Heterogeneity of Ca\textsuperscript{2+} handling among and within Golgi compartments

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The Golgi apparatus (GA) is a dynamic intracellular Ca\textsuperscript{2+} store endowed with complex Ca\textsuperscript{2+} homeostatic mechanisms in part distinct from those of the endoplasmic reticulum (ER). We describe the generation of a novel fluorescent Ca\textsuperscript{2+} probe selectively targeted to the medial-Golgi. We demonstrate that in the medial-Golgi: (i) Ca\textsuperscript{2+} accumulation takes advantage of two distinct pumps, the sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase and the secretory pathway Ca\textsuperscript{2+} ATPase; (ii) activation of IP\textsubscript{3} or ryanodine receptors causes Ca\textsuperscript{2+} release, while no functional two-pore channel was found; (iii) luminal Ca\textsuperscript{2+} concentration appears higher than that of the trans-Golgi, but lower than that of the ER, suggesting the existence of a cis- to trans-Golgi Ca\textsuperscript{2+} concentration gradient. Thus, the GA represents a Ca\textsuperscript{2+} store of high complexity where, despite the continuous flow of membranes and luminal contents, each sub-compartment maintains its Ca\textsuperscript{2+} identity with specific Ca\textsuperscript{2+} homeostatic characteristics. The functional role of such micro-heterogeneity in GA Ca\textsuperscript{2+} handling is discussed.

Keywords: Golgi apparatus, Ca\textsuperscript{2+} homeostasis, FRET-based Ca\textsuperscript{2+} probes, SERCA, SPCA1, intracellular Ca\textsuperscript{2+}-releasing channels

Introduction

The Golgi apparatus (GA) is an intracellular organelle with a very highly organized structure: in the vast majority of cells, it is composed of individual stacks, formed by several cisternae, ordered in a polarized (cis-to-trans) fashion, that are linked and generate long connected ribbons near the nucleus.

From a functional point of view, the GA is the key cellular organelle involved in sorting and processing of newly made secretory and membrane proteins, as well as lipids, that move from the site of their synthesis, the endoplasmic reticulum (ER), to the plasma membrane or other cell compartments. Evidence from different groups suggests that the GA also represents, together with the ER, a dynamic Ca\textsuperscript{2+} storage compartment. Indeed, the GA expresses Ca\textsuperscript{2+}-releasing channels (inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and Ryanodine (Ry) receptors, IP\textsubscript{3}Rs and RyRs), Ca\textsuperscript{2+} pumps (the classical sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase, SERCA, and the secretory pathway Ca\textsuperscript{2+} ATPase, SPCA1), and luminal Ca\textsuperscript{2+} binding proteins (Calnuc, Cab45, p54/Nef, and Calumenin; (Pizzo et al., 2011)). In particular, it has been shown that the GA, as a whole, can contribute to the release of Ca\textsuperscript{2+} during cell activation, and indirect evidence suggested that Ca\textsuperscript{2+} handling within the GA is heterogeneous (Missiaen et al., 2004; Vanoevelen et al., 2004, 2005). More recently, using a novel GFP-based Ca\textsuperscript{2+} probe, Go-D1cpv, selectively localized in the trans-Golgi, we have directly demonstrated that the trans-Golgi takes up Ca\textsuperscript{2+} upon cell stimulation with IP\textsubscript{3} generating agonists, while it releases Ca\textsuperscript{2+} upon activation of RyRs; as far as Ca\textsuperscript{2+} uptake is concerned, the trans-Golgi relies solely on the activity of SPCA1 while SERCA appears to be excluded from this compartment (Lissandron et al., 2010; Pizzo et al., 2011).

Due to these features, the GA has a potential strategic importance for the generation of local cytosolic Ca\textsuperscript{2+} signals and may contribute, together with other intracellular Ca\textsuperscript{2+} handling organelles (ER, mitochondria, peroxisomes), to finely tune the overall cytosolic Ca\textsuperscript{2+} response to different stimuli (Missiaen et al., 2001; Vanoevelen et al., 2005). In addition, the complex Ca\textsuperscript{2+} toolkit may serve intra-Golgi specific functions, including modulating the post-translational modification of lipids and proteins, and folding and/or sorting of cargo proteins. Indeed, not only mutations in the gene coding for SPCA1 lead to a dominant human genetic pathology, the Hailey-Hailey disease (Hu et al., 2000), but it has also been shown that ablation or reduction in SPCA1 activity causes Golgi ribbon structure disruption and dysfunctions in vesicle trafficking and associated sorting of proteins through the secretory pathway (Lissandron et al., 2010; Micaroni et al., 2010). Along these lines, a particularly interesting and new observation is that the sorting of a certain class of secretory vesicles at the trans-Golgi network (TGN) requires not only the activity of SPCA1, but also the specific...
interaction between the pump and the two cytosolic proteins (ADF/ coflin) involved, via dynamic actin, in the transport of the above mentioned cargoes (von Blume et al., 2011). Similarly, the soluble Golgi-resident Ca\(^{2+}\)-binding protein Cab45 seems to be involved, in a Golgi Ca\(^{2+}\)-dependent way, in binding and sorting of several cargoes to the TGN (von Blume et al., 2012). It has been also suggested that Ca\(^{2+}\) released from the Golgi could lead to localized cytosolic Ca\(^{2+}\) rises that stimulate local vesicle fusion, thus contributing to intra-Golgi cargo transport, mainly in the late Ca\(^{2+}\)-dependent phase of SNARE-regulated fusion between the different Golgi sub-compartments (Micaroni et al., 2003).

Nevertheless, it is clear that specific functions of the GA are influenced by local Ca\(^{2+}\)-handling properties. For this reason, it appears urgent to further investigate whether additional heterogeneities in Ca\(^{2+}\) homeostasis exist among distinct GA sub-compartments.

We here describe a new fluorescent Ca\(^{2+}\) probe localized to the medial-part of the GA and show that this Golgi sub-compartment has characteristics that are distinct from those of the trans- and cis-Golgi. In particular, we demonstrate that the medial-Golgi expresses SERCA and SPCA1, as well as IP\(_3\)Rs and RyRs, and maintains within its lumen a steady state [Ca\(^{2+}\)] that is lower than that in the ER, but higher than that in the trans-Golgi. We have also obtained evidence suggesting that the cis-part of the GA, instead, more closely resembles the Ca\(^{2+}\) homeostatic properties of the ER. No other organelle investigated thus far appears to possess the heterogeneity in terms of Ca\(^{2+}\) handling here revealed for the GA and the possible functional implications of these characteristics are discussed.

**Results**

**Localization of the new Golgi Ca\(^{2+}\) probe**

We have recently demonstrated that the trans-Golgi is endowed with unique Ca\(^{2+}\)-handling characteristics (Lissandron et al., 2010), different from those described for the organelle as a whole (Pinton et al., 1998; Missiaen et al., 2001, 2004; Callewaert et al., 2003; Vaneevelen et al., 2004, 2005). To further elucidate GA Ca\(^{2+}\) heterogeneity, we generated a new probe aiming to specifically monitor the Ca\(^{2+}\) levels in the cis- medial-Golgi. We took advantage of the first 32 amino acids (C2gnT) of the resident Golgi enzyme 1,6 N-acetyl glucosaminyl transferase that has been shown to represent the minimal cis- to medial-Golgi targeting determinant when fused to GFP (Zerfaoui et al., 2002). This sequence was cloned upstream of the cDNA coding for the cameleon D1cpv (Lissandron et al., 2010) and the obtained construct (Figure 1A) was then transiently expressed in the human neuroblastoma cell line SH-SYSY. Figure 1B shows that the probe localizes in a perinuclear region that appears composed of cisternae and small vesicles. Figure 2 shows that, when probe-expressing cells (Figure 2A, D, and G) were fixed and immunolabelled for bona fide markers of the cis- (GM130; Figure 2B), cis/medial- (giantin; Figure 2E) and trans- (TGN46; Figure 2H) Golgi compartments, there was a quite good overlap of the fluorescence from the probe and the cis/medial-Golgi marker giantin (Figure 2F), while a partial separation was observed between the signals of GM130 (Figure 2C) and TGN46 (Figure 2I) and that of the novel probe. From a confocal Z-stack analysis to quantify the signal co-localization of the new probe versus the three markers, a partial overlap of the probe and the three endogenous markers was observed (Supplementary Figure S1A). However, a partial overlap was also observed when the same analysis was carried out for the three endogenous markers (Supplementary Figure S1B), most likely because the dimension of the GA cisternae is significantly smaller than the spatial resolution of confocal microscopy (especially in the z-axis).

We have also carried out a similar co-localization analysis between the probe and the SPCA1 protein that is known to be expressed in the trans-Golgi (Lissandron et al., 2010). Probe-expressing SH-SYSY cells immunolabelled with anti-SPCA1 antibody show a main, perinuclear structure positive for both signals, and within this structure, a very nice co-localization between the probe and the pump was observed (Supplementary Figure S2A–C). In particular, a large part of the probe-positive

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**Figure 1** cDNA construction and intracellular localization of the new Golgi probe. (A) Design of the new construct codifying the cameleon protein D1cpv fused to the first 32 amino acids of the resident Golgi enzyme 1,6 N-acetyl glucosaminyl transferase (C32). (B) Fluorescence image corresponding to a Z-projection of 10 confocal sections of a SH-SYS5Y cell expressing the new Ca\(^{2+}\) sensor. Scale bar, 10 \(\mu\)m.
structures is also positive for anti-SPCA1 antibody, while part of the cisternae positive for SPCA1 is not stained by the probe fluorescence. It should be mentioned, however, that SPCA1 is not solely expressed in the GA, since the pump is also present in post-Golgi vesicles of the secretory pathway (Mitchell et al., 2001, 2004). Accordingly, the Pearson’s co-localization index was significantly different when calculated in the whole cell or only in the perinuclear area (Supplementary Figure S2D).

To more accurately investigate the localization of the new probe within the GA, cells were treated with the microtubule depolymerizing agent nocodazole, which results in the accumulation of dispersed individual Golgi stacks in the cytoplasm and can facilitate the distinction among the different GA sub-compartments by confocal microscopy (Cole et al., 1996; Thyberg and Moskalewski, 1999). SH-SY5Y cells were treated with nocodazole, fixed, and then triple-immunolabelled for GM130, giantin, and TGN46. In this case (Figure 3A), the localization of the three endogenous markers appears distinct and, particularly when the vesicles were imaged en face, a typical three-colour staining was evident. Probe-expressing cells were similarly treated and immunolabelled for the cis- and the trans-Golgi markers (Figure 3B), and the cis- and the cis-/medial-Golgi markers (Figure 3C), respectively. Figure 3B shows that the spatial separation among the probe, GM130, and TGN46 signals was evident in several structures, indicating that the probe is largely located in structures distinct from those hosting both GM130 and TGN46. On the contrary, in Figure 3C, the probe signal appears again almost completely separated from GM130, but largely overlapped with giantin, indicating that the probe preferentially targets to the medial-Golgi hosting giantin. Similar results concerning the sub-cellular localization of the probe were obtained in HeLa and BHK cells (data not shown).

In conclusion, the new probe appears to be almost completely excluded from the compartment containing GM130, while its best co-localization was observed with the GA structures containing giantin. Moreover, although the distribution of the probe partially overlapped with the compartment hosting TGN46 and SPCA1, functional evidence (see below) demonstrates that the new probe is localized in vesicles distinct from those of the trans-Golgi. Accordingly, for simplicity, the new probe from now on is named medialGo-D1cpv.

**Ca**\(^{2+}\) **handling in the medial-Golgi**

Functional experiments were then carried out in living SH-SY5Y cells transiently transfected with the cDNA encoding medialGo-D1cpv. The Dcpv-probe family responds to [Ca\(^{2+}\)] changes with variations in fluorescence resonance energy transfer (FRET) between the two GFP variants housed in the molecule (Palmer et al., 2006). In practical terms, the ratio (R) between the fluorescence emitted at 540 nm (cpv-YFP) and that at 480 nm (CFP) upon excitation at 430 nm is a function of the [Ca\(^{2+}\)] within the lumen.

Figure 4 shows typical \(\Delta R/R_0\) changes in medialGo-D1cpv-expressing SH-SY5Y cells (Figure 4A) after the addition of different stimuli. The perfusion of an IP\(_3\)-generating stimulus, such as bradykinin (BK; Figure 4B), in the Ca\(^{2+}\)-free, EGTA-supplemented medium typically results in small decreases (a drop of 10% ± 0.4%; \(n = 14\); Figure 4E) of the average \(\Delta R/R_0\) value within the GA region. The subsequent addition of the Ca\(^{2+}\) ionophore ionomycin causes a rapid and large decrease (a drop of 70% ± 2.1%; \(n = 37\); Figure 4E) of the Golgi \(\Delta R/R_0\) down to a level that was not significantly affected by the subsequent permeabilization of the plasma membrane with digitonin.
We then tested the effect of the SERCA inhibitor cyclopiazonic acid (CPA), added alone (Figure 4C) or in combination with BK (Figure 4D) on medial-Golgi Ca\(^{2+}\) handling. CPA alone resulted in a slow decrease of \( \Delta R/R_0 \) down to a new steady-state value approximately halfway between the value at rest and the minimum value obtained after cell permeabilization with digitonin in Ca\(^{2+}\)-free, EGTA-containing medium (\(-40\% \pm 3.4\%\); \(n = 21\); Figure 4E). However, when the addition of CPA was rapidly followed by BK, CPA-induced slow \( \Delta R/R_0 \) decrease was strongly accelerated (Figure 4D) until a new steady-state value, similar to that reached upon CPA alone, was reached (\(-40\% \pm 1\%\); \(n = 9\); Figure 4E). The addition of ionomycin after CPA induced, in both cases, a further large drop in \( \Delta R/R_0 \) signal (Figure 4C and D). Figure 4E reported the averaged decreases in \( \Delta R/R_0 \) values obtained in SH-SY5Y cells expressing the medialGo-D1cpv construct, but for a smaller drop in medial-Golgi signal observed in these cell lines in response to CPA.
In some BHK cells (30%), especially those strongly expressing the probe, the medialGo-D1cpv probe appeared partially mis-sorted into a fine reticular network, most likely the ER (Supplementary Figure S3A). We took advantage of this feature to compare the changes in [Ca^{2+}] within the Golgi and the ER in the very same cell with the same probe. Specific regions of interest (ROIs) encompassing solely the Golgi or ER structures in the same cell upon different stimuli were thus separately analysed (see Supplementary Figure S3A for a BHK cell image). Upon BK application, there was a small decrease in the average ΔR/R₀ in the medial-Golgi ROI (Supplementary Figure S3B, black trace), while a more marked and rapid drop was revealed in the ER ROI (Supplementary Figure S3B, grey trace). The SERCA inhibitor CPA added after BK caused an almost complete emptying of the organelle Ca^{2+} in the ER, while CPA caused only a partial decrease of the ΔR/R₀ signal, with the following addition of ionomycin inducing a further large drop in the medial-Golgi. ΔR/R₀ values occurring in the two ROIs, for the two Ca^{2+} stores medial-Golgi and ER, respectively, in the same cell upon different subsequent stimulations are compared (Supplementary figure S3C). Noteworthily, the maximal Ca^{2+}-dependent change of the signal (the difference between the value in resting cells and that obtained after ionomycin) was a bit larger in the Golgi than in the ER.

We have shown previously that, if cells expressing the trans-Golgi probe Go-D1cpv are treated with brefeldin A (BFA) that blocks the forward but not the backward movement of vesicles in the organelle, the probe slowly flows back into the ER (Lissandron et al., 2010). In order to obtain further evidence on the Ca^{2+}-handling characteristic of GA regions closer to the ER, we performed the same experiment in parallel cells expressing the medialGo-D1cpv (Supplementary Figure S4). In 12 out of 20 analysed cells upon only 5 min of BFA treatment, the transfer of the probe into the ER was marginal (no significant increase in CFP and cpv-YFP fluorescence in the extra-Golgi ROI was detected; Supplementary Figure S4A and B). However, addition of BK caused a very rapid decrease of ΔR/R₀ (a drop of 20% ± 1%, mean ± SEM, n = 12; Supplementary Figure S4C), i.e. twice that caused by the same stimulus in control cells (see also Figure 4B) in the Golgi region.

**Ca^{2+} uptake mechanisms in the medial-Golgi**

The partial depletion of medial-Golgi Ca^{2+} content by SERCA inhibitors suggests that this Golgi compartment is endowed with two mechanisms of Ca^{2+} uptake, only one of which being sensitive to the drugs. The existence of a Ca^{2+} uptake mechanism other than the SERCA in the medial-Golgi was investigated by two different approaches. First, cells expressing the medialGo-D1cpv were treated with ionomycin in Ca^{2+}-free, EGTA-containing medium to completely deplete Ca^{2+} from the lumen; after washing away ionomycin, cells were treated with the irreversible SERCA inhibitor thapsigargin (Tg, 1 μM) and finally 1 mM CaCl₂ was added to the medium. For comparison, the same procedure was employed in parallel cells expressing an ER-targeted Ca^{2+} probe, ER-D4. Figure 5A shows that, while a substantial reuptake of Ca^{2+} was observed in the medial-Golgi (black trace), no Ca^{2+} refilling was observed in cells expressing the ER Ca^{2+} sensor (grey trace). Similar results were obtained when the refilling of pre-empted stores was followed in cells permeabilized with digitonin and perfused with an intracellular-like medium containing fixed [Ca^{2+}] (Figure 5B).

Second, we took advantage of the drug Bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane (bis-phenol) that has been recently shown to effectively inhibit SPCA1, but inhibit SERCA only at higher concentrations (Lai and Michelangeli, 2012). As this drug was poorly permeable to the plasma membrane, SH-SYSY cells were treated with Tg (1 μM) to fully and irreversibly inhibit the SERCA, permeabilized with digitonin in Ca^{2+}-free, EGTA-containing medium, and finally treated with bis-phenol (25 μM; Figure 5C). Cell perfusion with a medium containing 0.1 μM free Ca^{2+} (buffered with EGTA/H-EDTA) resulted in no increase in the medial-Go-D1cpv signal (Figure 5C, black trace). When bis-phenol was washed away, however, despite the presence of Tg, a rapid increase in medial-Golgi [Ca^{2+}] occurred that reached about 40% of the maximum value obtained by exposing the cells to 1 mM CaCl₂.

To confirm the specific action of bis-phenol on SPCA1 pumps, we performed the same experiment in SH-SYSY cells expressing the trans-Golgi probe Go-D1cpv, as SPCA1 has been shown to be the only Ca^{2+} uptake mechanism in this GA sub-compartment

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**Figure 5** Mechanism of Ca^{2+} uptake in the medial-Golgi. (A) Representative medial-Golgi (black trace) and ER (grey trace) Ca^{2+}-refilling kinetics in pre-empted intact SH-SYSY cells treated with thapsigargin (1 μM). Where indicated, Ca^{2+}-free, EGTA (300 μM)-containing medium was changed with the mKRB containing 1 mM CaCl₂. (B) Representative medial-Golgi (black trace) and ER (grey trace) Ca^{2+}-refilling kinetics in pre-empted, digitonin-permeabilized SH-SYSY cells treated with thapsigargin (1 μM). Where indicated, intracellular-like, Ca^{2+}-free, EGTA (300 μM)-containing medium was changed with an intracellular-like medium with fixed [Ca^{2+}] (0.1 μM). (C) The medial-Golgi Ca^{2+}-refilling plateau (expressed as percentage of ΔR/R₀) reached in permeabilized SH-SYSY cells treated with bis-phenol (bis-P, 25 μM) and thapsigargin (1 μM) following the protocol described in B.
(Lissandron et al., 2010). Accordingly, Ca\(^{2+}\) uptake was completely blocked by bis-phenol, while removal of the drug resulted in a rapid Ca\(^{2+}\) refilling of the trans-Golgi (data not shown).

Finally, we verified the effect of SPCA1 knockdown in SH-SY5Y cells expressing the medial-Golgi probe (Supplementary Figure S5). SH-SY5Y cells treated with specific siRNA against SPCA1 and expressing medialGo-Dcpv were analysed by Western blotting with an anti-SPCA1 antibody, and a reduction of 54% \pm 9% (n = 3) in the total SPCA1 protein level was revealed when compared with control scramble siRNA-transfected cells (Supplementary Figure S5A). Taken into consideration the transfection efficiency in these cells (~70%), this should correspond to a reduction of about 70% of SPCA1 level in the transfected cells.

The effects of SPCA1 down-regulation on medial-Golgi Ca\(^{2+}\) handling were then tested. Supplementary Figure S5B and C shows that SPCA1 knockdown caused, compared with controls, a lower steady-state FRET level within the medial-Golgi at rest (63.3% \pm 2.6% and 76.5% \pm 1.9%, respectively; n = 16), a bigger ∆FRET change upon CPA addition (−39% \pm 3.2% and −35% \pm 2.3%, respectively; n = 16), and accordingly, a smaller additional drop induced by ionomycin (−40% \pm 1.6% and −23.4% \pm 2.1%, respectively; n = 16).

**Homogeneous distribution of the Ca\(^{2+}\) toolkit within the medial-Golgi**

The observation that the medial-Golgi [Ca\(^{2+}\)] is only partially reduced by SERCA blockade could reflect either the presence of two pumps in the same compartment or a sub-heterogeneity in the Ca\(^{2+}\) handling of the structures hosting the probe, i.e. one sub-region could be endowed with only SPCA1 and thus totally CPA-insensitive, while another one with only SERCA and thus completely sensitive to CPA, as in the ER. To address this question, we analysed the dynamics of ∆R/\(R_0\) as well as the absolute R value changes in sub-regions of the medial-GoD1cpv-labelled structures. The Golgi structures undergo substantial movements and remodeling during an experiment; therefore, only in a few cases the same structure with minimal ROI repositioning can be followed for sufficient time. If the partial drop in the medial-Golgi signal induced by CPA is due to a diffuse expression of a CPA-insensitive Ca\(^{2+}\) pump in the whole compartment hosting the probe, the Ca\(^{2+}\) decrease caused by SERCA inhibition should be observed in all the labelled structures. On the contrary, if a significant fraction of the medialGo-D1cpv is localized in a compartment expressing solely SPCA1 or the probe is mis-sorted to the trans-Golgi, a highly non-homogeneous drop of the ∆R/\(R_0\) (or absolute R) signal should be observed upon SERCA inhibition. Figure 6 shows pseudocolor images of the same medial-Golgi-labelled structure in a SH-SY5Y cell taken before (Figure 6B), during (Figure 6C), and after (Figure 6D) 6 min of CPA treatment and after the addition of ionomycin (Figure 6E), as the time points indicated in Figure 6A by coloured symbols. A substantial decrease in the absolute R value, caused by CPA addition, was observed in all labelled regions (compare Figure 6B and D). As expected, ionomycin induced a larger and more homogeneous drop of R (Figure 6E). However, a dramatic change in the morphology of the labelled structure was also observed (Figure 6E). In Figure 6A and F, the dynamics of ∆R/\(R_0\) and R value variations of four identified ROIs (as depicted in Figure 6B–E) of the medial-GoD1cpv-labelled structure are presented, respectively. The drop in R and ∆R/\(R_0\) induced by CPA in the four ROIs is

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**Figure 6** Homogeneous SERCA distribution within different sub-regions of the medial-Golgi. (A) Representative kinetics of the ∆R/\(R_0\) changes of medialGo-D1cpv in SH-SY5Y cells. Where indicated, 1 mM CaCl\(_2\), 20 μM CPA, 300 μM EGTA, and 1 μM ionomycin (lono) were perfused. Coloured symbols report the individual mean ∆R/\(R_0\) values of the different ROIs depicted in B–E. (B–E) Pseudocolor images of a typical medialGo-D1cpv-labelled structure in SH-SY5Y cells showing R value variations in different medial-Golgi sub-regions before (B), during (C), and after (D) CPA treatment and after ionomycin addition (E). Images were taken at the time points indicated by the coloured symbols in A. Scale bar, 5 μm. (F) Mean R values at the different time points indicated by the coloured symbols in A, in distinct sub-regions (ROIs depicted in B–E) of the medialGo-D1cpv-labelled structure in a SH-SY5Y cell treated as in A.
quantitatively similar and no qualitative difference was ever observed among medial-Golgi sub-regions, i.e. no CPA-insensitive region was ever detected. Similar results were obtained in all cells analysed (six cells in three independent experiments).

A similar analysis for the SH-SYSY medial-Golgi response to BK was then carried out (Figure 7). In the analysed cells (seven cells in three independent experiments), the IP$_3$-generating agonist induced a small average change in $\Delta R/R_0$ (Figure 7A) with different sub-regions of the probe-labelled structure undergoing similar drops in both $\Delta R/R_0$ and the $R$ values (Figure 7A and E). In the fraction of cells showing a null average response to IP$_3$-linked stimuli (25%), no change in different sub-regions was ever detected.

**Other Ca$^{2+}$-releasing channels in the medial-Golgi**

The question then arises as to whether the medial-Golgi is endowed with Ca$^{2+}$-releasing channels other than the IP$_3$-R, i.e. two-pore channels (TPCs) (Calcraft et al., 2009) and/or RyRs. To investigate the expression of TPCs in the medial-Golgi, SH-SYSY cells transiently expressing medialGo-D1cpv were permeabilized with digitonin in an intracellular-like medium and stimulated with nicotinic acid adenine dinucleotide phosphate (NAADP), a potent Ca$^{2+}$-releasing second messenger that should specifically activate TPCs (Calcraft et al., 2009). The addition of NAADP (from 10 up to 100 nM) had no appreciable effect on $\Delta R/R_0$ and the $R$ values (Figure 7A and E). In the fraction of cells showing a null average response to IP$_3$, no CPA-insensitive region was ever detected. Similar results were obtained in all cells taken at the time points indicated by the coloured symbols in Figure 7A and E. Images were taken at the time points indicated by the coloured symbols in A, in distinct sub-regions (ROIs depicted in B–D) of the medialGo-D1cpv-labelled structure in a SH-SYSY cell treated as in A.

Given that RyRs2 are also expressed in the trans-Golgi (Lissandron et al., 2010), the question arises as to whether the medialGo-D1cpv fluorescence changes reflect a drop of [Ca$^{2+}$] in the medial-compartment or a partial, or complete, mis-localization of the probe in the trans-Golgi. To address this question, HL-1 cells were transfected with either the medial- or the trans-Golgi probe and challenged with caffeine and CPA. Figure 8C shows that the trans-Go-D1cpv (grey trace) responded upon addition of caffeine with a clear $\Delta R/R_0$ decrease ($-13\% \pm 0.7\%$; $n = 8$), but did not respond to a following addition of CPA, confirming the lack of SERCA pumps in this compartment (Lissandron et al., 2010). On the contrary, in HL-1 cells expressing the medialGo-D1cpv probe (Figure 8C, black trace), CPA induced a clear decrease of $\Delta R/R_0$ ($-30\% \pm 3\%$; $n = 8$) after caffeine stimulation.

**Calibration of the new medial-Golgi Ca$^{2+}$ probe**

In order to determine the absolute value of [Ca$^{2+}$] within the medial-Golgi compartment, we utilized the calibration procedure previously used for the trans-Golgi (Lissandron et al., 2010). A pre-requisite for this calibration was the determination of the luminal pH of the compartment, which was found to be 7.05 (Supplementary Figure S7; Llopis et al., 1998; Lissandron et al., 2010). Passive Ca$^{2+}$-loading experiments were then performed in cells permeabilized with digitonin in an intracellular-like medium at pH 7.05, containing different [Ca$^{2+}$] and no energy source.
obtained by other groups using the same probe in different cell lines (Missiaen et al., 2001, 2004; Callewaert et al., 2003; Vanoevelen et al., 2004, 2005; Zatti et al., 2006). More recently, using a novel GFP-based Ca\(^{2+}\) probe Go-D1cpv that selectively localized in the trans-Golgi, we have demonstrated that this GA sub-compartment is endowed with unique characteristics that differ completely from those of the ER (Lissandron et al., 2010). We here describe the generation of another Ca\(^{2+}\) probe aiming to measure the [Ca\(^{2+}\)] in the cis/trans-Golgi, thus revealing additional complexities in the Ca\(^{2+}\)-handling characteristics of the organelle.

The immunocytochemical evidence obtained by confocal microscopy suggests that the new probe, medialGo-D1cpv, is localized in structures that are clearly distinct from those hosting GM130, while there is an excellent overlap of the probe distribution with that of the endogenous (cis) medial-Golgi marker giantin. Based on morphological criteria, the localization of the new probe can be only partially distinguished from that of TGN46. However, the characteristics of Ca\(^{2+}\) handling within the lumen reported by the medialGo-D1cpv are totally distinct from those revealed by a bona fide trans-Golgi Ca\(^{2+}\) probe (Lissandron et al., 2010). In fact, unlike the trans-Golgi that utilizes solely SPCA1 for Ca\(^{2+}\) uptake (Lissandron et al., 2010), the medial compartment appears to accumulate Ca\(^{2+}\) within its lumen taking advantage of both SPCA1 and SERCA. In particular, we observed that the inhibition of the SERCA results in a partial signal decrease of the whole labelled structure, while its complete Ca\(^{2+}\) depletion required the addition of ionomycin.

It could be argued that the complexity of Ca\(^{2+}\) handling here revealed by the medialGo-D1cpv reflects the sum of the characteristics of the cis- and trans-Golgi rather than a characteristic of a specific GA sub-compartment, due to a partial mis-sorting of the probe into these regions. Although we cannot exclude a small spillover of the probe in these GA compartments, evidence suggests that indeed the Ca\(^{2+}\)-handling structure labelled by the novel probe is endowed with unique functional characteristics since: (i) all medialGo-D1cpv-labelled structures undergo a similar drop in response to SERCA inhibitors, indicating that this pump is diffusely and homogeneously expressed in the compartment; on the contrary, blockade of the SERCA causes no appreciable decrease of luminal [Ca\(^{2+}\)] in the trans-Golgi (Lissandron et al., 2010); (ii) upon receptor-activated production of IP\(_3\), the decrease in medial-Golgi [Ca\(^{2+}\)] appears again rather homogeneous. Most importantly, upon treatment with BFA to allow the back diffusion of the probe to a more cis-Golgi compartment, the response to IP\(_3\) generation increased and approached that of the ER.

Another unique characteristic of this medial-Golgi compartment is the contemporary presence of two Ca\(^{2+}\) pumps, SERCA and SPCA1. This conclusion is based on morphological, pharmacological, and genetic evidence: (i) the SPCA1 signal revealed by specific antibodies partially overlapped with that of the probe; (ii) blockade of SERCA causes only a partial depletion of the medial-Golgi Ca\(^{2+}\) level and, similarly, (iii) down-regulation of SPCA1, or its pharmacological inhibition, only in part impairs the capacity of the compartment to accumulate Ca\(^{2+}\) in its lumen. This is in clear contrast with the ER where Ca\(^{2+}\) uptake is solely dependent on SERCA and with the trans-Golgi where Ca\(^{2+}\) uptake all depends on SPCA1.

Typical traces are presented in Supplementary Figure S8A and B, while Supplementary Figure S8C shows the in situ calibration curve for the medialGo-D1cpv. The calculated apparent K\(_S\) for the new probe is 27.4 μM. The mean Ca\(^{2+}\) level that matched the cpv-YFP/CFP fluorescence emission ratio of SH-SYSY cells at rest before permeabilization was 235 ± 30 μM (n = 24), a value higher than that of the trans-Golgi (Lissandron et al., 2010), but lower than those measured in the ER of the same cells according to the above curve. All the major FRET variations obtained in SH-SYSY cells expressing the medialGo-D1cpv upon different stimulations and treatments are presented in terms of absolute average [Ca\(^{2+}\)] values in Supplementary Figure S8F–G.

**Discussion**

Direct information on the Ca\(^{2+}\) homeostatic mechanisms in the GA of living cells was obtained for the first time in 1998 using an aequorin chimeric construct localized in the organelle lumen. It was demonstrated that GA Ca\(^{2+}\) handling (as a whole) resembles that of the ER (Pinton et al., 1998). Similar results have been...
We have also tested whether the medial-Golgi is sensitive to other Ca\(^{2+}\)-mobilizing agents. But no evidence for the existence of a NAADP-sensitive Ca\(^{2+}\) release mechanism was observed. On the contrary, in a cardiomyocyte cell line the opening of RyRs (elicited by either Ca\(^{2+}\)-induced Ca\(^{2+}\) release or caffeine) resulted in a clear drop of [Ca\(^{2+}\)] in the medial-Golgi. Interestingly, the amplitude of the response to caffeine is quantitatively similar in the medial- and trans-Golgi, but smaller than that in the ER (unpublished data).

Thus, functionally the medial-Golgi appears to be a rapidly mobilizable Ca\(^{2+}\) store responding to IP\(_3\) and endowed, at least in some cell types, with a classical RyR mediated, Ca\(^{2+}\) release response. Similarly to the ER, one could hypothesize that the medial-Golgi released Ca\(^{2+}\) sustains a preferential cross-talk of this GA compartment with a subset of mitochondria, but no evidence for a privileged apposition of mitochondria to this or other Golgi compartnents was detected (unpublished data).

The final and most intriguing question is the functional role of the unpredicted, inter-compartment heterogeneity of the Ca\(^{2+}\)-handling toolkit in the GA, far larger than that observed in any other organelle (Pizzo et al., 2011). Our data indicate that the repertoire of Ca\(^{2+}\)-handling mechanisms is indeed strikingly different in the different Golgi regions: SERCA pumps and IP\(_3\)/Rs predominate in the compartment closer to the ER; in an intermediate region, SERCA and SPCA1 co-exist as Ca\(^{2+}\) uptake mechanisms, and IP\(_3\)/Rs are still expressed, while in the most trans-part of the organelle, both SERCA and IP\(_3\)/Rs are excluded, and Ca\(^{2+}\) uptake is totally controlled by SPCA1; finally, the RyRs, in cells that express them, appears to be present, based on the effects of caffeine on luminal [Ca\(^{2+}\)], at similar levels in both the medial- and trans-compartment. Thus, in a space of a few microns, despite its continuous inter-mixing dynamics, the Golgi Ca\(^{2+}\) toolkit undergoes major changes and the organelle appears to be formed by three separate functional entities with clearly distinct Ca\(^{2+}\)-handling mechanisms.

It has been suggested that the trans-Golgi Ca\(^{2+}\) plays a key role in the process of membrane and cargo proteins flowing into the secretory pathway. Indeed, down-regulation of SPCA1, or of the luminal Golgi Ca\(^{2+}\)-binding protein Cab\(_{45}\), results in alterations of the transfer of different proteins to the plasma membrane or to the TGN (Lissonandr et al., 2010; Micaroni et al., 2010; von Blume et al., 2011, 2012). Moreover, the haploinsufficiency of the gene coding for SPCA1 results in skin malfunctioning in the Hailey-Hailey disease, a genetic disease that exists in humans (Hu et al., 2000). However, given that SPCA1 is expressed also in post-Golgi vesicles (Mitchell et al., 2001, 2004), the conclusion that these defects depend solely, or primarily, on altered medial/trans-Golgi Ca\(^{2+}\) homeostasis is presently unwarranted. Similarly, at present, we have no genetic or pharmacological tool that allows to interfere specifically with cis- and medial-Golgi Ca\(^{2+}\) homeostasis, as the pump and the Ca\(^{2+}\) release channels of these Golgi regions appear to be indistinguishable from those of the ER (Zampese and Pizzo, 2012) and any attempt to modulate these Ca\(^{2+}\) mechanisms would inevitably affect both organelles. Thus, the functional significance of the complexity of Ca\(^{2+}\) handling in the Golgi still remains a fascinating mystery and novel tools should be invented to solve this puzzle.

Materials and methods

Materials
Restoration and modification enzymes were purchased from NEB Inc. CPA, digitonin, BK, caffeine, and bis-phenol were purchased from SIGMA-Aldrich. Ionomycin was from Calbiochem and NAADP was from Tocris. All other materials were analytical or of the highest available grade.

Constructs
The medialGo-D1cpv was obtained by introducing the first 32 amino acids of the resident Golgi enzyme 1,6-acetylglucosaminyl transferase (C2gnt) (MHTNLKKFSCCVLVFLFVAVCIVKKEKKGSY YDSFKLQTKEQVLKSGLAKMGSDSQ5VSSSTQ) upstream of the first codon of the D1cpv cDNA (kindly provided by Dr R. Tsien), upon its isolation from the GFP-fused construct described in Zerfaoui et al. (2002), kindly provided by Dr A. El-Battari. Some experiments used an ER-targeted D4 camleleon probe, the ER-D4, described in Kipanyula et al. (2012).

Cell culture and transfection
SH-SY5Y, HeLa, and BHK cells were grown in DMEM containing 10% FCS, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), in a humidified atmosphere containing 5% CO\(_2\). HL-1 cells were grown in Claycomb medium (SLD #6467; Sigma) containing 10% FBS, norepinephrine (100 μM), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were seeded onto glass coverslips (18-mm diameter) coated with fibronectin (25 μg/mL) in 0.2% gelatine for HL-1 cells. Transfection was performed at 60% confluence, using Lipofectamine™ 2000 Transfection Reagent (Life Technologies) for SH-SYSY and HL-1 cells and TransIT®-LT1 transfection reagent (Mirus Bio LCC) for HeLa and BHK cells, with 2 μg of DNA (0.5 μg of camleleon codifying DNAs and 1.5 μg of pcDNA3). FRET measurements were usually performed 24 h after transfection.

Cell treatments
Cells expressing the fluorescent probes were analysed as described in details in Supplementary material. Cells were mounted into an open-topped chamber and maintained in an extracellular medium (modified Krebs-Ringer Buffer, mM; in mM: 135 NaCl, 5 KCl, 1 MgCl\(_2\), 0.4 KH\(_2\)PO\(_4\), 1 MgSO\(_4\), 20 HEPES, 11 glucose, pH 7.4). Classical experiments started in 1 mM CaCl\(_2\); after perfusion with 300 μM EGTA, cells were stimulated with bradykinin (BK, 100 nM) and/or CPA (20 μM); thereafter, the Ca\(^{2+}\) ionophore ionomycin (1 μM) was applied to completely discharge the stores; digitonin (50 μM) in Ca\(^{2+}\)-free medium was applied to permeabilize plasma membrane, followed with a saturating concentration of CaCl\(_2\) (3 mM) in order to verify the dynamic range of the probe. Different cell media were used for pH determination, NAADP response and medialGo-D1cpv calibration experiments in permeabilized cells, and Ca\(^{2+}\)-pumping experiments (see Supplementary material). All media were perfused through a temperature controller (Warner Instruments, TC-324B) to have a constant temperature of 37°C in the chamber.

FRET measurements
FRET experiments were analysed with ImageJ (Wayne Rasband). Cpf-YFP and CFP images were subtracted of background signals and distinctly analysed after selecting proper ROIs on each cell; subsequently, a ratio between cpv-YFP and CFP emissions was calculated (R = F540/F480). Data are presented as ΔR/R\(_0\) values,
where \( \Delta R \) is the change of the cpv-YFP/CFP emission intensity ratio at any time and \( R_0 \) is the value at time of the first drug addition, as the percentage of the initial one. Data were analysed using Origin 7.5 SR5 (OriginLab Corporation).

**Immunocytochemistry**

Cells decorated with different antibodies were treated as described in details in Supplementary material. Images were collected using a Leica TCS-SP5 II confocal system, with a Planapo 100X/1.4 NA objective. The Argon laser line (488 nm) was used to excite the cameleon sensors or AlexaFluor488, the He/Ne 543 nm laser was used to excite the AlexaFluor555/568, and the AlexaFluor647 was excited by the HeNe 633 nm laser line. Z-stacks were acquired with a step of 0.42 \( \mu \)m. Images were elaborated with ImageJ (Wayne Rasband).

**SPCA1 down-regulation and western blotting**

The day before transfection, SH-SY5Y cells were plated in order to ensure 50% confluence on the day of transfection. Functionally validated siRNA directed against SPCA1 (CATCGAACATGGCCCTTA) and siGENOME RISC-Free Control siRNA were from Thermo scientific. A first hit of siRNAs (100 nM each) was added to the cells the day after plating. Twenty-four hours later, siRNAs were co-transfected with mediaGo-D, were from Transfection Reagent (Life Technologies). FRET measurements were performed 48 h after transfection. Proteins were resolved by SDS-PAGE, blotted onto a PVDF membrane, and probed with mouse monoclonal anti-ATP2C1 and mouse monoclonal anti-\( \beta \)-actin (Sigma-Aldrich).

**Statistical analysis**

Unless otherwise stated, numerical results presented throughout the text refer to mean \( \pm \) SEM. \( n \), number of independent experiments; *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \), unpaired Student’s \( t \)-test.

**Supplementary material**

Supplementary material is available at [Journal of Molecular Cell Biology online](http://www.jmcb.org).

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