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Protection by GTP from the effects of aluminum on the sodium efflux in barnacle muscle fibers

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The idea that guanine nucleotides act as chelators of Al^{3+} and that Al interrupts the mechanism by which GTP or Gpp(NH)p stimulates the Na efflux in single muscle fibers from the barnacle *Balanus nubilus* has been tested. As a rule, injection of GTP or Gpp(NH)p into unpoisoned and ouabain-poisoned fibers produces a rise in the ^{22}Na efflux that is usually transitory in nature. Fibers preinjected with GTP show a fall in the Na efflux following the injection of $AlCl_3$ in an equimolar concentration. If, however, the concentration of Al for injection is halved, then GTP is found to be fully protective. Fibers preinjected with $AlCl_3$ show little or no response to the injection of GTP. This is also the case with ouabain-poisoned fibers. Ouabain-poisoned fibers preinjected with GTP also show little or no response to the injection of $AlCl_3$. The stimulatory response to the injection of $AlCl_3$ into fibers preinjected with 0.5 M GTP is dose-dependent. A graded response is also found when 0.5 M $AlCl_3$ is injected into fibers preinjected with GTP in varying concentrations. Gpp(NH)p is fully protective against the inhibitory effect of Al injection in unpoisoned fibers. Further, Gpp(NH)p abolishes the biphasic effect of Al injection on the ouabain-insensitive Na efflux. To strengthen the argument that GTP acts as a chelator of Al, a solution mixture of 0.5 M GTP / 0.5 M $AlCl_3$ (pH 1–2) was injected into unpoisoned fibers. This is found to lead to a smaller fall in the resting Na efflux than that obtained by injecting $AlCl_3$ alone or injecting $AlCl_3$ after GTP. It is thus quite clear that the barnacle muscle fiber is a useful preparation for studies of this type.

Introduction

Earlier studies have shown that the effect on the Na efflux of injecting $AlCl_3$ into fibers from the barnacle *Balanus nubilus* is either biphasic with inhibition following transitory stimulation, or monophasic with inhibition taking about an hour to reach a maximum [1]. These studies have also shown that preinjection of the Al^{3+} chelator, deferoxamine, into unpoisoned fibers leads to complete abolition of the inhibitory effect of Al, while injection of the chelator after Al is without effect. More recently, experiments carried out with ouabain-poisoned fibers have led to clear-cut evidence that the injection of $AlCl_3$ causes a stimulatory response of the ouabain-insensitive Na efflux, which is sustained or transitory in nature, or biphasic with inhibition following stimulation [2]. In sharp contrast, however, ouabain-poisoned fibers preinjected with deferoxamine show an augmented stimulatory response to the injection of Al. An explanation for this result is not yet available.

The main purpose of the following communication is to describe experiments which were carried out with GTP and its non-hydrolyzable analogue, Gpp(NH)p, in an attempt to verify the hypothesis that both guanine nucleotides are able to stop the inhibitory and stimulatory effects of injected $AlCl_3$ from occurring. There are several reasons why GTP and Gpp(NH)p were chosen. In the first place, the stability constant (log K_s) for Al^{3+} and GTP is now known to be 10.9 [3], which is practically the same as that for Al^{3+} and ATP but is 6.7 units more than the K_s value for Mg^{2+} and GTP [4a]. Second, as a rule, injection of GTP or Gpp(NH)p into these fibers increases the resting Na efflux [5]. Hence, the Na efflux is regarded as a reliable and reproducible parameter in studies of this type. And third, although the connection between the action of GTP or Gpp(NH)p and the membrane adenylate cyclase system is not yet fully understood, current thinking holds that GTP in

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid; Gpp(NH)p, 5′-guanylyl imidodiphosphate.

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the form of GTPMg acts by replacing GDPMg on the α-subunit of the G_s protein (i.e., the subunit possessing intrinsic GTPase activity), and that there is an absolute requirement for Mg^{2+} at mmolar rather than μmolar concentrations in the absence of hormone [6]. It is, therefore, likely that the injection of $GTPNa_2$ into barnacle fibers produces its stimulatory effect on the Na efflux as the result of both its conversion to GTPMg and the ability of this complex to replace GDPMg. In the former case, there is ample evidence that the internal free Mg^{2+} in barnacle muscle fibers lies in the range of 4–5 mmol per kg wet weight [7,8]. In the latter case, there is compelling evidence for the involvement of newly formed cAMP in the response of the Na efflux to GTP: first, it is known that the injection of Gpp(NH)p into barnacle fibers leads to a sharp rise in internal cAMP [9]. The other is that the pre- or post-injection of PKI (protein kinase inhibitor) reduces considerably the magnitude of the response of the Na efflux to injected GTP or Gpp(NH)p [5]. If these are valid considerations, it is then reasonable to consider the possibility that GTP or Gpp(NH)p might exert a protective action against the effects of Al injection in both unpoisoned and ouabain-poisoned fibers. Further, if GTPMg but not any formed GTPAl is the specific activator of membrane G_s, and if Al^{3+} competes with Mg^{2+} for binding to proteins [3], then it is also reasonable to envisage the possibility that fibers preinjected with Al would show a reduced response to GTP (or Gpp(NH)p) injection. Thus, the main objective of the present work was to test both possibilities.

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 the same as those described by Bittar [10] and Bittar, Nwoga and Huang [1]. 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 GTP and Gpp(NH)p used for injection were prepared using 3 mM Hepes (pH 7.2). Solutions containing $AlCl_3$ in varying concentration were prepared using double-distilled, de-ionized water at a low pH (e.g., 0.5 M $AlCl_3$ in water, pH 1.9). The pH of these solutions was lowered by adding HCl and adjusted as necessary by adding KOH. No visible turbidity was present in these solutions and their pH was checked prior to their use. Some information about the speciation of Al in aqueous solutions at varying pH is available. Briefly, the equilibrium solubility of Al in an aqueous solution is described by the following relation:

$$c(\mathrm{Al}) = c(\mathrm{Al}^{3+}) + c(\mathrm{AlOH}^{2+}) + c(\mathrm{Al(OH)}_2^{+}) + c(\mathrm{Al(OH)}_4^{-})$$

where the concentration of the individual species is a direct function of pH viz. $K_{sol} = [Al^{3+}]/[H^+]^3$, and can be calculated from the Debye-Hückel equation. In a solution with a pH of 4.0 almost 90% of the total Al occurs in the form of the octahedral hexahydrate $Al(H_2O)_6^{3+}$ (abbreviated as Al^{3+}). Increasing the pH of the solution to 5.0 reduces the concentration of Al^{3+} to about 10%, and raises the concentration of $Al(OH)^{2+}$ and $Al(OH)_2^+$ to about 10% and 60%, respectively, of the total Al. At pH 7.0, the species $Al(OH)_3^0$ precipitates, and represents about 90% of the total Al, with the remaining Al largely occurring as $Al(OH)_2^+$ and $Al(OH)_4^-$ species. However, in a basic solution, $Al(OH)_3^0$ dissolves to form $Al(OH)_4^-$; at pH 8.0, for example, the predominant species are $Al(OH)_3^0$ and $Al(OH)_4^-$ with each representing slightly less than 50% of the total available Al [4a,4b].

The volume of test fluid, water or a 3 mM Hepes solution injected into a fiber was about 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 to 24 °C.

The results are presented as the mean ± standard error. 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 approx. 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. If, however, the transitory rise in the Na efflux exceeds 20%, then the decision is made to repeat all the experiments using another barnacle specimen. 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 and rate constant 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), and $GTPNa_2$ were obtained from Sigma Chemical Company, St. Louis, MI. 5'-Guanylyl imidodiphosphate, sodium was obtained from P.L. Biochemicals, Inc., Milwaukee, WI. $AlCl_3$ was purchased from Fisher Scientific Company, Fair Lawn, New Jersey.

Results

Effect of injecting $GTPNa_2$

Experiments involving the injection of 0.5 M GTP into unpoisoned fibers show a prompt rise in the resting Na efflux which is often transitory in nature. They also show that external application of 10^{-4} M ouabain following the onset of peak stimulation by GTP is either ineffective or slightly effective. As illustrated in Fig. 1A (a plot which is a composite of three efflux plots), the injection of 0.5 M GTP elicits a prompt stimulatory response, the magnitude of which in 24 fibers averages $43 \pm 6\%$. This also illustrates that external application of 10^{-4} M ouabain subsequent to the onset of peak stimulation fails to promptly reduce the Na efflux. The composite rate constant plot given in Fig. 1B confirms the lack of effect of ouabain. Notice, however, that the decay of the GTP-induced stimulatory response slows down rather drastically after $t = 90$ min. Kinetics of this kind showing a rapid decline in the rate of decay of the response to GTP injection are rather frequent. Such behavior is not surprising since it is also seen following the injection of cAMP and Ca^{2+} (see Ref. 10).

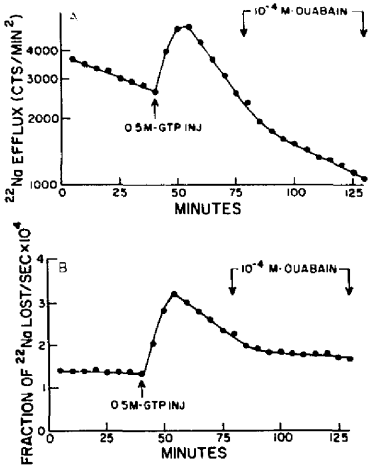

Fig. 1. The stimulatory effect on the resting Na efflux produced by injecting 0.5 M GTP (in 3 mM Hepes, pH 7.2) into an unpoisoned fiber. Notice that external application of 10^{-4} M ouabain following the onset of peak stimulation is without effect and that there is a slope change 5–10 min later (A, semilog efflux plot), indicative of a sharp decline in the decay rate of the response to GTP injection. This is indeed the case as shown in the rate-constant plot (B).

The finding by Bittar and Nwoga [5] that the injection of 0.25 M or 0.5 M $GTPNa_2$ causes a prompt rise in the Na efflux in fibers poisoned with 10^{-4} M ouabain beforehand and that this response is often transitory in nature as seen in unpoisoned fibers has now been repeatedly confirmed. For example, the injection of 0.5 M GTP produces a rise in the ouabain-insensitive Na efflux, the magnitude of which averages $196 \pm 19\%$ ($n = 49$).

Injection of GTP before and after $AlCl_3$

The injection of $AlCl_3$ into unpoisoned fibers is known to reduce the resting Na efflux in a dose- and time-dependent manner [1]. The results of experiments involving the injection of 0.5 M $AlCl_3$ before and after 0.5 M GTP are as follows: (i) Injection of 0.5 M $AlCl_3$ causes a $24 \pm 3\%$ fall in the Na efflux($n = 4$), a value which is practically the same as the fall obtained by injecting 0.5 M $AlCl_3$ 30 min after injecting 0.5 M GTP ($26 \pm 3\%$, $n = 4$). (ii) Injection of 0.5 M GTP causes a $24 \pm 1\%$ increase in the Na efflux ($n = 4$), a value which is significantly greater than the $15 \pm 1\%$ increase obtained by injecting a 3 mM Hepes solution ($n = 3$) but not significantly different from the $12 \pm 7\%$ stimulation obtained by injecting 0.5 M GTP 80 min later ($n = 4$). Upon repetition of these experiments, the results show: (i) $AlCl_3$ injection causes a $42 \pm 2\%$ fall ($n = 4$), a value which is not significantly greater than the $26 \pm 8\%$ fall ($n = 4$) obtained by injecting 0.5 M $AlCl_3$ 30 min after GTP (P being > 0.05). And (ii) GTP injection 80 min after Al causes a transitory stimulation of the order of $11 \pm 6\%$ ($n = 4$), which is significantly less than the $57 \pm 10\%$ stimulation ($n = 4$) obtained by injecting GTP into companion controls not injected with Al 80 min beforehand. Two representative experiments are given in Fig. 2. As is seen, the response to 0.5 M $AlCl_3$ injection after 0.5 M GTP is dual: inhibition which follows stimulation is rather marked (upper panel). A marked inhibitory effect is also produced by injecting Al into a companion fiber (lower panel). The subsequent injection of GTP into this fiber produces a small, transitory rise in the remaining Na efflux. Such results raised the possibility that the injection of GTP before Al is followed by substantial hydrolysis of the guanine nucleotide, thereby leaving an inadequate amount for Al buffering. To check this possibility, experiments were performed in which 0.25 M $AlCl_3$ was injected after 0.5 M GTP. The results show a biphasic response

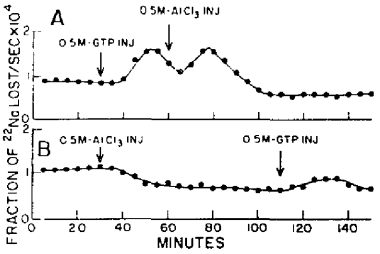

Fig. 2. (A) The biphasic effect produced by injecting 0.5 M $AlCl_3$ into a fiber preinjected with 0.5 M GTP. (B) The monophasic inhibitory effect on the Na efflux produced by injecting 0.5 M $AlCl_3$ into an unpoisoned fiber, followed 80 min later by a prompt, transitory rise in the remaining Na efflux produced by injecting 0.5 M GTP.

to Al, viz. a transitory stimulation of the order of $24 \pm 8\%$ ($n = 4$), which is not significantly different from $17 \pm 10\%$ ($n = 4$) stimulation obtained by injecting water (pH 1.9) after GTP or $18 \pm 4\%$ ($n = 4$) stimulation obtained by injecting 0.25 M $AlCl_3$ without prior addition of GTP. Stimulation is followed by inhibition of the order of $6 \pm 3\%$ ($n = 4$) obtained by injecting 0.25 M $AlCl_3$ into test fibers, but not significantly different from a value of $0.5 \pm 0.5\%$ ($n = 4$) obtained by injecting water after GTP. It thus seems that GTP in this concentration is protective against the inhibitory effect of Al injection.

Injection of $AlCl_3$ into ouabain-poisoned fibers before and after GTP

Injection of $AlCl_3$ into ouabain-poisoned fibers is found to cause a monophasic stimulatory response or a biphasic response viz. stimulation followed by inhibition. Since this stimulatory response is abolished by Ca^{2+} channel blockers, e.g., verapamil and its analogue devapamil, the inference has been drawn that the response is due to a fall in myoplasmic pCa resulting from the activation of voltage-dependent Ca^{2+} channels [2].

The protocol followed in this type of experiment was to inject 0.5 M $AlCl_3$ into fibers pretreated with 10^{-4} M-ouabain 30 or 40 min prior to or after injecting 0.5 M GTP. Companion control fibers were injected with water (pH 1.9) followed by GTP, or a 3 mM Hepes solution (pH 7.2) followed by $AlCl_3$. The results obtained clearly indicate a lack of effect of GTP in fibers preinjected with $AlCl_3$, as well as a lack of effect with $AlCl_3$ in fibers preinjected with GTP. Occasionally, however, small effects are seen, as illustrated by the composites given in Figs. 3A and B. Each is based on the efflux plots of four experiments.

Concentration-response relation for Al after GTP

In this type of experiment, fibers with relatively similar dimensions were isolated from a barnacle specimen and treated with 10^{-4} M ouabain 30 min before injecting $AlCl_3$ in varying concentration into them. Thirty minutes later they were injected with 0.5 M GTP. Companion controls were injected with water (pH 1.9) in lieu of $AlCl_3$. As summarized in Fig. 4, the magnitude of the stimulatory response elicited by GTP de-

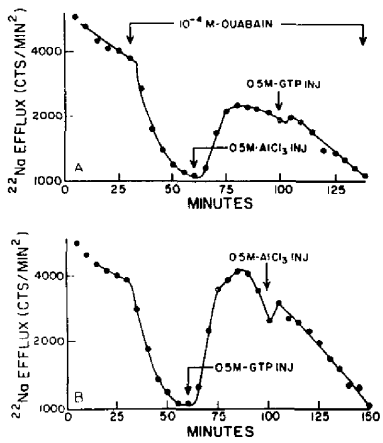

Fig. 3. (A) The time-course of the Na efflux before and after external application of 10^{-4} M ouabain and the injection of 0.5 M $AlCl_3$, and 0.5 M GTP in succession. This semilog plot is a composite of four efflux plots. The fibers used in this type of experiment were isolated from the same barnacle specimen. (B) The time-course of the Na efflux before and after external application of 10^{-4} M ouabain and following the injection of 0.5 M GTP and 0.5 M $AlCl_3$ in succession. This semilog plot is a composite of four efflux plots. The fibers used were isolated from the same barnacle specimen as that used in the preceding experiments.

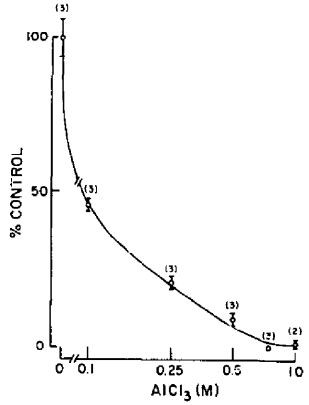

Fig. 4. The relationship between the stimulatory effect of injected 0.5 M GTP on the ouabain-insensitive Na efflux in fibers preinjected with a solution of $AlCl_3$ in varying concentration. The three companion controls were injected with water (pH 1.9), followed by 0.5 M GTP. Injection of water was without effect. Vertical bars span ± S.E. The fibers used were isolated from the same barnacle specimen.

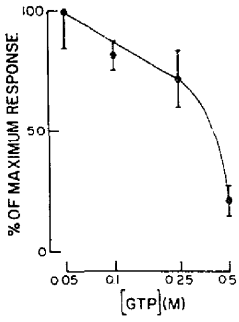

Fig. 5. The magnitude of the stimulatory response (expressed as % of maximum response) obtained by injecting 0.5 M $AlCl_3$ into ouabain-poisoned fibers preinjected with GTP in varying concentration. Abscissa: GTP concentration of solution before injection plotted on a logarithmic scale. Vertical bars indicate ± S.E. of triplicate measurements. The fibers used were isolated from the same barnacle specimen.

pends on the $AlCl_3$ concentration of the solution injected. Notice that the preinjection of 0.75 M and 1 M solutions of $AlCl_3$ completely abolishes the response to the injection of 0.5 M GTP.

Al action as a function of preinjected GTP

In this type of experiment, GTP in varying concentration was injected into fibers treated with 10^{-4} M ouabain, followed 30 min later by the injection of 0.5 M $AlCl_3$. Three companion controls were injected with water (pH 1.9) 30 min after injecting 0.5 M GTP. The results obtained, as summarized in Fig. 5, indicate a graded response to 0.5 M $AlCl_3$ injection, and that the preinjection of 0.5 M GTP is somewhat insufficient to abolish the effect produced by injecting 0.5 M $AlCl_3$. However, in parallel experiments, the injection of water (pH 1.9) after GTP into companion controls causes a transitory rise in the Na efflux averaging $18 \pm 11\%$ ($n = 3$), a value which is not significantly different from $30 \pm 18\%$ stimulation ($n = 3$) obtained by injecting 0.5 M $AlCl_3$ after 0.5 M GTP (P being > 0.4). Hence, it can be concluded that the preinjection of 0.5 M GTP is enough to stop the stimulatory effect of injected $AlCl_3$ from taking place.

Injection of Gpp(NH)p before and after $AlCl_3$

As demonstrated by Bittar and Nwoga [5], the injection of 5′-guanylyl imidodiphosphate [Gpp(NH)p] into ouabain-poisoned fibers leads to a stimulatory response resembling that obtained with GTP; that is, the response is prompt in onset, and reaches a maximum 20–25 min later. After which, there is a gradual decay. In the light of these considerations and the fact that this analogue is not readily hydrolyzable, experiments were undertaken to determine whether this analogue is able to stop injected Al from producing a monophasic inhibitory effect or biphasic effect on the Na efflux, and whether it can stop injected Al from producing a monophasic stimulatory effect or biphasic effect on the ouabain-insensitive Na efflux.

In the first series of experiments, 0.5 M $AlCl_3$ was injected into unpoisoned fibers, followed 90 min later by 0.5 M Gpp(NH)p. In a second group, 0.5 M Gpp(NH)p was injected, followed 30 min later by 0.5 M $AlCl_3$. And in a group of companion controls, water (pH 1.9) was injected, followed by 0.5 M Gpp(NH)p. The results of these experiments show: (i) only a monophasic inhibitory effect after injecting Al, which averages $19 \pm 7\%$ ($n = 4$) in magnitude, and a transitory stimulatory effect of the order of $28 \pm 13\%$ ($n = 4$) obtained by injecting Gpp(NH)p 90 min after Al. The latter value is significantly less than the $113 \pm 14\%$ stimulation ($n = 8$) obtained by injecting 0.5 M Gpp(NH)p prior to Al. And (ii) the lack of a stimulatory or inhibitory effect following the injection of Al into fibers preinjected with Gpp(NH)p ($n = 4$).

Repetition of these experiments led to essentially similar results; that is, whereas Al injection produces a monophasic inhibitory effect of the order of $46 \pm 4\%$, $n = 4$), the injection of Al after Gpp(NH)p is without effect ($n = 4$). In companion control fibers, the injection of 3 mM Hepes is without effect, while the injection of 0.5 M $AlCl_3$ 30 min later produces $50 \pm 3\%$ inhibition ($n = 4$). Thus, the only conclusion possible is that Gpp(NH)p is fully protective, and that its stimulatory action is significantly reduced in fibers preinjected with Al.

In the second series of experiments, ouabain-poisoned fibers were injected with 0.25 M Gpp(NH)p, followed 40 min later by injecting 0.25 M $AlCl_3$. The results obtained show a stimulatory response to $AlCl_3$ injection of the order of $27 \pm 11\%$ ($n = 3$), a value significantly less than $152 \pm 22\%$ stimulation obtained by injecting 0.25 M $AlCl_3$ after 3 mM Hepes (pH 7.2) into companion controls ($n = 3$). Next, this type of experiment was repeated but this time 0.5 M Gpp(NH)p was preinjected. The results obtained show a stimulatory response to the injection of 0.5 M $AlCl_3$ of the order of $17 \pm 10\%$ ($n = 3$), as compared with $134 \pm 10\%$ stimulation found in companion controls, $n = 3$. The difference is significant. This type of experiment is shown in Figs. 6 A and B, which is a composite of three experiments. However, the more usual result is that 0.5 M Gpp(NH)p is sufficient on preinjection to prevent $AlCl_3$ injection from stimulating the ouabain-insensitive Na efflux. This is apparent from the representative experiment given in Fig. 7. The gradual decline in the efflux after $t = 140$ min reflects decay of the response to Gpp(NH)p rather than an Al effect on the ouabain-insensitive Na efflux, since three companion controls injected with 3 mM Hepes followed by 0.5 M $AlCl_3$ show

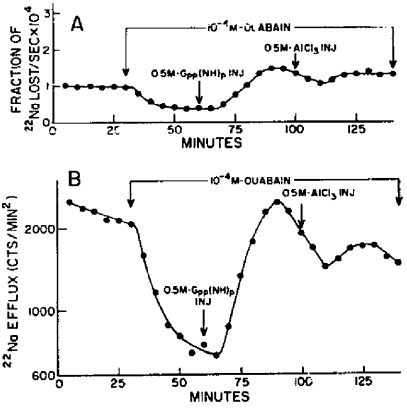

Fig. 6. To illustrate that on occasion the injection of 0.5 M $AlCl_3$ into a ouabain-poisoned fiber preinjected with 0.5 M Gpp(NH)p leads to a delayed stimulatory response which is sustained. (A) Rate-constant plot for ^{22}Na efflux. (B) Semilog plot of ^{22}Na efflux.

a monophasic transitory rise in the efflux rather than a biphasic effect.

The final step was to construct a concentration-response curve for the stimulatory effect of injecting 0.5 M $AlCl_3$ into ouabain-poisoned fibers preinjected with Gpp(NH)p in graded concentration. This is shown in Fig. 8. Two features stand out: one is that the concentration of Gpp(NH)p which produces a half-maximal reduction in the response to Al is 0.1 M and the other that the preinjection of 0.5 M Gpp(NH)p almost completely abolishes the response to 0.5 M Al.

A comparison of GTP with Gpp(NH)p

This argument runs thus: If the amount of GTP broken down following its injection varies rather widely, and this is the only reason why GTP is not as effective as Gpp(NH)p in stopping Al from inhibiting the Na efflux in unpoisoned fibers, one should then be able occasionally to produce data showing that they are equipotent, in particular, if the test fibers are not *hypersensitive* to the metal. The available evidence indicates that this is the case. (i) In the first group of experiments, unpoisoned fibers were injected with 0.5 M $AlCl_3$, and in two other groups, 0.5 M $AlCl_3$ was injected after 0.5

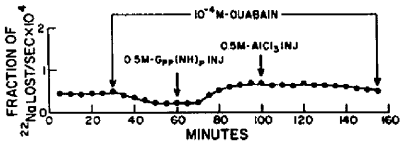

Fig. 7. To illustrate the lack of effect of the injection of 0.5 M $AlCl_3$ on the ouabain-insensitive Na efflux in a fiber injected with 0.5 M Gpp(NH)p beforehand.

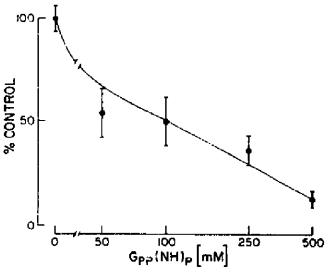

Fig. 8. The magnitude of the stimulatory response (expressed as % control) obtained by injecting 0.5 M $AlCl_3$ into ouabain-poisoned fibers as a function of preinjected Gpp(NH)p in varying concentration. Companion controls were injected with a solution of 3 mM Hepes (pH 7.2), followed by water (pH 1.9). Abscissa: Gpp(NH)p concentration of solution before injection plotted on a logarithmic scale. Vertical bars indicate ± S.E. of triplicate measurements. The fibers used were isolated from the same barnacle specimen.

M GTP and 0.5 M Gpp(NH)p. Controls were injected with 3 mM Hepes prior to 0.5 M $AlCl_3$. The results show: (i) a reduction in Na efflux by Al of the order of $31 \pm 2\%$ ($n = 4$), a value practically the same as that obtained with fibers injected with Hepes, followed by Al (i.e., $28 \pm 4\%$, $n = 4$) but significantly greater than the $5 \pm 4\%$ ($n = 4$) reduction brought about by Al injection after GTP. And (ii) a lack of effect of Al injection into fibers preinjected with Gpp(NH)p ($n = 4$).

A comparison of GTP with Gpp(NH)p in fibers hypersensitive to Al

There is ample evidence that certain groups of unpoisoned fibers are *hypersensitive* to Al injection; for example, Al injection may reduce the Na efflux by as much as 50%. It therefore seemed desirable to compare the actions of GTP and Gpp(NH)p in such fibers and see if Gpp(NH)p is a more effective binder than GTP because it is not broken down. (i) In the first group, unpoisoned fibers were injected with 0.25 M GTP, followed by 0.25 M $AlCl_3$ 40 min later, while in the second group, 0.25 M Gpp(NH)p was injected, followed by 0.25 M $AlCl_3$ 40 min later. The results obtained indicate that the injection of $AlCl_3$ after GTP and Gpp(NH)p produces a monophasic inhibitory effect, the magnitude of which averages $43 \pm 10\%$ ($n = 4$) and $45 \pm 4\%$ ($n = 4$), respectively. (ii) In the second group, fibers poisoned with 10^{-4} M ouabain were injected with 0.25 M GTP, followed 40 min later by 0.25 M $AlCl_3$ and with 0.25 M Gpp(NH)p followed by 0.25 M $AlCl_3$. The results show only a monophasic stimulatory effect following the injection of $AlCl_3$, which averages $30 \pm 3\%$ ($n = 4$) and $34 \pm 13\%$ ($n = 4$) in size, respectively. Together, these results are taken to mean that GTP and Gpp(NH)p are relatively ineffective in reducing the inhibitory effect of Al in unpoisoned fibers showing

hypersensitivity to the metal. Both nucleotides, however, are effective in stopping the Al inhibitory effect from occurring in ouabain-poisoned fibers.

Injection of a solution of GTP and $AlCl_3$ (pH 1–2)

The rationale for performing such experiments was that, at equilibrium, the species of GTP complexes existing in solution at pH 2 would include $[GTPAlH]^0$, $[GTPAl]^{1-}$ and $[GTPH_2]^{2-}$ and Al^{3+} in the free form. Al^{3+} in this solution would occur as a fairly sizeable fraction of the total Al^{3+}. Further, one would expect Al^{2+} to displace protons bound to GTP as the solution undergoes buffering and dilution by the myoplasm following injection into the myoplasm and as the proton pump responds to the proton load. The results obtained show that the injection of 0.5 M GTP-0.5 M $AlCl_3$ (pH 1–2) into unpoisoned fibers leads to a negligible stimulatory response of the order of $5 \pm 3\%$ ($n = 4$). This is followed 25–30 min later by a decline in the Na efflux of the order of $18 \pm 5\%$ ($n = 4$). Additional experiments were done by dividing the fibers into four groups: fibers in group 1 were injected with 0.25 M $AlCl_3$, followed 80 min later by 0.25 M GTP. Fibers in group 2 were injected with 0.25 M $AlCl_3$-0.25 M GTP, followed 30 min later by 0.25 M GTP. Fibers in group 3 were injected with 0.25 M GTP, followed 30 min later by 0.25 M $AlCl_3$. And fibers in group 4 were injected only with water, pH 1–2. The results are as follows: (i) Group 1 data indicate that the injection of 0.25 M $AlCl_3$ causes a monophasic inhibitory effect averaging $31 \pm 6\%$ ($n = 4$), which is significantly greater than the $11 \pm 5\%$ inhibition ($n = 4$) obtained after injecting 0.25 M $AlCl_3$-0.25 M GTP, $n = 4$ (group 2). Injection of 0.25 M GTP subsequent to Al causes $12 \pm 7\%$ stimulation ($n = 4$), a value not significantly different from $24 \pm 1\%$ ($n = 4$) obtained by injecting 0.25 M GTP after 0.25 M $AlCl_3$-0.25 GTP (group 2). (ii) Group 3 data indicate that the inhibitory effect obtained by injecting 0.25 M $AlCl_3$ after GTP averages $26 \pm 3\%$ ($n = 4$), a value significantly greater than $11 \pm 5\%$ obtained in fibers injected with Al-GTP, followed by GTP (group 2). (iii) Group 4 data indicate that the injection of water (pH 1–2) causes only a transitory rise in the Na efflux averaging $15 \pm 1\%$ ($n = 3$). This is not significantly different from the $25 \pm 5\%$ stimulation ($n = 4$) found after injecting 0.25 M $AlCl_3$-0.25 M GTP but is significantly less than the $24 \pm 1\%$ stimulation ($n = 4$) observed in fibers injected with GTP followed by Al (group 3). Collectively, it is thus clear that the size of the inhibitory effect produced by injecting Al is larger than that caused by injecting $AlCl_3$-GTP. Furthermore, a notable kinetic feature of the present results is that whereas the onset of the inhibitory effect of Al injection is almost immediately, the onset of the effect of $AlCl_3$-GTP injection is delayed by almost an hour. Such a delay falls within the time-frame of the occurrence of a small inhibitory effect in group 2 fibers injected with GTP following GTP-Al.

Experiments with ouabain-poisoned fibers were also carried out. The results indicate a lack of effect following the injection 0.5 M GTP-0.5 M $AlCl_3$ ($n = 3$), whereas companion controls injected with 0.5 M GTP show a stimulatory response averaging $135 \pm 4\%$ ($n = 4$). The latter value is significantly greater than $47 \pm 17\%$ stimulation ($n = 3$) obtained by injecting 0.5 M GTP after GTP-Al.

Discussion

The general working hypothesis that GTP exerts a protective action because it is able to bind Al^{3+} and that GTP fails to appreciably stimulate the Na efflux in fibers preinjected with $AlCl_3$ primarily because Al interrupts the mechanism by which GTP activates membrane G_s protein provides a framework within which to discuss the results of the experiments reported here. Ample justification for holding the view that the GTPAl complex is poorly hydrolyzed is to be found in the work of MacDonald, Humphries and Martin [3] who employed a cell-free GTP-tubulin complex from bovine brain. What these workers were able to establish is that GDP–GTP exchange in the presence of Al^{3+} is very slow, and that the hydrolysis of GTPAl is one-hundredth slower than GTPMg. Thus, a plausible explanation of why the injection of Al into ouabain-poisoned fibers preinjected with GTP fails to produce a large stimulatory response is that Al^{3+} is readily bound by GTP and that the GTPAl formed not only replaces GDP poorly but is also a poor substitute for GTPMg. The validity of this interpretation is strengthened by evidence that GTP-induced activation of G_s is highly Mg^{2+}-dependent [6] and that Al^{3+} binds to the tubulin-GTP complex $10^{7.5}$ times more strongly than Mg^{2+} [3,4a]. Moreover, the lack of a substantial effect upon the Na efflux of injecting GTP into ouabain-poisoned fibers preinjected with $AlCl_3$, is ascribable to the formation of GTPAl and reduced replacement of GDP by any formed GTPMg or GTPAl, resulting from competition between free Al^{3+} and Mg^{2+} for binding to the G_s protein site which has a high affinity for Mg^{2+}. The validity of this interpretation depends in part on the assumption that a fraction of the injected Al occurs in the free form in barnacle myoplasm whose pH is known to lie in the range of 7.1 to 7.3 [11,12]. According to Martin [4a], the highest obtainable free Al^{3+} concentration in an aqueous system at pH 7.4 is 3 pM. In blood plasma, for example, the predominant complex is $Al(OH)_4^-$ at 8 μM and only 3 pM is free Al^{3+}.

The experiments carried out by injecting Al after GTP into unpoisoned fibers make it reasonably clear that GTP is only effective if it exceeds the concentration of the Al injected, e.g., if it is double. However, the

same is not true of the non-hydrolyzable analogue, Gpp(NH)p. Thus, the question may well be put, Is there any evidence that injected GTP undergoes hydrolysis? The answer comes from measurements that had been made of internal GTP and GDP using isotachophoresis (Bittar, E.E. and Chiang, L., unpublished data). These show that there is: (i) a prompt fall in the total internal GTP content of fibers injected with 0.5 M GTP and that it declines with a half-time of 41 min, and (ii) a prompt rise in the internal GDP content after GTP injection, followed by an exponential decline with time which is somewhat slower than that of GTP. The validity of this interpretation that GTP is not sufficiently protective against the inhibitory effect of Al because a significant portion of it is hydrolyzed prior to the injection of Al is not in doubt in view of evidence which shows that GTP and Gpp(NH)p are relatively ineffective in unpoisoned fibers that are *hypersensitive* to the inhibitory effect of Al. The failure of both guanine nucleotides to stop Al from reducing the Na efflux by 45% is obviously a matter for surprise. The more difficult question can now be asked: What is the meaning of this finding? Clearly, more light on the problem of inhibition by Al of the Na efflux in unpoisoned fibers and, in particular, in *hypersensitive* fibers is required. For example, Al^{3+} may act rather rapidly by precipitating the available inorganic P to form $[Al(PO_4)_2H]^{2-}$ [13]. This is possible since internal P_i in fresh barnacle fibers averages 2.7 mmol/kg fiber water and rises slowly with time, as demonstrated in ^{31}P-NMR studies by Hansen, Sharpe and Bittar [14].

Two other key findings in these studies are, first, that the preinjection of Al reduces or abolishes the response of the remaining Na efflux to the injection of GTP and Gpp(NH)p. This is in keeping with the view that Al interrupts the G_s membrane transducer and that any formed GTPAl fails to act as an effective substitute for GTPMg. And second, that both GTP and Gpp(NH)p are not only able to reduce or abolish the stimulatory action of injected Al on the Na efflux in ouabain-poisoned fibers, but also to prevent a reduction in the ouabain-insensitive Na efflux from taking place. GTP or Gpp(NH)p, as will be recalled, stimulates the ouabain-insensitive Na efflux as the result of two mechanisms: one is a fall in myoplasmic pCa resulting from the activation of voltage-dependent Ca^{2+} channels, and the other is activation of cyclic AMP-dependent protein kinase by newly formed cAMP [5]. This observation raises the possibility that Gs protein is involved in Ca^{2+}-channel activation [15,16], a view which upholds current thinking [6]. If this be true, then the evidence obtained with ouabain-poisoned fibers may be taken as an indication that GTP or Gpp(NH)p reduces the stimulatory action of injected Al by binding Al^{3+}.

The view that GTP is protective emerges most clearly from evidence that the injection of the GTP/$AlCl_3$ mixture (pH 1–2) into unpoisoned fibers produces a biphasic response which is significantly smaller than that found in companion controls, and by evidence that the injection of this mixture into ouabain-poisoned fibers is without effect. In the latter case, it is noteworthy that a delayed inhibitory effect on the ouabain-insensitive Na efflux following the injection of GTP-$AlCl_3$ is not seen. One is therefore justified in drawing the conclusion that the protective action of GTP in ouabain-poisoned fibers is more complete than it is in unpoisoned fibers. Why this should be so is not yet clear. But it will be remembered that the opposite is found with deferoxamine; that is, whereas deferoxamine stops the inhibitory effect of Al in unpoisoned fibers from occurring, it augments the stimulatory effect of Al in ouabain-poisoned fibers [2].

The injection of GTP into unpoisoned or ouabain-poisoned fibers preinjected with a GTP/$AlCl_3$ mixture or preinjected with only $AlCl_3$, produces little or no effect. This finding accords well with the hypothesis that the addition of Al to the myoplasm results in the disruption of the GTP–GDP exchange system of the G_s protein. In the complete absence of information about the conditional stability constant for GTP and Al^{3+} and for GTP and H^+ at a low pH [17–19], it is impossible to specify the distribution of $[GTPAlH]^0$, $[GTPAl]^{1-}$, $[GTPH_2]^{2-}$ and Al^{3+} in the solution mixture used for injection. Nonetheless, it is reasonable to consider the inhibitory effect as being due to free Al^{3+}, since this is a mixture which would be expected to contain some free Al^{3+}. If this is the correct view, then the demonstration of an inhibitory effect with this mixture in unpoisoned fibers suggests that the $[GTPH_2]^{2-}$ species added to the myoplasm fails to stop Al^{3+} from acting. The reason for this is not clear.

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