Short Communication

Nonspecific Effects of the Selective κ -Opioid Receptor Agonist U-50,488H on Dopamine Uptake and Release in PC12 Cells

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Abstract. κ -Opioid receptor agonists decrease the levels of extracellular dopamine in vivo and in vitro. However, the mechanism(s) underlying these actions are unclear. The objective of this study was to distinguish between an effect of the selective κ -opioid receptor agonist U-50,488H ((*trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide methanesulfonate) on secretion and reuptake of dopamine by PC12 cells. The data show that U-50,488H has both a modest effect to increase dopamine release and a more pronounced effect to inhibit dopamine uptake. Neither effect was sensitive to nor-binaltorphimine or naloxone, suggesting that they are not mediated through an opioid receptor.

Keywords: κ -opioid receptor agonist, dopamine uptake, PC12 cell

The mesocorticolimbic dopaminergic system, arising in the ventral tegmental area and projecting to the nucleus accumbens, striatum, and cortex, has been suggested to play a crucial role in the behavioral and rewarding effects of drugs of abuse (1). The presence of μ , κ , and δ opioid receptors on neurons within the mesocorticolimbic dopaminergic system provides a basis for its role in the actions of drugs of abuse as well as a means for opioids to modulate dopaminergic transmission. U-50,488H ((*trans*-($\pm$)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide methanesulfonate), one of the first selective κ -opioid receptor agonists to be developed, and its congeners, such as U-69,593 ((+)-(5 α ,7 α ,8 β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneacetamide), have been widely used to delineate actions of that class of opioid receptors. κ -Opioid receptor agonists decrease extracellular dopamine content in the nucleus accumbens and the striatum in rats (2, 3) and K⁺-stimulated dopamine release from slices of rat striatum and nucleus accumbens (4, 5). Also, activation of κ -opioid receptors inhibits extracellular dopamine level in PC12 cells (6). Interestingly, the extent to which dopamine levels were inhibited in vivo was greater at lower doses of κ -opioid receptor agonists than at higher doses (2). In light of these observations,

the mechanisms underlying the effects of κ -opioid receptor agonists on extracellular dopamine are unclear. Actions on both secretion and reuptake of dopamine could contribute to the effects of κ -opioid receptor agonists. The objective of this study was to distinguish between these possibilities using the selective κ -opioid receptor agonist U-50,488H and PC12 cells as an experimental model.

PC12 cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 supplemented with 10% horse serum, 5% fetal calf serum, 25 units/ml penicillin, and 25 mg/ml streptomycin at 37°C in 5% CO₂/95% air. For measurement of dopamine release, cells were harvested and suspended in medium containing 30 nM [³H]dopamine (53.2 μ Ci/nmol), 0.1 mM ascorbate, and 10 μ M pargyline, a monoamine oxidase inhibitor. Cells (approximately 250 μ g protein) were placed in 12-well collagen-coated plates and allowed to adhere for 5–6 h at 37°C. Next, medium was replaced with Earle's balanced salt solution (EBSS) containing 10 mM HEPES (pH 7.4), 0.1 mM ascorbate, 10 μ M pargyline, and 1 μ M mazindol, a non-selective monoamine transporter inhibitor. After 10 min, buffer was replaced, with or without addition of U-50,488H, nor-BNI, or both, and the incubation was continued for 6 min. Medium was then collected and centrifuged (1,000 $\times$ g) for 5 min. The radioactivity of the supernatant was measured by liquid scintillation

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counting (Packard Instrument Co., Meriden, CT, USA).

For measurement of dopamine uptake, adherent PC12 cells were washed twice and harvested in EBSS-HEPES/0.1 mM ascorbate buffer. Cells (approximately 250 μ g protein) were aliquoted to individual tubes, warmed to 37°C, and incubated for 6 min in the presence of 30 nM [3 H]dopamine (53.2 μ Ci/nmol), with or without U-50,488H and/or nor-BNI. Uptake was stopped by collection of cells on glass filters. Filters were washed 5 times with ice cold EBSS-HEPES, placed in scintillation liquid, and counted for radioactivity. Since the norepinephrine transporter is primarily responsible for dopamine uptake in undifferentiated PC12 cells (7), transporter-independent (nonspecific) uptake was measured in parallel by incubating cells with 100 nM nisoxetine, a specific norepinephrine transporter blocker. Nisoxetine was added 10 min before other drugs. Transporter-mediated uptake of [3 H]dopamine under each experimental condition was calculated by subtracting uptake in the presence of nisoxetine from uptake in the absence of nisoxetine.

κ -Opioid receptor binding in PC12 cells was assayed as previously described (8) and compared to binding in the rat striatum, a tissue known to contain κ -opioid receptors. Animal care and management were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. PC12 cells or rat striata were homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, and the homogenate was centrifuged at $1,000 \times g$ for 5 min. The supernatant was centrifuged at $25,000 \times g$ for 30 min at 4°C, and the resulting membrane pellet was resuspended in Tris-EDTA buffer containing 5 mM $MgCl_2$. Membranes (250 μ g protein) were incubated for 60 min at 25°C with [3 H]U-69,593 (5 nM, 41.4 μ Ci/nmol). For non-specific binding, membranes were incubated with 1 μ M unlabeled U-69,593. The reaction was terminated by collection of cells on a glass filter. Filters were washed 5 times with ice cold 50 mM Tris buffer, and the radioactivity on each filter was measured by liquid scintillation counting.

Isotopes were purchased from Perkin Elmer Life Science, Inc. (Boston, MA, USA). Sera and antibiotics were from Gibco (Gaithersburg, MD, USA). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

In contrast to the results of previous studies (6), data from our laboratory (9) indicate that exposure of PC12 cells to high concentrations (20–80 μ M) of a κ -opioid agonist (i.e., U-50,488H) increased extracellular dopamine levels through a mechanism that was not

blocked by the selective μ -opioid receptor antagonist nor-binaltorphimine. Measurement of extracellular dopamine alone, however, cannot distinguish between an effect of U-50,488H on dopamine release and dopamine uptake. Based on the observation that reduction of extracellular Ca^{2+} abolished the increase in $[Ca^{2+}]_i$, but not the increase in extracellular dopamine, caused by U-50,488H, we proposed that inhibition of dopamine uptake, rather than stimulation of exocytotic secretion, is the primary mechanism by which U-50,488H increases extracellular dopamine; this premise is supported by the results reported herein. Although U-50,488H (40 μ M) increased dopamine release from PC12 cells by approximately 15% (Fig. 1A), the effect can account for no more than 25% of the increase in extracellular dopamine reported previously (9). Although the mechanism of this action is presently unknown, it does not appear to be mediated by the κ -opioid receptor. It might reflect displacement of dopamine from the secretory vesicle by U-50,488H or another nonselective action of the agonist. In addition to increasing dopamine release, U-50,488H had a more prominent effect to inhibit dopamine uptake (Fig. 1B). The ability of nisoxetine to block this latter effect, and the fact that PC-12 cells predominantly express the norepinephrine transporter (7), suggests that U-50,488H blocks dopamine uptake through direct interaction with the norepinephrine transporter. In that regard, the inhibition of dopamine uptake by U-50,488H was insensitive to the selective κ -opioid receptor antagonist nor-binaltorphimine (nor-BNI) as well as the nonselective opioid receptor antagonist naloxone.

It has been suggested that undifferentiated PC12 cells express κ -opioid receptors. However, PC12 cells that were cultured for more than 3 weeks changed morphology and lost their inhibitory responses to the κ -opioid receptor agonist U-69,593 (6). In the study reported herein, cells were subcultured for at least six weeks before being used in an experiment, but an increase in extracellular dopamine in response to U-50,488H was consistently noted. As shown herein (Fig. 2) and in previous studies (10–12), PC12 cells exhibit relatively low binding of a selective κ -opioid receptor ligand. These observations together with the failure of nor-BNI to block the effects of U-50,488H support the premise that its effects on PC12 cells to increase extracellular dopamine are not mediated through κ -opioid receptors.

It can be argued that U-50,488H elicits its effects through another subtype of opioid receptor. Inhibition of dopamine uptake by U-50,488H, however, was not affected by the non-selective opioid receptor antagonist naloxone (Fig. 3). These results are in accordance with those of a previous report that U-50,488H inhibited

Fig. 1. Effects of U-50,488H on release and uptake of dopamine in PC12 cells. A: Cells were incubated with 30 nM [3 H]dopamine and pargyline (a monoamine oxidase inhibitor) in RPMI medium for 5–6 h at 37°C. Then, medium was removed and replaced with EBSS-HEPES containing pargyline and 1 μ M mazindol. After 10 min, fresh medium with inhibitors and with or without U50,488H (40 μ M), nor-BNI (20 μ M), and/or U-50,488H plus nor-BNI was added, and the incubation was continued for 6 min. Medium was collected, and the radioactivity of cells and medium were determined. Values represent the mean $\pm$ S.E.M. of 7 experiments. B: PC12 cells were incubated in the presence of 30 nM [3 H]dopamine with or without experimental drugs for 6 min. Uptake was stopped by filtration. Washed filters were placed in scintillation liquid and counted for radioactivity. [3 H]Dopamine uptake by the norepinephrine transporter was calculated by subtracting uptake in the presence of 100 nM nisoxetine, a specific norepinephrine transporter blocker, from uptake in the absence of nisoxetine. Values represent the mean $\pm$ S.E.M. of 4 experiments. In each experiment, triplicate samples were averaged to give a single value for each condition. Data were analyzed by two-way repeated measures ANOVA. * P <0.05, compared to the respective control.

dopamine uptake in slices of striatum or nucleus accumbens through a nonopioid receptor-mediated

Fig. 2. Low specific binding of κ -opioid receptor agonist in PC12 cells. Membranes from PC12 cells or rat striata were prepared and incubated with 5 nM [3 H]U-69,593 at 25°C for 60 min. Specific binding was defined as the difference between nonspecific binding (measured in the presence of 1 μ M concentration of unlabeled U-69,593) and total binding. Values represent the mean $\pm$ S.E.M. of 3 experiments (n =3, performed in duplicate in each experiment). * P <0.05, compared to rat striatum; unpaired t -test.

Fig. 3. Nonopioid receptor-mediated effects of U-50,488H on dopamine uptake. The dopamine uptake in PC12 cells was measured using the same protocol as described in Fig. 1B except naloxone (20 μ M) was used to antagonize the effect of U-50,488H. Values represent the mean $\pm$ S.E.M. of 3 experiments (n =3, performed in triplicate in each experiment). * P <0.05, compared to the respective control group; two-way repeated measures ANOVA.

mechanism (13).

The dopaminergic nervous system is known to be involved in the perception of pleasure and reward. An increase in extracellular dopamine in specific regions of the brain caused by drugs of abuse is believed to be responsible for their addictive potential. Conversely, a reduction of extracellular dopamine is associated with

the decrease in self-administration of cocaine and other drugs of abuse caused by κ -opioid receptor agonists (14). Although κ -opioid receptor agonists decreased extracellular dopamine in the nucleus accumbens and the striatum of rats in vivo (3), the extent of inhibition was greater at lower doses of agonist than it was at higher doses. κ -Opioid receptor agonists elicited a similar phenomenon in studies of aversive behavior (15). Extrapolation of the data reported herein to observations in vivo suggest that the apparent loss of an inhibitory effect on dopamine release at higher doses of κ -opioid receptor agonists results from an inhibition of dopamine uptake as well as an increase in dopamine release. This evidence emphasizes the need to appropriately adjust dosage when using a κ -opioid receptor agonist in the management of symptoms associated with drug abuse and withdrawal.

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