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SPONTANEOUS CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM

A RE-EXAMINATION

MARK L. ENTMAN *, W. BARRY VAN WINKLE, EDWARD BORNET and CHARLOTTE TATE

Departments of Medicine and Biochemistry, Baylor College of Medicine and the Fondren-Brown Cardiovascular Research and Demonstration Center of The Methodist Hospital, Houston, TX 77030 (U.S.A.)

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Summary

A recent study by Blayney and co-workers (Blayney, L., Thomas, H., Muir, J. and Henderson, A. (1977) Biochim. Biophys. Acta 470, 128—133) purported to demonstrate that apparent spontaneous calcium release in sarcoplasmic reticulum is an artifact of the uptake of murexide dye. This report demonstrates that spontaneous calcium release (1) takes place despite equilibration of murexide sarcoplasmic reticulum to a stable baseline; (2) may be reversed by addition of ATP or oxalate after release has begun. The identical phenomenon can be demonstrated utilizing the indicator arsenazo III or Millipore filtration methods. The results suggest that equilibration of the murexide with sarcoplasmic reticulum vesicles must occur prior to ATP addition in order to achieve a stable baseline but that spontaneous calcium release is not an artifact.

Introduction

A recent paper in this journal by Blayney and co-workers [2] has demonstrated the caution which must be taken when using chelometric dyes in measuring calcium metabolism. In the study described, these workers demonstrated that murexide (ammonium purpurate) is apparently taken up in sarcoplasmic reticulum vesicles and, in their experiment, this uptake of murexide appears as an apparent spontaneous release of calcium from the sarcoplasmic reticulum

* To whom reprint requests should be sent at Department of Medicine, Section of Cardiovascular Sciences, Baylor College of Medicine, 1200 Moursund Avenue, Houston, TX 77030, U.S.A.

vesicles. They pointed out that this 'apparent' calcium release was not dependent on the presence of ATP at all. As a result of their experiments, they suggested that calculations of ATP-dependent calcium binding must include corrections for this apparent, and presumed artifactual, spontaneous release.

The experiments of Blayney et al. [2] were carried out in such a manner that murexide was not mixed with the sarcoplasmic reticulum vesicles until the moment of the reaction initiation utilizing a stopped flow apparatus. Utilizing this apparatus, these authors observed that an equilibration time of about 6 min was necessary before the apparent spontaneous release resulting from murexide uptake was dissipated. These findings are in themselves interesting and represent an important scientific contribution with regard to problems in methodology. The authors, however, have chosen to interpret their data as evidence that spontaneous calcium release does not occur in isolated sarcoplasmic reticulum at all and that, indeed, all reports describing this phenomenon have been victimized by an artifact of experimental design. The present report presents evidence to the contrary.

Methods

Canine cardiac sarcoplasmic reticulum was isolated by the method of Harigaya and Schwartz [5] as modified by Entman et al. [3,4]. Calcium binding was measured as demonstrated in the figures utilizing a dual wavelength spectrophotometer and the calcium chelometric dye, murexide [3—10,12]. The routine calcium binding media contained 10 mM $MgCl_2$, 0.1 M murexide, 0.1 M KCl, 40 mM Tris-maleate (pH 6.8) and other reactants as illustrated in the figures. When present, the ATP regenerating system utilized was 2.5 mM phospho*enol*pyruvate and 10 units/ml pyruvate kinase. The volume of reaction was 3 ml and the temperature utilized was 30°C in all experiments shown. Experiments done in the range of 20—37°C show the same qualitative results in all cases. Experiments were carried out in an Aminco-Chance dual wavelength spectrophotometer measuring changes in the wavelength pair 507—542 nm as previously described [3—5]; use of the pair 472—542 nm resulted in greater Ca^{2+} sensitivity but also a much larger light scatter artifact. There were no qualitative differences using the latter wavelength pair. As demonstrated in all figures, there was a 5 min pre-incubation time of sarcoplasmic reticulum vesicles and murexide before any reactions were measured unless specifically demonstrated in the figure. Experiments utilizing arsenazo III as the chelometric indicator were identical to those with murexide except that the wavelength pair was 675—685 nm and the arsenazo III concentration was 40 μM.

Results

In Fig. 1, two traces show that after a 5 min pre-incubation the addition of 40 μM calcium results in a stable baseline with no apparent calcium release over a 5 min period. ATP is then added and a rapid calcium accumulation begins which is followed by calcium release. If ATP is added during spontaneous calcium release, as demonstrated by the dotted lines (showing data from separate experiments), some calcium is re-accumulated and then spontaneous release

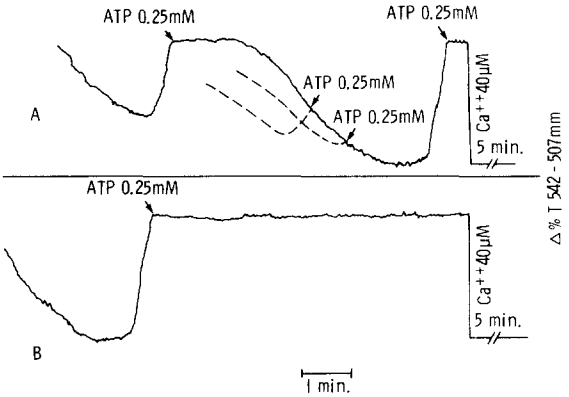

Fig. 1. In this, as in the subsequent figures, the calcium binding is carried out in an Aminco-Chance dual wavelength spectrophotometer at 30°C. Each cuvette contains 0.1 M KCl, 10 mM $MgCl_2$, 40 mM Tris-maleate (pH 6.8). All reactions were temperature equilibrated for 5 min prior to reaction initiation. In the upper trace (A) in which the reaction medium contains 0.8 mg sarcoplasmic reticulum/ml, 40 μM Ca^{2+} addition results in a shift to a stable (as seen in B) baseline. ATP is added at the arrow and Ca^{2+} binding is followed by spontaneous release (upswing of trace). Subsequent ATP addition re-stimulates Ca^{2+} binding and its release. ATP added during the spontaneous release phase (included as dotted lines, but performed as separate experiment), re-stimulated, but to a lesser degree.

resumes. If the spontaneous release reaction is allowed to go to completion, ATP addition results in calcium accumulation again. As previously described [3], this re-stimulation of calcium accumulation does not result in 100% accumulation. Past evidence has demonstrated that one of two classes of calcium binding sites in the sarcoplasmic reticulum are inhibited progressively by repeated addition of ATP [4] in a way suggesting that ADP is the inhibitor. In the lower trace (Fig. 1B), it is obvious that calcium accumulation and calcium release are not inhibited by extended pre-incubation. It is important to note that ATP depletion itself does not result in the spontaneous calcium release since we have shown identical release with much higher ATP concentrations [3] and with regenerating systems. These higher ATP concentrations or the presence of a regenerating system were not chosen for this figure since they hinder ATP re-stimulation of calcium binding [3].

If a passive uptake of murexide is responsible for spontaneous release, one would presume that this passive uptake would be independent of ATP concentration and dependent only on calcium and murexide concentration. Fig. 2 represents an experiment which demonstrates that this is not so. In this case, 0.8 mg of sarcoplasmic reticulum protein is pre-incubated 5 min in a cuvette with murexide. In contrast to Fig. 1, only 15 μM calcium is then added which is not sufficient to saturate the binding sites on the sarcoplasmic reticulum. ATP addition results in complete removal of all calcium from the medium, but in the upper tracing, all ATP has been consumed at the arrow (average time of ATP depletion is 100 s in the conditions demonstrated in Figs. 1 and 2) and spontaneous calcium release then ensues. In the lower tracing, an ATP regenerating system is present and no apparent spontaneous release occurs. If, however, sufficient calcium is added to saturate all binding sites, then spontaneous release occurs as normally observed in the presence or absence of ATP when calcium is not limiting. This suggests that spontaneous release has indeed

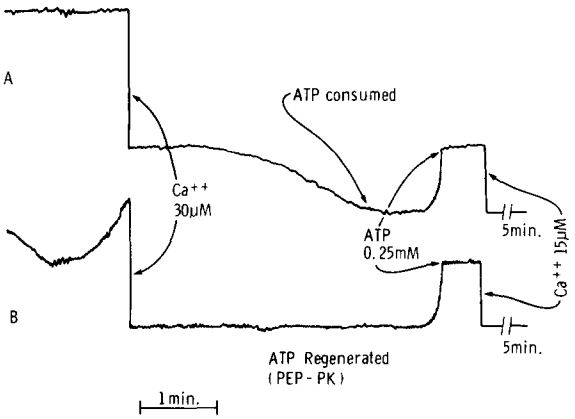

Fig. 2. Reaction conditions and reactants as in Fig. 1 except that a non-saturating concentration of Ca^{2+} is used (15 μM). In trace A, all added ATP is hydrolyzed, and bound Ca^{2+} is released. Further addition of Ca^{2+} results in no binding. In B, non-saturating Ca^{2+} is added, but this time to a reaction containing at ATP-regenerating system. Bound Ca^{2+} is released, but re-bound to unoccupied sites so that no net release occurs. Further addition of saturating Ca^{2+} produces binding and release (see text).

occurred in the lower tracing, but that the calcium released is being re-bound by previously unoccupied calcium binding sites. Saturation of all calcium binding sites then results in spontaneous calcium release. Additional evidence for this hypothesis is presented in a previous publication [3].

If no actual calcium release occurred, then one would presume that apparent spontaneous calcium release would be irreversible. In Fig. 1, it was demonstrated that, after spontaneous calcium release, calcium binding could be stimulated by subsequent additions of ATP and this, in itself, would be reasonable

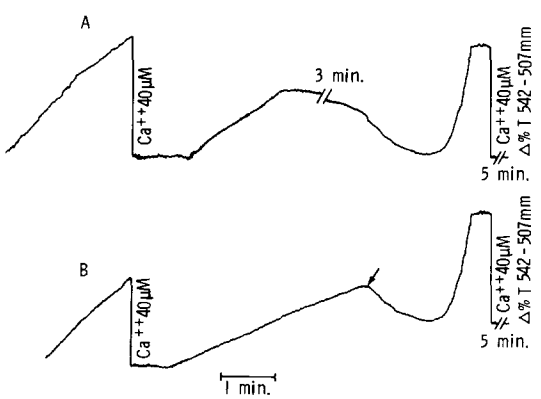

Fig. 3. Effect of phosphate and oxalate on calcium binding. Reaction cuvette contains ATP-regenerating system, additions after 5 min equilibration are 40 μM Ca^{2+} and 5 mM ATP. Binding and release are normal. However, inorganic phosphate (A) after 5 min stimulates uptake of Ca^{2+} (downard slope). After all Ca^{2+} is taken up, further uptake is started by addition of another 40 μM Ca^{2+}. In B reaction conditions are identical except that 5 mM sodium oxalate is added at the arrow. Following binding, a short portion of the Ca^{2+} release curve is observed. The addition of oxalate then results in the onset of calcium uptake. (Following oxalate addition, the instrument baseline is re-adjusted.) As in A, a re-addition of 40 μM Ca^{2+} results in further calcium uptake.

evidence that actual calcium release is occurring. The experiments in Fig. 3 provide further support. In Fig. 3A, calcium binding is initiated with an excess of ATP sufficient to optimally drive all sarcoplasmic reticulum ATPases and the continued optimum concentration of ATP is assured by the presence of the regenerating system. Calcium binding occurs as usual and spontaneous release begins despite the presence of ATP as previously emphasized [3]. However, after 5 min, phosphate build-up from ATP hydrolysis is suffient so that calcium release is the reversed and steady-state calcium uptake resulting from the presence of a precipitating anion occurs in the presence of constant ATP concentration. This suggests that, under these circumstances, the build-up of precipitating anion has changed spontaneous calcium release to a calcium uptake process untill calcium has been completely removed from the solution. Addition of more calcium results in continued calcium uptake as previously described [3]. Addition of the permanent anion oxalate (Fig. 3b) also results in a prompt onset of calcium uptake during the calcium release phase; it is important to emphasize here that an initial spectrophotometric change resulting from instantaneous association of oxalate with calcium is not shown, the steady-state rate which ensues as a result of calcium uptake has been illustrated after the baseline is corrected.

Since Blayney and colleagues [2] made their observations under conditions in which murexide and sarcoplasmic reticulum were being mixed for the first time at the initiation of the reaction, we attempted to examine these conditions. After 5 min incubation, calcium was added to the reaction cuvette which at this time contained no protein (Fig. 4). After 5 min, 0.8 mg/ml of sarcoplasmic reticulum protein was added and a large addition artifact occurred. In Fig. 4, a continuous trace shows that there was then a slow downward drift of the tracing which reached equilibrium in about 10 min. At this time, ATP was added and the typical calcium binding and release curve occurred. This binding and release could be re-stimulated as previously shown in Fig. 1. The identical experiment done at wavelengths 472—542 nm (as used by Blayney et al. [2]) at 25, 30, and 35°C resulted in identical qualitative findings.

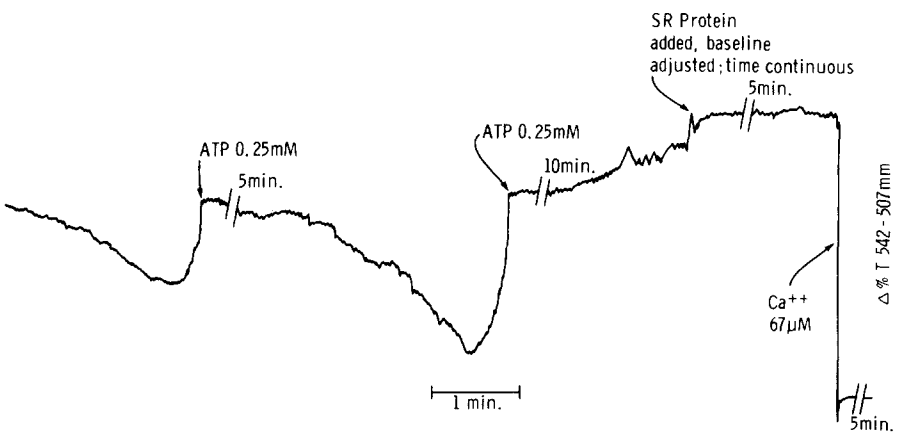

Fig. 4. Reaction cuvette initially contains no sarcoplasmic reticulum, Ca^{2+} or ATP. Ca^{2+} (67 μM) is added and, after 5 min, 0.8 mg sarcoplasmic reticulum/ml is added. ATP addition initiates Ca^{2+} binding and releas. Further stimulation by ATP is possible.

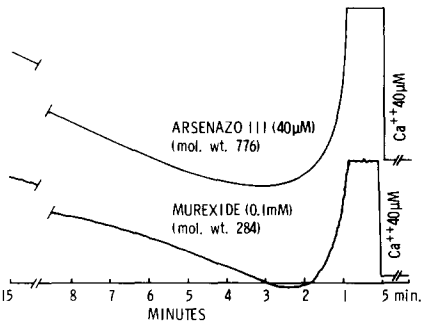

Fig. 5. Reaction as in Fig. 1 except upper trace contains arsenazo III (40 μM) instead of murexide (0.1 mM) (lower trace). Calcium calibration and reaction in presence of arsenazo III is followed using the wavelength pair 675—685 nm.

The chelometric dye arsenazo III (mol. wt. 776) has also been used as an accurate index of calcium concentration. It differs from murexide (mol. wt. 284) in molecular weight, charge, and affinity for calcium; therefore it would not be expected to diffuse with identical characteristics. In addition, it can be used at lower concentrations thus reducing its diffusion rate. Fig. 5 shows a comparison of calcium binding and release measured in identical media by murexide and arsenazo III demonstrating the almost identical configuration.

Discussion

In the work demonstrated in this study, the chelometric dye, murexide was satisfactorily utilized to demonstrate the movements of calcium resulting from ATP-dependent sarcoplasmic reticulum calcium accumulation. Calcium binding and spontaneous release were demonstrated as previously described [3—5,12]. It was also demonstrated that the calcium release could be reversed by readdition of ATP once ATP is depleted. However, calcium release occurs in the presence of ATP as well (Fig. 3), and could be reversed with accumulation by addition of phosphate or oxalate if ATP is persistent. In all of these cases it seems unlikely that spontaneous calcium release could be an artifact since it can be readily reversed by maneuvers known to result in calcium accumulation in sarcoplasmic reticulum.

The experiments of Blayney and colleagues [2] did not include pre-incubation of sarcoplasmic reticulum vesicles with murexide and thus the shifts which ultimately resulted in an equilibrium after 6 min might well have accounted for what they interpreted as spontaneous release. In the experiments conducted in our laboratory utilizing the wavelength pair 542—507 and 472—542 nm, addition of protein to murexide results in a drift toward apparent calcium accumulation (see Fig. 4) rather than towards apparent calcium release. We have in the past found that the wider wavelength pair, although giving greater calcium sensitivity, is much more sensitive to light scattering artifacts as would be expected. The importance of this light scattering in measuring ATP-dependent calcium accumulation by spectrophotometric means has been previously emphasized by our laboratory [8]. We have included data from experiments done only at 30°C in this manuscript, but similar data are obtainable at any temperature between 20 and 37°C [3,4].

It must be pointed out that spontaneous calcium release has also been previously demonstrated utilizing the Millipore filtration technique with ^{45}Ca by ourselves in isolated cardiac and skeletal muscle sarcoplasmic reticulum [3,5,8] and by Ash and colleagues [1] and Sorensen and de Meis [11] in skeletal muscle sarcoplasmic reticulum. We only wish to emphasize in this communication that spontaneous calcium release in isolated sarcoplasmic reticulum appears to be a genuine phenomenon. Experiments reported are those most persuasive of the presence of spontaneous calcium release as a true phenomenon and do not in any way represent all the existing evidence. The sensitivity of spontaneous calcium release to various agents such as NaN_3 [4], dantrolene [12] in which it inhibits spontaneous calcium release in skeletal muscle sarcoplasmic reticulum but not cardiac sarcoplasmic reticulum, and its sensitivity to pathologic conditions such as ischemia [9], cardiomyopathy [7] and congestive heart failure [6] which should in no way interfere with the passive diffusion of murexide also suggests that spontaneous calcium release is a genuine phenomenon. Aging of sarcoplasmic reticulum has been demonstrated to inhibit spontaneous calcium release [3]; whereas, if the aged vesicles become leaky as many have suggested, one would expect that aging would augment release where it is due to changes in murexide diffusion. Additional evidence for the genuine nature of this process is its high energy activation [3,4] of 17—18 kcal/M which is much in excess of that expected for a passive diffusion process. Finally, an identical release phase is observed using another chelometric dye, arsenazo III, which has a different affinity for calcium, molecular weight, and is present in much lower concentrations again suggesting that passive diffusion is an unlikely source of the calcium release phase in sarcoplasmic reticulum.

Acknowledgements

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References

1 Ash, A.S.F., Besch, H.R., Harigaya, S. and Zaimis, E. (1972) J. Physiol. London 224, 1—19
2 Blayney, L., Thomas, H., Muir, J. and Henderson, A. (1977) Biochim. Biophys. Acta 470, 128—133
3 Entman, M.L., Bornet, E.P. and Schwartz, A. (1972) J. Mol. Cell. Cardiol. 4, 155—170
4 Entman, M.L., Snow, T.R., Freed, D. and Schwartz, A. (1973) J. Biol. Chem. 248, 7762—7772
5 Harigaya, S. and Schwartz, A. (1969) Circ. Res. 25, 781—794
6 Lindenmayer, G.E., Sordahl, L.A., Harigaya, S., Allen, J.C., Besch, H.R. and Schwartz, A. (1971) Am. J. Cardiol. 27, 277—283
7 McCollum, W.B., Crow, C., Harigaya, S., Bajusz, E. and Schwartz, A. (1970) J. Mol. Cell. Cardiol. 1, 445—457
8 McCollum, W.B., Besch, H.R., Entman, M.L. and Schwartz, A. (1972) Am. J. Physiol. 223, 608—614
9 Schwartz, A., Wood,, J.M., Allen, J.C., Bornet, E.P., Entman, M.L., Goldstein, M.A., Sordahl, L.A., Suzuki, M. and Lewis, R.M. (1973) Am. J. Cardiol. 32, 46—61
10 Sordahl, L.A., McCollum, W.B., Wood, W.G. and Schwartz, A. (1973) Am. J. Physiol. 224, 497—502
11 Sorensen, M.M. and de Meis, L. (1977) Biochim. Biophys. Acta 465, 210—223
12 Van Winkle, W.B. (1976) Science 193, 1130—1131