The Quantal Nature of Calcium Release to Caffeine in Single Smooth Muscle Cells Results from Activation of the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase*

(Received for publication, November 20, 1995)

Josef M. Steenbergen and Fredric S. Fay‡

From the Biomedical Imaging Group, Program in Molecular Medicine, Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Calcium release from intracellular stores occurs in a graded manner in response to increasing concentrations of either inositol 1,4,5-trisphosphate or caffeine. To investigate the mechanism responsible for this quantal release phenomenon, 

\[ \text{[Ca}^{2+}\text{]} \]

changes inside intracellular stores in isolated single smooth muscle cells were monitored with mag-fura 2. Following permeabilization with saponin or \( \alpha \)-toxin the dye, loaded via its acetoxy-methyl ester, was predominantly trapped in the sarcoplasmic reticulum (SR). Low caffeine concentrations in the absence of ATP induced only partial Ca\(^{2+}\) release; however, after inhibiting the calcium pump with thapsigargin the same stimulus released twice as much Ca\(^{2+}\). When the SR Ca\(^{2+}\)-ATPase was rendered non-functional by depleting its “ATP pool,” submaximal caffeine doses almost fully emptied the stores of Ca\(^{2+}\). We conclude that quantal release of Ca\(^{2+}\) in response to caffeine in these smooth muscle cells is largely due to the activity of the SR Ca\(^{2+}\)-ATPase, which appears to return a portion of the released Ca\(^{2+}\) back to the SR, even in the absence of ATP. Apparently the SR Ca\(^{2+}\)-ATPase is fueled by ATP, which is either compartmentalized or bound to the SR.

Cells respond to increasing concentrations of many hormones and neurotransmitters with graded changes in cytosolic [Ca\(^{2+}\)]. The rise in [Ca\(^{2+}\)] arises in many cells due to release of Ca\(^{2+}\) from internal stores through activation of the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor by IP\(_3\) (1). The graded nature of Ca\(^{2+}\) release from internal stores is known as quantal release (2). Internal Ca\(^{2+}\) stores in many cells also contain ryanodine receptors (RyR), which are activated by cytoplasmic calcium (3) and possibly cyclic ADP-ribose (4). Calcium release due to activation of this receptor with caffeine also occurs in a quantal fashion (5, 6). Three principal hypotheses have been proposed to explain the partial emptying of Ca\(^{2+}\) from internal stores by submaximal levels of IP\(_3\) or caffeine. 1) Individual store elements have different sensitivities to agonists and empty in an all or none fashion (7); 2) all stores partially empty at certain agonist concentrations due to diminished positive feedback effects of luminal [Ca\(^{2+}\)] on Ca\(^{2+}\) release (7); and 3) individual store elements differ not in the sensitivity of their IP\(_3\) or possibly ryanodine receptors but rather in the density of these receptors (8).

An alternative to these explanations is that Ca\(^{2+}\) release is graded in response to increasing levels of agonists simply because at different levels of stimulation a new steady state is achieved reflecting IP\(_3\) - or caffeine-induced stimulation of release and Ca\(^{2+}\) sequestration by pumps associated with these stores. This explanation has been dismissed (9, 10) as IP\(_3\) -induced Ca\(^{2+}\) release in permeabilized cells still occurred in a “quantal fashion” under conditions where Ca\(^{2+}\) pumps were nominally blocked due to ATP removal. However, evidence is emerging that the ATP consumed by some ion pumps (11, 12) is compartmentalized and might not be readily lost either by washing away ATP or even by incubation with ATP scavengers used in some studies (9, 10). If Ca\(^{2+}\) pumps on internal stores also have a compartment of inaccessible ATP then the role of such pumps in quantal release phenomena may have to be reconsidered. Recent work showing that inhibition of the Ca\(^{2+}\) -ATPase with thapsigargin abolishes IP\(_3\)-induced quantal release in permeabilized pancreatic acinar cells (13) suggests that activity of Ca\(^{2+}\) pumps may well be required for quantal Ca\(^{2+}\) release. As thapsigargin caused a significant Ca\(^{2+}\) leak from intracellular stores in that study, the interpretation of those results may be difficult. Furthermore this study was carried out on populations of cells, and hence graded release may have arisen not because release of Ca\(^{2+}\) in each cell was graded but because of variations in the responses of cells within the population. We thus developed a technique to monitor [Ca\(^{2+}\)] changes in the sarcoplasmic reticulum in single isolated smooth muscle cells by loading these cells with the low affinity calcium indicator mag-fura 2 AM and permeabilizing the cell membrane to remove cytosolic dye and gain access to the cytoplasm, a general strategy first described by Hofe and Machen (14).

EXPERIMENTAL PROCEDURES

Isolation and Loading of Smooth Muscle Cells—Smooth muscle cells were enzymatically dissociated from the stomach of the toad Bufo marinus (15) and loaded at a density of 10\(^5\)-10\(^6\) cells/ml with acetoxy-methyl ester of mag-fura 2 (1 \(\mu\)M, 25 \(^\circ\)C) for at least 2 h.

Solutions—Buffer A consisted of (in mM): 130 KCl, 114 Cl\(^-\), 1 dithiothreitol, 5 EGTA, 2 Ca\(^{2+}\), 20 HEPES, and 1 \(\mu\)M leupeptin (pH 7.2). Buffer B was similar but also contained 3 mM MgATP and 0.1 mM Na\(_2\)GTP. Calculated [Ca\(^{2+}\)] in these buffers was 100 nM (16). Caffeine, \(\alpha\)-toxin, and saponin were dissolved in buffer A.

Permeabilization of Smooth Muscle Cells—After loading, cells were suspended in buffer A and placed in a perfusion chamber (200 \(\mu\)l). To exchange media the chamber was perfused at 2.5 ml/min for 30 s. After the cells settled, they were permeabilized with saponin (100 \(\mu\)g/ml, 30–60 s) or \(\alpha\)-toxin (100 units/ml, 30–60 min), washed with buffer A, and resuspended in buffer B. Prior to an experiment, cells were washed with buffer A (ATP free). The ability of the sarcoplasmic reticulum (SR) to store and release Ca\(^{2+}\) was retained following permeabilization with either agent. Saponin was used in most studies, as it acted more rapidly.

Three-dimensional Imaging of Fluorescence—To assess the three-dimensional distribution of mag-fura 2 a series of fluorescent images...
were obtained at 0.5-μm intervals through focus with a digital imaging microscope (17). Images were obtained at 380 nm excitation and 510 nm emission with a water immersion objective (Nikon, ×60, N.A. = 1.3). A constrained deconvolution algorithm (18) was applied to the images to reduce noise and distortion inherent to the acquisition process.

**Calcium Measurements—Fluorescence in a non-nuclear region of single cells was measured with a microfluorimeter (19).** Fluorescence was recorded alternately at 340 and 380 nm, at up to 330 Hz, and after correction for background fluorescence was converted to a calcium ratio (340/380). Minimum fluorescence ratio (Rmin) was determined in vitro in buffer A without CaCl2.

**Materials—Mag-fura 2 AM was from Molecular Probes (Eugene, OR), saponin from ICN Biochemicals (Cleveland, OH), α-toxin from Life Technologies, Inc., and all other chemicals from Sigma.**

**Statistics—Data are given as mean ± S.E. and were subjected to Student’s paired t test. Statistical significance was achieved at p < 0.05.**

**RESULTS AND DISCUSSION**

In permeabilized cells, mag-fura 2 was distributed in a punctuate manner principally beneath the cell membrane and around the nucleus and was almost absent from the cytoplasm (Fig. 1). The pattern of dye distribution beneath the cell membrane was similar to that of calcein-AM (18), a calcium-binding protein present in the SR of these cells, indicating that the loaded intracellular stores have a distribution pattern similar to this cellular compartment.

Caffeine (25 mM, no ATP), which activates the RyR in these cells (20), decreased the fluorescence ratio of mag-fura 2, and thus the [Ca2+]i, in the intracellular stores from 2.42 to 0.58. These stores apparently released their total free calcium in response to this high caffeine concentration as the final fluorescence ratio was virtually identical to Rmin in vitro. In the presence of ATP these stores were capable of calcium uptake, achieving a fluorescence ratio at least 80% of that prior to the initial caffeine challenge. As can be seen in Fig. 2A the response to caffeine was quite reproducible. Refilling of internal stores following caffeine stimulation was almost completely blocked by the specific inhibitor of the SR Ca2+-ATPase thapsigargin (5 min, 2 μM; Fig. 2B). Thus, based on the observations that the loaded intracellular stores have an anatomical distribution similar to the SR, contain a functional RyR and Ca2+-ATPase, and seem to release their total free calcium content in response to caffeine, we believe that the majority of mag-fura 2 signal originates from a SR-like compartment.

While high caffeine concentration (25 mM) caused the stores to release virtually all stored Ca2+, lower caffeine concentrations (5 mM) released less Ca2+ in a reproducible manner (Fig. 2C). Increasing the caffeine concentration from 5 to 25 mM in the absence of ATP caused additional Ca2+ release in the same cell (Fig. 3). The resting fluorescence ratio before caffeine application averaged 1.86 ± 0.13 and decreased to 1.18 ± 0.13 and 0.63 ± 0.05 in the presence of 5 and 25 mM caffeine, respectively (n = 7; Fig. 3B). This dose-dependent graded release of Ca2+ from stores in the absence of ATP is a fundamental characteristic of the "quantal response" (12) (Fig. 3A).

What is responsible for the graded nature of Ca2+ release with these submaximal doses of caffeine? It has been suggested that partial emptying of stores under such conditions may result from the presence of receptors on individual stores with different sensitivities to caffeine or alternatively that individual stores may have different densities of receptors with equivalent sensitivities to caffeine. In either case release of Ca2+ in response to supramaximal doses of caffeine should be described by a multieponential process. We found, however, that a single-exponential function was sufficient to fit the caffeine-induced Ca2+ release (Fig. 2A), implying that there is only one functional store from which calcium is released. Therefore, these data suggested that the mechanism of quantal release may have some other basis. As shown in Fig. 2B, in the presence of thapsigargin but in the absence of exogenous ATP caffeine not only released more Ca2+ from the SR but also at a faster rate compared with the caffeine response in the absence of thapsigargin. This suggested that perhaps the SR Ca2+-ATPase was active during the first caffeine response, even...
though caffeine was applied in the absence of ATP and the cells were washed with buffer A (60 s, no ATP/GTP). Thus the graded release of Ca$^{2+}$ at submaximal caffeine concentrations might result from opposing effects of the SR pumps and caffeine-activated Ca$^{2+}$ release channels, which may be operating to some extent even in an ATP-free medium, perhaps because the SR Ca$^{2+}$-ATPase contains a tightly bound "ATP pool."

To investigate this hypothesis 5 and 25 mM caffeine was applied incrementally to the same cell before and after blocking the SR Ca$^{2+}$-ATPase with thapsigargin (Fig. 4). As shown in Fig. 4A, caffeine at the lower concentration released more calcium from the SR and at a faster rate after thapsigargin. In six cells treated in this manner, the mean fluorescence ratio at rest was 2.07 ± 0.16 and decreased to 1.50 ± 0.18 and 0.65 ± 0.04 in the presence of 5 and 25 mM caffeine, respectively. The fluorescence ratio increased to 1.79 ± 0.16 in the presence of ATP (buffer B) for 5 min. After thapsigargin (2 μM) incubation for 5 min the resting ratio was 1.76 ± 0.17 and decreased to 0.92 ± 0.06 and 0.71 ± 0.04 in the presence of 5 and 25 mM caffeine, respectively (Fig. 4B). While the fluorescence ratio recovered to a variable degree after initial exposure to caffeine (range, 65–100%), the conversion of the response to caffeine following thapsigargin from being decidedly graded to largely all-or-none was not correlated with the extent of this recovery ($r^2 = 0.21$). These data supported our hypothesis that the SR Ca$^{2+}$-ATPase was active during the first caffeine applications, even though ATP was absent from the medium. However, after blocking the SR calcium pumps the stores were still capable of releasing more calcium in the presence of a high caffeine concentration. As noted in Fig. 2, thapsigargin was not 100% effective in blocking the SR calcium pumps at this concentration. Therefore, a small fraction of the pumps might have remained active and been able to establish an equilibrium between calcium influx and efflux from internal stores, albeit at a lower store [Ca$^{2+}$]$.\small\text{-}$ However, higher thapsigargin concentrations were not used to avoid nonspecific membrane leakiness seen at high concentrations (21). The fluorescence ratio throughout the 5-min incubation with thapsigargin remained constant, indicating that the stores have a very low endogenous Ca$^{2+}$ leak rate.

An additional experiment was performed to test the contribution of the SR Ca$^{2+}$ pumps to the quantal release phenomenon in response to caffeine without resorting to the use of pump inhibitors. In this series of experiments the putative "ATP pool" for SR Ca$^{2+}$ pumps was depleted by activation of these pumps at low caffeine concentration, and then the cells were tested for graded Ca$^{2+}$ release. For this, cells were stimulated with 5 mM caffeine, put in buffer A (no ATP) for 5 min, and then stimulated with 5 and 25 mM caffeine, during which time fluorescence ratio changes were monitored (Fig. 5). The resting ratio was 2.15 ± 0.17 and decreased to 1.41 ± 0.09 in the presence of 5 mM caffeine but in the absence of ATP. After buffer A (no ATP) incubation for 5 min the ratio had increased...
Underlying Mechanism of Quantal Release Revised

to 1.70 ± 0.11 but then decreased to 0.86 ± 0.03 and 0.79 ± 0.03 (n = 7) in the presence of 5 and 25 mM caffeine, respectively (Fig. 5B). After the first caffeine stimulus the cells were capable of calcium uptake as indicated by an increased ratio, supporting the notion that a small amount of ATP remained in the cell and was capable of fueling the SR calcium pump. The second caffeine response released virtually all free Ca\(^{2+}\) from the SR, but higher caffeine concentration induced still a small but significant release of Ca\(^{2+}\). It is not clear why this additional Ca\(^{2+}\) release occurred. It is possible that mitochondria are capable of producing a small amount of ATP and thus fuel the SR Ca\(^{2+}\) pump, or the mitochondria may directly take up some Ca\(^{2+}\) and pass it on to the SR. These data clearly indicate that the SR Ca\(^{2+}\)-ATPase is active during the first caffeine application, partially returning some of the released calcium back to the SR, ultimately resulting in a steady state where not all Ca\(^{2+}\) has been released. In addition, the second 5 mM caffeine application was capable of lowering the final calcium concentration in the SR more than the first stimulus, regardless of the initial luminal calcium concentration.

In conclusion, graded increases in caffeine concentrations released calcium from the SR in a quantal manner in isolated smooth muscle cells permeabilized with saponin. These data cannot be reconciled with the current three hypotheses to explain the underlying mechanism of quantal release. Calcium release could be fit by a single exponential function, which is inconsistent with the notion that 1) discrete stores have different sensitivity to agonists and empty in an all or none fashion or that 2) ryanodine receptors are heterogeneously distributed among the stores. In addition, a second caffeine application of equal magnitude was capable of releasing more calcium from the SR regardless of the luminal calcium concentration, contrary to the notion that partial emptying of stores results from an effect of luminal [Ca\(^{2+}\)] on the sensitivity of ryanodine receptors. We have shown that the phenomenon of quantal release at least in these cells in response to caffeine is largely due to the activity of the SR Ca\(^{2+}\)-ATPase, which appears to return a portion of the released Ca\(^{2+}\) back to the SR, even though these applications were in the absence of ATP and the cells were washed with an ATP-free buffer prior to these stimuli. Apparently, the SR Ca\(^{2+}\)-ATPase is fueled by ATP, which is either compartmentalized or bound to the SR membrane.

While the current data may not be directly relevant to the mechanism underlying quantal release of Ca\(^{2+}\) in response to IP\(_3\) or even caffeine in other cells, they highlight that Ca\(^{2+}\) pumps on internal stores are quite effective, at least acutely, in resequestering Ca\(^{2+}\) as it leaves those stores even after exogenous ATP has been removed and with Ca\(^{2+}\) buffers in the cytosol. Certainly this fact should be kept in mind in interpreting quantal release phenomena. Recent work on other single cells has shown that the ability to demonstrate quantal release phenomena is a function of other experimental conditions as well (Ca\(^{2+}\) buffering, Ca\(^{2+}\) indicator affinity, and Ca\(^{2+}\) indicator location); extent of fragmentation of the internal store system (23, 24)), suggesting that "quantal release" may reflect the interplay of the Ca\(^{2+}\) release mechanism with other aspects of Ca\(^{2+}\) store function in the cell. While it may still be that quantal release of Ca\(^{2+}\) at the cellular level is an inherent property of the release mechanism per se, as suggested by the work of Ferris et al. (22), further carefully controlled work on this intriguing phenomenon needs to be carried out.

REFERENCES
1. Berridge, M. J. (1993) Nature 361, 315–325
2. Mualem, S., Pandol, S. J., and Beeker, T. G. (1989) J. Biol. Chem. 264, 205–212
3. Xu, L., Lai, F. A., Cohn, A., Etter, E., Guerrero, A., Fay, F. S., and Meissner, G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3294–3298
4. Meszaros, L. G., Bak, J., and Chu, A. (1993) Nature 364, 76–79
5. Cheek, T. R., Moreton, R. B., Berridge, M. J., Stauderman, K. A., Murawsky, M. M., and Bootman, M. D. (1993) J. Biol. Chem. 268, 27076–27083
6. Cheek, T. R., Berridge, M. J., Moreton, R. B., Stauderman, K. A., Murawsky, M. M., and Bootman, M. D. (1994) Biochem. J. 301, 879–883
7. Bootman, M. D. (1994) Mol. Cell. Endocrinol. 98, 157–166
8. Hirose, K., and Iino, M. (1994) Nature 372, 791–794
9. Meyer, T., and Stryer, L. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3841–3845
10. Taylor, C. W., and Potter, B. V. L. (1990) Biochem. J. 266, 189–194
11. Mercer, R. W., and Dunham, P. B. (1981) J. Gen. Physiol. 78, 547–568
12. Hardin, C. D., Rayemaekers, L., and Paul, R. J. (1992) J. Gen. Physiol. 99, 21–40
13. Van de Put, F. H. M., De Pont, J. J. H. M., and Willems, P. H. G. M. (1994) J. Biol. Chem. 269, 12438–12443
14. Hofer, A. M., and Machen, T. E. (1992) Proc. Natl. Acad. Sci. U.S.A. 90, 2598–2602
15. Fay, F. S., Hoffmann, R., Lediar, S., and Merriam, P. (1982) Methods Enzymol. 85, 284–292
16. Brooks, S. P. J., and Storey, K. B. (1992) Anal. Biochem. 201, 119–126
17. Floyd, F. W., Carrington, W., and Fagarty, K. E. (1989) J. Microsc. (Oxf.) 153, 133–149
18. Carrington, W., Lynch, R. M., Moore, E. D. W., Isenberg, G., Fagarty, K. E., and Fay, F. S. (1995) Science 268, 1483–1487
19. Yagi, S., Beeker, P. L., and Fay, F. S. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4109–4113
20. Guerrero, A., Fay, F. S., and Singer, J. J. (1994) J. Gen. Physiol. 104, 375–394
21. Favoro, T. G., and Abramson, J. J. (1994) Cell Calcium 15, 183–189
22. Ferris, C. D., Cameron, A. M., Huganir, R. L., and Snyder, S. H. (1992) Nature 356, 350–352
23. Hajnoczy, G., Lin, C., and Thomas, A. P. (1994) J. Biol. Chem. 14, 10280–10287
24. Renard-Rooney, D. C., Hajnoczy, G., Setz, M. B., Schneider, T. G., and Thomas, A. P. (1993) J. Biol. Chem. 31, 23601–23610

\(^{2}\) T. Cheek and C. Taylor, personal communication.
The Quantal Nature of Calcium Release to Caffeine in Single Smooth Muscle Cells
Results from Activation of the Sarcoplasmic Reticulum Ca-ATPase
Josef M. Steenbergen and Fredric S. Fay

*J. Biol. Chem.* 1996, 271:1821-1824.
doi: 10.1074/jbc.271.4.1821

Access the most updated version of this article at [http://www.jbc.org/content/271/4/1821](http://www.jbc.org/content/271/4/1821)

Alerts:
- [When this article is cited](http://www.jbc.org/content/271/4/1821.alerts)
- [When a correction for this article is posted](http://www.jbc.org/content/271/4/1821.alerts)

[Click here](http://www.jbc.org/content/271/4/1821.alerts) to choose from all of JBC's e-mail alerts

This article cites 24 references, 13 of which can be accessed free at [http://www.jbc.org/content/271/4/1821.full.html#ref-list-1](http://www.jbc.org/content/271/4/1821.full.html#ref-list-1)