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The actions of ouabain and lithium chloride on cytosolic Ca^{2+} in single chromaffin cells

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Abstract

The effects of ouabain, Li^+ and veratridine on the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) were studied in single fura-2-loaded bovine adrenal chromaffin cells. Superfusion of cells with ouabain (10 μM for 60 min) caused only a delayed mild increase of the $[Ca^{2+}]_i$, from around 0.1 μM to 0.2–0.3 μM; this increase was Na_o^+-dependent. Replacement of all NaCl of the Krebs-Hepes solution by LiCl (144 mM) produced a gradual increase of $[Ca^{2+}]_i$, which remained elevated at a stable plateau of 0.4–0.5 μM for 40–50 min. When ouabain (in the presence of normal Na_o^+) or Li^+ (in the absence of Na_o^+) was given in Krebs-Hepes solution containing no Ca^{2+}, the reintroduction of 2.5 mM Ca^{2+} produced a fast elevation of the $[Ca^{2+}]_i$. In the case of ouabain-treated cells, the $[Ca^{2+}]_i$ curve exhibited an initial phasic component which inactivated to a tonic component. ω-Conotoxin MVIIC (3 μM) and R56865 (10 μM) inhibited the phasic but not the tonic component. Veratridine (30 μM) induced large $[Ca^{2+}]_i$ oscillations. Both ouabain or Li^+ abolished such oscillations. These results are compatible with ouabain causing elevation of $[Ca^{2+}]_i$ in bovine chromaffin cells through a dual mechanism, i.e. cell depolarisation and slowing down of the Na^+-Ca^{2+} exchanger of their plasmalemma. Through its binding to the Na^+ site on the Na^+-Ca^{2+} exchanger, Li^+ ions generate powerful Ca_i^{2+} signals that might be relevant to its known effects on neurosecretory mechanisms.

Keywords: Ouabain; Cytosolic Ca^{2+}; Chromaffin cell; Lithium chloride; Veratridine

1. Introduction

Banks (1967) first observed that ouabain enhanced the rate of spontaneous catecholamine release from perfused bovine adrenal glands. Secretion induced by ouabain was also observed in isolated bovine chromaffin cells (Aunis and García, 1981; Pocock, 1983a). Ouabain binding sites (Aunis and García, 1981; Castillo et al., 1989) associated with Na^+, K^+-activated ATPase activity (Aunis and García, 1981) and to Na^+/K^+ pumping (García et al., 1981; Pocock, 1983b) were found in adrenal medullary chromaffin cells. Also a Na^+-Ca^{2+} exchanger (Liu and Kao, 1990; Chern et al., 1992) and a Ca^{2+} pump (Pocock, 1983b; Kao and Cheung, 1990) have been characterised in these cells. The enhanced catecholamine release from perfused adrenal glands (Banks, 1967; Abajo et al., 1987, 1989; Esquerro et al., 1980) or isolated chromaffin cells (Aunis and García, 1981) exposed to ouabain exhibits an absolute requirement for external Ca^{2+} ions (Ca_o^{2+}) (but see Pocock, 1983a). Furthermore, a parallel increase of catecholamine and dopamine β-hydroxylase release support the exocytotic nature of the ouabain secretory effects (García et al., 1980). However, the effect of replacement of Na^+ by Li^+ was also shown to mimic the secretory effects of ouabain (Abajo et al., 1987).

The exocytotic release of catecholamines triggered by acetylcholine or high K^+ in adrenal medullary chromaffin cells is accompanied by an enhanced Ca_o^{2+} entry (Douglas and Poisner, 1962) and by an increase in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (Knight and Kesteven, 1983). Therefore, the question arises as to whether similar changes of the $[Ca^{2+}]_i$ occur in chromaffin cells exposed to ouabain or Li^+. The study presented here was planned to define the effects of ouabain or Li^+ on the $[Ca^{2+}]_i$ in

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single fura-2-loaded bovine chromaffin cells, with the aim of contributing to the understanding of the ionic basis of the well-established secretory effects of both ouabain and Li^+.

2. Materials and methods

2.1. Culture of bovine adrenal chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated as previously described (Livett, 1984), with the modifications introduced by Moro et al. (1990, 1991). Cells were plated on uncoated plastic culture wells (24-well Costar plates) containing 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Determination of total cell number was carried out according to Wilson (1987). Cultures were maintained for 1–6 days at 37°C in a water-saturated atmosphere with 5% CO_2. After 24 h, the medium was aspirated and replaced by serum-free medium, which was subsequently changed every 2 days. Trypan blue exclusion yielded cell viability values greater than 95%.

2.2. Measurement of changes of the $[Ca^{2+}]_i$ in fura-2-loaded bovine chromaffin cells

Chromaffin cells were loaded with fura-2 by incubating them with fura-2/AM (4 μM) for 30 min at room temperature in Krebs-Hepes solution (pH 7.4) containing (in mM): NaCl, 145; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2.5; sodium Hepes, 10; glucose, 10. The loading incubation was terminated by washing the coverslip containing the attached cells several times with Krebs-Hepes. Then, cells were kept at 37°C in the incubator for 15–30 min. The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of the $[Ca^{2+}]_i$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40 × fluorite objective. Emitted light was transmitted through a 425 nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated

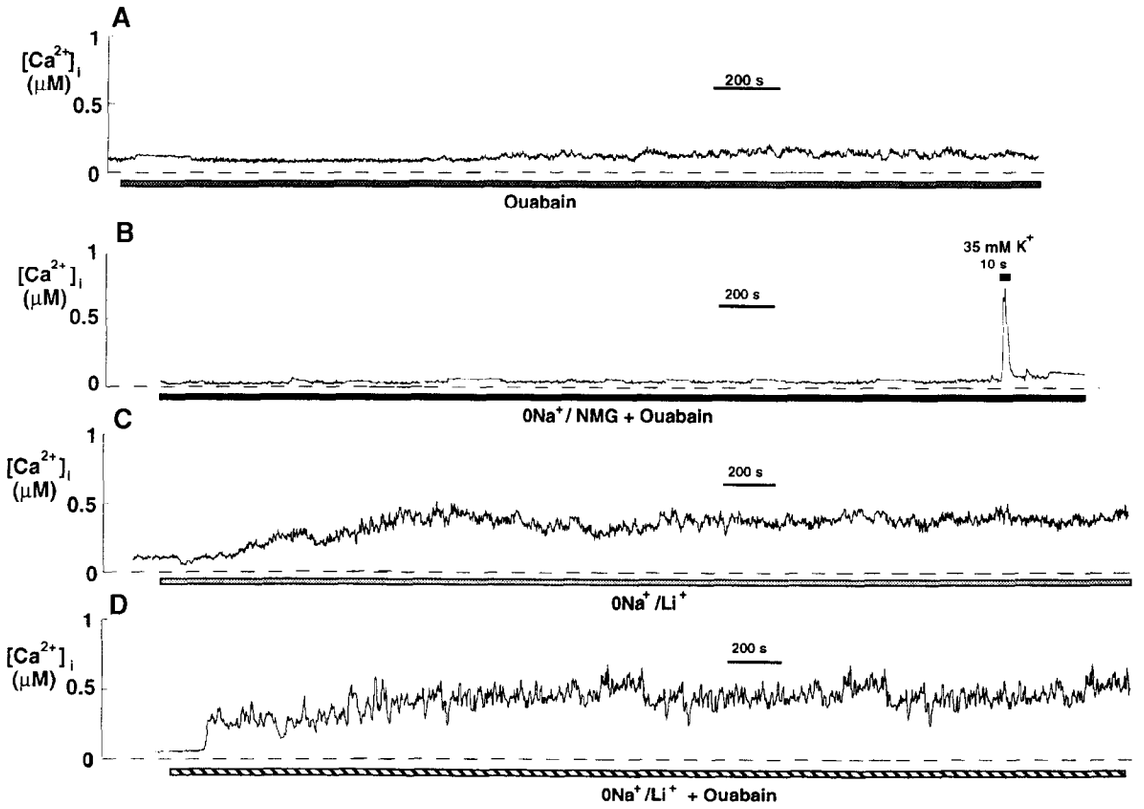

Fig. 1. Effects of ouabain and Li^+ on $[Ca^{2+}]_i$. In A, a fura-2-loaded chromaffin cell was superfused continuously with ouabain in a normal Krebs-Hepes solution for 1 h. After the basal $[Ca^{2+}]_i$ reached a steady state (usually around 100 nM), the selected cell was superfused with a Na^+-free Krebs-Hepes solution (B, C and D); NaCl was replaced by equimolar concentrations of LiCl (C and D) or *N*-methylglucamine chloride (NMG) (B). In A, B and D, ouabain (10 μM) was present throughout the time of recording. In B, a pulse of 35 mM K^+ was applied for 10 s at the end of the experiment. Similar results were observed in 3 (A), 4 (B) and 3 (C and D) cells.

from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz et al., 1985). Three intracellular calibration measurements were made in different cells dialysed in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981), with various EGTA-Ca^{2+} buffers added to a pipette filling solution containing (in mM): K-glutamate 135; NaCl 8; $MgCl_2$ 1; GTP 0.3; MgATP 0.5; fura-2-pentapotassium salt 0.1; KOH-Hepes 10; pH 7.2. The additions were either 10 mM EGTA or 10 mM $CaCl_2$ and the values of R_0 (fluorescence ratio at zero Ca^{2+}) and R_1 (limiting ratio at high Ca^{2+}) were measured directly in the cells. The K_{eff} (effective binding constant for Ca^{2+}) was calculated by making a third measurement of the fluorescence ratio from a cell injected with a solution containing 6.6 mM Ca-EGTA plus 3.3 mM free EGTA, assuming that the $[Ca^{2+}]_i$ is, under this condition, clamped to an apparent value of 0.3 μM (implying a K_D for EGTA at pH 7.2 of 0.15 μM). Experimental fluorescence data were sampled every 0.5 s by a computer which continuously provided the $[Ca^{2+}]_i$ values in nM.

2.3. Solutions

The basic equilibration solution superfusing the cells during the beginning of each experiment was the Krebs-Hepes solution used to load the cells with fura-2 (its composition was described in the previous section). The ionic composition of this solution was changed as indicated in Results for each experimental protocol. Thus, on some occasions 145 mM NaCl was replaced by 145 mM *N*-methylglucamine chloride. In other cases the Krebs-Hepes solution was simply deprived of Ca^{2+} ($0Ca^{2+}$ solution). From now on, solutions will be named following the abbreviations given here.

2.4. Statistical analysis

Averaged data are means ± S.E.M. The statistical significance of differences between means was determined by Student's t-test for paired or group data. Differences were considered significant at the level of $P < 0.05$.

2.5. Drugs and chemicals

Collagenase from *Clostridium histolyticum* was obtained from Boehringer-Manheim, Spain; bovine serum albumin fraction V, soybean trypsin inhibitor, cytosine arabinoside, veratridine and ouabain were from Sigma; Percoll was from Pharmacia, Spain; DMEM and fetal calf serum were from GIBCO, Madrid, Spain; fura-2/AM from Molecular Probes, USA. ω-Conotoxin MVIIC was from Bachem, UK. R56865, *N*-[1-(4-(4-fluorophenoxy)-butyl)]-4-piperidinyl-*N*-methyl-2-benzo-thiazolamine, was a kind gift from Janssen Farmaceutica, Madrid, Spain.

3. Results

3.1. Effects of ouabain and lithium on the basal $[Ca^{2+}]_i$

First we used two manipulations that lead to inhibition of the Na^+ pump, i.e. ouabain and Na_o^+ deprivation. Fig. 1A shows the time course of the $[Ca^{2+}]_i$ in a fura-2-loaded bovine chromaffin cell superfused continuously with 10 μM ouabain in normal Krebs-Hepes solution for a 60-min period. This ouabain concentration was selected for all experiments because it causes maximum inhibition of Na^+, K^+-ATPase activity (Aunis and García, 1981) and of $^{86}Rb^+$ influx and $^{24}Na^+$ efflux (Pocock, 1983b) in bovine chromaffin cells. Ouabain was introduced after a 5-min period of initial equilibration. In resting conditions, untreated bovine chromaffin cells did not change their basal levels of $[Ca^{2+}]_i$ during a 1-h period (around 100 nM). In the cell of panel A, the resting levels of $[Ca^{2+}]_i$ remained stable for the first 20 min of ouabain treatment. After this, mild elevations (0.2–0.3 μM) of the $[Ca^{2+}]_i$ were seen, with $[Ca^{2+}]_i$ adopting a kind of oscillatory pattern for the rest of the experiment. Such small oscillatory increments of $[Ca^{2+}]_i$ disappeared when ouabain was given in a $0Na^+$/*N*-methylglucamine solution (panel B). The values of $[Ca^{2+}]_i$ remained below the normal basal levels of 100 nM during the entire period of superfusion with the $0Na^+$/*N*-methylglucamine/ouabain solution. That the cell remained viable at the end of the experiment was demonstrated by the application for 10 s of a K^+-enriched Krebs-Hepes solution (35 mM NaCl replaced by 35 mM KCl). A sharp elevation of $[Ca^{2+}]_i$ to around 0.8 μM was observed upon the K^+ challenge (right part of the trace shown in panel B).

Panel C in Fig. 1 shows the effects of replacing Na_o^+ by Li^+. After a 3- to 4-min delay period, the $0Na^+/Li^+$ solution caused a gradual increase of $[Ca^{2+}]_i$ from resting levels of 0.1 μM to a plateau around 0.4–0.5 μM. The plateau demonstrated small oscillations which arose from the elevated $[Ca^{2+}]_i$ and never declined to resting levels. A similar pattern was observed when ouabain was given in the $0Na^+/Li^+$ solution, but with some differences (panel D): instead of the gradual increase of $[Ca^{2+}]_i$ seen in panel C, here once the cell was superfused with the $0Na^+/Li^+$/ouabain solution, a brisk increase of $[Ca^{2+}]_i$ to a plateau was seen. Also, under these experimental conditions the oscillations of $[Ca^{2+}]_i$ seemed to be greater, and the level of the plateau higher.

3.2. Effects of Ca_o^{2+} reintroduction on $[Ca^{2+}]_i$ in cells treated with ouabain or Li^+

The experiments described in the previous section explored the gradual development of changes in the $[Ca^{2+}]_i$ in the continued presence of 2.5 mM $[Ca^{2+}]_o$. In the experiments described here, the treatment of the cells with ouabain or Li^+ was performed first in the absence of Ca_o^{2+}

and the presence of Na_o^+, to allow the gradual accumulation of Na_i^+ ($0Ca^{2+}$ solution). Initially a cell was continuously superfused with a $0Ca^{2+}$ solution containing 10 μM ouabain. Ca^{2+} (2.5 mM) was reintroduced for 2-min periods at 15-min intervals. During the first Ca_o^{2+} introduction the $[Ca^{2+}]_i$ increases slowly from 0.1 μM to a plateau around 0.2 μM. The second reintroduction produced a higher plateau (around 0.4 μM). The third increased the $[Ca^{2+}]_i$ to 1 μM and the fourth the $[Ca^{2+}]_i$ to 1.2 μM. It should be noted that the increases of $[Ca^{2+}]_i$ were sustained and $[Ca^{2+}]_i$ remained at the increased or at slightly decreased level during the 2-min period of Ca_o^{2+} application. It is also worth noticing that the $[Ca^{2+}]_i$ changes seen during the third and fourth Ca_o^{2+} reintroductions were of the same magnitude. So, after 30-min treatment with ouabain, the reintroduction of Ca_o^{2+} increased the $[Ca^{2+}]_i$ to a peak of 1.16 ± 0.15 μM ($n = 8$ cells).

An experiment similar to that made with ouabain was performed with a $0Ca^{2+}/0Na^+/Li^+$ solution. Cells were continuously superfused with this Li^+ solution; at 15, 30, 45 and 60 min the Ca^{2+}-containing solution (in $0Na^+/Li^+$) was introduced for 2-min periods. Some differences emerged when comparisons with the ouabain traces were made: (i) the initial $[Ca^{2+}]_i$ elevation was sharper; (ii) the maximum increase was seen at the second Ca_o^{2+} reintroduction; (iii) the plateau seemed to be more stable. In Li^+-treated cells, the reintroduction of Ca_o^{2+} increased the $[Ca^{2+}]_i$ to a peak of 0.55 ± 0.05 μM ($n = 7$ cells).

3.3. Effects of wide-spectrum Ca^{2+} channel blockers on the increase of $[Ca^{2+}]_i$ induced by Ca_o^{2+} reintroduction in ouabain-treated cells

The two cells shown in panels A and B of Fig. 2 were pretreated for 30 min with ouabain (10 μM, in a $0Ca^{2+}$ solution) and then Ca^{2+} (2.5 mM) was reintroduced in the absence of Na^+ (*N*-methylglucamine) for a 2-min period. In A, no drug was given between the first and the second Ca^{2+} reintroductions. The profile of the increases in $[Ca^{2+}]_i$ were quite similar, exhibiting phasic and tonic components. In B, ω-conotoxin MVIIC (3 μM) was present for 5 min before and during the 2-min period of the second Ca^{2+} reintroduction. The first Ca_o^{2+} pulse gave the usual phasic and tonic components of $[Ca^{2+}]_i$; however, ω-conotoxin MVIIC suppressed the phasic component but left untouched the tonic plateau at around 0.5 μM $[Ca^{2+}]_i$.

R56865 has been shown to be an organic wide-spectrum Ca^{2+} channel blocker in bovine chromaffin cells (Garcez-do-Carmo et al., 1993). Therefore, it was tested in a protocol similar to that for ω-conotoxin MVIIC. Fig. 2C shows a cell initially treated with ouabain in $0Ca^{2+}$ for 30 min. The first Ca^{2+} reintroduction produced an elevation of $[Ca^{2+}]_i$ to 1.7 μM followed by a plateau at around 1.2 μM. The second Ca^{2+} reintroduction was done in the presence of 10 μM R56865; both the peak and the plateau were markedly reduced.

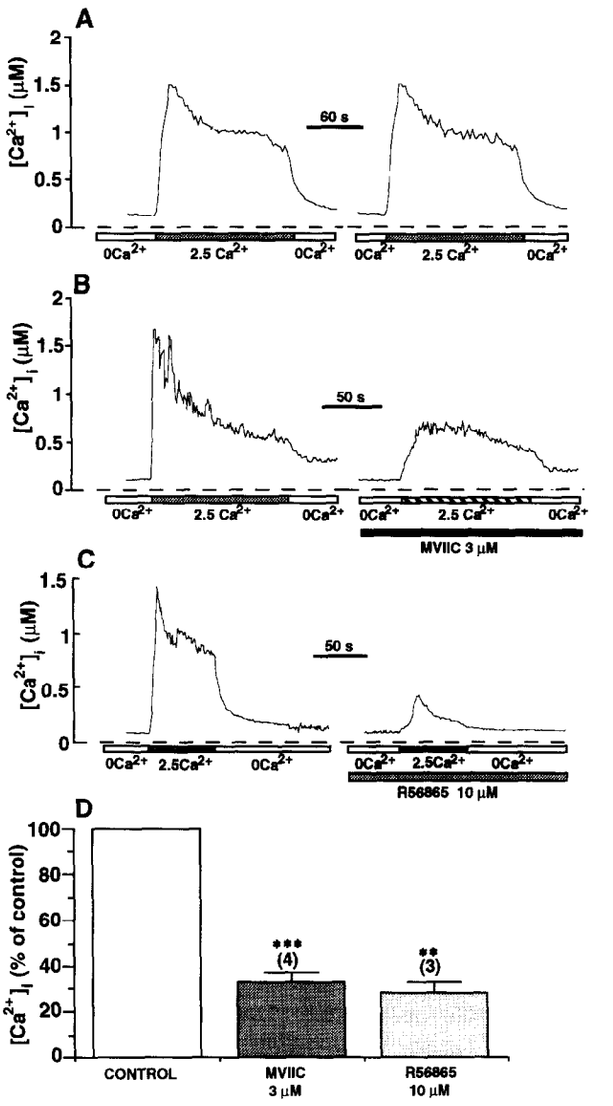

Fig. 2. Changes of $[Ca^{2+}]_i$ induced by Ca^{2+} reintroduction and effects of ω-conotoxin MVIIC (MVIIC) and R56865 on such changes. Cells were superfused with ouabain (10 μM) in a Ca^{2+}-free solution during 30 min. Then 2.5 mM Ca^{2+} was introduced in a Na^+-free Krebs-Hepes solution (NaCl was replaced by equimolar concentrations of *N*-methylglucamine). Ca^{2+} reintroductions were done at 15-min intervals (as shown on the bottom horizontal bars). In B, MVIIC 3 μM was present 5 min before and during the second Ca^{2+} reintroduction. Panel C shows the effects of R56865 on $[Ca^{2+}]_i$ responses induced by Ca^{2+} reintroduction in ouabain-treated cells. R56865 (10 μM) was applied 5 min before and during the second Ca^{2+} reintroduction, as shown on the bottom horizontal bar below the record. Panel D shows the averaged values concerning the effects of R56865 and MVIIC on $[Ca^{2+}]_i$ signals. Values are means ± S.E.M. Control represents peak values obtained after the Ca^{2+} reintroduction. Values were normalised to % of peaks and are means ± S.E.M. of the number of experiments shown in parentheses. ** $P < 0.01$, *** $P < 0.001$ with respect to the control response.

Fig. 2D shows averaged results from various cells treated with ω-conotoxin MVIIC or R56865, using the Ca_o^{2+} reintroduction protocols in ouabain-treated cells. ω-Conotoxin MVIIC reduced the $[Ca^{2+}]_i$ peak by 67 ± 4%

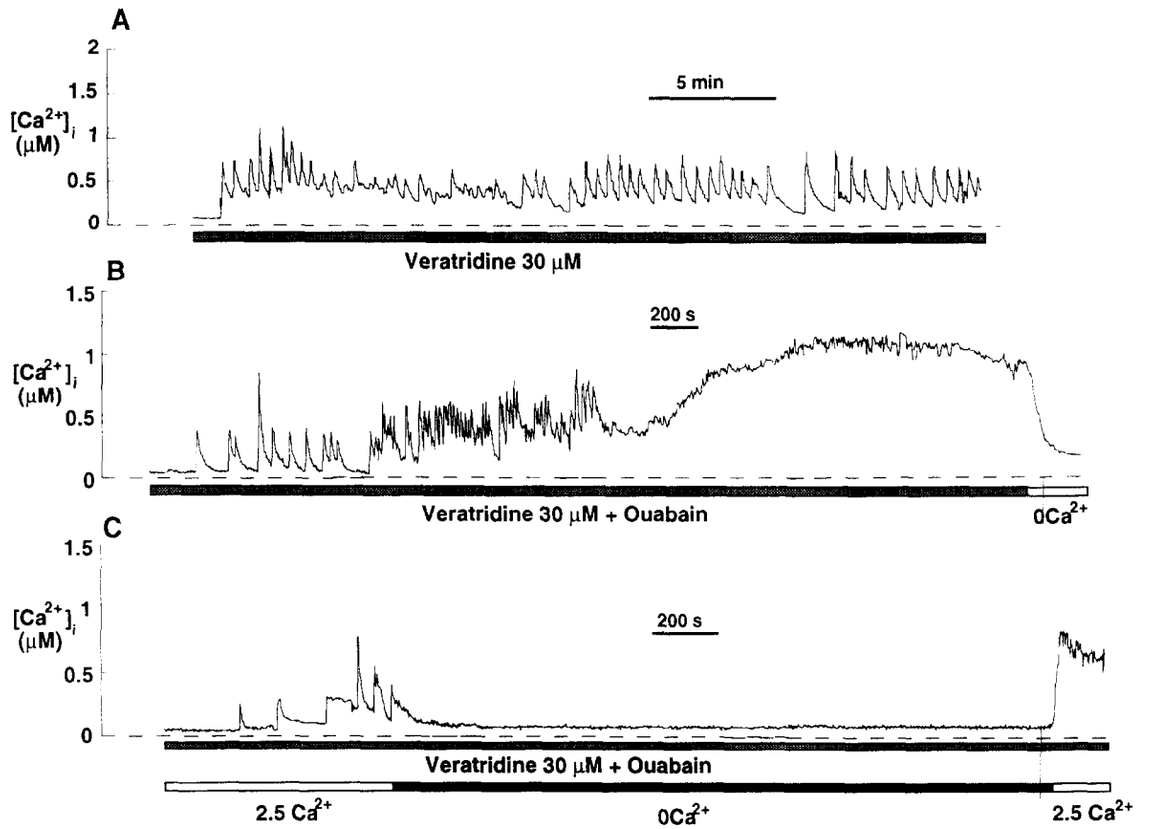

Fig. 3. Oscillation of $[Ca^{2+}]_i$ induced by veratridine (A) and the effect of ouabain (B and C) on this oscillatory pattern. Veratridine was applied to three different cells loaded with fura-2 as shown by the horizontal bars. In B, ouabain (10 μM) was introduced along with veratridine. In C, a third cell was superfused first with veratridine (30 μM) + ouabain (10 μM) in a normal Krebs-Hepes solution and then with a Ca^{2+}-free solution, as shown by the horizontal bottom bar. At the end, extracellular Ca^{2+} was reintroduced again. Similar results were observed in 4 (A), 4 (B) and 3 (C) cells.

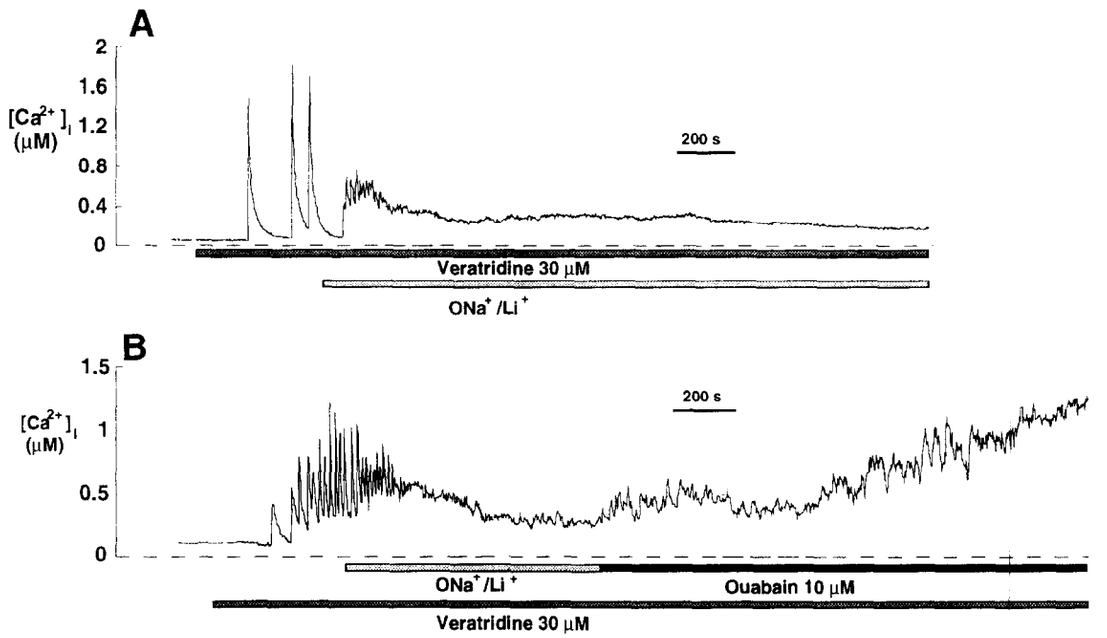

Fig. 4. Effect of lithium on the oscillations of $[Ca^{2+}]_i$ induced by veratridine (30 μM). The cell was incubated with veratridine 30 μM; once the oscillatory pattern was established, a Na^+-free Krebs-Hepes solution was introduced (NaCl was replaced by equimolar concentrations of LiCl). In a second cell (B), ouabain 10 μM was introduced in a Krebs-Hepes normal solution, after perfusion with LiCl (144 mM). Similar results were observed in 3 cells.

($n = 4$ cells) and R56865 caused a reduction of 72 ± 5% ($n = 3$ cells).

3.4. Effects of ouabain and Li^+ on the oscillations of $[Ca^{2+}]_i$ induced by veratridine

By delaying the inactivation of the Na^+ channel (Ohta et al., 1973), veratridine causes cyclic cell depolarisation and an oscillatory pattern of $[Ca^{2+}]_i$ in bovine chromaffin cells (López et al., 1995). These oscillations usually start 2–3 min after of initiation of the superfusion with 30 μM veratridine (Fig. 3A). They are irregular in magnitude (0.5–1.2 μM Ca_i^{2+}) and of low frequency (0.05–0.1 Hz).

When ouabain and veratridine were added simultaneously (Fig. 3B), the oscillatory pattern changed considerably. During the first 10 min, the cell started to fire irregular and infrequent $[Ca^{2+}]_i$ oscillations, starting at baseline. During the following 10–15 min the baseline $[Ca^{2+}]_i$ rose to 0.3–0.4 μM and the oscillations were more frequent but smaller. Finally, the oscillations disappeared as the baseline $[Ca^{2+}]_i$ increased to a stable plateau at around 1 μM. At the end of the 60-min period of superfusion with veratridine plus ouabain, the cell was still viable since Ca_o^{2+} removal quickly decreased the $[Ca^{2+}]_i$ from 1 μM to almost the initial basal level.

In the third cell (Fig. 3C), treatment with veratridine plus ouabain was initiated in normal Krebs-Hepes solution containing 2.5 mM Ca^{2+}. Some small and infrequent oscillations were observed. After 10 min, Ca_o^{2+} was removed and the oscillations disappeared immediately. Then, after superfusion with $0Ca^{2+}$ for 55 min, Ca_o^{2+} (2.5 mM) was reintroduced. The $[Ca^{2+}]_i$ rapidly rose from a basal level below 0.1 μM to a sustained, non-oscillatory plateau at around 0.8 μM.

Fig. 4 shows the effects of Li^+ on the veratridine-induced $[Ca^{2+}]_i$ oscillations. The cell of panel A was initially superfused with veratridine in normal Krebs-Hepes solution. After 2–3 min, large $[Ca^{2+}]_i$ oscillations of 1.5–1.7 μM started. After 5 min, all NaCl of the veratridine superfusing solution was replaced by LiCl. The baseline $[Ca^{2+}]_i$ immediately rose to around 0.4 μM, the oscillations were drastically reduced and after 1 min they were fully suppressed. The $[Ca^{2+}]_i$ remained elevated at 0.3–0.4 μM for the 30-min period of superfusion with the $0Na^+/Li^+$ solution.

In another cell (Fig. 4B) veratridine initially caused frequent and large $[Ca^{2+}]_i$ oscillations. After replacement of Na^+ by Li^+, the oscillations were reduced in size and soon disappeared. After 14 min of superfusion with the Li^+ solution, ouabain was then added. After 10–15 min, in which time the $[Ca^{2+}]_i$ was maintained at around 0.5 μM, the $[Ca^{2+}]_i$ started to increase gradually to become higher than 1.2 μM at the end of the experiment. It is worth noticing that some oscillations reappeared during treatment with combined ouabain plus veratridine.

4. Discussion

The main findings of this study are that ouabain and replacement of Na_o^+ by Li^+ elevated the $[Ca^{2+}]_i$ in single fura-2-loaded bovine chromaffin cells. The elevation of $[Ca^{2+}]_i$ was mild with ouabain alone, greater with $0Na^+/Li^+$ and faster and higher with combined ouabain plus $0Na^+/Li^+$ (Fig. 1). This suggests that the activity of the Na^+ pump indirectly controls the $[Ca^{2+}]_i$, and that $[Ca^{2+}]_i$ is a function of the concentration of monovalent cations in the cytosol: the higher their concentration the greater the $[Ca^{2+}]_i$ changes. The fact that the affinity of Li^+ for the Na^+ binding site in the Na^+ pump is lower than that for Na^+ (Keynes and Swan, 1959) will prevent Li^+ extrusion to the extracellular space once it enters the cytosol. This will lead to a faster accumulation of Li^+ in the cytosol, and also to decrease of the Li^+ gradient. The question now arises as to which mechanisms link Na^+ and Li^+ changes to Ca_i^{2+} changes. Two possible mechanisms might be involved: one relates to changes in the activity of the Na^+-Ca^{2+} transporter, the other to cell depolarisation secondary to accumulation of monovalent cations in the cytosol.

Evidence for the depolarising effects of Na^+ and Li^+ in ouabain-treated cells comes from two sets of experiments. One set of experiments demonstrated the selective blockade by ω-conotoxin MVIIC or R56865 of the phasic component of the $[Ca^{2+}]_i$ elevations produced by Ca_o^{2+} reintroduction. ω-Conotoxin MVIIC and R56865 are known to inhibit most of Ca_o^{2+} entry through voltage-dependent Ca^{2+} channels in bovine chromaffin cells depolarised with high K^+ concentrations (López et al., 1994; Garcez-do-Carmo et al., 1993). These channels open only under depolarising conditions, and thus the effects of ω-conotoxin MVIIC and R56865 in ouabain-treated cells can be explained best if these cells were depolarised. The second set of experiments relates to the rapid suppression by Li^+ or ouabain of the veratridine-induced $[Ca^{2+}]_i$ oscillations. Such oscillations were shown to be due to oscillatory depolarisations of the membrane potential, and were abolished when the cell was mildly depolarised with high K^+ or with current injection through a patch-clamp pipette (López et al., 1995). The fact that ouabain (Fig. 3) and Li^+ (Fig. 4) also suppressed the veratridine-induced $[Ca^{2+}]_i$ oscillations strongly supports the notion that these two manoeuvres cause cell depolarisation. Such depolarisation might cause the triggering of action potentials that will induce alternative opening and closing of Ca^{2+} channels, thus explaining the oscillatory nature of the observed $[Ca^{2+}]_i$ changes (see particularly panel D in Fig. 1).

But the Ca_i^{2+} signal in response to Ca^{2+} reintroduction in ouabain-treated cells had phasic and tonic components. As discussed above, the selective inhibition by ω-conotoxin MVIIC and R56865 of the phasic component indicates the participation of cell depolarisation and voltage-dependent Ca^{2+} channels in its generation. However, in

the presence of these channel blockers, the tonic plateau component of the $[Ca^{2+}]_i$ elevation remained. This component is likely due to a slowing down of Na^+-Ca^{2+} exchanger activity due to a gradual decrease of the Na_o^+/Na_i^+ gradient. In ouabain-treated cells, the interruption of Na_i^+ pumping will allow a time-dependent accumulation of Na_i^+. The fact that the $[Ca^{2+}]_i$ increase upon Ca_o^{2+} reintroduction was greater the longer the exposure time to ouabain suggests that the Na^+-Ca^{2+} exchanger was being gradually inactivated as Na^+ accumulated in the cytosol. Thus, Ca^{2+} extrusion will be delayed and the generated $[Ca^{2+}]_i$ increase will be greater.

The rapid and sharp elevation of $[Ca^{2+}]_i$ induced by Ca^{2+} reintroduction in cells superfused with a $0Na^+/Li^+$ solution strongly suggests that Li^+ readily binds to the Na^+ site in the Na^+-Ca^{2+} exchanger. That the chromaffin cell Na^+-Ca^{2+} exchanger can recognise Li^+ was already suggested on the basis of functional experiments measuring catecholamine release from perfused cat adrenal glands. Abajo et al. (1987) demonstrated that Li^+ loading of chromaffin cells caused a sharp secretory response upon Ca^{2+} reintroduction; this experimental protocol is similar to that used here to demonstrate the elevation of $[Ca^{2+}]_i$ by Ca_o^{2+} reintroduction in Li^+-loaded chromaffin cells. Given the fact that Li^+ exhibits poorer affinity than Na^+ for the Na^+ site on the Na^+ pump (Keynes and Swan, 1959), the Li^+ that had gained access to the cell interior would leave the cell with more difficulty than Na^+. Thus, in a way, treatment of cells with a $0Na^+/Li^+$ solution might create a situation similar to that after ouabain treatment in the presence of Na_o^+; in the first case Li^+ will accumulate in the cytosol, in the second cytosolic Na^+ accumulation will be produced. In both cases, cell depolarisation and a slowing down of the $Na^+(Li^+)$-Ca^{2+} exchanger will be the final outcome.

Our results provide evidence in favour of the ionic mechanisms proposed to be involved in the secretory effects of Li^+ and ouabain. For instance, Abajo et al. (1987) concluded that 'Li^+ causes an increase in the rate of catecholamine release 'and suggest that' the main mechanism for this response could involve Li^+-Ca^{2+} exchange'. The suggestion in that earlier report, that activation of a Li^+-Ca^{2+} exchange mechanism leads to an increase of $[Ca^{2+}]_i$ that subsequently promotes catecholamine secretion, is now clearly confirmed by the results shown in Figs. 1 and 2. Our results also agree with an earlier study showing that ouabain causes catecholamine release from perfused cat adrenal glands through a dual mechanism, chromaffin cell depolarisation and slowing down of the Na^+-Ca^{2+} exchanger (Artalejo and García, 1986). However, our results disagree with the observation of Pocock (1983a) that the secretory effects of ouabain in bovine chromaffin cells are maintained in the nominal absence of Ca^{2+}, since ouabain certainly increased the $[Ca^{2+}]_i$ and this effect was Ca_o^{2+}-dependent. As stated in the Introduction, several other studies have shown that the ouabain-induced catecholamine release from perfused adrenal glands (Banks, 1967; Abajo et al., 1987, 1989; Esquerro et al., 1980) or isolated chromaffin cells (Aunis and García, 1981) has an absolute requirement for Ca_o^{2+}.

In conclusion, the results presented here are compatible with ouabain causing elevation of $[Ca^{2+}]_i$ in bovine chromaffin cells by a dual mechanism, i.e. cell depolarisation and slowing down of the Na^+-Ca^{2+} exchanger activity of the plasmalemma. Depolarisation suggests that the Na^+ pump in these cells is electrogenic. In addition, the experiments with Li^+ indicate that this cation readily binds to the Na^+ site on the Na^+-Ca^{2+} exchanger, thereby generating powerful Ca_i^{2+} signals. These signals might be relevant to the therapeutic actions of Li^+, which are probably linked to its effects on neurosecretion mechanisms (Abajo et al., 1987, 1989).

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