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CHARACTERISTICS OF RECEPTORS FOR EXCITATORY NEUROTRANSMITTERS

ANNUAL SUMMARY REPORT

CARL W. COTMAN

AUGUST 15, 1984

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The purpose of this research is to investigate the properties of receptors for excitatory neurotransmitters in the central nervous system (CNS), particularly those for acidic amino acids and related derivates. Most of the synapses in the CNS appear to use these molecules as transmitters but little is known about their receptor properties Various phosphonic acid derivatives and toxins appear to act directly or indirectly on these receptors. This research uses a newly developed anatomical method to localize the various receptor subtypes in the rodent brain. Electrophysiological analysis is used to record the physiological

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Our results have shown, for the first time, that glutamate receptors are most concentrated in the cerebral cortex and hippocampus in addition to key pathways from the peripheral nervous system e.g./ to the central nervous system. Electrophysiological analysis indicates that select antagonists (e.g., 2-amino-4-phosphonobutyric acid) will block synaptic transmission at some pathways, while other antagonists in this series (e.g., 2-amino-5-phosphonovaleric acid) will block a type of synaptic short-term memory at other pathways without interfering with normal synaptic transmission. These compounds are thus highly selective in their mode of action. Other new antagonists related to piperazine-2,3-dicarboxylic acid are more general antagonists blocking synaptic transmission at most hippocampal pathways, though they are not as potent.

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The purpose of this research is to investigate the properties of receptors for excitatory neurotransmitters in the central nervous system (CNS), particularly those for acidic amino acids and related derivatives. Most of the synapses in the CNS appear to use these molecules as neurotransmitters but little is known about their receptor properties. Various phosphonic acid derivatives and toxins appear to act directly or indirectly on these receptors. This research uses a newly developed anatomical method to localize the various receptor subtypes in the rodent brain. Electrophysiological analysis is used to record the physiological response to various agonists and antagonists that act on these receptors.

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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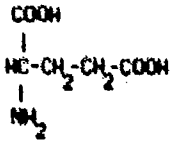
BACKGROUND

It now appears that the acidic amino acids glutamate and/or aspartate serve as the major excitatory neurotransmitters in the central nervous system (CNS). These transmitters certainly play a major role in brain and spinal cord function, mediating the inflow of information from the periphery and the processing and transformation of signals within the CNS prior to motor output via cholinergic pathways. Much is known about the structure-activity relationships of cholinergic synaptic transmission but very little is known about these relationships for the main excitatory pathways in the CNS. Compounds including phophonate derivates and several neurotoxins are active at such receptors, emphasizing the needs for better data on receptor properties. The goal of our work over the past year has been to define the types of acidic amino acid receptors that mediate synaptic transmission at various excitatory pathways in the CNS.

There appear to be at least four different classes of receptors for acidic amino acids that are best characterized by their selective interaction with different acidic amino acid analogues. These are the N-methyl-D-aspartate (NMDA) site, the quisqualate (QA) site, the kainate (KA) site, and the L-(+)-2-amino-4-phosphonobutyric acid (L-APB) site(1,2). We have localized several of the receptor subtypes in the rodent brain using a newly developed autoradiographic method(3), and studied the electrophysiological properties of NMDA and L-APB receptors primarily in the hippocampus.



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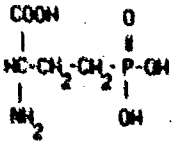
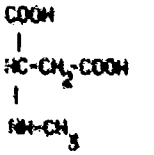


2-AMINO-4-PHOSPHONO-BUTYRIC ACID



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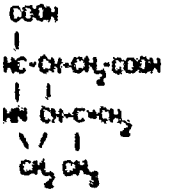
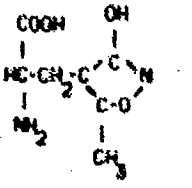
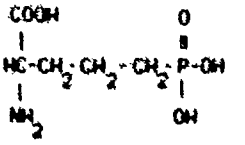


KAINIC ACID



α-AMINO-3-HYDROXY-5-METHYL 4-ISOAZOLE PROPIONIC ACID



2-AMINO-5-PHOSPHONO-PENTANOIC ACID

RESULTS AND DISCUSSION

1. Properties of L-APB Receptors in the Hippocampus

Previously we reported that L-APB is a potent antagonist at the lateral perforant path in the rodent hippocampus(4). Others subsequently showed that L-APB also acted on components of the monosynaptic pathway of the spinal cord and the rod-bipolar synapses in the rabbit(5,6). At present L-APB is the most potent antagonist available for excitatory pathways in the CNS. We also began to explore the development of other antagonists active at this receptor, including serine-O-phosphate (SOP). SOP will selectively discriminate between brain and spinal cord receptors in that it is relatively impotent at the spinal cord but is active in the brain. We also reported recently that L-

APB acts on hippocampal pathways in a species-specific manner(7). The acidic amino acid antagonist DL-APB is a potent blocker of synaptic transmission at guinea pig but not rat mossy fiber-CA3 synapses in hippocampal slices. The L-isomer of APB is responsible for the potent inhibition at the guinea pig synapse. The L-APB analogue L-SOP also is more potent against the guinea pig response. These differences may reflect a difference in a synaptic acidic amino acid receptor in these two species. Other acidic amino acid antagonists are less potent than L-APB or L-SOP and do not discriminate between the mossy fiber responses in the two species. Thus, L-APB appears to be an interesting and highly specific antagonist for CNS pathways. At present, the site of action of L-APB is unknown. It may work presynaptically, postsynaptically, or both. It is reasonably clear that it does not work as a conventional postsynaptic antagonist. Most evidence, such as that cited below, favors a novel presynaptic locus of action, possibly involving chloride ions. Work is still in progress on the mechanism of action.

2. NMDA Receptors in the Brain and the Action of Naturally Occurring NMDA-like Molecules

We have used radioligand binding techniques to determine the anatomical distribution of NMDA-displaceable ^{3}H-L-glutamate binding sites in rat brains. Young adult (45-60 day) male and female Sprague-Dawley rats were sacrificed by decapitation, and 6µ-thick brain sections were sliced on a freezing cryostat for quantitative autoradiography, as previously described(3). The highest concentrations are found in the outer layers of cerebral cortex, pyriform cortex, anterior olfactory nuclei, nucleus accumbens, and stratum radiatum and stratum oriens of hippocampus (see Fig. 1). High levels are found within the caudate/putamen, middle and deep cerebral cortical layers, nucleus reuniens, lateral septum and external plexiform layer of the olfactory bulb. Moderate levels are found in thalamus, granule cell layer of the cerebellum, inferior olive, medial vestibular nucleus, nucleus solitary tract, cuneate nucleus, dorsal cochlear nucleus, dorsal horn of spinal cord, and medial septum. Low levels are found in the globus pallidus, habenula, hypothalamus, midbrain, and the molecular layer of the cerebellum(8).

We have used extracellular and intracellular analyses of the excitations produced by several amino acid agonists to characterize the electrophysiological response in hippocampal area CA1, one of those regions containing the highest concentration of NMDA receptors in the brain. Previously we examined the relative potency of various agonists on CA1 pyramidal neurons(9). The relative potency of analogues of excitatory amino acids to produce depolarization when applied in the apical dendritic field of CA1 cells was studied in the hippocampal slice. The effect of these compounds was measured by recording focal potentials (FPs), the shift in the extracellular d.c. potential produced by the compounds applied. The ability of FPs to measure neuronal responses was evaluated. NMDA-type agonists were 10-20 times more potent, relative to L-glutamate, than reported from investigations in spinal cord. QA, ± α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and KA exhibited potencies on CA1 cells similar to those reported for spinal neurons. These data indicate that elements in CA1 cells possess a receptor with an affinity for NMDA-type agonists. We have examined several endogenous agonists, including quinolinic acid, S-sulpho-L-cysteine, and L-homocysteine sulfinate. These compounds, as well as the potent excitants NMDA and ibotenic acid, produce burst firing of sodium action potentials, tetrodotoxin-resistant recurrent spikes and apparent increases in input resistance. These excitants also show the profile mentioned above for antagonism by phosphono amino acids. Thus, it appears to be possible to differentiate NMDA-type agonists from other excitant amino acids by physiological as well as pharmacological criteria. Other agonists, including glutamate, QA, and KA do not elicit the responses listed above. The latter compounds elicit steady

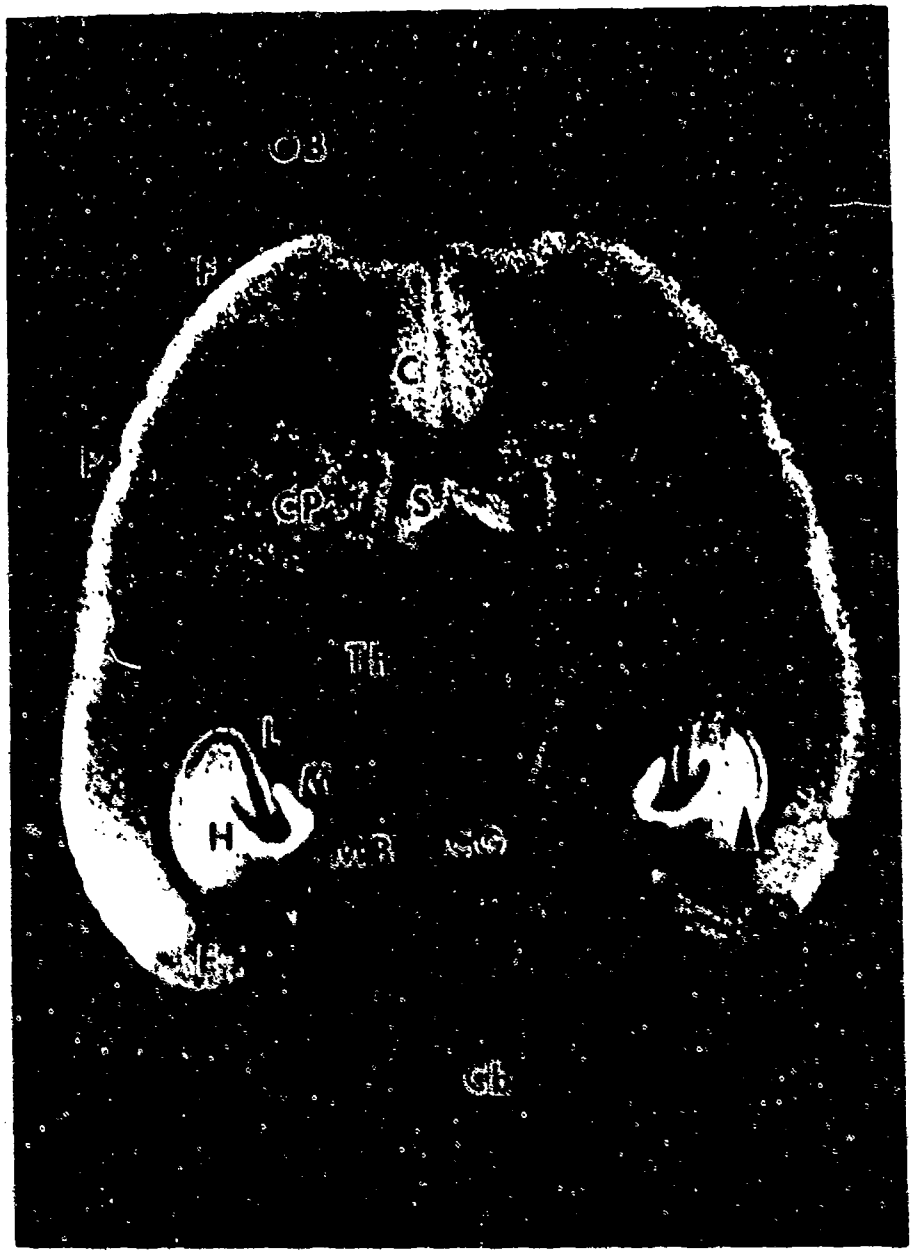


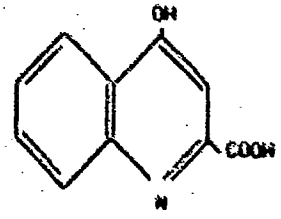
Figure 1. Distribution of NMDA-displaceable ^{3}H-glutamate binding sites in a horizontal section of a rat brain. The light areas correspond to maximal binding sites. Note that the heaviest labeling is found in the outer layers of the cerebral cortex and the hippocampus. Abbreviations, upper panel: C, cingulate cortex; CB, cerebellum; Cg, central gray; CP, caudate putamen; E, entorhainal cortex; F,frontal cortex; H, hippocampus; L, lateral geniculate; M medial geniculate; MB, mammillary body; OB, olfactory bulb; P, parietal cortex; S, septum; T, temporal cortex; Th, thalamus.

firing and decreases in input resistance; these compounds are also resistant to inhibition by phosphono amino acids. These data suggest that NMDA receptors play a special role in the hippocampus.

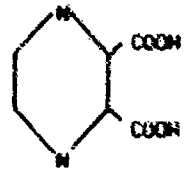
Recently there has been speculation that aspartate- or glutamate-containing dipeptides are excitatory neurotransmitters in the hippocampus. The dipeptide N-acetyl-aspartylglutamate (NAAG) has been found in high concentrations in rat brain and has been reported to bind to glutamate receptors(10). Further, it has been reported that excitations produced by NAAG in the rat olfactory cortex are selectively blocked by L-APB(11). However, our preliminary experiments with NAAG in the hippocampal slices indicate that this compound does not have excitatory actions on hippocampal neurons. Further work will be required to clarify the actions of this endogenous excitant in the hippocampus and other CNS structures.

3. Nature of the Receptor Involved in Synaptic Transmission

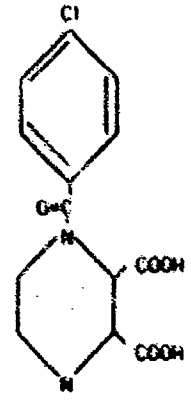
Our results and those of others suggest that synaptic receptors in the hippocampus may be of the KA or the QA type, while other receptors are of the L-APB or the NMDA type. Compounds such as GDEE (gamma-D-glutamyl glycine) and D(-)-2-amino-5-phosphono-pentanoic acid (D-AP5; also referred to as D-APV or -AP5) which are potent antagonists of applied excitatory amino acids(12) are rather poor blockers of CNS synaptic transmission(1,3,13). Previously we discovered that the naturally occurring compound kynurenic acid is an effective, though only moderately potent, antagonist in the hippocampus(14). Work is in progress using newly synthesized compounds to search for drugs that will prove to be more potent antagonists of these receptors, and will thereby aid in differentiating between these various receptor classes.



KYNURENIC ACID



PIPERAZINE-2,3-DICARBOXYLIC ACID



N-(p-CHLOROBENZOYL)-PzDA

Recently(15), we examined the effects of a new series of compounds, piperazine-2,3-dicarboxylic acid (PzDA) derivatives, on excitatory synaptic transmission in hippocampal slices. These compounds have been shown(16) to be potent antagonists of applied excitatory amino acids and provide a series of structurally-related comounds useful for examining structure-activity relationships. We examined the effects of these compounds on the pathway from hippocampal region CA3 to region CA1 (the Schaffer-collateral-commissural pathway) in slices obtained from young adult male Sprague-Dawley rats using standard procedures as described previously(4,7,9,14). Schaffer collaterals were stimulated electrically and evoked synaptic potentials were recorded extracellularly from region CA1. PzDA at 1mM inhibited the Schaffer collateral-commissural synaptic

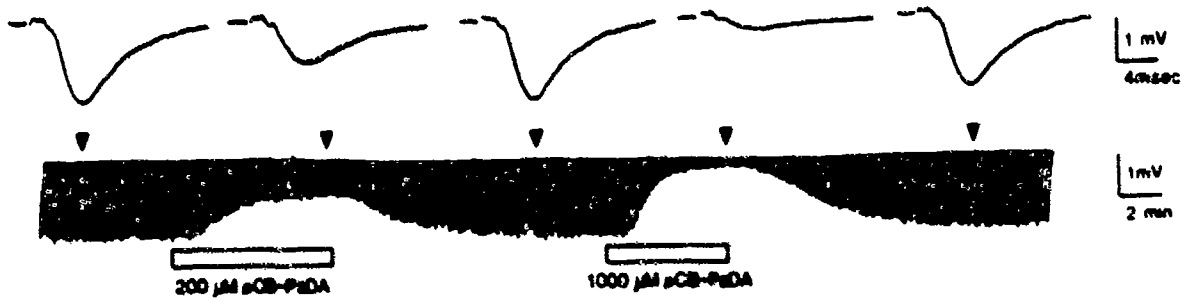


Figure 2. Depression of Schaffer commissural-collateral synaptic field potentials by N-p-CB-PzDA. The lower portion of the figure shows the amplitude of the peak negativity of the synaptic field potential. The waveforms in the upper portion of the figure are taken at the points indicated by asterisks. Solutions of p-CB-PzDA were introduced to the recording chamber during the intervals indicated by the open bars. The waveforms are photographs of oscilloscope traces of individual synaptic potentials.

responses less than 10%. Other PzDA analogues were more potent as Schaffer-CA1 synaptic blockers. Benzoyl-PzDA, N-(m-chlorobenzoyl)PzDA, N-(3',4'-dichlorobenzoyl)PzDA, and N-(o-chlorobenzoyl)PzDA depressed synaptic field potentials in that order of potency, with 50% depression occurring with 0.4-2 μM solutions. The most potent PzDA derivatives were N-(p-chlorobenzoyl)PzDA (N-p-CB-PzDA) and N-(p-bromobenzoyl)PzDA (N-p-BB-PzDA); half-maximal inhibition of Schaffer-CA1 responses was near 0.2 mM for these two derivatives. Figure 2 illustrates the depression of this synaptic response.

These PzDA derivatives will be helpful for clarifying CNS receptors in two ways. First, this series of analogues contains antagonists of synaptic transmission with a wider range of potency than that previously available. Second, several PzDA derivatives (p-CB-PzDA and p-BB-PzDA) are the most potent acidic amino acid antagonists yet available for the Schaffer-CA1 synaptic response, a prototypical pathway in the CNS. We have also examined the actions of the more potent antagonists on excitatory neurotransmission at the other hippocampal pathways (Fig. 3). Medial and lateral perforant path responses and mossy fiber synaptic responses were also inhibited by about 50% by 0.05-0.2 mM solutions of these two compounds.

Focal depolarizations induced by ionophoretic application of excitatory amino acids in stratum radiatum of field CA1 were also antagonized by solutions of N-p-CB-PzDA and N-p-BB-PzDA. These PzDA derivatives at 1000μM solutions blocked NMDA responses by 60-80% but were less effective against KA and especially QA responses (Fig. 4).

The similar effect of PzDA analogues against synaptic responses in different hippocampal pathways may indicate that neurotransmission in these pathways is mediated by similar excitatory amino acid receptors. The weak depression of hippocampal pathways by specific NMDA antagonists indicates that the primary synaptic receptor is not of the NMDA class. Although agonist-induced focal potentials and synaptic field potentials are difficult to compare quantitatively, the apparent greater potency of PzDA analogues against synaptic compared with agonist-induced responses suggests the possibility of a postsynaptic receptor type not selectively activated by NMDA, KA, or QA.

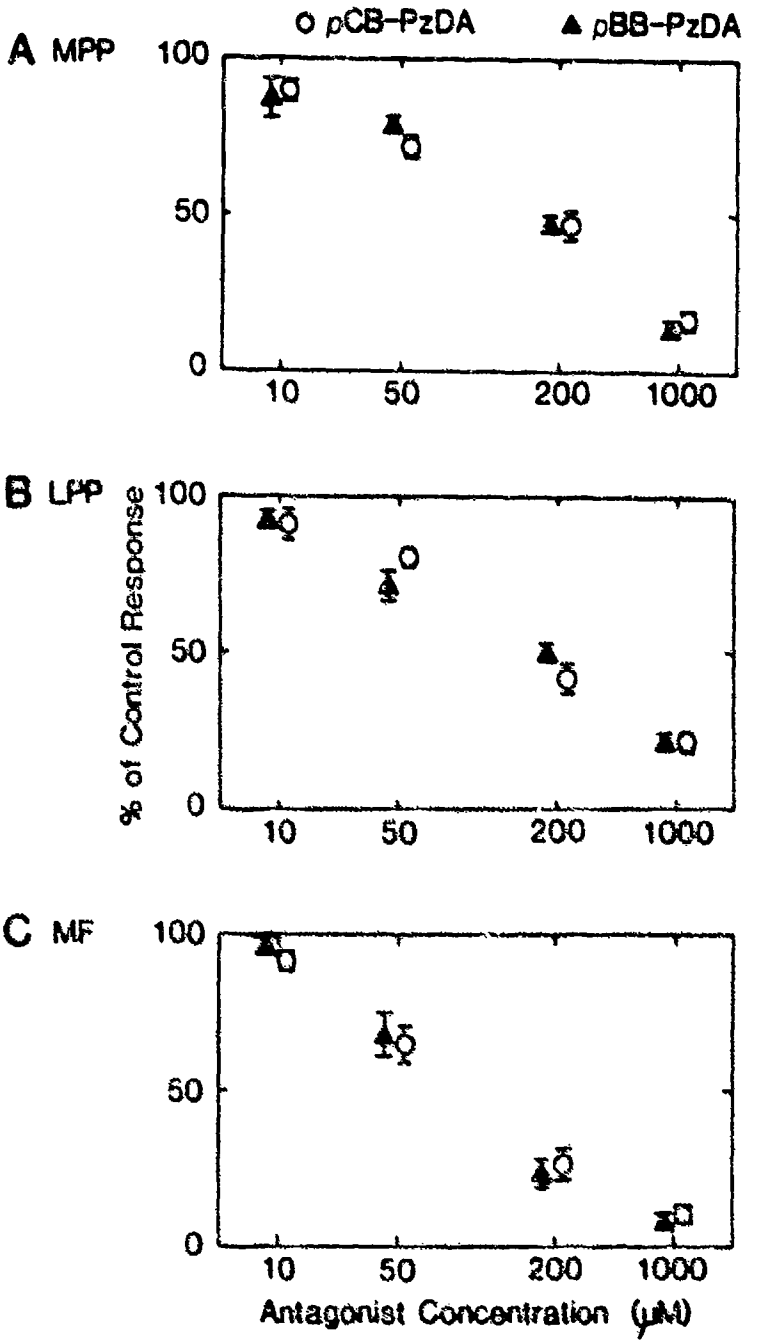


Figure 3. Antagonism by PzDA derivatives of hippocampal synaptic responses. Ordinate is the amplitude of field potentials recorded in antagonist solitions compared with the control response. Each point represents the mean and S.E.M. for data from at least four slices. A. MPP; Medial perforant path. B. LPP; Lateral perforant path. C. MF; Mossy fiber pathway. pCB-PzDA, N-(p-chlorobenzoyl)piperazine-2,3-dicorboxylate. pBB-PzDA, N-(p-bromobenzoyl)piperazine-2,3-dicarboxylate.

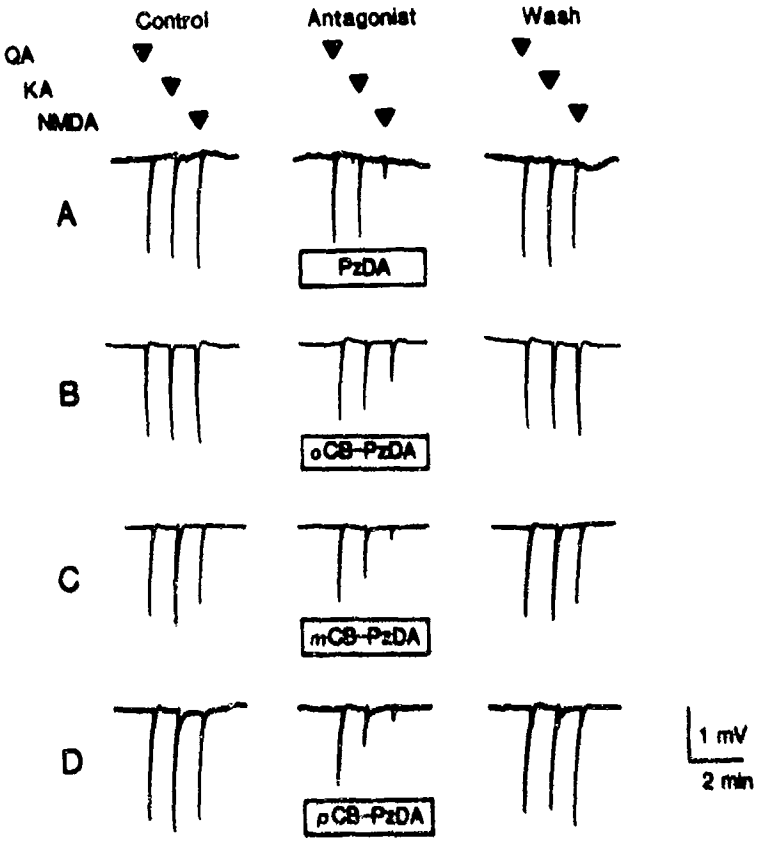


Figure 4. Antagonism by PzDA derivatives of responses to excitatory acidic amino acids. Changes in extracellular DC potential (focal potentials; FPs) evoked by iontophoretic application of excitants were recorded in hippocampal region CA1. Records are from a chart recorded with gaps representing intervals of 10-20 minutes. Slices were completely submerged in medium which included 1μM tetrodotoxin. The bars indicate the presence of antagonist at 1000μM. QA, Quisqualate. KA, Kainic acid. NMDA, N-methyl-D-aspartate. PzDA, piperazine-2,3-dicarboxylic acid. oCB-PzDA, N-(o-chlorobenzoyl)PzDA. mCB-PzDA, N-(mchlorobenzoyl)PzDA. pCB-PzDA, N-(pchlorobenzoyl)PzDA.

4. Structure Activity Studies on Hippocampal Synaptic Plasticity

a. Actions of L-APB and other analogues on short-term potentiation at CNS synapses.

The normal function of CNS pathways involves the operation of plasticity mechanisms. A ubiquitous form of synaptic plasticity is elicited by stimulation with pairs of stimuli separated by 20-400 milliseconds. Depending on the specific pathway, this produces potentiation of the second response as compared to the first (paired pulse potentiation, PPP) or depression (paired-pulse depression, sometimes also referred to as habituation). Previous studies on the effects of APB on habituation and PPP in the hippocampal formation(17) were carried out before differences between lateral and medial perforant paths were reported(4,18), and before the discovery of a new antagonist, kynurenic acid, that appears to block perforant path postsynaptic receptors(14). For these reasons, a reexamination of the effects of APB on perforant path was undertaken(19). Application of L-APB causes a reduction in lateral perforant path responses, but also an increase in the percent PPP of these responses. The effect does not result simply from reducing response size, because the amount of potentiation of matched first responses increases, and also because APB reduces the potentiated response proportionately less than a comparable first response. A similar effect is seen by decreasing extracellular calcium, which would decrease presynaptic release. Reducing lateral perforant path responses with kynurenic acid, which apparently acts on postsynaptic sites, does not have a similar effect on PPP; although the synaptic responses are reduced in amplitude, there is no change in the percent PPP. These results may indicate a presynaptic action of APB, possibly mediated via an effect on presynaptic calcium availability. One possible mechanism is suggested by the findings that Chloride ion affects APB-sensitive glutamte binding(2), and that APB increases a Chloride conductance in retina(5). APB may increase chloride conductance presynaptically at the lateral perforant path, thereby clamping the presynaptic terminal near resting membrane potential. This could then decrease the voltage-dependant Calcium ion influx (and therefore decrease presynaptic release) evoked by lateral perforant path fiber activation by decreasing the amount of presynaptic terminal depolarization.

In a study just completed, the effects of different synaptic antagonists on paired pulse depression of medial perforant path responses were examined in rat hippocampal slices(20). Kynurenic acid reduces the first and second responses to paired stimulation in the same proportions, having, therefore, no effect on the percent paired-pulse depression. This contrasts with the effects of the potent medial perforant path antagonist baclofen(20), which does not have the same net effect on the first and second responses to paired stimulation, and decreases the percent paired-pulse depression. Furthermore, at doses that reduced the medial perforant path response by half, the paired-pulse plasticity changed from depression to potentiation. A similar effect on medial perforant path paired-pulse plasticity is produced by decreasing the extracellular calcium concentration. These results suggest that baclofen reduces the synaptic response presynaptically, possibly via reducing presynaptic calcium availability, whereas kynurenate acts postsynaptically. This hypothesis was tested further using adenosine, which has been found to be a potent antagonist at other hippocampal pathways(21) and probably acts primarily via a presynaptic mechanism. Adenosine was also found to be a potent antagonist of medial perforant path responses, with effects on paired-pulse plasticity similar to those of baclofen. Finally, we examined the effects of the most potent of the PzDA derivatives, p-CB-PzDA, which we believe is a postsynaptic antagonist. Like kynurenate, p-CB-PzDA blocks the synaptic response without changing the %PPP. These results clearly show that precise selection of antagonists can be used to act on only certain aspects of hippocampal synaptic transmission.

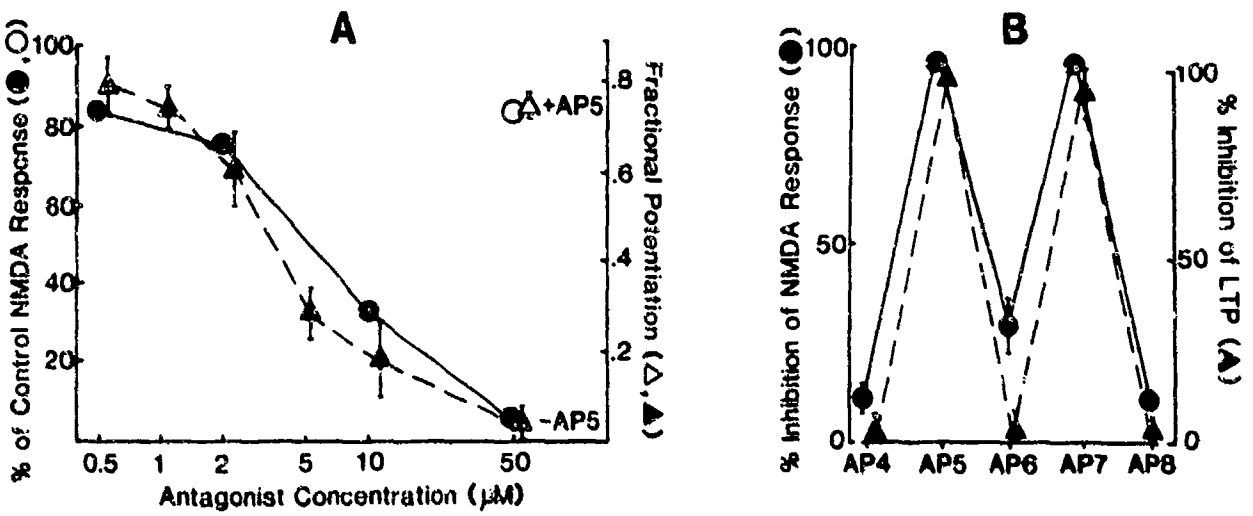


Figure 5. Comparison of the effects of antagonists on NMDA responses (circles) and on LTP (triangles). A. Dose-response data for -(D)AP5 (solid symbols) and +(L)AP5 (open symbols). Fractional LTP is that fraction of the total LTP resulting from two sets of high frequency stimulus trains (one given during and one after superfusion with the drug) that was produced by the first train (in the presence of the drug). B. Antagonism by 100 uM ω-phosphonate solutions of NMDA focal depolarizations. Each point represents the mean (± S.E.M.) averaged from at least 3 (for LTP data) or at least 4 (for NMDA data) different slices (in some cases slices from the same animal were used in LTP and agonist depolarization experiments). Inhibition of LTP is relative to the LTP obtained in 5 slices superfused with control medium containing no drug. (Error bars were not drawn if they fell on or within the corresponding symbol.)

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b. Actions of phosphonic acid analogues on long-term potentiation.

A striking change in synaptic efficacy along hippocampal pathways can be produced by delivering brief trains of high frequency stimulation(22). The resulting increase in synaptic efficacy may last for many hours, or even weeks and has been termed long-term potentiation (LTP)(23). LTP has been proposed to be a synaptic analogue of memory. There is evidence for NMDA receptors throughout the CNS(3,24), but their functional significance remains unclear, especially since the potent NMDA antagonists have not yet been found to block any identified central synaptic pathway in the vertebrate brain. As mentioned above, one of the highest concentrations of NMDA receptors was found in stratum radiatum of hippocampal subfield CA1. Recently, D,L-(±)-AP5 was reported to interfere with the induction of LTP in the hippocampus(13,25,26). We have examined the involvement of NMDA receptors in LTP using ±AP5, ±AP7 (D,L-(±)-2-amino-7-phosphono-heptanoic acid) and other longer- and shorter-chain phosphono acidic amino acid analogues(27). These compounds were chosen because NMDA receptors show a distinctive spectrum of sensitivity to this series of structurally related drugs(2,24; and see Fig. 5).

We have tested these analogues against NMDA response in the hippocampus and

also examined their effects on LTP of synaptic responses. The high frequency stimulation we used elicited near maximal LTP under control conditions. Under control conditions, a second high frequncy stimulation produced little further LTP. To control for variations in the maximal amount of LTP that could be elicited in different slices, LTP was quantified by comparing the amount of LTP obtained by a first high frequency stimulation in the presence of a test compound to the amount of LTP produced by stimulation after the drug was washed out. The 5- and 7-phosphono compounds (±AP5 and ±AP7) blocked NMDA depolarizations and also reversibly prevented the induction of LTP of Schaffer collateral responses. After these drugs were washed out, LTP could be elicited. These compounds did not reduce responses to applied KA or QA and did not affect unpotentiated synaptic response amplitude, however. APB and other closely related phosphonates ±AP6 (D,L-(±)-2-amino-6-phosphonohexanoic acid) and ±AP8 (D,L-(±)-2-amino-8-phosphonooctanoic acid) did not block either amino acid excitant responses or LTP (Fig. 5). This pharmacological profile is diagnostic for NMDA receptors(2,24) and these results demonstrate that NMDA receptors present in the hippocampus are identical to those found elsewhere. Furthermore, although these receptors are not necessary for normal synaptic transmission, they are involved in the initiation of long-term synaptic plasticity. This is the first clear evidence for the involvement of NMDA receptors in brain synaptic transmission.

CONCLUSION

Research over the first year has focused on the definition of acidic amino acid receptor types and their properties in the CNS. Phosphonic acid derivatives have proven to be among the most potent antagonists of excitatory synaptic transmission. With minor adjustments in their structures, they will block synaptic transmission or interfere with the development of synaptic plasticity at brain pathways. New compounds are also becoming available which are more general antagonists and which have the ability to generally depress brain activity. Piperazine derivatives are the most potent general antagonists yet identified. L-APB, however, is the most potent specific antagonist of acidic amino acids known.

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