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PERPETUATION OF MISINTERPRETATIONS DUE TO LACK OF METHODICAL INSIGHT. A CRITICAL RE-EVALUATION OF THE DETERMINATION OF ^{45}Ca RELEASE FROM INTACT GUINEA-PIG ATRIA

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1. The evaluation of still more pretentious and complicated methods is accompanied by a decline of methodical knowledge outside of the own technical field. Interpretations or extrapolations are taken as granted without critical examination of the methodical steps applied. An example is given by re-evaluating the ^{45}Ca release from isolated cardiac tissue and the possible interpretations.

2. ^{45}Ca release and tissue Ca content were measured in isolated guinea-pig left atria during Ca equilibrium and under conditions known to induce net Ca movements.

3. At equilibrium condition (1.8 mM Na^{2+}_o) 3 exponential phase of ^{45}Ca release from the atria were observed. The compartments contained 61%, 29% and 10% of total ^{45}Ca; the $t_{1/2}$ were 2, 12 and 90 min, respectively.

4. The release of ^{45}Ca from the slowly exchanging compartment ($t_{1/2}$ 90 min) decreased during incubation in nominal Ca-free solution, although a net loss of tissue Ca occurred. Addition of EGTA (5×10^{-5} M) to the washout medium abolished this retardation of ^{45}Ca release.

5. At external Na^+ concentrations below 40 mM (substituted by sucrose), the ^{45}Ca release from the slowly exchanging compartment decreased. Simultaneously, the tissue Ca content increased massively. The ^{45}Ca release was further reduced in Na-poor, nominal Ca-free solution. Under both conditions, the presence of EGTA in the washout medium normalized the rate of ^{45}Ca release.

6. The results suggest that the apparent decline of ^{45}Ca release from intact atria upon reduction of the external Ca and Na concentration does not reflect a decrease of the cellular efflux rate, but is the consequence of an enhanced re-uptake of ^{45}Ca from the extracellular space into the myocardial cells. The probability for the released ^{45}Ca either to escape into the organ bath or to become reabsorbed depends on the specific radioactivity of ^{45}Ca in the extracellular space during the washout phase. Thus, this experimental procedure is not suited to demonstrate a Na-Ca exchange at the cardiac sarcolemma.

Key words: *calcium release, cardiac muscle, sodium-calcium exchange*

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INTRODUCTION

The invention of new technical methods provides a major contribution to continuous progress in science. With every technical improvement the knowledge increases overproportionally. However, this progress has disadvantages: the methods become more and more complicated leading to an increased specialization which in turn reduces the ability to be familiar with other pretentious methodical procedures. The result of such a methodical specialization is that published findings and their interpretations are taken as granted without a critical evaluation of the used method. It can therewith easily happen that unsound hypotheses based on sophisticated methodical approaches are „generally accepted". In the following an example for a misinterpretation of this kind is given by a critical re-evaluation of the origin of the Na-Ca exchange hypothesis.

Measurements of ^{45}Ca release from intact atria have demonstrated a Ca efflux which is dependent on the extracellular Ca^{2+} and Na^{+} concentration; this led to the concept of a Na-Ca exchange operating at the sarcolemmal membrane (1, 2, 3). In these studies a rather slowly exchangeable Ca fraction was taken as indicating transsarcolemmal Ca fluxes. If dealing with the isotope technique, the complexity of an isolated organ gives rise to several problems, e.g. the difficulty to assign the release of radioactivity from the organ into the bath medium to a transport process across cellular membranes.

In the present study we have investigated whether or not the release of ^{45}Ca from intact beating atria is an adequate measure of the cellular Ca efflux. It is demonstrated that the release of tracer from the tissue is dependent on the probability of internal re-uptake of radioactive tracer within the tissue before reaching the organ bath. A preliminary report of some of the results has been published in abstract form (4).

MATERIALS AND METHODS

Determination of ^{45}Ca release

The method for simultaneously measuring the ^{45}Ca release and the contractile force of isolated guinea-pig atria is a modification of the experimental setup described by Reuter and Seitz (1). Briefly, guinea-pigs weighing 200—350 g were killed by a blow on the neck. The auricles were dissected from the left atria and mounted to a force transducer in a small organ bath containing 2 ml of oxygenated Tyrode solution (pH 7.4, 35°C). The atria were electrically stimulated via two platinum electrodes at a frequency of 3 Hz (impulse duration 5 ms, double threshold intensity). Twitch tension was registered isometrically with a strain gauge and recorded continuously on a pen writer. After an initial equilibration period the atria were incubated for 60 min in Tyrode solution containing trace amounts of ^{45}Ca. After the labelling period the atria were thoroughly rinsed for 15 sec in non-radioactive Tyrode solution. This procedure proved to be sufficient to

remove more than 97% of the adhering ^{45}Ca. The release of ^{45}Ca was followed for 300 min by transferring the atria through a series of test-tubes containing 2 ml of inactive solution. The radioactivity lost from the atria into the bath medium during each incubation period was counted in a liquid scintillation counter (Packard Tri-Carb). At the end of the experiments the atria were removed from the organ bath and gently blotted six times on a filter paper (Schleicher & Schuell) to remove any adherent solution. The atria were weighed and dissolved in 1 ml of Soluene-350 (Packard Instruments) for 12h at 45°C. After addition of scintillation fluid (Hi-ionic Fluor) the radioactivity was counted. The ^{45}Ca release curves were obtained by adding the amount of radioactivity lost during each incubation period to the radioactivity remaining in the atria at the end of the experiments. The ^{45}Ca release from the slowly exchanging compartments is expressed as a fraction of ^{45}Ca lost per min from the muscle into the washout medium. All results are expressed as means $\pm$S.D.

Determination of tissue Ca content

In a separate set of experiments up to 5 atria were kept under identical conditions in an organ bath containing 500 ml of oxygenated Tyrode solution. The atria were loaded with a weight of 500 mg and electrically stimulated at a frequency of 3 Hz. After the equilibration period the atria were incubated in modified Tyrode solutions: control (1.8 mM Ca^{2+}_0), nominal Ca-free, and reduced extracellular Na^+ concentration. The tissue Ca content was determined using the spectrofluorometric method of Zepf (5). Briefly, the atria were subjected to a wet ashing procedure using HNO_3 and $HClO_4$. Afrte heating at 210°C for 8 hr the dry residue was redissolved in 0.1 M HCl. After addition of calcein (Sigma), the Ca content of the samples was determined by measuring the fluorescence intensity of the Ca-calcein complex at 540 nm.

Solutions

The Tyrode solution had the following composition (in mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.0; $NaHCO_3$ 12; NaH_2PO_4 0.2; glucose 5.5. The pH of the solution was maintained at 7.4 by gassing with a mixture of 95% oxygen and 5% carbodioxide. A nominal Ca-free medium was obtained by omitting the $CaCl_2$ from the stock solution, resulting in a Ca^{2+}_0 concentration of about 1×10^{-5} M. In some experiments the Ca^{2+}_0 concentration was further reduced by addition of the Ca^{2+} chelator ethyleneglycol-bis(β-aminoethyl-ether)-N,N′-tetraacetic acid (EGTA, 5×10^{-4}—5×10^{-3}M). For reduction of the extracellular Na^+ concentration the NaCl was partially omitted and replaced by isoosmotic amounts of sucrose.

RESULTS

Release of ^{45}Ca during Ca equilibrium

Guinea-pig atria beating at a frequency of 3 Hz were incubated for 1 h in a ^{45}Ca containing Tyrode solution. This time period is sufficient to almost completely label the tissue Ca (6). The release of ^{45}Ca was followed up for 300 min simultaneously recording the contractile force which declined by 8%/h. *Fig. 1* depicts the ^{45}Ca release curve in a semilogarithmic plot. By graphical analysis (7) three exponential phases of ^{45}Ca release can be distinguished: a fast phase with a $t_{1/2}$ of about 2 min representing 61% of the total ^{45}Ca content, a second phase with a $t_{1/2}$ of about 12 min amounting to 29%, and finally a slow phase amounting to only 10% of the total ^{45}Ca with a $t_{1/2}$ of 90 min.

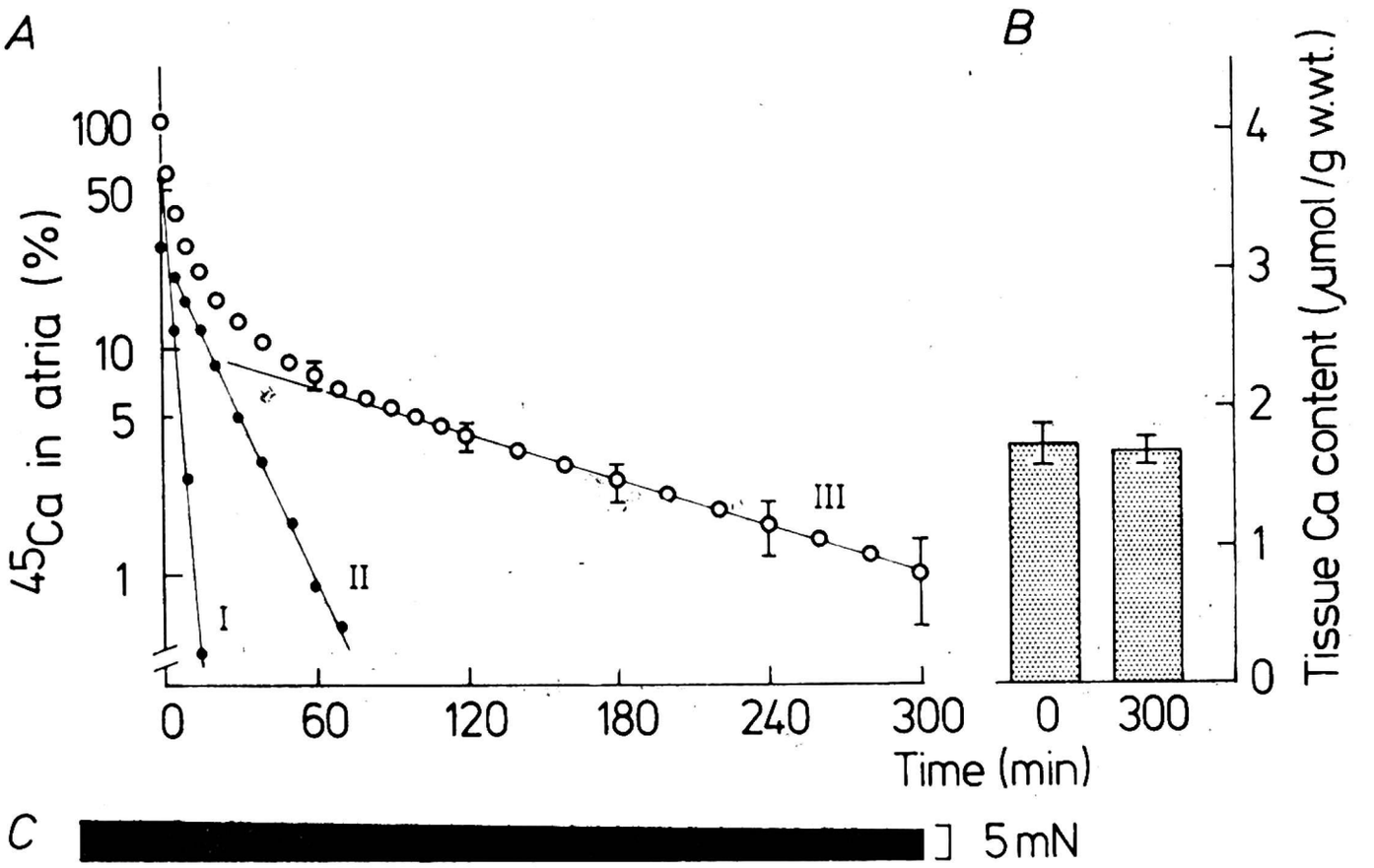

Fig. 1. ^{45}Ca release and tissue Ca content of electrically stimulated guinea-pig left atria at Ca equilibrium (1.8 mM Ca^{2+}_0). *A*, ^{45}Ca content of the atria plotted against washout time. Three phases of ^{45}Ca release (I—III) were determined by graphical analysis according to the method of Solomon (7). *B*, tissue Ca content at the beginning and at the end of the washout period. The points and columns are means of *5* determinations; vertical bars show S.E. *C*, isometric tension recording, redrawn from a single experiment on an electrically stimulated auricle incubated in in 1.8 mM Ca^{2+}_0.

Using non-stimulated resting atria the ^{45}Ca release curve significantly differed only with respect to the first two phases: the $t_{\frac{1}{2}}$ remained unchanged while the size of the fast component declined to 53% and the second component increased to 35% of the total ^{45}Ca Content. Both the size and the $t_{\frac{1}{2}}$ of the slow component were not affected. Therefore, the following experiments are based on the third component of ^{45}Ca release, which presumably reflects a deep cellular compartment.

Release of ^{45}Ca from the slowly exchanging compartment during Ca net movements

Alteration of extracellular Ca^{2+} concentration

The labelling of tissue Ca by ^{45}Ca was performed under control conditions, i.e. 1.8 mM Ca^{2+}_0. At the beginning of the release phase the extracellular Ca^{2+} concentration was changed within the range of 9 mM to nominal Ca-free

and additionally by adding different concentrations of EGTA. In *Fig. 2* the ^{45}Ca release curves are shown for the following conditions: controls (1.8 mM Ca^{2+}_0), nominal Ca-free ($1—3\times10^{-5}$ M Ca^{2+}_0) and Ca^{2+}_0 complexed by 5×10^{-3} M EGTA. The release of ^{45}Ca in nominal Ca-free solution was found to be retarded ($t_{1/2}$ increased from 90 to 120 min). To illustrate the net loss of tissue Ca at the reduced extracellular Ca^{2+} concentration the tissue

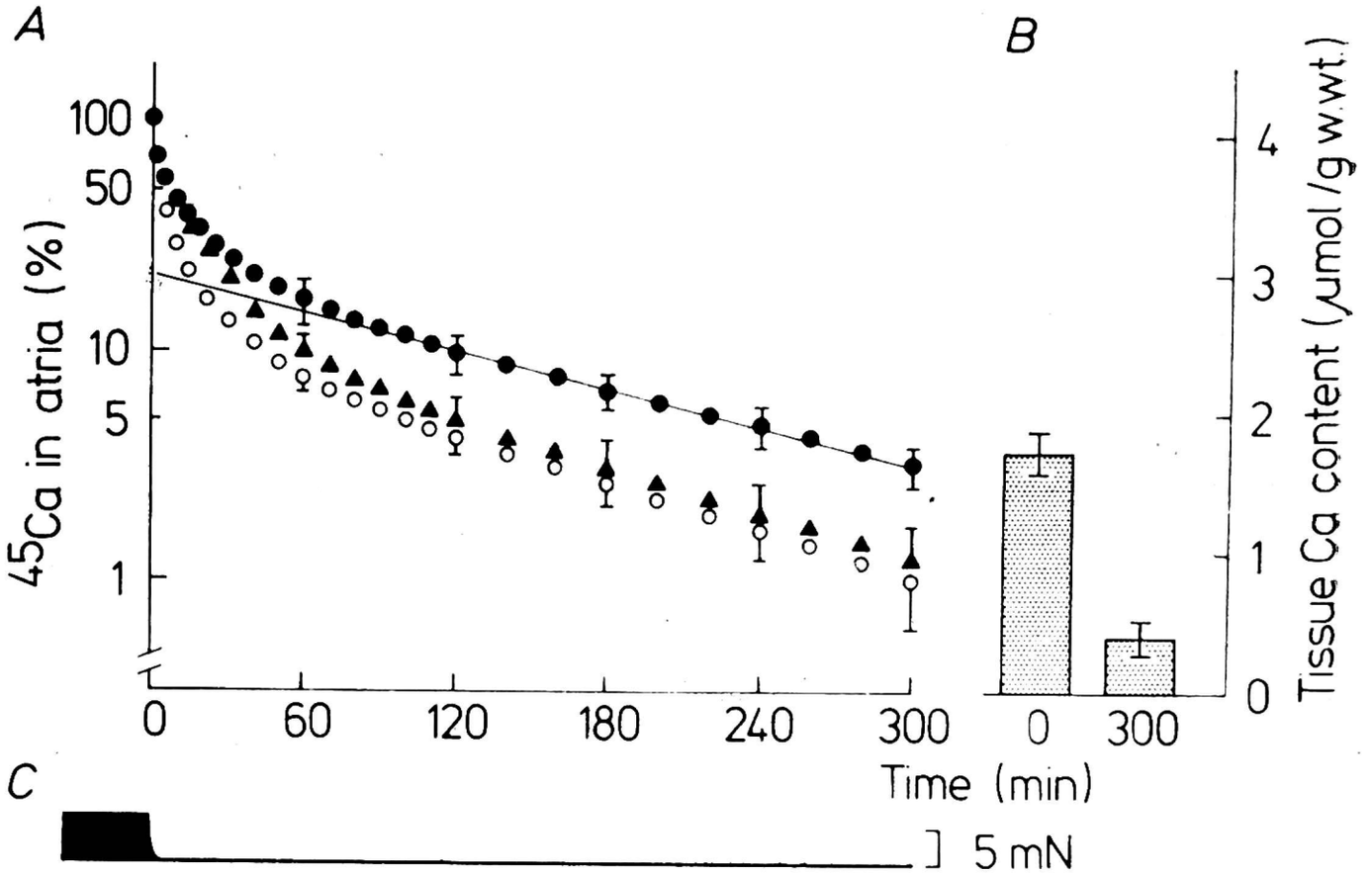

Fig. 2. The effect of incubation in nominal Ca-free medium on ^{45}Ca release and tissue Ca content of guinea-pig left atria. The atria were loaded with ^{45}Ca for 1 hr at 1.8 mM Ca^{2+}_0. During the washout period the atria were incubated in nominal Ca-free Tyrode solution. *A*, ^{45}Ca content of the atria plotted against the washout time for (○) control condition (see Fig. 1), (●) nominal Ca-free solution and (▲) nominal Ca-free medium containing 5 mM EGTA. *B*, tissue Ca content during incubation in nominal Ca-free solution for 300 min. The points and columns are means of 5 determinations; vertical bars show S.E. *C*, isometric tension recording of an auricle incubated in nominal Ca-free solution.

Ca content was determined after 5 h of incubation in nominal Ca-free medium. The calcein fluorescence method demonstrated a loss of Ca from 2.2 to 0.4 µmol Ca/g wet wt. It was remarkable that in spite of a massive loss of Ca from the tissue the ^{45}Ca release seemed to be retarded during incubation in nominal Ca-free medium. This retardation of ^{45}Ca release was almost completely abolished by EGTA (*Fig. 2*). The findings covering the entire range of extracellular Ca^{2+} concentrations are depicted in *Fig. 3* using the fractional ^{45}Ca loss of the slow component plotted against the actual Ca^{2+}_0 concentration of the

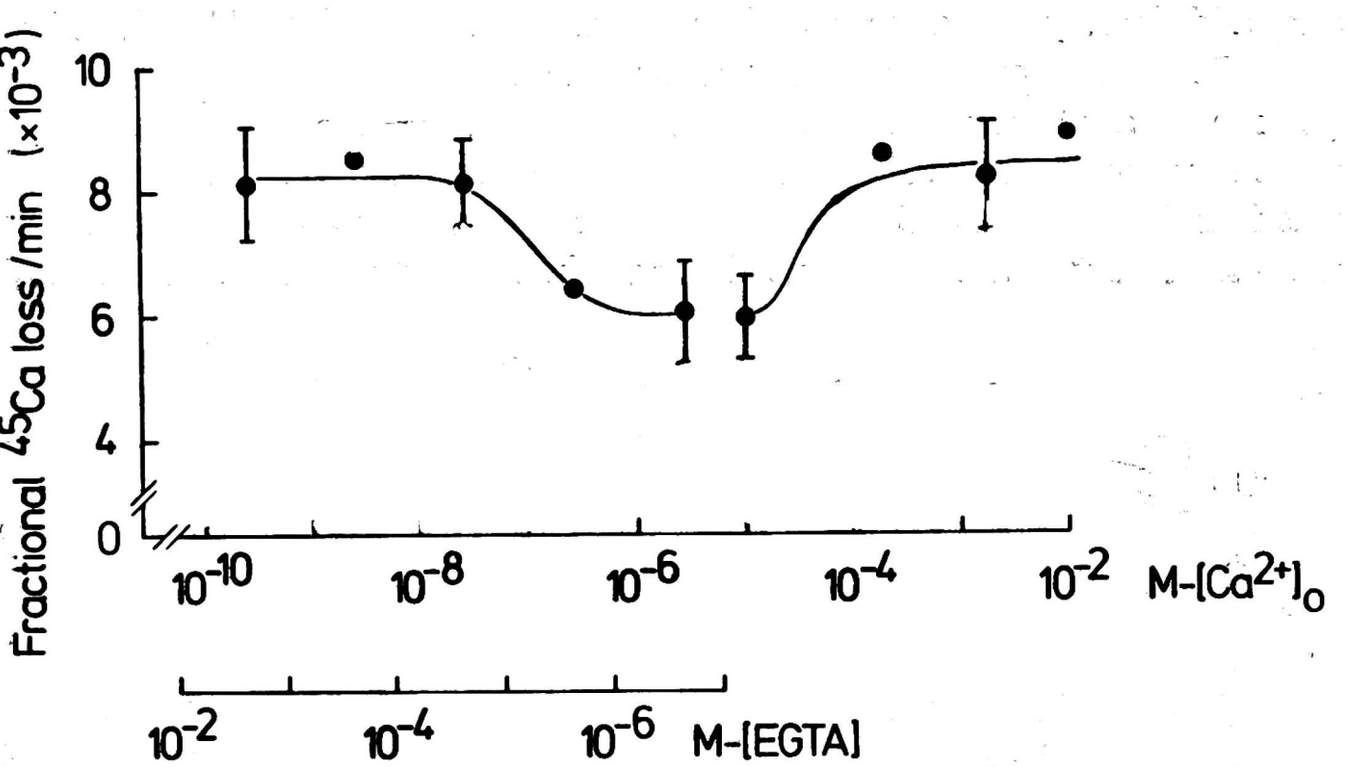

Fig. 3. The effect of the extracellular Ca^{2+} concentration and of EGTA on fractional ^{45}Ca release from the slowly exchanging compartment. EGTA was added to the medium to inhibit re-uptake. The Ca^{2+}_0 concentration was calculated using the apparent association constant for Ca-EGTA reported by Portzehl et al. (18). Atria were loaded for 1 hr with ^{45}Ca in 1.8 mM Ca^{2+}_0 solution. Ca^{2+}_0 was changed at the beginning of the washout period. Points are means; vertical bars show S.E. in case $n > 4$.

medium. This resulting curve displays a remarkable trough. The fractional loss of ^{45}Ca declined from about 0.008 to 0.006 min^{-1} at an extracellular Ca^{2+} concentration of 10^{-5}—10^{-6} M. Upon addition of EGTA in increasing concentration, i.e. reduction of the free Ca^{2+}_0 concentration, the rate of ^{45}Ca release rose again and reached the control level. It should be kept in mind that at all extracellular Ca^{2+} concentrations below 1.8 mM a net loss of tissue Ca occurred, whereas incubation at 9 mM Ca^{2+}_0 led to anet uptake of Ca.

Reduction of the extracellular Na^+ concentration

In the following experiments the Na^+ concentration of the medium was systematically lowered from 149 to 11 mM by replacing with isoosmotical concentrations of sucrose. At 11 mM Na^+_0 the atria stopped beating,developed a contracture and accumulated Ca (*Fig. 4*). The tissue Ca content rose to about 22 μmol/g wet wt. within 5 h. Plotting again the fractional loss of ^{45}Ca versus extracellular Na^+ concentration *Fig. 5* shows that at extracellular Na^+ concentrations between 149 and 60 mM the rate of ^{45}Ca loss remained constant. The Na^+_0 concentration has to be reduced below 40 mM to influence the ^{45}Ca release from the slowly exchanging compartment. A reduction to 20 and 11 mM Na^+_0 markedly retarded the ^{45}Ca release. In order to test

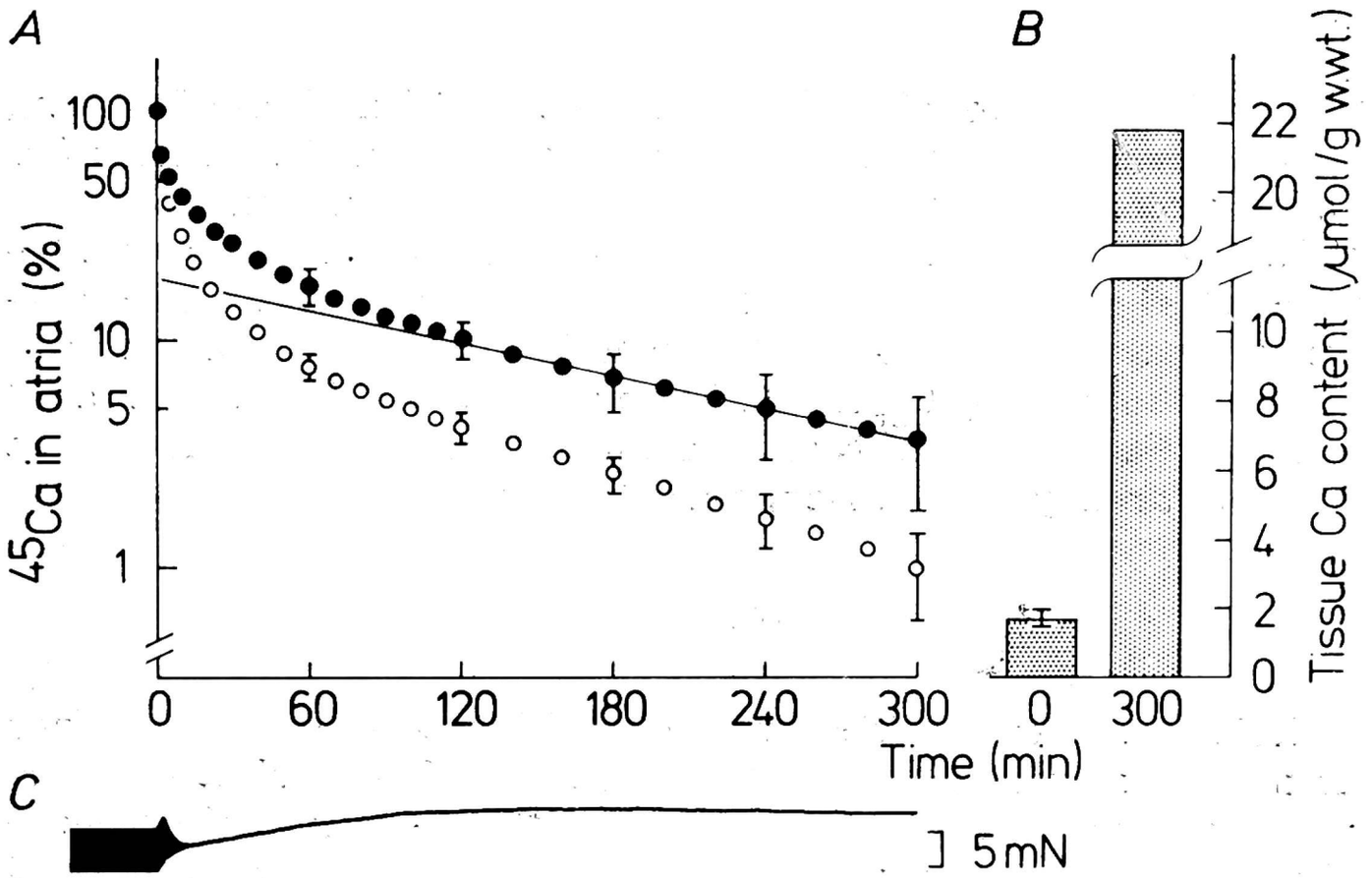

Fig. 4. ^{45}Ca release and tissue Ca content at reduced extracellular Na^+ concentration. *A*, ^{45}Ca content of the atria plotted against washout time. (●) 11 mM Na^+_o; substituted by sucrose. (○) control curve at 1.8 mM Ca^{2+}_o and 149 mM Na^+_o. Points are means of 5 determinations. Vertical bars show S.E. *B*, tissue Ca content during immersion in Na-poor solution. *C*, isometric tension recording illustrating the contracture of an auricle incubated in 11 mM Na^+ solution.

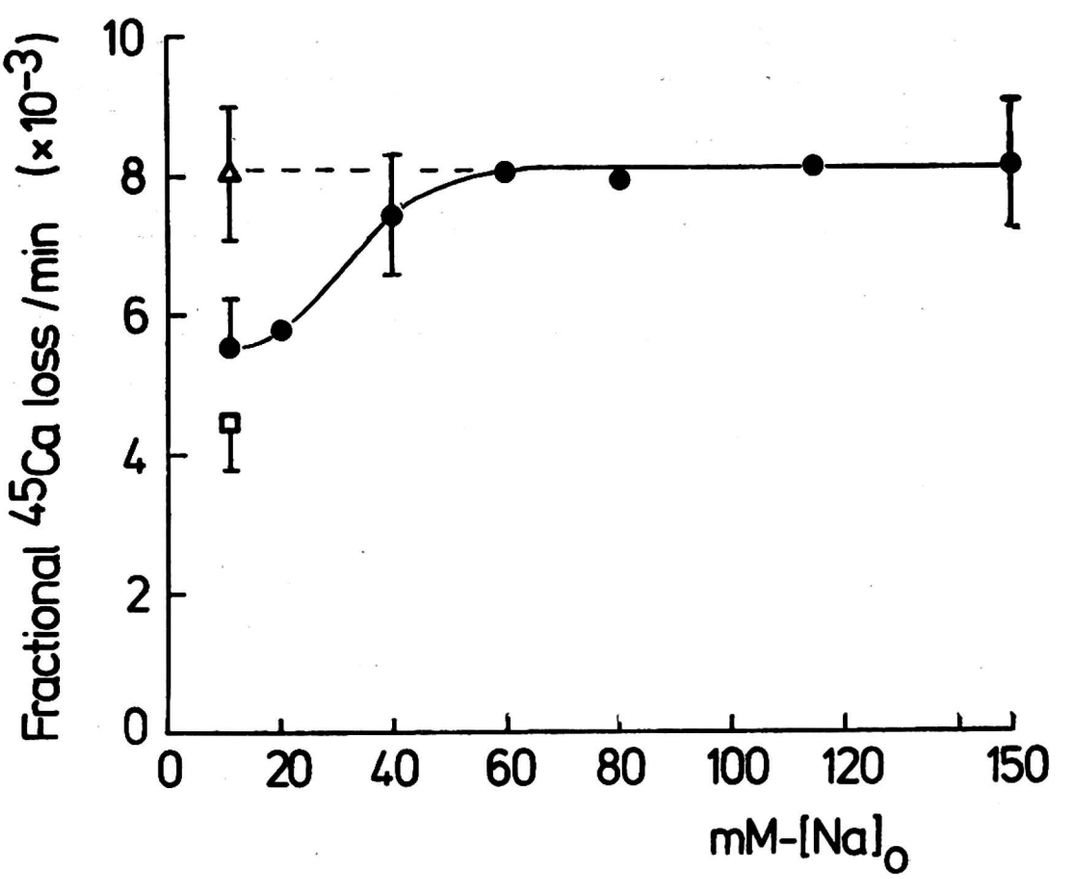

Fig. 5. The effect of varying Na^+_o concentration in solutions containing 1.8 mM Ca^{2+}_o (●), in nominal Ca-free solution (□) and in the presence of 5 mM EGTA (△). Fractional ^{45}Ca loss is plotted against the extracellular Na^+ concentration. Na is substituted by sucrose. Points are means; vertical bars show S.E. in case $n \geq 4$.

whether the net uptake of Ca per se caused a reduction of the ^{45}Ca release, two additional sets of experiments were performed, i.e. incubation in nominal Ca-free media with or without EGTA. Under these conditions net uptake of Ca does not occur (data not shown). As demonstrated in Fig. 5 the fractional ^{45}Ca loss was further depressed to 0.0043 min^{-1} in nominal Ca-free solution, whereas in the presence of 5×10^{-3} M EGTA the ^{45}Ca release rate rose to the control value (0.008 min^{-1}). These results clearly indicate that a net uptake of Ca by the tissue is not responsible for the depression of the ^{45}Ca release at strongly reduced Na^+_0 concentrations.

DISCUSSION

Isolated atria or ventricles have been widely used to study the cellular Ca metabolism by means of radiolabeled Ca (1—3, 8—11). While the actual measurements of ^{45}Ca (^{47}Ca) are performed on the tissues or on the bath medium, these authors have made extrapolations concerning transsarcolemmal transport processes. One typical example for this type of procedure is the Na^+_0-dependency of ^{45}Ca release from intact atria which led to the hypothesis of the Na-Ca exchange across the sarcolemma (1—3). This hypothesis was based on observations of ^{45}Ca release from a very slowly exchanging compartment ($t_{1/2}$ 70—90 min) (1), without explicit evidence to prove that the slow phase correctly represented a plasmalemmal process. This prompted us to re-investigate the matter in the present series of experiments, which combine measurements of Ca fluxes with the determination of tissue Ca content. Particular attention was paid as to whether the muscle maintained a Ca equilibrium, or whether net movements of Ca occurred during the release of ^{45}Ca.

Under control conditions, where net movements of Ca did not occur, the analysis of the ^{45}Ca release curve yielded three distinguishable phases. The fast component of the ^{45}Ca release ($t_{1/2}$ about 2 min) exceeded the size of the extracellular space by far, suggesting that a fraction of Ca (about 30% of tissue Ca) exchanged more rapidly than diffusion in the extracellular space takes place (12, 13). Thus, in intact atria this seemingly is the only fraction of tissue Ca the exchange of which is diffussion limited. The present experiments, however, demonstrate, that even the exchange rate of the slowely exchanging compartment ($t_{1/2}$ about 90 min) might be falsly retarded by an extracellular event, namely the re-uptake of tracer from the extracellular space. Every individual ^{45}Ca ion having left the cell and diffusing within the extracellular space will have a certain chance to get reabsorbed into a cellular compartment (Fig. 6). The chance for this re-uptake will depend on the readiness to escape into the bath medium and on the dilution of the labelled ions by unlabelled

Ca ions in the extracellular space. In isolated organs not perfused via the vascular system the diffusion distances are rather long and the diffusion effectivity is diminished by the maze-like geometry of the extracellular space.

Under control conditions, i.e. 1.8 mM Ca^{2+}_0, the labelled ^{45}Ca ions become highly diluted in the extracellular space, resulting in a negligible chance to become re-absorbed. A reduction of the extracellular Ca^{2+} concentration during the washout period induces a net loss of tissue Ca, and an increased loss of ^{45}Ca from the tissue might be anticipated, too. But as shown in *Fig. 2* and *3* the opposite was found: the ^{45}Ca release into the bath was retarded in nominal Ca-free solution although there was a net loss of Ca. This apparently parado-

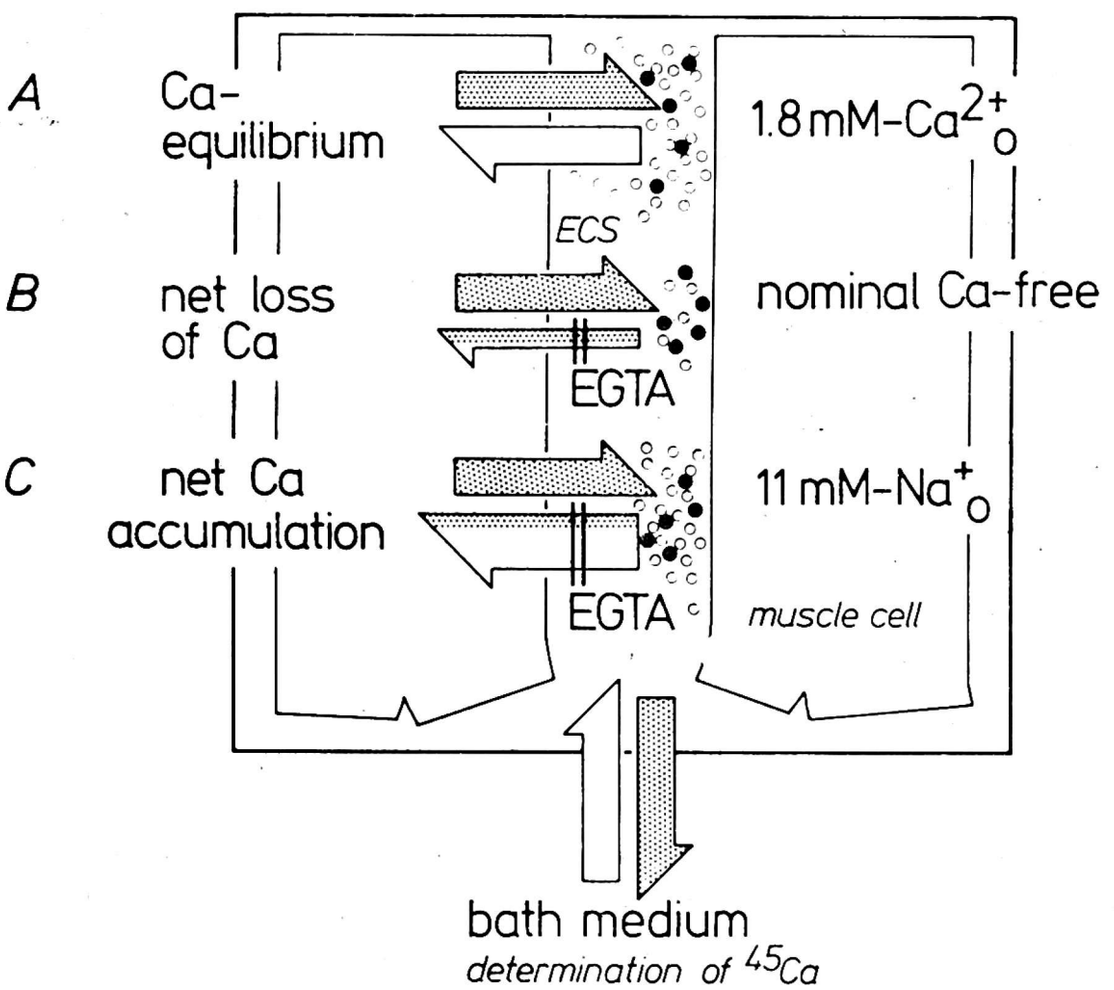

Fig. 6. Schematic drawing of the events influencing the ^{45}Ca release from an isolated atrium into the incubation medium. ^{45}Ca release from the slowly exchanging ^{45}Ca fraction is depicted for the following three conditions: *A*, ^{45}Ca efflux into the extracellular space (ECS) under equilibrium condition (1.8 mM Ca^{2+}_0). The tracer ions (●) are highly diluted by the non-radioactive ^{40}Ca ions (○) in the extracellular space, resulting in a negligible chance to become reabsorbed. *B*, ^{45}Ca efflux into the extracellular space under nominal Ca-free condition. The tracer ions (●) are only little diluted by non-labelled Ca ions. The re-uptake of ^{45}Ca, is, therefore, high and the ^{45}Ca release from the organ into the bath is retarded. Tracer re-uptake is prevented by complexing the ^{45}Ca ions with EGTA. *C*, reduction of the extracellular Na^+ concentration results in a net uptake of tissue Ca and reduces the washout of ^{45}Ca from the organ into the bath. EGTA completely inhibits this retardation of ^{45}Ca release. Movement of unlabelled Ca, open arrows; movement of labelled Ca, closed arrows.

xical effect can be explained by the increased probability for ^{45}Ca re-uptake from the extracellular space under a condition of strongly reduced dilution. If the re-uptake of ^{45}Ca is completely inhibited in the presence of the complexing agent EGTA, the rate of ^{45}Ca release from the organ reflects the actual cellular efflux. *Fig. 3* shows that the same amount of ^{45}Ca leaves the cardiac cells independent of whether there is an equilibrium or a net loss of cellular Ca. The depression of the ^{45}Ca release rate (the „through" in *Fig. 3*) at low extracellular Ca^{2+} concentrations does not reflect a cellular event but is caused by an enhanced chance for ^{45}Ca to become re-absorbed from the extracellular space.

Under conditions where a net uptake of Ca occurs, the chance for re-uptake of released ^{45}Ca also seems to play a role. As outlined in *Fig. 4* and *5*, lowering of the extracellular Na^+ concentration provides a possibility to induce a Ca net uptake by the tissue. Only extreme reduction of the Na^+_0 concentration to 20 or 11 mM, evoking a contracture of the atria, reduced the loss of ^{45}Ca from the organ. The release of ^{45}Ca was even more retarded when the atria were incubated in a nominal Ca-free solution additionally (see *Fig. 5*), a condition where no net uptake of Ca took place. Thus the net uptake of Ca induced by low extracellular Na^+ concentrations can be excluded as the reason for the decline of the rate of ^{45}Ca release. If not only the net uptake but also the re-uptake of ^{45}Ca from the extracellular space was inhibited by addition of EGTA, the rate of ^{45}Ca release was equivalent to values obtained under control condition (see *Fig. 5*). This suggests that the apparent decline of the ^{45}Ca release rate upon reduction of the extracellular Na^+ concentration does not reflect a decreased ^{45}Ca loss from the cardiac cells but is again the consequence of an extracellular event, namely an increased probability for re-uptake.

Our results demonstrate that the rate of ^{45}Ca-release from an isolated cardiac preparation depends upon the extracellular concentrations of Na^+ and Ca^{2+}. This agrees with the observations of previous authors (1, 2, 10) and was the basis for the Na-Ca exchange hypothesis. The present re-investigation, however, prompts us to propose a different interpretation. The determination of ^{45}Ca in the bath medium is not necessarily equivalent to the cellular efflux of ^{45}Ca into the extracellular space. Every individual ^{45}Ca ion is subject to a probability either of escaping into the bath medium or becoming re-absorbed. The variations of the experimental conditions may lead to nothing else but alterations of this probability rather than to true changes of cellular efflux. It is therefore not easy to justify the previous claim which was based on similar observations that there is a Na-Ca exchange operating at the sarcolemma. This is not to say that a Na-Ca exchange does not exist. It can, however, not be unequivocally detected by using a very

slowly exchanging fractions of tissue Ca, the exchange of which is probably not rate-limited by the sarcolemma. Our interpretation is supported by experimental results obtained with cardiac cell cultures: by using the slow exchange of Ca, no reduction of ^{45}Ca efflux could be observed when the Na^+_0 concentration was lowered (14—17).

In conclusion, ^{45}Ca release curves obtained by means of isolated organs are inevitably influenced by the probability by which the tracer either escapes into the bath (and thus becomes measurable) or is subject to cellular re-uptake. This is demonstrated in the present investigation by altering the probability of re-uptake of ^{45}Ca from the extracellular space.

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