PHYSIOLOGY

Synapse-Specific Plasticity in Command Neurons during Learning of Edible Snails under the Action of Caspase Inhibitors

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The effect of caspase inhibitors on long-term synaptic facilitation induced by nociceptive sensitization (a simple form of learning) was studied on the defensive behavior command neurons (left pleural neuron-1) in edible snail. Acquisition of sensitization under conditions of threatment with caspase-3 or caspase-8 inhibitors selectively inhibits synaptic transmission in the responses of the left pleural neuron-1 to tactile stimulation of the snail head, but not in responses to chemical stimulation of the head or tactile stimulation of the foot. Application of a wide-spectrum caspase inhibitor z-VAD-fmk to neurons of sensitized snails suppressed facilitation of responses evoked by chemical stimulation of the head. Probably, various caspases could be selectively involved into induction of long-term synapse-specific plasticity during learning.

Key Words: *mollusk; neuron; nociceptive sensitization; synapse-specific plasticity; caspases*

Cysteine-containing proteases (caspases) are the key factors controlling apoptotic death of the cells [1,6,13]. Accumulating data indicate the involvement of caspases in various non-apoptotic physiological processes, including learning-related plastic changes in the nervous system. In rats, caspase-3 inhibitors suppress consolidation of long-term memory in the Morris water maze [8] and stimulate the development of conditioned freezing response to the key sonic stimulus, but produce no effect on the development, consolidation, and retrieval of conditioned contextual fear [2]. In birds, habituation to the species-specific song is accompanied by accumulation of caspase-3 in the synaptic regions of neuronal dendrites in subdivisions of the forebrain involved into habituation processes [11]. Caspase-1 inhibitor impairs contextual fear conditioning in rats [9].

Caspases are found in the pre- and postsynaptic compartments of neurons where they can be activated in response to the afferent stimulation and activation of some receptors [4,6]. Experiments performed on CA1 neurons in hippocampal sections showed that specific inhibitor of caspase-3 suppressed induction of long-term potentiation [1]. At the same time, caspase-3 inhibitor did not prevent disturbances in induction of long-term memory in CA1 neurons caused by transient cerebral ischemia in rats [10]. Pan-caspase or caspase-1 inhibitors (but not caspase-6 inhibitor) considerably facilitated acquisition of long-term potentiation in CA1 neurons on sections [12]. The studies of the

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nervous system of edible snail carried out with specific antibodies revealed caspase-3-immunoreactive proteins with a molecular weight of 29 kDa [4]. Incubation of snail nervous system with selective caspase-3 inhibitor prevented the development of long-term facilitation of synaptic transmission in identified neurons [4]. Therefore, the available data indicate the key role of caspase in synaptic plasticity, although some of them are controversial.

The studies on defensive behavior command neurons in edible snail (LPl1, the left pleural neuron-1 and RPl1, the right pleural neuron-1) showed that during acquisition of sensitization different molecular and genetic mechanisms are selectively involved in the plasticity processes in the individual synaptic inputs of neurons. For example, cyclic AMP (cAMP) and corresponding cAMP-dependent transcriptional factor C/EBP (CAAT/enhancer binding protein) are involved in induction mechanisms of long-term facilitation in the sensory inputs of these neurons related to cephalic chemoreceptors, while fine adjustment of other sensory input related to cephalic mechanoreceptors is performed by protein kinase C with the corresponding transcriptional factor SRF (serum response factor) [3].

Our aim was to study the effects of specific caspase-3 and caspase-8 inhibitors as well as unselective caspase inhibitor z-VAD-fmk on synaptic plasticity of various sensory inputs in defensive behavior command neurons LPl1 in edible snail during acquisition of nociceptive sensitization.

MATERIALS AND METHODS

The experiments were carried out on semi-intact preparation of edible snail *Helix lucorum.* Standard electrophysiological methods were applied [3,4]. First, the animals were anesthetized by cooling in an ice-water mixture for 30-40 min and then with 100-150 mg $MgCl_2$ injected immediately before surgery (in 2 ml bolus of physiological saline).

Sensitization was induced by triple application of 100 μl concentrated (10%) quinine hydrochloride to the skin of snail head, the interval between the applications being 15 min. The neural responses to sensory stimuli were tested with weak quinine solution (0.25%) and with mechanical stimulation. Quinine (600 μl) was applied onto the anterior part of the head for 30 sec. Mechanical stimuli were applied to the head or to the middle part of the foot with an electromechanical device. The test stimuli were presented before the development of sensitization and on postsensitization minutes 120-150. At this postsensitization time, synaptic transmission in LPl1 neuron attained a stable level maintained for more than 24 h and depended on protein and RNA synthesis [3]. The responses evoked by sensory stimulation were assessed by the area under the plot of slow excitatory postsynaptic potentials (sEPSP).

In this study, we used specific caspase-3 inhibitor Ac-DEVD-CHO (N-Acetyl-Asp-Glu-Val-Asp-al), specific caspase-8 inhibitor z-IETD-fmk (Z-ile-Glu(O-ME)-Thr-Asp(O-ME) fluoromethyl ketone), and wide-spectrum caspase inhibitor z-VAD-fmk (N-benzyloxy-carbonyl-Val-Ala-Asp-(O-ME) fluoromethyl ketone) dissolved first in DMSO and then in physiological saline for molluscs at pH 7.6 (all preparations were from Sigma). The final concentration of caspase inhibitors and DMSO were 100 μM and 0.2 μM, respectively [1].

The tip of a microelectrode (diameter 20-30 μ) was positioned at a distance of 30-50 μ from the soma of LPl1 neuron. The microelectrode contained the test substances, which were applied under a pressure of 0.3-0.5 kg/cm^2 with a Neuro Phore BH-2 injector (Medical System Corp.). The mean rate of infusion was 5 μl/min. The injections were started 45 min before the development of sensitization and terminated 15 min before it. During application of caspase inhibitors dissolved in DMSO and control inhibitor-free solution of DMSO (0.2%) the membrane was depolarized by 5-8 mV. The membrane potential returned to the initial value after washout with physiological saline for 15 min before the development of sensitization.

The experimental data were normalized in each experiment (the values before sensitization were taken as 100%). These data were averaged and plotted in percents ±SEM. Significance at differences was assessed by Student's *t* test.

RESULTS

Application of 0.25% quinine solution to the snail head evoked sEPSP in LPl1 neurons with the area of 346±53 mV×sec (n=35). The corresponding values for the responses evoked by mechanical stimuli applied to the head and middle part of the foot were 183±39 mV×sec (n=35) and 118±30 mV×sec (n=35).

On minutes 120-150 after sensitization of LPl1 neurons in control (application of 0.2% DMSO) and experimental snails (application of Ac-DEVD-CHO, z-IETD-fmk, and z-VAD-fmk), the areas of sEPSP evoked by chemical stimulation of snail head differed from the presensitization value (taken for 100%): 108±19% (n=8), 95±15% (n=14), 106±18% (n=8), and 21±11% (n=16), respectively (Fig. 1). The corresponding normalized values of sEPSP area for mechanical stimulation were 72±19% (n=8), 12±11% (n=14), 1±12% (n=8), and 83±21% (n=16,

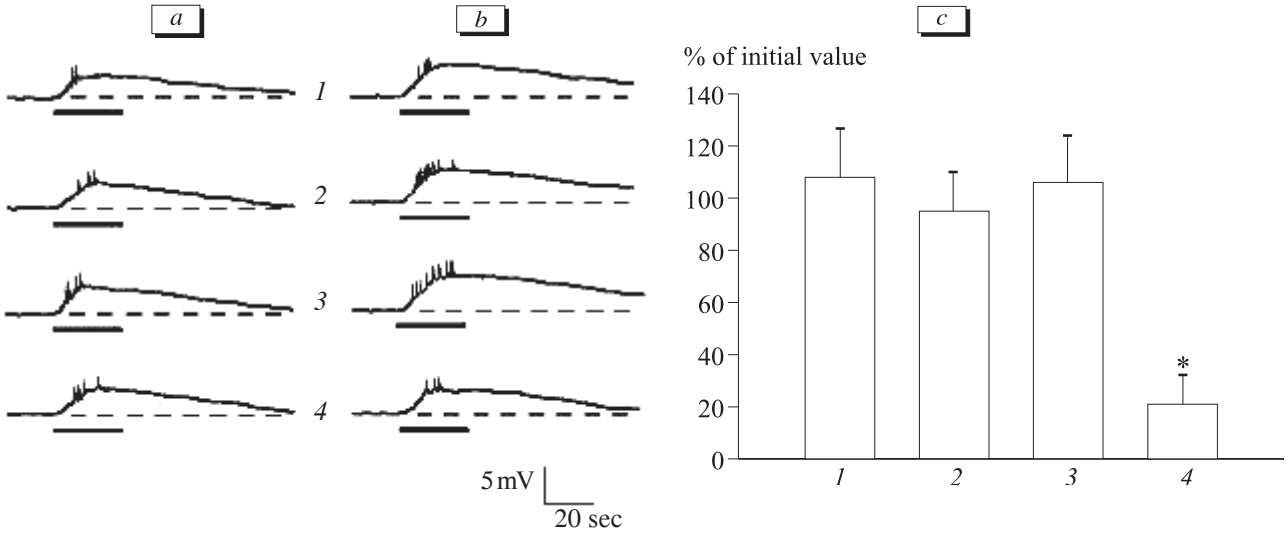

Fig. 1. Effects of caspase inhibitors on facilitation of responses of LPl1 neuron in edible snail induced chemical stimulation. Facilitation resulted from the development of nociceptive sensitization, and the responses were evoked by stimulation of the head with weak quinine solution (0.25%). Here and in Figs. 2 and 3: *a*) neurogram of initial responses to sensory stimulation; *b*) the responses of neurons evoked in 120-150 min after presentation of the first sensitizing stimulus; *c*) sEPSP area of sEPSP evoked under the control conditions (*1*) and after the action of caspase inhibitors (*2-4*). *1*) 0.2% DMSO; *2*) Ac-DEVD-CHO; *3*) z-IETD-fmk; *4*) z-VAD-fmk. The horizontal bar under the neurograms marks the moment of application of chemical stimulus (0.25% quinine) to the head. **p*<0.001 compared to the control (0.2% DMSO).

Fig. 2). Under the same sensitization and stimulation conditions, the corresponding responses of LPl1 neurons to mechanical stimulation of the middle part of the foot were 64±15% (*n*=8), 59±13% (*n*=14), 53±12% (*n*=8), and 69±21% (*n*=15, Fig 3).

When sensitization procedure was performed in snails whose LPl1 neurons were treated with Ac-DEVD-CHO or z-IETD-fmk, facilitation of the responses to tactile stimulation of the head was inhibited ($p<0.001$ in comparison with control), although changes of responses to chemical stimulation of the head or mechanical stimulation of the foot were the same as after application of 0.2% DMSO. When LPl1 neurons were treated with z-VAD-fmk, faci-

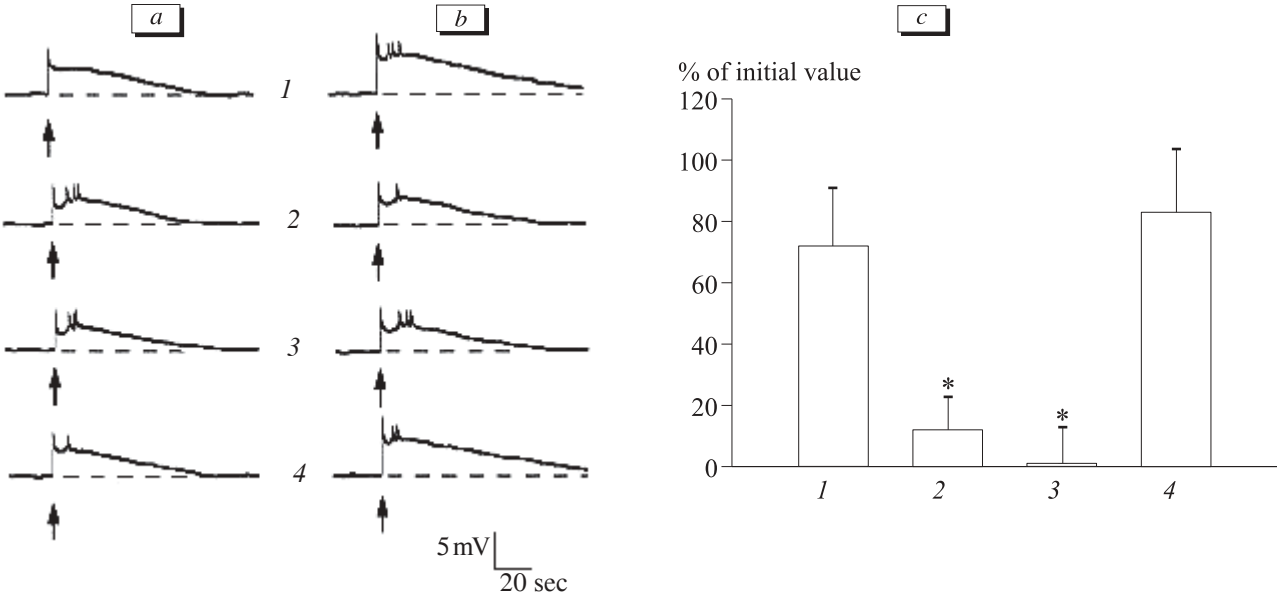

Fig. 2. Effects of caspase inhibitors on facilitation of responses of LPl1 neuron in edible snail revealed in responses to mechanical stimulation of the head. Facilitation resulted from the development of nociceptive sensitization. Arrow indicates the moment of tactile stimulation.

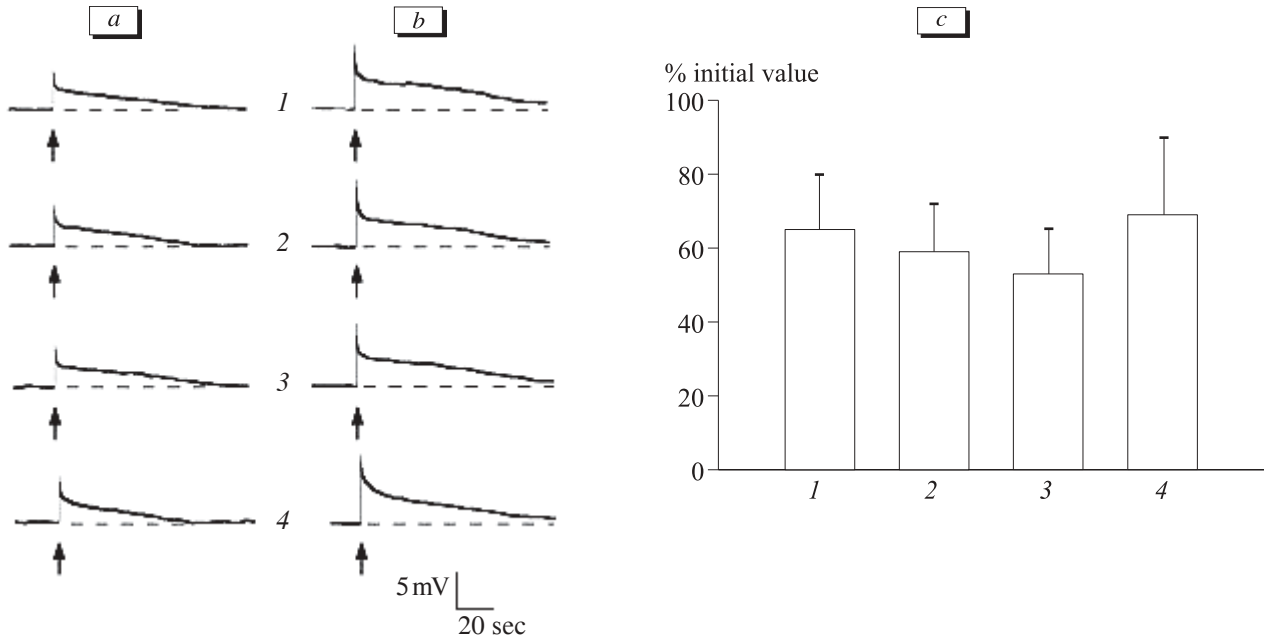

Fig. 3. Effects of caspase inhibitors on facilitation of the responses of LPl1 neuron in edible snail revealed in the responses to mechanical stimulation of the foot. Facilitation resulted from the development of nociceptive sensitization.

litation of synaptic transmission was inhibited in responses evoked by chemical stimulation of the head ($p<0.001$), but not in responses evoked by mechanical stimulation.

Therefore, the development of sensitization in snails with LPl1 neurons exposed to caspase-3 or caspase-8 inhibitor was modified: facilitation of the responses to mechanical stimulation of the head was selectively suppressed. In contrast, z-VAD-fmk selectively suppressed facilitation of responses evoked by chemical stimulation of the snail head.

It was established that protein kinase C and SFR transcription factor are involved in the regulation of sensory inputs from the cephalic mechanoreceptors [3]. In various organs and tissues, caspase-3 affects protein kinase C [1,6]. Partial proteolysis of protein kinase C by caspase-3 leads to its activation. In addition, caspase-3 can inhibit calpastatin, which suppresses calpain activity [6]. Similar to caspase-3, calpain activates protein kinase C [6]. In some tissues, activated caspase-3 cleaves SRF transcriptional factor, which can result in amplification of its effect [7]. Thus, the selective effect of caspase-3 inhibitor on the plasticity mechanisms of sensory inputs from the cephalic mechanoreceptors is probably explained by the control of activity of protein kinase C and SRF transcriptional factor by caspase-3. The effect of caspase-8 inhibitor on these inputs can result from the regulating effect of this enzyme on caspase-3 activity [1,6].

Pan-caspase inhibitor z-VAD-fmk suppressed synaptic facilitation in the sensory input from chemoreceptors of the head, but had no effect on facilitation in the sensory inputs from mechanoreceptors of the head and foot. It can be hypothesized that the effect of z-VAD-fmk on synaptic pathways from the head is realized via not yet identified caspase(s) involved in the mechanisms of regulation of this input. cAMP and C/EBR transcriptional factor are involved into these mechanisms. Some data including those obtained in the studies of CNS showed that caspases 1, 2, 9, and 10 are involved in modulation of cAMP level and activity of cAMP-dependent protein kinase A in cells [14,15]. Activity of C/EBP transcriptional factor can be regulated by caspase-1 [5]. Since caspases 2, 9, and 10 belong to the initiator-type enzymes and caspase-1 is an effector enzyme, it can by hypothesized that the latter is involved in the regulation of plasticity of synaptic inputs from chemoreceptors of snail head.

Pan-caspase inhibitor suppresses activity of caspase-3 and exerts the effects on various tissue process similar to those produced by specific inhibitors of this enzyme [6,13]. Paradoxically, in our experiments z-VAD-fmk produced no effect on synaptic input from head mechanoreceptors, which attests to the involvement of some other caspase(s) in the regulation of this sensory input in addition to caspase-3 and caspase-8. Hypothetically, the diverse effects of the wide-spectrum caspase inhibitor on various enzymes can compensate and eliminate the effects resulted from inhibition of caspase-3 thereby potemtiating synaptic transmission. Moreover,

enzymes of the caspase-3 family structurally differ from those in mammals and mollusks [4]. It cannot be excluded that caspase-3 enzyme of edible snail is characterized by low sensitivity to the pan-caspase inhibitor used in this study.

Our findings and published data suggest that each neuron employs only its "own" intrinsic and specific mechanisms of caspase action among a wide variety of possible molecular effects of caspases. Specifically, during the development of sensitization in edible snail, long-term plasticity of the synaptic input of defensive behavior command neurons LPl1 from head mechanoreceptors is specifically controlled by caspase-3 and caspase-8, as well as by protein kinase C and the corresponding SRF transcriptional factor. At the same time, a non-identified caspase functionally related to cAMP system and cAMP-dependent C/EBP transcriptional regulators induces the synthesis of RNA and the proteins, which control synaptic input from the head chemoreceptors in snail.

It should be also taken into consideration that in addition to systems of signal transduction, caspases also modify the structure and function of many cytoskeleton proteins [1]. During learning, caspases are locally activated in the pre- and postsynaptic areas in the cells, which results in selective morphological rearrangements in synapses and neurons [11]. These local morphofunctional alterations are considered as manifestations of the "synaptic apoptosis" developed during the reversal initial stages of the programmed cell death [13].

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