

# Ca<sup>2+</sup>-triggers massive exocytosis in Chinese hamster ovary cells

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**We have tracked the cell surface area of CHO cells by measuring the membrane capacitance,  $C_m$ . An increase in cytosolic  $[Ca^{2+}]_i$ , increased the cell surface area by 20–30%. At micromolar  $[Ca^{2+}]_i$  the increase occurred in minutes, while at 20  $\mu$ M or higher  $[Ca^{2+}]_i$  it occurred in seconds and was transient. GTP $\gamma$ S caused a 3% increase even at 0.1  $\mu$ M  $[Ca^{2+}]_i$ . We conclude that CHO cells, previously thought capable only of constitutive exocytosis, can perform Ca<sup>2+</sup>-triggered exocytosis that is both massive and rapid. Ca<sup>2+</sup>-triggered exocytosis was also observed in 3T3 fibroblasts. Our findings add evidence to the view that Ca induces exocytosis in cells other than known secretory cells.**

**Keywords:** endocytosis/flash photolysis/GTP $\gamma$ S/plasmalemmal repair/3T3 fibroblasts

## Introduction

All eukaryotic cells continually perform exocytosis that is thought to be 'constitutive' in that it is not subject to known regulation (Alberts *et al.*, 1994). Some cells, such as neurons and endocrine and exocrine cells, in addition perform 'regulated' exocytosis. Such cells have two secretory pathways, one constitutive, the other regulated (Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). The mechanisms used to sort cargo proteins into one pathway or the other are beginning to be understood (Bauerfeind and Huttner, 1993).

One can determine whether a cell has a regulated exocytic pathway by testing whether the release of its secretory cargo is triggered by a stimulus. However, this approach will not recognize all regulated exocytosis, since one must know the identity of the cargo before one can detect it in an assay. Indeed, it is possible that all cells can regulate their exocytosis and that this fact has been missed only because our assays are limited. Evidence for previously unrecognized regulated exocytosis was obtained by Poo and co-workers. They inserted a 'false' cargo molecule, acetylcholine (ACh), into exocytosis-competent vesicles of muscle cells (Dan and Poo, 1992), fibroblasts (Girod *et al.*, 1995) and CHO cells (Morimoto *et al.*, 1995) and demonstrated Ca-stimulated release of single packets of acetylcholine by using embryonic muscle cells as electrophysiological sensors. An alternative approach is to detect the increase in cell surface area that invariably results from exocytosis. This is done most conveniently by measuring the plasma membrane capacit-

ance,  $C_m$  (Neher and Marty, 1982). The approach can detect exocytosis even if the cargo carried by the exocytosing vesicle is unknown, indeed even if no cargo is secreted at all.  $C_m$  measurements have provided evidence for previously unknown, Ca-triggered exocytic pathways in mast cells (Almers and Neher, 1987; Kirillova *et al.*, 1993).

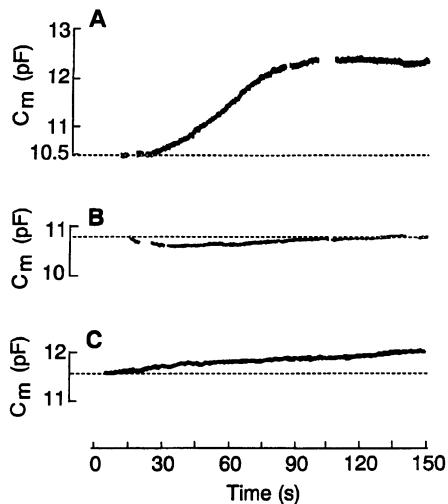
In CHO cells, an epithelial-fibroblastoid cell line, vesicles carrying pinocytic contents (Adams *et al.*, 1982) and transferrin receptors (Galli *et al.*, 1994) are capable of exocytosis that is thought to be constitutive. Secretion is independent of cytosolic  $[Ca^{2+}]_i$  up to 1  $\mu$ M (Miller and Moore, 1991). Here we provide evidence that CHO cells nevertheless contain a large reservoir of intracellular membranes that can insert into the plasmalemma in seconds as a result of Ca-stimulated exocytosis. The rate of exocytosis is strongly Ca sensitive and the inserted membrane can be retrieved within 1 min.

## Results

To track the cell surface area we measured the plasma membrane capacitance,  $C_m$ , of single, solitary CHO cells. The mean capacitance was  $11.2 \pm 0.4$  pF ( $n = 39$ ). Since biological membranes generally have a specific capacitance of  $\sim 1$   $\mu$ F/cm<sup>2</sup>, this corresponds to 1100  $\mu$ m<sup>2</sup> of cell surface. In 32 cells both  $C_m$  and the diameter were measured.  $C_m$  reported cell surfaces that were  $1.3 \pm 0.3$  times larger than expected if the cells were smooth spheres of the measured diameters. Evidently the surface of CHO cells is not smooth. Indeed, electron micrographs of rapidly frozen sections (H.Horstmann, unpublished results) show that CHO cells are sparsely covered with microvilli.

To see whether  $[Ca^{2+}]_i$  influences the cell surface area we used glass micropipettes to perfuse the cytosol with solutions of different  $[Ca^{2+}]_i$ . Figure 1A shows a trace with a  $[Ca^{2+}]_i$  of 4  $\mu$ M in the pipette;  $C_m$  increased by  $\sim 2$  pF. The average increase was by  $26 \pm 6\%$  after 2 min perfusion and reached a peak of  $30 \pm 6\%$  at  $234 \pm 53$  s after patch break ( $n = 9$ ). This change was significant ( $P < 0.01$ ) and corresponded to 3.3 pF or 330  $\mu$ m<sup>2</sup> of surface area for an average CHO cell. The maximal rate of increase was  $0.5 \pm 0.1\%/s$  ( $n = 9$ ). In contrast, when the pipette solution contained only a  $[Ca^{2+}]_i$  of 100 nM, a value close to the concentration in most resting cells, no visible exocytosis took place (Figure 1B) and  $C_m$  did not change significantly (by  $-0.3 \pm 0.2\%$  after 2 min perfusion,  $n = 9$ ). In summary, micromolar cytosolic Ca<sup>2+</sup> stimulates a large increase in the cell surface area. We attribute the increase to exocytosis. Such behavior is well known in neuroendocrine cells (Thomas *et al.*, 1990; Augustine and Neher, 1992), but is unexpected for CHO cells.

In cells of the hematopoietic lineage (Fernandez *et al.*, 1984; Coorsen *et al.*, 1990; Gomperts, 1990; Lindau

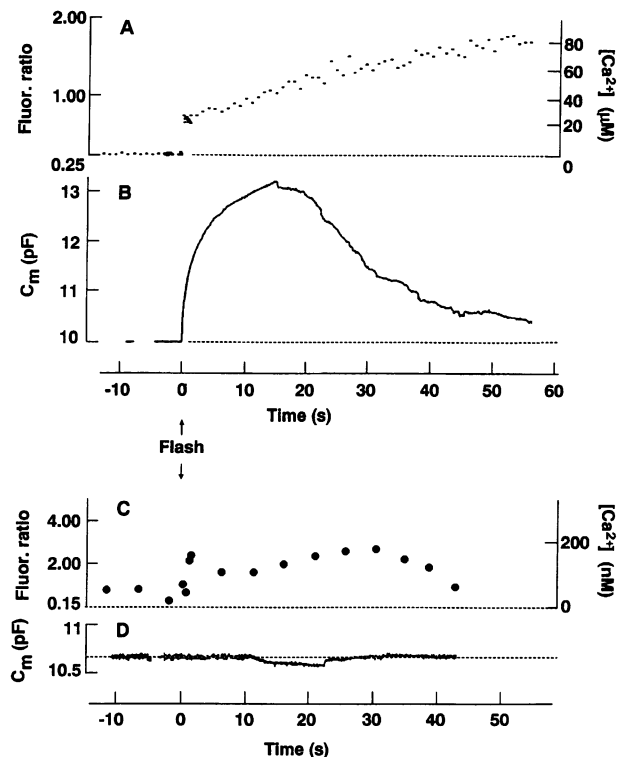


**Fig. 1.** Micromolar  $[Ca^{2+}]_i$  triggers increases in CHO cell membrane capacitance. Representative  $C_m$  traces during perfusion with pipette solutions containing strongly buffered  $[Ca^{2+}]_i$ . (A) At a  $[Ca^{2+}]_i$  of  $4 \mu M$  (solution A, see Materials and methods); (B) at a  $[Ca^{2+}]_i$  of  $100 nM$  (solution B); (C) solution B with  $0.1 mM$  GTP $\gamma$ S added. The origin marks the time when the pipette became continuous with the cytosol (patch break); dashed lines mark initial capacitance. Small sections of each trace corresponding to phase checks, calibration pulses and  $C_m$  compensation have been excised for clarity. Trace (C) was recorded with the CapTrak facility of an EPC-9 and traces (A) and (B) with a lock-in amplifier.

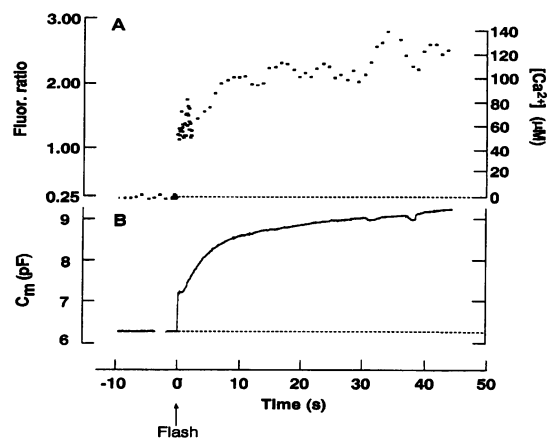
*et al.*, 1993) and in endocrine cells (Okano *et al.*, 1993; Burgoyne and Handel, 1994) exocytosis may also be induced by non-hydrolyzable GTP analogs. Indeed, GTP $\gamma$ S stimulated exocytosis in CHO cells when added to the  $100 nM$  Ca solution (Figure 1C).  $C_m$  changed by  $3.2 \pm 0.3 \%$  ( $n = 4$ ) after 2 min, corresponding to an increase of  $350 fF$ . Though statistically different from the control ( $P < 0.001$ ), the value is about seven times less than with  $4 \mu M [Ca^{2+}]_i$ . We tentatively attribute the finding to stimulation of exocytosis by GTP $\gamma$ S. The effect could also represent a block of ongoing endocytosis on a background of continuing constitutive exocytosis. However, the constitutive exocytosis of glycosaminoglycans in CHO cells is blocked by GTP $\gamma$ S at the concentration used here (Miller and Moore, 1991).

To test whether a higher  $[Ca^{2+}]_i$  produces more rapid exocytosis, we raised the  $[Ca^{2+}]_i$  by flash photolysis of caged Ca. Single cells were perfused for  $\sim 2$  min ( $137 \pm 4$  s,  $n = 9$ ) with a solution containing the photolabile Ca/Mg chelator DM-nitrophen. Then Ca or Mg were released from the chelator by a 10 ms UV flash. Figure 2 shows representative traces. When the chelator had been loaded with Ca,  $[Ca^{2+}]_i$  rose to an initial value of  $29 \pm 4 \mu M$  (range  $16 - 61 \mu M$ ,  $n = 9$ ; Figure 2A) and triggered a robust increase in  $C_m$  (Figure 2B). In most cells  $C_m$  rose to a peak and then declined (Figure 2B). We attribute the decline to membrane retrieval. In four cells out of nine  $C_m$  returned close to the pre-flash value, with half the membrane being retrieved in  $20 \pm 4$  s. In three of the remaining cells a brief period of decline was followed by further exocytosis and  $C_m$  did not return to baseline. Two of nine cells showed no evidence of membrane retrieval.

The largest  $C_m$  increase was measured in each cell and averaged  $23 \pm 5 \%$  ( $n = 9$ ), corresponding to  $2.6 pF$ . A similar increase ( $30 \pm 12 \%$ ,  $n = 3$ ) was also observed in

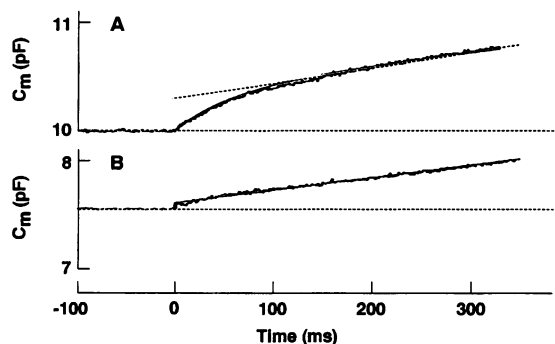


**Fig. 2.**  $Ca^{2+}$  but not  $Mg^{2+}$  triggers rapid exocytosis in CHO cells. (A)  $[Ca^{2+}]_i$  and (B)  $C_m$  measured from the same cell upon flash photolysis of Ca-loaded DM-nitrophen.  $[Ca^{2+}]_i$  (right ordinate) is calculated from the fluorescence ratio (Fura2, left ordinate) based on calibration constants measured in single melanotrophs (see Materials and methods). (C and D) As in (A) and (B) but from a cell containing  $Mg^{2+}$ -loaded DM-nitrophen.  $[Ca^{2+}]_i$  was measured with Fura-2; each point is the mean of five measurements. The calibration constants used to convert the fluorescence ratios into  $[Ca^{2+}]_i$  were measured in microcuvettes. In (B) and (D) a small section of each trace was excised to remove the electrical interference caused by the high voltage discharge of the flash lamp.



**Fig. 3.**  $Ca^{2+}$  triggers rapid exocytosis in 3T3-fibroblasts. Experimental details as in Figure 2A and B.

NIH 3T3 fibroblasts (Figure 3). When the chelator had been loaded with  $Mg^{2+}$ ,  $[Ca^{2+}]_i$  did not rise significantly (Figure 2C, note the 200-fold higher gain) and neither did  $C_m$  (Figure 2D). Again the largest  $C_m$  change was measured; it was statistically insignificant ( $0.3 \pm 0.3 \%$ ,  $n = 5$ ). Evidently the rise in  $C_m$  in Figure 2B was triggered



**Fig. 4.** Multiple kinetic components are observed in some CHO cells. (A) Expanded version of the trace in Figure 2B. The continuous line shows the fit of an exponential lag (time constant 56 ms) added to a linear rise; the latter is also shown separately (dashed). (B) Recording from another cell under identical conditions that showed no rapid component.

specifically by the rise in  $[Ca^{2+}]_i$ ; neither an increase in  $Mg^{2+}$  nor the UV flash was sufficient.

To explore the initial kinetics of exocytosis, traces as in Figure 2B were viewed at higher magnification. About half the traces were well fitted by the sum of a linear rise and an exponential (Figure 4A), suggesting an initial fast phase ('exocytic burst') followed by a slower phase. Other traces showed no evidence of an initial fast phase (Figure 4B). When results from both sets of cells were averaged  $C_m$  increased by  $4.2 \pm 0.4\%$  ( $n = 9$ ) in the first 140 ms and the initial rate of increase was  $39 \pm 8\%/s$  ( $n = 10$ ). These values may be compared with those in melanotrophs (an increase of 5.8% or 250 fF in a 4.7 pF cell at a rate of 43%/s at 27  $\mu M$   $[Ca^{2+}]_i$ ; Thomas *et al.*, 1993). In both melanotrophs and chromaffin cells the increase in  $C_m$  shows multiple kinetic phases (see also Neher and Zucker, 1993; Heinemann *et al.*, 1994; Parsons *et al.*, 1995).

## Discussion

We have shown that raising cytosolic  $Ca^{2+}$  stimulates an increase in the cell surface of CHO cells. When  $Ca^{2+}$  was raised to micromolar levels by diffusion from micropipettes the cell surface increased by ~30% within 4 min. When  $[Ca^{2+}]_i$  was raised to tens of micromolar by flash photolysis of DM-nitrophen the cell surface increased by ~20% in 7 s. As in previous work, we attribute the increase in cell surface to exocytosis. Comparison of results under the two conditions suggests that the rate of exocytosis increased strongly with  $[Ca^{2+}]_i$ , while the amount did not. Ca-stimulated exocytosis was also observed in 3T3 fibroblasts.

Our results may be compared with changes seen under comparable conditions in endocrine cells. When  $[Ca^{2+}]_i$  was raised to micromolar levels through internal perfusion  $C_m$  increased by 89% in melanotrophs (Parsons *et al.*, 1995) and by 103% (Parsons *et al.*, 1995) or 50–100% (Burgoyne and Handel, 1994) in chromaffin cells. With flash photolysis of caged Ca the  $C_m$  increase was 52% in melanotrophs and 41% in chromaffin cells (Parsons *et al.*, 1995; both 40 s after the flash). If referred to initial surface area, the membrane available for Ca-stimulated exocytosis in CHO cells is apparently one-third to half that in melanotrophs or chromaffin cells. Apart from being some-

what less, however, Ca-stimulated exocytosis in CHO cells seems remarkably similar to that in endocrine cells. In both cases it is rapid and can show multiple kinetic components. Furthermore, exocytosis is also stimulated by GTP $\gamma$ S. However, the 'dense core' granules so prominent in endocrine cells have not been found in CHO cells.

Evidence for Ca-stimulated exocytosis in CHO cells and fibroblasts was also recently obtained by a different approach. Ca influx through voltage-gated Ca channels stimulated the quantal release of ACh from ACh-loaded CHO cells (Morimoto *et al.*, 1995) and the release of ACh quanta was accelerated when  $[Ca^{2+}]_i$  was raised in fibroblasts (Girod *et al.*, 1995). Taken together, the quantal release observed earlier and the increase in  $C_m$  seen here leave little doubt that both CHO cells and fibroblasts are capable of Ca-stimulated exocytosis. There is one difference between the two sets of results. The earlier work detected single quanta. If quanta are contained in vesicles of 100 nm diameter, then the release of each would be accompanied by a surface area increase of ~0.008  $\mu m^2$ . The observed release of 10–100 quanta would increase the cell surface by only 0.08–0.8  $\mu m^2$ , or 0.01–0.1%. In the present work the Ca stimulus was probably stronger, with  $[Ca^{2+}]_i$  remaining at several micromolar for minutes, and exocytosis increased the cell surface by 30%. Even this large value may be an underestimate if exocytosis stimulates significant endocytosis, as it did in the experiments depicted in Figure 2B. Clearly, the Ca-stimulated increase in cell surface seen here was massive.

We do not know which cytosolic organelles are adding their membrane to the cell surface nor what cargo molecules, if any, they release physiologically. However, the size of the surface change suggests that the organelle(s) in question must be abundant. For instance, a constitutive secretory vesicle of 100 nm diameter would have a 0.008  $\mu m^2$  surface; a cell surface increase of 30%, or 330  $\mu m^2$ , would require the exocytosis of 42 000 such vesicles, or 38 vesicles/ $\mu m^2$  of plasmalemma. Abundant membranous compartments are seen in electron micrographs and many of them can fill with horseradish peroxidase as they recycle to and from the cell surface (Morimoto *et al.*, 1995). However, in the absence of quantitative morphometry it seems unlikely that such large numbers of small vesicles are available in the immediate vicinity of the plasmalemma. Perhaps the exocytosed organelle is larger and/or subsurface organelles are replaced after exocytosis by others that arrive from deeper regions of the cytosol.

In summary,  $Ca^{2+}$  stimulates large  $C_m$  increases even in cells other than neurons or neuroendocrine cells. This effect was seen wherever it has been looked for, first in non-endocrine secretory cells such as mast cells (Almers and Neher, 1987; Kirillova *et al.*, 1993) and now in CHO and 3T3 cells.  $Ca^{2+}$  also stimulates the quantal release of ACh and, hence, exocytosis in muscle cells (Dan and Poo, 1992). Evidently, Ca-stimulated exocytosis is also found in cells that are not called upon for rapid release of transmitters or hormones. Homologs of synaptotagmin, the putative Ca sensor protein for exocytosis in neurons (Geppert *et al.*, 1994), are found in cells of many non-neuronal tissues and may be expressed in all cells (Hudson and Birnbaum, 1995; Li *et al.*, 1995). These findings

suggest that many and perhaps all eukaryotic cells are capable of rapid Ca-stimulated exocytosis.

What purpose would this mechanism serve in non-secretory cells? In some cells Ca-stimulated exocytosis may serve the rapid insertion of membrane proteins into the plasma membrane, as with H<sup>+</sup> transporters in urinary epithelium (Schwartz and Al-Awqati, 1986). A more general answer is suggested by the recent demonstration (Steinhardt *et al.*, 1994; Bi *et al.*, 1995; Miyake and McNeil, 1995) that the healing of plasma membrane tears in a variety of cell types requires Ca<sup>2+</sup>-stimulated exocytosis. The exocytosing organelles may be endosomes and lysosomes (Miyake and McNeil, 1995) that are recruited to the lesion sites in seconds. Clearly, all cells must quickly repair breaches in the plasmalemma, lest a prolonged increase in [Ca<sup>2+</sup>]<sub>i</sub> cause irreversible damage. The most immediate intracellular signal of a plasmalemmal lesion is a local and probably large increase in [Ca<sup>2+</sup>]<sub>i</sub>. Hence, one may expect a mechanism for Ca-triggered exocytosis to be present in many or all eukaryotic cells. Ca-triggered secretion in neurons and neuroendocrine cells may have evolved from a universal and ancient cellular mechanism for the repair of plasmalemmal damage.

## Materials and methods

NIH 3T3 fibroblasts (ATCC, Rockville, MD) and CHO cells were plated on 12 mm diameter poly-L-lysine-coated glass coverslips at a density of 3000–5000 per coverslip. NIH 3T3 cells were maintained in DMEM (Gibco BRL) plus 10% fetal calf serum (Sigma) and 2 mM L-glutamine. CHO cells were maintained in modified Eagle's medium (MEM,  $\alpha$ -modification; Gibco BRL) with fetal calf serum and L-glutamine as above, plus 8  $\mu$ M methotrexate (Sigma). The clone used here (a generous gift from Dr Judy White) expressed the influenza virus haemagglutinin (HA) on its cell surface, but this is not expected to affect the results since HA mediates only ectoplasmic fusion, not exocytosis. Cultures were incubated in a 5% CO<sub>2</sub> atmosphere at 37°C. Both CHO and 3T3 cells were used between passages 2 and 16 and always 1–5 days after plating.

During experiments cells were bathed in a solution containing 125 mM NaCl, 20 mM tetraethylammonium (TEA) HCl, 5.5 mM glucose, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 1  $\mu$ M tetrodotoxin and 10 mM Na-HEPES, pH 7.4, and maintained at room temperature (21–25°C). For capacitance (C<sub>m</sub>) measurements a 40 mV peak-to-peak sine wave (800 Hz) was superimposed on a holding potential of –60 mV as previously described (Thomas *et al.*, 1993). Results are given as mean  $\pm$  SE unless indicated otherwise.

Cytosolic [Ca<sup>2+</sup>]<sub>i</sub> was controlled through a glass micropipette by internal perfusion of the cell with a Ca chelator buffer mixture. The pipette contained either solution A (3.66 mM MgCl<sub>2</sub>, 0.465 mM CaCl<sub>2</sub>, 120 mM glutamate, 2 mM ATP, 0.3 mM GTP, 2 mM Ca/Mg chelator HEDTA, 45 mM HEPES, pH 7.4, all as Na salts) or solution B (1 mM MgCl<sub>2</sub>, 2 mM MgATP, 5 mM CaCl<sub>2</sub>, 120 mM glutamate, 8 mM Cl, 0.3 mM GTP, 10 mM EGTA, 10 mM HEPES, pH 7.2, all as Na salts). Free Mg<sup>2+</sup> concentrations were calculated to be 0.4 mM for solution A and 0.8 mM for solution B (program SOL ID, Eric A.Ertel, University of Washington). A Ca-sensitive electrode was used to measure [Ca<sup>2+</sup>]<sub>i</sub> of the two solutions. For solution A [Ca<sup>2+</sup>]<sub>i</sub> = 4  $\mu$ M; the [Ca<sup>2+</sup>]<sub>i</sub> established in a cell, however, is likely to be only 2–3  $\mu$ M (Parsons *et al.*, 1995). For solution B [Ca<sup>2+</sup>]<sub>i</sub> = 100 nM. Pipettes containing these solutions made 4.64  $\pm$  0.2 M $\Omega$  ( $n$  = 17) connections with the cytosol (range 2.04–5.26 M $\Omega$ ).

For experiments involving flash photolysis micropipettes contained 76 mM Cs glutamate, 16 mM TEA HCl, 10 mM Na<sub>4</sub>DM-nitrophen (Calbiochem, La Jolla, CA), 7.5 mM CaCl<sub>2</sub>, 0.2 mM Fura-2 or Fura3 (Molecular Probes, OR) and 50 mM Cs HEPES, pH 7.2. They made 3.70  $\pm$  0.18 M $\Omega$  ( $n$  = 27) connections with the cytosol (range 2.44–4.95 M $\Omega$ ). Before use the [Ca<sup>2+</sup>]<sub>i</sub> of this solution was measured in 20  $\mu$ m thick glass microcuvettes (Vitro Dynamics, New York) using Fura-2; values ranged from 50 to 50 nM (41  $\pm$  2 nM,  $n$  = 11). In melanotrophs this solution was estimated to result in a cytosolic [Mg<sup>2+</sup>]

of <10 nM (Parsons *et al.*, 1995). Rates and amplitudes of the initial C<sub>m</sub> changes after a flash were analyzed as in Thomas *et al.* (1993).

Ratiometric measurements of [Ca<sup>2+</sup>]<sub>i</sub> were carried out with Fura3 or Fura-2 as described (Thomas *et al.*, 1993; Parsons *et al.*, 1995). Calibration constants for Fura-2 were measured in 20  $\mu$ m glass microcuvettes, those for Fura3 were measured in dye-filled melanotrophs.

## Acknowledgements

We thank I.Wunderlich, A.Migala and B.Küchler for technical assistance and Drs M.Lindau and T.Soldati for helpful discussions. J.R.C. was supported by fellowships from the Max-Planck-Society, the Natural Sciences and Engineering Research Council of Canada and by the Deutsche Forschungsgemeinschaft (SFB 317). H.S. was supported by the Deutsche Forschungsgemeinschaft (SFB 317).

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Received on February 8, 1996; revised on March 11, 1996