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EFFECTS OF ANODAL POLARIZATION ON PROTEIN KINASE Cγ (PKCγ) IN THE RAT BRAIN

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Abstract : An anodal direct current of 0.3 μA or 30.0 μA was unilaterally applied for 30 min or 3 hr to the surface of the sensorimotor cortex of rats, and the effects of anodal polarization on protein kinase C (PKCγ) activity were examined. The brains were processed by means of immunocytochemistry using the monoclonal antibody 36G9 raised against purified PKCγ. In sham-operated animals, PKCγ-like-immunoreactivity (PKCγ LI) was noted in neuronal cytoplasm, as well as in processes in the cerebral cortex and in the hippocampus. Anodal polarization with 3.0 μA for 30 min resulted in a pronounced increase in the number of PKCγ-like-positive neurons in accordance with the intensity of immunostaining in the cerebral cortex, and an increase in the polarized hemispheres was highlighted by repeated applications of the currents. Polarization with 0.3 μA for 3 hr also increased the PKCγ LI, but 0.3 μA for 30 min or 30.0 μA for any duration had no effects. The effect of polarization on PKCγ activity, as evaluated by the intensity of immunostaining and the number of neurons, began to increase 1 h after polarization, peaked at 3 hr and thereafter decreased to the control levels by 72 hr. These results indicated the involvement of the γ-isoform of PKC in the neurochemical mechanism of long-standing central and behavioral changes induced by anodal polarization.

Key words : anodal polarization, immunocytochemistry, rat, sensorimotor cortex, PKCγ, direct current

INTRODUCTION

Anodal polarization, the passage of constant weak currents to restricted cortical areas such as the motor cortex, has been regarded as a useful experimental model with which to study the mechanisms of central plastic changes, as it produces long-lasting neuronal functional changes by means of a small temporary alteration in their physical environment (1). Polarization causes characteristic changes in the electrophysiological activity of the cortex (1-4) and peripheral manifestations (4-6). Anodal polarization increases the neuronal firing rate, and the number and the size of

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evoked potentials with persistent after-effects (1-3, 7), which progressively increase with repeated applications of the current (2). The changes of characteristic peripheral motor activity, such as contralateral forelimb flexions of rabbits induced by anodal polarization, reportedly persist for several hours (4), or even as long as several weeks (5, 8) with reproducible results. Glial and neurochemical changes, such as cyclic AMP generation, accumulation of reactive phosphate groups of RNA, and protein synthesis, also occur in the polarized cortex (9-12). Although the phenomenon has been attributed to the formation of a chronic excitation focus (called "dominant focus") at the polarized point (13), the underlying basic mechanism remains obscure.

Phosphorylation reactions are important mechanisms by which extracellular signals are transmitted into the cells to regulate their functions (14). Among the many kinases, the calcium/phospholipid-dependent kinase, protein kinase C (PKC), appears to play a major role as the transmembrane signal of many extracellular agonists (15). In the brain where it is particularly abundant. (16,17), this enzyme has been implicated in neurotransmitter release, regulation of ion channels and neuronal plasticity (15, 18). Studies on other forms of synaptic plasticity, such as long-term potentiation (LTP) or kindling, have suggested the involvement of PKC in the mechanisms of central plastic changes (19, 20). These findings indicate that PKC may play an important role in the modulation of neuronal functions, ultimately leading to a hyperexcitability state responsible for the long-lasting electrical and behavioral manifestations following anodal polarization. However, little is known about the occurrence, despite the extensive clinical and experimental use of anodal polarization (1-13, 21). In this study, we used qualitative immunocytochemistry to examine the activation and subcellular distribution of PKC following anodal polarization of the sensorimotor cortex in the rat brain, in relation to the cellular and molecular bases of the polarization phenomenon.

METHODS

The animal preparations were essentially as described in our previous report (22). Briefly, male adult Wistar rats weighing 180-230 g were allowed to free access to tap water and food pellets, and they were maintained on a 12 hr light/dark cycle at –20°C. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital 35 mg/kg, and placed in a stereotaxic frame. The scalp was incised along the midline and the subcutaneous tissue was removed to expose the cranium. Two silver electrodes (1 mm in diameter) were bilaterally implanted into the cranial bone so as to set the tips on the dura mater over the sensorimotor cortex at symmetrical points of 1.5 mm rostral and 3.5 mm lateral to the bregma. A stainless steel electrode was implanted in the midline of the nasal bone. These electrodes were secured in place with dental resin.

After a post-operative recovery period of 7 days or more, the rats were divided into experimental and control groups. For anodal polarization, the experimental rats were transferred from their home cages to a large cage with no food and water and left for at

least 1 hr for acclimatization. The polarization was performed without anesthesia or restraint. A 0.3 μA, 3.0 μA or 30.0 μA current from a dry battery was applied continuously for 30 min or 3 hr to the surface of the left sensorimotor cortex, using the cortical and nasal electrodes as the anode and cathode, respectively. In another group of rats, anodal polarization (3.0 μA for 30 min) was repeated five times at an interval of 24 hr for successive days. The control rats received no current (sham -operated).

Tissue preparation

At various periods following polarization (1-72 hr), the rats were transcardially perfused with 400 ml of a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) under ether anesthesia. After careful removal from the cranial cavity, the perfused brains were stored in 0.1 M PB containing sucrose for cryprotection (4°C). Frontal sections (30 μm) were cut on a freezing cryostat while immersed in 0.1 M phosphate buffer saline (PBS).

Immunocytochemistry

After washing with PBS containing 0.3% Triton X-100 (PBS-T), the sections were incubated for 30 min in 10% normal sheep serum (NSS) in PBS at room temperature (RT) to block nonspecific binding sites. The free floating sections were then gently agitated overnight at 4°C with the primary antibody solution in PBS (mouse anti PKCγ IgG; 36G9, 1:200). After rinsing in PBS-T, the sections were exposed to biotinylated sheep anti-mouse IgG (1:200; Amersham) for at least 1 hr at RT. The sections were rinsed in PBS-T, then incubated with strepavidin-HRP (1:200; Zymed) for 1 hr at RT. Finally, the sections were washed in PBS and stained with diaminobenzidine containing glucose oxidase and nickel (23). The sections were then mounted and cover slipped for light microscopic inspection. Specificity was confirmed by means of preabsorption with purified protein kinase C, by omission of the primary antibody in the incubation cycle, or by using normal serum instead of primary antibody.

Quantitation of PKC-γ positive neurons

We initially checked all the brain structures for polarization-induced changes in PKC-γ expression. However, after it became clear that the main structures of changes were invariably found in the cerebral cortex, polarization induced changes in PKC-γ activity were quantified by counting the number of PKC-γ positive neurons in this region. The sampling areas are shown as hatched square in Fig. 1 (inset), where an area of 520×520 μm was counted by means of an ocular grid, which was moved with side-to-side and/or end-to-end inside the cerebral cortex, at X125 magnification. The sections at the level of the bregma were quantified. Statistical analysis was performed by means of the analysis of variance, followed by Student's 't'-test.

RESULTS

In all sham-operated control rats to

which no current was applied, PKCγ-positive neurons were consistently observed throughout the entire cerebral cortex (Fig. 2A) and hippocampus. PKCγ LI was found in the cytoplasm and processes of neurons. Occasionally, there was weak immunoreaction in the nuclei of a few pyramidal neurons, but almost never in the nucleus. The intensity of PKCγLI increased when an anodal current of 0.3 μA or 3.0 μA was applied for 3 hr or 30 min, respectively, to the surface of the sensorimotor cortex (Fig. 2B). The increase mostly occured in the plasma membrane of the cell bodies and processes of neurons in layer 2/3, and 5 and 6 of the neocortex (Fig. 2C). An increase in PKCγ LI caused by anodal polarization was found in the hemisphere ipsilateral to the polarization, but not in the contralateral hemisphere. In the ipsilateral hemisphere, PKCγLI was most pronounced in the neocortex and cingulate cortex. A moderate level of immunostaining was noted in the piriform cortex.

The number of PKCγ-positive neurons begun to increase 1 hr after anodal polarization with 0.3 or 3.0 μA for 3 hr or 30 min, respectively, and peaked by 3 hr (Fig. 1). This increase was more pronounced after repeated polarization. Thereafter, the number gradually decreased, reaching control levels 72 hr after one polarization trial. In contrast, after repeated polarization, the number of PKCγ-positive neurons remained almost constant at the elevated level until 24 hr after the last of 5 trials (Fig.1). Thereafter, the number of PKCγ-positive neurons reached control levels 1 week after the last trial. The time course of changes in the intensity of

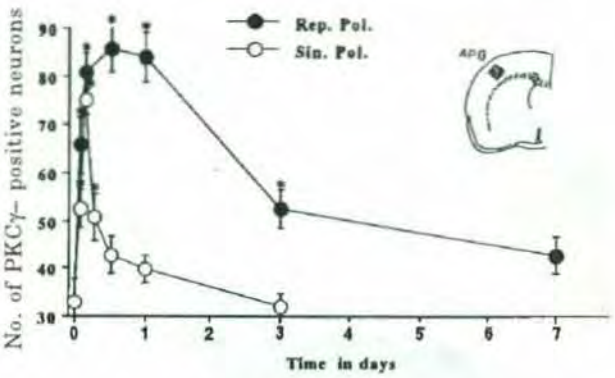

Fig. 1: The time course of changes in the number of PKCγ–positive neurons in the polarized neocortex following anodal polariazation with 3.0 μA for 30 min after a single (0) and the last of 5 repeated polarization (●). Values show the number of PKCγ–positive neurons expressed as the mean ±SEM of 5-6 animals. 'O' on abscissa corresponds to control data. Inset: A schematic drawing of the section selected for neuronal quantitation at the level of the bregma (AP 0). *P<0.05, comared to the control.

immunostaining in individual neurons during the progressive period was parallel.

A trial of one polarization with 0.3 μA or 3.0 μA caused a significant increase in the number of PKCγ-positive neurons in the polarized cortex. During repeated polarization trials, the number of PKCγ-positive neurons remained virtually constant at the elevated level in the polarized cortex (Fig. 3). In the contralateral cortex, there were no changes in the number of PKCγ-positive neurons before, and after several trials of polarization.

Fig. 4 shows the effects of one polarization trial with different intensities (0.3, 3.0 or 30.0 μA) or durations (30 min or 3 hr) on the number of of PKCγ-positive

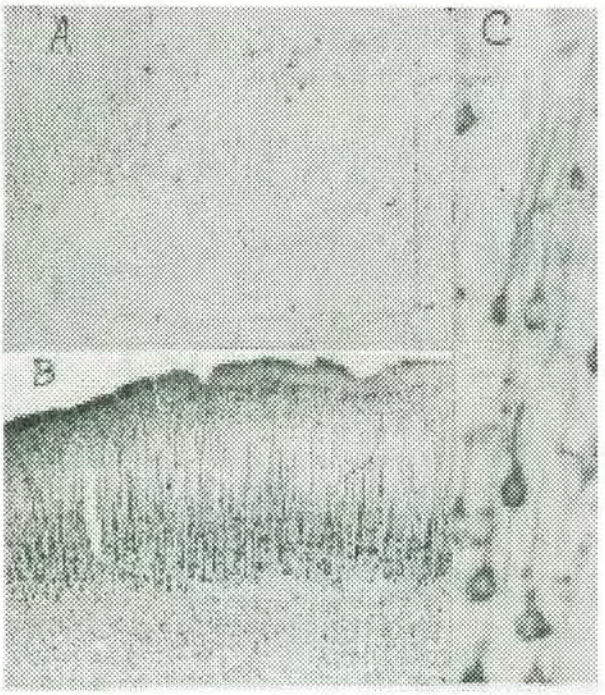

Fig. 2: PKCγ-like-positive neurons in the neocortex. A. Control. B. A clear increase in PKCγ LI was observed 3 hr after anodal polarization with 3.0 μA for 30 min. C. Large pyramidal neurons of layer 5 in B are shown at a higher magnification. Bar: A, B=130 μm; C=40 μm.

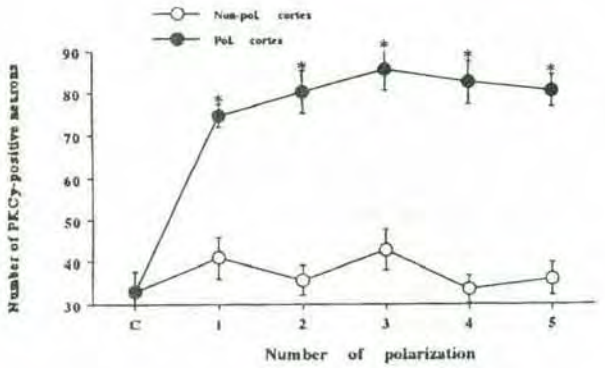

Fig. 3: The number of PKCγ-positive neurons in the polarized (●) and nonpolarized (○) neocortex following various trials of repeated anodal polarization with 3.0 μA for 30 min. Each value represents the mean ±SEM of 5-6 animals. *Significantly different (P<0.05) from the value of non-polarized control rats (C).

neurons. When applied for 30 min, the polarizing current of 3.0 μA invariably produced a significant increase in the number of PKCγ-positive neurons. A current of 0.3 or 30.0 μA had no effect for this duration. When applied for 3 hr, a polarizing current of 0.3 μA effectively enhanced the PKCγ activity but to a lesser extent than that of the polarization paradigm with 3.0 μA for 30 min. But, a current of 3.0 or 30.0 μA had no effects for this duration.

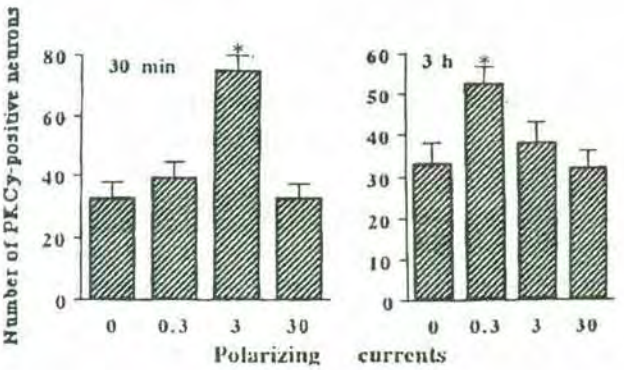

Fig. 4: The effects of a polarizing current with different intensities or durations on the number of PKCγ-positive neurons in the polarized neocortex. Anodal direct current of 0.3, 3.0 or 30.0 μA was applied for 30 min (left) or 3 hr (right)

DISCUSSION

These results showed that the intensity of neuronal immunostaining of PKCγ with antibody 36G9 and the number of PKCγ-positive neurons were enhanced after anodal polarization, providing evidence for the involvement of PKC activation in the polarization phenomenon. After polarization, PKCγ-staining was predominantly increased along the plasma membrane of the cell

bodies and processes. This finding suggested that synaptic transmission, by which PKCγLI is enhanced, shifts from the cell body to the membranes and processes. Another explanation for the enhanced PKCγLI is that de novo synthesis and degradation are changed, thus enlarging the total pool of PKCγ. Newly synthesized PKCγ might be time-dependently transported from the cell body to the membranes of processes. A similar time dependent redistribution of PKC from the cell somata to dendrites due to *de novo* synthesis of PKC has been suggested after conditioning (24). Activated PKC is reportedly involved in neurotransmitter release and in controlling ion channels (15,18). Anodal polarization may also influence the migration of divalent ions (25), membrane resistance and neurotransmitter release (26), in addition to protein synthesis and trans-synaptic modulation (10, 27). The enhancement of PKCγ activity found in the polarized cortex therefore, might be due to receptor activation, an increase in intracellular calcium concentration, proteolysis of PKC or the formation of PKC activators.

There were regional differences in PKC activation in cortices polarized with 0.3 μA for 3 hr and 3.0 μA for 30 min. Other polarization paradigms had no effect on PKCγLI. These results suggested that a polarizing current had different effects on the PKC activation system depending on intensity and duration; they either enhance and/or are ineffective. Earlier studies on cyclic AMP generation and behavioral activity using several paradigms of anodal polarization support this notion (6, 12). Polarization with 3.0 μA for 30 min profoundly increased the number or PKCγ-positive neurons in the polarized cortex. It has been suggested that current intensities of around 3.0 μA are optimum for establishing peripheral motor behavior and higher and lower current intensities are mostly inconvenient or have rather disturbing effects (5, 6). Thus, our results seem to provide neurochemical evidence for the optimum intensity of polarizing currents in establishing the behavioral manifestation induced by anodal polarization.

The increased behavioural motor activity induced by anodal polarization lasts for several weeks, during which repeated polarization is necessary to establish stable motor activity (6, 8). Repeated applications of the currents are also significant in highlighting their effects on cyclic AMP accumulation in the polarized cortex (11). The present resuls showed that a similar highlighting in the activtion of PKC in the polarized cortex appeared during repeated applications of polarizing currents, where the activation lasted for one week (Fig. 1). Thus, it is conveicible that the repeated anodal polarization makes activation of PKC sharp for a longer duration and leads to the establishment of long-lasting behavioral manifestations.

In conclusion, the application of polarizing currents to the restricted cortical point activated the γ-isoform of PKC. The effects of anodal polarization were biphasic. The most stimulatory effects occured when a polarizing current of 3.0 μA was applied and repeated application of the currents maintained the activation for longer duration. Thus, it is likely that the activation of PKCγ plays a fundamental role in the neurochemical mechanisms of cortical excitation induced by anodal polarization.

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