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Excitatory Amino Acid-Induced Release of ^{3}H-GABA from Cultured Mouse Cerebral Cortex Interneurons

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A newly developed continuous superfusion model was used for studies of ^{3}H-GABA release from cultured mouse cerebral cortex neurons. It was found that a series of excitatory amino acids (EAAs) representing all receptor subtypes evoked Ca^{2+}-dependent release of ^{3}H-GABA from the neurons. Quisqualate was the most potent agonist tested, with an EC_{50} value of 75 nM. L-Glutamate, *N*-methyl-D-aspartate (NMDA), and kainate showed EC_{50} values of 12, 16 and 29 μM, respectively. The EAA-evoked ^{3}H-GABA release could be blocked by a series of EAA antagonists. The highly selective NMDA antagonist D-2-amino-5-phosphonovaleric acid (D-APV) was found to block NMDA responses, whereas the nonselective antagonists *cis*-2,3-piperidine dicarboxylic acid (PDA) and γ-D-glutamyl-aminomethyl sulphonic acid (GAMS) blocked responses to all agonists. NMDA responses were found to be sensitive to Mg^+ blockade. EAA- as well as potassium-induced ^{3}H-GABA release from the neurons could be detected as early as day 5 in culture. However, during the culture period up to 12 d, the responses to K^+, quisqualate, and NMDA were increased. The ontogenetic development of binding sites for quisqualate, kainate, and NMDA in mouse cortex was studied using the radioligands ^{3}H-α-amino-3-hydroxy-5-methyl-4-isoxasole propionate (^{3}H-AMPA), ^{3}H-kainate, and ^{3}H-L-glutamate, respectively. The development of binding sites for the different EAA-receptor subtypes showed a good correlation with the development of neuronal ^{3}H-GABA release evoked by the excitatory amino acids. A study of ^{3}H-L-glutamate binding to membranes prepared from the cultures revealed pharmacological selectivities of the ^{3}H-L-glutamate-binding sites similar to the corresponding binding sites on rat cerebral cortex membranes.

Neuronal cultures obtained from mouse or rat embryo cerebral cortex hemispheres are widely used as model systems for neurochemical and functional studies of the central nervous system (cf. Schousboe et al., 1985). Such neurons have been shown to develop neurites after a few hours *in vitro,* and synaptogenesis and myelinogenesis appear after 5–8 d *in vitro* (Yavin and Yavin, 1974, 1977; Dichter, 1978). Within the first 2 weeks in culture, the cells develop electrophysiological properties similar to those of their *in vivo* counterparts, with resting membrane potentials around 65 mV (Dichter, 1978, 1980; Brodie et al., 1986). The extensive synaptic connections seen in the cultures include both excitatory and inhibitory synapses (Dichter, 1978).

On the basis of electrophysiological (Dichter, 1980) and biochemical (Snodgrass et al., 1980) evidence, it has been suggested that GABA is the main and possibly the exclusive inhibitory transmitter in the cultures. Furthermore, autoradiographic studies on the distribution of the GABA-synthesizing enzyme glutamate decarboxylase (GAD) and of ^{3}H-GABA uptake indicate that at least 50% of the neurons in the cultures are GABAergic (White et al., 1980). Depolarization of the cultured neurons, e.g., by high potassium, has been shown to induce release of ^{3}H-GABA from the neuronal cultures (Snodgrass et al., 1980; Larsson et al., 1983; Yu et al., 1984).

The excitatory transmitters in the cultures are unknown, but evidence from electrophysiological (Stone, 1973, 1976) and release studies (see references in Fagg and Lane, 1979) suggests that excitatory amino acids (EAAs) may be responsible for the excitation in major parts of the cortex. If EAAs are also the excitatory transmitter(s) in the cultured cortex neurons, they should be able to release GABA by depolarizing the GABAergic interneurons. In order to clarify whether the cultured mouse cortex neurons respond to EAAs, we investigated both the possible presence of ^{3}H-L-glutamate-binding sites in the cultures and whether ^{3}H-GABA would be released from the cultures by stimulation with EAAs. For the release studies, a newly developed continuous superfusion model was used.

Materials and Methods

Materials. NMRI mice, from embryonic day 17 to postnatal day 7 in age, were obtained from animal quarters at Ferrosan Ltd. Plastic tissue culture dishes were purchased from NUNC A/S (Denmark), and Dulbecco's minimum essential medium (DMEM) and horse serum were purchased from Gibco/Biocult Lab. (Scotland). Quisqualate, *N*-methyl-D-aspartate (NMDA), kainate, kynurenic acid, glutamate diethylester (GDEE), *cis*-2,3-piperidine dicarboxylic acid (PDA), γ-vinyl-γ-aminobutyric acid (γ-vinyl-GABA), cytosine arabinoside, poly-L-lysine, trypsin, soybean trypsin inhibitor, and DNAse were obtained from Sigma Chemical Co., St. Louis, MO; D-2-amino-5-phosphonovaleric acid (D-APV), DL-2-amino-7-phosphonoheptaoic acid (DL-APH), γ-D-glutamyl-aminomethyl sulphonic acid (GAMS) from Tocris Neuramin, (UK); penicillin from Leo (Denmark); and insulin from NOVO (Denmark); and ^{3}H-GABA (sp act, 71 Ci/mmol), ^{3}H-α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (^{3}H-AMPA) (sp act, 25 Ci/mmol), ^{3}H-kainate (sp act, 60 Ci/mmol), and ^{3}H-L-glutamate (sp act, 41.5 Ci/mmol) were obtained from NEN (Frankfurt, FRG). Amino-2-trifluormethoxy-6-benzothiazole (PK 26124) was a gift from Pharmuka (Gennevilliers, France). All other chemicals were of the purest grade available from regular commercial sources.

Cell cultures. Cerebral cortex neurons were cultured essentially as described by Yavin and Yavin (1974), with minor modifications (Larsson et al., 1983). In brief, the cerebral hemispheres were removed from 16-d-old mouse fetuses, cut into cubes of 0.4 × 0.4 mm, dissociated

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by mild trypsinization [0.1% (wt/vol) trypsin at 37°C for 15 min], and subsequently inoculated into poly-L-lysine-coated 3 cm petri dishes containing slightly modified DMEM (24.5 mM KCl, 30 mM glucose) supplemented with *p*-aminobenzoate (7 μM), insulin (100 mU/liter), and 10% (vol/vol) horse serum. Cells were maintained in culture for 2–12 d, with the addition of the antimitotic agent cytosine arabinoside (40 μM) from day 2 *in vitro* to prevent glial proliferation.

Membrane preparation and binding assays

Cultured neurons. ^{3}H-glutamate binding to membranes prepared from cultured cerebral cortex neurons was performed essentially as described by Honoré et al. (1986). Cultures (8 d *in vitro*) were frozen overnight, thawed, and homogenized in Tris-HCl (30 mM), $CaCl_2$ (2.5 mM), pH 7.1. They were then centrifuged for 15 min at 40,000 × *g* and the pellet washed 3 times. The homogenate was incubated for 30 min at 37°C, which was followed by centrifugation at 25°C for 10 min (40,000 × *g*). After washing and centrifugation, the final pellet was resuspended in buffer (5000 ml/gm of original tissue) and 2.5 ml aliquots in duplicate were mixed with 25 μl of displacer and 125 μl of ^{3}H-L-glutamate (final concentration, 2 nM). Samples were incubated for 20 min at 37°C. Nonspecific binding was determined using L-glutamate (final concentration, 0.68 mM) as the displacer. Bound radioactivity was determined after the addition of 5 ml ice-cold buffer, filtration through Whatman GF/C glass fiber filters, and washing twice with 5 ml of ice-cold buffer.

It has been found that the freezing of intact tissue (Honoré et al., 1986), in contrast to the freezing of homogenates (Foster and Fagg, 1984), only slightly (37%) reduces total ^{3}H-L-glutamate binding. Thus the present assay conditions favor the L-APB-sensitive binding sites (Honoré et al., 1986).

Mouse cortex. Cerebral cortices (0.5–1 gm/sample) were dissected from the brains of mice at different ontogenetic stages (embryonic day 16 to postnatal day 14) and the tissue frozen overnight. The next day the cortices were thawed and homogenized by an Ultra-Turrax homogenizer in 2 × 5 ml, 30 mM Tris-HCl buffer, pH 7.1, containing 2.5 mM $CaCl_2$, and centrifuged at 30,000 × *g* for 15 min. The pellet was washed 3 times by homogenization in the above buffer and by centrifugation at 40,000 × *g* for 15 min. The washed pellet was homogenized in 10 ml of buffer and incubated at 37°C for 30 min, followed by centrifugation at 25°C for 10 min. The resulting pellet was washed once by homogenization in buffer and by centrifugation at 40,000 × *g* for 10 min. After homogenization in buffer, the homogenate was frozen for 1–4 d at −20°C. On the day of the binding experiments the homogenate was thawed and centrifuged at 48,000 × *g* for 10 min, then washed twice by homogenization in 30 mM Tris-HCl, 2.5 mM $CaCl_2$ buffer, pH 7.1, and by centrifugation at 48,000 × *g* for 10 min. The final pellet was homogenized in buffer (see below) (50 ml/gm of original tissue) and used for binding assays. Aliquots (0.5 ml) in triplicate were incubated for 30 min at 0°C with 25 μl of the radioligand (see below) in the absence and presence of 0.68 mM L-glutamate to determine nonspecific binding. After the addition of 5 ml of ice-cold buffer, free and bound radioactivity were separated by filtration through Whatman GF/C glass fiber filters, and then washed with 2 × 5 ml ice-cold buffer.

^{3}H-AMPA binding studies were performed in a buffer containing 30 mM Tris-HCl, pH 7.1, 2.5 mM $CaCl_2$, and 100 mM KSCN using a ^{3}H-AMPA concentration of 5 nM; kainate binding was studied at 5 nM ^{3}H-kainate in Tris-citrate buffer (50 mM), and ^{3}H-L-glutamate binding (final concentration, 2 mM) was studied in Tris-HCl (30 mM), $CaCl_2$ (2.5 mM), pH 7.1, in the presence of 15 μM quisqualate.

Release experiments. Release experiments were performed using the model described by Drejer et al. (1986), which represents a further development of the original method of Drejer et al. (1983). To cerebral cortex interneurons cultured in petri dishes (30 mm) were added 100 μM γ-vinyl-GABA 1 hr before the experiment in order to inhibit degradation of GABA in the neurons. Thirty minutes before the experiment, 5 μCi ^{3}H-GABA was added to each culture, and after the preincubation period the cells were washed twice with HEPES-buffered saline (HBS) containing 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 6 mM D-glucose, pH 7.4, and placed in a superfusion system. This system, which is illustrated in Figure 1, consists of a peristaltic pump continuously delivering thermostated (37°C) superfusion medium (flow, 2 ml/min) from a reservoir to the top of the slightly tilted petri dish. The cell monolayer at the bottom of the dish was covered with a piece of nylon net (Nitex; 100 μm mesh) to facilitate dispersion of the medium over the cell layer. The medium was continuously collected from the lower part of the dish and delivered to a fraction collector. In order to wash out excess radioactivity, the cells were superfused with HBS for 15 min before the fraction collector was connected (30 sec collection periods). The cells were subsequently stimulated for 30 sec every 4 min by changing the superfusion medium from HBS to a corresponding medium containing an EAA agonist. In dose–response experiments, increasing concentrations of agonist were used. The radioactivity of every fourth fraction was determined using liquid scintillation counting, and the total release rate in the presence (stimulated release) and absence (basal release) of agonist was calculated in counts per minute. One experiment included 6 stimulation periods.

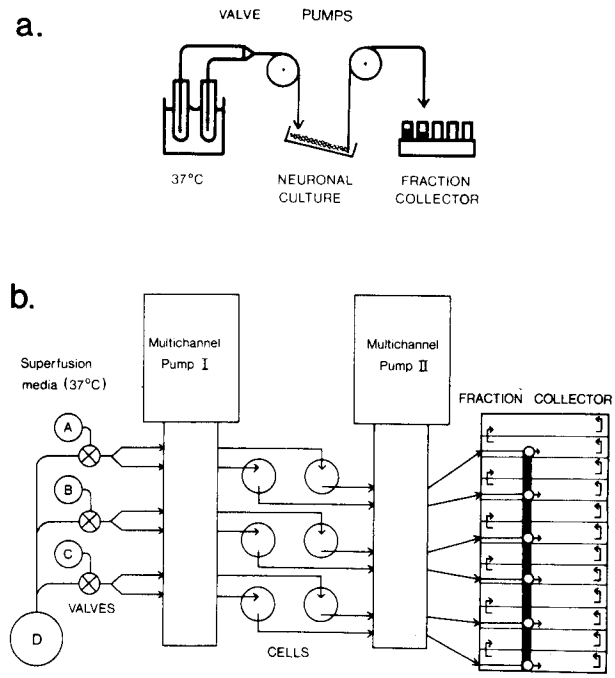

Figure 1. Schematic drawings of the superfusion system used for release experiments on monolayer neuronal cultures. *a,* The slightly tilted (5°) petri dish with neurons attached to the bottom. The cell layer is covered with a nylon net and fresh medium is continuously dripped (2 ml/min) onto the net (dripping distance, 7 mm). The superfusion medium is collected from the lower part of the petri dish and delivered to a fraction collector. The suction speed is slightly higher than the delivery rate in order to avoid buildup of medium in the dish. The dead volume of the dish is around 200 μl, which means that a complete change of extracellular medium is obtained within 5–10 sec. The superfusion medium can be changed from control to stimulation medium by turning the valve. In *b* is shown how 6 petri dishes can be run in parallel using 2 Autoclude (10-500 A) multichannel pumps and a LKB (2211) superrac fraction collector fitted with 6 drop heads on a horizontal acrylic bar. If 30 sec fractions are collected in minivials (Rack type A) and cells are stimulated every 4 min, it is possible to include 6 stimulations in each experiment.

Recovery of ^{3}H-GABA. The amount of radioactivity that remained as ^{3}H-GABA was determined in the last stimulation periods in 3 individual experiments, using ion-exchange chromatography (Kemel et al., 1979). Briefly, samples were diluted 1:1 with 0.4 mM NaAc, pH 4.95, and 0.5 ml was added to a 0.5 × 3 cm column of Dowex 50W, equilibrated 4 times with 0.2 M NaAc, pH 5.00. The column was first eluted 3 times with 2 ml of 0.02 M NaAc, pH 4.0 (eluting ^{3}H-H_2O, ^{3}H-glutamate, and ^{3}H-glutamine) and then 3 times with 1 ml of 0.4 M Tris-HCl, pH 7.4 (eluting ^{3}H-GABA). The results were corrected for column recovery by adding tracer ^{14}C-GABA to each sample.

Results

Binding studies

In order to determine the pharmacological specificities of ^{3}H-L-glutamate-binding sites on the cultured neurons, binding assays were performed in the presence of various standard in-

Table 1. Inhibition of ^{3}H-L-glutamate binding (final concentration, 2 nM) by various reference compounds

Compound	Cultured neurons IC_{50} (μM)	Cerebral[a] cortex IC_{50} (μM)
L-Glutamate	0.07	0.15
L-Aspartate	0.26	2.0
Quisqualate	0.18	0.10
AMPA	7.4	11.0
NMDA	400	>500
Kainate	>500	>500
D-APV	5.1	2.5
DL-APH	3.4	2.0
GDEE	15	—
PK 26124	28	—

IC_{50} values were estimated by Hill analysis, using at least 3 different concentrations of inhibitors. For abbreviations, see Materials and Methods.

[a] From Honoré et al., 1986.

hibitors of glutamate binding. In Table 1, the results of these displacement studies are shown, together with the results from ^{3}H-L-glutamate-binding studies on whole cortex membranes prepared in a similar manner (Honoré et al., 1986). It can be seen that inhibition of ^{3}H-L-glutamate binding by the tested agonists and antagonists correlates well for the 2 preparations, except that L-aspartate seems to be somewhat more potent as a displacer of glutamate binding in the cultured neurons, as compared to the whole cortex.

The ontogenetic development of the 3 classical EAA receptors—the quisqualate, kainate, and NMDA receptors—was studied in membranes prepared from frozen mouse cerebral cortex using the radioligands ^{3}H-AMPA (quisqualate receptor; Honoré and Nielsen, 1985), ^{3}H-kainate (kainate receptor; Honoré et al., 1986), and ^{3}H-L-glutamate in the presence of 15 μM quisqualate (NMDA receptor; Fagg and Matus, 1984). A similar study using the above-mentioned methods was not performed on the cultured neurons because of the limited amount of tissue available. In Figure 2, the specific binding of ^{3}H-AMPA, ^{3}H-kainate, and ^{3}H-L-glutamate (plus quisqualate) to membranes prepared from frozen mouse cortex is shown as a function of the pre- and postnatal developmental time period. The amount of ^{3}H-AMPA bound remained constant until about postnatal day 1. After this time, ^{3}H-AMPA binding increased rapidly to about 2 times the original level of ^{3}H-AMPA binding. ^{3}H-kainate binding remained almost constant over the developmental period studied, whereas ^{3}H-L-glutamate binding in the presence of 15 μM quisqualate (NMDA binding sites) showed a slow increase over the developmental period from postnatal days 1 to 14.

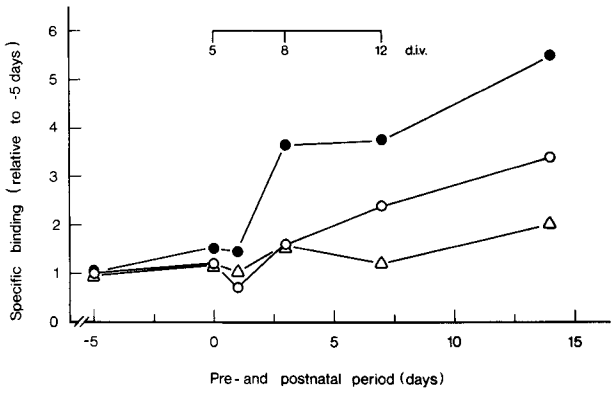

Figure 2. Specific binding of ^{3}H-AMPA (●), ^{3}H-kainate (△), and ^{3}H-L-glutamate (plus quisqualate) (○) to membranes prepared from mouse cerebral cortices of different ontogentic ages. Results are expressed relative to the binding at −5 d (16-d-old embryos). The same membrane preparation was used for the 3 different radioligands. The original binding results were expressed as cpm (total minus nonspecific)/gm of original tissue, corrected for membrane loss during the membrane preparation, using specific ^{3}H-flunitrazepam binding as the membrane marker. Under the assay conditions described in Materials and Methods, the specific binding in membranes prepared from 16-d-old mouse embryo cortex was as follows: for ^{3}H-AMPA, 22.8 cpm/gm; for ^{3}H-kainate, 56.3 cpm/gm; and for ^{3}H-L-glutamate (plus quisqualate), 11.2 cpm/gm. The *bar* at the top of the figure indicates the culture periods (days in vitro: *d.i.v.*) for neuronal cultures started from 16-d-old embryo cerebral cortex and may be used for comparisons with the release studies shown, for example, in Figure 4.

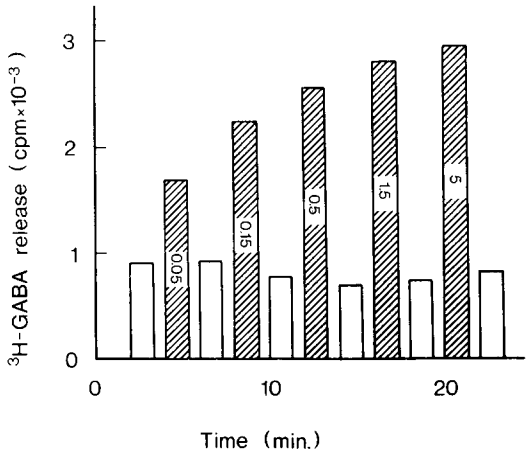

Figure 3. Time course of a typical dose–response experiment of stimulated ^{3}H-GABA release. Cells were stimulated every 4 min with increasing concentrations of quisqualate. *Open bars,* basal release during superfusion with control buffer; *hatched bars,* stimulated release during superfusion with quisqualate (concentrations in μM indicated in the bars).

Release experiments

In the present release studies, preloaded ^{3}H-GABA was used as a tracer of endogenous GABA. The GABA transaminase inhibitor γ-vinyl-GABA was included in the preincubation medium to avoid degradation of ^{3}H-GABA. It was found, using ion-exchange chromatography, that more than 90% of the radioactivity released from the cultures was GABA (results not shown).

Superfusion of the cultured neurons with media containing elevated potassium concentrations or excitatory amino acids led to a rapid and marked increase in the release rate of preloaded ^{3}H-GABA from the neurons.

Figure 3 shows an example of a release experiment where increasing concentrations of quisqualate were used to stimulate ^{3}H-GABA release from the neurons. Similar dose–response experiments were performed with L-glutamate, NMDA, kainate, and potassium on neurons cultured for different periods of time (Fig. 4).

^{3}H-GABA release could be induced as early as 5 d *in vitro* (d.i.v.) (Fig. 4). The potassium-stimulated release of ^{3}H-GABA increased gradually during the culture period (5–12 d.i.v.). Similarly, quisqualate was able to induce a significant release of ^{3}H-GABA at 5 d.i.v., but the release at 8 and 12 d.i.v. was increased

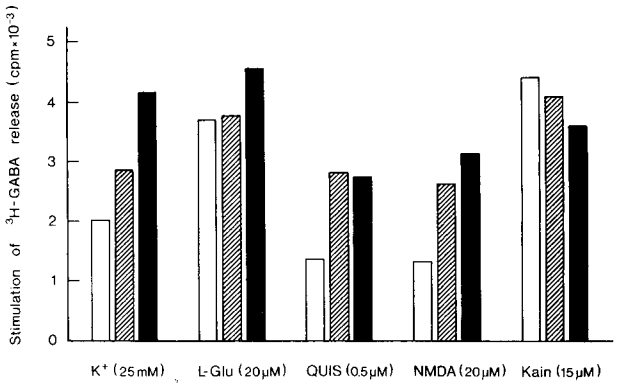

Figure 4. Stimulation by elevated potassium concentration (K^+), L-glutamate (L-Glu), quisqualate (QUIS), *N*-methyl-D-aspartate (NMDA), and kainate (Kain) of ^{3}H-GABA release from neuronal cultures grown for different periods of time. Concentrations are shown. *Open bars,* cultures of 5 d *in vitro* (d.i.v.); *hatched bars,* 8 d.i.v.; *filled bars,* 12 d.i.v. Experiments were performed in duplicate, essentially as illustrated in Figure 3, and results are expressed as stimulated release [i.e., total release (cpm) minus basal release (cpm)].

to about twice the original level. The stimulation of ^{3}H-GABA release by NMDA showed a gradual increase over the culture period, whereas L-glutamate- and kainate-induced release was almost constant during the culture period between 5 and 12 d.i.v.

Figure 5 shows dose–response curves for potassium-induced ^{3}H-GABA release from the cultured neurons at 5, 8, and 12 d.i.v. It is seen that only the maximal release is increased with time in culture, whereas the concentration needed for half-maximal stimulation of release is similar for the 3 cultures ($EC_{50} \simeq$ 30 mM). Also, when L-glutamate, quisqualate, NMDA, or kainate was used to stimulate ^{3}H-GABA release, the EC_{50} values were found to be constant during the culture period from 5 to 12 d.i.v. (results not shown). Dose–response curves for the effects of the 4 excitatory amino acids on ^{3}H-GABA release from neurons (6–8 d.i.v.) are shown in Figure 6. It is shown that quisqualate is by far the most potent agonist, with an EC_{50} of 75 nM. L-Glutamate, NMDA, and kainate are about equipotent, with EC_{50} values in the low micromolar range.

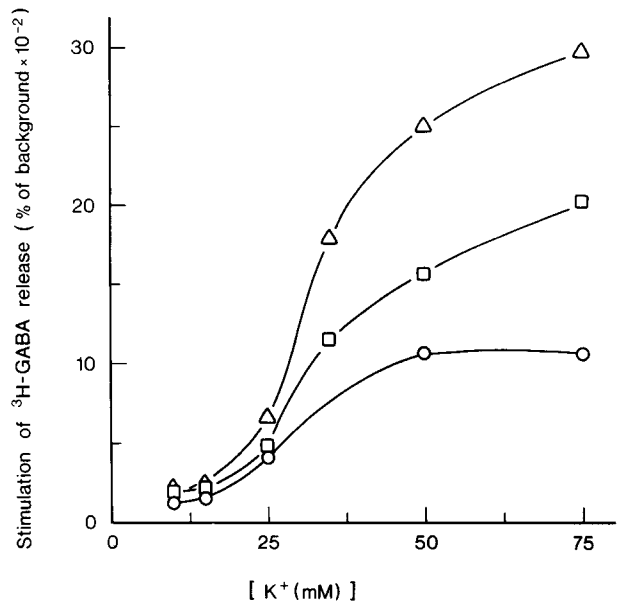

Figure 5. Dose–response curves for the stimulation of ^{3}H-GABA release from neuronal cultures grown for different periods of time by elevated potassium concentration (K^+ was substituted for Na^+ in order to preserve isotonicity). The culture periods were 5 (○), 8 (□), and 12 (△) d. Experiments were performed on 2 individual cultures run in parallel, similar to the experiments illustrated in Figure 3. Stimulations were expressed relative to the basal release (percentage), and the experimental points represent means from the 2 experiments.

Table 2. Antagonism of excitatory amino acid-induced ^{3}H-GABA release from cultured cortex neurons

Antagonist (IC_{50} values) (μM)	Quisqualate (0.3 μg/ml)	NMDA (3 μg/ml)	Kainate (3 μg/ml)
D-APV	110	1.5	150
Kynurenic acid	550	15	320
PDA	1165	150	360
GAMS	>200	236	185
PK 26124	17	5.1	14
GDEE	>1000	>1000	>1000

Experiments were performed as illustrated in Figure 7. Values in the table are IC_{50} (the concentration of antagonist estimated to give half-inhibition of agonist-induced ^{3}H-GABA release). The IC_{50} values were obtained by interpolation from dose–response curves using 3–5 different ligand concentrations, of which at least 2 gave inhibition in the 25–75% interval. for further details, see Materials and Methods and the legend to Figure 3. Abbreviations are given in Materials and Methods.

The stimulation of ^{3}H-GABA release by excitatory amino acids could be blocked by glutamate antagonists. Figure 7 illustrates the time course of an experiment in which the effects of the NMDA antagonist D-APV on NMDA-induced ^{3}H-GABA release was tested. The experiment starts and ends with 2 control stimulations by NMDA (20 μM). During stimulations 3–5, D-APV (1.5 μM) was included with NMDA. At this concentration, D-APV inhibited about half of the NMDA response and the effect was clearly reversible. Increasing the D-APV concentration to 5 μM led to a complete block of NMDA effects (results not shown). Table 2 shows the results of similar experiments, in which different concentrations of antagonists were tested against stimulated ^{3}H-GABA release by using fixed concentrations of NMDA, quisqualate, and kainate. D-APV is seen to be a highly selective antagonist of NMDA responses; kynurenic acid is also a rather potent blocker of NMDA stimulation, with only weak effects on kainate responses. PDA was found to be a weak and nonselective antagonist. GAMS was also weak but showed some selectivity against the kainate responses, whereas the compound PK 26124 (Benavides et al., 1985) was a quite potent antagonist of NMDA-, kainate-, and quisqualate-induced GABA release from the neurons.

The release of ^{3}H-GABA induced by potassium or excitatory amino acids was found to be calcium-dependent. When the external calcium concentration was lowered to 0.1 mM, approximately 70% of the stimulated release was blocked, and no further decrease was observed when external calcium concentrations were decreased below 0.1 mM or when the inorganic calcium-channel blocker Co^{2+} was added (results not shown).

The responses to NMDA were also found to be magnesium-dependent. Figure 8 shows the NMDA-induced ^{3}H-GABA release from the neurons as a function of the external magnesium concentration. It is seen that 1.2 mM magnesium causes an almost complete block of the NMDA response. At the physiological magnesium concentration of 0.6 mM, which was used

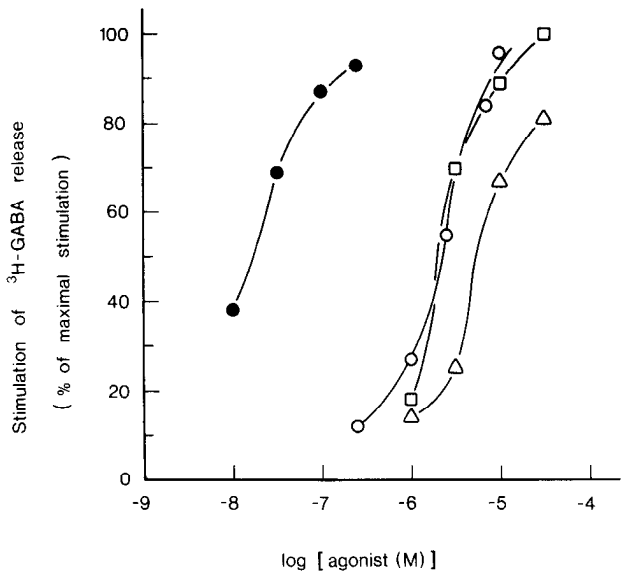

Figure 6. Dose–response curves for the stimulation of ^{3}H-GABA release from neuronal cultures (6–7 d.i.v.) by quisqualate (●), L-glutamate (○), NMDA (□), and kainate (△). Experiments were performed in duplicate, as illustrated in Figure 3, and were repeated twice with similar results. Results are expressed as percentage of the maximal stimulation obtained with 100 μM of the agonists. The EC_{50} values estimated from the figure are as follows: quisqualate, 0.075 μM; L-glutamate, 12 μM; NMDA, 16 μM; kainate, 29 μM.

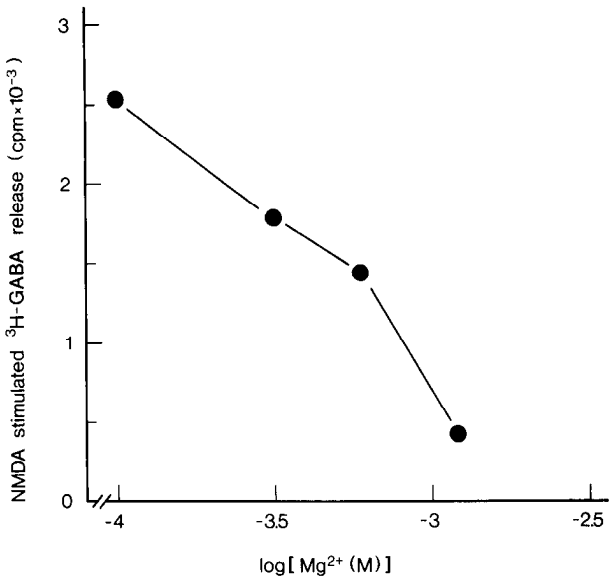

Figure 8. Effect of the external Mg^{2+} concentration on NMDA-stimulated ^{3}H-GABA release from cultured neurons. Experiments were performed in duplicate, essentially as illustrated in Figure 7, except that Mg^{2+} concentrations were varied both in basal and stimulation media.

in all other experiments, the responses to NMDA were about half-maximal.

Discussion

The release of GABA from brain tissue *in vitro* has been studied using several different techniques and preparations, including synaptosomes (e.g., Levy et al., 1976; Redburn et al., 1976), brain slices (e.g., Katz et al., 1969; Srinivasan et al., 1969; Arnfred and Hertz, 1971), and neurons in cell culture (Dichter, 1980; Pearce et al., 1981; Larsson et al., 1983). In most studies ^{3}H-GABA has been used as an exogenous label of transmitter GABA, and in all preparations a calcium-dependent induced release of GABA was demonstrated, indicating that a major part of the released GABA originates from a transmitter pool (for reviews, see Fagg and Lane, 1979; Schousboe, 1982).

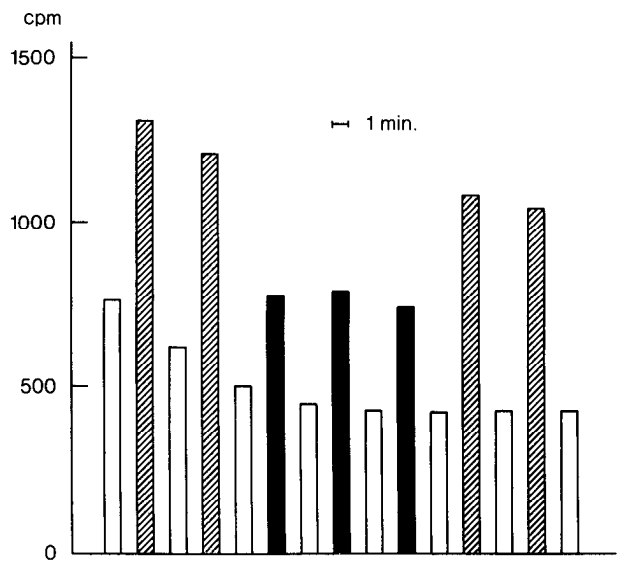

Figure 7. Time course of a typical ^{3}H-GABA-release experiment for study of the effects of EAA antagonists. Every 4 min, cells were stimulated for 1 min by changing the superfusion medium from HBS to HBS containing 20 μM NMDA. The *bars* represent tritium counts in every fourth fraction. The fractions representing basal release are indicated by *open bars*, control stimulations (20 μM NMDA) by *hatched bars*, and antagonized stimulations (20 μM NMDA + 20 μM D-APV) by *filled bars*. The results are means from experiments on 2 individual cultures.

The synaptosomal model has been useful in demonstrating that GABA is actually released from nerve terminals, but the preparation cannot be used to study the effect of external stimuli acting through dendritic or somatic receptors. In brain slices and neuronal cell cultures, most neurons are preserved intact, and these preparations may be used for studies of transmitter release induced via stimulation with excitatory agents.

As described in the present study, we have developed a technique with which transmitter release from monolayer neuronal cultures can be studied in a simple superfusion system. The system, in which up to 10 samples can be run in parallel, offers the advantage that the medium superfusing the cells can be changed within a few seconds. Since fresh medium is continuously washed over the cell layer (2 ml/min) and collected almost instantaneously, a buildup of, and possible reuptake of, released substances is avoided. Because of the rapid changes of medium, and because the monolayer of cells offers no diffusion barriers, the effects of added drugs on the neruons reach their maximum within a few seconds. This means that stimulation periods can be kept very short (30 sec or less). Following stimulation, the cells soon (within 1–2 min) return to their basal steady state. If stimulations are repeated, e.g., every 4 min, an almost constant stimulation of release can be obtained. In spite of the fact that the flow in the system is rather high (2 ml/min), there is almost no mechanical impact on the neurons because the medium is spread gently over the cell layer without external pressure. Experiments can be continued for at least 1 hr without any significant decrease in responses, and a preservation of the neuronal network is observed when cultures are examined microscopi-

cally after the experiments. A corresponding continuous release model cannot be exchanged for models using brain slices. Here the diffusion problems due to the thickness of the slices offer serious limitations with respect to time resolution (minutes instead of seconds) and buildup and/or possible uptake of drugs and released substances. The slices can only be stimulated for a very limited number of times—sometimes only once—and the responses tend to decrease drastically between stimulations. Functional studies of EAA effects in brain slices may be difficult to interpret because of the excessive uptake and release of L-glutamate. The same problems may be encountered in cell culture models, where a discontinuous setup is used.

It has previously been shown that cultured cortex neurons exhibit potassium-induced, calcium-dependent release of GABA (Snodgrass et al., 1980; Larsson et al., 1983). Here we demonstrate that GABA release could also be induced from the neurons by interacting with excitatory amino acid receptors.

The pharmacological specificities of the ^{3}H-L-glutamate-binding sites on the cultured neurons (Table 1) appeared to be similar to those described for rat cortex membranes (Honoré et al., 1986). The finding that quisqualate, NMDA, and kainate induced ^{3}H-GABA release from the neuronal cultures indicates that all 3 classical excitatory amino acid receptors are expressed and functionally active in the neurons. In agreement with this, it has been shown that these glutamate analogs, with the same potency rank order, are highly toxic to these neurons (Frandsen and Schousboe, 1987). However, this is in contrast to what has been found in cerebellar granule cell cultures, where L-glutamate, but neither quisqualate, NMDA, nor kainate, was able to induce transmitter release from the cultures (Drejer et al., 1986).

The development of receptors for quisqualate, NMDA, and kainate in the *in vivo* mouse cortex (Fig. 2) appears to parallel the development of functionally active receptors on the cultured neurons (Fig. 4). The finding that the effects of quisqualate on ^{3}H-GABA release were doubled from days 5 to 8 in culture (Fig. 4) is in agreement with the development of ^{3}H-AMPA binding sites *in vivo* (Fig. 2). Also, the progressive increase in the effect of NMDA over the culture period from days 5 to 12, and the almost constant effect of kainate on ^{3}H-GABA release as a function of development in culture (Fig. 4), is in agreement with findings on the development of binding sites for kainate and NMDA (Fig. 2). It should, however, be taken into account that the total amount of K^+-stimulated ^{3}H-GABA release increased markedly over the culture period (Figs. 4, 5), indicating the formation of an increasing amount of releasable transmitter GABA as a function of time in culture. This may reflect increased synaptogenesis and an increase in the formation of the GABA-synthesizing enzyme GAD in the cultures as a function of culture time (Yu et al., 1984).

The EAA-induced release of GABA from the neurons is assumed to result from a direct interaction with EAA receptors on the GABAergic neurons, but it could also be explained by an indirect action via other excitatory neurons in the cultures. A polysynaptic response via cholinergic neurons can be excluded, since the responses to NMDA and quisqualate could not be blocked by 10 μM atropin (results not shown). Moreover, we have found that all neurons in the cultures are sensitive to the excitotoxic action of, for example, kainate and NMDA (Frandsen and Schousboe, 1987). This is strong evidence for the presence of EAA receptors directly on the GABAergic neurons.

Unlike previous studies (Snodgrass et al., 1980; Yu et al., 1984), the present study showed K^+-stimulated ^{3}H-GABA release in the cultures from as early as day 5 *in vitro*. Moreover, the K^+-stimulated release of ^{3}H-GABA is much higher in the present study than has been found by others (Snodgrass et al., 1980; Yu et al., 1984). These discrepancies may be explained by the higher sensitivity in the release model used in the present study compared to in the other 2 studies, where a discontinuous change of media was used. The finding by Yavin and Yavin (1974, 1977) and Dichter (1978) of an intensive synaptogenesis and myelinogenesis in similar cultures after only 5 d would support the development of GABA release as early as this time period.

The high potency of quisqualate in inducing ^{3}H-GABA release from the neurons is remarkable; it should be noted that the EC_{50} value of 75 nM found in the present study is close to the IC_{50} value of around 20 nM found for quisqualate as a displacer of ^{3}H-AMPA binding from rat cortex membranes (Honoré and Nielsen, 1985). Kainate also shows some affinity for the ^{3}H-AMPA binding site (IC_{50} = 8.7 μM; Honoré and Nielsen, 1985), and it is possible that at least some of the effects of kainate on ^{3}H-GABA release could be mediated via the quisqualate receptor. Since the pharmacological selectivities of antagonists on quisqualate- and kainate-induced ^{3}H-GABA release (Table 2) are quite similar, it is not unlikely that quisqualate receptors are involved in some of the kainate responses. NMDA, however, is not able to displace ^{3}H-AMPA binding from mouse cortex membranes (Honoré and Nielsen, 1985) and the effects of the tested excitatory amino acid antagonists on NMDA responses are quite different when compared to the effects on quisqualate and kainate responses. D-APV and kynurenic acid were found to be potent and selective blockers of NMDA-induced ^{3}H-GABA release from the cultured neurons (Table 2), in agreement with generally accepted pharmacological selectivities (see, e.g., Watkins and Evans, 1981).

The ionophores coupled to NMDA receptors are distinct from the quisqualate and kainate ionophores (Nowak et al., 1984). As a consequence, Mg^{2+} ions are able to block NMDA responses without affecting electrophysiological responses to quisqualate or kainate. In support of this, Mg^{2+} was able to block NMDA-stimulated ^{3}H-GABA release from the neurons (Fig. 8) with an almost complete block at 1.2 mM Mg^{2+}. Mg^{2+} did not affect responses to quisqualate (results not shown). As in previous findings (Snodgrass et al., 1980; Larsson et al., 1983), K^+ stimulation of ^{3}H-GABA release was found to be calcium-dependent. This was also true for excitatory amino acid-induced ^{3}H-GABA release, indicating that the released ^{3}H-GABA originates from a transmitter pool of GABA. In this context it should be noted that we have recently found potassium-evoked release of endogenous, as well as exogenous, GABA in similar cultures (O. M. Larsson and A. Schousboe, unpublished observations).

In summary, we have described a simple superfusion model for studies of transmitter release from a large number of parallel neuronal cultures. The low dead volume of the system and the immediate change of superfusion medium give a high time resolution and make the model very sensitive in the detection of minor changes in transmitter release. In the present study, the model has been used to demonstrate the presence of functionally active excitatory amino acid receptors on cultured cerebral cortex neurons responding to quisqualate, NMDA, kainate, and L-glutamate.

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