A rise in the intracellular Ca^{2+} concentration of isolated rat supraoptic cells in response to oxytocin

Regis C. Lambert, Govindan Dayanithi*, Françoise C. Moos and Philippe Richard

Laboratoire de Neurobiologie Endocrinologique, URA 1197-CNRS, Université Montpellier II, 34095 Montpellier cedex 5, France

1. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored in single cells isolated from adult rat supraoptic (SO) nuclei. The great majority of cells (85%) were neurones and most were immunoreactive to oxytocin or to vasopressin (AVP).
2. The resting $[Ca^{2+}]_i$ of the majority (80%) of the neurones remained stable while 20% of the neurones displayed spontaneous $[Ca^{2+}]_i$ oscillations which disappeared in low-Ca^{2+} (100 nM) EGTA buffer.
3. Addition of 100 nM oxytocin increased the $[Ca^{2+}]_i$ in both stable and oscillating cells. Two types of responses were observed: (i) a sustained response with $[Ca^{2+}]_i$ being maintained at an elevated level and (ii) a brief response with $[Ca^{2+}]_i$ quickly returning to a near-resting level. Responses were reproducible, dose dependent and blocked with a specific oxytocin antagonist.
4. Removal of extracellular Ca^{2+} did not block the oxytocin response. In EGTA buffer, application of thapsigargin (200 nM) onto oxytocin-sensitive cells induced an increase in $[Ca^{2+}]_i$ and inhibited the oxytocin response. These effects were not induced by other intracellular Ca^{2+} mobilizers such as tBuBHQ (see Methods) or caffeine.
5. In conclusion, half of the SO cells respond to oxytocin with a rise in $[Ca^{2+}]_i$. The effect is mediated by oxytocin receptors and results from release of Ca^{2+} from thapsigargin-sensitive stores.

It is well established that oxytocin facilitates the periodic bursting activity (high-frequency bursts of spikes) displayed by magnocellular oxytocin neurones during suckling (Freund-Mercier & Richard, 1984). Several lines of evidence indicate that oxytocin exerts a crucial part of this facilitatory control directly in the hypothalamic magnocellular nuclei, containing the oxytocin neurones. Indeed, during suckling, local injections of oxytocin into the supraoptic (SO) or paraventricular nuclei increases the total number of spikes per burst and decreases the interburst interval (Moos & Richard, 1989). Furthermore, the local release of oxytocin within the magnocellular nuclei increases during the milk-ejection reflex, as demonstrated *in vivo* with push–pull perfusion and microdialysis techniques (Moos, Poulain, Rodriguez, Guerné, Vincent & Richard, 1989; Neumann, Russell & Landgraf, 1993). This raises the possibility of direct action of oxytocin on oxytocin neurones. *In vitro*, electrophysiological studies have demonstrated that oxytocin increases the firing rate of putative oxytocin neurones in hypothalamic slices of male rats (Inenaga & Yamashita, 1986; Yamashita *et al.* 1987), and decreases the firing rate in virgin female rats (Kuriyama, Nakashima, Kawarabayashi & Kiyohara, 1993), both effects persisting in Ca^{2+}-free medium (Yamashita *et al.* 1987; Kuriyama *et al.* 1993). As Ca^{2+}-free medium is assumed to block the synaptic transmission, the above results suggest direct action of oxytocin on oxytocin neurones.

Since it has been demonstrated in other cell types that the oxytocin response is dependent on extracellular Ca^{2+} (mammary myoepithelial cells; Olins & Bremel, 1984) or involves Ca^{2+} release from intracellular pools (myometrial cells: Batra, 1986; Anwer & Sanborn, 1989; and corticotrophs: Link, Dayanithi, Föhr & Gratzl, 1992), we have studied the effect of oxytocin on intracellular Ca^{2+} concentration in freshly dissociated SO cells.

*To whom correspondence should be addressed.

METHODS

Dissection

In each experiment, two male or two female Wistar rats (180–220 g body weight) were killed by decapitation. After removing the brain, two blocks of basal hypothalamic tissues (2 mm long, 1 mm thick) were dissected. They comprised part of the optic tract to the optic chiasm and a strip of tissue lateral to the optic tract (500 μm width). The exact position and extent of the blocks were chosen in order to contain the SO nuclei. After dissection, the tissues were transferred into Locke buffer containing (mM): NaCl, 140; KCl, 5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 1·8; glucose, 10; Hepes, 10. The Locke buffer was adjusted to pH 7·2 with NaOH. The osmolarity of the solution was maintained between 290 and 300 mosmol l^{-1}.

Isolation of cells from the SO nuclei

After washing, the dissected tissues were enzymatically dissociated by incubation in Locke buffer supplemented with 5 μg ml^{-1} deoxyribonuclease II (DNase II; Sigma Chemical Co., St Louis, MO, USA), 0·21% trypsin (Worthington Biochemical Corp., Freehold, NJ, USA), and 0·025% bovine serum albumin (BSA) tissue culture grade (Miles Laboratories, Kankakee, IL, USA) for 30 min at 34 °C in a shaking water bath. The tissues were further dispersed by gentle mechanical trituration for about 10 min in the same medium. The cell suspension was filtered through a 100 μm nylon mesh and centrifuged for 5 min at 100 *g*. The pellet was resuspended in 5 ml of Locke buffer containing 0·05% trypsin inhibitor (Sigma) and centrifuged as above. The final pellet was resuspended in Locke buffer for dye loading or immunostaining procedures.

Dye loading and measurement of intracellular free Ca^{2+}

The Ca^{2+}-sensitive dye fura-2 AM (acetoxymethyl form; Molecular Probes Inc., Eugene, OR, USA) was first dissolved in pure, water-free dimethyl sulphoxide (DMSO) and then diluted to 2 μM fura-2 AM and 0·01% DMSO with 5 ml of Locke buffer, containing the cells. For $[Ca^{2+}]_i$ measurement experiments, the above cell–dye mixture was distributed onto non-polylysine-coated coverslips (35 mm diameter, 0·13–0·17 mm thick; Poly Labo, Paul Block & Cie, Strasbourg, France). Dye loading was carried out for 35 min at 37 °C in a humidified atmosphere (5% CO_2–95% O_2). The incubation period allowed adhesion of cells on the coverslip. Subsequently, the unadhered cells were washed off with Locke buffer. $[Ca^{2+}]_i$ measurements were performed up to 4–8 h after dye loading.

Fluorescence measurements of $[Ca^{2+}]_i$ were performed with the Zeiss Microscope Photometer System (Fast Fluorescence Photometer-FFP; Zeiss, Oberkochen, Germany), based on an inverted microscope (Axiovert 10; Zeiss) equipped for epifluorescence (×100 oil immersion objective lens). The preparations were alternately (200 Hz) illuminated at wavelengths of 340 and 380 nm. In order to minimize the background noise of the fura-2 signal, successive values were averaged to a final time resolution of 300 ms.

Fura-2 calibration

For the *in vitro* calibration of $[Ca^{2+}]_i$ measurements, we used Ca^{2+}–EGTA buffer containing (mM): NaCl, 140; $MgCl_2$, 2; glucose, 10; Hepes, 10 at pH 7·2, adjusted with NaOH. Various standards were used for system calibration, i.e. (1) fura-2 with Ca^{2+} (10, 100, 1000 and 10000 nM) and (2) fura-2 without Ca^{2+} (see 'Solutions and drugs' below). The concentration of fura-2 in these standards was 20 μM and the standards gave fluorescence intensities similar to those of the cells being measured. The calibration of the fluorescence signal, in terms of free-Ca^{2+} concentration, was based on the procedure described by Grynkiewicz, Poenie & Tsien (1985):

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

with:

$$R = FI_1 - AFI_1 / FI_2 - AFI_2,$$

where R_{max} is the ratio of fluorescence intensities from wavelengths 340 and 380 nm for a maximum concentration of Ca^{2+} in an artificial medium; R_{min} is the ratio of fluorescence intensities from wavelengths 340 and 380 nm for a minimum concentration of Ca^{2+} in an artificial medium. These values were obtained by adding an excess of either $CaCl_2$ or Tris base–EGTA, pH > 8·3. K_d represents the dissociation constant of the complex of the ion with the indicator dye at 37 °C in an artificial ionic medium with the ratio (Sf_{380}/Sb_{380}) representing fluorescence intensities at 380 nm of fura-2-free (Sf_{380}) and Ca^{2+}-bound fura-2 (Sb_{380}) solutions. R is the ratio of (autofluorescence − corrected) fluorescence intensities. FI_1 and FI_2 are the raw fluorescence intensities measured at (excitation) wavelengths 1 (340 nm) and 2 (380 nm). AFI_1 and AFI_2 are the autofluorescence intensities measured at (excitation) wavelengths 1 and 2. β is the ratio of fluorescence intensity at 380 nm of the free dye to fluorescence intensity at 380 nm of the bound dye.

The intensities FI_1 and FI_2, and autofluorescence values AFI_1 and AFI_2 were determined during measurement. To calculate $[Ca^{2+}]_i$ values from measured data the parameters $K_d = 224$, $R_{min} = 0{\cdot}350$, $R_{max} = 6{\cdot}050$ and $\beta = 7{\cdot}750$ were specified in the calibration menu. The calibration menu allows input of the parameters of Tsien's equation either via keyboard or loading them from a file.

Solutions and drugs

Each experiment was performed on single cells on individual coverslips containing 500 μl of buffer. The Locke buffer, described above, served as a control medium. To test the effects of extracellular Ca^{2+}, an EGTA buffer was used which contained (mM): ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma), 0·1; NaCl, 140; KCl, 5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; glucose, 10; Hepes, 10, adjusted to pH 7·2 with NaOH. The osmolarity of the solution was maintained between 290 and 300 mosmol l^{-1}. Free Ca^{2+} concentration of the solution was in the 100 nM range which corresponds to the resting $[Ca^{2+}]_i$ typically observed in neurones.

Oxytocin and vasopressin (AVP; Boehringer Mannheim, Meylan, France) and [$d(CH_2)_5$,Tyr(Me)^2,Orn^8]-vasotocin ($d(CH_2)_5$OVT; Peninsula Lab, Belmont, CA, USA) were directly dissolved in the recording buffer. Stock solutions of 2,5-di-(ter-butyl)-1,4-benzohydroquinone (tBuBHQ; Alomone Labs, Jerusalem, Israel), and thapsigargin (TG; Alomone Labs) were first disolved in DMSO and further diluted in EGTA buffer at appropriate concentrations. Caffeine (Alomone Labs) was directly dissolved in EGTA buffer and osmolarity was adjusted by reducing the NaCl concentration. K^+ solution contained (mM): NaCl, 97; KCl, 48; KH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 1·8; glucose, 10; Hepes, 10, adjusted to pH 7·2 with NaOH.

Drops of test substances (10 μl) were applied in the proximity of the recorded cell. This method allowed rapid and highly reproducible changes in the medium surrounding a selected cell. Other methods, such as rapid perfusion of the test

substances, resulted in washing away of the selected cell. The drug concentrations indicated in the results were 1/50th of their concentration in the drop (10 in 500 μl). However, for K^+ application, 250 μl of the K^+ solution was added to the recording medium and the actual concentration of the solution is indicated in Results (since Locke buffer already contains 5 mM of this ion).

Immunostaining

Immunostaining was performed on cells dissociated from the SO nuclei of adult female rats (four SO nuclei in 2 ml buffer). The cell suspension (300 μl) was plated onto coverslips coated with polylysine (Sigma, 1% in distilled H_2O) and left at room temperature for 30 min to allow adhesion to the coverslips. Plated cells were then rinsed twice with phosphate-buffered saline (PBS, 0·1 M; pH 7·3) before fixation and incubation, the procedures of which varied according to the antibody used.

For immunostaining of oxytocin or AVP, cells were fixed with a paraformaldehyde solution (4% in PBS for 10 min). Following three washes in PBS, the cells were incubated overnight at 4 °C in PBS containing 0·1% saponin (Sigma), 5% BSA (Sigma), and specific antibodies directed against either oxytocin or AVP (both at 1:400 dilution). The specificity of these antibodies (generously supplied by Dr P. Siaud, Université Montpellier II, Montpellier, France) has been reported elsewhere (Alonso, 1988; Siaud, Denoroy, Assenmacher & Alonso, 1989). Control staining (blank) was performed by adding an anti-rabbit non-specific immunoserum (NIS; 1:400 dilution; Biosys, Compiègne, France) instead of the oxytocin or AVP antibody. Cells were then rinsed 3 times with PBS before incubation (for 60 min) with PBS containing the fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (1:100 dilution; Biosys) and 5% BSA. After three rinses in PBS, coverslips were mounted on glass slides in glycerin buffer (1%) and conserved in darkness at 4 °C.

For immunostaining of glial cells or neurones, fixation was performed with a methanol–ether solution (3/2; v/v) for 10 min at −80 °C. Following three washes in PBS, cells were incubated for 2 h in PBS containing 5% BSA and specific anti-

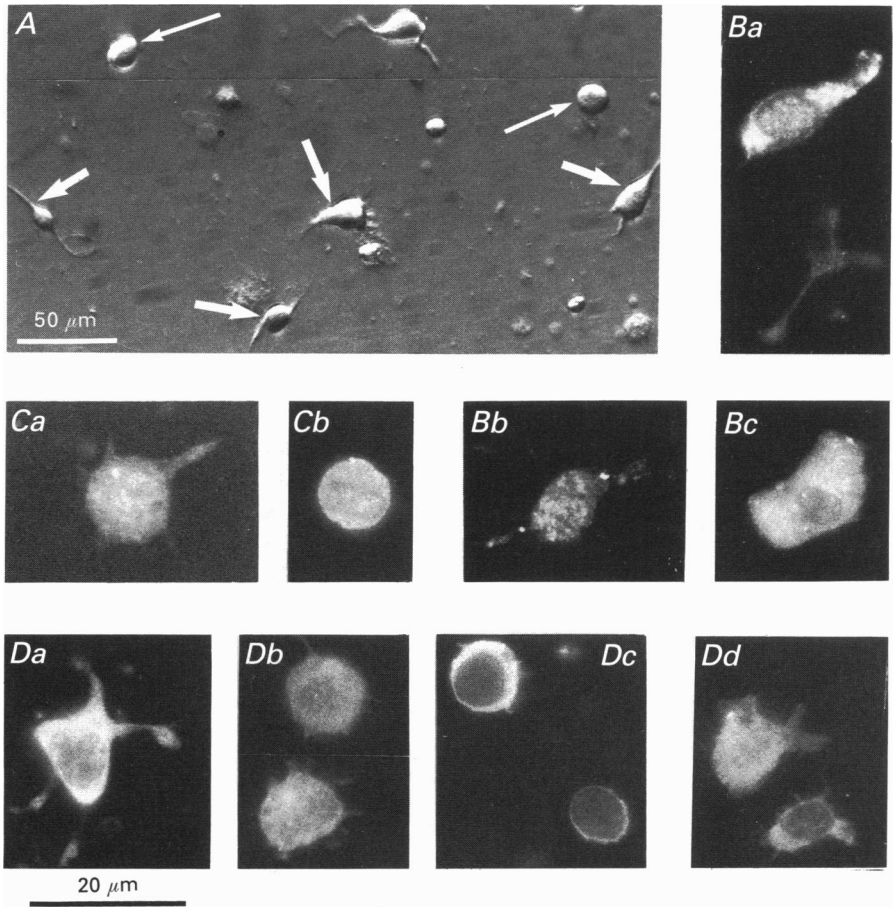

Figure 1. Morphology of dissociated supraoptic cells

A, field of plated cells under Hoffman modulation contrast optics. Note the bright appearance of viable cells with (thick arrows) or without (thin arrows) processes or neurites. Cells having a granular drab aspect (arrowheads) were considered as dead or unhealthy. *Ba–c*, different types of neurones immunostained with an antibody directed against neurofilaments and revealed by the appropriate fluorescent (Cy 3) conjugate. *Ca* and *b*, two examples of immunofluorescent AVP neurones revealed by the appropriate fluorescent (FITC) conjugate. *Da–d*, examples of immunofluorescent oxytocin neurones using the same conjugate as in *C*. Neurones retain either two short, large (*Da*) or multiple short fine (*Db*) processes. In *Dc* the larger cell is an oxytocin cell.

bodies directed against glial fibrillary acidic protein (GFAP; 1:1000 dilution; monoclonal anti-mouse anti-immunoglobulin-g (IGg anti-GFAP, Sigma) or against neurofilaments (1:1000 dilution; rabbit anti-NF-M, gift from Dr J. F. Leterrier, INSERM, Angers, France). Respective control stainings were performed by adding 5% BSA or anti-rabbit NIS (1:1000 dilution) instead of the specific antibodies. After three rinses in PBS, cells were incubated for 60 min respectively with the rhodamine-conjugated goat anti-mouse immunoglobulin (1:200 dilution; Chemicon International Inc., Temecula, CA, USA) in PBS containing 5% BSA and 1% sodium azide, or with the Cyanin dye, Cy 3-conjugated anti-rabbit immunoglobulin (1:500 dilution; Chemicon International Inc.) in PBS containing 5% BSA. Thereafter, coverslips were rinsed 3 times with PBS and mounted on glass slides in glycerin buffer and conserved in darkness at 4 °C. In the different controls, lack of immunofluorescent cells attested for the specificity of the stainings.

After depositing 300 μl of suspension on a coated coverslip, several hundred cells consistently adhered. However, when considering cells over 10 μm diameter or length (for ovoid cells) only, the mean number of cells per coverslip was 223 ± 3 cells ($n = 7$) indicating a rather homogenous repartition of cells on the different coverslips. Counting each type of immunostained cell revealed (per coverslip): 182 neurones, 74 oxytocin cells, 83 AVP cells and 31 glial cells. Considering that one adult rat SO nucleus contains about 5000 oxytocin/AVP neurones (Léránth, Záborszky, Marton & Palkovits, 1975), our procedures (dissection of 4 SO nuclei, dissociation in 2 ml buffer, plating of 300 μl) allowed 5·2% of magnocellular neurones to consistently adhere to the coverslips. This yield is comparable with that obtained by Oliet & Bourque (1992) using similar procedures.

All results given as means ± S.E.M. unless stated otherwise.

RESULTS

Experiments were performed on SO cells isolated from virgin female rats ($n = 139$) or male rats ($n = 25$). Resting $[Ca^{2+}]_i$ and responses to oxytocin were measured in cells from both sexes. However, pharmacological studies of the oxytocin response were performed only on cells dissociated from SO nuclei of female rats.

Immunocytochemical observations

The morphological characteristics of all types of neurones (oxytocin, AVP or unidentified) were variable making them indistinguishable from each other (Fig. 1). About 25% of the neurones clearly displayed two to three short ($\leqslant 20$ μm) but thick processes or retained numerous very short, thin processes all around the cellular body. The diameter or length of the neurones rarely exceeded 22 μm (maximum 24 μm), the majority of the neurones being between 12 and 18 μm in diameter but glial cells measured less than 12 μm. Based on these observations, the cells used for $[Ca^{2+}]_i$ measurements in this study were restricted to those more than 12 μm in diameter (many having processes).

Resting $[Ca^{2+}]_i$ of supraoptic cells

A total of 139 SO cells from female rats were analysed for $[Ca^{2+}]_i$ under resting conditions in Locke buffer, for a period of 1–5 min. In 78% of the cells, the resting $[Ca^{2+}]_i$ remained stable around 43 ± 3 nM ($n = 109$) throughout the measuring period. However, thirty cells (22%) displayed spontaneous oscillations of $[Ca^{2+}]_i$ from 44 ± 6 to 118 ± 12 nM (Figs 2 and 7*B*). The characteristics of this phenomenon varied from cell to cell, oscillations being more or less regular with a mean periodicity of 56 ± 6 s. A spontaneous rise in $[Ca^{2+}]_i$ occurred within 5 s with a slower decay to base line. Incubation of the SO oscillating cells ($n = 4$) in EGTA buffer (low-Ca^{2+} medium) reversibly abolished the spontaneous oscillations of $[Ca^{2+}]_i$ (Fig. 2). The effect of the EGTA buffer was also investigated in fifteen cells with stable resting $[Ca^{2+}]_i$. The resting $[Ca^{2+}]_i$

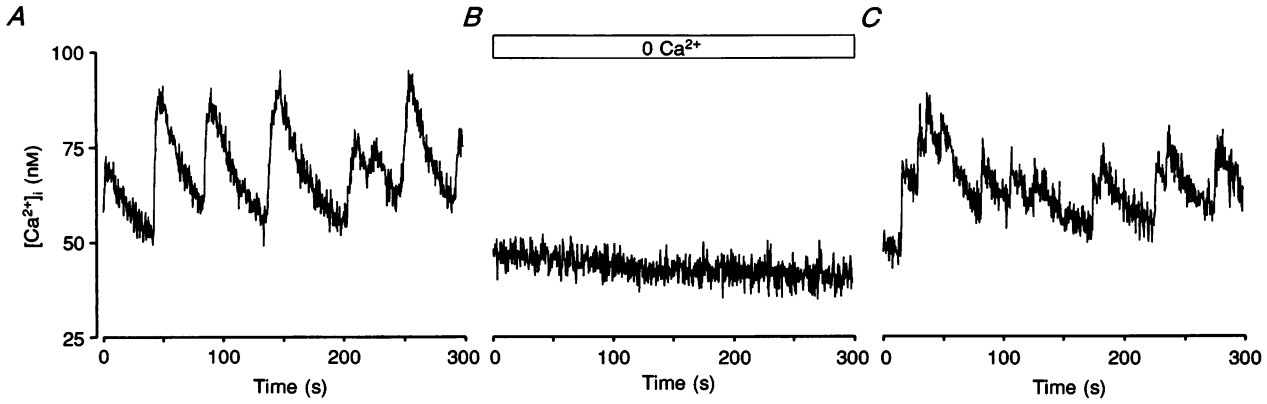

Figure 2. Effect of removing extracellular Ca^{2+} on spontaneous oscillations of $[Ca^{2+}]_i$ in a SO cell

A, the cell was observed under normal conditions for 5 min in Locke buffer (containing 1·8 mM Ca^{2+}). *B*, the medium was changed to EGTA buffer in which free Ca^{2+} was maintained at approximately 100 nM. *C*, after measurement of $[Ca^{2+}]_i$ for 5 min, the Locke buffer was reintroduced. Note that, in low extracellular Ca^{2+} concentration, the spontaneous oscillations disappeared and the effect is reversible.

was similar in high or low extracellular Ca^{2+} concentration (29 ± 3 or 31 ± 4 nM, respectively).

Of the twenty-five SO cells studied from male rats, twenty-one displayed a stable resting $[Ca^{2+}]_i$ of 53 ± 4 nM, and four showed spontaneous $[Ca^{2+}]_i$ oscillations from 50 ± 9 to 120 ± 20 nM with a mean periodicity of 55 ± 7 s.

For male or female rats, both types of cells (with stable or oscillating resting $[Ca^{2+}]_i$) were found in a given preparation and no morphological distinction could be made between them under microscopic observation.

Oxytocin-induced $[Ca^{2+}]_i$ rise

One hundred and thirty-five cells from female rats were tested for responses to oxytocin about 1 min after the beginning of the recording. Of these, twenty-six cells were spontaneously oscillating and 109 had a stable resting $[Ca^{2+}]_i$. In sixty (55%) of the latter cells, oxytocin (100 nM) caused a marked increase in $[Ca^{2+}]_i$ (peak, 339 ± 27 nM). The peak $[Ca^{2+}]_i$ rise was reached within 18 ± 2 s after oxytocin application (Fig. 3*A*). Similarly, in thirteen (50%)

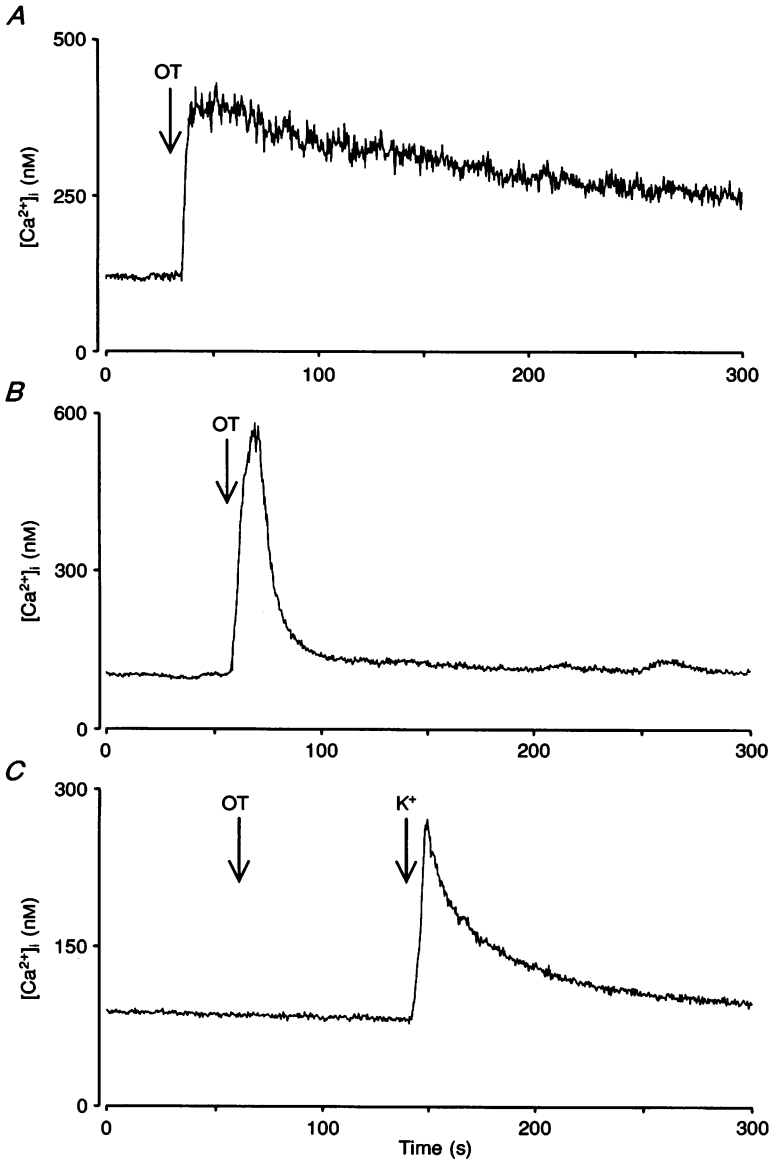

Figure 3. Distinct patterns of oxytocin-induced rise in $[Ca^{2+}]_i$ in SO cells
In all cases, the cells were tested with 100 nM oxytocin (OT), and the stimulus was maintained up to 5 min. *A*, example of sustained response: oxytocin induced a sharp increase in $[Ca^{2+}]_i$ which remained elevated, decaying slowly during the 4 min recording. *B*, example of brief response: this cell displayed a sharp rise in $[Ca^{2+}]_i$ with a fast increase but a return to resting level within 1 min. *C*, absence of response to oxytocin: the viability of the cell was checked by K^+ application (48 mM) which induced a sharp increase in $[Ca^{2+}]_i$ with fast response.

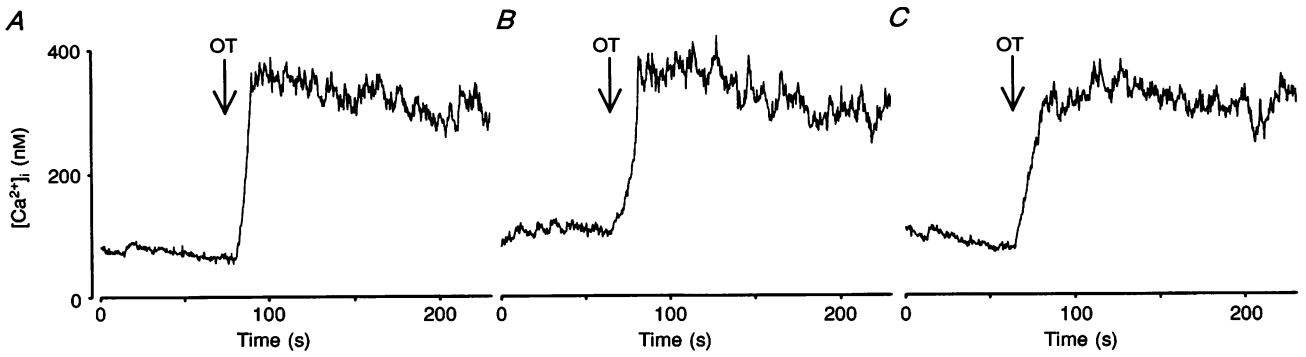

Figure 4. Reproducibility of the response to oxytocin
The cell was subjected to three successive stimulations with oxytocin (100 nM), separated by a 5 min interval after washing. The intracellular free Ca^{2+} level had returned to prestimulation level before application of each stimulus. Note that the oxytocin-induced rise in $[Ca^{2+}]_i$ is highly reproducible.

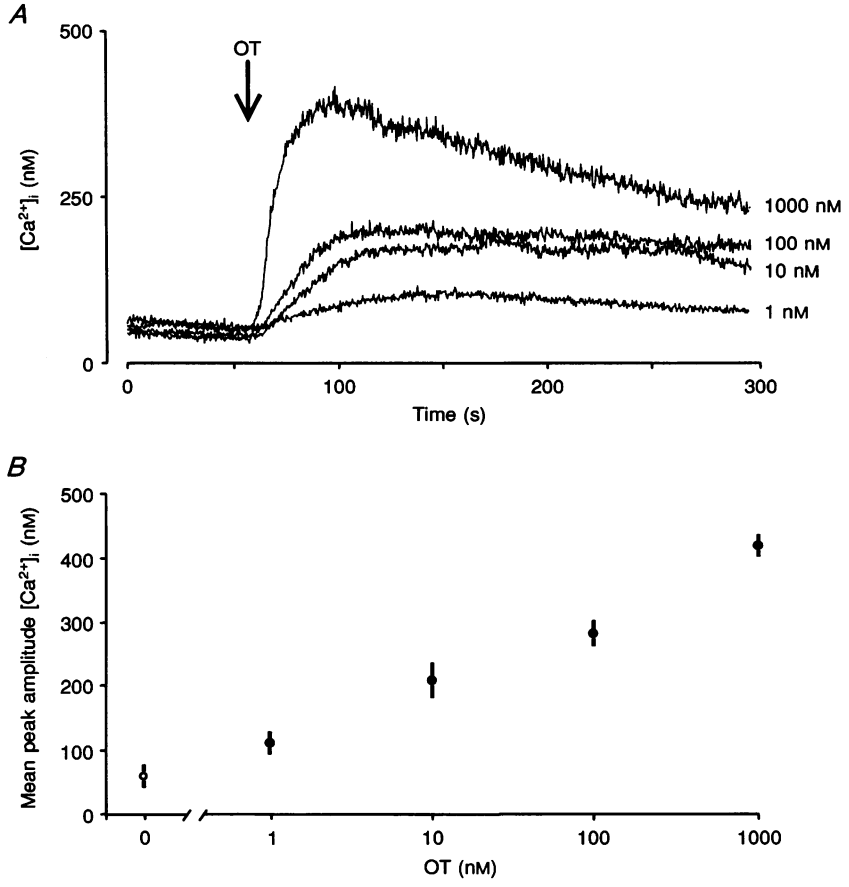

Figure 5. Dose-dependent effect of oxytocin
A, the superimposed traces correspond to increases in $[Ca^{2+}]_i$ induced by increasing doses of oxytocin, ranging from 1 to 1000 nM. The four doses of oxytocin were tested successively, each one being preceded by 5 min intermission in control medium. In these conditions, $[Ca^{2+}]_i$ had returned to prestimulation resting level before application of each stimulus. Note the slight but clear $[Ca^{2+}]_i$ rise already observed with the lowest concentration of oxytocin and the dose-dependent increase of the responses. Whatever concentration of oxytocin was used, the increase in $[Ca^{2+}]_i$ was sustained, with a slow decay until washing 4 min after oxytocin application. *B*, the graph shows the peak amplitude (means ± S.E.M.) of the $[Ca^{2+}]_i$ rise *versus* the dose of oxytocin. Data were obtained from 5 cells, with stable resting $[Ca^{2+}]_i$ (○), tested using the same protocol as in *A*.

out of the twenty-six oscillating cells, oxytocin evoked $[Ca^{2+}]_i$ increase to 341 ± 46 nM with a mean rise time of 13 ± 2 s. In both types of cells (oscillating or stable), two types of $[Ca^{2+}]_i$ responses were recorded.

(i) A sustained response consisting of a single rise in $[Ca^{2+}]_i$, with a plateau phase, decaying slowly throughout the measuring period (Fig. 3*A*). The $[Ca^{2+}]_i$ always returned to resting level after washing. This sustained response was exhibited by the majority of cells (9 oscillating and 54 stable cells).

(ii) A brief response consisting of a single rise in $[Ca^{2+}]_i$ which decayed to near-resting level in less than 1 min, even in the continued presence of oxytocin (Fig. 3*B*). This brief response was displayed by a minority of cells (4 oscillating and 6 stable cells).

Of the sixty-two cells which did not respond to 100 nM oxytocin, ten of them were also tested with 1 μM oxytocin and thirty-two of them with 48 mM K^+. In all cases the high dose of oxytocin was inefficient. However, K^+ application induced a sharp increase in $[Ca^{2+}]_i$ in all thirty-two cells (Fig. 4*C*), attesting the viability of the oxytocin-insensitive cells. The increase in $[Ca^{2+}]_i$ always returned to near-resting levels even in the continued presence of elevated K^+. Oxytocin-sensitive and -insensitive cells, respectively, had similar resting $[Ca^{2+}]_i$ (38 ± 3 and 43 ± 5 nM in stable cells, from 52 ± 10 to 115 ± 15 nM; and from 34 ± 7 to 120 ± 19 nM in spontaneously oscillating cells).

Thirteen out of twenty-five cells from male rat SO nucleus tested for oxytocin (12 cells with stable resting $[Ca^{2+}]_i$ and 1 spontaneously oscillating cell) displayed a marked increase in $[Ca^{2+}]_i$ after oxytocin application. The Ca^{2+} transient peaked to 353 ± 31 nM within 30 ± 5 s, and only two cells showed a quick decay to resting $[Ca^{2+}]_i$ in less than 1 min.

Reproducibility of the responses (Fig. 4) allowed the use of successive oxytocin applications on single cells to characterize further the oxytocin response. However, due to the fact that cells which displayed oxytocin-induced $[Ca^{2+}]_i$ rise with rapid kinetics were seldom observed, no attempt was made to characterize this oxytocin response pharmacologically. All the following experiments were performed on SO cells isolated from female rats.

Oxytocin elicited a dose-dependent increase in $[Ca^{2+}]_i$ within the range of doses tested (1–1000 nM, Fig. 5). In all five cells tested with different doses of oxytocin, the evoked increase in $[Ca^{2+}]_i$ was already detectable at 1 nM.

Incubation of oxytocin-sensitive cells for 10 min with 100 nM of the oxytocin antagonist, $d(CH_2)_5OVT$, completely abolished the response induced by 100 nM oxytocin ($n = 7$, Fig. 6). In order to ascertain whether this effect was due to loss of responsiveness, the cells were further subjected to K^+ (48 mM) in the presence of $d(CH_2)_5OVT$. K^+ evoked a sharp increase in $[Ca^{2+}]_i$ with a fast decay to resting level, attesting to the viability of the cells. Furthermore, three out of the seven cells incubated with $d(CH_2)_5OVT$ were again tested with oxytocin in Locke buffer. In no case was the Ca^{2+} response due to oxytocin restored.

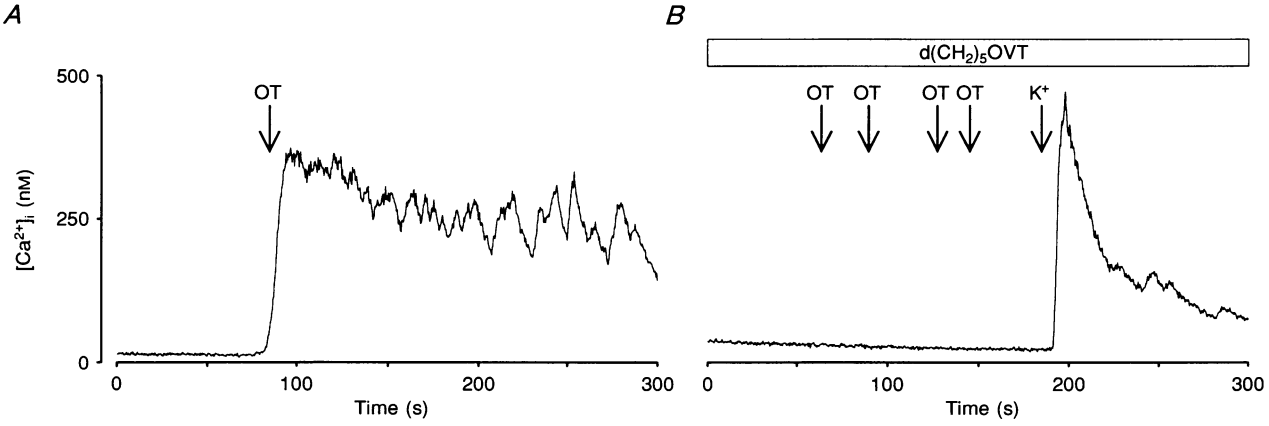

Figure 6. Block of the oxytocin-induced $[Ca^{2+}]_i$ rise by the specific oxytocin antagonist, $d(CH_2)_5OVT$

A, a cell, with stable resting $[Ca^{2+}]_i$, was subjected to 100 nM oxytocin (arrow) which induced a marked, sustained rise in $[Ca^{2+}]_i$. The return to resting level was accompanied by successive small $[Ca^{2+}]_i$ oscillations, a phenomenon observed in a few tested cells. *B*, after washing, the same cell was incubated with 100 nM $d(CH_2)_5OVT$ for 10 min, and subjected to four successive oxytocin applications (100 nM each) which did not induce any rise in $[Ca^{2+}]_i$. At the end of the recording, to check the viability of the cell, K^+ (48 mM) was applied inducing a marked increase in $[Ca^{2+}]_i$ with a fast response.

Nature of the $[Ca^{2+}]_i$ rise induced by oxytocin

In order to investigate whether the oxytocin-induced $[Ca^{2+}]_i$ rise was dependent on the presence of external Ca^{2+}, sixteen cells (15 stable cells and 1 oscillating cell) which had already responded to oxytocin in Locke buffer were tested with oxytocin in EGTA buffer. Ca^{2+} removal did not affect the kinetics of the $[Ca^{2+}]_i$ transients evoked by 100 nM oxytocin (Fig. 7). The mean $[Ca^{2+}]_i$ rise time was 17 ± 4 s in Locke buffer and 11 ± 3 s in EGTA buffer. In both conditions, the $[Ca^{2+}]_i$ remained elevated during the measuring period, with a slow decay. However, in ten of sixteen cells, the peak amplitude of the rise in $[Ca^{2+}]_i$ evoked by oxytocin was slightly reduced in EGTA buffer and only one cell showed a greater oxytocin-induced $[Ca^{2+}]_i$ rise in low extracellular Ca^{2+} concentration. The mean value of the oxytocin-evoked increase in $[Ca^{2+}]_i$ was 384 ± 59 nM in Locke buffer and 300 ± 52 nM in EGTA buffer ($n = 16$). This slight reduction in the peak value, is nevertheless significant ($P < 0.02$, Student's paired t test) and suggests that a small amount of extracellular Ca^{2+} is involved in the $[Ca^{2+}]_i$ rise induced by oxytocin. However, the oxytocin-induced rise in $[Ca^{2+}]_i$ is mainly due to Ca^{2+} release from intracellular stores. Therefore, we further tested the nature of the intracellular releasable pool of Ca^{2+} in oxytocin-sensitive cells using various intracellular Ca^{2+} mobilizing agents: thapsigargin, tBuBHQ and caffeine. Experiments were performed in EGTA buffer to limit Ca^{2+} influx, under which conditions the resting $[Ca^{2+}]_i$ was 37 ± 6 nM ($n = 28$) and remained stable.

Fourteen cells which displayed a robust oxytocin-induced $[Ca^{2+}]_i$ rise, (329 ± 50 nM; Fig. 8*A*, left panel) were thereafter tested with 200 nM thapsigargin. In all fourteen cells, thapsigargin elicited a $[Ca^{2+}]_i$ rise of 299 ± 44 nM, which peaked within 33 ± 6 s (Fig. 8*A*, middle panel). The thapsigargin-induced increase in $[Ca^{2+}]_i$ was always a single large transient decaying to resting level in less than 4–5 min. After prolonged incubation (about 10 min) with thapsigargin, oxytocin did not elicit any further $[Ca^{2+}]_i$ rise (Fig. 8*A*, right panel).

Neither tBuBHQ (25 μM) nor caffeine (10 mM) elicited $[Ca^{2+}]_i$ transients when applied onto cells which had

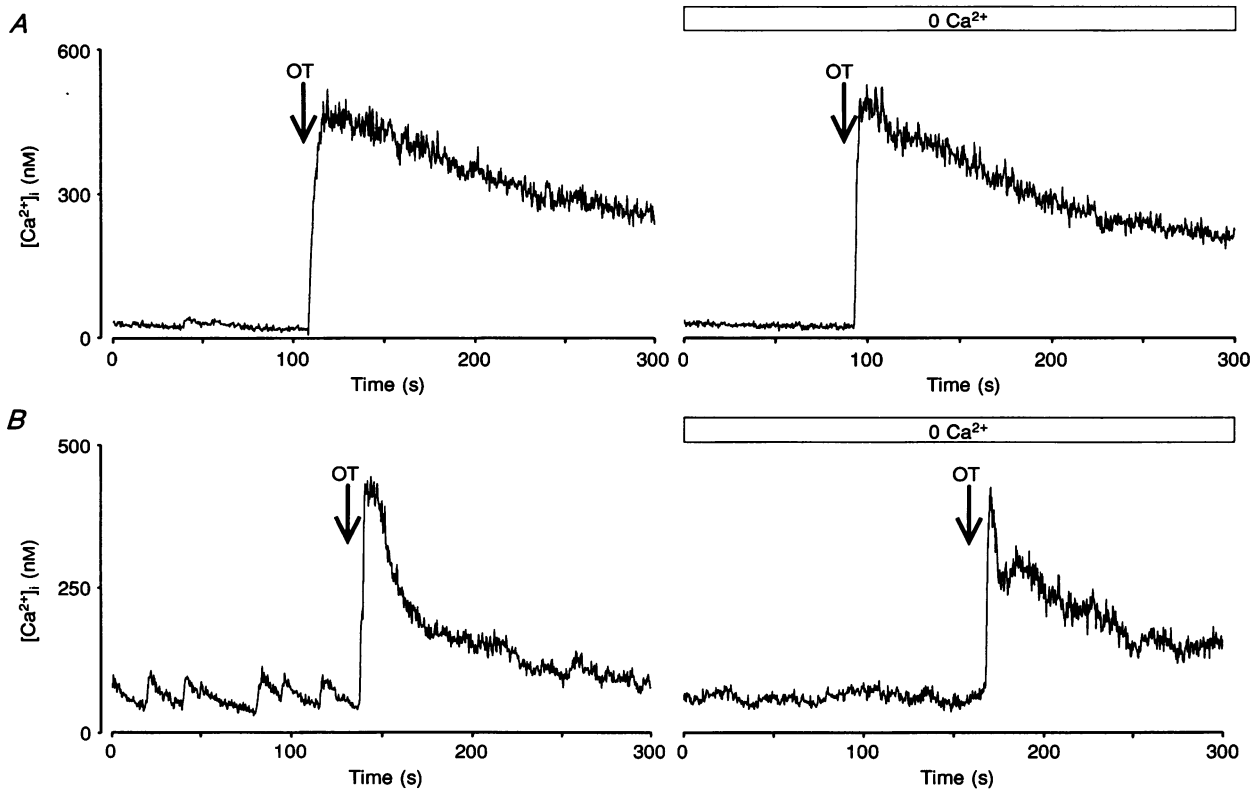

Figure 7. Response of SO cells to oxytocin in EGTA buffer
A, the left panel represents $[Ca^{2+}]_i$ responses to 100 nM oxytocin from a selected cell with stable resting $[Ca^{2+}]_i$, maintained in Locke buffer (1·8 mM Ca^{2+}). After washing (right panel), the same cell was pre-incubated for 5 min in EGTA buffer (approximately 100 nM Ca^{2+}) and then tested with oxytocin. *B*, response of a cell exhibiting spontaneous $[Ca^{2+}]_i$ oscillations. The $[Ca^{2+}]_i$ transients are shown in Locke buffer (left panel) and in EGTA buffer (right panel). Note that spontaneous oscillations disappeared in EGTA buffer as in Fig. 3. In *A* and *B*, the response to oxytocin persisted in low extracellular Ca^{2+} concentration.

previously shown a $[Ca^{2+}]_i$ rise evoked by oxytocin in EGTA buffer (Fig. 8*B* and *C*, middle panels). Furthermore, incubation of cells with tBuBHQ or caffeine for longer periods (15 min) never impeded the oxytocin-induced rise in $[Ca^{2+}]_i$ (Fig. 8*B* and *C*, right panels). The mean peak amplitude of oxytocin responses was similar in the absence or presence of tBuBHQ (357 ± 65 and 350 ± 60 nM, respectively; $n = 6$) as was the mean rise time (10 ± 3 and 11 ± 3 s, respectively; $n = 8$). Caffeine was tested in six cells under similar experimental conditions, and in no case was the response to oxytocin modified. The mean peak amplitude in $[Ca^{2+}]_i$ and mean rise time of oxytocin responses were, respectively, 312 ± 68 nM and 14 ± 4 s in control and 307 ± 69 nM and 15 ± 3 s in the presence of caffeine.

DISCUSSION

The present results provide evidence for direct action of oxytocin on freshly dissociated cells from the SO nucleus, and the measurement of $[Ca^{2+}]_i$, using the fluorimetric technique, provides new information on the cellular mechanisms of oxytocin action. Although direct proof was not possible, immunocytochemical controls indicate that the recordings were performed on a neuronal population, primarily comprising oxytocin and AVP neurones.

Most of the neurones recorded displayed a stable resting $[Ca^{2+}]_i$ whereas 20% displayed spontaneous $[Ca^{2+}]_i$ oscillations dependent on extracellular Ca^{2+}. Addition of oxytocin to the medium induced a specific, dose-dependent $[Ca^{2+}]_i$ rise in 50% of the recorded cells, regardless of their

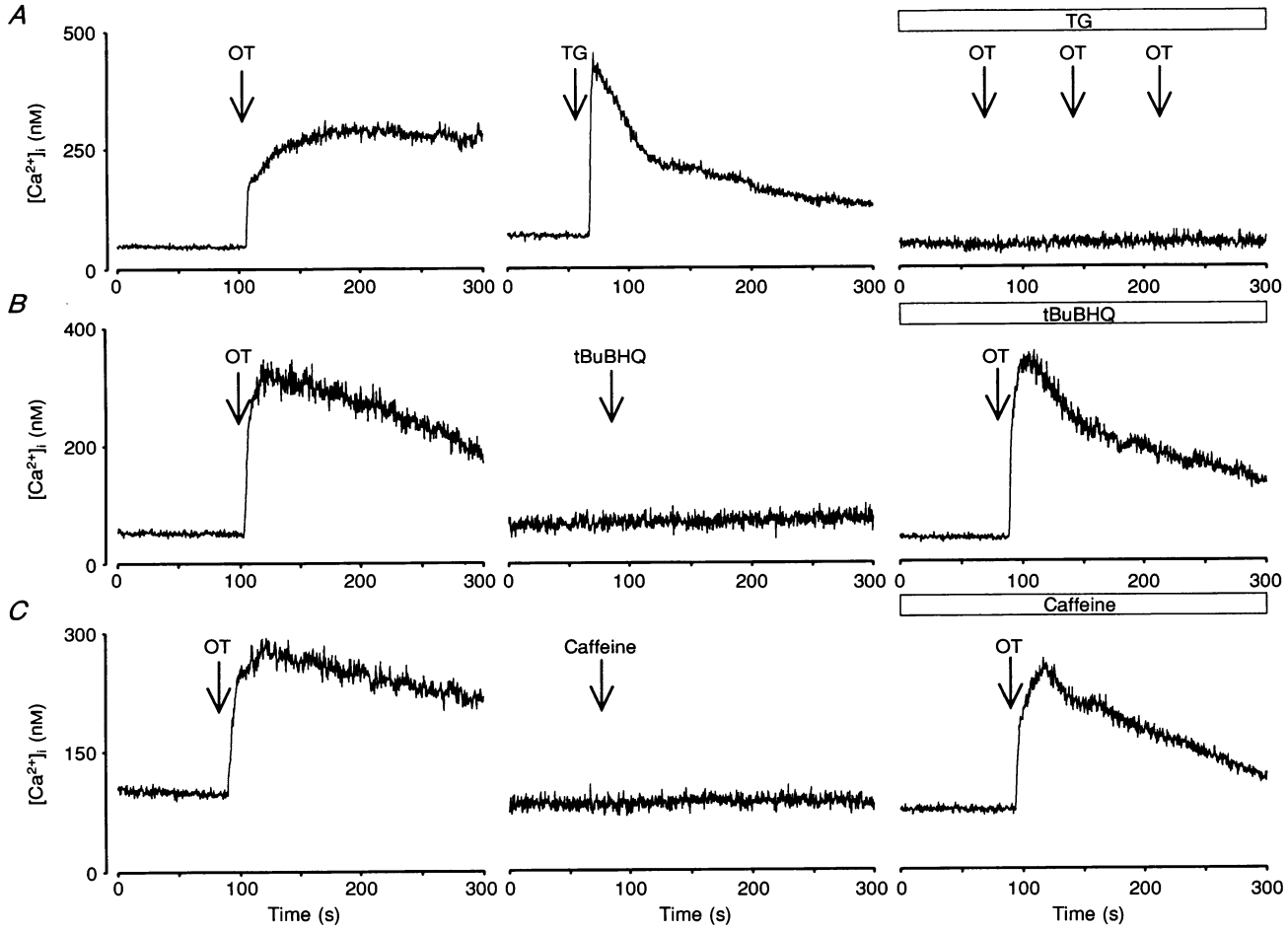

Figure 8. Effect of thapsigargin, tBuBHQ and caffeine on $[Ca^{2+}]_i$ in SO cells
A, oxytocin (100 nM) induced $[Ca^{2+}]_i$ rise from a SO cell maintained in EGTA buffer (left panel). The same cell, after washing in the same medium, was exposed to 200 nM thapsigargin (TG). Note the robust rise in $[Ca^{2+}]_i$ with fast response (middle panel). The subsequent trace (right panel) shows the inefficiency of three successive oxytocin applications (100 nM) while maintaining the thapsigargin concentration (200 nM) throughout the measuring period. Similar results were obtained in 14 cells tested with this protocol. *B* and *C*, respective effects of tBuBHQ (25 μM) and caffeine (10 mM) on 2 selected SO cells sensitive to oxytocin (left panel). tBuBHQ or caffeine neither induced any change in $[Ca^{2+}]_i$ (middle panel) nor prevented the response to oxytocin (right panel).

resting $[Ca^{2+}]_i$. Responses were reproducible and, in a majority of cases, consisted of a sharp increase in $[Ca^{2+}]_i$ which remained elevated throughout the recording period, with a slow decay. This $[Ca^{2+}]_i$ rise was independent of the presence of extracellular Ca^{2+}, and pharmacological study demonstrated that the oxytocin response implied Ca^{2+} release from thapsigargin-sensitive intracellular stores.

Cell population

The immunocytochemical data indicated that neurones constitute the great majority of the cell population, most of them being immunoreactive to oxytocin or AVP. Therefore, considering the similarity of the morphological characteristics of oxytocin/AVP neurones and of the recorded cells, the probability that we studied magnocellular neurones was high. However, as only half of our cell population was sensitive to oxytocin, the problem then arising concerns the identity of SO neurones affected by oxytocin. In the cell preparation, the numbers of oxytocin- or AVP-immunoreactive neurones were quite similar. The hypothesis of an oxytocin action on AVP neurones is unlikely when considering results from electrophysiological studies. In lactating rats, the effect of oxytocin injections into the third ventricle is restricted to oxytocin neurones (Freund-Mercier & Richard, 1984) and, *in vitro*, oxytocin only modifies the activity of continuously firing neurones, which are generally considered to be oxytocin neurones (Inenaga & Yamashita, 1986; Yamashita *et al.* 1987; Kuriyama *et al.* 1993). Although it may not be justified to link the oxytocin effects to $[Ca^{2+}]_i$ and firing rate, these results suggest that in our preparation, the oxytocin-sensitive cells could be oxytocin neurones. However, action on other neuronal types cannot be excluded. Indeed, SO neurones send collaterals to interneurones in an area adjacent and dorsal to the SO nucleus (Leng, 1982; Mason, Ho & Hatton, 1984). These neurones could constitute part of the non-oxytocin/AVP neurones which could be present in our preparations and may be sensitive to oxytocin.

Resting $[Ca^{2+}]_i$ level

This study is the first analysis of free cytosolic Ca^{2+} concentration in SO cells. The great majority of the cells (80%) presented a stable resting $[Ca^{2+}]_i$ throughout the measuring period. This resting $[Ca^{2+}]_i$ was not influenced by removal of extracellular Ca^{2+}, and was lower than 100 nM, a typical situation in neurones. However, nearly 20% of the isolated cells displayed more or less regular spontaneous fluctuations in $[Ca^{2+}]_i$. These spontaneous fluctuations reversibly disappeared in the absence of extracellular Ca^{2+}. Therefore, it is possible that spontaneous oscillations require a flux of extracellular Ca^{2+} into the cell, and we can suspect that the relatively large increase in $[Ca^{2+}]_i$ occurring spontaneously and periodically may be an example of Ca^{2+}-induced Ca^{2+} release. This kind of spontaneous oscillation has been well characterized in the clonal rat pituitary cell strains GH4C1 (Wagner, Yacono, Golan & Tashjian, 1993) and GH3B6 (Schlegel *et al.* 1987) in which spontaneous $[Ca^{2+}]_i$ fluctuations are dependent on the entry of extracellular Ca^{2+}, via voltage-activated Ca^{2+} channels, which in turn induces Ca^{2+} release from intracellular stores. A similar mechanism may also be involved in our cells, since action potentials of magnocellular neurones have a large Ca^{2+} component (Mason & Leng, 1984) and some of the isolated cells in our preparation generate action potentials. Nevertheless, similar sensitivity to oxytocin of stable and oscillating cells suggests that they do not constitute two different types of cells.

Oxytocin induces $[Ca^{2+}]_i$ rise

Two types of responses were observed. For the majority of cells (86%), the $[Ca^{2+}]_i$ remained elevated throughout the measuring period but, for 14% of the cells, the $[Ca^{2+}]_i$ transient returned to the basal level in less than 1 min. Since these two patterns of response were found in different cells of the same preparation, they could not be induced by differences in cell dissociation. Furthermore, as the oxytocin responses were highly reproducible throughout the recordings, it is unlikely that the brief responses resulted from an artifact, but that the difference between sustained and brief responses was due to differences in $[Ca^{2+}]_i$ regulatory mechanisms. Although no morphological distinction could be made between these cells, we can suspect that the two different types of oxytocin-induced rises in $[Ca^{2+}]_i$ could be present in two different types of cells.

Rise in $[Ca^{2+}]_i$ was detectable after application of as little as 1 nM oxytocin, and this effect increased in a dose-dependent manner within the range tested (1–1000 nM). The effectiveness of 1 nM oxytocin is in agreement with results of *in vitro* electrophysiological studies performed in different hypothalamic nuclei. Indeed, the threshold concentration necessary to induce any modification of the neuronal firing rate is between 0·05 and 1 nM oxytocin (Inenaga & Yamashita, 1986; Yamashita *et al.* 1987; Kawarabayashi, Kuriyama, Nakashima, Kiyohara & Sugimori, 1993; Kuriyama *et al.* 1993).

Under our experimental conditions, the oxytocin response was blocked by $d(CH_2)_5OVT$, a specific oxytocin antagonist the characteristics of which were defined by Bankowski, Manning, Seto, Halder & Sawyer (1980). These data are in agreement with previous *in vivo* electrophysiological studies in lactating rats (Freund-Mercier & Richard, 1984) demonstrating that $d(CH_2)_5OVT$ antagonizes the oxytocin action on magnocellular neurones. In addition, it suggests that the oxytocin action on $[Ca^{2+}]_i$ is mediated by specific oxytocin receptors. In fact, the direct oxytocin action on SO cells implies the presence of oxytocin receptors. However, the presence of these

receptors in the SO nucleus is not clearly established, and the detection of oxytocin binding sites could depend on the strain of rat studied (for review see Richard, Moos & Freund-Mercier, 1991). A recent work of interest demonstrates a moderate expression of oxytocin receptor mRNA in a group of magnocells in both the supraoptic and paraventricular nuclei (Yoshimura *et al.* 1993).

Regarding the specificity of oxytocin *versus* AVP on SO neurones, our results reveal that AVP, like oxytocin, evoked $[Ca^{2+}]_i$ rise in the SO cells tested. However, the cells responded to either AVP or oxytocin but never to both peptides (G. Dayanithi, unpublished observations). These observations strengthen the notion that the actions of AVP or oxytocin on magnocellular neurones of the supraoptic nuclei are independent and specific. Nevertheless, further immunocytochemical identification of the cells recorded and receptor characterization using specific agonists and antagonists would confirm the specificity of each action on a particular type of cell.

If we compare our results further with previous *in vitro* electrophysiological studies investigating oxytocin effects on SO neurones, it seems interesting to note that the cells obtained from male rat SO nucleus displayed Ca^{2+} responses to oxytocin similar to those from female rat SO nucleus. Indeed, oxytocin increases the electrical activity of putative oxytocin neurones of male rats (Inenaga & Yamashita, 1986; Yamashita *et al.* 1987) but decreases that of cyclic female rats (Kuriyama *et al.* 1993). However, the possible link between the oxytocin effect on firing rate and $[Ca^{2+}]_i$ is unknown. We could consider that, whatever the effects of oxytocin on basal electrical activity (excitation or inhibition), it necessitates a rise in $[Ca^{2+}]_i$, but the subsequent intracellular mechanisms involved in each case could differ. In addition, recent *in vitro* electrophysiological data have demonstrated that, in virgin female rats, the oxytocin action on the SO neurone is independent of the cycle state of the animal (Kuriyama *et al.* 1993). This result is in agreement with our present observation indicating that the oxytocin-induced $[Ca^{2+}]_i$ rise is similar in different virgin cyclic female rats.

Mobilization of intracellular stored Ca^{2+}

Although a slight but significant reduction in the peak amplitude of the Ca^{2+} response was observed in Ca^{2+}-free medium, the major part of the response to oxytocin was independent of the presence of extracellular Ca^{2+}. Therefore, we cannot exclude the hypothesis that a small amount of extracellular Ca^{2+} entered the cell during oxytocin stimulation, but this phenomenon was not limiting. The oxytocin response mainly involved Ca^{2+} release from intracellular stores and similar observations have been made in two other oxytocin-sensitive cell types: the myometrial cells (Batra, 1986; Anwer & Sanborn, 1989) and corticotrophs (Link *et al.* 1992).

Our results demonstrate that SO cells which respond to oxytocin are sensitive to thapsigargin but not to tBuBHQ and caffeine. In addition, prolonged incubation with thapsigargin, which is supposed to deplete at least a part of the sensitive pool (Jackson, Patterson, Thastrup & Hanley, 1988), antagonized the oxytocin-induced rise in $[Ca^{2+}]_i$, suggesting that oxytocin-sensitive intracellular Ca^{2+} stores are also sensitive to thapsigargin. Similarly, in corticotrophs, oxytocin-induced $[Ca^{2+}]_i$ rise results from liberation of intracellular Ca^{2+} from thapsigargin-sensitive pools, and caffeine or inositol 1,4,5-trisphosphate are ineffective (G. Dayanithi, unpublished results). These pharmacological results cannot be directly compared with other data in the literature. In myometrial cells, the oxytocin mobilization of intracellular Ca^{2+} stores is assumed to be dependent on a pertussis toxin-sensitive protein and to involve inositol phosphate (Anwer & Sanborn, 1989). tBuBHQ has been claimed to mobilize the same Ca^{2+} stores as inositol 1,4,5-trisphosphate, but the extent of its specificity is unclear (Duddy, Kas & Orrenius, 1989; Oldershaw & Taylor, 1990; Robinson & Bourgoyne, 1991). Caffeine-sensitive stores which are present in different cell types, i.e. bovine adrenal chromaffin cells (Cheek, O'Sullivan, Moreton, Berridge & Bourgoyne, 1990) and in several neuronal populations (Thayer, Hirning & Miller, 1988; Thayer, Perney & Miller, 1988; Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988; Irving, Collingridge & Schofield, 1992*a*,*b*), are distinct from the inositol phosphate-sensitive pools only in certain cell types (Kostyuk & Kirischuk, 1993). Therefore, as neither tBuBHQ nor caffeine are strictly specific for a particular intracellular Ca^{2+} pool, no further conclusion could be drawn from the lack of effect observed in our cells. However, thapsigargin, which has been shown to release Ca^{2+} from intracellular stores in many cell types (Cheek & Thastrup, 1989; Thastrup *et al.* 1989; Thastrup, Cullen, Drobak, Hanley & Dawson, 1990) including neurones in hippocampal slices (Harvey & Collingridge, 1992), seems to have a specific effect. Indeed, thapsigargin induces Ca^{2+} release by specifically inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al.* 1990). This $[Ca^{2+}]_i$ rise occurs without hydrolysis of inositol phospholipids (Thastrup, Linnebjerg, Bjerrum, Knudsen & Christensen, 1987; Jackson *et al.* 1988), but the thapsigargin-releasable pools include the pools sensitive to inositol 1,4,5-trisphosphate. Therefore, our data suggest that oxytocin mobilizes intracellular Ca^{2+} from the endoplasmic reticulum pools in SO cells. Further experiments will be necessary to determine whether inositol phospholipids are implicated in the oxytocin response.

In conclusion, our observations demonstrate that oxytocin can induce a $[Ca^{2+}]_i$ rise in a population of SO neurones obtained from male and female rats. The oxytocin-induced rise in $[Ca^{2+}]_i$ involves Ca^{2+} release from thapsigargin-sensitive intracellular stores. Further

experiments are now in progress to investigate any link between oxytocin effects on $[Ca^{2+}]_i$ and on electrical activity of SO neurones.

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