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Spontaneous miniature hyperpolarizations affect threshold for action potential generation in mudpuppy cardiac neurons. J Neurophysiol 88: 1119–1127, 2002; 10.1152/jn.00935.2001. Mudpuppy parasympathetic neurons exhibit spontaneous miniature hyperpolarizations (SMHs) that are generated by potassium currents, which are spontaneous miniature outward currents (SMOCs), flowing through clusters of large conductance voltage- and calcium (Ca$^{2+}$)-activated potassium (BK) channels. The underlying SMOCs are initiated by a Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) mechanism. Perforated-patch whole cell voltage recordings were used to determine whether activation of SMHs contributed to action potential (AP) repolarization or affected the latency to AP generation. Blockade of BK channels by iberiotoxin (IBX, 100 nM) slowed AP repolarization and increased AP duration. Treatment with ω-conotoxin GVIA (3 μM) or nifedipine (10 μM) to inhibit Ca$^{2+}$ influx through N- or L-type voltage-dependent calcium channels (VDCCs), respectively, also decreased the rate of AP repolarization and increased AP duration. Elimination of CICR by treatment with either thapsigargin (1 μM) or ryanodine (10 μM) produced no significant change in AP repolarization or duration. Blockade of BK channels with IBX and inhibition of N-type VDCCs with ω-conotoxin GVIA, but not inhibition of L-type VDCCs with nifedipine, decreased the latency of AP generation. A decrease in latency to AP generation occurred with elimination of SMHs by inhibition of CICR following treatment with thapsigargin. Ryanodine treatment decreased AP latency in three of six cells. Apamin (100 nM) had no effect on AP repolarization, duration, or latency to AP generation, but did decrease the hyperpolarizing afterpotential (HAP). Inhibition of L-type VDCCs by nifedipine also decreased HAP amplitude. Inhibition of CICR by either thapsigargin or ryanodine treatment increased the number of APs generated with long depolarizing current pulses, whereas exposure to IBX or ω-conotoxin GVIA depressed excitability. We conclude that CICR, the process responsible for SMH generation, represents a unique mechanism to modulate the response to subthreshold depolarizing currents that drive the membrane potential toward the threshold for AP initiation but does not contribute to AP repolarization. Subthreshold depolarizations would not activate sufficient numbers of VDCCs to allow Ca$^{2+}$ influx to elevate [Ca$^{2+}$], to the extent needed to directly activate nearby BK channels. However, the elevation in [Ca$^{2+}$], is sufficient to trigger CICR from ryanodine-sensitive Ca$^{2+}$ stores. Thus CICR acts as an amplification mechanism to trigger a local elevation of [Ca$^{2+}$], near a cluster of BK channels to activate these channels at negative levels of membrane potential.

INTRODUCTION

Potassium efflux through large conductance, voltage- and calcium-activated potassium channels (BK channels) contributes to action potential repolarization in many autonomic neurons (Adams and Harper 1995; Clark et al. 1990; Rudy 1988; Sah 1996). BK channel activation occurs during membrane depolarization coupled with a rise in intracellular Ca$^{2+}$ ([Ca$^{2+}$]), near BK channels. A rise in [Ca$^{2+}$], commonly is produced as voltage-dependent Ca$^{2+}$ channels (VDCC) open, allowing Ca$^{2+}$ influx (Adams et al. 1982; MacDermott and Weight 1982). Release of Ca$^{2+}$ from internal stores via calcium-induced calcium release (CICR) also can contribute to the rise in [Ca$^{2+}$], (Berridge 1998; Henzi and MacDermott 1992; Kuba 1994; Verkhovsky and Shmigol 1996). In bullfrog sympathetic neurons, BK channel and N-type VDCC inhibitors slow action potential (AP) repolarization, as do agents such as ryanodine that inhibit CICR (Akita and Kuba 2000). This suggested, for amphibian sympathetic neurons, that BK channel activation underlying spike repolarization was in part determined by a rise in [Ca$^{2+}$], caused by the influx of Ca$^{2+}$ through N-type VDCC and by CICR.

Activation of BK channels also contributes to repolarization of APs recorded from mudpuppy parasympathetic cardiac neurons (Konopka et al. 1989). In addition, mudpuppy cardiac neurons exhibit spontaneous miniature hyperpolarizations (SMHs) at resting values of membrane potential (Hartzell et al. 1977). The SMHs are caused by spontaneous miniature outward currents (SMOCs) that represent K$^+$ currents flowing through K$^+$ channels (Scornik et al. 2001). SMOCs are generated by a CICR mechanism, with the frequency and amplitude of SMOCs as a function of membrane potential (Merriam et al. 1999; Satin and Adams 1987).

SMHs or SMOCs have been recorded from a number of different neuron types (Arima et al. 2001; Fletcher and Chippinelli 1992; Hartzell et al. 1977; Mathers and Barker 1981, 1984; Merriam et al. 1999; Satin and Adams 1987), but their function is not established. Given that BK channel activation is an important determinant of AP repolarization (Adams and Harper 1995; Clark et al. 1990), it is plausible that activation of SMOCs contribute to the repolarizing K$^+$ current in some cardiac neurons.
cells. SMH (or SMOC) amplitude increases with depolarization, whereas SMOC frequency exhibits a bell-shaped voltage dependence; the SMOC frequency increases initially with membrane depolarization but then decreases with membrane voltages beyond 0 mV (Merriam et al. 1999; Satin and Adams 1987). Given that SMOC frequency increased markedly with small depolarizations, SMHs also might play a role in determining membrane excitability, especially at membrane voltages near the threshold potential for spike generation.

This study was undertaken to determine the function of SMHs in mudpuppy cardiac neurons. In particular, the studies test whether SMH generation contributes to AP repolarization and/or modulates cell excitability near the threshold potential for AP initiation.

METHODS

All experiments were performed on parasympathetic neurons dissociated from mudpuppy (Necturus maculosus) cardiac ganglia. Mudpuppies were killed by rapid decapitation, following procedures approved by the University of Vermont Institutional Animal Care and Use Committee. The method of dissociation used a combination of type I collagenase (Sigma Chemical Co., St. Louis, MO) and neutral protease (Roche Molecular Biochemicals, Indianapolis, IN), following methods described previously (Merriam and Parsons 1995). All experiments were completed at room temperature (21–22°C).

Electrophysiological methods

Whole cell voltage recordings were made using the perforated-patch configuration of the whole cell patch-recording technique (Horn and Marty 1988) and were controlled using the current-clamp bridge configuration of the whole cell patch-recording technique (Horn and Marty 1988) and were controlled using the current-clamp bridge mode of an Axoclamp 2A/Digidata 1200/pClamp 6.0.3 acquisition system (Axon Instruments, Union City, CA). Voltage responses were digitized at 1 kHz and acquired on-line. Previously we determined that the average resting membrane of the dissociated neurons was approximately −50 mV (Scornik et al. 2001). Although similar values were obtained in the present study, the resting membrane potential did vary between cells. However, in all experiments, control and test results were obtained from the same cell with the membrane potential maintained at a similar level.

Two protocols were used to elicit APs. Single APs were elicited by applying 1-ms suprathreshold depolarizing currents and multiple APs were generated with 500-ms depolarizing current steps. The large suprathreshold depolarizing current pulses used to elicit single APs altered the rising phase of the AP. Consequently, for single APs, analysis focused on AP duration and the maximum rate of fall (MRF) of the AP. The AP duration for control and test conditions was determined at −30 mV, and MRF was obtained by differentiating the repolarization phase of the AP (Clampfit, pClamp version 6.0.3). Mudpuppy cardiac neurons generate multiple APs when long-duration suprathreshold stimuli are applied (Konopka et al. 1989). Excitability was determined by comparing the number of APs produced during 500-ms depolarizing current pulses of progressively greater stimulus intensities (10–100 pA) (Konopka et al. 1989). Current ramps (400–500 ms) were applied to determine the latency to AP generation. The rate of depolarization with the current ramp was adjusted to elicit at least one AP under control conditions. The latency, determined as the time interval from onset of the current ramp to the point at which the rising phase of the AP crossed 0 mV, was compared in the same cell prior to and after drug application.

SMOCs were recorded in voltage-clamped cells using the perforated-patch configuration of the whole cell patch-clamp technique (Horn and Marty 1988) and controlled by an Axopatch 200/Digidata 1200/pClamp 6.0.3 acquisition system (Axon Instruments). Currents were filtered at 2 kHz, stored on tape using a PCM recorder (A. R. Vetter Co., Rebersburg, PA), and digitized (200 μS) for further analysis using the SCAN program (Strathclyde Electrophysiology Software, John Dempster, University of Strathclyde, Glasgow, Scotland). To demonstrate the voltage dependence of SMOCs, currents were recorded for 2–2 min at holding potentials ranging between −50 and −20 mV (Scornik et al. 2001).

Data analysis

Control and test results were averaged from different cells, and the averaged values from a number of cells were expressed as the mean ± SE of the control or test group. Data were analyzed with the Students paired t-test with P < 0.05 considered statistically significant.

Solutions for action potential, SMH, and SMOC recordings

The bath solution contained the following (in mM): 110 NaCl, 3.6 CaCl2, 2.5 KCl, and 10 NaHEPES, pH 7.3. In a few experiments, in which the effect of depolarization on SMOC frequency and amplitude were determined, 0.3 μM tetrodotoxin (TTX) was added to the bath solution to block AP generation. The pipette solution was (in mM) 80 Kaspartate, 40 KCl, 5 MgCl2, and 10 HEPES-KOH, pH 7.2. The patch pipettes were backfilled with 0.2 mg/ml amphotericin B (Sigma).

Drugs

All drugs used in this study were obtained from commercial sources: pamiparin, caffeine, nifedipine (Sigma); ibiotixin (IBX) and ω-conotoxin GVIA (Alomone Labs, Jerusalem, Israel); and thapsigargin and ryanodine (Calbiochem, La Jolla, CA). All drugs were used at concentrations that were used in our previous studies and were in excess of the established Ks of each drug. Thapsigargin was prepared as a 1,000× concentrated stock solution in DMSO and frozen until use. Ryanodine was prepared daily as a 1,000× concentrated stock solution in DMSO. Nifedipine was prepared in acetone as a 10 mM stock and stored at −20°C until used. As a control, vehicle was added at the final concentration to the control solution; there was no obvious effect on AP threshold or repolarization.

RESULTS

SMOC amplitude and frequency increase when dissociated mudpuppy cardiac neurons are depolarized

Previously, Hartzell et al. (1977) reported that the frequency and amplitude of the SMHs commonly seen in voltage recordings from mudpuppy cardiac neurons increased as the neurons were depolarized. We also showed (Merriam et al. 1999; Scornik et al. 2001) that the frequency and amplitude of SMOCs, the spontaneous outward currents generating SMHs, were voltage dependent. However, our previous studies used cadmium to reduce Ca2+ influx through VDCC and thus to decrease SMOC frequency. Consequently, in the present study, we recorded SMOCs, when cadmium was not present, in dissociated neurons voltage clamped between −50 and −20 mV (Fig. 1A) to demonstrate the effect of voltage on SMOC amplitude and frequency. The cells were treated with TTX to block activation of voltage-gated sodium currents. Consistent with previously reported results, both SMOC frequency and amplitude increased with depolarization from −50 to −20 mV (Fig. 1A). Similar results were obtained in eight cells.

We also demonstrated that SMOCs were absent when CICR was eliminated by treatment with 1 μM thapsigargin. Thapsi-
ApD duration is increased by blockade of BK channels and inhibition of VDCCs but not by conditions that eliminate CICR

Experiments tested whether AP duration and MRF of mudpuppy cardiac neurons were affected by drugs that 1) specifically block BK channels, 2) interfere with CICR, or 3) preferentially inhibit different VDCCs.

First, we established the effect of treatment with 100 nM IBX, a specific blocker of BK channels (Galvez et al. 1990). As shown in Fig. 2A1, AP duration increased and MRF decreased during exposure to IBX, confirming that activation of BK channels contributed to spike repolarization in these cells (Konopka et al. 1989). For eight cells, AP duration increased by 65 ± 6% (P < 0.0001) and MRF decreased by 44 ± 3% (P < 0.0001; Fig. 2, B and C).

We next tested whether inhibition of VDCCs also increased AP duration and decreased MRF. Treatment with 3 μM ω-conotoxin GVIA to inhibit N-type VDCCs (Merriam and Parsons 1995) effectively increased AP duration by 39 ± 7% (P = 0.01) and decreased MRF by 39 ± 8% (P = 0.002) in six cells (Fig. 2, A2, B, and C). Treatment with 10 μM nifedipine to block L-type VDCCs (Merriam and Parsons 1995) increased AP duration by 18 ± 3% (P = 0.015) and decreased MRF by 22 ± 3% (P = 0.004) in six cells. The changes in AP duration and rate of repolarization were significant but smaller than those noted with ω-conotoxin GVIA (Fig. 2, B and C).

The results with ω-conotoxin GVIA and nifedipine suggested that Ca2+ influx through both N-type and L-type VDCCs was involved in the activation of BK channels that contributed to AP repolarization. Previously, Merriam et al. (1999) demonstrated that Ca2+ influx through both N-type and L-type VDCCs contributed to the generation of SMOCs through a CICR mechanism. To determine the potential role of CICR in activating BK channels involved in AP repolarization, APs were recorded prior to and during exposure to either 1 μM thapsigargin or 10 μM ryanodine after caffeine challenges. Caffeine (10 mM) was included with either thapsigargin or ryanodine in an initial 3- to 5-min challenge to stimulate release of Ca2+ from endoplasmic reticulum (ER) stores after thapsigargin inhibits the smooth endoplasmic reticulum calcium ATPase (SERCA ATPase) responsible for replenishing Ca2+ stores; with time, calcium stores gradually become depleted (Thomas and Hanley 1994). Recordings were made at −30 mV prior to and after exposure to thapsigargin. Caffeine (10 mM) was included for the initial 4-min exposure to thapsigargin (Fig. 1B1) but not recorded during the thapsigargin treatment (Fig. 1B2).

**FIG. 1.** Spontaneous miniature outward currents (SMOCs) increased as mudpuppy cardiac neurons were depolarized and were eliminated by thapsigargin treatment. Tetrodotoxin (0.3 μM) was added to the bathing solution to block action potential (AP) generation. A: examples of SMOCs recorded from the same cell at −50, −40, −30, and −20 mV. B: recordings of SMOCs from a dissociated neuron held at −30 mV before (Control) and during treatment with 1 μM thapsigargin (Thapsigargin). Caffeine (10 mM) was included for the initial 4-min exposure to thapsigargin. Calibration: y axis, 100 pA in A and 180 pA in B; x axis, 400 ms.

**FIG. 2.** AP duration is increased and maximum rate of fall (MRF) is decreased during exposure to iberiotoxin (IBX) and ω-conotoxin GVIA, but not by treatment with thapsigargin or ryanodine. A: in each example, control and test APs were recorded from the same neuron. A1: APs recorded prior to and during exposure to 100 nM iberiotoxin for 15 min. A2: APs recorded prior to and during exposure to 10 nM ω-conotoxin GVIA for 15 min. A3: APs recorded prior to and during exposure to 1 μM thapsigargin and ryanodine treatments, APs were recorded after depletion of stores by caffeine challenge (see text). In each panel, the top of the AP is truncated for display. Calibration: y axis, 10 mV; x axis: 5 ms. B and C: average AP duration measured at −30 mV (B) and MRF (C) in the presence of 100 nM IBX (IBX), 3 μM ω-conotoxin GVIA (CTX), 10 μM nifedipine (Nifed), 1 μM thapsigargin (Thaps), 10 μM ryanodine (Ryan), and 100 nM apamin. In each treatment, control and test recordings were made from the same cells. The results are presented as the mean ± SE for averaged values from ≥3 cells. Asterisks indicate significance (P < 0.05) between control and test condition.
which the cells were exposed to either thapsigargin or ryanodine alone. Neither thapsigargin treatment \((n = 5 \text{ cells}, P = 0.58, P = 0.42)\) nor ryanodine treatment \((n = 3 \text{ cells}, P = 0.24, P = 0.22)\) significantly affected AP duration or MRF, respectively (Fig. 2, A3, A4, B, and C).

To test whether IBX and \(\omega\)-conotoxin GVIA still could alter AP repolarization even after CICR was inhibited, we exposed cells to IBX or \(\omega\)-conotoxin GVIA after thapsigargin or ryanodine treatment. Exposure to IBX \((n = 2 \text{ cells})\) or \(\omega\)-conotoxin GVIA \((n = 3 \text{ cells})\) following thapsigargin treatment increased AP duration \((51\% \text{ with IBX, } 32\% \text{ with } \omega\text{-conotoxin GVIA})\) and decreased MRF \((47\% \text{ with IBX, } 34\% \text{ with } \omega\text{-conotoxin GVIA})\). Similarly, exposure to IBX \((n = 3 \text{ cells})\) following ryanodine treatment increased AP duration by 49\% and decreased MRF by 35\%. These results suggest that the effect of IBX and \(\omega\)-conotoxin GVIA on AP duration and MRF is not related to BK channels activated by CICR but by BK channels directly activated by \(\text{Ca}^{2+}\) influx through VDCCs.

The hyperpolarizing afterpotential following the AP is reduced by inhibition of small conductance \(\text{Ca}^{2+}\)-activated \(K^+\) channels, but not by inhibition of BK channels or inhibition of CICR

The results of the previous experiments demonstrated that direct activation of BK channels, rather than activation through a CICR mechanism, contributed to AP repolarization in mudpuppy parasympathetic neurons. In addition, the results indicated that \(\text{Ca}^{2+}\) influx through both N-type and L-type VDCCs contributed to direct activation of BK channels participating in AP repolarization. In mudpuppy cardiac neurons, as in most neurons, there is a period of hyperpolarization following each AP, the hyperpolarizing afterpotential (HAP) (Konopka et al. 1989). Current through small conductance \(\text{Ca}^{2+}\)-activated potassium (SK) channels, which can be specifically blocked by apamin, commonly contributes to the generation of the HAP (Rudy 1988; Sah 1996). In four cardiac neurons, treatment with 100 nM apamin decreased the peak amplitude of the HAP by 25 ± 4\% \((P = 0.01; \text{Fig. 3} \text{. A and B})\). Apamin had no effect on the rate of AP repolarization or duration (Figs. 2, B and C) and also did not eliminate SMHs in these cells; this latter result confirms earlier observations (Merriam et al. 1999).

Treatment with 100 nM IBX to inhibit BK channels \((n = 8 \text{ cells})\) or exposure to 3 \(\mu\text{M} \omega\)-conotoxin GVIA to inhibit N-type VDCCs \((n = 6 \text{ cells})\) had no significant effect on the HAP amplitude (IBX, \(P = 0.7\); \(\omega\)-conotoxin GVIA, \(P = 0.1\); Fig. 3B). In contrast, treatment with 10 \(\mu\text{M} \text{nifedipine to block L-type VDCCs decreased the peak HAP amplitude by } 44 \pm 10\% \left(n = 6 \text{ cells}, P = 0.02; \text{Fig. 3} \text{. A and B} \right)\). In five of the six cells, we tested whether exposure to 100 nM apamin following treatment with 10 \(\mu\text{M} \text{nifedipine would produce a further decrease in HAP amplitude. In these five cells, nifedipine decreased the peak HAP amplitude by approximately } 47 \pm 11\% \text{ from } 20 \pm 2 \text{ mV to } 12 \pm 3 \text{ mV. Subsequent exposure to apamin along with nifedipine further reduced the peak HAP amplitude by } 32 \pm 8\% \text{ to } 8 \pm 2 \text{ mV } (P = 0.049)\).

We next tested whether CICR activation by \(\text{Ca}^{2+}\) influx through L-type VDCCs might contribute to HAP generation. We recorded the HAP prior to and during exposure to 1 \(\mu\text{M} \text{thapsigargin or } 10 \mu\text{M ryanodine. As in the previous experiments with these drugs, } 10 \mu\text{M caffeine was present during the first } 3–5 \text{ min of exposure to facilitate depletion of the internal } \text{Ca}^{2+}\text{ stores. The peak HAP amplitude was not significantly altered by either thapsigargin } (n = 5 \text{ cells}, P = 0.7; \text{Fig. 3, A and B}) \text{ or ryanodine treatment } (n = 3 \text{ cells}, P = 0.4; \text{Fig. 3B})\).

The latency to AP generation is altered by inhibition of BK channels and by elimination of CICR

Previously, Konopka et al. (1989) demonstrated that, for mudpuppy cardiac neurons with resting membrane potentials of approximately −50 mV, the threshold for AP generation is −25 mV. Results presented in Fig. 1A and those reported previously by Hartzell et al. (1977) demonstrate that the amplitude of SMOCs (or SMHs) increases as cells are progressively depolarized from the rest potential toward the threshold for AP generation. As SMHs result from the synchronous activation of a cluster of BK channels (Merriam et al. 1999; Satin and Adams 1987; Scomnik et al. 2001), it was considered possible that the increased SMH activity could oppose depolarizing stimuli and thus affect the threshold for AP generation. To test this possibility, we determined the latency to initiate the first AP from the resting potential during depolarizing current ramps. The latency to AP generation was determined in the same cells prior to and following exposure to IBX to directly inhibit BK channels, to \(\omega\)-conotoxin GVIA or nifedipine to inhibit N-type or L-type VDCCs, respectively, and to thapsigargin.
The latency to AP generation with depolarizing ramp currents is decreased by IBX, ω-conotoxin GVIA, and thapsigargin, but not by nifedipine or ryanodine. For all 4 panels, the solid line indicates control traces and the light line indicates traces during drug treatment. Calibration: x-axis, 50 ms; y-axis, 10 mV. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 100 nM IBX for 15 min. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 10 µM ω-conotoxin GVIA for 15 min. A: AP generation by a 500-ms depolarizing current ramp prior to and during exposure to 10 µM nifedipine for 15 min. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 1 µM thapsigargin. With thapsigargin treatment, the latency was determined after depletion of Ca^{2+} stores by caffeine challenge (see text). B: latency to AP generation during depolarizing currents measured prior to and during exposure to 100 nM IBX (IBX), 3 µM ω-conotoxin GVIA (CTX), 10 µM nifedipine (Nifed), 1 µM thapsigargin (Thaps), 10 µM ryanodine (Ryan), or 100 nM apamin. In each treatment, control and test recordings were made from the same cell. The results are presented as the mean ± SE for averaged values from ≥4 cells. *Significance (P < 0.05) between control and test condition.

gargin or ryanodine to eliminate CICR. The latency was determined as the time interval from the start of the current ramp to the time when the rising phase of the first AP crossed 0 mV.

During exposure to IBX, the latency to AP generation in five cells was decreased by 16 ± 5% (P = 0.045; Fig. 4B). Example results are shown in Fig. 4A1. The latency to AP generation was also decreased by 16 ± 3% in six cells (P = 0.002) exposed to 3 µM ω-conotoxin GVIA to block N-type VDCCs (Fig. 4, A2 and B). The SMH frequency was also greatly reduced in these cells during ω-conotoxin GVIA treatment. In contrast, during exposure of four cells to 10 µM nifedipine to inhibit L-type VDCCs, the latency to AP initiation did not significantly decrease (P = 0.3; Fig. 4, A3 and B) and SMHs remained.

Additional experiments were done to test whether treatment with 1 µM thapsigargin or 10 µM ryanodine (both treatments accompanied with an initial 3- to 5-min challenge with 10 mM caffeine) affected the latency to AP generation. In 12 cells, during exposure to thapsigargin, the latency to AP generation was decreased by 32 ± 6% (P < 0.0006; Fig. 4, A4 and B). Also, SMHs were consistently not observed after thapsigargin treatment.

The effect of ryanodine treatment on the latency to first AP was tested in six cells. In three of the six cells, there was a decrease in latency (37 ± 9%), whereas in the three other cells, the latency to first AP was not changed (0.6 ± 5% decrease) during ryanodine treatment. Consequently, although the mean latency for all six cells was decreased by 18 ± 10% after ryanodine, the difference was not significant (P = 0.11; Fig. 4B). In this series of experiments, SMHs appeared to be eliminated in those cells in which the latency was decreased. In contrast, in the three cells in which the latency was not changed, either small SMHs were still evident, or the voltage trace was noisy. Therefore we suggest that in these cells CICR was not eliminated (see DISCUSSION).

Experiments were also completed to test whether, during exposure to 100 nM apamin to inhibit SK channels, the latency to the first AP was altered. Our results indicated, in seven cells, that exposure to apamin did not significantly affect the latency to the first AP (P = 0.3; Fig. 4B).

Direct inhibition of BK channels and elimination of SMHs by inhibition of CICR produce different effects on excitability curves

Mudpuppy cardiac neurons fire multiple APs when stimulated with long duration suprathreshold depolarizing current pulses (Fig. 5A) (Konopka et al. 1989). An excitability curve can be generated for these neurons by plotting the number of APs initiated during a 500-ms depolarizing current pulse of

FIG. 4. The latency to AP generation with depolarizing ramp currents is decreased by IBX, ω-conotoxin GVIA, and thapsigargin, but not by nifedipine or ryanodine. For all 4 panels, the solid line indicates control traces and the light line indicates traces during drug treatment. Calibration: x-axis, 50 ms; y-axis, 10 mV. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 100 nM IBX for 15 min. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 3 µM ω-conotoxin GVIA for 15 min. A: AP generation by a 500-ms depolarizing current ramp prior to and during exposure to 10 µM nifedipine for 15 min. A: AP generation by a 400-ms depolarizing current ramp prior to and during exposure to 1 µM thapsigargin. With thapsigargin treatment, the latency was determined after depletion of Ca^{2+} stores by caffeine challenge (see text). B: latency to AP generation during depolarizing currents measured prior to and during exposure to 100 nM IBX (IBX), 3 µM ω-conotoxin GVIA (CTX), 10 µM nifedipine (Nifed), 1 µM thapsigargin (Thaps), 10 µM ryanodine (Ryan), or 100 nM apamin. In each treatment, control and test recordings were made from the same cell. The results are presented as the mean ± SE for averaged values from ≥4 cells. *Significance (P < 0.05) between control and test condition.

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During exposure to IBX, the latency to AP generation in five cells was decreased by 16 ± 5% (P = 0.045; Fig. 4B). Example results are shown in Fig. 4A1. The latency to AP generation was also decreased by 16 ± 3% in six cells (P = 0.002) exposed to 3 µM ω-conotoxin GVIA to block N-type VDCCs (Fig. 4, A2 and B). The SMH frequency was also greatly reduced in these cells during ω-conotoxin GVIA treatment. In contrast, during exposure of four cells to 10 µM nifedipine to inhibit L-type VDCCs, the latency to AP initiation did not significantly decrease (P = 0.3; Fig. 4, A3 and B) and SMHs remained.

Additional experiments were done to test whether treatment with 1 µM thapsigargin or 10 µM ryanodine (both treatments accompanied with an initial 3- to 5-min challenge with 10 mM caffeine) affected the latency to AP generation. In 12 cells, during exposure to thapsigargin, the latency to AP generation was decreased by 32 ± 6% (P < 0.0006; Fig. 4, A4 and B). Also, SMHs were consistently not observed after thapsigargin treatment.

The effect of ryanodine treatment on the latency to first AP was tested in six cells. In three of the six cells, there was a decrease in latency (37 ± 9%), whereas in the three other cells, the latency to first AP was not changed (0.6 ± 5% decrease) during ryanodine treatment. Consequently, although the mean latency for all six cells was decreased by 18 ± 10% after ryanodine, the difference was not significant (P = 0.11; Fig. 4B). In this series of experiments, SMHs appeared to be eliminated in those cells in which the latency was decreased. In contrast, in the three cells in which the latency was not changed, either small SMHs were still evident, or the voltage trace was noisy. Therefore we suggest that in these cells CICR was not eliminated (see DISCUSSION).

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Direct inhibition of BK channels and elimination of SMHs by inhibition of CICR produce different effects on excitability curves

Mudpuppy cardiac neurons fire multiple APs when stimulated with long duration suprathreshold depolarizing current pulses (Fig. 5A) (Konopka et al. 1989). An excitability curve can be generated for these neurons by plotting the number of APs initiated during a 500-ms depolarizing current pulse of

FIG. 5. Mudpuppy cardiac neurons generate multiple APs during suprathreshold depolarizing current pulses: example AP recordings from a dissociated neuron stimulated with progressively greater intensity, 500-ms depolarizing current pulses. B: excitability curve for cardiac neurons determined from a plot of the number of APs produced with increasing stimulus intensities. Results are the mean ± SE of averaged responses obtained in 33 dissociated neurons. Data points are fit to a 2nd order polynomial.
increasing magnitude (Fig. 5B). In the final series of experiments, we tested whether the excitability curve was affected by direct inhibition of BK channels with IBX, blockade of N-type or L-type VDCCs by ω-conotoxin GVIA or nifedipine, respectively, or by inhibition of CICR by thapsigargin or ryanodine to eliminate SMHs.

Treatment with 100 nM IBX to directly inhibit BK channels (n = 6 cells) and exposure to 3 μM ω-conotoxin GVIA to inhibit N-type VDCCs (n = 9 cells) produced a similar complex change in the excitability curve (Fig. 6, A and B). With small amplitude depolarizing current pulses, the number of APs produced was similar to control during IBX or ω-conotoxin GVIA exposure (Fig. 6, A and B). However, with larger amplitude depolarizing pulses, the number of APs initiated in the presence of the drugs was less than in the absence of drug treatment (Fig. 6, A and B). Thus excitability initially was not affected but became depressed, and with a further increase in the amplitude of the stimulating current pulse, no additional increase in numbers of APs generated was observed.

In another five cells, the effect on the excitability curve of inhibiting L-type VDCCs by treatment with 10 μM nifedipine was determined. With small amplitude depolarizing current pulses, there was no change in the number of APs initiated, whereas with larger amplitude depolarizing current pulses, the number of APs generated was greater in the presence of nifedipine than prior to drug application (Fig. 6C).

We next tested, in 10 cells, whether elimination of SMHs by a challenge with 1 μM thapsigargin would change the excitability curve. As previously described, 10 mM caffeine was included during the initial 3- to 5-min exposure to thapsigargin. During thapsigargin exposure, the number of APs produced with each depolarizing current pulse was increased (Fig. 6D). Thus elimination of CICR and SMHs by thapsigargin treatment increased excitability. In 11 additional cells, we tested the effect of treatment with 10 μM ryanodine on excitability. A 5-min challenge with 10 mM caffeine accompanied the initial period of ryanodine exposure. During ryanodine exposure, excitability was increased with all depolarizing current steps (Fig. 6E). However, although the effect on excitability with thapsigargin and ryanodine treatment was similar, the increase in excitability was more variable with ryanodine treatment than with thapsigargin treatment.

Five additional cells were exposed to 100 nM apamin to inhibit SK channels and excitability tested. In these five cells, there was no change in the excitability curve (Fig. 6F).

**Discussion**

A number of interesting observations were obtained in this study. First, the results demonstrated that, in mudpuppy parasympathetic neurons, activation of BK channels by different mechanisms contributed to the repolarizing phase of the AP or modulated the latency to spike generation. Repolarization of the AP involved a direct activation of BK channels by Ca²⁺ influx, whereas AP latency was modulated by a CICR mechanism. Thus we propose that SMHs, generated by CICR, affected the latency to AP generation but did not participate in AP repolarization. Second, Ca²⁺ influx through N-type VDCCs participated in both activation of BK channels directly and by CICR. Third, Ca²⁺ influx through L-type VDCCs contributed, along with but to a lesser extent than that through N-type VDCCs, to direct BK channel activation involved in AP repolarization and had no measurable affect on the latency to AP generation. Fourth, Ca²⁺ influx through L-type VDCCs appeared to be more critical than Ca²⁺ influx through N-type VDCCs in activating channels that contributed to HAP generation.

Evidence for BK channel participation in both AP duration and latency to spike generation was derived from results with IBX. In IBX, the latency to AP generation was significantly shortened, the MRF of the AP was markedly decreased and AP duration increased. Exposure to ω-conotoxin GVIA, to inhibit
N-type VDCCs, also decreased the latency to AP generation, while the MRF and duration of APs were decreased and increased, respectively. Treatment with nifedipine to inhibit L-type VDCCs also decreased the rate of AP repolarization and increased AP duration but had no significant effect on the latency to AP generation. Thus Ca\(^{2+}\) influx through L-type VDCCs was sufficient to directly activate some BK channels involved in the repolarizing phase of the AP, but not great enough to generate sufficient SMHs by CICR to affect the latency to AP initiation.

In mudpuppy cardiac neurons, a CICR-type mechanism is required for the activation of SMOCs, which represent currents through synchronously activated clusters of 20–30 BK channels (Merriam et al. 1999; Scornik et al. 2001). BK channels are voltage- and Ca\(^{2+}\)-gated, thus both membrane depolarization and a rise in intracellular Ca\(^{2+}\) commonly regulate BK channel activation (Barrett et al. 1982). We suggest that the use of CICR to activate SMOCs is a strategic amplification mechanism by which BK channels can be activated at negative membrane potentials between the resting potential and the threshold for AP generation. With CICR, a limited Ca\(^{2+}\) influx that would occur with small depolarizations, elevates intracellular Ca\(^{2+}\) sufficient to trigger the release of Ca\(^{2+}\) from ryanodine sensitive stores in the ER (Santana et al. 1996). This released Ca\(^{2+}\) produces a rapid rise in Ca\(^{2+}\) locally near the plasma membrane to levels >40 μM, which is sufficient to activate a cluster of BK channels at negative membrane potentials giving rise to a SMOC (Scornik et al. 2001). SMOC frequency increases with depolarization as more VDCCs are activated and the elevation in intracellular Ca\(^{2+}\) increases proportionately.

SMOCs generate the SMHs recorded in current clamp (Hartzell et al. 1977; Satin and Adams 1987). Results from the present study demonstrate that elimination of SMHs by reducing CICR in thapsigargin-treated cells significantly decreased the latency to spike generation, but not the rate of AP repolarization or AP duration. Ryanodine treatment also did not affect AP repolarization rate or duration. Ryanodine treatment decreased the latency to AP generation in three of six cells. In the remaining three cells, the latency was not changed. Ryanodine effects on the Ca\(^{2+}\) release channel are complex and concentration dependent; it has been proposed that ryanodine, in the range of nanomolar to micromolar, locks the Ca\(^{2+}\) release channels in a subconductance state (Lai et al. 1992; Meissner 1986, 1994; Suiko and Airey 1996; Zucchi and Ronca-Testoni 1997). More recent observations show that, rather than locking the channels in an open state, ryanodine causes the probability of opening of the Ca\(^{2+}\) release channel to approach unity with the activated channel having a reduced conductance state (Du et al. 2001; Masumiya et al. 2001). With either mechanism, during treatment with 10 μM ryanodine, Ca\(^{2+}\) could be continually leaving intracellular stores. Ryanodine also increases the release channel sensitivity to Ca\(^{2+}\), thus fostering channel activation by elevations of intracellular Ca\(^{2+}\) (Du et al. 2001; Masumiya et al. 2001). Previously, we noted that, during continuous recordings of SMOCs at depolarized potentials, ryanodine altered the kinetics of individual SMOCs so that SMOC amplitude was decreased while at the same time the duration was increased (Merriam et al. 1999). Discrete SMOCs eventually disappeared in our initial study. In the present study, the cardiac neurons were intermittently stimulated to produce APs, and as the SERCA ATPase was not blocked, Ca\(^{2+}\) entering the cell during each AP could potentially be sequestered back into the intracellular Ca\(^{2+}\) store. It is quite likely Ca\(^{2+}\) was continuously leaving the internal stores through ryanodine-activated release channels, but Ca\(^{2+}\) uptake into the release stores also continued. We propose that in the present study, CICR, and thus SMH activity, should be reduced markedly in those cells where depletion of stores predominated, whereas Ca\(^{2+}\) release through a CICR mechanism would still be present in other cells in which sufficient Ca\(^{2+}\) was returned to the stores. Therefore we attribute the variability in results with ryanodine noted in this study to differences in ability of ryanodine to effectively deplete ER Ca\(^{2+}\) stores, a mechanism required to eliminate SMH generation by CICR.

Because thapsigargin treatment had no effect on AP repolarization and duration, we conclude that SMHs generated by CICR did not participate in AP repolarization. Rather, during the AP, the cell must be depolarized sufficiently and the Ca\(^{2+}\) influx adequate to elevate the [Ca\(^{2+}\)], near the inner surface of the plasma membrane enough to directly activate the BK channels that contribute to AP repolarization. An intriguing question raised by these observations is whether the BK channels activated by CICR and the BK channels activated directly by Ca\(^{2+}\) influx represent distinct pools of channels as schematically presented in Fig. 7. For instance, the BK channels generating SMHs might be clumped in local regions opposite ER ryanodine release sites, but not adjacent to N-type or L-type VDCCs, whereas the BK channels contributing repolarizing current might be BK channels adjacent to these VDCCs (Fig. 7). Alternatively, the same channels might be activated by these two different mechanisms, but the local rise in [Ca\(^{2+}\)], due to Ca\(^{2+}\) influx associated with subthreshold depolarizations might be insufficient to directly activate BK channels in this voltage range. Results generated to date do not distinguish between these two alternatives.

Activation of BK channels by CICR contributes to AP

![FIG. 7. Schematic view of a proposed model for Ca\(^{2+}\) influx through N- and L-type voltage-dependent calcium channels (VDCCs) and Ca\(^{2+}\) release through ryanodine receptors (RyR) by Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR): mechanisms that elevate [Ca\(^{2+}\)], and activate BK and other K\(^+\) channels responsible for spontaneous miniature hyperpolarizations (SMH) generation, AP repolarization, or HAP generation, respectively. Both apamin-sensitive SK channels and another K\(^+\) channel are suggested to contribute to HAP generation with the latter presumably closely coupled to L-type VDCCs. The arrangement of BK channels could explain the different effects of blockers on various aspects of the AP and on cell excitability. For example, BK channels activated by CICR and those activated directly by Ca\(^{2+}\) influx via VDCC may represent 2 distinct pools of channels thereby explaining the effects of IBX on the AP and on SMHs.](http://jn.physiology.org/Downloaded from http://jn.physiology.org/ by 10.220.22.246 on September 29, 2016)
repolarization in bullfrog sympathetic neurons (Akita and Kuba 2000). In amphibian sympathetic neurons, treatment with thapsigargin or ryanodine to eliminate CICR decreased the MRF of APs and increased AP duration although not to the same extent as direct BK channel blockade by IBX (Akita and Kuba 2000). In the mudpuppy neurons, thapsigargin or ryanodine treatment did not significantly alter AP repolarization or duration. Thus the role of CICR in AP repolarization differed between these two amphibian neuron types. However, the primary role of Ca\(^{2+}\) influx through N-type VDCCs in activating CICR was similar in the two types of amphibian neurons.

Previously, Merriam et al. (1999) showed that Ca\(^{2+}\) influx through both N-type and L-type VDCCs contribute to SMOC generation although inhibition of N-type VDCCs channels more effectively decreases SMOC generation. In the present study, inhibition of N-type VDCCs, but not inhibition of L-type VDCCs, markedly decreased SMHCs and decreased latency for AP generation. The apparent discrepancy between the results obtained in these two studies is most likely due to the fact that only 10–15% of the total Ca\(^{2+}\) current in these mudpuppy cells is contributed by L-type VDCCs (Merriam and Parsons 1995). Consequently, we suggest that in this study, SMH generation due to Ca\(^{2+}\) influx through L-type VDCCs was not sufficient to affect the latency to AP generation and thus during exposure to nifedipine, the latency to AP generation was not significantly changed. In contrast, during an AP, sufficient L-type VDCCs were activated so that Ca\(^{2+}\) influx directly activated some BK channels that participated in AP repolarization.

Apamin treatment to inhibit SK channels did not affect SMH generation, the latency to AP generation, or AP repolarization. A lack of effect of apamin treatment on SMH generation is consistent with earlier observations, which demonstrated that apamin-sensitive SK channels do not participate in SMOC generation in mudpuppy neurons (Merriam et al. 1999). In contrast, SMOCs in mammalian nucleus of Meynert neurons are potassium currents generated by activation of SK channels (Arima et al. 2001). Activation of apamin-sensitive SK channels did contribute to HAP generation in the mudpuppy cardiac neurons. However, CICR was not required for HAP generation in mudpuppy neurons, which occurs in some other neuron types (Cohen et al. 1997; Jobling et al. 1993; Kawai and Watanabe 1989; Moore et al. 1998; Sah and McLachlan 1991).

Inhibition of L-type, but not of N-type, VDCCs significantly decreased the HAP amplitude. This observation suggested for mudpuppy cardiac neurons that Ca\(^{2+}\) influx through L-type VDCCs is more essential to activation of channels responsible for HAP generation than Ca\(^{2+}\) influx through N-type VDCCs (Fig. 7). Also, our results suggest that some of the HAP channels activated by Ca\(^{2+}\) influx through L-type VDCC must not be apamin-sensitive SK channels, because apamin had a similar effect on HAP amplitude by itself or following nifedipine treatment. Preferential coupling between VDCC types and either BK or SK channels has been demonstrated in a number of different neurons (Davies et al. 1996; Marrion and Tavalin 1998; Wisigira and Dryer 1994). In the case of the mudpuppy cardiac neurons, although not absolute, we suggest that N-type VDCCs may be preferentially located near BK channels, whereas L-type VDCCs might be more closely associated with HAP channels. Excitability, determined by the number of APs generated (Konopka et al. 1989), was increased with thapsigargin or ryanodine treatment to minimize CICR and thus reduce SMH generation, but not with IBX or \(\omega\)-conotoxin GVIA exposure to block direct BK channel activation. During thapsigargin or ryanodine treatment, the number of APs generated continued to increase in proportion to the stimulus strength, with the number of APs greater in thapsigargin or ryanodine than prior to drug exposure. In contrast, in the presence of IBX or \(\omega\)-conotoxin GVIA, as the stimulus strength was increased, the number of APs generated reached a maximum value and a further increase in stimulus intensity did not initiate a greater number of APs.

We attribute the difference in effect of these drug treatments on excitability to differences produced in AP duration, which in turn affects the refractory period. The refractory period depends on the conversion of voltage-gated sodium channels from an inactivated state to an activatable state following each AP, a transition that is voltage dependent. Consequently, slowing of AP repolarization and consequent increased AP duration would decrease recovery from inactivation and thus prolong the refractory period. With treatments that significantly increased AP duration, the refractory period would be lengthened and the maximum frequency of AP generation decreased as seen following treatment with IBX and \(\omega\)-conotoxin GVIA.

Following nifedipine treatment, excitability was progressively enhanced. This unexpected effect could be related to the nifedipine-induced decrease in HAP amplitude, which was almost twice than that produced by apamin treatment. Presumably the extent of increase in duration of the AP in nifedipine was not sufficient to affect the refractory period as was suggested to occur with IBX and \(\omega\)-conotoxin GVIA treatment.

In summary, we propose that SMH generation by CICR represents a unique mechanism to modulate the response to subthreshold depolarizing currents that drive the membrane potential toward the threshold for AP initiation, but does not contribute to AP repolarization mechanisms. Subthreshold depolarizations would not activate sufficient numbers of VDCCs to allow Ca\(^{2+}\) influx to elevate [Ca\(^{2+}\)]\(_i\) near a cluster of BK channels (Cohen and Prunuske 1997). Thus CICR acts as an amplification mechanism to trigger a local elevation of [Ca\(^{2+}\)]\(_i\) near a cluster of BK channels, with the local elevation of Ca\(^{2+}\) exceeding 40 \(\mu\)M, the concentration required to activate these channels at negative levels of membrane potential (Scornik et al. 2001). SMH generation by CICR might ensure that subthreshold excitatory post synaptic potentials (EPSPs) do not initiate spike activity.

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