Neuroscience 357 (2017) 20–36

FORSKOLIN AND PROTEIN KINASE INHIBITORS DIFFERENTIALLY AFFECT HAIR CELL POTASSIUM CURRENTS AND TRANSMITTER RELEASE AT THE CYTONEURAL JUNCTION IN THE ISOLATED FROG LABYRINTH

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Abstract—The post-transductional elaboration of sensory input at the frog semicircular canal has been studied by correlating the effects of drugs that interfere with phosphorylation processes on: (i) potassium conductances in isolated hair cell and (ii) transmitter release at the cytoneural junction in the intact labyrinth. At hair cells, delayed potassium currents (IKD) undergo voltage- and time-dependent inactivation; inactivation removal requires ATP, is sensitive to kinase blockade, but is unaffected by exogenous application of cyclic nucleotides. We report here that forskolin, an activator of endogenous adenylyl cyclase, enhances IKD inactivation removal in isolated hair cells, but produces an overall decrease in IKD amplitude consistent with the direct blocking action of the drug on several families of K channels. In the intact labyrinth, forskolin enhances transmitter release, consistent with such depression of K conductances. Kinase blockers – H-89 and KT5823 – have been shown to reduce IKD inactivation removal and IKD amplitude at isolated hair cells. In the labyrinth, the effects of these drugs on junctional activity are quite variable, with predominant inhibition of transmitter release, rather than the enhancement expected from the impairment of K currents. The overall action of forskolin and kinase inhibitors on K conductances is similar (depression), but they have opposite effects on transmitter release: this indicates that some intermediate steps between the bioelectric control of hair cell membrane potential and transmitter release are affected in opposite ways and therefore are presumably regulated by protein phosphorylation.

Keywords: Semicircular canal, Hair cells, Potassium currents, Sensory discharge, Forskolin, Protein kinase inhibitors.

INTRODUCTION

Synaptic activity at the cytoneural junction of the frog semicircular canal is governed by the electrical activity at the hair cell, which is dynamically influenced by the receptor current (activated by stereocilia displacement) and basolateral currents (mainly potassium and calcium currents).

The bioelectric properties of hair cells have been studied in a number of preparations from different species. They display a remarkable variability among species and among hair cell subtypes in the same species, in terms of degree of expression of the various conductances, voltage- and time-dependence of channel kinetics and sensitivity to drugs (Fuchs and Evans, 1990; Kros and Crawford, 1990; Martini et al., 2009a; Meredith and Rennie, 2016). Regarding frog semicircular canals, a thorough and systematic characterization of each expressed conductance has been carried out in our laboratory. Furthermore, the bioelectric properties of hair cells and their sensitivity to experimental manipulations, studied by patch-clamping isolated hair cells from frog semicircular canals, have been correlated with the analysis of transmitter release and spike generation at the cytoneural junction of the posterior semicircular canal in the intact frog labyrinth. A number of specific observations were made in the past and some of them will be highlighted to provide the conceptual framework for the work presented here.

Hair cells isolated from frog semicircular canals display a voltage-dependent calcium current, ICa, and three K currents: a fast inactivating, transient IA and a delayed K current complex, IKD, that is comprised of a Ca-dependent IKCa and a purely voltage-dependent IKV. The IKD current displays slow depolarization-induced inactivation and biphasic removal of inactivation by hyperpolarization (Martini et al., 2009a).

In the absence of an adequate supply of ATP, K channels undergo a marked run-down (Martini et al., 2013), thereby suggesting that an active process must be operating to maintain their function. Given that K channels undergo a slow but relevant process of inactivation (with different kinetics for the various subtypes) even at normal resting potentials (less negative than −100 mV), ATP might be needed to sustain the process of K channel inactivation removal. Accordingly, blockers of kinases such as H-89 and KT5823 specifically interfere with such

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http://dx.doi.org/10.1016/j.neuroscience.2017.05.039
0306-4522/

Abbreviations and drugs

ForskolinH-89	(N-[2-(p-Bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide)
KT5823	(2,3,10,11,12hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H diindolo[1,2,3-fg:3 9,29,19-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester)
DMSO	dimethyl sulfoxide
PDE	phosphodiesterase
mEPSP	miniature excitatory postsynaptic potential
IRC	inactivation removal coefficient
IKD	compound delayed outward rectifier potassium current
IKV	purely voltage-dependent component of IKD
IKCa	voltage- and calcium-dependent component of IKD

process of inactivation removal (Martini et al., 2013); the effects of both drugs were selective since they did not interfere with IA or ICa. This suggests that inactivation removal depends on a phosphorylation process. So, channel phosphorylation may preserve the fraction of K channels that can be activated by depolarization, at any value of membrane potential. This process participates in defining the balance between the depolarizing receptor current and the repolarizing basolateral K currents, which is expected to control the dynamics of membrane potential and consequently transmitter release at the cytoneural junction.

In a previous study, direct application of cyclic nucleotides or synthetic analogs was shown to be unable to enhance the process of inactivation removal (Martini et al., 2013). This might be due to the reported compartmentalization of second messenger signaling pathways (McCormick and Baillie, 2014; Brescia and Zaccolo, 2016). In fact, cyclic nucleotides seem to be produced at specific localizations, close to their target, and their diffusion appears to be restricted, so that their levels are spatially heterogeneous in the cell. As a result, responses to exogenous cyclic nucleotides do not mimic endogenous production (Rich et al., 2000). The production of cAMP at specific sites may thus be needed to maintain kinases active and keep the channel phosphorylated.

Thus, we explored here the effects of forskolin, which is presumed to provide a continuous source of cAMP exactly where it is supposed to be physiologically produced and maintain a significant level of PKA activation at appropriate sites. We actually observed a direct effect of the drug, a voltage-independent block of K channels, that is consistent with similar effects reported for forskolin on other K channels in various preparations (Coombs and Thompson, 1987; Zünkler et al., 1988; Garber et al., 1990; Herness et al., 1997; Angel-Chavez et al., 2015). In addition to this, however, a specific enhancement of IKD inactivation removal was detected, as would be expected upon phosphorylation of the channel.

Since forskolin and kinase inhibitors markedly modulate IKD currents in isolated hair cells, we turned to the intact labyrinth to examine the effects of the same drugs on cytoneural activity. In particular, mEPSP size and frequency, as well as the corresponding spike rate, were analyzed by recording intracellularly the afferent activity, at rest and during rotation, from single fibers of the posterior canal in the isolated and intact frog labyrinth, before and after the application of forskolin, H-89 or KT5823 to the bathing solution.

The underlying assumption was that the sensory discharge in the intact preparation should be determined by hair cell membrane potential, which depends on the opposing actions of the transduction current and the basolateral repolarizing K-currents, the latter displaying complex sensitivity to the drugs used in this study. The results reported here suggest that the effects on the cytoneural activity of drugs that interfere with phosphorylation cannot be predicted from their actions on the sole bioelectric properties of the isolated hair cell, because further steps in the transmitter release process presumably are also sensitive to phosphorylation.

EXPERIMENTAL PROCEDURES

Experimental treatments

Our procedures followed the Principles of Laboratory Animal Care (86/609/EEC Directive of 1986), and were approved by the Italian Ministry of Health as well as by the Animal Care and Use Committee of the University of Ferrara. The experiments were performed at room temperature (20–22 °C) on frogs (*Rana esculenta*, 25–30 g body weight) purchased from authorized dealers. Animals were of the same geographical origin (Albania), and the experiments were preferably carried out in spring to minimize seasonally related variability. About one hundred frogs have been utilized in our experiments.

The frogs were anaesthetized in tricaine methane sulfonate solution (1 g/l in tap water) and subsequently decapitated.

PATCH CLAMP EXPERIMENTS

The composition of the solution employed in the present experiments to record K or Ca currents, the procedures to dissect the two labyrinths and isolate the hair cells, as well as the recording techniques, have been reported in previous papers (Martini et al., 2000, 2007, 2009a, 2009b, 2013, 2015).

The patch pipette solution always contained 8–10 mM ATP to avoid K current rundown during long-lasting recordings. Cadmium chloride (200 μM) was used as a blocker of voltage-dependent calcium channels. It was applied by rapidly changing (typically < 50 ms) the external solution through the computer-controlled