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Regulation of the functional activity of the human dopamine transporter by the arachidonic acid pathway

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Abstract

The role of arachidonic acid was examined in the regulation of dopamine transport in C6 glioma cells stably expressing the human dopamine transporter. Exogenously added arachidonic acid (20–160 μM) stimulated [^{3}H]dopamine uptake when pre-incubated for short times (15–30 min); 160 μM arachidonic acid inhibited following longer pre-exposures (45–60 min). Under the same conditions, only decreases were observed in the binding of the cocaine analog [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([^{3}H]WIN 35,428). The reduction in dopamine transporter activity by arachidonic acid (at 160 μM for 60 min) was caused by a decrease in the V_{max} (from 202 to 44 pmol/mg/min) opposed by a smaller reduction in K_m (from 1.2 to 0.8 μM), whereas the effect of arachidonic acid (at 160 μM for 15 min) on [^{3}H]WIN 35,428 binding was caused by a reduction in the B_{max} (from 1.8 to 1.3 pmol/mg) without a change in K_d (7.2 nM). Upon 15-min exposure, melittin, an activator of phospholipase A_2, and nordihydroguaiaretic acid, a lipooxygenase inhibitor, both expected to cause enhanced endogenous arachidonic acid, inhibited [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding with an IC_{50} value close to 1 μM, whereas thimerosal, which raises arachidonic acid by inhibiting lipid reacylation, caused similar reductions at the sub-millimolar level. Co-presence of staurosporine (0.3–2 μM), an inhibitor of protein kinase C, had little or no effect on the melittin- or arachidonic acid-induced inhibition of [^{3}H]dopamine uptake. Both the melittin- and arachidonic acid-, but not phorbol 12-myristate 13-acetate-induced inhibition of uptake were counteracted by bovine serum albumin (0.1 and 1 mg/ml) which binds arachidonic acid. The data taken together suggest that the inhibitory effects of arachidonic acid activators and those of protein kinase C activators on dopamine uptake are mediated by separate mechanisms.

Keywords: Dopamine transporter; Dopamine uptake; WIN 35,428 binding; Arachidonic acid; Protein kinase C; Second messenger

1. Introduction

The dopamine transporter plays an important role in dopamine neurotransmission by mediating uptake of released neurotransmitter into pre-synaptic nerve terminals (Uhl and Johnson, 1994; Garris et al., 1994). Homozygous dopamine transporter knockout mice are spontaneously hyperactive and show extremely slow clearance of neuronally released dopamine (Giros et al., 1996), indicating the importance of the dopamine transporter in maintaining normal dopamine tone. The dopamine transporter is also a target for drugs of abuse, such as cocaine (Ritz et al., 1987; Giros et al., 1996), and endogenous neurotoxins (Pifl et al., 1993) that have been speculated to contribute to the development of Parkinson's disease (Uhl et al., 1994). This protein is a member of a larger Na^+,Cl^--dependent neurotransmitter transporter family (Hoffman, 1994; Uhl and Johnson, 1994) and so far only one type has been found of each monoamine transporter (Amara and Kuhar, 1993). Regulation of monoamine uptake at the transporter level by phosphorylation appears to be a possibility given the existence of consensus sites for the action of protein kinase A and C in the amino-acid sequences of Na^+,Cl^--dependent transporters (Giros and Caron, 1993; Hoffman, 1994). Indeed, activation of protein kinase A or C in various systems has been reported to affect transport of (γ-aminobutyric acid (GABA) (Tian et al., 1994; Corey et al., 1994; Osawa et al., 1994) and serotonin (Myers et al., 1989; Cool et al., 1991; Anderson and Horne, 1992; Ramamoorthy et al., 1993), but effects at steps distal from the transporter may be involved. Kitayama et al. (1994) have

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shown reduced uptake of dopamine and binding of [^{3}H]WIN 35,428 (2β-carbomethoxy-3β-(4-fluorophenyl)tropane), a tropane analog of cocaine, in COS cells expressing the cloned rat dopamine transporter upon treatment with phorbol 12-myristate 13-acetate (PMA), a well-known activator of protein kinase C. These findings are extended in our recent study on C6 glioma cells expressing the cloned human dopamine transporter which further implicate the involvement of protein kinase C (Zhang et al., 1996).

It has become increasingly clear that intracellular signaling pathways have multiple points of interaction (Asaoka et al., 1992; Nishizuka, 1995; Naor et al., 1995). The same signals that induce phospholipase C (leading to protein kinase C activation among other events) frequently cause the release of arachidonic acid through phospholipase A_2 activation; the responsible event linking the two pathways probably consists of activation by protein kinase C of mitogen-activated protein kinase which in turn phosphorylates phospholipase A_2 (for references, see Nishizuka, 1995). Another connection between the two pathways is the amplification of diacylglycerol-stimulated protein kinase C activity by arachidonic acid (see Asaoka et al., 1992; Nishizuka, 1995). Therefore, two possibilities can be distinguished for the reduction in dopamine transporter function upon activation of protein kinase C by the phorbol ester PMA. First, PMA could cause protein kinase C-mediated phosphorylation of the dopamine transporter, resulting in a reduced uptake function unrelated to effects on the arachidonic acid pathway. Second, PMA-induced protein kinase C activation could stimulate phospholipase A_2 causing release of arachidonic acid which could inhibit dopamine transporter function as a simple reversible inhibitor. Indeed, synaptosomal dopamine uptake has been reported to be inhibited by arachidonic acid (L'hirondel et al., 1995); it is not known whether this is a protein kinase C-mediated effect. The present experiments examine the effects of arachidonic acid on the function of the dopamine transporter in C6 cells expressing the human dopamine transporter, either exogenously by adding it to the cells, or endogenously by stimulating phospholipase A_2, which releases arachidonic acid from membrane phospholipids, or inhibiting lipooxygenase, one of enzymes that breakdown arachidonic acid, or inhibiting acyltransferases that reacylate lipids by reesterification of fatty acids including arachidonic acid into lipids (see Volterra et al., 1992). If arachidonic acid regulates the dopamine transporter through protein kinase C-mediated phosphorylation, one would expect a similar pattern for arachidonic acid- and protein kinase C-induced effects on [^{3}H]dopamine uptake/[^{3}H]WIN 35,428 binding, and one would expect reversal of the arachidonic acid effect by protein kinase C inhibition. Alternatively, if protein kinase C activation reduces dopamine transport by the release of arachidonic acid, one would again expect a similar pattern for arachidonic acid- and protein kinase C-induced effects on [^{3}H]dopamine uptake/[^{3}H]WIN 35,428 binding, but no reversal of the arachidonic acid effect by protein kinase C inhibition.

2. Materials and methods

2.1. Materials

Chemicals used in these experiments were obtained from Sigma (St. Louis, MO, USA), except [^{3}H]dopamine (37.54 Ci/mmol) and [^{3}H]WIN 35,428 (83.5 Ci/mmol) which were purchased from Dupont-New England Nuclear (Boston, MA, USA) and cocaine hydrochloride which was obtained from Mallinckrodt (St. Louis, MO, USA). All test compounds were dissolved in purified deionized H_2O prior to addition to buffers, except for arachidonic acid, nordihydroguaiaretic acid, phorbol 12-myristate 13-acetate (PMA) and thimerosal, which were dissolved in dimethylsulfoxide. The final concentrations of dimethylsulfoxide in the assays were $< 0.5\%$ which by itself had no effect on [^{3}H]dopamine uptake or [^{3}H]WIN 35,428 binding.

2.2. C6 glioma cells expressing human dopamine transporter

Human dopamine transporter cDNA was cloned in the laboratory of Dr. Aaron Janowsky (Oregon Health Sciences University, Portland, OR, USA) by screening a human substantia nigra cDNA library with a PCR-amplified probe based on the rat cDNA sequence, and stably transfected into rat C6 glioma cells, as described previously (Eshleman et al., 1994, 1995). Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 5% fetal bovine serum, 5% bovine calf serum, and 0.0001% puromycin (with pBabePuro confering resistance to puromycin), and aliquots were frozen at a density of 10^7 cells/ml with 5% dimethylsulfoxide. For each series of experiments, one freezing vial (1 ml) was rapidly thawed and seeded into a 75-cm^2 flask with the above medium. After 3 days of culturing ($\approx 3 \times 10^7$ cells total), the cells were collected by trypsinization and counted with a hemocytometer. Cells were then seeded into 96-well culture plates. Plates seeded at a density of 45 000 and 30 000 cells/well gave similar protein recoveries per well after 2 and 3 days of culturing, respectively.

2.3. Uptake of [^{3}H]dopamine

The uptake assay was performed on cells grown on 96-well culture plates, as described above. The culture medium was removed from each well in the plate. Cells were washed with 200 μl wash buffer at room temperature (in mM concentrations: 122 NaCl, 5 KCl, 1.2 $MgSO_4$, 10 glucose, 1 $CaCl_2$, 15 Na_2HPO_4 and enough 0.85% H_3PO_4 to achieve a pH of 7.4). Subsequently, 70 μl of 'uptake'

buffer (wash buffer containing 10 μM nialamide) and 10 μl of water containing test drug were added. The cells were pre-incubated for 15 min at 21°C with protein kinase C activator/inhibitor, or phosphatase inhibitor, on a plate shaker, followed by the addition of 20 μl assay buffer containing [^{3}H]dopamine (11 nM final concentration) plus, where indicated, 100 nM (final concentration) nonradioactive dopamine (i.e., 111 nM total dopamine), and incubation for another 8 min. For saturation analysis, the concentrations of unlabeled dopamine varied, and the incubation was for only 3 min. Uptake was terminated by putting the plate on ice and adding 100 μl ice-cold wash buffer. The assay medium was removed immediately by aspiration, followed by two washes with 200 μl ice-cold wash buffer. The cells in each well were incubated with 200 μl 3% (w/v) ice-cold trichloroacetic acid for 30 min. The entire liquid content of each well was transferred to a scintillation vial and assayed for radioactivity with 5 ml cytoscint cocktail (ICN, Costa Mesa, CA, USA) by liquid scintillation counting in a Beckman model LS 6000 IC spectrometer. Non-specific uptake was defined with 100 μM cocaine. The cells in four separate wells were dissolved with 100 μl 1N NaOH, and the Folin phenol reagent method was used to determine the concentration of protein. ≈ 20 μg of protein was obtained from one well.

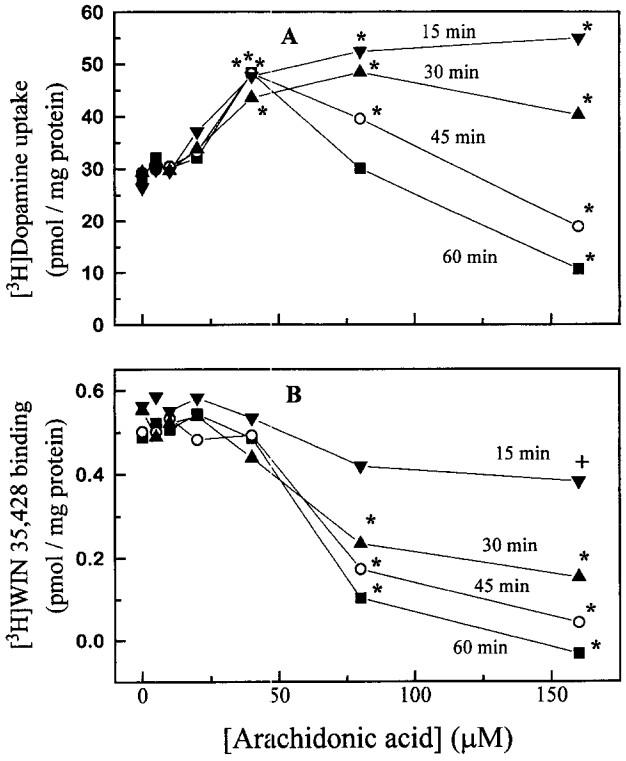

Fig. 1. Effect of varying concentrations of arachidonic acid as a function of pre-incubation time on [^{3}H]dopamine uptake (A) and [^{3}H]WIN 35,428 binding (B). Cells were pre-incubated for the time indicated with arachidonic acid at 21°C and [^{3}H]dopamine (111 nM) uptake or [^{3}H]WIN 35,428 binding was allowed to occur for 8 min. Results are the mean of three observations with the same cell preparation; the entire experiment was replicated once with a different cell preparation. The S.E. in the data shown was on the average 8%. $^*P < 0.001$ compared with zero-concentration of arachidonic acid (Tukey's test following one-way analysis of variance); $^+P < 0.05$ (ibid.).

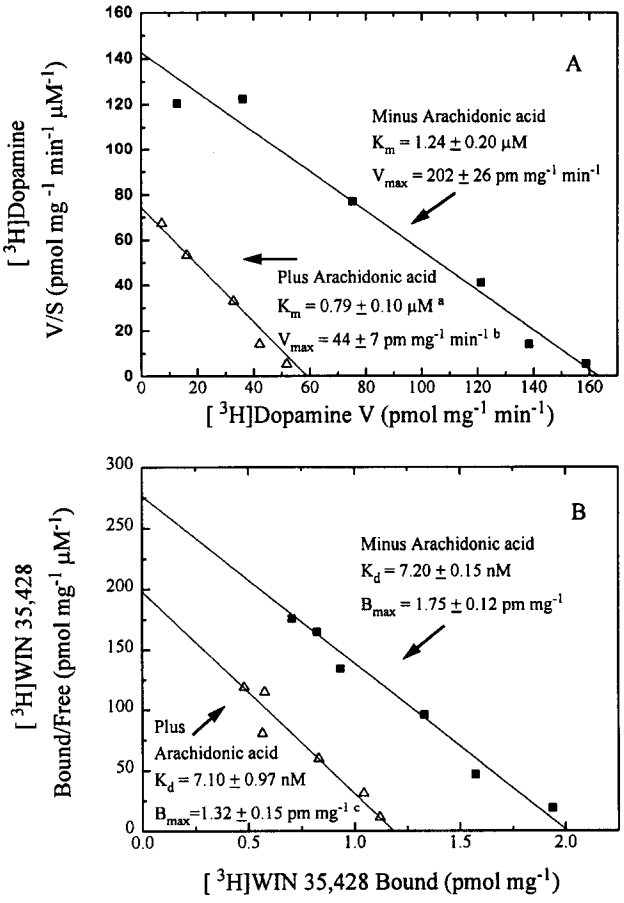

Fig. 2. Saturation analysis of [^{3}H]dopamine uptake (A) and of [^{3}H]WIN 35,428 binding (B) in the presence or absence of arachidonic acid (160 μM) added 60 (A) or 15 (B) min prior to start of assay. (A) [^{3}H]dopamine was present at 11 nM and increasing concentrations of unlabeled dopamine (0.1, 0.3, 1, 3, 10 and 30 μM) were added for a total assay time of 3 min. (B) [^{3}H]WIN 35,428 was present at 4 nM and increasing concentrations of unlabeled WIN 35,428 (1, 3, 10, 30 and 100 nM) were added for a total assay time of 8 min. The straight line represents the best fit chosen by the LIGAND program. Shown are typical experiments, assayed in triplicate, that were carried out 6 (A) or 5 (B) times. The insets show the mean ± S.E. of these results. ■, no drug; Δ, arachidonic acid. $^aP < 0.05$ compared with corresponding measure of other group in same panel (paired Student's t-test); $^bP < 0.001$ compared with corresponding measure of other group in same panel (paired Student's t-test); $^cP < 0.05$ compared with corresponding measure of other group in same panel (unpaired Student's t-test).

2.4. Binding of [^{3}H]WIN 35,428

Binding assays were carried out as the uptake measurements described above with the following changes. [^{3}H]WIN 35,428 was added (4 nM final concentration) instead of [^{3}H]dopamine, and the incubation was continued on a plate shaker for 8 min at 21°C. The termination of the binding assays was identical to that of the uptake assays.

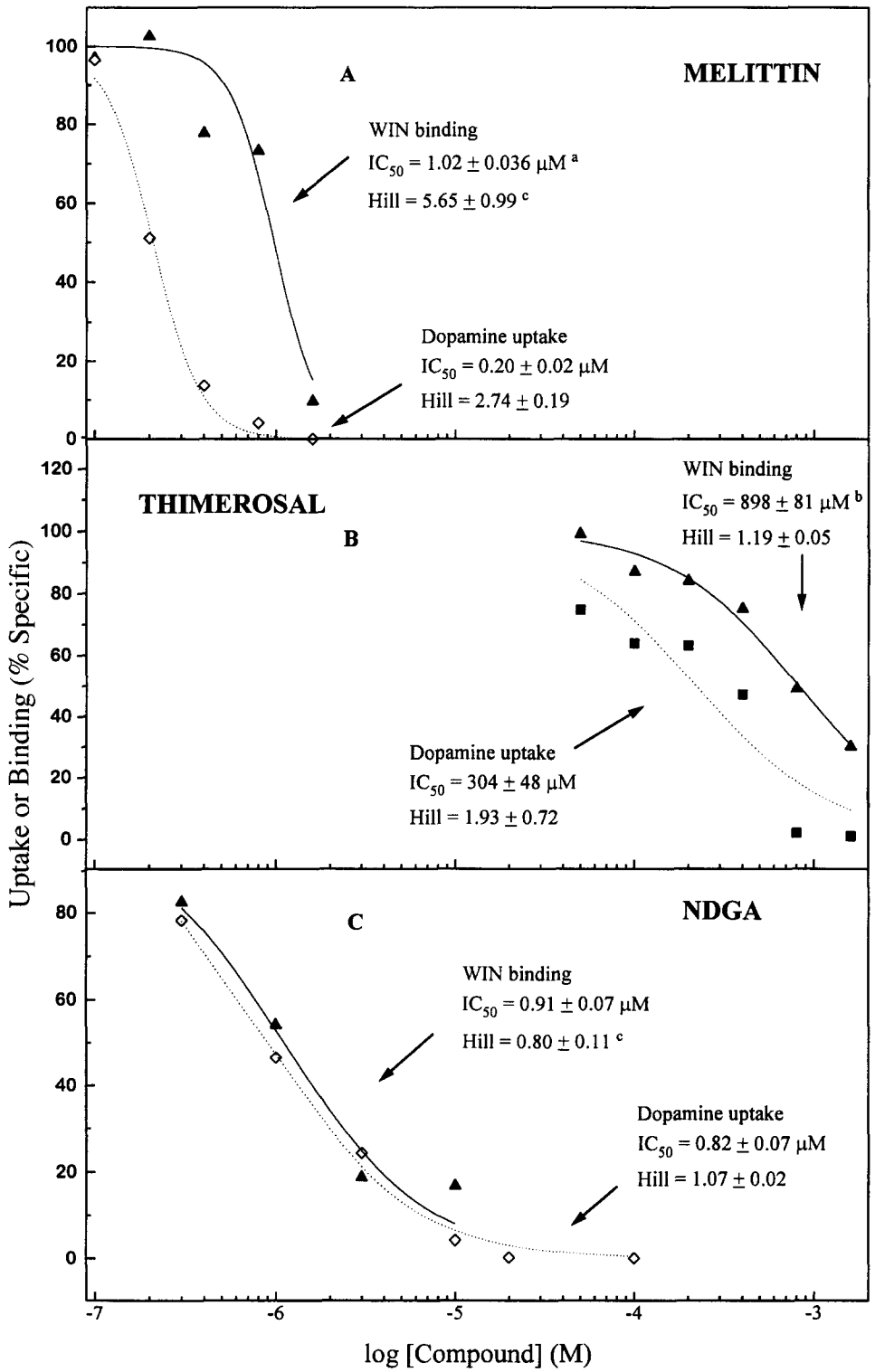

Fig. 3. Concentration-dependent inhibition of [^{3}H]dopamine (11 nM) uptake (————) and [^{3}H]WIN 35,428 binding (—) by melittin (A), thimerosal (B) and nordihydroguaiaretic acid (NDGA) (C). Cells were pre-incubated with various concentrations of the compound for 15 min at 21°C prior to an 8-min exposure to [^{3}H]dopamine. Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out 4 times. The insets show the mean ± S.E. of these results. The mean control uptake in the absence of inhibitor was 1.7 pmol/mg of protein and the corresponding binding was 0.3 pmol/mg of protein. [a] $P < 0.0001$ compared with corresponding measure of other group in same panel (unpaired Student's t-test); [b] $P < 0.001$ (ibid.); [c] $P < 0.05$ (ibid.).

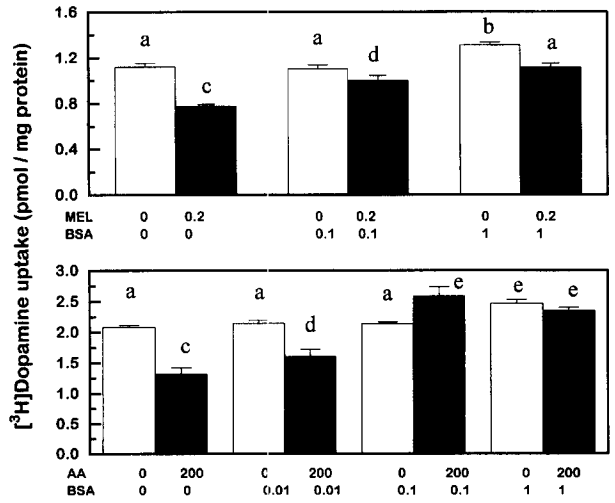

Fig. 4. Effect of bovine serum albumin on melittin (MEL) (top panel)- or arachidonic acid (AA) (bottom panel)-induced inhibition of [^{3}H]dopamine (11 nM) uptake. Cells were pre-treated with MEL/AA or vehicle in the absence or presence of bovine serum albumin at the concentrations indicated at 21°C for 15 min prior to an 8-min exposure to [^{3}H]dopamine. Numbers under bars denote the micromolar (MEL/AA) or mg/ml (bovine serum albumin, BSA) concentrations of each agent present. Results are mean ± S.E. (vertical bar) for 3-4 experiments, each in triplicate, with the same cell preparation. $^{a-d}P < 0.05$ for comparisons between groups; groups labeled with different letters are statistically not from the same population, whereas for groups sharing a letter the null hypothesis (that they are not different) is not rejected (one-way analysis of variance followed by the least significant difference multiple range test).

In one set of experiments, this method was compared with dissolving cell material with 1% (w/v) sodium dodecyl sulphate; the two methods gave identical results. Non-specific binding was defined with 1 μM WIN 35,428 to exclude the low-affinity portion of [^{3}H]WIN 35,428 found to be present in intact cell assays (Reith et al., 1996).

2.5. Data analysis

One-way analysis of variance was used to assess differences among treatment groups, with follow-up by the least significant difference multiple range test or Tukey test for multiple comparisons. Where appropriate, paired Student's *t*-tests were used. Deviations of pseudo-Hill numbers from unity were assessed with Student's one-sample *t*-test. Differences were considered to be significant when $P \leq 0.05$. Saturation uptake data were analyzed with the non-linear computer fitting program LIGAND (Munson and Rodbard, 1980), as described previously (Xu et al., 1995). The IC_{50} values and pseudo-Hill numbers were computed with the equation of ALLFIT program of DeLean et al. (1978) entered into the Microsoft ORIGIN curve-fitting and plotting software. This non-linear regression program was run with total and non-specific uptake (binding) entered as constants.

3. Results

3.1. Raising exogenous arachidonic acid

Pre-incubation of C6 transporter-containing cells with arachidonic acid (20–160 μM) for short times (15–30 min) caused an increase in [^{3}H]dopamine uptake (Fig. 1A). With longer pre-incubation times (45–60 min), arachidonic acid still enhanced uptake at low concentrations (40–80 μM) but decreased uptake at the highest concentration examined, 160 μM. In contrast, under the same conditions only decreases were observed for [^{3}H]WIN 35,428 binding, with greater effects occurring following longer pre-incubation times (Fig. 1B).

The reduction in [^{3}H]dopamine uptake observed following pre-incubation for 60 min with 160 μM arachidonic acid was due predominantly to a decrease in the V_{max} (from 202 ± 26 to 44 ± 7 pmol/mg protein/min, $P < 0.001$), counteracted by a small decrease in the K_m (from 1.24 ± 0.20 to 0.79 ± 0.10, $P < 0.05$) (Fig. 2A). The reduction in [^{3}H]WIN 35,428 binding observed following pre-incubation for 15 min with 160 μM arachidonic acid was due to a decrease in the B_{max} (from 1.75 ± 0.12 to 1.32 ± 0.15 pmol/mg protein, $P < 0.05$) whereas the K_m was unchanged (7.2 ± 0.2 compared with 7.1 ± 1.0) (Fig. 2B).

3.2. Raising endogenous arachidonic acid

Upon 15-min exposure, melittin, an activator of phospholipase A_2, inhibited [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding with an IC_{50} value in the (sub)micromolar range and a Hill number appreciably higher than unity ($P < 0.03$) (Fig. 3A). Thimerosal, a reacylation inhibitor blocking acyltransferases that reesterify fatty acids, such as

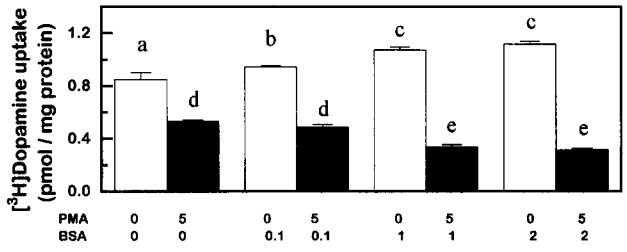

Fig. 5. Effect of bovine serum albumin (BSA) on PMA-induced inhibition of [^{3}H]dopamine uptake. Cells were pre-treated with PMA or vehicle in the absence or presence of BSA at the concentrations indicated at 21°C for 15 min prior to an 8-min exposure to [^{3}H]dopamine (11 nM). Numbers under bars denote the micromolar (PMA) or mg/ml (BSA) concentrations of each agent present. Results are mean ± S.E. (vertical bar) for 3-4 experiments, each in triplicate, with the same cell preparation. $^{a-e}P < 0.05$ for comparisons between groups; groups labeled with different letters are statistically not from the same population, whereas for groups sharing a letter the null hypothesis (that they are not different) is not rejected (one-way analysis of variance followed by the least significant difference multiple range test).

arachidonic acid into lipids (see above), inhibited both uptake and binding with an IC_{50} value in the sub-millimolar range with Hill slopes closer to unity (Fig. 3B). In another attempt to raise endogenous arachidonic acid, C6 transporter-containing cells were pre-incubated for 15 min with varying concentrations of nordihydroguaiaretic acid, a lipooxygenase inhibitor, and both [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding were inhibited with IC_{50} values close to 1 μM and Hill numbers close to unity (Fig. 3C).

Under identical assay conditions, the IC_{50} values for inhibition of [^{3}H]dopamine uptake were greater than those for inhibition of [^{3}H]WIN 35,428 binding in the case of melittin ($P < 0.0001$) and thimerosal ($P < 0.001$).

3.3. Trapping arachidonic acid by bovine serum albumin

The co-presence of bovine serum albumin (0.1 and 1 mg/ml) counteracted the melittin (0.2 μM for 15 min)-induced inhibition of [^{3}H]dopamine uptake (Fig. 4, top panel). Similarly, bovine serum albumin at a low concentration (0.01 mg/ml) attenuated the effect of exogenously added arachidonic acid (200 μM for 1 h) on [^{3}H]dopamine uptake ($P < 0.05$) whereas higher concentrations (0.1 and 1 mg/ml) fully restored control uptake levels (Fig. 4, bottom panel). In both sets of experiments, the highest concentration of bovine serum albumin tested (1 mg/ml) caused, by itself, a slight increase in [^{3}H]dopamine uptake ($P < 0.05$).

The reduction in uptake caused by the phorbol ester PMA (5 μM for 15 min) was not attenuated by the co-presence of bovine serum albumin (0.1–2 mg/ml) (Fig. 5). In fact, bovine serum albumin (1–2 mg/ml) appeared to slightly enhance the effect of staurosporine ($P < 0.05$).

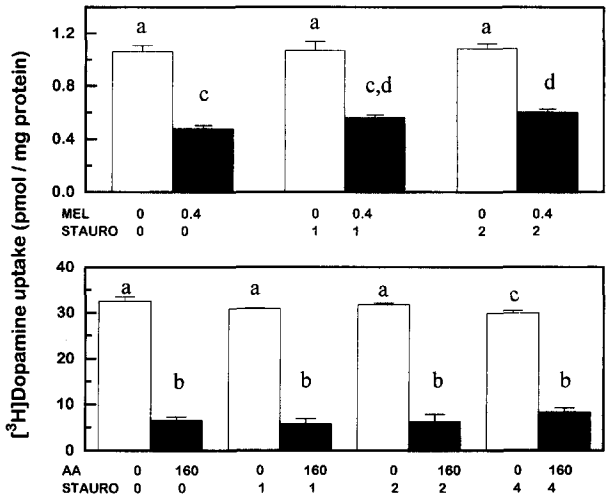

Fig. 7. Effect of staurosporine (STAURO) on melittin (MEL) (top panel)- or arachidonic acid (AA) (bottom panel)-induced inhibition of [^{3}H]dopamine uptake. Cells were pre-treated with MEL/AA or vehicle in the absence or presence of STAURO at the concentrations indicated at 21°C for 15 min prior to an 8-min exposure to [^{3}H]dopamine (11 nM in top panel and 111 nM in bottom panel). Numbers under bars denote the micromolar concentrations of each agent present. Results are mean ± S.E. (vertical bar) for 3-4 experiments, each in triplicate, with the same cell preparation. $^{a-d}P < 0.05$ for comparisons between groups; groups labeled with different letters are statistically not from the same population, whereas for groups sharing a letter the null hypothesis (that they are not different) is not rejected (one-way analysis of variance followed by the least significant difference multiple range test).

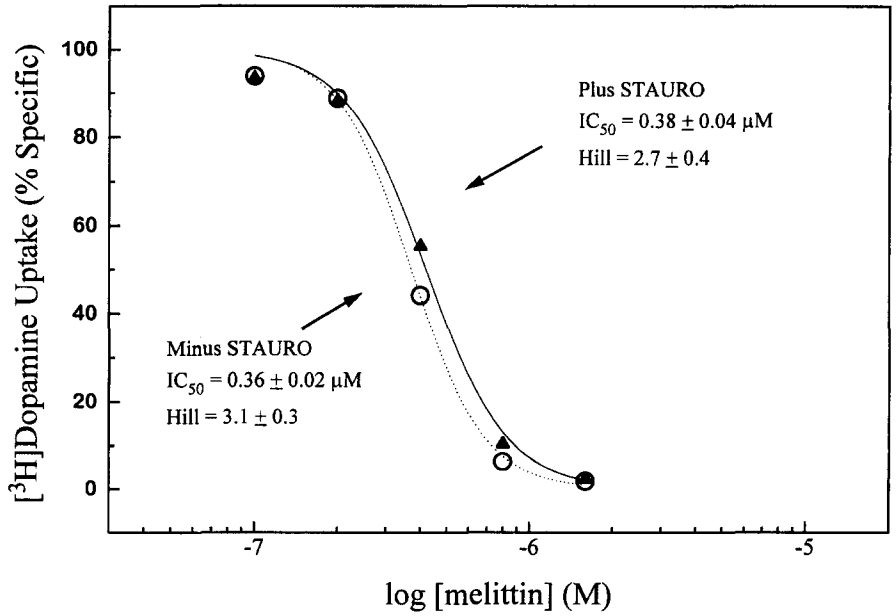

Fig. 6. Concentration-dependent inhibition of [^{3}H]dopamine uptake by melittin in the presence (▲ ——— ▲) or absence (○ · · · ○) of staurosporine (STAURO) (0.3 μM). Cells were pre-incubated with various concentrations of the compound for 15 min at 21°C prior to an 8-min exposure to [^{3}H]dopamine (11 nM). Points shown are those obtained in a single experiment, assayed in triplicate, that was carried out 4 times. The insets show the mean ± S.E. of these results. The mean control uptake in the absence of inhibitor was 1.7 pmol/mg of protein. There were no statistically significant differences in IC_{50} values and Hill numbers between the presence and absence of STAURO.

3.4. Blocking protein kinase C by staurosporine

Staurosporine (0.3 μM) did not shift the curve of melittin (0.1–1.8 μM for 15 min) for inhibition of [^{3}H]dopamine uptake (Fig. 6). Again, the Hill numbers for melittin inhibition were appreciably higher than unity ($P \leq 0.02$). A higher concentration of staurosporine (1 μM) still did not affect the melittin (0.4 μM)-induced inhibition of uptake, whereas a marginal effect was noted with 2 μM staurosporine ($P < 0.05$) (Fig. 7, top panel). Staurosporine (1–4 μM) had no effect at all on the inhibition of uptake caused by exogenously added arachidonic acid (160 μM for 1 h) (Fig. 7, bottom panel).

4. Discussion

4.1. Methodological considerations

The time course of [^{3}H]dopamine uptake under the present conditions was essentially linear for the first 8 min as reported in our previous study (Reith et al., 1996). Other support for the existence of initial velocity conditions during that time frame comes from our finding that inhibitor potencies were identical with [^{3}H]dopamine accumulation times of 3, 6 or 8 min; in addition, the K_m and B_{max} were the same in 3- and 8-min uptake assays with the present cell preparations (Reith et al., 1996). These observations make it also less likely that reversed transport of [^{3}H]dopamine plays an important role in the current uptake assay, because this penomenon is expected to increase with time as more intracellular [^{3}H]dopamine becomes available for back-transport, causing deviation from linearity at longer times. Furthermore, reversed transport is expected to play a greater role at high than low medium concentrations of dopamine shifting the K_m to a lower value which was not observed in experiments comparing 3- and 8-min uptake times. Under conditions similar to the present ones, [^{3}H]dopamine has been reported to be stable during 5-min incubations of brain synaptosomes in the presence of a monoamine oxidase inhibitor and ascorbate (Snyder and Coyle, 1969). The presence of ascorbate did not affect the present results (data not shown), and one would again expect that longer exposure times would cause more metabolism of [^{3}H]dopamine causing different kinetic constants at 3 and 8 min, which was not observed. All data taken together suggest that the current assay measures initial velocity of [^{3}H]dopamine uptake without the confounding occurrence of reverse transport or substrate metabolism.

4.2. Do similar inhibitory effects of arachidonic acid and protein kinase C activators represent a common mechanism?

There are similarities between the present effects of arachidonic acid and those previously observed for PMA (Zhang et al., 1996). In both cases, the inhibition of [^{3}H]dopamine uptake is due to a decrease in the V_{max}, counteracted somewhat by an increase in K_m, whereas the inhibition of [^{3}H]WIN 35,428 binding is due entirely to a reduction in B_{max}. In addition, arachidonic acid and protein kinase C activators display, in general, a smaller IC_{50} value for inhibiting [^{3}H]dopamine uptake than in inhibiting [^{3}H]WIN 35,428 binding. Because arachidonic acid is known in many systems to activate protein kinase C (Asaoka et al., 1992; Nishizuka, 1995), the data appear to be consonant with an endpoint involving protein kinase C-mediated phosphorylation. However, staurosporine, a potent inhibitor of protein kinase C, did not counteract the inhibitory effect of added arachidonic acid or melittin which is expected to raise endogenous arachidonic acid. Therefore, in the current C6 glioma expression system, the arachidonic acid effect is not mediated by protein kinase C. Is it possible, then, to postulate that in both the present experiments with arachidonic acid and our previous study with protein kinase C activators, the endpoint is the production of arachidonic acid, which has been reported to enhance protein kinase C activity in some systems (Asaoka et al., 1992; Nishizuka, 1995)? Evidence against this comes from the present observation that the inhibitory effect of PMA on [^{3}H]dopamine uptake is not at all affected by the presence of bovine serum albumin which is known to trap arachidonic acid. In contrast, the same treatment with bovine serum albumin did counteract the inhibitory effect of arachidonic acid and that of melittin. The data taken together suggest that the inhibitory effects of arachidonic acid activators and those of protein kinase C activators on dopamine uptake are mediated by separate mechanisms.

The effect of bovine serum albumin at 1 mg/ml in slightly enhancing [^{3}H]dopamine uptake by itself is reminiscent of the effect reported for the same concentration of bovine serum albumin on glutamate uptake into both synaptosomes and astrocytes from rat cerebral cortex (Volterra et al., 1992). It may indicate that dopamine uptake rate in vivo is under regulatory control by arachidonic acid.

4.3. What mechanisms could be involved in the effect of adding exogenous arachidonic acid and raising endogenous arachidonic acid?

Previous discussions have addressed the pitfalls in comparing the concentrations of added arachidonic acid with those generated by activation of endogenous arachidonic acid (Cass et al., 1991; Volterra et al., 1992). Although earlier work emphasized the importance of penetration of lipophilic arachonic acid into the membrane, altering the lipidic environment of the transporter studied (see below) (Yu et al., 1986; Zafra et al., 1990; Volterra et al., 1992), a recent study by Trotti et al. (1995) provides evidence for an action at the membrane protein or the protein-lipid boundary by arachidonic acid present in the water phase in

the case of the glutamate transporter. The present experiments do not provide evidence to distinguish the effect of membrane- vs. water phase-located arachidonic acid, but the fact that high concentrations of exogenous arachidonic acid are needed for transport effect in the current study are consonant with the suggested existence of binding sites in the membrane that act like a sink for arachidonic acid, making it unavailable for interaction with the transporter (Trotti et al., 1995).

If arachidonic acid inhibits dopamine transport by acting from the water phase, what is the underlying mechanism? The fact that the inhibitory effect on [^{3}H]dopamine uptake and [^{3}H]WIN 35,428 binding is based on a V_{max} or B_{max} reduction rather than a K_m or K_d increase indicates that arachidonic acid is not a simple competitive inhibitor. Although it could be thought that the longer time needed for the development of the uptake inhibitory effect indicates a more complex mechanism, the binding inhibitory effect developed more readily. It is possible that the stimulatory effect observed on uptake at short times (see below) overshadows the inhibitory effect, and that only at later times, when the stimulatory effect has dissipated, one can observe the inhibition of [^{3}H]dopamine uptake. The latter effect is either a direct effect on the dopamine transporter or an indirect activity involving other features needed for transport, such as enzyme pumps for maintaining ion gradients, correct polarization of the membrane, or integrity of membrane environment embedding the transporter. The present experiments do not directly address these possibilities, but the observed changes in [^{3}H]WIN 35,428 binding concurrent with those in [^{3}H]dopamine uptake (at longer times and higher arachidonic acid concentrations) argue in favor of an action at the dopamine transporter itself as far as the inhibitory effect is concerned. A plasma membrane vesicle system containing the dopamine transporter will be helpful in addressing these issues because in such a system there is no need for enzyme activities to maintain ion gradients.

The reduction in both the V_{max} of [^{3}H]dopamine uptake and the B_{max} of [^{3}H]WIN 35,428 binding could be thought to result from a decrease in expression of dopamine transporter protein by the C6 glioma cells. However, this possibility becomes less likely if one considers that the reported half-life for the dopamine transporter in the porcine kidney epithelial cell line LLC-PK$_1$ is 23 h (Mantel et al., 1996), which is appreciably longer than the time scale of $\approx$ 1 h (for the inhibitory effect on uptake) or less (binding) in the present experiments. A related possibility is the occurrence of subcellular redistribution, reported to occur in the case of the GABA transporter (GAT1 subtype) (Corey et al., 1994). Again, however, the time scale of the current experiments is much shorter than that (days) described for redistribution (Corey et al., 1994) unless one postulates the existence of exo- and endocytotic-like mechanisms for transporter relocation as shown for the glucose transporter in response to insulin (Simpson and Cushman, 1986). To our knowledge, no such evidence exists for the dopamine transporter.

In the present experiments aimed at raising endogenous arachidonic acid, the possibility should be considered that the agents used inhibit, by themselves, the dopamine transporter, unrelated to their effects on arachidonic acid metabolism. In fact, for one of the compounds used here, nordihydroguaiaretic acid, it has been suggested that dopamine transporter inhibition can occur with concentrations in the micromolar range (Cass et al., 1991). This is only slightly higher than the potency for inhibition observed in the present study (0.8–0.9 μM), and it is, therefore, possible that the underlying mechanism involved dopamine transporter inhibition by nordihydroguaiaretic acid itself. However, the effect of melittin in the present study is not likely to be mediated by direct transporter inhibition, because it could be counteracted by bovine serum albumin, which traps arachidonic acid, at a concentration that was without effect by itself (0.1 mg/ml). Thimerosal, another compound used in this study, is closely related to aspirin, which has been shown to be extremely weak ($IC_{50} > 1$ mM) in inhibiting the dopamine transporter (Cass et al., 1991).

4.4. How does the effect of arachidonic acid on transport of dopamine compare with that on transport of other neurotransmitters?

In general, arachidonic acid-induced decreases in neurotransmitter transport have been reported, such as for glutamate (Volterra et al., 1992, 1994; Trotti et al., 1995), GABA (Yu et al., 1986), and glycine (Zafra et al., 1990). L'hirondel et al. (1995) describe inhibition of dopamine uptake by rat striatal synaptosomes by concentrations of arachidonic acid (30–100 μM) that also stimulate dopamine release. Interestingly, the two effects are sepate because the release effect is also observed in the presence of dopamine uptake block by nomifensin (L'hirondel et al., 1995). The latter effect appears to be mediated by protein kinase C, whereas the mechanism of the dopamine uptake effect has not been further investigated by the group of Glowinski (L'hirondel et al., 1995). To our knowledge, there is only one other example of a stimulatory effect of arachidonic acid on transport, reported by Zerangue et al. (1995) for *Xenopus* oocytes expressing one of the human brain glutamate transporter subtyptes (human excitatory amino-acid transporter 2, EAAT2). As opposed to the other subtype, EAAT1, which displays the commonly observed decrease in glutamate uptake, EAAT2 takes up substrate with a higher affinity in the presence of arachidonic acid (Zerangue et al., 1995). So far, no subtypes for the dopamine transporter have been described (Amara and Kuhar, 1993), and it is unlikely that the stimulatory effect we observe in the present study is associated with one subtype and the inhibitory effect with another subtype. Further characterization of this stimulatory effect is neces-

sary to decide whether it involves a direct action at the dopamine transporter or an indirect activity, such as an effect on membrane integrity or ion pumping. It is possible that arachidonic acid metabolites activate ion channels causing membrane hyperpolarization, as suggested for receptor-linked K^+ channels in *Aplysia* (Buttner et al., 1989), and it is known that dopamine transport is electrogenic, being stimulated by membrane potential (negative inside) (Rudnick, 1996).

In the case of glutamate transmission, it has been speculated that arachidonic acid is released upon synaptic activation of glutamate receptors. In dopamine terminal areas in the brain, glutamate released from glutamate afferents can act on glutamate receptors located on GABA cells to release arachidonic acid that in turn can act on dopamine terminals (see L'hirondel et al., 1995). The resulting dopamine releasing activity, presumably mediated by protein kinase C (L'hirondel et al., 1995), in combination with both stimulatory and inhibitory effects of arachidonic acid on dopamine uptake (present work), would make complex regulation possible with a role for transmitters that act on G-protein-coupled receptors altering phospholipase A_2 activity.

4.5. Concluding remarks

The present experiments focused on the inhibitory effects on dopamine uptake observed by raising endogenous arachidonic acid or applying high exogenous concentrations of arachidonic acid in C6 glioma cells expressing the human dopamine transporter. Whereas staurosporine had little or no effect on the melittin- or arachidonic acid-induced inhibition of [^{3}H]dopamine uptake, these latter inhibitions were counteracted by bovine serum albumin, in contrast to the lack of effect of albumin on the inhibition of dopamine uptake induced by PMA. The data taken together suggest that the inhibitory effects of arachidonic acid activators and those of protein kinase C activators on dopamine uptake are mediated by separate mechanisms.

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