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CALCIUM-DEPENDENT RELEASE OF PUTATIVE NEUROTRANSMITTERS IN THE CHICK VISUAL SYSTEM

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Abstract—The calcium-dependent release of putative neurotransmitters has been studied in the chick brain. Several radioactive compounds were taken up into chick optic lobe homogenates by a high-affinity sodium-dependent transport mechanism. At a concentration of potassium sufficient to cause extensive depolarization, a large proportion of the efflux of the accumulated labeled compounds was calcium dependent. Calcium-stimulated release was antagonized by magnesium. The calcium ionophore, A-23187, enabled calcium-related release to occur in the absence of depolarizing conditions, except after accumulation of [^3H]choline. Veratridine was used in order to bring about depolarization in low external potassium concentrations. In this case magnesium was able to replace calcium in inducing the efflux of labeled compounds. Tetrodotoxin inhibited the veratridine-stimulated release of compounds in the presence of calcium. The rate of release of accumulated leucine was affected to a much lesser extent by the presence of calcium. Calcium-stimulated release was also found in homogenates of the optic nerve, indicating that this process is not confined to synapses. Calcium-stimulated release of different compounds varied according to the developmental age and temperature of incubation. This suggests that some dissimilarities in the nature of their release mechanisms exist.

Our results suggest that much calcium-dependent efflux is related to presynaptic neurotransmitter release mechanisms. However, a similar release process may exist in non-synaptic regions of the neuron and in glia. Furthermore, the release of non-transmitter compounds under depolarizing conditions can also be enhanced by the presence of calcium.

THE ADDITION of depolarizing concentrations of potassium to brain tissue results in an efflux of a variety of compounds. This release appears to be less specific than that which also requires the presence of calcium (COTMAN, HAYCOCK & WHITE, 1976). Such calcium-dependent release of neurotransmitter compounds may be predominantly confined to neurons and especially the synapse (COTMAN *et al.*, 1976; HAMMARSTAD & LYTLE, 1976; SELLSTROM & HAMBERGER, 1977; OLSEN, LAMAR & BAYLESS, 1977). However, Ca^{2+} -dependent release of putative neurotransmitters from glia has been reported (ROBERTS, 1974; MINCHIN & IVERSEN, 1974).

Calcium-requiring release from brain slices or synaptosomes has been shown for several putative neurotransmitters (DE BELLEROCHÉ & BRADFORD, 1977; VARGAS, DE LORENZO, SALDATE & ORREGO, 1977; OSBORNE, BRADFORD & JONES, 1973), while Ca^{2+} does not greatly affect the efflux of non-neurotransmitter amino acids from such preparations (BRADFORD, 1975). There is much evidence of the

essential role of calcium in neurotransmitter release mechanisms (SIMPSON, 1968). The electricity stimulated release of γ -aminobutyrate (GABA), glycine and glutamate from nerve tissue is calcium dependent (ROBERTS & MITCHELL, 1972). It is not clear whether such substances are always released from synaptic vesicles since there is evidence that some amino acid neurotransmitter candidates can be directly released from the synaptic cytoplasm by depolarization (DE BELLEROCHÉ & BRADFORD, 1977).

We have examined the characteristics of the Ca^{2+} -dependent release process in regions of the maturing chick CNS. The method we have developed ensures that Ca^{2+} requiring release is specifically determined. Care was taken to ensure that preparations were subjected to no significant osmotic changes in the applied media.

The use of radiolabeled neurotransmitter candidates is not as satisfactory as the measurement of the liberation of endogenous unlabeled compounds. However, the release patterns of endogenous and exogenous neurotransmitters in response to ion changes often appear to parallel each other (COTMAN *et al.*, 1976). On the other hand, the intracellular pool into

Abbreviation: GABA, γ -aminobutyrate.

which exogenous compounds are accumulated may have characteristics that do not always correspond to the pool that is responsive to Ca^{2+} -stimulation (NADLER, WHITE, VACA & COTMAN, 1977a; RYAN & ROSKOWSKI, 1975).

The specificity of calcium-related release has been determined using several radiolabeled postulated neurotransmitter and non-neurotransmitter chemicals. We have also examined the effects of a depolarizing agent (veratridine), a calcium ionophore (A-23187, Eli Lilly Corp.), and magnesium ions on the release of accumulated labeled compounds.

EXPERIMENTAL PROCEDURES

Uptake of labeled compounds

Chick and chick embryos of the White Leghorn strain were used. After decapitation, tissues were rapidly dissected out and weighed. Retinae were carefully removed largely free of the pigment layer. Tissues were then homogenized in 19 vols of 0.32 M sucrose using a conical glass homogenizer.

The standard incubation medium consisted of Krebs-Ringer buffer containing 121 mM NaCl, 4.9 mM KCl, 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , 1 mM ascorbic acid, 10 mM glucose and 40 mM Tris HCl, pH 7.4. An inhibitor of monoamine oxidase (pargyline) was also present (10^{-4} M) as was amino oxyacetic acid (1×10^{-5} M), an inhibitor of GABA transaminase. To this medium was then added a single radioactive compound (New England Nuclear, Boston, MA). Final μM concentrations of each compound were: L-[3- ^3H]glutamic acid 0.01 (3.7 Ci/mmol); [2,3- ^3H]GABA, 0.01 (10 Ci/mmol); [methyl- ^3H]choline chloride, 0.017 (8.4 Ci/mmol); [ethyl-1- ^3H]3,4-dihydroxyphenylethylamine (dopamine) 0.0125 (8.0 Ci/mmol); [2- ^3H]glycine 0.01 (9.4 Ci/mmol); and [4,5- ^3H]leucine 0.008 (58 Ci/mmol). Radioactive choline was used (rather than acetylcholine) since it is relatively stable and specifically transported into cholinergic neurons (KUHA, SETHY, ROTH & AGHAJANIAN, 1973). This medium was gassed with 95% O_2 -5% CO_2 and 0.9 ml of this was mixed with 0.1 ml of a 5% (v/v) tissue homogenate in 0.32 M sucrose. This represented 5 mg wet tissue (320 μg protein) per sample. Incubation was at 37°C for 5 min with continuous shaking. Inhibitors of the uptake process, when used, were added to the medium prior to the addition of tissue preparations. In the case of dopamine, incubations were performed in dim light in order to retard photodecomposition.

All samples were then centrifuged at 28,000 g for 10 min at 0°C. Supernatants were drawn off for determination of the radioactivity remaining unbound to particulate matter.

Release of accumulated materials

Pellets that had taken up labeled postulated neurotransmitters or choline were resuspended in 4 ml of a high-potassium, calcium-free wash buffer containing 75 mM NaCl, 55 mM KCl, 1.2 mM MgSO_4 , 1 mM ascorbic acid, 10 mM glucose, 1 mM ethylene glycol-bis-(β -aminoethyl ether)*N,N*-tetra-acetate and 20 mM tris HCl, pH 7.4. This suspension was centrifuged (28,000 g for 10 min) and the pellets rewashed twice more in this matter. The final precipitate was taken up in 4 ml of release buffer which was identical to the wash buffer except that no ethylene glycol-bis-(β -aminoethyl ether)*N,N*-tetra-acetate or MgSO_4 was

present and 2 mM CaCl_2 was added. Similar control tubes contained the calcium-free wash buffer. These were incubated at 37°C for 5 min and then centrifuged at high speed. One ml of the supernatant was then taken to determine released radioactivity. The pellet was dissolved in 0.5 ml tissue solubilizer (NCS, Amersham-Searle, Arlington Heights, IL) at 45°C, prior to counting. Calcium-independent release control values were determined in matched samples where the release incubation was carried out in the calcium-free wash buffer. It was thus possible to calculate the amount of radioactive compound whose release was dependent on the presence of calcium ions. The use of a series of washes in a high-potassium, calcium-free medium ensured the prior removal of much label that was released in a calcium-independent manner.

The relatively short incubation time used minimizes any metabolic conversion of labeled compounds (IVERSEN & SCHON, 1965; KUHA, SHASKAN & SNYDER, 1971; ROBERTS, 1974; SNYDER, GREEN & HENDLEY, 1975). But during depolarization-induced release after uptake of [^3H]choline *in vitro*, the bulk of the label liberated into the medium is in the form of acetylcholine (MULDER, YAMAMURA, KUHA & SNYDER, 1974).

The basic release method was on occasion modified by the addition of pharmacological agents (veratridine, tetrodotoxin or the calcium ionophore A-23187) or by the addition or substitution of magnesium for calcium in the release buffer. In the case of experiments involving pharmacological agents, the washes and the release incubation were carried out with standard Krebs-Ringer buffer rather than the high potassium buffers. The incubation time for release in the veratridine studies was extended to 20 min in view of the time required for depolarization by this alkaloid (ULBRICHT, 1969). This longer time was also used in experiments with the calcium ionophore.

Each data point presented in Tables or Figures represents the mean of values derived from 6 to 14 individual animals. Standard errors of the mean are given for these means.

RESULTS

Release evoked by potassium-enriched medium

The quantity of each isotopically labeled compound incorporated by chick retina, optic nerve and optic lobe under these conditions has been previously reported (BONDY & PURDY, 1977). The percentage of incorporated label remaining after three washes at 0°C that was released by incubation with calcium-free buffer was as follows: 18.1 ± 1.6 (glutamate), 23.8 ± 1.0 (glycine), 18.9 ± 2.9 (GABA), 13.8 ± 1.5 (dopamine), and 13.8 ± 0.9 (choline). The amount of putative neurotransmitters released during incubation with calcium ions was over twice that released in the absence of calcium. When 10 mM magnesium was substituted for calcium no additional release over control values was produced (Fig. 1). Furthermore, 10 mM MgCl_2 inhibited the release brought about by 2 mM Ca^{2+} (Fig. 1). Thus, Mg^{2+} could not serve in place of Ca^{2+} in effecting release and could also antagonize Ca^{2+} -related release events. Leucine release was also elevated in the presence of calcium. However, the

FIG. 1. Release of radioactive compounds accumulated by homogenates of chick brain. Columns indicate excess release above that occurring in the absence of divalent cations. Standard errors of percentage released radioactivity are shown.

Abbreviations: DA, dopamine; GABA, γ -aminobutyrate; GLU, L-glutamate; GLY, glycine; Leu, leucine.

efflux of this non-neurotransmitter was lower than corresponding values for putative neurotransmitters.

Release evoked by veratridine or a calcium ionophore

In another series of experiments, the washes following accumulation of isotopes were carried out in Ca-free Krebs-Ringer buffer and the release incubation was performed in this buffer or in calcium-containing Krebs-Ringer (Fig. 2). No Ca-mediated release could be evoked under these non-depolarizing conditions where potassium concentration was maintained at a low level. A further indication that depolarization was needed for Ca-stimulated release was the finding that, in the presence of veratridine, 2 mM Ca²⁺ could provoke the release of several accumulated labeled compounds even at low K⁺ levels (Fig. 2). However, the release of choline and of leucine, a compound not

suspected of having a transmitter role, was not enhanced significantly. When tetrodotoxin was added to the incubation medium in addition to veratridine, release was inhibited. Since veratridine increases membrane permeability to Na⁺ while tetrodotoxin blocks the sodium channels of the neuronal membrane (KAO, 1966), the release stimulating effects of veratridine may have involved selective opening of sodium channels and consequent membrane depolarization. Thus, depolarization rather than the specific presence of high external K⁺ concentrations was sufficient to allow Ca²⁺-stimulated efflux of labeled compounds. REDBURN, SHELTON & COTMAN (1976) found a similar effect of tetrodotoxin on veratridine-stimulated release of GABA by synaptosomes. However, veratridine also increased the amounts of labeled compounds effluxing in the presence of 10 mM Mg²⁺

FIG. 2. Effect of veratridine on release of labeled compounds from chick brain homogenate using low potassium (4.9 mM) buffer in the presence of 2 mM Ca²⁺. Columns indicate release in excess of that occurring in the absence of Ca²⁺. TTX, tetrodotoxin; for other abbreviations see legend to Fig. 1.

FIG. 3. Effect of the calcium-ionophore A-23187 (25 mg/ml) on the release of transported radioactive compounds from chick brain homogenate using low potassium (4.9 mM) buffer. Columns represent release above that occurring in the absence of divalent cations. For abbreviations see legend to Fig. 1.

(Fig. 2). Thus, the calcium requiring specificity was not maintained in the presence of veratridine. Ca^{2+} -independent release of transmitter amino acids in the presence of veratridine has been reported by NADLER, WHITE, VACA, REDBURN & COTMAN (1977b). The calcium ionophore A-23187 also enabled Ca^{2+} -dependent liberation of radioactive putative neurotransmitters from brain homogenates (Fig. 3). The liberation of labeled compounds after choline or leucine accumulation was not greatly stimulated by the ionophore. Magnesium was not able to substitute for calcium in the presence of A-23187, except in the case of dopamine (Fig. 3). Therefore, release stimulated by the ionophore was more specifically related to Ca^{2+} than was the veratridine-induced release. The use of this ionophore may increase experimental selectivity toward the neurotransmitter-related process.

Release of substances from homogenates of optic nerve and retina

The Ca^{2+} -dependent liberation of compounds in optic nerve homogenates was measured in order to estimate the extent to which observed release was of non-synaptic origin (Table 1). Using a series of isotopically labeled putative neurotransmitters, the proportion of accumulated radioactivity that could be released was roughly as high in such preparations as in optic lobe homogenates. This efflux may have been from micelles of axonal or glial origin but was obviously not from synapses. However, it should be noted that since the high-affinity uptake capacity of optic nerve homogenates is relatively low (BONDY & PURDY, 1977), the absolute amounts released from the optic nerve is much less than that of a corresponding chick brain homogenate. Calcium-related release of labeled compounds from the optic nerve also occurred if veratridine or A-23187 was substituted for high K^+ as a means of enabling Ca^{2+} to enter into cytoplasmic particles and encapsulated cellular frag-

ments. Thus, it was not possible pharmacologically to separate out the contributions of non-synaptic material to calcium-mediated release. Non-synaptic release of amino acids from nerve trunks has been previously reported as being independent of K^+ and Ca^{2+} concentrations (WEINREICH & HAMMARSLAG, 1975). A pronounced Ca^{2+} -dependent release of all compounds studied other than dopamine was also found in retinal homogenates

TABLE 1. CALCIUM-STIMULATION RELEASE OF LABELED COMPOUNDS FROM HOMOGENATES

Transported compound	Per cent released by 2 mM Ca^{2+}		
	Optic lobe	Optic nerve	Retina
L-glutamate	37.1 ± 4.4	29.3 ± 3.8	27.7 ± 3.1
Glycine	27.7 ± 3.0	22.4 ± 5.0	23.4 ± 3.4
GABA	30.1 ± 2.9	23.3 ± 2.9	27.3 ± 3.0
Dopamine	14.1 ± 2.8	5.0 ± 1.6	3.0 ± 1.2
Choline	25.4 ± 3.7	20.1 ± 1.8	22.2 ± 1.9

Labeled compounds were transported into homogenates from 10^{-7} M to 10^{-8} M solution and the proportion of accumulated radioactivity that was released by the addition of calcium to the potassium-enriched solution was calculated as described in Experimental Procedures. Standard errors of the mean are presented.

Influence of temperature on the release of dopamine and γ -aminobutyrate

After accumulation of [^3H]GABA, the Ca^{2+} -dependent efflux of radioactivity was unaffected by variations in the temperature of the release incubation (Table 2). However, the release of label after dopamine transport was more temperature sensitive with a Q_{10} of around 1.9. This value suggests facilitated diffusion rather than an energy-dependent release process (SNYDER, GREEN & HENDLEY, 1968).

Influence of the stage of development

The proportion of radioactivity released by Ca^{2+}

TABLE 2. EFFECT OF TEMPERATURE ON THE CALCIUM-DEPENDENT EFFLUX OF LABELED COMPOUNDS FROM HOMOGENATES OF CHICK BRAIN

Transported compound	Per cent release by 2 mM Ca^{2+} at:			
	0°C	15°C	25°C	37°C
GABA	28.3 $\pm$ 1.2	24.5 $\pm$ 3.0	27.2 $\pm$ 3.3	24.3 $\pm$ 3.0
Dopamine	1.5 $\pm$ 0.3	6.1 $\pm$ 1.1	6.9 $\pm$ 2.0	14.3 $\pm$ 1.8

Incubation and release conditions are described in the text and Table 1.

was tested at various developmental stages in optic lobe homogenates (Table 3). While the rate of high-affinity uptake is lower in embryonic brain (BONDY & PURDY, 1977), the proportion of the label released in response to Ca^{2+} was relatively constant after [^3H]choline or [^3H]GABA accumulation. On the other hand, the Ca^{2+} -related release of radioactivity after dopamine transport was not clearly demonstrable in embryos.

DISCUSSION

Importance of calcium ions

In these studies calcium-mediated release of labeled compounds subsequent to high-affinity transport of neurotransmitter-related compounds was brought about using conditions where Ca^{2+} could be expected to enter subcellular fragments within brain homogenates. Such calcium entry can occur by depolarization of membranes either with a high concentration of external K^+ or by pharmacologically increasing the membrane permeability to Na^+ . Alternatively, the presence of a calcium ionophore can allow Ca^{2+} entry in the absence of membrane depolarization. The use of homogenates rather than partially purified synaptosomal fractions allows the overall Ca^{2+} -related release capacity of brain areas to be compared.

The specificity of calcium-dependent release both for neurotransmitter compounds and for the neuronal location of such release has been suggested by several workers. The efflux of amino acids that are not thought to be neurotransmitters from synaptosomes or brain slices has been described as independent of Ca^{2+} (BRADFORD, 1975) and is not stimulated by veratridine (ADAIR & DAVIDOFF, 1977; BLAUSTEIN, JOHNSON & NEEDLEMAN, 1972). The neuronal origin of much Ca^{2+} -mediated release is suggested by failure

of Ca^{2+} to stimulate [^3H]GABA release from isolated glia (SELLSTROM & HAMBERGER, 1977) and failure of Ca^{2+} or veratridine to release labeled putative neurotransmitters from C-6 glioma cells (LEVY, REDBURN & COTMAN, 1973; WEDEGE, LUQMANI & BRADFORD, 1977). However, the criterion of Ca^{2+} -dependent release may not be absolute for putative neurotransmitters. Some release of alanine may also be triggered by Ca^{2+} (DE BELLEROCHE & BRADFORD, 1977). Also, Ca^{2+} -dependent release of GABA has been described in glial cells of the dorsal root ganglion (MINCHIN & IVERSEN, 1974). In their report, Mg^{2+} was able to partially substitute for, rather than antagonize, Ca^{2+} . Magnesium or manganese ions effectively abolish the transneuronal passage of the action potential (DEL CASTILLO & KATZ, 1954). *In vitro* these ions usually have little or no stimulatory capacity in the release of putative neurotransmitters (ADAIR & DAVIDOFF, 1977; ROBERTS & MITCHELL, 1972; NADLER *et al.*, 1977; REDBURN *et al.*, 1976) (see Fig. 1). Release of postulated transmitters from neuronal preparations may, however, not always be responsive solely to Ca^{2+} . A sodium-dependent release of neurotransmitter has been described that is independent of Ca^{2+} and somewhat facilitated by Mg^{2+} (CHARLTON & ATWOOD, 1977; SWENARCHUK & ATWOOD, 1975). Paradoxical inhibition of the action potential and increase of neurotransmitter release at the neuromuscular junction has been described for the manganese ion (BALNARVE & GAGE, 1973). Such anomalous events may be due to displacement of internally bound calcium or minor calcium agonist activity of other ions. A requirement for magnesium as well as calcium for bringing about neurotransmitter release from synaptic vesicles has been reported (DE LORENZO & FREEDMAN, 1978). Calcium-independent neurotransmitter release may also occur if nerve endings are exposed to hyperosmotic conditions (CHAN & FISHMAN, 1977) but this effect has not always been found (NADLER *et al.*, 1977).

The bulk of the Ca^{2+} -dependent efflux may be relevant to presynaptic neurotransmitter release because the sodium-dependent high-affinity uptake of labeled putative neurotransmitters that precedes release in our system may be predominantly synaptosomal in homogenates (KUCHAR, 1973; BONDY & PURDY, 1977).

The means used to allow Ca^{2+} entry into cerebral homogenates can alter the selectivity of the Ca^{2+} -stimulated release. Thus, when 55 mM KCl was used as a depolarizing agent, specificity of release by Ca^{2+}

TABLE 3. DEVELOPMENT OF CALCIUM-DEPENDENT RELEASE IN HOMOGENATES OF CHICK BRAIN

Transported compound	Per cent released by 2 mM Ca^{2+}		
	9-day-old embryo	14-day-old embryo	1-day-old chick
GABA	26.8 $\pm$ 2.7	25.9 $\pm$ 2.9	27.0 $\pm$ 4.1
Choline	19.8 $\pm$ 3.1	27.8 $\pm$ 2.2	26.8 $\pm$ 3.2
Dopamine	0.7 $\pm$ 0.4	—	12.8 $\pm$ 0.6

Incubation and release conditions are described in the text and in Table 1.

rather than Mg^{2+} was absolute, but release of accumulated leucine was also Ca stimulated. Veratridine causes a selective increase of neuronal permeability to the sodium ion (OHTA, NARAHASHI & KELLER, 1973). The resulting depolarization causes increasing entry of Ca^{2+} into the nerve terminal (ISHIDA & YONEDA, 1974; CATTERALL, 1975) and a specific release of postulated neurotransmitter compounds (ADAIR & DAVIDOFF, 1977; BLAUSTEIN *et al.*, 1972; REDBURN *et al.*, 1976). Non-neurotransmitter amino acids are not released in this manner and no such release from C-6 glioma cells in culture or from microsomes was observed (WEDEGE *et al.*, 1977; LEVY *et al.*, 1973). However, in our study, specificity for Ca was lost in the presence of veratridine since release in the presence of Mg was also stimulated by this alkaloid. The greatest specificity for Ca in effecting the release of putative neurotransmitters was found with the calcium ionophore A-21387. In this case Mg could not replace Ca (except for the release of dopamine) and Ca did not enhance release of the non-transmitter leucine.

Release of substances from optic nerve homogenates

Under all conditions used, we found considerable Ca-dependent release of accumulated putative neurotransmitters from homogenates of the optic nerve. While this efflux may have been from neuronal or glial fragments, it could not have originated from nerve endings. Tetrodotoxin blocked the veratridine-stimulated release of labeled compounds from optic nerve homogenates (unpublished results). Since this toxin is thought to act primarily on the neuronal membrane (BENJAMIN & QUASTEL, 1972), the release of substances from optic nerve preparations may be predominantly from axonal fragments.

Influence of temperature and of developmental age

The Ca^{2+} -mediated release of dopamine differed from that of GABA both in its temperature sensitivity and according to the age of the embryo. Therefore, the precise mechanism of release of various compounds is not uniform. Calcium-dependent release may develop either concurrently with, or after maturation of high-affinity uptake systems. REDBURN, BROOME, FERKARY & ENNA (1978) found a more gradual development of Ca-dependent release in rat brain than we are reporting for chick brain. These authors suggest that the ontogenesis of release mechanisms correlates more closely with synaptogenesis than does high-affinity uptake. DAVIES, JOHNSTON & STEPHANSON (1975) found little ontogenic difference in Ca^{2+} -related glycine release from mouse spinal cord but an increase in GABA release from mouse cortex with maturation. The energy requirement for the Ca^{2+} -stimulated liberation of accumulated compounds is unclear. While temperature did not affect [3H]GABA release in our system, NELSON-KRAUSE & HOWARD (1978) found that K^+ or calcium-ionophore related release of [3H]GABA from rat brain synaptosomes was reduced but not abolished by metabolic inhibitors. We have found that dibutyl lead acetate, a neurotoxic compound, may actually enhance release of accumulated compounds when it is present at low concentrations (below 10^{-6} M, BONDY, HARRINGTON, ANDERSON & PRASAD, 1978).

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