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ELECTROPHYSIOLOGY OF GUINEA-PIG SUPRAOPTIC NEURONES: ROLE OF A HYPERPOLARIZATION-ACTIVATED CATION CURRENT IN PHASIC FIRING

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SUMMARY

1. Immunocytochemically identified magnocellular neurosecretory cells (MNCs) in the guinea-pig supraoptic nucleus (SON) were studied using the *in vitro* intracellular recording technique. Cells were identified as containing arginine vasopressin (AVP) or oxytocin (OT) following recordings made with biocytin-filled electrodes. Both AVP and OT MNCs demonstrated a fusiform or pyramidal shape (15–20 μm by 26–39 μm), with two to three processes. There were no significant differences in the proportion of AVP and OT cells in the retrochiasmatic (caudal) *versus* the rostral slices.

2. No significant differences in passive membrane properties were observed between AVP and OT cells, except that AVP cells exhibited a significantly broader action potential width ($1{\cdot}51 \pm 0{\cdot}1$ ms, $n = 11$) than did OT cells ($1{\cdot}01 \pm 0{\cdot}08$ ms, $n = 7$).

3. Firing patterns were recorded for 100 MNCs, 41% of which fired in a phasic manner (repeated clustering of action potentials into bursts). Of the seventy-seven cells which were immunocytochemically identified, only AVP-containing MNCs displayed phasic firing. Phasic firing occurred only in MNCs demonstrating a depolarizing potential which followed hyperpolarizing after-potentials (HAPs). The presence of the depolarizing potential was not always associated with phasic firing, however, as both OT cells and non-phasic AVP cells sometimes exhibited a depolarizing potential.

4. In 160 MNCs examined for the presence of the time-dependent inward rectification (TDR in current clamp, or I_h in voltage clamp), a significant difference in the proportion of cells expressing the I_h was observed in the two cell types. The I_h was expressed in forty-five of fifty-four AVP MNCs (83%) and in six of fifteen OT MNCs (40%). No significant association was found with firing pattern.

5. The I_h exhibited properties similar to those found in other CNS and peripheral tissues. It appeared on steps to potentials more hyperpolarized than -65 mV. It was augmented by raising the extracellular potassium concentration, blocked by 2 mM CsCl, and insensitive to 100–500 μM $BaCl_2$. Activation followed a single exponential, and the time constant of activation was voltage dependent.

6. The adenylate cyclase activator forskolin increased the I_h and shifted its

activation curve to more depolarized levels. In cells recorded for several hours, the I_h varied in amplitude, suggesting intrinsic modulation, possibly by intracellular second messenger systems. The I_h in guinea-pig SON MNCs appears to serve an excitatory role, bringing cells closer to firing threshold.

INTRODUCTION

The supraoptic nucleus (SON) in mammals chiefly contains magnocellular neurosecretory cells (MNCs) which secrete arginine vasopressin (AVP) or oxytocin (OT) into the bloodstream via axon terminals in the neurohypophysis (Silverman & Zimmerman, 1983). Studies in the rat utilizing *in vivo* (Dutton & Dyball, 1979; Moos & Richard, 1983; Cirino & Renaud, 1985; Belin & Moos, 1985; Blackburn, Leng & Russell, 1987), or *in vitro* (Armstrong & Sladek, 1982; Mason, 1983; Bourque & Renaud, 1983, 1984; Andrew & Dudek, 1983, 1984) techniques, have characterized the AVP-secreting neurones as firing in repeated bursts of action potentials (phasic firing), whereas OT-secreting neurones fire in a slow, irregular pattern. OT cells also fire in continuous high-frequency bursts during milk ejection. Recent studies combining electrophysiological recordings with immunocytochemical staining methods have further supported the differences in firing pattern (Cobbett, Smithson, & Hatton, 1986; Cobbett, Legendre & Mason, 1989). The studies describing intrinsic membrane conductances in these cells have been confined to rat SON. These have employed standard ionic solutions, or Lucifer Yellow, in the recording electrodes, without immunocytochemical verification of the type of MNC (AVP- or OT-containing).

The phasic firing pattern of putative rat AVP neurones has been attributed to several cell properties: (1) the depolarizing after-potential (DAP) which (a) follows an action potential, (b) is calcium dependent, (c) activates at potentials positive to -70 mV, and (d) can summate into a plateau potential (Andrew & Dudek, 1983; Andrew, 1987); (2) an interburst slow depolarization (Dudek & Gribkoff, 1987), which also activates positive to -70 mV; and (3) an after-hyperpolarization (AHP) following bursts of action potentials (Andrew & Dudek, 1984; Bourque, Randle & Renaud, 1985; Legendre, Poulain & Vincent, 1988), which is thought to terminate the burst. The AHP is dependent upon the calcium-dependent potassium current ($I_{K,Ca}$), and reverses at the potassium equilibrium potential (Bourque *et al.* 1985).

The ionic bases of the DAP and the slow depolarization, however, have not been established. Andrew (1987) has argued that calcium plays the primary role in mediating this conductance. However, he reports that conductance decreases during the DAP, suggesting that reduction in an outward current (such as a calcium-dependent potassium current) may play a role in its generation.

No analyses of the intrinsic conductances of guinea-pig SON MNCs have been reported. The few descriptive intracellular studies of guinea-pig SON have not assessed firing patterns, examined membrane properties beyond the resting membrane potential (RMP) and input resistance (R_{in}), or utilized immunocytochemical identification of AVP- *versus* OT-containing neurones (Abe & Ogata, 1982; Abe, Inoue, Matsuo & Ogata, 1983; Ogata & Matsuo, 1986; Ogata, 1987).

In the present study the passive and active membrane properties of immunocytochemically identified AVP and OT guinea-pig SON cells have been described.

A depolarizing potential contributing to phasic firing (Erickson, Ronnekleiv & Kelly, 1991) has been further elucidated. The I_h reported in our preliminary study in guinea-pigs (Erickson, Ronnekleiv & Kelly, 1990) has been characterized in greater detail, and its expression in identified AVP and OT MNCs has been compared.

METHODS

Animal preparation

Male and female Topeka guinea-pigs weighing 350–650 g were used for the study. At least one week prior to the experiment, female guinea-pigs were ovariectomized, then injected with oestradiol (25 μg) 24 h before being killed to provide a uniform hormone level, thereby controlling for oestradiol fluctuations in the 16- to 18-day oestrous cycle of this species. Serum oestradiol levels varied from 80–150 pg/ml as measured by radioimmunoassay (Resko, Ellinwood, Pasztor & Buhl, 1980) by the Steroid RIA Core (P 30 HD18185).

Slice preparation

Guinea-pigs were decapitated with a guillotine, in accordance with the 'NIH Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 80–23, revised 1978). The brain was removed and three 450–500 μm coronal hypothalamic slices were cut on a vibratome. The slices were maintained in an auxiliary chamber at room temperature until they were transferred to the recording chamber, in which they were kept submerged and perfused at 1–2 ml/min in a warmed (36 ± 1 °C), oxygenated (95% O_2, 5% CO_2) environment with a modified Krebs–Ringer buffer (in mM: NaCl, 124; KCl, 5·0; NaH_2PO_4, 1·25; $MgSO_4$, 2; $CaCl_2$, 2; $NaHCO_3$, 26; dextrose, 10; HEPES, 5; pH, 7·35–7·45). For some experiments, a low-potassium medium containing 2·5 mM KCl, or a high-potassium medium containing 10 mM KCl and 117 mM NaCl was used.

Drug solutions were delivered via stopcock-controlled, gravity-fed syringes. Solutions (all in the artificial cerebrospinal fluid (ACSF) medium above) contained tetrodotoxin (TTX, 1 μM, Sigma), bicuculline methiodide (10–50 μM, Sigma), barium chloride ($BaCl_2$, 100 μM–1 mM, Sigma), caesium chloride (CsCl, 2 mM, J. T. Baker), or forskolin (10–20 μM, Sigma).

Recording methods

Intracellular recordings were made from one of the three coronal slices containing the supraoptic nucleus, using borosilicate glass micropipettes (i.d. 0·58 mm, o.d. 1·0 mm; AM Systems) pulled on a Brown–Flaming P-87 puller (Sutter Instruments). These were filled with KCl–potassium citrate (3 M/1·5 M, 20/80 v/v) or with 1–3% biocytin (Sigma) in 1·75 M KCl with 0·025 M Tris buffer (pH 7·2) (Ronnekleiv, Loose, Erickson & Kelly, 1990). Resistances ranged from 90 to 150 MΩ. Intracellular potentials were amplified and current was passed through the electrode using an Axoclamp-2A (Axon Instruments). Initially following penetration and stabilization, the action potential height, threshold and width (at $\frac{1}{3}$ × height) were measured on a Tektronix 2230 digital storage scope. Input resistance (R_{in}) and membrane time constant (τ) were measured by delivering 250 ms hyperpolarizing current pulses (to achieve a 10–20 mV deflection in the voltage trace from RMP) after the bridge was balanced. In some experiments, the cell was held at RMP to determine the firing pattern (e.g. phasic *versus* continuous). Absolute RMP was obtained by subtracting the extracellular potential reading obtained upon withdrawing the electrode 2–5 μm from the cell or following a 'positive clear' pulse, from the RMP measured during intracellular recording.

Voltage clamp recordings utilized the single-electrode voltage clamp with a 2–2·5 kHz sampling frequency, 30% duty cycle, and gain of 0·07–0·15 nA/V. The discontinuous mode was utilized. The potential at the amplifier headstage was monitored on a separate oscilloscope. Data were continuously recorded on a strip chart recorder (Gould 2200). Only cells exhibiting overshooting action potentials, $\tau > 5$ ms, and $R_{in} > 50$ MΩ were analysed.

In *current clamp*, the time-dependent inwardly rectifying conductance was measured by delivering 800 ms hyperpolarizing current pulses from a holding potential (V_h) between −55 and −70 mV, to determine the range of activation. The conductance was identified as an initial hyperpolarization followed by a repolarizing 'sag' in the voltage trace when the membrane potential was stepped below −70 mV (Fig. 3*A*). This protocol was repeated in 100 μM–1 mM $BaCl_2$ and in 2 mM CsCl, in low (2·5 or 5·0 mM) or high (10·0 mM) extracellular KCl, or in 10–20 μM forskolin.

In *voltage clamp*, step *I–V* recordings were made by holding the membrane potential at rest or at slightly hyperpolarized levels (mean V_h of $-58{\cdot}7 \pm 2{\cdot}0$ mV, $n = 14$) to prevent firing, then stepping the potential over the range of -60 to -110 mV for 1–1·3 s. Current was measured by subtracting the current value prior to each step from the steady-state current at the end of each step. Steady-state *I–V* ramps were formed by varying the voltage from RMP to a potential 60 mV hyperpolarized to RMP, at a rate of 1 mV/s. The I_h was identified as a slowly developing inward current at membrane potentials below -65 to -70 mV, activating over several hundred milliseconds and showing no inactivation during a 1–1·3 s command step (Fig. 3*B*). This conductance was further characterized by measuring its response to 2 mM CsCl and 100 μM–1 mM $BaCl_2$. The activation threshold and amplitude of the conductance was also examined in low (2·5 or 5 mM) and high (10 mM) extracellular potassium, to determine the relative contribution of this ion to the overall conductance. Forskolin (10–20 μM) was applied during step or steady-state ramp *I–V*s to examine modulation of the I_h. Experiments were conducted in 5 mM KCl unless otherwise noted.

Reversal potential was estimated in four cells using the method of Mayer & Westbrook (1983). In this method, the instantaneous current values were measured during depolarizing voltage steps from a V_h of -90 to -100 mV (I_h fully activated) and during hyperpolarizing steps from -60 to -50 mV (I_h fully deactivated). Least-squares regression lines were fitted to the two sets of current values. The point of intersection of these lines was extrapolated, and taken as the I_h reversal potential for that MNC.

A measure of the contribution of the I_h to the spontaneous firing pattern of guinea-pig SON MNCs was made by holding the membrane potential (V_m) near RMP during current clamp. The least hyperpolarized V_m at which the cell would fire from a smooth baseline was measured. This was termed the spontaneous steady-state firing threshold (SSFT). More negative values of the SSFT were taken as an indication of the capacity of that MNC to generate action potentials from more hyperpolarized levels, i.e. a measure of the cell's excitability.

Streptavidin fluorescein isothiocyanate (FITC) staining

Following intracellular recordings made with biocytin-filled electrodes, the 450–500 μm slices were fixed in 4% paraformaldehyde in 0·03 M Sorensen's phosphate buffer (PB) (pH 7·4; 80% potassium phosphate, 20% sodium phosphate, each mixed at 0·067 M) for 90–100 min, and then soaked overnight in phosphate buffer (PB; 0·1 M, pH 7·2) containing 30% sucrose. Each slice was then rapidly frozen and sectioned at 16 μm thickness on a cryostat, mounted on poly-L-lysine-coated slides and stored frozen at a temperature of -20 °C. All of the slides from a given slice were then washed with buffer, and streptavidin-FITC (1:600, Jackson Laboratories) was applied for 2 h. The reaction was terminated by washing with 0·1 M PB. The slides were scanned for the injected neurone with a Leitz Dialux 20 microscope (Rockleigh, NJ, USA) fitted with incident-light epifluorescence generated by a 100 W mercury vapour lamp through an I_2 filter cube (excitation filter bandpass 450–490 nm).

Immunocytochemical identification

After localization of the biocytin-filled neurones, slides containing the soma of fibres were processed for the presence of AVP or OT, using fluorescence immunocytochemistry as we have described in detail (Ronnekleiv *et al.* 1990). Alternate sections were incubated with OT (Morris, Stevens & Adams, 1980) or AVP (Dave, Rubinstein & Eskay, 1985) antisera at 1:1000 and 1:2000 dilutions, respectively, for 16–32 h. The antisera for each hormone (AVP and OT) showed no cross-reactivity with the other hormone. For evaluation of the specificity of the immunocytochemical reactions, some of the tissue sections were reacted with AVP and OT antisera that had been incubated for 24 h with synthetic AVP and OT, respectively (each 7·5 μg/ml). Absorption controls demonstrated that the AVP and OT antisera were completely absorbed (no immunostaining observed) by incubating them with their respective peptide. Sections were washed in 0·1 M sodium phosphate buffer (pH 7·4) for 30 min, then incubated for at least 2 h with a combination of donkey anti-rabbit γ-globulin conjugated to Texas Red (ARGG-TR; Jackson Immunoresearch Labs) diluted 1:100 and streptavidin-FITC at 1:300. The streptavidin-FITC was added again at this stage to ensure preservation of the labelling of the biocytin inside the cells. The sections were rinsed five times for 30 min each in PB, covered with a glycerin solution (2 parts v/v glycerin and 1 part v/v glycine buffer, pH 8·0) containing 5% *N*-propyl gallate (Sigma), and a coverslip was applied.

RESULTS

Neuroanatomical and morphological features of identified MNCs

Intracellular recordings were made in a total of 233 magnocellular neurones from the supraoptic nucleus. Sixty-two of these were recorded using the single-electrode voltage clamp. One hundred and thirty-one (61%) were recorded in a rostral slice containing the SON just lateral and adjacent to the optic chiasm, and eighty-five (39%) in a retrochiasmatic slice in which the SON just medial to the optic tract was utilized. Following immunocytochemical staining, the rostral slice displayed a cluster of OT-immunoreactive (OT-ir) and AVP-ir neurones lateral to the optic chiasm. The retrochiasmatic slice displayed a group of OT-ir and AVP-ir neurones distributed medially to the optic tracts which extended in a thin band along the ventral surface. The distribution of AVP-ir and OT-ir cells was nearly identical, although AVP-ir cells displayed a wide ventrodorsal pattern, and outnumbered OT-ir MNCs by approximately 2:1 (authors' unpublished observations).

One hundred and twenty-four of the MNCs were recorded with biocytin-filled electrodes. Seventy-seven of these were identified following streptavidin-FITC. Fifty-eight (75%) were immunoreactive for AVP, and nineteen (25%) for OT. The remaining forty-seven neurones were either not located following streptavidin-FITC ($n = 27$), or were not clearly immunoreactive for AVP or OT ($n = 20$). Staining of neurones which were recorded with biocytin-filled electrodes and identified with streptavidin-FITC revealed that all of the recordings were made from magnocellular neurones with fusiform or pyramidal somata with a short axis diameter of 15–20 μm and a long axis diameter of 26–39 μm (Fig. 1). Two to three fibres were observed, which could be traced up to 200 μm through several sections.

Basic electrophysiological features of AVP and OT MNCs

The membrane properties derived from the total group of SON MNCs were as follows: RMP $= -55{\cdot}5 \pm 0{\cdot}4$ mV ($n = 214$); $R_{in} = 421{\cdot}3 \pm 17{\cdot}6$ MΩ ($n = 195$); $\tau = 20{\cdot}7 \pm 0{\cdot}6$ ms ($n = 205$); action potential (AP) height $= 52{\cdot}4 \pm 0{\cdot}7$ mV ($n = 55$); AP width $= 1{\cdot}40 \pm 0{\cdot}07$ ms ($n = 35$); and AP threshold $= -51{\cdot}6 \pm 0{\cdot}8$ mV ($n = 115$). Cells displayed chiefly linear *I–V* steady-state curves in the range -55 to -95 mV, with outward rectification at potentials positive to -45 mV and inward rectification negative to -95 mV. Hyperpolarizing after-potentials (HAPs) followed each AP with an average amplitude of $10{\cdot}8 \pm 0{\cdot}6$ mV ($n = 14$, range 7·0–16·0 mV) and duration of 87 ± 17 ms ($n = 9$, range 40–200 ms) (Fig. 2). Depolarizing potentials (DPs) often followed single APs or groups of APs (Fig. 2). DP amplitude varied from 4·0 to 7·0 mV (mean of $4{\cdot}3 \pm 0{\cdot}3$, $n = 17$) and in duration from 40 to 500 ms (mean of 188 ± 33 ms, $n = 16$).

Differences by sex, location (slice) and cell type

Sex. Sixty-two neurones (27%) were recorded in male guinea-pigs and 167 (73%) in females. More male than female guinea-pig cells exhibited phasic firing (62 *versus* 34%, $\chi^2 = 4{\cdot}1$, $P = 0{\cdot}04$, d.f. = 2). In comparing cell membrane properties by sex, a statistically significant difference was observed in τ (males: $18{\cdot}4 \pm 1{\cdot}1$ ms, $n = 54$; *versus* females: $21{\cdot}4 \pm 0{\cdot}73$ ms, $n = 147$; $P = 0{\cdot}03$, d.f. = 199). No differences in R_{in},

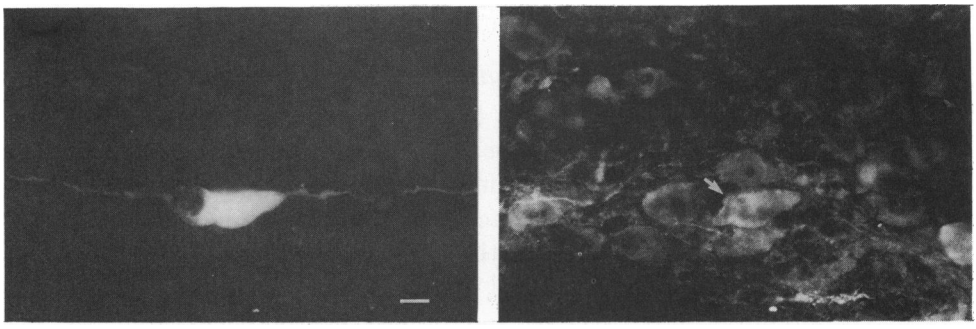

Fig. 1. Guinea-pig SON AVP-containing MNC. At least two prominent fibres can be seen. Left photograph demonstrates the biocytin-filled MNC, while the right photograph demonstrates the MNC following immunostaining for AVP (arrow). The cell exhibited an I_h. Calibration bar, 25 μm.

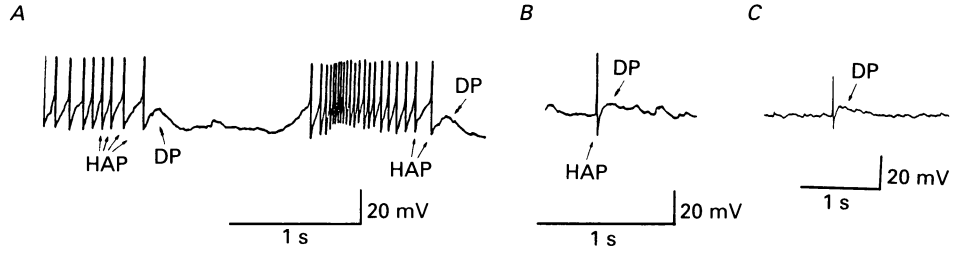

Fig. 2. Sample of recording from a phasically firing MNC, demonstrating the hyperpolarizing after-potential (HAP) that follows single action potentials (*A–C*), and the depolarizing potential (DP) that followed multiple (*A*) or single (*B* and *C*) action potentials. Recordings were at RMP (−64 mV). Full amplitude of the spike has not been reproduced by the pen recorder.

RMP or spike amplitude were observed between male and female guinea-pigs, however.

Anatomical location. No significant differences were observed in membrane properties between neurones in the rostral *versus* the retrochiasmatic slice.

Cell type. AVP-ir MNCs demonstrated a 50% greater AP width than did OT-ir cells ($1{\cdot}51 \pm 0{\cdot}12$ ms, $n = 11$, *versus* $1{\cdot}01 \pm 0{\cdot}08$ ms, $n = 7$; $P = 0{\cdot}008$, d.f. = 16). No statistically significant difference was observed between AVP-ir and OT-ir MNCs in R_{in} (487 ± 43 MΩ, $n = 48$, *versus* 381 ± 63 MΩ, $n = 16$). RMP ($-55{\cdot}9 \pm 0{\cdot}7$ mV, $n = 54$, *versus* $-55{\cdot}9 \pm 1{\cdot}4$ mV, $n = 19$) or τ ($21{\cdot}7 \pm 1{\cdot}4$ ms, $n = 48$, *versus* $18{\cdot}6 \pm 1{\cdot}8$ ms, $n = 15$).

Firing patterns and immunocytochemical cell type

Of the fifty-eight identified AVP-ir neurones, seventeen were observed at RMP to determine firing pattern. Of these, seven (41%) showed a pattern of repeated bursts of action potentials, or phasic firing. Bursts were defined as groups of at least five action potentials, occurring on a repeated basis. Burst duration ranged from 100 ms to 180 s, and interburst intervals ranged from 100 ms to 50 s. Bursts were most commonly seen without any alteration in underlying RMP. None of the six OT-

containing neurones examined for firing pattern demonstrated phasic bursts. AVP-ir MNCs and putative AVP cells (phasic firing) exhibited a greater firing frequency (21·2±3·3 Hz, $n = 11$; range 3·3–39 Hz) than non-phasic OT-ir cells (6·5±2·1 Hz, $n = 6$; range 0·8–16·0 Hz). Of the total group of 100 MNCs examined for firing pattern

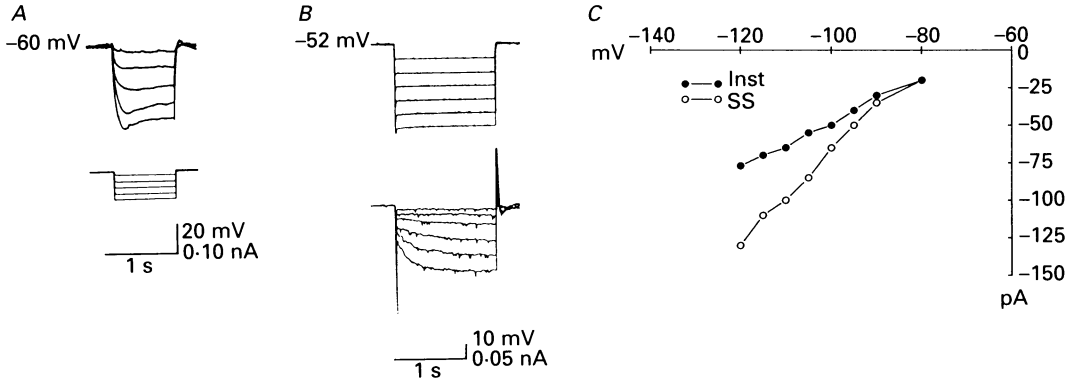

Fig. 3. *A*, time-dependent rectification, observed in current clamp. Hyperpolarizing pulses of 800 ms duration and 0·01–0·09 nA were delivered, resulting in the voltage responses shown. Note that the TDR 'sag' begins to appear below −70 mV. Membrane potential was −60 mV (RMP). The MNC was not identified immunocytochemically, but fired phasically. *B*, prominent I_h, measured in voltage clamp in an AVP-ir MNC. Voltage steps (1300 ms) from −10 to −60 mV were applied, from a holding potential of −52 mV. The I_h began to be evident at the −82 mV step. Activation rate increased with size of the hyperpolarizing step. Steady-state inward current at the largest step (to −112 mV) reached 240 pA, approximately 100 pA more than the instantaneous current. Bicuculline (10 μM) was applied in the perfusate to reduce postsynaptic currents. *C*, *I–V* curves generated by delivering 1 s hyperpolarizing steps (−10 to −50 mV) from a holding potential of −70 mV. Inst, instantaneous current value, measured at outset of voltage step; SS, steady-state current value, measured at end of voltage step. Note the onset of prominent rectification at around −90 mV in the SS trace only. The cell was an AVP-containing MNC perfused with 1 μM TTX and ACSF containing 10 mM KCl during the experiment.

at rest, forty-one showed repeated phasic firing, while fifty-nine did not. The two groups did not differ significantly on any measures of passive cell properties. However, a striking association was found between firing pattern and the presence of the depolarizing potential (DP). Of the thirty-four MNCs in this group tested for presence of a DP, 76% of those with a DP (19 of 25) exhibited phasic firing, while none of the nine cells without a DP fired phasically ($\chi^2 = 12·6$, $P = 0·0004$, d.f. = 1). DPs were observed in both AVP-ir ($n = 7$) and OT-ir ($n = 3$) MNCs, but the latter cells did not fire in bursts.

The time-dependent rectification/I_h

Characterization

In current clamp the time-dependent rectification (TDR) appeared on steps to membrane potentials below −70 mV (Fig. 3*A*). The rate of activation of this depolarizing current increased at more hyperpolarized potentials. In voltage clamp,

the current was observed at steps to membrane potentials below −65 mV, activating over several hundred milliseconds and showing no inactivation during a 1–1·3 s hyperpolarizing command step (I_h, Fig. 3*B*).

Relationship of I_h to membrane properties and other parameters

One hundred and sixty neurones were tested for the presence of the TDR or I_h, and 102 (64%) exhibited the feature. Forty-five of these 102 MNCs were identified as AVP-containing and six as OT-containing. Eight additional biocytin-labelled cells were not identified, but displayed phasic firing patterns, and therefore were considered to probably represent AVP neurones. The TDR was expressed in a significantly greater percentage of the AVP-ir MNCs (83%) than the OT-ir MNCs (40%) ($\chi^2 = 9{\cdot}3$, $P = 0{\cdot}0023$, d.f. = 1).

No significant differences were observed in RMP, R_{in}, AP height, AP width, or firing threshold for MNCs expressing *versus* those not expressing the TDR. However, the mean steady-state firing threshold (see Methods) for the former group was $-53{\cdot}9 \pm 1{\cdot}0$ mV ($n = 32$) *versus* $-50{\cdot}1 \pm 1{\cdot}3$ ($n = 39$) for the latter group. The mean difference of 3·8 mV was significant ($P = 0{\cdot}03$, d.f. = 69). This would support the role of the TDR in depolarizing the SON MNC from more hyperpolarized membrane potentials, permitting the cell to approach firing threshold following IPSPs (see Discussion).

MNCs expressing the TDR did not show a significantly greater likelihood of firing phasically than did MNCs which did not express the TDR (48 *versus* 28%). The TDR was expressed in a significantly greater percentage of neurones from male (78%, $n = 31$) *versus* female (60%, $n = 71$) guinea-pigs ($\chi^2 = 4{\cdot}5$, $P = 0{\cdot}03$, d.f. = 2). No relationship between expression of the TDR and cell location (rostral *versus* retrochiasmatic slice) was observed.

Characterization of the I_h in voltage clamp

The conductance (I_h) underlying the TDR was examined in voltage clamp in 52 of the 102 cells exhibiting this feature, to determine its time and voltage dependence and response to ionic and pharmacological agents.

Voltage and time dependence of activation. On *I–V* steps, the I_h began to appear as a gradually activating inward current at membrane potentials more hyperpolarized than −65 to −70 mV (Fig. 3*B*). Activation followed a single exponential (Fig. 4*B* and *C*), with the time constant of activation dependent upon voltage (700 ms at −80 mV to 200 ms at −110 mV; Fig. 4*D*).

However, evidence that the underlying conductance was activated at more depolarized levels was obtained in the following way: a caesium-sensitive current was discernible on steady-state *I–V* ramps between −55 and −60 mV (at or within 5–10 mV of RMP) in four of nine cells thus tested (Fig. 6*A*). Since extracellular caesium is not reported to block other conductances, this caesium-sensitive conductance was postulated to be the time-dependent inward rectifier, or I_h.

If the I_h is partially activated at or near rest in voltage clamp, this would result in a larger instantaneous (chord) value for the current at the onset of a hyperpolarizing voltage step than if the I_h was completely deactivated. This observation was used to

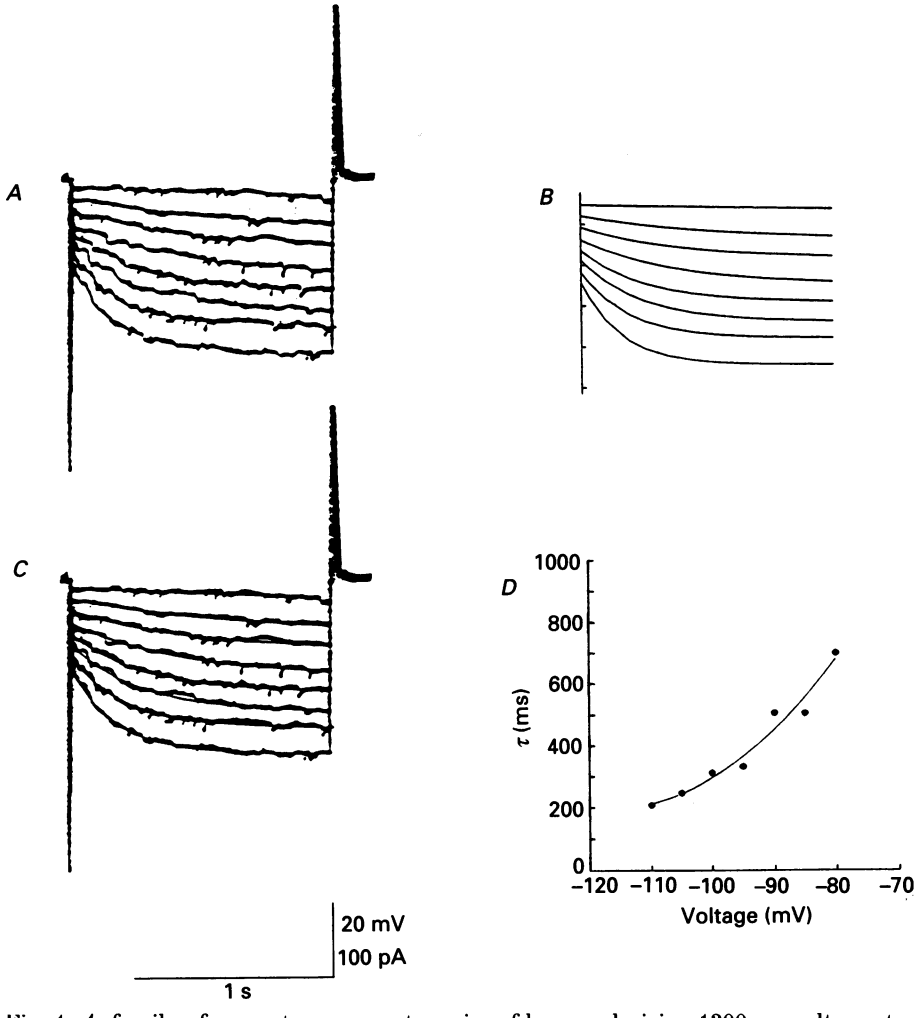

Fig. 4. *A*, family of current responses to series of hyperpolarizing 1300 ms voltage steps, in same AVP-ir MNC as in Fig. 3. *B*, series of computer-generated curves, all described by the single exponential equation:

$$f(t) = A_0 + A_1 \times (e^{-t/\tau}),$$

where τ is the activation time constant in milleseconds, A_0 and A_1 are coefficients, and $f(t)$ is the curve function value at time t (ms). *C*, all the current responses could be well fitted by such curves, suggesting that the time course of activation of the I_h follows a single exponential in guinea-pig SON MNCs. Another MNC displayed the same match of current traces to single exponential curves (not shown). *D*, the time constant of activation (calculated from the equation above) was voltage dependent, decreasing with greater hyperpolarization.

obtain two additional lines of evidence that the I_h is active at or near RMP in guinea-pig SON MNCs: (1) a caesium inhibition of the current at the onset of the voltage step was evident on 5–10 mV hyperpolarizing step commands from RMP in six out of twelve cells thus tested (the other six MNCs showed no caesium inhibition until

larger hyperpolarizing steps were delivered); and (2) experiments in low (2·5 mM) and high (10·0 mM) KCl (see below) demonstrated augmentation of the current measured at the onset of 5–10 mV hyperpolarizing steps in four MNCs.

Voltage dependence of deactivation. The I_h exhibited deactivation following the offset of hyperpolarizing steps. The time constant of deactivation was voltage

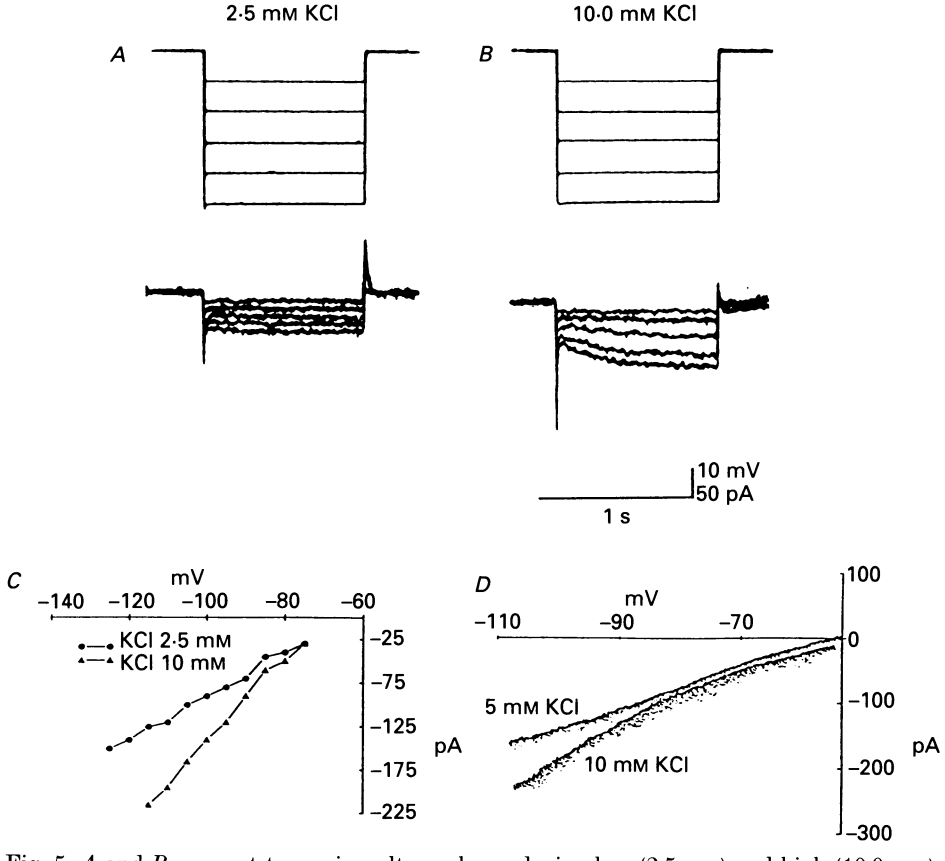

Fig. 5. *A* and *B*, current traces in voltage clamp during low (2·5 mM) and high (10·0 mM) KCl. The medium contained 100 μM $BaCl_2$ and 1 μM TTX. Note the prominent increase of the I_h in the high KCl medium, in contrast to its near absence in the low KCl medium. $V_h = -65$ mV (RMP = −59 mV). The cell was AVP containing. *C*, *I–V* curve generated from the current traces in *A* and *B*, using the steady-state current values to 1 s hyperpolarizing voltage steps from the holding potential of −65 mV. Note the increase in inward rectification in high KCl, beginning at −75 mV. *D*, steady-state *I–V* ramp in another AVP-containing MNC, demonstrating an observable increase in inward current, and augmentation of the inward rectification at approximately −85 mV, with a doubling of extracellular potassium concentration.

dependent, decreasing after the offset from larger hyperpolarizing steps. It varied from 80 ms following steps to −110 mV, to 200 ms following steps to −70 mV.

Response to raising extracellular potassium. Raising the extracellular KCl concentration ($[KCl]_o$) increased the I_h magnitude in all nine cells tested (Fig. 5*A* and *B*). Raising $[KCl]_o$ from 2·5 to 10 mM ($n = 4$) increased instantaneous and steady-

state current values on step commands from RMP, suggesting a partial activation of I_h at rest (although an increased leak conductance or shift in activation threshold of the I_h could also contribute). The I_h was also augmented on steady-state ramp *I–V* curves in the presence of high $[KCl]_o$ (Fig. 5*D*). Therefore, the conductance underlying I_h is dependent, in part, upon the extracellular potassium concentration.

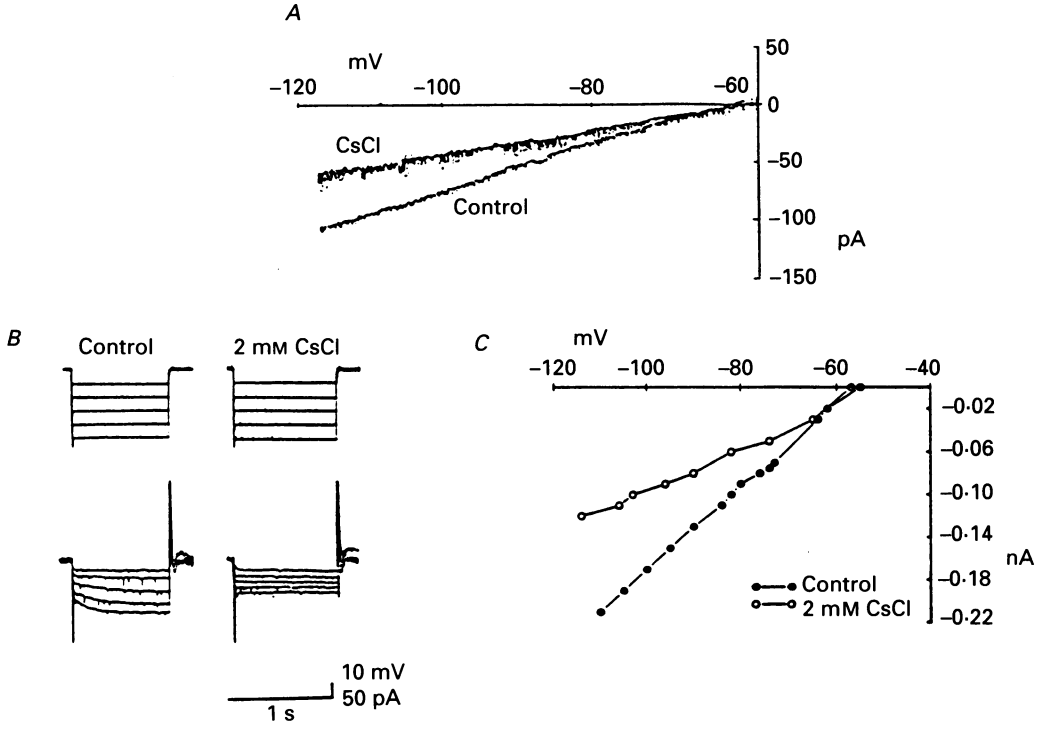

Fig. 6. *A*, steady-state ramp *I–V* demonstrating the effects of 2 mM CsCl on the I_h in an AVP-containing MNC. Caesium blockade begins to appear at approximately −65 mV. *B*, current traces in voltage clamp in an AVP-containing MNC, in response to 1 s hyperpolarizing pulses (−10 to −50 mV), in control medium and 2 mM CsCl. $V_h = -50$ mV. *C*, *I–V* curve generated in same cell, from steady-state current values at the end of 1 s hyperpolarizing steps, from holding potentials of −50 mV in each instance. Note the prominent blockade by 2 mM CsCl, beginning at −65 mV.

Reversal potential. Reversal potential was estimated in four MNCs by plotting the intersection of slopes for the instantaneous current–voltage relationships for (a) hyperpolarizing steps from a holding potential of −55 to −60 mV, and (b) depolarizing steps from a holding potential of −90 to −100 mV (Mayer & Westbrook, 1983). Results were as follows (all in 5 mM KCl): −56, −52, −28 and −65 mV (mean of -50 ± 8 mV). The wide range of values obtained in these cells may reflect partial activation of the I_h at RMP, or the presence of other conductances that would confound this measure.

Response to ionic blockers (caesium and barium). The response of the TDR (or I_h)

was tested to CsCl ($n = 24$, 9 in current clamp, 16 in voltage clamp). The addition of 2 mM CsCl to the bathing medium completely abolished the sag associated with the TDR in the nine MNCs tested in current clamp. In voltage clamp, the I_h was inhibited in sixteen MNCs (Fig. 6*B*). In seven of these cells, step *I–V* curves were

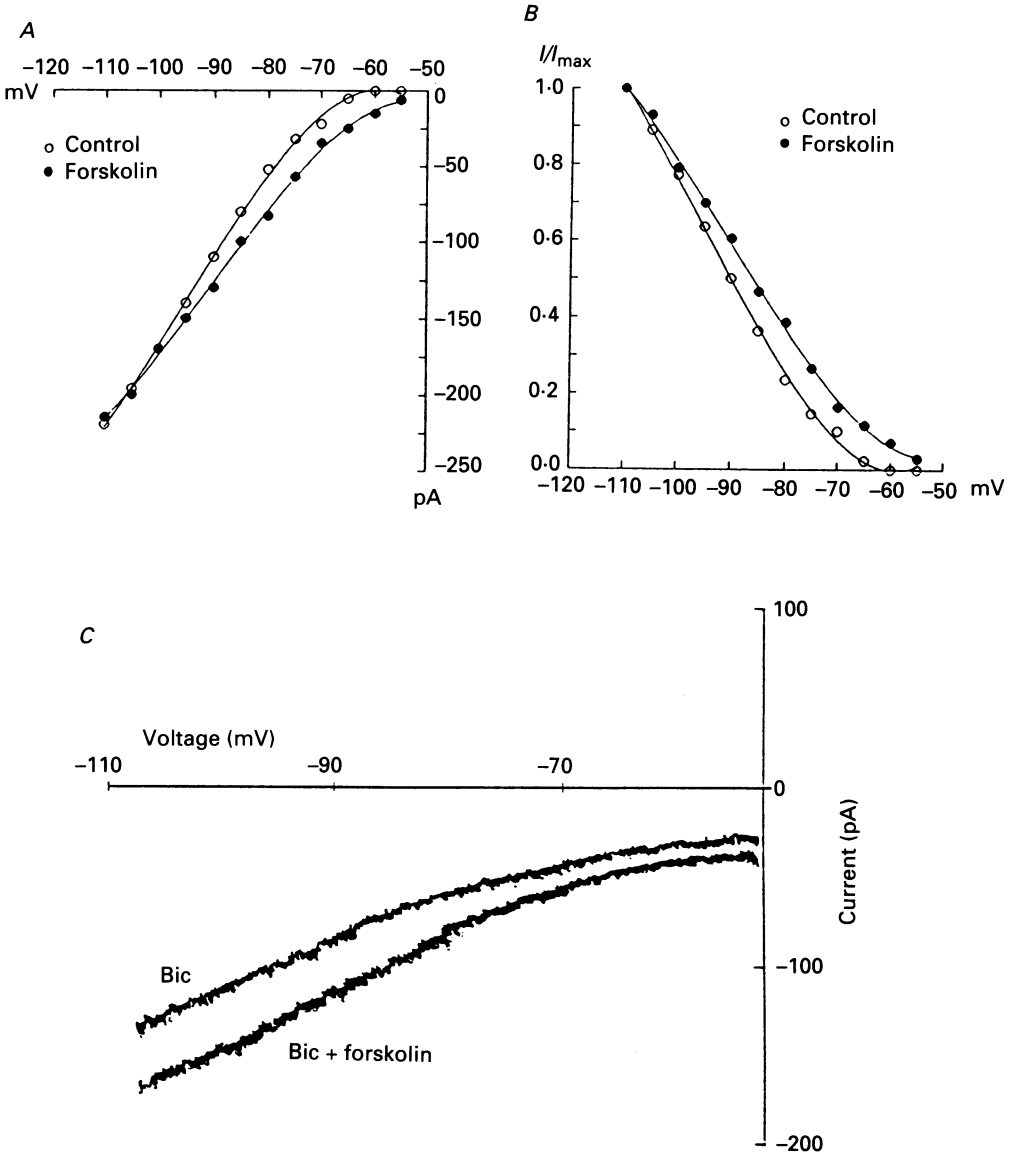

Fig. 7. *A*, I_h steady-state *I–V* curve in forskolin. Forskolin increases the I_h steady-state current, chiefly at less hyperpolarized levels. *B*, I_h steady-state activation in forskolin. The effect of forskolin in this cell appeared to be due primarily to a shifting of the I_h activation curve to more depolarized potentials. *C*, steady-state *I–V* ramp in the same AVP-ir MNC. Note that 10 μM forskolin results in a visible increase in inward rectification beginning at approximately −80 mV. The cell was recorded in the presence of 10 μM bicuculline methiodide (Bic) to reduce postsynaptic currents.

constructed prior to and during application of 2 mM CsCl, and demonstrated inhibition of the steady-state current values beginning at steps to −65 mV or more hyperpolarized (Fig. 6*C*). This inhibitory effect was also observed on steady-state *I–V* ramps in nine cells (Fig. 6*A*), in which the mean membrane potential at which the caesium effects became apparent (−64 mV) was 8 mV hyperpolarized to the mean RMP (−56 mV).

The caesium-sensitive current (I_h) ranged in amplitude from 17 to 107 pA at −100 mV in nine cells (mean of 52·9 ± 9·8 pA) as determined by subtraction after 2 mM CsCl was given. While relatively small in absolute terms, its contribution to the overall cell conductance was substantial, ranging from 20 to 60% in these experiments (total current in 5 mM KCl rarely exceeded 200 pA at −100 mV in the MNCs measured).

In contrast, only two of ten cells ($n = 5$ in current clamp, $n = 5$ in voltage clamp) demonstrated a partial inhibition of the TDR of I_h in response to higher concentrations of $BaCl_2$. The magnitude of inhibition was 50% (500 μM, in a phasic MNC) and 15% (1 mM, in an AVP-ir MNC). The remaining eight MNCs (seven AVP-ir, one OT-ir) showed no effects of $BaCl_2$.

Modulation of the I_h

The I_h has been reported to be modulated by intracellular calcium levels (Hagiwara & Irisawa, 1989), noradrenaline and serotonin (Pape & McCormick, 1989), by the adenylate cyclase activator forskolin (Tokimasa & Akasu, 1990), and by direct G protein application in cardiac sino-atrial node pacemaker cells (Yatani, Okabe, Codina, Birnbaumer & Brown, 1990). In the present study, the effects of the adenylate cyclase activator forskolin on I_h were investigated.

Forskolin (10 or 20 μM) was administered to six AVP-ir MNCs. In four, the I_h was increased and its activation curve shifted to a more depolarized level (from 5 to 10 mV positive, at step potentials from −60 to −70 mV, Fig. 7*A* and *B*). Steady-state *I–V* ramps yielded similar results, demonstrating an increase in the inwardly rectifying current below -80 mV (Fig. 7*C*). In the two other MNCs, 10–20 μM forskolin appeared to have no effect on I_h.

In three cells not given forskolin, the I_h was observed to vary in its degree of expression. Hyperpolarizing steps delivered to all three MNCs initially suggested that no time-dependent inward rectifier was present. However, the I_h appeared later – at 1, 2 or 3 h after penetration (depending upon the cell). No relationship to any other change in the cell's electrophysiological properties, or to drugs applied, could be determined.

DISCUSSION

Cell distribution and morphology in guinea-pig SON

As in earlier descriptions of guinea-pig SON (Sofroniew, Weindl, Schinko & Wetzstein, 1979), the present study demonstrated that AVP-ir neurones and OT-ir neurones share a similar distribution. However, in both rostral and retrochiasmatic slices, AVP-ir MNCs outnumbered OT-ir MNCs in an approximately 2:1 ratio, and extended a greater distance in the ventrodorsal axis. Cell size, morphology and

distribution were similar to that which has been reported using streptavidin-HRP immunocytochemical staining techniques in guinea-pig (Sofroniew *et al.* 1979).

Basic membrane properties

The membrane properties reported here for MNCs in the guinea-pig SON exhibit some differences from those described in the rat (Mason, 1983) and cat (Fagan & Andrew, 1991) as well as earlier studies in guinea-pig (Abe & Ogata, 1982; Abe *et al.* 1983; Ogata & Matsuo, 1986; Ogata, 1987). The RMP was more depolarized, with a mean of −55·5 mV in the present study *versus* −59 mV or −67 mV in rats (Mason, 1983; Fagan & Andrew, 1991), and −61 mV in cats (Fagan & Andrew, 1991). Earlier studies in guinea-pig reported RMP values from −61·7 mV (Ogata, 1987) to −57·2 mV (Ogata & Matsuo, 1986), which are closer to those of the present study. The more depolarized RMP and firing threshold in guinea-pig SON cells suggest that distinct groups of voltage-dependent conductances to those in rat may operate to control cell firing pattern.

Action potential width exhibited a nearly 50% greater duration in AVP-ir *versus* OT-ir neurones in the guinea-pig. The basis of this difference, and its relevance to phasic firing in AVP neurones, remains to be established. However, one consequence would be that during the action potential, AVP-containing neurones could permit a greater degree of calcium influx than OT-containing MNCs, with important effects on calcium-dependent conductances, second messenger systems, or other calcium-dependent intracellular events.

A major difference observed in the present experiments compared to earlier guinea-pig SON studies was the large input resistance (mean of 421 MΩ *versus* 140–157 MΩ; see Abe *et al.* 1983 and Ogata & Matsuo, 1986). In those studies, potassium acetate was used in the recording electrodes, as opposed to a combination of KCl and potassium citrate in this study. Finally, membrane time constant in the guinea-pig was significantly longer than in rat (mean of 20 ms *versus* 14 ms). Therefore, while brief EPSPs and other fast events would be attenuated by a longer time constant, longer EPSPs and depolarizing phenomena such as the DP would summate to a greater extent in the guinea-pig.

Firing patterns

Forty-one per cent of MNCs in this study exhibited phasic firing ($n = 100$). The bursts were considerably shorter than those reported in the rat SON (Poulain & Wakerley, 1982), ranging from 100 ms to 3 s in most cases (13 and 170 s in two MNCs). A DP followed action potentials in both AVP-containing and OT-containing MNCs, resembling the DAP which triggers burst firing in rat SON. In AVP-containing neurones it appeared to be an essential (although not sufficient) feature for bursts of action potentials to occur. However, it was much shorter (100–300 ms) and smaller (4–7 mV) than the DAP reported in rat SON, which has a duration of 1–2 s (Andrew & Dudek, 1983; Andrew, 1987) and amplitude of 10–15 mV (Bourque, 1986). Moreover, in association with the more depolarized RMP observed in these guinea-pig MNCs, the DP appeared at more depolarized membrane potentials (i.e. over the range of the RMP, from −65 to −55 mV). In contrast, the DAP in rat SON appears at membrane potentials from −80 mV (Bourque, 1986) to −70 mV (Andrew, 1987).

In rat, the DAP is thought to summate to form plateau potentials. No clear summation of this type was observed in these experiments, the DP being present in a number of both AVP-ir and OT-ir neurones lacking plateau potentials.

The percentage of phasically firing neurones in these experiments is similar to that reported for rat SON (30% in Andrew & Dudek, 1984; 42% in Fagan & Andrew, 1991). As in the rat, phasic firing was found only in AVP-ir MNCs (Cobbett *et al.* 1989). Significantly, the DP was the only membrane property observed to vary between phasic and non-phasic MNCs, suggesting that it plays a critical role in eliciting or permitting phasic activity (Erickson *et al.* 1991). Its role may be to depolarize the cell to firing threshold following the preceding AP and HAP. The DP in guinea-pig is large enough (4–7 mV, mean of 4·3 mV) to often bring the cell from the RMP (mean of −55·5 mV) to firing threshold (mean of −51·6 mV). A single action potential is capable of producing a robust DP, as demonstrated in Fig. 2*B* and *C*. When the DP is expressed, it may be buried in a long train of APs (burst). Therefore, it may only be observed at the burst termination when hyperpolarizing influences hold the cell potential below firing threshold throughout its depolarizing course. In cat SON, no DAP has been observed, consistent with the absence of phasic firing in this species (Fagan & Andrew, 1991).

To achieve the membrane potentials in which the DP is most influential for shaping cell firing (i.e. −65 to −55 mV), guinea-pig SON MNCs may make use of the time-dependent rectification (I_h). This cationic current, discussed below, depolarizes cells from hyperpolarized levels.

The time-dependent rectification

The time-dependent inwardly rectifying current, or I_h, has been described in numerous excitable cells including rat nucleus accumbens (Uchimura, Cherubini & North, 1990), guinea-pig thalamic relay neurones (McCormick & Pape, 1990), rat dorsal raphe neurones (Williams, Colmers & Pan, 1988), rat cerebellar Purkinje cells (Crepel & Penit-Soria, 1986), guinea-pig hippocampal CA1 pyramidal cells (Halliwell & Adams, 1982), mouse dorsal root ganglion neurones (Mayer & Westbrook, 1982), rabbit sino-atrial (SA) node and cardiac Purkinje cells (Brown & DiFrancesco, 1980; DiFrancesco & Ojeda, 1980; Yanagihara & Irisawa, 1980; Ginneken & Giles, 1991; DiFrancesco, 1991), and rabbit jejunum longitudinal smooth muscle (Benham, Bolton, Denbigh & Lang, 1987).

The time-dependent inwardly rectifying current in this study resembled the I_h described in other CNS regions and in the periphery in a number of respects. Its time constant of activation approximated that reported in the thalamus with regard to magnitude and voltage dependence (Pape & McCormick, 1989). As in the studies cited earlier, I_h activation occurred at potentials hyperpolarized to −70 mV, and no inactivation was observed at these voltage ranges (Crepel & Penit-Soria, 1986; Williams *et al.* 1989; McCormick & Pape, 1990). The estimated mean for the reversal potential (−50 mV) was consistent with that observed in other CNS regions (−34 to −70 mV), and approximated that reported in the thalamus (Pape & McCormick, 1989). Consistent with findings in CNS and periphery, a partial cationic contribution to the I_h from potassium was observed, blockade by 2 mM CsCl was marked, and the current was relatively insensitive to $BaCl_2$, in eight of ten cells (distinguishing it from

the rapid inward rectifier). In general, its relative contribution to total cell conductance at hyperpolarized potentials appears smaller than in other CNS neurones (Crepel & Penit-Soria, 1986; Williams *et al.* 1988; McCormick & Pape, 1990).

At RMP, its contribution to cell firing behaviour or membrane potential was small or absent. However, at potentials 5–10 mV hyperpolarized to rest it generated a significant depolarizing influence, as was clearly observed during experiments in which I_h was blocked by CsCl or augmented by increasing extracellular potassium concentration. The resulting possible contribution to cell firing patterns is discussed further below.

Expression and modulation of I_h in SON MNCs

The time-dependent inwardly rectifying current has not been previously characterized in magnocellular neurones of the SON except in our initial guinea-pig experiments (Erickson *et al.* 1990). In guinea-pig SON, over 60% of the magnocellular neurones (both AVP- and OT-containing cells) express the TDR (I_h). A factor of possibly critical importance in determining whether a neurone expresses this conductance is suggested by a recent study demonstrating that intracellular calcium levels modulated both the amplitude and voltage dependence of the I_h (Hagiwara & Irisawa, 1989). Expression of the I_h in AVP and OT neurones of the SON may also depend on intracellular calcium levels or other second messenger substances. The effects of forskolin on AVP-ir MNCs in the present experiments are similar to those reported in the bullfrog sympathetic ganglion (Tokimasa & Akasu, 1990), and suggest that cyclic AMP may serve such a second messenger function.

Functional role for I_h in guinea-pig SON MNCs

The time-dependent inward rectifier plays a prominent role in modulating the pacemaker activity of neurones in the cardiac conduction system (Brown & DiFrancesco, 1980; DiFrancesco & Ojeda, 1980; Yanagihara & Irisawa, 1980), and in increasing firing rate in phasically firing thalamic relay neurones (McCormick & Pape, 1990). During the after-hyperpolarization following single or multiple action potentials, it exerts a repolarizing influence, reducing the time to return the membrane to RMP or until the next action potential (Halliwell & Adams, 1982; Mayer & Westbrook, 1983; Crepel & Penit-Soria, 1986; McCormick & Pape, 1990).

In a guinea-pig SON MNC in which I_h is expressed, this inward cationic current would result in a more rapid repolarization of the membrane potential towards RMP following any prolonged hyperpolarizing input, such as a group of IPSPs or the after-hyperpolarization following a burst of action potentials. Thus, the net effect would be to increase the excitability of both AVP and OT neurones, and enhance hormone release in response to excitatory synaptic input. This hypothesis is supported by the observation that the steady-state firing threshold for cells expressing I_h (TDR) was 4 mV more hyperpolarized than for those which did not. Thus, MNCs in which the I_h (TDR) was expressed were able to generate action potentials from a significantly more hyperpolarized membrane potential. It is probable that I_h acts in this instance to augment other depolarizing influences, such as excitatory postsynaptic potentials, increasing the likelihood that these will reach firing threshold. The net result would

be to increase firing rate, and in phasically firing MNCs, to induce or to enhance burst firing by bringing the membrane potential into a range in which the depolarizing potential can influence firing patterns. Such a role for the I_h has been described in bursting guinea-pig and cat thalamic relay neurones (McCormick & Pape, 1990). In that system, the I_h interacts with a low-threshold calcium current and calcium-dependent potassium current to bring about phasic firing. We have described such an interaction of the I_h with a T-type calcium current in guinea-pig SON MNCs (Ronnekleiv, Erickson & Kelly, 1991).

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