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Concerning stimulation by injected fluoroaluminate of the sodium efflux in barnacle muscle fibers

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Single barnacle muscle fibers from *Balanus nubilus* were used primarily to examine the validity of two ideas: first, that the injection of KF stimulates the ouabain-insensitive Na^+ efflux, and that this action is potentiated by adding $AlCl_3$ (Al) in a low concentration to the solution of KF prior to injection. And second, that the injection of a KF-$AlCl_3$ solution into ouabain-poisoned, $K^{\pm}$-depolarized fibers elicits a stimulatory response resembling that obtained by injecting GTP. The results of this study are as follows: injection of 0.5 M KF into unpoisoned fibers causes a sustained rise in the resting Na^+ efflux. However, injection of a 0.5 M KF, 10^{-3} M $AlCl_3$ solution leads to a reduced rather than an augmented response. Whereas injection of 0.5 M KF into ouabain-poisoned fibers elicits a marked stimulatory response, the injection of 0.5 M KF, 10^{-3} M $AlCl_3$ reduces the remaining Na^+ efflux. Injection of KF-$AlCl_3$ in equimolar concentrations, e.g., 0.25 M, elicits a response that is significantly larger than that obtained by injecting 0.25 M KF. A dose-response curve indicates that a 0.2 M solution of fluoroaluminate probably represents an optimal concentration. Injection of 0.3 M KF following peak stimulation by injecting 0.3 M $AlCl_3$ completely reverses this response to Al. In sharp contrast, injection of a 0.3 M KF, 0.3 M $AlCl_3$ mixture following peak stimulation by injecting 0.3 M $AlCl_3$ is ineffective. Injection of KF into ouabain-poisoned, K^+depolarized fibers does not always cause sustained stimulation of the remaining Na^+ efflux. But injection of KF-$AlCl_3$ in equimolar concentration always seems to cause a delayed sustained stimulatory response. Sustained stimulation is also observed after injecting Na_2GTP. Although these results provide evidence in support of the hypothesis that the primary point of action of equimolar KF-$AlCl_3$ solutions following injection into barnacle fibers is the membrane adenylate cyclase system, they raise doubts about the validity of the concept that trace amounts of Al are required for KF to act as a positive effector of this system.

Introduction

The starting point of this work is the finding of Rall and Sutherland [1] that fluoride (F^-) is an activator of membrane-bound adenylate cyclase in cell-free preparations, and that of Sternweis and Gilman [2] that activation of this enzyme depends on the presence of traces of aluminum (Al). The aluminum dependence of the action of F^- was explained away by these workers by considering that a fluoroaluminate complex, such as AlF_4^- serves as the activator. Just how it brings about activation of G_s of membrane adenylate cyclase is not yet known with certainty, but one model put forward by Bigay et al. [3] holds that AlF_4^- mimics the terminal γ-phosphate of GTP and hence, promotes activation of membrane-bound adenylate cyclase. More recently, however, Martin [4] has recalculated the equilibria of fluoroaluminate complexes and reached the conclusion that at neutral pH the predominant species is AlF_3^0 and $AlF_3(OH)^-$ rather than AlF_4^- [5,33]. Nelson and Martin [6] also produced evidence based on ^{19}F-NMR and ^{1}H-NMR methods which indicates the existence of ternary complexes in aqueous solutions when nucleoside diphosphates (NDP), e.g., ADP and GDP, are used. Only (NDP)-AlF_x ($x = 1$–3) could be found. Support of this new view comes from the work of Jackson [7] who has taken the position that, under conditions where inorganic P_i is present in an aqueous solution, the AlF_4^- species does not exist. This is because Al binds P_i more powerfully than F^- does. This line of thinking has been pursued by Bittar and Huang [8] in trying to understand in part why the injection of Al into barnacle muscle fibers results in a reduction of the resting Na^+ efflux. Proceeding, then, on this view that Al binds P_i more strongly than F^- does [4,9], it seemed worthwhile to determine the behavior of the Na^+ efflux following the injection of not only KF but also of mixtures of KF-$AlCl_3$. This strategy has several distinct advantages. One is that it involves the introduction of

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substances into a relatively intact preparation. Another is that it allows the testing of the validity of a number of concepts, most notably the concept that a mixture containing AlF species where F^- occurs in excess stimulates the Na^+ efflux. This paper provides information which throws some light on this and other inter-related questions and raises doubts about the validity of the notion that the AlF_4^- species behaves as an activator.

Materials and Methods

The species of barnacles, the methods of dissection, cannulation, microinjection and counting of Na^+ activity in the effluent and the fiber were essentially the same as those described by Bittar [10]. The artificial sea water (ASW) used had the following composition (mM): NaCl 465, KCl 10, $MgCl_2$ 10, $CaCl_2$ 10, $NaHCO_3$ 10 (pH 7.8). ASW containing 100 mM K^+ was prepared by reducing NaCl in an osmotically equivalent amount. Solutions containing KF and KF-$AlCl_3$ mixtures were prepared using double-distilled, de-ionized water at a low pH. For example, a 0.3 M KF solution (pH 2.7); a 0.3 M KF, 0.3 M $AlCl_3$ solution (pH 2.7); and H_2O (pH 2.7) as a control solution. The pH of these solutions was reduced by adding HCl and adjusted as necessary by adding KOH. No visible turbidity was present in these solutions for injection. The volume of test fluid or water injected into a cannulated 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. Significance levels were determined using Student's unpaired *t*-test. Differences between means were considered significant at $P < 0.05$. Estimates of the size of the observed effects on the Na^+ efflux were calculated on the basis of the rate constant plots (the fractional rate constant being efflux rate/fiber count + 1/2 efflux).

Agents

Ouabain, KF and guanosine triphosphate as the sodium salt were purchased from Sigma (St. Louis, MO, USA). $AlCl_3$ was purchased from Fisher (Fair Lawn, NJ, USA). All reagents used were analytical grade.

Results

Injection of 0.5 M KF into unpoisoned fibers

The work of Bittar and Nwoga [11] showed that the injection of KF into ouabain-poisoned fibers leads to stimulation of the remaining Na^+ efflux. However, this work did not include the use of unpoisoned fibers. Experiments therefore were done in which 0.5 M KF was injected into such fibers. The results obtained

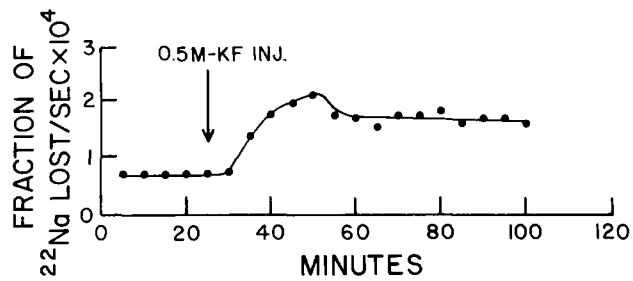

Fig. 1. The stimulatory response of the resting Na^+ efflux to the injection of a 0.5 M KF (pH 5.5) solution.

show a stimulatory response, the magnitude of which averages 90 ± 34% ($n = 5$). As illustrated in Fig. 1, stimulation is followed by a 10–15-min period of decay, and thence a period of sustained stimulation. Since F^- is known to bind Mg^{2+} [4,12], the simplest interpretation of this finding is that it is the result of the removal of internal free Mg^{2+} (see Discussion). Further, the ability of F^- to bind Ca^{2+} [4,12] provides a plausible explanation as to why the response is relatively moderate.

To check whether Al potentiates the action of F^-, or is required for F^- to activate a system such as membrane adenylate cyclase [2], which is thought to be involved here in the response of the resting Na^+ efflux to KF injection, a solution of 0.5 M KF, 10^{-3}M $AlCl_3$ (pH 5.2) was injected into unpoisoned fibers isolated from the same bundle as those used in the preceding experiments. Injection of this mixture is found to exert a negligible rise in the resting Na^+ efflux (averaging 11 ± 7%, $n = 5$). This is obviously the opposite of what was expected. And when this value is compared with 90 ± 34%, ($n = 5$), P turns out to be equal to 0.05. Additionally, in parallel experiments, injection of 10^{-3} M $AlCl_3$ (pH 4.7) is found to cause a rise in the resting Na^+ efflux averaging 21 ± 6% ($n = 5$). However, inhibition following stimulation [13] fails to occur in these fibers. Clearly, this surprising data are a telling argument against the view that membrane-bound adenylate cyclase is activated by injecting F^- with a trace of Al. However, it very well may be asked why this is the case. A possible clue comes from work carried out on adenylate cyclase preparations from the liver fluke *Fasciola hepatica* [14]. This study reveals that when the F concentration is 5 mM or more, a trace of Al exerts little stimulatory action on adenylate cyclase activity.

Injection of KF and KF-$AlCl_3$ into ouabain-poisoned fibers

The finding that the injection of a mixture of KF-$AlCl_3$ into unpoisoned fibers exerts a rather negligible stimulatory effect raised the question whether a similar situation occurs when the Na^+ gradient across the

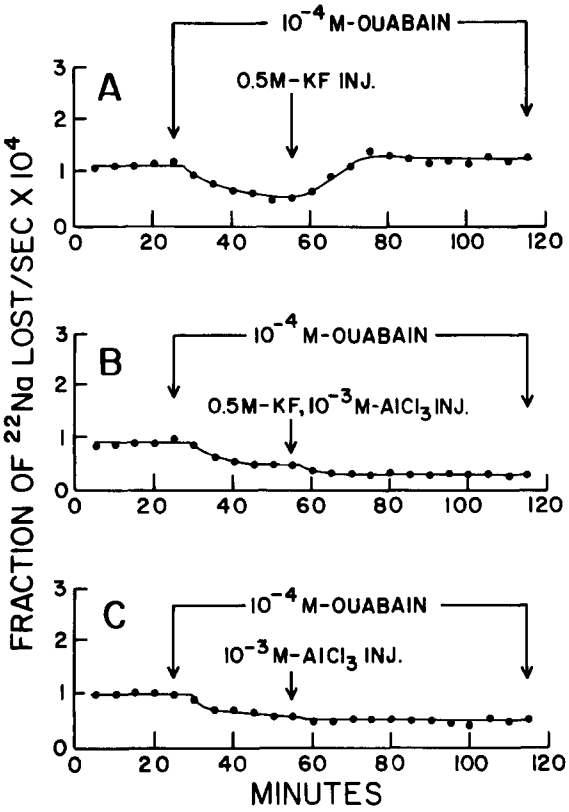

Fig. 2. (A) Sustained stimulation of the remaining Na^+ efflux by injection of a 0.5 M KF solution (pH 5.5) into a fiber pretreated with 10^{-4} M ouabain. (B) Inhibition of the remaining Na^+ efflux by injection of a 0.5 M KF, 10^{-3} M $AlCl_3$ solution (pH 5.2) into a fiber pretreated with 10^{-4} M ouabain. (C) Inhibition of the remaining Na^+ efflux by injection of a 10^{-3} M $AlCl_3$ solution (pH 4.7) into a fiber pretreated with 10^{-4} M ouabain. In this type of experiment the fibers used were isolated from the same muscle bundle.

fiber membrane is reduced, e.g., by ouabain [15]. As illustrated in Fig. 2A–C, injection of 0.5 M KF into a fiber poisoned with 10^{-4} M ouabain beforehand causes sustained stimulation of the ouabain-insensitive Na^+ efflux (averaging $211 \pm 49\%$, $n = 5$) (Fig. 2A), whilst the injection of 0.5 M KF, 10^{-3}M $AlCl_3$ (Fig. 2B), and injection of 10^{-3}M $AlCl_3$ (Fig. 2C) cause a small reduction in the remaining Na^+ efflux, averaging $33 \pm 2\%$, ($n = 5$) and $16 \pm 5\%$ ($n = 5$), respectively. The difference between these two last values is significant. The reason for a large response to the injection of 0.5 M KF into ouabain-poisoned fibers is not hard to find. The removal of internal free Mg^{2+} in the presence of a reduced Na^+ gradient across the fiber membrane would be expected to activate Na^+/Ca^{2+} exchange in the reverse mode (see, e.g., Ref. 16). Inhibition by F^- with a trace of Al underpins at least two possibilities, namely that such a mixture may exert an inhibitory action on basal adenylate cyclase activity and/or activate Gi, the GTP-binding protein that inhibits membrane adenylate cyclase. In regard to the inhibition caused by injecting 10^{-3}M $AlCl_3$, this is not unexpected, since it could be due to reduced basal adenylate cyclase activity resulting from the displacement of some essential Mg^{2+} associated with this enzyme.

The next step seemed to be to try the effect of KF and $AlCl_3$ in equimolar concentrations; for example, a solution of 0.25 M of the mixture. The results of these experiments are as follows: (i) Injection of 0.25 M KF-0.25 M $AlCl_3$ (pH 3.1) into ouabain-poisoned fibers causes $284 \pm 61\%$ stimulation ($n = 5$), a value significantly larger than $149 \pm 25\%$ obtained by injecting 0.25 M KF (pH 3.1) ($n = 5$), and $48 \pm 8\%$ ($n = 5$) obtained by injecting 0.25 M $AlCl_3$ (pH 3.1). Such results are in keeping with the concept that a fluoroaluminate mixture in which F^- and Al are present in equimolar concentrations acts as a positive effector of the Na^+ efflux in ouabain-poisoned fibers as the result of activation of membrane-bound adenylate cyclase. In regard to stimulation by injecting 0.25 M $AlCl_3$, this confirms earlier work and is accounted for by assuming a rise in internal free Ca^{2+} as a consequence of activation of voltage-dependent Ca^{2+} channels [13].

Concentration-response relation

Summarized in Fig. 3 are the results of experiments involving the injection into ouabain-poisoned fibers of solutions of KF-$AlCl_3$ in varying equimolar concentrations. At least two points are notable: first, that the largest response is obtainable with a 0.2 M solution, and second, that the injection of mixtures in a higher

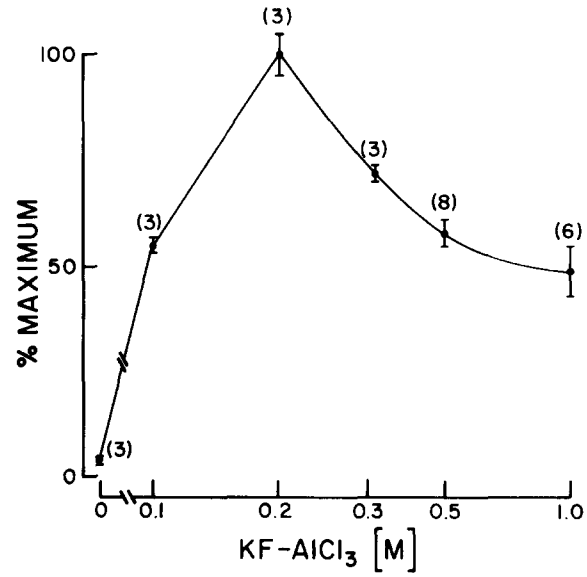

Fig. 3. The magnitude of the stimulatory response (expressed as % of maximum) obtained by injecting KF-$AlCl_3$ in equimolar concentration over a wide range into ouabain-poisoned fibers. The pH of the solution was 2.5. This was also the case with H_2O (control). Abscissa: Molarity of KF-$AlCl_3$ solution before injection plotted on a logarithmic scale. Vertical bars span ±S.E. The number of measurements done is recorded in parentheses. The fibers used were isolated from the same barnacle specimen.

concentration elicits a reduced response. For example, the difference between the values obtained by injecting a 0.2 M solution and a 1 M solution is significant. It is worth mentioning that the injection of water (pH 2.7) into companion controls causes a small rise in the ouabain-insensitive Na^+ efflux but this is not an unusual happening, since the procedure of injection itself causes a transitory rise in the internal free Ca^{2+} concentration (see, e.g., Ref. 10).

Al injection before and after KF

Knowing already that adding Al in low concentration to a solution containing KF is not the way to achieve a large response, it seemed of special interest to determine if injection of Al, e.g., a 0.3 M solution before 0.3 M KF would produce an overall kinetic picture resembling that obtained by injecting a mixture of 0.3 M KF, 0.3 M $AlCl_3$ (pH 2.7). Thus, ouabain-poisoned fibers were divided into four groups. In the first, 0.3 M KF (pH 2.7) was injected 40 min prior to 0.3 M $AlCl_3$ (pH 2.7). No effect with KF was obtained ($n = 4$). However, Al injection caused a stimulatory response of $108 \pm 19\%$ ($n = 4$). In the second group, a mixture of 0.3 M KF, 0.3 M $AlCl_3$ (pH 2.7) was injected. This caused $890 \pm 123\%$ stimulation ($n = 4$), a value that is significantly larger than that obtained with Al. In the third group, 0.3 M KF was injected 40 min after the injection of 0.3 M $AlCl_3$ (i.e., after peak stimulation by Al averaging $70 \pm 8\%$ ($n = 4$). This caused complete reversal of the response to Al ($n = 4$). And in the fourth group, 0.3 M KF was injected 30 mins. after H_2O (pH 2.7) injection. Neither H_2O nor KF had an effect ($n = 4$). Evidence of KF being able to reverse the response to Al is consistent with the hypothesis that the injection of $AlCl_3$ in high concentrations leads to a raised internal free Ca^{2+} because of activation of voltage-dependent Ca^{2+} channels [13] and that F^- binds free Ca^{2+} to form CaF^+ [4,12].

Al injection before a mixture of KF-$AlCl_3$

The finding that KF injection reverses the response to Al led to the question whether or not the injection of a mixture after Al would be less effective. In other words, if Al acts in part by displacing Mg^+ bound to membrane adenylate cyclase, and if F^- acts by chelating increased Ca^{2+}, then a mixture of KF-Al is not likely to have a significant effect. That this prediction is borne out is shown in Fig. 4. As can be seen, injection of 0.3 M $AlCl_3$ causes a sharp but delayed rise in the remaining Na^+ efflux (averaging $322 \pm 137\%$, ($n = 4$), which is neither reversed nor potentiated by the subsequent injection of 0.3 M KF, 0.3 M $AlCl_3$ ($n = 4$). This kinetic result contrasts rather sharply with that observed in companion controls, where the injection of H_2O (pH 2.7) is followed 50 min later by the injection of a 0.3 M KF, 0.3 M $AlCl_3$ mixture, which causes a

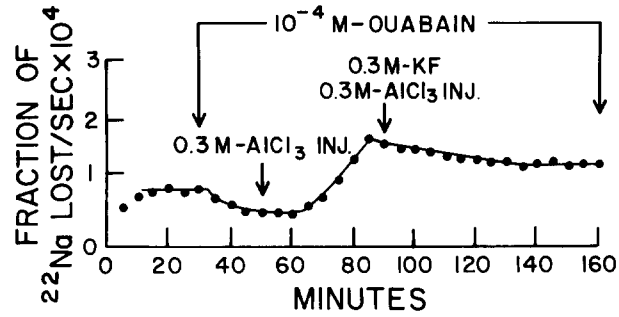

Fig. 4. Lack of effect of injection of a 0.3 M KF, 0.3 M $AlCl_3$ solution (pH 2.7) on the stimulatory response of the ouabain-insensitive Na^+ efflux to the injection of a 0.3 M $AlCl_3$ solution (pH 2.7).

stimulatory response averaging $600 \pm 94\%$ ($n = 4$). Collectively, these results are in line with the hypothesis that Al acts in part at two primary sites, viz., membrane adenylate cyclase, and the voltage-dependent Ca^{2+} channel, and that prior injection of a significant amount of Al stops a mixture of KF-Al from activating the former system.

Injection of KF and KF-$AlCl_3$ following sudden elevation in Ke

The work of Bittar and Nwoga [11] revealed that the injection of KF, e.g., 0.25 M into ouabain-poisoned fibers depolarized beforehand by raising Ke, e.g., to 100 mM leads to a stimulatory response, the size of which appears to depend on the degree of sensitivity of the fiber to high Ke. That is, fibers moderately sensitive to 100 mM Ke show a larger response to KF injection than fibers that are very sensitive to high Ke. In an effort to gain some insight into the problem of whether ouabain-poisoned fibers depolarized by high K^+ are sensitive to the injection of a mixture of KF-$AlCl_3$, it seemed desirable to extend this work to include experiments involving the injection of KF and GTP following sudden elevation of Ke. As will be recalled, the stimulatory response resulting from the injection of Na_2GTP into ouabain-poisoned fibers is considered as being due to two mechanisms: one is a fall in myoplasmic pCa resulting from activation of voltage-dependent Ca^{2+} channels, and the other activation of membrane adenylate cyclase [17]. Thus, in the latter case, according to current thinking, the formed Mg/GTP complex acts by replacing MgGDP on the α-subunit of the G_s protein (see, e.g., Refs. 18,19).

On this basis, then, the aim of the following experiments was 3-fold: first, to verify the finding of Bittar and Nwoga [11] that the injection of KF into K-depolarized fibers brings about stimulation of the ouabain-insensitive Na^+ efflux. Second, to explore the possibility that the injection of a KF-$AlCl_3$ mixture into $K^{\pm}$-depolarized, ouabain-poisoned fibers leads to a stimulatory response. And third, to test for the effect

of injected Na_2GTP, and see whether the kinetics of the response bear any resemblance to the results obtained with the injection of KF and a KF-$AlCl_3$ mixture. The results of the experiments are as follows.

(i) Shown in Fig. 5 are three representative experiments relating to the use of ouabain-poisoned fibers. The first (Fig. 5A) indicates that the injection of H_2O (pH 2.7) exerts a negligible rise in the ouabain-insensitive Na^+ efflux, whereas the subsequent injection of a 0.3 M KF, 0.3 M $AlCl_3$ mixture (pH 2.7) causes a sharp rise in the remaining efflux ($n = 4$). The second (Fig. 5B), which is that of a fiber isolated from the same muscle bundle as that used in the preceding experiment, shows that it is moderately sensitive to sudden elevation of Ke from 10 mM to 100 mM, and that the injection of 0.3 M KF (pH 2.7) following almost complete decay of the response to high Ke is without effect ($n = 4$). And the third (Fig. 5C) shows that the injection of 0.3 M KF (pH 2.7) following almost complete decay of the response to high Ke into a fiber isolated from a barnacle specimen belonging to another batch of barnacles causes a delayed but distinct rise in the

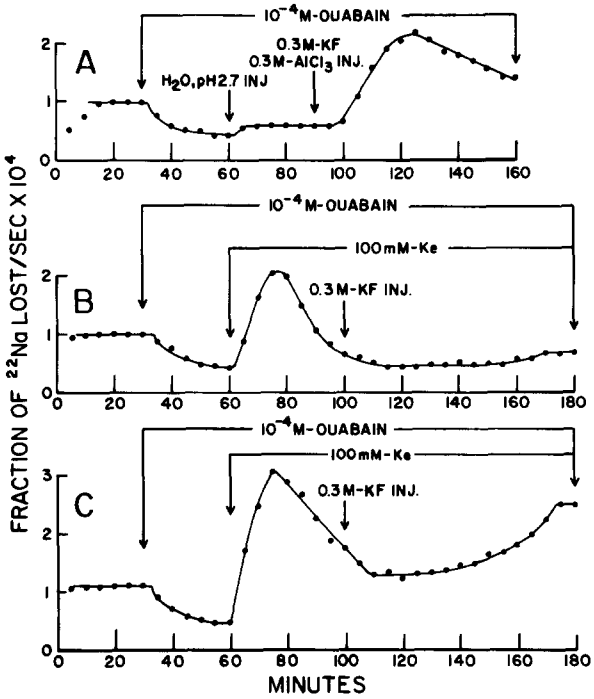

Fig. 5. (A) Marked stimulation of the ouabain-insensitive Na^+ efflux by the injection of a 0.3 M KF, 0.3 M $AlCl_3$ solution. Notice that the preinjection into the fiber of H_2O exerts a negligible effect. (B) A delayed but negligible effect on the ouabain-insensitive Na^+ efflux following the injection of 0.3 M KF into a fiber depolarized by suddenly raising Ke 10-fold. Notice that injection of KF was carried out after decay of the response to high Ke. (C) A delayed but marked rise in the ouabain-insensitive Na^+ efflux following the injection of 0.3 M KF into a fiber depolarized by suddenly raising Ke 10-fold. Notice that injection was carried out 25 min after peak stimulation by high Ke, and that the response to KF injection assumes a sustained nature close to $t = 180$ min.

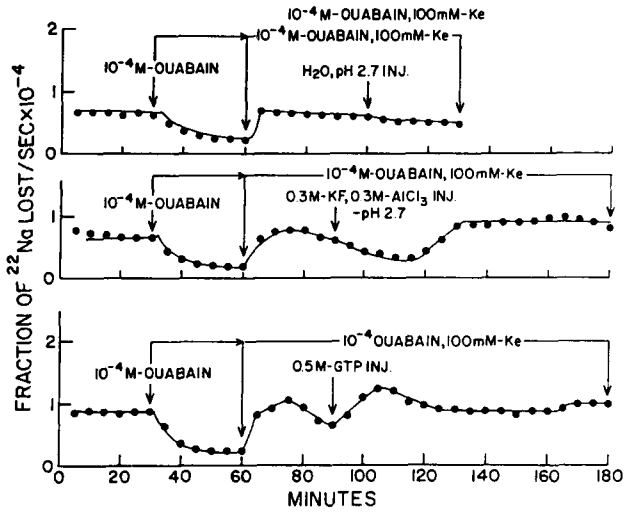

Fig 6. Upper panel: Lack of effect of injection of H_2O into a fiber treated with 10^{-4} M ouabain and depolarized by high Ke beforehand. Notice the rather small response of the remaining Na^+ efflux to 100 mM Ke. Middle panel: A delayed but sustained stimulatory response of the ouabain-insensitive Na^+ efflux to the injection of a 0.3 M KF, 0.3 M $AlCl_3$ solution into a fiber depolarized by high Ke beforehand. Lower panel: A prompt and sustained stimulatory response of the ouabain-insensitive Na^+ efflux to the injection of a 0.5 M Na_2GTP solution (in 3 mM Hepes (pH 7.2)) into a fiber depolarized by high Ke beforehand.

ouabain-insensitive Na^+ efflux, which reaches a peak about an hour after injection ($n = 4$). The inference thus drawn is that depolarized, ouabain-poisoned fibers are not always sensitive to the injection of KF.

(ii) Presented in Fig. 6 are three representative experiments also relating to the use of ouabain-poisoned fibers. The first (upper panel) indicates low sensitivity to sudden elevation of Ke to 100 mM, and that the injection of H_2O (pH 2.7) is ineffective ($n = 3$). The second (middle panel) shows that the injection of a 0.3 M KF, 0.3 M $AlCl_3$ mixture (pH 2.7) 15 min after peak response to high Ke causes a delayed but sustained stimulation of the ouabain-insensitive Na^+ efflux ($n = 4$). And the third (lower panel) shows that the injection of 0.5 M GTP into a depolarized, ouabain-poisoned fiber (isolated from the same bundle as the fibers in the preceding experiments) causes the expected sustained stimulation of the ouabain-insensitive Na^+ efflux. Notice that the onset of the response to GTP injection is prompt. Such actions by the mixture and GTP could arise if both are positive effectors of membrane adenylate cyclase.

Discussion

The main picture which emerges is that only the injection of mixtures of KF-$AlCl_3$ in equimolar concentration into barnacle muscle fibers causes stimulation of the ouabain-insensitive Na^+ efflux, and that the

optimal concentration lies in the region of 0.2 M. In sharp contrast, injection of F with a trace of Al causes a fall in the ouabain-insensitive Na^+ efflux. This surprising observation suggests that an additional mechanism may exist causing the Na^+ efflux to fall. Several explanations can be suggested: one is that excess F reduces the internal free Ca^{2+}, and the other that Al released by the complex forms AlGTP, which is a dead-end complex, since it cannot replace MgGTP as the activator of membrane G_s protein. The latter possibility is likely in view of evidence that GTP levels in barnacle fibers are rather low, e.g., 0.27 ± 0.04 mmol/kg fiber water as measured by isotachophoresis [20] and that the log K_s for GTP and Al is 10.9 [21]. An additional explanation of the anomalous result obtained with KF and a trace of Al is that such a mixture may exert a dual effect on basal adenylate cyclase activity [14], or that it activates G_i [22].

The far more important outcome of this study is, of course, the fact that the injection of mixtures of KF and $AlCl_3$ in equimolar concentration into ouabain-poisoned fibers always stimulates the remaining Na^+ efflux, and that this response is not only sustained but also follows the shape of an inverted V. A parsimonious but tentative scheme that could account for such results is based on four concepts. The first assumes that the response obtained by injecting mixtures with a 1:1 stoichiometry represents an overall effect. Second, that the AlF_x complex(es) acts as a genuine activator of membrane-bound adenylate cyclase. Third, that F stimulates the Na^+ efflux as the result of the removal of internal free Mg^{2+}. And fourth, that Al binds free P_i, PP_i, ATP and GTP far more strongly than does F^- [21,23]. Consider now, for example, the injection of a mixture of 0.25 M KF, 0.25 M $AlCl_3$ into ouabain-poisoned fibers. On the one hand, release of some Al by the AlF complex(es) would be expected to lead to binding of P_i, and hence the formation of the insoluble $AlPO_4$ complex. Whilst this represents an inhibitory action, the binding by Al of PP_i would lead to maintenance of persistent activation of membrane adenylate cyclase by AlF_x complex(es) because of the absence of product inhibition. On this view, the overall effect on Na^+ efflux would depend on which of the two is the dominant mechanism. On the other hand, what emerges is that injection of mixtures of equimolar KF and $AlCl_3$ that do not exceed 0.2 M leads to stimulation of the ouabain-insensitive Na^+ efflux, whereas injection of mixtures in excess of 0.2 M produces a graded reduction in the size of the response (or more precisely the overall effect). Thus, in the latter case, one plausible explanation is that precipitation of myoplasmic free P_i is the dominant mechanism, and that it occurs in direct proportion to the rising levels of internal free P_i. Thus, the question arises as to whether myoplasmic P_i levels in barnacle fibers fall in a range that would allow a significant amount of Al to be released. The answer is that measurements of internal P_i in freshly dissected fibers have been made using ^{31}P-NMR [24]. These indicate that free P_i can be as low as 1.4 mM and as high as 5.9 mM about 30 min after cannulation, and that these levels rise rather slowly with time. It is, therefore, scarcely surprising that the injection of mixtures exceeding 0.2 M KF, 0.2 M $AlCl_3$ about 1 h or 1 1/2 h after cannulation of the fibers produces a graded reduction in the stimulatory response of the ouabain-insensitive Na^+ efflux. Although this provides a conceptual basis for an inhibitory effect resulting from the abolition of P_i, it takes no account of the excess F^- resulting from the release of Al. The significance of F^- in excess lies in the ability of F to bind Mg^{2+} and hence, to stimulate the Na^+ efflux as the result of reducing internal free Mg [16,25], but it must also be borne in mind that F^- is a much weaker binder of Mg^{2+} than ATP or GTP, the K_s for F^- and Mg^{2+}, and for F and ATP or GTP being 2.0 and 4.3, respectively [26,27]. Now, assuming that internal free Mg^{2+} is about 5 mM [28,29], it can be readily deduced on the strength of the available data that the magnitude of the response to the injection of 0.25 M KF, 0.25 M $AlCl_3$ would be greater than that obtained with 0.25 M KF (see Results). The reason why on occasion the injection of KF is ineffective is unclear, but one possible reason could be that F may not remove enough free internal Mg^{2+} in these particular fibers to activate Na^+/Ca^{2+} exchange in the reverse mode. Support for this interpretation is provided by the experiments showing a characteristic lag phase preceding the onset of a stimulatory response to the injection of KF into ouabain-poisoned, K^+-depolarized fibers. An alternative explanation of the lack of an effect is batch-to-batch or seasonal variation.

It is not without significance that the kinetics of the response to KF-$AlCl_3$ bear a striking resemblance to those observed after injecting Na_2GTP; that is, the response is of a sustained nature. This is consistent with the widely accepted view that both the mixture and MgGTP irreversibly activate membrane adenylate cyclase. To what extent inhibition of phosphatases by F^- (see, e.g., Ref. 30) slows down the protein dephosphorylation process is impossible to state but the problem bears investigation. As for the absence of a lag phase following the injection of $GTPNa_2$, this is not unexpected, since as mentioned earlier, GTP binds internal free Mg^{2+} quite strongly.

It remains to consider whether stimulation by injected F^- may be due to a raised internal free Ca^{2+} concentration, resulting from an elevation in Ins(1,4,5)P_3 levels, e.g., in hepatocytes [31] and supraorbital nasal gland cells [32]. That this is highly unlikely is shown by the fact that the injection of D-Ins(1,4,5)P_3 into ouabain-poisoned fibers is without effect on the

remaining Na^+ efflux. This is also rendered unlikely by the fact that F^- acts as a binder of Ca^{2+}.

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