We are indebted to Professor F. G. YOUNG for his advice and encouragement. This work was supported by the British Diabetic Association and the Theodore Chapin Beebe Fund. R.D.G.M. holds the Stanley Elmore Senior Research Scholarship, Sidney Sussex College, Cambridge.

The Department of Biochemistry,
University of Cambridge,
Cambridge (Great Britain)

R. D. G. MILNER
C. N. HALES

1 R. D. G. MILNER AND C. N. HALES, *Diabetologia*, 3 (1967) 47.
2 R. D. G. MILNER AND C. N. HALES, *Biochim. Biophys. Acta*, 135 (1967) 375.
3 R. D. G. MILNER AND C. N. HALES, *Biochem. J.*, in the press.
4 W. W. DOUGLAS, D. W. LYWOOD AND R. W. STRAUB, *J. Physiol. London*, 156 (1961) 515.
5 W. W. DOUGLAS AND R. P. RUBIN, *Nature*, 203 (1964) 305.
6 H. G. COORE AND P. J. RANDLE, *Biochem. J.*, 93 (1964) 66.
7 C. N. HALES AND P. J. RANDLE, *Biochem. J.*, 88 (1963) 137.
8 H. A. KREBS, *Biochim. Biophys. Acta*, 4 (1950) 249.

Received September 18th, 1967

Biochim. Biophys. Acta, 150 (1968) 165–167

BBA 71012

Sodium-dependent uptake of calcium by crab nerve

The influx of Ca^{2+} into squid axons is dependent both on the Na^+ content of the sea water and also on the Na^+ concentration in the axoplasm[1]. These observations have been repeated and extended on nerves obtained from the limbs of the spider crab *Maia squinado*. In this preparation the nerves are too small to sample the axoplasm directly. The measurements of Ca^{2+} uptake were made on whole nerve trunks and presumably include contributions from axons and Schwann cells.

After dissection, nerves were kept for up to 2 h in artificial sea water (ASW) of composition (in mM): NaCl, 460; KCl, 10; $MgCl_2$, 55; $CaCl_2$, 11; $NaHCO_3$, 2.5. Nerves were loaded with Na^+ by tetanization at 30 impulses/sec for 5 min in K^+-free sea water. This increased the Na^+ content from about 25 mmoles/kg nerve to about 80 mmoles/kg nerve. Exposure to test solutions was made in a shaking water bath maintained at 16.5°. As ouabain (10^{-3} M) inhibits the Na^+–K^+ pump in crab nerve[2] without affecting Ca^{2+} uptake (see below), this drug was included in all solutions in order to slow down the rate of loss of Na^+ from the cells. Even in the presence of ouabain, the Na^+ content fell by about 25 % during a 7-min exposure to Li^+-ASW. Ca^{2+} uptake was followed by flame photometry and by use of ^{45}Ca. In both measurements great care was taken before analysis to wash the nerves free of extracellular Ca^{2+}. For flame photometry, nerves were washed for 10 min at 0° in 3 changes of Ca^{2+}-free choline–ASW followed by 2 washes in buffered isotonic choline and for tracer uptake the nerves were given five 2-min washes in K^+-free sea water at 0°. After being washed, the nerves were blotted on filter paper and their middle portions taken for analysis.

The Ca^{2+} content of nerves immersed in Na^+-ASW averaged 1.01 ± 0.18

Abbreviation: ASW, artificial sea water.

mmoles/kg nerve. During Na^+ loading there was no significant increase in Ca^{2+} content. However, the Ca^{2+} content of these Na^+-loaded nerves doubled in 10 min after transfer to sea waters in which the NaCl had been replaced isosmotically by LiCl, choline chloride or dextrose. This increase was unaffected by inclusion of 10^{-3} M ouabain in the medium. A net gain of Ca^{2+} must result from either an increased influx or a decreased efflux or a mixture of these two factors. Satisfactory measurements of efflux have not proved possible with crab nerve; however, measurements of Ca^{2+} influx have been made. In Na^+-loaded nerves, the influx from K^+-free sea waters based on Li^+, choline or dextrose was 3–8 times higher than that from Na^+ ASW. This suggests that much of the net gain in Ca^{2+} results from an increased Ca^{2+} influx.

The shapes of the curves relating net and tracer uptake of Ca^{2+} to the external Na^+ concentration are shown in Fig. 1a. The increase in uptake occurs mainly between 230 and 2.5 mM Na^+. Fig. 1b shows the Ca^{2+} influx at different external Ca^{2+} concentrations. At the four Na^+ concentrations examined, the curves relating Ca^{2+} influx to external Ca^{2+} concentration approximate to sections of rectangular hyperbolae. Progressive replacement of Na^+ by Li^+ increases the affinity of the uptake process for Ca^{2+}. Similar sets of curves have not been determined for mixtures of Na^+ and choline or Na^+ and dextrose, but in isotonic dextrose and choline the shapes of the curves relating Ca^{2+} influx to external Ca^{2+} concentration are similar to those obtained in Li^+.

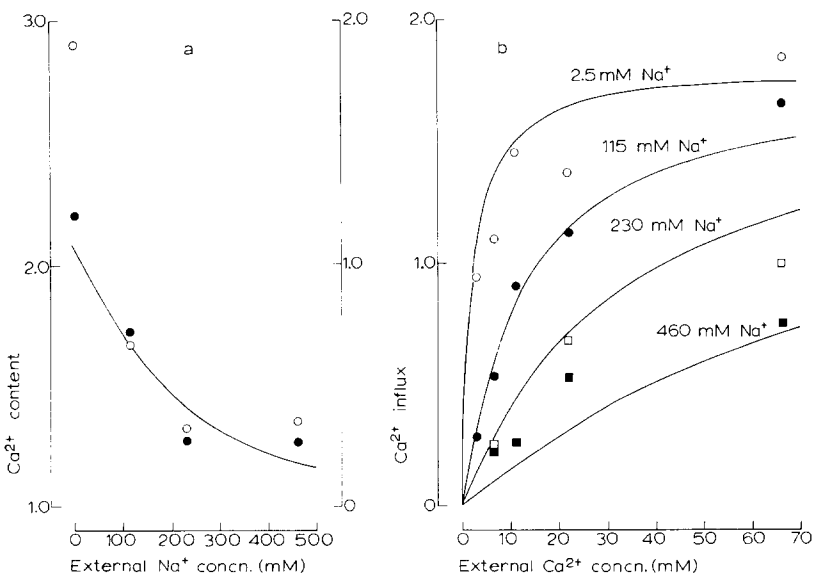

Fig. 1a. Ca^{2+} content (○) (mmoles/kg nerve after 10 min) and Ca^{2+} influx (●) (mmoles/kg nerve during a 7-min exposure to ^{45}Ca) as a function of the external Na^+ concentration (mM). Na^+-ASW was replaced isosmotically by Li^+-ASW. The Ca^{2+} content and Ca^{2+} influx were obtained on different crabs. Each point is the mean of 3 determinations. An essentially similar curve was obtained when Na^+-ASW was replaced by dextrose-ASW.

Fig. 1b. Ca^{2+} influx (mmoles/kg nerve during a 7-min exposure to ^{45}Ca) as a function of the external Ca^{2+} concentration (mM). The solutions were Mg^{2+}-free and Na^+ was replaced isosmotically by Li^+. Each point is the mean of at least 3 separate determinations. The curves in Figs. 1a and 1b have been calculated as described in the text.

The influence of Mg^{2+} has been examined in a few experiments in Li^+-ASW. At low, but not high Ca^{2+} concentrations, isosmotic replacement of Mg^{2+} by Li^+ increased the Ca^{2+} influx. These results suggest that external Mg^{2+}, like external Na^+, can displace Ca^{2+} from the sites which facilitate Ca^{2+} uptake. The data are consistent with a substrate constant of 2–3 mM for Ca^{2+} and an inhibitor constant of 20–30 mM for Mg^{2+}. It is not known whether the Ca^{2+} uptake process can also transport Mg^{2+}.

In frog cardiac muscle there is evidence that the uptake of 1 Ca^{2+} ion is inhibited competitively by 2 Na^+ ions[3,4]. If the Ca^{2+} influx into crab nerve is governed in a similar way—with each Na^+ ion acting independently of the other—and assuming that Mg^{2+} also inhibits competitively, it can be shown that the rate of Ca^{2+} uptake (v) should be given by:

$$v = \frac{V}{1 + \frac{K_{Ca}[Mg^{2+}]}{K_{Mg}[Ca^{2+}]} + \frac{K_{Ca}}{[Ca^{2+}]}\left(1 + \frac{[Na^+]}{\bar{K}_{Na}}\right)^2}$$

where $\bar{K}_{Na}$ is related by the Langmuir principle[5] to the true inhibitor constants K_i and K_i' for the combination of the first and second Na^+ ions by the relation $\bar{K}_{Na} = \sqrt{K_i' K_i} = 2 K_i$. The curves in Figs. 1a and 1b have been calculated from this equation with $V = 1.8$; $K_{Ca} = 2$ mM; $K_{Mg} = 20$ mM and $\bar{K}_{Na} = 75$ mM. While the curves roughly fit the experimental points, the fit is not adequate to exclude other possibilities. It might be improved if Ca^{2+} uptake is governed by a higher power of the external Na^+ concentration.

In order to examine the effects of varying the internal Na^+ concentration, nerves were tetanized for 5 min at 30 impulses/sec either in Ca^{2+}-free Li^+-ASW or in Ca^{2+}-free Na^+-ASW. Lowering the internal Na^+ content by tetanization in Li^+ had no effect on the Ca^{2+} uptake from Na^+-ASW; but markedly reduced that from sea waters based on Li^+, choline or dextrose (Table I). Both the net and tracer uptakes were reduced to the same level as that from Na^+-ASW. Nerves tetanized in Ca^{2+}-free Na^+-ASW behaved normally. There was no evidence for an inhibitory action of internal Li^+ as essentially similar results were obtained both when unstimulated nerves were used and when nerves were depleted of Na^+ by soaking in Ca^{2+}-free choline for 30 min.

With Na^+-loaded nerves, the Ca^{2+} influx from Na^+-ASW was unaffected by pH (6.0–8.5) and by temperature (0–16°), whereas the extra influx from Li^+-ASW tripled with both a rise in pH from 6.0 to 8.5 and an increase in temperature from 0° to 16°.

TABLE I

EFFECT OF CHANGING THE INTERNAL Na^+ CONCENTRATION ON THE Ca^{2+} INFLUX FROM Li^+-ASW

Ca^{2+} uptake (mmoles/kg nerve during a 7-min exposure to ^{45}Ca) is expressed as mean ± S.E. of the mean. Two crabs were used and the number of nerves exposed to each treatment is given in parentheses.

Pretreatment of nerve	*Ca^{2+} uptake*
Unstimulated	0.352 ± 0.030 (3)
Stimulated in Ca^{2+}-free Na^+-ASW	0.796 ± 0.096 (7)
Stimulated in Ca^{2+}-free Li^+-ASW	0.192 ± 0.033 (3)
Soaked for 30 min in Ca^{2+}-free choline–ASW	0.213 ± 0.031 (3)
Soaked for 30 min in Ca^{2+}-free choline–ASW and then stimulated in Ca^{2+}-free Na^+-ASW	0.834 ± 0.053 (3)

The complete unresponsiveness of the Ca^{2+} influx from Na^+-ASW to a wide variety of conditions suggests that much of it might represent some kind of non-specific adsorption which is not easily reversed. While some of the uptake from Na^+-free solutions might reflect a similar phenomenon, that part which is dependent on internal Na^+ seems to represent a rather specific uptake process. There is a striking similarity both qualitatively and quantitatively between the properties of the Ca^{2+} influx into crab nerve and the properties of the Ca^{2+}-dependent Na^+ efflux recently described in squid axons[6]. There is no evidence in crab nerve that Ca^{2+} uptake is necessarily associated with a net loss of Na^+.

Perhaps the most interesting feature of this work is that it provides further evidence to show that Ca^{2+} uptake by nerve is very dependent on the Na^+ concentration inside the cells. If a similar mechanism exists in other cells, marked changes in the level of free intracellular Ca^{2+} might result from quite small changes in internal Na^+. These changes in Ca^{2+} might, in turn, exert very powerful effects on metabolism.

The Physiological Laboratory, P. F. BAKER
Cambridge (Great Britain) M. P. BLAUSTEIN

1 P. F. BAKER, M. P. BLAUSTEIN, A. L. HODGKIN AND R. A. STEINHARDT, *J. Physiol. London*, 192 (1967) 43P.
2 P. F. BAKER, *J. Physiol. London*, 180 (1965) 383.
3 W. WILBRANDT AND H. KOLLER, *Helv. Physiol. Acta*, 6 (1948) 208.
4 H. C. LÜTTGAU AND R. NIEDERGERKE, *J. Physiol. London*, 143 (1958) 486.
5 W. H. FORBES AND F. J. W. ROUGHTON, *J. Physiol. London*, 71 (1931) 229.
6 P. F. BAKER, M. P. BLAUSTEIN, J. MANIL AND R. A. STEINHARDT, *J. Physiol. London*, 191 (1967) 100P.

Received October 27th, 1967