Methcathinone and 2-methylamino-1-(3,4-methylenedioxyphenyl) Propan-1-one (methylone) selectively inhibit plasma membrane catecholamine reuptake transporters.

Nicholas V. Cozzi,[1,2] Michael K. Sievert,[2] Alexander T. Shulgin,[3]
Peyton Jacob III,[4] Arnold E. Ruoho[2]

[1]Department of Pharmacology, East Carolina University School of Medicine, Greenville, NC 27858. [2]Department of Pharmacology, University of Wisconsin Medical School, Madison, WI 53706. [3]1483 Shulgin Road, Lafayette, CA 94549. [4]Departments of Medicine and Psychiatry, University of California San Francisco, San Francisco, CA 94110.

INTRODUCTION

Our research in monoamine neurotransmitter pharmacology and in photoaffinity labeling technology suggested to us that methcathinone (2-methylamino-1-phenylpropan-1-one, MCAT; Fig. 1) and methylone (2-methylamino-1-[3,4-methylenedioxy-phenyl]propan-1-one, MDMCAT; Fig. 1) might be useful photoaffinity labels for the substrate binding sites on the plasma membrane monoamine uptake transporters and the vesicular monoamine transporter.

Methcathinone is the *N*-methyl derivative of cathinone, a naturally occurring psychostimulant found in the leaves of *Catha edulis*, the khat bush. Methcathinone was first synthesized in 1928,[1] but its psychopharmacology remained unexplored until Parke, Davis, and Company developed methcathinone as an analeptic in the late 1950's.[2] More recently, methcathinone has been used outside of medical settings for its euphoric properties and is now designated a Schedule I controlled substance in the United States.[3,4] Behavioral studies have shown that methcathinone will substitute for cocaine or amphetamine in animals trained to discriminate either cocaine or amphetamine from saline in stimulus generalization tests.[5] Baboons will self-administer methcathinone, confirming that the drug has reinforcing properties.[6]

Dal Cason *et al*. recently reported behavioral data for methylone, the benzylic ketone analog of 3,4-methylenedioxymethamphetamine (MDMA).[7] In this account, methylone substituted for MDMA in rats trained to discriminate MDMA from saline, with an ED_{50} of 1.6 mg/kg. At similar doses, methylone did not substitute for (+)amphetamine in amphetamine-trained animals, nor did it substitute for the hallucinogen 2-amino-1-(2,5-dimethoxy-4-methylphenyl)propane (DOM) in DOM-trained rats.

From these reports and others it seems likely that methcathinone and methylone act on monoaminergic systems, specifically through monoamine uptake transporters. We presented a preliminary report on the effects of these componds on serotonin uptake.[8] In anticipation of the future development of these drugs as photoaffinity probes for the monoamine carrier proteins, we have extended our earlier work and now report the first study of methcathinone and methylone in catecholamine and vesicular transporter systems. We compared the potencies of methcathinone, methamphetamine (MA), methylone, and MDMA to inhibit the monoamine plasma membrane uptake transporters and to inhibit the vesicular monoamine transporter, VMAT2. We also synthesized radiolabeled methcathinone and tested whether it was photoactivable.

MATERIALS AND METHODS

Drugs

The test drugs were synthesized in our laboratories; all analytical data were consistent with the assigned structures. [^{14}C]Methcathinone was synthesized as described for [^{3}H]methcathinone,[9] except [^{14}C]methyl iodide was used as the radioactive labeling reagent. All other drugs and reagents were obtained from commercial sources or as noted.

Serotonin plasma membrane uptake transporter: [^{3}H]5-HT uptake into human platelets

Outdated human platelets were obtained from the blood bank at the University of Wisconsin Clinical Sciences Center or from the Veteran's Administration Hospital, Madison, WI. Platelets from 5-10 donors were pooled, 10% DMSO was added, and aliquots were stored frozen at -80°C until use. For assays, 5 ml of platelets were thawed and added to 20 ml ice-cold Krebs-Ringer-HEPES (KRH) buffer containing (mM): NaCl (124.0), KCl (2.9), $MgSO_4$ (1.3), KH_2PO_4 (1.2), $CaCl_2$ (2.4), *d*-glucose (5.2), HEPES (25.0), Na ascorbate (0.1), pargyline (0.1), pH = 7.4. The platelet suspension was subjected to centrifugation (4340 x g, 4 °C, 10 min) and the supernatant was discarded. The pellet was washed twice by resuspension in KRH and centrifugation. The final pellet was suspended in 70 ml ice-cold KRH using a polytron (setting 7, 10 s) and stored on ice until use. The ability of platelets to accumulate [^{3}H]5-HT was measured in the absence and presence of various concentrations of test drugs as follows: a 400 µl aliquot of the platelet suspension was added to glass tubes containing 50 µl test drugs (dissolved in KRH) or 50 µl KRH (for total and nonspecific determinations). The assay tubes were preincubated in a 37 °C shaking water bath for 5 min. The tubes were then returned to the ice bath and chilled for 15 min. [^{3}H]5-HT was added (50 µl of stock solution; final concentration, 10 nM), giving a total incubation volume of 500 µl. All tubes except nonspecific tubes were returned to the 37 °C shaking water bath for 5 min to initiate neurotransmitter uptake. Uptake was terminated by chilling the test tubes in the ice bath. After adding 3 ml ice-cold KRH, each assay tube was vacuum filtered through glass fiber filters (Whatman GF/B) pretreated with 0.1% polyethyleneimine (PEI). Filters were washed with 2 x 3 ml ice-cold KRH, allowed to dry briefly under vacuum, then placed in liquid scintillation vials. Eight ml scintillation cocktail was added and the vials were sealed, vortexed, and allowed to stand overnight. Radioactivity was measured using liquid scintillation spectroscopy (Packard Tri-Carb 1600 CA). Specific uptake was defined as uptake at 37 °C minus uptake at 0 °C in the absence of drugs. Under these conditions, specific [^{3}H]5-HT uptake was typically greater than 85%. The IC_{50} ± SEM for each test drug was determined from displacement curves from 3-5 experiments using 6-11 drug concentrations, each run in triplicate. Data were transformed from dpm to % specific uptake and fit to a four-parameter logistic curve using commercial computer software. The IC_{50} values were calculated from the derived equations and were compared using Student's *t*-test.

Dopamine plasma membrane uptake transporter: [^{3}H]DA uptake into transfected cells

C6 glial cells stably expressing the rat dopamine transporter (C6-DAT) were a gift from Dr. Susan Amara (Oregon Health Sciences University). Cells were maintained in a humidified atmosphere (5% CO_2 in air) in selective culture medium, Dulbecco's Modified Eagle's Medium

(DMEM) containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml geneticin). For assays, 3 x 100 mm dishes of confluent cells were washed with phosphate buffered saline, pH = 7.1. The cells were then treated with trypsin/EDTA and split into 4 x 6-well (35 mm) plates in 2 ml/well culture medium minus geneticin. After passage, the cells were allowed to grow overnight and were used for uptake assays the following day. The ability of the test drugs to inhibit [3H]DA uptake was measured as follows: the DMEM was aspirated from the 6-well plates containing confluent C6-DAT cells. The cells were washed with 2 x 2 ml 37 °C KRH, then 490 μl 37 °C KRH was added to each well. This was followed by the addition of 5 μl KRH (for "total" determinations), 5 μl 10 mM cocaine hydrochloride (for "nonspecific" determinations; final concentration, 100 μM), or 5 μl of test drug solution. The plates were preincubated at 37 °C for 15 min, then 5 μl of [^{3}H]DA (final concentration, 20 nM) was added to each well to initiate uptake. Uptake was allowed to proceed for 20 min, then the incubation buffer was discarded and the cells were washed with 3 x 2 ml ice-cold KRH. The cells were solubilized in 500 μl 37 °C 1% sodium dodecyl sulfate, then the solubilized well contents were transferred to liquid scintillation vials containing 3 ml scintillation cocktail. Radioactivity was measured and data were analyzed as described for platelets.

Norepinephrine plasma membrane uptake transporter: [^{3}H]NE uptake into transfected cells

C6 glial cells stably expressing the human norepinephrine transporter (C6-NET) were also a gift from Dr. Susan Amara. Assays and work-up were performed as described for dopamine uptake, except [^{3}H]NE was used as the radiolabeled substrate.

Vesicular monoamine transporter (VMAT2): [^{3}H]5-HT uptake into bovine chromaffin granules

Chromaffin vesicles were prepared from bovine adrenal medullae as follows: 150 bovine adrenals from freshly slaughtered animals were collected at Peck's slaughterhouse in Milwaukee, WI. The adrenal glands were bisected and the medullae were scraped out and put into ice-cold 0.3 M sucrose, 10 mM HEPES, pH = 7.0. The medullae were minced with a polytron (setting 10, 3 s) then homogenized with a motor-driven Potter-Elvehjem tissue grinder (setting 10, 10 passes). After removal of the unbroken cells and nuclei by centrifugation, the chromaffin granule membranes were pelleted through 1.6 M sucrose at 100,000 x g for 60 min. Chromaffin vesicle ghosts were prepared by osmotically lysing these crude granules in hypotonic buffer containing (mM): HEPES (5), $MgSO_4$ (2), $CaCl_2$ (10), dithiothreitol (0.1), pH = 7.5. The membranes were recovered by centrifugation at 100,000 x g for 60 min. The vesicles were resealed by suspending the pellets in storage buffer containing (mM): sucrose (300), HEPES (10), $MgSO_4$ (2), dithiothreitol (0.1), pH = 7.0 at a concentration of 5-10 mg/ml. Aliquots were snap-frozen in liquid N_2 and stored at -80 °C. For uptake assays, chromaffin vesicle ghosts were diluted in assay buffer containing (mM): sucrose (300), HEPES (10), ATP (5), $MgSO_4$ (5), pH = 7.8 to a final volume of 0.5 ml. The samples were incubated at 37 °C for 10 min and then cooled on ice for 10 min. Uptake was tested in the absence or presence of various concentrations of the test drugs added to the assay buffer. Nonspecific uptake was defined by 10 μM reserpine. [^{3}H]5-HT was added to a final concentration of 10 nM and the samples were incubated for 5 min at 37 °C to initiate uptake. The samples were then transferred to an ice bath and individual samples were removed, diluted with 4 ml of ice-cold assay buffer minus ATP and $MgSO_4$ then collected on cellulose acetate filters (Millipore type HAWP, 0.45 mm pore size) by vacuum filtration. The filters were washed with 2 x 4 ml of ice-cold assay buffer. Filters

were transferred to scintillation vials and 4 ml scintillation cocktail was added. Radioactivity was measured and data were analyzed as described for platelets.

Photolysis of [^{14}C]methcathinone

[^{14}C]Methcathinone was photolyzed both in methanol solution and on PEI-impregnated cellulose plates. For photolysis in methanol solution, 15 nCi of [^{14}C]methcathinone (specific activity = 10 mCi/mmol) was placed into each of two UV-transparent acrylic cuvettes containing 100 μl methanol. The test cuvette was exposed to ca. 21 watts of 300 nm UV light for 60 min and the control cuvette was kept in the dark. Following photolysis, the contents of the cuvettes were individually deposited onto a silica gel thin layer chromatography (TLC) plate and the spots were allowed to dry. The TLC plate was developed using *n*-butanol:water:acetic acid (4:1:1) as the eluant. The TLC plate was dried, then used to produce an autoradiogram using a phosphor imager (Molecular Dynamics, Sunnyvale, CA) following a 72 hour exposure. Photolysis on solid phase was accomplished by depositing two aliquots of 15 nCi [^{14}C]methcathinone directly onto a PEI-impregnated cellulose TLC plate, allowing the spots to dry, and then exposing the plate to 300 nm UV light for 60 min; the dark control was covered with aluminum foil to block the UV light. After photolysis, the TLC plate was developed using 0.4 M LiCl as the eluant. The plate was dried under a stream of warm air, then visualized with the phosphor imager as described above.

RESULTS AND DISCUSSION

Drug inhibition curves, illustrated for [^{3}H]5-HT uptake into platelets, are shown in Fig. 2. IC_{50} values for all drugs and transporter systems are presented in Table 1. All inhibition curves had slope coefficients not different from unity, indicating that the drugs interacted with a single site on the transporter proteins. Methcathinone and methylone were threefold less potent ($P < 0.05$) than MA or MDMA at inhibiting [^{3}H]5-HT uptake into human platelets, with IC_{50}'s of 31.4 ± 7.3 μM and 5.8 ± 0.7 μM, respectively (Table 1). In C6 glial cells stably expressing the rat dopamine transporter, methcathinone and methylone were similar in potency to MA and MDMA, with IC_{50}'s for [^{3}H]DA uptake of 0.36 ± 0.06 μM and 0.82 ± 0.17 μM, respectively. Methcathinone and methylone were also similar in potency to MA and MDMA in C6 cells expressing the human norepinephrine transporter, with IC_{50}'s for [^{3}H]NE accumulation of 0.51 ± 0.10 μM and 1.2 ± 0.1 μM, respectively. The benzylic ketone moiety of methcathinone and methylone had a large negative impact on the capacities of these drugs to inhibit the vesicular monoamine transporter compared to MA and MDMA. In VMAT2-containing bovine chromaffin granules, the IC_{50} values for [^{3}H]5-HT uptake were 103 ± 15 μM for methcathinone and 125 ± 16 μM for methylone. These values are tenfold less potent ($P < 0.05$) than MA and MDMA, respectively.

These results indicate that methcathinone and methylone are potent and selective inhibitors of plasma membrane catecholamine uptake transporters, with more modest effects at the serotonin uptake transporter. With respect to methcathinone, the observed selectivity is about 100-fold for the dopamine transporter and about 60-fold for the norepinephrine carrier compared to the serotonin transporter. In contrast, these drugs are essentially inactive at the vesicular monoamine transporter compared to the nonketo drugs MA and MDMA. The plasma membrane catecholamine transporters are more permissive than the serotonin transporter in this series of drugs; the benzylic ketone moiety is well-tolerated by the dopamine and norepinephrine carriers, but decreases potency by threefold at the serotonin transporter. This effect is even more pronounced at VMAT2, where methcathinone and

methylone are tenfold weaker than the corresponding nonketo drugs. The benzylic position of these molecules thus carries important structural information for recognition by the neurotransmitter transporter proteins.

We hypothesized that methcathinone would be photolabile. Our results demonstrate that this is indeed true. When incubated in methanol (Fig. 3, left panel), [^{14}C]methcathinone migrates as one spot in the absence of light. Exposure to light results in the complete photolysis of [^{14}C]methcathinone and the appearance of at least four new radiolabeled substances. When photolyzed on solid phase (Fig. 3, right panel), much of the [^{14}C]methcathinone remains covalently bound at the origin. In the dark control, no radioactivity remains at the origin. Instead, the entire sample migrates as an amorphous spot near the solvent front. These results confirm the photolysis of [^{14}C]methcathinone both in organic solution and on solid phase.

Together, these data indicate that we have developed the first photoaffinity label for amphetamine binding sites on the plasma membrane monoamine uptake transporters. We have synthesized and are presently testing carrier-free radioiodinated derivatives of methcathinone as new, high specific activity photoaffinity labels for these sites. We believe that these new photoprobes will prove useful in identifying binding sites on the monoamine uptake carriers for neurotransmitter substrates, as well as binding sites for exogenous substances such as methamphetamine, methcathinone, and MDMA.

ACKNOWLEDGMENTS

N.V.C. was supported by a grant from the National Alliance for Research on Schizophrenia and Depression. M.K.S. was supported by a grant from the American Heart Association. A.E.R. was supported by NIH Grant NS33650.

REFERENCES

1. Hyde, J.F.; Browning, E.; Adams, R. Synthetic homologs of d,l-ephedrine. *J. Amer. Chem. Soc., 50*, 2287-2292 (1928)

2. L'Italien, Y.J.; Park, H.; Rebstock, M.C. Methylamino-propiophenone compounds and methods for producing the same. *U.S. Patent # 2802865*, August 13, 1957

3. Rosen, D.S. Methcathinone. *J. Adoles. Health, 14*, 426 (1993)

4. *Federal Register, 58* (198), 53404-53406 (1993)

5. Glennon, R.A.; Yousif, M.; Naiman, N.; Kalix, P. Methcathinone: a new and potent amphetamine-like agent. *Pharmacol. Biochem. Behav., 26*, 547-551 (1987)

6. Kaminski, B.J.; Griffiths, R.R. Intravenous self-injection of methcathinone in the baboon. *Pharmacol. Biochem. Behav., 47*, 981-983 (1994)

7. Dal Cason, T.A.; Young, R.; Glennon, R.A. Cathinone: an investigation of several *N*-alkyl and methylenedioxy-substituted analogs. *Pharmacol. Biochem. Behav., 58*, 1109-1116 (1997)

8. Cozzi, N.V.; Shulgin, A.T.; Ruoho, A.E. Methcathinone (MCAT) and 2-methylamino-1-(3,4-methylenedioxyphenyl)propan-1-one (MDMCAT) inhibit [^{3}H]serotonin uptake into human platelets. *Am. Chem. Soc. Div. Med. Chem. Abs., 215*, 152 (1998)

9. Cozzi, N.V.; Ruoho A.E. Radiosynthesis of [^{3}H]methcathinone, an inhibitor of monoamine reuptake transporters. *J. Labelled Cpd. Radiopharm., XLI*, 927-933 (1998)