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Stimulation by High External Potassium of the Sodium Efflux in Barnacle Muscle Fibers

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Summary. Single barnacle muscle fibers from *Balanus nubilus* were used to study the effect of elevated external potassium concentration, $[K]_o$, on Na efflux, membrane potential, and cyclic nucleotide levels. Elevation of $[K]_o$ causes a prompt, transient stimulation of the ouabain-insensitive Na efflux. The minimal effective concentrations is ~20 mM. The membrane potential of ouabain-treated fibers bathed in 10 mM Ca^{2+} artificial seawater (ASW) or in Ca^{2+}-free ASW decreases approximately linearly with increasing logarithm of $[K]_o$. The slope of the plot is slightly steeper for fibers bathed in Ca^{2+}-free ASW. The magnitude of the stimulatory response of the ouabain-insensitive Na efflux to 100 mM K_o depends on the external Na^+ and Ca^{2+} concentrations, as well as on external pH, but is independent of external Mg^{2+} concentration. External application of 10^{-4} M verapamil virtually abolishes the response of the Na efflux to subsequent K-depolarization. Stabilization of myoplasmic-free Ca^{2+} by injection of 250 mM EGTA before exposure of the fiber to 100 mM K_o leads to ~60% reduction in the magnitude of the stimulation. Pre-injection of a pure inhibitor of cyclic AMP-dependent protein kinase reduces the response of the Na efflux to 100 mM K_o by ~50%. Increasing intracellular ATP, by injection of 0.5 M ATP-Na_2 before elevation of $[K]_o$, fails to prolong the duration of the stimulation of the Na efflux. Exposure of ouabain-treated, cannulated fibers to 100 mM K_o for time periods ranging from 30 sec to 10 min causes a small (~60%), but significant, increase in the intracellular content of cyclic AMP with little change in the cyclic GMP level. These results are compatible with the view that the stimulatory response of the ouabain-insensitive Na efflux to high K_o is largely due to a fall in myoplasmic *p*Ca resulting from activation of voltage-dependent Ca^{2+} channels and that an accompanying rise in internal cAMP accounts for a portion of this response.

Key Words. High K, Na efflux, barnacle muscle fibers

Danielson et al. (1971) showed that the Na efflux in barnacle muscle fibers is stimulated by increased K_o and by increased myoplasmic Ca^{2+}. The present paper extends these findings to an investigation of the mechanism by which high K_o stimulates the ouabain-insensitive Na efflux. It includes new information about the use of pure protein kinase inhibitor to define the role of cAMP-dependent protein kinase in the response of the Na efflux to high K.

Materials and Methods

Specimens of *Balanus nubilus* were obtained from Mr. David King at Friday Harbor, Washington. They were kept in a filtered aerated 150-gallon aquarium containing seawater made from Instant Ocean salts. The temperature of the aquarium water was maintained at 12 °C.

Dissection and Cannulation

Single fibers measuring 3–4 cm in length and 1–2 mm in width were isolated by dissection from the depressor muscle bundles and then cannulated. A weight of approximately 70 mg was attached to the tendon so as to keep the fiber in a vertical position while suspended in artificial seawater (ASW).

Solutions

The artificial seawater (ASW) and Li^+-ASW used in the experiments had the following compositions, respectively (mM): NaCl, 465; KCl, 10; $MgCl_2$ 10; $CaCl_2$, 10; $NaHCO_3$, 10 at pH 7.8; and LiCl, 475; $MgCl_2$, 10; $CaCl_2$, 10; $KHCO_3$, 10 at pH 7.8. Solutions with varying K^+, Ca^{2+} and Mg^{2+} concentrations were prepared by raising or lowering NaCl in osmotically equivalent amounts. In some experiments 3 mM-N-2-hydroxyl ethylpiperazine-N'-2-ethane-

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sulphonic acid (HEPES) was used instead of HCO_3^- as the buffer. The pH of all bathing solutions was adjusted as necessary by adding HCl or NaOH.

Microinjector

The microinjector used was of the type described by Bittar and Tallitsch (1975). The volume of fluid discharged per cm excursion of the micromanipulator was 0.8–1.0 µl. In this work the volume of test fluid usually injected into the fibers was 0.4–0.5 µl. Since the intrafiber fluid volume was on average 40 µl, dilution by the myoplasm of the injected test fluid may be taken as about 100-fold.

Radioactivity Measurements

^{22}NaCl in aqueous solution was obtained from Amersham-Searle Corp. The solution was dried and then redissolved in distilled water so that volumes of about 0.3 µl gave ~700,000 counts per min. An automated Biogamma gamma counter was used for counting the samples. The flux data was processed and plotted as a fractional rate constant for ^{22}Na efflux on linear paper (fraction of ^{22}Na lost/sec *vs.* time). Stimulation of Na efflux was computed by taking the difference between the maximum rate constant and the value immediately preceding treatment and expressing this as a percentage change. Where comparisons have been made, the experiments always included companion fibers as controls. These were isolated from the same barnacle specimen and usually from the same bundle as that from which test fibers were obtained. The results given in this paper are means ± SEM. Student's *t*-test was used to compute significance levels.

Radioimmunoassay of Cyclic AMP and Cyclic GMP

Single muscle fibers were dissected, cannulated, and suspended in ASW as described for fibers used in the flux experiments. Test fibers were then bathed in 10^{-4} M-ouabain ASW for 30 min prior to exposure to 100 mM K-ASW for $\frac{1}{2}$, 1, 2, 5 and 10 min, and control fibers were bathed in 10^{-4} M ouabain-ASW for 30, 32, and 35 min. The fibers were frozen in liquid nitrogen prior to homogenization in 8 ml of cold (~4 °C) 0.3-N perchloric acid (PCA) containing tritiated cyclic AMP (<0.125 pmol/ml PCA) and tritiated cyclic GMP (<0.005 pmol/ml PCA) to enable recoveries to be followed during the subsequent extraction procedures. The acid-precipitated proteins were pelleted by centrifugation. After removal of the supernatant the protein content of the pellets was measured by the method of Lowry et al. (1951). The supernatant was neutralized with 6-N-KOH and centrifuged. The supernatant was then applied to a 1.25 ml column of Bio-Rad AG 1-X8 (200–400 mesh, formate form) equilibrated in 0.1-N-formic acid. After a wash with 10 ml of 0.1-N-formic acid, cyclic AMP was eluted with 12 ml 2-N-formic acid and cyclic GMP was eluted with 16 ml 4-N-formic acid. Following evaporation of these fractions to dryness, the cyclic AMP residues were dissolved in 18.8 ml and the cyclic GMP residues in 0.8 ml of 50-mM sodium acetate, pH 6.2. 100-µl aliquots of these samples were assayed for cyclic AMP and cyclic GMP using the reagents supplied in the New England Nuclear cyclic nucleotide radioimmunoassay kits. The method used is based on the procedure of Steiner, Parker and Kipnis (1972) as modified by Harper and Brooker (1975) and Frandsen and Krishna (1976). This modification involves the acetylation of the standards and samples which increases the sensitivity of the assays several-fold. The [^{125}I] antigen-antibody complex was harvested by absorption onto Millipore filters (type HASP 025 00) after the addition of 1.0 ml of cold 50-mM sodium acetate, pH 6.2. The filters were then counted in an automated Biogamma counter.

The specificity of the method for measurement of cyclic AMP and cyclic GMP in our samples was validated by the following criteria: (1) treatment of samples with cyclic nucleotide phosphodiesterase prior to acetylation destroyed more than 95% of the immunoreactive material present; (2) the assays responded in a linear manner to increasing amounts of tissue extract, and (3) the addition of a known amount (20 fmol) of the appropriate cyclic nucleotide to replicate assay tubes of samples resulted in the expected increment in cyclic nucleotide detected by the assay. The average recovery of exogenous cyclic GMP and cyclic AMP, added to the fiber homogenates, was 88 and 89%, respectively.

Em Measurements

The method of membrane potential measurement was essentially as described by Bittar and Sharp (1979). In the studies presented here the electrode tip was positioned ~1 cm below the tip of the cannula in order to avoid penetration of the sarcolemma by the electrode when contraction of the fiber occurred following exposure to elevated $[K]_o$. The cannulated fibers were allowed to equilibrate for 35 min in either 10^{-4} M ouabain-ASW or Ca^{2+}-free ouabain-ASW prior to increasing $[K]_o$.

Agents

Guanosine 3′, 5′-monophosphoric acid (cyclic GMP), ouabain, HEPES, ethylene glycol-bis-(β-aminoethylether) N,N′-tetraacetic acid (EGTA), nitrilotriacetic acid, 2′-deoxyadenosine-3′-monophosphoric acid, bovine serum albumin and Folin and Ciocalteus phenol reagent were obtained from Sigma Chemical Company, St. Louis, Mo. The ionophore A23187 was supplied gratis by Eli Lilly Company, Indianapolis, Ind. Verapamil HCl and D-600 HCl were obtained from Knoll Pharmaceutical Company, Whippany, N.J. Cyclic AMP- and cyclic GMP-[^{125}I] Ria Kits, [8, 5′-^{3}H]-cyclic GMP (sp act 38.7 Ci/mmol) and Biofluor Scintillation fluid were purchased from New England Nuclear, Boston, Mass. Anion exchange resin AG 1-X8 (200–400 mesh, formate form) was obtained from Bio-Rad Laboratories, Richmond, Calif., and beef heart cyclic nucleotide phosphodiesterase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The inhibitor of cyclic AMP-dependent protein kinase was a gift from Drs. J. Demaille and E. Fischer of the Department of Biochemistry, University of Washington, Seattle. Unless otherwise stated, all agents used for microinjection were dissolved in distilled deionized water and the pH adjusted to 7.0–7.2 by adding HCl or KOH. All experiments were carried out at room temperature, 20–24 °C.

Results

Stimulation by Raised external K of the Ouabain-Insensitive Na Efflux

Earlier work showed that exposure of barnacle muscle fibers (isolated from Monterey Bay specimens) to 30 mM K stimulates the Na efflux in ouabain-treated and untreated fibers (Danielson et al., 1971; Bittar et al., 1972). This work has been confirmed (Bittar et al., 1973). In addition, it has been established that the kinetic response of fibers pretreated with 10^{-3} M ouabain to 100 mM K_e is the same as that of fibers pretreated with 10^{-4} M ouabain (E.E. Bittar and D.M. Sharp, *unpublished*). In view of this, all the

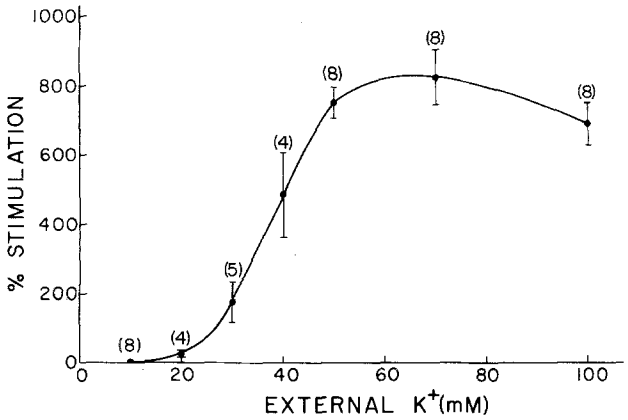

Fig. 1. The concentration-response curve for the effect of graded external K concentrations on the Na efflux in fibers suspended in 10^{-4} M ouabain-ASW, pH 7.8. The number of observations made at each concentration tested is indicated in parenthesis. Vertical bars indicate ± SEM

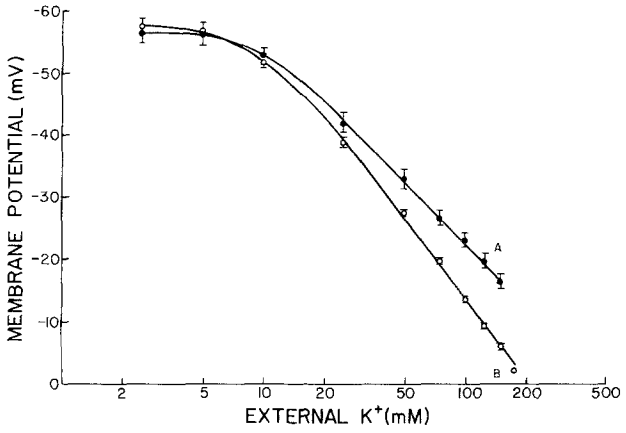

Fig. 2. The relationship between membrane potential and external K concentration. K was varied in normal ASW (pH 7.8) containing 10^{-4} M ouabain (*A*), and in Ca^{2+}-free ASW containing 10^{-4} M ouabain (*B*). Both lines represent the mean of data obtained from four fibers. The fibers used to obtain line *A* were taken from a barnacle different from those used to obtain *B*. Vertical bars indicate ± SEM

experiments described here were carried out on fibers treated with 10^{-4} M ouabain.

In order to gain a more complete picture of the sensitivity to K_o, experiments were first carried out to establish the relationship between $[K]_o$ and the magnitude of stimulation of the ouabain-insensitive Na efflux. The results obtained are summarized in Fig. 1. It can be seen that a twofold increase in K_o is sufficient to stimulate the Na efflux. At this $[K]_o$ there was no visible shortening of the fibers. However, a sustained contracture occurred at all higher $[K]_o$ tested.

In order to determine the depolarization caused by K over the range of concentrations tested, the membrane potential was also measured in fibers under conditions identical to those used in the flux experiments. The results of these measurements are shown in Fig. 2 (curve *A*). It can be seen that the membrane potential decreases approximately linearly with increasing logarithm of $[K]_o$. The slope of the straight-line relationship is approximately 34 mV for a 10-fold increase in $[K]_o$. When comparing this value with those reported by Hagiwara, Chichibu and Naka (1964) (58 mV) and Hinke (1970) (55 mV), it should be kept in mind that the conditions used by these workers were quite different from those used in this laboratory. For example, both Hagiwara et al. (1964) and Hinke (1970) used untreated fibers and $[K]_o$ was increased in such a manner that the product of K_o and Cl_o was kept constant. In contrast, the experiments described here were carried out on ouabain-treated fibers, and $[K]_o$ was increased by replacing NaCl with equimolar KCl. Both of these factors would tend to decrease the slope of the relationship between membrane potential and logarithm of $[K]_o$.

Dependence on External Cations of the Response to Raised K

(i) External pH. In order to see if the response of the Na efflux to 100 mM K-ASW is dependent on external pH (pH_e), the pH of ASW was lowered to 5.8 prior to depolarization of the fibers. Since reducing the pH of HCO_3^--containing ASW below 6.8 stimulates the Na efflux but not when HEPES is used as a substitute for HCO_3 (Bittar et al., 1977), experiments were done in which the fibers used were bathed in ASW containing HEPES as the buffer. In the presence of an external pH of 5.8, exposure to 100 mM K caused an average stimulation of 312.8 ± 44.8%, $n=6$. This is somewhat greater than the value of 189.3 ± 15.8%, $n=6$, obtained with control fibers bathed in HEPES-ASW at pH 7.8 ($P<0.05$). To determine the concentration dependence of this effect, pH_e was varied over the range 5.3–7.8. The results of these experiments are summarized in Fig. 3. It is apparent that the K_o-stimulated sodium efflux exhibits a dependence on pH_e over the range tested. However, in the physiological pH range it seems to be relatively independent of pH_e. The response of the ouabain-insensitive Na efflux to injection of 0.1 M $CaCl_2$ is enhanced to a similar extent (~60%) by lowering pH_e from 7.8 to 5.8. After reducing pH_e, injection of $CaCl_2$ caused an average stimulation of 383.4 ± 20.5%, $n=5$, compared with a value of 241.2 ± 29.8%, $n=5$, seen in controls ($P<0.005$).

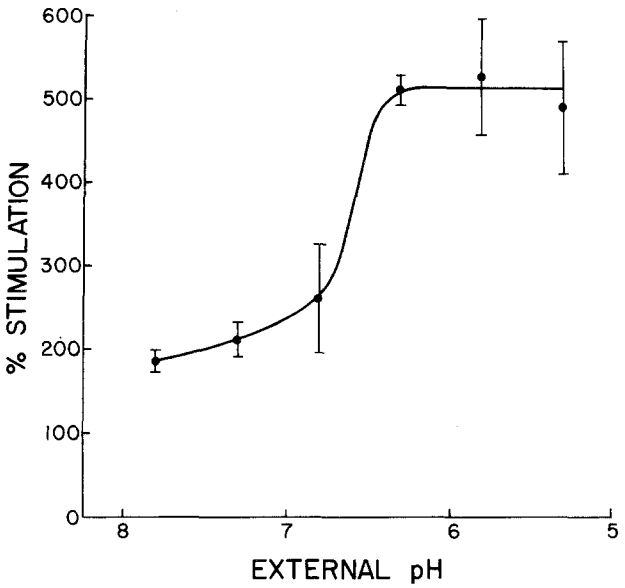

Fig. 3. The effect of varying external pH on the response of the ouabain-insensitive Na efflux to 100 mM K^+. Each point represents the mean of data obtained from four fibers. Vertical bars indicate ± SEM

(ii) External Na. To test whether stimulation of the Na efflux by elevated $[K]_o$ is dependent on external Na^+, experiments were carried out using Na^+-free ASW. Following iso-osmotic replacement of the Na^+ with Li^+ (Li^+-ASW), 10^{-4} M ouabain was applied externally and the fibers were subsequently depolarized with 100 mM K. Thirty-five minutes later the external Na^+ was restored. The results of such an experiment are shown in Fig. 4*A*. Replacement of the external Na^+ with Li^+ caused a fall in the Na efflux which was further reduced by ouabain. There was also a considerable reduction in the response of the ouabain-insensitive Na efflux to raised $[K]_o$. The value for the average stimulation was 243.8±61.3%, $n=8$, compared with a value of 963.9±119.4%, $n=8$, obtained with control fibers bathed in normal Na^+-containing ASW ($P<0.001$). Restoration of external Na^+ following the peak of stimulation caused a further rise in the efflux.

The reduction in the response of the sodium efflux to elevated $[K]_o$ when Li^+ is substituted for external Na^+ might be caused by either the addition of Li^+ or the removal of Na^+, or possibly a combination of these two aspects of the substitution procedure. There have in fact been several reports indicating that Li^+ can influence a variety of membrane systems including the Na-Ca exchange mechanism in squid axons (Baker et al., 1969), the Na-K pump in frog muscle (Beaugé, 1975), and adenylate cyclase in a number of tissues (Birnbaumer, Pohl & Rodbell, 1969; Marcus & Aurbach, 1971). In order to resolve this problem the type of experiment shown in Fig. 4*A* was repeated, but this time the NaCl in the ASW was replaced with choline chloride (choline-ASW). The results obtained were similar to those shown in Fig. 4*A*. Following substitution of choline for external Na^+ the average magnitude of the K_o-induced stimulation of the ouabain-insensitive Na efflux was only 33.4±6.7%, $n=7$, compared with a value of 661.4±76.5%, $n=8$, obtained with control fibers bathed in normal Na^+-containing ASW ($P<0.001$). A further rise in the Na efflux was also seen when Na^+ was restored following the peak of stimulation.

These results suggest that while the addition of Li^+ and choline may not be entirely without effect on the Na efflux, the reduction in the response to raised $[K]_o$ is largely due to removal of external Na^+. (It should be noted that there was frequently noticeable shortening throughout the length of fibers bathed in choline-ASW. Contraction was seen less frequently in fibers bathed in Li^+-ASW and was always restricted to a region, 1.2 mm long, immediately below the tip of the cannula.) In order to gain a more complete picture of this dependency on external sodium, experiments were carried out in which the external Na concentration was varied by Li^+ replacement prior to exposure to 100 mM K. The results of these experiments are summarized in Fig. 4*B*. It is quite clear that the response of the Na efflux to elevated $[K]_o$ shows a dependence on external Na at concentrations less than 50% of normal.

Following replacement of external Na^+ with Li^+ the kinetics of the response of the ouabain-insensitive Na efflux to injection of $CaCl_2$ differed from those seen in control fibers bathed in Na^+-containing ASW. In control fibers the stimulation was transient, peaking 25–35 min after $CaCl_2$ injection ($n=10$). In contrast, following $CaCl_2$ injection of fibers bathed in Li^+-ASW the Na efflux either rose gradually and plateaued after ~50 min, or continued to rise slowly throughout the remainder of the experiment (85 min) ($n=10$). Consequently, a quantitative comparison of the responses seen in control and test fibers was difficult or impossible. However, injection of 0.1 M $CaCl_2$ caused a transient stimulation of the ouabain-insensitive Na efflux in fibers bathed in choline-ASW. Under these conditions the magnitude of the response was considerably smaller than that seen in control fibers bathed in Na^+-containing ASW: 37.2±6.3%, $n=5$, stimulation compared with a value of 231.4±42.3%, $n=5$ ($P<0.005$). Thus, although this suggests that the response to injection of $CaCl_2$ is dependent on external Na, it is not clear why the kinetics of the response are altered when external Na^+ is replaced with Li^+.

(iii) External Ca^{2+}. Bittar et al. (1972) reported that the Na efflux in fibers isolated from Monterey Bay

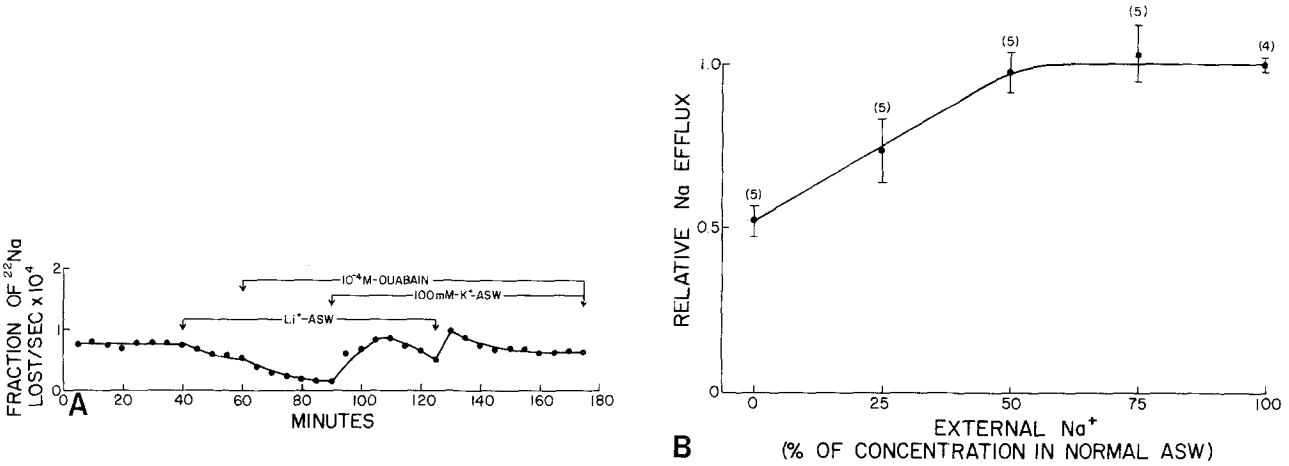

Fig. 4. The dependence on external Na^+ of the response of the Na efflux to elevated K concentration. (*A*): The effect of Na^+-free ASW on the response of the ouabain-insensitive Na efflux to 100 mM K. (*B*): The effect of varying external Na^+ on the response of the ouabain-insensitive Na efflux to 100 mM K. The ordinate is the average maximum rate constant for the stimulated Na efflux relative to that measured separately in control experiments using normal (100%) Na^+-ASW. The number of observations made at each concentration tested is indicated in parenthesis. Vertical bars indicate ± SEM

specimens is stimulated even when external Ca^{2+} is removed. Experiments carried out with fibers isolated from Puget Sound specimens in which Ca^{2+} was omitted from the bathing medium and 10^{-4} M ouabain applied externally 40 min before exposure of the fibers to 100 mM K_o show that Ca^{2+} omission virtually abolished the response to K [8.5±1.4%, $n=6$, stimulation compared with a value of 1,085.5±149.1%, $n=6$, obtained with control fibers bathed in 10 mM Ca^{2+}-ASW ($P<0.001$)]. Treatment with 100 mM K_o caused no visible shortening of the fibers bathed in Ca^{2+}-free ASW. In contrast, the control fibers contracted to 20–30% of their original length.

Hagiwara and Naka (1964) have reported the occurrence of irreversible changes in membrane function in barnacle muscle fibers bathed in Ca^{2+}-free solutions for long periods of time. It was therefore important to see whether the response of the Na efflux to elevated $[K]_o$ was abolished following shorter exposure to Ca^{2+}-free ASW. Thus, the experiments described above were repeated, but this time the Ca^{2+} was omitted from the bathing medium 15 min before increasing $[K]_o$. The results obtained were similar to those obtained with longer exposure to Ca^{2+}-free ASW. Following omission of external Ca^{2+}, 100 mM K caused an average stimulation of the Na efflux of 16.5±5.0%, $n=10$, compared with a value of 753.9±145.0%, $n=9$, obtained for controls ($P<0.001$). This suggests that the lack of response of the Na efflux to elevated $[K]_o$ following omission of external Ca^{2+} is not due to adverse effects on the membrane of exposure of the fibers to Ca^{2+}-free solutions. Further evidence in support of this conclusion was obtained from experiments involving replacement of Ca^{2+} with equimolar Mg^{2+} rather than Na^+. Hagiwara and Naka (1964) found that the irreversible changes in membrane function seen following exposure to Ca^{2+}-free ASW could usually be prevented by increasing the Mg^{2+} concentration. Therefore, the type of experiment described above was repeated, except in this case Mg^{2+} was substituted for Ca^{2+} in the bathing medium. The results were similar to those obtained with Ca-free ASW. Replacement of external Ca^{2+} completely abolished the response to subsequent elevation of $[K]_o$, whereas the average stimulation seen in control fibers was 479.3±96.7%, $n=3$ ($P<0.025$).

The results of membrane potential measurements made with fibers bathed in Ca^{2+}-free, 10^{-4} M ouabain-ASW and exposed to a range of external K concentrations are summarized in Fig. 2 (curve *B*). At a $[K]_o$ of 10 mM the membrane potential of fibers in Ca^{2+}-free ASW does not differ significantly from that seen in fibers bathed in 10 mM Ca^{2+}-ASW. However, as $[K]_o$ is increased the membrane of fibers in Ca^{2+}-free ASW is depolarized to a somewhat greater degree than that of fibers in 10 mM Ca^{2+}. The slope of the straight line realtionship obtained in Ca^{2+}-free ASW is about 43 mV for a 10-fold increase in $[K]_o$. Thus, at 100 mM K the membrane potential of fibers in Ca^{2+}-free ASW is ~10 mV lower. It seems unlikely therefore that the effect on the membrane potential of omitting Ca^{2+} from the bathing medium is in any way responsible for the lack of stimulation of the Na efflux by 100 mM K.

In view of the effect of Ca^{2+}-free solutions on the response of the ouabain-insensitive Na efflux to elevated $[K]_o$, experiments were carried out in which external Ca^{2+} was varied over the range 0–20 mM prior to depolarization with a 200 mM K^+-ASW. The

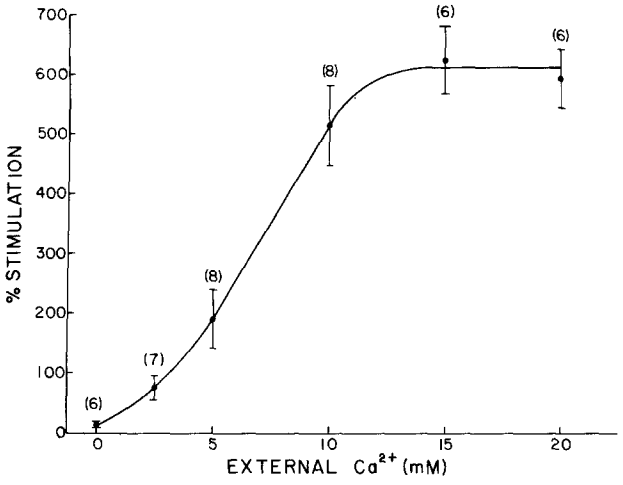

Fig. 5. The effect of varying external Ca^{2+} on the response of the ouabain-insensitive Na efflux to 200 mM K. The number of observations made at each concentration tested is indicated in parenthesis. Vertical bars indicate ± SEM

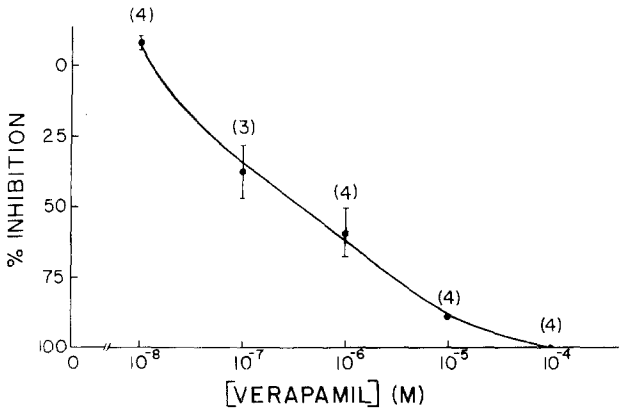

Fig. 6. The effect of varying concentrations of verapamil on the response of the ouabain-insensitive Na efflux to 100 mM K. The number of observations made at each concentration tested is indicated in parenthesis. Vertical bars indicate ± SEM

results are summarized in Fig. 5. It can be seen that there is a definite sigmoidal relationship between the response of the Na efflux to elevated $[K]_o$ and the external Ca^{2+} concentration.

(iv) Lack of Dependence on External Mg^{2+}. In order to see if the response of the Na efflux to 100 mM K is dependent on the external Mg^{2+} concentration, Mg^{2+} was omitted from the bathing medium 25 min before raising $[K]_o$. The average stimulation of the ouabain-insensitive Na efflux seen following K depolarization of fibers bathing in Mg^{2+}-free ASW was 789.4 ± 108.3%, $n=9$. This is comparable to the value of 828.0 ± 75.3%, $n=9$, obtained with control fibers bathed in 10 mM Mg^{2+}-ASW ($P<0.5$). It is hence concluded that the response of the Na efflux to elevated $[K]_o$ is independent of external Mg^{2+}.

The Role of Calcium in the Response of the Na Efflux to Elevated Potassium

Three possible explanations for the dependency on external Ca^{2+} of the response of the Na efflux to elevated K are: (1) that the K_o-stimulated Na efflux involves an exchange between external Ca^{2+} and internal Na^+, (2) that the system responsible for the increase in Na efflux is activated by a rise in myoplasmic Ca^{2+} caused by the entry of external Ca^{2+} via the voltage-dependent calcium "channels" which have been demonstrated in barnacle muscle fibers (Hagiwara, Hayashi & Takahashi, 1969; Keynes et al., 1973), and (3) that the K_o-stimulated Na efflux requires Ca^{2+} bound externally to the plasma membrane. The experiments carried out to test these possibilities are described in this section.

(i) The Effect of Ca^{2+}-Channel Blocking Agents. The group of drugs referred to as "calcium-antagonists" has been demonstrated to block voltage-dependent Ca^{2+} channels in a variety of excitable tissues (*see* Fleckenstein, 1977) including barnacle muscle fibers (Rojas & Luxoro, 1974; Ashley & Lea, 1978). If the response of the Na efflux to elevated $[K]_o$ is mediated by a rise in myoplasmic Ca^{2+} (explanation *2* above), the external application of one of these drugs should then reduce or abolish the response to subsequent K-depolarization of fibers bathed in 10 mM Ca^{2+}-ASW. Thus, experiments were carried out in which 10^{-4} M verapamil was applied externally to ouabain-treated fibers prior to their exposure to 100 mM K. The results of these experiments indicate that the response of the Na efflux to elevated $[K]_o$ is practically abolished by verapamil: 3.0 ± 2.0%, $n=7$, stimulation compared with a value of 876.8 ± 86.6%, $n=5$, seen in control fibers that had not been treated with verapamil ($P<0.001$). Verapamil reduced the degree of visible shortening caused by depolarization by ~ 60%.

In order to see if the drug was acting in a dose-dependent manner, experiments were carried out in which varying concentrations of verapamil were applied externally prior to exposure of ouabain-treated fibers to 100 mM K. The results summarized in Fig. 6 indicate that at a concentration as low as 10^{-7} M verapamil causes a considerable reduction in the mag-

nitude of the response of the Na efflux to elevated $[K]_o$. The concentration range over which the drug is effective is similar to that seen in other tissues (Kaumann & Uchitel, 1976; Fleckenstein, 1977). Additional experiments have shown that the response to potassium-depolarization is also markedly reduced by external application of 10^{-4} M D-600, a methoxy derivative of verapamil (88% reduction, $n=4$, $P<0.05$), and by 3 mM Mn^{2+} (63% reduction, $n=3$, $P<0.001$) which has been shown to suppress the Ca^{2+} conductance increase in barnacle muscle fibers (Hagiwara & Nakajima, 1966).

The results obtained with these Ca^{2+}-channel blocking agents suggest that the K-induced stimulation of the Na efflux involves a mechanism activated by a rise in myoplasmic Ca^{2+} (explanation *2* above). However, this interpretation assumes that these agents act in a highly specific manner and do not interfere with normal functioning of other systems that might be involved in the response to raised $[K]_o$. In order to test the possibility that verapamil prevents the response to K depolarization by a means other than blocking the voltage-dependent Ca^{2+} channels, the following experiment was carried out: cyclic GMP, which, in barnacle muscle fibers causes a stimulation of the ouabain-insensitive Na efflux that is dependent on external Ca^{2+} but does not involve a change in membrane potential (Bittar & Sharp, 1979), was injected into fibers following external application of 10^{-4} M verapamil. In verapamil-treated fibers injection of 10^{-3} M cyclic GMP caused an average stimulation of the ouabain-sensitive Na efflux of $67.2 \pm 13.2\%$, $n=5$. This is similar to the value of $51.2 \pm 11.2\%$, $n=5$, obtained with control fibers that had not been exposed to verapamil ($P<0.4$). This suggests that verapamil does not interfere in a nonspecific way with processes exhibiting a dependency on external Ca^{2+}.

(ii) The Effect of Ca^{2+}-chelating Agents. The second type of experiment carried out to test whether a rise in myoplasmic Ca^{2+} is involved in mediating the response of the Na efflux to elevated $[K]_o$ is shown in Fig. 7. 250 mM EGTA was injected throughout the length of ouabain-treated fibers which were subsequently exposed to 100 mM K-ASW containing 10 mM Ca^{2+}. The magnitude of stimulation seen in the EGTA-treated fibers was less than 50% of that seen in control fibers that had not been pre-injected with EGTA: $311.7 \pm 39.4\%$, $n=6$, stimulation compared with $769.7 \pm 105.8\%$, $n=6$ ($P<0.005$). 250 mM EGTA also, when injected 30 min before raising K_e, reduced the degree of visible shortening caused by K depolarization by ~90%. However, 100 mM EGTA failed to significantly reduce the response of the Na efflux

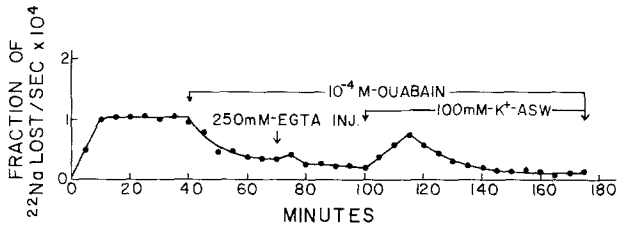

Fig. 7. The reduced response of the ouabain-insensitive Na efflux to 100 mM K following injection of 250 mM EGTA

to 100 mM K, although it decreased the magnitude of contraction by ~70%.

The effectiveness of EGTA as a "Ca^{2+}-buffer" decreases as the free Ca^{2+} concentration rises above 10^{-8} M. Since nitrilotriacetic acid has been reported to be a more effective Ca^{2+}-buffer at higher free Ca^{2+} concentrations (Reed & Bygrave, 1975), the type of experiment shown in Fig. 7 was repeated with 250 mM nitrilotriacetic acid injected instead of EGTA. Like EGTA, nitrilotriacetic acid caused a significant ($P<0.05$) reduction in the response of the ouabain-insensitive Na efflux to 100 mM K ($n=4$) although the magnitude of the reduction (~25%) was less than that observed with EGTA.

(iii) The Effect of Raising Myoplasmic Ca^{2+} by Microinjection of $CaCl_2$ and by External Application of A23187. The results obtained with calcium-antagonists and calcium-chelators strongly suggest that the stimulation of the Na efflux caused by elevated $[K]_o$ involves a mechanism activated by the rise in myoplasmic Ca^{2+} that occurs following depolarization. As previously demonstrated (Danielson et al., 1971), and confirmed by the studies described here, raising internal Ca^{2+} by microinjection also stimulates the Na efflux. An alternative method of raising myoplasmic Ca^{2+} involving external application of the ionophore A23187 (Reed & Lardy, 1972; Desmedt & Hainaut, 1976) had a similar effect in barnacle muscle fibers. Exposure to 10^{-5} M A23187 caused an average stimulation of the ouabain-insensitive Na efflux of $152.3 \pm 35.0\%$, $n=4$. This response exhibited an absolute requirement for external Ca^{2+}.

(iv) The Effect of External Ca^{2+} on the Response to Injection of $CaCl_2$. On the basis of the data described above, it is not possible to determine whether the K_o-stimulated Na efflux involves an exchange between external Ca^{2+} and internal Na^+, or if it requires Ca^{2+} bound externally. Since the stimulation caused by $[K]_o$ appears to be chiefly triggered by a rise in myoplasmic Ca^{2+}, experiments were carried out to see whether the response to injected $CaCl_2$ is dependent on external Ca^{2+}. As illustrated in

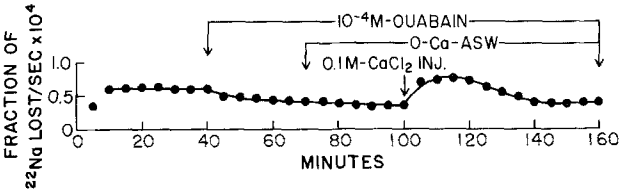

Fig. 8. The response to injection of 0.1 M $CaCl_2$ of the ouabain-insensitive Na efflux into Ca^{2+}-free ASW

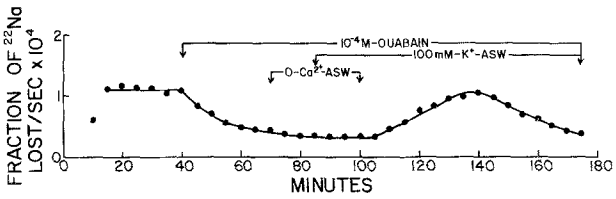

Fig. 9. The response to restoration of external Ca^{2+} of the ouabain-insensitive Na efflux in a K-depolarized fiber

Fig. 8, injection of 0.1 M $CaCl_2$ 30 min after omission of external Ca^{2+} resulted in a prompt and transitory rise of the efflux, the magnitude of which averages $131.3 \pm 21.8\%$, $n=9$. Not only is this similar to the value of $131.5 \pm 20.1\%$, $n=8$, obtained with control fibers bathed in 10 mM Ca^{2+}-ASW ($P<0.5$) but also confirmatory of an earlier unpublished finding by E.E. Bittar and R. Schultz that injected $CaCl_2$ stimulates the Na efflux into Ca-free ASW. However, the objection may be raised that in this particular experiment the resting Na efflux had a low rate-constant value. The fact is that the rate constants for Na efflux in both test and control fibers were in the region of 0.7×10^{-4} sec^{-1} prior to the application of ouabain. Thus, the provisional conclusion drawn from these experiments is that the response to $CaCl_2$ injection is independent of external Ca^{2+}, which suggests that Na-Ca exchange is not involved.

(v) Time-Course of Dependence on Inward Ca^{2+} Current. Unlike the Na^+ channel of most nerves, the voltage-dependent Ca^{2+} channel in barnacle muscle fibers does not exhibit appreciable inactivation (Keynes et al., 1973). Since the peak of the K_o-induced stimulation of Na^+ efflux normally occurs 15–25 min after elevating $[K]_o$, this raised the question of whether a sustained inward Ca^{2+} current is required throughout this time period. In order to check this, depolarization of ouabain-treated fibers with 100 mM K-ASW was followed, after varying time intervals, by external application of 10^{-4} M verapamil. No reduction in the magnitude of the K_o-induced stimulation of the Na efflux was seen until the time period between depolarization and treatment with verapamil was reduced to 10 min. Under these conditions the value for the average stimulation was $486.6 \pm 70.7\%$, $n=8$, compared with a value of $717.3 \pm 91.6\%$, $n=8$, obtained with control fibers that were not treated with verapamil ($P<0.05$).

The second type of experiment carried out to see whether a sustained influx of Ca^{2+} is necessary, involved repolarization of the fibers with 10 mM K-ASW following depolarization with 100 mM K-ASW. The magnitude of stimulation of the ouabain-insensitive Na efflux was not significantly reduced even when the fibers were repolarized 5 min after raising $[K]_0$ ($n=8$). Thus, it is apparent that the response of the Na efflux to increased $[K]_o$ is not dependent on the inward Ca^{2+} current being maintained throughout the rising phase of the stimulation.

Although it appears that the voltage-dependent Ca^{2+} channels play a key role in the response to $[K]_o$ only during the first 5–10 min after K-depolarization, this does not exclude the possibility that Ca^{2+} conductance remains high for a longer time period. To test this possibility, fibers bathed in 10^{-4} M ouabain, Ca^{2+}-free ASW were depolarized with 100 mM K and the Ca^{2+} was then restored 15 min or longer after raising $[K]_o$. The results of an experiment of this type are shown in Fig. 9. It can be seen, as described earlier, that there is virtually no response to elevation of $[K]_o$ in the absence of external Ca^{2+}. However, restoration of external Ca^{2+} caused an appreciable stimulation of the ouabain-insensitive Na efflux, with an average value of $154.9 \pm 41.6\%$, $n=4$. The magnitude and kinetics of the response of K-depolarized fibers to restoration of external Ca^{2+} varied noticeably from one barnacle specimen to the next. (Thus, the average value given above was obtained from fibers taken from the same barnacle.) Several trends were apparent, however. In all cases where Ca^{2+} was restored within 45 min of K-depolarization there was a stimulation of the Na efflux, although in some cases the response was very small ($n=20$). The average value of the response to restoration of Ca^{2+} was always less than the average value of the response to 100 mM K seen in control fibers from the same barnacle that were bathed in 10 mM Ca^{2+}-ASW. For example, the average value of the magnitude of stimulation caused by 100 mM K in the control fibers of the experiment shown in Fig. 9 was $418.6 \pm 32.0\%$, $n=5$ ($P<0.005$). In addition, the stimulation caused by Ca^{2+} restoration generally took longer to reach its peak value than that seen in control fibers.

Since, as indicated earlier, prolonged bathing of fibers in Ca^{2+}-free ASW has been reported to cause some changes in membrane function (Hagiwara &

Naka, 1964), it was necessary to ascertain whether the response to restoration of external Ca^{2+} occurs only in fibers that are depolarized. Thus, experiments were carried out in which ouabain-treated fibers were bathed in Ca^{2+}-free ASW for varying time periods, but were not exposed to elevated $[K]_o$. When the Ca^{2+} was restored, after periods of up to 75 min, no stimulation of the Na efflux was seen ($n=6$). It is thus apparent that the mechanism stimulated by elevated $[K]_o$ is not inactivated rapidly following the onset of a sustained K-depolarization.

Mechanism of Activation of the Quabain-Insensitive Na Efflux by Elevated Potassium

(i) The Effect of an Inhibitor of cAMP-dependent Protein Kinase. It has been shown that the responses of the ouabain-insensitive Na efflux to injection of cyclic AMP (Bittar et al., 1979) and cyclic GMP (Bittar & Sharp, 1979) are markedly reduced by preinjection of a pure inhibitor of cyclic AMP-dependent protein kinase, PKI (Demaille, Peters & Fischer, 1977). The results of a similar experiment carried out to see whether PKI also causes a reduced response to elevated $[K]_o$ are shown in Fig. 10. As summarized in Table 1, the average response of fibers injected

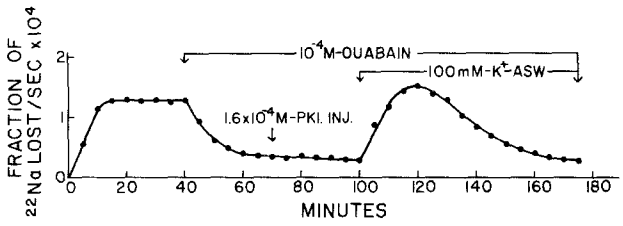

Fig. 10. The reduced response of the ouabain-insensitive Na efflux to 100 mM K following injection of a pure inhibitor (1.6×10^{-4} M) of cyclic AMP-dependent protein kinase

Table 1. (*a*): Rate constants ($sec^{-1}\times10^{-4}$) for Na efflux in experiments involving the injection of pure protein kinase inhibitor before and after raising K_e and microinjection of $CaCl_2$

Barnacle #	Fiber #	Experiments with 100 mM K_e						Experiments involving $CaCl_2$ injection					
		Test fibers			Control fibers			Test fibers			Control fibers		
		A	B	C	A	B	C	A	B	C	A	B	C
1	1	1.2	0.51	2.56	0.85	0.39	2.80	0.91	0.39	0.84	0.80	0.18	0.52
	2	0.97	0.38	3.0	0.79	0.19	2.40	0.92	0.34	0.71	0.95	0.35	0.92
	3	0.99	0.49	1.73	1.15	0.47	3.97	0.89	0.28	0.77	0.85	0.44	1.27
	4	1.0	0.38	2.0	0.90	0.47	1.96	0.89	0.36	0.83	–	–	–
2	5	1.24	0.29	1.60	1.18	0.28	3.0	1.0	0.20	0.38	1.0	0.27	0.90
	6	1.10	0.29	2.21	1.30	0.27	2.56	1.0	0.30	0.80	1.0	0.28	0.76
	7	1.29	0.27	1.52	1.11	0.28	3.78	0.94	0.23	0.50	1.1	0.28	0.85
	8	1.18	0.27	1.54	1.0	0.28	3.35	1.0	0.26	0.57	1.0	0.24	0.65
3	9	1.37	0.28	1.24	1.0	0.29	1.90						
	10	1.30	0.29	1.02	1.28	0.32	3.60						
	11	1.15	0.27	1.46	1.32	0.33	3.18						
	12	–	–	–	1.35	0.31	3.10						

Key: A – Value before ouabain application. B – Value before raising external K. C – Value obtaining at peak stimulation.

(*b*): Effect of pure protein kinase inhibitor on stimulation of the ouabain-insensitive Na efflux by high K-ASW, microinjection of $CaCl_2$, cGMP and cAMP

	Average magnitude of stimulation caused by			
	External application of 100 mM K^+-ASW (%)	Microinjection of 0.1 M $CaCl_2$ (%)	Microinjection of 10^{-3} M cGMP (%)	Microinjection of 10^{-4} M cAMP (%)
Control fibers	862.3 ± 77.9 ($n=12$)	210.7 ± 23.7 ($n=7$)	184.3 ± 23.6 ($n=10$)	530.3 ± 103.9 ($n=3$)
Fibers preinjected with 1.6×10^{-4} M–protein kinase inhibitor	441.2 ± 42 ($n=11$)	127.1 ± 10.4 ($n=8$)	43.4 ± 11.9[a] ($n=11$)	102.8 ± 32.0[b] ($n=4$)
Reference	This paper	This paper	Bittar & Sharp, 1979	Bittar et al., 1979

[a] $P<0.001$. [b] $P<0.025$. Values are $\pm$SEM.

with PKI to subsequent exposure to 100 mM K was markedly reduced compared with controls that had not been injected with PKI but injected with distilled water. It is worthy of emphasis that, except in one experiment which was discarded, the injection of 1.6×10^{-4} M PKI had no effect on the ouabain-insensitive Na efflux. Similarly, injection of distilled water (~ 0.3 µl) into companion control fibers was without effect. Thus, mention of the rate constant values found following the injection of PKI and distilled water has been omitted here. Table 1 additionally shows that preinjection of 1.6×10^{-4} M PKI caused a reduction in the magnitude of the response of the ouabain-insensitive Na efflux to injection of 0.1 M $CaCl_2$. Again, companion control fibers were injected with ~ 0.3 µl distilled water. This had no effect on the course of the ouabain-insensitive Na efflux. A similar situation was seen following the injection of PKI. Alternatively, a more valid comparison would be to examine only those results where the rate constants for the ouabain-insensitive Na efflux prior to raising K_e or injecting $CaCl_2$ in test and control fibers are about equal, e.g., experiments 5–8 from the left (Barnacle # 2) and 5–8 from the right (barnacle # 2). Such a comparison confirms that PKI does reduce the size of the response to 100 mM K-ASW and to injection of 0.1 M $CaCl_2$. Moreover, a comparison of the results of both types of experiments with those obtained for the effect of PKI on the response of the Na efflux to cyclic AMP and cyclic GMP (*vidé* Table 1*b*) makes it quite clear that this protein inhibitor is able to reduce the responses to all of these agents. However, it is also apparent that the degree of inhibition varies, depending on the method of stimulation.

(ii) The Lack of Effect of 2′-Deoxyadenosine-3′Monophosphate, a Reputed Inhibitor of Adenylate Cyclase. Sahyoun et al. (1976) have reported the presence of a naturally occurring inhibitor of adenylate cyclase in a variety of tissues. This compound, which has been isolated and characterized as 2′-deoxyadenosine-3′-monophosphate (2′-deoxy-3′-AMP) was found to cause a marked inhibition of adenylate cyclase in most, but not all, of the preparations tested. In order to see if the response of the Na efflux to 100 mM K^+ is modulated by this reputed adenylate cyclase inhibitor, 10^{-2} M-2′-deoxy-3′-AMP was injected into ouabain-treated fibers 30 min before K-depolarization. Injection of 2′-deoxy-3′-AMP caused either a very small stimulation or had no effect on the Na efflux. Subsequent elevation of $[K]_o$ caused an average stimulation of $359.5 \pm 41.4\%$, $n=10$, which was slightly, but not significantly, smaller than the value of $529.8 \pm 73.1\%$, $n=10$ obtained with controls ($P<0.1$). This suggests either that the inhibitor is ineffective in barnacle muscle or that the response to elevated $[K]_o$ is not mediated by a change in adenylate cyclase activity.

(iii) Lack of Effect of Lowering the pH of HCO_3^--ASW. It has been shown that the response of the ouabain-insensitive Na efflux to injection of cyclic GMP or cyclic AMP is significantly enhanced by lowering the pH of HCO_3^--ASW shortly before injection of the cyclic nucleotide (Bittar et al., 1979). Since Boron (1977) has demonstrated that raising the pCO_2 of ASW results in a rapid fall in internal pH in barnacle muscle fibers, these results were interpreted as indicating that lowering the internal pH enhances the response of the Na efflux to cyclic nucleotides. In order to see if a similar enhancement of the response to elevated $[K]_o$ occurs, the pH of HCO_3^--ASW was reduced from 7.8 to 7.0 five min before exposure of the fibers to 100 mM K. After lowering the pH, the average K_o-induced stimulation obtained was $1{,}069.5 \pm 68.6\%$, $n=10$, which is comparable to the value of $1{,}103.6 \pm 69.2\%$, $n=10$, obtained with controls ($P<0.5$). However, the response of the ouabain-insensitive Na efflux to injection of 0.1 M $CaCl_2$ was enhanced somewhat by lowering the pH of HCO_3^--ASW shortly before injection: $293.2 \pm 33.3\%$, $n=10$, stimulation compared with a value of $201.6 \pm 23.7\%$, $n=10$, obtained with control fibers bathed in HCO_3^--ASW at pH 7.8 ($P<0.05$). It is apparent that lowering the pH of HCO_3^--ASW has no effect on the response to elevated $[K]_o$ and only a moderate effect on the response to $CaCl_2$ injection.

(iv) Dependence of Response on Intracellular ATP levels. A more general question relating to the mechanism of the response of the Na efflux to elevated $[K]_o$ concerns the possibility of dependence on ATP. A notable feature of the response is that it is transient despite the fact that the $[K]_o$ was kept elevated until the end of most experiments.

If the K-stimulated Na efflux is dependent on internal ATP, a possible explanation for the transient nature of the response is that the internal ATP concentration has fallen below a critical level due to its utilization in both contraction and increased Na transport. In order to test this possibility, intracellular ATP was increased by injection of 0.5 M ATP-Na_2. Following this, the fibers were treated with 10^{-4} M ouabain and subsequently exposed to 100 mM K. A typical result of an experiment of this kind and of a control experiment, carried out at the same time, are shown in Fig. 11 *A* and *B*, respectively. It is apparent that the response of the ouabain-insensitive Na efflux to elevated $[K]_o$ is not prolonged by prior injection of the fibers with ATP-Na_2.

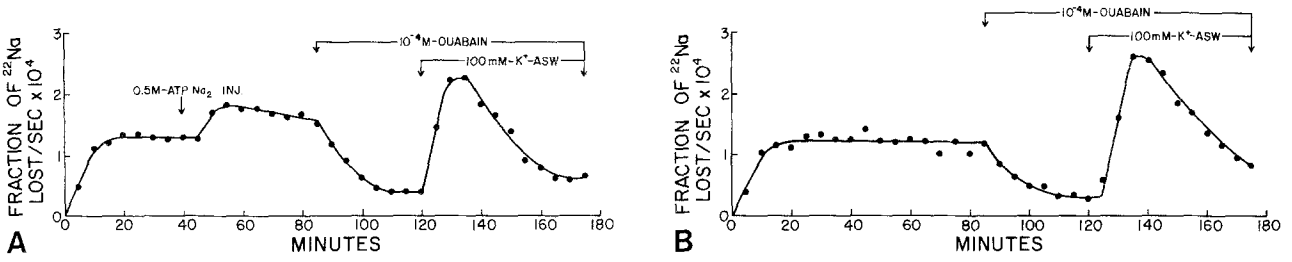

Fig. 11. The lack of effect of ATP-Na_2 on the duration of the response of the Na efflux to elevated K concentration. (*A*): The response of the ouabain-insensitive Na efflux to 100 mM K following injection of 0.5 M ATP-Na_2. (*B*): The response to 100 mM K of the ouabain-insensitive Na efflux from a control fiber

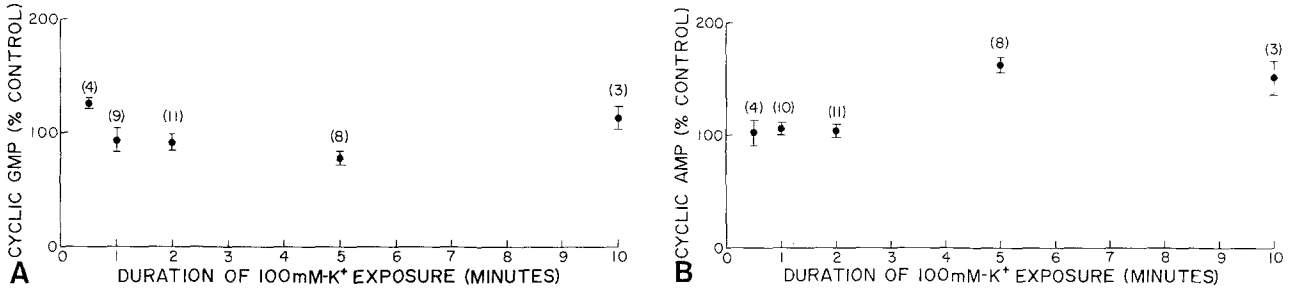

Fig. 12. The influence of elevated K concentration on cyclic nucleotide levels in barnacle muscle fibers. (*A*): The virtual lack of effect of 100 mM K on the cyclic GMP content of ouabain-treated, cannulated fibers. (*B*): The increase in cyclic AMP content of ouabain-treated, cannulated fibers following exposure to 100 mM K-ASW. The number of observations made at each time is indicated in parenthesis. Vertical bars indicate ± SEM

(v) Cyclic Nucleotide Levels Following Elevation of External K. Beam, Nestler and Greengard (1977) reported an approximately threefold increase in the cyclic GMP content of whole muscle bundles following their exposure to 100 mM K-ASW. The rise in cyclic GMP peaked 2 min after the onset of K depolarization and then declined rapidly towards the basal level even when exposure to elevated $[K]_o$ was maintained. They detected little effect (19% increase above control) of 100 mM K on cyclic AMP levels with exposures ranging from 30 sec to 2 min.

In order to see whether similar results are obtained under the conditions used for the Na efflux experiments described above, radioimmunoassay of both cyclic GMP and cyclic AMP was carried out on single muscle fibers which were cannulated and bathed in 10^{-4} M ouabain-ASW for 30 min prior to exposure to 100 mM K for varying time periods. Cannulated control fibers were assayed after bathing in 10^{-4} M ouabain-ASW for 32 min. The results of the cyclic GMP and cyclic AMP assays are shown in Fig. 12*A* and *B*, respectively. It can be seen that there is little change in the cyclic GMP level following exposure of fibers to 10 mM K for up to 10 min. However, K-depolarization caused a small increase in the cyclic AMP content of cannulated fibers. No significant difference between the control and test values was seen with 100 mM K exposure of less than 5 min. The values obtained for the cyclic AMP content at 5 and 10 min were ~160% ($P<0.005$) and ~150% ($P<0.025$), respectively, of the control value. This suggests that exposure of cannulated, ouabain-treated barnacle muscle fibers to 100 mM K_o causes a relatively slow rise in the cyclic AMP level which reaches a maximum in about 5 min and then remains reasonably constant over the next 5 min. However, it is possible that the cyclic AMP content actually peaks between 2 and 5 min or between 5 and 10 min after the onset of K-depolarization, in which case the true maximal value would be greater than that shown in Fig. 12*B*.

Discussion

Raised K_o causes a pronounced stimulation of the ouabain-insensitive component of the Na efflux in barnacle muscle fibers. This is also the case with Na efflux in squid axon (Baker 1969) but not with frog muscle (Edwards & Harris, 1957; Horowicz & Gerber, 1965) and erythrocytes (Garrahan & Glynn, 1967), since the response to K_o in these systems is mediated by the Na-K pump. The kinetics of this response indicate that the stimulation is always transitory, a situation reminiscent of the effect of injected cAMP, cGMP and $CaCl_2$ (Bittar, Chambers & Schultz, 1976; Schultz & Bittar, 1978; Bittar & Sharp, 1979). Additionally, a transitory response im-

plies that in barnacle muscle fibers a raised K_o fails to lessen the inhibitory effect of ouabain (10^{-4} M). The present results also indicate that the K-stimulated Na efflux is reduced when external Na is replaced by Li. However, dependency on external Na is only apparent at Na concentrations less than 50% of normal. This may be taken to mean that the transport mechanism is saturated at higher external Na concentrations or that another ion(s) is able to substitute for external Na^+ to some degree. These interpretations are, of course, made with caution, since it is quite possible that the reduction seen in the response to 100 mM K_o is not a direct effect of Na^+ removal but is secondary to some other change caused by the replacement. Thus, for example, Na-free ASW causes both the free internal Ca^{2+} and the Ca influx in barnacle muscle fibers to rise to new steady levels (Ashley, Ellory & Hainaut, 1974; Ashley & Lea, 1978). Therefore, the reduced response of the Na efflux to elevated K_o seen following replacement of Na^+ might be due to a rise in internal free Ca^{2+} rather than removal of external Na^+.

The absolute requirement for external Ca^{2+} exhibited by the K_o-stimulated Na efflux need not be attributed to an exchange between external Ca^{2+} and internal Na. Such a line of argument is greatly strengthened by evidence that the Na efflux into Ca-free ASW is stimulated by injected $CaCl_2$. The requirement itself that external Ca^{2+} be present is in keeping with similar findings concerning the effect of K_o on metabolism in frog muscle (Kaye & Mommaerts, 1960; Van der Kloot, 1967) and on the ouabain-insensitive Na efflux in squid axon (Baker et al., 1969). However, in considering the role of external Ca^{2+} in the response to elevated K_o in these three tissues, it must be kept in mind that barnacle muscle differs from frog muscle and squid axon in that the inward current associated with excitability is carried by Ca^{2+} ions only.

The results of the experiments with Ca-antagonists, Ca-chelators and $CaCl_2$ injection support the view that stimulation of the ouabain-insensitive Na efflux by raised K_o is triggered by a rise in internal free Ca^{2+} resulting from the entry of external Ca^{2+} via voltage-dependent Ca^{2+} channels. Verapamil, D-600 and Mn^{2+}, for example are all capable of reducing or abolishing the response of the Na efflux to K-depolarization, indicating that Ca^{2+} entry is an essential step in the activation process. Although nonspecific effects of these Ca^{2+} antagonists cannot be excluded, it is noteworthy that Ashley and Lea (1978) have found that D-600 (5×10^{-4} M) has no apparent effect on the Ca^{2+} influx in unstimulated barnacle muscle fibers, and also that many reported attempts to demonstrate an effect with verapamil on cardiac myofibrillar ATPase and on Ca^{2+} movements in isolated cardiac sarcoplasmic reticulum and mitochondria have failed (*see* Fleckenstein, 1977).

The above formulation, however, does not go far enough. Why is it that EGTA fails to completely abolish the response to depolarization? One possibility is that the system activated by Ca^{2+} is intramembrane in location and hence inaccessible to EGTA. Another possibility is that EGTA acts as a chelator of trace elements (Holloway & Reilley, 1960) and, hence, acts by removing Fe and Zn that are inhibitory to the membrane mechanism, e.g., phosphoprotein phosphatase (Li, Hsiao & Chan, 1978). To date, evidence is unavailable that barnacle fibers possess a phosphoprotein phosphatase that is inhibited by trace elements. However, trace elements such as Fe or Zn are able, following injection, to reduce markedly the verapamil-insensitive stimulatory response to injected GTP of the Na efflux in ouabain-treated fibers (Bittar, Nwoga & Bárány, 1980).

There are two reasons for thinking that a fall in myoplasmic *p*Ca following depolarization by high K_o leads to stimulation of the ouabain-insensitive Na efflux as the result of newly formed cyclic nucleotide. *Firstly,* the results of radioimmunoassay indicate a rise in total cAMP (and not cGMP). And *secondly,* preinjection of pure protein kinase inhibitor causes a reduced response to high K_o. At first sight of the data presented here, one is tempted to reason in the following way: First, 5 min after the onset of K depolarization, the total internal cAMP is elevated to ca. 0.4×10^{-6} M (taking the intracellular fluid phase and the protein content of barnacle muscle fibers as being 74 and 12%, respectively, and the average fiber wet wt as 50 mg). Second, from Table 1 it can be seen that the average magnitude of stimulation of the Na efflux caused by 100 mM K_o is greater than 800%. Assuming 100-fold dilution by the myoplasm, a conservative estimate of cAMP concentration following injection of a 10^{-4} M solution would lie in the 10^{-7} – 10^{-6} M range (similar to the concentration of cAMP 5 min after the onset of K-depolarization). Third, as can be appreciated from Table 1, the average stimulation (530%) of the Na efflux caused by injection of 10^{-4} M cAMP is considerably less than that observed following exposure to 100 mM K. The question then is: What is the underlying reason for this discrepancy? Two possibilities suggest themselves: one is that high K depolarization in the presence of external Ca^{2+} triggers not only voltage-dependent Ca^{2+} channels but also a phosphoprotein phosphatase system. The other is that an increase in myoplasmic Ca^{2+} following depolarization results in direct activation by Ca^{2+}

of Ca^{2+}-dependent protein kinase, which instead is conceivably calmodulin (Schulman & Greengard, 1978) in addition to activation by newly formed cAMP of cAMP-dependent protein kinase. This seems reasonable in the light of evidence that preinjection of pure kinase inhibitor reduces but does not abolish the response to high K or injected $CaCl_2$.

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