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Protein kinase C expression and activity in the human brain after ischaemic stroke

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Abstract. We examined the expression of protein kinase C isoforms in infarcted tissue, penumbra and contralateral brain tissue from 10 patients who died between 1-52 days after ischaemic stroke. Ten patients aged 61-89 years were used in the study. Tissue samples were assayed for protein kinase C activity using a non-radioactive method, and specific isoforms expression determined by Western blotting and staining with anti-PKC polyclonal antibodies. There was a 2-24 fold increase in PKCγ in the ischaemic penumbra of nine out of 10 patients compared to contralateral tissue. In infarcted tissue expression of PKCγ was not significantly changed in any of 10 samples but the βI isoform increased in eight and the βII in nine patients. There was no significant change in expression in PKCα or in infarct or penumbra. Differences in total PKC activity were not specific in seven out of eight patients and it is difficult to estimate their significance. In conclusion after ischaemia there was an altered expression of PKC isoforms with an increase of PKCγ in the surviving penumbra and βI and βII in the infarcted core.

Key words: protein kinase C, ischaemic stroke

INTRODUCTION

Activation of protein kinase C (PKC) is an early signal transduction event in the inflammatory process and immune response involving platelets, fibroblasts and endothelial cells (Nishizuka 1986). Continued activation of PKC is essential for subsequent responses such as cell proliferation and differentiation through activation of MAP kinases and transcription factors, eg. AP-1, c-fos and c-jun (Bishop and Bell 1988, Presta et al. 1991, Moriya et al. 1996). Signalling through PKC is an important pathway for the control of gene expression and the cell cycle. In the cell membrane PKC has a central role in down-regulation of receptors, modulation of ion-channels, release of hormones and neurotransmitters and exocytosis (Nishizuka et al. 1986). PKC consists of a family of at least 12 isoforms which phosphorylate serine and threonine residues on their target proteins (Wetsel et al. 1992). The isoforms are composed of regulatory and catalytic subunits on a single polypeptide chain and with a molecular mass of around 80 kDa. PKC isoforms have specific functions within a cell or tissue type. For example, PKCα mediated bFGF (basic fibroblast grwoth factor) protection of endothelial cells against radiation-induced apoptosis (Haimovitz-Friedman et al. 1994) whilst PKCβ_I activation corresponded with differentiation of human melanoblasts (Park et al. 1993) and PKCε mRNA increased in human neuroblastoma cell lines after treatment with IFNγ (interferon gamma) (Ponzoni et al. 1993). PKC regulates neuronal excitability (Kaczmarek 1987), neurotransmitter release (Pozzan et al. 1984), synaptic plasticity (Akers et al. 1986), apoptosis (Forbes et al. 1992, Rajotte et al. 1992) and brain cell proliferation (Singh et al. 1994). PKC may be involved in the survival of neurons since many neuronal trophic growth factors, including nerve growth factor, function partly through PKC activation (Clarke and Lee 1991). In neurodegenerative diseases such as Alzheimers, PKC is thought to play an important role in glia and in neurons. There is a significant increase in the number of glia and associated sprouting neurites together with secretion of many different neurotransmitters in senile plaques, consistent with the idea that neurotrophic factors might be secreted by glia and that this process is controlled by PKC. Recent observations suggest that PKC, especially the α isoform, constitutes an important mediator in the initiation of myelin formation (Young et al. 1994). Activation of PKC can be mediated by release of endothelins from human cerebrovascular endothelium. Endothelins increase the permeability of small blood vessels to low molecular weight molecules and thus may contribute to the formation of cerebral oedema (Stanimirovic et al. 1994). Studies of the distribution of the PKC β_I and β_{II} isoforms show that there is more of the former in the brain and reveal idiosyncratic cell expression. For example, in rat cerebellar cortex, the β_I species is present mainly in the granule cell bodies and the β_{II} in the molecular layer -localised in the presynaptic nerve endings terminating on the dendrites and cell bodies of Purkinje cells (Nishizuka 1988). Interestingly the γ isoform appears to be expressed solely in the brain and spinal cord and is concentrated in the hippocampus, cerebral cortex and amygdaloid complex. In the cerebellum the γ isoform is localised in the nerve endings of Purkinje cell axons that terminate in the deep cerebellar nuclei. Thus PKC may be important in specialised neuronal processes, such as long-term potentiation in the hippocampus (Akers et al. 1986, Suzuki 1994). We have previously shown that brain ischaemic stroke is followed by dynamic changes, for example increased angiogenesis in the penumbra (Krupiński et al. 1994). This may prove to be of therapeutic interest. In this study we demonstrate differences of PKC isoform expression in stroke, penumbra and normal-looking brain tissue.

METHODS

Patients and tissue samples

Brain tissue samples from human thromboembolic stroke were obtained from 10 patients (see Table I). The patients, for male and six female, were aged between 61-89 years and lived between 1-52 days following the stroke. Death was due to occlusion of the middle or posterior cerebral artery. Immediately after death, the body was put in a cold chamber and kept for next two to six hours. Tissue samples were collected only from grey matter, either the infarct region, the penumbra or the contralateral hemisphere which was used as a control. Tissue was immediately frozen in liquid nitrogen, kept at -70°C and a section was stained with haematoxylin and eosin (Eke et al. 1990) to determine tissue morphology.

Extraction of proteins from tissue samples

Tissue samples (1 g) were washed in ice-cold PBS and homogenised at 4°C with a Teflon homogeniser in 3 ml

TABLE I

Details of patients used in the study

Patient	Sex	Age	Survival time	Hemisphere	Artery affected
1	F	89	25	L	MCA/PCA
2	M	63	14	L	MCA
3	F	85	16	R	MCA
4	F	65	52	L	MCA
5	M	65	22	L	MCA
6	F	61	16	R	MCA
7	F	73	7	R	MCA
8	M	71	1	R	MCA
9	F	78	21	L	MCA
10	M	74	1	R	MCA

of a buffer containing 10 mM Tris-HC1, pH 7.5, 50 mM NaC1, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 0.1% SDS, 1mM Na_3VO_4 and 5 μg/ml aprotinin (Vainikka et al. 1994). Extracts were sonicated, centrifuged (33,000 g; 10 min) and the supernatant containing soluble enzymes removed and stored at -70°C. Protein concentration was determined using the Biorad microassay (Bradford 1976).

PKC activity assay

A non-radioactive PKC detection kit was obtained from Upstate Biotechnology Incorporated. Triplicate 10 μg samples of cell extract (12 μl) were mixed with 108 μl of a mixture containing Tris-HC1 (25 mM), $MgC1_2$ (2 mM), ATP (0.1 mM), $CaC1_2$ (0.8 mM) and phosphatidylserine (PS, 50 μg) pH 7.0. EGTA (200 mM, pH 7.0) was added instead of PS as a negative control. Samples were preincubated for 5 min at 27°C and 100 μl of the reaction mixture added to a well precoated with porcine glial fibrillary acidic protein (GFAP). The PKC was allowed to bind to and phosphorylate GFAP (5 min, 27 °C) and the reaction stopped (20% phosphoric acid). After washing, 100 μl of mouse monoclonal antibody to phosphorylated GFAP was added to each well and the samples incubated (30 min, 27 °C). After washing, 100 μl of peroxide conjugated anti-mouse antibody was added to each well and allowed to incubate (1 h, 27 °C). After washing, peroxidase substrate (100 μl) was added and the absorbance measured at 405 nm on an ELISA plate reader. A range of PKC standards (0.05-1.0 U) were processed at the same time and PKC activities determined from the standard curve. Significant differences ($P<0.05$) were determined by Students *t*-test.

Western blotting

Samples of protein (10 μg) were mixed with 5x Laemmli sample buffer, vortex mixed and boiled for 15 min before application and separation along with prestained molecular weight markers by 12% SDS PAGE. The proteins were electroblotted onto nitrocellulose filters (Hoefer, California USA) and the filters blocked overnight with 5% defatted milk in TBS-Tween (50 mM Tris-HC1, pH 7.4, 200 mM NaC1 and 1% Tween 20) for PKC analysis. After blocking, filters were stained for 4 h at ambient temperature with PKC isoform specific polyclonal antibodies (1:100 in blocking buffer, Santa Cruz Laboratories). Filters were washed in TBS-Tween (5 x 3 min) before staining with the appropriate peroxidase conjugated secondary antibody (anti-rabbit, 1:1,000, Sigma) for 1 h at room temperature. After washing in TBS-Tween (5 x 3 min), blots were developed using the ECL Western blotting detection system. The relative amounts of PKC were estimated from the X-ray film by densitometric scanning using an LKB 2202 ultrascan densitometer. PKC isoform expression in infarct or penumbra were compared to the control sample (normal contralateral tissues) assigned an arbitrary value of 1.0.

Antibody specificity studies

Equal amounts of protein (10 μg) from total cell lysates were resolved in duplicate by 12% SDS-PAGE and blotted onto nitrocellulose as described for western blotting. Antibodies (1 μg/ml) were incubated with or without blocking peptide (10 μg/ml) for 4 h on a rotating mixer. Antibodies were then further diluted in the appropriate blocking buffer and identical blots stained with antibody with or without peptide treatment using the method described in Western blotting.

RESULTS

Brain tissue from the contralateral hemisphere of stroke patients was shown to express the α, β_I, β_{II}, γ and ε but not Θ or δ isoforms of PKC (data not included).

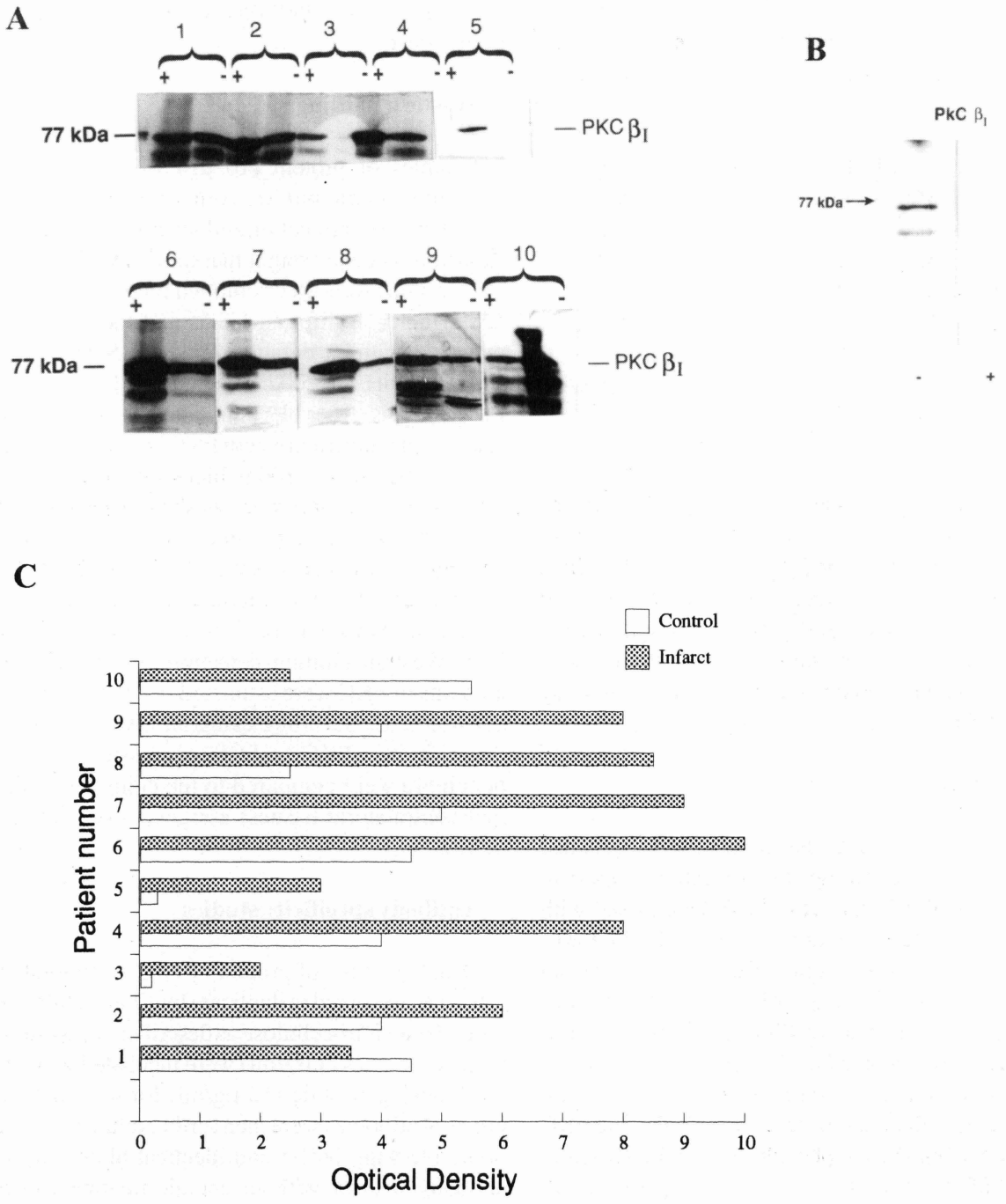

Fig. 1. A, 10 μg of protein was separated by SDS-PAGE using a 12% running gel and stained with polyclonal anti-PKCβI antibody. The arrow indicates the position of PKC as determined by comparison with molecular weight markers and peptide inhibition studies shown in (B) + indicates infarct tissue and - contralateral control tissue. C, optical densitometry scanning of the PKC bands visualised as described as above. Numbers refer to patients described in table I. Experiments were repeated at least twice and a representative result is shown.

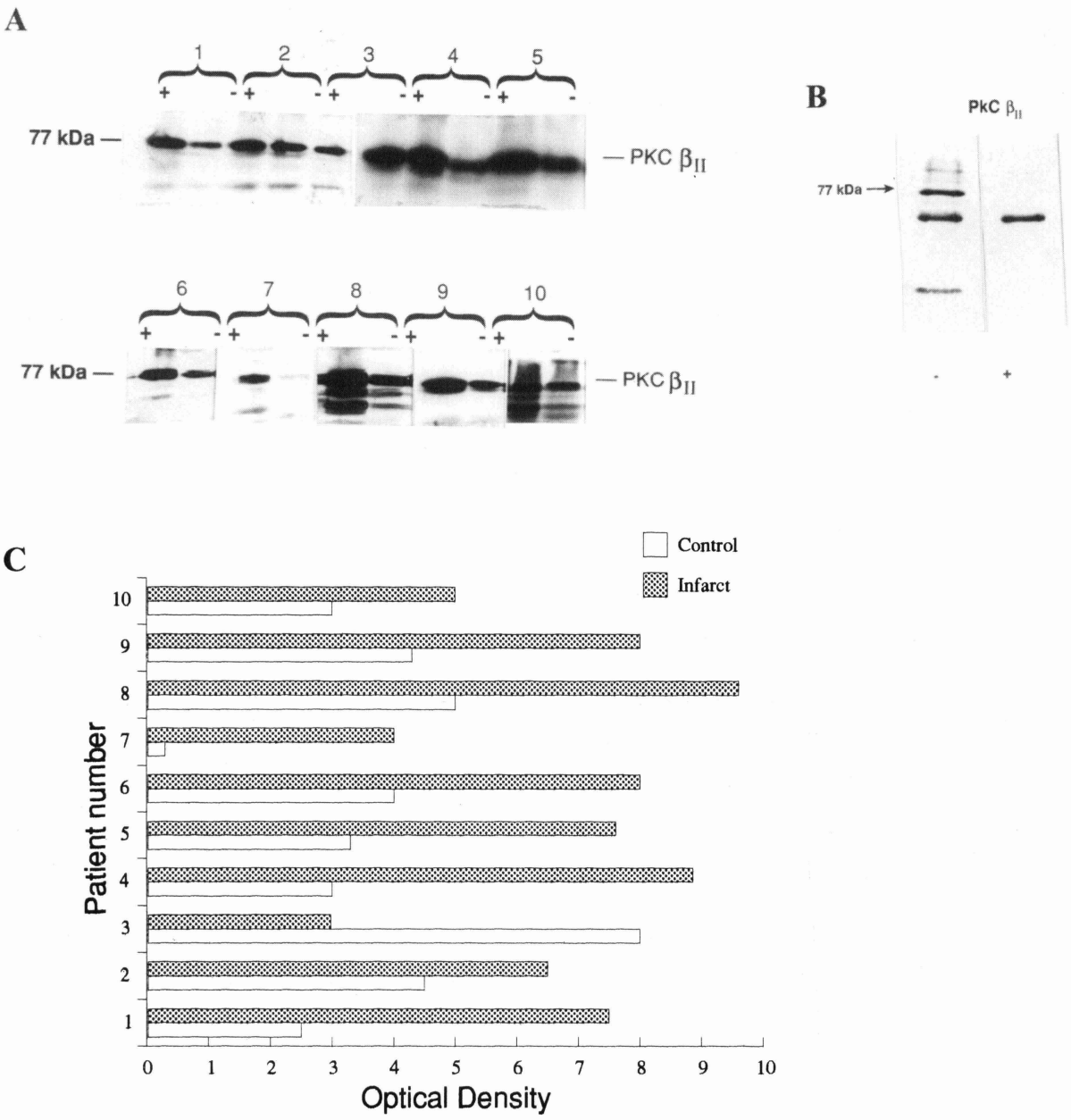

Fig. 2. A, experimental details are as described in Fig. 1 but gels were stained with polyclonal anti-PKCβII antibody. B, + indicates infarct tissue and - contralateral control.

Changes in PKC isoform expression in the grey matter of patients after stroke

There was no significant change in the expression of PKCα or isoforms in infarcted tissue or penumbra compared to the control (results not included). In infarcted tissue there was an increase in total PKCβI in nine out of ten patients (Fig. 1). The increase varied between 1.5 (patient 2) and 10 (patient 5) times the control. In penumbra PKCβI increased to between 1.4-5.7 times the control in only four out of nine patients (figure not included). However, PKCβII increased between 1.5-12 fold in nine out of 10 patients in infarct (Fig. 2). In the penumbra there was an increase in PKCβII in only three

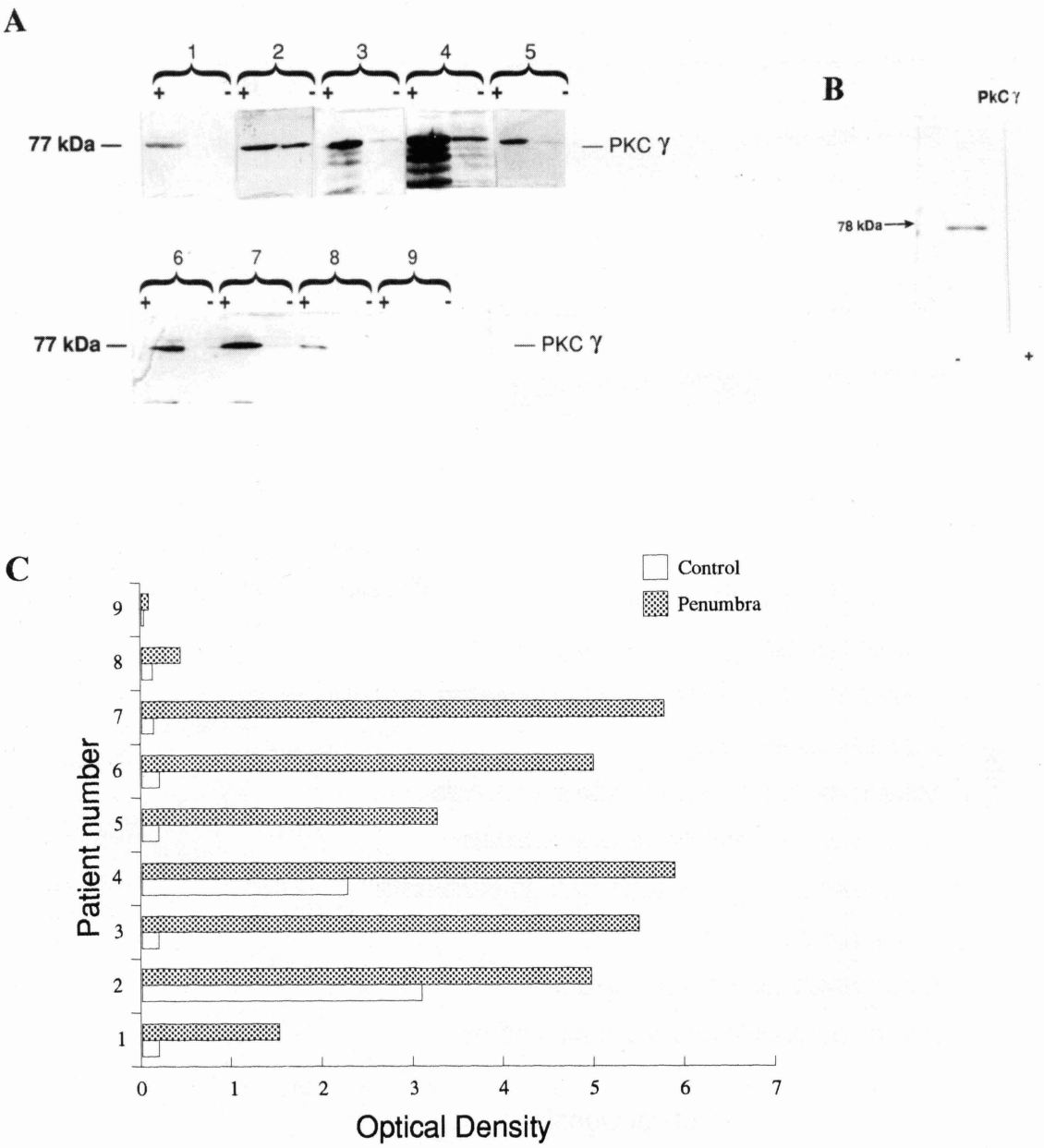

Fig. 3. A, experimental details are as described in Fig. 1 but gels were stained with polyclonal anti-PKCγ antibody. B, + indicates penumbra and - contralateral control.

out of nine patients. PKCγ increased between 2-24 fold in the penumbra of nine patients compared to the control (Fig. 3) but there was no change in expression in infarcted tissue. The specificity of anti-PKC antibodies was determined by peptide inhibition studies (Figs. 1B; 2B and 3B).

Total PKC activity in tissue samples

The total PKC activity was compared in the contralateral hemisphere, penumbra and infarct tissue of eight patients. Total PKC activity was significantly increased in the penumbra of only one patient and decreased in three.

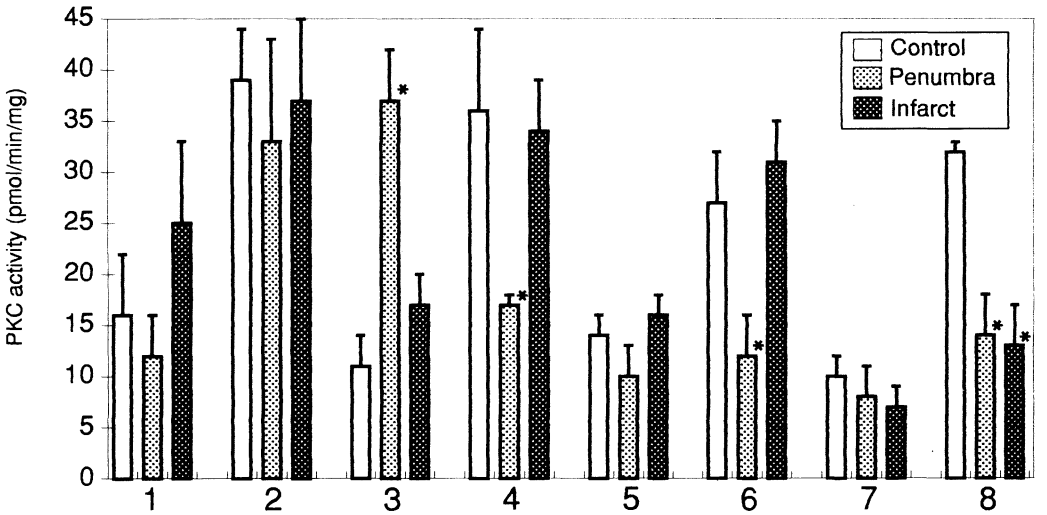

Fig. 4. PKC activity was determined as described in the materials and methods. Each bar represents the mean PKC activity of three determinations. The experiment was repeated twice and a representative result is shown. *$P<0.05$ as determined by Students test.

In infarcted tissue total PKC activity was significantly decreased in only one patient compared to the control (Fig. 4).

DISCUSSION

The results presented in this report indicate that brain ischaemia in humans is followed by differential changes in PKC isoform expression but the overall activity is constant. We have demonstrated a dramatic upregulation of PKCγ in the penumbra of all nine patients studied. In a sample of 10 patients $PKC\beta_I$ increased in eight and $PKC\beta_{II}$ in nine samples of infarcted tissue. The α and isoforms showed no variation in stroke or penumbra samples. This may reflect both a different sensitivity of isoforms to molecules secreted during ischaemia and reperfusion, as well as the fact that individual isoforms of PKC may be activated by different factors and have distinct functions within the central nervous system. Post-mortem hypoxia and ischaemia might also influence both activity and expression of individual isoforms of PKC. In rat models the subcellular distribution and enzymatic activity of PKC is markedly affected during and following cerebral ischaemia (Louis et al. 1988). PKC isoforms were differentially translocated to the cell membrane during ischaemia, although the total PKC activity was reduced (Cardell and Wieloch 1993). The latter report demonstrated that early after the onset of ischaemia there was an increase of PKCγ activity, changes in its subcellular redistribution followed by later transient increases in β_{II} and α isoforms. This especially applies to the PKCγ isoform where more extensive tissue damage was followed by a dramatic rise in its expression in the penumbra. PKC down-regulation may be detrimental to neuronal function as the expression of this enzyme is thought to be important in the maintenance of neuronal activity and survival (Nelson et al. 1990). It is possible that ischaemia-induced damage produces an increase in intracellular calcium concentration which contributes to PKC translocation to the membrane (Kass and Lipton 1986). PKC may also be dephosphorylated and thus down-regulated after ischaemia, rendering the enzyme inactive (Cardell and Wieloch 1993). Another possibility exists of dual activation of PKC after ischaemia, i.e. early overactivation followed by abnormal degradation which can lead to neuronal degeneration. In this case neurotrophic factors which can regulate and normalize PKC phosphorylation could be of therapeutic value. In a rabbit model of coronary ischaemia, early activation of PKC induced a delayed cytoprotective response (Baxter et al. 1995). Islam et al. (1995) demonstrated an increase in the intensity of staining and the number of PKCγ positive neurons in both transient and permanent common artery occlusion models in rats. Changes in the subcellular distribution of PKCγ during ischaemia correlate with massive neurotransmitter release, membrane depolarization and activation of phospholipid degradation suggesting a role for PKCγ in neurotransmission and modulation of synaptic efficacy after ischaemia (Barrie et al. 1991, Islam et al. 1995). In

hippocampal lesions there is different PKC redistribution, depending on whether there is deefferentiation and regeneration in the CNS. Transient cerebral ischaemia in gerbil hippocampus enhances PKCα and β immunoreactivity, suggesting that these isoforms are activated early after brain ischaemia (Gajkowska et al. 1994). Our study suggests a potential role for PKC in the pathology of ischaemic brain stroke in humans. Dramatic changes in PKCγ expression on the border of the still-surviving penumbra and the dying stroke core, point to this isoenzyme as a possible target for future therapies and normalization of its activation and hyperphosphorylation could be of benefit for tissue survival.

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