Use of a trapped fluorescent indicator to demonstrate effects of thyroliberin and dopamine on cytoplasmic calcium concentrations in bovine anterior pituitary cells

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The fluorescent calcium indicator 'quin2' was used to demonstrate changes in cytoplasmic calcium concentrations in bovine anterior pituitary cells. The basal calcium concentration was 0.21 ± 0.02 μM (mean of 4 cell preparations). Thyroliberin (TRH) (10^{-10}–10^{-6} M) rapidly and at the higher concentrations transiently increased the concentration. Dopamine (10^{-10}–10^{-7} M) decreased the concentration transiently and more slowly. At 10^{-5} M, dopamine prevented the increase in calcium concentration caused by 10^{-9} M TRH, and partially inhibited the increase caused by higher concentrations of the peptide. The data support the hypothesis that calcium is the second messenger for TRH, and suggest that dopamine inhibits TRH-induced prolactin secretion by preventing the calcium concentration from exceeding the level necessary to increase secretion.

Anterior pituitary *Cytoplasmic calcium* *Calcium concentration* *Dopamine* *Thyroliberin* *Prolactin secretion*

1. INTRODUCTION

The secretion of prolactin from the anterior pituitary is stimulated by TRH [1] and inhibited by dopamine [2]. Both effectors bind to the plasma membrane [3,4], and are therefore presumed to act through internal secondary messengers. Some data indicate that TRH increases and dopamine decreases pituitary cyclic nucleotide concentrations [1,5], although not all data support the suggestion that this mediates changes in hormone secretion [1,6]. Other data suggest that TRH increases [1,7–9] and dopamine decreases [6,10] cytoplasmic calcium concentrations, but although the indirect evidence is generally accepted for TRH, the effect of dopamine is controversial [10,11].

The difficulty in deciding whether calcium is a second messenger in the anterior pituitary is in part due to the inaccessibility of cytoplasmic calcium to direct measurements in small cells. Recently a fluorescent dye, quin2, has been used as an internal calcium indicator in lymphocytes [12]. The dye is added as quin2 acetoxymethyl ester which permeates the cells and is de-esterified within them. The de-esterified quin2, which cannot leak out of the cells, binds calcium with a K_d of 0.115 μM (within the range expected for cytoplasmic calcium) and increases its fluorescence when bound to calcium. Thus changes in cytoplasmic calcium concentration are reflected in changes in quin2 fluorescence. This paper reports that the technique is applicable to pituitary cells and that TRH increases and dopamine decreases cytoplasmic calcium concentrations in them.

2. MATERIALS AND METHODS

2.1. *Quin2*

Quin2 acetoxymethyl ester was obtained from Dr T.J. Rink of the Physiological Laboratory (Downing Street, Cambridge CB2 3EG). It was dissolved in dimethylsulphoxide at 20 mM, and stored at −20°C over desicant.

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2.2. *Incorporation of quin2 into pituitary cells*

Bovine anterior pituitary glands were dispersed and the cells centrifuged through Percoll as in [13]. The pellet, enriched in lactotrophs and somatotrophs, was resuspended in the HEPES-buffered salt solution used for dispersion (containing NaCl (131 mM), KCl (6.0 mM), KH_2PO_4 (1.2 mM), $MgCl_2$ (0.24 mM), $CaCl_2$ (0.25 mM), sodium 3-hydroxybutyrate (1.2 mM), glucose (2.8 mM), bovine serum albumin (Sigma, fraction V, 1 mg/ml) and buffered with HEPES (20 mM) adjusted to pH 7.4 with NaOH). About 6×10^7 cells were incubated for 60 min at 37°C in 6.5 ml of this buffer containing quin2 acetoxymethyl ester (30 M). The cells were then recovered by centrifugation and resuspended in incubation buffer (identical to the dispersion buffer except that the concentrations of $MgCl_2$ and $CaCl_2$ were 1.2 mM and 1 mM, respectively). The cell suspension was stored in ice until use.

2.3. *Measurement of quin2 fluorescence*

To measure the fluorescence of internal quin2 an aliquot of the cell suspension (4×10^6 cells) was centrifuged briefly (1 s, Eppendorf 5412 centrifuge $12000 \times g$), and resuspended in 3 ml incubation buffer at 37°C. This removed any quin2 leaked from the cells during storage on ice, which would affect the calibration procedure described below. If this precaution is taken, the basal calcium concentration of the cells measured by fluorescence remains constant for at least 3 h. The fluorescence of the cell suspension was measured using 339 nm excitation and 490 nm emission [12], in a plastic cuvette maintained at 37°C and mixed either by a rotating paddle or by a magnetic stirrer. The fluorescence of the cells fell to a stable basal level within 5 min of warming the cells in the HEPES incubation buffer.

The observed fluorescence (F_{obs}) can be converted into an apparent calcium concentration by the calibration procedure in [12]. Digitonin (20 μM final conc.) was added to lyse the cells and give the fluorescence when all the quin2 is saturated with calcium (F_{max}), and EGTA (25 mM final conc., final pH 7.65) was added to give the fluorescence when all the quin2 is free (F_{min}). The concentrations of free and calcium-bound quin2 which would give the observed fluorescence can then be calculated, and the calcium concentration can be estimated using the K_d of quin2 for calcium [12]. The addition of digitonin to unloaded cells causes a small increase in light scattering for which the values of F_{max} and F_{min} must be corrected before the calculations are made. This artefact can be avoided by careful selection of the excitation wavelength by double monochromators or by inclusion of a Wratten 18B filter, and by using Barr and Stroud MS2 interference filters combined with Kodak Wratten no.2B filters to select emitted light. Under these conditions none of the hormones altered the fluorescence of unloaded cells.

3. RESULTS

Fig.1 shows the changes in fluorescence in 3 cuvettes prepared from a single batch of cells. Fig.1a shows that increasing the TRH concentration in the cuvette in 10-fold increments from 10^{-10}–10^{-6} M increased the fluorescence of the loaded cells. The increases at low concentrations (10^{-10}–10^{-8} M) were maintained but at high concentrations (10^{-7} and 10^{-6} M) they were transient, rising rapidly within the mixing time and falling over the next 2 min.

Fig.1b shows that increasing the dopamine concentration in a single cuvette by 10-fold increments from 10^{-10} to 10^{-7} M caused the fluorescence of the internal quin2 to decrease. The fall in fluorescence caused by dopamine was slower than the rise after TRH, taking about 1 min to reach its nadir, and was transient especially at the lower dopamine concentrations.

Fig.1c shows the effects of dopamine on the responses to TRH; in this cuvette dopamine was added at the higher concentration of 10^{-5} M, because we have previously found that this concentration blocks the stimulation of prolactin secretion by TRH (10^{-7} M; [13]). Dopamine at 10^{-5} M caused a pronounced and prolonged fall in fluorescence. Subsequently, TRH at 10^{-10} or 10^{-9} M had little effect on the fluorescence but the peptide clearly increased fluorescence at 10^{-8}, 10^{-7} and 10^{-6} M, in the presence of dopamine.

The experiments shown in fig.1 cannot be used to give dose–response relationships for TRH because the timing of the successive additions of TRH affects the peak responses. In fig.1a, for example, the peak response to 10^{-6} M TRH was lower than that to 10^{-7} M, whereas in fig.1b the

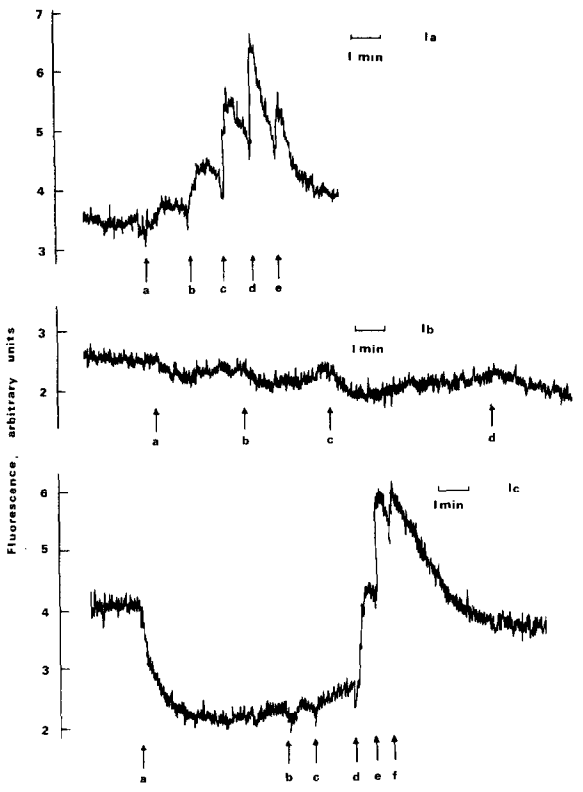

Fig.1. The figure contains 3 chart recorder traces showing changes in fluorescence of quin2-loaded bovine pituitary cells. In each, the abscissa is increased fluorescence in arbitrary units and the ordinate is time; the inset bar in each panel represents 1 min. In fig.1a, TRH was added at the times shown by the arrows to give final concentrations from the left of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M. The rapid transient fall in fluorescence is an artefact caused by insertion of the injection needle into the light pathway. In fig.1b, dopamine was added at the times shown by the arrows to give final concentrations from the left of 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M. In fig.1c, dopamine was added at the first arrow to bring the final concentration to 10^{-5} M, and then 5 successive additions of TRH added to give concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M.

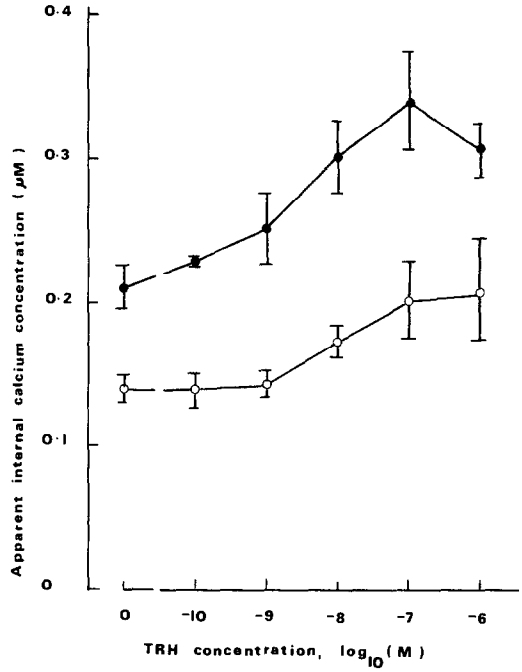

Fig.2. The figure shows the effect of increasing TRH concentrations on the apparent cytoplasmic [Ca^{2+}] of quin2-loaded pituitary cells in the absence (—●—) or presence (—○—) of dopamine (10^{-5} M). Each point is the mean and the vertical bars SEM of 4 cell preparations.

two responses were identical. In the latter case there was a shorter time lag between the two TRH additions so the fluorescence had less time to fall after the response to 10^{-7} M TRH. In other experiments, therefore, only one addition of TRH was made to each cuvette either in the presence or absence of dopamine (10^{-5} M), and the maximum rise in calcium concentration determined. Fig.2 shows that the basal calcium concentration was increased from 0.21 ± 0.02 to 0.34 ± 0.03 μM by TRH (10^{-7} M), and was decreased to 0.14 ± 0.01 μM by dopamine (10^{-5} M). Dopamine decreased the sensitivity of the system to TRH: in the absence of dopamine, TRH (10^{-9} M) increased the calcium concentration by 19 ± 3.4% whereas in the presence of dopamine it had no effect. Dopamine also decreased the maximum response to TRH: in the presence of dopamine, TRH (10^{-7} M) only increased the calcium concentration to 0.20 ± 0.03 μM, a value not significantly different from the basal level, of 0.21 ± 0.02 μM.

4. DISCUSSION

The data show that quin2 acetoxymethyl ester permeates pituitary cells and is hydrolysed within them, and that the fluorescence of intracellular quin2 responds to two physiological effectors of prolactin secretion, TRH and dopamine. The calculation of apparent calcium concentrations from the fluorescence assumes that the calcium

concentration changes uniformly throughout the cytoplasm, and in all the cells in the suspension [12]. This permits data from stimulated cells in different experiments to be combined, but the values obtained probably do not have much physiological significance. In stimulated secretory cells calcium is probably non-uniformly distributed [14] and lactotrophs but not somatotrophs in the cell dispersion respond to TRH and dopamine under these conditions [13,15]. The calcium concentration in restricted regions of lactotrophs probably changes more than the calculated values in fig.2 indicate.

With this proviso, the data in fig.2 provide direct evidence that TRH at concentrations within the physiological range increases cytoplasmic calcium concentrations. This confirms earlier indirect evidence [7,9]. Both the secretory [13,15] and the fluorescence responses to TRH are transient. The transience of secretion could reflect the fluctuation in cytoplasmic calcium, possibly due to exhaustion of a rapidly releasable calcium pool since indirect evidence indicates TRH mobilizes internally stored calcium [8,16].

The data also show that dopamine at concentrations within the physiological range decreased the intracellular calcium concentration. This could account for its ability to inhibit basal prolactin secretion [2,10], although the fact that the decrease in calcium was only transient whereas inhibition of secretion is sustained [2] rather complicates the interpretation. Dopamine at 10^{-5} M prevented the increase in calcium in response to 10^{-9} M TRH and decreased the response to higher TRH concentrations. The cell dispersions used in this work contain both somatotrophs and lactotrophs, but lactotrophs predominate since the ratio of the prolactin to growth hormone contents of the dispersions is 1.85 ± 0.16. However, in the presence of tetraethylammonium [15] or a methylxanthine [13] TRH can stimulate growth hormone secretion and dopamine fails to inhibit this stimulation [13]. The rise in calcium seen at high TRH concentrations in the presence of dopamine could therefore occur in somatotrophs. Further purification of the cells is needed to determine whether this is so, but we have not observed any stimulatory effect of TRH on growth hormone secretion in the presence of 10^{-5} M dopamine (unpublished) and have therefore no reason to suppose that TRH increases the calcium concentration in somatotrophs in the presence of dopamine. Thus it is probable that dopamine inhibits prolactin secretion in response to TRH without completely preventing the rise in calcium in the lactotrophs. Either the calcium concentration fails to reach the threshold necessary to increase secretion, or dopamine must prevent the action of calcium on the secretory pathway as suggested in [10,11].

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