Modulation of stimulus–secretion coupling in single rat gonadotrophs

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1. Exocytosis and intracellular [Ca^{2+}] were determined simultaneously in single anterior pituitary gonadotrophs from ovariectomized female rats. Dispersed cells were cultured for 2–4 days with or without 0·2 nM oestradiol-17β (E_2) before use. Cells were stimulated with either gonadotrophin releasing hormone (GnRH) or by membrane depolarization. Exocytosis was determined from the change in membrane capacitance (C_m) using the perforated-patch whole-cell recording technique. Intracellular [Ca^{2+}] was measured using fura-2 fluorescence.

2. The exocytotic response to 1 nM GnRH was characterized by a wide spectrum of responses, ranging from exocytotic bursts to relatively slow, graded increases that were dependent on the evoked intracellular Ca^{2+} pattern. A kinetic model is presented that incorporates the observed steep dependence of exocytosis on measured intracellular [Ca^{2+}]; simulated exocytosis reasonably approximated observed exocytotic responses, both kinetically and quantitatively. The model also suggests that the modulatory effects of E_2 are brought about either by a change in the Ca^{2+} sensitivity of exocytosis or by a preferential clustering of docked-secretory granules close to sites of Ca^{2+} release. The results suggest that in gonadotrophs an oscillatory Ca^{2+} signal is sensed by the exocytotic apparatus in a modified form of digital encoding.

3. Exocytosis in E_2-treated cells was 3-fold greater than in non-treated cells for GnRH-evoked secretion, and 38% greater for depolarization; however, there was no effect of E_2 on the intracellular Ca^{2+} response to either stimulus. The results show that maximum expression of the effect of E_2 on exocytosis requires activation of GnRH-dependent pathways.

Gonadotrophs of the anterior pituitary gland secrete the gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone under the control of the hypothalamic hormone gonadotrophin releasing hormone (GnRH). In turn, the gonadotrophins control mammalian reproductive function. Beginning with the work of Samli & Geschwind (1968), the intimate relationship between Ca^{2+} and GnRH-stimulated LH secretion has been repeatedly demonstrated and extensively studied. Studies of single gonadotrophs by several laboratories have revealed a complex pattern of intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) responses to GnRH (reviewed in Stojilkovic & Catt, 1995). It has been shown that at low concentrations of GnRH ($\leqslant$ 0·1 nM), many cells show a 'subthreshold' response with small amplitude increases in [Ca^{2+}]$_i$ ($\leqslant$ 0·4 μM). At higher GnRH concentrations (0·1–10 nM), most cells show very dramatic, large amplitude oscillations in [Ca^{2+}]$_i$ ($\geqslant$ 1 μM), and at yet higher concentrations of GnRH ($\geqslant$ 10 nM), these oscillations appear to increase in frequency and ultimately fuse to form characteristic 'biphasic' increases in [Ca^{2+}]$_i$.

Oscillations in [Ca^{2+}]$_i$ have been observed in several cell types (Tsien & Tsien, 1990; Meyer & Stryer, 1991), but in many cases the functional significance of these oscillations remains obscure. The major obstacle to understanding the function of [Ca^{2+}]$_i$ oscillations has been the lack of adequate techniques to investigate the Ca^{2+}-dependent physiological activity of single cells. Correspondingly, there have been few studies of the relationship between the complex patterns of [Ca^{2+}]$_i$ and hormone secretion that permit a direct, quantitative correlation between exocytosis and [Ca^{2+}]$_i$ in individual gonadotrophs. Qualitatively, however, a closely coupled temporal relationship between [Ca^{2+}]$_i$ oscillations and exocytosis, as determined by changes in membrane capacitance (C_m), has been observed in single gonadotrophs (Tse, Tse, Almers & Hille, 1993). Examination of the quantitative temporal relationship between [Ca^{2+}]$_i$ and C_m has been addressed in other secretory cell types, principally the chromaffin cell (Augustine & Neher, 1992), both experimentally and theoretically (Heinemann, von Rüden, Chow & Neher, 1993; von Rüden & Neher, 1993).

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The objective of this study was to examine the relationship between $[Ca^{2+}]_i$ and exocytosis at the level of the single female rat gonadotroph in a physiologically relevant context. We chose to examine the effect of oestradiol-17β (E_2) because of the important and profound effect this steroid hormone has on female gonadotroph function, including gonadotrophin secretion. We used the perforated-patch configuration of the whole-cell patch-clamp recording technique for the measurement of C_m, to ensure minimal disturbance of the intracellular environment and thus the pathways responsible for the induced changes in $[Ca^{2+}]_i$ and exocytosis. Finally, because of the complex nature of the $[Ca^{2+}]_i$ response of gonadotrophs to GnRH, we have used quantitative modelling to analyse the relationship between $[Ca^{2+}]_i$ and exocytosis, and to examine the modulation of exocytosis by E_2.

METHODS

Materials

Adult female Sprague–Dawley rats (Simonsens, Gilroy, CA, USA) were maintained in controlled light conditions (14 h light, 10 h dark). Media, trypsin/EDTA and sera for cell culture were obtained from Gibco BRL; oxoid tablets for Dulbecco's phosphate-buffered saline were from Unipath Co. (Ogdensburg, NY, USA). Kanamycin sulphate, bovine serum albumin fraction V, DMSO, amphotericin B, apamin and GnRH were from Sigma, and tetrodotoxin (TTX), fura-2 sodium salt (Na_5fura-2), Hepes free acid and oestradiol-17β were purchased from Calbiochem. Fura-2/acetoxymethyl ester (fura-2 AM) and pluronic F-127 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). General chemicals were from either Sigma or Fisher Scientific (Pittsburgh, PA, USA).

Ovariectomy and pituitary cell culture

The protocols used in these experiments were reviewed and approved by the University of California Davis Animal Use and Care Administrative Advisory Committee. Removal of the ovaries was performed on ether-anaesthetized rats (deep surgical anaesthesia) in a fume hood under aseptic conditions, and required approximately 5 min per animal. Following alcohol swabbing, bilateral flank incisions (approximately 1·5 cm) were made just below the kidneys. Each ovary was exteriorized, ligatures placed at the utero-isthmus junctions, and the ovaries, oviducts and attendant fascia excised. The incision was repaired by suture of the muscle wall and wound clips in the skin. Starting with the latter quarter of this study, immediately post-operatively rats were administered an analgesic, buprenorphine, injected subcutaneously (0·02 mg kg^{-1}). The administration of buprenorphine had no effect on the experimental results (data not shown). Ovariectomized rats were maintained for 2–3 weeks before use.

Individually, rats were placed in a chamber and the CO_2 concentration gradually increased to render them unconscious, after which they were immediately decapitated and the anterior pituitary gland removed. Anterior pituitary tissue was enzymatically dispersed and prepared for cell culture as described (Turgeon & Waring, 1990). Cells were plated into modified 35 mm dishes in which a hole had been cut and over which a glass coverslip was affixed with Sylgard 184 (Dow Corning Corp., Midland, MI, USA). Cells were maintained in Eagle's minimal essential medium containing D-valine supplemented with 200 μM kanamycin sulphate and 10% (v/v) fetal bovine serum (FBS) that had been charcoal treated to remove endogenous steroids (ctFBS; Horwitz & McGuire, 1978). Cells were incubated with or without 0·2 nM E_2 in a humidified atmosphere (37 °C) of 5% CO_2 in air. Cells were used for experiments from day 2 to day 4 of culture (day of plating was day 0). As reported by Stutzin, Stojilkovic, Catt & Rojas (1989), gonadotrophs in cultures obtained from ovariectomized rats are readily identified on morphological criteria; in our experience, > 95% of cells thus identified respond to GnRH (data not shown).

Experimental procedure

For the experiments, culture medium was replaced with an extracellular medium consisting of 135 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 5·6 mM glucose, 0·5 μM TTX and 1 mg ml^{-1} BSA; pH 7·4 with 10 mM Hepes/NaOH. In some experiments BSA was omitted; no differences were observed between recordings with or without BSA (data not shown). TTX was included to prevent the generation of action potentials by the sinusoidal voltage used to determine C_m (Thomas, Surprenant & Almers, 1990; Tse *et al.* 1993). The experimental set-up was as described (Thomas, Mellon, Turgeon & Waring, 1996). Briefly, dishes with attached cells were transferred onto a temperature-regulated microscope stage maintained at 22 °C, and the dish was superfused with extracellular medium using a peristaltic pump (approximately 1 ml min^{-1}). Appropriate dilutions of GnRH in extracellular medium were made from a stock solution on the day of the experiment. GnRH was applied to the cells for either 1 or 5 min; no differences were observed in the $[Ca^{2+}]_i$ responses or the secretory responses with either treatment protocol (data not shown), and so the data were pooled.

Measurement of $[Ca^{2+}]_i$. The determination of $[Ca^{2+}]_i$ was as described (Thomas *et al.* 1996). Briefly, $[Ca^{2+}]_i$ was determined ratiometrically with the fluorescent probe fura-2. Cells in extracellular medium were loaded with fura-2 by incubation with 2 μM fura-2 AM for 20 min at 37 °C. Fluorescence was alternately excited at 340 and 380 nm and the ratio calculated every 100 ms. Cell fluorescence was collected using a ×40 CF Fluor (1·3 NA) oil-immersion objective and passed thorough a bandpass filter to the photomultiplier.

$[Ca^{2+}]_i$ was calculated according to Almers & Neher (1985) using eqn (1):

$$[Ca^{2+}] = K^* \left(\frac{R - R_{min}}{R_{max} - R} \right). \quad (1)$$

The calibration constants R_{min}, R_{max} and K^* were measured in 20 μm thick microslides (Vitro Dynamics Corp., Rockaway, NJ, USA) using the following solutions, each of which contained 75 μM Na_5fura-2: R_{min}, 10 mM K-EGTA, 100 mM KCl and 20 mM Hepes/NaOH at pH 7·2; R_{max}, 1 mM $CaCl_2$, 100 mM KCl and 20 mM Hepes/NaOH at pH 7·2; K^*, 10 mM K-EGTA, 5 mM $CaCl_2$, 100 mM KCl and 20 mM Hepes/NaOH at pH 7·2. The free $[Ca^{2+}]$ in the latter solution was assumed to be 151 nM (Almers & Neher, 1985).

Electrophysiology. Electrophysiological measurements were carried out using the perforated-patch variant (Marty & Neher, 1995) of the tight-seal patch-clamp recording technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The pipette filling solution consisted of 48 mM potassium glutamate, 75 mM K_2SO_4, 10 mM NaCl, 5 mM $MgSO_4$ and 0·5 mM K-EGTA; pH 7·2 with 10 mM Hepes/KOH. Perforation of the patch was achieved by inclusion of a pore-forming antibiotic, amphotericin B, as described (Thomas *et al.* 1996). Pipettes had resistances of 2–10 MΩ and attained access resistances (R_a) of less than 35 MΩ (mean 22·7 ± 0·5 MΩ, $n = 92$). Cells were chosen for patch-clamp analysis

Scheme 1. Kinetic model of exocytosis

$$S_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S_1 \xrightarrow{k_2} P \xrightarrow{k_3} I$$

S denotes the distal intracellular vesicle compartments (S_1 are 'readily releasable' vesicles and S_2 are 'reserve' vesicles). P denotes the change in plasma membrane area (ΔC_m). I denotes retrieved membrane (endocytosis). k values are rate constants (k_{-1} is $[Ca^{2+}]$ independent, and k_1, k_2 and k_3 are $[Ca^{2+}]$ dependent).

based on the criteria that they were: (i) single cells, (ii) firmly attached to the dish, and (iii) $< 20\ \mu m$ in diameter (initial $C_m = 9{\cdot}0 \pm 0{\cdot}2$ pF, $n = 92$). The cell size was chosen because the signal-to-noise ratio in measurements of C_m is inversely related to the product of R_a and C_m. Under our recording conditions, we have found that C_m measurements require an R_aC_m time constant (τ_{RC}) of less than 350 μs (in these experiments τ_{RC} was $203 \pm 6\ \mu s$, $n = 92$). Recordings were made with an Axopatch-1C patch-clamp amplifier via a TL-1 computer interface (Axon Instruments). C_m data were acquired and analysed using Axobasic software programs based on phase-tracking (Fidler & Fernandez, 1989) as described (Thomas *et al.* 1996). Measurements were made by superimposing an 833 Hz voltage sinusoid (40 mV peak-to-peak) on the holding potential (−80 mV). C_m was monitored at a phase angle of $\alpha + 90$ deg by phase-tracking with a 47 Ω resistor (equivalent to 470 fF), which was dithered under computer control in the C_{slow} compensation circuitry of the patch-clamp amplifier (modified DC-1, Axon Instruments). The phase angle α was chosen to minimize any effects changes in membrane conductance might have on the C_m signal. The phase angle was tracked every 2000 points, i.e. every 42 s. Changes in C_m were confirmed by recompensation of the patch-clamp amplifier following each treatment.

For synchronization of simultaneously acquired data, acquisition of $[Ca^{2+}]_i$ was triggered by a TTL (transistor-transistor logic) pulse at the start of the C_m data acquisition. Likewise for hormone addition, event markers were simultaneously acquired to both data files via TTL pulses. Minor differences in computer clock speed were corrected during data analysis after importing both data files into SigmaPlot (SPSS, Inc., Chicago, IL, USA).

Data analysis

Baseline $[Ca^{2+}]_i$ and oscillation amplitudes were determined after traces were smoothed using a buffer size of 15 points (Oscar or Felix software, Photon Technology International, South Brunswick, NJ, USA). Rates of secretion (dC_m/dt) of cells treated with GnRH were determined by calculating the slopes of regression lines fitted to short, overlapping segments of the C_m trace. The length of these lines varied with the rate of secretion. For rates below 7·5 fF s^{-1} the lines were 2 s long, for rates between 7·5 and 25 fF s^{-1} they were 1 s long, for rates between 25 and 100 fF s^{-1} they were 0·5 s long, and for rates > 100 fF s^{-1} they were 0·25 s in length. The dC_m/dt traces were then smoothed with a buffer size of 21 points (Savitzky & Golay, 1964).

Modelling. To evaluate the Ca^{2+} dependence of exocytosis, we used the model of Heinemann *et al.* (1993) and adapted it to include endocytosis (Scheme 1). First-order differential equations based on this model were used to derive simulated secretory responses from $[Ca^{2+}]_i$ data obtained in experiments with simultaneously determined C_m.

The equations are as follows (see Scheme 1 for definitions of symbols):

$$\frac{dS_2}{dt} = k_{-1}S_1 - k_1S_2 \quad (2)$$

$$\frac{dS_1}{dt} = k_1S_2 - (k_{-1} + k_2)S_1 \quad (3)$$

$$\frac{dP}{dt} = k_2S_1 - k_3P \quad (4)$$

$$\frac{dI}{dt} = k_3P \quad (5)$$

$$k_1 = \frac{a_1[Ca^{2+}]^{c_1}}{b_1^{c_1} + [Ca^{2+}]^{c_1}} \quad (6)$$

$$k_{-1} = a_2 \quad (7)$$

$$k_2 = a_3[Ca^{2+}]^{c_2} \quad (8)$$

$$k_3 = \frac{a_4[Ca^{2+}]}{b_2 + [Ca^{2+}]} \quad (9)$$

The equations were solved using either a first-order Euler method (Mathematica; Wolfram Research, Inc., Champaign, IL, USA) or a Runge-Kutta method (ModelMaker; Cherwell Scientific, Oxford, UK) and an iteration interval of 0·1; the results were indistinguishable. Increasing the iteration interval to 0·01 did not substantially change or improve the results. In equations 6–9, the a, b and c terms are empirically determined constants. The values of a_1, a_3, c_1 and c_2 are given in the figure legends. The numbers of vesicles in S_1 and S_2 (expressed in units of C_m, i.e. fF) were varied as shown in the figure legends; however, the proportion of vesicles in each compartment was maintained constant ($S_1 = 18{\cdot}5\%$ of the total number of vesicles in $S_1 + S_2$). Because k_1 is Ca^{2+} dependent and k_{-1} is not, and because the baseline $[Ca^{2+}]$ varied from cell to cell, the value of a_2 was adjusted in each simulation to maintain the initial values of S_2 and S_1 at baseline $[Ca^{2+}]$; typically a_2 ranged from 0·0001 to 0·005 s^{-1}. The Ca^{2+} dissociation constant for the S_2 to S_1 transition (b_1) was initially 1·6 μM; however, it was adjusted to reflect changes in the Ca^{2+} sensitivity of exocytosis (see Fig. 4*C*). In the absence of specific information, we assumed the Ca^{2+} dissociation constant for endocytosis (b_2) to be 1·6 μM. In these experiments endocytosis retrieved membrane at maximal rates of approximately 15 fF s^{-1}; therefore, we made $a_4 = 0{\cdot}09$ s^{-1}, a value that gave rise to similar maximal rates in the simulations.

In Scheme 1, we have used the term 'P' to distinguish our model from earlier models of this type that refer to this parameter as 'E' (exocytosis) (Heinemann *et al.* 1993; Thomas, Wong, Lee & Almers, 1993); this was required because of our inclusion of endocytosis ('I') in the model. Thus dP/dt is equivalent to dC_m/dt,

and so ΔC_m is given by the integral of eqn (4). Accordingly, the value $k_2 S_1$ is equal to the rate of exocytosis and $k_3 P$ is equal to the rate of endocytosis, and the time integral of $dP/dt + dI/dt$ is equal to total exocytosis. In contrast, exocytosis estimated from the C_m data can be an underestimate, since non-zero values of exocytosis are not accounted for when the rate of endocytosis exceeds the rate of exocytosis, i.e. when the slope of the C_m data trace is zero or negative. Therefore, to generate values comparable to exocytosis estimated from the C_m data, we summed the positive steps in C_m traces obtained in simulations; these are the model output data that are compared with the observed secretion results.

Secretory granule morphology. Rat gonadotrophs do not have secretory granules of uniform size, rather the majority range in size from 150 to 250 nm (reviewed in Farquhar, 1971; Pelletier & Labrie, 1982). Qualitatively, there is no suggestion that the distribution in gonadotroph secretory granule sizes is affected by castration or E_2 replacement in the female rat (Farquhar, 1971). Using a specific capacitance of ~10 fF μm^{-2}, vesicles of 250 nm diameter would have a capacitance of about 2 fF, a value that we have assumed to interconvert vesicle number and C_m.

Statistical analyses. Student's t test (paired or unpaired as appropriate) was used for comparisons that involved two groups with equal variances. When the variances of the two groups differed significantly, we used Welch's t test for unpaired comparisons and the non-parametric Wilcoxon signed rank test for paired comparisons. For comparisons involving more than two groups, data were analysed by ANOVA. Multiple pairwise comparisons were made following one-way ANOVA with the Student–Newman–Keuls method (GraphPad Software, San Diego, CA, USA).

RESULTS

GnRH-evoked $[Ca^{2+}]_i$ responses in patched *vs.* non-patched gonadotrophs

We first established whether patched and non-patched gonadotrophs exhibited qualitatively similar $[Ca^{2+}]_i$ responses to GnRH, particularly with regard to the general categories of subthreshold, oscillatory and biphasic $[Ca^{2+}]_i$ responses. In these and all subsequent experiments, cells were exposed to GnRH only once. As previously defined (Tomic, Cesnjaj, Catt & Stojilkovic, 1994), subthreshold responses occur at low, possibly subphysiological, GnRH concentrations; they are of low amplitude (< 400 nM) and transient. Oscillations from the baseline are seen in response to intermediate GnRH concentrations. The $[Ca^{2+}]_i$ response to high GnRH concentrations is biphasic: an initial peak or spike is followed by a longer low amplitude plateau.

The upper panels of Fig. 1 show representative recordings from individual gonadotrophs that were patch-clamped using the perforated-patch technique and exposed to 1 nM GnRH. The cell in Fig. 1*A* gave a subthreshold response to GnRH, the middle panels of Fig. 1*B* and *C* show typical oscillatory $[Ca^{2+}]_i$ responses, and the cell in Fig. 1*D* exhibited the prominent $[Ca^{2+}]_i$ peak and plateau characteristic of a biphasic response. Within the latter two categories, we found some overlap in that many cells exhibited oscillations that did not return to baseline (e.g. Fig. 1*B* and *C*), and some cells showed distinct oscillations superimposed on a

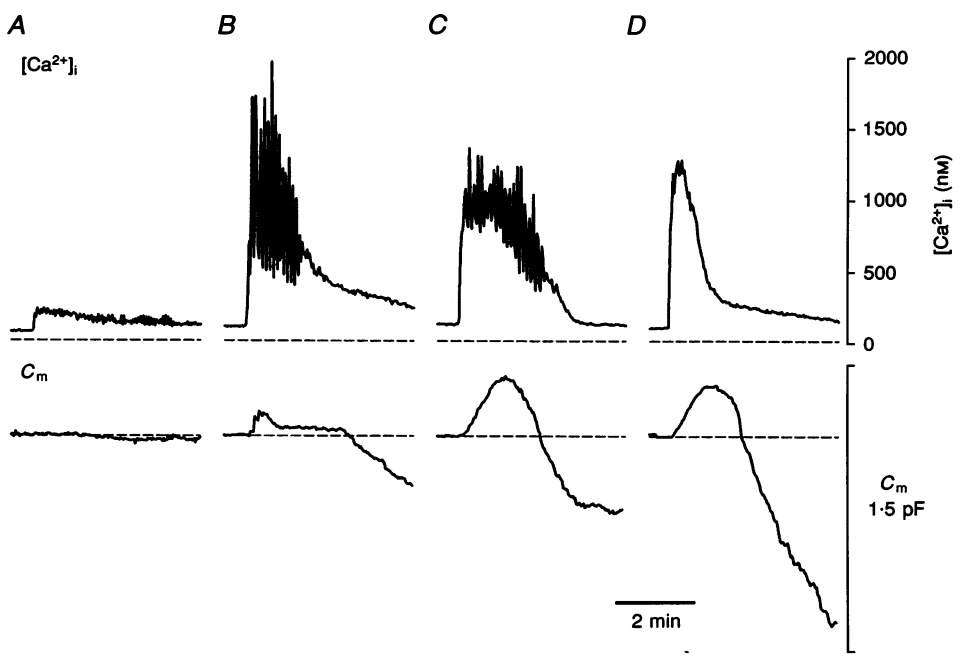

Figure 1. GnRH-evoked increases in $[Ca^{2+}]_i$ and exocytosis in single gonadotrophs

Representative traces are shown from four different gonadotrophs that were exposed to 1 nM GnRH beginning within 10 s of the start of each record. C_m traces were smoothed with a smoothing buffer of 11 points. The cells were cultured for 3–4 days in E_2 and charcoal-treated fetal bovine serum (E_2-ctFBS). The initial C_m of each cell was: *A*, 11·8 pF (τ_{RC} = 294 μs); *B*, 8·7 pF (τ_{RC} = 204 μs); *C*, 8·5 pF (τ_{RC} = 190 μs); *D*, 7·6 pF (τ_{RC} = 113 μs).

Table 1. $[Ca^{2+}]_i$ responses of individual gonadotrophs treated with GnRH

Cells	[GnRH]	n	Type of $[Ca^{2+}]_i$ response (%)		
			Subthreshold	Oscillatory	Biphasic
Unpatched	0·1 nM	6	17	83	0
	1 nM	9	0	100	0
Patched	0·1 nM	7	14	86	0
	1 nM	30	13	77	10

Cells were cultured in E_2-ctFBS for 2–4 days. Cells were treated with GnRH for 1 or 5 min. Patched cells were voltage clamped at a holding potential of −80 mV.

Table 2. Oscillatory $[Ca^{2+}]_i$ responses of patched and unpatched gonadotrophs

Characteristic	Baseline $[Ca^{2+}]_i$ (nM)	Oscillation frequency (min^{-1})	No. of oscillations	Peak amplitude (nM)	n
Unpatched	73 ± 6	8·2 ± 0·7	11·9 ± 1·8	1548 ± 143	9
Patched	85 ± 6 [a]	9·6 ± 0·6	19·0 ± 2·0*	1300 ± 114	12

Cells were cultured in E_2-ctFBS for 2–4 days. The cells were then treated with 1 nM GnRH for 1 min. Patched cells were voltage clamped at a holding potential of −80 mV. *a*, before patching $[Ca^{2+}]_i = 73 \pm 7$ nM. * $P < 0·05$ with respect to unpatched cells.

biphasic pattern (e.g. Fig. 1*B*). Because a focus of our study was on the relationship between exocytosis and $[Ca^{2+}]_i$ oscillations, it was necessary to distinguish further the oscillatory and biphasic categories. We classified cells as oscillatory, rather than biphasic, if (i) there were more than three oscillations within the first 30 s of the Ca^{2+} response or (ii) if the mean inter-oscillation $[Ca^{2+}]_i$ (in the first 30 s) was < 85% of the mean peak amplitude. These criteria unambiguously categorized the cells as either oscillatory (e.g. Fig. 1*B* and *C*) or biphasic (e.g. Fig. 1*D*); at the GnRH concentrations used, the majority of cells were oscillatory.

The data presented in Table 1 demonstrate the qualitative similarities between the GnRH-evoked $[Ca^{2+}]_i$ responses in patched *vs.* unpatched gonadotrophs. Under either condition, a distribution of $[Ca^{2+}]_i$ response patterns is observed from subthreshold through to biphasic, with biphasic responses generally being associated with increased GnRH concentration. We used only two GnRH concentrations, but based on the pattern in Table 1 and in other studies (Tomic *et al.* 1994), it is anticipated that a higher GnRH concentration (e.g. 10 nM) would shift the $[Ca^{2+}]_i$ response pattern to be predominantly biphasic. In sum, individual gonadotrophs can respond with strikingly different patterns of activity to the same concentration of GnRH, and each of the three patterns of $[Ca^{2+}]_i$ may be observed at the same GnRH concentration (Fig. 1 and Table 1). Furthermore, the responses of the patched gonadotrophs emphasize that on this time scale the main effect of GnRH on $[Ca^{2+}]_i$ occurs via the release of Ca^{2+} from intracellular stores, since the patched cells are held under voltage clamp and thus voltage-gated Ca^{2+} channels (VOCCs) will remain closed.

More quantitative comparisons of the patched and unpatched gonadotrophs (Table 2) revealed that, among those gonadotrophs that showed an oscillatory response to 1 min applications of 1 nM GnRH, only the number of oscillations increased in patched *vs.* unpatched gonadotrophs. There was no difference in the delay in the start of the GnRH response in the two groups of cells (data not shown); thus the increase in the number of oscillations was due to a prolonged oscillatory episode. Minor differences in the baseline $[Ca^{2+}]_i$ and in the amplitude and frequency of the oscillations were not statistically significant.

We chose to study oscillatory cells and we used 1 nM GnRH for the remainder of the studies, as this GnRH concentration produced an oscillatory response in the large majority of cells.

GnRH-evoked exocytosis

The lower panels of Fig. 1 show the simultaneously acquired C_m traces that reflect the exocytotic activity of the cells. The secretory responses varied with the type of $[Ca^{2+}]_i$ response (Table 3); more secretion occurred in the oscillatory and biphasic cells than in the cells showing subthreshold responses. However, due to the small sample size for biphasic cells and the large variation in the exocytotic response, it was not possible to determine whether the biphasic $[Ca^{2+}]_i$ response resulted in a further increase in secretory activity over that observed in the oscillatory cells. The integral of the $[Ca^{2+}]_i$ response also increased in oscillatory and biphasic cells *vs.* subthreshold cells. However, as with the secretory response, there was no difference in the $[Ca^{2+}]_i$ integral in the oscillatory compared with the biphasic cells.

Table 3. Comparison of the exocytotic and Ca^{2+} responses of individual gonadotrophs

	Type of $[Ca^{2+}]_i$ response		
	Subthreshold	Oscillatory	Biphasic
Exocytosis (fF)	51 ± 35^{a}	377 ± 44	589 ± 194
Integrated Ca^{2+} (μM s)	$33 \pm 10^{b,c}$	89 ± 5	107 ± 43
n	4	23	3

a, significantly different from both oscillatory and biphasic, $P < 0{\cdot}01$; *b*, significantly different from oscillatory, $P < 0{\cdot}01$; *c*, significantly different from biphasic, $P < 0{\cdot}05$.

Steroid effects on GnRH-evoked responses

All of the responses described thus far were obtained in cells incubated in E_2-ctFBS. We also examined the $[Ca^{2+}]_i$ and secretory responses of gonadotrophs incubated in the absence of steroids (ctFBS). Qualitatively, the ctFBS cells gave similar calcium responses to E_2-ctFBS cells (8% subthreshold, 76% oscillatory and 16% biphasic when treated with 1 nM GnRH, $n = 25$; cf. patched cells in Table 1). We have restricted our comparison of the E_2-ctFBS and ctFBS cells to those cells demonstrating oscillatory behaviour when treated with 1 nM GnRH. Quantitative comparisons between the ctFBS and E_2-ctFBS cells revealed no significant differences between the integrated $[Ca^{2+}]_i$ responses in the ctFBS and E_2-ctFBS cells (Fig. 2); there also were no differences in the baseline $[Ca^{2+}]_i$ and the number, frequency or amplitude of $[Ca^{2+}]_i$ oscillations (data not shown). Nevertheless, significant differences were observed between these two groups of cells when the secretory responses were compared. All of the E_2-ctFBS cells gave a secretory response, whereas 16% of ctFBS cells failed to secrete. Furthermore, the mean exocytotic response of the E_2-ctFBS cells was approximately 3-fold larger than that of the ctFBS cells (Fig. 2).

Modelling of exocytosis

Rhythmic bursts of exocytosis (C_m) that occur in synchrony with $[Ca^{2+}]_i$ oscillations have been observed in male rat pituitary gonadotrophs using the conventional whole-cell recording technique (Tse *et al.* 1993). We also observed exocytotic bursts in perforated-patch recordings in female rat gonadotrophs cultured in the presence of E_2 (Fig. 3*A*). These bursts are most clearly seen in the time derivative of the C_m record (dC_m/dt; lower traces in Fig. 3). Tse *et al.* (1993) reported that for the majority of cells they examined the maximum rate of exocytosis occurred during the rising phase of a $[Ca^{2+}]_i$ oscillation; in the remaining cells, the maximum rate of exocytosis coincided with the maximum $[Ca^{2+}]_i$. For our data, the temporal resolution and uncertainties concerning the synchronization of acquisition programs does not allow this distinction; within these uncertainties, peak rates of exocytosis were observed to occur with the maximum $[Ca^{2+}]_i$. A wide spectrum of kinetic behaviour was observed, and some cells showed little evidence of exocytotic bursts (Fig. 3*B*). Indeed, analysis of the maximal rates of exocytosis in the oscillatory cells revealed that some cells (e.g. Fig. 3*A*) had much higher rates of exocytosis than others (e.g. Fig. 3*B*), despite the fact that the $[Ca^{2+}]_i$ peaks were similar. In cells such as the one shown in Fig. 3*B* it was

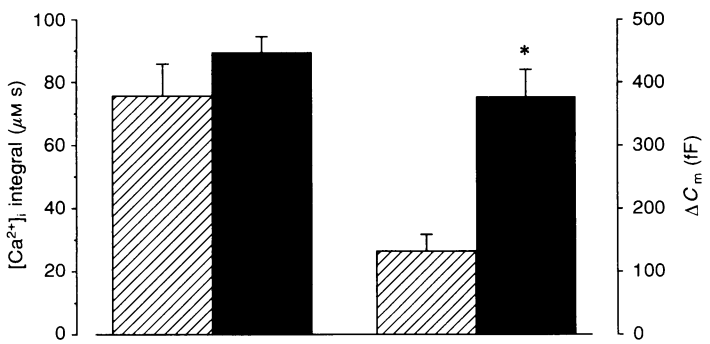

Figure 2. Comparison of the effect of oestradiol-17β on the $[Ca^{2+}]_i$ and exocytotic responses of single gonadotrophs

Left, means (± S.E.M.) of the integral of the $[Ca^{2+}]_i$ response of individual gonadotrophs that gave oscillatory responses when treated with 1 nM GnRH; right, means (± S.E.M.) of the total amount of exocytosis observed in the same cells (ctFBS, $n = 19$; E_2-ctFBS, $n = 23$). * Significantly different from ctFBS ($P < 0{\cdot}05$). ▨, ctFBS; ■, E_2-ctFBS.

difficult to associate particular rates of exocytosis with individual $[Ca^{2+}]_i$ oscillations; furthermore, in order to measure exocytotic rates in such cells, we found it necessary to use longer stretches of the C_m trace, which consequently underestimated the true rates of secretion. We therefore restricted our analysis of the Ca^{2+} dependency of the rate of exocytosis to those cells giving well-defined bursts of exocytosis (peak rates > 15 fF s^{-1}). When we examined the relationship between the rate of exocytosis evoked by individual $[Ca^{2+}]$ oscillations (Fig. 4*B*) and the peaks in $[Ca^{2+}]_i$ measured during those oscillations (Fig. 4*A*), it was clear that the apparent Ca^{2+} dependency of secretion was extraordinarily steep (Fig. 4*C*). The curves in Fig. 4*C* are the least-squares fits to the equation:

$$\frac{dC_m}{dt} = A[Ca^{2+}]^n, \quad (10)$$

where A is a constant of proportionality and $n = 10$. Apparently small differences in the amplitudes of the Ca^{2+} oscillations may lead to large differences in the rates of exocytosis. This steep relationship between the rate of secretion and $[Ca^{2+}]_i$ suggests that the two disparate kinetic responses shown in Fig. 3 could be reconciled by a single mechanism if the observed rates of exocytosis are solely a function of the different patterns of $[Ca^{2+}]_i$ oscillations.

Additionally, in those cells showing bursts of exocytosis, the pattern of the exocytotic bursts was very distinctive. The first $[Ca^{2+}]_i$ spike gave rise to little or no exocytosis; the rate of exocytosis then increased from the second to the third spike and then decreased with subsequent oscillations (see Figs 3*A* and 4*B*).

In an attempt to understand these distinctive kinetics and the varied secretory responses, we determined whether a single model of exocytosis that incorporated the observed dependence of exocytosis on $[Ca^{2+}]_i$ could explain the different kinetic responses that we obtained in these experiments. For this analysis, we used the simple two-step model of secretion developed by Neher and colleagues to describe secretion in chromaffin cells (Heinemann *et al.* 1993). To this model we have added an additional step to include the endocytotic activity we often observed experimentally (Fig. 1). The basic assumptions of this model are shown in Scheme 1.

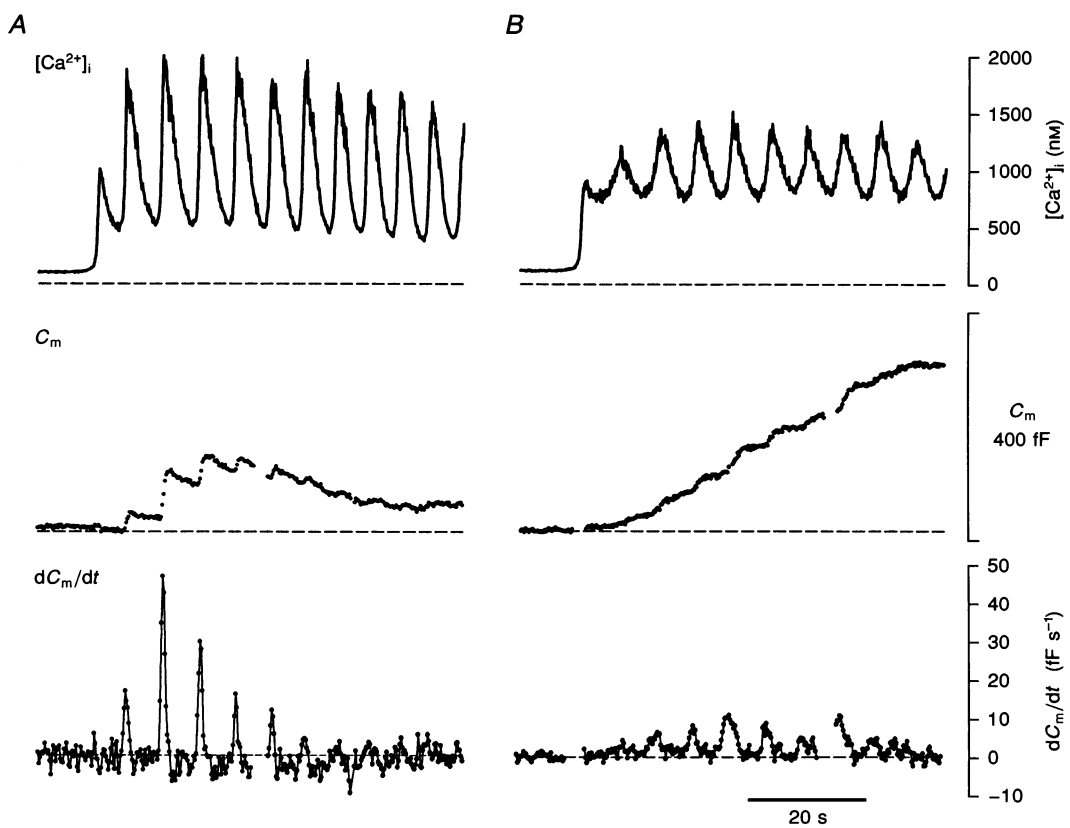

Figure 3. Two types of exocytotic response in oscillatory cells

The upper ($[Ca^{2+}]_i$) and middle (C_m) panels of *A* and *B* show the traces from Fig. 1*B* and *C*, respectively, on expanded scales. The lower panel shows the time derivatives (dC_m/dt) of the C_m traces. Each point is the slope of a line fitted to a short segment of the C_m trace; in *A* this line extended 0·25 s on either side of the corresponding C_m data point, in *B* the line extended $\pm$ 0·5 s. Breaks in the traces are due to phase-tracking episodes (~2 s every 42 s).

Essentially, there is a small pool of 'readily releasable' vesicles (S_1) that can be exocytosed into a 'secreted' pool (P), and S_1 can be replenished by movement of vesicles from a larger 'reserve' pool (S_2). Additionally, the secreted pool can be retrieved into an 'internalized' pool (I). The two forward rate constants that govern exocytosis (k_1 and k_2) are Ca^{2+} dependent and, because the final exocytotic reaction is thought to be governed by a protein that binds multiple calcium ions, k_2 is proportional to the third or fourth power of the $[Ca^{2+}]$ (Heinemann *et al.* 1993; Thomas *et al.* 1993). Since secretory vesicle membrane retrieval in chromaffin cells is Ca^{2+} dependent (Artalejo, Elhamdani & Palfrey, 1996), we have also assumed that k_3 is Ca^{2+} dependent. For simplicity, the reverse rate constant k_{-1} is assumed to be Ca^{2+} independent (Heinemann *et al.* 1993). Simulated exocytotic events were then obtained by using the $[Ca^{2+}]_i$ acquired in experiments on individual gonadotrophs in eqns (6), (8) and (9). Preliminary analysis showed that the rate constants and numbers of vesicles used to model chromaffin cells gave larger amplitude responses and slower kinetics than we observed in gonadotrophs (data not shown). Possible explanations of these differences are, first, that gonadotrophs have less available secretory vesicle membrane (i.e. fewer or smaller vesicles), and, secondly, the original model assumes that k_2 is a function of the third power of the $[Ca^{2+}]$, whereas from Fig. 4*C* it would seem that the relationship between the measured $[Ca^{2+}]$ and exocytosis is much steeper in our experiments (~10th power). In an initial attempt to account for these differences, we made k_2 a function of the sixth power and k_1 a function of the square of the $[Ca^{2+}]$, and we reduced the number of vesicles in S_2 and in S_1. Simulations using this modified model show many of the characteristics of the experimental recordings (cf. Fig. 5*A* and Fig. 3*A*), e.g. little or no secretion with the first Ca^{2+} oscillation, distinctive bursts of exocytosis (see dC_m/dt traces, Figs 3*A* and 5*A*), and an overall secretory response that exhausts after six or seven Ca^{2+} oscillations. Additionally, this model also predicts, qualitatively, the slower kinetic response observed in the cell in Fig. 3*B* (Fig. 5*B*; C_m).

Having arrived at model parameters that qualitatively reproduce the kinetics of the secretory response, we attempted to model the data more quantitatively. In order to emulate the amount of exocytosis in the examples in Fig. 5, we had to assume that individual cells have different numbers of available secretory vesicles. Indeed, in some cells, apparently small amplitude $[Ca^{2+}]_i$ oscillations led to large C_m increases, whereas in other cells large amplitude $[Ca^{2+}]_i$ oscillations led to small C_m increases. Consideration of this observation requires a more complex model than the simple Ca^{2+}-dependent one with which we started. Nevertheless, to characterize the 'average' cell, we determined the number of vesicles required to emulate the mean

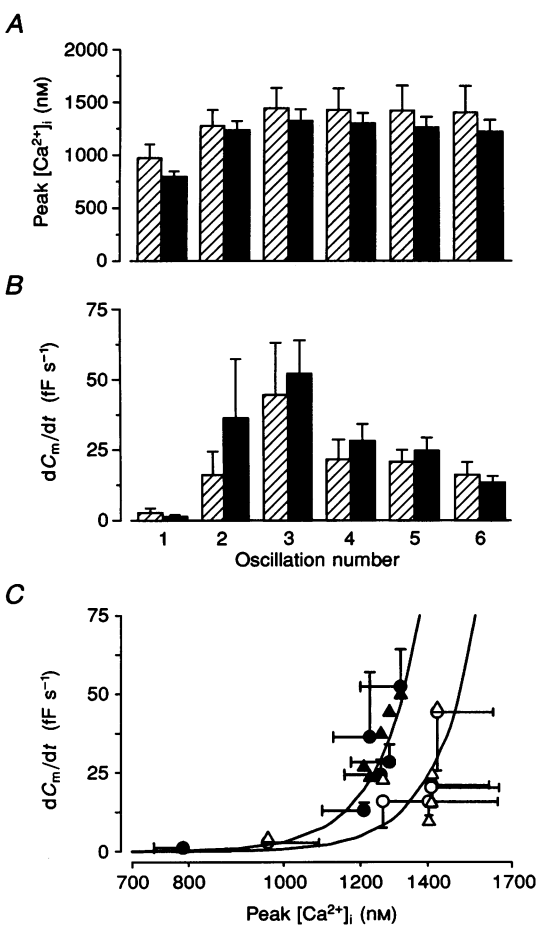

Figure 4. Comparison of the peak rates of secretion and peak $[Ca^{2+}]_i$

A, peak $[Ca^{2+}]_i$ reached during the first six oscillations stimulated by 1 nM GnRH in cells showing bursts of exocytosis (peak rates > 15 fF s^{-1}). ▨, means ± S.E.M. from cells cultured in ctFBS ($n = 8$); ■, values from cells cultured in E_2-ctFBS ($n = 11$). *B*, peak exocytotic rates measured during these same oscillations. *C*, plots of the data from *B vs.* the data in *A* (○, ctFBS-cultured cells; ●, E_2-ctFBS-cultured cells). The continuous lines are least-squares fits to eqn (10) with $A = 2{\cdot}66 \times 10^{-30}$ fF $(s\ \mu M^n)^{-1}$ and $n = 10{\cdot}02$ (E_2-ctFBS cells, $r = 0{\cdot}88$), or $A = 8{\cdot}24 \times 10^{-31}$ fF $(s\ \mu M^n)^{-1}$ and $n = 10{\cdot}00$ (ctFBS cells, $r = 0{\cdot}80$). The triangles are the simulated results of modelling the data. ▲, E_2-ctFBS cells: $S_2 = 1830$ fF; $S_1 = 415$ fF; $a_1 = 0{\cdot}15\ s^{-1}$; $a_3 = 0{\cdot}01\ \mu M^{-6}\ s^{-1}$; $b_1 = 1{\cdot}6\ \mu M$; $c_1 = 2$; $c_2 = 6$. The error bars have been omitted for the modelled data for clarity; they were of the same magnitude as for the experimental data. △, ctFBS cells: all constants were the same as for the E_2-ctFBS cells, except $b_1 = 7{\cdot}0\ \mu M$.

exocytotic response obtained experimentally. For these calculations the remaining model parameters were unchanged from those stated in the legend to Fig. 5. When we examined all the Ca^{2+} traces obtained in cells cultured in E_2-ctFBS, we found that a pool of 2245 fF ($S_2 = 1830$ fF and $S_1 = 415$ fF, i.e. 1122 vesicles) gave a mean exocytotic response (370 ± 78 fF) equivalent to the mean observed in the experiments (377 ± 44 fF; $n = 23$). When the E_2-ctFBS data were divided into the subset of cells that was used to develop the model (i.e. peak rates of exocytosis > 15 fF s^{-1}) and those cells not used, these parameters still gave exocytotic responses of similar amplitude to the experimental data (rates > 15 s^{-1}: simulations, 460 ± 128 fF *vs.* experimental, 369 ± 58 fF, $n = 11$; rates < 15 s^{-1}: simulations, 287 ± 93 fF *vs.* experimental, 384 ± 68 fF, $n = 12$). The validity of the model is further supported by the fact that the S.E.M. of the simulations is of the same order of magnitude as the experimental results. Additionally, using these parameters, the model also accurately predicted the Ca^{2+} dependency of the exocytotic response (▲, Fig. 4*C*).

Regarding cells cultured in the absence of steroid, as shown in Fig. 2, cells cultured in ctFBS gave almost 3-fold less secretion than the E_2-ctFBS-cultured cells. As suggested by the data in Fig. 4*C*, this smaller response could reflect a shift in the Ca^{2+} sensitivity of exocytosis. To examine this, we determined the effect of increasing the Ca^{2+} dissociation constant of the S_2 to S_1 transition (b_1) on the simulated exocytotic response. Indeed, when we increased b_1 from 1·6 to 7·1 μM, the model faithfully reproduced both the amplitude of the secretory response (simulations, 135 ± 40 fF *vs.* experimental, 128 ± 28 fF; $n = 19$) and the Ca^{2+} dependency of secretion observed in the ctFBS cells (△, Fig. 4*C*). The reduced response in the absence of added steroid could also reflect fewer available vesicles. Indeed, reducing the vesicle number 3-fold had a similar effect to increasing the value of b_1 (data not shown); however, the small effect of E_2 treatment on depolarization-evoked exocytosis (see below) suggested that a shift in Ca^{2+} sensitivity might be a more reasonable explanation of the effect of E_2 treatment.

Endocytosis

A number of cells, as illustrated by three of the cells in Fig. 1, showed very dramatic declines in C_m following the episodes of exocytosis. These decreases in cell surface area presumably reflect the retrieval of secretory vesicle membrane; nevertheless, many of the cells retrieved more membrane than was added to the cell surface during exocytosis. We could find no correlation between the type of $[Ca^{2+}]_i$ response and the amount of endocytosis, nor was

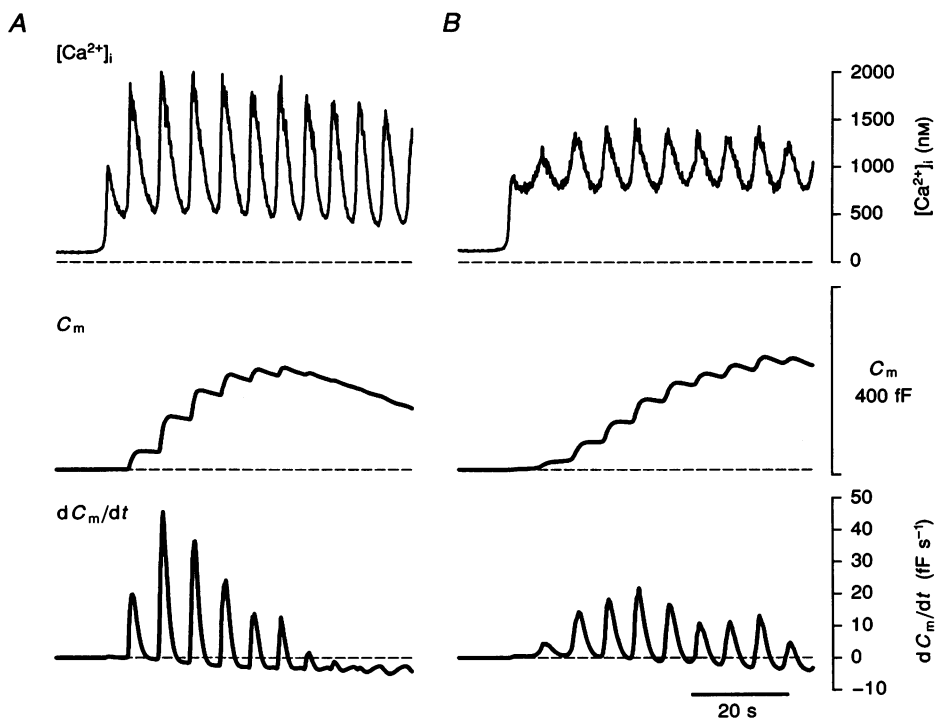

Figure 5. Simulations of GnRH-evoked secretion

The upper part of panel *A* shows the $[Ca^{2+}]_i$ recording from Fig. 3*A*, and the upper part of panel *B* is the $[Ca^{2+}]_i$ recording from Fig. 3*B*. The middle parts of each panel represent the simulated changes in C_m derived from the $[Ca^{2+}]_i$ signal shown above, and the lower parts show the time derivatives of these simulations. The constants used to derive the C_m and dC_m/dt traces in *A* were as follows: $S_2 = 387$ fF; $S_1 = 88$ fF; $a_1 = 0{\cdot}15\ s^{-1}$; $a_3 = 0{\cdot}01\ \mu M^{-6}\ s^{-1}$; $b_1 = 1{\cdot}6\ \mu M$; $c_1 = 2$; $c_2 = 6$. For the C_m and dC_m/dt traces in *B*, S_2 was increased to 774 fF and S_1 to 176 fF.

there a significant difference in the extent of endocytosis observed in cells cultured in ctFBS or in E_2-ctFBS (data not shown). In forty-four of the fifty-five cells giving a secretory response to GnRH, the C_m stabilized sufficiently following stimulation, so that we were able to recompensate the patch-clamp amplifier and confirm the change in cell surface area. Of the forty-four cells, only 16% returned to their pre-GnRH surface area (+0·1 ± 0·3% of initial surface area), 14% showed an increase in surface area (+4·5 ± 1·2%), while 70% of the cells were smaller (−10·5 ± 1·5%) than before the application of GnRH. It is not clear why the majority of cells should retrieve more membrane than that added by exocytosis. This observation may be due to a retrieval process that removes plasma membrane components as well as secretory vesicle membrane from the cell surface, or it may reflect an additional endocytotic process that occurs in parallel with secretory vesicle membrane retrieval.

Depolarization-evoked exocytosis

In addition to stimulating exocytosis with GnRH, we have also examined the effect of membrane depolarization on exocytosis. To open VOCCs and cause an influx of extracellular Ca^{2+}, gonadotrophs were briefly (1 s) depolarized to +20 mV; this was repeated five times in rapid succession. After depolarization, a GnRH exposure protocol was used to verify that the cell was indeed a gonadotroph. Figure 6 shows examples of the results of the depolarization protocol on a cell cultured either in ctFBS (Fig. 6*A*) or in E_2-ctFBS (Fig. 6*B*). As with GnRH stimulation, we could detect no significant differences between the depolarization-evoked peak $[Ca^{2+}]_i$ in ctFBS cells (1255 ± 127 nM, $n = 12$) and E_2-ctFBS cells (1166 ± 153 nM, $n = 12$). However, the total amount of exocytosis achieved in the E_2-ctFBS cells was significantly greater than that in the ctFBS cells (242 ± 41 *vs.* 175 ± 24 fF; $P < 0·05$), although the magnitude of the effect was markedly less than the 3-fold difference seen with GnRH stimulation.

We also modelled the depolarization-evoked secretory responses. We used the same number of vesicles ($S_1 + S_2 =$ 2245 fF) and the same rate constants as used previously for GnRH-evoked secretory responses of E_2-ctFBS cells (see legend to Fig. 5) together with the $[Ca^{2+}]_i$ responses evoked by depolarization in E_2-ctFBS cells. To adequately emulate the experimental results we found we had to decrease the Ca^{2+}-dissociation constant of the S_2 to S_1 transition (b_1) to 1 μM. Under these conditions the model predicted a total increase in C_m of 250 ± 70 fF; this value compares well with the increase in C_m observed experimentally. The same parameters, when applied to the ctFBS cells, overestimated the responses to depolarization obtained in these cells (not shown); however, we could obtain good agreement between the model (176 ± 57 fF) and the empirical results if we increased the Ca^{2+}-dissociation constant, b_1, to 1·8 μM.

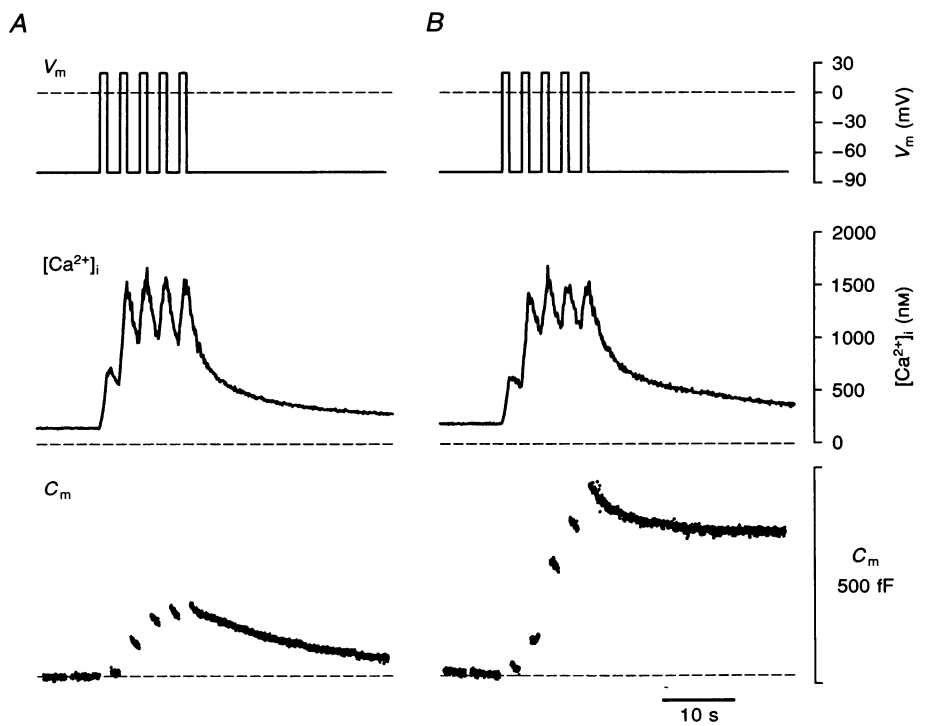

Figure 6. Depolarization-evoked secretion in individual gonadotrophs

Representative traces are shown from gonadotrophs that were depolarized five times (1 s each time) to +20 mV (holding potential, −80 mV). The middle panels show the $[Ca^{2+}]_i$ recordings, and the lower panels show the simultaneously acquired C_m traces. The cells were cultured for 3 days in ctFBS (*A*) or in E_2-ctFBS (*B*). The initial C_m of each cell was: *A*, 8·2 pF ($\tau_{RC} = 132$ μs); *B*, 6·3 pF ($\tau_{RC} = 134$ μs).

DISCUSSION

Agonist-stimulated, repetitive $[Ca^{2+}]_i$ oscillations have been observed in several cell types; nevertheless, direct evidence for the relevance of these oscillations to physiological function is sparse. Electrophysiological methods, such as patch-clamping and amperometry, are amongst the few techniques that have both adequate temporal resolution and the sensitivity to address this question. One of the first studies to provide evidence for a correlation between $[Ca^{2+}]_i$ oscillations and endocrine cell function was that of Tse *et al.* (1993) who, using the simultaneous measurement of $[Ca^{2+}]_i$ and C_m, were able to show that bursts of secretory activity in rat pituitary gonadotrophs can be driven by $[Ca^{2+}]_i$ oscillations. This study used the conventional whole-cell recording technique in which the cytosol is dialysed with the contents of the patch pipette, a condition known to lead in a time-dependent manner to the loss of substances crucial for continued exocytosis (Heinemann *et al.* 1993). Because of the potential for distortion of the relationship amongst the signalling and exocytotic components, the conventional whole-cell technique is of limited usefulness in the investigation of the physiological and endocrinological modulation of exocytosis.

We have adopted the perforated-patch configuration of the whole-cell recording configuration that avoids many of the shortcomings of the conventional whole-cell technique. Two advantages are particularly important for the study of $[Ca^{2+}]_i$ and exocytosis. First, except for the dialysis of monovalent ions, the cytosolic composition is affected little by the patch-clamp procedure, thus minimizing the procedure-induced, time-dependent changes of constituents with direct or modulatory roles in the exocytotic process. Second, the cytosolic Ca^{2+} buffering system remains intact. Thus the time-dependent oscillations in $[Ca^{2+}]_i$ will more closely represent, both qualitatively and quantitatively, those of the intact cell.

We have carried out experiments on gonadotrophs from ovariectomized rats using the perforated-patch technique to address the relationship between calcium oscillations and exocytosis. Furthermore, in order to gain more insight into the regulation of secretion in these cells, we have investigated both GnRH- and depolarization-evoked changes in $[Ca^{2+}]_i$ and exocytosis, together with the modulation of these processes by the steroid hormone E_2.

$[Ca^{2+}]_i$ oscillations

A previous study has called into question the relevance of $[Ca^{2+}]_i$ oscillations to secretory activity in pituitary gonadotrophs (Leong & Thorner, 1991). However, our results, and those of Tse *et al.* (1993), clearly demonstrate that such oscillations lead to exocytosis in these cells. Possibly the reverse haemolytic plaque assay used to monitor secretion in this previous study was not sensitive enough to detect secretion from the oscillatory cells.

The physiological advantage of oscillations over graded, sustained increases in $[Ca^{2+}]_i$ are a matter of some speculation. It has been suggested that oscillations with a constant amplitude provide an intracellular message with a much better signal-to-noise ratio than sustained increases of varying amplitude (Berridge, 1988). Additionally, oscillations may provide a way for the cell to optimize its physiological output while preventing desensitization or fatigue (Law, Pachter & Dannies, 1989). The manner in which this digital signal is converted into a cellular response is also a subject of debate. Berridge (1988) and Tsien & Tsien (1990) have proposed that information is transmitted in the timing of the oscillations (frequency encoding), whereas Meyer & Stryer (1991) have suggested that information is instead carried in the number of oscillations (digital encoding). In the model presented by Berridge (1988), increasing frequency extends the time that a protein spends in an active form and thus leads to a smoothly graded cellular response. Clearly, the bursting patterns of secretion that we have observed in gonadotrophs would not support such a model. On the other hand, in digital encoding, $[Ca^{2+}]_i$ spikes generate small cellular responses that sum together giving a response whose amplitude is thus dependent on the number of spikes in the train. Meyer & Stryer (1991) have proposed three different mechanisms by which spikes could be counted; they have termed these the 'solitary spike detector', the 'spike counter', and the 'calcium integrator'. Our experimental results most closely resemble the latter model. Indeed, the model we have used to simulate exocytotic activity is strikingly similar to the calcium integrator model (see Fig. 9 of Meyer & Stryer, 1991), e.g. the S_1 pool of vesicles may be considered equivalent to the activated target protein of their model and the S_2 pool could be equivalent to the inactivated form. However, one difference between our results and true digital encoding is that the response to individual Ca^{2+} oscillations is graded depending on the size of the Ca^{2+} spike (as opposed to an all-or-none phenomenon). Thus secretory activity is only partially digitally encoded, i.e. the extent of secretion is determined not only by the number of spikes but also by the amplitude of each Ca^{2+} oscillation (and the number of vesicles available for exocytosis at the time of the spike; see below).

We do not consider digital encoding to be an exclusive mechanism in transmitting the action of GnRH. One should bear in mind that GnRH activates multiple intracellular pathways subserving its pleiotropic actions in the gonadotroph, and that oscillations in $[Ca^{2+}]_i$ are observed to increase in frequency with increasing GnRH concentration. Thus $[Ca^{2+}]_i$ oscillations probably serve to transmit multiple signals within the cell, and these could be either frequency or digitally encoded.

Modelling of exocytosis

The patterns of $[Ca^{2+}]_i$ oscillations evoked in different cells by GnRH are extremely varied, and the secretory responses evoked by these oscillations are equally complex. Determining how exocytosis is related to the changes in $[Ca^{2+}]_i$ is therefore difficult, and understanding how modulators, such as E_2, affect this relationship is even more

problematic. To address these questions we have developed a simple model which suggests that the variability in the pattern of secretion can be explained solely on the basis of differences in the $[Ca^{2+}]_i$ profiles that are evoked by GnRH in individual cells. The ability of the model to emulate the empirical data clearly depends upon the assumption that the relationship between the rate of exocytosis and $[Ca^{2+}]_i$ is extremely steep. This assumption seems justified based upon the results shown in Fig. 4*C*; however, to our knowledge, such a steep relationship has not been observed previously in secretory cells. Implicit in our analysis is the presumption that the measured $[Ca^{2+}]$ is the concentration that drives exocytosis. Theoretical considerations of the spatial distribution of cytosolic $[Ca^{2+}]$ at varying distances from Ca^{2+} release sites (Smith, Wagner & Keizer, 1996) suggest that, when myo-inositol 1,4,5-trisphosphate (IP_3) receptor channels (IP_3Rs) are open, large $[Ca^{2+}]$ gradients are formed. Furthermore, these gradients are highly non-linear. Since Ca^{2+} stores (and presumably release sites) are not homogeneously distributed throughout the cytoplasm of gonadotrophs (see Fig. 1 of Tse, Tse & Hille, 1994), then the relationship between the spatially averaged (i.e. measured) $[Ca^{2+}]$ and the $[Ca^{2+}]$ at any defined point in the cytosol will be similarly non-linear. This disparity between the spatially averaged $[Ca^{2+}]$ and the local $[Ca^{2+}]$ will be most pronounced for regions in close vicinity (< 1 μm) to the release sites (see Fig. 2*A* of Smith, 1996). At these short distances, such spatial $[Ca^{2+}]$ gradients would be expected to dissipate less than 1 ms after the IP_3Rs close (Smith *et al.* 1996). However, the rise times of the Ca^{2+} oscillations in our experiments are between 300 and 500 ms. These slow rise times imply that during the rising phase of the oscillation, Ca^{2+} is being continuously released into the cytosol, a phenomenon that might lead to persistent $[Ca^{2+}]_i$ gradients over this 300–500 ms period. Thus the high power functions, both observed experimentally and required by the model, may be explained if there is a co-localization of Ca^{2+} release sites with exocytotic sites and if exocytosis occurs during periods when there are large spatial gradients in $[Ca^{2+}]_i$, i.e. during the rising phase of the oscillation (see Fig. 3*A*). Indeed, since the relationship between the local $[Ca^{2+}]$ and the spatially averaged $[Ca^{2+}]$ is dependent on diffusion of Ca^{2+}, and diffusion is a function of the square of the distance, then this contention may explain why a doubling of the power functions in the model adequately emulates the experimental results. Such a hypothesis is also consistent with a recent report that exocytosis in gonadotrophs is driven by localized increases in $[Ca^{2+}]_i$ (Tse, Tse, Hille, Horstmann & Almers, 1997).

The hypothesized dependence of exocytosis on localized increases in $[Ca^{2+}]_i$ may also account for some of the differences between the modelling results and the empirical data. Better modelling could be obtained by inclusion of a more detailed mathematical consideration of the spatial $[Ca^{2+}]_i$ gradients. However, such an approach would require knowledge of the relative locations of Ca^{2+} release sites and exocytotic sites, together with an estimate of the degree of loading of the stores and the number of IP_3 channels contributing to Ca^{2+} release at the individual sites, and this information is not yet available. Experimentally, measurement of the $[Ca^{2+}]_i$ with high temporal-resolution confocal imaging using low-affinity, fluorescent Ca^{2+} probes might give a better estimate of the $[Ca^{2+}]$ driving exocytosis.

Another feature that could contribute to the steepness of the Ca^{2+} dependency would be a limited availability of secretory vesicles, i.e. as the number of vesicles is exhausted by multiple oscillations, the rate of exocytosis will also decline. Indeed, accurate emulation of the experimental results required that we restrict the number of available secretory granules to less than 1200. In flash photolysis experiments in melanotrophs and chromaffin cells, apparently all of the vesicles morphologically docked to the plasma membrane can be exocytosed in three phases of secretion (Parsons, Coorssen, Horstmann & Almers, 1995). These phases of secretion have been interpreted to result from the fact that there are distinct pools of vesicles at three stages of readiness for exocytosis, i.e. not all morphologically docked vesicles are immediately prepared for exocytosis (Thomas *et al.* 1993). There is a small pool of 'readily releasable' vesicles (S_1) that, on raising $[Ca^{2+}]_i$, is released in a rapid burst of exocytosis. Secondly, there is a larger pool of vesicles (S_2) that is secreted in a phase that is delayed and slower than the exocytotic burst because these vesicles must pass through an additional reaction to reach S_1. Finally, there is a third pool of vesicles that can be recruited for secretion as long as $[Ca^{2+}]_i$ remains high. Vesicles in this latter pool represent about half of the docked vesicles and are secreted in an ultra-slow phase of exocytosis that is extremely temperature sensitive (Thomas *et al.* 1993). Because our experiments were carried out at room temperature, we have assumed that the vesicles in the latter pool would not play a significant part in the secretory response; therefore, our model only considers vesicles S_1 and S_2. The total number of vesicles (1122) in the combined S_1 and S_2 pools represents about half of the docked vesicles, and, therefore, the model predicts ~2244 docked granules in the gonadotrophs used for this investigation (~2·5 granules per square micrometre of cell surface). Considering the differences in secretory granule sizes, this value is comparable to the estimated number of docked vesicles in rat melanotrophs and bovine chromaffin cells (Parsons *et al.* 1995).

Effects of E_2

The secretory responses to GnRH were markedly affected by the steroid hormone background to which the cells had been exposed. Cells that had been incubated in the presence of E_2 for 2–4 days showed 3-fold more exocytosis than cells cultured in the absence of steroids. As observed previously (Ortmann, Stojilkovic, Cesnjaj, Emons & Catt, 1992*a*), incubation with E_2 had no discernible effect on the Ca^{2+} signal elicited by GnRH; thus E_2 must exert its effect at a step downstream of intracellular Ca^{2+} release. E_2 pre-

treatment also augmented depolarization-evoked exocytosis, but to a lesser extent (~38%), without affecting the stimulated increase in $[Ca^{2+}]_i$.

Previous studies on cell populations have shown a similar potentiating effect of E_2 on LH secretion (Turgeon & Waring, 1990; Ortmann, Tilse & Emons, 1992*b*); this effect depends in part on increased GnRH receptor (GnRH-R) expression (Emons *et al.* 1988), but is independent of any changes in LH synthesis (Ortmann *et al.* 1992*b*). There are several possibilities that either singly or in combination could account for the effect of E_2 on exocytosis. The apparent E_2-dependent increase in the Ca^{2+} sensitivity of exocytosis could be achieved at several steps in the exocytotic pathway. E_2 might promote secretory granule maturation and thus increase the number of vesicles that are competent of undergoing exocytosis, or E_2 might increase rate-limiting Ca^{2+}-dependent proteins, thus increasing the sensitivity of the secretory apparatus to $[Ca^{2+}]$. Alternatively, E_2 might stimulate synthesis of Ca^{2+}-independent proteins involved in the regulation of the secretory pathway and so increase the efficiency of stimulus–secretion coupling. An increase in vesicle number would be expected to affect the secretory response to GnRH and membrane depolarization similarly, and yet we observed that the magnitude of E_2 augmentation of GnRH- and depolarization-evoked exocytosis were markedly different. Therefore, an increase in the number of available vesicles cannot explain fully the effect of E_2 on exocytosis. The relatively minor effect of E_2 on depolarization-evoked secretion shows that expression of the major effect of E_2 on exocytosis depends upon GnRH-R occupation. Such a receptor-dependent effect is most easily explained by sensitization of components of the secretory apparatus that are targeted specifically by GnRH-R activation. Interestingly, we have observed a similar GnRH-R-dependent, steroid-induced shift in the Ca^{2+} sensitivity of exocytosis in the clonal gonadotroph LβT2 (Thomas *et al.* 1996).

One way in which E_2 might change the Ca^{2+} sensitivity of the secretory apparatus in a GnRH-R-dependent manner would be by up-regulating other receptor-activated signalling pathways. Protein kinase C (PKC) plays an important role in the control of secretion in gonadotrophs (Stojilkovic, Iida, Merelli, Torsello, Karsmanovic & Catt, 1991), and this kinase has been demonstrated to increase the Ca^{2+} sensitivity of exocytosis in other systems (Knight & Baker, 1983; Bittner & Holz, 1993; Gillis, Mössner & Neher, 1996). Furthermore, one report has also proposed that, under certain circumstances, the levels of PKC in gonadotrophs might be increased by E_2 (Drouva, Gorenne, Laplante, Rérat, Enjalbert & Kordon, 1990). Thus the diacylglycerol/PKC arm of the GnRH-R-activated signalling pathway could be more active in E_2-treated cells and so enhance Ca^{2+}-dependent exocytosis. Our simulations of exocytotic responses also support the hypothesis that a GnRH-dependent change in Ca^{2+} sensitivity might underlie the potentiating effect of E_2 on GnRH-evoked exocytosis (see Figs 4*C* and 5*A*), although modelling does not establish this as a specific mechanism. The data could equally well be explained by modelling an E_2-dependent 3-fold increase in vesicle availability (data not shown). Still, the much greater effect of E_2 on GnRH-evoked exocytosis suggests a pathway-specific change in vesicle availability. One reasonable hypothesis that would impart GnRH specificity would be that E_2 treatment causes a preferential colocalization of docked-secretory vesicles with IP_3-sensitive stores.

A complicating factor in these studies is that C_m measurements only monitor the net change in cell surface area, and so reliable determination of exocytotic activity depends upon the temporal separation of exo- and endocytotic activity. Clearly, in our experiments the later parts of the C_m traces can be dominated by membrane internalization (see Fig. 1). Such activity precludes accurate estimation of the secretory activity that may be ongoing during this period; if E_2 potentiates the slower secretory phases, its effects could be obscured by vigorous endocytotic activity. The excess retrieval sometimes occurs after a pronounced delay (see Fig. 1*B*). Additionally, it is not obviously Ca^{2+} dependent, with much of the retrieval often occurring when $[Ca^{2+}]_i$ has returned almost to baseline (see Fig. 1*D*). Such properties make modelling this phenomenon somewhat problematic; however, since our modelling of exocytosis has concentrated on the early portion of the secretory response, we have included in our model an endocytotic component that only retrieves the membrane added to the cell surface by exocytotic activity. Modelling of the excess retrieval, which is not unique to gonadotrophs (Artalejo, Henley, McNiven & Palfrey, 1995), will obviously require further information on the mechanisms responsible for this novel internalization process.

Ca^{2+} dependency

The term 'Ca^{2+} sensitivity' as it relates to the modelling studies warrants some consideration. As discussed above, the assumption that the measured $[Ca^{2+}]_i$ is the concentration that drives exocytosis seems unlikely. Thus the Ca^{2+} affinities used in the simulations must reflect not only the inherent Ca^{2+} sensitivities of the proteins that regulate exocytosis but also the relationship between the measured $[Ca^{2+}]_i$ and the actual $[Ca^{2+}]$ at the site of exocytosis. Of particular interest is the fact that the model requires that the Ca^{2+} affinity of exocytosis be higher for depolarization- than for the GnRH-evoked responses, i.e. experimentally, for the same spatially averaged $[Ca^{2+}]_i$, there is more exocytosis when Ca^{2+} enters through VOCCs than when Ca^{2+} is released from intracellular stores. Assuming there is no difference in the Ca^{2+} sensitivity of exocytosis under the two stimulation conditions, this observation would imply that, for a given spatially averaged $[Ca^{2+}]_i$, the $[Ca^{2+}]$ at the site of exocytosis is higher when Ca^{2+} enters the cytosol through VOCCs than through IP_3Rs. This observation may be explained either by the fact that release of Ca^{2+} from

intracellular stores leads to a more uniform elevation of $[Ca^{2+}]_i$ (and thus the measured $[Ca^{2+}]$ is a closer estimate of the $[Ca^{2+}]$ at the site of exocytosis) or that VOCCs are closer than IP_3Rs to secretory vesicles. This latter possibility also underscores the fact that the Ca^{2+} sensitivity used in the model is dependent upon the relative localities of exocytotic and Ca^{2+} release sites. Indeed, the observation that in some cells small amplitude $[Ca^{2+}]_i$ oscillations led to large C_m increases whereas in other cells large amplitude oscillations led to small exocytotic responses, suggests that either the Ca^{2+} sensitivity of the secretory apparatus varies greatly or, more likely, the proximity of Ca^{2+} stores and exocytotic sites differs from cell to cell. Likewise, the hypothesized effect of E_2 on the Ca^{2+} sensitivity of exocytosis could be brought about, not only by a change in the inherent Ca^{2+} affinity of a protein that controls exocytosis, but also by structural changes in the cell that alter the proximity of exocytotic and Ca^{2+} release sites. For the increase in Ca^{2+} sensitivity that is dependent on GnRH, however, such a structural change either must have no effect on location of exocytotic sites relative to VOCCs or must occur within seconds of receptor occupation.

In summary, the GnRH-induced exocytosis in single gonadotrophs is characterized by a wide spectrum of responses, ranging from exocytotic bursts to relatively slow, graded increases that are dependent on the evoked intracellular Ca^{2+} pattern. A kinetic model is presented that incorporates the observed steep dependence of exocytosis on measured intracellular $[Ca^{2+}]$; simulated exocytosis reasonably approximated observed exocytotic responses, both kinetically and quantitatively. The model also suggests that the modulatory effects of E_2 are brought about either by a change in the Ca^{2+} sensitivity of exocytosis or by a preferential clustering of docked-secretory granules close to sites of Ca^{2+} release. The results suggest that in gonadotrophs, an oscillatory Ca^{2+} signal is sensed by the exocytotic apparatus in a modified form of digital encoding. Furthermore, exocytosis in E_2-treated cells showed that maximum expression of the effect of this steroid on exocytosis requires activation of GnRH-dependent pathways.

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