Calmodulin (CaM) has previously been implicated in regulated exocytosis, transcytosis, and receptor recycling. We have investigated the role of CaM in endocytic transport by examining the effects of several CaM antagonists in intact cells. We present evidence indicating that the mixing of sequentially internalized ligands is inhibited by CaM antagonists, indicating that CaM may play a general role in regulating endosomal membrane trafficking. To address the specific events that are affected by CaM we studied its role in an in vitro assay that reconstitutes fusion among endosomes. CaM antagonists inhibited endosome fusion, and the inhibition was reversed by the addition of CaM. Moreover, we found that Ca\(^{2+}\) stimulates fusion among endosomes and that addition of CaM stimulates fusion beyond that produced by Ca\(^{2+}\) alone. Our data indicate that one of the possible targets for CaM in endosome fusion is the CaM-dependent kinase II. We propose that CaM regulates endocytic transport by modulating an essential component(s) of the membrane traffic machinery.

Calmodulin (CaM)\(^1\) activates numerous proteins in response to Ca\(^{2+}\) after a conformational change in the CaM molecule. CaM plays a central role in many important signaling pathways in the cell by modulating the activity of key regulatory enzymes, ion pumps, and proteins implicated in motility function (for a review, see Refs. 1 and 2). In several cell types CaM has been shown to be involved in secretion by studies using CaM antagonists in intact cells (3–5). In permeabilized cells CaM stimulates triggering of regulated exocytosis (6, 7). Moreover, recently it has been shown that injection of CaM into adrenal chromaffin cells (by patch-clamp) increases exocytosis (8). Although CaM has long been associated with calcium-regulated exocytosis, its precise site of action has not yet been identified. There have been few reports of the involvement of CaM in regulating membrane trafficking events along the endocytic pathway, in some cases, with controversial results. CaM has been implicated in phagocytosis (9), receptor recycling, and transcytosis (10–13). Thus, CaM may play a general role in regulating membrane traffic. However, the putative direct or indirect targets of CaM and the mechanism by which CaM regulates specific transport events are largely unknown.

\(^{1}\) The abbreviations used are: CaM, calmodulin; DNP, dinitrophenol; PBS, phosphate-buffered saline.

To examine the role of calmodulin in endocytic transport we have studied the effect of CaM inhibitors in intact cells. We found that colocalization of sequentially internalized endocytic markers is inhibited by CaM antagonists, suggesting that fusion among endosomes might be regulated by CaM. To conclusively establish a role for CaM in endosome fusion, we studied the effect of CaM antagonists in an in vitro assay that reconstitutes fusion among endosomes. In this report we present evidence that a CaM activity regulates endosome fusion, suggesting that CaM plays a more general role in vesicular trafficking than previously thought.

**MATERIALS AND METHODS**

**Cells and Materials**—J774 E-clone (mannose receptor-positive), a macrophage cell line, was grown to confluence in minimum essential medium containing Earle’s salts and supplemented with 10% fetal calf serum. HDPI-1, a mouse IgG1 monoclonal antibody specific for dinitrophenol (DNP), was isolated as described previously (14, 15). β-Glucuronidase was isolated from rat preputial glands and derivatized with DNP using dinitrofluorobenzene (14). Cytoxol from the J774 cells was the high-speed supernatant of a cell homogenate obtained as described (14) and stored at –80 °C. Cytoxol samples (200 μl) were gel-filtered through 1 ml Sephadex G-25 spin columns just before use in the fusion assay. Protein concentration after filtration was 3–5 mg/ml. KN-93 and the calmodulin kinase II peptide 281–291 (calmodulin kinase II subunit) were obtained from Calbiochem. All other chemicals were obtained from Sigma.

**Preparation of Endocytic Vesicles**—Early endosomes were loaded with either aggregated anti-DNP IgG or DNP-β-glucuronidase by a 5-min uptake at 37 °C as described previously (14, 15). After ligand uptake, the macrophages (1 × 10\(^6\) cells) were washed sequentially with 150 mM NaCl, 5 mM EDTA, and 10 mM phosphate buffer (pH 7.0) and with 250 mM sucrose, 0.5 mM EGTA, and 20 mM Hepes-KOH (pH 7.0; homogenization buffer) and homogenized in the latter buffer (2 ml) using a cell homogenizer (15). Homogenates were centrifuged at 800 × g for 5 min to eliminate nuclei and intact cells. Postnuclear supernatants were quickly frozen in liquid nitrogen and stored at −80 °C.

**In Vitro Fusion Assay**—Postnuclear fractions were quickly thawed, diluted with homogenization buffer, and then pelleted for 1 min at 37,000 × g in a Beckman L 100 centrifuge. The supernatants were centrifuged for an additional 5 min at 50,000 × g. The pellets of this second centrifugation were enriched with 5-min endosomes. Endosomal fractions containing each probe were mixed with fusion buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-KOH (pH 7.0); homogenization buffer) and homogenized in the latter buffer (2 ml) using a cell homogenizer (15). Homogenates were centrifuged at 800 × g for 5 min to eliminate nuclei and intact cells. Postnuclear supernatants were quickly frozen in liquid nitrogen and stored at −80 °C.

**Ligand Mixing Assay**—In a standard assay, DNP-β-glucuronidase activity was measured using 4-methylumbelliferyl β-D-glucuronide as substrate in a Microplate fluorometer 7600 (Cambridge Technology, Inc; Ref. 15). Fusion was expressed in arbitrary fluorescence units.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
was bound to J774 macrophages suspended at 4 °C for 30 min in uptake media (16, 17). Cells were incubated at 37 °C for 5 min to allow ligand internalization and then washed twice with PBS-EDTA (5 mM) containing 50 mM mannose to remove ligand bound to cell surface mannose receptors. Internalized DNP-β-glucuronidase was chased along the endocytic pathway by a 10-min incubation at 37 °C. During this incubation 1 mg/ml mannan was added to avoid the reinternalization of DNP-β-glucuronidase recycled to the extracellular media. After cooling to 4 °C, cells were washed twice with PBS-EDTA-mannose and once with uptake media (Hanks’ balanced saline and 10% bovine serum albumin). The cells were allowed to bind rabbit anti-mouse IgG/anti-DNP complexes (aggregated anti-DNP IgG) to cell surface Fc receptors at 4 °C for 30 min. After an incubation at 17 °C for 5 min (at this temperature ligand is internalized, but fusion is blocked), cells were incubated at 37 °C for different periods of time in the presence or the absence of the calmodulin antagonists. The cells were then transferred to 4 °C and resuspended in solubilization buffer to measure the immunocomplexes formed as described (see above).

RESULTS

CaM Antagonists Inhibit Endocytic Transport—CaM is a highly conserved intracellular protein expressed in all cell types. CaM acts by interacting with its target proteins through two hydrophobic patches that are exposed after the binding of Ca$^{2+}$. These regions were shown to be involved in the binding of CaM to protein receptor molecules. Naphtalenesulfonamide derivatives such as W7, W12, and W13 are CaM antagonists that bind to CaM in a Ca$^{2+}$-dependent manner through hydrophobic and electrostatic interactions (18).

It has previously been reported that CaM is required for transcytosis and recycling of endocytosed transferrin receptors (12, 13) and low density lipoprotein receptors (19). To assess whether CaM is involved in other steps in the endocytic pathway we studied the colocalization of endocytic markers in vivo. This approach takes advantage of the observation that sequentially internalized ligands, when appropriately timed, catch up to each other within the endosomal pathway. Catch-up is a reflection of the competency of the pathway and the fusion events that govern it (16, 17). Cells were labeled with DNP-β-glucuronidase as the first ligand for 3 min at 37 °C, washed, and then further incubated at 37 °C for 10 min (chase). The second ligand, aggregated anti-DNP IgG, was bound to the cell surface at 4 °C for 30 min and then internalized by incubating the cells for 5 min at 17 °C. At this temperature ligand is endocytosed, but fusion is blocked. Cells were then washed and incubated at 37 °C in the presence of the potent calmodulin antagonist N-(4-aminobutyl)-5-chloro-2-naphtalenesulfonamide (W13) for additional periods of time. As a control, we used W12 (N-4-aminobutyl-naphtalesulfonamide), a compound that is structurally related to W13 but significantly less potent in inhibiting calmodulin activity. Therefore, W12 was used to distinguish calmodulin-specific inhibitory effects from nonspecific drug effects (12, 20). After solubilization, the amount of immune complex formed as a result of delivery of both ligands to the same intracellular compartment was determined as described under “Materials and Methods.” Data represent one of three independent experiments.

Perhaps one of these steps is fusion among endocytic vesicles. CaM Antagonists Inhibit Endosome-Endosome Fusion—To study the possibility that W13 affects endosome fusion we have used an in vitro assay that reconstitutes fusion among endosomes (see “Materials and Methods”). As shown in Fig. 2, the addition of increasing concentrations of W13 completely blocked endosomal fusion (closed circles). Similar to the results obtained in vivo, little effect was observed with W12 at the concentrations indicated (open circles). However, at higher concentrations W12 completely inhibited endosome fusion (data not shown). This is in agreement with the reduced potency of this analog. Similar inhibitory effects were observed with other calmodulin inhibitors such as chlorpromazine and calmidazolium (data not shown).

As mentioned above, naphtalenesulfonamide derivatives
such as W12 and W13 bind to CaM in a Ca\(^{2+}\)-dependent manner through hydrophobic interactions. These compounds block the stimulation of calmodulin-dependent enzymes in a competitive fashion with CaM (18). Therefore, we decided to examine whether the addition of purified CaM would reverse the inhibition caused by the CaM antagonists. Fig. 3 shows that CaM can reverse the inhibitory effect of the CaM antagonist W13. Similar results were obtained when other CaM inhibitors were used (data not shown). These results confirm that the process is CaM-specific and rule out the possibility that the inhibition seen represents CaM antagonist interaction with other proteins.

Interestingly, we found that the addition of Ca\(^{2+}\) (500 nM free Ca\(^{2+}\)) stimulates endosome fusion, even in the absence of cytosol (Fig. 4). This stimulatory effect was inhibited by adding the CaM antagonist W13, implicating CaM in the Ca\(^{2+}\)-stimulated fusion observed in the absence of cytosol. However, it is possible that other Ca\(^{2+}\)-binding proteins may be involved in the process because fusion was not completely abrogated by W13. The further addition of CaM to the assay stimulated fusion beyond that produced by Ca\(^{2+}\) alone (Fig. 5A), but CaM at higher concentrations seems inhibitory, indicating that a strict ratio among factors is critical for endosome fusion. This stimulatory effect was inhibited by W13 (data not shown). Fig. 5B shows the stimulation of endosome fusion by CaM at different cytosol concentrations. It is interesting to note that the stimulatory effect of CaM is mainly observed at limiting cytosol concentrations.

The hydrophobic domains that are exposed after the binding of Ca\(^{2+}\) were shown to be involved in the binding of CaM to protein receptor molecules. CaM modulates the function of the target proteins by exposing sites that either are substrates for catalytic modifications or result in displacement of autoinhibitory domains from catalytic sites (21). This mechanism allows the activation of enzymes such as CaM kinase II, a ubiquitous and multifunctional enzyme that phosphorylates numerous proteins. To study the possibility that CaM kinase II might be involved in fusion among endosomes, we have tested the effect of peptides known to interfere with the mechanism of CaM action. Fig. 6A shows the inhibitory effect of a peptide corresponding to the CaM-binding domain of CaM-dependent kinase II (290–309), a peptide expected to sequester CaM and render it incapable of interacting not only with CaM kinase II but also with other proteins. Therefore, this peptide is considered a strong CaM antagonist. We also tested another peptide from calmodulin-dependent kinase II corresponding to amino acids 281–291. This peptide antagonizes the kinase activity of CaM kinase II by acting as a pseudosubstrate but has very weak anti-CaM activity and consequently does not block the interaction between CaM and other proteins. The inhibitory effect obtained with both peptides suggests that, in our endosome fusion assay, one of the molecules regulated by CaM is CaM-dependent kinase II. Although caution must be exercised when using peptides, we have found that a variety of other peptides...
have no effect on the in vitro fusion assay (data not shown). Moreover, we cannot rule out the possibility that other kinases or other proteins may also be modulated by CaM.

We next tested a nonpeptide inhibitor, KN-93, which is a specific inhibitor of CaM-dependent kinase II (22). Because this inhibitor is insoluble in water but soluble in Me$_2$SO, we carried out a control experiment by the addition of Me$_2$SO alone. As shown in Fig. 6$^B$, the Ca$^{2+}$-mediated stimulation of endosome fusion was to some extent inhibited by KN-93, indicating that CaM kinase II activity is likely to be involved in regulating in vitro endosome fusion.

**DISCUSSION**

Ca$^{2+}$ is an important player in exocytosis of both secretory granules and synaptic vesicles, a process that involves the fusion of these vesicles with the plasma membrane. The role of Ca$^{2+}$ in this special transport event has been extensively studied in intact and permeabilized cell preparations (for a review, see Refs. 23 and 24). Ca$^{2+}$ is also involved in other transport events such as the transfer of proteins from the endoplasmic reticulum to the Golgi (25–27), the fusion of nuclear membrane vesicles after mitosis (28), and the fusion of transcytotic vesicles (29). Recently, it was shown that recycling from the endocytic compartment to the plasma membrane can be up-regulated by elevating the Ca$^{2+}$ concentration (30). Taken together, these observations suggest that Ca$^{2+}$ may be required for intracellular transport as it is required for neurotransmitter release at the nerve terminals.

It is well documented that Ca$^{2+}$ exerts its effect through one or more Ca$^{2+}$-binding proteins that undergo a conformational change upon interaction with the ion. Annexins, a family of Ca$^{2+}$-binding proteins, have been reported to mediate membrane-membrane interactions leading to secretory vesicle fusion (31). It has been shown that annexin II is a major component of fusogenic endosomal vesicles (32), suggesting a role for annexins in intracellular transport. In fact, we have previously shown that annexin II participates in endosome fusion (33). In polarized cells, a role for annexin XIII in transport to the apical membrane has been proposed (34). In a very recent report, a
novel Ca\(^{2+}\)-binding protein, p22, has been implicated in the fusion of transcytotic vesicles (29). Therefore, it is likely that the role of Ca\(^{2+}\) in vesicular transport is a complex matter involving multiple Ca\(^{2+}\)-binding proteins.

Evidence for a role of the Ca\(^{2+}\)-binding protein CaM in various intracellular transport events has been presented. CaM inhibitors were found to have marked effects on phagocytosis, transcytosis, receptor recycling, and lysosomal transport (9, 10, 12, 13). It has been shown that CaM is essential for assembling the links necessary for exocytotic membrane fusion in Paramecium (35). CaM antagonists are potent inhibitors of endothelium tubulation (36), a process that may be involved in the recycling of receptors back to the plasma membrane. In addition, it has been shown that CaM is required for both endocytosis in yeast (37) and rapid endocytosis in adrenal chromaffin cells (38). However, the specific events regulated by CaM are largely unknown. In this report we present evidence that CaM regulates endosome fusion both \textit{in vivo} and \textit{in vitro}. We found that several CaM antagonists inhibit fusion among endosomes, an inhibitory effect that was reversed by purified bovine brain CaM. Furthermore, we observed that Ca\(^{2+}\) stimulates \textit{in vitro} endosome fusion and that the further addition of CaM stimulates fusion above that produced by Ca\(^{2+}\) alone, strongly implicating CaM in the regulation of endocytic transport. These observations are in agreement with recent findings that CaM enhances the fusion of isolated parotid secretion granules with plasma membrane vesicles (39). Our data are also consistent with the stimulatory role for CaM in triggering exocytosis (40–42), suggesting that CaM is a common regulatory molecule governing multiple fusion events.

CaM might act directly in membrane trafficking or act indirectly by modulating an essential component of the vesicular transport machinery. The putative downstream effectors for CaM in endosome fusion remain to be identified. However, several CaM-binding proteins have been identified on endosomes (41). EEA1 is an early endosome-associated protein that has a calmodulin-binding domain (42). Although the function of EEA1 remains to be determined, a role in membrane trafficking has been proposed (42).

The current consensus in the field of intracellular trafficking is that cells use essentially the same or similar machinery in both intracellular transport and neurotransmitter exocytosis (24, 43, 44). Therefore, other putative effectors for CaM arise from the role of this protein in neurotransmitter release. Phosphorylation of synapsin, a synaptic vesicle protein, by CaM-dependent protein kinase II promotes dissociation of synapsin from vesicles, facilitating synaptic vesicle fusion (45, 46). Perhaps a similar mechanism is involved in endosome fusion. Indeed, our results suggest that a CaM-dependent kinase activity seems to be involved in endosome fusion. Our assay was sensitive to KN-93, a CaM kinase II antagonist (22), and to a peptide from CaM kinase II amino acids 281–291 that antagonizes the kinase activity by acting as a pseudosubstrate (47).

Another possible target is the vesicle protein synaptotagmin, which is a Ca\(^{2+}\)-binding protein and also a CaM-binding protein (48, 49). Synaptotagmin is likely to be a Ca\(^{2+}\) sensor in the final fusion step of exocytosis (23, 24). Indeed, Ca\(^{2+}\) regulates the interaction between synaptotagmin and syntaxin 1 (50), one of the components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex (51), which is thought to comprise the core of the exocytic fusion machine (24, 52). It has recently been shown that \(\beta\)-soluble N-ethylmaleimide-sensitive factor attachment protein binds synaptotagmin and recruits N-ethylmaleimide-sensitive factor, suggesting that this complex may link the process of membrane fusion to a Ca\(^{2+}\) signal (53). In a recent publication we have shown that the association of N-ethylmaleimide-sensitive factor with endosomal membranes is regulated by Ca\(^{2+}\) (54). Moreover, we have found that CaM is one of the Ca\(^{2+}\)-binding proteins that regulates the association of factors involved in vesicular transport. These findings suggest that CaM may be required for proper binding and recycling of factors during the normal cycle of vesicle budding, docking, and fusion.

Further understanding of the role of CaM and other Ca\(^{2+}\)-binding proteins in membrane fusion events awaits the identification of key interacting molecules and the specific steps in which these proteins are involved. Further investigation is needed to determine the mechanism by which CaM regulates endosome fusion and other fusion events. However, the results presented in this manuscript suggest a more general role for CaM in vesicular traffic than previously thought.

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