Biochimica et Biophysica Acta, 470 (1977) 128–133
© Elsevier/North-Holland Biomedical Press

BBA Report

BBA 71311

CRITICAL REEVALUATION OF MUREXIDE TECHNIQUE IN THE MEASUREMENT OF CALCIUM TRANSPORT BY CARDIAC SARCOPLASMIC RETICULUM

LYNDA BLAYNEY, HUW THOMAS, JOHN MUIR and ANDREW HENDERSON
Department of Cardiology, Welsh National School of Medicine, Cardiff (U.K.)
(Received May 31st, 1977)

Summary

Calcium transport by cardiac sarcoplasmic reticulum preparations appears biphasic when measured by dual wavelength spectrophotometry with murexide, initial binding being followed by the supposedly contingent process of "release". We present evidence that this "release" phase is a methodological artefact due to murexide penetration into the sarcoplasmic reticulum.

Dual wavelength spectrophotometry with murexide has been developed [1—3] and widely applied [4—6] to study Ca transport by cardiac sarcoplasmic reticulum. The spontaneous release phase of the biphasic reactions observed with this method has previously been ignored in studies of Ca accumulation, and has been of uncertain physiological relevance, though it provides the basis of the suggestion that cardiac sarcoplasmic reticulum accumulates Ca by a different mechanism from skeletal muscle [1—3] and is cited as evidence in the continuing controversy about the source of activating Ca in cardiac muscle [7].

Preparations rich in sarcoplasmic reticulum were prepared from rabbit myocardium [4] and studied the same day. Ca accumulation was studied with the "murexide" method [1—3]. Changes in free Ca concentration were followed using a purpose-built dual wavelength spectrophotometer fitted with an Aminco Morrow stopped-flow device. One reservoir syringe normally contained sarcoplasmic reticulum preparation and buffer, while the other normally contained Mg, Ca, ATP, ATP-regenerating system, murexide and buffer: final concentrations after mixing: 0.4 mg protein/ml sarcoplasmic reticulum preparation, 100 mM KCl, 20 mM Tris/maleate, 10 mM $MgCl_2$, 30 μM $CaCl_2$, 0.2 mM ATP, 5 mM phospho*enol*pyruvate and 14 μg/ml

pyruvate kinase, 0.2 mM murexide, pH 6.8, 35°C. ATP-regenerating system was included in order to avoid any possibility that Ca release might be occurring because of inadequate ATP [3,5], though its addition made no difference to the experimental findings with our preparations. Murexide was freshly prepared each day. The instrument was calibrated for Ca^{2+} (15—120 μM) at each murexide concentration (0.1—0.4 mM), in similar buffer. Mixing of the two syringes occurred in < 4 ms into an observation chamber of light path 1 cm. The light sources were set at 474 and 542 nm and the signal recorded on a Bryants flat bed recorder. Observed release curves were analyzed by linear regression of log ($Ca_t - Ca_\infty$) vs. time ($r > 0.99$, $n = 6$). Ca accumulation was studied also by timed millipore filtration with ^{45}Ca [8] under identical experimental conditions: 1-ml aliquots were passed through 0.45 μm diameter pore-size HA Millipore filters, the filters were washed with 2 ml buffer and their ^{45}Ca content measured by liquid scintillation spectrometry. ATPase activity was measured in 2 ml reaction mixture, similar except in containing 5 mM NaN_3 and only approx. 0.1 mg protein/ml: liberated phosphate (P_i) was measured [9], the reaction being started by adding ATP and stopped after 5 min with tricarboxylic acid (to 5% v/v). Endogenous total Ca of sarcoplasmic reticulum preparations was measured by atomic absorption spectrometry of "ashed" samples. The relative accumulation of murexide by sarcoplasmic reticulum vesicles was measured after incubation for 10 min as compared with 30 s. Preparations (5 mg protein/ml) were incubated at 35°C with 0.4 mM murexide in KCl-Tris/maleate buffer. 0.2 ml aliquots were rapidly mixed with 3.8 ml buffer and passed through 0.45 μm diameter pore size HA Millipore filters. The filters were washed with 2 ml 120 μM $CaCl_2$ solution for 30 min to lyze trapped vesicles, and the murexide content of the solution was measured by reading the absorbance (calcium murexide signal) at 542 nm against a blank (120 μM $CaCl_2$ solution) using the spectrophotometer in its split-beam mode (full scale deflection 0.01 absorbance units). Results are presented as mean ± standard error and are compared by Student's t test.

Fig. 1 shows that "release" occurred equally whether or not it was preceded by ATP-dependent "binding" (curves A and B). Analysis of "release" curves gives similar rate constants ($4.9 \pm 0.4 \cdot 10^{-3}$ and $4.3 \pm 0.5 \cdot 10^{-3}$ s^{-1}, $n = 13$) in the presence and absence of ATP respectively, with intercepts 120 ± 14 and 69 ± 8 nmol Ca/mg protein (the difference of 51 nmol Ca/mg protein reflecting ATP-dependent Ca accumulation*). Furthermore, preincubation with murexide affected the measured curve of Ca accumulation (Fig. 1, curve C), though murexide does not alter total or Ca-activated ATPase activity (773 ± 25 and 381 ± 84 nmol P_i/mg protein per min, $n = 5$, with murexide; 766 ± 66 and 327 ± 56, without murexide). This "release" curve was not seen when Ca transport was studied under identical conditions with the millipore method and ^{45}Ca (Fig. 2, B and C). This suggests that it is an artefact of the murexide method.

The findings are consistent with the possibility that murexide slowly

*cf. 53.8 nmol Ca/mg protein, derived from corrected Ca accumulation curve.

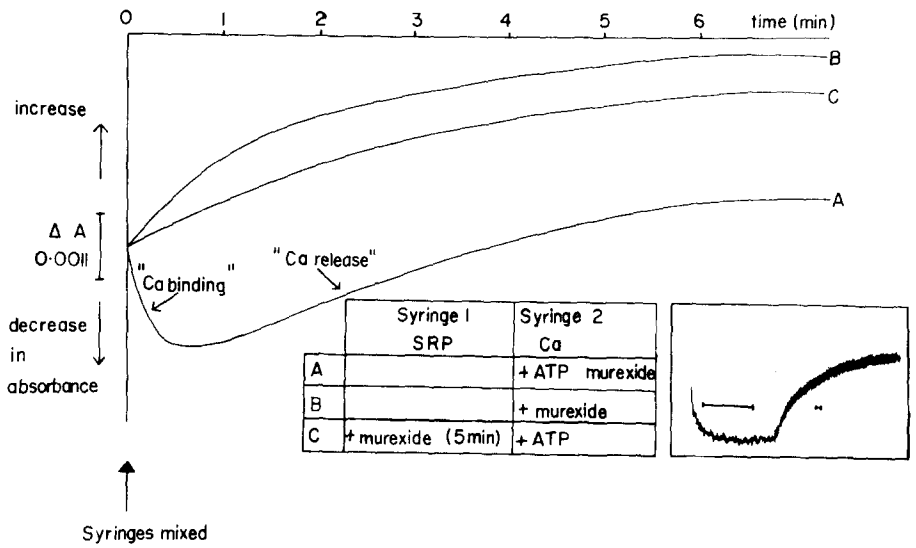

Fig. 1. Representative traces of Ca transport by sarcoplasmic reticulum preparations (dual wavelength spectrometry with murexide), showing (A) conventional biphasic "binding/release" curve, (B) persistence of apparent "release" phase in absence of ATP, (C) apparent loss of "binding" by short preincubation with murexide (e.g. 5 min). (ΔA = change in absorbance). Inset is an actual recording from which traces are drawn (time bar = 20 s; note change in paper speed).

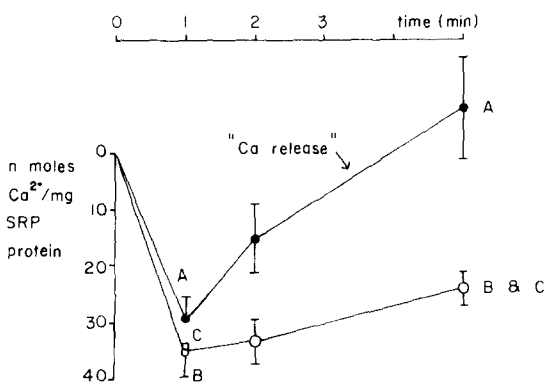

Fig. 2. Ca transport by sarcoplasmic reticulum preparations (SRP) (mean ± S.E., n = 6). (A) Measured by dual wavelength spectrophotometry with murexide (as in Fig. 1A). (B) Measured by millipore filtration with $^{45}Ca^{2+}$ in the absence and (C) in the presence of murexide.

penetrates sarcoplasmic reticulum and interacts with endogenous intravesicular Ca, so contributing to the total calcium murexide signal. The presence of endogenous Ca was confirmed by measurement of ashed samples which showed 57.4 ± 5.0 nmol Ca/mg protein (n = 10). It was also shown that sarcoplasmic reticulum preparations accumulate murexide over a 10 min period, comparable to the duration of the observed "release" phase. The murexide content after rapid washing was 33.6 ± 2.5 nmol/mg protein (n = 5), after 10 min incubation, compared with 20.5 ± 1.7 after 30 s incubation, $p < 0.025$. Fig. 3 shows that the "release" phase (in the absence

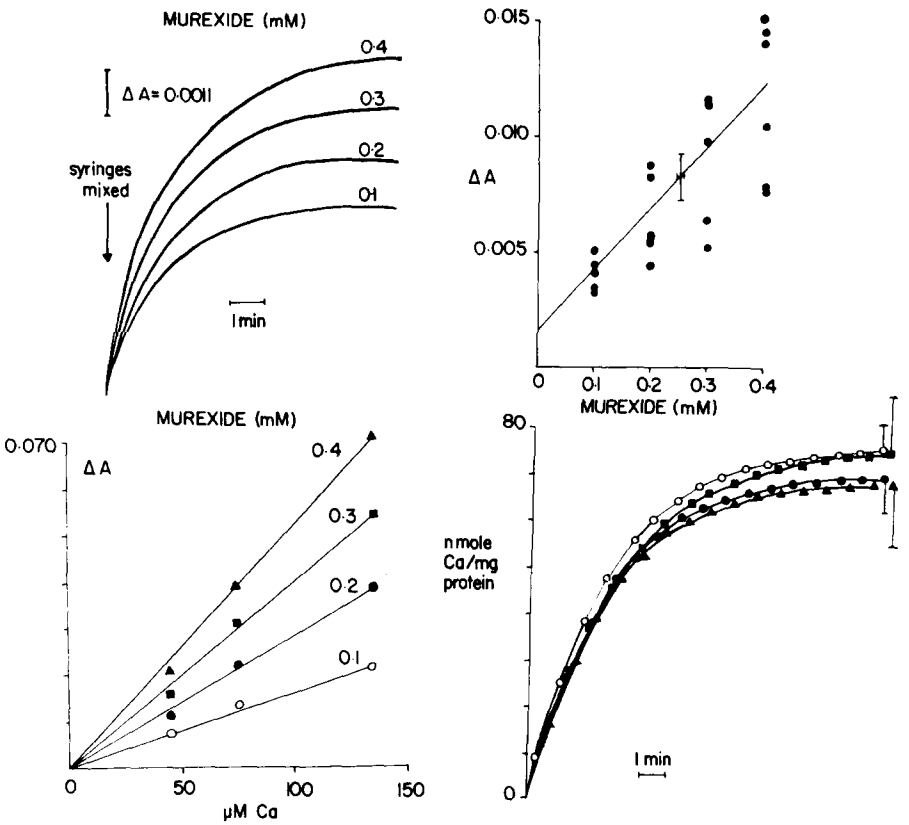

Fig. 3. Murexide accumulation by sarcoplasmic reticulum preparations: Upper left, representative traces (dual wavelength spectrophotometry) obtained by adding different concentrations of murexide without ATP (as in Fig. 1B); upper right, final absorbance levels from similar traces (n = 5 at each concentration) plotted against external murexide concentration (linear regression r = 0.74); lower left, calibration of Ca signal at different murexide concentrations (linear regressions through mean values, n = 6 at each point); lower right, normalisation of experimental traces (each experimental curve analysed as exponential function, curves normalised to give Ca/mg protein, and reconstructed as mean curves).

of ATP) was related to murexide concentration but that normalisation of the observed curves yielded the same "Δ Ca" curve. This curve presumably represents murexide inward diffusion at constant endogenous Ca (estimated from these curves as 74.3 ± 5.2 nmol Ca/mg protein (n = 23), assuming similar calcium murexide signal calibration inside and outside the vesicles).

Correction for the artefactual "release" phase is therefore necessary to obtain true net Ca accumulation which is then susceptible to kinetic analysis (Fig. 4). The validity of the correction receives implicit support from the exponential nature of the corrected curve (linear regression, log Ca vs. time, $r > 0.99$). The corrected Ca accumulation curve is characterised by an intercept, B, 53.8 ± 6.8 nmol Ca/mg protein, and rate constant, k_B, 0.06 ± 0.008 s^{-1} (n = 13).

Contrary to earlier conclusions based on observations of the "release" phase [1–3], the present findings with cardiac sarcoplasmic reticulum preparations are consistent with the mechanism for Ca accumulation which is proposed for skeletal muscle [10–13] whereby Ca is actively pumped across the sarcoplasmic reticulum membrane and accumulated within its lumen. Indeed, there is now evidence that the protein constituents of cardiac and skeletal muscle sarcoplasmic reticulum are similar [14].

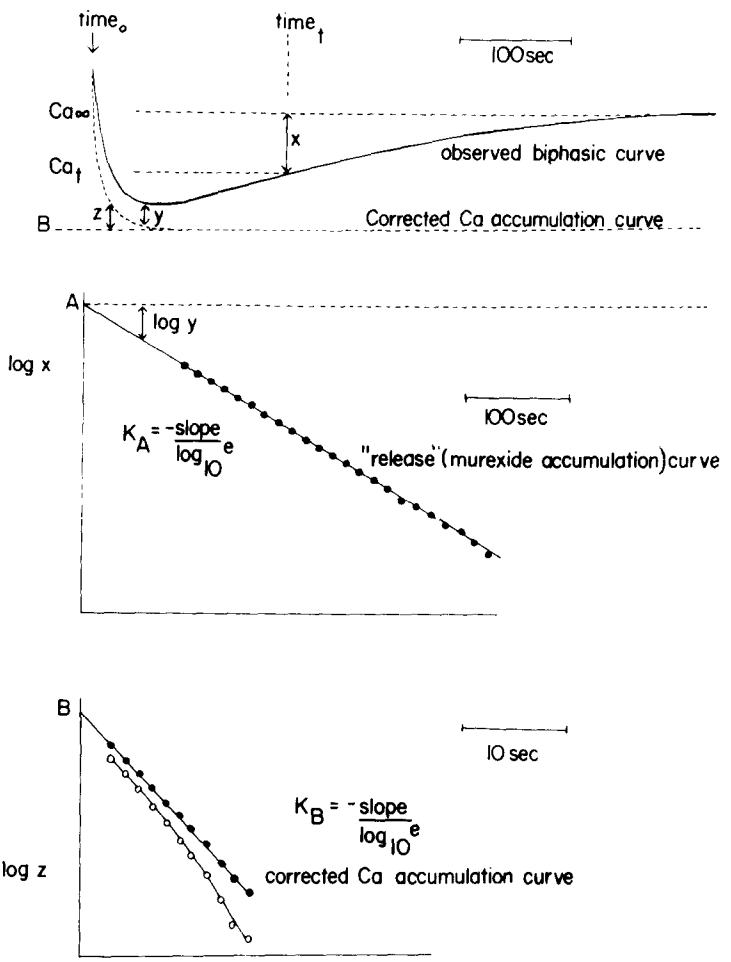

Fig. 4. Analysis of biphasic curves obtained with "murexide" method. Top panel: representative observed biphasic curve; x = difference between measured Ca concentration at any time t (Ca_t) and at final steady state (Ca_∞) during the apparent release phase, y (derived from middle panel) is added to observed curve to give corrected Ca accumulation curve, and z = difference between corrected Ca accumulation curve at any time t and at calculated steady state (B). Middle panel: plot of log x vs. time to characterize the exponential apparent "release" (murexide accumulation) curve, with intercept A and rate constant k_A; y = difference between Ca concentration at any time t and A. Bottom panel: plot of log z vs. time to characterize the exponential corrected Ca accumulation curve, with intercept B and rate constant k_B (also shown is log plot of uncorrected "Ca binding" phase, which appears non-exponential).

Acknowledgements

We thank A.K. Campbell, A.C. Grey and S.A. Hodson for helpful discussion. This study was completed with the aid of grants from the British Heart Foundation and Welsh Cardiac Research Society.

References

1 Entman, M.L. and Schwartz, A. (1972) Recent Adv. Cardiac Struct. Metab. 4, 437—450
2 McCollum, W.B., Besch, H.R., Entman, M.L. and Schwartz, A. (1972) Am. J. Physiol. 223, 608—614
3 Entman, M.L., Bornet, F.P. and Schwartz, A. (1972) J. Mol. Cell Cardiol. 4, 155—169
4 Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 781—794
5 Nayler, W.G., Dunnet, J. and Burian, W. (1975) J. Mol. Cell. Cardiol. 7, 663—675
6 Entman, M.L., Snow, T.R., Freed, D. and Schwartz, A. (1973) J. Biol. Chem. 248, 7762—7772
7 Fabiato, A. and Fabiato, F. (1977) Circ. Res. 40, 119—129
8 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648—657
9 Fiske, C.F. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—405
10 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518—528
11 Kanazawa, T., Yamada, S. and Tonomura, Y. (1970) J. Biochem. (Tokyo) 68, 593—595
12 Inesi, G. (1972) Annu. Rev. Biophys. Bioeng. 1, 191—210
13 Martonosi, A., Lagwinska, E. and Oliver, M. (1974) Ann. N.Y. Acad. Sci. 227, 549—567
14 Levitsky, D.O., Alieu, M.K., Kuzmin, A.V., Leuchenko, T.S., Smirnov, V.N. and Chazov, E.I. (1976) Biochim. Biophys. Acta 443, 468—484