Acute Regulation of Norepinephrine Transport: I. Protein Kinase C-Linked Muscarinic Receptors Influence Transport Capacity and Transporter Density in SK-N-SH Cells

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ABSTRACT

Using SK-N-SH cells, we observe that muscarinic acetylcholine receptor activation by methacholine (MCh) rapidly and selectively diminishes l-NE transport capacity (Vmax) with little or no change in norepinephrine (NE) Km, and without apparent effects on membrane potential monitored directly under current clamp. Over the same time frame, MCh exposure reduces the density of [3H]nisoxetine binding sites (Bmax) in intact cells but not in total membrane fractions, consistent with a loss of transport capacity mediated by sequestration of transporters rather than changes in intrinsic transport activity or protein degradation. Similar changes in NE transport and [3H]nisoxetine binding capacity are observed after phorbol ester (β-PMA) treatment. Inhibition of PKC by antagonists and downregulation of PKC by chronic treatment with phorbol esters abolishes β-PMA-mediated effects but produce only a partial blockade of MCh-induced effects. Neither muscarinic acetylcholine receptor nor PKC activation require extracellular Ca2+ to diminish NET activity. In contrast, treatment of cells with the Ca2+/ATPase antagonist, thapsigargin in Ca2+-free medium, eliminates the staurosporine-insensitive component of MCh regulation. These findings were further corroborated by the ability of [1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(ace-toxyethyl)ester application in Ca2+-free medium to abolish NET regulation by MCh. Although they may contribute to basal NET expression, we could not implicate CaMKII-, PKA- or nitric oxide-linked pathways in MCh regulation. Together, these findings 1) provide evidence in support of G-protein coupled receptor-mediated regulation of catecholamine transport, 2) reveal intracellular Ca2+-sensitive, PKC-dependent and -independent pathways that serve to regulate NET expression and 3) indicate that the diminished capacity for NE transport evident after mACHR and PKC activation involves a redistribution of NET protein.

L-NE is the principal catecholamine released from presynaptic sympathetic and central noradrenergic neurons (Axelrod and Kopin, 1969). Active transport of l-NE into presynaptic nerve terminals by Na+- and Cl−-dependent NETs represents the primary means of inactivation of l-NE at noradrenergic synapses (Axelrod and Kopin, 1969). The contribution of l-NE clearance to synaptic activity is evident in the prominent overflow of transmitter following blockade of reuptake with aripiprazole or tricyclic antidepressants and the subsequent alteration in peripheral vascular resistance and heart rate (Furchgott et al., 1963; Eisenhofer et al., 1990; Barker and Blakely, 1995). NETs also clear NE from extracellular spaces in the brain and, in brain regions such as the prefrontal cortex where dopaminergic innervation is sparse relative to noradrenergic fibers, may participate in DA clearance (Carboni et al., 1990). The prominent contribution of catecholamine reuptake to synaptic homeostasis is evidenced by compensatory biochemical changes in DA transporter knockout mice (Giros et al., 1996). Alterations in l-NE clearance or NET density have been reported in hypertension, cardiomyopathy and depression necessitating attention to endogenous mechanisms that determine the appropriate level of NE transport in vivo (Barker and Blakely, 1995). In this regard, multiple studies suggest that

ABBREVIATIONS: BAPTA, [1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, BAPTA-AM, [1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(ace-toxyethyl)ester; CaMK, Ca2+/calmodulin-dependent kinase; CTX, cholea toxin; DA, dopamine; 4-DAMP, 4-diphenylacectoxy-N-methylpiperidine methiodide; KN-93, [2-(N-2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamul)-N-methylbenzylamine; GPCR, G-protein coupled receptor; mACHR, muscarinic acetylcholine receptor; MCh, methacholine; NE, norepinephrine; NET, norepinephrine transporters; NOS, nitric oxide synthase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PTX, pertussis toxin; K RH, Krebs-Ringers HEPES buffer; SDS, sodium dioctyl sulfate; U-5521, 3,4-dihydroxymethylpropyphenone.
NET activity can be regulated by diverse stimuli, including neuronal activity, and peptide hormones as well as second messengers elevated after receptor activation (Barker and Blakely, 1995; Kaye et al., 1997). However, molecular mechanisms coordinating NET regulation remain to be defined.

Increasing evidence suggests a role for PKC in acute regulation of multiple members of the Na+ and Cl–-coupled neurotransmitter transporter gene family including GABA, DA and 5HT transporters (Corey et al., 1994; Qian et al., 1997; Zhang et al., 1997). As with these carriers, the hNET bears multiple consensus sites for PKC phosphorylation on putative cytoplasmic domains (Pacholczyk et al., 1991). Although PKC phosphorlates NET cytoplasmic domains in vitro and NETs are phosphorylated in transfected cells after PKC activation (Apparsundaram S, preliminary studies), we lack an understanding of whether PKC participates in NET regulation in native cells. We have taken advantage of the presence of both NETs (Pacholczyk et al., 1991) and mAChRs coupled to PLC and PKC activation in the noradrenergic neuroblastoma SK-N-SH (Peralta et al., 1996; Lambert et al., 1989; Baird et al., 1989), to explore the participation of GPCRs in the acute regulation of catecholamine transporters. We discuss our findings with regard to contributions from intracellular Ca2+ stores, PKC-dependent and -independent pathways after receptor activation and the participation of kinase-linked membrane trafficking pathways in catecholamine transporter regulation.

Methods

Materials. Reagents used to manipulate receptors, second messengers and protein kinases were obtained from the following sources: actinomycin D, atropine sulphate, cholera toxin, cycloheximide, desipramine, dopamine, L-NE, methacholine, pertussis toxin, pirenzepine and staurosporine (Sigma Chemical Co., St. Louis, MO); KN-93, β-PM and thapsigargin (Alexis Biochemicals, San Diego, CA); BAPTA and BAPTA-AM (Calbiochem, San Diego, CA); 4-DAMP (RBI, Natick, MA); U-0521 (Upjohn, Kalamazoo, MI); L-[7,8-3H]noradrenaline (37 Ci/mmol) and [N-methyl-3H]nisoxetine (86 Ci/mmol), 1989; Baird et al., 1995; Kaye et al., 1997; Zhang et al., 1997; Zhang et al., 1997) from intracellular Ca2+ stores, NET-dependent pathways after receptor activation and the participation of kinase-linked membrane trafficking pathways in catecholamine transporter regulation.

Cell culture and L-NE uptake assays. SK-N-SH cells (ATCC) were maintained in culture medium containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 I.U/ml penicillin and 100 μg/ml streptomycin. For uptake studies, SK-N-SH cells were plated on 300,000 cells/well in 24-well plates 2 days before experiments. Uptake assays were carried out as previously described (Melikian et al., 1994). Briefly, culture medium was removed by aspiration and cells were washed with 2 ml KRH buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, pH 7.4). Cells were then preincubated at 37°C in KRH containing 10 μM t-glucose, 100 μM pargyline, 10 μM U-0521 and 100 μM ascorbic acid for 10 min. After the equilibration period, cells were incubated at 37°C with assay buffer containing either modulating agents or appropriate vehicle. L-NE transport assays were initiated by the addition of [3H]-L-NE for 5 to 10 min at 37°C and terminated by three rapid washes with ice-cold KRH buffer. In some experiments, assays was carried out using Ca2+ free medium by eliminating Ca2+ salts. Cells were lysed in Optiphase Supernix scintillation cocktail (Wallac) and accumulated radioactivity directly quantified in a microparticle liquid scintillation counter (Microbeta, Wallac, Gaithersburg, MD). Nonspecific [3H]-L-NE uptake, defined as the accumulation in the presence of 1 μM desipramine (see figure legends), was subtracted from total uptake to define hNET-specific accumulation. Nonlinear curve fits of data (Kaleidagraph, Synergy Software, Reading, PA) for uptake used the generalized Michaelis-Menten model V = Vmax[S]/[S]+[K]. Results presented on the effects of modulators on NE uptake arise from experiments using vehicle treated cells, assayed in parallel. Statistical analyses were performed comparing mean transport values or kinetic constants using INSTAT software (GraphPad Software, San Diego, CA).

Electrophysiological measurements on SK-N-SH cells. The effects of MCh on resting membrane potential (Vm) of SK-N-SH cells was performed using current clamp whole-cell recordings. An Axopatch 200A amplifier band-limited at 5000 Hz was used for electrophysiological measurements. Data were stored digitally on a video recorder and analyzed on a Nicolet 4094 oscilloscope connected to an IBM-AT computer. Prior to electrical recording, SK-N-SH cells were plated at a density of 50,000 cells in 35-mm culture dish. After 48 hr attached cells were washed three times with bath solution at room temperature. All other steps were carried out at 37°C. The bath solution (pH 7.35; 300 nMol/liter) contained (in mM): 130 NaCl, 1.3 KCl, 1.3 KH2PO4, 0.5 MgSO4, 1.5 CaCl2, 10 HEPES, 34 g glucose. Pipette solutions (pH 7.35; 270 nMol/liter) contained (in mM): 130 KCl, 0.1 CaCl2, 2 MgCl2, 1.1 EGTA, 10 HEPES, 30 g glucose. A whole cell current clamp configuration was used to record changes in membrane potential. After making a seal and going to whole-cell configuration, membrane potential was recorded in the absence and presence of MCh using concentrations and application conditions that trigger maximal reduction of NE uptake.

Quantitative estimation of hNET surface density. To assess changes in hNET surface density in SK-N-SH cells, radioligand binding assays with a high affinity NET-selective ligand, [3H]nisoxetine, were performed using both intact cells and isolated membrane fractions. For radioligand binding assays in intact cells, SK-N-SH cells were grown to confluence and treated with modulating agents in duplicate as described in uptake assays, with experiments repeated three times. After drug treatment, cells were transferred to 4°C and washed with ice-cold binding buffer (100 mM NaCl, 50 mM Tris, ascorbic acid 100 μM, pH 8). After 10 min, cells were incubated in 0.5 ml of ice-cold binding buffer containing [3H]nisoxetine (0.01–30 nM) at 4°C for 2 hr. Binding assays were terminated by washing the cells five times with ice-cold binding buffer. Cell extracts were prepared with 0.5 ml of 1% sodium dioctyl sulfate (SDS) or 1 ml of Optiphase Supermix scintillation cocktail and bound radioactivity quantified using scintillation spectrometry. A portion of the cell extracts were analyzed for protein content (Bradford assay, BioRad, Hercules, CA). Nonspecific binding was determined using 100 μM DA which gave the same values as 10 μM desipramine.

For radioligand binding in SK-N-SH membranes, cells were plated in 35-mm culture dishes and grown to confluence, after which cells were incubated in the presence and absence of modulating agents. After treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and then scraped off the dishes in ice-cold PBS. Cells from 12 culture dishes were pooled and pelleted at 1600 × g. The supernatant was discarded and the pellet resuspended in 3 ml of ice-cold binding buffer and the cells were resuspended at 20,000 × g. Supernatant was discarded and cells were resuspended in binding buffer and homogenized with a Polytron (Brinkman, Westbury, NY) at 25000 rev/min for 5 sec. Centrifugation, resuspension and homogenization were repeated and an aliquot of the sample was used for protein determination by the Bradford method (BioRad, Hercules, CA). Initial studies with total cell membranes isolated from SK-N-SH cells demonstrated linearity of specific binding (10 nM [3H]nisoxetine) up to 100 μM membrane protein per tube and subse-quent assays used 80 μg/tube. Cells were incubated with [3H]nisoxetine (0.01–30 mM) for 4 hr at 4°C. Membrane bound radioactivity was recovered by rapid filtration (Brandel, Gaithersburg, MD) over GF/B glass-fiber filters (Whatman, Clifton, NJ), presoaked in 0.3% polyethyleneimine (Sigma, St. Louis, MO). Filters were washed in
brane potential current clamp configuration (MCh-induced change in membrane potential in whole-cell that could act to slow transport rates. However, we find that membrane depolarization or effects on other ion gradients induced reductions in l-NE uptake could be secondary to the mRNA synthesis inhibitor, actinomycin D or the transduction inhibitor cycloheximide (table 2), consistent with a posttranslational event leading to reduction in NET activity.

### Methacholine-induced reductions in NE uptake in SK-N-SH cells involve changes in surface transporter density.

MCh-evoked reductions in l-NE transport could arise from a reduction in transport efficiency of a fixed population of carriers, a reduction in the number of functional carriers in the plasma membrane, or both. To assess this issue, we sought to determine the effect of MCh (0.1 μM; 30 min) on the expression of hNETs resident on the plasma

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### Results

**Activation of muscarinic acetylcholine receptors regulate hNET activity in SK-N-SH cells.** SK-N-SH cells possess both M<sub>1</sub> and M<sub>3</sub> muscarinic acetylcholine receptors (mACHRs) coupled to phospholipase C, leading to Ca<sup>2+</sup> mobilization and PKC activation (Peralta et al., 1996; Baird et al., 1989; Lambert et al., 1989). To examine whether mACHRs regulate NE transport, we tested the effects of the mAChR agonist MCh on desipramine-sensitive NE uptake. Treatment of cells with MCh produces a time- and concentration-dependent inhibition of l-NE transport (fig. 1, A and B). Reductions in NE transport are apparent within 5 min of MCh (100 nM) treatment and maximal reductions are achieved by 30 min treatment. Using the 30-min incubation time, we find that reductions in NE transport are observed at low nanomolar concentrations of MCh, with a maximal effect apparent at 100 nM. The nonselective mAChR antagonist atropine (1 μM) has no effect on NE transport (data not shown). However, coapplication of atropine with MCh abolishes the MCh-induced reduction in l-NE transport (fig. 1B; table 1). In contrast, the nicotinic receptor antagonist hexamethonium fails to blunt the MCh effect (table 1), suggesting that MCh actions are mediated by muscarinic and not nicotinic receptors. Moreover, the MCh-induced reduction in l-NE uptake is abolished by the M<sub>1</sub>/M<sub>3</sub> antagonist 4-DAMP but not by the M<sub>1</sub>-selective antagonist pirenzepine (table 1), consistent with M<sub>3</sub> mAChR participation in NET regulation. With time and concentration profiles of NET regulation established, we turned to a kinetic analysis of the MCh-induced reduction in NET activity. As previously described (Richards and Sadee, 1986), we find transport of l-NE in SK-N-SH cells to display single-site, saturable kinetics with a K<sub>m</sub> of 462 ± 43 nM, and a Vmax of 5.2 pmol/10<sup>6</sup> cells/min for l-NE (fig. 1C). Treatment of SK-N-SH cells with MCh under conditions (0.1 μM; 30 min) that diminish NE uptake by ~30% using low NE concentrations leads to a significant (27%) decrease in transport capacity (Vmax) with little or no change in K<sub>m</sub> of l-NE transport (fig. 1C).

The transport of NE by hNET is achieved with the movement of net positive charge across the plasma membrane coupled to the transport cycle and transport is accompanied by an inward ion flux (Galli et al., 1996). Possibly, MCh-induced reductions in l-NE uptake could be secondary to membrane depolarization or effects on other ion gradients that could act to slow transport rates. However, we find that 100 nM MCh fails to alter membrane potential in whole-cell current clamp configuration (MCh-induced change in membrane potential = −5.6 ± 6.9 mV, n = 4). Moreover, the effect of MCh is selective for l-NE transport, because sodium-coupled alanine, glutamate, and glycine transport as well as sodium-independent leucine transport are unaffected by MCh exposure (table 1). Finally, reductions in NET activity induced by MCh are insensitive to pretreatment of cells with the mRNA synthesis inhibitor, actinomycin D or the transport.
TABLE 1
Specificity of MCh- and β-PMA-mediated effects on NE transport in SK-N-SH cells

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[3H]NE</th>
<th>MCh (0.1 μM)</th>
<th>MCh + Atropine (1 μM)</th>
<th>MCh + 4-DAMP (0.1 μM)</th>
<th>MCh + Pirenzepine (1 μM)</th>
<th>MCh + Hexamethonium (30 μM)</th>
<th>β-PMA (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Control</td>
<td></td>
<td>70 ± 2*</td>
<td>99 ± 1</td>
<td>98 ± 1</td>
<td>74 ± 3*</td>
<td>69 ± 4*</td>
<td>68 ± 2*</td>
</tr>
</tbody>
</table>

TABLE 2
Reduction in NE transport evoked by MCh and β-PMA in SK-N-SH cells is protein and mRNA synthesis independent

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>NE Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>% of Control</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>Cyclohexamide</td>
<td>106 ± 3</td>
</tr>
</tbody>
</table>

Fig. 2. MCh reduces Bmax of [3H]nisoxetine binding in intact SK-N-SH cells. A, Cells were incubated with vehicle or MCh (0.1 μM; 30 min), followed by incubation with [3H]nisoxetine (0.01–30 nM) as described in “Methods.” Nonspecific binding was defined by using 100 μM dopamine, and was subtracted from total binding. B, Cells were incubated with vehicle or MCh (0.1 μM; 30 min), followed by preparation of membrane fractions as described in “Methods.” A total of 80 μg of membrane suspension was incubated with 0.5 ml of [3H]nisoxetine (0.01–30 nM) at 4°C for 4 hr as described in “Methods.” Nonspecific binding was defined by using 100 μM dopamine, and was subtracted from total binding. Fluo-4 and [3H]nisoxetine binding in a representative experiment. Bmax and Kd of [3H]nisoxetine binding in the presence and absence of drug treatment obtained in three separate experiments are presented as mean ± S.E.M. Asterisks indicate statistically significant changes compared to vehicle controls (P < .05, Student’s t test).

membrane. The low expression levels of hNET protein in SK-N-SH cells (Melikian et al., 1994) precludes analyses of hNET protein trafficking as achieved in biontination and immunofluorescence experiments using transfected cells (Qian et al., 1997; Apparsundaram et al., accompanying manuscript). Therefore, we established an intact cell radioligand binding paradigm using the NET-selective antagonist, [3H]nisoxetine (Tejani-Butt, 1992; Cheetham et al., 1996) to explore whether MCh-induced reductions in NE uptake capacity are related to changes in surface density of hNET proteins. Comparisons were made between [3H]nisoxetine binding to intact cells and to total membrane fractions isolated from lysed cells (fig. 2). Scatchard analysis of nisoxetine binding to intact SK-N-SH cells reveals single-site kinetics with a Kd of 6.0 nM and Bmax of 1.05 pmol/mg protein. In intact SK-N-SH cells, MCh treatment (0.1 μM; 30 min) reduces the Bmax of [3H]nisoxetine binding (~30% reduction) (fig. 2A). Nisoxetine Kd appeared to be reduced following MCh exposure, although this effect did not achieve statistical significance. As observed with intact cells, Scatchard analysis of [3H]nisoxetine binding to membrane fractions indicates single-site kinetics with a Kd equivalent to that observed in intact cells (6.7 nM). The density of [3H]nisoxetine sites was greater than that found with intact cell measurements (Bmax of 2.2 pmol/mg protein) suggesting ~55% of NETs are inaccessible in intact cell assays. Unlike intact cell assays, MCh treatment failed to alter either the Bmax of [3H]nisoxetine binding in membrane fractions or the apparent affinity of nisoxetine binding to membrane fractions (fig. 2B).

Direct activation of PKC diminishes hNET activity via altered surface distribution of hNETs. M3 muscarinic receptor activation in SK-N-SH cells leads to the activation and translocation of PKC (Baumgold and Dyer, 1994). To evaluate M3 linked pathways involved in NET regulation, we triggered PKC activation directly using phorbol esters and examined the consequences on hNET-mediated NE transport. As with MCh, β-PMA produces a rapid, time- and concentration-dependent reduction in 1-NE uptake (fig. 3, A and B). Significant reductions in 1-NE uptake are observed within 10 min of β-PMA treatment (1 μM) and maximal
reductions (39 ± 2%) achieved with 30-min exposure. β-PMA concentrations as low as 1 nM (30 min incubation) significantly reduce NE transport and maximal inhibition of NE uptake is obtained with 1 mM β-PMA. As with MCh, β-PMA-induced reductions in NET activity are insensitive to actinomycin D (10 µM; 20 min) or cycloheximide (10 µM; 1 hr) (table 2). The reduction in NE transport induced by β-PMA is abolished by coapplication of staurosporine (1 µM) (fig. 3B). As with MCh application, the effects of β-PMA (1 µM; 30 min) are resolved in kinetic studies to reflect a decrease in transport capacity (Vmax) with little or no change in the Km of l-NE transport (fig. 3C). The effects of PMA are stereospecific as the PKC-inactive isomer α-PMA failed to alter l-NE uptake under the same conditions (data not shown). Similarly, sodium-coupled alanine, glutamate and glycine transport and sodium-independent leucine transport are unaffected by β-PMA exposure (table 1). Finally, as with MCh, β-PMA treatment reduces the Bmax of [3H]nisoxetine binding (27% reduction) with no change in nisoxetine \( K_d \) in intact cells, but the phorbol ester does not affect [3H]nisoxetine binding assayed in total membrane fractions (fig. 4B).

MCh-induced reductions of l-NE transport involves both PKC dependent and independent pathways. MCh-induced reductions in NE transport are not mimicked by treatment of SK-N-SH cells with cholera toxin (50 µg/ml; 2 hr) or abolished by pertussis toxin (500 ng/ml, 8 hr), though the latter treatment significantly diminishes basal NE uptake capacity (Vmax) with little or no change in the Km of l-NE transport (fig. 3C). The effects of PMA are stereospecific as the PKC-inactive isomer α-PMA failed to alter l-NE uptake under the same conditions (data not shown).

**Fig. 3.** Inhibition of l-NE uptake by β-PMA in SK-N-SH cells. A, Time-dependent effect of β-PMA on l-NE uptake in SK-N-SH cells. Cells were treated with 1 µM β-PMA for indicated times and assayed for l-NE (1 µM, 5 min) uptake as described in “Methods.” B, Concentration-dependent effect of β-PMA in the presence and absence of 1 mM staurosporine on l-NE uptake. Maximal inhibition of NET uptake was observed after a total incubation time of 30 min with 1 µM of β-PMA. C, Effect of β-PMA (1 µM; 30 min) on l-NE transport kinetics. β-PMA produces a significant decrease in transport capacity (Vmax) with no effect on NE \( K_m \) (control: \( K_m = 564 ± 92 \text{ nM, } Vmax = 3.4 ± 0.16 \text{ pmol/10}^6 \text{ cells/min; β-PMA-treated: } K_m = 608 ± 60 \text{ nM, } Vmax = 2.55 ± 0.13 \text{ pmol/10}^6 \text{ cells/min; } n = 3, P < .05, \text{ Student’s } t \text{ test). Data are presented as means ± S.E.M. of three experiments performed in triplicate. Parallel assays were carried out in the presence of 1 µM desipramine to define specific uptake. Data presented are means ± S.E.M. of three separate experiments performed in triplicate. Asterisks indicates statistically significant changes as compared to vehicle controls (P < .05, Student’s t test).**

**Fig. 4.** β-PMA reduces Bmax of [3H]nisoxetine binding in intact SK-N-SH cells. A, Cells were incubated with vehicle or β-PMA (1 µM; 30 min), followed by incubation with [3H]nisoxetine (0.01–30 nM) as described in “Methods.” Nonspecific binding was defined by using 100 µM dopamine, and was subtracted from total binding. B, Cells were incubated with vehicle or β-PMA (1 µM; 30 min), followed by preparation of membrane fractions as described in “Methods.” A total of 80 µg of membrane suspension was incubated with 0.5 ml of [3H]nisoxetine (0.01–30 nM) at 4°C for 4 hr as described in “Methods.” Nonspecific binding was defined by using 100 µM dopamine, and was subtracted from total binding. Plots show Scatchard analysis of the [3H]nisoxetine binding of a representative experiment. Bmax and \( K_d \) of [3H]nisoxetine binding in the presence and absence of drug treatment obtained in three separate experiments are presented as means ± S.E.M. Asterisks indicates statistically significant changes as compared to vehicle controls (P < .05, Student’s t test).
take by 28 ± 4% (n = 3). M₃ mACHRs are known to couple to phospholipase C leading to the generation of inositol trisphosphate, a rise in intracellular Ca²⁺, and the activation of PKC in these cells (Fisher et al., 1988; Baird et al., 1989). To determine whether the mACHR-mediated regulation of NETs proceeds solely through PKC-dependent pathways, we tested the additive effects of MCh and β-PMA on NE transport and the sensitivity of MCh and β-PMA actions to PKC inhibitors staurosporine and bisindolylmaleimide I. We found that β-PMA can augment the reduction in NE uptake achieved by a maximally effective concentration of MCh (MCh, 30 ± 3% reduction; MCh + β-PMA, 48 ± 2% reduction) (fig. 5), suggesting that MChRs linked to NET regulation may use pathways distinct from phorbol ester-sensitive PKCs. This latter position is supported by the inability of staurosporine (1 μM) to abolish the MCh-induced reduction in l-NE uptake whereas staurosporine under identical conditions abolishes the β-PMA-induced reduction in NE transport (figs. 3 and 5). Another more specific PKC inhibitor, bisindolylmaleimide I produced effects comparable to staurosporine (MCh, 29 ± 4% reduction; MCh + bisindolylmaleimide I, 16 ± 4% reduction; n = 3; P < .05). Finally, we attempted to downregulate PKC expression by prolonged treatment of SK-N-SH cells with β-PMA (1 μM; 16 hr) produces a 13 ± 2% increase (n = 5, P < .05) in basal NE activity. More importantly, β-PMA treatment markedly diminishes the β-PMA-mediated reduction of NET activity whereas the same conditions produce only a partial blockade of MCh-evoked effects (fig. 6).

**MCh-evoked reduction of NET activity requires internal but not external Ca²⁺: studies with thapsigargin and BAPTA-AM.** M₃ muscarinic receptor activation leads to elevations in intracellular Ca²⁺ in addition to elevations in PKC activity (Baird et al., 1989). To explore the alternative pathways by which mACHR activation may trigger changes in NE uptake, we tested the requirements of extracellular and intracellular Ca²⁺ for basal NET expression and NET regulation by MCh and β-PMA. Our normal assay buffer contains 2.2 mM external Ca²⁺. Incubation of cells in Ca²⁺-free medium reduces basal NE transport (16 ± 1%; table 3). However, MCh and β-PMA reduce l-NE uptake in Ca²⁺-free buffer to a similar extent as observed in the presence of external Ca²⁺ (fig. 7; table 3). Moreover, β-PMA reductions remained staurosporine-sensitive to the same extent as observed in normal medium (fig. 7; table 3).

To determine whether intracellular Ca²⁺ pools are involved in MCh and β-PMA effects, we treated cells in Ca²⁺-free medium with the endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin. Thapsigargin has previously been shown in SK-N-SH cells to elevate Ca²⁺ acutely but deplete IP₃-sensitive Ca²⁺ stores upon prolonged application in Ca²⁺-free medium (Moore et al., 1991). Acute treatment (5 μM; 3 min) of cells with thapsigargin in standard medium

### TABLE 3

<table>
<thead>
<tr>
<th>Agent</th>
<th>Normal buffer (2.2 mM Ca²⁺)</th>
<th>Ca²⁺-free buffer (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>84 ± 1b</td>
</tr>
<tr>
<td>β-PMA (1 μM)</td>
<td>69 ± 2a</td>
<td>74 ± 4a</td>
</tr>
<tr>
<td>Methacholine (0.1 μM)</td>
<td>69 ± 2a</td>
<td>74 ± 3a</td>
</tr>
<tr>
<td>BAPTA-AM (50 μM)</td>
<td>88 ± 2</td>
<td>71 ± 4ab</td>
</tr>
<tr>
<td>BAPTA</td>
<td>97 ± 1</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>KN-93 (3 μM)</td>
<td>51 ± 2a</td>
<td>62 ± 4ab</td>
</tr>
<tr>
<td>Thapsigargin (5 μM)</td>
<td>98 ± 2</td>
<td>90 ± 3</td>
</tr>
</tbody>
</table>

Cells were preincubated in assay buffer containing either 0 or 2.2 mM Ca²⁺ for 1 hr. After 1 hr, cells were incubated with β-PMA or MCh for 20 min and with other modulating agents for 40 min before the addition of labeled l-NE. Data are presented as mean ± S.E.M. of three experiments performed in triplicate.

a Significant difference as compared with control in the same column (P < .05, Student’s t test).

b Significant difference (P < .05, Student’s t test) as compared with normal buffer.

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**Fig. 5.** Effect of β-PMA- and staurosporine on MCh-induced decrease in l-NE uptake in SK-N-SH cells. Cells were treated with 1 μM β-PMA, and 0.1 μM MCh for 20 min either alone or in combination followed by assay of l-NE uptake (1 μM l-NE; 10 min) as described in “Methods.” Staurosporine (1 μM) was added 20 min prior to the addition of β-PMA or MCh. Parallel assays were carried out in the presence of vehicle and 1 μM desipramine to define effects of vehicle and specific uptake. Data presented are means ± S.E.M. of three experiments performed in triplicate. Asterisks indicate P < .05 (two-tailed Student’s t test) as compared to l-NE uptake in untreated cells whereas the daggar indicates P < .05 (two-tailed Student’s t test) as compared to l-NE uptake in cells treated with MCh alone.

**Fig. 6.** Effect of chronic phorbol ester exposure on β-PMA- and MCh-induced reduction of l-NE uptake in SK-N-SH cells. Cells were treated with 1 μM β-PMA or vehicle for 16 hr. After treatment, cells were incubated with 1 μM β-PMA or 0.1 μM MCh for 20 min followed by assay of l-NE uptake (1 μM l-NE; 10 min) as described in “Methods.” Parallel assays were carried out in the presence of 1 μM desipramine to define nonspecific uptake. Data presented are means ± S.E.M. of five experiments performed in triplicate. Asterisks indicate a significant difference (P < .05, two-tailed Student’s t test) as compared to vehicle-treated cells.

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**Fig. 6.** Effect of chronic phorbol ester exposure on β-PMA- and MCh-induced reduction of l-NE uptake in SK-N-SH cells. Cells were treated with 1 μM β-PMA or vehicle for 16 hr. After treatment, cells were incubated with 1 μM β-PMA or 0.1 μM MCh for 20 min followed by assay of l-NE uptake (1 μM l-NE; 10 min) as described in “Methods.” Parallel assays were carried out in the presence of 1 μM desipramine to define nonspecific uptake. Data presented are means ± S.E.M. of five experiments performed in triplicate. Asterisks indicate a significant difference (P < .05, two-tailed Student’s t test) as compared to vehicle-treated cells.

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caused a significant reduction in NE transport (16 ± 3% reduction; n = 3). With chronic application in Ca\(^{2+}\)-free medium, thapsigargin (5 μM; 40 min) failed to alter NE uptake either in the presence or absence of external Ca\(^{2+}\) (table 3), suggesting a lack of involvement of IP\(_3\)-sensitive Ca\(^{2+}\) pools in basal NET expression. In addition, the effect of β-PMA on NETs was not significantly perturbed (untreated, 34 ± 2%; thapsigargin-treated, 28 ± 2%, n = 3, P < .05). However, thapsigargin treatment in the absence of external Ca\(^{2+}\) significantly diminishes the MCh-induced reduction of NE uptake (fig. 7). Furthermore, coapplication of thapsigargin and staurosporine essentially eliminated the MCh-induced reduction of NET activity (fig. 7).

Although clear loss of MCh effects on NET expression were observed with thapsigargin treatment, we did not achieve complete blockade even in Ca\(^{2+}\)-free buffers with thapsigargin alone. This suggested to us that thapsigargin-insensitive Ca\(^{2+}\) stores might provide sufficient Ca\(^{2+}\) to support MCh-induced effects. We thus tested the activity of the Ca\(^{2+}\) chelator BAPTA and its membrane permeant derivative BAPTA-AM in modulating MCh responses. Addition of the membrane impermeant Ca\(^{2+}\)-chelator BAPTA (50 μM, 40 min) to Ca\(^{2+}\)-free incubation media failed to reduce NE transport below the reduction (~16%) observed in Ca\(^{2+}\)-free media alone (table 3) and confirms a lack of effect of the chelator directly on the transporter. However, treatment of cells with the membrane permeant analog BAPTA-AM (50 μM, 40 min) significantly reduces l-NE uptake. Restoration of Ca\(^{2+}\) to the external media reduces the BAPTA-AM effect (table 3). Whereas thapsigargin treatment only partially eliminated the MCh-reduction of NET activity, pretreatment of SK-N-SH cells in Ca\(^{2+}\)-free medium with BAPTA-AM abolishes the MCh-induced reduction in l-NE transport (fig. 7).

The PKC-independent component of muscarinic receptor-mediated reduction of NE uptake does not involve PKA, NOS and CaMKII. In SK-N-SH cells, activation of mAChR is reported to cause accumulation of cAMP via a PLC-linked pathway (Baumgold and Fishman, 1988). Therefore, we examined the effects of a membrane permeant cAMP analog, 8-pCT-cAMP (1 μM; 40 min) and the inhibitory analog of cAMP, Rp-8-pCT-cAMP (1 μM; 40 min) on NE transport and MCh-induced regulation. Both cAMP analogs failed to alter either basal or MCh-triggered reduction in l-NE uptake (data not shown), suggesting that activated PKA is unlikely to be involved in mAChR regulation of NET. Activation of mAChRs augments generation of free radicals (Kaye et al., 1997) and recent data suggest that exogenous NO donors reduce catecholamine uptake in PC12 cells (Naarala et al., 1997). NOS is regulated by Ca\(^{2+}\)/calmodulin and could be a target for the thapsigargin-insensitive, but thapsigargin and BAPTA-AM-diminished regulation of NE uptake. However, pretreatment of SK-N-SH cells with the NOS inhibitor l-NMMA (1 μM; 40 min) fails to affect MCh-induced reduction of NET activity (% reduction: control 28 ± 2%; l-NMMA-treated 30 ± 3%). Moreover, unlike PC12 cells (Naarala et al., 1997), treatment of SK-N-SH cells with the NO donor SNAP (1 μM, 20 min) augments, rather than inhibiting l-NE uptake (% increase: 36 ± 4%). Thus, though there may be regulatory NO-linked pathways in SK-N-SH cells, it is unlikely that activation of NOS lies in the pathway from activated mAChRs to internalized NETs. Last, given suggestions that Ca\(^{2+}\)/CaMKII regulates homologous DA and 5HT transporters (Uchikawa et al., 1995; Yura et al., 1996), we tested the role of this enzyme in mAChR-triggered diminution of NET activity. Pretreatment of cells with the CaMKII inhibitor, KN-93 (3 μM; 40 min) significantly reduces l-NE uptake, independent of external Ca\(^{2+}\) (table 3). However, a further 26 ± 2% (n = 3) reduction in activity is evident with MCh coapplication with KN-93, comparable to that seen in the absence of KN-93, suggesting that CaMKII may participate in basal NET expression but is unlikely to be required in MCh-linked pathways leading to NET sequestration.

**Discussion**

Muscarinic receptors are located presynaptically on sympathetic and central noradrenergic neurons where they can modulate release of l-NE (Starke et al., 1989). We speculate that regulation of l-NE clearance may provide another avenue for the tight control of noradrenergic neurotransmission. mAChRs are found to affect NE release through PKC-dependent and -independent pathways (Murphy et al., 1992). In our studies, we demonstrate that NETs expressed in the neuroblastoma SK-N-SH can be rapidly regulated by mAChR activation independent of changes in transporter mRNA transcription or protein translation. SK-N-SH cells express nicotinic and two classes of mAChRs (M\(_1\) and M\(_3\)) (Lambert et al., 1989; Peralta et al., 1988). We chose MCh as an agonist for these studies due to its preferential activation of muscarinic receptors and indeed found no blockade of MCh responses by the nicotinic receptor antagonist hexamethonium. Moreover, our antagonist studies suggest the M\(_3\) subtype of
mAChRs is largely responsible for MCh actions to reduce NET activity and surface expression, and these effects are mediated by coupling to cholinergic and pertussis toxin-insensitive G-proteins (Neer, 1995). Although activation of M₃ mAChRs can cause release of NE (Murphy et al., 1992), we find that MCh decreases the maximal capacity (Vmax) of l-NE transport without significantly altering l-NE Km. This change in transport capacity is unlikely to reflect an increase in unlabeled NE in the external medium as a result of release because we should have primarily detected an elevation in the NE Km as a consequence of isotopic dilution which does not occur. Furthermore such an explanation would not lead to the alterations observed in transporter density (see below).

NET activity involves the translocation of charged substrates and is sensitive to membrane potential (Galli et al., 1996). Thus, reduction of NE uptake could reflect M₃-receptor mediated depolarization. In our studies mAChR activation regulates NETs independently of changes in membrane potential and does not appear to be linked to global changes in ion gradients as other Na⁺-dependent transport activities are unaffected by MCh. Therefore we focussed our attention on second-messenger linked pathways downstream of mAChR activation. Previous studies have established that mAChRs in SK-N-SH cells are coupled to multiple signaling pathways (Baumgold and Fishman, 1988; Fisher et al., 1988; Akil and Fisher, 1989; Noronha-Blob et al., 1989; Felder, 1995; Naarala et al., 1997). Stimulation of mAChRs results in activation of adenylate cyclase, and phospholipase C via a G-protein coupled pathway (Peralta et al., 1994; Qian et al., 1994; Zhang et al., 1997) and which have been shown to abolish muscarinic receptor elevations in intracellular Ca²⁺ (Grudt et al., 1996). Our findings suggest that muscarinic regulation of NET either involves a Ca²⁺-independent component or that thapsigargin-insensitive Ca²⁺ pools may participate in NET regulation. Our studies with BAPTA-AM, a membrane permeant Ca²⁺ chelator (Lew et al., 1988), are most consistent with the latter explanation. In fact, BAPTA-AM is the only agent we have found to completely eliminate MCh regulation of NET. We found BAPTA-AM, but not the membrane impermeant analog BAPTA, to abolish MCh-induced diminution of NET activity. BAPTA-AM in Ca²⁺-free medium also attenuated basal NE transport activity, an effect diminished by supplementation with external Ca²⁺, suggesting that Ca²⁺ participates in both tonic NET regulation and pathways triggered by GPCR activation.

Our results with thapsigargin and BAPTA-AM prompted us to consider other Ca²⁺-sensitive pathways downstream of M₃ receptor activation that could contribute to the staurosporine-insensitive reduction of NET activity. A Ca²⁺-dependent increase in cAMP accumulation has been demonstrated in SK-N-SH and SH-SY5Y cells following muscarinic receptor activation (Baumgold and Fishman, 1988). However, this effect is PLC- and PKC-dependent and we find that treatment of SK-N-SH cells with various agents to modulate cAMP pathways (8-Br-cAMP, forskolin, Rp-8-pCT-cAMPS and 8-pOT-cAMP) fails to alter either basal l-NE uptake or MCh-induced reductions in l-NE transport, suggesting that adenylate cyclase-linked pathways do not contribute to the regulation we observe. A Ca²⁺-modulated NOS pathway has been described in SK-N-SH cells (Liu and Shaw, 1997) and might subserve the staurosporine-insensitive pathway for NET regulation. Indeed, exogenous nitric oxide donors have been found to reduce l-NE uptake in PC12 cells (Kay et al., 1997) and activation of mAChRs has been argued in SK-N-SH cells to lead to the generation of free radicals, such as NO (Naarala et al., 1997). However, the inability of the NOS inhibitor l-NMMA to alter either basal or MCh-evoked l-NE uptake points away from this pathway. We did find that pertussis toxin diminished basal NE transport, consistent with a G₁-linked pathway involvement in NET expression (Bunn et al., 1992), though we could not implicate this pathway in MCh modulation. Similarly, we did not find the CaMKII inhibitor KN-93 to blunt the MCh regulation of NET, but it did substantially decrease basal l-NE uptake, suggesting that the Gi-linked tonic pathway may be influenced by CaMKII. Similar conclusions of CaMKII participation in biogenic amine transporter regulation have been advanced for the homologous serotonin transporter (Uchikawa et al., 1998; Puhl et al., 1997). Since removal of external Ca²⁺ from our assay buffer reduces basal NE transport but does not suppress MCh-induced effects, Ca²⁺ influx may support tonic expression of NET but is likely not be required for mAChR-mediated reduction in NET activity. However, intracellular Ca²⁺ stores appear to be vital for NET modulation by MCh. Chronic (40 min) treatment of cells with thapsigargin (Thastrup et al., 1990) in Ca²⁺-free media, significantly diminishes MCh-induced reduction in l-NE uptake. The blockade by thapsigargin of the MCh-evoked reduction in NET activity is not complete, though we used concentrations of thapsigargin that deplete IP₃-sensitive Ca²⁺ stores (Moore et al., 1991) and which have been shown to abolish muscarinic receptor elevations in intracellular Ca²⁺ (Grudt et al., 1996). Our findings suggest that muscarinic regulation of NET either involves a Ca²⁺-independent component or that thapsigargin-insensitive Ca²⁺ pools may participate in NET regulation. Our studies with BAPTA-AM, a membrane permeant Ca²⁺ chelator (Lew et al., 1988), are most consistent with the latter explanation. In fact, BAPTA-AM is the only agent we have found to completely eliminate MCh regulation of NET. We found BAPTA-AM, but not the membrane impermeant analog BAPTA, to abolish MCh-induced diminution of NET activity. BAPTA-AM in Ca²⁺-free medium also attenuated basal NE transport activity, an effect diminished by supplementation with external Ca²⁺, suggesting that Ca²⁺ participates in both tonic NET regulation and pathways triggered by GPCR activation.

Activation of mAChRs produces a rapid and significant elevation of intracellular Ca²⁺ in SK-N-SH cells (Fisher et al., 1988; Baird et al., 1989; Puhl et al., 1997). Elevation of intracellular Ca²⁺ that occurs as a result of influx of extracellular Ca²⁺ and mobilization of Ca²⁺ from intracellular stores is involved in mAChR signal transduction pathways (Fish-
et al., 1995). Preliminary studies in our lab indicate that PI-3-kinase activation may also participate in MCh-mediated NET regulation (Apparsundaram and Blakely, 1997). Further studies are warranted to explore whether the latter kinase, known to be involved in vesicular trafficking of GLUT4 glucose transporters (Yang et al., 1996), may act downstream or in parallel with PKC to modulate NET expression.

The mechanism of MCh-mediated diminution of NET activity depends on the process by which NE transport capacity is lost following receptor activation. NETs may become less stable and catalytically less efficient as they do when N-glycosylation is prevented (Melikian et al., 1994). Alternatively, NETs may be sequestered or internalized as occurs with G-protein coupled receptors after heterologous and homologous down-regulation (Ferguson et al., 1996). Unlike heterologous models (Apparsundaram et al., accompanying paper), hNET expression in SK-N-SH cells is insufficient to achieve immunolocalization or biochemical analysis of transporter proteins (Melikian et al., 1994; Qian et al., 1997). Therefore, we established a radioligand binding paradigm that could report surface expression of NET proteins. [3H]Nisoxetine binds NETs with high affinity and single site kinetics (Tejani-Butt, 1992) in a Na+-dependent manner. Although nisoxetine is not a hydrophilic ligand, the significant Na+-dependence of its binding and the reduced pH of intracellular compartments, particularly endosomes, may preclude nisoxetine binding to NETs sequestered in intracellular compartments. To further constrain this paradigm, we conducted intact cell binding experiments on ice to prevent endocytosis of the ligand and defined nonspecific binding using the hydrophilic substrate dopamine. Our estimate of surface density for NETs in SK-N-SH cells (~42,000 sites/cell) coupled with our Vmax measurements, yields a turnover rate of ~2.2 cycles/sec that is consistent with estimates derived from PC12 plasma membrane vesicle studies (Friedrich and Bönisch, 1986). However, comparisons between [3H]nisoxetine binding to intact SK-N-SH cells and isolated SK-N-SH membranes suggests that only ~45% of NETs are resident at the cell surface under basal conditions. MCh and β-PMA induce a reduction in the Bmax in intact cells (but not the Kd) of [3H]nisoxetine binding that is of comparable magnitude to the loss in transport capacity (Vmax). These agents failed to alter NET density in membranes isolated from MCh-treated cells, suggesting that MCh- and β-PMA-induced reductions in NE transport capacity arise from a sequestration of NETs, with no loss in total NET protein as might occur if NETs were degraded. This conclusion is also supported by preliminary studies indicating reversibility of MCh regulation of NETs after agonist washout and the involvement of clathrin-dependent mechanisms in β-PMA effects (Bauman, P., Apparsundaram, S., Blakely, R.D., unpublished observation). Analogous to our findings with NETs, DATs expressed in Xenopus laevis oocytes become inaccessible to [3H]mazindol after PKC activation (Zhu et al., 1997). Whether individual transporters are inactivated catalytically before sequestration is unknown but is a subject of ongoing investigation using detached patch recordings of transport-associated currents (Galli et al., 1996) after kinase exposure.

Our studies with phorbol esters and kinase inhibitors point to an important role for PKC in acute hNET regulation as proposed for other transporters (Corey et al., 1994; Qian et al., 1997; Quick et al., 1997; Zhang et al., 1997; Zhu et al., 1997). PKC is a family of enzymes consisting of at least 12 members, divided in three subgroups: Ca++- and DAG-dependent classical PKCs (α, βI, βII, γ); Ca++-independent novel...
PKCs (δ, ε, η, θ, ρ) and atypical PKCs (ζ, τ, and υ) that are activated by IP_3 but are Ca^{2+}-independent and -insensitive to DAG or phorbol esters (Casabona, 1997). However, all PKC isoforms are sensitive to staurosporine which fails to inhibit DAG or phorbol esters (Casabona, 1997). However, all PKC isoforms are sensitive to staurosporine which fails to reduce MCH-induced reductions in NE transport suggesting a PKC-independent component. Although the MCH-induced reduction of NETs exhibits complete Ca^{2+}-dependence, this requirement may reflect steps downstream of PKC activation. Indeed, mACHR activation in SK-N-SH cells has been shown to induce translocation of PKCo and activation of PKCζ isoforms (Baumgold and Dyer, 1994). Future studies using isoform specific antagonists or mutants may help clarify the role of different PKCs in NET regulation.

Based on our findings, we propose a model for M_3 mACHR-mediated internalization of NETs involving activated PKC, perhaps causing phosphorylation of transporter and/or proteins associated with the carrier’s sequestration, and a Ca^{2+}-dependent reduction in NE transport capacity mediated by loss of the carrier from the cell surface (fig. 8). Canonical phosphorylation sites for PKC, PKA and PKG are located on the putative cytoplasmic domains of hNETs (Pacholczyk et al., 1991). Using in vitro phosphorylation, we are able to demonstrate phosphorylation of both NH_2 and COOH termini of hNETs by PKC, PKA and PKG (Apparsundaram and Blakely, 1996). PKC activation leads to the phosphorylation of dopamine and serotonin transporters (Huff et al., 1997; Ramamoorthy et al., 1998), and our initial studies present evidence of NET phosphorylation after PKC activation and PIP2/PA1 inhibition in transfected LLC-PK1 cells in parallel with PKC-mediated changes in surface expression (Apparsundaram S, preliminary studies). Future studies using mutant NETs transfected into the native context provided by SK-N-SH cells and their derivatives should help clarify the role of direct NET phosphorylation in acute regulation. Because NE clearance is altered by behavioral stress, hormonal stimuli and disease process (Barker and Blakely, 1995), further analysis of mechanisms of NET regulation may provide important insights into modulation of noradrenergic signaling and loss of compromised regulation in autonomic dys- function and mental illness.

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References


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