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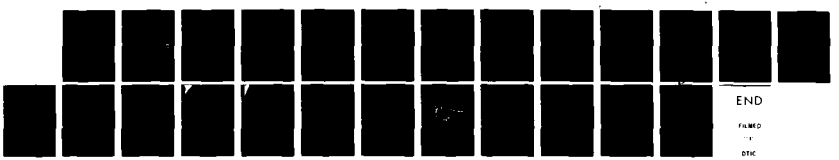

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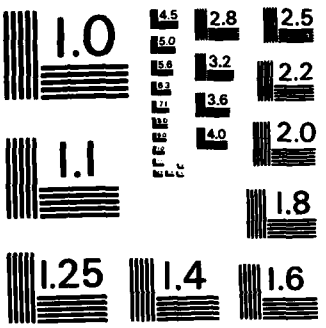

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Molecular Characteristics of Membrane Glutamate Receptor-Ionophore Interaction

Elias K. Michaelis and Hsuan Hung Chang

October 15, 1982

U.S. Army Research Office

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The view, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

receptor, ionophore, synaptosomes, synaptic membrane vesicles, glutamate binding protein, L-glutamic acid, Na^+ fluxes, SCN^- uptake, electrogenicity, reconstitution, neuroexcitatory amino acids and its analogs, antagonists.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The excitatory responses produced in mammalian central nervous system neurons and in invertebrate muscles by the action of L-glutamic acid and L-aspartic acid are apparently the result of a glutamate- or aspartate-induced increase in membrane conductance of Na^+. In our studies, we have used rat brain synaptosomal and synaptic membrane vesicular preparation to study this process. The synaptosomes and resealed synaptic plasma membrane vesicle preparations from brain tissue are thought to consist largely of

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20. ABSTRACT CONTINUED

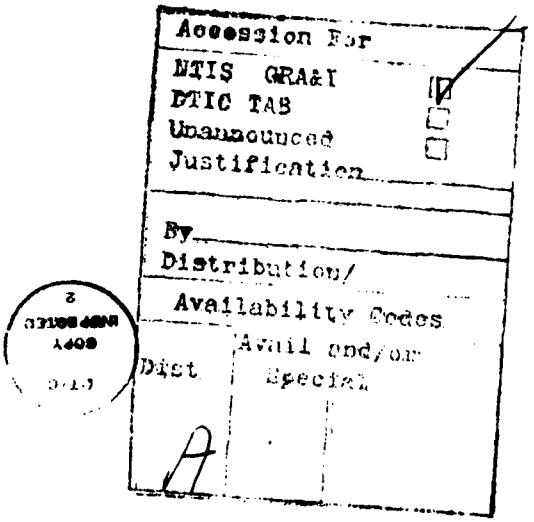
presynaptic membrane sacs and of a few postsynaptic membrane vesicles. These plasma membrane fractions apparently retain a high degree of functional and structural integrity. In our laboratory, we have shown that these neuronal plasma membrane subfractions are enriched in L-[^{3}H] glutamic acid binding sites.

Synaptosomes incubated at room temperature exhibited a diphasic base-line Na^+ uptake pattern, whereas the resealed synaptic plasma membrane vesicles exhibited only a rapid biphasic ^{22}Na diffusion. Both phases of synaptosomal Na^+ diffusion were enhanced by pretreatment of the preparations with ouabain (0.5 mM), gramicidin D (10μM) , or glutamate (1μM). Ouabain and gramacidin D caused strong inhibition of synaptosomal and synaptic membrane (Na^+-K^+)-ATPase activity, whereas glutamate caused a small to moderate enhancement of the enzyme activity. Ouabain did not alter the ^{22}Na diffusion in synaptic membrane vesicles. Maximal Na^+ uptake stimulation by glutamate was obtained by exposure of the synaptosomes and synaptic membrane to 10^{-6} to 10^{-5} and 10^{-7} to 10^{-6}M glutamate concentrations respectively. Glutamic acid stimulation of Na^+ uptake was not altered by prior treatment of the membranes with tetrodotoxin or with p-chloromecuribenzene sulfonate. When synaptic membrane vesicles were preloaded with Na^+ ions, gramicidin D and L-glutamic acid also stimulated the efflux of Na^+ ions from these vesicles. The rate and magnitude of passive Na^+ efflux were dependent on the initial intravesicular NaCl concentration. Gramicidin D markedly enhanced Na^+ efflux in a concentration dependent manner and at 10μM it caused total loss of intravesicular ^{22}Na. The neuroexcitatory amino acids L-glutamate and D-glutamate, and the amino acid analogs kainic acid, N-methyl-D-aspartic acid and Homocysteic acid also stimulated the Na^+ efflux. The mechanism of glutamate stimulation of Na^+ flux is presumed to be through the activation of the glutamate receptor·Na channel complex in these membranes.

The electrogenic nature of the L-glutamate-stimulated Na^+ flux was examined by measuring the distribution of the lipophilic anion [^{35}S] thiocyanate (SCN^-) into synaptic membrane vesicles that were incubated in a NaCl medium. Concentrations of L-glutamate from 10^{-7} to 10^{-4}M added to the incubation medium caused an enhnaced intravesicular accumulation of SCN^-. Based on the SCN^- distribution in synaptic membrane vesicles it was concluded that 10μM L-glutamate induced an anverage change in the membrane potential of +13 mV. L-Glutamate enhanced both the Na^+ and K^+ co[illegible]nce of these membranes as determined by increases in SCN^- influx. [illegible]neuroexcitatory amino acid analogs, D-glutamate, L-aspartate, L-cystei[illegible]finate, kainate, ibotenate, quisqualate, N-methyl-D-aspartate and DL-homocysteate also increased SCN^- accumulation in synaptic membrane vesicles. These observations are indicative of the activation by L-glutamic acid and by some of its analogs of excitatory amino acid receptor ion channel complexes in synaptic membranes.

In more recent studies we have attempted to explore the possibility that the purified glutamate binding protein obtained from synaptic plasma membranes can function both as a glutamate recognition site and as an ion channel protein. The techniques for reconstitution of this protein into liposomes have been developed and the reconstitution of the isolated glutamate binding protein was accomplished. The glutamate binding characteristics and ion channel properties of this reconstituted protein were determined and it was shown that the binding activity remained fairly intact but that the ion channel response appeared to be more labile.

3. Table of Contents



2. Foreword

L-glutamic acid may function as a rather universal excitatory agent in the mammalian CNS and may also function as an important agent in cellular communication in prokaryotes and eukaryotes alike. We have recently accumulated evidence that in one type of abnormal brain excitatory state, that which is associated with withdrawal seizures in chronic alcoholism, there appears to be an abnormal hypersensitivity of glutamate receptors (Michaelis et al., 1981; Michaelis et al., 1981; Freed and Michaelis, 1977). Even partial blockade of these receptors by the glutamate antagonist glutamate diethylester can bring about a remarkable amelioration of these seizure phenomena induced by chronic ethanolism (Freed and Michaelis, 1977). In addition, we have demonstrated that excess activity of L-glutamate in the CNS may disrupt normal learning activities and prolong the necessity for the training of a new behavior (Freed and Michaelis, 1976) even though it does not bring about any changes in general locomotor activity.

Based on this rather widespread level of glutamate effects in nature it appears that any progress made in understanding the molecular interactions which lead to the appearance of responses to glutamate would be of scientific and potentially of practical benefit. The research results described in this report may eventually provide us with models of L-glutamate's activity in the CNS which may prove useful in future attempts to design agents which can specifically alter this amino acid's excitatory effects in this tissue.

4. List of Figures

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5. Report

a) Statement of the Problem Studied

The excitatory action of glutamate is mediated by depolarization and decreased resistance of the postsynaptic membrane. This depolarization is thought to be brought about by the binding of glutamic acid to its receptor sites which is accompanied by an increase in membrane permeability to sodium ions and to a lesser extent to potassium ions (Anwyl, 1977; Krnjevic, 1974). Clearly the issues surrounding receptor-ionophore junction are quite complex and too difficult to resolve totally on the basis of electrophysiologic and neuropharmacologic studies. Advances in the biochemical characterization of specific glutamate receptor sites and receptor·ionophore interaction might provide some answers to the abnormal electrical activity and nerve cell damage in the brain. In our research, we have set our goals to examine the molecular characteristics of the L-glutamate·receptor interaction in nerve cell membranes which leads to nerve cell excitation. In particular, the issues which have been examined in the last three years are:

(1) Whether L-glutamic acid does activate a sodium ionophore in synaptic membranes from brain. Na^+ fluxes across these resealed synaptic membrane vesicles were used to monitor this process.

(2) Whether the L-glutamic acid induced Na^+-flux is an electrogenic process which can produce the depolarization effect.

(3) Whether the L-glutamic acid stimulatory effect has similar pharmacological characteristics as reported in the literature.

(4) Whether the synaptic membrane glycoprotein which binds L-glutamic acid with a high degree of affinity and steroselectivity could indeed function as the glutamate receptor. This purified glycoprotein has been reconstituted into liposomes in order to study whether it can function both as a receptor and as a Na^+ ionophore system.

b) Summary of the Most Important Results

Two membrane preparations have been used in this study, synaptosomes and synaptic membrane vesicles.

Synaptosomes

Investigations conducted in our laboratory over the last few years have allowed us to develop expertise in the use of preparations which provide us with material enriched in pinched-off nerve endings (synaptosomes). These particles are known to contain mitochondria, synaptic vesicles and endoplasmic reticulum. A biphasic, passive Na^+ uptake pattern was observed in rat brain synaptosomal preparations. The maximum ^{22}Na uptake for the rapid phase was determined to be 0.31 ± 0.06 (SEM) μeq Na^+/mg protein (n=11), and for the slow phase it was 0.36 ± 0.8 μeq Na^+/mg protein (n=10). The peak of ^{22}Na uptake for the fast phase usually occurred at 30-50 seconds of incubation, and that for the slow phase usually took place

in about 2.5 to 8.5 minutes. By using the equilibrium level of Na^+ uptake at the slow phase, an approximate intravesicular volume was estimated to be 4.6 ± 0.28 (SEM) µl/mg protein (n=11). The overall uptake pattern was not changed by pretreatment of synaptosomes with a Na^+-K^+ ATPase inhibitor-ouabain. Only a slightly larger accumulation of Na^+ in both rapid and slow phases was observed. Both phases of synaptosomal Na^+ diffusion were also enhanced by L-glutamic acid (1 µM). However, L-glutamate at the same concentration did not inhibit Na^+-K^+ ATPase activity. The glutamate induced stimulation ranged from 0.05 to 0.4 µeq Na^+/mg protein for the fast phase and 0.06-0.33 µeq Na^+/mg protein (n=5) for the slow phase. The stimulation of ^{22}Na uptake by L-glutamate showed dose-response characteristics (Fig. 1). Maximal stimulation of Na^+ uptake was observed in the range of 10^{-6}-10^{-5}M L-glutamic acid.

Synaptic Membrane Vesicles

The synaptic plasma membrane fraction obtained following osmotic rupture of these nerve endings has been found to be enriched in glutamate binding activity which has many of the expected characteristics of the excitatory amino acid receptors (Michaelis, et al., 1974).

1. L-glutamic acid induced Na^+ fluxes:

These synaptic membrane vesicles exhibited a simple Na^+ uptake which was similar to the fast phase of Na^+ diffusion in the synaptosomal fraction. Uptake of Na^+ into synaptic membrane vesicles was temperature dependent. The rate of uptake was slower at 4°C. The magnitude of the peak activity of this Na^+ uptake was 0.46 ± 0.17 µeq Na^+/mg protein when measured at 25°C (n=8). The anion selective sequence for the Na^+ diffusion process was

$$Cl^- >> SCN^- \cong Br^- > F^- > SO_4^{=} \cong I^-$$

It was also found that maximal activity of Na^+ influx was linearly dependent on the external NaCl concentration up to 100 mM. Variation of medium osmolarity resulted in a progressive decrease in Na^+ uptake. This suggested that Na^+ ions diffused into a vesicular space and little binding was observed. Ouabain had no effect either on the pattern or the amount of Na^+ trapped. This was thought to be due to the lack of endogenous ATP in these synaptic membrane vesicles. Preexposure of these membrane vesicles to 0.1 µM L-glutamate also caused an increase of $^{22}Na^+$ uptake which ranged between 0.08-1.04 µeq Na^+/mg protein, depending on the batch of membranes being used in these studies. This glutamate induced stimulation of Na^+ influx was found to be insensitive to prior treatment with tetrodotoxin or p-chloromercuribenzene sulfonate. These results suggested that this glutamate-induced Na^+ influx was different from the neuronal voltage dependent Na^+ flux which is sensitive to tetrodotoxin. The stimulation of Na^+ uptake in these synaptic membranes is maximal at L-glutamate concentrations in the range of 10^{-7}M-10^{-6} (Fig. 2). The effectiveness of various L-glutamate analogs in stimulating Na^+ influx was L-glutamate > cysteine sulfinic acid > L-aspartic acid.

In the second phase, we have characterized the Na^+ efflux from NaCl preloaded synaptic membrane vesicles in the presence and absence of L-glutamic acid. The average amount of Na^+ trapped within these vesicles following storage at 4°C for 48h was 236 ± 16 nmoles/mg protein. The intravesicular space is calculated to be 1.96 ± 0.33 µl/mg protein. This represents the internal volume to which Na^+ has access. The efflux of Na^+ from these synaptic membrane vesicles was rapid and temperature dependent. The presence of any of the cations Li^+, Cs^+ · K^+ and Na^+ in the external medium enhanced the rate of Na^+ efflux. Exposure of Na^+-loaded synaptic membrane vesicles to gramicidin D (10 µM) led to a very rapid release of all of the intravesicular Na^+ ions within 15 sec of incubation (Fig. 3). This result indicates that the intravesicular Na^+ was freely mobile. The rate of Na^+ efflux was also enhanced to a moderate extent by exposure of the vesicles to the putative excitatory neurotransmitter L-glutamic acid (Fig. 4). Under the same condition, no uptake of L-[^{3}H]-glutamic acid into the synaptic membrane vesicles was observed. It seems reasonable to state that the activation by glutamate of Na^+ fluxes is not the result of Na^+ co-transport with L-glutamate in these synaptic membrane vesicles. The neuroexcitatory amino acids, D-glutamic acid, kainic acid, N-methyl-D-aspartic acid (NMDA) and D,L-thomocysteic acid also stimulated Na^+ efflux (Fig. 4 and 5). Based on these studies, the L-glutamic acid induced Na^+ flux in these synaptic membrane vesicles has many similar features to that measured in brain slices or in in vivo electrophysiologic studies (Luni et al., 1981; McIlwain, et al., 1969).

2. Electrogenicity of the Na^+ uptake: In order for L-glutamic acid to produce a membrane depolarization of neuronal cells, one would have to assume that the flux of Na^+ across the plasma membrane is an electrogenic process. The electrogenic nature of the L-glutamate-stimulated Na^+ flux was examined by measuring the distribution of the lipophilic anion [^{35}S] thiocynate (SCN^-) into synaptic membrane vesicles that were incubated in a NaCl medium. Based on the SCN^- distribution in synaptic membrane vesicles it was calculated that 10 µM L-glutamic acid induced an average change in the membrane potential of + 13mV. This value is comparable to the values obtained in cerebral cortical slices (Gibson and McIlwain, 1965) and brain synaptosomes (Chang and Michaelis, 1980) (Table I). Maximal stimulation of SCN^- uptake by L-glutamate was obtained following exposure of the synaptic membrane to 10^{-7} to 10^{-5}M L-glutamic acid (Fig. 6). L-Glutamic acid enhanced both the Na^+ and K^+ conductance of these membranes as determined by increases in SCN^- influx. Other neuroexcitatory amino acids and amino acid analogs produced increases in SCN^- accumulation similar to those observed with L-glutamic acid. The relative order of potency is shown in Table II and Fig. 7 with control SCN^- uptake arbitrarily set at 100. The effectiveness in inducing SCN^- uptake by various neuroexcitatory agents is D-glutamic acid > L-glutamic acid ≥ NMDA > kainic acid ~ quisqualic acid ~ Ibotenic acid > L-aspartic acid > L-cysteine sulfinic acid ~ DL-Homocysteic acid > L-glutamine. On the other hand, the neuroinhibitory transmitters GABA and glycine, which are thought to increase permeability to Cl^- in neuronal membranes, either had no effect or produced a small decrease in SCN^- accumulation in synaptic membranes (Table II). However, since neuropharmacologic and biochemical studies have previously shown that D-glutamic acid and NMDA interact with the same receptors while L-glutamate and kainic acid activate distinct receptors, the specificity of the synaptic membrane receptor sites

involved in the depolarization response was explored further. L-Glutamic acid-induced SCN^- accumulation was strongly antagonized by 100 μM glutamate diethylester (Fig. 8) and D,L-α-methyl glutamate (Fig. 9), and more weakly blocked by 100 μM 2-amino-4-phosphono butyric acid (2-APB) and 2-amino-3-phosphono-propionic acid (2-APP) (Table III). Both 2-APB and 2-APP also exhibited agonist-like activity.

3. Reconstitution of GBP into liposomes: This phase of our work was centered primarily on testing the possibility that the glutamate binding protein is the glutamate receptor and the glutamate-activated ionophore. This synaptic plasma membrane fraction has been found to be enriched in glutamate binding protein (GBP) (Michaelis et al., 1974). This protein has been purified by means of affinity chromatographic steps. Previous reports from this laboratory have shown that the pharmacologic characteristics of L-glutamic acid binding of the liposomes reconstituted with the GBP were very similar to those observed in synaptic membranes. Glutamate-stimulated Na^+ uptake of the GBP-reconstituted liposomes was also investigated. An increase in Na^+ uptake was induced by 8 μM L-glutamic acid in this preparation, however, L-glutamic acid did not stimulate the Na^+ uptake process in the control liposome fraction (Fig. 10). This may suggest that the GBP protein could be functioning as the receptor recognition site and as the ion channel. However, the magnitude of the L-glutamic acid stimulation is variable from preparation to preparation. It would appear that the binding activity of the protein is preserved through the purification and reconstitution steps, but that the ion channel properties of the protein are more labile.

c) List of All Publications

Articles Published

Chang, H. H. and Michaelis, E. K. (1980) Effects of L-glutamic acid on synaptosomal and synaptic membrane Na^+ fluxes and (Na^+-K^+) ATPase, J. Biol. Chem. 255, 2411-2417.

Chang, H. H. and Michaelis, E. K. (1981) L-Glutamate stimulation of Na^+ efflux from brain synaptic membrane vesicles, J. Biol. Chem. 256, 10084-10087.

Michaelis, E. K., Michaelis, M. L., Chang, H. H., Grubbs, R. D., and Kuonen, D. R. (1981) Molecular characteristics of glutamate receptors in the mammalian brain, Molec. Cell. Biochem. 38, 163-179.

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Articles in Press

Michaelis, E. K., Michaelis, M. L., Stormann, T. Chittenden, W. L., and Grubbs, R. D. (1982) Purification and molecular characterization of the brain synaptic membrane glutamate binding protein, J. Neurochem., in press.

Abstracts Published

Chang, H. H. and Michaelis, E. K. (1979) Characterization of the glutamate receptor ionophore interaction of brain synaptic membranes. Neurosc. Abstr. 5, 301.

Belieu, R. M., Michaelis, E. K., Michaelis, M. L., and Chang, H. H. (1979) Characteristics of the glutamate receptor function of brain synaptic membranes and of the purified binding protein. Neurosc. Abstr. 5, 584.

Grubbs, R. D. and Michaelis, E. K. (1980) Characterization of the glutamate receptor-like protein reconstitution into liposomes. Neurosc. Abstr. 6, 301.

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d) List of Participating Scientific Personnel

Dr. Elias K. Michaelis, Professor Human Development and Biochemistry.

Dr. Hsuan H. Chang, Courtesy Assistant Professor of Human Development and Research Scientist, Center for Biomedical Research.

Ms. Julia Lau, M.S. degree obtained, 1981.

Mr. Sherrell Early, M.S. degree obtained, 1980.

Ms. Sabita Roy

Mr. W. Leroy Chittenden, Ph.D. degree candidate, 1982.

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7. Appendix

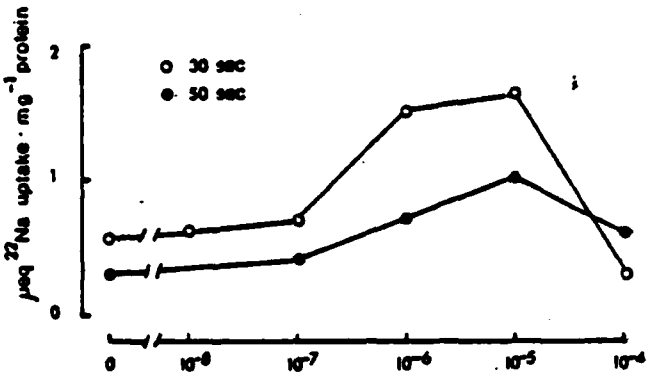


Fig. 1

Dose-response characteristics of L-glutamate-induced increase in synaptosomal Na^+ influx. A synaptosomal preparation was preincubated with various concentrations of L-glutamic acid and Na^+ diffusion into the synaptosomes was measured under conditions identical to those described for Fig. 2. Samples were obtained following either a 30- or 50-s incubation in the presence of ^{22}Na and 22°C. The glutamate concentrations shown represent the final concentration of the agent in the assay. Each value is the mean of triplicate determinations.

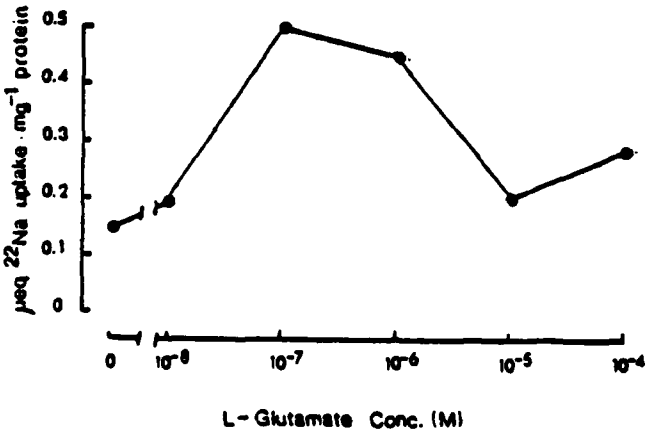


Fig. 2

Dose-response relationship of glutamate-induced Na^+ influx in synaptic membrane vesicles. Time kinetics of Na^+ influx from 15 s to 2 min in the presence of each of the concentrations of L-glutamate shown were obtained. The maximum point of Na^+ influx obtained from each time kinetic determination in the presence or absence of glutamate was used to plot the data for the dose-response relationship. Each value is the mean of two such determinations for each glutamate concentration. The point of maximum Na^+ influx occurred usually between 15 and 30 s of incubation.

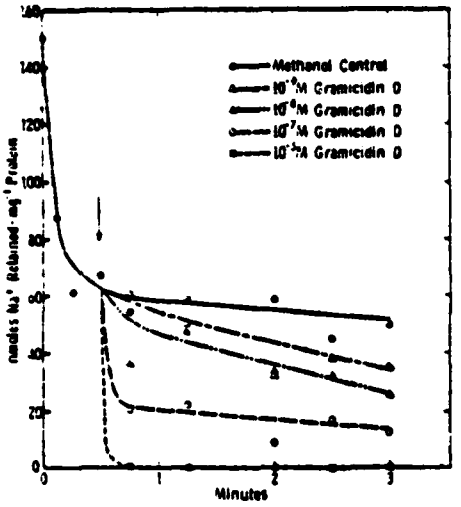


Fig. 3

The effect of various concentrations of gramicidin D on the efflux of Na^+ from membrane vesicles. The synaptic membrane vesicles were loaded with ^{22}Na and the efflux of this ion was determined according to the procedures described in Fig. 1. All efflux measurements were done at 24 °C. Methanol (5 μl) or various concentrations of gramicidin D in methanol were added to the incubation medium at the time indicated by the *arrow*. Each point is the mean of duplicate determinations.

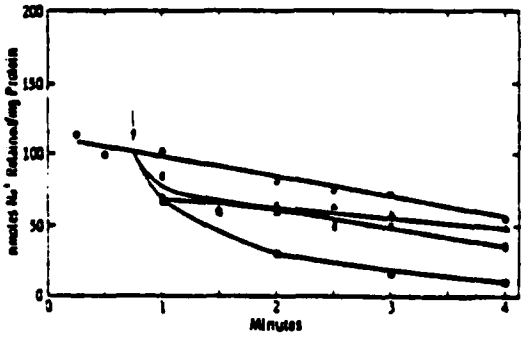
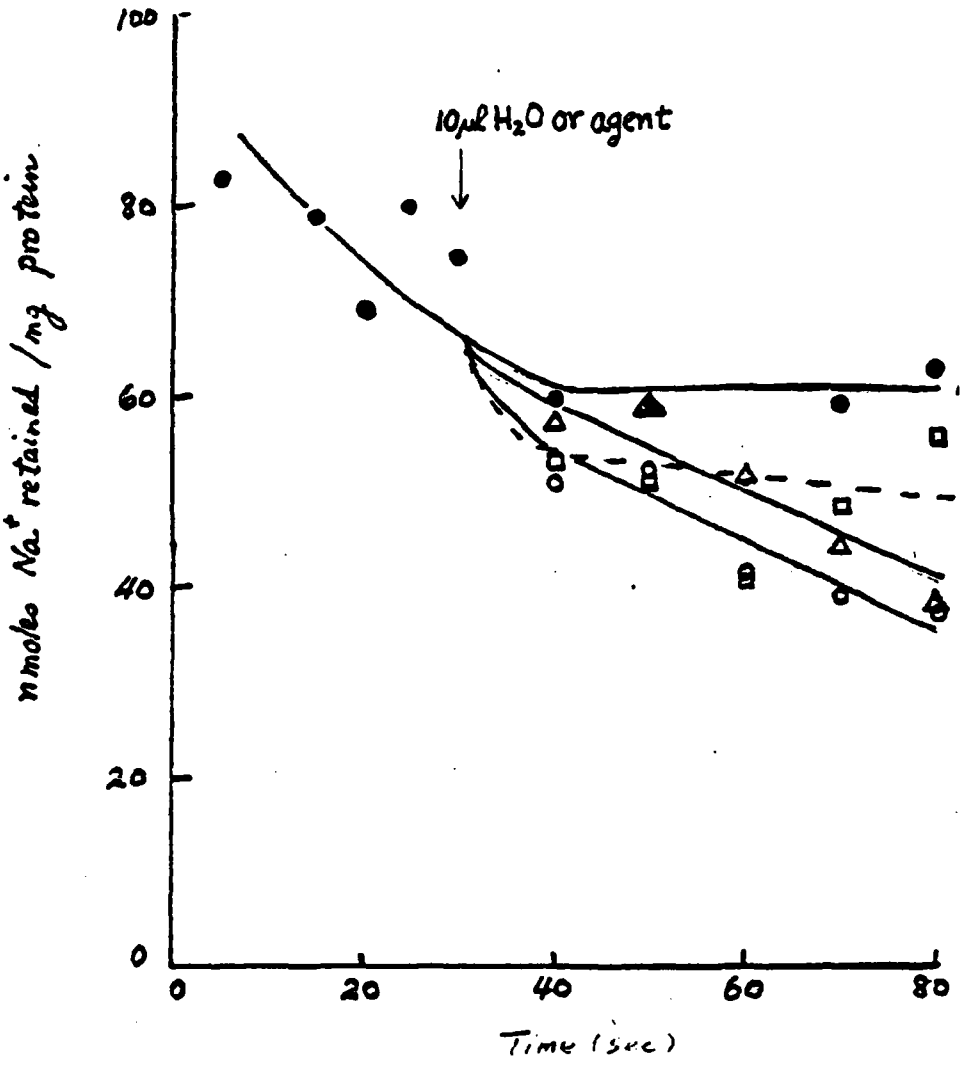


Fig. 4

Stimulation of Na^+ efflux from synaptic plasma membrane vesicles by 1 μM L-glutamic, D-glutamic, and kainic acid. Synaptic membrane vesicles were internally loaded with 50 mM Na_2SO_4 by incubating them with this medium at 37 °C for 5 min, followed by a 2-h incubation at 4 °C according to the procedures described under "Methods." The efflux of ^{22}Na was initiated by diluting 20 μl of the vesicles into the choline-Cl medium (4 °C). At the time indicated by the *arrow*, either 10 μl of H_2O (●) or of L-glutamate (■), or of D-glutamate (▲), or of kainic acid (▣) solution was added. Each point is the mean of duplicate determinations.



Stimulation of Na^+ efflux from synaptic plasma membrane vesicles by 1μM L-glutamic acid, 10 μM N-methyl D-aspartic (NMDA) and Homocysteic acid. Synaptic membrane vesicles were internally loaded with 50 mM Na_2SO_4 by incubating them with this medium at 37°C for 5 min, followed by a 2-h incubation at 4°C. The efflux of ^{22}Na was initiated by diluting 20 μl of the vesicles into choline-cl medium (4°C). At the time indicated by the arrow, either 10 μl of H_2O (●) or of L-glutamate (▲), or of NMDA (○), or of Homocysteic acid (□) solution was added. Each point is the mean of duplicate determinations.

Figure 5

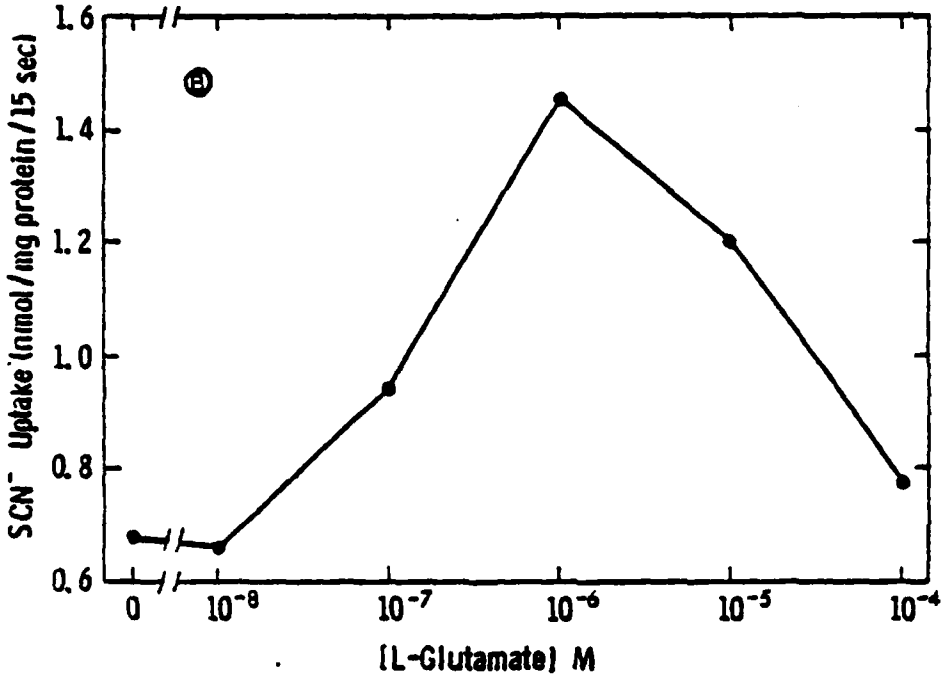


Fig. 6 Dose-response characteristics of L-glutamate-induced increases in SCN^- influx into synaptic membranes. The SCN^- uptake at 15 sec of incubation is shown.

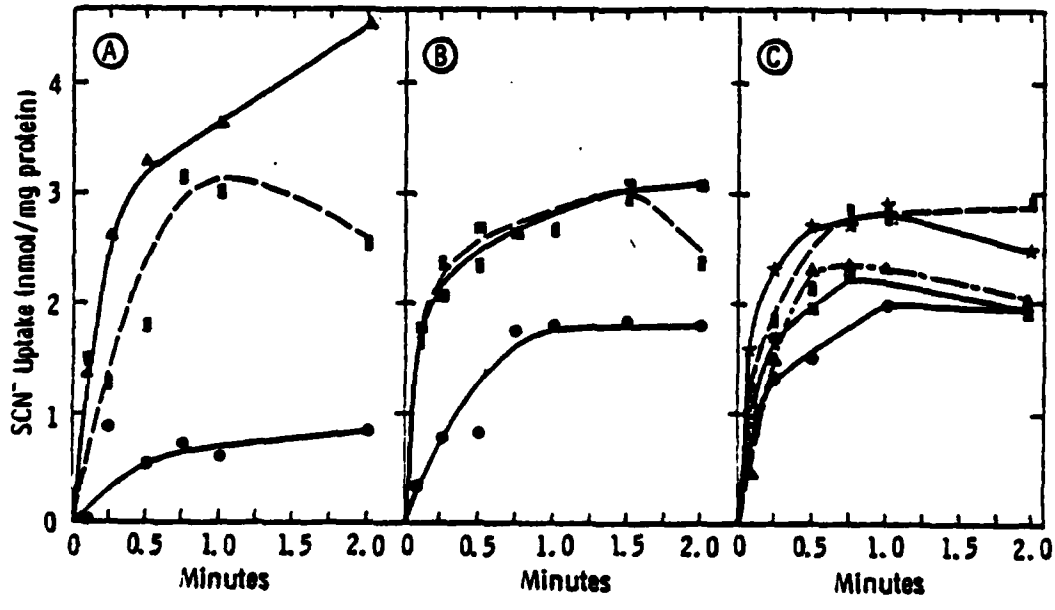


Fig. 7 Effects of various excitatory amino acids and amino acid analogs on SCN^- accumulation by synaptic membrane vesicles. (A) Uptake of SCN^- in the presence of 10 μM D-glutamate (▲) or L-glutamate (▮) or in the absence of either agent (●). (B) Uptake of SCN^- in the presence of 10 μM kainic acid (■) or L-glutamate (▮) was compared to the basal SCN^- uptake (●). (C) Influx of SCN^- in the presence of 10 μM L-glutamate (▮), N-methyl-D-aspartate (*), D,L-homocysteic acid (▲), ibotenic acid (■), and in the absence of any of these agents (●).

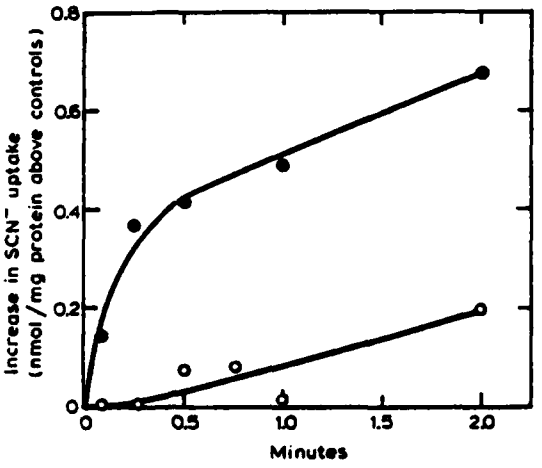


Fig. 8

Inhibition of L-glutamate-induced SCN^- uptake by L-glutamate diethyl ester. The net increase in SCN^- accumulation above control influx brought about by exposure of the membranes to 5 μM L-glutamic acid is shown. Both control and L-glutamate-exposed membrane vesicles were incubated in a NaCl medium in the absence (●) or presence (○) of 100 μM L-glutamate diethyl ester. Each point is the mean of duplicate determinations from two membrane preparations.

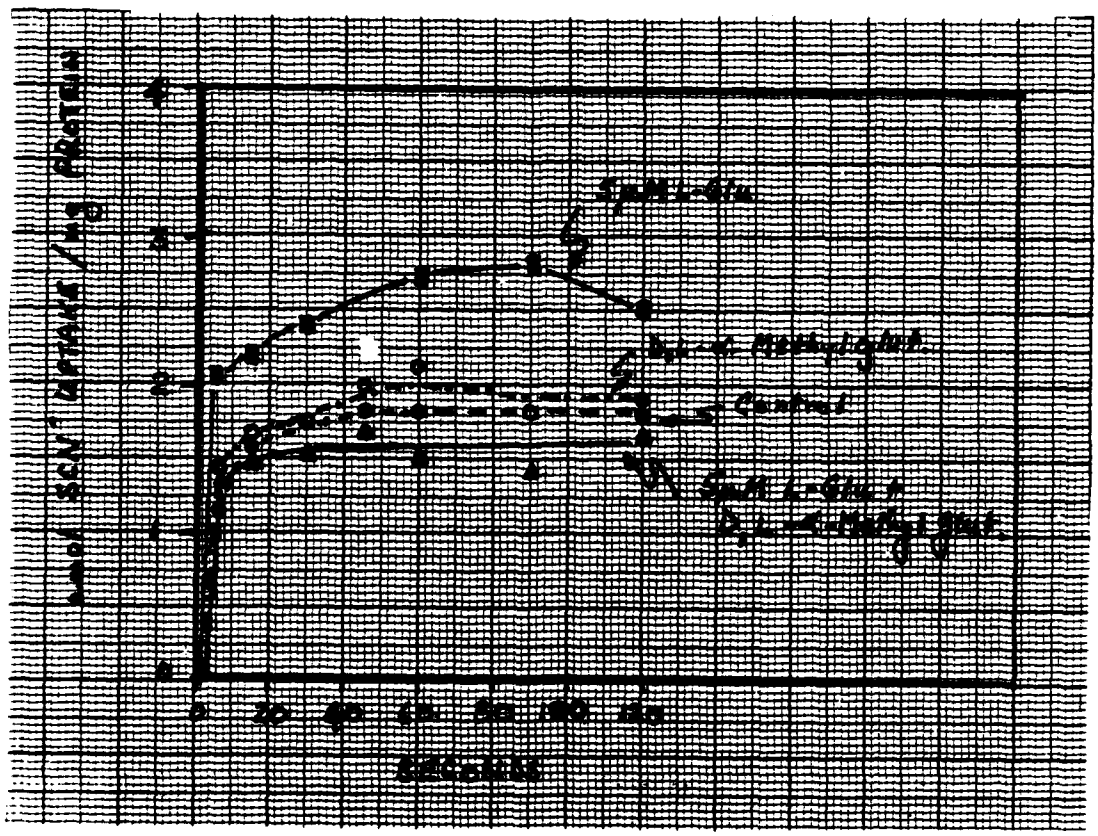


Fig. 9

Inhibition of L-glutamate-induced SCN^- uptake by 100μM D,L-α-methyl glutamate.

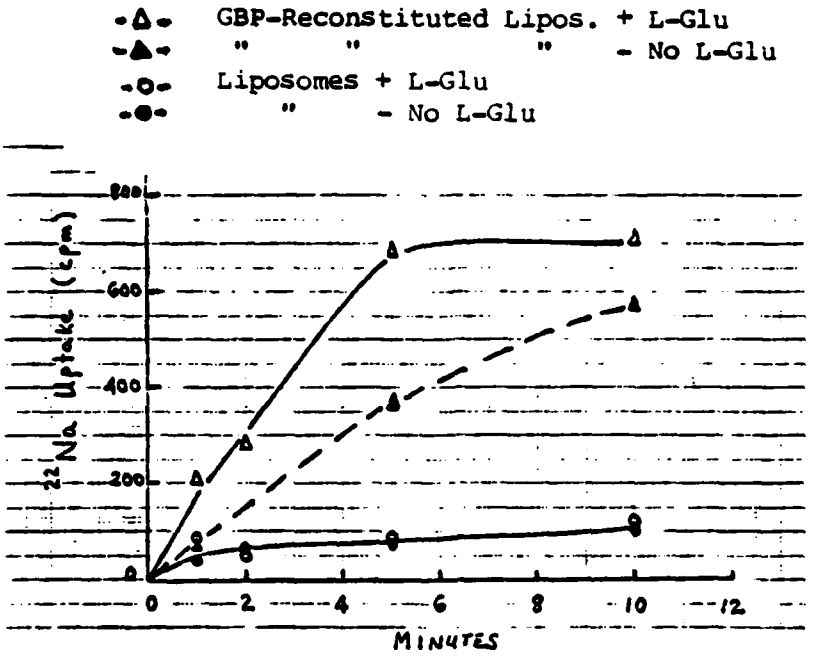


Fig. 10 Reconstitution of GBP into PC vesicles and determination of ^{22}Na influx. Liposome formation and protein reconstitution was conducted according to the procedures described in the text. Influx of ^{22}Na following incubation for various time periods at 24°C in the presence or absence of 10 μM L-glutamate was determined by the Dowex chromatographic procedure.

Table I

$$\text{Membrane potential} = \frac{RT}{nF} \ln \frac{[\text{ion}]_{\text{in}}}{[\text{ion}]_{\text{out}}}$$

Cerebral Cortical slices (Gibson and McIlwain, 1965)	+10 to +30mV
Brain Synaptosomes	+20mV
Resealed Synaptic membrane vesicles	+13mV

Table III

Antagonists	% Inhibition of L-glutamic acid induced SCN^- uptake	
Glutamate diethyl ester	71%	(n=2)
D,L-α-methyl glutamate	100%	(n=2)
2-amino-4-phosphonobutyric acid	14%	(n=3)
2-amino-3-phosphono propionic acid	12%	(n=1)

Table II

Amino Acids (10μM)	% of control SCN^- diffusion		
Control	100	100	100
Excitatory Amino Acids			
L-glutamic acid	200	196	158
D-glutamic acid		223	
Kanic acid		180	
L-aspartic acid		165	
L-cysteine sulfinic acid		141	
L-glutamine		125	
Quisqualic acid	173		
N-metheyl-D-aspartic acid			151
Ibotenic acid			125
DL-homocysteic acid			114
Ihibitory Amino Acids			
Y-amino butyric acid	100		
Glycine	84		