Spontaneous ryanodine-receptor-dependent Ca\(^{2+}\)-activated K\(^{+}\) currents and hyperpolarizations in rat medial preoptic neurons

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The aim of the present study was to clarify the identity of slow spontaneous currents, the underlying mechanism and possible role for impulse generation in neurons of the rat medial preoptic nucleus (MPN). Acutely dissociated neurons were studied with the perforated patch-clamp technique. Spontaneous outward currents, at a frequency of ~ 0.5 Hz and with a decay time constant of ~ 200 ms, were frequently detected in neurons when voltage-clamped between ~ -70 and -30 mV. The dependence on extracellular K⁺ concentration was consistent with K⁺ as the main charge carrier. We concluded that the main characteristics were similar to those of spontaneous miniature outward currents (SMOCs), previously reported mainly for muscle fibres and peripheral nerve. From the dependence on voltage and from a pharmacological analysis, we concluded that the currents were carried through small-conductance Ca²⁺-activated (SK) channels, of the SK3 subtype. From experiments with ryanodine, xestospongin C and caffeine we concluded that the spontaneous currents were triggered by Ca²⁺ release from intracellular stores via ryanodine receptor channels. An apparent voltage dependence was explained by masking of the spontaneous currents as a consequence of steady SK-channel activation at membrane potentials > -30 mV. Under current-clamp conditions, corresponding transient hyperpolarizations occasionally exceeded 10 mV in amplitude and reduced the frequency of spontaneous impulses. In conclusion, MPN neurons display spontaneous hyperpolarizations triggered by Ca²⁺ release via ryanodine receptors and SK3-channel activation. Thus, such events may affect impulse firing of MPN neurons.
INTRODUCTION

The preoptic area plays important roles in the control of sexual behaviour (Larsson 1979), thermoregulation (Boulant 1994) and slow-wave sleep (Sterman and Shouse 1985). The medial preoptic nucleus (MPN) is the largest nucleus in the preoptic area and has been reported to contain an electrophysiologically homogenous population of neurons (Hoffman et al. 1994). Nevertheless, in the absence of external stimulation, neurons of the MPN show a variety of firing patterns, including regular, irregular and bursting firing. The spontaneous firing of Ca\(^{2+}\)- and Na\(^{+}\)-dependent action potentials in MPN neurons has previously been described and analysed (Sundgren-Andersson and Johansson 1995, 1998). In the present investigation, we show that MPN neurons also generate spontaneous miniature outward currents (SMOCs) and corresponding voltage responses. We also analyse the underlying mechanism. Although SMOCs have previously been observed and analysed in peripheral neurons (Satin and Adams 1987) and in smooth muscle fibres (here called spontaneous transient outward currents, STOCs) (Bolton et al. 1988), there are very few reports on SMOCs in the central nervous system (CNS). There, SMOCs have been observed in retinal amacrine cells (Mitra and Slaughter, 2002), in neurons of the Meynert nucleus (Arima et al. 2001) and in midbrain dopamine neurons (Cui et al. 2004), but the possible generation of SMOCs in other neuronal populations is unknown. In one case (Cui et al. 2004), SMOCs have been shown to affect the firing pattern, but in general, the functional role in neurons is unclear.

SMOCs are caused by Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)) channels and reflect the oscillations in concentration of intracellular Ca\(^{2+}\), \([Ca^{2+}]_i\) (Satin and Adams 1987; Merriam et al. 1988).
1999), released from intracellular stores. In peripheral neurons and smooth muscle, the majority of the channels involved in SMOCs are voltage- and Ca\(^{2+}\)-gated large-conductance (BK or \(K_{\text{Ca}1}\)) channels (Merriam et al. 1999; Mitra and Slaughter 2002), while in CNS neurons voltage-independent Ca\(^{2+}\)-gated small-conductance (SK or \(K_{\text{Ca}2}\)) channels have been suggested as mediators (Arima et al. 2001; Cui et al. 2004). The SK-channel subtype (SK1, SK2, and SK3 or \(K_{\text{Ca}2.1}, K_{\text{Ca}2.2}\) and \(K_{\text{Ca}2.3}\); Köhler et al. 1996) involved in SMOC generation has so far not been determined.

The \([Ca^{2+}]_i\) oscillations in neurons are due to release of Ca\(^{2+}\) from internal stores, mainly from the endoplasmic reticulum (ER), via two types of ER channels, the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP\(_3\)R) (Henzi and MacDermott, 1992; Kostyuk and Verkhratsky, 1994; Simpson et al. 1995). SMOCs appear mainly to be caused by Ca\(^{2+}\) release via RyRs (Arima et al. 2001; Cui et al. 2004; Merriam et al. 1999; Mitra and Slaughter, 2002). In some cases, coordinated release via clusters of RyRs or IP\(_3\)Rs has been reported (Cheng et al. 1993; Yao et al. 1995). Released Ca\(^{2+}\) can constitute a localized “Ca\(^{2+}\) spark” (see Imaizumi et al. 1999, for review) that in some cases triggers a “global” Ca\(^{2+}\) wave propagating throughout the cell (Berridge et al. 2000). The activation of Ca\(^{2+}\)-gated K\(^+\) channels causes a hyperpolarization of the neuron, in some cases regulating the “global” cellular Ca\(^{2+}\) concentration by a reduced inflow of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels (Perez et al. 1999).

In the present study, we demonstrate spontaneous slow miniature currents in mechanically dissociated neurons from the MPN of rat. The results suggest that the channels involved are of SK3 type and that their activation is triggered by Ca\(^{2+}\) released from the ER via RyRs. We also show that caffeine triggers such spontaneous slow
currents, but also larger, more continuous currents. Under current-clamp conditions, caffeine evokes corresponding transient or oscillatory membrane hyperpolarizations, sometimes exceeding 10 mV in amplitude, with a reduction in firing frequency in spontaneously active neurons. Thus, our findings suggest that the spontaneous slow miniature currents have an impact on the impulse firing in MPN neurons.

METHODS

Cell preparation

Ethical approval of the procedures described was given by the local ethics committee for animal research. Male Sprague-Dawley rats of weight 50 - 150 g, corresponding to an age of approximately 20 - 35 days, were used. They were kept in a 12 h light and 12 h dark cycle. Food and water was supported ad libitum. Decapitation was performed without anaesthetics. Within one minute, the brain was removed and put into ice-cold, pre-oxygenated incubation solution (see below). The brain was cut into a block containing the medial preoptic area and sliced in 300 μm thick coronal slices by using a vibro-slicer (752 M, Campden Instruments, Leicestershire, UK). The slicing procedure was performed in ice-cold, pre-oxygenated incubation solution. The preoptic area and the MPN were localized using the description by Swanson (1999). The brain slices were removed from the chamber by using a soft brush and placed in the oxygenated incubation solution for at least one hour in room temperature (21 - 23 °C). After the incubation, the slices were transferred to a small petri dish, which also
contained the same incubation solution. Neurons were acutely dissociated by using a vibrating glass rod placed on the MPN, a method modified after Vorobjev (1991) and described more thoroughly by Johansson et al. (1995) and Karlsson et al. (1997). The slice was removed while the remaining dissociated cells were allowed to sink to the bottom of the dish, for about 20 minutes.

As a complementary preparation, slices prepared as above, but of thickness ~ 150 μm, were used directly for electrical recording. In these cases, neurons of the MPN were cleaned by a stream of extracellular solution from a glass pipette during microscopic inspection before recording (cf. Malinina et al. 2005; Edwards et al. 1989).

Electrophysiology

Borosilicate glass capillaries (GC150-10, Harvard Apparatus, Edenbridge, UK) were used in a Flaming/Brown P-97 puller (Sutter Instrument, Novato, CA, USA). The tips of the pipettes were filled with intracellular solution by capillary forces. Pipettes were subsequently back-filled with amphotericin B-containing intracellular solution. The resistance, when immersed in extracellular solution was 1.8 - 3.5 MΩ. Recordings of neuronal whole-cell currents were performed in the perforated-patch configuration (Rae et al. 1991). In a few experiments (six cells; see Results), amphotericin was not included in the pipette and conventional whole-cell recordings were performed. Series resistance compensation was not used, due to its introduction of extra noise and due to the small amplitudes of the measured currents. All potential values given have been
compensated for the liquid-junction potential of -14 mV, measured as described by Neher (1992). All recordings were performed at room temperature (21 - 23 °C).

An Axopatch 200A amplifier, a Digidata 1200 A/D, D/A-converter and the pCLAMP software (versions 7.0 to 9.0; electronic equipment as well as software from Axon Instruments, Foster City, CA, USA) were used for computerised electrophysiological recordings and analysis.

Solutions and chemicals

The incubation solution, used during the preparational procedures, contained (in mM): 150 NaCl, 5.0 KCl, 2.0 CaCl₂, 10 HEPES, 10 glucose, 4.9 Tris-base. pH was 7.5. The intracellular solution, used for filling pipettes contained in mM: 140 K-gluconate, 3.0 NaCl, 1.2 MgCl₂, 1.0 EGTA, 10 HEPES. The pH was adjusted to 7.2 with 1 - 2 M KOH. The standard extracellular solution contained (in mM): 137 NaCl, 5.0 KCl, 1.0 CaCl₂, 10 HEPES, 10 glucose. Glycine (3.0 μM) was added routinely, and pH was adjusted to 7.4 with 1 - 2 M NaOH. In some cases, an extracellular solution with a K⁺ concentration raised to 35, 71, or 105 mM was used. In those cases, the Na⁺ concentration was reduced to 107, 71, and 37 mM respectively, to keep iso-osmolarity, and pH was adjusted to 7.4 by using a mixture (1/1) of 2.0 M NaOH and 2.0 M KOH. Picrotoxin (100 μM) and MK-801 (2.0 μM) were added to the extracellular solutions in a majority of experiments, to prevent currents through GABA_A-receptor channels and NMDA-receptor channels. Basic chemicals were purchased either from Sigma-Aldrich, St Louis, MO, USA or from a local supplier at Umeå University. ATP, bicuculline
methiodide (BMI), carbamylcholine, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), (5R,10S)-(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX), nimodipine, picrotoxin, ryanodine, serotonin and *Streptomyces* amphotericin B were purchased from Sigma-Aldrich. Caffeine was purchased from Research Biochemicals International, Natick, MA, USA, apamin and scyllatoxin from Latoxan, Valence, France, and xestospongin C from Cayman Chemicals, Ann Arbor, MI, USA. *Streptomyces* amphotericin B was added to the intracellular solution used for back-filling of pipettes (see above) except for those used for conventional whole-cell recording. A stock solution of 18 mg amphotericin B dissolved in 30 μl dimethylsulphoxide (DMSO) was used for dilution in pre-filtered (Millipore; pore size 0.22 μm) intracellular solution to a final amphotericin B concentration of 7.8 mM. The final Amphotericin B-containing intracellular solution was used within 12 h. Substances with specific effects on different ion channels or receptors were added to the extracellular solution as described in the Results section. Ryanodine was dissolved in ethanol before being added to the test solution, giving a final ethanol concentration of 0.015 - 0.03 % (v/v). Nimodipine and, in some experiments, ryanodine were prepared from a stock solution (10 mM in DMSO) and xestospongin C from a stock solution (100 μM in DMSO). In those cases, all solutions were complemented with the same final concentration of DMSO. The effects of ethanol and DMSO per se were tested in separate experiments. No effect at the relevant concentrations was noted.
Analysis

The equilibrium potentials ($E$) for potassium ($E_K$) and chloride ($E_{Cl}$) were calculated from the Nernst equation:

$$E = \frac{(R \times T)}{(z \times F)} \times \ln \left( \frac{[C]_o}{[C]_i} \right)$$

(1)

where $R$ is the gas constant, $T$ the temperature, $z$ the ionic valence and $F$ the Faraday constant. $[C]_o$ and $[C]_i$ are the ion (K$^+$ or Cl$^-$) concentrations on extracellular and intracellular sides, respectively.

The K$^+$ conductance ($G_K$) at the peak of the current ($I_{peak}$) was calculated from:

$$G_K = \frac{I_{peak}}{(V - E_K)}$$

(2)

where $V$ is the membrane potential and $E_K$ the equilibrium potential for K$^+$. The coefficient of variation ($C_V$) for SMOC inter-event intervals was calculated by:

$$C_V = \frac{\text{S.D.}}{\text{mean}}$$

(3)

where mean and S.D. are the mean and the standard deviation of inter-event intervals.

The index of dispersion (ID) of the SMOC rate at different bin widths was calculated by:

$$ID = \frac{\text{S.D.}^2}{\text{mean}}$$

(4)
where S.D. and mean for the number of SMOCs in each bin (time interval) was used. The ID is distinct from the $C_V$ in that a random pattern would be expected to have an ID of 1, but with a more clustered pattern ID would be > 1 and with a more regular pattern < 1 (Bull et al. 2006; cf Brown et al. 2000; Leng et al. 2004).

The experimental results are presented as mean ± S.E.M. from a number (n) of cells. Statistical significance was evaluated by using the Wilcoxon matched-pairs signed-ranks test or Wilcoxon two-sample test, as appropriate, with $p < 0.05$ chosen to imply significance.

RESULTS

Characterization of the slow spontaneous currents

Several types of spontaneous currents may be recorded from mechanically isolated MPN neurons under voltage-clamp conditions. Functional presynaptic nerve terminals adhere to the dissociated neurons and release transmitter that give rise to spontaneous postsynaptic currents. The currents due to GABA release and GABA$_A$-receptor activation have been characterized in some detail (Haage et al. 1998; Haage and Johansson 1999; Druzin et al. 2002). Similarly, spontaneous postsynaptic currents due to AMPA- and NMDA-receptor activation have been observed (G. Klement and S. Johansson, unpublished observations). In the present work, we describe another type of spontaneous current, corresponding to the SMOCs described for some other
preparations (see Introduction). As described in detail below, in MPN neurons these
currents differ from the spontaneous postsynaptic currents by showing a considerably
slower time course, deviating pharmacological features and a different charge carrier.
Similar currents were observed in acutely dissociated MPN neurons and in MPN
neurons in slice preparations (see Methods). However, in the slice preparation the
currents were more difficult to detect due to background current fluctuations. With
standard extracellular and pipette-filling solutions and at a potential of -54 mV, such
currents were clearly detected in only 6 of 61 (9.8 %) cells in intact slices. The
frequency of currents, analyzed from four cells with relatively low background noise,
was 0.58 ± 0.01 Hz (in the remarkably narrow range 0.55 – 0.62 Hz) and the decay time
constant 110 ± 12 ms. Although the mean decay time constant was faster for the four
cells in slices, these values are within the ranges observed also for dissociated cells (see
below). Since the acutely dissociated cell preparation provided superior accessibility for
pharmacological substances and less interference by other current types, it was used for
the main part of the analysis.
A first analysis of the slow spontaneous currents (Fig. 1A-B) recorded from
dissociated cells in standard extracellular solution (see Methods) showed that they were
more easily detected in a limited potential range, between ~ -70 to -30 mV. Thus, in six
studied neurons, such currents were observed in all six cells at -44 mV, but in none at -4
mV. Further, measurements of the reversal potential were difficult due to instability of
cells at potentials negative to ~ -90 mV, but from five cells, a reversal potential of -79 ±
4 mV (n = 5) was obtained.
The reversal potential suggested a possible underlying conductance to either K⁺ or
Cl⁻. (Eₖ and Eₖ were calculated from Eq. 1 to -85 mV and -83 mV, respectively.) To
clarify the selectivity, we recorded corresponding currents in three different external K⁺ concentrations ([K⁺]₀; 5.0, 35 and 71 mM, see Methods). When [K⁺]₀ was 71 mM, the amplitudes were well described by a Gaussian distribution (Fig. 2A, B) and in cells where currents were detected over a wide range of membrane potential, the amplitudes showed a roughly linear relation to potential (Fig. 2C). The reversal potential versus [K⁺]₀ curve was well described by the Nernst equation for K⁺ (Eq. 1) (Fig. 2D), suggesting that the slow spontaneous currents were carried by K⁺. The lack of effect of the GABA_A-receptor blocker picrotoxin (100 μM; n = 4; not shown) was also consistent with this idea. Since a [K⁺]₀ of 71 mM increased the driving force at negative potentials (-84 to -74 mV) where background noise is small, it facilitated the general characterization of the currents and was used in the following description unless otherwise stated. Except for the altered amplitude and direction, the features of the currents were intact in 71 mM [K⁺]₀ (See below.) Under these conditions, the slow
FIG. 2. Current amplitudes – dependence on potential and \([K^+]_o\). A: Example currents recorded with \([K^+]_o = 71\) mM at the potentials indicated. B: Amplitude distribution for slow currents from 23 neurons, recorded with \([K^+]_o = 71\) mM at -84 mV. The smooth curve is a fitted Gaussian with peak at -57 pA and a width of 38 pA at half-maximal amplitude. C: Current amplitude versus membrane potential in 71 mM \([K^+]_o\). (Mean amplitudes from 7 cells.) D: Reversal potentials of the slow spontaneous currents, recorded with \([K^+]_o = 5\) mM, 35 mM and 71 mM from the indicated number (n) of cells. The straight line indicates the expected relation for a perfect selectivity to K\(^+\) (calculated from Eq. 1).

Spontaneous currents, now inwardly directed at -84 mV, were observed in 43 out of 104 studied cells (41 %). In 23 cells analysed in detail, the currents showed a fast rising phase, with a 10 – 90 % rise time of 38 ± 2 ms, followed by a slower, roughly exponential decay, with a time constant of 217 ± 11 ms. The peak K\(^+\) conductance at -84 mV, calculated from Eq. 2, was 782 ± 55 pS (n = 23).

While the experiments described above were obtained with the amphotericin B-perforated patch technique, we attempted to record similar currents also with
conventional whole-cell recording (with 1.0 mM EGTA and nominally no Ca\(^{2+}\) in the pipette; see Methods). However, in none of the six cells studied in the conventional whole-cell configuration did we observe slow spontaneous currents of the type described above. The probability of obtaining this outcome by chance would be < 5 % (4.07 %) for a neuronal population with the above reported fraction (~ 41 %) of cells capable of generating such currents. This suggests that the observed currents depend on the relatively preserved intracellular environment provided by the perforated-patch technique.

The frequency of slow spontaneous currents at -84 mV was 0.44 ± 0.03 Hz. At -44 mV, where such currents were seen with either \([K^+]_o = 5\) mM or \([K^+]_o = 71\) mM, the frequency did not depend on \([K^+]_o\) (0.64 ± 0.12 Hz, \(n = 6\), for \([K^+]_o = 5\) mM and 0.65 ± 0.09 Hz, \(n = 5\), for \([K^+]_o = 71\) mM). In the high-[K\(^+\)] solution, as in standard solutions, the slow currents were often not seen at potentials more positive than -30 mV. However, their presence at such potentials in a few cells enabled a characterization of the potential dependence of the frequency. Only cells that showed such currents over a potential range of at least 30 mV were used and only potentials where slow currents were clearly detectable were included. To be able to cover a wider potential range, data obtained with \([K^+]_o = 5.0\) mM and \([K^+]_o = 71\) mM were pooled. The analysis indicated some potential dependence: The frequency increased with potential up to -44 mV (Fig. 3A).

Since earlier reports have suggested that SMOCs are generated at random intervals (Satin and Adams 1987; Cui et al. 2004), but our initial impression was that the slow spontaneous currents often were generated at relatively regular intervals (see e.g. Fig. 5B, below), suggesting a non-random mechanism of generation, we
FIG. 3. Frequency and regularity of slow spontaneous currents. A: Potential-dependence of slow spontaneous current frequency. Pooled data from recordings with \([K^+]_o = 5 \text{ mM}\) and \([K^+]_o = 71 \text{ mM}\). Only potentials where slow currents were clearly detectable were included. Note that frequency increases with potential up to -44 mV. Data from 3–11 neurons. Note that current traces at potentials without slow spontaneous currents were excluded from the above analysis. The justification for this is the “masking” of slow spontaneous currents at relatively positive potentials, described in the main text. B: ID of slow spontaneous current occurrence plotted against the bin width. (One-minute traces at -84 mV; pooled data from 17 neurons; \([K^+]_o = 71 \text{ mM}\).) Note that ID < 1 and varies with bin width.

characterized the degree of regularity. In 17 neurons studied over 1-minute intervals, the coefficient of variation \((C_V)\) for inter-event intervals was 0.68 ± 0.05, thus lower than expected for exponentially distributed intervals where a \(C_V\) closer to 1 is expected. A mechanism that was more regular than a random process was further supported by plotting the index of dispersion (ID) of frequency against the bin width (Fig. 3B), showing that the ID depends on the bin width and is < 1. For a random (Poisson) process, an ID of 1 that does not vary with bin width is expected (Brown et al. 2000; Leng et al. 2004; Bull et al. 2006).
The slow spontaneous currents are mediated by SK3 channels

The close similarity between the $E_K - [K^+]_o$ relation and the reversal potential - $[K^+]_o$ relation (see above) implied that the slow spontaneous currents were carried by $K^+$. This was further supported by experiments with $\text{Ba}^{2+}$, a relatively non-selective $K^+$-channel blocker (Århem 1980; Armstrong and Taylor 1980; Elinder and Århem 2003). 5.0 mM $\text{Ba}^{2+}$ reversibly reduced the current amplitude to $44 \pm 8\%$ (range $0.16 - 0.70\%$; $n = 8$; $p = 0.0078$) of control, while the frequency was nearly unaffected (Fig. 4A).

Assuming a first order reversible reaction between $\text{Ba}^{2+}$ and the channel causing the spontaneous slow currents, we obtained an IC$_{50}$ value of 4.5 mM. Notably, SK channels in other preparations show a similar sensitivity to $\text{Ba}^{2+}$ (Hanselmann and Grissmer 1996; Wittekindt et al. 2004). Since the present currents were regularly recorded under voltage-clamp conditions at -84 mV, a major contribution of voltage-dependent $K_V$ or $K_{Ca}$ (BK; $K_{Ca1}$) channels did not seem likely under these experimental conditions.

The slow spontaneous currents in MPN neurons are in many respects similar to spontaneous miniature outward currents (SMOCs) described for other preparations (e.g. they are carried by $K^+$ and show a similar time course). The SMOCs are carried through BK or/and SK channels (Merriam et al. 1999; Arima et al. 2001). Since voltage-dependent $K_{Ca}$ (BK) channels or $K_V$ channels did not seem likely, we investigated the effects of different specific SK channel blockers. Bicuculline methiodide (BMI) has previously been shown to block SK channels in MPN neurons, with an IC$_{50}$ value of 12 μM (Johansson et al. 2001). In the present investigation, 200 μM BMI reversibly reduced the amplitude of the slow spontaneous currents by $94 \pm 4\%$ ($n = 3$) of the control value (Fig. 4B), thus in agreement with the effects on SK channels.
FIG. 4. Pharmacology of the slow spontaneous currents. A: Reversible reduction by 5.0 mM Ba\textsuperscript{2+}. B: Reversible block by 200 μM BMI. C: Block by 5.0 nM apamin. D: Reduction by 10 nM scyllatoxin. All substances were added to the external solution for the indicated time intervals. [K\textsuperscript{+}]\textsubscript{o} = 71 mM, V = -84 mV (in A – D). Insets in C and D show part of the traces in the absence (left) and presence (right) of blocker at an expanded time scale.

To determine which SK channel subtype is the major cause of the current events, we used the arthropod toxins apamin and scyllatoxin, known to differentially affect subtypes of SK channels. Apamin gradually blocked the amplitude of the spontaneous currents. At steady-state, reached after ~ 4 minutes, 0.5 nM apamin reduced the current amplitudes by 23 ± 9 % (n = 3), whereas 5.0 nM apamin blocked the currents completely (n = 5) (Fig. 4C). Scyllatoxin was also found to slowly block the spontaneous currents. Within 2 minutes, 10 nM scyllatoxin induced a steady-state
reduction by 45 ± 5 % (n = 4) (Fig. 4D). Lower concentrations of apamin (0.1 nM) and scyllatoxin (1.0 nM), reported to affect SK2 channels (Strobaek et al. 2000), did not significantly affect the current amplitudes when tested in 4 and 2 cells, respectively. Comparing these results with the pharmacological profiles of the SK subtypes (- the used toxin concentrations do not affect SK1 channels; Vergara et al. 1998, Pedarzani et al. 2000; Strobaek et al. 2000 -), we conclude that SK3 (KCa2.3) channels are most likely mediating the spontaneous currents.

A possible role of voltage-gated Ca\(^{2+}\) channels in regulating spontaneous slow miniature currents?

The above findings suggest that cytoplasmic Ca\(^{2+}\) triggers the slow spontaneous currents. Since the currents were recorded mainly at -84 mV, where steady Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels is small, we did not expect that such channels were involved in the triggering process. Nevertheless, since Ca\(^{2+}\) currents through voltage-gated channels have been shown to contribute to the SMOC activity in peripheral and central neurons (Merriam et al. 1999; Mitra and Slaughter 2002; Cui et al. 2004), we applied Cd\(^{2+}\), which blocks high-threshold Ca\(^{2+}\) channels (L, N or P/Q type) in several types of neuron (Fox et al. 1987; Ellinor et al. 1993; Sather et al. 1993) including MPN neurons (Sundgren-Andersson and Johansson 1998) and also, with lower affinity, blocks low-threshold Ca\(^{2+}\) channels (T-type) (Huguenard 1996). We found no effect of 200 µM Cd\(^{2+}\) on the frequency or on the amplitude of the spontaneous slow currents (n = 3) recorded at -84 mV.
We have previously shown that BMI-sensitive SK channels in MPN neurons contribute to a steady $K^+$ current at membrane potentials $> \sim -50$ mV (Johansson et al. 2001). This is most likely a consequence of the steady $Ca^{2+}$ influx through high-threshold $Ca^{2+}$ channels in these neurons (Sundgren-Andersson and Johansson 1998). We therefore hypothesized that steady SK-channel activation and possible saturation may mask the spontaneous slow currents at more positive potentials, and that this may be the reason that such currents were usually not seen at potentials more positive than $\sim -30$ mV. If this hypothesis is correct, unmasking of slow spontaneous currents at more positive potentials should be possible by reducing or blocking the $Ca^{2+}$ influx. To test this hypothesis, we studied neurons that did not generate clear slow spontaneous currents at -4 mV in standard extracellular solution (with $[K^+]_o = 5$ mM). We subsequently applied blockers of voltage-gated $Ca^{2+}$ channels or reduced the free extracellular $Ca^{2+}$ concentration, $[Ca^{2+}]_o$, by applying $Ca^{2+}$ buffers or replacing $Ca^{2+}$ with $Co^{2+}$. This frequently caused a reduction in baseline current accompanied by the generation of slow spontaneous currents. Thus, substituting $Co^{2+}$ for $Ca^{2+}$ revealed currents in 47 % of neurons (9 of 19) tested (Fig. 5A), EGTA (2.3 mM, expected to give a free $[Ca^{2+}]_o$ of $\sim$100 nM) revealed currents in 55 % of neurons (12 of 22) (Fig. 5B), 200 $\mu$M $Cd^{2+}$ revealed spontaneous currents in 61 % of neurons (11 of 18) (Fig. 5C), and the L-type-channel blocker nimodipine (100 $\mu$M) revealed currents in 19 % of neurons (3 of 16) (Fig. 5D). Overall, 69 % of the neurons (18 of 26) tested in one or several of these conditions with the common property of reducing $Ca^{2+}$ influx, responded by generating slow spontaneous currents.

To clarify whether reducing $Ca^{2+}$ influx unmasked the slow spontaneous currents, rather than trigger their generation, we also applied EGTA at -44 mV, when slow
FIG. 5. Unmasking of slow spontaneous currents in conditions with reduced Ca\textsuperscript{2+} influx. Example traces with application of different external solutions expected to reduce Ca\textsuperscript{2+} influx. At the start of the traces, the cells were exposed to standard extracellular solution ([K\textsuperscript{+}]\textsubscript{o} = 5.0 mM) and clamped at -4 mV, with no clear slow spontaneous currents. Some faster current events seen have the characteristics of GABA\textsubscript{A}-receptor-mediated mIPSCs. (Picrotoxin was not used in these experiments.) A: Exposure to Ca\textsuperscript{2+}-free, Co\textsuperscript{2+}-containing solution. B: Addition of 2.3 mM EGTA. C: Addition of 200 μM Cd\textsuperscript{2+}. D: Addition of 100 μM nimodipine. E: The effect of EGTA on slow spontaneous currents varies with potential. EGTA (2.3 mM) was added at -4 mV and at -44 mV as indicated. Current scale is absolute, but time periods at steady-state conditions shown as adjacent for facilitation of comparison and to avoid artifacts at the solution exchange. Note that at -4 mV, clear slow currents are not detectable, whereas they are easily detected upon addition of EGTA. On the contrary, at -44 mV, slow currents are clearly visible in control solution and the frequency is reduced upon addition of EGTA. Note also the large reduction in baseline current upon addition of EGTA at -4 mV.

Currents were already present in standard extracellular solution and no significant masking K\textsuperscript{+} current was expected. At this potential, EGTA caused a reduction (by 53 ±
16 \%, n = 5) in the frequency of slow spontaneous currents (Fig. 5E). This supports the conclusion that the slow spontaneous currents are not triggered by reducing Ca\(^{2+}\) influx, but rather unmasked by the reduction in steady SK channel activation at relatively positive membrane potentials.

Is a synaptic induction mechanism involved in the generation of the slow spontaneous currents?

Activation of SK channels may result as a consequence of Ca\(^{2+}\) influx through Ca\(^{2+}\)-permeable ligand-gated channels, such as nicotinic acetylcholine receptors or NMDA receptors, which may be activated synaptically (see e.g. Shah and Haylett 2002; Ngo-Anh et al. 2005; Faber et al. 2005; Kong et al. 2006). The dissociated neurons used in the present study retained attached functional synaptic terminals (Haage et al. 1998), and it therefore seemed possible that synaptically released ligands were involved in the generation of the slow spontaneous currents. To test this hypothesis, we applied antagonists and agonists to a number of common receptor types to the MPN neurons. Thus, we tested compounds affecting receptors to glutamate, acetylcholine, serotonin and ATP. The substances and the concentrations used are listed in Table 1. None of the substances tested did significantly affect the slow spontaneous currents (neither frequency nor amplitude nor time course of individual events), suggesting that none of the classical ligand-activated channels are involved in triggering or shaping these currents in MPN neurons.
TABLE 1. Tested substances without effect on slow spontaneous currents

<table>
<thead>
<tr>
<th>Channel/Receptor</th>
<th>Agonist*/antagonist**</th>
<th>Concentration</th>
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<tr>
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MK-801 is (5R,10S)-(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, CPP is (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, NBQX is 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium salt.

The spontaneous currents are triggered by release of Ca\(^{2+}\) via RyR channels

Previous studies of SMOCs in peripheral as well as in central neurons suggest that the SK or BK channels are activated by Ca\(^{2+}\) released from internal stores via RyRs (Merriam et al. 1999; Arima et al. 2001). This was confirmed also for the slow spontaneous currents in MPN neurons. Thus, 50 µM ryanodine, at this concentration a RyR blocker, completely blocked the slow spontaneous currents within 5 minutes (n = 5) (Fig. 6), whereas the IP\(_3\)R blocker xestospongin C (1.0 µM) did not significantly affect the current amplitudes when tested for a minimum of 5 minutes on 4 cells.

![FIG. 6. Slow spontaneous currents are blocked by ryanodine. Example trace showing that the slow currents are slowly blocked by addition of 50 µM ryanodine to the external solution, for the indicated interval. Insets show part of the traces in the absence (left) and presence (right) of ryanodine at an expanded time scale. [K\(^+\)]\(_o\) = 71 mM, V = −84 mV.](image)
Neither did 1.0 μM xestospongin C block the caffeine-evoked slow currents described below, when applied for 6 – 12 minutes to two cells.) In control experiments, slow spontaneous currents were readily detected without any significant change in amplitude during > 20 minutes.

Caffeine is known to potentiate Ca\(^{2+}\) release via RyRs (McPherson et al. 1991; Miller 1991), and was therefore predicted to affect the slow spontaneous currents. When the MPN neurons were superfused with caffeine in high-[K\(^+\)] solution and studied at a membrane potential of -84 mV, three different types of response were noted: First, in cells that did not show spontaneous slow currents under control conditions, 1.0 - 3.0 mM caffeine triggered such currents in 78% of the cases (21 out of 27 cells) (Fig. 7A).

Second, in cells that generated spontaneous slow currents in the absence of caffeine, 1.0 – 3.0 mM caffeine increased the frequency as well as the amplitude of such currents (Fig. 7B). Thus, in 3.0 mM caffeine, the current amplitude increased to 270 ± 30% of control (n = 7; p = 0.016) and the frequency increased to 650 ± 220% of control (n = 7; p = 0.016). Third, in 63% (24 out of 38) of the cells with spontaneous slow currents, 3.0 mM caffeine induced larger and more long-lasting continuous inward currents (Fig. 7C). The latter currents showed variable kinetics, sometimes with a substantial inactivation within 30 s. The peak current evoked by 3.0 mM caffeine was -138 ± 19 pA (n = 24) at -84 mV. In some cases, the caffeine-evoked current was relatively noisy and appeared to be the sum of many spontaneous slow current events. In other cases, the current was less noisy and was accompanied by extinction of the spontaneous slow currents preceding the caffeine application (Fig. 7C). The large continuous currents were carried by K\(^+\); the reversal potential being -19 ± 2 mV (n = 7) in high-[K\(^+\)] solution (71 mM) and thus close to the E\(_K\) (-17 mV). The caffeine-evoked
FIG. 7. Caffeine-evoked currents in high external $K^+$ concentration ([K$^+$]$_o$ = 71 mM, -84 mV). A: Slow spontaneous currents were reversibly induced by adding 3.0 mM caffeine to the external solution. B: Caffeine increased the frequency and amplitudes of slow currents. (Three traces from one neuron.) C: Caffeine-evoked large continuous current. D, E: Neither caffeine-evoked slow currents ($D$) nor caffeine-evoked large continuous currents ($E$) were blocked by 100 μM picrotoxin (PTX), but both current types were blocked by 100 μM BMI.
“spontaneous” currents (Fig. 7D) as well as the caffeine-evoked continuous currents (Fig. 7E) were blocked by 100 or 200 μM BMI: the amplitude of the peak current evoked by 3.0 mM caffeine was reduced 96 ± 2 % (n = 7) in the presence of 200 μM BMI. (In the presence of BMI, caffeine often evoked a small outward current, as can be seen in Fig. 7E, that was not further investigated in the present study.) In conclusion, the caffeine-induced large, long-lasting continuous currents had many properties in common with the spontaneous slow currents, suggesting a similar underlying mechanism. The effects of caffeine were in most cases reversible within 30 s.

While the above described effects of caffeine were recorded in the perforated-patch mode, caffeine (3.0 mM) was applied also to the cells studied with the conventional whole-cell technique (-84 mV, [K+]o = 71 mM; see Methods). However, none of these six cells showed any significant response to caffeine. In summary, the results from the experiments with ryanodine, xestospongin C and caffeine suggest that the spontaneous slow currents were triggered by Ca2+, released from intracellular stores via caffeine-sensitive RyRs.

Role of spontaneous currents in shaping membrane potential and impulse firing

The functional role of spontaneous slow miniature currents is poorly understood. Spontaneous hyperpolarizations, caused by currents similar to the present spontaneous slow currents, have been described for a number of preparations (Satin and Adams 1987; Cui et al. 2004; Arima et al. 2001), but their effect on impulse firing has only
been analysed for midbrain dopamine neurons from neonatal rats, where it was shown that they were involved in creating an irregular firing pattern (Cui et al. 2004).

To clarify the role of the spontaneous slow miniature currents in the MPN neurons under more physiological conditions, we performed current-clamp experiments in low $[\text{K}^+]_o$ (5.0 mM). We also added 100 $\mu$M picrotoxin to the external solution to avoid GABA$_A$-receptor-mediated hyperpolarizations. As demonstrated in Fig. 8A, we found neurons that showed repetitive hyperpolarizations, sometimes exceeding -10 mV, the mean frequency in three neurons being $0.39 \pm 0.04$ Hz, the amplitude $-5.7 \pm 1.4$ mV from a preceding/resting potential of $-51 \pm 2$ mV, the rise time (10 - 90 %) $114 \pm 19$ ms and the decay time constant $457 \pm 109$ ms. The mean frequency of the hyperpolarizations was close to the frequency of the slow current events recorded in voltage-clamp mode under corresponding conditions, thus supporting the notion that the hyperpolarizations reflect the same mechanisms that cause the spontaneous slow miniature currents. (A slower time course of hyperpolarizations in current-clamp conditions relative spontaneous currents in voltage-clamp conditions is expected from the capacitive properties of the membrane and from the gradual change in $V$ relative to $E_K$ under current-clamp conditions.) Similar hyperpolarizations were also triggered by 3.0 mM caffeine applied to neurons that did not generate hyperpolarizations spontaneously (Fig. 8B; n = 10). These hyperpolarizations were blocked by 100 $\mu$M BMI (n = 4), but resistant to 100 $\mu$M picrotoxin (Fig. 8C), which provides further evidence for their correspondence to the slow spontaneous currents. When such hyperpolarizations were triggered in spontaneously firing neurons, the impulse frequency was clearly reduced in 5 out of 6 neurons, (Fig. 8B). On average, the firing frequency during the first 30 s in caffeine was reduced to $39 \pm 16$ % (n = 6) of that the
FIG. 8. (For legend, see next page.)
FIG. 8. Spontaneous and caffeine-evoked hyperpolarizations. Membrane potential records under current-clamp conditions. A: Slow spontaneous hyperpolarizations. B: Application of 3.0 mM caffeine triggers hyperpolarizations and leads to cessation of impulse generation. C: Hyperpolarizations are resistant to picrotoxin (PTX), but sensitive to BMI. D: Transient, continuous hyperpolarization and block of impulse activity upon addition of 3.0 mM caffeine. E: Application of 50 μM ryanodine induces first reduced and subsequently increased firing frequency in a cell that generated slow spontaneous currents under voltage-clamp conditions. Lower traces show selected portions at an expanded time scale. A – E from five different MPN neurons, with [K⁺]o = 5.0 mM in all cases, and resting membrane potentials (or most stable “baseline” potential between impulses or hyperpolarizations) of -50, -57, -50, -60 and -72 mV, respectively in A - E.

Last 30 s before caffeine application. Further, in several cells (n = 10), 3.0 mM caffeine transiently reduced or completely abolished the spontaneous firing without giving rise to clear spontaneous hyperpolarizations (Fig. 8D). The continuous caffeine-evoked current observed under voltage-clamp conditions (see above) is likely mediating this effect.

We have previously reported the effects of blocking a steady SK channel-mediated conductance on impulse firing (Johansson et al. 2001). With the hope that blocking RyRs rather than SK channels would more selectively affect the repetitively activated conductance underlying the slow spontaneous currents, we here applied 50 μM ryanodine to spontaneously firing cells. Although ryanodine changed the firing frequency (e.g. inducing an initial decrease followed by an increase in firing frequency; Fig. 8E), such changes were seen in cells without as well as in cells with slow spontaneous currents under voltage-clamp conditions, and, in similarity with the previously reported effects of blocking a steady SK channel-mediated conductance (Johansson et al. 2001), the direction of change in firing frequency varied between cells. Since the observed effects were likely partly due to block of a steady conductance rather
than a selective block of the repetitive conductance underlying the slow spontaneous currents, we did not further quantify the effects of ryanodine on impulse firing.

DISCUSSION

In the present work, we have (i) demonstrated that rat MPN neurons show spontaneous slow miniature currents, similar to SMOCs in other preparations; (ii) presented evidence that the slow currents are caused by activation of \( K^+ \)-channels with a pharmacological profile similar to SK3 channels; (iii) demonstrated that the activation mainly depends on \( Ca^{2+} \) release via RyR receptors; (iv) shown that the currents may be masked by steady SK-channel activation when the membrane potential is above -30 mV and (v) shown corresponding hyperpolarizations that may exceed 10 mV in amplitude and reduce the rate of spontaneous firing under current-clamp conditions.

Mechanisms of slow spontaneous current generation

The mechanisms of slow spontaneous currents here described show many similarities, but also differences from the SMOCs or STOCs described in other preparations. Thus, in common with those in other reports, they are generated by the release of \( Ca^{2+} \), via RyRs, from caffeine-sensitive intracellular \( Ca^{2+} \) stores and the subsequent activation of \( K_{Ca} \) channels. However, in contrast to the STOCs in smooth muscle (Bolton and Imaizumi 1996) and the SMOCs in cardiac neurons (Merriam et al.
and bullfrog neurons (Satin and Adams 1987; Marrion and Adams 1992), the K+ channels involved are not BK channels but rather SK channels. This is, however, also the case for the SMOCs in Meynert neurons (Arima et al. 2001) and midbrain dopamine neurons (Cui et al. 2004), the only two other types of central mammalian neurons reported to generate SMOCs. The present report is, to our knowledge, the first to suggest the subtype of involved SK channel as SK3. This is in line with results from an in situ hybridization study, showing a moderate concentration of SK3, a low concentration of SK2 and a very low concentration of SK1 in the preoptic area (Stocker and Pedarzani 2000).

Another difference from some earlier reports is that Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels was not required for the slow spontaneous currents in the present study. The frequency was reduced when Ca\(^{2+}\) influx was blocked, but on the other hand, the slow spontaneous currents were clearly generated in nominally Ca\(^{2+}\)-free external solution as well as at potentials where neither low-threshold nor high-threshold Ca\(^{2+}\)-channels are activated in MPN neurons (Sundgren-Andersson and Johansson 1998). This is in contrast to the SMOCs in salamander retinal amacrine cells, where L-type Ca\(^{2+}\) channels were involved (Mitra and Slaughter 2002), in mudpuppy cardiac neurons, where mainly N-type Ca\(^{2+}\) channels were involved (Merriam et al. 1999), and in rat midbrain dopamine neurons, where T-type Ca\(^{2+}\) channels were involved in the generation of large SMOCs (Cui et al. 2004). Thus, we may conclude that among different types of neuron, even within the mammalian brain, the mechanisms underlying SMOC generation are heterogeneous.

Although the presynaptic terminals attach to the neurons studied, and although Arima et al. (2001) found SMOCs predominantly in preparations that spared the
synaptic terminals, we did not find any evidence for a synaptic contribution to the slow spontaneous miniature currents. Thus, the common types of Ca$^{2+}$-permeable AMPA receptors, NMDA receptors, nicotinic acetylcholine receptors, 5-HT$_3$ receptors or P2X receptors are not critically involved in SMOC generation in MPN neurons.

Masking explains apparent voltage dependence

A notable finding in several reports on SMOCs is that, in addition to the increase with membrane potential at the most negative potentials, the frequency of occurrence shows a dramatic voltage dependence, with a significant drop or complete absence of SMOCs at potentials more positive than -30 to -20 mV (see e.g. Merriam et al. 1999; Mitra and Slaughter 2002; Cui et al. 2004). This has been paradoxical since the driving force ($V - E_K$) increases with potential for physiological K$^+$ concentrations. The present work provides an explanation for this apparent voltage dependence by demonstrating that at more positive potentials the slow spontaneous currents are masked by steady activation of the K$_{Ca}$ channels. At the same time, a plausible explanation to why the slow currents/SMOCs are not masked in all preparations, or not even in all cells of a similar preparation, is provided: Masking is expected in cases where there is a sufficient steady influx of Ca$^{2+}$ to achieve steady activation of the K$_{Ca}$ channels. Thus “unmasking” may be achieved, as demonstrated in the present study, by reducing Ca$^{2+}$ influx. In cells with insufficient steady Ca$^{2+}$ influx, slow currents/SMOCs may be expected also at positive voltages, as occasionally seen in the present work. It seems likely that “unmasking” also explains the increasing effect of Ca$^{2+}$-free solutions on
SMOC amplitude in the study by Arima et al. (2001). This is what should be expected if there is some, but not maximal, steady activation of SK channels.

Notably, the unmasking here demonstrated also implies that the molecular machinery underlying the slow currents/SMOCs is active also at positive membrane potentials. It also implies that this machinery is active not in a small fraction, but in a majority of MPN neurons. (Unmasking was seen in ~ 70 % of tested neurons in the present study.) The most likely molecular interpretation is that the RyRs do release Ca\(^{2+}\) in a spark-like manner from the internal stores (cf. Imaizumi et al. 1999) more or less spontaneously under a variety of conditions in a majority of cells.

Regularity of slow current generation

SMOCs in previous studies have been described as stochastic phenomena, generated at random intervals (see e.g. Satin and Adams 1987; Cui et al. 2004). The present study implies that this may be different for MPN neurons. The occasional remarkable regularity (Fig. 5B), the overall ID < 1 and the variation of ID with bin width (Fig. 3B) all suggest (cf. Brown et al. 2000; Leng et al. 2004; Bull et al. 2006) that the slow spontaneous currents are not generated at random intervals. Therefore, it seems likely that some feedback mechanism controls the interval between the slow spontaneous currents. We speculate that the sensitivity of RyRs to cytoplasmic or luminal Ca\(^{2+}\) in combination with the Ca\(^{2+}\) concentration changes that result from RyR activation may provide the basis for such a feedback system. Plausible regulators of the interval may be the Ca\(^{2+}\) transport and buffer mechanisms that restore changes in Ca\(^{2+}\)
concentration in the vicinity of the RyRs. It is also clear from the present study, that the frequency can be modulated, e.g. by alterations in membrane voltage, Ca\textsuperscript{2+} influx or by caffeine, suggesting that physiological modulation of the 0.5-Hz signal is possible. In line with the speculations above, the effect of membrane voltage on the frequency of slow spontaneous currents may be due to altered Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} permeable channels in the cell membrane and consequent changes in the Ca\textsuperscript{2+} dynamics in vicinity of RyRs.

How ubiquitous are slow spontaneous currents/SMOCs in the CNS?

As noted above, slow spontaneous currents may be generated by a majority of MPN neurons. However, to our knowledge, there are only two previous reports on SMOCs from central mammalian neurons (Arima et al. 2001; Cui et al. 2004). The reason for the relative scarceness of reported SMOC generation in CNS neurons may originate from the experimental conditions. Several methodological procedures used in the present study may account for the relatively high incidence of neurons with such currents. The mechanical dissociation does not require enzymes and therefore may spare structures that are possibly damaged with more common enzymatic dissociation techniques. Further, the perforated-patch technique implies that wash-out of functionally important molecules from the cytoplasm was avoided, whereas conventional whole-cell recording may be expected to interfere with e.g. the fluctuations in [Ca\textsuperscript{2+}]. Neither in the present study nor in that by Arima et al. (2001) were slow spontaneous currents/SMOCs detected with conventional whole-cell
recording although such currents were present with perforated-patch recording. Arima et al. (2001) also reported SMOC generation mainly when non-enzymatic dissociation was used. On the other hand, Cui et al. (2004) detected SMOCs by whole-cell recordings in slice preparations. Possibly, this was a consequence of the very low concentration (25 μM) of EGTA used and thus a more freely fluctuating [Ca^{2+}]_i in the study by Cui et al. (2004). Further, cytoplasmic wash-out may be less complete in slices than in dissociated cells, due to better sparing of dendritic structures. The apparent sensitivity to wash-out by the commonly used conventional whole-cell technique and possibly also sensitivity to enzymatic treatment give reason to expect that, in spite of the limited number of reports, slow spontaneous currents/SMOCs may be a relatively ubiquitous phenomenon in central mammalian neurons, a phenomenon that may be revealed by proper methodological conditions.

**The functional role of spontaneous slow currents**

The functional role of SMOCs and of the postulated fluctuations in [Ca^{2+}]_i in MPN neurons remains elusive, as do the functions of Ca^{2+} sparks and SMOCs in other cell types. The various differences in the underlying machinery, such as involvement of BK or SK channels, requirement or not of Ca^{2+} influx through specific channel types etc., to achieve a similar phenomenon may be taken to suggest that this phenomenon does reflect a particular function. The STOCs described in smooth muscle have been suggested to contribute to the control of vascular tonus (Imaizumi et al. 1999), but for central neurons other functions must be considered. The voltage recordings in the
present study clearly revealed that the spontaneous fluctuations in membrane potential, corresponding to the SMOCs, were of amplitude sometimes > 10 mV, and thus could be larger and more long-lasting than evoked inhibitory postsynaptic potentials in MPN neurons (Malinina et al. 2005). Consistent with this, we also showed that the hyperpolarizing membrane potential fluctuations may clearly reduce the firing frequency. One previous study (Cui et al. 2004) has also reported effects on the impulse firing, with SMOCs contributing to the typical irregular firing of dopamine neurons from neonatal rats.

Remarkably, all studies of SMOCs in the mammalian CNS report a relatively similar average frequency, within the range ~ 0.2 Hz to 0.5 Hz (Arima et al. 2001; Cui et al. 2004; present study). Thus, one may speculate that the frequency near 0.5 Hz may be of particular significance for central neuronal function. Clearly, more information will be needed to elucidate this function.
ACKNOWLEDGEMENTS

The current address of G. Klement is: AstraZeneca R&D Södertälje, Local Discovery Research Area CNS & Pain Control, Molecular Pharmacology, SE-151 85 Södertälje, Sweden.

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FIGURE LEGENDS

FIG. 1. Slow spontaneous currents in standard extracellular solution. A: Slow spontaneous currents when \([K^+]_o = 5\) mM, at the potentials indicated. Note that current amplitudes decrease when potential is changed from -44 to -74 mV, but that the currents are not clearly seen at -34 mV. B: Slow spontaneous current at -44 mV \([K^+]_o = 5\) mM with superimposed fitted exponential of time constant 198 ms. (From another cell than used for A.)

FIG. 2. Current amplitudes – dependence on potential and \([K^+]_o\). A: Example currents recorded with \([K^+]_o = 71\) mM at the potentials indicated. B: Amplitude distribution for slow currents from 23 neurons, recorded with \([K^+]_o = 71\) mM at -84 mV. The smooth curve is a fitted Gaussian with peak at -57 pA and a width of 38 pA at half-maximal amplitude. C: Current amplitude versus membrane potential in 71 mM \([K^+]_o\). (Mean amplitudes from 7 cells.) D: Reversal potentials of the slow spontaneous currents, recorded with \([K^+]_o = 5\) mM, 35 mM and 71 mM from the indicated number (n) of cells. The straight line indicates the expected relation for a perfect selectivity to \(K^+\) (calculated from Eq. 1).

FIG. 3. Frequency and regularity of slow spontaneous currents. A: Potential-dependence of slow spontaneous current frequency. Pooled data from recordings with \([K^+]_o = 5\) mM and \([K^+]_o = 71\) mM. Only potentials where slow currents were clearly detectable were included. Note that frequency increases with potential up to -44 mV. Data from 3 – 11 neurons. Note that current traces at potentials without slow spontaneous currents were
excluded from the above analysis. The justification for this is the “masking” of slow spontaneous currents at relatively positive potentials, described in the main text. B: ID of slow spontaneous current occurrence plotted against the bin width. (One-minute traces at -84 mV; pooled data from 17 neurons; $[K^+]_o = 71$ mM.) Note that ID < 1 and varies with bin width.

FIG. 4. Pharmacology of the slow spontaneous currents. A: Reversible reduction by 5.0 mM Ba$^{2+}$. B: Reversible block by 200 μM BMI. C: Block by 5.0 nM apamin. D: Reduction by 10 nM scyllatoxin. All substances were added to the external solution for the indicated time intervals. $[K^+]_o = 71$ mM, $V = -84$ mV (in A – D). Insets in C and D show part of the traces in the absence (left) and presence (right) of blocker at an expanded time scale.

FIG. 5. Unmasking of slow spontaneous currents in conditions with reduced Ca$^{2+}$ influx. Example traces with application of different external solutions expected to reduce Ca$^{2+}$ influx. At the start of the traces, the cells were exposed to standard extracellular solution ($[K^+]_o = 5.0$ mM) and clamped at -4 mV, with no clear slow spontaneous currents. Some faster current events seen have the characteristics of GABA_A-receptor-mediated mIPSCs. (Picrotoxin was not used in these experiments.) A: Exposure to Ca$^{2+}$-free, Co$^{2+}$-containing solution. B: Addition of 2.3 mM EGTA. C: Addition of 200 μM Cd$^{2+}$. D: Addition of 100 μM nimodipine. E: The effect of EGTA on slow spontaneous currents varies with potential. EGTA (2.3 mM) was added at -4 mV and at -44 mV as indicated. Current scale is absolute, but time periods at steady-state conditions shown as adjacent for facilitation of comparison and to avoid artifacts at the solution exchange. Note that at -4 mV, clear slow currents are not detectable, whereas they are easily detected upon addition of EGTA. On the contrary, at -44 mV, slow currents are clearly visible.
in control solution and the frequency is reduced upon addition of EGTA. Note also the large reduction in baseline current upon addition of EGTA at -4 mV.

FIG. 6. Slow spontaneous currents are blocked by ryanodine. Example trace showing that the slow currents are slowly blocked by addition of 50 μM ryanodine to the external solution, for the indicated interval. Insets show part of the traces in the absence (left) and presence (right) of ryanodine at an expanded time scale. $[K^+]_o = 71$ mM, $V = -84$ mV.

FIG. 7. Caffeine-evoked currents in high external $K^+$ concentration ($[K^+]_o = 71$ mM, -84 mV).

A: Slow spontaneous currents were reversibly induced by adding 3.0 mM caffeine to the external solution. B: Caffeine increased the frequency and amplitudes of slow currents. (Three traces from one neuron.) C: Caffeine-evoked large continuous current. D, E: Neither caffeine-evoked slow currents ($D$) nor caffeine-evoked large continuous currents ($E$) were blocked by 100 μM picrotoxin (PTX), but both current types were blocked by 100 μM BMI.

FIG. 8. Spontaneous and caffeine-evoked hyperpolarizations. Membrane potential records under current-clamp conditions. A: Slow spontaneous hyperpolarizations. B: Application of 3.0 mM caffeine triggers hyperpolarizations and leads to cessation of impulse generation. C: Hyperpolarizations are resistant to picrotoxin (PTX), but sensitive to BMI. D: Transient, continuous hyperpolarization and block of impulse activity upon addition of 3.0 mM caffeine. E: Application of 50 μM ryanodine induces first reduced and subsequently increased firing frequency in a cell that generated slow spontaneous currents under voltage-clamp conditions. Lower traces show selected portions at an expanded time scale. $A – E$ from five different MPN neurons, with $[K^+]_o = 5.0$ mM in all cases, and resting membrane potentials (or most stable
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A

50 pA

-34 mV

-44 mV

-54 mV

-64 mV

-74 mV

-84 mV

2.0 s

B

5 pA

\( \tau = 198 \text{ ms} \)

500 ms
A. Ca\(^{2+}\) replaced by Co\(^{2+}\)

B. 2.3 mM EGTA

C. 200 \(\mu\)M Cd\(^{2+}\)

D. 100 \(\mu\)M nimodipine

E. -4 mV