Calcium cations play a critical role in regulating vesicular transport between different intracellular membrane-bound compartments. The role of calcium in transport between the Golgi cisternae, however, remains unclear. Using a well characterized cell-free intra-Golgi transport assay, we now show that changes in free Ca$^{2+}$ concentration in the physiological range regulate this transport process. The calcium-chelating agent 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid blocked transport with an IC$_{50}$ of approximately 0.8 mM. The effect of 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid was reversible by addition of fresh cytosol and was irreversible when performed in the presence of a Ca$^{2+}$ ionophore that depletes calcium from lumenal stores. We demonstrate here that intra-Golgi transport is stimulated by low Ca$^{2+}$ concentrations (20–100 nM) but is inhibited by higher concentrations (above 100 nM). Furthermore, we show that calmodulin antagonists specifically block intra-Golgi transport, implying a role for calmodulin in mediating the effect of calcium. Our results suggest that Ca$^{2+}$ efflux from intracellular pools may play an essential role in regulating intra-Golgi transport.

Although Ca$^{2+}$ is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner. Calcium sequestration into the ER lumen is the most abundant cation in vertebrates, eukaryotic cells sequester Ca$^{2+}$ efficiently, mainly by uptake into intracellular stores, and thus display low cytosolic concentrations of about 100 nM (for reviews see Refs. 1 and 2). Fast and short (transient) increases in the cytosolic Ca$^{2+}$ concentration play a pivotal role in many physiological processes, and the dynamic characteristics of these pools are regulated in a well defined manner.

In this study we have characterized the requirement for calcium in transport within the Golgi. We present evidence that intra-Golgi transport requires low free Ca$^{2+}$ concentrations within the physiological range and that above a critical level (100 nM) Ca$^{2+}$ has the reverse effect and inhibits transport. We thus show that free Ca$^{2+}$ is required in a very narrow concentration range for this process and suggest a dual role for Ca$^{2+}$ in intra-Golgi transport. In addition, we provide evidence that links CaM to the stimulatory effect of Ca$^{2+}$ on transport.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rat Brain Cytosol—** Rat brain cytosol was prepared essentially as described previously for bovine brain cytosol (15).

**Intra-Golgi Transport Assay—** The standard intra-Golgi transport was performed as described previously (16). A standard intra-Golgi transport assay (25 μl) contained 2.5 mM Hepes buffer, pH 7.0, 15 mM KCl, 2.5 mM magnesium acetate, 0.4 μCi of UDP-[3H]N-acetylgalactosamine, 5 μl of a 1:1 mixture of donor and acceptor Golgi membranes (2–3 μg of protein), 0.25 μl (30 μg) of His$_6$-SNAP, 10 μM palmitoyl-coenzyme A, and ATP and UTP regenerating systems. Transport activity was dependent upon addition of cytosol, and in this study 27.5 μg of rat brain cytosol was added to achieve optimum conditions for the transport activity. The transport reactions were incubated at 30 °C for 2 h. [3H]N-Acetylgalactosamine incorporated into VSV-G protein was determined as described previously (17). Each of the transport assay experiments shown in this study represent at least three independent experiments performed in duplicate. (Where standard errors are absent the results represent 3 independent experiments with duplicates. Standard errors did not exceed 5%).

**Glycosylation Assay—** For the glycosylation assay, “wild type donor” membranes were prepared as described by Taylor et al. (18). Briefly Golgi membranes were isolated from wild type Chinese hamster ovary cells infected with VSV, after which the isolated “wild type donor” membranes were treated with N-ethylmaleimide (1 mM) for 15 min on ice, at which time diithiothreitol (2 mM) was added to quench any remaining N-ethylmaleimide. The glycosylation assay was performed under identical conditions as those described for the standard transport assay.

**Free Ca$^{2+}$ Concentration Measurements—** The free cation concentrations were determined using the Bound and Determined (BAD) software (33).
RESULTS

To address the role of Ca\(^{2+}\) in intra-Golgi transport we tested the effects of EGTA and BAPTA, two Ca\(^{2+}\) chelators, on the well characterized cell-free intra-Golgi transport assay (16). Addition of increasing amounts of BAPTA inhibited transport with an IC\(_{50}\) of approximately 0.8 mM, whereas EGTA showed only minimal effect (Fig. 1A). The inhibition of transport observed in the presence of BAPTA is not due to chelation of Mg\(^{2+}\) because a similar pattern was observed when BAPTA was added in the presence of 3 mM Mg\(^{2+}\) (Fig. 1B). BAPTA inhibition is blocked by calcium. BAPTA (2 mM) was added to the transport assay in the presence or absence of molar equivalence CaCl\(_2\) as indicated. C, efflux of calcium is required for intra-Golgi transport. Golgi membranes were incubated (15 min, 25°C) with either transport buffer containing BAPTA (5 mM) or BAPTA (5 mM) and ionomycin (100 \(\mu\)M) as indicated. The membranes were reisolated, supplemented with transport buffer in the presence or absence of rat brain cytosol, and assayed for transport using standard conditions. D, calcium chelators do not affect N-acetylglucosamine transferase I activity. The glycosylation assay was performed using wild type VSV Golgi membranes (see “Experimental Procedures”) in the absence or presence of either 5 mM BAPTA or 5 mM EGTA.

The effect of BAPTA on intra-Golgi transport was fully reversible when the Golgi membranes were reisolated and incubated with fresh untreated cytosol (Fig. 1C). However, when the membranes were incubated with BAPTA in the presence of 100 \(\mu\)M ionomycin, a Ca\(^{2+}\) ionophore that causes release of Ca\(^{2+}\) from lumenal stores, fresh cytosol failed to restore transport. These results are consistent with the notion that low and transient Ca\(^{2+}\) effluxes are essential for intra-Golgi transport.

To exclude the possibility that the inhibition of the assay signal by BAPTA resulted from decreased glycosylation activity of GlcNAc transferase rather than from inhibition of transport, we used a glycosylation assay that determines GlcNAc activity when membrane transport is inactivated (for details see “Experimental Procedures”). As shown in Fig. 1D, neither BAPTA nor EGTA affected the glycosylation of VSV-G protein, indicating that BAPTA indeed exerts its effect on the process of intra-Golgi transport.

To examine at which stage BAPTA acts in inhibiting intra-Golgi transport, we examined the effect of BAPTA when combined with two other inhibitors of transport (15, 22). In the experiment described in Fig. 2, the transport assay was either

![Diagram A](image1.png)

**A** Local calcium is required for intra-Golgi transport. A, effect of calcium chelators is shown. Increasing amounts of BAPTA and EGTA were added as indicated, to the intra-Golgi transport assay in the presence or absence of 3 mM Mg\(^{2+}\). B, BAPTA inhibition is blocked by calcium. BAPTA (2 mM) was added to the transport assay in the presence or absence of molar equivalence CaCl\(_2\) as indicated. C, efflux of calcium is required for intra-Golgi transport. Golgi membranes were incubated (15 min, 25°C) with either transport buffer containing BAPTA (5 mM) or BAPTA (5 mM) and ionomycin (100 \(\mu\)M) as indicated. The membranes were reisolated, supplemented with transport buffer in the presence or absence of rat brain cytosol, and assayed for transport using standard conditions. D, calcium chelators do not affect N-acetylglucosamine transferase I activity. The glycosylation assay was performed using wild type VSV Golgi membranes (see “Experimental Procedures”) in the absence or presence of either 5 mM BAPTA or 5 mM EGTA.
terminated at different time points by placing the reaction on ice, or at these time points the inhibitors BAPTA, anti-SBP56 antibodies, or GTP\(\gamma\)S were added as indicated, and the reaction was allowed to proceed for 2 h. Control samples treated with buffer only were incubated likewise at 30° C until the end of the 2-h incubation period and served to represent 100% of transport activity. All three inhibitors, when added at the onset of the reaction, produced 90% inhibition of transport. The reaction became resistant to BAPTA after the inhibition by GTP\(\gamma\)S and anti-SBP56 antibodies, indicating that Ca\(^{2+}\) is required late in the transport process, possibly at the fusion stage.

It has been demonstrated that different organelles, including the Golgi apparatus, accumulate Ca\(^{2+}\) in their lumen (8). To test the role of the luminal Ca\(^{2+}\) pool in intra-Golgi transport, Golgi membranes were incubated in the presence of increasing concentrations of either ionomycin (a Ca\(^{2+}\) ionophore) or thapsigargin (a tumor-promoting sesquiterpene lactone that binds with high affinity and irreversibly inhibits all sarcoplasmic reticulum adenosine triphosphatase pumps). These two compounds are known to reduce luminal Ca\(^{2+}\) levels selectively from intracellular organelles. As shown in Fig. 3, both ionomycin and thapsigargin inhibited intra-Golgi transport with IC\(_{50}\) values of 35 and 50 \(\mu\)M, respectively, with no effect on glycosylation by GlcNac transferase. These results clearly indicated that luminal Ca\(^{2+}\) was essential for this process.

Having demonstrated that release of Ca\(^{2+}\) from intracellular stores may play a role in regulating transport, we examined the concentration of free Ca\(^{2+}\) required for intra-Golgi transport. For that purpose we added increasing CaCl\(_2\) concentrations to the cell-free transport assay in the presence of 5 mM BAPTA or EGTA (Fig. 4, A and B). Interestingly addition of 100 nM free Ca\(^{2+}\) caused the assay signal to return to almost maximal levels (88% of the control determined in the absence of any Ca\(^{2+}\) chelator), but addition of higher free Ca\(^{2+}\) concentrations significantly inhibited the cell-free transport assay. When tested, neither Mn\(^{2+}\) nor Cu\(^{2+}\) was able to substitute Ca\(^{2+}\) (Fig. 4A). We then tested the inhibitory effect of Ca\(^{2+}\) using EGTA as cation chelator. Under these conditions the stimulatory effect of Ca\(^{2+}\) could not be observed. However, as found in the presence of BAPTA, free Ca\(^{2+}\) concentrations above 100 nM inhibited transport, reaching a maximum effect at about 200 nM free Ca\(^{2+}\) (Fig. 4B). These results clearly demonstrated that within the physiological range, Ca\(^{2+}\) plays a dual role in regulating intra-Golgi transport. Furthermore, the ability of BAPTA but not EGTA to inhibit transport by chelating Ca\(^{2+}\) suggests that the effect of Ca\(^{2+}\) in regulating membrane fusion is localized to the vicinity of the membrane.

A number of studies using endosomal fusion (12) or yeast vacuole homotypic fusion (11) have demonstrated a requirement for the cytosolic Ca\(^{2+}\) effector, CaM. We therefore examined the involvement of CaM in intra-Golgi transport. For that purpose, increasing concentrations of two specific CaM inhibitors, W7 or trifluoperazine dimaleate, were added to the cell-free transport assay. As shown in Fig. 5A, 25 \(\mu\)M either W7 or trifluoperazine dimaleate inhibited up to 90% of the total transport activity with an IC\(_{50}\) of about 10 \(\mu\)M. Addition of W5, a much weaker CaM antagonist, however, only partially inhibited transport (IC\(_{50}\) > 250 \(\mu\)M) thus indicating that the inhibition observed in the presence of the different antagonists is CaM-mediated. Notably, W7 or trifluoperazine dimaleate failed to significantly inhibit the glycosylation of VSV-G protein as determined by the glycosylation assay (data not shown).

To further verify the involvement of CaM in this process, we performed a two-stage transport assay in which Golgi membranes were first treated with or without W7 (30 min on ice) in transport assay conditions. The membranes were then reisolated, washed, and tested for transport activity in the presence of fresh cytosol (Fig. 5B). Addition of fresh cytosol to the control membranes recovered most of their transport activity, whereas W7-treated membranes could restore up to 90% of transport only when purified CaM was added together with the fresh cytosol. Hence, CaM appears to be involved in intra-Golgi transport. These results also indicate that the low levels of CaM in the cytosol are insufficient to reconvert the Golgi membranes after treatment with W7. CaM-dependent transport was strongly inhibited by BAPTA (Fig. 5B) indicating that Ca\(^{2+}\) is required for the CaM activation of transport.

**DISCUSSION**

It has been well demonstrated that Ca\(^{2+}\) regulates vesicular transport between a number of different intracellular or-
ganelles, but its involvement in intra-Golgi transport remains poorly understood. Here, we have investigated the role Ca\(^{2+}\) plays in this process by using the well established intra-Golgi transport assay. We found that low cytosolic Ca\(^{2+}\) concentrations (approximately 100 nM) are optimal for intra-Golgi transport whereas higher Ca\(^{2+}\) concentrations inhibit this process. Moreover, it appears that Ca\(^{2+}\) efflux from intracellular stores (possibly from the Golgi complex itself) is essential for intra-Golgi transport. Our results indicated that Ca\(^{2+}\) played a role in late stages of transport, possibly in the fusion of vesicles with their target membrane. Finally we demonstrate that CaM is involved in intra-Golgi transport.

Several studies have documented that Ca\(^{2+}\) plays an important role in regulated exocytosis of secretory granules and synaptic vesicles (reviewed in Refs. 23 and 24). More recently it was reported that Ca\(^{2+}\) participates in other intracellular transport events, including ER to Golgi transport (25), assembly of nuclear membrane (10), transcytotic vesicle fusion (26), vacuolar membrane fusion (11), and fusion between endosomes (12, 13). The requirement for Ca\(^{2+}\) in intra-Golgi transport has remained unclear, however, because early reports using a cell-free intra-Golgi transport assay demonstrated no effect of EGTA (14), whereas in semi-intact cells, EGTA was able to inhibit this transport process (9). In agreement with the early reports, we now show that intra-Golgi transport is indeed resistant to EGTA. However, this transport step is highly sensitive to the fast acting Ca\(^{2+}\) chelator, BAPTA. This phenomenon of sensitivity to BAPTA but resistance to EGTA has been observed for other systems of membrane fusion, including homotypic vacuolar fusion (11) and endosomal fusion (13). This suggests that transient, probably local, changes in Ca\(^{2+}\) concentration mediate the fusion process. Indeed, our results (Fig. 3) are consistent with the notion that intra-Golgi transport depends upon Ca\(^{2+}\) efflux from inner membrane stores.

Several studies have indicated that the Golgi complex may function as a Ca\(^{2+}\) storage organelle (8, 27–29). Thus it is feasible that Ca\(^{2+}\) from the lumen of the Golgi directly participated in regulating intra-Golgi transport.

In most cells the free Ca\(^{2+}\) concentration rests at approxi-
mately 100 nM, and following activation of cellular signaling pathways Ca\(^{2+}\) levels are elevated about 1000-fold (30, 31). We have found that intra-Golgi transport is optimal in the presence of about 100 nM free Ca\(^{2+}\), but that higher Ca\(^{2+}\) concentrations strongly inhibit transport. This may imply that constitutive membrane transport is optimal when the cytosolic Ca\(^{2+}\) concentration remain at resting levels, but is inhibited upon elevation of cytosolic Ca\(^{2+}\). Because exocytosis depends critically on high Ca\(^{2+}\) levels, this may provide a mechanism for the cell to conserve the fusion machinery for the transient and more immediate requirement of exocytosis at the expense of constitutive transport.

What are the downstream targets of Ca\(^{2+}\) in this system? It has recently been demonstrated that the Ca\(^{2+}\)-binding protein CaM plays an important role in fusion between yeast vacuoles (11). It was postulated that CaM acts in this system at a late step of the fusion reaction, probably after docking of the transport vesicle with its target membrane (11). CaM antagonists were found to inhibit endosome fusion in vitro (12). The strong inhibition of intra-Golgi transport by the CaM antagonist reported here suggests that CaM may regulate various intracellular fusion processes in a Ca\(^{2+}\)-dependent manner. Recently Mayer and co-workers (32) suggested that protein phosphatase 1 may be involved in late stages of vacuolar fusion, possibly in a CaM-dependent manner. Future studies will determine whether protein phosphatase 1 or another target represents the effector of Ca\(^{2+}\) in constitutive membrane transport and how this effector acts on the transport machinery.

Acknowledgment—We thank Simone Fishburn for critical reading of the manuscript and for stimulating discussions.

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