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RAPID COMMUNICATION

COCAINE-SENSITIVE, ATP-DEPENDENT DOPAMINE UPTAKE INTO STRIATAL SYNAPTOSOMES

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The synaptic action of biogenic amines, in the central and peripheral nervous systems, is terminated by reuptake of the amine transmitters into the presynaptic nerve terminals (1). This neuronal uptake is temperature-dependent and exhibits saturation kinetics and stereospecificity for substrates and inhibitors (2). Striking properties of neuronal amine uptake are its complete dependency on Na^+ (1-3) and its sensitivity to the cardiac glycoside ouabain (3). Thus, it was hypothesized that neuronal amine uptake was a Na^+-dependent active transport process like uptake of sugars and amino acids (1) and that Na^+,K^+-ATPase provided the energy to drive amine transport. This model prevailed for the last 20 years despite serious challenges early on (4,5), and ATP-dependent uptake was never observed.

Neuronal amine transporters are the pharmacological receptors for tricyclic antidepressants and central stimulants (e.g. cocaine). Although cocaine inhibits uptake of norepinephrine and 5-hydroxytryptamine as well as dopamine (DA), it is the last effect which correlates best with cocaine's behavioral actions (6-8). In this study, we provide evidence that the cocaine-sensitive uptake of DA into striatal synaptosomes is regulated by ATP.

MATERIALS AND METHODS

Tissue preparation. Striata from fresh bovine brains (from a local slaughterhouse) were homogenized in buffer I (0.32 M sucrose, 10 mM Na_2HPO_4, pH 7.4) using the method of Krueger *et al.* (9) with slight modification. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant fraction was recentrifuged at 20,000 *g* for 20 min. The pellets were suspended in buffer I, layered over 0.8 M sucrose and centrifuged at 9000 *g* for 30 min. The 0.8 M sucrose layer was diluted with 3 vol. of buffer I and centrifuged at 20,000 *g* for 20 min. This protocol eliminates synaptic vesicles except those inside the synaptosomes (10). Synaptosomes were further fractionated following lysis using the Gray and Whittaker method (11). The pellets were suspended in buffer II (0.3 M sucrose, 11 mM glucose, pH 7.4) and protein concentration was determined by the method of Lowry *et al.* (12).

[^{3}H]Dopamine uptake assay. The uptake of 2-4 nM [^{3}H]DA (3,4-[7-^{3}H]dihydroxyphenylethylamine; 40 Ci/mmol, NEN) into striatal synaptosomes was measured by a filtration assay at 37° as described previously (6) with modifi-

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cations. Aliquots (100 µL) of the membrane preparations were added to 0.9 mL of the uptake buffer (in mM concentration: NaCl, 122; KCl, 4.8; $CaCl_2$, 1.3; $MgSO_4$, 1.2; Na_2HPO_4, 1.58; $NaHCO_3$, 20) containing glucose (2 mg/mL), ascorbic acid (0.2 mg/mL), nialamide (12.5 µM) and 0.1 M sucrose (pH 7.4). Uptake was initiated by addition of [^{3}H]DA and terminated by vacuum filtration over GF/B filters. Radioactivity retained on the filters was measured by liquid scintillation. Nonspecific uptake was determined as that measured at 0-4° in uptake buffer containing 122 mM choline chloride in place of NaCl. All experiments were run in triplicates.

ATPase assay. ATPase activity was determined by a calorimetric method that measures the hydrolysis of inorganic phosphate from ATP (13). The assay medium which was free of Ca^{2+} contained 1 mM EDTA, phosphoenolpyruvate and pyruvate kinase to regenerate ATP from ADP produced during the reaction. All measurements were made in triplicate and means ± SEM were obtained from two experiments.

Chemicals, drugs and reagents. All the drugs, ATPase inhibitors and substrates were obtained from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

Saturable cocaine-sensitive [^{3}H]DA uptake into striatal synaptosomes was observed in the absence of added ATP. However, it was stimulated significantly when ATP was added in a concentration-dependent manner (Fig. 1). The EC_{50} for ATP was slightly higher than 1 mM, and maximal uptake was obtained in the presence of 3 mM ATP. The stimulation of [^{3}H]DA uptake by added ATP was seen best when the uptake buffer had a high buffering capacity and the same osmolarity as the synaptosomal preparation. ATP increased the V_{max} of [^{3}H]DA uptake from 66.7 to 333.3 pmol/mg protein/min but did not change its K_m value significantly. Inhibition of [^{3}H]DA uptake by ouabain in the absence of added ATP was significant ($p < 0.05$) and in the presence of ATP was highly significant at 0.5, 1 and 3 mM ATP ($p < 0.001$). Inhibition by cocaine was highly significant at all ATP concentrations ($p < 0.001$).

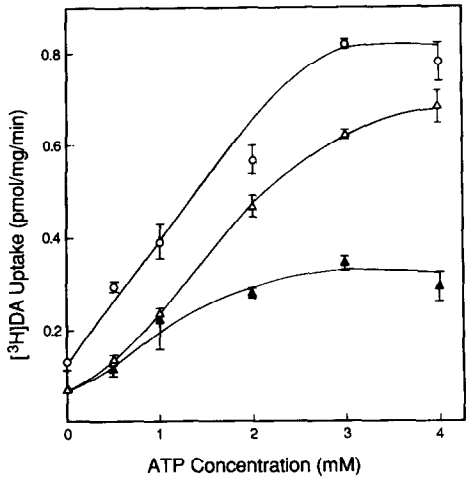

Fig. 1. Inhibition of ATP-dependent [^{3}H]DA (4 nM) uptake into striatal synaptosomes (○) by 10 µM ouabain (△) and 100 µM cocaine (▲). Incubation time, 5 min at 37°. Values are means ± SEM, N=9.

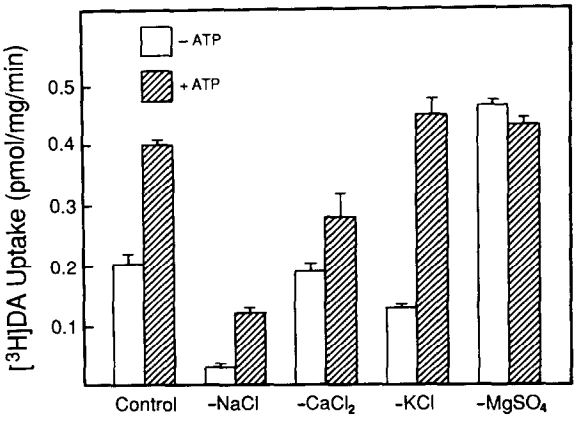

Fig. 2. Effects of cations on [^{3}H]DA (2 nM) uptake in the absence and presence of 5 mM ATP. Incubation time, 5 min at 37°. Values are means ± SEM, N=6.

The cation requirement for [^{3}H]DA uptake did not change when ATP was added. [^{3}H]DA uptake was reduced by 90% when NaCl was replaced by choline chloride (the uptake buffer still contained 20 mM $NaHCO_3$ and 1.58 mM Na_2HPO_4), but addition of ATP still stimulated [^{3}H]DA uptake (Fig. 2). Deletion of K^+ reduced uptake significantly ($p < 0.05$), but did not reduce uptake in the presence of ATP. Deletion of Ca^{2+}, on the other hand, reduced uptake in the presence of ATP significantly ($p < 0.05$). Deletion of Mg^{2+} resulted in a highly significant increase in uptake ($p < 0.001$), but ATP failed to stimulate uptake in this buffer.

The time courses of [^{3}H]DA uptake, in the presence of added ATP, were significantly faster than without ATP ($p < 0.001$) (Fig. 3). The ATPase inhibitor ouabain and the proton ionophore carbonyl cyanide *p*-trifluoro-methoxy-phenyl-hydrazone (FCCP) inhibited the ATP-dependent [^{3}H]DA uptake. Interestingly, their effects were more apparent when uptake reached steady state and the measurement indicated total accumulation of [^{3}H]DA uptake (Fig. 3). Both compounds also inhibited [^{3}H]DA uptake in the absence of added ATP. When crude synaptosomes (i.e. rich in free mitochondria) were pretreated with 50 µM FCCP, control uptake decreased from 38.69 ± 7.2 to 23.59 ± 9.6 fmol/mg/min ($p < 0.001$), whereas in the presence of 3 mM ATP control uptake was not reduced significantly (control = 416.58 ± 18 *vs* FCCP-treated 407.86 ± 45.2 fmol/mg/min). These results suggest that FCCP was effective only when ATP supply was limited and addition of 3 mM ATP overcame that effect.

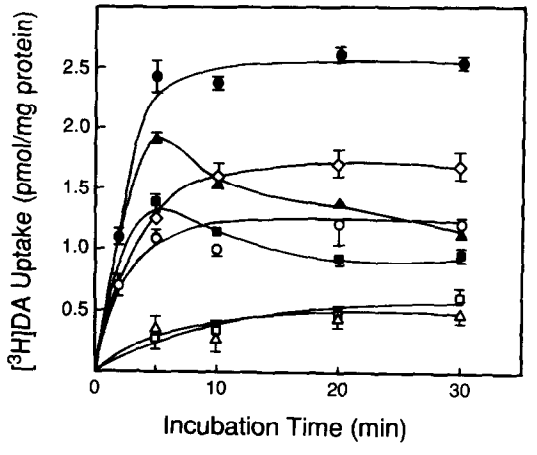

Fig. 3. Time course of [^{3}H]DA (2 nM) uptake into striatal synaptosomes at 37° in the absence of ATP (○), but in the presence of 100 µM FCCP (□), 100 µM ouabain (△) or 3 mM AMP-PNP (◇); and in the presence of 3 mM ATP alone (●) or in addition to 10 µM ouabain (▲) or 100 µM FCCP (■). Values are means ± SEM, N=9.

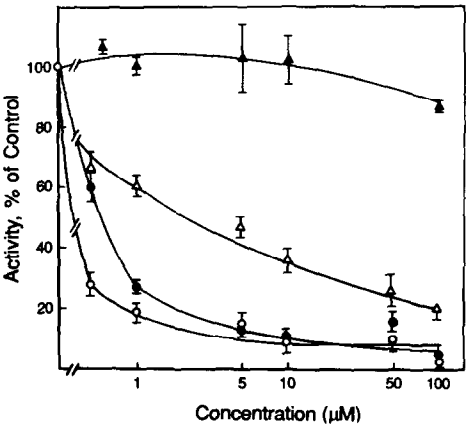

Fig. 4. Inhibition of [^{3}H]DA (2 nM) uptake (closed symbols) and Na^+,K^+-ATPase (open symbols) by ouabain (○,●) and vanadate (△,▲). Inhibitors were preincubated with synaptosomes for 10 min prior to assay. Control [^{3}H]DA uptake was 0.23 ± 0.003 pmol/mg protein. Control ATPase activity was 0.39 ± 0.008 µmol P_i/mg/min. Values are means ± SEM, N=6.

[^{3}H]DA uptake had variable sensitivities to ATPase inhibitors. It was most sensitive to ouabain, and almost insensitive to vanadium (Fig. 4). The Na^+,K^+-ATPase activity of the crude synaptosomal preparation (250 µmol P_i/mg protein/min) was inhibited totally by 100 µM ouabain, and 80% by 100 µM vanadium

sulfate. Since ATP is also known to regulate [^{3}H]DA uptake into synaptic vesicles (14,15), the presence of free synaptic vesicles in the synaptosomal preparation would contribute to the observed ATP-stimulated uptake. However, after careful removal of synaptic vesicles by sucrose gradient centrifugation, following lysis of the synaptosomes (11), there was no significant change in the ATP-stimulated [^{3}H]DA uptake. Nor was there a difference in [^{3}H]DA uptake by synaptosomes and synaptosomal ghosts which had been exposed to 10 μM reserpine, a known inhibitor of vesicular uptake.

DISCUSSION

Stimulation of [^{3}H]DA uptake into striatal synaptosomes with ATP in a dose- (Fig. 1) and time-dependent (Fig. 3) manner suggests a role for ATP in regulating [^{3}H]DA uptake. The ATP-regulated uptake was sensitive to Na^+ (Fig. 2), but had much higher capacity than uptake in the absence of added ATP (V_{max} was 5-fold higher). [^{3}H]DA uptake was inhibited by ouabain even in the absence of added ATP (Figs. 1, 3 and 4), as previously reported (4). This was considered evidence for the involvement of Na^+,K^+-ATPase (13). However, vanadate, which is a potent Na^+,K^+-ATPase inhibitor, did not inhibit [^{3}H]DA uptake significantly. This is difficult to explain unless vanadate, which inhibits Na^+,K^+-ATPase from inside the cell, does not cross the cell membrane easily. It is unlikely that a Na^+,K^+-ATPase contributes to the ATP-regulated uptake, since ATP in the assay medium is inaccessible to the intracellular Na^+,K^+-ATPase. Also, more importantly, the significant stimulation of [^{3}H]DA uptake by the nonhydrolyzable ATP analog 5'-adenylimidodiphosphate (AMP-PNP) argues against the involvement of an ATPase. Alternatively, ATP may be acting on an ATP receptor associated with the DA transporter. Although other ATP receptors have been described (16, 17), none were presynaptic.

Inhibition of synaptosomal uptake by the proton ionophore FCCP (Fig. 3) is difficult to explain, unless we assume that free mitochondria in the synaptosomal preparation produces ATP that contributes to the uptake. Antagonizing the action of FCCP on crude synaptosomal uptake of [^{3}H]DA by ATP supports this suggestion. It may explain why crude synaptosomal preparations, which are rich in mitochondria, have higher uptake activities than purified synaptosomes (18).

Observing reserpine-insensitive, ATP-stimulated [^{3}H]DA uptake into synaptosomal ghosts after removal of the vesicles proves that the synaptosomal ATP-regulated [^{3}H]DA uptake is not vesicular in nature. It is believed that the observed ATP-regulated uptake is physiologically relevant. In intact tissue, ATP is stored with DA and the ATP needed for regulating transport would be provided by the transmitter release process. It is well documented that ATP is co-stored and co-released with catecholamines (19,20). The present findings fit a model of an ATP-dependent active transport. It is suggested that the DA transporter in the striatum, i.e. the cocaine receptor, carries an ATP-binding site and is a target for modulation by ATP.

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REFERENCES

1. Iversen LL, Uptake processes for biogenic amines. In: *Handbook of Psychopharmacology* (Eds. Iversen LL, Iversen SD and Snyder SH), Vol. 3, pp. 381-442. Plenum Press, New York, 1975.
2. Gillis CN and Paton DM, Effects of hypothermia and anoxia on retention of noradrenaline by the cat perfused heart. *Br J Pharmacol* **26**: 426-434, 1966.
3. Bogdanski DF, Tissari AH and Brodie BB, Mechanism of transport and storage of biogenic amines, III. Effects of sodium and potassium on kinetics of 5-hydroxytryptamine and norepinephrine transport by rabbit synaptosomes. *Biochim Biophys Acta* **219**: 189-199, 1970.
4. White TD and Keen P, Effects of inhibitors of (Na^+ + K^+)-dependent adenosine triphosphate on the uptake of norepinephrine by synaptosomes. *Mol Pharmacol* **7**: 40-45, 1971.
5. White TD and Paton DM, Effects of external Na^+ and K^+ on the initial rates of noradrenaline uptake by synaptosomes prepared from rat brain. *Biochim Biophys Acta* **266**: 116-127, 1972.
6. Richelson E and Pfenning M, Blockade by antidepressants and related compounds of biogenic amine uptake into rat brain synaptosomes: Most antidepressants selectively block norepinephrine uptake. *Eur J Pharmacol* **104**: 277-286, 1982.
7. Ritz MC, Lamb RJ, Goldberg SR and Kuhar MJ, Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science* **237**: 1219-1223, 1987.
8. Calligaro DO and Eldefrawi ME, High affinity stereospecific binding of [^{3}H]cocaine in striatum and its relationship to the dopamine transporter. *Membr Biochem* **7**: 87-106, 1988.
9. Krueger BC, Ratzlaff RW, Strichartz GR and Blaustein MP, Saxitoxin binding to synaptosomes, membranes, and solubilized binding sites from rat brain. *J Membr Biol* **50**: 287-310, 1979.
10. Slotkin TA, Seidler FJ, Whitmore WL, Lau C, Salvaggio M and Kirksey DF, Rat brain synaptic vesicles: Uptake specifications of [^{3}H]norepinephrine and [^{3}H]serotonin in preparations from whole brain and brain regions. *J Neurochem* **31**: 961-968, 1978.
11. Gray EG and Whittaker VP, The isolation of nerve endings from brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J Anat* **96**: 79-87, 1962.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265-275, 1951.
13. Jones LR and Besch HR, Isolation of canine cardiac sarcolemmal vesicles. In: *Methods in Pharmacology* (Ed. Schwartz A), Vol. 5, pp. 1-12. Plenum Press, New York, 1984.
14. Beers MF, Carty SE, Johnson RG and Scarpa A, H^+-ATPase and catecholamine transport in chromaffin granule. *Ann NY Acad Sci* **402**: 116-133, 1982.

15. Phillipu A and Beyer J, Dopamine and noradrenaline transport into subcellular vesicles of the striatum. *Naunyn Schmiedebergs Arch Pharmacol* **278:** 387-402, 1973.

16. Burnstock G and Kennedy C, Is there a basis for distinguishing two types of P_2-purine receptors? *Gen Pharmacol* **16:** 433-440, 1985.

17. Burnstock G, Purinergic transmission. In: *Handbook of Psychopharmacology* (Eds. Iversen LL, Iversen SD and Snyder SH), Vol. 5, pp. 131-194. Plenum Press, New York, 1975.

18. Tuomisto J and Tuomisto L, The accumulation of dopamine into striatal synaptosomes, and its inhibition: Comparison of crude, partially purified and purified synaptosomal preparations. *Med Biol* **57:** 238-245, 1979.

19. Baldessarini RJ, Release of catecholamines. In: *Handbook of Psychopharmacology* (Eds. Iversen LL, Iversen SD and Snyder SH), Vol. 3, pp. 1-35. Plenum Press, New York, 1975.

20. Reichardt LF and Kelly RB, A molecular description of nerve terminal function. *Annu Rev Biochem* **52:** 871-926, 1983.