Use of quin 2 to measure calcium concentrations in ovine anterior pituitary cells and the effects of quin 2 on secretion of growth hormone and prolactin

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Intracellular free Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured in ovine anterior pituitary cells using the quin 2 technique. Thyrotropin-releasing hormone (TRH) increased, dopamine decreased and growth hormone-releasing hormone (GHRH) had no detectable effect on $[Ca^{2+}]_i$. Loading the cells with quin 2, at an intracellular concentration less than that used during calcium determination, reduced both basal growth hormone (GH) and (to a small extent) prolactin secretion. Loading cells with quin 2 also markedly reduced GHRH-stimulated GH secretion. However, TRH-stimulated prolactin secretion was 3-times basal irrespective of quin 2 loading. The results indicate that the use of quin 2 to measure $[Ca^{2+}]_i$ in some cell types may be complicated by actions of quin 2 on cellular function.

Quin 2 *Thyrotropin-releasing hormone* *Dopamine* *Growth hormone-releasing hormone* *(Ovine pituitary cell)* *Ca^{2+}* *Growth hormone* *Prolactin* *Hormone secretion*

1. INTRODUCTION

The fluorescent indicator quin 2 has been used extensively to measure intracellular free calcium concentrations ($[Ca^{2+}]_i$) in rat pituitary tumour (GH) cells [1–3] and bovine anterior pituitary cells [4]. These studies suggest that TRH, a potent stimulator of prolactin secretion [5], elevates $[Ca^{2+}]_i$ [1–4] and that dopamine, an inhibitor of prolactin secretion [6], lowers $[Ca^{2+}]_i$ [4]. The $[Ca^{2+}]_i$ response to TRH has been most fully characterized [1–4].

Quin 2, whose structure resembles EGTA, is added to a cell suspension in its ester form (quin 2 acetoxymethyl ester; quin 2/AM) which is readily taken into the cells. It is subsequently hydrolysed by non-specific esterases within the cell and trapped. When quin 2 binds intracellular calcium its fluorescence increases, and changes in $[Ca^{2+}]_i$ can therefore be calculated by measuring changes in fluorescence. However, the effect of loading normal pituitary cells with quin 2 has not been well characterized, particularly with respect to actions on secretion.

Here we show, using the quin 2 technique, that TRH elevates and dopamine lowers $[Ca^{2+}]_i$ in ovine anterior pituitary cells. GHRH, a potent stimulator of GH secretion (whose action is mediated primarily via the intracellular second messenger cyclic AMP [7,8]) had no detectable effect on $[Ca^{2+}]_i$. However, we also report that loading ovine pituitary cells with quin 2, at an intracellular concentration lower than that used during $[Ca^{2+}]_i$ determinations, has specific and differing effects on basal and stimulated GH and prolactin secretion.

Abbreviations: TRH, thyrotropin-releasing hormone; GHRH, growth hormone-releasing hormone; GH, growth hormone; HIM buffer, Hepes-buffered incubation medium; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration

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2. MATERIALS AND METHODS

2.1. *Materials*

Quin 2/AM (Sigma (London), Poole, England) was dissolved in dimethyl sulphoxide at 5 mM and stored at −20°C over desiccant. Synthetic GHRH (44-NH_2) was from Universal Biologicals, Cambridge. TRH and dopamine were from Sigma.

2.2. *Quin 2 fluorescence experiments*

Ovine anterior pituitary cells were prepared according to [9]. Glands were dispersed (75 min, 37°C) using collagenase (Boehringer, 3 mg/ml), hyaluronidase (Sigma, type-1S; 1 mg/ml) and deoxyribonuclease I (Boehringer, grade II; 0.25 mg/ml) in medium containing bovine serum albumin (Sigma fraction V; 30 mg/ml), NaCl (137 mM), KCl (5 mM), Na_2HPO_4 (0.7 mM), glucose (10 mM) and Hepes (25 mM) which was adjusted to pH 7.4 with NaOH. Cells were collected by centrifugation (1600 rpm, 3 min) and washed 5 times. Dispersed cells were plated (5×10^6 cells/dish) into 5 cm bacteriological grade plates (Sterilin) and incubated in Dulbecco's modified Eagle medium (15 ml/dish) containing 5% foetal calf serum, 10% horse serum and antibiotics [9]. Under these conditions cell viability is maintained but cells do not attach. After 48 h at 37°C under 95% air/5% CO_2 cells were pelleted and resuspended 3 times in a Hepes-buffered incubation medium (HIM) containing NaCl (125 mM), KCl (5 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1 mM), KH_2PO_4 (1 mM), $NaHCO_3$ (3.5 mM), Hepes (25 mM) and glucose (10 mM) which was adjusted to pH 7.4 with NaOH.

About 20×10^6 cells were incubated in 5 ml HIM buffer containing 20 μM quin 2/AM for 45 min at 37°C with occasional agitation. The cells were then recovered by centrifugation, rinsed twice and resuspended in 10 ml HIM plus bovine serum albumin (0.1 mg/ml). The fluorescence of the cell suspension was measured using a Perkin Elmer LS-3 fluorimeter (λ emission = 492 nm, λ excitation = 338 nm) at 37°C with stirring. After addition of test substances the fluorescence measurements were converted into $[Ca^{2+}]_i$ using the calibration procedure in [10]. Digitonin (20 μM) was added to lyse the cells and determine the fluorescence when all of the quin 2 in the cells was bound to Ca^{2+} (quin 2 − Ca^{2+}) (F_{max}). EGTA (1.5 mM, pH > 8) was then added to determine the fluorescence of unbound quin 2 (F_{min}). The concentrations of quin 2 trapped intracellularly were measured by comparing the fluorescence of total bound quin 2 (F_{max}) with a standard curve constructed with quin 2 (allowance being made for cell autofluorescence). The cell volume was estimated to be 0.697 μl/10^6 cells by measuring the average cell diameter.

Corrections were made for the increase in fluorescence on addition of digitonin to unloaded cells. None of the test substances altered the fluorescence of unloaded cells.

2.3. *Secretion experiments*

Cells were dispersed, as in section 2.2, and maintained in culture (1×10^6 cells/dish) in 3.5 cm tissue culture plates (Sterilin) in 3 ml Dulbecco's modified Eagle medium with 5% foetal calf serum, 10% horse serum and antibiotics. After 72 h the incubation medium was removed, and the cells (which had attached to the plates) were rinsed 3 times with HIM buffer and incubated in 1 ml HIM buffer with or without quin 2/AM for 45 min at 37°C. The medium was discarded, the cells rinsed 3 times with HIM buffer and incubated for 60 min with 1 ml HIM buffer + 0.1 mg/ml bovine serum albumin with or without test substances. At the end of the incubation period medium was removed and centrifuged and the supernatants were stored at −20°C. Media were assayed for GH and prolactin using radioimmunoassay procedures as described in [9]. Hormones were iodinated using the iodogen method of Fraker and Speck [11]. All samples and standards were dissolved in buffer containing sodium phosphate (0.05 M, pH 7.6), merthiolate (0.6 mM), bovine serum albumin (0.05%) and Tween 20 (0.05%).

The concentrations of quin 2 trapped intracellularly were measured, as in section 2.2, by comparing the fluorescence of loaded cells, scraped from the culture dishes and lysed with digitonin, with a standard curve constructed with quin 2 (allowance being made for cell autofluorescence).

2.4. *Treatment of results*

All experiments were performed at least twice, with different preparations of pituitary cells, and consistent results were obtained. Experimental values are shown as means ± SE and were com-

pared with controls for determination of statistical significance using Student's *t*-test.

3. RESULTS

3.1. $[Ca^{2+}]_i$ determinations

The basal level of $[Ca^{2+}]_i$ in cultured ovine anterior pituitary cells, estimated from the basal level of quin 2 fluorescence, corresponded to 310 ± 35 nM (mean ± SE, n = 12). TRH (0.01 μM) stimulated a rapid increase in fluorescence over 10 s corresponding to a doubling of $[Ca^{2+}]_i$ to 208 ± 16% (n = 3) of basal (fig.1A). $[Ca^{2+}]_i$ levels remained elevated for at least 10 min. Subsequent addition of dopamine (0.01 μM) after TRH caused a slow decrease in $[Ca^{2+}]_i$ to reach 88 ± 16% (n = 2) of basal over a period of about 1 min.

Dopamine alone caused a slow decrease in $[Ca^{2+}]_i$ (fig.1B) over several minutes to 61 ± 3% (n = 3) of basal. $[Ca^{2+}]_i$ remained at this level for at least 10 min. Subsequent addition of TRH (0.01 μM) after dopamine caused a rapid increase in $[Ca^{2+}]_i$ to 102 ± 10% (n = 4) of basal. GHRH (1 nM) had not detectable effect on $[Ca^{2+}]_i$ (fig.1C).

The intracellular quin 2 concentration during these measurements was estimated to be 2.14 mM using the method described in section 2.2.

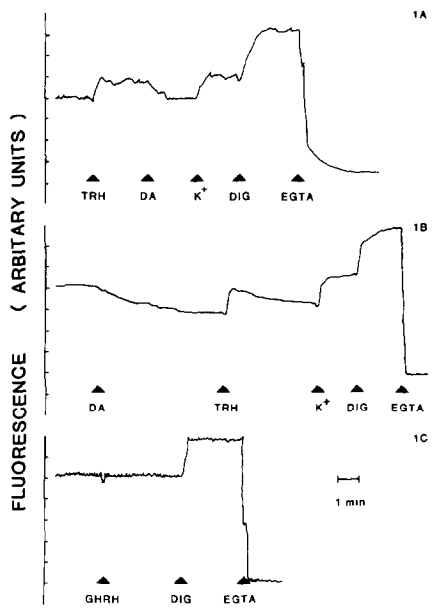

Fig.1. Changes in quin 2 fluorescence observed in ovine anterior pituitary cells treated with various secretagogues. Cells were cultured overnight and incubated with 20 μM quin 2/AM for 45 min at 37°C; the fluorescence was then measured (λ emission = 492 nm, λ excitation = 338 nm). (A) TRH (0.01 μM) was added, followed by dopamine (DA, 0.01 μM) and then K^+ (25 mM). (B) Dopamine (0.01 μM) was added, followed by TRH (0.01 μM) and then K^+ (25 mM). (C) GHRH (1 nM) was added. In each experiment an estimate of fluorescence was finally obtained after lysis of cells by digitonin (DIG, 20 μM) (when all quin 2 present would become bound to Ca^{2+}) and then in the presence of EGTA (when all quin 2 present would be dissociated from Ca^{2+}). Time is shown on the abscissa.

3.2. *Effects of loading cells with quin 2 on secretion of GH and prolactin*

The effects of various quin 2/AM concentrations (0–30 μM) on basal and stimulated GH and prolactin secretion are shown in fig.2A and B. Loading cells with increasing concentrations of quin 2 caused a slight, but not significant, reduction in basal prolactin secretion and a small but

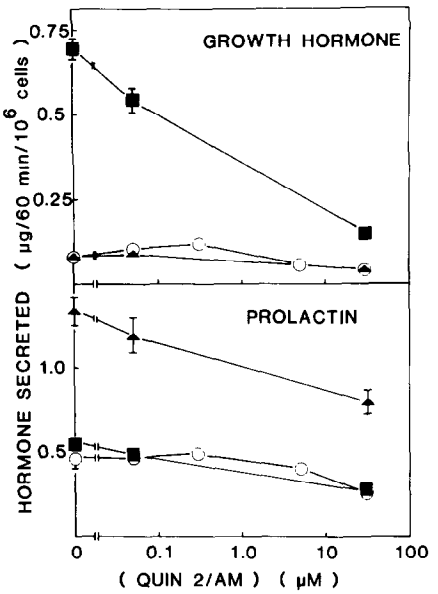

Fig.2. Effect of incubating cultured sheep pituitary cells with increasing concentrations of quin 2/AM (0–30 μM) on basal and stimulated growth hormone (top) and prolactin (bottom) secretion. After incubation with quin 2/AM for 45 min cells were rinsed and treated with 0.1 nM GHRH (■), 0.1 μM TRH (▲) or no additions (○). Error bars indicate ± SE; where no bar is shown SE falls within the limits of the symbol.

significant reduction in TRH-stimulated secretion. However, TRH (0.1 μM) significantly stimulated prolactin release 3-fold, irrespective of quin 2 loading ($P < 0.01$).

Loading cells with increasing concentrations of quin 2 reduced basal GH secretion in a dose-dependent manner. Cells incubated with 30 μM quin 2/AM released 60% less GH than unloaded cells ($P < 0.01$). Incubating cells with 30 μM quin 2/AM also reduced GHRH (0.1 nM)-stimulated GH secretion from 9-times basal to 4-times basal ($P < 0.001$ vs unloaded cells). The intracellular [quin 2] during these experiments was estimated to be 1.2 mM, on incubation with 30 μM quin 2/AM as described in section 2.3.

4. DISCUSSION

4.1. *$[Ca^{2+}]_i$ determinations*

Our data, using cultured ovine pituitary cells, show a basal $[Ca^{2+}]_i$ of 310 ± 35 nM compared with 210 ± 20 nM reported using bovine anterior pituitary cells [4]. Basal $[Ca^{2+}]_i$ reported for the rat pituitary tumour-derived cells (GH cells) varied considerably: 118 ± 18 nM [1], 350 ± 80 nM [2], 195 ± 10 nM [3], 148 ± 8.6 nM [12].

Our data show clearly a rapid increase in $[Ca^{2+}]_i$ in response to TRH and a slow decrease in $[Ca^{2+}]_i$ in response to dopamine. The results are similar to those obtained by Schofield [4] using bovine cells. The biphasic response induced by TRH reported in rat pituitary tumour-derived cells [2] was not observed. The data also show that dopamine (0.01 μM) lowers the TRH (0.01 μM)-induced elevation of $[Ca^{2+}]_i$ to near basal levels, as has been reported using bovine cells [4].

Our data also show no detectable effect of GHRH on $[Ca^{2+}]_i$ based on quin 2 fluorescence. This is interesting in view of a recent report that GHRH produces a significant stimulation of calcium efflux, with GH release, from perifused rat anterior pituitary cells [13]. Calcium has been shown to be required for the actions of GHRH on release of GH [7,14]. It should be noted, however, that our dispersed ovine pituitary preparation is heterogeneous and contains only 15–20% somatotrophs, as determined by immunofluorescent staining (J.J. Gomm, personal communication). Thus, any changes in $[Ca^{2+}]_i$ may be masked by other cell types present in the preparation.

4.2. *Effect of quin 2 loading on secretion*

Our results indicate that when pituitary cells are loaded with quin 2 there is a slight, but not significant, reduction in basal prolactin secretion, but no lowering in the extent of TRH-stimulated prolactin secretion over basal. Similar effects have been observed using rat pituitary tumour-derived (GH) cells by Albert and Tashjian [2] who reported that loading cells with quin 2 at intracellular concentrations up to 0.25 mM had no effect on prolactin secretion. Above this level inhibition of basal release occurred but THR-stimulated prolactin release was unaffected. On the other hand, Gershengorn and Thaw [1,12] found that intracellular concentrations of quin 2 up to 1.5 mM had no effect on basal or TRH-stimulated prolactin release from GH_3 cells.

Different effects on basal and stimulated prolactin secretion have also been observed using cultured ovine anterior pituitary cells in the presence of 'low Ca^{2+}' buffer [15]. If external Ca^{2+} concentration was lowered to less than 30 μM, basal prolactin secretion was inhibited by 58%. TRH-stimulated prolactin release was also reduced, but the incremental effect of TRH did not change significantly [15]. The similarity between this result and the effect obtained with quin 2 suggests that the latter may act by lowering effective Ca^{2+} levels.

Both basal and GHRH-stimulated GH secretion were greatly reduced, in a dose-dependent manner, by loading the pituitary cells with quin 2. A similar effect has been observed using blockers of Ca^{2+} uptake [7,13]. Verapamil, $CoCl_2$, $CdCl_2$ and also pimozide, an inhibitor of Ca^{2+}-calmodulin-dependent activities [7] and $CoCl_2$ [14] have been shown to reduce markedly GHRH-stimulated GH secretion from rat anterior pituitary cells. Law [16] also reported the reduction in GHRH-stimulated GH secretion to near basal levels when Ca^{2+} was omitted from the extracellular medium. It is interesting to note that lowering of extracellular Ca^{2+} concentration appeared to enhance GHRH-stimulated intracellular cyclic AMP levels [16], a phenomenon also noted with quin 2-loaded cells (unpublished).

Our data thus suggest that the 'Ca^{2+} buffering' effect of the intracellular quin 2 may affect both GH and prolactin secretion from the cells. However, quin 2 may alter $[Ca^{2+}]_i$ in other ways.

Thus, a recent report suggests that loading squid axons with quin 2 can almost abolish sodium-dependent Ca^{2+} inflow which occurs via Na^+/Ca^{2+} exchange [17]. It is also possible that the by-products of quin 2/AM hydrolysis (protons, acetate and formaldehyde) may affect hormone release.

In conclusion, the quin 2 technique has been used to show the effect of TRH, dopamine and GHRH on $[Ca^{2+}]_i$ using ovine anterior pituitary cells. However, loading cells with quin 2 can alter both GH and (to a lesser extent) prolactin secretion from the cells. The exact method by which it does this remains to be established.

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