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J Clin Invest. 1980;66(2):254-259. https://doi.org/10.1172/JCI109851.

Research Article

Dog erythrocytes (RBC) have a system for passive Ca and Na movements that resembles the Ca-Na exchanger first described in cardiac muscle. Amrinone, a new cardiotonic drug active in humans with congestive heart failure, is shown to stimulate net Ca uptake by dog RBC. Amrinone's action is on Ca influx rather than efflux. The influence of Amrinone on Ca uptake is enhanced when the cells are placed in low Na media; raising external Na or lowering intracellular Na both abolish the effect of the drug. The data suggest that amrinone potentiates passive Ca entry into the cells by a Na-dependent pathway. If Ca moves through myocardial sarcolemma as it does through dog RBC membranes, then the inotropic action of amrinone can be explained on the basis that the drug increases intracellular Ca levels.

Effects of Amrinone, a Cardiotonic Drug, on Calcium Movements in Dog Erythrocytes

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ABSTRACT Dog erythrocytes (RBC) have a system for passive Ca and Na movements that resembles the Ca-Na exchanger first described in cardiac muscle. Amrinone, a new cardiotonic drug active in humans with congestive heart failure, is shown to stimulate net Ca uptake by dog RBC. Amrinone's action is on Ca influx rather than efflux. The influence of Amrinone on Ca uptake is enhanced when the cells are placed in low Na media; raising external Na or lowering intracellular Na both abolish the effect of the drug. The data suggest that amrinone potentiates passive Ca entry into the cells by a Na-dependent pathway. If Ca moves through myocardial sarcolemma as it does through dog RBC membranes, then the inotropic action of amrinone can be explained on the basis that the drug increases intracellular Ca levels.

INTRODUCTION

In searching for useful agents for the treatment of congestive heart failure, Farah and Alousi (1) found that a bipyridine derivative, amrinone (5-amino-3,4′-bipyridine-6(1H)-one), had selective inotropic activity both in isolated cat myocardium and in intact dogs. The drug was found to be effective in humans with severe cardiac decompensation (2). Unlike cardiac glycosides, amrinone had no effect on Na,K-ATPase and did not appear to influence catecholamine or cyclic nucleotide metabolism (1, 3, 4).

It occurred to us that amrinone might exert its inotropic effect by altering cell Ca levels (5). Dog erythrocytes (RBC)[1] have a system for passive Ca transport that is dependent on both intracellular and extracellular Na concentrations and, thus, resembles the Ca-Na exchanger of myocardium (6). We thought it would be interesting to test the effect of amrinone on Ca movements in dog RBC. The action of digitalis as a Na-K pump inhibitor was first discovered in human RBC (7). We felt dog RBC might be particularly discriminative tissue in which to study amrinone's action because these cells lack the nearly ubiquitous Na-K pump and are unresponsive to digitalis (6).

METHODS

Amrinone (lots R-011-LE and R-011-SJ) was the generous gift of Drs. A. E. Farah and A. E. Soria of the Sterling-Winthrop Research Institute, Rensselaer, N. Y. The drug was soluble with some difficulty in all media used; no vehicle was required. Its calculated molecular weight was 184.

Blood was drawn into heparinized syringes from healthy mongrel dogs on the day of each experiment. The plasma and buffy coat were removed and the cells washed by centrifugation three times in 10 vol of a solution containing (mM): NaCl 120, Hepes 5, pH 7.4 at 37°C. If Na-free media were to be used, the wash medium contained LiCl in place of NaCl. Detailed procedures for each experiment are given in the figure legends. Most experiments were done in "NaCl buffer" or "LiCl buffer" which contained (mM): NaCl or LiCl 100, Hepes 10, Tris 10, glucose 5, free EDTA 0.1, with additions of $CaCl_2$ and amrinone as noted in the legends. The pH of all media was adjusted to 7.4 at 37°C with tris-hydroxide. All incubations were at 37°C in a thermostatted shaker bath. The volume ratio of cells to media in all suspensions was from 1:10 to 1:20. Hemolysis was <2%.

Procedures for determining cell Ca by flameless atomic absorption spectrophotometry (8, 9), cell Na and K by flame photometry (8), and cell water content by drying to constant weight (8) are detailed in previous publications from this laboratory. Alteration of cell Na and K content by incubation with extracellular ATP is described in the legend to Fig. 6 and in more detail in previous reports (10, 11).

There was some variation in the absolute amount of Ca accumulation from dog to dog and from day to day. For this reason, the data are presented either as duplicate or representative studies. In many cases (e.g., Figs. 1, 9, and 10; 2 and 5; 3 and 6), the same effects are shown in different experimental contexts.

RESULTS

The actions of amrinone are influenced by the time of exposure of cells to the drug, the cell volume and

Presented in part at the Alfred Benzon Symposium No. 14, August 1979, Copenhagen, Denmark. 1979. *Clin. Res.* **27**: 770A. (Abstr.)

Received for publication 31 January 1980 and in revised form 28 March 1980.

[1] *Abbreviation used in this paper*: RBC, erythrocytes.

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* · *0021-9738/80/08/0254/06 $1.00*
Volume 66 August 1980 254–259

cation content, and the composition of the medium. Fig. 1 shows the basic observation.

Previous work has shown that dog RBC suspended in a hypotonic NaCl medium accumulate Ca during the early hours of incubation, whereas at later time points the cell Ca falls (8). Fig. 1 shows that with amrinone present, Ca accumulation is increased while the biphasic net flow of Ca between cells and medium is preserved. The concentration of amrinone used (0.54 mM or 100 μg/ml) was shown by Farah and Alousi (1) to increase strongly the contractile force of cat papillary muscle in vitro. The first time point at which amrinone has an effect on cell Ca in Fig. 1 is 3 h. When earlier samples are taken (Fig. 2, upper panels), it is clear that the effect of amrinone is delayed. Some reduction in the delay was seen in circumstances leading to more rapid Ca uptake, e.g., with all external Na replaced by Li (Fig. 2, lower left) or with increased medium Ca (Fig. 2, lower right). Pretreatment of cells with amrinone for as long as 4 h before addition of Ca to the medium did not hasten the onset of the drug's effect. The mechanism of the delay is discussed below.

Dose-response curves for amrinone are given in Fig. 3. A steady rise in cell Ca with increasing drug concentration is shown, consistent with data published for cat atria and papillary muscle, showing contractile force increasing with amrinone levels up to 5.4 mM (1,000 μg/ml) (3). The sensitivity of dog RBC to the drug may be underestimated by the assay of Fig. 3 because, as will be discussed, the cell Ca level is the resultant of at least two processes, passive Ca influx and active Ca efflux.

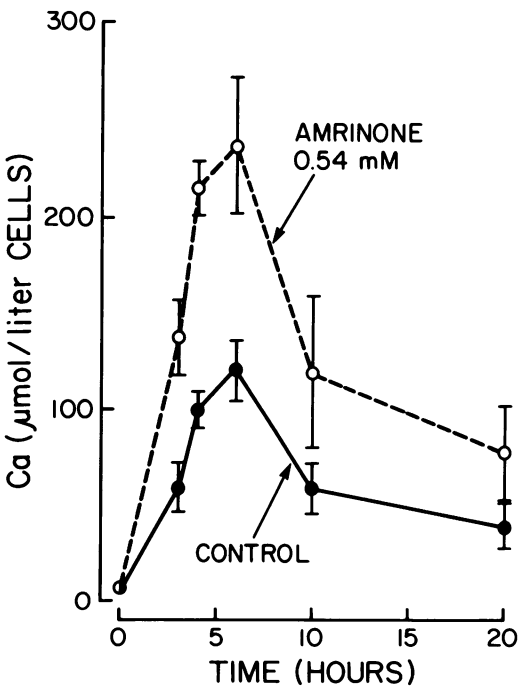

FIGURE 1 Cell Ca as a function of incubation time in the presence (○) and absence (●) of 0.54 mM amrinone. 100 mM NaCl buffer with 5 mM Ca. Mean±SD for four experiments.

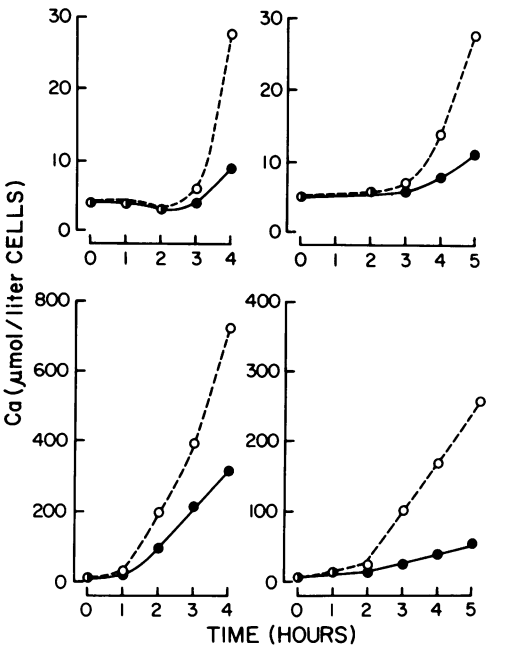

FIGURE 2 Cell Ca as a function of incubation time in the presence (○) and absence (●) of 1.3 mM amrinone. Upper two panels show duplicate studies on cells from separate dogs in 100 mM NaCl buffer with 2.0 mM Ca. Lower left panel shows a study with 100 mM LiCl buffer, 1 mM Ca. Lower right panel shows a study with 100 mM NaCl buffer, 5 mM Ca.

Figs. 4 and 5 show the influence of cell volume (as measured by cell water content) and medium Na concentration on the amrinone effect. Previous studies had shown that Ca entry into dog RBC is greatly retarded by cell shrinkage and a high external Na (12).

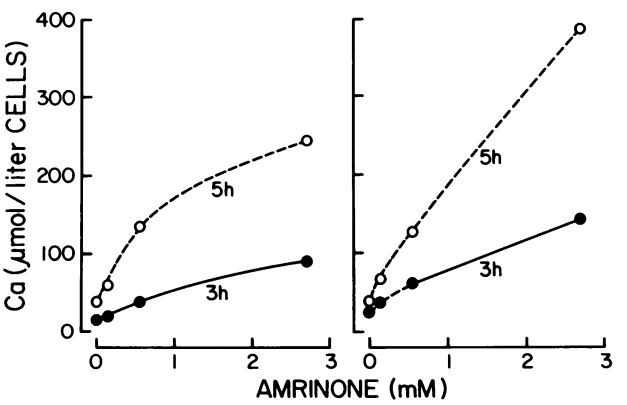

FIGURE 3 Cell Ca after 3 (●) and 5 (○) h incubation as a function of medium amrinone concentration. 100 mM NaCl buffer with 2.1 mM Ca. Duplicate studies are with blood from separate dogs.

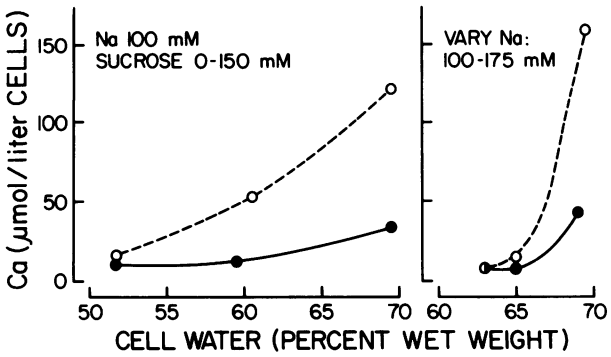

FIGURE 4 Cell Ca after 5 h incubation as a function of final cell water content in the presence (○) and absence (●) of 1.45 mM amrinone. NaCl buffer with 2.1 mM Ca and modifications to alter tonicity as follows: sucrose added at 0, 75, and 150 mM; NaCl added at 0, 37.5, and 75 mM. Each panel shows a single study representing two others.

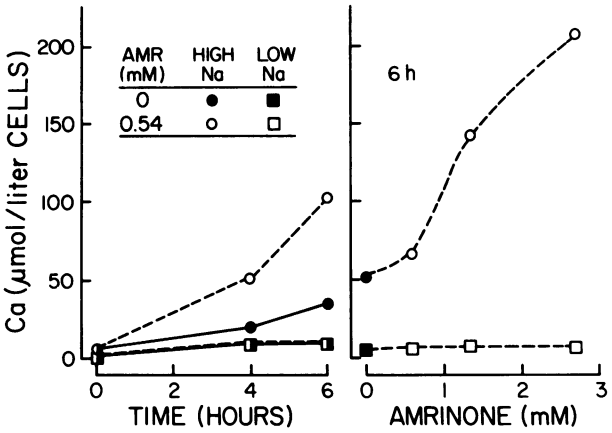

FIGURE 6 Ca content of high (circles) and low (squares) Na cells as a function of incubation time (left), and medium amrinone (AMR) concentration (right) in the presence (open symbols) and absence (closed symbols) of amrinone. High and low Na cells were prepared by preincubating cells 45 min at 37°C in media containing (mM): NaCl or KCl 140, ATP 1, Hepes 10, glucose 5, adjusted to pH 7.4 at 37°C with Tris-OH (see Methods). Cell ion and water contents after this incubation and a wash with 150 mM LiCl were (millimoles or milliliters per kilogram dry cell weight): for high Na cells Na 306, K 4.2, water 2,497, and for low Na cells Na 42, K 266, water 2,487. All cells were washed free of external ATP and placed in 140 mM NaCl buffer with 5 mM Ca and amrinone concentrations as indicated in the figure. Each panel shows a single study representing two others.

The stimulatory effect of amrinone on Ca accumulation is opposed by the same conditions. Na is a more potent amrinone antagonist than sucrose when used as an agent to raise medium tonicity (Fig. 4); at isovolume, replacement of Na by Li amplifies the effect of amrinone (Fig. 5). The study in Fig. 5 was done with an external Ca of 1 mM, a concentration at which little or no amrinone effect is seen in a high Na medium at 3 h (see Figs. 7 and 10). This Ca concentration was selected to emphasize how much the action of amrinone is augmented at lower external Na levels.

Previous work has shown that, whereas Na in the external medium inhibits Ca influx in dog RBC, Na in the cytoplasm stimulates Ca accumulation. When the normally high Na content of dog RBC is reduced by replacement with K or Li, Ca influx is inhibited (12). Fig. 6 shows that the stimulatory effect of amrinone on Ca accumulation in dog RBC is likewise dependent on a high cell Na. Low Na (high K) cells are sparingly permeable to Ca and unresponsive to amrinone. High Na cells subjected to the same preincubation conditions continue to show the amrinone effect.

Fig. 7 shows that the effect of amrinone is augmented when the external Ca concentration is increased.

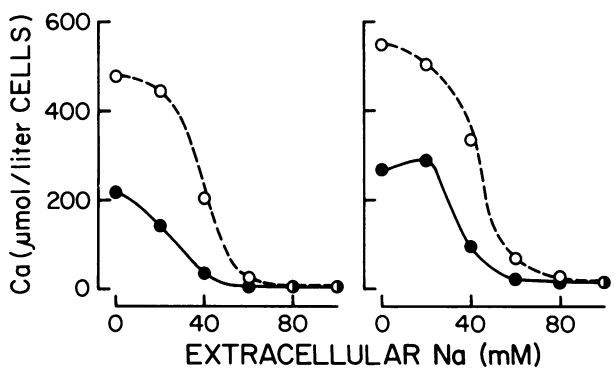

FIGURE 5 Cell Ca after 3 h incubation as a function of medium Na concentration in the presence (○) and absence (●) of 0.65 mM amrinone. 100 mM NaCl and 100 mM LiCl buffers mixed to give Na concentration indicated on abscissa. All incubation media contained 1 mM Ca. Duplicate studies are with blood from separate dogs.

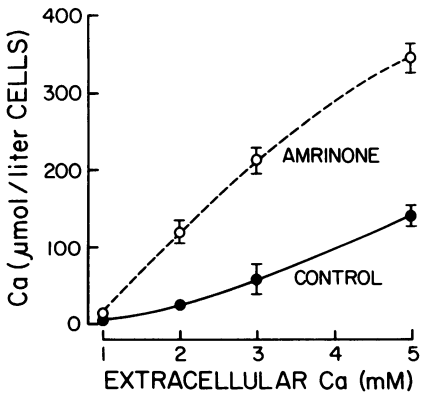

FIGURE 7 Cell Ca content as a function of medium Ca concentration after a 6-h incubation in the presence (○) and absence (●) of 0.54 mM amrinone. 100 mM NaCl buffer with Ca as noted on the figure. Mean and range for two studies from the same dog's blood.

Another interpretation of this figure would be that amrinone increases the affinity of dog RBC for Ca, but such a conclusion requires that amrinone be shown to affect entry rather than exit of Ca. All the effects of amrinone shown above could have come about through stimulation of Ca entry or by inhibition of Ca exit. To separate these two possibilities, Ca efflux studies were done according to a procedure in which dog RBC were preloaded with Ca by incubation at low temperature in a low Na solution (9). The Ca-loaded cells were then washed and incubated in Ca-free media with rapid and frequent sampling as reported previously (9). No effects of amrinone on net Ca efflux were seen in these experiments (data not shown). However, as noted in connection with Fig. 2, amrinone has a delayed effect on Ca accumulation. Therefore, it seemed necessary to do the efflux experiments in circumstances where the cells had been clearly influenced by the drug.

Fig. 8 shows duplicate experiments that indicate that (*a*) the effect of amrinone on cell Ca accumulation is reversible and (*b*) the drug does not inhibit Ca efflux into a Ca-free medium. Cells were preincubated with and without amrinone for 3 h to demonstrate that the drug had influenced cell Ca (zero time in Fig. 8). The cells that had been exposed to amrinone were then centrifuged and washed in the four solutions in which they were to be reincubated, i.e., in the presence and absence of both Ca and amrinone. The upper two curves in the graphs in Fig. 8 show that, upon reincubation in Ca-containing media, the effect of amrinone is reversible, inasmuch as the cells washed free of the drug maintained lower Ca values than the cells continuously exposed. The lower two curves, showing the fall of cell Ca in Ca-free media, indicate that amrinone has no retarding action on Ca efflux.

Finally, it seemed important to inquire whether amrinone might have an effect on active Na transport in dog RBC. These cells, lacking an ouabain-sensitive Na-K exchange pump, extrude Na—their most abundant cation—by a process requiring external Ca plus an energy source (13). This active Na extrusion constitutes the main mechanism by which the cells adjust from a swollen state to a normal volume (8, 14). The nature of the Ca requirement for Na pumping in dog RBC has never been clear. Fig. 9 shows the time course for Na and Ca movements in hypotonically swollen dog RBC incubated for 20 h in the presence of 2.0 mM Ca, plus and minus amrinone. As shown before (13), Na efflux under these conditions is against an electrochemical energy gradient. Although amrinone potentiates Ca accumulation during this incubation, the drug has no effect on net Na efflux. Fig. 10 summarizes similar 20 h incubations done at various external Ca concentrations. Cell Ca concentration as a function of time is shown in the upper panels; net uphill Na extrusion over 20 h as a function of medium Ca is shown in the lower panels. As previously demonstrated (13), Na extrusion is a

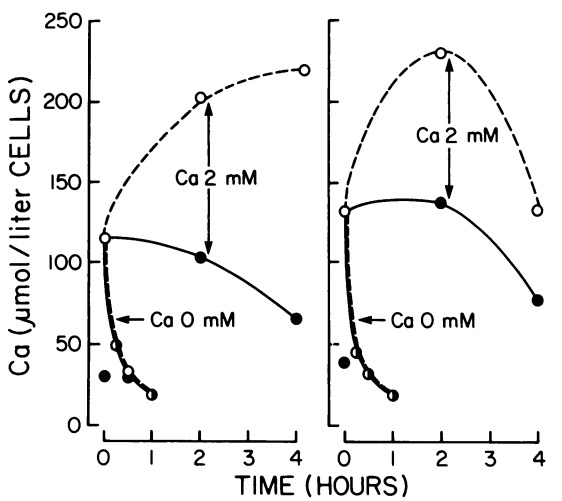

FIGURE 8 Cell Ca as a function of time under conditions designed to test (*a*) the reversibility of the amrinone effect and (*b*) the influence of amrinone on Ca efflux into a Ca-free medium. Cells preincubated 3 h in 100 mM NaCl buffer with 5.2 mM Ca in the presence (○) and absence (●) of 1 mM amrinone. Cell Ca at the end of this incubation is shown at zero time on the figures. Amrinone-preexposed cells were then divided into four lots and washed at 5°C in solutions in which they were to be incubated at 37°C: 100 mM NaCl buffer with 0 or 2 mM Ca, each in the presence and absence of 1 mM amrinone. Duplicate studies are with blood from separate dogs.

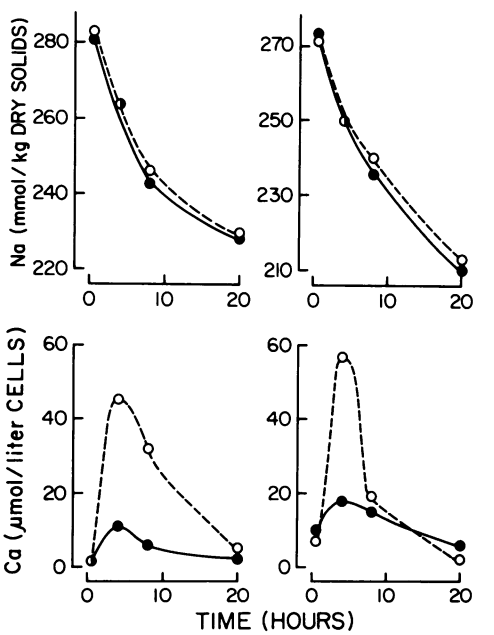

FIGURE 9 Effects of amrinone on cell Na (upper panels) and Ca (lower panels) content as a function of incubation time at 37°C. 100 mM NaCl buffer was used with 2.0 mM Ca plus (○) and minus (●) 0.54 mM amrinone. Duplicate studies are with blood from separate dogs.

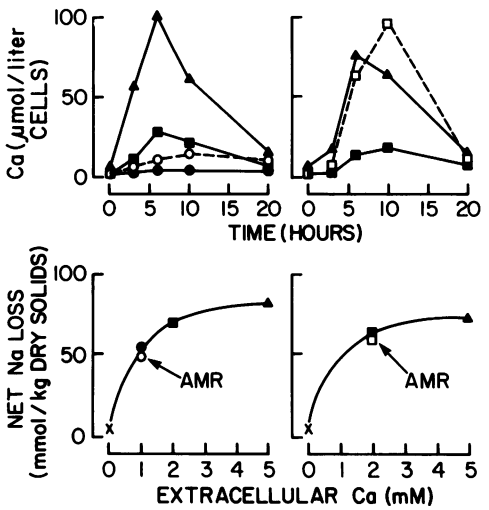

FIGURE 10 Effects of Ca and amrinone (AMR) on cell Ca content and net cell Na extrusion over 20 h at 37°C. 100 mM NaCl buffer was used with $CaCl_2$ additions of 0 mM (×), 1 mM (circles), 2 mM (squares), and 5 mM (▲). Open symbols indicate presence of 0.54 amrinone in addition to $CaCl_2$. Upper panels show cell Ca as a function of incubation time; lower panels show net Na extrusion over the 20-h period. Left hand panels show effects of amrinone added to 1 mM Ca; right hand panels show effects of amrinone added to 2 mM Ca.

saturable function of *external* Ca; but amrinone, although it causes an increase in *internal* Ca, does not stimulate Ca-dependent Na pumping.

DISCUSSION

A close relationship between Na and Ca movements in dog RBC was first suggested by Omachi et al. (15) who found that Ca moved from medium to cells when the Na in the external medium was replaced by K. The authors noted the similarity of their findings to observations made in frog hearts by Niedergerke (16). Subsequent work (12) has shown that passive Na and Ca movements in dog RBC follow many predictions for a model of Ca-Na exchange first described in cardiac (17) and neural (18) tissues. Ca entry into dog RBC is favored by a low external and a high internal Na. The Na-dependent Ca fluxes in dog RBC are shown to best advantage when the cells are swollen, perhaps because this transport pathway is involved in cell volume regulation (12).

All of the stimulatory effects of amrinone on Ca entry into dog RBC are augmented by conditions favoring passive Na-dependent Ca influx, and opposed by a reversal of those conditions. Thus, for amrinone to have a maximal effect, the cells must be swollen, there must be little Na in the medium, and the cytoplasmic Na must be at its normal high level. When the cells are caused to shrink, when the external Na is high, or when cell Na is low, the amrinone effect is diminished or abolished. Amrinone does not inhibit Ca efflux. All these observations suggest that amrinone stimulates Ca influx via a Na-dependent pathway that has the characteristics of Ca-Na exchange.

A possible mechanism for the action of the drug might be that it modifies the membrane so as to give Ca an advantage in its competition with Na for a position on the external face of the transporter that mediates Ca entry. Quantitation of the affinities of the inward Ca transport system is difficult in the present experimental system because the dependent variable—cell Ca concentration—is a function both of passive Ca influx and ATP-dependent Ca extrusion (9). The effects of amrinone on Ca influx per se could only be discerned if there were a way to inhibit selectively the Ca pump, but this has not been possible. This consideration affects the interpretation of the dose-response curve (Figs. 3 and 6); small increments in Ca influx might be compensated for by increases in the Ca pump rate. At some point, however, cell Ca concentration may exceed the level at which the Ca pump runs maximally and thus give rise to the accumulations described herein. Such an explanation would account for the apparent delay in onset of the amrinone effect as shown in Fig. 2 and for the shortening of the delay by maneuvers that raise the cell Ca more rapidly. With these reservations in mind, the data can be interpreted in a qualitative way as indicating that at constant external Na (Fig. 7), amrinone increases the affinity of the inward transport system for Ca, and at constant external Ca (Fig. 5) the effect of amrinone is such as to decrease Na inhibition of Ca entry.

The action of amrinone in raising cell Ca offers some insight into the mechanism of active Na transport in these cells. Dog RBC have an internal Na concentration of ~150 mmol/kg cell water—virtually the same as that of plasma; the membrane potential of −8 to −10 mV constitutes a driving force for Na entry (11, 13). Despite their lack of a K- or digitalis-sensitive Na efflux, and although they have no Na,K-ATPase, dog RBC can pump Na outward provided Ca is present in the medium (6). Net uphill Na extrusion is a saturable function of external Ca (13, Fig. 10). Just how Ca activates Na pumping in these cells is not clear, but one can divide the possible explanations into three categories: (*a*) Ca acts as a catalyst at some intracellular site to stimulate a Na pump; (*b*) the action of Ca is dependent on its crossing the membrane, e.g., in exchange for Na; (*c*) Ca activates a Na pump from the outside, again as a catalyst, without having to enter the cell. A priori, there is no reason to prefer any one of these mechanisms; the external Ca activation curve would be consistent with all three. The effects of amrinone (Figs. 9 and 10) would appear to weigh against the first possibility, (*a*), because the drug fails to stimulate Na pumping, whereas it raises cell Ca. Amrinone's action can be used as evidence against the

second mechanism, (*b*), as well. The attractive notion that Ca-dependent Na pumping involves Ca-Na exchange has been difficult to test. Because there is no specific inhibitor for the Ca pump, one cannot measure the postulated unidirectional counterflows of Ca and Na during active Na transport. The net movements of Ca in experiments, such as shown in Fig. 9, are three orders of magnitude less than those of Na. To the extent that amrinone activates Na-dependent Ca influx, the drug's failure to influence Ca-dependent Na pumping weighs against an association of the two processes. Perhaps Ca activates the Na pump by the third mechanism, (*c*), from the outer membrane surface, without necessarily entering the cell. Attempts are currently underway to approach this possibility by measuring ATP metabolism in resealed ghosts as a function of Ca and Na concentrations on either side of the membrane.

The role of alternative Na pumps may have wider applications than for dog RBC. Recent evidence suggests that the volume regulatory system in cardiac muscle involves an ouabain-insensitive Na pump that acts independently of Na-K exchange (19). Possibly the Ca-dependent Na pump identified in dog RBC is present in sarcolemma as well.

If amrinone acts on the myocardium as it does on dog RBC to raise cytoplasmic Ca, then the inotropic effect of the drug is well accounted for (5). Studies of the action of amrinone on Ca-Na exchange in cardiac sarcolemma vesicles (20) would be of interest.

ACKNOWLEDGMENTS

We are indebted to Paula Glosson for technical assistance.

This study was supported by grant AM 11356 from the National Institutes of Health.

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