_3$ was obtained from Fisher Scientific, Fair Lawn, NJ.

Results

Injection of 1 M K_2HPO_4 prior to 0.5 M $AlCl_3$

Bittar, Nwoga and Huang [5] showed that injection of Al into unpoisoned fibers causes a biphasic effect, viz. stimulation followed by inhibition of the basal Na efflux or a monophasic inhibitory effect. Illustrated in Fig. 1 is that whereas the injection of 1 M K_2HPO_4 elicits a negligible and transitory rise in the basal Na efflux, the injection of 0.5 M $AlCl_3$ 40 min later produces a biphasic response. Stimulation which is preceded by a 5-min lag period is followed by inhibition, which is evident at about $t = 120$ min. The magnitude of the fall averages 15 ± 3% ($n = 4$), a value which is significantly less than 23 ± 1% ($n = 4$) obtained by injecting 0.5 M $AlCl_3$ into unpoisoned control fibers

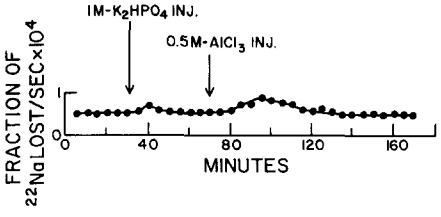

Fig. 1. The biphasic response of the basal Na efflux to the injection of a 0.5 M solution of $AlCl_3$ (pH 1.9) in a fiber injected with 1 M K_2HPO_4 (pH 7.3) beforehand (rate constant plot).

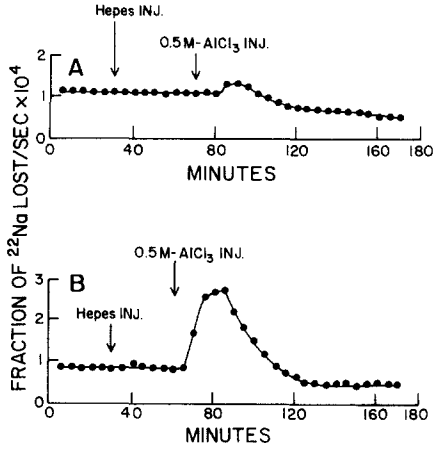

Fig. 2. (A) A biphasic response of the basal Na efflux to the injection of 0.5 M $AlCl_3$ into a control fiber preinjected with 3 mM Hepes beforehand. (B) Marked stimulation of the basal Na efflux preceding inhibition in a control fiber caused by injecting 0.5 M $AlCl_3$ that had been injected with 3 mM Hepes beforehand.

(e.g., Ref. 5). Subsequent injection of a 1 M solution of K_2HPO_4 into the control fibers produces the expected small and transitory rise in the residual Na efflux ($n = 4$).

Concentration–response relation

In experiments of this type, a 3 mM solution of Hepes was injected into unpoisoned control fibers prior to 0.5 M $AlCl_3$. It is quite apparent from Figs. 2a and b that Al elicits the characteristic biphasic response, whereas injection of the buffer is ineffective. Judging by the magnitude of the inhibitory effect, viz. 48 ± 3% ($n = 3$), the fibers obtainable from this barnacle specimen were considered as being hypersensitive to Al, and hence ideal for the establishment of a dose–response curve. The curve obtained by injecting K_2HPO_4

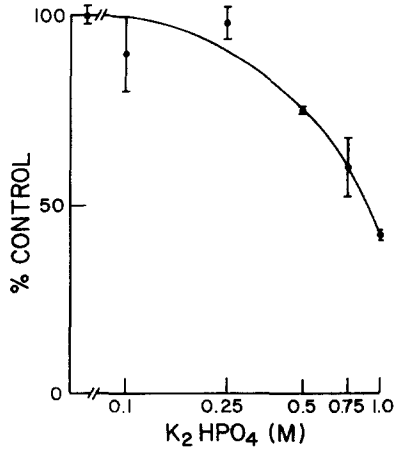

Fig. 3. The concentration response curve for the reduction of the basal Na efflux by injecting 0.5 M $AlCl_3$ into unpoisoned fibers preenriched with K_2HPO_4 30 min beforehand. Each plotted point is the mean value of three measurements. Vertical bars indicate ± S.E. The fibers used were isolated from the same barnacle specimen.

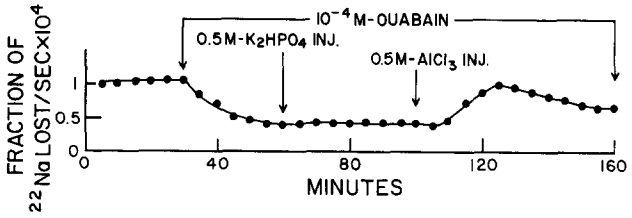

Fig. 4. Lack of effect on the ouabain-insensitive Na efflux of injection of 0.5 M K_2HPO_4. Injection of 0.5 M $AlCl_3$ 40 min later elicits a stimulatory response.

in varying concentration into unpoisoned fibers prior to 0.5 M $AlCl_3$ shown in Fig. 3 indicates that the magnitude of the inhibitory effect of Al is reduced by K_2HPO_4. For example, with a 1 M solution of K_2HPO_4 the fall in Na efflux is roughly half of the control value. Assuming a dilution factor of 100, the amount of phosphate being added to the myoplasmic pool following equilibration is, to a first approximation, 10 mM. This turns out to be 5-fold larger than the endogenous P_i content of single fibers measured by ^{31}P-NMR an hour or so after isolation from the muscle bundle (see Fig. 4, Hansen et al. [3]).

Injection of 0.5 M K_2HPO_4 into ouabain-poisoned fibers prior to 0.5 M $AlCl_3$

Characteristically, injection of Al into ouabain-poisoned fibers produces stimulation of the remaining Na efflux which often is of a transitory nature. On occasion, the response is biphasic; that is, stimulation is followed by a small inhibitory effect [6]. It therefore seemed of some interest to ascertain whether the preinjection of K_2HPO_4 would modify the kinetics of this stimulatory response in ouabain-poisoned fibers. The results obtained by injecting 0.5 M K_2HPO_4, illustrated in Fig. 4, strengthen the finding that a sudden rise in myoplasmic inorganic phosphate is without effect. Subsequent injection of 0.5 M $AlCl_3$ produces a transitory rise, the magnitude of which averages $113 \pm 15\%$ ($n = 4$), a value which is not very different from that of $173 \pm 47\%$ obtained by injecting 0.5 M $AlCl_3$ into control fibers ($n = 4$).

Injection of 0.5 M K_2HPO_4 after 0.5 M $AlCl_3$

Shown in Fig. 5 is that the injection of 0.5 M K_2HPO_4 following peak stimulation by injecting 0.5 M

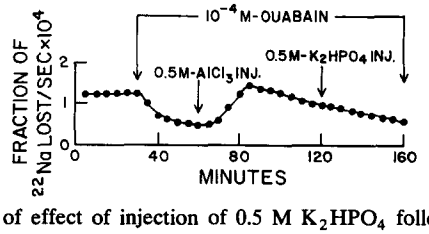

Fig. 5. Lack of effect of injection of 0.5 M K_2HPO_4 following the onset of peak stimulation of the ouabain-insensitive Na efflux by injecting 0.5 M $AlCl_3$.

$AlCl_3$ is completely ineffective on the decay phase of the stimulatory response to the injection of 0.5 M $AlCl_3$. Thus, the only conclusion possible is that the mechanism responsible for raising the ouabain-insensitive Na efflux by injecting Al is unaffected by loading these fibers with inorganic phosphate beforehand.

Discussion

Especially significant in this study is the finding that the hypersensitivity of the basal Na efflux to the injection of Al can be reduced but not abolished by prior injection of K_2HPO_4. In striking contrast to this, preinjection of ATP does not at all protect the Na efflux in unpoisoned fibers from falling, as demonstrated by Huang and Bittar [1]. One reasonable explanation of this result is provided by the work of Jackson (Ref. 2 and Jackson, G.E., private communication), indicating that the stability constants for HPO_4^{2-} and Al^{3+}, and for ATP and Al^{3+} are 11.81 and 8.96, respectively, and that only the species $[Al(PO_4)_2H]^{2-}$ is formed in the presence of 1 mM PO_4^{3-} and 10^{-7} M Al^{3+} over a wide pH range. Although conditional rather than stability constants would be the more relevant information in this regard, the question arising is this: Why is the injection of as much as 1 M K_2HPO_4 (i.e., adding 10 mM HPO_4^{2-} to the myoplasm after equilibration is reached) not enough to stop the injection of 0.5 M $AlCl_3$ from reducing the Na efflux in unpoisoned fibers? A satisfactory answer is not yet possible; clearly, HPO_4^{2-} is half as effective as deferoxamine [1], and it is too early to tell whether other ligands, notably phosphoinositide metabolites [7,8] and tripolyphosphate (TPP_4) might prove more effective than HPO_4^{2-} on an equimolar basis.

Another intriguing observation is that the injection of K_2HPO_4, e.g., a 0.5 M or 1 M solution fails to alter the course of the basal Na efflux in both unpoisoned and ouabain-poisoned fibers. Failure of the Na efflux in unpoisoned fibers to rise suggests that the Na pump is poised optimally in terms of ATP and pH (e.g., Ref. 4). And if it be true that a reduced phosphorylation potential leads to a raised internal ATP, it follows then that the resulting increase in ATP is insufficient to activate reverse Na/Ca exchange. This holds particularly true of ouabain-poisoned fibers in which a reduced Na gradient would be expected to promote activation of the Na/Ca exchanger in the reverse mode [9]. However, such a result could be important in suggesting that total internal ArP (arginine phosphate) rather than ATP undergoes an increase.

An alternative possibility is, of course, that much of the injected HPO_4^{2-} lies in the myoplasm in the form of $KHPO_4^-$ (e.g., see Rosing and Slater [10]) and that a fraction is sequestered. In the latter case, several tissues including perfused heart are known to sequester

P_i; for example, only 40% of the total phosphate in the myoplasm of perfused hearts is detectable by ^{31}P-NMR spectroscopy [11]. Sequestration by mitochondria mediated by a P_i-malate shuttle is one real possibility. Furthermore, a case could be made for binding but not for bulk loss into the bathing medium. Evidence in favor of binding comes from experiments carried out with barnacle fibers loaded with $[^{32}P]PO_4$ by injection [12]. These show that the efflux curve is a composite of three exponential phases, with the first having a $t_{1/2}$ of 3.7 min which is consistent with binding. Calculations of the absolute efflux of ^{32}P give a value as low as 65 fmol/cm^2 per s at 24°C, which is practically one-tenth that reported for crab muscle fibers [13].

Finally, a simplifying idea as to why a considerably raised myoplasmic P_i fails to stimulate the Na efflux, especially in ouabain-poisoned fibers is that myoplasmic pMg remains relatively unchanged while ArP increases. This could happen if the ATP released into the myoplasm following activation by P_i of oxidative phosphorylation and glycolysis [14,15] occurs in the form of the ATPMg complex, and not as free ATP^{4-}, and if its phosphoryl group is immediately transferred to L-arginine by arginine kinase [16]. Such an interpretation is in accord with current thinking of the role of ArP as an 'energy shuttle' in addition to that of an 'energy reserve'.

Acknowledgement

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