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Polyamines Contribute to Calcium-Stimulated Release of Aspartate from Brain Particulate Fractions

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The calcium-stimulated release of several neurotransmitters has been studied in crude synaptosomal preparations following accumulation of various radioactively labeled neurotransmitters or their precursors. The high affinity uptake of D-aspartate, γ -aminobutyric acid (GABA) and dopamine was not modulated in the presence of 5 mM of difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (ODC). Depolarization-induced calcium influx caused release of labeled compounds. In the case of D-aspartate, this process was inhibited by DFMO. This major inhibition was completely reversed in the presence of 0.5 mM putrescine, the product of ODC-mediated catabolism of ornithine. This effect could not be shown for the depolarization-related release of dopamine, GABA or acetylcholine. In our preparation, a direct effect of the depolarizing medium upon levels of synaptosomal ODC could not be demonstrated, and the 50 mM K^+ -stimulated entry of ^{45}Ca into the synaptosomal fraction was not inhibited by DFMO. These data suggest that polyamines are involved in the regulation of neurotransmitter release within certain classes of neurons.

INTRODUCTION

Ornithine decarboxylase (ODC) is an enzyme associated with cell division, hypertrophy or growth and is found at high levels in dividing or hormonally activated tissues¹⁰. ODC is also found at low levels in nerve tissue, and its role in mature brain, which has a very low mitotic rate, is unclear. However, it is known that under certain conditions, such as surgical intervention, cold injury or various pharmacological treatments, the level of cerebral ODC can be greatly elevated^{4,6}. In addition, electrical stimulation can cause dramatic increases in ODC levels of adult rat brain¹⁹. These latter results suggest that neuronal activation may involve alterations in polyamine levels. This concept is supported by the ability of brain ODC levels to fluctuate in a rapid and transient manner¹¹.

Recent work of H. Koenig et al. has implicated the polyamines, putrescine, spermine and spermidine in mediating calcium fluxes in a variety of tissues^{11–13}. These changes in calcium transport have been attrib-

uted to rapid alterations in the levels of ODC, the rate limiting enzyme for the synthesis of these amines. It has been proposed that the mechanism of action of testosterone or β -adrenergic agonists in mobilizing renal calcium involves a receptor-dependent stimulation of preexisting ODC¹².

The purpose of this study was to determine whether synaptic neurotransmitter translocations, high affinity uptake or calcium-stimulated release could be regulated by polyamines.

MATERIALS AND METHODS

High affinity uptake

The uptake of several radioactively labelled neurotransmitters or their precursors was studied in frontal cortical tissue derived from 8–12-week-old male rats (Fischer strain); 0.1 ml of a 1% (w/v) whole tissue homogenate in 0.32 M sucrose was added to 0.9 ml of an incubation mixture containing (in mM): Tris-HCl (pH 7.4), 40; glucose, 25; KCl, 5; NaCl,

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150; CaCl_2 , 1.5; MgSO_4 , 1.4; ascorbate, 1.2; aminoxyacetic acid, 0.1; and 10^{-8} M of a labeled compound. These were [methyl- ^3H]choline chloride (80 Ci/mmol), D-[2,3- ^3H]aspartic acid (14 Ci/mmol), γ -[2,3- ^3H (N)]aminobutyric acid (34.9 Ci/mmol) or [8- ^3H (N)]dopamine (41.1 Ci/mmol). Isotopes were from New England Nuclear, Boston, MA. It was found that results were unaltered after oxygenation of the reaction mixture, or in the presence of 10^{-4} M nialamide and thus these were omitted. Incubation was at 37 °C for 5 min. Parallel incubations were run at 0 °C as controls. These controls were always below 17% of corresponding incubated values. The reaction was stopped by the addition of 5 ml buffer at 0 °C and filtration through glass fiber discs (25 mm diameter, 0.3 μm pore size, Gelman, Ann Arbor, MI). Samples were washed with 3×5 ml buffer before filters were dried and assayed for trapped radioactivity. At the low concentration of homogenate used (1 mg tissue/ml), uptake was proportional to the amount of tissue used and the course of uptake was linear throughout incubation.

Release assay

Radioactive compounds were accumulated into frontal cortical homogenates as described for the uptake procedure except that 0.1 ml of a 5% homogenate in 0.32 M sucrose was used. While not giving uptake rates, linearly proportional to tissue content, this higher tissue content allowed the transport of a greater amount of isotope. At the end of a 5-min incubation at 37 °C, samples were filtered into glass fiber filters and washed slowly at a flow rate of 6–10 ml/min under partial vacuum with 7×3 ml of wash buffer containing (in mM): NaCl, 150; KCl, 5; MgSO_4 , 1.2; ascorbate, 1; glucose, 10; EGTA, 1.3. Then 2.0 ml release buffer were added to the filters and the filtrate counted in 18 ml scintillation fluid (Aquasol, New England Nuclear). The composition of release buffer was similar to the wash buffer except that 2 mM CaCl_2 was substituted for MgSO_4 , EGTA was omitted, NaCl concentration was reduced to 75 mM and 55 mM KCl was present. Control values were obtained when wash buffer was substituted for release buffer in the final step.

^{45}Ca transport

A 5% homogenate of frontal cortex in 0.32 M su-

crose was centrifuged (1000 g, 10 min) and the supernatant from this was recentrifuged (20,000 g, 15 min). The final P2 fraction was resuspended in the original volume of 0.32 M sucrose. Of this suspension 0.1 ml was mixed with 0.8 ml of the incubation buffer, and incubated (20 min, 30 °C), sometimes in the presence of various chemicals. Then 0.1 ml of ^{45}Ca (0.5 μCi , 55.9 Ci/mmol) in either 0.5 M KCl or 0.5 M NaCl was mixed into the suspension and incubation was continued for fifteen further seconds. Then ^{45}Ca uptake was stopped by the addition of 5 ml ice-cold wash buffer followed by rapid filtration on glass fiber filters²². The filter discs were then washed with 2×5 ml buffer and trapped radioactively assayed. ^{45}Ca taken up in the presence of 0.05 M NaCl was used as a control value representing uptake not related to depolarization. This was always less than 35% of label taken up in the presence of 0.05 M KCl.

Ornithine decarboxylase

This enzyme was assayed by the measurement of evolved CO_2 from carboxyl[1- ^{14}C]-labeled ornithine as originally described by Russell and Snyder²⁰. The incubation was for 30 min at 37 °C in a sealed tube and the incubation mixture contained 10^{-2} M Tris-HCl (pH 7.2), 2×10^{-3} M dithiothreitol, 5×10^{-5} M pyridoxal phosphate and 5×10^{-6} M L-[1- ^{14}C]ornithine (55.9 mCi/mmol, New England Nuclear, Boston, MA). Labeled CO_2 was trapped on a paper wick containing hyamine that was suspended above the reaction mixture. Background decarboxylation, not attributable to ODC was determined by a parallel incubation in the presence of 5×10^{-3} M difluoromethylornithine (DFMO), a specific inhibitor of ODC¹⁵.

RESULTS

After a 5 min preincubation of cortical suspensions in the presence of 5 mM DFMO, a selective inhibitor of ODC¹⁸, levels of high affinity uptake of all labeled compounds studied were unaltered (Table I). Putrescine (0.5 mM) also had no effect on the uptake of these compounds. In the combined presence of DFMO and putrescine, choline uptake was depressed whereas the other uptake systems studied did not differ significantly from control values. Depolarization-related, calcium-stimulated release of pre-

TABLE I

Effect of DFMO and putrescine on high affinity uptake

Means $\pm$ S.E.M. of 6 replicates are presented. Values represent pmol compound taken up/5 min/g tissue.

	Control	5 mM DFMO	0.5 mM Putrescine	DFMO + Putrescine
Dopamine	118 $\pm$ 11	103 $\pm$ 3	117 $\pm$ 9	94 $\pm$ 11
Choline	9.2 $\pm$ 1.9	8.6 $\pm$ 0.7	7.0 $\pm$ 0.3	6.0 $\pm$ 0.5*
D-Aspartate	113 $\pm$ 7	122 $\pm$ 8	111 $\pm$ 2	114 $\pm$ 5
GABA	73 $\pm$ 4	77 $\pm$ 7	77 $\pm$ 4	76 $\pm$ 4

* Value differs from corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance).

viously accumulated radioactive GABA or choline showed no significant change when uptake was preceded by preincubation with DFMO or putrescine. However, the release of D-aspartate was strongly inhibited after preincubation with DFMO. If 0.5 mM putrescine was also present, the inhibitory effect of DFMO was abolished (Table II). Preincubation with putrescine in the absence of DFMO had no significant effect on D-aspartate release. DFMO-inhibited depolarization-dependent aspartate release was completely restored by putrescine but not by spermine or spermidine at the same concentration (0.5 mM) (Fig. 1). The efflux of labeled D-aspartate in the absence of Ca^{2+} remained unaltered after preincubation with DFMO prior to isotope accumulation (data not shown). While DFMO had a lesser inhibitory effect on dopamine release, this was not reversed by putrescine.

The depolarization-induced entry of ^{45}Ca into a crude synaptosomal P2 fraction was not inhibited by a 20 min preincubation at 30 °C with 5 mM DFMO

TABLE II

Effect of DFMO and putrescine on K^+ and Ca^{2+} -stimulated release

Results are expressed as percentage of control values $\pm$ S.E.M.

	5 mM DFMO	0.5 mM Putrescine	DFMO + Putrescine
Dopamine	86 $\pm$ 3*	98 $\pm$ 3	77 $\pm$ 3*
Choline	89 $\pm$ 11	89 $\pm$ 6	88 $\pm$ 11
D-Aspartate	51 $\pm$ 5*	93 $\pm$ 3	93 $\pm$ 6
GABA	88 $\pm$ 7	89 $\pm$ 6	65 $\pm$ 8*

* Value differs from corresponding control ($P < 0.05$, Student's *t*-test after analysis of variance).

Fig. 1. Inhibition of depolarization-induced [^3H]aspartate release by DFMO in the presence of various polyamines. Concentration of DFMO was 5×10^{-3} M and of each polyamine was 5×10^{-4} M. Each bar represents the mean of 5–8 separate determinations $\pm$ S.E.M. * $P < 0.05$; value differs from inhibition effected by DFMO alone (Student's two-tailed *t*-test after analysis of variance).

and was not altered when the preincubation mixture contained DFMO and 0.5 mM putrescine (Table III).

In order to test the possibility that Ca^{2+} entry might directly stimulate putrescine synthesis, the P2 fraction was incubated in the presence of varying concentrations of Ca^{2+} and K^+ in an ODC assay (Fig. 2). Ca^{2+} levels were inhibitory to ODC in a concentration-dependent manner and this inhibition was greater in the concurrent presence of 50 mM K^+ .

DISCUSSION

The lack of effect of putrescine on high affinity transport systems is in agreement with a recent report¹⁵ indicating no effect of physiological levels of polyamine on uptake processes. The data suggest

TABLE III

Effect of DFMO and putrescine on depolarization-stimulated ^{45}Ca entry into the crude synaptosomal fraction (P2)^a

Addition	pmol ^{45}Ca taken up in presence of 50 mM KCl
None	6.63 $\pm$ 0.32
5 mM DFMO	6.49 $\pm$ 0.16
0.5 mM putrescine	6.89 $\pm$ 0.22
5 mM DFMO + 0.5 mM putrescine	6.62 $\pm$ 0.73

^a Non-depolarization dependent background entry of ^{45}Ca was 2.15 ± 0.14 pmol/5 mg original tissue.

Fig. 2. Effect of potassium and calcium on particulate ODC in P2 fraction of rat brain. Each point represents the mean of 3 determinations $\pm$ S.E.M. ODC values were taken as decarboxylation activity inhibited by 5 mM DFMO.

that the Ca^{2+} -mediated release of D-aspartate may in part be regulated by biogenic polyamines. D-Aspartate is non-metabolizable amino acid that is selectively taken up by both aspartate or glutamate terminals⁵. It is not clear whether this role for amines is specific for acidic amino acid neurotransmitter systems or whether other reasons exist for failure to demonstrate this phenomenon in release processes of other neurotransmitters. The absence of an effect of DFMO upon K^{+} -stimulated Ca^{2+} -dependent release of dopamine, choline and GABA suggests that the effects of polyamine on transmitter release may be confined to a limited class of neurons. While the possibility of high affinity uptake into non-synaptic sites

exists, even in purified synaptosomal fractions^{2,9}, D-aspartate release in response to depolarizing stimuli appears to be a valid marker for synaptic termini of glutamate and aspartate neurons¹⁷.

The reversal of the observed effect of DFMO by putrescine does not necessarily imply that this amine is directly responsible for the effects described, since putrescine can be rapidly metabolized to spermine and spermidine¹⁶. However, the failure to neutralize the inhibitory effect of DFMO by spermine or spermidine suggests that putrescine is the active component in facilitating aspartate release. Endogenous levels of putrescine, spermine and spermidine in mature rat brain total 0.2–0.6 $\mu\text{mol/g}$ tissue, but putrescine constitutes only around 1% of this^{7,21}. This may enable putrescine levels to respond more rapidly to altered physiological conditions than the other polyamines¹⁰.

It has been shown that ODC elevation in rat glioma cells, induced by serum, can be selectively blocked by EGTA⁸, suggesting that the synthesis of this enzyme may be regulated by calcium levels in this isolated system. However, more rapid changes in ODC activity are more likely to be brought about by activation of preexisting ODC, perhaps by altered antizyme inhibition¹⁴. The results presented here, together with the report of Koenig et al.¹¹ suggest that a role of polyamines in nerve tissue may be the mobilization of intracellular calcium and consequent stimulation of neurotransmitter release.

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