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Ryoichi Teruyama  
*University of Tennessee*

William E. Armstrong  
*University of Tennessee*

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Teruyama, R., & Armstrong, W. (2007). Calcium-dependent fast depolarizing afterpotentials in vasopressin neurons in the rat supraoptic nucleus. *Journal of Neurophysiology, 98* (5), 2612-2621. [https://doi.org/10.1152/jn.00599.2007](https://doi.org/10.1152/jn.00599.2007)

This Article is brought to you for free and open access by the Department of Biological Sciences at LSU Scholarly Repository. It has been accepted for inclusion in Faculty Publications by an authorized administrator of LSU Scholarly Repository. For more information, please contact ir@lsu.edu.
Calcium-Dependent Fast Depolarizing Afterpotentials in Vasopressin Neurons in the Rat Supraoptic Nucleus

Ryoichi Teruyama and William E. Armstrong

Department of Anatomy and Neurobiology, University of Tennessee, Health Science Center, Tennessee

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Teruyama R, Armstrong WE. Calcium-dependent fast depolarizing afterpotentials in vasopressin neurons in the rat supraoptic nucleus. J Neurophysiol 98: 2612–2621, 2007. First published August 22, 2007; doi:10.1152/jn.00599.2007. Oxytocin (OT) and vasopressin (VP) synthesizing magnocellular cells (MNCs) in the supraoptic nucleus (SON) display distinct firing patterns during the physiological demands for these hormones. Depolarizing afterpotentials (DAPs) in these neurons are involved in controlling phasic bursting in VP neurons. Our whole cell recordings demonstrated a Cs+-resistant fast DAP (fDAP; decay tau = ~200 ms), which has not been previously reported, in addition to the well-known Cs+-sensitive slower DAP (sDAP; decay tau = ~2 s). Immuno-identification of recorded neurons revealed that all VP neurons, but only 20% of OT neurons, expressed the fDAP. The activation of the fDAP required influx of Ca2+ through voltage-gated Ca2+ channels as it was strongly suppressed in Ca2+-free extracellular solution or by bath application of Cd2+. Additionally, the current underlying the fDAP (fDAP) is a Ca2+-activated current rather than a Ca2+ current per se as it was abolished by strongly buffering intracellular Ca2+ with BAPTA. The I-V relationship of the fDAPs was linear at potentials less than ~60 mV but showed pronounced outward rectification near ~50 mV. I_{fDAP} is sensitive to changes in extracellular Na+ and K+ but not Cl−. A blocker of Ca2+-activated nonselective cation (CAN) currents, flufenamic acid, blocked the fDAP, suggesting the involvement of a CAN current in the generation of fDAP in VP neurons. We speculate that the two DAPs have different roles in generating after burst discharges and could play important roles in determining the distinct firing properties of VP neurons in the SON neurons.

INTRODUCTION

The secretion of neurohormones into the circulation largely depends on the pattern of neuronal activity of the synthesizing neurons. The neurohyophysial hormones vasopressin (VP) and oxytocin (OT) are synthesized in magnocellular cells (MNCs) within the supraoptic nuclei (SON), and these two neuron types show distinct firing patterns when physiological demands for their hormones are high. Preceding each milk ejection in lactating rats, the firing pattern in OT neurons changes dramatically, characterized by a short (2–4 s), high-frequency (≤80 Hz) burst of action potentials (Poulain and Wakerley 1982). In contrast, VP neurons respond to hyperosmolality (Brimble and Dyball 1977) and hypovolemia (Harris et al. 1975) by increasing their firing rate and adopting a phasic firing pattern comprising alternating periods of activity (7–15 Hz) and silence, each lasting tens of seconds. The release of VP is maximized by stimulation patterns mimicking phasic firing (Cazalis et al. 1985; Dutton, and Dyball 1979). The emergence of these firing patterns is therefore an important part of the response of MNCs during their hormonal demands.

The firing pattern of a neuron is generally a result of the interaction between synaptic and intrinsic membrane properties of the neuron. The depolarizing afterpotential (DAP) is an intrinsic membrane property of MNCs, originally observed after a single spike or a brief spike train in a subpopulation of MNCs (Andrew and Dudek 1983). Summation of DAPs induces a plateau potential that underlies the burst of action potentials in phasic neurons (Andrew and Dudek 1984a; Ghamari-Langroudi and Bourque 1998) and is found in most VP neurons and a minority of OT neurons (Armstrong et al. 1994; Smith and Armstrong 1993). However, DAPs may also play a role in the short bursting activity in OT neurons as their expression in these neurons is increased during pregnancy and lactation (Stern and Armstrong 1996; Teruyama and Armstrong 2002).

Although, there is strong agreement that DAPs in MNCs are triggered by Ca2+ influx during spikes (Andrew and Dudek 1984a; Greffrath et al. 1998; Li and Hatton 1997a; Smith and Armstrong 1993), their ionic basis is not fully understood. One study suggested that DAPs may result from the Ca2+-dependent reduction of a resting K+ conductance (Li and Hatton 1997b). In that study, DAPs were attenuated by tetrodotoxin (TTX) or tetraethyl ammonium (TEA) but were relatively insensitive to external Cs+. In other studies, DAPs were not blocked by TTX (Andrew 1987) or TEA (Greffrath et al. 1998), but they were blocked by external Cs+ (Ghamari-Langroudi and Bourque 1998). More recent work suggested the involvement of a Ca2+-activated nonselective cation (CAN) channel because the CAN channel blocker, flufenamic acid (FFA), reversibly inhibited DAPs and phasic firing in MNCs (Ghamari-Langroudi and Bourque 2002). These disparate results may imply that multiple currents underlie DAPs. Recently, when blocking the apamin-sensitive medium AHP (mAHP), we unmasked a DAP that was faster than that typically described in MNCs. The present study was conducted to investigate the properties of this fast DAP in MNCs and has previously been published in abstract from (Teruyama and Armstrong 2005b, 2006).

METHODS

Animals and slice preparation

Brain slices containing the SON were prepared from random cycling, virgin female adult rats (180–210 g body wt; Sprague-
Dawley, Harlan Laboratories, Indianapolis, IN). The rats were deeply
anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused
through the heart with a sucrose solution (an artificial cerebrospinal
fluid (ACSF) solution (see following text) in which NaCl was re-
placed by an equiosmolar amount of sucrose). The brains were
removed and sliced in the coronal plane at a thickness of 250 μm in
the ice-cold sucrose solution. Slices were maintained in ACSF, which
was bubbled continuously with 95% O₂-5% CO₂, containing (in mM)
124 NaCl, 3 KCl, 2.0 CaCl₂, 1.3 MgCl₂, 1.24 NaH₂PO₄, 25 NaHCO₃,
0.2 ascorbic acid, and 10 d-glucose (pH 7.4). Slices were stored at
room temperature prior to recording. Animal procedures were per-
formed under protocols approved by the Institutional Animal Care and
Use Committee at University of Tennessee.

Electrophysiology

Whole cell patch-clamp recordings were obtained with an Axon
200B amplifier (Axon Instruments, Foster City, CA). Traces were
acquired digitally at 20 kHz and filtered at 5 kHz with a Digidata 1320A
(Axon Instruments) in conjunction with pClamp 9 software
(Axon Instruments). Axograph 4.9 (Axon Instruments) was used to
analyze the recordings. The current-voltage relationships were ana-
yzed using Igor Pro Carbon 4.07 (WaveMetrics, Lake Oswego, OR).
Averaged data are presented as the means ± SE, where n is the
number of cells. Statistically significant difference between means
was set to P < 0.05, using paired Student’s t-test, unless otherwise
stated.

For analyzing the tail currents, K- and Cs-glucuronate pipette solu-
tions were used. The K-glucuronate pipette solution consisted of (in
mM) 135 K-glucuronate, 2 MgCl₂, 10 HEPES, 10 phosphocreatine,
10 myo-inositol (no phosphate), 0.1 EGTA, 0.4 GTP (Na), and 2 ATP
(Mg). The pipette solutions were adjusted to a pH of 7.3 with KOH.
In some experiments, bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic
acid (BAPTA, 10 mM) was added to this pipette solution. The Cs-
glucuronate pipette solution consisted of (in mM) 100 CsOH, 10
glucuronic acid, 10 HEPES, 2 MgCl₂; 2 ATP (Mg); 0.4 GTP (Na),
10 phosphocreatine, 10 myo-inositol (no phosphate), and 0.1 EGTA.
The pipette solutions were adjusted to a pH of 7.3 with HCl. Phosphocre-
atine was included because “rundown” of Ca²⁺ currents was effect-
ively reduced by such inclusion to allow regeneration of ATP
(Foehring and Armstrong 1996). Myo-inositol was added to prevent
possible rundown of the fDAP because phosphatydilinositol
4,5-bisphosphate plays a central role in the activation of several CAN
channels (Rohacs et al. 2005). Both pipette solutions contained 0.2%
biocytin (Sigma, St. Louis, MO) to identify the patched cell (see
Immunocytochemistry). Patch electrodes were drawn from borosili-
cate glass tubing (G150T-3, Warner Instruments, Hamden, CT) to have resistances of 4 – 8 MΩ. Averaged data were not corrected for LJPs. To isolate the current under-
lying the fast DAP, Ca²⁺-sensitive and -resistant DAPs are both present in MNCs

Prominent DAPs were observed following a train of action potentials in a subpopulation of MNCs. Among those cells expressing DAPs, repetitive single action potentials evoked by intracellular current injections (20 × 5 ms depolarizing pulses, 100–250 pA, 20 Hz) generated a DAP after the AHPs (Fig. 2A). The AHP generated with this protocol appeared to be mostly the medium AHP (mAHP) from its decay time course (~500 ms). The current underlying the mAHP is mediated by the small-conductance Ca²⁺-activated K⁺ channels (SK channels) in MNCs because it is blocked by bee venom apamin, a known blocker of SK channels (Armstrong et al. 1994; Bourque and Brown 1987; Greffrath et al. 1998; Kirkpatrick and Bourque 1996; Teruyama and Armstrong 2005a). As expected, bath application of apamin (100 nM) strongly suppressed the

Ca²⁺-sensitive and -resistant DAPs are both present in MNCs

FIG. 1. Immunocytochemical identification of cell types in magnocellular
neurons (MCNs). The patched neuron was filled with biocytin and visualized by
7-aminomethylcoumarin (3-acetic acid (avidin-AMCA; Vector Labs, Burlington, CA) 4–6 h at room

temperature. The secondary antibodies used were fluorescein isothiocya-
nate (FITC)-conjugated goat anti-rabbit (Vector Labs) and Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR). Avidin-AMCA was used to visualize the recorded cells. Neurons were considered as either OT or VP types only if positive staining of one
antibody was complemented by a negative reaction with the other

Patched Neuron  

VP-NP ir-Neurons  

OT-NP ir-Neurons

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FIG. 2. Cs⁺-sensitive and -resistant depolarizing afterpotentials (DAPs) in MNCs. Afterpotentials in a MNC were generated by a train of action potentials evoked by intracellular current injections (20 × 5 ms depolarizing pulses, 100–250 pA, 20 Hz). A: in artificial cerebrospinal fluid (ACSF), the train of action potentials was followed by a distinct medium afterhyperpolarization (mAHP) that was subsequently followed by the slow DAP (sDAP). B: bath application of apamin (100 nM) completely blocked the mAHP and unmasked the presence of the fast DAP (fDAP), which was followed by the sDAP. C: additional application of Cs⁺ (5 mM) blocked the sDAP that revealed the sAHP. D: superimposed traces of A–C illustrate the temporally overlapping, multiple afterpotentials. E: expanded portion of the trace in C (indicated by underline) revealed that the onset of the fDAP occurred after the 1st action potential and its amplitude increased with each subsequent action potential until a plateau was reached after 12 spikes. F: inward tail current thought to be underlying the DAPs (I_{fDAP}) was generated by 50 ms steps to 0 mV from a holding potential of −60 mV. Tail currents with similar time courses as the fast and slow DAPs were obtained and the application of 5 mM Cs⁺ blocked only I_{fDAP}.

mAHP, enhanced the DAP, and shifted its peak to the left (Fig. 2B). It has been known that Cs⁺ blocks the DAP in MNCs (Ghamari-Langroudi and Bourque 1998). However, in the presence of apamin, bath application of Cs⁺ (5 mM) blocked only a slower part of the DAP and not the peak (Fig. 2C). Inhibition of the slow part of the DAP with Cs⁺, in turn, revealed the presence of the slow aHP (sAHP) described previously in MNCs (Ghamari-Langroudi and Bourque 2004; Greffrath et al. 1998; Teruyama and Armstrong 2005a). The time course of the faster part of the DAPs could not be observed easily with this protocol unless the mAHP and the slower part of the DAPs were both suppressed. Because the time course of the Cs⁺-resistant DAP is faster than the Cs⁺-sensitive DAP, we refer to them as fast DAP (fDAP) and slow DAP (sDAP), respectively. The superimposed images from Fig. 2, A–C (Fig. 2D) illustrate the presence of multiple, temporally overlapping afterpotentials in the MNC. It is clear that time courses of the mAHP and sAHP overlap considerably with those of the fDAP and sDAP, respectively.

The fDAP showed strong activity dependence. More detailed observation in the expanded portion of the trace during repetitive spike activation (Fig. 2E) revealed that the onset of the fDAP was seen after the first action potential and its amplitude continued to increase with each subsequent action potential until a plateau was reached after ~15 spikes.

The inward tail currents thought to underlie the DAP (I_{fDAP}) were generated by 50 ms steps to 0 mV from the holding potential of −60 mV in the presence of apamin (100 nM) in the bathing solution (Fig. 2F). Tail currents with time courses similar to the fDAP and sDAP were obtained. Bath application of 5 mM Cs⁺ blocked only the slow part of the I_{fDAP}, similar to its effect on the sDAP in current clamp (Fig. 2F). The time course of the I_{fDAP} was fitted with a single-exponential function with a time constant of decay of 283.0 ± 12.7 ms (n = 29) for a pulse duration of 50 ms at a holding potential of −60 mV.

Because the fDAP showed strong activity dependence, the dependence of the I_{fDAP} on the duration of depolarizing steps was evaluated in voltage clamp. These steps would allow a progressive increase in [Ca²⁺]. This protocol was chosen over mimicking spikes in current clamp because it better isolated the fDAP from the sAHP, which increases with spike number during a train, because it provides a better space clamp than transient depolarizations that would be heavily filtered, and because the activity dependence in current clamp is heavily dependent on spike frequency. The amplitude of I_{fDAP} increased with the duration of the stimulus, and the maximum amplitude I_{fDAP} reached with the pulse duration of 150 ms (Fig. 3A; n = 5). The relationship between peak I_{fDAP} amplitude and pulse duration was fitted with a single-exponential function with a time constant of ~100 ms (n = 4; Fig. 3B).

Under our recording conditions, fDAPs were seen in the vast majority of VP neurons. Only 1 in 69 immunolabeled VP neurons failed to express fDAP. In contrast, only 13 of 65 (20%) immunolabeled OT neurons expressed the fDAP. To study the fDAP and its underlying current, we analyzed the recordings exclusively from the immunolabeled VP neurons. To isolate the fDAP, experiments were conducted in the presence of apamin (100 nM) and Cs⁺ (5 mM) unless otherwise stated.

Ca²⁺ dependence of the fDAP

Although the mechanisms underlying the DAP in MNCs are controversial, previous studies agree they are Ca²⁺ dependent (Andrew and Dudek 1984b; Ghamari-Langroudi and Bourque 1998; Li and Hatton 1997a). Ca²⁺ influx appeared to be an
FIG. 3. Dependence of the \( I_{fDAP} \) on the duration of the depolarizing pulses. A: amplitude of the \( I_{fDAP} \) increased with stimulus duration. B: relationship between amplitude of the peak current and pulse duration could be fitted with a single-exponential function. The amplitude reached a maximum value at 150 ms of duration (\( n = 4 \)).

important determinant for the activation of the fDAP as well. Lowering extracellular \( Ca^{2+} \) concentration reversely inhibited the fDAP (Fig. 4A; \( n = 6 \)). A similar effect on \( I_{fDAP} \) was observed when \( Ca^{2+} \) channels were blocked by bath application of 400 \( \mu M \) Ca\(^{2+}\) (Fig. 4B; \( n = 5 \)). These results indicate that the fDAP is also \( Ca^{2+} \)-dependent.

To distinguish between \( I_{fDAP} \) as a Ca\(^{2+}\)-activated current versus a Ca\(^{2+}\) current per se, recordings were made with high intracellular buffering of Ca\(^{2+}\). If the underlying current is a Ca\(^{2+}\) current, the \( I_{fDAP} \) should be observed despite strongly buffering Ca\(^{2+}\) with intracellular BAPTA (10 mM). As illustrated in Fig. 4, C and D, the \( I_{fDAP} \) was never observed in the recordings with an intracellular solution containing BAPTA (\( n = 9 \)). These results showed that the \( I_{fDAP} \) is probably not a voltage-gated Ca\(^{2+}\) current but rather is a Ca\(^{2+}\)-activated current.

\( Na^{+} \) influx through TTX-sensitive channel is not required for the fDAP production

Because the application of TTX reduced \( I_{fDAP} \), in MNCs in the study of Li and Hatton (1997), the possibility that the fDAP is a result of TTX-sensitive \( Na^{+} \) channels was evaluated. A train of action potentials was evoked by a 200 ms stimulus pulse (0.2 nA) to generate a fDAP in the presence of amiparin (100 nM) and Cs\(^{+}\) (5 mM). Subsequent bath application of TTX (0.5 \( \mu M \)) blocked sodium spikes and a presumptive persistent \( Na^{+} \) current (not shown), but the fDAP evoked by calcium spikes remained constant (Fig. 5; \( n = 8 \)). Therefore the activation of TTX-sensitive sodium channels is not required for generation of the fDAP in VP neurons.

Current-voltage relationship of the \( I_{fDAP} \)

The current-voltage relationship of the \( I_{fDAP} \) was examined at various holding potentials under voltage clamp. In this experiment, activation of \( I_{fDAP} \) was conducted in VP neurons filled with Cs\(^{+}\)-gluconate pipette solution. The tail current thought to underlie the fDAP was evoked by a 50 ms depolarizing voltage step to 0 mV from the holding potential of \(-60 \) mV, then returned to different membrane potentials (Fig. 6A). To isolate this Ca\(^{2+}\)-dependent inward current from the influence of other voltage-dependent currents, traces taken without the conditioning pulse (used to produce Ca\(^{2+}\) influx) were subtracted from the traces obtained from those with test pulses (Fig. 6, A and B). The \( I-V \) relationship of the \( I_{fDAP} \) was characterized by a relatively linear relation between \(-90 \) and \(-50 \) mV and pronounced outward rectification above \(-50 \) mV (Fig. 6C). The outward rectification suggested that the channel is permeable to Cs\(^{+}\) and is probably regulated by membrane voltage as well as Ca\(^{2+}\), although a more thorough characterization of voltage dependence would require a more extended
Because a mixed cation current was suggested as the ionic basis of the current, we tested Na\(^{+}\) permeability by lowering [NaCl]\(_o\) to 27 mM in VP neurons filled with Cs\(^{+}\)-gluconate-filled pipettes. The Nernst equation indicated that this treatment would shift \(E_{Na}^{+}\) negative by 46 mV and \(E_{Cl}^{-}\) positive by 87 mV. As shown in Fig. 7, reduction of the [NaCl]\(_o\) in the bathing solution resulted in significant reduction in the amplitude of \(I_{fDAP}\) at potentials between \(-90\) and \(-50\) mV (\(n = 5\); \(P < 0.05\)). This suggests the involvement of Na\(^{+}\) in generation of fDAP.

To test whether \(I_{fDAP}\) is also carried by K\(^{+}\) ions, the effect of raising extracellular K\(^{+}\) concentration to 10 mM from control of 2.5 mM was examined in VP neurons filled with K-gluconate pipette solution. This treatment would shift \(E_{K}^{+}\) positive by 37 mV (Nernst equation). To minimize the effect of voltage-dependent K\(^{+}\) conductances, TEA and 4-AP (10 mM each) were added along with apamin, Cs\(^{+}\), and TTX (100 nM, 5 mM, and 500 nM, respectively) in the bathing solution. Despite these treatments, a transient outward current opposing the \(I_{fDAP}\) appeared at potentials above \(-50\) mV (indicated in Fig. 8A), indicating the presence of a probable K\(^{+}\) current (which was blocked by the internal Cs\(^{+}\) in the previous experiment). However, the amplitudes obtained for plotting the \(I-V\) relationship were taken from the point (indicated in arrowheads) where the outward current subsided, therefore minimizing contamination, and in control solution, produced an \(I-V\) curve similar to that when Cs\(^{+}\) was used intracellularly to block other K\(^{+}\) currents in the experiments testing for Na\(^{+}\) dependence (Fig. 7). Raising the extracellular concentration of K\(^{+}\) significantly increased amplitudes of the \(I_{fDAP}\) (\(n = 6\); \(P < 0.05\)) and shifted its reversal potential in the depolarizing direction. Thus K\(^{+}\) also contributes to the amplitude of the \(I_{fDAP}\).

Ca\(^{2+}\)-activated Cl\(^{-}\) currents have been reported to mediate depolarizing afterpotentials in other cell types, such as dorsal root ganglion, spinal cord, and autonomic neurons (reviewed in Hartzell et al. 2005). In VP neurons filled with our K-gluconate pipette solution, \(E_{Cl}^{-}\) would be approximately \(-63\) mV. Opening of Ca\(^{2+}\)-activated Cl\(^{-}\) channels could contribute to depolarization when the membrane potential is more positive than

**Fig. 6.** The current-voltage (\(I-V\)) relationship of the \(I_{fDAP}\). A1: tail current was generated by a 50 ms depolarizing voltage step to 0 mV from a holding potential of \(-60\) mV, then returned to different test membrane potentials from \(-90\) to \(-40\) mV. A2: control traces were obtained without the conditioning depolarizing step. B: to isolate the Ca\(^{2+}\)-dependent inward current of \(fDAP\), control traces (A2) were subtracted from test traces (A1). \(x\) where current amplitudes were measured. C: \(I-V\) relationship was relatively linear between \(-90\) and \(-50\) mV but rectified outwardly above \(-50\) mV, suggesting a voltage dependence of the current. These recordings were conducted with Cs\(^{+}\)-gluconate intracellular solution in the presence of extracellular Cs\(^{+}\), apamin, and TTX (5 mM, 100 nM, and 500 nM, respectively). D: fDAPs were generated by evoking action potentials with a 50 ms square pulse in the presence of apamin (100 nM), Cs\(^{+}\) (5 mM), TEA (10 mM), and TTX (500 nM). The amplitudes of the fDAP became larger when the holding potential was hyperpolarized, complementing the \(I-V\) relationship in voltage clamp.

**Fig. 7.** Effect of lowering [Na\(^{+}\)]\(_o\) on \(I_{fDAP}\). A: \(I_{fDAP}\) at membrane potentials from \(-90\) to \(-40\) mV was obtained by the subtraction method described in the previous figure. B: plotted \(I-V\) showing that lowering [Na\(^{+}\)]\(_o\) significantly decreases the amplitude of the \(I_{fDAP}\) throughout the voltage range \(-90\) to \(-50\) mV (\(n = 5\); \(P < 0.05\)).

**Ionic dependence of the current underlying fDAP**

Because a mixed cation current was suggested as the ionic basis of the current, we tested Na\(^{+}\) permeability by lowering [NaCl]\(_o\) to 27 mM in VP neurons filled with Cs\(^{+}\)-gluconate-filled pipettes. The Nernst equation indicated that this treatment would shift \(E_{Na}^{+}\) negative by 46 mV and \(E_{Cl}^{-}\) positive by 87 mV. As shown in Fig. 7, reduction of the [NaCl]\(_o\) in the bathing solution resulted in significant reduction in the amplitude of \(I_{fDAP}\) at potentials between \(-90\) and \(-50\) mV (\(n = 5\); \(P < 0.05\)). This suggests the involvement of Na\(^{+}\) in generation of fDAP.

To test whether \(I_{fDAP}\) is also carried by K\(^{+}\) ions, the effect of raising extracellular K\(^{+}\) concentration to 10 mM from control of 2.5 mM was examined in VP neurons filled with K-gluconate pipette solution. This treatment would shift \(E_{K}^{+}\) positive by 37 mV (Nernst equation). To minimize the effect of voltage-dependent K\(^{+}\) conductances, TEA and 4-AP (10 mM each) were added along with apamin, Cs\(^{+}\), and TTX (100 nM, 5 mM, and 500 nM, respectively) in the bathing solution. Despite these treatments, a transient outward current opposing the \(I_{fDAP}\) appeared at potentials above \(-50\) mV (indicated in Fig. 8A), indicating the presence of a probable K\(^{+}\) current (which was blocked by the internal Cs\(^{+}\) in the previous experiment). However, the amplitudes obtained for plotting the \(I-V\) relationship were taken from the point (indicated in arrowheads) where the outward current subsided, therefore minimizing contamination, and in control solution, produced an \(I-V\) curve similar to that when Cs\(^{+}\) was used intracellularly to block other K\(^{+}\) currents in the experiments testing for Na\(^{+}\) dependence (Fig. 7). Raising the extracellular concentration of K\(^{+}\) significantly increased amplitudes of the \(I_{fDAP}\) (\(n = 6\); \(P < 0.05\)) and shifted its reversal potential in the depolarizing direction. Thus K\(^{+}\) also contributes to the amplitude of the \(I_{fDAP}\).

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**Pharmacological characterization of fDAP**

Together, the previous results suggest that a Ca\textsuperscript{2+}-activated nonselective cation (CAN) channel is responsible for generation of the fDAP in VP neurons. Therefore the effects of the several blockers of cation channels on the fDAP were tested. A nonsteroidal antiinflammatory drug, flufenamic acid (FFA), has been reported to block two closely related Ca\textsuperscript{2+}-activated cation channels, the melastatin-related subfamily of transient receptor potential channels (TRP channels) (TRPM4 and TRPM5 channels) (Ullrich et al. 2005). As shown in Fig. 10A, bath application of FFA (100 μM) significantly and reversibly reduced the amplitude of fDAP by 69.7 ± 10.6% (n = 4; P < 0.05). The blockade of the DAP by FFA had a slow onset (~12 min) and reversed only slowly (~15 min) on washout.

Another family of TRP channels, TRP vanilloid type 1, 2, and 4 (TRPV1, TRPV2, and TRPV4) were reported to be expressed in VP neurons (Wainwright et al. 2004). Therefore the effect of a TRPV blocker, the inorganic polycationic dye ruthenium red (RuR), on fDAP was examined. Bath application of RuR, even at 10 μM concentration, did not significantly affect the amplitude of fDAP (n = 4; Fig. 10B). Moreover, capsaizpine, a competitive antagonist of capsaicin at the TRPV1 channel (Dickenson and Dray 1991), did not affect the amplitude of fDAP (n = 2; Fig. 10C). These results suggest the generation of fDAP is not mediated by TRPV channels.

In addition, SKF 96365, a commonly used cation channel blocker that blocks TRP canonical type 3 (TRPC3) (Zhu et al. 1998) and type 6 (TRPC6) (Boulay et al. 1997; Inoue et al. 2001; Tseng et al. 2004) channels, did not affect the amplitude of fDAP (n = 4; Fig. 10D), indicating that TRPC3 and TRPC6 channels are not involved in the generation of fDAP in VP neurons. Although U50488H is not a cation channel blocker but rather a synthetic κ-opioid receptor agonist, the effect of this compound on the fDAP was tested because both endogenous dynorphin and U50488H have been reported to inhibit DAPs and decrease burst duration in MNCs (Brown and Bourque 2004; Brown et al. 1999). However, as seen in Fig. 10E, bath application of U50488 did not affect the amplitude fDAP (n = 3). Thus like external Cs\textsuperscript{+}, κ-receptor activation probably targets the sDAP, but not the fDAP, in VP neurons.

**DISCUSSION**

**Supraoptic VP neurons generate a fast DAP**

The present study demonstrated that essentially all VP neurons in the SON generate a Ca\textsuperscript{2+}-dependent fDAP following a train of action potentials, whereas only minority of OT neurons expressed this afterpotential. Many studies have attempted to elucidate the mechanisms underlying DAPs in MNCs with disparate results. It has been suggested that discrepancies between labs may arise from the multiple mechanisms underlying the expression of DAPs in MNCs (Ghamari-Langroudi and Bourque 2002; Li and Hatton 1997b). Most previous studies of DAPs in MNCs were done in the presence of multiple overlapping voltage- and Ca\textsuperscript{2+}-dependent currents. Results will likely be biased to mechanisms that can be observed best under particular experimental conditions. Activation of each afterpotential may thus differ between experimental conditions (e.g., patch-clamp or sharp electrode experiments). For example, Li and Hatton (1997b) suggested that the DAP may result from the Ca\textsuperscript{2+}-dependent reduction of a resting K\textsuperscript{+} conductance. The DAP in that study was attenuated by TTX or TEA and insensitive to external Cs\textsuperscript{+}. In contrast, DAPs in other studies were not blocked by TTX (Andrew
Cells were filled with a Cs+-dependent reduction of a resting K+-conductance. However, the fDAP was still present as its rectification at depolarized potentials. Although a fDAP was studied, the amplitude of fDAP increased with hyperpolarization, and a relationship of the I-V

Our results indicate that I_{fDAP} is generated by Ca^{2+}-dependent ion channels that are permeable to Na^+ and K^+, but not to Cl^- (Partridge 1993). When the I-V relationship of the I_{fDAP} was studied, the amplitude of I_{fDAP} increased with hyperpolarization, and a pronounced outward rectification was observed at potential above −50 mV. This rectification was still observed when the cells were filled with a Cs^+-glucuronate pipette solution that should inhibit the majority of K^+ conductances. Despite eliminating these conductances, however, the I_{fDAP} was still present as was its rectification at depolarized potentials. Although a more thorough examination is required before making a conclusion, these findings indicate that the conductance that underlies the I_{fDAP} is probably permeable to intracellular Cs^+, and the I_{fDAP} is not only Ca^{2+}-dependent but also voltage-dependent. To our knowledge, the only ion channel classes known to meet these criteria are Ca^{2+}-activated nonselective cation (CAN) channels.

It has been shown that DAPs and plateau potentials can result from the activation of CAN channels in MNCs (Ghamari-Langroudi and Bourque 2002) as well as in other cell types of mammalian cells. These data suggest that these conductances are Ca^{2+}-activated nonselective cation (CAN) channels.

Ionic nature of fDAP and possible channels mediating the generation of fDAP in VP neurons

FIG. 10. Effect of the nonselective cation channel blockers on fDAP. The fDAP was generated with a train of spikes during a 200-ms current injection in the presence of extracellular Cs^+ and apamin (5 and 100 nM, respectively). The number of spikes evoked was carefully monitored to ensure the same number of spikes (16–20 spikes) were generated before and after any 1 drug application. A, Cs^+-activated nonselective cation (CAN) channel blocker, FFA, effectively and reversibly blocked the fDAP (n = 4). B, transient receptor potential (TRP) vanilloid type (TRPV) channel blocker RuR did not effectively reduce the fDAP (n = 4). C, competitive antagonist of the TRPV1 channel, capsazepine, did not affect the fDAP (n = 3). D, blocker of TRP canonical type 3 and type 6 channels, SKF 96365, did not affect fDAP (n = 4). E, dynorphin agonist, U-50488, known to suppress DAPs in MNCs, did not affect the fDAP (n = 3).
nels in a range of tissues including the voltage-gated Na\(^+\) (Lee et al. 2003) and K\(^-\) (Lee and Wang 1999) channels and Ca\(^{2+}\)-activated chloride currents (Kim et al. 2003). FFA also causes a transient release of intracellular Ca\(^{2+}\)-release from stores (Partridge and Valenzuela 1999). Moreover, the molecular identification and the biophysical properties of these channels must be elucidated by RT-PCR or a comparable technique, and single channel recordings, respectively.

VP neurons in SON are directly osmosensitive, and this osmosensitivity is mediated by stretch-inhibited cation channels (Oliet and Bourque 1993). A recent study showed that SON neurons express an N-terminal splice variant of the TRPV vanilloid type-1 (TRPV1) channel but not full-length TRPV1 (Sharif Naeini et al. 2006). In that study, the SON neurons in TRPV1 knockout mice could not generate increases in membrane conductance in response to hyperosmotic stimulation (Sharif Naeini et al. 2006). Thus the N-terminal splice variant of the TRPV1 has been suggested as a functional stretch-inhibited cation channel (Sharif Naeini et al. 2006). In addition, other studies have indicated that TRPV4 channel may contribute to the detection of osmotic signals (Liedtke et al. 2000; Strotmann et al. 2000) and to the osmotic control of VP release (Liedtke and Friedman 2003; Mizuno et al. 2003). The inorganic polycationic dye, ruthenium red (RuR), appears to be one of the most selective blockers of currents through the TRPV channels (Tominaga et al. 1998) as all TRPV channels are reportedly blocked by RuR (Watanabe et al. 2003). Moreover the increase in membrane conductance provoked by hyperosmolality was significantly attenuated in the presence of RuR in MNCs of mice (Sharif Naeini et al. 2006). In the present study, an application of RuR did not affect the fDAP in VP neurons. This strongly suggests that the currents underlying fDAP are not mediated by TRPV channels and the \(I_{\text{fDAP}}\) probably plays little role in the osmosensitive activation of VP neurons.

Possible functional role of the fDAP in MNCs

Although the precise physiological functions of the fDAP are unknown at this point, the fact that essentially all VP neurons, whereas only a minority of OT neurons, possess this property implicates the involvement of fDAP in the generation of the specific firing pattern observed in VP neurons. In MNCs, Ca\(^{2+}\) influx through high-voltage-gated channels typically evoked by spikes, but not sub-threshold events, is required to generate a plateau potential on which phasic bursts ride (Andrew and Dudek 1984a). Therefore VP neurons require the ability to depolarize from negative potentials to the voltage range where bursts may occur through the activation of the current underlying the sDAP. Thus the fDAP may serve to bootstrap the sDAP, but this would clearly depend on the nature of the interaction between the fDAP and the medium AHP.

The DAPs and phasic bursting activity can be observed in brain slice preparations when synaptic activity has been blocked (Andrew 1987; Bourque and Renaud 1984; Hatton 1982) and even somewhat in dissociated cells (Oliet and Bourque 1992). Therefore phasic bursting is largely an intrinsic property of VP neurons. However, the phasic bursting in vivo is clearly triggered from synaptic inputs because excitatory amino acid receptor antagonists prevent bursts (Brown et al. 2004; Nissen et al. 1994). Therefore phasic bursts in MNCs are not intrinsically regenerative in vivo. This discrepancy may be due to the steep voltage sensitivity of the sDAP. Phasic firing in vitro is observed only within a relatively narrow range of membrane potential (~48 to ~55 mV). Slightly more depolarized potentials results in continuous firing, whereas a slightly hyperpolarized potentials results in slow irregular firing or no firing at all (Inenaga et al. 1993). In contrast, most VP neurons in vivo exhibit a slow irregular discharge in absence of stimulation (Wakerley et al. 1978), suggesting the resting potential in vivo is below the range of sDAP activation. This probably prevents excessive firing that would result in inappropriate hormone secretion at rest. The fDAP may also be needed for the initiation of bursting to occur appropriately in response to strong excitatory synaptic inputs from osmosensitive areas (Denton et al. 1996; McKinley et al. 1996; Richard and Bourque 1995) in response to osmotic challenge. Interestingly, it has been shown that N-methyl-d-aspartate (NMDA) induced burst via activation of CAN current in subthalamic neurons (Zhu et al. 2004) and in neocortical cells (Schiller 2004). In addition, Ca\(^{2+}\) influx responsible for activation of CAN current was mediated by both NMDA-receptor channels and voltage-gated calcium channels and to lesser extent internal calcium stores (Schiller 2004). These reports further support the notion in the synaptic activation of the fDAP in VP neurons. Indeed, NMDA receptor activation in SON neurons also contributes to rhythmic burst firing (Hu and Bourque 1992). Therefore we suggest that the Ca\(^{2+}\)-activated fDAP plays a role in the instigation of phasic firing, the latter relying in turn on the subsequent activation of the sDAP.

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FAST DEPOLARIZING AFTERPOTENTIALS IN VASOPRESSIN NEURONS


