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

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The slow \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) 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 (Blitzer 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 \( sI_{\text{AHP}} \). 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 \( \text{Ca}^{2+} \) current and the delayed rectifier current \( I_K \) are modulated by a tonic balance between the basal activity of kinases and phosphatases (Frace and Hartnell 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-\( \mu \)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 room temperature (21–24°C). Bicuculline (10 \( \mu \)M), tetrodotoxin (0.5 \( \mu \)M), and tetrathylammonium (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 (Blanton 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-
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 (\( sI_{\text{AMP}} \)) (Lancaster and Adams 1986) followed a \( \mathrm{Ca}^{2+} \) current elicited by a short depolarizing step. After the initial run-up period (see METHODS and Fig. 1A), \( sI_{\text{AMP}} \) 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 \( sI_{\text{AMP}} \) 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 \( sI_{\text{AMP}} \) amplitude when applied in the patch pipette. At the relatively high concentrations used, microcystin and cantharidic acid can also inhibit the \( \mathrm{Ca}^{2+} \)-calmodulin–dependent protein phosphatase 2B (PP-2B or calcineurin; \( I_{\text{Ca}} = 200 \) nM for microcystin and 30 \( \mu \)M for cantharidic acid) (Honkanen 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 \( sI_{\text{AMP}} \), we used two specific PP-2B inhibitors, FK-506 and calcineurin autoinhibitory peptide. When intracellularly applied, neither FK-506 (10–200 \( \mu \)M; \( n = 11; \) Fig. 1, G and H) nor the calcineurin autoinhibitory peptide (1 mM; \( n = 6; \) not shown) produced any effect on \( sI_{\text{AMP}} \), suggesting a lack of involvement of PP-2B in the basal modulation of \( sI_{\text{AMP}} \).

To determine whether the effects of the broad spectrum phosphatase inhibitors microcystin, calyculin A, and cantharidic acid cause run-down of the slow \( \mathrm{Ca}^{2+} \)-activated \( \mathrm{K}^{+} \)current, \( sI_{\text{AMP}} \). Under control conditions, the \( sI_{\text{AMP}} \) amplitude was stable for the duration of the recording, except for the initial increase (run-up) during the first 10–15 min (A). In contrast, cells dialyzed with microcystin (B; 10 \( \mu \)M, ●; 50 \( \mu \)M, ■), calyculin A (C and D; 5 \( \mu \)M, ●; 10 \( \mu \)M, ■), and cantharidic acid (E and F; 10 \( \mu \)M) exhibited a gradual run-down in the \( sI_{\text{AMP}} \) amplitude during 30–80 min (13.1 ± 6%; 35.7 ± 3%, and 45.8 ± 12.4% \( sI_{\text{AMP}} \) left, respectively). On the contrary, intracellular application of the calcineurin (PP-2B) inhibitor FK-506 (G and H; 10 \( \mu \)M, ●; 200 \( \mu \)M, ■) did not significantly affect the \( sI_{\text{AMP}} \) amplitude during ≤60 min (85 ± 3.6%). Time ‘0 min’ in A, B, D, and F indicates the 1st current trace recorded after establishing the whole cell configuration. In C, calyculin A was 10 \( \mu \)M; in G, FK-506 was 200 \( \mu \)M. Scale bars: C, 50 pA and 4 s; E, 20 pA and 2 s; G, 20 pA and 4 s.
phosphatase inhibitors were solely a consequence of PP-1 inhibition, we tested a specific PP-1 peptide inhibitor, phospho-DARPP-32 peptide (Hemmings et al. 1990). The PP-1 peptide inhibitor failed to show a significant effect on the \( s_{I_{AHP}} \) (not shown).

The involvement of protein phosphorylation in the basal modulation of \( s_{I_{AHP}} \) was further tested with the ATP analogue ATP-\( \gamma \)-S, a substrate for protein kinases causing thio-phosphorylation of target proteins (Eckstein 1985). ATP-\( \gamma \)-S (0.5–1 mM) caused a gradual run-down in the \( s_{I_{AHP}} \) amplitude (\( n = 4; P = 0.0002; \) Fig. 2, A and B), thus supporting an involvement of protein phosphorylation in the basal modulation of \( s_{I_{AHP}} \).

To identify the protein kinase responsible for the basal phosphorylation, we tried to prevent \( s_{I_{AHP}} \) run-down caused by phosphatase inhibitors by coapplying 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 \( s_{I_{AHP}} \) was observed (\( n = 13; \) Fig. 2, C–E), in contrast to the marked reduction of \( s_{I_{AHP}} \) 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 \( s_{I_{AHP}} \) in the absence of an exogenous stimulation of the cAMP cascade. This conclusion is consistent with the larger amplitude of \( s_{I_{AHP}} \) 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 \( s_{I_{AHP}} \) 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 \( s_{I_{AHP}} \) 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 \( s_{I_{AHP}} \) (\( n = 4; P = 0.06; \) Fig. 3, A and C), suggesting that \( s_{I_{AHP}} \) 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 \( s_{I_{AHP}} \) (\( P = 0.02 \)). 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
basal modulation of $s_{\text{AHP}}$
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 $s_{\text{AHP}}$ 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, cantharidic acid, and microcystin, the results obtained with these protein phosphatase inhibitors argue against the possibility that $s_{\text{AHP}}$ inhibition is due to some unspecific effects of these drugs on $s_{\text{AHP}}$ or Ca$^{2+}$ channels. We used relatively high concentrations of the inhibitors compared to 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 $s_{\text{AHP}}$ probably due to limited diffusion (Pedarzani and Storm 1993). Consequently, we could not rely on the different specificity of microcystin, calyculin A, and cantharidic acid 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 $s_{\text{AHP}}$. 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 $s_{\text{AHP}}$. 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 $s_{\text{AHP}}$ (Pedarzani and Storm 1996). Nevertheless, we cannot categorically exclude that PP-1 or PP-2B contribute to the basal modulation of $s_{\text{AHP}}$.

The results obtained by selectively inhibiting PKA with Rp-cAMPS indicate that this kinase is mainly responsible for the run-down of $s_{\text{AHP}}$ during phosphatase inhibition and therefore for the steady-state modulation of $s_{\text{AHP}}$ under basal conditions. This result, which adds to the function of PKA in the modulation of $s_{\text{AHP}}$ by monoamine transmitters (Blitzer et al. 1994; Pedarzani and Storm 1993, 1995; Torres et al. 1995), is consistent with the gradual increase in $s_{\text{AHP}}$ induced by bath application of the broad-spectrum kinase inhibitor staurosporine (Gerber et al. 1992; Sim et al. 1992) and of Rp-cAMPS in cultured CA3 neurons (Gerber and Gähwiler 1994).

Our data, however, do not provide any direct indication of the target molecule being phosphorylated or dephosphorylated, thereby leading to the basal modulation of $s_{\text{AHP}}$. It recently has been proposed, for example, that serotonin suppresses $s_{\text{AHP}}$ by modulating intracellular calcium-induced calcium release (CICR) through the phosphorylation of CICR channels by PKA (Torres et al. 1996). Direct evidence to solve this issue can be provided only by biochemical and electrophysiological studies on AHP channels in isolation after purification or cloning.

Inhibition of adenylyl cyclase and of cAMP-phosphodiesterase type IV activity led to an increase or decrease in the $s_{\text{AHP}}$ amplitude, respectively, in agreement with results obtained with conventional microelectrode recordings on AHP (Madison and Nicoll 1986). These observations suggest that a tonic activity of adenylyl cyclase leads to the production of sufficient levels of cAMP to reduce $s_{\text{AHP}}$ under resting conditions. This cyclase activity does not seem to be due to the release of monoamine transmitters from presynaptic terminals because neither antagonists of various monoamine receptors, alone or in combination (Pedarzani and Storm, unpublished data), nor the G-protein inhibitor GDP-β-S (Krause and Pedarzani, unpublished data) enhanced $s_{\text{AHP}}$. Furthermore, the hypothesis of a tonic adenylyl cyclase activity is supported by the enhancement of $s_{\text{AHP}}$, sAHP, and spike frequency adaptation previously observed in response to adenosine (A1) 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 $s_{\text{AHP}}$ due to a reversible and balanced activity of phosphorylating and dephosphorylating systems constituting a cyclic cascade (Shacter et al. 1988).

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