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Inhibition by arachidonic acid and other fatty acids of dopamine uptake at the human dopamine transporter

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Abstract

It is known that arachidonic acid, in addition to promoting release of dopamine, can inhibit its transport. The present study provides preliminary information on structure–activity relationships for uptake inhibition by rotating disk voltammetry in human embryonic kidney-293 cells expressing the human dopamine transporter. Except for anandamide, all other fatty acids studied at a pretreatment concentration of 80 μM caused significant reductions in V_{max} but not K_m. Increasing saturation of the hydrocarbon tails (partial saturation: oleic acid, linoleic acid; full saturation: arachidic acid, stearic acid, stearic acid ethyl ester) removed inhibitory activity incrementally, suggesting a role for cis-unsaturation (folding/bending of hydrocarbon tails). The relative lack of effect of 5,8,11,14-eicosatetraynoic acid also supports the idea that less linear structures are less inhibitory on dopamine uptake. Esterification of the free carboxylic group (arachidonic acid ethyl ester) prevented most of the inhibitory activity, arguing against mere membrane lipid disruption. Finally, the endogenous cannabinoid anandamide greatly reduced uptake V_{max} accompanied by a small decrease in K_m, a potentially important effect on dopaminergic neurotransmission.

Keywords: Dopamine transport; Voltammetry, rotating disk; Arachidonic acid; Fatty acid; Eicosanoid

1. Introduction

The dopamine transporter plays a crucial role in dopaminergic neurotransmission by taking up extracellular dopamine into nerve cells, thereby terminating action of the released transmitter (Iversen, 1971). The mechanisms that regulate dopamine uptake are of medicinal importance as potential sites of action for Parkinson's and other neurological diseases, and for psychiatric and other complex diseases involving dopamine (Hahn and Blakely, 2002). Dopamine transporter function is modulated by experimental manipulation of dopamine D2 receptors, nitric oxide, tyrosine kinase, and protein kinase C (Zahniser and Doolen, 2001; Chen and Reith, 2002; Cao and Reith, 2002). As far as protein kinase C is concerned, its activation also stimulates phospholipase A_2 which releases arachidonic acid (Nishizuka, 1995); in turn, arachidonic acid can inhibit dopamine uptake as first shown by L'hirondel et al. (1995). Subsequent studies with the cloned human dopamine transporter in a heterologous expression system have shown inhibitory or stimulatory effects on dopamine uptake of arachidonic acid depending on concentration and time of treatment (Zhang and Reith, 1996) or voltage clamping (Ingram and Amara, 2000). Only inhibitory effects occur upon raising concentrations of endogenous arachidonic acid, and dopamine uptake inhibition resulting from arachidonic acid activation is independent of the protein kinase C pathway (Zhang and Reith, 1996). These results suggest that the arachidonic acid pathway can regulate the (extra)synaptic concentration of dopamine. The inhibition or stimulation of dopamine uptake by arachidonic acid is consistent with reported effects on other neurotransmitter transporters. The rate of glycine uptake in C6 glioma cells is selectively reduced by cis-unsaturated arachidonic acid (Zafra et al., 1990). Uptake of glutamate by the human excitatory amino-acid transporter 2

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(EAAT2) is enhanced by arachidonic acid, but uptake by EAAT1 is reduced (Zerangue et al., 1995). Arachidonic acid inhibits the uptake of glutamate by rat brain synaptosomes (Manzoni and Mennini, 1997) and by glial cells expressing the glutamate transporter (Barbour et al., 1989); this inhibition is displayed also by the glutamate transporter integrated into liposomes implying that the inhibition is a direct action on the transporter itself or its lipidic environment (Trotti et al., 1995). In addition to modulating substrate transport, arachidonic acid has been shown to elicit transporter-associated currents. Thus, in the EAAT4 glutamate transporter, arachidonic acid increases the amplitude of a substrate-activated proton-selective conductance without affecting substrate transport (Fairman et al., 1998). In addition, in oocytes expressing the human dopamine transporter, arachidonic acid stimulates an exclusive nonselective cation conductance apart from conductances connected with ion or dopamine translocation (Ingram and Amara, 2000).

The present experiments focus on the inhibitory effect of arachidonic acid on dopamine uptake in cells heterologously expressing the human dopamine transporter. One method to monitor dopamine concentration outside of cells is rotating disc voltammetry. Rotating disc voltammetry is a convenient technique for measuring changes in concentration of easily oxidizable substrates, such as dopamine. The free solution dopamine concentration is related to the detected oxidation current signal, and thus, the rate of dopamine uptake can be assessed by monitoring the disappearance of dopamine from the medium with negligible signal from other compounds present (Danek Burgess and Justice, 1999; Earles et al., 1998; Earles and Schenk, 1998). The present study addresses the structure–activity relationships for the inhibitory effect of arachidonic acid on dopamine uptake. The arachidonic acid molecule has a high degree of unsaturation of the hydrocarbon tail with four double bonds, raising the question as to what the impact is on dopamine uptake of decreasing the degree of unsaturation. Furthermore, what is the impact of esterification of the free carboxylic acid group? This is of interest as alkyl esters of arachidonic acid can still partition into the lipid phase of the plasma membrane (Trotti et al., 1995) and it is unknown whether arachidonic acid acts at the dopamine transporter from within the lipidic membrane environment or from the water phase bathing the cells. Finally, the present results are discussed in the context of the question as to whether dopamine uptake inhibition is a direct effect of arachidonic acid or a second messenger effect with arachidonic acid serving as the precursor for eicosanoid metabolites.

2. Materials and methods

2.1. Materials

The fatty acids were purchased from Aldrich (Milwaukee, WI, USA) All other chemicals were purchased from Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

2.2. Treatment of cells expressing transporter

The source of the human embryonic kidney cells (HEK-293) expressing the human dopamine transporter was Janowsky and co-workers (Eshleman et al., 1997). Cells were grown in 150-cm^2 flasks and one flask per treatment was used. The following description applies to one flask; more flasks were used in each experiment depending on the scheduled number of treatments. Cells were harvested approximately 3 days after seeding by first removing media, washing with HANKS solution, and incubation with 2 ml of 0.25% (w/v) trypsin–EDTA for less than 3 min at 37 °C. After addition of 2 ml of bovine calf serum to the flask, and washing out with 5 ml of HANKS solution, the suspension was collected and centrifuged at $1000 \times g$ for 2 min in a DAMON/IEC UV centrifuge (International Equipment, Needham, MA). The supernatant was discarded, the pellet was resuspended in 20 ml of "Assay buffer" (130 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, and 10 mM glucose, pH 7.4 at room temperature) and the suspension was centrifuged. The resulting pellet was re-suspended in 15 ml of assay buffer containing 5% (v/v) ethanol with 80 μM fatty acid or without ("vehicle") in an Erlenmeyer flask. The sample was shaken for 15 min at room temperature, and centrifuged at $1000 \times g$ for 2 min in a tabletop centrifuge ("Centrific" of Fisher Scientific). The pellet was resuspended in 15 ml of assay buffer, and after centrifugation, the resulting pellet was gently mixed with 0.8 ml of assay buffer and allowed to stand at room temperature for approximately 30 min. These conditions were chosen to remove the ethanol (less dense than water and in supernatant upon centrifugation) while retaining fatty acid partitioned into the membranes (in pellet); among several procedures tried, the adopted one maximized uptake activity and cell viability.

In order to test the viability of cells treated with vehicle or test compound, aliquots of cells after treatment were examined for the ability to extrude Trypane Blue dye. The proportion of viable cells was assessed by cell counting with a hemocytometer. For each treatment condition, a total of 20–60 cells were examined.

2.3. Uptake assay with rotating disk electrode voltammetry

The procedures were a modification of the previously published method (Chen et al., 1998). A Teflon-shielded glassy carbon working electrode (1.3-mm diameter; Pine Instrument, Grove City, PA) was mounted at the top of an electrochemical cell. A Petit Ampere LC-3D Amperometric Detector (Bioanalytical Systems, West Lafayette, IN) was used as the potentiostat, with settings 200 nA for the current range and 200 ms for the time constant. The

Table 1
Dopamine uptake into HEK-293 cells expressing human dopamine transporter assessed by rotating disk voltammetry

Treatment	Dopamine K_m^a (μM)	Dopamine V_{max}^b (pmol/mg/s)	Dopamine V_{max} (%decrease)
Set 1			
None	1.36 ± 0.27	42.2 ± 11.4	
Vehicle	1.16 ± 0.21	29.0 ± 5.0	0
Arachidonic acid	1.51 ± 0.60	6.39 ± 1.37	78
Arachidic acid	1.06 ± 0.48	20.5 ± 6.6	29
Stearic acid	1.16 ± 0.40	26.7 ± 3.6	8
Oleic acid	1.07 ± 0.04	16.4 ± 3.3	44
Linoleic acid	0.85 ± 0.23	12.4 ± 5.0	57
Arachidonic acid ethylester	1.32 ± 0.21	18.9 ± 6.4	35
Stearic acid ethylester	1.03 ± 0.21	23.2 ± 4.2	20
Set 2			
Vehicle	2.28 ± 0.22	56.8 ± 9.2	0
5,8,11,14-eicosatetraynoic acid	2.00 ± 0.16	49.9 ± 3.8	12
Anandamide	0.85 ± 0.08	10.4 ± 0.8	82

Cells were incubated with 80 μM test drug for 15 min, washed to remove drug, and assessed for dopamine uptake activity by rotating disk voltammetry. Results are mean ± S.E. for three experiments with independent cell preparations.

[a] Set 1: $P > 0.05$ and Set 2: $P < 0.005$ (one-way analysis of variance on all K_m values within set).

[b] Set 1 and Set 2: $P < 0.01$ (one-way analysis of variance on all V_{max} values within set).

potential of the working electrode was +400 mV relative to the Ag/AgCl reference electrode. The cell suspension (50 μl) was combined with 250 μl assay buffer (37 °C) in the electrochemical cell (37 °C), the electrode lowered into the solution and rotated at 4000 rpm with an AFMSRX Analytical Rotator System (Pine Instrument). Upon reaching a stable baseline for 60 s, dopamine (6.1 μl, for a final concentration ranging from 0.25 to 12 μM in the electrochemical cell) was added, and the rate of uptake was recorded for 20 s or more (up to 60 s) depending on the rate of disappearance. Voltammetric measurements were acquired at a frequency of 4 Hz on a computer through a PCI-MIO-16XE-50 DAQ board (National Instruments, Austin, TX) controlled by custom programed LabView software (National Instruments). The acquired data were imported into Origin software (OriginLab, Northampton, MA) for further analysis as described previously (Chen and Justice, 1998). Proteins were determined by the Lowry method with absorption measurement on a DU 640 Spectrophotometer (Beckman, Fullerton, CA).

2.4. Data analysis

Transport velocities were analyzed as described by us previously (Chen et al., 1998; Chen and Justice, 1998). Briefly, current after dopamine addition was corrected for baseline current extrapolated from before addition. The time interval from 1 to 6 s after peak signal was used for linear regression analysis providing the initial rate of dopamine uptake. Corrected current at time zero gave the current corresponding to the initial dopamine concentration. Uptake as a function of dopamine concentration was graphed as Eadie-Hofstee plots of V vs. V/S, and analyzed by nonlinear least-squares regression analysis with the LIGAND program (Munson and Rodbard, 1980) adopted by KELL (Biosoft, Ferguson, MO). Group differences were tested by one-way analysis of variance, with an accepted level of significance of 0.05.

3. Results

3.1. Cell viability

After treatment with Vehicle, arachidonic acid (80 μM) or arachidonic acid ethyl ester (80 μM), a majority of cells were found to be viable with the Trypane Blue exclusion test (70–89%). There was no difference in cell viability between Vehicle and fatty acid at 80 μM. At 160 μM, arachidonic acid had a deleterious effect leaving approximately 30% of treated cells viable. In all subsequent experiments, test compounds were studied at a concentration of 80 μM.

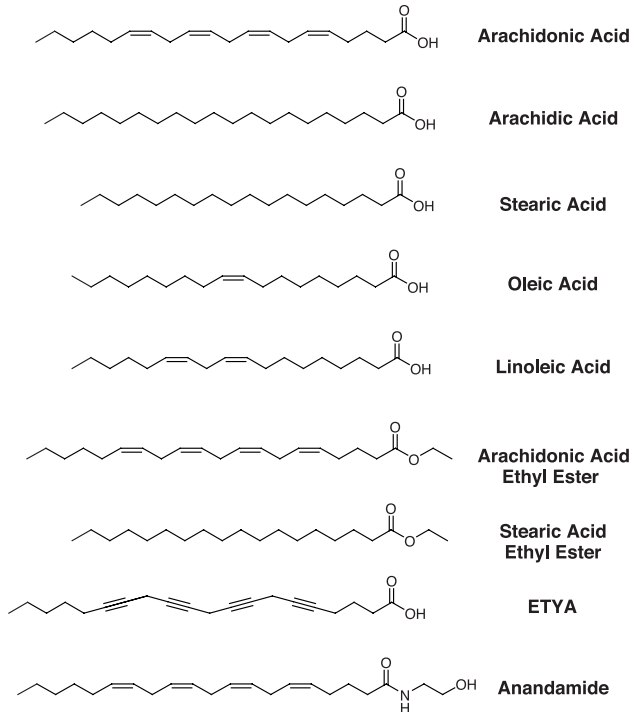

Fig. 1. Structures of fatty acids studied. The cartoon does not depict the folding and bending introduced by cis-unsaturation (arachidonic acid, arachidonic acid ethyl ester, linoleic acid, oleic acid, anandamide). ETYA is 5,8,11,14-eicosatetraynoic acid.

3.2. Kinetics of dopamine uptake and effect of vehicle

Except for anandamide, the fatty acids studied at 80 μM did not affect the K_m of dopamine uptake (Table 1). The presence of 5% ethanol by itself, necessary for keeping the fatty acids in solution during pretreatment, reduced the V_{max} (Table 1, Vehicle in set 1). For comparison of test compounds, effects on V_{max} were expressed as %decrease compared with Vehicle.

3.3. Saturation of hydrocarbon tails

Arachidic acid (20-carbon-long), which is saturated arachidonic acid, and stearic acid (18-carbon-long), which is also saturated but two carbons shorter than arachidonic acid (Fig. 1), caused considerably less dopamine uptake inhibition (29% and 8%) than arachidonic acid itself (78%) (Table 1, Set 1 and Fig. 2A). The 18-carbon-long oleic and linoleic acid, which have some degree of unsaturation (one or two double bonds), caused inhibition (44% and 57%, Table 1, Set 1 and Fig. 2B) but not as much as arachidonic acid which is more unsaturated (four double bonds). Overall, the inhibitory potency of the compounds had the following rank order: arachidonic acid>linoleic acid>oleic acid>arachidic acid>stearic acid (Table 1).

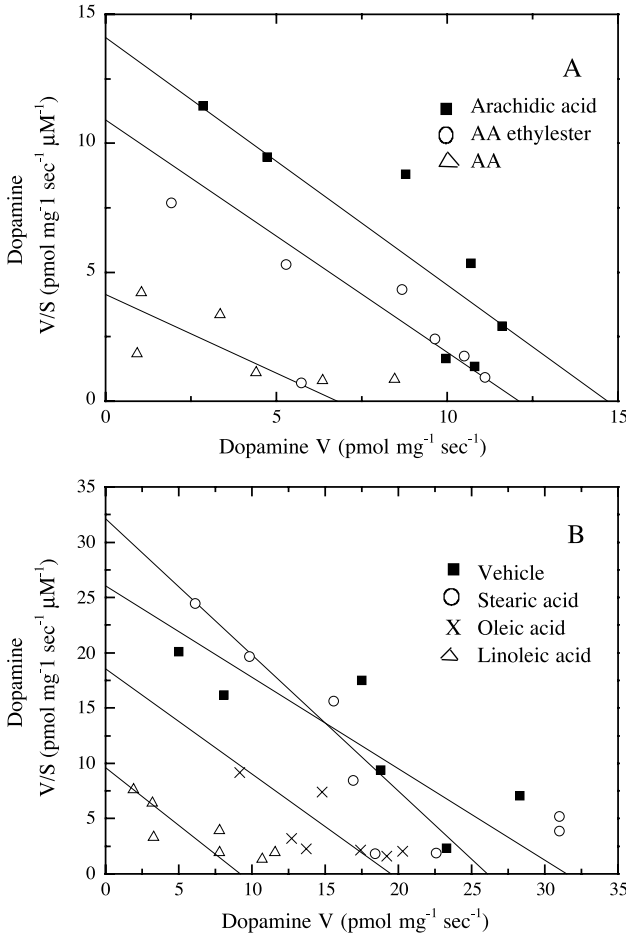

Fig. 2. Eadie–Hofstee plots of dopamine uptake. Representative examples are shown of single experiments which are part of the results shown in Table 1. Straight lines represent the results of analysis with the KELL curve fitting program which is based on the original LIGAND method (see Materials and methods). AA stands for arachidonic acid.

3.4. Esterification of free carboxylic acid group

Esterification of the free carboxylic group in arachidonic acid as in arachidonic acid ethyl ester prevented most of the inhibition (35% compared with 78%, Table 1, Set 1 and Fig. 2A). Esterification of the free carboxyl group in stearic acid (stearic acid ethyl ester) did not substantially alter the lack of inhibition exerted by this completely saturated compound (20% vs. 8%, Table 1, Set 1 and Fig. 2B). Overall, the rank order of the inhibitory potencies was: arachidonic acid> arachidonic acid ethyl ester>stearic acid ethyl ester.

3.5. Anandamide

Conversion of the free carboxylic acid group to carboxylethanolamide as in anandamide did not interfere with the inhibitory effect of arachidonic acid (82% vs. 78%, Table 1, Set 2). In addition to the reduction in V_{max}, there was an approximately twofold decrease in K_m with anandamide compared with Vehicle.

3.6. Nonmetabolizable arachidonic acid analog

There was little or no effect (12%, Table 1, Set 2) by the presence of 5,8,11,14-eicosatetraynoic acid, an arachidonic acid analog that cannot be metabolized by lipooxygenase, epoxygenase, or cyclooxygenase.

4. Discussion

The present results point to an *inhibitory* effect of arachidonic acid at 80 μM on dopamine uptake. In a similar dopamine transporter expressing heterologous cell system, we observed that arachidonic acid (20–160 μM) *stimulated* [^{3}H]dopamine uptake when preincubated for short times (15–30 min), whereas at 160 μM, it *inhibited* following longer pre-exposures (45–60 min) (Zhang and Reith, 1996). Thus, higher concentrations and longer exposures made arachidonic acid inhibitory. Under the present conditions, the inhibitory effect of arachidonic acid (80 μM) prevailed; the possibility can be entertained that there was a mixture of stimulatory and inhibitory influences, through separate mechanisms, with the inhibitory action being the dominant one. In both the previous study (Zhang and Reith, 1996) and the present work, the main component of the dopamine uptake inhibitory effect of arachidonic acid was the reduction in the V_{max}, accompanied by a smaller change, or no change at all, in K_m. The modest increase in dopamine uptake by non-voltage-clamped oocytes expressing the

dopamine transporter was also due to an increase in V_{max} and not K_m (Ingram and Amara, 2000). The inhibitory effect of arachidonic acid on glutamate uptake by glial cells, cultured astrocytes, or purified synaptosomes was again characterized primarily by the reduction in V_{max} (Barbour et al., 1989; Volterra et al., 1992b). It is possible that arachidonic acid affects substrate translocation efficiency rather than substrate recognition.

The arachidonic acid second messenger pathway involves metabolism of arachidonic acid to eicosanoid metabolites by lipooxygenase, epoxygenase, or cyclooxygenase. In our previous work (Zhang and Reith, 1996), nordihydroguiaretic acid, an inhibitor of lipooxygenase preventing the formation of hydroxyeicosatetraenoic acids or leukotrienes and increasing endogenous arachidonic acid, caused dopamine uptake inhibition in the heterologous cell system used for expressing the dopamine transporter. This points to a role for endogenous arachidonic acid itself rather than eicosanoid metabolites generated by lipooxygenase. Furthermore, oleic and linoleic acid, which are cis-unsaturated as arachidonic acid but to a lesser degree, could still inhibit dopamine uptake, albeit to a lesser extent. This also argues against arachidonic acid serving as precursor for eicosanoid metabolites in a second messenger fashion. The results taken together rather point to an involvement of some aspect of the unsaturated fatty acid nature of arachidonic acid in its ability to inhibit dopamine uptake.

Clearly, removing unsaturation from arachidonic acid (arachidic acid, stearic acid) abolished most of its dopamine uptake inhibitory effect. In addition, reducing the degree of unsaturation (linoleic acid, oleic acid) decreased the uptake effect. Most likely, the degree of cis-unsaturation is important for inhibiting dopamine uptake, with cis-unsaturated fatty acids being folded and bent in contrast to saturated or trans-unsaturated fatty acids being linear. Such folded cis-unsaturated fatty acids have been described to target more fluid domains of membranes as opposed to linear fatty acids preferring gel-like domains (Klausner et al., 1980). Thus, it could be thought that the present uptake effects result from fatty acids intercalating in the lipidic microenvironment surrounding the dopamine transporter. However, against this notion is the present observation that dopamine uptake was inhibited much more weakly by arachidonic acid ethyl ester, which shows the same degree of cis-unsaturated folding as arachidonic acid and is even more lipophilic by the esterification of the carboxyl group. It appears likely that a more specific effect is involved than a disruption in membrane lipid organization resulting from the introduction of fatty acid into the membrane. Possibly, fatty acid-binding domains on proteins are implicated, similar to those on serum albumin and protein kinase C (Shearman et al., 1989). Thus, arachidonic acid could interact with specific domains on the dopamine transporter itself or proteins that are associated with the transporter, and esterification of the free carboxyl group of arachidonic acid interferes with this interaction.

We do not interpret the relative lack of effect of 5,8,11,14-eicosatetraynoic acid to be related to the fact that it cannot generate eicosanoid metabolites, for reasons explained above (see previous paragraph). Rather, we think that the large zig–zag structure (each zig and zag having a length of three carbon bonds) interferes with the interaction with specific protein domains as alluded to above (previous paragraph).

There is an ongoing debate as to whether arachidonic acid acts as a free fatty acid outside the plasma membrane or from within, in inhibiting glutamate transport. Barbour et al. (1989) pointed to the correlation between lipid disrupting potency and uptake inhibitory potency as supporting interacalation within the membrane, but Volterra et al. (1992a) argued for an action of free fatty acid from the water phase based on three lines of evidence. First, the inhibitory effect of arachidonic acid was counteracted by the presence of bovine serum albumin suggesting that free arachidonic acid was responsible rather than accumulated arachidonic acid in the membrane interior, because bovine serum albumin chelates only free fatty acids and does not cross cell membranes. Second, dilution of arachidonic acid-pretreated preparations prior to the uptake assay obliterated the uptake effect despite the continuing presence of 95% of the total arachidonic acid content inside the lipidic environment of the preparations. Third, arachidonic acid ethyl ester was inactive despite its ability to penetrate membranes. In our previous study with dopamine transporter expressing C6 glioma cells (Zhang and Reith, 1996), bovine serum albumin also counteracted the uptake inhibition induced by arachidonic acid suggesting a role for free fatty acid. In these experiments, arachidonic acid was not removed prior to initiating [^{3}H]dopamine uptake. In contrast, in the current experiments, cells were preincubated with arachidonic acid for 15 min, washed by centrifugation, and allowed to stand with buffer for another 30 min. If the findings of Trotti et al. (1995) for liposomes apply to the current cells, the bulk of the added arachidonic acid resides in the lipid compartment of the cells regardless of the dilution applied during washing, and the free arachidonic acid concentration in the final uptake assay is likely to be appreciably less than 80 μM. It is likely, then, that arachidonic acid acts from within the plasma membrane to affect dopamine uptake rather than as a free fatty acid, contrary to the effect on glutamate transport suggested to be caused by free fatty acid (Volterra et al., 1992a). The effect of albumin on dopamine uptake observed previously (Zhang and Reith, 1996) could have been caused by chelation of arachidonic acid prior to entering the membrane. Alternatively, in the ongoing partitioning process of arachidonic acid, distributing itself between the membrane and the bathing medium, it may be the more externally situated fatty acid in the membrane that interferes with uptake and is susceptible to chelation by albumin in water crevices in the outer membrane in direct contact with the medium.

Arachidonic acid has been linked to excitatory amino receptors, in particular those of the *N*-methyl-D-aspartate

(NMDA) subtype. Stimulation of NMDA receptors leads to Ca^{2+} entry, activating phospholipase A_2, which in turn releases arachidonic acid (Lazarewicz et al., 1990). In dopamine terminal areas in the brain, NMDA receptors reside on γ-amino-butyric acid (GABA) neurons and on dopamine fibers, with both receptor populations mediating arachidonic acid release (for refs see L'hirondel et al., 1999). Thus, endogenous arachidonic acid has the opportunity to regulate dopamine neurotransmission either by diffusing from GABAergic neurons as an intercellular communicator, or by acting on dopamine terminals subsequent to its release from these terminals. Previous studies have demonstrated the possibility of arachidonic acid regulating a nonselective cation conductance through the dopamine transporter, possibly producing local depolarizations altering dopamine signaling (Ingram and Amara, 2000). The group of Glowinsky has shown effects of both exogenous (L'hirondel et al., 1995) and endogenous (L'hirondel et al., 1999) arachidonic acid on dopamine release, in synergy with activation of muscarinic receptors. The present experiments add more complexity to the regulation of dopaminergic neurotransmission by arachidonic acid, in advancing evidence for an additional contribution of changes in dopamine uptake activity. "Classical" initial velocity assays with [^{3}H]dopamine are difficult to achieve at 37 °C in our HEK cells expressing human dopamine transporter because uptake occurs so fast that linearity with time is not observed beyond 1 min, technically the shortest interval that can be measured accurately with the radiolabeled substrate method. In contrast, the rotating disk voltammetry assay used in the present experiments measures uptake of dopamine on a second scale and can easily monitor initial uptake velocity during an interval (5 s) where uptake is linear with time. Thus, arachidonic acid, in addition to stimulating dopamine release (L'hirondel et al., 1995, 1999), can enhance the effectiveness of extracellular dopamine by inhibiting its uptake by the transporter. It is of interest that anadamide, an arachidonic acid-like fatty acid, also inhibits dopamine uptake. In addition to reducing V_{max}, it also decreases K_m; the latter effect would serve to somewhat dampen the inhibitory effect on dopamine uptake at levels of dopamine less than, or in the range of, the K_m for normal dopamine uptake. Anandamide, originally identified as arachidonylethanolamide (Devane et al., 1992), is an endogenous cannabinoid acting on the same cannabinoid receptors that are the target of Δ^9-tetrahydrocannabinol, the active ingredient of marijuana or hashish. The euphoric effect of Δ^9-tetrahydrocannabinol may be mediated by its ability to increase extracellular dopamine (Chen et al., 1993). The mechanism underlying the dopamine increase is subject to debate (see Ameri, 1999). One possibility is a local effect at dopamine terminals, involving dopamine uptake inhibition as inferred from in vitro studies with synaptosomes and [^{3}H]dopamine (Banerjee et al., 1975). The present results indicate that anandamide, the endogenous cannabinoid, also inhibits dopamine uptake as measured by rotating disk voltammetry.

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