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Biochimica et Biophysica Acta 1195 (1994) 51–54

BBA Biochimica et Biophysica Acta

Zinc is an inhibitor of the stimulatory response of the sodium efflux to the microinjection of cyclic AMP and forskolin in single barnacle muscle fibers

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Received 28 December 1993; revised 21 June 1994

Abstract

The main aim of the experiments with which this paper deals was to test the hypothesis that Zn is an inhibitor of the stimulatory response of the ouabain-insensitive component of the Na efflux to the injection of cAMP, DcAMP (dibutyryl-cAMP) and FD (forskolin derivative) in barnacle muscle fibers. The results obtained were as follows: (1) External application of Zn caused a fall in the Na efflux in fibers poisoned with ouabain beforehand; (2) external application of Zn prior to the injection of cAMP, DcAMP and FD led to a marked reduction in the response of the Na efflux in fibers pre-treated with ouabain; (3) the response obtained by the injection of DcAMP and FD into ouabain-poisoned fibers pre-exposed to Zn was small but sustained; and (4) external application of Zn following peak stimulation by injecting DcAMP or FD led to reversal of this response. (Parallel experiments involving the injection of cAMP were not done, since the response following the onset of peak stimulation decays quite rapidly.) Taken together, these results support the hypothesis that Zn behaves as an inhibitor of the stimulatory response obtained by the injection of cAMP, DcAMP and FD.

Keywords: Sodium ion efflux; Zinc; Microinjection; cAMP; Forskolin; Barnacle fiber

1. Introduction

Recent work carried out in this laboratory has led to persuasive evidence that zinc (Zn) is a powerful inhibitor of the resting Na efflux in both unpoisoned and ouabain-poisoned fibers isolated from the barnacle *Balanus nubilus* [1]. Because the injection of cAMP caused a rise in the resting efflux (e.g., [2]) which was abolished entirely by preinjecting protein kinase inhibitor (e.g., [3]), and because the injection of forskolin, a known activator of membrane adenylate cyclase (e.g., [4]) caused a sustained stimulation in fibers poisoned with ouabain [5], it seemed worthwhile to verify the validity of the hypothesis that Zn is able to reduce or abolish the response obtained by injecting cAMP, dibutyryl cyclic AMP (DcAMP), and forskolin derivative (FD) into these fibers.

2. Materials and methods

The methods of dissection, cannulation, microinjection and counting of ^{22}Na activity in the effluent and in the fiber were essentially as described by Bittar [6]. The artificial seawater (ASW) used had the following composition (mM): NaCl 465; KCl 10; $MgCl_2$ 10; $CaCl_2$ 10, Hepes 10, and pH 7.3. The solutions of cAMP and dibutyryl cAMP for injection were prepared using 3 mM Hepes (pH 7.3). In the case of forskolin, the vehicle used was DMSO (50 mg forskolin per 1 ml DMSO, this being the maximally soluble stock solution). The volume of test solution injected into cannulated fibers was 0.3–0.4 μl. This is diluted by the myoplasm by a factor of approx. 100. All the experiments were done at a room temperature of 22°C to 24°C.

Abbreviations: cAMP, adenosine 3′:5′ cyclic monophosphate; DcAMP, dibutyryl adenosine 3′:5′ cyclic monophosphate; FD, forskolin derivative; DMSO, dimethylsulfoxide; Hepes, (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)).

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SSDI 0005-2736(94)00168-5

The results given in this paper are expressed as the mean ± S.E. of the mean, and significant levels were determined by using Student's t-test. Values for $P < 0.05$ were considered as being significant. Estimates of the magnitude of the observed effects on the course of the Na efflux were computed on the basis of the rate constant plots, i.e., fraction of ^{22}Na lost per second vs. time (min).

All reagents used were analytical grade. Ouabain, Hepes, cyclic AMP and DcAMP were obtained from Sigma, St. Louis, MO. Forskolin derivative (FD) which is 7β-deacetyl-7β-(*N*-methyl piperazine) was purchased from Calbiochem, San Diego, CA. ^{22}NaCl (0.2 mCi per ml-SKS.1) was supplied by Amersham-Searle, Arlington Heights, IL.

3. Results

Shown in the upper panel of Fig. 1 is that the injection of 10^{-2} M cAMP into a companion control fiber that was pretreated with 10^{-4} M ouabain resulted in a sharp but transitory rise in the remaining Na efflux, the magnitude of which averages 613 ± 44% ($n = 4$). Shown in the lower panel is that the injection of 10^{-2} M cAMP into a test companion fiber, pretreated with 10^{-4} M ouabain and followed by external application of 2 mM Zn caused a stimulatory response averaging 106 ± 9% ($n = 3$). The difference is significant. Notice that 2 mM Zn caused the expected fall of the ouabain-insensitive component of the Na efflux [1].

DcAMP is widely known to mimic the action of cAMP but to have a lower affinity for cAMP-protein kinase than cAMP (e.g., [7]). For these two reasons experiments were done where 10^{-2} M DcAMP was injected into fibers pretreated with 10^{-4} M ouabain. As can be seen in Fig. 2, injection of the analog elicited a prompt and sustained rise in the ouabain-insensitive Na efflux (averaging 259 ± 7% ($n = 4$), which was partially reversed by external application of 2 mM Zn (viz. 44 ± 3% reversal, $n = 4$). As illustrated in the lower panel, injection of 10^{-2} M DcAMP caused a prompt and sustained rise in the ouabain-insensitive Na efflux (averaging 104 ± 4%; $n = 4$).

The vehicle used for the injection of FD was DMSO. DMSO injection is known to be without effect on the Na efflux in ouabain-poisoned fibers (e.g., [8]). Illustrated in the upper panel of Fig. 3 is that the injection

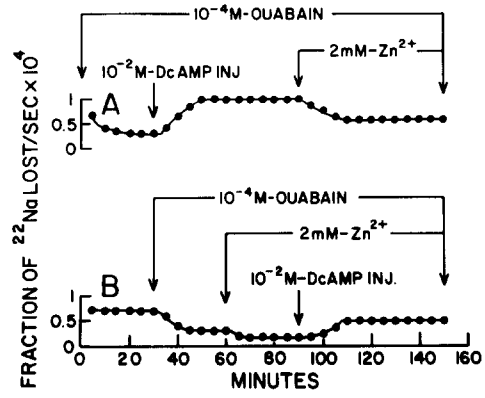

Fig. 2. (A) To illustrate the sustained stimulatory response of the ouabain-insensitive Na efflux to the injection of 10^{-2} M DcAMP and its reversal by the sudden addition to the bathing medium of 2 mM Zn. (B) To illustrate the reduced stimulatory response of the ouabain-insensitive Na efflux to the injection of 10^{-2} M DcAMP in the presence of 2 mM Zn in the bathing medium. Notice that the fall in the remaining Na efflux caused by Zn is prompt, while the onset of stimulation by DcAMP is somewhat slow (lower panel).

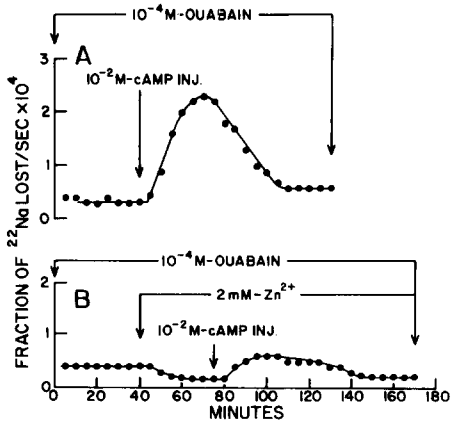

Fig. 1. (A) To illustrate the transitory stimulation of the ouabain-insensitive Na efflux as the result of the injection of 10^{-2} M cAMP. (B) To illustrate the fall of the ouabain-insensitive Na efflux following the addition of 2 mM Zn to the bathing medium, as well as the reduced stimulatory response obtained by the injection of 10^{-2} M cAMP.

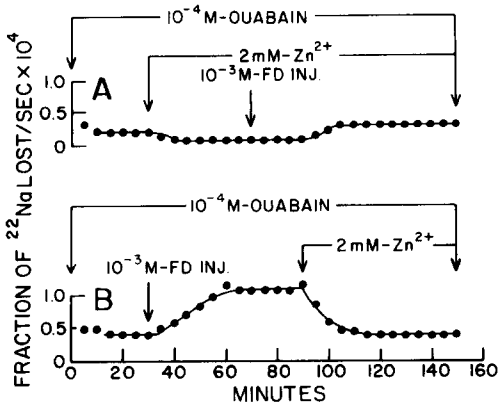

Fig. 3. (A) To illustrate the response of the ouabain-insensitive Na efflux to the sudden addition of 2 mM Zn to the bathing medium and that a lag period precedes the onset of the stimulatory response to the injection of 10^{-3} M FD, and that this response is sustained in nature. (B) To illustrate the stimulatory response to the injection of 10^{-3} M FD and its complete reversal by adding 2 mM Zn to the bathing medium.

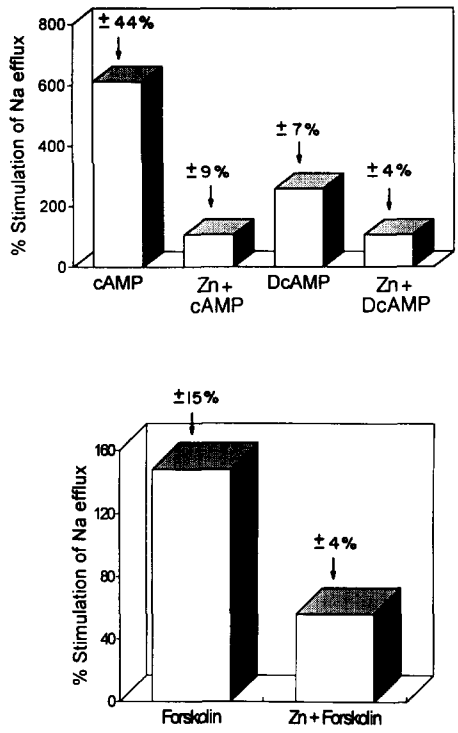

Fig. 4. The histogram shown in the upper panel indicates the extent of the reduction obtained by external application of Zn of the stimulatory response of the ouabain-insensitive Na efflux obtained by injecting cAMP and DcAMP (upper panel). Indicated in the lower panel is the size of the response obtained by injecting FD into fibers exposed to Zn beforehand.

of 10^{-3} M FD into a fiber preexposed to 2 mM Zn produced a delayed but sustained stimulation in the remaining Na efflux, the magnitude of which averages $56 \pm 4\%$ ($n = 6$). In sharp contrast, as shown in the lower panel, injection of 10^{-3} M FD prior to external application of 2 mM Zn caused a sustained stimulatory response averaging $148 \pm 15\%$ ($n = 6$). This value is significantly greater than the value of $56 \pm 4\%$. Notice that external application of 2 mM Zn completely reversed the response to FD ($n = 6$).

In summary, then, the histograms presented in Fig. 4 indicate quite dramatically that Zn is a potent inhibitor of the responses obtained by injecting cAMP and DcAMP (upper panel), and by injecting FD (lower panel).

4. Discussion

Several points arise when considering the results of these experiments. First, external application of 2 mM Zn resulted in a fall of the ouabain-insensitive component of the Na efflux. This finding substantiates the conclusion drawn from earlier work that Zn behaves as an inhibitor of the Na efflux in ouabain-poisoned fibers. Second, the stimulatory response obtained by the injection of 10^{-2} M cAMP, 10^{-2} M DcAMP, and 10^{-3} M FD into fibers poisoned with 10^{-4} M ouabain was drastically reduced by external applications of 2 mM Zn prior to the injection of these three effectors. Incomplete abolition is a surprising result which can be accounted for by assuming that Zn reaching the myoplasm was not enough to completely abolish the response obtained by injecting the three effectors. Alternatively, the preferred explanation is that a significant fraction of the Zn reaching the myoplasm was rapidly buffered by several proteins, most notably metallothionein (MT), whose stability constant (log K_s) for Zn is approx. 12 (e.g., [9]) Whether MT occurs in barnacle fibers in not yet known but its distribution in the animal kingdom is ubiquitous (e.g., [9,10]). For example, it is also present in human skeletal muscle [11]. Third, the Zn reaching the myoplasm may act by inhibiting the activity of the catalytic subunit of cAMP-protein kinase. However, to obtain such an effect may require the presence of a very high concentration of Zn viz. 10 mM (e.g., [12]). Fourth, a striking feature of the results is that the response obtained by injecting cAMP was considerably greater than that obtained by injecting DcAMP in an equimolar concentration. Such a finding is not wholly unexpected in view of evidence that permeant analogs of cAMP such as DcAMP bind less avidly to the regulatory subunit of cAMP-protein kinase than cAMP (e.g., [13,14]). Binding is known to occur in the following order: cAMP > N^6-monobutyryl > N^6-2′O-dibutyryl cAMP (e.g., [13]). To what extent cAMP in a high concentration activates cGMP-protein kinase (e.g., [15]) and hence, stimulates Na efflux (e.g., [16]) is impossible to tell. And fifth, DcAMP (and FD) but not cAMP are able to bring about in the presence of 2 mM Zn in the bathing medium a sustained but small response. A plausible explanation is that DcAMP is a poor substrate for the isoforms of phosphodiesterase (PDE), whereas cAMP is rapidly degraded to 5′-AMP.

The evidence brought forward also indicates that external application of Zn prior to the injection of FD fails to completely abolish the response of the ouabain-insensitive Na efflux, whereas application of Zn following peak stimulation by injecting FD completely reverses the response (vidé Fig. 3B). The reason for this difference in sensitivity to FD arising from the treatment order is somewhat unclear. One possible explanation is that Zn gains greater access to its site(s) of action in fibers already undergoing a phosphorylation-dephosphorylation reaction triggered by newly formed cAMP than in fibers injected with FD after treatment with Zn. Keeping this in mind, and that FD is known to cause persistent activation of membrane adenylate cyclase, one would predict only partial reversal by Zn of the response obtained by injecting DcAMP. This is indeed found to be the case here.

Acknowledgment

This work was supported by NIH Grant 5142.

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