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# Involvement of Na,K-pump in SEPYLRFamide-mediated reduction of cholinosensitivity in *Helix* neurons

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**Abstract**

SEPYLRFamide acts as an inhibitory modulator of acetylcholine (ACh) receptors in *Helix lucorum* neurones. Ouabain, a specific inhibitor of Na,K-pump, (0.1 mM, bath application) decreased the ACh-induced inward current (ACh-current) and increased the leak current. Ouabain decreased the modulatory SEPYLRFamide effect on the ACh-current. There was a correlation between the effects of ouabain on the amplitude of the ACh-current and on the modulatory peptide effect. Ouabain and SEPYLRFamide inhibited the activity of *Helix aspersa* brain Na,K-ATPase. Activation of Na,K-pump by intracellular injection of 3 M Na acetate or 3 M NaCl reduced the modulatory peptide effect on the ACh-current. An inhibitor of Na/Ca-exchange, benzamil (25 μM, bath application), and an inhibitor of $Ca^{2+}$-pump in the endoplasmic reticulum, thapsigargin (TG, applied intracellularly), both prevented the effect of ouabain on SEPYLRFamide-mediated modulatory effect. Another inhibitor of $Ca^{2+}$-pump in the endoplasmic reticulum, cyclopiazonic acid (applied intracellularly), did not prevent the effect of ouabain on SEPYLRFamide-mediated modulatory effect. These results indicate that Na,K-pump is responsible for the SEPYLRFamide-mediated inhibition of ACh receptors in *Helix* neurons. Na/Ca-exchange and intracellular $Ca^{2+}$ released from internal pools containing TG-sensitive $Ca^{2+}$-pump are involved in the Na,K-pump pathway for the SEPYLRFamide-mediated inhibition of ACh receptors.


*Keywords:* SEPYLRFamide; Na,K-pump; Acetylcholine receptors; Released $Ca^{2+}$; Na/Ca-exchange; *Helix* neurons

## 1. Introduction

A large number of endogenous peptides with an RFamide carboxyl terminal (FMRFamide-related peptides/FaRPs/), occur in molluscs and other invertebrate phyla and vertebrates including mammals [50] which strongly consider their importance as extracellular signalling molecules. The FMRFamide family has important roles both as neurotransmitters and as neuromodulators in the central and peripheral nervous system [14,26,30,53].

Seven FMRFamide-related peptides were extracted and sequenced in the land snail, *Helix aspersa* [43]. There are two endogenous *Helix* tetrapeptides (FMRFamide, FLRFamide) and five heptapeptides (NDPFLRFamide, SDPFLRFamide, pQDPFLRFamide, NDPYLRFamide, SEPYLRFamide) [43].

Endogenous molluscan FMRFamide-related peptides (FaRPs): FMRFamide, SKPYMRFamide, SEPYLRFamide and its N-terminally modified analogues, EPYLRFamide, SEGYLRFamide, SRPYLRFamide, SKPYLRFamide and acetyl-SKPYMRFamide act as inhibitory neuromodulators of cholinergic transmission in *H. aspersa* [1,38,41] and *Helix lucorum* [36,39,40,42] parietal ganglia.

The modulation of synaptic transmission by FaRPs can occur through a change in the sensitivity of the postsynaptic membrane to transmitters since it was found that all the FaRPs reduced reversibly the inward current in *H. aspersa* and *H. lucorum* identified neurons to local acetylcholine (ACh) application onto the soma. Reduction of ACh-induced current (metabotropic effect) by SEPYLRFamide and its N-terminally modified analogues at concentrations 0.01–10.0 μM was not associated with any changes in the leak current. At concentration of 50 μM the peptides evoked an electrogenic effect, i.e., an increase (by 15–25%) of inward leak current with full recovery after wash. [39].

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Elevation of free intracellular $Ca^{2+}$ reduces ACh-induced inward $Cl^-$ currents in *Lymnaea stagnalis* [12,52] and *Helix pomatia* [3] dialysed neurons. This means that an artificial increase of intracellular free $Ca^{2+}$ in the cytoplasm has a similar effect to that of FaRPs on somatic acetylcholine receptors. Hence FaRPs may inhibit cholinosensitivity in *Helix* neurons via an elevation of intracellular $Ca^{2+}$. This assumption is supported by the observation that FMRFamide increases basal intracellular $Ca^{2+}$ in *Helix* neurons since FMRFamide amplifies a fluorescent signal from *H. aspersa* identified neurons loaded with $Ca^{2+}$-sensitive dye fluo-3 [36].

Our electrophysiological data show that intracellular free $Ca^{2+}$ is involved in the intracellular mechanism for the reduction of cholinosensitivity in *Helix* neurons by FaRPs [36,38,41]. Ryanodine receptors and $IP_3$ receptors may be involved in the inhibitory metabotropic effects of FaRPs on somatic cholinergic receptors of identified *Helix* neurons [36,38,41,42].

We searched the literature for a possible cellular regulator to explain the inhibitory metabotropic effect evoked by the FaRPs on ACh receptors. One possible regulator is the Na,K-pump. It is known from the literature that inhibition of the Na,K-pump by ouabain evokes a decrease of ACh-induced currents of *H. pomatia* dialysed neurons [3]. Ouabain can elevate intracellular calcium via the activation of the reverse mode of Na/Ca-exchange ($Na^+$-efflux, $Ca^{2+}$-influx) [1,11,13,15,20,31,45,46] and stimulate $Ca^{2+}$ release from internal stores [4,32,46,47].

Previous work has shown that FMRFamide increases intracellular $Ca^{2+}$ via inhibition in $Na_{out}$-dependent $Ca^{2+}$-efflux (Na/Ca-exchange in the normal mode) [16,24,51] and activation of $Ca^{2+}$-mobilization via ryanodine receptors and $IP_3$ receptors [18,19,36,38,41,54].

Ouabain reduces reversibly ACh-induced inward currents in identified *H. lucorum* neurons (LPa2, LPa3, RPa3, RPa2) [33,35,37] that were used in our investigations with FaRPs [36,38,39,40,42]. This ouabain-mediated metabotropic effect is $Ca^{2+}$-dependent.

There are identical effects of FaRPs and ouabain on the amplitude of ACh-induced currents in *Helix* neurons and on intracellular $Ca^{2+}$ level. This provides the basis to postulate a working hypothesis: FaRPs may inhibit postsynaptic cholinosensitivity in *Helix* neurons partly via initial Na,K-pump inhibition by means of G-proteins followed by elevation of intracellular free $Ca^{2+}$.

In this study we investigated the participation of Na,K-pump in the modulatory effect of endogenous molluscan heptapeptide, SEPYLRFamide, on cholinosensitivity in *Helix* neurones. The role of the following intracellular regulators were investigated, G-proteins, Na/Ca-exchange and intracellular calcium released from internal pools in the modulatory peptide-mediated cascade involving inhibition of Na,K-pump.

## 2. Materials and methods

### 2.1. Recording of transmembrane currents

#### 2.1.1. Animals

Snails, *H. lucorum*, were collected locally in the Sevastopol region, Crimea, Ukraine. Experiments were performed using an isolated ganglia preparation at room temperature (18–22 °C) during 2002–2004.

Animals were anaesthetized in cold saline and the circumoesophageal nerve ring was removed for study. Circumoesophageal ganglia were pinned down, dorsal side up, on a silicon rubber-coated flow chamber with a bath volume of 1.0 ml. The connective tissue sheath covering neurons was removed prior to the experiments after preliminary enzymatic preparation (digestase/Seatec/, 0.5%, 30–40 min at room temperature (18–22 °C). Ganglia were constantly superfused (0.5–0.8 ml/min) with normal *Helix* saline containing (in mM): NaCl, 100; KCl, 4; $CaCl_2$, 10; $MgCl_2$, 4; Tris buffer, 10; adjusted to pH 7.5 with HCl.

#### 2.1.2. Electrophysiological recording

Experiments were carried out on identified LPa2, LPa3, RPa3 and RPa2 neurons from left and right parietal ganglia of *H. lucorum*. These cells are command elements of withdrawal responses to noxious stimuli [22].

Single-barrel glass microelectrodes were pulled using a PUL-1 puller (World Precision Instruments) from Pyrex glass (1.5 mm outer diameter) and filled with 2 M potassium acetate; resistance 8–120 MΩ. The electrodes were connected by a Ag–AgCl microelectrode holder (World Precision Instruments design) to a Micro-Electrode Amplifier MEZ-8101 (Nihon Kohden) and Voltage Clamp Amplifier CEZ-1100 (Nihon Kohden) that were used in Virtual Ground Mode for two-electrode voltage clamp experiments. A briquette of Ag–AgCl (Medicor) was used as reference electrode. The neurons were clamped at −75 mV. Currents were entered on a PC through the analogous-digital interface L-154 (L-CARD, Moscow) and recorded using CONAN 3.5 software (InCo, Moscow, Russia).

ACh was applied ionophoretically (interstimulus interval 5 min) onto the neuronal soma using the current source isolated from the ground. The ionophoretic solution was as follows: 1 M ACh chloride (Sigma) in distilled water (pH 7.0). The resistance of the ionophoretic pipette was 14–46 MΩ. The reference pipette was filled with normal *Helix* saline with a resistance of 1–3 MΩ. Cationic currents (685–877 nA; 0.1–3.0 s) were used for ionophoresis. A Laboratory Electrostimulator ESL-2 (Kaunas Research Institute of Radiometrical Engineering) was used to control the duration of the ionophoretic current. Ejection of positive currents through an ionophoretic pipette filled only with distilled water had no effect on the cells. A negative retention current (10 nA) was passed continuously through the ionophoretic pipette in order to prevent the spontaneous diffusion of ACh.

For an estimation of changes of stationary membrane conductance the holding potential in voltage clamp mode was shifted periodically in a negative direction (rectangular pulses, 10 mV, 5 s) for 9 s up to the start of ionophoretic ACh application. Change of amplitude of the inward leak current evoked by the negative shift of a holding potential was directly proportional to the change in resting membrane conductance.

#### 2.1.3. Drugs

SEPYLRFamide (Department of Biochemistry, University of Southampton; Alta Bioscience, University of Birmingham) was locally applied under pressure at a concentration of 5 mM