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Influences of Temperature, Detergents, and Enzymes on Glutamate Receptor Binding and Its Regulation by Calcium in Rat Hippocampal Membranes

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SUMMARY

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The effects of various physical or chemical treatments which cause modifications of protein-protein or protein-lipid interactions on basal and calcium-stimulated [^{3}H]glutamate receptor binding have been studied in rat hippocampal membranes. Increasing temperature increased the number of binding sites without changing their affinity for [^{3}H]glutamate; in addition, no significant stimulation by calcium of this binding was observed below 15–20°. An Arrhenius-like plot of the data indicated a marked discontinuity at 21° and yielded activation energies of 20–60 kcal·mole^{-1}. Treatment of membranes with the detergents Triton X-100 or sodium deoxycholate resulted in a dose-dependent reduction in [^{3}H]glutamate binding accompanied by a loss of the stimulatory effect of calcium ions. Treatment with phospholipase C decreased basal binding without altering the percentage increase in binding produced by calcium, whereas treatment with phospholipase D did not affect basal binding, but instead reduced the effect of calcium. Trypsin and chymotrypsin caused an increased basal binding and did not change the stimulatory effect of calcium. These data support the hypothesis that calcium increases the number of [^{3}H]glutamate binding sites by inducing local changes in membrane fluidity.

INTRODUCTION

During the past 5 years, the development of ligand binding techniques in neurochemistry has resulted in a substantial increase in information about neurotransmitter receptors and the mechanisms involved in their regulation (1). Recently, we developed a binding assay for hippocampal glutamate receptors using [^{3}H]glutamate as a ligand (2, 3), and found that the sodium-independent binding sites exhibit several properties of postsynaptic glutamate receptors (for a review, see ref. 4). We reported that [^{3}H]glutamate binding to hippocampal membranes is regulated by cations (5); monovalent cations decrease the number of binding sites, whereas divalent cations, calcium in particular, increase the maximal number of binding sites without changing either their apparent affinity for glutamate (5) or their pharmacological properties.[1] This effect of calcium is partially irreversible and is observed in some brain areas but not in others (6). On the basis of pharmacological experiments using protease inhibitors, we suggested that calcium ions stimulate [^{3}H]glutamate binding by activating a membrane-bound protease, thus revealing sites which were otherwise inaccessible (6). Subsequent experiments indicated that low levels of calcium cause the breakdown of a high molecular weight protein in cortical and hippocampal synaptic membranes, and the effect is relatively specific to this protein (7). These results raise the question of how calcium-activated proteolysis might increase the number of glutamate binding sites in hippocampal and cortical membranes. The enzyme could remove an occluding protein and thereby allow the ligand access to its receptor, as is the case for γ-aminobutyric acid receptors (8, 9). Alternatively, the uncovering of additional sites could be a secondary event reflecting a general change produced by the protease on membrane properties. There is evidence that proteases affect cytoskeletal elements associated with membranes (10) and in this way they may produce rather broad alterations in membrane organization. Furthermore, treatments which are known to perturb the membrane organization reduce the binding of a variety of ligands including opiates (11, 12), muscarinic agents (13), and γ-aminobutyric acid (14, 15) to their respective receptors. In light of these observations, it

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[1] M. Baudry, E. Smith, and G. Lynch, unpublished data.

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seems that an understanding of mechanisms by which calcium-activated processes influence the number of glutamate receptors would be furthered by a study of the effects of physical or chemical manipulations of hippocampal synaptic membranes on basal and on calcium-stimulated [^{3}H]glutamate binding.

MATERIALS AND METHODS

Rat hippocampal membranes were prepared according to the method of Enna and Snyder (16) as previously described (3, 5, 6). The final crude synaptic membrane pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, at a protein concentration of about 0.5–1.0 mg of protein per milliliter.

For the standard [^{3}H]glutamate-binding assay procedure, aliquots of the membrane preparation (50–90 μl) were incubated in the presence or absence of the indicated drugs at 30° for 10 min. The incubation was started with the addition of 0.1 ml of [^{3}H]glutamate (final concentration 100 nM, 0.4 μCi) in a final volume of 0.2 ml. After 15 min, the reaction was terminated by dilution with 3 ml of cold Tris-HCl buffer and filtration on Millipore cellulose filters (pore size, 0.45 μm); the tubes were rinsed with an additional 3 ml of cold buffer and finally the filters were washed with 4 ml of cold buffer (for a discussion of the validity of the filtration procedure, see ref. 3). The radioactivity in the filter was extracted with 3.5 ml of an aqueous scintillation cocktail (ACS; Amersham Corporation, Arlington Heights, Ill.) and counted in a liquid scintillation spectrophotometer with an efficiency of 40%. Nonspecific binding was determined in the presence of an excess (0.1 mM) of cold glutamate; binding under these conditions was never significantly different from that in blanks obtained by omitting tissue from the incubation. Therefore, under standard conditions, specific binding represents total binding minus the values for the blanks.

In experiments using enzymes or detergents, the synaptic membranes were incubated in a final volume of 1 ml with the appropriate amounts of enzymes or detergents for 30 min at 30°. Following the incubation, the suspension was diluted 10 times with cold Tris-HCl buffer and centrifuged at 40,000 × *g* for 30 min; the pellet was resuspended in 1 ml of cold buffer for the standard [^{3}H]glutamate binding assay.

Proteins were measured according to the method of Lowry *et al.* (17) with bovine serum albumin as standard. [^{3}H]Glutamate (specific activity, 21 Ci/mmole) was obtained from Amersham Corporation. Triton X-100, sodium deoxycholate, phospholipase C (from *Clostridium perfringens*, 7.7 units/mg of solid) and phospholipase D (from cabbage, 49 units/mg of solid), trypsin, and chymotrypsin were purchased from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Effects of temperature on basal and calcium-stimulated [^{3}H]glutamate binding. We previously reported that calcium ions do not stimulate [^{3}H]glutamate binding when the incubation temperature is 4° (6). We re-examined in greater detail the effects of temperature on both basal and calcium-stimulated [^{3}H]glutamate binding in the range of 15–45°. Basal [^{3}H]glutamate binding doubled between 15° and 25° and then increased slightly between 25° and 35°; beyond 35° there was a significant reduction in binding (Fig. 1a). When plotted as an Arrhenius-like plot (Fig. 1b), the curve showed a marked discontinuity at a temperature of 25°; the Arrhenius activation energy between 15° and 25° was 10.6 kcal·mol^{-1}, whereas it was only 3.3 kcal·mol^{-1} between 25 and 35°. The increase in [^{3}H]glutamate binding elicited by temperature did not seem to be due to a change in affinity, but rather to an increase in the maximal number of binding sites (Fig. 2). At 20°, the apparent affinity was 350 nM and the maximal number of sites was 3.0 pmoles/mg of protein, whereas the corresponding values at 30° were 400 nM and 6.0 pmoles/mg of protein.

The effects of various calcium concentrations on [^{3}H]glutamate binding were examined at various temperatures between 15° and 45° (Fig. 3a). Calcium in concentrations as high as 500 μM did not increase [^{3}H]glutamate binding at 15°. Increasing the temperature progressively increased the maximal effect of calcium without significantly modifying the EC_{50} for calcium (about 30 μM at each temperature tested). The influence of temperature on the maximal effect of calcium was also plotted as an Arrhenius-like plot (Fig. 3b). The curve shows a marked discontinuity between 20° and 25° with Arrhenius activation energies of 59.0 and 20.5 kcal·mol^{-1}, respectively, for the two parts of the curve. These effects of tempera-

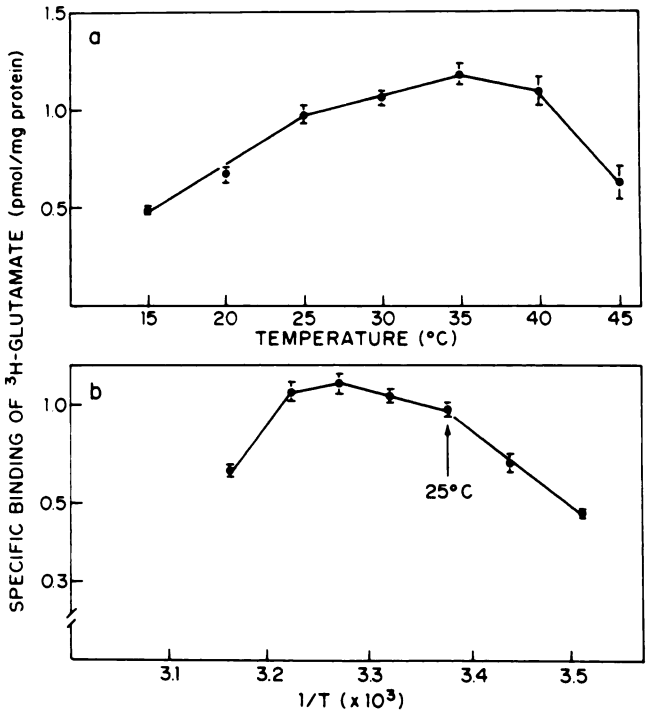

FIG. 1. *Effect of temperature on [^{3}H]glutamate binding to rat hippocampal membranes*

[^{3}H]Glutamate binding to hippocampal membranes was measured at various temperatures as described under Materials and Methods. Results are mean ± standard error of the mean of six different experiments.

a. Linear plot of the data.

b. Arrhenius-like plot of the same data.

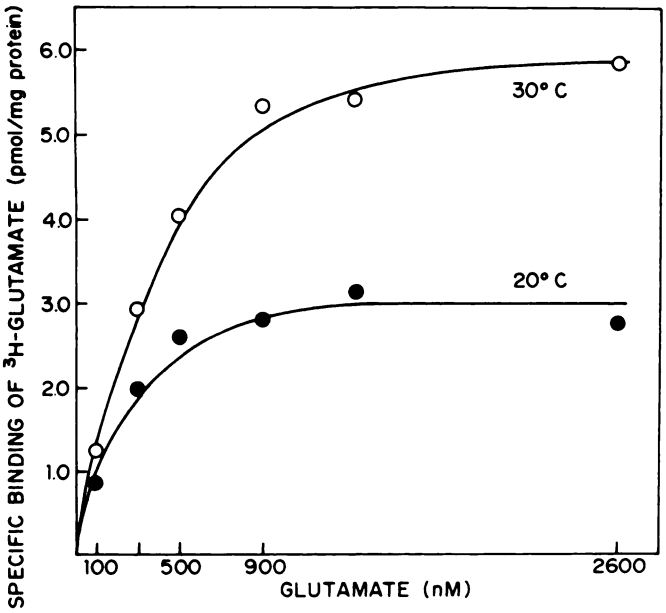

FIG. 2. *Saturation kinetics of [^{3}H]glutamate binding to hippocampal membranes at 20 and 30°*

The saturation of specific [^{3}H]glutamate binding was determined at 20° and at 30° by adding to a fixed amount of [^{3}H]glutamate (100 nM) increasing concentrations of unlabeled glutamate. Results are the mean of three different experiments which varied less than 10%.

ture on both basal and calcium-stimulated [^{3}H]glutamate binding are not likely to be due to changes in the pH of the Tris-HCl buffer used (about 0.5 pH unit between 20° and 40°), since no significant difference in these parameters are observed by varying the pH around 7.4 by 0.5 pH unit (data not shown).

Effects of detergents on basal and calcium-stimulated [^{3}H]glutamate binding. Treatment of membranes with Triton X-100 induced a dose-dependent decrease in specific [^{3}H]glutamate binding, representing a 50% decrease at a Triton concentration of 0.05% (v/v) (Fig. 4). A similar result has been previously reported by Sharif and Roberts (18) for cerebellar membranes. This effect of Triton X-100 was due to a decrease in the maximal number of [^{3}H]glutamate binding sites and occurred without significant change in their apparent affinity for [^{3}H]glutamate (data not shown). It should be noted that at this concentration of Triton X-100 about 80% of the protein remained in the particulate fraction.

The effect of treatment of membranes with Triton X-100 on the stimulatory effect of calcium was more dramatic than its actions on basal binding. [^{3}H]Glutamate binding was decreased by 30% in the presence of 0.02% Triton X-100, but the stimulatory effect of calcium (250 μM) was virtually eliminated (Fig. 4). Similarly, low concentrations of sodium ions were no longer able to decrease [^{3}H]glutamate binding after treatment of the membranes with Triton X-100 (Table 1).

Treatment of membranes with sodium deoxycholate (in the range 0.001–0.1%) also induced both a progressive decrease in basal [^{3}H]glutamate binding and a suppression of the ability of calcium to stimulate this binding (data not shown).

Since these detergents act mainly by perturbing the lipid environment of membranes, these results prompted us to study the effects of phospholipases on the calcium regulation of [^{3}H]glutamate binding.

Effects of Phospholipase C and D on basal and calcium-stimulated [^{3}H]glutamate binding. Treatment of membranes with phospholipase C resulted in a dose-

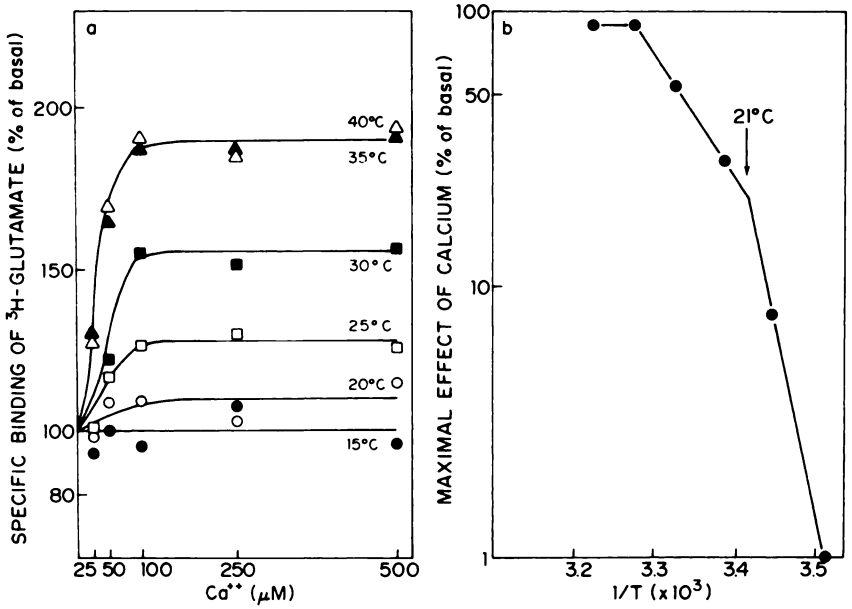

FIG. 3. *Effect of increasing calcium concentrations on [^{3}H]glutamate binding to hippocampal membranes at various temperatures*

The effects of various calcium concentrations on [^{3}H]glutamate binding were determined at various temperatures, at a [^{3}H]glutamate concentration of 100 nM.

a. The results are expressed as percentage of the respective basal binding (measured in the absence of added calcium; see Fig. 1).

b. Arrhenius-like plot of the same data, where the ordinate represents the logarithm of the maximal effect of calcium determined from *a*, and the absissa represents the reciprocal of absolute temperature. Results are the mean of six different experiments.

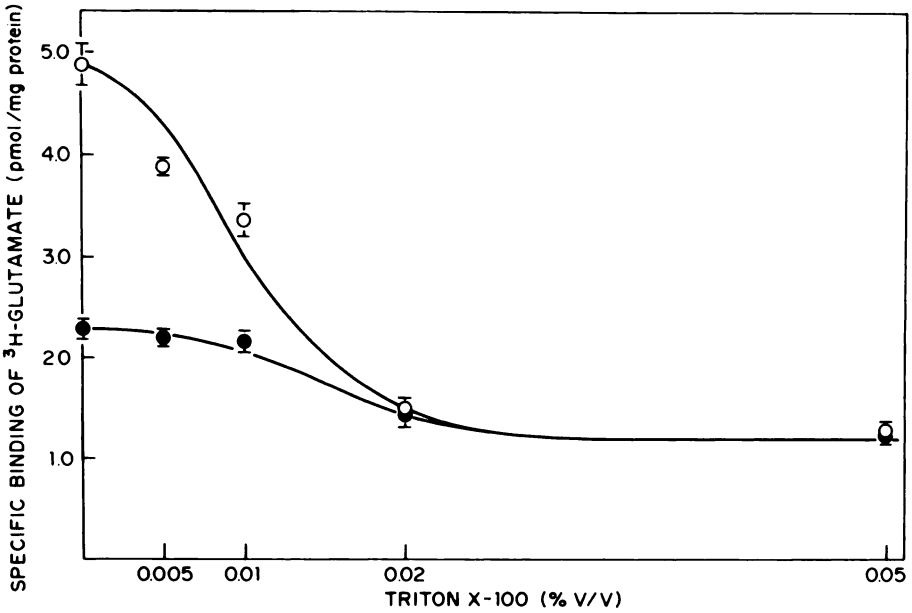

FIG. 4. *Effect of various concentrations of Triton X-100 on basal and calcium-stimulated [³H]glutamate binding to rat hippocampal membranes*

Hippocampal membranes were incubated with increasing concentrations of Triton X-100 and then were washed. The binding of [³H]glutamate binding (100 nM) was measured in the absence (●) or presence (○) of 250 μM $CaCl_2$. Results (in picomoles per milligram of protein) are the mean ± standard error of the mean of four to six experiments.

dependent decrease in [³H]glutamate binding (Fig. 5). However, under these conditions, the stimulatory effect of calcium on [³H]glutamate binding did not seem to be changed since the increase in binding elicited by 250 μM calcium, expressed as percentage over basal, remained constant at each concentration of phospholipase C tested (Fig. 5).

Phospholipase D produced an opposite pattern of results. Whereas concentrations of phospholipase D up to 200 μg/ml did not affect basal [³H]glutamate binding, they induced a progressive and dose-dependent inhibition of the stimulatory effect of calcium (Fig. 6). Under these conditions, only the maximal effect of calcium was decreased and there was no change in the EC_{50} for calcium.

TABLE 1

Effect of calcium and sodium ions on [³H]glutamate binding following Triton X-100 treatment

Hippocampal membranes were incubated at 30° for 30 min in the absence or presence of Triton X-100 (0.05%, v/v) and then were washed. The binding of [³H]glutamate (100 nM) was then measured in the presence of $CaCl_2$ (250 μM) or NaCl (2.5 mM). Results are expressed as percentage of the respective binding measured in the absence of added ions, which was 2.28 ± 0.15 pmoles/mg of protein in the control untreated membranes and 1.24 ± 0.08 pmoles/mg of protein in the Triton X-100-treated membranes. Results are the mean ± standard error of the mean of six experiments.

Incubation treatment	[³H]Glutamate binding	
	Ca^{2+} (250 μM)	Na^+ (2.5 mM)
	% of basal	
None	166 ± 6[a]	28 ± 1[a]
Triton X-100, 0.05% (v/v)	102 ± 5	99 ± 4

[a] $p < 0.001$ as compared with control (Student's *t*-test).

Effects of trypsin and chymotrypsin on basal and calcium-stimulated [³H]glutamate binding. We previously reported that the addition of trypsin and chymotrypsin to the [³H]glutamate binding assay resulted in an increased number of binding sites (6). Treatment of the membranes with trypsin or chymotrypsin followed by

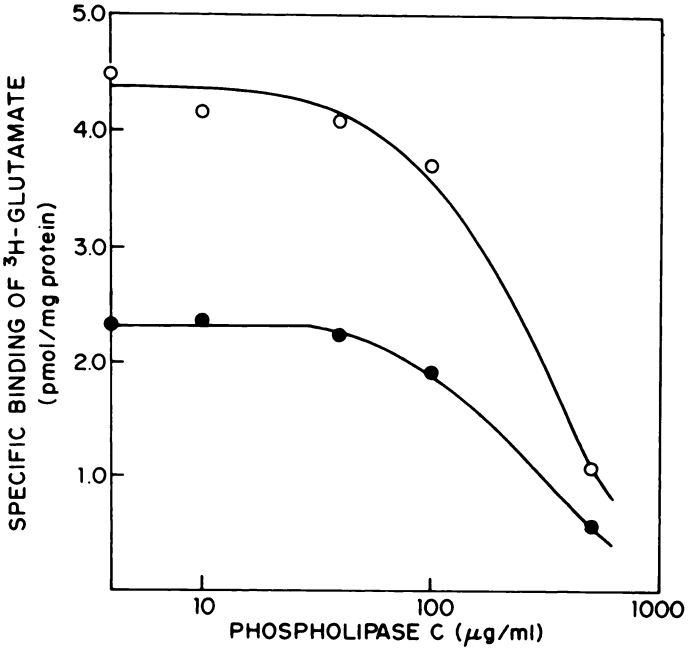

FIG. 5. *Effect of phospholipase C on basal and calcium-stimulated [³H]glutamate binding to rat hippocampal membranes*

Hippocampal membranes were incubated with various concentrations of phospholipase C and then were washed. The binding of [³H]glutamate (100 nM) was then measured in the absence (●) or presence (○) of 250 μM $CaCl_2$. Results (in picomoles per milligram of protein) are the mean of three different experiments which varied less than 15%.

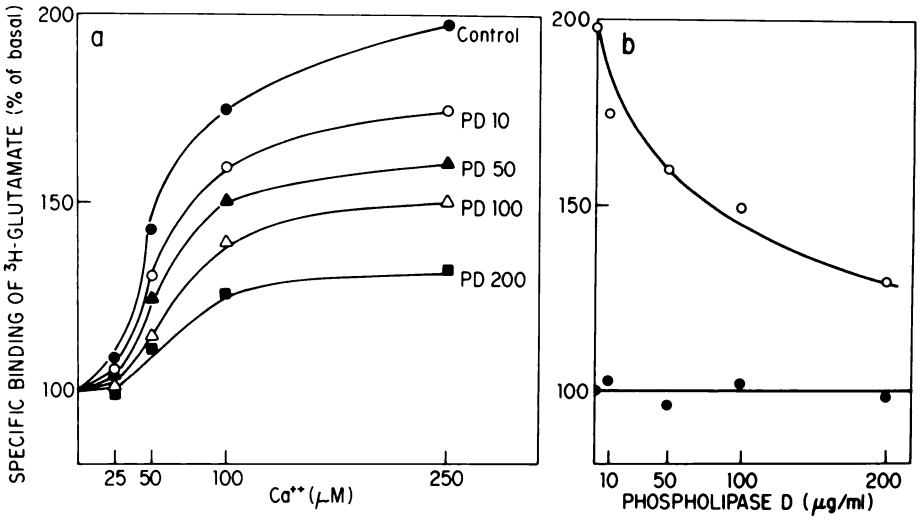

FIG. 6. *Effect of phospholipase D on basal and calcium-stimulated [^{3}H]glutamate binding to rat hippocampal membranes*

Hippocampal membranes were incubated with various concentrations of phospholipase D (*PD*) and then were washed. The binding of [^{3}H]-glutamate binding (100 nM) was measured in the presence of various calcium concentrations.

a. Results are expressed as percentage of the binding measured in the absence of calcium and are means of three experiments.

b. Binding in the absence (●) or presence (○) of 250 μM $CaCl_2$ is expressed as percentage of the binding in control, untreated membranes, which was 2.30 ± 0.15 pmoles/mg of protein.

washing to eliminate these enzymes also produced an increase in [^{3}H]glutamate binding (Table 2). Treatment with trypsin (1 μg/ml) induced a 30% increase, whereas treatment with chymotrypsin (5 and 20 μg/ml) increased basal [^{3}H]glutamate binding by 50 and 80%, respectively. However, treatment with either proteolytic enzyme did not modify the effects of calcium on [^{3}H]glutamate binding (Table 2). The maximal effect (expressed as percentage over binding in the absence of calcium) or the EC_{50} for calcium was not significantly changed in either case.

DISCUSSION

The present data show that physical or chemical treatments of hippocampal membranes, which cause perturbations of protein-protein or protein-lipid interactions, induce various changes in basal as well as calcium-stimulated [^{3}H]glutamate receptor binding.

Increasing temperature between 15° and 35° increases the number of binding sites without changing their apparent affinity for glutamate. This suggests that some binding sites are indeed inaccessible to glutamate at low temperature when the lipids are in a "solid state"; the Arrhenius-like plot of the effects of temperature on basal [^{3}H]glutamate binding reveals a discontinuity at about 25°, a temperature which has been shown to be the transition temperature from a solid to a liquid state of a variety of lipids (19). Furthermore, calcium ions do not stimulate [^{3}H]glutamate below 20° and the Arrhenius-like plot of the data shows a discontinuity between 20° and 25°. Similar effects of temperature have been reported on the activation of adenylate cyclase by catecholamines (20) or cholera toxin (21), and also on muscarinic receptor activation and desensitization (22). It is of interest that the activation energies for the effects of calcium (20–60 kcal·mole^{-1}) are similar to those reported for the activation and desensitization of muscarinic receptors on neuroblastoma cells (22). In all of these cases, it has been proposed that the fluid state of the membranes is responsible for the discontinuities in the Arrhenius plots. It is thus conceivable that a certain state of membrane fluidity is required for calcium ions to stimulate [^{3}H]glutamate binding; in particular, the activity of the postulated calcium-dependent protease could be markedly influenced by such a change in membrane fluidity as is the case for a number of membrane-bound enzymes (23).

The effects of the detergents Triton X-100 and sodium deoxycholate point to a similar conclusion. These detergents act mainly by peturbing the lipid environment of membranes, as well as by extracting some proteins loosely associated with membranes. Therefore, both the progressive inhibition of [^{3}H]glutamate binding and the suppression of the stimulatory effect of calcium could be

TABLE 2

Effect of trypsin and chymotrypsin on basal and calcium-stimulated [^{3}H]glutamate binding to hippocampal membranes

Hippocampal membranes were incubated with trypsin or chymotrypsin for 30 min at 30°, and then were washed. The binding of [^{3}H] glutamate (100 nM) was then measured in the presence of various calcium concentrations. Results are the mean ± standard error of the mean of four different experiments.

Incubation treatment	[^{3}H]Glutamate binding		
	Basal	Ca^{2+} (100 μM)	Ca^{2+} (250 μM)
	pmoles/mg protein	%	%
None	2.44 ± 0.14	156 ± 2	170 ± 2
Trypsin (1 μg/ml)	3.26 ± 0.21[a] (+ 34%)	151 ± 4	171 ± 3
α-Chymotrypsin (5 μg/ml)	3.60 ± 0.20[a] (+ 48%)	146 ± 4	163 ± 3
α-Chymotrypsin (20 μg/ml)	4.50 ± 0.20[b] (+ 84%)	145 ± 4	160 ± 4

[a] $p < 0.01$.

[b] $p < 0.001$ as compared with control, untreated membranes (Student's *t*-test).

due to a decreased fluidity of membranes elicited by the extraction of some essential lipid components. Alternatively, it is possible that the solubilization of specific membrane-bound proteins is responsible for the suppression of the effects of calcium. Studies on the pattern of the membrane proteins following treatment with these detergents are now in progress and should shed some light on these alternatives.

The fact that treatment with Triton X-100 also suppresses the inhibitory effects of low concentrations of sodium ions suggests that this effect of sodium is not likely to be a direct effect on the receptor molecule itself, but rather an indirect effect via some protein-protein or protein-lipid interaction. This in in agreement with our previously proposed hypothesis that the inhibitory effect of sodium is due to the existence of a link between the receptor and a sodium-conductance channel (5). Studies are now in progress to test this hypothesis.

Phospholipases C and D have different effects on baseline and calcium-stimulated binding. A variety of neurotransmitter receptors are susceptible to phospholipase C treatment (11–15) and this has been taken as evidence that acidic phospholipids form part of the receptor or, as membrane components, are intimately related to the receptor. Similar arguments might apply to our results, although the possibility that reaction products inhibit [^{3}H]glutamate binding has not been excluded. It is important to note that calcium ions stimulate [^{3}H]glutamate binding after phospholipase C treatment (when the results are expressed as percentage of basal binding).

Phospholipase D produces an opposite pattern of results: it does not inhibit basal binding but progressively reduces the stimulatory effect of calcium. Other investigators (10) have reported that phospholipase D treatment does not reduce the binding of muscarinic ligands to their receptors; however, it should be noted that we used higher concentrations of this enzyme in our study. It would seem then that the effect of phospholipase D is a rather selective one and may provide some insights into the link between calcium and stimulation of receptor binding. One possibility is that the sensitivity to calcium of the postulated membrane-associated protease is dependent upon interactions between this protein and the polar groups of exposed phospholipids. The membrane protease appears to respond to calcium levels which are much lower than those needed to activate soluble proteases (24) and it is conceivable that this is due to lipoprotein interactions.

As we previously reported, proteolytic treatments of membranes with trypsin or chymotrypsin, which have been shown to have marked effects on synaptic complexes (25), result in an increased number of [^{3}H]glutamate binding sites; however, these enzymes do not affect the ability of calcium ions to stimulate [^{3}H]glutamate binding. This result might indicate that these two serine proteases act at a site different from that of the postulated calcium-sensitive protease, which is in agreement with the hypothesis that the latter is a thiol protease (6, 7).

Our results agree in part with the results of Sharif and Roberts (18) on the effects of various enzymes or detergents on [^{3}H]glutamate binding to cerebellar membranes. They also found that treatment with Triton X-100 inhibited [^{3}H]glutamate binding whereas phospholipase D up to 20 μg/ml was ineffective. It should be noted that we also observed that incubation of hippocampal membranes for 30 min at 30° markedly enhanced basal [^{3}H]-glutamate binding (compare Fig. 4 with Fig. 1). Sharif and Roberts (26) suggested that this effect was due to the existence of an endogenous inhibitor which could be removed by incubation and washing of the membranes. An alternative explanation is that a temperature-dependent reorganization of the membranes exposes additional binding sites.

The interaction between calcium ions, membrane proteins, and cell surface receptors has been demonstrated in several systems (27). The best-documented example is the human erythrocyte, in which an inner surface protein network involving spectrin and several other membrane components restricts the mobility of the membrane glycoproteins. Calcium ions, by activating a transglutaminase and a protease, are able to modify the distribution of cell surface receptors (10). One of the most common mechanisms of the control of cell surface receptors has been proposed to involve membrane-associated cytoskeletal components such as microtubules and microfilaments (28). These elements are often associated with plasma membrane inner surfaces and are involved in the clustering, patching, or capping of cell surface receptors. Various models involving protein-lipid interactions have been proposed to explain the transmembrane control of cell surface receptors (27). Although our data do not provide a specific mechanism to explain the calcium regulation of glutamate receptors, they do indicate that this mechanism may ultimately present some similarities to those described for a variety of hormones, and in particular with the regulation of *beta*-adrenergic receptors (29). In agreement with the idea of coupling between receptors and membrane-bound enzymes, we recently found that calcium ions induce the proteolysis of a high molecular weight protein in hippocampal membranes which possesses some characteristics of neurofilament proteins (7). Therefore, it is possible that the breakdown of this protein results in a local change in the fluid state of the membranes which might lead to changes in the surface distribution of glutamate receptors.

In conclusion, by delineating the effects of various physical or chemical modifications of membrane organization on basal and calcium-stimulated [^{3}H]glutamate receptor binding, the present data offer a means of further dissecting the sequence of events relating fluctuations in calcium levels to changes in glutamate receptors.

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