Journal of Physiology (1991), **437**, *pp.* 477–494
With 6 figures
Printed in Great Britain

PHYSIOLOGICAL CHARACTERIZATION OF TWO FUNCTIONAL STATES IN SUBPOPULATIONS OF PROLACTIN CELLS FROM LACTATING RATS

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(*Received 14 September 1990*)

SUMMARY

1. Lactotroph cells from lactating female rat pituitary glands were dissociated, separated and enriched on a continuous gradient of bovine serum albumin at unit gravity. Two lactotroph subpopulations were observed in the light (F(3–5)) and the heavy (F(7–9)) fractions of the gradient. Both populations were maintained for at least 6 days in culture before experiments were performed.

2. Patch-clamp recordings, in the whole-cell mode, were performed on both lactotroph subpopulations in order to measure passive membrane properties and Ca^{2+} currents. Resting membrane potential as well as membrane capacitance values were found to be lower in light fraction cells. The two components of Ca^{2+} currents, called fast and slow deactivating (FD and SD) currents, were present with different proportions in each subpopulation; the ratio of current amplitudes, SD/FD, was $2{\cdot}42 \pm 0{\cdot}41$ ($n = 18$) in light fraction cells and $1{\cdot}17 \pm 0{\cdot}27$ ($n = 17$) in heavy fraction cells ($P < 0{\cdot}02$).

3. Reverse haemolytic plaque assay showed that in the light and heavy fractions, 68 and 47 % of the lactotroph cells, respectively, were secreting. Population analysis of the plaque areas revealed a bimodal frequency distribution of plaque sizes consisting of small (1500 μm^2) and large plaques (3995 μm^2). A majority of light fraction cells produced large plaques whereas most of the heavy fraction cells produced small plaques.

4. Perifusion experiments performed on enriched prolactin cells showed that (1) basal prolactin (PRL) release was higher in light fraction than in heavy fraction cells, (2) the dopamine (10^{-8} M)-induced inhibition of PRL release was greater in light fraction cells (86 ± 15 %) than in heavy fraction cells (41 ± 21 %), and (3) the thyrotrophin-releasing hormone (TRH, 10^{-8} M)-induced increase of PRL release was 150 ± 60 % in light fraction *versus* 330 ± 82 % in heavy fraction cells.

5. Current-clamp recordings were performed using the intracellular technique.

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Lactotrophs were categorized according to their electrophysiological response following application of dopamine or TRH (both 10^{-8} M). In the light fractions, the majority of the cells tested were hyperpolarized by dopamine (68%), whereas only 7% were depolarized by TRH application. In the heavy fractions, most of the cells (63%) responded to TRH application, while only 13% were dopamine sensitive.

6. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements with the fluorescent probe Indo-1 revealed two lactotroph subtypes. Most cells in the light fractions (sixteen of twenty-two tested cells) exhibited an unstable level of $[Ca^{2+}]_i$ with values fluctuating between $114{\cdot}1 \pm 34{\cdot}3$ and 221 ± 50 nM (mean $\pm$ S.D.). Application of dopamine or of the D_2 receptor agonist RU 24213 (10^{-8} M) resulted in the disappearance of these fluctuations and in an accompanying decrease in basal $[Ca^{2+}]_i$ level. Cells showing a block of spontaneous $[Ca^{2+}]_i$ transients in response to dopamine agonists were TRH insensitive ($n = 7$). Cells from the heavy fractions (thirteen cells tested) showed a lower basal $[Ca^{2+}]_i$ level (61 ± 13 nM; means $\pm$ S.D.). Many of these cells were quiescent, exhibited a $[Ca^{2+}]_i$ increase in response to TRH (10^{-8} M) but were dopamine insensitive prior to TRH application.

7. Together, these results show that lactotrophs exist as two subpopulations: (i) the low-density cells present a high basal release of PRL associated with spontaneous electrical activity and a basal $[Ca^{2+}]_i$ level that is high and oscillating; (ii) the high-density cells exhibit low basal PRL release accompanied by a high resting membrane potential and a low and stable $[Ca^{2+}]_i$ level. Dopamine may suppress preferentially the activity of the lactotroph subpopulation with a high basal release, while TRH appears effective on the subpopulation characterized by a low basal release. We discuss the possible existence of a cell cycle and the way in which it might be regulated.

INTRODUCTION

Lactotrophs, the secretory cells of the anterior pituitary which synthesize and release prolactin (PRL; Baker, 1974), can be divided into two subtypes.

Israel, Kirk & Vincent (1987) separated dissociated pituitary cells from lactating female Wistar rats on a continuous density gradient of bovine serum albumin (BSA) and found cells immunoreactive for PRL in two distinct sections of the gradient: among 'light' fractions, 3–5, and among 'heavy' fractions, 7–9 (Israel *et al.* 1987). Ninety-five per cent of the cells in the light fractions (F(3–5)) and up to 70% in the heavy fractions (F(7–9)) were lactotrophs. Lactotrophs were not present in the heavy fractions of cells prepared from adult males (Zhang, Chen, Kukstas, Verrier, Vincent & Israel, 1990) or from virgin females (Israel *et al.* 1987). However, treatment of males with 17-β-oestradiol led to the appearance of lactotrophs in the heavy fractions (Zhang *et al.* 1990). It has subsequently been shown that both light and heavy fractions are present during proestrous (Kukstas, Verrier, Zhang, Chen, Israel & Vincent, 1990). It therefore appears that these two populations, which differ according to the donor, may reveal different physiological properties.

In this respect, some differences in the physiological properties of the two populations have already been reported. The effects of the two major physiological regulators of PRL release, thyrotrophin-releasing hormone (TRH) and dopamine, on the membrane potentials of lactotrophs have been examined in our laboratory (Israel

et al. 1987; Israel, Kukstas & Vincent, 1990). It was found that individual cells responded either to TRH (by a depolarization) or to dopamine (by a hyperpolarization). In the light fractions, the majority of cells responded to dopamine, and few responded to TRH, while in the heavy fractions the majority responded to TRH.

We have now examined the electrical membrane properties of cells of the two populations, mainly using voltage-clamp techniques. Since these are secretory cells, it was to be expected that Ca^{2+} would play an important part in their physiology (see Douglas, 1981) and indeed we found Ca^{2+} conductances which differed in the two populations, notably in the distribution of fast and slow deactivating (FD and SD respectively) Ca^{2+} currents. This led us to measure cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, and PRL release from single cells. We will show that the observed differences in the Ca^{2+} dynamics of the two types of cells can explain the different rates of hormone release.

METHODS

Preparation of the cells

Pituitary glands were dissected from lactating female Wistar rats, at the end of the last week of lactation. The animals were rapidly decapitated and the pituitaries were removed and placed in a Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Gibco). Cell dissociation, separation and primary culture methods have been described previously (Israel *et al.* 1987). Cells from both fractions were plated at a density of 10^5 cells per dish for electrophysiological experiments (onto 35 mm Petri dishes, Nunc, Denmark) and intracellular Ca^{2+} measurements (onto glass cover-slides), and at 10^6 cells per dish for perifusion experiments. Culture medium consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum and 3·7 g l^{-1} $NaHCO_3$ (pH 7·3). The Petri dishes were placed in a humidified incubator (37 °C, 94% air:6% CO_2). Antibiotics (35 mg l^{-1} penicillin and 50 mg l^{-1} streptomycin, pH 7·4) were added during the first 2 days of culture; the medium was then renewed every 2 days. Cells from both light and heavy fractions were only examined in the 6- to 15-day *in vitro* cultures; there were no significant time-dependent effects during this period.

Reverse haemolytic plaque assay (RHPA)

Single lactotrophs were identified using the RHPA according to the method of Neill & Frawley (1983) and as described previously (Lledo, Legendre, Israel & Vincent, 1990*a*). Plaque counts and areas were analysed with a Leitz microscope and the Biocom 200 image analyser (Imagerie Instrumentation Biotechnologique, Les Ulis, France). Counts of plaque-forming and non-plaque-forming cells were carried out by using the touch count mode of the system. Percentages were determined by counting 100–150 cells per cover-slip. Areas were measured by projecting the microscope image onto a screen and using a cursor to outline the plaques. The computer then calculated the plaque area (μm^2). At least ten plaque areas per cover-slip were measured when possible.

Electrophysiological recordings and analysis

Lactotrophs were resuspended on the day of electrophysiological studies and then subjected to a plaque assay. A suspension of monodispersed cells was obtained by a brief retrypsinization (5 min incubation with 0·025% trypsin–EDTA). Intracellular recordings were performed as described by Israel *et al.* (1987). The Petri dish containing the cells was fixed on the stage of an inverted phase-contrast microscope (Nikon TMD). The temperature was maintained at 36 ± 1 °C by a warm-air system. The resistance of the intracellular microelectrodes varied from 30 to 60 MΩ (3 M-potassium chloride). Measurements of membrane currents were made with a single-electrode sample-and-hold amplifier (Axoclamp 2, Axon Instruments, Burlingame, CA, USA). The amplifier switched between

voltage measuring (70%) and current passing (30%) at a frequency which was adjusted according to the electrode characteristics (usually 1 kHz). The recording medium was slightly modified from the initial culture medium and consisted of DMEM + 15 mM-HEPES + 12 mM-$NaHCO_3$ (pH 7·4).

Lactotrophs were voltage clamped using the whole-cell mode of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with a high-gain voltage-clamp amplifier (RK 300 Biologic, Grenoble, France). Patch pipettes were fabricated from borosilicate capillary tubing and pulled on a BB-CH micropipette puller (Mécanex, Geneva, Switzerland). They presented resistances of 2·0–4·5 MΩ when filled with the patch pipette solution (see below). The junction potential between the electrode solution and the bath was adjusted to zero and series resistances were compensated using the procedure described in the RK 300 manual.

Membrane currents were stored in digital form on magnetic videotape (Lamb, 1985) at a recording bandwidth of 16 kHz using a digital audio processor (PCM-701 ES, Sony). For analysis, records were first filtered using an eight-pole Bessel low-pass filter (3 kHz, −3 dB, Frequency Devices, Haverhill, MA, USA). Signals were digitized at 5 kHz using a PDP 11/73 computer and a Cambridge Electronic Design 502 analogue–digital interface with direct memory access to a 160 MB Winchester hard disc. Computer acquisition of data, current averaging and subtraction of capacitive and linear leakage currents were performed using the ANADISK and DA 23 Library programs which were compiled by Dr T. D. Lamb (Physiological Laboratory, University of Cambridge, UK). All current signals (each current trace was an average of five consecutive records elicited at a frequency of 0·2 Hz) were measured after subtraction of capacity current and linear leakage current determined from hyperpolarizing command pulses.

Passive cell membrane properties were calculated from the current response to 10 mV hyperpolarizing pulses from a holding potential of −80 mV. After electronic compensation for the electrode capacitance, the integral of the current transient in response to these small hyperpolarizing pulses was divided by steps to yield the cell membrane capacitance. From the steady-state current, the sum of the membrane resistance, in parallel with the seal resistance and the electrode series resistance, also could be calculated. The series resistance between the pipette and the cell was between 4·5 and 9·5 MΩ (evaluated from the time constant of the capacitive current and of the cell capacitance).

When tail currents consisted of two exponential components, a single exponential was fitted to the data points 0·6 ms after the onset of the repolarization, and extrapolated back to the end of the pulse in order to measure the slow-component amplitude. The fitted slow component was then subtracted from the total tail current to isolate the fast component.

In order to isolate Ca^{2+} currents, the pipette (internal) solution used in this study consisted of (mM): CsCl, 120; tetraethylammonium chloride (TEA-Cl), 20; $MgCl_2$, 1·3; $CaCl_2$, 1; EGTA, 11; HEPES, 10; glucose, 10; MgATP, 4; cyclic AMP, 0·2; GTP, 0·4 (pH 7·3, 295 mosM). The extracellular solution contained (mM): NaCl, 120; KCl, 5; $CaCl_2$, 5; $MgCl_2$, 0·5; glucose, 10; HEPES, 10; TEA-Cl, 20 (pH 7·3, 320 mosM). Substitutions or additions to these solutions are indicated where appropriate. All tested substances were dissolved in recording medium just prior to use, and were ejected close to the cell tested through a micropipette (1–3 μm tip diameter) with a micropressure ejection system (BH-2, Medical System Corp, USA). Culture medium and sera were obtained from Gibco (95051 Cergy-Pontoise, France); all other salts and drugs were purchased from the Sigma Chemical Company (St Louis, MO, USA). The bath solution was continuously perfused using gravity flow at a rate of 0·5–1 ml min^{-1} (bath solution volume around 1 ml). Patch-clamp experiments were conducted at room temperature (21–24 °C).

When statistical comparisons were made, Student's two-tailed t test was applied to the means of the measurements.

Optical recordings of cytosolic free Ca^{2+} concentration

Lactotroph cells were grown on glass cover-slides in the nutrient medium described above. $[Ca^{2+}]_i$ was determined in individual cells by dual emission microspectrofluorimetry using Indo-1 as the intracellular fluorescent calcium probe (Grynkiewicz, Poenie & Tsien, 1985; Mollard, Guérineau, Audin & Dufy, 1989). Before loading the cells with Indo-1, the nutrient medium was replaced by modified Hank's solution containing 142·6 mM-NaCl, 5·6 mM-KCl, 2 mM-$CaCl_2$, 0·8 mM-$MgCl_2$, 5 mM-glucose and 10 mM-HEPES; adjusted to pH 7·3 with NaOH. The cells were loaded with Indo-1 by exposure to 5 μM-Indo-1 penta-acetoxymethylester (Indo-1/AM, Calbiochem, USA) and 0·02% Pluronic F-127 (Molecular Probes, USA) in Hank's solution for 30 min at 20 °C. $[Ca^{2+}]_i$ was

estimated in individual cells from Indo-1 fluorescence by the ratio method, using single-wavelength excitation (355 nm) and dual emission (405 and 480 nm), as previously described (Mollard *et al.* 1989). The ratio of the fluorescence at 405 nm to the fluorescence at 480 nm ($R = F_{405}/F_{480}$) can be expressed as $[Ca^{2+}]_i$ according to the formula (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}]_i = K_d\beta\,(R-R_{min})/(R_{max}-R),$$

where K_d is the dissociation constant for the Ca^{2+}–Indo-1 complex at 37 °C, β is the ratio of F_{480} at minimal versus maximal $[Ca^{2+}]_i$, R_{min} is the value of R at limiting low $[Ca^{2+}]_i$ and R_{max} is the value of R at saturating $[Ca^{2+}]_i$. R_{max}, R_{min} and $K_d\beta$ were determined in lactotroph cells in primary culture. Recorded cells were characterized as lactotrophs due to their responsiveness to either dopamine or TRH.

Hormone-release studies

Hormone-release experiments were carried out as previously reported by Israel *et al.* (1987). In brief, hormone-release experiments were performed on cells in the original culture dish. A continuous-flow perifusion system accommodating four dishes at a time was maintained at constant temperature (37 ± 0·5 °C) by a water-heated platform. The fractions were collected after a stabilization period of an hour. The medium was fractionally collected every 2 min (flow rate: 0·5 ml min^{-1}). The medium used during the perifusion was slightly modified from the initial culture medium, and consisted of DMEM + 15 mM-HEPES + 12 mM-$NaHCO_3$ (pH 7·4).

RESULTS

Passive membrane properties

After identification of lactotrophs by RHPA, 'giga-seal' patch-clamp recordings were performed using the whole-cell configuration. Thus, passive membrane properties from both the light and heavy fraction lactotrophs were measured (Table 1). The resting membrane potential values (V_m) reported here are the zero-current potentials. The V_m of heavy fraction cells was greater (−66·5 ± 10·2 mV, mean ± S.E.M., $n = 49$) than that of light fraction cells (−47·4 ± 8·4 mV, $n = 82$; $P < 0·001$), while membrane resistance values (R_m) were not significantly different in heavy fraction *versus* light fraction cells. Assuming that specific membrane capacitances are constant between the two lactotroph subtypes, the membrane capacitance value (C_m), which is directly linked to the membrane surface area, can be considered as a suitable parameter of size variations. According to the differences between membrane capacitance data, cells from heavy fractions appeared to be larger (9·3 ± 3·18 pF in heavy fraction cells *versus* 7·4 ± 2·25 pF in light fraction cells; $P < 0·02$). This observation has been confirmed in recent work in which a fluorescence-activated cell sorter has been used (L. A. Kukstas, personal communication).

Two voltage-activated Ca^{2+} currents in lactotrophs

Figure 1 shows the properties of two voltage-activated Ca^{2+} currents (I_{Ca}) recorded from a light fraction cell, under voltage-clamp conditions in the whole-cell recording configuration. Figure 1*A* shows two Ca^{2+} current recordings elicited by 10 ms of test pulses of −30 mV (top trace) and +10 mV (bottom trace), from a holding potential of −80 mV. Following small depolarizations, the decay of tail currents displayed a slow time course. This current may be called the slowly deactivating (SD) current component. Larger depolarizations activated an additional fast component termed the fast deactivating (FD) current component, since the total tail current was biexponential for membrane potentials greater than −20 mV. An exponential was

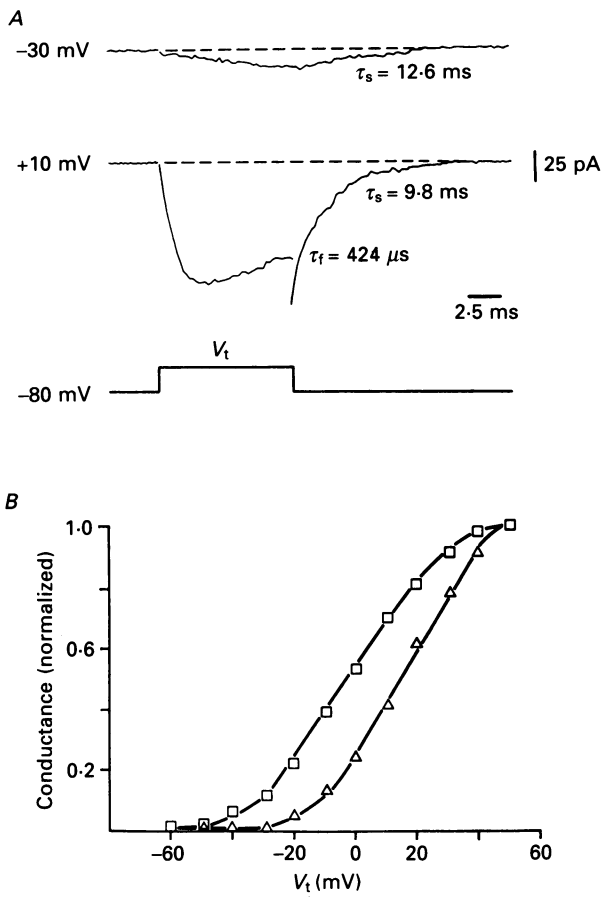

Fig. 1. Two voltage-activated Ca^{2+} currents in light fraction lactotroph cell. *A*, Ca^{2+} current recordings were elicited by 10 ms step depolarizations to −30 mV (top trace) and +10 mV (bottom trace) from a holding potential of −80 mV, followed by repolarizations to −80 mV. Time constants of fast (τ_f) and slow (τ_s) components in the tail current are given near the recordings. *B*, normalized amplitudes of the FD (△) and SD (□) components of the tail currents are plotted as a function of the activating potential (V_t). Ca^{2+} tail currents were recorded at −80 mV after 10 ms activating pulses of various amplitudes (see text for more details).

TABLE 1. Passive membrane properties

	V_m (mV)	R_m (GΩ)	C_m (pF)
F(3–5)	−47·4±8·4 (82)	4·1±2·8 (56)	7·4±2·25 (26)
F(7–9)	−66·5±10·2 (49)**	4·9±3·2 (37)	9·3±3·18 (24)*

Solutions used to measure passive membrane properties are: pipette solution (mM): KCl, 130; $MgCl_2$, 1·3; $CaCl_2$, 1; EGTA, 11; HEPES, 10; glucose, 10; MgATP, 4; cyclic AMP, 0·2; GTP, 0·4 (pH 7·3, 295 mosM); bath solution (mM): NaCl, 125; KCl, 5; $CaCl_2$, 1·5; $MgCl_2$, 1·3; glucose, 10; HEPES, 10 (pH 7·3, 320 mosM). Values are expressed as mean±S.E.M. with the number of cells in parentheses.

F(7–9) values are significantly different from F(3–5) values with $*P < 0·02$ $**P < 0·001$.

fitted to the slow phase of each tail record, and its amplitude was extrapolated to the onset of repolarization. The fast component was fitted after computer subtraction of the slow component from the total tail current. The amplitudes of both exponentials were linked to the instantaneous conductance of the respective Ca^{2+} channels at the

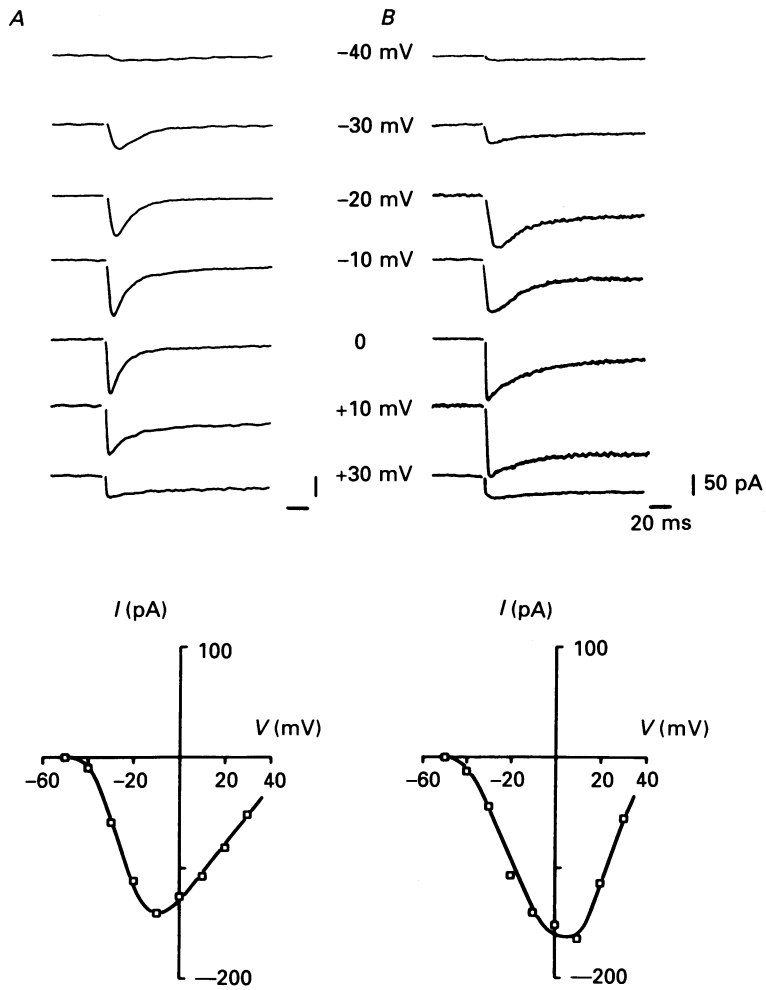

Fig. 2. Comparison of Ca^{2+} currents between the two lactotroph subtypes. *I–V* relationships of peak Ca^{2+} currents elicited from −80 mV, in a light (*A*) and a heavy (*B*) fraction cell. For each cell, current records at different potentials are shown with the test potential indicated (upper traces). Note that the current in part *A* strongly inactivated following activation, whereas the current in part *B* showed little inactivation.

time of repolarization. Conductance–voltage curves for both Ca^{2+} current components, as determined from tail currents, are given in Fig. 1*B*. It can be noted that FD and SD current components activate over different voltage ranges, i.e. −50 mV for the SD current component and −20 mV for the FD current component. The half-maximal activation levels for SD and FD current components were -4 ± 4 and

$+13 \pm 5$ mV ($n = 44$) respectively, whereas maxima of both FD and SD current activation occurred at a similar voltage level of +50 mV. Conductance–voltage curves, as well as kinetic properties corresponding to the two Ca^{2+} current components, were similar in both light and heavy fraction cells (data not shown).

These observations provide further evidence for the existence of two major types of Ca^{2+} channels, differing in their closing kinetics and voltage range of activation, as previously reported in dispersed lactotrophs (DeRiemer & Sakmann, 1986; Lingle, Sombati & Freeman, 1986; Cobbett, Ingram & Mason, 1987; Lewis, Goodman, Saint John & Barker, 1988; Lledo *et al.* 1990*a*), in clonal pituitary cells (Armstrong & Matteson, 1985; Cohen & McCarthy, 1987) or pars intermedia cells (Cota, 1986).

Differing contributions of the two voltage-activated Ca^{2+} currents

As stated above, two components of I_{Ca} could be activated by voltage pulses: the SD and the FD Ca^{2+} current components. The contribution of each component of the total Ca^{2+} current of lactotrophs varied between cells, and could be determined by comparing Ca^{2+} currents elicited from a holding potential of −80 mV (Fig. 2). The contribution of the FD current, that appeared as a sustained component during a 150 ms test pulse, was small in light fraction cells (Fig. 2*A*, top) and large in heavy fraction cells (Fig. 2*B*, top). Furthermore, current–voltage (*I–V*) relationships determined by measuring the peak of total I_{Ca} showed different maxima: −10 mV for the light fraction cell shown in Fig. 2*A* (bottom) and +10 mV for the heavy fraction cell shown in Fig. 2*B* (bottom). Figure 3 illustrates the difference in maxima by displaying the distribution of membrane potential values corresponding to the *I–V* curve peak from the populations of light (Fig. 3*A*) and heavy (Fig. 3*B*) fraction cells tested. *I–V* curves peaked at −10 mV in most lactotrophs from the light fractions (32·3%, $n = 34$), while the majority of lactotrophs from the heavy fractions (30·6%, $n = 31$) were characterized by a maximum at +5 mV. Therefore, although both lactotroph subtypes possess the two types of I_{Ca}, the strength of each component differs between the subtypes. In order to reiterate these differences, amplitudes of both fast and slow components in Ca^{2+} tail currents recorded at −80 mV, after 10 ms activating pulses to +50 mV, were divided by the cell membrane capacitance. From these data, it is apparent that the normalized SD Ca^{2+} current predominates in eighteen of the light fraction cells tested ($8{\cdot}35 \pm 0{\cdot}8$ *versus* $3{\cdot}45 \pm 0{\cdot}7$ pA/pF for SD and FD Ca^{2+} current components respectively; mean ± S.E.M., $P < 0{\cdot}001$), while in the heavy fractions ($n = 17$) there is no significant difference in the amplitudes of the SD and FD Ca^{2+} current components ($6{\cdot}1 \pm 0{\cdot}7$ *versus* $5{\cdot}2 \pm 0{\cdot}7$ pA/pF for SD and FD components, respectively). The ratio of current amplitudes SD/FD was $2{\cdot}42 \pm 0{\cdot}41$ ($n = 18$) in light fraction cells and $1{\cdot}17 \pm 0{\cdot}27$ ($n = 17$) in heavy fraction cells, suggesting a different ratio of functional channel densities or single-channel conductances ($P < 0{\cdot}02$).

Reverse haemolytic plaque assay (RHPA)

RHPA has been previously shown to be a powerful means of identifying single lactotroph cells in primary cultures prior to electrophysiological studies (Lledo *et al.* 1990*a*). We also used, here, the RHPA technique as a tool to quantify PRL secretion from individual cells. Several controls were performed in separate experiments to

ensure the specificity of the complement-mediated lysis of the erythrocytes. Omission of antisera or complement, or replacement of antisera with normal rabbit serum, abolished haemolytic plaque formation. In the light and heavy fraction cells, about 68 and 47% of the cells, respectively, secreted PRL spontaneously. Analysis of the

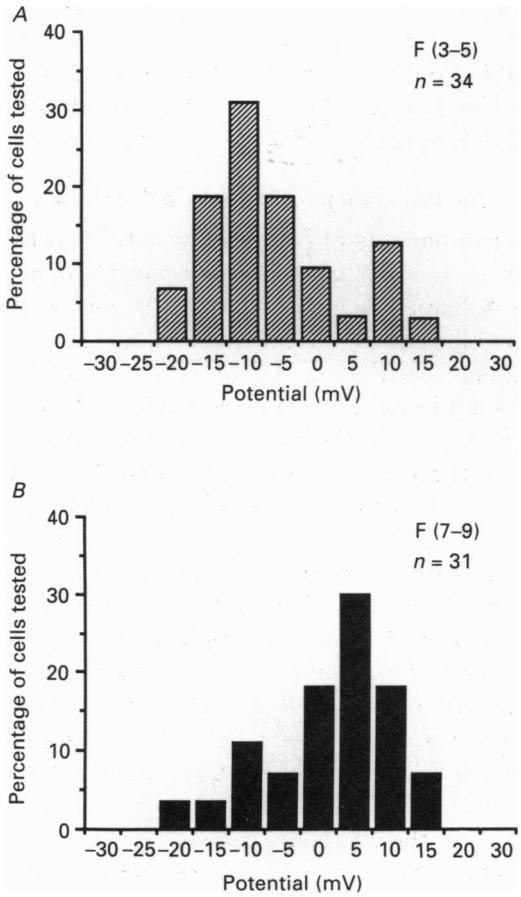

Fig. 3. Distribution of potential values eliciting the maximum peak Ca^{2+} current in light (*A*) and heavy fraction cells (*B*). Potential values were measured from the peak of the *I–V* relationships obtained at −80 mV and expressed as a percentage of cells tested. The number of cells tested (*n*) is indicated.

sizes of plaques produced by lactotrophs revealed a bimodal frequency distribution consisting of small and large modes (Fig. 4). The large plaques were 2·7 times greater in area than the small plaques as the two average modes, from all data, were 1500 and 3995 μm^2. Light fraction cells produced mainly large plaques (22% of plaque-forming cells) and heavy fraction cells mostly small plaques (21% of plaque-forming cells).

Prolactin release from perifusion experiments

In order to examine the characteristics of PRL secretion from lactotroph-enriched cultures, perifusion experiments were performed on cells from both fractions. Figure 5 shows representative experiments from which three differences between the two

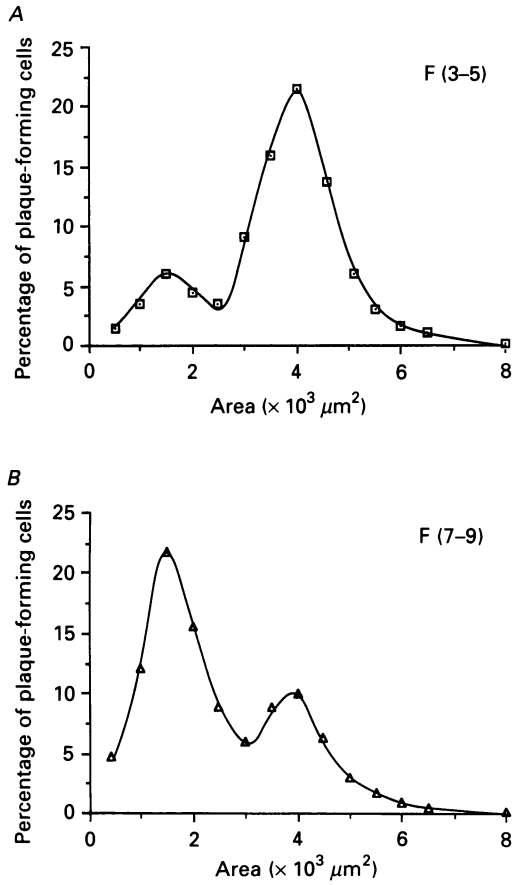

Fig. 4. Subpopulations of lactotroph cells detected with the RHPA. The measurement of plaque areas revealed a bimodal frequency distribution. *A*, distribution of plaque areas in light (⊡) fraction cells ($n = 94$). *B*, distribution of plaque areas in heavy (△) fraction cells ($n = 82$). Curves are the fit of a 10th degree polynomial regression. These data are representative of four independent experiments.

populations can be noted: (1) spontaneous PRL release from light fraction cells was greater than from heavy fraction cells (Fig. 5*A*) (a mean ± S.E.M. of 5·83 ± 0·91 and 3·11 ± 0·73 ng $(10^6$ cells$)^{-1}$ ml^{-1}, respectively, from five experiments; $P < 0·05$); (2) the dopamine inhibitory response was more pronounced in light fraction cells than in heavy fraction cells (a mean ± S.E.M. of 86 ± 15 and 41 ± 21 %, respectively; $n = 5$) (Fig. 5*A* and *B*); (3) the TRH-induced stimulation of secretion was greater for heavy fraction cells than for light fraction cells (330 ± 82 compared to 150 ± 60 %; $n = 5$) (Fig. 5*A* and *B*).

Intracellular recordings: dopamine- and thyrotrophin-releasing hormone-induced effects

The electrical responses induced by dopamine and TRH (Ingram, Bicknell & Mason, 1986; Israel *et al.* 1987, 1990) were systematically recorded from seventy-four light and eighty-five heavy fraction cells. Sensitivity was assessed as hyper-

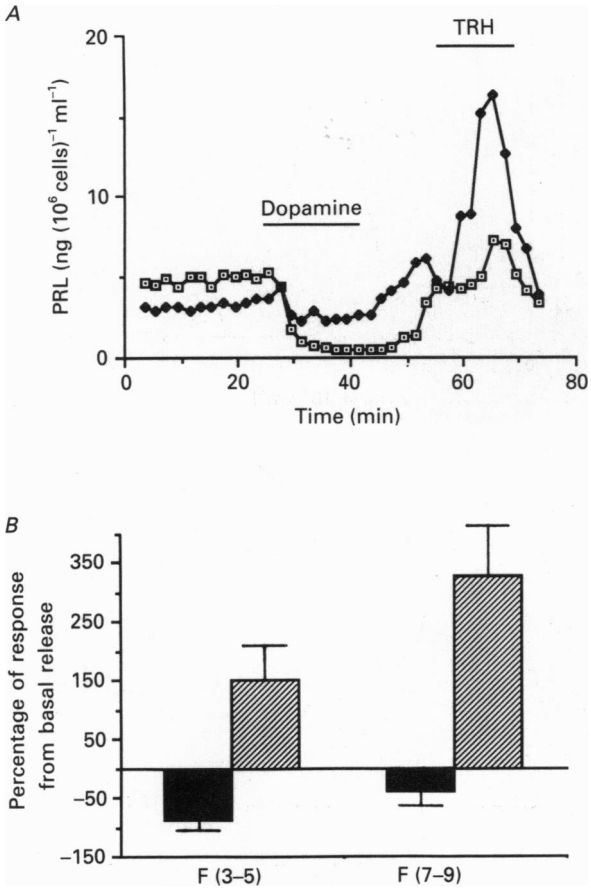

Fig. 5. Effects of dopamine and TRH on spontaneous PRL release from lactotroph cells. *A*, light (⊡) and heavy (◆) fraction cells were continuously perifused, and medium was collected every 2 min (0·5 ml min^{-1}; 1 ml $aliquot^{-1}$). Dopamine and TRH (both 10^{-8} M) were applied during 18 and 17 min periods respectively (bars). The light fraction cells showed a high basal secretion and a large dopamine-induced inhibition of PRL release, while the TRH-induced increase of PRL release was lower compared to heavy fraction cells. This curve is representative of three experiments. *B*, comparison of the effects of 10^{-8} M-dopamine (■) and 10^{-8} M-TRH (▨) on basal PRL release (mean ± S.D.). PRL release in the presence of dopamine or TRH is expressed as a percentage of the basal value. Data are from the two lactotroph subpopulations measured in three independent experiments.

polarization (induced by dopamine) or depolarization (induced by TRH) of the membrane resting potential recorded in current-clamp experiments during intracellular recordings. A majority (68%) of light fraction cells gave a response to

dopamine (10^{-8} M), whereas only 7% were sensitive to TRH delivery (10^{-8} M). Contrasting data were obtained from heavy fraction cells, where 63% were depolarized in response to TRH application, whereas only 13% were hyperpolarized by dopamine.

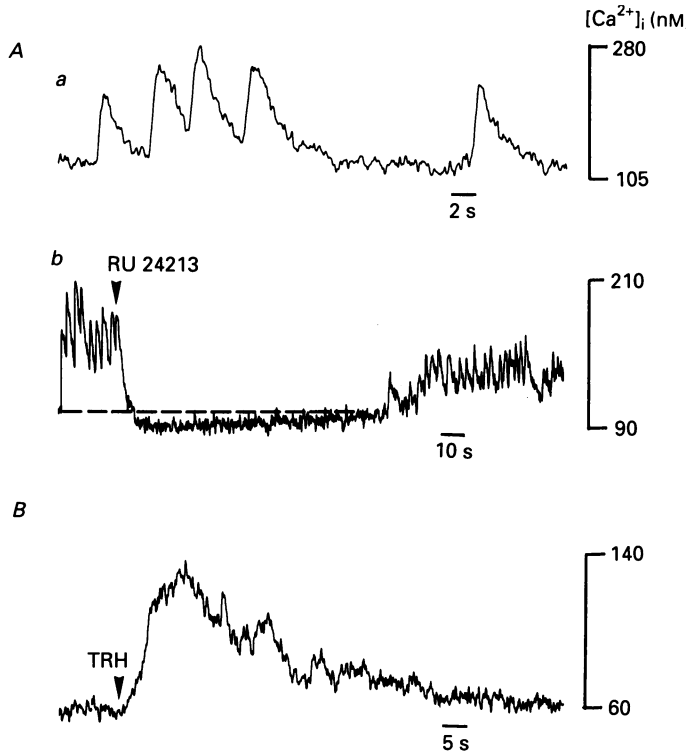

Fig. 6. Single-cell $[Ca^{2+}]_i$ recordings: basal characteristics and responses to the D_2 receptor agonist RU 24213 and to TRH. *A*, two separate cells from the light fractions recorded using different time scales. *Aa*, detailed kinetics of spontaneous $[Ca^{2+}]_i$ oscillations. *Ab*, application of RU 24213 (10 nM) induced a reversible block of spontaneous $[Ca^{2+}]_i$ transients. Note that RU 24213 also caused a significant decrease in the basal $[Ca^{2+}]_i$ level (dashed line). Basal $[Ca^{2+}]_i$ level from oscillating cells was estimated during silent phases between individual or grouped $[Ca^{2+}]_i$ transients. *B*, application of TRH (10 nM) evoked a prolonged transient rise in $[Ca^{2+}]_i$ of an otherwise stable cell from the heavy fractions.

Single-cell monitoring of cytosolic free Ca^{2+} concentration

Cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, was measured in individual cells from both fractions. Single-cell $[Ca^{2+}]_i$ recordings addressed the question of whether $[Ca^{2+}]_i$ is different between the subtypes and whether it might be differentially regulated in the lactotroph subpopulations.

A total of twenty-two cells from the light fractions were recorded in a Ca^{2+}-containing saline solution. They showed a basal $[Ca^{2+}]_i$ level of $114{\cdot}1 \pm 34{\cdot}3$ nM (mean $\pm$ S.D.) often associated with spontaneous $[Ca^{2+}]_i$ oscillations (73% of cells; Fig. 6*A*). The latter showed well-defined kinetic features, i.e. a rapid onset with a

transition time of 458 ± 107 ms to reach the peak (221 ± 50 nM; $n = 16$), and a return to the basal $[Ca^{2+}]_i$ level within a few seconds (Fig. 6*Aa*). Spontaneous oscillations of $[Ca^{2+}]_i$ were primarily, if not exclusively, due to Ca^{2+} channel opening during spontaneous action potentials, since $[Ca^{2+}]_i$ transients were blocked by removal and chelation, by the use of 1 mM-EGTA, of external Ca^{2+} ($n = 7$, not shown). In such cells showing spontaneous $[Ca^{2+}]_i$ oscillations, dopamine or the D_2 receptor agonist RU 24213 induced an inhibition of spontaneous $[Ca^{2+}]_i$ transients (Fig. 6*Ab*) at concentrations effective in reducing basal hormone release (Israel *et al.* 1987; 70% of light fraction cells tested), whereas TRH application (10 nM) was ineffective in producing any Ca^{2+} response ($n = 5$). This inhibition of spontaneous $[Ca^{2+}]_i$ transients by D_2 receptor activation could be associated with a decrease in basal $[Ca^{2+}]_i$ levels (Fig. 6*Ab*). D_2 receptor stimulation was ineffective in lowering $[Ca^{2+}]_i$ levels in cells which did not display oscillations ($n = 6$), or in experiments where external Ca^{2+} was removed and chelated by 1 mM-EGTA ($n = 7$, data not shown).

$[Ca^{2+}]_i$ was recorded in thirteen cells from the heavy fractions. Seventy-nine per cent of these cells were characterized by a stable and lower basal $[Ca^{2+}]_i$ level (61 ± 13 nM; mean $\pm$ S.D.). Many responded only to 10 nM-TRH application (62% of heavy fraction cells tested) by a massive $[Ca^{2+}]_i$ rise (Fig. 6*B*) often followed with a series of modest $[Ca^{2+}]_i$ oscillations. However, a few cells from these fractions (28%), which showed spontaneous $[Ca^{2+}]_i$ oscillations, were TRH insensitive but exhibited a dopamine responsiveness similar to the light fraction cells.

DISCUSSION

This study was performed on two lactotroph subtypes isolated from lactating rat pituitary glands. It has been suggested that calcium, which is required for the secretory process, enters the cell via membrane calcium channels activated during electrical activity such as action potentials or membrane potential fluctuations (Douglas, 1981). In view of this stimulus–secretion concept, we have used electrophysiological techniques (i.e. whole-cell patch-clamp and intracellular microelectrode recordings) in parallel with assessment of PRL secretion and $[Ca^{2+}]_i$ measurements on the two lactotroph subtypes. The present results show that rat lactotroph cells could serve as a model for investigating functional differences between hormone-secreting cells of a given type.

Two types of voltage-activated Ca^{2+} currents expressed in lactotrophs

Two voltage-activated Ca^{2+} currents are present in light fraction lactotrophs (Lledo *et al.* 1990*a*). Here, using tail current analysis, we show that Ca^{2+} currents possess two distinct types of Ca^{2+} currents, namely slowly deactivating (SD) and fast deactivating (FD), according to the nomenclature proposed by Armstrong & Matteson (1985). The SD Ca^{2+} current component is also named the 'transient' or 'T-type' Ca^{2+} current, while the FD Ca^{2+} current component is called the 'long-lasting' or 'L-type' Ca^{2+} current (for review, see Tsien, Lipscombe, Madison, Bley & Fox, 1988).

With a constant repolarizing level, the amplitude of the tail current is directly proportional to the number of open FD and SD channels at the end of the activating

pulse (Armstrong & Matteson, 1985). Analysis of tail currents is a convenient way of separating the contribution of FD and SD current components to the total Ca^{2+} current, since there is a considerable difference in the time course between the two types of Ca^{2+} current. Interestingly, average values for the amplitudes of SD and FD currents show that the SD current is more prominent in light fraction cells, while no significant difference between the currents' amplitudes is observed in heavy fraction cells. These observations corroborate data obtained from *I–V* relationships, which show different maxima according to the contribution of each Ca^{2+} current (the SD current peaks at more negative potentials than the FD current).

Physiological function of slow and fast deactivating Ca^{2+} current components

FD and SD current components may be associated with different functions in endocrine cells. We have considered the role of each channel in controlling the electrical activity of the cell. SD current component may support action potentials since rat lactotrophs are known to be devoid of functional Na^+ channels (DeReimer & Sakmann, 1986; Lingle *et al.* 1986; Lledo *et al.* 1990*a*). Because of its high threshold, the FD current component is well adapted to allow Ca^{2+} entry during plateau activity (Armstrong & Matteson, 1985) which is spontaneous or triggered by a stimulating agent such as TRH (Israel *et al.* 1990). However, the role of Ca^{2+} channels in calcium influx must be evaluated also in the absence of electrical activity. In this respect, we have previously shown that the curves describing steady-state activation and inactivation of the SD Ca^{2+} current overlap between -50 mV (threshold of SD component) and -30 mV (value at which SD component is completely inactivated), and this may lead to a steady inward flux of Ca^{2+} within this voltage range (Lledo *et al.* 1990*a*). Thus, Ca^{2+} channels may not only be involved in calcium influx during electrical activity, but may also allow a large basal calcium influx at rest, and in this way contribute to basal secretory activity of lactotrophs.

Differences in amplitudes of slow and fast deactivating current components among lactotroph subtypes

Current-clamp recordings using the whole-cell configuration showed two electrophysiologically distinct types of lactotroph: silent and spontaneously active cells. In the light fractions, we noted that 65% of cells were spontaneously active, whereas 35% were silent but exhibited membrane potential fluctuations of up to 20 mV (Cobbett *et al.* 1987; Lledo, Legendre, Zhang, Israel & Vincent, 1990*b*). In the heavy fractions, many cells tested (88% of cells, $n = 38$) were silent and were characterized by a high resting membrane potential value (Table 1). These different electrical characteristics suggest that SD current components may participate in the spontaneous electrical activity, as mainly seen in cells from light fractions, but its contribution may be minimal in most of the heavy fraction cells. Moreover, if the data in Table 1 are pooled, we can note that, in light fraction cells, the mean resting membrane potential value ($-47{\cdot}4 \pm 8{\cdot}4$ mV) is in the voltage range where a steady inward flux of Ca^{2+} through the SD current component might be expected. In contrast, the resting membrane potential of heavy fraction cells ($-66{\cdot}5 \pm 10{\cdot}2$ mV) is below the activation threshold of the SD current (close to -50 mV, see Fig. 1),

confirming the insignificant participation of the SD current component in Ca^{2+} influx seen in these cells. Concerning the FD current components, their sustained activation may be related to the characteristic plateau potentials previously recorded in most of the lactotrophs from the heavy fractions (Israel *et al.* 1990). Their contribution to the spontaneous electrical activity recorded in many cells from the light fractions cannot be excluded. Further experiments will be necessary to assign roles to both SD and FD current components in the electrical behaviour of lactotrophs and thus in hormone release.

$[Ca^{2+}]_i$ states related to electrophysiological characteristics and basal prolactin release

The present results have revealed the heterogeneity of lactotrophs in terms of their $[Ca^{2+}]_i$ states in agreement with data previously reported (Malgaroli, Vallar, Elahi, Pozzan, Spada & Meldolesi, 1987; Lewis *et al.* 1988). We found two groups of lactotrophs according to the fractions from which they originated. The first group, with high and fluctuating $[Ca^{2+}]_i$, was common in the light fractions. In other experiments performed on individual clonal PRL-secreting cells (GH_3 line), $[Ca^{2+}]_i$ recordings showed that similar fluctuations in $[Ca^{2+}]_i$ were due to spontaneous electrical activity (Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim & Dufy, 1987). As both spontaneous electrical activity and $[Ca^{2+}]_i$ oscillations are blocked by Ca^{2+} removal (Lledo *et al.* 1990*b*; present $[Ca^{2+}]_i$ recordings), we can speculate that fluctuations in $[Ca^{2+}]_i$ occurring spontaneously in normal lactotrophs are linked to action potential firing. Furthermore, a relationship between oscillating $[Ca^{2+}]_i$ state and basal PRL release can be established. Many cells from the light fractions exhibit a high rate of $[Ca^{2+}]_i$ oscillations as well as a high level of basal secretion, as observed in single cells or in whole populations. In this respect, the frequency and amplitude of $[Ca^{2+}]_i$ oscillations may control PRL release as previously proposed for growth hormone release (Holl, Thorner, Mandell, Sullivan, Sinha & Leong, 1988). The second group of cells, predominant in the heavy fractions, exhibited a low and stable resting $[Ca^{2+}]_i$, which may be associated with their observed high and constant resting membrane potential. This low $[Ca^{2+}]_i$ level is unlikely to support a Ca^{2+}-dependent secretory process, as suggested by the Ca^{2+} sensitivity of PRL release determined in permeabilized GH_3 cells (Ronning & Martin, 1986).

Differential responsiveness of lactotroph subtypes to dopamine and thyrotrophin-releasing hormone

Population analysis of the secretory activity of individual cells revealed the existence of two subpopulations: one secreting low basal amounts of PRL, predominant in the heavy fractions, and the other one secreting large amounts, mainly found in the light fractions. Similar observations were made from unseparated lactotroph cells (Luque, Munoz de Toro, Smith & Neill, 1986). These differences in basal secretion may account for differences in size (indicated by membrane capacitance measurement) and in density of the cells, as has been reported recently (Kukstas *et al.* 1990). In order to relate hormone release to the physiological state of these cells, we analysed the effects of dopamine and TRH on the two cell subtypes. According to intracellular recordings and PRL release measurements from cell

populations, it appeared that light fraction cells were more sensitive to dopamine application than cells from heavy fractions, while the converse was true for sensitivity to TRH application. $[Ca^{2+}]_i$ measurements at the single-cell level supported this observation. Only the cells that showed an elevated and oscillating $[Ca^{2+}]_i$ responded reversibly to D_2 receptor stimulation by a decrease in $[Ca^{2+}]_i$. Such cells were unresponsive to a TRH application. In contrast, cells exhibiting a low and quiescent $[Ca^{2+}]_i$ level, which was unaltered by dopamine, showed a characteristic $[Ca^{2+}]_i$ rise in response to TRH stimulation. The mechanisms by which dopamine or TRH induce their effects on $[Ca^{2+}]_i$ were not investigated in the present work in detail, as they have been described extensively in other studies (Malgaroli *et al.* 1987; Winiger, Wuarin, Zahnd, Wollheim & Schlegel, 1987).

The significance of the existence of two subpopulations of lactotrophs preferentially responsive to dopamine and TRH is still not known. The first indication was provided by studies performed on rat anterior pituitary cells separated by velocity sedimentation at unit gravity (Snyder, Wilfinger & Hymer, 1976). Using this method, subgroups of lactotrophs were isolated on the basis of their size and secretory granule content. Today, several methods give further evidence for the functional heterogeneity of lactotrophs, including: (1) immunocytochemical studies (Saint John, Dufy-Barbe & Barker, 1986); (2) pulse-chase experiments with isotope labelling, used to compare the release of newly synthesized and older PRL (Walker & Farquhar, 1980; Morin, Rosenbaum & Tixier-Vidal, 1984); (3) the reverse haemolytic plaque assay (RHPA) method for the detection of hormone secretion by individual cells (Boockfor, Hoeffler & Frawley, 1986; Frawley & Clark, 1986; Luque *et al.* 1986; Boockfor & Frawley, 1987); (4) radioimmunoassay combined with *in situ* hybridization (Velkeniers, Hooghe-Peters, Hooghe, Belayew, Smets, Claeys, Robberecht & Vanhaelst, 1988); (5) the use of fluorescent intracellular calcium probes such as Fura-2, which allows measurement of $[Ca^{2+}]_i$ (Malgaroli *et al.* 1987; Winiger *et al.* 1987; Lewis *et al.* 1988).

The question of the origin of such functional heterogeneity among lactotrophs thus becomes a matter of considerable importance. The differences could consist of a change in the cells over time, in accordance with an intrinsic secretory cell cycle, as has been reported in histological observations (Severinghaus, 1937), and/or in a peripheral hormone regulation (Zhang *et al.* 1990). We can also suppose that these differences arose from topographic location within the gland. Hence, cells derived from the outer zone of the pituitary gland are reported to be highly responsive to TRH but only moderately so to dopamine; contrasting data occurred for cells derived from the inner pituitary zone (Boockfor & Frawley, 1987).

Further experiments will be needed in order to establish the link between $[Ca^{2+}]_i$ and its influence on PRL release. Such a study might be attempted by a direct measurement of Ca^{2+}-dependent exocytosis, using membrane capacitance measurements, combined with simultaneous Ca^{2+} current recordings, as recently reported for nerve terminals (Lim, Nowycky & Bookman, 1990).

The authors particularly wish to thank Mrs D. Verrier for excellent technical assistance, Dr E. Arnauld for her help in computer measurements of plaque areas and Mr R. Miguelez for artwork. We also thank Dr S. Luckman and Dr J. Coles for critically reading the manuscript. This work was supported by grants from the 'Ministère de la Recherche et de la Technologie', the 'Institut

National de la Santé et de la Recherche Médicale', 'Centre National pour la Recherche Scientifique' and 'Université de Bordeaux II'.

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