Changes in cytoplasmic calcium determine the secretory response to extracellular cations in human parathyroid cells: a confocal microscopy study using FM1-43 dye

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Whether activation of the calcium receptor (CaR) modulates secretory events was investigated by real-time fluorescence and confocal microscopy using fura 2 and FM1-43 fluorescent dye. Two paradigms were used: human parathyroid cells, which are stimulated by a step from a high to a low extracellular calcium concentration ($[Ca^{2+}]_{ext}$), and rMTC6-23 cells, a rat medullary thyroid carcinoma cell line whose secretion is stimulated by an increase in $[Ca^{2+}]_{ext}$. Parathyroid cells were dispersed from parathyroid adenomas removed from 18 patients with primary hyperparathyroidism. In both cell types, incubation with FM1-43 (2 μ M) resulted in staining of the plasma membranes, which was rapidly increased following changes in $[Ca^{2+}]_{ext}$ known to stimulate secretion. A high $[Ca^{2+}]_{ext}$ and lanthanum (La^{3+}) decreased the membrane-associated FM1-43 fluorescence. Prolonged incubation (5–30 min) in the presence of FM1-43 resulted in accumulation of the dye in the cytoplasm, its granular distribution suggesting targeting of the secretory compartment. These data suggest that FM1-43 fluorescence is determined by:

(i) changes in cell membrane surface area associated with secretion-associated events, (ii) displacement/quenching by extracellular cations and (iii) endocytosis of the dye. In parathyroid cells, a rise in FM1-43 fluorescence occurred during incubation in a high (inhibitory) $[Ca^{2+}]_{ext}$ if the cytoplasmic calcium concentration ($[Ca^{2+}]_i$) was decreased by the calcium chelator BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] (10–50 μ M). Alternatively, the expected rise in FM1-43 fluorescence did not occur during incubation in a low (stimulatory) $[Ca^{2+}]_{ext}$ if $[Ca^{2+}]_i$ was increased by addition of the calcium ionophore A23187 (10–25 μ M). These data suggest that $[Ca^{2+}]_i$, rather than the absolute value of $[Ca^{2+}]_{ext}$, is the main modulator of secretion from parathyroid cells.

Key words: Calcium receptor (CaR), exocytosis, endocytosis, hyperparathyroidism, rMTC6-23 cells.

INTRODUCTION

A key role in the homeostasis of the extracellular calcium concentration ($[Ca^{2+}]_{ext}$) is played by the parathyroid glands, secretion from which is stimulated by a decrease in $[Ca^{2+}]_{ext}$ and inhibited by hypercalcaemia [1]. Expression of a plasma-membrane calcium receptor (CaR) [2] confers upon parathyroid cells the ability to recognize and respond to small changes in $[Ca^{2+}]_{ext}$. Stimulation of this CaR by extracellular calcium and other agonists (e.g. lanthanum, gadolinium, neomycin) activates the G-protein/phospholipase C pathway, inducing a rise in the cytoplasmic inositol 1,4,5-trisphosphate concentration, followed by release of Ca^{2+} from intracellular stores [3,4]. This mechanism leads to an elevation of the cytoplasmic calcium concentration ($[Ca^{2+}]_i$) during hypercalcaemia, and therefore the secretion of parathyroid hormone (PTH) is inhibited by a rise in $[Ca^{2+}]_i$. Since in other endocrine cells secretion is stimulated rather than inhibited by an increase in $[Ca^{2+}]_i$, this 'parathyroid paradox' remains a challenging question. Furthermore, morphological studies reported that PTH is stored in cytoplasmic granules [5] found under the cytoplasmic membrane of cells from parathyroid adenomas [6] and which are lost during secretion induced by low $[Ca^{2+}]_{ext}$ [7]. These data suggest that, despite its inhibition by high $[Ca^{2+}]_i$, PTH secretion occurs through regulated exocytosis.

In patients with primary hyperparathyroidism, secretion of PTH continues uninhibited in the face of hypercalcaemia. Most patients with primary hyperparathyroidism have a single parathyroid adenoma whose cells are monoclonal [8–10], have a lower $[Ca^{2+}]_i$ in response to $[Ca^{2+}]_{ext}$ [11,12] and a higher set point (defined as the $[Ca^{2+}]_{ext}$ necessary for half-inhibition of PTH secretion) [13]. Cloning of the CaR from bovine parathyroid cells [2] and its isolation from human parathyroid adenomas [4] raised hopes that the molecular basis of primary hyperparathyroidism would be better understood. Specific mutations of the CaR explain an altered sensitivity to $[Ca^{2+}]_{ext}$ in some diseases (e.g. familial hypocalciuric hypercalcaemia) [14–16], but no such mutations have been identified in parathyroid adenomas [17,18]. Although lower CaR expression occurs in parathyroid adenomas compared with normal glands [19,20], it remains likely that an abnormality of the signalling pathways of the CaR or of the secretory machinery is involved in the pathogenesis of primary hyperparathyroidism. The present study was initiated in order to investigate further the role of $[Ca^{2+}]_{ext}$ in modulating secretion from parathyroid cells in patients with primary hyperparathyroidism.

Imaging techniques were used in the present study because they allow simultaneous examination of $[Ca^{2+}]_i$ and secretion in multiple cells. Changes in $[Ca^{2+}]_i$ were monitored using quant-

Abbreviations used: BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); $[Ca^{2+}]_{ext}$, extracellular calcium concentration; $[Ca^{2+}]_i$, cytosolic calcium concentration; CaR, calcium receptor; fura 2/AM, fura 2 acetoxymethyl ester; PTH, parathyroid hormone.

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itative fluorescence microscopy in fura 2-loaded cells [21]. Secretion-coupled membrane trafficking was explored using FM1-43 [*N*-3-(triethyl ammoniopropyl)-4-(*p*-dibutylaminostyryl)pyridium dibromide], a lipophilic membrane probe which becomes fluorescent on incorporation into membranes [22,23]. Use of FM1-43 as a probe for membrane trafficking stems from its ability to reversibly stain membranes, its inability to penetrate membranes and its fluorescence. Furthermore, the absorption and emission wavelengths of FM1-43 (502 and 625 nm respectively) allow it to be used simultaneously with measurement of $[Ca^{2+}]_i$ using fura 2 (whose optimal absorption and emission wavelengths are 340/380 nm and 510 nm respectively) [21]. A dual-labelling technique with FM1-43 and fura 2 was used previously in our laboratory to study secretion from bovine anterior pituitary cells [24,25].

The present study aimed to provide further evidence for a link between $[Ca^{2+}]_i$ responses to CaR activation and secretion in human parathyroid cells. Parallel experiments were undertaken using rMTC6-23 cells, a rat medullary thyroid carcinoma cell line derived from calcitonin-secreting cells of the thyroid (C-cells), which are responsive to changes in $[Ca^{2+}]_{ext}$ and secretion from which is stimulated by a rise in $[Ca^{2+}]_{ext}$ [26]. It was anticipated that CaR activation by extracellular cations should have opposite effects on secretion from parathyroid cells and rMTC6-23 cells, and that these differences could be demonstrated and quantified by monitoring the uptake of FM1-43.

MATERIALS AND METHODS

Preparation of dispersed human parathyroid cells

A single parathyroid adenoma was removed from 18 patients with primary hyperparathyroidism. The patients comprised 14 women and four men, aged 24–75 years (median 54 years). All patients had hypercalcaemia (mean serum calcium 2.96 ± 0.31 mmol/l, range 2.61–3.77 mmol/l; reference range 2.20–2.60 mmol/l) and uninhibited intact PTH concentrations (18.3 ± 7.6 pmol/l, range 8.2–46.0 pmol/l; reference range 2.6–7.0 pmol/l). Intact PTH was measured using a chemiluminescent assay (Immulate; DPC) (coefficient of variance 4.7% and 5.5% for intra-assay and interassay precision respectively). Histological examination of paraffin-embedded sections confirmed the diagnosis of parathyroid adenoma in all patients.

The adenomas were collected from the operating theatre and stored in ice-cold culture medium [RPMI-1640, supplemented with 10% (v/v) newborn calf serum, 1% glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B]. The outer capsule was removed, and the tissue was minced with scissors into 1 mm fragments and digested for 45 min at 37 °C in RPMI-1640 with 2 mg/ml collagenase (type 2) and 0.2 mg/ml DNAase (Sigma). The digest was thereafter mechanically dispersed by aspiration through needles of decreasing gauge (nos. 19–22). The cell suspension was filtered through a 100 μ m mesh stainless steel gauze and centrifuged at 200 *g* for 5 min. Trypan Blue exclusion vital staining was used to assess cell viability (> 90%) and yield. Cell cultures were incubated at 37 °C, under a CO₂/air mixture (5:95) for 24–72 h. All culture surfaces (glass coverslips and Petri dishes) were coated with Pronectin F (TCS Biologicals, Botolph Claydon, Bucks., U.K.).

Culture of rMTC6-23 cells

The rMTC6-23 cells (European Cell Culture Collection; no. 87042206, clone 6, passage 23) were grown in Dulbecco's modified Eagle's medium with high glucose (4500 mg/l) and sodium pyruvate (110 mg/l) (DMEM6540; Sigma) supplemented with

glutamine (2 mM) and 10% (v/v) foetal bovine serum. Cells were grown on a Matrigel Basement Membrane Matrix (Becton Dickinson, Bedford, MA, U.S.A.) according to the manufacturer's instructions.

Confocal microscopy on FM1-43-loaded cells

A Leica DM-IRBE inverted epifluorescence microscope with phase contrast ($\times 40$ Leica objective) was connected to a laser scanning confocal microscope (Leica TCS-NT) equipped with a krypton/argon mixed gas laser, generating two wavelengths (568 and 488 nm). The scan head filters were selected to optimize the wavelength for FM1-43, and the rhodamine filter set was always used (bandpass filter 514 nm, dichroic 510 nm, emission above 515 nm).

The glass coverslips with cells attached were immersed in an experimental medium containing: NaCl 129 mM, KCl 4.7 mM, Hepes 11 mM, NaHCO₃ 4.7 mM, NaH₂PO₄ 2.7 mM, MgCl₂ 1.2 mM and glucose 5.5 mM, at pH 7.4. Cells were incubated for 1–2 min with 2 μ M FM1-43 at 3 mM $[Ca^{2+}]_{ext}$ before imaging. Unless specified otherwise, the FM1-43 dye was present throughout the experiment at the same concentration (2 μ M).

Images were collected at equal time intervals (30–60 s) using the confocal software. The intensity of fluorescence was represented by a computer-generated pseudo-colour scale running from black (low intensity) via blue, green and red to white (high intensity). All fluorescence measurements were expressed in arbitrary units generated from the pixel grey scale. The imaging parameters were constant for data presented in the same Figure, but not between Figures.

Fluorescence microscopy for double labelling with fura 2 acetoxymethyl ester (fura 2/AM) and FM1-43 dyes

Quantification of the two dyes in the same cell was achieved by using a Nikon Diaphot inverted epifluorescence microscope with a Nikon CF-series UV-Fluor $\times 40$ oil-immersion objective (numerical aperture 1.3). The cells were excited at 340 nm and 380 nm (for fura 2) and at 460 nm and 510 nm (for FM1-43) using band-pass interference filters (Omega Optics) mounted on a wheel turned by a computer-controlled stepper motor (Improvision; IonVision). A Nikon 455 nm dichroic mirror reflected the 340 and 380 nm light to the stage, but allowed the fluorescent signal emitted by fura 2 (510 nm) to pass. The fluorescent emission was passed through a barrier filter into an intensified CCD camera (Extended ISIS-M; Photonic Sciences). Sufficient incident light to excite FM1-43 (460 and 510 nm) was reflected by the dichroic mirror. Neutral density filters were placed in front of the exciting light to decrease the intensity of the FM1-43 emission to that of the fura 2 emission. Autofluorescence was negligible at all wavelengths.

The glass coverslips with the cells attached were immersed in experimental medium and incubated with 2 μ M fura 2/AM at room temperature for 45–60 min, and then washed with medium and stored at room temperature until experiments were performed (not more than 2 h). The coverslips were mounted in a perfusion chamber (working volume 400 μ l) on the stage of the Nikon Diaphot microscope. All experiments were performed at ambient room temperature (22–25 °C). The medium in the bath was changed by pipetting, and drugs were added as 100 μ l of 5-fold concentrated solutions.

An Improvision IonVision analysis system was used for the acquisition, quantification and presentation of $[Ca^{2+}]_i$ as the ratio of the intensity of fluorescence at 340 nm and 380 nm. Cells were incubated in darkness and exposed alternatively to short (300 ms) periods of excitation at the appropriate wavelength to reduce

photobleaching. The intervals between excitations (1–10 s) were controlled by the software. No deleterious effect during prolonged exposure to FM1-43 or photobleaching of the two dyes was observed. At the beginning of each experiment, a background image was recorded from an empty field and subtracted from each sample image on a pixel-by-pixel basis. The intensity of the FM1-43 fluorescence was quantified and expressed in arbitrary units generated from the pixel grey scale using the same 'regions of interests' as for the $[Ca^{2+}]_i$ measurements.

Chemicals and reagents

RPMI-1640 culture medium, glutamine, penicillin, streptomycin and amphotericin B were from Gibco BRL (Paisley, Scotland, U.K.). Experimental medium was prepared using chemicals of standard purity from Sigma (Poole, Dorset, U.K.) and BDH (Poole, Dorset, U.K.). FM1-43 was purchased from Molecular Probes (Eugene, OR, U.S.A.). Fura 2/AM, BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis-(acetoxymethyl ester)] and A23187 were from Calbiochem (Beeston, Notts., U.K.).

Statistical analysis

Student's *t*-test for pairs was used to compare the fluorescent signals from individual cells at different time points of the experiments.

RESULTS

$[Ca^{2+}]_{ext}$ -dependent uptake of FM1-43

Parathyroid cells incubated in the presence of 2 μ M FM1-43 at high $[Ca^{2+}]_{ext}$ (3 mM, to inhibit secretion) appeared on both fluorescence and confocal microscopy as narrow annuli, with cells becoming visible 1–2 min after the addition of FM1-43. A step in $[Ca^{2+}]_{ext}$ from 3 mM to 0.5 mM induced a progressive increase in the fluorescent signal, with a new steady state reached within 3–10 min (Figure 1). Stepping from 3 mM to 0.5 mM $[Ca^{2+}]_{ext}$ induced a decrease in $[Ca^{2+}]_i$ and a simultaneous increase

Figure 1 Increase in FM1-43 fluorescence associated with plasma membranes in human parathyroid cells in response to stepping to low $[Ca^{2+}]_{ext}$

Dispersed human parathyroid cells were incubated in the presence of 2 μ M FM1-43 at 3 mM $[Ca^{2+}]_{ext}$ and imaged by fluorescence microscopy (see the Materials and methods section). The Figure illustrates the fluorescence of cells 5 min after stepping to 0.5 mM $[Ca^{2+}]_{ext}$ and demonstrates that, shortly after stepping to low $[Ca^{2+}]_{ext}$, FM1-43 remains restricted mainly to the plasma membranes.

Figure 2 Changes in $[Ca^{2+}]_i$ and FM1-43 fluorescence in parathyroid cells in response to a step in $[Ca^{2+}]_{ext}$

Symbols represent average responses (means $\pm$ S.D.) for $n = 21$ cells. Horizontal bars correspond to changes in $[Ca^{2+}]_{ext}$ from 3 mM (upper bars) to 0.5 mM (lower bars), in the presence (filled bars) or in the absence (empty bars) of 2 μ M FM1-43 in the extracellular medium.

Figure 3 Changes in FM1-43 fluorescence in rMTC6-23 cells in response to a step in $[Ca^{2+}]_{ext}$

Symbols represent average responses (means $\pm$ S.D.) for $n = 17$ cells. Horizontal bars correspond to changes in $[Ca^{2+}]_{ext}$ from 0.5 mM (lower bars) to 3 mM (upper bars), in the presence (filled bars) or in the absence (empty bars) of 2 μ M FM1-43 in the extracellular medium.

in FM1-43 uptake, which stopped if cells were incubated again in 3 mM $[Ca^{2+}]_{ext}$ and 'restarted' when they were re-exposed to 0.5 mM $[Ca^{2+}]_{ext}$ (Figure 2). This was consistently observed in

Figure 4 Relative potency of 100 μM lanthanum and 3 mM $[\text{Ca}^{2+}]_{\text{ext}}$ in changing $[\text{Ca}^{2+}]_{\text{i}}$ and FM1-43 fluorescence in parathyroid cells

Symbols represent average responses (means $\pm$ S.D.) for $n = 38$ cells. Horizontal bars correspond to changes in $[\text{Ca}^{2+}]_{\text{ext}}$ from 3 mM (upper bars) to 0 mM (lower bars). The addition of 100 μM La^{3+} is marked by the horizontal line. Numbers indicate different periods of the incubation protocol (as referred to in the Results section).

repeated experiments with cells from five different parathyroid tumours. Both $[\text{Ca}^{2+}]_{\text{i}}$ and FM1-43 responses were preserved during culture of human parathyroid cells for up to 7 days.

In rMTC6-23 cells, a step from 0.5 to 3 mM $[\text{Ca}^{2+}]_{\text{ext}}$ induced a decrease in FM1-43 fluorescence (Figure 3, section 2 of the curve). However, on returning to low $[\text{Ca}^{2+}]_{\text{ext}}$, the FM1-43 fluorescence was significantly higher than basal levels (Figure 3, compare section 3 with section 1; 41.9 ± 16.2 compared with 32.6 ± 14.1 arbitrary units; $P < 0.001$), with an increase of $33 \pm 15\%$ (range 11–67%) of the FM1-43 fluorescence in individual cells. When FM1-43 was removed from the extracellular medium, the fluorescent signal decreased dramatically (Figure 3, sections 4 and 5), suggesting that most of the initial increase in FM1-43 fluorescence (i.e. Figure 3, section 3) was due to uptake of dye associated with the plasma membranes.

Effect of the trivalent cation lanthanum on FM1-43 fluorescence

A large fall in FM1-43 fluorescence was seen during incubation with the trivalent cation lanthanum (La^{3+} ; an agonist for the CaR). In parathyroid cells, 100 μM La^{3+} induced a large transient rise in $[\text{Ca}^{2+}]_{\text{i}}$ and a rapid fall in the FM1-43 signal (Figure 4, lower panel, section 5 of the curve). Compared with 3 mM $[\text{Ca}^{2+}]_{\text{ext}}$, 100 μM La^{3+} was more effective at displacing FM1-43 than at increasing $[\text{Ca}^{2+}]_{\text{i}}$; it induced a $[\text{Ca}^{2+}]_{\text{i}}$ response similar to that caused by 3 mM $[\text{Ca}^{2+}]_{\text{ext}}$, but a significantly larger fall in

Figure 5 Dose-dependent effects of lanthanum on FM1-43 fluorescence in parathyroid cells

Symbols represent average responses (means $\pm$ S.D.) for $n = 31$ cells. Horizontal lines mark the addition of lanthanum (10 and 100 μM respectively). Horizontal bars correspond to changes in $[\text{Ca}^{2+}]_{\text{ext}}$ from 3 mM (upper bars) to 0 mM (lower bars), in the presence (filled bars) or in the absence (empty bars) of 2 μM FM1-43 in the extracellular medium. Numbers indicate different periods of the incubation protocol (as referred to in the Results section).

FM1-43 fluorescence (Figure 4, compare section 5 with section 3). This effect on FM1-43 fluorescence was only partially reversible after removing La^{3+} from the medium (Figure 4, section 6). The time courses of the changes in $[\text{Ca}^{2+}]_{\text{i}}$ and FM1-43 fluorescence in response to 100 μM La^{3+} were also different: the $[\text{Ca}^{2+}]_{\text{i}}$ response was transient, whereas the fall in FM1-43 fluorescence was sustained.

The effect of La^{3+} on FM1-43 fluorescence was dose-dependent. During incubation with 10 μM La^{3+} , FM1-43 fluorescence decreased slowly (Figure 5, lower panel, section 2 of the curve); it partially 'recovered' when La^{3+} was removed (Figure 5, section 3). In contrast, when 100 μM La^{3+} was added (Figure 5, section 4), FM1-43 fluorescence fell sharply to a lower plateau level at which the dye had been almost completely displaced from the membranes. Removal of the FM1-43 dye from the incubation medium had a minimal further effect on the fluorescence (Figure 5, section 5), suggesting that most of the FM1-43 dye had already been displaced from the plasma membranes. It should be noted that during this experimental protocol the step to 100 μM La^{3+} did not cause an incremental rise in $[\text{Ca}^{2+}]_{\text{i}}$, because the intracellular Ca^{2+} stores had been emptied by the previous exposure to 10 μM La^{3+} , suggesting that

Figure 6 Dose-dependent effects of lanthanum on FM1-43 fluorescence in rMTC6-23 cells

Symbols represent average responses (means $\pm$ S.D.) for $n = 45$ cells. Horizontal lines mark the addition of lanthanum (10, 25 and 50 μ M respectively). Horizontal bars correspond to changes in $[Ca^{2+}]_{ext}$ from 0 mM (lower bars) to 3 mM (upper bars), in the presence (filled bars) or in the absence (empty bars) of 2 μ M FM1-43 in the extracellular medium. Numbers indicate different periods of the incubation protocol (as referred to in the Results section).

10 μ M La^{3+} caused a maximal increase in $[Ca^{2+}]_i$ but a sub-maximal displacement of FM1-43 associated with the plasma membranes.

Similar effects of La^{3+} on FM1-43 fluorescence were observed in rMTC6-23 cells (Figure 6). In the presence of 10 μ M La^{3+} the fluorescent signal decreased from 114.5 ± 18.6 to 42.6 ± 12.8 arbitrary units ($n = 45$ cells, $P < 0.001$) (Figure 6, section 1 of the curve). Further falls to 34.5 ± 11.8 and 29.1 ± 13.2 arbitrary units occurred in the presence of 25 μ M and 50 μ M La^{3+} respectively (Figure 6, sections 2 and 4). Transfer into a La^{3+} -free medium restored only partially the FM1-43 fluorescence, with the signal remaining much lower than before the addition of La^{3+} .

Figure 7 Effects of BAPTA/AM on $[Ca^{2+}]_i$ and FM1-43 uptake in parathyroid cells

Symbols represent average responses for changes in $[Ca^{2+}]_i$ (■) and FM1-43 fluorescence (□) for $n = 17$ cells. Horizontal bars mark changes in $[Ca^{2+}]_{ext}$ from 3 mM (upper bar) to 0.5 mM (lower bar). The presence of 25 μ M BAPTA/AM in the incubation medium is indicated by the horizontal line.

(Figure 6, compare sections 3 and 5 with section 1). It appeared that 50 μ M La^{3+} displaced most/all of the FM1-43 associated with the plasma membranes, since no further decrease in fluorescence occurred when cells were incubated in a medium without FM1-43 and with high $[Ca^{2+}]_{ext}$ (Figure 6, sections 6 and 7).

Effects of dissociating $[Ca^{2+}]_i$ from $[Ca^{2+}]_{ext}$ on FM1-43 uptake by parathyroid cells

Whatever its cause, the direct decrease in FM1-43 fluorescence caused by extracellular La^{3+} or Ca^{2+} cations made difficult the interpretation of fluorescence responses as being due strictly to changes in the rate of membrane turnover associated with secretion. To avoid this complication, experiments were conducted in which $[Ca^{2+}]_i$ was modulated while maintaining a stable $[Ca^{2+}]_{ext}$.

Effects of the calcium chelator BAPTA/AM

Low concentrations of BAPTA/AM (5 μ M) induced a transient fall in $[Ca^{2+}]_i$ ($R_{340/380}$ decreased from 1.2 ± 0.2 to 0.65 ± 0.1 ; $n = 16$) within the first 2 min, with a gradual return to control levels during the subsequent 10 min (results not shown). Addition of 25 μ M BAPTA/AM during incubation in 3 mM $[Ca^{2+}]_{ext}$ induced a decrease in $[Ca^{2+}]_i$ and initiated a slow rise in FM1-43 fluorescence (Figure 7). Whereas the large fall in $[Ca^{2+}]_i$ caused by 25 μ M BAPTA/AM induced only a modest change in FM1-43 fluorescence, a subsequent step to low $[Ca^{2+}]_{ext}$ caused a significantly larger rise in FM1-43 fluorescence (81.9 ± 18.4 compared with 65.6 ± 14.7 arbitrary units; $n = 17$, $P < 0.02$).

Effects of the calcium ionophore A23187

During incubation with 10 μ M A23187 at 0.5 mM $[Ca^{2+}]_{ext}$, $[Ca^{2+}]_i$ increased to levels above those attained during incubation at 3 mM $[Ca^{2+}]_{ext}$ in the absence of A23187 (Figure 8). There was no increase in FM1-43 fluorescence in response to low $[Ca^{2+}]_{ext}$ until A23187 was removed from the extracellular medium and $[Ca^{2+}]_i$ decreased.

Cytoplasmic uptake of FM1-43

In experiments described in previous sections, the rapid uptake of FM1-43 was evaluated as a marker of membrane turnover associated with exocytosis. After a 10–30 min incubation at 0.5 mM $[Ca^{2+}]_{ext}$ in the presence of 2 μ M FM1-43, parathyroid cells observed by confocal microscopy showed fluorescence concentrated in discrete areas, generating a 'spotted' aspect over the entire cytoplasm, but not the nucleus. When transferred into medium without FM1-43 and with 3 mM $[Ca^{2+}]_{ext}$ most of these fluorescent spots appeared to be confined to the cytoplasm. A subsequent step in $[Ca^{2+}]_{ext}$ from 3 mM to 0.5 mM induced movement of some of these fluorescent spots towards the plasma membranes and an increased rate of loss of the dye from the cytoplasm; the intensity of the fluorescent signal decreased to $59.6 \pm 5.9\%$ of the initial signal (range 47.2–66.5%). A similar 'destaining' was induced by incubation with 20 μ M BAPTA/AM.

Confocal microscopy was used to provide morphological evidence of whether uptake of FM1-43 dye in rMTC6-23 cells can be induced by a rise in $[Ca^{2+}]_{ext}$ (i.e. a stimulus for secretion from these cells). Figure 9 shows the results of such an experiment. In the left-hand panel, the two cells were imaged during incubation in a medium with 0.5 mM $[Ca^{2+}]_{ext}$ and 2 μ M FM1-43.

Figure 8 Effects of A23187 on $[Ca^{2+}]_i$ and FM1-43 uptake in parathyroid cells

Symbols represent average responses (means $\pm$ S.D.) for $n = 39$ cells. Horizontal bars mark changes in $[Ca^{2+}]_{ext}$ from 3 mM (upper bar) to 0.5 mM (lower bar). The presence of 10 μ M A23187 in the incubation medium is indicated by the horizontal line.

Subsequent panels are time-lapse images recorded at 3 and 10 min after increasing $[Ca^{2+}]_{ext}$ to 3 mM in the presence of 2 μ M FM1-43. Morphological details demonstrate that, initially, the FM1-43 fluorescence increased over areas corresponding

to the plasma membranes; subsequently the dye accumulated in the cytoplasm of the cells.

DISCUSSION

This study investigated *in vitro* the relationship between changes in $[Ca^{2+}]_i$ and secretion-coupled membrane turnover in two types of cells expressing the CaR: human parathyroid cells and rat rMTC6-23 cells. Real-time imaging techniques using the fluorescent dye FM1-43 were used to monitor secretion-coupled events in response to extracellular cations, because this allowed simultaneous quantification of $[Ca^{2+}]_i$ responses in the same cell. Previous studies exploring the secretory behaviour of parathyroid cells *in vitro* were based on quantification of PTH by radioimmunoassay, and evaluated secretion from a cell population over time intervals of minutes. In addition, reverse haemolytic plaque assays were used by others to quantify PTH release from individual parathyroid cells, and revealed heterogeneity in the set points for inhibition by $[Ca^{2+}]_{ext}$ [27] and recruitment of normal parathyroid cells from an 'off' to an 'on' secretory state upon stimulation by low $[Ca^{2+}]_{ext}$ [28]. The disadvantage of this technique, however, is that it necessitates long-term exposure. In contrast, unrivalled time resolution (milliseconds) for monitoring secretory events is allowed by measurements of membrane capacitance (reviewed in [29]), but this method is limited in that it can monitor only one cell in a given field during an experiment.

FM1-43 is one of the styryl dyes developed as fluorescent membrane markers, and it has been used extensively to study movement and recycling of synaptic vesicles [30–34]. No previous studies have explored whether FM1-43 can be used to monitor secretion in response to extracellular cations. In experiments using parathyroid cells, preincubation was at high $[Ca^{2+}]_{ext}$ in order to inhibit secretion, so that a pool of secretory granules remained 'available' for exocytosis during the imaging experiment (i.e. in response to low $[Ca^{2+}]_{ext}$). Parathyroid cells were dispersed from adenomas removed from patients with primary hyperparathyroidism, as normal human parathyroid cells are not available due to ethical restrictions related to the severity of symptoms following the surgical removal of normal parathyroid glands. Although the set-point for calcium-controlled PTH secretion is reported to be higher in cells from patients with primary hyperparathyroidism, the absolute values are similar with normal cells (i.e. 1.2–1.5 mM). A step from 3 mM to

Figure 9 Time-lapse confocal microscopy demonstrating cytoplasmic uptake of FM1-43 dye by rMTC6-23 cells in response to an increase in $[Ca^{2+}]_{ext}$ from 0.5 to 3.0 mM

Shown are the fluorescent images of two cells incubated in the presence of 2 μ M FM1-43 at 0.5 mM $[Ca^{2+}]_{ext}$ (left panel) and after stepping to 3 mM $[Ca^{2+}]_{ext}$ for 3 min (middle panel) and 10 min (right panel). The scale bar corresponds to 5 μ m.

0.5 mM $[Ca^{2+}]_{ext}$ was therefore used as a standard stimulus for secretion, because these concentrations would correspond to the maximum inhibitory and maximum stimulatory parts respectively of the sigmoidal relationship between $[Ca^{2+}]_{ext}$ and PTH secretion in both normal parathyroid cells [1] and hyperparathyroid cells.

In contrast with parathyroid cells, secretion from rMTC6-23 cells is inhibited at low $[Ca^{2+}]_{ext}$ and stimulated by high $[Ca^{2+}]_{ext}$. These experiments therefore started at 0.5 mM $[Ca^{2+}]_{ext}$, and stepping to 3 mM $[Ca^{2+}]_{ext}$ was used as a stimulus for secretion.

Confocal microscopy using human parathyroid cells showed that, at high $[Ca^{2+}]_{ext}$ (3 mM, to inhibit secretion), the FM1-43 dye equilibrated rapidly on the plasma membranes and the fluorescent signal remained stable. A fall in $[Ca^{2+}]_{ext}$ from 3 mM to 0.5 mM was followed by a progressive increase in FM1-43 fluorescence in most cells. These data support a model in which lowering $[Ca^{2+}]_{ext}$ induces the fusion of secretory granules with the plasma membranes (i.e. secretion) and increases the surface of plasma membranes. More FM1-43 molecules would therefore insert in the lipid bilayer, and the FM1-43 fluorescent signal increases. This model has been suggested in other cell models [30–34], but has the limitation of not considering the possibility of competitive binding between cations (i.e. Ca^{2+} and La^{3+}) and FM1-43.

The results presented demonstrate that high $[Ca^{2+}]_{ext}$ and trivalent cations are very effective in decreasing membrane-associated FM1-43 fluorescence, possibly by displacing FM1-43 from its binding sites. The converse of this situation is that lowering $[Ca^{2+}]_{ext}$ will favour binding, and could explain a rise in FM1-43 fluorescence independent of secretion-coupled events. The rapid and dramatic decrease in FM1-43 fluorescence after addition of La^{3+} indicates that the dye (which is positively charged) has been prevented from inserting into the plasma membranes or has had its fluorescence quenched by the cation. The same effect, rather than an increase in secretion, could therefore explain the increase in FM1-43 fluorescence in parathyroid cells in response to a fall in $[Ca^{2+}]_{ext}$. Alternative explanations are that the decrease in fluorescence caused by La^{3+} , and to a lesser extent by Ca^{2+} , could be due to: (i) quenching of FM1-43 fluorescence when the dye is in the membranes, possibly by affecting the mobility or 'flexibility' of the dye, or (ii) preventing access of the dye to the membrane by a charge screening effect: association of the inorganic multivalent cations with the charged surface layer close to the plasma membranes increases the positive charge on it, repels the positively charged FM1-43 and prevents its insertion.

Similar data were obtained using rMTC6-23 cells. A step to high $[Ca^{2+}]_{ext}$ was used to stimulate secretion, and was anticipated to induce an increase in FM1-43 fluorescence. In contrast with this anticipated pattern of response, a decrease, rather than an increase, in FM1-43 fluorescence was observed. When returning to low $[Ca^{2+}]_{ext}$, however, the cell-associated FM1-43 signal was significantly higher than that before stimulation of secretion by exposure to 3 mM $[Ca^{2+}]_{ext}$. This confirmed the model suggested by the data from parathyroid cells: high $[Ca^{2+}]_{ext}$ stimulates secretion from rMTC6-23 cells and increases the cell surface, but has a simultaneous direct effect of diminishing FM1-43 fluorescence, so that the real increase in cell surface can only be quantified on return to low $[Ca^{2+}]_{ext}$.

To explore further the mechanisms behind an increase in FM1-43 fluorescence, experiments were designed to 'dissociate' changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ext}$ (i.e. $[Ca^{2+}]_i$ was altered pharmacologically while $[Ca^{2+}]_{ext}$ was maintained stable).

During incubation in high (inhibitory) $[Ca^{2+}]_{ext}$, FM1-43 fluorescence increased in response to a fall in $[Ca^{2+}]_i$ induced by

the intracellular calcium chelator BAPTA/AM. In such experiments, changes in FM1-43 fluorescence were variable between cells. This could be explained by the findings of other studies reporting that parathyroid cells 'cycle' between secretory and non-secretory phases [35], with cells being recruited from an 'off' to an 'on' secretory state by repeated episodes of hypocalcaemia [36]. A similar increase in FM1-43 uptake during incubation in high $[Ca^{2+}]_{ext}$ was seen in response to high extracellular potassium (results not shown), confirming previous reports that a high extracellular K^+ concentration decreases $[Ca^{2+}]_i$ and stimulates PTH release in parathyroid cells [37].

In order to increase $[Ca^{2+}]_i$ during incubation at 0.5 mM (low, stimulatory) $[Ca^{2+}]_{ext}$, cells were incubated with the calcium ionophore A23187. This induced an increase in $[Ca^{2+}]_i$ to a plateau level similar to that attained at 3 mM $[Ca^{2+}]_{ext}$, and prevented the expected increase in FM1-43 fluorescence on decreasing $[Ca^{2+}]_{ext}$. Similar inhibition of FM1-43 uptake at low $[Ca^{2+}]_{ext}$ was seen in cells incubated with extracellular ATP, which induced an initial peak in $[Ca^{2+}]_i$ followed by a prominent plateau (results not shown), confirming previous reports that activation of purinergic receptors increases $[Ca^{2+}]_i$ in parathyroid cells [38]. The data also suggest that both $[Ca^{2+}]_i$ and $[Ca^{2+}]_{ext}$ should decrease in order to observe a rise in FM1-43 fluorescence in parathyroid cells.

Following longer incubations with FM1-43, morphological data suggested that the intracytoplasmic uptake of FM1-43 corresponds to endocytotic events, and that the rise in FM1-43 fluorescence is not due only to increased binding on the plasma membranes. At low $[Ca^{2+}]_{ext}$ the dye was internalized in the cytoplasm of parathyroid cells, and this phenomenon was considered to illustrate endocytotic events which follow each burst of exocytosis. Uptake of FM1-43 dye was observed in rMTC6-23 cells during incubation in high $[Ca^{2+}]_{ext}$. Such data are similar to those reported by others, who have shown that FM1-43 dye is trapped during endocytosis in recycled synaptic vesicles of nerve terminals [39]. It was anticipated that such a cell model should be functional, because electron microscopy morphometry in rat parathyroid cells showed that a transient depression in $[Ca^{2+}]_{ext}$ induces initial centrifugal membrane shifting, indicating enhanced exocytosis, followed by centripetal membrane shifting, indicating endocytotic retrieval of plasma membrane [6,7].

Furthermore, when FM1-43 fluorescence microscopy was coupled with membrane capacitance monitoring to measure exocytosis (quantified as changes in fluorescence) and endocytosis (quantified as the difference between changes in capacitance and fluorescence) [40], it was found that, with relatively small stimuli, exocytosis ceases before endocytosis begins, and that during prolonged stimulation the onset of endocytosis is delayed by 2–3 min [40]. The delayed onset of endocytosis may be an emergency defence against catastrophic cell swelling. Similar data were obtained using another fluorescent dye [TMA-DPH; 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] in single gonadotropes: it was shown that gonadotropin-releasing hormone induces exocytosis and endocytosis within 10 s and 60 s respectively, and that recycling of the plasma membrane starts at 180–300 s [41]. Those studies report, therefore, that the time scale for exocytotic and endocytotic events followed by confocal/fluorescence microscopy is similar to that observed in the present study.

The specific intracellular compartment(s) in which the dye will be concentrated are difficult to predict, but some of the endocytosed dye could target the secretory machinery and the newly synthesized secretory granules. This mechanism should therefore generate a cell model in which the secretory granules are stained

by FM1-43, and exocytosis could be visualized as a loss of fluorescence. This model is suggested by studies in presynaptic neuronal boutons under sustained membrane depolarization: the dye is trapped during endocytosis in recycled synaptic vesicles of nerve terminals which become available again for exocytosis within 30 s and can be released subsequently by repeating nerve stimulation [31,39]. During such an experimental protocol, exocytosis is visualized as a diminution of fluorescence. We have observed that some of the dye that accumulates in cytoplasmic granules in parathyroid cells can be mobilized in response to a further step in $[Ca^{2+}]_{ext}$ (results not shown), but quantification of the movement of these fluorescent granules against the background fluorescence was difficult, and we can only hypothesize that the loss of fluorescence from FM1-43-loaded parathyroid cells represents recycling of the secretory granules.

In summary, the increase in the uptake of FM1-43 dye in response to changes in $[Ca^{2+}]_{ext}$ known to stimulate secretion from parathyroid and rMTC6-23 cells appeared to be the result of three phenomena, namely (i) an increase in cell surface area during exocytotic events, (ii) increased uptake of the dye into intracellular domains by endocytosis, and (iii) a direct effect of extracellular cations on the plasma-membrane-bound FM1-43 either by 'charge screening' or a quenching effect. The direct effect of extracellular cations on the fluorescence of membrane-bound FM1-43 is a new observation, and further confocal microscopy studies using this fluorescent dye are required to evaluate its use for monitoring of exocytotic/endocytotic events in endocrine cells that are responsive to changes in $[Ca^{2+}]_{ext}$.

The use of alternative methods of controlling $[Ca^{2+}]_i$ at constant $[Ca^{2+}]_{ext}$ during FM1-43 imaging showed that, in human parathyroid cells, $[Ca^{2+}]_i$ rather than the absolute value of $[Ca^{2+}]_{ext}$ is the main regulator of secretion from parathyroid adenoma cells. According to this model, in patients with primary hyperparathyroidism, a defective Ca^{2+} -sensing mechanism would allow parathyroid cells to maintain low $[Ca^{2+}]_i$ during incubation in high $[Ca^{2+}]_{ext}$, and thus uninhibited PTH secretion would continue despite hypercalcaemia.

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