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The primary acute, toxic effect of cholinergic agonists in the central nervous system is the production of seizures which are often fatal. One of the major signal transducing systems in the brain activated by cholinergic agonists is the hydrolysis of phosphoinositides. This system is a major site of action of lithium which also potentiates the convulsions and death associated with administration of cholinergic agonists. Therefore, the overall goal of the project is to determine how phosphoinositide hydrolysis, as well as protein phosphorylation, is modulated, especially by excitatory amino acids which mediate many types of seizures and by an inhibitory amino acid which blocks seizures, and how these processes are affected by cholinergic agonist-induced seizures, with an emphasis on those induced by coadministration of lithium and pilocarpine.

Modulation of phosphoinositide hydrolysis by excitatory amino acids was found to be due to a specific subtype of receptors, those activated by the selective agonist quisqualate. Two major effects of quisqualate were identified, activation by itself and

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inhibition of the effects of other agonists, especially norepinephrine. A variety of agents were found to affect these responses to the excitatory amino acid, most notably the concentrations of calcium and sodium. The inhibitory amino acid GABA was found to have effects opposite those of quisqualate, as GABA potentiated phosphoinositide hydrolysis induced by other agonists. The excitatory amino acid antagonist MK-801 also enhanced phosphoinositide hydrolysis. Seizures induced by lithium and pilocarpine caused changes similar to those of excitatory amino acids as there was selective impairment of phosphoinositide hydrolysis. The activity of protein kinase C was unaltered by seizures but tyrosine kinase activity was increased.

These results are consistent with the hypothesis that cholinergic agonist-induced seizures cause major impairments of the important second messenger-generating system of phosphoinositide hydrolysis and this may be mediated in part due to responses to excitatory amino acids, which in turn are influenced by a number of factors, most notably the calcium concentration.

FOREWORD

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Introduction

Cholinergic agonist-induced seizures

In the mammalian central nervous system the major acute toxic effect of high doses of inhibitors of acetylcholinesterase is the stimulation of convulsions. This effect is due to accumulation of excessively high concentrations of the neurotransmitter acetylcholine that build-up when acetylcholinesterase is inhibited, and the subsequent large amount of stimulation of cholinergic receptors (1). Seizures can also be produced by administration of direct-acting cholinergic agonists which mimick the action of acetylcholine on cholinergic receptors. Cholinergic agonists which are charged cannot cross the blood brain barrier to enter the central nervous system if they are administred peripherally, but central administration of such drugs (e.g., carbachol) induces seizures (2,3). In addition, central administration of carbachol is used widely to induce the important "kindling" model of seizures (4). Cholinergic agonists that are not charged, such as pilocarpine, can cross the blood brain barrier and can cause seizures when administered either peripherally or centrally (5). Such seizures are often lethal, even when peripheral cholinergic effects are blocked by an antagonist, such as methylatropine, indicating that the centrally-induced convulsant effects are sufficient to cause death. Pretreatment with a cholinergic antagonist that enters the brain (e.g., atropine) prevents both seizures and death (5), confirming that these are mediated by cholinergic receptors.

The mechanisms whereby cholinergic receptor stimulation leads to seizures and death is still not clear. Direct neurochemical studies with anticholinesterase drugs or cholinergic agonists are impaired by the great interindividual variation in the responses to these agents (6). Thus, a single dose of one of these agents can induce rapid seizures and death, prolonged seizures, or no seizure response in apparently identical groups of subjects, such as a single strain of rats. The use of lithium in conjunction with cholinergic agonists has provided in two ways an advantageous system to study: this cotreatment regimen provides a very reproducible convulsant response resulting in all rats demonstrating seizures over very similar time courses, and the fact that lithium potentiates cholinergic agonist-induced seizures suggests that lithium affects an important biochemical system mediating this response, thus providing a lead as to the biochemical basis of the lethal seizures.

Lithium potentiates responses to cholinergic agonists

Lithium was first reported to potentiate the convulsions and lethality of physostigmine (7,8). These studies used an acute dose of LiCl given several hours before administration of a normally subconvulsive dose of physostigmine. Honchar et al. (9) extended this work by reporting that lithium also had a proconvulsant effect on the responses to two cholinergic agonists, pilocarpine and arecoline. They also reported (9) that administration of lithium and cholinergic agonists resulted in large increases in the brain concentration of inositol monophosphate (IP1), a metabolite produced from hydrolysis of inositol-containing lipids (phosphoinositides), an important receptor-coupled second messenger generating system (10).

Our laboratory extended this previous work in a number of reports (11-17). We utilized EEG recordings from electrodes implanted in specific regions of rat brains to identify seizures induced by pilocarpine, arecoline or physostigmine, each in the absence and presence of pretreatment with LiCl. Complete dose-response curves for each of these was reported (15). The seizures induced by coadministration of LiCl and cholinergic agonists appear identical with those induced by high doses of cholinergic agonists alone using both EEG and behavioral criteria, except that the utilization of LiCl plus a cholinergic agonist produces extremely consistent and reproducible seizure responses in contrast to the variability obtained with the use of cholinergic agonists alone. The seizures are manifested as generalized convulsive status epilepticus, the seizures appear approximately simultaneously in all brain regions that we have tested, and status epilepticus continues without abatement for several hours, until death occurs. We have also reported that cortical and hippocampal concentrations of acetylcholine are increased to unprecedented levels (higher even than after administration of soman) in rats treated with lithium and pilocarpine even though acetylcholinesterase was not inhibited (14). Such a massive accumulation of acetylcholine after treatment with lithium and pilocarpine emphasizes the important alterations occuring in the cholinergic system in response to this treatment. Two papers have been published which report that lithium treatment potentiates the convulsant effect of two other anticholinesterases, paraoxon and soman (18,19). Increased brain levels of IP1 were reported with these treatments (18,19) as had previously been reported with coadministration of lithium and other cholinergic agonists (9). The seizure-potentiating effect of lithium is, as far as we know, selective for cholinergic agonists because screening of several other convulsants showed little or no influence of lithium treatment (17). Thus, lithium is affecting a critical regulatory site that is specifically important in modulating the response to cholinergic stimuli. Identification of the initiating mechanisms should be extremely useful in the development of drugs to block this toxic, lethal response to cholinergic agonists. Examination of many of the classical anticonvulsants after lithium and pilocarpine treatment revealed that once seizures began these drugs generally had little or no anticonvulsive effects (13). Thus, lithium has the interesting effect of selectivity potentiating the seizurogenic effects of all tested cholinergic agonists and once seizures begin they are difficult to control. Therefore, identification of the effects of lithium on the biochemical response to cholinergic agonists should be indicative of the biochemical mechanisms responsible for seizures initiated by cholinergic agonists.

Modulation of receptor-coupled second messenger systems

For receptor stimulation to induce a cellular response, the receptor must be coupled to a system that alters the intracellular biochemistry of neurons. During the last few years, it has become obvious that a major second messenger system in brain and many other tissues is the receptor-coupled hydrolysis of phosphoinositides. Several recent reviews have covered this in detail (10,20). Muscarinic receptors in brain are coupled to a guanine nucleotide binding protein (G-protein) which mediates the binding of an agonist to a receptor and the subsequent hydrolysis of phosphoinositides. There are three phosphoinositides, phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP), and phosphatidylinositol bisphosphate (PIP2). It is still not clear exactly which of these lipids is hydrolyzed following receptor activation, but current evidence suggests that both PI and PIP2 can be hydrolyzed by receptor activation (21). Hydrolysis of PIP2 results in the generation of two second messengers, diacylglycerol and inositol trisphosphate

(IP3). Hydrolysis of PI produces only one second messenger, diacylglycerol. Diacylglycerol activates protein kinase C, which then phosphorylates a variety of substrate proteins. Several small reports have shown that lithium treatment alters protein phosphorylation in the brain (22-24). IP3 stimulates the intracellular release of calcium from bound stores, resulting in an increase of the intracellular calcium concentration. There are many more complexities associated with this system that are covered in recent reviews (10,20) and cannot be adequately detailed here.

Lithium, at low concentrations (1 mM), has been shown to have a major effect on the phosphoinositide system. Sherman's group (25,26) has shown that lithium (both in vitro and in vivo) inhibits myo-inositol-1-phosphatase. Inhibition of this enzyme results in the accumulation of its substrate, IP1, and depletion of its product, inositol, in brain, as well as in many other tissues. Lithium administration to rats was shown to cause a dose-dependent, several-fold increase of the in vivo concentration of IP1 (26, 27). Administration of a cholinergic agonist along with lithium resulted in a synergistic larger increase of the concentration of IP1 (9). Treatment with atropine or scopolamne blocked the effects of lithium alone or with a cholinergic agonist (9,27) indicating that even in the absence of a cholinergic agonist, much of the in vivo phosphoinositide hydrolysis was mediated by muscarinic receptors. Recent reviews have covered in greater detail this, and further, evidence that has led many investigators to believe that one of the primary in vivo effects of lithium is this interaction with the phosphoinositide system (10,20,26,28,29).

In summary, it is logical to hypothesize that the interaction of lithium with the phosphoinositide system may provide the mechanism whereby lithium potentiates cholinergic agonist-induced seizures. Furthermore, it is logical to surmise that lithium is potentiating excitatory responses or attenuating inhibitory influences to reduce the seizure threshold to cholinergic agents and that these actions may also play a role in the subsequent long duration, severity, and lethality of the seizures. However, little more is presently known, especially about how this important systeme is regulated. Two types of regulation have been described: modulation by protein kinase C and modulation by neurotransmitter (or neuromodulator) interactions.

Agents which stimulate phosphoinositide hydrolysis increase the production of diacylglycerol, which activates protein kinase C (10). Activation of protein kinase C by some agents, but not others, has been shown to cause the translocation of protein kinase C from a soluble (cytoplasmic) to a particulate (membrane) fraction (30-32). Activation of protein kinase C, in turn, induces a feedback inhibition of agonist-stimulated phosphoinositide metabolism (33,34). The mechanism for this modulating inhibition is not clear, involvement of phosphorylation of receptors, G-proteins and phospholipase C have each been supported (10,20). This regulatory process is perhaps the major mechanism that has been found to date for modulation of phosphoinositide metabolism. Therefore, it would be logical to examine the effects of seizures on both the soluble and particulate fractions of protein kinase C.

The second type of modulation of phosphoinositide hydrolysis that has been identified consists of interactions among neurotransmitter systems or neuromodulators. Perhaps those most widely studied are the interactions among excitatory amino acid agonists and amine neurotransmitters (35,36). For example, we and others have reported that quisqualate, a receptor subtype selective excitatory amino acid agonist, effectively inhibits phosphoinositide hydrolysis induced by norepinephrine (37,38). The modulatory effects of the excitatory amino acids are especially relevant to the present investigation because excitatory amino acids are believed to play a major stimulatory role in many types of seizures. During the last few years a great number of reports have documented the seizure-inducing effects of excitatory amino acids and have shown that much of the neurodegeneration associated with seizures is due to activation of these pathways (35,39). As added evidence of their importance, many studies have shown that antagonists of excitatory amino acid receptors, such as MK-801, are effective anticonvulsants and protect the brain from seizure-induced damage (39). It is important to note that these anticonvulsant effects of excitatory amino acid antagonists apply to seizures induced by cholinergic agonists. For example, we recently reported (16) an extensive study of the anticonvulsant effects of MK-801 using the model of seizures induced by coadministration of lithium and pilocarpine. Pretreatment with MK-801 blocked the cholinergic agonist induced production of generalized convulsive status epilepticus and also prevented death which otherwise always occurred with this treatment. Equally impressive was the finding that administration of MK-801 after seizures had begun resulted in termination of the seizures and blocked the death that normally occurs as a result of seizure-induced brain damage (16). These and other studies clearly demonstrate that activation of excitatory amino acid systems plays a major role in seizures, brain damage, and death associated with seizures induced by cholinergic agonists. These results emphasize the necessity of identifying the interactions of excitatory amino acids with second messenger systems, such as receptor-coupled phosphoinositide hydrolysis. As noted above, excitatory amino acids can inhibit phosphoinositide hydrolysis. It is interesting to note that in the kindling model of seizures, there is also reduced phosphoinositide hydrolysis (40). Furthermore, lithium, which potentiates cholinergic agonist-induced seizures, also reduces phosphoinositide hydrolysis (41). Thus, there is accumulating evidence that seizures are associated with inhibition of the normal activity of the phosphoinositide second messenger-generating system.

Studies of inhibitory amino acid neurotransmitters, such as GABA, provide evidence of effects opposing those of the excitatory amino acids. GABA agonists generally have anticonvulsant effects and a preliminary report found that GABA enhanced phosphoinositide hydrolysis induced by norepinephrine (42). Thus, GABA and the excitatory amino acids appear to have opposing effects on both seizures and phosphoinositide metabolism, but very little research has been applied towards clarifying the modulatory properties of GABA on phosphoinositide hydrolysis.

The results summarized above indicate that studies of the modulation of phosphoinositide hydrolysis by excitatory and inhibitory amino acids are critical to understanding their modulatory roles in seizures. Furthermore, they indicate that use of the lithium plus pilocarpine model of seizure generation is most appropriate to identify the influences of phosphoinositide metabolism on seizures and vice versa, and to understand the CNS toxicity of cholinergic agonists.

EXPERIMENTAL PROCEDURES

Brain slice preparation. Male, Sprague-Dawley rats (200-250g) were decapitated and the brains were rapidly dissected in ice-cold 0.32 M sucrose. Slices (0.3 x 0.3 mm) from the cerebral cortex, or other regions where indicated, were prepared with a McIlwain tissue slicer and washed thoroughly with incubation media (NaCl, 122 mM; KCl, 5 mM; $MgCl_2$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.3 mM; $NaHCO_3$, 3.6 mM; glucose, 11 mM; HEPES, 30 mM; freshly bubbled with 95% O_2/5% CO_2; adjusted to pH 7.35). The slices were incubated in the same media at 37 C for 60 min for regeneration, followed by several washes with fresh media.

Assays of [^{3}H]inositol incorporation into slices and [^{3}H]inositol phosphate production. Two experimental protocols were used to expose slices to [^{3}H]inositol and to test the effects of different agents on [^{3}H]phosphoinositide synthesis and/or hydrolysis.

Data in Figures 1-7 and Tables 1-3 are from experiments in which slices were simultaneously exposed to [^{3}H]inositol and test agents to measure modulation of both [^{3}H]phosphoinositide synthesis and [^{3}H]inositol phosphate production as described previously (38). Aliquots of slices were incubated in a final vol of 500 μl incubation media containing 0.5 μM myo-[2-^{3}H]inositol (15 Ci/mmole), 10 mM LiCl and the indicated concentrations of test agents for 60 min (or other times where indicated) at 37°C. For those experiments in which proposed inhibitors of receptors, ion channels or enzymes were used, these agents were added 10 min before adding other agents. At the end of the incubation, samples were rapidly washed two times with 3 ml of ice-cold media and were mixed with 1.5 ml of$CHCl_3$:MeOH:12 N HCl (1:2:0.01).

For experiments shown in further figures and tables, preincubated slices were prelabelled by incubation at 37°C for 1 hr in fresh buffer containing 0.5 μM [^{3}H]inositol and 10 mM LiCl. After the incubation, the slices were rapidly washed several times to remove exogenous free [^{3}H]inositol. Aliquots of [^{3}H]inositol-prelabelled slices were incubated in 500 μl for 60 min or the indicated times in the presence of agonists or other agents. The reaction was stopped by adding 1.7 ml of $CHCl_3$:MeOH:12 N HCl (1:2:0.01).

Samples obtained from either assay were transferred to extraction tubes and mixed with 1 ml of $CHCl_3$ and 0.5 ml H_2O. The lipid phase was separated from the aqueous phase by centrifugation. Aqueous fractions were mixed with 0.5 ml of a 50% slurry of AG1-X8 resin and [^{3}H]inositol phosphates were separated by the method of Berridge et al. (43) as described previously (34) and the radioactivity in each samples was measured. The lipid phase was dried overnight at room temperature and the radioactivity was measured. Data were analyzed using 2-way ANOVA or Student's t-test.

Animal treatments

Male Sprague-Dawley rats initially weighing approximately 130 g were fed pelleted rat chow containing LiCl (1.696 g/kg diet; Teklad-Mills, Madison, WI) or commercial rat chow for 30 days. Food, water and 0.9% saline (to prevent lithium toxicity) were provided ad libitum. This method of administration of lithium is advantageous because it produces plasma lithium concentrations (0.86 ± 0.06 mM) that are within the range considered to be therapeutic in human patients and because rats maintain body weights not significantly different from controls, unlike some lithium treatment protocols. In these studies, initial and weekly body weights were 134 ± 3, 164 ± 4, 186 ± 5, 220 ± 6, and 254 ± 5, for controls, and they were 136 ± 4, 164 ± 4, 194 ± 6, 234 ± 5 and 267 ± 7 for the lithium-treated rats (n = 10 for each group).

Other rats were treated with acute LiCl (3 mmole/kg; ip; 20 hr prior to experiments). Seizures were induced by administration of pilocarpine (30 mg/kg; sc) to lithium-treated rats. This results in paroxysmal spike activity after approximately 15 min, which develops into spike trains within the next 5 min, and finally status epilepticus develops shortly thereafter (15). Rats remain in status epilepticus for several hours until death occurs.

Preparation of subcellular fractions

Each rat was decapitated, and the brain was rapidly removed and placed in ice-cold 0.32 M sucrose. The hippocampus was isolated and homogenized in 10 volumes of 10 mM tris-Cl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 20 µg/ml leupeptin, 10 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The homogenate was centrifuged at 100,000 x g for 1 hour at 4°C. After collection of the supernatant (soluble fraction) on ice, the pellet (particulate fraction) was resuspended in an equivalent volume of buffer A containing 0.2% Nonidet P-40 (NP-40) and stirred for 30 min at 4°C (44). Protein concentration was determined (45) using bovine serum albumin (BSA) as the standard. Protein concentrations of each fraction were adjusted to 0.1 mg/ml for the protein kinase C assay and 2 mg/ml for the assay of endogenous protein phosphorylation.

Assay of protein kinase C activity

Protein kinase C activity was measured at 30°C using assay procedures adapted from Kikkawa et al. (44), Castagna et al. (46), and Zatz (47). Phosphorylation was performed in a reaction mixture (0.2 ml, final volume) which contained 25 mM tris-Cl, pH 7.4, 6 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 1 mM ß-mercaptoethanol, 1.5 mM $CaCl_2$, 50 μg of histone (Type IIIS, Sigma Chemical Co., St. Louis, MO), 2 μg sample protein, and 30 μM [γ-^{32}P]ATP (40 Ci/mole) in the presence or absence of 10 μg phosphatidylserine (PS) and 1 μM phorbol 12-myristate 13-acetate (PMA). The reaction mixture without [γ-^{32}P]ATP was preincubated for 30 sec, followed by addition of [γ.-^{32}P]ATP to initiate phosphorylation. After 2 min, phosphorylation was stopped by addition of 0.5 ml ice-cold 10% trichloroacetic acid (TCA) containing 5 mM ATP and 20 mM NaH_2PO_4. BSA (100 μg) was added and the precipitated protein was collected onto Whatman GF/B membrane filters. The filters were washed three times with 5 ml 5% TCA, dried, and radioactivity was measured by liquid scintillation spectrometry. Protein kinase C activity was expressed as nanomole ^{32}P incorporated/min/mg protein and calculated as the difference between phosphorylation measured in the presence or absence of PS and PMA.

Phosphorylation of endogenous proteins and SDS gel electrophoresis

Phosphorylation of endogenous proteins was conducted at 30°C in a reaction mixture (0.2 ml, final volume) containing 25 mM tris-Cl, pH 7.4, 6 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 1 mM ß-mercaptoethanol, and 100 μg sample protein in the presence of either (a) no further additions, (b) 1.5 mM $CaCl_2$, (c) 1.5 mM $CaCl_2$,10 μg PS, and 1 μM PMA, (d) 1 mM theophylline, or (e) 1 mM theophylline and 50 μM cAMP. After preincubation for 30 sec, phosphorylation was initiated by the addition of 2 μM [γ-^{32}P]ATP (2500 Ci/mole). Phosphorylation was stopped after 30 sec by addition of 100 μl of SDS stop solution containing (final concentrations) 93.8 mM Tris-Cl, pH 6.8, 25% glycerol, 3% SDS, 10% ß-mercaptoethanol, with bromphenol blue as the dye front marker, and each sample was placed in a boiling water bath for 2 min.

A portion of each sample (containing 20-30 μg protein) was subjected to one-dimensional SDS-polyacrylamide (6.5% or 12%) gel electrophoresis (SDS-PAGE) as described by Laemmli (48). The gels were stained for 2 hrs with Coomassie Brilliant Blue R-250, destained overnight, and dried. Dried gels were exposed to Kodak X-OMAT AR film, and the autoradiographs were scanned with a GS 300 transmittance/reflectance scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA). Protein kinase C-mediated phosphorylation was measured by subtracting peak heights obtained with calcium alone from those in the presence of calcium, PS, and PMA. Cyclic AMP-dependent protein phosphorylation was measured by subtracting peak heights obtained with theophylline alone from those in the presence of theophylline and cAMP. These data were expressed as percent of control values (peak height of lithium-treated/peak height of control X 100). Apparent molecular masses of the phosphoproteins in kilodaltons (kD) were determined from protein standards: lysozyme (14.4 kD), soybean trypsin inhibitor (21.5 kD), carbonic anhydrase (31 kD), ovalbumin (45 kD), BSA (66.2 kD), phosphorylase B (92.5 kD), ß galactosidase (116 kD), and myosin (200 kD) (BioRad; Richmond, CA).

Protein tyrosine phosphorylation

The brain regions indicated were rapidly removed, weighed, and homogenized in 10 vol of 125 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% ß-mercaptoethanol and 1 mM sodium vanadate. The homogenates were sonicated for 30 sec and placed in a boiling water bath for 5 min. The protein concentration was measured (49) after acid precipitation of the protein. Samples were diluted with Laemmli sample buffer and 80 μg protein were run on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose (50) and probed with a monoclonal antibody to phosphotyrosines (1:1000, PY 20; ICN Immunobiologicals, Lisle, IL). After incubation with the primary antibody, the blots were incubated with horseradish peroxidase labeled goat-antimouse IgG and color developed with 3,3'-diaminobenzidine in the presence of hydrogen peroxide. The immunoblots were dried and the bands were quantitated using a BioRad Video Densitometer 620. Band areas of samples from control and treated rats were compared and the results from several preliminary experiments were used to choose proteins for quantitative analysis, including those in which possible changes occurred and some which appeared not to be affected by the treatments under study. Statistically significant differences were determined by analysis of variance.

RESULTS

Our initial experiments established optimal conditions for quantifying the activity of the phosphoinositide second messenger generating system in slices from regions of rat brain. We characterized the concentration-dependence for carbachol and norepinephrine to induce phosphoinositide hydrolysis and in preliminary studies showed that glutamate inhibited agonist-induced phosphoinositide hydrolysis. This inhibitory effect of glutamate was found to be due to activation of the quisqualate-selective receptor. Therefore, the initial portion of the present investigation was undertaken to examine in more detail the modulation of phosphoinositide hydrolysis by excitatory amino acids.

Effects of calcium concentration on [^{3}H]phosphoinositide synthesis and hydrolysis and the inhibitory effect of glutamate.

Figure 1 shows that calcium had two opposing effects on the phosphoinositide system in cortical slices. Increasing the calcium concentration reduced the incorporation of [^{3}H]inositol into [^{3}H]phosphoinositides (Figure 1A), as reported previously (34). This inhibitory effect occurred irrespective of whether or not norepinephrine (NE) or carbachol was included in the incubation medium. Glutamate significantly inhibited the synthesis of [^{3}H]phosphoinositides in all conditions except in the presence of EGTA, and the percent inhibition by glutamate increased as the calcium concentration was increased (e.g., glutamate in the presence of NE inhibited [^{3}H]phosphoinositide synthesis by 16, 27, 33, and 37% in EGTA, no added calcium, 0.1 mM and 1.3 mM calcium, respectively).

In contrast to [^{3}H]phosphoinositide synthesis, agonist-stimulated [^{3}H]IP$_1$ production required calcium and was severely reduced as the concentration of calcium was decreased (Figure 1B). Glutamate significantly inhibited NE-stimulated [^{3}H]IP$_1$ production under all conditions except in the presence of EGTA, but had no significant effect on carbachol-stimulated [^{3}H]IP$_1$ production. As with [^{3}H]phosphoinositide synthesis, the percent inhibition of NE-stimulated [^{3}H]IP$_1$ production caused by glutamate increased as the calcium concentration was increased, resulting in 6, 31, 37 and 49% inhibition in the four conditions.

A calcium channel blocker, verapamil, in media containing 1.3 mM calcium, resulted in a concentration-dependent increase of the synthesis of [^{3}H]phosphoinositides in the absence or presence of NE (Figure 2A) which is consistent with the finding that reduced calcium enhances [^{3}H]phosphoinositide synthesis. Glutamate significantly ($p < 0.05$) reduced [^{3}H]phosphoinositide synthesis in the presence of NE at all concentrations of verapamil.

Verapamil caused a significant inhibition of NE-induced [^{3}H]IP$_1$ production (Figure 2B), in contrast to its stimulation of [^{3}H]phosphoinositide synthesis. NE-stimulated [^{3}H]IP$_1$ production was significantly reduced by glutamate in the absence of verapamil or with 10, 30 and 50 μM verapamil. However, with 75 or 100 μM verapamil there was not a statistically significantly inhibition of glutamate as the inhibitory effect of verapamil had reduced the response to NE to near that observed in the presence of NE and glutamate.

Effects of quisqualate on [^{3}H]phosphoinositide synthesis and hydrolysis. Previous observations indicated that the inhibitory effect of glutamate on NE-stimulated [^{3}H]IP$_1$ production was mimicked by quisqualate, but not by kainate or NMDA (38). Quisqualate produced a concentration-dependent inhibition of [^{3}H]phosphoinositide synthesis (Figure 3A). As reported by others (51), 100 μM quisqualate caused a small but statistically significant stimulation of [^{3}H]IP$_1$ formation, both in the presence and absence of NE, whereas higher concentrations of quisqualate inhibited this process (Figure 3B). This biphasic effect of quisqualate makes it difficult to obtain a clear measurement of the inhibition of NE-stimulated [^{3}H]IP$_1$ production, as both quisqualate-mediated responses may be activated at concentrations near 10^{-4} M. The biphasic response to quisqualate also necessitates the use of relatively high concentrations to study the inhibitory portion of its action.

None of several excitatory amino acid receptor antagonists blocked quisqualate-mediated inhibition of [^{3}H]phosphoinositide synthesis or of the response to NE (Figure 4), including the relatively specific quisqualate receptor-selective antagonist CNQX. AMPA, which is an agonist apparently selective for a subpopulation of quisqualate receptors (35), did not alter [^{3}H]phosphoinositide synthesis or NE-induced [^{3}H]IP$_1$ production. These observations support previous suggestions that the inhibitory effect of quisqualate is mediated by a unique receptor that is insensitive to the known excitatory amino acid receptor antagonists (36) and is not activated by AMPA.

Effects of arachidonic acid on [^{3}H]phosphoinositide synthesis and hydrolysis. Excitatory amino acids and calcium can stimulate phospholipase A_2 (52), resulting in the liberation of arachidonic acid. Therefore, we tested the possibility that arachidonic acid may modulate [^{3}H]phosphoinositide synthesis and its hydrolysis stimulated by NE. Concentrations of arachidonic acid of 50 μM and greater significantly reduced the synthesis of [^{3}H]phosphoinositides (Figure 5A) and NE-stimulated [^{3}H]IP_1 production (Figure 5B). Both effects of arachidonic acid were prevented by inclusion of BSA (1 mg/ml), which binds free fatty acids in the incubation medium (data not shown). Thus, as found with glutamate and quisqualate, arachidonic acid caused concomitant inhibition of both [^{3}H]phosphoinositide synthesis and the hydrolysis stimulated by NE.

The time course of these inhibitory effects of arachidonic acid (Figure 6) was similar to those of glutamate and quisqualate, as reported previously (38). [^{3}H]Phosphoinositide synthesis was inhibited by arachidonic acid within 10 min and synthesis remained depressed through 60 min of incubation. NE did not significantly stimulate [^{3}H]IP_1 production until 40 min, as reported previously due to the need to significantly label the pool of lipids available to the receptor (38), and arachidonic acid significantly depressed this response to NE. Arachidonic acid produced similar inhibitory effects on NE-stimulated production of [^{3}H]IP_2 and [^{3}H]IP_3 (data not shown).

In the presence of carbachol, arachidonic acid also inhibited the synthesis of [^{3}H]phosphoinositides and the stimulated production of [^{3}H]IP_1 (Figure 7). This indicates that the inhibitory effect of arachidonic acid is not specific for only adrenergic receptor-containing cells.

Calculation of the ratio [^{3}H]IP_1/[^{3}H]IP_1 + [^{3}H]phosphoinositides provides an indication of the activity of receptors coupled to phosphoinositide hydrolysis. This ratio was unchanged by arachidonic acid compared with either NE or carbachol alone (Table 1) suggesting that the inhibitory effect of arachidonic acid is nonselective with regards to pools of phosphoinositides and that its inhibitory effect on [^{3}H]IP$_1$ hydrolysis is likely to be due to the impaired synthesis of [^{3}H]phosphoinositides rather than to impaired function of the receptor complex.

To examine if [^{3}H]phosphoinositide synthesis and the production of [^{3}H]IP$_1$ were inhibited directly by arachidonic acid or by products of its metabolism, we tested several inhibitors of arachidonic acid metabolism, including indomethacin (cyclooxygenase inhibitor), nordihydroguaiaretic acid (NDGA, lipoxygenase inhibitor) and phenidone (inhibitor of both cyclooxygenase and lipoxygenase), each at a final concentration of 100 μM. Indomethacin and phenidone, but not NDGA, had weak inhibitory effects of their own, and none of these agents reduced the inhibition caused by 200 μM arachidonic acid (n=2; data not shown). This indicates that arachidonic acid itself may cause the inhibition of [^{3}H]phosphoinositide synthesis and hydrolysis.

Agents known to modulate the activity of phospholipase A$_2$ were tested for direct effects on [^{3}H]phosphoinositide synthesis and NE-stimulated [^{3}H]IP$_1$ formation, and for modulatory effects on the inhibition of these processes induced by quisqualate. Table 2 shows that activation of phospholipase A$_2$ activity by melittin caused large reductions of [^{3}H]phosphoinositide synthesis and [^{3}H]IP$_1$ production. This was apparently a widespread effect, not localized to certain cells, since the calculated ratio did not change, similar to the response to arachidonic acid. Inhibitors of phospholipase A$_2$ including chloroquine, bromphenacyl bromide (BPB), and dexamethasone were tested in this system although none of these agents is highly specific for phospholipase A$_2$. Each of these inhibitors produced some inhibition of [^{3}H]phosphoinositide synthesis and chloroquine and BPB significantly reduced NE-stimulated [^{3}H]IP$_1$ production. None of these agents blocked the inhibitory effects of quisqualate.

Experiments were carried out to determine if any of several agents altered the incorporation of [^{3}H]inositol into phosphoinositides mediated by the Mn^{2+}-activated base exchange reaction. Mn^{2+} increased the incorporation of [^{3}H]inositol into phosphoinositides (53) and NE did not alter the rate of the base exchange reaction (Table 3). Glutamate, quisqualate, and arachidonic acid each inhibited [^{3}H]phosphoinositide synthesis in the absence of Mn^{2+}. Only arachidonic acid reduced the base exchange-mediated incorporation of [^{3}H]inositol into phosphoinositides, but the percent inhibition was much less than that obtained in the absence of Mn^{2+}.

Effects of amino acids on agonist-stimulated [^{3}H]phosphoinositide hydrolysis in prelabelled slices. The previous experiments utilized a method in which slices were exposed simultaneously to [^{3}H]inositol and test agents to allow measurements of both synthesis and hydrolysis of [^{3}H]phosphoinositides. In the following experiments, slices were prelabelled with [^{3}H]inositol and subsequently thoroughly washed before agonists and other drugs were added in an attempt to dissociate modulatory effects on [^{3}H]IP$_1$ production from those on [^{3}H]phosphoinositide synthesis.

Because there is greater synthesis of [^{3}H]phosphoinositides in the absence than in the presence of added calcium (34), each condition was used during the prelabelling incubation prior to the measurement of [^{3}H]IP$_1$ production (which was always carried out in media containing 1.3 mM calcium). These experiments revealed further complexities in the role of calcium in phosphoinositide hydrolysis. Although the synthesis of [^{3}H]phosphoinositides was higher in the absence of calcium (as shown in Figure 1), the subsequent response to NE was depressed (Figure 8). Additionally, the modulatory effects of the amino acids on NE-stimulated [^{3}H]IP$_1$ production were blunted in slices prelabelled without calcium, suggesting that reduced calcium attenuates the inhibitory effects of the modulatory amino acids. However, in the slices incubated continuously in the presence of calcium there were clear modulatory influences by the amino acids. [^{3}H]IP$_1$ production induced by NE was inhibited by both glutamate and quisqualate, unaltered by kainate, surprisingly enhanced by NMDA, and inhibited by homocysteic acid and L-cysteine. Thus, this condition was used in further studies reported below.

Examination of the NE concentration-dependent stimulation of [^{3}H]IP$_1$ production revealed that quisqualate significantly reduced the maximal response to NE (Figure 9).

The inhibitory effects of excitatory amino acids on both phosphoinositide synthesis and NE-stimulated inositol phosphate production raised the question of the size of the pool of phosphoinositides available to the α1-adrenergic receptor and its rate of turnover in the presence of

NE. That is, in prelabelled slices is it possible to inhibit NE-stimulated [^{3}H]IP$_1$ production by inhibition of further synthesis of [^{3}H]phosphoinositides? To examine this, following prelabelling of slices with [^{3}H]inositol and washing away extracellular [^{3}H]inositol, unlabelled inositol (1,5, or 20 mM final concentration) was equilibrated with slices for 15 min followed by incubation with NE for 15 or 30 min. Figure 10 shows that there was an inositol-induced concentration-dependent and time-dependent reduction of NE-stimulated [^{3}H]IP$_1$ production. The results with 20 mM inositol may be the most relevant because high concentrations have been reported to be required to dilute endogenous [^{3}H]inositol (54). With 20 mM inositol, during the first 15 min of incubation with NE the production of [^{3}H]IP$_1$ was reduced by 53%, and by 30 min it was reduced by over 75%. These results suggest that phosphoinositides available to the α1-adrenergic receptor complex turn-over to a significant extent during these incubation periods and that inhibition of [^{3}H]phosphoinositide synthesis may contribute to inhibition of [^{3}H]IP$_1$ production even in prelabelled slices. Alternatively, however, it is possible that high concentrations of inositol are capable of directly displacing [^{3}H]inositol from labelled phosphoinositides through homologous base exchange reactions.

Excitatory amino acids have been reported to induce chloride influx (55) as well as polyamine synthesis (56), so the effects of antagonists of these processes were measured on the response to NE and its inhibition caused by quisqualate. Neither furosemide nor DIDS blocked the inhibitory effect of quisqualate, although each of these agents slightly increased the response to NE (Table 4). Similarly, DFMO, an inhibitor of ornithine decarboxylase, did not block the inhibition induced by quisqualate.

Modulation by GABA of agonist-stimulated [^{3}H]phosphoinositide hydrolysis in prelabelled slices. Because GABA has been reported to enhance [^{3}H]IP$_1$ production in response to NE (42), the effects of GABA on stimulation by agonists and inhibition by excitatory amino acids and arachidonic acid were examined. GABA produced a biphasic concentration-dependent modulation of NE-stimulated [^{3}H]IP$_1$ production in cortical slices (Figure 11). The response to a maximal concentration of NE (200 μM) was enhanced by 30 and 100 μM GABA and was inhibited by 1 and 10 mM GABA. There was a greater enhancement of the response to a submaximal concentration of NE (2 μM). GABA did not alter carbachol-stimulated $[^3H]IP_1$ production, nor did it alter inhibition by glutamate of $[^3H]IP_1$ produced in response to 200 μM NE.

The effects of GABA were found to be regional specific. In agreement with previous reports (42), GABA alone caused no stimulation of $[^3H]IP_1$ production in any of the brain regions that were tested. In striatal slices GABA enhanced the response to carbachol, but did not affect that of NE or ibotenate (Figure 12). In hippocampal slices, GABA enhance the response to carbachol and to ibotenate, although different concentrations of GABA were effective with each of these agonists (Figure 13). GABA also enhanced the response to NE, and this increase in hippocampal slices was greater than that observed in cortical slices. The maximal effect of GABA was obtained at a concentration of 300 μM which increased the response to NE throughout the range of effective concentrations of NE, producing from 69% to 30% increases (Figure 14).

The effects on NE-stimulated $[^3H]IP_1$ production of combinations of GABA and inhibitory agents were tested. Quisqualate and arachidonic acid significantly inhibited NE-induced $[^3H]IP_1$ production in slices that had been prelabelled with $[^3H]$inositol and baclofen, a specific $GABA_B$ agonist, was as effective as GABA in enhancing the response to NE (Figure 15). Neither GABA nor baclofen significantly blocked the inhibitory effect of quisqualate, but baclofen reduced the inhibitory effect of arachidonic acid.

Effects of NMDA receptor antagonists on phosphoinositide hydrolysis

MK-801 is an NMDA receptor antagonist which is an effective anticonvulsant in many seizure models, including seizures induced by administration of lithium and pilocarpine (16). In experiments using MK-801 to block the modulatory effects of excitatory amino acids on phosphoinositide hydrolysis, we noted that MK-801 itself was able to activate phosphoinositide metabolism. Therefore, this response to MK-801 was investigated in further detail.

MK-801 induced a concentration-dependent (EC_{50} ~ 380 μM) increase of phosphoinositide hydrolysis (Fig. 16). The maximum effect occurred at 1 mM MK-801 and was 4.5-fold of basal $[^3H]$-IP_1 accumulation. AP-7, another NMDA antagonist, did not affect phosphoinositide turnover and PCP did not induce phosphoinositide hydrolysis until 1 mM. Phosphoinositide hydrolysis stimulated by 500 μM MK-801 was not significantly affected by NMDA (1 mM) or glycine (0.1, 1.0 mM) alone, or in combination, or by Zn^{2+} (0.5 mM) (Fig. 17). Removal of Mg^{2+} from the assay medium did not significantly affect the MK-801-induced phosphoinositide hydrolysis (Fig. 17). The MK-801-induced response in the absence of Mg^{2+} was not significantly affected by NMDA (1 mM) or glycine (0.1, 1.0 mM), alone or in combination. The MK-801 response was unaffected by either prazosin (10 μM) or atropine (10 μM) (data not shown).

Modulation of phosphoinositide hydrolysis by $Na^{\pm}$

Excitatory amino acids function in part by activating ion channels, which can result in the influx of Na^+ (35). To examine if Na^+ influx was necessary for the modulatory effects of excitatory amino acids on phosphoinositide hydrolysis, the concentration of Na^+ in the extracellular incubation medium was reduced. This was observed to have number of important effects on phosphoinositide hydrolysis.

The accumulation of $[^3H]IP_1$ was measured after a 60 min incubation of $[^3H]$inositol-prelabelled cerebral cortical slices in media containing variable concentrations of Na^+ (Figure 18). In the absence of added agonist (basal), reduction of the Na^+ concentration from 120 mM Na^+ in controls resulted in enhanced accumulation of $[^3H]IP_1$, with maximal increases of approximately 7-fold occurring in media with 0 or 5 mM Na^+. Norepinephrine (100 μM) significantly stimulated the production of $[^3H]IP_1$ in 120 mM Na^+, and this response was increased incrementally as the Na^+ concentration was reduced. The inset in Figure 18 shows the difference in the $[^3H]IP_1$ produced in media with reduced Na^+ compared with that in 120 mM Na^+, demonstrating that the enhanced response to norepinephrine is greater than, and independent of, the increase in the basal rate of $[^3H]IP_1$ production. Prazosin (10 μM), an α-adrenergic receptor antagonist, completely blocked the response to norepinephrine in 120 and 5 mM Na+ (data not shown).

Incubation with the sodium channel blocker tetrodotoxin (TTX) did not cause the same effects as did removal of Na^+ from the medium indicating that Na^+ influx through voltage-dependent channels is not responsible for the depressed phosphoinositide hydrolysis observed in 120 mM Na^+. Basal and norepinephrine-stimulated $[^3H]IP_1$ production were unaffected by inclusion of TTX in the medium, while the response to carbachol was significantly reduced by TTX concentrations of 0.3 μM and greater (Figure 19).

Figure 20 shows the agonist-selectivity of the influence of Na^+ on phosphoinositide hydrolysis. Phosphoinositide hydrolysis stimulated by norepinephrine was enhanced in low Na^+ and this increase was more than additive with the increased $[^3H]IP_1$ produced under basal conditions. In contrast, the responses to carbachol and ibotenate were only slightly enhanced in low Na^+ and these increases appeared to be additive with the larger basal response, indicating that phosphoinositide hydrolysis induced by these two agents was not sensitive to the Na^+ concentration. The most impressive Na^+-sensitivity was observed with quisqualate which produced a large increase in $[^3H]IP_1$ accumulation in media with low Na^+.

We recently reported that glutamate, arachidonate, and quisqualate significantly inhibited norepinephrine-stimulated phosphoinositide hydrolysis in rat cerebral cortical slices (57). Figure 20 shows that glutamate (0.5 mM) reduced $[^3H]IP_1$ produced in response to norepinephrine by approximately 50% and that this degree of inhibition was not altered in media with low Na^+. Arachidonate (0.2 mM) inhibited the response to norepinephrine by approximately 40% in 120 mM Na^+ and this inhibition was only slightly reduced (to approximately 25%) in 5 mM Na^+. In contrast, whereas quisqualate (0.5 mM) inhibited the response to norepinephrine by 60% in 120 mM Na^+, there was no inhibition by quisqualate evident in 5 mM Na^+. TTX did not affect the inhibitory effects of glutamate, arachidonate, or quisqualate.

To examine if the apparent absence of an inhibitory effect of quisqualate in low Na^+ was actually due to its being masked by enhanced quisqualate-stimulated phosphoinositide hydrolysis, we employed another excitatory amino acid agonist, L-BOAA. As reported previously (58), L-BOAA mimicks the inhibition caused by quisqualate but does not itself induce $[^3H]IP_1$ production in 120 mMNa^+ (Figure 21). In 5 mM Na^+, inhibition by L-BOAA of the response to norepinephrine was totally blocked, while L-BOAA (1 mM) alone produced only a small rise in [^{3}H]IP$_1$ which was much below that caused by quisqualate (Fig. 20). These results reinforce the conclusion that L-BOAA is more selective than is quisqualate in causing inhibition of norepinephrine-stimulated phosphoinositide hydrolysis without activating this process. Furthermore, the data indicate that the two responses to quisqualate are differentially affected by low Na^+, supporting the hypothesis that they are mediated by different receptors or mechanisms.

The enhancement by low Na^+ of quisqualate-stimulated [^{3}H]IP$_1$ production was examined further by measuring the concentration-dependence of this response. In the presence of 5 mM Na^+ there was a remarkable enhancement of the potency of quisqualate so that 10^{-7}M quisqualate significantly elevated [^{3}H]IP$_1$ (Figure 22). This is about a 100-fold lower concentration than is required to activate phosphoinositide hydrolysis in 120 mM Na^+. Also, the maximal response to quisqualate was almost 6-fold higher in 5 mM Na^+ than in 120 mM Na^+. These large effects of Na^+ on quisqualate response contrast with the lack of Na^+-sensitivity of ibotenate-stimulated phosphoinositide hydrolysis. Additionally, the inhibition caused by the higher concentrations of quisqualate in 120 mM Na^+ are greatly attenuated in 5 mM Na^+.

Phosphoinositide hydrolysis in hippocampal slices was also influenced by the Na^+ concentration. Figure 23 shows that compared with 120 mM Na^+, in 5 mM Na^+ [^{3}H]IP$_1$ production was increased under basal conditions and in response to norepinephrine and quisqualate. Also shown in Figure 23 are the findings that in 5 mM compared with 120 mM Na^+ there were much greater accumulations of [^{3}H]IP$_2$ and slightly increased accumulations of [^{3}H]IP$_3$ in response to quisqualate or norepinephrine.

Protein kinase C activity

Stimulation of phosphoinositide hydrolysis results in the production of diacylglycerol which activates protein kinase C. Therefore, we established methods to study protein kinase C, including measurements of the enzyme activity and of the phosphorylation of proteins present in particulate (membrane) or soluble (cytosolic) preparations from rat brain regions. Initial experiments were carried out to make these measurements in brain samples from control and lithium-treated rats in preparation for studying changes following induction of seizures by administration of lithium and cholinergic agonists.

The activity of protein kinase C in the soluble and particulate fractions from hippocampus of control and lithium-treated rats is summarized in Table 5. The relative distribution of protein kinase C activity in control soluble and particulate fractions was 39% and 61%, respectively. This is in agreement with previously published data (44). Chronic lithium treatment did not alter the relative distribution of this enzyme activity. Also, there were no statistically significant differences (Student's t-test, $p > 0.05$) in specific activity or total protein kinase C activity in the hippocampal particulate or soluble fractions prepared from chronic lithium treated rats when compared with control values.

Endogenous protein phosphorylation

Particulate and soluble fractions prepared from hippocampus of control and chronic lithium-treated rats were incubated with either calcium alone and calcium with phosphatidylserine (PS) and the phorbol ester PMA (to activate protein kinase C), or theophylline alone and theophylline with cAMP. This allowed measurement of phosphorylation of endogenous proteins by protein kinase C and cAMP-dependent protein kinase, respectively. Basal phosphorylation was measured in the absence of any of these additions. Electrophoresis was performed on 6.5% or 12% polyacrylamide gels to separate phosphoproteins of apparent molecular mass greater than 45 kD or less than 45 kD, respectively.

Particulate fraction: Endogenous and stimulated protein phosphorylation

In the particulate fraction, under basal conditions a major band of phosphoprotein was observed at an apparent molecular mass of 47 kD, intermediate bands were observed at 50, 115, and 138 kD, and several minor bands appeared (Fig. 24). There were no significant differences in the phosphorylation of these proteins between control and chronic lithium-treated rats (data not shown).

W [illegible] addition of calcium, phosphorylation of at least three proteins of apparent molecular masses 45, 58, and 72 kD was increased compared with basal conditions (Fig. 24). After chronic lithium treatment, phosphorylation of these proteins was slightly reduced (to 90 ± 17%, 84 ± 7%, and 88 ± 7%, of controls, respectively) but the differences were not statistically significant.

Activation of protein kinase C with PS and PMA increased the phosphorylation of a number of proteins in the particulate fraction (Fig. 24). The phosphorylation of several high molecular mass proteins of 45, 76, 87, 158, and 200 kD (Fig. 24), and three low molecular mass proteins at 16, 18, and 19 kD (Fig. 24) was most clearly increased. Phosphorylation of most of the protein kinase C substrate proteins was unaltered by chronic lithium treatment, but there were statistically significant decreases in the phosphorylation of the 18, 19, and 87 kD proteins (Table 6).

In the presence of the theophylline alone (Fig. 25), no significant change in particulate protein phosphorylation was observed compared with basalconditions. Incubation with theophylline and cAMP stimulated the phosphorylation of several proteins in the particulate fraction (Fig. 25). Chronic lithium treatment significantly reduced the phosphorylation of the phosphorylation of the 54 and 71 kD proteins (Table 6).

Soluble fraction: Endogenous and stimulated protein phosphorylation

Upon incubation of the soluble fraction under basal conditions, phosphorylation was limited to a few faint bands (data not shown). No significant differences in the phosphorylation of soluble proteins between the control and lithium-treated samples were observed in these samples.

Incubation with calcium increased the phosphorylation of proteins with apparent molecular masses of 46 and 56 kD (Fig. 26). As in the particulate fraction, incubation of soluble fractions with calcium had little effect on the phosphorylation of lower molecular mass proteins (Fig. 3B). In samples from chronic lithium-treated rats, no significant alterations in phosphorylation were observed in comparison with controls.

Activation of protein kinase C led to increased phosphorylation of a large number of proteins in the soluble fraction (Fig. 26). In samples from chronic lithium-treated rats, phosphorylation was increased significantly in four proteins of apparent molecular masses of 16, 17, 20 and 22 kD (Table 7). The phosphorylation of several other proteins also appeared to be increased, but, due to the large variations in the percent stimulation of phosphorylation after chronic lithium treatment compared with controls, the increases were not statistically significant.

Incubation of the soluble fraction with cAMP primarily increased phosphorylation of a 260 kD protein band, and to a lesser extent, the 48, 49, 69, 70, and 126 kD phosphoproteins. Chronic lithium treatment did not significantly alter the phosphorylation of these proteins compared with controls (data not shown).

Effects of seizures on phosphoinositide hydrolysis

Following the identification of modulators of phosphoinositide hydrolysis, we investigated if seizures induced in rats by administration of lithium and pilocarpine affected phosphoinositide metabolism.

Phosphoinositide hydrolysis was measured in hippocampal slices from control, lithium-treated rats, or lithium plus pilocarpine-treated rats, the latter being either 25 min post-pilocarpine, near the initiation of status epilepticus, or after 90 min, when status epilepticus had continued for just over one hour. Treatment with only lithium or pilocarpine (data not shown) did not alter the response to any of the agonists that were tested. However, seizures induced by lithium plus pilocarpine caused decreased responses to ibotenate and to norepinephrine (Figure 27). There were significant decreases with these two agonists in the samples from rats where seizures had just initiated (25 min) as well as further decrements after an hour of continuous seizures (90 min). In contrast to these effects of seizures, there were no changes in phosphoinositide hydrolysis induced by carbachol, carbachol plus 15 mM K^+ (which potentiates the response to carbachol), or 55 mM K^+, in any of the treatment groups.

The reduced response to norepinephrine in hippocampal slices after seizures was examined in greater detail by measuring the concentration-dependence of norepinephrine-induced phosphoinositide hydrolysis (Figure 28). These results demonstrated that after seizures there were decreased responses to norepinephrine at concentrations of 3 x 10^{-6}M and greater, and that the major effect of seizures was the reduction of the maximal response to norepinephrine. It is also evident that the largest deficit occurred rapidly after the initiation of seizures (25 min) and that further decreases (90 mins after pilocarpine) were relatively smaller.

Similar neurotransmitter-specific impairments of phosphoinositide hydrolysis were observed in cortical slices from rats undergoing seizures induced by lithium plus pilocarpine. Figure 29 shows that phosphoinositide hydrolysis induced by carbachol was similar in cortical slices from control and seizing rats, while the response to norepinephrine was reduced by seizures Quisqualate, another excitatory amino acid agonist, caused less stimulation of phosphoinositide hydrolysis in cortical slices from seizing rats than from controls. Quisqualate also inhibits norepinephrine-induced phosphoinositide hydrolysis, and this inhibitory influence was not altered by seizures, as 0.5 mM quisqualate reduced the response to norepinephrine by approximately 50% in control and treated samples.

As detailed earlier in this report phosphoinositide hydrolysis stimulated by norepinephrine and quisqualate (but not carbachol) is enhanced when the concentration of Na^+ in the incubation medium is reduced to 5 mM. The results in Figure 29 demonstrate these effects of low Na^+ and show that in slices from seizing rats the responses to norepinephrine and quisqualate remain below controls even when measured in 5 mM Na^+. Lowered Na^+ greatly reduces the inhibitory influence of quisqualate on norepinephrine-stimulated phosphoinositide hydrolysis, and this effect was evident in slices from control and seizing rats. Thus, the stimulatory response to quisqualate as well as to norepinephrine was reduced by seizures but the inhibitory effect of quisqualate on norepinephrine-induced phosphoinositide hydrolysis was not altered by seizures.

Since the responses to ibotenate and norepinephrine were altered by seizures, we examined whether the effects of modulators of these two agonists were also altered. Table 8 shows that, as reported previously quisqualate greatly inhibited the response to norepinephrine. This inhibitory effect of quisqualate was unaltered by lithium or seizures. Table 8 also shows that AP-4, an antagonist at specific excitatory amino acid receptors, greatly inhibits the response to ibotenate and that lithium and seizures did not alter this modulatory effect.

NaF activates phosphoinositide hydrolysis purportedly by activation of the associated G-protein by AlF_4^- (59). There was no significant effect of seizures on NaF-stimulated phosphoinositide hydrolysis (Figure 30) which agrees with the findings given above that the effects of seizures were neurotransmitter-selective.

Effects of seizures on protein kinase C

The activity of protein kinase C was determined by measuring protein phosphorylation in membrane and cytosolic fractions prepared from control or treated rats using $[^{32}]$ATP and the specific activators of this enzyme, calcium, phosphatidylserine, and the phorbol ester PMA. Protein phosphorylation catalyzed by kinases other than protein kinase C was measured in the presence of only calcium, and this background, which was typically 20-25% of total phosphorylation, was subtracted from the measurement of total phosphorylation to calculate specific phosphorylation catalyzed by protein kinase C.

In the cortex, the activity of protein kinase C in controls was 5.60 ± 0.75 and 4.17 ± 0.59 nmoles/min/mg protein (n=17) in the cytosolic and membrane fractions, respectively. In the hippocampus, control protein kinase C activity was 11.47 ± 2.07 and 4.77 ± 0.75 nmoles/min/mg protein (n=16) in the cytosolic and membrane fractions, respectively. Thus, in control cortex 43% of the protein kinase C was membrane-bound, while in the hippocampus this value was 29%.

Seizures were induced in rats by administration of pilocarpine (30 mg/kg; sc) to lithium-treated (3 mmole/kg/ ip; 20 hours prior) rats. Assays were carried out at several times after pilocarpine treatment, including 10 minutes (prior to seizure activity), 20 minutes (coincident with the initiation of seizures), 60 minutes (after 35 to 40 minutes of status epilepticus) and 120 minutes (after approximately 100 minutes of status epilepticus). Figures 31 and 32 show the effects of seizures on cytosolic and membrane bound protein kinase C activity in the cortex and hippocampus, respectively. In the cortex, treatment with lithium and pilocarpine and the resultant seizures caused no statistically significant changes in the protein kinase C activity in the cytosolic or membrane fractions. Thus, there was no evidence of translocation of protein kinase C to the membrane which is sometimes taken as an indication of activation of protein kinase C. To check these results we also induced seizures with the excitatory amino acid agonist, kainic acid (10 mg/kg). With this agent we tested periods of 20 minutes (n=4; when seizure activity begins) and of 96 minutes (n=2, when status epilepticus begins). Some rats were pretreated with lithium (3 mmole/kg; ip; 20 hour prior) followed by kainic acid and assays at 20 minutes (n=4) and 96 minutes (n=2). As with the lithium and pilocarpine-induced seizures, there was little change in the protein kinase C activity in cortical cytosolic or membrane fractions after treatment with kainic acid alone or in combination with lithium. Additionally, lithium treatment alone did not alter protein kinase C activity.

In the hippocampus (Figure 32) results were obtained very similar to those observed in the cortex. Thus, there was no alteration of protein kinase C activity in the cytosolic or membrane fractions 10, 20 or 120 minutes after pilocarpine administration to lithium-treated rats. There was an indication of decreased protein kinase C activity in both fractions 60 minutes after pilocarpine treatment, but this single alteration is difficult to explain considering the other lack of changes. Notably, treatment with lithium alone also tended to reduce the protein kinase C activity in both fractions.

As in the cortex, the results with the seizures induced by lithium and pilocarpine were checked using administration of the convulsant excitatory amino acid agonist kainic acid. Again there was little indication of any changes in protein kinase C activity. Additionally, calculation of the percent membrane-bound protein kinase C activity indicated that no significant translocation of protein kinase C to the membranes had occurred during seizures.

To ensure that our assays of protein kinase C activity were providing accurate measurements, we also employed an alternative method. With this method, the specific binding of a labelled phorbol ester ([^{3}H]PDBμ) was measured in membrane preparations. This ligand binds specifically to protein C kinase and thus measurement of its binding provides a quantitative assay of the protein kinase C present. Table 9 shows the results of the [^{3}H]PDBμ binding experiments.

These were carried out in membrane fractions from cortex and hippocampus from controls and from seizing rats treated previously with lithium and pilocarpine(25 or 60 minutes prior). There were no significant effects of seizures on [^{3}H]PDBµ binding at either time period and in either region. Thus, these results confirm our findings using protein phosphorylation as a measure of protein kinase C activity.

Protein tyrosine phosphorylation

Although many proteins are phosphorylated on serine or threonine amino acids, several critical proteins are phosphorylated on tyrosine amino acids. Specific tyrosine phosphoproteins can be detected using antibodies directed towards the phosphotyrosine residues. Therefore, this approach was utilized to identify the effects of seizures on the phosphorylation of tyrosines in endogenous proteins in rat brain.

Figure 33 shows phosphotyrosine immunoblots from homogenates of hippocampus and cortex prepared from rats that had been sacrificed by decapitation or microwave irradiation. Similar patterns were evident in both regions, with several prominent bands. Equivalent bands were present in samples using each method of sacrifice, indicating that microwave irradiation is appropriate for this method of analysis. Therefore microwave irradiation was used in all further experiments (except for those in which subcellular fractions were prepared) to optimize the detection of in vivo alterations which might be transient and sensitive to postmortem alterations after decapitation.

Status epilepticus was induced in rats by administration of pilocarpine (30 mg/kg; ip) to LiCl-treated (3 mmole/kg; ip; 20 hr prior) rats. This treatment induces paroxysmal spike activity and spike trains 15 to 20 min after pilocarpine, followed by generalized convulsive status epilepticus which continues unabated for several hours (11). Figure 34 shows that 60 min after pilocarpine treatment (during status epilepticus) there was increased phosphotyrosine associated with a 40 kD protein in the hippocampus, cerebral cortex, and striatum, while, at most, only slight changes were observed in the other phosphotyrosine proteins. The increases in the tyrosine phosphorylation of the 40 kD protein resulted in values greater than 3-fold those of controls in all three regions.

Figure 35 shows the time course of the increased tyrosine phosphorylation of the 40 kD protein in the hippocampus of acute lithium plus pilocarpine-treated rats. Samples were analyzed prior to paroxysomal spiking (10 min), during a period of intermittent spike trains (20 min), immediately after the first tonic-clonic seizure which signals the initiation of status epilepticus (which averaged 25 min), and early or late during status epilepticus. There was an abrupt increase in the phosphotyrosine content of the 40 kD protein at a time (25 min) coincident with the initiation of status epilepticus, and the increase was maintained to at least 120 min. No significant differences were observed in the 45, 60, and 180 kD proteins, while relatively slight increases in tyrosine phosphorylation occurred in the 50 kD protein during seizures and in the 110 kD protein at 60 min. Similar changes were observed in the cortex and acute lithium administration alone caused no significant changes (data not shown).

DISCUSSION

Modulation of phosphoinositide hydrolysis by excitatory amino acids

Glutamate inhibited [^{3}H]phosphoinositide synthesis and NE-stimulated [^{3}H]IP$_1$ production in rat cerebral cortical slices. Examination of selective agonists indicated that this effect was likely to be mediated by quisqualate-selective receptors. Stimulation of neuronal quisqualate-selective receptors has been shown to result in calcium influx and in mobilization of bound intracellular calcium, likely as a result of stimulation of phosphoinositide hydrolysis (35). The latter response is especially prominent in the neonatal brain, but is still evident in adults (36,37). Due to the lack of effective antagonists it is not known if the inhibitory effects of quisqualate reported in this study are mediated by one of the receptors mediating these processes or by another receptor. We suggested that the quisqualate-induced rise of intracellular calcium may be an intermediary step in the inhibitory effect of quisqualate in part because calcium also reduces the synthesis of [^{3}H]phosphoinositides from [^{3}H]inositol in brain slices (38), and calcium inhibits two key enzymes, phosphatidylinositol synthase and phosphatidylinositol phosphate kinase (60,61). Therefore it was necessary to examine if calcium mediated the inhibitory effects of glutamate or quisqualate, either directly or indirectly, such as through activation of phospholipase A$_2$ with release of arachidonic acid. Simultaneous examination of the effects of calcium on [^{3}H]phosphoinositide synthesis and agonist-stimulated [^{3}H]IP$_1$ production showed quite clearly that exogenous calcium had two opposing effects, as calcium reduced [^{3}H]phosphoinositide synthesis but promoted the production of [^{3}H]IP$_1$ in response to agonists. The limited responses to agonists in medium with reduced concentrations of calcium obstructed direct examination of the calcium requirement for the inhibitory response to glutamate. The results obtained, i.e. that increasing calcium resulted in increased glutamate-induced inhibition of [^{3}H]phosphoinositide synthesis and of NE-stimulated [^{3}H]IP$_1$ production, were consistent with the hypothesis that calcium mediates this inhibitory effect, but the dual effects of calcium limit the clarity of these results. It is noteworthy that under these conditions of both glutamate- and calcium-induced suppression of [^{3}H]phosphoinositide synthesis (Figure 1), there was not a significant inhibition of carbachol stimulated [^{3}H]IP$_1$ production. This suggests several possibilities, e.g., that the relevant receptors (i.e., quisqualate-selective) may be colocalized with α1-adrenergic receptors but not with phosphoinositide-coupled muscarinic receptors, or that the regulation of adrenergic and cholinergic-coupled phosphoinositide systems may differ.

Similarly, the experiments with verapamil were consistent with the calcium-dependence of the inhibitory effects of glutamate, since increasing concentrations of verapamil reduced glutamate-induced inhibition of NE-stimulated [^{3}H]IP$_1$ production. However, the direct inhibitory effects of verapamil on the response to NE obstructed a definitive conclusion. Overall, though, consistent effects of increased calcium were reduction of [^{3}H]phosphoinositide synthesis, enhanced production of [^{3}H]IP$_1$ in response to NE, and greater inhibition of both processes by glutamate.

We attempted to dissociate the complex actions of calcium by studying the response to agonists in slices that had been prelabelled with [^{3}H]inositol. [^{3}H]Phosphoinositide synthesis was enhanced in slices that had been prelabelled in the absence of added calcium (compared with the of addition of 1.3 mM calcium), but the response to NE was severely impaired. Slices exposed continuously to calcium displayed a strong response to NE and selective inhibitory effects by excitatory amino acids were observed. These data show again that inhibition of NE-stimulated [^{3}H]IP$_1$ production is selectively inhibited by quisqualate.

The quisqualate-selective receptor mediating this inhibitory effect is apparently a different subtype than those classically studied because neither the selective antagonist CNQX nor less selective antagonists blocked the inhibition. We have previously reported that L-BOAA also appears to selectively activate this inhibitory receptor (58), if indeed this is a receptor-mediated effect which cannot be finally concluded until an antagonist is available.

Excitatory amino acid-induced inhibition of NE-stimulated [^{3}H]IP$_1$ production in prelabelled slices raised the question whether this could be due to inhibition of [^{3}H]phosphoinositide synthesis. Even after thoroughly washing slices prelabelled with [^{3}H]inositol, much free [^{3}H]inositol is retained within the cells which can be used for [^{3}H]phosphoinositide synthesis. To test if [^{3}H]IP$_1$ generated by NE was dependent on continued synthesis of [^{3}H]phosphoinositides, high concentrations of unlabelled inositol were added to the medium to attempt to dilute the [^{3}H]inositol and thus block any further synthesis of [^{3}H]phosphoinositides. With 20 mM inositol, the generation of [^{3}H]IP$_1$ induced by NE was already reduced by 53% after 15 min. Since complete elimination of [^{3}H]phosphoinositide synthesis cannot be assured, this should be taken as a minimum estimate of the required regeneration of [^{3}H]phosphoinositides for maximal NE response. This suggests that the pool of [^{3}H]phosphoinositides coupled to the α1-adrenergic receptor is relatively small and that it can be depleted rather quickly leading to a diminished response to NE. Thus, although this result does not show that this is the mechanism of the inhibitory effect of amino acids, it demonstrates that such a sequence of events can occur even in prelabelled slices.

Modulation of phosphoinositide hydrolysis by arachidonic acid

There are many mechanisms by which calcium might mediate the inhibitory effects of glutamate on the phosphoinositide system. It was important to examine if arachidonic acid might mediate this process. This hypothesis was based on the following evidence: (i) glutamate has been reported to stimulate phospholipase A$_2$ in brain tissue (52), possibly as a consequence of elevated intracellular calcium concentration, and (ii) arachidonic acid is one of the only other effectors besides excitatory amino acids that has been reported to inhibit the synthesis of phosphoinositides (62). Although a cause-and-effect relationship cannot be established in the heterogenous brain slice preparation, by testing the effects of arachidonic acid we were able to confirm that it is a potential mediator of the inhibitory effects of glutamate. In fact, the effects of arachidonic acid were extremely interesting because it caused a more global, less specific, inhibitory effect than did glutamate. Arachidonic acid caused a concentration-dependent reduction of [^{3}H]phosphoinositide synthesis, and [^{3}H]IP$_1$ produced in response to either NE or carbachol was inhibited. When the inhibition of [^{3}H]phosphoinositide synthesis was taken into account by calculating the ratio of [^{3}H]IP$_1$/([^{3}H]IP$_1$ + [^{3}H]phosphoinositides), this value remained constant throughout the arachidonic acid concentration range that was examined. This shows that the reduction of [^{3}H]IP$_1$ production caused by arachidonic acid was matched by the reduction of

[^{3}H]phosphoinositide synthesis. This strongly suggests that the two effects are directly associated, as well as that the effect of arachidonic acid applies to [^{3}H]phosphoinositide synthesis uniformly (rather than a cell-specific effect) as might be expected from a modulator not acting through cell-specific receptors. This calculated ratio provides an interesting contrast to the same value calculated following application of quisqualate with NE, which reduced the ratio by approximately half. We postulate that this is because quisqualate does not inhibit [^{3}H]phosphoinositide synthesis globally, but its effects are restricted due to the limited distribution of quisqualate-selective receptors which are apparently at least partly colocalized with α1-adrenergic receptors. Thus, [^{3}H]IP$_1$ produced in response to NE is reduced by quisqualate to a greater extent than is the overall [^{3}H]phosphoinositide synthesis, resulting in a reduced ratio.

An obvious test of the possibility of a connection between glutamate-activated phospholipase A$_2$ activity and its inhibitory effects on the phosphoinositide system would be to measure the effects of inhibitors of phospholipase A$_2$. However, the inhibitors that are available are known not to be specific and we found that chloroquine, BPB and quinacrine (not shown) each inhibited NE-induced [^{3}H]IP$_1$ formation, possibly due to direct inhibition of phospholipase C. Alternatively, we investigated if activation of phospholipase A$_2$ by melittin affected phosphoinositide metabolism and found that this agent produced a significant inhibition of [^{3}H]phosphoinositide synthesis and [^{3}H]IP$_1$ production. This inhibitory effect of melittin could be due to liberation of arachidonic acid, but it also could be due to other perturbations of the membrane caused by melittin.

We also attempted to examine whether the effects of arachidonic acid were direct or were mediated by a product of its metabolism. In the concentrations tested, neither inhibitors of cyclooxygenase nor of lipoxygenase blocked the inhibition by arachidonic acid, suggesting that this may be a direct effect of arachidonic acid. However, solubility limitations with these drugs limited the concentrations that could be tested (we find significant inhibitory effects of NE-stimulated [^{3}H]IP$_1$ production by low concentrations of ethanol and DMSO), and their effects on arachidonic acid metabolism were not measured, although similar concentrations of these drugs have been reported to effectively inhibit arachidonic acid metabolism (63).

These results indicate that the inhibitory effects of the excitatory amino acids may be mediated by activation of phospholipase A_2 and liberation of arachidonic acid. However, a direct demonstration of this relationship remains to be tested.

Modulation of phosphoinositide hydrolysis by GABA

The inhibitory neurotransmitter, GABA, had modulatory effects on phosphoinositide that in general contrasted with those of the excitatory amino acids. In agreement with previous reports (42), GABA caused little or no direct activation of [^{3}H]phosphoinositide hydrolysis, but it enhanced the response to NE, especially in hippocampal slices. Enhancement by GABA (10 mM) of [^{3}H]inositol phosphate produced in response to 1 μM NE was reported previously (42). However, we found that lower concentrations of GABA effectively enhanced the response to NE in cortical and hippocampal slices and that 300 μM GABA potentiated the response to NE throughout its effective concentration range. In cortical slices, baclofen also potentiated the response to NE, suggesting that the modulatory effect of GABA is mediated by the $GABA_B$ receptor. Baclofen did not alter the inhibitory effect of quisqualate, but it reduced that of arachidonic acid. While GABA produced a substantially enhanced response to NE, and smaller, but in some regions significant, enhanced responses to carbachol and ibotenate, it is interesting to note that GABA and baclofen have been reported to inhibit phosphoinositide hydrolysis induced by histamine (64) or serotonin (65) in cortical slices from rat and mouse, respectively.

The inhibitory effects of excitatory amino acids on phosphoinositide synthesis and NE-stimulated hydrolysis of phosphoinositides may provide a mechanism for enhancement of the stimulatory effect of glutamate, by reduction of inhibitory influences on NE in specific neuronal networks. Also, if calcium is indeed the mediator of this interaction, this may provide a protective negative feedback mechanism whereby relatively long-term increases of intracellular calcium impair further production of IP_3, and its release of bound calcium, even in the face of continued receptor activation. On the other hand, the observed effects of the excitatory amino acids may represent one of the neurotoxic consequences of these agents which eventually leads to cell death.

Thus, these results are consistent with the hypothesis that modulatory effects of excitatory amino acids on the phosphoinositide system may play an important role in neurotoxicity associated with neurodegenerative conditions, such as seizures. Furthermore, since GABA is well known to cause inhibitory, anticonvulsive responses, the finding that GABA has oppossing effects to those of the excitatory amino acids on phosphoinositide metabolism further substantiates the conclusion that modulation of this system plays an important role in seizure-induced brain damage.

Modulation of phosphoinositide hydrolysis by MK-801, an NMDA antagonist

MK-801 is one of the most widely used NMDA receptor antagonists, both as an anticonvulsant and to block brain damage induced by seizures and other insults. MK-801 caused an increase in phosphoinositide hydrolysis in a concentration-dependent manner. Although the concentration of MK-801 required to produce this effect appears relatively high, it is notable that similar concentrations of classical neurotransmitter agonists, such as carbachol, are required to produce maximal phosphoinositide hydrolysis in brain slices (10). Additionally, the maximal stimulation of phosphoinositide hydrolysis induced by MK-801 was similar to that produced by other agonists that induce this response. In contrast to the noncompetitive NMDA antagonist MK801, the competitive NMDA antagonist AP-7 had no effect on $[^3H]$-IP_1 production. Also PCP, another non-competitive antagonist which is much less potent than NMDA, produced a relatively small stimulation and only at high concentrations. A number of drugs known to affect MK-801 binding to the NMDA receptor ion channel (66,67) including NMDA, glycine (alone or in combination), Mg^{2+}, and Zn^{2+} did not alter the response to MK-801. Interestingly, administration of relatively high doses of MK-801 has been reported to cause seizure activity (16,68) and also vacuolization of particular neurones in the rat brain (69). Thus, there is increasing evidence that the effects of MK-801 are complex, and the findings reported here may be related to some of these poorly understood responses to MK-801. These observations are not without precedent as the competitive NMDA antagonist AP-5, at similar concentrations to those of

MK-801 used in this study, was shown to act at a non-NMDA receptor site (70). Further experiments are needed to identify this novel MK-801 binding site, its properties, and distribution, but they are beyond the goals of this project. It is notable that the stimulatory effects of MK-801 on phosphoinositide hydrolysis support our previous observations and conclusions with excitatory and inhibitory amino acids. Thus, agents which are protective towards brain damage, GABA and MK-801, each enhanced phosphoinositide hydrolysis though clearly by different mechanisms. On the contrary, the excitatory amino acids which appear to participate in the induction of brain damage under a variety of neurotoxic conditions, are able to inhibit agonist-induced phosphoinositide hydrolysis.

Modulation of phosphoinositide hydrolysis by Na^+

Na^+ was found to have a strong modulatory influence on phosphoinositide hydrolysis in rat brain slices and this modulation was selective for only some of the agonists which activate this system. Most notable was the Na^+-sensitivity of the responses to quisqualate. Quisqualate can directly stimulate phosphoinositide hydrolysis and it also inhibits norepinephrine-stimulated phosphoinositide hydrolysis (38,57). Reduction of the Na^+ concentration increased both the efficacy and the potency of the stimulatory response to quisqualate. Thus, low Na^+ converted what was a small quisqualate response requiring relatively high concentrations of quisqualate in normal Na^+ to a strong response which was greater than that of ibotenate and was evident even at a very low quisqualate concentration. The suboptimal stimulation by quisqualate of phosphoinositide hydrolysis observed in high concentrations of Na^+ suggests that Na^+ limits this interaction in many situations and that under appropriate conditions quisqualate can activate phosphoinositide metabolism to a much greater extent than was recognized previously. In contrast to the enhancement by low Na^+ of the stimulatory response to quisqualate, low Na^+ eliminated quisqualate-induced inhibition of norepinephrine-stimulated phosphoinositide hydrolysis. These two opposing effects of quisqualate precluded simple delineation of whether the inhibitory modulation was blocked by low Na^+ or if it was masked by the enhanced stimulation produced by quisqualate in low Na^+. Thus, we utilized the excitatory amino acid L-BOAA to solve this problem. L-BOAA mimicks the inhibitory, but not the stimulatory, response to quisqualate (58). The finding that low Na^+ also blocked the inhibition induced by L-BOAA indicates that the two responses to quisqualate are separate processes and they are differentially influenced by the concentration of Na^+. Thus, low Na^+ enhanced the stimulation of phosphoinositide hydrolysis induced by quisqualate and blocked its inhibitory effect on the response to norepinephrine. These results indicate that if the Na^+ concentration is altered it will have an important influence on the interactions between quisqualate and phosphoinositide metabolism. The results with quisqualate also suggest that responses measured in 120 mM Na^+ are due to a summation of the stimulatory and inhibitory responses to quisqualate and that lowered Na^+ serves to separate these two processes so the stimulatory response is more evident at lower quisqualate concentrations while the inhibitory effect is only observed at 1 mM quisqualate. Others have also reported that omission of Na^+ from the incubation medium reduced inhibition by N-methyl-D-aspartate of carbachol-stimulated phosphoinositide hydrolysis in hippocampal slices (71,72). Thus, physiological concentrations of Na^+ may be a common requirement for one mechanism by which excitatory amino acids inhibit agonist-stimulated phosphoinositide hydrolysis and the selective interactions among excitatory amino acid agonists and other neurotransmitter systems may be dependent upon the receptor distributions. Glutamate also inhibited norepinephrine-stimulated phosphoinositide hydrolysis but, unlike quisqualate, this effect of glutamate was not blocked by reduced Na^+. This may be because glutamate induces inhibition by a different mechanism, such as by activation of phospholipase A_2 and subsequent inhibition of phosphoinositide hydrolysis by arachidonate (57), or because the effect of Na^+ is only evident with the more structurally restricted excitatory amino acid analogs.

The major observed effects of lowered Na^+ concentration on phosphoinositide metabolism, including increased basal hydrolysis, increased stimulatory responses to norepinephrine and quisqualate, and blocked inhibitory effects of quisqualate and L-BOAA may be due to multiple mechanisms. The binding characteristics of a number of neurotransmitter and hormone receptors are influenced by the Na^+ concentration. For example, Na^+ significantly reduced the quisqualate-sensitive [^{3}H]glutamate binding in a soluble preparation from rat adrenal (73). Other investigators have suggested that enhanced phosphoinositide hydrolysis occurring in low Na^+ may be due to Na^+/Ca^{2+} exchange resulting in increased intracellular Ca^{2+} (74). This may be especially relevant to the increased basal rate of phosphoinositide hydrolysis in low Na^+ since increased intracellular Ca^{2+} caused by depolarizing concentrations of K^+ also increases phosphoinositide hydrolysis. However high K^+ has little effect on phosphoinositide hydrolysis induced by norepinephrine or quisqualate, suggesting that an additional mechanism is required to explain the enhanced responses with these agonists. Furthermore, the response to carbachol is greatly potentiated by high K^+ but is unaffected by low Na^+, indicating that increased intracellular Ca^{2+} may not be responsible for the enhanced responses to agonists in low Na^+. Simply reducing the Ca^{2+} in the media cannot be used to test the Ca^{2+}-dependency of the effect of low Na^+ because the agonist responses are greatly impaired in the absence of Ca^{2+} in brain slices.

The present results may be due in part to modulation by Na^+ of G-proteins mediating phosphoinositide hydrolysis, as G-proteins associated with cyclic AMP production have previously been shown to be influenced by Na^+. The inhibitory G-protein, G_i, associated with the cyclic AMP system has previously been reported to be Na^+-dependent, so that physiological concentrations of Na^+ are required for optimal activity of neurotransmitters or hormones which inhibit adenylate cyclase (75,76). Complex tissue- and brain region-dependent effects of Na^+ have been reported in adenylate cyclase studies and precise mechanisms of action of Na^+ are not clarified (77). Nevertheless, it is interesting to speculate that an inhibitory G-protein may be associated with some phosphoinositide systems in the brain, such as the inhibition of norepinephrine-stimulated phosphoinositide hydrolysis by quisqualate, and that such an inhibitory G-protein may be inactive in low Na^+ in analogy with the cyclic AMP system. As suggested by Duman et al. (77) Na^+ flux associated with neuronal activity may modulate second messenger activity by actions on G-proteins.

Tetrodotoxin blocks voltage-dependent Na^+ channels. It was found that tetrodotoxin selectively inhibited carbachol-induced phosphoinositide hydrolysis while not affecting that induced by norepinephrine or quisqualate. This may signify that cholinergic muscarinic receptor responses are uniquely influenced by Na^+ channel activity in the brain.

These findings may be directly relevant in identifying the neurochemical responses to seizures induced by cholinergic agonists or other convulsants and the subsequent brain damage that occurs. Influx of Na^+ accompanies seizures, so it seems highly likely that the modulation by Na^+ of phosphoinositide hydrolysis induced by quisqualate and norepinephrine that we identified will induce altered responses of this important second messenger-inducing system.

Protein kinase C in brain of control and lithium-treated rats

Methods were established to measure the activity of protein kinase C since this enzyme is believed to be regulated in concert with phosphoinositide metabolism. Two procedures were used, including measurements of the total activity of protein kinase C in membrane and cytosolic preparations from rat brain and measurements of the degree of phosphorylation of endogenous proteins by activation of protein kinase C. Initial experiments were carried out to establish the methods for these assays and to determine if treatment with lithium alone altered these processes.

Following lithium treatment, no change in either the distribution or activity of protein kinase C was detected. The enzyme distribution, which was about 40% cytosolic and 60% particulate, was comparable to previous reports of protein kinase C distribution in brain (44).

The pattern of endogenous protein phosphorylation in particulate and soluble fractions corresponded closely with previous characterizations of protein kinase C and cAMP-dependent phosphorylation (78-80). Under basal conditions or with addition of calcium, no effects of chronic lithium treatment were observed.

Lithium treatment resulted in significant reductions of the protein kinase C-mediated phosphorylation of three proteins and of the cAMP-dependent phosphorylation of two proteins in the hippocampal particulate fractions. The selectivity of these changes suggests that they are not due to a global, non-specific effect of lithium on the phosphorylation-dephosphorylation process, but may reflect specific responses to lithium treatment. The mechanisms involved and the identities of the affected proteins remain to be established. These changes may coincide with the selective effects of chronic lithium treatment on phosphoinositide hydrolysis and cyclic AMP synthesis, as the effects of lithium treatment on these second messenger systems appear to be specific for certain cells and neurotransmitter systems. Additionally, lithium treatment could influence specific protein kinase C isozymes present in brain (81). Differential effects on protein kinase C-mediated phosphorylation are certainly not without precedent, as previous studies have reported selectivity of the proteins phosphorylated by protein kinase C dependent upon the experimental conditions (82).

Examination of protein phosphorylation in hippocampal soluble fractions revealed less distinct effects of lithium treatment compared with the particulate fractions. There were no significant changes of cAMP-dependent phosphorylation and a widespread, and somewhat variable, increase of protein kinase C-mediated phosphorylation of proteins in the soluble fraction following lithium treatment. The variable nature of this response may be due to inherent interindividual variations in the response to chronic lithium treatment in combination with the complex interactions that modulate protein phosphorylation and its measurement.

In summary, protein kinase C activity was measured in cytosolic and membrane prepartions from rat brain. Additionally, endogenous proteins that were phosphorylated by either protein kinase C or cAMP-dependent protein kinase were identified. Lithium treatment had very limited effects on these processes.

Effects of seizures on phosphoinositide hydrolysis and protein kinase C

The results detailed previously in this report indicated that seizures may be associated with reduced phosphoinositide hydrolysis. Thus seizures were induced by coadministration of lithium and pilocarpine. It was found that the ensuing seizures were associated with significant reductions of phosphoinositide hydrolysis induced by norepinephrine or ibotenate. The impairment in the response to norepinephrine was especially prominent and occurred rapidly upon induction of seizures.

These findings are consistent with our previous results. Thus, excitatory amino acids which play a prominent role in seizure-induced brain damage impair norepinephrine-stimulated phosphoinositide hydrolysis and this process is also impaired by in vivo seizure activity. Norepinephrine is well-known to have inhibitory, anticonvulsive effects and it appears likely that inhibition of this function of norepinephrine plays an important role in seizures and the resultant brain damage. Also consistent with this conclusion are our findings that the inhibitory neurotransmitter GABA enhances norepinephrine-stimulated phosphoinositide hydrolysis. Thus, the modulation of the activity of this second messenger system appears to be closely linked with seizure activity.

In contrast to the clear effects of seizures on agonist-induced phosphoinositide hydrolysis, seizures did not alter the activity or intracellular localization of protein kinase C. This lack of effect of seizures induced by cholinergic stimulation was confirmed in two ways: (i) seizures induced the excitatory amino acid agonist kainate also did not alter protein kinase C activity, and (ii) another method to assay protein kinase C, [^{3}H]PDBu binding, also indicated no significant effect of seizures on protein kinase C.

This extensive series of experiments on protein kinase C activity clearly rules out major alterations in the activity or intracellular location of this enzyme in playing an important role in cholinergic agonist-induced seizures. It remains possible, however, that there are selective alterations in protein kinase C activity in a very restricted number of cells and that this limited change is impossible to detect over the unaltered activity of the majority of the protein kinase C present in brain regions. It is presently not possible to study protein kinase C activity after seizures in individual cells, so this possibility, though unlikely, cannot entirely be ruled out.

Protein tyrosine phosphorylation in rat brain

For a complete analysis of the effects of seizures on protein phosphorylation in rat brain, we also employed methods to specifically detect the phosphorylation of tyrosine amino acids on endogenous proteins. Although most proteins are phosphorylated on serine or threonine amino acids, the activity of several proteins which are critical for signal transduction have been shown to be modulated by phosphorylation of tyrosines.

Using immunoblots probed with a monoclonal antibody to phosphotyrosines, several protein bands containing phosphotyrosine residues were observed in rat brain. There were no major differences in the phosphotyrosine proteins that were detectable among the three regions that were studied, the cerebral cortex, hippocampus, and striatum. Most of these phosphotyrosine proteins were unaffected by seizures induced by administration of lithium plus pilocarpine. This general lack of response emphasizes the specificity of the large increases in the tyrosine phosphorylation of the 40 kD protein that were observed. This increase did not preceed seizure activity induced by lithium and pilocarpine, but occurred rapidly upon initiation of generalized convulsive status epilepticus. After the initial abrupt rise there was little further increase, and no decrease, during continued seizures. This response was evident in all regions that were examined and occurred with two convulsive stimuli having different mechanisms of action. Thus, the increased tyrosine phosphorylation of the 40 kD protein is a specific response, and it is maintained for long periods of time during status epilepticus, whereas most phosphotyrosine proteins were unaltered by seizures.

The molecular mass and subcellular location of the responsive 40 kD phosphotyrosine protein suggests that it is the same one as observed by Stratton et al. (83,84) which responded to carbachol and to protein kinase C activation in hippocampal slices and to maximal electroconvulsive shock. Those authors concluded that the specific tyrosine phosphorylation of the 40 kD protein occurred in response to phosphoinositide hydrolysis-induced activation of protein kinase C. This is consistent with other results from this laboratory in which we have found a massive stimulation of phosphoinositide hydrolysis associated with initiation of seizures induced by lithium plus pilocarpine (Jope, unpublished data). The association of this process to muscarinic receptor activation as found by Stratton et al. (83) in hippocampal slices, is also supported by the observation that administration of the cholinergic agonist pilocarpine alone induced increased tyrosine phosphorylation of the 40 kD protein. The increased tyrosine phosphorylation of the 40 kD protein associated with seizures may be due to protein kinase C-induced activation of a specific tyrosine kinase as suggested by Stratton et al. (83). The tyrosine phosphorylation of other proteins was also increased by pilocarpine, notably a 50% increase in the 45 kD protein in the cortex, indicating further associations between muscarinic receptor activation and tyrosine phosphorylation.

CONCLUSIONS

Although this work is still in progress, several firm conclusions can be drawn from the studies completed to date and recommendations for emphasis of further investigations can be formulated.

First, it is very evident that excitatory and inhibitory amino acid receptor agonists have important modulatory influences on phosphoinositide metabolism. The effects of the excitatory amino acids were generally opposite to those of the inhibitory amino acid GABA. This finding complements very well the knowledge that excitatory amino acids promote seizures and brain damage whereas inhibitory amino acids are protective. The mechanisms whereby these agents act are not completely clear, but supporting evidence was obtained that the effects are mediated in part by increased intracellular calcium and possibly by production of arachidonic acid. Na^+ was also implicated as a modifying influence. However, further clarification of the precise mechanisms is needed.

It is also evident that there is a close association between cholinergic agonist-induced seizures and alteration of phosphoinositide metabolism. This is important since phosphoinositide hydrolysis is such a critical second messenger-producing system in the brain and it functions in part to modulate the intracellular concentration of calcium. Further work must be done to identify the mechanistic association between altered phosphoinositide metabolism and seizures and brain damage.

Protein kinase C activity was not sensitive to cholinergic-agonist induced seizures. This was confirmed in a number of ways and indicates that further investigations of this system are unlikely to be productive.

In contrast, activation of tyrosine-specific kinases was discovered to be linked with seizures induced by cholinergic agonists. Phosphorylation of tyrosine amino acids is known to be an important method of modulation of the activities of several proteins critical in signal transduction. Thus, further study of this aspect of protein phosphorylation is clearly warranted.

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Table 1. Comparison of inhibitory effects of arachidonic acid on inhibition of [^{3}H]phosphoinositide synthesis and of [^{3}H]IP$_1$ production.

Rat cortical slices were incubated with [^{3}H]inositol and arachidonic acid (AA), at the concentrations indicated, with either NE (100 µM) or carbachol (2 mM). [^{3}H]Phosphoinositides and [^{3}H]IP$_1$ values are calculated from the data shown in Figures 5 and 7.

Ratio = [^{3}H]IP$_1$/[^{3}H]IP$_1$+[^{3}H]phosphoinositide

Agonist	AA	% Inhibition		Ratio
	(µM)	PI	IP$_1$	
NE	-	-	-	16.7±0.9
NE	10	3±2	3±3	16.7±0.7
NE	20	10±6	10±8	17.4±0.2
NE	50	22±10	16±10	17.9±0.8
NE	100	28±9	17±9	18.8±1.1
NE	200	43±7	35±9	18.7±0.7
NE	500	55±7	55+9	16.6±0.9
Carbachol	-	-	-	7.9±0.5
Carbachol	200	45±2	40±3	8.8±0.9

Table 2. Effects of agents which alter phospholipase A_2 activity on $[^3H]$phosphoinositide synthesis and $[^3H]IP_1$ production.

Cortical slices were incubated simultaneously with $[^3H]$inositol, 100 µM NE where indicated (+), 500 µM quisqualate (QA) where indicated (+), and the indicated agents (final concentrations were 100 µM, except for melittin which was 20 µg/ml) for 60 min, and $[^3H]$phosphoinositide synthesis and $[^3H]IP_1$ production were measured as described in Methods. Each value represents the mean ± SE of two experiments measured in triplicate. (BPB = bromphenacyl bromide). Ratio = $[^3H]IP_1/[^3H]IP_1 + [^3H]$phosphoinositides.

Addition	NE	QA	$[^3H]$phosphoinositide (cpm x 10^{-3})	$[^3H]IP_1$ (cpm x 10^{-3})	Ratio
-	-	-	31.5±0.9	0.9±0.1	2.8
-	+	-	32.8±1.4	7.8±0.4	19.1
Melittin	+	-	15.2±0.9	3.8±0.2	20.1
Chloroquine	+	-	25.3±0.6	4.8±0.1	15.9
BPB	+	-	21.7±1.7	4.2±0.3	16.4
Dexamethasone	+	-	26.9±1.1	7.1±0.2	20.8
-	+	+	17.1±0.7	1.5±0.1	7.8
Chloroquine	+	+	14.6±0.6	1.0±1.0	6.5
BPB	+	+	13.4±1.0	1.2±0.1	8.3
Dexamethasone	+	+	14.8±1.0	1.4±0.1	8.8

Table 3. Comparison of inhibitory effects on [^{3}H]phosphoinositide synthesis and on Mn^{2+}-activated incorporation of [^{3}H]inositol into lipids by the base exchange reaction.

Rat cortical slices were incubated with [^{3}H]inositol, and where indicated, 100 µM NE,1 mM glutamate (Glu), 0.5 mM quisqualate (QA), or 200 µM arachidonic acid (AA), in the absence (control) or presence of 1 mM $MnCl_2$ (to stimulate the base exchange reaction) for 60 min followed by measurement of [^{3}H]phosphoinositides as described in the Methods. Values are means ± SE of three experiments, each measured in triplicate. *$p < 0.05$ compared with the value obtained with NE.

	[^{3}H]Phosphoinositide			
Addition	Control (cpm x 10^{-3})	Percent Inhibition	$MnCl_2$ (cpm x 10^{-3})	Percent Inhibition
NONE	26.6±1.1	-	434.2±16.5	-
NE	29.6±1.7	-	445.0±14.1	-
NE + Glu	15.0±0.8*	48	399.6±18.0	10
NE + QA	19.2±0.9*	35	411.8±12.7	7
NE + AA	10.6±0.7*	64	357.1±18.9*	20

Table 4. NE-stimulated [^{3}H]IP$_1$ production in the presence of various modulators.

Cortical slices were prelabelled with [^{3}H]inositol for 60 min followed by several washes. Prelabelled slices were then incubated for 60 min in the presence of NE (100 μM) or NE and quisqualate (QA; 500 μM) in the absence or presence of furosemide (500 μM), diisothiocyanotostilbene-2,2-disulfonic acid (DIDS; 500 μM), or α-difluoromethylornithine (DFMO; 10 mM), and [^{3}H]IP$_1$ was measured. The results are expressed as [^{3}H]IP$_1$ formed as a percentage of total {^{3}H]. Each value represents the mean ± SE of two experiments measured in triplicate.

Addition	NE	QA	[^{3}H]IP$_1$
-	-	-	0.3±0.1
-	+	-	3.2±0.3
Furosemide	+	-	3.8±0.2
DIDS	+	-	4.0±0.2
DFMO	+	-	4.1±0.3
-	+	+	1.5±0.1
Furosemide	+	+	1.2±0.1
DIDS	+	+	1.8±0.1
DFMO	+	+	1.0±0.1

Table 5. Protein kinase C activity in soluble and particulate fractions from hippocampus of control and chronic lithium-treated rats.

Protein kinase C activity was measured as described in the Methods and was calculated as the difference between activity measured in the presence of 1.5 mM $CaCl_2$ from that observed in the presence of $CaCl_2$,10 µg PS, and 1 µM PMA. Results are expressed as means ± SEM (n = 8-9) of quadruplicate determinations.

Fraction	Specific Activity (nmol/min/mg protein)		Total Activity (nmol/min)		Distribution (%)	
	Control	Lithium	Control	Lithium	Control	Lithium
SOLUBLE	41 ± 2	36± 2	75 ± 7	74 ± 8	39%	39%
PARTICULATE	15 ± 1	14 ± 1	116 ± 4	113 ± 6	61%	61%

Table 6. Effects of chronic lithium treatment on protein phosphorylation mediated by protein kinase C or cAMP-dependent kinase in the hippocampal particulate fraction.

Phosphorylation of particulate proteins was measured as described in the legends to Fig. 1 and 2 and the Methods. Phosphorylation of proteins after chronic lithium treatment is expressed as the percent of control values (peak height of lithium-treated/peak height of control X 100) after subtracting phosphorylation observed in the presence of calcium alone for protein kinase C or theophylline alone for cAMP-dependent phosphorylation. (Mean ± SEM; $n = 5\text{-}9$ for protein kinase C, $n=8\text{-}10$ for cAMP; *$p < 0.05$, Wilcoxon signed-rank test).

PROTEIN KINASE C		CYCLIC AMP	
PHOSPHOPROTEIN MOLECULAR MASS (kD)	PERCENT CONTROL	PHOSPHOPROTEIN MOLECULAR MASS (kD)	PERCENT CONTROL
16	101 ± 2	47	97 ±14
18	84 ± 6*	54	92 ± 3*
19	94 ± 2*	71	87 ± 6*
45	96 ± 7	76	87 ± 5
76	92 ± 9	38	88 ± 7
87	81 ± 6*	260	92 ± 5
158	99 ± 9		
200	101± 8		

Table 7. Effects of chronic lithium treatment on protein kinase C-mediated protein phosphorylation in the hippocampal soluble fraction

Phosphorylation of soluble proteins mediated by protein kinase C was measured as described in the legend to Fig. 3 and the Methods. Phosphorylation of proteins after chronic lithium treatment is expressed as the percent of control values (peak height of lithium-treated/peak height of control X 100) after subtracting phosphorylation observed in the presence of calcium alone. (Mean ± SEM; n = 7 - 8; **p < 0.05; *p = 0.06, (n = 5); Wilcoxon signed-rank test.

PHOSPHOPROTEIN MOLECULAR MASS (kD)	PERCENT CONTROL
16	156 ± 24**
17	225 ± 34**
20	203 ± 29**
22	240 ± 35*
24	188 ± 43
26	141 ± 27
28	145 ± 30
30	148 ± 33
36	160 ± 43
43	100 ± 17
45	120 ± 15
50	95 ± 7
56	102 ± 26
71	119 ± 14
80	106 ± 4
250	94 ± 8

Table 8. Effects of modulators on phosphoinositide hydrolysis

Hippocampal slices prelabelled with [^{3}H]inositol were incubated with norepinephrine (100 μM) in the absence or presence of quisqualate (300 μM) or with ibotenate (1 mM) in the absence or presence of AP-4 (1 mM). Quisqualate and AP-4 inhibited each response similarly in each treatment group. The number of experiments measured in triplicate are indicated in parenthesis.

	Phosphoinositide Hydrolysis (% Inhibition)		
	Control	Lithium	Li + Pilocarpine (25 min)
NE + QA	67 ± 6 (4)	71 ± 2 (3)	71 ± 9 (4)
IBO + AP-4	82 ± 4 (3)	91 ± 7 (2)	88 ± 2 (3)

Table 9. Protein kinase C associated with membranes as measured by $[^3H]$PDBμ binding.

Membranes were prepared from the cortex and hippocampus of rats, including controls and rats given LiCl (3 mmole/kg; ip; 20 hours prior) and pilocarpine (25 or 60 minutes prior to sacrifice). The treated rats were in the initial stages of seizures (25 minutes) or were undergoing status epilepticus (60 min). Means ± SEM (n=3 per group).

$[^3H]$PDBμ Bound

	Control	Li + Pilo (25 min)	Li + Pilo (60 min)
		B_{max} (pmole/mg protein)	
Hippocampus	33.6 ± 2.3	35.4 ± 5.2	39.1 ± 4.1
Cortex	36.6 ± 3.2	36.3 ± 7.0	35.5 ± 4.6
		K_D (nM)	
Hippocampus	0.99 ± 0.10	1.02 ± 0.10	1.13 ± 0.10
Cortex	1.30 ± 0.20	1.45 ± 0.20	0.97 ± 0.20

FIGURE LEGENDS

Figure 1. Calcium dependence of [^{3}H]phosphoinositide synthesis and hydrolysis.

Rat cerebral cortical slices were incubated simultaneously with [^{3}H]inositol and the indicated agents for 60 min, with the incubation media containing: EGTA (1 mM) without added calcium; no added calcium; 0.1 mM calcium; or 1.3 mM calcium. With each incubation condition, the slices were treated with no addition (basal), NE (100 μM), NE and glutamate (500 μM), carbachol (2 mM), or carbachol and glutamate (500 μM). [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate. For basal, NE and carbachol, each increase of the calcium in the incubation medium produced significantly ($p < 0.05$) increased production of [^{3}H]IP$_1$ and significantly decreased synthesis of [^{3}H]phosphoinositides. *$p < 0.05$ indicates significant effects of glutamate compared with the results with each agonist in the absence of glutamate.

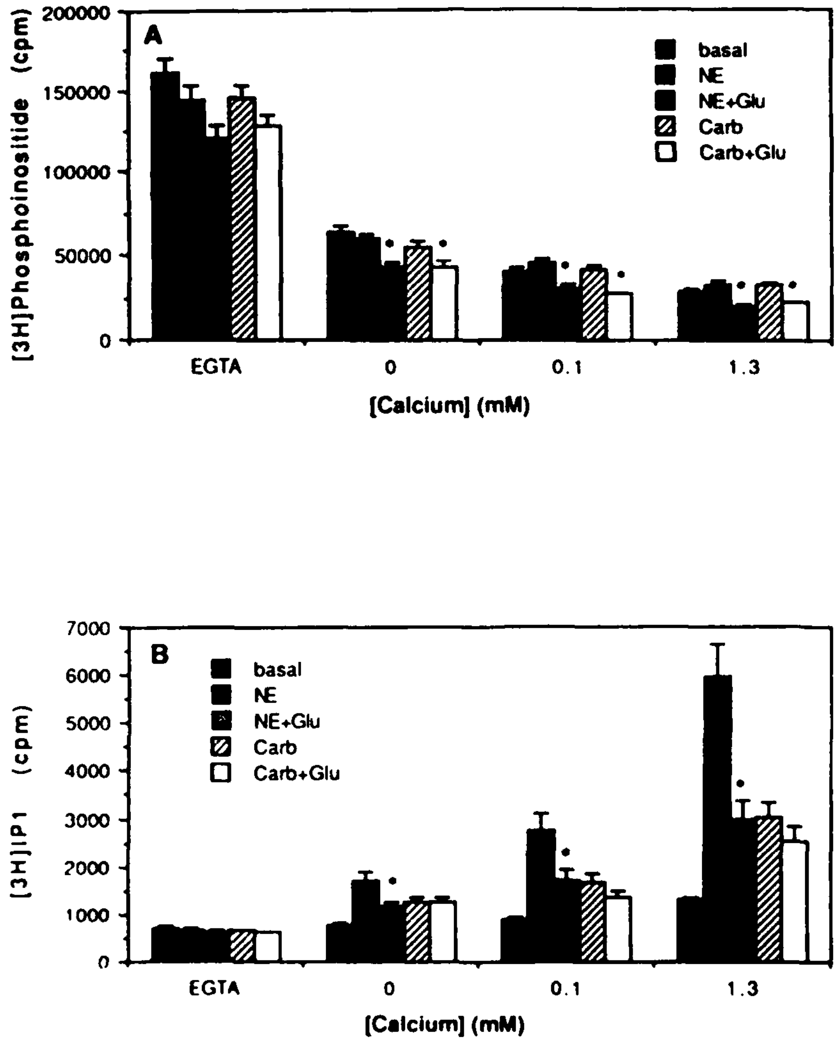

Figure 2. Effects of varapamil on [^{3}H]phosphoinositide synthesis and hydrolysis.

Rat cortical slices were incubated simultaneously with [^{3}H]inositol and verapamil (0-100 μM) for 10 min followed by 60 min incubation with no addition (□), NE (100 μM) (○) or NE (100 μM) plus glutamate (500 μM) (●). [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate. All values obtained with NE plus glutamate were significantly ($p < 0.05$) less than those obtained with NE, except for [^{3}H]IP$_1$ in the presence of 75 or 100 μM verapamil.

*$p < 0.05$ indicates significant effects of verapamil compared with the results obtained in the absence of verapamil.

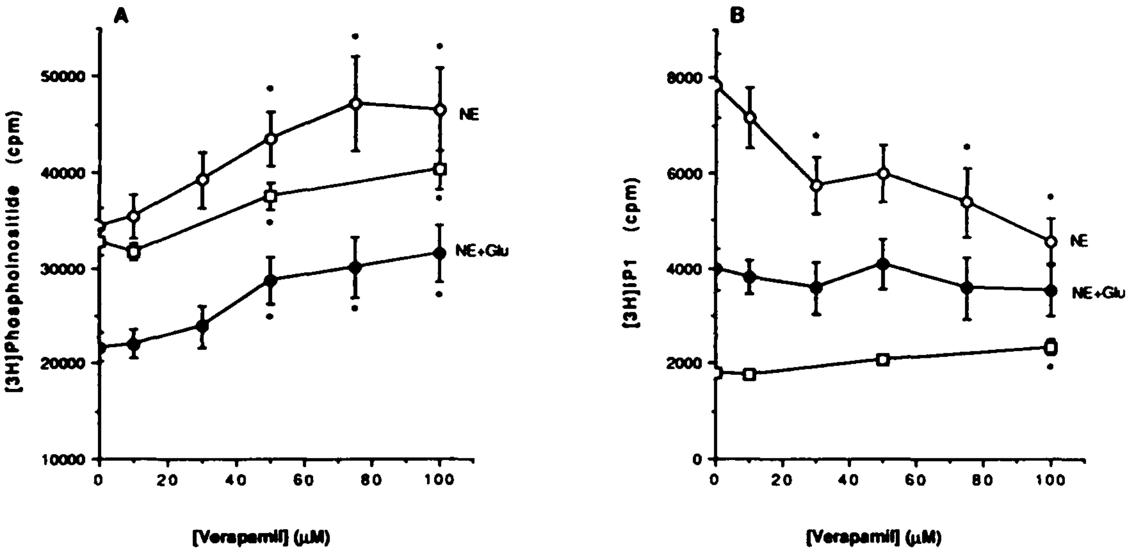

Figure 3. Quisqualate concentration-dependent inhibition of [^{3}H]inositol synthesis and hydrolysis.

Rat cortical slices were incubated simultaneously with [^{3}H]inositol and quisqualate (○) or NE (200 μM) and quisqualate (●) for 60 min. [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate.

*p < 0.05 compared with the response in the absence of quisqualate.

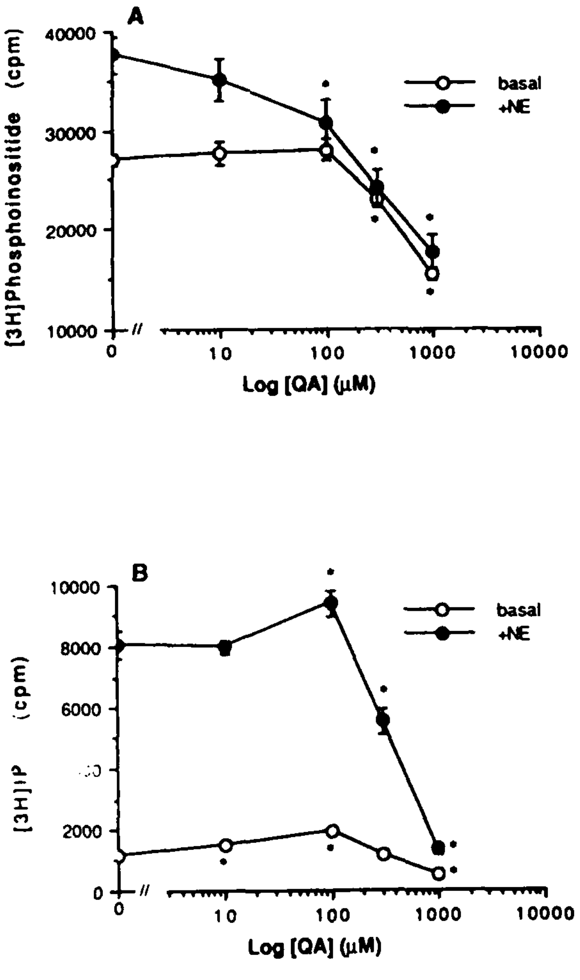

Figure 4. Excitatory amino acid antagonists do not modify quisqualate-inhibited [^{3}H]phosphoinositide hydrolysis.

Rat cortical slices were incubated with [^{3}H]inositol for 60 min and antagonists, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 500 μM), glutamate diethyl ether (GDEE; 500 μM), r-glutamylglycine (r-GG; 500 μM), and DL-2-amino-4-phosphonobutyric acid (AP$_4$; 500 μM), for the final 10 min, followed by addition of NE (100 μM), NE and quisqualate (500 μM), or NE and AMPA (500 μM), and a further 60 min incubation period. [^{3}H]IP$_1$ was measured as described in Methods. Each value represents the mean ± SE of two experiments measured in triplicate.

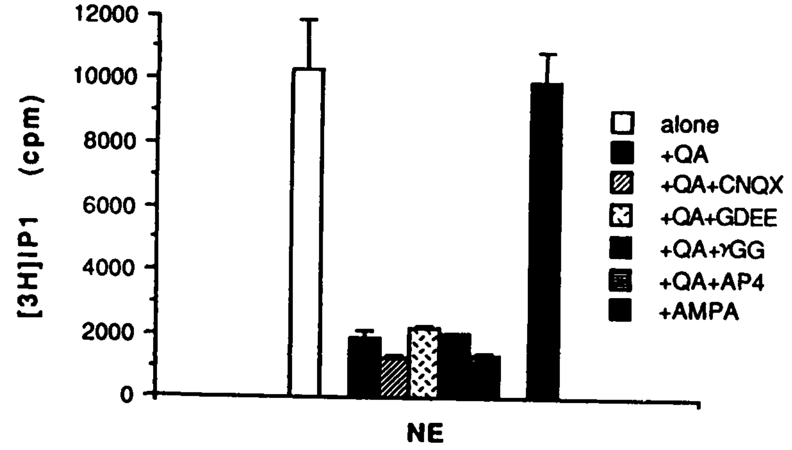

Figure 5. Concentration-response of arachidonic acid on [^{3}H]phosphoinositide synthesis and hydrolysis.

Rat cortical slices were incubated simultaneously with [^{3}H]inositol and arachidonic acid (○), or NE (100 μM) and arachidonic acid (●) for 60 min. [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate. * $p < 0.05$ compared with the response in the absence of arachidonic acid.

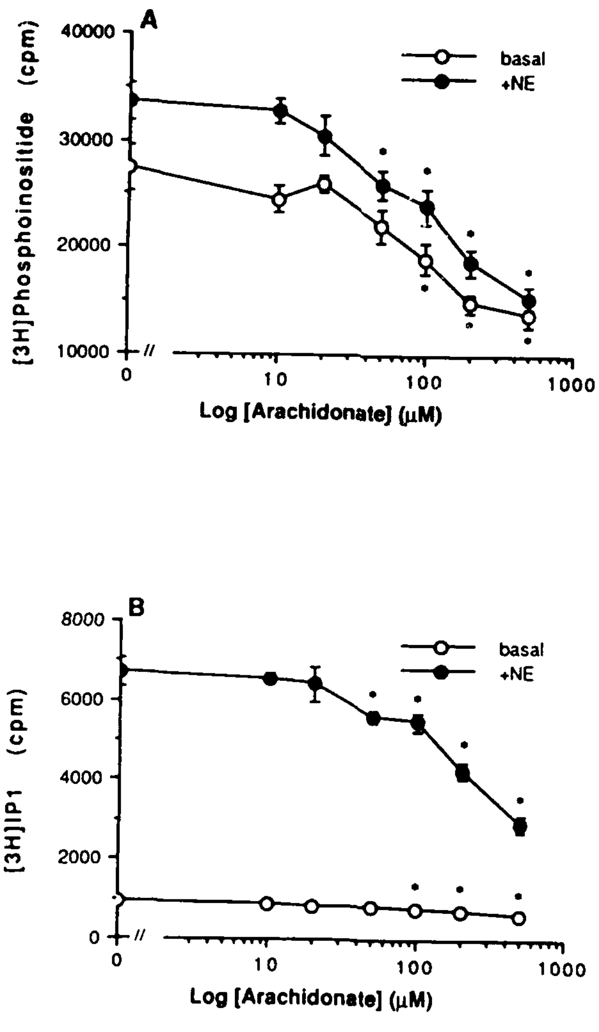

Figure 6. Time course of the effects of arachidonic acid on [^{3}H]phosphoinositide synthesis and hydrolysis.

Rat cortical slices were incubated simultaneously with [^{3}H]inositol and no addition (basal: □), 100 μM NE (○), or 100 μM NE plus 200 μM arachidonic acid (AA: ●), for 5, 10, 20, 40 or 60 min. [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate. *$p < 0.05$ indicates significant differences in samples incubated with NE and arachidonic acid compared with those incubated with NE.

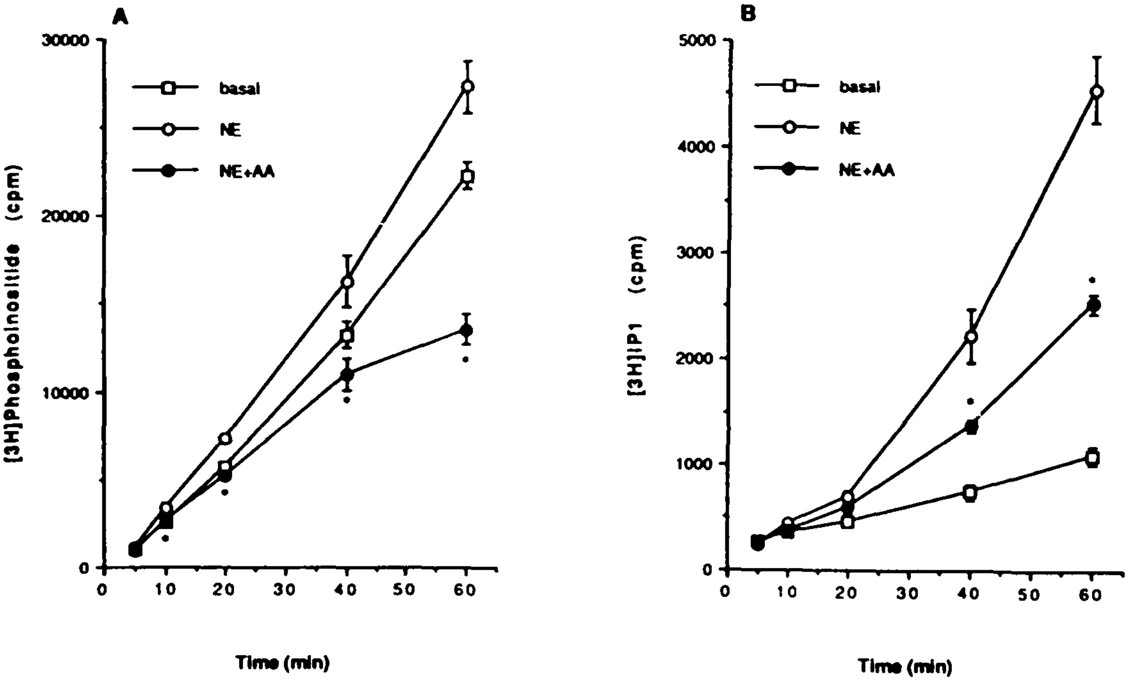

Figure 7. Arachidonic acid inhibits carbachol-stimulated [^{3}H]phosphoinositide hydrolysis.

Rat cortical slices were incubated simultaneously with [^{3}H]inositol and no addition (B; basal), carbachol (2 mM; Carb), or carbachol plus arachidonic acid (200 μM; AA) for 60 min. [^{3}H]Phosphoinositides (Figure A) and [^{3}H]IP$_1$ (Figure B) were measured as described in Methods. Each value represents the mean ± SE of three experiments measured in triplicate. *$p < 0.05$ compared with the value with carbachol alone.

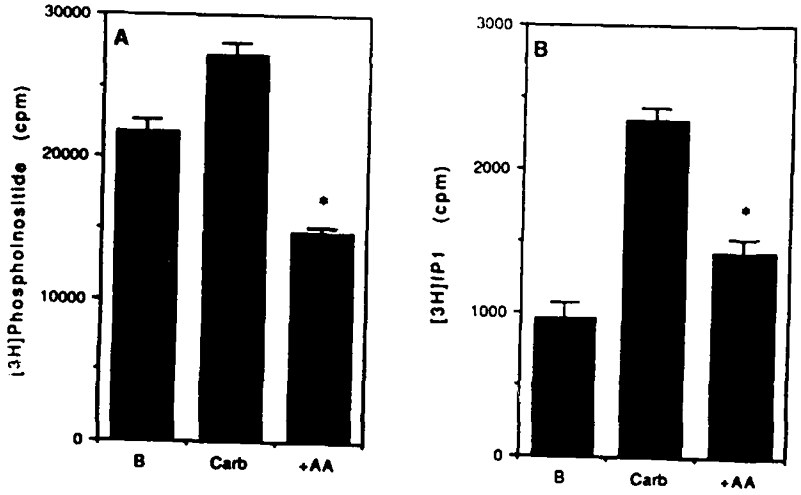

Figure 8. The effects of amino acids on NE-stimulated $[^3H]IP_1$ production in cortical slices that were prelabelled with $[^3H]$inositol in the presence of 1.3 mM Ca^{2+} or in the absence of added Ca^{2+}.

Rat cortical slices were prelabelled with $[^3H]$inositol for 60 min with the incubation media containing 1.3 mM calcium (+Ca) or no calcium (-Ca). After prelabelling, the slices were washed thoroughly with incubation media containing 1.3 mM calcium, and then incubated with 200 μM NE and the indicated additions for 60 min in the calcium-containing media. The left panel shows the results from slices prelabelled in calcium-containing media. The right panel shows the results from slices prelabelled in media without added calcium. The results are expressed as $[^3H]IP_1$ formed as percentage of total $[^3H]$ (which was calculated as $[^3H]$inositol + $[^3H]$phosphoinositides + $[^3H]IP_1$). Each value represents the mean ± SE of three experiments measured in triplicate. $*p < 0.05$ compared with NE.

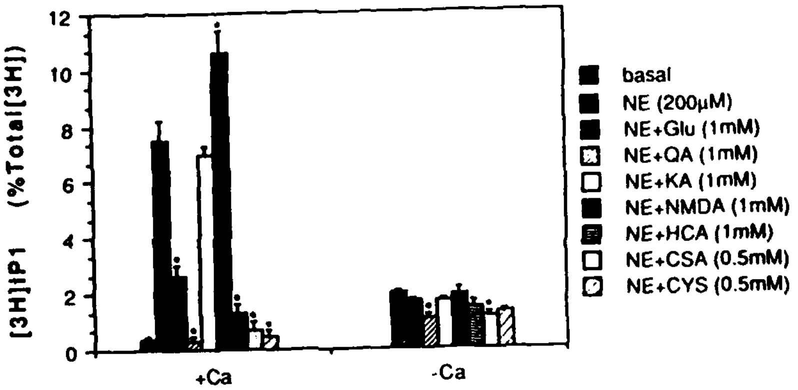

Figure 9. Inhibition by quisqualate of NE-induced [^{3}H]IP$_1$ production.

Cortical slices were prelabelled with [^{3}H]inositol for 60 min and washed, as described in the Methods. Slices were incubated for 60 min with the indicated concentrations of NE in the absence (○) or presence (●) of 500 μM quisqualate. Values are means ± SE of three experiments measured in triplicate. Quisqualate significantly ($p < 0.05$) reduced the production of [^{3}H]IP$_1$ at all concentrations of NE.

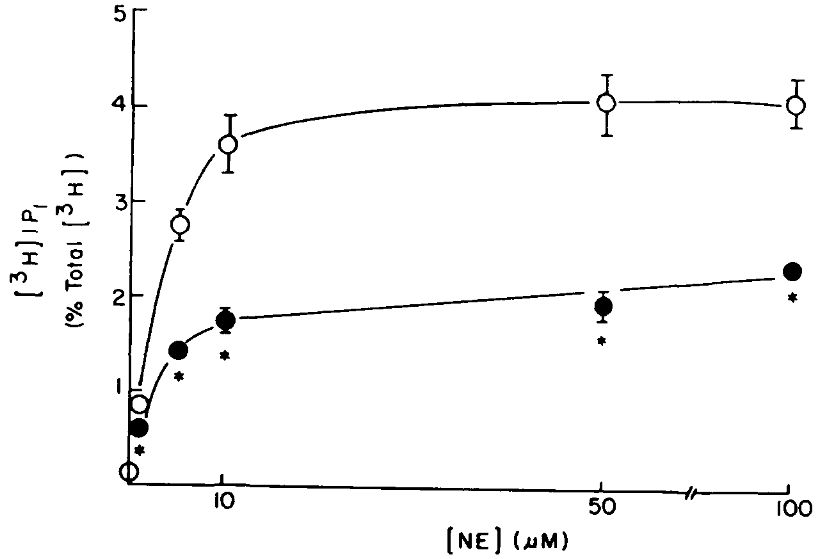

Figure 10. Incubation with unlabelled inositol reduces NE-stimulated $[^3H]IP_1$ production.

Cortical slices were prelabelled with $[^3H]$inositol for 60 min and washed, as described in the Methods. Slices were incubated for 15 min in incubation medium containing 0, 1, 5, or 20 mM inositol followed by addition of NE (200 μM) and incubation of 15 min (open bars) or 30 min (solid bars) and measurement of $[^3H]IP_1$. Values are given as % of $[^3H]IP_1$ produced by NE in the absence of unlabelled inositol, which were 1608 ± 308 cpm at 15 min and 3562 ± 430 cpm at 30 min (mean ± SE of 3 experiments carried out in triplicate). $*p < 0.05$

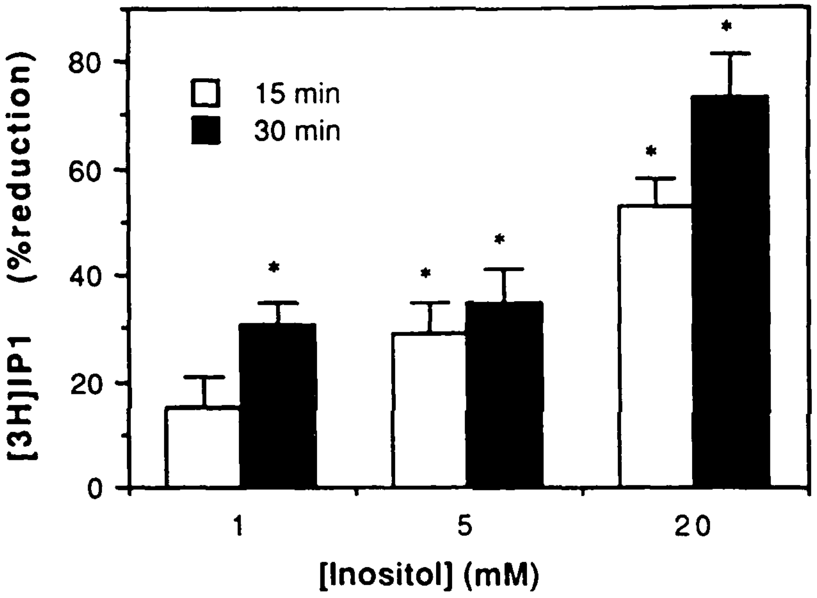

Figure 11. Effects of GABA on agonist-stimulated [^{3}H]phosphoinositide hydrolysis in cortical slices.

Cortical slices were prelabelled with [3H]inositol for 60 min followed by several washes. The labelled slices were incubated for 60 min with the indicated concentrations of GABA and 2 µM NE (□), 200 µM NE (○), 200 µM NE plus 1 mM glutamate (●), or 2 mM carbachol (△), followed by measurement of [^{3}H]IP$_1$. Each value is the mean ± SEM of 3-5 experiments. $^*p < 0.05$ compared with no GABA.

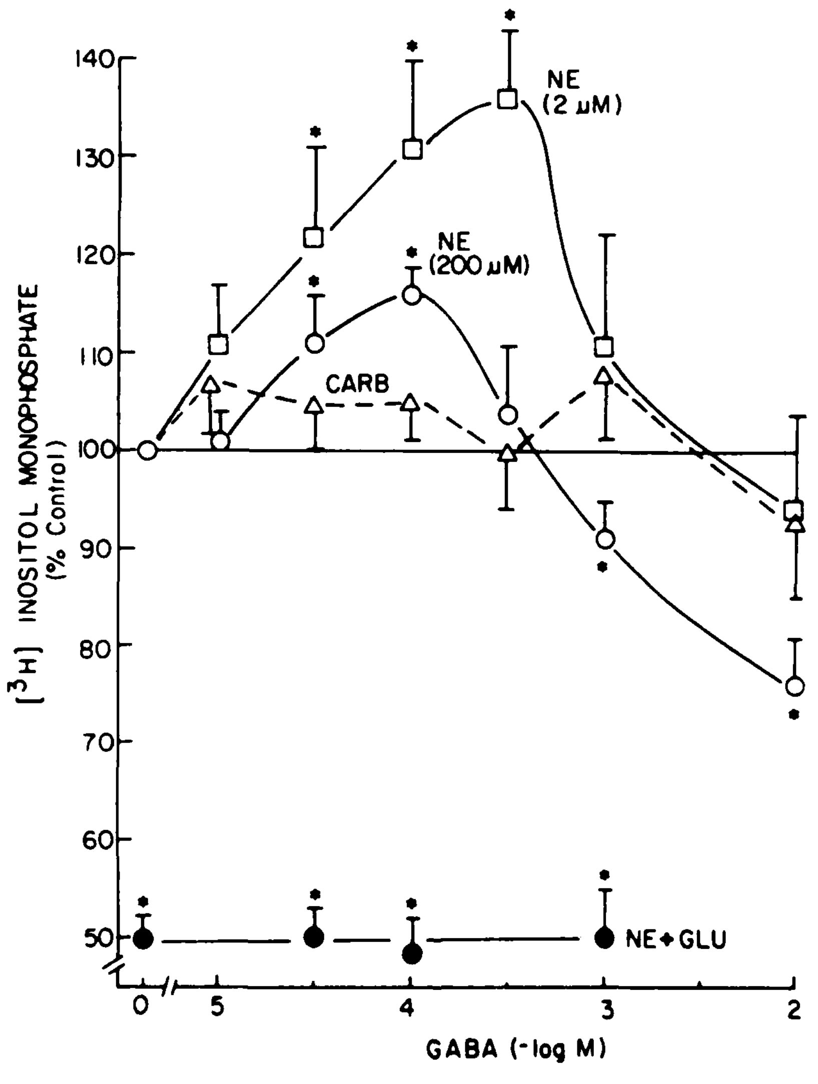

Figure 12. GABA enhances carbachol-stimulated [^{3}H]IP$_1$ production in striatal slices.

Rat striatal slices were prelabelled with [^{3}H]inositol for 60 min and then thoroughly washed. The labelled slices were incubated with 0, 100 µM, or 1 mM GABA and 200 µM NE, 2 mM carbachol, or 1 mM ibotenate (IBO) for 60 min and [^{3}H]IP$_1$ was measured as described in Methods. Values are given as the % of [^{3}H]IP$_1$ produced by each agonist in the absence of GABA, means ± SE of three experiments each measured in triplicate. *$p < 0.05$ compared with no added GABA.

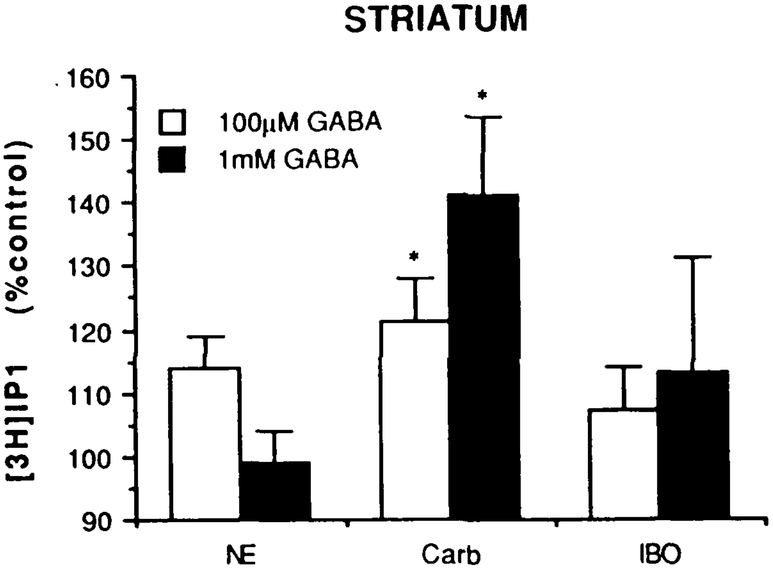

Figure 13. Modulation by GABA of agonist stimulated [^{3}H]IP$_1$ production in hippocampal slices.

Rat hippocampal slices were prelabelled with [^{3}H]inositol for 60 min followed by several washes. The labelled slices were incubated for 60 min with the indicated concentrations of GABA and 2 mM carbachol, 1 mM ibotenate, or 200 µM, and [^{3}H]IP$_1$ was measured as described in Methods. Values are given as the % of [^{3}H]IP$_1$ produced by each agonist in the absence of GABA, means ± SE of three experiments measured in triplicate. *$p < 0.05$ compared with no added GABA.

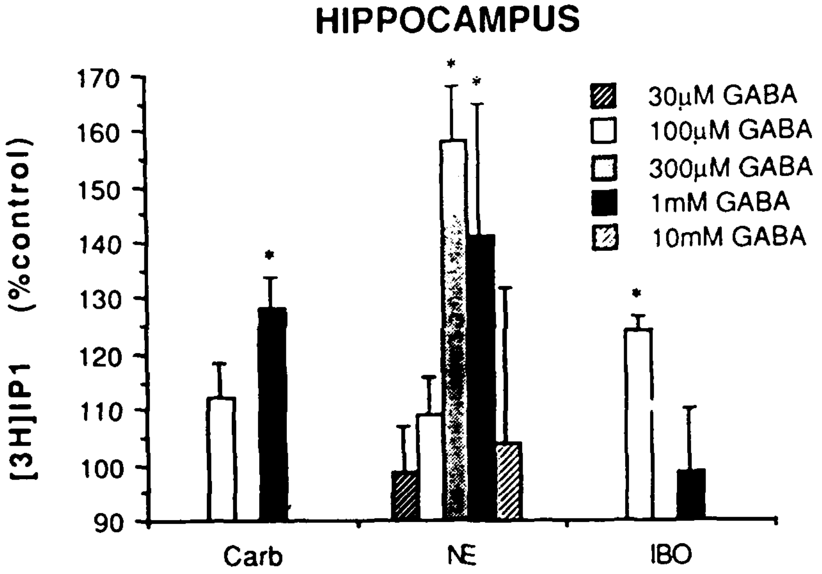

Figure 14. Potentiation by GABA of NE-stimulated $[^3H]IP_1$ production in hippocampal slices.

Rat hippocampal slices were prelabelled with $[^3H]$inositol for 60 min followed by several washes. The labelled slices were incubated with the indicated concentrations of NE and either 300 μM GABA (●) or no added GABA (○), for 60 min and $[^3H]IP_1$ was measured as decribed in Methods. Values are given as $[^3H]IP_1$ produced as percent of the total $[^3H]$ in the slices, means ± SE of three experiments measured in triplicate. All results with GABA were significantly ($p < 0.05$) greater than those in the absence of GABA.

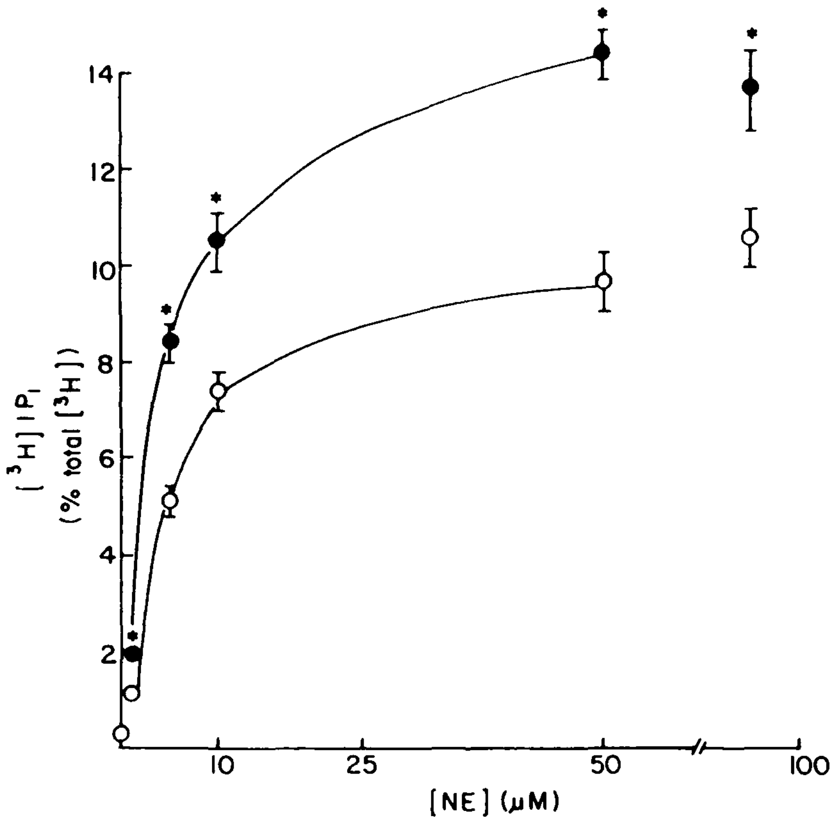

Figure 15. Effects of multiple modulators on NE-stimulated $[^3H]IP_1$ production.

Cerebral cortical slices were prelabelled with $[^3H]$inositol for 60 min followed by several washes. The labelled slices were incubated with no addition (basal), 300 μM baclofen (BACL), 200 μM NE, 500 μM quisqualate (QA), 200 μM arachidonic acid (AA), 300 μM GABA in the combinations indicated for 60 min and $[^3H]IP_1$ was measured as described in Methods. Values are given as $[^3H]IP_1$ produced as a percent of the total $[^3H]$ in the slices, means ± SE of three experiments measured in triplicate. $^*p < 0.05$ compared with NE alone. The response with NE, baclofen and AA was significantly ($p < 0.05$) greater than with NE and AA.

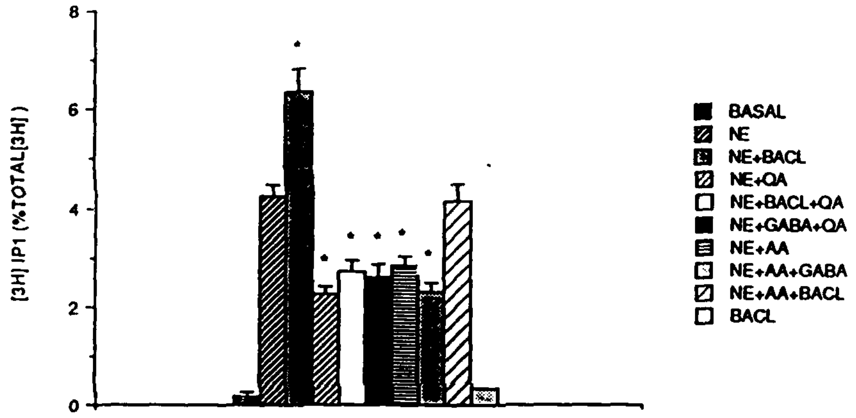

Figure 16. Effect of NMDA antagonists on phosphoinositide hydrolysis. Prelabelled cortical slices were incubated (1 hr, 37°C) with 10^{-5}-2 x 10^{-3}M MK-801 (Δ), PCP (O) or AP-7 (●). The points represent ± SEM of three experiments measured in triplicate.

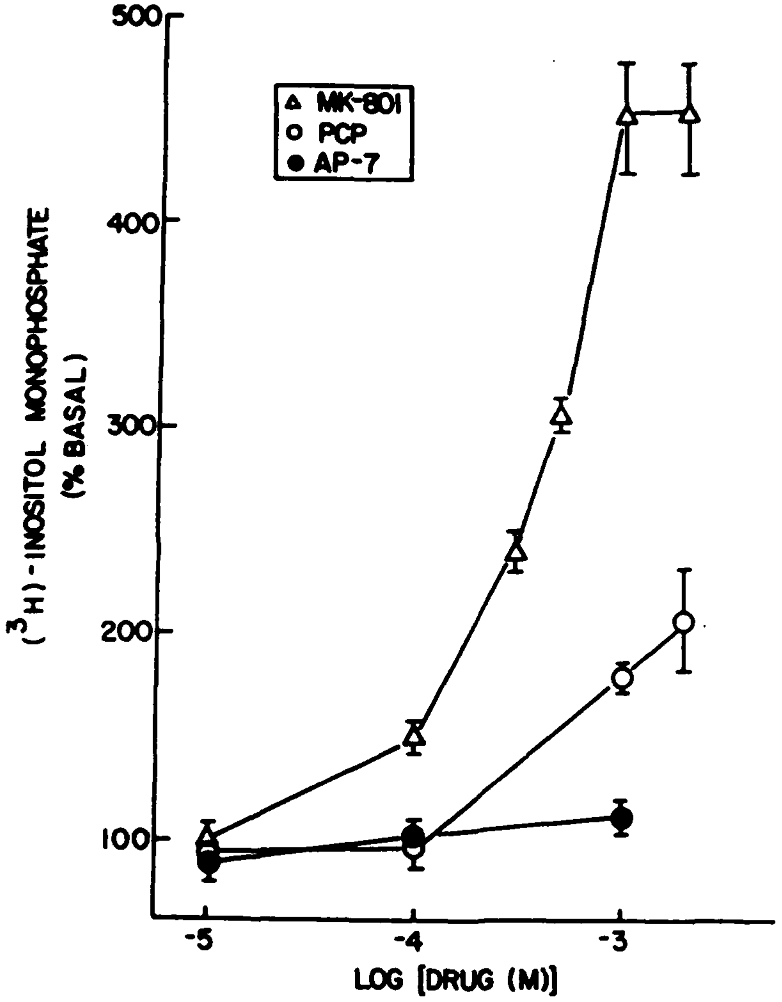

Figure 17. Effect of NMDA and glycine on MK-801-stimulated phosphoinositide hydrolysis.

Prelabelled slices were incubated with MK-801 (500 μM, shaded bars) and/or glycine (0.1, 1.0 mM), NMDA (1 mM) or Zn^{2+} (500 μM). The assay was carried out in Mg^{2+}-containing (panel A) or Mg^{2+}-free (panel B) buffer. Glycine or Zn^{2+} alone did not affect (^3H)-inositol monophosphate accumulation (not shown). The bars represent mean ± SEM of three experiments measured in triplicate.

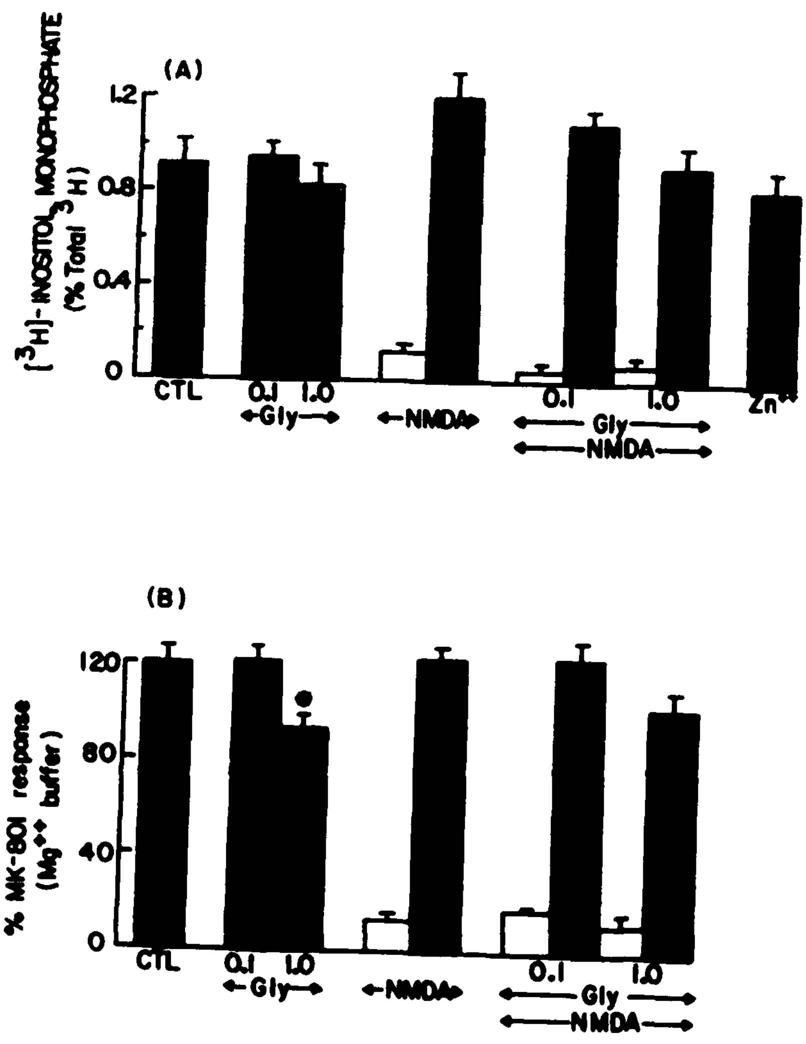

Figure 18. Na$^+$-dependence of [^{3}H]IP$_1$ production.

Cortical slices were prelabelled with [^{3}H]inositol for 60 min and washed, as described in the Methods. Slices were incubated for 60 min in media with the indicated concentrations of Na$^+$ in the absence (Basal) or presence of 100 μM norepinephrine (NE). Values are means ± S.E.M. from four experiments measured in triplicate. The inset shows the values for (○) basal and (●) norepinephrine-stimulated [^{3}H]IP$_1$ production after subtraction of the values obtained with 120 mM Na$^+$. These calculated values show that reduced Na$^+$ independently enhanced the response with each condition.

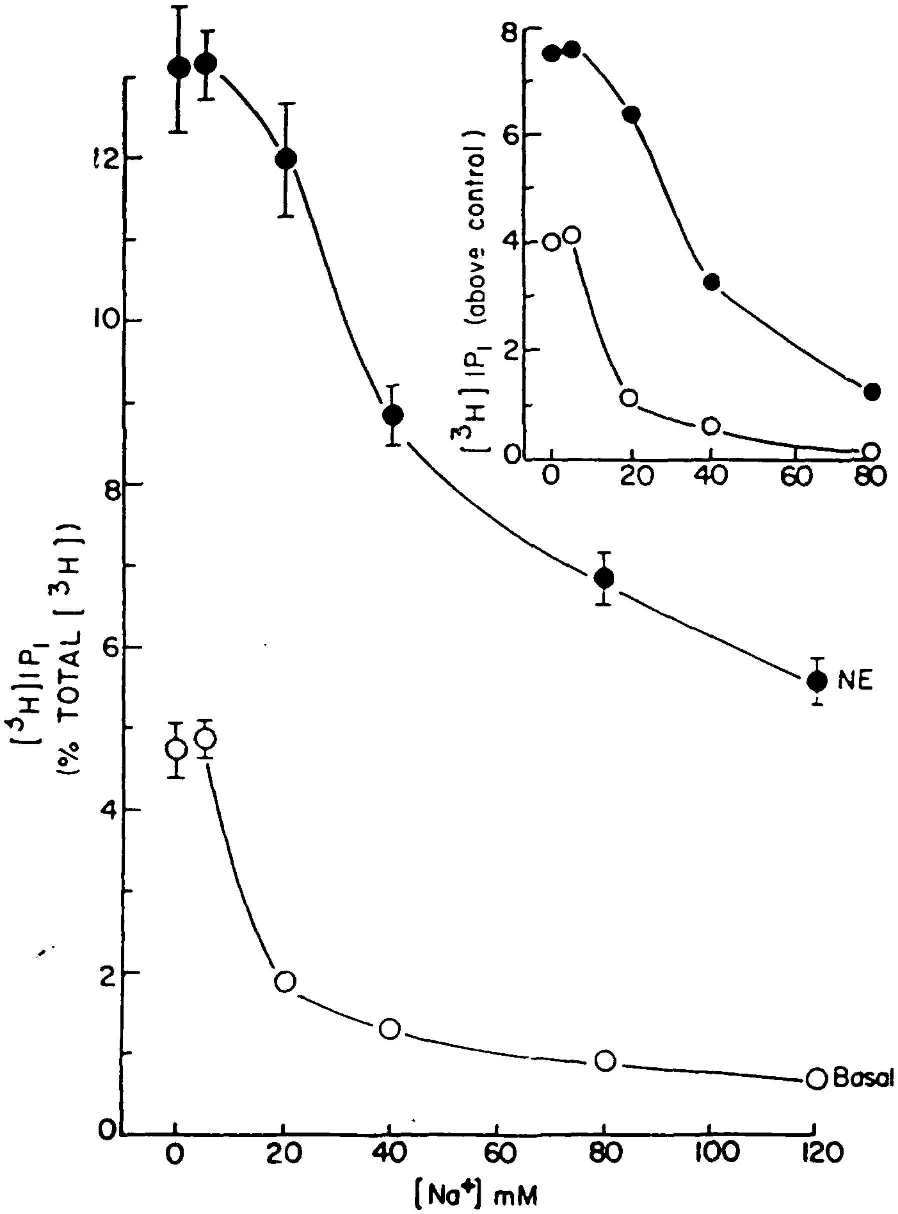

Figure 19. Effects of tetrodotoxin on [^{3}H]IP$_1$ production.

Cortical slices that had been prelabelled with [^{3}H]inositol and washed were incubated for 60 min with 100 µM norepinephrine (▲)or 2 mM carbachol (●) in the presence of varying concentrations of tetrodotoxin. Values are means ± S.E.M. from three experiments measured in triplicate. *$p < 0.05$ compared with no tetrodotoxin.

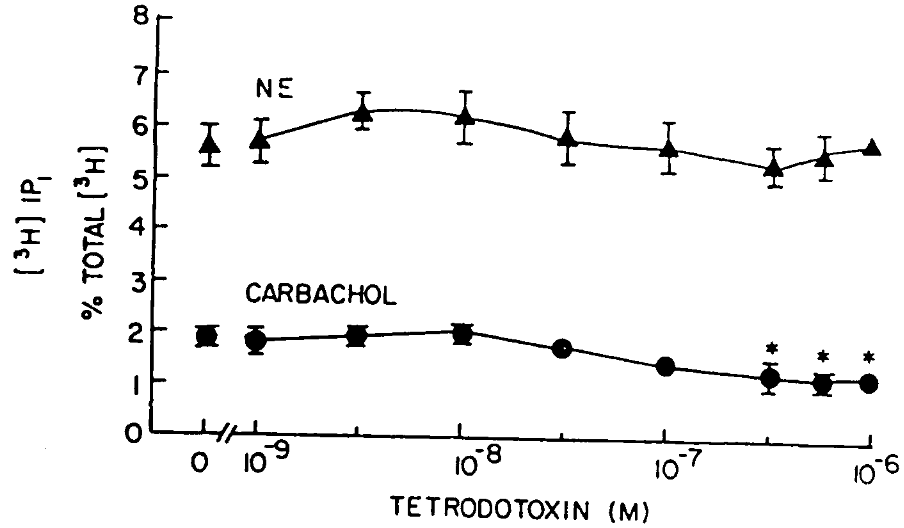

Figure 20. Effects of reduced Na+ or tetrodotoxin on the modulation of phosphoinositide hydrolysis.

Cortical slices were prelabelled with [^{3}H]inositol for 60 min and washed, as described in the Methods. Slices were incubated for 60 min in normal media containing 120 mM Na+ (control; open bars), media with 5 x 10^{-7} M tetrodotoxin (TTX; hatched bars), or media with 5 mM Na+ (low Na+; solid bars). The production of [^{3}H]IP$_1$ was measured after incubation with no added agonist (Basal), 100 µM norepinephrine (NE), 500 µM quisqualate (QA), NE plus QA, NE plus 500 µM glutamate (Glu), NE plus 200 µM arachidonic acid (AA), 2 mM carbachol (CARB), or 0.1 or 1.0 mM ibotenate (IBO). Values are means ± S.E.M. from three experiments (except n = 2 for IBO) measured in triplicate.

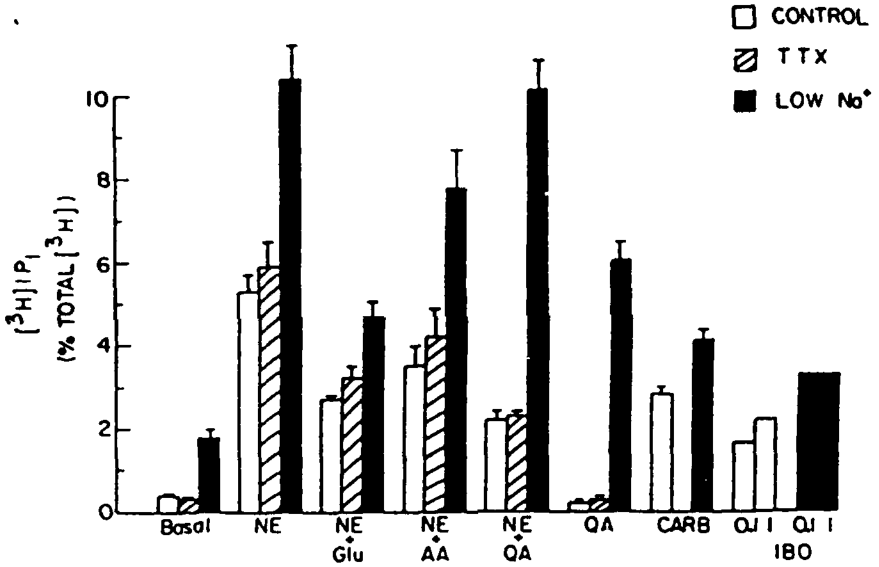

Figure 21. Low Na+ blocked L-BOAA-induced inhibition of norepinephrine-stimulated [^{3}H]IP$_1$ production.

Cortical slices that had been prelabelled with [^{3}H]inositol and washed were incubated for 60 min with no addition (BSL), 100 μM norepinephrine (NE; hatched bars), 0.3 or 1 mM L-BOAA, or L-BOAA and norepinephrine (hatched bars), in media containing 5 mM Na+ (Low Na+) or 120 mM Na+. (Normal Na+). Values are means ± S.E.M. of three experiments measured in triplicate.

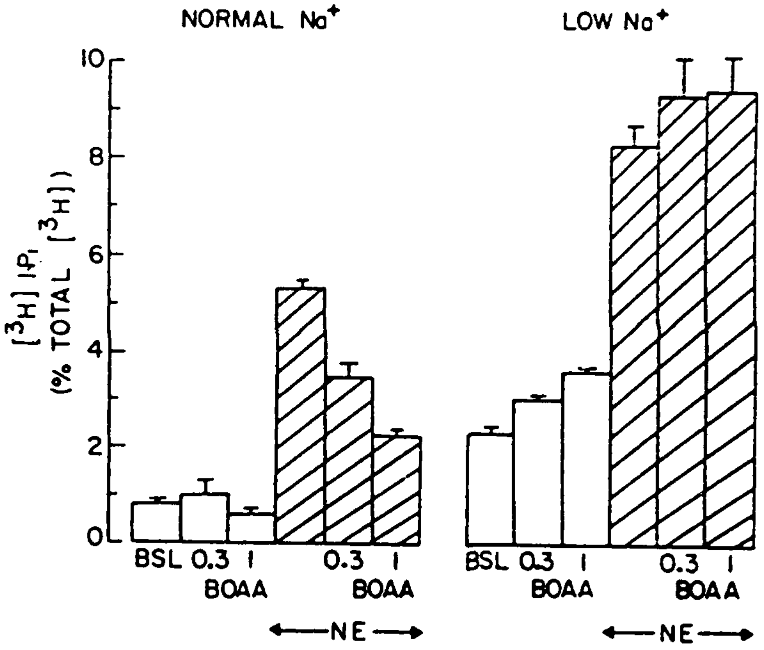

Figure 22. Effect of Na$^+$on [^{3}H]IP$_1$ produced in response to quisqualate.

Cortical slices that had been prelabelled with [^{3}H]inositol and washed were incubated for 60 min with no agonist (BSL) or with concentrations of quisqualate (QA) varying from 10^{-7} to 10^{-3}M. Hatched bars indicate [^{3}H]IP$_1$ produced in response to quisqualate after subtraction of the corresponding basal value. Values are means ± S.E.M. of three experiments measured in triplicate.

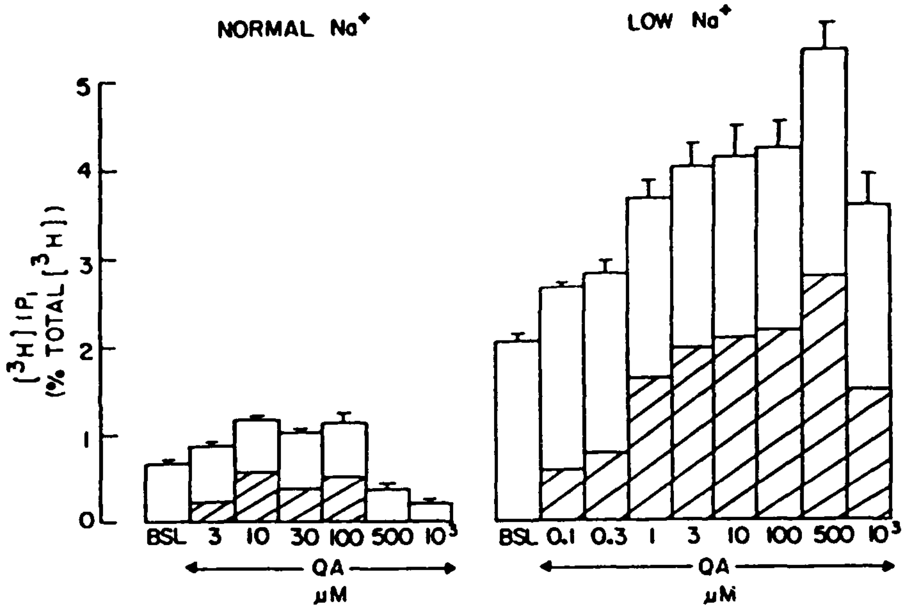

Figure 23. Effect of Na+ on phosphoinositide hydrolysis in hippocampal slices.

Hippocampal slices that had been prelabelled with [^{3}H]inositol and washed were incubated for 60 min in the absence of added agonist (basal, B, open bars), with 100 µM quisqualate (Q, hatched bars), or with 100 µM norepinephrine (N, solid bars) in medium containing 120 mM Na+ (Normal Na+) or 5 mM Na+ (Low Na+). [^{3}H]IP$_1$ (left ordinate), [^{3}H]IP$_2$, and [^{3}H]IP$_3$ (right ordinate) were measured as described in the Methods. Values are means ± S.E.M. of three experiments measured in triplicate.

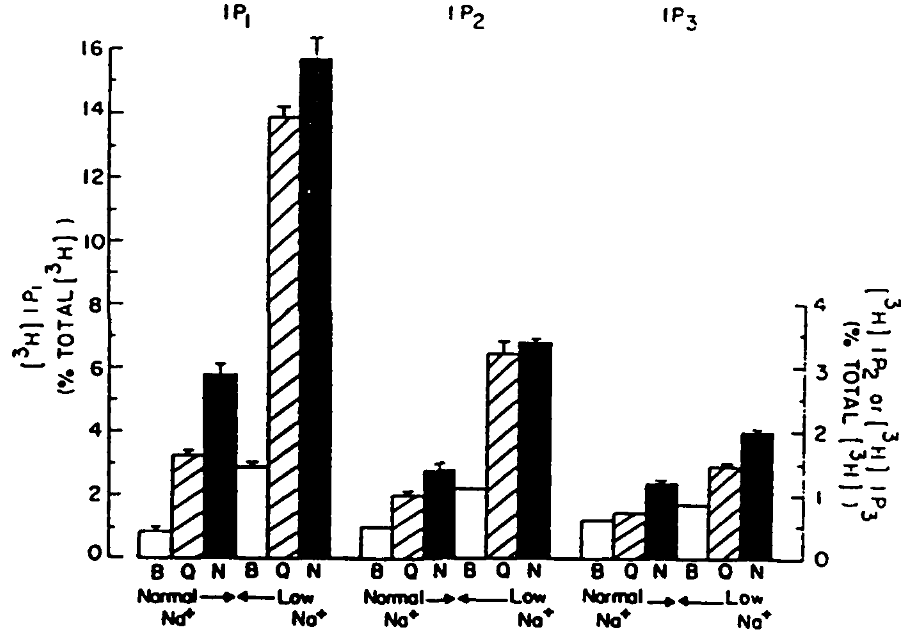

Figure 24. Basal, calcium-dependent, and protein kinase C-dependent protein phosphorylation in hippocampal particulate fractions of high (A) and low (B) molecular mass proteins from control (-) and chronic lithium-treated (+) rats. Assays and SDS-PAGE were conducted as described in the Methods, using 6.5% gels to separate proteins > 45 kD and 12% gels to separate proteins < 45 kD. Autoradiographs show representative results from one control and one lithium-treated rat: (A) phosphorylation measured under basal conditions (lanes 1 and 2), in the presence of 1.5 mM $CaCl_2$ (lanes 3-6), or in the presence of $CaCl_2$, 10 µg PS, 1 µM PMA (lanes 7 and 8); and (B) phosphorylation in the presence of $CaCl_2$ (lanes 1 and 2) or in the presence of $CaCl_2$, 10 µg PS, and 1 µM PMA (lanes 3 and 4). Apparent molecular masses (kD) of major phosphoproteins are indicated.

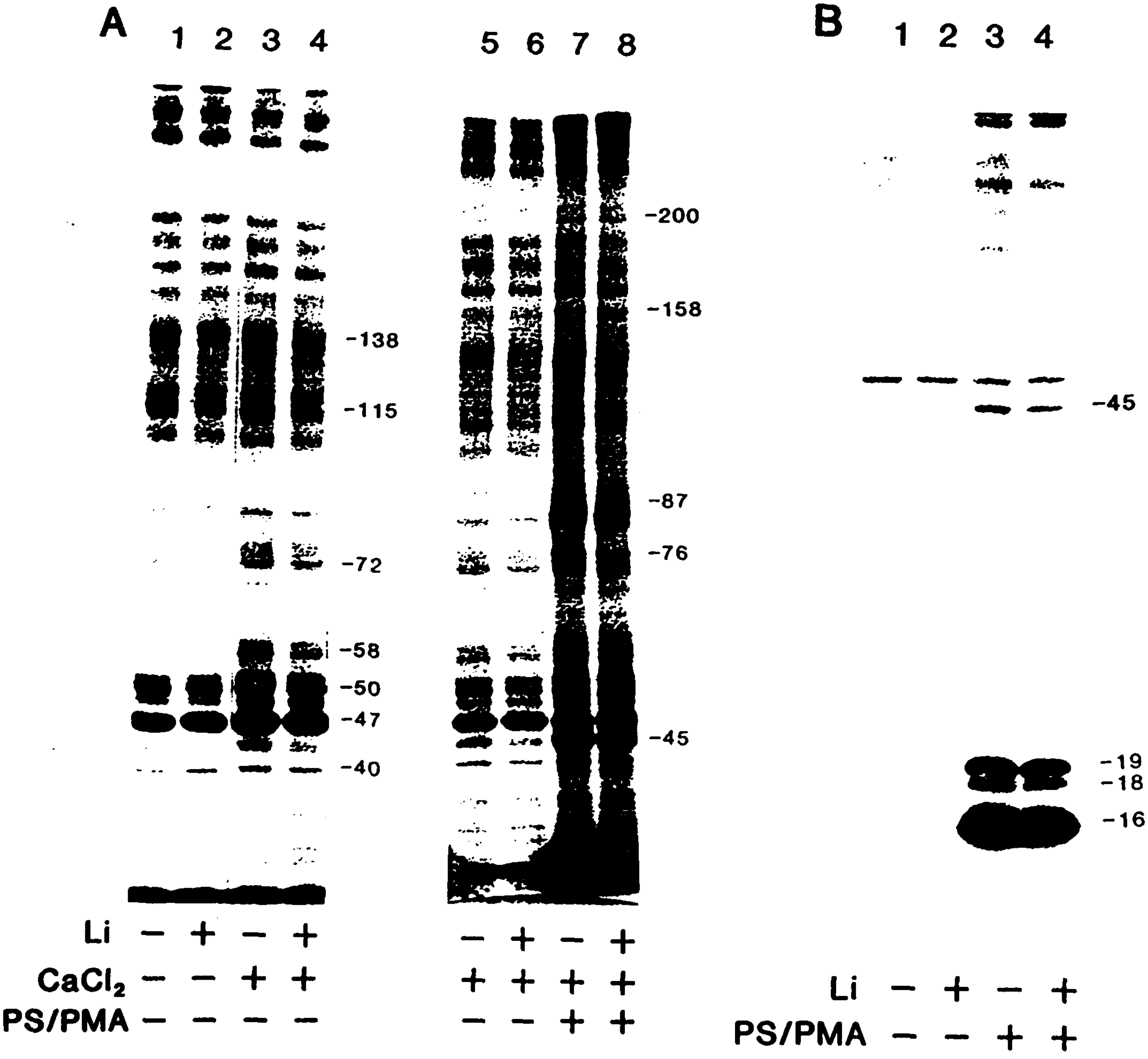

Figure 25. Cyclic AMP-dependent phosphorylation of hippocampal particulate proteins from control (-) and chronic lithium-treated (+) rats. Particulate proteins were phosphorylated as described in the Methods and separated by SDS-PAGE on 6.5% gels. Phosphorylation was measured in the presence of 1 mM theophylline (lanes 1 and 2) or in the presence of theophylline and 50 μM cAMP (lanes 3 and 4). Apparent molecular masses (kD) of major phosphoproteins are indicated.

Figure 26. Calcium-dependent and protein kinase C-dependent protein phosphorylation in hippocampal soluble fractions of high (A) and low (B) molecular mass proteins from control (-) and chronic lithium-treated (+) rats. Assays and SDS-PAGE were conducted as described in the Methods, using 6.5% gels to separate proteins > 45 kD and 12% gels to separate proteins < 45 kD. Phosphorylation was measured in the presence of 1.5 mM $CaCl_2$ (lanes 1 and 2), or in the presence of $CaCl_2$, 10 μg PS, and 1 μM PMA (lanes 3 and 4). Apparent molecular masses (kD) of major phosphoproteins are indicated.

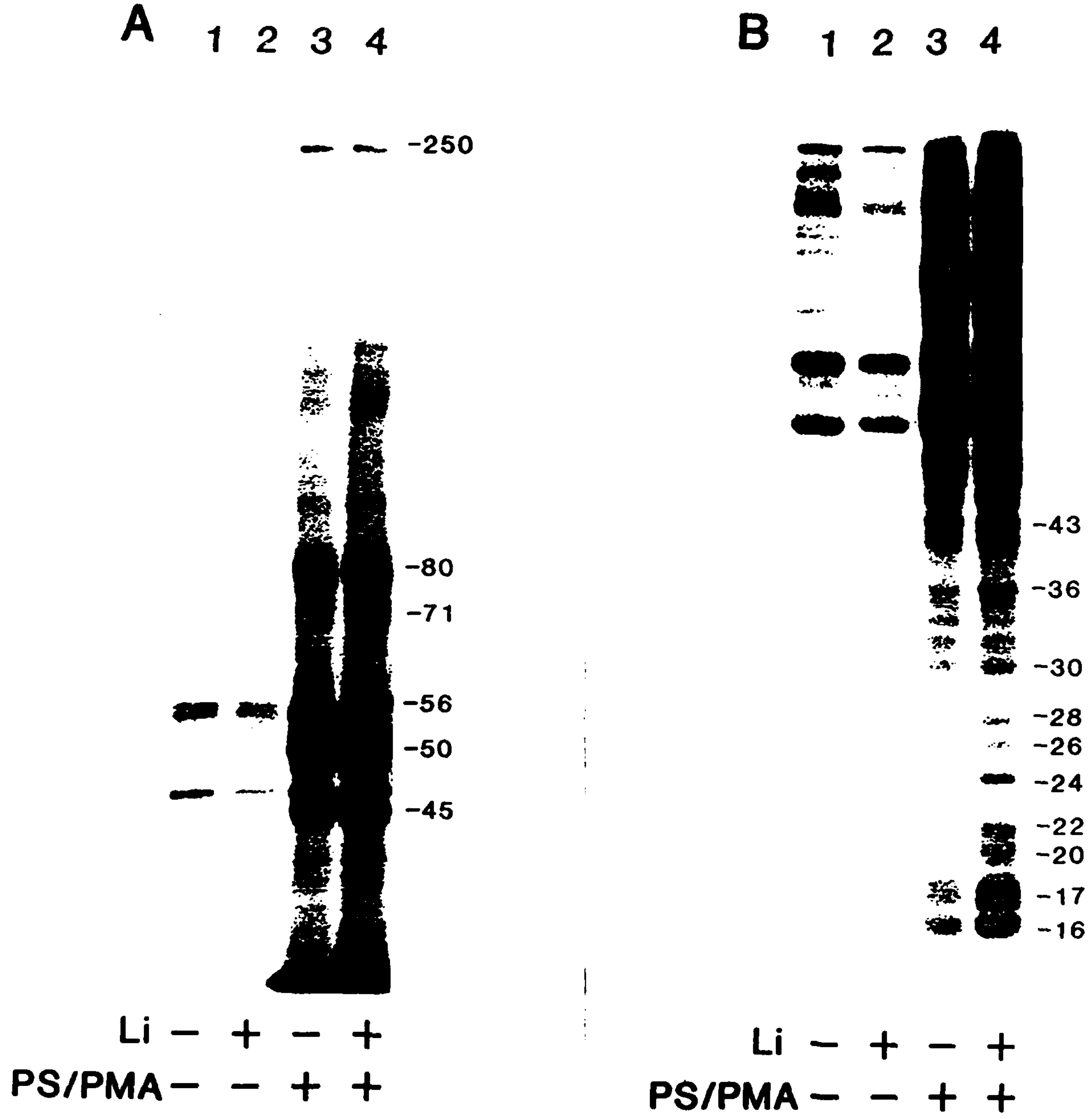

Figure 27. Phosphoinositide hydrolysis in hippocampus.

Rats were treated with LiCl (L; 3 mmole/kg; ip; 20 hr prior) or LiCl plus pilocarpine (LP; 30 mg/kg; sc; 25 or 90 min prior) and then hippocampal slices were prepared and prelabelled with [^{3}H]inositol as described in the Methods. Slices were incubated with 1 mM ibotenate (Ibo), 2 mM carbachol (Carb), 2 mM carbachol plus 15 mM KCl, 100 μM norepinephrine (NE), or 55 mM KCl, followed by measurement of [^{3}H]inositol monophosphate [^{3}H]IP$_1$). Values are means ± SEM and are given as a percent of total [^{3}H] incorporated into the slices. n = 3-4, *$p < 0.05$ compared with controls.

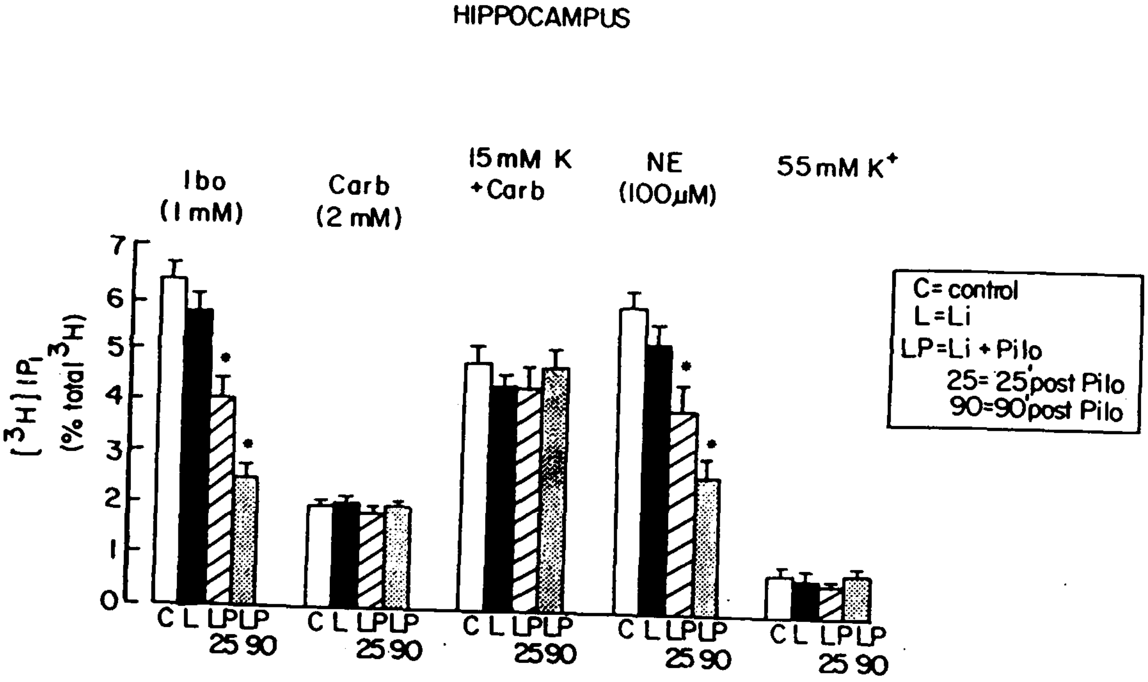

Figure 28. Norepinephrine-stimulated phosphoinositide hydrolysis.

Rats were treated as described in the legend to Figure 27 followed by measurement of [^{3}H]inositol monophosphate production in the presence of 10^{-6} to 10^{-4} M norepinephrine (NE) as described in the Methods. All values from rats treated with LiCl plus pilocarpine (Li + Pilo) at concentrations of 3 x 10^{-6} M NE and greater were significantly less than controls, ($p < 0.05$), n = 3.

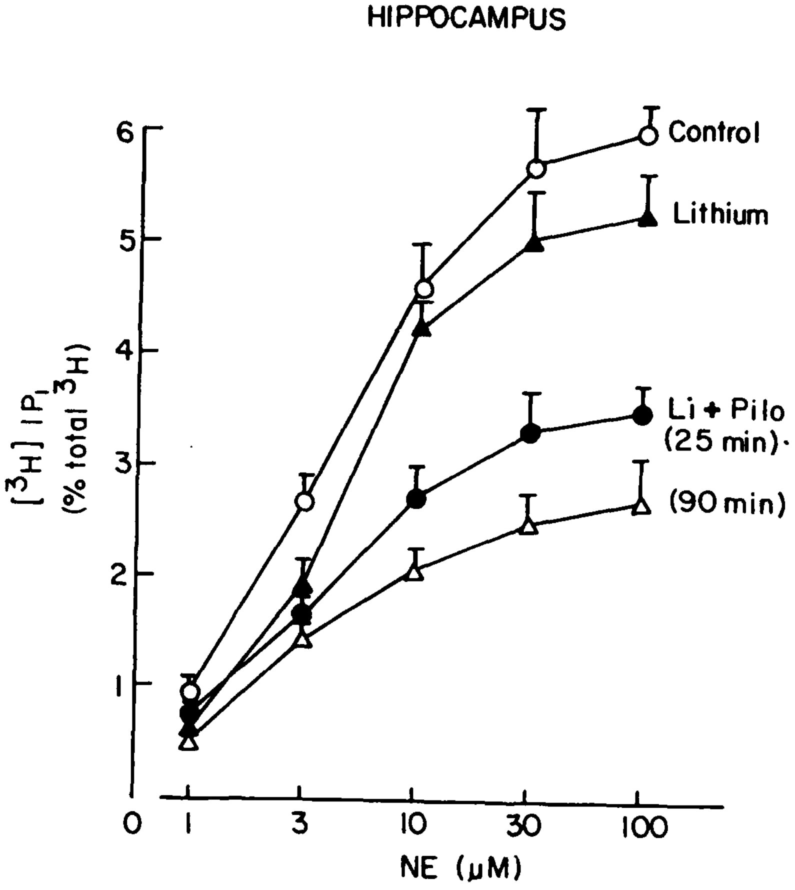

Figure 29. Phosphoinositide hydrolysis in cortical slices.

Cortical slices prepared from control (C) or treated (S; seizing) rats (LiCl plus pilocarpine; 60 min) were prelabelled with [^{3}H]inositol as described in the Methods. Slices were incubated in normal buffer containing 120 mM Na$^+$ or in buffer containing 5 mM Na$^+$ (NaCl replaced with choline chloride) and with 2 mM carbachol (CARB), 100 μM norepinephrine (NE), 100 μM quisqualate (QA), or 100 μM NE plus 500 μM QA. n = 3.

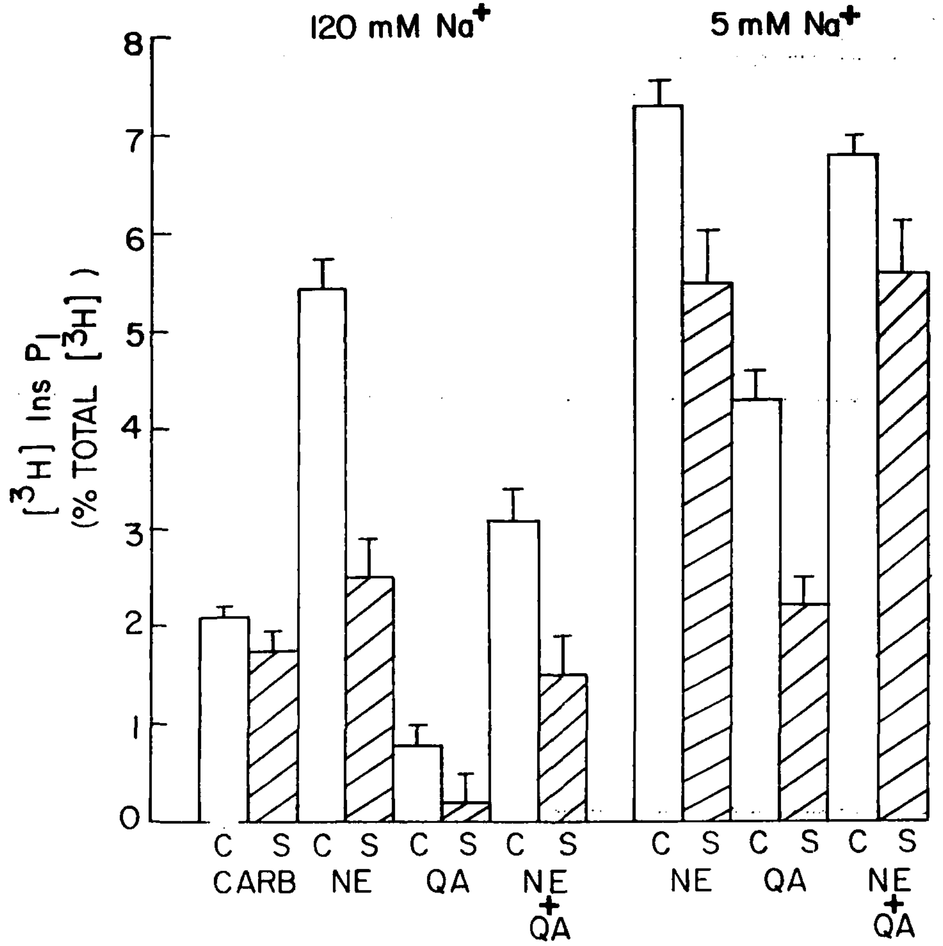

Figure 30. NaF-stimulated phosphoinositide hydrolysis.

Cortical slices prepared from control or treated (seizing) rats (LiCl plus pilocarpine; 60 min) were prelabelled with [^{3}H]inositol as described in the Methods. Slices were incubated with the indicated concentration of NaF in the absence of Ca^{2+} (which enhances the response to NaF) followed by measurement of [^{3}H]inositol monophosphate ([^{3}H]InsP$_1$). n = 3

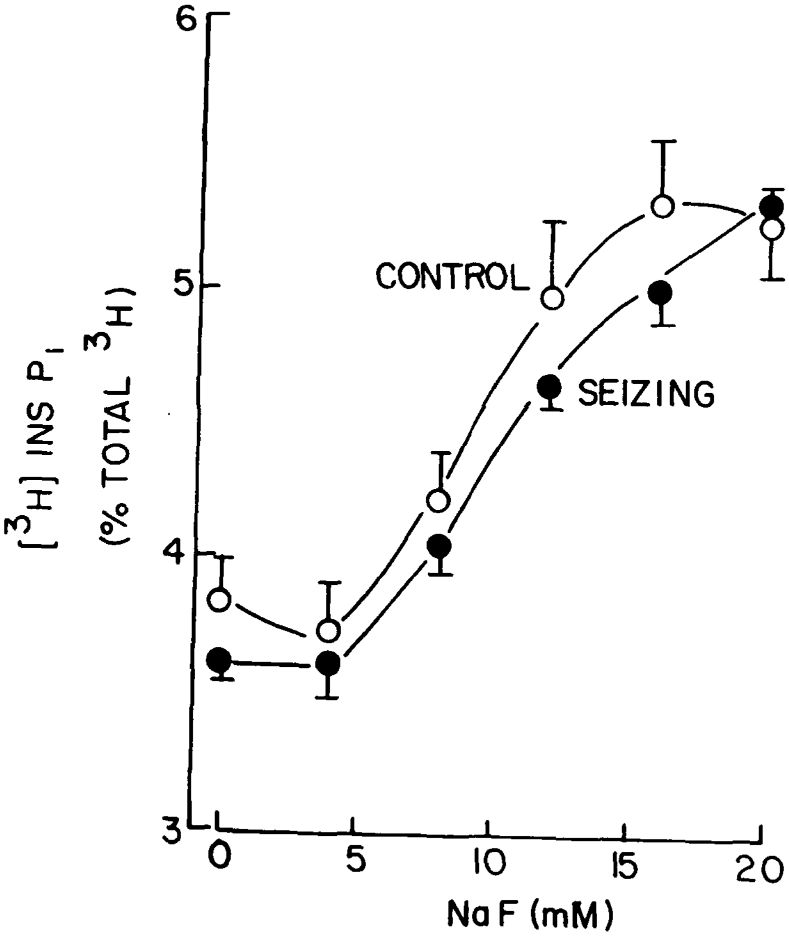

Figure 31. Protein kinase C activity in rat cerebral cortex.

The activity of protein kinase C was measured in the cytosolic (top) or membrane (middle) fraction prepared from rat cerebral cortex. Rats were treated with (i) the combination of LiCl (3 mmole/kg; ip; 20 hr prior) plus pilocarpine (30 mg/kg; sc; 10, 20, 60 or 120 min prior), (ii) LiCl alone, (iii) kainate (10 mg/kg; sc; 20 or 96 min prior), or LiCl plus kainate. The bottom panel shows the % of total protein kinase C activity associated with the membrane fraction.

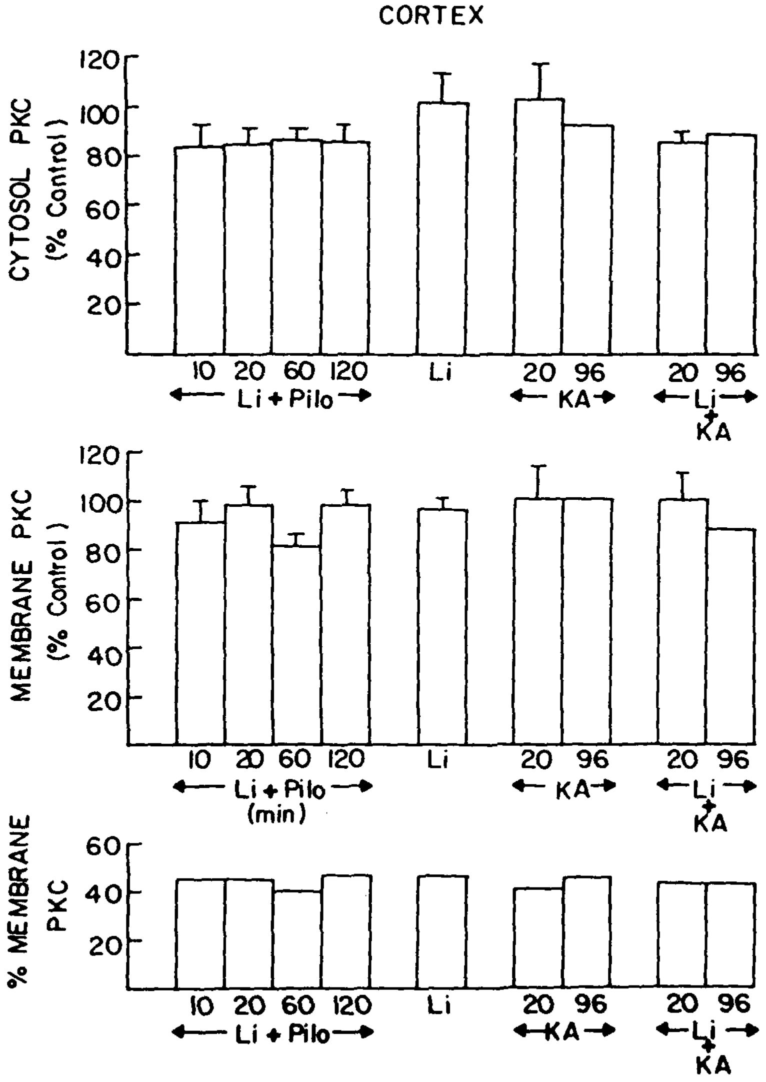

Figure 32. Protein kinase C activity in rat hippocampus.

The activity of protein kinase C was measured in the cytosolic (top) or membrane (middle) fraction prepared from the hippocampus of rats treated as described in the legend to Figure 5. The bottom panel shows the % of total protein kinase C activity associated with the membrane fraction.

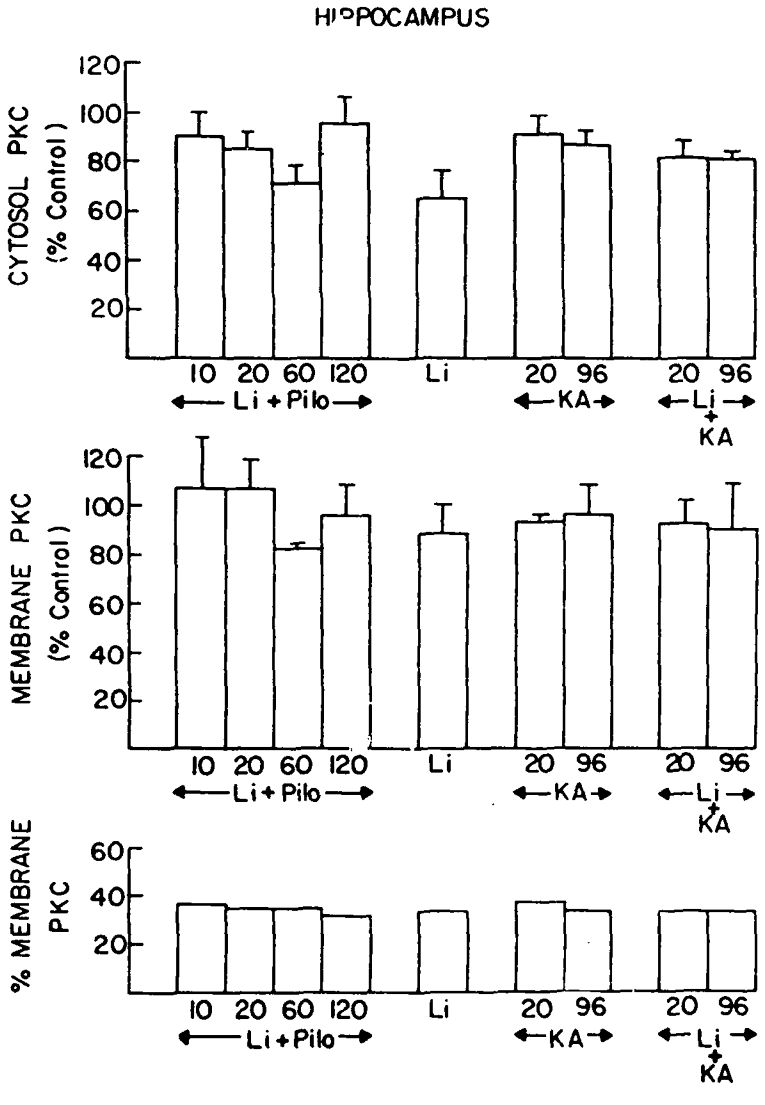

Figure 33. Immunoblots of phosphotyrosine proteins in rat cortex and hippocampus after sacrifice by decapitation (D) or focussed-beam microwave irradiation (M). Molecular masses (kD) of phosphotyrosine proteins analyzed quantitatively are indicated.

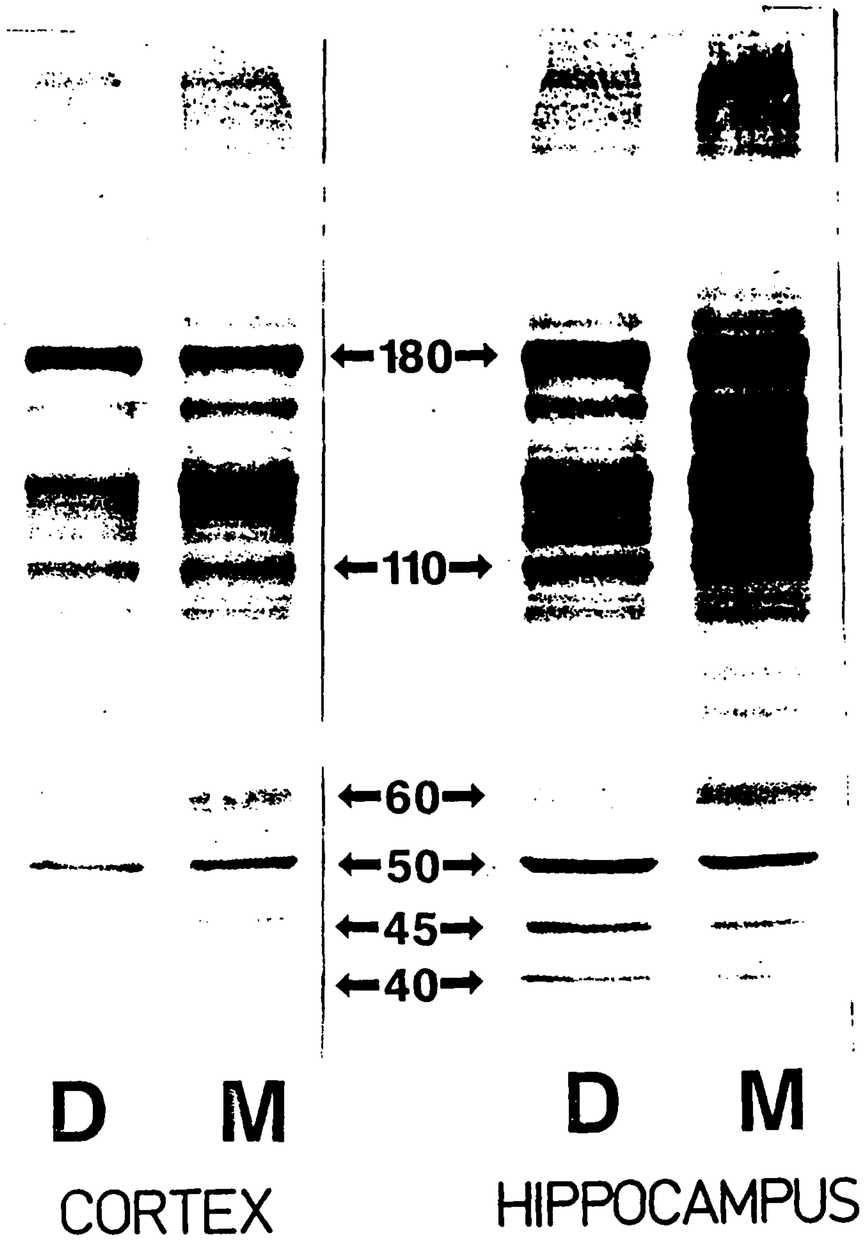

Figure 34. Immunoblots of phosphotyrosine proteins in rat brain regions.

Saline-treated controls (-) or rats in which seizures were induced (+) by administration of LiCl (3 mmole/kg, ip, 20 hr prior) and pilocarpine (30 mg/kg, sc, 1 hr prior) were sacrificed by focussed-beam microwave irradiation and phosphotyrosine proteins were identified as described in the Methods, in the cerebral cortex (CTX), hippocampus (HIP), and striatum (STM). Molecular masses (kD) are indicated for the phosphotyrosine proteins which were analyzed quantitatively in further experiments.

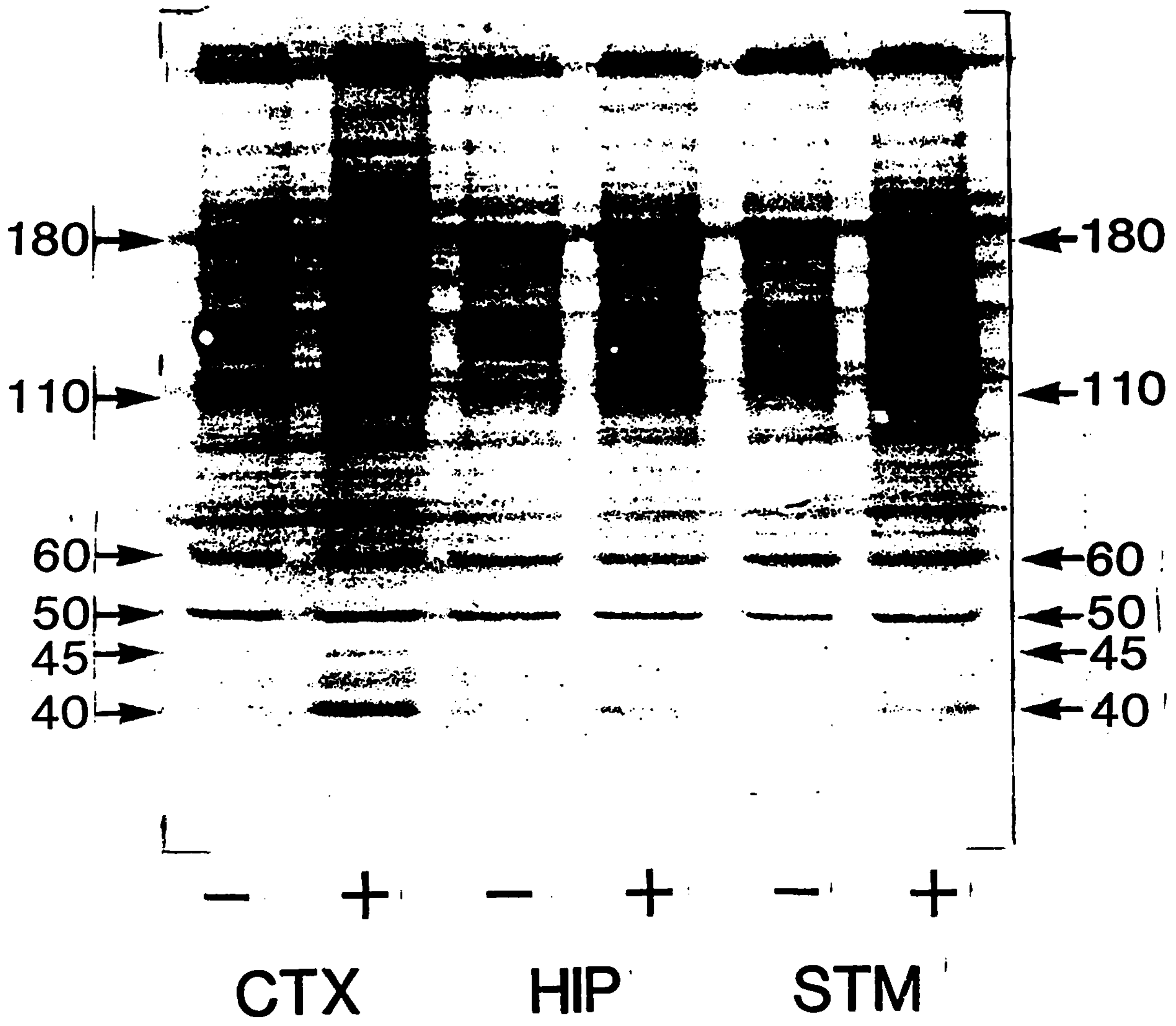

Figure 35. Quantitative analysis of hippocampal phosphotyrosine proteins during initiation and maintenance of status epilepticus.

Rats were treated with LiCl (3 mmole/kg, ip, 20 hr prior) and pilocarpine (30 mg/kg, sc) and sacrificed by focussed-beam microwave irradiation after 10 min (before paroxysmal spikes), 20 min (when spike trains are observed by EEG analysis), 25 min (immediately after the first tonic-clonic seizure signalling the initiation of status epilepticus), 60 min (during status epilepticus) and 120 min (during status epilepticus). Values were compared with controls run in parallel and are means ± SEM of 3-4 experiments.