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Phospholipase A_2 Modulates Different Subtypes of Excitatory Amino Acid Receptors: Autoradiographic Evidence

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Abstract: Exogenous phospholipases have been used extensively as tools to study the role of membrane lipids in receptor mechanisms. We used in vitro quantitative autoradiography to evaluate the effect of phospholipase A_2 (PLA_2) on *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors in rat brain. PLA_2 pretreatment induced a significant increase in α-[^{3}H]amino-3-hydroxy-5-methylisoxazole-4-propionate ([^{3}H]AMPA) binding in the stratum radiatum of the CA1 region of the hippocampus and in the stratum moleculare of the cerebellum. No modification of [^{3}H]AMPA binding was found in the stratum pyramidale of the hippocampus at different ligand concentrations. [^{3}H]-Glutamate binding to the metabotropic glutamate receptor and the non-NMDA-, non-kainate-, non-quisqualate-sensitive [^{3}H]glutamate binding site were also increased by PLA_2 pretreatment. [^{3}H]Kainate binding and NMDA-sensitive [^{3}H]glutamate binding were minimally affected by the enzyme pretreatment. The PLA_2 effect was reversed by EGTA, the PLA_2 inhibitor *p*-bromophenacyl bromide, and prolonged pretreatment with heat. Bovine serum albumin (1%) prevented the increase in metabotropic binding by PLA_2. Arachidonic acid failed to mimic the PLA_2 effect on metabotropic binding. These results indicate that PLA_2 can selectively modulate certain subtypes of excitatory amino acid receptors. This effect is due to the enzymatic activity but is probably not correlated with the formation of arachidonic acid metabolites. Independent of their possible physiological implications, our results provide the first autoradiographic evidence that an enzymatic treatment can selectively affect the binding properties of excitatory amino acid receptors in different regions of the CNS. **Key Words:** Phospholipase A_2—Glutamate—α-Amino-3-hydroxy-5-methylisoxazole-4-propionate—Metabotropic—Autoradiography. **Catania M. V. et al.** Phospholipase A_2 modulates different subtypes of excitatory amino acid receptors: Autoradiographic evidence. *J. Neurochem.* **60,** 236–245 (1993).

Phospholipase A_2 (PLA_2; EC 3.1.1.4; phosphatidylcholine 2-acylhydrolase) is a calcium-dependent, ubiquitous enzyme whose activation leads to the release of unsaturated fatty acids from the sn-2 position of membrane phospholipids (Van den Bosch, 1980). Increasing evidence suggests the involvement of PLA_2 in mechanisms underlying various aspects of neuronal function. PLA_2 activation and the subsequent production of different metabolites play an important role in the regulation of receptor binding (Pasternak and Snyder, 1973; Limbird and Lefkowitz, 1975; Aronstam et al., 1977; Andreasen et al., 1979; Oliveira et al., 1984; Yoneda et al., 1985; Havoundjian et al., 1986; Massicotte and Baudry, 1990; Radja et al., 1992), membrane transport (Saltarelli et al., 1990), neurotransmitter release (Ohmichi et al., 1989), and channel activation (Schwartz et al., 1988). Previous studies performed in telencephalic membranes indicate that PLA_2 can modulate the properties of the (*RS*)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/quisqualate subtype of excitatory amino acid (EAA) receptors (Massicotte and Baudry, 1990; Baudry et al., 1991). This modulation has been correlated with a possible involvement of PLA_2 activation in the expression of long-term potentiation, a putative synaptic substrate of learning and memory (Massicotte et al., 1990, 1991).

EAA receptors are the principal neurotransmitter receptors mediating synaptic excitation in the mammalian CNS. Different subtypes of EAA receptors

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; ANOVA, analysis of variance; BSA, bovine serum albumin; cAMP, cyclic AMP; EAA, excitatory amino acid; $GABA_A$, γ-aminobutyric $acid_A$; KSCN, potassium thiocyanate; NMDA, *N*-methyl-D-aspartate; NNKQ, non-NMDA-, non-kainate-, non-quisqualate-sensitive; pBPB, *p*-bromophenacyl bromide; PLA_2, phospholipase A_2.

have been described on the basis of differential pharmacological properties, distinct mechanisms of intracellular signal transduction, different localizations in the CNS (Greenamyre et al., 1985; Monaghan et al., 1989; Watkins et al., 1990), and heterogeneity of cloned cDNAs (Hollmann et al., 1989; Keinänen et al., 1990; Egebjerg et al., 1991; Masu et al., 1991; Moriyoshi et al., 1991; Tanabe et al., 1992). The EAA receptors are generally classified into at least three ionotropic receptor subtypes linked to cation channels: *N*-methyl-D-aspartate (NMDA), AMPA, and kainate (non-NMDA) receptors, named for the preferential affinity for these agonists. Recently, however, a new non-NMDA receptor subtype coupled through a GTP-binding protein to activation of inositol phospholipid hydrolysis and inhibition of cyclic AMP (cAMP) formation has been discovered (the metabotropic receptor) (Sladeczek et al., 1985; Nicoletti et al., 1986; Sugiyama and Ito, 1987; Schoepp et al., 1992). In autoradiographic studies, a unique glutamate recognition site, distinct from any known receptor type, has been described (Higgins et al., 1989). This site is non-NMDA-, non-kainate-, and non-quisqualate-sensitive (NNKQ) and is transiently expressed in the globus pallidus and thalamus during postnatal development (Greenamyre et al., 1990).

We used in vitro quantitative autoradiography to evaluate the effect of PLA_2 on the EAA receptor subtypes in three regions of rat brain—hippocampus, striatum, and cerebellum—where NMDA and non-NMDA receptors are widely and differentially localized.

MATERIALS AND METHODS

Materials

(*RS*)-[^{3}H]AMPA (60 Ci/mmol) and [^{3}H]glutamate (45–56 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Quisqualate, nonradioactive AMPA, and NMDA were obtained from Cambridge Research Biochemicals (Wilmington, DE, U.S.A.). Potassium thiocyanate (KSCN) was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). PLA_2 from porcine pancreas (600 U/mg of protein) and from bee venom (1000 U/mg of protein) and all other reagents and compounds were purchased from Sigma (St. Louis, MO, U.S.A.).

Tissue preparation

Male Sprague–Dawley rats (175–195 g) were decapitated and the brains were quickly removed, mounted with Lipshaw embedding matrix on a cryotome pedestal, and frozen under powdered dry ice. Twenty-micrometer-thick horizontal sections were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides. Sections were stored for less than 24 h at −20°C.

Phospholipase A_2 pretreatment

Before performing [^{3}H]AMPA and metabotropic binding, sections were incubated with PLA_2 at the indicated concentrations for 20 min at 37°C in 50 m*M* Tris-HCl with 30 m*M* KSCN in the presence of 4 or 25 m*M* $CaCl_2$, pH 8. Sections were then transferred to the same buffer for 10 min at 4°C, pH 7.2, in the presence of 2.5 m*M* $CaCl_2$. For [^{3}H]-kainate binding and NMDA-sensitive [^{3}H]glutamate binding, 50 m*M* Tris-acetate in the presence of 25 m*M* $CaCl_2$, pH 8, was used during pretreatment with the enzyme at 37°C. Cold, Ca^{2+}-free Tris-acetate, pH 7.4, was then used for the wash. Control sections were treated following the same procedure in the absence of the enzyme.

Autoradiography

After the above-described pretreatment, the sections were immersed in incubation buffer at 4°C with tritiated ligand and appropriate blocking agents for 45 min. After incubation, sections were rinsed quickly three times with cold buffer, then rinsed with cold 2.5% (vol/vol) glutaraldehyde in acetone and blown dry under a stream of hot air. The rinsing/drying procedure took no more than 10 s. Slides were placed in x-ray cassettes with appropriate radioactive standards and apposed to tritium-sensitive film (Hyperfilm, Amersham Corporation), then stored at 4°C for varying lengths of time. Films were developed in Kodak D-19 and analyzed using the MCID image processing system (Imaging Research, St. Catharines, Ontario, Canada). Ten to twenty-five readings were averaged for each region. Film density was converted into bound radioactivity using a computer-generated polynomial regression curve derived from the radioactive standards. A computer algorithm with higher resolution was used to measure film density in the stratum pyramidale of the hippocampus.

For [^{3}H]AMPA binding, after pretreatment with/without PLA_2, slides were incubated for 45 min at 4°C in 50 m*M* Tris-HCl containing 2.5 m*M* $CaCl_2$, 30 m*M* KSCN, pH 7.2, in the presence of different concentrations (2, 5, 30, and 500 n*M*) of [^{3}H]AMPA (specific activity, 60, 50, and 2.7 Ci/mmol). Nonspecific binding was defined as [^{3}H]AMPA binding occurring in the presence of 1 m*M* unlabeled glutamate.

We also examined the putative metabotropic receptor using a recently described quantitative autoradiographic assay (Cha et al., 1990). Briefly, slides were incubated for 45 min in 50 m*M* Tris-HCl, pH 7.2, containing 2.5 m*M* $CaCl_2$, 30 m*M* KSCN, and 100 n*M* [^{3}H]glutamate (specific activity, 45–56 Ci/mmol) in the presence of 100 μ*M* NMDA and 10 μ*M* AMPA at 4°C. Nonspecific binding was determined by the addition of 2.5 μ*M* quisqualate.

For [^{3}H]kainate and NMDA-sensitive [^{3}H]glutamate binding, sections were incubated for 45 min at 4°C in 50 m*M* Tris-Ac, pH 7.4, containing 65 n*M* [^{3}H]kainate (specific activity, 7.8 Ci/mmol) or 65 n*M* [^{3}H]glutamate (specific activity, 45–56 Ci/mmol), 2.5 μ*M* quisqualate, and 1 μ*M* kainate. Nonspecific binding was defined by the addition of 100 μ*M* kainate and 1 m*M* NMDA, respectively.

A unique [^{3}H]glutamate binding site has been described, the NNKQ site (Higgins et al., 1989; Greenamyre et al., 1990). Sections were incubated for 45 min with 65 n*M* [^{3}H]-glutamate (specific activity, 45–56 Ci/mmol) in 50 m*M* Tris-HCl, pH 7.2, containing 2.5 m*M* $CaCl_2$ and 30 m*M* KSCN in the presence of 100 μ*M* NMDA, 2.5 μ*M* quisqualate, and 1 μ*M* kainate. Nonspecific binding was obtained by adding 1 m*M* glutamate.

RESULTS

[^{3}H]AMPA binding

Prewash conditions. [^{3}H]AMPA binding was performed using different concentrations of [^{3}H]AMPA:

TABLE 1. *Variation of conditions during the prewash affects [^{3}H]AMPA binding [p = 0.0001 by two-way analysis of variance (ANOVA); magnitude = −20%] and NMDA-sensitive [^{3}H]glutamate binding (p = 0.0001 by two-way ANOVA; magnitude = +32%) but not metabotropic binding (p = 0.1 by two-way ANOVA)*

	AMPA binding		Metabotropic binding		NMDA binding	
Structure	4°C, pH 7.2, 2.5 m*M* Ca^{2+}	37°C, pH 8, 25 m*M* Ca^{2+}	4°C, pH 7.2, 2.5 m*M* Ca^{2+}	37°C, pH 8, 25 m*M* Ca^{2+}	4°C, pH 7.4	37°C, pH 8, 25 m*M* Ca^{2+}
DG	24.1 ± 2.86	18.9 ± 1.54	0.90 ± 0.06	0.94 ± 0.15	1.60 ± 0.30	2.10 ± 0.11[a]
CA1 SR	31.9 ± 3.17	24.6 ± 1.52[a]	0.72 ± 0.07	0.80 ± 0.11	1.75 ± 0.29	2.21 ± 0.06[a]
CA1 SO	22.4 ± 1.60	18.9 ± 1.61	0.71 ± 0.15	0.86 ± 0.10	1.47 ± 0.21	1.90 ± 0.20[a]
CA3SR	20.0 ± 3.00	16.5 ± 2.23	0.70 ± 0.08	0.70 ± 0.09	1.02 ± 0.18	1.30 ± 0.08[a]
CA3 SO	17.9 ± 1.81	14.8 ± 1.65	0.76 ± 0.10	0.75 ± 0.08	1.00 ± 0.19	1.31 ± 0.07
CB MOL	13.5 ± 2.1	10.7 ± 0.71	1.28 ± 0.17	1.49 ± 0.15	0.07 ± 0.03	0.04 ± 0.03
CB GCL	1.42 ± 0.25	1.39 ± 0.28	0.55 ± 0.12	0.58 ± 0.04	0.26 ± 0.08	0.50 ± 0.05
STR	9.75 ± 0.62	7.43 ± 1.44	1.02 ± 0.36	0.93 ± 0.22	0.49 ± 0.01	0.80 ± 0.11

[^{3}H]AMPA binding was carried out in 500 n*M* [^{3}H]AMPA (sp act, 2.7 Ci/mmol). All values are expressed as pmol/mg of protein. DG, dentate gyrus; CA1 SR, stratum radiatum of the CA1 region of the hippocampus; CA1 SO, stratum oriens of the CA1 region of the hippocampus; CA3 SR, stratum radiatum of the CA3 region of the hippocampus; CA3 SO, stratum oriens of the CA3 region of the hippocampus; CB MOL, cerebellar molecular layer; CB GCL, cerebellar granular cell layer; STR, striatum. Results represent the mean ± SD for three (AMPA) and four (metabotropic and NMDA) animals in separate experiments for each assay.

[a] $p < 0.05$ by Tukey's (*a*) test.

2, 5, 30, and 500 n*M* (the latter had a specific activity of 2.7 Ci/mmol). The possibility that the prewash performed under different conditions could modify the results of binding studies was tested. Pretreatment at 37°C, pH 8, in the presence of 25 m*M* $CaCl_2$ led to a slight decrease in [^{3}H]AMPA binding in all regions examined (80% of [^{3}H]AMPA binding performed on standard prewash condition-pretreated sections: 4°C, pH 7.2, and 2.5 m*M* $CaCl_2$) when experiments were carried out at 500 n*M* (Table 1). The different prewash conditions did not affect [^{3}H]AMPA binding when it was performed at 5 n*M* [^{3}H]AMPA (data not shown).

Regional distribution. The regional distribution of [^{3}H]AMPA binding was similar to the localization found by other authors (Nielsen et al., 1988). The highest amounts of binding were detected in the dentate gyrus and in the strata radiatum and oriens of the hippocampus (Table 1). However, a different pattern of distribution of [^{3}H]AMPA binding was found in the hippocampus when extreme concentrations of ligand were used to study the high- and low-affinity binding sites selectively. More binding was detected in the stratum pyramidale of the hippocampus than in the strata radiatum and oriens at 2 and 5 n*M* concentrations of ligand (124 and 115%, respectively, of the binding in the stratum radiatum). In contrast, less binding (57% of the stratum radiatum) was detected in the stratum pyramidale at 500 n*M* (Fig. 1).

PLA_2 effect. PLA_2's from porcine pancreas and from bee venom were used. Pretreatment with PLA_2 from porcine pancreas (0.008–0.08 U/ml) increased the low-affinity (500 n*M*) component of [^{3}H]AMPA binding. Post hoc pairwise Tukey's (*a*) test revealed a significant increase only in the stratum radiatum of the CA1 (34%) at 0.08 U/ml PLA_2. A consistent, but not statistically significant increase in binding was caused by 0.08 U/ml PLA_2 in the CA3 region of the hippocampus (39%) and in the molecular layer of the cerebellum (31%), whereas binding in the stratum pyramidale of the hippocampus, the striatum, or the granular cell layer of the cerebellum was not affected (Fig. 2A and B). Pretreatment with porcine pancreatic PLA_2 (0.05 U/ml) did not modify the high-affinity component (5 n*M*) in any region (Fig. 3A and B).

The pretreatment of the sections with PLA_2 from bee venom (0.5 U/ml) induced a significant increase in [^{3}H]AMPA binding in the strata radiatum and oriens of the CA1 region of the hippocampus (56% at 2

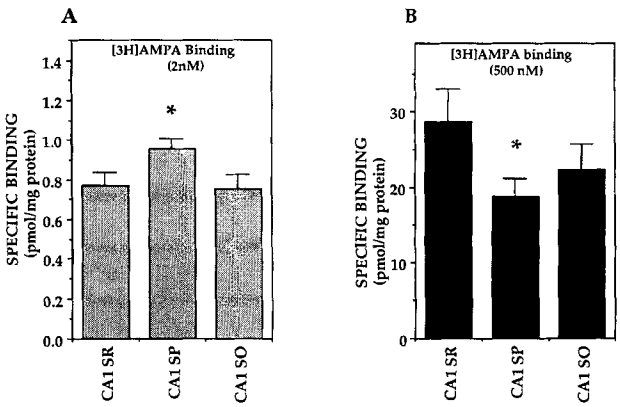

FIG. 1. Differential distribution of [^{3}H]AMPA binding in the hippocampus at 2 n*M* (**A**) and 500 n*M* (**B**). CA1 SR, stratum radiatum of the CA1 region of the hippocampus; CA1 SP, stratum pyramidale of CA1 region of the hippocampus; CA1 SO, stratum oriens of the CA1 region of the hippocampus. Columns represent the mean ± SD values of four animals. $p < 0.001$ by one-way ANOVA; *post hoc Scheffé *F*-pairwise comparison.

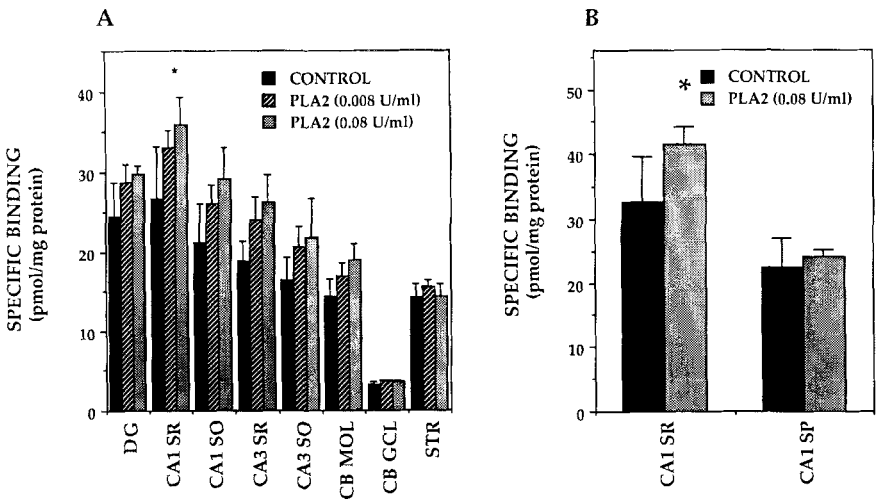

FIG. 2. PLA_2 (porcine pancreas) effect on [^{3}H]AMPA specific binding to low-affinity binding sites (500 n*M* [^{3}H]AMPA) ($p < 0.001$ by two-way ANOVA; magnitude = 20% at 0.008 U/ml PLA_2 and 26% at 0.08 U/ml PLA_2). *PLA pretreatment in individual region is different from control [$p < 0.05$ by Tukey's (*a*) test] (**A**). Using a computer algorithm with a higher resolution, no increase was revealed in the hippocampal pyramidal layer by PLA_2 [$p < 0.05$ by two-way ANOVA; *$p < 0.05$ by Tukey's (*a*) test] (**B**). DG, dentate gyrus; CA1, CA1 region of the hippocampus; CA3, CA3 region of the hippocampus; SR, stratum radiatum; SO, stratum oriens; CB MOL, cerebellar molecular layer; CB GCL, cerebellar granular cell layer; STR, striatum. PLA_2 pretreatment carried out at 25 m*M* $CaCl_2$. Columns represent the mean ± SD values of four animals.

n*M* and 34% at 30 n*M*) and in the molecular layer of the cerebellum (98% at 2 n*M* and 34% at 30 n*M*). [^{3}H]AMPA binding was not modified by the same pretreatment in the stratum pyramidale of the hippocampus, the striatum, or the granule cell layer of the cerebellum.

Metabotropic binding

Increased temperature (37°C instead of 4°C), basic pH (8 instead of 7.2), and $CaCl_2$ concentration (25 m*M*) during the prewash failed to alter metabotropic binding (Table 1). Pretreatment of the rat brain slices with PLA_2 from porcine pancreas led to a significant increase in metabotropic binding at concentrations of 0.1 U/ml but not lower (Fig. 4). Although a certain increase in binding was detected in the hippocampus (30%), only in the striatum (50%) did the post hoc comparison test reach statistical significance (Fig. 4).

PLA_2 from bee venom induced a significant increase in binding in the hippocampus and in the molecular layer of the cerebellum only at concentrations above 0.5 U/ml. No increase was found in the granule cell layer of the cerebellum (Table 2).

[^{3}H]Kainate and NMDA-sensitive [^{3}H]glutamate binding

Pretreatment without PLA_2 at 37°C, pH 8, in the presence of 25 m*M* $CaCl_2$ increased binding of [^{3}H]-glutamate to the NMDA receptor in every region except the cerebellar molecular layer, where binding was negligible (Table 1). A slight increase in [^{3}H]kainate and NMDA-sensitive [^{3}H]glutamate binding (9%) was caused by PLA_2 pretreatment at the concentrations indicated (Table 3). Statistical analysis with post hoc Tukey's (*a*) test indicated that the increase in [^{3}H]-kainate binding was due to a main effect in the stratum lucidum of the CA3 region of the hippocampus. No main regional effect was revealed by post hoc test for NMDA-sensitive [^{3}H]glutamate binding. At higher concentrations (0.9 U/ml) no further increase

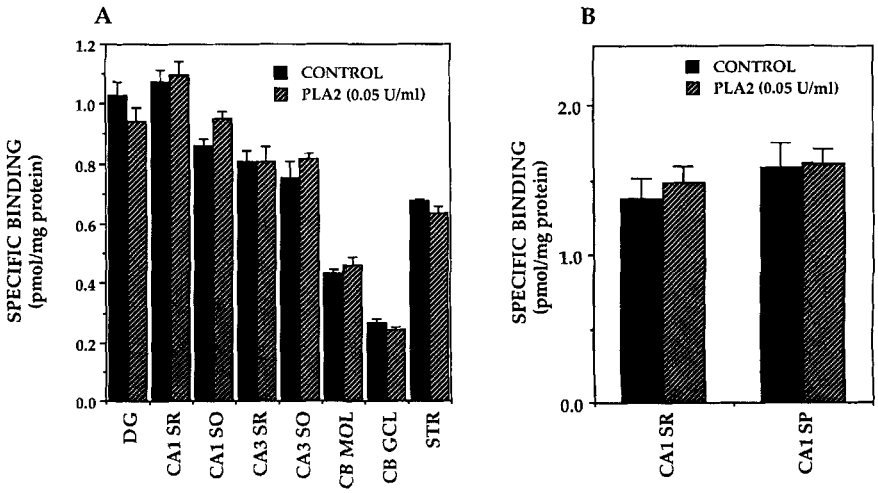

FIG. 3. PLA_2 (porcine pancreas) does not affect [^{3}H]AMPA specific binding to high-affinity binding sites (5 n*M* [^{3}H]-AMPA) in any region examined ($p > 0.5$ by two-way ANOVA) (**A**). Analysis of the same film using a computer algorithm with a higher resolution confirmed no increase also in the stratum pyramidale of the hippocampus ($p > 0.5$ by two-way ANOVA) (**B**). DG, dentate gyrus; CA1, CA1 region of the hippocampus; CA3, CA3 region of the hippocampus; SR, stratum radiatum; SO, stratum oriens; CB MOL, cerebellar molecular layer; CB GCL, cerebellar granular cell layer; STR, striatum. PLA_2 pretreatment carried out at 25 m*M* $CaCl_2$. Columns represent the mean ± SD values of four animals.

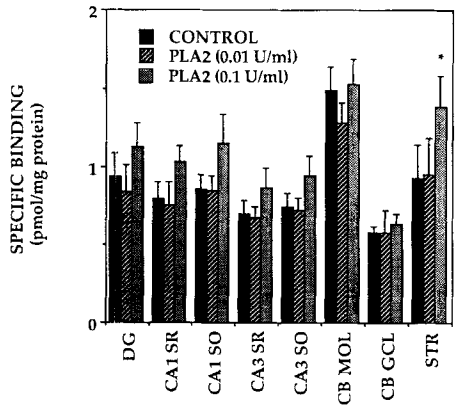

FIG. 4. Effect of PLA_2 (porcine pancreas) pretreatment on metabotropic binding ($p < 0.001$ by two-way ANOVA; magnitude = 24% for 0.1 U/ml PLA_2). *PLA_2 pretreatment in individual region is different from control [$p < 0.05$ by Tukey's (*a*) test]. DG, dentate gyrus; CA1, CA1 region of the hippocampus; CA3, CA3 region of the hippocampus; SR, stratum radiatum; SO, stratum oriens; CB MOL, cerebellar molecular layer; CB GCL, cerebellar granular cell layer; STR, striatum. PLA_2 pretreatment carried out at 25 m*M* $CaCl_2$. Columns represent the mean ± SD values of four animals.

in NMDA-sensitive [^{3}H]glutamate binding was detected in any region (data not shown).

NNKQ [^{3}H]glutamate binding site

PLA_2 pretreatment (bee venom, 0.5 U/ml) increased binding to the NNKQ site. Significant increases (50%) were observed in the dentate gyrus and the striatum (Table 2).

Characterization of PLA_2 enzymatic activity

PLA_2 is a calcium-dependent enzyme. The addition of EGTA (10 m*M*) to the preincubation buffer to chelate the calcium (4 m*M*) prevented the enzyme's enhancing effect on binding (Fig. 5A). *p*-Bromophenacyl bromide (pBPB) is an alkylating agent that inactivates PLA_2 by acting at the catalytic site on the enzyme (Volwerk et al., 1974). After treatment with pBPB (100 μM) for 8 h at 0°C in calcium-free medium, PLA_2 lost the ability to increase metabotropic binding (Fig. 5A).

PLA_2 is an enzyme that is quite difficult to denature (Brockerhoff and Jensen, 1974). However, heat pretreatment of the enzyme (100°C for 30 min) blocked its ability to increase metabotropic binding (Fig. 5B).

Exposure of PLA_2-pretreated slices to 1% bovine serum albumin (BSA), which binds fatty acids (Goodman, 1958), prevented the activation of metabotropic binding normally induced by PLA_2 (Fig. 6A). The main product generated by PLA_2 activation is arachidonic acid. Thus, the sections were exposed to arachidonic acid (300 μM–1 m*M*) either in the presence or in the absence of the enzyme. Arachidonic acid failed to increase metabotropic binding but, when added in the presence of PLA_2, completely reversed the increment in binding (Fig. 6B), in accord with its ability to inhibit the enzyme (Dennis et al., 1989).

DISCUSSION

The present autoradiographic study indicates that PLA_2 pretreatment can selectively modify AMPA and metabotropic receptors without considerably affecting NMDA and kainate receptors. In fact, at similar concentrations of PLA_2 (0.05–0.09 U/ml), the effect on [^{3}H]AMPA binding and metabotropic binding was much higher than the effect on [^{3}H]kainate- and NMDA-sensitive [^{3}H]glutamate binding. A selective action of PLA_2 on AMPA receptors, but not on NMDA and kainate receptors, has been shown in telencephalic membranes (Massicotte et al., 1990). Our results are in accord with this previous report, because it is conceivable that such a modest increase in NMDA and kainate binding is detectable only with autoradiography.

The use of an autoradiographic technique allowed us to elucidate interesting differences in the regional regulation of AMPA receptors by PLA_2. Two [^{3}H]-

TABLE 2. *PLA_2 (bee venom) effect on metabotropic binding (p = 0.0001 by two-way ANOVA; magnitude = +45%) and NNKQ binding (p = 0.0001 by two-way ANOVA; magnitude = +42%)*

	Metabotropic binding		NNKQ binding	
Structures	Control	PLA_2 (0.5 U/ml)	Control	PLA_2 (0.5 U/ml)
DG	1.36 ± 0.02	1.85 ± 0.02[a]	0.98 ± 0.28	1.5 ± 0.16[a]
CA1 SR	1.34 ± 0.22	2.04 ± 0.04[a]	0.71 ± 0.19	1.03 ± 0.12
CA1 SO	1.38 ± 0.06	2.24 ± 0.17[a]	0.75 ± 0.18	1.09 ± 0.13
CA3 SR	1.32 ± 0.07	2.26 ± 0.1[a]	0.52 ± 0.15	0.64 ± 0.07
CA3 SO	1.34 ± 0.13	2.27 ± 0.19[a]	0.48 ± 0.15	0.61 ± 0.08
CB MOL	1.94 ± 0.08	3.39 ± 0.34[a]	0.25 ± 0.13	0.39 ± 0.09
CB GCL	0.84 ± 0.06	0.96 ± 0.23	0.16 ± 0.14	0.21 ± 0.05
STR	1.33 ± 0.2	2.00 ± 0.12[a]	0.94 ± 0.28	1.48 ± 0.10[a]

All values are expressed as pmol/mg of protein. Abbreviations as in the first footnote to Table 1. PLA_2 pretreatment carried out at 4 m*M* $CaCl_2$ (metabotropic binding) and 25 m*M* $CaCl_2$ (NNKQ binding). Results represent the mean ± SD for four animals in separate experiments for each assay.

[a] PLA_2 pretreatment in individual region is different from control [$p < 0.05$ by Tukey's (*a*) test].

TABLE 3. *PLA_2 (porcine pancreas) pretreatment minimally affects [^{3}H]kainate binding to kainate receptors (p = 0.001 by two-way ANOVA; magnitude = 9%) and [^{3}H]glutamate binding to NMDA receptors (p = 0.004 by two-way ANOVA; magnitude = 9%)*

	Kainate binding		NMDA binding	
Structure	Control	PLA_2 (0.08 U/ml)	Control	PLA_2 (0.09 U/ml)
DG	0.99 ± 0.1	1.04 ± 0.06	2.12 ± 0.11	2.33 ± 0.12
CA1 SR	0.44 ± 0.10	0.54 ± 0.05	2.20 ± 0.06	2.43 ± 0.24
CA1 SO	0.51 ± 0.03	0.60 ± 0.06	1.90 ± 0.20	2.14 ± 0.21
CA3 SL	2.21 ± 0.09	2.36 ± 0.13[a]	ND	ND
CB MOL	0.47 ± 0.05	0.49 ± 0.03	0.04 ± 0.03	0.07 ± 0.01
CB GCL	0.95 ± 0.04	1.02 ± 0.05	0.50 ± 0.04	0.39 ± 0.07
STR	1.02 ± 0.09	1.12 ± 0.06	1.25 ± 0.08	1.34 ± 0.18

All values are expressed as pmol/mg of protein. CA3 SL, stratum lucidum of the CA3 region of the hippocampus; ND, not determined. Other abbreviations as in the first footnote to Table 1. Results represent the mean ± SD for four animals in separate experiments for each assay.

[a] PLA_2 pretreatment in individual region is different from control [$p < 0.05$ by Tukey's (a) test].

AMPA binding sites, with a high and a low affinity, have been described in the presence of KSCN in rat forebrain membranes (Honoré and Drejer, 1988). In autoradiographic studies the existence of two different binding sites in the presence of KSCN has been confirmed repeatedly (Nielsen et al., 1988; Makowiec et al., 1991; Cha et al., 1992). In rat brain, a K_D of 14 nM and B_{max} of 1.9 pmol/mg of protein for the high-affinity site and a K_D of 1 μM and B_{max} of 21 pmol/mg of protein for the low-affinity site have been found in the molecular layer of the cerebellum (Cha et al., 1992). Similar results were obtained for the stratum radiatum of the hippocampus, where saturation data indicated a K_D of 31.3 nM and B_{max} of 6.1 pmol/mg of protein for the high-affinity site and a K_D of 2.6 μM and B_{max} of 69 pmol/mg of protein for the low-affinity site (unpublished observations). According to these values we chose extreme concentrations to estimate low- and high-affinity binding (2–5 and 500 nM). The percentage contribution of the high- and low-affinity components of [^{3}H]AMPA binding at equilibrium is about 90 and 10% at 2 nM and 80 and 20% at 5 nM but 20 and 80% at 500 nM. It has been suggested that these two sites represent different conformational states of the same receptor (Honoré and Drejer, 1988). No difference in the relative distribution of the two binding sites has been found previously. Surprisingly, at 500 nM [^{3}H]AMPA we observed lower levels of binding in the stratum pyramidale of the hippocampus compared to the stratum radiatum. A different pattern of binding was detected in the hippocampus at low concentrations of ligand (2–5 nM). The latter concentrations favor binding to the high-affinity site.

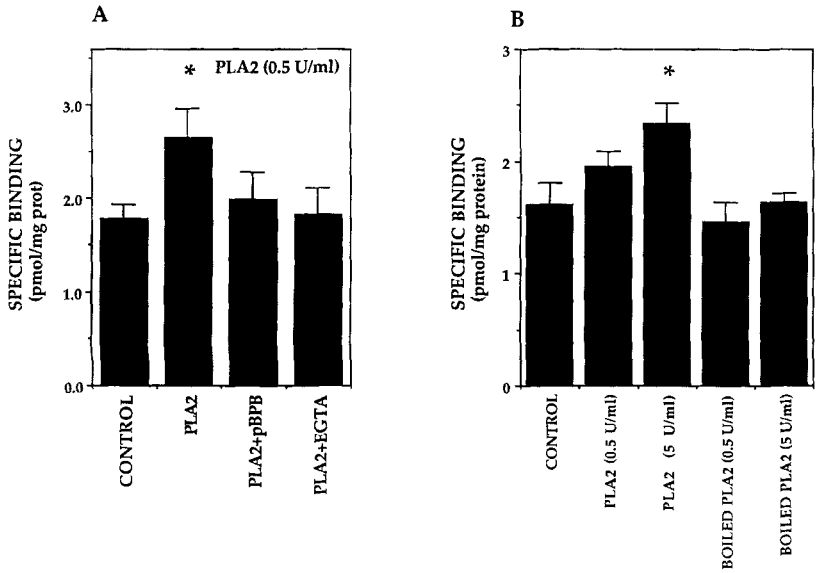

FIG. 5. A: pBPB (100 μM) and EGTA (10 mM) prevent PLA_2 (bee venom) enhancement of metabotropic binding in the cerebellar molecular layer. Enzymatic pretreatment was performed in 4 mM $CaCl_2$. Similar results were found in the other regions examined. Columns represent the mean ± SD values of four animals. $p < 0.01$ by one-way ANOVA; *$p < 0.05$ by post hoc Scheffé F-pairwise comparison. **B:** Heat treatment abolishes PLA_2 (bee venom) enhancement of metabotropic binding in the cerebellar molecular layer. PLA_2 pretreatment carried out at 25 mM $CaCl_2$. Similar results were found in the other regions examined. Columns represent the mean ± SD values of four animals. $p < 0.001$ by one-way ANOVA; *$p < 0.05$ by Scheffé F-post hoc pairwise comparison.

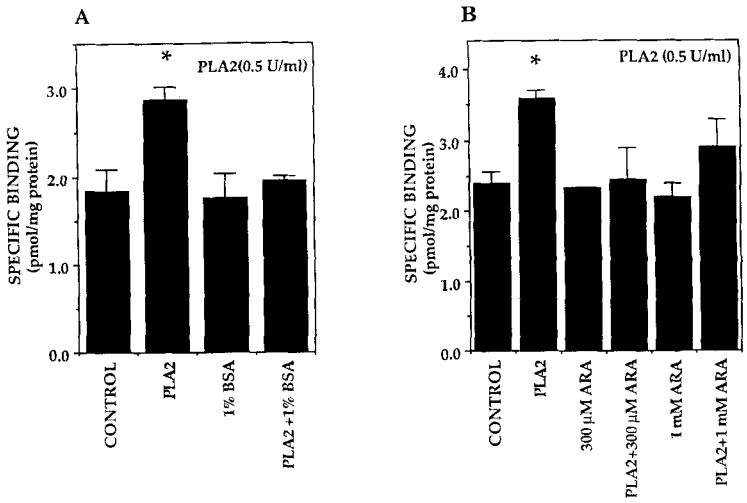

FIG. 6. A: BSA (1%) prevents PLA_2 (bee venom) enhancement of metabotropic binding in the cerebellar molecular layer. Similar results were found in the other regions examined. PLA_2 pretreatment carried out at 25 m*M* $CaCl_2$. Columns represent the mean ± SD values of four animals. $p < 0.001$ by one-way ANOVA; $^*p < 0.05$ by Scheffé *F*-post hoc pairwise comparison. **B:** Arachidonic acid (ARA) does not affect metabotropic binding in the cerebellar molecular layer but prevents PLA_2 enhancement of metabotropic binding. Similar results were found in the other regions examined. Enzymatic pretreatment was performed in 4 m*M* $CaCl_2$. Columns represent the mean ± SD values of four animals. $p < 0.001$ by one-way ANOVA; $^*p < 0.05$ by post hoc Scheffé *F*-pairwise comparison.

We observed that pretreatment with PLA_2 from porcine pancreas appeared to increase the low-affinity binding sites in the stratum radiatum without affecting the high-affinity binding sites. In addition, in the hippocampal pyramidal layer no significant effect of PLA_2 was observed on either high- or low-affinity binding sites. When the bee venom enzyme was used, an increase in the binding at 2 and 30 n*M* was observed, suggesting the possibility that both the high- and the low-affinity binding sites were affected by PLA_2. Furthermore, bee venom PLA_2 increased binding in the same regions affected by porcine PLA_2, indicating that the biochemical effect on the receptors was the same for both enzymes. The differences observed using porcine and bee venom PLA_2 may be because we used lower concentrations of porcine PLA_2. A differential sensitivity to the action of PLA_2 at different concentrations has been shown for different ligand binding sites on the γ-aminobutyric $acid_A$ ($GABA_A$) receptor complex (Havoundjian et al., 1986). It is possible that the high-affinity binding site is less sensitive to PLA_2, and at higher concentrations of PLA_2 both sites would be affected. The different pattern of distribution in the hippocampus and the differential response to PLA_2 suggest the possibility that the two sites represent separate receptors. On the basis of our results, however, we cannot exclude the possibility that low- and high-affinity AMPA binding sites represent two conformational states of the same protein whose molecular properties and interaction with the lipid environment change in the different layers of the hippocampus. The high levels of low-affinity binding sites in the stratum radiatum and in the stratum oriens of the hippocampus and the sensitivity to lower concentrations of PLA_2 support the hypothesis that the low-affinity binding sites represent the physiological receptor. Our suggestion is in accord with the report that, in the molecular layer of the cerebellum of mice lacking Purkinje cells, only the low-affinity binding sites are reduced (Makowiec et al., 1991).

PLA_2 pretreatment is accompanied by a marked increase in metabotropic binding. As the metabotropic specific binding is an indirect measure derived from [^{3}H]glutamate binding performed in the presence of NMDA (100 μM) and AMPA (10 μM), the increase in metabotropic binding that we observed in the presence of PLA_2 could potentially be ascribed to the increase in AMPA binding. However, no increase in metabotropic binding was detected at concentrations of PLA_2 from porcine pancreas (0.01 U/ml) that are partially active on [^{3}H]AMPA binding. In addition, a significant increase in metabotropic receptor binding was found in the striatum, where there is no modification of [^{3}H]AMPA binding. Metabotropic receptors appear to be less sensitive than AMPA receptors to the PLA_2 effect. In particular, metabotropic receptors localized in the cerebellum seem to be less sensitive to the membrane modification operated by the enzymatic treatment than metabotropic receptors localized in the striatum. Different subtypes of metabotropic receptors with distinct localizations in the brain have been discovered recently (Tanabe et al., 1992). The regional difference in receptor sensitivity might be explained by the existence of different receptor subtypes or by a differential chemical composition of neuronal membrane in discrete brain regions. In addition to metabotropic and AMPA receptors, the unique NNKQ [^{3}H]glutamate binding site, insensitive to NMDA (100 μM), kainate (1 μM), and quisqualate (2.5 μM), was also modulated by PLA_2. The nature of this binding site is obscure and its role in synaptic transmission is still unknown.

Exogenous PLA_2's from various sources have been used extensively to study how changes in the lipid environment affect the binding and functional properties of membrane-associated receptors (for a review see Loh and Law, 1980). In some of these studies

PLA_2 concentrations effective on binding were lower (0.5–10 mU/ml) than those used in our study (Barden and Labrie, 1973; Pasternak and Snyder, 1973; Azhar et al., 1976). In other studies, however, PLA_2 concentrations up to 1–5 U/ml were used (Limbird and Lefkowitz, 1975; Aronstam et al., 1977; Saltarelli et al., 1990). It is possible that intact sections, instead of homogenates, require higher concentrations of enzyme. Recently, however, a very strong decremental effect on binding has been shown for 5-hydroxytryptamine$_{1A}$ receptors with 0.008 U/ml PLA_2 in a quantitative autoradiographic study (Radja et al., 1992). In our study, the same concentrations produced a modest, although not significant, increase in AMPA binding and did not affect metabotropic binding. In addition, concentrations of PLA_2 from porcine pancreas and bee venom active on AMPA binding performed in homogenates are consistent with the concentrations used in our study (Massicotte and Baudry, 1990; Baudry et al., 1991). Thus, more than different binding techniques, an intrinsic resistance of EAA receptor binding properties to modifications of the membrane lipids might account for this discrepancy. It has been demonstrated that the chloride channel of the $GABA_A$ receptor is much more sensitive to the PLA_2 effect than the binding to the constituent recognition sites (Havounjian et al., 1986). Thus, on the basis of our binding study, we cannot exclude the possibility that EAA receptor function might be affected by lower concentrations of PLA_2.

Calcium is an essential cofactor for the catalytic activity of PLA_2. The ion is bound to the porcine pancreatic enzyme—the best characterized among extracellular PLA_2's from various sources—with an affinity constant of about 4 m*M* at pH 7–8 (De Haas et al., 1971). $CaCl_2$ concentrations ranging from 10 to 100 m*M* are generally used in enzymatic assays (Nieuwenhuizen et al., 1974; De Geus et al., 1987; Bekkers et al., 1991). Therefore, a $CaCl_2$ concentration of 25 m*M* was chosen to obtain the full activity of the porcine enzyme on membrane phospholipids, after demonstration that such a high $CaCl_2$ concentration did not enhance binding to metabotropic and AMPA receptors per se. When PLA_2 from bee venom was used, however, some experiments were performed also at a lower $CaCl_2$ concentration (4 m*M*). Variation of the $CaCl_2$ concentration did not affect the action of PLA_2 from bee venom (data not shown) and allowed us to use reasonable concentrations of EGTA to block enzymatic activity.

It is interesting to note that different prewash conditions have a significant opposite effect on AMPA and NMDA binding. It is possible that the increase in NMDA binding is due to the more effective removal of endogenous glutamate at 37 than at 4°C. Explaining the effects of different prewash conditions on AMPA binding is more difficult. The decrease we observed could be ascribed to the removal of an endogenous inhibitor or to a modification of the structural properties of the AMPA receptor.

The effect of PLA_2 is probably due to its enzymatic activity because the addition of EGTA at concentrations that chelate calcium in the incubation buffer blocked the effect of PLA_2 on binding. In addition, heat- and pBPB-treated PLA_2's were ineffective.

The effect of PLA_2 might be due to the action of free fatty acids that are released from the membrane phospholipids. To evaluate such a possibility, we pretreated the sections with PLA_2 in the presence of 1% BSA (Goodman, 1958). The removal of free fatty acids by BSA prevented the increase in metabotropic binding, suggesting the involvement of free fatty acids and/or their metabolites in the action of PLA_2.

Degradation of membrane-bound phospholipids and production of free fatty acids occur rapidly in the brain after decapitation (Bazán et al., 1971; Rehncrona et al., 1982). It is possible that the differential effect of PLA_2 on binding could be ascribed to a limited additional phospholipid breakdown by exogenously adding PLA_2 in some regions and not in others. If this were the case, however, we would expect an effect always in the same regions, despite the ligand tested. In contrast, we observed a constant increase in metabotropic binding in the striatum, where no increase in AMPA binding was ever found.

Arachidonic acid, the main product of PLA_2 enzymatic activity, failed to mimic PLA_2 action and reversed the enzyme effect. This result is in accord with the inhibiting action of arachidonic acid on PLA_2 activity (Dennis et al., 1989) and suggests that arachidonic acid and, presumably, its metabolites are not involved in the PLA_2 effect. Because we could not wash the slices extensively after PLA_2 pretreatment without affecting the integrity of the tissue, we cannot exclude the possibility that the effect of PLA_2 was due to the formation of phospholipid metabolites different from those derived from the arachidonic cascade. Lysophospholipids, produced by cleavage of the membrane phospholipids by PLA_2, could be the agents responsible for the PLA_2 effect on binding for their surface-active properties. Oliveira et al. (1984) reported that exogeneous added lysophosphatidylcholine mimics the PLA_2 effect on dopamine receptor binding in caudate nucleus microsomal membranes. Those authors found that albumin prevented the effect of PLA_2 only when it was included in the incubation medium, and not when it was included in the washing buffer. This is consistent with an action by lysophosphatides, which are known to be sequestered by albumin but not easily released from the membrane (Loh and Law, 1980). In analogy with this previous study, our results suggest that lysophosphatides could mediate PLA_2 action on EAA binding.

Taken together, our results indicate that PLA_2 modulates the binding of AMPA and metabotropic receptors through a modification of the lipid environment

that surrounds the receptors. It is possible that the increased binding obtained following enzyme treatment in some regions but not in others is due to a differential interaction of the receptors with the membrane environment. This could depend on the differential lipidic composition in different regions of the brain as well as in different parts of the neuron, but also on the heterogeneity of EAA receptor subtypes as demonstrated at the mRNA level.

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