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ORIGINAL ARTICLE

Prolactin Induces a Hyperpolarising Current in Rat Paraventricular Oxytocinergic Neurones

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Prolactin and oxytocin are important reproductive hormones implicated in several common adaptive functions during pregnancy, pseudopregnancy and lactation. Recently, extracellular recordings of supraoptic neurones have shown that prolactin may modulate the electrical activity of oxytocinergic neurones. However, no study has been conducted aiming to establish whether prolactin directly influences this activity in oxytocinergic paraventricular neurones. In the present study, we addressed this question by studying the effects of prolactin on the electrical activity and voltage–current relationship of identified paraventricular neurones in rat brain slices. Whole-cell recordings were obtained and neurones were classified on the basis of their morphological and electrophysiological fingerprint (i.e. magnocellular or parvicellular) and neuropeptide phenotype (i.e. oxytocinergic or non-oxytocinergic). We report that prolactin elicited a hyperpolarising current in 37% of the neurones in this nucleus, of which the majority (67%) were identified as putative magnocellular oxytocin neurones and the reminder (33%) were regarded as oxytocin-negative, parvicellular neuroendocrine neurones. Our results suggest that, in addition to the well-established negative feedback loop between prolactin-secreting lactotrophs and dopaminergic neurones in the arcuate nucleus, an inhibitory feedback loop also exists between lactotrophs and oxytocinergic paraventricular neurones.

Key words: oxytocin, prolactin, paraventricular nucleus, whole-cell recordings, immunocytochemistry, feedback.

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The pituitary hormone prolactin plays an essential role in the reproductive function of female rats (1). It is important for maintaining the structural and functional integrity of the corpus luteum that, in turn, is responsible for progesterone synthesis during pregnancy (1). In the early phase of pregnancy in rats, prolactin from lactotrophs located in the anterior lobe of the pituitary gland shows a rhythmic secretion pattern characterised by nocturnal and diurnal surges (at approximately 03.00 h and 17.00 h, respectively) (1). In addition, the secretion of this hormone appears to be under circadian control (2–4).

Prolactin secretion is controlled by the balanced supply of hypothalamic stimulatory and inhibitory factors delivered to the lactotrophs via long and short portal vessel systems (1). The major inhibiting factor is dopamine, which is released primarily by tuberoinfundibular neurones of the arcuate nucleus (1,5,6). Dopamine tonically inhibits the secretion of prolactin; thus, the hormone can only be released when dopamine levels in the portal blood are reduced (7,8). However, the temporary reduction of dopamine does not fully account for the entire amount of prolactin released, suggesting that additional prolactin-releasing factors of hypothalamic origin may be involved in the generation of the surges. Once prolactin is secreted, it stimulates dopamine synthesis and metabolism in tuberoinfundibular neurones with a time delay (9,10). This so-called short feedback loop between lactotrophs and tuberoinfundibular dopaminergic neurones (11,12) has been proposed to be largely responsible for prolactin homeostasis (5).

Recent studies from our group and others demonstrated a stimulatory effect of oxytocin in the regulation of prolactin secretion

(13–16). We showed that bath application of oxytocin to cultured lactotrophs elevates intracellular Ca^{2+} levels and induces prolactin release (14). In addition to the neurones in the supraoptic nuclei (SON), those in the paraventricular nucleus (PVN) of the hypothalamus are major production sites for oxytocin (17). Importantly, oxytocinergic PVN neurones in rats display daily activity rhythms that correlate with the prolactin-releasing pattern of pregnant/pseudopregnant rats (18). PVN neurones have been classified morphologically into magnocellular and parvicellular populations, and electrophysiologically into at least two types of neurones (19). It has been proposed that magnocellular and parvicellular neurones correspond to types I and II, respectively (20,21).

Using immunocytochemical techniques, we have shown that oxytocinergic neurones in the PVN express prolactin receptors (15). This is in line with other studies that have found prolactin receptor mRNA in neurones of the PVN (22–25). Because prolactin may gain access to the brain through a carrier-mediated transport system (26), which probably involves prolactin-binding sites in the choroid plexus (27–29), the fact that oxytocin stimulates prolactin secretion from lactotrophs and that prolactin receptors can be found on oxytocin neurones in the PVN raises the possibility that a prolactin-oxytocin feedback loop exists that is substantially involved in the regulation of prolactin secretion, in addition to the well known prolactin-dopamine feedback loop.

The present study aimed to determine whether and how prolactin affects the electrical activity of oxytocinergic neurones of the PVN. The study was carried out on acute brain slices of the hypothalamic PVN using a combination of electrophysiological (whole-cell patch clamp) and immunocytochemical techniques. Responses to prolactin were thus obtained from neurones carefully characterised by immunohistochemistry, pharmacology and electrophysiology.

Materials and methods

Chemicals

Isoflurane used for anaesthesia was purchased from Baxter (Volketswil, Switzerland). For the voltage clamp experiments, tetrodotoxin (TTX; Ascent Scientific, Bristol, UK) was diluted in artificial cerebrospinal fluid to a final concentration of 1 μM, to prevent spike-mediated synaptic transmission. For the perfusion protocols (see below), we used a δ opioid receptor agonist [D-Ala^2, D-Leu^5]-enkephalin (DADLE; 10 μM; Sigma-Aldrich, St Louis, MO, USA) and rat prolactin [100 ng/ml; kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases and Dr A. F. Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA)].

Hypothalamic brain slice preparation

PVN brain slices were obtained from 49 juvenile female Sprague–Dawley rats (90–100 g body weight) hosted at the animal facility (Brain Research Institute, University of Zurich). All animal procedures were approved by the local animal ethics committee and complied with the Swiss regulations for care and use of animals. The slices were cut as described previously (20). In brief, deeply anaesthetised (isoflurane) animals were decapitated, their brains removed and placed in ice-cold, oxygenated artificial cerebrospinal fluid composed of (mM): NaCl 124, KCl 2.8, $MgSO_4 \cdot H_2O$ 2, $NaH_2PO_4 \cdot H_2O$ 1.25, $NaHCO_3$ 26, D-glucose ($C_6H_{12}O_6$) 10, ascorbic acid ($C_6H_8O_6$) 0.4, $CaCl_2 \cdot 2\ H_2O$ 2 (pH adjusted to 7.4 and osmolarity in the range 290–300 mOsmol/l). Thereafter, the brains were trimmed to tissue sections of the hypothalamus (1 cm^2) before cutting 300-μm thick slices of the PVN on a vibratome (VT 1200; Leica, Microsystems, Wetzlar, Germany). Immediately thereafter, the brain sections were incubated in oxygenated artificial cerebrospinal fluid at 37 °C for 30 min and kept for equilibration at room temperature for an additional 1 h. For the experiments, slices were placed into a 1-ml recording chamber mounted on an upright microscope (Axioscope FS; Zeiss, Oberkochen, Germany). A bath perfusion system enabled continuous chamber perfusion with either artificial cerebrospinal fluid or test substances (1–2 ml/min flow rate). A total of 87 neurones were analysed in the PVN slices obtained from all 49 animals. No distinct patterns were observed among slices of different animals.

Whole-cell recordings

Patch-clamp electrodes were pulled from borosilicate glass capillaries (1.5 mm; Harvard Apparatus, Edenbridge, UK) resulting in a resistance in the range 5–8 MΩ. Patch pipettes were filled with a solution containing (in mM): potassium D-gluconate 105, KCl 30, Hepes 10, ethylene glycol tetraacetic acid 1.1, $MgCl_2 \cdot 6H_2O$ 4, Na_2ATP 2, Na_2GTP 0.3. The patch-pipette solution was pH adjusted to 7.3 using KOH, and to an osmolarity in the range 300–310 mOsmol/l. Biocytin (1%) was added to the pipette solution for subsequent immunocytochemical analysis in most of the experiments. Whole-cell patch-clamp recordings were carried out on PVN neurones in slices superfused with artificial cerebrospinal fluid at 32 °C, under visual control. Recordings were performed in both current-clamp and voltage-clamp configurations using an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA, USA). All currents were low-pass filtered at 2 kHz and sampled at 5 kHz using pCLAMP9 software (Molecular Devices). Membrane voltage data were corrected for the liquid-junction potential (17 mV). Series resistance was in the range 10–20 MΩ, and was compensated up to 70–80%.

Electrophysiological and pharmacological identification

According to the biocytin staining, 49 neurones were identified as PVN neurones. Type I and type II PVN neurones were distinguished on the basis of the presence or absence, respectively, of a transient I_A-mediated rectification, as described by Swanson and Sawchenko (30), Tasker and Dudek (19) and Hoffman *et al.* (21). To activate I_A, a current-clamp protocol was used which hyperpolarised the cell first (−400 pA, 300 ms) and then depolarised it with currents in the range 0–100 pA in 10 pA steps. In this fashion, 24 out of 49 (49%) PVN neurones analysed were classified as type I and the remaining 25 cells (51%) as type II. Substantial evidence supports the notion that electrophysiologically defined type I and type II cells correspond to morphologically categorised magnocellular and parvicellular neurones (20,21), and will thus be henceforth termed 'putative magnocellular' and 'putative parvicellular'. Additional analysis of the electrophysiological recordings of putative parvicellular neurones allowed us to distinguish between neurosecretory and non-neurosecretory cells. We adopted the criteria of Luther *et al.* (31) in which non-neurosecretory parvicellular neurones generate a low-threshold spike and robust T-type Ca^{2+} current, whereas neurosecretory cells show no low-threshold spike and only a small T-current. In our preparation, we identified 80% of putative parvicellular PVN neurones as neurosecretory cells and 20% as pre-autonomic non-neurosecretory cells.

Cells were initially monitored under current clamp conditions to evaluate their firing activity and membrane potential. PVN neurones that exhibited a continuous (nonphasic) firing pattern were classified as putative oxytocinergic neurones. Those that exhibited a phasic firing pattern (characterised by a bursting pattern of action potentials with a mean intraburst firing rate

range of 4–15 spikes/s, a mean burst duration between 10 and 30 s, a mean silence duration between 5 and 20 s, minimum number of spikes per burst of nine and a minimum burst and silent duration of 3 s and 2 s, respectively) were classified as putative vasopressin neurones (32,33). In addition, PVN neurones were identified as oxytocinergic if their firing activity showed a transient inhibition in response to D-ala-D-leu-enkephalin (DADLE; see below) (32). Further identification was defined on the basis of subsequent immunocytochemical analysis.

Drug application and I–V protocol

The drug application protocol was conducted in the presence of TTX (1 μM) under voltage clamp conditions and included an initial control period of 3 min during which the conditions before drug application were recorded. Thereafter, prolactin (100 ng/ml) was applied to the recording chamber for 2 min. Control artificial cerebrospinal fluid was then superfused for 10–12 min to remove prolactin (washout). Finally, the δ opioid receptor agonist DADLE was added (10 μg/ml, 2 min) to indicate whether or not the investigated neurone was oxytocinergic. The electrical properties of the patched neurones were determined at the end of exposure to prolactin, washout or DADLE treatment period by applying a voltage-clamp step protocol: from a holding potential of −60 mV, step to −105 mV for 350 ms, and then to a test potential in the range of −105 to −35 mV (in 10 mV steps) for 350 ms.

Electrophysiological parameters and curve fitting

The following electrophysiological parameters were recorded to further characterise PVN neurones and to describe prolactin-induced changes: resting membrane potential (V_m), membrane conductance ($G_m = 1/R_m$ – input resistance R_m of the cell was measured with a weak current pulse, usually −50 or −100 pA), action potential amplitude (AP) and mean firing rate (MFR). Under control conditions (unstimulated, before TTX application), those values (± SEM) agreed with those previously reported (20), and were: for putative magnocellular neurones, $V_m = -54 \pm 3$mV, $G_m = 0.94 \pm 0.10$ nS, AP = 78 ± 11 mV and MFR = 8.6 ± 1.2 Hz (n = 24); for putative parvicellular neurones, $V_m = -59 \pm 8$ mV, $G_m = 2.52 \pm 0.60$ nS (n = 25), of which only 11 were spontaneously active with AP = 75 ± 20 mV and MFR = 3.8 ± 3.4 Hz.

For each type of PVN neurone studied, a number of I–V curves were selected and averaged to obtain mean I–V curves representative of the neuronal type and of the different phases of the experiment. Each of the averaged curves was then fit to an Ohmic current function with a Boltzmann conductance function:

$$I(V) = g(V)(V - V_0), \quad g(V) = \frac{A}{1 + \exp\left(-\frac{V - V_{1/2}}{s}\right)} + C$$

where V_0 is the voltage at zero current and g(V) is the observed membrane conductance for a voltage V, A is the amplitude parameter, $V_{1/2}$ is the half-activation voltage, s is the slope parameter, and C is the conductance offset.

Statistical analysis

Results are expressed as the mean ± SEM. For each neuronal population, statistically significant differences between the I–V relationships acquired in three sequential experimental conditions (control, 2 min pulse of prolactin and 10 min after hormone withdrawal) were evaluated at the level of the whole I–V relationship using a nonparametric multiple comparison test for repeated measurements (Friedman's test): Friedman Q statistics were obtained for each potential value recorded, and the results were summed. Under the null hypothesis of independent errors for differential potential values and absence of effect of the treatments, this summed Q-statistics follows an approximate chi-squared distribution with $n \times (K - 1)$ degrees of freedom, where n is the number of points in each I–V curve (n = 8) and K = 2 or 3 according to the number of experimental conditions being compared. Reported P-values were computed according to this distribution. Equivalent parametric analysis (one-way analysis of variance followed by post-hoc Tukey's test) was also conducted with comparable results. Comparisons of the conductances measured after prolactin application and removal versus the control were analysed using the nonparametric Wilcoxon's signed rank test for correlated samples (similar results were obtained using paired Student's t test). $P < 0.05$ was considered significant.

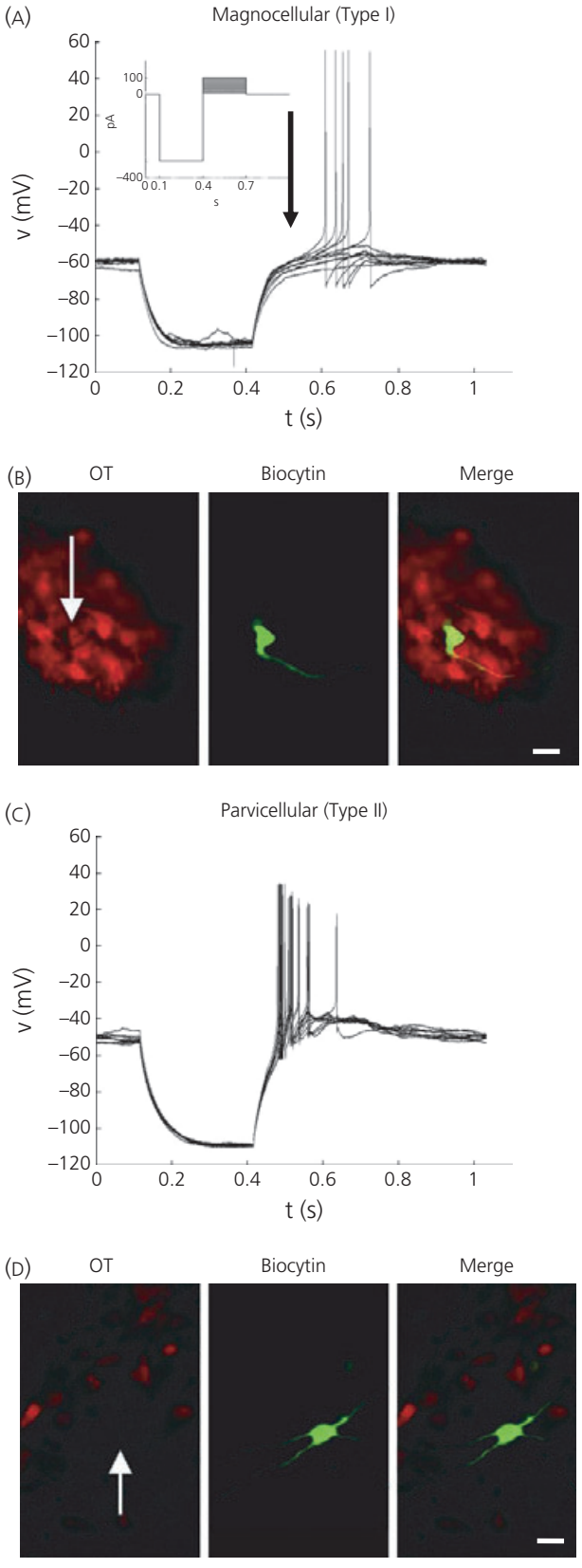
Immunocytochemistry

Each of the recorded neurones was immunocytochemically investigated after the electrophysiological recordings, allowing both localisation of the cell and detection of the intracellular content. To allow morphological identification of the cells, biocytin (1%) was added to the pipette solution to diffuse into the recorded neurones. After the experiments, the slices were removed from the chamber and left overnight for fixation in 4% paraformaldehyde and 0.15% picric acid diluted in phosphate-buffered saline (PBS) at 4 °C. The next day, the slices were placed in cryoprotective solution (30% sucrose, 12% glycerol, 0.1 M phosphate buffer) and frozen at −20 °C. For further analysis, slices were washed three times in PBS, pre-incubated overnight to block nonspecific antigen binding in 0.4% Triton-X-100 containing 10% horse-serum (blocking solution), and incubated for 72 h at 4 °C in the presence of monoclonal mouse antibodies raised against oxytocin-related neurophysin in fresh blocking solution (dilution 1 : 20 000; Chemicon International, Temecula, CA, USA) (34). Afterwards, the slices were washed three times in PBS for 1 h and then incubated overnight at 4 °C in a mixture containing Cy3-conjugated AffiniPure Goat Anti-mouse IgG (H+L) secondary antibodies (1.5 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA USA) and AlexaFluor-488-conjugated streptavidin (dilution 1 : 1000; Molecular Probes, Carlsbad, CA, USA) for the detection and amplification of oxytocin-related neurophysin and biocytin staining, respectively. To test for the specificity of labelling, slices were incubated in buffer without primary antibody. Finally, slices were mounted on a glass slide, covered with Aqua-Poly/Mount (Poly-sciences, Warrington, PA, USA) and digitally photographed using a SPOT camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The acquired images were contrast-adjusted and visualised using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Thus, double-stained cells indicated oxytocin accumulation in electrophysiologically investigated neurones.

Results

Electrophysiological, morphological and pharmacological characterisation of PVN neurones

Magnocellular neurones were characterised by the presence of an A-type potassium current (I_A), which is absent in putative parvicellular neurones (see Materials and methods). The typical delayed onset of action potentials of magnocellular neurones, which is a result of the I_A current, could be demonstrated in these neurones (Fig. 1A, arrow) but not in parvicellular (Fig. 1C) neurones. Indeed, the two groups of neurones could be distinguished not only by the presence/absence of I_A, but also by their activity level. Putative



magnocellular neurones were typically active, with a mean overall firing rate of 8.6 ± 1.2 Hz (n = 24), whereas only a fraction of the 25 parvicellular neurones analyzed were spontaneously active, with a mean overall firing rate of 3.8 ± 3.4 Hz (n = 11).

Morphological analysis with biocytin labelling revealed further differences between putative magnocellular and parvicellular neurones, consistent with those described in the literature (35). The majority of magnocellular neurones were bipolar with long and extended somata (Fig. 1B, centre panel). There were two main neuronal branches leaving the cell body on opposite sides. By contrast, the somata of parvicellular neurones were mostly compact, and there were typically three to five neuronal branches in addition to the axon (Fig. 1D, centre panel).

Oxytocin neurones were considered as cells that showed positive immunostaining for oxytocin-neurophysin (Fig. 1B) and that hyperpolarised under DADLE (not shown). Under these criteria, 50% of the magnocellular neurones studied were regarded as oxytocin neurones. The other half was thus regarded as vasopressin-producing neurons. Figure 1(B) shows an example of one of the magnocellular oxytocinergic cells used in the recordings. None of the putative parvicellular cells investigated showed positive immunostaining for oxytocin-neurophysin (Fig. 1D).

Prolactin evokes a hyperpolarising current in responsive PVN neurones

The effects of prolactin on membrane currents of PVN neurones were determined with a voltage-clamp protocol under basal conditions and at the end of each treatment period (prolactin 100 ng/ml for 2 min; a 10-min washout to remove prolactin, and DADLE 10 μg/ml for 2 min) yielding a series of I–V curves that were analysed by Boltzmann fits for each experimental condition (see Materials and methods). Prolactin had an effect on 12 out of 24 (50%) of the putative magnocellular and six out of 25 (24%) of the putative parvicellular PVN neurones. Interestingly, all prolactin-sensitive magnocellular neurones corresponded to the entire population of oxytocin magnocellular cells studied. The results from this analysis are shown for only those neurones that exhibited a significant response to prolactin.

Figure 2 shows the corresponding I–V plots for putative oxytocinergic, magnocellular (Fig. 2A) and non-oxytocinergic, parvicellular (Fig. 2B) PVN neurones before and after a 2-min application of prolactin. In both neuronal populations, prolactin induced an outward

Fig. 1. Electrophysiological, morphological and immunohistochemical characterisation of PVN neurones. (A) Magnocellular (type I) neurones. Typical traces recorded from a type I neurone showing prominent action potential onset delay (I_A current, arrow). Inset displays the current protocol used. (B) Left panel: Immunocytochemical staining for oxytocin-neurophysin (OT; arrow pointing to the location of the recorded cell). Center panel: Biocytin labelling of the recorded neurone (dye applied through the patch-pipette). Right panel: Merged images. Scale bar = 20 μm. (C) Parvicellular (type II) neurones. Typical traces recorded from a type II neurone with no onset delay. (D) OT and biocytin staining as shown in (B).

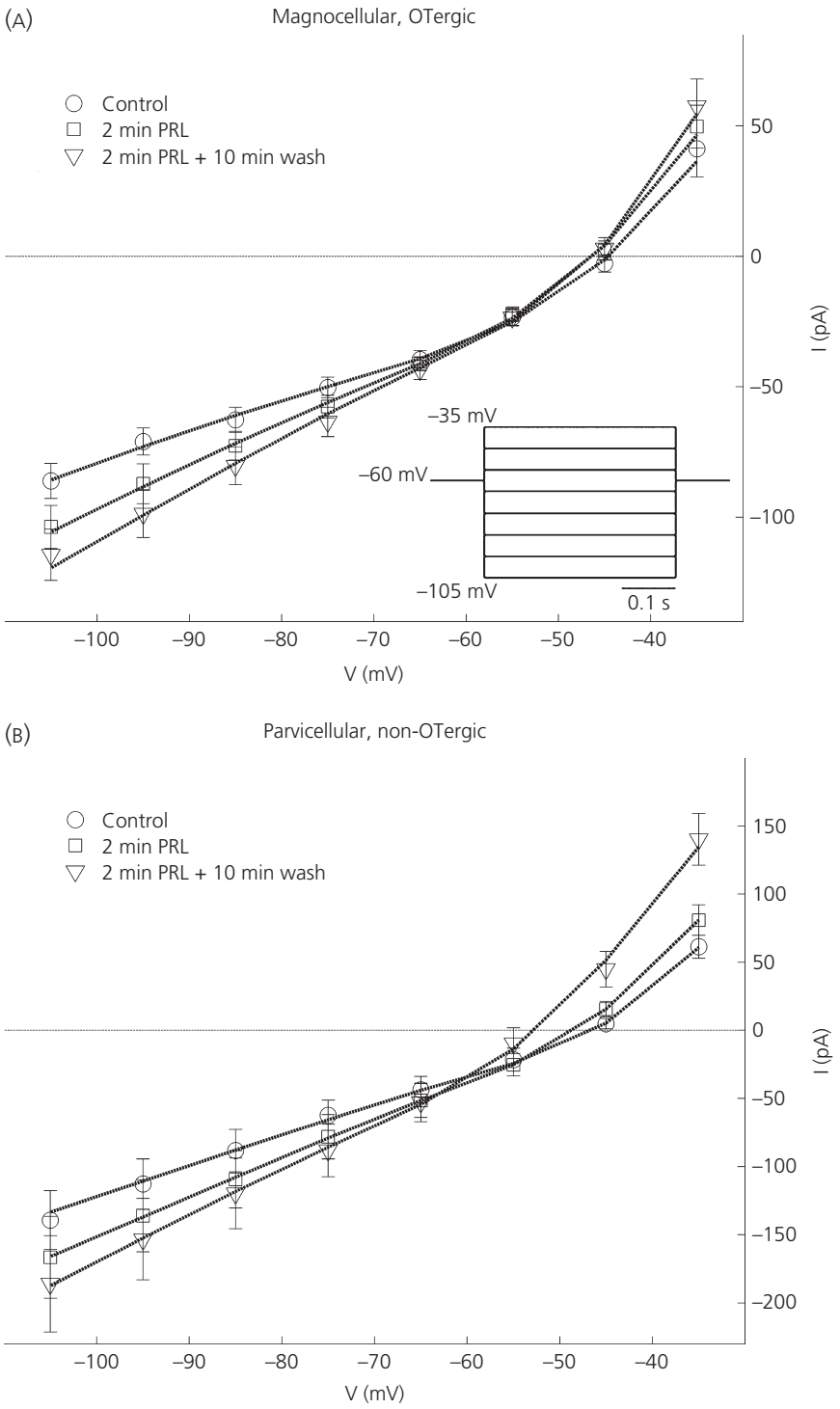


Fig. 2. Representative I–V curves of prolactin-sensitive magnocellular and parvicellular PVN neurones before (circles), at the end of a 2-min pulse of prolactin (PRL) 100 ng/ml (squares) and after 10 min of hormone withdrawal (triangles). Neurones responded to prolactin with a hyperpolarising current that reversed at a potential more negative than the control current and persisted after 10 min of removal of the agonist. (A) 50% of putative magnocellular neurones were immunoreactive to oxytocin-neurophysin (OT) and were prolactin-responsive (n = 12). Inset: voltage step protocol used for all I–V curves. (B) Twenty-four percent of putative parvicellular neurones were prolactin-responsive (n = 6). None of these neurones were immunoreactive to oxytocin-neurophysin (OT). Non-parametric multiple comparison test for repeated measures (Friedman's test) followed by Dunn's post-hoc analysis showed that the I–V relationship was significantly different after prolactin treatment in both neuronal cell types (for further details, see text). Errors bars indicate the SE. Note the different current amplitudes in magnocellular and parvicellular neurones.

(hyperpolarising) current for potentials above −60 mV, resulting in a left shift of the reversal potential.

In all oxytocinergic, putative magnocellular PVN neurones (Fig. 2A) (n =12), the current slope conductance of the membrane (G_m) increased significantly in response to prolactin between −95 and −75 mV (prolactin: 1.48 ± 0.2 nS versus control: 0.9 ± 0.1 nS, P = 0.02, paired Wilcoxon's signed rank test), as well as in the interval −55 to −35 mV (prolactin: 3.6 ± 0.4 nS versus control: 3.0 ± 0.5 nS, P = 0.04). Friedman's test followed by post-hoc analysis showed that the I–V relationship recorded at the end of the prolactin pulse was significantly different from that obtained in control conditions when comparing the whole curve ($P < 10^{-4}$). The average difference current $\Delta I = I_{PRL} - I_{ctl}$ obtained by subtracting control currents from currents measured at the end of the PRL treatment (Fig. 3A) reversed at −56 mV and was positive (hyperpolarising) for larger potentials. The difference current was almost linear, suggesting that PRL activates a V-independent current. To evaluate the impact of such a current on the electrical activity of oxytocinergic magnocellular neurones, the effect of prolactin was investigated in one such cell under current clamp conditions. As shown in Fig. 4, prolactin application elicited hyperpolarisation of the membrane potential with inhibition of its firing activity that was sustained for several minutes (see below).

As indicated earlier, prolactin introduced qualitatively similar changes to the electrophysiological properties of a fraction (24%) of putative parvicellular PVN neurones (Fig. 2B, n = 6). The I–V relationship obtained after the 2-min prolactin application was found to be significantly different from the control when both curves were analysed with nonparametric multiple comparison tests (P = 0.034, Friedman's post-hoc test). However, the extent of the increase of the G_m after prolactin treatment was not statistically significant, probably as a result of the comparatively smaller size of the sample. The reversal potential of the I–V curve was slightly left shifted after 2 min of prolactin application and the average difference current was increasing almost linearly (Fig. 3B) with a reversal potential of −57 mV.

Effects of prolactin on membrane potential are long-lasting

Unexpectedly, the effects of prolactin persisted at least 10 min after the hormone was removed. In putative magnocellular neurones (Fig. 2A), the I–V curve obtained 10 min after prolactin washout was significantly different from that obtained under control conditions and after the 2-min prolactin application ($P < 10^{-4}$ versus control, $P = 2 \times 10^{-4}$ versus 2-min prolactin, Friedman post-hoc test). This result is consistent with the sustained hyperpolarisation shown earlier after prolactin application under current clamp mode (Fig. 4). It was determined that G_m measured 10 min after prolactin withdrawal in the voltage range −95 to −75 mV was significantly larger (1.72 ± 0.3 nS) than in the control group (0.9 ± 0.1 nS) (P < 0.005 versus control, paired Wilcoxon's signed rank test). For voltages closer to the resting potential (between −55 and −35 mV), the G_m was found to be augmented as well (washout: 4.1 ± 0.6 nS versus control: 3.0 ± 0.5 nS, P = 0.001). The reversal potential for these neurones remained more negative than the one recorded previously under control conditions, although it was no different from that observed at the end of the prolactin pulse (not shown). Similarly, the reversal potential of the average difference current obtained after 10 min of hormone washout (Fig. 3A) was approximately −57 mV, which is very similar to the one previously determined at the end of the prolactin pulse.

In those putative parvicellular PVN neurones responsive to prolactin, the I–V relationship obtained after 10 min of prolactin withdrawal (Fig. 2B) was significantly different from that recorded under control conditions (P = 0.008, Friedman's post-hoc test), although it did not reach statistical significance compared to the one obtained after 2 min of prolactin treatment (P = 0.082). The G_m 10 min after hormone withdrawal was larger than in control conditions over the whole range of voltages tested (Fig. 2B). Indeed, the increase was statistically significant (washout: 7.5 ± 1.4 nS versus control: 4.1 ± 1.0 nS, P = 0.03) for voltages above −55 mV. Furthermore, the reversal potential obtained after prolactin withdrawal was further shifted to the left than at the end of the hormonal pulse. In line with this, the reversal potential of the difference current (Fig. 3B) was approximately −68 mV compared to −57 mV observed at the end of the prolactin pulse (Fig. 3B). In addition, the current–voltage relationship of the difference current was no longer linear 10 min after washout, suggesting the introduction of a voltage-dependent current.

Discussion

Several lines of evidence support the idea that prolactin exerts critical modulatory actions at different hypothalamic areas involved in its regulation. First, systemic prolactin may gain access to the brain by means of a transport system at the choroid plexus (1,26,28). Second, prolactin receptor mRNA (22–25) and protein (36) have been identified in both the SON and PVN hypothalamic nuclei. In the latter nucleus, we have further demonstrated prolactin receptor immunoreactivity in oxytocinergic neurones (15). Third, both the SON and PVN undergo spectacular changes during pregnancy and lactation, and prolactin receptors are specifically up-regulated in the PVN during lactation (16,36,37). Fourth, these same nuclei may produce and release prolactin locally (38,39). Fifth, in the hypothalamic-neurohypophyseal axis prolactin specifically increases oxytocin mRNA expression (40–43) and stimulates oxytocin secretion *in vitro* (44) and *in vivo* (45). Because oxytocin releases prolactin from pituitary lactotrophs and the daily activity rhythm of oxytocinergic PVN neurones correlates with the prolactin-releasing pattern of pregnant/pseudopregnant rats (18), these observations support the hypothesis that prolactin may elicit modulatory roles on the activity of oxytocinergic PVN neurones. The present study reports, for the first time, prolactin-induced biophysical effects on identified PVN neurones.

Forty-nine PVN neurones were electrophysiologicaly identified as type I, putative magnocellular neurones (n = 24; 49%) and type II, putative parvicellular neurones (n = 25; 51%), using the definitions outlined by Tasker and Dudek (19) and Hoffman *et al.* (21). Among the magnocellular neurones investigated, 50% were classified as oxytocinergic, as indicated by immunocytochemical staining and

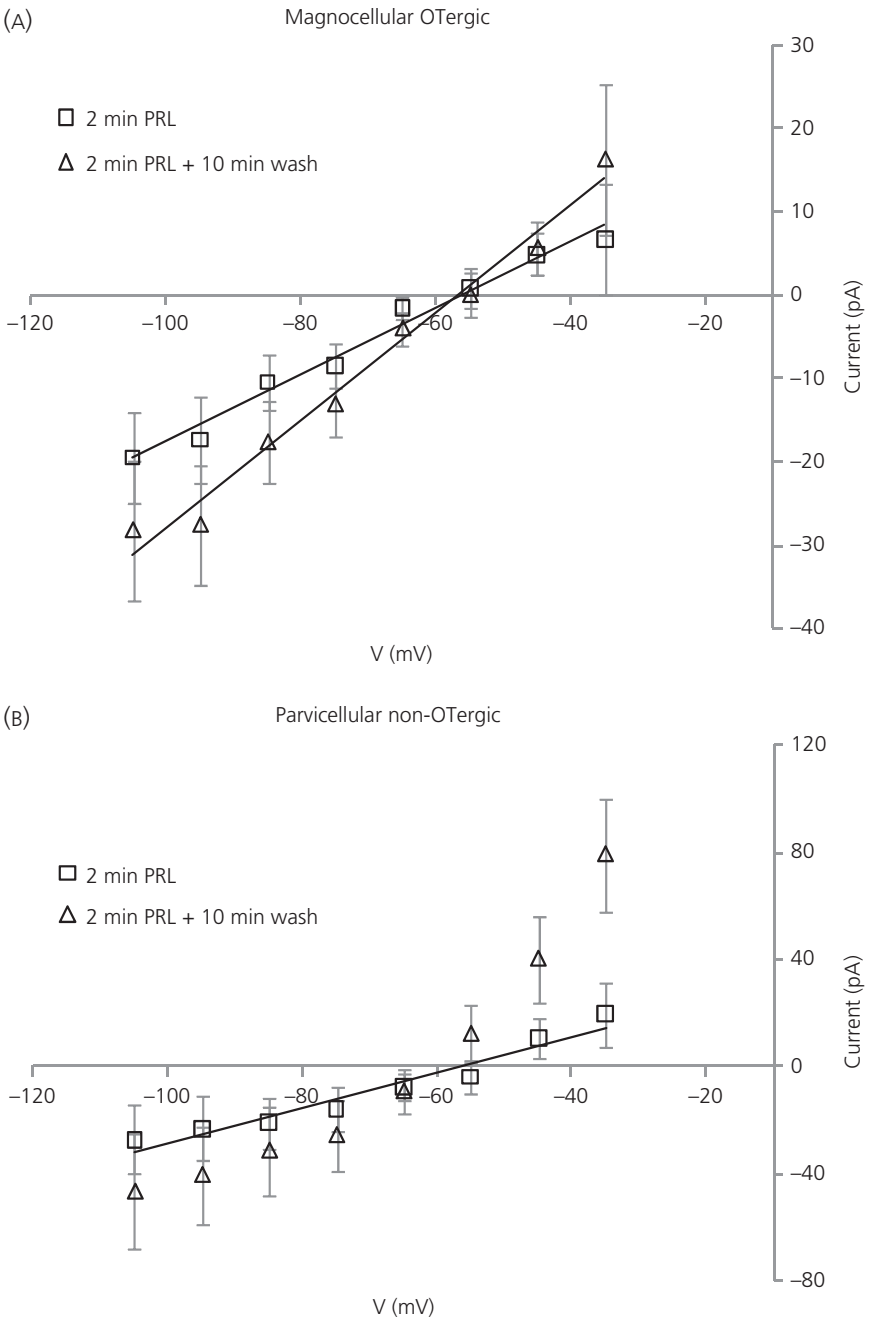


Fig. 3. Average current difference. Difference currents were obtained by subtracting control current from currents measured at the end of a 2-min prolactin (PRL) application (squares) and after 10 min of hormone removal (triangles). (A) Average difference current obtained from prolactin-responsive, oxytocinergic magnocellular cells. Data were calculated from I–V plots shown in Fig. 2(A). The reversal potential of this difference current did not significantly change between these two conditions and was approximately −56 to −57 mV. (B) Average difference current obtained in the prolactin-responsive parvicellular cells shown in Fig. 2(B). The reversal potential of this difference current after the 2-min prolactin treatment was −57 mV but shifted to −68 mV after 10 min of removal of the agonist. Error bars indicate the SE. Note the different current amplitudes in magnocellular and parvicellular neurones. OT, oxytocin-neurophysin.

their hyperpolarising response to DADLE application. Interestingly, all magnocellular oxytocinergic neurones were responsive to prolactin application. For membrane potentials greater than −60 mV, prolactin elicited a hyperpolarising current that persisted at least 10 min after hormone washout. This effect resulted in sustained hyperpolarisation and clear inhibition of firing activity in a magnocellular oxytocinergic neurone when evaluated under current clamp mode (Fig. 4). The inhibitory effects of prolactin are in line with the *in vivo* extracellular recordings reported by Kokay *et al.* (16) from magnocellular oxytocin neurones in the SON showing a decrease of neuronal activity after prolactin application. Of note, it was previously reported that supraoptic oxytocin neurones of male rats show

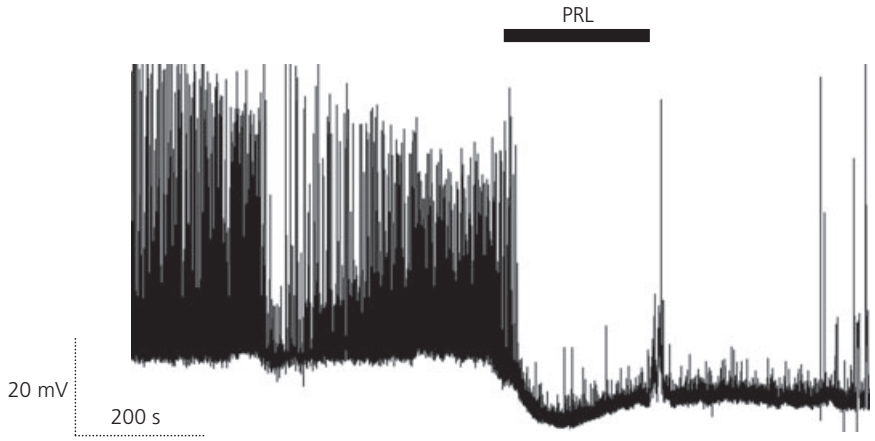


Fig. 4. Response of a magnocellular oxytocinergic paraventricular neurone to prolactin (PRL). The neuronal activity decreases as soon as prolactin-containing artificial cerebrospinal fluid (100 ng/ml prolactin) reaches the cell. Horizontal bar indicates time of PRL application.

both excitatory and inhibitory responses to prolactin in horizontal hypothalamic slices (46).

Type I neurones are generally regarded as magnocellular neuroendocrine cells that supply their products to the posterior pituitary gland by means of the hypothalamic-hypophyseal tract (17). Electrophysiological studies on oxytocin neurones showed that an increase in firing activity facilitates peptide secretion (47). Because prolactin induced a hyperpolarising current in oxytocinergic PVN neurones, we hypothesise that this decreases the neuronal activity resulting in less oxytocin release from the axon terminals of the posterior pituitary. This would result in a reduction of prolactin secretion from the anterior lobe of the pituitary gland. Such a relationship between prolactin-secreting lactotrophs and oxytocinergic neurones of the PVN would thus provide a negative-feedback loop where prolactin suppresses its own secretion indirectly via a reduction of oxytocin release. However, as noted above, prolactin has been shown to stimulate oxytocin synthesis (40–43) and secretion (44,45) in lactating and steroid-primed ovariectomised female rats. This apparent discrepancy might be explained by a differential regulation of somatic versus nerve terminal release of oxytocin, as has been shown for α-melanocyte-stimulating hormone (48). Thus, it is possible that prolactin-induced effects in the cell bodies of oxytocin neurones are dissociated from those at their terminals in the posterior pituitary. Alternatively, the effect of peptides may vary according to the experimental condition (*in vitro*, *in vivo*), gender and physiological status. Indeed, central prolactin has been shown to release oxytocin in plasma in virgin female rats, but inhibit stress-induced oxytocin release (43,49).

The electrophysiological analysis of putative parvicellular neurones showed that approximately one-quarter of these cells responded to prolactin. Based on their lack of low-threshold spike and small T-current (31), these parvicellular prolactin-responsive cells are assumed to be neuroendocrine cells and represent approximately 30% of the population of parvicellular neurones that project to the median eminence. No pre-autonomic (nonneurosecretory) parvicellular cells responded to prolactin. However, in contrast to previous studies (18,50,51), we failed to observe oxytocin-positive stained cells among putative parvicellular neurones. The reason for this is not immediately clear, although it should be noted that only a minority of the oxytocinergic fibres found in the external layer of the median eminence originate in the parvicellular cell bodies in the PVN (50). Additionally, it is possible that this cell population is less abundant in the juvenile female rats used for our studies and that we would have found oxytocin-positive parvicellular neurones if our sampling size had been larger. Notwithstanding, the fact that there were non-oxytocinergic, prolactin sensitive parvicellular neurones with electrophysiological characteristics of neurosecretory cells raises the possibility that prolactin may influence the secretion of adrenocorticotrophic hormone and growth hormone through modulation of corticotrophin-releasing hormone, arginine-vasopressin, growth hormone-releasing hormone, somatostatin and thyrotrophin-releasing hormone cell bodies (35). A well-known correlation exists between suckling-stimulated prolactin secretion and reduced hypothalamic-pituitary-adrenal axis responses in lactation, and our findings may add to the accumulating evidence suggesting a potential role of central prolactin in such diminished responses (52).

As mentioned earlier, prolactin has been shown to be synthesised and expressed locally in both magnocellular and parvicellular PVN and SON neurones (38, 39, 53). Although such evidence is not apparent in the present study, if locally produced prolactin were present in significant amounts in our PVN preparations, it might have masked, to some extent, the hyperpolarising current elicited by exogenously added prolactin.

Our data show that prolactin induces a hyperpolarising current in putative magnocellular oxytocin neurones in the PVN that may result in inhibition of firing activity. A number of previous studies have shown that prolactin induces rapid positive (46,54) and negative (16,46) changes in the excitability of hypothalamic neurones. These diverse effects of prolactin (within and among different subsets of hypothalamic neurones) might be achieved by operating through its different prolactin receptor isoforms (1, 55). In rats, short, intermediate and long forms of the receptor have been found, which differ with respect to the length of their intracellular domains and signalling capabilities (1, 55). As a result, the short form of the prolactin receptor uses mainly signalling pathways mediated through the mitogen-activated protein kinase. However, only the long form, which in the PVN is preferentially expressed by

oxytocin neurones, retains the ability to fully activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway (16). In contrast to the stimulation/inhibition of gene transcription, the activation of ion channels is one of the primary proximal (rapid) events in prolactin receptor signal transduction. For this reason, it is unlikely that such rapid effects involve the JAK/STAT pathway. Nevertheless, JAK2 tyrosine kinase activity has been shown to be required for prolactin-induced phosphorylation and activation of ion channels (56, 57), transporters (58, 59) and kinases (60, 61).

The prolactin-induced hyperpolarising current reported in the present study was sustained for at least 10 min. Indeed, prolactin has been shown to elicit a slow K^+ current with a time lapse to peak effect of approximately 10 min and an incomplete return to control levels after 10 min of washing through a mechanism that involves tyrosine phosphorylation in CHO (62), as well as in human breast MCF-7 (57) and prostate LNCaP (63) cancer cells. Although the persistence of the effects of prolactin reported in the present study is comparable to these studies, our data do not rule out the participation of other hyperpolarising conductances that may also be modulated by prolactin (64) in the PVN. In the magnocellular system, prolactin may facilitate the sensitivity of $GABA_A$ receptors to inhibitory inputs because these receptors are known to be modulated by locally produced neurohormones in particular physiological stages (65).

Recently, studies by Townsend *et al.* (46) and Kokay *et al.* (16) have reported modulatory effects of prolactin on the electrical activity of oxytocinergic supraoptic neurones. Because their data were based on extracellular recordings, it is not known whether the effects of prolactin involved direct action within the supraoptic nucleus or indirect interaction with its perinuclear afferent circuitry. In the present study, we have provided the first direct evidence that prolactin elicits a rapid and long-lasting hyperpolarising current in putative magnocellular (type I) oxytocinergic neurones and putative parvicellular (type II) neurosecretory neurones of the PVN. This means that the neuronal activity of PVN neurones, particularly oxytocin-immunostained cells, is likely reduced by prolactin. Thus, besides the well known negative-feedback loop between prolactin and tuberoinfundibular dopaminergic neurones (1,5), an inhibitory feedback loop exists between lactotrophs of the anterior pituitary gland and PVN neurones that supply the prolactin-releasing factor oxytocin.

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