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Hippocampal Phosphoinositide Turnover Is Altered by Hippocampal Sympathetic Ingrowth and Cholinergic Denervation

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HARRELL, L. E., V. AYYAGARI, D. S. PARSONS, D. J. CONNOR AND A. PEAGLER. *Hippocampal phosphoinositide turnover is altered by hippocampal sympathetic ingrowth and cholinergic denervation.* PHARMACOL BIOCHEM BEHAV 42(2) 277–284, 1992.—Cholinergic denervation of the hippocampus, by medial septal (MS) lesions, results in an unusual neuronal rearrangement in which peripheral sympathetic nerves, which originate from the superior cervical ganglia, grow into the hippocampal formation. To assess the functional significance of hippocampal sympathetic ingrowth (HSI), hydrolysis of phosphoinositides was examined in three groups: control, MS lesions + sham ganglionectomy (HSI group); and MS lesions + ganglionectomy (MSGx; no ingrowth). Four months after surgery, both norepinephrine (NE) and carbachol were found to produce a dose-dependent increase in the hydrolysis of hippocampal phosphoinositides in all groups. However, the presence of HSI, when compared to control and MSGx groups, significantly enhanced the turnover of phosphoinositides when stimulated by carbachol, but not NE. In further studies, the time course of this effect was studied. One week after surgery, carbachol-stimulated phosphoinositide turnover was equivalent among all groups; by 2 weeks, phosphoinositide turnover was enhanced in the HSI and MSGx group; by 4 weeks, PI turnover was markedly diminished in the MSGx group when compared to both the HSI and control groups, which were equivalent to each other. To ensure that the ganglionectomy alone did not alter phosphoinositide turnover, a ganglionectomy-alone group was studied at the 4-week time point. In this group, phosphoinositide turnover was equivalent to controls, suggesting no influence of the superior cervical ganglia on this response. In all groups, atropine inhibited carbachol-stimulated phosphoinositide turnover. These results suggest that both cholinergic denervation (i.e., MSGx group) and HSI produce marked functional alterations in hippocampal metabolic activity.

Phosphoinositol metabolism Hippocampus Sympathetic ingrowth Medial septum

FOLLOWING destruction of some central cholinergic neuronal populations, peripheral sympathetic fibers originating in the superior cervical ganglia and usually confined to cranial blood vessels, choroid plexus, and pineal gland, grow into the CNS and appear to replace the degenerating cholinergic nerve terminals (8). This reorganization has been described in the neocortex after lesions of the nucleus basalis magnocellularis (16), the origin of the neocortical cholinergic projection (20), and in the habenula and hippocampus after interruption of the cholinergic septohippocampal pathway (8). Although this neuronal reorganization has been most extensively studied in the rat hippocampus, it has also recently been reported to occur in the neocortex of Alzheimer's disease patients (3). The functional significance of this neuronal rearrangement, however, is unclear.

Recent investigations in the rat brain have demonstrated that some neurotransmitters mediate their effects through phosphoinositide-linked second messenger systems (4,11). Thus, stimulation of muscarinic and α-adrenergic receptors results in the hydrolysis of phosphoinositides to form inositol-1,4,5-trisphosphate and diacylglycerol (11). These substances, in turn, have been suggested to play a pivotal role in subsequent intracellular events (i.e., increased release of intracellular calcium and activation of protein kinase C) (4,) that may underlie synaptic plasticity and learning/memory (1).

In a previous study (7), we found that 4 months after medial septal lesions hydrolysis of phosphoinositides in the hippocampus was increased by carbachol, but not norepinephrine (NE), stimulation. However, because ganglionectomized animals were not studied it was unclear whether this effect was

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due to brain injury alone or occurred secondary to hippocampal sympathetic ingrowth (HSI). In an attempt to differentiate between these two possibilities, we examined the effect of carbachol- and NE-stimulated hydrolysis of phosphoinositides in the hippocampus 4 months after medial septal (MS) lesions in animals with and without HSI.

EXPERIMENT 1

METHOD

Animals and Surgery

Forty-six male Sprague-Dawley rats, initial body weight of 180–200 g, were housed communally in a light- (12 L:12 D) and temperature-controlled colony. Following 1 week of adaptation, animals were randomly assigned to one of three surgical groups (18 animals/group): controls (CON)—sham MS lesions and sham ganglionectomy (GX); MS lesions + sham Gx (ingrowth group), and MS lesions + Gx. Bilateral MS lesions [AP + 0.8 mm (from bregma); DV-5.8 mm (from dura); LAT = 0.0 mm] were made by passing a direct current (3 mA × 25 s) through a bipolar Teflon-coated stainless steel electrode. Sham-operated animals were treated in a similar fashion except no current was passed. Bilateral superior cervical ganglionectomies were performed by making a midline neck incision, with blunt dissection utilized to expose the bifurcation of the carotid artery. The superior cervical ganglia were visualized and removed. Sham-operated animals were treated in a similar manner except ganglia were left in place. All surgery was performed at one time utilizing a combination of ketamine (110 mg/kg) and xylazine (13 mg/kg) for anesthesia.

Procedure

Animals were sacrificed 130–140 days after surgery and brains removed. A septal block was taken to assess lesion placement, while the hippocampi were dissected and pooled from two animals for subsequent measurement of phosphoinositide hydrolysis employing a modification of Berridge et al. (2) utilizing a "batch" procedure.

Assay of [^{3}H]Inositol Metabolites

Immediately after dissection, the hippocampi were cross-chopped on a McIlwain tissue slicer (350 × 350 μm) and washed in cold HEPES-bicarb. buffer (in mM: NaCl, 122; KCl, 4.9; $MgSO_4$, 1.2; $NaHCO_3$, 3.6; KH_2PO_4, 1.2; $CaCl_2$, 1.3; dextrose, 11; HEPES, 30; pH 7.4; oxygenated in 95% O_2/5% CO_2 × 30 min). Slices were then incubated in buffer at 37°C for 60 min. The tissue was further washed and labeling of the slices was performed by adding 0.053 mmol myo[^{3}H]inositol (19 Ci/mM; Amersham Corp., Arlington Heights, IL) in 0.5 ml to 3.5 ml buffer and incubated at 37°C for 60 min. Following this step, 25 μl tissue was added to a biovial containing Li^+ (10 mM), agonist (carbachol 0.05, 0.125, 0.5, 5.0 mM; NE 1, 5, 200 μM) and buffer and incubated for 60 min at 37°C. The incubation was stopped with 1.7 ml chloroform:methanol:HCl (1:2:0.01) mixture. Reaction products were transferred to extraction tubes containing 1 ml chloroform and 0.5 ml H_2O and vortexed. The mixture was centrifuged and the aqueous layer removed for analysis on dowex columns. The lipid layer was transferred to scintillation vials and allowed to dry overnight, after which scintillation solution (5 ml Budget-Solve; Fisher Scientific Co., Fair Lawn, NJ) was added. Dowex-1-formate resin (0.5 ml; Bio Rad, Richmond, CA) was added with 1 ml H_2O to the aqueous samples and vortexed. Ten minutes later, the dowex and samples were transferred to 10-ml disposable minicolumns and washed with 8 ml of a 5 mM disodium tetraborate/60 mM sodium formate solution to remove free inositol and glycerol phosphoinositol. Inositol monophosphate (IP_1) was eluted with 6 ml 0.2 M ammonium formate/0.1 M formic acid solution into scintillation vials and 10 ml Budget-Solve added. The data was expressed as a percent over basal stimulation, and to compensate for the variability associated with pipetting slice suspensions the radioactivity accumulating in the IP_1 fraction was also expressed as IP_1/IP_1 + lipid (12).

Histology

To ensure appropriate lesion placement and completeness of ganglionectomy, coronal sections (20 μm) were taken at 100-μm intervals throughout the septum on an AO cryostat. Adjacent sections were stained with cresyl violet and for acetylcholinesterase (25). The third set were processed for catecholamine histofluorescence by the method of de la Torre and Surgeon (9).

Data Analysis

To assess the effect of surgical manipulations on phosphoinositide hydrolysis, analyses of variance (ANOVAs), followed by Duncan's multiple-range test or least-square means, were performed.

RESULTS

Anatomy

Examination of the lesions revealed complete destruction of the medial septal region including the cholinergic cell bodies within the medial septal region and the vertical limb of the diagonal band of Broca (Figs. 1A and B). The lesions were also found, in some animals, to extend into the region of the lateral septum. There was sparing of the overlying cortical regions and the most posterior aspects of the septum. No tissue destruction was evident in control animals, although gliosis was observed along the electrode tract. In controls and animals that were sham ganglionectomized, typical peripheral sympathetic fibers were observed surrounding blood vessels. These fibers were not present in ganglionectomized animals (Figs. 1C and D).

Biochemistry

Basal labeling of the inositol phosphates and inositol lipids was fairly consistent from preparation to preparation, varying from 16,607–22,950 dpm/mg protein for the phospholipids and from 1,045–1,332 dpm/mg protein for the inositol phosphates. NE-stimulated hydrolysis of phosphoinositides was found to be dependent on the concentration of NE employed. Thus, little hydrolysis was observed at 1 μM while near maximal hydrolysis was found at 200 μM. No differences were observed among groups of animals at any of the concentration levels (Fig. 2).

Like NE, carbachol (Fig. 2) was found to stimulate phosphoinositide hydrolysis in a dose-dependent fashion. No differences were observed among the different groups of animals at the lower concentrations of carbachol. However, at the maximal concentration of carbachol employed in this study a significant group effect was observed on the accumulation of

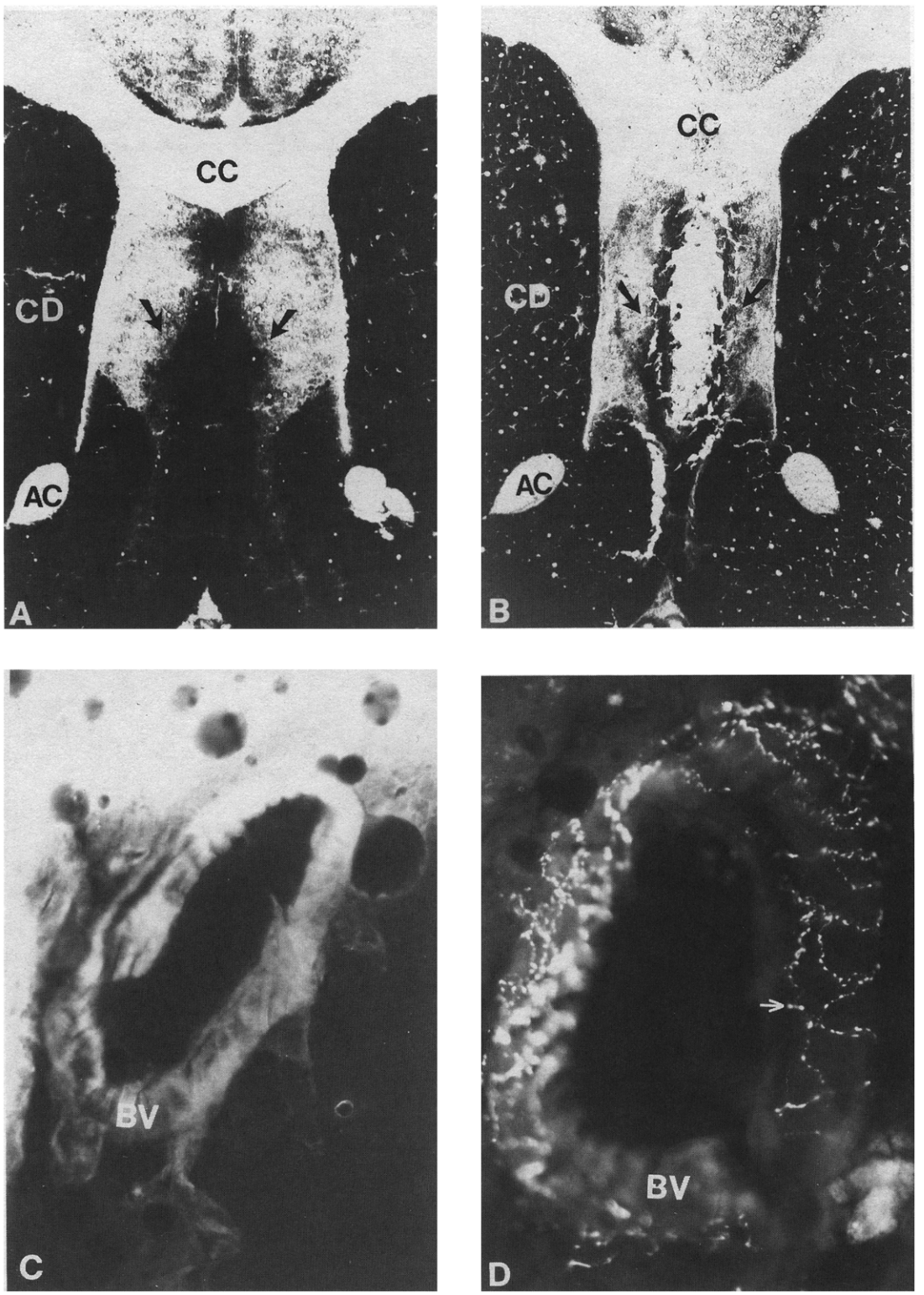

FIG. 1. Photomicrograph (×3.5) of the medial septal area (black arrows) stained for acetylcholinesterase from (A) control and (B) lesioned animals. Note the almost total destruction of the cholinergic neurons in this region. Panels C and D are dark field photomicrographs (×83) of blood vessels processed for NE histofluorescences. Panel C is from a MSGx animal while panel D is from an MS animal. Note the lack of NE fibers on the blood vessel (BV) in the MSGx animal when compared to the MS animal (NE fibers delineated by white arrow). AC, anterior commissure; CC, corpus callosum; CD, caudate nucleus.

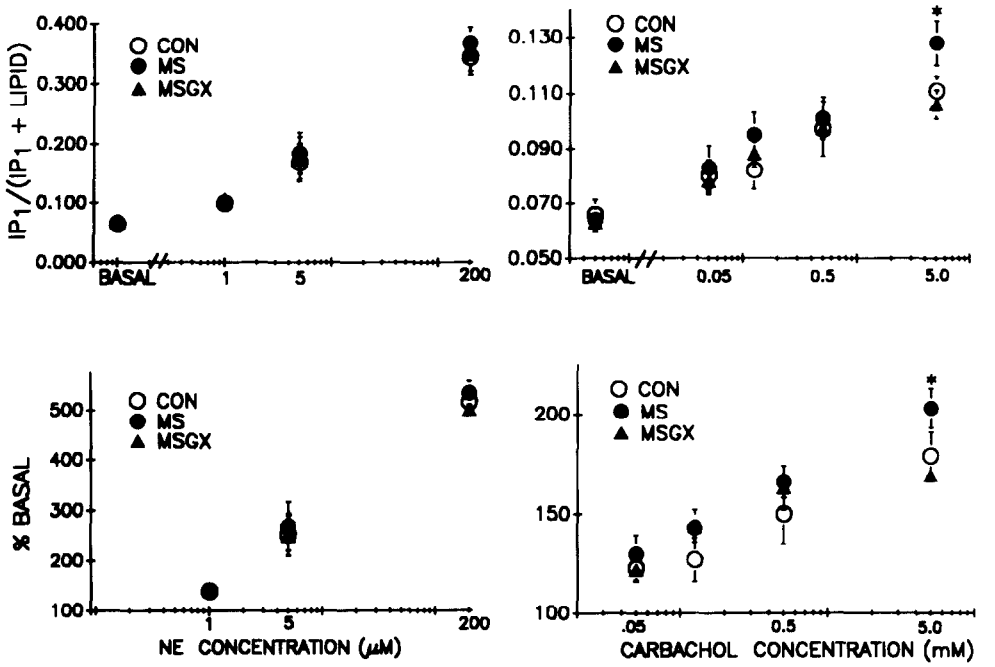

FIG. 2. NE- and carbachol-stimulated accumulation of [^{3}H]IP_1 presented as the ratio of IP_1/IP_1 + lipid and % basal stimulation in hippocampal slices from CON, MS, and MSGx groups. In the carbachol stimulation experiments, each point represents six experimental runs with hippocampi combined from two animals, while in the NE experiments each point represents three experimental runs. Values are means ± SEM. *$p < 0.05$. See the text for futher discussion.

IP_1 [ratio, $F(2, 24) = 3.2$, $p < 0.05$; % basal, $F(2, 24) = 3.5$, $p < 0.05$]. Further posthoc analysis revealed that IP_1 accumulation was significantly greater in the MS group ($p < 0.05$) than either the CON or MSGx groups, which did not differ from each other.

DISCUSSION

The findings of the present study suggest that HSI, rather than brain injury alone, is responsible for the alteration of phosphoinositide turnover 4 months after septal lesions. Furthermore, the findings confirm that HSI mediates its effects through cholinergic, rather than adrenergic, mechanisms.

The time course of HSI is well known (8). Peripheral sympathetic fibers are first appreciated, by histofluorescent techniques, 9 days after cholinergic denervation. By 30 days, the distribution and total number of NE fibers is complete (8). However, hippocampal NE concentration continues to increase throughout the life of the animal after induction of HSI (22). In an attempt to further define when HSI begins to mediate its effect on phosphoinositide metabolism, we assessed the effect of HSI on carbachol-stimulated phosphoinositide hydrolysis at different time intervals after medial septal lesions.

EXPERIMENT 2

METHOD

Animal and Subject Groups

One-hundred male Sprague-Dawley rats were maintained under the same conditions and randomized into the same three surgical groups as described in Experiment 1, with the exception that 12 animals underwent only Gx. These animals were included to ensure that Gx alone did not induce any changes in phosphoinositide metabolism.

Procedure

Animals from the CON, MS, and MSGx groups were randomly selected for sacrifice at 1, 2, or 4 weeks following surgical procedures. Ganglionectomized animals were sacrificed only at the 4-week time point. The histological, biochemical, and data analysis procedures were performed as outlined in Experiment 1 with the exception that only carbachol was employed for stimulation of phosphoinositide hydrolysis and atropine (10^{-6} M) was employed as an antagonist in some assays. In addition, inositol biphosphate (IP_2) and inositol trisphosphate (IP_3; also contains tetrakisphosphate) were eluted from the dowex columns in a stepwise fashion employing 6 ml 0.4 M ammonium formate in 0.1 M formic acid followed by 6 ml 1.0 M ammonium formate in 0.1 M formic acid.

RESULTS

Anatomically, the findings were similar to those described in Experiment 1. Overall ANOVAs of the ratio data (Fig. 3) revealed significant group, $F(3, 57) = 3.0$, $p < 0.05$, concentration, $F(6, 57) = 11.81$, $p < 0.0001$, week, $F(2, 57) = 17.2$, $p < 0.0001$, and group × week, $F(4, 260) = 2.99$, $p < 0.05$, effects. Analysis of the percent of stimulation over basal metabolism data (Fig. 4) revealed significant group, $F(3, 57) = 3.22$, $p < 0.02$, concentration, $F(6, 57) = 24.46$, $p < 0.0001$, week, $F(2, 57) = 35.53$, $p < 0.0001$, and group × week, $F(4, 260) = 3.19$, $p < 0.01$, effects.

As expected, the accumulation of IP_1 was concentration dependent. Thus, lower concentrations of carbachol led to

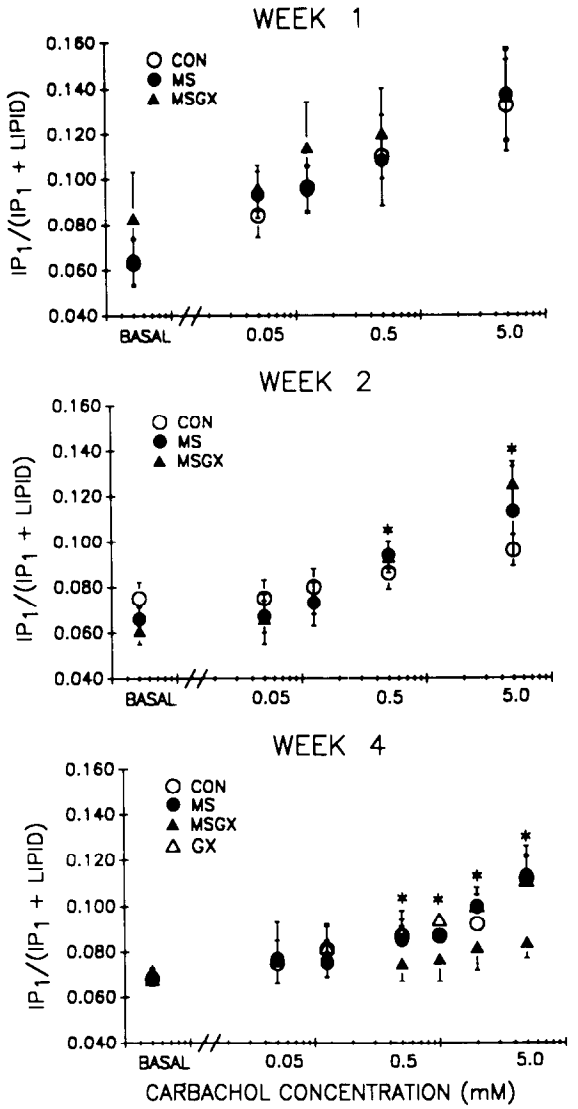

FIG. 3. Carbachol-stimulated accumulation of [^{3}H]IP presented in ratio form in hippocampal slices at 1, 2, and 4 weeks postlesions. Each point represents four to six experimental runs with hippocampi combined from two to three animals. Values are means ± SEM. Two-week *$p < 0.05$; 4-week *$p < 0.01$. See the text for further discussion.

little accumulation of IP_1 while higher concentrations produced greater hydrolysis of phosphoinositides. This effect was observed within all groups at each of the various time points.

As one of the major objectives of this study was to assess the effect of HSI on IP_1 accumulation, the data was analyzed at each time point. One week following MS lesions, no differences were found among the groups at any carbachol concentration (Figs. 3 and 4). However, by week 2 group differences in carbachol-stimulated IP_1 accumulation had begun to emerge [ratio, $F(2, 6) = 3.3$, $p < 0.05$; % basal stimulation, $F(2, 6) = 3.6$, $p < 0.03$]. At the higher concentrations of carbachol (i.e., 0.5 and 5 mM), IP_1 accumulation was significantly greater in animals with MS lesions, regardless of the presence of HSI, than controls ($p < 0.05$). However, no differences were observed between the MS and MSGx groups.

Group effects were still appreciated at the week 4 time point [ratio, $F(3, 9) = 3.2$, $p < 0.05$; % basal stimulation, $F(4, 9) = 10.28$, $p < 0.0001$]. As expected, IP_1 accumulation was dose dependent in all groups. However, overall IP_1 accumulation was significantly diminished in the MSGx group as compared to the other three experimental groups ($p < 0.01$), which did not differ among themselves. This effect was first appreciated with the 0.5-mM concentration of carbachol and then observed with all subsequent concentrations of carbachol (i.e., 1, 2, and 5 mM). This effect was found when the data was analyzed as percentage of basal stimulation as well as the ratio of IP_1/IP_1 + lipid.

To ensure that this effect was being mediated at the cholinergic receptor, experiments were performed at the 4-week time point, employing the muscarinic antagonist atropine. Atro-

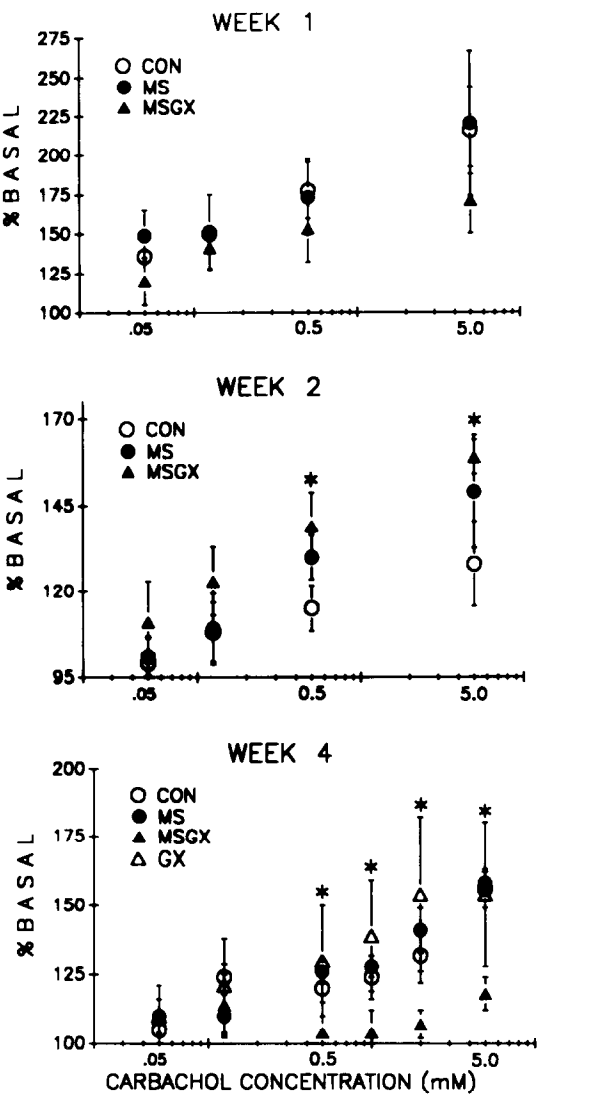

FIG. 4. Carbachol-stimulated accumulation of IP_1 presented as % basal stimulation in hippocampal slices at 1, 2, and 4 weeks postlesions. Each point represents four to six experimental runs with hippocampi combined for two to three animals. Values are means ± SEM. Two-week *$p < 0.05$; 4-week *$p < 0.01$. See the text for further discussion.

pine was found to totally inhibit the accumulation of IP_1 in all experimental groups when 1, 2, or 5 mM of carbachol was employed to stimulate hydrolysis of phosphoinositides. In addition, neither MS lesions nor HSI appeared to alter the metabolism of phosphoinositides as IP_2 and IP_3 accounted for less than 10% of the total inositol phosphates and their amounts were similar among all experimental groups.

GENERAL DISCUSSION

When the results of our experiments are taken together, they suggest that both cholinergic denervation (i.e., MSGx group) and HSI produce function alterations in the hippocampal phosphoinositide second messenger system. This effect, however, was time dependent. Thus, 2 weeks following MS lesions hydrolysis of inositol phosphoinositides was enhanced in the hippocampus regardless of the presence or absence of HSI. At 4 weeks, there was normalization of phosphoinositol metabolism in HSI animals, with marked diminution of metabolism in cholinergically denervated animals (MSGx). At 4 months, phosphoinositide metabolism returned to control levels in cholinergically denervated animals, while enhanced accumulation of IP_1 was observed in HSI animals. These results suggest a complex interaction among cholinergic denervation, neuronal reorganizations, and phosphoinositol metabolism.

As in our study, previous investigators (31) also observed an increased turnover of polyphosphoinositides 2 weeks after cholinergic denervation of the hippocampus. Since no alterations in number or affinity of either M_1 or M_2 muscarinic receptors were observed in this (31) or other studies (28,37) after hippocampal cholinergic denervation, the mechanism for this enhancement is unknown. However, several hypotheses can be advanced. First, it is known that in neonatal animals, where acetylcholine levels are low, this same type of enhanced coupling of muscarinic receptors and phosphoinositide turnover occurs (17). Since previous studies (11) demonstrated that a relationship exists between the conformational state of the muscarinic receptor and phosphoinositide metabolism, perhaps low levels of acetylcholine led to an alteration in the conformation of the muscarinic receptor. Second, cholinergic denervation may alter the number of M_3 muscarinic receptors. Since these receptors are coupled to the inositol phospholipid second messenger system (36), an increase in their number or affinity could theoretically increase the hydrolysis of phosphoinositides. Third, cholinergic denervation could in some way alter the coupling between the muscarinic receptor and its G-protein, thereby enhancing turnover of phosphoinositides (13). Fourth, an increase in glial elements associated with the phagocytosis of denervating nerve terminals could enhance phosphoinositide turnover, as these cellular elements also contain muscarinic receptors coupled to phosphoinositide metabolism (29).

Interestingly, hydrolysis of phosphoinositides was markedly diminished in the MSGx group 4 weeks after cholinergic denervation. The mechanism underlying this change is unknown. As discussed above, changes in proportion, type, number, or affinity of muscarinic receptors, conformational change in the muscarinic receptor, or changes in G-proteins could all lead to this finding. At this same time point, HSI was found to "normalize" phosphoinositide turnover. However, it is unclear whether this is a pre- or postsynaptic event since muscarinic receptors are present on peripheral sympathetic nerves (5,6) and neural growth cones (33). Further studies are under way to address this issue.

By 4 months after MS lesions, phosphoinositide turnover had returned to normal in the MSGx group. This suggests that even though presynaptic cholinergic activity, as reflected by measurement of choline acetyltransferase level, is still reduced (27) postsynaptic mechanisms are capable of compensation, at least at the level of the second messenger. Whether this occurs by an increased number or changed affinity of the postsynaptic receptor or through alterations in some other part of the receptor complex is unknown.

In animals with HSI, phosphoinositide responsiveness was found to be increased by 4 months after MS lesions. This finding is unlikely to be secondary to the presence of growth cones since the ingrowth of peripheral sympathetic fibers appears to be complete by 6 weeks after lesions (8). Thus, alterations in either pre- or postsynaptic muscarinic receptors could lead to this finding. Presynaptic modifications could be as simple as an increase in the number of muscarinic receptors associated with peripheral sympathetic nerves (5,6). Recently, however, it has been demonstrated that the coculturing of hippocampus and sympathetic neurons results in induction of cholinergic function within the sympathetic neuron (18). Perhaps this is also occurring in this in vivo situation.

Since previous research (24) failed to demonstrate changes in hippocampal adrenergic of cholinergic receptor number or affinity following HSI induction, if changes are occurring postsynaptically then some other mechanism would need to be invoked. It is known that NE levels associated with HSI continue to increase throughout the life of the animal (22) and that NE can inhibit the release of acetylcholine (ACh) (23,34). Perhaps HSI, with its associated increased levels of NE, inhibits ACh release from spared cholinergic interneurons or spared septohippocampal cholinergic fibers, resulting in a state of relative "increased" cholinergic denervation and subsequently supersensitivity of the cholinergic receptor. Or, perhaps, HSI in some manner inhibits the regrowth of spared cholinergic fibers. This, however, is unlikely since prior studies have not demonstrated this phenomenon (19). Other studies demonstrated that noradrenergic and cholinergic systems can coregulate each other as 1) NE levels in the hypothalamus can be altered by treatment of animals with either muscarinic agonist or antagonist (30), 2) parasympathetically denervated salivary glands develop supersensitivity not only to cholinergic agents but also to α-adrenergic agonists (10), and 3) cholinergic agonists, in cardiac tissue, can alter binding affinity for the NE agonists [^{3}H]WB4101 and [^{3}H]dihydroalprenotolol (35). Whatever the mechanism (i.e., pre- or postsynaptic), it does seem clear that HSI somehow alters cholinergically mediated PI metabolism. Although it could be argued that a 20% enhancement (at the 4-month time point) is relatively small, the fact that second messenger systems serve as an amplification step in cell signaling means that HSI could have a significant effect on subsequent cellular metabolic events.

Gx alone produced no alteration in hippocampal phosphoinositide metabolism. This finding supports our previous behavioral work (14), which demonstrated that Gx alone produced no alteration in spatial learning, while HSI was detrimental to learning of these tasks (15). This suggests that both behavioral and biochemical effects of HSI are due to some interaction within the hippocampus.

Due to a lack of specific controls, the effects of neuronal reorganizations on brain physiology, biochemistry, and subsequent behavior has, at times, been difficult to fully delineate. Despite this, other investigators demonstrated that the rearrangement in the hippocampus that follows entorhinal cor-

tex lesions is physiologically competent (32) and appears to be associated with recovery of some behavioral tasks (21). Moreover, other studies demonstrated an increase in phosphoinositide turnover evoked by excitatory amino acids in the hippocampus following lesions of either the CA_3 area, CA_4 area, or the dentate granule cells (26) of the hippocampal formation. Unfortunately, it is difficult to ascertain whether the observed effects are due to the primary tissue injury or the subsequent rearrangements. Our study does not have this type of difficulty since tissue injury is controlled (i.e., MS and MSGx groups have the same type of brain damage), making it clear that HSI is responsible for the observed effects.

The results of this study suggest a very dynamic interaction between brain injury, neuronal rearrangements, and the phosphoinositide second messenger system. The results of studies assessing cholinergic denervation should be interpreted cautiously if peripheral sympathetic ingrowth is not controlled. Further studies are under way to define how both HSI and cholinergic denervation mediate their effects on phosphionositide metabolism.

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