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SELECTIVE INHIBITION OF EXCITATORY AMINO ACID-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN THE RAT HIPPOCAMPUS BY ACTIVATION OF PROTEIN KINASE C

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Abstract—The relative roles of protein kinase C in regulating excitatory amino acid-, cholinoceptor-, and adrenoceptor-stimulated phosphoinositide hydrolysis were studied. Slices of rat hippocampus were prelabeled with [^{3}H]-*myo*-inositol, and agonist-induced [^{3}H]-phosphoinositide hydrolysis was measured by the formation of [^{3}H]-inositol monophosphate ([^{3}H]-IP) in the presence of lithium ion. Activation of protein kinase C with phorbol 12,13-dibutyrate (PDB) (10^{-6} M) completely inhibited ibotenate (IBO) (10^{-3} M)-induced [^{3}H]-phosphoinositide hydrolysis. Half-maximal inhibition was observed at about 10^{-7} M PDB. Higher concentrations of PDB were required to inhibit stimulation of [^{3}H]-IP by either carbachol (CARB) (10^{-3} M) or norepinephrine (NE) (10^{-4} M), and only partial inhibition could be attained. Preincubation with staurosporine (STAURO) (10^{-5} M) or 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (10^{-4} M), inhibitors of protein kinase C, potentiated IBO- but not CARB- or NE-induced stimulation of [^{3}H]-IP. PDB inhibition of IBO- or NE-stimulated [^{3}H]-phosphoinositide hydrolysis was reversed by co-addition of STAURO or H-7. In the case of IBO + STAURO, this reversal was to the potentiated level observed with STAURO alone. Enhanced agonist stimulation and reversal of PDB inhibition were also produced by STAURO when [^{3}H]-phosphoinositide hydrolysis was stimulated by either L-glutamate or quisqualate. These experiments show that direct activation of protein kinase C by PDB leads to inhibition of phosphoinositide hydrolysis mediated by excitatory amino acid receptors, cholinoceptors, or adrenoceptors. However, the enhanced agonist-stimulated phosphoinositide hydrolysis elicited by inhibitors of protein kinase C suggests that, when protein kinase C is indirectly activated, only excitatory amino acids rapidly inhibit further receptor-coupling.

The hydrolysis of cellular phosphoinositides is a receptor-coupled event which mediates tissue responses to a variety of neurotransmitters and hormones. The products of hydrolysis, inositol phosphates (i.e. inositol 1,4,5-trisphosphate) and diacylglycerol, act as second messengers which mediate changes in intracellular metabolism [1, 2]. Inositol 1,4,5-trisphosphate has been shown to mobilize intracellular calcium from a variety of peripheral cells including pancreatic acinar cells [3], hepatocytes [4], vascular smooth muscle [5], and adrenal chromaffin cells [6, 7]. Recently, Ghandi and Ross [8] have reported that inositol 1,4,5-trisphosphate also releases calcium from presynaptic nerve endings.

Diacylglycerol serves a second messenger role by activating protein kinase C, a calcium phospholipid-dependent protein kinase that is present in high concentration in the brain [9–11]. A variety of cytosolic as well as membrane-bound proteins can serve as substrates for the activated enzyme [12, 13]. Phorbol esters are substances that mimic diacylglycerol second messenger and thus bypass the cell surface receptor to directly activate protein kinase C [14, 15]. In a number of tissues, phorbol esters have been shown to inhibit agonist-induced phosphoinositide hydrolysis [16, 19]. This suggests that at least one role of protein kinase C is to provide negative feedback by uncoupling the receptor, possibly to prevent excessive stimulation of the cell.

Receptor types that utilize this second messenger system in the brain include the alpha$_1$-adrenoceptor [20, 21], the muscarinic cholinoceptor [22, 23], the histamine H_1 receptor [24], and various peptide receptors including substance P [25], neurotensin [26], and vasopressin [27]. Excitatory amino acids also stimulate phosphoinositide hydrolysis in brain tissue. In slices of the rat hippocampus, agonists such as IBO†, quisqualate, and L-glutamate act at excitatory amino acid receptors that are specifically antagonized by aminophosphonobutyrate (AP4) [28–30].

Phorbol ester-induced inhibition of muscarinic cholinoceptor- and alpha-adrenoceptor-stimulated phosphoinositide hydrolysis in brain tissue has been shown previously [16]. The study reported here utilizes phorbol dibutyrate (PDB) to directly activate protein kinase C, and staurosporine (STAURO) and H-7, inhibitors of protein kinase C [31, 32], to examine a possible role for protein kinase C in regulating excitatory amino acid recep-

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† Abbreviations: IBO, ibotenate; PDB, phorbol 12,13-dibutyrate; CARB, carbachol; NE, norepinephrine; STAURO, staurosporine; IP, inositol monophosphate; and H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine.

tors coupled to phosphoinositide hydrolysis in brain.

METHODS

Measurement of phosphoinositide hydrolysis in slices of rat hippocampus. Male Sprague–Dawley rats (175–250 g) were decapitated. The brains were removed, and the hippocampi were dissected while bathed in Krebs-bicarbonate buffer of the following composition (mM): NaCl, 117.9; KCl, 4.72; $CaCl_2$, 2.54; $MgSO_4$, 1.18; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; dextrose, 11.1 (equilibrated with 95% O_2–5% CO_2, pH 7.4). Generally, the hippocampal tissue from eight rats was pooled in each experiment. This tissue was blotted on filter paper, weighed (about 0.12 g of tissue from each rat), and then chopped twice each in perpendicular directions using a McIlwain tissue chopper (0.3-mm intervals). Tissue slices were suspended in 10 vol. of the same buffer and centrifuged at 800 *g* for 5 min (4°). The resultant tissue pellet was resuspended in 5 vol. of cold buffer. An aliquot of this tissue suspension was added to a tube on ice containing [^{3}H]-*myo*-inositol for a final concentration of 10 μCi per ml of tissue (0.714 μM). Tissue was then incubated in a shaking water bath at 37° for 60 min to prelabel phosphoinositides. Following incubation, the labeled tissue was centrifuged (800 *g* for 5 min, 4°) and the supernatant fraction was discarded. The tissue pellet was washed twice by resuspension in 10 vol. of a modified Krebs-bicarbonate buffer which also contained 10 μM LiCl (in place of equimolar NaCl) and 10 μM nonlabeled *myo*-inositol. Samples were centrifuged as above following each resuspension. The final washed tissue pellet was resuspended in 5 vol. of modified Krebs-bicarbonate buffer. Aliquots of tissue suspension (240 μl, 2–3 mg protein) were added to 12 × 75 mm polypropylene tubes on ice. Tubes were incubated at 37° for 20 min to "chase" the [^{3}H]-inositol label. A 5-μl aliquot of the receptor agonist or water vehicle was added, and the incubation continued for 60 min. In preliminary studies, the effects of PBD were observed when it was added 20 min, 10 min, or at the same time as the receptor agonist. For convenience, PDB and/or STAURO, H-7, or dimethyl sulfoxide (DMSO) vehicle (5 μl) were added to tubes on ice prior to the 20-min "chase" incubation. The final concentration of DMSO vehicle (2%) had no significant effect on either basal or agonist-stimulated phosphoinositide hydrolysis. Preliminary experiments also showed that quisqualate (10^{-4} M) or IBO (10^{-3} M) stimulated increases in [^{3}H]-IP that were linear for up to 120 min of incubation. NE (10^{-4} M) or CARB (10^{-3} M) stimulated [^{3}H]-IP for up to 60 min of incubation.

The incubation was terminated by adding 1 ml of ice-cold 10 mM LiCl solution and placing the tubes on ice. Samples were homogenized using a Tekmar Tissuemizer and then centrifuged at 10,000 *g* for 10 min. The supernatant fraction was decanted and analyzed for [^{3}H]-IP using anion exchange column chromatography as previously described [33]. Protein in the resultant pellet from each sample (solubilized in 3% NaOH) was determined by the Biuret method [34]. Data were expressed as disintegrations per minute of [^{3}H]-IP per milligram protein or were converted and expressed as a percent of the basal value [no drug(s) added] in each experiment.

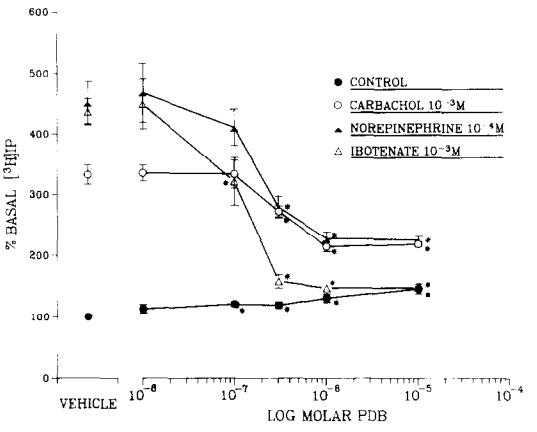

Fig. 1. Dose-related inhibition of agonist-stimulated [^{3}H]phosphoinositide hydrolysis by PDB. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and differing concentrations of PDB or DMSO vehicle. Receptor agonists were added, and the incubation was continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data were expressed as percent of the basal value (drug vehicles only) in each experiment. The absolute basal value from all experiments was 1928 ± 126 dpm [^{3}H]IP/mg protein (mean ± SE, N = 4). Key: (*) $p < 0.05$ when compared to the corresponding DMSO vehicle treatment group.

Statistical analysis. Statistical significance was determined using either a Student's *t*-test or ANOVA in conjunction with Duncan's New Multiple Range Procedure for comparison of multiple means [35]. A $P < 0.05$ was considered significant. The concentrations of agonists that stimulated 50% of maximal in each experiment (EC_{50}) were calculated by converting the data to probits. The EC_{50} concentration (probit 5) was then obtained by linear regression [35].

Drugs and chemicals. myo-[2-^{3}H]Inositol (14 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). The following substances were all purchased from the Sigma Chemical Co. (St. Louis, MO): quisqualic acid, ibotenic acid, L-glutamate monosodium, L-homocysteic acid, carbamylcholine (carbachol), norepinephrine bitartrate, H-7, 4α-phorbol, and phorbol 12,13-dibutyrate. Staurosporine was obtained from Dr. Jeffry Howbert (Eli Lilly & Company, Indianapolis, IN).

RESULTS

The effects of PDB on basal, excitatory amino acid-, cholinoceptor-, and adrenoceptor-stimulated phosphoinositide hydrolysis are shown in Fig. 1. In the absence of receptor agonists, PDB produced a very modest, but statistically significant and dose-related increase in [^{3}H]-IP above the basal value

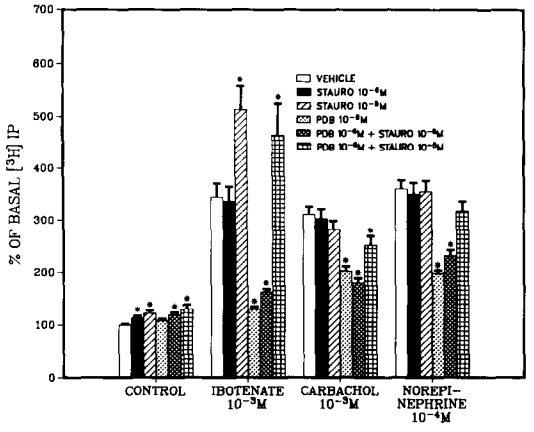

Fig. 2. Effects of STAURO and/or PDB on basal, IBO-, CARB-, and NE-stimulated [^{3}H]phosphoinositide hydrolysis. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and STAURO, PDB and/or DMSO vehicle. Receptor agonists were added, and the incubation was continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data were expressed as percent of the basal value (drug vehicles only) in each experiment. The absolute basal value from all experiments was 2225 ± 170 dpm [^{3}H]IP/mg protein (mean ± SE, N = 5). Key: (*) $P < 0.05$ when compared to the corresponding DMSO vehicle treatment group.

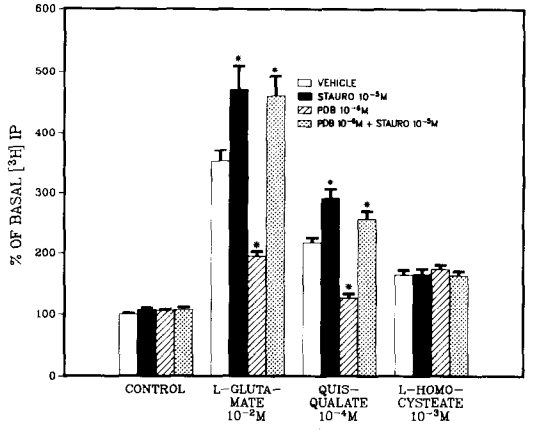

Fig. 3. Effects of STAURO and/or PDB on basal and excitatory amino acid-stimulated [^{3}H]phosphoinositide hydrolysis. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and STAURO, PDB and/or DMSO vehicle. Receptor agonists were added, and the incubation was continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data were expressed as percent of the basal value (drug vehicles only) in each experiment. The absolute basal value from all experiments was 2358 ± 75 dpm [^{3}H]IP/mg protein (mean ± SE, N = 4). Key: (*) $P < 0.05$ when compared to the corresponding DMSO vehicle treatment group.

(131% of basal at 10^{-6} M). IBO (10^{-3} M), NE (10^{-4} M), or CARB (10^{-3} M) each significantly increased [^{3}H]-IP by 3- to 4-fold in the absence of PDB. Incubation with PDB produced a dose related inhibition of receptor agonist-stimulated phosphoinositide hydrolysis. Of the three receptor agonists examined, IBO stimulation was the most sensitive to inhibition by PDB. At 10^{-7} M and 3×10^{-7} M PDB, IBO-stimulated [^{3}H]-IP above basal was decreased significantly to 66% and 17% of the IBO + DMSO vehicle control respectively. At 10^{-6} M PDB, IBO stimulation was inhibited completely, and values were not significantly different from PDB treatment alone. Relatively higher concentrations of PDB were required to inhibit stimulation by either CARB or NE. Significant inhibition was not observed until 3×10^{-7} M PDB, a concentration which inhibited NE and CARB stimulation to 50 and 75% of their respective agonist + DMSO vehicle controls. At 10^{-6} M PDB, NE and CARB stimulations of [^{3}H]-IP above basal were maximally inhibited to 36 and 50% of the agonist + DMSO vehicle controls respectively.

The effect of STAURO, a protein kinase C inhibitor, on IBO-, NE-, and CARB-stimulated phosphoinositide hydrolysis is shown in Fig. 2. STAURO alone very modestly enhanced the basal values of [^{3}H]-IP (122% of basal at 10^{-5} M). Also at 10^{-5} M, STAURO caused a potentiation of IBO-induced phosphoinositide hydrolysis. This potentiating effect of STAURO was selective for the excitatory amino acid agonist. STAURO alone did not alter stimulation by either CARB or NE. When IBO stimulation was maximally inhibited by PDB (10^{-6} M), STAURO reversed this inhibition in a concentration-related manner. At 10^{-5} M STAURO, the inhibitory effect of PDB on IBO stimulation was reversed completely to the potentiated value observed with STAURO alone. The higher concentration of STAURO (10^{-5} M) also significantly reversed PDB inhibition of either CARB- or NE-stimulated [^{3}H]-IP. In the case of CARB, this reversal was only partial and [^{3}H]-IP was still reduced significantly when compared to CARB alone. STAURO (10^{-5} M) significantly reversed PDB inhibition of NE stimulation to a value less than, but not significantly different from, NE alone.

STAURO also potentiated the effects of other excitatory amino acid agonists. As shown in Fig. 3, the agonists L-glutamate (10^{-2} M), quisqualate (10^{-4} M), and L-homocysteate (10^{-3} M) significantly increased [^{3}H]-IP to 352, 217, and 165% of the basal values respectively. STAURO (10^{-5} M) enhanced stimulation by L-glutamate and quisqualate to 470 and 291% of the basal values respectively. PDB (10^{-6} M) treatment resulted in near complete inhibition of quisqualate-stimulated phosphoinositide hydrolysis, and this inhibition was reversed to the potentiated values by STAURO. PDB (10^{-6} M) only partially inhibited stimulation by L-glutamate. Nevertheless, PDB inhibition of L-glutamate stimulation was reversed by STAURO to the potentiated value observed with STAURO alone. STAURO or PDB, either alone or in combination, had no effects on stimulation by L-homocysteate. In contrast to

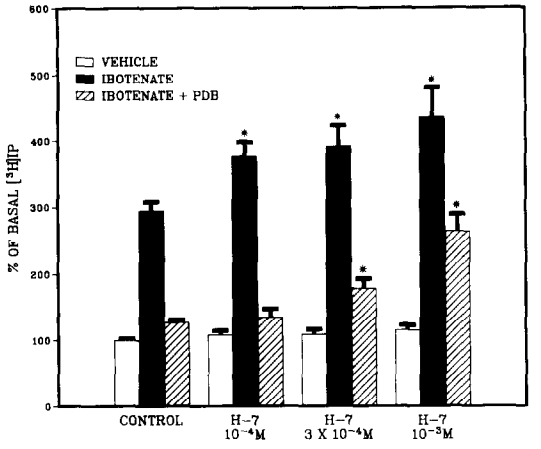

Fig. 4. Effects of H-7 and/or PDB on basal and IBO-stimulated [^{3}H]phosphoinositide hydrolysis. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and H-7, PDB (10^{-6} M final concn) and/or DMSO vehicle. IBO (10^{-3} M final concn) or water vehicle was added and the incubation continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data were expressed as percent of the basal value (drug vehicles only) in each experiment. The absolute basal value from all experiments was 1907 ± 74 dpm [^{3}H]IP/mg protein (mean ± SE, N = 4). Key: (*) $P < 0.05$ when compared to the corresponding control treatment group.

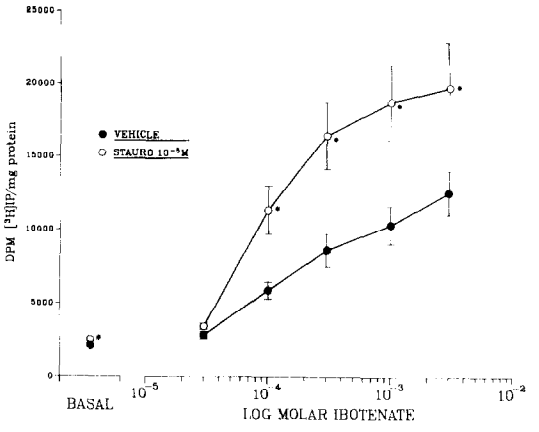

Fig. 5. Effect of STAURO on IBO dose–response stimulation of [^{3}H]phosphoinositide hydrolysis. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and STAURO (10^{-5} M final concn) and/or DMSO vehicle. IBO was added and the incubation continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data represent mean ± SE of six experiments. Key: (*) $P < 0.05$ when compared to the corresponding DMSO vehicle treatment group.

PDB, 4α-phorbol (10^{-5} M) which is an inactive phorbol ester did not alter stimulation by either IBO (10^{-3} M) or quisqualate (10^{-4} M) (data not shown).

Also, when IBO (10^{-3} M) was used as agonist, H-7 at 10^{-4} M or greater potentiated IBO stimulation (Fig. 4). H-7 significantly reversed PDB inhibition of IBO stimulation, but only at concentrations of 3×10^{-4} M or greater (Fig. 4). When NE (10^{-4} M) was used as agonist, H-7 (10^{-3} M) partially reversed the inhibitory effect of PDB (10^{-6} M), but did not potentiate agonist stimulation (data not shown). In the case of CARB (10^{-3} M) H-7 (10^{-3} M) did not potentiate agonist stimulation nor reverse the inhibitory effect of PDB (10^{-6} M) (data not shown).

The concentrations of receptor agonists used in the above studies were those which produce maximal or near-maximal stimulation of phosphoinositide hydrolysis under these conditions [30]. Figure 5 shows the effect of STAURO (10^{-5} M) on IBO dose–response stimulation. Again, basal values of [^{3}H]-IP were elevated modestly by STAURO (117% of the vehicle treatment). STAURO potentiated the effects of IBO at all concentrations where significant IBO stimulation was observed (10^{-4} M to 3×10^{-3} M). Using 3×10^{-3} M as the maximal effect (100% stimulation), the EC_{50}% values for IBO in the presence and absence of STAURO were 306 ± 50 and 152 ± 13 μM respectively. STAURO (10^{-5} M) had a similar potentiating effect on the quisqualate dose–response curve. As shown in Fig. 6, STAURO increased [^{3}H]-IP at all stimulatory concentrations of quisqualate to greater values than can be accounted for by the modest STAURO-induced increase in basal. Apparent EC_{50} values for quisqualate in the presence and absence of STAURO were 8.6 ± 0.9 and 10.5 ± 2.0 μM respectively.

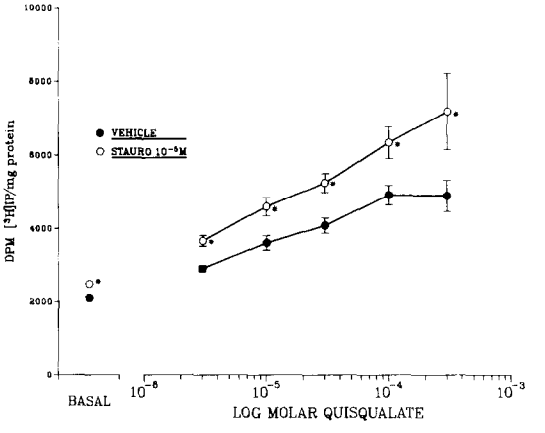

Fig. 6. Effects of STAURO on quisqualate dose–response stimulation of [^{3}H]phosphoinositide hydrolysis. Rat hippocampal slices were incubated with [^{3}H]*myo*-inositol for 60 min to prelabel [^{3}H]phosphoinositides. Tissue was washed and further incubated for 20 min in buffer with non-labeled *myo*-inositol (10 mM), LiCl (10 mM), and STAURO (10^{-5} M final concn) and/or DMSO vehicle. Quisqualate was added and the incubation continued for 60 min. [^{3}H]IP was isolated from tissue extracts using anion exchange column chromatography. Data represent mean ± SE of six experiments. Key: (*) $p < 0.05$ when compared to the corresponding DMSO vehicle treatment group.

DISCUSSION

The present study shows that excitatory amino acid receptors, like various other receptors that are linked to the enhanced hydrolysis of cell phosphoinositides, can be inhibited by a phorbol ester that activates protein kinase C. In agreement with Labarca *et al.* [16], PDB also induced the inhibition of alpha-adrenoceptor and muscarinic cholinoceptor coupling to phosphoinositide hydrolysis. However, the effect of PDB on IBO-and quisqualate-stimulated phosphoinositide hydrolysis was quantitatively greater when compared to the other receptor agonists. IBO and quisqualate stimulations could be inhibited completely with 10^{-6} M PDB. Moreover, relatively higher concentrations of PDB were required for significant inhibition of either carbachol or norepinephrine stimulation, and at the highest PDB concentrations only partial inhibition was seen. The inability of phorbol esters to inhibit completely receptor coupling has been observed in the other systems as well. For example, bradykinin-stimulated phosphoinositide hydrolysis in neuroblastoma-glioma cells can be inhibited by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), but only maximally by 50% [36]. The greater effect and sensitivity of PDB for excitatory amino acid receptor responses suggest that activated protein kinase C in some way exerts a greater negative influence on receptor coupling in excitatory amino acid sensitive cells.

It should be noted that direct activation of protein kinase C by phorbol esters would differ from indirect activation via receptors. Phorbol esters activate protein kinase C in a persistent fashion since they are not readily metabolized by the cell. However, activation of protein kinase C by diacylglycerol formed from phosphoinositide hydrolysis would be transient since endogenous diacylglycerol is readily metabolized. The effects of PDB in this study are due to direct activation of protein kinase C, and inhibitors of protein kinase C reversed PDB inhibition of excitatory amino acid, adrenoceptor, and cholinoceptor responses. If indirect activation of protein kinase C following receptor coupling also leads to inhibition, directly inhibiting protein kinase C should likewise prevent it, and effectively enhance stimulation by the agonist during the course of the incubation. Stimulation of phosphoinositide hydrolysis by IBO, L-glutamate, or quisqualate was selectively potentiated by STAURO. Thus, in the case of this neurotransmitter system, indirect "receptor" activation of protein kinase C and subsequent protein kinase C-mediated inhibition does appear to occur. However, this did not appear to be the case for adrenoceptor or cholinoceptor coupling, since inhibitors of protein kinase C did not enhance carbachol or norepinephrine response. Alternatively, in excitatory amino acid-, but not adrenoceptor- or cholinoceptor-sensitive cells, the basal activity of protein kinase C may be sufficient to maintain the cell in a partially inhibited state, and inhibition of protein kinase C thus leads to a potentiated response.

Although STAURO is a potent inhibitor of protein kinase C, its selectivity is relative and it will also inhibit cAMP-dependent protein kinase at about 10-fold higher concentration [37]. Furthermore, greater concentrations of STAURO and H-7 were required to reverse PDB inhibition in brain slices than those which will inhibit isolated protein kinase C. However, as also noted for phorbol esters [16], this discrepancy in potency between effects on the isolated enzyme versus that in brain slices is due, at least in part, to the high lipid solubility of these compounds. Sequestration of compounds such as STAURO into lipids of the brain slice would make the free concentration of the compound that is available to interact with the enzyme lower than that which was added. We do not know to what degree other kinases might be inhibited under these conditions and what influence this inhibition would have. Thus, the mechanism by which protein kinase C inhibitors selectively potentiate excitatory amino acid agonist receptor coupling warrants further study.

Multiple forms or families of protein kinase C termed alpha, beta, and gamma, have now been shown to exist in brain and other tissues [38–40]. Distinct forms of the enzyme in brain can also be differentially regulated by constituents of the membrane such as fatty acids [41]. Moreover, immunologically distinct protein kinase C isozymes have been shown to be localized in different cell types and cell populations of the brain [42–44]. It is possible that these forms of protein kinase C serve different functions in the various cell types. However, it is not presently known if a specific isozyme of protein kinase C mediates receptor inhibition phenomenon such as that shown here.

The biochemical mechanism involved in phorbol ester-induced receptor inhibition has been studied by others. Decreases in adrenoceptor coupling can be induced in vascular smooth muscle in a heterologous manner following bradykinin receptor stimulation or by direct activation of protein kinase C with phorbol ester [45]. This inhibitor effect occurs without changes in adrenoceptor ligand binding, but is associated with phosphorylation of serine and threonine residues of membrane proteins. Orellana *et al.* [46] have also shown that phorbol esters inhibit guanine nucleotide-stimulated [^{3}H]-phosphoinositide hydrolysis in membranes from 1321Ni astrocytoma cells. The addition of purified protein kinase C to the membrane mimics this effect of the phorbol ester. Therefore, protein kinase C may be altering the function of a membrane bound G-protein that regulates the phospholipase C enzyme.

Stimulation of phosphoinositide hydrolysis by L-homocysteate differed from all other agonists used in this study. Its stimulation, although quantitatively less than the other agonists, was not inhibited by PDB nor potentiated by STAURO. Since stimulation by this agonist is apparently not regulated in a similar manner when compared to other excitatory amino acid agonists, its effect on phosphoinositide hydrolysis could be mediated by a different population of receptors or may be due to a non-receptor mechanism. L-Homocysteate also has some affinity for the *N*-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptor [47, 48], and NMDA receptor agonists can stimulate phosphoinositide hydrolysis [49]. However, NMDA responses are

greatly inhibited by magnesium ions [50], and the present study used a magnesium-containing buffer. In agreement with Nicoletti *et al.* [29], our laboratory has observed that NMDA at concentrations up to 10^{-3} M does not stimulate phosphoinositide hydrolysis under these assay conditions [30]. Thus, the stimulatory effect of L-homocysteate is probably not due to NMDA receptor activation. It will require additional investigation to elucidate why the effect of this endogenous excitatory amino acid on phosphoinositide hydrolysis was not also regulated by protein kinase C.

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