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Excitatory amino acid uptake and *N*-methyl-D-aspartate-mediated secretion in a neural cell line

(*N*-methyl-D-aspartate receptor/neurosecretion/2-amino-4-phosphonobutyrate/phorbol esters/protein kinase C)

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ABSTRACT **A functional *N*-methyl-D-aspartate (NMDA) receptor has been identified on HT-4 cells, a clonal neural cell line, in which glutamate activates the receptor to elicit neurotransmitter secretion. Specific inhibitors of the NMDA receptor block glutamate-mediated secretion, and the characteristics of NMDA-mediated secretion parallel the reported properties of the NMDA receptor. Excitatory amino acid secretion can be elicited by potassium-evoked depolarization and is not the simple reversal of the uptake system. 2-Amino-4-phosphonobutyrate (APB) inhibits depolarization-induced secretion of excitatory amino acids but has no effect on excitatory amino acid uptake, suggesting that the APB binding protein in the brain represents a component involved in the secretion of excitatory amino acids.**

Glutamate neurotransmission is important to a number of basic neuronal processes such as learning, memory, and development and plays a role in neurodegenerative diseases such as Alzheimer and Huntington diseases and ischemia (1, 2). Postsynaptic glutamate neurotransmission can be classified into four major receptor subtypes, *N*-methyl-D-aspartate (NMDA), kainate, quisqualate, and 2-amino-4-phosphonobutyrate (APB). This classification originates from the pharmacological specificity as determined by iontophoresis and radioligand-binding studies (3).

It has been proposed that presynaptic excitatory neurosecretory mechanisms are calcium-dependent, calcium-independent, vesicular, and nonvesicular, and there is disagreement on the mechanism by which this neurotransmitter is released (4). Part of the difficulty involved in studying the biochemistry of excitatory amino acid release is the lack of a suitable model system. Just as the use of chromaffin cells has provided a consensus for an exocytotic mechanism in catecholamine secretion, a model system to study excitatory amino acid secretion is desirable.

To discern the molecular mechanism of excitatory amino acid neurotransmission, an experimental system amenable to biochemical manipulation is needed. The immense complexity and heterogeneity of cells and cell types in the brain make it extremely difficult to study the biochemical components in neural signal transduction by electrophysiological procedures alone. Although primary cultures of neurons and synaptosomes have been used extensively, such preparations are not homogeneous, and complications occur in the interpretation of the biochemical mechanisms determined in these populations. An experimental system, such as a clonal culture of cells in which the biochemical properties can be studied, is needed to elucidate the signal transduction mechanisms mediated by glutamate receptors and the mechanisms for glutamate secretion.

Frederiksen *et al.* (5) have immortalized precursor stem cells derived from the rat cerebellum with a temperature-sensitive retroviral vector. Using this same strategy, McKay and co-workers have constructed a similar cell line, HT-4, derived from mouse neuronal tissue (6). HT-4 cells have the advantage of a homogeneous population, and growth at an elevated, nonpermissive temperature allows the cells to take on properties of differentiated neurons.

We therefore tested HT-4 cells for the presence of both glutamate receptors and a neurosecretion pathway so as to study the signal transduction mechanisms involved in glutamatergic neurotransmission. Properties of excitation and secretion mediated by the glutamate receptor have been revealed by the exploitation of this clonal cell culture system.

MATERIALS AND METHODS

Chemicals and Reagents. Tissue culture media and supplements were from Whittaker M.A. Bioproducts. Radiolabeled compounds were obtained from Amersham. (±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) was from Research Biochemicals (Natick, MA). All other chemicals were from Sigma. HT-4 cells were a generous gift from Ronald McKay at Massachusetts Institute of Technology.

Cell Growth. HT-4 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 33°C. For all experiments, cells were grown for 3–5 days at 39°C, the nonpermissive temperature for the simian virus 40 tumor (T) antigen.

Uptake Studies. HT-4 cells were cultured on poly(L-ornithine)-coated six-well dishes (Falcon). Tissue culture medium was removed from the cells, and the cells were washed twice with modified Krebs solution buffered with 20 mM Hepes (pH 7.4) (7). The cells were then placed in modified Krebs medium containing the radiolabeled neurotransmitter and inhibitors as indicated. At various times the medium was removed and the cells were washed four times with buffered saline. Radioactive neurotransmitter was released from the cells with 0.1% Triton X-100, and the radioactivity was determined by scintillation counting.

Secretion Studies. HT-4 cells were grown on microcarrier beads (Cytodex 2, Pharmacia) for 3–5 days at 39°C. The cells on beads were collected and washed twice with modified Krebs buffer to remove the tissue culture medium. These cells were incubated for 4–6 hr in modified Krebs buffer containing 4 μCi (1 Ci = 37 GBq) of D-[^{3}H]aspartate. The cells on beads were perifused with different media essentially as described by McFadden and Koshland (8). Briefly, approximately 10^6 cells were placed in a perifusion chamber, this chamber was connected to a peristaltic pump, and radioactivity in the effluent was monitored on-line with a flow-through scintillation counter (Flo-One, Radiomatic Instru-

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Abbreviations: NMDA, *N*-methyl-D-aspartate; APB, 2-amino-4-phosphonobutyrate; PMA, 4β-phorbol 12-myristate 13-acetate; CPP, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid.

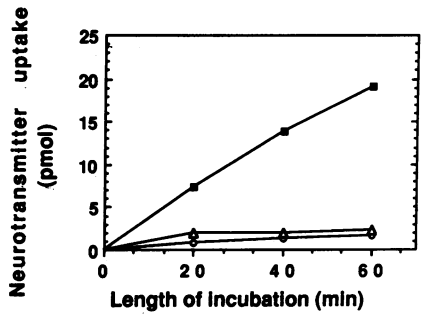

FIG. 1. Neurotransmitter uptake in HT-4 cells. HT-4 cells were incubated for various lengths of time at 39°C in the presence of 0.16 mM (4 μCi/ml) radioactive neurotransmitter. The cells were washed with modified Krebs buffer, and the radioactive neurotransmitter was released with 0.1% Triton X-100. Release of radioactivity was determined by scintillation counting. ■, D-Aspartate; △, γ-aminobutyric acid; ○, norepinephrine.

ments and Chemical, Tampa, FL). The perfusion medium was changed by using a computer-controlled solenoid valve. At the end of each experiment, the cells were lysed with 1% Triton X-100, and the total radioactive counts were determined. The amount of radioactive neurotransmitter secreted was corrected for the ongoing loss and is expressed as a percentage of the remaining radiolabel.

RESULTS

Neurotransmitter Uptake. To characterize HT-4 cells, we sought to identify the neurotransmitter they utilized. Uptake of radiolabeled γ-aminobutyric acid, norepinephrine, and D-aspartate is shown in Fig. 1. Of the three compounds tested, only D-aspartate was taken up at an appreciable rate. Since D-aspartate is not metabolized by the cells, it provided a good substitute for glutamate in the uptake and secretion studies.

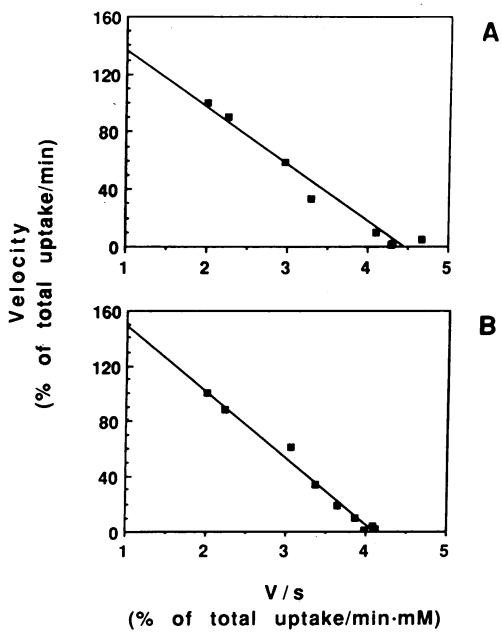

FIG. 2. Neurotransmitter uptake kinetics. (*A*) Initial velocities of D-aspartate uptake were determined in the presence of various concentrations of radiolabeled D-aspartate. Data are presented in Eadie–Hofstee plots, where the slope of the line is equal to the K_m for uptake. (*B*) Initial velocities of L-glutamate uptake were determined in the presence of various concentrations of radiolabeled L-glutamate. Data are presented in Eadie–Hofstee plots. s, Substate; V, velocity.

Table 1. Effect of glutamate analogs on the rate of D-aspartate uptake

Addition	Rate of D-aspartate uptake, pmol per min
None	0.218 ± 0.023
NMDA	0.200 ± 0.003
Kainate	0.205 ± 0.010
Quisqualate	0.224 ± 0.021
APB	0.240 ± 0.024
Glutamic diethyl ester	0.198 ± 0.007
CPP	0.216 ± 0.004
Glutamate	0.032 ± 0.002

About 10^6 HT-4 cells were grown in one well of a six-well dish. The initial rate of radioactive D-aspartate (0.16 μM) uptake was determined in the presence of 100 μM of the listed glutamate analogs. The results are expressed as the mean ± SEM for $n = 3$.

To analyze this excitatory amino acid uptake system, various concentrations of D-aspartate and L-glutamate were used, and the kinetic properties of uptake were determined (Fig. 2). The K_m for D-aspartate uptake was 40 μM (Fig. 2*A*), and the K_m for L-glutamate uptake was 48 μM (Fig. 2*B*). The similarity between D-aspartate and L-glutamate uptake kinetics suggests a common mechanism. Subsequent experiments utilized the nonmetabolizable excitatory amino acid, D-aspartate.

Glutamate analogs at a concentration of 100 μM each had no effect on D-aspartate uptake (Table 1). The glutamate receptor agonists, NMDA, kainate, quisqualate, and APB, and the receptor antagonists, CPP and glutamyl diethyl ester, did not alter the rate of D-aspartate uptake. Only nonradioactive glutamate competed for D-aspartate uptake. The HT-4 uptake system could also utilize L-aspartate, suggesting that the excitatory amino acid uptake system is specific for both glutamate and aspartate and is not affected by glutamate receptor agonists or antagonists.

D-Aspartate uptake in HT-4 cells requires an intact membrane potential. Raising the potassium ion concentration from 5 mM to 55 mM (while compensating the ionic strength with a reciprocal reduction in the sodium concentration) inhibited excitatory amino acid uptake (Table 2). In the absence of depolarization, uptake of D-aspartate was not affected by calcium. The inhibition of D-aspartate uptake by potassium ion depolarization was also unaffected by extracellular calcium ion concentrations in the range from 0 to 4 mM.

Neurotransmitter Secretion. To establish glutamate/aspartate as a neurotransmitter in HT-4 cells, we characterized the

Table 2. Effect of membrane potential and calcium ion concentrations on the rate of D-aspartate uptake

Treatment	Rate of D-aspartate uptake, pmol per min
Normal saline, 2.5 mM Ca^{2+}	0.182 ± 0.008
Normal saline, no Ca^{2+}	0.174 ± 0.009
Depolarizing, no Ca^{2+}	0.050 ± 0.002
Depolarizing, 0.2 mM Ca^{2+}	0.058 ± 0.001
Depolarizing, 0.4 mM Ca^{2+}	0.051 ± 0.003
Depolarizing, 0.8 mM Ca^{2+}	0.054 ± 0.002
Depolarizing, 1.5 mM Ca^{2+}	0.056 ± 0.003
Depolarizing, 4.0 mM Ca^{2+}	0.053 ± 0.002

About 10^6 HT-4 cells were grown in one well of a six-well dish. The initial rate of radioactive D-aspartate uptake was determined and is expressed as the mean ± SEM for $n = 3$.

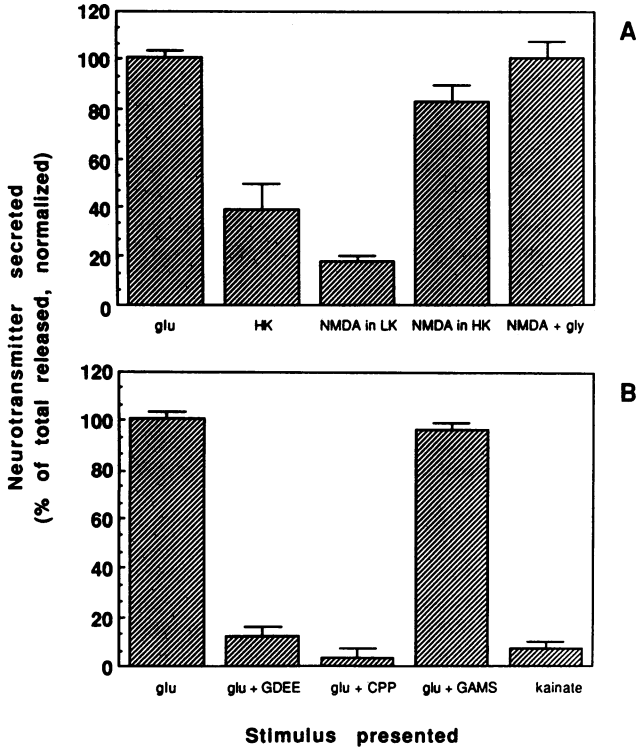

FIG. 3. Effect of various excitatory amino acid analogs on neurotransmitter secretion. HT-4 cells were labeled with 4 μCi of D-[^{3}H]aspartate. The cells were washed and placed in a perifusion device as described. Radioactive neurotransmitter secretion was determined by using an on-line scintillation counter, and the amount of neurotransmitter secreted per stimulus was integrated and normalized to the amount of secretion by 100 μM glutamate (glu). All compounds were presented for 2 min at a concentration of 100 μM. Results are expressed as means ± SEM for n = 5. HK, high-potassium medium; LK, low-potassium medium; GDEE, glutamyl diethyl ester; GAMS, γ-D-glutamylaminomethylsulfonic acid.

secretion of radioactive D-aspartate. Membrane depolarization achieved by raising the extracellular potassium concentration from 5 mM to 55 mM resulted in the secretion of radiolabeled D-aspartate. HT-4 cells were presented with a 2-min stimulus of depolarizing media, and the calcium-dependent secretion of radioactive D-aspartate was monitored on-line with a flow-through scintillation counter. The amount of neurotransmitter released was 1.83 ± 0.06% of total × min. We were able to inhibit this depolarization-induced secretion 41% by introducing 100 μM APB, which reduced the amount of secretion to 1.08 ± 0.04.

Secretion of D-aspartate can be elicited by the presentation of glutamate and seems to be mediated by the NMDA-type receptor. The NMDA receptor–channel complex is blocked by physiological Mg^{2+} concentrations at normal resting membrane potentials (9, 10). Depolarization of the plasma membrane relieves this blockage, and together with the presentation of ligand, allows the receptor–channel complex to signal (9, 10). The secretion of D-aspartate has some of the same characteristics described for NMDA receptor activation. NMDA was ineffective in stimulating secretion at 100 μM in low potassium nondepolarizing medium (LK medium) (Fig. 3A). However, the presentation of NMDA in high potassium (55 mM) depolarizing medium (HK medium) resulted in D-aspartate secretion that was twice as great as the high-potassium (HK) control. The copresentation of glycine with NMDA increased D-aspartate release by an additional 25% above NMDA/HK secretion. This is consistent with the regulatory role for glycine on the NMDA receptor as reported by Johnson and Ascher (11).

Antagonism of the NMDA receptor by CPP effectively blocked glutamate-mediated secretion, as did the broader glutamate receptor antagonist glutamyl diethyl ester (Fig. 3B). Neither of these antagonists had any effect on HK-mediated secretion. The presentation of kainate did not result in neurosecretion. Similarly, the non-NMDA-receptor antagonist γ-D-glutamylaminoethylsulfonic acid had no effect on glutamate-mediated secretion, further suggesting that secretion involves the specific activation of the NMDA receptor. The midpoint of the dose–response curve for glutamate-stimulated secretion was 1.1 μM and for NMDA-stimulated secretion was 18.5 μM. This is similar to the K_d obtained by radioligand binding studies (12).

Modulation of Neurosecretion. At this point we looked for other conditions that could modulate neurosecretion. We found the presentation of 4β-phorbol 12-myristate 13-acetate (PMA) was able to potentiate subsequent secretion events transiently (Fig. 4). HT-4 cells were presented with a 2-min depolarizing stimulus every 5 min for 10 repetitions, and the amount of radioactive D-aspartate released per stimulus was integrated and normalized to give a value of 100.0 ± 5.1. All subsequent secretion was normalized to this value. After the initial 10 stimulations, a 1-min presentation of 0.1 μM PMA increased the first five-subsequent depolarizations 50–75%, after which the amount of secretion returned to the basal level. An hour after the first PMA presentation, HT-4 cells were exposed to a second 1 min of 0.1 μM PMA. This

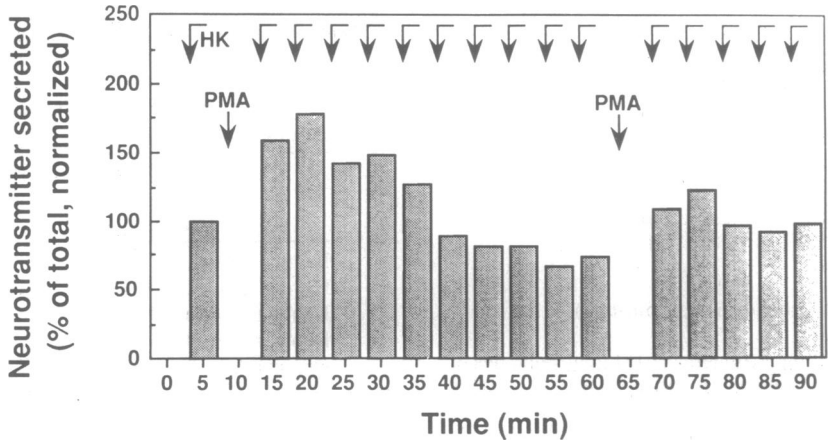

FIG. 4. Effect of phorbol esters on depolarization-induced neurosecretion. D-[^{3}H]Aspartate-loaded cells were stimulated with 55 mM potassium ion (HK) for 2 min every 5 min. The amount of neurotransmitter secreted was integrated and normalized to the value of secretion prior to PMA treatment. At the indicated times, 0.1 μM PMA was applied to the cells for 2 min.

additional presentation of PMA increased secretion only 15% over that of the initial pre-PMA stimulus, possibly because of adaptation or down-regulation of protein kinase C.

DISCUSSION

The search for a system that followed the biochemical characteristics of glutamate activity and secretion led us to characterize the properties of HT-4 cells. Properties of this cell line were consistent with the presence of NMDA receptors. Specific NMDA inhibitors block glutamate-mediated neurosecretion, and the characteristics of NMDA-mediated secretion were consistent with the reported properties of the NMDA receptor. Thus, HT-4 cells possess a complete transduction pathway from NMDA receptor stimulation to a specific output response, which can be monitored by D-aspartate secretion.

The coupling of the NMDA receptor to secretion may involve an increase in intracellular calcium, since it is known that NMDA receptor activation results in calcium influx. The extent of glutamate-stimulated secretion is greater than that of depolarization-induced secretion. This suggests that either (*i*) parallel pathways for depolarization- and glutamate-mediated secretion exist or (*ii*) the NMDA receptor is able to activate some component of depolarization-induced secretion. Secretion is abolished by blocking the NMDA component of glutamate-mediated secretion, and introduction of kainate results in a minor amount of secretion, suggesting glutamate-mediated secretion is a pathway independent of depolarization-induced secretion. The kainate and quisqualate glutamate receptors are reported to result in membrane depolarization (9, 13). If glutamate-mediated secretion in HT-4 cells is composed of both a depolarization and a NMDA component, then the antagonism of the NMDA receptor should still allow secretion by the depolarization component. However, when HT-4 cells are presented with glutamate in the presence of the NMDA-specific antagonist CPP, secretion is reduced virtually to zero.

In HT-4 cells, D-aspartate secretion is not the simple reversal of the electrogenic uptake system. Although depolarization of the plasma membrane reduces the uptake of D-aspartate, this reduction cannot completely explain the depolarization-induced secretion. The presence of calcium in the depolarizing medium has no effect on the rate of D-aspartate uptake; however, depolarization-induced secretion was greatly affected by calcium concentrations. Additionally, the glutamate analog APB did not affect neurotransmitter uptake but can effectively reduce the depolarization-induced neurosecretion. This suggests that the uptake and release of excitatory amino acids are mediated by functionally distinct mechanisms.

Of the four classes of glutamate receptors, only the APB binding activity displays unique ion requirements (3). This binding activity requires the presence of both chloride and calcium ions and has been described as a novel, sodium-independent glutamate carrier (14). Our results suggest that in HT-4 cells this APB binding activity is not associated with glutamate uptake but may be involved in the presynaptic neurosecretory pathway. The unusual sensitivity of APB binding to osmotic stress, the freeze/thaw cycle, and low temperatures (15) would be explained if the APB binding protein were part of the glutamate neurosecretory system, since an intact membrane would be necessary for optimal APB binding.

The modulation of secretion by phorbol esters suggests the involvement of protein kinase C in the regulation of excitatory amino acid neurosecretion. The activation of protein kinase C by phorbol esters does not result in neurosecretion, implying a modulatory role for protein phosphorylation. Protein kinase C may phosphorylate a component of excitatory amino acid secretion, thereby increasing that component's sensitivity to intracellular calcium. This phosphorylated component may be the APB binding protein that mediates calcium-dependent neurosecretion. The rise in intracellular calcium associated with neurosecretion may result from the activation of either voltage-dependent calcium channels or the NMDA receptor complex.

Excitatory amino acid neurotransmission is an important component in long-term potentiation and neurodegenerative diseases (2). Understanding the interactions between the molecular components in excitatory neurotransmission may provide insight about its role in neural circuitry. To achieve this goal, an experimental system amenable to biochemical manipulation is necessary to isolate and characterize the elements involved. This work shows that HT-4 cells, a clonal homogeneous population of neural cells, possess NMDA receptors and a functional secretory pathway.

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