Rapid transient elevations of cytosolic calcium triggered by thyrotropin releasing hormone in individual cells of the pituitary line GH₃B₆

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The kinetic features of the changes in the cytosolic free Ca²⁺ concentration, [Ca²⁺]_i, following stimulation by thyrotropin releasing hormone (TRH) were analysed in single cells of a pituitary line (GH₃B₆) by dual excitation microfluorimetry [Tsien, Rink & Poenie (1985) *Cell Calcium* 6, 145–157], using fura 2 as intracellular Ca²⁺ probe. Two phases were observed: initially, [Ca²⁺]_i is raised in a single rapid transient to a maximum averaging 8.0 μM, and in a second phase TRH causes a series of rapid [Ca²⁺]_i oscillations with maxima around 1.0 μM, which are probably due to the enhanced firing of action potentials. TRH triggers both phases independently, i.e. it can elicit either the first or the second phase exclusively. This is also the case in those cells in which [Ca²⁺]_i undergoes rhythmic oscillations due to the firing of spontaneous action potentials [Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wolheim & Dufy (1987) *Nature* (London) 329, 719–721]. The sudden onset of the first phase of TRH action on [Ca²⁺]_i shows that Ca²⁺ mobilization due to enhanced production of inositol phosphates may occur as rapidly as the firing of action potentials, i.e. in the ms time range. Due to a marked response type heterogeneity and to the randomness of the rapid events, previous monitoring of [Ca²⁺]_i in cell populations had misleadingly suggested small and maintained changes due to TRH. It is concluded that stimulatory regulation of secretion is provided by the generation of rapid [Ca²⁺]_i transients, the frequency of which determines secretory rate. Furthermore, it is demonstrated that the regulation of [Ca²⁺]_i by hormones and neurotransmitters in pituitary and many other cell types will have to be studied at the single cell level in order to appreciate its role in cell activation.

INTRODUCTION

During the last years, our understanding of the mechanism of action of thyrotropin releasing hormone, TRH, has advanced markedly (for recent review, see [1,2]). Like many other hormones, TRH initially stimulates the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate [3–6], leading to the enhanced generation of two intracellular second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] [7] and 1,2-diacylglycerol [8], the former causing the mobilization of Ca²⁺ [9,10], the latter the stimulation [11] and possibly the redistribution [12,13] of protein kinase C [14]. Hence a rise in the cytosolic free Ca²⁺ concentration, [Ca²⁺]_i, and the concomitant activation of protein kinase C are currently thought to cause cell activation.

Recently, methods have been developed which allow the monitoring of [Ca²⁺]_i in individual small cells [15,16]. From the single cell work so far available, it has become obvious that sizeable rapid changes in [Ca²⁺]_i occur randomly in individual cells [17–19]. For pituitary cells which are, like many other endocrine cells, electrically excitable, we have recently demonstrated that the spontaneous firing of action potentials creates [Ca²⁺]_i transients of a magnitude sufficient to trigger [Ca²⁺]_i-dependent cell activation [20]. These shortlived events

are not apparent when monitoring 'average' [Ca²⁺]_i in populations of GH₃ cells [21–23]; yet, their abolition by somatostatin [20] provides a straightforward explanation for the effects of this and possibly other inhibitory factors to reduce basal hormone secretion.

The impact of the single cell measurements for the elucidation of inhibitory regulation of hormone secretion prompted us to investigate the real kinetics of the alterations of [Ca²⁺]_i in pituitary cells in response to a stimulatory hormone. To this end, TRH effects at maximal dose on [Ca²⁺]_i were analysed in individual cells of the pituitary line GH₃B₆. The present paper describes the rapid randomly occurring alterations in [Ca²⁺]_i associated with stimulation of pituitary cells by this hypothalamic releasing factor.

EXPERIMENTAL

GH₃B₆ cells (kindly provided by Dr. D. Gourdj, Paris, France), a subclone of the pituitary line GH₃ [24], were cultured on glass coverslips with HamF10 culture medium supplemented with 15% horse and 2.5% fetal calf serum for 3–5 days, loaded with fura 2 by exposure to 1 ml of 2 μM-fura 2/AM in HEPES-buffered (pH 7.4) Krebs–Ringer bicarbonate buffer, KRB (for composition see [25]) with 0.1% bovine serum albumin for 30 min at

Abbreviations used: TRH, thyrotropin releasing hormone; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; Ins(1,4,5)P₃, myo-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P₄, myo-inositol 1,3,4,5-tetrakisphosphate.

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37 °C, washed and maintained in the same buffer at room temperature prior to the fluorescence measurements. This procedure results in an intracellular fura 2 concentration of between 10 μ M and 50 μ M, estimated from comparison with the loading via a patch pipette [20]. Dual excitation microfluorimetry was performed essentially as described previously [16, 26] on an inverted microscope (NIKON) in the epifluorescence mode (objective NIKON F100, λ_{crit} of the dichroic mirror 400 nm); monochromatic excitation light alternating rapidly (50 Hz) between λ_1 350 nm and λ_2 380 nm was provided by a SPEX fluorimeter (Glen Creston, London, U.K.). Fluorescence of single cells is monitored through a pinhole diaphragm slightly larger than the cells and an interference filter (λ_c 500 nm) by a photon counting tube, photons being sampled in separate channels for the two excitation wavelengths with a time constant of 100 or 200 ms. During the $[\text{Ca}^{2+}]_i$ monitoring, the cells are kept at 37° and continuously superfused with KRBH; TRH was injected from a 500-fold concentrated solution into the superfusion chamber to yield 10^{-7} M final concentration. From the ratio $R = F_{350}/F_{380}$, the cytosolic calcium was calculated using the formula derived previously [27]:

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

Calibration constants ($R_{\min.}$, $R_{\max.}$, β) were determined in the same experimental setup in separate experiments with fura 2 loaded GH_3B_6 cells exposed to the calcium ionophore ionomycin to allow equilibration of $[\text{Ca}^{2+}]_i$ with ambient $[\text{Ca}^{2+}]$ kept at either below 10 nM ($R_{\min.}$) or at 5 mM ($R_{\max.}$); β , i.e. the ratio between fluorescence at 380 nm at minimal or maximal $[\text{Ca}^{2+}]_i$, was monitored during a change between either of these conditions, correcting for eventual loss of fura 2 (evident from the fluorescence measured simultaneously at the crossover

wavelength, 360 nm); K_d was 225 nM, taken from previous work [27]. $R_{\min.}$, $R_{\max.}$, and β averaged 0.42 ± 0.03 ($\pm$ S.E.M., $n = 24$), 4.3 ± 0.3 ($n = 24$) and 4.7 ± 0.3 ($n = 17$), respectively, and were distinct from the corresponding constants obtained from calibration solutions [26].

RESULTS

The real kinetics of the biphasic $[\text{Ca}^{2+}]_i$ response to TRH

In Fig. 1, a biphasic response to TRH in a single GH_3 cell is presented. Following the addition of the stimulus there is a rapid rise in $[\text{Ca}^{2+}]_i$, then the cell recovers to prestimulatory level. After a certain period there is a second phase in the response, characterized by repetitive transient elevations of $[\text{Ca}^{2+}]_i$. As is shown in more detail below, the $[\text{Ca}^{2+}]_i$ spike in the first phase has characteristic rapid kinetics of onset; the duration of the first phase, which depends on the maximal $[\text{Ca}^{2+}]_i$ reached and the speed of recovery, is variable such that, in some cells, basal $[\text{Ca}^{2+}]_i$ is reached within seconds whereas, in others, $[\text{Ca}^{2+}]_i$ has not completely returned to basal levels prior to the initiation of the second phase.

The oscillations of $[\text{Ca}^{2+}]_i$ in the second phase are likely to represent the effects of electrical activity triggered by TRH [28, 29]. As we have shown previously by combining electrophysiological recording with the monitoring of $[\text{Ca}^{2+}]_i$ [20], spontaneous action potential firing in cells of the same line leads to $[\text{Ca}^{2+}]_i$ transients characterized by a rapid onset, shortlived maxima and a return to prespike level within seconds. The oscillations in the second phase of TRH action share these features (Fig. 1, insert). Due to the enhanced frequency of these transients during the second phase of TRH action,

Fig. 1. TRH-induced biphasic change in cytosolic free $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was monitored in a single cell of the pituitary line GH_3B_6 by dual excitation microfluorimetry using fura 2 as intracellular Ca^{2+} probe as described in the Experimental section. Shown is the ratio of fura 2 fluorescence (F_{350}/F_{380}) calibrated for $[\text{Ca}^{2+}]_i$. TRH, added at the time indicated by the arrow to yield 0.1 μ M final concentration, elicited a rapid transient elevation of $[\text{Ca}^{2+}]_i$ immediately following its addition (first phase) and a series of rapid $[\text{Ca}^{2+}]_i$ transients after 60 s (second phase). The insert shows the beginning of the second phase in more detail.

$[Ca^{2+}]_i$ may not always return to prespike levels between oscillations. Duration and intensity of the second phase are variable; usually there is a gradual increase in spiking frequency as in the example shown in Fig. 1, and spiking can continue for as long as several minutes; thereafter, cells return to the silent state or the spontaneous spiking frequency (see below) recorded prior to TRH stimulation.

The first and second phases of TRH action can be triggered independently

When analysing $[Ca^{2+}]_i$ in individual rat lactotrophs in primary culture, we have encountered a marked heterogeneity in the type of responses to TRH and dopamine [30]. As is illustrated in Fig. 2, response type heterogeneity is also observed in the clonal cell line GH₃B₆. Indeed, it was found that the responsive cells (92%) react with three distinct patterns to maximal TRH stimulation, showing either both phases as shown in Fig. 1 (24%), only the first phase as in Fig. 2(a) (25%), or only the second phase as in Fig. 2(b) (43%). The second phase is thus more likely to be observed than the first phase. The different response patterns can be well distinguished given the significant ($P < 0.001$) difference in the lag time between TRH addition and response, which averaged 4.8 ± 1.2 s ($\pm$ S.E.M., $n = 29$) and 28.1 ± 3.7 s ($\pm$ S.E.M., $n = 23$) for first and second phase, respectively. Independent triggering of the two phases of TRH action on $[Ca^{2+}]_i$ in pituitary cells suggests that TRH activates distinct pathways of signal transduction during each phase.

Fig. 2. Independent triggering of first and second phase of TRH action

$[Ca^{2+}]_i$ in individual GH₃B₆ cells which respond to TRH stimulation with either the first phase (panel a) or the second phase (panel b). Experimental conditions as in Fig. 1; for the relative abundance of the response types displayed in Figs. 1 and 2 we refer to the text.

TRH response in spontaneously spiking GH₃ cells

In 24 cells TRH was added while $[Ca^{2+}]_i$ was oscillating, due to spontaneously occurring action potentials [20]. An example of such a reaction is shown in Fig. 3. Again two types of reactions were observed. In Fig. 3(a) it is shown that some cells responded with a first phase, which rapidly produced a massive rise in $[Ca^{2+}]_i$; in this instance, cells were transiently inactivated, due probably to the hyperpolarization following TRH action described previously [22,31]. When the spiking resumed, its frequency was markedly increased when compared with the frequency prior to TRH addition. A second type of response is illustrated in Fig. 3(b), namely that some cells did not display a first phase response, but increased the spiking frequency, indicating the onset of the second phase of TRH action.

The frequency of $[Ca^{2+}]_i$ oscillations occurring spontaneously averages 6.4 ± 0.6 ($\pm$ S.E.M., $n = 24$)/min, whereas after TRH action the average is 18.7 ± 2.0 /min ($\pm$ S.E.M., $n = 24$), a highly significant ($P < 0.0001$) difference. Taken together, the evidence from spontaneously active cells underlines that the two phases are triggered independently; furthermore, it is shown that spontaneous $[Ca^{2+}]_i$ spiking is not affecting the nature of the TRH responses.

The magnitude of the $[Ca^{2+}]_i$ rise in both phases is sufficient to stimulate secretion

Data on a large number of $[Ca^{2+}]_i$ responses to TRH are summarized in Table 1. It can be seen that the fluctuations in $[Ca^{2+}]_i$ during both phases of TRH action

Fig. 3. TRH response in cells spontaneously oscillating in $[Ca^{2+}]_i$

TRH ($0.1 \mu\text{M}$ final concentration) was added to cells undergoing oscillations in $[Ca^{2+}]_i$ due to spontaneous action potentials [20]. Experimental conditions as in Fig. 1. Shown is an example of a cell displaying both first and second phase (panel a) and a cell which shows only the second phase of the TRH response (panel b).

Table 1. Peak $[Ca^{2+}]_i$ during first and second phases of TRH action in GH_3B_6 cells

This Table summarizes all observations; no significant differences in the peak $[Ca^{2+}]_i$ was found between the different response types depicted in Figs. 1–3.

	Ratio F_{350}/F_{380} (mean $\pm$ S.E.M.)	$[Ca^{2+}]_i$ (μM) (mean, 95 % confidence limits)
First phase ($n = 29$)	3.88 ± 0.32	8.7 (4.5–40)
Second phase ($n = 38$)	2.39 ± 0.15	1.1 (0.9–1.3)

permit calcium-dependent cell activation. The $[Ca^{2+}]_i$ dependency for prolactin secretion determined in permeabilized GH_3 cells shows that stimulation occurs over the range of $0.1 \mu M$ – $10 \mu M$ free $[Ca^{2+}]_i$ [32]. The maximum in $[Ca^{2+}]_i$ reached during the first phase described here ($8 \mu M$) is thus close to the $[Ca^{2+}]_i$ required for full stimulation of secretion; moreover, the average maximal $[Ca^{2+}]_i$ reached during the second phase ($1 \mu M$) is well above the $[Ca^{2+}]_i$ (approx. $0.6 \mu M$) required for half-maximal stimulation of secretion in the absence of any other factor [32].

Speed of onset of the first phase: a threshold for Ca^{2+} mobilization?

Monitoring fura 2 fluorescence from the whole cell implies that the diffusion of Ca^{2+} in the cytosol has to be complete before the maximum change in signal can be seen. The speed of the onset of the $[Ca^{2+}]_i$ response is thus determined by the diffusion of Ca^{2+} after the mobilizing event. This was nicely demonstrated for the transients due to action potentials: enhanced Ca^{2+} influx during 5–15 ms causes a transient which has an onset time of around 400 ms. The mean time for the transition from prestimulatory levels to maximum $[Ca^{2+}]_i$ during the first phase of the response to TRH is also very short; it averages 840 ± 70 ms ($\pm$ S.E.M., $n = 29$) and is hence only twice as long as the corresponding transition time observed upon the firing of action potentials [20], despite the fact that the peak $[Ca^{2+}]_i$ reached is 10-fold higher. It can thus be concluded that the Ca^{2+} mobilization during the first phase of TRH action could occur within a very short time period, similar to the action potential duration, i.e. in the ms time range.

The speed of onset of the first phase of TRH action is the same irrespective of the lag time between the addition of the stimulus and the onset of the response (Fig. 4). This lag is determined in part by the time required to build up a sufficient concentration of TRH at the cell under study, and is variable due to the experimental setup. It is striking that the speed of onset reflected by the slope during the upswing in the traces in Fig. 4 in a cell which responded within the first second (first trace) is identical to the onset speed of a cell which reacts only after 5 s (middle trace), a circumstance reflecting a slow buildup of the TRH concentration in the vicinity of the cell. It thus appears that the first phase of TRH action is not triggered unless a 'critical' level of stimulation is reached; once started, this part of the response proceeds with high and uniform speed. These features indicate strongly the presence of a threshold for Ca^{2+} mobilization

Fig. 4. Onset speed of the TRH response

$[Ca^{2+}]_i$ monitored in single GH_3B_6 cells which reacted after various lag times (trace 1, 1 s; trace 2, 5 s; trace 3, 25 s) following the addition of TRH (arrow, $0.1 \mu M$ final concentration).

during the first phase of the response to TRH; potential sites of such a threshold in the signal transduction pathway for TRH will be discussed below.

DISCUSSION

The introduction by Tsien *et al.* of fluorescent intracellular Ca^{2+} probes [33] marked the beginning of a new era in the studies concerning the regulatory role of $[Ca^{2+}]_i$, since these markers allowed direct monitoring of the key variable in small mammalian cells. Thanks to further progress made by the same group [16,27], fluorescent dyes now permit monitoring of $[Ca^{2+}]_i$ at the single cell level. This may prove to be as important as the initial description of quin 2, since monitoring of $[Ca^{2+}]_i$ in individual cells can now reveal the kinetics of the changes due to external stimuli (e.g. this study) or electrical activity [20].

For the stimulation of pituitary cells with TRH, the difference between the data reported here and what was observed previously on cell populations is indeed very striking. In studies using mainly quin 2 as a probe for $[Ca^{2+}]_i$ in populations of GH_3 cells, the response to TRH has been described as a biphasic elevation of $[Ca^{2+}]_i$, composed of a first phase in which maxima of 0.3 – $0.6 \mu M$ were reached and which were slowly reduced, either via a 'nadir' or directly to a new maintained 'steady state' $[Ca^{2+}]_i$, slightly above prestimulatory levels [1,2,22,23]. The data presented above show that the real changes observed at the single cell level are very different. As is illustrated in Fig. 5, this discrepancy is the consequence of the averaging of the changes occurring randomly in the population: the summation of the alterations occurring in the individual cells with distinct response types results in a fluorescence trace in which none of the real kinetic features (speed of onset, short duration of the first phase and oscillatory pattern in the second phase) are reflected. In addition, monitoring of cell populations leads to marked underestimation of the magnitude of the changes in $[Ca^{2+}]_i$. Thus, the first conclusion from this study is that the analysis of $[Ca^{2+}]_i$ regulation in cell populations can yield misleading information.

The most striking feature of the TRH response in

Fig. 5. Averaged information on $[Ca^{2+}]_i$ resulting from monitoring $[Ca^{2+}]_i$ in a cell population does not reflect kinetics and magnitude of the response to TRH

GH_3B_6 cells respond to TRH with distinct types of alterations of $[Ca^{2+}]_i$ shown in Figs. 1–3 and depicted schematically here (panels *a–e*). In panel *f*, a fluorescence trace monitored from a monolayer of GH_3B_6 cells in a cuvette ($\lambda_{ex.} = 340$ nm; $\lambda_{em.} = 505$ nm) loaded with fura 2 as described in the Experimental section is shown. It can be observed that the apparent change in $[Ca^{2+}]_i$ is small but maintained; similar results are obtained when monitoring $[Ca^{2+}]_i$ with quin 2 in suspensions of $(1-3) \times 10^6$ cells/ml [21–23]. This results from the averaging of heterogeneous responses consisting of rapid $[Ca^{2+}]_i$ transients with random onset occurring in individual cells.

GH_3B_6 cells is that $[Ca^{2+}]_i$ is raised in rapid transients during both phases of the response. This has several implications. In view of the large Ca^{2+} -binding capacity of the cytosol the most efficient way to raise $[Ca^{2+}]_i$ beyond a certain threshold is by a rapid event (Ca^{2+} influx or mobilization); in this manner, the signalling can take place with minimal quantities of Ca^{2+} , all of which has eventually to be extruded or exported by energy-requiring mechanisms. The control of Ca^{2+} -dependent intracellular processes (secretion, protein synthesis, movement, etc.) can in theory be exerted by the regulation of the steady state $[Ca^{2+}]_i$ level or by the frequency of rapid $[Ca^{2+}]_i$ transients. We have demonstrated previously that

frequency regulation is likely to control basal secretion [20]; the present data suggest strongly that stimulation of secretion is also based on increased frequency of rapid $[Ca^{2+}]_i$ transients. The principle of regulating cellular activities by short transient elevations of $[Ca^{2+}]_i$ has been demonstrated previously: using a Ca^{2+} -sensitive photoprotein, aequorin, it was shown that activation of hepatocytes with various agonists leads to oscillations of $[Ca^{2+}]_i$, the frequency of which is dependent on hormone dose [18,34].

Distinct molecular mechanisms are involved in the generation of rapid $[Ca^{2+}]_i$ transients in the first and second phase of TRH action. During the latter phase, a

rapid surge in Ca^{2+} influx is achieved by the concerted opening of voltage-dependent Ca^{2+} channels during the action potentials [20], the frequency of which is enhanced by TRH [28,29] via mechanisms possibly involving the protein kinase C pathway [35]. Hence, endocrine cells use the molecular machinery of electrical excitation for the purpose of intracellular signalling. The generation of the rapid transient during the first phase is more difficult to envisage: shortly after TRH addition, pituitary cells are hyperpolarized [22,30,35] and voltage-dependent Ca^{2+} channels are thus closed, such that the Ca^{2+} mobilized must be derived from intracellular stores or from a yet undefined Ca^{2+} influx pathway. $\text{Ins}(1,4,5)\text{P}_3$ [7,9,10] and possibly $\text{Ins}(1,3,4,5)\text{P}_4$ [36] have been recognized to play a central role in these early events. The sudden onset of the first phase could be due to a rapid onset of production and/or action of these inositol phosphate isomers. Receptor activation of phospholipase C has never been studied in single cells; the kinetics of onset of the response to $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ microinjected into individual cells are not known. It can thus at present not be decided at which level the 'pacemaker' function is exerted. Based on a comparison of the transients generated with various agonists in hepatocytes it was suggested that the stimulation of phospholipase C would be the step with a threshold to be passed prior to proceeding rapidly to full activation [34]. Feedforward mechanisms potentially contributing to the early phase include Ca^{2+} activation of phospholipase C [37,38] and of $\text{Ins}(1,4,5)\text{P}_3$ kinase [38–40], as well as the synergistic action of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ [41]. It is thus possible to envisage that the events at early times following hormone stimulation occur at a speed comparable to the opening and closing of ion channels, i.e. that the cascade of events resulting in Ca^{2+} mobilization is complete within a few ms. Analysis of inositol phosphates in a population of GH cells with rapid mixing techniques [42] yielded a slow onset of $\text{Ins}(1,4,5)\text{P}_3$ production which did not precede the increase in the breakdown product $\text{Ins}(1,4)\text{P}_2$. As is the case for the monitoring of $[\text{Ca}^{2+}]_i$, the analysis of rapid events with random onset in a population of cells may be misleading and unable to reflect the actual changes. Thus, we would suggest that the speed and random onset of receptor stimulation of inositol phosphate production preclude its analysis in the GH model system.

Termination of the $[\text{Ca}^{2+}]_i$ transients in the second phase is provided by the activation of a Ca^{2+} -dependent K^+ current causing a transient hyperpolarization and thus the closing of voltage-gated Ca^{2+} channels. Ca^{2+} mobilization by $\text{Ins}(1,4,5)\text{P}_3$ may be stopped by negative feedback mechanisms, for which there is a wide range of possibilities, and/or by exhaustion of substrate for phospholipase C or of the mobilizable Ca^{2+} pool. Both in the first and the second phase $[\text{Ca}^{2+}]_i$ transients, the reduction in $[\text{Ca}^{2+}]_i$ probably involves extrusion as well as transfer to the mitochondria [10].

It has been known for a long time that pituitary hormone secretion is a discontinuous phenomenon: hormones are released in bursts at various intervals. This allows raising of peripheral levels beyond a certain threshold with minimal quantities of hormones secreted. In addition it facilitates feedback control. The present paper provides the correlate of discontinuous secretion at the cellular level. It raises the question of the internal organization of the pituitary which will optimize regu-

lation of discontinuous secretion by, e.g., coupling and co-ordination of individual pituitary cells.

The authors are indebted to Ms. I. Puiz and Ms. I. Hritier for excellent technical and secretarial assistance. We thank Drs. Gaston R. Zahnd and Claes B. Wollheim for helpful discussions. This work was supported by the Fonds National Suisse (grant Nr. 3.824-0.86) and by the Fondation Carlos and Elsie de Reuter.

REFERENCES

1. Drummond, A. H. (1986) *J. Exp. Biol.* **124**, 337–358
2. Gershengorn, M. C. (1986) *Annu. Rev. Physiol.* **48**, 515–526
3. Rebecchi, M. J. & Gershengorn, M. C. (1983) *Biochem. J.* **216**, 287–294
4. Martin, T. F. J. (1983) *J. Biol. Chem.* **258**, 14816–14822
5. Schlegel, W., Roduit, C. & Zahnd, G. R. (1981) *FEBS Lett.* **168**, 54–60
6. MacPhee, C. H. & Drummond, A. H. (1984) *Mol. Pharmacol.* **25**, 193–200
7. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
8. Nishizuka, Y. (1983) *Trends Biochem. Sci.* **8**, 13–16
9. Gersengorn, M. C., Geras, E., Purrello, V. S. & Rebecchi, M. J. (1984) *J. Biol. Chem.* **259**, 10675–10681
10. Biden, T. J., Wollheim, C. B. & Schlegel, W. (1986) *J. Biol. Chem.* **261**, 7223–7229
11. Drust, D. S. & Martin, T. F. J. (1984) *J. Biol. Chem.* **259**, 14520–14530
12. Drust, D. S. & Martin, T. F. J. (1985) *Biochem. Biophys. Res. Commun.* **128**, 521–537
13. Fearon, C. W. & Tashjian, A. H., Jr. (1985) *J. Biol. Chem.* **260**, 8366–8371
14. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979) *J. Biol. Chem.* **254**, 3692–3695
15. Cobbold, P. H., Cuthbertson, K. S. R., Goyns, M. H. & Rice, V. (1983) *J. Cell Sci.* **61**, 123–136
16. Tsien, R. Y., Rink, T. J. & Poenie, M. (1985) *Cell Calcium* **6**, 145–147
17. Cuthbertson, K. S. R. & Cobbold, P. H. (1985) *Nature (London)* **316**, 541–542
18. Woods, N. V., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602
19. Poenie, M., Alderton, J., Tsien, R. Y. & Steinhardt, R. A. (1985) *Nature (London)* **315**, 147–150
20. Schlegel, W., Winiger, B. P., Mollard, P., Vacher, P., Wuarin, F., Zahnd, G. R., Wollheim, C. B. & Dufy, B. (1987) *Nature (London)* **329**, 719–721
21. Gershengorn, M. C. & Thaw, C. (1983) *Endocrinology (Baltimore)* **113**, 1522–1524
22. Schlegel, W. & Wollheim, C. B. (1984) *J. Cell Biol.* **99**, 83–87
23. Albert, P. R. & Tashjian, A. H., Jr. (1984) *J. Biol. Chem.* **259**, 5827–5832
24. Tashjian, A. H., Jr. (1979) *Methods Enzymol.* **58**, 527–535
25. Schlegel, W., Wuarin, F., Wollheim, C. B. & Zahnd, G. R. (1984) *Cell Calcium* **5**, 223–236
26. Schlegel, W., Winiger, B. P., Wuarin, F., Zahnd, G. R. & Wollheim, C. B. (1988) *J. Receptor Res.* **8**, 493–507
27. Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
28. Taraskevich, P. S. & Douglas, W. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4064–4067
29. Dufy, B., Vincent, J. D., Fleury, H., du Pasquier, P., Gourdji, D. & Tixier-Vidal, A. (1979) *Science* **204**, 509–511

30. Winiger, B. P., Wuarin, F., Zahnd, G. R., Wollheim, C. B. & Schlegel, W. (1987) *Endocrinology* (Baltimore) **121**, 2222–2226
31. Ozawa, S. & Sand, O. (1986) *Physiol. Rev.* **66**, 887–952
32. Ronning, S. A. & Martin, T. F. J. (1986) *J. Biol. Chem.* **261**, 7840–7845
33. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
34. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1987) *Cell Calcium* **8**, 79–100
35. Dufy, B., Jaken, S. & Barker, J. L. (1987) *Endocrinology* (Baltimore) **121**, 793–802
36. Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
37. Lucas, D. O., Bajjalieh, S. M., Kowalchuk, J. A. & Martin, T. F. J. (1985) *Biochem. Biophys. Res. Commun.* **132**, 721–728
38. Biden, T. J., Peter-Riesch, B., Schlegel, W. & Wollheim, C. B. (1987) *J. Biol. Chem.* **262**, 3567–3571
39. Biden, T. J. & Wollheim, C. B. (1986) *J. Biol. Chem.* **261**, 11931–11934
40. Zilberman, Y., Howe, L. R., Moore, J. P., Hesketh, T. R. & Metcalf, J. C. (1987) *EMBO J.* **6**, 957–962
41. Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, O. H. (1987) *Nature* (London) **330**, 653–655
42. Tashjian, A. H., Jr., Heslop, J. P. & Berridge, M. J. (1987) *Biochem. J.* **243**, 305–308

Received 26 February 1988/3 May 1988; accepted 19 May 1988