Electrophysiological evidence for mutual excitation of oxytocin cells in the supraoptic nucleus of the rat hypothalamus

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1. Using the ventral surgical approach *in vivo*, extracellular recordings were made from seventy-nine cells in the supraoptic nucleus of urethane-anaesthetized male, virgin female or lactating female rats while stimulating the pituitary stalk. Cells were classed according to their spontaneous firing activity as: continuous (putative oxytocin), phasic (putative vasopressin) and silent.

2. Stimulation of the neural stalk produced an excitation (up to 25 ms poststimulus) in eleven of the seventy-nine antidromically identified magnocellular neurones, consistent with the existence of excitatory collaterals or dendritic contacts between such cells. In these recordings a second spike could frequently be seen, following the antidromic spike, with a variable latency. Such spikes consistently collided with subsequent antidromically evoked spikes. Poststimulus excitation was only seen in silent and continuously firing (putative oxytocin) cells, suggesting that oxytocin and vasopressin cells have different connections.

3. Excitatory connections were seen more frequently in lactating females (8 out of 22 cells) than in males (1 out of 15 cells) or virgin females (2 out of 10 cells), and thus may make an important contribution to the bursts of firing which precede reflex milk ejection.

In the lactating rat, suckling evokes bursts of activity in the magnocellular oxytocin neurones of the hypothalamus. These bursts consist of 1–2 s of intense discharge and occur at intervals of 1–10 min (Wakerley & Lincoln, 1973). Since oxytocin given centrally facilitates bursting behaviour (Freund-Mercier & Richard, 1984) it has been speculated that oxytocin release within the hypothalamus provides a positive feedback signal to promote bursting. Oxytocin also induces changes in the synaptic morphology of the adult hypothalamo– neurohypophysial system, which are similar to those seen in lactating rats (Theodosis, Montagnese, Rodriguez, Vincent & Poulain, 1986*b*). However, to date there has been no direct electrophysiological evidence for excitatory interconnections between oxytocin cells.

By contrast, the first studies of intercommunication between magnocellular neurosecretory cells suggested the existence of inhibitory collaterals. Following electrical stimulation of the neural stalk (Dreifuss & Kelly, 1972) the magnocellular neurones of the supraoptic nucleus were quiescent for 30–50 ms after antidromic invasion. It emerged subsequently (Leng & Dyball, 1983) that the period following an antidromic spike, during which the probability of the cell firing a second spike was reduced, had the same duration as that for spontaneous spikes. It corresponded approximately with the duration of the hyperpolarizing after-potential seen in intracellular recording (see, e.g. Bourque, 1988). This made it unnecessary to introduce the concept of an inhibitory collateral to explain the behaviour of the system.

The hyperpolarizing after-potential is almost certainly the mechanism which normally restricts the observed interval between successive action potentials to between 40 and 50 ms for both oxytocin and vasopressin cells (Dyball & Leng, 1986). In this earlier study, we showed that, during milk ejection bursts, the modal interspike interval ranged from 9 to 18 ms. It was thus clear that, to allow all the oxytocin cells to fire a synchronous burst of spikes at a sufficiently high frequency, the mechanism which normally prevents short interspike intervals in magnocellular neurones must be modified during milk ejection, possibly by mutual excitation of oxytocin neurones. Such excitation might involve collaterals. Evidence for the existence of collaterals in the magnocellular system was provided for the supraoptic nucleus by Mason, Ho & Hatton (1984) and for the paraventricular nucleus by Hatton, Cobbett & Salm (1985). These authors provided evidence for connections between the cells of the magnocellular nuclei and cells elsewhere in the hypothalamus. Hatton's group has also provided evidence for dye coupling between cells within the magnocellular nuclei (Hatton, Yang & Smithson, 1988) and evidence for electrical coupling between rat supraoptic neurones (Yang & Hatton, 1988). We know of no reports of

direct evidence for such connections within the nuclei using recording studies *in vivo*. However, we have recently seen evidence in the paraventricular nucleus for poststimulus excitation when the neural stalk was stimulated under conditions in which the occurrence of an antidromic spike was prevented by collision (McKenzie & Dyball, 1994). This led us to look for a similar excitation in recordings from magnocellular neurones of the supraoptic nucleus. The presence of excitation in either or both nuclei would suggest the existence of excitatory interconnections between the magnocellular neurones.

METHODS

Extracellular recordings were made from the supraoptic nucleus of urethane-anaesthetized (1·3 g kg^{-1}, I.P.) lactating female, virgin female and male Wistar rats using the ventral surgical approach (Leng, 1980). The recordings were made using conventional electrophysiological techniques with glass microelectrodes which were orientated under direct visual control using a dissecting microscope. The electrodes were filled with 0·5 M sodium acetate containing 2% Pontamine Sky Blue for marking the recording sites and had a tip resistance of 10–15 MΩ. Spike trains were recorded on a digital tape-recorder (DTR 1203, Biologic Science Instruments, Claix, France) and subsequently analysed using an intelligent interface (1401 plus, Cambridge Electronic Design, Cambridge, UK) and Spike 2 software (Cambridge Electronic Design). Stimuli were applied to the neural stalk through a side-by-side bipolar electrode placed on it under visual control. The stimulus pulses were biphasic with a matched square waveform and a total duration of 2 ms. Stimulus intensity at threshold for antidromic activation varied between 0·5 and 0·7 mA.

A cell was classified as showing poststimulus excitation if more spikes occurred following a stimulus presented 5 ms after a spontaneous spike than in the absence of the stimulus. The number of spikes which occurred within 25 ms of the spontaneous spike was compared with a comparable control period and significance was determined by the binomial test. A period of 25 ms was chosen since previous work (Dyball & Leng, 1986) had shown that intervals shorter than this were extremely rare outside milk ejection bursts.

RESULTS

The occurrence of 'double' antidromic spikes

Recordings were made from a total of seventy-nine neurones in the supraoptic nucleus (47 continuously firing, 28 phasic and 4 silent cells). In two recordings, a suprathreshold stimulus pulse applied to the neural stalk frequently evoked a pair of constant latency antidromic spikes (Fig. 1). There was no evidence for collision between the second of the evoked spikes and an antidromic spike

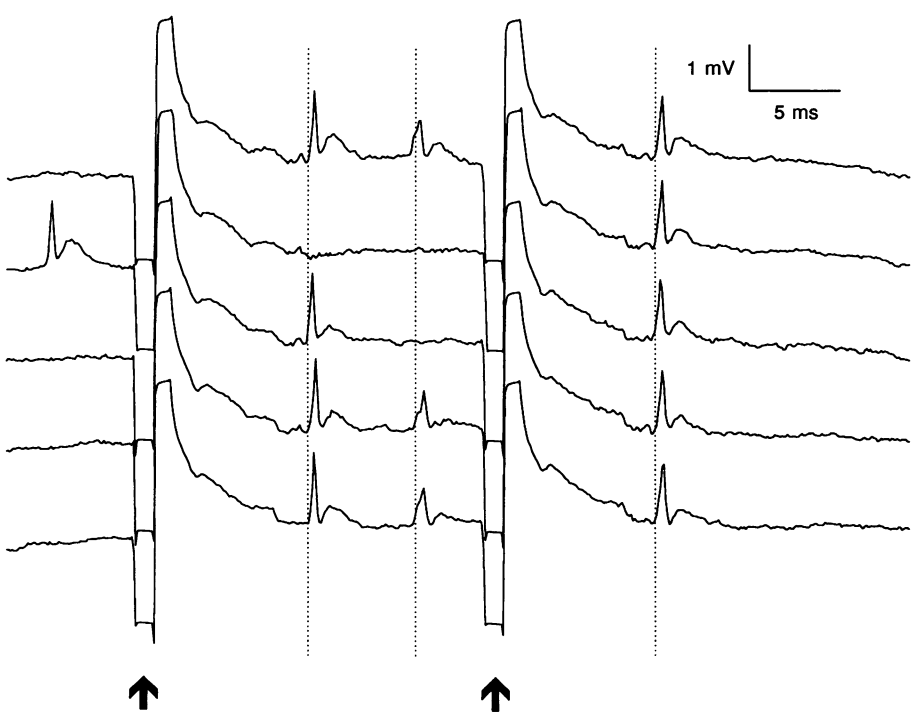

Figure 1. Double antidromic spikes following neural stalk stimulation

Five consecutive traces recorded from the supraoptic nucleus of a male rat to show 1 or 2 antidromic spikes following each of a pair of stimulus pulses (arrowed) applied to the neural stalk with an interval of 20 ms, except where the spike was eliminated by collision with a spontaneous spike. In 3 out of the 5 sweeps, a second spike also followed the first pulse. Note that the second spike of each pair must have been antidromically evoked because the second spike after the first pulse never eliminated the spike following the second pulse by collision.

evoked by a second stimulus pulse. The second spike was not seen when stimulus intensity was lowered to close to the threshold for antidromic activation. The latencies of both the first and second evoked spikes were constant. Such observations are consistent with a number of possible explanations. Action potentials might have been elicited at two separate sites on the axon or, after the stimulus pulse, the stimulus site may have remained depolarized for longer than the absolute refractory period of the axon so that a second antidromic spike was evoked following the single pulse. Such observations provide no evidence for the existence of axon collaterals.

In some other cells (11), two spikes were sometimes seen after a single pulse but the second spike followed the stimulus pulse inconsistently and with a variable latency.

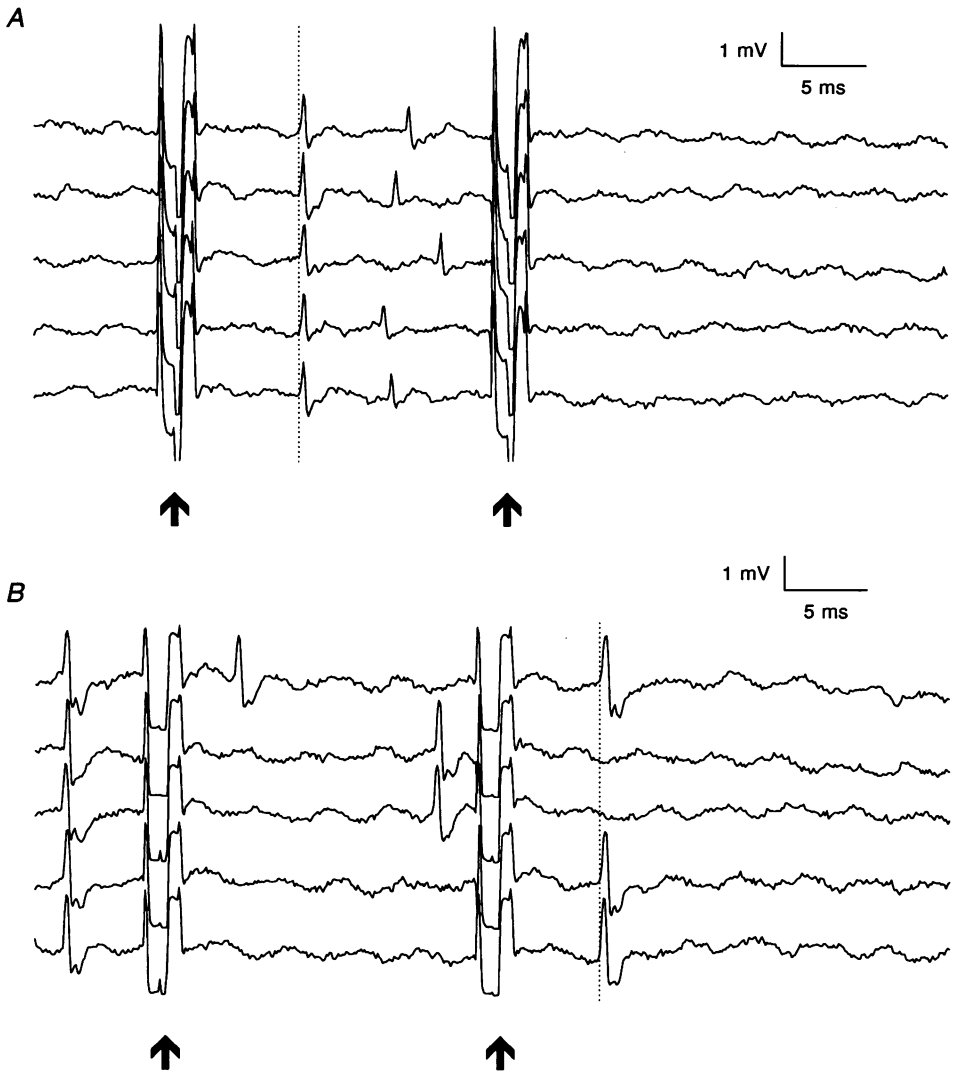

Figure 2. Collision of stimulus-evoked orthodromic and antidromic spikes

A, 5 traces recorded from the supraoptic nucleus of a virgin female rat following pairs of stimulus pulses (arrowed) applied to the neural stalk with an interval of 20 ms. The first spike following the first stimulus pulse was almost certainly antidromically evoked since it followed the pulse at a constant latency. In each sweep, the antidromic spike following the second pulse was eliminated by collision with the presumably orthodromically propagated spike which followed activation of the putative mutual excitatory path. *B*, 5 traces recorded from the same cell as *A* following pairs of stimulus pulses (arrowed) applied to the neural stalk with an interval of 20 ms but triggered 5 ms after a spontaneous spike. As expected, the antidromically evoked spike following the first stimulus pulse was eliminated by collision. In 3 sweeps, a spike followed the first pulse with a variable latency. The spike in the top trace must have propagated orthodromically because it was not eliminated by collision. The spikes between the pulses in traces 2 and 3 must have propagated orthodromically since they prevented the occurrence of an antidromic spike following the second pulse. These orthodromically propagated spikes may also have resulted from activation of the putative mutual excitatory path.

In the second type of 'double-spike' recording, the second spike following the stimulus pulse applied to the neural stalk occurred at threshold but was not always present, even with very high stimulus intensity.

When pairs of stimulus pulses were applied to the neural stalk, in which the second stimulus followed a late second spike, the antidromic spike following the second pulse was always extinguished (presumably) by collision, demonstrating the orthodromic nature of the late spike. This is illustrated in Fig. 2*A* in which an antidromic spike was never seen after the second of a pair of pulses applied to the neural stalk because the second (putative orthodromic) spike was present very regularly. The 'second' spike always eliminated the expected antidromic spike following a second stimulus pulse applied 20 ms after the first. The 'second' spike also occurred if the first antidromic spike was intentionally eliminated by collision (Fig. 2*B*) and, if it occurred just before a second stimulus pulse, eliminated the expected antidromic spike. Such behaviour suggests that the second spike propagated orthodromically. It thus appeared that stimuli to the neural stalk evoked a variable latency orthodromic spike, in addition to an antidromic spike. Since these orthodromic spikes frequently occurred within the normal duration of the hyperpolarizing afterpotential it is likely that a substantial orthodromic excitation must have occurred.

The occurrence of the presumed orthodromically propagated spike could be seen more clearly if a single

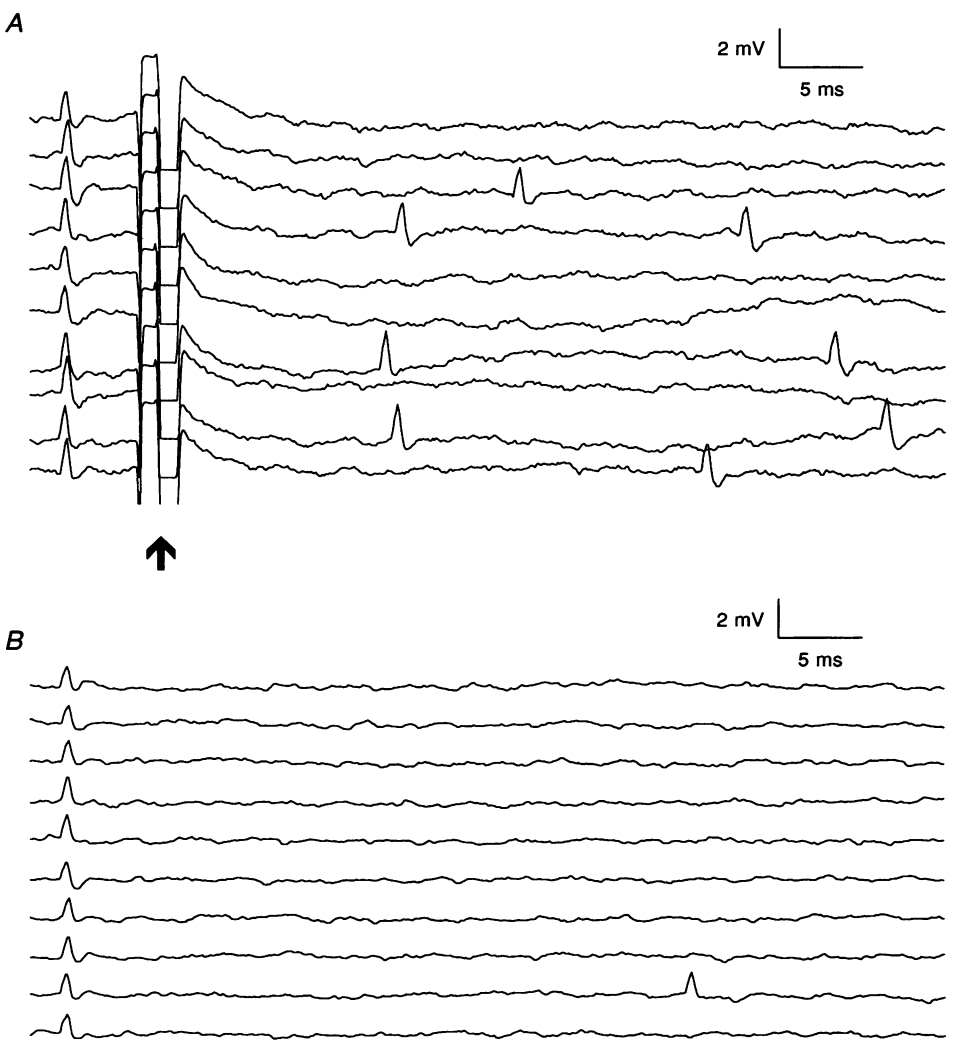

Figure 3. Orthodromic spikes evoked by neural stalk stimulation

Traces from a single cell in the supraoptic nucleus of a lactating rat showing that the cell fires more frequently following electrical stimulation of the neural stalk. *A*, 10 traces to show the effects of stimulating the neural stalk on the subsequent firing of the cell. Each trace was triggered by a spontaneously occurring spike. *B*, 10 traces from the same cell without stimulation. As before, each trace was triggered by a spontaneously occurring spike. It is clear that the application of the stimulus pulse increased the number of spikes which occurred after the spike which triggered the sweep. The latency for antidromic activation of this cell was 12 ms.

stimulus pulse was applied to the neural stalk following the occurrence of a spontaneous spike. Figure 3*A* shows recording traces to illustrate this point. In each trace a spontaneous spike triggered a stimulus pulse applied to the neural stalk. It should be compared with Fig. 3*B* in which the traces were triggered in exactly the same way but with the stimulus isolation units switched off. This controls for spontaneously occurring (unstimulated) short intervals.

To demonstrate the effect more clearly, poststimulus histograms were created. Figure 4*A* shows the poststimulus histogram following the application of 115 stimuli to the neural stalk at 5 ms after the occurrence of a spontaneous spike. The pulses were delivered at approximately 0·5 Hz since they were triggered (with a 5 ms delay) by the next spontaneous spike which occurred after 2 s had elapsed since the previous stimulus pulse. The poststimulus excitation should be contrasted with the histogram in Fig. 4*B*, which was prepared in exactly the same way but with the stimulus isolation units turned off. Excitation between 10 and 50 ms following the stimulus pulse was

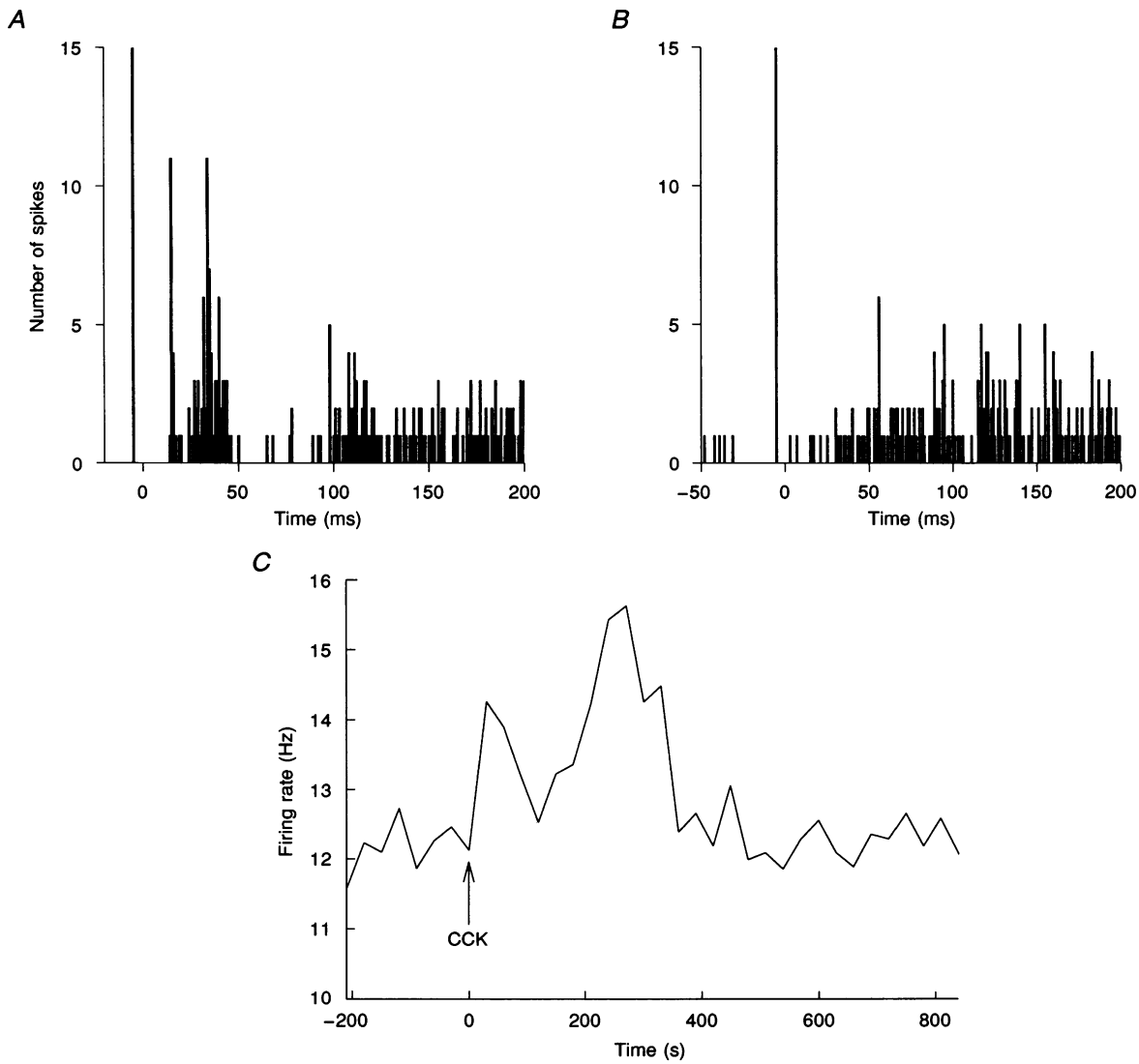

Figure 4. Orthodromic excitation of an oxytocin cell by neural stalk stimulation

Recordings from a single oxytocin cell in the supraoptic nucleus of a lactating rat to show that the cell was excited by electrical stimulation of the neural stalk, even though the antidromic spikes were eliminated by collision. *A*, poststimulus histogram (115 sweeps; ≤ 0·5 Hz) showing the effect of application of a stimulus pulse to the neural stalk (at time 0) triggered 5 ms after the occurrence of a spontaneous spike. A clear excitation occurred after the stimulus pulse with peaks at 15 and 34 ms poststimulus; these occur later than the antidromic latency of 12 ms. Note that the peak at −5 ms is truncated and represents 115 spikes. The biphasic nature of this response was not a consistent feature of all such responses. *B*, this excitation was not seen when the stimulus isolation units were switched off. Again, the peak at −5 ms has been truncated from 115 spikes. *C*, ratemeter record to show that intravenous injection of cholecystokinin (CCK; 20 μg kg^{-1} at time 0) excited the cell. The effect was significant ($P < 0{\cdot}01$; Student's t test).

seen clearly in Fig. 4A but not in Fig. 4B. The early (15 ms) peak in Fig. 4A occurred at a longer latency than the latency for antidromic activation (12 ms) in this cell. It should be noted that the biphasic poststimulus excitation illustrated in Fig. 4A was not a feature of all the cells tested.

Identity of the cells showing mutual excitation

Short latency (< 20 ms) poststimulus excitation following the application of a pulse to the neural stalk at 5 ms after a spontaneous spike was not seen in any of the twenty-eight of the seventy-nine recorded cells which fired phasically.

Poststimulus excitation, however, was a feature of eleven cells which did not fire phasically. Two of the twenty-four cells in virgin female (1 continuous, 1 silent), one (continuous) of the twenty-five cells in male and eight (all continuous) of the twenty-eight cells recorded from lactating female rats showed such a response. It was not possible to test all such cells with intravenous injections of cholecystokinin (20 μg kg^{-1}) which activates oxytocin but not vasopressin cells (Leng, Way & Dyball, 1991), but two of the eight continuously firing cells in lactating animals were excited (Fig. 4C) and a further two did not fire phasically during constant collision stimulation which is a useful additional method for identifying vasopressin cells (Leng & Dyball, 1991). The remaining four cells in lactating animals were not tested. One of the two cells in virgin female rats, which showed poststimulus excitation, was also excited by cholecystokinin. Considering the continuously firing and silent cells only, the proportion of cells which showed poststimulus excitation was significantly greater in lactating rats than in the others ($P < 0{\cdot}05$; Fisher's exact probabilities test, including males and virgin females as a single group). It seems that the poststimulus excitation was confined to oxytocin neurones, although it is possible that some vasopressin neurones which do not fire phasically also show the phenomenon.

DISCUSSION

Recording studies *in vivo* have revealed two types of characteristic firing pattern for the cells of the supraoptic nucleus, classified as phasic (bursting) and continuous (Poulain & Wakerley, 1982). Although these patterns are very different from one another, electrophysiological studies *in vitro* have not revealed two distinct classes of cell. This suggests that at least some of the differences found *in vivo* between the two cell types might be due to features of the cells which are suppressed or not present in slices. A particular feature of the difference is the capacity of the cells to fire a short burst of spikes at high frequency (interval < 20 ms) during reflex milk ejection, which must mean that the usual hyperpolarizing after-potential (duration 25–35 ms), which is characteristic of magnocellular cells recorded intracellularly *in vitro* (Bourque, 1988) or *in vivo* (Bourque & Renaud, 1991; Dyball, Tasker, Wuarin & Dudek, 1991), must be overridden for a short time. The difference might be due to a change in the excitability of the cell membrane and there is evidence that oxytocin might exert such an influence (Freund-Mercier & Richard, 1984); however, another possibility might be the existence of excitatory collaterals causing the cells to mutually excite their neighbours. There is good evidence that such excitatory connections can form between cultured magnocellular cells *in vitro* (Gähwiler & Dreifuss, 1979). In that report the activity of the 'pacemaker' and 'follower' cells was intermittent but the duration of the bursts was rather shorter than that recorded from vasopressin cells *in vivo* so that the intermittently active cells may not have been vasopressin cells. Until now, however, no good evidence has emerged for the existence of such connections between oxytocin cells *in vivo*.

Our experiments have shown that while high intensity stimuli applied to the axons of the neural stalk can evoke two spikes which are both antidromically propagated, stimuli just above threshold can also evoke two spikes in some neurones. The first of these can be eliminated by collision with an orthodromic spike. It would be hard to set up an experimental situation in which the second of the two spikes might collide with an orthodromic spike. However, it is almost certain that such spikes are not antidromically evoked because a stimulus pulse applied just after the second spike failed to evoke an antidromic spike itself in these cells. It must therefore have been propagating orthodromically and have collided in mid-axon with the antidromic spike evoked by the second stimulus pulse. If it had been an antidromic spike, it would not have eliminated the antidromic spike following the second stimulus pulse (Figs 1 and 2). The construction of poststimulus histograms for cells which showed excitation following stimuli to the neural stalk showed that, when the stimulus pulse was applied after a spontaneous spike to eliminate the antidromic spike which would otherwise have occurred, there was an increased likelihood of the occurrence of spikes up to 25 ms after the stimulus pulse. It is possible that the poststimulus excitation seen in such circumstances was not due to excitation of axon collaterals or other excitatory interaction between magnocellular cells but to accidental excitation of excitatory input to the supraoptic nucleus from the basal region of the brain just dorsal to the neural stalk. This seems unlikely for several reasons. First, the spread of stimulus current from stimulating electrodes of the type used in this study only extends for approximately 0·1 mm for each doubling of stimulus intensity (Dyball & Leng, 1992) and the stimulus intensities used for this study were just above threshold. Second, if such excitation occurred, there is no reason to believe that it would have been more frequent in lactating animals than in virgin females or male animals. If the excitation had been accidental it might also have occurred in vasopressin cells. Third, even if accidental stimulation had occurred, the

expected effect would have been inhibition. Leng, Yamashita, Dyball & Bunting (1988) showed that stimulation of the region of the arcuate nucleus (which is just dorsal to the stimulus site) inhibited seventeen and excited none of nineteen putative oxytocin cells in the supraoptic nucleus.

Coles & Poulain (1991) reported significant changes in extracellular potassium concentration associated with bursts of 160 antidromic spikes. It seems unlikely, however, that a single antidromic spike would change the extracellular potassium concentration in the vicinity of oxytocin cells sufficiently to alter the excitability of the cells for a period of up to 25 ms, especially since Coles & Poulain also conclude that potassium clearance around oxytocin cells is more effective than it is around vasopressin cells. This makes it unlikely that the poststimulus excitation we saw was due to factors such as local changes in extracellular ion concentration. It is also possible that the phasic firing pattern somehow prevents the excitation but it seems more likely that there is a distinct difference in connectivity between oxytocin and vasopressin cells. This suggestion is supported by the reports of structural changes in the supraoptic nuclei of lactating rats (increased cell membrane apposition and an increased number of double synapses bridging two cells) which are confined to oxytocin neurones (Theodosis, Chapman, Montagnese, Poulain & Morris, 1986*a*). While mutual excitation is clearly a feature that might have been expected between neuroendocrine cells which fire together to produce an increase in the plasma concentration of the hormone they secrete, it is also clear that the cytoarchitecture and local connections required to support the phenomenon are not restricted to lactating animals since it can also be seen in male and virgin female animals as well as lactating females.

Since antidromic activation has been used for many years to identify cells of the neurohypophysial system, the question arises as to why the phenomenon has not been reported earlier. Our suggestion is that the phenomenon was in fact seen but since it is not common in non-lactating animals it was only seen occasionally. When it was seen, any orthodromically evoked spikes which occurred very soon after the stimulus pulse would have appeared as 'antidromic' spikes with an inconstant latency. Spikes evoked later would have interfered with the second antidromic spike in the '2 shock collision test' frequently used by those working on the neurohypophysial system. Cells displaying the phenomenon may thus have been discarded as unclassifiable and not recorded.

Not all cells which showed poststimulus excitation were recorded for long enough to be tested with cholecystokinin in order to identify continuously firing cells as oxytocin cells. However, we never observed evidence of mutual excitation in identified vasopressin cells although such cells were recorded close to and tested in the same way and in the same animals as the putative oxytocin cells. Whatever the reason for their not having been reported previously, the excitatory interactions we have observed may be extremely important functionally if they contribute to the orchestrated activity of the oxytocin cells during the milk ejection burst.

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