Calcium Efflux
from Barnacle Muscle Fibers

Dependence on External Cations

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ABSTRACT   Calcium-45 was injected into single giant barnacle muscle fibers, and the rate of efflux was measured under a variety of conditions. The rate constant (k) for ^{45}Ca efflux into standard seawater averaged 17×10^{-4} min^{-1}, which corresponds to an efflux of about 1–2 pmol/cm$^2 \cdot$s. Removal of external Ca (Ca_o) reduced the efflux by 50%. In most fibers about 40% of the ^{45}Ca efflux into Ca-free seawater was dependent on external Na (Na_o); treatment with 3.5 mM caffeine increased the magnitude of the Na_o-dependent efflux. In a few fibers removal of Na_o, in the absence of Ca_o, either had no effect or increased k; caffeine (2–3.5 mM) unmasked an Na_o-dependent efflux in these fibers. The Na_o-dependent Ca efflux had a Q_{10} of about 3.7. The data are consistent with the idea that a large fraction of the Ca efflux may be carrier-mediated, and may involve both Ca-Ca and Na-Ca counterflow. The relation between the Na_o-dependent Ca efflux and the external Na concentration is sigmoid, and suggests that two, or more likely three, external Na$^+$ ions may activate the efflux of one Ca^{+2}. With a three-for-one Na-Ca exchange, the Na electrochemical gradient may be able to supply sufficient energy to maintain the Ca gradient in these fibers. Other, more complex models are not excluded, however, and may be required to explain some puzzling features of the Ca efflux such as the variable Na_o-dependence.

INTRODUCTION

Evidence that there is a large calcium electrochemical gradient across the surface membrane of resting giant barnacle muscle fibers has recently been reviewed (Blaustein, 1974). This gradient favors the inward movement of Ca, and since Ca is known to enter the fibers at rest as well as during depolarization (Hagiwara and Naka, 1964; and see Blaustein, et al., 1971) barnacle muscle must possess a mechanism to extrude Ca against the electrochemical gradient.

To obtain information about Ca efflux mechanisms in barnacle muscle, single fibers were injected with ^{45}Ca and the rate of efflux was measured under a variety of conditions; the methods were very similar to those used recently by Ashley et al. (1972) in their study of ^{45}Ca efflux from crustacean giant muscle fibers. They observed a resting Ca efflux of about 1–2 pmol/cm$^2 \cdot$s in barnacle muscle; external application of potassium-rich solutions and external or internal application of caffeine increased Ca efflux. An identical value for the resting Ca efflux has been obtained in the present study, and the effect of externally applied caffeine has been tested and confirmed.

The primary purpose of the present investigation has been to examine the response of Ca efflux from barnacle muscle to changes in external cation concentrations; the main observation is that in most fibers about 40% of the ^{45}Ca efflux into Ca-free seawater requires the presence of sodium in the external solution. This finding complements the results of influx experiments (Blaustein et al. 1971; DiPolo, 1973 a; Blaustein and Russell, unpublished data) which show that the resting Ca influx into barnacle muscle is enhanced by increasing internal Na and is inhibited by external Na. Taken together, these results provide evidence that the large electrochemical gradient for Ca may at least partially depend upon a Na-Ca countertransport mechanism similar to the type which has been implicated in Ca transport in a variety of nerve and muscle preparations (e.g. Baker and Blaustein, 1968; Baker et al., 1969; Blaustein and Hodgkin, 1969; Reuter and Seitz, 1968; Glitsch et al., 1970; Reuter, et al., 1973; Blaustein and Weismann, 1970). A preliminary report of some of these findings has been communicated to the Biophysical Society (Blaustein et al., 1971).

METHODS

Barnacle Muscle Fiber Preparation

Single giant muscle fibers from large specimens of *Balanus nubilus* or *Balanus aquila* were prepared as described by Hoyle and Smyth (1963). Fibers not used immediately after dissection were stored in Na SW (Table I) at 12°C, for use the following day; all experiments were completed within 48 h of dissection. The cation content remained constant (Blaustein and Russell, unpublished observation), and no differences in Ca efflux were noted between freshly dissected fibers and day-old fibers.

External Solutions

The Tris-buffered solutions employed in early experiments were based on those used by Hoyle and Smyth (1963). For later experiments the composition was changed somewhat (Table I) to conform more closely to the ionic composition of barnacle hemolymph (Blaustein and Russell, unpublished data). No significant differences were noted between the behavior of muscle fibers incubated in bicarbonate-buffered and in the Tris-buffered seawaters (see Results). The osmolarities of these solutions

TABLE I
EXTERNAL SOLUTIONS*

	NaCl	CaCl₂	MgCl₂	LiCl	SrCl₂	MnCl₂
			mmol/liter			
Na SW	440	11	32	—	—	—
Li SW	—	11	32	440	—	—
Ca-free Na SW	440	—	43	—	—	—
Ca-free Li SW	—	—	43	440	—	—
Ca-free Mg-free Li SW	—	—	—	483	—	—
Na SW + Sr	440	11	21	—	11	—
Ca-free Na SW + Sr	440	—	32	—	11	—
Ca-free Na SW + Mn	435	—	32	—	—	11

* All solutions except Ca-free Na SW + Mn contained 7 mM KCl and 3 mM KHCO₃. The pH at 20°C was 7.8. The Ca-free Na SW + Mn contained 10 mM KCl and 5 mM tris(hydroxy-methyl)aminomethane base buffered to pH 7.8 at 20°C with HCl. Glass distilled water was used throughout.

were routinely measured by means of a freezing point depression osmometer and were in the range of 955 ± 5 mosmol/kg.

⁴⁵Ca Efflux

Single fibers from the scutal depressors were isolated and cut from the basal plate after washing a fiber bundle in Ca-free Na seawater (SW) for 30 min. A glass cannula was inserted into the cut end and tied in place, and a 100-mg weight was attached to the tendon with a silk thread. The fiber was then mounted vertically in a bath containing Na SW with the tendon end immobilized.

The method of fiber injection was similar to that used previously in squid axons (Hodgkin and Keynes, 1956; Caldwell et al., 1960) and in giant crab muscle fibers (Caldwell and Walster, 1963), except that a 1 μl syringe (Hamilton Co., Whittier, Calif.) was employed in the present study (cf. Baker et al., 1969; Ashley et al., 1972). The syringe, which delivered approximately 0.18 μl/cm of plunger travel, was attached to a 150-μm (outside diameter) glass capillary. Under microscopic examination, the glass capillary was guided through the cannula and down the center of the fiber. The injected region was always 10 mm in length and located at least 10–15 mm from the cannula.

The injection solution contained 2.5 mCi/ml ⁴⁵Ca (sp act = 11 mCi/mg) so that about 500,000 cpm were injected in each fiber. Thus, even at the lowest rates of ⁴⁵Ca efflux encountered in these experiments the samples always contained a minimum of 10–20 times background counts. The injection solution had the following composition, in millimoles/liter: K₂SO₄, 306; K₂HPO₄, 50; EGTA, 2.35; and Tris base, 10; the pH was adjusted to 7.5 by addition of H₂SO₄. The calculated ionized Ca concentration was 5 × 10⁻⁷ M, a level low enough to avoid contractures (Hagiwara and Nakajima, 1966) when the fluid was injected.

The injected fiber was removed from the cannula and mounted vertically in a glass tube (2.5-mm internal diameter) with a side arm 1.5 cm below the top of tube. The fiber was positioned with the upper margin of the injected region just below the entrance of the side arm. The injured basal region was located above the opening of

the side arm and was surrounded by Vaseline Petroleum Jelly (Chesebrough-Ponds Inc., New York) to prevent seawater from contacting it; the Vaseline also served to seal off the top of the tube. The small weight was left attached to the tendon end to maintain approximate resting length.

Appropriate solutions entered the side arm and flowed down past the fiber and out the open bottom of the tube at a constant rate maintained by a Holter pump (model 907) (Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.). Initially, flow rates of 0.5–1.5 ml/min ewere compared; since there was no discernible difference in ^{45}Ca efflux, a flow of 0.5 ml/min was used for all subsequent experiments. At the latter flow rate, a dye-dilution study indicated that within 1.5 min of the time a new solution was introduced into the sidearm tube, exchange in the effluent solution was 63 % complete.

An Isco fraction collector (Instrumentation Specialties Co., Lincoln, Neb.) was used to collect the superfusate over timed intervals, and a 2.0 ml aliquot was added to 12 ml of scintillation cocktail (Bray, 1960) for determination of ^{45}Ca activity using a Packard Tricarb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). At the end of the experiment, the fiber was quickly removed from the sidearm tube, blotted lightly, and placed in a scintillation counting vial with 0.5 ml of 1 N NaOH. After digestion, 0.5 ml of 1 N HCl, 1 ml of Ca-free Na SW and 12 ml of liquid scintillation cocktail were added, and the contents counted.

In a few experiments the injection chamber was fitted with moveable blocks which were slid together after the injection cannula was in place inside the fiber. Thus, a small (3.0-ml), watertight chamber was formed around the fiber. The fiber could then be injected and the entire bathing contents (2.8 ml) removed and replaced by fresh solutions at timed intervals. The bathing solutions were prepared for liquid scintillation counting by adding them to 15 ml of scintillation cocktail. At the end of the experiment the fiber was prepared for counting as above, except that 15 ml of scintillation cocktail were added.

Temperature Control

The temperature of the solution running through the sidearm tube was controlled by passing it through a coil immersed in a 40 % (V/V)-solution of methanol and water. The temperature of the methanol-H_2O mixture was maintained by a constant temperature bath and circulator. A thermistor was implanted in the side-arm tube wall, at the level of the injected region of the fiber, for continuous temperature monitoring.

Calculation of Rate Constants

Rate constants (k's) for ^{45}Ca efflux were calculated as the fraction of ^{45}Ca (in cpm) leaving the fiber per unit time:

$$k(\text{min}^{-1}) = \frac{\text{cpm/min (in sample)}}{\text{mean cpm in fiber}}.$$

RESULTS

^{45}Ca Efflux into Na SW

In agreement with the observations of Ashley et al. (1972), when ^{45}Ca is injected into barnacle muscle fibers, the isotopic efflux reaches a peak within

15–20 min and then slowly declines to a steady level over the next 1–2 h (see Fig. 7). This behavior probably reflects slow diffusion and compartmentalization of Ca within the sarcoplasm (see Ashley et al., 1972). In two fibers in which the early time-course of ^{45}Ca efflux was studied, the peak efflux rate constants (k's) were 26 × 10^{-4} and 37 10^{-4} min $^{-1}$, respectively. These values are considerably lower than those reported by Ashley et al. (1972), and reflect the fact that in the present experiments the injected ^{45}Ca was buffered with EGTA (see Methods); contractures were never observed as a consequence of the injection procedures (cf. Ashley et al., 1972).

Three fibers were superfused with Na SW for 120–170 min, and in these experiments the steady k averaged 16.9 min^{-1} (Table II, column 2). In most experiments Ca, and in some instances, Na, were removed from the bathing medium before the efflux into Na SW had reached a steady level; in the six experiments in which the fibers were returned to Na SW an average of 12 h after injection (range = 6–17 h), the mean k in Na SW was 17.4 min^{-1} (Table II, column 6). When the ^{45}Ca efflux into Na SW was measured early on, and again late in the same experiment (fibers 1113 and 2133 of Table II, and Fig. 7), the two k's were essentially the same.

The overall mean k for efflux into Na SW for the seven fibers was therefore about 17 × 10^{-4} min^{-1}, a value identical to that obtained for *Balanus* by Ashley et al. (1972). If complete mixing of the ^{45}Ca with the unlabeled Ca (2.1 mmol/l fiber water; Blaustein and Russell, unpublished data) in the injected region is assumed, this rate constant corresponds to an efflux of 1–2 pmol/cm^2·s, which is similar to the resting ^{45}Ca influx in intact barnacle muscle fibers (Blaustein and Russell, unpublished data).

The Na SW in experiments 11282 and 11292 contained 20 mM Ca and Tris buffer, all the other fibers were bathed in 11 mM Ca and bicarbonate buffer (Table I, and see Methods). Although the sample size is small, there does not appear to be any difference in the efflux rate constant between the two groups. This may indicate that the external Ca-dependent component of the Ca efflux is saturated by a Ca concentration of less than 11 mM.

Dependence of ^{45}Ca Efflux upon External Ca

When external Ca (Ca$_o$) was replaced by Mg after the k had reached a steady level in Na SW, the ^{45}Ca efflux reversibly declined to a new steady level (fibers 1103 *b*, 1113 *a*, and 2133 of Table II, and see Fig. 7). However, in most experiments Ca was removed before a steady k was attained; this invariably led to a marked increase in the rate of decline of k (Figs. 1 and 2). The steady level of k in Ca-free SW averaged 8.4 × 10^{-4} min $^{-1}$ (38 determinations in 20 fibers; Table II, columns 3 and 5 show most of these data). When Na SW (containing Ca) was reintroduced after varying periods of time in Ca-free solutions, a large increase in k was always observed (six fibers; cf. Table II). In the seven fibers where the data could be directly compared, the rate constants
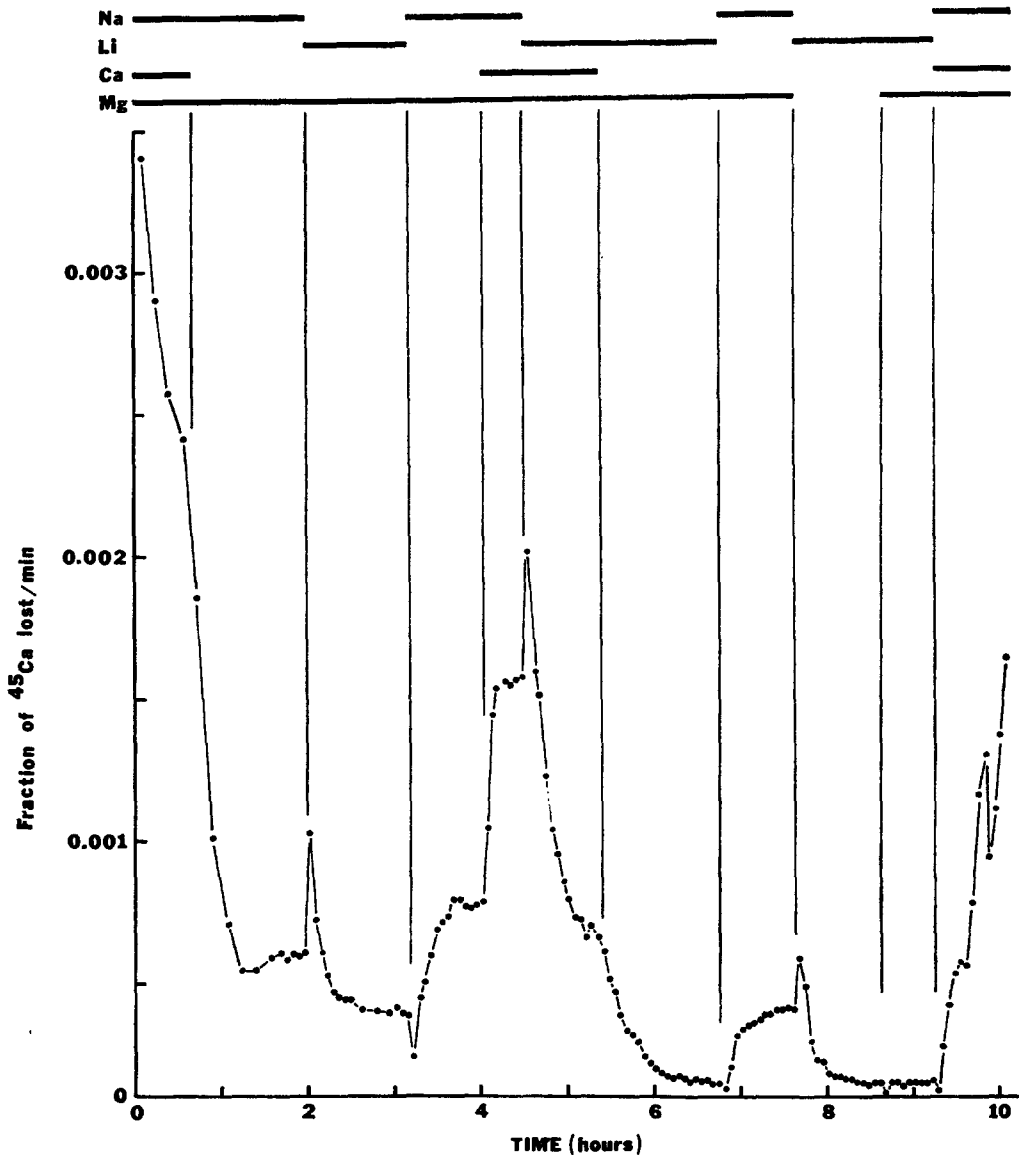
FIGURE 1.   Effects of external cations on the rate constant of ^{45}Ca efflux from a barnacle muscle fiber. In this and subsequent figures, the bars at the top of the figure indicate the presence of Na, Li, or Ca (top to bottom, respectively) in the bathing medium. This fiber (11282 of Table II) was injected with ^{45}Ca 115 min before the first point shown on the graph. The fiber went into contracture shortly after Li SW superfusion was started; complete relaxation occurred within 15 min of the reintroduction of Na (see text). Fiber diameter 1.46 mm; temperature = 19 ± 1°C.

TABLE II

EFFECT OF EXTERNAL CATIONS ON ^{45}Ca EFFLUX FROM BARNACLE MUSCLE FIBERS

Solution sequence:....	Na SW	Ca-free Na SW	Ca-free Li SW	Ca-free Na SW	Na SW	Time of Na SW determination (Hours after Ca^{++} injection)
Fiber		Steady rate constant ($\times 10^{-4}$ min^{-1})				
1042	—	9.4	6.9	9.1	—	
10302	—	7.1	5.5	7.8	—	
10312	—	9.8	7.6	—	—	
11282	—	6.1	3.9	7.9	16.8	6
11292	—	9.0	3.4	8.6	16.1	17
12192	—	—	10.0	11.9	—	
1103 a	—	10.6	8.6	9.6	24.0	17
1103 b	16.5	8.7	—	—	—	
1113 a	20.1	8.0	2.0	7.2	19.8	11
1113 b	—	7.2	2.8	—	—	
163	—	5.4	2.6	—	—	
Mean ± SE			5.3±1.1 (10)			
1092*	—	7.7	11.4	8.2	—	
10102*	—	5.0	5.2	5.9	—	
12132*	—	10.0	13.5	12.9	—	
1173*	—	8.9	8.0	6.7	—	
Mean ± SE			9.5±1.8 (4)			
			Ca-free Choline SW			
11292	—	8.4	4.7	8.0	—	
2133	14.3	6.1	3.5	—	13.9	8.5
2143 a	—	8.7	5.4	—	14.0	12
2143 b	—	10.5	5.3	10.1	—	
Mean ± SE	16.9±1.6 (3)	8.2±0.6 (18)	4.7±1.3 (4)	8.8±0.7 (13)	17.4±1.6 (6)	

Means of rate constant ratios:

$$\frac{\text{Ca-free Na SW}}{\text{Na SW}} = 0.49 \ (7)\ddagger$$

$$\frac{\text{Ca-free Li SW}}{\text{Na SW}} = 0.23 \ (4) \qquad \frac{\text{Ca-free Choline SW}}{\text{Na SW}} = 0.31 \ (3)$$

$$\frac{\text{Ca-free Li SW}}{\text{Ca-free Na SW}} = 0.61\S \ (10) \qquad \frac{\text{Ca-free Choline SW}}{\text{Ca-free Na SW}} = 0.57 \ (3)$$

* Denotes fibers which had no Na-dependent Ca efflux.

‡ The number of values used in calculating each of the mean ratios is shown in parentheses.

§ Excludes the values from the four fibers (denoted by asterisks) not demonstrating an Na$_o$-dependent Ca efflux.

in Ca-free Na SW averaged 49% of those in control Na SW (Table II). These observations all indicate that removal of Ca$_o$, a maneuver which creates a gradient favorable to passive Ca efflux, actually reduces the rate of ^{45}Ca efflux, a result consistent with a Ca-Ca exchange mechanism.

The long duration of these experiments, already alluded to (and see Figs.

1–4, 6, and 7), was a consequence of the relatively slow rate of change of k following an alteration of the bathing medium composition. The approach of k to the new steady level followed first order kinetics with a time constant of 7–9 min. This presumably reflects washout of the extracellular space since the time constant for washout of the sulfate space is virtually identical (Blaustein and Russell, unpublished data).

Ca was replaced by Mg in all Ca-free solutions used in these experiments (with one exception; see below) since it has been noted that the resistance and excitability of barnacle muscle fiber membranes can be maintained in Ca-free solutions by increasing external Mg (Hagiwara and Naka, 1964). The fact that periodic measurements of k in Na SW (e.g. Table II, columns 2 and 6) or in Ca-free Na SW (e.g. Table II, columns 3 and 5; and see Figs. 1–4 and 7) gave reproducible results, is evidence that the fiber membrane may retain functional integrity during long periods in Ca-free media containing increased Mg. In addition, fibers incubated for 10–11 h in Ca-free Na SW at 15°C, 24 h after initial dissection, always contracted when exposed to 200 mM K SW (Na SW with 190 mmol/l NaCl replaced by KCl) (Russell and Blaustein, unpublished data).

Since Mg may slightly inhibit Ca influx (Blaustein and Russell, unpublished data), it was important to know if inhibition by Mg could account for the difference between the k in Na SW and in Ca-free Na SW (Table II). Fig. 1 shows data from an experiment in which Mg was removed and later returned to the Ca-free bathing solutions. Since no influence on k was noted, inhibition of ^{45}Ca efflux by Mg cannot account for the 50% reduction of k when Ca_o was replaced by Mg (Table II).

Dependence of ^{45}Ca Efflux upon External Na

The dependence upon external cations of the remaining 50% of ^{45}Ca efflux (i.e. the efflux into Ca-free Na SW) was tested by replacing Na with either Li (15 fibers) or choline (4 fibers) after the efflux into Ca-free Na SW had attained a steady level. When external Na (Na_o) was removed, a transient increase in ^{45}Ca efflux was invariably seen in samples collected within the first 10 min of the solution change; when Na was added back, the efflux transiently decreased (Figs. 1–4, 6, and 7). The cause of these transients is unknown.

Upon removal of Na_o, the usual response (15 fibers), after the transient, was a further decline of k, relative to that in Ca-free Na SW. A new steady k was approached with a time constant of 7–9 min (see Figs. 1, 2, 4, and 6); the mean k for these 14 fibers fell from 8.2×10^{-4} min^{-1} in Ca-free Na SW, to 5.2×10^{-4} min^{-1} in Ca-free Na-free SW with no difference between the effects of Li- and choline-substituted solutions. The effect was fully reversible when Na was added back. Thus, the Na-dependent fraction of ^{45}Ca efflux represents about 18% of the total efflux or about 38% of the efflux into Ca-free
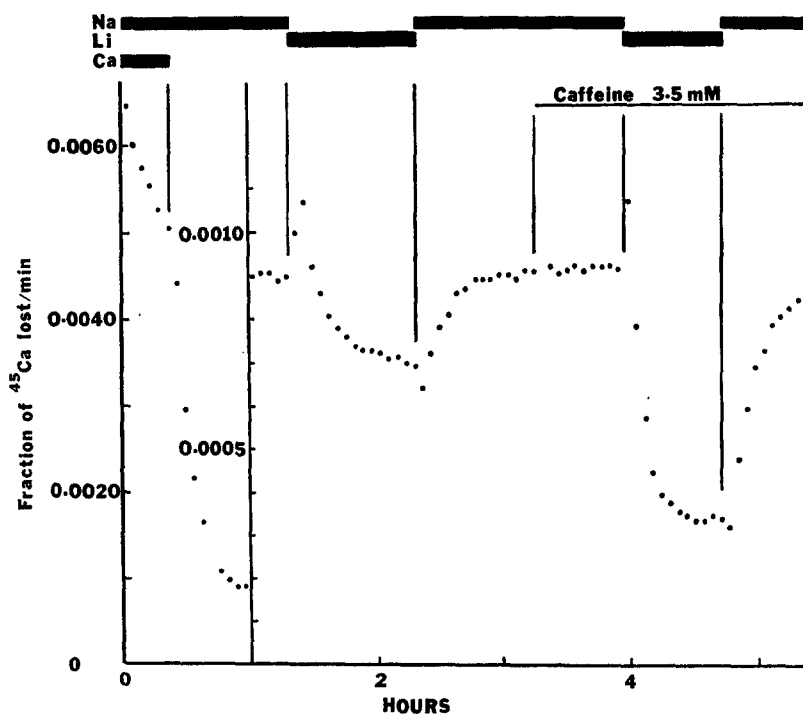
FIGURE 2. Effects of external cations on the rate constant of ^{45}Ca efflux from a barnacle muscle fiber before and during caffeine treatment. This fiber (1042 of Table II) was injected 40 min before the first point on the graph. No contracture was visible in the muscle during the exposure to caffeine. Fiber diameter, 1.28 mm; temperature = 21 ± 1°C.

Na SW. The absolute magnitude of the efflux rate constant in Ca-free Na-free SW varied considerably from fiber to fiber (Table II, column 4).

The effect of Na removal in the presence of external Ca was investigated in one fiber (Fig. 1); a marked decrease in k, from 15.7 × 10^{-4} min^{-1} to 6.8 × 10^{-4} min^{-1}, was observed. When this fiber was later exposed to Ca-free Na SW, the steady k (4 × 10^{-4} min^{-1}) was considerably lower than it had been in Ca-free Na SW prior to the treatment with Li SW (7 × 10^{-4} min^{-1}). This inhibition of k could not be attributed to the Li ion itself, because, as noted above, exposure to Ca-free Li SW had no apparent long-term effects. A similar "irreversible" decline in the k for ^{45}Ca efflux into Na SW was observed in squid axons exposed to Li SW (Blaustein and Hodgkin, 1969); the magnitude of this decline of k was directly related to the duration of exposure to Li SW, and was attributed to possible saturation or inhibition of the Ca extrusion mechanism due to Ca entry from Li SW.

In barnacle muscle, the low k in Ca-free Na SW late in the experiment (Fig.

1) could result from an inhibition of the Ca efflux mechanism, with a consequent reduction in the absolute magnitude of the Ca efflux.

Alternatively, the reduced k could occur with no change, or even an increase in Ca efflux, relative to the efflux into Ca-free Na SW earlier in the experiment. Since Ca influx increases in fibers exposed to Na-free SW (Blaustein et al., 1971; DiPolo, 1973 a) thereby reducing sarcoplasmic ^{45}Ca-specific activity, if the Ca transport sites at the inner surface of the membrane were near saturation in relaxed muscle (free $Ca^{+2} < 10^{-6}$ M), an increase in internal Ca could decrease k without decreasing the absolute Ca efflux. An estimate of the dilution of intracellular ^{45}Ca can be made by assuming that the difference between the k's in Ca-free Na SW before (7×10^{-4} min^{-1}) and after (4×10^{-4} min^{-1}) exposure to Ca-containing Li SW was due solely to the decrease in specific activity, rather than to a decrease in the absolute value of the Ca efflux. On the basis of this assumption, when Li SW replaced Na SW, k should have fallen to a value of about 9×10^{-4} min^{-1}, rather than to the observed value of 6.8×10^{-4} min^{-1}. The difference between these two values may then represent the Na_o-dependent component of the Ca efflux; if the absolute Ca efflux actually increased, despite the decline of k, this difference would underestimate the apparent Na_o-dependent Ca efflux.

Another way to account for the "irreversibly" decreased k after Li SW is to assume that the Ca sequestration sites within the sarcoplasm (sarcoplasmic reticulum and/or mitochondria) have a variable capacity to store Ca. In this regard, Carvalho and Leo (1967) have shown that under some conditions, the amount of Ca bound to rabbit muscle arcoplasmic reticulum may increase more steeply than the free Ca^{+2} concentration ($[Ca^{+2}]_i$). If the sequestration sites in barnacle muscle stored nearly all of the Ca which entered the fiber from Li SW so that the rise in $[Ca^{+2}]_i$ was not directly proportional to the net gain of Ca by the fiber, the lower k in Ca-free Na SW late in the experiment might not necessarily indicate a reduced Ca efflux.

All of the foregoing possibilities assume a uniform distribution of isotope in the exchangeable sacroplasmic Ca (including storage sites), since the relationship between k and Ca efflux is unpredictable in the absence of isotopic equilibrium.

Although lack of direct evidence makes it difficult to select the most likely explanation for the inhibition of k, several factors suggest that the first two proposals, inhibition and saturation of Ca efflux, may be relatively unlikely. Firstly, caffeine, which presumably increases $[Ca^{+2}]_i$ (see below), does not reduce the Ca efflux, and may increase it (Ashley et al., 1972; Chen et al., 1972). Also, since depolarization appears to increase Ca entry and induce a transient rise in $[Ca^{+2}]_i$ (Hagiwara et al., 1968; Ashley and Ridgeway, 1968), it seems unlikely that these normal physiological changes in fiber Ca should interfere with Ca extrusion. Finally, on the chance that the mechanisms of Ca

efflux from barnacle muscle and squid axon may be similar (see Discussion), it should be noted that axons in which $[Ca^{+2}]_i$ was increased by cyanide poisoning exhibited significantly increased Ca_o-dependent and Na_o-dependent Ca effluxes (Blaustein and Hodgkin, 1969).

The fiber of Fig. 1, like several others which were studied in another context (Blaustein, unpublished data), went into contracture when exposed to Li SW, and did not relax when Ca_o was subsequently removed. (Contracture does not occur if Na_o is replaced in the absence of Ca_o; Blaustein and Russell, unpublished data.) The fact that these fibers relaxed only when Na SW or Ca-free Na SW was reintroduced provides evidence that Na_o may play a significant role in net Ca efflux and in relaxation, since *Balanus* fibers have a relatively sparse sarcoplasmic reticulum (Hoyle et al., 1973).

In four fibers, removal of Na_o caused either little change (Table II, fibers 10102 and 1173; see Fig. 3), or a significant increase (Table II, fibers 1092 and 12132) in k. The reason for the qualitatively different response in these fibers is unknown. However, the phenomenon may be related to differences between individual barnacles, since it was noted that if one fiber from an individual animal gave this response, all those tested from the same animal responded similarly. For example, in Table II experiments 1092 and 10102 were performed on fibers from the same animal and neither had an Na_o-de-
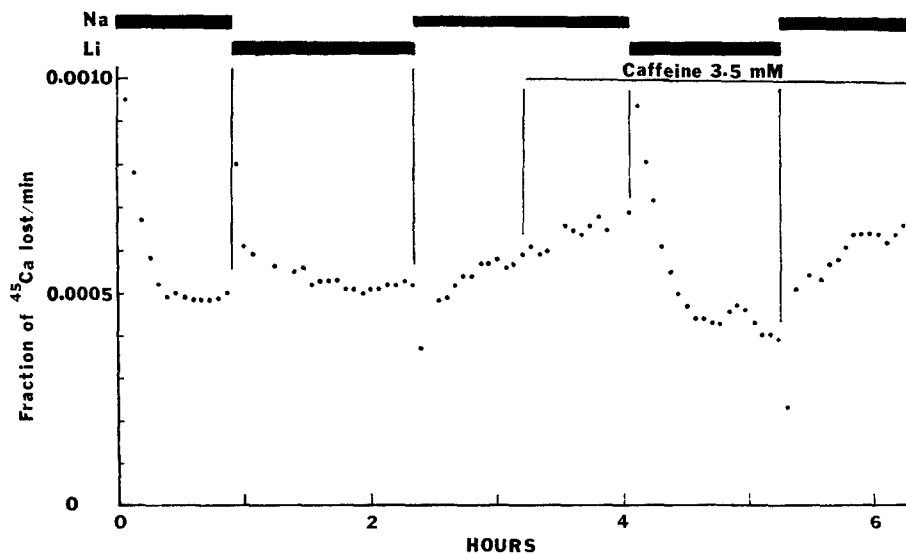

FIGURE 3. Effect of removal of external Na from Ca-free media before and during caffeine treatment on the rate constant of ^{45}Ca efflux. This fiber (10102 of Table II) was injected with ^{45}Ca 160 min before the first point on the graph, and Ca_o was removed 20 min before the first point. No signs of contracture were observed during the exposure to caffeine. Fiber diameter, 1.33 mm; temperature $= 19 \pm 1°C$.

pendent Ca efflux, whereas experiments 10302 and 10312 were from another animal and both fibers had an Na_o-dependent Ca efflux.

Effects of Caffeine on External Cation-Dependent Ca Effluxes

External application of caffeine (2–10 mM) is known to cause contractures and increase the rate constant for ^{45}Ca loss from injected barnacle fibers bathed in Ca-containing fluids (Ashley et al., 1972; Chen et al., 1972; Russell and Blaustein, unpublished observations). This presumably reflects release of Ca from the sarcoplasmic reticulum (Weber and Herz, 1968). In the present experiments, caffeine was tested for its effects on fibers bathed in Ca-free sea-waters.

When caffeine (2–3.5 mM) was applied to eight fibers bathed in Ca-free Na SW there was never any visible evidence of contraction. Moreover, the rate constant for ^{45}Ca efflux was essentially unaffected (Table III and Figs. 2 and 3) which is quite unlike the situation observed in Ca-containing solutions. It is well known that in the absence of Ca_o, invertebrate muscle fibers (Zacharová and Zachar, 1968, quoted in Zachar, 1971; Chiarandini 1970), like vertebrate muscle fibers (Frank, 1960), contract when treated with caffeine. After 1-2 h in Ca-free Na SW, *Balanus* fibers shortened rapidly upon exposure to Ca-free Na SW containing 3.5 mM caffeine; however, after 10–11 h in Ca-free Na SW, addition of 3.5 mM caffeine had no effect although these same

TABLE III

EFFECT OF CAFFEINE ON ^{45}Ca EFFLUX FROM BARNACLE MUSCLE FIBERS BATHED IN Ca-FREE SOLUTIONS*

| | Steady rate constant ($\times 10^{-4}$ min^{-1}) | | | | | |
| | A (4 fibers) | | B (4 fibers) | | C (2 fibers) | |
Group: Solution sequence	Control	During caffeine (2–3.5 mM)	Control	During caffeine (2–3.5 mM)	Control	During caffeine (2–3.5 mM)
Ca-free Na SW	8.8±0.8	9.4±1.0	7.9±1.0	7.6±0.4	8.4±1.3	—
Ca-free Li SW	7.5±1.0	3.4±0.3	9.5±1.9	4.5±0.4	6.5±1.1	6.1±1.1
Ca-free Na SW	9.6±1.2	9.2±0.9	8.4±1.5	6.8±0.6	—	8.1±0.3
Ca-free Li SW	—	—	—	—	—	3.7±0.4
$\dfrac{\text{Ca-free Li SW}‡}{\text{Ca-free Na SW}}$	0.82	0.37	1.17	0.63	0.77	0.75 (a)
						0.46 (b)

* Prior to caffeine treatment, k decreased in all fibers in Groups A and C when exposed to Ca-free Li SW, whereas in Group B two fibers (10102 and 1173 of Table II) exhibited little change and two fibers (1092 and 12132 of Table II) exhibited a slight increase in k.

‡ Ratio of k in Ca-free Li SW to the mean k in Ca-free Na SW except Group C where (a) is the ratio of k in the first exposure to Ca-free Li SW + Caffeine to that in Ca-free Na SW + caffeine and (b) is the ratio of the k during the second exposure to Ca-free Li SW + caffeine to that in Ca-free Na SW + caffeine.

fibers contracted vigorously when treated with 200 mM K SW (Russell and Blaustein, unpublished data).

Of the eight fibers tested, four had an Na_o-dependent Ca efflux prior to caffeine treatment (Table III, group A and Fig. 2) and four did not (Table III, group B and Fig. 3). However, after caffeine was applied, all eight fibers exhibited a large Na_o-dependent Ca efflux (Table III). That is, caffeine apparently uncovered an Na_o-dependent efflux in those fibers which previously had none, and increased the size of the already present Na_o-dependent efflux in the remaining fibers.

In two fibers (Table III, group C) caffeine was applied while the fiber was in Ca-free Li SW; again, no contracture was observed and no change in k occurred. Returning the fibers to Ca-free Na SW + caffeine caused no change in k relative to pretreatment levels. However, when these caffeine-treated fibers were tested in Ca-free Li SW a second time, the Na_o-dependent ^{45}Ca efflux was increased.

Quantitative Relation Between $[Na]_o$ and ^{45}Ca Efflux

The results described in the foregoing sections show that under appropriate conditions, when external Na is completely removed from the bathing medium, all fibers exhibit a reduction in ^{45}Ca efflux. Although the magnitude of this Na_o-dependent efflux is variable and may not be apparent in the absence of caffeine, it usually accounts for about 40% or more of the total efflux into Ca-free Na SW. To further define the relationship between Na_o and ^{45}Ca efflux, the influence of intermediate Na concentrations was examined in three fibers; all of the Na_o was replaced by Li, and Na was then added back stepwise. Fig. 4 shows the data from one fiber in which Na was added back sequentially, 5 or 10% at a time. The main point is that increasing Na_o from 0 to 22 mM caused only a slight increment in k, whereas increasing Na_o from 22 to 44 mM caused a much larger increment in k.

Results similar to those shown in Fig. 4 were obtained in the other two fibers, when a more random pattern of Na addition was employed. The mean values for the relative magnitude of the Na_o-dependent ^{45}Ca efflux are shown as a function of $[Na]_o$ in Fig. 5. The S-shaped relationship suggests that more than one Na^+ ion is required to activate the efflux of one Ca^{+2} ion.

Temperature Dependence of ^{45}Ca Efflux

The effect of temperature was examined in three experiments in order to obtain information about the activation energy for Ca efflux. Fig. 6 shows data from one fiber in which the effects of cooling on k in Ca-free media in the presence and absence of Na were tested. Similar results were obtained on two other fibers in which three temperatures in the range of 6–20°C were tested. The time constant for changing the temperature of the bathing solution was
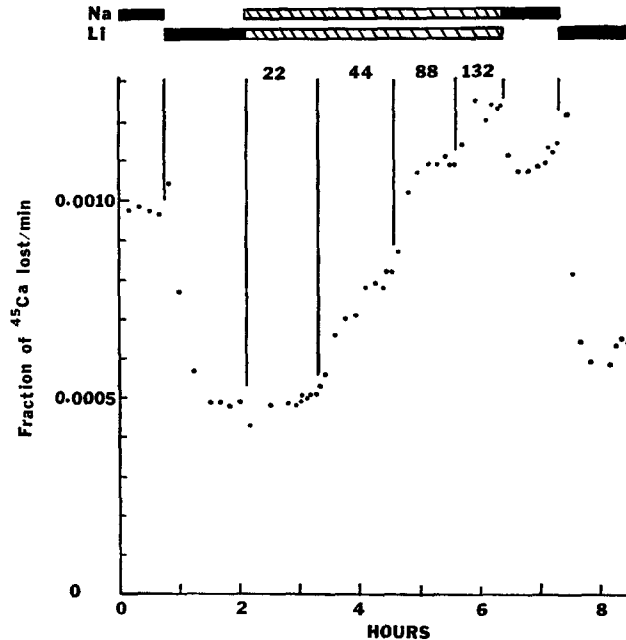
FIGURE 4. Effect of step-wise addition of Na to Ca-free media on the rate constant of ^{45}Ca efflux. The hatched bars at the top show the period that the fiber was superfused with various mixtures of Ca-free Na SW and Ca-free Li SW; the numbers immediately below the hatched bars indicate the Na concentrations (in millimoles/liter) present during the intervals between the vertical lines. The fiber (1103 *a* of Table II) was injected with ^{45}Ca 360 min before the first point, and Ca_o was removed 200 min before the first point on the graph. Fiber diameter, 1.09 mm; temperature $= 19 \pm 1°C$.

about 1 min, and the change in k followed rapidly, with a time constant of about 2 min; the latter is clearly much shorter than the time constant for the changes in k due to alterations in the composition of the bathing medium (7–9 min; see Fig. 6).

When the fibers were cooled, the initial, rapid decline of k was always followed by a slow upward "creep." Furthermore, upon rewarming there was always an overshoot of the rate constant before it returned to precooled levels. These two observations may indicate that during cooling, isotopically labeled Ca was released from some intracellular sites capable of active Ca uptake, possibly sarcoplasmic reticulum (Hasselbach and Makinose, 1961) and/or mitochondria (but see Drahota et al., 1965).

In order to avoid possible complications due to these secondary effects, the temperature dependence was calculated using the k's obtained about 10 min after cooling was begun. The Q_{10} for ^{45}Ca efflux into Ca-free Na SW was 2.4 ± 0.2, and 1.8 ± 0.3 for efflux into Ca-free Li SW. In these three experiments, the Q_{10} for the Na_o-dependent component of the Ca efflux averaged
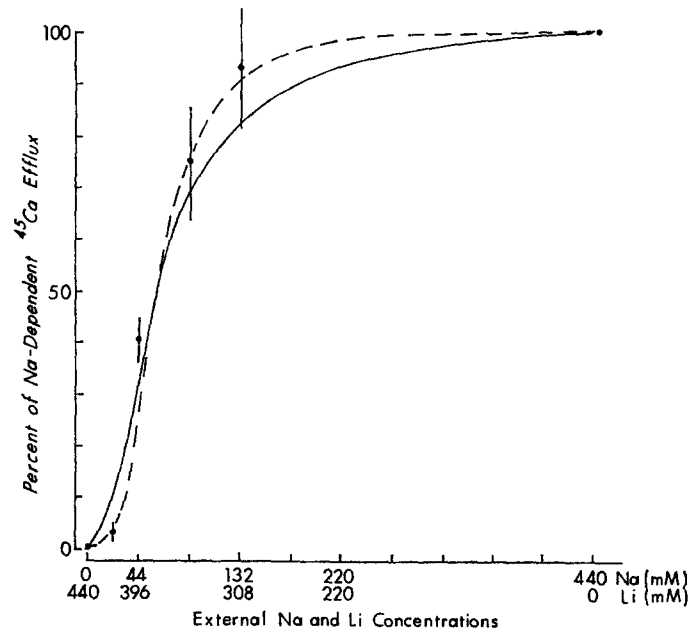
FIGURE 5.   Percent of the Na_o-dependent ^{45}Ca efflux in Ca-free seawaters plotted as a function of the external Na and Li concentrations; the Na_o-dependent Ca efflux into Ca-free Na SW is taken as 100%. The graphed points are the mean values from three experiments similar to (and including) the one shown in Fig. 4; the vertical bars through the points represent the standard errors. All experiments were conducted at temperatures of 18 ± 1°C. The data have been fitted to an equation of the form, $v = V/[1 + (K_{Na}/[Na]_o{}^n)]$, where v is the Na_o-dependent ^{45}Ca efflux at any external Na concentration ($[Na]_o$), relative to that in Ca-free Na SW (V); K_{Na} is the mean affinity constant for Na_o. The mean rate constant for the Na_o-dependent ^{45}Ca efflux in Ca-free Na SW (i.e. k in Ca-free Na SW minus k in Ca-free Li SW) for the three experiments was 5.3 ± 0.4 × 10^{-4} min^{-1}. The exponent (n) had a value of 2 (solid line) or 3 (dashed line); K_{Na} was 60 mM in both cases. See text for further discussion.

3.7 ± 0.8. However, if the Ca transport model of Fig. 8 B has any validity for barnacle muscle (see Discussion), the fact that barnacle muscle is depolarized by about 1 mV/° of cooling (DiPolo and Latorre, 1972) may mean that the Q_{10} for the Na_o-dependent Ca efflux has been somewhat overestimated.

Influence of Strontium and Manganese Ions on ^{45}Ca Efflux

The effects of external Sr and Mn were tested in four experiments to ascertain whether these ions could substitute for Ca_o and stimulate ^{45}Ca efflux, or could inhibit ^{45}Ca efflux. Fig. 7 shows that 11 mM Sr stimulated ^{45}Ca efflux into Ca-free and Ca-containing seawaters; in one experiment (not shown), Sr also increased the ^{45}Ca efflux into Li SW. In quantitative terms, 11 mM Sr only increased k by about one seventh as much as did 11 mM Ca, above the level in
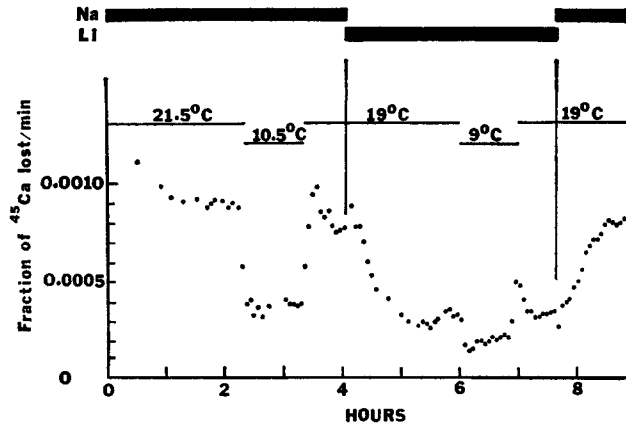
FIGURE 6. Effect of cooling on the rate constant of ^{45}Ca efflux in Ca-free Na SW and Ca-free Li SW. This fiber (11292 of Table II) was injected with ^{45}Ca 175 min before and Ca_o removed 50 min before the first point on the graph. The time constant for the temperature change in the medium was 1 min. Fiber diameter, 1.28 mm.
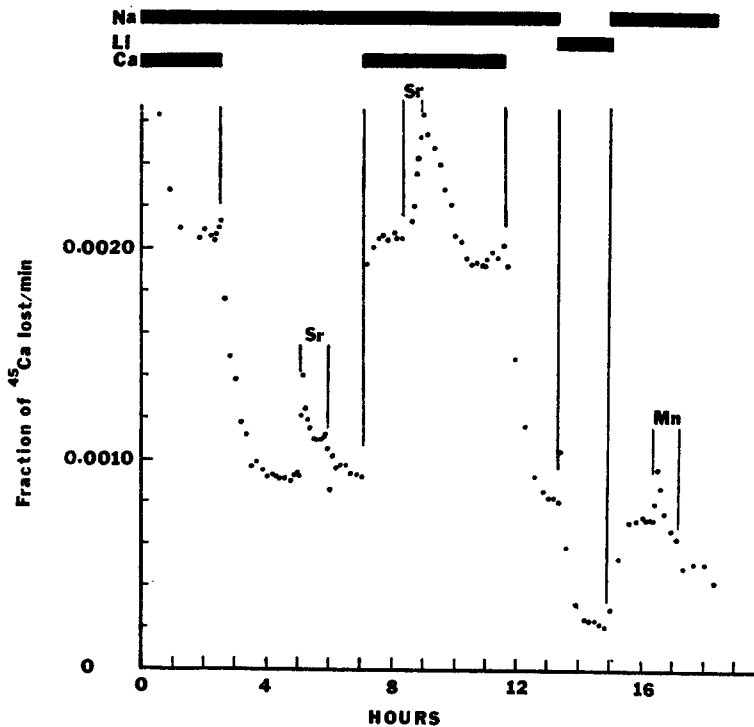

FIGURE 7. Effects of strontium and manganese on ^{45}Ca efflux. The fiber (1113 a of Table II) was injected at zero-time. Fiber diameter, 1.28 mm; temperature = 16.5 ± 0.5°C.

Ca-free Na SW (Fig. 7). Similar results were obtained on two other fibers, although the stimulatory effect of Sr was somewhat greater in the latter experiments. These data may indicate that Sr can partially replace Ca_o.

When 11 mM Mn was added to the Ca-free Na SW (Fig. 7), the effect, after an initial stimulation, was an apparently irreversible inhibition of ^{45}Ca efflux. The two other fibers tested, showed similar responses to Mn. Manganese (11 mM) was added to the Ca-free Li SW bathing one fiber, and in this case the steady k in Ca-free Li SW + Mn was about the same as in the Ca-free Li SW without Mn.

DISCUSSION

Ca-Ca Exchange: Evidence for a Ca Carrier

The experiments described above show that a considerable fraction of the ^{45}Ca efflux from injected *Balanus* muscle fibers is dependent upon external Ca and Na. Calcium efflux is reduced by about half when external Ca is removed. Since Ca influx must also be decreased when external Ca is removed, these observations provide strong evidence for Ca-Ca exchange diffusion (cf. Ussing, 1947), a phenomenon which indicates that a mobile carrier mechanism may be involved (Wilbrandt and Rosenberg, 1961).

In the course of these experiments, several other divalent cations were tested for their ability to replace external Ca as promoters of ^{45}Ca efflux. Magnesium was found to be ineffective in this regard, while Mn actually inhibited ^{45}Ca efflux into Ca-free Na SW. On the other hand, externally applied Sr stimulated ^{45}Ca efflux. The latter observation may be additional evidence in favor of a carrier mechanism, since it suggests the possibility of a counterflow exchange of Ca for Sr (cf. Rosenberg and Wilbrandt, 1957; Wilbrandt and Rosenberg, 1961).

Na_o-Dependent Ca Efflux: Evidence for Na-Ca Exchange

Clearly, a carrier which is involved in one-for-one Ca-Ca exchange cannot be directly participating in the net transfer of Ca across the muscle fiber surface membrane. However, the foregoing experiments also show that a significant, but variable, fraction of the ^{45}Ca efflux from *Balanus* fibers is dependent upon external Na; on the average, this Na_o-dependent efflux accounted for about 40% of the total ^{45}Ca efflux into Ca-free Na SW. The large activation energy for the Na_o-dependent efflux (about 17 kcal·mol^{-1}) although perhaps overestimated due to cooling-induced depolarization, is consistent with the possibility of a carrier-mediated process.

The most straightforward explanation for these observations is that the Na_o-dependent Ca efflux may be coupled with a counter flow of Na ions. A carrier-mediated Na-Ca exchange could participate in net extrusion of Ca

from barnacle muscle, and similar mechanisms have been implicated in "up-hill" Ca transport in a variety of other tissues (see Introduction). Unfortunately, direct evidence for countertransport of Na is lacking and may be difficult to obtain from tracer studies because any Na influx linked to Ca efflux (less than 1 $pmol/cm^2 \cdot s$) should account for only a small fraction of the normal barnacle muscle resting Na influx (about 50 $pmol/cm^2 \cdot s$; Brinley, 1968). Nevertheless, the evidence that external Na may compete with Ca for carrier sites, and that Ca influx is likely coupled with Na efflux (Blaustein et al., 1971; DiPolo, 1973 *a*; Blaustein and Russell, unpublished data) when considered in conjunction with these ^{45}Ca efflux data, is consistent with the idea that a mobile carrier mechanism may serve to transfer Na and/or Ca in either direction across the sarcolemma.

Additional support for this hypothesis comes from the observations by Vogel and Brinley (1973) on internally dialyzed barnacle muscle fibers. They, too, found that ^{45}Ca efflux was partially dependent upon external Na; furthermore, they obtained evidence that Na and Ca may compete at sites on the inner surface of the membrane, since ^{45}Ca efflux was increased by 50% when internal Na was reduced from 80 to 2 mM.

Model for Na-Ca Counterflow

A simple carrier model which takes many of these observations and ideas into account is illustrated in Fig. 8A. The carrier (X^{-2}) is assumed to operate in parallel with the sodium pump (Brinley, 1968) which serves to maintain the Na gradient across the sarcolemma. Since the activation curve of the Na_o-dependent ^{45}Ca efflux is S-shaped with respect to the external Na concentration (Fig. 5), it seems likely that two or more Na^+ ions may exchange for one Ca^{+2}. According to this model, which assumes that the carrier mediates the electrically neutral exchange of two Na^+-for-one Ca^{2+}, the energy released by inward diffusion of Na_2X (Na moving down its electrochemical gradient) may be coupled with the uphill movement of Ca (outward diffusion of CaX). In the steady state, the equilibrium potentials for Ca and Na (E_{Ca} and E_{Na}, respectively) should then be equal:

$$E_{Ca} = \frac{RT}{2F} \ln \frac{{}^a Ca_o}{{}^a Ca_i} = E_{Na} = \frac{RT}{F} \frac{{}^a Na_o}{{}^a Na_i}, \tag{1}$$

or,

$$\frac{{}^a Ca_o}{{}^a Ca_i} = \frac{({}^a Na_o)^2}{({}^a Na_i)^2}, \tag{1 a}$$

where R, T, and F are the gas constant, absolute temperature and Faraday's number, respectively. The thermodynamic activities of Ca and Na are indi-

cated by ^aCa and ^aNa, respectively, and the subscripts refer to the sarcoplasm (i) and the hemolymph (o).

The Debye-Hückel-Güntelberg equation (as modified by Guggenheim; cf. Robinson and Stokes, 1968) was used to calculate single ion activity coefficients for both Na^{+2} and Ca^{+2} in seawater, 0.7 and 0.25, respectively, on the assumption that the activity coefficients for Na and Cl are equal. Similar values for the Na and Ca activity coefficients can be independently derived without this assumption, on the basis of the Stokes-Robinson hydration theory (Bates et al., 1970).

Taking the hemolymph Na concentration as 460 mM (Blaustein and Russell, unpublished data), and the activity coefficient of 0.7, a value of 322 mM is obtained for ^aNa_o. A value of about 10 mM for the activity of Na in *Balanus* sarcoplasm (California specimens) has been obtained by Gayton et al. (1969) with Na-selective microelectrodes.

Calculation of the blood Ca activity is somewhat more complex. *Balanus* hemolymph contains 11 mM Ca (Blaustein and Russell, unpublished data), of which at least 7–8% is nondialyzable (Blaustein, unpublished data) and therefore presumably protein-bound. However, marine invertebrate hemolymph also contains about 29 mM SO_4 (Hayes and Pelluet, 1947), and much of it must be in the form of undissociated $CaSO_4$ and $MgSO_4$ since the dissociation constants for both sulfates are about 5×10^{-3} mol/liter (Sillén, 1964). Taking these factors into account, as well as the activity coefficient for Ca (0.25; see above), we calculate that ^aCa_o is about 1.3 mM.

Substitution of these cation activities into equation 1 *a*:

$$\frac{1.3}{^aCa_i} = \frac{(322)^2}{(10)^2},$$

(1 *b*)

leads to the conclusion that the Na gradient may only provide sufficient energy for the two Na^+-for-one Ca^{+2} exchange mechanism of Fig. 8 A to maintain the activity of Ca^{+2} in barnacle muscle at about 1.3×10^{-6} M. This value is considerably greater than the values for resting Ca^{+2} activity, $5–20 \times 10^{-8}$ M, obtained from the data of Hagiwara and Nakajima (1966). Clearly, the mechanism depicted in Fig. 8 A is inadequate to maintain the low sarcoplasmic Ca level in *Balanus* muscle.

Since the data of Fig. 5 best fit a cubic, rather than a square-law, equation, they indicate that a more complex model than that of Fig. 8 A, perhaps involving 3 Na^+-for-1 Ca^{2+} exchange (cf. Baker et al., 1969; Blaustein and Hodgkin, 1969; Baker, 1972) should be considered. One possibility, illustrated in Fig. 8 B (and see Blaustein, 1974, for a more detailed discussion), is that the carrier is trivalent (X^{-3}) and can exchange three Na^+ for one Ca^{+2} and one negative charge (Blaustein and Hodgkin, 1969). For this model, the
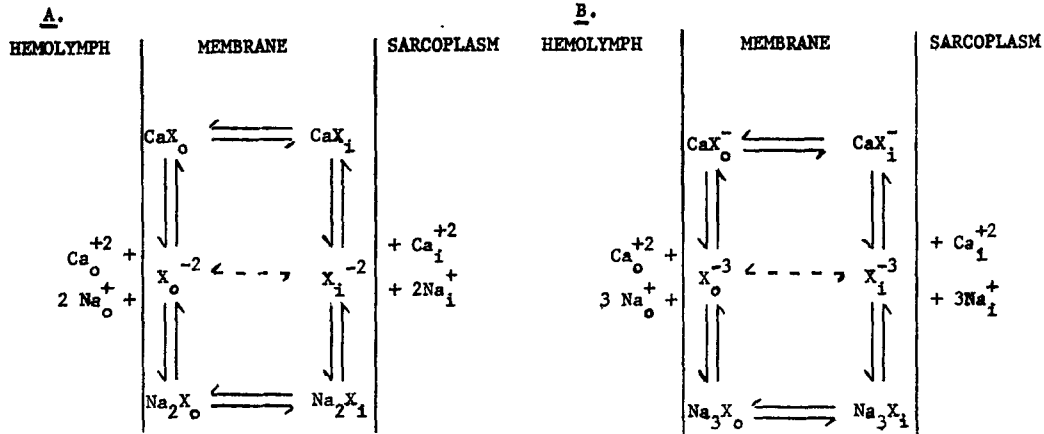
FIGURE 8. Two models of sodium-calcium countertransport carrier mechanisms. (A) Model for an electrically neutral exchange of two Na^+-for-one Ca^{+2}. (B) Model involving the exchange of three Na^+-for-one Ca^{+2} and one free negative charge. The free carrier (X^{-2} or X^{-3}) is assumed to be a divalent or trivalent anion, respectively, with specificity for Na or Ca ions. The chemical reactions between carrier and counter ions are assumed to be very rapid, while the diffusion of the carrier-cation complexes (CaX or Na_2X in the case of the divalent carrier, and CaX^- or Na_3X for the trivalent model) across the membrane are rate limiting; free carrier presumably diffuses very slowly, if at all. The subscripts, i and o, refer to the sarcoplasm and hemolymph, or internal and external surfaces of the membrane, respectively.

steady-state Ca gradient would be given by:

$$\frac{{}^aCa_o}{{}^aCa_i} = \frac{({}^aNa_o)^3}{({}^aNa_i)^3} e^{-VF/RT}. \tag{2}$$

This mechanism could, in principle, reduce ${}^aCa_i$ to about 4×10^{-9} M, which is even lower than the values calculated from the data of Hagiwara and Nakajima (1966). Although support for this model is limited to the data in Fig. 5, recent experiments in squid axons (Blaustein et al., unpublished data; F. J. Brinley, Jr. and L. J. Mullins, personal communication) indicate that the Na_o-dependent Ca efflux declines approximately e-fold or a little less when the membrane is depolarized by 25 mV, consistent with a net charge of -1 on the outwardly moving Ca-carrier complex.

The foregoing speculation does not, of course, exclude the possibility that another energy source such as ATP or the outward K gradient (Blaustein and Hodgkin, 1969) may also play a role in the extrusion of Ca from barnacle muscle. However, of considerable interest in this regard is the recent observation (DiPolo, 1973 b) that the Na_o-dependent Ca efflux from dialyzed squid giant axons is unaffected by the reduction of the axoplasmic ATP concentration to 2–4 μM.

Variations in the Magnitude of the Na_o-dependent Ca Efflux: Possible Effect of $[Ca^{+2}]_i$

One of the more puzzling features of the Ca efflux from barnacle muscle concerns the variation observed in the magnitude of the Na_o-dependent fraction. In two fibers, ^{45}Ca efflux actually increased when Na_o was removed from the Ca-free seawater. However, exposure to 2–3.5 mM caffeine unmasked an Na_o-dependent Ca efflux in these fibers and significantly increased the magnitude of the Na_o-dependent Ca efflux in fibers in which this dependency was demonstrable even before caffeine treatment. Since caffeine is known to release Ca from sarcoplasmic reticulum (Weber and Herz, 1968), the enhancement of the Na_o-dependent Ca efflux in the presence of caffeine may be the consequence of a rise in the level of free Ca^{+2} in the barnacle muscle sarcoplasm. The fact that these fibers, after prolonged superfusion with Ca-free media, never contracted when treated with caffeine, may indicate that even in the presence of caffeine the sarcoplasmic Ca^{+2} concentration never exceeded about 8×10^{-7} M (the threshold for contraction; Hagiwara and Nakajima, 1966). This, in turn, may provide an indication of the sensitivity of the Na_o-dependent Ca efflux to the internal Ca concentration.

Additional evidence that the level of internal Ca may help to regulate the magnitude of the Na_o-dependent Ca efflux is seen in the experiment of Fig. 1. Prior to the incubation in Li SW, the Na_o-dependent efflux accounted for about 54% of the total ^{45}Ca efflux into Ca-free Na SW. The exposure to Li SW presumably reduced Ca efflux (see Results) and markedly stimulated Ca influx as evidenced by the fact that the fiber went into contracture (Fig. 1, and see Blaustein et al., 1971; DiPolo, 1973 a). The Na_o-dependent Ca efflux was subsequently observed to account for 82% of the ^{45}Ca efflux into Ca-free Na SW. The feature which is probably common to this experiment and the caffeine experiments is an increased $[Ca^{+2}]_i$ as a result of the Li SW or caffeine treatments, respectively. The rise in the level of free Ca^{+2} may perhaps stimulate Na_o-dependent Ca efflux as a manifestation of Ca-Na competition at the inner surface of the sarcolemma (see above), although why this should cause a simultaneous and approximately equal reduction in the external cation-independent Ca efflux remains uncertain. One untested possibility is that muscle Ca may be extruded directly from the sarcoplasmic reticulum into the extracellular space (ECS) by an external cation-independent mechanism, and from the sarcoplasm (free Ca^{+2}) into the ECS by an Na_o-dependent route (Na-Ca exchange). Caffeine, by releasing Ca from the sarcoplasmic reticulum, might thereby enhance the Na_o-dependent Ca efflux at the expense of the external cation-independent efflux.

Residual, Cation-Independent Ca Efflux

In most of these experiments, although the magnitude of the ^{45}Ca efflux into Ca-free SW was relatively constant, the apportionment of this flux between Na_o-dependent and Na_o-independent fractions was obviously quite variable. The residual Na_o-independent Ca efflux is rather enigmatic since its magnitude was reduced by conditions which presumably increased $[Ca^{+2}]_i$. In squid axons, too, the external cation-dependent and -independent fractions of the Ca efflux responded to increased $[Ca^{+2}]_i$ in different ways: during cyanide poisoning, the magnitudes of the Na_o-dependent and Ca_o-dependent Ca effluxes increased markedly, while the external cation-independent efflux generally remained constant or declined slightly (Blaustein and Hodgkin, 1969).

In barnacle muscle (as in squid axons), this residual flux was not an insignificant fraction of the total Ca efflux into Na SW. It was dependent neither upon alkali metal nor upon alkaline earth cations; furthermore, in one experiment (fiber 2133 of Table II), when all of the external medium was replaced by isotonic sucrose containing 2 mM $LaCl_3$ (Blaustein and Russell, unpublished observation), the ^{45}Ca efflux rate constant fell only to 2.7 $\times$ 10^{-4} min^{-1} (about 20% of the value in Na SW). This represents an efflux of about 0.2–0.4 pmol/cm$^2 \cdot$s, which is far more than one would expect for passive diffusion on the basis of flux-ratio equation (Ussing, 1950) calculations. The mechanisms underlying this external cation-independent Ca efflux remain unknown.

The tentative conclusion from these experiments is that a sodium-calcium countertransport mechanism which can utilize energy from the Na gradient is likely involved in the extrusion of Ca from barnacle muscle fibers. A simple two Na^+-for-one Ca^{+2} exchange will not provide sufficient energy to maintain the observed Ca gradient, and a three-for-one exchange, which would be more than adequate, may give a better fit to some of the available data. Further speculation seems unwarranted, however, until more information about the Ca efflux is obtained; it will be necessary to test the effect of membrane potential, and to precisely control $[Ca^{+2}]_i$ and $[Na^+]_i$ in order to resolve many of the uncertainties and clarify the mechanism of "active" calcium transport in barnacle muscle.

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