Biochimica et Biophysica Acta, 389 (1975) 506–515

BBA 76943

Ca^{2+}-DEPENDENT INHIBITORY EFFECTS OF Na^+ AND K^- ON Ca^{2+} TRANSPORT IN SARCOPLASMIC RETICULUM VESICLES

CERLI ROCHA GATTASS and LEOPOLDO DE MEIS

Instituto de Biofísica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, GB (Brazil)

(Received October 25th, 1974)

SUMMARY

Effects of Na^+ and K^+ on Ca^{2+} transport by sarcoplasmic reticulum vesicles were studied in a medium containing high Mg^{2+} and ATP (2mM) and low Ca^{2+} (0.44μM) concentrations. Under these conditions, Na^+ and K^+ inhibit Ca^{2+} uptake, ATPase activity and membrane phosphorylation by ATP. Since the concentrations of ATP and Ca^{2+} used are consistent with relaxation in vivo, the results suggest that under physiological resting conditions the Ca^{2+} pump of the sarcoplasmic reticulum operates below its maximal capacity.

INTRODUCTION

A highly efficient ATP-dependent system for Ca^{2+} transport has been described in sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle homogenates. This system plays a key role in the process of excitation-contraction coupling in muscle cells [1–4].

It has been shown that Na^+ and K^+ activate the Ca^{2+} pump in the presence of 0.1 – 4.0 mM ATP and Ca^{2+} concentrations above 10 μM [5–7]. This concentration of Ca^{2+} is in the range of that required to fully activate a muscle fiber [3, 8–9]. In this report, the effects of alkali ions on Ca^{2+} transport were studied, using concentrations of MgATP and Ca^{2+} in the range of those expected to be found in a relaxed living fiber [10–11].

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles

These were prepared from rabbit skeletal muscle as previously described [12]. Ca^{2+} uptake was determined, unless stated otherwise, using a medium consisting of 10mM Tris/maleate buffer (pH 6.85) 2 mM $MgCl_2$, 2 mM ATP, 2 mM Ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 4 mM potassium oxalate,

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

0.2 mM $^{45}CaCl_2$, 0.25 mg sarcoplasmic reticulum vesicles protein/ml and the specified concentrations of NaCl or KCl. The control medium, to which no monovalent cation was added, contained approximately 15 mM K^+ from the other reagents used. The reaction was started by the addition of sarcoplasmic reticulum vesicles and stopped after different incubation intervals at 37 °C by removing the vesicles with Millipore filters [13].

The concentrations of $MgCl_2$ and protein were chosen after 2 sets of preliminary experiments in which the Mg concentration was varied from 0.25 to 4.0 mM and sarcoplasmic reticulum vesicles protein varied from 0.05 to 0.4 mg/ml. The percentage of Ca^{2+} removed from the medium after 12 min was essentially constant in the range of 0.1–3.0 mM Mg and 0.2–0.4 mg sarcoplasmic reticulum vesicles protein/ml, respectively.

Different values for the Ca-EGTA dissociation constant (K_d) have been reported [2, 12, 14–16]. In this paper, the value of $3.95 \cdot 10^{-6}$ M was used [12, 16]. Thus the calculated free Ca^{2+} concentration in the control medium was 0.44 uM. The apparent K_d of the Ca-EGTA complex in the presence of oxalate is not modified by Na^+, K^+ or Li^+ [12].

Ca^{2+} release

Ca^{2+} release was determined after preloading the vesicles in a medium containing 10 mM Tris/maleate buffer (pH 6.85), 2 mM ATP, 2 mM $MgCl_2$, 4 mM potassium oxalate, 1.0 mM EGTA, 1.0 mM $^{45}CaCl_2$ and 2 mg sarcoplasmic reticulum vesicles protein/ml. After a 3-min incubation, the vesicles had taken up more than 95 % of the Ca^{2+}. Calcium release was initiated by diluting the loaded vesicles 20-fold in a medium similar to that described for loading but without Ca^{2+} or ATP. Ca^{2+} efflux was assayed by filtering aliquots of the reaction mixture at different times following dilution and measuring the radioactivity of the filtrate as in the determination of Ca^{2+} uptake.

ATPase activity

ATPase activity assayed as described previously [13] using the medium described for Ca^{2+} uptake, after stopping the reaction by filtration through Millipore filters. The Ca^{2+}-dependent ATPase was calculated as the difference between the activity of the $(Ca^{2+}+Mg^{2+})$dependent ATPase (assayed in the presence of Ca^{2+} and Mg^{2+}) and the activity of the Mg^{2+}-dependent ATPase (assayed in a medium with Mg^{2+} and 2 mM EGTA but without added Ca^{2+}).

E-P formation from [γ-^{32}P]ATP

E-P formation from [γ-^{32}P]ATP was assayed as described by de Meis [17]. The reaction was started by the addition of sarcoplasmic reticulum vesicles protein and stopped after 20 s by adding perchloric or trichloroacetic acid.

RESULTS

The inhibitory effects of Na^+ and K^+ on Ca^{2+} transport by sarcoplasmic reticulum vesicles in a medium containing low Ca^{2+} and high ATP are shown in Fig.1. In this experiment, the Ca^{2+} uptake was measured after a prolonged incubation inter-

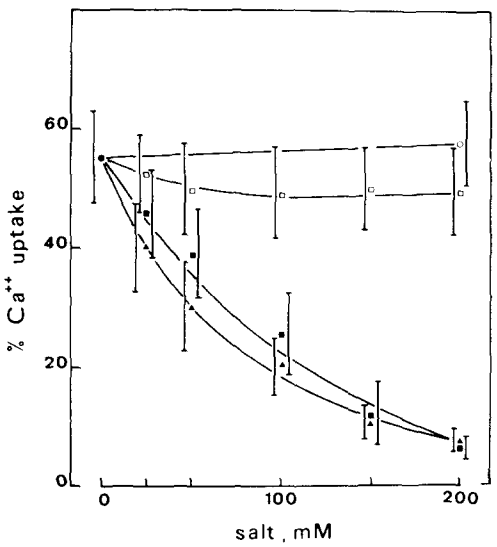

Fig. 1. Effect of alkali ions and sucrose on the Ca^{2+} uptake at the steady state. The assay medium contained 10 mM Tris/maleate buffer (pH 6.85), 2 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA, 4 mM potassium oxalate, 0.2 mM $^{45}CaCl_2$, 0.25 mg sarcoplasmic reticulum vesicles protein/ml and the specified concentrations of salts or sucrose. Incubation time was 12 min. The values represent the average ±S.E. of 4 experiments. Ca^{2+} incorporated in the control medium was 0.43 μmol/mg protein. (●) control, control plus (▲) Na^+, (■) K^+, (□) Li^+ or (○) sucrose.

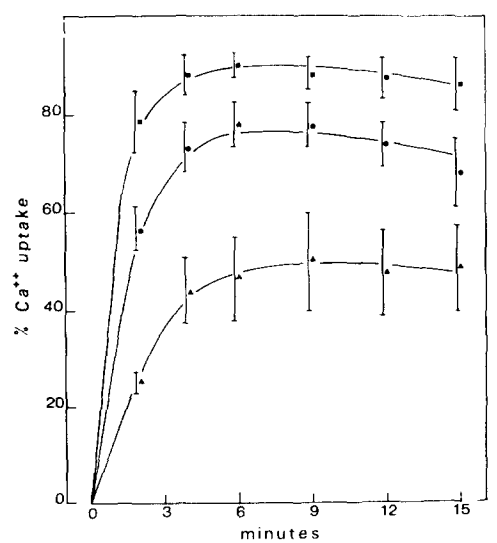

Fig. 2. Ca^{2+} uptake as a function of the initial free Ca^{2+} concentration in the assay medium. For the Ca^{2+} concentrations of (■) 0.98 μM, (●) 0.44 μM and (▲) 0.22 μM, the assay medium contained 0.2 mM $^{45}CaCl_2$ and 1.0, 2.0 and 4.0 mM EGTA, respectively. Other conditions were as described for the control medium in Fig. 1. Each value represents the average ± S.E. of 4 experiments.

val to ensure that maximal Ca^{2+} accumulation was attained, i.e. at the steady state. No inhibition was observed when LiCl or sucrose was added to the assay medium.

In the next experiments, we examined the parameters controlling the percentage of Ca^{2+} removed from the medium by sarcoplasmic reticulum vesicles in the steady state.

Fig. 2 is a control experiment which shows that the percentage of Ca^{2+} removed from the medium by sarcoplasmic reticulum vesicles in the steady state decreases with the initial free Ca^{2+} concentration [3, 19, 20]. After 6 min, maximal accumulation was obtained, implying a balance between the calcium influx due to active transport (i_a) and the calcium efflux due to passive diffusion (e_p). Since oxalate was included in the asssay medium, the free Ca^{2+} concentration inside the vesicles will be fixed by the calcium oxalate solubility product [4]. Table I shows that, independently of the initial free Ca^{2+} concentration of the medium, the equilibrium between i_a and e_p is reached at the same free Ca^{2+} concentration in the medium, i.e. 0.1 μM. Therefore, if the rate efflux depends only on the Ca^{2+} concentration gradient, it should be identical in the 3 steady-state conditions shown in Fig. 2. On the other hand, the rate of active transport decreases with a decrease in the Ca^{2+} of the medium. Thus, as Ca^{2+} is removed by the vesicles, the rate of i_a falls progressively until it reaches that of e_p [4, 20].

The inhibitory effects of Na^+ and K^+ shown in Fig. 1 could be related to a modification of the balance between i_a and e_p, so that the steady state is reached at higher external Ca^{2+} concentrations. In fact, this appears to be the case, as increasing concentrations of Na^+ or K^+ in the assay medium lead to a progressively less effective removal of Ca^{2+} from the medium in the steady state (Fig. 3). Fig. 4 shows that if Na^+ or K^+ is added to the control incubation medium after the equilibrium between i_a and e_p is attained, part of the Ca^{2+} that was accumulated by sarcoplasmic reticulum vesicles is released, and subsequently a new steady state is reached as the rates of i_a and e_p tend to equilibrate at a higher external Ca^{2+} concentration.

In the experiments of Figs 5 and 6, we attempted to distinguish an effect on i_a from an increase in the efflux. Since Na^+ and K^+ have no effect on the passive efflux (Fig. 5), but decrease the initial rate of Ca^{2+} uptake (Fig. 6), we infer that the inhibitory effect of these ions shown in Figs 1, 3 and 4 are due to a depression of the active Ca^{2+} influx.

Active Ca^{2+} transport is mediated by a membrane-bound ATPase which is highly sensitive to changes in the free Ca^{2+} concentration of the medium. In the process of ATP hydrolysis, the γ-phosphate of ATP is covalently bound to a membrane

TABLE I

IONIC Ca^{2+} IN THE ASSAY MEDIUM AT THE STEADY STATE

The experimental conditions were as described for Fig. 2. The incubation time was 6 min. Each value represents the average $\pm$S.E. of 8 experiments.

Initial free Ca^{2+} in the medium (μM)	0.22	0.44	0.98
Ionic Ca^{2+} at the steady state (μM)	0.10$\pm$0.01	0.10$\pm$0.01	0.09$\pm$0.02

protein. This phosphoprotein (E-P) represents an intermediate product in the sequence of reactions leading to Ca^{2+} transport and P_i liberation [16, 19, 21]. If Na^+ and K^+ interfere with active Ca^{2+} transport, they should also modify the Ca-dependent ATPase and E-P formation.

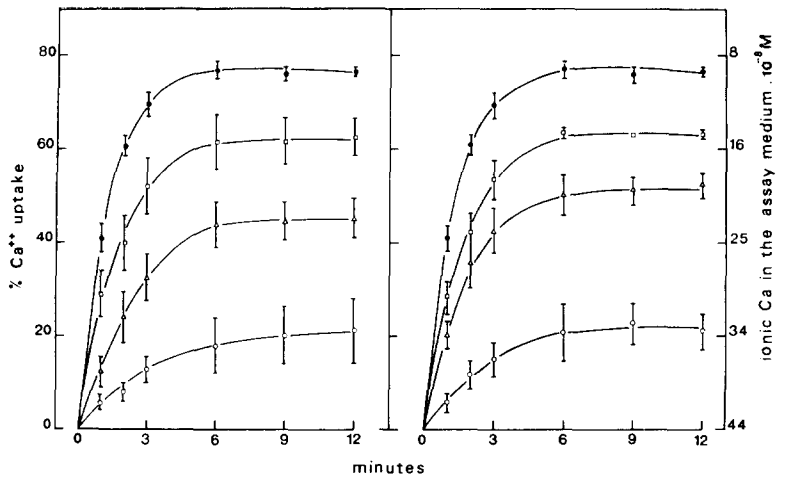

Fig. 3. Effects of different Na^+ and K^+ concentrations on the time course of Ca^{2+} uptake. The assay medium as described in the legend to Fig. 1 contained in addition (●) none, (□) 50 mM, (△) 100 mM, (○) 200 mM NaCl (left) or KCl (right). The values represent the average ±S.E. of 3 experiments.

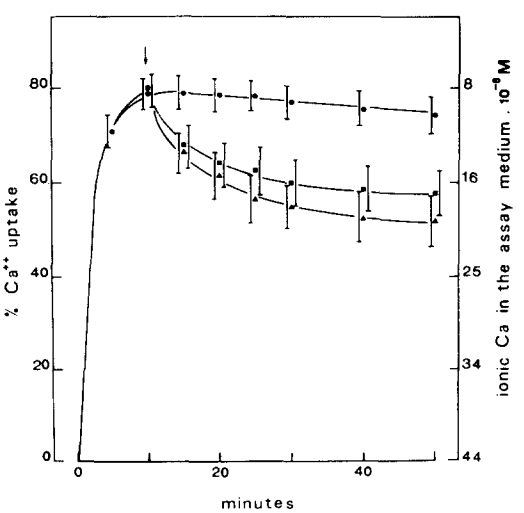

Fig. 4. Effects of the addition of Na^+ or K^+ to a system in which the Ca^{2+} transport is in the steady state. After 10 min incubation in the control medium described in Fig. 1 (●), 200 mM NaCl (▲) or KCl (■) was added to the medium. Each value represents the average ±S.E. of 5 experiments.

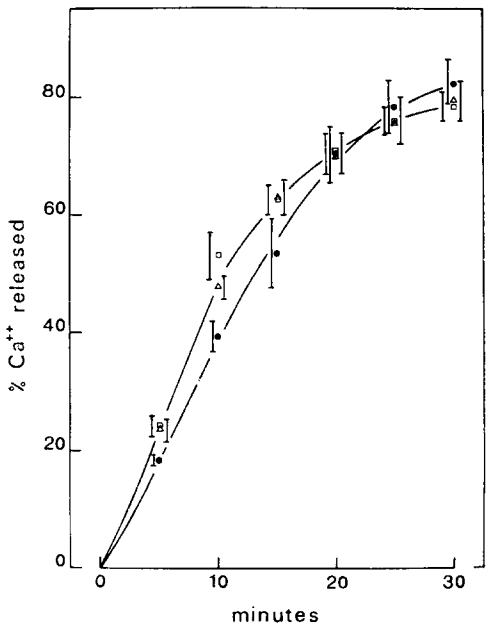

Fig. 5. Passive efflux of Ca^{2+} in the presence of Na^+ and K^+. Vesicles pre-loaded as described in Methods were diluted to a final concentration of 0.1 mg protein/ml in media containing 10 mM Tris/maleate buffer (pH 6.85), 2 mM $MgCl_2$, 4 mM potassium oxalate, 1 mM EGTA, and (●) no added ions, (□) 200 mM NaCl or (△) 200 mM KCl. Other experimental conditions were as described under Methods. Each value represents the average ±S.E. of 4 experiments.

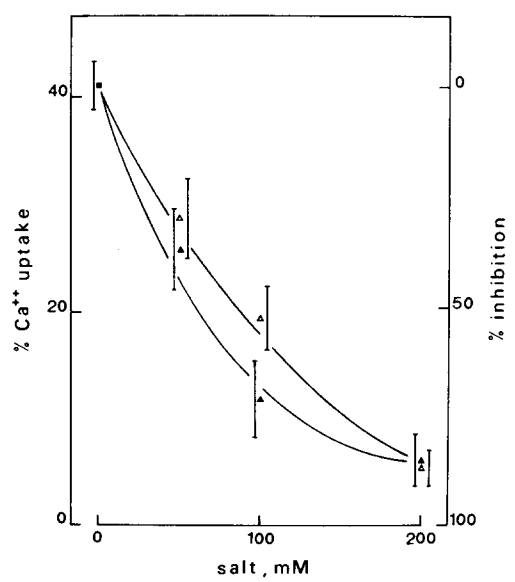

Fig. 6. Effects of Na^+ and K^+ on the initial rate of Ca^{2+} uptake. NaCl (▲) or KCl (△) in the concentrations indicated were added to the control medium (■) described in Fig. 1. The reaction was started by the addition of sarcoplasmic reticulum vesicles and stopped after a 1-min incubation by Millipore filtration. Each value represents the average ±S.E. of 4 experiments.

Fig. 7 shows that the addition of 200 mM Na^+ or K^+ to the assay medium results in inhibition of the Ca-dependent ATPase activity. The inhibition is evident in the first minutes of the reaction. As the steady state is reached (after 6 min, cf. Figs 2 and 3), the effect of the alkali ions virtually disappears. At this point, the higher ex-

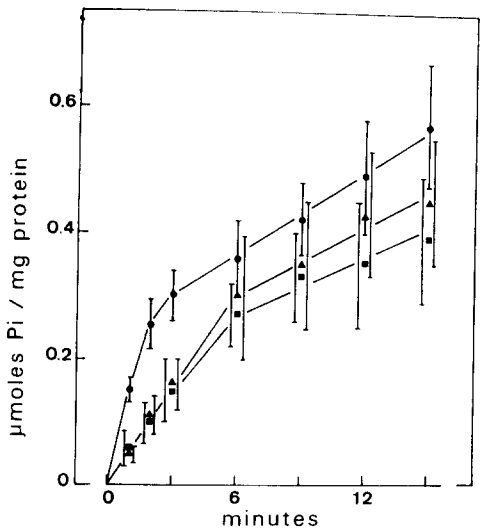

Fig. 7. Effects of Na^+ and K^+ on the time course of the Ca^{2+}-dependent ATPase. The Ca-dependent ATPase shown was the difference between the activities of the (Ca^{2+} + Mg^{2+})-dependent ATPase and Mg^{2+}-dependent ATPase assayed as described in Methods. The values represent the average ±S.E. of 6 experiments. Control (●), control plus 200 mM NaCl (■) or KCl (▲).

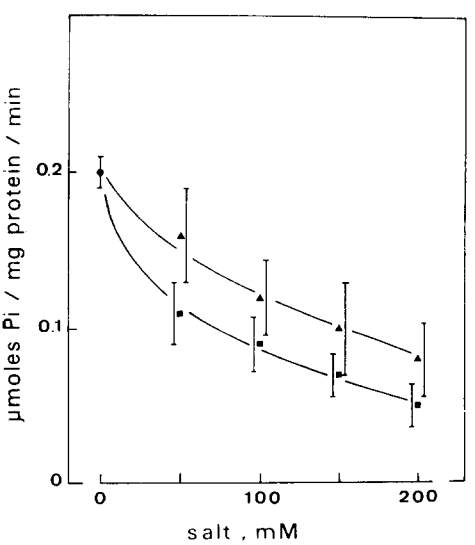

Fig. 8. Inhibition of Ca^{2+}-dependent ATPase activity by different Na^+ and K^+ concentrations. The experimental conditions were as described for Fig. 7 but with the indicated concentrations of (■) NaCl or (▲) KCl added to the control (●) medium. Incubation time was 1 min. Each value represents the average ±S.E. of 4 experiments.

ternal Ca^{2+} concentration in media containing Na^+ or K^+ is probably compensating for their inhibitory effects on the rate of hydrolysis.

The effect of a range of Na^+ and K^+ concentrations on the initial rate of ATPase activity is shown in Fig. 8.

TABLE II

EFFECTS OF ALKALI IONS ON E-P FORMATION

The assay media (see Methods) contained 0.2 mM $CaCl_2$ plus 1 mM EGTA (free $Ca^{2+} = 0.98\,\mu M$) or 0.1 mM $CaCl_2$ and no EGTA. The incubation time was 20 s. The values represent the average ±S.E. of the number of experiments indicated in parentheses.

Additions to assay medium	μmol E-P/g protein	
	0.98 μM initial Ca^{2+}	100 μM initial Ca^{2+}
none	0.58±0.06 (14)	1.68±0.10 (5)
50 mM NaCl	0.35±0.07 (9)	
100 mM NaCl	0.14±0.06 (6)	1.86±0.12 (5)
50 mM KCl	0.41±0.07 (7)	
100 mM KCl	0.29±0.11 (6)	1.81±0.09 (5)

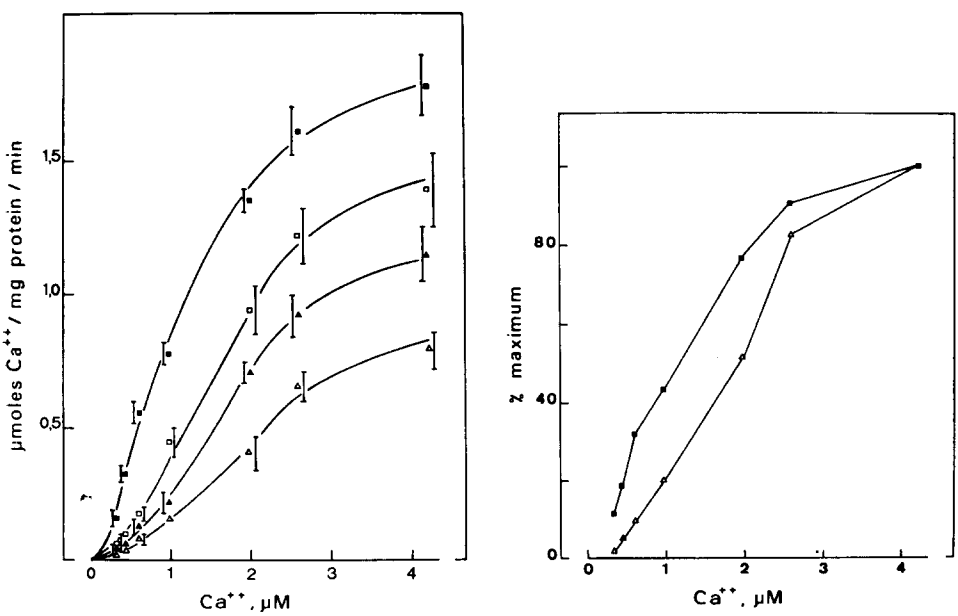

Fig. 9. Initial rate of Ca^{2+} uptake as a function of free Ca^{2+} concentration; effect of NaCl. (a) The incubation medium described for Fig. 1 was altered by the addition of different concentrations of EGTA (3.0–0.38 mM) to obtain the indicated free Ca^{2+} concentrations in the presence of (■) zero, (□) 50 mM, (▲) 100 mM or (△) 200 mM NaCl. The reaction was started by the addition of 0.2 mg sarcoplasmic reticulum vesicles protein/ml for the two lowest Ca^{2+} concentrations, 0.1 mg protein ml, for the next three Ca^{2+} concentrations and 0.05 mg protein/ml for the higher Ca^{2+} concentrations. The incubation time was 30 s. Each value represents the average ±S.E. of 5 experiments. (b) The data obtained for the initial rates of Ca^{2+} uptake in zero (■) or 200 mM NaCl (△) in the experiment of Fig. 9 are normalized to the highest initial rate shown for each condition (at 4.2 μM Ca^{2+}).

Na^+ and K^+ also inhibit the E-P formation when the free Ca^{2+} concentration of the medium is below 1.0 μM (Table II). As has also been shown by de Meis [22], this inhibition is no longer observed in high Ca^{2+} concentrations.

The differences observed in high and low Ca^{2+} in the effects of Na^+ and K^+ on i_a (Figs 6–8 and Table II) suggest that they act by altering the Ca^{2+} sensitivity of the pump. To test this possibility we measured the initial rate of Ca^{2+} accumulation over a range of Ca^{2+} concentrations on the presence of various concentrations of one of the alkali ions.

Three effects of NaCl can be seen in Fig. 9a; a depression of the maximal rate of Ca^{2+} uptake at 4.2 μM Ca^{2+}, a decrease in sensivitity to Ca^{2+} and a change in the form of the dependence on Ca^{2+}. All three effects must contribute to the inhibition of Ca^{2+} uptake. At low Ca^{2+} concentrations, however, the inhibition is proportionally greater: at 4.2 μM Ca^{2+}, the rate in 200 mM NaCl is 45 % of that in the absence of Na^+, but at 0.4 μM Ca^{2+} this rate is only 12 % of the control. When the effect on the rate at 4.2 μM is eliminated graphically by normalizing each curve to its maximum (Fig. 9b), it becomes obvious that the marked inhibitory effects observed at low Ca^{2+} can be attributed mainly to the increased sigmoidicity or decreased sensitivity to Ca^{2+}.

DISCUSSION

Previous reports have shown that in the presence of 0.1 – 4.0 mM ATP and Ca^{2+} concentrations above 10 μM, Na^+ and K^+ do not inhibit Ca^{2+} transport but rather activate the Ca^{2+} pump [5–7]. Conversely, when the ATP concentration is between 2 and 10 μM, Na^+ and K^+ inhibit both Ca^{2+} uptake and ATPase activity [5, 17]. In this paper, it is shown that in low Ca^{2+} concentrations, Na^+ and K^+ significantly inhibit Ca^{2+} uptake (Figs 1 and 3), Ca-dependent ATPase (Figs 7 and 8) and E-P formation (Table II) when the ATP concentration in the medium is 2 mM. As was also observed for the high Ca^{2+} and low ATP condition [5], the inhibitory effects of Na^+ and K^+ appear to be specific and not related to the osmotic or ionic strength of the medium (Fig. 1). As shown in Fig. 9, these inhibitory effects are probably related to a decrease in the apparent Ca^{2+} affinity of the pump. One result is a decrease in the ability of the sarcoplasmic reticulum vesicles to maintain a low Ca^{2+} concentration in the medium.

The Ca^{2+} and ATP concentrations at which an inhibition by the alkali ions is observed are in the range of those expected to be found in the sarcoplasm of a relaxed muscle fiber. Therefore in a resting muscle fiber, the Ca^{2+} pump of the sarcoplasmic reticulum may be operating below maximal capacity due to the K^+ concentration of the sarcoplasm. A possible drawback to this hypothesis is the use in our reaction medium of oxalate concentrations which are not found in psysiological conditions. Several reports have shown that oxalate increases the calcium storage capacity of the vesicles, providing a sink for the entering calcium, without modifying the properties of the pump [18, 23–25]. Oxalate merely serves to reduce the concentration of the vesicles required to lower the Ca^{2+} in a solution to the level found in the cytoplasm of a relaxed muscle cell. Relaxation of myofibrils by sarcoplasmic reticulum vesicles has been obtained 'in vitro' both in the presence and in the absence of oxalate [2, 3, 23, 26, 27].

ACKNOWLEDGEMENTS

This investigation was supported in part by the Conselho Nacional de Pesquisas, Brasil, Conselho de Ensino para Graduados da U.F.R.J., Brasil and by the Banco Nacional de Desenvolvimento Econômico (Funtec 241). The authors are grateful to Dr Martha Sorenseon for helpful discussion of the manuscript. C.R.G. is a recipient of a Fellowship from the Coordenação do Aperfeiçoamento de Pessoal de Nível Superior (Capes).

REFERENCES

1 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518–528
2 Ebashi, E. (1961) J. Biochem. (Tokyo) 50, 236–244
3 Hasselbach, W. (1964) Progr. Biophys. Mol. Biol. 14, 167–222
4 Hasselbach, W. (1972) in Molecular Bioenergetics and Macromolecular Biochemistry (Weber, H. H., ed.), pp. 149–171, Springer Verlag, Berlin
5 de Meis, L. (1971) J. Biol. Chem. 246, 4764–4773
6 Rubin, B. B. and Katz, A. M. (1967) Science 158, 1189–1190
7 Duggan, P. F. (1968) Life Sci. 7, 913–919
8 Brandt, P. W., Reuben, J. P. and Grundfest, H. (1972) J. Gen. Physiol. 59, 305–317
9 Ebashi, S. and Endo, M. (1968) Progr. Biophys. Mol. Biol. 18, 123–183
10 Portzehl, H., Caldwell, P. C. and Ruegg, J. C. (1964) Biochim. Biophys. Acta 79, 581–591
11 Infante, A. A. and Davies, R. E. (1962) Biochem. Biophys. Res. Commun. 9, 410–415
12 de Meis, L. and Hasselbach, W. (1971) J. Biol. Chem. 246, 4759–4763
13 de Meis, L. (1969) J. Biol. Chem. 244, 3733–3739
14 Schwarzenbach, G., Senn, H. and Anderegg, G. (1957) Helv. Chim. Acta 40, 1886–1900
15 Holloway, J. H. and Reilley, C. N. (1960) Anal. Chem. 32, 249–256
16 Ogawa, Y. (1968) J. Biochem. (Tokyo) 64, 255–257
17 de Meis, L. (1972) Biochemistry 11, 2460–2565
18 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329–369
19 Friedman, Z. and Makinose, M. (1970) FEBS Lett. 11, 69–72
20 Inesi, G. (1972) Annu. Rev. Biophys. Bioeng. 1, 191–210
21 Inesi, G., Maring, E., Murphy, A. J. and McFarland, B. H. (1970) Arch. Biochem. Biophys. 138, 285–294
22 de Meis, L. and de Mello, M. C. F. (1973) J. Biol. Chem. 248, 3691–3701
23 Hasselbach, W. (1964) Fed. Proc. 23, 909–912
24 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648–658
25 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 659–668
26 Baird, G. D. and Perry, S. V. (1960) Biochem. J. 77, 262–271
27 Weber, A., Herz, R. and Reiss, I. (1963) J. Gen. Physiol. 46, 679–702