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Carbon Dioxide or Bicarbonate Ions Release Ca^{2+} from Internal Stores in Crustacean Myofibrillar Bundles

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Summary. The paper describes an investigation into the increase in intracellular free Ca^{2+} and resting tension of barnacle muscle fibers when exposed to CO_2. Isometric tension was recorded in isolated myofibrillar bundles prepared from barnacles and crabs. On replacement of a low relaxing bathing solution (free Ca^{2+}: 20 nM) at pH 7.1 with a similar one containing 100% CO_2 and 130 mM HCO_3^-, also at pH 7.1, the bundles developed a phasic contraction, which aequorin experiments confirmed was due to a release of Ca^{2+} from a store within the bundles. The source of this Ca^{2+} is tentatively identified as the sarcoplasmic reticulum (SR) for the following reasons: (1) prior exposure to 20 mM caffeine depleted this Ca^{2+} store, (2) procaine (10 mM) inhibited the response, and (3) the extracellular space or "clefts" and the mitochondria could be eliminated as possible sources. An effect of the $CO_2+HCO_3^-$ on the free Ca^{2+}/Mg^{2+} ratio in the bathing solution was excluded as a possible mechanism. The diuretic furosemide (1 mM) enhanced the response to $CO_2+HCO_3^-$. Both furosemide and SITS (1–10 mM), by themselves, also released Ca^{2+} in myofibrillar bundles. A scheme is put forward to explain these results: it is suggested that diffusion of dissolved CO_2 into the SR produces an acidification of the SR lumen, which modifies either the Ca^{2+}/-ATPase or the Ca^{2+}-induced release process in such a way to release Ca^{2+}.

Key words: Sarcoplasmic reticulum, calcium release, carbon dioxide, bicarbonate ions, crustacean myofibrils

We have previously shown that extracellular CO_2 increases the intracellular free calcium concentration (free Ca_i^{2+}) and the resting isometric tension of muscle fibers of the barnacle, *Balanus nubilus*, (Lea & Ashley, 1978). The release of Ca^{2+} from some intracellular store was put forward as a possible cause of this effect. It is known that extracellular CO_2 readily lowers the sarcoplasmic pH, (pH_i), of crustacean muscle fibers (Caldwell, 1958; Aickin & Thomas, 1975; Boron, 1977), and changes greater than one pH unit have been recorded in crab fibers exposed to 100% CO_2 saline (Aickin & Thomas, 1975). Another consequence of the diffusion of gaseous CO_2 into the sarcoplasm is an increase in the intracellular HCO_3^- concentration (Boron, 1977). It is thus conceivable that an increase in the sarcoplasmic concentration of either gaseous CO_2 or H^+ or HCO_3^- could be responsible for the release of Ca^{2+}. In order to examine directly the effects of these factors on intracellular Ca^{2+} stores, we have used the myofibrillar bundle preparation from mechanically "skinned" fibers of the barnacle and crab (Ashley & Moisescu, 1974, 1977). In this paper we demonstrate that by increasing the concentrations of CO_2 and HCO_3^- in the solution bathing a myofibrillar bundle, while at the same time maintaining the solution pH constant, one can elicit a transient increase in the isometric tension of the bundle. The evidence suggests that this is due to Ca^{2+} release from the sarcoplasmic reticulum.

Materials and Methods

The dissection and preparation of the myofibrillar bundle, the recording of tension, and the changing of solutions were performed in similar ways to those previously described (Hellam & Podolsky, 1969; Ashley & Moisescu, 1974, 1977). Briefly, single fibers were isolated from the barnacle and in a few experiments from the legs of the spider crab *Maia*, rinsed in Ca^{2+}-free saline for 1 min, blotted and immersed in "light" mineral oil. Bundles of 200–400 μm diameter and ~5 mm length were pared from fibers which had been longitudinally divided with forceps. The ends of the myofibrillar preparation were clamped between two pairs of steel, jeweller's forceps, one of which was attached directly to the anode peg of an RCA 5734 mechanoelectric transducer. The length of

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the bundle could be adjusted. The output from the transducer circuit was fed into a pen recorder (Devices M2 or Tekman, TE220). The sensitivity of the modified transducer was about 1000 V/N.

The bathing solutions (2 ml volume) were contained in a series of cylindrical wells drilled out of a circular perspex block, which could be rotated horizontally about its axis and also readily raised or lowered. Solutions in the wells were maintained at a temperature of 14–16 °C.

Measurement of Aequorin Light in Myofibrillar Bundles

A photomultiplier tube (EMI 9635A) was fitted directly beneath the solution well to measure light output from myofibrillar bundles containing aequorin (Ashley & Moisescu, 1974; Ashley et al., 1976). The photocathode was maintained at −900 V by a stabilized high voltage power supply. The output from the anode was passed through a current-to-voltage converter (time constant 20 msec) into the pen recorder. Bundles were either prepared from single fibers which had been injected with a saturated aequorin solution (0.3 µl) (Campbell, Lea & Ashley, 1979) two hours earlier or were "loaded" in air by ejecting 100 nl of aequorin solution, diluted in LR-Cl solution, directly onto the bundles and allowing a 10-min equilibration under oil.

Solutions

Table 1 gives the composition of solutions. Other chemicals used were obtained from the following: Furosemide: Hoechst; SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid): BDH; procaine: Sigma; caffeine: BDH; FCCP (carbonyl cyanide *p*-trifluoromethoxy phenyl hydrazone): Sigma. Solutions (10 mM) of the first four chemicals listed were analyzed on the atomic absorption spectrometer for total Ca content: all had negligible amounts with the exception of SITS, which contained 52 µM total Ca^{2+}. $LaCl_3$ (4 mM) was added to all solutions to be analyzed.

Results

$CO_2 + HCO_3^-$ at pH 7.1 Release Ca^{2+}

Balanus myofibrillar bundles, which had first been equilibrated in a low relaxing solution, LR-Cl, pH 7.1 (*S*1, Table 1), developed a phasic contraction when bathed in solution LR-HCO_3 (*S*2), which contained 100% CO_2 and was buffered at pH 7.1 with 130 mM HCO_3^- (Fig. 1). Typically, several phasic contractions could be produced from a single myofibrillar preparation when exposed repeatedly to this solution, although usually the first contraction was larger than the later ones (as measured by the area under the tension curve). A recovery period of more than 2 min and less than 6 min was required between these successive contractions, indicating a refractoriness of the response (Fig. 1), which was not due to a decrease in the CO_2 content of the bathing solution. Solutions containing 20 and 50% CO_2 and buffered to pH 7.1 with 26 and 65 mM HCO_3^-, respectively, were prepared from solutions *S*1 and *S*2; these gave proportionately smaller contractions than LR-HCO_3, containing 100% CO_2. Previous workers using skinned muscle fibers (Godt, 1974; Ashley & Moisescu, 1977) have included an ATP-regenerating system in their bathing solutions to ensure that the ATP concentration within the interior of the skinned fiber, where ATP hydrolysis by contractile proteins and the SR is occurring, is the same as that in the bathing solution. Modified LR-HCO_3 solutions which contained either an ATP-regenerating system (10 mM creatine phosphate +20 u·ml^{-1} creatine kinase) or 15 mM total ATP also elicited phasic contractions from the myofibrillar bundles. This result makes it unlikely that the contractions, induced by LR-HCO_3 solutions, are in some way due to ATP gradients within the myofibrillar bundles.

Myofibrillar bundles which were prepared from the legs of the spider crab, *Maia*, also gave phasic contractions on immersion in CO_2-containing solutions (Fig. 2), although these were characteristically different from those of *Balanus* bundles. Whereas in response to immersion in LR-HCO_3 *Balanus* preparations gave a single phasic contraction lasting no longer than about 5–10 min, the *Maia* bundles gave a series of contractions of different amplitudes, continuing for up to at least 15 min. In contrast to the *Balanus* bundle, the response (area under tension curve) obtained by the first exposure to LR-HCO_3 was not always larger than subsequent ones.

Use of Aequorin to Demonstrate Ca^{2+} Release

The calcium-sensitive photoprotein aequorin has been previously used successfully to demonstrate Ca^{2+} release in myofibrillar bundles exposed to caffeine, detergents, and ionophores (Ashley, Griffiths, Moisescu & Rose, 1974). It was therefore used to investigate the phasic contractions produced by CO_2-containing solutions. Myofibrillar bundles from barnacle and crab fibers were "loaded" with aequorin solution either by first injecting cannulated muscle fibers or by injecting aequorin solution in LR-Cl directly onto the clamped bundle (*see* Methods). After a short equilibration in LR-Cl, exposure to LR-HCO_3 resulted in a transient increase in the aequorin light output, indicating an increase in the free Ca^{2+} concentration within the myofibrillar bundles. This experiment was repeated with similar results for solutions which had been adjusted to pH 6.3. In *Maia* bundles, the seemingly random series of contractions in the CO_2-containing solution was accompanied by a series of light responses, which decreased in size as aequorin was utilized and lost from the bundles by diffusion (Fig. 3). The transient increase in free Ca^{2+} within the bundles from both *Maia* and *Balanus* is direct evidence that Ca^{2+} is being released from some intracellular site within the bundle.

Table 1. Composition of bathing solutions

No.	Name	K^+	Na^+	Cl^-	HCO_3^-	$MeSO_4^-$	TES	ATP	Mg_t^{2+}	Mg_{free}^{2+}	$EGTA_t$	Ca_{free}^{2+}	Sucrose	pH
S1[a]	LR-Cl	~150	10	140	~0	0	18	5.0	5.4	1.0	0.1	20 nM[b]	1000	7.1
S2[a]	LR-HCO_3	~150	10	10	130	0	18	5.0	5.4	1.0	0.1	<20 nM[b]	1000	7.1[d]
S3	*A*	~150	10	140	~0	0	18	5.0	5.4	1.0	0	16 μM[c]	1000	7.1
S4	A^{CO_2}	~150	10	10	130	0	18	5.0	5.4	1.0	0	<16 μM[c]	1000	7.1
S5	LR-Cl (no sucrose)	~150	10	140	~0	0	18	5.0	5.4	1.0	0.1	20 nM[b]	0	7.1
S6	LR-$MeSO_4$ (no sucrose)	~150	10	10	~0	130	18	5.0	5.4	1.0	0.1	20 nM[b]	0	7.1

Subscript "*t*" refers to total amount in solution. All columns are expressed as mM, except Ca_{free}^{2+} and pH.

[a] An ATP-regenerating system was added to *S1* and *S2* in a few experiments. It consisted of 10 mM creatine phosphate (Na_2) and 20 u/ml creatine kinase (Godt, 1974; Ashley & Moisescu, 1977). This increased total Na^+ to 30 mM.

[b] Ca^{2+} was present only as a contaminant from the other constituents. Atomic absorption spectrometry gave a value for Ca_t^{2+} as about 10 μM (range = 5–13 μM). The results of an experiment which is described later indicate that due to complexing of Ca^{2+} with the $CO_2+HCO_3^-$ the free Ca^{2+} in LR-HCO_3 may be as low as about 10 nM. The reagents used in the solutions were of Analar grade.

[c] To solutions *S3* and *S4* was added 40 μM $CaCl_2$ to give a nominal free Ca^{2+} of 16 μM.

[d] LR-HCO_3 was bubbled with 100% CO_2 for 1 min immediately before it was used.

The values for the apparent affinity constants (K^{app}) of Ca^{2+} and Mg^{2+} for the ligands in the solutions for 22 °C and pH 7.1 were taken from Ashley and Moisescu (1977). They were as follows: $EGTA_{Ca}=6\times10^6\ M^{-1}$, $EGTA_{Mg}=46\ M^{-1}$, $ATP_{Ca}=3900\ M^{-1}$, $ATP_{Mg}=7500\ M^{-1}$, $TES_{Mg}\leqq1\ M^{-1}$, $TES_{Ca}\leqq1\ M^{-1}$.

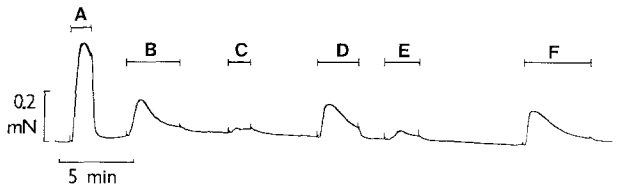

Fig. 1. (*A*)-(*F*): Phasic contractions of a *Balanus* myofibrillar bundle on repeatedly replacing a low relaxing Cl^- solution at pH 7.1 (LR-Cl) with one containing 100% CO_2 also at pH 7.1 (LR-HCO_3). The first response is typically larger than later ones, and the abortive responses *C* and *E* demonstrate a refractory period. Bundle diameter = 300 μm

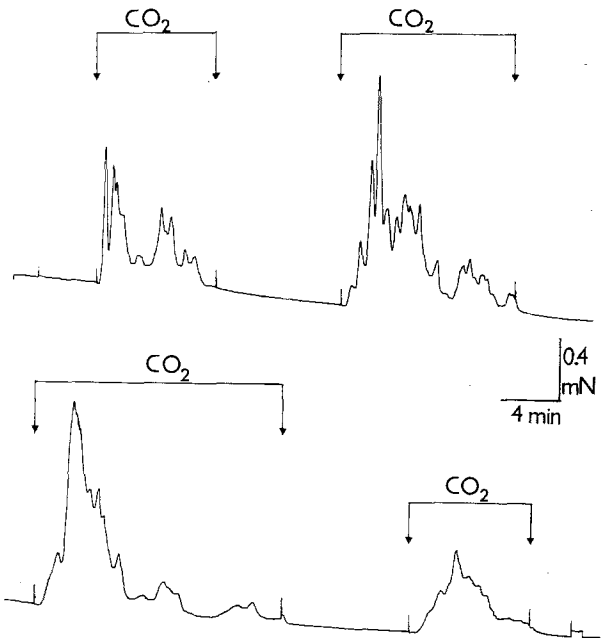

Fig. 2. Typical spontaneously recurring contractions of a *Maia* myofibrillar bundle on replacing LR-Cl with the $CO_2+HCO_3^-$ solution (LR-HCO_3). Bundle diameter = 500 μm

The Identification of the Site of Ca^{2+} Release

Caffeine was employed to investigate the identity of the sites within the myofibrillar bundles responding to $CO_2+HCO_3^-$. This compound is known to release Ca^{2+} from internal stores in this preparation (Ashley et al., 1974) and in vertebrate muscle fibers, in which the stores have been shown to form part of the SR (Weber & Herz, 1968). Exposure of a *Balanus* bundle to LR-Cl containing 20 mM caffeine caused a contraction which decreased in amplitude over 10–20 min. Exposure to LR-HCO_3 afterwards failed to produce any response, even though a further caffeine application did produce a small contraction (Fig. 4*A*). Control bundles gave the normal phasic response to LR-HCO_3 when it was applied first, but then again failed to do so following caffeine application (Fig. 4*B*); in this case the caffeine contraction was reduced in amplitude. These results are consistent with the idea that the fraction of Ca^{2+} released by LR-HCO_3 is also releasable by caffeine but that there may be in addition a calcium store which is sensitive mainly or only to caffeine.

In all the myofibrillar bundles examined from both *Balanus* and *Maia*, caffeine produced a marked tension response, whereas responses to LR-HCO_3 were variable ranging from total absence to contractions equal in amplitude to those of caffeine. There

appeared to be a seasonal variability in the response to LR-HCO_3 and it was at its best in the June to December period. When the response of myofibrillar bundles to LR-HCO_3 was absent, the response of intact fibers to extracellular CO_2 (an increase in free Ca_i^{2+} and resting tension) was still present. One possible explanation for this behavior was a variation in the activity of the Ca^{2+}-ATPase pump in the SR membrane. Increasing the solution free Ca^{2+} to 200 nM, and prior treatment of bundles in solutions of higher free Ca^{2+} in order to "load" Ca^{2+} stores both failed to restore the response to LR-HCO_3 in unresponsive bundles. However, the inhibitor of anion transport, furosemide, was successful in restoring the response (*see* below, Fig. 9).

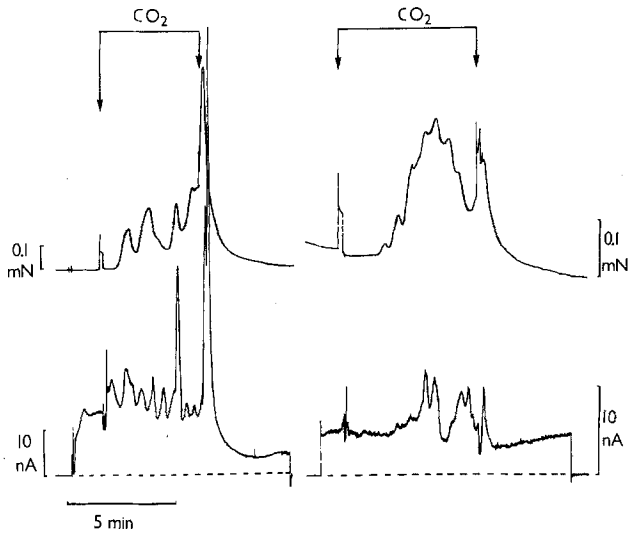

Fig. 3. Isometric tension (upper trace) and aequorin light (lower trace) responses on replacing LR-Cl by LR-HCO_3(CO_2) in *Maia* myofibrillar bundle (diameter, 270 μm). First artefact on light trace is due to switching on of high voltage for the photomultiplier. Dashed line is background light in the absence of aequorin. Note change in calibrations after first CO_2 application

Although the results with caffeine strongly suggested that part of the SR was involved in the LR-HCO_3 response, it was of importance to exclude the possibility that the calcium responsible for the tension development might be derived from other sites. In the process of "skinning" these large muscle fibers, elements which are extensions of the external space (clefts) may be retained and these could reseal, enclosing pockets of extracellular calcium, (11.8 mM), despite the brief rinse in a Ca-free saline (OCa_o). As CO_2 can cause surface membrane depolarization, a depolarization of a resealed cleft system might release calcium into the myofibrillar space. Experiments with isolated intact muscle fibers under current-clamp indicated that CO_2/HCO_3^- artificial seawater (ASW) (pH 7.3), containing 200 mM K^+ tended to hyperpolarize the surface membrane and yet still caused a contraction (J. Lignon and C.C. Ashley, *unpublished*). Single fibers were exposed for 10–15 min to OCa_o, and then to 200 mM K^+/OCa_o for 10 min, and subsequently "skinned". Control fibers were exposed either to OCa_o or to ASW before "skinning". In all three cases the LR-HCO_3 response was present and of a similar magnitude. This finding, together with the observation that in intact muscle fibers, voltage-clamped at the resting membrane potential, the CO_2/HCO_3^- response was still observed (Ashley, Franciolini, Lea & Lignon, 1979), indicates that depolarization of the surface or cleft membranes cannot be the mechanism by which calcium is released by LR-HCO_3. In addition, intact fibers soaked in OCa_o/La^{3+} ASW for 5 hr, to deplete the cleft calcium but retain the internal calcium (Ashley, Ellory & Griffiths, 1977), when "skinned" gave tension responses to LR-HCO_3, although these were reduced in magnitude

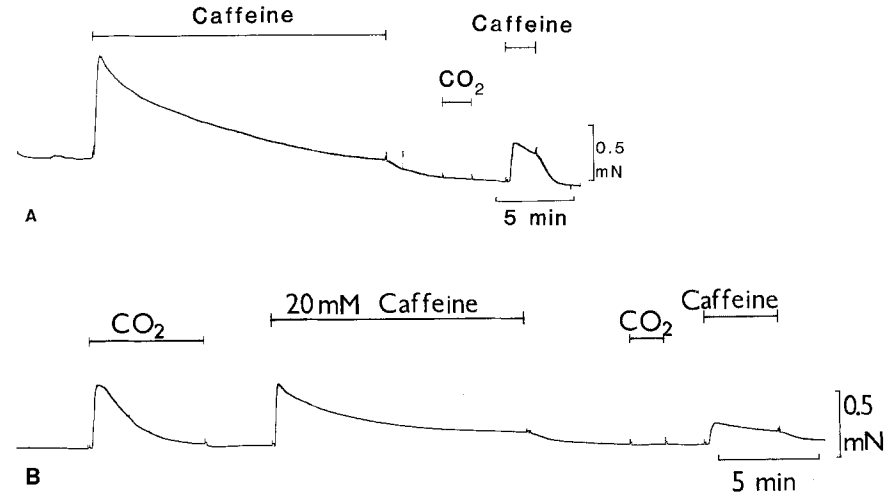

Fig. 4. (*A*): Prior exposure to 20 mM caffeine in LR-Cl abolishes the response to $CO_2+HCO_3^-$ (LR-HCO_3). (*B*): A ($CO_2+HCO_3^-$)-induced contraction reduces the size of the subsequent caffeine response. Successive experiments on two separate bundles of diameter 250–300 μm, taken from the same *Balanus* muscle fiber. Note the residual caffeine response at the end of each trace

as were the caffeine responses. Furthermore, measurements with $^{45}Ca^{2+}$ in intact fibers showed no large net influx of Ca^{2+} during exposure to CO_2-saline (Lea & Ashley, 1978).

The possible release of Ca^{2+} from mitochondria by LR-HCO_3 was examined by employing the mitochondrial uncoupler and protonophore, FCCP (Scarpa, 1975; Scarpa, Brinley & Dubyak, 1978). At a concentration of 8 μM this agent produced no contraction and presumably no release of Ca^{2+} in bundles which were responsive to LR-HCO_3, yet this concentration is effective at releasing the total accumulated Ca^{2+} from rat liver mitochondria. Thus, the tension produced in LR-HCO_3 cannot easily be attributed to release of the mitochondrial Ca^{2+}. In addition, there is no evidence to suggest that either caffeine (Weber, 1968) or CO_2 can release Ca^{2+} from isolated mitochondria; in fact 5% CO_2 stimulates a net Ca^{2+} uptake into isolated mitochondria (Elder & Lehninger, 1973; Harris, 1978).

Comparison of the Release by ($CO_2+HCO_3^-$) with Other Known Mechanisms

The mechanism of the Ca^{2+} release, induced by LR-HCO_3, was explored further in the context of the two best documented release mechanisms in skinned fibers, namely "depolarization-induced" release (Costantin & Podolsky, 1967; Ford & Podolsky, 1970; Endo & Nakajima, 1973) and "calcium-induced" release (Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1970; Stephenson & Williams, 1980). A "depolarization-induced" release could be demonstrated in the present work in barnacle myofibrillar preparations by changing from a methylsulphate-containing solution (*S6*, Table 1) to a chloride-containing solution (*S5*), as long as sucrose was omitted from the solutions (Fig. 5). The contractions were, however, not only faster than those induced by LR-HCO_3 but were also inhibited by sucrose. A similar effect has been reported in vertebrate "skinned" fibers where as little as 40 mM sucrose can inhibit the "depolarization-induced" release (Endo, 1977). It is thus difficult to explain the release of Ca^{2+} by LR-HCO_3 in the presence of 1 M sucrose in terms of the "depolarization-induced" release mechanism. In addition, it would require the SR membrane to be more permeable to HCO_3^- than Cl^-, and this seems unlikely. A true change in the membrane potential of the SR cannot be entirely excluded as a determining factor though, since some workers attribute "depolarization-induced" release simply to an osmotic change in the SR (Meissner & McKinley, 1976). It is possible that an increased H^+ concentration in the SR lumen, following entry of CO_2, could generate a diffusion potential across the SR membrane, as has been reported in SR vesicles (Meissner & Young, 1980).

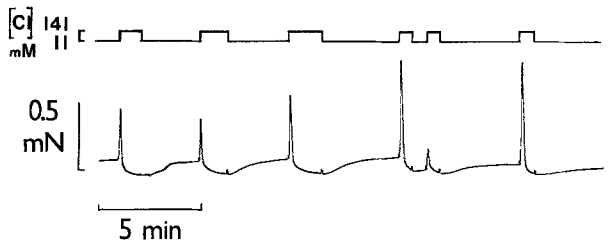

Fig. 5. Successive "depolarization-induced" Ca^{2+} releases produced by replacing a methylsulphate-containing solution (*S6*) with a Cl^--containing one (*S5*); neither solution contained sucrose. *Balanus* bundles diameter = 300 μm

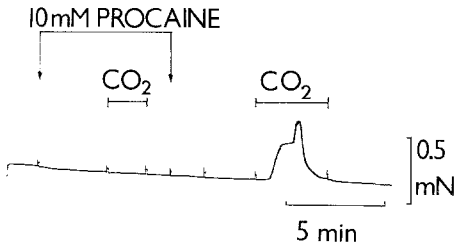

Fig. 6. $CO_2+HCO_3^-$ (LR-HCO_3) fails to produce a response in the presence of 10 mM procaine but does so after its removal. (*Balanus* bundle)

"Calcium-induced" release has been demonstrated to occur in "skinned" fibers of barnacles and crabs when the free Ca^{2+} concentration is raised to about 10^{-6} M (Stephenson & Williams, 1980). The local anesthetic procaine inhibits this type of release as well as caffeine-induced release both in barnacle (T.J. Lea and C.C. Ashley, *unpublished*) and in frog (Endo, 1977). Figure 6 shows that 10 mM procaine also reversibly inhibits the Ca^{2+} release induced by LR-HCO_3 in barnacle myofibrillar bundles. This result not only supports the earlier conclusion that the release by $CO_2+HCO_3^-$ occurs from the SR, but it also suggests that the Ca^{2+}-induced release pathway may be involved in the release by $CO_2+HCO_3^-$.

Does $CO_2+HCO_3^-$ Affect the Free Ca^{2+}/Mg^{2+} Ratio?

In view of the similarity between the Ca^{2+}-induced release and the ($CO_2+HCO_3^-$)-induced release, effects of the $CO_2+HCO_3^-$ system on the free Ca^{2+} and Mg^{2+} concentrations in solution were examined in detail, since Ca^{2+}-induced release in frog "skinned" fibers can be enhanced by reducing the free Mg^{2+} concentration (Endo, 1977). The amplitude of the Ca^{2+}-activated tension responses of a myofibrillar bundle were used to compare the ratios of free Ca^{2+} and free Mg^{2+} concentrations in the solu-

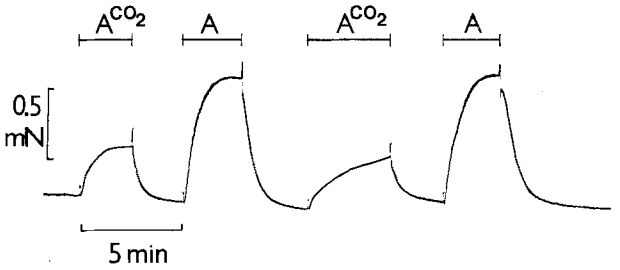

Fig. 7. Tonic contractions due to activating solutions A and A^{CO_2} in the presence of Cl^- and $CO_2+HCO_3^-$, respectively (*S*3 and *S*4, Table 1). Free Ca^{2+} was nominally increased to 16 μM by adding $CaCl_2$, and 10 mM procaine was also present. The bundle was first immersed in LR-Cl. Reduced amplitude in A^{CO_2} suggests that some of the solution Ca^{2+} is complexed to $CO_2+HCO_3^-$. *Balanus* bundle diameter = 360 μm. (*See* text for further details)

tions A and A^{CO_2} (*S*3 and *S*4, Table 1) (Fig. 7). These were modified from solutions LR-Cl and LR-HCO_3, respectively, in that $CaCl_2$ was added and EGTA was omitted in order to raise the free Ca^{2+} concentration in each (calculated assuming ATP is the only Ca^{2+} buffer), to a nominal value of 16 μM. Any Ca^{2+}-induced Ca^{2+} release from the SR was inhibited by 10 mM procaine, so that the resulting tonic contractions could be assumed to be due to activation of the contractile proteins by the free Ca^{2+} in the bathing solutions alone. The outcome of the experiment was that the contraction produced by solution A^{CO_2} was only about half the amplitude of that produced by A (Fig. 7). This either suggests that the free Ca^{2+} concentration in A^{CO_2} is less than that in A or, since it is known that Mg^{2+} competes with Ca^{2+} for the functional unit for tension (Ashley & Moisescu, 1977), that the free Mg^{2+} in A^{CO_2} is greater than that in A. Whichever is the case, the effect of CO_2 and HCO_3^- on the ratio of free Ca^{2+} to free Mg^{2+} in solution A^{CO_2} and presumably also in LR-HCO_3, is to *raise* the threshold for Ca^{2+}-induced release from the SR. In other words, the action of $CO_2+HCO_3^-$ in releasing Ca^{2+} in myofibrillar bundles exposed to LR-HCO_3 must be mediated by some process other than a modification of the free Ca^{2+}/Mg^{2+} ratio in the bathing solutions. The reduced Ca^{2+} activation in solution A^{CO_2} compared with that in A can in fact be explained by the formation of a complex $CaHCO_3^+$. An apparent affinity constant of ~0.26 M has been reported (Pedersen, 1971) which would reduce the calculated value of the free Ca^{2+} in A^{CO_2} from 16 μM (assuming ATP to be the only buffer) to about 10 μM.

In connection with the idea that the ($CO_2+HCO_3^-$)-induced Ca^{2+} release may involve the Ca^{2+}-induced release pathway, for example by lowering the free Ca^{2+} threshold, the results of a repeat of the above experiment in the *absence* of procaine are of some importance. This time, the tension response in solution A^{CO_2} was bigger than that in A, by a factor of as much as 60%. In the absence of procaine, about 75% of the response to a free Ca^{2+} of 16 μM is the result of Ca^{2+}-induced release (T.J. Lea and C.C. Ashley, *unpublished*), assuming that procaine completely abolishes release but has no effect on tension (Ashley & De Clerck, 1981). Therefore, this experiment demonstrates an enhancement, by at least 100%, of Ca^{2+}-induced release in the presence of $CO_2+HCO_3^-$, despite the unfavorable effects on free Ca^{2+} in solution.

The Effects of Agents which Could Affect the SR Intraluminal pH

One simple scheme for the mechanism of ($CO_2+HCO_3^-$)-induced Ca^{2+} release suggested itself early in the investigation (Fig. 11). Gaseous CO_2 entering the SR by diffusion would be hydrated and, on dissociation, would release protons within the SR lumen, in the same way that extracellular CO_2 is thought to lower the intracellular pH of muscle and nerve cells (e.g., Boron, 1977). The reduction in the SR intraluminal pH might then release Ca^{2+} from the SR by some, as yet unknown, pathway. To test this proposal, we examined in myofibrillar bundles the calcium-releasing ability of other agents capable of reducing the intraluminal pH.

As described previously, the protonophore FCCP caused no contraction at 8 μM in LR-Cl and this is in agreement with the reported failure of protonophores to release Ca^{2+} from Ca^{2+}-loaded SR vesicles (Scarpa & Inesi, 1972; Katz et al., 1980). This does not necessarily disprove the above scheme, for if protons were already in electrochemical equilibrium across the SR membrane, an increase in proton permeability would not change the intraluminal pH. High concentrations of both FCCP (120 μM) and DNP (5 mM) did, however, cause a slow contraction of bundles. Weak acids are known to be able to reduce the intracellular pH of cells by crossing the cell membrane in the protonated form and then releasing protons upon dissociation (e.g., Caldwell, 1958). A similar reasoning suggested that a weak acid could reduce the intraluminal pH of the SR. Potassium hydrogen phthalate (20 mM) added to a modified LR-Cl solution at pH 6.3 did produce contractions in 50% of the bundles tested, but they were slower and smaller than those obtained with LR-HCO_3. It may be that CO_2 is more effective at reducing the intraluminal pH than 20 mM phthalate, which is certainly slower than CO_2 in reducing the sarcoplasmic pH in muscle (Caldwell, 1958).

If the entry of gaseous CO_2 does acidify the SR

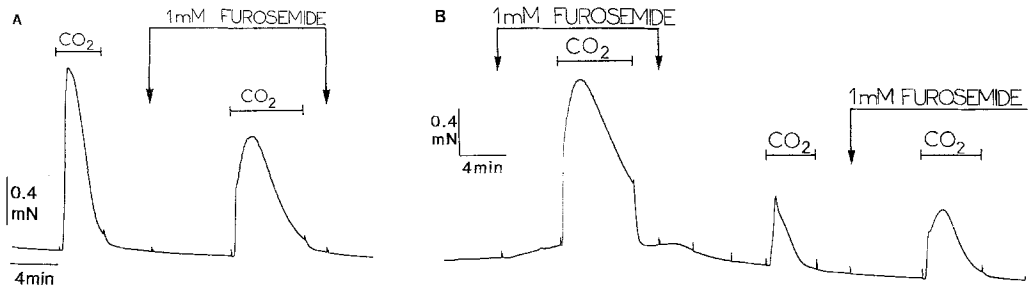

Fig. 8. Enhancement of the response to $CO_2+HCO_3^-$ (solution LR-HCO_3) by 1 mM furosemide. In *A*, the second response, in the presence of furosemide, is 1.3 times the first, whereas in control bundles it is 0.4 times the first (taking response as the area under the tension curve). In *B*, furosemide approximately doubles the third response with respect to the second. Note in *B* the slow rise in tension due to furosemide alone. Separate bundles were from separate *Balanus* fibers; diameters of *A* and *B* were both 400 μm

lumen in the manner proposed in Fig. 11, then the presumably slower entry of HCO_3^- from the solution LR-HCO_3 would be expected to buffer this pH change. An analagous effect has been recorded in intact muscle fibers of the barnacle, in which the acidification of the sarcoplasm brought about by extracellular CO_2 is attenuated by increasing the extracellular HCO_3^- concentration (Boron, 1977). In an attempt to inhibit the HCO_3^- entry across the SR membrane and thereby increase or prolong the intraluminal acidification, we used the diuretic furosemide, which is an inhibitor of anion exchange across the surface membranes of red blood cells (Brazy & Gunn, 1976) and muscle fibers (Ashley, Ellory, Lea & Ramos, 1978; Boron et al., 1978). Of particular relevance is the ability of furosemide (0.6 mM) to inhibit an acid extrusion mechanism, in the surface membrane of muscle fibers, which appears to involve a HCO_3^-–Cl^- exchange (Boron et al., 1978). When tested on the myofibrillar bundles from both *Balanus* and *Maia*, 1 mM furosemide was found to enhance the response induced by LR-HCO_3 (Fig. 8*A*, *B*). Both the time to peak and the decay half-time of the responses for *Balanus* were prolonged, so that the area under the tension response was at least doubled by furosemide. In comparing successive responses to LR-HCO_3 in the same bundle from *Balanus* fibers, due allowance was made for the observation in control bundles that the second complete response was on average only 37% of the first (measured as the area under the tension curve) and that the second and third responses were about equal. For example, the second response in Fig. 8*A* in the presence of furosemide is 130% of the first response, whereas in its absence the second response would be 37% of the first.

Further confirmation for the potentiating effect of furosemide was found in those myofibrillar bundles which were initially unresponsive to LR-HCO_3. Figure 9 shows that in such a bundle from *Maia*, 1 mM furosemide restored the response to LR-HCO_3, when applied either first in LR-Cl or later in LR-HCO_3.

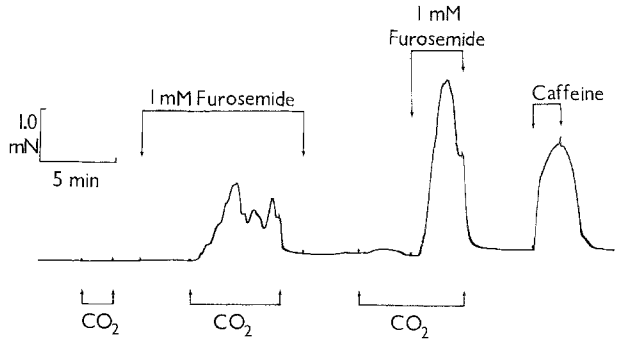

Fig. 9. In this *Maia* bundle, $CO_2+HCO_3^-$ (solution LR-HCO_3) failed to produce a response except in the presence of 1 mM furosemide, which could also restore the response even after 3 min exposure to LR-HCO_3. The release due to 20 mM caffeine is shown for comparison. Bundle diameter = 300 μm

Another well-known inhibitor of anion transport, SITS, was also used. In addition to having similar effects to furosemide on cell membranes (Knauf & Rothstein, 1971; Boron et al., 1978), it has been shown to inhibit net anion movements across the membranes of SR vesicles (Kasai & Kometani, 1979). At concentrations of 0.1–1.0 mM, SITS was relatively ineffective in prolonging the tension responses of bundles to LR-HCO_3.

Both furosemide and SITS when applied in higher concentrations (1–10 mM) to both *Balanus* and *Maia* myofilbrillar bundles in LR-Cl, produced a slow phasic contraction lasting for up to 20 min, following which both LR-HCO_3 and 20 mM caffeine failed to elicit any response (Fig. 10). Experiments using aequorin showed a transient increase in free Ca^{2+} concentration in the myofibrillar bundle in the presence of 10 mM furosemide, suggesting a release of Ca^{2+} from stores in the bundle. This release was not inhibited by 10 mM procaine. Whereas furosemide consistently produced the effects just described in a series of preparations, SITS was more unpredictable and often gave a biphasic tension response. This indicated a second site of action, perhaps an inhibitory one,

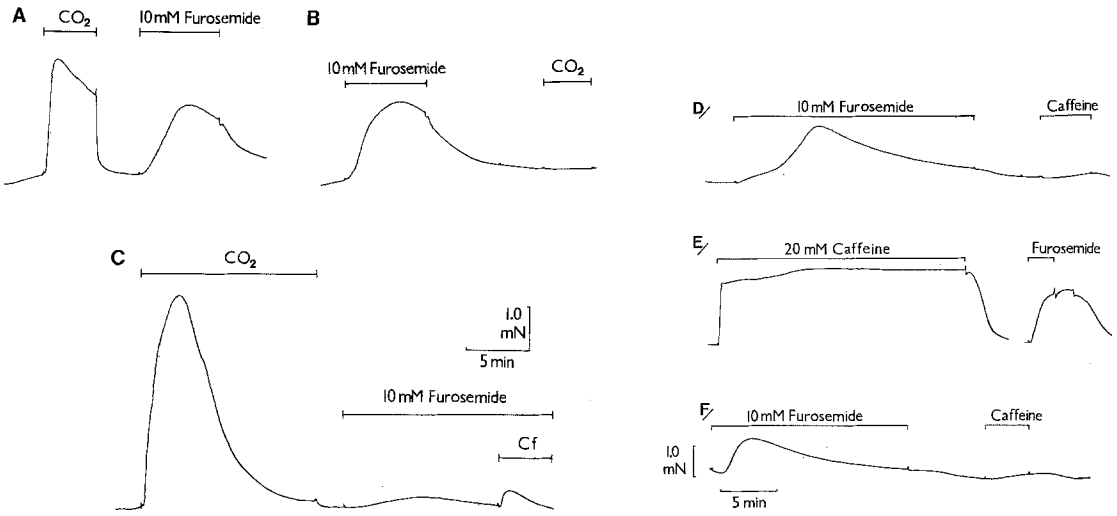

Fig. 10. (*A*)–(*C*): Results from three separate bundles, all diameter of 270 μm, from a *Balanus* fiber, demonstrating that $CO_2+HCO_3^-$ and furosemide (10 mM) release Ca^{2+} from the same store. A partial response to $CO_2+HCO_3^-$ is followed by a detectable response to furosemide (*A*), whereas the full response to CO_2 abolishes any subsequent furosemide-induced release (*C*). Conversely, an initial furosemide application abolishes any subsequent ($CO_2+HCO_3^-$)-induced release (*B*). (*D*)–(*F*): Similarly, prior exposure to furosemide (10 mM) abolishes any subsequent caffeine-induced release (*D* and *F*). In *E*, 20 mM caffeine applied first uncharacteristically produced a tonic contraction, following which furosemide was able to still release Ca^{2+}. This may indicate a reuptake of released Ca^{2+} was occurring in the presence of caffeine. Two bundles from *Balanus* fiber: (*D*) 1st bundle, (*E, F*) second; diameters were both 300 μm

on the Ca^{2+} binding-sites of the contractile proteins. This could be the result of indiscriminate binding of SITS to the myofibrillar proteins, since SITS can react covalently with amino groups. The failure of 0.1–1.0 mM SITS to affect the response to CO_2 may therefore be because very little of the reagent actually reaches the SR membrane. Furosemide also has effects on sites other than the anion transport: it inhibits both ($Na^+ + K^+$) ATPases (Hook & Williamson, 1965; Sachs, 1971) and Ca^{2+}-ATPases (Moore & Landon, 1979). It may exert its effect on the response to LR-HCO_3 by inhibiting the Ca^{2+}-ATPase pump in the SR membrane, especially since quercetin (0.5 mM), an inhibitor of the Ca^{2+}-ATPase from rabbit SR (Fewtrell & Gomperts, 1977), mimicked the effect of furosemide in restoring the response of bundles to LR-HCO_3.

Discussion

The effects of $CO_2+HCO_3^-$ solutions on crustacean myofibrillar bundles described in this paper provide strong evidence for the existence of a novel intracellular Ca^{2+} releasing mechanism. The lack of evidence implicating either the cleft space or mitochondria in this release, coupled with the effectiveness of two SR-active agents on the response, namely caffeine and procaine, support the idea that part of the SR is involved. The variability of the response to $CO_2+HCO_3^-$ in myofibrillar bundle preparations, in which caffeine consistently released Ca^{2+} is a problem to which there is no clear answer. One explanation is that there are two caffeine-sensitive Ca^{2+} stores in these bundles, one of which is sensitive to $CO_2+HCO_3^-$ and is more labile than the other. The terminal cisternae and the longitudinal tubules could be these stores.

An interesting feature of the response to $CO_2+HCO_3^-$ in bundles from *Maia* but not from *Balanus* was the spontaneously recurring contractions. Similar contractions, which can be observed in frog skinned fibers in caffeine or 10^{-6} M free Ca^{2+}, have been attributed to a cycle of events in which Ca^{2+} release from the SR is followed by a reuptake of the Ca^{2+} from the buffered bathing solution (Endo et al., 1970).

A simple scheme to account for the release by $CO_2+HCO_3^-$ is presented in Fig. 11: it relies upon an inward diffusion of dissolved CO_2 across the SR membrane, leading to an acidification of the SR lumen. As yet, the value of the intraluminal pH is unknown, but it is assumed that CO_2 will lower this pH by analogy with the effect of extracellular CO_2 on the intracellular pH of intact cells. The results with other possible modifiers of the intraluminal pH, such as protonophores and weak acids, were not marked, although reasons to account for them have been discussed in the Results. It may be that it is the *rate* of change of intraluminal pH which is important in releasing Ca^{2+}, and this would help to explain why phthalate was less potent than CO_2 as a Ca^{2+} releaser.

How a reduction in intraluminal pH might result

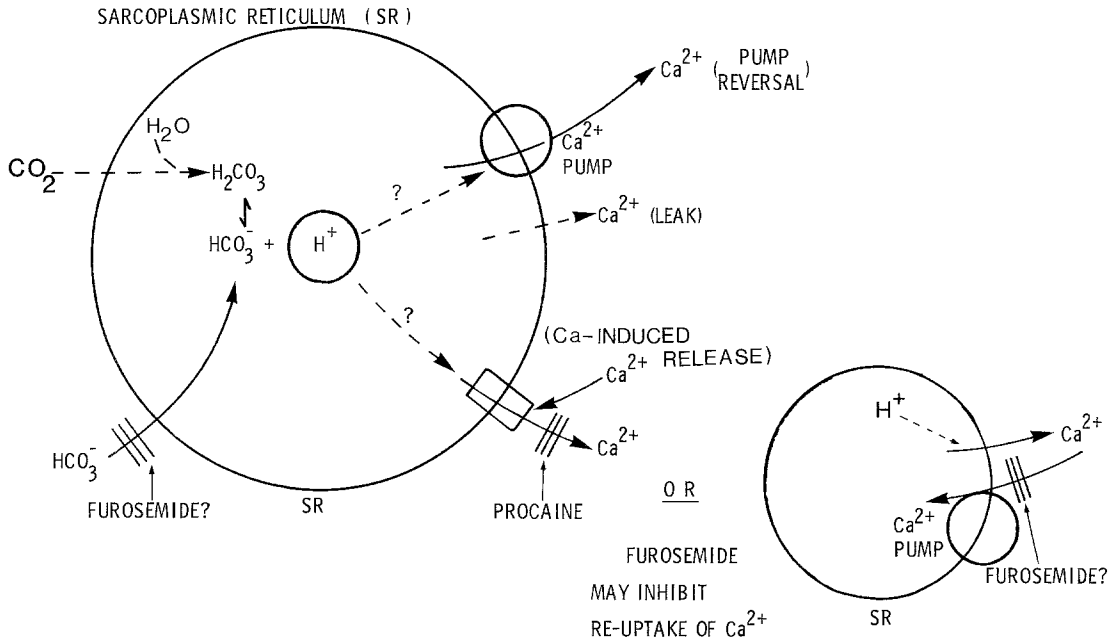

Fig. 11. A suggested mechanism of the $(CO_2+HCO_3^-)$-induced Ca^{2+} release from the SR, with possible sites of action for procaine and furosemide (*see* text for further explanation)

in a net Ca^{2+} efflux from the SR is uncertain. One proposal already put forward is that the change in pH lowers the threshold value of the solution free Ca^{2+} at which Ca^{2+}-induced release occurs. The effect of procaine and the observed enhancement by $CO_2+HCO_3^-$ of the Ca^{2+} release induced by high free Ca^{2+} concentrations are consistent with this idea. The lowest free Ca^{2+} concentration in a bathing solution which has been reported to release Ca^{2+} from crustacean "skinned" muscle fibers is less than 10^{-6} M at a free Mg^{2+} concentration of 1 mM (Stephenson & Williams, 1980). Since the free Ca^{2+} concentration of the $CO_2+HCO_3^-$ solution (LR-HCO_3) is estimated to be 10 nM, it is evident that a shift in the threshold of two orders of magnitude would be required to achieve a Ca^{2+} release by this means. It remains to be determined whether Ca^{2+}-induced release from barnacle SR is pH-sensitive; decreasing the pH of the bathing solution has been reported to decrease Ca^{2+}-induced release from cardiac SR (Fabiato & Fabiato, 1978). One possible way in which intraluminal pH might affect the Ca^{2+} releasing process is as follows: if most of the Ca^{2+} in the SR were bound to a protein, similar to the calsequestrin of vertebrate muscle (MacLennan & Wong, 1971), a reduction in intraluminal pH could liberate Ca^{2+} ions from this protein, thus increasing the intraluminal free Ca^{2+} available for the release from the SR.

A second site of action for protons on the SR membrane must be considered, and that is the Ca^{2+} pump (Fig. 11). The activity of the Ca^{2+}-ATPase from barnacle fragmented SR is pH-sensitive, having an optimum at 7.4 and falling to undetectable levels at 6.5 and 8.0 (Garcia, Lennon & Hidalgo, 1975). An inhibitory action of protons on the inner-facing side of the Ca^{2+}-ATPase could conceivably produce a release of Ca^{2+} via a leak pathway. Generally, Ca^{2+} release mechanisms are thought of as separate entities from the calcium pump (Endo, 1977), but one exception is the case of the reversal of the pump, in which ATP synthesis from ADP and P_i together with Ca^{2+} release occurs in SR vesicles under appropriate conditions. This has also been demonstrated following the imposition of a proton gradient (inside alkaline) across the membrane of the SR vesicle (de Meis & Tume, 1977).

A third possibility is that acidification of the SR lumen releases Ca^{2+} through an effect on the SR membrane potential, either by creating a proton diffusion potential as has been measured in SR vesicles (Meissner & Young, 1980) or by altering a pH-sensitive conductance in the SR membrane. It should be added though that the idea of a "depolarization-induced release" (Endo & Nakajima, 1973) has become less convincing in the light of recent work with SR vesicles (Meissner & McKinley, 1976; Beeler & Martonosi, 1979).

At first sight, the ability of furosemide, an inhibitor of anion transport, to enhance the response of myofibrillar bundles to $CO_2+HCO_3^-$ supports the idea that a reduction in intraluminal pH is a necessary step in the release mechanism. The existence of an

anion transport system in the SR membrane is fairly certain (Kometani & Kasai, 1978; Kasai & Kometani, 1979), so that the inward movement of CO_2 across the SR membrane from the $CO_2+HCO_3^-$ solution is probably followed by the influx of HCO_3^- which would tend to buffer the acidification. Furosemide, by inhibiting this influx of HCO_3^-, can be imagined to enhance the acidification and thus the Ca^{2+} release. Unfortunately there is an alternative explanation, since furosemide has been found to inhibit Ca^{2+}-ATPase (Moore & Landon, 1979): by inhibiting the reuptake of released Ca^{2+} back into the SR, furosemide could enhance the response to $CO_2+HCO_3^-$. This is supported by the similar effect of quercetin, a compound which has been definitely shown to inhibit the activity of Ca^{2+}-ATPase from the SR (Fewtrell & Gomperts, 1977). The question of furosemide's site of action could not be resolved by using SITS, since this reagent appears no more specific an inhibitor of anion transport than furosemide, probably because of nonspecific binding to the myofibrillar proteins.

In summary therefore, the site of furosemide's action on the response to $CO_2+HCO_3^-$ could either be the anion transport or the Ca^{2+}-ATPase in the SR membrane. The release of calcium by high concentrations of furosemide and SITS themselves is more easily explained by assuming inhibition of the Ca^{2+} pump and a release via the leak pathway, rather than an inhibition of the anion transport. In the latter case it would be necessary for this anion system to be capable of regulating the intraluminal pH in the same way that Cl^--HCO_3^- exchange in the sarcolemma is thought to regulate pH_i (Boron et al., 1978). Inhibition of HCO_3^- movements into the SR would then lead to acidification of the lumen and the consequent release of Ca^{2+}.

There seems little doubt that the observed effects of CO_2 on the free Ca_i^{2+} and resting tension of intact muscle fibers (Lea & Ashley, 1978) can be explained by the proposed Ca^{2+} release due to $CO_2+HCO_3^-$. Whether the accompanying decrease in pH_i in fibers exposed to CO_2 contributes to the effect has not been investigated in the present work. The results of injecting acid buffers into barnacle muscle fibers suggested that acidification of the sarcoplasm can increase the free Ca_i^{2+} (Lea & Ashley, 1978). One effect that intracellular acidification may have is to enhance the ($CO_2+HCO_3^-$)-induced Ca^{2+} release from the SR, because at pH values below 6.5 the SR Ca^{2+}-ATPase activity is very low (Garcia et al., 1975). Extracellular CO_2 also increases the free Ca_i^{2+} of nonmuscle cells such as neurones (Connor & Ahmed, 1979) and salivary glands (Rose & Rick, 1978), but is is not known whether this is due to a direct effect of CO_2 on intracellular Ca^{2+} stores or a consequence of the reduction in pH_i.

The results described in this paper pose the more general question whether a change in intraluminal pH plays a role in the physiological working of the SR. In this context, it is interesting that Madeira (1980), using SR vesicles, has observed an intravesicular pH change of about 0.2 units prior to active Ca^{2+} uptake, although Dupont (1979) was unable to confirm this. Of course, the SR *in situ* will possess different properties from SR vesicles, the main one being the intricate structure of the triad. If proton movements play a role in the function of the SR, then perhaps they occur following the opening of a voltage-dependent channel in the SR membrane and lead to the release of Ca^{2+} during muscle activation.

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