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CHARACTERISTICS OF EARLY- AND LATE-RECRUITED OXYTOCIN BURSTING CELLS AT THE BEGINNING OF SUCKLING IN RATS

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SUMMARY

1. Paired or single recordings of paraventricular and/or supraoptic oxytocin cells at the beginning of suckling in urethane-anaesthetized rats enabled us to study cell recruitment and compare the characteristics of the early- and late-recruited cells. This was done under different experimental conditions, i.e. when the reflex was triggered in less than 1 h suckling (control), and when its triggering was facilitated either by the intraventricular (I.C.V.) injection of oxytocin, of apomorphine (a dopamine agonist) or by the intravenous (I.V.) injection of propranolol (a β-adrenoceptor antagonist) into suckled rats with no milk ejection.

2. Under control conditions, the amplitude (total number of spikes) of the successive bursts of the early-recruited cells progressively increased, generally reaching maximum by the 6th burst. This increase was more rapid and greater after oxytocin than under control conditions or after apomorphine injection, and was delayed and reduced after propranolol. The burst frequency was higher after oxytocin and apomorphine injections than under control conditions and very low after propranolol.

3. Late-recruited cells were observed under all experimental conditions, except after oxytocin injection, since all cells displayed bursts right away. Moreover, when injected during the recruitment period of a control reflex, oxytocin greatly speeded up the recruitment of the late-recruited cells. These cells generally displayed smaller amplitude bursts than the early-recruited cells. Moreover, the increase in burst amplitude was less marked for the late- than for the early-recruited cells and often was not sustained.

4. Neither the likelihood of recruitment of an oxytocin cell nor its burst amplitude could be correlated with background activity level and there was no clear relationship between the recruitment period or the bursting characteristics on one hand and the background activity on the other.

5. In conclusion, the differences between the early- and late-recruited cells in recruitment time and in burst amplitude reflected differences in cell excitability which may depend mainly on the presence of oxytocin in the magnocellular nuclei.

INTRODUCTION

A series of data published since 1973 has clearly established that milk ejection is not an immediate response to suckling in the rat. The mean latency for the first milk ejection is 10 min in conscious rats and 20–40 min in anaesthetized rats (Lincoln, Hill & Wakerley, 1973). These values substantially exceed the time taken for nerve impulses from cutaneous mammary receptors to reach the neurohypophysis plus the time for oxytocin to reach the myoepithelial cells via the blood system. Each milk ejection is preceded (15–20 s) by a characteristic brief increase in the firing rate of oxytocin cells referred to as a 'neurosecretory burst'.

Simultaneous recordings of at least two oxytocin cells in different nuclei (supraoptic or paraventricular nuclei) showed that some of the cells began to display a regular pattern of bursts early in the suckling period (early-recruited cells) whereas their counterparts did not do so immediately (late-recruited cells) but simultaneously with the 3rd, 4th or 5th burst of early-recruited cells (Belin & Moos, 1986). During the recruitment period, burst periodicity did not change with time, but burst amplitude (total number of spikes) increased progressively, to remain constant after the 5th or 6th burst. Milk ejections only occurred when the burst amplitude and the number of cells recruited were high enough to induce the release of sufficient amounts of oxytocin.

Thus, not all oxytocin cells have the same ability to burst at the beginning of suckling; bursting develops progressively, more or less rapidly according to the cells. In some cases, no milk ejection and no burst had occurred more than 1 h after the young rats had begun to suck while in others the regular pattern of milk ejections or bursts stopped unexpectedly (Tribollet, Clarke, Dreifuss & Lincoln, 1978). It was then possible to trigger the milk ejection reflex with propranolol (Tribollet *et al.* 1978), dopamine (Moos & Richard, 1982), or oxytocin (Freund-Mercier & Richard, 1984). To study the mechanisms controlling the recruitment phenomenon and the progressive increase in burst amplitude, the characteristics of early- and late-recruited cells were compared under control conditions (bursts occurring within 1 h suckling) and after injecting the above-mentioned substances, known to facilitate the triggering of the milk ejection reflex.

METHODS

Experiments were performed on lactating Wistar rats beween the 8th and 12th day of lactation which had been separated from all but one young rat 15 h before anaesthesia (urethane 1·2 g/kg I.P.). A thoracic mammary gland was cannulated for pressure recording and a catheter was inserted into the jugular vein for I.V. injections. Using the dorsal approach, a concentric bipolar steel electrode was inserted into the neurohypophysis. The extracellular activity of several (up to three) neurosecretory cells was recorded simultaneously with glass micropipettes located in the paraventricular (PV) and supraoptic (SO) nuclei as already described by Belin & Moos (1986). Three hours after anaesthesia, ten young rats were allowed to suck to induce reflex milk ejection. Neurosecretory cells were first identified by means of their antidromic response to electrical stimulation of the neurohypophysis and thereafter by their characteristic brief (1–4 s) and high-frequency burst of spikes (neurosecretory burst) 10–20 s before each increase in intramammary pressure.

The following parameters were considered in this study: (a) The delay (in minutes) between the beginning of suckling and the occurrence of the first neurosecretory burst (or milk ejection). (b) The

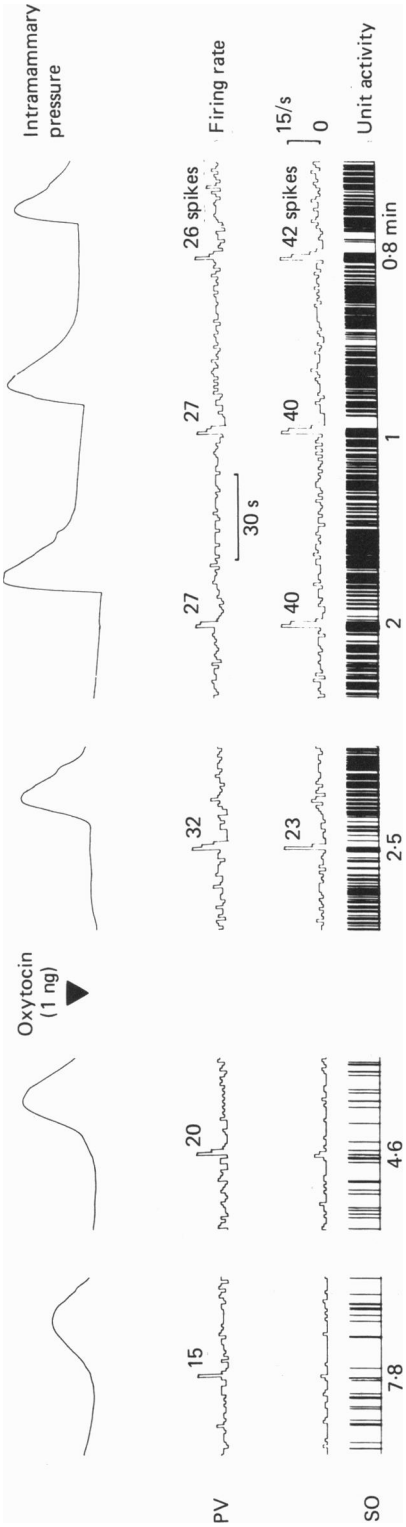

Fig. 1. Effect of oxytocin on oxytocin cell recruitment. The unit activity and the firing rate (in spikes/s) of all SO cells were recorded simultaneously with the firing rate of a PV cell and with the intramammary pressure. The number beside each neurosecretory burst indicates its amplitude (total number of spikes). The numbers under the bursts of the SO cell indicate the intervals (in minutes) since the previous bursts. During the first four bursts of the PV cell (only the 3rd and 4th bursts are shown) no burst emerged from the background activity of the SO cell. Injecting 1 ng oxytocin I.C.V. allowed the recruitment of the SO cell which then displayed bursts clearly emerging from background activity and simultaneous with those of the PV cell. The amplitude of the intramammary pressure peaks correlated with the number of cells recruited and burst amplitude which increased after oxytocin injection.

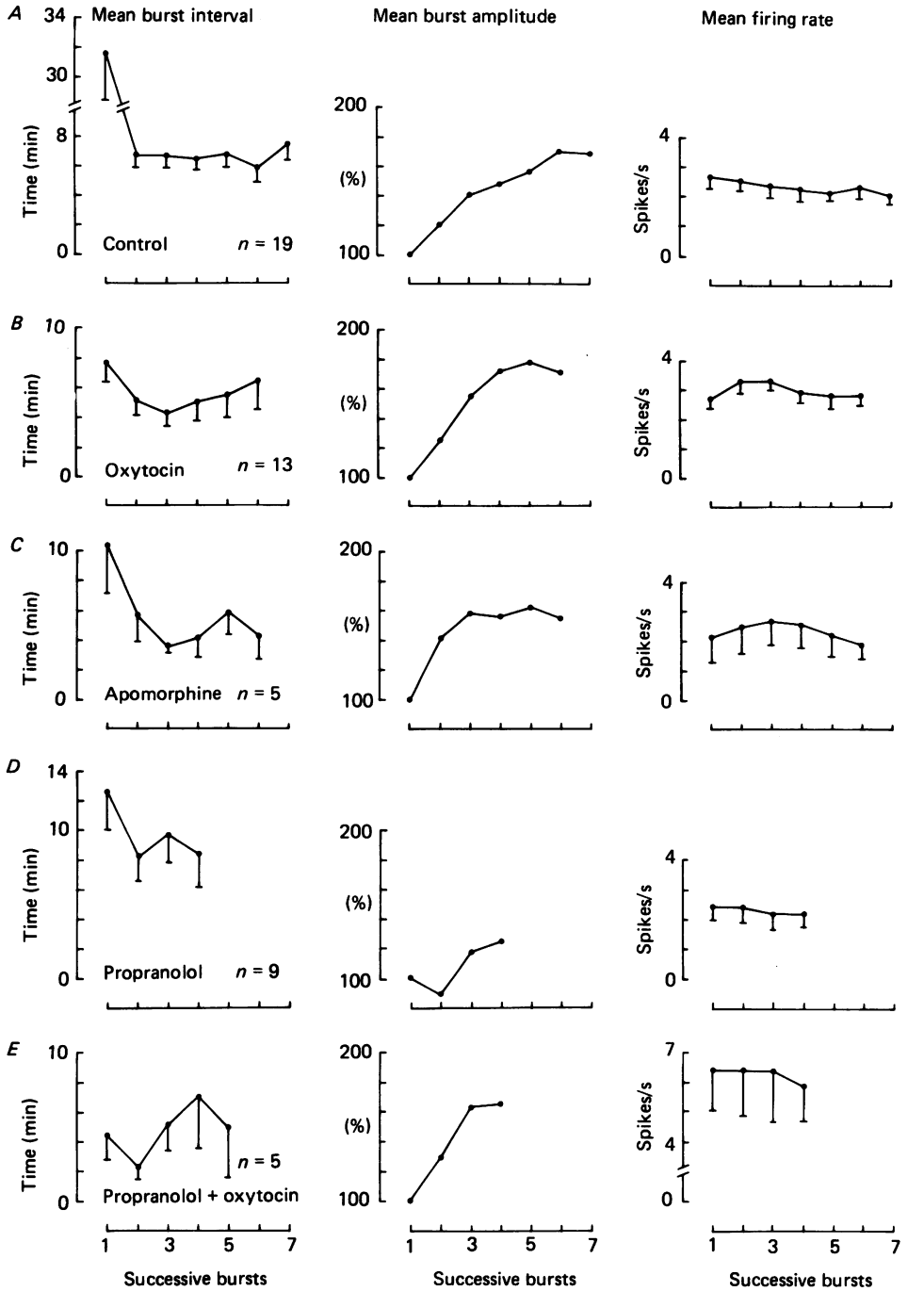

Fig. 2. Bursting and firing characteristics of the early-recruited cells when the reflex occurred spontaneously in less than 1 h suckling (*A*), or when its triggering was facilitated by I.C.V. injection of oxytocin (*B*), or of apomorphine (*C*), or by I.V. injection of propranolol (*D*), or of oxytocin when propranolol did not work (*E*). The graphs in the first column show the evolution of the mean delays in minutes (mean + S.E.M.) between the

interval (in minutes) between successive neurosecretory bursts during suckling. (c) The amplitude of the neurosecretory bursts, i.e. the total number of spikes per burst, expressed as a percentage of the first control burst. (d) The background activity, expressed in spikes per second and calculated either per 5 min period (excluding the neurosecretory burst and the following silent period) or during the 100 s before each neurosecretory burst.

Oxytocin (Sandoz) was injected I.C.V. (3rd ventricle) at a dose of 1 ng, i.e. 1 μl of a 10^{-6} M solution (solvent: 0·9% NaCl solution). Apomorphine hydrochloride (Sandoz), a dopamine agonist, was injected I.C.V. at a dose of 5 μg, i.e. 1 μl of a $1{\cdot}5 \times 10^{-2}$ M solution (solvent: 0·2% ascorbic acid solution). Propranolol (ICI and Ciba Geigy), a β-adrenoreceptor antagonist, was injected I.V. at a dose of 1·2 mg/kg (solvent: 0·2% ascorbic acid solution).

RESULTS

In most cases, oxytocin cell recruitment was observed directly via paired PV–SO or SO–SO cell recordings (see example in Fig. 1) and sometimes indirectly by comparison with the increases in intramammary pressure (e.g. the cell recorded began to display neurosecretory bursts at the 3rd milk ejection after the beginning of suckling).

The functional characteristics of oxytocin cell bursts were studied when the reflex occurred spontaneously during suckling (control conditions) or was facilitated either with oxytocin, apomorphine or propranolol. Since it has been shown that oxytocin cells of both PV and SO nuclei react similarly whatever the experimental conditions (Belin & Moos, 1986), the results obtained from PV or SO cells were pooled and comparisons were made between the early- and late-recruited cells.

Functional characteristics of the early-recruited cells under various experimental conditions

Under control conditions, i.e. without any injection, only fifty-seven suckling rats out of 137 spontaneously displayed regular milk ejections within 1 h of the start of suckling. For nineteen early-recruited cells, the first neurosecretory burst occurred 31·4 ± 3 min after suckling began (Fig. 2*A*). Thereafter, the mean burst intervals varied from 6 to 8 min over more than 40 min. The amplitude of the successive bursts increased progressively to reach maximum at the 6th bursts (170%; Fig. 2*A*). The background activity was not affected by the occurrence of bursts: the means, calculated per 5 min period, were 2·6 ± 0·2 spikes/s before the first burst and 2·4 ± 0·2 spikes/s 20 min later. The mean background activity during the 100 s before each burst did not change from the first to the 7th (Fig. 2*A*).

In suckled rats with no milk ejection reflex after more than 1 h suckling, I.C.V. injections of oxytocin (1 ng) or apomorphine (5 μg) or I.V. injections of propranolol (1·2 mg/kg) were used to trigger the reflex.

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successive bursts of *n* cells, the first value being the mean latency for the first burst since the beginning of suckling (control) or since the drug injection. The graphs in the 2nd column show the evolution of the mean amplitude of the successive bursts of *n* cells. For each cell, the amplitude of the successive bursts is expressed as a percentage of that of the first burst. The graphs in the 3rd column show the evolution of the mean firing rate (in spikes/s) during the 100 s before each successive burst of *n* cells. For comments see text.

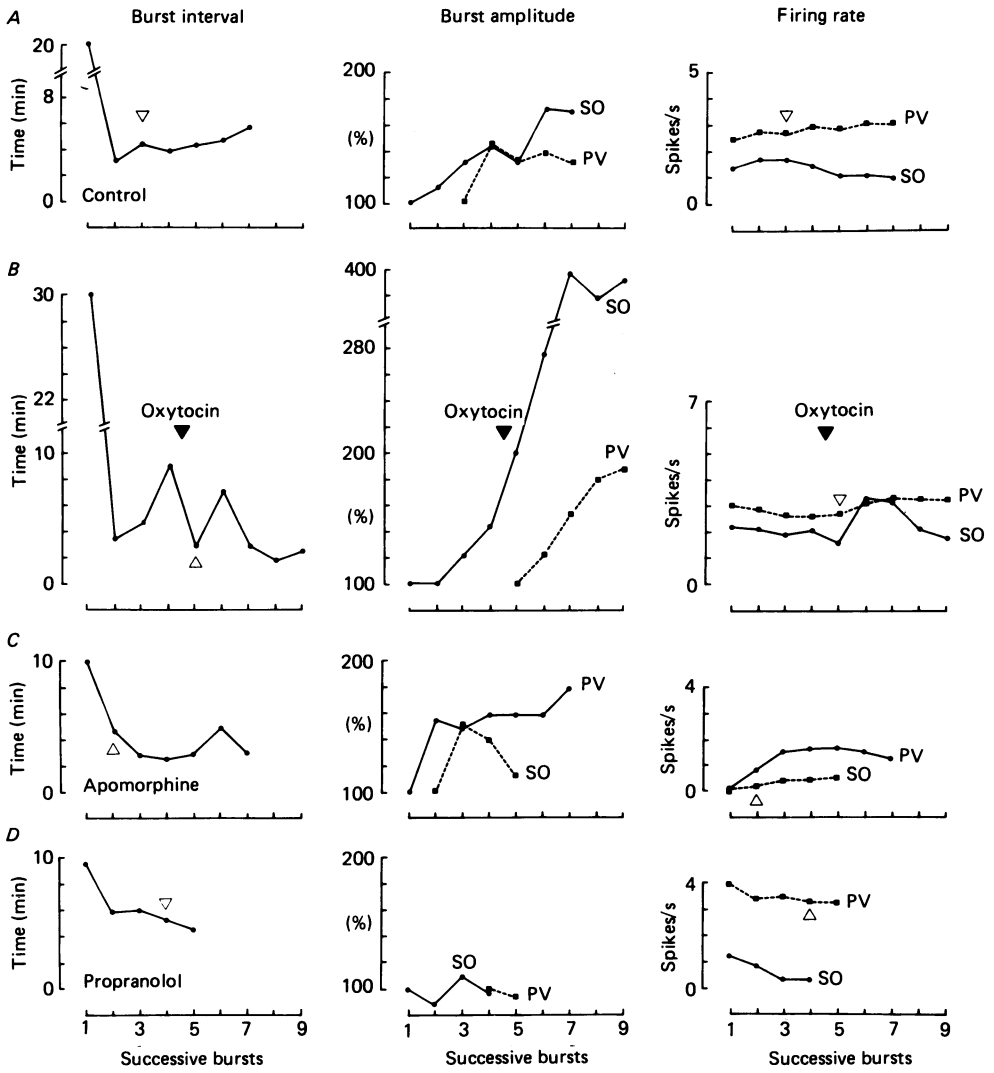

Fig. 3. Comparison of bursting and firing characteristics between early- and late-recruited cells when the reflex occurred spontaneously in less than 1 h suckling (*A*), when oxytocin was injected I.C.V. during the recruitment period (*B*), when the triggering of the reflex was facilitated by I.C.V. injection of apomorphine (*C*) or by I.V. injection of propranolol (*D*). In each case, paired PV–SO cells were recorded simultaneously. The graphs in the first column show the evolution of the delays between the successive bursts of the early-recruited cell, the first value being the latency for the occurrence of its first burst. The open arrow-head indicates the occurrence of the first burst on the late-recruited cell. The graphs in the 2nd column show the evolution of the amplitude of the successive bursts of the early- and late-recruited cells. For both cells, the amplitude of each burst was expressed as a percentage of that of the first burst. The graphs in the 3rd column show the evolution of the firing rates (in spikes/s) of the early- and late-recruited cells, during the 100 s preceding each successive burst, the open arrow-head indicating the occurrence of the first burst on the late-recruited cell. The moment when the late-recruited cells were recruited varied according to the cells, their first bursts occurring between the 2nd and 5th bursts of the early-recruited cells. As soon as they were recruited, all their successive

Oxytocin injections induced a reflex in thirty-one rats out of fifty-one, which confirms previous data of Freund-Mercier & Richard (1984). On thirteen paired oxytocin cells recorded, no recruitment phenomenon was observed, all cells displaying bursts as soon as the reflex began. For these cells, the first burst occurred $7{\cdot}6 \pm 1{\cdot}2$ min after the injection (Fig. 2*B*). Thereafter, the mean burst intervals varied from 4 to 5 min and increased with time to reach 6–8 min on and after the 6th burst. The amplitude of the successive bursts increased rapidly, reaching maximum at the 4–5th bursts (178%; Fig. 2*B*). The mean background activity, calculated per 5 min period, increased significantly after the I.C.V. injection of oxytocin from $2{\cdot}1 \pm 0{\cdot}2$ spikes/s (5 min before) to $3{\cdot}2 \pm 0{\cdot}3$ spikes/s (15 min after) ($P < 0{\cdot}001$, Students' paired t test). However, the mean background activity during the 100 s before each burst did not differ significantly from the first to the 6th although it was highest when the delays between bursts were shortest (Fig. 2*B*).

Apomorphine injections induced a reflex in twelve rats out of seventeen. On five early-recruited cells, the first burst occurred $10{\cdot}3 \pm 3{\cdot}1$ min after the injection (Fig. 2*C*). Thereafter, the burst intervals varied from 3 to 6 min. The amplitude of the successive bursts increased as under control conditions and the maximum was recorded at the 5th bursts (162%; Fig. 2*C*). After apomorphine injection, the mean background activity increased significantly from $1{\cdot}4 \pm 0{\cdot}6$ spikes/s, 5 min before injection, to $2{\cdot}4 \pm 0{\cdot}7$ spikes/s, 20 min later ($P < 0{\cdot}05$). The mean background activity during the 100 s before each burst did not differ significantly from the first to the 6th burst, but, as after oxytocin injection, it was highest when apomorphine was facilitating burst occurrence (Fig. 2*C*).

In five rats unaffected by the I.C.V. injection of apomorphine, oxytocin was injected I.C.V. A milk ejection reflex occurred in two cases but was limited to two to four successive bursts and/or milk ejections.

Propranolol was injected twenty-one times, either at the beginning of suckling in rats with no milk ejection for more than 1 h, or after an unexpected interruption of the reflex for more than 40 min. Propranolol triggered a reflex in eight rats out of fourteen and seven out of seven respectively. Since in both categories the evolution of the functional characteristics of oxytocin cells was similar, the results were pooled. For nine early-recruited cells, the first burst occurred within $12{\cdot}7 \pm 2{\cdot}7$ min after injection (Fig. 2*D*). Thereafter, the mean burst intervals ranged from 8 to 10 min, which was higher than under control conditions or after injecting oxytocin or apomorphine. The mean amplitude of bursts increased slightly and slowly, the mean for the 4th bursts being 124% of the first bursts (Fig. 2*D*). The mean background activity, per 5 min period, was not affected by the occurrence of the neurosecretory bursts: the means, 5 min before and 30 min after propranolol, were $2{\cdot}5 \pm 0{\cdot}3$ and $2{\cdot}6 \pm 0{\cdot}4$ spikes/s respectively. Similarly, background activity did not vary over 100 s before each successive burst (Fig. 2*D*).

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bursts were simultaneous with those of the early-recruited cells. The increase in burst amplitude of the late-recruited cells remained lower than that of the early-recruited one. After propranolol, burst amplitude remained very low in both cells. The recruitment did not correlate with the background activity level of oxytocin cells and there was no clear relationship between the recruitment period or the bursting characteristics on one hand and background activity level on the other.

For the six rats unaffected by propranolol, the I.C.V. injection of oxytocin, 30 min later, triggered the reflex in five cases. For the five early-recruited cells, the first burst occurred, on average, 4·3 ± 1·6 min after oxytocin injection (Fig. 2*E*). Thereafter, the mean burst interval varied from 3 to 7 min. The amplitude of the successive bursts increased rapidly, reaching 164% of control values at the 3rd–4th burst (Fig. 2*E*). This increase was similar to that observed for the reflex triggered by oxytocin alone (Fig. 2*B*). Background activity did not change over 100 s before each successive burst (Fig. 2*E*).

In nine rats where oxytocin did not trigger the milk ejection reflex, propranolol was injected I.V. 30 min later. Only in one did propranolol trigger the milk ejection reflex and then only four bursts occurred.

Functional characteristics of the late-recruited cells

Under control conditions, recruitment was observed in six out of sixteen paired recordings of PV–SO or SO–SO cells: the late-recruited cells, whether PV or SO cells, displayed their first neurosecretory burst at the 2nd, 3rd or 4th burst of the early-recruited cells (one example is given in Fig. 3*A*). Once the late-recruited cells had displayed a burst, they were recruited definitively and bursts were always simultaneous with those of the counterpart cells. For the six late-recruited cells, the first burst was of small amplitude (mean: 18 ± 2 spikes *versus* 36 ± 4 spikes for the early-recruited cells) and sometimes scarcely emerged from the background activity. The amplitude of the following bursts increased progressively but the increase was less marked and less sustained than in the early-recruited cells (see example in Fig. 3*A*). Moreover, in most cases, all the successive bursts of the late-recruited cells were of lower amplitude than in the early-recruited ones. The mean background activity of the six late-recruited cells (2·1 ± 0·7 spikes/s; range from 0·5 to 5 spikes/s) was similar to that of the early-recruited cells and did not change during or after the recruitment period (example in Fig. 3*A*).

As previously reported, when oxytocin was injected to trigger the milk ejection reflex, all the paired cells recorded were recruited right away and displayed bursts at the first milk ejection. To check whether oxytocin facilitated cell recruitment or not it was injected 3 times during the recruitment period of a reflex induced spontaneously by suckling. Injections were given after two to four bursts on the early-recruited cells when the paired cells had not yet been recruited (examples in Figs 1 and 3*B*). Oxytocin largely speeded up the recruitment of the non-responsive cells which then displayed bursts right away, simultaneously with those of the early-recruited cells. For the cells recruited after oxytocin injection (see the example in Fig. 3*B*), the bursts occurred without any noticeable change in background activity and the increase in burst amplitude was as rapid and large as in Fig. 2 (reflex triggered by oxytocin). For the early-recruited cells (see the example in Fig. 3*B*), the background activity and burst amplitude increased after oxytocin injection as reported by Freund-Mercier & Richard (1984).

After apomorphine injection, recruitment was observed twice in five paired recordings. As under control conditions, the late-recruited cells displayed short-lived mini-bursts with few spikes. Figure 3*C* gives an example: the late-recruited cell (SO cell) began to display bursts at the 2nd burst of the early-recruited cell (PV cell);

the increase in burst amplitude was maintained for the early-recruited cell but not for the late-recruited one, its 4th burst being as low as the first one. Moreover, the background activity of the early-recruited cell increased after injection whereas that of the late-recruited cell was not modified when the successive bursts occurred (Fig. 3*C*).

Recruitment was also observed twice after propranolol injection. In the example of Fig. 3*D*, the late-recruited cell did not display its first burst until the 4th burst of the paired early-recruited cell. Its amplitude was low (fifteen to sixteen spikes) and scarcely emerged from the background activity. For both the early- and late-recruited cells, the amplitude of the successive bursts did not vary much (Fig. 3*D*) and there was no significant change in their background activities with the successive bursts (Fig. 3*D*).

DISCUSSION

Whether spontaneous or facilitated by drugs, the triggering of the milk ejection reflex in response to suckling appeared as a peculiar period during which oxytocin cells more or less rapidly acquired the capacity to burst periodically. The different bursting characteristics of the early- and late-recruited cells will be discussed in relation with the effects of three substances (apomorphine, propranolol and oxytocin) known to facilitate the triggering of the milk ejection reflex and so liable to speed up the cell recruitment.

Cell recruitment

Under control conditions, the oxytocin cells were recruited progressively and the amplitude of the successive neurosecretory bursts increased. This period lasted 30–40 min, i.e. until the 5–6th burst on the early-recruited cells. From then on, a great number, if not all, oxytocin cells were recruited and displayed synchronous bursts of almost constant amplitude at regular intervals. Oxytocin and apomorphine had similar facilitatory effects on reflex triggering, though the peptide acted more rapidly and proved more efficient than the amine: the mean delay for the first burst was shorter, the increase in burst amplitude was more rapid and the maximum burst amplitude was higher after oxytocin than after apomorphine or in control rats. Propranolol also triggered the reflex either at the beginning of suckling or after a long unexpected interruption of the reflex, but the bursting characteristics of oxytocin cells were lower than under control conditions: the successive bursts occurred at longer intervals and the increase in burst amplitude was delayed and less marked. Moreover, a reflex triggered by propranolol rarely lasted beyond 1 h, as Tribollet *et al.* (1978) reported. So, in all these respects, propranolol was less efficient than apomorphine and, *a fortiori*, oxytocin. As regards cell recruitment, the three substances also had different effects. After apomorphine or propranolol, recruitment was progressive and comparable to that observed during a control reflex. On the other hand, after oxytocin, all the oxytocin cells were recruited right away since no late-recruited cells were ever observed in any of the paired recordings. The strong facilitatory role of oxytocin in burst triggering was corroborated by the fact that I.C.V. oxytocin injection during the recruitment period of a control reflex substantially speeded up the recruitment of the late-recruited cells.

The increase in cell recruitment and in burst amplitude at the beginning of suckling might reflect increasing excitability of all the oxytocin cells, if the ability to burst and the burst amplitude itself are taken as an index of excitability. However, this excitability differs between cells: the late-recruited cells probably had very low excitability since their potential capacity to burst was acquired later and their bursts were always of lower amplitude than those of the early-recruited cells. Moreover, the increase in burst amplitude was less marked and less sustained for the late- than for the early-recruited cells. The change in oxytocin cell excitability, underlying the changes in cell recruitment and in burst characteristics, was not accompanied by any systematic increase in background activity, except after oxytocin, which substantially facilitated cell recruitment. However, no relationship was found between cell recruitment and the firing rate. Indeed, even if the background activities of oxytocin cells were heterogeneous, there was no difference between the means of the late- and the early-recruited cells. Moreover, paired recordings clearly show that the firing rate of an early-recruited cell was often below that of a late-recruited one. Injecting apomorphine increased background activity as did oxytocin, but without facilitating cell recruitment. Under control conditions or after propranolol, cell recruitment occurred without any noticeable change in background activity. So, the level of firing itself cannot be taken as an index of bursting capacity. This might parallel the observations on firing rate and burst amplitude: paired recordings have clearly shown that there is no correlation between these two parameters for either type of cell at a given time during the milk ejection reflex (Belin & Moos, 1986). However, for a given cell recorded throughout the reflex, background activity and burst amplitude generally vary in a parallel manner (Lincoln & Wakerley, 1975; Belin & Moos, 1986). Changes in excitability and progressive acquisition of the capacity to burst are probably associated with changes in membrane properties in response to excitatory afferents.

Hypotheses on the sites and mechanisms of action of the three drugs on cell recruitment

The present results provide clear evidence that oxytocin is the main factor favouring the recruitment of oxytocin cells. In the hypothalamus, the oxytocin required to activate oxytocin cell bursting is released inside the magnocellular nuclei, as shown by *in vitro* experiments (Chapman, Hatton, Ho, Mason & Robinson, 1983; Moos, Freund-Mercier, Guerné, Guerné, Stoeckel & Richard, 1984; Mason, Hatton, Ho, Chapman & Robinson, 1986; Di Scala-Guenot, Strosser & Richard, 1987) and by push–pull perifusions in suckled rats (Moos, Strosser, Poulain, Di Scala-Guenot, Guerné, Rodriguez, Richard & Vincent, 1987). This release, corroborated both morphologically and electrophysiologically (see discussion in Belin & Moos, 1986) might occur by exocytosis along the plasmalemma or from dendrites and axons outside active synapse zones of terminals as was reported for dense-cored vesicles in the trigeminal subnucleus caudalis (Zhu, Thureson-Klein & Klein, 1986). The oxytocin thus released might act either on the oxytocin cell afferents or on the oxytocin cells themselves. There is some corroboration for the latter. *In vitro*, oxytocin depolarizes the magnocellular neurones (Abe, Inoue, Matsuo & Ogata, 1983) and depolarization by current injection facilitates the occurrence of non-

synaptic depolarizing potentials that may help initiate bursts of spikes (Bourque, Randle & Renaud, 1986). Such changes in membrane potential could only exist during suckling: the increase in oxytocin cell firing in response to I.C.V. oxytocin injection (indicating change in membrane properties) only occurs if the young are sucking (Meyer, Freund-Mercier & Richard, 1987). During suckling, the release of oxytocin inside the magnocellular nuclei probably results from excitatory afferents continually reaching the oxytocin cells. The dopamine fibres are among these afferents since the injection of apomorphine has a similar effect to that of oxytocin, but delayed and less marked (present result and Moos, Freund-Mercier & Richard, 1983). Dopamine probably acts directly on oxytocin cells. Indeed, axon terminals labelled with 6-hydroxydopamine (5-OHDA) make synaptic contact with neurophysin-immunoreactive neurones in the magnocellular nuclei (Nakada & Nakai, 1985) and ionophoretic or local applications of dopamine modify the background activity of oxytocin cells (Mason, 1983; Honda, Negoro, Fukuoka, Higuchi & Uchide, 1985). Inhibitory control is exerted by noradrenaline, via the β-receptors, either on the ascending pathways at the supraspinal level (Poulain & Dyer, 1984) or on oxytocin cells themselves (Day & Renaud, 1984; Day, Randle & Renaud, 1985; Honda *et al.* 1985) by raising their threshold for spikes (Ogata & Matsuo, 1986).

Thus, the characteristic bursting response of oxytocin cells during suckling may result from changes in their membrane properties due both to the excitatory inputs from the mammary glands and to the release of oxytocin in the nuclei, oxytocin then allowing the progressive recruitment and bursting of oxytocin cells. The oxytocin cells receiving these afferents and releasing oxytocin into the extracellular spaces might be the early-recruited cells. The late-recruited cells then become able to burst in response to the oxytocin released by the early-recruited cells. A complementary effect of oxytocin on the oxytocin cell afferents, enhancing the excitatory inputs, might also intervene, but this possibility requires further investigation.

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