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STIMULUS-SECRETION COUPLING PROCESSES IN BRAIN: ANALYSIS OF NORADRENALINE AND GAMMA-AMINOBUTYRIC ACID RELEASE

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SUMMARY

1. Brain synaptosomal fractions released both endogenous and exogenously loaded noradrenaline and γ-aminobutyric acid (GABA) in response to calcium. Elevation of magnesium concentrations in the release media decreased the calcium-dependent release.

2. The release of noradrenaline and GABA occurred within 250 msec following the application of calcium. Following the initial response to calcium, release progressively decreased with continued application of calcium. GABA release declined more rapidly than noradrenaline release, consistent with a noradrenaline distribution having greater accessibility to the release process.

3. Sodium was required for the loading of noradrenaline and GABA into pools released by calcium. On the other hand, the presence of sodium was not required for release from previously loaded pools.

4. Microsomal fractions did not exhibit calcium-dependent release of noradrenaline or GABA. Furthermore, exogenously loaded lysine was not released from synaptosomal fractions in response to calcium.

5. Barium and strontium, but not magnesium, stimulated noradrenaline and GABA release in the absence of calcium. The ordering of alkaline earth efficacies was barium > strontium > calcium.

6. Manganese inhibited calcium-dependent release of noradrenaline and GABA to a greater extent than magnesium.

7. Release, in response to 1 mM calcium, increased linearly with the log. $[K^+]_o$, suggesting that a voltage-dependent calcium ionophore limits

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release. The slope of release *vs.* log. $[K^+]_o$ was greater for noradrenaline than for GABA.

8. For a given $[K^+]_o$ less than 55 mM, increases in external calcium concentration above 1 mM increased noradrenaline release but decreased GABA release. These data suggest that calcium can decrease its own permeation and that differences in the release process may exist for different neurotransmitters.

9. In the presence of the artificial calcium ionophore, A23187, both noradrenaline and GABA release increased linearly with the log. $[Ca^{2+}]_o$. The slope for noradrenaline release was greater than that for GABA release.

10. Stimulus–secretion coupling in brain is suggested to be regulated at the level of a voltage dependent calcium permeation mechanism. However, basic differences in the interaction of calcium with the release process may exist between the noradrenaline and GABA systems.

INTRODUCTION

Although much is known about the release of neurotransmitters from the peripheral nervous system, very little by comparison is known about the release process in brain. In part, this has been due to the lack of a suitable approach. Investigations on the intact perfused brain restrict the temporal collection of released substances by presenting long distances over which the substances must pass before collection, during which catabolism or reuptake could take place. In addition, manipulations of the immediate extracellular environment cannot be achieved rapidly or accurately, making detailed kinetic analyses almost impossible. An approach is required in which stimulus–secretion coupling processes for C.N.S. transmitters can be studied *in vitro*. As indicated by the pioneering studies of deBelleroche & Bradford (1972*a*, *b*), Blaustein, Johnson & Needleman (1972) and Levy, Redburn & Cotman (1973), synaptosomes appear to offer many advantages as an experimental system for the study of neurotransmitter release in brain. Release can be studied independent of multineuronal or neural–glial interactions; substances can be rapidly applied without intervening diffusional barriers; and released transmitter can be collected quickly and directly. Thus both the transmitter substance and the release process can be quantitatively analysed. Furthermore, synaptosomes provide a cell-free system in which neurotransmitter synthesis or uptake, for example, may be studied in relationship to the release process.

In order for synaptosomes to serve as an *in vitro* system suitable for the study of brain transmitter release, certain criteria, established for well-studied stimulus–secretion coupling systems, must be fulfilled: (1) calcium

should be both necessary and sufficient for release. As discussed by Douglas (1968) and Rubin (1970), the absolute requirement for calcium has been described for all vesicle-containing exocytotic secretion systems. (2) Magnesium should inhibit the calcium-dependent release. The interaction between magnesium and calcium upon the release process appears to be competitive for many systems (see Krnjević, 1974, for review). (3) Release should ensue rapidly following the application of a stimulus. The synaptic delay interpolated by chemical transmission is well recognized to be on the order of millisecond(s), and quantal responses are recorded within this time range. Thus the release of transmitter *per se* should be detectable within the earliest measurable time frame.

Investigation of other phenomena characteristic of stimulus–secretion coupling processes becomes feasible following establishment of the above criteria. For example, in well-studied stimulus–secretion coupling systems the alkaline earths strontium and barium can support release in the absence of calcium. Magnesium, on the other hand, not only inhibits secretory responses in the presence of calcium, but usually fails to support stimulus–secretion coupling by itself (see Douglas, 1968; Hubbard, 1970; Rubin, 1970; Krnjević, 1974).

An additional characteristic of most stimulus–secretion coupling systems is that in the physiological situation depolarization provides a necessary but insufficient stimulus for release. That is, the depolarizing consequences of invading action potentials (or elevated potassium concentrations) subsequently allow calcium to react with the release process. At least for the squid, the depolarization is understood to control a potential-dependent calcium ionophore (Katz & Miledi, 1967; Baker, Meves & Ridgway, 1973*a*, *b*). Depolarization, then, facilitates calcium entry into the cell such that calcium permeation appears to be a limiting factor for the secretory response in most stimulus–secretion coupling systems.

In this paper we analyse in detail the requirements for the participation of calcium as the stimulus for noradrenaline and GABA secretion from mouse brain synaptosomes. Our goal is to determine the degree to which stimulus–secretion coupling of these neurotransmitters resembles stimulus–secretion coupling processes described for other neural systems. In addition, it is not presently known whether the properties and characteristics of calcium-dependent release are distinct for different C.N.S. neurotransmitters. Although noradrenaline and GABA systems both serve in a largely inhibitory capacity in mammalian brain, the nature and characteristics of each system's post-synaptic response appear distinct (Dreifuss, Kelly & Krnjević, 1969; Siggins, Oliver, Hoffer & Bloom, 1971). In this study we compare properties of the release process for these two systems.

METHODS

Forebrains from adult, male Swiss Webster mice (Simonsen Laboratories, 60–90 days old) were used in this study. Crude mitochondrial fractions containing synaptosomes were prepared by the method of Cotman & Matthews (1971). The brains were homogenized in cold 0·32 M sucrose in a Teflon-glass homogenizer. A crude nuclear fraction was removed by centrifugation at 5500 rev/min in a Spinco 30 rotor to a cut-off force of $2 \times 10^7\ \omega^2 t$ (2200 g for 120 sec). The supernatant was similarly centrifuged at 13,000 rev/min to a cut-off force of $80 \times 10^7\ \omega^2 t$ (14,000 g for 12 min). The crude mitochondrial fraction (P_2) was washed in 0·32 M sucrose and again pelleted as the crude mitochondrial fraction (P_2'). About 45 % of the particles in this fraction were synaptosomes as determined by electron microscopic examination. This fraction will be termed the synaptosomal fraction. Microsomal fractions (P_3) were obtained by pelleting the supernatant (S_2') from P_2 crude mitochondrial fractions.

TABLE 1. Composition of experimental solutions

Chemical	Solution composition (mM)			
	*A** (incubation)	*B** (wash)	*C*†	*D*†
NaCl	150·0	150·0	124·0	124·0
$NaHCO_3$	—	—	26·0	26·0
HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane sulphonic acid)	20·0	20·0	—	—
D-glucose	10·0	10·0	10·0	—
KOH	5·0	5·0	—	—
KCl	—	variable (5–50)	5·0	15·0
Na_2HPO_4	1·2	—	1·2	—
$MgSO_4$	1·2	—	1·2	—
$MgCl_2$	—	1·0	—	1·0
AOAA (aminooxy acetic acid)	0·1	0·1	0·1	—
Pargyline HCl	0·1	0·1	0·1	—
L-ascorbic acid	1·0	1·0	1·0	—

* pH 7·4 with Tris base. † pH 7·4 with O_2:CO_2 (95 %:5 %).

The preparations were resuspended in incubation solution (Solution *A* unless otherwise noted, Table 1), pelleted, and kept as the pellet at 0–4° C until incubation. Although in the present study pellets were never kept for more than 30 min, tissue can be stored as the pellet for up to 2 hr at 0–4° C without apparent change in uptake or release characteristics. Tissue pellets were resuspended in incubation solution at protein concentrations of 0·6–0·9 mg/ml. (Lowry, Rosenbrough, Farr & Randall, 1951) and incubated for 10 min at 37° C under hydrated O_2:CO_2 (95:5 %) with 0·26 μM [^{3}H](−)noradrenaline and 0·44 μM [^{14}C]GABA (unless otherwise noted). Baseline efflux and stimulated release were studied, based on the technique of Levy *et al.* 1973. Immediately following incubation, tissue aliquots (usually 1 ml.) were plated on to four filters. Following suction of the tissue suspension, seven 2 ml. washes of calcium-deficient wash solution (Solution *B* unless otherwise noted,

Table 1) were applied to the filters. Wash solution was applied for 30 sec every 60 sec. As can be seen from Fig. 1, washout in the presence of elevated potassium was stabilized by the seventh wash. Stimulating alkaline earth cations (as the chloride salt) were introduced only in the seventh wash. Investigation of the time course of release stimulation was essentially as above except that filters with previously incubated and plated tissue were placed in a positive-pressure perfusion apparatus (Redburn, Biela, Shelton & Cotman, 1975) and samples were collected every 5 sec or 250 msec. All release measurements were conducted at room temperature (22–24° C).

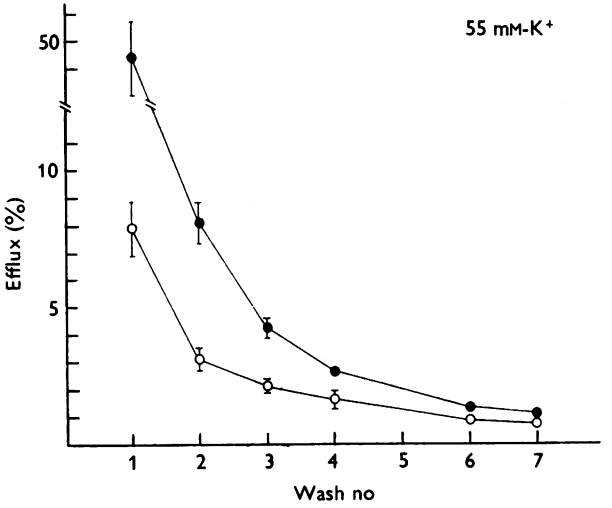

Fig. 1. Efflux during washout in Ca^{2+}-deficient media. Following incubation with [^{3}H]noradrenaline and [^{14}C]GABA, synaptosomal tissue aliquots were plated on to filter units and successive 2 ml. washes of 55 mM-K^+ Solution *B* were collected every 60 sec. As in subsequent Figures, the darkened circles indicate [^{3}H]noradrenaline and the open circles indicate [^{14}C]GABA. Efflux in a given filtrate is represented as the % of the total radioactivity present on the filters immediately before that given wash. Each point represents the mean and S.E. of the mean of four determinations. Where no error bars are presented, the S.E. was smaller than the radius of the circle.

Double-isotope radioactivity in 1 ml. wash filtrate was counted in 10 ml. Triton X-100/toluene/phosphor medium (Patterson & Greene, 1965) and corrected for quenching with external standard ratios. In the presence of aminooxy acetic acid, 95 % of the [^{14}C] had been previously demonstrated to be associated with GABA (Levy *et al.* 1973). In the presence of pargyline 96·5 ± 0·8 % (± S.E. of the mean, n = 4) of the [^{3}H] in the seventh wash filtrates was associated with noradrenaline as assessed by ascending paper chromatography (butanol:acetic acid:water, 12:3:5; Smith, 1969). Radioactivity in the filters was counted in the same manner in 20 ml. of the same medium following overnight solubilization in 2 ml. of 1 % sodium dodecyl sulphate, 20 mM EDTA, pH 8 with NaOH.

Efflux for a given collection period is expressed as that % of the total radioactivity present on the filters immediately before the wash of interest. Importantly, release (measured from the seventh wash) is expressed as the difference between efflux

from simultaneously run filters treated identically except for the additional alkaline earth. Inhibition of release is expressed as

$$\left(\begin{matrix}\text{Release without}\\ \text{inhibitor}\end{matrix} - \begin{matrix}\text{Release with}\\ \text{inhibitor}\end{matrix}\right) \Big/ \left(\begin{matrix}\text{Release without}\\ \text{inhibitor}\end{matrix}\right) \times 100.$$

Two problems are overcome by expression of the data as described above. Ca^{2+}-dependent release is analysed independently from K^+-dependent, Ca^{2+}-independent effects. This is important in that the effects of K^+ pulses alone can be quite large in these systems (D. A. Redburn, D. Shelton & C. W. Cotman, 1975). Thus: (1) the alkaline earth pulse is not presented until the washout in K^+ media has flattened (see Fig. 1) and (2) the Ca^{2+}-independent effects are subtracted out. Although there may be a contribution of spontaneous release to baseline efflux, spontaneous and K^+-induced lysis and leakage may also contribute to these values.

The method utilized to express release is important in that release is expressed relative to the transmitter pool at the time of stimulation. When these pools are differentially undergoing dynamic changes, such as during widely differing release conditions, failure to express release relative to these differentially changed pools can lead to incorrect estimates of release with respect to its then present state of the transmitter pool. For example, Levi & Raiteri (1974*a*) express the efflux of GABA in a given collection relative to the total radioactivity from filter and all washes. Initial release in a Na^+-free condition was elevated fifteenfold relative to Na^+ conditions. Many factions later, efflux in the Na^+-free condition, calculated in their fashion, reached an asymptote lower than that for the Na^+ condition. The authors have concluded that their system prevents reuptake of released substances (Levi & Raiteri, 1974*a*, *b*). However, calculated relative to the radioactivity on the filters immediately before a given collection, efflux in the Na^+-free condition reaches an asymptotic value two to three times higher than efflux in the Na^+ condition. Release expressed in this fashion leads then to a diametrically opposed conclusion: reuptake is in fact ongoing.

Membrane potential was indirectly assessed using the cyanine dye diO-C_6 (3,3′-dihexyloxacarbocyanine iodide) (Sims, Waggoner, Wang & Hoffman, 1974). Standard tissue aliquots were brought to room temperature, incubated at 1 μM diO-C_6 for 5 min, brought to the appropriate KCl concentration, incubated for another 6 min, and then read for fluorescence (excitation 465 nm, emission 506 nm; uncorrected).

Endogenous noradrenaline in the filtrates and filters was assayed according to Coyle and Henry (1973) and Cuello, Hiley & Iversen (1973) with slight modifications. In order to elevate the amount of noradrenaline per filter unit, glass fibre filters previously plated with tissue were stacked such that each filter unit held tissue from the equivalent of one mouse forebrain (approximately 10 mg synaptosomal protein). Following the seventh wash, filters were homogenized in 0·1 N perchloric acid. Parallel filters were run with [^{3}H]noradrenaline for comparison.

Endogenous GABA in the filtrates and filters was assayed according to Graham & Aprison (1966) with slight modifications. For this experiment tissue pellets were resuspended in Solution *C* (Table 1) and incubated with [^{14}C]GABA as described above. Following washout and Ca^{2+}-stimulation in Solution *D* (Table 1), the GABA remaining on the filters was eluted by hypo-osmotic shock in two 2 ml. H_2O washes. [^{14}C]GABA release was measured from aliquots of the same filtrates and lysates as endogenous GABA. In this situation efflux in the lysates contained over 95 % of the radioactivity previously present on the filters. Thus, release was expressed relative to totals in filtrates plus lysates for these experiments.

7-[^{3}H](−)noradrenaline (3·7 Ci/m-mole) and 4,5-[^{3}H]-(L)-lysine (55 Ci/m-mole) were obtained from New England Nuclear. [U-^{14}C]-4-aminobutyric acid (228 mCi/-m-mole)

was obtained from Amersham/Searle. A23187 was supplied by Lilly; diO-C_6 was provided by Dr A. Waggoner. (A23187 was added to wash solutions 1:1000 from a 10 mM stock in DMSO. The final 10 μM concentrations were maximal figures as A23187 did not go completely into solution. 1:1000 additions of DMSO alone had no effect on calcium-dependent release in this situation.) All other chemicals were reagent grade.

RESULTS

Stimulus-coupled secretion of noradrenaline and GABA. The demonstration of stimulus–secretion coupling for a given transmitter system requires that release be Ca^{2+}-dependent and Mg^{2+}-inhibited and that release rapidly follow stimulation. As illustrated in Fig. 2 (right) exogenously loaded [^{3}H]noradrenaline and [^{14}C]GABA were released in response to 1 mM-Ca^{2+} in the presence of 15 mM-K^+. Elevation of Mg^{2+} concentrations in the wash solution inhibited the Ca^{2+} stimulation by 70–80%. As will be described in more detail below, release occurred within at least 250 msec following the application of Ca^{2+}.

The Ca^{2+}-dependent, Mg^{2+}-inhibited release of endogenous noradrenaline and GABA (in situations either parallel or identical, respectively, to those in which the release of exogenously loaded transmitter was investigated above) accompanied at least qualitatively the release of [^{3}H]noradrenaline and [^{14}C]GABA (Fig. 2, left). Clearly both endogenous and exogenously loaded pools release transmitter in response to Ca^{2+}; and Mg^{2+} inhibited this release.

Calcium-dependent release: physical specificity. Although it appeared that the release of exogenously loaded noradrenaline and GABA would provide a valid measure of endogenous noradrenaline and GABA release, it is essential to determine the extent of specificity of this release to the specific transmitter candidates and to the synaptosomal fraction.

Lysine was chosen as a molecule which could be accumulated by the synaptosomal tissue, yet was not considered to be a transmitter candidate (Mulder & Snyder, 1974). As shown in Table 2, there was no change in the efflux of [^{3}H]lysine when either Ca^{2+} or Mg^{2+} was introduced into the wash solution. Simultaneously, however, GABA exhibited both Ca^{2+}-dependent and Mg^{2+}-inhibited release. Furthermore, the baseline efflux (efflux in the absence of Ca^{2+}) of lysine was greater than that for GABA, i.e. accumulated lysine was not retained as well as GABA. Thus, the Ca^{2+}-dependent release of exogenously loaded molecules is not general to any sequestered molecule.

Although synaptic boutons are the major locus of noradrenaline and GABA accumulation for incubation situations similar to these (Iversen & Bloom, 1972; Descarries & Droz, 1970), microsomes have been shown to take up noradrenaline and GABA and are recognized as a contaminant of synaptosomal fractions (Cotman & Matthews, 1971; Morgan, Wolfe,

Mandel & Gombos, 1971). However, microsomes should not release the accumulated molecules since they do not contain vesicles, an essential feature of stimulus–secretion coupling systems. As illustrated in Table 3, microsomal fractions had a much reduced capacity to accumulate and retain noradrenaline and GABA compared to the synaptosomal fractions. But more importantly, microsomal fractions did not support Ca^{2+}-dependent release. Although both preparations were similar in protein

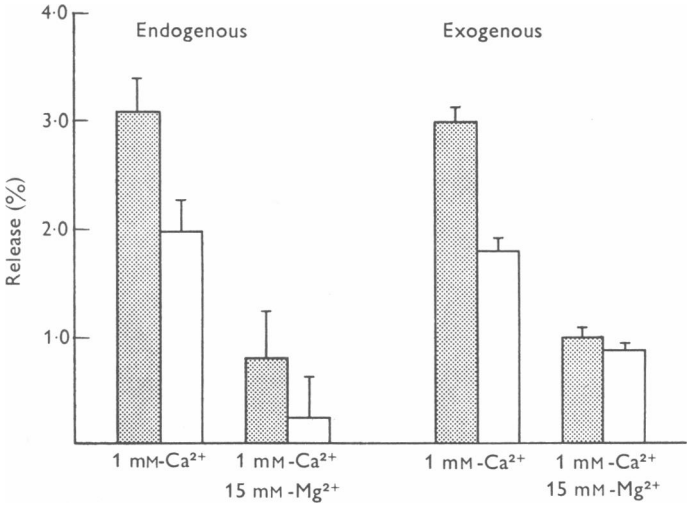

Fig. 2. The influence of Mg^{2+} upon the Ca^{2+}-dependent release of endogenous and exogenously loaded noradrenaline (stippled bars) and GABA (open bars). Noradrenaline release: synaptosomal tissue was plated on to filter units as described in Methods after incubation with or without [^{3}H]noradrenaline. Following washout in 15 mM-K^+ media, Ca^{2+}, Mg^{2+}, or both was introduced into the wash solution. Filters were homogenized in 0·1 N perchloric acid following stimulation. Filtrates and filter extracts were analysed either for noradrenaline or radioactivity (depending on the incubation situation). Values represent the means and S.E. of the mean of two determinations. The amount of [^{3}H]noradrenaline in the filtrates and extracts was approximately 1/100 of the endogenous noradrenaline. Baseline effluxes of noradrenaline and [^{3}H]noradrenaline were $2{\cdot}0 \pm 0{\cdot}1$ and $1{\cdot}44 \pm 0{\cdot}06$ %, respectively. GABA release: synaptosomal tissue was plated on to filter units as described in Methods after incubation in Solution *C* with [^{14}C]GABA. Following washout in 15 mM-K^+ media, Ca^{2+}, Mg^{2+}, or both, was introduced into the wash solution (Solution *D*). Following stimulation, tissue was lysed with water. Aliquots of the filtrates and lysates were analysed for GABA and radioactivity. Values represent the means and S.E. of the mean of three determinations. The amount of [^{14}C]GABA in the filtrates and lysates was approximately 1/1000 of the endogenous GABA. Baseline effluxes of GABA and [^{14}C]GABA were $0{\cdot}36 \pm 0{\cdot}06$ and $1{\cdot}28 \pm 0{\cdot}02$ %, respectively.

TABLE 2. Comparison of GABA and lysine efflux from synaptosomal fractions and the influence of Ca^{2+} and Mg^{2+}

	[^{3}H]Lysine		[^{14}C]GABA	
Wash solution	Efflux (%)	Release (%)	Efflux (%)	Release (%)
—	2·14 ± 0·19		1·27 ± 0·02	
		−0·16 ± 0·22		1·75 ± 0·04
Ca^{2+}	1·98 ± 0·18		3·02 ± 0·08	
Mg^{2+}	2·29 ± 0·07		0·92 ± 0·06	
		0·00 ± 0·11		0·98 ± 0·11
Mg^{2+}, Ca^{2+}	2·28 ± 0·09		1·90 ± 0·06	

Synaptosomal tissue was incubated with [^{3}H]lysine and [^{14}C]GABA and washed in 15 mM-K^+ media. Following washout, 1 mM-Ca^{2+}, 15 mM-Mg^{2+}, or both was introduced into the wash solution. Values represent the means and S.E. of the mean of four determinations.

concentrations during incubation with the labelled transmitters (and hence on the filters) following washout only 13 % as much [^{3}H]noradrenaline and 34 % as much [^{14}C]GABA were retained by the microsomal fractions compared to the synaptosomal fractions. The decreased retention could have resulted from a relative lack of uptake sites and/or a lower capacity uptake system; but the relatively high baseline efflux from the microsomal tissue also suggests a more labile storage for the substances in the microsomal tissue. Ca^{2+}-dependent release in this situation then is not a general property of any subcellular particle which can accumulate noradrenaline or GABA. In addition, it is unlikely that microsomal contamination contributes to the observed Ca^{2+}-dependent release from the synaptosomal fractions.

Synaptosomes possess high-affinity, Na^+-dependent uptake systems for noradrenaline and GABA (Colburn, Goodwin, Murphy, Bunney & Davis, 1968; Varon, Weinstein, Baxter & Roberts, 1965). And it has been suggested that brain slices accumulate amino-acid neurotransmitters for release via such high-affinity, Na^+-dependent processes (Mulder & Snyder, 1974). As shown in Table 4, equimolar substitution for Na^+ by choline in the incubation solution resulted in only 8 % [^{3}H]noradrenaline and 0·7 % [^{14}C]GABA retention following washout. Clearly then, the majority of the noradrenaline and GABA accumulated required Na^+. Furthermore, that which was accumulated in the absence of Na^+ was not retained as well as that accumulated in the presence of Na^+, as indicated by the elevated baseline effluxes of noradrenaline and GABA from tissue incubated in the choline media. Importantly, when noradrenaline and GABA were loaded in the Na^+-deficient media, Ca^{2+} no longer stimulated

TABLE 3. Comparison of synaptosomal and microsomal fractions

	[^{3}H]noradrenaline			[^{14}C]GABA		
Tissue fraction	D.p.m. on filter before release	Baseline efflux (%)	Release (%)	D.p.m. on filter before release	Baseline efflux (%)	Release (%)
Synaptosomal	425,000 ± 11,000	1·58 ± 0·12	6·88 ± 0·28	168,000 ± 2000	2·00 ± 0·35	2·76 ± 0·25
Microsomal	61,600 ± 1200	5·60 ± 0·28	0·92 ± 0·32	57,600 ± 900	4·46 ± 0·24	0·00 ± 0·03

Synaptosomal and microsomal fractions (prepared as described in Methods) were incubated with [^{3}H]noradrenaline and [^{14}C]-GABA and washed in 55 mM-K^+ media. Following washout, 5 mM-Ca^{2+} was introduced into the wash solution. Values represent the means and S.E. of the mean of four determinations.

TABLE 4. The influence of Na^+ during incubation

	[^{3}H]noradrenaline			[^{14}C]GABA		
Incubation solution	D.p.m. on filter before release	Baseline efflux (%)	Release (%)	D.p.m. on filter before release	Baseline efflux (%)	Release (%)
Na^+ buffer	458,000 ± 14,000	0·93 ± 0·24	6·40 ± 0·32	182,000 ± 3500	0·69 ± 0·04	2·60 ± 0·07
Choline buffer	41,600 ± 600	10·36 ± 0·65	0·50 ± 0·47	1180 ± 20	4·76 ± 0·74	−0·09 ± 0·35

Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA either in Solution *A* (see Table 1) or in a modified Solution *A* in which the NaCl and Na_2HPO_4 was replaced with equimolar choline chloride and K_2HPO_4 respectively (KOH was adjusted to maintain 5 mM-K^+). Following washout in 55 mM-K^+ media, 2 mM-Ca^{2+} was introduced into the wash solution. Values were obtained as in Table 3.

increases in release. Thus within synaptosomal fractions, Ca^{2+}-dependent release of noradrenaline and GABA arises from pools loaded via high-affinity, Na^+-dependent transport systems.

The physical specificity of the Ca^{2+}-dependent release of noradrenaline and GABA with respect to both sequestered molecule and sequestering particle apparently accrues from the association of stimulus–secretion coupling processes and high-affinity, Na^+-dependent uptake processes in synaptosomes. The non-stimulus–secretion coupling processes are associated with either low-affinity and/or low-capacity uptake systems which exhibit relatively poor retention of the accumulated pools. Although baseline efflux may differ for different transmitters, the existence of such uptake systems specific to both transmitter substance and synaptosomal fractions suggests a further characteristic common to at least these stimulus–secretion coupling systems in brain: a high-affinity, Na^+-dependent uptake system obligatory for the subsequent Ca^{2+}-dependent release.

It should be pointed out that the forebrain synaptosomes used in this study are composed of mixed transmitter types, and noradrenaline and GABA may be sequestered and released by types of synaptosomes which do not normally utilize the transmitter. For example, in regions such as the corpus striatum, which is predominantly dopaminergic, [^{3}H]noradrenaline is accumulated and released quite effectively (J. W. Haycock, J. Meligeni & C. W. Cotman, unpublished). Thus some portion of the [^{3}H]noradrenaline uptake and release presented here is probably contributed by dopaminergic terminals.

Calcium-dependent release: quantifiability. In the physiological situation, the previously described uptake systems have been implicated as major mechanisms by which the synaptic actions of released noradrenaline and GABA may be terminated (Iversen, 1971). Should the released transmitter reach sufficient concentrations at the synaptosomal membrane to be taken up before collection of the sample, *apparent* release would be an underestimate of the actual release. Furthermore, at least in the case of noradrenaline, such extracellular concentrations could stimulate presynaptic α-adrenergic receptors which, when activated, decrease the amount of *actual* release (cf. Langer, 1974). Interactions such as these would vitiate attempts to quantify kinetics of the release process *per se*. Therefore it is essential to assess the extent of participation of such regulatory processes, if any, for the present situation.

If the reuptake systems for the plated synaptosomes are similar to the previously described Na^+-dependent uptake systems utilized to initially load the synaptosomal fractions, then (as demonstrated above) omission of Na^+ from the wash media should inhibit the reuptake processes. As

TABLE 5. The influence of Na^+ during washout and release

	[^{3}H] noradrenaline			[^{14}C] GABA		
Wash solution	D.p.m. on filter before release	Baseline efflux (%)	Release (%)	D.p.m. on filter before release	Baseline efflux (%)	Release (%)
Na^+ buffer	426,000 ± 25,000	0·98 ± 0·04	5·96 ± 0·50	183,000 ± 1100	0·69 ± 0·04	2·13 ± 0·05
Choline buffer	423,000 ± 24,000	0·98 ± 0·02	5·95 ± 0·50	182,000 ± 1400	0·62 ± 0·02	2·06 ± 0·02

Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA and washed in either 55 mM-K^+ Solution *B* or in a 55 mM-K^+ Solution *B* in which the NaCl was replaced with equimolar choline chloride. Following washout, 2 mM-Ca^{2+} was introduced into the wash solution. Values were obtained as in Table 3.

TABLE 6. The influence of aminooxy-acetic acid (AOAA), pargyline and ascorbate during washout and release

	[^{3}H] noradrenaline			[^{14}C] GABA		
Wash solution	D.p.m. on filter before release	Baseline efflux %	Release (%)	D.p.m. on filter before release	Baseline efflux (%)	Release (%)
Solution B	467,000 ± 30,000	0·87 ± 0·04	6·48 ± 0·20	185,000 ± 2000	0·75 ± 0·02	2·27 ± 0·15
Solution B w/o AOAA pargyline ascorbate	469,000 ± 19,000	0·90 ± 0·03	6·34 ± 0·28	183,000 ± 2000	0·78 ± 0·02	2·21 ± 0·17

Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA and washed either in 55 mM-K^+ Solution *B* or in 55 mM-K^+ Solution *B* from which the AOAA, pargyline and ascorbate had been omitted. Following washout, 2 mM-Ca^{2+} was introduced into the wash solution. Values were obtained as in Table 3.

shown in Table 5, substitution of Na^+ with choline in the wash solutions (but with prior incubation in Na^+ incubation solutions) did not influence baseline efflux, Ca^{2+}-dependent secretion, or the amount of noradrenaline or GABA retained on the filters following washout. For this system, then, it appears that the released noradrenaline and GABA are being diluted or removed (via diffusion or flow through) sufficiently to prevent such interactions. Additionally, Na^+–Ca^{2+} exchange appears to be minimally involved in the present release situations.

Another factor which might influence the quantitation of release is the presence of pargyline, ascorbate and AOAA in the wash solutions, included previously to minimize differential metabolism of endogenous and exogenously loaded pools. Blakeley, Powis & Summers (1973) reported, for example, that pargyline increased the stimulation-induced release of noradrenaline from cat spleen, independent of the monamine oxidase inhibition. As indicated in Table 6, however, these agents apparently did not exert direct effects upon the release process in this situation. Following incubation in the presence of the inhibitors, omission of pargyline, ascorbate and AOAA from the wash solutions did not affect baseline efflux, Ca^{2+}-dependent secretion, or the amount of label retained by the tissue following washout.

Calcium-dependent release: temporal characteristics. As described above, an additional requirement for stimulus–secretion coupling is the rapid efflux of transmitter in response to stimulation. The previous figure indicated that Ca^{2+} induces release within at least 30 sec following application (Fig. 2). As indicated in Fig. 3*A* the onset of Ca^{2+}-dependent release of noradrenaline and GABA is detectable within the first 5 sec following introduction of Ca^{2+}. In the absence of Ca^{2+}, efflux remains low and gradually continues to decline. Addition of 1 mM-Ca^{2+} induced an abrupt increase in both noradrenaline and GABA efflux. Fig. 3*B* illustrates the Ca^{2+}-dependent release of noradrenaline and GABA (i.e. that part of the efflux from the Ca^{2+} condition calculated by subtracting baseline efflux). Although release in this situation peaks in the second collection period, higher perfusion rates (to overcome mixing of the Ca^{2+} solution with a 0·3 ml. dead volume) and shorter collection times, as illustrated in Fig. 4, demonstrate that Ca^{2+}-dependent release of noradrenaline and GABA can be detected within 250 msec and that release peaks much sooner than 5 sec following the application of Ca^{2+}.

As reported for the mammalian neuromuscular junction (Elmqvist & Feldman, 1966), Ca^{2+}-dependent release of noradrenaline and GABA from the synaptosomal fraction in the presence of elevated K^+ levels exhibited a rapid but transient rise following stimulation (Figs. 3*B*, 4). Subsequent release gradually fell to levels slightly above base line. Baker *et al.* (1973*b*) have described a similar shape for Ca^{2+} influx in the presence of elevated

K^+ across squid axonal membrane, implying that in these situations Ca^{2+} influx could be the limiting factor for release. However, Llinàs & Nicholson (1975) did not observe concurrent decreases in Ca^{2+} influx with decreases in release suggesting that factors such as transmitter depletion from releasable pools can supersede Ca^{2+} influx in limiting release.

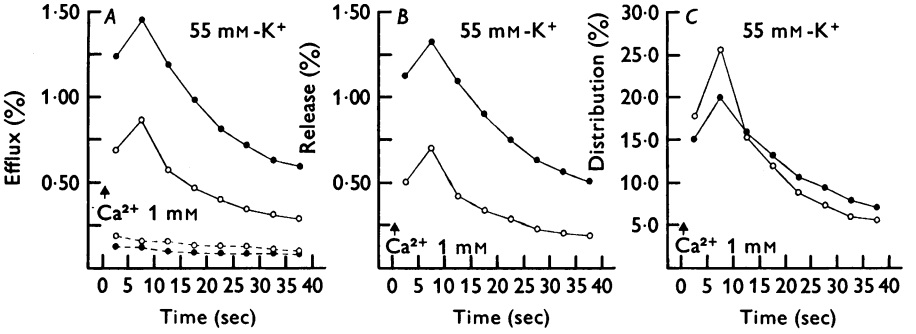

Fig. 3. Temporal aspects of noradrenaline and GABA release. Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA, plated on to filter units and placed in a positive-pressure perfusion apparatus (Redburn *et al.* 1975). Filled circles indicate noradrenaline efflux and release and open circles indicate GABA efflux and release. Solutions were perfused at a rate of 1 ml./5 sec and samples were collected every 5 sec. Following washout in 55 mM-K^+ media for 30 sec, filters were perfused with 55 mM-K^+ wash solution either with or without the addition of 1 mM-Ca^{2+}. Zero time was taken as the beginning of the collection immediately following washout. Each point represents the mean of four determinations. S.E. of the mean have been omitted for clarity here and in subsequent figures describing the temporal aspects of release, but were typically less than 5% of the mean. *A*, efflux of noradrenaline and GABA following washout from filters administered 1 mM-Ca^{2+} (upper) and from filters administered the wash solution without the addition of Ca^{2+} (lower). *B*, Ca^{2+}-dependent release of noradrenaline and GABA, calculated by subtracting the efflux during equivalent collection periods from filters not receiving the addition of Ca^{2+}. *C*, relative temporal distribution of noradrenaline and GABA released in response to Ca^{2+}, calculated by expressing the d.p.m. of noradrenaline and GABA released in response to Ca^{2+} during a given collection period as % total d.p.m. released in response to Ca^{2+} over the 40 sec stimulation.

A direct comparison of the temporal profiles for release of noradrenaline and GABA is illustrated in Fig. 3*C*. Here the total d.p.m. of noradrenaline and GABA released in response to Ca^{2+} are normalized to 100% and the d.p.m. per collection expressed as a percent of that total. Analysis in this manner revealed that noradrenaline release, independent of differences in the actual percent release, was sustained relatively longer than was GABA release. Although the apparently greater capacity of the noradrenaline system to respond to a given Ca^{2+} concentration may represent a

differential ability of the two systems to support Ca^{2+} influxes over time, other alternatives such as differences in the sizes of releasable pools and rates of mobilization are also consistent with the shapes of the temporally extended release curves.

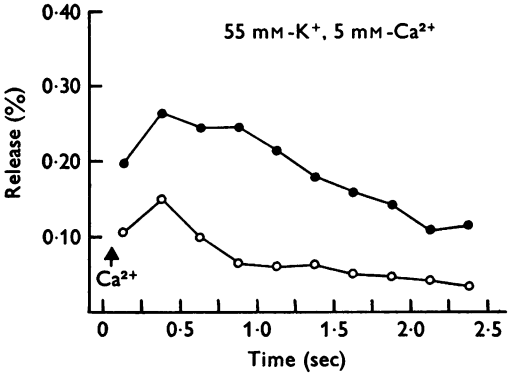

Fig. 4. Release of noradrenaline and GABA over short stimulation times. Filled circles indicate noradrenaline release and open circles indicate GABA release. Solutions were perfused at a rate of 1 ml./sec and samples were collected every 250 msec. Synaptosomal tissue was treated essentially as described in Fig. 3. Following washout in 55 mM-K^+ media for 10 sec, filters were perfused with either 55 mM-K^+ wash solution or 55 mM-K^+ wash solution containing 5 mM-Ca^{2+}. Data is presented as in Fig. 3*B*. Each point represents the mean of four determinations.

Calcium-dependent release: other divalent cations. A characteristic of stimulus–secretion coupling systems is that the alkaline earths strontium and barium, but not magnesium, substitute for calcium in stimulating secretion (Douglas & Rubin, 1964; Elmqvist & Feldman, 1965). In the presence of 15 mM-K^+, Ba^{2+} and Sr^{2+} were more effective than Ca^{2+} for producing release of either noradrenaline or GABA (Fig. 5). The ordering of alkaline earth efficacy was identical for noradrenaline and GABA release ($Ca^{2+} < Sr^{2+} < Ba^{2+}$); however, the relative potencies differed more widely for noradrenaline release. The approximate ratios for stimulating noradrenaline release were $Ca^{2+}/Sr^{2+}/Ba^{2+} = 1/2/4$ whereas for GABA release $Ca^{2+}/Sr^{2+}/Ba^{2+} = 1/1{\cdot}5/2$. Thus not only was noradrenaline release in response to Ca^{2+} larger (and more sustained) than GABA release, but noradrenaline release in response to Ba^{2+} was increased relatively more than was GABA release. As illustrated in Fig. 6, Mg^{2+} failed to support release, and Mn^{2+} only minimally induced release.

The ordering of divalent cations observed is in general agreement with that for peripheral stimulus–secretion coupling systems. The $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ ordering is seen at the adrenal medulla (Douglas & Rubin, 1964); however, at the neuromuscular junction and spleen, Ca^{2+} and Sr^{2+} are

reversed (Elmqvist & Feldman, 1965; Kirkepar & Misu, 1967). In addition, the ability of Mn^{2+} to support release in some situations has been described by Balnave & Gage (1973). These differences in divalent cation release-stimulating efficacy, as in other stimulus–secretion coupling systems, may be based on differences in membrane permeation and intracellular binding site affinities (Blioch, Glagoleva, Liberman & Nenashev, 1968), but indirect participation in stimulus–secretion coupling processes may also occur.

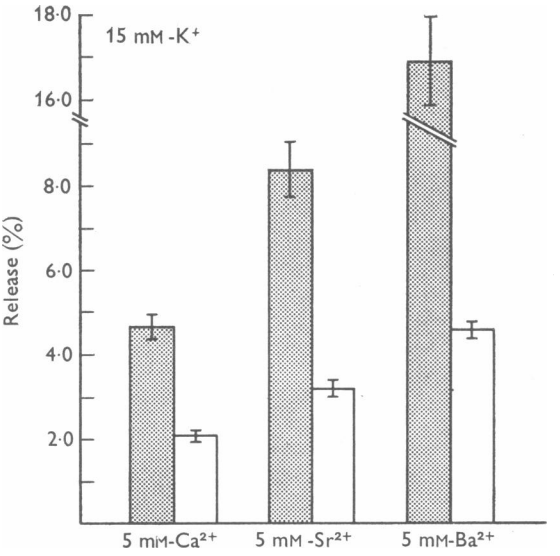

Fig. 5. Alkaline earth stimulation of release. Synaptosomal tissue was incubated in Solution *A* containing [^{3}H]noradrenaline and [^{14}C]GABA and washed in 15 mM-K^+ Solution *B*. Following washout, 5 mM-Ca^{2+}, $-Sr^{2+}$, or $-Ba^{2+}$ was introduced into the wash solution. Stippled bars indicate noradrenaline release and open bars indicate GABA release. Values represent the means and S.E. of the mean of four determinations.

In peripheral stimulus–secretion coupling systems, the ability of Ca^{2+} to stimulate release is reduced when Mn^{2+} or Mg^{2+} are introduced in combination with Ca^{2+}. In this situation, Mn^{2+} and Mg^{2+} are thought to compete with Ca^{2+} for presynaptic permeation sites (Balnave & Gage, 1973; Cooke, Okamoto & Quastel, 1973). Thus, elevation of Mg^{2+} concentrations or addition of Mn^{2+} to the wash solutions should relatively reduce the ability of Ca^{2+} to promote stimulus–secretion coupling. As indicated in Table 7, both Mg^{2+} and Mn^{2+} inhibited Ca^{2+}-dependent release. These inhibitory divalent cations decreased Ca^{2+}-dependent release when present before the introduction of Ca^{2+} or even when added simultaneously

TABLE 7. Antagonism of Ca^{2+}-dependent release by Mg^{2+} and Mn^{2+}

Treatment	Concentration (mM)	Inhibition (%) [^{3}H] noradrenaline	Inhibition (%) [^{14}C] GABA
Washes 1–7			
$MgCl_2$	15	41·6 ± 5·6	58·9 ± 8·6
$MnCl_2$	1	39·5 ± 2·4	55·4 ± 2·4
	15	98·0 ± 0·7	94·2 ± 3·1
Wash 7 only			
$MgCl_2$	15	35·6 ± 4·2	50·1 ± 2·3
$MnCl_2$	1	30·6 ± 1·2	30·3 ± 1·5

Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA. Mg^{2+} or Mn^{2+} was introduced at the indicated concentration into the 55 mM-K^+ wash solution either during the washout and stimulation (Washes 1–7) or just during the stimulation with 5 mM-Ca^{2+} (Wash 7). In all cases the design was similar to that presented in Table 2. Mg^{2+} or Mn^{2+} inhibition of 5 mM-Ca^{2+} stimulation of release was calculated as described in Methods. Each value is the mean and S.E. of the mean of four determinations. The individual effects of Mg^{2+} and Mn^{2+}, in comparison to Ca^{2+}, are presented in Fig. 6.

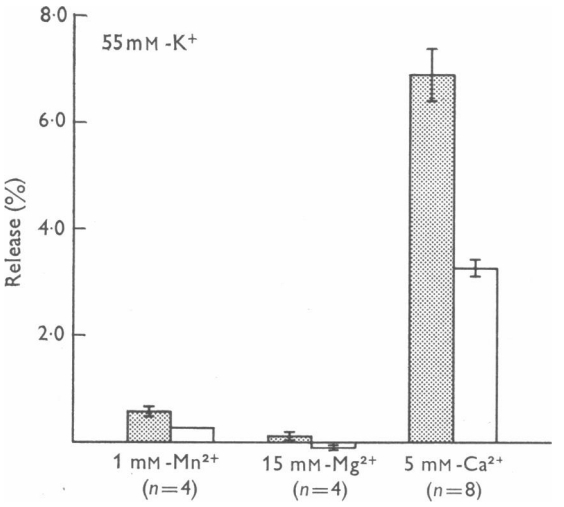

Fig. 6. Comparison of the influence of Mg^{2+}, Mn^{2+} and Ca^{2+} upon efflux. Synaptosomal tissue was incubated as in Fig. 5 and washed in 55 mM-K^+ media. Following washout, 5 mM-Ca^{2+}, 15 mM-Mg^{2+}, or 1 mM-Mn^{2+} was introduced into the wash solution. Stippled bars indicate noradrenaline release and open bars indicate GABA release. Values represent mean and S.E. of the mean of the indicated determinations.

with Ca^{2+}. Mn^{2+} was approximately fifteen times more potent than Mg^{2+} in inhibiting the effects of Ca^{2+}.

Calcium-dependent release: depolarization and Ca^{2+} permeation. K^+ is well known to facilitate Ca^{2+}-dependent release, presumably via depolarization of the presynaptic membrane by increasing Ca^{2+} permeation through a voltage-sensitive Ca^{2+} ionophore (Katz & Miledi, 1967; Baker *et al.* 1973*a*, *b*). The development of cyanine dyes as indicators of membrane polarization (Sims *et al.* 1974) allows relative fluorescence measurements as indirect measures of polarization in situations, such as for

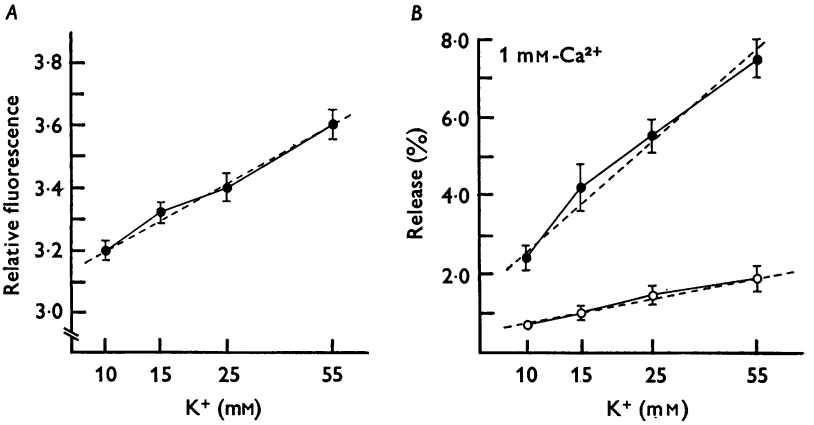

Fig. 7. The relationship of external K^+ concentration to diO-C_6 fluorescence and Ca^{2+}-dependent release. *A*, synaptosomal tissue was incubated with 1 μM diO-C_6 for 5 min. Aliquots were brought to the indicated K^+ concentrations, incubated for another 6 min and fluorescent readings were taken (as described in Methods). Each point represents the mean and S.E. of the mean of four determinations. *B*, synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA and washed with Solution *B* at the indicated K^+ concentrations. Following washout, 1 mM-Ca^{2+} was introduced into the wash solution. Each point represents the mean and S.E. of the mean of four determinations. Filled circles indicate noradrenaline release and open circles indicate GABA release. The dashed lines are the least squares best fits for the continuous lines described by the points.

synaptosomes, where direct electrophysiological measurements are presently impossible. As can be seen in Fig. 7*A*, fluorescence of diO-C_6-treated synaptosomal fractions increased linearly with the $\log_{10}[K^+]_o$ ($r = 0{\cdot}998$) in accordance with the prediction given by the Nernst equation for the relationship between membrane potential and $[K^+]_o$. Furthermore, as illustrated in Fig. 7*B*, release in response to 1 mM-Ca^{2+} also increased linearly with $\log_{10}[K^+]_o$ (noradrenaline, $r = 0{\cdot}992$; GABA, $r = 0{\cdot}990$).

In that release, as presented here, constitutes only that efflux dependent upon Ca^{2+}, the relationships in Fig. 7 converge upon a central principle in

the control of transmitter release: the state of depolarization can directly control release in the presence of Ca^{2+} by controlling Ca^{2+} influx. Depolarization and release are coupled by Ca^{2+} influx through the activation of a potential-dependent Ca^{2+} ionophore. That is, it appears as if in synaptosomes, Ca^{2+} influx can limit the quantity of transmitter secreted.

TABLE 8. The influence of A23187 upon Ca^{2+}-dependent release.

Treatment	K^+ (mM)	Release (%)	
		[^{3}H] NA	[^{14}C] GABA
0·1 % DMSO	15	4·41 ± 0·26	1·02 ± 0·12
	55	9·84 ± 0·62	2·82 ± 0·04
10^{-5} M A23187,	15	9·68 ± 0·52	3·36 ± 0·62
0·1 % DMSO	55	12·14 ± 1·01	4·50 ± 0·22

Synaptosomal tissue was incubated with [^{3}H]noradrenaline and [^{14}C]GABA and washed in either 15 mM or 55 mM-K^+ Solution *B* to which either DMSO or 10^{-2} M A23187 in DMSO was added (1:1000 dilution). Following washout, 1 mm-Ca^{2+} was introduced into the wash solution. Each value is the mean and S.E. of the mean of four determinations.

In a situation of depolarization-limited Ca^{2+} influx, increased depolarization (higher $[K^+]_o$) should, and did, produce more release (Fig. 7). In addition, treatments which increase Ca^{2+} permeation without producing changes in membrane potential should also increase release. A23187, an artificial Ca^{2+} ionophore (cf. Pfeiffer, Reed & Lardy, 1974), provides a by-pass to the normal voltage control of membrane permeability to Ca^{2+} in synaptosomes (Holz, 1975). At a given state of depolarization, then, A23187 should increase release to the extent that Ca^{2+} influx has been limited by that given level of depolarization. As shown in Table 8, noradrenaline and GABA release in response to 1 mM-Ca^{2+} were enhanced by A23187, even in the presence of 55 mM-K^+. For the K^+ concentrations tested, it would appear that Ca^{2+}-dependent release in the presence of K^+ is at least in part controlled at the level of Ca^{2+} permeation and that the control of Ca^{2+} influx is similar to that for the K^+-sensitive, voltage-dependent Ca^{2+} ionophore described by Baker *et al.* (1973*a*, *b*).

Calcium-dependent release: calcium dependency. Increases in $[Ca^{2+}]_o$ at a given $[K^+]_o$ should eventually saturate permeation sites. In 55 mM-K^+, as illustrated in Fig. 8, both noradrenaline and GABA release approached asymptotic values at higher $[Ca^{2+}]_o$. However, GABA release required lower $[Ca^{2+}]_o$ to reach asymptote: $[Ca^{2+}]_o$ above 1 mM produced little additional effect upon GABA release but noradrenaline release continued to increase. Even at 10 mM-Ca^{2+}, noradrenaline release only approached asymptotic values. At high $[Ca^{2+}]_o$, then, the differences in noradrenaline

and GABA release are most dramatically illustrated. At 1 mM-Ca^{2+} noradrenaline release was fourfold larger than GABA release, whereas at 10 mM-Ca^{2+} the difference approached sixfold.

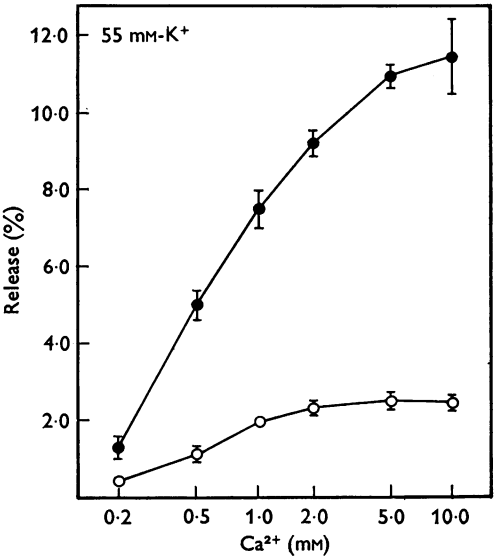

Fig. 8. The influence of external Ca^{2+} upon release. Filled circles indicate noradrenaline release and open circles indicate GABA release. Synaptosomal tissue was incubated as described in Fig. 5, and washed in 55 mM-K^+ Solution *B*. Following washout, Ca^{2+}, at the indicated concentrations, was introduced into the wash solution. Each point represents the mean and S.E. of the mean of four to eight determinations. No error bar is shown for a S.E. less than the radius of the circle.

Parametric analysis of the interaction between $[Ca^{2+}]_o$ and $[K^+]_o$ revealed additional differences in the responses of the noradrenaline and GABA systems to variations in the stimulation situation (Fig. 9). For a given $[Ca^{2+}]_o$, increases in $[K^+]_o$ always increased release of both noradrenaline and GABA, and in lower $[K^+]_o$ relatively lower $[Ca^{2+}]_o$ were required to produce asymptotic release of noradrenaline (Fig. 9*A*). However, GABA release (Fig. 9*B*) in the presence of $[K^+]_o$ lower than 55 mM apparently decreased, rather than approaching an asymptote, with higher $[Ca^{2+}]_o$. That is, Ca^{2+} appeared to inhibit its own stimulation of GABA but not noradrenaline release. This differential effect of Ca^{2+} upon noradrenaline and GABA release is demonstrated more clearly in Fig. 10. From identical filters, noradrenaline release increased but GABA release decreased with the higher $[Ca^{2+}]_o$ (GABA, paired *t* test: 10 mM-K^+, $t = 2{\cdot}44$; 15 mM-K^+, $t = 8{\cdot}01$; 25 mM-K^+, $t = 3{\cdot}72$). The decrease in release with elevated Ca^{2+} concentrations described previously for GABA release from rat brain

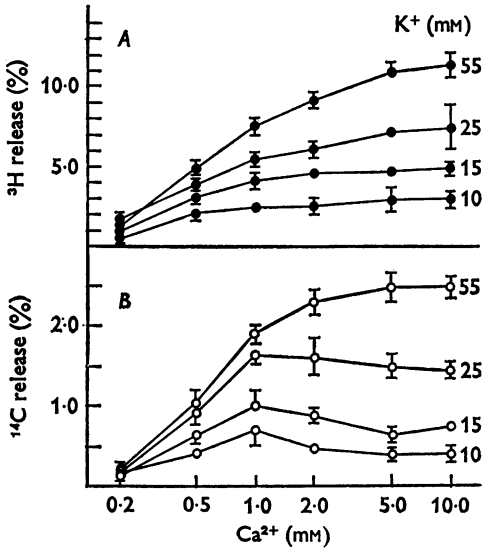

Fig. 9. Release as a function of K^+ and Ca^{2+} concentration. Filled circles indicate noradrenaline release and open circles indicate GABA release. Synaptosomal tissue was incubated as described in Fig. 5 and washed in Solution *B* at the K^+ concentrations indicated. Following washout, Ca^{2+} was introduced into the wash solution at the indicated concentrations. Each point represents the mean and S.E. of the mean of four to eight determinations. Note scale differences between noradrenaline release, *A* and GABA release, *B*.

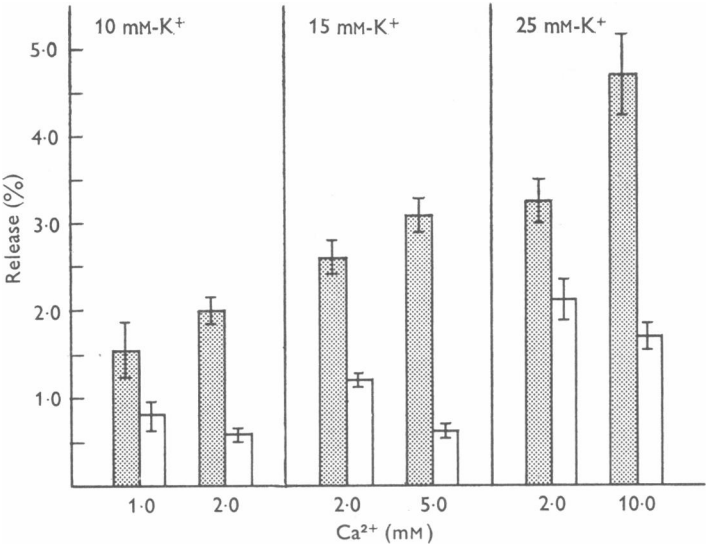

Fig. 10. Ca^{2+} auto-inhibition of release. Synaptosomal tissue was incubated, washed and stimulated as in Fig. 9. Each point represents the mean and S.E. of the mean of four determinations. Stippled bars indicate noradrenaline release and open bars indicate GABA release.

synaptosomes has been termed 'calcium autoinhibition' (Levy, Haycock & Cotman, 1974).

An analysis of the time course of 15 mM-K^+-facilitated, Ca^{2+}-dependent release at 1 mM ('normal') and 5 mM ('auto-inhibited') Ca^{2+} is illustrated in Fig. 11. The early phasic release of GABA was qualitatively altered such that the peak normally seen during the second 5 sec collection was eliminated. The release of noradrenaline, on the other hand, followed the same general shape for 1 and 5 mM-Ca^{2+} and did not exhibit Ca^{2+} auto-inhibition.

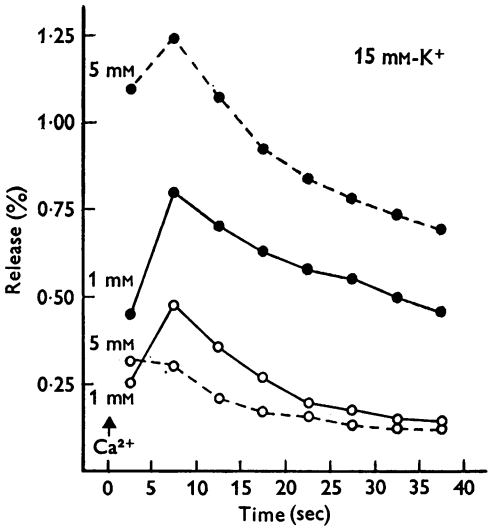

Fig. 11. Temporal characteristics of Ca^{2+} auto-inhibition. Experimental details and calculations were as presented for Fig. 3*B*. Each point represents the mean of four determinations. Filled circles indicate noradrenaline release and open circles indicate GABA release.

Calcium-dependent release: calcium auto-inhibition. Three possible mechanisms can be suggested from current theories to explain calcium auto-inhibition of GABA release: (*a*) Ca^{2+} may decrease its own membrane permeability; (*b*) Ca^{2+}, once having permeated, may decrease its own ability to promote exocytosis and (*c*) Ca^{2+} may partially reverse the depolarizing effects of K^+ thereby indirectly decreasing its own membrane permeability. Importantly, calcium auto-inhibition is observed for the GABA system but not the noradrenaline system, a distinction not accommodated *a priori* by any of these alternatives.

In support of the third alternative, 5 mM-Ca^{2+} slightly decreased diO-C_6 fluorescence induced by 15 mM-K^+. This Ca^{2+} effect, however, was indistinguishable from 5 mM-Mg^{2+} (which does not support release) or 5 mM-Ba^{2+} (which does not produce auto-inhibition). Thus, even

though Mg^{2+} and Ba^{2+} might be expected to antagonize K^+ effects in their own right, the possibility exists that the slight Ca^{2+} effects observed arose from non-specific divalent cation effects upon diO-C_6 binding (Sims *et al.* 1974).

On the other hand, A23187, the Ca^{2+} ionophore, might be expected to distinguish between alternatives *a* and *b*. If calcium auto-inhibition were due to Ca^{2+}-induced decreases in Ca^{2+} permeation, A23187 should either eliminate Ca^{2+} autoinhibition or increase the external Ca^{2+} concentration

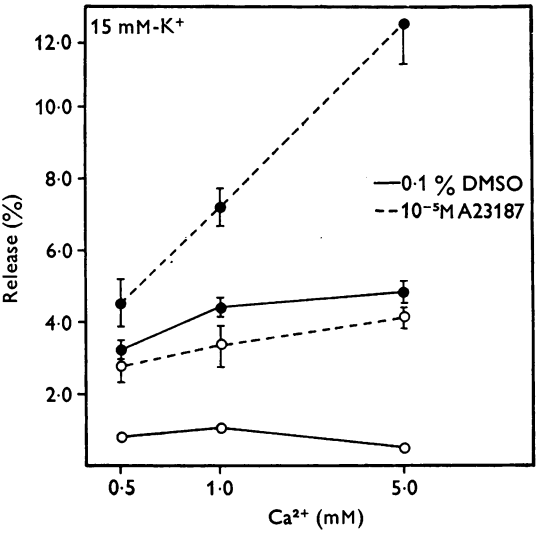

Fig. 12. Influence of A23187 upon Ca^{2+} auto-inhibition. Synaptosomal tissue was incubated as in Fig. 5 and washed either in 15 mM-K^+ media with 0·1 % DMSO or in 15 mM-K^+ media with 10^{-5} M A23187 and 0·1 % DMSO. Following washout, Ca^{2+} was introduced into the wash solution at the indicated concentrations. Each point represents the mean and S.E. of the mean of four determinations. Filled circles indicate noradrenaline release and open circles indicate GABA release.

required to produce auto-inhibition. If, however, a relative excess of intracellular Ca^{2+} were producing the effect, increasing Ca^{2+} influx with A23187 should lower the external Ca^{2+} concentration necessary to produce auto-inhibition. As can be seen in Fig. 12, 10^{-5} M A23187 elevated release of both noradrenaline and GABA for all Ca^{2+} concentrations tested. A23187 not only shifted the Ca^{2+}-release curve to the left but eliminated the decrease in GABA release observed normally between 1 mM and 5 mM-Ca^{2+}. These data converge then on the hypothesis that calcium auto-inhibition arises from a Ca^{2+}-induced decrease in its own permeation, but do not distinguish between a direct or indirect action.

DISCUSSION

Stimulus–secretion coupling processes in brain

These data demonstrate that noradrenaline and GABA release from brain synaptosomes meet the criteria of stimulus–secretion coupling established in other well-studied systems. Noradrenaline and GABA were rapidly released from endogenous stores in a Ca^{2+}-dependent manner which was antagonized by Mg^{2+}. The requirement for Ca^{2+} in the release of noradrenaline and GABA from endogenous synaptosomal stores confirms previous reports for a variety of transmitter substances (deBelleroche & Bradford, 1972*a*, *b*; Blaustein *et al.* 1972; Holz, 1975). However, Mg^{2+} antagonism has been reported only for the release of acetylcholine from brain slices (Molenaar & Polak, 1970). In that the other alkaline earths (Sr^{2+}, Ba^{2+}) stimulate release, the demonstration of Mg^{2+}'s ability to inhibit release without itself supporting release constitutes an important criterion for stimulus–secretion coupling. In other systems Mg^{2+} competes with Ca^{2+} and should antagonize Ca^{2+}-dependent release, but in addition, the failure of Mg^{2+} itself to support release eliminates possible interpretations of generalized divalent cation effects upon the synaptosomal tissue unassociated with the release process *per se*.

Previously, we showed that GABA accumulated by synaptosomes from exogenous pools was released in a Mg^{2+}-antagonized, Ca^{2+}-dependent manner, suggesting the functional coupling of transport and release in isolated synaptosomes (Levy *et al.* 1973, 1974). The present data further demonstrate stimulus-secretion coupling for both noradrenaline and GABA accumulated by synaptosomal tissue which appears quite comparable to the release of endogenous transmitter.

As in brain slices (Mulder & Snyder, 1974; Mulder, Yamamura, Kuhar & Snyder, 1974), our data illustrate the association of stimulus–secretion coupling systems with high-affinity, Na^{+}-dependent uptake systems. The transport–release processes were restricted to synaptosomal fractions and the transmitter candidates studied. Other particles accumulated transmitter substances and synaptosomal fractions accumulated other molecules, but the transport–release processes appeared coupled only for transmitter candidates and only within the synaptosomal preparations. Taken together, these observations suggest that the release of exogenously-loaded transmitter provides a valid method for the investigation of stimulus–secretion coupling in synaptosomes.

The role of Ca^{2+} in K^+-stimulated stimulus–secretion coupling processes in brain

In the physiological situation, the depolarizing influence of invading action potentials couple calcium influx to the secretory event. In synaptosomes and brain slices, K^+ is recognized as a depolarizing agent which increases Ca^{2+} influx (cf. Blaustein, 1974) and has been implicitly applied as such in many release situations. K^+ is also recognized to exert other effects upon release not directly related to calcium fluxes (Cooke & Quastel 1973). Thus consideration of stimulus–secretion coupling when K^+ is used as the stimulus requires the distinction between efflux dependent upon external Ca^{2+} (release) and efflux deriving from other sources. This provides the rationale for our paradigm in which Ca^{2+} is pulsed into a predepolarized system and release is calculated as that above baseline efflux. Only when release is distinguished from these other sources of efflux can a clear interpretation within the concepts of stimulus–secretion coupling be made.

The release process in synaptosomes is associated with the influx of Ca^{2+} (Blaustein *et al.* 1972; Holz, 1975). The present study further suggests that the influx of Ca^{2+} associated with the release process is controlled by a voltage-dependent Ca^{2+} ionophore, as suggested for other systems (Katz & Miledi, 1967, 1969; Baker *et al.* 1973*a*, *b*). For low levels of Ca^{2+}, release of noradrenaline and GABA was related directly to log $[K^+]_o$ and, by inference from the diO-C_6 fluorescent probe measurements, directly to depolarization. Such control of Ca^{2+} influx by a voltage-sensitive ionophore could actually be rate limiting for release over short intervals. Even in the presence of 55 mM-K^+, A23187 increased the release of both noradrenaline and GABA at all Ca^{2+} concentrations tested. Consistent with a release process limited by a Ca^{2+} influx controlled by membrane depolarization, in the presence of 55 mM-K^+, increases in Ca^{2+} concentrations appeared to saturate the release process. And, for the noradrenaline system, lower concentrations of Ca^{2+} were required to saturate the release process in the presence of lower K^+ concentrations.

When the depolarization-limited influx of Ca^{2+} was by-passed with A23187, release increased linearly with the log $[Ca^{2+}]_o$. Even though part of this relationship may be due to A23187-limited permeation, such a situation may represent a relatively direct Ca^{2+}-release site interaction. The notion of a release site (X) with which Ca^{2+} interacts ($Ca + X \rightleftharpoons CaX$, del Castillo & Katz, 1954) has been applied in analogy to a Michaelis–Menton analysis of steady-state enzyme kinetics (Jenkinson, 1957; Dodge & Rahamimoff, 1967). For a reaction $nCa + X \rightleftharpoons Ca_nX$, the maximum slope of the relationship ln release *vs.* ln $[Ca^{2+}]_o$ estimates the parameter n.

Linearity of release with log $[Ca^{2+}]_o$ has been described for other systems (Hubbard, 1961; Kirkepar & Misu, 1967); however interpretation within this analogy is not immediately obvious.

Characteristic of other stimulus–secretion coupling systems, Sr^{2+} and Ba^{2+}, but not Mg^{2+}, substitute for Ca^{2+} in supporting secretion from the synaptosomal fraction. The ordering of the alkaline earths for noradrenaline and GABA secretion ($Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$) is similar to that for the secretion of catecholamines from the adrenal medulla. These and the previously mentioned similarities between the release process for noradrenaline and GABA in synaptosomes and the release process in other well-studied stimulus–secretion coupling systems argue that the underlying release processes for these systems are the same. For the peripheral stimulus–secretion coupling systems, convincing evidence has been presented that Ca^{2+}-dependent secretion represents exocytosis of transmitter substance from vesicular storage organelles (cf. Heuser & Reese, 1973; Kagayama & Douglas, 1974; Grynszpan-Winograd, 1971). Recently, in fact, van Harreveld & Trubatch (1975) have demonstrated an increase in exocytotic profiles for brain in the presence of calcium and elevated potassium. However, although secretion is clearly dependent on Ca^{2+}, a direct association of Ca^{2+}-dependent release and an exocytotic process as yet remains to be demonstrated for the synaptosomal system.

Differences between stimulus–secretion coupling of noradrenaline and GABA

The observed differences in the release of noradrenaline and GABA may reflect inherently distinct input–output functions for these two systems. In all stimulation conditions, noradrenaline release was larger than GABA release and for the more intense stimuli the differences became greater, suggesting that the noradrenaline system has a wider response flexibility than the GABA system.

A consistent observation throughout these studies was that the % noradrenaline release was always greater than the % GABA release. An initial interpretation could be that GABA was loaded into more 'irrelevant' pools, i.e. pools not accessible to the release process. However, this reservation does not apply to the release of endogenous noradrenaline and GABA. Furthermore, if, for example, only 50 % of the GABA loaded into the tissue were in 'relevant' pools, then the % release of GABA should actually be twice as large as calculated. In most situations, however, to equate noradrenaline and GABA release via this correction would require that over 50 % of the GABA be unreleasable, a value higher than allowed by previous data (Levy *et al.* 1973). Thus, the differences in percent release between noradrenaline and GABA are probably not accounted for solely on the basis of differential distribution of the accumulated pools.

Not only was noradrenaline release greater, but the noradrenaline system appeared to have a greater capacity to respond to the more intense stimulation conditions. Release of noradrenaline in response to Ba^{2+} increased fourfold relative to Ca^{2+} whereas the release of GABA only increased twofold. Release of noradrenaline also approached larger asymptotic release values with increases in Ca^{2+}, and higher Ca^{2+} concentrations were required to approach an asymptote. Of particular interest the slope of noradrenaline release *vs.* K^+ concentration and *vs.* Ca^{2+} concentration in the presence of A23187 was greater than the slope of GABA release. In these cases, even if the lower release values were equalized on the basis of differential distribution of the transmitter, the slope of noradrenaline release was still larger than the slope of GABA release! These data suggest that, independent of the exact kinetic interpretation of the $[Ca^{2+}]_o$-release relationship, the noradrenaline release process somehow generates a more efficient input–output function. Furthermore, the basis of the difference between the noradrenaline and GABA system could be as basic as the stoichiometry between Ca^{2+} and the postulated release site. Clearly, at least at a qualitative level, these differences exist since Ca^{2+} produced auto-inhibition only for GABA release.

Since a given 30 sec Ca^{2+} pulse produces a fractional release on the order of 10^{-2} of the available store from the synaptosomal tissue (compared to 10^{-5} for single electrical pulses in peripheral systems, cf. Hughes & Roth, 1974; Stjarne, 1973), transmitter availability may present a limiting condition for release, especially for the later stages of release when secretion is gradually decreasing. If, for example, the initial release of both noradrenaline and GABA in these situations is limited by Ca^{2+} influx interacting with some 'releasable' pool whereas release at more extended periods of time reflects an interaction between Ca^{2+} influx and the ability of the transmitter systems to 'mobilize' transmitter into the releasable pool, the observed maintenance of noradrenaline release compared to GABA release at the more extended time periods (Figs. 3, 4) may reflect basic differences in the storage properties for these two systems. At present, however, the contributions of Ca^{2+} influx and transmitter availability to the levels of noradrenaline *vs.* GABA release are unknown.

Stimulus–secretion coupling processes in brain synaptosomes provide a forceful analogy to other well-studied stimulus–secretion coupling systems. In addition to fulfilling the criteria for stimulus–secretion coupling and exhibiting other processes characteristic of stimulus–secretion coupling systems, the study of transmitter release from synaptosomes provides technical advantages not obtained from other systems. Because the synaptosomal preparation minimizes diffusional barriers and cell–cell interactions, the release process and released substance can be quantitatively analysed.

Demonstration of release within 250 msec represents for biochemical measurements the most rapid detection of Ca^{2+}-dependent secretion. In certain applications, though, the heterogeneity of synaptosomal fractions with respect to type, functional integrity, and contamination may limit their usefulness. With this preparation, however, the possibility exists of further resolving the release process into molecular components and perhaps ultimately reconstituting the release process. More importantly, the synaptosomal preparation allows a cogent approach for studying the properties of release processes in brain.

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