

Protein Kinase A Activity May Kinetically Upregulate the Striatal Transporter for Dopamine

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The neuronal dopamine transporter (DAT) plays a key role in terminating dopaminergic chemical neurotransmission; thus, the study of the regulation of DAT activity is important in defining parameters relevant to the control of dopaminergic neurotransmission. Interpretation of the results from previous work of this laboratory suggests that occupation of presynaptic autoreceptors increases DAT activity. Second messenger signaling related to kinetic upregulation of DAT has not been examined previously. However, others have shown that protein kinase C activity may downregulate DAT activity, whereas protein kinase A has shown variable results. Herein it is shown that protein kinase A activity mediates the kinetic upregulation of DAT. Quinpirole increased DAT activity that was blocked by sulpiride and the protein kinase A selective inhibitor H-89. Brief

incubations with forskolin and 8-bromo-cAMP (8-Br-cAMP) were found to stimulate striatal DAT activity by increasing the V_{max} of transport without affecting the K_m . Exposures >15 min had no effect. The 8-Br-cAMP-stimulated increases in DAT activity were blocked by pre-exposure to H-89. Thus, second messenger signaling via the cAMP cascade may mediate kinetic upregulation of DAT. Kinetic analyses of the results suggest that either insertion of DAT into the membrane or activation of pre-existing DAT within the membrane mediates the regulation.

Key words: dopamine transporter; protein kinase A; rotating disk electrode voltammetry; striatum; 8-Br-cAMP; forskolin; quinpirole; sulpiride; H-89; H-7; H-9

It has been suggested in recent reports that the activity of the transporter for dopamine (DAT) may be regulated (for review, see Reith et al., 1997). Various stimuli have been shown to produce changes in the kinetic activity of DAT including: increases in DAT activity after receptor occupation of the presynaptic receptors of the D₂ type (Meiergerd et al., 1993; Cass and Gerhardt, 1994); persistent kinetic changes after withdrawal from treatments with cocaine (Yi and Johnson, 1990; Meiergerd et al., 1994a,b; Meiergerd et al., 1997); and decreases and increases in DAT activity by a variety of compounds involved in second messenger signaling (Pierce and Kalivas, 1997; Reith et al., 1997; Zhu et al., 1997). In addition, high and low responding animals in a behavioral model exhibit differences in DAT activity (Hooks et al., 1994). The cloned rat and human DATs possess consensus sites for phosphorylation by cAMP-dependent protein kinases and by protein kinase C in the N- and C-terminal domains and third extracellular loop (Giros and Caron, 1993). Most studies of the effects of second messenger systems on DAT kinetic activity have focused on the effects of protein kinase C activity on DAT activity by studying the intracellular accumulation of [³H]dopamine in cell culture systems expressing DAT and in synaptosomes (Huff et al., 1997; Vaughn et al., 1997; Zhang et al., 1997; Zhu et al., 1997). The results suggest that stimulation of protein kinase C

downregulates the kinetic activity of DAT by decreasing the V_{max} of accumulation. Others have reported that stimulation of protein kinase C activity increases DAT-mediated release of dopamine (see Giambalvo, 1992; Kantor and Gnegy, 1998 and references therein). A couple of reports suggesting that calcium-calmodulin-dependent protein kinases may upregulate DAT have appeared recently (Uchikawa et al., 1995; Pierce and Kalivas, 1997), and an earlier report has shown that the cAMP-dependent protein kinase, protein kinase A, upregulates [³H]dopamine accumulation by DAT (Kadawaki et al., 1990). Other investigations report that this protein kinase failed to affect DAT activity (Tian et al., 1994; Copeland et al., 1996; Zhu et al., 1997). Because DAT can be upregulated by a variety of stimuli, and others have shown kinetic upregulation of DAT by protein kinase A, we examine this issue further. Herein we describe results of studies of the effects of protein kinase A on the kinetically resolved inwardly directed activity of striatal DAT as measured by rotating disk electrode voltammetry. Because this approach monitors the clearance of extracellular concentrations of dopamine ($[DA]_o$), it provides a window for observing what presynaptic and postsynaptic receptors may “see” after alterations in DAT kinetic activity and a much shorter window of observation of the kinetic activity of DAT than traditional assays with [³H]dopamine. As will be shown, it was found that protein kinase A activity transiently upregulates DAT activity. Kinetic analyses suggest that insertion of DAT into the neuronal membrane or activation of previously inactive DAT within the membrane likely mediates the kinetic upregulation.

MATERIALS AND METHODS

Rotating disk electrode (RDE) voltammetric monitoring of the velocity of the transporter for dopamine into striatal suspensions in 500 μ l of physiological buffer were conducted as previously described (Meiergerd

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and Schenk, 1995; Earles et al., 1998). Tissue suspensions were prepared from striata obtained from rapid decapitation of male, 275–325 gm, Sprague Dawley rats. Rats were not pretreated with any drug before experimentation, and the animal protocols used were reviewed and approved by the University Animal Care and Use Committee. The potentiostat was a Bioanalytical Systems Inc. (West Lafayette, IN) model LC3D (Petite Ampère) with a time constant (5RC) changed (details available on request) to 200 msec. The RDE controller and glassy carbon electrode were purchased from Pine Instruments Inc. (Grove City, PA), and the electroanalytical parameters used were: $E_{app} = +450$ mV versus Ag–AgCl and a rotation rate of 2000 rpm with current outputs fed to a Nicolet (Madison, WI) model 2090 digital oscilloscope.

The initial velocities of transport (v) were defined and measured as previously described (Meiergerd and Schenk, 1995), and values of K_m and V_{max} were estimated by fitting experimentally observed values of v versus $[DA]_o$ to the Michaelis–Menten expression using commercially available nonlinear curve-fitting software (GraphPad Prism, San Diego, CA). Indicators of the precision of the resulting kinetic parameters are standard errors of regression (SER), and statistical comparisons of the results were made using a z test (Havlicek and Crain, 1988). More detailed analyses of changes observed in the kinetic parameters were based on treatments by Fersht (1985) and Segel (1993).

All concentrations reported are final molarities in the incubation chamber. Inhibition studies were conducted by pre-exposing the tissue to an inhibitor dissolved in physiological buffer at a concentration 10-fold higher than its IC_{50} . Exposure times were between 12 and 15 min. In experiments with 8-bromo-cAMP (8-Br-cAMP), 8-Br-cAMP (50 μ M) was added 1 min before dopamine. Forskolin and 1,9-dideoxyforskolin experiments were performed at 50 μ M. Stock concentrations of these agents were made in dimethylsulfoxide. The quinpirole experiments were conducted at 0.1 μ M quinpirole by adding it 30 sec before dopamine, as described previously (Meiergerd et al., 1993). Sulpiride was added 5 min before dopamine.

Chemicals, solutions, and drugs. All solutions used in these studies were made in university-supplied deionized water that was purified further with a Nanopure (Barnstead, Dubuque, IA) water purification system. The common buffer salts were purchased from Baker Chemical Co. (Philipsburg, NJ). Forskolin, 1,9-dideoxyforskolin, dopamine (DA), 8-Br-cAMP, quinpirole (QUIN), sulpiride (SULP), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine HCl (H-7), and N -[2-(methylamino)ethyl]-5-isoquinolinesulfonamide HCl (H-9) were purchased from Research Biochemicals (Natick, MA). Bisindolylmaleimides and N -[2-(*p*-bromocinnamyl)amino]ethyl]-5-isoquinolinesulfonamide HCl (H-89) were purchased from Calbiochem-Novabiochem (La Jolla, CA). A bicarbonate-based physiological buffer at 37°C and pH 7.4 was used in these experiments (composition in mM: 124 NaCl, 1.80 KCl, 1.24 KH_2PO_4 , 1.30 $MgSO_4$, 2.50 $CaCl_2 \cdot 2H_2O$, 26.0 $NaHCO_3$, and 10.0 glucose).

RESULTS

Figure 1 shows that 100 nM quinpirole increases the inwardly directed transport of dopamine in striatal suspensions and that the increased velocity of transport is blocked by 5 μ M sulpiride and pre-exposure to the nonselective protein kinase inhibitor H-9. H-89, a selective inhibitor of protein kinase A, at 500 nM, also blocked the effect of quinpirole. Thus, the effect of the cAMP pathway on DAT activity was examined in more detail by measuring the kinetic parameters of dopamine transport under different treatment conditions. The control values of K_m and V_{max} were found to be close to those reported previously for this type of transport experiment (Meiergerd and Schenk, 1994; Wayment et al., 1998). The numerical results for the control studies and other analyses estimated from data obtained under the other treatment conditions along with the values of SER are listed in Table 1. In preliminary studies ($n \geq 5$) to set conditions for these experiments it was found that forskolin (at 50 μ M) increased DAT activity at 4 μ M dopamine from 793 ± 25 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$ in controls to 903 ± 72 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$ after a 1 min incubation, whereas at a longer incubation time (15 min as used by others in the study of protein kinase C effects, *vide ante*) the transport value was lower at 786 ± 110 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$. Values ob-

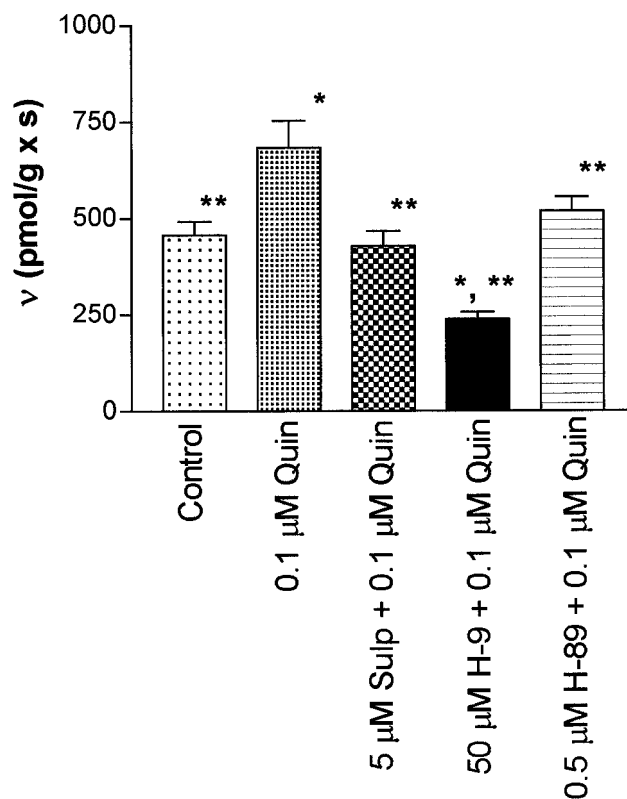


Figure 1. Upregulation of DAT activity by QUIN is blocked by SULP and protein kinase inhibitors. The transport velocity of an instantaneous pulse of 1.0 μ M dopamine was measured. The kinetic upregulation of striatal DAT activity by the dopamine agonist QUIN (684 ± 70 ; $n = 5$) is reversed by the dopamine antagonist SULP (428 ± 38 ; $n = 6$), the nonselective protein kinase inhibitor H-9 (238 ± 18), and the selective protein kinase A inhibitor H-89 (519 ± 36 ; $n = 15$). The bars represent values of SEM. The single asterisks indicate a difference from the control at $p \leq 0.01$, and the double asterisks indicate a difference from QUIN at $p \leq 0.01$ via a z test. Each n value represents a single experiment conducted with a single striatum.

served at 12 min of incubation were intermediate in value at 714 ± 99 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$. Thus, the greatest numerical difference (the largest increased observed velocity) with the best precision was observed at the 1 min incubation time, and this condition was used throughout the remainder of the study. Figure 2 shows that forskolin, when compared with results obtained with the inactive forskolin derivative 1,9-dideoxyforskolin increases the inwardly directed transport of dopamine by increasing the V_{max} of transport. No statistically significant change in the K_m of the transport of dopamine was observed, and the V_{max}/K_m quotient was not different from controls (Table 1).

In preliminary experiments ($n \geq 3$) to set conditions for studies with 50 μ M 8-Br-cAMP, it was found that DAT activity at 1.0 μ M dopamine was increased to 579 ± 41 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$ from the control value of 457 ± 35 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$. However, when incubations with 8-Br-cAMP were conducted for 12 min before adding dopamine, the observed velocity, 469 ± 27 pmol \cdot sec $^{-1} \cdot$ gm $^{-1}$, was found to be statistically indistinguishable from the control value. Thus, in subsequent experimentation 8-Br-cAMP was added 1 min before dopamine. Figure 3 illustrates that 8-Br-cAMP kinetically upregulates the striatal transport of dopamine by increasing the value of V_{max} . No change in the value of K_m or the V_{max}/K_m quotient was observed (Table 1).

Table 1. Effects of agents modifying the activity of protein kinases on kinetic parameters of the inwardly directed transport of dopamine into striatal suspensions

Treatment ^a	V_{\max} ($\text{pmol} \cdot \text{gm}^{-1} \cdot \text{sec}^{-1}$)	K_m (μM)	V_{\max}/K_m
Control	976 ± 67	0.98 ± 0.18	996 ± 195
Forskolin	1152 ± 92*	1.10 ± 0.23	1047 ± 234
1,9-Dideoxyforskolin	927 ± 54	0.82 ± 0.13	1130 ± 190
8-Br-cAMP	1294 ± 83**	1.30 ± 0.20	995 ± 165
8-Br-cAMP + H-7	672 ± 60**	2.04 ± 0.38**	329 ± 68**
8-Br-cAMP + H-9	742 ± 91**	2.20 ± 0.55**	337 ± 93**
8-Br cAMP + H-89	1072 ± 53	1.31 ± 0.16	818 ± 108
H-7	662 ± 82**	1.58 ± 0.47	419 ± 135**
H-9	703 ± 46**	1.56 ± 0.23	450 ± 72**
H-89	777 ± 57**	0.89 ± 0.18	873 ± 188

*Different from its control, 1,9 dideoxyforskolin, at the 99% confidence level via a z test.

**Difference from control at the $\geq 97\%$ confidence level via a z test.

^aAll agents were used at 50 μM except H-89, which was used at a protein kinase A-selective concentration of 500 nM.

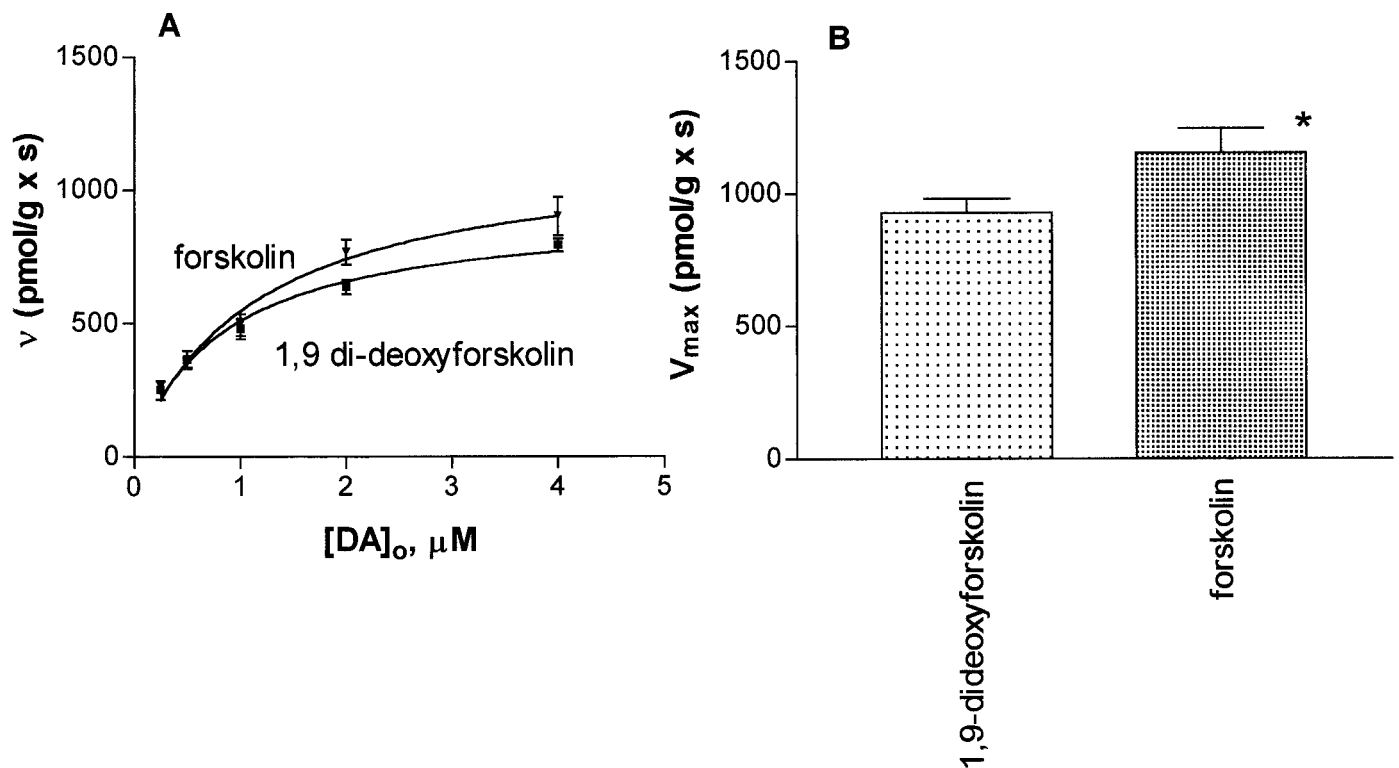


Figure 2. The striatal transporter for dopamine is kinetically upregulated by forskolin by increasing the V_{\max} of transport. *A* shows the Michaelis-Menten curves observed in the absence (1,9-di-deoxyforskolin) and presence of forskolin. *B* compares the value of V_{\max} in the presence of forskolin ($1152 \pm 92 \text{ pmol} \cdot \text{sec}^{-1} \cdot \text{gm}^{-1}$) to that of 1,9-dideoxyforskolin ($927 \pm 54 \text{ pmol} \cdot \text{sec}^{-1} \cdot \text{gm}^{-1}$). The asterisk represents a difference from the control at $p \leq 0.02$ via a z test. Each point represents n values of 9–23. Each n value represents a single experiment conducted with a single striatum.

H-89 was also found to reverse the upregulation mediated by 8-Br-cAMP. Furthermore, it was found (Fig. 3) that H-89 alone reduced the velocity of the striatal transport of dopamine accompanied by a decrease in V_{\max} with no change in K_m (Table 1). When H-89 was used in the presence of 8-Br-cAMP, the reduction in the kinetic parameters was reversed and returned to control values. In contrast to the results with the protein kinase A inhibitor, Figure 4 shows that bisindolylmaleimide I, an inhibitor of protein kinase C, increased the transport velocity of the striatal transport of dopamine. In these experiments a close to saturating [dopamine] was used to provide results reflecting how the V_{\max} of

DAT activity may have changed. The nonselective protein kinase inhibitors H-7 and H-9 reduced the values of V_{\max} with no effect on K_m and the ratio, V_{\max}/K_m was reduced relative to controls (Table 1).

DISCUSSION

The findings of this study are that kinetic upregulation of DAT results from activation of protein kinase A. Quinpirole, as in previous studies (Meiergerd et al., 1993; Cass and Gerhardt, 1994), was found to kinetically upregulate the transport of dopamine. The upregulation could be blocked by sulpiride and the

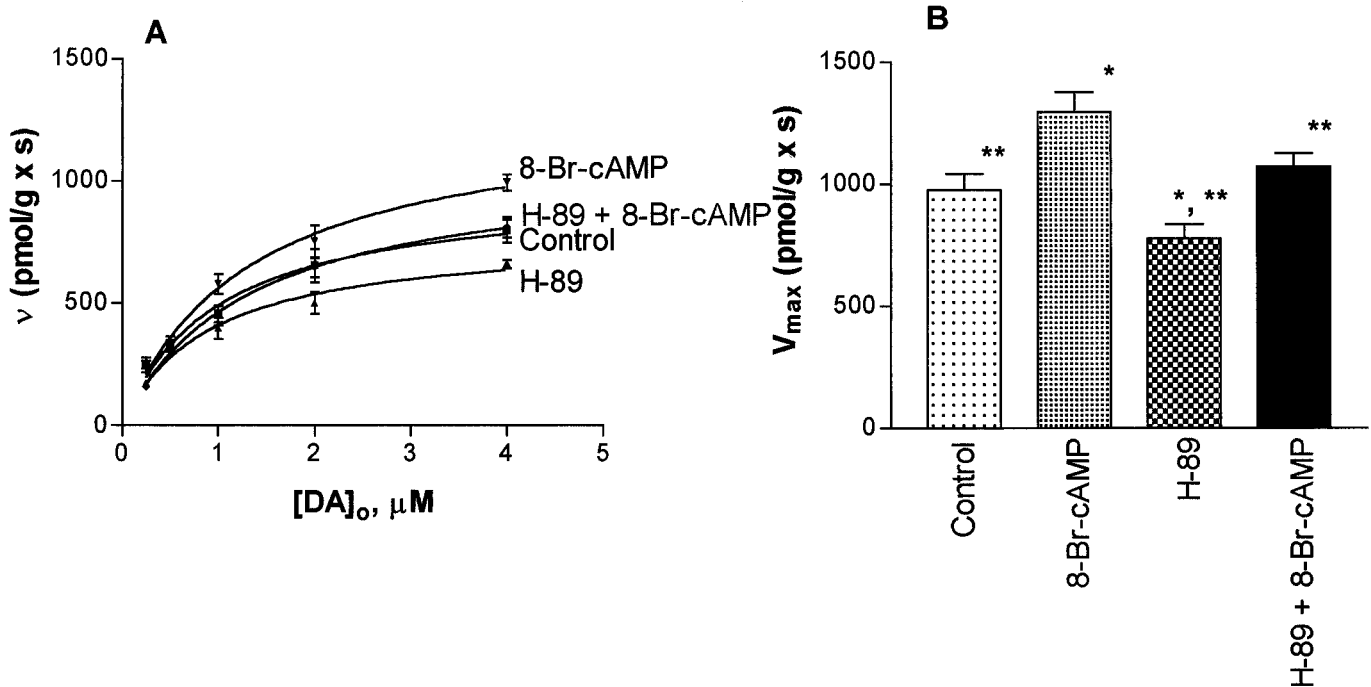


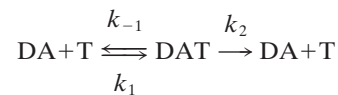
Figure 3. Protein kinase A mediates a kinetic upregulation of DAT. *A* shows Michaelis–Menten curves obtained in the presence of 8-Br-cAMP alone and in the presence of 8-Br-cAMP and H-89. Each point represents *n* values of 10–20. Each *n* value represents a single experiment conducted with a single striatum. *B* shows the effects of protein kinase A on the V_{max} of transport. The experimental conditions and numerical results for the estimated $V_{max} \pm \text{SER}$ are (in $\text{pmol} \cdot \text{sec}^{-1} \cdot \text{gm}^{-1}$ wet weight): control, 976 ± 67 ; 8-Br-cAMP, (50 μM , 1 min before DA) 1294 ± 83 ; H-89 (0.5 μM , exposure ~ 15 min before DA) plus 8-Br-cAMP (50 μM , 1 min before DA), 1072 ± 53 ; and H-89, (0.5 μM , exposure ~ 15 min before DA) 777 ± 57 . The error bars represent the SER, and the single asterisks indicate differences from controls at $p \leq 0.02$ via a *z* test. The double asterisks represent differences from 8-Br-cAMP at $p \leq 0.02$ also via a *z* test.

nonselective protein kinase inhibitor H-9, as well as by the protein kinase A inhibitor H-89. Further work showed that activation of adenylate cyclase with forskolin or the addition of the membrane-soluble cAMP analog 8-Br-cAMP also produced an H-89-sensitive kinetic upregulation of the striatal transport of dopamine. H-89 alone was found to kinetically downregulate the transport of dopamine, however, this effect could be modulated by addition of 8-Br-cAMP. Taken together, these results suggest that a cAMP-dependent protein kinase, protein kinase A, functions to upregulate the striatal transporter for dopamine. The lack of effect on the ratio, V_{max}/K_m , is taken as evidence that the kinetic upregulation is not the result of a change in the kinetic state of DAT. However, this conclusion may be a result of propagation of error in the analyses. The nonselective inhibitors of protein kinases H-7 and H-9 reduced V_{max} and the V_{max}/K_m ratio, suggesting that the downregulation observed was accompanied by a change in the state DAT. When protein kinase C activity was inhibited selectively the transport activity of DAT was found to increase, a finding consistent with those previously reported for the effects of altering protein kinase C activity on DAT activity (vide ante) in which activation of protein kinase C resulted in a reduction of V_{max} .

Comments on the changes in DAT mediated by protein kinase activity

An analysis of the observed kinetic results can suggest what changes in interactions between dopamine and DAT occur as result of the activity of protein kinases. The possibilities for a change in kinetic activity include changes in K_m , V_{max} , or both. The results obtained here with protein kinase A indicate that only

V_{max} is changed by the activity of the kinase. The significance of this change can be examined in more detail. In treatments of Michaelis–Menten kinetics the catalytic activity is depicted by the reaction sequence:



where T signifies the transporter, DA signifies dopamine, DAT signifies the dopamine-occupied transporter, k_1 is the second order rate constant of association of DA with T, k_{-1} is the first order dissociation rate constant of DA from DAT, and k_2 is the first order rate constant of the catalytic cycle of the transport of dopamine across the membrane. In the classical kinetic treatment of this model the interaction of DA with T is considered to be rapid and reversible, and the transport event occurs as a second step signified by k_2 (Fersht, 1985). The well known steady state analysis yields the Michaelis–Menten expression where $K_m = [\text{DA}][\text{T}]/[\text{DAT}] = k_{-1}/k_1$, the dissociation constant of DA from DAT (K_{DA}) and $V_{max} = k_2 \times$ the concentration (or density) of the transporter. In the case in which $k_2 \gg k_{-1}$ (the Briggs–Haldane kinetic condition) the $K_m = K_{\text{DA}} + k_2/k_1$, the V_{max} has the same significance as before, and the rate constants of association of DA with T should be in the 10^7 – $10^8 \text{ sec}^{-1} \text{ M}^{-1}$ range (Fersht, 1985). Results of previous work by this laboratory (Meiergerd and Schenk, 1994; Meiergerd et al., 1994b) suggest that the kinetics of striatal DAT follows the Briggs–Haldane model (Fersht, 1985; Segel, 1993) because the second order rate constant of association of dopamine with DAT was in the range

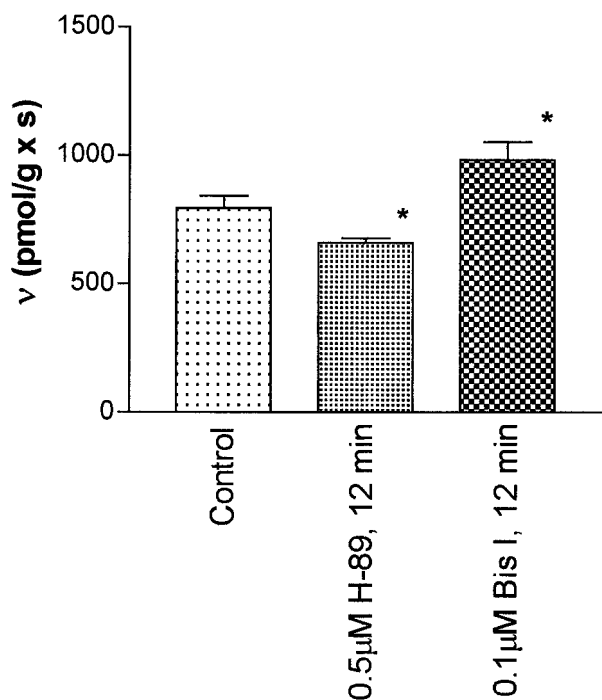


Figure 4. Protein kinase C and protein kinase A exhibit opposing effects on the velocity of DAT. Bisindolylmaleimide I (exposure ≥ 15 min), a protein kinase C inhibitor, produced an increase in DAT activity. The results with H-89, a selective protein kinase A inhibitor, were statistically lower than the controls under similar experimental conditions. Each experiment was conducted by measuring the transport velocity of an instantaneous pulse of 4.0 μM dopamine, to estimate an effect on the value of V_{max} . The numerical values (in $\text{pmol} \cdot \text{sec}^{-1} \cdot \text{gm}^{-1}$ wet weight) were: control, 796 ± 47 , $n = 23$; bisindolylmaleimide I, 983 ± 70 , $n = 7$; and H-89, 661 ± 17 , $n = 5$. The bars represent values of SER. The single asterisks indicate a difference from the controls at $p \leq 0.03$ via a z test. The n values represent a single experiment conducted with a single striatum.

indicated above for the Briggs–Haldane condition. Indeed, similar conclusions from a different line of argument have been made by Prof. Reith's laboratory (Zimanyi et al., 1989). Thus, if the kinetics of transport are changed, but no change in the dissociation constant occurs, then k_2 should be the only parameter changed, and both the values of K_m and V_{max} should be altered by the change in kinetic state. Alternatively, if the density of DAT is altered by a regulatory event but not the dissociation constant for dopamine, then a change in V_{max} would be the result with no change in the K_m . Finally, a change in K_{DA} as an effect would be reflected by a change in K_m only. Thus, in examining the values of the kinetic parameters in Table 1, it can be hypothesized with reasonable kinetic support that upregulation of DAT activity by forskolin and 8-Br-cAMP may be mediated by a change in the density of active DAT because no change in K_m was observed. In one model in an expression system it has been shown that changes in V_{max} of human DAT result from activation of previously inactive DAT within the membrane (Pristupa et al., 1998). In the apparent downregulation of DAT activity by H-89, it may be concluded that the density of active DAT within the membrane was altered as well because there was a change (in the opposite direction) involving V_{max} only. These results suggest that protein kinase A may mediate control of membrane trafficking of DAT or activate otherwise inactive DAT within the membrane. In contrast to the findings with inhibition of protein kinase A, it was observed that the use of nonselective protein kinase inhibitors

H-7 and H-9, presumably inhibiting both protein kinase A and protein kinase C, results in a diminution of V_{max} as well as an increase in K_m , suggesting that binding of dopamine to the transporter as well as a reduction in the density and/or the value of k_2 occurred.

Comparison to the findings of other investigators

These findings agree with those of Kadawaki et al. (1990), Uchikawa et al. (1995), and Pierce and Kalivas (1997) in that protein kinase activity can kinetically upregulate DAT activity and appear to contrast with those of some others investigating the effects protein phosphorylation on the activity DAT (Tian et al., 1994; Copeland et al., 1996; Zhu et al., 1997). These later investigators have focused on the effects of protein kinase C activity on DAT activity. However, those investigating the effects of protein kinase A within their paradigms (i.e., incubation times, periods of monitoring effects, the study of accumulation, etc.) used in the study of protein kinase C have found no effects of forskolin and/or 8-Br-cAMP on DAT activity. We observe here that the effects of these agents occur rapidly (within a minute or so), and appear to be transient because the effect of DAT activity is diminished or unobservable after ~ 12 –15 min. Furthermore, data consistent with others on effects of protein kinase C activity were observed in the present study. The cited investigators used paradigms outside the time window of 1 min used here for exposure times to protein kinase A activators, and it may be assumed that the results here are not in direct contrast with those of others; the differences observed may simply be a consequence of the different time windows of observation.

In conclusion, it is hypothesized that protein kinase A upregulates DAT by increasing the density of active DAT within the membrane. These results could be confirmed with binding experiments. However, binding experiments are difficult to perform on very short time scales at the present time. As studied by others and consistent with the data presented here, protein kinase C has the opposite effect on DAT. Combined, DAT may be regulated by protein kinase A in addition to protein kinase C, but in apparent opposite directions. These two phosphorylating systems are thought to act independently and, when present simultaneously, not on the same substrate. Indeed, Vaughn et al. (1997) have shown that 8-Br-cAMP and forskolin-stimulated protein kinase A activity did not result in observable phosphorylation of DAT. Taken together it may be assumed, as has been suggested by Reith et al. (1997), that the substrates acted on by these two systems are different and that one, the protein kinase C system, may be involved in downregulating the activity of DAT via direct phosphorylation of DAT protein. In contrast, the protein kinase A system may upregulate DAT activity by phosphorylation of members of another biochemical control pathway.

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