Lithium-induced decrease in spontaneous Ca^{2+} oscillations in single GH₃ rat pituitary cells

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1 Measurement of $[\text{Ca}^{2+}]_i$ in single rat pituitary GH₃ cells by dynamic single cell imaging techniques demonstrated that under basal conditions there is a large variation in the temporal pattern of $[\text{Ca}^{2+}]_i$ signalling between individual cells ranging from high frequency asynchronous oscillations to quiescence.

2 We have reported previously that treatment of GH₃ cells with 1 mM Li^+ (a concentration used therapeutically in the treatment of manic depression) for 7 days reduces basal and thyrotrophin-releasing hormone (TRH)-stimulated levels of mass inositol 1,4,5-trisphosphate $[\text{Ins}(1,4,5)\text{P}_3]$. In the present study, we show that this is associated with a reduction in the number of cells exhibiting basal Ca^{2+} oscillations over a sampling period of 60 s, whereas the maximum amplitude of oscillations is unaffected.

3 The pattern of $[\text{Ca}^{2+}]_i$ responses to the agonist TRH varied considerably between individual cells, making quantitation of the responses difficult; however, data obtained from measurements made on a population of cells showed that increases in peak $[\text{Ca}^{2+}]_i$ induced by high concentrations of TRH were reduced in cells treated with 1 mM Li^+ for 7 days relative to control cells.

4 The sensitivity of the phosphoinositide pathway to $[\text{Ca}^{2+}]_i$ was investigated by loading GH₃ cells with BAPTA/AM at a concentration sufficient to lower 'basal' $[\text{Ca}^{2+}]_i$ in a population of cells and to inhibit agonist-stimulated increases in $[\text{Ca}^{2+}]_i$. Under these conditions, basal and TRH-stimulated mass $\text{Ins}(1,4,5)\text{P}_3$ levels were unaffected.

5 These results demonstrate that a 7-day Li^+ treatment leads to an alteration in Ca^{2+} signalling, in particular by reducing the number of cells exhibiting high frequency Ca^{2+} oscillations under basal conditions. The significance of these results to the clinical effectiveness of Li^+ in the treatment of manic depression is discussed.

Keywords: Rat pituitary; GH₃ cells; lithium; Ca^{2+} oscillations; inositol 1,4,5-trisphosphate; TRH; BAPTA

Introduction

Lithium ions are used therapeutically at a serum level of approximately 1 mM in the treatment of manic depression to alleviate both the manic and depressive phases of this disorder (reviewed in Wood & Goodwin, 1987). The mechanism by which Li^+ produces its therapeutic effects is not known although there is evidence for inhibition of the adenylyl cyclase (Newman & Belmaker, 1987) and phosphoinositide pathways (Berridge *et al.*, 1982; 1989) and it has been proposed that these effects are mediated at the level of G-proteins (Avisar *et al.*, 1988). However, this does not provide an explanation for the selectivity of action of Li^+ to manic depressive disease and a more widely accepted hypothesis is the ability of Li^+ to inhibit metabolism of certain inositol phosphates. At therapeutic concentrations, Li^+ uncompetitively inhibits inositol monophosphatase (Hallcher & Sherman, 1980), a key enzyme in the metabolism of inositol monophosphates. Inhibition of this enzyme by Li^+ leads to a large accumulation of inositol monophosphates in rat brain, and a corresponding reduction in inositol levels (Allison & Stewart, 1971; Allison *et al.*, 1976). In tissues which have access to limited supplies of inositol, such as brain because of the relatively poor ability of inositol to cross the blood brain barrier, reduced inositol levels may lead to reduced synthesis of phosphoinositides, and ultimately impair receptor-stimulated phosphoinositide activity by reducing production of the Ca^{2+} -mobilizing second messenger $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol which activates protein kinase C (Berridge *et al.* 1982; 1989). In support of this, the majority of studies have reported a reduction in agonist-stimulated generation of inositol phosphates and mass $\text{Ins}(1,4,5)\text{P}_3$ in brain slices following chronic treatment with

Li^+ (reviewed by Nahorski *et al.*, 1991), although an enhanced accumulation of $\text{Ins}(1,4,5)\text{P}_3$ has been described in a few cases (Whitworth *et al.*, 1990; Lee *et al.*, 1992; Dixon *et al.*, 1992).

In order to investigate the interaction of Li^+ with the phosphoinositide cycle in further detail, we have previously set up a model using rat pituitary GH₃ cells. These cells were chosen because, like neurones, they are electrically excitable neurosecretory cells giving robust Ca^{2+} and phosphoinositide responses. Using these cells we have previously shown that chronic treatment with 1 mM Li^+ reduces both basal and agonist-stimulated levels of mass $\text{Ins}(1,4,5)\text{P}_3$ and that this is correlated with a reduction in Ca^{2+} signalling and prolactin secretion (Varney *et al.*, 1992). The magnitude of the inhibitory effects observed, however, were relatively small (~20% inhibition), although we suggested that heterogeneity may exist between cells in their susceptibility to Li^+ . In this study we have addressed this by examining the effect of Li^+ treatment on Ca^{2+} signalling in individual GH₃ cells using dynamic Ca^{2+} imaging techniques.

Methods

Li⁺ treatment of rat pituitary cells

Rat pituitary GH₃ cells were grown in Hams F10 medium supplemented with 16% foetal calf serum. For experiments however, cells were seeded into either flasks or 16-well plates and grown in inositol-free Hams F10 medium (Gibco BRL) supplemented with 5% foetal calf serum either in the absence or presence of 1 mM LiCl as described previously (Varney *et al.*, 1992). All experiments were performed after a period of 7 days of treatment with Li^+ . Measurements of mass $\text{Ins}(1,4,5)\text{P}_3$ was generally performed in multiwell plates,

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although essentially identical results were obtained with cellular suspensions following removal of cells from flasks by incubation with trypsin.

Where appropriate, cells were loaded with BAPTA either by incubation with 100 μ M BAPTA/AM alone or with 100 μ M BAPTA/AM and 1 μ M fura-2/AM (for Ca²⁺ measurements). After 45 min incubation cells were washed, resuspended in fresh buffer, and mass Ins(1,4,5)P₃ and population [Ca²⁺]_i levels measured as described previously (Varney *et al.*, 1992).

Measurement of cytosolic free Ca²⁺ in single GH₃ cells

Cytosolic Ca²⁺ levels ([Ca²⁺]_i) were measured in GH₃ cells using the Ca²⁺-sensitive dye fura-2. Measurements of [Ca²⁺]_i were carried out in cells grown on borosilicate glass coverslips. After 4 to 5 days in culture, cells were loaded with 3 μ M fura-2/AM in 3 ml of Hanks buffer in the presence of 0.1% Pluronic for 45 min at room temperature. Coverslips were washed twice in Hanks buffer, left for a further 10 min, and transferred to a Teflon coverslip holder (Digitimer Ltd.; Welwyn Garden City, Herts). All [Ca²⁺]_i measurements were performed at 22°C. The coverslip dish was placed on the stage of a Zeiss Axiovert 35 inverted microscope equipped for fluorescence microscopy. A 75 W xenon arc lamp combined with interference filters of 350 and 380 nm, present in a filter wheel changer (Ludl Electronic Products Ltd., Hawthorne, New York) was used for excitation of the loaded fura-2. Excitation light was deflected by a dichroic mirror (Zeiss FT 395 nm) into the microscope objective ($\times 40$ Achromatig oil immersion lens; n.a. 1.30) and onto the sample. Excited light was collected from the sample through the dichroic mirror and a 510 nm interference filter and directed into an intensified CCD camera (Extended ISIS-M; Photonic Science, U.K.). Care was taken to attenuate the excitation

light as much as possible to avoid bleaching of the dye and photodamage to cells. Pairs of images were captured every 2–3 s with an image grabbing board (PixelPipeline; Perceptics Corp., Knoxville, TN, U.S.A.) fitted to an Apple Macintosh Quadra 900 and controlled by the software IonVision (Improvision, Coventry). Four frames were normally averaged at each excitation wavelength, and the 350/380 nm ratio calculated for each pair of images after subtraction of the backgrounds. The mean [Ca²⁺]_i levels were calculated from each ratio image using the software IonVision. Calibration of the signals as a function of [Ca²⁺]_i was performed with standard Ca²⁺ solutions (Williams & Fay, 1990), and a viscosity correction factor determined and applied as described by Poenie (1990).

Agonists were added directly to the coverslip chamber at the appropriate time with a Gilson pipette. A relatively large volume of agonist was added to the chamber so as to ensure rapid mixing (i.e. 0.5 ml agonist added to 2.5 ml bath volume).

Statistical analysis

Results are shown as mean $\pm$ s.e.mean of at least three separate experiments. Statistical analysis were performed by the Wilcoxon Rank sum test, or Student's *t* test following a non-significant Wilk-Shapiro Normality test. Statistical significance was taken as $P < 0.05$.

Materials

[³H]-Ins(1,4,5)P₃ (specific activity 17.54 Ci mmol⁻¹) was obtained from New England Nuclear or Amersham International. Fura-2/AM and BAPTA/AM were obtained from Molecular Probes. Thyrotrophin-releasing hormone (TRH) and Ins(1,4,5)P₃ were purchased from Sigma. Tissue culture

Figure 1 Heterogeneity of [Ca²⁺]_i in individual GH₃ cells under non-stimulated conditions, observed by dynamic video-imaging of fura-2 at various time points (indicated on each time frame). The images are the ratios of those observed at 350 nm and 380 nm, following background subtraction, and calibrated against Ca²⁺ standards, as described in the Methods section. Images are displayed in pseudocolour, with red representing high Ca²⁺, and blue low Ca²⁺.

reagents such as medium (including custom-made inositol-free Hams F10) were obtained from GIBCO, and plastic tissue culture equipment from Griffiths & Neilson. All other standard laboratory reagents were of Analytical grade.

Results

Single cell $[Ca^{2+}]_i$ measurements

Measurement of Ca^{2+} revealed a wide variation in basal levels of $[Ca^{2+}]_i$ between individual cells as exemplified in Figure 1; some cells appeared quiescent since $[Ca^{2+}]_i$ levels did not change significantly over the recording period, whilst others exhibited frequent, irregular spontaneous oscillations in $[Ca^{2+}]_i$. Two extreme examples of this behaviour are shown in Figure 2a. In order to examine the action of Li^+ on individual cells, the mean $[Ca^{2+}]_i$ throughout a 60 s recording period was measured in 370 cells treated with 1 mM Li^+ for 7 days and compared to the same number of control cells. The frequency distribution histogram of mean $[Ca^{2+}]_i$ levels over a 60 s recording period revealed a clear shift to the left in the Li^+ -treated group (Figure 2b). When results in individual cells were pooled, cells treated with Li^+ had a significantly ($P < 0.05$) lower mean $[Ca^{2+}]_i$ of 160 ± 7 nM as compared to 195 ± 9 nM in control cells (Figure 3a) a result which is similar to values previously obtained in whole cell populations (Varney *et al.*, 1992). This reduction in intracellular Ca^{2+} did not appear to be due to a change in cell viability as

Figure 2 (a) A time-course of $[Ca^{2+}]_i$ levels in 2 individual cells, one of which displays $[Ca^{2+}]_i$ oscillations (○), whilst the other is quiescent (●). A pair of images were acquired every 2 s, and $[Ca^{2+}]_i$ levels calculated by image analysis of the time-sequence of images. (b) A frequency distribution histogram of mean $[Ca^{2+}]_i$ levels in GH₃ cells under non-stimulated conditions, recorded over a 60 s period, with images acquired every 2 s. Mean $[Ca^{2+}]_i$ levels were assigned into groups which differed by 25 nM (i.e. 0–25, 26–50, etc) for a total of 370 control cells (open columns) and 370 chronic Li^+ -treated cells (solid columns). Numbers on the abscissa scale refer to the upper limit of the range.

Figure 3 Summary of $[Ca^{2+}]_i$ measurement in control GH₃ cells (open columns) and chronic Li^+ -treated cells (solid columns). (a) Mean basal $[Ca^{2+}]_i$, (b) numbers of cells showing $[Ca^{2+}]_i$ oscillations, and (c) the mean maximum amplitude of these $[Ca^{2+}]_i$ oscillations. Data are summarised from 370 control and Li^+ -treated cells, obtained from 21 experiments; values are mean with s.e.mean. *represents a significantly different value from the control value ($P < 0.05$).

Figure 4 $[Ca^{2+}]_i$ measurements in GH₃ cells. (a) Example traces of basal $[Ca^{2+}]_i$ levels, and 1 μ M TRH-stimulated levels (indicated by the solid bar), in both control (a) and chronic Li^+ -treated cells (b). Note the variability of $[Ca^{2+}]_i$ levels between cells under basal conditions, and in response to TRH. Calibration bars indicating $[Ca^{2+}]_i$ and time are shown, and apply to all traces.

both groups of cells exhibited a similar ability to exclude trypan blue (not shown).

A more detailed analysis of basal $[Ca^{2+}]_i$ levels was performed through the study of Ca^{2+} oscillations in individual GH₃ cells. Since the Ca^{2+} oscillations were not easily defined into individual oscillations or patterns (see Figure 2a), their frequency could not be reliably calculated, and instead, cells were defined as either oscillating or quiescent. A change in $[Ca^{2+}]_i$ of greater than 100 nM over a 60 s recording period was taken to define a cell as oscillating. The maximum amplitude (maximum minus minimum $[Ca^{2+}]_i$ level) was also measured from cells which were defined as oscillating. This analysis revealed that under non-stimulated conditions $48 \pm 4\%$ of control cells showed oscillations in Ca^{2+} and that following Li^+ treatment this number was significantly reduced to $24 \pm 8\%$ ($P < 0.05$; Figure 3b). The maximal amplitude of oscillations, however, was unaffected in cells which displayed Ca^{2+} oscillations in the two treatment regimes (Figure 3c). This decrease in frequency of basal Ca^{2+} oscillations can also be seen by comparison of the representative Ca^{2+} traces in single GH₃ cells before agonist stimulation (Figure 4).

TRH-stimulated $[Ca^{2+}]_i$ responses varied considerably between individual GH₃ cells. Although, the majority of cells responded to TRH with a rapid peak increase in $[Ca^{2+}]_i$, this was followed by more variable behaviour with some cells showing an increase and some a decrease in frequency of oscillations, while in others, a maintained increase in $[Ca^{2+}]_i$ was observed (see Figure 4 for example traces). This heterogeneity in $[Ca^{2+}]_i$ responses to TRH prevented a detailed quantitative analysis of traces from single cell experiments. However, measuring the mean of TRH-stimulated $[Ca^{2+}]_i$ responses from many individual cells revealed that a high concentration of TRH (1 μ M) induces a larger peak Ca^{2+} response in control cells (658 ± 59 nM), as compared to Li^+ -treated cells (492 ± 49 nM, $n = 5$, 15–20 observations per experiment; $P < 0.05$). This small ($\sim 25\%$) inhibitory effect of Li^+ against high concentrations of TRH is similar to that described previously in a population of cells (Varney *et al.*, 1992).

Effect of $[Ca^{2+}]_i$ levels on mass *Ins(1,4,5)P₃*

When cells were loaded with 100 μ M BAPTA/AM for 30 min, there was a reduction in basal $[Ca^{2+}]_i$ in cell suspensions from

Figure 5 Effect of TRH (100 nM) on $[Ca^{2+}]_i$ responses in control (a) and BAPTA-loaded cells (b) obtained from recordings made from a whole coverslip. Traces are representative of two further experiments. The addition of the agonist is shown by the arrow head, and the relevant $[Ca^{2+}]_i$ calibration bar is shown alongside the traces.

209 ± 17 to 57 ± 7 nM ($n = 3$, 12 observations) and little or no detectable increase in $[Ca^{2+}]_i$ was observed in BAPTA-loaded cells on challenge with TRH (100 nM) or K^+ (70 mM) (see Figure 5 for example traces). In contrast, mass *Ins(1,4,5)P₃* levels were similar in control (12.4 ± 1.4 pmol mg^{-1} protein) and BAPTA-treated cells (11.3 ± 1.2 pmol mg^{-1} protein), and in control (36.6 ± 3.2 pmol mg^{-1} protein) and BAPTA-loaded cells (43.7 ± 3.5 pmol mg^{-1} protein) challenged with 100 nM TRH for 10 s.

Discussion

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

In a previous study we demonstrated that chronic treatment of GH₃ cells with a therapeutic concentration of Li^+ reduced basal and agonist-stimulated intracellular Ca^{2+} levels in a suspension of GH₃ cells (Varney *et al.*, 1992). In this study we have extended these observations to single cells using dynamic video imaging techniques. The results demonstrate a marked heterogeneity in $[Ca^{2+}]_i$ signalling between single GH₃ cells. A sub-population of cells exhibit spontaneous, irregular $[Ca^{2+}]_i$ oscillations under basal conditions which are asynchronous with respect to neighbouring cells. Although

the limited sampling period used in this study (60 s) may underestimate the actual percentage of cells displaying $[Ca^{2+}]_i$ oscillations, our result that approximately 50% of the GH₃ cell population display oscillations within this period is in agreement with results obtained for GH₄C₁ cells where considerably longer sampling times were used (Wagner *et al.*, 1993). The number of cells exhibiting $[Ca^{2+}]_i$ oscillations in the recording period was reduced in cells treated with Li⁺, although the amplitude of the oscillations in the remaining cells was not changed. The present results, therefore, demonstrate that the previously reported decrease in 'basal' Ca²⁺ observed in a suspension of GH₃ cells following Li⁺ treatment (Varney *et al.*, 1992) is mediated by either a reduction in the frequency of spontaneous $[Ca^{2+}]_i$ oscillations or an increase in the sub-population of cells exhibiting quiescent behaviour.

We have shown previously that chronic Li⁺ treatment of GH₃ cells results in a decrease in basal and TRH-stimulated levels of mass Ins(1,4,5)P₃. Since the role of Ins(1,4,5)P₃ is to release Ca²⁺ from intracellular stores, the altered Ca²⁺ signalling in GH₃ cells following Li⁺ treatment might be explained by a Li⁺-induced decrease in phosphoinositide turnover. An alternative explanation that the Li⁺-induced decrease in Ca²⁺ signalling results in a decreased phosphoinositide pathway activity seems unlikely as BAPTA-loaded cells did not exhibit reduced basal or TRH-stimulated levels of Ins(1,4,5)P₃ despite a marked reduction in intracellular Ca²⁺. Consistent with this, increasing $[Ca^{2+}]_i$ levels using high K⁺ or addition of a Ca²⁺ ionophore did not alter basal or TRH-stimulated formation of [³H]-inositol phosphates (M.A.V., unpublished observations). These data argue against a role for Ca²⁺ in the regulation of phospholipase C in GH₃ cells.

The reduction in $[Ca^{2+}]_i$ oscillations under non-stimulated conditions may underlie the decrease in 'basal' prolactin secretion reported previously following Li⁺-treatment (Varney *et al.*, 1992) as $[Ca^{2+}]_i$ oscillations are believed to control basal secretion in GH₃ cells (Schlegel *et al.*, 1987). The previously reported reduction in basal level of Ins(1,4,5)P₃ following Li⁺-treatment (Varney *et al.*, 1992) may be responsible for the reduced $[Ca^{2+}]_i$ oscillations. In GH₃ cells, Ca²⁺ oscillations are completely dependent on the presence of extracellular Ca²⁺ since they are blocked by addition of dihydropyridine calcium channel blockers e.g. nifedipine, or by the lowering of extracellular Ca²⁺ (Schlegel

et al., 1987; Wagner *et al.*, 1993). Ca²⁺ oscillations are thus driven by entry of Ca²⁺ through voltage-dependent Ca²⁺ channels. Recent models of Ca²⁺ oscillations suggest that Ca²⁺ enhances its own release through a process of Ca²⁺-induced Ca²⁺ release (CICR) through ryanodine and Ins(1,4,5)P₃ receptors (reviewed by Berridge, 1993). Ins(1,4,5)P₃-sensitive stores become more sensitive to Ins(1,4,5)P₃ as they fill up with Ca²⁺, and once the Ca²⁺ reaches a critical amount the basal levels of Ins(1,4,5)P₃ may be sufficient to release Ca²⁺ through a process of CICR (Missiaen *et al.*, 1991). Thus in GH₃ cells, the voltage-dependent entry of Ca²⁺ through spontaneous action potentials may serve to prime the Ins(1,4,5)P₃-sensitive stores such that basal levels of Ins(1,4,5)P₃ are sufficient to cause Ca²⁺ release, resulting in the initiation of an oscillation. However, in Li⁺-treated cells, lower basal levels of Ins(1,4,5)P₃ effectively reduce the sensitivity of the stores, resulting in a longer period between Ca²⁺ discharge and therefore a reduction in the frequency of $[Ca^{2+}]_i$ oscillations.

In conclusion, these results show that treatment of GH₃ cells with low concentrations of Li⁺ reduces the likelihood of spontaneous $[Ca^{2+}]_i$ oscillations in single rat pituitary GH₃ cells. An inhibitory action of an acute treatment with Li⁺ on oscillatory changes in a Ca²⁺-activated Cl⁻ current in blowfly salivary gland has also been described (Galione & Berridge, 1988). It can be speculated that the reduced levels of Ins(1,4,5)P₃ observed in rat cerebral cortex following chronic treatment with Li⁺ may also lead to a decrease in Ca²⁺ signalling (Varney *et al.*, 1992). The observation that $[Ca^{2+}]_i$ oscillations occur in neurones (Murphy & Miller, 1989; Friel & Tsien, 1992) and glia (Cornell-Bell *et al.*, 1990), and that CICR may play an important role within neurones (Marrion & Adams, 1992), suggest that changes in Ca²⁺ mobilization will induce a change in neuronal activity. Changes in basal Ca²⁺ levels can also influence responses to agonists. For example, in acinar cells the pattern of agonist-induced Ca²⁺ oscillations is dependent on basal Ca²⁺ levels (Toescu *et al.*, 1993), while hippocampal neurones with higher resting levels of $[Ca^{2+}]_i$ show increased responses to NMDA and K⁺ depolarization (Nakajima *et al.*, 1993). An altered Ca²⁺ signalling may therefore be an important modulatory mechanism in Li⁺-sensitive cells.

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