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Calcium currents and exocytosis in single isolated pars intermedia cells from tilapia (*Oreochromis mossambicus*)

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Abstract

The role of calcium influx through voltage-dependent calcium ion channels in the exocytotic response of single isolated pars intermedia cells from the teleost tilapia (*Oreochromis mossambicus*) was investigated by means of the whole-cell patch-clamp technique and high resolution electrical measurements. Calcium currents differed from barium currents in several ways: the peak I_{Ca} was smaller, the current-voltage relationship for calcium attained its maximum at +10 mV instead of 0 mV, and the inward calcium current inactivated more rapidly. Electron micrographs showed that pars intermedia cells possess dense, encored vesicles with an average diameter of 140 nm. Influx of calcium resulted in an increased cell membrane capacitance (C_m) after the depolarizing period, indicating a fast exocytotic response. Comparison with "late" recordings revealed the presence of a transient in C_m , presumably attributable to movement of ion channel gates. The average increase in C_m was 13.4 fF, suggesting the fusion of at least 23 vesicles with the plasma membrane during the depolarizing pulse. In contrast to calcium, barium did not support significant exocytosis. We conclude that calcium entry through voltage-dependent calcium channels rapidly leads to the exocytosis of secretory vesicles from tilapia pars intermedia cells.

Introduction

Control of the secretory activity of the pituitary gland must and does occur rapidly during adaptation of an animal to changes in the environment. Traditional experimental approaches, e.g., superfusion of isolated pituitary lobes, focussed on longer-term modulation of the secretory response. The actual event of hormone secretion from neuroendocrine cells – fusion of a secretory vesicle with the plasma membrane – occurs within milliseconds (Almers 1990). Using whole-cell voltage-clamp and a phase-sensitive detection unit, changes in plasma membrane electrical capacitance (C_m) can be monitored in real time (Lindau and Neher 1988; Gillis 1995). Alternatively, suitable secretory products can be detected

electrochemically by an amperometric detection method (Chow et al. 1992; Paras and Kennedy 1995).

We have studied hormone release from the MSH cells in the intermediate lobe of the pituitary of the Mozambique tilapia. These cells are involved in background adaptation and in adaptation to stressors (Lamers et al. 1992, 1994). In a separate study we have characterized the voltage-dependent calcium channels of isolated intermediate lobe cells (Schoenmakers et al. unpublished data). We here report on the exocytotic response of single cells by means of fast C_m measurements before and after depolarizing pulses. Exocytosis proved to be specifically dependent on entry of extracellular calcium.

Materials and methods

Cell isolation

Pars intermedia cells were isolated according to Kaneda et al. (1988) with slight modifications. Briefly, fish were killed by spinal transection. A dorsosagittal incision was made in the skull and forceps were used to lift the brain out partly by the spinal cord. The pituitary was subsequently removed. The pars distalis was separated from the neurointermediate lobe (NIL) and the NIL was split along the mid-sagittal plane to facilitate subsequent enzyme access. The NIL pieces were placed in 1 ml of oxygenated incubation solution and washed twice with 2 ml of incubation buffer. The NIL fragments were then incubated in 2 ml of ice-cold buffer containing 1 mg ml⁻¹ trypsin and 1 mg ml⁻¹ of collagenase for 15 min. The tube was then incubated at 28 °C for 20 min, after which the medium was changed to ice-cold buffer in which the pieces were left for 15 min. A drop of liquid containing the pieces was transferred to a coverslip coated with poly-L-lysine. The pieces were subsequently disrupted with 15 to 20 passes through each of three Pasteur pipettes fire-polished to three decreasing sizes. Finally, the cell suspension was distributed over poly-L-lysine-coated coverslips and the cells were given 30 min to adhere. After adhesion was complete, 2 ml of Leibovitz L15-medium (Gibco Laboratories) was added to each petri dish. The cells were kept in primary culture for 1 to 5 days before use.

Measurement of calcium currents and membrane capacitance

The whole-cell mode of the patch-clamp technique was used to record calcium or barium currents through calcium ion channels (Hamill et al. 1981). Whole-cell currents were recorded with an intrapipette solution of (in mM) CsCl (125), TEACl (10), MgCl₂ (1), EGTA (0.1), MgATP (0.5), GTP (0.1), and HEPES (10), adjusted to pH 7.4 with CsOH. The extracellular solution consisted of TEACl (145), CaCl₂ or BaCl₂ (10), MgCl₂ (1) and HEPES (10), buffered to pH 7.4, with TEAOH. In some instances an extracellular solution was used consisting of NaCl (142), KCl

(5), CaCl₂ (2, 5 or 10; osmolarity was kept constant by adjusting the NaCl concentration) and HEPES (10), buffered to pH 7.4 with Tris. All solutions were filtered through 0.2 µM cellulose acetate filters before use.

Cells were superfused with extracellular solution at a rate of 0.5 ml min⁻¹ in a total volume of 0.4 ml extracellular solution. The bath was grounded via an agar bridge. Patch electrodes were pulled from borosilicate capillaries (GC150-15, Clark Electromedical Instruments, Pangbourne, UK) on a Narishige PP-83 patch pipette puller (Narishige Scientific Instruments Lab, Tokyo, Japan) and had a typical resistance of 6 MΩ. Pipettes were coated with Sylgard (Dow Corning Corp., Midland, MI 48640, USA.) to reduce pipette capacitance. When comparing calcium and barium currents the superfusion tubing used to administer the extracellular solutions (Intramedic PE-50 polyethylene tubing, Clay Adams, Becton Dickinson, NJ 07054, USA) was placed 200 µm from the cell under study. Solution applications were performed by operating a four-way valve that switched the continuous flow between calcium-containing and barium-containing solutions. The "Lindau-Neher" technique was used for measuring membrane capacitance changes (Lindau and Neher 1988; Gillis 1995): a sinusoidal voltage with a frequency of 800 Hz and a peak-to-peak amplitude of 25 mV was superimposed on the holding voltage of -80 mV. Step depolarizations of various length and amplitude were applied to stimulate exocytosis. Capacitance measurements were not possible during this period due to the highly non-linear membrane conductances activated by the depolarization (Lindau and Neher 1988). Pulse generation (16 sample points per sine cycle) and data acquisition were performed on an Apple Macintosh® PowerPC 7100/66 computer using HEKA's Pulse/Pulsefit software with the Lock-in extension (version 8.03; HEKA Elektronik Dr. Ing. P. Schulze GmbH, Lambrecht/Pfalz, Germany). Data were filtered with the 4-pole Bessel filter built into the EPC-9 at 2 kHz and sampled at at least 3 times the filter frequency to avoid aliasing. The resulting sinusoidal and steady-state membrane current were analyzed with the software-implemented lock-in amplifier of the EPC9.

Table 1. Differences between calcium and barium currents

Property	Calcium	Barium	n
Inactivation halftime (ms)	42 ± 4	61 ± 7**	7
Inactivation ratio at 0 mV	2.1 ± 0.3	0.8 ± 0.1**	7
Peak current (pA)	62 ± 15	82 ± 18*	5
Plateau current (pA)	30 ± 11	56 ± 15*	5

All data are given as mean ± SEM. * $p < 0.05$; ** $p < 0.01$, as tested with a paired Student's t-test. Inactivation ratio is the ratio of inactivating current size over plateau current size. Current-voltage plots yielded maximal peak and plateau currents for calcium at +10 mV, while maximal barium currents were recorded at 0 mV.

Results

Figure 1 shows typical calcium and barium currents measured from a tilapia pars intermedia cell. The current carried by calcium differed from that carried by barium in several aspects, as summarized in Table 1. The maximal current carried by calcium ions was smaller than that carried by barium. Inactivation of barium currents was less pronounced during the depolarization period. When fitted with a single exponential, the relaxation time for calcium currents was 42 ± 4 ms, while that for barium currents was 61 ± 7 ms (Fig. 1A). The calcium currents were also inactivated to a greater extent. The ratio of inactivating current amplitude ($I_{\text{peak}} - I_{\text{plateau}}$) over noninactivating current amplitude (I_{plateau}) for calcium was almost three times that found for barium. Finally, the voltage-dependence of the current was different as well. The current-voltage relationship for barium showed a maximum at 0 mV, while calcium currents were maximal at +10 mV (Fig. 1B). Tested at their respective maximal voltage, both peak and plateau currents for calcium were significantly smaller than for barium (cf. Table 1).

Barium was, however, far less effective in inducing exocytosis than calcium. Figure 2A shows calcium and barium currents in a single cell during a 200-ms depolarizing pulse to +10 mV. Total barium entry equalled 4.0 pC, while total charge carried by calcium was 0.9 pC. Nonetheless, calcium induced an 8 fF-rise in membrane capacitance at the end of the 250 ms following the depolarization, while barium influx resulted in only a 2 fF increase (Fig. 2B). Thus, calcium was about sixteenfold more effective in inducing exocytosis than barium.

As shown in Figure 3A and B, 200-ms depolarizations to +10 mV induced exocytosis without significantly altering G_{total} (a possible source of artefacts). G_{total} was mainly composed of the access conductance (G_s), as membrane conductance (G_m) at the holding potential typically was 100 pS or less. In approximately 40% of the cells, we observed an increased G_m (≤ 2 times the pre-pulse G_m) after repolarization to -80 mV, which returned to control levels within 50 ms. This increased membrane conductance might reflect a slight activation of calcium-dependent potassium channels. Its effect on G_{total} was negligible. An increase in C_m was observed in 11 out of 33 cells tested. Exocytotic response was loosely coupled to calcium current magnitude; by and large, only cells with peak calcium currents larger than 50 pA displayed changes in membrane capacitance. After repolarization to -80 mV the C_m did not continue to increase. Instead, the C_m trace invariably showed a decreasing transient directly after the depolarization. Exocytotic responses were absent in traces from a "late" stage of the whole-cell recording, but the transient could still be observed (Fig. 3C). Also, the transient was still present when cells were depolarized to highly positive potentials (+80 mV), where calcium entry was minimal and exocytosis was not observed. The capacitive transient was fitted with a single exponential with a decay time constant of 53 ± 7 ms (mean ± SEM; $n=11$). We tentatively attribute the transient to sodium channel gating-current relaxation (Horrigan and Bookman 1994). Records corrected for this transient by subtracting the curve fitted on corresponding "late" traces showed a step response in membrane capacitance (Fig. 3B; lowermost trace). In our further analysis, we routinely ignored the first 100 ms following the depolarization. The average step increase observed in response to a 200-ms depolarization to +10 mV was 13.4 ± 1.3 fF (mean ± SEM; $n=8$ cells, multiple records per cell). The increase in C_m was positively correlated with the integral of inward calcium current (Fig. 3D). Half-maximal activation of exocytosis occurred at 1.1 pC with a Hill-coefficient of 0.97. The average dense-core vesicle diameter of tilapia pituicytes, as seen in electron-micrographs (Fig. 4), was 140 nm. Taking into account a membrane capacitance of 1 pF $100 \mu\text{m}^{-2}$, the contribution of one vesicle fusing with

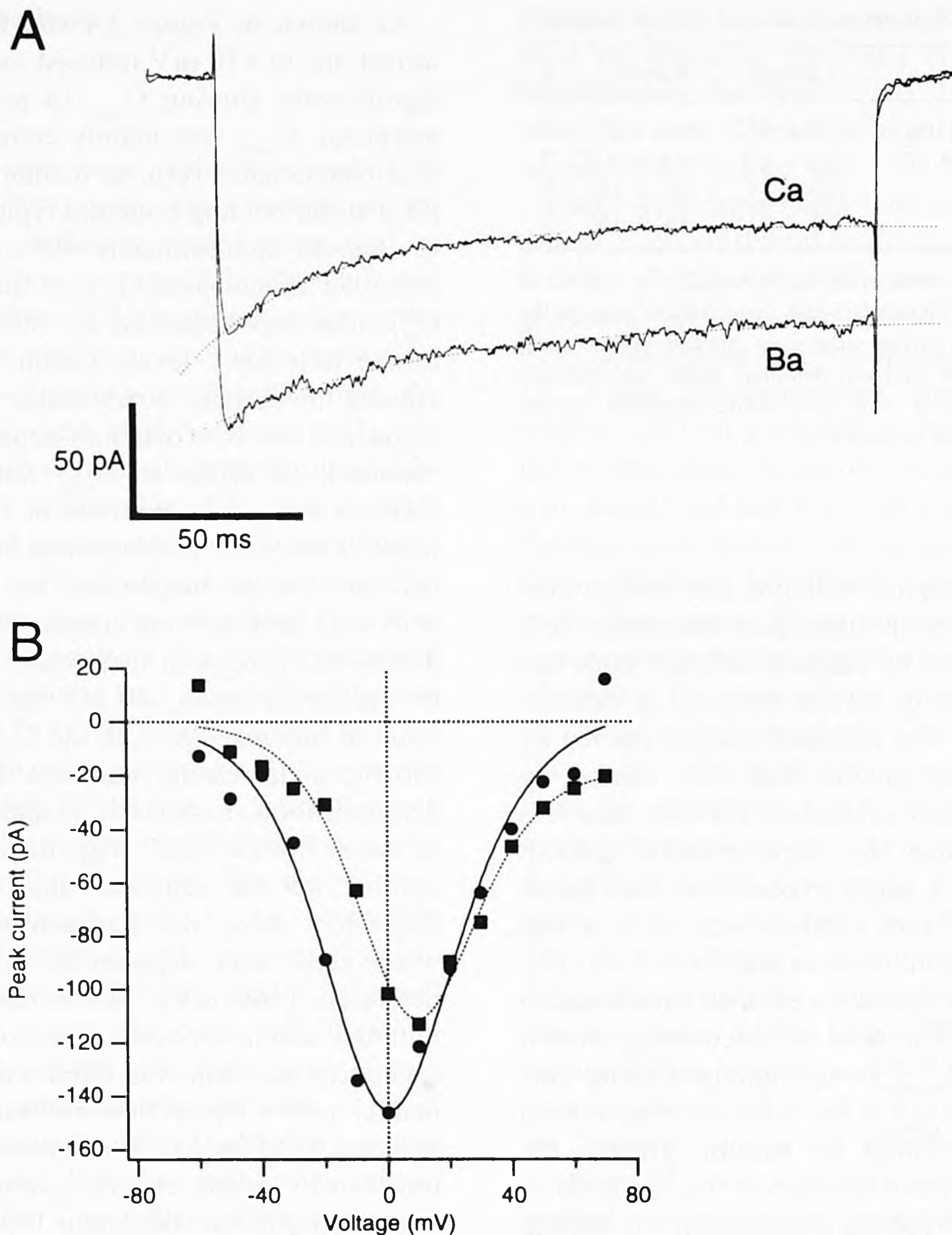


Fig. 1. (A) shows recordings from a single pars intermedia cell superfused with extracellular solutions containing either 10 mM calcium or 10 mM barium. Calcium and barium current traces are averages of 6 and 3 recordings from the same cell, respectively. A 200-ms depolarization to 0 mV induced a maximal calcium current of 117 pA and a maximal barium current of 161 pA. The calcium current decayed to a plateau level of 68 pA with a halftime of 39 ms; the barium current inactivated more slowly ($\tau = 66$ ms) to a 110 pA plateau current. (B) shows the current-voltage relationships for calcium and barium currents of this cell. The maximal current with calcium as permeant ion (filled squares and dotted line) is attained at a V_m of +10 mV. It is smaller than the barium current, which is maximal at 0 mV (filled circles and solid line). The continuous curves are fits of a combined Boltzmann and Goldman-Hodgkin-Katz equation to the data.

the plasma membrane is calculated to be 0.58 fF. Therefore, we conclude that on average, a 200-ms depolarization to +10 mV resulted in the fusion of at least 23 secretory vesicles with the cell membrane.

Discussion

The study demonstrates that high-voltage activated calcium channels showed different characteristics when calcium rather than barium is the permeant cation. Maximal calcium currents when compared to barium currents were smaller, inacti-

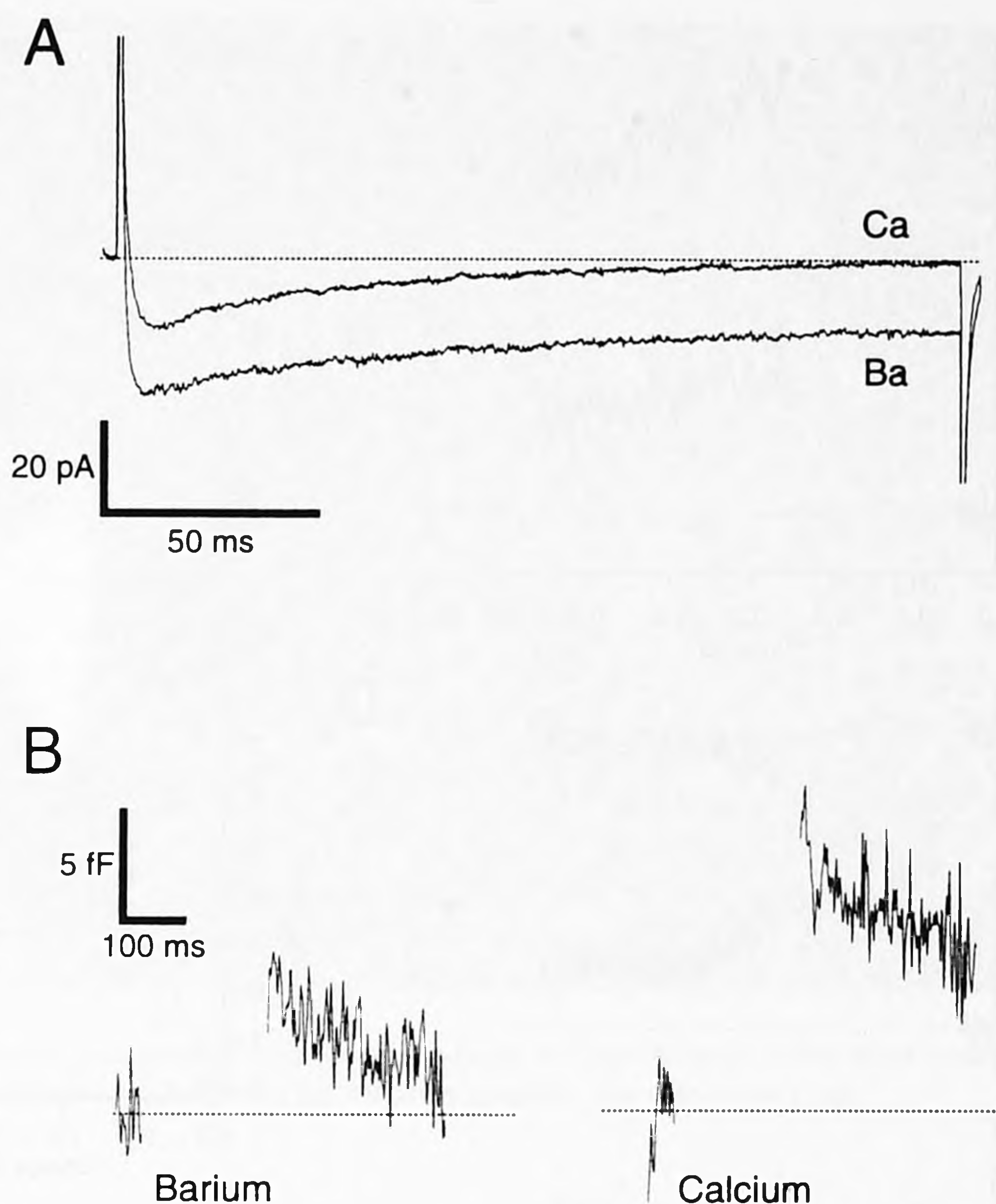


Fig. 2. A: Currents recorded from a single pars intermedia cell superfused with solutions containing either 10 mM calcium (upper trace) or 10 mM barium (lower trace). Membrane capacitance measurements preceded and followed the 200-ms depolarizations to +10 mV. Integrated charge carried into the cell was 0.88 pC for calcium and 4.0 pC for barium. B: C_m measurements accompanying the above currents. ΔC_m was 2.0 fF for barium, and 8.0 fF for the calcium current immediately afterwards.

vated more rapidly and their voltage dependence shifted towards more positive voltages. Further, although barium was more permeant than calcium, its potency for inducing exocytosis was minimal. Calcium entry into pars intermedia cells, however, induced a dose-dependent increase in C_m , which would correspond to the exocytosis of approximately 23 secretory vesicles.

The teleostean part intermedia consists of several cell types. Using antibodies raised against chum salmon somatolactin (kindly provided by Dr. T. Kaneko, Ocean Research Institute, Tokyo, Japan) and tilapia α -MSH, we observed that more than 80% of the pars intermedia cells are melanotrophs. Therefore, it remains a possibility that the results obtained here pertain not only to melanotrophs, but also to somatolactin cells. The involve-

ment of stellate cells and other nerve tissue can be ruled out on the basis of their different morphology.

Barium versus calcium

The fact that barium produces larger currents and less inactivation than calcium, is well established (Hille 1991). The rate of inactivation of the calcium current is somewhat decreased by the experimental protocol (depolarizations to 0 mV) underlying Figure 1A. When comparing barium and calcium currents evoked at 0 and 10 mV respectively (cf. Fig. 1B), the inactivating component of the calcium current is much more prominent. Depolarizations to even higher voltages resulted in

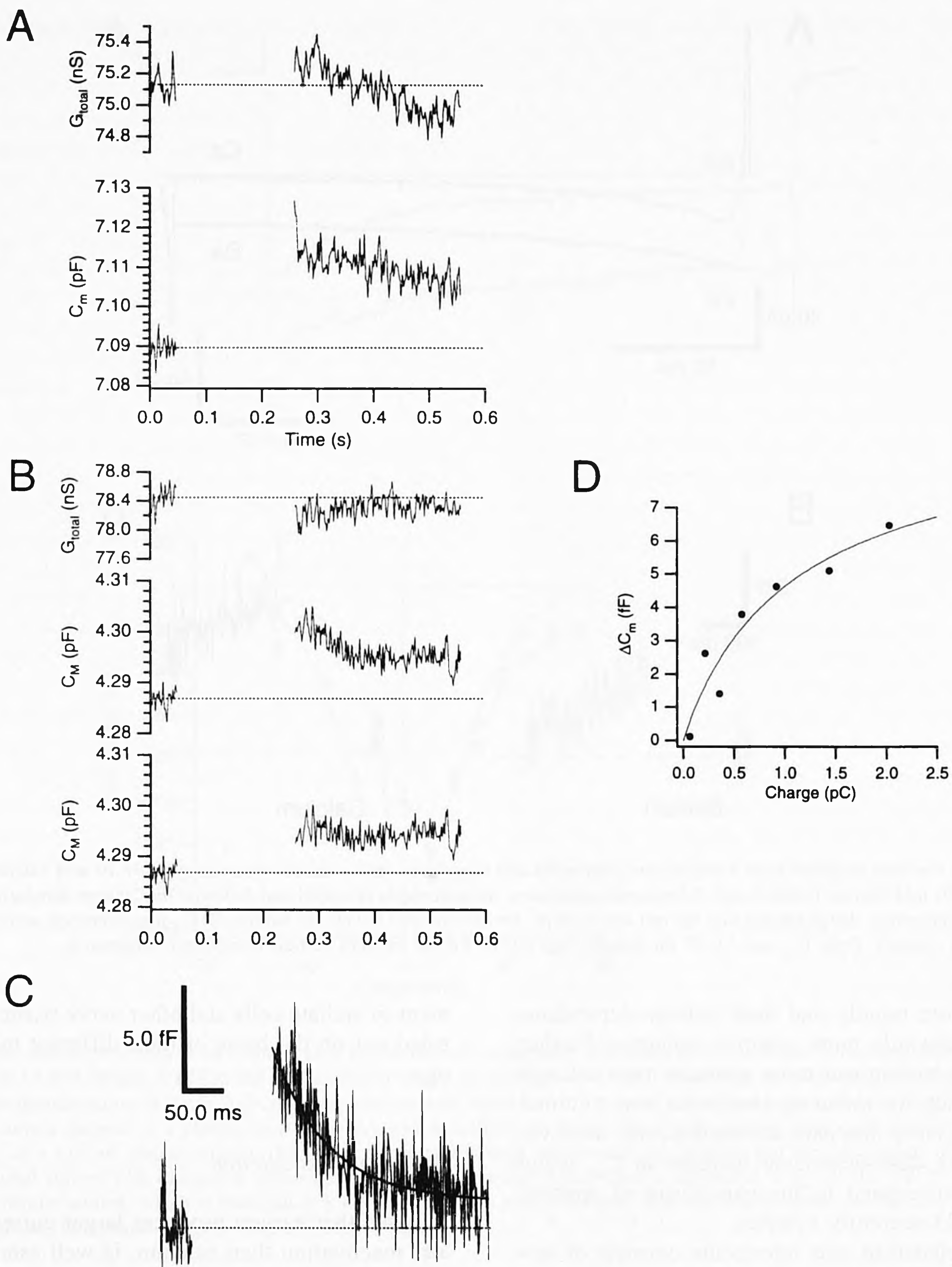


Fig. 3. A: typical recording of an exocytotic response. G_{total} does not change, while C_m increases with 19 fF. B: recording from another cell, in which C_m increased by 8 fF. The capacitive transient was further characterized (shown enlarged in C) at the end of the experiment, when the cell showed no more exocytotic activity. It was fitted by a single exponential with a maximum of 58 fF and a half-time constant of 76 ms. Subtracting this fitted curve from the middle C_m trace yields a step response in C_m , shown as the bottom trace in 3B. This cell was further subjected to depolarizations to +10 mV of increasing length and the calcium currents were integrated to give total charge. The average of 4 series is shown in D. Exocytosis appears to be a saturable function of the amount of calcium ions entering the cell.

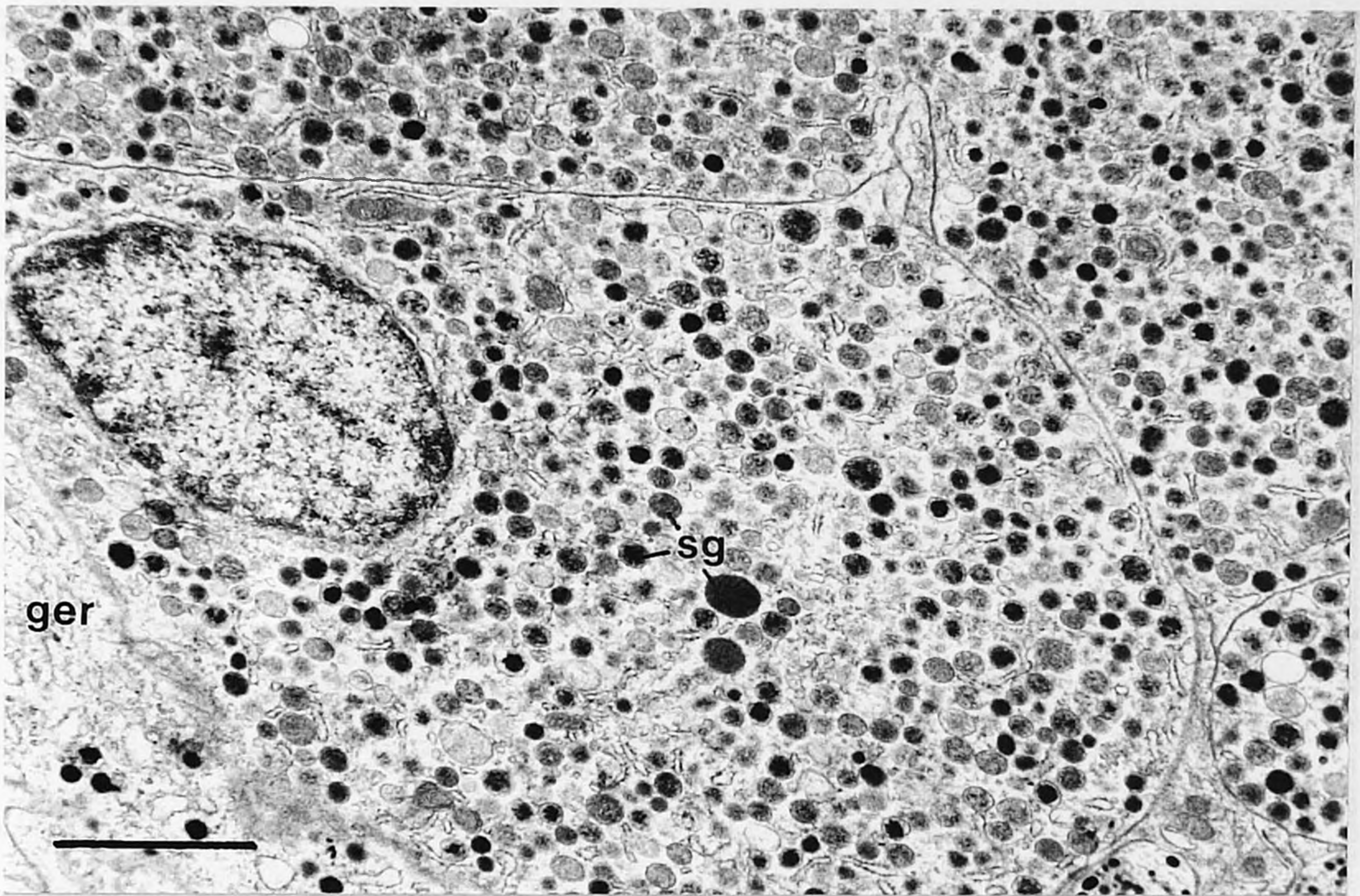


Fig. 4. Transmission electron micrograph of a tilapia melanotroph. Average diameter of the dense-cored vesicles was 140 nm; Ger, strands of the granular endoplasmic reticulum; sg, secretory granules. Bar represents 1 μ m.

less calcium influx and show a lower rate of inactivation. This suggests that inactivation of calcium current in these cells is, at least partly, calcium-dependent, as was shown previously for other high-voltage activated calcium channels (Hille 1991; Kasai and Neher 1992).

Figure 2 shows that exocytosis was stimulated to a much greater extent by calcium than by barium in these cells. Evidently, although barium more readily permeated through the calcium channels, it was notably ineffective in inducing vesicle fusion. A similar discrepancy was described for bovine chromaffin cells (von Rüden et al. 1993). Clearly, the process of vesicle fusion is more specific for calcium than the ionic sieve of the calcium channel itself. The entry of calcium appears to be essential for the exocytotic process. This observation is congruous with the relationship between intracellular calcium concentration and secretory activity observed in pituitary cells from other vertebrate groups such as amphibians (Shibuya and Douglas 1993; Scheenen et al. 1994) and mammals (Tse et al. 1993).

Exocytosis

Depolarizations to +10 mV resulted in an increased membrane capacitance, as illustrated in Figure 3. Our experiments indicated that the capacitive transient following the depolarizing pulse was not caused by a change in the other parameters – G_m and/or G_s – derived by the software lock-in amplifier, but presumably originated from the movement of charged residues of plasma membrane proteins, such as ion channels, as originally suggested for rat adrenal chromaffin cells by Horrigan and Bookman (1994). First, cells displaying little or no calcium current showed no increase in C_m at the end of the high-resolution capacitance measurement. The capacitive transient, however, could still be observed. Second, cells with an intracellular EGTA concentration of 10 mM (*cf.* below) showed no exocytosis, but did display the C_m transient. Third, depolarizations to +80 mV did not induce calcium influx nor a sustained increase in C_m . The capacitive transient increased approximately twofold in size compared

with the transient evoked by depolarizations to +10 mV, although its decay time constant remained constant. The time constant for the capacitive transient lies between those reported for rat (606 ms) and bovine (16 ms) adrenal chromaffin cells (Horrigan and Bookman 1994; Chow et al. 1996).

When corrected for the capacitive transient, C_m had increased by an average of 13.4 fF, corresponding to the fusion of at least some 23 secretory vesicles with the plasma membrane. This observation is in line with values reported for other neuroendocrine cells (Horrigan and Bookman 1994). C_m traces showed virtually no change during the 300 ms following the depolarizing pulse (Fig. 3B, lowermost trace), suggesting that exocytosis and endocytosis proceed at equal rates. Long-term recordings made in the intervals between the high-resolution measurements shown here indicated that endocytosis became predominant after many seconds, returning the cell membrane capacitance to a near-initial value. In our hands, loss of endocytotic activity did not precede that of exocytosis during experiments, as commonly observed for chromaffin cells (Chow et al. 1996). Endocytosis may be coupled to intracellular calmodulin and the phosphorylation state of membrane proteins (Artalejo et al. 1996). As we did not include an ATP-regenerating system in our intrapipette solutions, decreased phosphorylation state of intracellular proteins may partly underlie the decreased endocytotic and exocytotic activity in prolonged whole-cell recordings. We did not further investigate this particular issue.

Cells with a sufficiently large calcium current displayed distinct C_m steps of 3–5 fF 300 ms after depolarizing pulses as short as 10 ms when an intrapipette EGTA concentration of 0.1 mM was used. When we used higher EGTA concentrations (1 and 10 mM), exocytotic activity was absent in almost all cells tested. Further reducing the EGTA concentration led to a rapid deterioration in cell and seal quality after attaining the whole-cell configuration. The lack of exocytotic activity at higher EGTA concentrations indicates that this “slow” calcium buffer (Harrison and Bers 1987) chelates most of the calcium ions entering the cell before they can interact with the secretory machinery. This argues against the existence of “active zones”, where calcium ion channels and secretory

vesicles are tightly colocated (Robinson et al. 1995). Recent electrochemical and patch-clamp data on chromaffin cells appear to contradict the “active zone” theory as well (Chow et al. 1996).

A dose-dependent relationship between calcium entry and exocytotic activity, as shown in Figure 3D, was also apparent when extracellular calcium concentrations were less than 10 mM. Exocytosis could still be observed in some cells tested with 5 mM extracellular calcium, but we did not find exocytosis with 2 mM extracellular calcium. The curve shown in Figure 3D has a Hill coefficient near 1. This apparently contrasts with the “power relationship” commonly observed in neurons and neuroendocrine cells, where coefficients from 2 to 4 have been reported (Augustine and Neher 1992; Thomas et al. 1993; Dunlap et al. 1995). Perhaps, the close temporal spacing of the depolarizing pulses (1 Hz) in this particular experimental protocol caused a sustained elevated intracellular calcium concentration, resulting in a distortion of the actual concentration-effect relationship.

Our present results demonstrate the possibility to study rapid secretory events using a high time resolution in single neuroendocrine cells from the fish pituitary pars intermedia. They indicate a significant role in the exocytotic process for calcium entering across the plasma membrane. Recently, it has been shown that secretory products from melanotrophs can also be detected electrochemically (Paras and Kennedy 1995). We are presently investigating the regulation of these calcium currents by hypothalamic factors and we will extend these experiments to include hormone effects on the secretory activity of these cells, possibly by a combination of electrochemical and whole-cell patch-clamp techniques.

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