



Different types of potassium channels underlie the long afterhyperpolarization in guinea-pig sympathetic and enteric neurons

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Abstract

Ca²⁺-activated K⁺ channels play an important role in the control of neuronal excitability via the generation of the afterhyperpolarization. While both small and large conductance Ca²⁺-activated K⁺ channels underlie afterhyperpolarizations in different neuron types, the role of intermediate conductance Ca²⁺-activated K⁺ channels (IK_{Ca}) in the generation of afterhyperpolarizations remains unclear. The effects of blockade of IK_{Ca} on guinea pig coeliac and ileal myenteric neurons were studied using single microelectrode current and voltage clamp. In coeliac neurons, TRAM-39, a selective blocker of IK_{Ca}, depressed the amplitude of the prolonged conductance underlying the slow afterhyperpolarization, (gKCa2) by 57%. In contrast, the conductance underlying the prolonged afterhyperpolarization in AH-type myenteric neurons was unaffected by TRAM-39, although it has been suggested that this AHP is mediated by IK_{Ca}. In both types of neurons, TRAM-39 did not alter the resting cell properties or the properties of the action potential. TRAM-39 had no effect on the amplitude of the fast component of the afterhyperpolarization present in sympathetic LAH neurons. The results of this study suggest that in sympathetic LAH neurons, activation of IK_{Ca} underlies at least part of the prolonged afterhyperpolarization while the nature of the channel underlying the AHP in enteric neurons remains unclear.

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Following the action potential all neurons exhibit afterhyperpolarizations (AHPs), ranging in duration from as little as tens of milliseconds up to 20 s. In many cases, AHPs are generated by the opening of K⁺ channels activated by calcium influx during action potentials (Sah and Davies, 2000) or the consequent release from internal Ca²⁺ stores (Jobling et al., 1993). These Ca²⁺-activated K⁺ channels can be divided into three broad families based on their conductance, kinetics and pharmacology (Sah and Faber, 2002). Large conductance type channels (BK) are blocked by toxins such as charybdotoxin or iberiotoxin or by low concentrations of TEA, are activated during the upstroke of

the action potential and contribute to action potential repolarization and the initial fast part of the AHP. Small conductance type channels (SK), blocked by apamin, typically underlie the initial fast to intermediate component of the AHP (Sah and Faber, 2002). In many neurons, the Ca²⁺-activated K⁺ channels underlying prolonged AHPs are resistant to blockers of BK- and SK-type channels. However, in some peripheral autonomic neurons, like the long-afterhyperpolarizing (LAH) neurons of the celiac ganglion, the slower component of the AHP is partially sensitive to blockers of BK- and SK-type channels (Martinez-Pinna et al., 2000). In LAH neurons, a resistant component remains after blockade of both BK- and SK-type channels (Martinez-Pinna et al., 2000) suggesting the presence of a third channel type. Further, it has been proposed on the basis of single channel analysis and its blockade by clotrimazole, that an intermediate conductance

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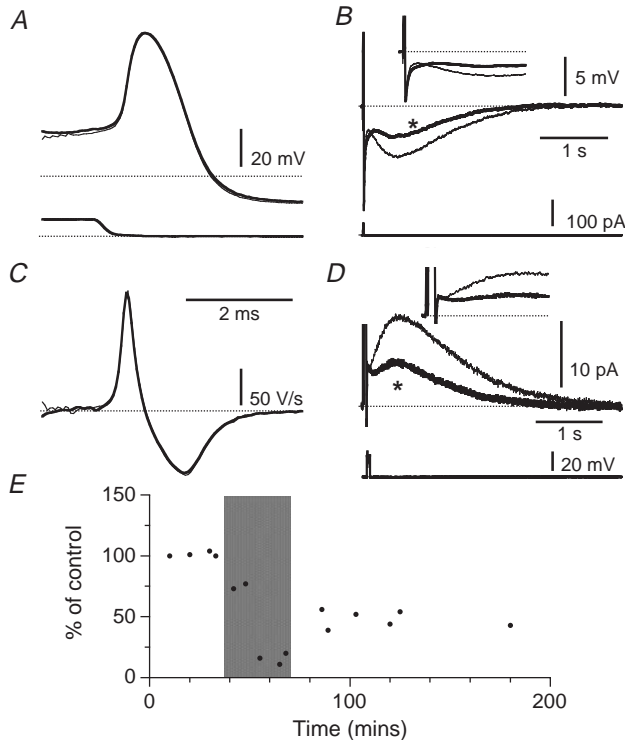


Fig. 1. Effect of TRAM-39 on an LAH neuron in the guinea pig coeliac ganglion. Records show action potentials (A), afterhyperpolarizations (AHP) (B), voltage derivative of the action potentials (C), and the outward tail currents at a holding potential of -50 mV (D). Control and drug responses are superimposed with the latter shown as thicker lines (asterisk). (A) $1 \mu\text{M}$ TRAM-39 had virtually no effect on the threshold or amplitude of the action potential (average of 5 trials). (B) The AHP after a single action potential (AP) evoked at -55 mV (average of 5 trials). The peak of the AP is truncated. The lower trace shows the current step used to trigger the AP. The slow component of the AHP is reduced in amplitude and time course after addition of TRAM-39. (C) The rate of rise or repolarization of the action potential is unchanged after addition of TRAM-39. (D) Outward tail current (upper trace) recorded in voltage clamp at a holding potential of -50 mV following a brief depolarizing voltage step (lower trace) that initiated a single active response (averages of 12–15 trials). TRAM-39 reduced the amplitude of gKCa_2 but not the amplitude of gKCa_1 . Insets in (B) and (D) show expanded traces (700 ms) scaled in amplitude by 50%. RMP in (A) and (B) was -60 mV. Time scale in (C) also applies in (A). (E) Plot showing the time course of blockade of gKCa_2 by TRAM 39 and its partial recovery following washout. Grey rectangle indicates period during which TRAM-39 was present.

Ca^{2+} -activated K^+ channel (IK_{Ca}) underlies the prolonged AHP in myenteric neurons (IK_{Ca}) (Vogalis et al., 2001, 2002b), although earlier work indicated that this AHP is sensitive to BK channel blockers. However, clotrimazole has actions on channels other than IK_{Ca} (Shah et al., 2001). Although IK_{Ca} has been described in other cell types, the absence of a selective blocker has hampered investigation of its role in neuronal excitability (e.g. Hay and Kunze, 1994). Recently, clotrimazole analogs that inhibit the IKCa_1 channel more selectively, have been developed (Wulff et al., 2000). In this study, we report the effects of a selective blocker of both cloned and native IK_{Ca} channels, TRAM-39 (Wulff et al., 2000), on myenteric AH and

sympathetic LAH neurons using single electrode current- and voltage-clamp recordings.

Preparations of the myenteric plexus or the left coeliac ganglion were obtained from guinea pigs (either sex; 170–400 g). Animals were killed by stunning, followed immediately by exsanguination and severing of the spinal cord. All procedures were approved by the Animal Care and Ethics Committee of the University of Melbourne. Preparations of myenteric plexus were prepared from segments of ileum as described previously (Bornstein et al., 1991). The preparations were pinned in a shallow tissue bath, continuously superfused with physiological salt solution [composition (in mM): NaCl, 118; KCl, 4.8; NaH_2PO_4 , 1.0; MgSO_4 , 1.2; CaCl_2 , 2.5; NaHCO_3 , 25; glucose 11, pH 7.2–7.4 gassed with 95% O_2 and 5% CO_2] at 3 ml min^{-1} and warmed to $35\text{--}37^\circ\text{C}$.

Intracellular recordings were made from ganglion cells using microelectrodes filled with 0.5 M KCl (resistance 120–180 $\text{M}\Omega$) and records taken in bridge mode, single electrode current clamp (SECC), and single electrode voltage clamp (SEVC) with a single electrode amplifier (Axoclamp-2A, Axon instruments). All measured electrical properties were from neurons in which impalements were stable for at least 15 min before recordings were taken. Passive electrical properties (see Cassell et al., 1986) were monitored regularly throughout the recording. Data were only included if the passive electrical properties in control solution remained stable and action potentials had amplitudes >60 mV. All voltage and current records were digitized at 1–20 kHz (PowerLab 4SP; ADI Instruments, NSW, Australia) using Chart/Scope (v. 3.6) software and analysed using Igor Pro software (v. 3, WaveMetrics, Oregon, USA). Steady-state voltage–current relationships were determined from a holding potential of -65 mV. Outward tail currents were generated after a voltage command step (50 ms) from a holding potential of -50 mV to a potential that elicited a single “action current” (Cassell and McLachlan, 1987). This tail current has been characterized in sympathetic ganglion cells (Cassell and McLachlan, 1987; Davies et al., 1996). Resting membrane potential (RMP) was determined as the difference between the voltage immediately before and after withdrawal of the microelectrode from the cell. Coeliac LAH and myenteric AH neurons were distinguished from other electrophysiological classes by the presence of a prolonged AHP lasting several seconds (Hirst et al., 1974; Cassell and McLachlan, 1987; Bornstein et al., 1994).

TRAM-39 (2-(2-chlorophenyl)-2,2-diphenylacetonitrile) was made fresh on the day of each experiment and was added to the perfusate ($1 \mu\text{M}$) after control measurements had been taken. This concentration is a saturating dose, fully blocking all IK_{Ca} channels in multicellular preparations (Haddock and Hill, 2002; Hinton and Langton, 2003). Measurements were made every 5–10 min to monitor the effects of TRAM-39 and to ensure that steady state block had occurred. Perfusion with TRAM-39 was continued for

at least 30–40 min to ensure that any lack of effect was not due to slow access. Increasing the concentration of TRAM-39 to 3 μM in three LAH neurons caused no further reduction in the amplitude of gKCa2. TRAM-39 was generously supplied by Dr. Heike Wullf and Dr. George Chandy (University of California Irvine) and dissolved in 20% dimethyl sulfoxide (DSMO) in 0.9% sodium chloride. The effect of the DSMO vehicle was tested in four LAH sympathetic neurons and had no effects on any of the measured parameters reported in this study. All values are expressed as mean \pm standard error of the mean (S.E.M.). All reported significant differences had P values <0.05 .

The RMPs of LAH neurons in this study were -58 ± 1 mV and the passive cell properties determined between -60 and -70 mV were similar to those previously reported (cell input resistance 92 ± 9 M Ω ; cell time constant 12 ± 2 ms, $n=13$) (Cassell and McLachlan, 1987). Brief (10 ms) depolarizing current steps initiated an action potential at a threshold of -27 ± 2 mV with an amplitude of 85 ± 2 mV and maximum rate of rise of 191 ± 12 V/s ($n=13$; Fig. 1A,C) (see Davies et al., 1999). This was followed by an AHP with an amplitude of 14 ± 1 mV and lasting 2.1 ± 0.3 s ($n=9$) (Fig. 1B). Current–voltage relations determined from a holding potential of -65 mV were linear between -90 and -60 mV (Davies et al., 1999). The RMP of enteric AH neurons was more positive than that of LAH neurons being -51 ± 3 mV ($n=6$). AH neurons had similar passive membrane properties to those previously reported (cell input resistance 230 ± 25 M Ω ; cell time constant 14 ± 3 ms, $n=7$) (Jobling et al., 1993; Martinez-Pinna et al., 2000).

In LAH cells, a depolarizing step that initiated a single action current in voltage clamp, was followed by an outward current with two components: a fast decaying component, and a second component which rose to a peak at around 500 ms and then decayed slowly (Fig. 1D). These currents are referred to here as gKCa1 and gKCa2, respectively, and their properties (Table 1) were similar to those previously reported (Cassell and McLachlan, 1987).

The action potentials of all AH neurons had characteristic ‘humps’ on their falling phases (Fig. 2A,C) as previously reported (Schutte et al., 1995). A single action potential was initiated at a threshold voltage of -33 ± 3 mV and had an

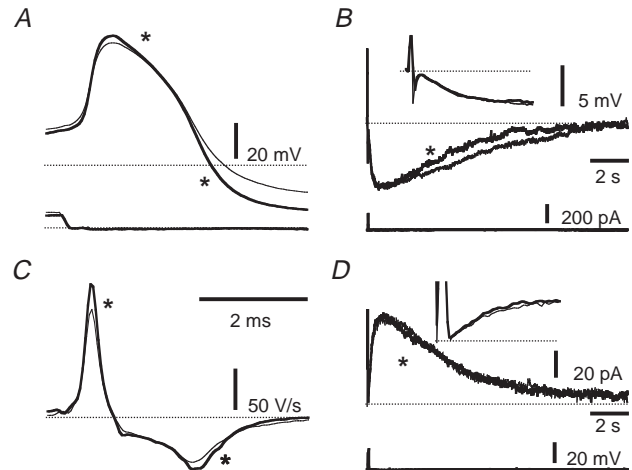


Fig. 2. Effect of TRAM-39 in an AH neuron in a guinea pig myenteric ganglion. Records show action potentials (A), afterhyperpolarizations (AHP) (B), voltage derivative of the action potentials (C), and the outward tail currents at a holding potential of -50 mV (D). Control and drug responses are superimposed with the latter shown as thicker lines (asterisk). (A) Addition of 1 μM TRAM-39 had little effect on the threshold or amplitude of the action potential. (B) The AHP after a single action potential (AP) evoked at -55 mV. Both the fast and slow component of the AHP in TRAM-39 are similar to that in control solution. (C) The rate of rise or repolarization of the action potential is unchanged by TRAM-39. (D) Outward tail current (upper trace) recorded in voltage clamp at a holding potential of -50 mV. TRAM-39 had no effect on gKCa2. RMP in (A) and (B) was -56 mV. Insets in (B) and (D) show expanded traces (700 ms) scaled in amplitude by 50%. Time scale in (C) also applies in (A).

amplitude of 69 ± 3 mV and a maximum rate of rise of 97 ± 22 V/s ($n=8$; Fig. 2A,C). The action potential was followed by an AHP with an amplitude of 12 ± 2 mV which lasted 6.5 ± 2.1 s ($n=7$) (Fig. 2B). In AH neurons, the AHP usually consisted of a fast transient component that decayed rapidly towards RMP, followed by a smaller more slowly developing component with a mean amplitude of 7 ± 1 mV ($n=7$). The peak of the slower component occurred 980 ± 170 ms ($n=7$) after the end of the action potential. In voltage-clamped AH neurons, a depolarizing step (50 ms) from a holding potential of -50 mV, which initiated a single action current produced an outward current with only one component which rose to a peak and then decayed slowly

Table 1

Effects on the properties of the AHP and underlying currents of addition of blocking IK_{Ca} channels of sympathetic LAH and enteric AH neurons

	Sympathetic LAH		Enteric AH	
	Control	TRAM-39	Control	TRAM-39
Peak AHP (mV)	14 ± 1	13 ± 1 (11)	12 ± 2	13 ± 2 (7)
AHP half-width (ms)	170 ± 90	130 ± 40 (11)	3540 ± 1110	3990 ± 760 (7)
AHP duration (ms)	2110 ± 320	2140 ± 320 (11)	8220 ± 2430	11120 ± 1640 (7)
gKCa1 amplitude (pA)	196 ± 33	153 ± 22 (12)	Absent	Absent
gKCa1 decay τ (ms)	130 ± 5	120 ± 6 (12)	Absent	Absent
gKCa2 amplitude (pA)	118 ± 35	$62 \pm 19^*$ (12)	357 ± 154	304 ± 75 (6)
gKCa2 decay τ (ms)	1200 ± 150	930 ± 120 (12)	4400 ± 570	5840 ± 1420 (6)

Numbers in brackets indicate the number of experiments. P values derived using paired t -tests.

* $P < 0.05$.

(Fig. 2D). The rise and decay of this current was well described by a double exponential function with time constants of 375 ± 160 ms and 4220 ± 580 ms ($n=7$). The delay for the start of the double exponential in AH neurons, was shorter (27 ± 5 ms, $n=7$) than that for sympathetic LAH neurons (cf. (Hirst et al., 1985)). Due to its similarity to the current observed in sympathetic LAH neurons, it is also referred to here as gKCa2 (see also Hirst et al., 1974). In a few neurons, an additional small, rapidly decaying component could also be observed immediately after the voltage step, but was difficult to accurately measure due to its small size. When this fast current could be identified, it had a time constant of decay of around 100 ms ($n=2$). As in sympathetic neurons (Cassell and McLachlan, 1987), the relationship between amplitude of gKCa2 and the number of action currents in myenteric neurons was linear up to 3 action potentials (1 action current, 304 ± 75 pA; 2 action currents 720 ± 303 pA; 3 action currents 985 ± 230 pA).

TRAM-39 (1 μ M) reduced the peak amplitude of gKCa2 in sympathetic LAH neurons (mean percentage change $56 \pm 23\%$ $n=12$; Fig. 1D), but not in myenteric AH neurons (Table 1, Fig. 2D). The magnitude of the reduction in gKCa2 was variable between LAH ganglion cells and there was no change in the time course of gKCa2 (Table 1) (Fig. 1D). However, TRAM-39 had no significant effects on RMP, passive properties or the action potential in either coeliac LAH or myenteric AH neurons, suggesting it had no effects on resting conductances or the voltage and calcium-activated currents associated with the action potential (Davies et al., 1999). Steady-state voltage–current relationships were unchanged by addition of TRAM-39. The block of gKCa2 in LAH neurons by TRAM-39 reached a steady state within 30 min of exposure and was partially reversible (recovery of $>50\%$ after 30 min) for those neurons ($n=5$) in which impalements were maintained for 30–40 min after return to control physiological salt solution (Fig. 1E). In three LAH neurons, increasing the concentration of TRAM-39 from 1 to 3 μ M produced no additional reduction in the amplitude of gKCa2, ($P>0.05$), confirming that 1 μ M was a saturating dose. We also tested the effect of TRAM-39 on the firing properties of LAH sympathetic neurons during a maintained depolarizing current step (500 ms). While TRAM-39 did not alter the firing patterns in the majority of coeliac ganglion cells, in 3 of 7 neurons tested, the addition of TRAM-39 increased the number of action potentials generated during a prolonged depolarizing current step.

As an additional test of the selectivity of the block by TRAM-39, we tested its effects in 1 phasic and 3 tonic neurons located in the coeliac ganglia. These neuron classes lack gKCa2 and the associated prolonged AHP (Cassell et al., 1986; Cassell and McLachlan, 1987) and display a single fast decaying outward current with a time constant around 100 ms. This conductance, called gKCa1 to distinguish it from the slower conductance (Cassell and McLachlan, 1987), is blocked by apamin and so is probably

mediated by SK-type channels (Jobling et al., 1993). In these cells, TRAM-39 had no effect on the properties of the AHP or gKCa1, nor was there any detectable change in the passive properties or the properties of the action potential.

These results indicate that blockade of IK_{Ca} -type channels reduces the prolonged current underlying the AHP in sympathetic neurons, but not the prolonged slow current underlying the AHP in enteric AH neurons. This suggests the channels underlying the slow AHP differ between these two neuron types.

This conclusion relies on the selectivity of TRAM-39 for IK_{Ca} -type Ca^{2+} -activated K^+ channels. We have not observed any evidence of nonspecific actions of TRAM-39 as has been seen with other IK_{Ca} blockers such as clotrimazole (Shah et al., 2001). TRAM-39 did not block voltage-dependent Na^+ or Ca^{2+} channels activated during the action potential as there was no detectable effect in either neuron type on the amplitude, threshold or maximum rate of depolarization of the action potential. TRAM-39 also did not affect the repolarization rate of the action potential suggesting that it did not block voltage-activated or BK-type K^+ channels activated during the action potential. Finally, TRAM-39 had no effect on the fast component of the AHP, gKCa1, due to activation of SK-type channels, in either LAH cells or phasic and tonic cells. The partial recovery following washout of TRAM-39 also further support the notion that the reduction in gKCa2 observed in LAH cells was dependent on TRAM-39 rather than time-dependent effects.

Blockade of IK_{Ca} type channels in sympathetic LAH neurons produced a partial and reversible reduction in the amplitude of gKCa2 suggesting that, at least in part, this conductance results from the activation of IK_{Ca} type channels. However, the block was only partial with just under half of gKCa2 attributable to the activation of IK_{Ca} channels, suggesting that other channel types are involved. The size of the TRAM-39-resistant component matches the proportion of gKCa2 previously shown to remain after combined block of SK- and BK-type channels in guinea pig coeliac LAH neurons (Martinez-Pinna et al., 2000). Further experiments using combined blockade of IK- and SK- and BK-type channels may resolve this. However, these results suggest the generation of the AHP in sympathetic neurons involves a diverse range of Ca^{2+} -activated K^+ channel types.

Calcium influx via L-, N- and P-type voltage-gated Ca^{2+} channels has been demonstrated to evoke the LAH in sympathetic coeliac ganglion cells (Davies et al., 1999; Martinez-Pinna et al., 2000). While it is not presently known whether all three Ca^{2+} channels contribute to the TRAM-39-sensitive component of the LAH, Ca^{2+} influx via each channel does not directly activate the channels underlying the LAH but rather the rise in cytoplasmic Ca^{2+} activates release from ryanodine-sensitive internal Ca^{2+} stores (Jobling et al., 1993).

Myenteric AH neurons express, in addition to BK-type channels, a TEA- and apamin-resistant IK_{Ca} channel with a

conductance of around 40 pS (Vogalis et al., 2002a). The classification of this channel on the basis of its kinetic and biophysical properties is at odds with our pharmacological approach using a specific blocker of IK_{Ca} channels. These channels are also blocked by addition of clotrimazole (Vogalis et al., 2002a). It is difficult to resolve these discrepancies on the basis of available evidence, unless the channels underlying the long AHP in myenteric AH neurons are IK_{Ca} type channels with novel pharmacological properties or at least properties different from those underlying $gKCa2$ in sympathetic LAH cells. This idea is consistent with previous findings that these channels in myenteric AH neurons are blocked by either iberiotoxin or charybdotoxin (Kunze et al., 1994) whereas native IK_{Ca} in other cell types are blocked by charybdotoxin, but are resistant to iberiotoxin (see Sah and Faber, 2002). Further work examining the pharmacological properties of these channels is clearly needed.

This study has provided evidence that using a blocker of IK_{Ca} channels, at least part of the slow AHP in sympathetic neurons may be due to activation of IK_{Ca} channels. Together with our previous observations of the pharmacological sensitivity of the slow AHP in sympathetic LAH neurons, Martinez-Pinna et al. (2000) suggest that it is due to the activation of multiple Ca^{2+} -activated K^+ channels.

Acknowledgments

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