Ca^{2+} uptake by the endoplasmic reticulum Ca^{2+}-ATPase in rat microvascular endothelial cells

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In non-excitable cells, many agonists increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by inducing an inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from the intracellular stores. Ca^{2+} influx from the extracellular medium may then sustain the Ca^{2+} signal. $[Ca^{2+}]_i$ recovers its resting level as a consequence of Ca^{2+}-removing mechanisms, i.e. plasma-membrane Ca^{2+}-ATPase (PMCA) pump, Na^+/Ca^{2+} exchanger (NCX) and sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump. In a study performed in pancreatic acinar cells, evidence has been provided suggesting that, during the decay phase of the agonist-evoked Ca^{2+} transients, the Ca^{2+} concentration within the intracellular stores remains essentially constant [Mogami, Tepikin and Petersen (1998) EMBO J. **17**, 435–442]. It was therefore hypothesized that, in such a situation, intracellular Ca^{2+} is not only picked up by the SERCA pump, but is also newly released through IP_3-sensitive Ca^{2+} channels, with the balance between these two processes being approximately null. The main aim of the present work was to test this hypothesis by a different experimental approach. Using cardiac microvascular endothelial cells, we found that inhibition of the SERCA pump has no effect on the time course of agonist-evoked Ca^{2+} transients. This result was not due to a low capacity of the SERCA pump since, after agonist removal, this pump proved to be very powerful in clearing the excess of intracellular Ca^{2+}. We showed further that: (i) in order to avoid a rapid removal of Ca^{2+} by the SERCA pump, continuous IP_3 production appears to be required throughout all of the decay phase of the Ca^{2+} transient; and (ii) Ca^{2+} picked up by the SERCA pump can be fully and immediately released by agonist application. All these results support the model of Mogami, Tepikin and Petersen [(1998) EMBO J. **17**, 435–442]. Since the SERCA pump did not appear to be involved in shaping the decay phase of the agonist-evoked Ca^{2+} transient, we inhibited the PMCA pump with carboxyeosin, and NCX with benzamil and by removing extracellular Na^+. The results indicate that, during the decay phase of the agonist-evoked Ca^{2+} transient, the intracellular Ca^{2+} is removed by both the PMCA pump and NCX. Finally, we provide evidence indicating that mitochondria have no role in clearing intracellular Ca^{2+} during agonist-evoked Ca^{2+} transients.

Key words: calcium, fura-2, Na^+/Ca^{2+} exchanger, plasma membrane Ca^{2+}-ATPase, UTP.

INTRODUCTION

In endothelial cells (ECs), an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the triggering event in the synthesis and release of a number of vasoactive compounds, such as prostacyclin, nitric oxide and endothelins [1,2]. Several local mediators (histamine, ATP, UTP, acetylcholine and bradykinin) are able to promote $[Ca^{2+}]_i$ increases by elevating the intracellular level of inositol 1,4,5-trisphosphate (IP_3), thus causing Ca^{2+} release from endoplasmic-reticulum Ca^{2+} stores through IP_3-sensitive channels [3]. Subsequently, even if Ca^{2+} influx from the extracellular medium usually occurs [4,5], $[Ca^{2+}]_i$ decreases and approaches the basal level in about 5–10 min. In fact, prolonged $[Ca^{2+}]_i$ increase can be cytotoxic [6], and long-lasting Ca^{2+} signalling might be better accomplished by $[Ca^{2+}]_i$ oscillations [7].

The decrease in $[Ca^{2+}]_i$ is due to the activity of Ca^{2+}-clearing mechanisms, such as the plasma membrane Ca^{2+}-ATPase (PMCA) pump, the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump [8] and the plasma membrane Na^+/Ca^{2+} exchanger (NCX) [9]. In ECs, the roles of these mechanisms are not well established. Participation of the PMCA pump has not been widely investigated [10,11], and conflicting evidence has been reported about the presence and function of NCX [12–14]. As far as the SERCA pump is concerned, it seems to have a relevant role in clearing intracellular Ca^{2+} after agonist removal [14]. Furthermore, it has been suggested that the SERCA pump, by quickly picking up Ca^{2+} entering the cell, is part of the so-called 'superficial buffer barrier' [15,16].

In the present study, the role of the SERCA pump is investigated in cardiac microvascular ECs (CMECs) in more detail. We focused our attention on the hypothesis, formulated for pancreatic acinar cells, that, during agonist-evoked Ca^{2+} transients, the SERCA pump is active and IP_3-sensitive channels are open, so that Ca^{2+} picked up by the SERCA pump is newly released and the $[Ca^{2+}]_i$ decrease is mainly due to SERCA-pump-independent mechanisms [17,18]. We tested this hypothesis, and the results reported here add new supporting evidence. Since the SERCA pump does not appear to participate in clearing agonist-released Ca^{2+}, the possible roles of two alternative Ca^{2+}-removing mechanisms, namely the PMCA pump and NCX, were analysed. Our results show that both mechanisms are active in CMECs. Finally, in order to test whether mitochondria might be a major 'Ca^{2+}-handling' mechanism during

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; PMCA, plasma-membrane Ca^{2+}-ATPase; NCX, Na^+/Ca^{2+} exchanger; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; EC, endothelial cell; CMEC, cardiac microvascular EC; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks balanced salt solution; PSS, physiological salt solution; CPA, cyclopiazonic acid; PLC, phospholipase C; PKC, protein kinase C; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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the decay phase of the Ca^{2+} transient [19], the effect of a mitochondrial protonophore was investigated.

MATERIAL AND METHODS

Isolation and culture of CMECs

The procedure for cell isolation has been described previously [20,21], and is very similar to the procedure originally developed by Nishida et al. [22]. Adult Wistar rats (250–350 g) were anaesthetized with ether, and the heart was rapidly excised and perfused in retrograde with Dulbecco's modified Eagle's Medium (DMEM) saturated with an O_2/CO_2 mixture (19:1). After perfusion, the left ventricle was isolated, and epicardial mesothelial cells and endocardial cells were devitalized by immersion in 70 % (v/v) ethanol for 30 s and then washed with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS). Approximately one-third of the outer ventricular wall and the septum were dissected away to remove epicardial arteries and larger penetrating vessels. The remaining tissue was then minced in HBSS and incubated in 0.2 % collagenase (type II) for 20 min at 37 °C in a shaking water bath. The tissue was sheared in a 10 ml pipette, incubated for 20 min at 37 °C in the shaking bath after addition of 0.027 % trypsin, and then sheared again. Dissociated cells were filtered through a 100 μm mesh filter, suspended in 10 ml of HBSS, and centrifuged at 750 rev./min (102 ***g***) for 10 min. The pellet was again re-suspended and centrifuged for 5 min. The pellet was then re-suspended in 1.6 ml of DMEM supplemented with 20 % (v/v) foetal bovine serum, antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and antimycotic (25 μg/ml amphotericin B). CMECs were plated on coverslips pre-treated with 2 % gelatin, which were placed in a multiwell dish (1.9 cm^2 growth area) with 0.3 ml of the previously described medium. After plating (2 h), non-adherent cells were removed by washing with DMEM. Cells usually reached confluence after 7–9 days, and displayed uniform 'cobblestone' morphology.

Identification of CMECs

Cultured cells were tested for their endothelial origin by three distinct endothelial cell markers: (i) factor VIII-related antigen; (ii) lectin I from *Griffonia (Bandeiraea) simplicifolia*; and (iii) uptake of acetylated low-density lipoprotein. More than 90 % of the cells were positively identified, as estimated by cell counting in microphotographs of randomly chosen visual fields [21].

Microfluorimetry

The microfluorimetric technique here employed has been described previously [21]. Briefly, CMECs were loaded with 2 μM fura-2 acetoxymethyl ester in normal physiological salt solution (PSS) for 25 min at room temperature (22–24 °C), and then visualized by an upright epifluorescence Zeiss Axiolab microscope (Carl Zeiss, Germany) equipped with a Zeiss ×40 Achroplan water-immersion objective; 1.7 mm working distance, 0.75 numerical aperture). Excitation was at 340 and 380 nm, with emission detected at 510 nm. The exciting filters (Chroma Technology, Brattleboro, VT, U.S.A.) were mounted on a filter-wheel equipped with a shutter (Lambda 10; Sutter Instruments, Novato, CA, U.S.A.). A neutral density filter (0.6 optical density) was coupled with the 380 nm filter to approach the intensity of the 340 nm light. Emitted light was collected by a camera (Extended-ISIS Camera; Photonic Science, Robertsbridge, East Sussex, U.K.) interfaced by a frame grabber (CX100; Image-Nation, Beaverton, OR, U.S.A.) to a personal computer. Home-developed software was used to drive the camera, the filter-wheel and the shutter, and to measure and plot the fluorescence on-line from a number of rectangular regions of interest. $[Ca^{2+}]_i$ was usually monitored by evaluating the ratio of fluorescence signals emitted at 510 nm when exciting at 340 and 380 nm, respectively. Ratio measurements were performed every 1.5, 2 or 3 s.

Isolated or 'semi-confluent' cells were employed. Unless stated explicitly, the experiments were performed at room temperature (22–24 °C).

Chemicals

Acetylated low-density lipoprotein labelled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate was from Biomedical Technologies, Inc. (Stoughton, MA, U.S.A.). FITC-conjugated antibody raised against human factor VIII-related antigen was from Atlantic Antibodies (Scarborough, ME, U.S.A.). Fura-2 acetoxymethyl ester, 5-(and 6)-carboxyeosin diacetate and succinimidyl ester were obtained from Molecular Probes (Eugene, OR, U.S.A.). DMEM was purchased from Gibco–BRL (Milan, Italy) and HBSS was from Mascia Brunelli (Milan, Italy). All other chemicals were from Sigma.

Solutions

PSS had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. The osmolality of these solutions, as measured with an osmometer (Wescor 5500, Logan, UT, U.S.A.), was 300–310 mosmol·kg^{-1} solution. U73122, carboxyeosin and thapsigargin were dissolved in DMSO and stored as 5 mM stock solutions. Cyclopiazonic acid (CPA) was dissolved in DMSO and stored as a 10 mM stock solution. Benzamil was dissolved in methanol and stored as a 100 mM stock solution.

Drug administration

Medium exchange and administration of agonists or other drugs were performed by removing the bathing medium (2 ml) and adding the desired solution. The medium could be exchanged quickly without producing artefacts in the fluorescence signal because a small meniscus of liquid remained between the tip of the objective and the facing surface of the coverslip.

Loading of CMECs with carboxyeosin was accomplished by incubating the cells in 25 μM carboxyeosin ester for 25 min and, subsequently, in PSS for 20 min. PMCA pump inhibition by carboxyeosin was sensitive to the incubation time in PSS.

Data analysis and presentation

The decay time of agonist-evoked Ca^{2+} signals was computed as the time in which $[Ca^{2+}]_i$ decayed from 80 % to 20 % of its peak amplitude. This parameter was named t_{80-20}. The results are expressed as means ± S.E.M. Mean values have been compared by means of the Student's t test for unpaired or paired data, depending on the experimental protocol. The percentage variation in decay time, named Δt_{80-20}, was computed as [(treated − control)/control]·100.

RESULTS

In order to exclude the contribution of Ca^{2+} influx, the experiments described here were performed, unless otherwise stated explicitly, in Ca^{2+}-free extracellular solution.

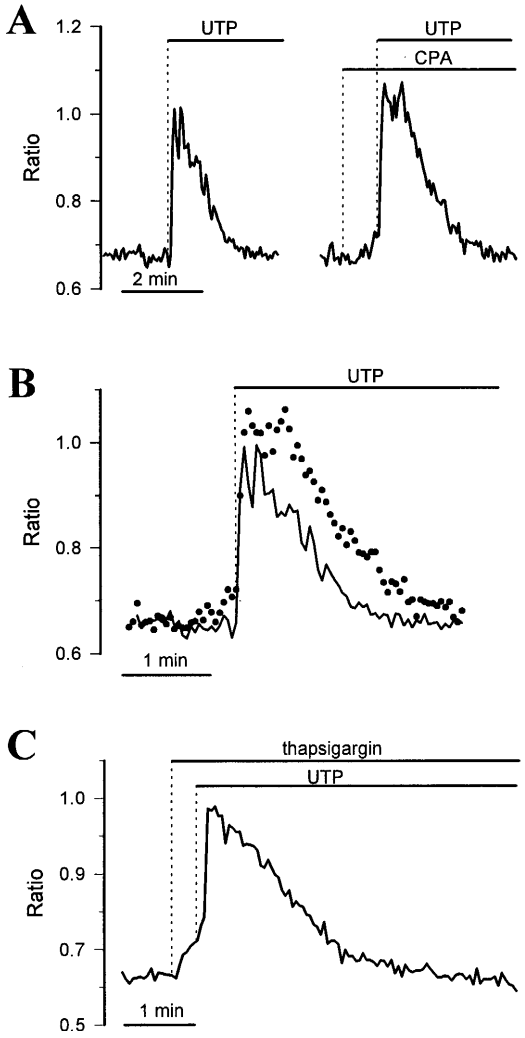

Figure 1 Effect of SERCA pump inhibition on UTP-evoked Ca^{2+} transient

(**A**) Ca^{2+} transients evoked by 10 μM UTP in the absence (left) or in the presence (right) of 10 μM CPA. To ensure that CPA actually inhibits the SERCA pump, UTP was added after the occurrence of the CPA-induced increase in $[Ca^{2+}]_i$ (single cell tracing). (**B**) The two Ca^{2+} transients shown in (**A**) have been superimposed (solid line and dots), adjusting the time base so that agonist application occurs simultaneously. (**C**) Ca^{2+} transient evoked by 10 μM UTP in the presence of 1 μM thapsigargin (single cell tracing). In (**A**), (**B**) and (**C**), all tracings were recorded in Ca^{2+}-free solution.

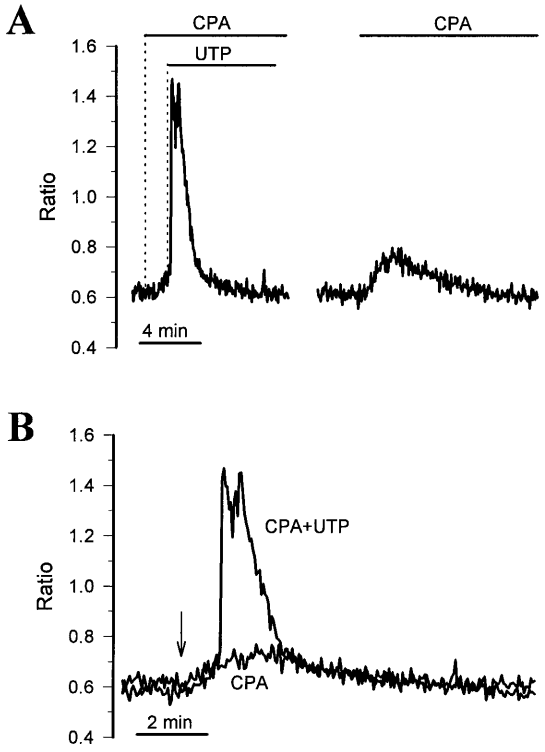

Figure 2 Effect of CPA-evoked Ca^{2+} release on the kinetics of the decay phase of the UTP-evoked Ca^{2+} signal

(**A**) CPA + UTP-evoked signal (left panel) and, after washing in PSS, CPA-evoked signal alone (right panel). Single cell tracing in Ca^{2+}-free solution. (**B**) The CPA-evoked signal has been superimposed on the CPA + UTP-evoked signal, adjusting the time base in order to make $[Ca^{2+}]_i$ increase due to CPA application (shown by the arrow) occur simultaneously in the two tracings.

SERCA pump does not affect the decay phase of agonist-evoked Ca^{2+} transients during continuous agonist application

As shown in Figure 1, UTP, a P_{2Y2} receptor agonist [21], evoked intracellular Ca^{2+} transients, the decay phase of which was not affected by treatment with SERCA inhibitors, namely CPA or thapsigargin. Indeed, the decay time (t_{80-20}) of the Ca^{2+} transients averaged 74.7 ± 3.07 s in control cells ($n = 64$), 77.2 ± 3.6 s in CPA-treated cells ($n = 32$) and 78.4 ± 8.6 s in thapsigargin-treated cells ($n = 11$). These mean values were not statistically different.

It should be noted that CPA and thapsigargin not only inhibited the SERCA pump, but were also able to induce a slow, long-lasting Ca^{2+} release (Figure 2A, right panel). The decay times measured in the presence of CPA or thapsigargin might therefore reflect not only the kinetics of the UTP-evoked signal, but also the kinetics of the CPA- or thapsigargin-evoked Ca^{2+} release. In order to evaluate the possible contaminating influence of Ca^{2+} increase evoked by SERCA inhibitors, both the global signal (UTP + CPA) and the CPA-evoked signal alone, observed in the same cell, were recorded and superimposed, as exemplified in Figure 2 ($n = 5$). This comparison clearly showed that, due to its small amplitude and slow kinetics, the CPA signal did not exert a major effect on the fast part of the decay phase, the importance of which is largely prevalent in the t_{80-20} measurement. Furthermore, the CPA-evoked signal underlying the UTP signal can be expected to be smaller than the CPA-signal alone, because part of the Ca^{2+} contained in the stores is released quickly by UTP.

With respect to thapsigargin, Ca^{2+} transients evoked by this SERCA inhibitor were similar to those elicited by CPA (not shown). However, since the effect of thapsigargin is irreversible, it was not possible to record, in the same cell, both the UTP + thapsigargin signal and the thapsigargin signal.

These data suggest therefore that the SERCA pump is not a major factor in shaping the decay phase of the UTP-evoked Ca^{2+} response.

SERCA rapidly picks up Ca^{2+} when UTP is removed during the decay phase of the Ca^{2+} transient

As shown in Figure 3, agonist removal during the decay phase of the Ca^{2+} transient caused a sharp acceleration of the $[Ca^{2+}]_i$ decrease, both in PSS and in Ca^{2+}-free solution. This also occurred when the agonist was withdrawn during the final part

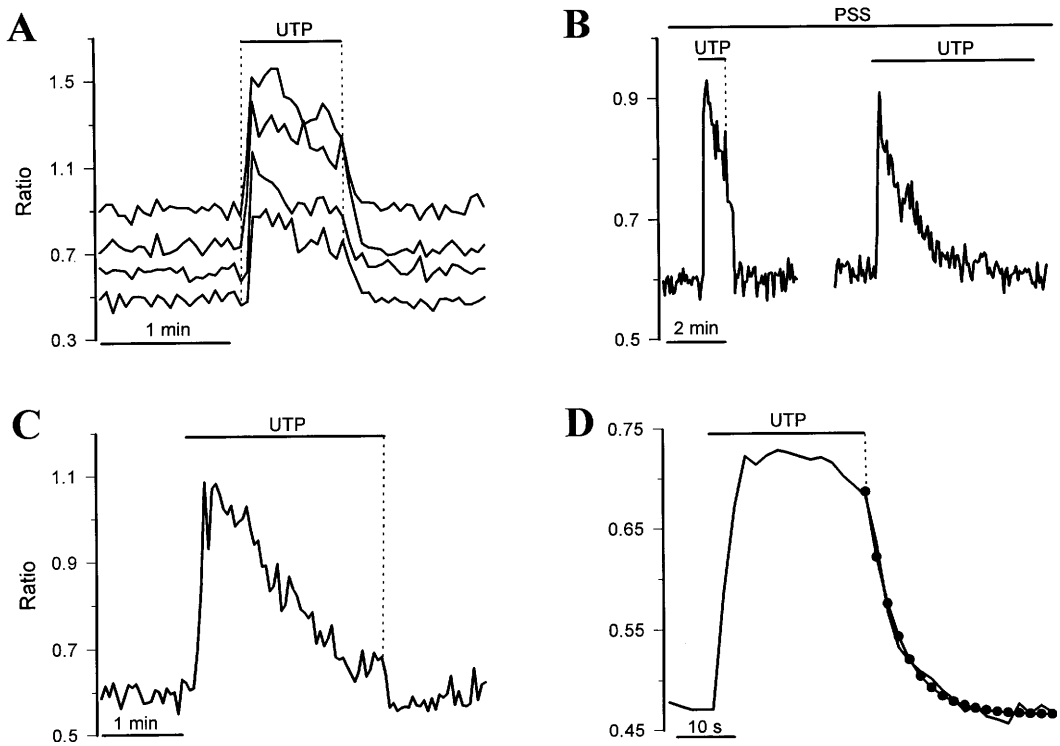

Figure 3 Effect of agonist removal during the decay phase of the Ca^{2+} transient

(**A**) UTP (10 μM) has been applied and the agonist removed during the decay phase of the Ca^{2+} transient (four single cell tracings recorded in Ca^{2+}-free solution). The y-axis of two cells has been slightly shifted to avoid tracing overlapping. (**B**) In normal PSS, 10 μM UTP was applied and the agonist was removed during the decay phase of the transient. After washing, a control transient was evoked in the same cell (single cell recording). (**C**) UTP (10 μM) was applied and the agonist was removed during the later part of decay phase of the $[Ca^{2+}]_i$ transient (single cell recording in Ca^{2+}-free solution). (**D**) The kinetics of the decay of the Ca^{2+} transient after agonist removal (continuous line) has been fitted by an exponential curve (dots). Mean of four single cell signals recorded in Ca^{2+}-free solution.

of the Ca^{2+} transient (Figure 3C). Under these conditions, the kinetics of $[Ca^{2+}]_i$ decay could be adequately fitted by a single exponential function plus a constant (the steady-state $[Ca^{2+}]_i$), with an average time constant (τ_d) of 5.75 ± 0.42 s ($n = 26$; Figure 3D). At 34 C°, τ_d was 3.05 ± 0.41 s ($n = 7$), with a Q_{10} of 1.89.

Differently from the decay phase recorded in the presence of UTP, Ca^{2+} clearing after agonist removal was dramatically sensitive to SERCA inhibition. Indeed, in the presence of CPA, agonist withdrawal made the decay kinetics of $[Ca^{2+}]_i$ marginally faster (Figures 4A and 4B), with an average t_{80-20} of 62.6 ± 4.5 s ($n = 30$). This small decrease in t_{80-20} was, however, statistically significant ($P < 0.05$) when compared with both control t_{80-20} and t_{80-20} measured in the presence of CPA + UTP. This observation, discussed more thoroughly in the Discussion, might be explained by the finding of Mogami et al. [18] that a net Ca^{2+} efflux from the stores appears to occur during the initial part of the decay phase of the Ca^{2+} transient.

An estimate of the relative efficacy of SERCA-dependent versus SERCA-independent mechanisms may be obtained by considering that, after agonist removal, SERCA + non-SERCA mechanisms clear 63 % of the intracellular Ca^{2+} in one τ_d, i.e. 5.75 s, with a clearing rate (1/5.75) of 0.174 s^{-1}. In the presence of CPA, again after agonist removal, non-SERCA mechanisms need to clear 63 % of the intracellular Ca^{2+}, 51.2 ± 6.4 s ($n = 21$) with a clearing rate of 0.0195 s^{-1}. The difference between the two clearing rates (0.154 s^{-1}) is the clearing rate of the SERCA, which is therefore 7.9 times more powerful than non-SERCA mechanisms.

Effect of phospholipase C (PLC) inhibition and protein kinase C (PKC) activation on Ca^{2+} transient decay phase

In order to gain a further insight into the mechanisms that cause fast $[Ca^{2+}]_i$ decrease following agonist removal, we inhibited, during the decay phase of the Ca^{2+} transient, the agonist-evoked PLC activity using the PLC blocker U73122. A high concentration of U73122 (200 μM), applied simultaneously with 10 μM UTP, was able to fully inhibit the response to the agonist in 15 out of 24 cells (Figure 5A), the remaining nine cells showing a very small response (results not shown). With a delay of more than 30 s, an increase in the fluorescence ratio, with variable amplitude, was usually observed (Figure 5A). This high concentration of U73122, about one order of magnitude higher than the dose usually employed to inhibit PLC activity [23], was required in order to instantaneously block the UTP-induced PLC stimulation (lower concentrations of U73122 require preincubations). Application of UTP + U73122 during the decay phase of the Ca^{2+} transient always caused a prominent acceleration of the $[Ca^{2+}]_i$ decrease (Figure 5B; $n = 25$), with an average τ_d of 6.6 ± 0.7 s ($n = 12$). This occurred even when U73122 was applied during the final part of the Ca^{2+} signal (results not shown). Substitution of UTP with UTP + DMSO (4 %) did not cause any fast decrease in $[Ca^{2+}]_i$ (results not

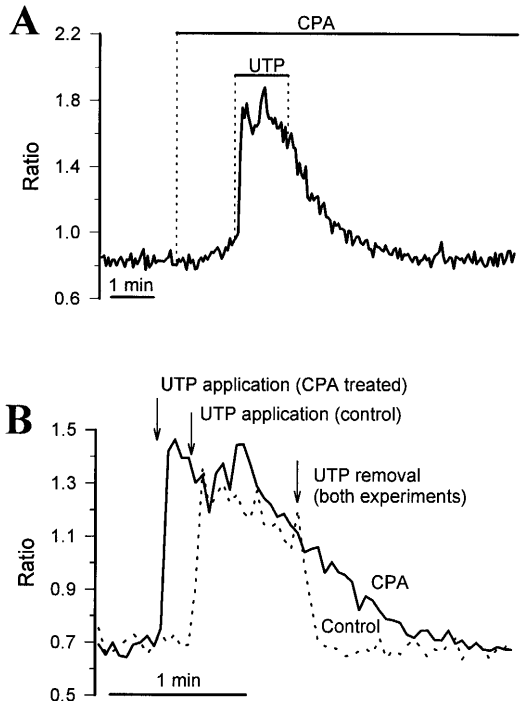

Figure 4 Decay phase of the Ca^{2+} transient after agonist removal: effect of CPA

(**A**) CPA was applied, followed by 10 μM UTP. Then UTP was removed during the decay phase of the transient. (**B**) A CPA-treated transient and a control transient (continuous and broken lines respectively) have been superimposed to highlight the difference in the relaxing kinetics after agonist removal. The time bases have been adjusted in order to make UTP removal occur simultaneously in the two tracings. In (**A**) and (**B**), all tracings were recorded in Ca^{2+}-free solution.

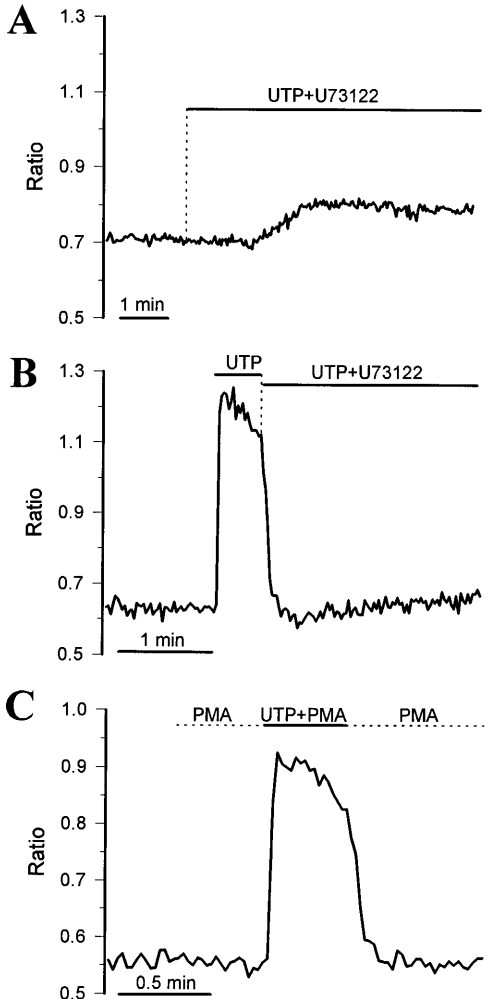

Figure 5 Effect of PLC inhibition and PKC activation on the decay phase of the Ca^{2+} transient

(**A**) U73122 (200 μM), simultaneously applied to UTP (10 μM), is able to fully inhibit the response to UTP. A delayed increase in the fluorescence ratio, in this tracing particularly evident, may occur. (**B**) Substitution of UTP (10 μM) with UTP (10 μM) plus U73122 (200 μM) during the decay phase of the transient causes a fast decrease in $[Ca^{2+}]_i$. (**C**) Agonist removal during acute application of PMA causes a fast decrease in $[Ca^{2+}]_i$. Single cell tracings recorded in Ca^{2+}-free solution.

shown; $n = 11$). These results suggest that continuous UTP-evoked PLC activity is required to sustain the decay phase of the Ca^{2+} transient.

It is well known that PLC produces both IP_3 and diacylglycerol, the latter of which is a PKC activator. We therefore tested if PKC activation could substitute for UTP during the decay phase of the Ca^{2+} transient. To activate PKC, an acute application of PMA was employed [24]. As shown in Figure 5(C), PMA application was not able to prevent fast Ca^{2+} decrease after agonist removal ($n = 20$), with a τ_d of 5.4 ± 0.64 ($n = 12$). Taken together, these data suggest that IP_3, but not PKC activation, is required in order to sustain the decay phase of the Ca^{2+} transient.

Intracellular Ca^{2+} turnover during agonist application

Our finding that SERCA activity is prominently involved in fast Ca^{2+} clearing following agonist removal (in which circumstance its function overwhelms that of non-SERCA mechanisms) might appear to be in contradiction with the observation that SERCA inhibition has no major effect on the decay rate of Ca^{2+} transients evoked in response to continuous agonist application. However, this apparent discrepancy may be resolved if it is hypothesized that, during the Ca^{2+} transient's decay phase, Ca^{2+} picked up by SERCA is recycled through IP_3-sensitive channels, which remain open owing to the presence of the agonist [18]. Such a viewpoint is confirmed further by our finding that PLC inhibition (and hence IP_3-production block) mimics the effect of agonist withdrawal in terms of Ca^{2+}-transient decay acceleration. The experiments shown in Figure 6 were designed to test above hypothetical process of continuous Ca^{2+} uptake/Ca^{2+} release occurring in the presence of agonist. The continuous UTP application was substituted with a sequence of UTP removal/UTP application cycles, a protocol that, in principle, should make the two concomitant processes of Ca^{2+} uptake/Ca^{2+} release alternate in distinct time intervals, thereby making them apparent. The tracings clearly show that: (i) re-application of agonist releases an amount of Ca^{2+}, which closely approaches that previously picked up by Ca^{2+}-clearing mechanisms (i.e. almost exclusively SERCA) following agonist removal; and (ii) the cycle of agonist removal/agonist application can be repeated many times, up to the end of the transient decay ($n = 45$). As the cycle becomes shorter and shorter, we may suppose that we would obtain a closer approximation to the continuous Ca^{2+} uptake/Ca^{2+} release process that actually occurs.

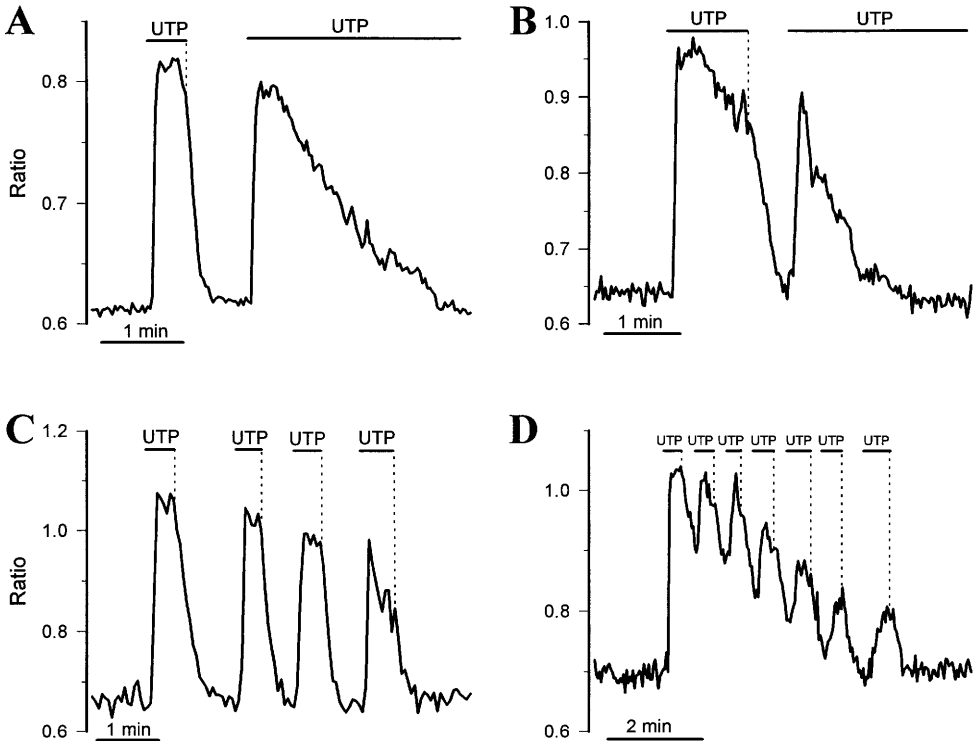

Figure 6 Effect of the UTP removal/UTP application-cycle on $[Ca^{2+}]_i$

(**A**) UTP was removed and reapplied in the initial part of the decay phase. (**B**) UTP was removed and reapplied in the middle part of the decay phase. (**C**) The cycle of UTP removal/UTP application was repeated three times, allowing $[Ca^{2+}]_i$ to reach the baseline. (**D**) The cycle of UTP removal/UTP application was repeated six times. UTP was applied before $[Ca^{2+}]_i$ reached the baseline. Single cell tracings recorded in Ca^{2+}-free solution.

These results appear to be compatible with the hypothesis that, during the decay phase of the Ca^{2+} transient, Ca^{2+} is continuously picked up by SERCA, and all this Ca^{2+} is again released through IP_3-sensitive channels. The actual $[Ca^{2+}]_i$ decrease should therefore be accomplished by non-SERCA mechanisms, such as NCX and the PMCA pump.

Effect of NCX on the kinetics of the agonist-evoked Ca^{2+} transient

The hypothesis that, during the decay phase of the Ca^{2+} transient, NCX contributes to cytosolic Ca^{2+} extrusion was first tested by removing extracellular Na^+. In non-stimulated cells, Na^+ removal is expected to cause NCX to work in the Ca^{2+} influx mode and, therefore, to increase $[Ca^{2+}]_i$ and decrease the intracellular Na^+ concentration. Owing to the progressive intracellular Na^+ depletion, NCX becomes less and less active and the PMCA pump can restore the basal $[Ca^{2+}]_i$. In the presence of extracellular Ca^{2+}, extracellular Na^+ removal caused the expected transient increase in $[Ca^{2+}]_i$ in 48.5 % of cells (Figure 7A; $n = 198$). Interestingly, 86.4 % of the cells not responding to Na^+ withdrawal became responsive if the PMCA pump was inhibited by carboxyeosin (Figure 7B; $n = 84$). Furthermore, in carboxyeosin-treated cells, Na^+ removal always elicited very long Ca^{2+} transients, showing a plateau in 80 % of the cells (Figure 7C). Under these conditions, UTP stimulation was almost ineffective (Figure 7C), possibly due to the permanent elevation in $[Ca^{2+}]_i$. Na^+ removal did not increase $[Ca^{2+}]_i$ in Ca^{2+}-free solution (results not shown; $n = 70$).

The effect of Na^+ removal on the Ca^{2+} transient kinetics was investigated in PSS and in Ca^{2+}-free solution. In PSS the UTP-evoked Ca^{2+} transient becomes slightly longer ($t_{80-20} = 89.1 \pm 4.3$ s; $n = 50$), but no plateau was observed [21]. As shown in Figure 8(A), Na^+ removal made the kinetics of the Ca^{2+} transient slower, with a measured Δt_{80-20} of 50.1 % ($P < 0.01$, $n = 52$). A similar result was also obtained in Ca^{2+}-free solution (Figure 8B), with a Δt_{80-20} equal to 32 % ($P < 0.01$, $n = 70$). In these experiments (and in the subsequent experiments in which benzamil was used), the control signal was recorded before treatment in 50 % of the tested cells. The recording sequence was reversed in the other 50 %.

Benzamil (100 μM), an inhibitor of NCX [25], slowed the Ca^{2+} transient kinetics (Figure 8C), with a Δt_{80-20} of 39 % ($P < 0.01$; $n = 22$). These experiments were performed in Ca^{2+}-free solution. Benzamil is a fluorescent molecule, which implies that its signal adds to the fura-2 signal and modifies the baseline fluorescence. However, since benzamil background fluorescence remains constant during the recording periods preceding and following agonist application (see Figure 8C), kinetic (but not amplitude) analysis should be allowed.

Effect of the PMCA pump on the kinetics of the agonist-evoked Ca^{2+} transient

Incubation with carboxyeosin made the decay phase of the agonist-evoked Ca^{2+} transients slower (Figure 9), both in PSS ($\Delta t_{80-20} = 81$ %, $P < 0.01$; $n = 42$) and in Ca^{2+}-free solution ($\Delta t_{80-20} = 119$ %, $P < 0.01$; $n = 20$).

Since the effect of carboxyeosin appears to be irreversible [26], experiments with reversed recording sequence were not performed. However, Ca^{2+} transients recorded before and after

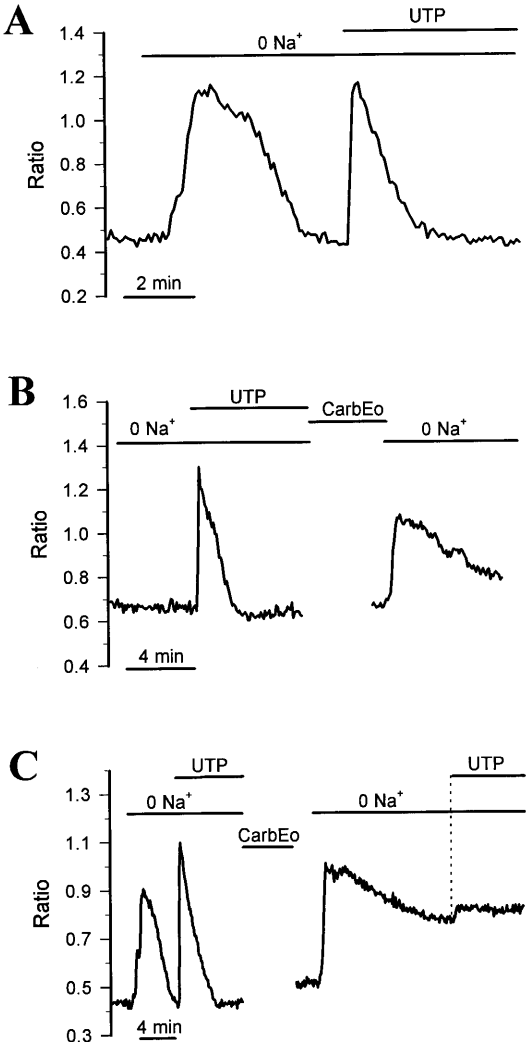

Figure 7 Effect of Na^+ removal on $[Ca^{2+}]_i$

(**A**) Na^+ removal (0 Na^+) causes a transient increase in $[Ca^{2+}]_i$. A UTP-evoked Ca^{2+} transient, recorded in the absence of extracellular Na^+, is also shown. (**B**) Na^+ removal does not modify $[Ca^{2+}]_i$ in cells which respond to UTP. After loading by carboxyeosin and washing in PSS (CarbEo), Na^+ removal causes an increase in $[Ca^{2+}]_i$. (**C**) Na^+ removal causes a transient increase in $[Ca^{2+}]_i$. A UTP-evoked Ca^{2+} transient, recorded in the absence of extracellular Na^+, is also shown. After loading by carboxyeosin and washing in PSS, the Ca^{2+} transient following Na^+ removal becomes very long and reaches a plateau. Subsequent UTP stimulation is almost ineffective. All tracings have been recorded in the presence of extracellular Ca^{2+}, and are the means of four single cell recordings.

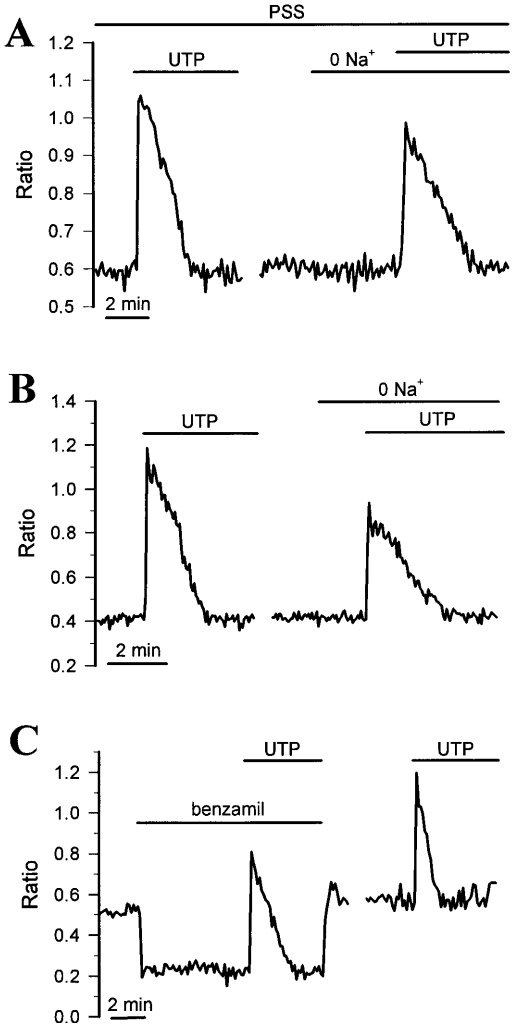

Figure 8 Effect of NCX inhibition on the kinetics of the agonist-evoked Ca^{2+} transient

(**A**) Na^+ removal (0 Na^+) makes the 10 μM UTP-evoked Ca^{2+} transient slower in normal Ca^{2+} solution (PSS). (**B**) Na^+ removal also makes the 10 μM UTP-evoked Ca^{2+} transient slower in Ca^{2+}-free solution. (**C**) Benzamil (100 μM) makes the 10 μM UTP-evoked Ca^{2+} transient slower (Ca^{2+}-free solution). During the gaps shown in the traces, cells were washed with PSS. All traces are the means of three single cell recordings.

30 min of incubation in PSS (i.e. without any treatment) showed no statistically significant difference in the decay time (results not shown, $n = 33$).

Effect of inhibiting both the PMCA pump and NCX

As observed previously, incubation with carboxyeosin and subsequent Na^+ removal make CMECs in PSS scarcely sensitive to UTP stimulation. This does not occur in Ca^{2+}-free solution, so the effect of inhibiting both the PMCA pump and NCX can be investigated. Figure 10 shows that inhibition of both Ca^{2+}-extrusion mechanisms dramatically slows down the agonist-evoked Ca^{2+} transients. In 70.9 % of the cells ($n = 39$), the Ca^{2+} transient reached a plateau, and in the remaining cells ($n = 16$) $[Ca^{2+}]_i$ recovered to the baseline level with a Δt_{80-20} of 323 %.

Effect of the mitochondrial protonophore on the kinetics of the agonist-evoked Ca^{2+} transient

Mitochondria have been reported to strongly affect the time course of stimulus-evoked $[Ca^{2+}]_i$ signals [19]. The effect of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [27] on the kinetics of the UTP-evoked Ca^{2+} transients was therefore tested. CCCP (2 μM) caused a transient $[Ca^{2+}]_i$ increase in non-stimulated cells. This occurred in both normal (results not shown; $n = 29$) and Ca^{2+}-free solution (Figure 11A; $n = 17$). When UTP was applied after CCCP, the agonist-evoked Ca^{2+} transient, which was superimposed on the CCCP-evoked Ca^{2+} transient, showed no apparent kinetic change (Figure 11B). Indeed, under these conditions, the decay time of the agonist-evoked transient showed a Δt_{80-20} of 13.2 % or -1.3 % ($n = 16$), depending on whether the baseline level used to measure the transient amplitude was the tracing baseline or the higher $[Ca^{2+}]_i$ reached at the time of agonist application.

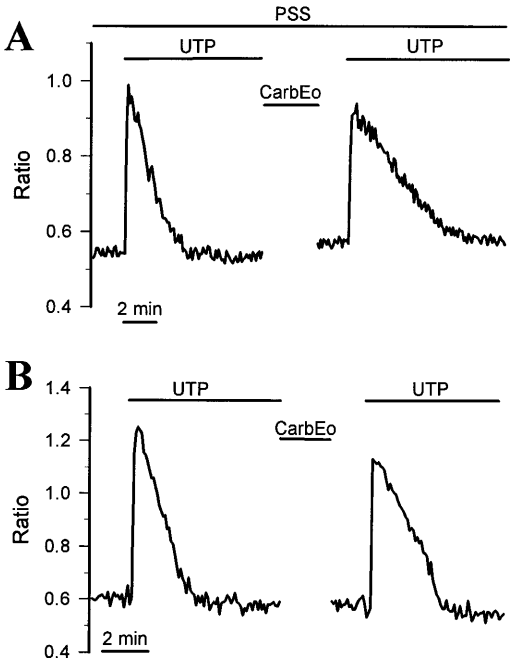

Figure 9 Effect of the PMCA pump inhibition on the kinetics of the agonist-evoked Ca^{2+} transient

Loading by carboxyeosin (CarbEo) makes the UTP (10 μM)-evoked Ca^{2+} transient slower both in PSS (**A**) and in Ca^{2+}-free solution (**B**). During the tracing gaps, cells were washed by PSS. All tracings are the means of four single cell recordings.

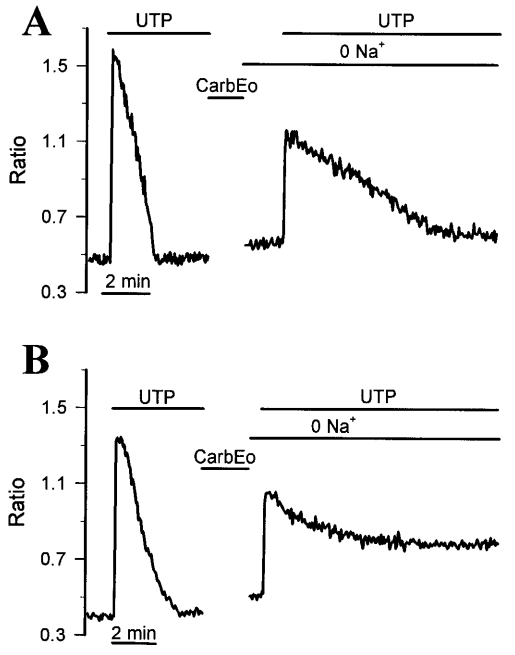

Figure 10 Effect of simultaneous inhibition of the PMCA pump and NCX on the kinetics of the agonist-evoked Ca^{2+} transient

Loading by carboxyeosin (CarbEo) and subsequent Na^+ removal makes the decay phase of the UTP (10 μM)-evoked Ca^{2+} transient dramatically longer. In (**B**), the Ca^{2+} transient reaches a plateau. During the gaps in the traces, cells were washed with PSS. In (**A**) and (**B**), all tracings are the means of three single cell signals recorded in Ca^{2+}-free solution.

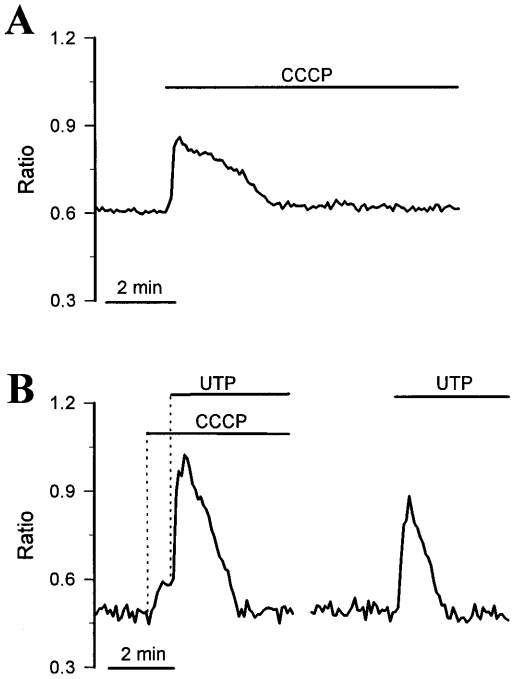

Figure 11 Effect of mitochondrial protonophore CCCP on the basal $[Ca^{2+}]_i$ and during agonist stimulation

(**A**) A particularly prominent increase in $[Ca^{2+}]_i$ caused by CCCP (2 μM) application (means of three single cell tracings). (**B**) UTP (10 μM) was applied during the CCCP-evoked Ca^{2+} transient. After washing in PSS, a control transient was evoked (mean of four single cell recordings). In (**A**) and (**B**), all experiments were performed in Ca^{2+}-free solution.

The above results indicate that mitochondria have no major role in removing cytosolic Ca^{2+} during agonist stimulation. Finally, prolonged application of CCCP was found to make the UTP stimulation ineffective (results not shown; $n = 15$).

DISCUSSION

Role of the SERCA pump after agonist removal

After agonist removal, the SERCA pump appears to be an efficient Ca^{2+}-removing system, since 87% of the intracellular Ca^{2+} is cleared in a time equal to 2 τ_d, i.e. about 10 s. Since IP_3-degradation time is of the order of 1 s [28], this estimate is probably not biased by the residual opening of IP_3-sensitive channels. The physiological meaning of fast intracellular Ca^{2+} clearing is obviously to allow the cell to quickly (in comparison with the duration of the Ca^{2+} transient) curtail the Ca^{2+} signal in the absence of extracellular agonist. The SERCA pump proved to be the most important mechanism in this process, as directly shown by both Figure 4(B) and the reported estimate of the relative power of SERCA pump versus non-SERCA pump mechanisms (about 8:1). Since the relative contribution of the Ca^{2+} clearing systems might vary as a function of $[Ca^{2+}]_i$ [17,29], our estimate should be considered as a mean value computed for a $[Ca^{2+}]_i$ ranging from approximately the peak value to 37% of the peak. The actual amplitude of the transients has been measured in CMECs as described by Grynkiewicz et al. [30]. The baseline level averaged 0.092 ± 0.012 μM, and the peak amplitude averaged 0.71 ± 0.13 μM ($n = 6$), so that 37% of the transient amplitude is about 0.3 μM/l. In pancreatic acinar cells, at $[Ca^{2+}]_i = 0.35$ μM, the SERCA pump activity has been reported to be 14 times higher than non-SERCA pump mechanisms [17],

whereas the relative contribution of the SERCA pump is much lower in Purkinje neurons, being approx. 0.5 at a $[Ca^{2+}]_i$ of 0.5 μM [29].

Role of the SERCA pump during agonist-evoked Ca^{2+} transient

Mogami et al. [18] and Barrero et al. [31], using pancreatic acinar or EM26 cells respectively, showed by directly measuring the mean $[Ca^{2+}]$ within the stores that, during a large part of the Ca^{2+} transient's decay phase, $[Ca^{2+}]$ within the stores tends to remain constant. On the basis of this observation, it was suggested that during the Ca^{2+} transient's decay phase IP_3-sensitive channels are open and the SERCA pump is active, and the balance between Ca^{2+} release and Ca^{2+} uptake is approximately null [18]. The data reported in the present study lend further support to this hypothesis, since it has been shown that: (i) SERCA pump inhibition exerts no remarkable effect on the time course of the decay phase, in agreement with the notion that, because of the continuous opening of IP_3-sensitive channels, Ca^{2+} extrusion is the only effective factor in clearing Ca^{2+} from the cytoplasm; (ii) the lack of effect of SERCA pump inhibition is not due to the fact that the pumping power is too low to affect $[Ca^{2+}]_i$ in the time scale of the Ca^{2+} signal; (iii) all of the Ca^{2+} picked up by the SERCA pump is immediately and fully available for being newly released, if the agonist is re-applied (Figure 6); and (iv) PLC activity, but not PKC activation, is required in order to avoid fast Ca^{2+} clearing, which indirectly indicates that IP_3-sensitive channels must be open throughout all of the decay phase. Consistently with our conclusions, Oldershaw et al. [32], in a study on the quantal nature of the IP_3 response, showed that the IP_3 receptor does not become desensitized following stimulation for several minutes. Furthermore, it has been shown that, in rabbit microvascular ECs, IP_3 production by ATP stimulation may last for more than 5 min [33]. Mogami et al. [18] also showed that, during the initial part of the decay phase of the Ca^{2+} transient, the $[Ca^{2+}]_i$ within the stores shows a small decrease (i.e. there is a small net Ca^{2+} efflux from the stores). This observation might explain our result that, in the presence of SERCA pump inhibition, removing the agonist just after the Ca^{2+} peak makes the decay phase of the Ca^{2+} transients slightly faster, since in this case less Ca^{2+} has to be extruded out of the cell.

The lack of effect of SERCA pump inhibition on agonist-induced Ca^{2+}-transient decay might be explained by another model, where again Ca^{2+} release mainly occurs in the very initial part of the transient, but agonist stimulation also results in the production of a hypothetical factor that exerts an almost complete inhibitory effect on SERCA pump activity. Ca^{2+} decrease would therefore occur due to non-SERCA pump mechanisms only, and the level of Ca^{2+} within the stores would remain constant. According to our data, this hypothetical SERCA-pump-inhibiting factor should be a product of the agonist-activated PLC activity not related to PKC. Moreover, it should be Ca^{2+}-independent, since fast Ca^{2+} decrease is a consequence of its disappearance. Nitric oxide [34] can therefore be excluded. Furthermore, this messenger has been reported to exert a limited degree of inhibition (no more than 58 %), which would be completely insufficient to avoid a fast decrease of $[Ca^{2+}]_i$ in the absence of open IP_3 channels. Owing to the lack of evidence indicating the existence of a factor fully inhibiting SERCA pump activity, the continuous Ca^{2+}-uptake/Ca^{2+}-release model seems to be more likely.

Ca^{2+}-handling mechanisms other than the SERCA pump

The PMCA pump and NCX are ubiquitously expressed Ca^{2+}-handling mechanisms, and appear to be essential for cellular Ca^{2+} homoeostasis [8,9]. In ECs, the role of the PMCA pump has not been widely investigated. Iijima and co-workers [10], by using vanadate and La^{3+}, suggested that PMCA pump inhibition affects the resting level of $[Ca^{2+}]_i$ and makes the agonist-induced Ca^{2+} transient slower, a result partially confirmed by the use of antisense nucleotide against the PMCA pump isoform 1 [11]. With respect to NCX, several pieces of evidence suggest that it is present in a variety of ECs [10,13,14,35,36], but a more detailed characterization of its physiological role is needed. Our results show that, during the decay phase of the Ca^{2+} transient, Ca^{2+} is extruded by both the PMCA pump and NCX. As to the relative power of the PMCA pump and NCX, the result that a fraction of cells does not respond to Na^+ removal indicates that, in CMECs, their relative power is not homogeneous. This hypothesis is confirmed by the fact that non-responding cells become responsive after carboxyeosin loading. Indeed, non-responding cells might be hypothesized to quickly balance Ca^{2+} influx secondarily to Na^+ removal by means of a PMCA pump endowed with a higher relative power. Carboxyeosin, by reducing the PMCA pump efficiency, would unveil the NCX activity in these cells too. We did not investigate the basis of this variability, which could derive from cell-culturing conditions or might be present in ECs *in situ*. Kaye and Kelly [36] showed, in CMECs, that Na^+ removal caused a consistent increase in $[Ca^{2+}]_i$, but in that study average measurements on cell populations were performed, so that possible variability among cells could not be detected. The result that Na^+ removal is more effective in normal solution ($\Delta t_{80-20} = 50.1\,\%$) than in Ca^{2+}-free solution ($\Delta t_{80-20} = 32\,\%$) may appear unexpected, since the electrochemical gradient shift caused by Ca^{2+} removal should favour the Ca^{2+}-efflux mode. However, Ca^{2+}-efflux mode is activated by non-transported intracellular Ca^{2+} [9]. Since CMECs are endowed with a basal Ca^{2+} permeability (F. Tanzi, unpublished work), extracellular Ca^{2+} removal might conceivably decrease the sub-plasmalemmal $[Ca^{2+}]$, thus reducing the exchanger activity.

Carboxyeosin might also inhibit (at a concentration much higher than that required to inhibit the PMCA pump) the activity of NCX [37]. The question could therefore be raised of whether the decrease in the Ca^{2+}-transient kinetics operated by carboxyeosin might be due to inhibition of NCX. Our results, however, strongly argue against this possibility, since simultaneous carboxyeosin loading and Na^+ removal shows a potentiating effect. The effect of carboxyeosin on cells not responding to Na^+ removal agrees with the hypothesis that carboxyeosin does not inhibit NCX.

Block of both the Na^+-dependent and Na^+-independent Ca^{2+}-extrusion mechanisms, as obtained by extracellular Na^+ removal after carboxyeosin loading, makes the cell unable to maintain a normal $[Ca^{2+}]_i$ in the presence of PSS or to extrude the Ca^{2+} released by the endoplasmic reticulum following UTP stimulation. CMECs, therefore, still retain some capability to cope with an intracellular Ca^{2+} increase when either the PMCA pump or NCX are inhibited, but the simultaneous inhibition of both extrusion mechanisms completely perturbs Ca^{2+} homoeostasis.

No attempt has been made to assess the role of the PMCA pump and NCX in controlling the basal $[Ca^{2+}]_i$. It may be noted that the effect of Na^+ removal is transient or absent, suggesting that the PMCA pump alone is able to maintain the basal $[Ca^{2+}]_i$ to a normal level. Loading with carboxyeosin often causes a slight increase in the fluorescence ratio, which might suggest that PMCA pump inhibition increases $[Ca^{2+}]_i$. However, a drift from the base-line level may spontaneously occur during long-term recordings (25 min loading by carboxyeosin plus 20 min washing). Furthermore, carboxyeosin itself is fluorescent, causing an

increase in the fluorescence ratio (results not shown). Therefore, the small increase in the base-line level observed after carboxyeosin ester loading might also be due to the intrinsic carboxyeosin fluorescence.

Mitochondria have been shown recently to be involved in Ca^{2+} signalling and homoeostasis, not only in pathological but also physiological conditions [19]. Protonophores, such as CCCP and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which collapse mitochondrial membrane potential, are widely used to inhibit mitochondrial Ca^{2+} handling, even if it has been proposed that they may act primarily as acidic stimuli [38]. In CMECs, CCCP causes, as expected, a transient increase in $[Ca^{2+}]_i$, but no major change occurs in the kinetics of UTP-evoked Ca^{2+} transients in the presence of CCCP. Since mitochondria are a low-affinity Ca^{2+} uptake system [39], the UTP-evoked Ca^{2+} increase (up to about 700 nM) might be too low to be influenced by mitochondrial activity. Nevertheless, incubation with CCCP makes CMEC insensitive to UTP. A response to protonophores possibly related to this result has been reported by Landolfi et al. [40] in BHK-21 cells, where FCCP decreases the rate of Ca^{2+} release from the stores.

In conclusion, mitochondria do not have a role in modulating the kinetics of the decay phase of the agonist-evoked Ca^{2+} transient, which appears to entirely depend on the activity of the PMCA pump and NCX.

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