Role for Calcium Signaling and Arachidonic Acid Metabolites in the Activity-Dependent Increase of AHP Amplitude in Leech T Sensory Neurons

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INTRODUCTION

The afterhyperpolarization (AHP) following action potentials and its modulation in amplitude and duration play an important role in determining changes in neuronal excitability (Schwind et al. 1988; Wu et al. 2004) often related with learning (Moyer et al. 2000; Oh et al. 2003; Saar et al. 1998; Sanchez-Vives et al. 2000; Seroussi et al. 2002). In the leech Hirudo medicinalis, tactile (T) cells exhibit an AHP after the discharge of action potentials. This AHP is mainly generated by the activation of molecular pathways, which finally may alter the activity of critical enzymes such as the Na+/K+ ATPase, that sustains the generation of the AHP and its increase during repetitive stimulation. These data indicate that a sophisticated cascade of intracellular signals links the repetitive discharge of T neurons to the activation of molecular pathways, which finally may alter the activity of the AHP, and we provide evidence that not only PLA2 but also the recruitment of arachidonic acid metabolites generated by the 5-lipoxygenase pathway are necessary for the induction of AHP increase. These data indicate that a sophisticated cascade of intracellular signals links the repetitive discharge of T neurons to the activation of molecular pathways, which finally may alter the activity of critical enzymes such as the Na+/K+ ATPase, that sustains the generation of the AHP and its increase during repetitive stimulation. These results also suggest the potential importance of the poorly studied 5-lipoxygenase pathway in forms of neuronal plasticity.

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the molecular mechanisms that link the repetitive neuronal activity of T cells with the increase of AHP. First, we analyzed a putative role for calcium as intracellular messenger. We found out that the AHP increase is triggered by calcium influx during neural activity and requires the release of calcium from intracellular stores. Because the AHP increase during repetitive stimulation is a phenomenon that is intrinsic to the single T cell and it depends on elevation of the cytosolic calcium concentration, we tested the involvement of the phospholipase A2 (PLA2) in its induction. We provide evidence that the AHP increase requires the activation of PLA2 and the recruitment of arachidonic acid metabolites generated by the 5-lipoxygenase pathway.

**METHODS**

**Animals**

Adult leeches, *H. medicinalis* (10-mo old), were obtained from Ricarimpex (Eysines, France). Animals were kept in an aquarium at 16°C in commercially available mineral water (Acqua Panna, Firenze, Italy) under natural daylight rhythm.

**Solutions**

All the experiments were performed in leech saline solution, which consisted of (in mM) 115 NaCl, 4 KCl, 1.8 CaCl2, and 10 glucose, buffered at pH 7.4 with 10 Tris-maleate.

To interfere with specific steps in the molecular cascades investigated in this work, we selected drugs previously tested on leech neurons, such as nifedipine, a L-type Ca2+ channel antagonist (Dierkes et al. 2004), and thapsigargin, a Ca2+ ATPase inhibitor (Schoppe et al. 1997; Trueta et al. 2004). We also applied blockers widely employed in both vertebrate and invertebrate, such as 8-((N,N-diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an inhibitor of intracellular Ca2+ release (Birkenbeil and Dedos 2002), 4-bromo phenacyl bromide (4-BPB), a blocker of the PLA2 (Kits et al. 1997; van Tol-Steyte et al. 1999), NDGA, an inhibitor of lipoxygenases (Kits et al. 1997; van Tol-Steyte et al. 1999), indomethacin, an inhibitor of cyclooxygenases (Kits et al. 1997; van Tol-Steyte et al. 1999), baicalein, an inhibitor of 12-lipoxygenase (Miller and Stanley 2004; Wang et al. 2004), and 3-[3-tert-butylthio-1-(4-chlorobenzil)-5-isopropyl-1H-indol-2-yl]-2,2-dimethylpropionio acid (MK886), a 5-lipoxygenase inhibitor (Mancini et al. 1998; Penzo et al. 2004). All drugs were from Sigma (St. Louis, MO). Except for TMB-8 and thapsigargin, which were directly added to the saline solution, all the other drugs used were previously dissolved in dimethylsulfoxide (DMSO) 1:1,000 and then added to saline solution. At this concentration, DMSO did not affect either the electrical properties of T cells or the increase of AHP during repetitive intracellular stimulation (data not shown).

**Electrophysiological recordings**

A short chain of ganglia was removed from mid-body level of anesthetized animals (dipped for 10 min in 10% ethanol in tap water) by cutting the body wall along the mid-line and by opening the ventral sinus. The surgery was carried out at room temperature. Ganglia were kept at 16°C in saline solution (see following text) for ≥1 h before the recording started.

For each experiment, a single ganglion was placed ventral side-up in a silicone elastomer (Sylgard)-lined (Dow Corning, Midland, MI) recording chamber. T cells were identified by the size and location of their cell bodies as well as by the profile of their firing pattern (Nicholls and Baylor 1968). Intracellular recordings from the soma were made with conventional current-clamp techniques using borosilicate sharp microelectrodes (Hilgerberg GmbH, Germany) filled with 4 M potassium acetate and having impedances of 60–80 MΩ. The signals were amplified with an appropriate electrometer. The data were filtered and digitized for analysis using National Instruments data-acquisition software with a BNC-2090 National Instruments series interface (National Instruments S.r.l., Milano, Italy). All experiments were carried out at room temperature.

The experiments focused on the modulation of the AHP in T neurons by repetitive intracellular stimulation (RIS). The AHP was induced by injecting 3-s trains of intracellular depolarizing impulses (200-ms, 2.5 Hz). During a single 200-ms impulse, T neurons fired a burst of seven to eight spikes, which reliably reproduced the pattern of discharge evoked by stimulation of their receptive field. The discharge frequency of a T neuron was kept constant during each series of trials by adjusting the amount of injected current ranging from 0.4 to 0.8 nA (Scuri et al. 2002). Because AHP amplitude is very sensitive to the quality of the impalement, only neurons with a resting membrane potential greater than −40 mV and input resistance (measured with 200-ms hyperpolarizing impulses, 0.5 nA) >70 MΩ were selected for this study. Unless otherwise stated, T neuron RIS consisted of a series of 10 3-s trains of depolarizing impulses of 200 ms at 2.5 Hz, spaced 15 s apart. This pattern of stimulation has been shown to reliably induce a sustained increase in the AHP amplitude (Scuri et al. 2002). After each train, the AHP amplitude was measured from the baseline of the resting potential before the stimulation to the peak of maximal hyperpolarization (Scuri et al. 2002).

The protocol used to test the effect of a selective drug on the increase of the AHP consisted of subsequent sessions of RIS. The AHP increase was first induced during perfusion with saline. After 20 min of rest, the ganglion was perfused for 15 min with saline containing the selected drug, and then a second session of RIS was delivered. Additional sessions of RIS were also presented at different time points during washout. AHP amplitude was measured after each train during RIS sessions, and the increase of the AHP was calculated as percentage increment of the amplitude between the AHP recorded after the 1st train (AHP1) and that recorded after the 10th train (AHP10) for each RIS session.

**Statistical evaluation**

Descriptive statistics are given as means ± SE. Statistical analysis was done using the Wilcoxon test for dependent groups because the data were not normally distributed. Statistical analysis was performed by using the Statistica software package (StatSoft, Tulsa, OK).

**RESULTS**

**Role of calcium in induction of AHP increase during RIS**

In this study, we performed a series of experiments to clarify the cascade of intracellular signals that might link repetitive neural activity with the induction of the increase of AHP during RIS. Because the activation of molecular pathways in response to neural activity can be triggered by an increase in the cytosolic calcium (for reviews see Berridge 1998; Blackstone and Sheng 1999), we tested a putative role for Ca2+ in our model. Bath application of 25 μM nifedipine, a blocker of L-type calcium channels both in vertebrates (Miller 1992) and leech neurons (Dierkes et al. 2004), for 15 min before RIS prevented RIS from inducing changes in AHP amplitude (n = 10 cells, −3.22 ± 2.93%), whereas RIS elicited an increment of 48.89 ± 6.79% in saline (Wilcoxon test: P = 0.005) and after 20-min washout (62.77 ± 14.4%, Wilcoxon test: P = 0.028; Fig. 1, A and B). Treatments with other blockers of voltage-dependent Ca2+ channels (e.g., verapamil or dilit-
whereas RIS elicited increments of 29.87 ± 2.12% occurred 10 min after the application of the drug, and of 24.35 ± 3.76% after 30-min washout, respectively, which were both statistically significant when compared with the one obtained after TMB-8 treatment (Wilcoxon test: \( P = 0.005 \), and \( P = 0.028 \), respectively; Fig. 2A). Moreover, when 1 \( \mu \text{M} \) thapsigargin, a blocker of the calcium uptake in the intracellular stores (Schoppe et al. 1997), was bath-applied for 15 min, RIS induced an increase of the AHP amplitude (\( n = 11 \) cells, 39.3 ± 6.89%) that was significantly greater than that obtained both in saline (24.79 ± 5.5%, Wilcoxon test: \( P = 0.0015 \)) and after 20-min washout (17.8 ± 3.5%, Wilcoxon test: \( P = 0.0027 \); Fig. 2B). Taken together, these results suggest that both extra- and intracellular calcium play a role in the AHP increase during RIS.

**Role of the phospholipase A2 in induction of AHP increase during RIS**

In various model systems, an elevation of the intracellular concentration of calcium can activate cytosolic isoforms of the phospholipase A2 (PLA2) (Farooqui et al. 1997; Massicotte 2000; Yoshinara and Watanabe 1990). Therefore the activation of PLA2 seemed to be a step in the molecular cascade linking the activity-dependent elevation in the cytosolic concentration of calcium with the induction of AHP increase during RIS.

We tested the effect of the PLA2 blocking agent 4-BPB (Massicotte et al. 1990; Piomelli et al. 1987) in the increase of AHP induced by RIS. Bath application of 10 \( \mu \text{M} \) 4-BPB for 15 min prevented the increase of AHP (\( n = 6 \) cells, \(-5.9 \pm 2.89\%\)) normally induced by RIS either in saline (29.61 ± 7.35%; Fig. 3A) or after 20 min wash in saline (34.58 ± 7.47%;
We performed two series of experiments in which lipoxygenases and cyclooxygenases were blocked by NDGA and indomethacin, respectively (Kits et al. 1997; Piomelli et al. 1987; van Tol-Steye et al. 1999). Fifteen-minute bath application of 5 μM NDGA prevented the increase of AHP amplitude \((n = 9 \text{ cells}, 1.23 \pm 1.23\%\)} normally induced by RIS either in saline \((36.27 \pm 5.8\%\}; \text{Wilcoxon test: mean percentage increment in NDGA vs. saline } P = 0.008\)} or after 20-min wash \((34.27 \pm 6\%\}; \text{Wilcoxon test: mean percentage increment in NDGA vs. washout } P = 0.027; \text{Fig. 4A})\). In contrast, after 15-min perfusion with 5 μM indomethacin, the RIS-induced AHP increase \((n = 7 \text{ cells}, 36.6 \pm 9.04\%\)) did not differ from that measured either in saline \((36.27 \pm 6.34\%, \text{Wilcoxon test: } P = 0.182)\) or after 20-min wash \((36.53 \pm 4.4\%, \text{Wilcoxon test: } P = 0.685; \text{Fig. 4B})\).

Lipoxygenases metabolites of AA appeared to be involved in AHP increase during RIS in T neurons and, in particular, the metabolites derived from the 5-lipoxygenase pathway resulted playing a pivotal role in the induction of AHP increase. In fact, 15-min bath application of 20 μM MK886, a specific blocker of 5-lipoxygenase, prevented AHP increase during RIS \((n = 9 \text{ cells}, 6.18 \pm 9.45\%\}; \text{Wilcoxon test: mean percentage increment in MK886 vs. saline } P = 0.021, \text{MK886 vs. washout } P = 0.042\)), whereas 15-min bath application of 20 μM baicalein, which selectively blocks the 12-lipoxygenase, did not interfere with the activity-dependent increase of the AHP \((n = 9 \text{ cells}, 32.75 \pm 7.61\%; \text{Fig. 5B})\).

Lipoxygenase metabolites of arachidonic acid are necessary for the induction of AHP increase during RIS

The next step was to investigate the intracellular cascade(s) of second messengers that was triggered by the increase of cytosolic Ca\(^{2+}\) concentration during RIS-induced increase of the AHP amplitude. Arachidonic acid (AA) and its metabolites were potential candidates as second messengers involved in the molecular mechanisms underlying this phenomenon. Previously, it has been shown that the second-messenger cyclic AMP (cAMP) mediates depression of AHP amplitude in leech T neurons (Catarsi et al. 1993) as part of the biochemical cascade activated by serotonin (5HT) (Belardetti et al. 1984; Catarsi and Brunelli 1991), whereas cyclic GMP or AMP did not affect AHP amplitude (Catarsi et al. 1993). Moreover, in several systems, molecules of AA are cleaved from the plasma membrane by cytosolic isoforms of PLA2 in response to an elevation in the intracellular Ca\(^{2+}\) concentration (Clark et al. 1991; Lin et al. 1992), thus providing the substrate for further degradation by enzymes such as lipoxygenases and cyclooxygenases (Shimizu and Wolfe 1990). Therefore we analyzed the potential involvement of AA and its metabolites in AHP increase during RIS.

**FIG. 3.** Phospholipase A2 (PLA2) activity is required for the induction of the AHP increase during RIS. A: raw data from a single T neuron illustrating that the increase of AHP amplitude occurred in saline (top) and did not occur after 15-min perfusion with 10 μM 4-bromo phenacyl bromide (4-BPB, bottom). B: histogram summarizing the effect of treatment with 4-BPB on AHP amplitude during RIS. Percent increments measured after 15-min bath application of 4-BPB were different from the one measured in saline and during washout.

**FIG. 4.** The metabolites of arachidonic acid (AA) derived from the lipoxygenase pathway are involved in the AHP increase during RIS. A: 15-min bath application of 5 μM nordihydroguaiaretic acid (NDGA) abolished the RIS-induced increase of AHP in T neurons. Percentage increments measured in saline and after NDGA treatment were different. B: AHP increase occurred during RIS in T cells treated for 15 min with 5 μM indomethacin as well as when RIS was performed in saline solution and during washout.
influx of Ca\(^{2+}\) through nifedipine-sensitive voltage-gated channels during bursts of action potentials (Fig. 1) and release of Ca\(^{2+}\) from intracellular stores (Fig. 2). Interestingly, blocking the release of Ca\(^{2+}\) from intracellular pools fully prevented the increase of AHP during RIS, thus suggesting a calcium-induced calcium release mechanism.

The role of extracellular Ca\(^{2+}\) in the induction of the AHP increase is not completely clear mostly because detailed data about calcium channels and their pharmacology in leech T neurons are still poor (Beck et al. 2001; Dierkes et al. 2004; Kleinhaus and Angstadt 1995; Stewart et al. 1989). Previous studies have shown that the high magnesium solution utilized to block the chemical synapses reduced the AHP amplitude (Catarsi and Brunelli 1991) but did not prevent the AHP increase during RIS (Scuri et al. 2002). Similarly, CdCl\(_2\), which was used to block K/Ca conductances and which reduced \(\sim 30\%\) of the AHP amplitude (i.e., the contribution of the AHP due to the K/Ca conductances), did not prevent the increase of the AHP amplitude (Scuri et al. 2002). In 2004, Dierkes et al. have shown that many if not all leech neurons possess voltage-gated Ca\(^{2+}\) channels that are akin to vertebrate L-type channels. However, this does not mean that there is a single type of Ca\(^{2+}\) channels operating in leech neurons. In the present study, we show that when L-type Ca\(^{2+}\) channels are blocked by means of nifedipine application, the increase of the AHP amplitude during RIS does not occur either. It is possible that the concentration of nifedipine used in T cells abolishes the influx of the extracellular Ca\(^{2+}\) necessary for preventing the AHP increase but does not affect the AHP produced by the Na\(^{+}\)/K\(^{+}\) ATPase activity during each burst of action potentials.

Elevated cytosolic calcium can activate PLA2 (Clark et al. 1991; Massicotte 2000; Yoshinara and Watanabe 1990), which cleaves AA from membrane lipids in response to receptor-dependent as well as receptor-independent activation (Axelrod et al. 1988; Choi et al. 1995; Lin et al. 1992). A mechanism of activation of PLA2 that does not depend on recruitment of membrane receptors seems to be a candidate step in the molecular cascade underlying AHP increase because the increase of the AHP in T neurons is independent of receptor

**FIG. 5.** The 5-lipoxygenase pathway is involved in the AHP increase during RIS. A: histogram illustrating that the percent increment measured after 15-min bath application of 20 μM 3-(3-tert-butylinthio-1-(4-chlorobenzil)-5-isopropyl-1H-indol-2-yl)-2,2-dimethylpropionioc acid (MK886) was significantly reduced in comparison with that obtained in saline and during washout. B: bath application of 20 μM baicalein did not affect AHP increase during RIS. The percentage increment in baicalein vs. saline \(P = 0.690\), baicalain vs. washout \(P = 0.079\).

**DISCUSSION**

This study reveals a chain of intracellular events that may link repetitive activity to changes in the properties of neuronal functioning.

The bursts of action potentials elicited in T sensory neurons by repetitive electrical stimuli bring about activity-dependent changes in the intrinsic properties (i.e., increase of AHP amplitude). In fact, the activity-dependent increase of AHP is due to an enhancement of the Na\(^{+}\)/K\(^{+}\) ATPase activity and leads to a lasting depression of the synapse between T neurons and their followers (Scuri et al. 2002).

**Increase of AHP requires calcium from external and internal sources and the activation of cytosolic isoform of PLA2**

What are the signaling pathways that link the repetitive discharge of action potentials in T neurons to the lasting increase of the AHP? The findings reported in this study provide evidence that an intracellular cascade of second messengers, which is summarized in the scheme of Fig. 6, underlies the activity-dependent increase of AHP amplitude in T neurons. Specifically, repetitive electrical stimulation leads to an activity-dependent elevation of the cytosolic Ca\(^{2+}\) through
stimulation (Scuri et al. 2002). The treatment with 4-BPB not only provided evidence that PLA2 is necessary for AHP increase during RIS (Fig. 3) but also indicated that AA and/or its metabolites are involved in this phenomenon.

**Involvement of AA and its metabolites in activity-dependent plasticity in invertebrate and vertebrate models systems**

AA and its metabolites are involved in the modulation of synaptic and neuronal activity in several model systems (e.g., Feinmark et al. 2003; Guan et al. 2003; Nishizaki et al. 1999; Piomelli et al. 1987). In *Aplysia*, for examples, AA and its 12-lipoxygenase metabolites are required for presynaptic inhibition (Piomelli 1991; Piomelli et al. 1987), which is mediated by the tetrapeptide FMRFamide (Mackey et al. 1987; Small et al. 1992). FMRFamide reduces the efficacy of the sensory to motor synapse through an increase of cytosolic AA (Guan et al. 2003) that is further degraded by 12-lipoxygenase. Metabolites such as 12-hydroxyeicosatetraenoic acid (12-HPETE) and heparoxin A3 act as second messengers affecting the properties of K+ channels (Belardetti et al. 1989; Piomelli et al. 1987) as well as activating the p38-mitogen-activated protein kinase (Guan et al. 2003). In rats, 12-lipoxygenase metabolites mediate a form of long-term depression at hippocampal CA3 to CA1 synapses that depends on activation of metabotropic glutamate receptors (Feinmark et al. 2003). Interestingly, a glutathione conjugate of heparoxin A3 increases the AHP amplitude of hippocampal CA1 neurons (Pace-Asciak et al. 1990). Our findings show that, in the leech, the block of the lipoxygenase pathway by NDGA and, more specifically, of 5-lipoxygenase by MK886 prevented the progressive increase in the AHP amplitude during RIS (Fig. 5), whereas the blockade of cyclooxygenases by indomethacin was ineffective (Fig. 4). The molecular cascades activated during repetitive stimulation in the leech have some similarity with those activated by FMRFamide during presynaptic inhibition in *Aplysia* (i.e., the activation of PLA2, and the activation of lipoxygenases, but not cyclooxygenases), the main difference being that in the leech it is the 5-lipoxygenase to be involved in the activity-dependent increase of AHP, whereas in *Aplysia* it is the 12-lipoxygenase to be activated during synaptic depression.

The present knowledge about the physiological function of 5-lipoxygenase metabolites in the brain is still rather limited. The 5-lipoxygenase metabolites might potentiate the activity of several substrates, including the Na+/K+ ATPase (Fig. 6), which is responsible for the increase of AHP amplitude in T neurons during RIS (Scuri et al. 2002). The activity of the Na+/K+ ATPase has been reported to be affected by free fatty acids (Swarts et al. 1990) as well as by cyclooxygenase (Cohen-Luria et al. 1993; Satoh et al. 1993) and P450 monoxygenase-derived metabolites of AA (Schwartzman et al. 1987), whereas the role of lipoxygenase-derived metabolites of AA as possible regulators of Na+/K+ ATPase activity is up to now poorly known. Foley (1997) demonstrated that 5-hydroxyeicosatetraenoic acid (5-HPETE) inhibits Na+/K+ ATPase in synapticosomal membrane preparations from rat cerebral cortex. This inhibition of Na+/K+ ATPase activity by 5-HPETE may result from an action of 5-HPETE either directly on the Na+/K+ ATPase or on a protein with a regulatory function on the Na+/K+ ATPase. Interestingly, AA has a positive effect on the Na+/K+ ATPase in the mouse diaphragm (Vyskocil et al. 1987) as well as in brain microsomes (Vyskocil et al. 1988). However, in the leech, further experiments are required to establish the actual mechanisms through which 5-lipoxygenase metabolites can modulate cellular substrates including the Na+/K+ ATPase activity.

**Is the bidirectional modulation of AHP a mechanism for the expression of different forms of synaptic plasticity?**

In T cells, widely arborized, the modulation of the AHP amplitude seems to play an important role in determining the propagation of the action potentials that travel along the extensively branched neurites (Yau 1976; for review, see Debanne 2004). In particular, a failure in the conduction of the action potentials reaching synaptic terminals may occur when the AHP amplitude is large and the pump rate increased (Cataldo et al. 2005; Lombardo et al. 2004).

The present study unveils some of the molecular steps underlying the activity-dependent increase of the AHP. In leech T neurons, the AHP is also modulated by 5HT (Belardetti et al. 1984). 5HT, which is involved in both sensitization and dishabituation in the leech (Belardetti et al. 1982; Catarsi et al. 1990; Sahley 1995; Zaccardi et al. 2004), depresses AHP amplitude via cAMP (Belardetti et al. 1984; Catarsi and Brunelli 1991; Catarsi et al. 1993), suppresses AHP increase induced by RIS (Scuri et al. 2002), and reduces conduction blocks in T neurons (Mar and Drapeau 1996), thus producing effects that are opposite to those induced by repetitive stimulation. Moreover, the elevation of cAMP induced by 5HT causes an inhibition of the Na+/K+ ATPase activity, resulting in the decrement of AHP (Catarsi et al. 1993). Therefore these results allow outlining an interesting general scheme, in which the bidirectional modulation of the AHP induced by the activation of different intracellular pathways may exert a role in different forms of synaptic plasticity.

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**References**


MOLEcular MECHANISms OF THE AHP INCRease IN THE LEECH


