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Deregulation by zinc of the sodium efflux in barnacle muscle fibers

Huiwen Xie and E. Edward Bittar *

Department of Physiology, University of Wisconsin, Madison, WI 53706 (USA)

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Single muscle fibers from the barnacle *Balanus nubilus* were employed to study the behavior of the resting Na^+ efflux toward external and internal application of zinc (Zn^{2+}). This involved both unpoisoned and ouabain-poisoned fibers. The results obtained are as follows: (i) External application of Zn^{2+}, e.g., 2 mM (a maximal dosage) in 10 mM Hepes-ASW (pH 7.3) causes a fall in the resting Na^+ efflux which exceeds that caused by 10^{-4} M ouabain in companion controls. (ii) The buffer of choice is found to be Hepes, rather than HCO_3^- or imidazole. (iii) The observed fall in the resting Na^+ efflux caused by external application of Zn^{2+} is concentration-dependent, the IC_{50} being 10 μM. (iv) The inhibitory effect of Zn^{2+} is partially reversible; occasionally, however, reversibility is not seen. (v) The Zn^{2+}-insensitive component of the Na^+ efflux is reduced by 10^{-4} M ouabain. (vi) The ouabain-insensitive component of the Na^+ efflux is reduced by external application of Zn^{2+}. This response is concentration-dependent. (vii) Preinjection of EGTA reduces the sensitivity of the Na^+ efflux to external application of Zn^{2+}. This is true of both unpoisoned and ouabain-poisoned fibers. (viii) (a) The resting Na^+ efflux is reduced by injecting Zn^{2+}. Ouabain application reduces the remaining Na^+ efflux. (b) Injection of Zn^{2+} reduces the ouabain-insensitive component of the Na^+ efflux. (c) External application of Zn^{2+} following the injection of Zn^{2+} reduces the remaining Na^+ efflux. Ouabain is ineffective when applied after both maneuvers. (d) Injection of Zn^{2+} after its external application is without effect. Subsequent application of ouabain is also without effect. (e) Injection or external application of Zn^{2+} reduces the ouabain-insensitive Na^+ efflux. Whereas in the former case subsequent external application of Zn^{2+} reduces the remaining Na^+ efflux, in the latter case Zn^{2+} injection after external application of Zn^{2+} is ineffective. Collectively, these results provide evidence in support of the hypothesis that Zn^{2+} is a potent inhibitor of the ouabain-sensitive and ouabain-insensitive components of the Na^+ efflux, and that the inhibitory effect is partly due to the entry of Zn^{2+} into the myoplasm. They also raise the possibility that the inhibitory effect caused by Zn^{2+} injection may be the result of Zn^{2+} leakage from the fiber interior.

Introduction

Practically nothing is yet known about the behavior of the resting Na^+ efflux towards zinc (Zn^{2+}). However, there are several reasons for thinking that the trace metal might prove to be a genuine inhibitor of active Na^+ transport. One is that Zn^{2+} is found to reduce the activity of the membrane Na^+/K^+-ATPase, e.g., in extracts of Littre cells, the IC_{50} being approx. 10 μM [1]. Another is that besides interacting with sulfhydryls, Zn^{2+} is known to interact with constituents of the plasma membrane such as phosphatidyl serine [2]. Thus, the purpose of the following communication is to give an account of some work that has been carried out using the barnacle muscle fiber as a preparation. It provides clear-cut evidence that Zn^{2+} is a powerful inhibitor of the Na^+ efflux in this single cell model system.

Materials and Methods

Materials. Specimens of the barnacle *Balanus nubilus* were supplied by the Pacific Biomarine Laboratory, Venice, CA and Bio-Marine Enterprises, Seattle, WA. They were maintained in an Instant Ocean aquarium at a temperature of 10–12°C. The composition of the aquarium seawater was as follows (mM): Na, 465; K, 10; Ca, 10; and Mg, 60. The pH was 7.8–8.0.

Dissection and cannulation. Single muscle fibers were isolated by dissection from the depressor muscle bundles, and cannulated as described by Caldwell and Walster [3] for crab muscle fibers. These fibers were generally 3–5 cm in length and 1–2 mm in width.

The microinjector used was similar to that described by Bittar and Tallitsch [4]. The volume of fluid released

* Corresponding author. Fax: +1 (608) 2622327.

into a fiber was 0.3–0.4 μl. If the average intrafiber fluid volume is considered to be about 40 μl, then dilution by the myoplasm of the injected solution may roughly be taken as being 100-fold.

Solutions. Dissection of the fibers and the experiments carried out involved the use of artificial seawater (ASW) as the bathing medium, the composition of which was as follows (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10, imidazole, 10 or Hepes, 10; the pH was 7.3.

Radioactivity measurements. $^{22}NaCl$ in aqueous solution was supplied by Amersham–Searle, Arlington Heights, IL. The solution was dried down and then redissolved in water so that volumes of 0.4 μl gave at least 700 000 counts per minute (cpm). The procedures used for collecting the effluent following the injection of $^{22}NaCl$ into the cannulated fiber and for counting its activity, as well as the activity remaining in the fiber at the end of the experiment, were those described by Bittar et al. [5]. Samples were counted in a Beckman 'Biogamma' counter and the data obtained was processed using an Apple II computer programmed to compute the $^{22}Na^+$ efflux in cpm and the fractional rate constant for $^{22}Na^+$ loss. Inhibition was estimated on the basis of the rate constant plots by extrapolating the last few points of the inhibitory phase back to the time of application of the agent, and the rate constant before the onset of inhibition. Estimates of a second inhibitory effect on the ouabain-insensitive Na^+ efflux were made by taking the difference between the rate constant found by extrapolating the last few points of the inhibitory phase back to the time of application of the agent and the rate constant before the onset of the second inhibitory phase. The results given in this paper are means $\pm$ S.E., and significant levels were estimated using Student's unpaired *t*-test. A significance level of $P < 0.05$ was chosen. All experiments were performed at an environmental temperature of 23 $\pm$ 1°C.

Agents. Ouabain, Hepes, imidazole and EGTA were purchased from Sigma, St. Louis, MO. $ZnCl_2$ was supplied by Aldrich Chemical, Milwaukee, WI.

Results

Effects of Zn^{2+} on the resting Na^+ efflux

As a rule, freshly dissected fibers suspended in ASW containing one of three buffers: HCO_3^-, Hepes or imidazole, were found to be sensitive to external application of Zn^{2+}. Preliminary trials revealed that a maximal effect was obtainable with 1–2 mM Zn. Thus, for example, application of 2 mM Zn to fibers suspended in ASW containing 10 mM Hepes as buffer at pH 7.3 caused a prompt fall in the resting Na^+ efflux, the magnitude of which averages 68 $\pm$ 1% ($n = 8$). This value is significantly greater than the 50 $\pm$ 2% fall which 10^{-4} M ouabain caused in companion controls

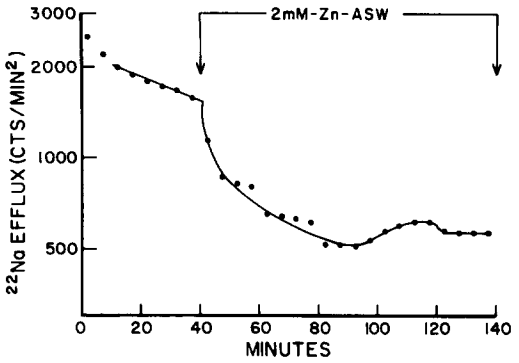

Fig. 1. The inhibitory effect on the resting Na^+ efflux of external application of 2 mM Zn (semilog plot).

($n = 8$). This result obtained with Zn^{2+} is illustrated in Fig. 1, where it can be seen that following the onset of the full inhibitory effect a small but transitory rise in Na^+ efflux occurred.

To ascertain whether the magnitude of the inhibitory effect depends in part on the buffer used, experiments were carried out with ASW containing HCO_3^-, Hepes or imidazole as the buffer, the concentration selected being 10 mM, and the pH 7.3. However, in the case of HCO_3^-, it should be borne in mind that lowering the pH of ASW from 7.8 to 7.3 reduces the bicarbonate level to about 7 mM in an open system [6]. The histogram shown in Fig. 2 indicates fairly clearly that the potency of Zn^{2+} as an inhibitor is greatest when Hepes is used as the buffer. The differences between the values obtained with HCO_3^- and Hepes, and between imidazole and Hepes are significant. The choice of Hepes as the buffer for further experiments with Zn^{2+} was thus based on these results.

Zn^{2+} concentration–response relation

Summarized in Fig. 3 are the results obtained by external application of Zn^{2+} in varying concentration

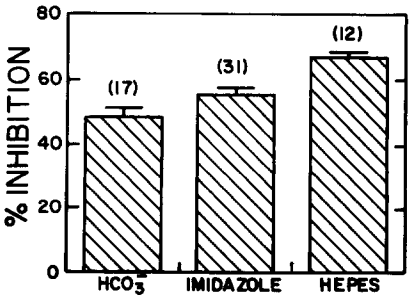

Fig. 2. Histogram providing a comparison of the magnitude of the inhibitory effect on the resting Na^+ efflux of external application of 2 mM Zn to fibers suspended in ASW containing three different buffers, viz., HCO_3^-, imidazole, and Hepes. Vertical bars span $\pm$S.E. (only upper shown). The number of measurements done is recorded in parentheses.

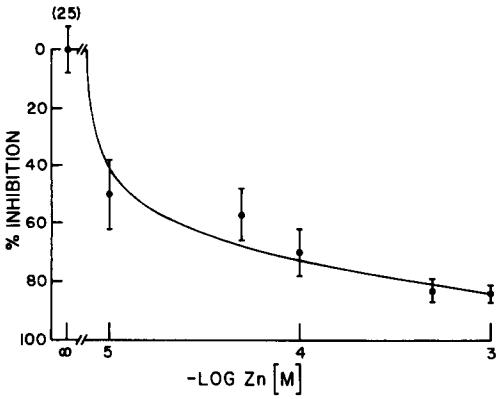

Fig. 3. Log concentration–response relation for the inhibitory effect of Zn^{2+} on the resting Na^+ efflux. Abscissa: $-\log$ scale. The curve was drawn according to best visual fit. Each plotted test point represents the mean value of three measurements carried out on fibers isolated from the same muscle bundle. Vertical bars span $\pm$S.E.

to unpoisoned fibers. The plot shows that the fibers were very sensitive to Zn^{2+}, half-maximal inhibition being obtained with 10 μM.

Omission of Zn^{2+} following its application

Next, experiments were designed to determine whether the inhibitory effect of Zn^{2+} on the resting Na^+ efflux is reversible. The results obtained indicate that the sudden omission of 2 mM Zn from the bathing medium following the onset of the full effect of Zn^{2+} resulted in partial restoration of the Na^+ efflux, but not always. An experiment showing partial reversal is given in Fig. 4. In this particular group of experiments, partial reversal averages $53 \pm 4\%$ ($n = 4$).

Application of ouabain after Zn^{2+}

Initially, this type of experiment was done using fibers suspended in 10 mM imidazole-ASW (pH 7.3) and 10 mM Hepes-ASW (pH 7.3). Characteristically, 2 mM Zn caused a prompt and sharp fall in the resting Na^+ efflux. Subsequent external application of 10^{-4} M ouabain (this being a maximally effective concentration [7]) caused a fall in the remaining Na^+ efflux. This effect is illustrated in Fig. 5a and b. As is seen, a small rise in Na^+ efflux took place at about $t = 60$ min in the

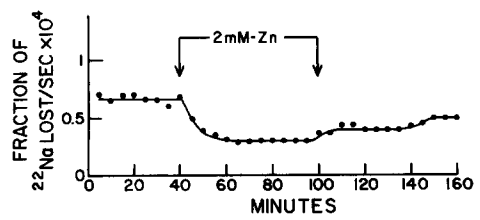

Fig. 4. Partial reversal of the inhibitory effect on the resting Na^+ efflux of external application of 2 mM Zn by sudden omission of Zn^{2+} from the bathing medium.

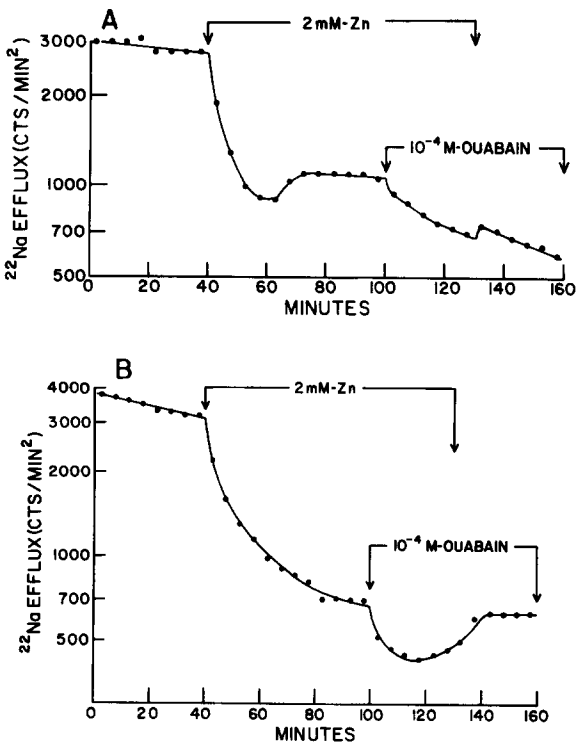

Fig. 5. (A) The biphasic response of the resting Na^+ efflux into 10 mM imidazole-ASW (pH 7.3) to external application of 2 mM Zn. Also shown is that subsequent application of 10^{-4} M ouabain reduces the remaining Na^+ efflux. This semilog plot represents a composite of five experiments. The fibers used were isolated from the same barnacle specimen. (B) The monophasic inhibitory response of the resting Na^+ efflux into 10 mM Hepes-ASW (pH 7.3) to external application of 2 mM Zn. Also shown is the biphasic effect obtained following external application of 10^{-4} M ouabain. This semilog plot represents a composite of five experiments. The fibers used were isolated from the same barnacle specimen.

fiber suspended in ASW containing 10 mM imidazole ($n = 5$). But in the case of the fiber suspended in ASW containing 10 mM Hepes, a rise in Na^+ efflux occurred only after the onset of the full inhibitory effect of ouabain ($n = 5$).

Application of Zn^{2+} after ouabain

To verify the idea that Zn^{2+} reduces the ouabain-insensitive component of the Na^+ efflux, 2 mM Zn was applied after the development of the full inhibitory effect of 10^{-4} M ouabain. Illustrated in Fig. 6 is that the ouabain-insensitive component of the Na^+ efflux was appreciably reduced by Zn^{2+}.

Such results prompted determination of a concentration-response relation for the inhibitory effect of Zn^{2+} on the ouabain-insensitive Na^+ efflux. As summarized in Fig. 7, the fall in the ouabain-insensitive component of the Na^+ efflux is concentration-dependent. It seems quite likely that the threshold concentration lies in the region of 10 μM and that the IC_{50} value falls in the region of 1 mM.

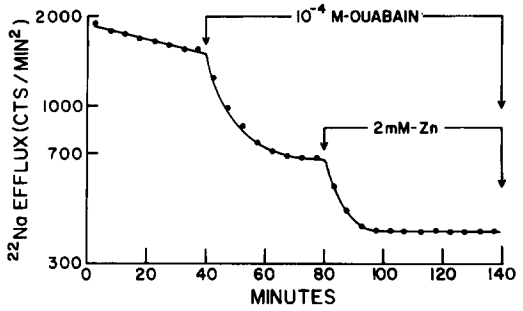

Fig. 6. The inhibitory effect on the ouabain-insensitive component of the Na^+ efflux caused by external application of 2 mM Zn.

Injection of EGTA before Zn^{2+} application

To rule in or out the possibility that the observed effect of Zn^{2+} on the resting Na^+ efflux is partially the result of the entry of Zn^{2+} into the myoplasm, EGTA, a chelator of Zn^{2+} with a stability constant (log K_s) of 12.6 [8,9] was injected in a concentration of 0.25 M into unpoisoned and ouabain-poisoned fibers approximately 1 h prior to loading them with radiosodium. Shown in Fig. 8A and B are two representative experiments: the fiber preinjected with EGTA (panel A) was less sensitive to 2 mM Zn than the control fiber (panel B) (i.e., $42 \pm 4\%$, $n = 4$ vs. $56 \pm 2\%$, $n = 4$, P being < 0.05. Also shown in Figs. 8C and D, is a similar situation where a fiber preinjected with EGTA and subsequently treated with 10^{-4} M ouabain was exposed to 2 mM Zn (panel C). A representative control is given in panel D (i.e., $8 \pm 3\%$, $n = 4$ vs. $33 \pm 2\%$, $n = 4$, P being $<$ 0.001).

Injection of Zn^{2+} before and after external application of ouabain, and injection of Zn^{2+} before and after external application of Zn^{2+} in unpoisoned and ouabain-poisoned fibers

As illustrated in Fig. 9A (upper panel), injection of a 0.1 M solution of Zn^{2+} led to a delayed fall in the Na^+

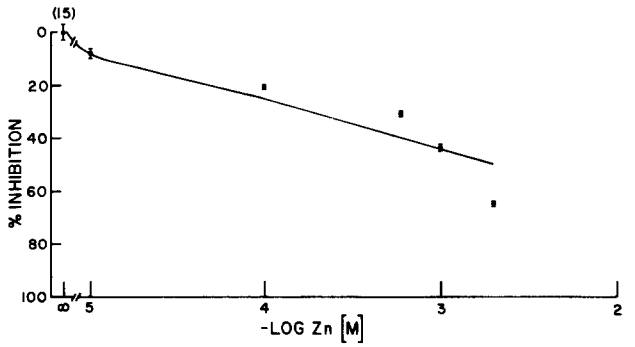

Fig. 7. Log concentration–response relation for the inhibitory action of Zn^{2+} on the remaining Na^+ efflux in fibers pre-treated with 10^{-4} M ouabain. Abscissa: $-\log$ scale. The curve was best fitted by eye. Each plotted test point is the mean value of three measurements. Vertical bars span $\pm$S.E. The fibers used were isolated from the same barnacle specimen.

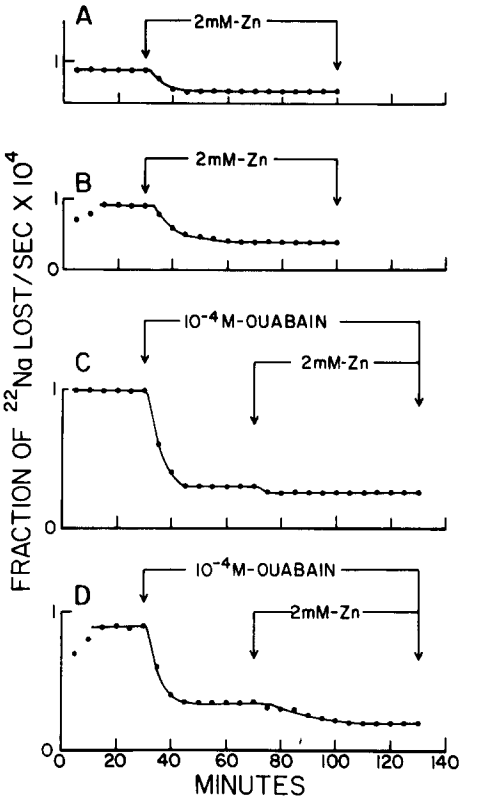

Fig. 8. (A) The reduced inhibitory effect on the resting Na^+ efflux of external application of 2 mM Zn in a fiber injected with 0.25 M EGTA 60 min prior to loading with radiosodium. (B) Companion control fiber. (C) The reduced inhibitory effect on the ouabain-insensitive Na^+ efflux of external application of 2 mM Zn in a fiber injected with 0.25 M EGTA 60 min prior to loading with radiosodium. (D) Companion control fiber.

efflux (averaging $32 \pm 3\%$), and that subsequent external application of 10^{-4} M ouabain reduced the remaining Na^+ efflux by $42 \pm 2\%$ ($n = 4$). That injection of Zn^{2+} is able to elicit a fall in the ouabain-insensitive component of the Na^+ efflux is illustrated in the lower panel, Fig. 9A (ouabain: $63 \pm 2\%$, and Zn^{2+}: $54 \pm 2\%$, $n = 4$). In both types of experiment, comparison of the second inhibitory phase was based on the magnitude of the fall after the new steady state had developed.

In the next group of experiments, an attempt was made to determine whether fibers preinjected with Zn^{2+} are sensitive to external application of Zn^{2+}, followed by ouabain and whether fibers preexposed to Zn^{2+} are sensitive to the injection of Zn^{2+} into them, as well as to subsequent external application of ouabain. The representative experiments presented in Fig. 9B show a fall in the resting Na^+ efflux following the injection of 0.1 M Zn (averaging $48 \pm 4\%$: upper panel) and that the remaining Na^+ efflux was reduced by external application of 2 mM Zn (averaging $47 \pm 5\%$, $n = 4$). However, ouabain application was ineffective. As illustrated in the lower panel of Fig. 9B, injection of 0.1 M Zn following a fall in the resting Na^+ efflux

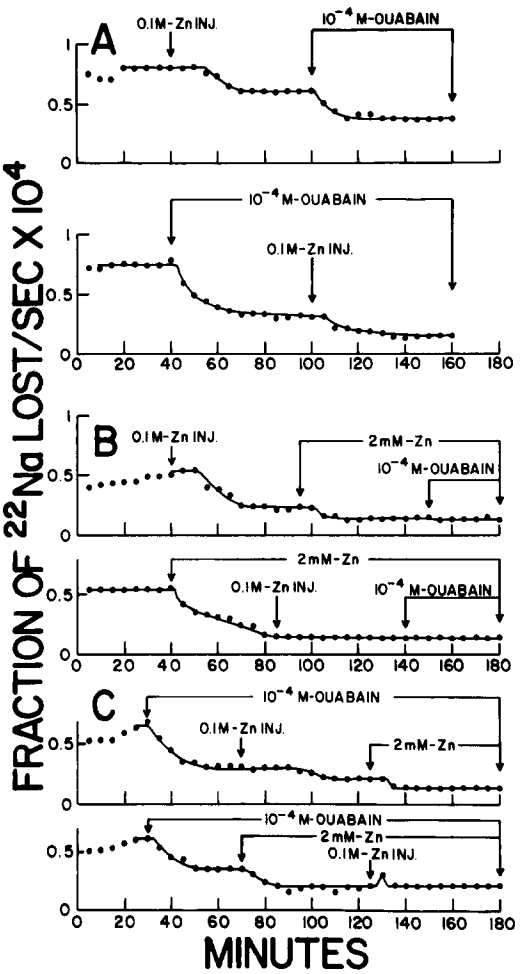

Fig. 9. (A) Upper panel: Delayed inhibitory effect on the resting Na^+ efflux of injection of 0.1 M Zn (pH 6.8) and the effect of external application of 10^{-4} M ouabain on the remaining efflux. Lower panel: Inhibition of the ouabain-insensitive Na^+ efflux by injecting 0.1 M Zn. (B) Upper panel: Delayed inhibitory effect on the resting Na^+ efflux of injection of 0.1 M Zn, followed by the effect of external application of 2 mM Zn and the lack of effect of externally applied ouabain (10^{-4} M). Lower panel: Lack of effect of injection of 0.1 M Zn and 10^{-4} M ouabain on the Na^+ efflux remaining after external application of 2 mM Zn. (C) Upper panel: Delayed inhibitory effects of injection of 0.1 M Zn and external application of 2 mM Zn on the ouabain-insensitive Na^+ efflux. Lower panel: Inhibition by external application of 2 mM Zn of the ouabain-insensitive Na^+ efflux and the ineffectiveness of injecting 0.1 M Zn on the remaining Na^+ efflux.

caused by external application of 2 mM Zn (averaging $61 \pm 5\%$, $n = 4$) was ineffective. This was also the case with ouabain application.

Fig. 9C shows two representative experiments where the fibers were pretreated with 10^{-4} M ouabain. The upper panel indicates that the injection of 0.1 M Zn caused a delayed fall in the ouabain-insensitive Na^+ efflux (averaging $23 \pm 6\%$, $n = 3$), whilst external application of 2 mM Zn afterwards also caused a fall (averaging $38 \pm 5\%$, $n = 3$). By contrast, injection of 0.1 M Zn after a fall in the ouabain-insensitive Na^+ efflux caused by external application of 2 mM Zn was ineffective – lower panel of Fig. 9C ($n = 4$). Thus, the inference that can be drawn from these experiments is that Zn^{2+} reduces the ouabain-insensitive Na^+ efflux not only by acting on the external side of the plasmalemma, but also by acting from the inside of these fibers following entry or injection. Leakage after injection could explain the observed inhibitory effect of Zn^{2+} (vidé infra).

Discussion

The results obtained provide evidence that Zn^{2+} acts as a potent inhibitor of the resting Na^+ efflux, and that the inhibitory phase involves both the ouabain-sensitive and ouabain-insensitive components of the Na^+ efflux. Although no inferences can be drawn about the chemical nature of the specific membrane sites of Zn^{2+} interaction, a likely possibility is that they involve SH groups of the membrane Na^+/K^+-ATPase that are essential for its activity [10] and are accessible to Zn^{2+}. Direct evidence linking the effect of Zn^{2+} with the membrane Na^+/K^+-ATPase in these fibers is not yet available, but as will be recalled, prior application of ouabain is found to drastically reduce the magnitude of the fall in the Na^+ efflux caused by Zn^{2+} application. This line of reasoning is in accord with indications that Zn^{2+} is an inhibitor of the transport enzyme; for example, cell-free preparations of Na^+/K^+-ATPase from rat and rabbit brain are inhibited by 10 μM Zn by about 50% [11]. This is also the case with rat kidney preparations [12]. The question as to whether Zn^{2+} interaction with membrane phospholipid reduces the activity of the transport enzyme is of course, important, but unanswerable.

The fact that the response to Zn^{2+} is monophasic and larger than that seen with ouabain emphasizes the conclusion that Zn^{2+} is able to inhibit the ouabain-insensitive component of the Na^+ efflux. This is also based on the observation that Zn^{2+} drastically reduces the remaining Na^+ efflux in fibers pretreated with ouabain. The requirement for a high concentration is not surprising, particularly if Zn^{2+} additionally acts as the result of entry into the fibers. Though evidence of Zn^{2+} uptake in barnacle fibers is unavailable, other cells, e.g., hepatocytes [13,14] possess an uptake system. An indication that Zn^{2+} is present in these fibers is provided by atomic absorption spectrometric measurements showing a value of 0.73 ± 0.03 mmol per kg fiber water, $n = 6$ (Chambers, G. and Bittar, E.E., unpublished data). Clearly, in order to understand why Zn^{2+} in the millimolar range is required, one needs to know something about internal thiols, notably metallothionein (MT) in these fibers. MT is known to avidly bind Zn^{2+} (e.g., Ref. 15) and to be found in practically

all tissues including skeletal muscle [16]. Perhaps what is more important at this particular stage of the present study is the evidence that the injection of Zn^{2+} leads to an appreciable fall in the resting Na^+ efflux. However, to elicit a fairly significant fall in Na^+ efflux, Zn^{2+} needs to be injected in a high concentration. This is governed not only by the fact that dilution of the injected Zn^{2+} by the myoplasm does occur, but also by the degree of binding by putative MT (and other Zn^{2+}-binding proteins), as well as by some leakage. Leakage of a fraction of the injected Zn^{2+} into the narrow channels of the transverse tubular system is a possibility that cannot be ignored. If it does occur, then it could account in part or wholly for the inhibition seen. The presence of a lag phase following injection of Zn^{2+} favors this interpretation.

It remains to consider whether the finding that Zn^{2+} in a concentration as low as 10 μM causes a large fall in the resting Na^+ efflux is of some biological significance. This particular matter is of more than academic interest in view of ample evidence that Zn^{2+} in seawater (e.g., Ref. 17), as well as in human plasma (e.g., Ref. 17) is present in a total concentration of 10–15 μM. However, because the toxicity of trace metals, e.g., Zn [18,19] is directly related to the activity of free Zn^{2+}, and because reliable information concerning this point is not yet available, one can only speculate as to whether the activity of free Zn^{2+} in hemolymph and plasma falls in a range which allows the trace metal to act as a modulator of the resting Na^+ efflux. The significance then of the present work must remain an enigma until a clearer view of these problems has been gained, and evidence that Zn^{2+} application to vertebrate tissues reduces Na^+ efflux is forthcoming.

Acknowledgement

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