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EFFECTS OF PENTOBARBITAL AND Ca^{2+} ON THE RESTING AND K^+-STIMULATED RELEASE OF SEVERAL ENDOGENOUS NEUROTRANSMITTERS FROM RAT MIDBRAIN SLICES*

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Abstract—The release of endogenous amino acids [γ-aminobutyric acid (GABA), glutamate, aspartate, glycine and alanine] and of 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) from rat midbrain slices was examined under various conditions of superfusion. Depolarization with high K^+ stimulated the release of all substances examined, but the K^+-stimulated release was Ca^{2+} dependent only for GABA, glutamate, aspartate, 5-HT and ACh. Pentobarbital, although not substantially affecting resting release, inhibited the K^+-stimulated release of GABA, glutamate, aspartate and ACh markedly and significantly. The effect of pentobarbital on K^+-stimulated 5-HT release was not statistically significant in this series of experiments. These results are consistent with the hypothesis that the barbiturate inhibits stimulated transmitter release by inhibiting Ca^{2+} influx during depolarization. The tissue content of amino acids and 5-HT decreased considerably from fresh tissue levels after 50 min of perfusion in regular low K^+ medium; in contrast, ACh tissue levels increased slightly during this time. K^+ stimulation resulted in an increased synthesis particularly of GABA, glutamate, glycine, alanine and ACh, for the amount released into the medium was far more than that lost from the tissue. 5-HT and possibly aspartate, on the other hand, were released into the medium on stimulation largely at the expense of tissue stores, under our experimental conditions.

In previous studies in our laboratory we demonstrated that barbiturates inhibit K^+-stimulated Ca^{2+}-dependent acetylcholine (ACh) release from rat midbrain slices [1, 2]. As a result of the findings of Blaustein and Ector [3] that these drugs inhibit depolarization-induced Ca^{2+} influx by nerve endings, it can be predicted that the Ca^{2+}-dependent stimulated release of all putative transmitters will be inhibited by them. But, data in the literature on transmitters other than ACh are meager and often conflicting.

Pentobarbital at a relatively high concentration reportedly enhanced electrically evoked release of radioactive γ-aminobutyric acid (GABA) [4]. Cutler *et al.* [5], however, also reported that pentobarbital and amobarbital (but not hexobarbital or phenobarbital) inhibited K^+-stimulated [^{3}H]GABA release. Furthermore, an inhibitory effect of pentobarbital on K^+-stimulated Ca^{2+}-dependent [^{3}H]GABA release from synaptosomes has been reported by Haycock *et al.* [6], but Olsen *et al.* [7] found no inhibition. To our knowledge the only study of stimulated endogenous GABA release was that by Cutler and Young [8], who demonstrated inhibition by several barbiturates including amobarbital and pentobarbital but not by phenobarbital or secobarbital. Pentobarbital also inhibited the stimulated release of glutamate, but amobarbital did not. In olfactory cortex slices *in vitro*, stimulated via the lateral olfactory tract, phenobarbital inhibited stimulated [^{14}C]glutamate release [9].

The diverse effects of the various barbiturates on amino acid release are not understood readily; K^+-stimulated ACh release was inhibited by all of the several barbiturates tested [2]. In the studies by Blaustein and Ector [3] on the inhibition of stimulated Ca^{2+} uptake by barbiturates, phenobarbital was not effective in one experiment at 0.9 mM. Sohn and Ferrendelli [10], however, observed an inhibition with 4.0 mM phenobarbital.

Relatively little work has been done on the effects of barbiturates on the release of other putative transmitters. Pentobarbital inhibited catecholamine release from perfused adrenal glands that had been stimulated by several different methods [11]. The electrically induced release of [^{3}H]norepinephrine (NE) from rat cerebral cortex slices was not inhibited by 10^{-4} M pentobarbital [12], but 2×10^{-4} M pentobarbital did inhibit K^+-stimulated release of [^{3}H]NE from rat cerebral cortical synaptosomes [6]. Phenobarbital inhibited the K^+-stimulated release of [^{3}H-5-hydroxytryptamine (5-HT) from brain slices [13, 14].

We decided it would be valuable to study the effects of pentobarbital on several putative transmitters at the same time so that, if differing effects

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on the various transmitters were found, they could not be the result of differing experimental conditions. Because radioactively labeled transmitters may not always simulate the behavior of endogenous substances and, because no more than two transmitters can be studied simultaneously by radioactive tagging procedures, we chose to measure the release of endogenous putative transmitters. Our results suggest that the K^+-stimulated release of GABA, glutamate, aspartate, 5-HT and ACh is at least partially Ca^{2+}-dependent and is inhibited by pentobarbital.

METHODS

Tissue preparation. Male Wistar rats (200–300 g) were decapitated for superfusion studies *in vitro*. The brain was removed and dissected on a petri dish on ice. The midbrain was isolated by removing the cerebral cortex, the hippocampi, the striatum and the septum. A transverse cut immediately caudal to the colliculi separated the midbrain from the cerebellum and pons-medulla. Each midbrain was weighed, and then prisms were prepared by cutting at 0.4 mm intervals in two planes at a 45° angle with a Brinkman–McIlwain tissue chopper. The slices were then suspended in 5 ml of incubation medium and the total suspension was loaded into a 25 mm Swinnex (Millipore Corp., Bedford, MA) and superfused with medium at 37° at 0.5 ml/min as described previously [15]. The medium contained (mM): 120 NaCl, 0.75 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose, either 5 or 50 KCl, and 5×10^{-5} M paraoxon (diethyl-*p*-nitrophenylphosphate, Sigma Chemical Co., St. Louis, MO) and was continuously bubbled with 5% CO_2/95% O_2 (final pH 7.5). Ascorbic acid (0.2 mg/ml of medium) was added to retard auto-oxidation of the monoamines. A Ca^{2+}-deficient medium was prepared by replacing the $CaCl_2$ with distilled water and by adding ethyleneglycol-bis (β-aminoethyl ether) tetraacetic acid (EGTA) (final concentration 2 mM) to chelate any remaining calcium. When 50 mM KCl or Ca^{2+}-deficient media were used, no adjustments were made in the concentration of the other ions. Sodium pentobarbital (5×10^{-4} M) was included in the medium, as indicated in Results. Samples of superfusate (2.5 ml) were collected as timed fractions (5 min) with a fraction collector. Tissue was recovered by forcing excess medium out of the Swinnex with a syringe filled with air and transferring the tissue with a spatula to a homogenizer containing 1 ml of the medium with which the tissue had last been perfused. The tissue was homogenized on ice with eight strokes of a pestle rotating at 840 rpm. To determine the amount of each putative neurotransmitter in unperfused tissue, rats were decapitated and the midbrain was dissected as described above. However, after chopping, the prisms were immediately homogenized in 1 ml of 5 mM KCl medium.

Assay of compounds. The acetylcholine in 500 μl superfusate samples and in 250 μl of the tissue homogenate was measured by the method of Goldberg and McCaman [16] with modifications [15]. In this radioenzymatic method, blanks and standards were prepared in low K^+ medium (since other medium constituents such as increased K^+ concentrations, pentobarbital and EGTA did not affect the assay) and then treated identically to experimental samples. An internal standard (0.2 μCi [^{3}H]ACh) was added to samples and standards to enable correction for recovery. The amount of ACh in the samples was determined by reference to the standard curve, which was linear over the range of our samples. Samples were counted in 12 ml of Aquasol (New England Nuclear Corp., Boston, MA) or ACS (Amersham–Searle Corp., Arlington Heights, IL) scintillation solution in a model 3375 Packard scintillation counter. Internal standards were used to determine counting efficiency and, because two isotopes were counted, the results were corrected for overlap.

A 400 μl superfusate sample and a sample of the tissue homogenate calculated to contain the equivalent of 20 mg tissue (100 μl) were assayed for alanine, glycine, γ-aminobutyric acid, glutamate and aspartate. The amino acids were quantified on a model 220 Tracor gas chromatograph after conversion to their respective 2,4-dinitrophenol-amino acid methyl ester derivatives [17]. Blanks and standards were prepared routinely in regular low K^+ incubation medium because preliminary tests showed that the concentrations of KCl, $CaCl_2$, paraoxon, pentobarbital, EGTA and ascorbic acid used did not affect the assays. These blanks and standards were treated identically to experimental samples in each experiment. Methyl stearate was added to all samples before chromatography to allow correction for variations in the sensitivity of the machine from sample to sample. The quantity of amino acids in the samples was calculated by reference to the results obtained for the standards, so that the results were corrected automatically for losses throughout the preparation. The assumption that the losses were equivalent in identically treated samples was verified (C.V. ≤ 8 per cent). Standards also demonstrated that the assay was linear with respect to the amount of amino acid added.

The norepinephrine and 5-hydroxytryptamine in a 1 ml superfusate sample and 500 μl of the tissue homogenate (equivalent to approximately 100 mg tissue) were isolated and measured concurrently by the methods of Anton and Sayre [18] and Maickel and Miller [19], respectively, using the modifications described previously [17]. The fluorescence of each sample was determined by an MKI Farrand spectrofluorometer. Blanks and standards prepared in low K^+ medium containing paraoxon and ascorbate were treated identically to experimental samples, and the amounts in experimental samples were calculated by reference to the standard curve. The assay was linear over the range of our samples.

Results are expressed as the amount of each compound in samples derived from 1 g (wet weight) of tissue ± S.E.M. The per cent inhibition of stimulated release by a treatment was calculated from the amount of transmitter released under the following conditions:

$$\frac{(\text{high } K^+_{\text{control}} - \text{low } K^+_{\text{control}}) - (\text{high } K^+_{\text{treatment}} - \text{low } K^+_{\text{treatment}})}{(\text{high } K^+_{\text{control}} - \text{low } K^+_{\text{control}})} \times 100$$

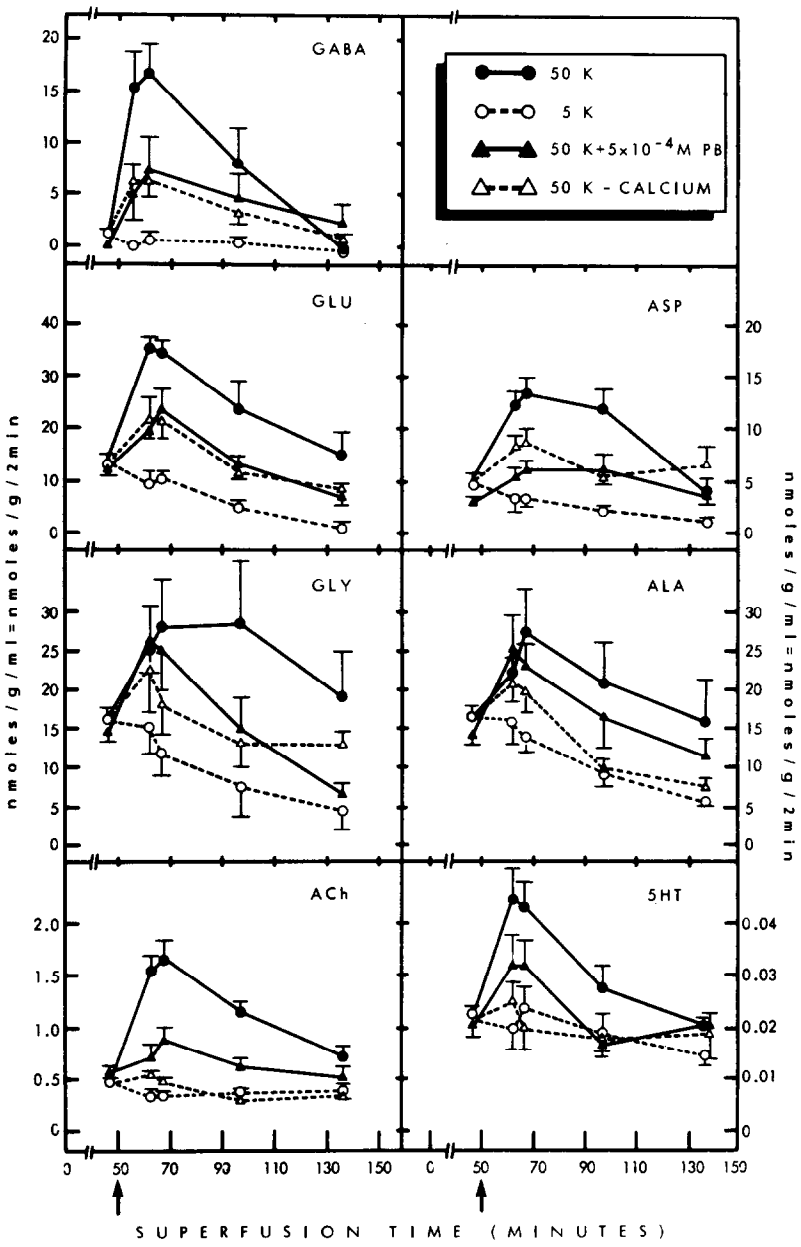

Fig. 1. Time courses of release of five amino acids, ACh and 5-HT during superfusion under various conditions. Rat midbrain slices were superfused and the substances that were released into the medium were assayed as described in Methods. Results are expressed as nmoles of substance released from 1 g of tissue in 2 min at the superfusion rate of 0.5 ml/min (mean ± S.E.M.).

Student's *t*-test was used to determine the statistical significance ($P < 0.05$) of the differences between the means.

RESULTS

The resting release from midbrain slices of the seven substances measured 45–50 min after superfusion began varied considerably (Table 1). The rate for 5-HT was lowest and that of ACh was next lowest. Among the amino acids, glutamate, glycine and alanine were the highest; aspartate was less than half that of glutamate, while GABA was by far the lowest of all the amino acids. When expressed as a fraction of the tissue levels at 50 min, however, the resting release rates between 45 and 50 min varied in a somewhat different order (Table 1). Glycine and alanine still had the highest rates, but 5-HT and ACh were the next highest. Aspartate was still less than glutamate, although not dramatically so, and GABA still had the lowest rate. Between 45–50 and 135–140 min of superfusion, resting release rates declined precipitously for GABA and glutamate, less dramatically for aspartate, glycine and alanine,

Table 1. Resting release of amino acids, ACh and 5-HT at two different times during superfusion with regular low K^+ medium

	Tissue content (nmoles/g)	Resting release (nmoles/g/min)	Fractional release rate* (min^{-1})	
	After 50 min superfusion	In low K^+ 45–50 min	At 45–50 min	
GABA	2260	0.595	0.00026	
Glutamate	2180	6.75	0.0031	
Aspartate	1100	2.54	0.0023	
Glycine	594	8.15	0.014	
Alanine	165	8.05	0.049	
ACh	28	0.24	0.0086	
5-HT	1.84	0.0111	0.0060	
	After 140 min superfusion	In low K^+ 135–140 min	At 135–140 min	Percent of 45–50 min
GABA	1760	0	0	0
Glutamate	1920	0.535	0.00028	9
Aspartate	1040	0.57	0.00054	23
Glycine	351	2.37	0.0067	48
Alanine	86	2.76	0.032	65
ACh	23.7	0.195	0.0082	95
5-HT	1.27	0.0075	0.0059	98

* Fractional release rate was calculated by dividing the resting release by the tissue content.

and relatively little for ACh and 5-HT (Table 1 and Fig. 1). Among the amino acids, the lower the fractional release rate at 45–50 min, the more rapid was the decline throughout the experiment. 5-HT and ACh, however, did not fit this pattern because they had only moderate fractional release rates at 45–50 min and showed essentially no change in the resting release between 45–50 and 135–140 min.

Raising the concentration of K^+ in the superfusing medium to 50 mM significantly increased the release of all these substances. This can be observed qualitatively in the graphs of the release with time (Fig. 1) and quantitatively by considering either the areas under the curves in these graphs or the peak release rate (60–65 or 65–70 min) relative to the resting release rate at 45–50 min just before high K^+ was introduced (Table 2). The magnitude of the increase in release rate caused by high K^+ varied considerably among the substances considered here. GABA release was stimulated to the greatest extent—some 15- to 20-fold. Glutamate, aspartate and ACh were stimulated 3- to 4-fold, and 5-HT, glycine and alanine release were stimulated to a lesser, but nevertheless significant, degree.

Similarly, the calcium dependence of the stimulated release was examined in several ways. From the data graphed in Fig. 1, the nearly complete dependence on Ca^{2+} of the stimulated release of ACh and 5-HT is evident, while the dependence of GABA, glutamate and aspartate, although partial, is clearly significant. The stimulated release of glycine and alanine, however, did not show a clear dependence on Ca^{2+} in the extracellular medium. This may in part be the result of the greater degree of variability. When just the peak time of stimulated release is considered (60–65 or 65–70 min, see Table 3), a similar conclusion is reached. In addition, the data in Table 3 show that there was no significant

Table 2. K^+ stimulation of release of amino acids, ACh and 5-HT*

	Resting release 45–50 min (nmoles/g/ml)	Peak K^+-stimulated release	Stimulated release/ Resting release (peak values)	Stimulated release/ Resting release (area under curve values)
GABA	1.19 ± 0.266 (37)	16.9 ± 2.73† (9)	15.9	22.7
Glutamate	13.5 ± 1.08 (38)	35.6 ± 1.83† (8)	2.5	3.8
Aspartate	5.08 ± 0.546 (36)	13.4 ± 1.63† (9)	2.5	3.8
Glycine	16.3 ± 1.34 (37)	28.1 ± 6.0† (9)	1.6	2.6
Alanine	16.1 ± 0.892 (38)	27.9 ± 5.10† (8)	1.6	1.9
ACh	0.48 ± 0.024 (35)	1.66 ± 0.170† (8)	3.5	3.0
5-HT	0.0223 ± 0.0017 (38)	0.045 ± 0.0058† (9)	1.7	1.6

* Release rates, in nmoles·g^{-1}·ml^{-1}, are given as means ± S.E.M. with the number of experiments in parentheses.
† Denotes values significantly greater than the corresponding resting release rate ($P < 0.05$, Student's t-test, one-tail).
The areas under the curves (from which the stimulated release/resting release was calculated) are given in Table 6.

Table 3. Effect of Ca^{2+} on resting and K^+-stimulated release of amino acids, ACh and 5-HT*

	High K^+	High K^+, No Ca^{2+}	Low K^+	Low K^+, No Ca^{2+}	% Inhibition of stimulated release (peak)	% Inhibition of stimulated release (area under curve)
GABA	16.9 ± 2.73 (9)	6.21 ± 1.59† (7)	0.64 ± 0.64 (5)	1.12 ± 0.71 (5)	69	63
Glutamate	35.6 ± 1.83 (8)	21.4 ± 4.56† (7)	9.33 ± 2.57 (5)	13.5 ± 3.74 (5)	70	67
Aspartate	13.4 ± 1.63 (9)	8.85 ± 1.14† (7)	3.41 ± 0.82 (5)	5.82 ± 2.37 (5)	70	60
Glycine	28.1 ± 6.0 (9)	18.4 ± 4.04 (6)	12.0 ± 3.04 (5)	20.2 ± 6.87 (5)	100	99
Alanine	27.9 ± 5.10 (10)	20.0 ± 2.60 (7)	14.0 ± 1.99 (5)	7.93 ± 2.97 (5)	13	59
ACh	1.66 ± 0.17 (9)	0.48 ± 0.030† (7)	0.35 ± 0.030 (4)	0.37 ± 0.028 (5)	92	94
5-HT	0.045 ± 0.0058 (9)	0.025 ± 0.0043† (7)	0.020 ± 0.0038 (5)	0.027 ± 0.0028 (4)	100	82

* Release data are given in nmoles · g^{-1} · ml^{-1}, mean ± S.E.M. (N), at the peak of K^+-stimulated release (60–65 or 65–70 min, see Fig. 1) for high K^+ and at the same time under the other conditions. The per cent inhibition of stimulated release was calculated as described in Methods, using the data in the first four columns or the appropriate areas under the curves (data not shown).

† Denotes high K^+ No Ca^{2+} values significantly different from the corresponding high K^+ value.

Table 4. Effect of pentobarbital on resting and K^+-stimulated release of amino acids, ACh and 5-HT*

	High K^+	High K^+ + PB	Low K^+	Low K^+ + PB	% Inhibition of stimulated release (peak)	% Inhibition of stimulated release (area under curve)
GABA	16.9 ± 2.73 (8)	7.23 ± 3.38† (6)	0.64 ± 0.64 (5)	0.81 ± 0.53 (5)	60	53
Glutamate	35.6 ± 1.83 (8)	19.6 ± 1.53† (5)	9.33 ± 2.57 (5)	8.54 ± 0.59 (5)	58	61
Aspartate	13.4 ± 1.63 (9)	5.90 ± 0.94† (4)	3.41 ± 0.82 (5)	1.93 ± 1.05 (5)	60	60
Glycine	28.1 ± 6.00 (9)	24.9 ± 4.97 (6)	12.0 ± 3.04 (5)	11.2 ± 3.02 (5)	15	51
Alanine	27.9 ± 5.10 (10)	23.3 ± 2.75 (5)	14.0 ± 1.99 (5)	10.0 ± 1.55 (5)	4	13
ACh	1.66 ± 0.17 (9)	0.89 ± 0.11† (6)	0.35 ± 0.03 (4)	0.56 ± 0.095 (5)	75	84
5-HT	0.045 ± 0.0058 (9)	0.032 ± 0.0066 (6)	0.020 ± 0.0038 (5)	0.027 ± 0.0065 (5)	80	76

* Release data are given in nmoles · g^{-1} · ml^{-1}, mean ± S.E.M. (N), at the peak of K^+-stimulated release (60–65 or 65–70 min, see Fig. 1) for high K^+ and at the same time under the other conditions. Pentobarbital (PB, 5×10^{-4} M) was included in the medium throughout the superfusion. The per cent inhibition of stimulated release was calculated as described in Methods, using the data in the first four columns or the appropriate areas under the curves (data not shown).

† Denotes high K^+ + PB values significantly different from the corresponding high K^+ value.

Table 5. Tissue levels of amino acids, ACh and 5-HT in fresh rat midbrain and in rat midbrain slices after superfusion under various conditions*

	Fresh tissue	50 min low K^+	50 min low K^+ + PB	140 min low K^+	140 min low K^+ + PB
			μmoles/g		
GABA	4.29 ± 0.174 (4)	2.26 ± 0.162 (7)	2.67 ± 0.370 (7)	1.76 ± 0.219 (5)	2.39 ± 0.268 (5)
Glutamate	11.2 ± 0.091 (4)	2.18 ± 0.233 (7)	2.81 ± 0.226 (7)	1.92 ± 0.238 (5)	2.58 ± 0.323 (5)
Aspartate	3.09 ± 0.097 (4)	1.10 ± 0.093 (6)	1.34 ± 0.202 (7)	1.04 ± 0.101 (5)	1.00 ± 0.098 (5)
Glycine	1.88 ± 0.054 (4)	0.594 ± 0.076 (6)	0.632 ± 0.067 (7)	0.351 ± 0.089 (5)	0.512 ± 0.107 (5)
Alanine	0.422 ± 0.014 (4)	0.165 ± 0.022 (7)	0.180 ± 0.019 (7)	0.086 ± 0.027 (5)	0.141 ± 0.039 (5)
			nmoles/g		
ACh	21.2 ± 1.12 (4)	28.0 ± 1.73 (6)	30.5 ± 1.66 (7)	23.7 ± 2.03 (5)	28.8 ± 2.22 (5)
5-HT	4.74 ± 0.481 (4)	1.84 ± 0.269 (6)	2.06 ± 0.198 (6)	1.27 ± 0.362 (5)	1.40 ± 0.236 (4)

	140 min low K^+, no Ca^{2+}	140 min high K^+	140 min high K^+ + PB	140 min high K^+, no Ca^{2+}
		μmoles/g		
GABA	1.98 ± 0.129 (5)	1.88 ± 0.145 (10)	2.49 ± 0.423 (6)	1.73 ± 0.188 (7)
Glutamate	2.31 ± 0.032 (5)	2.01 ± 0.123 (10)	2.78 ± 0.157 (6)	2.00 ± 0.144 (7)
Aspartate	0.847 ± 0.024 (5)	0.794 ± 0.039 (10)	0.858 ± 0.059 (6)	0.717 ± 0.046 (7)
Glycine	0.450 ± 0.040 (5)	0.260 ± 0.062 (10)	0.424 ± 0.066 (6)	0.310 ± 0.026 (6)
Alanine	0.107 ± 0.017 (5)	0.088 ± 0.017 (10)	0.146 ± 0.025 (6)	0.063 ± 0.0064 (7)
		nmoles/g		
ACh	26.0 ± 0.94 (5)	20.6 ± 1.49 (9)	23.0 ± 0.89 (6)	23.1 ± 1.23 (7)
5-HT	1.21 ± 0.145 (5)	0.849 ± 0.0858 (10)	1.80 ± 0.306 (6)	1.27 ± 0.164 (6)

* Values given are means ± S.E.M. (N). PB = pentobarbital, 5×10^{-4} M.

effect of removing Ca^{2+} on the resting release of any of these substances at that time point.

When 5×10^{-4} M pentobarbital was added to the superfusing medium, the stimulated release of GABA, glutamate, aspartate or ACh was clearly and significantly depressed (see Fig. 1 and Table 4). The stimulated release of 5-HT was decreased by the drug, but not significantly except at 95–100 min. Stimulated glycine release showed a somewhat different pattern, in that the rate of release with high K^+ was more variable and did not decline as dramatically with time; the effect of pentobarbital on stimulated glycine release, if any, appeared only at the very latest time examined (135–140 min). In Fig.

Table 6. Comparison of amount lost from the tissue with that recovered in the medium for amino acids, ACh and 5-HT*

	GABA	Glu	Asp	Gly	Ala	ACh	5-HT
(1) Tissue levels (low K^+ 50 min − low K^+ 140 min) = lost from tissue (nmoles/g)	500	260	60	243	79	4.3	0.57
(2) Area under low K^+ curve = nmoles/g released in 90 min	16.3	287	116	434	485	16.9	0.87
(3) Tissue levels (low K^+ 50 min − high K^+ 140 min) = lost from tissue (nmoles/g)	380	170	306	334	77	7.4	0.99
(4) Area under high K^+ curve = nmoles/g released in 90 min	370	1104	445	1128	950	51.4	1.37
(5) Tissue levels (low K^+ 140 min − high K^+ 140 min) = lost from tissue due to high K^+ alone, nmoles/g	−120	−90	246	91	−2	3.1	0.42
(6) Area under high K^+ curve − area under low K^+ curve = nmoles/g released by high K^+ alone	354	817	329	694	465	34.5	0.50

* Values for tissue levels at different times under different superfusion conditions are given in Table 5. Negative numbers in line 5 signify an increase in the tissue levels. Key: Glu, glutamate; Asp, aspartate; Gly, glycine; and Ala, alanine.

1, the data for resting release at 45–50 min are presented as either with or without pentobarbital, since the drug was added at zero time. At 45–50 min there was an apparent significant decrease in the resting release of GABA and aspartate by pentobarbital (Fig. 1). At 65–70 min (Table 4), however, there was no statistically significant effect of the drug on resting release although the trend was still there for aspartate. There were no other remarkable effects of pentobarbital on resting release.

Tissue levels of these seven substances were measured both at the end of 50 min of superfusion (at the point just before high K^+ would normally be introduced) and at the end of 140 min of superfusion with the various treatments; for comparison fresh tissue levels were also determined (Table 5). All of the amino acids and 5-HT decreased considerably from fresh tissue levels after the 50-min superfusion in low K^+ (or low K^+ and 5×10^{-4} M pentobarbital). Only ACh tissue levels did not decrease; they increased during this 50 min of superfusion by 32 per cent. The decrease in glutamate of 81 per cent was particularly large; the decrease in GABA was less marked (48 per cent). There were some further decreases in the tissue contents between 50 and 140 min of superfusion with normal low K^+ medium. ACh, in particular, returned almost to the fresh tissue level. Superfusion with high K^+ medium between 50 and 140 min caused little additional loss compared with low K^+ medium.

The amount lost from the tissue has been compared with the amount released into the medium (from the areas under the curves) (Table 6). During low K^+ superfusion, the amount of GABA released, i.e. collected in the medium, was considerably less than that lost from the tissue, whereas for the other amino acids and ACh and 5-HT, the amount lost was less than or similar to that recovered in the medium. When the tissue was superfused with high K^+ medium, the picture changed. K^+-stimulation caused increased synthesis of GABA, glutamate, glycine, alanine and ACh, for the amount released into the medium was more than that lost from the tissue (compare lines 5 and 6 in Table 6). For aspartate and 5-HT, however, there was, apparently, relatively little synthesis to compensate for the amounts released by high K^+. At one extreme, both in low K^+ and high K^+ medium, ACh synthesis kept pace with release, so there was little change in the tissue levels. On the other hand, 5-HT appeared to be lost to the medium from the tissue with little additional synthesis, especially under depolarizing conditions. This difference between ACh and 5-HT occurred in spite of the fact that the resting release of both transmitters remained relatively constant over the 50–140 min period (see Fig. 1). It should be noted, however, that the medium contained paraoxon to protect the released ACh from degradation, whereas no efforts were made to preserve the released 5-HT from reuptake and/or metabolism. Differences in the amounts of precursors available (choline and tryptophan) could also account for the difference in synthesis of ACh and 5-HT.

DISCUSSION

Our choice of rat midbrain slices for this study was dictated in part by previous data we had obtained with this preparation [1] and in part by the possibility that the barbiturates have a primary action on subcortical regions [20]. When extended to the several putative transmitters studied here, our results are consistent with the hypothesis that barbiturates block Ca^{2+}-dependent stimulated transmitter release. This is clearly shown for ACh, glutamate, aspartate and GABA. The results with 5-HT probably fit this pattern also, although the substantial inhibition by pentobarbital that was observed did not reach statistical significance. Other studies in our laboratory using rat midbrain slices incubated without superfusion confirm that K^+-stimulated [^{3}H]-5-HT release and endogenous 5-HT release (measured by high performance liquid chromatography) are both Ca^{2+}-dependent and inhibited by phenobarbital or pentobarbital [14].

It should be recalled that the barbiturates are not

very potent drugs. Thus, although the concentration of pentobarbital used in these experiments was relatively high, it resulted in slice concentrations only about ten times the estimated *in vivo* brain concentrations after a hypnotic dose (see Ref. 1). We have not tested other concentrations here, but the concentration-related nature of the inhibition of K^+-stimulated transmitter release has been shown for ACh [1, 2].

The results with glycine are more equivocal. If only the time of peak effect is examined, the K^+-stimulated release of glycine was neither Ca^{2+} dependent nor inhibited by pentobarbital. Nevertheless, the calculations of per cent inhibition suggest that there may be an effect of Ca^{2+} removal. The variability of these data precludes any stronger statements. A test of whether or not stimulated glycine release is indeed Ca^{2+} dependent and inhibited by barbiturates needs to be made in a system in which less variable data can be obtained, such as a region where glycine has a more prominent transmitter role (see below).

Alanine, included as a control amino acid, is not suspected of having a transmitter role [21]. Our results demonstrate that the small amount of alanine release stimulated by high K^+ was not very Ca^{2+} dependent and was not inhibited by pentobarbital. The specificity of the effect of the barbiturates on the depolarization-induced Ca^{2+} influx is further substantiated by the findings of Haycock *et al.* [6], who showed that pentobarbital did not affect [^{3}H]NE or [^{14}C]GABA release when the Ca^{2+} influx was induced by the ionophore A23187.

The inhibition of K^+-stimulated GABA release, however, is difficult to reconcile with the physiological findings that pre-synaptic inhibition, presumably mediated by GABA, is enhanced and prolonged by the barbiturates [22, 23]. It may be, as suggested by Haefely [24], that the depression of 'excitatory' transmission predominates at higher doses of the barbiturates and that the enhancement of GABA-induced synaptic inhibition occurs only at lower doses. Our results (even if at only one concentration of pentobarbital) do not reveal a weaker inhibitory effect on GABA release compared to the other transmitters examined (see Table 4). Such a distinction might better be sought in a more physiological preparation in which normal inputs can be stimulated electrically and the release of GABA as well as other transmitters can be examined simultaneously. Alternatively, the enhancement of GABAergic pre-synaptic inhibition may be related to an action on the GABA system other than GABA release, as is now thought to be the case for benzodiazepines [25].

There have been a large number of studies on the *in vitro* release of radioactive amino acids; the use of a variety of tissue preparations, methods of stimulation and other conditions have often made it difficult to determine whether or not the stimulated release was selective for transmitter candidates and/or Ca^{2+}dependent (e.g. see Refs. 9 and 26–39).

Furthermore, preincubating the tissue with radioactive amino acids and then looking at the release of radioactivity (even when it is identified as the amino acid in question and not a metabolite) can give rise to other problems: the radioactive material may not mix completely with, and thereby may not measure the behaviour of, the endogenous substance, or the radioactive material may be taken up and then released from sites that do not normally release it. In some *in vitro* preparations, radioactive amino acid release and endogenous amino acid release have been shown to give similar results [40], but in other cases they have not [41]. With glutamate and GABA, in particular, the possibility of the exogenously supplied amino acid being taken up into glia and then released on stimulation in a Ca^{2+}-dependent manner has been considered. Ca^{2+}-dependent K^+-stimulated release of radioactive GABA from glia has been found in some studies [41, 42] but not in others [43, 44]. K^+-stimulated, Ca^{2+}-dependent release of endogenous amino acids from non-neuronal sites may be less of a problem [41].

In spite of these considerations, the release of endogenous amino acids has been less thoroughly examined in preparations *in vitro*. K^+-stimulated, Ca^{2+}-dependent GABA release has been demonstrated in brain slices [40, 45, 46] and synaptosomes [47]. Ca^{2+}-dependent release of glutamate has also been shown in slices [48, 49], and glutamate release and aspartate release have been demonstrated in slices [46] and in synaptosomes [47]. Ca^{2+}-dependent release of endogenous glycine stimulated electrically and by high K^+ has been demonstrated only in synaptosome preparations from the spinal cord [50].

Identification of the transmitter utilized by a particular nerve tract or in a particular brain region can most readily be made also by measuring the release of the endogenous substance [49]. This has been a particular issue with glycine. Although some investigators have shown that labeled glycine is not released from cortical slices on stimulation [28, 51], others have released radioactive glycine with high K^+ or electrical stimulation from cortical preparations as well as spinal cord preparations [29, 32, 34–37], and in many of these cases it was also Ca^{2+} dependent. Endogenous glycine release from cortical synaptosomes, however, was not stimulated [52].

In stimulated slice preparations *in vitro*, the amount of radioactive glycine that is released from the cortex has generally been less than that of other amino acids released, such as GABA, as well as less than the amount of glycine released from the spinal cord. It has been suggested that the small amount of stimulated, Ca^{2+}-dependent glycine release from the more rostral portions of the neuroaxis might be occurring from a relatively small number of neurons in these regions utilizing glycine as a neurotransmitter [32]. In contrast to others [53–55], Davidoff and Adair [32] have observed a small amount of high affinity Na^+-dependent glycine uptake in the cortex, which might mediate the removal of released glycine from the synaptic cleft of these few glycinergic neurons. Our data on the midbrain may be consistent with the possibility that there are a few neurons using glycine as a transmitter in this region, for some methods of calculation indicate there is a release that is Ca^{2+} dependent (Table 3). The data, however, are too variable to make a more definitive statement.

Stimulated release of [^{3}H]-5-HT from brain slices

has been observed by others [51, 56], but when tested, the stimulated release was not Ca^{2+} dependent. Ca^{2+}-dependent, K^+-stimulated release from synaptosomes of [^{3}H]-5-HT [57] and endogenous 5-HT [58, 59] has been shown.

We were unable to detect dopamine (DA) in the superfusates, and NE was detected infrequently. Attempts to preserve the NE and DA released, use of a different brain region to increase the proportion of dopamine neurons, and use of a more sensitive assay method may be necessary to obtain reliable results with these transmitters. K^+-stimulated, Ca^{2+}-dependent release of endogenous NE and DA has been measured recently in slice preparations [60, 61].

Our data clearly show a Ca^{2+}-dependent, K^+-stimulated release for the amino acids GABA, glutamate and aspartate, as well as for 5-HT and ACh, which suggests that these substances play a transmitter role in the midbrain region. The relative degree of stimulation observed is in the range of that found by others, with the exception of GABA. The 15- to 20-fold stimulation of release by 50 mM KCl is greater than that seen in other systems. Others have found that, even without efforts to inhibit GABA transaminase, GABA released into the incubation medium is not greatly metabolized [42, 46]. Thus, the magnitude of the stimulated release might be attributed to the use of a superfusion system that would tend to remove the released GABA before it could be taken back up by the tissue again. The lesser degree of stimulation in the case of glutamate and aspartate, on the other hand, may be the result of rapid metabolism, as has been reported [42, 46]. 5-HT also could have been decreased by metabolism because, even though monoaminoxidase is an intracellular enzyme, broken cells in the slice preparation could have allowed the 5-HT to be metabolized. It would be of interest to determine if the stimulated rate of release of glutamate, aspartate or 5-HT can be enhanced by inhibitors of their metabolism. Since some reuptake could be occurring, even with the superfusion system, inhibition of this process could also be tested for its effect on release. ACh was protected from metabolism by including paraoxon in the medium, and ACh is not taken up readily by the choline transport system [62]. Pre-synaptic feedback mechanisms, however, have been shown to alter ACh release, so the release of this transmitter was also probably not at the highest possible rate [63, 64].

Expression of the release as a fractional efflux may not be entirely appropriate or very accurate because all the substances were not released from a finite store (synthesis of GABA, glutamate, glycine, alanine and ACh appears to occur during K^+ stimulation). In addition, Nadler *et al.* [46] have noted that the magnitude of the Ca^{2+}-dependent, K^+-stimulated release is independent of the tissue amino acid content and suggested that only a small pool was affected by the releasing conditions.

In our preparations, the amount of glutamate released was greater than the amount of aspartate released. The possibility of aspartate having a unique transmitter role, or serving only to modulate glutamate action, has been discussed [65]. In some situations, such as stimulation of the lateral olfactory tract, only glutamate is released [49]. The relative amounts of glutamate and aspartate released in the present experiments, however, probably have little particular significance since the tissue is so heterogeneous.

All of the substances examined were released at maximum rates within 10–15 min of the switch to superfusion with high K^+ medium, and then the rate declined. (The delayed peak is primarily the result of the dead space in the system; see Ref. 15). The tissue stores of GABA, glutamate and ACh were not diminished at the end of superfusion, which might suggest that sufficient transmitter should have been available for continued maximal release. However, if the releasable store is a small fraction of the total tissue content, depletion would not be detectable. A rapid rise and then fall of stimulated release of GABA, glutamate and aspartate were also noted when very short periods of stimulation were used [46]. These authors had additional data that argued against the suggestion that there is a decline in Ca^{2+} permeability with continuous stimulation, but they could offer no other explanation. In the case of ACh, it has been shown [15] that the presence of the precursor choline in the superfusing medium maintains the stimulated release near the maximal rate. The possibility that glutamine may serve as a precursor of glutamate and can enhance the amount of glutamate released has been investigated [48, 66, 67]. The minimal synthesis of aspartate during stimulated release may suggest that insufficient glutamate is available for transamination to releasable aspartate. The almost complete lack of synthesis during stimulated release of 5-HT might also simply reflect a relative lack of tryptophan available in the preparation. It would be of interest to determine whether or not providing glutamine or other suitable precursors would allow the stimulated release of GABA, glutamate and aspartate to be maintained at their highest rates.

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