Vasopressin-induced intracellular Ca^{2+} increase in isolated rat supraoptic cells

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1. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored in single magnocellular neurones freshly isolated from rat supraoptic nucleus. Application of 100 nM vasopressin increased $[Ca^{2+}]_i$. Two types of $[Ca^{2+}]_i$ responses were observed: (i) a transient response, displayed by 86% of the vasopressin-sensitive neurones, and (ii) a sustained response displayed by 14% of the vasopressin-sensitive neurones.

2. Among responding neurones, 52% were vasopressin sensitive, 44% were oxytocin sensitive and 4% were sensitive to both peptides.

3. Responses to vasopressin were dose dependent, showed a progressive desensitization after successive applications, were specifically blocked by the V_{1a} vasopressin receptor antagonist, SR 49059, and were unaffected by the oxytocin receptor antagonist, $d(CH_2)_5OVT$.

4. Vasopressin responses were completely suppressed by the removal of external Ca^{2+}.

5. The intracellular Ca^{2+} mobilizers, caffeine and tBuBHQ, did not affect resting or vasopressin-induced $[Ca^{2+}]_i$ changes. Thapsigargin (200 nM) on its own evoked an increase in $[Ca^{2+}]_i$, and reduced the $[Ca^{2+}]_i$ increase evoked by vasopressin by 52%, suggesting that thapsigargin-sensitive Ca^{2+} stores are partially involved in the vasopressin response.

6. Immunocytochemical identification revealed that vasopressin-responding neurones synthesize vasopressin whereas oxytocin-responding neurones synthesize oxytocin.

7. In conclusion, vasopressin- (partially external Ca^{2+} dependent) and oxytocin (totally external Ca^{2+} independent)-induced $[Ca^{2+}]_i$ changes are mediated by specific receptors. In addition, vasopressin and oxytocin neurones are specifically autoregulated by their own peptides.

Magnocellular hypothalamic neurones of the rat supraoptic (SO) and paraventricular (PV) nuclei constitute two subpopulations: one synthesizing and secreting vasopressin, and the other oxytocin. The peripheral roles of these peptides are well described; vasopressin is mainly involved in hydromineral regulation and oxytocin in parturition and the milk ejection reflex. In addition to their specific hormonal effects, both peptides play a role as neurotransmitters in the central nervous system (for a review see Richard, Moos & Freund-Mercier, 1991). A growing body of evidence is available from electrophysiological (Freund-Mercier & Richard, 1984; Moos & Richard, 1989; Lambert, Moos & Richard, 1993) and microdialysis/microperifusion studies (Moos, Poulain, Rodriguez, Guerné, Vincent & Richard, 1989; Neumann, Russell & Landgraf, 1993) concerning the facilitatory action of oxytocin on hypothalamic oxytocin magnocellular neurones. In addition, we have recently demonstrated that oxytocin directly increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly dissociated SO cells via oxytocin receptors (Lambert, Dayanithi, Moos & Richard, 1994). By contrast, vasopressin appears to have both inhibitory and excitatory actions. Electrophysiological studies on rat brain slices have demonstrated that vasopressin inhibits the firing of SO phasic neurones by reducing the length of the bursts (Leng & Mason, 1982). In guinea-pig brain slices, vasopressin is reported to have mixed effects on spontaneously firing SO neurones: the firing rate was decreased in about 69% of the cells, increased in 5% and not affected in the remaining neurones (Abe, Inoue, Matsuo & Ogata, 1983). In the same preparation, magnocellular neurones of the PV nucleus that exhibited a phasic firing activity were not affected by the application of vasopressin, whereas adjacent non-magnocellular neurones were depolarized (Carette & Poulain, 1989). Excitatory responses to vasopressin have been observed in magnocellular neurones recorded in rat brain slices containing PV nucleus and these effects were

mediated through V_1 type vasopressin receptors located on the magnocellular neurones (Inenaga & Yamashita, 1986). Furthermore, *in vivo* studies using the microdialysis/microperifusion technique have demonstrated that vasopressin is released into the extracellular fluid of the SO nucleus and facilitates its own local release by a receptor-mediated feedback mechanism (Wotjak, Ludwig & Landgraf, 1994). Based on these findings, one could assume that vasopressin effects are excitatory, inhibitory or both. In addition, the mechanism(s) by which vasopressin exerts its function at the cellular level is still unclear.

Vasopressin has been shown to mobilize Ca_i^{2+} in hepatocytes (Kawanishi, Blank, Harootunian, Smith & Tsien, 1989) and in pituicytes (Hatton, Bicknell, Hoyland, Bunting & Mason, 1992). In pituicytes, vasopressin and V_1 type vasopressin receptor agonists elicit a $[Ca^{2+}]_i$ increase which is blocked by V_1 type receptor antagonists (Hatton *et al.* 1992). However, no information is available on the effect of vasopressin on $[Ca^{2+}]_i$ within magnocellular neurones. Here we report the effects of vasopressin on $[Ca^{2+}]_i$ of freshly dissociated SO magnocellular neurones. The nature, specificity and mode of action of vasopressin are compared with those of oxytocin (see Lambert *et al.* 1994).

METHODS

Supraoptic nuclei dissection and cell dissociation

Two to four adult male Wistar rats (100–200 g body weight) were used in each experiment. The rats were killed by decapitation. The dissection of the supraoptic nuclei and the cell dissociation procedures are described elsewhere (Lambert *et al.* 1994). Briefly, after dissection, the tissue pieces 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; pH 7·2 (adjusted with NaOH) and the osmolarity of the buffer was 290–310 mosmol l^{-1}. The neurones 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 0·25% bovine serum albumin (BSA) tissue culture grade (Miles Laboratories, Kankake, IL, USA) for 30 min at 34 °C in a shaking water bath. After gentle mechanical trituration, the cell suspension was filtered through a 100 μm nylon mesh and centrifuged for 5 min at 100 *g*. The pellet was resuspended in Locke buffer containing 0·05% trypsin inhibitor (Sigma) and centrifuged as above. The final pellet was resuspended in Locke buffer for dye loading.

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

The dissociated SO cells were loaded by incubation in 3 μM of the Ca^{2+}-sensitive dye fura-2 AM (acetoxymethyl ester form of fura-2; Molecular Probes Inc., Eugene, OR, USA) in Locke buffer and plated onto polylysine-coated chambered coverglasses (Lab-tek no. 178565; Nunc, Inc., Naperville, IL, USA). Dye loading was carried out for 35 min at 37 °C in a humidified atmosphere (5% CO_2–95% O_2). Fluorescence measurements of $[Ca^{2+}]_i$ were performed using the Zeiss Microscope Photometer System (Fast Fluorescence Photometer (FFP); Zeiss, Oberkochen, Germany), based on an inverted microscope (Axiovert 100; Zeiss) equipped for epifluorescence (Plan-Neofluar ×100/1.30 oil-immersion objective lens). The cells were alternately illuminated (200 Hz) at 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 320 ms. For fast switching between different excitation wavelengths, a rotating filter wheel was mounted in the excitation light path. A measuring amplifier was synchronized to the filter wheel to measure the fluorescence intensities resulting from different wavelengths. The FFP software controlled acquisition of intensity data and provided functions for adjustment of signal values, display and storage of measured data, and calculations of ion concentrations. A CCD camera was used to visualize the cells in phase contrast together with the measuring diaphragm on the monitor screen. Graphics generated by the system were overlaid on the video image. This system was also equipped with a motorized microscope stage which was controlled by the FFP software and was used for automatic repositioning and measurement of preselected cells. This feature was used to relocate the recorded cells after immunocytochemical processing (see below). With fluorescence values corrected for background and dark current, $[Ca^{2+}]_i$ calculations were carried out from the ratio between recordings at 340 and 380 nm, in accordance with the equation given by Grynkiewicz, Poenie & Tsien (1985). Fura-2 calibration was performed with the actual instrument, following the same procedure described by Lambert *et al.* (1994), which gave values of R_{min} (fluorescence ratio in the absence of Ca^{2+}) of 0·203, R_{max} (fluorescence ratio in the presence of saturating Ca^{2+}) of 6·351, β (fluorescence ratio of the free to bound dye) of 3·418 and K_d of 218·780 at 24 °C.

Solutions and drug application

The Locke buffer described above was the control medium. To test the effects of extracellular Ca^{2+}, a different buffer was used, which contained (mM): EGTA (Sigma), 0·1; NaCl, 140; KCl, 5; KH_2PO_4, 1·2; $MgSO_4$, 1·2; glucose, 10; Hepes, 10; pH 7·2 (adjusted with NaOH), and the osmolarity was 290–310 mosmol l^{-1}. The Ca^{2+} concentration of the EGTA buffer was in the 100 nM range which corresponds to the resting $[Ca^{2+}]_i$ typically observed in neurones.

Vasopressin and oxytocin (Boehringer Mannheim, Meylan, France), $[d(CH_2)_5,Tyr(Me)^2,Orn^8]$-vasotocin ($d(CH_2)_5OVT$; Peninsula Laboratories, Belmont, CA, USA) and SR 49059 ((2*S*)1[(2*R*3*S*)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulphonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide; generously supplied by Sanofi Recherche, Centre de Toulouse, 31036-Toulouse cedex, France) were directly dissolved in the recording buffer. Thapsigargin (TG; Alomone Laboratories, Jerusalem, Israel) and 2,5-di-(ter-butyl)-1,4-benzohydroquinone (tBuBHQ; Alomone Laboratories) were dissolved first in DMSO (final concentration, 0·01%) and further diluted in EGTA buffer to give the appropriate concentrations. Caffeine (Alomone Laboratories) was directly dissolved in EGTA buffer and the osmolarity was adjusted by reducing the NaCl concentration. The 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; at pH 7·2 (adjusted with NaOH).

All test substances (25 μl) were added to the bath containing 250 μl measuring buffer with a motor-driven syringe (Hamilton Microlab 900; Bonaduz, Switzerland; speed setting 9) in the proximity of the recorded cell, unless otherwise stated. This method allowed rapid and highly reproducible changes of the medium surrounding a selected cell.

Results are given as means ± S.E.M.

Immunocytochemistry

In the experiments including immunocytochemical identification of the neurones tested, a video print of the cell was taken before $[Ca^{2+}]_i$ measurements and the localization of each cell was recorded by their *X* and *Y* co-ordinates with the help of the motorized microscope stage of the FFP system (see also Link, Dayanithi, Föhr & Gratzl, 1992). To identify the '0' position, a mark was made at the bottom of the coverglass using a diamond pencil. Conventional immunocytochemical photographs could not be obtained, since the Axiovert-100 microscope was only equipped with a CCD camera and a thermal paper printer (Multiscan Video Printer, UP-930; Sony). On the other hand, the previously recorded neurones could only be relocalized via their co-ordinates memorized in the computer driving the motorized plate. Therefore the original medium quality pictures were subsequently processed with a graphical computer program (Photostyler 1.0; U-Lead System, Inc., 680 Knox Street, Torrance, CA 90502, USA).

The identification of oxytocin- and vasopressin-synthesizing neurones was performed using monoclonal antibodies PS38 and PS41. These two monoclonal antibodies, raised against rat oxytocin- and vasopressin–neurophysin, respectively, have been well characterized by Ben-Barak, Russell, Whitnall, Ozato & Gainer (1985). According to these authors, the PS38 antibody (anti-oxytocin–neurophysin) displayed no cross-reactivity with vasopressin–neurophysin. Concerning the PS41 antibody (anti-vasopressin–neurophysin), a weak cross-reactivity could be observed in Herring bodies and neurosecretory vesicles in ultrathin sections of the pituitary, i.e. in regions in which the antigens were mostly concentrated. Such high concentrations are unlikely to be found in the soma of freshly dissociated neurones, suggesting that the probability of cross-reaction under our conditions was very low. These antibodies were kindly given to us by Dr David Pow (University of Queensland, Australia).

Immediately after $[Ca^{2+}]_i$ measurements, the cells were processed for immunocytochemical analysis. The coverglass supporting the cells was rinsed three times with phosphate-buffered saline (PBS) and fixed in a mixture of ethanol–acetic acid (95%–5% (v/v)) for 10 min at 4 °C. All of the subsequent labelling steps were performed at room temperature (20–22 °C). The cells were first pre-incubated in 10% goat serum (G-6767; Sigma; diluted in PBS in order to minimize non-specific antibody binding) for 30 min and then successively incubated in either PS38 or PS41 for 2 h, in the secondary anti-mouse IgG biotinylated antibody (RPN 1177; Amersham) for 30 min, and finally in a streptavidin–biotinylated horseradish peroxidase complex (RPN 1051; Amersham) for another 30 min. The primary and secondary antibodies, as well as the peroxidase complex, were diluted at 1/100 in PBS complemented with 10% goat serum. Peroxidase was revealed by incubation in diaminobenzidine (0·5 mg ml^{-1} in 0·05 M Tris–HCl buffer at pH 7·6) containing H_2O_2 (0·03%) for 7 min. No labelling was observed when the whole procedure was performed in the absence of the primary antibody.

RESULTS

All data presented in this study were obtained from SO cells of adult male rats. The neurones were chosen following the same criteria as described elsewhere (Lambert *et al.* 1994), i.e. the soma of the recorded neurones was larger than 12 μm, and in many cases, two to three short and thick processes could be observed extending from the soma. A total number of 403 cells were tested from fifty-seven preparations. In all of these cells, both vasopressin and oxytocin were routinely tested one after the other without any order of preference and a high proportion (227 cells out of 403, 56%) showed a response to one or other peptide; 176 cells (44%) responded to neither peptide. However, K^+ (48 mM) application induced a sharp rise in $[Ca^{2+}]_i$ in a great majority of the cells, attesting the viability of the non-responding cells. These cells were discarded from all subsequent analysis reported in this study. On the basis of the resting $[Ca^{2+}]_i$ levels, no difference was observed between responding and non-responding cell populations. Since a similar analysis had already been performed on oxytocin-sensitive neurones (Lambert *et al.* 1994), we focused here on the neurones that responded selectively to vasopressin, unless otherwise stated.

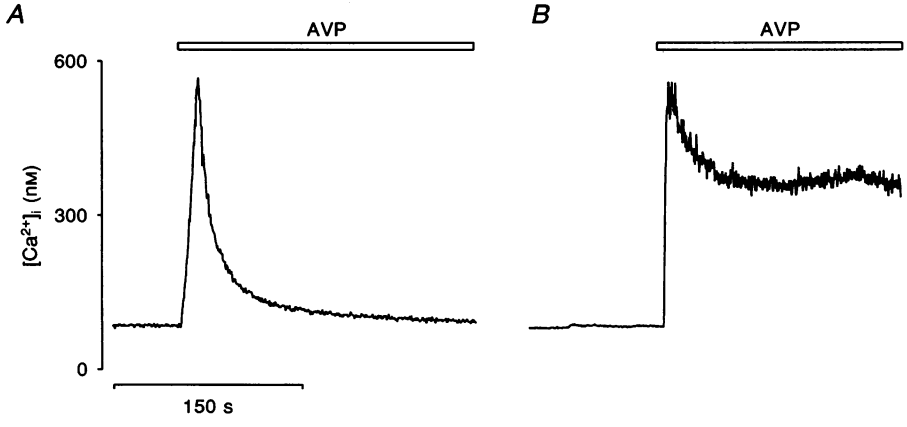

Figure 1. Patterns of vasopressin-induced $[Ca^{2+}]_i$ rise in SO cells

An oxytocin-insensitive cell was tested with 100 nM vasopressin (AVP). *A*, example of a transient response: this cell exhibited a rapid, sharp increase in $[Ca^{2+}]_i$ and returned to the resting level within 1 min. *B*, example of a sustained response: this cell also displayed a rapid and sharp rise in $[Ca^{2+}]_i$ which remained elevated throughout the recording period.

Table 1. An account of the different types of magnocellular neurone responses to oxytocin or vasopressin

	Vasopressin-sensitive neurones			Oxytocin-sensitive neurones		
Resting $[Ca^{2+}]_i$ level	Sustained $[Ca^{2+}]_i$ rise	Transient $[Ca^{2+}]_i$ rise	Total no. of cells tested	Sustained $[Ca^{2+}]_i$ rise	Transient $[Ca^{2+}]_i$ rise	Total no. of cells tested
Stable	11	78	89	74	7	81
Oscillating	6	23	29	14	5	19

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

Resting Ca_i^{2+} concentrations were analysed in Locke buffer for a period of 1–5 min. Two cell populations were classified based on their resting $[Ca^{2+}]_i$ levels: (i) silent cells with stable $[Ca^{2+}]_i$ (72 ± 2 nM; $n = 89$), and (ii) spontaneously oscillating cells with $[Ca^{2+}]_i$ varying between 67 ± 19 and 212 ± 12 nM ($n = 29$) (e.g. Fig. 4*B*). Spontaneous oscillations were mostly regular with a mean periodicity of 28 ± 12 s.

Vasopressin-induced $[Ca^{2+}]_i$ increase

Application of vasopressin (100 nM) evoked a marked increase in $[Ca^{2+}]_i$ reaching a peak within 11 ± 4 s (peak value, 482 ± 13 nM) in the eighty-nine cells with stable resting $[Ca^{2+}]_i$. Similarly, vasopressin induced an increase in $[Ca^{2+}]_i$ (peak value, 547 ± 22 nM), which peaked within 9 ± 2 s in the twenty-nine oscillating cells. In both classes of cells (stable or oscillating), two distinct types of $[Ca^{2+}]_i$ responses were recorded: (i) a transient rise in $[Ca^{2+}]_i$ which

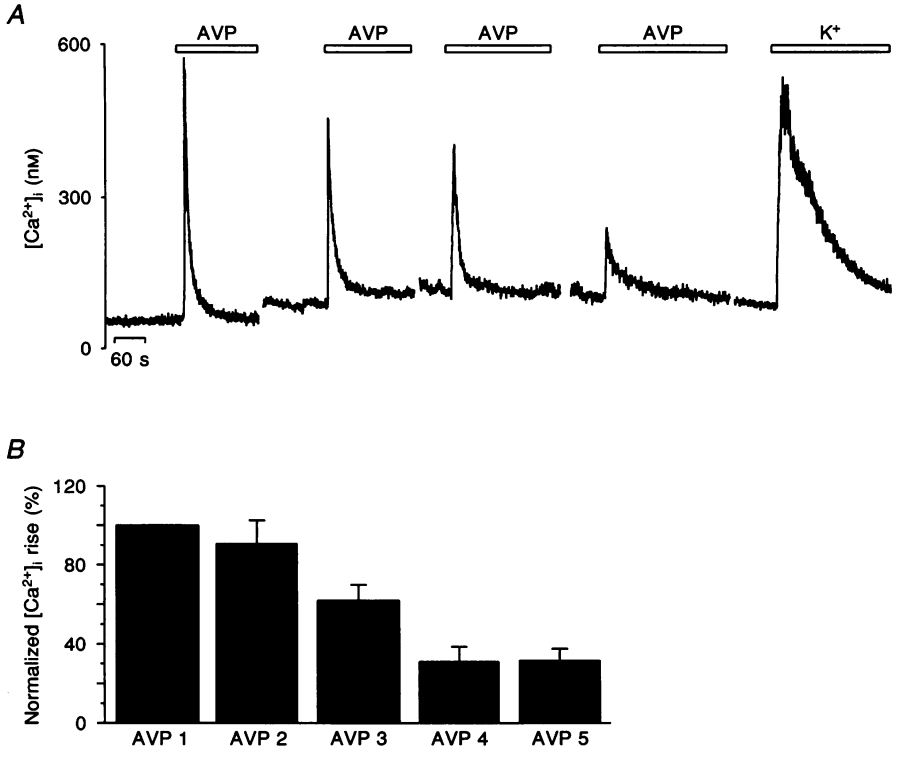

Figure 2. Effect of successive vasopressin applications on the $[Ca^{2+}]_i$ rise

A, the oxytocin-insensitive cell was subjected to four successive applications of vasopressin (100 nM), separated by 5–8 min intervals after washing. The resting $[Ca^{2+}]_i$ had returned to prestimulation level before each application. Note that the amplitude of the $[Ca^{2+}]_i$ response induced by vasopressin progressively decreased. At the end of the final measurement, the viability of the cell was tested by a K^+ depolarization (48 mM), which evoked a sharp increase in $[Ca^{2+}]_i$. Five cells were tested in this manner. *B*, diagram showing the percentage of the evoked increase in $[Ca^{2+}]_i$ induced by the first (AVP 1), second (AVP 2), third (AVP 3), fourth (AVP 4) and fifth (AVP 5) successive vasopressin (100 nM) application. Data were obtained from five cells.

decayed to the resting level in less than 1 min in the presence of the peptide (Fig. 1*A*), and (ii) a sustained rise in $[Ca^{2+}]_i$ which showed only a slight decay throughout the measuring period (Fig. 1*B*). The respective proportions of the different types of neurones are given in Table 1.

Desensitization and dose–response relationship

Repeated applications of vasopressin induced progressively smaller increases in $[Ca^{2+}]_i$. Nevertheless the response was not completely abolished even after four successive applications (Fig. 2*A*). Under these conditions, a consistent increase could still be evoked by high K^+ application. Figure 2*B* shows the decrease in the rise in $[Ca^{2+}]_i$ to five successive applications of vasopressin. For each cell, the increase in $[Ca^{2+}]_i$ elicited by successive applications was reported as the percentage of the increase elicited by the first application and then the percentages were averaged.

The amplitude of the $[Ca^{2+}]_i$ rise increased as a function of vasopressin concentration in the range 1 nM to 10 μM (Fig. 3*A*). In all four cells tested, 1 nM vasopressin had already induced a detectable increase in $[Ca^{2+}]_i$. Similar results were obtained from the SO cells which exhibited a transient $[Ca^{2+}]_i$ response. Since the maximal response was reduced due to the desensitization, a true dose–response curve could not be established; nevertheless a clear dose–response relationship was present (Fig. 3*B*).

External Ca^{2+} dependence

In order to investigate whether the vasopressin-induced $[Ca^{2+}]_i$ increase was dependent on the presence of

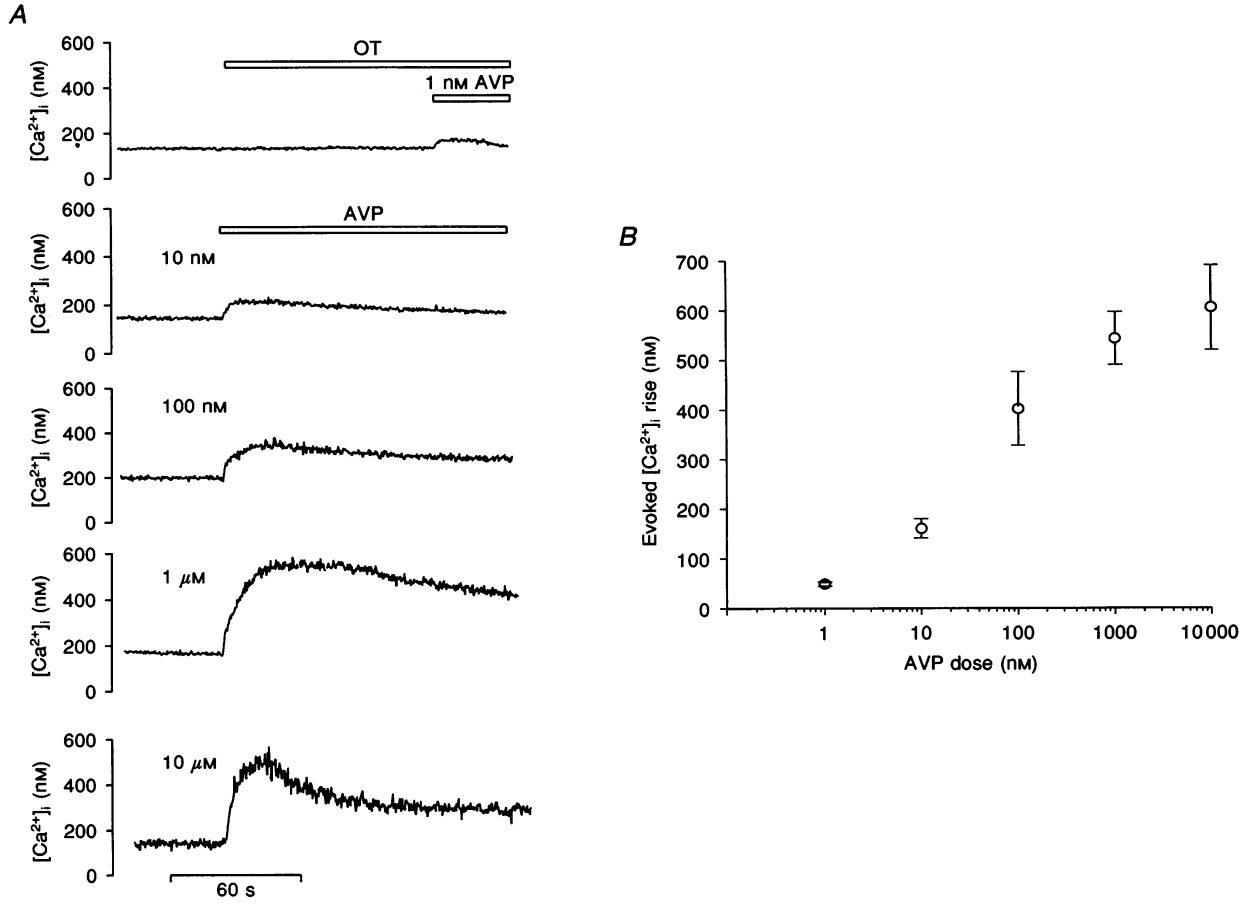

Figure 3. Dose-dependent effect of vasopressin

A, traces represent changes in $[Ca^{2+}]_i$ evoked by increasing concentrations of vasopressin (1 nM to 10 μM) measured in a SO cell. After each test the cell was washed three times with Locke buffer and under these conditions $[Ca^{2+}]_i$ had returned to the prestimulation resting level before the application of each stimulus. Note that this cell did not respond to oxytocin (OT; 100 nM). Regardless of the concentration of vasopressin employed, the increase in $[Ca^{2+}]_i$ was sustained with a slow decay until washing 1–4 min after vasopressin application. *B*, graph showing the dose–response relationship of the evoked $[Ca^{2+}]_i$ increase (means ± S.E.M.) as a function of vasopressin concentration. The basal $[Ca^{2+}]_i$ measured from each cell was subtracted from the peak amplitude evoked by each concentration of vasopressin. Data were obtained from four cells, using the same protocol as in *A*.

extracellular Ca^{2+}, thirteen cells (9 silent and 4 oscillating), which responded selectively to vasopressin (mean peak value, 648 ± 53 nM; $n = 13$), were tested in EGTA buffer. The increase in $[Ca^{2+}]_i$ induced by 100 nM vasopressin was abolished in EGTA buffer (Fig. 4), and this block was reversed after the reintroduction of Locke buffer (mean peak value, 616 ± 41 nM), suggesting that extracellular Ca^{2+} is necessary for the action of vasopressin on SO cells. The slight reduction in the peak value is probably due to the desensitization observed after the second vasopressin application (see Fig. 2*A*). It should be noted that the spontaneous oscillations of resting $[Ca^{2+}]_i$ were also reversibly abolished by EGTA buffer. The requirement of extracellular Ca^{2+} was further supported by the results in which a classical fluorescence quench by Mn^{2+} was observed after vasopressin (100 nM) application. In these experiments, the external Ca^{2+} was replaced by 2 mM $MnCl_2$ in the Locke buffer (illustrations not shown).

Effect of intracellular calcium mobilizers

Although the vasopressin-induced $[Ca^{2+}]_i$ increase is dependent on external Ca^{2+}, the involvement of intracellular Ca^{2+} stores during the action of vasopressin has not been excluded. To test this hypothesis, we investigated the nature of the intracellular releasable pool of Ca^{2+} in vasopressin-sensitive SO cells using various intracellular Ca^{2+} mobilizing agents: caffeine, tBuBHQ and thapsigargin. The action of these drugs on intracellular Ca^{2+} was first tested in EGTA buffer, then the subsequent action of vasopressin was tested after reintroduction of Locke buffer into the bath. Neither caffeine (20 mM) nor tBuBHQ (25 μM) elicited an increase in $[Ca^{2+}]_i$ in vasopressin-sensitive cells, nor did their presence alter vasopressin responses (Fig. 5*A* and *B*). The peak values obtained for the vasopressin response were as follows: control, 622 ± 56 nM ($n = 8$); in the presence of caffeine, 548 ± 47 nM ($n = 4$); in the presence of tBuBHQ, 560 ± 36 nM ($n = 4$). In contrast, thapsigargin (200 nM) on its own elicited a transient and marked increase in $[Ca^{2+}]_i$ in vasopressin-sensitive cells (Fig. 5*C*, middle panel). After prolonged incubation of about 15 min in thapsigargin, vasopressin evoked a $[Ca^{2+}]_i$ response (Fig. 5*C*, right panel). In these experiments, a mean $[Ca^{2+}]_i$ increase of 600 ± 40 nM ($n = 5$) was observed after the first application of vasopressin and thapsigargin on its own

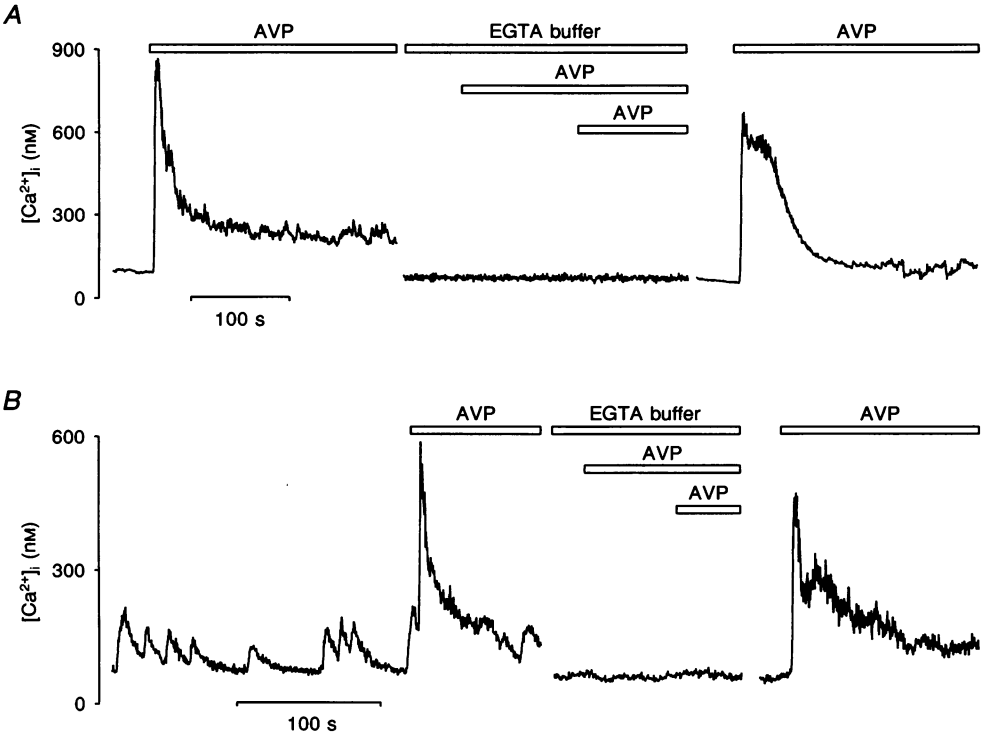

Figure 4. Reversible block of vasopressin-induced $[Ca^{2+}]_i$ rise by supression of extracellular Ca^{2+}

A, the left panel represents the $[Ca^{2+}]_i$ response induced by 100 nM vasopressin in the presence of Locke buffer containing 1·8 mM external Ca^{2+} from an non-oscillating cell with stable resting $[Ca^{2+}]_i$. Pre-incubation in low-Ca^{2+} EGTA buffer (see Methods) for 10 min abolished any $[Ca^{2+}]_i$ response to two successive applications of vasopressin (middle panel). Reintroduction of Locke buffer restored the $[Ca^{2+}]_i$ response induced by vasopressin (right panel). *B*, the same experimental protocol was applied to a spontaneously oscillating cell. Note that both spontaneous oscillations and the vasopressin-induced rise in $[Ca^{2+}]_i$ were suppressed in EGTA buffer. In these experiments, the $[Ca^{2+}]_i$ measurement was performed using a fast perifusion system to change the medium.

elicited an increase of 520 ± 55 nM. After incubation in thapsigargin, vasopressin still evoked an increase of 235 ± 46 nM. When reported as a percentage, the $[Ca^{2+}]_i$ response after thapsigargin was reduced by 61% compared with the control reponse. Desensitization experiments showed that a 9% reduction of the second vasopressin response was expected when neurones were allowed to recover for 5–8 min between vasopressin applications (see Fig. 2). Since more than 15 min elapsed between vasopressin applications in the above experiments, these results strongly suggest that pre-incubation with thapsigargin was responsible for an additional reduction of the response of at least 52%.

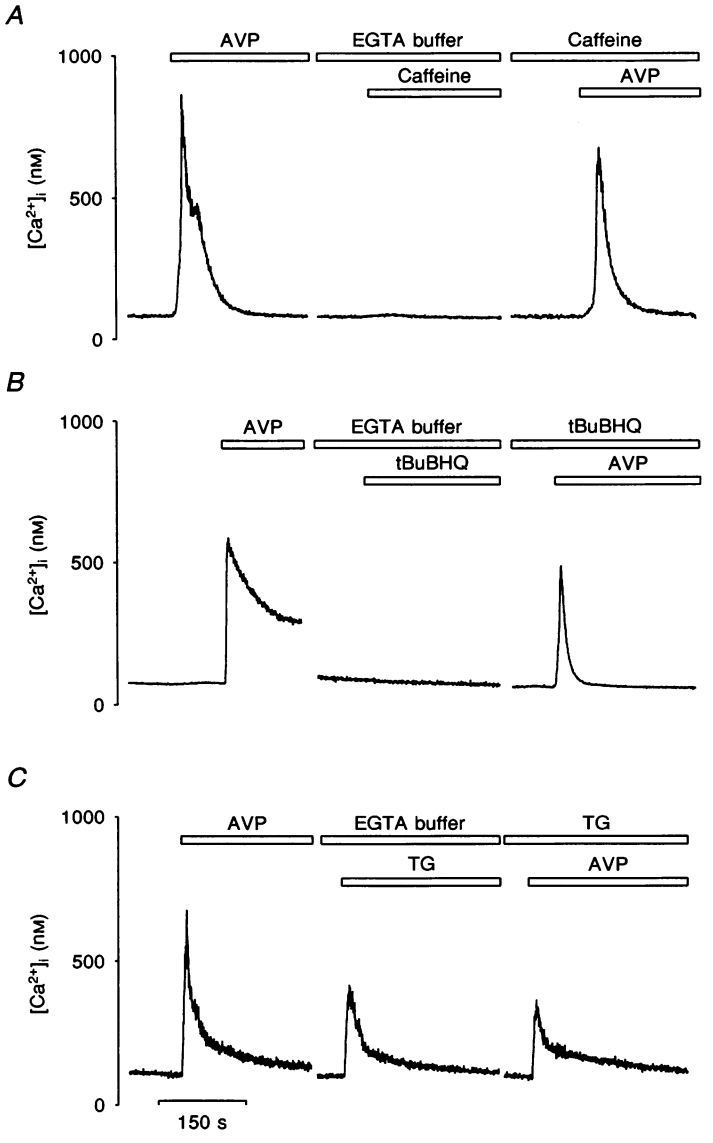

Figure 5. Effect of caffeine, tBuBHQ and thapsigargin on $[Ca^{2+}]_i$

A, a typical vasopressin (100 nM)-induced $[Ca^{2+}]_i$ rise from an oxytocin-insensitive SO cell maintained in Locke buffer (left panel). The same cell, after washing, was subjected to 20 mM caffeine in EGTA buffer (middle panel). Subsequently, EGTA buffer was replaced by Locke buffer containing 20 mM caffeine and the cell was then stimulated with vasopressin (right panel). Four cells showed a similar response. *B*, similarly, the effect of tBuBHQ (25 μM) was tested on vasopressin-sensitive SO cells which were insensitive to oxytocin. Note that neither caffeine nor tBuBHQ evoked any change in $[Ca^{2+}]_i$, nor prevented the response to vasopressin. *C*, under similar experimental conditions, an oxytocin-insensitive cell elicited a good response to 100 nM vasopressin (left panel). In the absence of external Ca^{2+}, application of 200 nM thapsigargin (TG) evoked a rise in $[Ca^{2+}]_i$ (middle panel). The trace in the right panel shows the residual vasopressin-induced $[Ca^{2+}]_i$ increase obtained in the continued presence of thapsigargin after reintroduction of Locke buffer.

Effect of V_{1a} type vasopressin receptor antagonist, SR 49059

The specific vasopressin receptor antagonist, SR 49059, applied at either 1 or 10 nM did not affect the resting $[Ca^{2+}]_i$ level (Fig. 6*A* and *B*, middle panels). In the presence of 1 nM SR 49059, the vasopressin-induced increase in $[Ca^{2+}]_i$ measured in three cells (peak value, 656 ± 57 nM) was reduced by about 50% (peak value, 340 ± 42 nM; Fig. 6*A*, right panel) whereas it was abolished by pre-incubation with 10 nM SR 49059 ($n = 6$; Fig. 6*B*, middle panel). Subsequent stimulation by K^+ depolarization evoked an increase in $[Ca^{2+}]_i$, demonstrating that these cells were still alive (Fig. 6*B*, right panel).

Selective cell sensitivity

Most cells responded selectively to one or other peptide applied at a concentration of 100 nM. Indeed, among 227 cells, vasopressin alone evoked a $[Ca^{2+}]_i$ rise in 118 cells (52%), whereas 100 cells (44%) responded only to oxytocin (Fig. 7*A* and *C*). In addition, the selective response to either peptide was not affected by the presence of the other in the bath (Fig. 7*B* and *D*). Nevertheless, in nine cells (4%), a $[Ca^{2+}]_i$ response to both peptides could be observed (Fig. 8). It should be noted that these cells displayed a much larger response to one peptide than to the other. Indeed, a small rise in $[Ca^{2+}]_i$ was elicited by oxytocin in three cells which displayed a consistent rise in $[Ca^{2+}]_i$ in response to vasopressin, whereas in six other cells, a small vasopressin response was observed, followed by a consistent response to oxytocin (Fig. 8*A* and *B*). As the number of neurones responding to both peptides was very small, no further pharmacological characterization was performed.

In a series of experiments, we investigated whether oxytocin and vasopressin responses were mediated by distinct receptors. The V_{1a} type vasopressin receptor antagonist SR 49059 applied at 10 nM onto three oxytocin-sensitive neurones did not affect resting $[Ca^{2+}]_i$ (Fig. 9*A*, middle panel) or the oxytocin-induced $[Ca^{2+}]_i$ increase (Fig. 9*A*, right panel). In another set of experiments, we tested the effect of the specific oxytocin receptor antagonist, $d(CH_2)_5OVT$, on the vasopressin response. The increase in $[Ca^{2+}]_i$ induced by 100 nM vasopressin, measured in four stable (peak value, 722 ± 76 nM; Fig. 9*B*, left panel) and four spontaneously oscillating (peak value, 568 ± 52 nM; Fig. 9*C*, left panel) neurones, was not altered by incubating the cells in 100 nM $d(CH_2)_5OVT$ (peak values, 669 ± 88 and 520 ± 64 nM, respectively; Fig. 9*B* and *C*, right panels). These results clearly demonstrate that distinct receptors mediate the selective responses to oxytocin and vasopressin.

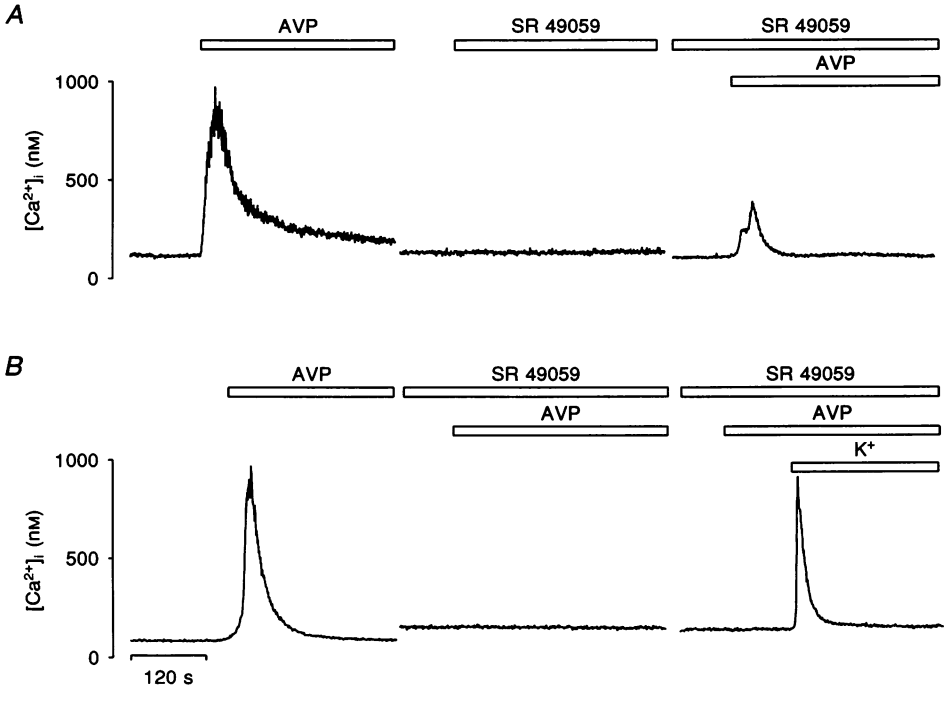

Figure 6. Effect of the specific V_1 type vasopressin receptor antagonist, SR 49059

A, trace showing vasopressin-induced $[Ca^{2+}]_i$ increase in an oxytocin-insensitive cell (left panel). This cell was subjected to 1 nM SR 49059, a specific V_1 type vasopressin receptor antagonist (middle panel), which by itself had no effect. The effect of vasopressin was strongly reduced in the presence of SR 49059 (right panel). *B*, a similar type of experiment was performed with 10 nM SR 49059. This concentration completely blocked the vasopressin-induced $[Ca^{2+}]_i$ rise, although the $[Ca^{2+}]_i$ increase induced by high K^+ attested the viability of the cell.

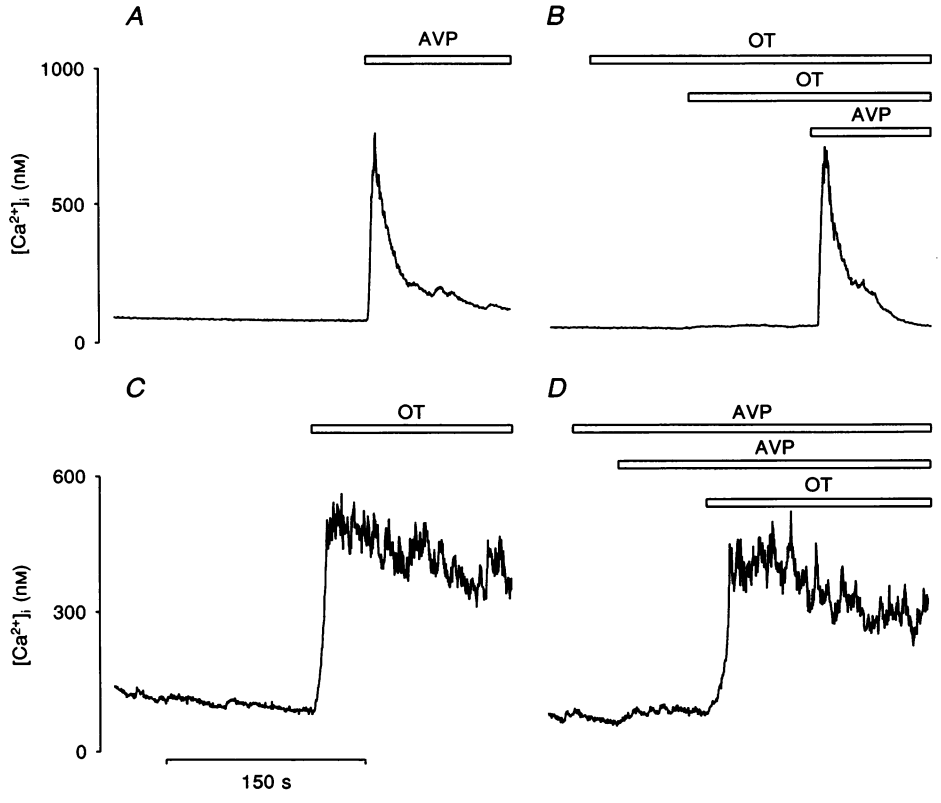

Figure 7. Comparison of responses to either vasopressin or oxytocin

A, a cell with stable resting $[Ca^{2+}]_i$ responded to 100 nM vasopressin with a marked and brief increase in $[Ca^{2+}]_i$. *B*, absence of effect when the same cell was exposed to two successive oxytocin applications (100 nM each). At the last minute of the recording, a robust increase in $[Ca^{2+}]_i$ was observed after vasopressin (100 nM) application. Note that the increase in $[Ca^{2+}]_i$ induced by vasopressin was not affected by the presence of oxytocin in the bath. *C*, using a similar experimental approach, a spontaneously oscillating cell was first subjected to 100 nM oxytocin, which resulted in a sustained $[Ca^{2+}]_i$ increase. *D*, absence of effect of vasopressin (100 nM) on the same cell. Note that the oxytocin-induced $[Ca^{2+}]_i$ response was not affected by the presence of vasopressin in the bath.

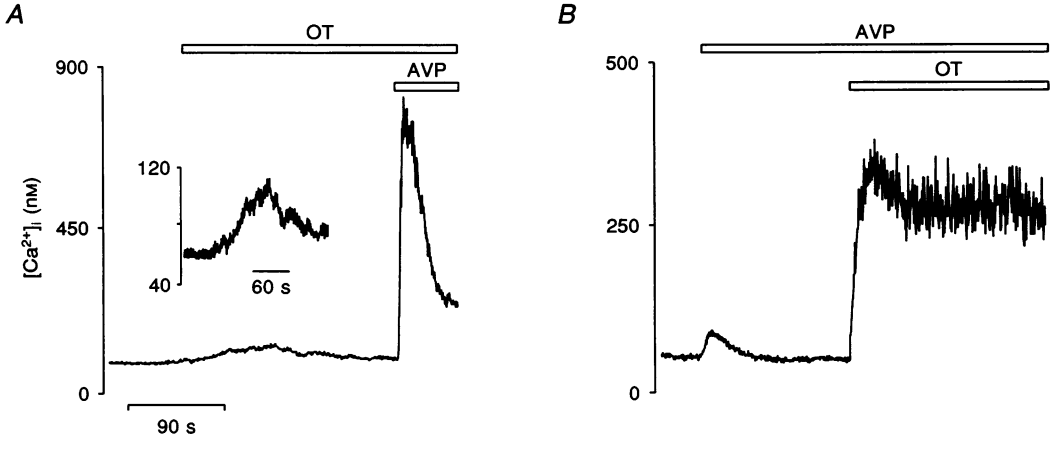

Figure 8. Cells responding to both vasopressin and oxytocin

A, a non-oscillating cell was first subjected to 100 nM oxytocin followed by vasopressin at the same concentration. Oxytocin induced a small and typical sustained increase, whereas vasopressin evoked a characteristic transient response. The trace shown in the inset is an enlargement of the oxytocin response. *B*, a second cell in which a small increase in $[Ca^{2+}]_i$ was observed upon vasopressin application, whereas oxytocin induced a large and sustained increase in $[Ca^{2+}]_i$ throughout the measuring period.

Immunocytochemical identification

In order to assess the hormonal identity of vasopressin- or oxytocin-responding neurones, immunocytochemical identification was performed after $[Ca^{2+}]_i$ measurements. In each of seven experiments, neurones were plated in four wells; in two wells the neurones were tested for vasopressin-induced $[Ca^{2+}]_i$ responses and in the other two for oxytocin-induced responses. Several neurones were tested in each well, some responding and others not responding and then the neurones were processed with specific antibodies raised against either oxytocin- or vasopressin–neurophysin. Despite the high level of labelling observed by Ben Barak *et al.* (1985) in hypothalamic sections with these antibodies and which we also obtained using our immunocytochemical procedure, less than half of the freshly dissociated magnocellular neurones in a well were strongly and unambiguously labelled. Therefore the lack of staining of a given neurone with oxytocin–neurophysin was not indicative of the presence of vasopressin, and vice versa. Consequently, only strongly immunopositive neurones were considered in the analysis of the correlation between peptide presence and receptor properties. Using this protocol, eight neurones which responded to oxytocin gave a positive reaction to oxytocin–neurophysin labelling,

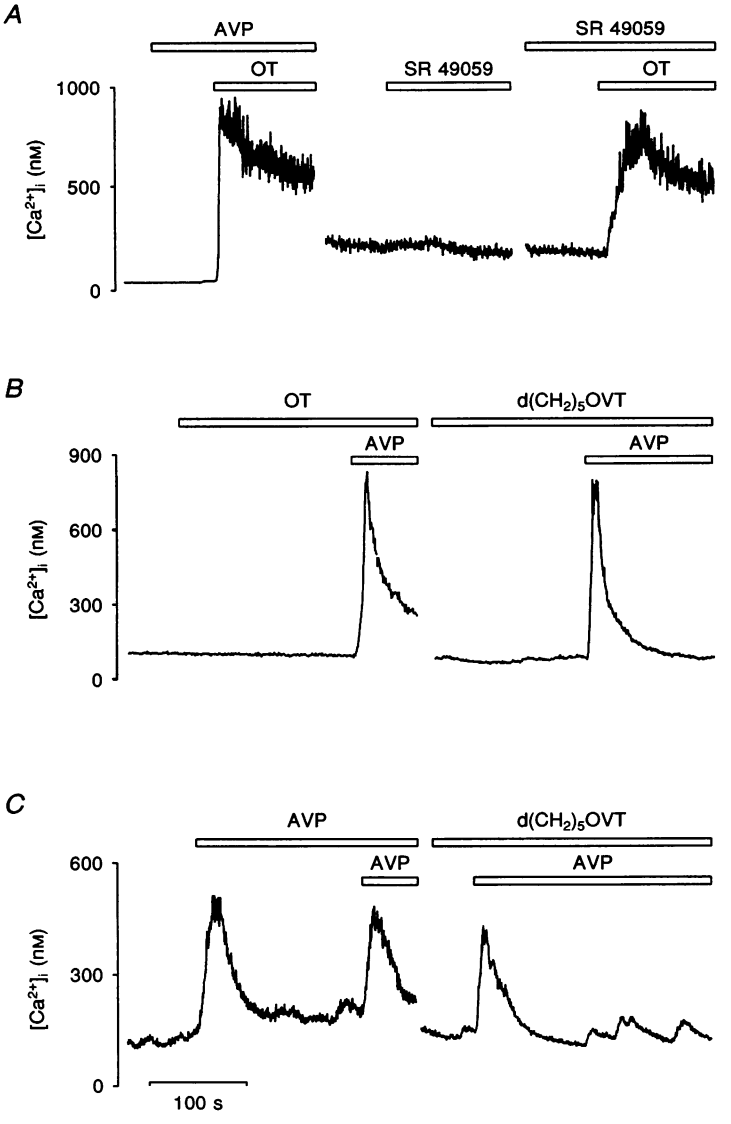

Figure 9. Selective actions of receptor antagonists on peptide-induced $[Ca^{2+}]_i$

A, oxytocin-induced increase in $[Ca^{2+}]_i$ in a vasopressin-insensitive cell (left panel). Application of SR 49059 (10 nM) affected neither the resting $[Ca^{2+}]_i$ (middle panel) nor the increase in $[Ca^{2+}]_i$ induced by oxytocin (right panel). *B*, vasopressin-induced $[Ca^{2+}]_i$ increase in an oxytocin-insensitive cell. Application of $d(CH_2)_5OVT$ (100 nM) did not affect both resting $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by vasopressin. *C*, using a similar experimental approach, the traces represent the effect of $d(CH_2)_5OVT$ recorded on an oscillating cell. Note that the oxytocin-induced $[Ca^{2+}]_i$ changes were not altered by the oxytocin receptor antagonist, $d(CH_2)_5OVT$.

whereas four neurones which responded to vasopressin gave a positive reaction to vasopressin–neurophysin labelling. Figure 10*A* shows an example of a neurone which responded to 100 nM oxytocin by an increase in $[Ca^{2+}]_i$ from 234 to 781 nM and subsequently gave a positive reaction to oxytocin–neurophysin labelling. Figure 10*B* shows the increase in $[Ca^{2+}]_i$ from 140 to 841 nM induced by 100 nM vasopressin in another neurone, which subsequently gave a positive reaction to vasopressin–neurophysin labelling. In addition, one neurone which responded to oxytocin (vasopressin was not tested) gave a positive reaction to vasopressin–neurophysin labelling. The mean oxytocin-induced $[Ca^{2+}]_i$ response was 624 ± 40 nM, whereas the mean vasopressin-induced response was 623 ± 70 nM.

DISCUSSION

In the present study, we have demonstrated a direct action of vasopressin on freshly dissociated magnocellular neurones from the SO nucleus. On the basis of direct measurements of changes in $[Ca^{2+}]_i$ using microspectrofluorimetry of fura-2, we have provided new information concerning the cellular mechanisms of vasopressin action. This paper follows our previous study on the direct effect of oxytocin on the same type of neurones (Lambert *et al.* 1994). In that study, it was demonstrated using immunocytochemical labelling that the freshly dissociated SO cell suspension primarily contains vasopressin and oxytocin magnocellular neurones, which can be identified by the large size of their soma, ranging between 12 and 18 μm, and the presence of processes. These criteria were used in the present study to select the cells for $[Ca^{2+}]_i$ measurements. Vasopressin evoked a transient increase in $[Ca^{2+}]_i$ in 86% of the vasopressin-sensitive cells, whereas the remaining cells displayed a sustained response (see Table 1). This feature is quite opposite to that observed in oxytocin-sensitive neurones in which a sustained response was observed in 88% of the cells, while the remaining neurones displayed transient responses (see Lambert *et al.* 1994). The physiological significance of these two types of $[Ca^{2+}]_i$ responses is still unclear. Nevertheless, more information about the fate of the oxytocin and vasopressin released in the extracellular medium of the SO nucleus is required to determine whether sustained responses are indeed relevant or whether they result from the dissociated state of the cells under investigation.

We observed that most of the sensitive cells responded selectively to only one peptide, either oxytocin or vasopressin (100 nM), except for 4% of the neurones, which

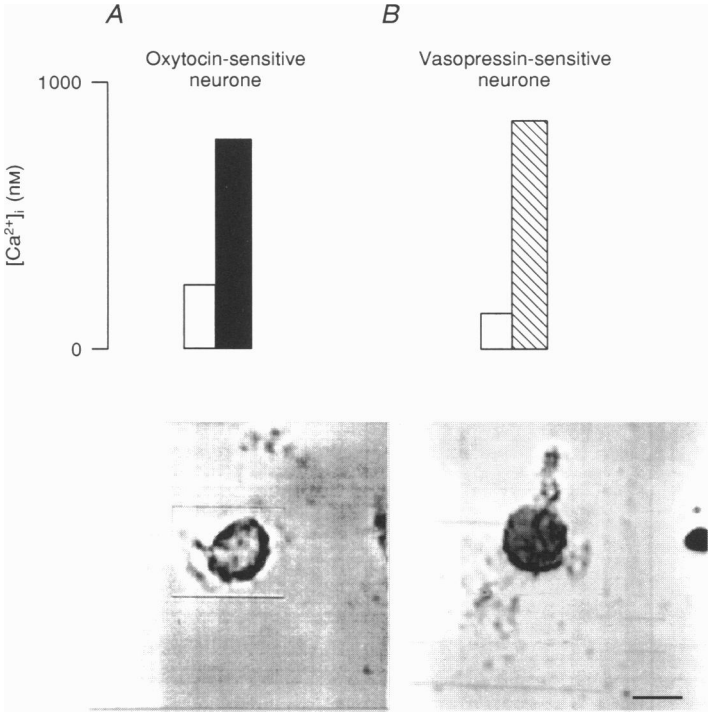

Figure 10. Immunocytochemical identification

A, increase in $[Ca^{2+}]_i$ elicited by 100 nM oxytocin (top) in a neurone which gave a positive reaction to subsequent labelling with oxytocin–neurophysin antibody (PS38; bottom). *B*, increase in $[Ca^{2+}]_i$ elicited by 100 nM vasopressin (top) in a neurone which subsequently gave a positive reaction to vasopressin–neurophysin antibody (PS41) (bottom). □, resting $[Ca^{2+}]_i$; ■, oxytocin-induced $[Ca^{2+}]_i$ increase; ▨, vasopressin-induced $[Ca^{2+}]_i$ increase. The bottom illustrations were obtained as described in Methods. Scale bar, 10 μm.

responded to both. Immunocytochemical labelling for oxytocin and vasopressin revealed that, except in one case, the cells which responded to vasopressin were immunoreactive to vasopressin–neurophysin, and those responding to oxytocin were immunoreactive to oxytocin–neurophysin, suggesting that oxytocin and vasopressin act on at least part of their respective neuronal populations by a rise in $[Ca^{2+}]_i$. These findings corroborate previous results on the central action of oxytocin and vasopressin. Indeed, immunocytochemical studies on male rats revealed that both peptides are released locally by the dendrites and the soma of SO magnocellular neurones (Pow & Morris, 1989). Concerning oxytocin, the presence of receptors and of receptor transcripts has been reported for magnocellular neurones (Yoshimura *et al.* 1993; Freund-Mercier, Stoeckel & Klein, 1994). In addition, local injections of oxytocin into the SO nucleus during suckling were shown to increase the electrical activity of oxytocin neurones *in vivo* (Moos & Richard, 1989), suggesting that the local release of oxytocin in the SO nucleus during the milk ejection reflex (Moos *et al.* 1989; Neumann *et al.* 1993) affects the electrical activity of oxytocin neurones via a rise in $[Ca^{2+}]_i$. Concerning vasopressin, a release of this peptide within the SO nucleus in response to osmotic stimulation (Landgraf & Ludwig, 1991) and local vasopressin infusion (Wotjak *et al.* 1994) has been reported. Our results provide a mechanism of action for vasopressin, whose presumptive role under physiological conditions is discussed at the end of this section.

In hypothalamic SO neurones, *in situ* hybridization revealed coexpression of the messenger RNA for oxytocin and vasopressin in a small population of cells (Kiyama & Emson, 1990), which increased to about 10% during lactation (Mezey & Kiss, 1991). In addition, in SO and PV nuclei of suckled rats, some electrophysiologically recorded neurones were shown to display both oxytocin-related (milk ejection bursts) and vasopressin-related (phasic activity) electrical activity, suggesting that some magnocellular neurones possess a mixed electrophysiological phenotype with the possibility of peptide coexistence (see Moos & Ingram, 1995). Therefore, it was speculated that electrophysiologically identified vasopressin–oxytocin neurones belong to the same population of magnocellular neurones which coexpress oxytocin and vasopressin. Our data could provide a functional support to this hypothesis, since we found some neurones which exhibited $[Ca^{2+}]_i$ responses to both oxytocin and vasopressin. It is noteworthy that the percentage of the cell population displaying this mixed phenotype was less than 10% in *in situ* hybridization, electrophysiological and microspectrofluorimetric studies.

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

The spontaneous $[Ca^{2+}]_i$ oscillations observed in a significant proportion of vasopressin-responding cells were suppressed by lowering the external Ca^{2+} concentration, as has already been reported in oxytocin-sensitive neurones (Lambert *et al.* 1994), suggesting that the entry of extracellular Ca^{2+} is a necessary step in these oscillations. However, there seems to be a distinction between oscillations observed in oxytocin- and vasopressin-responding neurones: oscillations in vasopressin-sensitive neurones were mostly regular with rather constant amplitudes, whereas in oxytocin-sensitive neurones, the oscillations were much more irregular and of variable amplitudes (G. Dayanithi, unpublished observations). In primary cultures of embryonic hypothalamic neurones, suppression of spontaneous oscillations was also reported in low external Ca^{2+} as well as under TTX application (Dayanithi, Rage, Richard & Tapia-Arancibia, 1995). This latter finding suggests that $[Ca^{2+}]_i$ oscillations might arise from Ca^{2+} entry via voltage-gated channels.

Receptor mediation of the vasopressin response

To date, three vasopressin receptor subtypes (V_{1a}, V_{1b} and V_2) have been characterized. The V_{1b} receptors were characterized in the anterior pituitary on the basis of their unique pharmacological profile, and their role is still unclear. V_2 receptors are known to activate the cyclic AMP pathway and exert a major role in the renal antidiuretic response to vasopressin. The V_{1a} receptors, whose activation results in an increase in $[Ca^{2+}]_i$ via activation of the phosphoinositide pathway, and the V_2 receptors have been cloned from liver and kidney libraries, respectively (Morel, O'Carroll, Brownstein & Lolait, 1992). *In situ* hybridization studies revealed that only the V_{1a} transcripts could be observed in the brain (Ostrowski, Lolait, Bradley, O'Carroll, Brownstein & Young, 1992; Ostrowski, Lolait & Young, 1994), challenging the presence of V_2 receptor antagonist binding sites described in the suprachiasmatic nucleus (van Leeuwen, van der Beek, van Heerihuize, Wolters, van der Meulen & Wan, 1987). This corroborates electrophysiological data which showed that vasopressin-induced excitation elicited in several regions of the brain is selectively blocked by V_1 receptor antagonists (Raggenbass, Tribollet, Dubois-Dauphin & Dreifuss, 1989; Dreifuss, Tribollet, Dubois-Dauphin & Raggenbass, 1989; Tribollet, Goumaz, Raggenbass, Dubois-Dauphin & Dreifuss, 1991). By contrast, in guinea-pig hypothalamic slices containing the SO nucleus region, Abe and co-workers (Abe *et al.* 1983) reported that vasopressin could depolarize the neurones and activate adenylate cyclase, suggesting that vasopressin binds to V_2 rather than to V_1 receptors. Nevertheless, no receptor antagonist was tested to give further support to this hypothesis. SR 49059, a new potent non-peptide antagonist for the V_{1a} type receptor has recently been characterized in several *in vivo* and *in vitro* models (Serradeil-Le Gal *et al.* 1993). SR 49059 showed a high affinity for V_{1a} receptors from rat liver (the concentration giving half-maximal inhibition (K_i) ranging from 1·65 to 2·2 nM) and human tissues such as platelets, adrenals and myometrium (K_i ranging from 1·1 to 6·3 nM). In the present

Table 2. A comparative statement of magnocellular neurone responses ($[Ca^{2+}]_i$ rise) to oxytocin or vasopressin application

Oxytocin-sensitive neurones	Vasopressin-sensitive neurones
Mostly sustained responses	Mostly transient responses
Dose dependent	Dose dependent
No desensitization	Desensitization
Responses blocked by oxytocin receptor antagonist	Responses blocked by V_{1a} type vasopressin receptor antagonist
External Ca^{2+} independent	External Ca^{2+} dependent
Mostly from thapsigargin-sensitive stores	Partly from thapsigargin-sensitive stores

study, we showed that SR 49059 abolished the vasopressin-induced $[Ca^{2+}]_i$ rise in the neurones responding with a transient response. This suggests that V_{1a} receptors mediate the Ca^{2+} response in most of the responsive magnocellular neurones, as is the case in the other regions of the brain. On the other hand, our preliminary results revealed that V_2 antagonist applied at a high concentration (100 nM) seems to have a non-specific effect on the vasopressin-induced $[Ca^{2+}]_i$ responses. However, it is noteworthy that the antagonist affects the spontaneous $[Ca^{2+}]_i$ oscillations of magnocellular neurones (G. Dayanithi, unpublished observations). Nevertheless, further pharmacological characterization using this antagonist would confirm the specificity of its action on magnocellular neurones.

A peculiarity of oxytocin and vasopressin receptors is that they can show cross-reactivity for oxytocin and vasopressin. Indeed, the work of Audigier & Barberis (1985) in the hippocampus showed that oxytocin receptors exhibit equal affinity for vasopressin and oxytocin while V_{1a} receptors, although exhibiting a higher affinity for vasopressin, are still capable of binding oxytocin. It should be pointed out that, under our experimental conditions, we demonstrated that application of 10 nM of the V_{1a} antagonist, SR 49059, did not alter the oxytocin-induced $[Ca^{2+}]_i$ response. In addition, the oxytocin receptor antagonist $d(CH_2)_5OVT$ did not affect the vasopressin-induced $[Ca^{2+}]_i$ increase. This suggests that there is no cross-reactivity between the respective receptors of oxytocin and vasopressin at the concentrations used in this study and that the vasopressin and oxytocin responses are mediated by specific receptors.

Ca^{2+} mobilization from intracellular stores

In the present study, we have shown, by means of the experiments involving thapsigargin, that the vasopressin-induced increase in $[Ca^{2+}]_i$ is due partly to an influx of Ca^{2+} and partly to release from internal stores. Thapsigargin, a selective blocker of the Ca^{2+}-ATPase located in the endoplasmic reticulum (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990) has been shown to mobilize intracellular Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores without hydrolysis of inositol phospholipids (Jackson, Patterson, Thastrup & Hanley, 1988), suggesting that the phosphoinositide pathway may be involved in the vasopressin response. Ca^{2+} mobilization from intracellular stores could occur through activation of Ca^{2+} channels gated by IP_3 or ryanodine receptors. Both IP_3 and ryanodine receptors have been involved in receptor- or depolarization-mediated Ca^{2+} signals in nerve cells (Friel & Tsien, 1992). Our results demonstrate that part of the vasopressin-induced $[Ca^{2+}]_i$ increase comes from thapsigargin-sensitive stores, since it is significantly reduced after pre-incubation in thapsigargin. Thapsigargin-sensitive stores are also involved in the oxytocin-induced $[Ca^{2+}]_i$ response, but in this case they represent the main source of the $[Ca^{2+}]_i$ increase (Lambert *et al.* 1994). Nevertheless, the vasopressin response differs markedly from the oxytocin response in that the former is dependent on external Ca^{2+} whereas the latter is not. Mobilization of intracellular Ca^{2+} has been demonstrated in hepatocytes (Kawanishi *et al.* 1989) and in pituicytes (Hatton *et al.* 1992), which was mediated by V_1 type receptors. However, the Ca^{2+} response observed in these preparations was shown to be independent of external Ca^{2+}.

The other compounds such as caffeine and tBuBHQ, which are used routinely as tools for defining intracellular Ca^{2+} stores as well as Ca^{2+} fluxes and Ca^{2+}-dependent processes (Cheek, O'Sullivan, Moreton, Berridge & Bourgoyne, 1990), were without effect on resting or vasopressin-induced $[Ca^{2+}]_i$ responses. Therefore, we can conclude that the Ca^{2+} stores sensitive to both compounds are not implicated in the vasopressin-induced response in SO neurones.

A characteristic feature of magnocellular neurones is that the vasopressin-induced increase in $[Ca^{2+}]_i$ is desensitized after successive applications, which was not reported for the V_1-mediated $[Ca^{2+}]_i$ response measured in both pituicytes and hepatocytes (Kawanishi *et al.* 1989; Hatton *et al.* 1992). In addition, no desensitization of the vasopressin-induced membrane current mediated via V_1 type receptors was observed in different regions of the brain in which such a current could be elicited (Raggenbass *et al.* 1989; Dreifuss *et al.* 1989; Tribollet *et al.* 1991).

Physiological role

The physiological role of the central action of oxytocin on magnocellular neurones is clearly established. Nevertheless, such consistencies are not evident when considering the previously reported contradictory effects of vasopressin on putative vasopressin neurones. Since vasopressin directly evokes an increase in $[Ca^{2+}]_i$ at the cellular level (present study), the most obvious and exciting consequence arising from the activation of a Ca^{2+} current in vasopressin neurones is that this will contribute to the activation of a Ca^{2+}-dependent K^+ current. It is possible, therefore, to speculate that during a phase of activity the vasopressin neurones release vasopressin from the dendrites, which, when it reaches a threshold concentration, activates a Ca^{2+} current. The vasopressin-induced Ca^{2+} current would add to the voltage-activated Ca^{2+} current and result in a more potent increase in $[Ca^{2+}]_i$. This will in turn activate the K^+ current more rapidly, which would hyperpolarize the neurone and stop the phasic bursts. Because the vasopressin-induced $[Ca^{2+}]_i$ increase is mainly transient, despite the continued presence of vasopressin, the hyperpolarizing current will subside as the $[Ca^{2+}]_i$ returns to normal. During this silent period, the extracellular vasopressin concentration will also decline as the peptide diffuses away from the neurone. The regenerative properties of the neurone will then cause the neurone to begin firing again to release vasopressin.

The percentage of neurones sensitive to either oxytocin or vasopressin is about 50%, the remaining 50% being insensitive to both peptides. This implies the existence of peptide-containing neurones which do not respond to peptide application. Indeed, immunocytochemical labelling reported in our previous study revealed that a great majority of magnocellular neurones were stained for either oxytocin or vasopressin (Lambert *et al.* 1994), suggesting the existence of distinct subpopulations of vasopressin neurones. Several hypotheses can be postulated concerning these subpopulations. First, non-responsiveness to either peptide could reflect the fact that oxytocin and vasopressin receptors are either absent or located in the remote dendritic processes, which are not entirely preserved by the dissociation procedure. Second, the neurones that do not respond to either oxytocin or vasopressin could possess different receptors, the activation of which would not affect $[Ca^{2+}]_i$. Alternatively, the responsive neurones could function as pacemakers, and the non-responsive neurones as follower cells.

In conclusion, the present results complement our previous study (Lambert *et al.* 1994), demonstrating that vasopressinergic and oxytocinergic neurones are subject to a selective autoregulation by their respective neuropeptide (see Table 2). This autocontrol is mediated by specific vasopressin and oxytocin receptors, which both induce a rise in $[Ca^{2+}]_i$ upon activation, although involving distinct intracellular mechanisms. The functional significance of these $[Ca^{2+}]_i$ increases in the regulation of the electrical activity of magnocellular neurones by vasopressin and oxytocin remains to be determined.

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