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CALCIUM TRANSPORT IN ISOLATED GUINEA-PIG ATRIA DURING METABOLIC INHIBITION*

HANS GÜNTHER LAHRTZ, HEINZ LÜLLMANN AND PIETER A. VAN ZWIETEN
Department of Pharmacology, Christian-Albrechts-University, Kiel (W.-Germany)

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SUMMARY

Investigations were performed in order to study whether in guinea-pig atrial tissue the transport of Ca^{2+} across the cell membrane into the extracellular space occurs by means of an energy-requiring active transport. The turnover of ^{45}Ca and the total calcium content were established after metabolic inactivation. Inhibition of energy metabolism was induced by cooling the isolated atria from 30° to either 15° or 1°. In order to determine whether metabolic inactivation was really achieved by this treatment, cellular potassium and sodium were also determined after cooling.

1. During hypothermia cellular sodium increased, whereas the potassium content was lowered. This effect indicates that metabolic inactivation has really taken place.

2. The total cellular calcium content of control preparations proved to be 345 ± 3 nequiv/100 mg tissue (wet wt.). Lowering of the temperature to 15° provoked an increase in the calcium concentration to a final level of 462 ± 28 nequiv/100 mg. At 1° this increase was even more pronounced and reached a value of 578 ± 44 nequiv/100 mg tissue after incubation for 150 min.

3. During hypothermia the release of ^{45}Ca was inhibited in comparison with that by control organs: at 30° electrically stimulated atria lost 50% of the initial ^{45}Ca after 5 min; for resting left auricles this value was 8 min. At 15°, 20 min were required for this percentage of release and at 1° even 45 min.

4. During hypothermia (15°) the uptake of ^{45}Ca by the cell was only slightly diminished.

5. The results obtained after hypothermia suggest that the transport of Ca^{2+} out of the heart-muscle cell may occur by means of an energy-requiring calcium pump mechanism.

INTRODUCTION

In heart-muscle tissue, the concentration of cellular Ca^{2+} is believed to increase

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considerably during the excitation process. The following two phenomena may be responsible for this rise in concentration: (1) during the excitation process the amount of Ca^{2+} within the cell rapidly rises, owing to the mobilization of these ions from firmly bound fractions (in analogy to observations in skeletal muscle see WEBER, HERZ AND REISS[1], PORTZEHL, CALDWELL AND RUEGG[2]) and (2) simultaneously an accelerated movement of Ca^{2+} from the extracellular space into the cell is taking place during excitation (see refs. 3–7). In order to restore the situation prior to the excitation process, *i.e.* the low Ca^{2+} content of the cells ($< 1\ \mu M$), a redistribution of these ions must necessarily take place. The reduction of the high cellular Ca^{2+} level to its normal low value at rest is effected by two different processes: (1) reabsorption and binding of Ca^{2+} by the endoplasmatic reticulum and (2) transport of Ca^{2+} across the membrane into extracellular space. HASSELBACH AND MAKINOSE[8,9] have given evidence that the former process, *i.e.* the storage of Ca^{2+} by the endoplasmatic reticulum, proceeds by means of an active transport mechanism.

The occurrence of the second process, *i.e.* the transport of Ca^{2+} out of the cells, has been demonstrated by means of efflux experiments in resting and contracting heart muscle[4,5]. This transport has to proceed against considerable chemical and electrical gradients, as shown in the schematic representation of the distribution and movement of calcium in heart muscle (Fig. 1). In view of this gradient, the existence of a calcium pump mechanism for this transport process might be assumed. Such a mechanism would be expected to be similar to the well-known sodium pump.

Recently, SCHATZMANN[10] was able to demonstrate that human erythrocytes may transport Ca^{2+} out of the cells against a concentration gradient by means of an ATP-requiring, active transport mechanism. The present paper deals with experi-

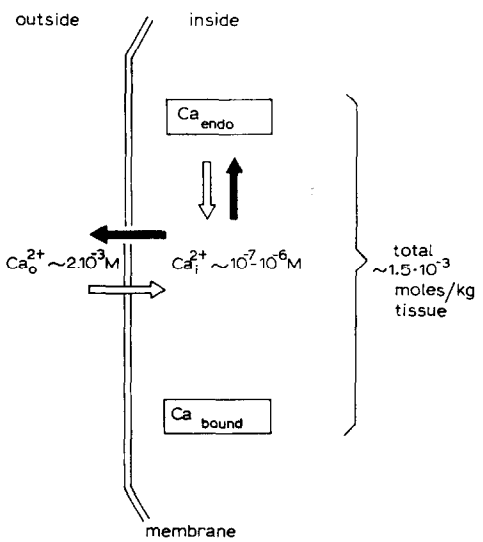

Fig. 1. Schematic representation of the various calcium fractions in heart-muscle cells. Ca_o^{2+}, extracellular Ca^{2+} (about 2 mM); Ca_i^{2+}, cellular calcium in ionized form (order of magnitude $1–0.1^{-1}\ \mu M$); Ca_{endo}, calcium in the endoplasmatic reticulum; Ca_{bound}, firmly bound calcium, which does not participate to exchange processes. Total calcium content of atrium-muscle tissue: about $1.57 \cdot 10^{-3}$ mole/kg wet wt. Open arrow, passive calcium flow; black arrow, active calcium transport.

ments carried out in order to obtain information on the characteristics of calcium transport out of heart-muscle cells. In order to establish whether a calcium pump may exist within the membrane, the calcium flow as well as the total calcium content of isolated guinea-pig atria were measured during metabolic inhibition, induced by cooling the organs to either 15° or 1°. If a calcium pump mechanism does indeed exist, a reduction in calcium outflow would be expected after inactivation of energy-producing reactions at the low temperatures involved. Logically, a reduced outflow could be expected to lead to a rise in the total cellular calcium content. The results obtained are in agreement with the existence of the postulated calcium pump mechanism.

METHODS

Isolated guinea-pig atria were prepared and incubated as described in detail by HODITZ AND LÜLLMANN[5]. Cooling of the organ bath to either 15° or 1° was carried out by means of a liquid-cooling device (Colora Messtechnik, model FK 2300). In the uptake experiments the atria were equilibrated for 60 min at 15° or at 1° and subsequently transferred to a $^{45}Ca^{2+}$-containing tyrode solution of the same temperature. The atria were incubated in the radioactive solution for various periods from 3 to 120 min. After incubation, the organs were blotted dry under standard conditions, weighed, and subjected to a wet-ashing procedure described below.

Electrically stimulated atria and resting left auricles incubated at 30° were used as controls. For the electrical stimulations, a Grass-Stimulator S-4H was used. Parameters: duration, 3 msec; frequency of beating, 180/min; supramaximal stimulation.

The release of calcium by isolated heart-muscle tissue was studied as follows. After equilibration, the atria were incubated for 90 min in a $^{45}Ca^{2+}$-containing tyrode solution which was cooled to either 15° or 1°. After this period the uptake of ^{45}Ca had reached equilibrium. Subsequently, the organs were transferred to a non-radioactive tyrode solution of the same temperature and incubated for various periods. After uptake or release experiments as described above the total calcium content and the concentration of ^{45}Ca were determined by methods described below.

Determination of ^{45}Ca. The wet-ashing of the weighed organs was carried out as follows: per 100 mg tissue (wet wt.) 0.75 ml of the digestion acid (65% HNO_3 and 60% $HClO_4$, ratio 1:1, v/v) was added. The tubes (Duran glass, height 130 mm, diameter 10 mm) which contained the atria and the acid were placed in holes in a block of aluminum. The holes were of such a depth that the glass tubes were almost entirely surrounded. The block was heated electrically in two stages: 2 h at 140° and subsequently 4–6 h at 210°. These temperatures are critical, since at higher temperatures polyphosphates are formed which may interfere with the calcium determination[11]. The dry residue was taken up in a mixture of 0.2 ml 0.1 M HCl and 2 ml ethanol. 1 ml of the solution thus obtained was transferred to a counting vial containing 5 ml dioxane *plus* 5 ml of a solution of 3 g 2,5-diphenyloxazole in 1 l of toluene. Radioactivity was determined by means of a Packard Tri-Carb liquid-scintillation spectrometer (model 3002); gain 28%, window aperture 50–1000. Under these conditions 10 mμC yielded approx. 15 000 counts/min. Background, 18–25 counts/min.

Determination of the total calcium content. The rest (1 ml) of the acid–alcoholic solution left after determination of ^{45}Ca (see above) was evaporated to dryness at 110°. The residue was redissolved in 0.1 M HCl. For the determination of calcium in the solution thus obtained, the spectrofluorimetric method of ZEPF[11] was employed (spectrofluorimeter: Zeiss ZFM 4 C with two monochromators; xenon lamp XBO 450 W/P). The sensitivity of this method allows the accurate determination of the calcium level in 10 mg tissue (wet wt.).

Determination of sodium and potassium. After wet-ashing as described above and appropriate dilution of the solution thus obtained, the two ions were determined by means of a flame-photometric procedure (flame photometer: Eppendorf Model, Netheler and Hinz, Ltd.).

Mechanograms. Isolated guinea-pig atria were suspended as usual in oxygenated tyrode solution. The spontaneously occurring contractions were recorded *via* a transducer and a Heleoscriptor (Type HE–86 t) recording device.

Extracellular space. For the calculation of the cellular calcium and ^{45}Ca concentrations, the value 0.35 ml/g, established by LÜLLMANN AND VAN ZWIETEN[12] by means of [^{14}C]saccharose was used for the organs.

RESULTS

Influence of cooling on the mechanical activity of guinea-pig atria

First the influence of cooling on the frequency of beating and on the amplitude of contraction was studied by means of mechanograms. A typical mechanogram is

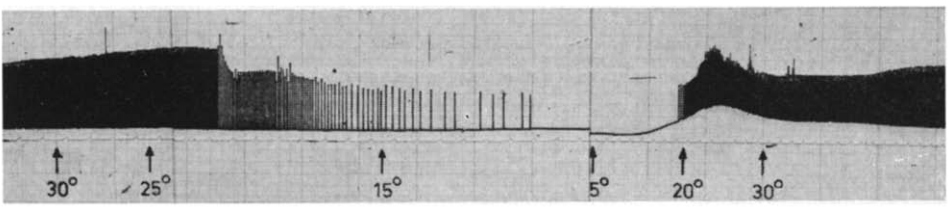

Fig. 2. Isometrically recorded mechanogram of a spontaneously beating guinea-pig atrium upon cooling and rewarming.

represented in Fig. 2. Cooling of the organ bath from 30° to 25° provoked an increase in the contraction amplitude and the frequency of the spontaneously beating atria was simultaneously diminished. At approx. 18° the contraction amplitude decreased. At that temperature only sluggish contractions occurred, which became slower and slower upon further cooling. The mechanical activity of the atria ceased at about 10°. Upon rewarming the organ bath a transient contracture developed when the mechanical activity restarted. The contracture disappeared with increasing temperature and the atria started to beat again. Soon after the original temperature (30°) had been reached again, the frequency and contraction amplitude attained their initial values. Even after repeated cooling and subsequent rewarming, no impairment of the normal contractile force and of the frequency could be demonstrated.

Influence of hypothermia on potassium and sodium content

The calcium flow and the total calcium content were measured both at 15° and at 1°. In order to be sure that metabolism was really inactivated by the cooling procedure, both the sodium and potassium concentrations of the atria thus treated were determined. The temperature-dependent changes in the concentrations of these two ions are represented in Fig. 3. Under control circumstances, *i.e.* at 30°, the sodium and potassium levels remained unchanged throughout the experiment (regression coefficients: 0.01 mequiv/min for K^+, 0.02 mequiv/min for Na^+; neither of the two values differs significantly from zero). At 30°, the following concentrations were obtained: Na^+, 16.2 ± 0.80 mequiv/l fibre water; K^+, 139.6 ± 0.85 mequiv/l. Upon cooling, however, the sodium content of the cell increased, whereas the potassium

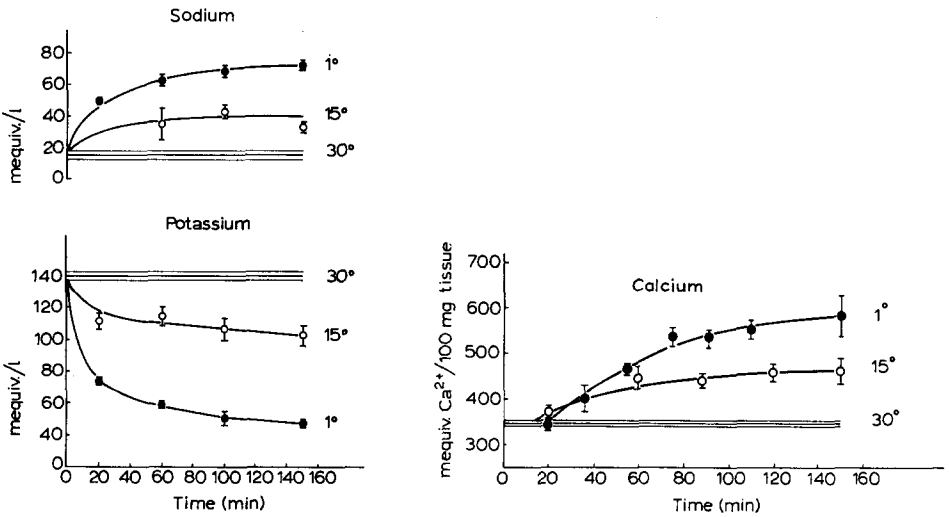

Fig. 3. Cellular concentrations of sodium and potassium in isolated atria after metabolic inactivation by hypothermia. Concentration (equiv/l cellular water) plotted against incubation time (min). At 30° the regression coefficients for both sodium (0.02 mequiv/min) and potassium (0.01 mequiv/min) do not significantly differ from zero. Each point on the curve represents the mean of at least 20 atria (mean ± S.E.M.). ○—○, atria cooled to 15°; ●—●, atria cooled to 1°.

Fig. 4. Total cellular calcium concentration of isolated atria at three different temperatures. Calcium content (nequiv/100 mg wet wt.) plotted against incubation time (min). Controls: resting left auricles and electrically stimulated atria. The regression coefficient $b = 0.05$ nequiv/min is not significantly different from zero. ○—○, non-stimulated atria at 15°; ●—●, non-stimulated atria at 1°. Each point represents the mean value of at least 20 atria (mean ± S.E.M.).

concentration decreased, both changes being related to the reduction in the temperature. From these findings it may be concluded that the active transport of these ions has been inhibited by the hypothermia: the cell has lost its ability to maintain the normal sodium and potassium concentration gradients.

Changes in total calcium content during hypothermia

The changes in total calcium content of the atria, induced by incubation up to 150 min at the three temperatures studied (30°, 15° and 1°) are represented in Fig. 4. The calcium content of the control atria (30°) proved to be 345 ± 3 nequiv/

100 mg tissue* (wet wt.). This concentration remained unchanged during the entire period (150 min) of incubation. The regression coefficient was 0.05 nequiv calcium/min. These findings confirmed the experiments described by WINEGRAD AND SHANES[13]. HODITZ AND LÜLLMANN[4,5] have already demonstrated that resting and contracting atria have the same calcium content.

Upon cooling of heart-muscle tissue its calcium concentration increased, dependent on the temperature. This observation implies that calcium from the tyrode solution is taken up by the cells. In the experiments carried out at 15° the calcium concentration reached equilibrium at a higher level after approx. 70 min. At 1°, however, the calcium content was still increasing slowly even after an incubation period of 150 min.

Uptake and release of ^{45}Ca during hypothermia

As described before (see HODITZ AND LÜLLMANN[4,5]), at 30° resting left auricles took up $^{45}Ca^{2+}$ more slowly than beating atria at the same temperature. Cooling to 15° hardly affected the uptake velocity of ^{45}Ca, although at 1° the accumulation was considerably retarded (Fig. 5).

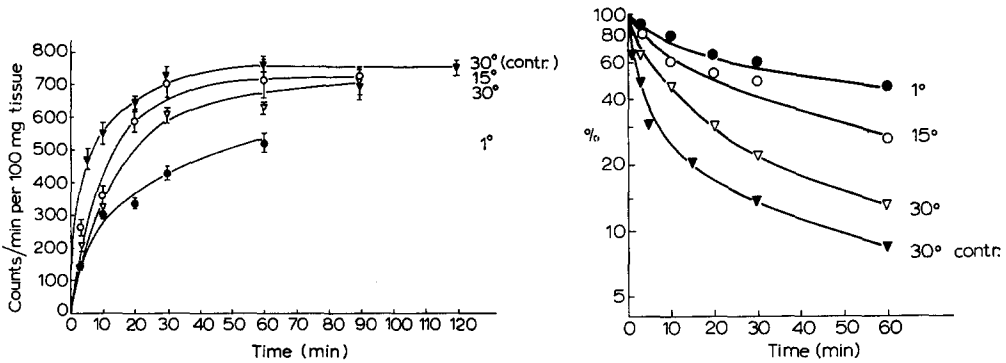

Fig. 5. Uptake of ^{45}Ca by isolated atria and by left auricles at different temperatures. Tissue radioactivity (counts/min per 100 mg tissue) plotted against incubation time in the radioactive solution. ▼—▼, electrically stimulated atria, 30°; ▽—▽, resting left auricles, 30°; ○—○, spontaneously beating atria, low frequency, 15°; ●—●, non-stimulated, resting atria, 1°.

Fig. 6. Release of ^{45}Ca by guinea-pig atria at three different temperatures, plotted in a semi-logarithmic system. Cellular content of ^{45}Ca (in % of initial level) plotted against the time of incubation of the atria in inactive tyrode solution. ▼—▼, electrically stimulated atria, 30°; ▽—▽, resting left auricles, 30°; ○—○, spontaneously beating atria, low frequency, 15°; ●—●, non-stimulated, resting atria, 1°.

The release of ^{45}Ca by isolated atria at the temperatures involved was of particular interest for our investigations. Fig. 6 shows the release of ^{45}Ca from atria at the three different bath temperatures studied. The contracting heart muscle at 30° rapidly lost ^{45}Ca. Even after about 2.5 min 50% of the ^{45}Ca originally taken up had been released. At the same temperature the outflow of ^{45}Ca from resting left

* The exceptionally low value of the S.E. (less than 1%) may be explained by the fact that the mean of an unusually large number of atria ($n = 395$) has been calculated.

auricles proved to be considerably slower than that from contracting organs: half of the initial amount of ^{45}Ca had been lost by the resting auricles after 8 min. The release of ^{45}Ca was retarded even more by cooling. At 15° approx. 20 min were necessary for the release of 50% of the initial ^{45}Ca; at 1° a period of 45 min was required for the same percentage of wash-out.

For all calculations concerning experiments at reduced temperature, the same value for extracellular space (0.35 ml/g wet wt.) was used as that determined at 15° or 30° (ref. 12).

DISCUSSION

Hypothermia, to the extent applied in our experiments did not provoke measurable damage of the guinea-pig atria. Even after repeated cooling and rewarming both the frequency and the contractile force regained entirely normal values. Moreover, no morphological alteration whatsoever could be observed under the electron microscope (E. LINDNER, personal communication). Only after incubation of the atria for at least 1 h at 1° was the cell membrane affected. In other investigations we have observed that after incubation for longer than 1 h at 1° [^{14}C]-saccharose penetrates into the cells considerably more rapidly than at either 15° or 30° (LÜLLMANN AND VAN ZWIETEN, unpublished experiments).

In order to demonstrate that in our experiments metabolism had been inactivated, the influence of hypothermia upon the cellular sodium and potassium contents was established. After cooling of the atria, the sodium concentration of the tissues markedly increased, whereas the potassium level diminished. These findings confirm that hypothermia as applied in our investigations really leads to the inactivation of metabolism. In heart-muscle tissue there exists a considerable chemical and electrical gradient for Ca^{2+} from the extracellular space towards the inside of the cells. Accordingly, the existence of an active outward transport of Ca^{2+} might be considered. The experiments described in the present paper are in agreement with such a hypothesis. After metabolic inactivation by means of hypothermia, the calcium outflow markedly decreased (Fig. 6). At 15° the uptake of $^{45}Ca^{2+}$ was somewhat retarded: more so, however, at 1°. Still, the considerable increase of total calcium as shown in Fig. 3 indicates that hypothermia has inhibited the outflow to a far greater extent than the passive inflow. Under normal circumstances an equilibrium exists between the in- and outflow of Ca^{2+}. Consequently, the inhibition of the outward transport is bound to cause a rise in the total cellular calcium content when the passive inflow is but slightly impaired. In our view, the alterations of calcium outflow and that of the total cellular calcium concentration during hypothermia support the assumption that the outward movement of Ca^{2+} proceeds by means of an energy-requiring, active transport mechanism (see Fig. 1).

A recent report by SCHATZMANN[10] suggests that an active outward transport mechanism for Ca^{2+} occurs in human erythrocytes. According to this author, erythrocytes maintain their cellular calcium content at a low level by means of an active transport mechanism, which obtains the energy required from hydrolysis of ATP. Its functioning seems to be independent of the sodium pump. From our experiments it cannot be concluded whether the presumed calcium pump mechanism is linked to the sodium pump. Previous investigations have demonstrated that a low concen-

tration of ouabain, which clearly influences cellular calcium distribution, does not alter the cellular sodium and potassium contents and fluxes[14].

We have also attempted to provoke metabolic inhibition by means of inhibitors like 2,4-dinitrophenol and monoiodoacetic acid. The results obtained with these two inhibitors were not too satisfactory, although they did not conflict with our assumption of active outward transport for Ca^{2+}.

As shown in Fig. 2, hypothermia led to an initial increase in the contraction amplitude, which was followed by a decrease in this parameter at temperatures below 20°. The positive inotropic effect of hypothermia which has been described previously by others (see refs. 15–17), may be attributed to an increased cellular concentration of Ca^{2+}. The decreased efflux of Ca^{2+} at low temperatures, as described in this paper, provokes such a rise in concentration, which is also reflected by the increased total cellular calcium level. Moreover, the energy-requiring reabsorption of of Ca^{2+} by the endoplasmatic reticulum is likely to be inhibited as well, thus preventing the deionization of Ca^{2+}. A transient contracture of the atria was observed when the temperature was rised after hypothermia. The considerably increased concentration of Ca^{2+} mentioned above probably leads to a maximal saturation of the heart-muscle cell with these ions, the deionization being inhibited. This increased cellular level of Ca^{2+} may be responsible for the contracture observed after rewarming. Further rewarming reactivates the energy-requiring storage of Ca^{2+} as well as the active outward transport mechanism. Consequently, the Ca^{2+} concentration is reduced and contracture disappears.

To recapitulate, the experiments with isolated guinea-pig atria, subjected to hypothermia, in our opinion indicate that the transport of Ca^{2+} across the cell membrane into the extracellular space occurs by means of an energy-requiring calcium pump mechanism.

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