Biochimica et Biophysica Acta, 1108 (1992) 210–214

BBAMEM 75694

Citrate as an aluminum chelator and positive effector of the sodium efflux in single barnacle muscle fibers

E. Edward Bittar, Zhuzai Xiang and Yong-Ping Huang

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

(Received 4 February 1992)

Key words: Citrate; Aluminum; Sodium ion efflux; Barnacle fiber

The injection of citrate produces considerably greater stimulation of the Na efflux in ouabain-poisoned fibers (from the barnacle *balanus nubilus*) than in unpoisoned fibers. When injected in excess together with Al into unpoisoned fibers it is without effect. Its injection is also without effect on the decline in the Na efflux elicited by injecting Al beforehand. Citrate injection into ouabain-poisoned fibers following peak stimulation by injecting Al produces a further rise which is a function of the Al concentration. Al injection after peak stimulation of the ouabain-insensitive Na efflux by citrate is usually without significant effect. Injection of aspartate into poisoned fibers causes a small rise in the remaining Na efflux and fails to prevent the response to Al injection from occurring. Taken together, these observations are in keeping with the view that citrate is not only a powerful chelator of Al but also a powerful activator of reverse Na^+/Ca^{2+} exchange in ouabain-poisoned fibers, presumably because of its ability to raise myoplasmic pMg.

This paper is a continuation of earlier papers [1,2] concerning work on aluminum (Al) chelators. Its aim is to bring forward evidence which strongly suggests: (i) that citrate binds Al inside barnacle fibers; (ii) that citrate injection into fibers in which the Na efflux is appreciably reduced by Al injection (i.e., hypersensitive fibers) fails to reverse this untoward effect; and (iii) that aspartate is a very weak chelator in this model system. The choice of citrate ($pK_a = 3$, 4.4 and 5.8, see Ref. 3) was based on the conditional log K value for citrate and Al^{3+} at pH 7.4 which (as reported by Martin [3] and T.L. Macdonald and R.B. Martin (private communication) is 1.6 log units more than the log K_c for ATP and Al^{3+} at this pH. Thus, one would expect an organoligand such as citrate to promote the formation in barnacle myoplasm (pH of 7.2, see Refs. 4 and 5) of $Al(OH)(H_{-1}Cit)^{2-}$ (see also Ref. 6). In other words, citrate competes effectively with ATP for Al^{3+}, and hence allows ATPMg to remain in this form. Furthermore, as pointed out by Martin [3] and Macdonald and Martin [7], a much overlooked point of clinical significance is that citrate which occurs in blood plasma in a concentration of about 0.1 mM may well complex Al, resulting in the entry of the complex into cells.

Materials and Methods

The species of barnacles, the method of dissection, cannulation, microinjection and counting of ^{22} Na activity in the effluent and the fiber were essentially the same as those described by Bittar [8] and Huang and Bittar [1]. The artificial seawater (ASW) used had the following composition (mM): NaCl 465, KCl 10, $MgCl_2$ 10, $CaCl_2$, $NaHCO_3$ 10 and pH 7.8. The solutions of citrate and aspartate used for injection were prepared using 3 mM Hepes (pH 7.2). Solutions of $AlCl_3$ e.g. 0.065 M and 0.5 M were prepared using double-distilled, de-ionized water at a pH of 2.9 and 1.9, respectively. The pH of these solutions was lowered by adding HCl and adjusted as necessary by adding KOH. Details concerning speciation of Al at varying pH are to be found in the paper by Huang and Bittar [1].

The volume of test fluid, 3 mM Hepes buffer solution or water injected into a fiber was about 0.4 μl. This is diluted roughly 100 times by the myoplasm. All experiments were carried out at an environmental temperature of 22 to 24°C.

The data are expressed as the mean value ±S.E. Student's t-test was used to compare the data statistically. Values for $P < 0.05$ were considered as being

Correspondence to: E.E. Bittar, Department of Physiology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA.

Abbreviation: Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid.

significant. Estimates of the size of the observed effects on the ^{22}Na efflux were based on the rate constant plots (i.e., fraction of ^{22}Na lost per s vs. time).

All reagents used were analytical grade. Ouabain, Hepes (4(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and citric acid were obtained form Sigma Chemical, St. Louis, MO. $AlCl_3$ was supplied by Fisher Scientific Company, Fair Lawn, NJ.

Results

Injection of 0.5 M-citrate prior to 0.5 M $AlCl_3$

Previous studies showed that the injection of Al into unpoisoned fibers causes a biphasic effect, i.e., stimulation followed by inhibition of the Na efflux or a monophasic inhibitory effect [9]. As illustrated in Fig. 1, the injection of 0.5 M $AlCl_3$ 30 min after the injection of 0.5 M citrate exerts a triple effect viz. prompt but transitory inhibition (the magnitude of which is $17 \pm 10\%$, $n = 4$), followed 10 min later by a transitory rise (the magnitude of which averages $22 \pm 13\%$, $n = 4$) and then a sustained fall which is of the order of $31 \pm 5\%$, $n = 4$. Notice that prior injection of citrate is ineffective ($n = 4$).

Injection of 0.5 M citrate after 0.5 M Al

Injection of 0.5 M $AlCl_3$ into four fibers causes a fall in the resting Na efflux of the order of $26 \pm 6\%$. Injection of 0.5 M citrate 10 min later is without effect on the course of the efflux.

Injection of 65 mM $AlCl_3$ before and after 100 mM citrate, as well as injection of a mixture of the two

Shown in Fig. 2 are three representative experiments. As seen in panel A, injection of 65 mM $AlCl_3$ (pH 2.9) leads to a biphasic response of the resting Na efflux: stimulation is followed by inhibition ($2 \pm 3\%$, $n = 4$). This is shown in panel A of Fig. 2. Also to be seen is that the subsequent injection of 100 mM citrate (pH 7.2) causes a rise in the Na efflux which reaches a peak some 10–15 min later (the rise being of the order of $54 \pm 68\%$, $n = 4$). Panel B shows that the injection of 100 mM citrate (pH 7.2) results in a rather small rise

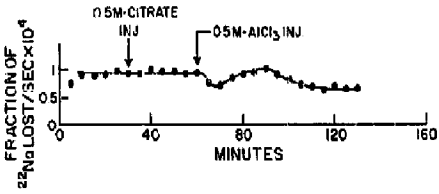

Fig. 1. The triphasic response of the resting Na efflux to the injection of 0.5 M $AlCl_3$ following the injection of 0.5 M citrate (rate constant plots).

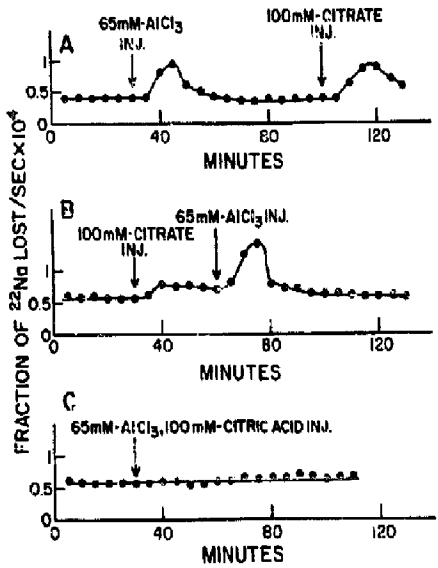

Fig. 2. (Panel A) Biphasic action following the injection of 65 mM $AlCl_3$ into an unpoisoned fiber. Injection of 100 mM citrate (pH 7.2) at $t = 100$ min causes a transitory rise in the efflux. (Panel B) Injection of 65 mM $AlCl_3$ shortly after 100 mM citrate elicits a biphasic response viz. stimulation followed by slow and persistent inhibition. (Panel C) The lack of effect of injection of a mixture of 65 mM $AlCl_3$ and 100 mM citric acid (pH 2.9) on the resting Na efflux.

in the resting Na efflux (of the order of $28 \pm 25\%$, $n = 4$) which declines slowly. Injection of 65 mM $AlCl_3$ 30 min later elicits a biphasic response: stimulation (of the order of $109 \pm 109\%$, $n = 4$) followed by inhibition at $t = 130$ min still persists and is small in size based on extrapolating the last few points back to the time of injecting Al ($n = 4$). These experiments were repeated. The results obtained show no effect with citrate injection in three fibers and a 30% rise in the fourth, whilst Al elicits stimulation in two fibers viz. 43% and 28% but none in the remaining two. Inhibition, however, is of the order of $27 \pm 20\%$ ($n = 4$). Panel C shows that the injection of a mixture of 65 mM $AlCl_3$ and 100 mM citric acid (pH 2.9) is completely ineffective ($n = 4$). This is also the case upon repeating the experiments ($n = 4$).

Injection of 0.5 M citrate before and after 0.5 M $AlCl_3$ into ouabain-poisoned fibers

(i) Injection of a 0.5 M citrate solution (pH 7.2) into fibers pretreated with 10^{-4} M ouabain causes a sharp rise in the ouabain-insensitive Na efflux, as illustrated by Fig. 3, which is a composite of four semilog efflux plots. The magnitude of the response averages $455 \pm 38\%$. Notice that the injection of 0.5 M $AlCl_3$ (pH 1.9)

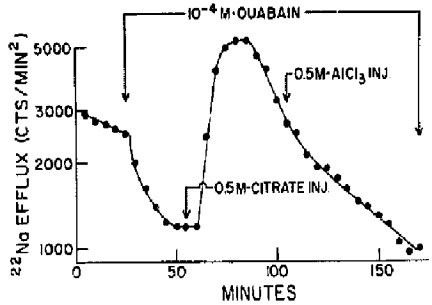

Fig. 3. To illustrate the sharp but transitory rise in the ouabain-insensitive Na efflux following the injection of 0.5 M citrate (pH 7.2), as well as the failure of the injection of 0.5 M $AlCl_3$ after the onset of peak stimulation to reverse this response (composite of four semilog plots).

following peak stimulation fails to cause a step-down but does somewhat reduce the rate at which the ^{22}Na efflux is falling. Inspection of the rate constant plots confirms that Al slows down the decay of the response to injected citrate. For example, the rate constant for ^{22}Na loss at $t = 175$ min is still appreciably greater than its value at $t = 55$ min ($n = 4$). This type of experiment was repeated. The results obtained were essentially the same ($n = 5$). But on repeating it again it is found that injection of 0.5 M $AlCl_3$ following peak stimulation by citrate ($458 \pm 147\%$ stimulation, $n = 5$) causes a $77 \pm 16\%$ reversal in four of the five test fibers, as based on the analysis of the rate constant plots.

(ii) If, however, 0.5 M citrate is injected 30 min after the injection of 0.5 M $AlCl_3$, two distinct modes of behavior are observed. These are illustrated in Figs. 4A and B where it will be seen that in panel A citrate elicits a monophasic stimulatory response, the magnitude of which is $118 \pm 59\%$ ($n = 4$). This value is significantly less than $455 \pm 38\%$ ($n = 4$) – vidé supra – P being < 0.01. The stimulation obtained by injecting 0.5 M $AlCl_3$ is of the order of $116 \pm 12\%$ ($n = 4$), as compared with $127 \pm 19\%$ ($n = 4$) which is obtained by injecting it after a 3 mM Hepes solution. The second mode of response to the injection of 0.5 M citrate following 0.5 M $AlCl_3$ injection is biphasic, as illustrated in panel B. Complete decay sets in 15 min after the onset of peak stimulation.

In order to be more certain that citrate is able to inhibit the efflux, experiments were designed in which a 1 M solution of $AlCl_3$ (pH 1.5–1.9) was injected prior to 0.5 M citrate. Shown in Fig. 5 is that citrate does in fact cause a small reduction in the stimulatory response to Al ($n = 4$). The size of this is hard to ascertain, since Al itself does not produce sustained stimulation.

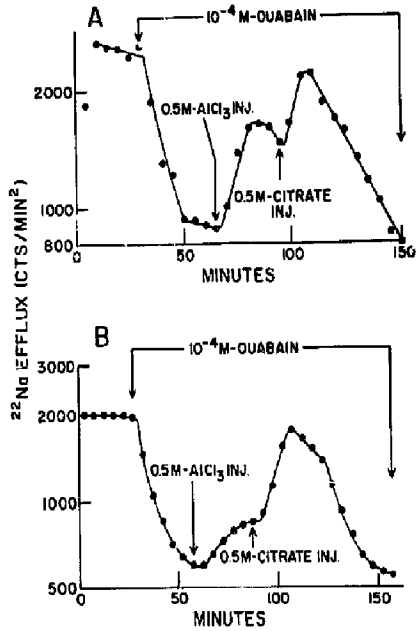

Fig. 4. (A) Stimulation caused by injecting 0.5 M citrate following the onset of peak stimulation by injecting 0.5 M $AlCl_3$. (B) The bimodal responses to the injection of 0.5 M citrate after peak stimulation by injecting 0.5 M $AlCl_3$. Notice that the step-down indicates complete reversal of the initial response to citrate.

Dose-response for Al effect using citrate in a fixed concentration

If the response to citrate injection after $AlCl_3$ into poisoned fibers is both prompt in onset and stimulatory in nature, it then seemed reasonable to raise the question whether the magnitude of this response is a function of the concentration of Al pre-injected. Summarized in Fig. 6 are the results obtained by injecting 0.5 M citrate 60 min after injecting Al in varying concentration. Close to 50% reduction in the magnitude of the response to citrate is obtained in fibers preinjected

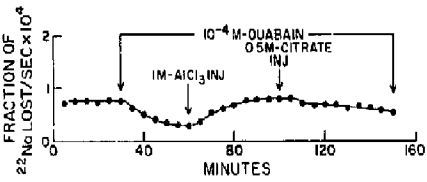

Fig. 5. A slight decline after injecting 0.5 M citrate into a poisoned fiber pre-injected with 1 M $AlCl_3$. Notice the absence of a prompt rise after citrate injection.

with 0.5 M $AlCl_3$. Control fibers injected with 3 mM Hepes after 1 M $AlCl_3$ show no effect ($n = 3$).

Next, attempts were made to do the opposite, namely inject 0.5 M $AlCl_3$ after citrate in varying concentration (0.1 to 1 M) but the plots failed to yield any meaningful relationship. However, some of the experiments, e.g. injection of 0.5 M $AlCl_3$ after 0.5 M citrate, show $53 \pm 26\%$ stimulation ($n = 4$), as compared to $394 \pm 104\%$ stimulation obtained by injecting 0.5 M $AlCl_3$ into control fibers ($n = 4$). The difference is significant.

Injection of 0.5 M $AlCl_3$ before and after 0.5 M aspartate

Inspection of both plots given in Fig. 7 which are composites of four semilog efflux plots indicates that the injection of 0.5 M aspartate after peak stimulation by injecting 0.5 M $AlCl_3$ elicits a prompt but small stimulatory response and that complete decay is not seen at $t = 150$ min. Panel B substantiates the view that the injection of 0.5 M aspartate leads to a small, transitory rise in the ouabain-insensitive Na efflux and that the response to 0.5 M $AlCl_3$ obtained by injecting it after aspartate does not readily decay as judged by its continued presence at $t = 150$ min. Thus, unlike citrate, aspartate fails to exert a large effect upon injection into poisoned fibers. Such a result is consistent with evidence that it has pK_a values of 3.9 and 10 [10]. In other words, one would not expect it to be an effective chelator of internal free Mg^{2+}.

Discussion

On the whole the present studies have brought to light several points. The first is that the injection of citrate into unpoisoned fibers is without effect on the resting Na efflux. This bears a striking resemblance to what often happens when ATPMg rather than $ATPNa_2$ is injected into such fibers [11]. The second is that like ATP, citrate fails to stop Al from reducing the resting Na efflux in hypersensitive fibers. Since citrate is known to bind Ca^{2+} and Mg^{2+} equally well [12], and since barnacle fibers possess considerably more free Mg^{2+} than free Ca^{2+} in the myoplasm (e.g., 5 mM Mg (e.g. Refs. 13 and 14) and 100 nM Ca^{2+} (e.g., Ref. 15)), the lack of a stimulatory response to the injection of 0.5 M citrate is puzzling, in particular because the injection of $ATPNa_2$ causes a transitory rise [11]. If, however, one accepts the view that the concentration of citrate used is too high, then the question arising is whether the addition of 100 mM citrate to the myoplasm elicits a response. As shown in Fig. 2, 100 mM citrate is able to increase the resting Na efflux. Such an effect can be explained by assuming that citrate reduces the internal free Mg^{2+} concentration. Thus, the observed response may reflect deinhibition of the Na-Ca exchanger.

The important piece of evidence in support of the view that citrate is a powerful chelator of Al is that the

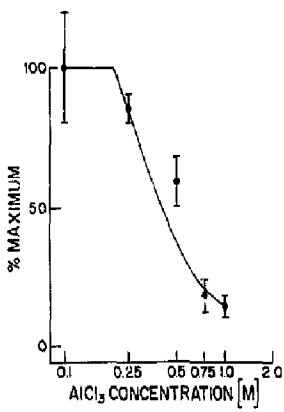

Fig. 6. The response of the ouabain-insensitive Na efflux to the injection of a 'fixed' concentration of citrate (0.5 M) 60 min after injecting $AlCl_3$ in varying concentration. Each point represents the mean value of three measurements. Vertical bars span ± S.E. The fibers used were isolated from the same barnacle specimen.

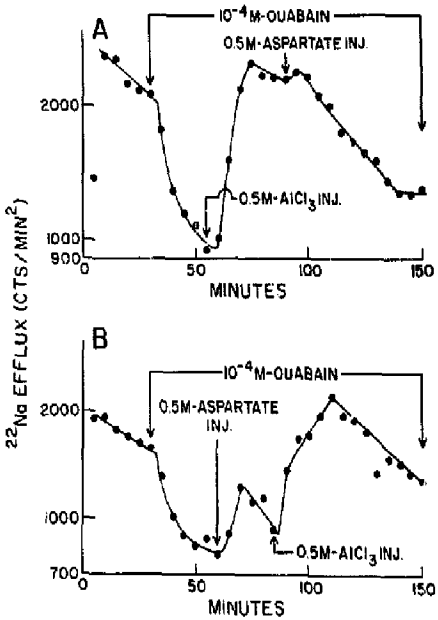

Fig. 7. (Panel A) Injection of 0.5 M aspartate (pH 7.2) following peak stimulation by injecting 0.5 M $AlCl_3$ causes slight stimulation of the ouabain-insensitive Na efflux. Notice that in these fibers, as in previous ones (e.g., Fig. 6) the response to Al fails to completely decay. (Panel B) A small transitory rise in the ouabain-insensitive Na efflux obtained by injecting 0.5 M asparate. Notice, again, that the response to $AlCl_3$ injection is prompt and large, and that at $t = 150$ min it has not yet decayed.

injection of a mixture of 65 mM Al and 100 mM citric acid fails to alter the course of the resting Na efflux in unpoisoned fibers, whereas the injection of 100 mM Al separately leads to a decline in the efflux. In this respect a lack of effect is somewhat similar to that obtained by injecting a mixture of $AlCl_3$-$ATPNa_2$ in that it causes less inhibition than $AlCl_3$ injection. It thus seems likely that citrate is a more potent chelator of Al than $ATPNa_2$. Similar experiments using ouabain-poisoned fibers were not done mainly because the criterion of inhibition by Al of basal Na efflux is considered to be a far more reliable one than stimulation by Al of the ouabain-insensitive Na efflux.

The experiments carried out with ouabain-poisoned fibers support the hypothesis that citrate activates the Na-Ca exchanger in the reverse mode by raising myoplasmic pMg. The observed response parallels that seen when $ATPNa_2$ is injected into poisoned fibers (see Fig. 3A). Moreover, the situation is not different when Al is injected after peak stimulation. When citrate or ATP is injected beforehand, Al fails to produce the expected step-up in the Na efflux. The explanation for this is that citrate rapidly chelates Al. If Ohman's data concerning speciation are correct, then one can suggest that at pH 3 shortly after injection of the $AlCl_3$ solution (pH 1.9) the following species exist: Al^{3+} (40%), $AlCit^0$ (40%), $Al(H_{-1}Cit)$ (5%) and $Al(HCit)^-$ (5%). And subsequently at pH 7, more than 90% of the Al occurs in the form of $Al(OH)(H_{-1}Cit)^{2-}$ and the remainder as $Al(H_{-1}Cit)^-$. It is a striking fact, however, that the injection of citrate following peak stimulation by Al leads to a marked rise in the ouabain-insensitive Na efflux, rather than an immediate step-down or reversal of the Al effect. This raises the possibility that the over-riding action of citrate is the activation of the Na-Ca exchanger in the reverse mode as the result of chelating internal free Mg^{2+}. Earlier studies, as will be recalled, revealed that preinjection of EGTA fails to interrupt the rise in Na efflux elicited by $ATPNa_2$ injection [11]. Use of EDTA was avoided in view of evidence that it causes a rise in the Na efflux which to a rather large extent involves activation of the Ca^{2+} channel [16]. The reason for a biphasic response such as that observed in Fig. 4B is thought to be related to excessive removal of internal free Mg^{2+} or to Al^{3+} action in view of the finding that the doubling of the concentration of Al used is associated with a slight decline in the Na efflux upon the injection of 0.5 M citrate. As a general conclusion, therefore, it may be suggested that reverse Na/Ca exhange is stimulated by the injection of citrate when the Na gradient in these fibers is reduced, e.g., by ouabain, because it is able to raise myoplasmic pMg.

Acknowledgement

This work was supported by NIH grant 5142.

References

1 Huang, Y.-P. and Bittar, E.E. (1991) Biochim. Biophys. Acta 1062, 255–263.
2 Hwang, Y.-P. and Bittar, E.E. (1992) Biochim. Biophys. Acta 1103, 77–84.
3 Martin, R.B. (1986) J. Inorg. Biochemistry 28, 181–187.
4 Bittar, E.E., Danielson, B.G., Lin, W. and Richards, J. (1977) J. Membr. Biol. 34, 223–246.
5 Boron, W. (1977) in Ph.D. Thesis, p. 115, Washington University, St. Louis, MO.
6 Ohman, L.-O. (1988) Inorg. Chem. 27, 2565–2570.
7 Macdonald, T.L. and Martin, R.B. (1988) Trends Biochem. Sci. 3, 15–19.
8 Bittar, E.E. (1983) Progr. Neurobiol. 20, 1–54.
9 Bittar, E.E., Nwoga, J. and Huang, Y.-P. (1990) Toxic Appl. Pharmacol 102, 174–185.
10 Pospichal, J., Gebauer, P. and Bocek, P. (1989) Chem. Rev. 89, 419–430.
11 Bittar, E.E. and Huang, Y.-P. (1991) Biochim. Biophys. Acta 1070, 332–342.
12 Hastings, A.B., McLean, F.C., Eichelberger, L., Hall, J.L. and Da Costa, E. (1934) J. Biol. Chem. 107, 351–370.
13 Ashley, C.C. and Ellory, J.C. (1972) J. Physiol. 226, 653–674.
14 Scarpa, A. and Brinley, F., Jr. (1981) Fed. Proc. 40, 2646–2652.
15 Ashley, C.C. (1970) J. Physiol. 210, 133–134P.
16 Bittar, E.E. and Nwoga, J. (1982) J. Physiol. 322, 389–397