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Age-Related Effects of Rolipram on [^{3}H]Quinuclidinyl Benzilate and [^{3}H]Phorbol 12, 13-Dibutyrate Binding in the Rat Brain

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CHEN, T., KATO, H., ARAKI, T., ITOYAMA, Y. and KOGURE, K. *Age-Related Effects of Rolipram on [^{3}H]Quinuclidinyl Benzilate and [^{3}H]Phorbol 12, 13-Dibutyrate Binding in the Rat Brain.* Tohoku J. Exp. Med., 1998, **185** (2), 107-118 —— Cholinergic neurotransmission and protein kinase C (PKC) in the brain play important roles in the processes of cognitive function. In this study, we examined the effect of chronic treatment with rolipram, a 3′, 5′-cyclic adenosine monophosphate (cyclic AMP)-selective phosphodiesterase inhibitor, on age-related changes in [^{3}H]quinuclidinyl benzilate (QNB) and [^{3}H]phorbol 12, 13-dibutyrate (PDBu) binding, which labeled brain muscarinic cholinergic receptors and PKC, respectively. Rolipram was administered per os to young (15 weeks old) and old (80 weeks old) Wistar rats at dosage of 0.01 mg/kg and 0.1 mg/kg once a day over 4 weeks. Then, quantitative in vitro autoradiography was performed. Control old rats showed elevations in [^{3}H]PDBu binding in the hippocampus and the cerebellum compared to young rats, but [^{3}H]QNB binding was largely unchanged. Chronic treatment of the old rats with the higher dose of rolipram led to reductions in [^{3}H]QNB and [^{3}H]PDBu binding in many brain regions. However, the same treatment of the young rats induced no or minimal effect. Thus, the response of the brain to rolipram was different between young and old rats. These results suggest that the cyclic AMP-selective phosphodiesterase system in the brain is modified during aging, modulating subsequently cholinergic neurotransmission and PKC activity exclusively in old rat brains. ———— rolipram; phosphodiesterase inhibitor; aging; acetylcholine; PKC

The central nervous system is a network of numerous neurons, and transmission of information is mediated by various neurotransmitters. Age-related decline in cognitive function, such as learning and memory (Bartus et al. 1980; Brizzee et al. 1980; Presty et al. 1987; Scarpace and Abrass 1988; Wenk et al.

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1989), is associated with a dysfunction of releases of a number of neurotransmitters (Gibson and Peterson 1982; Meyer et al. 1986; Freeman and Gibson 1987; McIntosh and Westfall 1987) and changes in their receptors (Misra et al. 1980; Giorgi et al. 1987; Haba et al. 1988; Gozlan et al. 1990; Najlerahim et al. 1990; Wenk et al. 1991). In particular, forebrain cholinergic systems have been considered to be involved in the processes of learning and memory, and the deficits of them in aging are attributable, at least in part, to a decline in the functional integrity of the forebrain cholinergic systems (Lippa et al. 1980; Bartus et al. 1982; Dunnett and Fibiger 1993; Nabeshima 1993). The dysfunction of neurotransmitter systems is also of paramount importance in age-related neurodegenerative disorders, such as Alzheimer's disease (Whitehouse et al. 1980; Mann and Yates 1984; Kellar et al. 1987; Chan-Palay and Asan 1989). Therefore, the effect of aging on neurotransmitter systems is of great interest with regard to age-related neuronal dysfunction and decline in cognitive function.

Many neurotransmitters in the brain are coupled to intracellular second messenger systems, the adenylate cyclase and phosphoinositide systems, which play critical roles in mediating the actions of various neurotransmitters in the brain (Worley et al. 1987; Nishizuka 1988). Biochemical and electrophysiological investigations have suggested that protein kinase C (PKC) of the phosphoinositide system modulates the number of neurotransmitters released by nerve cells as well as intracellular ion concentrations (Malenka et al. 1986; Nishizuka 1986). PKC plays a key role in modulating neuronal transmission, intracellular signal transduction, and synaptic plasticity (Nishizuka 1988), and is involved in the generation of long-term potentiation in the hippocampus and the process of learning and memory (Alkers et al. 1986; Hu et al. 1986; Malenka et al. 1986). PKC activity also changes during aging (Barnes et al. 1988; Friedman and Wang 1989). Thus, cholinergic neurotransmission and PKC are important mediators of cognitive function, and may undergo age-dependent modifications.

Rolipram is a specific inhibitor of a Ca^{2+}/calmodulin independent 3′, 5′-cyclic adenosine monophosphate (cyclic AMP)-selective phosphodiesterase isoenzyme (Schwabe et al. 1976; Ilien et al. 1982; Davis 1984), and leads to increased cyclic AMP levels in the brain (Schneider 1984). Rolipram has been known as a clinically effective antidepressant (Zeller et al. 1984; Bertolino et al. 1988; Bobon et al. 1988) and, in addition, has recently been demonstrated to have a neuroprotective effect against hippocampal neuronal damage following cerebral ischemia in gerbils (Kato et al. 1995). Since abnormalities in cyclic AMP signal amplification have been found in aged people and demented patients (Ebstein et al. 1986), rolipram can be a candidate for the medical treatment of age-associated disorders, such as Alzheimer's disease, in addition to depression and stroke. Therefore, it is very important to know how rolipram modifies the neurotransmission and transduction systems in the brain. The purpose of this study was therefore to investigate the effects of chronic treatment with rolipram on regional

age-related changes in muscarinic cholinergic receptors and PKC using in vitro quantitative autoradiography.

Materials and Methods

Experimental animals. We used male Wistar rats (Clea Japan Inc., Tokyo), 15 weeks old (young rats) and 80 weeks old (old rats), and weighing approximately 390 g and 520 g, respectively. They were allowed free access to food and water throughout the experiments. Rolipram (donated by Meiji Seika Kaisha Ltd., Tokyo) at dosage of 0.01 mg/kg and 0.1 mg/kg or its vehicle (distilled water) was administered orally once a day for 4 weeks. A previous paper has shown that an intraperitoneal injection of 0.1 mg/kg rolipram ameliorates the impairments of learning and memory induced by scopolamine or cerebral ischemia (Imanishi et al. 1997). Each group contained six rats. The rats were sacrificed 24 hours after the final administration, when the animals were 19 weeks and 84 weeks old. Their brains were removed quickly, frozen in powdered dry ice, and stored at $-80^{\circ}C$ until assay. Sagittal sections, 15 μm in thickness, were cut on a cryostat at the level of the dorsal hippocampus and thaw mounted onto gelatin-coated slides. Adjacent sections were stained with cresyl violet, and anatomic structures were verified by comparing the stained sections with a rat brain atlas.

[^{3}H]Quinuclidinyl benzilate (QNB) binding. Muscarinic cholinergic receptors were quantified using the radiolabeled antagonist [^{3}H]QNB (specific activity 43 Ci/mmol, Amersham Corp., Arlington, IL, USA) as reported previously (Araki et al. 1991). The sections were incubated with 1 nM [^{3}H]QNB in phosphate buffer (pH 7.4) for 90 minutes at room temperature. The sections were then washed in the buffer for 5 minutes at 4°C. Nonspecific binding was determined using 1 μmol unlabeled atropine (Sigma Chemical Co., St. Louis, MO, USA).

[^{3}H]Phorbol 12, 13-dibutyrate (PDBu) binding. Autoradiography with [^{3}H]PDBu was carried out essentially as described by Worley et al. (1986). The brain sections were incubated for 60 minutes at room temperature in the buffer (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 1 mM $CaCl_2$) containing 2.5 nM [^{3}H]PDBu (specific activity 20.7 Ci/mmol, New England Nuclear, Newton, MA, USA). Following incubation, the sections were washed twice for 2 minutes at 4°C in the same buffer. Non-specific binding was evaluated in the presence of 1 μM PDBu (Sigma) in the buffer.

Autoradiography. All procedures were performed under subdued lighting. The sections were dried under a stream of cold air and exposed to Hyperfilm-^{3}H (Amersham) for 2 weeks in x-ray cassettes with a set of tritium standards ([^{3}H] microscale, Amersham). The optical density of the brain regions was measured using a computer-assisted image analyzer (IBAS Image Analyser System, Zeiss, Oberkochen, Germany). The relationship between the optical density and radioactivity was obtained with reference to the [^{3}H]microscale exposed along with the tissue sections. The binding assay was performed in duplicate. Because non-

specific binding was negligible in this study, total binding values were used as specific binding values. The data were expressed as mean values ± S.D. (fmol/mg tissue). Statistical comparisons were made with the analysis of variance and the Bonferroni's multiple comparison test.

Results

[^{3}H]QNB binding. In vehicle-treated young rats, high [^{3}H]QNB binding was seen in the CA1 subfield of the hippocampus, the nucleus accumbens, the dentate gyrus, and the striatum, followed by the frontal cortex and the CA3 subfield of the hippocampus. [^{3}H]QNB binding was relatively low in the thalamus and the substantia nigra, and very low in the cerebellum (Table 1 and Fig. 1). No age-related changes in [^{3}H]QNB binding were observed in the forebrain, but significant reductions in [^{3}H]QNB binding were seen in the thalamus and the cerebellum of old rats (Table 1). Chronic treatment of the old rats with the higher dose, but not the lower dose, of rolipram induced significant reductions in [^{3}H]QNB binding in the frontal cortex, the striatum, and the CA3 subfield of the hippocampus. However, rolipram produced no changes in the young rats (Table 1).

[^{3}H]PDBu binding. In vehicle-treated young rats, the highest density of the [^{3}H]PDBu binding was found in the CA1 subfield of the hippocampus, the molecular layer of the cerebellum, the dentate gyrus, and the CA3 subfield of the hippocampus. The accumbens nucleus, the neocortex, the striatum, and the thalamus exhibited relatively high [^{3}H]PDBu binding (Table 2 and Fig. 1). In vehicle-treated old rats, significant elevations in [^{3}H]PDBu binding were seen in the CA1 subfield of the hippocampus, the dentate gyrus, and the molecular layer

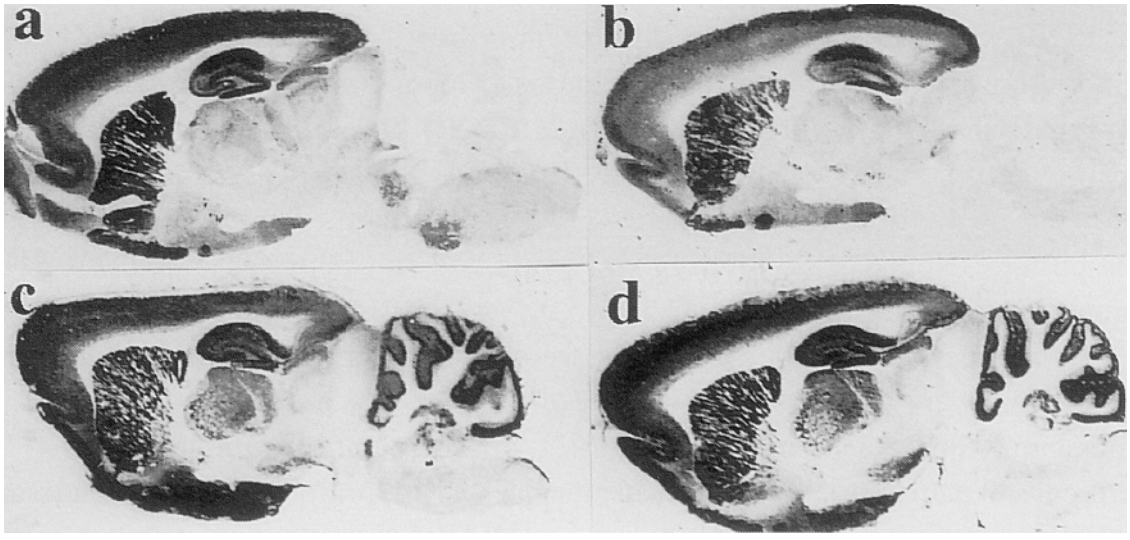

Fig. 1. Representative autoradiographs of [^{3}H]quinuclidinyl benzilate binding (a, b) and [^{3}H]phorbol 12, 13-dibutyrate binding (c, d) which label muscarinic cholinergic receptors and protein kinase C, respectively. a, c, brains of young rats (19 weeks old); b, d, brains of old rats (84 weeks old).

TABLE 1. *Effect of chronic rolipram treatment on age-related alterations in specific [^3H] quinuclidinyl benzilate binding in the rat brain*

	19 weeks old			84 weeks old		
	Vehicle	0.01 mg/kg	0.1 mg/kg	Vehicle	0.01 mg/kg	0.1 mg/kg
Frontal cortex	352±41	369±29	347±30	331±29	305±13	271±25[d]
Striatum	377±45	400±29	390±42	351±28	357±24	303±27[c]
Nucleus accumbens	444±40	455±47	447±23	411±27	402±38	386±38
Hippocampus						
CA1 sector	469±39	482±19	485±17	473±32	474±28	437±31
CA3 sector	307±35	303±14	287±37	282±29	274±20	245±13[c]
dentate gyrus	398±35	379±23	391±42	402±42	398±23	362±34
Thalamus	135±36	119±21	105±29	83±20[a]	80±22	67±19
Substantia nigra	83±40	52±21	50±15	43±11	50±15	45±17
Cerebellum	38±7	27±10	29±7	26±6[b]	35±7	32±6

Data are expressed as mean values±S.D. $n=5$–6, fmol/mg tissue

[a] $p<0.05$, [b] $p<0.01$ vs. vehicle-treated young animals

[c] $p<0.05$, [d] $p<0.01$ vs. vehicle-treated old animals (Bonferroni's multiple comparison test)

of the cerebellum compared with young rats (Table 2). Chronic treatment of the old rats with the higher dose of rolipram caused significant reductions in [^{3}H] PDBu binding in the CA3 subfield of the hippocampus, the accumbens nucleus, the molecular layer of the cerebellum, the frontal cortex, the striatum, the dentate gyrus, and the thalamus. The lower dose of rolipram reduced [^{3}H]PDBu binding only in the thalamus. The same treatment of the young rats induced no alterations in [^{3}H]PDBu binding except for an elevation in the dentate gyrus of rats treated with the lower dose of rolipram (Table 2).

DISCUSSION

Age-related changes in [^3H]QNB and [^3H]PDBu binding. Age-related alterations in neurotransmission may underlie age-related deficits in cognitive function and psychomotor performance (Freeman and Gibson 1987; Friedman and Wang 1989). Furthermore, the dysfunction of neurotransmitter systems has been thought to play a role in age-related neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Whitehouse et al. 1980; Kellar et al. 1987; Chan-Palay and Asan 1989). Therefore, the effect of aging on neurotransmitter systems is of great interest with regard to the age-related neuronal dysfunction and impaired cognitive functions. A number of neurochemical studies have addressed whether age-related changes occur in the cholinergic system in the brain. Earlier studies demonstrated significant reductions in acetylcholine receptors during aging (Freund 1980; Pedigo et al. 1984; Haba et al. 1988; Biegon et al. 1989). Several authors have demonstrated that impaired cognitive function in

TABLE 2. *Effect of chronic rolipram treatment on age-related alterations in specific [^3H] phorbol 12, 13-dibutyrate binding in the rat brain*

	19 weeks old			84 weeks old		
	Vehicle	0.01 mg/kg	0.1 mg/kg	Vehicle	0.01 mg/kg	0.1 mg/kg
Frontal cortex	876±95	925±94	862±56	897±81	839±79	748±99[c]
Parietal cortex	810±57	822±86	795±88	908±99	924±54	808±89
Striatum	756±95	794±99	728±21	783±80	692±75	657±50[c]
Accumbens nucleus	890±99	994±95	893±66	943±100	836±82	701±47[d]
Hippocampus						
CA1 sector	998±39	1052±55	988±66	1090±92[a]	1084±43	1086±69
CA3 sector	906±84	988±67	945±71	973±86	936±73	820±61[d]
Dentate gyrus	915±43	988±22[b]	927±30	1006±86[a]	937±99	860±57[c]
Thalamus	648±59	686±75	598±66	638±71	510±84[c]	446±62[d]
Brain stem	205±24	188±39	179±13	177±29	168±36	150±18
Cerebellum						
Molecular layer	933±89	1008±46	997±98	1068±95[a]	1018±55	897±38[d]

Data are expressed as mean values±S.D. $n=5$–6, fmol/mg tissue

[a] $p<0.05$, [b] $p<0.01$ vs. vehicle-treated young animals

[c] $p<0.05$, [d] $p<0.01$ vs. vehicle-treated old animals (Bonferroni's multiple comparison test)

aged animals is associated with a decrease in the size and number of cholinergic cells in the brain (Biegon et al. 1986; Gage et al. 1988; Fischer et al. 1991). Based on these observations, it has been proposed that the age-related decline in cognitive function is closely linked to the dysfunction of the cholinergic system. However, other studies have reported no changes (Morin and Wasterlain 1980; Lippa et al. 1985; Consolo et al. 1986; Decker 1987; Araki et al. 1995), and increases (Springer et al. 1987; Araki et al. 1993) in cholinergic receptors in aged animal brains. In the present study, we found no changes in [^{3}H]QNB binding in the forebrain between 19 weeks and 84 weeks of age. Decreased [^{3}H]QNB binding was seen in the thalamus and the cerebellum, but the significance of these reductions is uncertain because the binding activities in these regions are low. The different results among different authors may be in part due to different strains and species, different ages of rats both young and old ones, and the methods employed.

The second messenger systems are crucial to various aspects of cellular function. In particular PKC is a widespread family of kinases responsible for neurotransmitter release, neuronal activity, synaptic plasticity, long-term potentiation, and growth and differentiation (Nishizuka, 1984, 1986; Alkers et al. 1986). Thus, this enzyme is thought to play a major role in neuronal function. Reduced PKC activity in aged rat brain has been demonstrated (Barnes et al. 1988; Friedman and Wang 1989), and in addition, it has been suggested that the

amount of membrane-bound PKC activity may be a determinant of age-related decline in spatial learning (Fordyce and Wehner 1993). In this study, we observed significant elevations in [^{3}H]PDBu binding in old rats in the hippocampal CA1 sector, the dentate gyrus, and the cerebellar molecular layer compared with young animals. Earlier reports on age-related changes in [^{3}H]PDBu binding are only a few, but reported data include reductions (Araki et al. 1993) and no changes (Araki et al. 1995) in [^{3}H]PDBu binding during aging. The implication of the elevations of [^{3}H]PDBu binding in several brain regions seen in this study is uncertain at present. They are likely to be a response to age-related changes in neurotransmission.

Effects of chronic rolipram treatment. Since it has been known that a clinical response to antidepressant therapy requires several weeks, the neurochemical basis of their pharmacological effects should be studied after long-term treatment. Although changes in the levels of neurotransmitters and second messengers are induced in response to acute drug exposure, changes in receptor density, enzyme activity, and gene expression are observed after chronic, but not acute, administration of antidepressants, including rolipram (Schultz and Folkers 1988; Perez et al. 1989; Nibuya et al. 1996). This is the reason why chronic treatment was employed in this study.

We found that chronic treatment of the old rats with rolipram, especially of the higher dose, decreased [^{3}H]QNB and [^{3}H]PDBu binding in many brain regions. Of note, the effect of rolipram treatment was observed only in the old rats. The decrease in [^{3}H]QNB binding in the old rat brain could be a reflection of down-regulation of receptor density in response to the increased levels of cyclic AMP, which may mimic an increase in acetylcholine neurotransmission. Age-related alterations in neurotransmitter release have been considered to play an important role in age-related alterations in receptor density (Freeman and Gibson 1987). The reductions in [^{3}H]PDBu binding following rolipram treatment in the old rats could also be similar down-regulation, responding to increased cyclic AMP levels. These down-regulations may be induced by both presynaptic potentiation of neurotransmission and postsynaptic action via enhanced availability of the second messenger cyclic AMP (Kehr et al. 1985; Wachtel and Schneider 1986). Since such down-regulations were seen only in old rats, it is suggested that the cyclic AMP-selective phosphodiesterase system itself or its modulatory feedback system is altered in aged brains, causing further effects on other neurotransmission and transduction systems. Thus, age-related deficits of receptor density and receptor function are not restricted to a single system since there is a link among different systems. One has to be very careful in interpreting changes of binding as changes in receptor function or enzyme activity. No enzyme activity study, or behavioral study was available in this study. We could not determine whether the observed changes in [^{3}H]QNB and [^{3}H]PDBu binding were owing to changes in the affinity constant (K_d) or the number of binding sites (B_{max}). Therefore,

further studies are needed to investigate the precise biochemical mechanisms of our finding.

As has been demonstrated, rolipram is a promising member of a new class of antidepressants (Zeller et al. 1984; Bertolino et al. 1988; Bobon et al. 1988). Rolipram has selective cyclic AMP phosphodiesterase inhibiting properties, and leads to an increase in cyclic AMP levels in brain (Schneider 1984). It has been found that the changes in basal activity of adenylate cyclase occurs in the brains of senescent animals under different experimental conditions (Nomura et al. 1984). Autoradiographic data have shown the regional age-related changes in [^{3}H]cyclic AMP and [^{3}H]forskolin (which labels adenylate cyclase) binding sites in the brain (Araki et al. 1993, 1995). Since abnormalities in cyclic AMP signal amplification have been found in aged people and demented patients (Ebstein et al. 1986), tissue-specific phosphodiesterase inhibitors like rolipram may provide a therapeutic alternative to first messenger related drug therapy in age-related disorders, such as depression and dementia (Kaulen et al. 1989). There is abundant evidence that several neurotransmitters, their marker enzymes and any neurotransmitter receptors are significantly reduced in brain tissue from Alzheimer's disease. The dysfunction of intracellular second messenger systems is also known to occur in such age-related neurodegenerative disorders as Alzheimer's disease. Therefore, the impairment of cognitive function may reflect the dysfunction of neurotransmitter and second messenger systems occurring not only in aged brain, but also in age-related neurodegenerative disorders. Modification of age-related dysfunction of neurotransmission and second messenger systems might be an important aspect of the pharmacological properties of rolipram and other drugs effective in treating age-related deficits of mental performance, which certainly deserves further investigation.

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