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EXPERIMENTAL ARTICLES

Localization of the Atypical Protein Kinase Cζ in the Nervous System of the Terrestrial Snail *Helix*

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Abstract—The distribution of the atypical protein kinase Cζ in the nervous system of the adult and juvenile terrestrial snail *Helix aspersa* L. was studied immunocytochemically. Immunoreactive (IR) elements were detected in all CNS ganglia. The pattern of immunoreactivity included cytoplasmic staining of many CNS neurons, fibers in the CNS tracts and nerves, and ultrathin fibers and single varicosities inside the ganglia neuropil, which is the location of the major zones of synaptic contacts. The primary neurites and their proximal branches were not immunoreactive. The relatively low immunoreactivity of the fibers did not correspond to the presence of numerous Cζ-IR cell bodies. In the juvenile CNS, the IR neuropil was better developed than in the adult CNS, while immunoreactivity was practically absent in the cell bodies. We conclude that the atypical protein kinase Cζ is present in the snail CNS, while in the CNS neuropil it is predominantly located in varicosities.

Keywords: nervous system, snail, immunocytochemistry, atypical protein kinase Cζ

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INTRODUCTION

The protein kinase C family includes more than ten members, most of which are expressed in the brain [1]. Many of them participate in synaptic plasticity in the hippocampus; they become activated for a short period during the induction of long-term potentiation (LTP) [2]. Of particular significance is the atypical (it is activated by secondary lipid messengers [3]) protein kinase Cζ (PKCζ) which is activated for a long period during the development of LTP. This is accompanied by cleavage of an active catalytic domain, Mζ [4]. Protein kinase PKCζ has been found not only in vertebrates but also in the sea mollusk *Aplysia* [5], and calpain was shown to proteolytically cleave its Mζ domain (PKMζ). Recent studies have revealed an important role of Mζ in memory storage in both vertebrates [6, 7] and mollusks [5, 8]. It has been shown in cultured sensory neurons of *Aplysia* [5, 9] that PKCζ is expressed in the cell nucleus but its cleavage with the release of the PKMζ domain occurs in the cytoplasm within the cell body and processes of neurons (analogous to the neuronal dendritic tree of vertebrates). To our knowledge, there is has been no study concerning the distribution of PKCζ or PKMζ within the nervous system of any mollusk at the supercellular level; this is a serious drawback for studies on nervous systems that consist of a limited number of mostly identified neurons with known roles in behavior.

Recently, the sequence of the atypical protein kinase Cζ of the terrestrial snail (GenBank, no. KM875662) was published [10], which has 85% homology to the sequence that was described for rat, including a substantial similarity in the C-terminal part of the molecule. Therefore, in the present study we used an antibody against the C-terminal part of the PKCζ molecule of rat to investigate the occurrence and distribution of neuronal elements that are stained by this antibody within the nervous system of the *Helix* terrestrial snail (a classic object of neurobiological studies). These data may turn out to be important in studies of the potential function of PKCζ in learning and memory formation in mollusks.

MATERIALS AND METHODS

Immunocytochemistry. This study was performed using sections of the nervous system of the terrestrial snail, *Helix aspersa* L. Adult animals were obtained from a laboratory colony and juvenile animals were obtained from mating of the adult snails. In the pilot experiments, the nervous system was fixed using different fixatives: 4% paraformaldehyde in 0.1 M phosphate buffer (PFS), acetone, Bouin's solution, and Nikiforov solution (alcohol with chloroform, 50 : 50). The best results were obtained with 4% PFS; therefore, most of the study was made using this fixative.

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After cold anesthesia, the nervous system was excised and fixed for 1–2 hours at 4°C. After washing in 0.1 M PBS and dehydration in graded alcohols, the nervous system was embedded in paraplast and cut in 10-μm sections. After rehydration in alcohols and rinsing in PBS, the CNS was incubated in blocking solution for 1 hour. The blocking solution contained 0.5% Triton X-100, 0.01% sodium azide, 5% normal goat serum (Sigma), and 1% bovine serum albumin (Sigma) in 0.1 M PBS. Sections were incubated for 24 hours at 4°C in a solution of primary rabbit antibodies (1 : 500, Sc-216, Santa Cruz Biotechnology, Inc.) against the C-terminal end of protein kinase Cζ. Alexa-488-conjugated goat anti-rabbit immunoglobulin (1 : 100, Molecular Probes) was used as the secondary antibody. The sections were embedded in Aqua Poly/Mount medium (Polysciences, Inc) and observed under an AxioPlan fluorescent microscope (Zeiss, Germany). In the control experiments, the incubation with primary antibodies was omitted.

Western blotting. The western blotting method was used to verify the antibodies against PKCζ. As a control for snail CNS samples, we used 400 μm sections of the rat hippocampus. The hippocampus and previously homogenized samples of snail CNS were treated with ultrasound (duration 14 s, 90% spare cycles, 50% power; Bandelin Sonopuls) on ice in 150 μL of PBS that contained protease inhibitors (Roche, Complete-mini EDTA-free). A part of the sample was then used to measure the protein concentration with the Bradford assay and the remaining sample was modified by addition of an equal volume of 2× RIPA buffer (NaCl 300 mM, 2% NP-40, 1% sodium deoxycholate, 0.2% SDS, 100 μL Tris, pH 8.0) that contained protease inhibitors (Roche, Complete-mini EDTA-free). The mixture was incubated at constant shaking for 15 minutes on ice and then centrifuged at 14000 *g* and +4°C for 15 minutes. The supernatant was taken and frozen at –70°C for further use. Protein samples were heated at 95°C with 2× loading buffer (0.125 Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol; bromphenole blue) at a 1 : 1 ratio; standard proteins were used as molecular weight markers (Fermentas, #SM 1811). Protein electrophoresis was performed for 80 minutes at a constant voltage of 100 V in a polyacrylamide gel (SDS-PAGE) with a 5–10% concentration gradient. The samples were transferred to polyvinylidene fluoride membrane (Amersham Biosciences) for 60 minutes at a constant current of 100 mA (Bio-Rad). Nonspecific binding was blocked by 5% BSA in PBS with the addition of 0.1% Tween-20 overnight at +4°C; the same solution was used for antibody preparation. The membrane was then incubated with primary antibodies to PKCζ Sc-216 (Santa Cruz, 1 : 500) for 1 hour at room temperature and then rinsed four times for 10 minutes in PBS with Tween-20. The same protocol was used for incubation with secondary sheep anti-rabbit antibodies (G21240, Invitrogen, 1 : 1000) conjugated with horseradish peroxidase. Antigen–antibody complexes were visualized using the chemiluminescent method, for which Pierce ECL western blotting substrate (Thermo Scientific) reagent was applied on the membrane for 5 minutes. The signal was detected using blue-sensitive X-ray film. The pictures were processed using ImageJ software.

RESULTS

Immunoreactivity in the CNS of adult animals. Immunopositive elements were found in all CNS ganglia of adult snails. The type of immunoreactivity was very unusual: thin rims of cytoplasm were observed in many neuronal bodies (Figs. 1a, 1b), as well as filaments within nerves, commissures and connectives, binding the ganglia together (Figs. 1c, 1e, 1f) and virtually no immunopositive (IP) neuronal processes (neurites) in the neuropil (the central part of the ganglia). Instead, there were either very thin fibers or separate IP varicosities in the neuropil (Fig. 1d). Therefore, it was impossible to trace which identified neurons had IP fibers in the nerves. The general impression was that most of the nerve arborization of IP neuronal bodies (within the ganglia) was not immunopositive. Another unusual issue was that the density of IP fibers and varicosities was not high; thus, it did not correspond to the large number of cell bodies where IP cytoplasm was found. Within the parietal–pleural–visceral complex of the ganglia, many IP cell bodies were found, including the well-studied command neurons of withdrawal behavior [11], which are located in the rostral part of the parietal ganglia (Figs. 1a, 1b), whereas the well-defined thick primary neurites of these neurons were not immunopositive (Fig. 1b). Immunopositive tracts were defined within pedal ganglia, including the interpedal commissures (Figs. 1e, 1f), which provide IP fibers to the pedal muscular nerves (Fig. 1f). The number of IP cells in these ganglia was lower than in the pleural, parietal, and visceral ganglia. Local condensations of IP neuropil were defined in the pleural and pedal ganglia (Fig. 1e) and their location indicates their correspondence to pleural-pedal connectives. Within the buccal ganglia, which regulate the feeding behavior of the snail, IP cell bodies (i.e., the cytoplasm surrounding the non-stained nuclei) were abundant and the IP neuropil was well elaborated (Figs. 2a, 2b), including IP fibers at the border between the neuropil and the layer of the cell bodies (Fig. 2b). In the cerebral ganglia, IP neuronal bodies were sparse (Fig. 2d) but separate IP cells, such as the well-known serotonergic giant metacerebral neuron 1, which is the modulating interneuron of feeding behavior [12], were still present (Fig. 2e). The immunopositive neuropil of the cerebral ganglia was poorly elaborated but IP fibers were reliably visualized in the nerves and connectives of the cerebral ganglia (Fig. 2d).

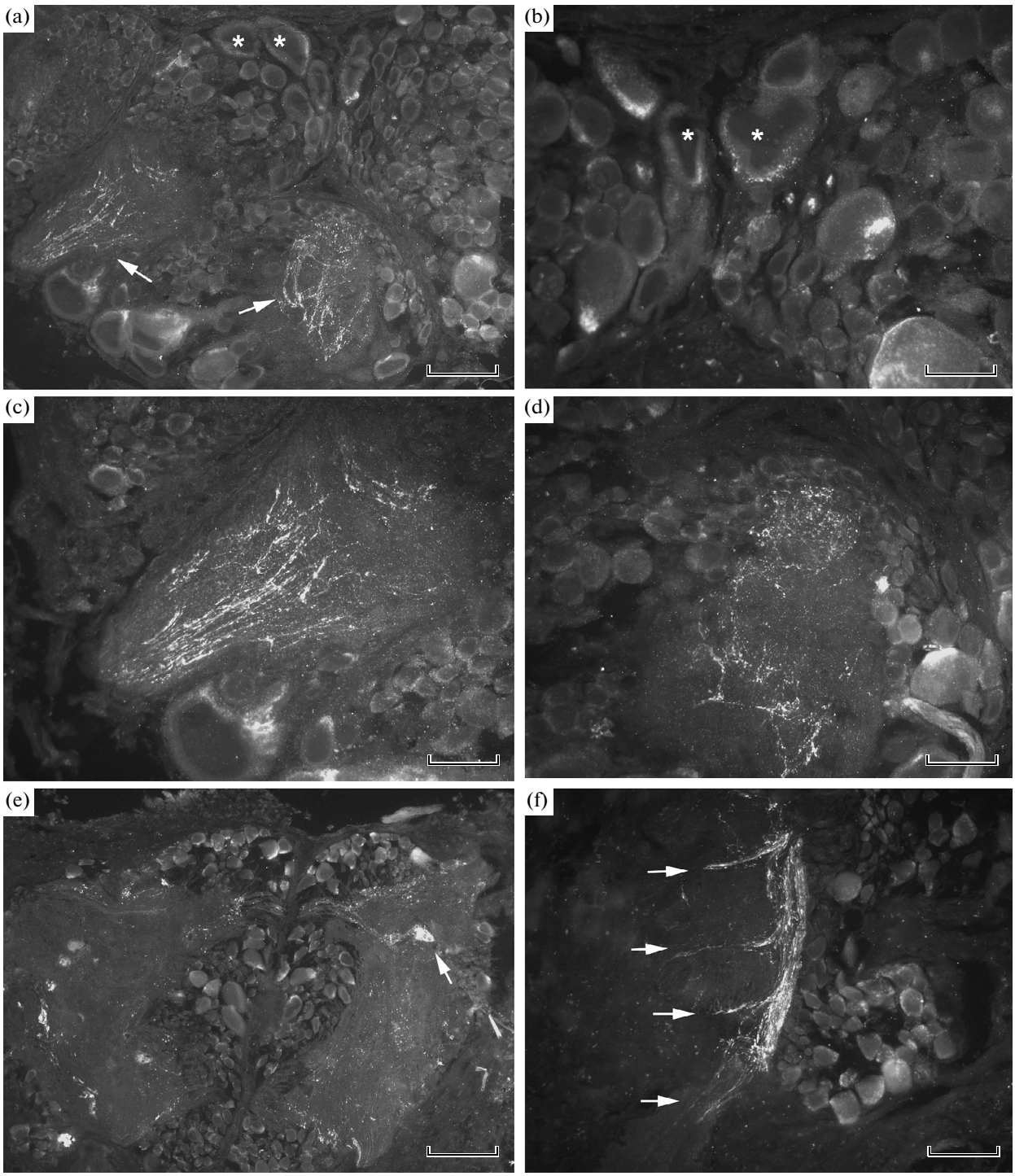

Fig. 1. The immunoreactive elements in the CNS of the adult snail: the pleural-parietal complex of ganglia (a–d) and pedal ganglia (e, f). (a) A general view of the parietal ganglia, the arrows indicate two areas of immunopositive neuropil: at left, the pallial nerve (the same area is presented at higher magnification in (c) and in the visceral ganglion (presented at higher magnification in (d). The asterisks in (a) and (b) indicate the interneurons of withdrawal behavior (command neurons). The arrow in (e) points at the density of immunopositive elements in the pedal ganglion. Immunoreactive fibers are detected in the nerves of the pedal ganglion (indicated by an arrow in (f). Bar: 200 μm (a, e), 100 μm (b, c, d, and f).

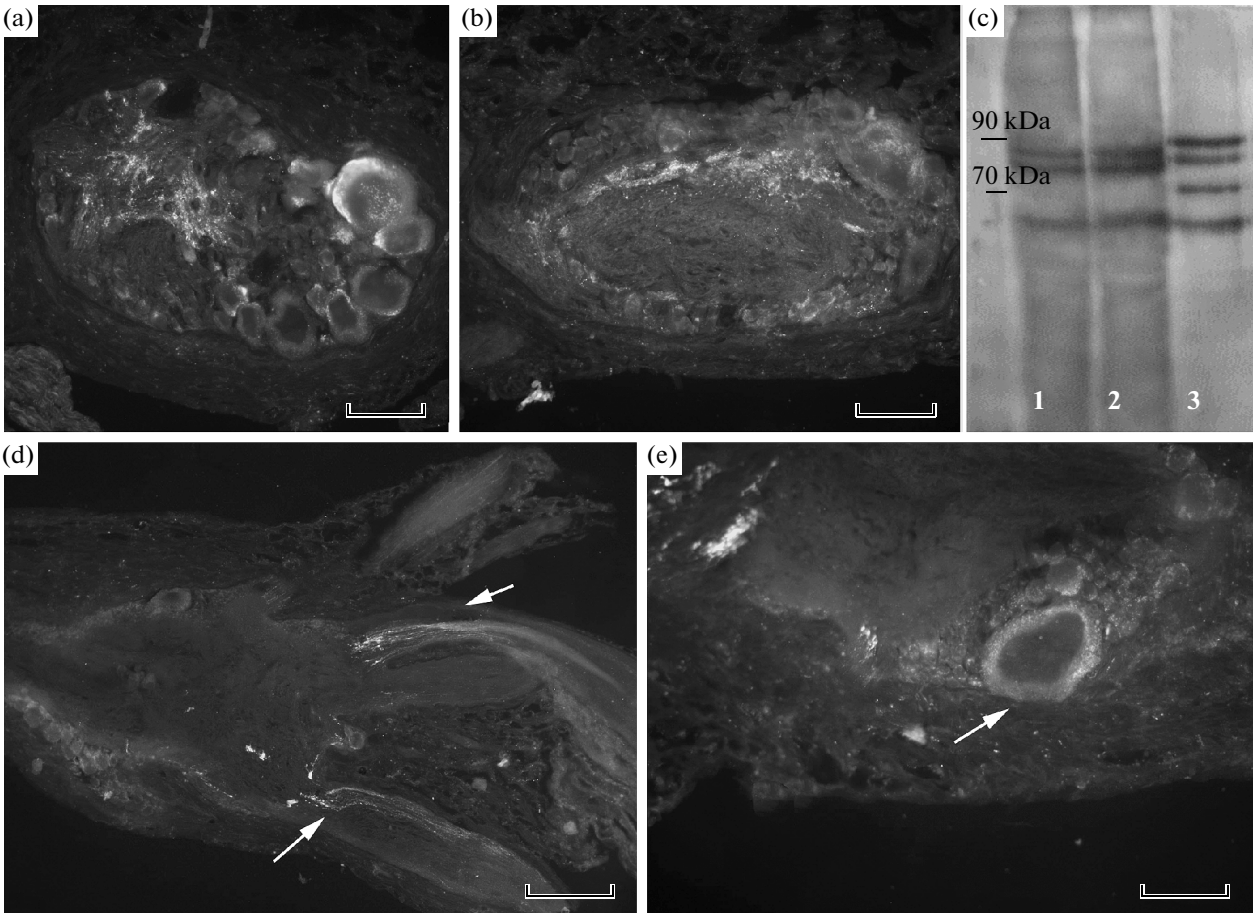

Fig. 2. The immunoreactive elements in the CNS of adult snails: buccal ganglion (slices made at different levels, (a, b) and cerebral ganglion (d, e). The arrows in (d) point to immunoreactive fibers in the middle labial nerve (upper arrow) and in the cerebral-pleural connective (lower arrow). In (e) (a part of the cerebral ganglion), the arrow indicates the giant metacerebral neuron with immunoreactive cytoplasmic rim. (c, a) Western blot assay of PKCζ isoforms in the samples of snail brain (1 and 2) and rat hippocampus (3). The immunoreactive products in the nervous system of the snail with a mass of approximately 80 kDa (two upper bands) presumably correspond to different isoforms of protein kinase Cζ, and product at 55 kDa (lower band), to a cleaved part of protein kinase Cζ (Mζ domain). Bar: 100 μm (a, b, and e) and 200 μm (e).

Immunoreactivity in the CNS in juvenile animals. The pattern of immunoreactivity in the CNS of juvenile animals was notably different from the pattern in adult animals. First, the immunoreactivity of neuronal bodies was virtually absent or very faint (Figs. 3a–3f). Second, in all ganglia of the juvenile CNS, the IP neuropil was substantially denser than in the adult CNS. In the juvenile CNS, the type of immunoreactivity per se was almost the same as in the adult CNS: sparse tracts in ganglia (Figs. 3b–3e), fibers in nerves and connectives, single thin fibers, and varicosities in the main part of the ganglion (Figs. 3a–3f). In the nerves and connectives of the juvenile CNS, the immunoreactivity was less intense than in the respective structures of the adult CNS.

Identification of the immunopositive product by western blotting. To confirm the presence of PKCζ-like proteins in *Helix* we analyzed the total protein lysates from the CNS of the adult snail by western blotting (Fig. 2c). Proteins from the mammalian brain (rat hippocampal slices), where the presence of PKCζ was repeatedly demonstrated, were used as positive control samples. The immunoreactivity assay of snail CNS lysates revealed three proteins; one of them has a molecular weight of 55 kDa and two others have similar masses of approximately 80 kDa (Fig. 2c, lanes 1, 2). In the samples of the rat hippocampus, we found four proteins; two of them have masses of 55 and 70 kDa and two have similar masses of 80 kDa (Fig. 2c, lane 3). The two upper groups with masses of approximately 80 kDa that were present in all of the samples correspond to the atypical protein kinase Cζ (possibly, to its isoforms). Since the primary antibodies that we used were against the C-terminus of PKCζ, the staining of the samples should reveal both the full molecule of protein kinase Cζ and its C-terminal fragment (protein kinase Mζ). Therefore, the staining of a lower band with a molecular weight of approximately 55 kDa in all of the samples may indicate the presence of a shortened isoform of protein kinase Cζ, viz., the atyp-

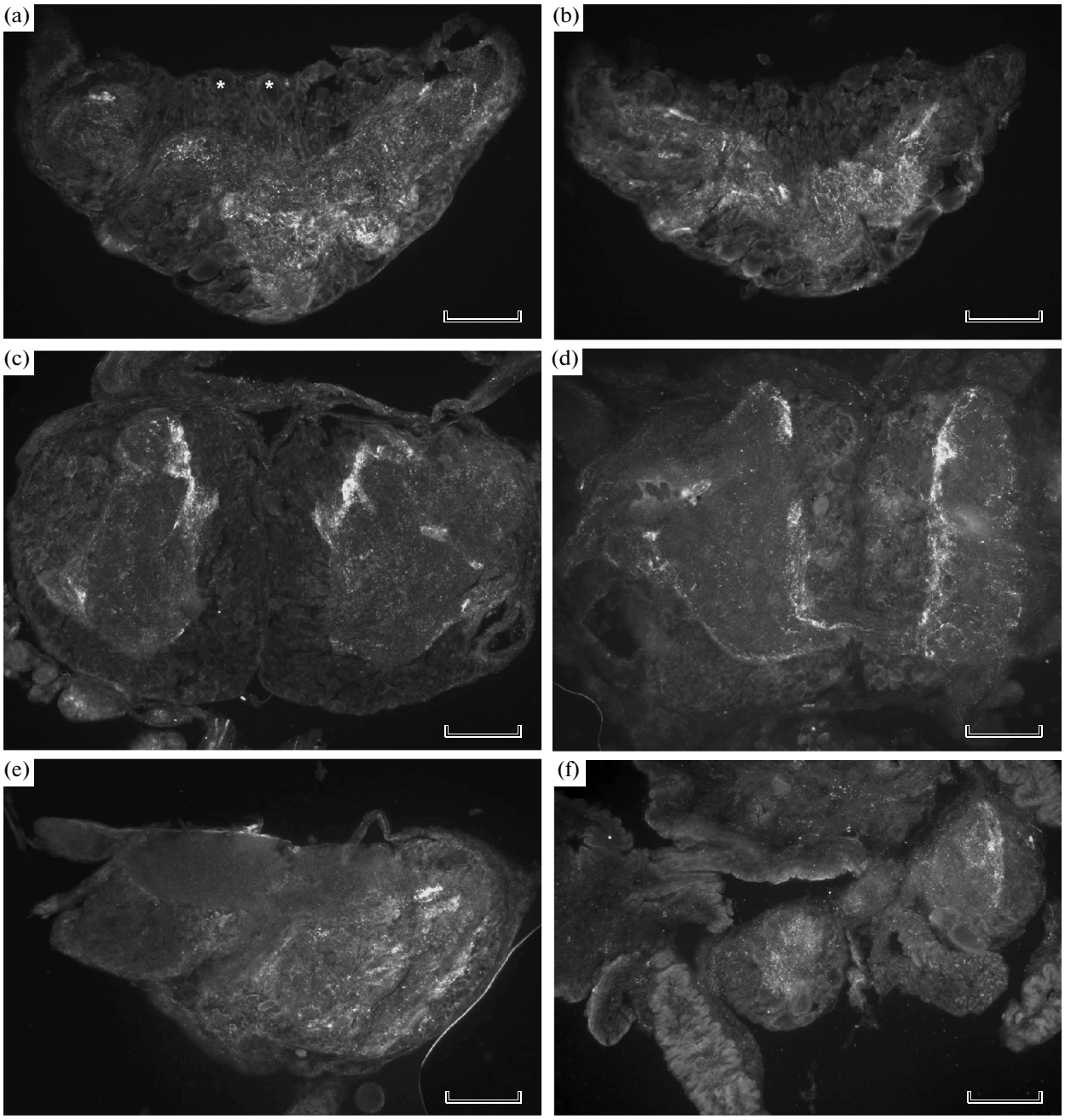

Fig. 3. The immunoreactive elements in the CNS of juvenile snails. (a and b) The sections of the pleural-parietal complex of ganglia (asterisks in (a) indicate the interneurons of withdrawal behavior, the command neurons). (c and d) The sections of pedal ganglia. (e) The section of the left cerebral ganglion. (f) The section of buccal ganglion. Bar: 100 μm.

ical protein kinase Mζ. The remaining band at 70 kDa that was detected in the rat brain extract but not in the snail brain extract seems to be related to cross reactivity of the antibody with one of the PKC isoforms. The data from the western blotting demonstrate similar staining of the samples that were taken from mammalian and snail brains with antibodies to PKCζ. Thus, our results indicate that antibody had no unspecific interactions with snail CNS proteins, which indirectly confirms that the revealed IP elements in the snail CNS do contain protein kinase Cζ.

DISCUSSION

It was shown previously that the spatial distribution and level of the expression of different protein kinase C forms vary in the vertebrate brain [13], for example, protein kinase Mζ is especially active in the hippoc-

ampus [1]. Typically, in the neurons of vertebrates different protein kinase C forms (including protein kinase PKCζ) are present mainly in the cytoplasm [1] but not in the nucleus or primary neurites, which completely agrees with our data that were obtained in snail neurons. The correspondence of the molecular weight of proteins that were stained with antibody to PKCζ in the CNS of the snail and rat hippocampus implies the identity of the detected antigens. In a recent study [14], it was shown that the distribution of PKMζ in the neurons of the rat brain is predominantly somato-dendritic; moreover, PKMζ is present in postsynaptic densities, and a punctate distribution of product was found in the dendritic tree, including dendritic bifurcations. In embryonic hippocampal neurons PKMζ is localized only in those processes that give rise to dendrites and its suppression leads to excessive generation of axons [15]. Thus, PKMζ is involved in the formation of dendrites. It seems that punctate immunoreactivity in the neuropil area of the snail CNS qualitatively coincides with the pattern that has been described in the vertebrate brain. Unlike the neurons of vertebrates, the pseudounipolar neurons of the snail combine the features of both an axon (the thick parts of the process) and the dendrites (thin branches) in their branching neurite. Therefore, the absence of large IP fibers in the neuropil of the snail, as well as the immunoreactivity in thin branches and dotted clusters that is described here, implies that PKCζ and its cleavage product (PKMζ) are located at *dendrites* of neurons in the snail, as they are in vertebrates. It is important to note that the relatively low density of IP neuropil clearly does not correspond to the large number of cell bodies that are immunoreactive to the antibody against PKCζ, which means that in most immunoreactive neurons the PKCζ is located in the neuronal soma and the distant processes with the majority of synapses on them. As well, the demonstrated pattern of staining did not correspond to any kind of identified network or neuronal group, although all of the known interneurons were immunopositive to PKCζ.

The only data on the distribution of PKCζ in mollusk neurons (localization in the cytoplasm of the cell bodies and thin processes) was obtained in cultured sensory neurons of *Aplysia* [5, 9]. Given that memory formation involves many interconnected neurons, our data that show the presence of PKCζ in the majority of cell bodies and thin branches of neurons may have important implications in further investigations of the role of PKCζ in the processes of learning and formation of memory.

CONCLUSIONS

An atypical protein kinase C (PKCζ) was immunohistochemically detected in the vast majority of the cell bodies of CNS neurons in the terrestrial snail. Thin branches of processes and single varicosities were immunoreactive in the synaptic area of the ganglia neuropil. The immunoreactivity in the neuronal bodies in the juvenile CNS was faint and the density of immunopositive neuropil was relatively higher than in the CNS of adult animals.

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