The caffeine-sensitive Ca^{2+} store in bovine adrenal chromaffin cells; an examination of its role in triggering secretion and Ca^{2+} homeostasis

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The effect of caffeine on catecholamine secretion and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in bovine adrenal chromaffin cells was examined using single fura-2-loaded cells and cell populations. In cell populations caffeine elicited a large (~200 nM) transient rise in $[Ca^{2+}]_i$ that was independent of external Ca^{2+}. This rise in $[Ca^{2+}]_i$ triggered little secretion. Single cell measurements of $[Ca^{2+}]_i$ showed that most cells responded with a large (>200 nM) rise in $[Ca^{2+}]_i$, whereas a minority failed to respond. The latter, whose caffeine-sensitive store was empty, buffered a Ca^{2+} load induced by a depolarizing stimulus more effectively than those whose store was full. The caffeine-sensitive store in bovine chromaffin cells may be involved in Ca^{2+} homeostasis rather than in triggering exocytosis.

Ca^{2+}; Secretion; Fluorescence imaging; Fura-2; Caffeine; Adrenal chromaffin cell

1. INTRODUCTION

Bovine adrenal chromaffin cells secrete catecholamine in response to the rise in cytosolic calcium concentration ($[Ca^{2+}]_i$) that results from Ca^{2+} influx induced by depolarizing stimuli such as nicotine or high K^+ [1]. Both of these stimuli lead to Ca^{2+} activation of the subplasmalemmal exocytotic sites [2,3]. In contrast, the $InsP_3$-mobilizing agonist angiotensin II results in either no secretion or a polarized secretory response as the $InsP_3$-sensitive store [3,5] and the intracellular Ca^{2+} signal [4] are spatially restricted. We have previously shown that, in addition to the $InsP_3$-sensitive store, these cells also possess a caffeine-sensitive Ca^{2+} store that is diffusely distributed throughout the cell [5]. Caffeine-sensitive Ca^{2+} stores have also been identified in smooth muscle cells [6,7], sympathetic [8,9] and sensory [10] neurons, pancreatic acinar cells [17] and rat chromaffin cells [11]. The role that $InsP_3$-insensitive stores play in Ca^{2+} signalling in cells is unknown but is the subject of intense speculation (e.g. [8–17]).

We have used video-imaging techniques in single fura-2 loaded cells to investigate some characteristics of the Ca^{2+} signal induced by caffeine in bovine chromaffin cells. The results show that the caffeine store is able to participate in buffering a Ca^{2+} load induced by a depolarizing stimulus, and that the rise in $[Ca^{2+}]_i$ elicited by caffeine occurs evenly throughout these cells and is relatively ineffective at triggering secretion. The caffeine-sensitive store may therefore be involved in intracellular Ca^{2+} homeostasis, rather than directly in triggering exocytosis from these cells.

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2. MATERIALS AND METHODS

Chromaffin cells were isolated from bovine adrenal medullas by enzymatic digestion using either the method of Knight & Baker [18] or a modification [19] of the method of Greenberg & Zinder [20]. Cells were isolated in Ca^{2+}-free Krebs-Ringer buffer consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes pH 7.4 (buffer A) and then washed in buffer A. Chromaffin cells were purified by differential plating [21]. Non-adherent chromaffin cells were then plated in 24 well trays at a density of 1.0×10^6 cells per well in culture medium (Dulbecco's modified Eagles medium with 25 mM Hepes, 10% foetal calf serum, 8 μm fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 U/ml penicillin, 25 μg/ml streptomycin) for 2–4 days.

The determination of catecholamine secretion was carried out on cultured cells at 37°C as described [2,22]. Determination of cytosolic free Ca^{2+} in cell populations was performed at 22°C using fura-2 as previously described [2,22]. The drop in fluorescence immediately following addition of EGTA (Fig. 1a trace 2) resulted from a decrease in the signal from external fura-2 rather than from $[Ca^{2+}]_i$ [22,24].

Imaging of intracellular Ca^{2+} in single cells was carried out as described in detail elsewhere [3,4]. After differential plating, cells were resuspended in fresh DMEM and seeded onto 22 mm diameter glass coverslips at a density of 1×10^5 cells/ml. After 1–2 days in culture the cells were washed in Ca^{2+}-containing Krebs and loaded with 1 or 2 μM fura-2 acetoxymethylester for 30 min at room

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temperature. The cells were washed once in Ca^{2+}-containing Krebs and imaged after equilibration to 37°C for 3 min. Experiments were carried out at 37°C with continual perfusion of Ca^{2+}-containing Krebs buffer. Stimuli were applied to cells via a U-tube positioned to within 2 mm of the cell. Using this method the cells were challenged with the stimulus within 1 s of the onset of application. Fluorescent images were obtained by alternate excitation at 340 nm or 380 nm (40 ms each wavelength) using an image-processing system (Imagine, Synoptics Ltd, Cambridge, UK) interfaced to a DEC microvax II microcomputer. The ratio image was obtained at video rate and filtered with a time constant of 200 ms. Determination of $[Ca^{2+}]_i$ was as described by equation 5 of Grynkiewicz et al. [25]. Three dimensional plots were generated by Imagine from the ratio image and depict either the resting distribution of $[Ca^{2+}]_i$ or the distribution and qualitative rise in $[Ca^{2+}]_i$ elicited by caffeine.

3. RESULTS AND DISCUSSION

Caffeine was able to elicit a dose-dependent rise in $[Ca^{2+}]_i$ as monitored in a population of fura-2 loaded chromaffin cells. Fig. 1a shows that challenging the cells with 10 mM caffeine resulted in an immediate increase in $[Ca^{2+}]_i$ to about 220 nM above basal both in the presence (trace 1) and absence (trace 2) of external Ca^{2+}. Both responses were transient over ~90 s. Because population measurements have been shown to mask the changes in $[Ca^{2+}]_i$ that occur at the single cell level [2], the changes in $[Ca^{2+}]_i$ that occurred in single fura-2 loaded cells exposed to caffeine were monitored. Fig. 1b shows the $[Ca^{2+}]_i$ changes in 13 single cells challenged either in the presence (traces 1–6) or absence (traces 7–13) of external Ca^{2+}. The caffeine response displayed considerable heterogeneity which was independent of the presence or absence of external Ca^{2+}. Some cells showed a large (>500 nM) Ca^{2+} transient (e.g. traces 1, 2, 7, 8), whereas other cells showed either a smaller (traces 3, 5, 6, 9, 12, 13) or indeed no rise in $[Ca^{2+}]_i$ (traces 4, 10, 11). It is likely that this heterogeneity reflects the varying degrees to which the caffeine stores are loaded with Ca^{2+} at the time of the challenge. A similar result was observed in response to muscarinic receptor activation of these cells [2], where it was also found that, in those cells that responded strongly, the Ca^{2+} signal was restricted to one pole of the cell. In response to caffeine (in the absence of external Ca^{2+}), however, no such restriction of the Ca^{2+} signal was observed (Fig. 2). The three-dimensional plots clearly show that the rise in $[Ca^{2+}]_i$ was initiated throughout the cell and that, at the peak of the response, the Ca^{2+} was uniformly distributed. This is consistent with the notion that the caffeine-sensitive store is itself diffusely distributed throughout these cells [5].

The Ca^{2+} response to caffeine is monophasic (Fig. 1b, traces 1–6). That is, it does not result in the phase of sustained Ca^{2+} entry that is seen following Ca^{2+} mobilization by $InsP_3$-mobilizing stimuli in these [3,4,24,26] and other (e.g. [27,28]) cell types. It has recently been shown that release of internal Ca^{2+} from the $InsP_3$-sensitive store is not the stimulus that causes Ca^{2+} entry into the cytosol of these cells [26]. The present result shows that release of Ca^{2+} from the caffeine-sensitive store is also unable to automatically trigger Ca^{2+} entry.

The caffeine-sensitive store may function to modulate the Ca^{2+} signal induced by the depolarizing agonist nicotine (Fig. 3). A field, consisting of three cells, was sequentially exposed to 0.3 mM methacholine, 10 mM caffeine and then 10 μM nicotine. No cell responded with a rise in $[Ca^{2+}]_i$ to the muscarinic agonist, consistent with our previous results which showed some chromaffin cells to be unresponsive to muscarinic stimuli [2]. In response to caffeine only cells 1 and 2 responded with a Ca^{2+} transient. Upon removal of caffeine the store refills rapidly, as a subsequent application of caffeine applied within 20 s triggers another rise in $[Ca^{2+}]_i$ (data not shown). It appears therefore that only in cell 3 were both the $InsP_3$- and caffeine-sensitive stores empty prior to the cells being depolarized with nicotine. Following a 10 s nicotinic stimulation, cell 3 was able to buffer the Ca^{2+} load more quickly than cells 1 and 2 (Fig. 3). In cell 3, the rise in $[Ca^{2+}]_i$ had returned to resting levels by 80 s, whereas $[Ca^{2+}]_i$ was still considerably elevated at this time in cells 1 and 2. Cell 3 may have been able to sequester cytosolic Ca^{2+} relatively quickly because its Ca^{2+} stores were empty, whereas the caffeine-sensitive stores in cells 1 and 2 were full. Alternatively, a Ca^{2+}-induced Ca^{2+} release mechanism similar to that seen after depolarization of sympathetic neurons [9], may have resulted in the Ca^{2+} entry induced by nicotine promoting release of the internally stored Ca^{2+} in cells 1 and 2, thereby prolonging the Ca^{2+} signal in those cells. Clearly, the Ca^{2+} signal induced by depolarizing stimuli can be considerably influenced by the state of the internal pools.

In view of the fact that concentrations of depolarizing stimuli that elevate $[Ca^{2+}]_i$ in cell populations to ~200 nM above basal trigger secretion of around 20% of total cellular catecholamine from these cells [2,22,24], the ability of caffeine to elicit a secretory response was tested. Fig. 4 shows the effect on catecholamine secretion of a dose of caffeine which elevates $[Ca^{2+}]_i$ to this same level (see Fig. 1a). Caffeine is a relatively weak secretagogue, triggering the release of only 0.5–1.0% of total cellular catecholamine. Fig. 4 also shows the effect of external Ca^{2+} and Mg^{2+} on caffeine-induced secretion. Release was at least partially independent of external Ca^{2+}, and was not significantly increased in the absence of external Mg^{2+}, as has been reported for cat chromaffin cells [29]. It is unlikely that the weak secretion due to caffeine is caused by elevated cAMP inhibiting the response because forskolin, cholera toxin and 8-brcAMP were all without effect on the K^+-induced rise in $[Ca^{2+}]_i$ and secretory response in these cells [30].

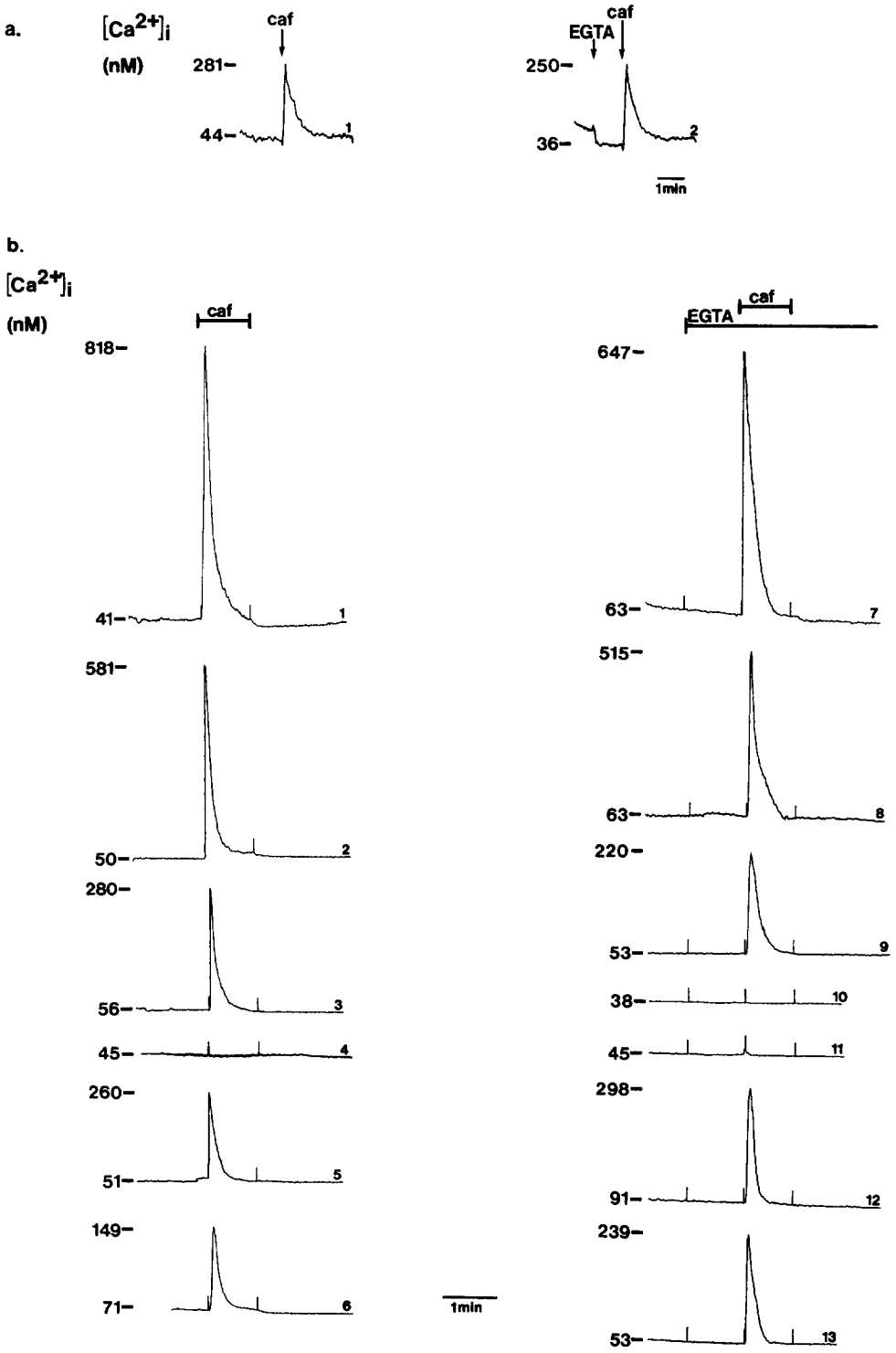

Fig. 1. Time courses of changes in $[Ca^{2+}]_i$ in chromaffin cells in response to caffeine. (a) Changes in $[Ca^{2+}]_i$ in populations of fura-2 loaded cells in response to 10 mM caffeine (caf) either in the presence of 3 mM external Ca^{2+} (trace 1) or in medium made essentially free of Ca^{2+} by addition of 4 mM EGTA (trace 2). (b) Changes in $[Ca^{2+}]_i$ in 13 single cells in response to 10 mM caffeine (caf) either in the presence of 3 mM external Ca^{2+} (traces 1–6) or in the presence of nominally Ca^{2+}-free medium containing 1 mM EGTA (traces 7–13).

Although it has been known for over 20 years that a rise in $[Ca^{2+}]_i$ is a necessary prerequisite for secretion from bovine chromaffin cells, recent results from us [2,4] and others [31,32] have demonstrated that, in intact cells responding to physiological agonists, a large (>200 nM) rise in $[Ca^{2+}]_i$ does not necessarily trigger a full secretory response. Depolarizing stimuli, which elevate $[Ca^{2+}]_i$ by inducing Ca^{2+} influx, are potent

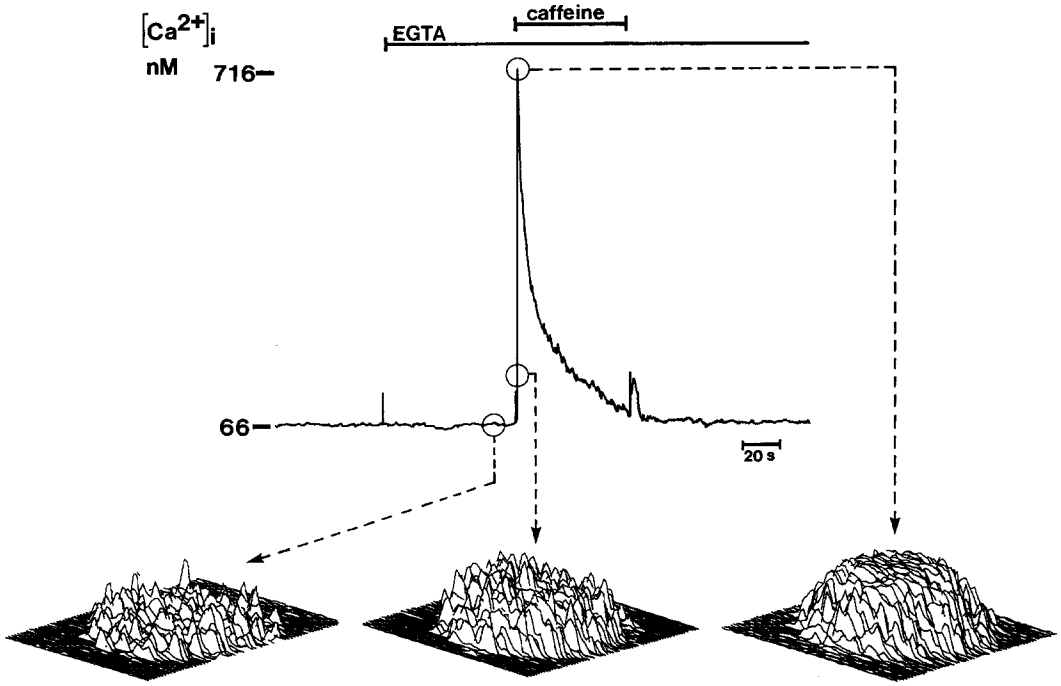

Fig. 2. Time course and spatial localization of changes in $[Ca^{2+}]_i$ in a single chromaffin cell in response to caffeine. The time course shows the response of a cell to 10 mM caffeine (caf) in nominally Ca^{2+}-free medium containing 1 mM EGTA. The three-dimensional plots show the subcellular localization of $[Ca^{2+}]_i$ at the times indicated.

secretagogues, whereas stimuli which release internally stored Ca^{2+} via mobilization of $InsP_3$ are weak secretagogues. The finding that caffeine can elicit a large rise in $[Ca^{2+}]_i$ in cells by releasing internally stored Ca^{2+}, but is only a weak secretagogue, is consistent with these results. It is also a further indication that Ca^{2+} sources can be functionally different in cells. In the bovine chromaffin cell, it appears that secretion requires external Ca^{2+}, whereas release of internal Ca^{2+} may be important for triggering alternative functions such as hormone biosynthesis or Ca^{2+}-dependent events within the nucleus [2,5,31].

Caffeine-sensitive stores that are $InsP_3$-insensitive have also been reported in smooth muscle cells [6,7],

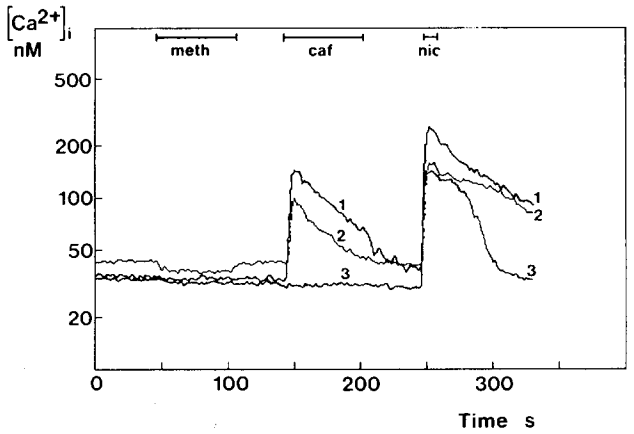

Fig. 3. Time courses of changes in $[Ca^{2+}]_i$ in chromaffin cells in response to methacholine, caffeine and nicotine. Data shown are from three cells in the same microscope field that were sequentially challenged with 0.3 mM methacholine (meth), 10 mM caffeine (caf) and then 10 μM nicotine (nic). The medium contained 3 mM external Ca^{2+}. Cell 3, whose $InsP_3$- and caffeine-sensitive stores were empty, was able to buffer a nicotine-induced Ca^{2+} load more effectively than cells 1 and 2 (see text).

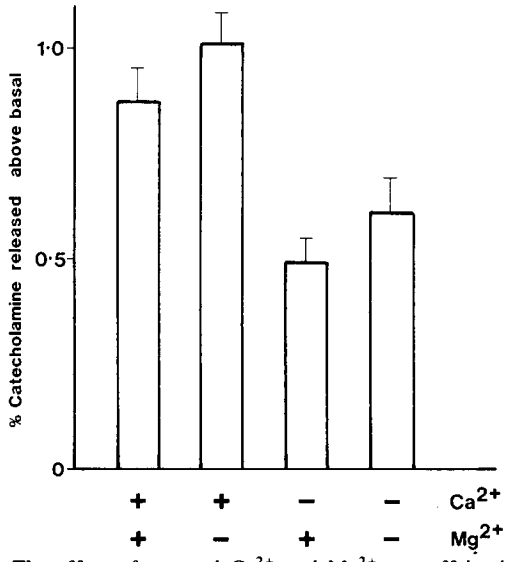

Fig. 4. The effect of external Ca^{2+} and Mg^{2+} on caffeine-induced catecholamine secretion. Cells were challenged with 10 mM caffeine in the presence of 3 mM Ca^{2+} or 1 mM EGTA (with no added Ca^{2+}), in the presence or absence of 1.3 mM Mg^{2+}. Catecholamine released above basal over a 10 min period was measured and is expressed as a percentage of the total catecholamine in the cell. Data are mean ± SE (n = 6) of a typical experiment. By comparison, stimulation with nicotine over a similar period would have released ~20% of total cellular catecholamine (see text and [24]).

sensory neurons [10] and rat chromaffin cells [11]. The physiological role of these stores in Ca^{2+} signalling is unknown, but the existence of two internal Ca^{2+} stores has been invoked in models for the mechanism of Ca^{2+} oscillations [15,16,33] seen in response to $InsP_3$-mobilizing agonists [34]. Whether physiological agonists are capable of releasing Ca^{2+} from the caffeine-sensitive store in bovine chromaffin cells or whether the role of the store is purely to help the cell buffer an agonist-induced Ca^{2+} load is currently under investigation.

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REFERENCES

[1] Burgoyne, R.D. (1984) Biochim. Biophys. Acta 779, 201–216.
[2] Cheek, T.R., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. (1989) FEBS Lett. 247, 429–434.
[3] O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. (1989) EMBO J. 8, 401–411.
[4] Cheek, T.R., Jackson, T.R., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J. and Burgoyne, R.D. (1989) J. Cell Biol. 109, 1219–1227.
[5] Burgoyne, R.D., Cheek, T.R., Morgan, A., O'Sullivan, A.J., Moreton, R.B., Berridge, M.J., Mata, A.M., Colyer, J., Lee, A.G. and East, J.M. (1989) Nature 342, 72–74.
[6] Kanaide, H., Shogakiuchi, Y. and Nakamura, M. (1987) FEBS Lett. 214, 130–134.
[7] Saida, K. and Van Breemen, C. (1983) Pflügers Arch. 397, 166–167.
[8] Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.Y. and Tsien, R.W. (1988) Proc. Natl. Acad. Sci. USA 85, 2398–2402.
[9] Lipscombe, D., Madison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) Neuron 1, 355–365.
[10] Thayer, S.A., Perney, T.M. and Miller, R.J. (1988) J. Neurosci. 8, 4089–4097.
[11] Malgaroli, A., Fesce, R. and Meldolesi, J. (1990) J. Biol. Chem. 265, 3005–3008.
[12] Gill, D.L., Ghosh, T.K. and Mullaney, J.M. (1989) Cell Calcium 10, 363–374.
[13] Ghosh, T.K., Mullaney, J.M., Tarazi, F.I. and Gill, D.L. (1989) Nature 340, 236–239.
[14] Schulz, I., Thevenod, F. and Dehlinger-Kremer, M. (1989) Cell Calcium 10, 325–336.
[15] Berridge, M.J. and Irvine, R.F. (1989) Nature 341, 197–205.
[16] Berridge, M.J. and Galione, A. (1988) FASEB J. 2, 3074–3082.
[17] Osipchuk, Y.V., Wakui, M., Yule, D.I., Gallacher, D.V. and Petersen, O.H. (1990) EMBO J. 9, 697–704.
[18] Knight, D.E. and Baker, P.F. (1983) Q. J. Exp. Physiol. 68, 123–143.
[19] Burgoyne, R.D., Morgan, A. and O'Sullivan, A.J. (1988) FEBS Lett. 238, 151–155.
[20] Greenberg, A. and Zinder, O. (1982) Cell Tiss. Res. 226, 655–665.
[21] Waymire, J.C., Bennett, W.F., Boehme, R., Hankins, L., Gilmer-Waymire, K. and Haycock, J.W. (1983) J. Neurosci. Meth. 7, 329–351.
[22] Cheek, T.R. and Burgoyne, R.D. (1985) Biochim. Biophys. Acta 846, 167–173.
[23] O'Sullivan, A.J. and Burgoyne, R.D. (1988) Biochim. Biophys. Acta 969, 211–216.
[24] O'Sullivan, A.J. and Burgoyne, R.D. (1989) Biosci. Rep. 9, 243–252.
[25] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[26] Stauderman, K.A. and Pruss, R.M. (1989) J. Biol. Chem. 264, 18349–18355.
[27] Yule, D.I. and Gallacher, D.V. (1988) FEBS Lett. 239, 358–362.
[28] Jacob, R. (1990) J. Physiol., in press.
[29] Yamada, Y., Teraoka, H., Nakazato, Y. and Ohga, A. (1988) Neurosci. Lett. 90, 338–342.
[30] Cheek, T.R. and Burgoyne, R.D. (1987) J. Biol. Chem. 262, 11663–11666.
[31] Kim, K.-T. and Westhead, E.W. (1989) Proc. Natl. Acad. Sci. USA 86, 9881–9885.
[32] Pruss, R.M. and Stauderman, K.A. (1990) J. Physiol., in press.
[33] Rooney, T.A., Sass, E. and Thomas, A.P. (1989) J. Biol. Chem. 264, 17131–17141.
[34] Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. (1986) Nature 319, 600–602.