Modulation of the Ca\(^{2+}\)-Activated K\(^{+}\) Current \(sI_{\text{AHP}}\) by a Phosphatase-Kinase Balance Under Basal Conditions in Rat CA1 Pyramidal Neurons

PAOLA PEDARZANI, MICHAEL KRAUSE, TRUDE HAUG, JOHAN F. STORM, AND WALTER STÜHMER

1Department of Molecular Biology of Neuronal Signals, Max-Planck-Institute for Experimental Medicine, 37075 Gottingen, Germany; and 2Institute of Physiology, University of Oslo, 0317 Oslo, Norway

Pedarzani, Paola, Michael Krause, Trude Haug, Johan F. Storm, and Walter Stühmer. Modulation of the Ca\(^{2+}\)-activated K\(^{+}\) current \(sI_{\text{AHP}}\) by a phosphatase-kinase balance under basal conditions in rat CA1 pyramidal neurons. J. Neurophysiol. 79: 3252–3256, 1998. The slow Ca\(^{2+}\)-activated K\(^{+}\) current, \(sI_{\text{AHP}}\), underlying spike frequency adaptation, was recorded with the whole cell patch-clamp technique in CA1 pyramidal neurons in rat hippocampal slices. Inhibitors of serine/threonine protein phosphatases (microcystin, calyculin A, cantharidin acid) caused a gradual decrease of \(sI_{\text{AHP}}\) amplitude, suggesting the presence of a basal phosphorylation-dephosphorylation turnover regulating \(sI_{\text{AHP}}\). Because selective calcineurin (PP-2B) inhibitors did not affect the amplitude of \(sI_{\text{AHP}}\), protein phosphatase 1 (PP-1) or 2A (PP-2A) are most likely involved in the basal regulation of this current. The ATP analogue, ATP-\(\gamma\)-S, caused a gradual decrease in the \(sI_{\text{AHP}}\) amplitude, supporting a role of protein phosphorylation in the basal modulation of \(sI_{\text{AHP}}\). When the protein kinase A (PKA) inhibitor adenine-3’,5’-monophosphorothioate, Rp-isomer (Rp-cAMPS) was coapplied with the phosphatase inhibitor microcystin, it prevented the decrease in the \(sI_{\text{AHP}}\) amplitude that was observed when microcystin alone was applied. Furthermore, inhibition of PKA by Rp-cAMPS led to an increase in the \(sI_{\text{AHP}}\) amplitude. Finally, an adenylyl cyclase inhibitor (SQ22,536) and adenine 3’,5’-cyclic monophosphate-specific type IV phosphodiesterase inhibitors (Ro 20–1724 and rolipram) led to an increase or a decrease in the \(sI_{\text{AHP}}\) amplitude, respectively. These findings suggest that a balance between basally active PKA and a phosphatase (PP-1 or PP-2A) is responsible for the tonic modulation of \(sI_{\text{AHP}}\), resulting in a continuous modulation of excitability and firing properties of hippocampal pyramidal neurons.

INTRODUCTION

The slow Ca\(^{2+}\)-activated K\(^{+}\) current, \(sI_{\text{AHP}}\), underlying the slow afterhyperpolarization and spike frequency adaptation in hippocampal and neocortical neurons (Alger and Nicoll 1980; Hotson and Prince 1980; Lancaster and Adams 1986; Schwartzkroin and Stafstrom 1980) represents one of the best-studied examples of neuromodulation in the vertebrate CNS. Many neurotransmitters, including various monoamines (norepinephrine, serotonin, histamine, and dopamine), acetylcholine, and glutamate, suppress \(sI_{\text{AHP}}\), thereby enhancing neuronal excitability, decreasing spike frequency adaptation (Sah 1996), and most likely contributing to shifts in the overall functional state of the brain (McCormick and Williamson 1989). Monoamine transmitters modulate \(sI_{\text{AHP}}\) via adenosine 3’,5’-cyclic monophosphate (cAMP) and protein kinase A (PKA) in CA1 pyramidal neurons (Blitz et al. 1994; Madison and Nicoll 1986; Pedarzani and Storm 1993, 1995; Torres et al. 1995), whereas other kinases have been proposed to mediate the suppression of \(sI_{\text{AHP}}\) by muscarinic and metabotropic glutamate receptor agonists (Abdul-Ghani et al. 1996; Gerber et al. 1992; Müller et al. 1992; Pedarzani and Storm 1996; Sim et al. 1992).

One line of evidence supporting the role of protein phosphorylation in the modulation of \(sI_{\text{AHP}}\) in hippocampal neurons was provided by experiments in which protein phosphatases were blocked. This resulted in a gradual suppression of \(sI_{\text{AHP}}\), suggesting the presence of an on-going phosphorylation-dephosphorylation turnover regulating \(sI_{\text{AHP}}\) in the absence of stimulation by neurotransmitters (Müller et al. 1992; Pedarzani and Storm 1993). A similar basal modulation has been proposed to take place for example in Aplysia sensory neurons (Ichinose and Byrne 1991) and in frog heart, where the L-type Ca\(^{2+}\) current and the delayed rectifier current \(I_{\text{K}}\) are modulated by a tonic balance between the basal activity of kinases and phosphatases (Frace and Hartzell 1993).

In the present study, we sought to investigate the molecular components involved in the basal modulation of \(sI_{\text{AHP}}\), i.e., in the absence of synaptic stimulation or exogenous neurotransmitter application, in hippocampal neurons.

METHODS

Transverse hippocampal slices (400- \(\mu\text{m}\) thick) were prepared from 18- to 28-day-old Wistar rats decapitated under halothane anesthesia, transferred to a humidified holding chamber, and allowed to recover for \(\approx 1\) h. During recording, the slices were superfused with extracellular medium (2.5 ml/min) containing (in mM) 125 NaCl, 25 NaHCO\(_3\), 1.25 KCl, 1.25 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), 1.5 MgCl\(_2\), and 16 glucose and saturated with 95% O\(_2\)-5% CO\(_2\) at 1-24°C. Bicuculline (10 \(\mu\text{M}\)), tetrodotoxin (0.5 \(\mu\text{M}\)), and tetraethylammonium (TEA; 5 mM) routinely were added to the medium. Whole cell recordings were obtained from CA1 pyramidal cells in the slice, using the “blind” method (Branton et al. 1989). The patch pipettes were filled with a control solution containing (in mM): 140 potassium gluconate (140 potassium methylsulfate in a subset of experiments), 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 2 Na\(_2\)-ATP, 3 MgCl\(_2\), and 0.4 Na\(_2\)-GTP (pipette resistance: 5–9 MΩ). ATP-\(\gamma\)-S (0.5–1 mM; Boehringer Mannheim) substituted Na\(_2\)-ATP in the corre-
sponding experiments described in RESULTS section. Using an EPC-9 amplifier (Heka Elektronik), the cells were voltage-clamped at −70 mV and depolarizing steps (100-ms long) of sufficient amplitude (typically +60 to +70 mV) to elicit a robust, unclamped Ca\(^{2+}\) action current were applied once every 30 s. The access resistance (range 15–35 MΩ) and the amplitude and time course of the Ca\(^{2+}\) current during the step were monitored and showed only minimal variations during the recordings included in this study. With the control pipette solution, \(s_{I_{AHP}}\) often showed a time-dependent increase in both amplitude and duration within a few minutes after establishing the whole cell recording mode (‘‘run-up’’) (see also Zhang et al. 1995). For comparisons, we therefore usually used the traces recorded after the first 10–15 min after achieving the whole cell configuration to allow completion of the run-up. The current traces were filtered at 250 Hz, sampled at 1 kHz, and stored on a Power Macintosh 7100/66 using Pulse v8.00 (Heka Elektronik) or filtered at 10 kHz and stored on videotape. Data analysis was performed using the programs Pulsefit v8.00 (Heka Elektronik) and Igor Pro 2.04 (WaveMetrics). Stock solutions of microcystin LR, calyculin A, FK-506, Ro 20–1724, and rolipram were prepared in dimethyl sulfoxide (DMSO), aliquoted, and kept frozen at −20°C until use. The final concentration of DMSO was invariably <0.5% and did not affect \(s_{I_{AHP}}\) in control recordings. Recordings with drugs and controls were normally intercalated. Microcystin-LR, rolipram, and calcineurin autoinhibitory peptide were obtained from Calbiochem; tetrodotoxin, bicuculline methylchloride, 9-(tetracydro-2-furyl)adenine (SQ22,536), Ro 20–1724, and isoproterenol hydrochloride from Research Biochemicals; calyculin A and cantharidic acid from LC Laboratories; adenosine-3',5'-monophosphorothioate, Rp-isomer (Rp-cAMPS) from BIOLOG Life Science Institute; tetraethylammonium from Sigma. FK-506 was a generous gift of Fujisawa. Dr. Angus Nairn generously provided the PP1 inhibitory peptide, phospho-DARPP-32 peptide [6–39] (Hemmings et al. 1990), which was synthesized in Dr. Nairn’s laboratory in Rockefeller University, NY. Values are reported as means ± SE. Two-tailed Student t-test and analysis of variance were used for statistical comparisons between groups (\(\alpha = 0.05\)).

RESULTS

Whole cell recordings (Blanton et al. 1989) were obtained from 80 CA1 pyramidal cells in rat hippocampal slices. In 98% of the cells, a typical slow afterhyperpolarization (AHP) current (\(s_{I_{AHP}}\)) (Lancaster and Adams 1986) followed a Ca\(^{2+}\) current elicited by a short depolarizing step. After the initial run-up period (see METHODS and Fig. 1A), \(s_{I_{AHP}}\) remained relatively stable for the duration of the recording, normally 1–2 h (12.6 ± 2.5% peak amplitude decrease after 60–75 min; \(n = 6\); Fig. 1A).

As previously shown (Pedarzani and Storm 1993), intracellular application of the phosphatase inhibitor microcystin (5–50 \(\mu\)M) caused a dose-dependent reduction in \(s_{I_{AHP}}\) amplitude (10–50 \(\mu\)M; \(n = 4\); Fig. 1B; 5 \(\mu\)M; \(n = 2\); not shown).

Two other protein phosphatase inhibitors, calyculin A (5–10 \(\mu\)M; \(n = 4\); Fig. 1, C and D) and cantharidic acid (10 \(\mu\)M; \(n = 3\); Fig. 1, E and F), also gradually reduced the \(s_{I_{AHP}}\) amplitude when applied in the patch pipette. At the relatively high concentrations used, microcystin and cantharidic acid can also inhibit the Ca\(^{2+}\)-calmodulin–dependent protein phosphatase 2B (PP-2B or calcineurin; \(IC_{50} = 200\) nM for microcystin and 30 \(\mu\)M for cantharidic acid) (Honkainen 1993; Honkanen et al. 1990), beside protein phosphatase 1 (PP-1) and 2A (PP-2A). To investigate the possible involvement of PP-2B in the basal modulation of \(s_{I_{AHP}}\), we used two specific PP-2B inhibitors, FK-506 and calcineurin autoinhibitory peptide. When intracellulary applied, neither FK-506 (10–200 \(\mu\)M; \(n = 11\); Fig. 1, G and H) nor the calcineurin autoinhibitory peptide (1 \(m\)M; \(n = 6\); not shown) produced any effect on \(s_{I_{AHP}}\), suggesting a lack of involvement of PP-2B in the basal modulation of \(s_{I_{AHP}}\).

To determine whether the effects of the broad spectrum

\[\text{FK-506 (10 ± 200 \(m\)M) often showed a time-dependent increase in both amplitude and duration within a few minutes after establishing the whole cell recording mode (‘‘run-up’’).} \]
Phosphatase inhibitors were solely a consequence of PP-1 inhibition, we tested a specific PP-1 peptide inhibitor, phospho-DARPP-32 peptide (Hemings et al. 1990). The PP-1 peptide inhibitor failed to show a significant effect on the $I_{AHP}$ (Fig. 2A; not shown).

The involvement of protein phosphorylation in the basal modulation of $I_{AHP}$ was further tested with the ATP analogue ATP-$\gamma$S, a substrate for protein kinases causing thio-phosphorylation of target proteins, which are then resistant to dephosphorylation by protein phosphatases. Further, we tried to prevent $I_{AHP}$ run-down by applying the specific protein kinase A (PKA) inhibitor Rp-cAMPS (500 $\mu$M) (Botelho et al. 1988). When this inhibitor was applied intracellularly together with microcystin (10 $\mu$M), no run-down in the $I_{AHP}$ was observed ($n=13$; Fig. 2C–E), in contrast to the marked reduction of $I_{AHP}$ produced by the same dose of microcystin applied alone ($P=0.0003$; Figs. 1B and 2C). This suggests that a balance exists between the basal activity of PKA and of a phosphatase (PP-1 or PP-2A) modulating $I_{AHP}$ in the absence of an exogenous stimulation of the cAMP cascade. This conclusion is consistent with the larger amplitude of $I_{AHP}$ observed in cells loaded with Rp-cAMPS (40.6 ± 8.1 pA; $n=5$) in comparison with control cells (26.2 ± 2.6 pA; $n=21$; $P=0.04$; Fig. 2F). Also with methylsulfate as main anion in the pipette solution, the amplitude of $I_{AHP}$ was more than twice as large (246%) in cells recorded with Rp-cAMPS ($n=18$) as in control cells ($n=12$; $P=0.0005$; not shown). To further test if the basal activity of the cAMP cascade tonically modulates the $I_{AHP}$ amplitude, we tried to inhibit production and breakdown of cAMP. Application of the adenylyl cyclase inhibitor SQ22,536 induced a small but consistent increase in the size of $I_{AHP}$ ($n=4$; $P=0.06$; Fig. 3A and C), suggesting that $I_{AHP}$ is inhibited tonically by cAMP produced by basally active adenylyl cyclase. Furthermore, two inhibitors of cAMP-phosphodiesterase type IV (Beavo and Reifsnyder 1990), Ro 20–1724 (200 $\mu$M; $n=2$; Fig. 3B) and rolipram (250 $\mu$M; $n=5$; Fig. 3B and D), caused a gradual decline of $I_{AHP}$ ($P=0.02$). The effect of rolipram was observed in four of five cells tested. Taken together, these observations support the hypothesis of a steady-state basal modulation of $I_{AHP}$.
sI_AHP due to a tonic level of activity of the cAMP-PKA system in CA1 neurons.

**Discussion**

The results presented in this study provide converging lines of evidence that basal phosphorylation and dephosphorylation activities exert a tonic modulatory effect on sI_AHP in the absence of exogenous stimulation in CA1 hippocampal pyramidal neurons. The basal phosphorylation activity is provided by the cAMP signaling cascade activating PKA, whereas the dephosphorylation is most likely provided by PP-1 or PP-2A.

Considering the different chemical structure of calyculin A, cantharidine, and microcystin, the results obtained with these protein phosphatase inhibitors argue against the possibility that sI_AHP inhibition is due to some unspecific effects of these drugs on sI_AHP or Ca^{2+} channels. We used relatively high concentrations of the inhibitors compared with the reported K_i values (Honkanen 1993; Honkanen et al. 1990; Ishihara et al. 1989; MacKintosh and MacKintosh 1994) because lower concentrations produced only a partial inhibition of sI_AHP probably due to limited diffusion (Pedarzani and Storm 1993). Consequently, we could not rely on the different specificity of microcystin, calyculin A, and cantharidine for PP-1, PP-2A, and PP-2B (PP-2C could be excluded because it does not seem to be inhibited by microcystin) to deduce which phosphatase is mainly responsible for the basal modulation of sI_AHP. However, our results with selective inhibitors of PP-1 and PP-2B do not support an involvement of these enzymes in the basal modulation of sI_AHP. Because PP-2B is calmodulin dependent, this conclusion also is supported by our previous observation that intracellular application of a calmodulin-binding peptide did not significantly reduce sI_AHP (Pedarzani and Storm 1996).

Nevertheless, we cannot categorically exclude that PP-1 or PP-2B contribute to the basal modulation of sI_AHP. The results obtained by selectively inhibiting PKA with Rp-cAMPS indicate that this kinase is mainly responsible for the run-down of sI_AHP during phosphorylation and therefore for the steady-state modulation of sI_AHP under basal conditions. This result, which adds to the function of PKA stimulated by adenosine (A_1) or G-protein inhibitor GDP-β-S (Krause and Pedarzani, unpublished data) and the G-protein inhibitor GDP-β-S (Krause and Pedarzani, unpublished data) enhanced sI_AHP. Furthermore, the hypothesis of a tonic adenyl cyclase activity is supported by the enhancement of sI_AHP, sAHP, and spike frequency adaptation previously observed in response to adenosine (A_1) or γ-aminobutyric acid-B receptor stimulation (Gerber and Gähwiler 1994; Haas and Greene 1984).

In conclusion, our results support the idea of a continuous modulation of neuronal excitability through sI_AHP due to a reversible and balanced activity of phosphorylating and dephosphorylating systems constituting a cyclic cascade (Shacter et al. 1988).

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Address for reprint requests: P. Pedarzani, Dept. of Molecular Biology of Neuronal Signals, Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, 37075 Göttingen, Germany.

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