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# Aging-Related Alterations in the Distribution of Ca<sup>2+</sup>-Dependent PKC Isoforms in Rabbit Hippocampus

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**ABSTRACT:** The immunocytochemical and subcellular localization of the Ca<sup>2+</sup>-dependent protein kinase C (cPKC) isoforms (PKC $\alpha$ ,  $\beta$ 1,  $\beta$ 2, and  $\gamma$ ) was examined in rabbit hippocampus of young (3 months of age; n = 11) and aging (36 months of age; n = 14) subjects. Detailed immunocytochemical analyses revealed a significant increase in PKC $\beta$ 1,  $\beta$ 2, and  $\gamma$  immunoreactivity in principal cell bodies and associated dendrites, and interneurons of the hilar region in the aging rabbits. The number of PKC $\alpha$ - and  $\gamma$ -positive interneurons in the aging stratum oriens declined significantly. PKC $\alpha$  was least affected in principal cells, showing an increase in immunostaining in granule cells only. Weakly PKC-positive principal cells intermingled between densely stained ones were seen in parts of the hippocampus in most of the aging rabbits, showing that the degree of aging-related alterations in PKC-immunoreactivity varies between neurons. Changes in PKC expression in the molecular and subgranular layer of the aging dentate gyrus suggested a reorganization of PKC-positive afferents to this region. Western blot analysis revealed a significant loss of PKC in the pellet fraction for all isoforms, and a tendency for increased levels of cytosolic PKC. However, no significant changes were found in total PKC content for any PKC isoform. A concurrent dramatic loss of the PKC anchoring protein receptor for activated C kinase (RACK1) in the pellet fraction was shown by Western blotting. These findings suggest that the loss of RACK1 contributes to the dysregulation of the PKC system in the aging rabbit hippocampus. The enhanced PKC-immunoreactivity might relate to reduced protein-protein interactions of PKC with the anchoring protein RACK1 leading to increased access of the antibodies to the antigenic site. In conclusion, the results suggest that memory deficits in aging rabbits are (in part) caused by dysregulation of subcellular PKC localization in hippocampal neurons. © 2004 Wiley-Liss, Inc.

**KEY WORDS:** immunocytochemistry; protein kinase C; RACK1; Western blot; memory

## INTRODUCTION

The hippocampus is known to be critically involved in mnemonic processes. This brain region is especially affected by aging and in diseases associated with aging (van Hoesen and Hyman, 1990; Barnes, 1994). The rabbit has been widely used in behavioral and biological studies of aging (Thomp-

son, 1988; Woodruff-Pak, 1988; Deyo et al., 1989; Woodruff-Pak and Trojanowski, 1996). The rabbit eyeblink conditioning model has been promoted for its direct behavioral parallels with studies in aging humans (Solomon et al., 1988; Woodruff-Pak, 1988; Deyo et al., 1989; Knuttinen et al., 2001). Trace eyeblink conditioning is a hippocampus-dependent learning task useful in studying behavioral, neurochemical, neurophysiological, and biophysical aspects of associative learning in the young and aged hippocampus (Moyer et al., 1992; Thompson et al., 1992; Weiss and Thompson, 1992; Disterhoft et al., 1993; Moyer and Disterhoft, 1994). A reduced acquisition rate and an increase in the proportion of rabbits failing to learn the eyeblink conditioning task were observed with advancing age, and at 36 months, a clear aging-related memory deficit is apparent (Thompson et al., 1996). However, less is known about possible neurochemical changes that may occur in the aging rabbit hippocampus. Alterations in calcium binding proteins in the hippocampus have been described previously, which may indicate altered calcium homeostasis in interneurons of the aging rabbit (De Jong et al., 1996).

The role of calcium/phospholipid-dependent protein kinase C (cPKC) in learning and memory has been well established during the past decade (for review, see Van der Zee and Douma, 1997). Following transsynaptic neurotransmission, PKC translocates from the cytosol to the cell membrane or to intracellular sites (Nishizuka, 1986, 1995; for review, see Liu and Heckman, 1998). Receptors for activated C kinase (RACKs) are crucial anchoring proteins that regulate translocation and subsequent function of PKC (Mochly-Rosen et al., 1991; Mochly-Rosen, 1995; for review, see Mochly-Rosen and Gordon, 1998). RACK1, in contrast to RACK2, specifically anchors activated cPKC, including PKC $\gamma$  (Schechtman and Mochly-Rosen, 2001; Chen et al., 2003). Activated PKC phosphorylates various substrates, such as cytoskeletal proteins, ion channels, receptors, and other elements involved in signal transduction (for review, see Liu, 1996). In this way, PKC plays a crucial role in converting extracellular signals to intracellular events underlying learning and memory processes. PKC $\gamma$  in particular contributes to hippocampus-dependent learning. For example, C57BL mice express more PKC $\gamma$  in the hippocampus than is found in DBA mice, and solve a spatial task easier than do the DBA mice (Bowers et al.,

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1995; Paylor et al., 1996). The involvement of PKC $\gamma$  in learning in mouse, rat, and rabbit hippocampus has been demonstrated extensively immunocytochemically (Van der Zee et al., 1992, 1995, 1997c; Douma et al., 1997, 1998; for review, see Van der Zee et al., 1997a).

The aim of the present study was to determine whether the aging-related memory deficit in hippocampus-dependent trace eyeblink conditioning observed in 36-month-old rabbits is reflected in age-dependent changes in the PKC system. Reduced hippocampal PKC activity (Fordyce and Wehner, 1993; Pascale et al., 1998), impaired PKC translocation (Battaini et al., 1995), enhanced hippocampal PKC $\gamma$  immunostaining (Colombo and Gallagher, 2002), and lack of PKC activation in response to various stimuli (Battaini et al., 1995, 1997) have been reported in the aging rat. Moreover, aged rats have increased levels of membrane-bound PKC $\gamma$  in the hippocampus (Battaini et al., 1995; Colombo et al., 1997), which is interpreted in terms of dysregulation of the translocation process. Especially in Alzheimer's disease, PKC activity levels are decreased in the aging human hippocampus (Clark et al., 1991; Battaini et al., 1994). A deficit in RACKs, regulating PKC activity, contributes to functional impairment in PKC activation, both in aging rats and in Alzheimer's disease patients (Pascale et al., 1996, 1998; Battaini et al., 1999). Therefore, we carried out a detailed examination of the expression of cPKC-isoforms  $\alpha$ ,  $\beta$ 1,  $\beta$ 2, and  $\gamma$ , as well as RACK1 in aging rabbit hippocampus, by means of immunocytochemical and biochemical methods.

## MATERIALS AND METHODS

### Animals

Eleven young (2–3-month-old) and 14 aging (36-month-old) New Zealand albino rabbits (*Oryctolagus cuniculus*) were used in this study. The animals were housed individually under a light/dark cycle of 12:12 h, with food and water available ad libitum. Animal use procedures were approved by Northwestern University's Animal Care and Use Committee, according to the standards of the United States Department of Agriculture.

### Immunocytochemical Procedure

Rabbits were deeply anesthetized with a mixture of ketamine and xylazine, and each was transcardially perfused with 150 ml saline (40 ml/min) followed by 860 ml (young rabbits) or 1,300-ml (old rabbits) fixative composed of 2.5% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed from the skull, immersion-fixed for 2 h in 3% paraformaldehyde, and cryoprotected by overnight storage in 30% sucrose in 0.1 M PB. Immunostaining was carried out on frozen sections coronally cut at a thickness of 20  $\mu$ m.

Free-floating sections were used to obtain a high-quality image of hippocampal PKC-immunoreactivity (PKC-ir) for photography, while sections thaw-mounted on gelatin-coated slides were used for quantitative immunocytochemistry because their immunostaining was not saturated. In this case, sections of young and

old animals were collected on a glass slide to guarantee identical incubation conditions. Eight levels through the dorsal and posterior part of the hippocampus were collected, with an interval of  $\sim$ 500  $\mu$ m. The range of the sampled hippocampal region corresponds approximately to level 53–66 in the brain atlas of Shek et al. (1986).

PKC isoforms were visualized by means of polyclonal rabbit IgG antibodies raised against peptides corresponding to C-terminal sequences contained in the catalytic domain of the  $\alpha$ ,  $\beta$ 1,  $\beta$ 2, and  $\gamma$  isoforms known to bind to mammalian PKC (C20, C16, C18, and C19, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal mouse IgG antibody, 36G9 (known to recognize rabbit PKC $\gamma$ ; Chemunex, Paris, France), raised against purified bovine PKC $\gamma$  and reactive with the V2 region of the regulatory domain (Cazaubon et al., 1989). The tissue sections were preincubated for 15 min in 0.1% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline (PBS), subsequently rinsed in PBS and immersed in 5% normal sheep serum (NSS) or normal goat serum (NGS) in PBS for 30 min for the monoclonal or polyclonal antibody, respectively, to reduce aspecific binding in the following incubation step. Next, the sections were incubated overnight at room temperature (RT) under gentle movement of the incubation medium containing the monoclonal antibody (36G9), diluted 1:200 in 1% NSS in PBS, or one of the polyclonal antibodies, diluted 1:200 in 1% NGS in PBS. After the primary incubation, sections were rinsed in PBS and again preincubated with 5% NSS or NGS for 30 min before the secondary incubation step in biotinylated sheep anti-mouse IgG (1:200; Amersham, Arlington Heights, IL) or biotinylated goat anti-rabbit (1:200; Amersham), in PBS for 2 h at RT. Thereafter, the sections were thoroughly rinsed in PBS and incubated in streptavidin-horseradish peroxidase (HRP) (Zymed, San Francisco, CA) diluted 1:200 in PBS for 2 h at RT. Finally, after subsequent rinsing in PBS and Tris buffer, the sections were processed by the diaminobenzidine (DAB)-H<sub>2</sub>O<sub>2</sub> reaction (30 mg DAB and 0.01% H<sub>2</sub>O<sub>2</sub>/100 ml Tris buffer), guided by a visual check. Control experiments were performed by the omission of the primary antibody from the incubation medium, yielding immunonegative results. Photomicrographs of the DAB-processed material were taken under identical exposure condition and all co-processed under similar printing conditions in the darkroom.

### Tissue Fractionation and Western Blot Procedure

Four young and four old rabbits were used for Western blot analysis. Animals were deeply anesthetized with a mixture of ketamine and xylazine and were then decapitated. The brain was quickly removed from the skull and put on ice; the hippocampi were rapidly dissected within 1.5–2 min. Blocks of the middle portion of the hippocampus (containing all subregions, i.e., dentate gyrus, cornu ammonis 3 (CA3), CA1, and subiculum), weighing  $\sim$ 0.1–0.2 g wet weight, were sampled. Blocks were each homogenized in a 1.0-ml volume of PPI buffer [protease/phosphatase inhibitor buffer, as described in Das et al. (1986)] containing (in mM) 80  $\beta$ -glycerophosphate, 10 KPO<sub>4</sub>, 1 EDTA, 5 EGTA, 10 MgCl<sub>2</sub>, 2 dithiothreitol (DTT), 1 Na-orthovanadate, and 100  $\mu$ g/ml pepstatin A, 21  $\mu$ g/ml leupeptin, 0.23 mM phenylmethyl-

sulfonyl fluoride (PMSF), and 10% ethanol, pH 7.25. Homogenization was performed using 20–30 strokes in a hand-held ground-glass Potter Elvehjem homogenizer, and the resulting homogenates were centrifuged for 70 min at 105,000g (Hogeboom, 1955; Thomas et al., 1987) corresponding to 33,000 rpm in a Beckman SW 50.1 swinging bucket rotor in a Beckman model L8-70 preparative ultracentrifuge. The high-speed supernatants were collected for use as cytosol fractions (Hunzicker-Dunn and Jungmann, 1978). The high-speed pellets, containing plasma membrane and pellet membrane-bound subcellular organelles, including nuclei, mitochondria, microsomes (Hunzicker-Dunn and Jungmann, 1978), synaptosomes (Kikkawa et al., 1983), and cytoskeletal components (Kiley and Jaken, 1990), were resuspended by homogenization in 1.0-ml volumes (corresponding to the original homogenization buffer) of PPI buffer, and the resulting suspensions were used as pellet fractions. Aliquots of cytosol and pellet fractions, respectively, were prepared for subsequent Western blot analysis by the addition of Laemmli sodium dodecyl sulfate (SDS) sample buffer, followed by 5 min of heat denaturation at 100°C. Additional aliquots of cytosol and pellet fractions were collected for protein determination according to Lowry et al. (1951), using crystalline bovine serum albumin (BSA) as a standard.

Cytosol and pellet fraction proteins were separated on SDS-polyacrylamide (8.5% for PKC; 10% for RACK1) gels and then transferred to Nytran membrane (Schleicher & Schuell, Keene, NH) for PKC blots or Hybond C-extra nitrocellulose membrane (Amersham) for RACK blots. Blots were probed with anti-PKC isoform antibodies or with a monoclonal antibody raised against the membrane-bound receptor for activated C kinase (RACK1; Transduction Laboratories, Lexington, KY) overnight at 4°C. [<sup>125</sup>I]Protein A (~1 µCi/lane) was used to detect antigen-antibody complexes in the PKC blots; enhanced chemiluminescence (ECL, according to the Amersham protocol, using HRP-conjugated anti-mouse as a secondary antibody) was used in the RACK1 blots.

### Data Analysis

Four immunostained brain sections of the dorsal and posterior hippocampus per animal were selected for each PKC-isoform, and both the left and right hippocampi were analyzed for optical densities (ODs). A thalamic (the dorsomedial thalamus) and a hypothalamic (the lateral hypothalamus) were selected within these sections as two non-hippocampal control regions. Analysis of the ODs described below were performed “blind” to the age of the animals. The ODs of PKC-ir of pyramidal cell bodies and their apical dendrites in the subiculum, CA1 and CA3, as well as the granule cell bodies in the dentate gyrus, were measured (see Fig. 4C in Van der Zee et al., 1997c, for schematic delineation of these areas of interest). OD measures were always taken from those areas with homogeneous staining intensity. The ODs of the dorsomedial thalamus and medial posterior hypothalamus were taken in an area of 250 × 250 µm.

The OD was expressed in arbitrary units corresponding to gray levels using a Zeiss IBAS image analysis system. The value of background labeling was measured in the corpus callosum, which was

relatively devoid of PKC-ir. The OD of the region of interest was related to the background value by the formula

$$(OD_{\text{area}} - OD_{\text{background}}) / OD_{\text{background}}$$

thus eliminating the variability in background staining among sections and correcting for different durations of the DAB reaction between animals.

Four sections from each animal were used for quantitative analysis of the number of PKC-positive interneurons in the CA1 stratum oriens. Cells were counted only when the nucleus was in the plane of section. The boundaries of the CA1 region were determined by the thickening of the pyramidal cell layer and the size of the pyramidal cells at the border with CA2 and subiculum, which are well recognizable in rabbit hippocampus (De Jong et al., 1996; Geinisman et al., 1996). Quantitation of the bound <sup>125</sup>I or ECL of the immunoblots was done with the aid of an Imaging Densitometer (BioRad, model GS-670). ODs were converted to the percentage of PKC, to compare the relative content in the cytosol and pellet fraction.

### Statistical Analysis

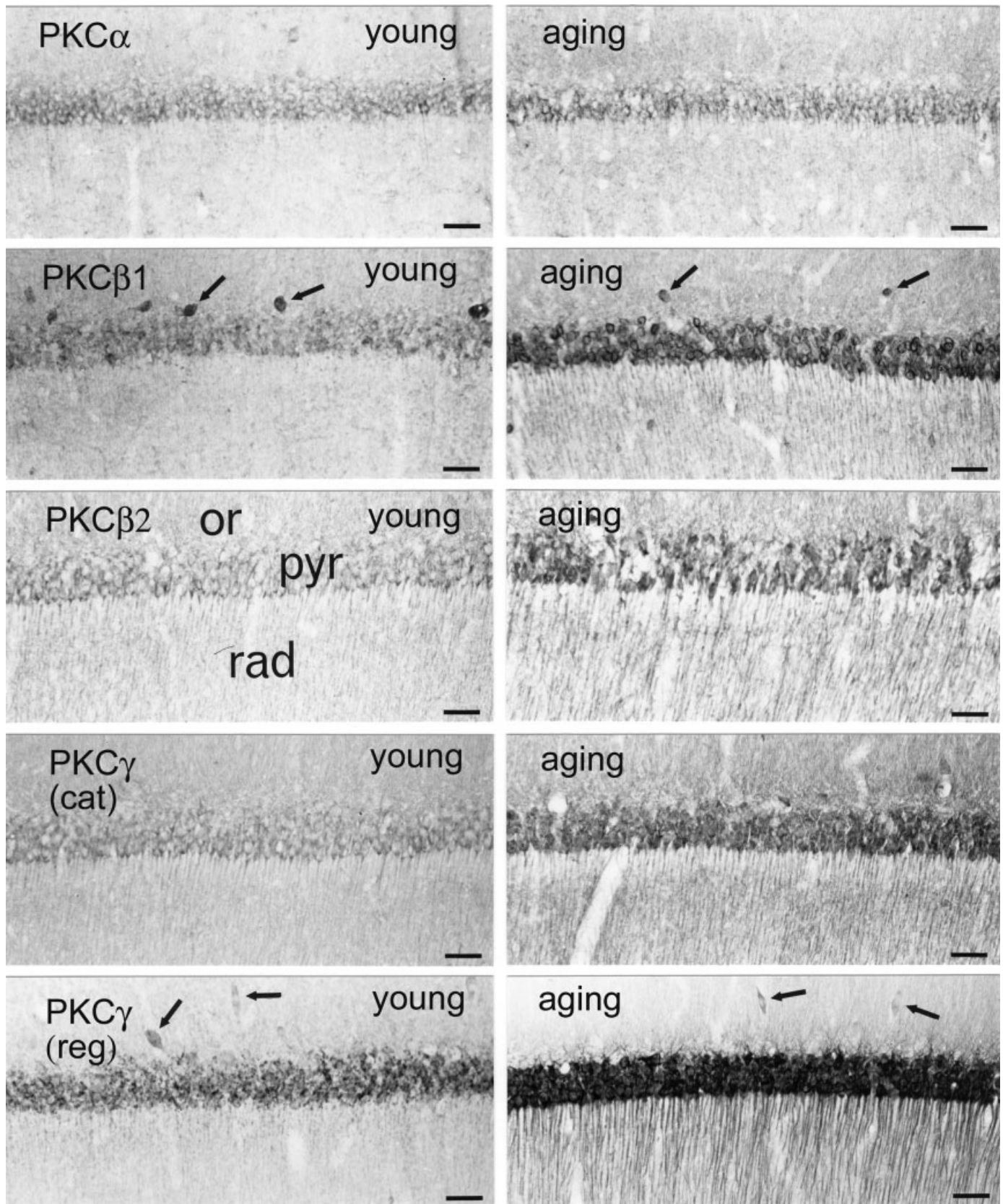
Analysis of variance (ANOVA), followed by a Tukey-Studentized range test (Student's *t*-test equivalent) or a Mann-Whitney U-test, was used when appropriate, with a probability level of *P* < 0.05 used as an index of statistical significance.

## RESULTS

### Analysis of PKC-Isoform Immunoreactivity in the Hippocampus of Young and Aging Rabbits

PKC-ir for all four isoforms is present in pyramidal neurons, granule cells, and diverse populations of interneurons. The immunoreactivity is relatively low in pyramidal cells (Fig. 1A,C,E,G,I) and granule cells (see Fig. 3A,C,E) of young animals. OD measures, taken from the cell bodies and apical dendrites of pyramidal cells located in the subiculum, CA1, and CA3, and the cell bodies of dentate gyrus granule cells, are shown in (Table 1). Dense PKC staining in axons was generally absent, but thin immunopositive fibers could be seen for PKC $\alpha$  and, to a lesser extent, PKC $\beta$ 1 in the middle-third layer of the dentate gyrus molecular layer (Fig. 3A). Weakly immunoreactive astrocytes scattered throughout the hippocampus were found for all isoforms, but were only rarely encountered with the anti-PKC $\gamma$  antibody 36G9. Numerous PKC-positive interneurons could be seen, notably in the stratum oriens. The number of labeled interneurons depended on the isoform, and in young rabbits ranked as follows in CA1 stratum oriens: PKC $\gamma$  > PKC $\beta$ 1  $\gg$  PKC $\alpha$  > PKC $\beta$ 2 (Fig. 2).

In general, PKC-ir was higher in the aging pyramidal cells (Fig. 1B,D,F,H,J), granule cells and interneurons in the hilar region (Fig. 3B,D,F), but somewhat lower in interneurons of the hippocampus proper (see, e.g., PKC $\beta$ 1-positive interneurons in Fig. 1D). The number of these interneurons in the stratum oriens declined significantly (*P* < 0.05) for PKC $\alpha$  and  $\gamma$ , but not for PKC $\beta$ 1 and  $\beta$ 2 (Fig. 2). OD measures revealed a significant in-



**FIGURE 1.** Alterations in protein kinase C (PKC)-immunoreactivity in the aging CA1. Immunostaining for the  $Ca^{2+}$ -dependent protein kinase C (cPKC) isoforms  $\alpha$ ,  $\beta 1$ ,  $\beta 2$ , and  $\gamma$  ( $\gamma$ (cat) and  $\gamma$ (reg)) in the CA1 region of young (left) and aging (right) rabbits. An increase in immunoreactivity is found for  $\beta 1$ ,  $\beta 2$ , and  $\gamma$ , but not for  $\alpha$ . Arrows point to immunolabeled interneurons. or, stratum oriens; pyr, stratum pyramidale; rad, stratum radiatum. Scale bars = 50  $\mu$ m.

TABLE 1.

Optical Density Measures ( $\pm$ SEM) for PKC Immunoreactivity in Various Hippocampal Subregions, Thalamus (Dorsomedial Thalamus) and Hypothalamus (Medial Posterior Hypothalamus) of Young (Y;  $n = 7$ ) and Aging (A;  $n = 10$ ) Rabbits

	Subiculum				CA1				CA3				DG		Thalamus		Hypothalamus	
	Cell bodies		Dendrites		Cell bodies		Dendrites		Cell bodies		Dendrites		Cell bodies	Cell bodies	Cell bodies			
	Y	A	Y	A	Y	A	Y	A	Y	A	Y	A	Y	A	Y	A		
PKC $\alpha$	1.33	1.46	0.83	1.06*	1.34	1.53	0.91	1.13	1.28	1.53	0.62	0.64	0.53	0.69*	0.80	0.90	0.41	0.44
	$\pm 0.16$	$\pm 0.18$	$\pm 0.09$	$\pm 0.15$	$\pm 0.20$	$\pm 0.19$	$\pm 0.15$	$\pm 0.15$	$\pm 0.28$	$\pm 0.19$	$\pm 0.17$	$\pm 0.11$	$\pm 0.11$	$\pm 0.11$	$\pm 0.10$	$\pm 0.14$	$\pm 0.14$	$\pm 0.09$
PKC $\beta$ 1	1.25	1.80**	0.64	1.05**	1.44	2.07**	0.80	1.15**	1.13	1.72**	0.53	0.63	0.52	1.01**	0.60	0.83*	0.57	0.74
	$\pm 0.22$	$\pm 0.25$	$\pm 0.13$	$\pm 0.16$	$\pm 0.27$	$\pm 0.27$	$\pm 0.16$	$\pm 0.16$	$\pm 0.23$	$\pm 0.24$	$\pm 0.14$	$\pm 0.12$	$\pm 0.11$	$\pm 0.15$	$\pm 0.16$	$\pm 0.18$	$\pm 0.18$	$\pm 0.20$
PKC $\beta$ 2	1.18	1.71**	0.77	1.38**	1.19	1.80**	0.84	1.46**	0.69	1.29**	0.47	0.66*	0.44	0.84**	0.48	0.61**	0.25	0.26
	$\pm 0.19$	$\pm 0.14$	$\pm 0.14$	$\pm 0.13$	$\pm 0.17$	$\pm 0.16$	$\pm 0.13$	$\pm 0.13$	$\pm 0.20$	$\pm 0.18$	$\pm 0.14$	$\pm 0.12$	$\pm 0.08$	$\pm 0.11$	$\pm 0.09$	$\pm 0.10$	$\pm 0.13$	$\pm 0.12$
PKC $\gamma$ (cat) C19	1.71	2.35*	0.84	1.46**	1.90	2.77*	1.01	1.75**	1.47	2.27*	0.65	0.82	0.70	1.19**	0.74	1.07**	0.43	0.34
	$\pm 0.16$	$\pm 0.21$	$\pm 0.08$	$\pm 0.14$	$\pm 0.25$	$\pm 0.25$	$\pm 0.14$	$\pm 0.16$	$\pm 0.28$	$\pm 0.25$	$\pm 0.14$	$\pm 0.09$	$\pm 0.09$	$\pm 0.10$	$\pm 0.13$	$\pm 0.16$	$\pm 0.04$	$\pm 0.06$
PKC $\gamma$ (reg) 36G9	2.00	3.75**	0.58	1.48**	2.56	4.08**	0.78	1.66*	2.48	3.91**	0.67	1.14*	0.75	1.55**	0.85	1.39**	0.05	0.06
	$\pm 0.19$	$\pm 0.35$	$\pm 0.11$	$\pm 0.21$	$\pm 0.19$	$\pm 0.37$	$\pm 0.12$	$\pm 0.25$	$\pm 0.35$	$\pm 0.27$	$\pm 0.11$	$\pm 0.12$	$\pm 0.14$	$\pm 0.20$	$\pm 0.16$	$\pm 0.20$	$\pm 0.02$	$\pm 0.03$

PKC, protein kinase C; cat, catalytic; reg, regulatory.

\* $P < 0.05$ ; \*\* $P < 0.01$  as compared with young rabbits.

crease in the staining-intensity in principal cells for PKC $\beta$ 1,  $\beta$ 2 and  $\gamma$  throughout the hippocampal subfields, with the exception of the dendrites of the CA3 pyramidal cells for PKC $\beta$ 1 and  $\gamma$ (cat), which, although enhanced, did not reach statistical significance (Table

1). PKC $\alpha$  gives a different picture, however. Although an increase in staining-intensity is seen in all aging hippocampal cells, it only reached significant levels in the dendrites of subicular pyramidal cells and the cell bodies of the granule cells. The dorsomedial thalamus and medial posterior hypothalamus served as a non-hippocampal control regions. None of the PKC isoforms revealed significant aging-related changes in the medial posterior hypothalamus, whereas all isoforms except PKC $\alpha$  were significantly enhanced in the dorsomedial thalamus in the aged individuals (Table 1).

A striking shift in presynaptic PKC $\alpha$ -ir (and PKC $\beta$ 1-ir to a lesser extent) is seen in the aging as compared with the young dentate gyrus. Young animals have a relatively dense band of PKC $\alpha$ -positive fibers in the middle-third layer (indicated with double-headed arrows in Fig. 3A,B) of the molecular layer. This band is nearly absent in old rabbits, while in these animals a thin layer of PKC $\alpha$ -positive fibers in the subgranular layer (Fig. 3B, small arrows).

A remarkable finding was the presence of (relatively large) areas with heterogeneity in PKC-ir in the principal cells in most of the animals; whereas PKC-ir was normally homogeneous among individual cells in most of the hippocampal formation, areas with heterogeneity were characterized by the mixture of intensely and lightly stained cells (Fig. 4). These areas varied in size, but generally covered several 100  $\mu$ m in tangential axis per section. The heterogeneity was observed for all PKC-isoforms (Table 2). In young animals, this was seen predominantly in the CA3a region (Fig. 4F), and only incidentally in other subregions (Table 2). In aging rabbits (Fig. 4A–E), however, this heterogeneity was seen in all hippocampal subfields covering much larger areas, but was not present in the CA3a area.

### Analysis of Immunoblots of the Hippocampus From Young and Aging Rabbits

Western blot analysis was performed to examine whether the increase in PKC-ir could be due to an increase in the total content

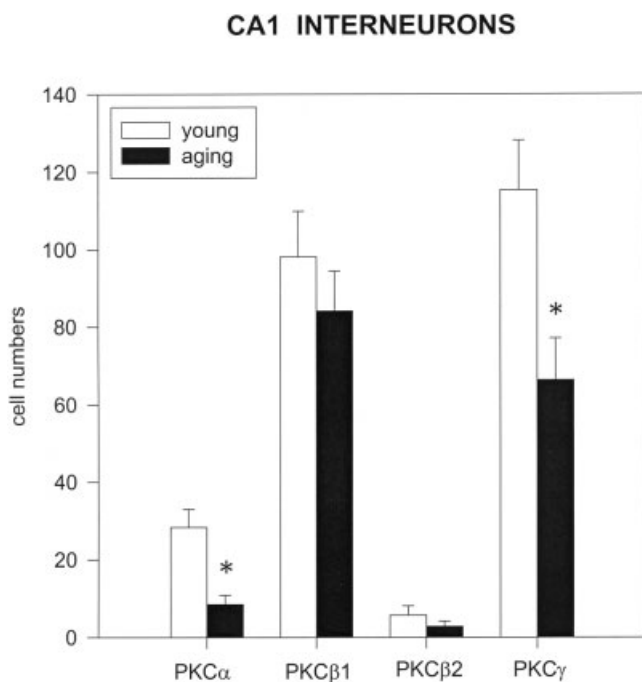
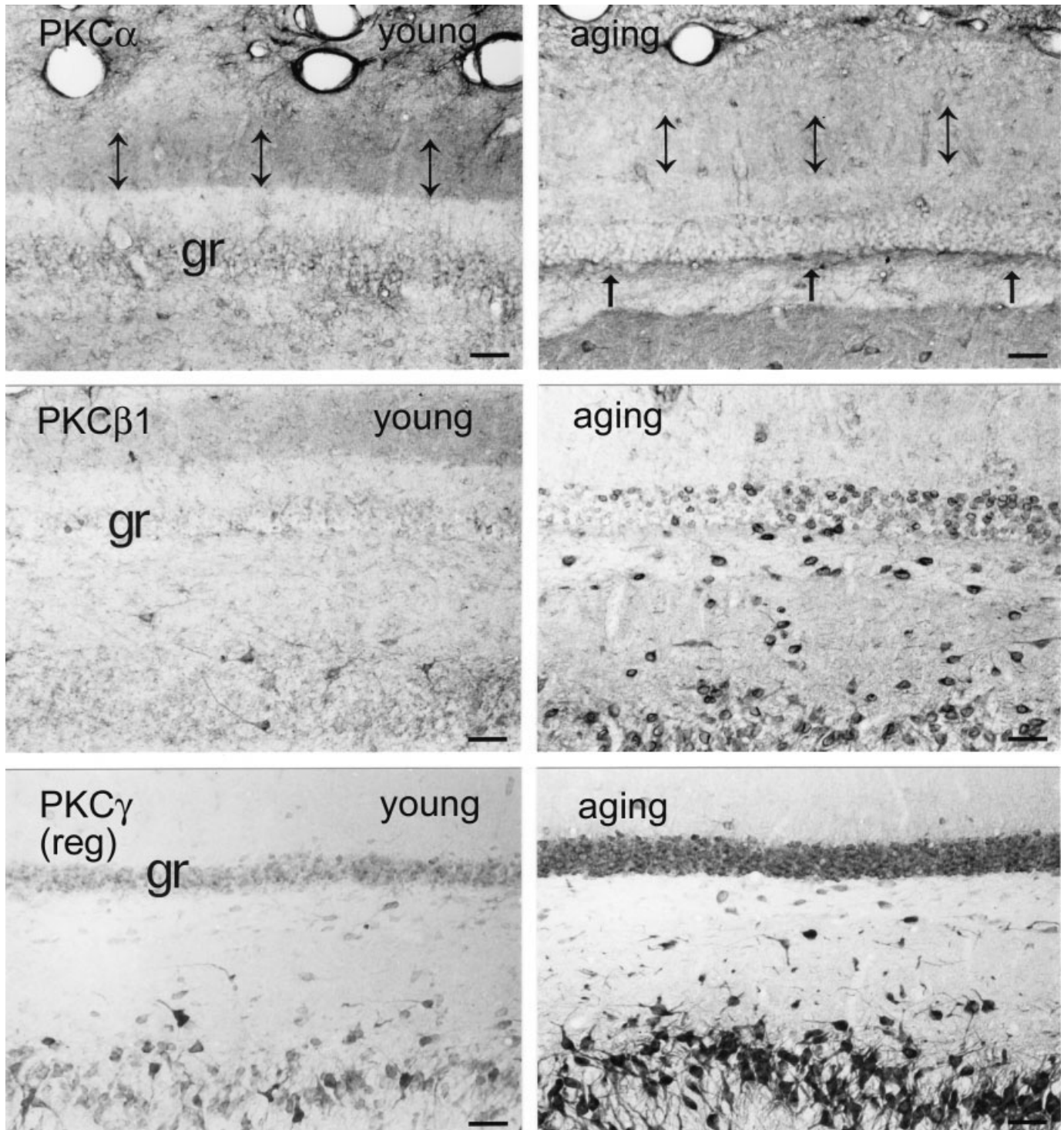


FIGURE 2. Number of protein kinase C (PKC)-immunoreactive interneurons in the CA1 of young and aging rabbits. A significant reduction ( $*P < 0.05$ ) in the number of PKC $\alpha$ - and PKC $\gamma$ -positive interneurons in the CA1 stratum oriens was found in aging rabbits. In contrast, no changes were found for PKC $\beta$ 1 or  $\beta$ 2. In the case of PKC $\gamma$ , immunocytochemical data of the antibodies directed against the catalytic domain (cat), C19 Santa Cruz, and regulatory domain (reg), 36G9 monoclonal, were pooled and averaged.

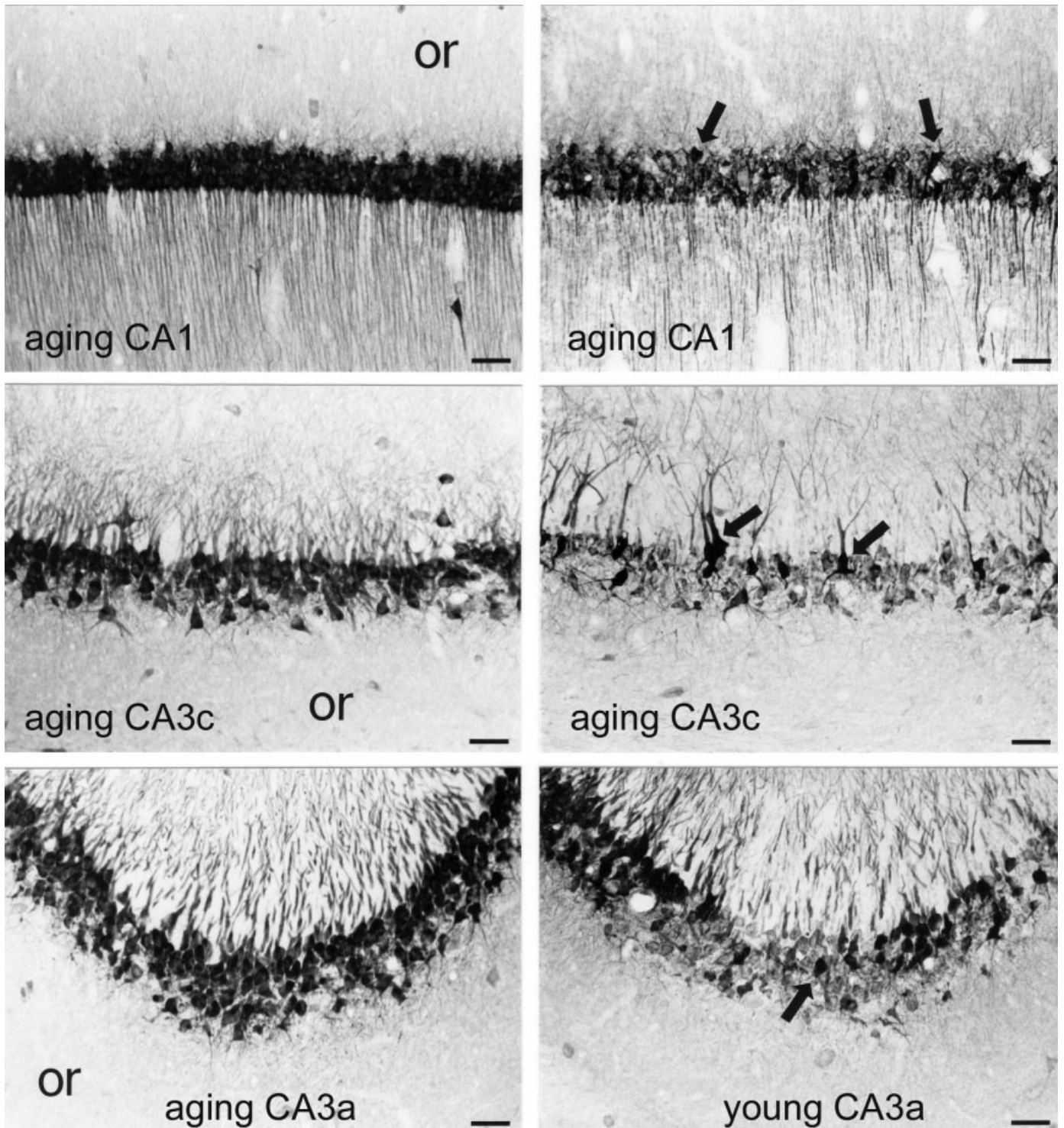


**FIGURE 3.** Alterations in protein kinase C (PKC)-immunoreactivity in the aging dentate gyrus. Immunostaining for PKC $\alpha$ ,  $\beta$ 1, and  $\gamma$  in the dentate gyrus of young (left) and aging (right) rabbits. Pre-synaptic labeling was reduced for PKC $\alpha$  and  $\beta$ 1 in the middle-third of the molecular layer (indicated with double-headed arrows) of the

aging dentate gyrus, while PKC $\alpha$ -ir was enhanced in a thin plexus of positive fibers just beneath the granule cells (small arrows in upper right). The immunoreactivity for PKC $\beta$ 1 and  $\gamma$  is enhanced in the aging granule cells and hilar interneurons. Gr, granule cell layer. Scale bars = 50  $\mu$ m.

of PKC, or shifts in the subcellular fraction. Figure 5 shows a typical immunoblot for all PKC isoforms. The immunoreactivity is seen at a migration position of  $\sim$ 80 kDa. In the case of PKC $\gamma$ ,

no immunoreactive band appeared around 30 kDa (indicative for the single regulatory domain, which could be detected by the 36G9 antibody), indicating that proteolytic activation of PKC $\gamma$  as a con-



**FIGURE 4.** Heterogeneity in protein kinase C (PKC) $\gamma$  immunostaining in principal cells. A striking heterogeneity in PKC-immunoreactivity can be seen throughout the hippocampal formation in aging (and occasionally young) animals. Typical examples of PKC $\gamma$  (reg) in the CA1 (upper), CA3c (middle), and CA3a (lower) region are shown. Similar results were found for PKC $\beta$ 1,  $\beta$ 2, and  $\gamma$  (cat). Left: an impression is presented of the increase of PKC-immunoreactivity

generally seen in the vast majority of aging hippocampal neurons. Right: areas with heterogeneity in immunostaining are depicted. Arrows point to the intensely immunopositive cells among lightly stained cells. Note that in the CA3a region (lower) heterogeneity is depicted in a *young* animal (see Table 2). or, stratum oriens. Scale bars = 50  $\mu$ m.



TABLE 2.

## Heterogeneity of PKC Immunoreactivity\*

Area	Young rabbits (n = 7)					Aging rabbits (n = 10)				
	PKC $\alpha$	PKC $\beta$ 1	PKC $\beta$ 2	PKC $\gamma$ (cat) C19	PKC $\gamma$ (reg) 36G9	PKC $\alpha$	PKC $\beta$ 1	PKC $\beta$ 2	PKC $\gamma$ (cat) C19	PKC $\gamma$ (reg) 36G9
Sub/CA1	—	1	1	—	1	4	7	5	5	5
CA3a	3	1	4	3	4	—	—	—	—	—
CA3c	—	—	—	1	1	3	4	4	6	6
DGgran	—	—	—	1	1	1	4	1	5	4

PKC, protein kinase C; Sub, subiculum; DGgran, granule cell bodies in dentate gyrus.

\*Number of young and aging rabbits showing heterogeneity of PKC immunoreactivity characterized by a mixture of lightly and intensely stained principal cells.

sequence of aging did not occur. Likewise, the absence of immunoreactive PKM catalytic fragments at 40–50 kDa on immunoblots performed with the PKC $\gamma$  antibody C19 raised against the catalytic domain supported this conclusion. Moreover, no immunoreactive bands at 40–50 kDa were seen for either PKC $\alpha$ ,  $\beta$ 1, or  $\beta$ 2, indicating that none of the isoforms was extensively proteolyzed. The ODs of the immunoreactive bands of the PKC isoforms in the immunoblots were measured (Table 3). This Western blot

analysis revealed a consistent and significant reduction ( $P < 0.01$ ) of PKC content in the pellet fraction of aging animals, and a nonsignificant increase in the cytosol fraction. Expressed in percentages, the total content of all four PKC isoforms was reduced in aging between 67.6% and 85.3%, as compared to young hippocampus [values for PKC $\gamma$  (reg) and PKC $\beta$ 2, respectively; see Table 4]. The loss of PKC in the pellet fraction, expressed in percentages, ranged from 66.7% to 90% [values for PKC $\alpha$  and PKC $\gamma$  (cat), respectively; see Table 4], whereas the increase in cytosolic PKC was limited as compared to a 100% level for each isoform in young rabbits (Table 4).

In order to study whether the dramatic reduction of PKC in the pellet fraction of the aging hippocampus was due to PKC binding conditions at the cell membrane, Western blotting was performed for RACK1. A representative immunoblot is shown in Figure 6. The blot shows a dramatic reduction (~25-fold) in the content of RACK1 in the pellet fraction of the aging rabbits (OD measures for young versus aging:  $30.8 \pm 2.1$  versus  $1.2 \pm 0.2$ ;  $P < 0.05$ ; Mann-Whitney U-test). No RACK1 expression was found in the cytosol fraction of either young or aging rabbits (data not shown).

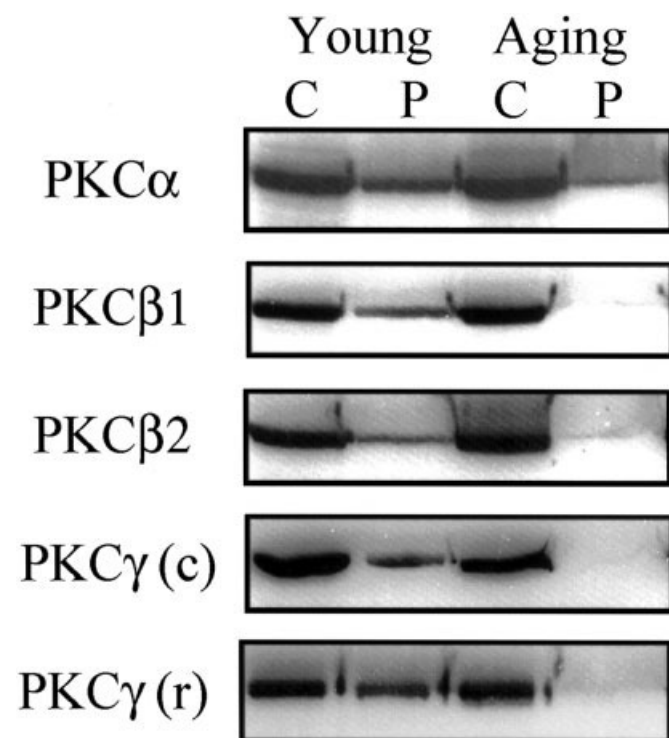


FIGURE 5. Protein kinase C (PKC) immunoblots of young and aging hippocampus. Representative immunoblots for PKC $\alpha$ ,  $\beta$ 1,  $\beta$ 2, and  $\gamma$  of hippocampal tissue homogenates of young and aging rabbits. 100  $\mu$ g protein was loaded per lane. Clear differences in the subcellular distribution, i.e., decreases in the pellet fraction, are found in the aging hippocampus for all four isoforms. C, cytosol; c, catalytic domain; P, pellet; r, regulatory domain.

TABLE 3.

Optical Density Measures ( $\pm$ SEM) of PKC Immunoblots of Young (n = 4) and Aging (n = 4) Rabbits\*

	Young		Aging	
	Cytosol	Pellet	Cytosol	Pellet
PKC $\alpha$	125 $\pm$ 18	92 $\pm$ 15	146 $\pm$ 22	31 $\pm$ 9**
PKC $\beta$ 1	119 $\pm$ 16	77 $\pm$ 24	144 $\pm$ 23	8 $\pm$ 2**
PKC $\beta$ 2	110 $\pm$ 20	58 $\pm$ 8	131 $\pm$ 28	13 $\pm$ 3**
PKC $\gamma$ (cat) C19	121 $\pm$ 17	60 $\pm$ 11	132 $\pm$ 21	6 $\pm$ 2**
PKC $\gamma$ (reg) 36G9	112 $\pm$ 14	101 $\pm$ 25	125 $\pm$ 18	19 $\pm$ 4**

PKC, protein kinase C; cat, catalytic; reg, regulatory.

\*Significantly lower levels of optical density were found for all PKC isoforms in the pellet fraction of aging hippocampi compared with the pellet fraction of young hippocampi; \*\* $P < 0.01$ .

TABLE 4.

**Quantification of PKC Immunoblots in Aging Hippocampus\***

	Total amount	Cytosol	Pellet
	(%)	(%)	(%)
PKC $\alpha$	81.6 $\pm$ 6.5	116.8 $\pm$ 10.4	33.7 $\pm$ 3.8
PKC $\beta$ 1	77.6 $\pm$ 5.0	121.0 $\pm$ 8.8	10.4 $\pm$ 2.4
PKC $\beta$ 2	85.3 $\pm$ 6.8	119.1 $\pm$ 11.9	22.4 $\pm$ 3.6
PKC $\gamma$ (cat) C19	76.2 $\pm$ 7.3	109.1 $\pm$ 13.6	10.0 $\pm$ 2.8
PKC $\gamma$ (reg) 36G9	67.6 $\pm$ 7.1	111.6 $\pm$ 12.2	18.8 $\pm$ 3.3

PKC, protein kinase C; cat, catalytic; reg, regulatory.

\*Total amount of the four PKC isoforms and their relative distribution in cytosol or pellet fraction in aging rabbits expressed in percentages ( $\pm$ SEM) and compared to values of young rabbits (=100%).

## DISCUSSION

### Alterations in the Localization of PKC Isoform Immunoreactivity in Aging Rabbit Hippocampus

Immunocytochemistry was used to localize which hippocampal cells and which hippocampal areas were affected by aging-related alterations in the PKC system. The present results show a clear immunocytochemical increase for PKC $\beta$ 1,  $\beta$ 2, and  $\gamma$  but only a limited increase for PKC $\alpha$  in principal cells and interneurons in the hilar region of the aging rabbit hippocampus. Immunostaining of the four different PKC-isoforms had a predominantly postsynaptic appearance as described previously (Van der Zee et al., 1996, 1997c), although PKC $\alpha$  (and PKC $\beta$ 1 to a lesser extent) was found presynaptically as well. A striking feature of PKC-ir in aging principal cells is the heterogeneity among neighboring principal cells in large areas of all hippocampal subregions (with the exception of the CA3a region): in such areas, a proportion of the aging neurons still retained a low level of PKC-ir such as seen in young animals, although the major part of the hippocampus always revealed a homogeneous aging-related increase in PKC-ir. It is worth noting that the heterogeneity in PKC-ir is not an immunocytochemical flaw due to, for example, antibody penetration, since other markers, such as calbindin and parvalbumin (De Jong et al., 1996) or several isoforms of neurofilament (Van der Zee et al., 1997b), never displayed this pattern in the aging rabbit hippocampus.

The remarkable finding that this heterogeneity is already present in the CA3a region of young animals, but no longer in the aging CA3a region, while appearing in all other hippocampal subfields, may suggest that this phenomenon reflects a progressive cellular aging process. Apparently the pace of a change in the PKC system is not identical for all cells, suggesting that cells do not age at the same speed. In the young rabbit hippocampus, CA3a pyramidal cells are apparently the first to be subject to PKC dysfunction, whereas in the aging hippocampus all CA3a cells are affected. In a proportion of aging rabbits, however, still unaffected hippocampal principal cells are present ( $\sim$ 40% in the case of PKC $\gamma$ ). Interestingly,  $\sim$ 40% of 36 months old rabbits still have a relatively high rate of acquisition in the hippocampus-dependent trace eyeblink

conditioning test (Thompson et al., 1996). It is tempting to speculate that the aging rabbits with a large proportion of PKC $\gamma$ -positive hippocampal principal cells resembling young ones belong to the group of good learners.

Recent biophysical data also support this suggestion. CA1 pyramidal neurons in rabbits that learn trace eyeblink conditioning, a hippocampus-dependent task, show reductions in the slow post-burst afterhyperpolarization that is comparable in amplitude to neurons from young animals who learn this task (Moyer et al., 2000). In contrast, CA1 pyramidal neurons from aging rabbits that are trained but are unable to learn the task do not show such reductions but rather enhanced afterhyperpolarizations (AHPs) characteristic of aging pyramidal neurons. We know that manipulations that enhance CA1 pyramidal neuron excitability and reduce the slow afterhyperpolarization, such as muscarinic cholinergic activation (Oh et al., 1999; Weiss et al., 2000) also activate PKC as demonstrated with immunocytochemical measurements (Van der Zee et al., 1993). Aging rabbits with a large proportion of CA1 pyramidal neurons with enhanced PKC-ir, presumably reflecting hippocampal regions with reduced ability to alter neuronal excitability by activation of the PKC pathway, would have difficulty learning hippocampus-dependent tasks and even after training those neurons would not have a reduced slow post-burst afterhyperpolarization. In contrast, those rabbits whose hippocampus has a normal complement of PKC would be able to learn tasks such as trace eyeblink conditioning and, after learning, their hippocampal neurons would have reduced slow AHPs as a result of the activation of PKC dependent neuronal signaling pathways.

The fact that some young rabbits have this patchy distribution of PKC-ir in CA3 could also imply that poor learners among young rabbits ( $\sim$ 46%, Van der Zee et al., 1997c) are those with already enhanced PKC $\gamma$ -ir in the CA3c region at the beginning of training. What can be the reason that CA3a neurons already reveal this phenomenon? CA3 cells are physiologically very active, characterized by recurrent collateral activation and synchronous discharge bursts in subgroups (Buzsáki, 1989). If the change in PKC-ir reflects removal of PKC from the membrane due to the loss of anchoring proteins (see below), this may prevent neurons from overexcitation through activation of the PKC signal transduction pathways. On the other hand, this also means that hippocampal



FIGURE 6. Receptor for activated C kinase (RACK1) immunoblots of young and aging hippocampus. Representative immunoblots for RACK1 of hippocampal tissue homogenates of the pellet fraction of two young and two aging rabbits. 40  $\mu$ g protein was loaded per lane. Note the dramatic reduction of RACK1 expression in aging rabbits. No RACK1 expression was found in the cytosol fraction in either young or aging rabbits (data not shown).

engagement in a learning task is more difficult due to impaired translocation of PKC to the membrane upon stimulation.

The PKC-isoform-selective loss of immunopositive interneurons in the CA1 region suggests the existence of different populations of nonprincipal cells expressing a certain PKC isozyme. Previously, it was demonstrated by De Jong et al. (1996) that the number of calbindin-positive neurons in the CA1, but not the number of parvalbumin-positive interneurons in this region, declined with advancing age. Although it remains to be determined which PKC isoforms are expressed by these interneurons immunopositive for  $Ca^{2+}$ -binding proteins, the present data indicate that it is more likely that calbindin-positive interneurons express the  $\alpha$ - and  $\gamma$ -isoform, instead of PKC $\beta$ 1 or  $\beta$ 2. Moreover, the loss of calbindin/PKC $\alpha$ / $\gamma$ -positive  $\gamma$ -aminobutyric acid (GABA)ergic neurons in the aging CA1 region may contribute to enhanced PKC $\gamma$ -ir in the pyramidal cells due to loss of GABAergic inhibition (and hence overexcitation) of the pyramidal cells. Such a mechanism has also been suggested to explain enhanced PKC $\gamma$ -ir in the hippocampus after stroke in spontaneously hypertensive stroke-prone rats (De Jong et al., 1993).

The present results indicate a reorganization of PKC-positive afferents to the aging dentate gyrus. A decrease in the middle molecular layer of the dentate gyrus coincides with an increase in PKC-positive fibers terminating directly underneath the granule cell layer. Although we can only speculate about the origin of these inputs, it is possible that a diminished PKC $\alpha$  (and  $\beta$ 1 to a lesser extent) positive input from layer II/III of the entorhinal cortex is counteracted by an enhanced input from the deeper layers (IV–VI) of the entorhinal cortex. Neurons in the latter region have previously been shown to project specifically subjacent to the granule cell layer (Deller et al., 1996) where the increase in PKC $\alpha$ -ir in the aging dentate gyrus is seen. This shift in PKC-positive input may be related to the observed reappearance of neurofilament-ir in the granule cells bordering the hilar region seen typically in rabbits aged  $\geq 36$  months (Van der Zee et al., 1997b). Notably, PKC $\alpha$  seems to be involved in the regulation of neurotransmitter release (Majewski and Iannazzo, 1998), and enhanced levels of presynaptic PKC $\alpha$  in fibers innervating these granule cells may trigger the remarkable upregulation in neurofilament expression. Taken together, these results imply a functional reorganization of the aging dentate gyrus not seen in the other hippocampal areas.

The OD measures of the dorsomedial thalamus and medial posterior hypothalamus revealed that the aging-related PKC changes were brain region specific, but not restricted to the hippocampus. Although not included in the analyses, several neocortical areas (e.g., somatosensory cortex, but much less so in the motor cortex) and entorhinal cortex also have enhanced PKC-ir in the aging rabbit. The regional selectivity of the enhanced PKC expression may suggest that notably those areas involved in learning and memory processes are especially affected by aging, with the hippocampus as one of the main substrates.

### Change in Subcellular Localization of PKC During Aging

The immunoblot studies clearly revealed a concurrent loss of RACK1 and cPKC (irrespective of isoform) in the pellet fraction of

the aging hippocampus. Since RACK has been suggested to be required for the translocation of PKC (Mochly-Rosen et al., 1991; Mochly-Rosen and Gordon, 1998), the most likely explanation of the loss of PKC in the pellet fraction is a reduction of membrane-bound PKC due to the severe loss of RACK1. RACK1 has a preference to interact with PKC $\beta$  at least in cardiac tissue (Ron et al., 1994), but also interacts with PKC $\gamma$  (Chen et al., 2003). In the aging rat brain, RACK1 content was decreased by 50 % compared with young adult animals (Pascale et al., 1996), implicating RACK1 deficits as a more general underlying cause of aging-dependent dysregulation of the PKC signal transduction system.

In a preliminary autoradiographic study, Woodruff-Pak et al. (1991) demonstrated a clear decrease in phorbol ester binding ( $[^3H]$ phorbol-12,13-dibutyrate ( $[^3H]$ PDBu)) in the hippocampus of older rabbits (30–48 months of age) compared with young rabbits (3 months of age).  $[^3H]$ PDBu most likely binds to membrane-associated PKC (Olds et al., 1989), suggesting a decrease in membrane-associated PKC in old rabbit hippocampus. These results corroborate our findings obtained with Western blotting and immunocytochemical methods.

No significant changes in the total content of the four PKC isoforms was found. The PKC immunoblots, therefore, also demonstrate that what would appear to be the most obvious explanation for the age-related increase in PKC-ir—a proportional increase in total content of PKC—does not occur. In fact, all isoforms showed a tendency toward reduced levels in total content with aging.

### Differences Between PKC Isoforms and Species in Aging-Related Alterations

The immunocytochemical data show that PKC $\alpha$ -ir is least affected in aging pyramidal cells. Also, PKC immunoblots reveal that the  $\alpha$ -isoform was the one with the least reduction in the pellet fraction in the aging hippocampus. Although it remains to be determined with which PKC isoforms RACK1 interacts in the rabbit hippocampus, our results suggest that PKC $\alpha$  is less regulated by RACK1 in comparison to the other  $Ca^{2+}$ -dependent isoforms. Instead of RACK1, other anchoring proteins or scaffold proteins such as AKAP79, RICK or PICK1 (Newton, 1996; Faux and Scott, 1996; for review, see Liu and Heckman, 1998; Mochly-Rosen and Gordon, 1998) may be more directly linked to PKC $\alpha$  functioning. Moreover, it should be noted that due to its more prominent presynaptic localization, PKC $\alpha$  seems to be involved in a larger variety of hippocampal functions than the three other  $Ca^{2+}$ -dependent isoforms.

Age-related changes in the PKC system appears to be species- and even strain-specific (Magnoni et al., 1991; for review, see Battaini et al., 1994). In contrast to the aging rabbit, in aging Wistar rat hippocampus an increase in membrane-bound PKC $\gamma$  has been reported (Battaini et al., 1995), a finding also observed in the aging Wistar rat using the PKC $\gamma$  antibody 36G9 (B.R.K. Douma and E.A. Van der Zee, unpublished observations). Apparently, although the induction of PKC translocation from cytosol to membrane is impaired in the old rat, more PKC $\gamma$  is already membrane bound in the unstimulated situation. This high concentra-

tion of membrane-bound PKC $\gamma$ , which is possibly due to high levels of intracellular Ca<sup>2+</sup>, does nevertheless not improve memory function (Colombo et al., 1997). Interestingly, old Long Evans rats with spatial memory impairments (but not those with intact spatial memory) have enhanced hippocampal PKC $\gamma$ -ir, like that seen in aging rabbits in the present study (Colombo and Gallagher, 2002).

### Possible Cause of Aging-Related Changes in PKC-Immunoreactivity

The main findings of the present study are (1) an increase in PKC-ir in pyramidal cells, granule cells, and hilar interneurons of the aging hippocampus; (2) a severe loss of PKC localization in the pellet fraction of the aging hippocampus; and (3) a dramatic loss of RACK1 in the pellet fraction of the aging hippocampus. Immunostaining for PKCs increases with aging. The data on heterogeneity suggest that this is a gradual effect, with increasing numbers of densely stained neurons with increasing age. PKC isoform-selective binding proteins like RACK1 direct the Ca<sup>2+</sup>-dependent isozymes to different subcellular localizations. The reduction in anchoring protein (RACK1) results in the loss of membrane-bound PKCs. Loss of these binding sites may result in dysregulation of the hippocampal PKC system with consequences for normal cell functioning, and hence learning and memory processes. Hippocampus-dependent trace eyeblink conditioning revealed an aging-related impairment in acquisition in rabbits. Sixty percent of 36-month-old rabbits failed to reach an 80% criterion of correct responses (Thompson et al., 1996). The altered subcellular PKC distribution at this age as found in this study may contribute to a reduced information processing capacity due to reduced excitability of aging hippocampal cells, possibly via an enhanced slow outward calcium-activated potassium current contributing to enhanced post-burst AHP (Power et al., 2002). Increased PKC-ir in experimentally naive animals, therefore, appears to be indicative of a malfunction in PKC-mediated signal transduction. We can only speculate on the cause of the increased immunoreactivity (which is not due to more PKCs in aging, as shown by Western blot analysis). Because much less PKC is bound to RACK1 (which occurs via protein-protein interactions), it is quite possible that epitopes on the PKCs are more accessible. This increase in accessible binding sites could explain the observed increase in PKC-ir (as previously demonstrated for general protein-protein interactions with PKC $\gamma$ ) (Van der Zee et al., 2000).

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