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INHIBITION OF RECEPTOR-COUPLED PHOSPHOINOSITIDE HYDROLYSIS BY SULFUR-CONTAINING AMINO ACIDS IN RAT BRAIN SLICES

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Abstract—Sulfur-containing amino acids were found to inhibit norepinephrine-stimulated [^{3}H]phosphoinositide hydrolysis in rat cortical slices. Of the amino acids tested, L-cysteine was the most potent, inhibiting the response by 42 and 85% at concentrations of 50 and 500 μM respectively. L-Cystine and L-serine-*O*-sulfate also inhibited the response to norepinephrine, but to a lesser degree than did L-cysteine. L-Homocysteic acid slightly potentiated phosphoinositide hydrolysis at a concentration of 100 μM, but caused inhibition at 500 μM. L-Cysteine sulfinate produced effects intermediate to those of L-cysteine and L-homocysteic acid, having no effect on the response to norepinephrine at 50 μM, but causing 84% inhibition at 500 μM. The D-isomers of cysteine and homocysteic acid were much less potent than were the L-isomers. Examination of the time course of the inhibition of norepinephrine-stimulated [^{3}H]phosphoinositide hydrolysis by L-cysteine showed that it was inhibited almost completely after 15, 30, 45 and 60 min of incubation. L-Cysteine and L-homocysteic acid caused similarly strong inhibitions of the production of [^{3}H]inositol monophosphate, [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate. The hydrolysis of [^{3}H]phosphoinositides stimulated by norepinephrine in slices from rat hippocampus and striatum were inhibited by L-cysteine to an extent similar to that occurring in cortical slices. These results demonstrate that several sulfur-containing amino acids, some of which have been proposed to be endogenous excitatory amino acid neurotransmitters, effectively modulate the response to norepinephrine of the phosphoinositide second messenger system in rat brain.

The second messengers generated from phosphoinositide metabolism have been shown to play important roles in neuronal function in the mammalian central nervous system [1, 2]. Agonists, such as norepinephrine, activate receptors coupled to a G-protein which stimulates phospholipase C to hydrolyze inositol-containing phospholipids. At least two second messengers are formed from this reaction, diacylglycerol, which activates protein kinase C, and inositol trisphosphate, which mobilizes intracellular calcium [3]. Only few modulatory mechanisms have been identified for this system in brain, and this is currently an area of great interest. Excitatory amino acids have been reported recently to produce stimulatory or inhibitory effects on phosphoinositide metabolism in brain tissue. Of interest for this study, excitatory amino acids have been found to inhibit norepinephrine-induced phosphoinositide hydrolysis in brain tissue by some investigators [4, 5], but not by others [6, 7].

Several sulfur-containing amino acids have been identified as endogenous constituents of mammalian brain, and have been shown to have excitatory properties and to bind to excitatory amino acid binding sites [8–11]. For example, L-homocysteic acid, which has been studied in relatively greater detail than most others, has been shown by several studies to fulfill most of the criteria required for it to be considered an excitatory amino acid neurotransmitter. Release of L-homocysteic acid can be stimulated from brain slices by depolarization in a calcium-dependent manner, and it binds excitatory amino acid receptors, mainly the *N*-methyl-D-aspartate and 2-amino-4-phosphonobutanoate (AP4) sites [11, 12]. There may also be a specific homocysteic acid binding site in the brain [13]. It has been reported that L-homocysteic acid can induce either excitatory or excitotoxic effects in brains [14–16]. The stereospecificity of homocysteic acid on its excitatory effect, binding, and uptake characteristics has been studied, with the L-enantiomer being more potent than the D-enantiomer [17–19]. Other sulfur-containing amino acids have been studied to varying degrees and some, such as L-cysteine sulfinate, have been shown to have excitatory properties [20–22]. L-Cysteine has been shown to bind to the AP4 excitatory amino acid binding site in rat brain [11], but it is not known if this occurs *in vivo*. Recently, cysteine was shown to be released from rat brain slices by depolarization in a calcium-dependent manner, and cysteine was suggested to be an endogenous neuromodulator [23].

Because inhibition by excitatory amino acids represents one of the few known modulators of the phosphoinositide response in the brain, we investigated whether sulfur-containing amino acids may play a role in modulating norepinephrine-stimulated phosphoinositide hydrolysis in rat brain slices.

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MATERIALS AND METHODS

Tissue preparation. Male, Sprague–Dawley rats

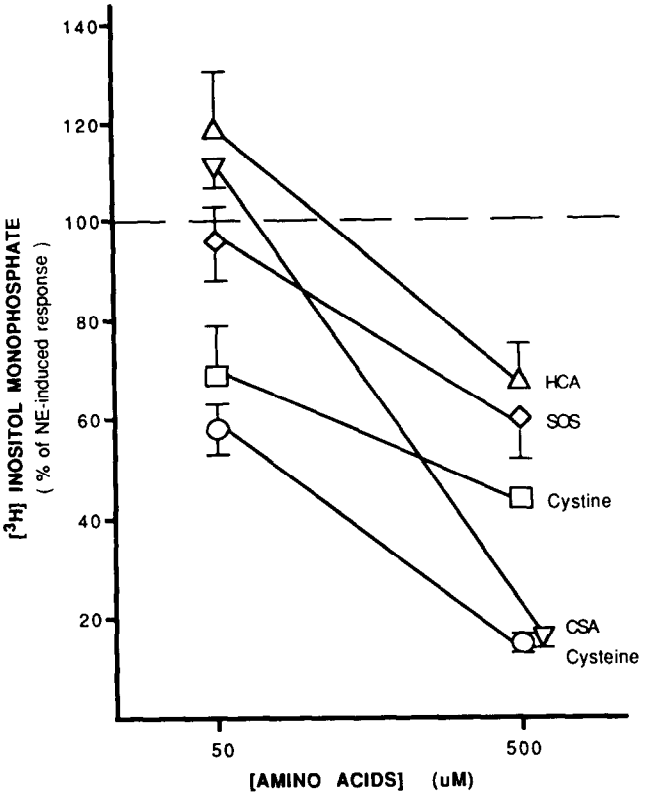

Fig. 1. Effects of sulfur-containing amino acids on norepinephrine-stimulated [^{3}H]inositol monophosphate production in rat cortical slices. Slices were washed and incubated with [^{3}H]inositol for 60 min, as described in Materials and Methods, and then the labeled slices were incubated for 60 min with 200 μM norepinephrine plus each of the following sulfur-containing amino acids: (○) L-cysteine, (□) L-cystine, (▽) L-cysteine sulfinate, (◇) L-serine-*O*-sulfate, and (△) L-homocysteic acid. [^{3}H]Inositol monophosphate was measured, and the results are expressed as a percentage of the norepinephrine-induced response (7800 ± 900 cpm). Each symbol represents the mean ± SE of three or four experiments measured in triplicate.

(200–250 g) were decapitated and the brains were dissected rapidly in ice-cold 0.32 M sucrose. Slices (0.3 mm × 0.3 mm) from cerebral cortex, hippocampus or striatum were prepared with a McIlwain tissue slicer, and washed thoroughly with Krebs–bicarbonate–HEPES medium (NaCl, 122 mM; $NaHCO_3$, 3.6 mM; $MgSO_4$, 1.2 mM; KCl, 5 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 1.3 mM; *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), 30 mM; glucose, 11 mM; freshly bubbled with 95% O_2/5% CO_2; adjusted to pH 7.35). The slices were incubated with the same medium at 37° for 45 min for regeneration, followed by several washes with fresh medium.

Assay of [^{3}H]*inositol phosphate production.* Slices were incubated at 37° for 1 hr in fresh buffer containing 0.5 μM *myo*-[2-^{3}H]inositol (15 Ci/mmol) and 10 mM LiCl. After the incubation, the slices were rapidly washed several times with cold medium to remove exogenous free [^{3}H]inositol. Aliquots of [^{3}H]inositol-labeled slices were incubated in a final volume of 500 μl for 1 hr in the presence of the indicated agonists and/or sulfur-containing amino acids. The reaction was stopped by adding 1.7 ml of $CHCl_3$:MeOH:12 N HCl (1:2:0.01). The samples were transferred to extraction tubes and mixed with 1 ml of $CHCl_3$ and 0.5 ml of 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 as described by Berridge *et al.* [24]. Organic fractions were dried overnight at room temperature, and the radioactivity was measured.

Statistical analysis. Data were analyzed using Student's *t*-test, and a P value of <0.05 was considered significant.

Materials. L-Norepinephrine, D- and L-cysteine, L-cystine, D- and L-homocysteic acid, L-serine-*O*-sulfate and L-cysteine sulfinate were from the Sigma Chemical Co. (St Louis, MO). *Myo*-[2-^{3}H(N)]Inositol (15 Ci/mmol) was obtained from American Radiolabelled Chemical Inc. (St Louis, MO). AG1-X8 anion exchange resin was from Bio-Rad (Richmond, CA).

RESULTS

Several sulfur-containing amino acids with different structures, chemical properties and excitatory

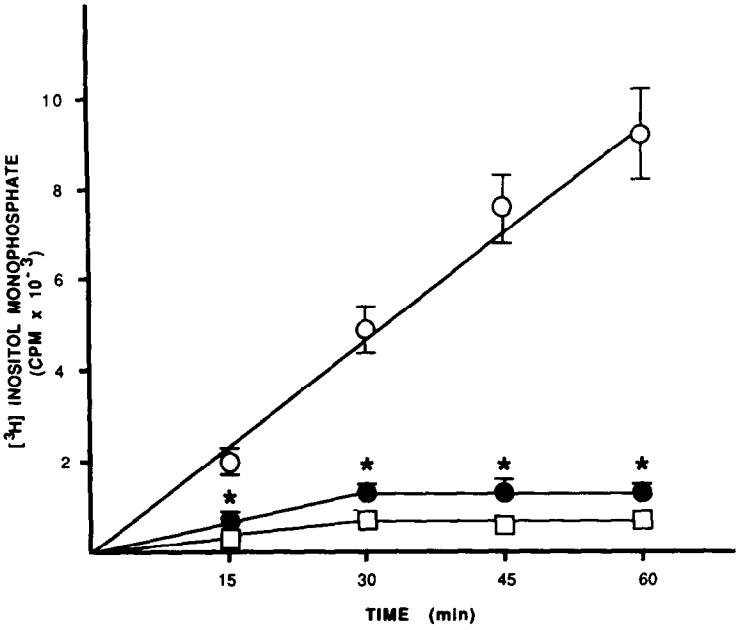

Fig. 2. Time course of [^{3}H]inositol monophosphate production in the presence of norepinephrine and L-cysteine in rat cortical slices. Slices prelabeled with [^{3}H]inositol were incubated for 15, 30, 45 or 60 min with (□) no addition, (○) 200 μM norepinephrine, or (●) 200 μM norepinephrine and 500 μM L-cysteine. Each symbol represents the mean ± SE of three experiments measured in triplicate. Key: (*)$P < 0.05$ compared with norepinephrine at each time point (Student's *t*-test).

amino acid receptor binding characteristics [11] were tested for effects on [^{3}H]inositol monophosphate formation stimulated by norepinephrine in cortical slices (Fig. 1). At a concentration of 500 μM, each of the sulfur-containing amino acids significantly ($P < 0.05$) inhibited norepinephrine-stimulated phosphoinositide hydrolysis. L-Cysteine was found to be the most potent inhibitor, causing a 42% inhibition at the lower concentration (50 μM) and 85% inhibition at the higher concentration (500 μM). L-Cystine was also inhibitory, but the effect was less than that of L-cysteine at 50 μM and the inhibition was not much greater when the concentration was increased to 500 μM. The lower concentration of L-cysteine sulfinate had no inhibitory effect, but a strong inhibitory effect (of 84%) was caused by 500 μM L-cysteine sulfinate, indicating a very steep concentration–response curve. L-Serine-*O*-sulfate was a relatively weak inhibitor compared to L-cysteine and L-cystine, but still inhibited the response by 40% at 500 μM. L-Homocysteic acid caused a biphasic effect; 50 μM L-homocysteic acid slightly potentiated [^{3}H]inositol monophosphate formation in the presence of norepinephrine, whereas a 32% inhibition was caused by 500 μM L-homocysteic acid.

L-Cysteine and L-homocysteic acid were studied further because the former was the most potent of the agents tested, and the latter has been suggested to be an endogenous excitatory amino acid neurotransmitter and produced both stimulation and inhibition of the norepinephrine response.

Figure 2 shows the time course of the response to norepinephrine and the inhibition by L-cysteine. Norepinephrine significantly stimulated the production of [^{3}H]inositol monophosphate, which increased linearly throughout the 60-min incubation period. L-Cysteine (500 μM) significantly ($P < 0.05$) inhibited norepinephrine-stimulated [^{3}H]inositol monophosphate production at all time points tested from 15 to 60 min of incubation.

The concentration-dependence of the effects of the D- and L-isomers of cysteine and homocysteic acid on norepinephrine-stimulated phosphoinositide hydrolysis in cortical slices is shown in Fig. 3. L-Cysteine was at least an order of magnitude more potent than was D-cysteine. D-Homocysteic acid caused no inhibition up to a concentration of 1 mM, in contrast to L-homocysteic acid which caused an 80% inhibition at that concentration. L-Cysteine, but not D-cysteine, also reduced the production of [^{3}H]inositol monophosphate in the absence of added agonists, whereas both D- and L-homocysteic acid slightly stimulated production at concentrations of 100 μM, but only the L-isomer caused inhibition at a concentration of 1 mM (data not shown).

The effects of L-cysteine and L-homocysteic acid on [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate production are shown in Fig. 4. The results were similar to those obtained with [^{3}H]inositol monophosphate. Norepinephrine significantly ($P < 0.05$) stimulated the production of [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate and 500 μM L-cysteine or L-homocysteic acid significantly ($P < 0.05$) inhibited the production of each of these products. The inhibitory effect of L-cysteine was greater than that of L-homocysteic acid on both products, as it was with [^{3}H]inositol monophosphate.

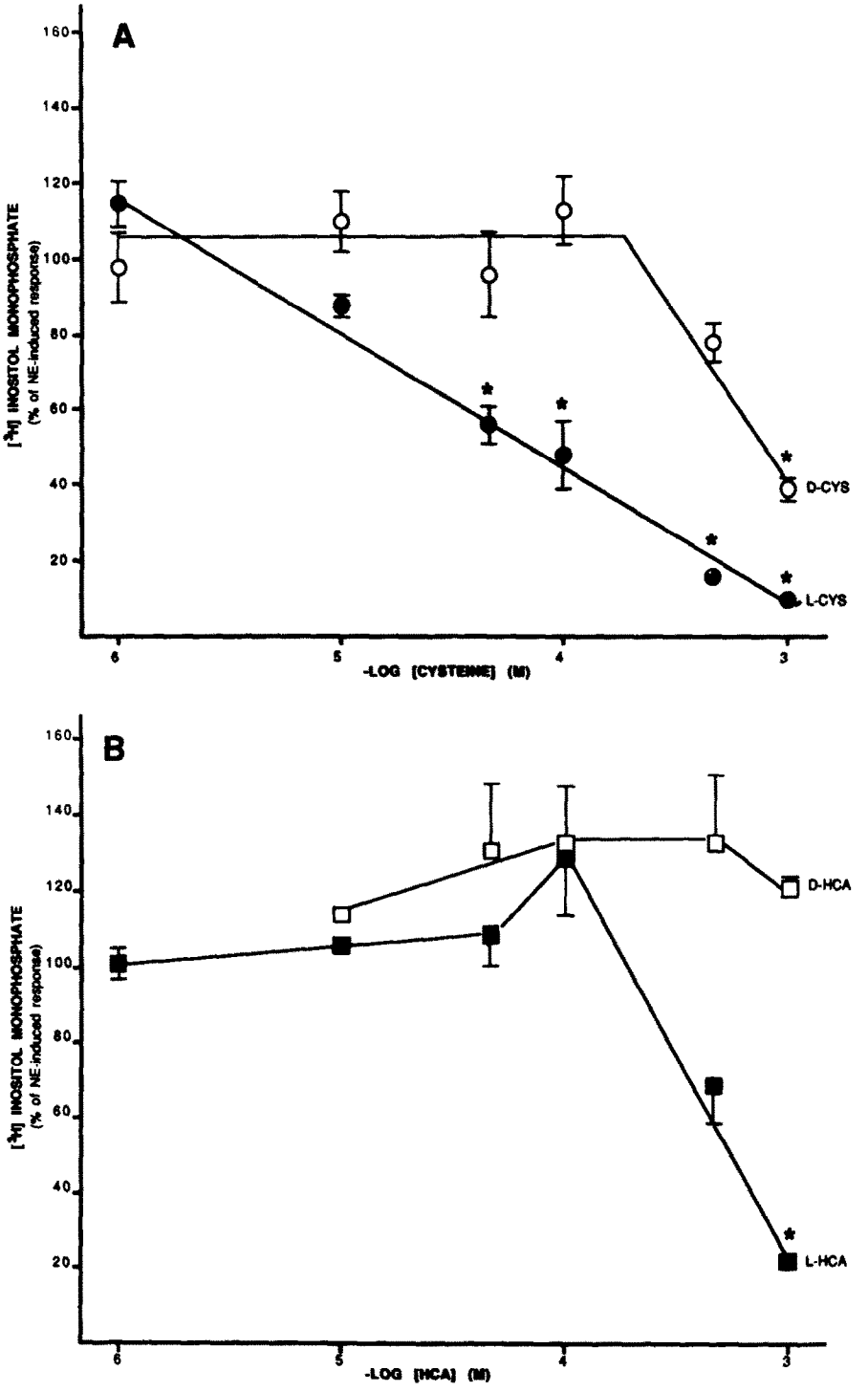

Fig. 3. Concentration–response of D- and L-isomers of cysteine and homocysteic acid on norepinephrine-stimulated [^{3}H]inositol monophosphate production in rat cortical slices. Prelabeled slices were incubated with D-cysteine (○) or L-cysteine (●) (panel A) or with D-homocysteic acid (□) or L-homocysteic acid (■) (panel B) in the presence of 200 μM norepinephrine. [^{3}H]Inositol monophosphate was measured, and the results are expressed as a percentage of norepinephrine-induced response (6500 ± 650 cpm). Each symbol represents the mean ± SE of three experiments measured in triplicate. Key: (*)$P < 0.05$ compared with norepinephrine (Student's t-test).

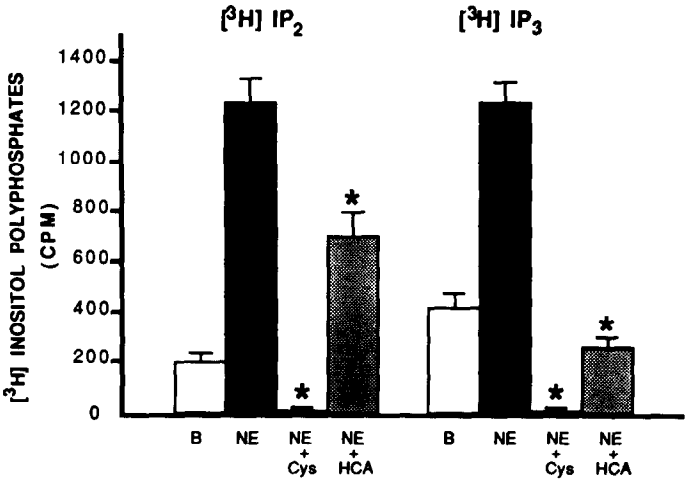

Fig. 4. Effects of norepinephrine and L-cysteine or L-homocysteic acid on [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate production in rat cortical slices. Prelabeled slices were incubated with no addition (B) or the indicated agents for 60 min using 200 μM norepinephrine (NE) and 500 μM L-cysteine (Cys) or L-homocysteic acid (HCA). [^{3}H]Inositol bisphosphate (IP_2) and [^{3}H]inositol trisphosphate (IP_3) were measured as described in Materials and Methods. Each value is the mean ± SE of three experiments measured in triplicate. Key: (*)$P < 0.05$ compared with norepinephrine (Student's t-test).

To test if the inhibitory effect of L-cysteine on phosphoinositide hydrolysis is also elicited in other brain regions, [^{3}H]inositol monophosphate production was measured in prelabeled slices from the hippocampus and the striatum (Fig. 5). Norepinephrine stimulated [^{3}H]inositol monophosphate to a higher degree in the hippocampus than in the cortex, but produced a lower response in the striatum. L-Cysteine (500 μM) significantly ($P < 0.05$) inhibited basal and norepinephrine-stimulated [^{3}H]inositol monophosphate production in slices from all three of the regions examined.

DISCUSSION

Each one of the five different sulfur-containing amino acids that were tested inhibited phosphoinositide hydrolysis stimulated by norepinephrine. Among these sulfur-containing amino acids, L-cysteine was the most potent inhibitor and, therefore, was studied in the greatest detail. The inhibition of the response to norepinephrine by 500 μM L-cysteine appeared to be rapid and almost complete, as there was little production of [^{3}H]inositol monophosphate over basal throughout the entire time course and there was no stimulation of the production of [^{3}H]inositol bisphosphate or [^{3}H]inositol trisphosphate. The IC_{50} value of approximately 50 μM for L-cysteine is about 10-fold lower than that reported for glutamate on norepinephrine-stimulated phosphoinositide hydrolysis in hippocampal slices [4] and is similar to those reported for several excitatory amino acids on carbachol-induced phosphoinositide hydrolysis in brain tissue [6, 7]. This effect was selective for L-cysteine, as the potency of D-cysteine was approximately 10-fold less. The functional significance of this effect of L-cysteine is unknown. Since L-cysteine is not commonly considered to be a neurotransmitter or neuromodulator, these effects may be the result of L-cysteine interacting with a site normally activated by another endogenous, perhaps sulfur-containing, modulator. Alternatively, cysteine was suggested recently to be a potential endogenous neuromodulator [23]. L-Homocysteic acid, on the other hand, has been shown in several studies to meet many of the criteria required for it to be considered as an excitatory amino acid neurotransmitter [10–12, 14–17]. L-Homocysteic acid has been reported previously to stimulate slightly phosphoinositide hydrolysis in the absence of other agonists and to inhibit carbachol-induced inositol phosphate formation in hippocampal slices [6, 25, 26]. We found that L-homocysteic acid appeared to have a biphasic effect, at 100 μM slightly stimulating (by 31%) the response to norepinephrine and causing inhibition at higher concentrations. In contrast to L-homocysteic acid, D-homocysteic acid caused no inhibition at the concentration tested. All three of the other sulfur-containing amino acids that were tested, L-cysteine sulfinate, L-cystine and L-serine-O-sulfate, significantly ($P < 0.05$) inhibited norepinephrine-stimulated phosphoinositide hydrolysis with varying potencies. L-Cysteine sulfinate, which is considered by some investigators to be a candidate excitatory amino acid neurotransmitter [20–22], appeared to possess a combination of the properties of L-homocysteic acid and L-cysteine. At 50 μM, L-cysteine sulfinate caused no inhibition, and perhaps a slight stimulation, of the norepinephrine-induced response, whereas at 500 μM L-cysteine sulfinate caused inhibition to an extent similar to L-cysteine.

It is interesting that, in binding studies reported by Pullan *et al.* [11], the K_i value of each of the five

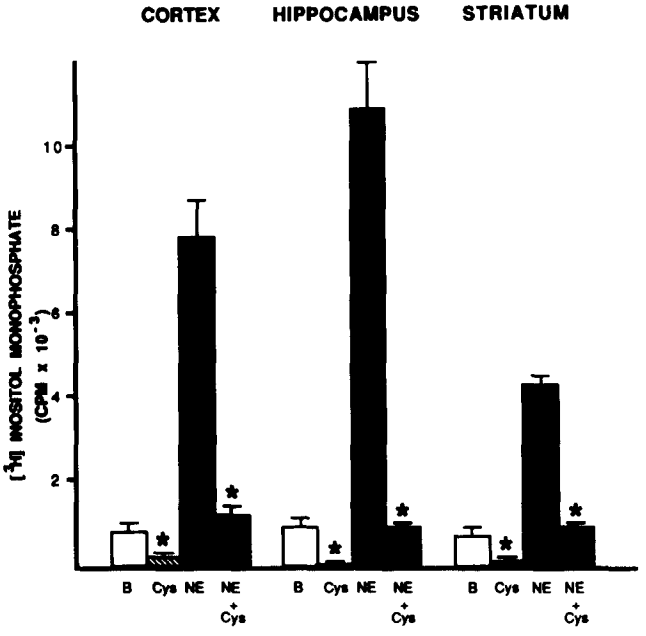

Fig. 5. Effect of L-cysteine on [^{3}H]inositol monophosphate production in the absence or presence of norepinephrine in rat cortical, hippocampal and striatal slices. Prelabeled slices from the three brain regions were incubated with no addition (B) or the indicated agents for 60 min using 200 μM norepinephrine (NE) and 500 μM L-cysteine (Cys). Each value is the mean ± SE of three experiments measured in triplicate. Key: (*)$P < 0.05$ compared with no addition for L-cysteine alone or compared with norepinephrine for norepinephrine plus L-cysteine (Student's t-test).

sulfur-containing amino acids studied here for the L-AP4 site was approximately equal to or lower than their K_i values for *N*-methyl-D-aspartate, kainate or quisqualate binding sites. Thus, it is possible that the AP4 site is associated with the modulatory effect on norepinephrine-induced phosphoinositide hydrolysis.

There are many potential mechanisms whereby sulfur-containing amino acids could inhibit norepinephrine-induced phosphoinositide hydrolysis, including: (i) excitotoxicity, as caused by excitatory amino acids, could result in cell damage causing the observed inhibitory effect, (ii) sulfur-containing amino acids could activate specific receptors which have modulatory functions, either directly interacting with the α_1-adrenergic receptor or indirectly producing inhibition mediated by second messengers, and (iii) sulfur-containing amino acids may be taken up by cells and modulate the phosphoinositide system at an intracellular site. Studies aimed at distinguishing among these possibilities should help to resolve the functional interactions between the sulfur-containing amino acids and the phosphoinositide system. Although the mechanism remains to be resolved, the significant inhibitory effect of sulfur-containing amino acids on norepinephrine-stimulated phosphoinositide hydrolysis may prove to be an important regulatory process of the phosphoinositide second messenger system.

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