JCB Report

Calcium Regulates the Association between Mitochondria and a Smooth Subdomain of the Endoplasmic Reticulum

Hui-Jun Wang,* Ginette Guay,* Liviu Pogan,‡ Remy Sauvé,‡ and Ivan R. Nabi*

*Department of Pathology and Cell Biology and ‡Department of Physiology, University of Montreal, Montreal, Quebec, Canada H3C 3J7

Abstract. Association between the ER and mitochondria has long been observed, and the formation of close contacts between ER and mitochondria is necessary for the ER-mediated sequestration of cytosolic calcium by mitochondria. Autocrine motility factor receptor (AMF-R) is a marker for a smooth subdomain of the ER, shown here by confocal microscopy to be distinct from, yet closely associated with the calnexin- or calreticulin-labeled ER. By EM, smooth ER AMF-R tubules exhibit direct interactions with mitochondria, identifying them as a mitochondria-associated smooth ER subdomain. In digitonin-permeabilized MDCK cells, the addition of rat liver cytosol stimulates the dissociation of smooth ER and mitochondria under conditions of low calcium. Using BAPTA chelators of various affinities and CaEGTA buffers of defined free Ca^{2+} concentrations and quantitative confocal microscopy, we show that free calcium concentrations <100 nM favor dissociation, whereas those >1 μM favor close association between these two organelles. Therefore, we describe a cellular mechanism that facilitates the close association of this smooth ER subdomain and mitochondria when cytosolic free calcium rises above physiological levels.

Key words: free calcium • autocrine motility factor receptor • AMF-R tubule • cytosol • semipermeabilized cell assay

Introduction

A close association between ER and mitochondria has been described in various cell types. Close contacts between the ER membrane and the mitochondrial outer membrane have been visualized by EM (Franke and Kartenbeck, 1971; Morre et al., 1971; Montisano et al., 1982), and recent electron tomography studies have identified 15-nm diameter sites of close contact between the membranes of these two organelles (Perkins et al., 1997). Subcellular fractionation of cells identified an ER membrane fraction that copurifies with mitochondria (Lewis and Tata, 1973; Shore and Tata, 1977) and ER-derived mitochondria-associated membrane (MAM)[1] fractions have been implicated in phospholipid transfer between the two organelles (Vance, 1990; Shiao et al., 1998; Achleitner et al., 1999).

Address correspondence to Dr. Ivan R. Nabi, Département de pathologie et biologie cellulaire, Université de Montréal, C. P. 6128, succursale A, Montréal, Québec, Canada H3C 3J7. Tel.: (514) 343-6291. Fax: (514) 343-2459. E-mail: ivan.robert.nabi@umontreal.ca

[1]*Abbreviations used in this paper:* AMF-R, autocrine motility factor receptor; IP3, inositol 1,4,5-triphosphate; MAM, mitochondria-associated membrane; Mt-HSP70: mitochondrial heat shock protein 70.

Interaction between ER and mitochondria has also been implicated in the sequestration and regulation of free cytosolic calcium (Ca^{2+}; for review see Pozzan et al., 1994). Ca^{2+} release due to the generation of inositol 1,4,5-triphosphate (IP3) in response to receptor activation results in the formation of high local concentrations of Ca^{2+} at sites of close contact between ER and mitochondria that results in increases in mitochondrial Ca^{2+} (Rizzuto et al., 1993, 1998; Simpson et al., 1997). ER-mediated Ca^{2+} uptake by mitochondria may activate mitochondrial metabolic activity by stimulating Ca^{2+}-dependent mitochondrial dehydrogenases (Denton and McCormack, 1990; Rutter et al., 1996; Jouaville et al., 1999), as well as regulate the subcellular pattern of Ca^{2+} oscillations generated by receptor activation (Rizzuto et al., 1994; Landolfi et al., 1998; Robb-Gaspers et al., 1998; Hajnoczky et al., 1999). Close contacts between ER and mitochondria, particularly under conditions of high local free cytosolic Ca^{2+}, are therefore of functional importance.

We have identified a smooth ER subdomain specifically labeled for autocrine motility factor receptor (AMF-R;

Benlimame et al., 1995). By EM, smooth AMF-R–labeled tubules exhibit continuity with the ribosome-studded tubules of the rough ER, and after treatment with ilimaquinone, form a highly fenestrated network of smooth tubules morphologically equivalent to the smooth ER of the hepatocyte (Benlimame et al., 1995; Wang et al., 1997). AMF-R tubules can be distinguished from the ER-Golgi intermediate compartment (ERGIC) by fluorescence microscopy and therefore represent a distinct smooth ER subdomain (Wang et al., 1997). We show here that smooth ER AMF-R tubules are intimately associated with mitochondria. Using a semipermeabilized cell assay, we further show that this association can be disrupted by cytosol in the presence of low cytosolic free Ca^{2+} levels. Elevated cytosolic free Ca^{2+} inhibits the ability of cytosol to dissociate the two organelles identifying a mechanism that facilitates the close association between the ER and mitochondria necessary for mitochondrial Ca^{2+} uptake.

Materials and Methods

Cells, Antibodies, and Chemicals

MDCK II cells were grown in DME supplemented with 10% FCS, nonessential amino acids, vitamins, glutamine, and a penicillin–streptomycin antibiotic mixture in an air 5% CO_2 incubator at constant humidity.

Monoclonal IgM antibody against AMF-R was used in the form of concentrated hybridoma supernatant (Nabi et al., 1990). Polyclonal antibody to calreticulin was kindly provided by Dr. Luis Rokeach (Department of Biochemistry, Université de Montréal, Quebec, Canada) and to calnexin by Dr. John Bergeron (Department of Cell Biology and Anatomy, McGill University, Montreal, Quebec, Canada). Antibody to mitochondrial heat shock protein 70 (Mt-HSP70; clone JG1) was purchased from Affinity Bioreagents, Inc. Secondary antibodies conjugated to either Texas red or FITC were purchased from Jackson ImmunoResearch Laboratories. DTT was purchased from Sigma-Aldrich, and digitonin was purchased from ICN. The K_2H_2EGTA and $K_2CaEGTA$ solutions (Calibration Buffer Concentrate Kit) used to prepare the CaEGTA buffers. 5,5′-dimethyl BAPTA-AM, BAPTA-AM, 5,5′-difluoro BAPTA-AM, and 5,5′-dibromo BAPTA-AM were purchased from Molecular Probes.

Confocal and Electron Microscopy

Immunofluorescence labeling of 80/20% (vol/vol) methanol/acetone fixed MDCK cells for AMF-R, calnexin, calreticulin, and Mt-HSP70, and postembedding immunoelectron microscopic labeling for AMF-R were performed as previously described (Benlimame et al., 1995; Wang et al., 1997). Confocal images were obtained with a BioRad MRC-600 confocal microscope and EM images with a Phillips 300 electron microscope. The minimal ER-mitochondria distance was quantified by measuring from 14 randomly taken EM images (21,000×), the distance between the point on each ER tubule, smooth or rough, labeled or not for AMF-R, closest to a mitochondrion within the same cell. Rough ER tubules were identified by the presence of a linear array of membrane-associated ribosomes.

Preparation of Rat Liver Cytosol

Rat liver cytosol was prepared from cleaned rat liver cut into small pieces by scissors in buffer A (25 mM Tris, 25 mM KCl, 1 mM DTT, pH 7.4) supplemented with 85 mM sucrose and protease inhibitors at 4°C and homogenized at 4°C in 1 vol of the same buffer. The homogenate was centrifuged at 11,060 *g* for 20 min and the supernatant recentrifuged at 100,000 *g* for 90 min at 4°C. The supernatant was collected and assayed for protein concentration. Cytosol was heat-inactivated by boiling for 5 min.

In Vitro Assay for Smooth ER Mitochondria Association

MDCK cells plated at 25,000–50,000 cells/35-mm plate on glass cover slips for 2 d were quickly washed twice with cytoskeleton stabilizing buffer (CSB; 130 mM Hepes, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) prewarmed to 20°C and then incubated with 10 μg/ml digitonin in buffer A for 1 min at 20°C. The cells were then washed with CSB and incubated in buffer A, supplemented as indicated in the text, for 1 h at 37°C. The cells were washed twice with CSB containing 0.2% BSA, fixed with methanol/acetone and labeled for AMF-R (Texas red) and Mt-HSP70 (FITC). The gain and black level of the confocal images were adjusted for each slide for both the FITC and rhodamine channels such that the full dynamic range of the instrument was used. For each experiment, confocal images from all slides were obtained in one sitting using equivalent pinhole settings and the extent of overlap of AMF-R tubule labeling with mitochondria was determined using Northern Eclipse software (Empix Imaging). The nonspecific AMF-R labeling of the nuclei was first cut from the image and the total number of cytoplasmic pixels labeled for AMF-R determined. Using a convolution filter, the mitochondrial labeling was enlarged to ensure that shifts between the FITC and Texas red images and cellular variations in the intensity of the mitochondrial image did not influence the measure of the extent of AMF-R tubule overlap with mitochondria. Using the enlarged mitochondrial labeling as a mask, AMF-R labeling that coincided with the enhanced mitochondrial labeling was deleted and quantification of the remaining AMF-R–labeled pixels provided a measure of smooth ER AMF-R tubules that had dissociated from mitochondria. This value was divided by the total AMF-R–labeled cytoplasmic pixels to generate the percent dissociation of AMF-R tubules from mitochondria.

Ca^{2+} Measurements

To prepare the CaEGTA buffers, 100 mM stock solutions of K_2H_2EGTA and $K_2CaEGTA$ were diluted in buffer A to 25 mM. The resulting buffers were then mixed to generate 8 different 25-mM CaEGTA buffers containing 0, 5, 10, 15, 20, 22.13, 22.92, and 25 mM total Ca^{2+} (see Table I). Predicted free Ca^{2+} concentrations were calculated using WEBMAXCv1.10 at the CPatton MaxChelator website.

The free Ca^{2+} concentration of the Ca^{2+}-EGTA buffer solutions ($[Ca^{2+}]_{SOL}$) was estimated with the Ca^{2+} sensitive fluorophor Fura-2 used as a potassium salt at a concentration of 2.5 μM. Fluorescence measurements were performed using a dual-excitation spectrofluorometer (Spex Fluorolog II; Spex Industries Inc.). The excitation wavelengths were set at 350 and 380 nm and emission was monitored at 505 nm with a standard bandpass filter (Andover Corporation 500FS10).

$[Ca^{2+}]_{SOL}$ was calculated using the following equation (Grynkiewicz et al., 1985): $[Ca^{2+}]_{SOL} = K_d \times (R - R_{MIN})(R_{MAX} - R) \times Sf_2/Sb_2$; with K_d equal to 224 nM, R the ratio of the fluorescence measured at 350 and 380 nm, respectively, and Sf_2/Sb_2, the ratio of fluorescence at 380 nm in low and high Ca^{2+}, respectively. The maximum fluorescence ratio (R_{MAX}) was determined using a 10 mM $CaCl_2$ solution to oversaturate the Fura-2, whereas R_{MIN} was obtained with a Ca^{2+} free solution containing 10 mM EGTA. Measurements of $[Ca^{2+}]_{SOL}$ were performed at a pH of 7.35 at room temperature (21–23°C).

Results

Close Association between Smooth ER AMF-R Tubules and Mitochondria

Specific labeling of smooth ER tubules with the anti–AMF-R antibody has been previously reported in MDCK and other cell types by postembedding immunoelectron microscopy (Benlimame et al., 1995, 1998; Wang et al., 1997). Using confocal microscopy, we compared the distribution of smooth ER AMF-R tubules with that of the ER markers calnexin and calreticulin. As seen in Fig. 1, the majority of the AMF-R label can be distinguished from that of the calnexin- and calreticulin-labeled ER although partial overlap of AMF-R tubules with the ER can be observed. AMF-R–labeled tubules are always seen in proximity of calnexin- or calreticulin-labeled elements of the ER and frequently appear as projections of calnexin- or calreticulin-labeled tubules (Fig. 1, C and F, arrowheads), consistent with AMF-R labeling of smooth projections of

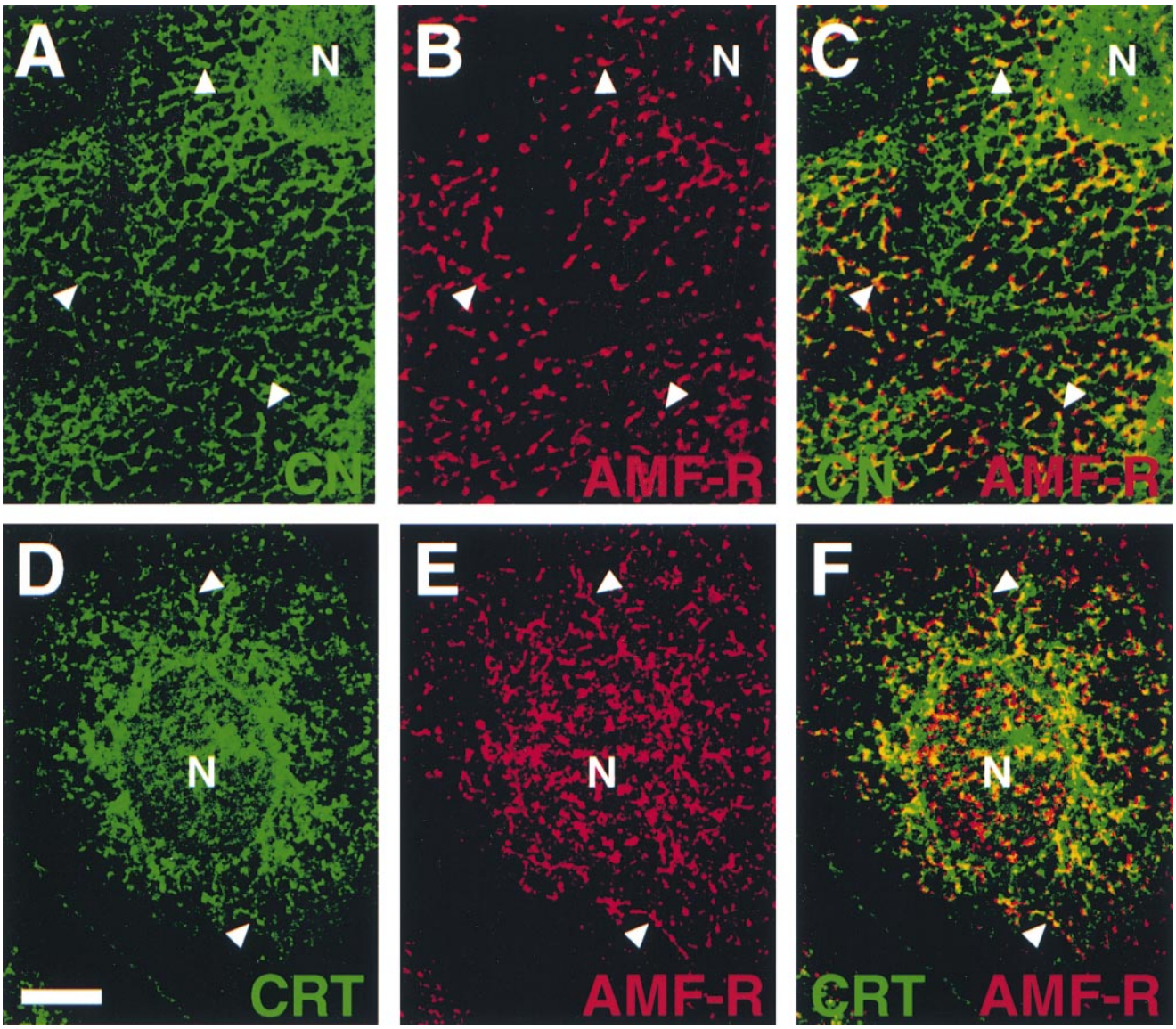

Figure 1. Smooth ER AMF-R tubules exhibit a close relationship to the calnexin- and calreticulin-labeled ER. MDCK cells were fixed and double-immunofluorescently labeled for either calnexin (A) or calreticulin (D) and AMF-R (B and E), and were imaged by confocal microscopy. Merged images (C and F) show that the distribution of AMF-R–labeled tubules is distinct from, but always in proximity to, the calnexin- or calreticulin-labeled ER (arrowheads). Bar, 10 μm.

rough ER tubules observed by EM (Benlimame et al., 1995; Wang et al., 1997).

Double labeling of MDCK cells for AMF-R and Mt-HSP70 revealed a high degree of colocalization between the two signals (Fig. 2, A–C). In contrast, the calnexin- and calreticulin-labeled ER exhibits only partial overlap with the mitochondrial signal (Fig. 2, D–I). Postembedding EM labeling of smooth ER tubules for AMF-R is shown in Fig. 3. Smooth ER AMF-R tubules are observed to exhibit direct interaction with mitochondria and to associate with more than one mitochondrion (Fig. 3, B and C). Quantification of the proximity of ER tubules to mitochondria on 14 EM micrographs of AMF-R–labeled MDCK cells taken in series with Fig. 3 A, revealed that AMF-R–labeled smooth ER tubules, and indeed smooth ER tubules in general, are more frequently observed within 200 nm of mitochondria than are rough ER tubules (Fig. 4). The fact that almost half of the AMF-R–labeled smooth ER tubules are located within 200 nm, the limit of optical resolution, of mitochondria in 80-nm ultrathin sections is consistent with the extensive colocalization of smooth ER AMF-R tubules with mitochondria observed by confocal microscopy. By subcellular fractionation, AMF-R is exclusively found within a high-speed membrane pellet and not within the 12,000 *g* mitochondrial pellet that contains Mt-HSP70, demonstrating that membranous smooth ER AMF-R tubules can be physically dissociated from mitochondria, similar to a previous study (Benlimame et al., 1995). Smooth ER AMF-R tubules are therefore a subdomain of the ER that is selectively associated with mitochondria.

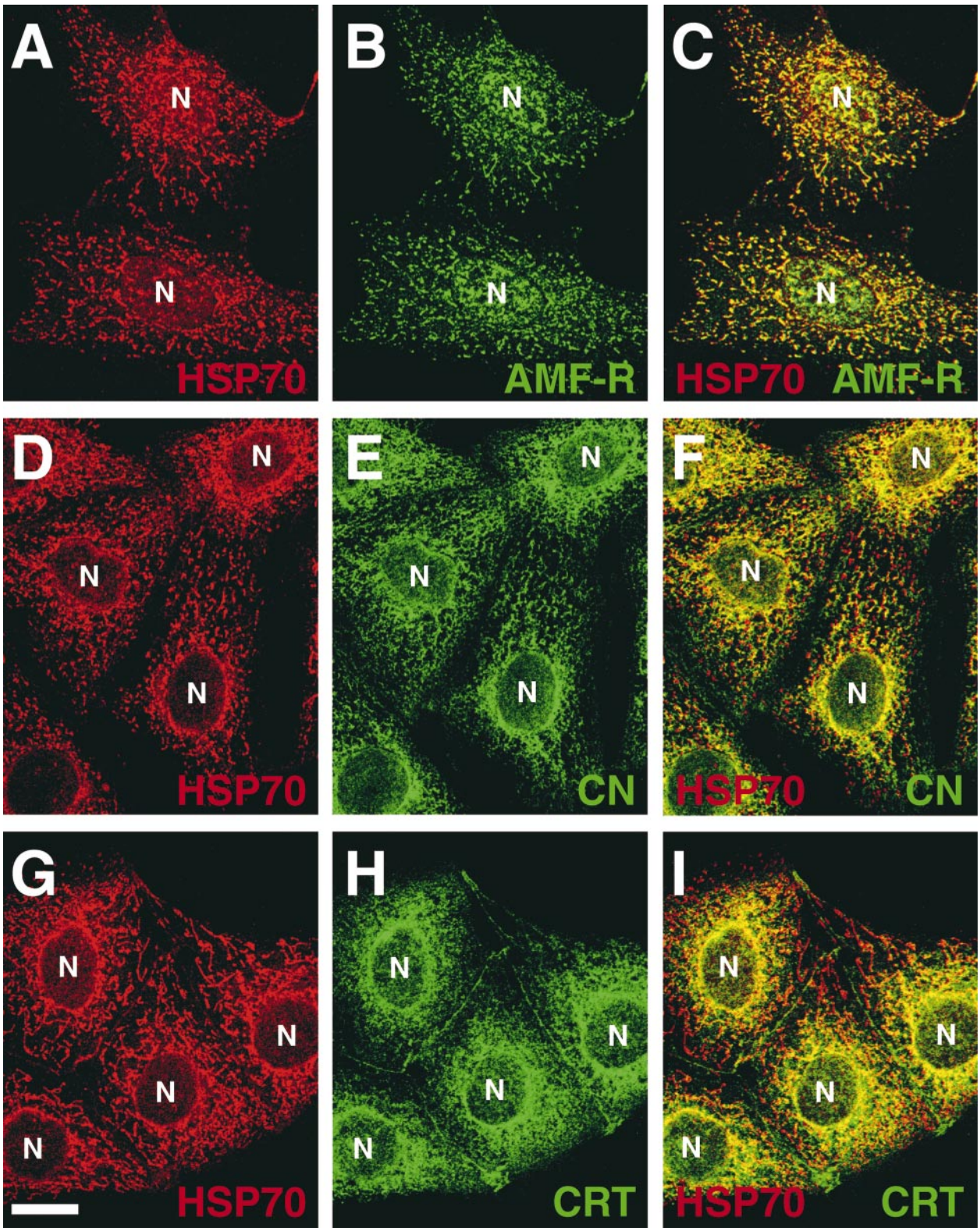

Figure 2. The AMF-R–labeled smooth ER subdomain is selectively associated with mitochondria. MDCK cells were fixed and double-immunofluorescently labeled for Mt-HSP70 (A,D, and G), together with AMF-R (B), calnexin (E), or calreticulin (N), and imaged by confocal microscopy. Merged images (C, F, and I) show that the association of smooth ER AMF-R tubules with mitochondria is significantly greater than either the calnexin- or calreticulin-labeled ER. Bar, 20 μm.

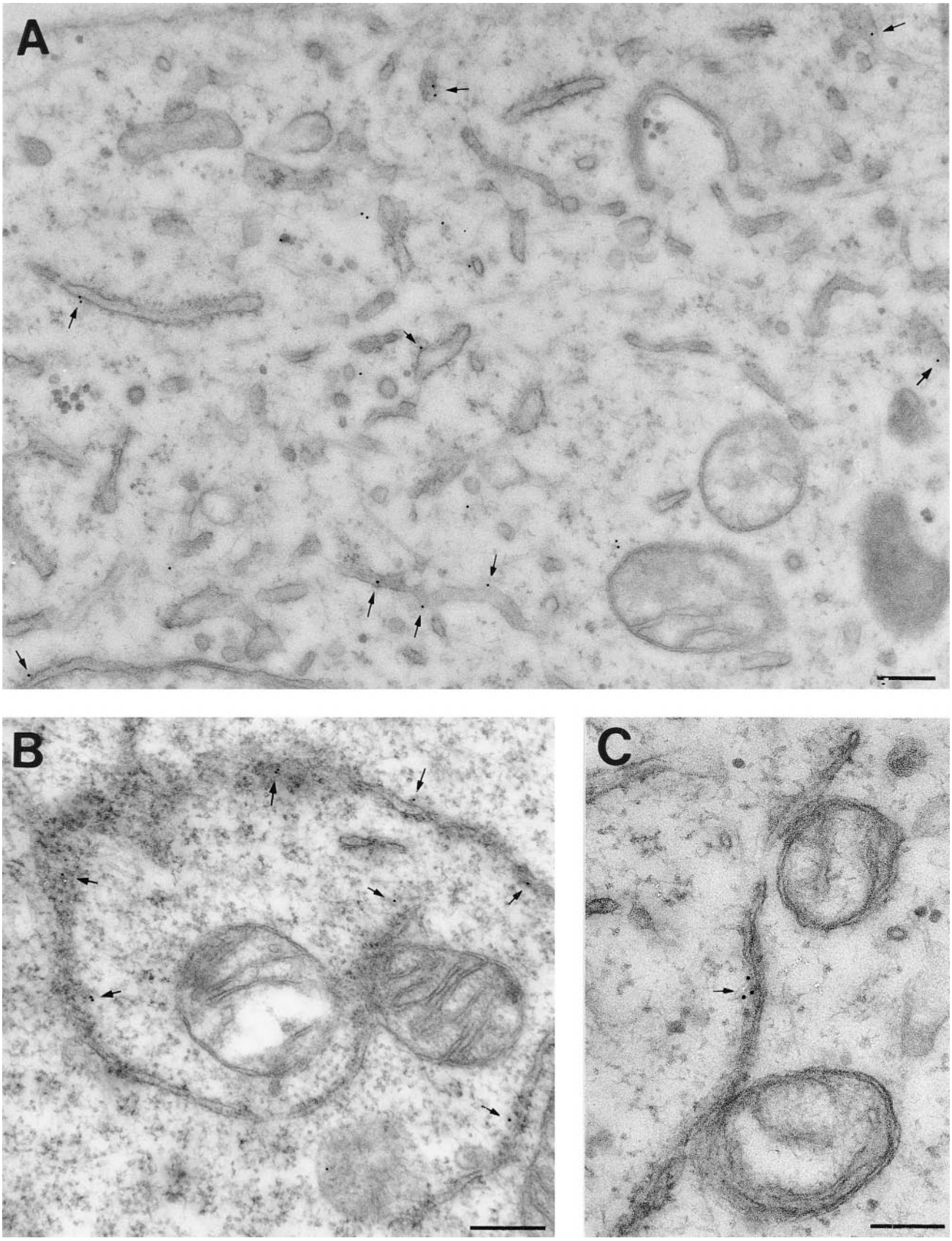

Figure 3. AMF-R tubules and mitochondria exhibit a close association by EM. MDCK cells were postembedding immunogold-labeled for AMF-R. AMF-R–labeling (arrows) is associated with smooth tubules closely associated with mitochondria. Bars: (A) 0.2 μM; (B and C) 0.1 μM.

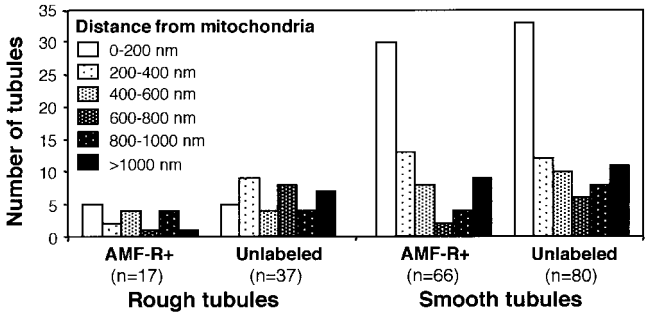

Figure 4. Quantitative analysis of the distance between ER tubules and mitochondria. The minimal distance of smooth and rough ER tubules labeled or not for AMF-R, as indicated, was determined from EM micrographs of AMF-R–labeled MDCK cells. The number of ER tubules located from 0–200, 200–400, 400–600, 600–800, 800–1,000, and >1,000 nm of a mitochondrion within the same cell is presented. The total number of tubules identified per condition (*n*) is indicated.

A Semipermeabilized Assay for Smooth ER–Mitochondria Dissociation

To better study the relationship between AMF-R tubules and mitochondria, we established a semipermeabilized assay based on digitonin permeabilization of subconfluent cultures of MDCK cells. Under the conditions of digitonin permeabilization used, upwards of 90% of the cells were permeabilized as determined by the ability to label cells with antibodies to cytoplasmic keratin filaments before fixation. Antibodies to calreticulin and to the lumenal domain of LAMP-2 did not label digitonin-permeabilized cells, indicating that intracellular organelles remained intact. Labeling with antibodies to tubulin revealed that the majority of microtubules were no longer intact. The association between smooth ER AMF-R tubules and mitochondria is maintained in semiintact MDCK cells since the two organelles exhibit essentially complete overlap in digitonin-permeabilized cells incubated only with buffer A (Fig. 5, A, B, and C). However, in cells incubated with

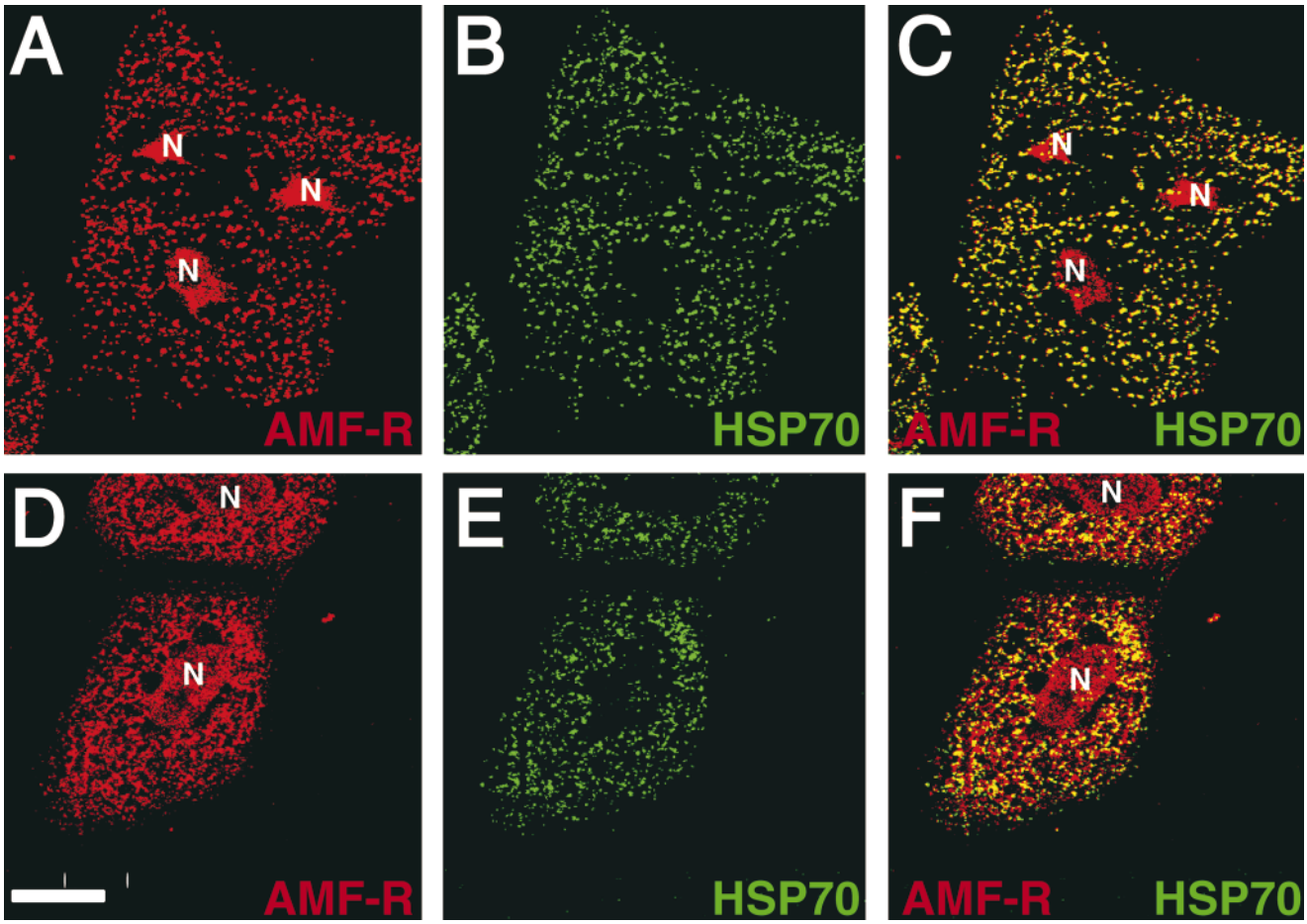

Figure 5. Rat liver cytosol dissociates AMF-R tubules and mitochondria in semipermeabilized MDCK cells. MDCK cells were permeabilized by digitonin and then incubated with buffer A (A, B, and C) or buffer A supplemented with 5 mg/ml rat liver cytosol (D, E, and F) at 37°C for 60 min. Fixed cells were immunofluorescently labeled for AMF-R (A and D; red) and Mt-HSP70 (B and E; green) and imaged by confocal microscopy. Dual color merged images are presented in C and F. Extensive overlap between smooth ER AMF-R tubules and mitochondria is observed in the presence of buffer alone (C), but following addition of cytosol (F) extensive AMF-R labeling not associated with mitochondria (in red) can be visualized. Bar, 20 μM.

buffer A supplemented with 5 mg/ml rat-liver cytosol, the AMF-R label was no longer completely associated with mitochondria and distinct regions of nonoverlap between the two organelles were observed (Fig. 5, D, E, and F) demonstrating the ability of cytosol to induce the dissociation of smooth ER from mitochondria.

Using confocal images of cells double-labeled with anti–AMF-R (Texas red) and anti–Mt-HSP70 (FITC), a mask of the mitochondria labeling was generated and used to quantify the extent of overlap of AMF-R tubule labeling with mitochondria (see Materials and Methods). Using this protocol we obtained consistent data of the extent of dissociation of smooth ER AMF-R tubules from mitochondria (Fig. 6 A). In the presence of buffer A alone, the majority of AMF-R labeling overlapped with mitochondria and AMF-R labeling not associated with mitochondria was limited, normally from 5–10%. The addition of 5 mg/ml rat liver cytosol resulted in a significant increase in the extent of AMF-R labeling that did not overlap with mitochondria (40–50%). Heat inactivated cytosol was unable to dissociate mitochondria and smooth ER implicating cytosolic proteins in the dissociation activity. The ability of cytosol to dissociate AMF-R tubules and mitochondria is not energy dependent and occurs equally well in the absence or presence of 2 mM ATP, 2 mM GTP, or a creatine kinase based ATP regenerating system (ARS; 2 mM ATP, 37 U/ml creatine kinase, 8 mM creatine phosphate; data not shown).

Ca^{2+}-dependent Association of Smooth ER Tubules and Mitochondria

The addition of millimolar concentrations of Ca^{2+} inhibited the ability of cytosol to dissociate AMF-R tubules from mitochondria (Fig. 6 B). The effect was concentration-dependent and the addition of 0.5 mM Ca^{2+} inhibited cytosol-induced dissociation by ~90%. In the absence of cytosol, Ca^{2+} alone did not affect the extent of association between smooth ER and mitochondria, demonstrating that Ca^{2+} specifically regulates the cytosolic dissociation activity. To determine the role of free Ca^{2+} in the regulation of the cytosol-dependent dissociation of smooth ER and mitochondria, we used BAPTA Ca^{2+} chelators with varying K_ds for Ca^{2+} (Fig. 6 C). 5-5′-dimethyl BAPTA (K_d = 40 nM) reversed by 70%, and BAPTA (K_d = 160 nM) by ~30% the ability of 0.5 mM Ca^{2+} to inhibit cytosol-dependent smooth ER-mitochondria dissociation. 5-5′-difluoro BAPTA (K_d = 635 nM) and 5-5′-dibromo BAPTA (K_d = 1.6 μM) reversed only minimally smooth ER–mitochondria dissociation. The ability of BAPTAs of various K_d's to differentially influence smooth ER mitochondria association demonstrates that free Ca^{2+} levels regulate ER–mitochondria association. The fact that these effects are observed in the presence of 100 μM BAPTA indicates that free Ca^{2+} in the presence of cytosol and cells must be below 100 μM, in spite of the fact that 0.5 mM Ca^{2+} was added to the reaction. Indeed, no effect was observed using 50 μM of the various BAPTAs, indicating that protein Ca^{2+} chelators in the cells and in the cytosol reduce free Ca^{2+} to between 50–100 μM.

To further define the free Ca^{2+} concentrations that regulate cytosol-mediated dissociation of smooth ER and mitochondria, we added cytosol in the presence of CaEGTA buffers of defined free Ca^{2+} concentrations. Free $[Ca^{2+}]_{SOL}$ was predicted using the WEBMAXCv1.10 program and measured using Fura-2 (Table I). Compared with the high degree of dissociation observed in the presence of free $[Ca^{2+}]_{SOL}$ of less than 50 nM (CaEGTA buffers 2 and 3), free $[Ca^{2+}]_{SOL}$ of ~100–400 nM (CaEGTA buffers 4 and 5) were associated with partial inhibition and free $[Ca^{2+}]_{SOL}$ concentrations >1 μM (CaEGTA buffers 6–8) associated with a significant inhibition of cytosol-mediated dissociation of smooth ER and mitochondria. Graphic representation of the data presented in Table I (CaEGTA buffers 2–7) reveals a logarithmic relationship between free $[Ca^{2+}]_{SOL}$ measured with Fura-2 and cytosol dissociation activity (Fig. 6 D). In particular, free $[Ca^{2+}]_{SOL}$ below 100 nM are associated with extensive dissociation of the two organelles, whereas increasing the free $[Ca^{2+}]_{SOL}$ above 100 nM results in the progressive inhibition of cytosol-mediated dissociation.

Discussion

The ER constitutes the largest intracellular membrane organelle of the cell, extends throughout the cytoplasm, and is composed of multiple functional domains (Sitia and Meldolesi, 1992). Confocal images of MDCK cells double-

Table I. Free Calcium and Smooth ER–Mitochondria Dissociation

Buffer	Total Ca^{2+}	Predicted free $[Ca^{2+}]$	Measured free $[Ca^{2+}]$	Cytosol added	Dissociation
	mM		*± SEM*		*% ± SEM*
Buffer A				−	10.0 ± 0.8 (7)
Buffer A				+	48.3 ± 3.8 (7)
CaEGTA buffer 1	0	0	8 ± 4 nM (3)	+	51.2 ± 3.7 (5)
CaEGTA buffer 2	5	14 nM	19 ± 5 nM (3)	+	37.7 ± 3.9 (4)
CaEGTA buffer 3	10	37 nM	39 ± 5 nM (3)	+	36.2 ± 3.4 (4)
CaEGTA buffer 4	15	83 nM	110 ± 20 nM (3)	+	29.9 ± 5.1 (4)
CaEGTA buffer 5	20	221 nM	350 ± 80 nM (3)	+	25.4 ± 4.8 (4)
CaEGTA buffer 6	22.13	700 nM	1.4 μM (1)	+	20.3 ± 7.4 (3)
CaEGTA buffer 7	22.92	1.0 μM	1.8 μM (1)	+	19.0 ± 6.1 (3)
CaEGTA buffer 8	25	38.0 μM	3.8 ± 0.2 μM (3)	+	15.9 ± 2.1 (5)

Free Ca^{2+} concentrations of 25 mM CaEGTA buffers containing the indicated amount of total Ca^{2+} as predicted by calculation and by measurement with Fura-2 (number of experiments indicated in parentheses) are indicated. Measured free $[Ca^{2+}]$ for CaEGTA buffer 8 is considered unreliable as it is outside Fura-2 sensitivity. The average percent dissociation of smooth ER and mitochondria was determined from the indicated number of experiments for each CaEGTA buffer.

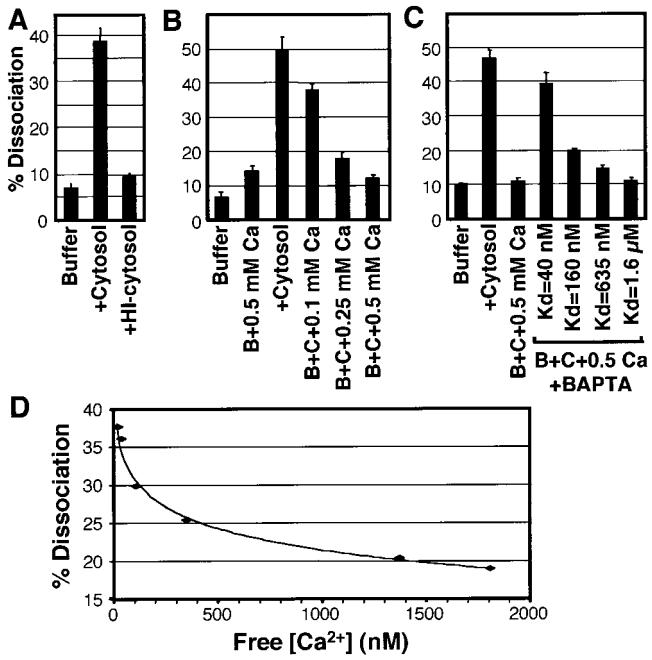

Figure 6. Ca^{2+} regulates cytosol-mediated dissociation of smooth ER AMF-R tubules and mitochondria. A, The percent dissociation of smooth ER tubules (± SEM) from mitochondria in semipermeabilized MDCK cells was quantified from confocal images of cells double-labeled for AMF-R and Mt-HSP70 (for details see Materials and Methods). MDCK cells were permeabilized with digitonin and incubated for 60 min in the presence of buffer A alone or buffer A supplemented with 5 mg/ml rat liver cytosol or 5 mg/ml heat-inactivated cytosol. B, Semipermeabilized MDCK cells were incubated for 60 min with buffer A supplemented with 0.5 mM Ca^{2+} (+0.5 mM Ca), with cytosol (+cytosol), or with cytosol (C) plus 0.1, 0.25, or 0.5 mM Ca^{2+}, as indicated. To assess the role of free Ca^{2+} in cytosol-mediated dissociation of smooth ER AMF-R tubules from mitochondria, semipermeabilized MDCK cells were incubated with buffer A alone, or with buffer A supplemented with cytosol (+cytosol), with cytosol containing 0.5 mM Ca^{2+} (+C+0.5 Ca), or with cytosol, 0.5 mM Ca^{2+}, and 100 μM of various BAPTAs (B+C+0.5 Ca+BAPTA) with Ca^{2+} dissociation constants (K_d) of 40 nM, 160 nM, 635 nM, and 1.6 μM, as indicated. Similar results were obtained from at least three independent experiments and data from representative experiments is presented. C, Using data presented in Table I, free $[Ca^{2+}]_{SOL}$ measured using Fura-2 in CaEGTA buffers 2-7 was plotted against the extent of dissociation of smooth ER AMF-R tubules from mitochondria. Free $[Ca^{2+}]_{SOL}$ above 100 nM is associated with increasing association between the two organelles.

labeled for AMF-R and calnexin or calreticulin show that while the AMF-R–labeled tubules can be clearly distinguished from the calnexin- and calreticulin-labeled ER, partial overlap is apparent and regions of close interaction between the two are evident. These images are therefore consistent with the identity of the AMF-R tubule as a smooth subdomain of the ER (Benlimame et al., 1995; Wang et al., 1997). The presence of AMF-R, the select sensitivity of smooth ER AMF-R tubules, but not the rough ER to ilimaquinone (Wang et al., 1997), and the fact that AMF-R labeling can be clearly distinguished from that of calnexin and calreticulin identify the smooth ER AMF-R tubule as a molecularly distinct subdomain of the ER. The high degree of association of AMF-R–labeled tubules with mitochondria by confocal and EM further identifies the AMF-R tubule as a mitochondria-associated subdomain of the ER.

The existence of ER subdomains that interact with mitochondria and which function to sequester cytosolic Ca^{2+} (Simpson et al., 1997; Rizzuto et al., 1998), together with the demonstrated role of cytosolic Ca^{2+} in regulating AMF-R tubule–mitochondria association, suggest a role for this smooth ER subdomain in the regulation of cytosolic Ca^{2+} levels. The diversity of Ca^{2+} concentrations and expression of Ca^{2+} binding proteins in different subdomains of the ER is well-established (Takei et al., 1992; Button and Eidsath, 1996; Rooney and Meldolesi, 1996; Golovina and Blaustein, 1997; Montero et al., 1997; Pezzati et al., 1997). Not unlike the smooth ER AMF-R tubule, a smooth membrane-bound organelle of 50–250 nm apposed to the ER and mitochondria, called the calciosome, has been described to be enriched in a nonmuscle calsequestrin-like protein and to represent the major IP3-sensitive Ca^{2+} storage organelle (Volpe et al., 1988). Both AMF-R and the IP3 receptor are associated with cell surface caveolae (Fujimoto et al., 1992; Benlimame et al., 1998), and the caveolar-like clathrin-independent internalization pathway to smooth ER defined by AMF-R (Benlimame et al., 1998; Le et al., 2000) may serve to regulate the delivery of Ca^{2+} regulatory proteins to this mitochondria-associated smooth ER subdomain.

In semipermeabilized MDCK cells in the absence of cytosol or energy and independent of Ca^{2+}, smooth ER AMF-R tubules and mitochondria maintain their close association, suggesting that interorganellar adhesion mechanisms are associated with the organelles. The ability of cytosol to dissociate the two organelles demonstrates the existence of a cytosolic mechanism that regulates ER–mitochondria association. The transfer of phosphatidyl serine between mitochondria and MAMs is prevented by proteolysis of mitochondrial surface proteins, but not of MAMs, suggesting that a mitochondrial surface protein is involved in the association between mitochondria and ER (Shiao et al., 1998). Whereas the relationship of smooth ER AMF-R tubules to MAMs remains unclear, cytosol may disrupt smooth ER AMF-R tubule–mitochondria association due to the presence of a Ca^{2+}-regulated cytosolic protein that inhibits the adhesive function of such a mitochondrial surface protein.

Our results using BAPTAs of various affinities and CaEGTA buffers of varying free $[Ca^{2+}]_{SOL}$ indicate that free Ca^{2+} regulates the ability of cytosol to stimulate dissociation of AMF-R tubules from mitochondria. While we hesitate to attribute precise free Ca^{2+} levels to our data, both the BAPTA and CaEGTA experiments are consistent with the ability of low free $[Ca^{2+}]_{SOL}$ (<100 nM) to favor dissociation and of high free $[Ca^{2+}]_{SOL}$ (>1 μM) to favor association of smooth ER tubules and mitochondria (Fig. 6; Table I). Therefore, these data argue that at physiological cytosolic Ca^{2+} levels, cytosol stimulates smooth ER-mitochondria dissociation. Whereas addition of cytosol increases the amount of smooth ER AMF-R tubules that do not colocalize with mitochondria, the two organelles still exhibit extensive association (Fig. 5 F) such

that even in the presence of cytosol the two organelles still interact. Dissociation of AMF-R tubules from mitochondria induced by cytosol therefore reflects a partial disruption of direct contacts between the two organelles. The increased overlap of the two fluorescent signals observed in the presence of increasing Ca^{2+} may be indicative of the increased formation of close contacts between the two organelles.

Ca^{2+} transients of 1.6–1.9 μM have been identified at high density patches of the ER enriched in the IP3 receptor and calreticulin (Simpson et al., 1997), and the mitochondrial surface has been shown to be exposed to 3.4 μM Ca^{2+} after IP3 stimulation (Rizzuto et al., 1998). Our data showing increased ER–mitochondria association at millimolar cytosolic Ca^{2+} indicates that the elevated Ca^{2+} levels present at these ER–mitochondria close contacts can actively regulate ER–mitochondria association. Ca^{2+}-dependent smooth ER–mitochondria association could therefore contribute to the establishment of segregated cytoplasmic domains between the two organelles in which the local elevated Ca^{2+} concentrations necessary for mitochondrial uptake can be established. A cellular mechanism that senses elevated cytosolic Ca^{2+} levels and then facilitates the establishment of ER–mitochondria contacts is therefore proposed to be implicated in the efficient sequestration of cytosolic Ca^{2+} to mitochondrial stores.

We thank Kevin Coonan and Nigel Banner of Empix Imaging (Mississuaga, Ontario) for developing the Northern Eclipse overlay program used to quantitate AMF-R tubule–mitochondria overlap. The photographic and digital reproductions were the work of Jean Leveillé and Gaston Lambert.

This study was supported by grants from the Medical Research Council of Canada.

Submitted: 24 May 2000
Revised: 5 July 2000
Accepted: 27 July 2000

References

Achleitner, G., B. Gaigg, A. Krasser, E. Kainersdorfer, S.D. Kohlwein, A. Perktold, G. Zellnig, and G. Daum. 1999. Association between the endoplasmic reticulum and mitochondria of yeast facilitates interorganelle transport of phospholipids through membrane contact. *Eur. J. Biochem.* 264:545–553.

Benlimame, N., D. Simard, and I.R. Nabi. 1995. Autocrine motility factor receptor is a marker for a distinct tubular membrane organelle. *J. Cell Biol.* 129:459–471.

Benlimame, N., P.U. Le, and I.R. Nabi. 1998. Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum. *Mol. Biol. Cell.* 9:1773–1786.

Button, D., and A. Eidsath. 1996. Aequorin targeted to the endoplasmic reticulum reveals heterogeneity in luminal Ca^{++} concentration and reports agonist- or IP3-induced release of Ca^{++}. *Mol. Biol. Cell.* 7:419–434.

Denton, R.M., and J.G. McCormack. 1990. Ca^{2+} as a second messenger within mitochondria of the heart and other tissues. *Annu. Rev. Physiol.* 52:451–466.

Franke, W.W., and J. Kartenbeck. 1971. Outer mitochondrial membrane continuous with endoplasmic reticulum. *Protoplasma.* 73:35–41.

Fujimoto, T., S. Nakade, A. Miyawaki, K. Mikoshiba, and K. Ogawa. 1992. Localization of 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J. Cell Biol.* 119:1507–1513.

Golovina, V.A., and M.P. Blaustein. 1997. Spatially and functionally distinct Ca^{2+} stores in sarcoplasmic and endoplasmic reticulum. *Science.* 275:1643–1648.

Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–3450.

Hajnoczky, G., R. Hager, and A.P. Thomas. 1999. Mitochondria suppress local feedback activation of inositol 1,4, 5- trisphosphate receptors by Ca^{2+}. *J. Biol. Chem.* 274:14157–14162.

Jouaville, L.S., P. Pinton, C. Bastianutto, G.A. Rutter, and R. Rizzuto. 1999. Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc. Natl. Acad. Sci. USA.* 96:13807–13812.

Landolfi, B., S. Curci, L. Debellis, T. Pozzan, and A.M. Hofer. 1998. Ca^{2+} homeostasis in the agonist-sensitive internal store: functional interactions between mitochondria and the ER measured in situ in intact cells. *J. Cell Biol.* 142:1235–1243.

Le, P.U., N. Benlimame, A. Lagana, A. Raz, and I.R. Nabi. 2000. Clathrin-mediated endocytosis and recycling of autocrine motility factor receptor to fibronectin fibrils is a limiting factor for cell motility. *J. Cell Sci.* 113:3227–3240.

Lewis, J.A., and J.R. Tata. 1973. A rapidly sedimenting fraction of rat liver endoplasmic reticulum. *J. Cell Sci.* 13:447–459.

Montero, M., J. Alvarez, W.J. Scheenen, R. Rizzuto, J. Meldolesi, and T. Pozzan. 1997. Ca^{2+} homeostasis in the endoplasmic reticulum: coexistence of high and low [Ca^{2+}] subcompartments in intact HeLa cells. *J. Cell Biol.* 139: 601–611.

Montisano, D.F., J. Cascarano, C.B. Pickett, and T.W. James. 1982. Association between mitochondria and rough endoplasmic reticulum in rat liver. *Anat. Rec.* 203:441–450.

Morre, D.J., W.D. Merritt, and C.A. Lembi. 1971. Connections between mitochondria and endoplasmic reticulum in rat liver and onion stem. *Protoplasma.* 73:43–49.

Nabi, I.R., H. Watanabe, and A. Raz. 1990. Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* 50:409–414.

Perkins, G., C. Renken, M.E. Martone, S.J. Young, M. Ellisman, and T. Frey. 1997. Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. *J. Struct. Biol.* 119:260–272.

Pezzati, R., M. Bossi, P. Podini, J. Meldolesi, and F. Grohovaz. 1997. High-resolution calcium mapping of the endoplasmic reticulum–Golgi exocytic membrane system. Electron energy loss imaging analysis of quick frozen-freeze dried PC12 cells. *Mol. Biol. Cell.* 8:1501–1512.

Pozzan, T., R. Rizzuto, P. Volpe, and J. Meldolesi. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* 74:595–636.

Rizzuto, R., M. Brini, M. Murgia, and T. Pozzan. 1993. Microdomains with high Ca^{2+} close to IP3-sensitive channels that are sensed by neighboring mitochondria. *Science.* 262:744–747.

Rizzuto, R., C. Bastianutto, M. Brini, M. Murgia, and T. Pozzan. 1994. Mitochondrial Ca^{2+} homeostasis in intact cells. *J. Cell Biol.* 126:1183–1194.

Rizzuto, R., P. Pinton, W. Carrington, F.S. Fay, K.E. Fogarty, L.M. Lifshitz, R.A. Tuft, and T. Pozzan. 1998. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science.* 280:1763–1766.

Robb-Gaspers, L.D., P. Burnett, G.A. Rutter, R.M. Denton, R. Rizzuto, and A.P. Thomas. 1998. Integrating cytosolic calcium signals into mitochondrial metabolic responses. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:4987–5000.

Rooney, E., and J. Meldolesi. 1996. The endoplasmic reticulum in PC12 cells. Evidence for a mosaic of domains differently specialized in Ca^{2+} handling. *J. Biol. Chem.* 271:29304–29311.

Rutter, G.A., P. Burnett, R. Rizzuto, M. Brini, M. Murgia, T. Pozzan, J.M. Tavare, and R.M. Denton. 1996. Subcellular imaging of intramitochondrial Ca^{2+} with recombinant targeted aequorin: significance for the regulation of pyruvate dehydrogenase activity. *Proc. Natl. Acad. Sci. USA.* 93:5489–5494.

Shiao, Y.J., B. Balcerzak, and J.E. Vance. 1998. A mitochondrial membrane protein is required for translocation of phosphatidylserine from mitochondria-associated membranes to mitochondria. *Biochem. J.* 331:217–223.

Shore, G.C., and J.R. Tata. 1977. Two fractions of rough endoplasmic reticulum from rat liver. I. Recovery of rapidly sedimenting endoplasmic reticulum in association with mitochondria. *J. Cell Biol.* 72:714–725.

Simpson, P.B., S. Mehotra, G.D. Lange, and J.T. Russell. 1997. High density distribution of endoplasmic reticulum proteins and mitochondria at specialized Ca^{2+} release sites in oligodendrocyte processes. *J. Biol. Chem.* 272: 22654–22661.

Sitia, R., and J. Meldolesi. 1992. Endoplasmic reticulum: a dynamic patchwork of specialized subregions. *Mol. Biol. Cell.* 3:1067–1072.

Takei, K., H. Stukenbrok, A. Metcalf, G.A. Mignery, T.C. Sudhof, P. Volpe, and P. De Camilli. 1992. Ca^{2+} stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the InsP3 receptor, Ca(2+)-ATPase, and calsequestrin. *J. Neurosci.* 12:489–505.

Vance, J.E. 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. *J. Biol. Chem.* 265:7248–7256.

Volpe, P., K.H. Krause, S. Hashimoto, F. Zorzato, T. Pozzan, J. Meldolesi, and D.P. Lew. 1988. Calciosome, a cytoplasmic organelle: the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store of nonmuscle cells? *Proc. Natl. Acad. Sci. USA.* 85:1091–1095.

Wang, H.-J., N. Benlimame, and I.R. Nabi. 1997. The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. *J. Cell Sci.* 110:3043–3053.