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Mitochondrial Ca^{2+} Uptake Requires Sustained Ca^{2+} Release from the Endoplasmic Reticulum*

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We analyzed the role of inositol 1,4,5-trisphosphate-induced Ca^{2+} release from the endoplasmic reticulum (ER) (i) in powering mitochondrial Ca^{2+} uptake and (ii) in maintaining a sustained elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). For this purpose, we expressed in HeLa cells aequorin-based Ca^{2+}-sensitive probes targeted to different intracellular compartments and studied the effect of two agonists: histamine, acting on endogenous H_1 receptors, and glutamate, acting on co-transfected metabotropic glutamate receptor (mGluR1a), which rapidly inactivates through protein kinase C-dependent phosphorylation and thus causes transient inositol 1,4,5-trisphosphate production. Glutamate induced a transient $[Ca^{2+}]_c$ rise and drop in ER luminal $[Ca^{2+}]$ ($[Ca^{2+}]_{er}$), and then the ER refilled with $[Ca^{2+}]_c$ at resting values. With histamine, $[Ca^{2+}]_c$ after the initial peak stabilized at a sustained plateau, and $[Ca^{2+}]_{er}$ decreased to a low steady-state value. In mitochondria, histamine evoked a much larger mitochondrial Ca^{2+} response than glutamate (~15 *versus* ~65 μM). Protein kinase C inhibition, partly relieving mGluR1a desensitization, reestablished both the $[Ca^{2+}]_c$ plateau and the sustained ER Ca^{2+} release and markedly increased the mitochondrial Ca^{2+} response. Conversely, mitochondrial Ca^{2+} uptake evoked by histamine was drastically reduced by very transient (~2-s) agonist applications. These data indicate that efficient mitochondrial Ca^{2+} uptake depends on the preservation of high Ca^{2+} microdomains at the mouth of ER Ca^{2+} release sites close to mitochondria. This in turn depends on continuous Ca^{2+} release balanced by Ca^{2+} reuptake into the ER and maintained by Ca^{2+} influx from the extracellular space.

Calcium-mobilizing agonists induce a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)[1] with a defined spatio-temporal pattern. In most cases, the $[Ca^{2+}]_c$ rise is composed of an initial peak, followed by repetitive $[Ca^{2+}]_c$ spiking or a sustained $[Ca^{2+}]_c$ elevation (for a review, see Ref. 1). Whereas the former is mostly contributed by the release of Ca^{2+} from intracellular stores (the ER and Golgi apparatus), the latter is sustained by Ca^{2+} entry from the extracellular space, which may be directly receptor activated or controlled by the filling state of the intracellular stores (store-operated Ca^{2+} influx (SOC); for a review, see Refs. 2 and 3). Indeed, removal of Ca^{2+} from the extracellular medium abolishes the sustained plateau phase but not the initial Ca^{2+} release. However, the necessity of a continuous influx from the extracellular medium for maintaining a prolonged Ca^{2+} rise does not imply that Ca^{2+} directly diffuses through the cytosol to the intracellular targets. Rather, Ca^{2+} entry could serve the purpose of filling ER cisternae in proximity of the plasma membrane; Ca^{2+} diffusion through the ER would then provide the driving force for continuous Ca^{2+} release in different cytosolic domains, including ER/mitochondria contact sites. Two recent observations support this scenario. First, examples of intracellular Ca^{2+} handling in different cell types show that influx-dependent ER refilling from the subplasma membrane space, followed by rapid diffusion of Ca^{2+} in the ER lumen, and IP_3-dependent Ca^{2+} release at distant sites may represent a general paradigm that allows the maintenance of sustained $[Ca^{2+}]$ rises in the cell body (4, 5) (for reviews, see Refs. 6 and 7). Second, some cytosolic effectors appear to "sense" very efficiently the release of stored Ca^{2+}, of which an excellent example is provided by mitochondria. Work from numerous laboratories has demonstrated that when a Ca^{2+} signal is elicited in the cytosol by the stimulation with IP_3-generating agonists, the cytosolic rise is always paralleled by Ca^{2+} uptake into the mitochondrial matrix (for reviews, see Refs. 8 and 9). A major increase in the mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) is thus observed (ranging from 5 μM in neuronal cells to 500 μM in chromaffin cells), which appears in contrast with the low affinity of the mitochondrial uptake mechanisms (the electrogenic uniporter of the inner membrane). The steep dependence of the mitochondrial Ca^{2+} uptake machinery on the extramitochondrial $[Ca^{2+}]$ has been well studied in isolated mitochondria (for reviews, see Refs. 10 and 11) and intact or digitonin-permeabilized cells (12–14). According to these studies, in order to obtain the $[Ca^{2+}]_m$ values observed in intact cells (*e.g.* 10–100 μM in HeLa cells) (15, 16), supramicromolar $[Ca^{2+}]$ should be generated at the

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[1] The abbreviations used are: $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum luminal Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial matrix Ca^{2+} concentration; cytAEQ, cytosolic aequorin; ER, endoplasmic reticulum; erAEQmut, low affinity aequorin mutant targeted to the endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; mGluR1a and mGluR5, metabotropic glutamate receptor type 1a and 5, respectively; mtAEQmut, low affinity aequorin mutant targeted to the mitochondria; PM, plasma membrane; PKC, protein kinase C; SERCA, sarcoendoplasmatic reticulum Ca^{2+} ATPase; SOC, store-operated Ca^{2+} influx; PMCA, plasmamembrane Ca^{2+} ATPase; tBHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; KRB, Krebs-Ringer modified buffer.

mitochondrial Ca^{2+} uptake sites. The apparent discrepancy was reconciled by the concept that high $[Ca^{2+}]$ microdomains generated at mouth of IP_3Rs during its activation are sensed by neighboring mitochondria (17), which are thus exposed to $[Ca^{2+}]$, that allow efficient Ca^{2+} uptake. Finally, the diffusion of Ca^{2+} through the outer mitochondrial membrane creates a lag time between the initial $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ rises into mitochondria (14, 18, 19). Thus, the properties of mitochondrial Ca^{2+} accumulation suggest that these organelles may represent the prototype of a cytosolic effector, which requires sustained release of Ca^{2+} from the ER (and thus maintenance of a high $[Ca^{2+}]$ microdomain at ER/mitochondria contacts) to be actively recruited in the calcium signaling pathway.

To investigate the relationship between the kinetics of ER Ca^{2+} release and mitochondrial Ca^{2+} accumulation, we carried out a study in the epithelial cell line HeLa, utilizing organelle-specific probes and agonists that induce different ER Ca^{2+} release patterns. These cells endogenously express the H_1 G-protein-coupled receptor coupled to phospholipase C activation and consequent continuous IP_3 production without detectable desensitization (20). Thus, histamine generates a typical biphasic cytosolic Ca^{2+} signal with sustained $[Ca^{2+}]_c$ elevation and parallel emptying of the ER until the agonist is present (21). Activation of SOC following ER emptying has been also shown after histamine stimulation (22, 23). In contrast to the H_1 receptor, the group I (Ca^{2+}-mobilizing) metabotropic glutamate receptors, such as mGluR1 or mGluR5, undergo receptor desensitization in the continuous presence of glutamate (for a review, see Ref. 24). In several cell types expressing either endogenous or recombinant mGluRs, the desensitization of these receptors was shown to be mediated by PKC (25). Feedback inhibition of the receptor by PKC phosphorylation results in inhibition of phosphoinositide hydrolysis; thus, application of glutamate induces only a transient IP_3 production (26). In the present study, we have taken advantage of the different properties of H_1 and mGluR1a receptors regarding the kinetics of IP_3 production for analyzing the role of IP_3-induced Ca^{2+} release in generating a sustained cytosolic Ca^{2+} response and its efficacy in inducing mitochondrial Ca^{2+} uptake.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, in 75-cm^2 Falcon flasks. For aequorin measurements, cells were seeded onto 13-mm glass coverslips and were cotransfected with 3 μg of mGluR1a/pcDM8 (27) and 2 μg of cytAEQ/VR1012, erAEQmut/VR1012, or mtAEQmut/VR1012, as previously described (28). Transient transfection was carried out using the Ca^{2+} phosphate precipitation technique. Luminescence analyses were carried out 36–48 h after transfection.

Aequorin Measurements—In the case of cytAEQ- or mtAEQmut-expressing cells, the coverslips with the cells were incubated with 5 μM wild type coelenterazine for 2 h in Krebs-Ringer modified buffer (KRB; 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 1 mM $CaCl_2$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) at 37 °C and then transferred to the perfusion chamber. In erAEQmut-expressing cells, the luminal $[Ca^{2+}]$ of the ER was reduced during aequorin reconstitution by incubating the cells for 1 h at 4 °C in KRB supplemented with 5 μM coelenterazine n, the Ca^{2+} ionophore ionomycin (5 μM), and 600 μM EGTA. After this incubation, cells were extensively washed with KRB without $CaCl_2$, supplemented with 2% bovine serum albumin and 1 mM EGTA. The ER Ca^{2+} store was refilled at the beginning of the experiments by perfusing the cells with KRB supplemented with 1 mM $CaCl_2$.

All aequorin measurements were carried out in KRB containing either 1 mM $CaCl_2$ (KRB/Ca^{2+}) or 100 μM EGTA (KRB/EGTA). Histamine (100 μM) or glutamate (100 μM) were added to the same medium. The aequorin experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signal was collected in a purpose-built luminometer and calibrated into $[Ca^{2+}]$ values as previously described (28). Chemicals and reagents were from Sigma or from Merck except for coelenterazine and coelenterazine n, which were from Molecular Probes, Inc. (Eugene, OR). Statistical data are presented as mean ± S.E.

Imaging Measurement of Cytosolic $[Ca^{2+}]$—To monitor $[Ca^{2+}]$ in the cytosol, mGluR1a-transfected HeLa cells were placed on 24-mm coverslips and loaded with 2 μM fura-2/AM in KRB/Ca^{2+} for 30 min at 37 °C. Cells were then washed in the same solution, and $[Ca^{2+}]_c$ changes were determined using a high speed, wide field digital imaging microscope. A Zeiss Axiovert 200 inverted microscope was used with a ×40 objective. Fura-2 was excited at 340 and 380 nm using a random access monochromator (Photon Technology International). Images were acquired by a Micromax 1300YHS camera (Princeton Instruments) using 4× binning in both the horizontal and vertical direction. Measurements were carried out at room temperature. Images were analyzed using the MetaFluor software (Universal Imaging Corp.).

RESULTS

Mitochondrial Ca^{2+} Uptake Depends on the Duration of ER Ca^{2+} Release—The start point of this work was to determine the correlation between the kinetics of the changes in cytosolic and mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, respectively) evoked in HeLa cells by the stimulation with a Ca^{2+}-mobilizing, IP_3-coupled agonist. For this purpose, HeLa cells were transfected with the appropriate targeted aequorin chimera (cytAEQ and mtAEQmut, respectively) (28). 36–48 h after transfection, functional aequorin was reconstituted by adding the prosthetic group. The coverslip with the cells was then transferred to the luminometer chamber and challenged with the agonist histamine. Light emission was collected and calibrated into $[Ca^{2+}]$ values, as described under "Experimental Procedures" and references therein. HeLa cells endogenously express the H_1 G-protein-coupled receptor, the stimulation of which leads to sustained IP_3 production. The results (Fig. 1) showed that agonist stimulation (100 μM histamine) evoked a cytosolic Ca^{2+} peak (2.5 μM), followed by a plateau phase (Fig. 1*A*). The cytosolic response was followed by an efficient mitochondrial Ca^{2+} uptake, reaching a peak level of ~70 μM (Fig. 1*B*). When the kinetics were compared, it was apparent that $[Ca^{2+}]_c$ peaked ~4 s after histamine addition (*i.e.* when the $[Ca^{2+}]_m$ rise was at <50% of the peak) and then rapidly declined toward a sustained plateau that was maintained throughout agonist stimulation. The $[Ca^{2+}]_m$ peak was reached when, through the activity of the SERCA and PMCA pumps, $[Ca^{2+}]_c$ was rapidly declining. As shown in Fig. 1, *C* and *D*, mitochondrial Ca^{2+} uptake depended almost entirely on Ca^{2+} release from internal stores, since stimulation of cells in Ca^{2+}-free extracellular medium led to the same mitochondrial Ca^{2+} uptake (64 ± 2.7 μM, $n = 5$ *versus* 66 ± 4.8 μM, $n = 35$ in control). We thus concluded that mitochondrial Ca^{2+} uptake depends on sustained release of Ca^{2+} from the ER.

To test this possibility, we stimulated HeLa cells through a G-protein-coupled receptor that rapidly undergoes PKC-dependent inactivation and thus causes a transient production of IP_3 and Ca^{2+} release from the ER. The receptor employed was mGluR1a (27), which was co-transfected with the targeted aequorin probes. Fig. 2 shows the functional properties of the transfected mGluR1a receptor. First, we analyzed the kinetics of Ca^{2+} release from the ER evoked by histamine and glutamate in parallel batches of mGluR1a-expressing cells, cotransfected with erAEQmut (28) (Fig. 2*A*). The data obtained highlight the fundamental difference between the two agonist responses: (i) stimulation of the endogenous histamine receptor caused a rapid initial $[Ca^{2+}]_{er}$ drop followed by a continuous and slower decrease reaching, ~2 min after the start of agonist stimulation, a low $[Ca^{2+}]_{er}$ steady-state value of ~50 μM, and (ii) in the case of glutamate, the initial rapid $[Ca^{2+}]_{er}$ decrease was followed by the refilling of the Ca^{2+} store, which was almost complete in about 2 min even in the continuous presence of glutamate.

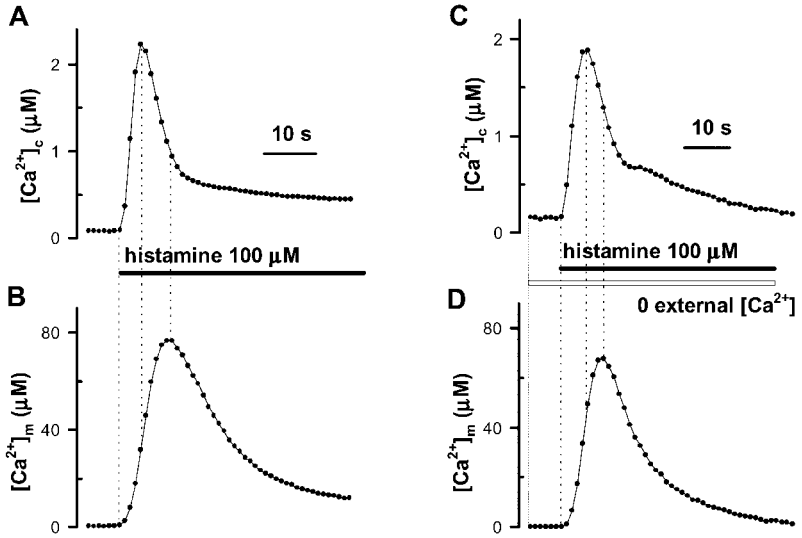

FIG. 1. **Cytosolic (*A* and *C*) and mitochondrial (*B* and *D*) $[Ca^{2+}]$ responses of HeLa cells stimulated with 100 μM histamine.** $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ was measured in cytAEQ- or mtAEQmut-expressing cells, respectively, 36 h after transfection. Aequorin luminescence was collected and calibrated into $[Ca^{2+}]$ values as described under "Experimental Procedures." Where indicated, HeLa cells were stimulated with the agonist, added to the perfusion medium (KRB in experiments shown in *A* and *B*; Ca^{2+}-free KRB plus 100 μM EGTA in *C* and *D*). The *dotted lines* indicate the start of stimulation and peak of cytosolic and mitochondrial Ca^{2+} signals, respectively. The traces are representative of >30 trials.

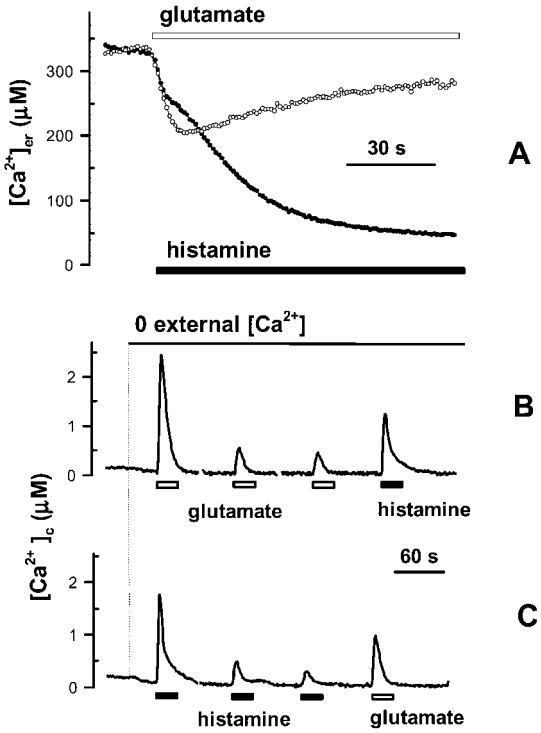

FIG. 2. **Transient Ca^{2+} release caused by glutamate in mGluR1a-expressing HeLa cells.** *A*, effect of glutamate (100 μM; *open circles*) and histamine (100 μM; *filled circles*) on $[Ca^{2+}]_{er}$ measured in HeLa cells co-transfected with erAEQmut. *B* and *C*, cytosolic Ca^{2+} transients measured in HeLa cells cotransfected with cytAEQ in response to glutamate (100 μM; *open circles*) and histamine (100 μM; *filled circles*) in Ca^{2+}-free medium (*dotted line*). The agonists were applied as shown by the *open* (histamine) and *filled* (glutamate) *bars*, respectively. The traces are representative of >10 trials.

Next, we applied another approach to explore the relationship between the stores controlled by the two agonists. We perfused cells co-transfected with cytAEQ and mGluR1a with a Ca^{2+}-free solution (KRB/EGTA; *i.e.* KRB supplemented with 100 μM EGTA instead of 1 mM $CaCl_2$) in order to prevent refilling of the stores, and we applied repetitive histamine pulses in order to deplete its intracellular Ca^{2+} store and then glutamate. It is apparent, as shown in Fig. 2*C*, that glutamate evoked substantially lower further release of Ca^{2+}, compared with that observed when glutamate was applied as first stimulus (see Fig. 2*B*). The reverse protocol (*i.e.* applying histamine after depleting the glutamate releasable pool) showed similar results (Fig. 2*B*). These experiments demonstrated that most of the glutamate- and histamine-releasable pools are overlapping; thus, the different kinetic behavior of the Ca^{2+} signal evoked by the two agonists cannot be ascribed to the use of separate intracellular stores.

We thus analyzed the amplitude and kinetics of $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ rises evoked by glutamate stimulation. Two major differences with the histamine responses are apparent. The first relates to the $[Ca^{2+}]_c$ rise. After the initial peak, which depends on the transient $[Ca^{2+}]_{er}$ decrease and is comparable in the two cases (2.1 ± 0.1 μM for histamine *versus* 1.75 ± 0.1 μM for glutamate; $n = 18$), there is no sustained plateau in the case of glutamate stimulation (*i.e.* $[Ca^{2+}]_c$ returns to the basal values in ~30 s in the continuous presence of the agonist) (Fig. 3*A*). The difference in $[Ca^{2+}]_m$ response was even more dramatic; the peak rise evoked by glutamate stimulation was drastically reduced (16.5 ± 1.8 μM ($n = 28$) *versus* 66 ± 4.8 μM ($n = 35$) during the histamine challenge) (Fig. 3*B*). Moreover, the difference between the time to peak of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ responses in this case was reduced (4.1 s for histamine *versus* 2.4 s for glutamate). In order to show that the same Ca^{2+} pools were used by both agonists to feed mitochondrial Ca^{2+} uptake, we applied histamine after 30 s of glutamate-induced Ca^{2+} release, before refilling of the ER (see Fig. 2*A*). In this case, the histamine-induced mitochondrial Ca^{2+} uptake was markedly reduced (23 ± 4.5 μM, $n = 5$) (Fig. 4*A*). Apparently, further depletion of the ER Ca^{2+} pool by histamine is responsible for the remaining $[Ca^{2+}]_m$ increase after glutamate stimulation as measured by erAEQmut (Fig. 4*B*).

The Kinetics of Initial Ca^{2+} Release Induced by Glutamate and Histamine Are Identical—We thus investigated the various possible reasons for the drastic reduction of the $[Ca^{2+}]_m$ rise in the glutamate response. The first possibility is that the kinetics of Ca^{2+} release from the ER is faster in histamine-stimulated cells, an effect that could be overlooked by the aequorin measurements. Indeed, given that the high rate of mitochondrial Ca^{2+} uptake depends on the exposure of Ca^{2+} microdomains generated at the mouth of IP_3Rs, one would envision that a faster release through the IP_3Rs would have a direct impact on mitochondrial Ca^{2+} accumulation. We thus evaluated the kinetics of the upstroke of $[Ca^{2+}]_c$ by fast digital imaging in fura-2-loaded HeLa cells expressing mGluR1a. As shown on Fig. 5, the kinetics of $[Ca^{2+}]_c$ elevation evoked by the two agonists appeared to be identical as analyzed by a time resolution of 200 ms ($t_{1/2}$ = 753 ± 47 ms for histamine, $t_{1/2}$ =

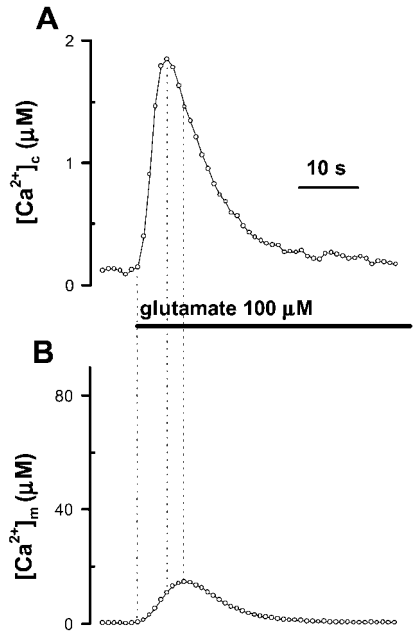

FIG. 3. **Cytosolic (*A*) and mitochondrial (*B*) $[Ca^{2+}]$ responses of HeLa cells co-transfected with mGluR1a stimulated with 100 μM glutamate.** $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were measured in cytAEQ- or mtAEQmut-expressing cells, respectively. Where indicated, HeLa cells were stimulated with the agonist, added to KRB. *Dotted lines* indicate the start of stimulation and peak of cytosolic and mitochondrial Ca^{2+} signals, respectively. The traces are representative of >10 trials.

712 ± 86 ms for glutamate; $n = 12$). Thus, the differences in mitochondrial Ca^{2+} uptake do not originate from differences in the velocity of the initial Ca^{2+} release. This conclusion is compatible with the preceding observations in cell populations transfected with the cytosolic and mitochondrial targeted aequorin probes (see above), where the mitochondrial Ca^{2+} uptake rate was slower than the cytosolic rise, and the mitochondrial uptake continued even after the cytosolic peak (see Fig. 1).

Alteration of the Duration of ER Ca^{2+} Release Modifies Mitochondrial Ca^{2+} Uptake—To further test the hypothesis that the $[Ca^{2+}]_m$ rise depends on a sustained release of Ca^{2+} from the ER, we decided to modify the kinetics of the cytosolic responses induced by the two agonists. As to glutamate, we applied the PKC inhibitor staurosporine, which was shown to prevent mGluR1a phosphorylation and to reduce the consequent receptor desensitization, ensuring sustained IP_3 production during glutamate stimulation. Preincubation of the cells with 400 nM staurosporine reversed the kinetics of both the ER and cytosolic Ca^{2+} signal; glutamate stimulation caused continuous Ca^{2+} release from the ER (Fig. 6*B*), similarly to the Ca^{2+} signal observed during histamine stimulation. As to $[Ca^{2+}]_c$, no difference was observed in the peak, but the $[Ca^{2+}]_c$ decrease after the peak was significantly slower, and a sustained plateau (maintained throughout agonist stimulation) was reached (Fig. 6*A*). We have to note that the addition of staurosporine does not prevent mGluR1a desensitization completely (24), as indicated by the slower kinetics of continuous ER Ca^{2+} release and the lower sustained $[Ca^{2+}]_c$ (see, for comparison, the sustained plateau following histamine stimulation) (Fig. 1*A*). Still, importantly, converting the glutamate-induced transient Ca^{2+} release into a more continuous response by staurosporine application, the $[Ca^{2+}]_m$ peak was markedly increased (25.6 ± 2.9 μM, compared with 16.5 ± 1.8 μM cells not treated with staurosporine; $n = 28$) (Fig. 6*C*).

The opposite experiment was also performed (*i.e.* the histamine-evoked release was made more transient) by reducing the duration of the agonist challenge to 2 s (Fig. 7). Under those conditions, the peak $[Ca^{2+}]_c$ response was marginally reduced (2.27 ± 0.1 *versus* 2.45 ± 0.1 μM cells receiving a 2-min histamine challenge; $n = 16$ for both groups) (Fig. 7*A*), but $[Ca^{2+}]_c$ rapidly returned to basal values, with disappearance of the sustained $[Ca^{2+}]_c$ plateau. Importantly, using the same protocol for $[Ca^{2+}]_m$ measurements, matching with the reduction of Ca^{2+} release time, the $[Ca^{2+}]_m$ peak was substantially reduced (Fig. 7*B*, from 66 ± 4.8 μM, $n = 35$, for 2-min stimulation to 29.1 ± 2.9 μM, $n = 16$, for 2-s stimulation), and $[Ca^{2+}]_m$ reached its peak earlier (9.6 ± 0.29 s control *versus* 4.5 ± 0.35 s for 2 s stimulation) (Fig. 7*B*).

Continuous Ca^{2+} Release from the Intracellular Ca^{2+} Stores Is Balanced by SOC and Ca^{2+} Recycling by SERCA—We thus concluded that prolonged release of Ca^{2+} from the ER is necessary to achieve maximal mitochondrial responses. But how can this release and the ensuing microdomains at ER/mitochondria contacts be maintained, given that PMCA efficiently reduces $[Ca^{2+}]_c$ by extruding Ca^{2+} in the extracellular space? To this end, it is necessary that ER be continuously refilled by Ca^{2+} entry and redistribute Ca^{2+} to the release sites, in keeping with the pathway of Ca^{2+} studied in depth in pancreatic acinar cells (29, 30). In other words, the steady-state phase of agonist-stimulated Ca^{2+} release from the ER, which is necessary for transferring the Ca^{2+} signal to mitochondria and other cytosolic effectors, must be sustained by the process of Ca^{2+} entry through the plasma membrane.

Thus, in the next set of experiments, we analyzed the contribution of SOC to the generation of the sustained Ca^{2+} signal. For this purpose, we released Ca^{2+} from the intracellular pools by applying either histamine (Fig. 8*A*) or glutamate (Fig. 8*B*) in KRB/EGTA to cells transfected with cytAEQ and mGluR1a. Then we evoked Ca^{2+} influx by changing the perfusion medium from KRB/EGTA to KRB/Ca^{2+} (in the continuous presence of the agonist). Ca^{2+} release from intracellular Ca^{2+} stores produced a transient peak upon stimulation with both agonists, which returned to the base line, showing that the presence of extracellular Ca^{2+} is essential to achieve sustained Ca^{2+} signal. Furthermore, depletion of the stores by histamine activated SOC, as observed from the increase of $[Ca^{2+}]_c$ after the readdition of external Ca^{2+} (see Fig. 8*A*). In contrast, Ca^{2+} reintroduction into KRB caused only a slight elevation in the presence of glutamate, probably due to smaller depletion of the stores caused by the transient IP_3 production. However, glutamate, after staurosporine preincubation, was able to activate SOC, since the readdition of Ca^{2+} caused a $[Ca^{2+}]_c$ elevation comparable with that of caused by histamine (see Fig. 8*B*). Thus, according to these data, we confirmed that both Ca^{2+} influx and continuous IP_3 production are necessary for maintaining the cytosolic Ca^{2+} signal.

Then we tested the effect of Cd^{2+}, a blocker of Ca^{2+} entry pathways of the plasma membrane, including store-operated channels, on $[Ca^{2+}]_{er}$ at different stages of cell stimulation (Fig. 9). As expected, if Cd^{2+} is added after the stimulation with histamine (*i.e.* when no Ca^{2+} release occurs and the ER is actively reaccumulating Ca^{2+}), the process of refilling is blocked, and a $[Ca^{2+}]_{er}$ steady state lower than in unstimulated cells is reached. Conversely, if Cd^{2+} is added in the presence of the agonist (*i.e.* when the ER is largely depleted and a steady state $[Ca^{2+}]_{er}$ value of ~50 μM is maintained), a rapid, further emptying of the ER is observed, leading to almost complete emptying of the ER. Thus, importantly, an equilibrium between refilling and Ca^{2+} release through IP_3Rs is maintained throughout the process of agonist stimulation.

If this is the case, one would expect that both blocking Ca^{2+} release through IP_3Rs or the process of ER refilling though store-operated channels should be equally effective in reducing

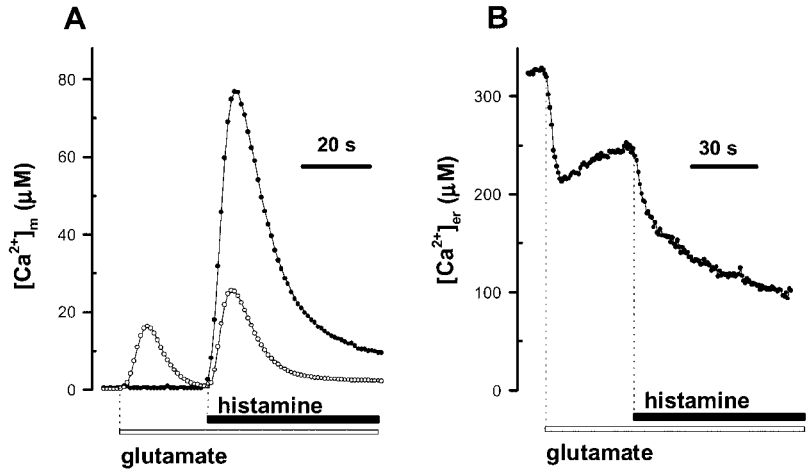

FIG. 4. **Ca^{2+} pools feeding mitochondrial Ca^{2+} uptake in case of glutamate and histamine stimulation.** *A*, mitochondrial Ca^{2+} transients measured in HeLa cells cotransfected with mtAEQmut and mGluR1a in response to 100 μM histamine alone (*open circles*) and after glutamate stimulation (*filled circles*). *B*, effect of 100 μM histamine on $[Ca^{2+}]_{er}$ added after 100 μM glutamate, as measured in HeLa cells cotransfected with erAEQmut and mGluR1a. The agonists were applied as shown by the *bars below* the *traces*. The *traces* are representative of >5 trials.

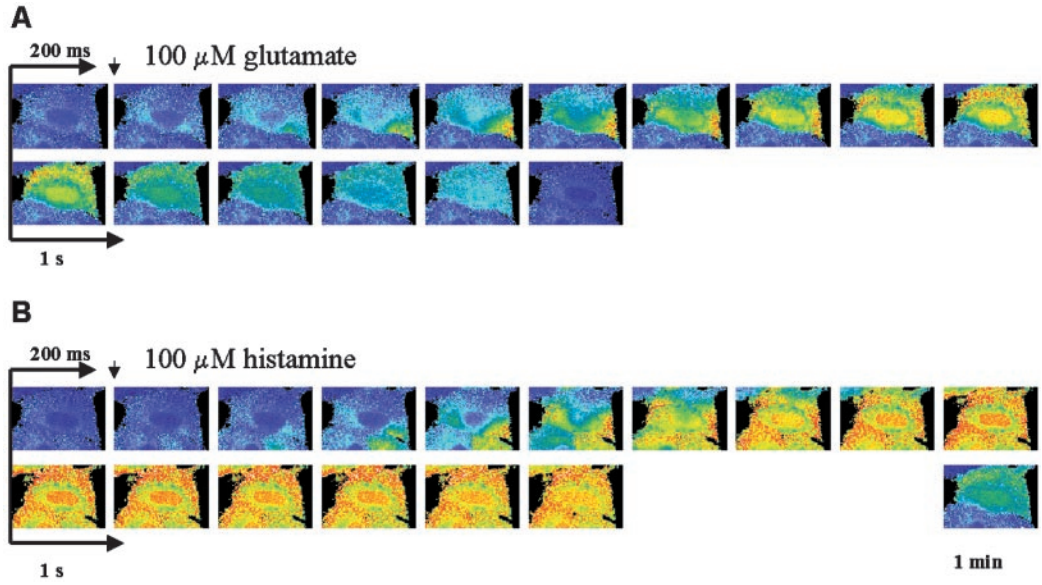

FIG. 5. **Cytosolic Ca^{2+} signal in a single HeLa cell expressing mGluR1a in response to glutamate (100 μM; *A*) and histamine (100 μM; *B*).** Cells were loaded with fura-2, and ratio images were acquired every 200 ms, as described under "Experimental Procedures." Conventional pseudocolor ratio images are shown every 200 ms in the *upper line* of each *panel* and in each second in the *lower line*. In case of histamine stimulation, elevated ratio levels were observed even after 1 min of stimulation (*lower right image*). Cells were stimulated by the addition of the agonist into the bath, as indicated by *arrows*. The series of *images* are representative of >10 experiments.

the sustained $[Ca^{2+}]_c$ rise observed during agonist stimulation. Moreover, the termination of the $[Ca^{2+}]_c$ signal in the former way should be more rapid, whereas the effect of Ca^{2+} entry blockade should occur only after the ER is depleted of the remaining Ca^{2+}. This was directly investigated in the experiment of Fig. 10, where we compared the kinetics of terminating the sustained cytosolic signal after histamine stimulation with two experimental protocols. In the first, we applied Cd^{2+} to block Ca^{2+} influx still in the presence of histamine and IP_3-induced Ca^{2+} release. In the second, we washed out histamine rapidly in order to terminate IP_3 production (*i.e.* to stop Ca^{2+} release). As shown in Fig. 9*A*, the half-decay time of the cytosolic Ca^{2+} signal was about 2 times longer during blockade of influx by 1 mM Cd^{2+} ($t_{½}$ = 15.4 ± 0.98 s, n = 12) than in the case of histamine washout ($t_{½}$ = 8.5 ± 0.49 s, n = 11, $p < 0.01$). These results show that Ca^{2+} release from the internal stores has the major role in the generation of $[Ca^{2+}]_c$ elevation, whereas the Ca^{2+} entry maintains the state of filling of the Ca^{2+} store, thus counteracting the forces of Ca^{2+} extrusion by PMCA.

Finally, we compared $[Ca^{2+}]_c$ changes during ER refilling in the presence and in the absence of IP_3. As shown in Fig. 11*A*, after emptying the intracellular Ca^{2+} pools by 100 μM histamine in the absence of extracellular Ca^{2+}, Ca^{2+} readdition to the medium exerted a significant $[Ca^{2+}]_c$ increase only if histamine (*i.e.* IP_3) was present. If histamine was washed out 30 s before Ca^{2+} readdition, refilling of the Ca^{2+} stores was accompanied by only a small $[Ca^{2+}]_c$ elevation. The efficiency of refilling in this case was demonstrated by a second application of histamine, which elicited a Ca^{2+} release comparable with the one exerted by the first stimulation (Fig. 11*A*, *filled circles*). Furthermore, prolonged histamine stimulation and IP_3-induced release caused further depletion of the Ca^{2+} stores even after Ca^{2+} readdition and following robust Ca^{2+} influx. This is illustrated by the small amount of releasable Ca^{2+} remaining in the pools even after a 20 s washout of histamine before its reapplication (Fig. 11*A*, *open circles*). Similarly, the readdition of 1 mM extracellular Ca^{2+} after depleting the Ca^{2+} stores by the reversible SERCA inhibitor 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBHQ; 10 μM) led to efficient store refilling without significant $[Ca^{2+}]_c$ elevation (Fig. 11*B*, *filled circles*). In contrast, in the continuous presence of tBHQ, Ca^{2+} influx was conveyed to the cytosol, as shown by the vast $[Ca^{2+}]_c$ elevation following the readdition of Ca^{2+} (Fig. 11*B*, *open circles*).

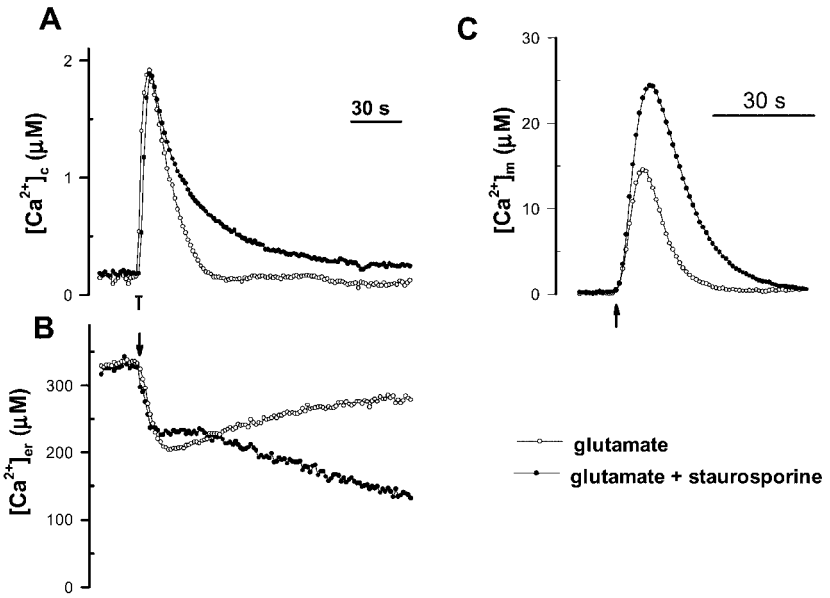

FIG. 6. **Effect of staurosporine (400 nM, added 5 min before cell stimulation) on Ca^{2+} signals elicited by glutamate (100 μM) in mGluR1a-expressing HeLa cells.** Control traces are shown with *open circles*, and traces of staurosporine-pretreated cells are shown with *filled circles*. $[Ca^{2+}]_c$ (*A*), $[Ca^{2+}]_{er}$ (*B*), and $[Ca^{2+}]_m$ (*C*) were measured in cytAEQ, erAEQmut, and mtAEQmut co-transfected cells, respectively. Glutamate was applied continuously in perfusion from the time point indicated by the *arrows*. The *traces* are representative of >10 trials.

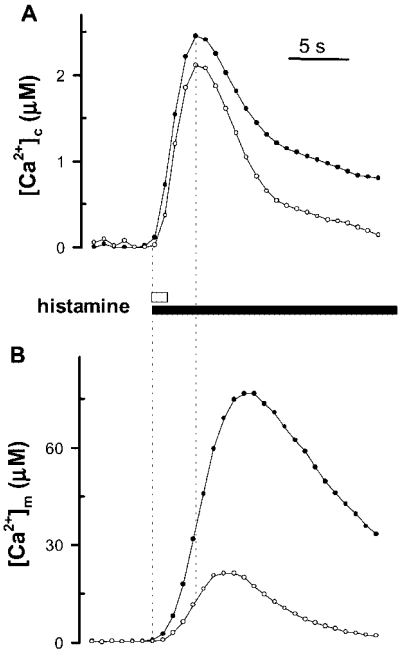

FIG. 7. **Cytosolic (*A*) and mitochondrial (*B*) $[Ca^{2+}]$ responses of HeLa cells to sustained (*filled circles*) and transient (2 s; *open circles*) histamine stimulations (100 μM).** $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were measured in cytAEQ- or mtAEQmut-expressing cells, respectively. Where indicated (*filled bar* for sustained, *open bar* for transient stimulation) HeLa cells were stimulated with the agonist, added to the perfusion medium. *Dotted lines* indicate the start of stimulation and the peak of cytosolic signals, respectively. The *traces* are representative of >20 trials.

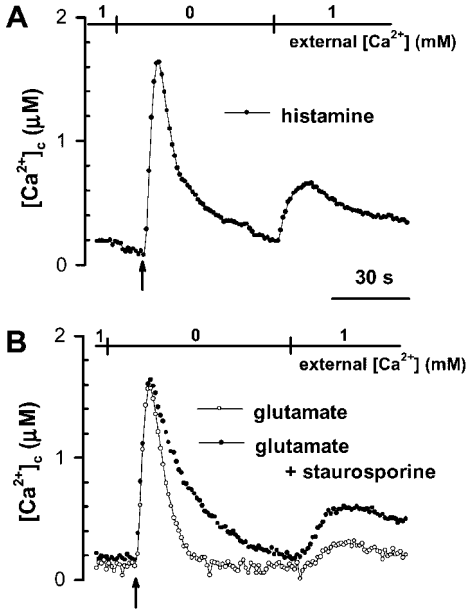

FIG. 8. **Dependence of Ca^{2+} signals evoked by histamine (100 μM; *A*, *filled circles*) and glutamate (100 μM; *B*, *open circles*) on the presence of extracellular Ca^{2+}.** The extracellular $[Ca^{2+}]$, either 0 (KRB plus 100 μM EGTA) or 1 mM, is shown on the *bars above* the *traces*. *B*, effect of staurosporine (400 nM, added 5 min before cell stimulation; *filled circles*) on glutamate-induced cytosolic Ca^{2+} signals in the absence and presence of extracellular Ca^{2+}. The starting points of the continuous stimulation by either agonist, added to the perfusion medium, are shown by *arrows*. The *traces* are representative of >10 trials.

These data clearly indicate that Ca^{2+}, after entering the cells, is taken up robustly by the ER without significantly elevating $[Ca^{2+}]_c$, and then Ca^{2+} is distributed by the ER to the entire cytosolic space. In this way, SOC is not directly responsible for the generation of the sustained cytosolic Ca^{2+} signal; nevertheless, it is necessary for its maintenance, by ensuring the continuous refilling of the internal Ca^{2+} stores. Furthermore, Ca^{2+} cycling at the vicinity of Ca^{2+} release sites maintains an equilibrium on both sides of the ER membrane (*i.e.* the steady state in the $[Ca^{2+}]_{er}$ and the sustained phase of the $[Ca^{2+}]_c$ elevation); thus, ER plays an important role as a redistribution pathway between plasma membrane Ca^{2+} entry and other intracellular organelles, such as the mitochondria.

DISCUSSION

In recent years, much information has been acquired on how specific spatio-temporal patterns of Ca^{2+} signaling can control different cell functions (or deleterious effects in pathological conditions). In particular, attention has been drawn to the properties and functional significance of local gradients (microdomains) and thus to the importance of the source and intracellular route of the $[Ca^{2+}]$ rise. Subplasmalemmal high $[Ca^{2+}]$ microdomains appear to regulate the activity of plasma membrane (PM) ion channels (such as voltage-dependent Ca^{2+}

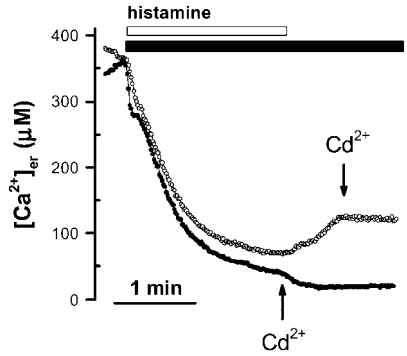

FIG. 9. **Comparison of the effect on $[Ca^{2+}]_{er}$ of Cd^{2+} added at different phases of the Ca^{2+} signal.** $[Ca^{2+}]_{er}$ was measured in cells transfected with erAEQmut as described under "Experimental Procedures." 100 μM histamine was added to the perfusion medium as indicated by the *bars below* the *traces*. The *arrows* mark the timing of Cd^{2+} addition. The *trace* with *filled circles* shows the effect of 1 mM Cd^{2+} added at steady state $[Ca^{2+}]_{er}$ in the presence of histamine (see the *filled bar*). The *trace* with *open circles* demonstrates the immediate effect of 1 mM Cd^{2+} added during store refilling after the removal of histamine from the perfusion medium (see *white bar*). The *traces* are representatives of >6 trials.

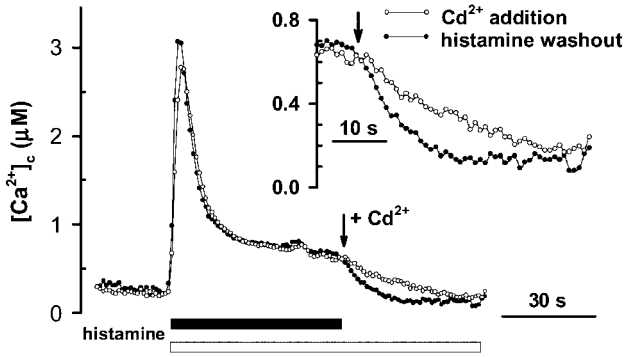

FIG. 10. **Comparison of the kinetics of cytosolic Ca^{2+} signal termination by washing out histamine (*traces* with *filled circles*) and by inhibiting Ca^{2+} entry with Cd^{2+} (*traces* with *open circles*).** $[Ca^{2+}]_c$ was measured in cells transfected with cytAEQ as described under "Experimental Procedures." 100 μM histamine was added to the perfusion medium as indicated by the *bars below* the *traces* (*filled bar*, in the case of histamine washout; *white bar*, in the case of the addition of 1 mM Cd^{2+}). The *inset* shows the magnification of the decay phases (*trace labeling* as in the *main panel*). The *traces* are representative of >12 trials.

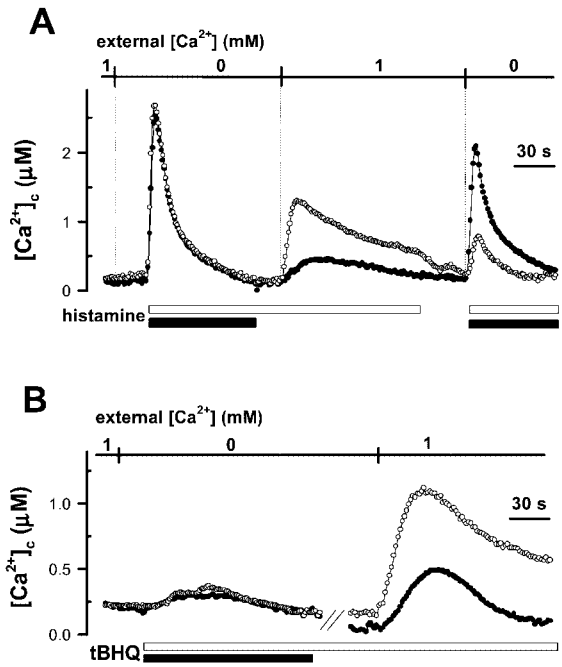

FIG. 11. **Analysis of Ca^{2+} reuptake by the ER during store-operated Ca^{2+} influx after store depletion by histamine stimulation (*A*) or by reversible inhibition of SERCA by tBHQ (*B*).** $[Ca^{2+}]_c$ was measured in cytAEQ-expressing HeLa cells. Store-operated Ca^{2+} influx was evoked by emptying the stores by perfusing 0 extracellular $[Ca^{2+}]$ (KRB + 100 μM EGTA) and then re-adding 1 mM $CaCl_2$, as shown on the *bars above* the *traces*. *A*, histamine (100 μM) was added to Ca^{2+}-free KRB and either removed before (*filled bar*, *trace* with *filled circles*) or maintained during (*open bar*, *trace* with *open circles*) the readdition of extracellular Ca^{2+}. An additional histamine stimulus was added in both cases in 0 extracellular $[Ca^{2+}]$ to show the filling state of the Ca^{2+} stores. *B*, tBHQ (10 μM) was added to the Ca^{2+}-free KRB and either removed before (*filled bar*, *trace* with *filled circles*) or maintained during (*open bar*, *trace* with *open circles*) the readdition of extracellular Ca^{2+}. The traces are representative of >6 trials.

and Na^+ channels, Ca^{2+}-activated K^+ channels, and SOC), polarity and excitability of the PM (neurons, smooth muscle), secretory responses, and neurotransmitter release. Ca^{2+} entry, by means of voltage-, ligand-, or store-operated channels, in most of the cases is necessary and sufficient to generate such a high $[Ca^{2+}]$ microdomain below the PM, also for longer periods of cell stimulation. Conversely, other long term processes regulated by sustained Ca^{2+} signals take place deeper in the cell interior. Among them, a well known example is the regulation of mitochondrial enzymes involved in ATP production or steroid synthesis, where Ca^{2+} taken up from microdomains generated at the mouth of ER Ca^{2+} release channels plays a fundamental role (15, 31). A transient $[Ca^{2+}]_m$ peak was shown to exert a long term effect at the level of ATP synthesis (32), and continuous mitochondrial $[Ca^{2+}]$ elevation, even at relatively lower extramitochondrial Ca^{2+} levels, was shown to increase the activity of dehydrogenases of the Krebs cycle (13, 33, 34), thus elevating the NADH level and the activity of the electron transport chain. At the same time, Ca^{2+} uptake by mitochondria has been shown to be involved in a radically different process (*i.e.* the release of proapoptotic factors and thus the induction of cell death) (35).

Mitochondria have thus recently emerged as key decoders of calcium signals, and the mechanism and timing of their recruitment control key decisions in cell life and death. In this contribution, we have analyzed the correlation between the kinetics of $[Ca^{2+}]_c$ increase and its different components and the $[Ca^{2+}]_m$ rises occurring in agonist-stimulated cells. For this purpose, we utilized a low affinity probe for $[Ca^{2+}]_m$ (that allows us to fully appreciate the large $[Ca^{2+}]_m$ swing) and two different agonist stimulations, through the endogenous histamine receptor and through a transfected metabotropic glutamate receptor, which undergoes rapid desensitization and thus causes transient IP_3 production. The first observation is that, upon cell stimulation, $[Ca^{2+}]_m$ peaks well after $[Ca^{2+}]_c$. As previously observed by various groups, there is a short delay in the upstroke, possibly due to the time needed for the diffusion of Ca^{2+} released by IP_3 receptors through the outer mitochondrial membrane, thus reaching the transport systems (uniporter) of the inner membrane (14, 18, 19). Then $[Ca^{2+}]_m$ rises and reaches its maximal value after ~10 s (*i.e.* when the $[Ca^{2+}]_c$ increase, through the activity of the Ca^{2+} pumps, is rapidly declining).

How can the slow kinetics of mitochondrial Ca^{2+} accumulation be reconciled with the notion that the low affinity of the mitochondrial uniporter requires, for rapid uptake, the high $[Ca^{2+}]$ gradient generated upon cell stimulation by the opening of IP_3 receptors? The most logical explanation is that, for maximal mitochondrial Ca^{2+} uptake, prolonged Ca^{2+} release from the ER must occur. Different experiments support this notion. Indeed, not only in the case of glutamate stimulation (in which

Ca^{2+} release from the ER is short lived) is the mitochondrial Ca^{2+} response drastically reduced, but the effect of the two stimuli (glutamate and histamine) on mitochondria can be reversed by modifying the time course of the Ca^{2+} release process. If desensitization of the mGluR is prevented by PKC inhibitors, ER release becomes sustained (with ensuing large amplitude emptying of the ER and activation of store-dependent Ca^{2+} influx), and mitochondrial responses are greatly enhanced. Conversely, a short (2-s) histamine pulse causes transient emptying of the Ca^{2+} store and drastically reduces the $[Ca^{2+}]_m$ rise evoked by the agonist. These data imply that in the late phases of agonist stimulation (*i.e.* when the activity of the pumps (SERCA and PMCA) counteracts the release of Ca^{2+} through the IP_3Rs), an equilibrium is attained between the two processes, as demonstrated by the direct measurement of $[Ca^{2+}]_{er}$; blocking Ca^{2+} release (*e.g.* by terminating cell stimulation) causes the rapid refilling of the store, while conversely interrupting the reaccumulation of Ca^{2+} in the ER (*e.g.* by blocking Ca^{2+} entry through SOC; see below) allows IP_3Rs to rapidly deplete the ER of Ca^{2+}.

Mitochondria, and possibly other cytosolic effectors, appear thus to be activated through the kinetic behavior of the ER release process. Conversely, Ca^{2+} entry, which is invariably essential for sustained Ca^{2+} signals, might not provide a direct supply for these localized Ca^{2+} regulatory events. Two considerations support this view. First, these processes rely on specific patterns of IP_3-dependent release of Ca^{2+} from the ER store, as discussed above. Second, Ca^{2+} entering the cytosol is strongly buffered by Ca^{2+}-binding proteins, such as parvalbumin, calbindin D_{28K}, and calretinin, rendering the diffusion rate of Ca^{2+} in the cytosol rather low (36). Thus, Ca^{2+} coming from the extracellular medium would reach slowly if at all the deeper regions of the cytosol. An ingenious solution for this challenge has been recently shown in a polarized cell model, the pancreatic acinar cells (30). At the initial phase of the physiological activation of these cells, focal Ca^{2+} release occurs exclusively at the secretory pole, serving as trigger for exocytosis and leading to local emptying of the Ca^{2+} store (30). The presence of Ca^{2+} signaling components and particularly TRP-like channels at the vicinity of the apical pole has been suggested to provide a straightforward route for local refilling of the depleted stores (37). On the other hand, given that the ER in these cells forms a continuous network (4), the resulting luminal $[Ca^{2+}]$ gradient has been shown to cause rapid diffusion of Ca^{2+} from the basolateral part of the cells, in a model where SOC is restricted to this area (38). The ground of this arrangement of Ca^{2+} signaling is the relatively high Ca^{2+} mobility in the ER tunnel compared with the cytosol, depending on the much lower binding capacity of the ER lumen (~20 *versus* ~2000 bound/free Ca^{2+} of the cytosol of mouse pancreatic acinar cells (39)). Similarly, in neurons, which is still a highly polarized cell type, it was proposed that subplasmalemmal ER cisternae of the cell body may be responsible for Ca^{2+} refilling from the extracellular space, and a continuous ER network would transport Ca^{2+} to the site of release in dendritic spines (40). In accordance with this idea, it has been shown that the cytosolic buffering capacity of Purkinje neurons is as high (~2000) as that of pancreatic acinar cells (41). On the other hand, in other cell types such as chromaffin cells (42), lower values of binding capacity have been found (~40), but we should emphasize that even if Ca^{2+} diffusion may occur at a similar velocity in the cytosol and the ER, another important advantage in the use of ER for distributing Ca^{2+} signals to the cell interior is that it may ensure localized Ca^{2+} release. Thus, in smooth muscle cells, even if the cytosolic buffering capacity is comparable with that of the ER (~30–40) (43), a superficial layer of SR rapidly buffers Ca^{2+} entering from the extracellular space, which is then distributed into the cell interior, causing contraction after its directed release (44).

Since our aim was to disclose the role of the ER in generating a sustained Ca^{2+} signal and in recruiting cytosolic effectors, in our work we did not characterize the exact nature of the Ca^{2+} entry pathway. However, some information on Ca^{2+} influx and store refilling can be obtained from the experimental data. Recently, a receptor-activated, arachidonic acid-mediated, non-capacitative mechanism for Ca^{2+} entry has been demonstrated that appears to operate in a PM domain distinct from that in which SOC operates in HEK293 cells (45). Two arguments suggest that this pathway does not contribute to the Ca^{2+} signals observed in our experiments. (i) We used maximal agonist concentrations for prolonged periods, producing a substantial depletion of Ca^{2+} stores; thus, we fully activated the SOC mechanism, which inhibits arachidonate-regulated channels (46). (ii) Ca^{2+} entry activated by store depletion in our system was clearly necessary for store refilling, in contrast to Ca^{2+} entering the cells by arachidonate-activated Ca^{2+} entry, which rather plays a role in potentiating the Ca^{2+} release induced by IP_3, thus increasing the frequency of oscillations (47).

The other issue concerns the way Ca^{2+} refills the Ca^{2+} stores. We demonstrated that in the case of transient IP_3 production (*i.e.* during glutamate stimulation), ER refilling occurs without detectable rise of $[Ca^{2+}]_c$ (compare Figs. 2*A* and 3*B*). However, based on our data, we cannot distinguish between refilling from the extracellular space through SOC channels and direct Ca^{2+} reuptake from the cytosol by SERCA. It appears that the buffering capacity of the cytosol determines the route by which Ca^{2+} released from the ER is eliminated, since increased buffering allows ER refilling by SERCA even in the absence of extracellular Ca^{2+} (48). Thus, in cells with inherent high cytosolic buffer capacity, such as the above mentioned pancreatic acinar cells or neurons, the SERCA pumps appear to dominate over PMCAs in rapidly reducing the $[Ca^{2+}]_c$ peak (49, 50). Thus, the oscillatory Ca^{2+} signals evoked by colecystokinin in these cells were shown to occur in the absence of Ca^{2+} entry, given that Ca^{2+} is taken back almost entirely from the cytosol after the Ca^{2+} spikes (see above). However, the situation differs with other types of stimulation; the Ca^{2+} oscillations evoked by carbachol strongly depend on Ca^{2+} influx in the proximity of the Ca^{2+} release sites of the apical pole (37). Up to now, there are no data concerning the cytosolic buffering capacity of HeLa cells, but evidence from the similar Chinese hamster ovary cell line shows that PMCA overexpression leads to larger reduction and faster termination of cytosolic Ca^{2+} signal compared with SERCA (51). Thus, it seems likely that the glutamate-induced Ca^{2+} transient is rapidly extruded from the cell, and the Ca^{2+} source of refilling in this case is the extracellular space.

In conclusion, the data presented in this paper indicate that mitochondria, important transducers of the Ca^{2+} signal, depend on the process of ER Ca^{2+} release, which in turn is sustained by continuous release through IP_3 receptors and refilling by SERCAs (with a primary role of Ca^{2+} influx in counteracting the extrusion of Ca^{2+} by PMCAs). Altogether, these data suggest that the ER provides a fast route for tunneling and releasing Ca^{2+} in the deeper portions of the cytoplasm (where mitochondria are only one of the numerous Ca^{2+} effectors) not only in the polarized pancreatic acinar cell (as proposed by Petersen *et al.* (30)) but also in different cell types. This provides an additional mechanism by which the selective placement and differential activation of Ca^{2+} channels in the ER and plasma membrane provide flexibility to the Ca^{2+} trans-

duction system, allowing this second messenger to play a key role in the modulation of virtually all cellular processes.

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