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The Golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store, with functional properties distinct from those of the endoplasmic reticulum

Paolo Pinton, Tullio Pozzan and Rosario Rizzuto[1]

Department of Biomedical Sciences and CNR Centre for the Study of Biomembranes, University of Padova, Via Colombo 3, 35121 Padova, Italy

[1]Corresponding author
e-mail: rizzuto@civ.bio.unipd.it

In the past few years, intracellular organelles, such as the endoplasmic reticulum, the nucleus and the mitochondria, have emerged as key determinants in the generation and transduction of Ca^{2+} signals of high spatio-temporal complexity. Little is known about the Golgi apparatus, despite the fact that Ca^{2+} within its lumen controls essential processes, such as protein processing and sorting. We report the direct monitoring of the $[Ca^{2+}]$ in the Golgi lumen ($[Ca^{2+}]_{Golgi}$) of living HeLa cells, using a specifically targeted Ca^{2+}-sensitive photoprotein. With this probe, we show that, in resting cells, $[Ca^{2+}]_{Golgi}$ is ~0.3 mM and that Ca^{2+} accumulation by the Golgi has properties distinct from those of the endoplasmic reticulum (as inferred by the sensitivity to specific inhibitors). Upon stimulation with histamine, an agonist coupled to the generation of inositol 1,4,5-trisphosphate (IP3), a large, rapid decrease in $[Ca^{2+}]_{Golgi}$ is observed. The Golgi apparatus can thus be regarded as a *bona fide* IP3-sensitive intracellular Ca^{2+} store, a notion with major implications for the control of organelle function, as well as for the generation of local cytosolic Ca^{2+} signals.

Keywords: Ca^{2+} store/Golgi apparatus/inositol 1,4,5-trisphosphate

Introduction

In the complex scenario of Ca^{2+} signalling, little or no attention is usually given to the Golgi apparatus. Indeed, while the total calcium content of the organelle is known to be high (Chandra *et al.*, 1994; Pezzati *et al.*, 1997), little is known about the $[Ca^{2+}]$ of this compartment, and its dynamic changes under physiological conditions. As far as Ca^{2+} signalling is concerned, it is widely accepted not only that the endoplasmic reticulum (ER) is the intracellular store of agonist-mobilizable Ca^{2+}, but also that Ca^{2+} handling by this organelle is characterized by marked functional heterogeneity (Montero *et al.*, 1997b). More recently, inositol 1,4,5-trisphosphate (IP3)-sensitive Ca^{2+} stores have been suggested to be present within the endomembrane system even in secretory vesicles (Gerasimenko *et al.*, 1996; Petersen, 1996). Based on these premises, the Golgi apparatus could also be expected to act as a Ca^{2+} store (Hofer *et al.*, 1995; Zha *et al.*, 1995) and, given its juxtanuclear localization, participate in determining the spatio-temporal complexity of the Ca^{2+} signal in a key region of the cell. Moreover, it has been reported that in the Golgi apparatus lumenal Ca^{2+} participates in controlling some key processes occurring in the organelle (post-translational modifications, protein sorting and trafficking, etc.) (Carnell and Moore, 1994; Austin and Shields, 1996; Duncan and Burgoyne, 1996), and thus dynamic variations of the $[Ca^{2+}]$ within the Golgi ($[Ca^{2+}]_{Golgi}$) could affect cell functions in a variety of ways.

To address this issue directly, we extended to this compartment the targeted aequorin approach which we have developed in the past and which proved useful for studying the Ca^{2+} handling of other intracellular compartments, such as the mitochondria (Rizzuto *et al.*, 1992), nucleus (Brini *et al.*, 1993), ER (Montero *et al.*, 1995), sarcoplasmic reticulum (Brini *et al.*, 1997) and subplasmalemmal space (Marsault *et al.*, 1997). We constructed a chimeric cDNA encoding a fusion protein including HA1-tagged aequorin (Brini *et al.*, 1995) and the transmembrane portion of sialyltransferase (ST), a resident protein of the lumen of the *trans*-Golgi and *trans*-Golgi network (TGN). This protein was used previously for studying protein sorting to the Golgi apparatus, and it was shown that the 17 amino acid membrane-spanning domain is responsible for the retention within the Golgi apparatus (Masibay *et al.*, 1993). This novel aequorin chimera, designated GoAEQ, is sorted selectively and provides novel, unexpected information on the Ca^{2+} handling by this organelle. Indeed, with this tool we demonstrate that a high $[Ca^{2+}]$ is maintained in the Golgi lumen in steady state due to the combined activity of a typical ER Ca^{2+} ATPase (SERCA) and of another Ca^{2+} pump, with distinct pharmacological properties. Furthermore, the Ca^{2+} content of the Golgi can be discharged rapidly and extensively following stimulation with an IP3-generating agonist.

Results

Subcellular localization of the GoAEQ chimera and reconstitution of the functional chemiluminescent protein

Figure 1A shows the schematic map of the GoAEQ chimera, which includes, from the N- to the C-terminus, a leader sequence, amino acids 1–69 of ST, the HA1 epitope (Field *et al.*, 1988) and aequorin (Inouye *et al.*, 1985). Two different GoAEQ constructs were prepared, including either wild-type aequorin or an aequorin mutant (Asp119→Ala) endowed with lower Ca^{2+} affinity (Kendall *et al.*, 1992), which were designated GoAEQwt and GoAEQmut, respectively. Figure 1B shows the immunocytochemical localization of GoAEQmut expressed transiently in HeLa cells, and revealed with the 12CA5

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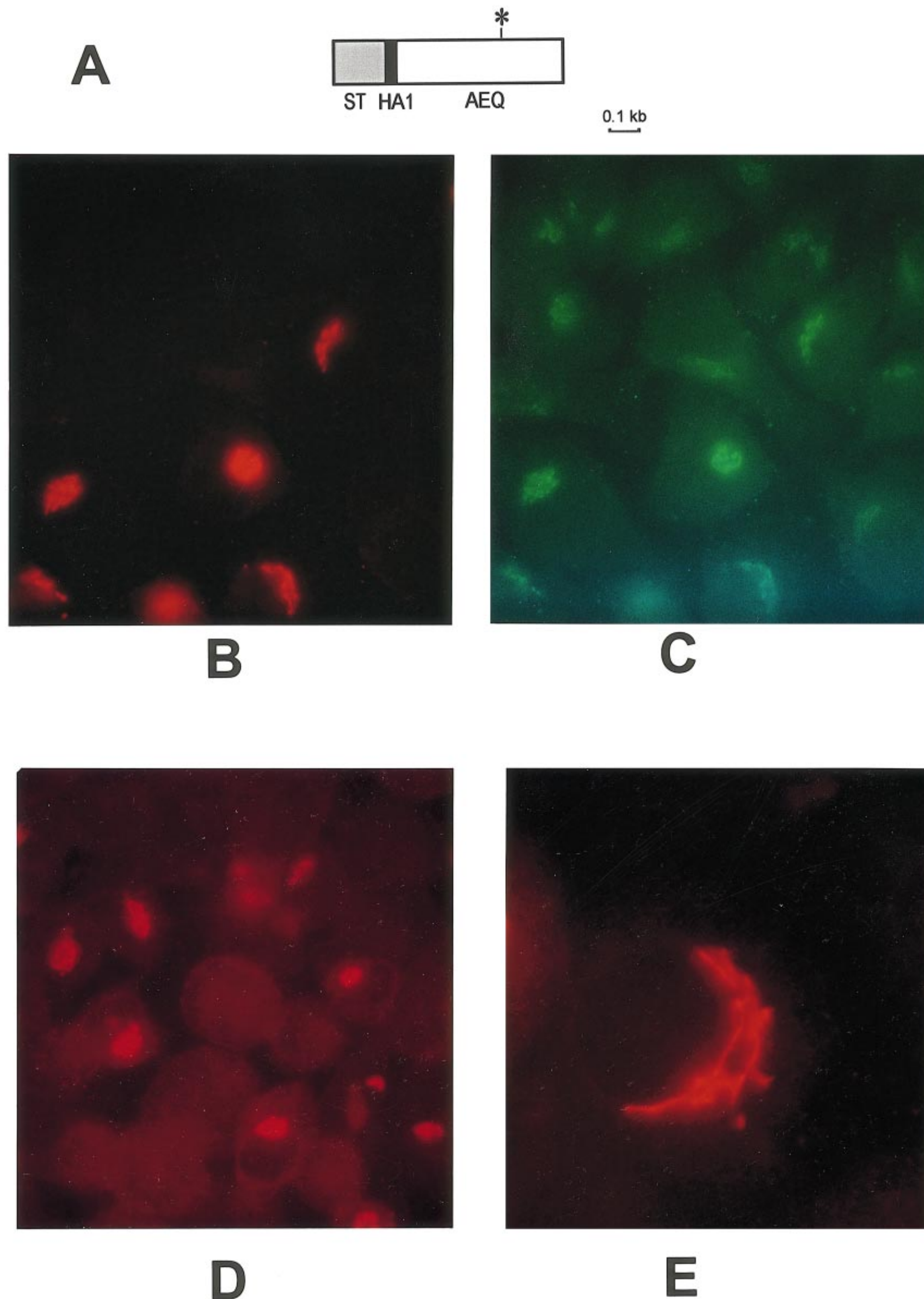

Fig. 1. (**A**) Schematic map of the GoAEQ cDNA. The chimeric cDNA was constructed by fusing in-frame the cDNAs encoding sialyltransferase (ST) and HA1-tagged aequorin (AEQ), as described in detail in Materials and methods. The portions encoding ST, the HA1 epitope and aequorin are hatched, black and white, respectively. An asterisk indicates the position of the Asp119→Ala mutation of the aequorin cDNA in the GoAEQmut (see text). (**B** and **C**) Immunolocalization of GoAEQmut and the Golgi apparatus marker α-D-mannosidase II in transiently transfected HeLa cells. Double staining with monoclonal anti-HA1 (B) and polyclonal anti-α-D-mannosidase II (C) antibodies, revealed by tetramethylrhodamine (TRITC)-conjugated and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, respectively. (**D** and **E**) Immunolocalization of the GoAEQmut after the Ca^{2+} depletion/aequorin reconstitution protocol (see text). The images in (D) and (E), which refer to transfected HeLa cells stained with a monoclonal anti-HA1 antibody and a TRITC-conjugated secondary antibody, were taken with a 63× and a 100× objective, respectively.

monoclonal antibody (recognizing the HA1 tag) and a tetramethylrhodamine isothiocyanate (TRITC) secondary antibody. A perinuclear convoluted structure was clearly revealed, while no significant difference (as shown by quantitative analysis of the fluorescence signal) was observed between non-transfected cells and non-Golgi regions of transfected cells, except for a small number ($<$5%) of cells in which the staining was more diffuse. The same results were obtained with GoAEQwt (data not shown). In order to confirm the Golgi localization of the expressed chimeric photoprotein, the cells were double labelled with an anti-α-D-mannosidase II (MAN) primary antibody and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The immunocytochemical staining pattern of GoAEQ and MAN shows an extensive overlap (Figure 1B and C). This result is not surprising given (i) the wide overlap of endogenous ST (from which the targeting sequence of GoAEQ is derived) and MAN, and (ii) the expected broader distribution of an over-expressed protein. Overall, these data confirm the correct sorting of the novel aequorin chimera and suggest that GoAEQ reports an average of the [Ca^{2+}] changes occurring in the different Golgi subcompartments.

We have shown previously that reconstitution with coelenterazine (to produce the functional Ca^{2+}-sensitive luminescent protein) is greatly dependent on the environment in which aequorin is located. In particular, when the recombinantly expressed protein is located in a compartment, such as the ER, endowed with a high Ca^{2+} concentration, reconstitution with coelenterazine is highly inefficient due to competition between consumption and reconstitution (Montero *et al.*, 1995). A similar situation applies also to the GoAEQ chimeras. Indeed, when reconstitution was carried out in Ca^{2+}-free medium after depleting the intracellular compartments of Ca^{2+} with the ionophore ionomycin, the light output of a transfected coverslip was $>$20-fold higher ($1.4 \pm 0.2\times10^6$ versus $4.8 \pm 0.8\times10^4$, $n = 5$), similarly to that reported previously for the ER ($11 \pm 3\times10^6$ versus $35 \pm 9\times10^4$, $n = 5$) (Montero *et al.*, 1995). Altogether, these data suggest that the lumenal Ca^{2+} concentration in the Golgi is similar to that of the ER, and highlight the importance of depleting the cells of Ca^{2+} prior to reconstitution in all the following experiments.

In the case of the ER-targeted aequorin, we demonstrated that the depletion protocol had no appreciable effect on the morphology and lumenal continuity of the organelle (Montero *et al.*, 1995, 1997a). On the contrary, it is known (and this was verified in a series of preliminary experiments) that the simple incubation of the cells in Ca^{2+}-free medium often results in a substantial modification of the Golgi structure, with fragmentation of the cisternae. In order to avoid this phenomenon, we devised a modification of the depletion protocol where the entire depletion and reconstitution period was at 4°C. The immunocytochemical analysis of transfected GoAEQ in these conditions (Figure 1D and E) shows that cells that have been treated with the depletion protocol, but at 4°C, maintained an intact Golgi morphology. In addition, we also employed another recombinant protein in which the aequorin cDNA was substituted with that encoding the S65T mutant of green fluorescent protein (GFP). In this case, the morphological appearance of the Golgi could be monitored before and after the depletion protocol, in the same living cells. Using this approach, we could confirm that, while the depletion protocol carried out at 37°C results in a substantial rearrangement of the Golgi structure, when the same experiment was carried out at 4°C, the morphology of the Golgi was unmodified (not shown). Last but not least, when Ca^{2+}-depleted cells at 4°C were rewarmed at 37°C and Ca^{2+} (or Sr^{2+}) rapidly readded to the medium, the Golgi appearance was not modified for at least the successive 30 min (not shown).

A final series of controls was carried out to ensure that the depletion protocol and the incubation at low temperature do not alter the basic Ca^{2+} homeostatic mechanisms. In particular, cells were transfected with aequorin constructs targeted to the mitochondrial matrix and to the cytosol and were subjected to the Ca^{2+} depletion protocol described above. After allowing a few minutes for refilling of the stores in Ca^{2+}-containing medium, the response of the cells to histamine was indistinguishable from that of controls (not shown).

[Ca^{2+}] in the Golgi

In all the following experiments, GoAEQmut reconstitution was thus carried out after depleting the organelle of Ca^{2+} with 5 μM ionomycin. After a 1 h incubation with 5 μM coelenterazine at 4°C, the cells were washed extensively with 2% bovine serum albumin (BSA) in Ca^{2+}-free medium (KRB supplemented with 1 mM EGTA), transferred to the luminometer chamber and perfused with medium at 37°C. When Ca^{2+} was added back to the perfusion buffer, a major increase was observed in light output, which consumed $>$90% of the photoprotein content in ~1–2 min (not shown). This result, while confirming the high resting [Ca^{2+}] within the Golgi, indicated that, as for the ER, procedures that reduce the rate of aequorin consumption must be employed in order to obtain an accurate estimate of the $[Ca^{2+}]_{Golgi}$. This can be obtained by two approaches: (i) substituting Ca^{2+} with Sr^{2+}, a Ca^{2+} surrogate which causes a much slower rate of photoprotein discharge (Montero *et al.*, 1995); or (ii) using the coelenterazine analogue, coelenterazine n (Barrero *et al.*, 1997).

In the experiment presented in Figure 2A and B, parallel batches of HeLa cells, transfected with either GoAEQmut or erAEQmut, were perfused with 1 mM $SrCl_2$. As previously observed (Montero *et al.*, 1995), due to the lower affinity of aequorin for Sr^{2+} than for Ca^{2+}, the rate of photon emission was much slower than upon Ca^{2+} addition (not shown). The conversion of aequorin luminescence values into [Sr^{2+}] (Figure 2A and B) revealed a qualitatively similar behaviour in the two compartments. Indeed, in the ER, a lumenal [Sr^{2+}] of ~2.5 mM (±0.3, $n = 6$) is reached in ~2 min, while in the Golgi [Sr^{2+}] increases, in the same time frame, to a value of ~1.4 mM (±0.2, $n = 6$) (Figure 2A). Given the relatively slow rate of aequorin consumption at these Sr^{2+} concentrations, the experiment could be continued for at least another 15 min (not shown).

In order to obtain a direct recording of $[Ca^{2+}]_{Golgi}$, we utilized the synthetic coelenterazine analogue, coelenterazine n, as recently described by Montero and co-workers (Barrero *et al.*, 1997). In these experiments, HeLa cells transfected with GoAEQmut were depleted of Ca^{2+} as

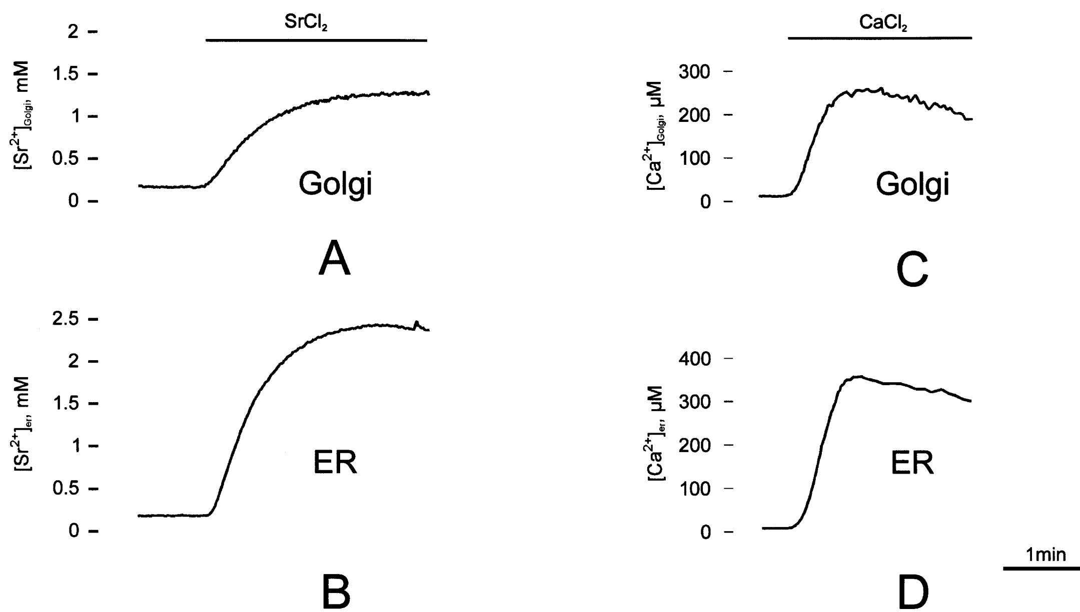

Fig. 2. Kinetics of $[cation^{2+}]_{Golgi}$ and $[cation^{2+}]_{er}$ increase upon readdition of $SrCl_2$ or $CaCl_2$ to Ca^{2+}-depleted cells. The experiments shown in this figure and in Figure 3 were performed utilizing HeLa cells, transiently expressing either GoAEQmut (**A** and **C**) or erAEQmut (**B** and **D**). The cells were depleted of Ca^{2+} by incubating them with 5 μM ionomycin in modified Krebs–Ringer buffer supplemented with 600 μM EGTA, then aequorin reconstitution with 5 μM coelenterazine (or coelenterazine n in the experiments with Ca^{2+}) was carried out in the same medium for 1 h at 4°C. After extensive washing with KRB supplemented with 2% BSA and 1 mM EGTA, the coverslip with the cells was placed in the thermostatted chamber of the luminometer and perfused with KRB, supplemented with 0.1 mM EGTA (KRB/EGTA). Where indicated, the EGTA was replaced with either 1 mM $SrCl_2$ (A and B) or 1 mM $CaCl_2$ (C and D). At the end of the experiment, the cells were lysed in a Ca^{2+}-rich hypotonic medium and the luminescence data were converted into $[Sr^{2+}]$ or $[Ca^{2+}]$ values, based on the Sr^{2+} or Ca^{2+} response curve of AEQmut. These data and those in the following figures are typical of at least five independent experiments, which gave the same results.

described above, and then incubated with 5 μM coelenterazine n. When the EGTA of the perfusion buffer was replaced with 1 mM $CaCl_2$, $[Ca^{2+}]_{Golgi}$ rapidly increased up to a concentration of ~0.3 mM (Figure 2C). For comparison, the kinetics of $[Ca^{2+}]_{er}$ are reported in Figure 2D. Qualitatively, the behaviour of $[Ca^{2+}]$ in the ER was remarkably similar to that of the Golgi, except for the maximum level eventually observed which was on average ~30% higher in the ER (440 ± 50 μM versus 310 ± 46 μM, $n = 5$). It should be noted that after consumption of >80% total aequorin, an apparent slow decrease of $[Ca^{2+}]$ is observed both in the ER and in the Golgi. As extensively discussed by Montero *et al.* (1997), this decrease is an artefact of the calibration, due to a minor fraction of the aequorin chimeras (~1–2%) retained in compartments at low Ca^{2+}.

Different Ca^{2+} accumulation pathways co-exist in the Golgi

In the following experiment, we investigated the type and properties of the pathway leading to Ca^{2+} accumulation in the Golgi. At first, we verified whether Ca^{2+} uptake could be attributed to the Ca^{2+} ATPase of the ER (SERCA) by testing the effect of the specific inhibitor thapsigargin (TG) (Thastrup *et al.*, 1990). For this purpose, at the end of the reconstitution step the cells were incubated with 1 μM TG. Although the effect of TG is known to be practically irreversible, the perfusion medium was also supplemented with 10 μM 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) (Kass *et al.*, 1989), another SERCA blocker which in our experience, unlike TG, can be perfused efficiently through plastic tubing. Under those conditions, a substantial increase in cytosolic $[Ca^{2+}]$ was observed upon Ca^{2+} readdition to the perfusion medium, which was larger than in untreated cells and slowly declined toward basal values (Figure 3A). As regards the Golgi, in TG-treated cells, the initial rate of $[Ca^{2+}]_{Golgi}$ increase following addition of 1 mM $CaCl_2$ to the perfusion medium was reduced to ~50% of the control value (Figure 3B). Under the same conditions, the initial rate of ER Ca^{2+} accumulation was reduced to 5% of controls (Figure 3C). The total aequorin consumption in GoAEQmut transfected cells in 5 min was 70% (64 ± 12%, $n = 7$) in controls and 35% (38 ± 9%, $n = 7$) in TG + tBuBHQ-treated cells. In cells expressing erAEQmut, the values were ~90% (87 ± 16%, $n = 7$) and 5% (7 ± 3%, $n = 7$), respectively. Qualitatively similar data were obtained when Sr^{2+} was used as the accumulated cation. It is interesting that (see also below) in the presence of TG after the peak (90 ± 22 μM, $n = 6$) $[Ca^{2+}]_{Golgi}$ declined to much lower values, eventually reaching a plateau hardly distinguishable from that of cells not refilled with Ca^{2+}. Taken together, the above described data indicate that in the Golgi a substantial part of the organelle can accumulate Ca^{2+} (or Sr^{2+}) by a mechanism independent of the SERCAs, while this fraction is much smaller in the ER. It is difficult to derive the relative contribution of the two accumulation pathways directly from an experiment such as that of Figure 3, given that, upon SERCA inhibition with TG, the Golgi is exposed transiently to higher cytosolic $[Ca^{2+}]$ than in control cells (Figure 3A; see Discussion).

The nature of this alternative mechanism of $cation^{2+}$ accumulation was next investigated. Figure 3D and E rules out the possibility that it is a H^+/Ca^{2+} exchanger, driven by the lower pH which is known to be maintained

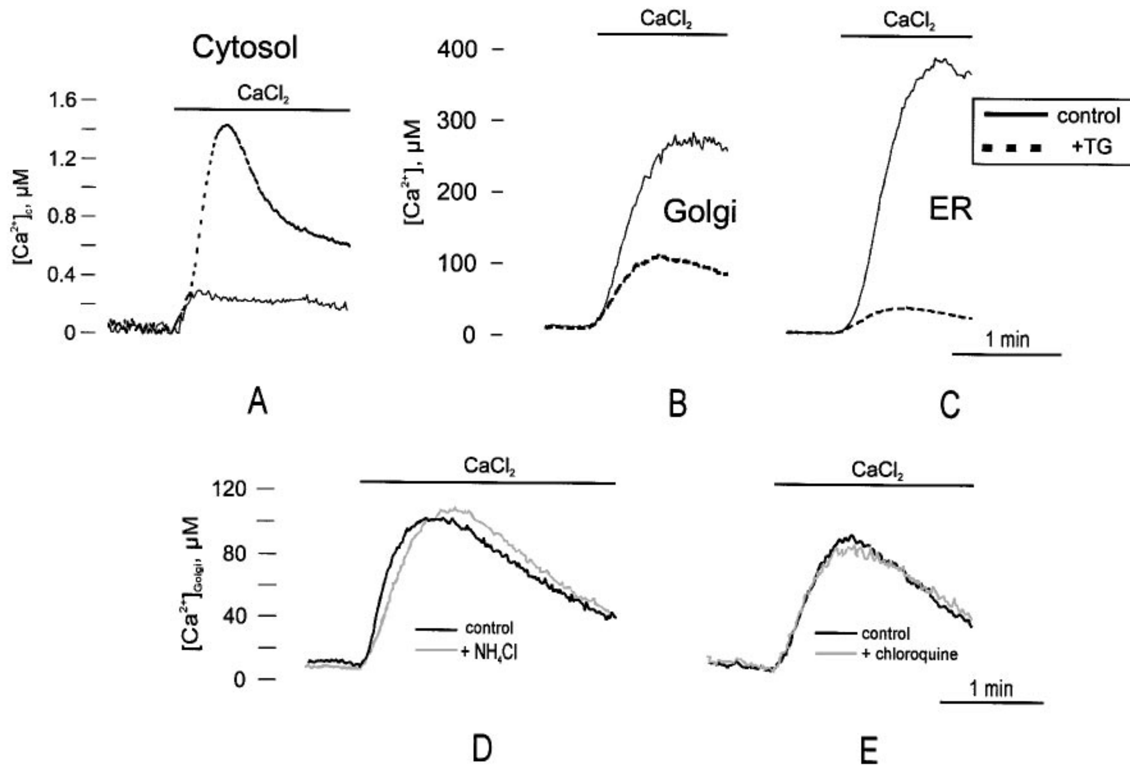

Fig. 3. (**A**–**C**) The effect of SERCA inhibition on the Ca^{2+} refilling of the cytosol, Golgi apparatus and the ER. After Ca^{2+} depletion and aequorin reconstitution (carried out as in the experiment of Figure 2), the cells were transferred to the luminometer chamber and perfused with KRB/EGTA. Where indicated, the EGTA in the KRB was replaced with 1 mM $CaCl_2$. In the dotted traces, the cells were incubated with TG in the final 5 min of reconstitution, and the perfusion medium was supplemented with tBuBHQ. In the continuous trace, TG and tBuBHQ were omitted. (**D** and **E**) The effect of NH_4Cl and chloroquine on the TG-insensitive Ca^{2+} uptake in the Golgi apparatus. All conditions, including SERCA inhibition with TG and tBuBHQ, were as in (B). In the appropriately labelled traces, 1 min prior to Ca^{2+} readdition the cells were incubated with either NH_4Cl or chloroquine, which was present in the perfusion medium throughout the experiment.

in the TGN (Kim *et al*., 1996). Indeed, the TG-independent Ca^{2+} accumulation was not affected by pre-treatment with agents such as NH_4Cl (Figure 3D) or chloroquine (Figure 3E) that collapse the H^+ gradient across cell membranes.

By using vanadate, a wide spectrum inhibitor of Ca^{2+} ATPases, we then verified whether the TG-insensitive Ca^{2+} uptake is due to an alternative pump. In order to avoid problems of equilibration of vanadate across the plasma membrane (and other side effects of this drug), this experiment was carried out in cells permeabilized with digitonin (Figure 4). Thus, after the aequorin reconstitution and TG treatment steps (carried out as above), the cells were transferred to the luminometer and permeabilized via a 1 min incubation with 100 µM digitonin added to a medium mimicking intracellular ion composition [intracellular buffer, IB (140 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM HEPES, pH 7.05 at 37°C)], supplemented with 100 µM EGTA (IB/EGTA). The perfusion medium was then changed to IB, with no added EGTA (thus containing ~5 µM free Ca^{2+}), in the presence of different vanadate concentrations. The initial rate of Ca^{2+} accumulation under these conditions was 70% inhibited by ~50 µM vanadate (Figure 4), while little or no effect was observed at concentrations of 10 µM, i.e. those sufficient to inhibit the Ca^{2+} ATPase of the plasma membrane (Caroni and Carafoli, 1981; Carafoli, 1991; Michelangeli *et al*., 1991; Dean and Quinton, 1995).

If different accumulation pathways co-exist in the Golgi

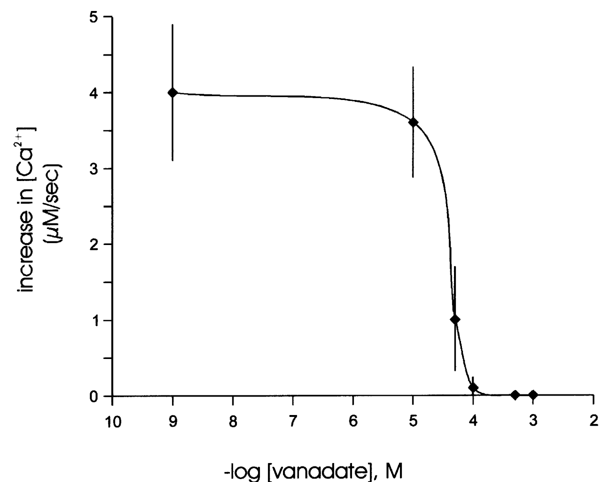

Fig. 4. Effect of vanadate on the thapsigargin-insensitive Ca^{2+} uptake of the Golgi apparatus. The plot refers to the rates of $[Ca^{2+}]_{Golgi}$ increase in permeabilized cells exposed to a buffered $[Ca^{2+}]$ and different vanadate concentrations. In all cases, Ca^{2+} depletion and GoAEQmut reconstitution was carried out as described in Figure 2, then the cells were transferred to the luminometer chamber and perfused with KRB/EGTA. The cells were then permeabilized via a 1 min incubation with 100 µM digitonin, added to an intracellular buffer, IB; after permeabilization, the perfusion medium was supplemented with the different vanadate concentrations and 5 µM $CaCl_2$.

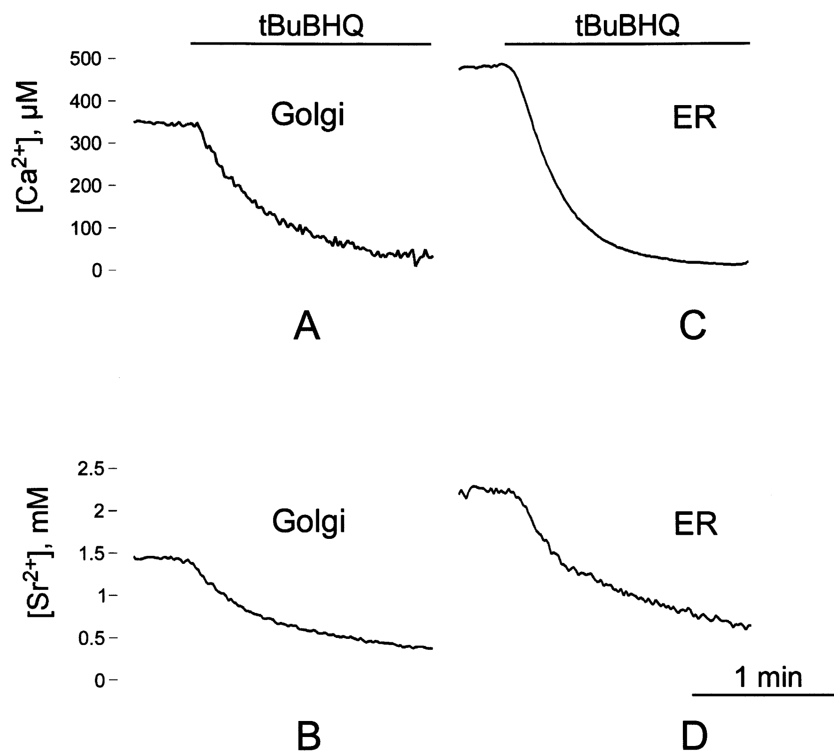

Fig. 5. Effect of tBuBHQ on the steady-state $[Sr^{2+}]$ and $[Ca^{2+}]_{er}$ values of the Golgi apparatus and the ER. Ca^{2+} depletion, aequorin reconstitution and refilling with either Ca^{2+} (**A** and **C**) or Sr^{2+} (**B** and **D**) were carried out as in Figure 2. Where indicated, the cells were challenged with 10 µM tBuBHQ.

apparatus, the question then arises as to whether they are located in separate, non-communicating subcompartments of the organelle or whether they are largely intermingled. To address this issue, GoAEQmut was reconstituted as described above, then the Golgi was refilled with either 1 mM Ca^{2+} (Figure 5A) or Sr^{2+} (Figure 5B). For comparison, the ER data are also shown (Figure 5C and D). After refilling, the cells were treated with 10 µM tBuBHQ which caused, with both Ca^{2+} and Sr^{2+}, a nearly complete release of the cations from the organelle, similarly to that observed in the ER. This result is in apparent contradiction to the results indicating that two accumulation mechanisms co-exist in the Golgi (Figure 3B). The discrepancy between the two experiments is, however, only apparent, since in the data shown in Figures 3 and 4 in the presence of TG, the increase in $[Ca^{2+}]_{Golgi}$ is transient and, at steady state, the mean $[Ca^{2+}]_{Golgi}$ is very low (<10 µM) (see Discussion).

Transfected calreticulin is not a major Ca^{2+} buffer in the Golgi apparatus

Calreticulin is a major Ca^{2+}-buffering protein in the ER (Bastianutto *et al.*, 1995; Mery *et al.*, 1996; Krause and Michalak, 1997) and, despite the fact that it possesses an ER retention signal, its glycosylation pattern indicates that it can progress up to the TGN (Van *et al.*, 1989). We thus investigated whether this protein could participate in Ca^{2+} buffering also in the Golgi apparatus. HeLa cells were co-transfected with calreticulin, using the previously described tagged calreticulin construct, tCR (Bastianutto *et al.*, 1995), and either erAEQmut or GoAEQmut. Ca^{2+} measurements were then carried out as above (Ca^{2+} depletion and aequorin reconstitution in Ca^{2+}-free medium). Figure 6 shows a comparison of the rate of ER or Golgi refilling in control and tCR-transfected cells. tCR expression reduced the rate of Ca^{2+} accumulation in the ER (6.7 ± 2.6 µM/s versus 15.2 ± 3.3 µM/s, $n = 5$), as expected for a protein playing a major buffering role in the ER lumen. Conversely, no major difference between tCR-transfected and control cells was observed in the rate of Golgi refilling (7.1 ± 2.6 µM/s versus 9.2 ± 2.8 µM/s, $n = 5$), thus (i) confirming that GoAEQmut is well sorted and does not report the $[Ca^{2+}]_{er}$ changes and (ii) indicating that calreticulin plays a minor, if any, role in buffering $[Ca^{2+}]_{Golgi}$. It should be noted that while in the case of $[Ca^{2+}]_{Golgi}$ the same steady-state plateau value was reached (303 ± 21 µM versus 320 ± 15 µM, $n = 5$), a lower steady-state $[Ca^{2+}]_{er}$ value appears to be reached in tCR-transfected cells (340 ± 27 µM versus 420 ± 33 µM, $n = 5$). This interesting observation, which involves ER Ca^{2+} handling and thus is outside the aims of the present study, will be investigated further. It should be remembered, however, that in tCR-co-transfected cells, the steady state is reached when the overall aequorin consumption exceeds 80%, i.e. in conditions in which the calibration of the aequorin signal becomes cumbersome (Montero *et al.*, 1997a).

The Golgi is part of the IP3-sensitive Ca^{2+} pools

A final question concerns the role of the Golgi in the Ca^{2+} mobilization triggered by IP3 production and, possibly even more important, the effect of IP3 on $[Ca^{2+}]_{Golgi}$. Figure 7 shows that addition of histamine, an agonist coupled to IP3 production, caused a rapid and extensive drop in the $[Sr^{2+}]$ (Figure 7A) or $[Ca^{2+}]$ (Figure 7C) of the Golgi apparatus. The amplitude and rate of the

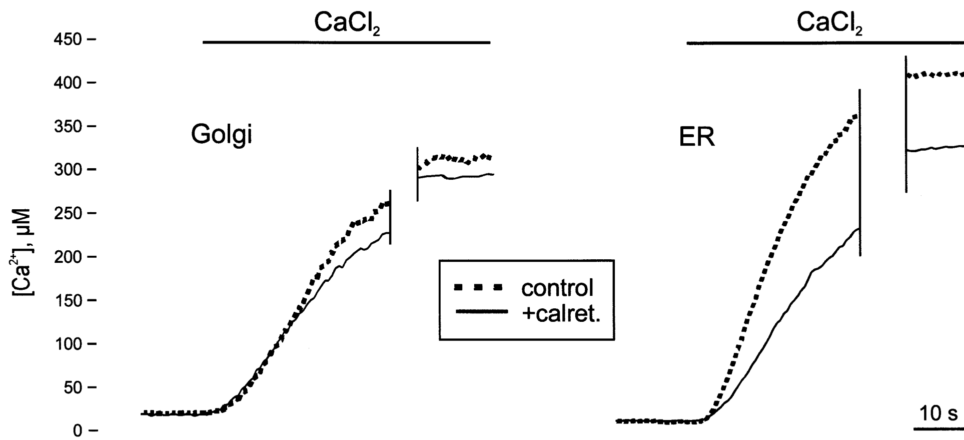

Fig. 6. Effects of calreticulin overexpression on the rates of Ca^{2+} refilling in the Golgi apparatus and the ER. The figure shows the Ca^{2+} refilling in the Golgi apparatus and the ER of cells expressing either the appropriately targeted aequorin chimera and exogenous calreticulin, tCR (continuous line), or only the aequorin chimera (dotted line). Ca^{2+} depletion and aequorin reconstitution were carried out as in Figure 2. Where indicated, the EGTA in the perfusion medium was replaced with 1 mM $CaCl_2$. Bars denote a 1 min interruption in the trace recording.

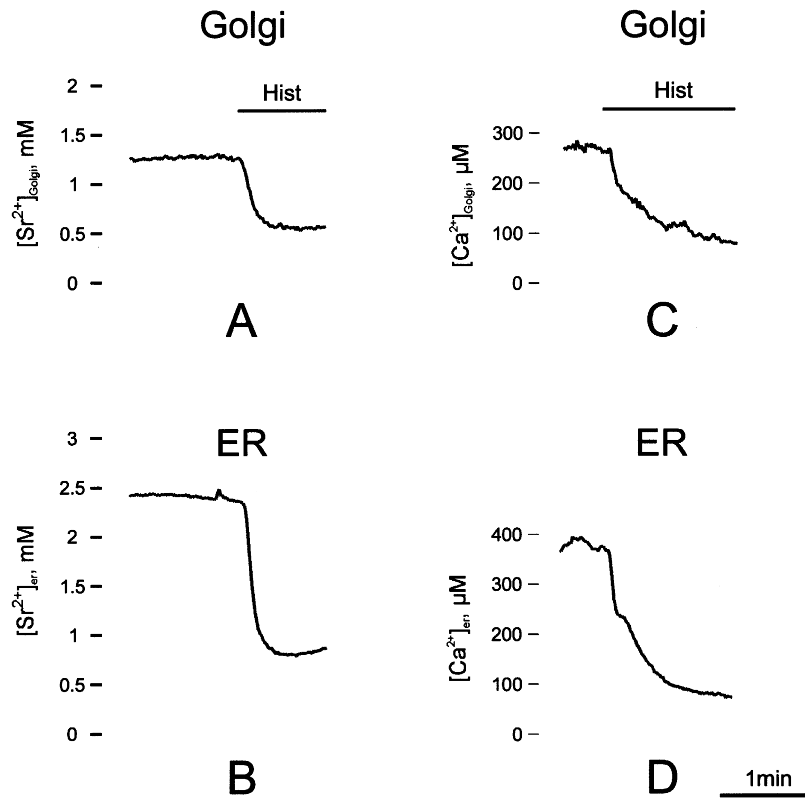

Fig. 7. The effect of the IP3-generating agonist on the $[Sr^{2+}]$ or $[Ca^{2+}]$ of the Golgi apparatus (**A** and **B**) and the ER (**C** and **D**). The $[cation^{2+}]$ of the two compartments was monitored in parallel batches of cells transfected with either GoAEQmut or erAEQmut. Ca^{2+} depletion, aequorin reconstitution and Ca^{2+} (or Sr^{2+}) refilling were carried out as in Figure 2; after a steady-state $[Ca^{2+}]$ or $[Sr^{2+}]$ was reached (as observed in Figure 2), the cells were challenged, where indicated, with 100 μM histamine.

$[cation^{2+}]$ drop caused by histamine in the Golgi is slightly smaller and slower than that observed in the ER (Figure 7B and D). It is of interest that no effect of histamine on $[Ca^{2+}]_{Golgi}$ was observed when the agonist was added to cells pre-treated with TG + tBuBHQ (not shown). In conclusion, the data show unambiguously that upon stimulation of a receptor coupled to IP3 production, not only does the Golgi contribute part of the released Ca^{2+}, but there are also major changes in $[Ca^{2+}]_{Golgi}$, with possible implications for the function of this organelle.

Neither Ca^{2+} uptake nor Ca^{2+} release in the Golgi are directly dependent on vesicular traffic

We then investigated whether the steady-state values of $[Ca^{2+}]_{Golgi}$, as well as the rapid decrease following stimulation with IP3-generating agonists, depended, at least in part, on the flow of vesicles between different Golgi subdomains and/or between the Golgi apparatus and the ER. To this end, we verified the effect of GTPγS, an inhibitor of vesicular traffic, on Ca^{2+} uptake and Ca^{2+} release in the Golgi. In these experiments, the cells were

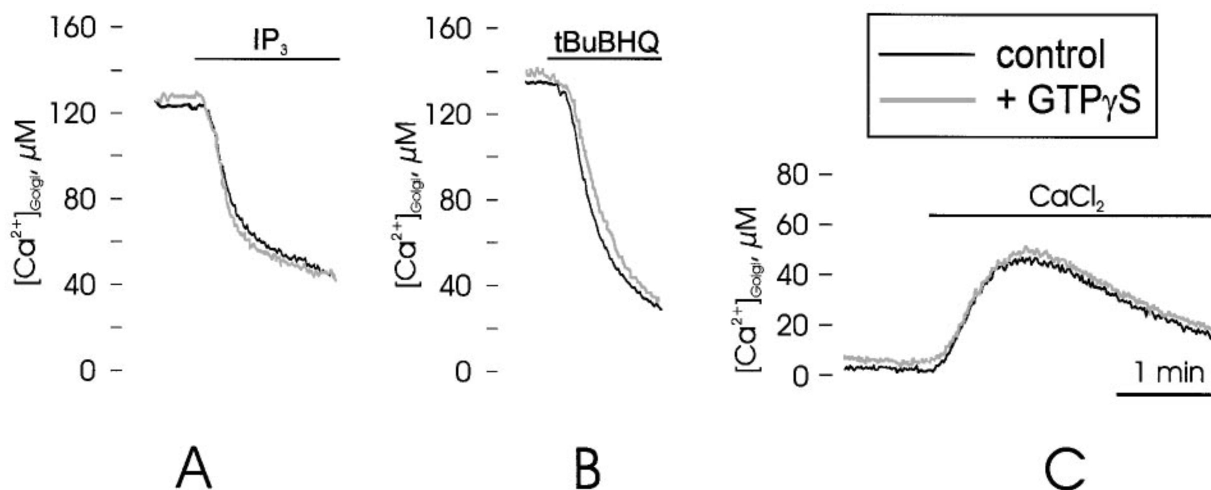

Fig. 8. Effect of GTPγS on Ca^{2+} release from the Golgi apparatus induced either by administering 5 μM IP_3 (**A**) or by blocking the SERCAs with 10 μM tBuBHQ (**B**), and on Ca^{2+} uptake in TG-treated cells (**C**). In all cases, Ca^{2+} depletion and GoAEQmut reconstitution were carried out as described in Figure 2. The cells were then permeabilized as described in Figure 4. After permeabilization, the perfusion medium was supplemented with 0.5 μM $CaCl_2$. In the appropriately labelled traces, 1 min prior to Ca^{2+} readdition the cells were incubated with 100 μM GTPγS.

first permeabilized via a 1 min incubation with digitonin (added to IB/EGTA), then Ca^{2+} uptake was initiated by perfusing an EGTA-buffered Ca^{2+} concentration of ~0.5 μM (IB/Ca^{2+}). A steady state was reached, which was lower that in intact cells (132 ± 16 μM, $n = 5$) and was unaffected by treating the cells with GTPγS (Figure 8A and B). Ca^{2+} release was then induced either by administering 5 μM IP3 (Figure 8A) or by blocking the SERCAs with 10 μM tBUBHQ (Figure 8B). In both cases, the kinetics and the amplitude of the $[Ca^{2+}]_{Golgi}$ decrease were the same in GTPγS-treated and control cells. We also verified that in intact cells the uptake of Ca^{2+} in the Golgi and the release induced by histamine were both insensitive to an acute treatment with brefeldin A (not shown). Finally, we tested whether vesicular traffic was involved in the rapid decline of the $[Ca^{2+}]_{Golgi}$ attained by TG-treated cells (Figure 3). In these experiments, TG-treated cells were permeabilized with digitonin in IB/EGTA (as above) then perfused with IB/Ca^{2+}. A biphasic rise was observed, which was unaffected by the presence of GTPγS in the buffer (Figure 8C)

Discussion

In the complex scenario of Ca^{2+} signalling, the role of the Golgi apparatus remains elusive, although several lines of evidence suggest that it may play a role in intracellular Ca^{2+} homeostasis. First, the total calcium content of the Golgi is high (>10 mmol/l) (Pezzati *et al*., 1997), which suggests that free Ca^{2+} may also be high, and thus the Golgi may be part of the intracellular stores of agonist-releasable Ca^{2+}. Secondly, *Saccharomyces cerevisiae*, a lower eukaryote which lacks SERCA-type ATPases, is endowed with an alternative pump located in the Golgi, PMR1 (Antebi and Fink, 1992; Cunningham and Fink, 1994); based on this observation, the Golgi apparatus has been proposed to be an evolutionarily 'ancient' Ca^{2+} store. Thirdly, accumulation and release of Ca^{2+} from the secretory vesicles has been demonstrated in exocrine pancreas and has been proposed to play a major role in the control of agonist-dependent granule discharge (Gerasimenko *et al*., 1996; Petersen, 1996). Although the inclusion of the secretory granules in the IP3-sensitive Ca^{2+} pool is still controversial, a logical and intriguing consequence is that the concept of a Ca^{2+} store could be extended to the whole endomembrane system, with distinct molecular and functional properties in the various portions (ER, Golgi, granules). Finally, it has been demonstrated that several processes which occur in the Golgi lumen are Ca^{2+}-dependent, thus suggesting that lumenal Ca^{2+} concentration, and physiological variations therein, may be key controllers of the functions of this organelle (Carnell and Moore, 1994; Austin and Shields, 1996; Duncan and Burgoyne, 1996). The direct measurement of $[Ca^{2+}]$ in the Golgi lumen allows us to address these issues, but so far this has been hampered by the lack of specific probes. We have reported here the construction of a chimera of the Ca^{2+}-sensitive photoprotein aequorin specifically targeted to the Golgi lumen (GoAEQ), and the first results obtained with this probe on Ca^{2+} homeostasis within this organelle.

Using GoAEQ, we could show that the concentration in the Golgi lumen ($[Ca^{2+}]_{Golgi}$) is high, comparable with that of the ER. Indirect evidence was first obtained from the reconstitution data, which revealed that reconstitution of GoAEQ was efficient only if the organelle previously was depleted of Ca^{2+}, similar to that reported for the ER. The direct monitoring of the $[Ca^{2+}]_{Golgi}$ after organelle refilling confirmed this observation and allowed an estimate of the steady-state values. Two different approaches were employed to reduce the rate of photoprotein consumption and thus obtain a reliable conversion of the luminescence signal into $[Ca^{2+}]$: the replacement of Ca^{2+} with a surrogate cation, Sr^{2+} (Montero *et al*., 1995), and the use of a modified prosthetic group (coelenterazine n) (Barrero *et al*., 1997; Montero *et al*., 1997b). The two approaches, while both demonstrating that, in resting cells, a high lumenal Ca^{2+} concentration is maintained in the Golgi apparatus, showed that Sr^{2+} leads to a significant overestimation of the steady-state value (1.4 mM with Sr^{2+} versus 0.3 mM with Ca^{2+}/coelenterazine n). A similar quantitative discrepancy was

also observed for the ER (Figure 4; Barrero *et al*., 1997; Montero *et al*., 1997b). Both approaches, however, reveal a difference between the resting $[cation^{2+}]_{Golgi}$ and $[cation^{2+}]_{er}$. In fact, the resting $[Ca^{2+}]_{Golgi}$ and $[Ca^{2+}]_{er}$ were ~0.3 and ~0.4 mM, while $[Sr^{2+}]_{Golgi}$ and $[Sr^{2+}]_{er}$ were ~1.4 and ~2.5 mM, respectively.

The differences between the Golgi apparatus and the ER, however, were not limited to the steady-state values (which could be explained, for example, by a different number of active pumps, different leak mechanisms or both) but, rather, reflected a more radical difference in the mechanisms of Ca^{2+} accumulation in these organelles. Indeed, TG pre-treatment nearly abolished the refilling with Ca^{2+} of the ER in intact cells, while it had only a partial effect on the Golgi apparatus. These data indicate the existence in the Golgi of a Ca^{2+} accumulation mechanism(s) other than the classical SERCAs. As to the nature of this alternative mechanism, our data exclude that it is a H^+/Ca^{2+} exchanger (based on the lack of effect of agents which collapse the proton gradient across cell membranes, such as chloroquine and NH_4Cl). Rather, the abolition of the TG-insensitive accumulation by vanadate demonstrates that it occurs via a classical Ca^{2+} ATPase, acting via the formation of a phosphorylated intermediate. The relatively high concentrations of vanadate necessary for complete inhibition suggest that, rather than plasma membrane Ca^{2+} ATPase (PMCA) in transit through the Golgi apparatus, this activity may be due to a novel, as yet unidentified Ca^{2+} ATPase, which could be the homologue of yeast PMR1.

While TG pre-treatment only partly reduces Ca^{2+} uptake in the Golgi, the administration of the SERCA blocker at equilibrium (i.e. after the organelle has been allowed to refill) causes the nearly complete depletion of Golgi Ca^{2+}. Several non-mutually exclusive possibilities may account for this apparent discrepancy. We first consider the TG-sensitive and TG-insensitive Ca^{2+} accumulation. If the TG-insensitive uptake system is diffused throughout the Golgi, but is of low Ca^{2+} affinity, it would allow substantial accumulation in the Golgi only at high cytosolic $[Ca^{2+}]$, such as those induced by the readdition of Ca^{2+} to TG-treated cells. Alternatively, the data could reflect a heterogeneity in Ca^{2+} handling within the Golgi apparatus. In fact, if the two pumps are concentrated in different, non-rapidly communicating domains of the Golgi, the different rates of aequorin consumption in these compartments upon refilling could easily explain the data. Namely, the addition of the SERCA blocker would prevent Ca^{2+} accumulation in the SERCA-dependent domain, whereas the SERCA-independent domain would consume its aequorin content progressively and thus become gradually irrelevant in the calibration. Overall, in either case, the predicted kinetics of Ca^{2+} accumulation in the presence of TG would be biphasic and the calibrated mean $[Ca^{2+}]$ would approach that of depleted cells. A third possibility is the occurrence of rapid Ca^{2+} diffusion between the TG-sensitive and TG-insensitive compartments. In this case, Ca^{2+} would be taken up by the TG-insensitive compartment and lost by the TG-sensitive one. At steady state, the mean $[Ca^{2+}]_{Golgi}$ should be higher than that of depleted cells, but its value is difficult to predict, given that it should be dependent on the relative rates of Ca^{2+} uptake, leak and diffusion. The data obtained in permeabilized cells with GTPγS indicate that such a diffusion, if actually occurring, is not due to vesicular traffic. The difference between the three above-mentioned possibilities is that while in the first and third hypothesis the steady-state $[Ca^{2+}]_{Golgi}$ is very low in the presence of SERCA blockers, in the second the TG-insensitive compartment maintains a high Ca^{2+} level but, due to aequorin consumption, its high $[Ca^{2+}]$ is not detected by the average luminescence signal. A similar reasoning applies to the effect of SERCA inhibitors after the steady-state $[Ca^{2+}]_{Golgi}$ is reached. Whichever model one assumes, the prediction is that addition of the drugs will eventually decrease the mean $[Ca^{2+}]_{Golgi}$ to levels hardly distinguishable from those of Ca^{2+}-depleted cells.

Surprisingly, the Golgi apparatus shares with the ER not only the property of accumulating Ca^{2+}, but also that of rapidly releasing it upon agonist-dependent IP3 generation. Indeed, when HeLa cells are challenged with histamine, $[Ca^{2+}]_{Golgi}$ drops from 0.3 to 0.2 mM in $<$10 s. The discrepancy with immunohistochemistry data, which never revealed IP3 receptors in the Golgi (for a review see Pozzan *et al*., 1994), is probably only apparent. (i) The immunocytochemical data (at the electron microscopy level) on the subcellular distribution of the IP3 receptors are available only for very few cell types and in particular for the Purkinje neurons, a cell type where specific sorting mechanisms could be operative. (ii) Very low levels of IP3 receptor expression, below the level of detection of the presently available electron microscopy techniques, are sufficient to ensure the rapid release of Ca^{2+} from the ER (e.g. in the hepatocytes). (iii) A lower density of the IP3 receptors in the Golgi compared with the ER could account for the reduced speed of Ca^{2+} release (2- to 3-fold lower in the Golgi than in the ER). In this respect, however, it should be pointed out that the lumenal $[Ca^{2+}]$ (lower in the Golgi) strongly affects the ion flux rate through the IP3 receptor (Tanimura and Turner, 1996; P.Pinton, T.Pozzan and R.Rizzuto, unpublished data). (iv) The two organelles appear to have different lumenal Ca^{2+} buffers. Indeed, the results of Figure 8 show that the predominant buffer of the ER lumen, the low-affinity high-capacity binding protein calreticulin, has no major buffering role in the Golgi lumen, even when recombinantly overexpressed. Obviously, this does not exclude the possibility that other resident proteins may play a similar role. If, however, the Ca^{2+}-buffering capacity of the Golgi is markedly lower, a smaller number of IP3 receptors could be necessary to cause a rapid and large decrease of $[Ca^{2+}]_{Golgi}$.

Finally, the experiments performed with inhibitors of vesicular traffic, such as GTPγS, suggest that the intriguing properties of Ca^{2+} homeostasis in the Golgi do not depend on exchange of vesicles between Golgi domains or with the ER. In particular, neither the loading of Ca^{2+} into the organelle nor the rapid emptying in TG-treated cells (see above) is affected. On one hand, this implies that the Golgi apparatus is endowed with resident mechanisms for Ca^{2+} accumulation (and does not depend on the flow of Ca^{2+}-rich vesicles from the ER); on the other hand, if Golgi subdomains differ in Ca^{2+} handling, they either maintain distinct properties or equilibrate by different mechanisms (e.g. lumenal continuity). Along the same lines, the agonist-dependent decreases in $[Ca^{2+}]_{Golgi}$ cannot

be considered a consequence of vesicular traffic between the Golgi and the ER emptied of Ca^{2+} by the release via IP3 receptors, but rather must rely on the activity of resident IP3 receptors.

In any case, the demonstration of a rapid agonist-dependent Ca^{2+} discharge from the Golgi has profound physiological implications. On one hand, the diffusion of the Ca^{2+} signal to an unsuspected domain (the Golgi lumen) may further extend the mechanisms by which Ca^{2+}-mediated signals exert their action. It can be envisaged, for example, that rapid, Ca^{2+}-dependent changes in the processing and sorting pattern of plasma membrane proteins (receptors, channels, enzymes) may trigger long-term effects of agonist stimulation. On the other hand, the observation that an organelle with a defined, restricted location within the cells (in close proximity to the nucleus, the location of important targets of the Ca^{2+} signal) is a bona fide store of rapidly mobilizable Ca^{2+} may provide further insight into the notion of 'local' Ca^{2+} signalling and its role in the control of cell functions.

Materials and methods

Construction of the GoAEQ and GoGFP chimeras

The chimeric cDNAs were constructed as follows. The coding region of the ST cDNA was amplified by PCR using specifically modified primers, which caused the addition of a *Hin*dIII site in the 5′ non-coding region and downstream of codon 69. This latter site allowed the fusion in-frame of the ST sequence to the aequorin and GFP cDNAs available in the laboratory (see below). This *Hin*dIII fragment was then inserted, in appropriately prepared pBS+ vectors, in front of one of the following cDNAs: (i) the *Hin*dIII–*Eco*RI fragment encoding HA1-tagged aequorin cDNA (Brini *et al*., 1995); (ii) the HA1-tagged Asp119→Ala aequorin mutant (Montero *et al*., 1995); and (iii) the HA1-tagged S65T mutant of GFP (Rizzuto *et al*., 1996). In all cases, the final chimeric cDNA, GoAEQwt, GoAEQmut and GoGFP(S65T), respectively, was then excised via *Not*I–*Xho*I digestion and inserted into the mammalian expression vectors pcDNAI or pcDNA3 (Invitrogen).

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), in 75 cm^2 Falcon flasks; before transfection, cells were seeded onto 13 mm (for aequorin measurements) or 24 mm (for GFP detection) glass coverslips and allowed to grow to 50% confluence. At this stage, transfection with 4 or 8 μg of plasmid DNA, for 13 or 24 mm coverslips respectively, was carried out as previously described (Rizzuto *et al*., 1995) and aequorin measurements or immunocytochemistry were performed 36 h after transfection.

Aequorin measurements

As discussed in the Results, before the reconstitution, it is necessary to reduce the Ca^{2+} content of the Golgi apparatus and the ER drastically. To this end, the cells were incubated for 1 h at 4°C, in KRB (Krebs–Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37°C) supplemented with 5 μM coelenterazine, the Ca^{2+} ionophore ionomycin (5 μM) and 600 μM EGTA. After this incubation, the cells were washed extensively with KRB supplemented with 2% bovine serum albumin (BSA) and 1 mM EGTA. In the experiments, additions (1 mM $SrCl_2$, 1 mM $CaCl_2$, histamine, etc.) were made to the same medium, as specified in the figure legends. In the experiments with permeabilized cells, a buffer mimicking the cytosolic ionic composition, ('intracellular buffer', IB) was employed: 140 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM HEPES (pH 7.05 at 37°C). The 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 and calibrated into $[Ca^{2+}]$ values as previously described (Brini *et al*., 1995; Montero *et al*., 1995; Rizzuto *et al*., 1995; Barrero *et al*., 1997). In brief, a 13 mm round coverslip with the transfected cells was placed in a perfused, thermostatted chamber placed in close proximity to a low-noise photomultiplier, with built-in amplifier-discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated off-line into $[Ca^{2+}]$ values, using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins, as previously described (Brini *et al*., 1995; Montero *et al*., 1995).

Immunolocalization of the HA1-tagged recombinant aequorin

At 36 h after transfection, HeLa cells were processed for immunofluorescence as follows. The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 20 min, washed three times with PBS and then incubated for 10 min in PBS supplemented with 50 mM NH_4Cl. Permeabilization of cell membranes was obtained with a 5 min incubation with 0.1% Triton X-100 in PBS, followed by a 1 h wash with 1% gelatin (type IV, from calf skin) in PBS. The cells were then incubated for 1 h at 37°C in a wet chamber with a 1:200 dilution (in PBS) of the monoclonal antibody 12CA5, which recognizes the HA1 tag (Field *et al*., 1988) and, for the double immunolocalization, with a 1:1000 dilution of a rabbit polyclonal antibody raised against the mannosidase II (Novikoff *et al*., 1983). Staining was then carried out with FITC-labelled anti-rabbit and TRITC-labelled anti-mouse secondary antibodies. After each antibody incubation, the cells were washed four times with PBS. Fluorescence was then analysed with a Zeiss Axioplan microscope and photographed using Kodak Ektachrome 200 ASA film.

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