

## CO-LOCALIZATION OF C-FOS PROTEIN AND PROTEIN KINASE C $\gamma$ IN THE RAT BRAIN FOLLOWING ANODAL POLARIZATION

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**Abstract:** The expression of protein kinase c  $\gamma$  (PKC $\gamma$ ) and c-fos protein was examined by means of double labeling in the rat brain in relation to the molecular mechanism of central plastic changes associated with anodal polarization. Under normal, non-polarized condition, approximately 75% of all fos positive neurons in the neocortex were immunopositive for PKC $\gamma$ . Conversely, nearly all PKC $\gamma$  positive neurons were fos immunopositive. Although both pyramidal and non-pyramidal neurons express both types of protein, the pyramidal cell type represents the vast majority. An anodal direct current of 3.0  $\mu$ A for 30 min to the surface of the left sensorimotor cortex resulted in a pronounced increase in the intensity of immunoreactivity for both PKC $\gamma$  and c-fos protein ipsilateral to the polarization. Approximately, 91% of fos positive neurons in the polarized neocortex was also intensely immunoreactive for PKC $\gamma$ . The high degree of codistribution of both transduction proteins in specific neurons following anodal polarization suggests the functional connection between PKC $\gamma$  activation and c-fos expression in polarization phenomenon.

**Key words:** transduction proteins  
C-fos protein

protein kinase C  
anodal polarization

### INTRODUCTION

Anodal polarization, the passage of a constant weak direct current to the sensorimotor cortex, is reported to cause characteristic changes in electrophysiological activity of the cortex and peripheral manifestations (1-5) with persistent after-effects. The phenomenon has been implied to be due to formation of a chronic excitation focus, which is called dominant focus, at the polarized point (6). Anodal polarization has been considered an important experimental model to study the mechanisms of central plastic changes (7) and has been thought to be useful for studying the mechanism of the formations of memory traces, learning and the process of conditioning (2). However, despite a number of electrophysiological and neurochemical studies (1-9), the basic molecular mechanism underlying the chronicity of cortical dominant focus remains unclear.

It has been suggested that immediate early genes might contribute to the maintenance of long-term responses in the central nervous system, such as long term potentiation (LTP), kindling and epilepsy (10-12). The cellular function of c-fos, a member of immediate early gene family, is not clear, but it has been proposed that this protein participates in the long-term cellular changes by regulating specific target gene expression, where it acts as a nuclear "third messenger" (13). Activation of c-fos is dependent (in cultured cells) upon cyclic AMP and protein kinase C (PKC) (14, 15). PKC, a key enzyme for signal transduction, induces c-fos (14) and may be involved in synaptic plasticity (16). Earlier study suggests a functional connection between PKC $\gamma$  activation and c-fos expression in neurons that are involved in learning (17). In view of these findings, we have speculated that there is an important functional

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connection between PKC $\gamma$  activation and c-fos expression in the modulation of neuronal functions, ultimately leading to long-lasting after-effects. Thus, in this study, we evaluated whether the expression of c-fos in the polarized neocortex is in neurons containing PKC $\gamma$ .

## METHODS

The operative and polarization procedures were essentially the same as those described (18). Briefly male Wistar rats weighing 180-230 g were allowed free access to food and water, and were housed under a 12 h light/dark cycle at 20-24°C. Under sodium pentobarbital anesthesia (35 mg/kg, i.p.), two silver electrodes (1 mm in diameter) were implanted bilaterally into the cranial bone with their tips set 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 into the midline of the nasal bone. All the electrodes were secured to the place with dental resin. The rats were allowed to recover for at least 1 week before polarization. An anodal direct current of 3.0  $\mu$ A was continuously applied to the surface of the left sensorimotor cortex for 30 min, or 3 h with a cortical electrode using the nasal one as the cathode. Polarization was performed without anesthesia or restraint. Polarization procedures were carried out between 09:00 h and 12:00 h. For the non-polarized control, rats were implanted with the electrodes but no current was applied.

At various times, the transcardiac perfusion proceeded with a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.5% glutaraldehyde in 0.1 M phosphate buffer solution (PB; pH 7.4) under ether anesthesia. For cryoprotection, the perfused brains were stored in PB solution containing 30% sucrose (4°C). Frontal sections (30  $\mu$ m) were cut using a cryostat. The method of double-labeling was only slightly modified from that reported by Ambalavanar et al (17). The sections were washed in 0.1 M PB-saline (PBS) containing 0.3% Triton X-100 (PBS-T), and incubated overnight at 4°C with mouse monoclonal anti-

fos (1:1000; Oncogene Science). After rinsing, the sections were processed using avidin-biotin complex horseradish peroxidase kit (Vector). The bound enzyme complex was visualised by diaminobenzidine (DAB) treatment (19), which gave a dense blue black reaction product. For double-labeling, the same sections were subsequently preincubated with 1% normal sheep serum for 1 h to reduce the nonspecific reactions, after treatment with avidin/biotin blocking solution (Zymed Kit). Then the sections were incubated overnight with mouse monoclonal anti-PKC- $\gamma$  IgG (36G9; 1:200; 4°C). Following rinsing with PBS-T, sections were incubated with secondary biotinylated sheep anti-mouse IgG (1:200; Amersham). Finally, after treatment with streptavidin-HRP (1:200; Zymed), the sections were subjected to a DAB reaction. The resultant brown coloured cell bodies with dendrites indicating PKC $\gamma$  were easily distinguishable from the dense blue black fos positive nuclei. Immunocytochemical control studies for the specificity were made by omission of either of the two or both antibody in the incubation cycle; or using normal mouse serum instead of primary antibody. In all cases, the controls yielded negative results, i.e., absence of any detectable labeling. The degree of co-expression was quantified by direct visual counting of 36G9 positive neuronal somata with processes and c-fos positive nuclei within an area of 450 x 450  $\mu$ m in the neocortex at the level of bregma at a magnification of X125.

## RESULTS

*Distribution patterns of fos and PKC $\gamma$  immunoreactivity:* The fos immunoreactivity was predominantly found in the nuclei of the neurons, whereas the neuronal somata, dendrites and nucleoli did not appear to be stained (Fig. 1A). In sham-operated control rats, there was faint staining of the cell nuclei in the cerebral cortex, hippocampus, piriform cortex and amygdala. Very weak staining was found in the midbrain, thalamus and septum. Immunoreactivity was only in the neurons, none was evident in glial cells. On the other hand, immunoreactivity to PKC $\gamma$  was present in the

cytoplasm of the cell bodies and associated dendrites (Fig. 1B). Occasionally, a few pyramidal neurons showed weak nuclear staining. In sham-operated control rats to which no current was applied, a consistent distribution pattern of PKC $\gamma$ -positive neurons was apparent throughout the entire cerebral cortex and hippocampus for all rats examined.

*Co-localization of fos and PKC $\gamma$  immunoreactivity:* Immunocytochemical double-labeling revealed a characteristic distribution of fos/PKC $\gamma$  double-labeled neurons, both markers were present, c-fos was in the nuclei, and PKC $\gamma$  was in the cell body and the dendritic processes (Fig. 2). The double-labeled neurons were most abundant and prominent in the large pyramidal neurons of layer V. Both pyramidal and non-pyramidal neurons appeared to be double-stained. However, the highest degree of coexistence was found in the large pyramidal neurons of layer V (Fig. 2). Of  $31.9 \pm 3.1$  large

C-fos-positive pyramidal neurons,  $23.9 \pm 2.4$  (75%) appeared to be double labeled. Conversely, approximately all PKC $\gamma$ -positive neurons were fos positive. Of all PKC $\gamma$ -positive neurons, 3% was found to be single labeled.

*C-fos and PKC $\gamma$  distribution characteristics following anodal polarization:* Polarized rats showed a massive increase in c-fos protein-like immunoreactivity in various layers of neocortex, cingulate cortex, piriform cortex, hippocampus and amygdala ipsilateral but not contralateral to the polarization. This increase was rapid in onset; being present as early as at 30 min, but not at 15 min after polarization, and transient. The number of neurons showing the response was almost back to baseline within 24 h. The number of c-fos positive neurons and intensity of immunostaining was maximal at 1 h in all layers of the neocortex (Fig. 3-A, B), the hippocampus and the piriform cortex ipsilateral to polarization.

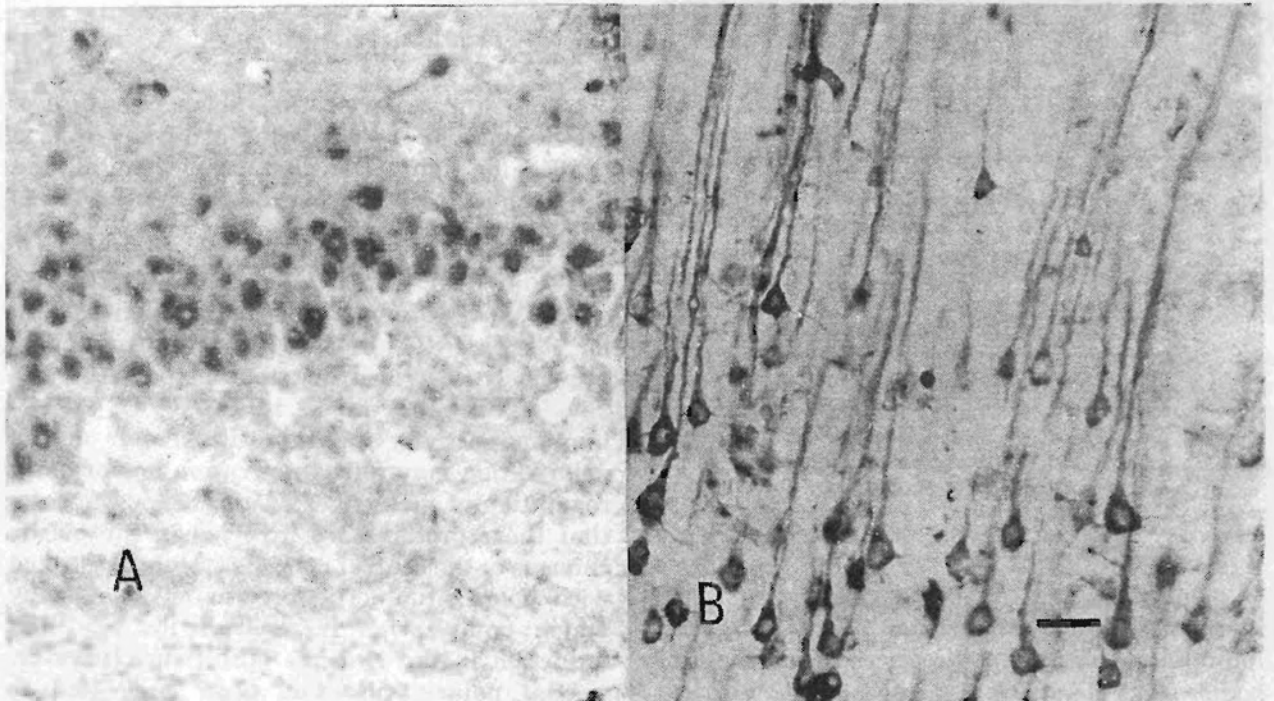


Fig. 1 : Photomicrograph showing c-fos protein-like immunoreactivity. Note the pronounced nuclear staining and the lack of staining in the nucleoli, cytoplasm and nerve cell processes. B : PKC $\gamma$ -like immunoreactivity. PKC $\gamma$ -like immunoreactivity is present in the cytoplasm of the cell bodies and dendritic arborizations. Bar=0.04 mm.



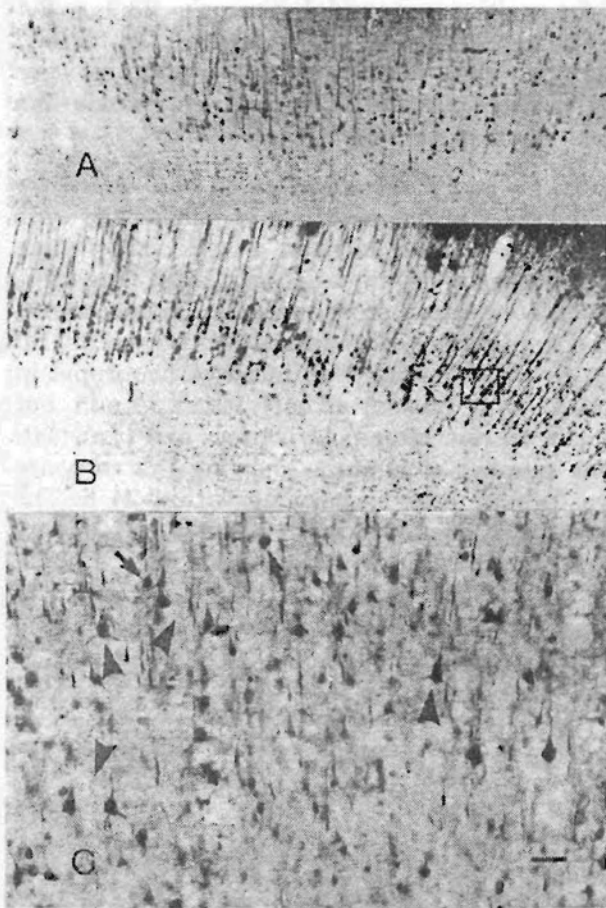


Fig. 2 : Immunocytochemical double-labeling for c-fos and PKC $\gamma$  in the neocortex. A: Double-labeled neurons are seen in sham-operated control; B: A clear increase in double-labeled immunoreactivity is found after polarization; C: The area within the square in B is shown at a higher magnification: A high degree of colocalization becomes apparent, fos in the nuclei and PKC $\gamma$  in the cytoplasm and nerve cell processes (arrow heads). However, besides double-labeled neurons, some neurons appear to be fos single-labeled [arrows]. Bar: A, B=0.13 mm; C=0.03 mm.

The intensity of PKC $\gamma$  immunoreactivity increased after polarization. The increased immunoreactivity mostly occurred in the cell bodies and dendritic processes of neocortical and hippocampal neurons ipsilateral to the polarization. In layer V, the large pyramidal neurons with associated dendrites were strongly

PKC $\gamma$  immunopositive. Increased immunoreactivity was observed at 1 h, became maximum at 3 h after polarization (Fig. 3-C, D). The number of PKC $\gamma$ -positive neurons decreased gradually, and decreased to control level 72 h after polarization. A parallel time course of changes in intensity of immunoreactivity in individual neuron during the progressive period was also observed.

*Co-localization of c-fos and PKC $\gamma$  immunoreactivity after anodal polarization:* After 1-3 h of polarization, double-labeling showed that majority of fos-positive neurons were immunoreactive for PKC $\gamma$  in the neocortex (Fig.2). However, due to the increase in the total number of c-fos-positive ( $135.1 \pm 8.7$  SEM) and PKC $\gamma$ -positive ( $122.7 \pm 7.9$  SEM) neurons in the neocortex following polarization, about 91% ( $\pm 1.2$  SEM) of neurons that expressed fos also stained positive for PKC $\gamma$ . This type of co-localisation of PKC $\gamma$  and c-fos was also seen in the cingulate and piriform cortices, and in the hippocampus.

## DISCUSSION

This study showed that about two-third of the neurons in the neocortex use the  $\gamma$ -isoform of PKC for signal transduction in general, and the number of these neurons increased (91%) following anodal polarization.

*Fos and PKC $\gamma$  immunoreactivity following anodal polarization :* This distribution pattern for fos or PKC $\gamma$  in the rat brain resembled previously described distribution patterns (20-24). The increase in fos or PKC $\gamma$  immunoreactivity in brain slices after anodal polarization may reflect activation and/or down regulation of messenger systems, and suggests that these proteins are involved in the plastic changes of the central nervous system associated with polarization. The precise mechanism by which polarization increases c-fos or PKC $\gamma$  induction in the neurons is unclear. However, anodal polarization has been suggested to influence the migration of divalent cations, membrane resistance or neurotransmitter release (25). In addition, protein synthesis and

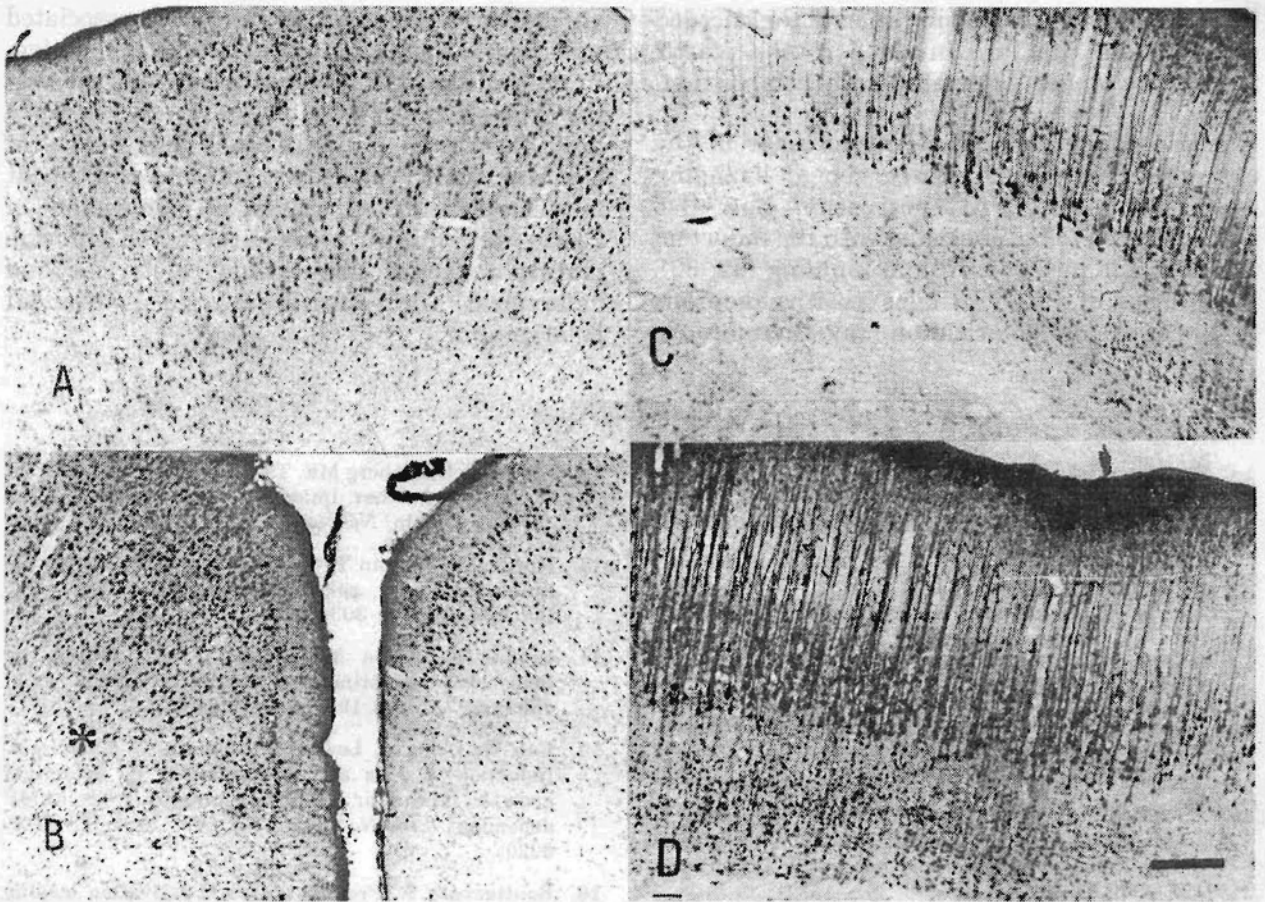


Fig. 3 : The effects of anodal polarization on the immunoreactivity for c-fos (B) and PKC $\gamma$  (D) compared to non-polarized brain slices (A, C respectively). A clear increase in fos (A versus B) or PKC $\gamma$  (C versus D) immunoreactivity is found following anodal polarization. Bar=0.1 mm. \*indicates polarized hemisphere.

transsynaptic modulation has also been reported to be involved in the increased cortical excitability by anodal polarization (26, 27). Role of Ca<sup>2+</sup>, c-AMP or neurotransmitter in fos or PKC activation is well documented (28, 29). The enhanced fos or PKC $\gamma$  immunoreactivity observed in the polarized cortex, therefore, may be due to receptor activation, an increase in intracellular Ca<sup>2+</sup> concentration or changes in neurotransmitter release.

*Co-localization of fos and PKC $\gamma$  following anodal polarization* : Among the 7 isoforms, one of the most abundant PKC isoform in the central nervous system is the  $\gamma$ -isoform (30), which is also the major isoform present in the cerebral

cortex (24). The PKC $\gamma$  negative neurons most likely utilize other PKC isoforms as the predominant second messenger system. The increased co-expression following anodal polarization indicates the functional connection between fos expression and PKC $\gamma$  activation. It has been suggested that proto-oncogenes, such as c-fos is transiently and rapidly induced by neuronal activation (31), and PKC dependent phosphorylation is required for the activation of these immediate early genes (29). PKC may serve to bridge the gap from a transient signal to a longer-lasting cellular change. Therefore, the increased co-localisation of c-fos protein with PKC $\gamma$  in the neurons following anodal polarization may indicate the notion that PKC $\gamma$

takes part in the phosphorylation of the encoded c-fos protein that are involved in the plastic neuronal changes associated with polarization.

In addition to a transient increase in the expression of various early genes, including c-fos, persistent PKC activation has also demonstrated to be associated with the induction and maintenance of LTP and kindling (32, 33). Expression of PKC $\gamma$  in c-fos positive neurons following anodal polarization may, therefore, be

an indicative common modification associated with long-lasting changes in neuronal function. In view of the present finding, we suggest that the expression of PKC $\gamma$  in c-fos positive neurons may provide a basis for the state of hyperexcitability in these rats, and this result may support the hypothesis of involvement of PKC $\gamma$  dependent c-fos activation in the maintenance of long-lasting electrical and behavioral after-effects induced by anodal polarization.

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