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WE showed previously that associative learning induced a twofold increase in protein kinase C γ -immunoreactivity (PKC γ -ir) in rabbit CA1 pyramidal neurons, whereas subicular neurons remained unchanged. Here, we investigated the effects of associative learning on PKC-positive astrocytes by determining their numerical density in the CA1 region and the subiculum of naive, pseudoconditioned and trace eyeblink conditioned rabbits. Associative learning induced a 70–80% reduction in the number of PKC β 2- and γ -positive astrocytes in the CA1 region, but not in the subiculum. No changes were found in the number of PKC α - or β 1-positive astrocytes in either hippocampal subregion. These results suggest a conditioning-specific downregulation of PKC β 2 and γ in a subset of astrocytes in a brain region where a simultaneous alteration in neuronal PKC γ was evident.

Key words: Astrocytes; Conditioning; Hippocampus; Immunocytochemistry; Isoforms; Learning and memory; Protein kinase C

Associative learning down-regulates PKC β 2- and γ -immunoreactivity in astrocytes

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Introduction

The involvement of protein kinase C (PKC) in learning and memory processes has been demonstrated extensively.¹ We recently demonstrated immunocytochemically that PKC is involved in hippocampally-dependent spatial and associative learning tasks.^{2–4} These studies focused primarily on hippocampal principal cells, and detailed analyses revealed that associative learning enhanced PKC γ -immunoreactivity (PKC γ -ir), in the dentate gyrus, CA3 and CA1, while the subiculum remained unchanged. No learning-specific changes were found for PKC α , β 1 or β 2. Astrocytes also express PKC, although to a much lesser degree.⁵ Astrocytes are thought to support neuronal function primarily by controlling the extracellular concentrations of neurotransmitters and ions. Endowed with neurotransmitter uptake sites, receptors and signal transduction proteins, astrocytes are fully geared to sense synaptic activity.^{6–8} For this reason, astrocytes might play a role in higher brain functions such as learning and memory. Recent studies using transgenic and knockout mice suggest a role for astrocytes in memory functions. Transgenic mice overexpressing the calcium-binding protein S100b, a PKC substrate solely expressed by astrocytes, were impaired in a hippocampus-dependent spatial learning task.⁹ Mutant mice lacking glial fibrillary acidic protein, the astrocyte-specific cytoskeletal protein, were shown to be impaired in associative learning when compared with normal littermates.¹⁰ Little evidence is available, however, demonstrating a role of astrocytes in

learning and memory functions in genetically intact animals.

Changes in astrocyte features, such as the buffering of extracellular K⁺ or the propagation of Ca²⁺ waves through gap junctions which support neuronal functioning, might underlie the suggested role of astrocytes in learning and memory. Phosphorylation of K⁺ channels and gap junctions by PKC reduces astrocyte functioning.^{11–13} The aim of the present study was to determine whether hippocampally-dependent trace eyeblink conditioning induced alterations in astroglial PKC-ir as seen in neurons, which would be indicative of a role for astrocytes in associative learning. If PKC-positive astrocytes, in concert with neurons, play a role in associative learning, one would expect these alterations to occur in the hippocampal subfields where neuronal PKC changes are found. We therefore analyzed both the rabbit CA1 region and the adjacent subiculum.

Materials and methods

Behavioral training procedure: Twenty-one young adult female New Zealand white albino rabbits (2–3 months of age) were randomly assigned to three groups: naive (N; $n = 7$), pseudoconditioned (P; $n = 7$) or trace conditioned (T; $n = 7$). Trace eyeblink conditioning trial presentation, data acquisition and data analysis procedures were similar to those previously described.² In short, subjects were trained in a sound attenuated chamber after being habituated to the apparatus for 60 min, at least 24 h prior to the first behavioral conditioning session. The trace

eyeblick conditioning paradigm was used, in which a tone conditioned stimulus (CS; 100 ms, 85 dB, 6 kHz) was followed by a 500 ms no-stimulus 'trace' period, and then the air puff unconditioned stimulus (UCS; 150 ms, 3 p.s.i. air puff). A conditioned response was defined as any blink occurring between CS onset and UCS onset, that was more than 4 s.d. above baseline. Rabbits were conditioned daily five times a week until a behavioral criterion of 80% conditioned responses in an 80-trial session was reached. Pseudoconditioned animals received equal numbers of explicitly unpaired presentations of the CS and UCS for equal numbers of trials over an equivalent time period. The naive animals, which were neither habituated nor exposed to the tone or air puff stimuli, served as cage controls.

Immunocytochemical procedure: Twenty-four hours after the last trial, a trace conditioned rabbit, its matched pseudoconditioned animal and a naive rabbit were deeply anesthetized and transcardially perfused with 150 ml saline followed by 800 ml fixative composed of 2.5% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer. The dissected brains were cryoprotected by overnight storage in 30% sucrose in 0.1 M phosphate buffer. Thereafter, immunostaining was carried out on frozen sections cut coronally at 20 μ m. Sections from all three animals were thaw-mounted on a gelatin-coated glass slide (three sections per slide; one section per animal) to guarantee identical incubation conditions. Eight levels through the dorsal and posterior part of the hippocampus were collected, with an interval of 500 μ m. Ca^{2+} -dependent PKC isoforms were visualized by overnight incubation (1:200; room temperature (RT)) with polyclonal rabbit IgG antibodies raised against the catalytic domain of the α , β 1, β 2 and γ isoforms (Santa Cruz Biotechnology Inc.), which are known to bind to rabbit PKC.² After the primary incubation, sections were incubated with biotinylated goat anti-rabbit IgG (2 h, RT, 1:200; Amersham), followed by streptavidin-HRP (2 h, RT, 1:200, Zymed). Finally, the sections were processed by the diaminobenzidine- H_2O_2 reaction, guided by a visual check. Control experiments were performed by the omission of the primary antibody from the incubation medium, which yielded immunonegative results.

Data analysis: The subicular and CA1 regions of the rabbit hippocampus were delineated. The CA1 was distinguished from the CA2/CA3 region by the absence of giant pyramidal cells (which are typical for CA3, and are also present in CA2), and from the subicular region by the abrupt termination of the bundle of perforant path fibers which pass through

the CA1 region. The stratum radiatum was delimited as the region between the stratum pyramidale and the bundle of perforant path fibers. The numerical density of PKC-positive astrocytes per mm^2 of the stratum radiatum was determined by counting immunoreactive astrocytes with the nucleus present in the section only identified at a light microscopic magnification of 400 $\times$, and by measuring the sampled surface areas. The areas of the sampled stratum radiatum were drawn on paper using a projection microscope, digitized and computed with the aid of NIH Image software.

Statistical analysis: Analysis of variance followed by a *post hoc* Student's *t*-test was used, with a probability level of $p < 0.05$ indicating statistical significance.

Results

The behavioral training showed that the seven trace conditioned rabbits reached the criterion of 80% conditioned responses in a training session at days 10 ($n = 2$), 12 ($n = 2$) and 15 ($n = 3$). The rate of acquisition differed significantly from that of pseudoconditioned rabbits ($p < 0.00001$), which displayed a low and stable baseline of conditioned responses by chance throughout the experiment.

Immunoreactivity for PKC α , β 1, β 2 and γ revealed the presence of all isoforms in hippocampal principal cells, interneurons and astrocytes. PKC-immunoreactive astrocytes were found scattered throughout the hippocampal formation (see Fig. 1A for an example of a PKC γ -positive astrocyte). The anatomical characteristics of PKC-labeled astrocytes in the stratum radiatum of the CA1 and subiculum were identical. The numerical density and localization, however, were isoform-dependent. PKC α -positive astrocytes were least abundant (approximately 25 cells mm^{-2}) and most frequently seen wrapped around arterioles. PKC β 2- and γ -positive astrocytes were most abundant, and these glial cells had similar distributions and numerical densities (approximately 102 and 98 cells mm^{-2} , respectively). The numerical density of PKC β 1-positive astrocytes (approximately 39 cells mm^{-2}) was slightly higher than that of PKC α . The processes of PKC β 1-positive astrocytes often had a somewhat granular appearance, which distinguished them from the other PKC-positive astrocytes.

Comparison of the numerical density of PKC-positive astrocytes in the CA1 and subicular stratum radiatum of naive, pseudoconditioned and trace conditioned rabbits is presented in Figure 2. No differences were found in the number of astrocytes immunoreactive for PKC α and β 1 between the

FIG. 1. Associative learning induces an increase in PKC γ -immunoreactivity in pyramidal cells (Pyr) of trace conditioned rabbits (B) over pseudoconditioned rabbits (A), but a decrease in the number of astroglial cells immunopositive for PKC γ (arrow in A). Bar = 15 μ m.

FIG. 2. Number of PKC-positive astrocytes per mm² in the CA1 and adjacent subiculum of naive (N), pseudoconditioned (P) and trace conditioned (T) rabbits. A significant ($p < 0.0001$) reduction in the numerical density of astrocytes immunoreactive for PKC β 2 and γ was found in the CA1 region of conditioned animals, but not in the subiculum. No changes were found for PKC α or β 1.

groups ($p > 0.5$ in all cases) in the CA1 and subiculum. The number of astrocytes immunoreactive for PKC β 2 and α in trace conditioned rabbits (approximately 28 and 29 cells mm⁻², respectively), was significantly reduced ($p < 0.0001$) compared with naive and pseudoconditioned controls in the CA1, but not in the subiculum. As reported previously,² PKC γ -ir was significantly increased in CA1 pyramidal neurons of trace conditioned animals (Fig. 1A,B), whereas no such changes were found in the subicular pyramidal cells or for the other PKC isoforms.

Discussion

Rabbit astrocytes are heterogeneous with respect to the expression of Ca²⁺-dependent PKC-isoforms. Based on the regional distribution, morphological appearance and numerical density, it is likely that PKC β 2 and γ are co-expressed in one subset of astrocytes, while PKC α and β 1-positive astrocytes each represent different subpopulations. The different subsets of astrocytes might have different functions. PKC α -positive astrocytes wrapped around blood vessels, for example, may play a predominant role in

metabolic support of neurons.⁸ The clear expression of PKC γ in rabbit hippocampal astrocytes is species-specific, and differs from that in chick, mouse and rat hippocampal tissue.^{3,14,15} In these species PKC γ is absent or, at best, weakly expressed in astrocytes.

The present results show a conditioning-specific reduction of 70% and 80% in the number of PKC β 2- and γ -positive astrocytes, respectively, which is only seen in a brain region (CA1) where a simultaneous twofold increase in neuronal PKC γ -ir occurs. This reduction is probably due to a downregulation of these PKC isoforms rather than the actual loss of the astroglial cells. The coincidental alterations in neuronal and glial PKC changes might be set in motion by an excess of transmitter release in areas with enhanced neuronal activity during the acquisition of trace eyeblink conditioning, stimulating astroglial receptors coupled to PKC. Activation of astroglial PKC readily induces a long-term downregulation of PKC.¹⁶

Differential conditioning-induced alterations in PKC-ir were found between neurons and astrocytes. We previously speculated² that in neurons only PKC γ changed due to its subcellular compartmentalization in spines (PKC α , β 1 and β 2 are not present in spines^{5,17,18}), which is one likely site for cellular processes underlying associative learning to occur. In astrocytes, however, no such compartmentalization is apparent, which might explain why in these cells both the β 2 and γ isoforms are affected by associative learning.

What might be the impact of altered PKC signal transduction in astrocytes? Two important functions of astrocytes modulated by PKC are buffering of extracellular K⁺ and the propagation of Ca²⁺ waves. Neuronal activity is enhanced in many hippocampal CA1 pyramidal cells during the acquisition of eyeblink conditioning (i.e. increased firing to the tone CS as it becomes behaviorally significant^{19,20}), which results in an increased extracellular K⁺ concentration.²¹ This excess of K⁺ is subsequently buffered by astrocytes, in part through Ca²⁺-dependent K⁺ channels.²² Phosphorylation of K⁺ channels by PKC reduces the K⁺ permeability of these channels.^{11,23} A reduced degree of phosphorylation of astroglial K⁺ channels due to PKC downregulation will, therefore, increase the K⁺ buffering capacity of astrocytes. In addition, reduced phosphorylation of gap junctions by PKCs might be crucial. Gap junction coupling is essential for astrocyte functions, including the spatial

buffering of extracellular K⁺ (i.e. the redistribution of excess of K⁺ through gap junctions²⁴) and the propagation of Ca²⁺ waves.^{12,25} Activation of PKC in astrocytes and subsequent phosphorylation of gap junctions reduces gap junction communication, and downregulation of PKC reverses this inhibition.^{12,13} The subset of astrocytes in which PKC-ir is down regulated might represent a specific circuit of astrocytes with active gap junction communication during the acquisition of trace eyeblink conditioning.

Conclusions

These results clearly demonstrate a conditioning-specific biochemical change in a subset of hippocampal astrocytes. These astroglial changes are region and PKC-isoform specific, and occur simultaneously with neuronal changes. This set of data adds to the currently growing number of observations about glia-neuron interactions in relation to brain function, and suggests a role for astrocytes in learning and memory processes through PKC-regulated mechanisms.

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