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Calcium-Dependent Norepinephrine Release from Presynaptic Nerve Endings *In Vitro*

(veratridine/K$^+$/tetrodotoxin/synaptosomes/rat/pargyline)

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ABSTRACT Potassium concentrations above 15–20 mM in the medium, or addition of 75 μM veratridine to medium that contains 5 mM K, stimulate calcium accumulation and Ca-dependent norepinephrine release by presynaptic nerve terminals (synaptosomes) incubated *in vitro*. The effects of veratridine, but not of K, are blocked by 0.2 μM tetrodotoxin. This association between Ca entry and norepinephrine release is consistent with the Calcium Hypothesis of transmitter release. These results add further evidence that synaptosomes may retain many functional properties of intact presynaptic endings.

Calcium ions are normally required for the depolarization-induced release of catecholamines by adrenal medulla (1) and sympathetic nerve endings (2). A similar Ca dependence is seen at cholinergic synapses (3) and in various other secretory processes (4). However, largely due to lack of suitable preparations for physiological investigation, little is known about transmitter release processes at synapses of central nervous system tissue. Furthermore, the precise role of Ca in the transmitter release process is unknown.

The present report provides evidence that potassium ions and veratridine, two agents that depolarize intact neurons (5), both stimulate Ca uptake and induce a Ca-dependent release of norepinephrine in preparations containing pinched-off presynaptic nerve endings (synaptosomes) from rat brain. These results indicate that, just as has been shown in regard to many other physiological properties (6–9), preparations containing isolated nerve endings retain the ability to release transmitter substances under appropriate conditions.

METHODS

Synaptosome preparations were obtained from adult rat brains by the techniques of Gray and Whittaker (10). Norepinephrine content was assayed fluorometrically after adsorption onto, and elution from, alumina, and after oxidation by the trihydroxyindole method (11). On the average, 55–65% of the norepinephrine and 30–35% of the protein of our brain homogenates were retained in the crude mitochondrial fraction ("P_2" of Gray and Whittaker). As shown in Table 1, after sucrose density gradient separation of the P_2 fraction, the highest activity (per gram of protein) of the norepinephrine-containing particles was found at the 0.8–1.2 M sucrose interface (fraction "P_2B" of Gray and Whittaker), the location of the most enriched preparation of synaptic bodies. Fraction P_2B (synaptosomes)* generally contained about 30% of the protein from fraction P_2, or about 10% of the protein from the original homogenate (about 100 mg of protein per brain). The similarity of the norepinephrine and nerve-ending distributions provides evidence that most of the norepinephrine in the P_2 fraction is associated with the nerve terminals. A number of norepinephrine-release experiments (see below) were performed with material from the P_2 fraction, rather than with the sucrose gradient fraction enriched for synaptosomes, in order to obtain sufficient norepinephrine for the multiple assays.

Pharmacological evidence that the fluorogenic material is norepinephrine is provided by the observation that its concentration was increased 2- to 4-fold in the brain subfractions of animals that had been treated with the monoamine oxidase inhibitor, pargyline (Table 1). Conversely, prior treatment with reserpine (1 mg/kg intraperitoneally, 36 and again 12 hr before death) reduced the norepinephrine content of the brain homogenate and subfractions to undetectable concentrations.

For Ca-uptake and norepinephrine-release experiments, synaptosomes, or unfractionated P_2 was slowly diluted (0.5 ml/min) to a final volume of 7 ml with a Ca-free physiological salt solution at 2°. The salt solution (Na + 5K) had the following composition (mM): NaCl, 132; KCl, 5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; glucose, 10; and Tris-maleate, 20 (final pH 7.4 at 30°). The tissue suspensions were centrifuged for 5 min at 10,000 × *g*; after the supernatant solutions were decanted, the particulate material (10–25 mg of protein) was resuspended in a small volume (0.4–0.6 ml) of a similar salt solution, and incubated for 12 min at 30° to allow internal cations to reach a steady concentration. Additional isotonic salt solution was added, bringing the final volume to 1.0 ml; this solution contained either $CaCl_2$ (final concentration = 1.2 mM) or ethylene glycol-bis[β-aminoethyl ether]*N*,*N*′-tetraacetic acid (EGTA; final concentration = 2 mM). In certain instances (see below), some of the Na in the medium was replaced isosmotically by K; alternatively, veratridine (K and K laboratories, Tarrytown, N.Y.) and/or tetrodotoxin (Sankyo Co., Ltd, Tokyo) was added to the medium. After a 10-min incubation at 30° (norepinephrine release was linear during this period), the suspension was centrifuged at 10,000 × *g* for 5

Abbreviation: EGTA, ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*′-tetraacetic acid.

* Although this fraction, located at the 0.8–1.2 M sucrose interface, is not a pure preparation of presynaptic nerve ending particles (see footnote to Table 1), for simplicity we will refer to it as the "synaptosome" preparation, in conformity with numerous other authors.

TABLE 1. *Distribution of norepinephrine, K-stimulated Ca uptake activity, and synaptosomes in various fractions of discontinuous sucrose density gradients*

Sucrose fraction	Norepinephrine concentration* (ng/mg protein)		Relative "specific" distribution†			
			Norepinephrine		K-Stimulated Ca uptake	Synaptosomes
	Normal	Pargyline treated	Normal	Pargyline treated		
0.32–0.8 M Interface	1.48	3.62	0.91	0.83	0.59	0.38
(predominantly membrane fragments)	1.07	3.69	0.71	1.04	—	—
	—	2.23	—	0.63	—	—
0.8–1.2 M Interface	2.14	6.12	1.32	1.40	1.38	1.50
(predominantly synaptosomes)	2.15	5.23	1.42	1.49	—	—
	—	5.09	—	1.44	—	—
Pellet	1.04	2.11	0.64	0.48	0.80	0.57
(predominantly mitochondria)	0.86	2.65	0.57	0.76	—	—
	—	1.30	—	0.37	—	—

The K-stimulated Ca uptake data are from Blaustein and Wiesmann (9). The synaptosome distribution values (*last column*) are calculated from the data of Michaelson and Whittaker (19, Fig. 2; also see ref. 8); their data indicate that about 70% of the recovered nitrogen in the region between 0.8 and 1.2 M sucrose is associated with "nerve-ending particles." Electron micrographs from our own laboratory also indicate that the 0.8–1.2 M sucrose interface is particularly enriched with synaptosomes, although no detailed quantitative distribution in the various gradient layers has been calculated.

* These data are from two gradient separations of the P_2 fractions from normal rats and three from the P_2 fractions from pargyline-treated rats. The third set of data in the "pargyline-treated" column is from a gradient prepared on a different day than the other four gradients. The treated rats were given 100 mg/kg of pargyline intraperitoneally, 12–15 hr before death.

† Concentration or activity (per mg protein) in the appropriate sucrose fraction, divided by the concentration or activity in the sum of the sucrose fractions. In our preparations, on the average, about 50% of the protein is at the 0.32–0.8 M sucrose interface, 30% at the 0.8–1.2 M interface, and 20% in the pellet.

min at 2°. The supernatant solution (1.0 ml) was immediately decanted and deproteinized with 0.2 ml of 1.17 M $HClO_4$–0.5 M Tris base–13.4 mM Na_2EDTA. In several experiments the pellet was also deproteinized, and both pellet and supernatant were assayed for norepinephrine content, although more frequently only the norepinephrine content of the supernatant solution was determined.

Pellet protein content was assayed by the Lowry method (12); bovine-serum albumin was used as a standard.

For ^{45}Ca-uptake studies, the incubation solution contained 0.2 mCi of ^{45}Ca/mmol of $CaCl_2$. Incubation was terminated, usually after 1 min (uptake was linear for 2–3 min—see ref. 9) by the addition of isotonic salt solution containing EGTA (final concentration = 7.5 mM). Procedures for removal of the extra-synaptosomal ^{45}Ca, and determination of the amount of ^{45}Ca sequestered within the particulate material have been described (8, 9).

RESULTS

Experiments (9) have shown that ^{45}Ca uptake is stimulated by elevation of the K concentration of the incubation medium ($[K]_o$) above 15–20 mM; the uptake curve levels off at about 60 mM K (Fig. 1). The particles that exhibit this activity distribute in the sucrose gradient in the same way as does norepinephrine, with the highest activity concentrated in the synaptosome fraction (Table 1).

An increase of $[K]_o$ also stimulates synaptosome norepinephrine release. Fig. 1 illustrates this effect in samples of a crude mitochondrial preparation. The stimulatory effect of K is abolished if Ca is omitted and 2 mM EGTA is added to the incubation medium (Fig. 1). It should be emphasized that the similarity between the ^{45}Ca-uptake and the norepinephrine release curves does not necessarily imply a linear relationship between Ca uptake and Ca-dependent norepinephrine release. Only a very small fraction of the pinched-off nerve endings in our preparations are noradrenergic endings, and many of the other types of endings may also respond to increased $[K]_o$ by increased Ca uptake (3, 4); preliminary data from our laboratory (unpublished) indicate that there is a large K-stimulated Ca uptake in synaptosome preparations obtained from both norepinephrine-rich (brainstem) and norepinephrine-poor (cerebellum) areas of brain. Another complicating factor is the rate of ^{45}Ca accumulation: while K-stimulated Ca influx in synaptosome preparations is linear for 5–10 min, Ca efflux increases markedly after 2–3 min, so that net Ca reaches a new steady concentration and ^{45}Ca uptake is no longer a good measure of Ca influx in incubations lasting longer than about 2 min (unpublished data). Thus, the similarities between the K-stimulated Ca accumulation and norepinephrine-release curves of Fig. 1 suggest an association between these two parameters in nerve endings, but additional data will be required to further define the quantitative relationship.

If these effects of increased $[K]_o$ on Ca uptake and norepinephrine release are a consequence of the depolarizing action of K^+ ion, other depolarizing agents should yield similar results. The alkaloid, veratridine, appears suitable for this purpose since it depolarizes nerve by selectively increasing Na permeability (13); this action is abolished by low concentrations of tetrodotoxin (T. Narahashi, personal communication). Tetrodotoxin selectively blocks the Na permeability of nerve (14); it does not affect K permeability and should not interfere with depolarization due to increased $[K]_o$.

The results of three representative experiments in which these agents were tested (Table 2) indicate that veratridine and 60 mM K both stimulate ^{45}Ca accumulation and norepinephrine release by synaptosomes (we assume that this is

TABLE 2. *Effects of potassium, Ca-free media, veratridine, and tetrodotoxin on Ca accumulation and norepinephrine release by synaptosomes and a crude mitochondrial (P_2) fraction from rat brain*

Incubation medium*	Synaptosome ^{45}Ca uptake		P_2 Norepinephrine release		Synaptosome norepinephrine release	
	μmol Ca/g protein × min	Percent change†	ng norepinephrine/g protein × min	Percent change†	ng norepinephrine/g protein × min	Percent change†
Na + 5K	0.56 ± 0.02	—	35.5 ± 1.8	—	44.9 ± 6.7	—
60 mM K	1.21 ± 0.09	+116	49.7 ± 4.9	+40	—	—
85 mM K	—	—	—	—	62.8 ± 3.1	+40
Na + 5K + veratridine	1.48 ± 0.03	+164	52.1 ± 1.6	+47	93.0 ± 2.1	+107
Na + 5K + tetrodotoxin	0.42 ± 0.01	−25	32.0 ± 1.1	−10	—	—
60 mM K + tetrodotoxin	1.23 ± 0.07	+120	62.3 ± 10.0	+75	—	—
Na + 5K + veratridine + tetrodotoxin	0.58 ± 0.01	+4	35.6 ± 4.7	0	—	—
Ca-free Na + 5K	—	—	—	—	28.5 ± 5.2	−37
Ca-free 85 mM K	—	—	—	—	37.5 ± 4.9	−16
Ca-free Na + 5K + veratridine	—	—	—	—	31.0 ± 0.8	−31

Each pair of columns represent a separate experiment. The ^{45}Ca-uptake values are the means of three determinations ±SE; norepinephrine release values are means of four determinations ±SE.

* Tetrodotoxin = 0.2 μM tetrodotoxin; veratridine = 75 μM veratridine. All Ca-free solutions contained 2 mM EGTA. The other solutions contained 1.2 mM Ca. All solutions for the norepinephrine-release experiments contained 260 μM pargyline and 165 μM cocaine.

† Relative to Ca uptake or norepinephrine release in Na + 5K medium (see *Methods*).

the "active" portion of the P_2 fraction—see Table 1). Secondly, the effects of veratridine, but not of increased $[K]_o$, are abolished by tetrodotoxin. And, thirdly, the stimulatory effects of K and veratridine on norepinephrine release both require the presence of Ca in the incubation medium.

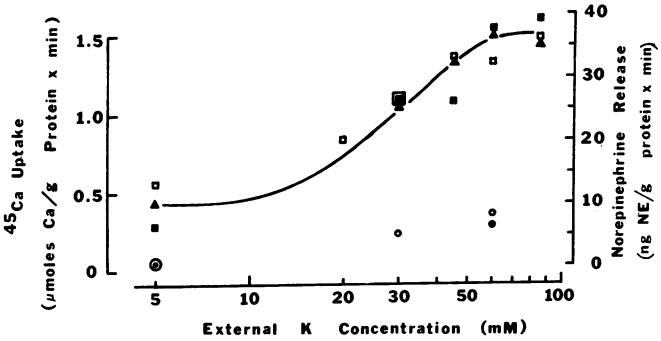

FIG. 1. Calcium-45 uptake and norepinephrine release by samples of crude mitochondrial (P_2) fraction. For the ^{45}Ca-uptake curve (▲), samples were incubated in the ^{45}Ca solution for 1 min. Each *point* represents the mean of three determinations, and the *solid curve* has been drawn through these points. Norepinephrine release data are shown for two experiments (*open* versus *solid* symbols); the P_2 preparation represented by the *solid symbols* is the same as that used for the ^{45}Ca-uptake curve. In order to obtain sufficient norepinephrine for assay, a 10-min incubation was used to measure norepinephrine release. Incubation was in Ca-free medium containing 2 mM EGTA (●, ○) or in the presence of 1.2 mM Ca (■, □); each *square* or *circle* represents the mean of four determinations. The amount of norepinephrine released into the Ca-free Na + 5K medium (see *Methods;* 23.2 ± 1.3 and 28.7 ± 1.5 ng of norepinephrine per gram of protein × min, in the experiments represented by the *solid* and *open* symbols, respectively) was substrated from all other values, as a "baseline." Cocaine (165 μM) and pargyline (260 μM) were present in all media.

The similarity of the K-stimulated ^{45}Ca accumulation and the Ca-dependent norepinephrine-release curves do not necessarily imply a linear relationship between these two parameters (see text).

DISCUSSION

The small size of synaptosomes (about 0.5 μm in diameter) precludes direct measurement of transmembrane potentials. However, the fact that they can accumulate and retain K against a concentration gradient (7, 15, and Blaustein, M. P., in preparation) suggests that, as in intact neurons (5), there may be a K diffusion potential across their surface membranes. If we assume that this is indeed the case, the Ca uptake and norepinephrine release data presented here are consistent with the Calcium Hypothesis (16): Depolarization of synaptosomes, whether induced by veratridine or elevated $[K]_o$, increases Ca permeability, allowing Ca to move into the terminals down an electrochemical gradient. This entering Ca, by an unknown mechanism, triggers the release of the transmitter substance (norepinephrine). This sequence of events will be interrupted if Ca is eliminated from the incubation medium, or if tetrodotoxin is added to prevent the veratridine-induced depolarization.

Synaptosomes prepared from rat brain are heterogeneous, and include cholinergic (10) as well as noradrenergic terminals. Since acetylcholine release from nerve terminals is also Ca dependent (3), it is not surprising that increased $[K]_o$ also stimulates a Ca-dependent acetylcholine release from rat brain synaptosomes (17). Furthermore, E. X. Albuquerque and S.-E. Jansson (personal communication) have recently shown that batrachotoxin also induces a Ca-dependent (at 28°), tetrodotoxin-inhibited release of acetylcholine from synaptosomes. Batrachotoxin, like veratridine, selectively increases Na permeability, and its action is thus subject to block by tetrodotoxin (18).

The observations described above show that depolarizing agents enhance Ca accumulation and Ca-dependent neurotransmitter release from preparations of presynaptic nerve endings. These data indicate that the synaptosome preparation may be functionally intact. It may, therefore, prove to be a useful tool for further investigation of presynaptic physiology and pharmacology.

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