L-Glutamate Increases Internal Free Calcium Levels in Synaptoneurosomes from Immature Rat Brain via Quisqualate Receptors

J. Benavides, Y. Claustre, and B. Scatton

Laboratoires d'Etudes et de Recherches Synthélabo (L.E.R.S.), Biology Department, 92220 Bagneux, France

Internal free calcium concentrations ($[Ca^{2+}]_i$) have been monitored in synaptoneurosomes from 8-d-old rat whole brain previously loaded with the calcium-sensitive fluorescent probe Fura 2. Under basal conditions, $[Ca^{2+}]_i$ was around 200 nM, this concentration increasing only slowly during storage of the synaptoneurosomes at room temperature (40% increase 2 hr after loading).

Opening of sodium channels with veratridine- (10 μ M) or KCl- (30 mM) induced depolarization caused rapid increases in synaptoneurosomal $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was also markedly increased by addition of the Ca^{2+} ionophore A23187 (10–100 nM). The effect of veratridine, but not of KCl was prevented by previous addition of TTX (1 μ M). KCl-induced $[Ca^{2+}]_i$ increase was dependent on external Ca^{2+} and was partially blocked by the dihydropyridine derivative PN 200-110 (IC_{50} 0.15 μ M, maximal inhibition 55% at 3 μ M).

L-Glutamate elicited a concentration-dependent fast increase in synaptoneurosomal $[Ca^{2+}]_i$ in the 8-d-old (but not in the adult) rat brain (EC_{50} = 2 μ M). The effect of glutamate was stereospecific, the EC_{50} of the D-isomer being 47 times higher than that of L-isomer. The magnitude of the L-glutamate response differed in several brain regions, being highest in the cerebral cortex and lowest in the cerebellum. The order of potency of excitatory amino acid receptor agonists in increasing synaptoneurosomal $[Ca^{2+}]_i$ in the whole brain (quisqualate > ibotenate > L-glutamate > AMPA > kainate $\approx$ NMDA) and the blockade of the L-glutamate effect by glutamate diethylester (1 mM) but not by 2-aminophosphonovalerate or $MgCl_2$ suggest that the L-glutamate response is mediated by a quisqualate-type receptor.

The L-glutamate-induced increase in whole-brain synaptoneurosomal $[Ca^{2+}]_i$ was completely dependent on external calcium and was slightly potentiated by previous depolarization with KCl (30 mM) but was unaffected by isosmotic substitution of NaCl by choline chloride. Moreover, the effect of glutamate on $[Ca^{2+}]_i$ was not sensitive to the calcium channel blocker PN 200-110 (1 μ M) or to the sodium channel blocker TTX (1 μ M).

The present results indicate that, in synaptoneurosomes

from the immature rat brain, activation of quisqualate-type receptors leads to an increase in $[Ca^{2+}]_i$ via the operation of dihydropyridine-resistant calcium channels.

The electrophysiological effects of glutamate and related excitatory amino acids appear to be mediated mainly by 3 receptor subtypes, which have been classified according to their preferential sensitivity to excitatory amino acid receptor agonists and antagonists as quisqualate, *N*-methyl-D-aspartate (NMDA), and kainate receptors (Watkins and Evans, 1981).

Biochemical and electrophysiological studies have shown that Ca^{2+} mobilization (from the internal stores or the extracellular medium) is a common consequence of the activation of the receptors for excitatory amino acids. Thus, excitatory amino acids diminish extracellular Ca^{2+} concentrations (reflecting Ca^{2+} influx into the neuron) *in vivo* and increase the uptake of ^{45}Ca into rat brain cortex cells or slices *in vitro* (Berdichevsky et al., 1983; Retz and Coyle, 1984; Pumain and Heinemann, 1985; Lazarewicz et al., 1986; Riveros and Orrego, 1986). Moreover, activation of NMDA receptors increases intracellular Ca^{2+} levels via Ca^{2+} influx in cultured spinal neurons (MacDermott et al., 1986) and activates a voltage-sensitive Ca^{2+} conductance in hippocampal pyramidal cells in the rodent (Dingledine, 1983).

Other studies have shown that a number of functional responses to excitatory amino acid receptor stimulation (e.g., NMDA-induced increase in cerebellar cyclic guanosyl 3',5'-monophosphate levels) are mediated by Ca^{2+} entry (Carter et al., 1987). Finally, the neurotoxic effects of high doses of excitatory amino acids (Schwarcz et al., 1978; Olney, 1980), which are considered to be an extension of the physiological effects of these agents on one or several excitatory amino acid receptor subtypes, appear to be related to increased intracellular levels of Ca^{2+} (Garthwaite et al., 1986).

The mechanism(s) whereby excitatory amino acid receptor activation leads to Ca^{2+} mobilization is as yet unclear. $[Ca^{2+}]_i$ elevation induced by excitatory amino acids may reflect activation of receptor-operated Ca^{2+} channels or may be secondary to biochemical alterations (e.g., increase in phosphatidyl inositol hydrolysis) (Sladeczek et al., 1985; Nicoletti et al., 1986a, b) or membrane permeability changes to other cations (Garthwaite et al., 1986), which will, in turn, increase the permeability to Ca^{2+} or release it from internal stores.

It is now possible to monitor rapid variations of $[Ca^{2+}]_i$ through the use of Ca^{2+} -sensitive probes such as Fura 2 (Grynkiewicz et al., 1985). Fura 2 acetoxymethyl ester (Fura 2 AM) is taken up by cells and hydrolyzed to its free acid form, which is trapped

Received Feb. 6, 1987; revised Feb. 10, 1988; accepted Feb. 22, 1988.

We wish to thank S. Bleasdale and V. Eninger for typing the manuscript.

Correspondence should be addressed to B. Scatton, Laboratoires d'Etudes et de Recherches Synthélabo (L.E.R.S.), Biology Department, 31 avenue Paul Vaillant Couturier, 92220 Bagneux, France.

Copyright © 1988 Society for Neuroscience 0270-6474/88/103607-09\$02.00/0

inside the cell and is fluorescent in relation to $[Ca^{2+}]_i$. This probe has recently been used to characterize the neuronal response to excitatory amino acids and Ca^{2+} channel blockers in isolated hippocampal neurons (Kudo and Ogura, 1986). We have now used this technique to investigate the mechanism of Ca^{2+} mobilization produced by excitatory amino acid receptor activation and the role of voltage-sensitive Ca^{2+} channels in the increase in $[Ca^{2+}]_i$ following depolarization. In this study, $[Ca^{2+}]_i$ was measured in synaptoneurosomes, a brain subcellular preparation enriched in synaptosomes with attached postsynaptic membrane fragments resealed in a vesicular fashion (Hollingsworth et al., 1986). Synaptoneurosomes constitute an interesting experimental model that has been used previously to characterize a number of biochemical responses to neurotransmitters and drugs, e.g., cyclic adenosine 3',5'-monophosphate synthesis and phosphatidyl inositol turnover activation (Hollingsworth et al., 1985, 1986), changes in chloride permeability induced by GABA receptor activation (Schwartz et al., 1984, 1985), or neurotransmitter release (Ebstein and Daly, 1982a, b; Ebstein et al., 1982).

Experiments were performed in synaptoneurosomes from 8-d-old rat brain because biochemical responses to excitatory amino acids are known to be higher in the developing than in the adult brain (Garthwaite and Balazs, 1978; Nicoletti et al., 1986a).

Materials and Methods

Eight-day-old Sprague-Dawley rats (IFFA CREDO, France) of either sex were used. Rats were sacrificed by decapitation, and the whole brains were removed. In some experiments the striatum, cerebral cortex, hippocampus, and cerebellum were dissected out at 4°C.

Whole-brain synaptoneurosomes were prepared by the technique of Hollingsworth et al. (1985), slightly modified. The brains from 4 rats were homogenized (4 strokes of a Teflon-glass homogenizer) in 30 ml of ice-cold Krebs-bicarbonate buffer (NaCl, 118 mM; KCl, 4.7 mM; $MgCl_2$, 1.18 mM; $CaCl_2$, 1.2 mM; $NaHCO_3$, 24.9 mM; KH_2PO_4 , 1.2 mM; and glucose, 10 mM) equilibrated with O_2/CO_2 (95:5). The homogenate was filtered through a 60 μm nylon mesh and the resulting suspension filtered again by applying a positive pressure through 8 μm SCWP Millipore filters. The filtered suspension was centrifuged at $1000 \times g$ for 15 min and the pellet gently resuspended in Krebs-bicarbonate buffer at a protein concentration (measured spectrofluorimetrically; Resch et al., 1971) of 3 mg/ml. Protein yield was about 10 mg/gm tissue. This suspension was allowed to reach room temperature under O_2/CO_2 (95:5) atmosphere and, after 10 min, Fura 2 AM (5 mM in dimethylsulfoxide) was added to a final concentration of 5 μM . Synaptoneurosomes were then incubated at 37°C for 10 min under an atmosphere of O_2/CO_2 , diluted with Krebs-bicarbonate buffer (22°C) to a final concentration of 0.6 mg/ml, and kept at room temperature (under O_2/CO_2 atmosphere) until used.

Immediately before the experiment, 1 ml of the synaptoneurosomal suspension was centrifuged for 10 sec in a desk Eppendorf Microfuge, resuspended in 3 ml of 37°C Krebs-HEPES buffer (20 mM HEPES substituting for $NaHCO_3$ and adjusted with NaOH to pH 7.4), and placed in the spectrofluorimetric cuvette. HEPES buffer was used instead of bicarbonate buffer because the latter caused the appearance of bubbles in the cuvette and thus increased the "noise." Experiments were carried out at 37°C under continuous stirring, and fluorescence was measured with an Aminco-Bowman 500 spectrofluorimeter equipped with a water-jacketed cuvette holder and a magnetic stirring system. Selected wavelengths were 340 nm (excitation) and 500 nm (emission). Drugs were added to the cuvette through a small hole in the sample compartment.

At the end of the experiment, the maximal fluorescence (F_{max}) was defined by the fluorescence level measured after addition of 10 μl 10% Triton-X 100 (a similar level was obtained after adding 30 μl 10% SDS). The minimal fluorescence (F_{min}) was then obtained by adding 30 μl of an alkaline (pH 9) solution of 0.5 M EGTA. Within the concentration range used, none of the drugs used interfered with the Fura 2 fluorescence. Moreover, none of these drugs, except A23187 at concentrations higher than 200 nM, exhibited measurable fluorescence per se. $[Ca^{2+}]_i$ was calculated using the equation $[Ca^{2+}]_i = K(F - F_{min})/(F_{max} - F)$, where

the K (stability constant of Fura 2- Ca^{2+} complex) value amounted to 225 nM (Grynkiewicz et al., 1985).

Reagents were obtained from the following sources: Fura 2 AM and Fura 2 free acid: Molecular Probes (Eugene, OR); A23187 and DL-2-amino-4-phosphonobutyrate: Calbiochem; L-glutamate, D-glutamate, L-aspartate, NMDA, ibotenate, quisqualate, carbachol, glutamic acid diethylester (GDEE), D-phosphoserine, L-phosphoserine, TTX, kainic acid, and veratridine: Sigma (St. Louis); DL-2-amino-5-phosphonopentanoate, γ -D-glutamyl-aminomethyl-sulfonic-acid (GAMS), γ -glutamyltaurine and γ -glutamylglycine: Tocris (Buckhurst Hill, England); RS- α -amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA): Research Biochemical Inc. (Wayland, MA). Secobarbital (Laboratoires Martinet, France), PN 200-110 (SANDOZ, Basel, Switzerland), and BAY K 8644 (Bayer, FRG) were kindly donated by the manufacturers.

Statistical analysis of the data was performed by ANOVA followed by the Dunnett's test.

Results

Preliminary studies

Preliminary experiments were carried out to optimize $[Ca^{2+}]_i$ measurement in synaptoneurosomes prepared from 8-d-old rat brain. Synaptoneurosomal $[Ca^{2+}]_i$ was found to be very sensitive to experimental conditions. Incubation of this preparation at 37°C for periods longer than 30 min had deleterious effects, as indicated by an irreversible increase in $[Ca^{2+}]_i$ to more than 2 μM . For this reason, the Fura 2 loading period had to be as brief as was compatible with a good loading and hydrolysis of the dye to provide the $[Ca^{2+}]_i$ -sensitive acid form. A good compromise was obtained by using a loading period of 10 min. After a 10 min incubation at 37°C, Fura 2 free-acid content (measured by comparison with a standard solution of Fura 2 acid) was $\sim 0.25 \pm 0.03$ nmol/mg protein, and basal $[Ca^{2+}]_i$ was $\sim 225 \pm 20$ nM (mean of 70 preparations). There was a negligible synaptoneurosomal autofluorescence (<3% of the loaded Fura 2 fluorescence). When loaded synaptoneurosomes were diluted and kept at room temperature under an O_2/CO_2 atmosphere, $[Ca^{2+}]_i$ increased only very slowly (40% and 75% increase after 2 and 3 hr, respectively), and the sensitivity to neurotransmitters and drugs was maintained for a least 3 hr. When the excitation wavelength was set at 380 nm (at which Fura 2 fluorescence is negatively related to $[Ca^{2+}]_i$), the basal $[Ca^{2+}]_i$ and the effect of KCl and L-glutamate on $[Ca^{2+}]_i$ were similar to those observed under standard (340 nm excitation wavelength) conditions.

Having established the optimal conditions for measuring $[Ca^{2+}]_i$ in synaptoneurosomes, further experiments were carried out to validate our model. When synaptoneurosomes were depolarized by the addition of KCl (30 mM), there was a very rapid increase in $[Ca^{2+}]_i$, which was maximal within less than 2 sec (Fig. 1). This increase was dependent on potassium concentration over the range of 0–50 mM; there was a 5-fold increase in $[Ca^{2+}]_i$ at the highest KCl concentration added (50 mM).

Veratridine, which depolarizes cells by opening sodium channels, also elicited an elevation of $[Ca^{2+}]_i$ (Table 1) but with a slower time course ($t_{1/2} = 3.2$ min at 10 μM veratridine). The effect of veratridine (10 μM) was totally prevented by previous addition of TTX (1 μM). At this concentration, TTX did not block the effect of 30 mM KCl on $[Ca^{2+}]_i$ but caused a small (statistically significant) potentiation of the KCl response (+22%, $p < 0.05$; Table 1).

KCl and veratridine effects on $[Ca^{2+}]_i$ were dependent on the external Ca^{2+} concentration. They were abolished when external levels were reduced to <0.1 μM by adding 0.2 mM EGTA to a medium without added Ca^{2+} (results not shown). The increase in $[Ca^{2+}]_i$ elicited by KCl (30 mM) was partially prevented by a calcium channel blocker of the dihydropyridine class, PN 200-

Figure 1. Effect of KCl-induced depolarization on synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain. The traces are representative of 4 experiments in which KCl was added from a 3 M solution. Note that the scale for $[Ca^{2+}]_i$ is sigmoidal. The calcium channel blocker PN 200-110 was added in a final volume of 3 μ l of ethanol 1 min before KCl. An equivalent volume of ethanol was without effect on KCl-induced $[Ca^{2+}]_i$ rise.

110 (Höf et al., 1984) with an IC_{50} of 0.15 μ M for the sensitive part of the curve (maximal inhibition 55% at 3 μ M; (Figs. 1 and 2, Table 1). KCl-induced elevation of $[Ca^{2+}]_i$ was slightly augmented (+24%, $p < 0.05$) by the previous addition of 100 nM of the calcium channel agonist BAY K 8644 (results not shown). A more detailed account of the modulation of calcium permeability by drugs of the dihydropyridine class will be published elsewhere.

$[Ca^{2+}]_i$ was also raised by the calcium ionophore A23187, which elicited a concentration-dependent increase in $[Ca^{2+}]_i$ ($\Delta = 150$ nM at 10 nM A23187, $\Delta = 400$ nM at 30 nM A23187, and $\Delta = 2500$ nM at 100 nM A23187). A transient increase in $[Ca^{2+}]_i$ was still detected after the addition of A23187 (30 nM) to a Ca^{2+} -free medium (0.2 mM EGTA added to a medium without added Ca^{2+}) (results not shown).

Similar basal $[Ca^{2+}]_i$ were observed in synaptoneurosomes prepared from brains of 3-month-old rats. In this preparation $[Ca^{2+}]_i$ was increased by KCl addition to a similar extent as that observed in 8-d-old rats (results not shown).

Effect of activation of receptors for excitatory amino acids on whole-brain synaptoneurosomal $[Ca^{2+}]_i$

L-Glutamate elicited a concentration-dependent increase in synaptoneurosomal $[Ca^{2+}]_i$ from 8-d-old rat brain (Fig. 3). This effect was concentration dependent over the range of 0.1–1000 μ M (EC_{50} 2.9 μ M; Fig. 4). The L-glutamate-induced increase was very fast, being complete within less than 3 sec at 100 μ M and slightly slower at lower L-glutamate concentrations (Fig. 3). At low L-glutamate concentrations (1 μ M), a lag period of 4 sec was observed between addition of the compound and the onset of $[Ca^{2+}]_i$ increase (Fig. 3). A moderate increase in $[Ca^{2+}]_i$ was also observed after addition of the muscarinic agonist carbachol. However, even at the most active concentration (10 μ M), the

Figure 2. Partial blockade by the calcium channel blocker PN 200-110 of the rise in synaptoneurosomal $[Ca^{2+}]_i$ induced by KCl. The points represent the means $\pm$ SEM of 4 experiments in which PN 200-110 was added 1 min before KCl (30 mM). Experimental conditions were as in Figure 1.

magnitude of the increase in $[Ca^{2+}]_i$ was much lower and the kinetics of the response was slower than those elicited by L-glutamate (Fig. 3).

The glutamate response was stereospecific, the D-isomer being 47 times less potent ($EC_{50} = 95$ μ M) than the L-isomer (Fig. 4). Moreover, the maximal effect elicited by 3 mM D-glutamate (highest concentration assayed) was smaller than that elicited by 100 μ M L-glutamate (Fig. 4).

L-Aspartate also caused an increase in synaptoneurosomal $[Ca^{2+}]_i$ (Figs. 3 and 4). However, the L-aspartate (100 μ M) response displayed biphasic kinetics, with an initial fast increase ($\Delta[Ca^{2+}]_i = 25$ nM) followed by a slow increase ($\Delta = 48$ nM) that reached a plateau 2.5 min after the addition of the amino acid (Fig. 3). This late slow increase phase was not observed after the addition of L-glutamate.

Other agonists with preferential affinity for the excitatory amino acid receptor subtypes were also tested (Figs. 3, 5). Quisqualate was the most active, eliciting a detectable increase in

Table 1. Effects of TTX and PN 200-110 on the increases in $[Ca^{2+}]_i$ elicited by KCl, veratridine, and L-glutamate in immature rat brain synaptoneurosomes

Depolarizing agent	$\Delta[Ca^{2+}]_i$ (nM)		
	—	TTX (1 μ M)	PN 200-110 (1 μ M)
KCl (30 mM)	220 $\pm$ 12	268 $\pm$ 14 ^a	108 $\pm$ 7 ^b
Veratridine (10 μ M)	280 $\pm$ 9	21 $\pm$ 4 ^b	—
L-Glutamate (100 μ M)	65 $\pm$ 3	67 $\pm$ 3	63 $\pm$ 4

Results are means $\pm$ SEM of data obtained from at least 4 experiments. TTX or PN 200-110 were added 1 min before the depolarizing agent.

^a $p < 0.05$.

^b $p < 0.001$ compared with the effect observed in the absence of the ionic channel blockers.

Figure 3. Effects of excitatory amino acids and carbachol on synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain. The traces are representative of 4 experiments in which agonists were added in a volume of 3 μ l of a concentrated solution. Note that the scale for $[Ca^{2+}]_i$ is sigmoidal. Note that both maximal increase and rate of increase are dependent on L-glutamate concentration and that there was a lag period between the addition of 1 μ M L-glutamate and the onset of the response. A lag time was also observed for carbachol. (Note the different time scale for carbachol, as well as for L-aspartate.)

synaptoneurosomal $[Ca^{2+}]_i$ at concentrations as low as 1 nM and a maximal effect at 1 μ M. The order of potency of the agonists in increasing $[Ca^{2+}]_i$ was as follows (EC_{50} , μ M within parentheses): quisqualate (0.05) > ibotenate (0.53) > L-glutamate (2) > AMPA (3.2) > NMDA (11) $\approx$ kainate (12). Ibotenate and AMPA displayed a potency similar to that of L-glutamate, but their maximal effects, as well as that of quisqualate, were always smaller than those found with L-glutamate. Addition of L-glutamate after these agonists did not produce a further increase in $[Ca^{2+}]_i$ (not shown). NMDA and kainate effects were detected only at high concentrations, and they elicited smaller increases in $[Ca^{2+}]_i$. The various excitatory amino acid receptor agonists failed to increase $[Ca^{2+}]_i$ in synaptoneurosomes prepared from 3-month-old rats.

Figure 4. Effects of D- and L-glutamate and L-aspartate on synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain. Compounds were added in a final volume of 3 μ l of a concentrated solution. Only the fast phase of the L-aspartate effect was considered. Each point represents the mean of 4 experiments with SEM of less than 10%. Error bars were omitted for the sake of clarity.

The L-glutamate (30 μ M) response in the immature rat brain was almost totally antagonized by GDEE (1 mM) and partially antagonized by 1 mM of GAMS (Table 2). γ -glutamyltaurine, secobarbital, γ -glutamylglycine, L- and D-serine O-phosphate, DL-2-aminophosphonobutyrate, and DL-2-aminophosphonovalerate were inactive up to 1 mM (Table 2).

Effects of L-glutamate on $[Ca^{2+}]_i$ in different brain areas of the immature rat

L-Glutamate (100 μ M) increased synaptoneurosomal $[Ca^{2+}]_i$ in several brain regions of the immature rat. Among the tested brain structures, the highest response was detected in the cerebral cortex followed by the striatum, hippocampus, and cerebellum (Fig. 6).

Table 2. Effects of putative antagonists of excitatory amino acid receptors on the L-glutamate-induced increase in synaptoneurosomal $[Ca^{2+}]_i$ in immature rat brain

Drug	% L-Glutamate response
GDEE (300 μ M)	100 $\pm$ 6 (5)
GDEE (1 mM)	46 $\pm$ 5 (3) ^a
GAMS (300 μ M)	25 $\pm$ 8 (3) ^a
GAMS (1 mM)	95 $\pm$ 4 (3) ^c
Secobarbital (1 mM)	71 $\pm$ 11 (4) ^b
2-Amino-phosphonobutyrate (1 mM)	92 $\pm$ 7 (4) ^c
γ -Glutamyltaurine (1 mM)	108 $\pm$ 4 (3) ^c
2-Aminophosphonovalerate (300 μ M)	77 $\pm$ 9 (3) ^c
2-Aminophosphonovalerate (1 mM)	101 $\pm$ 4 (3) ^c
L-Serine-O-phosphate (1 mM)	115 $\pm$ 3 (3) ^b
D-Serine-O-phosphate (1 mM)	103 $\pm$ 5 (3) ^c
D-Serine-O-phosphate (1 mM)	97 $\pm$ 5 (3) ^c

Results are means $\pm$ SEM of data obtained in the number of experiments indicated in parentheses. Drugs were added 1 min before L-glutamate (30 μ M).

^a $p < 0.001$.

^b $p < 0.05$.

^c Not significant vs. L-glutamate alone.

Figure 5. Effects of compounds with preferential affinity for the excitatory amino acid receptor subtypes on synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain. The compounds were added in a final volume of 3 μ l of a concentrated solution. Each point represents the mean of at least 3 experiments with SEM of less than 10%. Error bars were omitted for the sake of clarity.

Ionic dependence of the L-glutamate effect on whole-brain synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain

At Ca^{2+} concentrations lower than 0.1 μ M (obtained by adding 0.2 mM EGTA to a nominally Ca^{2+} -free medium), L-glutamate (100 μ M) failed to alter synaptoneurosomal $[Ca^{2+}]_i$ (Fig. 7). When EGTA was omitted (external $[Ca^{2+}]$ measured with Fura 2 $\approx$ 1.8 μ M), the effect of L-glutamate on $[Ca^{2+}]_i$ became apparent. Further increases in external Ca^{2+} resulted in larger L-glutamate effects, which plateaued at Ca^{2+} concentrations higher than 1.2 mM (Fig. 7). A half-maximal response to L-glutamate (100 μ M) was observed at an external Ca^{2+} concentration of 240 μ M. Basal $[Ca^{2+}]_i$ was also influenced by external Ca^{2+} ; it increased from 150 nM in the presence of 0.2 mM EGTA (external $[Ca^{2+}] < 0.1 \mu$ M) to 300 nM when external $[Ca^{2+}]$ amounted to 2.4 mM. The L-glutamate response was not modified by omitting Mg^{2+} from the medium or by increasing its concentration to 2.4 mM (not shown).

PN 200-110, added at a concentration of 1 μ M, which maximally blocked the KCl (30 mM)-induced rise in $[Ca^{2+}]_i$, did not affect the L-glutamate (100 μ M) response (Table 1). Moreover, the L-glutamate (100 μ M)-induced increase in $[Ca^{2+}]_i$ was not affected by previous addition of TTX (1 μ M) to the medium (Table 1).

Isosmotic substitution of Na^+ by choline, while greatly in-

creasing basal $[Ca^{2+}]_i$, was without effect on the $[Ca^{2+}]_i$ increase elicited by 100 μ M L-glutamate (Fig. 8).

The $[Ca^{2+}]_i$ increases elicited by KCl (30 mM) and L-glutamate (100 μ M) were supra-additive. Although mutual potentiating effects were observed whichever reagent was added first, $[Ca^{2+}]_i$ reached higher levels when L-glutamate was added before KCl (Table 3). In contrast, the increase in $[Ca^{2+}]_i$ elicited by NMDA was not potentiated by previous depolarization with KCl. Thus, the increase in $[Ca^{2+}]_i$ elicited by 100 μ M NMDA was 23 ± 4 nM in control conditions and 21 ± 5 nM when this agonist was added to a preparation previously depolarized by 30 mM KCl. This NMDA effect was also unaffected when the synaptoneurosomes were resuspended in a Mg^{2+} -free medium.

Discussion

The present study demonstrates the feasibility of using the fluorescent dye Fura 2 to monitor basal and drug- or neurotransmitter-induced changes in $[Ca^{2+}]_i$ in synaptoneurosomes from the immature rat brain. A drawback of synaptoneurosomes, compared with cultured neurons, is their poorer stability and probable heterogeneity in size and in surface receptors; however, the fact that it is an easily obtained preparation that may reflect

Table 3. Supra-additivity of the L-glutamate and KCl-induced elevation of synaptoneurosomal $[Ca^{2+}]_i$ in immature rat brain

$\Delta[Ca^{2+}]_i$ (nM)		Total $\Delta[Ca^{2+}]_i$ (nM)
1st addition	2nd addition	
L-Glutamate (100 μ M)	KCl (30 mM)	286.5 ± 2.4
63.1 ± 4.9	224.2 ± 9.4^b	
KCl (30 mM)	L-Glutamate (100 μ M)	250.0 ± 7.5
167.2 ± 5.1	84.2 ± 4.4^a	

Experiments were carried out as shown in Figures 1 and 3. The time elapsed between the first and second addition was 2 min. Results are means $\pm$ SEM of data from triplicate experiments. Note that the arithmetic addition of the separate effects of each agent ($63.1 + 167.2$ nM = 230.3 nM) was smaller than the effect of the 2 agents added sequentially. These differences were significant at $p < 0.05$.

^a $p < 0.05$ vs. $\Delta[Ca^{2+}]_i$ obtained when L-glutamate was added first.

^b $p < 0.01$ vs. $\Delta[Ca^{2+}]_i$ obtained when KCl was added first.

Figure 6. Effect of L-glutamate (100 μ M) on $[Ca^{2+}]_i$ in synaptoneurosomes prepared from several brain regions of the immature rat. Values are means $\pm$ SEM of triplicate experiments from 2 different preparations.

Figure 7. Influence of external $[Ca^{2+}]_i$ on basal synaptoneurosomal $[Ca^{2+}]_i$ and on the increase in $[Ca^{2+}]_i$ elicited by L-glutamate in the immature rat brain. Synaptoneurosomes were resuspended in Krebs-HEPES buffer containing the indicated $CaCl_2$ concentrations. Basal $[Ca^{2+}]_i$ were measured after a stable fluorescence level was recorded (approximately 5 min after resuspension) and L-glutamate ($100 \mu M$) was then added. Values are the means $\pm$ SEM of triplicate experiments. *, Without added Ca^{2+} (calculated external $[Ca^{2+}] = 1.8 \mu M$). +, In the presence of $0.2 mM$ EGTA and without added Ca^{2+} .

more closely *in vivo* conditions makes it a good experimental model for the study of Ca^{2+} homeostasis in the CNS. Although basal $[Ca^{2+}]_i$ was slightly higher than that reported for intact cells, it remained sufficiently low to allow studying the effects of neurotransmitters and drugs on $[Ca^{2+}]_i$ homeostasis. It is possible that the observed $[Ca^{2+}]_i$ represents the mean of tightly sealed synaptoneurosomes able to maintain $[Ca^{2+}]_i$ at physiological levels and of vesicles completely permeable to Ca^{2+} . Despite this, synaptoneurosomes have a remarkable ability to maintain a 5000-fold gradient of $[Ca^{2+}]_i$.

The validity of our model has been demonstrated by its sensitivity to agents (e.g., veratridine, A23187, and KCl) known to affect neuronal Ca^{2+} homeostasis. The marked rise in synaptoneurosomal $[Ca^{2+}]_i$ observed following KCl-induced depolarization indicates that voltage-sensitive Ca^{2+} channels are functional in this preparation. This view is substantiated by the

partial blockade of the KCl-induced increase in $[Ca^{2+}]_i$ by the dihydropyridine Ca^{2+} channel blocker PN 200-110 and by the small (but significant) potentiation of the KCl response by the Ca^{2+} channel agonist BAY K 8644. These results are in agreement with previous studies demonstrating that the early phase of Ca^{2+} uptake by KCl-depolarized synaptosomes was partially blocked by nifedipine, nimodipine, verapamil, and diltiazem (Turner and Goldin, 1985) and was stimulated by the Ca^{2+} channel activator BAY K 8644 (Woodward and Leslie, 1986). As the blocking effect of PN 200-110 reaches a plateau of inhibition at concentrations $> 3 \mu M$, it is possible that the KCl-induced rise in $[Ca^{2+}]_i$ is also partly mediated by other permeation systems, such as the dihydropyridine-insensitive Ca^{2+} channels (Nowicky et al., 1985; Hirming et al., 1986) and/or the Na^+/Ca^{2+} antiport (Gill et al., 1981). The existence of a Na^+/Ca^{2+} antiport system in synaptoneurosomes is further supported by the increase in $[Ca^{2+}]_i$ observed when choline was substituted for Na^+ in the external medium.

Internal Ca^{2+} levels were increased in a dose-dependent fashion by L-glutamate. The effect of this amino acid occurred in the concentration range expected for an interaction with a synaptic receptor. A possible metabolic effect of glutamate at the mitochondrial level (Harris, 1985) can be dismissed since the addition of D-glutamate, which is not a substrate for glutamate dehydrogenase but possesses excitatory amino acid receptor agonist properties, also provoked a $[Ca^{2+}]_i$ elevation.

The high potency of quisqualate at increasing $[Ca^{2+}]_i$ (minimal effective concentration $1 nM$) and the order of potency of the various excitatory amino acid agonists assayed suggest that the effect of L-glutamate on $[Ca^{2+}]_i$ is primarily mediated by the quisqualate-preferring receptor, as previously defined by electrophysiological (Watkins and Evans, 1981; Gardette and Crepel, 1986) and binding studies (Honoré et al., 1982; Krogsgaard-Larsen and Honoré, 1983; Fagg and Matus, 1984; Greenamyre et al., 1985; Monaghan et al., 1985) and by phosphatidylinositol turnover activation (Sladeczek et al., 1985; Nicoletti et al., 1986b). The higher potency of L-glutamate compared with L-aspartate and the 47-fold difference in potency between L- and D-glutamate are also consistent with this view.

Among all the compounds previously proposed as antagonists of the quisqualate receptor, only GDEE and GAMS (Davies and

Figure 8. Effect of isosmotic substitution of Na^+ by choline on basal and L-glutamate-induced increase in synaptoneurosomal $[Ca^{2+}]_i$ in the immature rat brain. Synaptoneurosomes were resuspended in Krebs-HEPES buffer containing the indicated concentration of Na^+ . Basal $[Ca^{2+}]_i$ were measured after a stable fluorescence level was recorded (approximately 5 min after resuspension) and L-glutamate ($100 \mu M$) was then added. Values are the means $\pm$ SEM of triplicate experiments. *, $p < 0.05$ compared with $143 mM$ Na^+ buffer.

Watkins, 1985) displayed antagonistic activity at high concentrations against L-glutamate $[Ca^{2+}]_i$ response. The fact that 2 specific blockers of NMDA receptor (2-aminophosphonovale and $MgCl_2$) failed to antagonize the L-glutamate response indicates that its effect is not mediated by NMDA receptors. The low potency and efficacy of kainate dismiss, in turn, the involvement of a kainate receptor in the glutamate-induced increase in $[Ca^{2+}]_i$.

It is somewhat surprising that NMDA and kainate induced only very small increases in $[Ca^{2+}]_i$. These compounds have previously been demonstrated to be extremely potent in activating Ca^{2+} uptake in brain slices (Berdichevsky et al., 1983), and studies with cultured neurons have also shown that NMDA-elicited increases in $[Ca^{2+}]_i$ were at least of the same magnitude as those elicited by quisqualate (Kudo and Ogara, 1986; Wroblewski et al., 1985). The failure of NMDA to increase $[Ca^{2+}]_i$ in synaptoneurosomes is not due to a blockade by Mg^{2+} of the NMDA receptor-associated channel (Nowak et al., 1984) since NMDA was not more effective in the absence of Mg^{2+} and the NMDA effect was not potentiated by previous depolarization with KCl, which is known to overcome the Mg^{2+} blockade of the NMDA receptor (Nowak et al., 1984). Neither can the low efficacy of NMDA be attributed to the absence of NMDA receptors in synaptoneurosomes as they could be detected in this preparation by measuring the binding of the NMDA receptor antagonist (Loo et al., 1986) 3H -TCP (120 fmol/mg protein at 2.5 nM TCP; J. Benavides et al., unpublished observations). Other explanations for the weak effects of NMDA and kainate on synaptoneurosomal $[Ca^{2+}]_i$ are that (1) there is an uncoupling of the NMDA and kainate receptors in this preparation or (2) sealed compartments in the synaptoneurosomes preparation are predominantly presynaptic.

One of the primary goals of the present study was to clarify the mechanism whereby quisqualate receptor activation leads to an increase in $[Ca^{2+}]_i$. The quisqualate-induced rise in $[Ca^{2+}]_i$ may be connected to activation of receptor-operated Ca^{2+} channels or may be subsequent to stimulation of biochemical processes (e.g., increase in phosphatidylinositol hydrolysis) or changes in membrane permeability to other cations. Another possibility is that the observed increases in $[Ca^{2+}]_i$ result from liberation of an unknown transmitter(s), which in turn increases $[Ca^{2+}]_i$. This latter possibility is, however, unlikely since (1) the L-glutamate effect on $[Ca^{2+}]_i$ was TTX insensitive and (2) similar increases in $[Ca^{2+}]_i$ could be observed at synaptoneurosomal protein concentrations ranging from 1.5 to 0.05 mg/ml in the cuvette.

The fast kinetics of the L-glutamate response, as well as its dependence on external Ca^{2+} suggests that the amino acid-induced increase in $[Ca^{2+}]_i$ is effected via an increase in plasma membrane permeability to Ca^{2+} . Ca^{2+} permeability increases seem to be operated by a channel specific for this cation since decreases in external Na^+ concentration, while increasing $[Ca^{2+}]_i$, failed to alter the ability of L-glutamate to increase $[Ca^{2+}]_i$. As this elevation could still be detected in the absence of Na^+ in the external medium and was not blocked by TTX, it can also be concluded that the L-glutamate effect on $[Ca^{2+}]_i$ is not mediated by the activation of a Na^+ channel which will subsequently activate Ca^{2+} entry mechanisms (i.e., voltage-sensitive Ca^{2+} channels, Ca^{2+}/Na^+ antiport). Furthermore, the failure of PN 200-110 to affect the L-glutamate-induced rise of $[Ca^{2+}]_i$ demonstrates that L-glutamate does not activate dihydropyridine-sensitive voltage-dependent Ca^{2+} channels. Nevertheless,

the facts that previous addition of L-glutamate potentiates the KCl effect on $[Ca^{2+}]_i$ and that prior depolarization by KCl increases the L-glutamate response favor the view that there is a coupling between the quisqualate receptor and a dihydropyridine-resistant (voltage-dependent) Ca^{2+} channel. However, a linkage of the quisqualate-operated receptor with the previously described dihydropyridine-resistant Ca^{2+} channels (Hirning et al., 1986) cannot be deduced with certainty from the present results. The role of the different types of Ca^{2+} channels in the mediation of the effects of excitatory amino acid agonists has been discussed extensively by Carter et al. (1987).

The involvement of the quisqualate receptor in the activation of phosphatidylinositol (PI) hydrolysis in cultured neurons and slices is now well documented (Sladeczek et al., 1985; Nicoletti et al., 1986a, b). A coupling between quisqualate-type receptors and inositol phospholipid metabolism has also been recently reported in synaptoneurosomes from the immature rat brain (Recasens et al., 1987). As the increase in $[Ca^{2+}]_i$ and the activation of PI hydrolysis elicited by excitatory amino acids display a similar pharmacological specificity, a link could exist between both phenomena. The PI hydrolysis product inositol triphosphate (IP_3) could provide such a link by mobilizing Ca^{2+} from internal stores, as has been proposed for other neurotransmitter and hormone receptors (Wollheim and Biden, 1986). However, the strict dependence of L-glutamate-induced increase in $[Ca^{2+}]_i$ on external $[Ca^{2+}]$ indicates a minor contribution, if any, of Ca^{2+} mobilization from internal stores. This view is supported by the fact that, in contrast to the L-glutamate-induced increase in $[Ca^{2+}]_i$, the L-glutamate-induced activation of PI hydrolysis in hippocampal slices (Y. Claustre et al., unpublished observations) and in synaptoneurosomes (Recasens et al., 1987) can still be detected in the absence of external Ca^{2+} . That Ca^{2+} mobilization is not mediated by an increase in PI turnover is also supported by the differential regional distribution of the stimulating effects of L-glutamate on synaptoneurosomal $[Ca^{2+}]_i$ (cortex > striatum > hippocampus > cerebellum) and on PI turnover in slices (striatum > hippocampus > cortex > cerebellum) (Nicoletti et al., 1986a, b). However, the possibility remains that the $[Ca^{2+}]_i$ increases observed at external Ca^{2+} concentrations between 1.8 and 100 μM (the range at which the L-glutamate effect appears insensitive to changes in external $[Ca^{2+}]$) may be mediated, at least in part by an increase in IP_3 formation.

The dependence of the L-glutamate-elicited increase in $[Ca^{2+}]_i$ on external $[Ca^{2+}]$, together with the fast kinetics of the L-glutamate response, strongly suggests a direct coupling between the glutamate receptor and the increase in $[Ca^{2+}]_i$ (possibly via the operation of a Ca^{2+} channel). Thus, the observed effects of L-glutamate on $[Ca^{2+}]_i$ are most probably the counterpart of the rapid membrane events detected electrophysiologically. In turn, PI turnover activation may mediate slower and more permanent synaptic changes (see Fagg et al., 1986). Alternatively, the increases in PI turnover elicited by L-glutamate may be, at least in part, the consequence (and not the cause) of the increase in $[Ca^{2+}]_i$ (Majerus et al., 1985). This view is supported by the fact that carbachol, a muscarinic agonist that in brain slices readily activates PI turnover (Jacobson et al., 1985) only elicited a very small increase in synaptoneurosomal $[Ca^{2+}]_i$.

Another point that deserves some comment is the inability of L-glutamate to increase $[Ca^{2+}]_i$ in synaptoneurosomes prepared from adult rats. Similar developmental changes have been noted for the NMDA receptor-mediated increases in cyclic

guanosyl 3',5'-monophosphate levels (Garthwaite and Balazs, 1978) and quisqualate receptor-mediated activation of PI turnover (Nicoletti et al., 1986a) and have been attributed to ontogenetic changes in the coupling between excitatory amino acid receptors and effector mechanisms (Nicoletti et al., 1986a) or in the capacity of the excitatory amino acid uptake systems (Garthwaite, 1985). The large dilution of the synaptoneurosomes in our experimental conditions (0.2 mg protein/ml) and the fact that excitatory amino acid agonists which are not taken up (e.g., ibotenate and quisqualate) also failed to increase $[Ca^{2+}]_i$ in the adult synaptoneurosomal preparation suggest that these developmental differences are unlikely to be related to changes in excitatory amino acid uptake capacity.

In conclusion, the results of the present study suggest that quisqualate-type receptors are positively coupled to dihydropyridine-resistant Ca^{2+} channels in immature rat brain synaptoneurosomes. The model depicted in this study provides a convenient and sensitive tool for investigating the effects of neurotransmitters or drugs on $[Ca^{2+}]$ homeostasis in brain and will be of help in the characterization of more potent and specific quisqualate receptor agonists and antagonists.

References

- Berdichevsky, E., N. Riveros, S. Sanchez-Armas, and F. Orrego (1983) Kainate, N-methylaspartate and other excitatory amino acids increase calcium influx into rat brain cortex cells in vitro. *Neurosci. Lett.* 36: 75–80.
- Carter, C., F. Noel, and B. Scatton (1987) Ionic mechanisms implicated in the stimulation of cerebellar cyclic GMP levels by N-methyl-D-aspartate. *J. Neurochem.* 49: 195–200.
- Davies, J., and J. C. Watkins (1985) Depressant actions of γ -D-glutamyl-aminomethyl sulfonate (GAMS) on amino acid-induced and synaptic excitation in the cat spinal cord. *Brain Res.* 337: 113–120.
- Dingledine, R. (1983) N-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells. *J. Physiol. (Lond.)* 343: 385–405.
- Ebstein, R. P., and J. W. Daly (1982a) Release of norepinephrine and dopamine from brain vesicular preparations: Effects of adenosine analogues. *Cell. Mol. Neurobiol.* 2: 193–204.
- Ebstein, R. P., and J. W. Daly (1982b) Release of norepinephrine and dopamine from brain vesicular preparations: Effects of calcium antagonists. *Cell. Mol. Neurobiol.* 2: 205–213.
- Ebstein, R. P., K. Seamon, C. R. Creveling, and J. W. Daly (1982) Release of norepinephrine from brain vesicular preparations: Effects of an adenylate cyclase activator, forskolin, and a phosphodiesterase inhibitor. *Cell. Mol. Neurobiol.* 2: 179–192.
- Fagg, G. E., and A. Matus (1984) Selective association of N-methyl aspartate and quisqualate types of L-glutamate receptor with brain postsynaptic densities. *Proc. Natl. Acad. Sci. USA* 81: 6876–6880.
- Fagg, G. E., A. C. Foster, and A. H. Ganong (1986) Excitatory amino acid synaptic mechanisms and neurological function. *Trends Pharmacol. Sci.* 7: 357–363.
- Gardette, R., and F. Crepel (1986) Chemosensitiveness of intracerebellar nuclei neurones to L-aspartate, L-glutamate and related derivatives in rat cerebellar slices maintained in vitro. *Neuroscience* 18: 93–100.
- Garthwaite, J. (1985) Cellular uptake disguises action of L-glutamate on N-methyl-D-aspartate receptors. *Br. J. Pharmacol.* 85: 297–307.
- Garthwaite, J., and R. Balazs (1978) Supersensitivity of cyclic GMP response to glutamate during cerebellar maturation. *Nature* 275: 328–329.
- Garthwaite, J., G. Garthwaite, and F. Hajos (1986) Amino acid neurotoxicity: Relationship to neuronal depolarization in rat cerebellar slices. *Neuroscience* 18: 449–460.
- Gill, D. L., E. F. Grollman, and L. D. Kohn (1981) Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. *J. Biol. Chem.* 256: 184–192.
- Greenamyre, J. T., J. M. M. Olson, J. B. Penney, Jr., and A. B. Young (1985) Autoradiographic characterization of N-methyl-D-aspartate-, quisqualate- and kainate-sensitive glutamate binding sites. *J. Pharmacol. Exp. Ther.* 233: 254–263.
- Gryniewicz, G., M. Poenie, and R. Y. Tsien (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440–3450.
- Harris, R. A. (1985) Effects of excitatory amino acids on calcium transport by brain membranes. *Brain Res.* 337: 167–170.
- Hirning, L. D., A. P. Fox, E. W. McCleskey, R. J. Miller, B. M. Olivera, S. A. Thager, and R. W. Tsien (1986) Dominant role of N-type calcium channels in K^{+} -evoked release of norepinephrine from sympathetic neurons. *Soc. Neurosci. Abstr.* 13: 12.8.
- Höf, R. P., G. Scholtysik, R. Loutzenhiser, H. J. Vuorela, and P. Neumann (1984) A new calcium antagonist: Electrophysiological, inotropic, and chronotropic effects on guinea pig myocardial tissue and effects on contraction and calcium uptake of rabbit aorta. *J. Cardiovasc. Pharmacol.* 6: 399–406.
- Hollingsworth, E. B., E. T. McNeal, J. L. Burton, R. J. Williams, J. W. Daly, and C. R. Creveling (1985) Biochemical characterization of a filtered synaptoneurosomal preparation from guinea pig cerebral cortex: Cyclic adenosine 3':5'-monophosphate-generating systems, receptors, and enzymes. *J. Neurosci.* 5: 2240–2253.
- Hollingsworth, E. B., E. B. Sears, R. A. de la Cruz, F. Gusovsky, and J. W. Daly (1986) Accumulations of cyclic AMP and inositol phosphates in guinea pig cerebral cortical synaptoneurosomes: Enhancement by agents acting at sodium channels. *Biochim. Biophys. Acta* 883: 15–25.
- Honoré, T., J. Lauridsen, and P. Krogsgaard-Larsen (1982) The binding of $[^3H]$ AMPA, a structural analogue of glutamic acid, to rat brain membranes. *J. Neurochem.* 38: 173–178.
- Jacobson, M. D., F. Wusteman, and C. P. Downes (1985) Muscarinic receptors and hydrolysis of inositol phospholipids in rat cerebral cortex and parotid gland. *J. Neurochem.* 44: 465–472.
- Krogsgaard-Larsen, P., and T. Honoré (1983) Glutamate receptor and new glutamate agonists. *Trends Pharmacol. Sci.* 4: 31–33.
- Kudo, Y., and A. Ogura (1986) Glutamate-induced increase in intracellular Ca^{2+} concentration in isolated hippocampal neurones. *Br. J. Pharmacol.* 89: 191–198.
- Lazarewicz, J. W., A. Lehmann, H. Hagberg, and A. Hamberger (1986) Effects of kainic acid on brain calcium fluxes studied in vivo and in vitro. *J. Neurochem.* 46: 494–498.
- Loo, P., A. Braunwalder, J. Lehmann, and M. Williams (1986) Radioligand binding to central phencyclidine recognition sites is dependent on excitatory amino acid receptor agonists. *Eur. J. Pharmacol.* 123: 467–468.
- MacDermott, A. B., M. L. Mayer, G. L. Westbrook, S. J. Smith, and J. L. Barker (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature* 321: 519–522.
- Majerus, P. W., D. B. Wilson, T. M. Connolly, T. E. Bross, and E. J. Neufeld (1985) Phosphoinositide turnover provides a link in stimulus-response coupling. *Trends Biochem.* 10: 168–171.
- Monaghan, D. T., D. Yao, and C. W. Cotman (1985) L- $[^3H]$ Glutamate binds to kainate-, NMDA- and AMPA-sensitive binding sites: An autoradiographic analysis. *Brain Res.* 340: 378–383.
- Nicoletti, F., M. J. Iadarola, J. T. Wroblewski, and E. Costa (1986a) Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: Developmental changes and interaction with α_1 -adrenoceptors. *Proc. Natl. Acad. Sci. USA* 83: 1931–1935.
- Nicoletti, F., J. L. Meek, M. J. Iadarola, D. M. Chuang, B. L. Roth, and E. Costa (1986b) Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. *J. Neurochem.* 46: 40–46.
- Nowak, L., P. Bregestovski, P. Ascher, A. Hebert, and A. Prochiantz (1984) Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307: 462–465.
- Nowicky, M. C., A. P. Fox, and R. W. Tsien (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440–446.
- Olney, J. W. (1980) Excitotoxic mechanisms of neurotoxicity. In *Experimental and Clinical Neurotoxicology*, P. S. Spencer and H. H. Schaumburg, eds., pp. 272–294, Williams & Wilkins, Baltimore.
- Pumain, R., and U. Heinemann (1985) Stimulus- and amino acid-induced calcium and potassium changes in rat neocortex. *J. Neurophysiol.* 53: 1–16.
- Récasens, M., I. Sasseti, A. Nourigat, F. Sladeczek, and J. Bockaert (1987) Characterization of subtypes of excitatory amino acid receptors involved in the stimulation of inositol phosphate synthesis in rat brain synaptoneurosomes. *Eur. J. Pharmacol.* 141: 87–93.

- Resch, K., W. Imm, E. Ferber, D. F. K. Wallach, and H. Fischer (1971) Quantitative determination of soluble and membrane protein through their native fluorescence. *Naturwissenschaften* 58: 220.
- Retz, K. C., and J. Coyle (1984) The differential effects of excitatory amino acids on uptake of $^{45}\text{CaCl}_2$ by slices from mouse striatum. *Neuropharmacology* 23: 89–94.
- Riveros, N., and F. Orrego (1986) N-Methylaspartate-activated calcium channels in rat brain cortex slices. Effect of calcium channel blockers and of inhibitory and depressant substances. *Neuroscience* 17: 541–546.
- Schwarcz, R., D. Scholz, and J. T. Coyle (1978) Structure-activity relations for the neurotoxicity of kainic acid derivatives and glutamate analogues. *Neuropharmacology* 17: 147–151.
- Schwartz, R. D., P. Skolnick, E. B. Hollingsworth, and S. M. Paul (1984) Barbiturate and picrotoxin-sensitive chloride efflux in rat cerebral cortical synaptoneuroosomes. *FEBS Lett.* 175: 193–196.
- Schwartz, R. D., J. A. Jackson, D. Weigert, P. Skolnick, and S. M. Paul (1985) Characterization of barbiturate-stimulated chloride efflux from rat brain synaptoneuroosomes. *J. Neurosci.* 5: 2963–2970.
- Sladeczek, F., J.-P. Pin, M. Récasens, J. Bockaert, and S. Weiss (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature* 317: 717–719.
- Turner, T. J., and S. M. Goldin (1985) Calcium channels in rat brain synaptosomes: Identification and pharmacological characterization. *J. Neurosci.* 5: 841–849.
- Watkins, J. C., and R. H. Evans (1981) Excitatory amino acid transmitters. *Annu. Rev. Pharmacol. Toxicol.* 21: 165–204.
- Wollheim, C. B., and T. J. Biden (1986) Second messenger function of inositol 1,4,5-triphosphate. Early changes in inositol phosphates, cytosolic Ca^{2+} , and insulin release in carbamylcholine-stimulated RINm5F cells. *J. Biol. Chem.* 261: 8314–8319.
- Woodward, J. J., and S. W. Leslie (1986) Bay K 8644 stimulation of calcium entry and endogenous dopamine release in rat striatal synaptosomes antagonized by nimodipine. *Brain Res.* 370: 397–400.
- Wroblewski, J. T., F. Nicoletti, and E. Costa (1985) Different coupling of excitatory amino acid receptors with Ca^{2+} channels in primary cultures of cerebellar granule cells. *Neuropharmacology* 24: 919–921.