Anesthesiology
77:109–113, 1992

Enflurane-induced Release of an Excitatory Amino Acid, Glutamate, from Mouse Brain Synaptosomes

Takuji Hirose, M.D., Masafumi Inoue, M.D.,† Morio Uchida, M.D.,‡ Chiyoko Inagaki, M.D.§*

To clarify the mechanisms of enflurane-induced convulsions, we examined the effects of enflurane, halothane, and diethyl ether on the release of an excitatory neurotransmitter, glutamate, from isolated pinched-off nerve terminals (synaptosomes) of the mouse cerebral cortex. At concentrations corresponding to those used clinically (0.75 and 1.25 mM), enflurane released more glutamate than did halothane. Diethyl ether (10 and 58 mM) had no effect on glutamate release. Enflurane (0.75–15 mM) increased glutamate and aspartate release in a dose-dependent manner but had little effect on the release of the inhibitory neurotransmitters glycine and γ-aminobutyric acid or on the release of glutamine. A glutamate uptake inhibitor, kainic acid (1 mM), did not affect enflurane-induced glutamate release. Replacement of the medium's Ca^{2+} by Co^{2+}, or exposure to cold (about 2° C), suppressed the enflurane-induced glutamate release. Depolarization caused by 40 mM K^+ increased the basal level of glutamate released, and enflurane-induced glutamate release was lower after depolarization. Enflurane had no effect in synaptosomes prepared from the cerebellum, diencephalon and pons, or medulla oblongata. Thus, enflurane increased Ca^{2+}- and temperature-dependent glutamate release, especially from synaptosomes of the cerebral cortex. These data provide a pathophysiologic explanation for enflurane-induced convulsions. (Key words: Anesthetic, volatile: diethyl ether; enflurane; halothane. Brain, synaptosomes: glutamate.)

VOLATILE ANESTHETICS are considered to depress neuronal excitation,[1] but some also have excitatory effects, including convulsions.[2,3] Enflurane is known to induce spikes and waves in the electroencephalogram and convulsions in some patients.[2,3] It also can cause epilepsy-like seizure activity in the electroencephalograms of cats[4] and dogs[5] and opisthotonus in mice.[6,¶,**] Enflurane-evoked seizure-like burst discharges from CA1 neurons in rat hippocampal slices are completely suppressed by a glutamate/N-methyl-D-aspartate receptor antagonist.[7,8] This suggests that enflurane stimulates the release of glutamate, an excitatory amino acid. In the present report we show that enflurane can enhance endogenous glutamate release from synaptosomes (isolated pinched-off nerve endings) of the mouse cerebral cortex.

* Anesthesiologist, Department of Anesthesiology.
† Instructor, Department of Pharmacology.
‡ Professor, Department of Anesthesiology.
§ Professor, Department of Pharmacology.

Received from Kansai Medical University, Osaka, Japan. Accepted for publication February 28, 1992. Supported by research grants from the Ministry of Education, Science and Culture, Japan; Japan Private School Promotion Foundation; Epilepsy Research Foundation, Japan; and a Katano Grant from the Alumni Association of Kansai Medical University.

Address reprint requests to Dr. Inagaki: Department of Pharmacology, Kansai Medical University, Fumizono-cho 1, Moriguchi City, Osaka 570, Japan.

¶ Komatsu H, Yokono S, Ogli K: The central nervous stimulating effect of four different halogenated ether anesthetics and halothane in mice. Journal of Anesthesia 2:115–117, 1988. Published by the Japan Society of Anesthesiology, ISSN 0913-8668.

** Komatsu H, Ohara T, Nogaya J, Tsukamoto I, Yokono S, Ogli K: The effect of age and anesthetic solubility on anesthetic-induced opisthotonus in mice. Journal of Anesthesia 5:228–232, 1991. Published by the Japan Society of Anesthesiology, ISSN 0913-8668.

Materials and Methods

Preparation of Synaptosomes

Experimental methods used in this study were approved by the animal research committee of Kansai Medical University. Male ddY mice (25–35 g) (Tokushima Animal Laboratory, Japan) were stunned and decapitated. The whole brains were rapidly removed and placed on an ice-cold glass plate. Then the brains were divided into four brain regions: cerebral cortex, cerebellum, diencephalon and pons, and medulla oblongata. In most of the experiments, the cerebral cortex was used. Synaptosomal fractions were prepared from brain tissues as described by Gray and Whittaker.[9] In brief, the brain tissues were homogenized in a Teflon–glass homogenizer in an ice-cold buffer solution (a homogenizing buffer, 2.0 ± 0.5° C, *p*H 7.4 ± 0.05) containing (millimolar concentrations) sucrose 250, Tris-2-(N-morpholino)-ethanesulfonic acid 12.5, ethylenediamine tetraacetic acid 1, and phenylmethylsulfonyl fluoride 1. The homogenate was centrifuged at 1,000 × g for 10 min, and the supernatant was centrifuged at 12,000 × g for 30 min. The resulting pellets were washed twice by centrifugation (12,000 × g, 30 min) with a homogenizing buffer. The final pellet was suspended in a modified Krebs-HEPES buffer solution (a standard solution, *p*H 7.3 ± 0.05) containing (millimolar concentrations): NaCl 128, KCl 2.5, $CaCl_2$ 2.7, $MgSO_4$ 1.2, Na_2HPO_4 1, HEPES 20, and glucose 20, to give a protein concentration of about 5 mg/ml, as measured by the method of Lowry *et al.*[10] They were used within 2 h of their preparation.

Assay of Amino Acid Release

Release of amino acid from synaptosomes was estimated according to the method described by Hardy *et al.*[11] and Inoue *et al.*[12] Aliquots of synaptosomal preparations (0.1

ml) were added to the standard solution (0.9 ml, control) or the solution containing 1 mM kainic acid, 2.7 mM Co^{2+} instead of Ca^{2+}, or 40 mM K^+ ($Na^+ + K^+ = 132.5$ mM), incubated at 32° C for 10 min, and then incubated for another 5 min following the addition of a volatile anesthetic (enflurane, halothane, or diethyl ether). To examine the effects of ice-cold conditions (about 2° C), we did the experiments in a cold room and kept test tubes in a box filled with crushed ice throughout the incubation period (15 min). Samples without anesthetics received an equivalent volume of a standard solution. To minimize synaptosomal damage and spontaneous release, incubation conditions included a high glucose concentration (20 mM) and low temperature (32° C). The incubation was terminated by the addition of four volumes of ice-cold standard solution (about 2° C), and the mixture was immediately centrifuged at 12,000 × g for 20 min. An aliquot (50 μl) of the supernatant was then mixed with 50 μl of 100 mM sodium borate containing o-phthalaldehyde/2-mercaptoethanol, and 10 μl of the mixture was injected into a high-performance liquid chromatograph with a spectrophotometer (Eikom Co. and Soma Kougaku Co., Japan), which was set at wavelengths of 360 and 450 nm for excitation and emission, respectively.

The concentrations of amino acids were calculated from the corresponding peak heights and were quantified with external standards and corrected for blank values. Anesthetic-induced glutamate release was calculated by subtracting the amount of glutamate released in the absence of anesthetic (basal level) from that released in the presence of the drug. Contents of intrasynaptosomal glutamate extracted by 0.1 N perchloric acid after the 5-min incubation with enflurane (5 mM) was estimated to be 4.73 ± 0.15 μg/mg protein (n = 8) and was about three to six times as high as the amount of glutamate released in the supernatant. This suggests that our preparations could be used to model the activities of intact nerve terminals.[13]

Materials

Drugs used were enflurane (Abbott Laboratory, Italy), halothane (Takeda Chemical Industries, Ltd., Japan), diethyl ether (Ishizu Pharmaceutical Co., Ltd., Japan). Other chemicals and solvents were of the highest grade commercially available. Volatile anesthetics were dissolved directly in a buffer solution by shaking.[14–16] To prevent evaporative loss, they were incubated in plugged glass test tubes. Millimolar-range concentrations of the volatile anesthetics are reportedly equivalent to those used clinically[16,17] (approximately corresponding to 1–2 MAC): 0.5–1.25 mM for enflurane[16,18] and halothane[16,19,20] and 10–20 mM for diethyl ether.[20,21]

Statistical Analysis

The data were analyzed with Student's unpaired *t* test. $P < 0.05$ was considered to be significant.

Results

Clinically used concentrations of diethyl ether (10 mM), halothane (0.75 mM, 1.25 mM) and enflurane (0.75 mM, 1.25 mM) were tested for glutamate release from cerebrocortical synaptosomes (table 1). Although both halothane and enflurane stimulated glutamate release, enflurane released more glutamate than did the same concentration of halothane. Diethyl ether at clinical and even at a much higher concentration (58 mM) did not stimulate glutamate release. Enflurane at 0.75 to 15 mM increased, in a dose-dependent manner, the amounts of glutamate and aspartate released, but it had only small effects on the release of γ-aminobutyric acid (GABA), glycine, and glutamine (fig. 1). In the following experiments, 5 mM was used to analyze quantitatively the effects of various treatments on enflurane-induced glutamate release. The amounts of glutamate released began to increase soon after enflurane was added, and reached a plateau within

TABLE 1. Effects of Volatile Anesthetics on Glutamate Release from Mouse Cerebrocortical Synaptosomes

Treatment		Amount of Glutamate Released (μg/mg protein)	Anesthetic-induced Glutamate Release (μg/mg protein)	Number of Brains
None		1.66 ± 0.09	—	10
Diethyl ether	10 mM	1.67 ± 0.11	0.10 ± 0.18	4
	58 mM	1.73 ± 0.15	0.16 ± 0.15	4
Halothane	0.75 mM	1.90 ± 0.05*	0.19 ± 0.05	6
	1.25 mM	1.99 ± 0.09*	0.28 ± 0.09	6
Enflurane	0.75 mM	2.06 ± 0.07*	0.36 ± 0.07†	6
	1.25 mM	2.19 ± 0.08*	0.48 ± 0.08†	6

Anesthetic-induced glutamate release was calculated as the difference between the amounts of glutamate released in the presence and absence of an anesthetic. Values are given as means ± SEM.

* $P < 0.05$: significant difference from the value in the absence of an anesthetic.

† $P < 0.05$: significant difference from each value with the same concentration of halothane.

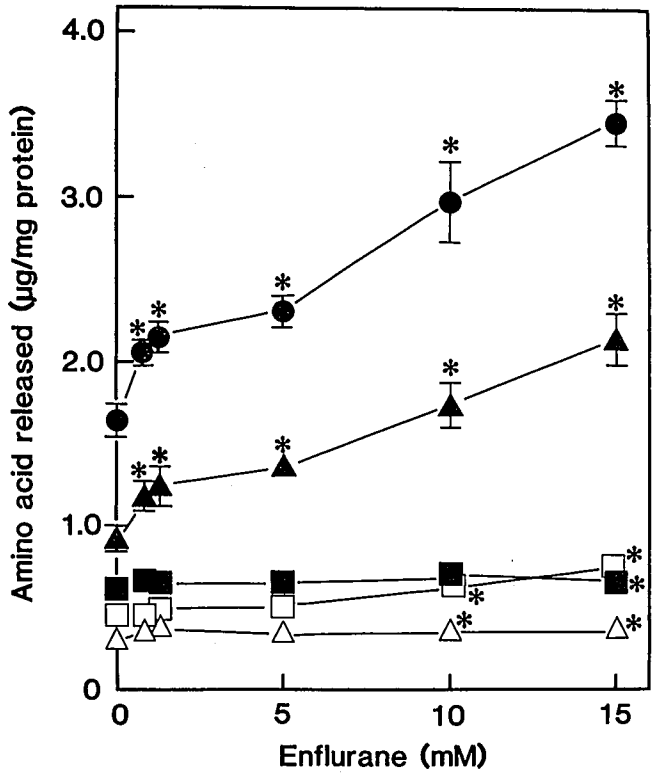

FIG. 1. Dose-dependent effects of enflurane on the glutamate (filled circles), aspartate (filled triangles), glycine (open triangles), γ-aminobutyric acid (open squares), and glutamine (filled squares) released from cerebrocortical synaptosomes of mice. Each point with bars represents a mean ± SEM of six brains. *$P < 0.05$, significantly different from the corresponding value without enflurane.

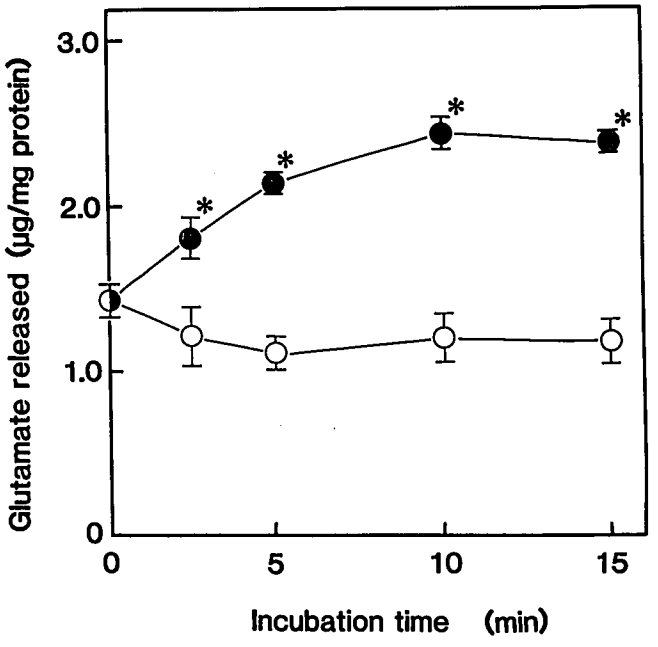

FIG. 2. Time course of glutamate released from cerebrocortical synaptosomes of mice in the absence (open circles) and presence (filled circles) of enflurane (5 mM). Each point with bars represents a mean ± SEM of four brains. *$P < 0.05$, significantly different from the corresponding value without enflurane.

10 min (fig. 2). In contrast, the amounts of glutamate recovered in the medium without enflurane did not change, which suggests that net spontaneous glutamate release during the incubation period was negligible.

A glutamate uptake inhibitor, kainic acid (1 mM), increased the amounts of glutamate released (basal level) but had no effect on enflurane-induced glutamate release (table 2). Replacement of the medium's Ca^{2+} by Co^{2+}, an inorganic Ca^{2+} entry blocker, reduced the enflurane-induced glutamate release without affecting the basal level. When synaptosomes were incubated under ice-cold conditions (about 2° C), the basal level did not change, but the enflurane-induced glutamate release was markedly lower than the value measured under control conditions (32° C). Depolarization with a high concentration of K^+ (40 mM) increased the basal level and reduced the enflurane-induced glutamate release.

In synaptosomes prepared from the cerebellum, diencephalon and pons, or medulla oblongata, depolarization with 40 mM K^+ increased the basal level, but enflurane did not stimulate glutamate release (fig. 3).

Discussion

The effects of anesthetics on the release of excitatory amino acids have received little attention. Amino acid release from synaptosomes can be used as a simple and useful indicator of release from intact nerve terminals.[13,22] The present data show that at concentrations equivalent to

TABLE 2. Effects of Different Treatments on Enflurane (5 mM)-induced Glutamate Release

Treatment	Amounts of Glutamate (μg/mg protein)			Number of Brains
	Basal Level	With Enflurane	Enflurane-induced Glutamate Release	
Control	1.74 ± 0.08	2.43 ± 0.09	0.69 ± 0.04	47
Kainic acid (1 mM)	2.05 ± 0.14*	2.76 ± 0.22*	0.72 ± 0.17	8
CO^{2+} (2.7 mM)	1.75 ± 0.09	2.05 ± 0.10*	0.30 ± 0.09*	4
Ice-cold conditions	1.70 ± 0.12	1.79 ± 0.09*	0.09 ± 0.05*	4
High K^+ (40 mM)	2.78 ± 0.13*	3.05 ± 0.18*	0.27 ± 0.06*	4

Enflurane-induced glutamate release was calculated as the difference between the amounts of glutamate released in the absence (basal release) and presence of enflurane. Values are given as means ± SEM.

* $P < 0.05$, significant difference from the corresponding control value.

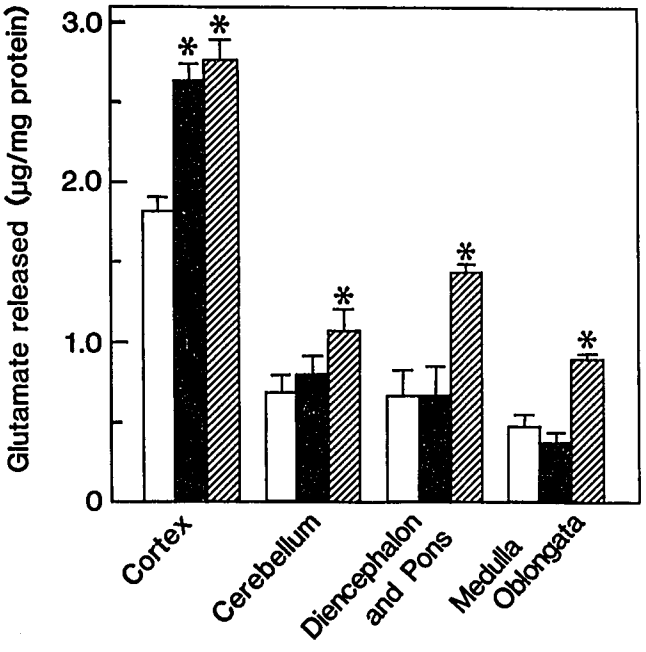

FIG. 3. Enflurane- and high K^+-induced glutamate release from the synaptosomes of four brain regions. Open columns represent the values in the absence of enflurane (control); filled and shaded columns represent the values in the presence of enflurane (5 mM) and high K^+ (40 mM), respectively. The data are means ± SEM of four brains. *$P < 0.05$, significantly different from the corresponding control value.

those used clinically,[16-21] enflurane released glutamate from mouse cerebrocortical synaptosomes more potently than did halothane. By contrast, diethyl ether did not affect glutamate release over a wide range of concentrations. This indicates that volatile anesthetic-related compounds do not necessarily increase glutamate release.

Enflurane, at clinically used concentrations and above, significantly increased the release of two excitatory amino acids, glutamate and aspartate, but only slightly stimulated the release of GABA, glycine, and glutamine at much higher concentrations (10 and 15 mM). The basal levels of glutamate and aspartate exceeded those of GABA, glycine, and glutamine, probably because of larger tissue contents of the former two than of the latter three in the brain cortex.[23] The effects of enflurane on the release of GABA, glycine, and glutamine might be underestimated because of their low basal levels. However, increases in the release of GABA and glycine from cerebrocortical synaptosomes were easily detected after depolarization with 40 mM K^+ (data not shown). Thus, enflurane appears to stimulate preferentially the release of excitatory rather than inhibitory amino acids from nerve endings. Because glutamine release, which is an indicator of nonspecific leakage of intrasynaptosomal substances,[12] was not affected by enflurane, the synaptosomal membranes appear not to have been so damaged by enflurane as to increase nonspecific leakage of glutamate. Kainic acid reportedly inhibits glutamate uptake in nerve endings without affecting glutamate exocytosis.[24] Kainic acid increased the basal level due to inhibition of glutamate uptake during the incubation period. Since enflurane increased the amount of glutamate released even in the presence of 1 mM kainic acid, this anesthetic probably directly stimulates exocytosis rather than inhibits the uptake of glutamate. This idea is also supported by the fact that enflurane-induced glutamate release was suppressed by Co^{2+}, an inorganic Ca^{2+} entry blocker,[25] and by cold, because exocytosis is known to be Ca^{2+}- and temperature-dependent.[22] Furthermore, after exposure to a high concentration of K^+, the enflurane-induced glutamate release was reduced, which suggests that enflurane and high concentrations of K^+ work *via* a common mechanism, which may be depolarization.

In all of the brain regions tested (cerebral cortex, cerebellum, diencephalon and pons, and medulla oblongata), a high K^+ solution increased glutamate release, probably through synaptosomal membrane depolarization. In contrast, enflurane stimulated glutamate release only from synaptosomes from the cerebral cortex. This may reflect the distribution of functional glutamatergic terminals in the brain. It has been reported that there are many [^{3}H]-glutamate binding sites in the cerebral cortex but few in the brain stem, and this corresponds to the distribution of the glutamatergic pathway.[26]

There is evidence that depression of postsynaptic neuronal responses may contribute to the action of general anesthetics.[16] In this context, diethyl ether–induced convulsions may be explained as effects of inhibition of inhibitory postsynaptic responses. Our results suggest that enflurane-induced convulsions occur when excitatory stimuli resulting from excessive release of excitatory amino acid may overcome postsynaptic inhibition. Because activation of glutamate receptors is known to cause convulsions *in vivo* and seizure discharge,[27] an increase in glutamate release is now believed to play an important role in the pathophysiology of convulsion-related disorders.[28] Consistent with the report that enflurane-induced seizure-like discharges in hippocampal neurons were completely inhibited by a glutamate/N-methyl-D-aspartate receptor antagonist,[7,8] our results provide direct evidence of potent glutamate release induced by enflurane and should help to clarify the mechanism of enflurane-induced convulsions.

References

1. Miller KW, Roth SH: Inside the "black box," Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KW. New York, Plenum Medical, 1986, pp 261–266

2. Drummond JC, Shapiro HM: Cerebral physiology, Anesthesia. Edited by Miller RD. New York, Churchill Livingstone, 1990, pp 621–658
3. Steen PA, Michenfelder JD: Neurotoxicity of anesthetics. ANESTHESIOLOGY 50:437–453, 1979
4. Stevens JE, Fujinaga M, Oshima E, Mori K: The biphasic pattern of the convulsive property of enflurane in cats. Br J Anaesth 56:395–403, 1984
5. Joas TA, Stevens WC, Eger II EI: Electroencephalographic seizure activity in dogs during anaesthesia. Br J Anaesth 43:739–745, 1971
6. Komatsu H, Ogli K: Opisthotonus during exposure to isoflurane, enflurane and halothane in mice. ANESTHESIOLOGY 67:771–774, 1987
7. MacIver MB, Roth SH: Enflurane-induced burst firing of hippocampal CA1 neurones. Br J Anaesth 59:369–378, 1987
8. MacIver MB, Kendig JJ: Enflurane-induced burst discharge of hippocampal CA1 neurones is blocked by the NMDA receptor antagonist APV. Br J Anaesth 63:296–305, 1989
9. Gray EG, Whittaker VP: The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. J Anat 96:79–88, 1962
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951
11. Hardy JA, Boakes RJ, Thomas DJE, Kidd AM, Edwardson JA, Virmani M, Turner J, Dodd PR: Release of aspartate and glutamate caused by chloride reduction in synaptosomal incubation media. J Neurochem 42:875–877, 1984
12. Inoue M, Hirose T, Inagaki C: Ethacrynic acid-induced glutamate release from mouse brain synaptosomes. Brain Res 543:160–162, 1991
13. Nicholls D, Attwell D: The release and uptake of excitatory amino acids. Trends Pharmacol Sci 11:462–468, 1990
14. Moody EJ, Suzdak PD, Paul SM, Skolnick P: Modulation of the benzodiazepine/γ-aminobutyric acid receptor chloride channel complex by inhalation anesthetics. J Neurochem 51:1386–1393, 1988
15. Ikemoto Y, Akaike N, Ono K: Differential effects of enflurane on Glu- and Ach-induced chloride currents in aplysia neurons. Life Sci 42:1557–1564, 1988
16. Nakahiro M, Yeh JZ, Brunner E, Narahashi T: General anesthetics modulate GABA receptor channel complex in rat dorsal root ganglion neurons. FASEB J 3:1850–1854, 1989
17. Klip A, Hill M, Ramlal T: Halothane increases cytosolic Ca^{2+} and inhibits Na^+/H^+ exchange in L6 muscle cells. J Pharmacol Exp Ther 254:552–559, 1990
18. Kimura K, Kami T, Satohne T, Kudoh M, Oyama T: Ethrane-N_2O-O_2 anesthesia and its arterial blood concentration in man. Masui 22:344–347, 1973
19. Atallah MM, Geddes IC: Metabolism of halothane during and after anaesthesia in man. Br J Anaesth 45:464–470, 1973
20. Meyers FH, Jawetz E, Goldfein A: General anesthetics, Review of Mediacal Pharmacology. Los Altos, California, Lange Medical Publications, 1980, pp 198–209
21. Jones RE, Linde HW, Deutsch S, Dripps RD, Price HL: Hemodynamic actions of diethyl ether in normal man. ANESTHESIOLOGY 23:299–305, 1962
22. Nicholls DG: Release of glutamate, aspartate, and γ-aminobutyric acid from isolated nerve terminals. J Neurochem 52:331–341, 1989
23. Curtis DR, Johnston GAR: Amino acid transmitters in the mammalian central nervous system. Ergebn Physiol 69:97–188, 1974
24. Pocock JM, Murphie HM, Nicholls DG: Kainic acid inhibits the synaptosomal plasma membrane glutamate carrier and allows glutamate leakage from the cytoplasm but does not affect glutamate exocytosis. J Neurochem 50:745–751, 1988
25. Sitges M: Effect of organic and inorganic calcium channel blockers on γ-amino-n-butyric acid release induced by monensin and veratrine in the absence of external calcium. J Neurochem 53: 436–441, 1989
26. Young AB, Fagg GE: Excitatory amino acid receptors in the brain: Membrane binding and receptor autoradiographic approaches. Trends Pharmacol Sci 11:126–133, 1990
27. Dinngledine R, McBain CJ, McNamara JO: Excitatory amino acid receptors in epilepsy. Trends Pharmacol Sci 11:334–338, 1990
28. Pearson J: Epilepsy, An Introduction to Neurotransmission in Health and Disease. Edited by Riederer P, Kopp N, Pearson J. Oxford, Oxford University Press, 1990, pp 366–374