Cell Calcium 33 (2003) 27–35

cell calcium

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Fura-2 antagonises calcium-induced calcium release

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Received 24 July 2002; received in revised form 6 September 2002; accepted 12 September 2002

Abstract

Calcium-induced calcium release (CICR) from the endoplasmic reticulum (ER) takes place through ryanodine receptors (RyRs) and it is often revealed by an increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) induced by caffeine. Using fura-2-loaded cells, we find such an effect in bovine adrenal chromaffin cells, but not in cerebellar granule neurones or in HEK-293 cells. In contrast, a caffeine-induced $[Ca^{2+}]_c$ increase was clearly visible with either fluo-3 or cytosolic aequorin. Simultaneous loading with fura-2 prevented the $[Ca^{2+}]_c$ increase reported by the other Ca^{2+} probes. Caffeine-induced Ca^{2+} release was also measured by following changes of $[Ca^{2+}]$ inside the ER ($[Ca^{2+}]_{ER}$) with ER-targeted aequorin in HEK-293 cells. Fura-2 loading did not modify Ca^{2+} release from the ER. Thus, fura-2, but not fluo-3, antagonises the generation of the cytosolic Ca^{2+} signal induced by activation of RyRs. Cytosolic Ca^{2+} buffering and/or acceleration of Ca^{2+} diffusion through the cytosol may contribute to these actions. Both effects may interfere with the generation of microdomains of high $[Ca^{2+}]_c$ near the ER release channels, which are essential for the propagation of the Ca^{2+} wave through the cytosol. In any case, our results caution the use of fura-2 to study CICR.

Keywords: Calcium-induced calcium release; Caffeine; Fura-2; Aequorin; Endoplasmic reticulum; Cerebellar granule neurones; HEK-293 cells

1. Introduction

Calcium-induced calcium release (CICR) is essential for proper cardiac beating [1]. CICR is mediated by sarco/endoplasmic reticulum (SR/ER) ryanodine receptors (RyRs). Three different isoforms, RyR1, RyR2 and RyR3, encoded by different genes, are expressed in mammalian tissues [2]. RyR1 and RyR2 are expressed predominantly in skeletal muscle and heart, respectively, where they play a crucial role in muscle contraction [3,4], but RyRs are widely distributed in several tissues [2,5,6]. The role of RyR in non-muscle cells is unclear [7,8]. It has been proposed that Ca^{2+} entry through voltage-dependent Ca^{2+} channels could trigger CICR in neurones, thus, amplifying the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) signal [9,10]. The existence of CICR has been clearly demonstrated in peripheral sensory and sympathetic neurones [11–14] and in cerebellar Purkinje cells [15]. In other central neurones, the amount of Ca^{2+} contained in the caffeine-sensitive stores is very small under resting conditions and CICR is difficult to demonstrate [9,10,13].

Caffeine triggers the opening of RyR at resting $[Ca^{2+}]_c$ levels and many important pieces of evidence for the CICR mechanism arose from investigation of caffeine-induced $[Ca^{2+}]_c$ elevation [13,16]. Reports in the literature on the existence of CICR are often controversial. For example, in cerebellar granule neurones loaded with the Ca^{2+} probe fura-2 [17], caffeine has been reported both, to increase $[Ca^{2+}]_c$ [18] and not to modify it [19]. Similar contradictions have been found in other cell systems. Since fura-2 (and other Ca^{2+} probes) are indeed Ca^{2+} chelators, we reasoned that they could buffer or otherwise modify $[Ca^{2+}]_c$ transients generated in the close proximity RyR, which are essential for the autoregenerative CICR propagation [8]. It is well known, for example, that buffering $[Ca^{2+}]_c$ modifies drastically Ca^{2+}-induced inactivation of voltage-gated Ca^{2+} channels [20]. On the other hand, fura-2 and other Ca^{2+} chelators accelerate Ca^{2+} diffusion through the cytosol, thus, tending to dissipate high $[Ca^{2+}]_c$ microdomains generated by the activity of Ca^{2+} channels [21–23]. If the effect of caffeine was dependent on the generation of such microdomains, it could certainly be disturbed by the Ca^{2+} probe, and subtle differences in experimental conditions, such as the degree of probe loading into the cells, could make big differences.

The above difficulties would not apply if the decrease of $[Ca^{2+}]_{ER}$ rather than the increase of $[Ca^{2+}]_c$ was used as the indicator of Ca^{2+} release from the ER. The fluorescent

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0143-4160/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0143-4160(02)00179-3

probes used to measure [Ca^{2+}] cannot be selectively targeted to inside ER (but see [24,25]), but this problem can be circumvented using protein probes, which can be directed to organelle by adding a targeting sequence. Aequorin, a Ca^{2+}-sensitive photoprotein, targeted to ER has been used recently for direct demonstration of CICR in adrenal chromaffin cells [26]. Here, we address the question of whether fluorescent Ca^{2+} probes can mask CICR in three different cell preparations, adrenal chromaffin cells, cerebellar granule neurones and HEK-293 cells, by comparing the results obtained with two different fluorescent cytosolic Ca^{2+} probes and with the cytosolic and the ER-targeted aequorin.

2. Materials and methods

2.1. Cell culture

Cerebellar granule neurones were prepared from 5- to 7-day-old Wistar rat pups killed by cervical dislocation followed by decapitation as described previously [19,27]. Cells were plated on poly-L-lysine-coated 11 mm diameter coverslips and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 500 μg/ml gentamicin. HEK-293 cells were plated on poly-L-lysine-coated coverslips and cultured in DMEM containing 10% foetal calf serum, penicillin and streptomycin. Bovine adrenal chromaffin cells were kindly provided by Dr. A.G. García, Instituto Teófilo Hernando, Madrid, Spain. They were prepared and handled as described previously [17,26,28,29].

2.2. Measurements of $[Ca^{2+}]_c$ with fluo-3 and fura-2

Measurements of $[Ca^{2+}]_c$ were performed by digital-imaging fluorescence microscopy in cells loaded with either fura-2 or fluo-3 by incubation with the corresponding acetoxymethyl (AM) esters. All experiments were conducted at 37 °C. The cells were under continuous perfusion with either control or test solutions at 2–3 ml/min. This rate of perfusion allowed >95% exchange of the medium within 5–10 s. The standard control solution had the following composition (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Na^+-HEPES, 10, pH, 7.4. The cells were epi-illuminated alternately at 340 and 380 nm and light emitted above 520 nm was recorded using a Magical Image Processor (Applied Imaging, Newcastle, UK). Pixel-by-pixel ratios of consecutive frames obtained at 340 and 380 nm excitation were produced and $[Ca^{2+}]_c$ was estimated from these ratios by comparison with fura-2 standards. Additional details can be found elsewhere [19,30–32].

2.3. Measurements of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ with aequorin

Expression of recombinant wild type (cytosolic) aequorin or mutated (low Ca^{2+} affinity) aequorin targeted to the ER [33,34] was achieved by infecting the cells with a defective herpes simplex virus type 1 containing the gene [26,35] 12–24 h prior to measurements. The cytosolic apoaequorin was reconstituted by incubation with coelenterazine h for 60–90 min [36]. For $[Ca^{2+}]_{ER}$ measurements, the ER was first depleted of Ca^{2+} by incubating the cells in Ca^{2+}-free medium (0.5 mM EGTA) containing 10 μM 2,5 di-*tert*-butyl-hydroquinone (an inhibitor of ER Ca^{2+}-ATPase) for 10 min at room temperature, and apoaequorin was reconstituted to aequorin by incubation for 1–2 h in Ca^{2+}-free medium containing 1 μM coelenterazine n. For measurements, the cells were placed in the luminometer (Cairn Research, Ltd.) and perfused at 5–7 ml/min with the different test solutions, as described in Section 3. Emitted light was integrated every 1-s period. The experiment was terminated by perfusion with a solution containing 100 μM digitonin and 10 mM $CaCl_2$ to release all the aequorin counts. Calibrations in $[Ca^{2+}]$ were done as described previously [34,37].

2.4. Chemicals

Fura-2/AM, fluo-3/AM and coelenterazines were obtained from Molecular Probes. Other reagents were of the highest quality available from Sigma, Calbiochem or Merck.

3. Results

Fig. 1A illustrates the increase of $[Ca^{2+}]_c$ induced by caffeine, as observed in fura-2-loaded chromaffin cells. Caffeine induced a large $[Ca^{2+}]_c$ increase which was very fast and transient and almost equal in height to the $[Ca^{2+}]_c$ peak induced by depolarisation with high K^+. It was prevented by Ry in a use-dependent fashion (not shown). When a second stimulation with caffeine was applied 1 min after the first one, almost no increase in $[Ca^{2+}]_c$ was observed (Fig. 1B). When the interval between caffeine pulses was increased, the $[Ca^{2+}]_c$ response also increased gradually, but restoration of the original response required at least 5 min (Fig. 1B). This likely reflects the time required for refilling the intracellular stores with Ca^{2+}. These observations are consistent with previous findings [27,38].

We were unable to detect an effect of caffeine on $[Ca^{2+}]_c$ in cerebellar granule neurones loaded with fura-2 (Fig. 2A). We then repeated these experiments in cells loaded with fluo-3. We were surprised to find that fluo-3 systematically reported a quite large $[Ca^{2+}]_c$ increase in most of the granule neurones stimulated with caffeine. The size of the $[Ca^{2+}]_c$ peak was comparable to the one obtained in cells depolarised with high K^+, even bigger in some individual cells, although it was always more transient (Fig. 2B).

The response to caffeine seemed bigger in cells cultured in vitro for shorter periods of time. For this reason we performed a systematical study, following the size of the responses to caffeine, acetylcholine and high K^+ after

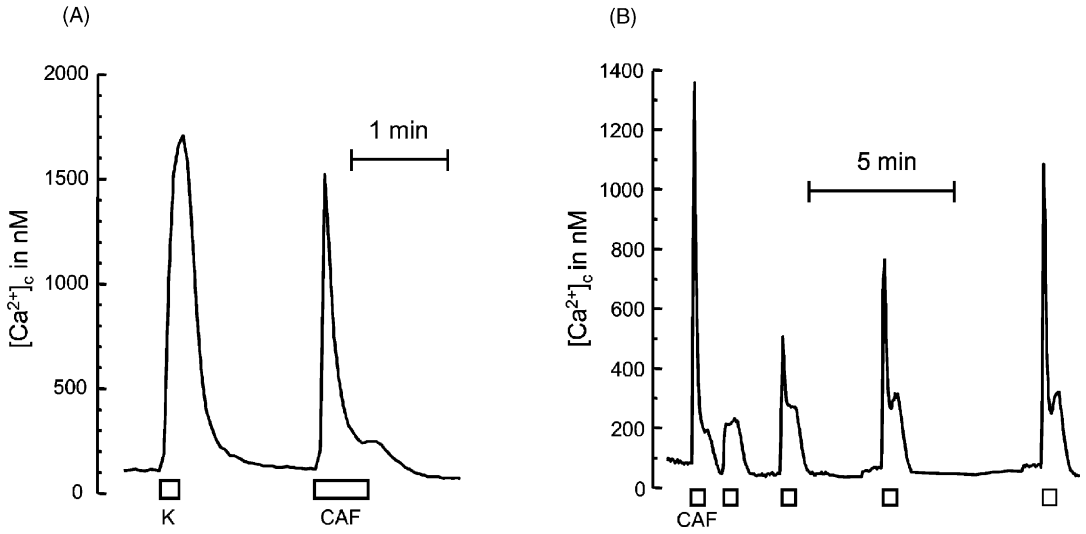

Fig. 1. Effects of caffeine in bovine adrenal chromaffin cells. Fura-2-loaded chromaffin cells were stimulated with high K^+ (70 mM) or caffeine (50 mM) as shown. In panel B, cells were stimulated repetitively with caffeine. The interval between successive stimuli was 1, 2, 3.5 and 5.5 min, respectively. The traces shown are the average of 40 (A) and 39 (B) individual cells present in the same microscope field.

different times of culture. Fig. 3 summarises the results of this study. There was a large decrease in the response to caffeine (about fourfold) with the time of culture in vitro (Fig. 3A). On the contrary, the response to high K^+ increased two to three times with culture, so that the difference between both responses was very much increased with time in culture. The response to acetylcholine was similar at all times in culture. Fig. 3B shows the changes with time of culture of the responses to caffeine and acetylcholine, expressed relative to the response to high K^+ measured in the same individual cell. The responses to caffeine tended to be larger when they were preceded by an stimulation with high K^+ (Fig. 2B). This response (after K^+) was somewhat better preserved after culture of the cells (Fig. 3B).

Once established the effects of cell culture, we re-addressed the specific effect of fura-2 and fluo-3 on the responses to caffeine. Both probes should be able to chelate Ca^{2+} similarly, so that it is intriguing why only one is unable to report the increase of $[Ca^{2+}]_c$. Does it just do not report or does it really prevent the increase of $[Ca^{2+}]_c$? We decided to investigate the effects of fura-2 in aliquots of the same cells loaded with either fluo-3, or fura-2 or with both probes at the same time. In the last case, we took advantage of the spectral differences among fura-2 and fluo-3 for measuring $[Ca^{2+}]_c$ simultaneously from both probes. Results are illustrated in Fig. 4. In cells loaded with fluo-3 only, there was a typical transient response to caffeine (Fig. 4A). In cells loaded with fura-2 only, there was no response to caffeine (as reported by fura-2, not shown). In cells loaded with both probes, fluo-3 still detected some response, although atypical (transient phase lost) and very much inhibited (Fig. 4B). Fura-2 did not detect any response in the same

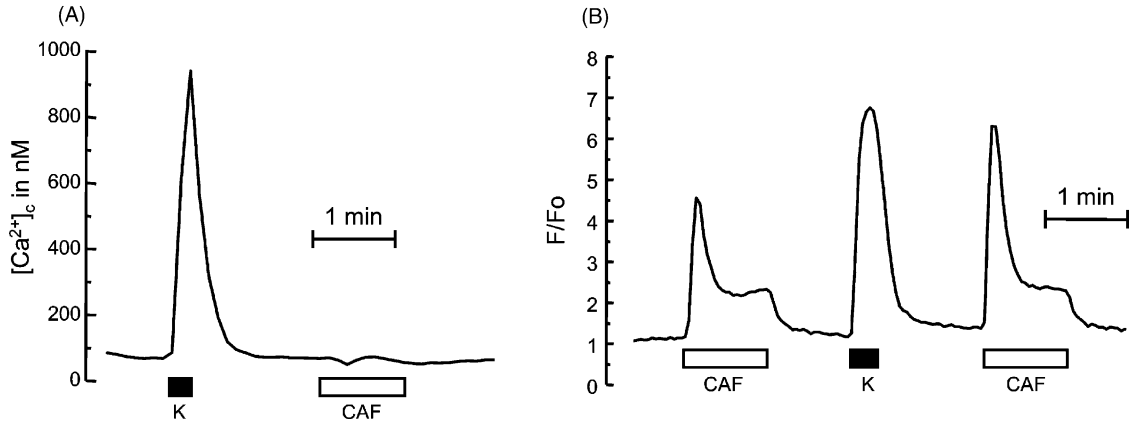

Fig. 2. Effects of caffeine in cerebellar granule neurones. Cells were stimulated with high K^+ (70 mM) or caffeine (50 mM) as shown. They had been loaded for 90 min with either 4 μM fura-2/AM (A) or 1 μM fluo-3/AM (B). Results are expressed either as $[Ca^{2+}]_c$ (A) or as F/F_0 (B).

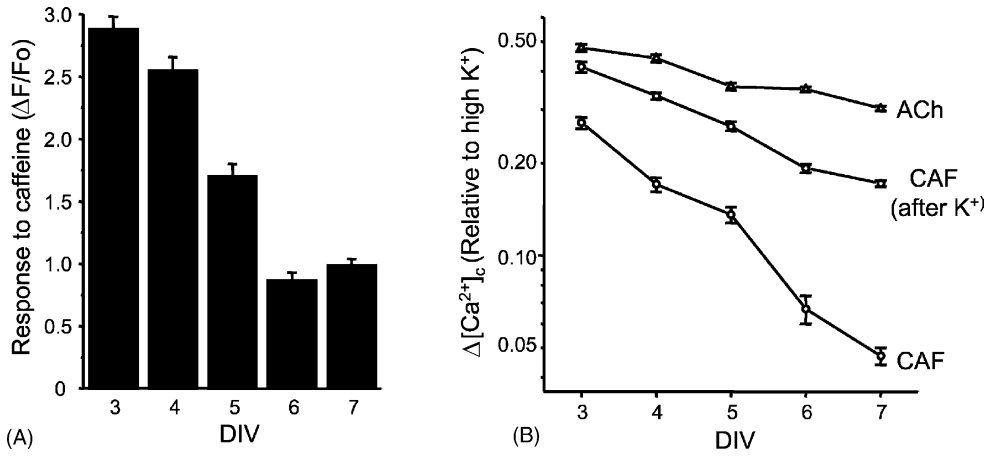

Fig. 3. Effects of caffeine in granule neurones cultured for different periods of time. Cells were tested after different times in culture (DIV, days in vitro). They were loaded with fluo-3 and stimulated sequentially with caffeine (50 mM, 30 s), high K^+ (70 mM, 15 s), caffeine again, and acetylcholine (10 μM, 30 s). The interval between stimuli was 1, 2, 5 and 5 min, respectively. In panel (A) responses were quantified as F/F_0. Results are mean ± S.D. of 163–460 individual cells from at least three different coverslips. In panel B the responses to caffeine and acetylcholine were divided by the response to high K^+ for every single cell. Note logarithmic scale.

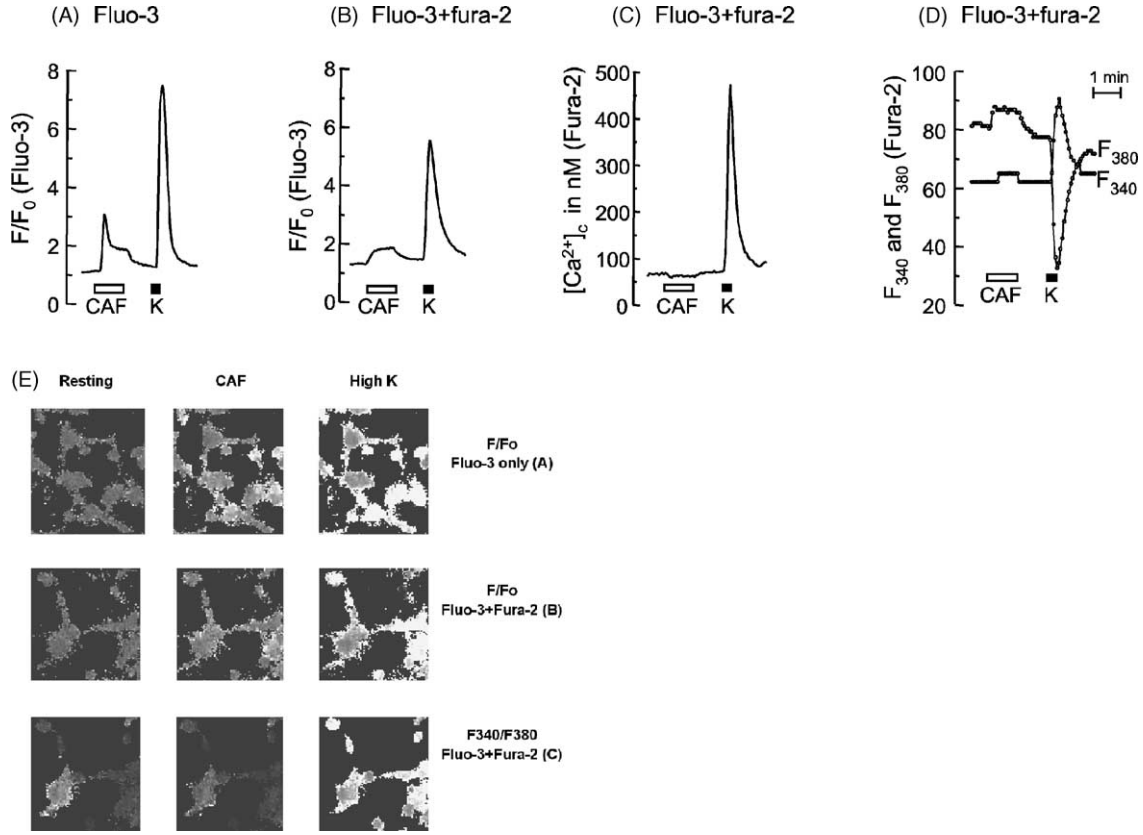

Fig. 4. Comparison of the effects of fura-2 and fluo-3 on the response to caffeine in granule neurones. Cells were loaded either with fluo-3/AM (1 μM, 90 min) (A) or with fluo-3/AM and fura-2/AM simultaneously (1 and 4 μM, respectively, 90 min) (B–D). Then they were stimulated with caffeine and high K^+ as shown. In panels B–D, fluorescence was excited alternately at 340, 380 and 485 nm and either F/F_0 (485 nm) (B) or $[Ca^{2+}]_c$ (estimated from F_{340}/F_{380}) (C) is shown on ordinates. In panel D, crude F_{340} and F_{380} fluorescences are shown. Traces shown are the average of 55–86 individual cells present in the same microscope field. Panel E shows grey images before stimulation (resting) or at the peak response with caffeine or high K^+ corresponding to the experiments of traces A–C.

cells (Fig. 4C). Fig. 4D shows the crude traces obtained for each of the two excitation wavelengths for fura-2. Perfusion with caffeine produced a small increase in both fluorescences, which cancelled out by doing the ratio. Panel E in Fig. 4 shows grey images taken at selected time points during these experiments. Caffeine is known to interact with fluorescent Ca^{2+} indicator dyes modifying the fluorescence emission with little effect on binding affinity for Ca^{2+} [39]. Fura-2 fluorescence is enhanced for both the Ca^{2+}-free and the Ca^{2+}-bound form, but most of the effect is cancelled on ratioing [39]. This effect is seen in Fig. 4E, where both fluorescences, F_{340} and F_{380}, were increased by caffeine, but the effect was cancelled on ratioing (Fig. 2B). Fluo-3 fluorescence is decreased by 25–30% by 60 mM caffeine [39], so that the $[Ca^{2+}]_c$-increasing effect induced by this drug could be slightly underestimated by the fluorescence changes. Overall, modifications of the fluorescence of the dyes induced by direct action of caffeine are unlikely to contribute important artefacts to the data presented here. In addition, the effects of fura-2 and fluo-3 on the $[Ca^{2+}]_c$-increasing ability of caffeine were confirmed by measurements with cytosolic aequorin (see Fig. 7 later). In summary, our results indicate that fura-2 really prevents the increase in $[Ca^{2+}]_c$ induced by caffeine.

At this stage, it became critical to investigate whether fura-2 was really preventing release of Ca^{2+} from the ER or just the resulting increase of $[Ca^{2+}]_c$. For these purposes we attempted to measure changes in $[Ca^{2+}]_{ER}$ after challenge with caffeine using ER-targeted aequorin. However, we were unable to perform these measurements because much of the aequorin was inside the glial cells contaminating the neuronal cultures. These cells can be easily removed from analysis in the imaging experiments with fluorescent probes described earlier, but this was not possible in the batch experiments with aequorin. We, therefore, decided to repeat the experiments using another cell type that permitted unbiased approach with the ER-targeted aequorins, such as HEK-293 cells.

Fura-2 reported an increase of $[Ca^{2+}]_c$ in HEK cells challenged with ATP, but caffeine did apparently give no response (Fig. 5A). When cells were loaded with fluo-3 instead of fura-2, the response to caffeine appeared (Fig. 5B). Fig. 6 compares the frequency distributions of the responses to caffeine and to ATP in HEK-293 cells loaded with either fluo-3 or fura-2. The loading with fura-2 did not make much difference in the distribution of responses to ATP (panels A and B). In contrast, it decreased dramatically the responses to caffeine (panels C and D). These results were confirmed in cells expressing the cytosolic aequorin (Fig. 7). The caffeine-induced $[Ca^{2+}]_c$ increase reported by aequorin was little modified by loading the cells with fluo-3, but it was inhibited by 80% by loading the cells with fura-2. The $[Ca^{2+}]_c$-increasing effect of caffeine was also observed in HEK cells loaded with larger concentrations of fluo-3/AM (4 and 10 μM, results not shown).

In order to study directly the effects of caffeine and ATP on the Ca^{2+} contents of ER, HEK-293 cells expressing the ER-targeted aequorin were challenged with these stimuli and $[Ca^{2+}]_{ER}$ was monitored by chemiluminescence emission (Fig. 8). The upper panels (A and B) compare the effects obtained in control and in fura-2-loaded cells in experiments performed at 20 °C. Aequorin was reconstituted by incubation with coelenterazine n in Ca^{2+}-free medium and the experiment was started by addition of Ca^{2+} in order to allow refilling of the ER. After 5 min, $[Ca^{2+}]_{ER}$ reached a stable level of about 800 μM. Stimulation with ATP at this stage produced a rapid Ca^{2+} release with decrease in $[Ca^{2+}]_{ER}$ to about 400 μM. On washing the stimulus $[Ca^{2+}]_{ER}$ increased to the original level. Stimulation with caffeine at this stage produced a slow and sustained release of Ca^{2+} from ER. Control and fura-2-loaded cells behaved similarly. The rates of $[Ca^{2+}]_{ER}$ decrease (in μM/s) in four similar experiments were (control versus fura-2-loaded cells; mean ± S.E.; $n = 4$): caffeine, 3.2 ± 0.5 versus 3.0 ± 0.4, and ATP, 72 ± 4 versus 74 ± 1, respectively. The bottom panels in Fig. 8 show results of similar experiments performed at 37 °C. At this

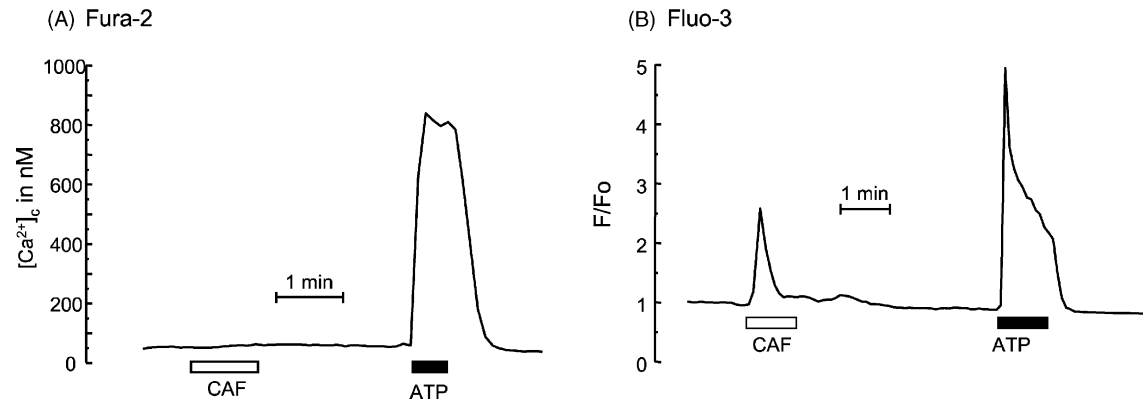

Fig. 5. Effects of fura-2 and fluo-3 on the response to caffeine in HEK-293 cells. Cells were stimulated with caffeine (50 mM) or ATP (10 μM) as shown. They had been loaded for 90 min with either 4 μM fura-2/AM (A) or 1 μM fluo-3/AM (B). Results are expressed either as $[Ca^{2+}]_c$ (A) or as F/F_0 (B). Traces are the average of 27–35 single cells present in the same microscope field.

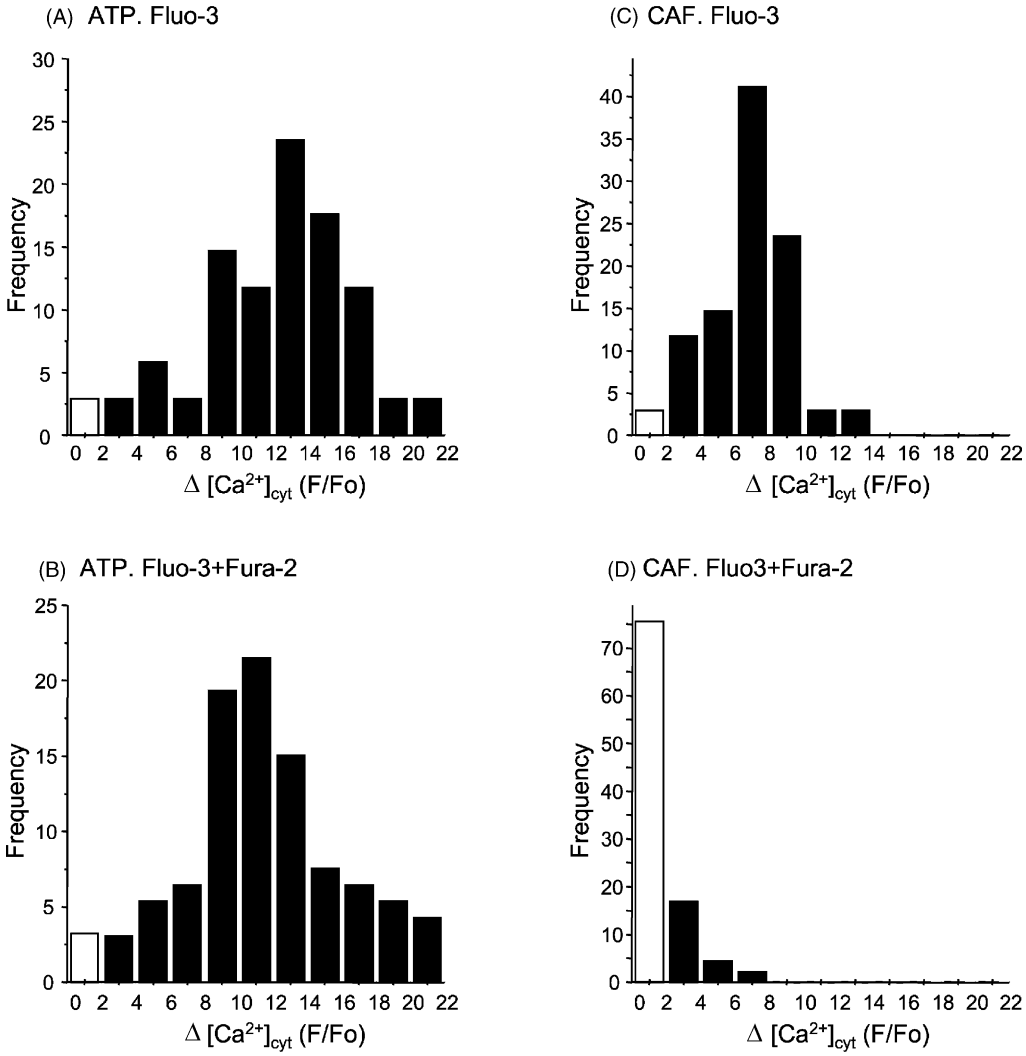

Fig. 6. Frequency distributions of the responses to caffeine and ATP in HEK-293 cells loaded with either fluo-3 only (upper row) or fura-2 and fluo-3 (lower row). In all the cases the changes of F/F_0 (from fluo-3 fluorescence) were used as the index of $[Ca^{2+}]_c$ increase. Other details are as in Fig. 5.

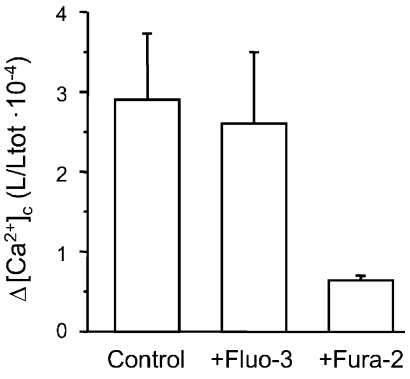

Fig. 7. Effects of loading with either fluo-3 or fura-2 on the responses to caffeine in HEK-293 cells expressing cytosolic aequorin. Cells were loaded with fura-2 or fluo-3 as in Fig. 5. Control cells were incubated in standard medium containing no Ca^{2+} probes for the same time. Aequorin was reconstituted with coelenterazine h for 60–90 min and $[Ca^{2+}]_c$ was estimated from chemiluminescence emission (see Section 2). Each bar represents the mean ± S.E. of four determinations.

temperature ER refilling was complete within 3 min after addition of Ca^{2+}. Addition of caffeine at this stage produced a decrease of $[Ca^{2+}]_{ER}$ which proceeded at a similar rate in control and in fura-2-loaded cells (Fig. 8C and D). In four similar experiments, the rates of $[Ca^{2+}]_{ER}$ decrease induced by caffeine were (in μM/s; mean±S.E.; $n = 4$) 6.2±0.2 and 6.7 ± 0.4 in control and in fura-2-loaded cells, respectively.

4. Discussion

Our results indicate that fura-2 masks caffeine-induced release of Ca^{2+} from the ER, at least in the cell types where this mechanism is not very prominent. This side effect may be responsible for many of the controversial results found in the literature on the presence or absence of CICR in several cell types (see Section 1). Modifications of the activity of the different Ca^{2+} transport systems with culture, as shown here for cerebellar granule neurones, may also

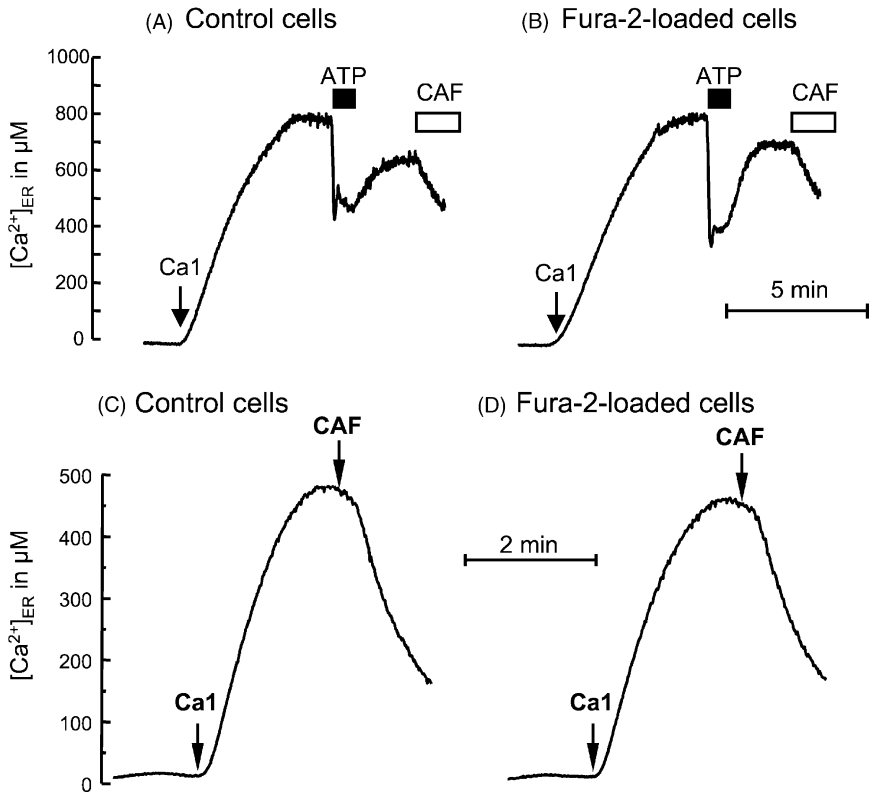

Fig. 8. Effects of caffeine and ATP on $[Ca^{2+}]_{ER}$ in control (left) and fura-2-loaded (right) HEK-293 cells. Cells expressing the ER-targeted aequorin (see Section 2) were incubated for 100–130 min at 20 °C in Ca^{2+}-free medium containing 2 μM coelenterazine n (control, left) or the same plus 4 μM fura-2/AM (+fura-2, right). The experiment in the upper panel was performed at 20 °C whereas the one in the bottom panel was performed at 37 °C. Calcium (1 mM, Ca1), ATP (10 μM) or caffeine (50 mM, CAF) were added as shown.

contribute to the discrepancies. Consistent with previous information [18], we find that CICR activity decreases with time in culture, whereas the activity of voltage-dependent Ca^{2+} channels increases (Fig. 3). It is difficult to decide whether this is the normal developing tendency of these cells or it should be considered as an artefact generated by the culture. Caffeine-induced Ca^{2+} release was increased in cells whose ER had been overloaded with Ca^{2+} by previous stimulation with high K^+ (Fig. 2B), as it should be expected and had been reported before [18]. In addition, release by caffeine from these overloaded stores was better preserved after primary culture of the granule neurones for several days (Fig. 3B). Again, it is difficult to decide which situation represents better the physiological state.

Fluo-3 allows detection of caffeine-induced Ca^{2+} release from the stores in the two cell types examined here, cerebellar granule neurones and HEK-293, in which fura-2 prevented observation. Therefore, use of this probe should be preferred over fura-2 for studies on CICR. It is intriguing, though, what could be the mechanistic difference among these two Ca^{2+} chelators. Fura-2 still prevented detection of CICR by fluo-3 in cells loaded with both dyes (Fig. 4), indicating that fura-2 does really prevent the generation of the $[Ca^{2+}]_c$ increase rather than just being unable to report it. Measurements with cytosolic aequorin confirmed the inhibitory effects of fura-2 (Fig. 7). In order to check whether the absence of a $[Ca^{2+}]_c$ increase was due to inhibition of release from ER, we measured directly changes in $[Ca^{2+}]_{ER}$ in HEK cells stimulated with caffeine using ER-targeted aequorin (Fig. 8). These experiments demonstrated that: (i) the release induced by caffeine is much slower than the one induced by inositol-trisphosphate-generating agonists, such as ATP, and (ii) ER release is indistinguishable in control and in fura-2-loaded cells.

Therefore, fura-2 interferes with the increase of $[Ca^{2+}]$ in the cytosol rather than with the release of Ca^{2+} from the ER. The simpler hypothesis is that fura-2 buffers the $[Ca^{2+}]_c$ increase to such an extent that it becomes undetectable. It is intriguing, though, why fluo-3, which is also a Ca^{2+} chelator, should not have the same effect. Either the cell loading or the 'in situ' K_d for both dyes may differ [40,41]. On the other hand, Ca^{2+} buffers do also increase the diffusion rate of calcium through the cytosol, thus, preventing the establishment of microdomains with different $[Ca^{2+}]$ concentrations [21,22]. This could somehow interfere with the generation of the $[Ca^{2+}]_c$ increase by release of Ca^{2+} from the stores. It has been repeatedly proposed that release of Ca^{2+} from the ER generates high Ca^{2+} microdomains near the release channels and that such microdomains are important for propagation of the $[Ca^{2+}]_c$ wave [8]. Under

this view, buffering by fura-2 could interfere in a subtle way with the progress of such waves. In any case, our results caution the use of this dye for studies of CICR, at least in cells where this mechanism is little prominent, and demonstrates the existence of CICR in cerebellar granule neurones and HEK cells, where it had been questioned previously.

Acknowledgements

This work was supported by Grant BFI2001-2073 from the Spanish Ministerio de Ciencia y Tecnología (MCyT). C. Villalobos is a recipient of a grant from the Ramón y Cajal Program, MCyT, Spain. P. Chamero holds a predoctoral fellowship from the Basque Government. We thank J. Fernandez for technical assistance.

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