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Effects of Polyvalent Cations on Stimulus-Coupled Secretion of [^{14}C]-γ-Aminobutyric Acid from Isolated Brain Synaptosomes

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SUMMARY

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The alkaline earths Ca^{++}, Sr^{++}, and Ba^{++} stimulated the secretion of [^{14}C]γ-aminobutyric acid from isolated brain synaptosomes at various K^+ and veratridine concentrations. K^+ and veratridine produced different orderings of alkaline earth secretion-stimulation efficacy. In addition, La^{+++} stimulated release in the presence of either K^+ or veratridine. Ca^{++} concentrations above physiological levels inhibited secretion in the presence of moderately elevated K^+ concentrations, but not in Na^+-free veratridine solutions. Tetrodotoxin inhibited Ca^{++}-stimulated secretion only when veratridine was present. Mn^{++} inhibited secretion in the presence of either K^+ or veratridine. These results agree well with comparable data from the neuromuscular junction and the adrenal medulla. Consideration of differential membrane permeability to divalent cations and differential interaction of the ions with intracellular binding sites provides a rationale for the relative efficacies of the alkaline earths.

INTRODUCTION

Recently it has become possible to study stimulus-coupled secretion of transmitters from brain synaptosomes (nerve ending particles) in a sufficiently unambiguous fashion to permit clear evaluation of the effects of chemical agents upon these processes. Previous demonstration of a rapid, calcium-stimulated, potassium-facilitated, magnesium-inhibited release of [^{14}C]γ-aminobutyric acid (1) from a preparation in which storage vesicles are present (2) fulfills for this system the conditions of stimulus-secretion coupling (3).

The isolated synaptosomal preparation provides advantages over some of the other techniques used to study transmitter function. Synaptosomes have been particularly useful in studying the localization (4–6), synthesis (4, 7), uptake (8, 9), metabolism (7), and recently release (1, 10, 11) of neurotransmitters.

The synaptosomal system is particularly well suited for the evaluation of the molecular mechanisms of, and drug effects upon, transmitter secretion. Release over short intervals of time may be investigated so that

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reuptake, short-term metabolic effects, and diffusional barriers may be minimized. In contrast to tissue slice preparations, there are no intact neural circuits that could mediate indirect release effects from interactions between neurons. Previously reported data (1) and the data reported here illustrate the suitability of this system for exploring a wide range of variables and demonstrate the close parallel of this system to the well-studied neuromuscular junction.

At the neuromuscular junction and in the adrenal medulla, elevated potassium accelerates calcium-dependent secretion (12, 13). To varying degrees other polyvalent cations (such as barium and strontium) can replace calcium in stimulating release (12–15). Divalent cations, including magnesium and manganese, inhibit this release (14, 16). At present, however, the site(s) of action is unidentified, and the mechanisms of divalent cation stimulation and inhibition are unknown. According to one prominent theory, developed by Blioch *et al.* (17), polyvalent cations that permeate the plasma membrane and neutralize the inner surface charge stimulate release. The inhibitory divalent ions (magnesium and manganese) are thought to be relatively impermeant. Instead, their extracellular presence inhibits calcium permeation. The decreased calcium permeation is then reflected by a decrease in the stimulus-secretion coupling process(es). The appeal of this particular theory also derives from its ability to account for temperature and osmotic effects. Furthermore, the proposed mechanism of the intracellular action of calcium subsequent to permeation is based on the well-specified property of calcium to induce flocculation and fusion in colloid systems (17, 18).

In this study we investigated the concentration dependence of calcium, strontium, barium, and lanthanum stimulus-coupled secretion of [^{14}C]γ-aminobutyric acid from brain synaptosomes. Potassium and veratridine facilitation of polyvalent cation-stimulated release, as well as the inhibition of these effects by manganese and tetrodotoxin, are interpreted on the basis of altered membrane permeability to the polyvalent cations.

METHODS

The following experimental protocol was observed for all results reported below. Tissue fractions were isolated, washed with incubation solution, and incubated in the presence of [^{14}C]-GABA[3] and aminooxyacetic acid [an inhibitor of GABA degradation (19) and possibly synthesis (20)]. The tissues then were loaded on filters and washed six times with the appropriate wash solution (calcium-free), followed by one wash with the appropriate experimental wash solution (with or without stimulating polyvalent cation added). Radioactivity was counted in the experimental filtrates and the material remaining on the filters.

Preparation of tissue. Forebrains from adult male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, Cal.) were used in this study. Crude mitochondrial fractions containing synaptosomes were prepared by the method of Cotman and Matthews (21).

Two types of synaptosomal fractions were derived from this preparation. The crude mitochondrial fraction was washed once in 0.32 M sucrose and again pelleted as the crude mitochondrial fraction (P_2 synaptosomes). Synaptosomes were separated from the crude mitochondrial fraction on a Ficoll-sucrose gradient according to Cotman and Matthews (21) (purified synaptosomes). The preparations were washed twice in incubation solution (see below) and kept as the pellet at 0–4° until incubation with [^{14}C]-GABA.

Solutions. Three general solutions were used following fractionation: incubation, wash, and experimental wash. All contained 5.0 mM KOH, 10.0 mM glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, and 0.10 mM aminooxy acetic acid adjusted to pH 7.4 ± 0.05 with Tris base. Incubation solutions also contained 150 mM NaCl, 1.2 mM Na_2HPO_4, and 1.2 mM $MgSO_4$ (no addition of calcium).

All wash solutions contained 1.0 mM $MgCl_2$ (sodium-free unless otherwise noted). Choline chloride and KCl were added to these solutions such that their total concen-

[3] The abbreviation used is: GABA, γ-aminobutyric acid.

tration was 150 mM (e.g., a solution termed 5.0 mM K^+ contained 5.0 mM KOH, no KCl, and 150 mM choline chloride; a solution termed 55.0 mM K^+ contained 5.0 mM KOH, 50.0 mM KCl, and 100 mM choline chloride). Some wash solutions contained veratridine (50–150 μM), $MnCl_2$ (15.0 mM), or tetrodotoxin (0.8 μM) as appropriate. When metabolic effects were investigated, inhibitors were added to 55.0 mM K^+ wash solutions.

A given experimental wash solution differed from its preceding wash solution only with respect to the addition of the appropriate polyvalent salt ($CaCl_2$, $SrCl_2$, $BaCl_2$, or $LaCl_3$ up to 10.0 mM).

Although KCl, choline chloride, or NaCl concentrations were altered, ionic and osmotic strength remained constant for all solutions.

All reagents were analytical reagent grade products of Mallinckrodt Chemical Works, with the following exceptions: aminooxy acetic acid, choline chloride, 2,4-dinitrophenol, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid, oligomycin, ouabain, and Tris base, from Sigma Chemical Company; veratridine, from Aldrich Chemical Company; [^{14}C]GABA, from Amersham/Searle; $LaCl_3$, from British Drug Houses; gramacidin D, from Calbiochem; and NaCN, from J. T. Baker.

Incubation with [^{14}*C*]*GABA.* Tissue pellets were resuspended in 5.0 ml of incubation medium containing 0.5 μM [^{14}C]GABA (203 mCi/mmole) and brought to room temperature (22–26°). Purified and P_2 synaptosomal fractions were incubated at a concentration of 1 mg/ml. Protein content was assayed according to Lowry *et al.* (22). During the 20-min incubation period the tissue suspensions were aerated with a hydrated O_2–CO_2 mixture (95:5%).

Filters and washes. As described previously (1), the apparatus consisted of four filter units resting on a vacuum box with an internal sliding shelf which allowed simultaneous, individual collection of filtrates from the four filter units. Each filter unit consisted of a modified, disposable Millipore Swinnex (25-mm) filter holder containing a glass fiber Whatman GF/A filter on top of a 0.4 μm Nucleopore filter.

Immediately following incubation the tissue was diluted with an additional 5.0 ml of incubation solution, and aliquots of the suspension were deposited onto the four filters (2 ml/filter). Following suction of the tissue suspension onto the filters, a total of seven washes (six washes and one experimental wash) were applied to the filters. (Specifically, one wash cycle consisted of 2.2 ml of solution added between 0 and 5 sec, vacuum applied at 25 sec, and release of vacuum at 55 to 60 sec. Suction of solutions was complete within approximately 10–15 sec following application of the vacuum.) The six washes applied to a given filter were identical, and were deficient in the appropriate polyvalent cation under investigation. In order to allow only stimulus-secretion coupling, only a pulse of polyvalent cation was used to excite release. The seventh wash, containing polyvalent cation, is thus referred to as the polyvalent experimental wash.

During the experimental wash at least one of the four filters received the same solution as in washes 1–6 and was referred to as the baseline experimental wash. The other filters received their appropriate experimental wash solution (identical wash solution plus polyvalent cation). Within a given four-filter run, only one variable was investigated (e.g., one polyvalent cation at different concentrations, or different polyvalent cations at one concentration).

Release. Radioactivity from the experimental wash was counted in a Triton-toluene (23) medium at approximately 80% efficiency and was corrected according to the channel ratio method. Radioactivity in the filters following the experimental wash was counted in the same medium following overnight solubilization in 2.0 ml of 1.0% sodium dodecyl sulfate and 20 mM EDTA. In the presence of aminooxy acetic acid 95% of the radioactivity was identified as GABA (1).

Baseline efflux of radioactivity, in filtrates from those experimental washes deficient in polyvalent cations, is expressed as a percentage of total radioactivity. Total radioactivity prior to the experimental wash was calculated by adding the radioactivity in the experimental filtrate to that left on the filter after the experimental wash. Re-

lease is expressed as a percentage of total radioactivity released above baseline efflux, according to the formula

$$\text{release} = \left[\left(\frac{\text{cpm in filtrate}}{\text{cpm in filtrate} + \text{filter}}\right)\text{in polyvalent experimental wash} - \left(\frac{\text{cpm in filtrate}}{\text{cpm in filtrate} + \text{filter}}\right)\text{in baseline experimental wash}\right] \times 100$$

Inhibition (as a percentage of normal release) was calculated from the formula

$$\text{inhibition} = \left(1 - \frac{\text{release in presence of inhibitor}}{\text{release in absence of inhibitor}}\right) \times 100$$

with standard errors of the mean calculated according to Topping (24).

All comparisons utilized a Kruskal-Wallis analysis of variance, *t*-test, or sign test (25, 26) as appropriate (significance level, $p < 0.05$, two-tailed). Two-point slope analysis was performed on different scores obtained from related samples by analysis of variance.

RESULTS

Metabolism and baseline. To a large extent the rapid release studied was independent of short-term metabolic effects. Addition of oligomycin (6 μM), NaCN (20 μM), gramacidin D (10 μM), ouabain (100 μM), or dinitrophenol (100 μM) to the Na^+-free, Ca^{++}-free wash solutions (55.0 mM K^+) did not produce appreciable effects on Ca^{++}-stimulated release. [In the presence of Na^+, it is expected that some of these compounds may have effects upon release (27).] The Na^+-free choline chloride wash solution was used here because it eliminates GABA reuptake (28) and any transmembrane Na^+-Ca^{++} exchange mechanism (29). As reported previously (1), replacement of Na^+ by choline ion does not change the amount of 1.0 mM Ca^{++}-stimulated release in 55.0 mM K^+.

Similarly, release when expressed as a percentage of total radioactivity over baseline (see METHODS) did not vary with changes in the baseline efflux. However, variations in treatment parameters, under certain circumstances, caused such baseline shifts. The effects of the various wash solutions upon baseline efflux (percentage of total [^{14}C]GABA present on filter) are presented in Table 1. Increasing the concentration of veratridine produced no appreciable effect upon baseline efflux in the Na^+-free condition. Increasing K^+ concentrations increased baseline efflux ($H = 17.2$, $df = 3$). The addition of $MnCl_2$ to either the K^+ or veratridine washes may slightly lower baseline efflux. This independence of release from short-term metabolism and baseline shifts renders this system suitable for examination of other variables.

Calcium stimulation of [^{14}C]GABA release in the presence of sodium-free, potassium, or veratridine wash solutions. Ca^{++}-stimulated release in the presence of 55.0 mM K^+ was demonstrated previously (1). Figure 1 (top) illustrates the effects of pulsing Na^+-free experimental wash solutions containing various concentrations of K^+ and Ca^{++}. For a given concentration of Ca^{++}, higher concentrations of K^+ produced greater release of [^{14}C]GABA. However, for certain concentrations of K^+, higher concentrations of $CaCl_2$ inhibited release (Fig. 2). As K^+ concentrations were raised, relatively higher concentrations of $CaCl_2$ were required to produce the inhibition.

Using the purified synaptosomal fraction, Ca^{++}-stimulated release in the presence of Na^+-free veratridine wash solutions was demonstrated in this study: 100 μM veratridine with a pulse of 1.0 mM $CaCl_2$ produced a significant increase in [^{14}C]GABA efflux compared to a similar pulse lacking $CaCl_2$ ($t = 2.8$, $df = 6$). Various concentrations of veratridine and $CaCl_2$ increased release of [^{14}C]GABA from P_2 synaptosomal preparations (Fig. 1, bottom). Although small, the effects of treatment with both 100 and 150 μM veratridine differed significantly from baseline (sign test: 150 μM, 11 of 12; 100 μM, 15 of 18). As illustrated in the

TABLE 1

Influence of various sodium-free wash solutions on baseline efflux

Baseline efflux is shown as a percentage of total radioactivity from those P_2 synaptosomes which received polyvalent-free experimental washes (baseline experimental wash). Wash solutions are described under METHODS. Results are the means ± standard errors of the number of determinations shown in parentheses.

Wash solution	Concentration	[^{14}C]GABA present on filter
	mM	*% total*
K^+	5.0	1.56 ± 0.14 (4)
	10.0	1.46 ± 0.10 (4)
	25.0	1.94 ± 1.00 (2)
	55.0	2.14 ± 0.24 (4)
K^+ with 15 mM Mn^{++}	55.0	1.84 ± 0.02 (2)
Veratridine	0.05	1.36 ± 0.04 (2)
	0.1	1.30 ± 0.10 (4)
	0.15	1.32 ± 0.22 (4)
Veratridine with 15 mM Mn^{++}	0.1	1.22 ± 0.06 (2)
	0.15	1.16 ± 0.04 (2)

logarithmic plot, increasing $CaCl_2$ concentrations increased release ($H = 14.2, df = 2$). In the case of release in the presence of the Na^+-free veratridine wash solutions, no indication of $CaCl_2$ inhibition was observed.

Na^+-free wash solutions and experimental washes were employed to minimize reuptake processes (see *Metabolism and baseline*). In this way data can be interpreted in terms of stimulus-secretion coupling. However, substitution of Na^+ for choline in the veratridine wash solution may have facilitated Ca^{++}-stimulated release (Table 2), in contrast to elevated K^+ conditions, under which Na^+ removal does not affect release (1).

Alkaline earth stimulation of [^{14}C]GABA release in the presence of sodium-free, potassium or veratridine wash solutions. Although Mg^{++} does not stimulate release in this system (1) and others (see ref. 3), Ca^{++}, Sr^{++}, and Ba^{++} were effective stimulants of [^{14}C]GABA release.

Table 3 illustrates the relative efficacy of 1.0 mM $CaCl_2$, $SrCl_2$, and $BaCl_2$ in stimulating the release of [^{14}C]GABA from purified synaptosomes in the presence of sodium-free

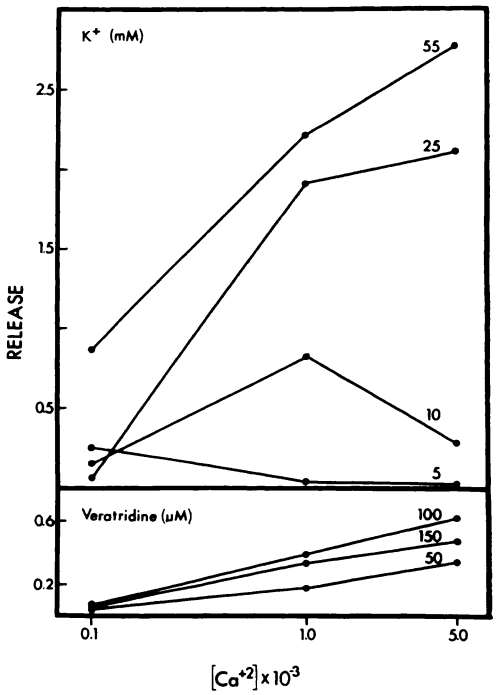

FIG. 1. *Calcium stimulation of [^{14}C]GABA release in the presence of sodium-free, potassium or veratridine wash solutions.*

Following six washes with the indicated solution concentration of K^+ or veratridine, P_2 synaptosomes received experimental washes containing the indicated concentration of $CaCl_2$ (except for Ca^{++}-free baseline filters). Each K^+ point represents two to four determinations; each veratridine point represents four to six determinations. Solutions and calculation of release are described under METHODS.

55.0 mM K^+ wash solutions. Identical treatment of P_2 synaptosomal fractions produced a similar order of efficacies (see also Table 3). Any differences between the efficacies of the ions is difficult to assess, however, because of the relatively large standard errors. Stimulation of release from P_2 synaptosomes in 55.0 mM K^+ at various divalent concentrations is shown in Fig. 3 (left). The effects of Ba^{++} and Ca^{++}, the more effective releasing agents at lower concentrations, appeared to approach an asymptote above 1.0 mM divalent cation concentration, while Sr^{++} effects continued to climb (logarithmic scale). An analysis of variance, in fact, demonstrated slope differences (see

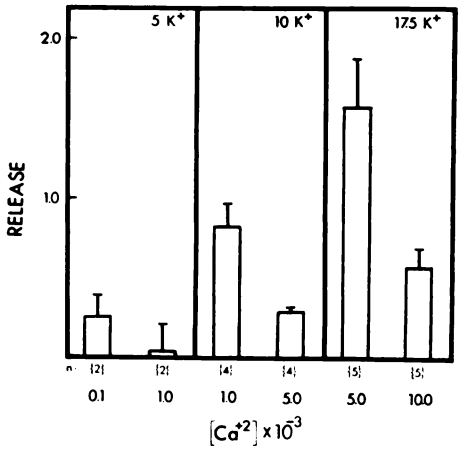

FIG. 2. *Calcium inhibition of calcium-stimulated release of [^{14}C]GABA*

P_2 synaptosomes were washed in the indicated Na^+-free, K^+ wash solutions. Experimental washes (except for baseline experimental washes) contained $CaCl_2$ as indicated. Notice that as K^+ concentrations were increased, a larger $CaCl_2$ concentration was necessary to produce inhibition. Solutions and calculation of release are described under METHODS. K^+, 5.0 mM: $t = 1.1$, $df = 3$. K^+, 10.0 mM: $t = 4.0$, $df = 7$. K^+, 17.5 mM: $t = 3.2$, $df = 9$. Error bars represent the standard error of the mean.

METHODS) from 1.0 to 5.0 mM alkaline earth ($H = 8.5$, $df = 2$). In view of the Ca^{++} inhibition of K^+-facilitated release at lower concentrations of K^+ (as seen in Fig. 2), it appears that the divalent cations which are more effective in stimulating release also have the greater inhibitory effects at higher concentrations.

A clearly different ordering of efficacies was obtained for the alkaline earths in the presence of Na^+-free 100 μM veratridine wash solutions (Fig. 4; compare with Table 3). Again, the divalent cations (5.0 mM in this case) produced an identical ordering of release efficacy for both the purified and P_2 synaptosomes. Furthermore, that the alkaline earth efficacy was ordered for veratridine is unambiguous (purified synaptosomes, $H = 21.9$, $df = 2$; P_2 synaptosomes, $H = 8.5$, $df = 2$). The effects of the divalent cations on [^{14}C]GABA release from the P_2 synaptosomal fraction are plotted for various divalent concentrations in Fig. 3 (right). Release in the presence of veratridine, as opposed to K^+, did not appear to approach an asymptote with increasing divalent concentration in the logarithmic plots.

The effects of the alkaline earths in veratridine-free (and Na^+-free) wash solutions are also shown in Fig. 3 (right, open circles). Clearly the effects of barium in veratridine, and in part the effects of strontium, were independent of the presence of veratridine in the wash solutions. However, 5.0 mM

TABLE 2

Influence of sodium on calcium-stimulated [^{14}C]GABA release in the presence of veratridine

Tissue aliquots from P_2 synaptosomes were washed six times with either Na^+-free (150 mM choline chloride) or Na^+ (150 mM NaCl) wash solutions (see METHODS) in the presence or absence of 100 μM veratridine. Release was calculated as described under METHODS, by subtracting the Ca^{++}-free baseline experimental wash from the experimental wash with 5.0 mM $CaCl_2$ added. Results are the means ± standard errors of two determinations.

Wash solution	[^{14}C]GABA release
	% total
Veratridine-free	
150 mM choline chloride	0.11 ± 0.24
150 mM NaCl	0.37 ± 0.10
100 μM veratridine in	
150 mM choline chloride	1.09 ± 0.01
150 mM NaCl	2.12 ± 0.62

TABLE 3

Alkaline earth stimulation of [^{14}C]GABA release in the presence of sodium-free, elevated potassium wash solutions

Purified and P_2 synaptosomes received experimental washes containing a 1.0 mM concentration of the indicated divalent cation added to the 55.0 mM K^+ wash solution. Solutions and calculation of release are calculated as described under METHODS. Results are the means ± standard errors of the number of determinations shown in parentheses.

Cation (1.0 mM)	Purified synaptosomal [^{14}C]GABA release	P_2 synaptosomal [^{14}C]GABA release
	% total	*% total*
Ca^{++}	3.60 ± 0.06 (2)	2.41 ± 0.46 (5)
Sr^{++}	2.87 ± 0.80 (2)	1.94 ± 0.61 (5)
Ba^{++}	4.28 ± 0.93 (2)	2.57 ± 0.50 (5)

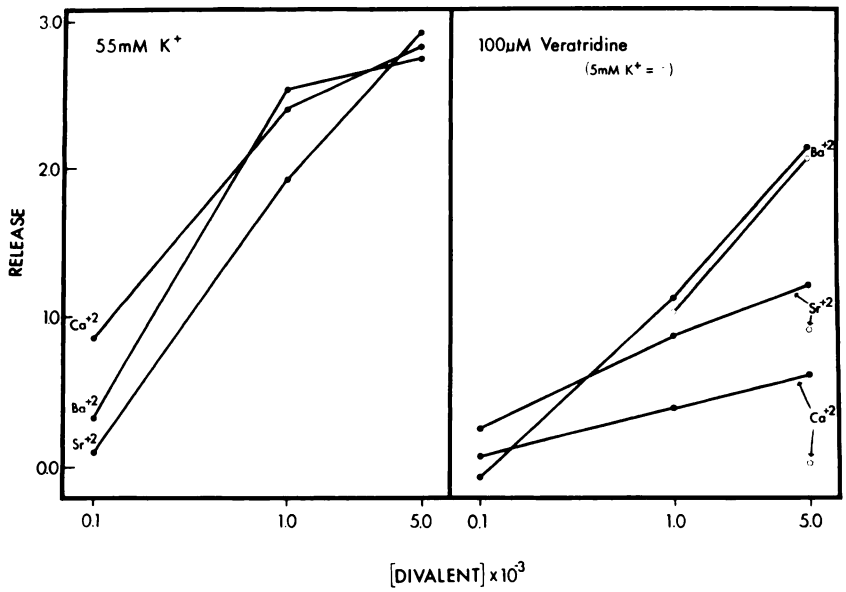

FIG. 3. *Alkaline earth stimulation of [^{14}C]GABA release*

Tissue aliquots from P_2 synaptosomal fractions were washed with Na^+-free, 55.0 mM K^+ (left) or 100 μM veratridine (right) solutions. Except for baseline experimental washes, experimental washes contained the indicated divalent at the indicated concentration. O (right), release when veratridine was omitted from the washes (thus representing divalent cation-stimulated release at 5.0mM K^+). Each point represents four to six determinations. Solutions and calculation of release are described under METHODS.

strontium and calcium released significantly more [^{14}C]GABA in the presence than in the absence of veratridine (strontium, $t = 2.9$, $df = 5$; calcium, $t = 3.7$, $df = 7$).

Manganese and tetrodotoxin inhibition of calcium-stimulated release. Previously we reported that either 1.0 mM $MnCl_2$ or 16.0 mM $MgCl_2$ decreased the release of GABA from the purified synaptosomal fraction, but in neither case was this inhibition complete (1). However, as shown in Table 4, at 15.0 mM $MnCl_2$, the inhibition of Ca^{++}-stimulated release was total in the presence of either 55.0 mM K^+ or 100 μM veratridine.

Tetrodotoxin (0.8 μM) in the Na^+-free wash solutions inhibited Ca^{++}-stimulated release in the presence of veratridine (100 μM veratridine, 5.0 mM Ca^{++}: 71 ± 24% inhibition, $n = 2$). In contrast, tetrodotoxin produced equivocal effects on release in the presence of K^+ (55.0 mM K^+, 1.0 mM Ca^{++}: 12 ± 21% inhibition, $n = 2$).

Lanthanum effects on release. $LaCl_3$ produced slight but significant stimulation in the presence of either the 55.0 mM K^+ or 100 μM veratridine, Na^+-free wash solution (Table 5) (K^+, $H = 16.3$, $df = 2$, veratridine, $H = 16.3$, $df = 2$). The release produced by lanthanum in 55.0 mM K^+ was much smaller than that produced by calcium ($t = 13.5$, $df = 5$).

DISCUSSION

Chemical agents may be categorized with regard to their facilitatory or inhibitory effects on stimulus-secretion coupling. Over the concentration ranges in this study, both K^+ and veratridine facilitated divalent cation-stimulated release in a concentration-dependent fashion. Although the effects of veratridine on spontaneous release have not been widely investigated (10),[4] elevated K^+ concentrations are reported to facilitate divalent cation-dependent release in several other systems, including the neuromuscular junction and the chromaffin cells of the adrenal medulla (12–15). Furthermore, the

[4] Personal communication by Albuquerque and Jansson cited in ref. (10).

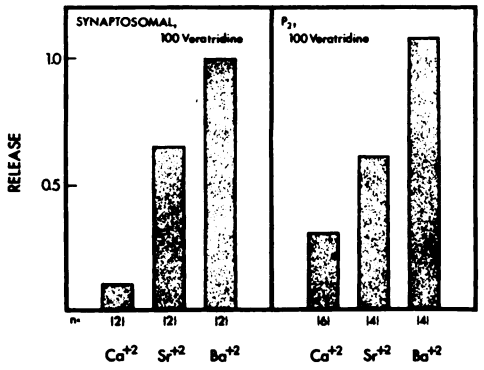

FIG. 4. *Alkaline earth stimulation of [^{14}C]GABA release in the presence of sodium-free, veratridine wash solutions*

Except for baseline experimental washes, 5.0 mM divalent cation was added to the experimental 100 μM veratridine wash solutions of both purified (left) and P_2 (right) synaptosomal fraction tissue aliquots. Solutions and calculation of release are described under METHODS.

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TABLE 4

Manganese inhibition of calcium-stimulated [^{14}C]GABA release

$MnCl_2$ (15.0 mM) was added to Na^+-free, K^+ and veratridine wash and experimental wash solutions. Experimental washes, except for the baseline experimental wash, contained the indicated $CaCl_2$ concentration. Inhibition is relative to release under identical conditions lacking $MnCl_2$, as described under METHODS. Results are the means ± standard errors of two determinations. See METHODS for calculation of percentage inhibition; standard errors were calculated according to Topping (24).

Wash solution (with 15 mM manganese)	Calcium concentration	Inhibition of [^{14}C]GABA release from P_2 synaptosomes
	mM	%
55.0 K^+	0.1	80 ± 11
	1.0	108 ± 4
	5.0	119 ± 3
100 μM veratridine	1.0	104 ± 12
	5.0	107 ± 13
150 μM veratridine	1.0	136 ± 59
	5.0	114 ± 23

TABLE 5

Influence of lanthanum on [^{14}C]GABA release

Tissue aliquots from P_2 synaptosomal fractions were washed with Na^+-free, K^+ or veratridine solutions. Except for the baseline experimental wash, the experimental washes contained the indicated $LaCl_3$ or $CaCl_2$ concentration. Solutions and calculation of release are described under METHODS. Results are the means ± standard errors of the number of determinations shown in parentheses.

Wash solution	Cation	Concentration	[^{14}C]GABA release
		mM	*% total*
55.0 mM K^+	La^{+++}	0.02	0.02 ± 0.05 (2)
		0.2	0.27 ± 0.04 (2)
		1.0	0.34 ± 0.02 (2)
	Ca^{++}	1.0	2.22 ± 0.15 (4)
100 μM veratridine	La^{+++}	0.02	0.03 ± 0.18 (2)
		0.2	0.08 ± 0.03 (2)
		1.0	0.39 ± 0.24 (2)
	Ca^{++}	1.0	0.40 ± 0.12 (6)

ordering of alkaline earth (group IIA) efficacies for stimulating release in the presence of K^+ ($Ba^{++} \geqq Ca^{++} \geqq Sr^{++}$, with Mg^{++} ineffective, all at 1.0 mM) observed in this system has also been reported for the neuromuscular junction and the adrenals (14, 15). In addition, Ba^{++} evoked substantial release without either elevated K^+ levels or the presence of veratridine. This effect has been observed at peripheral synapses and for chromaffin cells of the adrenal medulla (13, 30). La^{+++} stimulation of release must be considered separately. Although it did support release in our system, the effect in elevated K^+ was feeble compared to that of calcium. At the neuromuscular junction, although La^{+++} substantially increases spontaneous release, disagreement exists as to its ability to sustain evoked release (17, 31).

Various substances are able to inhibit release. As reported previously (1), 16 mM Mg^{++}, 1 mM Mn^{++}, and 1 mM Co^{++} partially inhibited Ca^{++}-stimulated, K^+-facilitated release in this system. In fact, the relative inhibitory potencies of Mn^{++} and Mg^{++} at the neuromuscular junction (16) and our system are quite similar. The present study also demonstrated that Mn^{++} can totally inhibit Ca^{++}-stimulated release whether

elevated K^+ or veratridine is present. It is suspected that Co^{++} and Mg^{++} may also inhibit veratridine-facilitated release. Tetrodotoxin, on the other hand, inhibited veratridine- but not K^+-facilitated release. Both Blaustein *et al.* (10) and Belleroche and Bradford (11) reported no inhibition of release by tetrodotoxin at elevated K^+ concentration.

Ca^{++}, the most well known of the stimulating divalent cations, is not usually considered an inhibitor of release. However, when the K^+ concentration is raised, an excessive concentration of Ca^{++} will lower the accelerated "spontaneous" release in the neuromuscular junction (12, 32). Thus a seemingly anomalous finding reported here is paralleled in one of the most extensively studied release systems. It is expected, in fact, that some of the other release stimulants could inhibit their own stimulation (as well as Ca^{++} stimulation) of release (see below), but tests of this hypothesis have not been performed.

The time course of K^+-controlled Ca^{++} influx in the squid axon (Fig. 4 of ref. 33) and K^+-controlled, Ca^{++}-stimulated release in the present system (Fig. 1 of ref. 1) also correlate. Both effects exhibit a rapid, transient rise in response to ionic alteration, followed by a rapid decay to levels slightly above the initial baseline. Although Baker *et al.* (33) used a pulse of K^+ and Levy *et al.* (1) employed Ca^{++}, we have found[5] that a K^+ pulse produces an effect similar to the Ca^{++} pulse. In addition, Elmqvist and Feldman (12) reported a similar transitory phenomenon at a mammalian neuromuscular junction when KCl was increased to 50 mM. That release does not return to the initial baseline suggests that in studies of Ca^{++}-stimulated release in the presence of elevated K^+, the time course of the experiment is critical. Observation of release for extended periods (past the initial pulse of release) may yield characteristically different results than observation of shorter periods.

The present study offers a systematic investigation of parameters influencing polyvalent cation-stimulated secretion. Polyvalent cation–veratridine interactions are not prevalent in the literature, and parametric examination of polyvalent cation–potassium interactions extends the previous observations in well-studied systems (see Table 6 for summary).

The basis for facilitation or inhibition of release depends upon the capability of a given treatment to support or inhibit the physicochemical reactions in stimulus-secretion coupling. In the physiological situation depolarization of the synaptic membrane increases the permeability of the membrane to Ca^{++} (35, 36). Ca^{++} permeates (primarily through the late Ca^{++} channel), interacts with an intracellular binding site(s), and

TABLE 6

Summary of analogies between stimulus-coupled [^{14}C]GABA secretion and other stimulus-coupled secretion systems

Characteristics of [^{14}C]GABA secretion from isolated brain synaptosomes in this system	Well-studied systems showing similar effects	
	Neuromuscular junction	Adrenal medulla
	ref. No.	
Facilitation		
K^+ facilitation of Ca^{++}-dependent stimulation (see also ref. 11)	12	13
Veratridine facilitation of Ca^{++}-dependent stimulation (see also ref. 10)	34[a]	
Comparative alkaline earth stimulation in presence of elevated K^+	15	14
Lanthanum stimulation in presence of elevated K^+	31[b]	
Inhibition		
Mg^{++} inhibition of Ca^{++} stimulation	16[c]	14
Mn^{++} inhibition of Ca^{++} stimulation	16[c]	
Ca^{++} inhibition of K^+ facilitation	33	
Tetodrotoxin inhibition of veratridine facilitation (see also ref. 10)		

[a] Batrachotoxin, a veratridine analogue, was used.

[b] In 2 mM KCl, pH 5.0.

[c] Electrically evoked Ca^{++} stimulation.

[5] D. Redburn and C. Cotman, unpublished observations.

stimulates transmitter secretion (17, 36, 37).

At the squid giant axon synapse, the influx of Ca^{++} that provides the stimulus for secretion is controlled by the membrane potential (36). For central nervous system synaptosomes Ca^{++} influx also appears to be controlled by membrane potential (10, 38). Increases in extracellular K^+ concentrations, which depolarize synaptosomes (38), increase Ca^{++} influx and also result in norepinephrine release (10). Our data illustrate a similar relationship for GABA release. At constant Ca^{++} concentrations, higher K^+ concentrations elicited greater release of [^{14}C]GABA. This was also the case for Sr^{++} and Ba^{++}. Increased external K^+ concentrations presumably depolarized the membrane, thereby increasing the permeability of a Ca^{++} channel. The involvement of the late Ca^{++} channel rather than the early Ca^{++} channel is directly supported by the observation that tetrodotoxin, which blocks the early Ca^{++} channel (35), did not alter K^+-facilitated release, whereas Mn^{++}, which blocks the late Ca^{++} channel (36), inhibited release.

The alkaline earths may differ in release-stimulating efficacy as a function of their ability to penetrate the membrane, access to the postulated intracellular binding site, and affinity for the intracellular binding site. However, indirect participation in the stimulus-secretion coupling processes may occur. The alkaline earths can affect membrane potential, thereby altering permeability through a voltage-dependent channel, or they may mobilize intracellular stores of Ca^{++} or competing ions.

The differential stimulation of release by the alkaline earths was more dramatic in 5.0 mM than in 55.0 mM K^+ medium. If at 5.0 mM K^+ the population of ionophores is only partially activated, an ordered permeation through the late ionophore could determine the differential ability of the ions to stimulate release. At high K^+ concentrations the ionophores may be "fully" activated, such that the similarity of stimulation by the alkaline earths may reflect large divalent cation influxes and saturation of intracellular binding sites.

At the lower concentrations of K^+, increases in Ca^{++} concentrations inhibited release. Direct effects of Ca^{++} on the membrane potential may account for these data. Ca^{++} is able to hyperpolarize excitable cells (39) possibly via screening or binding effects (40). Any decrease in membrane depolarization caused by an alkaline earth would reduce the activation of the late Ca^{++} channel, resulting in a decrease in alkaline earth influx, and hence secretion coupling. This antagonism of depolarization could also contribute to the asymptotic release produced by the high concentrations of the alkaline earths in 55.0 mM K^+.

In the *presence* of Na^+, veratridine depolarizes nervous tissue by activating a tetrodotoxin-blockable ionophore with characteristics identical with the Na^+ ionophore associated with the action potential (41). Thus veratridine should effect depolarization, which would increase Ca^{++} permeation through the late Ca^{++} ionophore. Facilitation of Ca^{++}-stimulated release in the presence of Na^+ was observed. In the *absence* of Na^+, no significant depolarization would be expected since veratridine is not expected to alter the normal membrane permeability to choline (40). Yet release was observed. It is possible that Ca^{++} permeates the membrane via the Na^+ ionophore (early Ca^{++} channel) (35, 36). If so, tetrodotoxin should inhibit veratridine-facilitated release, as in fact it did. Mn^{++} inhibition is also consistent with the involvement of the early Ca^{++} channel, in that Mn^{++} at sufficient concentrations can inhibit Ca^{++} entry at the Na^+-preferring ionophore (33). Thus, in the absence of Na^+ and the presence of veratridine, alkaline earths may penetrate the early Ca^{++} channel and stimulate release.

This paper is an initial attempt to define the chemical properties of stimulus-secretion coupling in the brain, using isolated synaptosomes. The similarity between this system and the release of acetylcholine at the neuromuscular junction and of norepinephrine at the adrenal medulla is striking, and argues for similar mechanisms controlling release of the central neurohumor GABA. Secretion produced by a polyvalent cation

pulse is influenced by K^+, veratridine, and polyvalent cation concentrations in the extracellular medium in a manner consistent with the general properties of axons and well-studied stimulus-secretion coupling. Ordering of alkaline earth release-stimulating efficacy is attributed to differences in membrane permeability and in release site affinities. Although the characteristics of Ca^{++} permeation are relatively well known, the nature of the intracellular binding site [recently implicated by Miledi (42)] is unknown. In view of the high presynaptic density of the late Ca^{++} ionophores (43) and associated negatively charged sites [as inferred from the general properties of Ca^{++}-preferring ionophores (44)], one possibility is that the postulated intracellular binding sites need not be different from some portion of the late Ca^{++} ionophores.

A synaptosomal system such as that presented here provides a means for analyzing membrane permeability properties in relationship to secretion processes, thus enabling an even more detailed analysis of the role of polyvalent cations in stimulus-secretion coupling.

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