Involvement of hyperpolarization-activated cation channels in synaptic modulation

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The frequency of miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons increases under the action of drugs that trigger a rise in the intracellular concentration of cyclicAMP. It is generally believed that this type of effect is mediated by protein kinase A. Here, we show that it largely depends on the activation of hyperpolarization-activated cation channels (Ih) by cyclicAMP. In mammals, Ih channels control membrane excitability, thanks to their function as ionic channels. Here, we show that the effect of Ih channels on glutamate release is not mediated by the depolarization induced by their activation and thus is not linked to the ionic channel aspect of Ih channels. This suggests that the Ih channel could be a bifunctional protein. *NeuroReport* 18:1231–1235 © 2007 Lippincott Williams & Wilkins.

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Introduction

Hyperpolarization-activated cation channels (Ih channels) are ionic channels that are, surprisingly, activated by hyperpolarization [1]. They were first identified in the heart, [2,3] but they are also widely distributed in the peripheral and central nervous system [1]. These channels activate slowly during hyperpolarization and become permeable to both Na^+ and K^+, which leads to some depolarization. As a result of these properties, Ih channels have been postulated to play a role in pacemaker activity [4,5] and in regulating neuronal excitability [6–8]. Moreover, Ih channels are activated not only by hyperpolarization but also by cAMP when this molecule binds to an intracellular site on the Ih channel [9,10].

The fact that Ih channels play an important role in regulating excitability and rhythmic activity of neurons is not disputed. By contrast, another of their postulated roles, their involvement in synaptic modulation and plasticity, is currently a matter for intense debate. Using ZD7288, a drug reputed to specifically block Ih channels; Beaumont and Zucker [11] proposed that serotonin enhanced synaptic transmission in crustacean neuromuscular junctions by triggering the generation of cAMP whose target was presynaptic Ih channels. These results on the crustacean neuromuscular junction prompted researchers to investigate the potential role of Ih in hippocampal mossy fiber long-term potentiation in mammals. The results of these experiments performed on slices, however, were contradictory [12,13].

Thus, a role of Ih channels in controlling neurotransmitter release has not yet been established in a central mammalian synapse. Here, we tackle this question using cultured mammalian hippocampal neurons and focusing on action-potential-independent neurotransmitter release.

Methods

All animal experiments were approved by the ethical committee of the University of Mons-Hainaut. They were carried out in accordance with the Council of the European Communities Directive of 24 November 1986 (86/609/EEC). Pregnant Wistar rats were euthanized with halothane and the 18-day-old embryos were removed. Hippocampi were dissected from these embryos [14]. Dissociated hippocampal cells were plated at a density of about 10 000 cells per cm^2 on coverslips coated with 0.01% poly-L-ornithine (Sigma, Saint Louis, Missouri, USA) for 30 min and with 10 μg/ml laminin (Roche Diagnostics Gmbh, Mannheim, Germany) for 2 h. We used a culture medium close to that set up by Brewer *et al.* [15], consisting of Neurobasal (Invitrogen, Carlsbad, California, USA) supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine (Invitrogen) and 1% fetal bovine serum (Invitrogen). All the experiments were carried out 10–16 days after plating.

Electrophysiology

Recordings were performed at room temperature (20–22°C) on neurons of pyramidal appearance, using the whole cell ruptured patch technique according to the procedure described by Ninan and Arancio [16]. The compositions of the patch electrode solution and extracellular perfusion solution (1 ml/min) were the same as those used by Ninan and Arancio [16]. Glycine (1 μM), picrotoxin (100 μM) and tetrodotoxin (1 μM) were added to the extracellular solution. The pH was 7.3. Osmolarity was adjusted to 330 mOsm with sucrose.

For miniature excitatory postsynaptic currents (mEPSCs) experiments, neurons were voltage clamped at −70 mV. Tetrodotoxin (1 μM) present in the extracellular perfusion suppressed action potentials. mEPSCs were recorded using an Axopatch 200 (Axon Instruments, Sunnyvale, California,

USA) and filtered at 1 kHz. The series resistance was monitored throughout all experiments. It ranged from 10 to 20 MΩ. Experiments varied by more than 20% were rejected. No electronic compensation for series resistance was employed. The input resistance of the recorded neurons ranged from 300 to 500 MΩ. The recordings were digitized (Digidata 1322A, Axon Instruments) and analyzed using the minianalysis program (version 4.0) from Synaptosoft, Inc. (Georgia, USA).

Drug treatments

The following compounds were used: forskolin (Alomone Labs Ltd, Jerusalem, Israel), IBMX (Sigma), tetrodotoxin (TTX, Latoxan, Valence, France), picrotoxin (Sigma), CNQX (Tocris Bioscience, Bristol, UK), D-APV (Tocris) and ZD 7288 (Tocris). Forskolin and IBMX were dissolved in dimethyl sulfoxide (DMSO); final concentrations of DMSO were 0.02% when forskolin and IBMX were present. Picrotoxin was dissolved in NaOH 0.1 mM. Other drugs were dissolved in distilled water.

Statistical analysis

The two variables checked above all were: (1) the frequency of mEPSCs (normalized to the basal values) and (2) the variation of membrane potential as a function of its baseline level. The results were expressed as the mean ± SEM. Statistical analyses were performed (if not specified otherwise) with analysis of variance (ANOVA). Each time the influence of a drug over time was studied, its statistical significance was assessed by one-way ANOVA with one repeated measure. Comparison of the influences over time caused by two or several different treatments on the two variables mentioned above was assessed statistically by two-way ANOVA with one repeated measure.

Results

Forskolin + IBMX-induced increase in frequency of the miniature excitatory postsynaptic currents

Application of forskolin (FSK, 50 μM), an activator of adenylate cyclase, and IBMX (30 μM), an inhibitor of phosphodiesterase, for 15 min produced a rapid increase in the frequency of spontaneous mEPSCs (Fig. 1a and b, $n=7$, $P<0.01$). The mEPSC frequency rose to 1053 ± 216% of baseline (mean ± SEM) at the end of the FSK + IBMX perfusion and reached a maximum of 1263 ± 193% of baseline 5 min later (Fig. 1b). Application of the FSK + IBMX solvent only, DMSO, at a concentration of 0.02%, did not significantly modify mEPSC frequency ($n=7$, $P=0.69$).

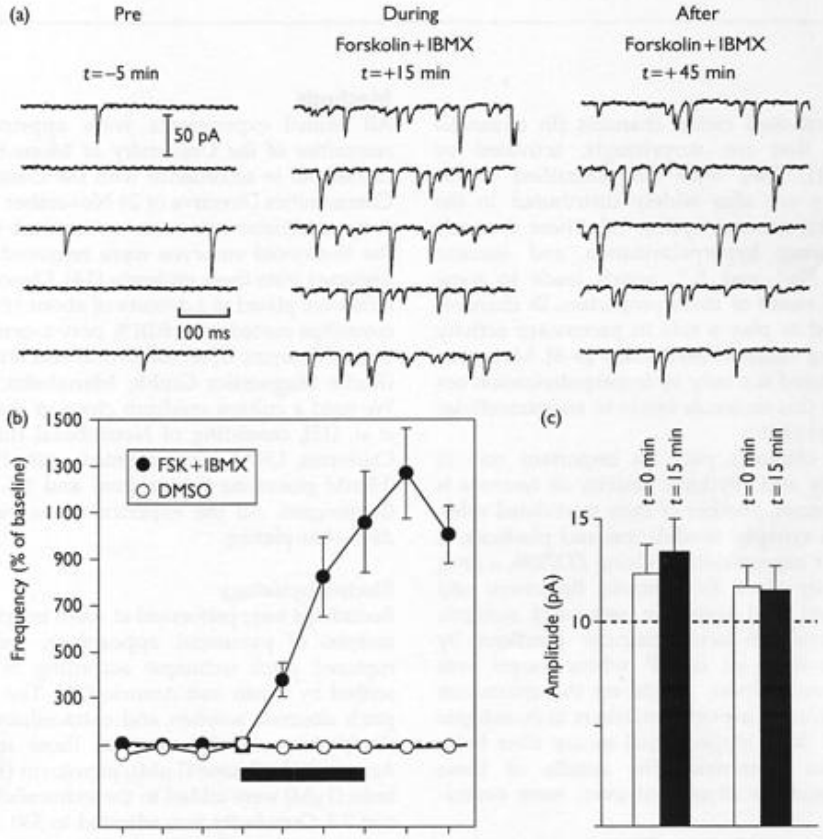

Fig. 1 Increase in mEPSC frequency triggered by pharmacological agents causing an increase in intracellular (cAMP). (a) Examples of spontaneous mEPSCs before (Pre), at the end of (During), and 30 min after (After) a 15-min coapplication of forskolin (FSK) and IBMX to cultured hippocampal neurons. (b) Average changes in mEPSC frequency during and after a 15-min perfusion of FSK + IBMX ($n=7$) and solvent DMSO 0.02% alone ($n=7$). For the sake of homogeneity, only neurons whose baseline mEPSC frequency ranged from 0.5/s to 5/s and remained stable during a 15-min control period were tested. (c) Mean mEPSC amplitudes before and just after application ($t=15$ min) of FSK + IBMX or DMSO alone.

The FSK + IBMX-induced increase in mEPSC frequency was significant compared with the DMSO condition ($P<0.001$). By contrast, FSK + IBMX did not induce any significant change in mEPSC amplitude (Fig. 1c). No difference was seen between the amplitudes of the mEPSCs measured just before and just after the end of FSK + IBMX perfusion (12.2 ± 1.4 pA versus 13.3 ± 1.6 pA, paired Student's *t*-test, $P=0.16$) or of a DMSO treatment (11.6 ± 1.0 pA versus 11.4 ± 1.2 pA, paired Student's *t*-test, $P=0.77$).

ZD 7288 and Cs^+ inhibit the increase in mEPSP frequency induced by Forskolin + IBMX

Then we tested the influence of ZD 7288 and Cs^+, two inhibitors of Ih channels, on the phenomenon described above [17]. Both ZD 7288 (10 μM) and Cs^+ (1 mM) caused a dramatic diminution in the increase in mEPSC frequency that would normally be triggered by FSK + IBMX ($n=7$, $P<0.001$ for ZD 7288 and $n=7$, $P<0.001$ for Cs^+) (Fig. 2a). Fifteen minute after the start of the perfusion of FSK + IBMX,

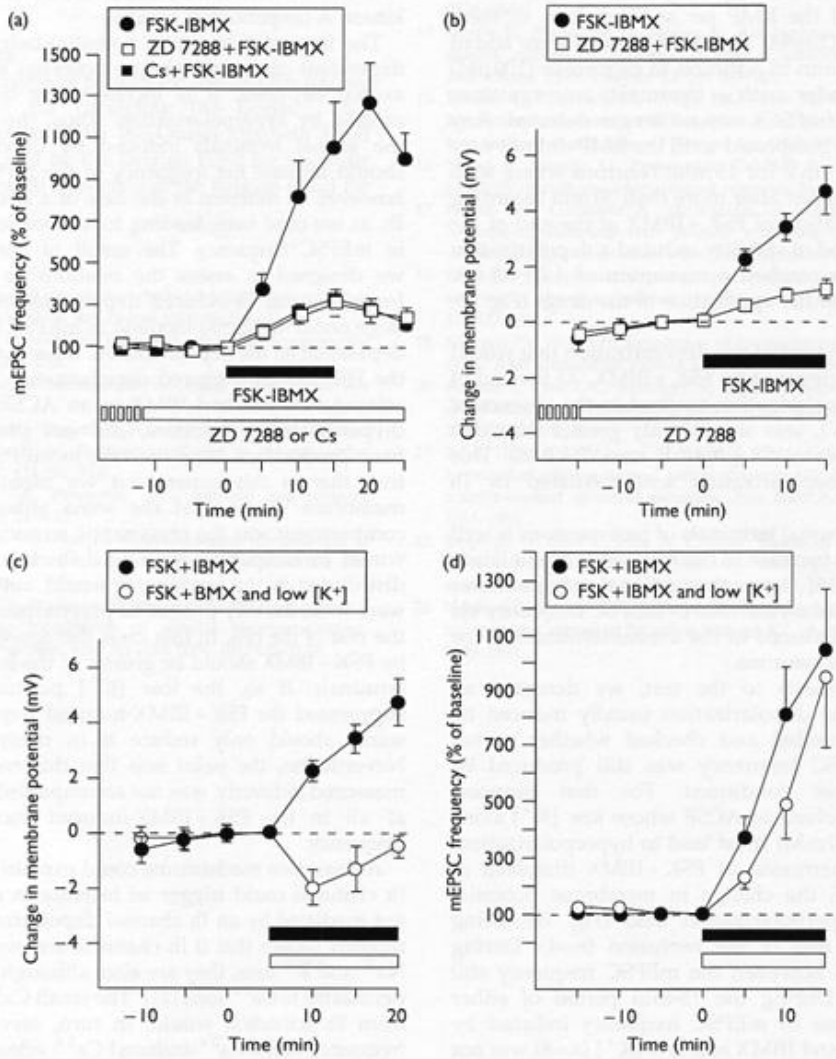

Fig. 2 ZD 7288, a membrane-permeable inhibitor of Ih channels, diminishes FSK + IBMX-induced increase in mEPSC frequency in cultured hippocampal neurons, but this effect is not due to the fact that ZD 7288 decreases the FSK + IBMX-induced depolarization. (a) Average changes in mEPSC frequency during and after a 15-min coapplication of FSK and IBMX ($n=7$, filled circles), ZD 7288 (10 μM) paired with FSK + IBMX ($n=7$, open squares) and Cs^+ (1 mM) paired with FSK + IBMX ($n=7$, filled squares). (b) Average change ($n=6$) in membrane potential induced by a 15-min coapplication of forskolin and IBMX in absence ($n=6$, filled circles) and in presence of ZD 7288 ($n=6$, open squares). Measurements were made in presence of TTX, APV, CNQX and picrotoxin. (c) Average changes in membrane potential induced by a 15-min application of FSK + IBMX dissolved in ASCF with the usual $[K^+]$ (5 mM, $n=6$ neurons, filled circles) or in ASCF where the $[K^+]$ was lowered (3.9 mM, $n=4$, open circles). When it was applied in the presence of low $[K^+]$, the perfusion of FSK + IBMX no longer caused a depolarization. Instead, a slight hyperpolarization was observed. (d) Average changes in mEPSC frequency induced by a 15-min application of FSK + IBMX in presence of 5 mM K^+ ($n=7$, filled circles) or of 3.9 mM K^+ ($n=8$, open circles).

the increase in mEPSC frequency was reduced to 315±71% of baseline under ZD 7288 influence and 326±85% under the action of Cs^+, two values significantly lower than the one measured in the absence of Ih antagonists (1053±216%, $P<0.05$ both for ZD 7288 and Cs^+).

Forskolin + IBMX-induced increase in neuro-mediator release is probably not mediated by the neuronal depolarization caused by Forskolin + IBMX

In another series of experiments, we measured the influence of FSK + IBMX application on the resting membrane potential (RMP). In such experiments, it was important to prevent the depolarization resulting from the FSK + IBMX-induced increase in mEPSC frequency from being mistaken for a modification of the RMP *per se*. Therefore, in these experiments, CNQX (20 μM) and APV (50 μM) were added to the perfusion medium in addition to picrotoxin (100 μM) and TTX (1 μM). Under such a treatment, any miniature postsynaptic current (mPSC) was no longer detected. Any drug application was postponed until the RMP did not vary outside a range of ±1 mV for 15 min. Neurons where such stability was not observed after more than 30 min recording were rejected. Application of FSK + IBMX at the end of the required 15-min period of stability induced a depolarization of the neuron, which reached a maximum of 4.7±0.8 mV 15 min after the start of the application of the drugs (Fig. 2b, $n=6$, $P<0.001$).

ZD 7288 (10 μM) prevented the depolarization that would normally have been triggered by FSK + IBMX. At the end of the perfusion, the depolarization reached in the absence of ZD 7288 (4.7±0.8 mV), was significantly greater than that measured in its presence (1.1±0.3 mV, $n=6$, $P<0.05$). This indicated that the depolarization was mediated by Ih channels.

Depolarization of axonal terminals of motoneurons is well known to produce an increase in the frequency of miniature end-plate potentials [18]. It was thus rational to hypothesize that FSK + IBMX caused an increase in mEPSC frequency via the depolarization it induced in the axonal terminals of the cultured hippocampal neurons.

To put this hypothesis to the test, we designed an experiment where the depolarization usually induced by FSK + IBMX was cancelled and checked whether or not an increase in mEPSC frequency was still produced by FSK + IBMX in these conditions. For that purpose, FSK + IBMX was dissolved in ACSF whose low $[K^+]$ alone (3.9 mM instead of 5.0 mM) must lead to hyperpolarization. During the 15-min perfusion of FSK + IBMX dissolved in that low $[K^+]$ ACSF, the change in membrane potential remained in the hyperpolarization field (Fig. 2c), being −0.5±0.4 mV at the end of the perfusion ($n=4$). During such a coapplication, however, the mEPSC frequency still increased (Fig. 2d). During the 15-min period of either treatment, the increase in mEPSC frequency induced by coapplication of FSK and IBMX and low $[K^+]$ ($n=8$) was not different from that observed when FSK + IBMX was administered in the usual ACSF ($n=7$, $P=0.34$). Thus, artificial cancellation of the FSK + IBMX-induced depolarization did not prevent FSK + IBMX from inducing a large increase in mEPSC frequency. This suggests that although the membrane potential was monitored at the soma level and not at the terminal level (Discussion), this effect was not dependent on the FSK + IBMX-induced depolarization.

Discussion

In cultured hippocampal neurons, we found that the action of FSK + IBMX (and hence cAMP generation) caused a large (10-fold) increase in the frequency of the mEPSCs without affecting their amplitude. This finding supports the assumption that the locus of action of FSK + IBMX is presynaptic.

This cAMP-triggered increase in mEPSC frequency being severely diminished by both ZD 7288 and Cs^+, two completely different Ih channel blockers, we conclude that this process is mediated by Ih channels. Nonetheless, this effect is not mediated exclusively by Ih channels but also by protein kinase A (PKA), another target of cAMP [19]. Indeed, the cAMP-triggered increase in mEPSC frequency was also diminished by KT 5720, an inhibitor of protein kinase A (unpublished results).

The frequency of action potential-independent mPSC is dependent on the membrane potential at the level of the axonal terminals: it is increased by depolarization, decreased by hyperpolarization. Thus, the depolarization of the axonal terminals induced by Ih channel activation should increase the frequency of the mPSCs [20]. It could, however, be different in the case of a strong stimulation of Ih, as we used here, leading to an increase of about 1000% in mEPSC frequency. The result of the experiment that we designed to assess the relationship between mEPSC frequency and Ih-induced depolarization showed that the large cAMP-triggered increase in mEPSP frequency was not dependent on the depolarization. Indeed, the suppression of the FSK + IBMX-triggered depolarization, achieved by dissolving forskolin and IBMX in an ACSF with a low $[K^+]$ (hyperpolarizing solution), did not prevent FSK + IBMX from producing a large increase in mEPSC frequency. It is true that in this experiment we monitored changes in membrane potential at the soma although the relevant compartment was the presynaptic terminal. This procedure would be completely accurate if Ih channels were equally distributed in the neurons; it would not be if Ih channels were more densely present in presynaptic terminals than in the rest of the cell. In this case, the depolarization induced by FSK + IBMX should be greater at the level of presynaptic terminals. If so, the low $[K^+]$ perfusion used, which suppressed the FSK + IBMX-induced depolarization at the soma, should only reduce it in presynaptic terminals. Nevertheless, the point was that this reduction, although measured indirectly, was not accompanied by any reduction at all in the FSK + IBMX-induced increase in mEPSC frequency.

At least two mechanisms could explain how activation of Ih channels could trigger an increase in mEPSC frequency not mediated by an Ih channel depolarization. First, it was recently shown that if Ih channels are overall permeable to Na^+ and K^+ ions, they are also, although to a lesser extent, permeable to Ca^{2+} ions [21]. The small Ca^{2+} influx resulting from Ih activation would, in turn, serve to increase the frequency of the Ca^{2+}-induced Ca^{2+} release from the stores present in the axonal terminals. This would explain the Ih activation-induced increase in mEPSC frequency, as spontaneous transmitter release is now known to be triggered by spontaneous Ca^{2+}-induced Ca^{2+} release [22]. In the dorsal root ganglion neurons, Yu *et al.* [21] also found that activation of Ih by hyperpolarization for 30 s, which was accompanied by a Ca^{2+} influx, resulted in an elevated action potential-induced secretion of neuro-mediator.

Second, as proposed for the crayfish neuromuscular junction, it could be that Ih was directly coupled to the release machinery [11]. In this case, like the ether-à-go-go (EAG) channel [23], Ih could be a bifunctional protein that not only forms an ion-conducting pore but also regulates an intracellular signaling pathway in a way that is completely independent of its job as an ionic channel.

Conclusion

Our results suggest that, in cultured hippocampal neurons, cAMP can act on presynaptic Ih channels to increase the spontaneous release of the neuro-mediator via a mechanism independent of the depolarization that is induced by Ih channel activation.

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