Exosome Release Is Regulated by a Calcium-dependent Mechanism in K562 Cells*

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Multivesicular bodies (MVBs) are endocytic structures that contain small vesicles formed by the budding of an endosomal membrane into the lumen of the compartment. Fusion of MVBs with the plasma membrane results in secretion of the small internal vesicles termed exosomes. K562 cells are a hematopoietic cell line that releases exosomes. The application of monensin (MON) generated large MVBs that were labeled with a fluorescent lipid. Exosome release was markedly enhanced by MON treatment, a Na\(^{+}/H\(^{+}\) exchanger that induces changes in intracellular calcium (Ca\(^{2+}\)). To explore the possibility that the effect of MON on exosome release was caused via an increase in Ca\(^{2+}\), we have used a calcium ionophore and a chelator of intracellular Ca\(^{2+}\). Our results indicate that increasing intracellular Ca\(^{2+}\) stimulates exosome secretion. Furthermore, MON-stimulated exosome release was completely eliminated by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM), implying a requirement for Ca\(^{2+}\) in this process. We have observed that the large MVBs generated in the presence of MON accumulated Ca\(^{2+}\) as determined by labeling with Fluor3-AM, suggesting that intraluminal Ca\(^{2+}\) might play a critical role in the secretory process. Interestingly, our results indicate that transferrin (TF) stimulated exosome release in a Ca\(^{2+}\)-dependent manner, suggesting that TF might be a physiological stimulus for exosome release in K562 cells.

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† The abbreviations used are: MVBs, multivesicular bodies; MON, monensin; TF, transferrin; TIR, TF receptor; AM, acetoxyethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; 2-APB, 2-aminoethoxydiphenylborate; N-Rh-Pe, N-lissamine rhodamine B sulfonyl-phosphatidylethanolamine; PBS, phosphate-buffered saline; PM, plasma membrane, AchE, acetylcholinesterase; TG, thapsigargin; IP\(_{3}\), inositol 1,4,5-triphosphate; N\(_{BAPTA}\), N,N,N',N'-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PM, plasma membrane, AchE, acetylcholinesterase; TG, thapsigargin; IP\(_{3}\), inositol 1,4,5-triphosphate.
late the Ca\textsuperscript{2+}-dependent exosome release, cells were incubated with transferrin (TF). Our results indicate that TF stimulates exosome release in a Ca\textsuperscript{2+}-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI cell culture medium and fetal calf serum were obtained from Invitrogen. EGTA-acetylcholinesterase (AM), BAPTA-AM, and A23187 were purchased from Molecular Probes (Eugene, OR). Fura-2 AM, Fluo-3 AM, xestopenogic C, cyclopiacizonic acid, and 2-aminoethoxy-diphenylborate (2-APB) were from Calbiochem. N-(Lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Acetylochloro-line (5,5′-dithiobis(2-nitrobenzoic acid)) were obtained from Sigma. Bodipy-TR ceramide and Hoechst 33342 were from Molecular Probes, Inc. (Aurora, OH).

**Cell Culture**—K562, a human erythroleukemia cell line, was grown in RPMI supplemented with 10% fetal calf serum, streptomycin (50 \(\mu\)g/ml), and penicillin (50 units/ml). Exosomes were collected from 10 ml of K562 media (15–20 x 10\(^6\) cells) cultured over 7–15 h. The culture media were collected on ice, centrifuged at 800 \(\times\) g for 10 min to sediment the cells, and then centrifuged at 12,000 \(\times\) g for 30 min to remove the cellular debris. Exosomes were separated from the supernatant by centrifugation at 100,000 \(\times\) g for 2 h. The exosome pellet was washed once in a large volume of PBS and resuspended in 100 \(\mu\)l of PBS (exosome fraction).

**Quantitation of Released Exosomes**—The amount of released exosomes was quantitated by measuring the activity of acetylcholinesterase, an enzyme that is specifically directed to these vesicles (12). Acetylcholinesterase activity was assayed following a previously described procedure (22). Briefly, 25 \(\mu\)l of the exosome fraction was suspended in 100 \(\mu\)l of phosphate buffer and incubated with 1.25 mM acetylcholine and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in a final volume of 1 ml. The incubation was carried out in cuvettes at 37 °C, and the change in absorbance at 412 nm was followed continuously. The data represent the enzymatic activity at 20 min of incubation.

As an independent assay, exosomes were quantitated by determining the levels of the protein Hsc70 by Western blot. Samples of the exosome fraction were solubilized in reducing SDS loading buffer, and then centrifuged at 12,000 \(\times\) g for 30 min to remove the cellular debris. Exosomes were separated from the supernatant by centrifugation at 100,000 \(\times\) g for 2 h. The exosome pellet was washed once in a large volume of PBS and resuspended in 100 \(\mu\)l of PBS (exosome fraction).

**Labeling MVBs with the Fluorescent Lipid N-Rh-PE and Fluo-3 AM for Imaging Calcium**—Fluo3-AM (15 \(\mu\)M) served by measuring, in the exosomal fraction, the activity of AChE (see "EXPERIMENTAL PROCEDURES"). As an independent assay, exosomes were quantitated by determining the levels of the protein Hsc70 by Western blot. Samples of the exosomal fraction (15 \(\mu\)l) were solubilized in reducing SDS loading buffer, incubated for 5 min at 95 °C, run on 10% polyacrylamide gels, and transferred to an Immobilon (Millipore) membrane. The membranes were blocked for 1 h in Blotto (5% nonfat milk, 0.1% Tween 20, and PBS) and subsequently washed twice with PBS with 0.1% Tween 20 or Tris-buffered saline with 0.1% Tween 20. Membranes were incubated with primary antibodies and peroxidase-conjugated secondary antibodies. The corresponding bands were detected using an enhanced chemiluminescence detection kit (Pierce) and quantitated by densitometric analysis.

**Labeling MVBs with the Fluorescent Lipid N-Rh-PE and Fluo-3 AM for Imaging Calcium**—The fluorescent phospholipid analog N-Rh-PE was inserted into the plasma membrane as described previously (23). Briefly, an appropriate amount of the lipid, stored in chloroform/methanol (2:1), was dried under nitrogen and subsequently solubilized in absolute ethanol. This ethanolic solution was injected with a Hamilton syringe into serum-free RPMI (<1%, v/v) while vigorously vortexing. The mixture was then added to the cells, which were incubated for 60 min at 4 °C. After this incubation period, the medium was removed, and the cells were extensively washed with cold PBS to remove excess unbound lipids. After the addition of complete RPMI medium and Fluor-3 AM (15 \(\mu\)M), labeled cells were cultured for 2–3 h under conditions as described and washed twice with ice-cold PBS. Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy. In some experiments, the cells were preloaded with Fluor-3 AM by incubating for 60 min at 37 °C before labeling with the fluorescent lipid. No major differences were observed between these experimental procedures.

**Fluorescence Microscopy**—K562 cells were analyzed using an inverted microscope (Nikon Eclipse TE 300, Japan) equipped with the following filter systems: excitation filter 450–490 nm, barrier filter 515 nm to visualize Fluor-3-AM; and excitation filter 510–560 nm, barrier filter 590 nm to localize N-Rh-PE. Images were captured with a CCD camera (Otsuka, Hamamatsu) and processed using the program MetaMorph (Universal Images Corporation). Some images were processed with a Nikon Confocal C1 and processed with the EZ-C1 program.

**Measurement of Intracellular Calcium Concentration**—Cells were incubated in the presence of 10 \(\mu\)M Fura2-AM for 60 min at 37 °C. They were washed to remove the extracellular dye and resuspended in complete RPMI medium containing 1 x 10\(^6\) cells/ml. Fura2-AM loaded cells were protected from light. Experiments were completed within 2 h. Changes in fluorescence after the addition of 7 \(\mu\)M MON or by adding 30 \(\mu\)M BAPTA-AM before MON were analyzed in a Hitachi F-2000 fluorescence spectrophotometer.

**RESULTS**

**Monensin Induces the Formation of Large MVBs and Stimulates Exosome Secretion**—K562 cells are human erythroleukemic cells that secrete exosomes (24), the small internal vesicles released into the extracellular media by fusion of MVBs with the plasma membrane (PM). It has been shown by electron microscopy that treatment of K562 cells with the ionophore MON causes the formation of dilated MVBs (25, 12). Therefore, exosomes were quantitated in exosomes that are eventually secreted into the extracellular medium (12, 26). The lipid N-Rh-PE was first bound to the PM at 4 °C, and cells were washed and subsequently incubated at 37 °C for 3 h in the absence or the presence of 7 \(\mu\)M MON. As shown in Fig. 1A, MON treatment caused the formation of large MVBs labeled by the fluorescent lipid. In Fig. 1B a confocal image of the MVBs formed is shown, with the internal vesicles labeled with the fluorescent lipid clearly depicted. Because fusion of the MVBs with the PM results in the release of exosomes, we tested the effect of MON on the release of exosomes from K562 cells. Exosomes were enriched in proteins such as the transferrin receptor (TfR), Hsc70, and acetylcholinesterase (AChE) (27). Therefore, exosomes were quantitated in the exosomal fraction by measuring the activity of AChE (see “EXPERIMENTAL PROCEDURES”). Also, the amount of Hsc70 and TfR was determined by Western blot as described previously (12). Exosomes were harvested from the extracellular media after 7-h incubations with different concentrations of MON and quantitated by determining the levels of the protein Hsc70 (Fig. 1C) and TfR (not shown) by Western blot. As shown in Fig. 1C, MON induced a marked increase in exosome release in a concentration-dependent manner. A similar increase was observed by measuring, in the exosomal fraction, the activity of AChE (Fig. 1D), which was maximal at 10 \(\mu\)M MON. At higher MON concentrations some alterations in cell viability were observed as assessed by trypan blue exclusion, for which reason a 7 \(\mu\)M concentration of MON was used in the rest of the experiments. At this concentration, cells were also assayed for apoptosis by staining the nucleus with Hoechst 33342 (Molecular Probes). No morphological evidence of apoptotic nuclei was observed (data not shown).

In some experiments, the amount of exosomes released was also quantified by assaying the fluorescent lipid N-Rh-PE. As mentioned above, this lipid accumulates in intracellular vesicles that are ultimately secreted into the extracellular medium as exosomes. As expected, MON also increased the release of exosomes labeled with the fluorescent lipid (data not shown). Taken together the results indicate that MON not only generates large MVBs but also increases the secretion of the internal vesicles termed exosomes.

**A Calcium-dependent Mechanism Is Involved in the Monensin-stimulated Exosome Release**—It has been shown that MON, a Na\textsuperscript{+} ionophore, can increase cytosolic Ca\textsuperscript{2+} by reversing the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange mechanism (14–16). Therefore, to assess whether in our system the enhanced exosome release induced by MON was due to an increase in intracellular Ca\textsuperscript{2+}, we first measured whether MON could modify the intracellular Ca\textsuperscript{2+}.
concentration in K562 cells. For this purpose, cells were loaded for 1 h at 37 °C with 10 μM Fura-2/AM. Subsequently, the intracellular Ca\(^{2+}\) concentration was measured by spectrofluorometry for different periods of time after the addition of MON. Fig. 2A shows that there was an initial Ca\(^{2+}\) peak and a subsequent marked rise in intracellular Ca\(^{2+}\) that was sustained over the 2-h period tested (Fig. 2B). The MON-induced Ca\(^{2+}\) rise was abolished by the previous addition of the intracellular Ca\(^{2+}\) chelator BAPTA-AM (Fig. 2A). Interestingly, in the presence of the extracellular Ca\(^{2+}\) chelator EGTA, MON induced the initial rise, which was likely due to Ca\(^{2+}\) release from intracellular stores. However, no sustained increase was observed, indicating that the latter is a result of Ca\(^{2+}\) influx from the extracellular environment (data not shown).

The results suggest that the increase in exosome release might be due to a Ca\(^{2+}\)-dependent mechanism. To test this hypothesis, we assessed whether the MON effect on exosome release could also be prevented by Ca\(^{2+}\) chelators. To chelate the Ca\(^{2+}\) present in the extracellular media, cells were incubated for several hours in the presence of 1.5 mM EGTA. Under these conditions, the free Ca\(^{2+}\) concentration was less than 10 nM as calculated with the Sliders program (see “Experimental Procedures”). BAPTA-AM was used to chelate intracellular Ca\(^{2+}\), because this is a membrane-permeable agent that efficiently chelates Ca\(^{2+}\). The released exosomes were collected from the media and quantitated by measuring AChE activity as indicated under “Experimental Procedures.” As shown in Fig. 2C, both EGTA and BAPTA-AM decreased, although slightly, the basal release of exosomes. Moreover, the MON-dependent increase was completely abrogated by the Ca\(^{2+}\) chelators, and no additive effects were observed when both chelators were added together (data not shown). The result clearly indicates that Ca\(^{2+}\) from the extracellular media and also from intracellular stores is required for the MON-induced exosome secretion.

Ca\(^{2+}\) involvement in exosome release was evaluated using the Ca\(^{2+}\) ionophore A23187. As shown in Fig. 3A, incubation with the Ca\(^{2+}\) ionophore stimulated exosome secretion to a similar extent as MON. No additive effects were observed when both agents were added together. As expected, the secretory effect of the Ca\(^{2+}\) ionophore was inhibited by the chelators EGTA or BAPTA-AM (Fig. 3B).

It is known that MON acts on acidic compartments by altering the proton gradient across vesicle membranes, resulting in a Ca\(^{2+}\) movement into the cytosol (28). We tested the effect of two agents known to alter the pH of vacuolar compartments, the weak base chloroquine and the vacuolar proton pump inhibitor bafilomycin A1 (29). Previous work has shown that these compounds may also discharge intracellular Ca\(^{2+}\) pools from acidic compartments (30, 31). We have evidence that chloroquine elevated intracellular Ca\(^{2+}\) in a similar manner to MON (not shown). As shown in Fig. 4A, chloroquine stimulated the release of exosomes, although to a lesser degree than MON. Non-additive effects were observed by the addition of chloroquine together with MON. Bafilomycin also increased the release of exosomes (Fig. 4B). Both chloroquine and bafilomycin effects were abrogated by clamping extracellular Ca\(^{2+}\) with the chelator EGTA, indicating that these compounds indeed act via a calcium-dependent mechanism.

Visualizing a MVB Calcium Pool by Fluo3-AM Imaging—Numerous reports indicate the existence of several intracellular Ca\(^{2+}\) pools (32); for a review see Refs. 33 and 34. Fluo3-AM is a membrane-permeant compound that accumulates in the cytoplasm where cytosolic esterases clip the AM groups, rendering the fluorescent probe membrane impermeable. However, it has been shown that, when used at higher concentrations, part of this indicator is capable of accumulating also in intracellular compartments and can be used as an indicator for intracellular Ca\(^{2+}\) stores (35). Cells were incubated with Fluo3-AM for 1 h at 37 °C to visualize the calcium-containing compartments. MVBs were labeled with the fluorescent lipid N-Rh-PE as mentioned above, and the cells were subsequently incubated with the indicated agents for 3 h at 37 °C. As shown in Fig. 5, the large MVBs induced by MON treatment were clearly labeled by Fluo3-AM, indicating that Ca\(^{2+}\) accumulates...
in these intracellular compartments. Similarly, Ca\(^{2+}\) was also present in the large MVBs formed by chloroquine treatment. The presence of BAPTA-AM depleted the MVBs calcium pool in both conditions. Strikingly, the size of the MVBs was markedly reduced, indicating that a calcium-dependent mechanism is involved in the development of the gigantic MVBs formed by MON or chloroquine treatment.

Calcium Levels Regulated by IP\(_3\) Receptors and a Thapsigargin-sensitive Ca\(^{2+}\) Pump Are Involved in the Release of Exosome—It is well established that thapsigargin (TG) causes a rapid inhibition of the calcium-ATPase pump present in the membranes of the endoplasmic reticulum (36), followed by a fast Ca\(^{2+}\) leak from other Ca\(^{2+}\) stores as well as influx from the extracellular media. This leads to a rapid and pronounced increase in the concentration of cytosolic-free calcium. As expected, treatment of K562 wells with this inhibitor stimulated exosome secretion in a similar manner as MON, and this effect was also blocked by EGTA (Fig. 6A). These findings confirmed a role for Ca\(^{2+}\) in the exosome secretory pathway and the participation of a TG-sensitive Ca\(^{2+}\) pump in the process.

Because TG stimulated exosome release, we were interested in knowing whether the large MVBs developed by MON were also formed by treatment with TG. As shown in Fig. 6B, TG neither generated large MVBs nor impaired the formation of the MON-induced gigantic structures that were filled with calcium. This suggests that an increase in cytosolic Ca\(^{2+}\) is not by itself enough to generate the enlarged MVBs, despite being sufficient to stimulate exosome secretion.

The phosphoinositide signaling cascade plays a prominent role in the regulation of exosome release. This pathway involves the activation of phospholipase C, which cleaves phosphatidylinositol bisphosphate to produce inositol triphosphate (IP\(_3\)) and diacylglycerol. IP\(_3\) then binds to IP\(_3\) receptors on the endoplasmic reticulum, causing calcium release. Diacylglycerol activates protein kinase C, which can also influence calcium release.

This dual mechanism of calcium mobilization provides a potential explanation for the observed effects of TG on exosome release. The rapid and pronounced increase in cytosolic Ca\(^{2+}\) elicited by TG is likely due to the inhibition of the calcium-ATPase pump, while the slower increase observed in response to MON might be mediated by other mechanisms, such as the release of calcium from intracellular stores or influx from the extracellular medium.
role in the mobilization of Ca\(^{2+}\) from intracellular stores (for a review see Refs. 37 and 38). The receptors for the second messenger, inositol 1,4,5-trisphosphate (IP\(_3\)), constitute a family of Ca\(^{2+}\) channels responsible for the mobilization of intracellular Ca\(^{2+}\) stores. The increase in the levels of IP\(_3\) result in the opening of Ca\(^{2+}\) channels present in the endoplasmic reticulum and the subsequent release of Ca\(^{2+}\) into the cytosol (39).

To test whether this type of channel might be involved in the calcium-induced exosome release, cells were incubated with 2-APB, a membrane-permeable inhibitor of IP\(_3\)-induced Ca\(^{2+}\) release. Fig. 6C shows that 2-APB inhibited monensin-stimulated exosome release. Similar results were obtained with xestospongin, a potent blocker of IP\(_3\) receptors (data not shown), indicating that a transient Ca\(^{2+}\) rise mediated by the stimulation of an IP\(_3\) receptor is critical for the MON-stimulated exosome release. However, the formation of the large Ca\(^{2+}\)-rich MVBs induced by MON was not completely abrogated by 2-APB (Fig. 6D), although there was a decrease in the size of the MVBs compared with the vesicles generated by MON in the absence of 2-APB. Vesicle area in the MON-treated cells was 436 ± 30 (relative units), whereas the addition of 2-APB reduced the size to 182 ± 16 (n = 50 vesicles counted). This suggests that the release of Ca\(^{2+}\) via the IP\(_3\)-sensitive Ca\(^{2+}\) channels contributes, at least in part, to the generation of the enlarged MVBs.

FIG. 6. Changes in the MVB size induced by calcium or calcium chelators. A. Cells were incubated for 7 h in the presence of 10 μM monensin (Chlor), 7 μM monensin (Mon), or 7 μM monensin/10 μM chloroquine (Chlor + Mon) (panel A) and with 10 μM chloroquine (Chlor), 10 μM bafilomycin A1 (Baf), 10 μM chloroquine/1.5 mM EGTA (Chlor + EGTA), or bafilomycin/1.5 mM EGTA (Baf + EGTA) (panel B). The secreted exosomes were collected and quantitated by measuring the AChE activity. Asterisk, significantly different from the control, p < 0.01. Fragmented diamond, significantly different from the Chlor- or Baf-treated cells, p < 0.01.
Fig. 6. Calcium from internal stores regulates exosome release. Cells were incubated for 7 h in the presence of 7 μM MON (Mon), 1 μM thapsigargin (Tg), 1 μM thapsigargin/7 μM monensin (Tg + Mon) or 1 μM thapsigargin/1.5 mM EGTA (Tg + EGTA) and with 7 μM MON (Mon), 100 μM 2-APB, or 7 μM MON/100 μM 2-APB (Mon + 2-APB). The secreted exosomes were collected and quantitated by measuring the AChE activity. Asterisk, significantly different from the control; p < 0.01. Fragmented diamond, significantly different from the thapsigargin-treated cells, p < 0.01 (panel A), or 2-APB-treated, p < 0.05 (panel C). B and D, K562 cells were loaded with 15 μM Fluo-3-AM for 1 h at 37 °C. Cells were then washed twice with PBS buffer and labeled with the fluorescent lipid analog N-Rh-PE and incubated for 3 h at 37 °C in the presence of 1 μM thapsigargin (Tg), 7 μM MON/1 μM thapsigargin (Tg MON), 100 μM 2-APB, or 7 μM MON/100 μM 2-APB (2-APB MON). Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy. Left panels, N-Rh-PE (red); middle panels, Fluo-3-AM (green); right panels, merged images.

DISCUSSION

In this study we have shown that the ionophore MON induces the formation of large MVBS and stimulates the release of the internal vesicles called exosomes. It has been shown that MON induces catecholamine secretion from adrenal chromaffin cells (14, 42). The release of regulated secretory granules is known to be Ca2+-dependent. Here, we present evidence that the MON-stimulated exosome secretion in K562 cells is indeed a calcium-dependent event. The application of MON generated a marked elevation of Ca2+ that was dependent on both an extracellular source and intracellular Ca2+ stores. The role for Ca2+ on exosome release was confirmed by the use of the Ca2+ ionophore A23187. Similar results were obtained with agents known to alter the pH of vacuolar compartments such as chloroquine and bafilomycin, an inhibitor of the proton pump. Both compounds stimulated exosome release via a Ca2+-dependent mechanism, because the effect was abrogated by BAPTA-AM. We have evidence that chloroquine elevated intracellular Ca2+ in a similar manner as MON. These data are in agreement with previous observations indicating that chloroquine causes a substantial Ca2+ release in the parasite Plasmodium chabaudi (30). Similarly, it has been shown that bafilomycin alters cytosolic Ca2+ by discharging intracellular Ca2+ pools in lizard red blood cells (31). Therefore, taken together, our results clearly indicate that Ca2+ is a key participant in the exosome release process. We believe that this is a relevant observation because, depending on their origin, exosomes can play roles in different physiological processes (8). For example, activated platelets release exosomes at sites of vascular injury where they may have a signaling/adhesion function (7). Antigen-presenting cells also secrete exosomes that carry peptide-loaded MHC molecules functioning as intercellular vehicles for antigenic material. Therefore, our observation that Ca2+ regulates exosome release suggests that a signal transduction mechanism is likely involved in the activation of exosome-carrying cells to release these small vesicles at the proper site.

The use of MON allowed us to assess that Ca2+ plays an important role in exosome secretion. It has been proposed that MON acts on acidic intracellular organelles such as endosomes and lysosomes, exchanging H+ for Na+ and causing swelling of these vesicles by passive water influx (44). Therefore, it would be possible that a similar mechanism might be involved in the generation of the large MVBS formed after MON treatment. However, our results indicate that the formation of the enlarged endosomes is a calcium-dependent event, because this process was completely abrogated by the Ca2+ chelator BAPTA-AM. It is tempting to speculate that the formation of these gigantic endosomes is not only due to swelling of the vesicles but requires also the contribution of membranes from other sources, implying fusion among vesicular compartments. It is becoming evident that many intracellular transport events depend on Ca2+ (45) and, thus, it is likely that Ca2+ might be required for the fusion events involved in the generation of the enlarged MVBS. Experiments are underway to address this possibility.

MON, as a Na+ ionophore, transports a molecule of Na+ inside the cells per molecule of H+ transported to the extracellular media. The increase in intracellular Na+ activates the
Calcium-dependent Exosome Release

**Fig. 7. Amiloride inhibits exosome release and the formation of the large MBVs.** A, K562 cells were incubated for 7 h in the presence of 7 μM MON (Mon), 15 mM amiloride (Amil), or 7 μM MON/15 mM amiloride (Mon + Amil). The secreted exosomes were collected and quantitated by measuring the acetylcholinesterase activity. Asterisk, significantly different from the control, p < 0.01. Fragmented diamond, significantly different from the MON-treated cells, p < 0.01. B, cells were loaded with 15 μM Fluor3-AM for 1 h at 37 °C. Cells were then washed twice with PBS buffer, labeled with the fluorescent lipid analog N-Rh-PE, and incubated for 3 h at 37 °C in the presence of 15 mM amiloride or 15 mM amiloride/7 μM MON. Samples were protected from light during the whole experimental period. Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy. Left panels, N-Rh-PE (red); middle panels, Fluo3-AM (green); right panels, merged images.

Na⁺/Ca²⁺ exchanger in a reverse mode, leading to an initial increase in cytosolic Ca²⁺ (16). Our data are in agreement with a requirement for an early entrance of Na⁺, because the MON-mediated effects were completely abrogated by amiloride, an inhibitor of the H⁺/Na⁺ and Na⁺/Ca²⁺ exchangers. Our data are also compatible with the idea that Na⁺ entry mediated by MON could activate the production of IP₃, which in turn releases Ca²⁺ from intracellular stores. Subsequently, the emptiness of these stores might trigger the opening of store-operated Ca²⁺ channels (SOC) at the plasma membrane that sustains a sustained Ca²⁺ rise consistent with the requirement for extracellular Ca²⁺ in our system. Data presented in this report indicate that IP₃-dependent channels are involved in MON-stimulated exosome release, indicating that a transient Ca²⁺ rise mediated by the stimulation of IP₃ receptors is critical for this event. However, the formation of the enlarged MVBs was only partially inhibited and not completely abrogated by inhibition of the IP₃ receptors, suggesting that an additional mechanism might be involved in the generation of these large endosomes.

Interestingly, we have observed that the large MVBs generated by MON-treatment are filled with Ca²⁺. It is likely that the MON-stimulated antiport activity might be exerted primarily at the plasma membrane, but it is also possible that a similar effect occurs on intracellular membranes. Indeed, it has been shown that MON selectively induces the secretion of azurophil granule contents by directly affecting the granule membrane (46). Swanson and co-workers (28) have reported that lysosomes contain high concentration of Ca²⁺ and therefore could function as an intracellular Ca²⁺ source. These authors have shown that changes in lysosomal pH resulted in the movement of vacuolar Ca²⁺ out of lysosomes into the cytoplasm, possible via pH-dependent calcium channels or pumps. Our results indicate that, even though a thapsigargin-sensitive Ca²⁺ pump is involved in the release of exosomes, inhibition of this pump does not block either the formation of the gigantic MVBs or the accumulation of Ca²⁺ inside the large membranous structures. Therefore, it is likely that another type of Ca²⁺ pump, present in intracellular compartments, might be responsible for filling the enlarged endosomes. PMR1, a Ca²⁺ ATPase present in yeast Golgi (47) similar to the plasma membrane Ca²⁺ ATPase (PMCA), is thapsigargin-insensitive. A Ca²⁺ ATPase in the Golgi of mammary tissue (48) with characteristics slightly different from the PMCA and the endoplasmic reticulum Ca²⁺ ATPase (ERCA) has also been identified. This mammary Golgi secretory pathway Ca²⁺-ATPase (SPCA) seems to be a homologue to the yeast PMR1 and is believed to be important for the function of the secretory pathway. Interestingly, we have recently shown that MVBs in K562 cells are formed, at least in part, by membrane influx from Golgi compartments (12). Furthermore, it has been shown that endocytic vesicles from reticulocytes possess a Ca²⁺-ATPase that pumps Ca²⁺ into the lumen of the vesicles (49). Therefore, it would not be unprecedented that a thapsigargin-insensitive Ca²⁺ pump
is involved in the formation of the enlarged MVBs and the accumulation of the lumenal Ca2+.

An interesting possibility is that the Ca2+ present inside the MVs is playing a critical role in the exosome secretory process. A role for intravesicular Ca2+ in several secretory and intracellular fusion events has been proposed. Fusion of early endosomes seems to require the release of luminal Ca2+ for fusion to occur (50). Also, in insulin-secreting cells it has been shown that Ca2+ depletion from granules inhibits exocytosis (21). Indeed, the presence of IP3 receptors on insulin and somatostatin secretory granules has been demonstrated, suggesting that these organelles represent a readily mobilizable IP3-regulated Ca2+ pool during the secretory process (52). As we mentioned above, it is known that MON disrupts proton gradients across the membranes, allowing the release of intravesicular Ca2+. Therefore, it is possible that the localized release of Ca2+ from the MVs at the docking site may play an active role in the fusion complex/pore formation. Even though our results clearly indicate that Ca2+ is a critical participant in the MON-stimulated exosome secretion, further experiments are required to elucidate whether the MVB luminal Ca2+ participates in this process.

Finally, we have presented data indicating that Tf increases intracellular Ca2+ in K562 cells and stimulated exosome release in a Ca2+-dependent manner, suggesting that the secretion of exosomes is under physiological control. Our results are consistent with previous observations that the binding of transferrin to its receptor increases intracellular Ca2+ and stimulates receptor recycling in L2C cells (40). Also, transferrin recycling was stimulated by Ca2+ in bovine chromaffin cells (53). Our results suggest that a component of the vesicular transport machinery involved in exosome secretion is regulated by Ca2+. Proteins such as synaptotagmins and calmodulin have been implicated in vesicular transport events where they function as Ca2+ sensors (54). Therefore, Tf, by increasing intracellular Ca2+, may modulate some critical components of the transport/fusion machinery. Interestingly, in a recent paper it has been shown that the binding of apotransferrin to its receptor increased, via a calcium-calmodulin-dependent kinase, the levels of tubulin and actin, proteins known to be involved in vesicular trafficking (41). In conclusion, the TfR seems to function as a signal transduction molecule that modulates not only its own recycling but also its shedding via the exosome pathway, and Ca2+ is one of the components of this signaling pathway triggered by Tf binding. It is important to mention that exosomes released to circulation from maturing red cells are the principal source of the soluble, circulating, truncated TfR (24). Soluble TfR levels 8-fold greater than normal have been reported in hemolytic anemias (51). Even though the physiological role of the soluble truncated TfR (43) has not been established yet, our observation that Tf stimulates exosome release and, as a consequence, the availability of soluble TfR, led us to speculate that this is a mechanism to regulate the free levels of circulating Tf.

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REFERENCES

1. Clayton, A., Court, J., Navalh, A., Adams, M., Mason, M. D., Hobt, J. A., Neyman, G. R., and Kabani, B. (2001) J. Immunol. Methods 247, 163–174.
2. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002) Traffic 3, 321–330.
3. Thiery, C., Zitvogel, L., and Amigorena, S. (2002) Nat. Immunol. 3, 569–579.
4. Johnston, R. M., Mathew, A., Mason, A. B., and Teng, K. J. (2001) J. Cell. Physiol. 147, 273–280.
5. Harding, C., Heuser, J., and Stahl, P. (1983) J. Cell Biol. 97, 329–339.
6. Harding, C., Heuser, J., and Stahl, P. (1984) Eur. J. Cell Biol. 35, 256–263.
7. Hejine, H. F., Schiel, A. E., Fijneheer, R., Geuze, H. J., and Sixma, J. J. (1999) Blood 94, 3791–3799.
8. Denzer, K., Kleijmeer, M. J., Heijine, H. F., Stoorvogel, W., and Geuze, H. J. (2000) J. Cell Sci. 113, 3365–3374.
9. Peter, P. J., Geuze, H. J., Van der Donk, H. A., Slot, J. W., Griffith, J. M., Slan, N. J., Clevers, H. C., and Borst, J. (1989) J. Exp. Med. 163, 1161–1172.
10. Savina, A., Vidal, M., and Colombo, M. I. (2002) J. Cell Sci. 115, 2505–2515.
11. Pressman, B. S. (1976) Ann. Rev. Biochem. 45, 501–530.
12. Nassar-Gentina, V., Rojas, E., and Luxoro, M. (1994) J. Biol. Chem. 269, 3745–3750.
13. Denzer, K., Kleijmeer, M. J., Heijne, H. F., Stoorvogel, W., and Geuze, H. J. (2001) J. Cell Biol. 152, 1079–1087.
14. Beraldo, F. H., Sartorello, R., Lanari, R. D., and Garcia, C. R. (2001) J. Biol. Chem. 276, 507–514.
15. Mills, I. G., Urbe, S., and Clague, M. J. (2001) J. Cell Sci. 114, 1959–1965.
16. Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1998) Nat. Med. 4, 594–600.
17. Rizzuto, R. (2001) Cell Calcium 29, 349–345.
18. Martinez, J. R., Willis, S., Puente, S., Wells, J., Helmke, R., and Zhang, G. H. (1996) J. Biol. Chem. 271, 1703–1709.
19. Vidal, M., Mangreat, P., and Hoekstra, D. (1997) J. Cell Biol. 138, 1863–1877.
20. Johnston, R. M., Bianchini, A., and Teng, K. (1989) Blood 74, 1844–1851.
21. Christensen, K. M., and Swanson, J. (2001) J. Cell Biol. 151, 599–607.
22. Passos, A. P., and Garcia, C. R. (1998) Biochem. Biophys. Res. Commun. 245, 155–160.
23. Rinaldi, B. F., Sartorello, R., Lanari, R. D., and Garcia, C. R. (1991) Cell Calcium 10, 439–445.
24. Martina, B. G., Hand, B. H., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470.
25. Takahashi, A., Cameron, F., Lechleiter, J. D., and Herman, B. (1999) J. Biol. Chem. 274, 11089–11125.
26.atheringn, A., and Bell, G. I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 517–521.
27. Tartakoff, A. M. (1983) Cell 32, 1026–1028.
28. Mayer, A. (1999) Curr. Opin. Cell Biol. 11, 447–452.
29. Pittschen, C., and Henson, P. M. (1991) J. Leukocyte Biol. 50, 517–528.
30. Sorin, A., Rosas, G., and Rao, R. (1997) J. Biol. Chem. 272, 9895–9901.
31. Reinhardt, T. A., Floten, A. G., Penniston, J. T., and Horst, R. L. (2000) Am. J. Physiol. Cell Physiol 279, C1595–C1602.
32. Kohgo, T., Torimoto, Y., and Kato, J. (2002) Int. J. Hematol. 76, 213–218.
33. Tartakoff, A. M. (1983) Cell 32, 1026–1028.
34. Mayer, A. (1999) Curr. Opin. Cell Biol. 11, 447–452.
35. Pittschen, C., and Henson, P. M. (1991) J. Leukocyte Biol. 50, 517–528.
36. Sorin, A., Rosas, G., and Rao, R. (1997) J. Biol. Chem. 272, 9895–9901.
37. Reinhardt, T. A., Floten, A. G., Penniston, J. T., and Horst, R. L. (2000) Am. J. Physiol. Cell Physiol 279, C1595–C1602.
38. Vidal, M., Leiferre, F., Rosat, B., Sainte-Marie, J., and Philippon, J. (1995) Biochim. Biophys. Acta 1247, 163–174.
39. Mills, I. G., Urbe, S., and Clague, M. J. (2001) J. Cell Biol. 151, 189–195.
40. Therien, K., and Romano, I. (1999) Stand. J. Clin. Lab. Invest. Suppl. 215, 113–120.
41. Blondel, O., Mordt, M. M., Depaoli, A. M., Sharp, A. H., Ross, C. A., Swift, H., and Bell, G. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7777–7781.
