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N-Methyl-D-aspartate and Quisqualate/DL-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptors: Differential Regulation by Phospholipase C Treatment

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SUMMARY

The effect of phospholipase C (PLC) treatment of rat brain membranes on the binding properties of excitatory amino acid receptors was investigated using both a phosphatidylcholine-hydrolyzing PLC from *Clostridium perfringens* and a phosphatidylinositol-specific PLC from *Bacillus thuringiensis*. PLC from *C. perfringens* produced an increased affinity of the quisqualate/DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor for its ligand, whereas kainate receptor binding was not affected. Both kinetic analysis and equilibrium saturation experiments indicated that PLC treatment produced a decrease in affinity for [^{3}H]*N*-(1-[thienyl]cyclohexyl)-piperidine ([^{3}H]TCP), a ligand for the *N*-methyl-D-aspartate (NMDA) receptor-associated ionic channel, when the channel was fully activated by high concentrations of glutamate and glycine but increased its binding under conditions in which the channel was presumably closed. This latter component of the binding was not due to an interaction of [^{3}H]TCP with non-glutamate receptor sites, such as σ opioid and histamine H_3 receptors. Binding of [^{3}H]glutamate and [^{3}H]glycine to the NMDA receptors was not modified by PLC treatment, but there was a large decrease in the binding of the NMDA antagonist [^{3}H]3-[(±)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid. Stimulation by glycine of [^{3}H]glutamate binding was also abolished following PLC treatment. In contrast to PLC from *C. perfringens*, phosphatidylinositol-specific PLC treatment did not detectably modify the binding properties of the quisqualate/AMPA receptor or the NMDA receptor channel. These data indicate that alterations in the lipid microenvironment of the glutamate receptors modulate both the conformation and the function of the receptors and suggest a possible role for phospholipases in the regulation of synaptic transmission at excitatory synapses.

Considerable progress has been made in characterizing the receptors that mediate the effects of the excitatory neurotransmitter glutamate. At least three types of receptors have been defined as a result of biochemical, pharmacological, and electrophysiological studies and named according to preferential agonists, i.e., kainate, quisqualate, and NMDA (1, 2). The recognition site of each subtype can be investigated using appropriate ligands such as [^{3}H]kainate, [^{3}H]AMPA, and [^{3}H]glutamate (1, 3–6). The NMDA receptor has received considerable attention because it appears to play a critical role in developmental plasticity, hippocampal long term potentiation, epilepsy, and neurodegenerative diseases as well as in various forms of brain damage (for review see Ref. 7). The activation of the NMDA receptor by an agonist leads to the opening of a cation conductance channel, which is blocked in a voltage-dependent manner by magnesium (8, 9). NMDA-evoked currents are blocked noncompetitively by dissociative anesthetics such as phencyclidine and ketamine (10, 11), whereas a strychnine-insensitive glycine site allosterically modulates the opening probability of the NMDA-gated channel (12). Current models of the NMDA receptor channel/complex suggest that [^{3}H]TCP, a phencyclidine analogue, binds inside the channel and that glutamate and glycine regulate the kinetics of [^{3}H]TCP binding by controlling the access of this ligand to the channel binding site (13, 14).

Activation of membrane-associated phospholipases represents an important component of the effects of both neurotransmitters and hormones (15). By cleaving membrane phospholipids, these enzymes not only modify the lipid microenvironment of cell membranes but also generate a number of metabolites that can play a second messenger function inside or outside the cell (16). Treatment of membranes with exoge-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, [(±)-2-carboxy-piperazine-4-yl]propyl-1-phosphonic acid; AP5, 2-amino-5-phosphonopentanoic acid; TCP, *N*-(1-[thienyl]cyclohexyl)piperidine; AMPA, DL-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; PLC, phospholipase C; Plase, phosphatidylinositol-specific phospholipase C; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

nous phospholipases has also been reported to alter the characteristics of the binding sites for various neurotransmitters or neuromodulators, such as norepinephrine (17, 18), γ-aminobutyric acid (19), and the opioid peptides (20, 21). In a number of cases it was shown that the nonpolar or polar moieties of membrane phospholipids, as well as the lysophospholipids and fatty acids generated by phospholipase treatment, were responsible for the changes in receptor binding. In other cases it appears more likely that changes in the lipid microenvironment of the receptor produce alterations in the conformation of the receptor, leading to changes in receptor binding (see Ref. 15).

Recent reports have suggested the existence of links between excitatory amino acid receptors and phospholipid metabolites generated by either PLC or phospholipase A_2 (22–24). Therefore, the present study investigated the effect of treatments with exogenous PLC on the binding characteristics of different subclasses of glutamate receptors in rat brain membrane preparations. Our results indicate that PLC treatment increases the affinity of agonist binding to the quisqualate site without changing agonist binding to the kainate and NMDA receptors or to the glycine site associated with the NMDA receptor. PLC also had marked effects on TCP binding to a site associated with the NMDA receptor channel, on the interaction between the glycine and NMDA receptors, and on the binding of an antagonist to the NMDA receptor. We conclude that the lipid microenvironment of the membrane influences the quisqualate receptor and the relationships between the various components of the NMDA receptor complex.

Materials and Methods

Membrane preparation and PLC treatment. Male Sprague-Dawley rats were sacrificed by decapitation and the telencephalon was rapidly dissected and homogenized in 10 ml of 0.32 M sucrose, 1 mM EGTA/Tris, pH 7.4, using a glass/Teflon homogenizer. Membranes were prepared as previously described (25, 26), using differential centrifugation and osmotic lysis. The final pellet was resuspended in Tris/acetate buffer (100 mM Tris/acetate, pH 7.4, 50 μM EGTA) at approximately 2 mg of protein/ml and the membrane preparation was incubated with 0.2% Triton X-100 at 0° for 25 min. Detergent treatment was performed in order to eliminate the Cl-dependent glutamate "binding sites", which have been shown to represent uptake into resealed vesicles (26, 27). The PLC effect reported in the present study was not modified by this detergent treatment (data not shown). Membranes were washed free of the detergent by four additional centrifugation steps and were resuspended at a final concentration of about 1.0 mg of protein/ml in Tris/acetate or Tris/HEPES buffer (100 mM Tris/HEPES, pH 7.4, 50 μM EGTA), as indicated in the figure legends. Membrane aliquots were frozen at −70° and resuspended by sonication on the day of the experiment.

Membrane suspensions (1.0 ml) were incubated with 2.0 mM $CaCl_2$ in the absence or presence of the indicated concentrations of PLC (*Clostridium perfringens*) for 30 min at 37°. As described previously, the hydrolysis of membrane phosphatidylinositol by a specific PIase (*Bacillus thuringiensis*) was performed in the absence of Ca^{2+} (21). The membranes were then diluted with 10 ml of the appropriate ice-cold Tris buffer, centrifuged, and resuspended in 1.0 ml of the same buffer. The proteolytic activity of PLC was determined using [^{14}C]casein as a substrate (28). Although the PLC preparation (*C. perfringens*) was found to contain a significant level of proteolytic activity, it was mostly Ca^{2+} independent. Because the PLC effects that are described below are calcium dependent, it is unlikely that the effects are due to the proteolytic activity of the PLC preparation.

Binding assays. Binding assays were performed in a final volume of 50–70 μl (approximately 30–40 μg of protein/assay) in Tris/acetate buffer containing the indicated concentrations of [^{3}H]glutamate, [^{3}H]CPP, [^{3}H]kainate, [^{3}H]AMPA, and [^{3}H]glycine. Incubations were performed at 0° for 30–50 min and were terminated by centrifugation of the samples at 48,000 × *g* for 15 min. The pellets were superficially washed with 200 μl of Tris/acetate buffer and were resuspended in 0.1 N NaOH; radioactivity was determined by liquid scintillation counting. In some experiments, [^{3}H]glutamate binding assays were terminated by filtration through GF/C filters; the samples were diluted with 3.0 ml of ice-cold buffer (100 mM Tris/acetate) and filtered as rapidly as possible. The filters were rinsed once with buffer. For [^{3}H]AMPA binding, 100 mM KSCN (potassium thiocyanate) was added to the assay buffer and washing solution. About 80% of [^{3}H]glutamate binding represents binding to the NMDA receptors, with the remaining sites being of the kainate type (6, 26); therefore, 5 μM kainate was included in the assay buffer to eliminate the kainate binding component.

Binding to the phencyclidine site was measured by incubating membranes (40 μg of protein/assay) with Tris/HEPES buffer, at 24° for 50 min, in the presence of 20 nM [^{3}H]TCP (or the indicated concentrations) and in the presence of 10 μM glutamate and 10 μM glycine. The assay was terminated by filtration through GF/C filters; the samples were diluted with 3.0 ml of stop solution (Tris/acetate buffer containing 500 mM KCl) and the filters were washed twice with 3.0 ml of stop solution. Binding measured in the presence of 200 μM ketamine was defined as the nonspecific binding. Further details concerning [^{3}H]TCP binding are described in the figure legends.

The binding data were analyzed using an analytical binding program (29). Protein content was determined according to the method of Bradford (30), with bovine serum albumin as the standard.

Chemicals. [^{3}H]Glutamate (specific activity, 50 Ci/mmol), [^{3}H]CPP (specific activity, 30 Ci/mmol), [^{3}H]glycine (specific activity, 40 Ci/mmol), [^{3}H]kainate (specific activity, 60 Ci/mmol), [^{3}H]AMPA (specific activity, 27 Ci/mmol), and [^{3}H]TCP (specific activity, 60 Ci/mmol) were obtained from NEN (Claremont, CA). PLC from *C. perfringens* (72 units/mg of protein; EC 3.1.4.3) was purchased from Sigma (St. Louis, MO) and PIase from *B. thuringiensis* (100 units/mg of protein; EC 3.1.4.10) from ICN (Cleveland, OH). All other drugs and reagents were obtained from commercially available sources.

Results

Effects of PLC on the characteristics of [^{3}H]TCP binding to the NMDA receptor. Pretreatment with PLC decreased specific [^{3}H]TCP binding to rat telencephalic membranes when the ionic channel associated with the NMDA receptor was maintained in an optimally open state by saturating concentrations of glutamate and glycine. The reduction reached a plateau at 10–20 μg of PLC/mg of protein (Fig. 1); at this PLC concentration [^{3}H]TCP binding was inhibited by about 40%. No protein solubilization was observed in PLC-treated samples. Scatchard analysis of the saturation curves showed that the reduction was due to a decrease in the binding affinity of the TCP binding sites after PLC treatment (Fig. 2; Table 1). This was further confirmed by kinetic analysis. Fig. 3 shows the time course for the association and dissociation of [^{3}H]TCP in the presence of glutamate and glycine. Equilibration was, in both cases, essentially complete after 30–50 min. Comparing control and PLC-treated membranes indicated that the phospholipase treatment decreased the [^{3}H]TCP association rate and clearly accelerated the dissociation, particularly at early time points (Fig. 3). To compare the effects of PLC treatment on the apparent association and dissociation rate constants, the initial portions of the dissociation curves were analyzed using a nonlinear curve-fitting program (29). The determination of the values for the rate constants k_{on} and k_{off} confirmed that the initial rate of dissociation was substantially

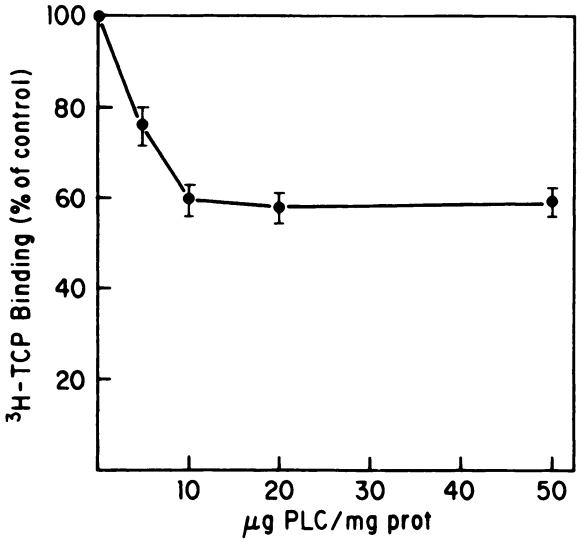

Fig. 1. Effects of various concentrations of PLC on specific [^{3}H]TCP binding to rat brain membranes. Membranes were preincubated with PLC from *C. perfringens* in the presence of 2.0 mM $CaCl_2$ for 30 min at 37°. After centrifugation, membranes were resuspended in Tris/HEPES buffer and incubated with [^{3}H]TCP (20 nM) in the presence of 10 μM glutamate and 10 μM glycine for 50 min at 24°. Data are expressed as percentage of specific binding measured in control membranes and are mean ± standard error of three experiments.

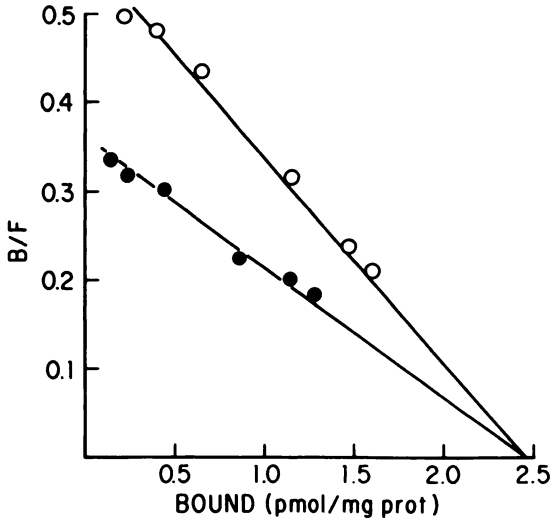

Fig. 2. Effect of PLC treatment on the characteristics of [^{3}H]TCP binding. Binding of increasing concentrations (5.0 to 150 nM) of [^{3}H]TCP to control and PLC-treated membranes (20 μg of PLC/mg of protein, 30 min. at 37°) was determined at 24° for 50 min in the presence of 10 μM glutamate and 10 μM glycine, as described in Materials and Methods. Data were calculated as pmol/mg of protein and are plotted as a Scatchard plot (B/F; pmol/mg of protein · pmol); the experiment was replicated three times with similar results. ●, Control membranes; ○, PLC-treated membranes.

increased (from 0.18 to 0.44 min^{-1}) and revealed further that the association rate constant was decreased after PLC treatment from 22 to 10 $\mu M^{-1} \cdot min^{-1}$ (Table 1). The value of the apparent dissociation constant (K_d) calculated from the ratio k_{off}/k_{on} was reduced in PLC-treated membranes, in agreement with the results of the Scatchard analysis; the difference in the magnitude of the change in apparent dissociation constant obtained by these two methods is likely due to the uncertainty in the determination of the rate constants, because dissociation

TABLE 1

Effect of PLC treatment on the kinetic and equilibrium constants for [^{3}H]TCP binding

The association (k_{on}) and dissociation (k_{off}) rate constants were calculated from the data presented in Fig. 3 and the kinetical K_d was determined by the ratio k_{off}/k_{on}. k_{on} was calculated according to published procedures (14, 29) from the following equation: $k_{on} = (k_{obs} - k_{off})/L$, where k_{obs} is the observed (experimental) association rate constant, k_{off} the dissociation rate constant of the fast dissociating component, and L the radioligand concentration (20 nM) used in the present experiments. However, because the dissociation kinetics are complex, these values should be viewed as approximate. Equilibrium constants (K_d) were calculated from the Scatchard plots presented in Fig. 2. Results are means ± standard errors of four to six experiments.

Binding constants	Control	PLC
k_{obs} (min^{-1})	0.61 ± 0.09	0.55 ± 0.07
k_{on} ($\mu M^{-1} \cdot min^{-1}$)	22.0 ± 1.5	10.0 ± 2.0*
k_{off} (min^{-1})	0.18 ± 0.02	0.44 ± 0.06*
K_d (kinetic) (nM)	8.0 ± 0.6	53.0 ± 2.0*
K_d (equilibrium) (nM)	53.0 ± 2.0	86.0 ± 4.0*

*$p < 0.05$ (Student's t test); PLC-treated versus control membranes.

data clearly show nonlinearity. The existence of two components with fast and slow dissociation rates has recently been described and it has been proposed that they correspond to the open and close states of the receptor/channel complex (31). Using a nonlinear curve-fitting program (29), we determined the percentage of binding sites exhibiting the two dissociation rates. Analysis of either association or dissociation data indicated that about 70% of the binding measured at 20 nM [^{3}H]TCP corresponded to the fast dissociating form of the receptor in control membranes; this value was not different following PLC treatment. Dissociation curve analysis also showed that the rate of dissociation of the slow component was accelerated from 0.15 min^{-1} in control to 0.30 min^{-1} in PLC-treated membranes.

We also measured [^{3}H]TCP binding to the NMDA receptor/channel complex under conditions of impaired access to its binding site. When glutamate and glycine are replaced by high concentrations of the glutamate antagonist AP5 (in the incubation medium), the rates of association and dissociation of [^{3}H]TCP to its binding sites are in general reduced to very small values and [^{3}H]TCP binding under these conditions has been assumed to represent binding to the closed state of the NMDA receptor channel (13, 14, 32). Interestingly, [^{3}H]TCP binding measured for 50 min in the presence of 100 μM AP5 was considerably increased in PLC-treated membranes (Table 2). When expressed in terms of percentage of total [^{3}H]TCP binding, the AP5-insensitive [^{3}H]TCP binding increased from 8% in control membranes to 40% following PLC treatment. The time course for the association of [^{3}H]TCP was evaluated in the presence of the glutamate antagonist AP5 in both control and PLC-treated membranes (Fig. 4). The association rate of [^{3}H]TCP to the PLC-treated membranes was clearly accelerated and the equilibrium was reached after about 8 hr. In control membranes, [^{3}H]TCP binding in the presence of AP5 did not reach equilibrium even after 24 hr of incubation, thus preventing the determination of kinetic constants at equilibrium. Nevertheless, binding of increasing concentrations of [^{3}H]TCP was measured in PLC-treated membranes for 15 hr in the presence of AP5 (20 μM) in order to determine whether the increased binding observed in the presence of AP5 was due to a change in apparent affinity. Scatchard analysis of the saturation curves indicated that the increase in [^{3}H]TCP binding observed following PLC treatment was not due to an increased affinity of the [^{3}H]TCP binding site. When compared

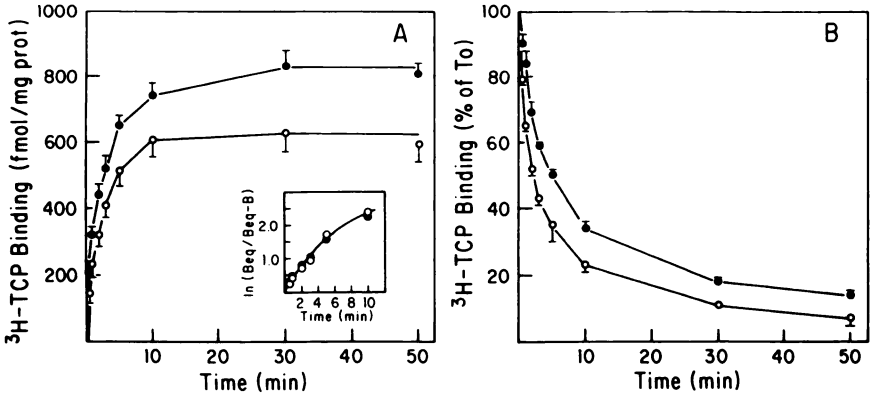

Fig. 3. Effect of PLC treatment on the time courses of association and dissociation of [^{3}H]TCP binding. A, The association of [^{3}H]TCP to control and PLC-treated (20 μg of PLC/mg of protein, 30 min. at 37°) membranes was measured by incubating 20 nM [^{3}H]TCP with membranes for the indicated periods of time, in the presence of 10 μM glutamate and 10 μM glycine. Incubations were terminated by rapid filtration through glass fiber filters, as described in Materials and Methods. Data are expressed in fmol/mg of protein and are mean ± standard error of five experiments. *Inset*, ln ($B_{eq}/B_{eq}-B$) versus time, where B_{eq} is the concentration of the ligand at equilibrium and B the concentration of ligand bound at time t. B, In dissociation experiments, the equilibrium binding was first established by incubating membranes with [^{3}H]TCP for 50 min at 24°. Aliquots of 50 μl were then diluted in 3.0 ml of Tris/HEPES buffer (at 24°) and the specific binding was measured as described in Materials and Methods. Data are expressed as percentage of the binding measured at the end of the equilibrium period and are mean ± standard error of five experiments. ●, Control membranes; ○, PLC-treated membranes.

TABLE 2

Effect of PLC treatment on the AP5-insensitive [^{3}H]TCP binding

Membranes were preincubated with PLC (20 μg/mg of protein 30 min at 37°) and washed by centrifugation. Following resuspension in Tris/HEPES buffer, specific [^{3}H]TCP binding was measured in the presence of 10 μM glutamate and 10 μM glycine or in the presence of 100 μM AP5. Results are expressed in fmol/mg of protein and are means ± standard errors of four experiments. The numbers in parentheses represent the fraction of the total binding that is AP5 insensitive.

Additions to binding assay	[^{3}H]TCP binding	
	Control	PLC
	fmol/mg of protein	
Glutamate/glycine, 10 μM	556 ± 86	381 ± 52*
AP5, 100 μM	42 ± 3 (8%)	154 ± 7* (40%)

*$p < 0.05$ (Student's t test); PLC-treated versus control membranes.

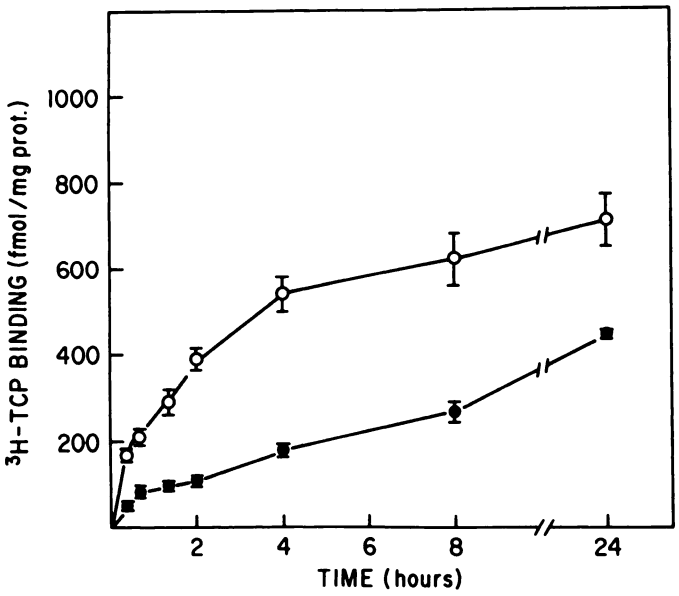

Fig. 4. Effect of PLC treatment on the time course of association of [^{3}H]TCP binding in the presence of AP5. The association of [^{3}H]TCP to control (●) and PLC-treated (○) (20 μg of PLC/mg of protein, 30 min at 37°) membranes was measured by incubating 20 nM [^{3}H]TCP with membranes for the indicated periods of time, in the presence of 20 μM AP5. Incubations were terminated by rapid filtration through glass fiber filters, as described in Materials and Methods. Data are expressed in fmol/mg of protein and are mean ± standard error of four experiments.

with the fully activated state of the NMDA channel in PLC-treated membranes (K_d = 86 ± 4.0 nM; B_{max} = 2.40 ± 0.20 pmol/mg of protein; see Table 1), the affinity determined in the presence of AP5 was slightly decreased (K_d = 135 ± 5.0 nM; B_{max} = 2.30 ± 0.10 pmol/mg of protein).

Because [^{3}H]TCP has been reported to interact also with binding sites on σ-opioid (33) and histamine H_3 (34) receptors, it was conceivable that the increase in binding measured in the presence of AP5 could be ascribed to the appearance of nonglutamate receptor sites. Two observations make this possibility unlikely. Neither 20 μM haloperidol, a compound that preferentially binds to the σ-opioid binding site (33), nor 1 mM histamine affected [^{3}H]TCP binding in the presence of AP5 (data not shown). Furthermore, the dissociation of [^{3}H]TCP that had been allowed to bind in the presence of AP5 was accelerated by glutamate (Fig. 5), indicating that this binding component most likely reflects binding to the NMDA receptor. We also tested the possibility that the NMDA receptors had a reduced affinity for AP5 after PLC treatment. The IC_{50} of AP5 for inhibiting [^{3}H]glutamate binding was not significantly changed following PLC treatment (Fig. 6).

Effects of PLC treatment on other excitatory amino acid receptor binding sites. The binding of different glutamate receptor-related ligands was determined in membranes treated with PLC (Fig. 7). In the presence of micromolar concentrations of kainate, more than 90% of [^{3}H]glutamate binding represents binding to NMDA receptors (6, 25). PLC treatment did not modify the binding of [^{3}H]glutamate under these conditions nor did it modify [^{3}H]glycine binding to the glycine site associated with the NMDA receptor or the binding of [^{3}H]kainate to the kainate binding site. In contrast, PLC treatment produced a marked reduction (by about 65%) of [^{3}H]CPP binding; this effect was due to a decreased affinity of [^{3}H]CPP for its binding site (K_d = 400 ± 50 nM and B_{max} = 4.10 ± 0.20 pmol/mg of protein in control membranes; K_d = 1240 ± 210 nM and B_{max} = 5.70 ± 0.20 pmol/mg of protein in PLC-treated membranes). Interestingly, the stimulation of [^{3}H]glutamate binding by D-serine (10 μM), a glycine site agonist (32),

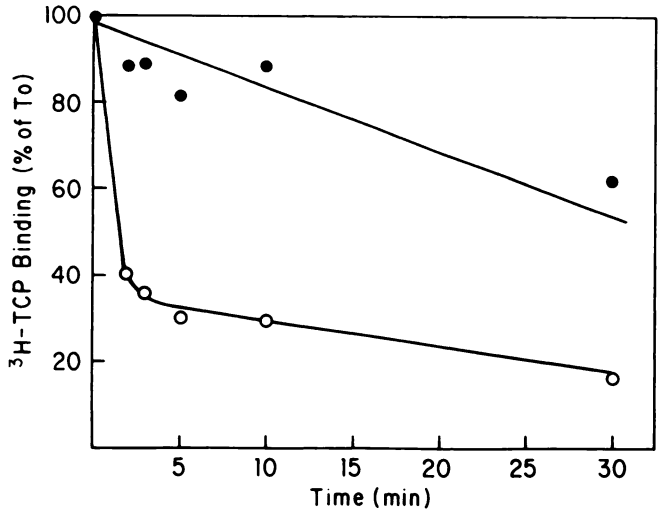

Fig. 5. Effect of PLC treatment on the rate of dissociation of the AP5-insensitive [^{3}H]TCP binding. PLC-treated membranes (20 μg of PLC/mg of protein, 30 min at 37°) were incubated for 50 min at 24° in presence of [^{3}H]TCP (20 nM) and AP5 (100 μM). Aliquots of 50 μl were then diluted in 3.0 ml of Tris/HEPES buffer (at 24°) in the absence (●) or presence (○) of glutamate (10 μM) and glycine (10 μM). The specific binding was measured at the indicated times, as described in Materials and Methods. Results are expressed as the percentage of the binding measured at the end of the equilibration period and are the mean of two experiments.

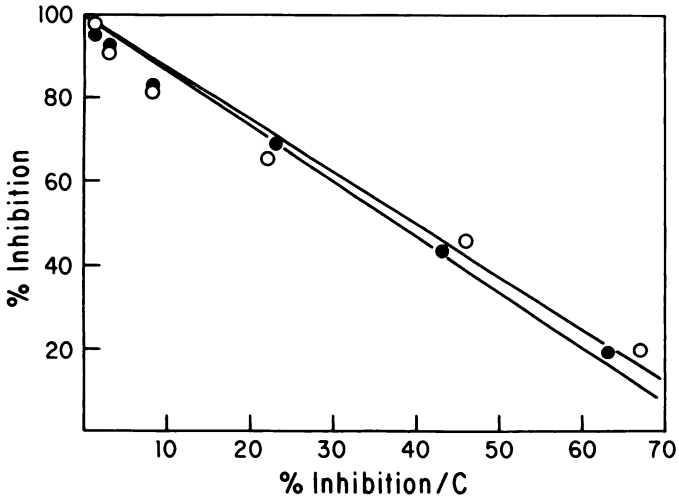

Fig. 6. Effect of PLC treatment on the inhibition by AP5 of [^{3}H]glutamate binding to the NMDA receptor. Rat brain membranes (1.0 mg of protein were pretreated with 20 μg of PLC at 37° for 30 min. After centrifugation, the membranes were resuspended in 1.0 ml of Tris/acetate buffer and the inhibition of [^{3}H]glutamate binding (50 nM, 0°, 30 min), by increasing concentrations of AP5, was determined, as described in Materials and Methods. The data are presented in a Scatchard-type inhibition plot, with *C* representing the AP5 concentration. The slopes of the regression lines represent the IC_{50} values [control (●) = 0.80 μM; PLC-treated (○) = 0.87 μM]. Results are mean ± standard error of three experiments.

was totally abolished after PLC treatment (−0.5 ± 0.3%), when compared with the untreated membranes (14 ± 2.0%).

[^{3}H]AMPA binding was increased in PLC-treated membranes (Fig. 7). In the detergent-treated membranes used for these experiments, analysis of the saturation kinetics provided a better fit of the data with a two-site model (see Ref. 35). However, after control and PLC-treated membranes were exposed to calcium, analytical discrimination of the two binding components was no longer possible, probably due to an increase

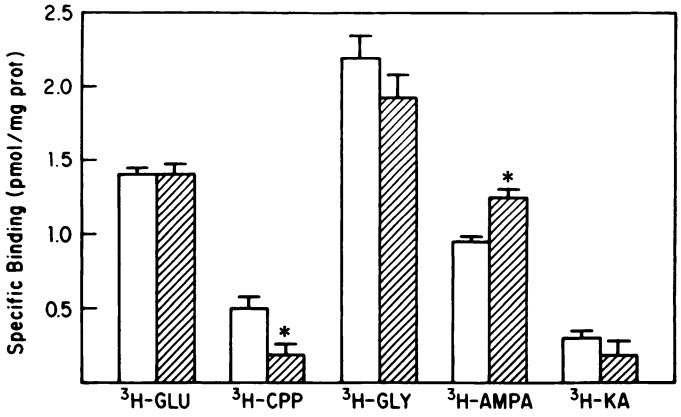

Fig. 7. Effect of PLC treatment on the binding of different glutamate receptor-related ligands. Membranes (1.0 mg of protein) were pretreated with 20 μg of PLC at 37° for 30 min. After centrifugation, the membranes were resuspended in 1.0 ml of Tris/acetate buffer and the binding of different ligands was determined as described in Materials and Methods. *GLU*, [^{3}H]glutamate (50 nM); *CPP*, [^{3}H]CPP (50 nM); *GLY*, [^{3}H]glycine (100 nM); *AMPA*, [^{3}H]AMPA (50 nM); *KA*, [^{3}H]kainate (20 nM). Results are mean ± standard error of three or four experiments. Only the [^{3}H]CPP and [^{3}H]AMPA binding were statistically different after PLC treatment (*$p < 0.05$; Student's *t* test).

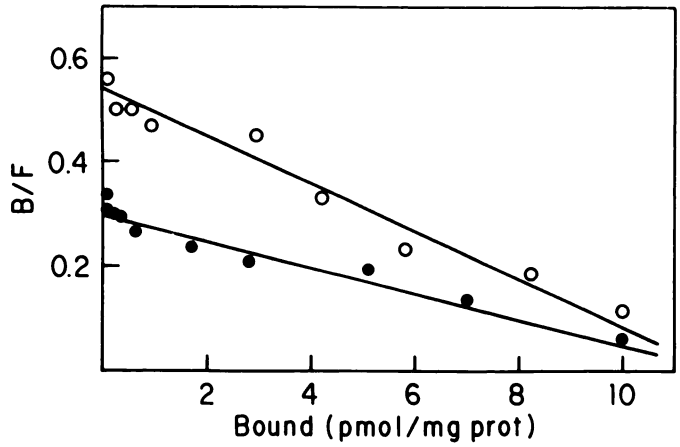

Fig. 8. Effect of PLC treatment on the characteristics of [^{3}H]AMPA binding. Binding of increasing concentrations (0.01 to 4.0 μM) of [^{3}H]AMPA to control (●) and PLC-treated (○) (20 μg of PLC/mg of protein, 30 min at 37°) membranes was determined at 0° for 40 min, as described in Materials and Methods. Data were calculated as pmol/mg of protein and are plotted as a Scatchard plot (B/F; pmol/mg of protein · pmol); the experiment was replicated twice with similar results (see Table 3).

in the affinity of the low affinity binding sites. The single-site analysis shown in Fig. 8 (see Table 3) indicated that the primary effect of PLC was to increase the affinity of the low affinity component, which accounts for more than 90% of the [^{3}H]AMPA binding sites (35).

Effects of a PIase on different glutamate receptor binding sites. [^{3}H]TCP and [^{3}H]AMPA binding under the conditions described above were repeated with membranes treated with a phospholipase C from *B. thuringiensis* that selectively cleaves inositol-phospholipids (Table 4). In marked contrast to the effects obtained with the less specific PLC from *C. perfringens*, [^{3}H]TCP binding to the NMDA receptor/channel was unchanged after PIase treatment. This was the case for both states of the channel, i.e., fully activated (in the presence of 10 μM glutamate and 10 μM glycine) and optimally closed (in the presence of 100 μM AP5). [^{3}H]AMPA binding

TABLE 3

Effect of PLC treatment on the characteristics of [^{3}H]AMPA binding

Rat brain membranes were preincubated with PLC (20 μg/mg of protein, 30 min at 37°) and washed by centrifugation. Following resuspension in Tris/acetate buffer, the binding of increasing concentrations of [^{3}H]AMPA was determined as described in Materials and Methods. Data from Fig. 7 were analyzed by the LIGAND program to generate K_d and B_{max}. Results are means ± standard errors of three experiments.

Conditions	K_d	B_{max}
	nM	*pmol/mg of protein*
Control	830 ± 60	15.0 ± 0.3
PLC	346 ± 37[a]	12.0 ± 0.3

[a] $p < 0.05$ (Student's t test); PLC-treated versus control membranes.

TABLE 4

[^{3}H]TCP and [^{3}H]AMPA binding following PIase treatment

Rat brain membranes were incubated in the presence and absence of PIase (500 mU/ml, 30 min at 37°) and washed by centrifugation. Following resuspension in Tris/HEPES buffer, specific [^{3}H]TCP (30 nM) binding was measured in the presence of 10 μM glutamate and 10 μM glycine or in the presence of 100 μM AP5. Membranes were also resuspended in Tris/acetate buffer (100 mM KSCN) and [^{3}H]AMPA binding was determined. The numbers in parentheses represent the fraction of the total [^{3}H]TCP binding that is AP5 insensitive.

Ligand/conditions	Binding	
	Control	PIase
	fmol/mg of protein	
[^{3}H]TCP (Glutamate/Glycine, 10 μM)	1025 ± 37	1064 ± 17
[^{3}H]TCP (AP5, 100 μM)	100 ± 17 (10%)	101 ± 10 (10%)
[^{3}H]AMPA	971 ± 14	877 ± 50

was slightly but not significantly decreased following PIase treatment.

Discussion

The present results indicate that treatment of rat synaptic membranes with PLC (from *C. perfringens*) produces a complex set of modifications of the different subtypes of glutamate receptors. Whereas the kainate binding sites do not appear to be affected, the quisqualate/AMPA receptors exhibit an increased affinity for the ligand. The binding of [^{3}H]glutamate and [^{3}H]glycine to the NMDA receptor is not modified but there is a large decrease in the binding of the NMDA antagonist [^{3}H]CPP. Finally, there is a decreased affinity of [^{3}H]TCP for the open state of the channel associated with the NMDA receptor but also an increased accessibility of TCP to the channel when it is closed.

It should be noted that treatment of membranes with a PLC from *B. thuringiensis*, which has a narrower specificity inasmuch as it cleaves mainly phosphatidylinositol rather than a whole range of phospholipids, does not produce the same modifications. Also, treatment of membranes with phospholipase A_2 results in a different pattern of effects on the different subtypes of glutamate receptors (data not shown). This suggests that a selective degradation of certain species of phospholipids is responsible for the observed effects. Moreover, the observed effects could not be due to the solubilization of proteins, because most modifications are of the apparent affinity of the receptors and not their maximal binding. Finally, because the membranes were extensively washed following the enzymatic treatment, the effects are not likely due to the formation of soluble metabolites that could directly interfere with the binding of different ligands. All these data point to the conclusion that the lipid microenvironment of the different receptors plays a critical role in determining the conformation and ligand binding affinity of the receptors.

Several effects of PLC treatment on the NMDA receptor complex were obtained. Both the kinetic analysis and the equilibrium saturation experiment indicate a decreased affinity of [^{3}H]TCP for its binding site when the channel is maintained in an open state by the combination of saturating concentrations of glutamate and glycine. This is apparently due to an increase in the dissociation rate of [^{3}H]TCP from its binding site and does not seem to be due to a modification of the relative percentage of [^{3}H]TCP binding exhibiting fast rather than slow kinetics. However, when the channel is closed (absence of glutamate and glycine plus presence of a glutamate antagonist), there is a marked increase in [^{3}H]TCP binding. Two arguments indicate that this increased binding represents binding to a site regulated by glutamate agonists and, therefore, is still associated with the NMDA receptor/channel complex. First, it is not affected by a number of compounds that are known to displace some of the phencyclidine ligands, such as haloperidol or histamine. Second, the dissociation of this binding is accelerated by glutamate. Finally, this increased binding was not due to an increased affinity for [^{3}H]TCP when the channel was closed, suggesting that the PLC treatment directly modified the accessibility of the NMDA channel binding site for [^{3}H]TCP. Thus, taken together, these results suggest that PLC treatment modified [^{3}H]TCP binding by two different mechanisms. When the channel is open, PLC treatment decreased [^{3}H]TCP binding by reducing the binding site affinity for this radioligand. When the channel is closed, the increased [^{3}H]TCP binding observed following PLC treatment might reflect an increased accessibility of the NMDA channel site for [^{3}H]TCP.

PLC treatment is accompanied by a marked decrease in the binding of [^{3}H]CPP, an NMDA antagonist, and by a suppression of the stimulation by glycine of [^{3}H]glutamate binding. In this case also, the decreased binding is due to a decreased affinity for CPP and not due to a change in the maximal number of sites. Using autoradiographic techniques, Monaghan *et al.* (36) have noted that the relative binding of [^{3}H]CPP versus [^{3}H]glutamate to the NMDA receptor varies across brain regions and that stimulation of glutamate binding by glycine covaries with the binding of [^{3}H]CPP. They ascribed this to the presence of two different populations of NMDA binding sites, termed agonist preferring and antagonist preferring. Our data could suggest that the NMDA binding sites have been converted to the agonist-preferring sites following PLC treatment. However, this interpretation is not consistent with the absence of effect of PLC treatment on [^{3}H]glutamate binding. The large decrease in [^{3}H]CPP binding affinity produced by PLC treatment was not associated with a change in the potency of AP5 to displace [^{3}H]glutamate binding. Such discrepancy could be ascribed to the difference in pharmacological properties obtained with various NMDA receptor radioligands. Antagonists generally display greater displacement potencies in ^{3}H-antagonist-binding assays than in ^{3}H-agonist assays (36). Thus, a change in the ^{3}H-antagonist binding might not be necessarily accompanied by modifications in the displacement potencies of the antagonists in ^{3}H-agonist binding assays. It is also possible that the two sites labeled by [^{3}H]glutamate and [^{3}H]CPP are not interconvertible and that PLC treatment selectively affects the antagonist-preferring site. Finally, one has to consider the possibility that NMDA receptors may be

variably assembled from a large number of different subunits, as has recently been shown to be the case for γ-aminobutyric acid receptors (37), and that each of the subunits responds differently to lipid modifications.

The pattern of results produced by PLC treatment may be relevant to certain properties of *in situ* glutamate receptors. Recent work has established that long term potentiation in hippocampus is expressed by an increase in the synaptic responses generated by quisqualate receptors with little change in those produced by NMDA receptors (38). Moreover, tetanic stimulation used to induce long term potentiation (39, 40), as well as NMDA receptor agonists (23, 24), activates at least some classes of phospholipases. There is, thus, a possibility that the increased quisqualate/AMPA receptor sensitivity observed in our experiments after perturbation of the lipid microenvironment of the membranes occurs *in situ* and produces important functional changes in synaptic operations. The overall effect of the phospholipid alteration on the NMDA receptors is unclear; if the agonist-preferring conformation is more responsive to agonists, we would also expect an increased sensitivity to agonists under these conditions. Because the phospholipid composition of membranes, as well as their turn-over rates, can be modified under a number of physiological and pathological conditions (41–43), it is likely that these conditions will be associated with alterations in glutamate receptors. This might be particularly important during the developmental period, in aging, or in various pathological states. In particular, it has been shown that a variety of neuronal insults result in an initial increase in phosphatidylinositol turn-over (44, 45), which would, therefore, create the condition for a self-perpetuating cycle of excitotoxicity.

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