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Melittin initiates dopamine transporter internalization and recycling in transfected HEK-293 cells

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Abstract

The dopamine transporter removes the neurotransmitter from the synapse, regulating dopamine availability. The transporter can be internalized and its function is blocked by cocaine and other ligands. Melittin inhibits dopamine transporter function and causes internalization of the recombinant transporter in stably transfected HEK-293 cells, but the specific pathways for internalization and disposition of the transporter are unknown. Here we report that melittin treatment increased both transporter internalization and colocalization with clathrin, effects that were blocked by pretreatment with cocaine. Density gradient centrifugation revealed that melittin treatment caused the dopamine transporter to associate with a density fraction containing the early endosome marker Rab 5A. Confocal microscopy revealed that melittin treatment also increased transporter colocalization with Rab 5A and decreased colocalization with the late endosome marker Rab 7 and the recycling endosome marker Rab 11. Following 60 min of melittin treatment, the transporter was trafficked back to the membrane. By comparison, phorbol ester treatment increased transporter colocalization with early endosome antigen 1 and Rab 7 in a time-dependent manner. Cocaine treatment alone does not affect transporter trafficking in these cells. Results indicate multiple dopamine transporter internalization and recycling pathways that depend on transporter-ligand interactions and post-translational modifications.

Keywords

Dopamine transporter; Melittin; Trafficking; Protein kinase

1. Introduction

The high affinity dopamine transporter removes neurotransmitter from the synapse (Parsons and Justice, 1994; Wightman and Zimmerman, 1990) and is a primary site of action of psychostimulants including cocaine and amphetamine (for review see (Zahniser and Doolen, 2001)). The dopamine transporter contains multiple *N*-glycosylation sites and putative protein kinase C (PKC) and protein kinase A (PKA) cytoplasmic phosphorylation sites (Amara and Kuhar, 1993).

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Dopamine transporter surface expression is regulated through PK-induced and constitutive trafficking pathways. Phorbol 12-myristate 13-acetate (PMA) activates PKC and increases transporter internalization in dopamine transporter-transfected cells (Daniels and Amara, 1999; Melikian and Buckley, 1999; Pristupa et al., 1998; Sorkina et al., 2003). The destination of internalized dopamine transporter varies across studies possibly due to differences in treatment conditions, transfected cell type, or visual detection tags fused to the transporters. Additionally, mitogen-activated PK regulates dopamine transporter function and surface expression in transfected cells and striatal preparations (Moron et al., 2003). Substrates (amphetamine, dopamine), which are actively transported, also decrease dopamine transporter function via decreases in surface expression in dopamine transporter-transfected cells and striatal synaptosomes (Chi and Reith, 2003; Saunders et al., 2000). Further, a model of constitutive dopamine transporter membrane trafficking involves a unique clathrin-dependent regulation pathway (Sorkina et al., 2005) distinct from transferrin receptor trafficking patterns (Loder and Melikian, 2003), and associated with endosomal Rab 7 or Rab 11, depending on cell type (Eriksen et al., 2010; Furman et al., 2009). However, uptake blockers that bind to the transporter, such as cocaine, either have no effect on or increase cell-surface expression of dopamine transporter (reviewed in Schmitt and Reith, 2010). While treatments with PMA and methamphetamine increase phosphorylation levels on the N-terminal region of the dopamine transporter (Vaughan et al., 1997), direct phosphorylation of the transporter is not necessary to mediate changes in transporter localization or function (Cervinski et al., 2005; Chang et al., 2001).

Melittin, a 26 amino acid amphiphilic peptide isolated from honeybee (*Apis mellifera*) venom, stimulates phospholipase A2 (PLA2) in neurons (Geddis et al., 2004; Muzzio et al., 2001) and multiple cell lines (Lee et al., 2001; Palomba et al., 2004; Tommasini et al., 2002), but not in our model HEK–dopamine transporter cells (Keith et al., 2011). Melittin inhibits dopamine uptake and causes an internalization of the dopamine transporter in our HEK-D₂-human dopamine transporter cells. Cocaine blocks this internalization but does not alter transporter surface expression. Thus, while melittin and cocaine both inhibit carbomethoxy-3 β -(4-iodophenyl)tropane ([¹²⁵I]RTI-55) binding to the transporter, they appear to affect transporter regulation through distinct mechanisms. Inhibition of dopamine transporter function by phorbol esters (Daniels and Amara, 1999) and amphetamine (Saunders et al., 2000) is mediated by endocytosis through dynamin-dependent clathrin-coated vesicles. To determine if a similar mechanism is involved in melittin-induced reduction of cell surface dopamine transporter, we used density gradient centrifugation and confocal microscopy to delineate the effects of melittin on dopamine transporter trafficking and localization in relation to clathrin and endosomal markers. We used confocal microscopy to directly compare the effects of melittin and PMA on pathways involved in transporter trafficking.

2. Materials and methods

2.1. Materials

Melittin, Optiprep, and PMA were purchased from Sigma-Aldrich (St Louis, MO USA). Antibodies used include rat anti-dopamine transporter purchased from Chemicon (Temecula, CA USA), mouse anti-Rab 11 and mouse anti-EEA purchased from Biodesign (Saco, ME USA), goat anti-clathrin, goat anti-Rab 7, and rabbit anti-Rab 5A purchased from Santa Cruz Biotechnologies (Santa Cruz, CA USA). The secondary antibodies goat anti-mouse, donkey anti-goat, and goat anti-rabbit conjugated to Alexa Fluora 568, and donkey anti-rat and goat anti-rat conjugated to Alexa Fluora 488 were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Stably transfected cell line

The cDNAs were cloned and human embryonic kidney-293 (HEK) cells were co-transfected with the cDNAs for the human dopamine transporter and the long form (D_{2L}) of the human dopamine D₂ receptor. The cloning and characterization of the human dopamine transporter and D₂ receptor cDNAs were described previously (Eshleman et al., 1995; Keith et al., 2011; Neve et al., 1989). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FetalClone (HyClone, Logan, UT USA), 0.05 U penicillin/streptomycin, 2 µg/ml puromycin, in a humidified 10% CO₂ incubator at 37 °C.

2.3. Density gradient centrifugation

A density gradient was generated using Optiprep (60% Iodixanol solution). Four solutions of Optiprep (2.5, 7.5, 12.5, and 17%) were prepared in HM buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4), layered in polyallomer centrifuge tubes (Beckman), and allowed to equilibrate overnight at 4 °C. Cells were grown on poly-D-lysine coated tissue culture plates until confluent, then rinsed and treated in PBS at 37 °C. Cells were then homogenized in 0.32 M sucrose containing protease inhibitors using a Teflon/glass homogenizer. The solution was centrifuged at 900×*g* for 10 min at 4 °C. The supernatant was then centrifuged at 12,000×*g* for 20 min at 4 °C. The resulting pellet was resuspended in HM buffer and layered above the prepared Optiprep gradient and centrifuged at 90,000×*g* for 3 h at 4 °C. Sixteen fractions of 750 µl were collected from each treatment condition. Fraction 1 represents the densest layer and fraction 16 is the least dense layer. Aliquots of each fraction, from the most (Fraction 1) to least (Fraction 16) dense, were separated by SDS-PAGE and transferred to PVDF membranes for western blotting and detection with the specified antibodies. Immunoreactivity was quantified by densitometry on a Typhoon phosphorimager using ImageQuant software (Molecular Dynamics; Sunnyvale CA, USA). Final densitometry of each band was corrected for the level of background in each lane.

2.4. [¹²⁵I]Carbomethoxy-3β-(4-iodophenyl)tropane (RTI-55) binding to the dopamine transporter

Experiments were performed as previously described (Eshleman et al., 1999) with some modifications. Total specific binding of [¹²⁵I]RTI-55 was determined in 25 µl aliquots of each fraction from the Optiprep density gradient. Assays were performed in triplicate in Krebs-Hepes buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1 µM pargyline, 100 µM tropolone, 2 mg glucose/ml, 1 mM ascorbic acid, pH 7.4), in a final volume of 250 µl. [¹²⁵I]RTI-55 (40 pM) was added and membranes were incubated for 90 min at room temperature in the dark. Nonspecific binding was determined in the presence of 10 µM mazindol. The assay was terminated by filtration through filtermat A filters (Perkin Elmer, Boston, MA, USA) using a 96-well Tomtec cell harvester (Hamden, CT, USA). Scintillation fluid was added to the filters and remaining radioactivity was determined using a Perkin Elmer 1205 betaplate scintillation counter.

2.5. Immunocytochemistry

For confocal microscopy, cells were treated and analyzed by a modification of previously described methods (Wang et al., 2005). Briefly, cells were plated on glass coverslips, treated with drugs or vehicle as indicated at 37 °C, fixed in 4% paraformaldehyde for 10 min at 37 °C, and then washed three times in 1 ml phosphate buffered saline (PBS: 136 mM NaCl, 25 mM KCl, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 0.5 mM CaCl₂, 2 mM MgCl₂) for 5 min at room temperature. To block non-specific reaction sites and permeabilize cell membranes, cells were incubated in PBS with 4% goat serum or donkey serum, 1% bovine serum albumin (BSA), and 0.2% Triton-X 100 for 1 h. Primary antibody was added in PBS with 0.25% BSA, 0.2% Triton-X 100 and the preparation was incubated for 1 h. The antibodies

were used at the following dilutions: rat anti-dopamine transporter, 1:250; goat anti-clathrin, 1:500; goat anti-Rab 7, 1:200; mouse anti Rab 11, 1:200; mouse anti-early endosome antigen 1 (EEA1), 1:200; rabbit anti-Rab 5A, 1:250. The cells were washed 3 times (1 ml, 5 min each) with PBS. Secondary antibodies were prepared in PBS with 1% goat or donkey serum and cells on coverslips were incubated at room temperature for 1 h. Coverslips were mounted on glass microscope slides using Prolong Antifade kit (Invitrogen, Carlsbad, CA USA), and cells were imaged using a laser-scanning confocal Leica TCS SP microscope with 100× objective. Consecutive sections were sequentially scanned alternating between 488 and 568 nm lasers, and sections were imaged at 300 µm intervals. Application of only the secondary antibodies to prepared cells did not produce detectable immunostaining.

2.6. Colocalization

The colocalization of dopamine transporters with each subcellular protein marker was determined by identifying the coordinates of each fluorescent pixel captured from the 488 nm laser and the 568 nm laser. The brightness intensity of each scanned image was divided into 256 Gy-scale values. The gray scale of fluorescently unsaturated images was proportional to the amount of fluorophore present. Green and red pixels with the same coordinates in each scan section were identified as dually labeled (colocalized). The extent of colocalization was determined from the ratio of colocalized fluorophore to the total fluorophores in the cell. The spatial resolution of colocalization was limited to the voxel measurements of 0.195 µm length×0.195 µm width× 0.25 µm depth. Colocalization was quantified for each image using IP Lab software (Fairfax, VA USA). One-way ANOVA was used to compare the changes in colocalization of dopamine transporters and marker proteins in control and treated cells. The ratio of total fluorescence in the green (Alexa Fluor 488) channel and the total fluorescence in the red (Alexa Fluor 568) channel was calculated for each image to verify that there were no changes in the levels of each protein. The Pearson's correlation coefficient was also calculated for each image using Image J.

2.7. Biotinylation of membrane proteins

To measure the dopamine transporters remaining on the cell surface following treatment, cells were treated with drugs or vehicle in DMEM at 37 °C, media was removed, and reaction plates were placed on ice. Cells were then incubated with 0.3 mg/ml NHS-SS-biotin (Pierce, Rockford, IL) in PBS at 4 °C with gentle agitation for 40 min. Unbound biotin was removed by rinsing with 0.1 M glycine in PBS. Cells were then rinsed with CHAPS buffer (1% CHAPS, 25 mM Tris, pH 7.4, 150 mM NaCl) and lysed using a glass-glass homogenizer. The samples were centrifuged at 14,000 g, and the supernatants were reserved. The protein concentration of each sample was determined using the BCA Assay kit (Pierce, Rockford, IL). Equal protein amounts from each sample were incubated with ImmunoPure™ Immobilized streptavidin (Pierce, Rockford, IL) for 40 min at 4 °C with gentle agitation to isolate biotinylated from nonbiotinylated proteins. Proteins were eluted from the streptavidin beads with 30 µl of Laemmli sample buffer (Sigma, St Louis, MO) and constant mixing for 20 min. Samples were separated by SDS-PAGE and transferred to PVDF membranes for western blotting and detection with anti-dopamine transporter antibody (Chemicon, Temecula, CA). Immunoreactivity of human dopamine transporter was quantified by densitometry on a Typhoon phosphorimager using ImageQuant software (Molecular Dynamics; Sunnyvale CA).

3. Results

3.1. Melittin modulates dopamine transporter distribution in equilibrium density gradients

Using a cell line stably transfected with the dopamine transporter, we previously discovered that 30 min of treatment with melittin induced transporter internalization (Keith et al., 2011).

In order to more fully understand the internalization pathway we utilized equilibrium density gradient centrifugation to determine which subcellular organelle associates with the dopamine transporter following melittin (1.3 μ M) treatment. The cell lysates were separated into 16 fractions using Optiprep density gradient centrifugation. For all fractions, binding of the cocaine analog [125 I]RTI-55 was used to determine the distribution of the dopamine transporter and immunoblotting was used to determine the distribution of each subcellular organelle marker protein. Following a 30 min melittin treatment [125 I]RTI-55 binding was significantly decreased ($P<0.05$) in fraction 12, while there was a significant increase ($P<0.05$) in [125 I]RTI-55 binding in fraction 7 (Fig. 1A). Following 60 min of melittin treatment (1.3 μ M) there were no detectable changes in dopamine transporter distribution in the equilibrium density gradient fractions, compared to vehicle treated control cells, (Fig. 1B).

Optiprep samples indicated that fractions 7 and 8 included subcellular organelle marker protein Rab 5A immunoreactivity (Fig. 2A). The distribution of the early endosome markers Rab 5A and EEA1 only overlapped in the least dense fractions, 15 and 16 (Fig. 2A, B, E, and F). The recycling endosome marker Rab 11 was predominantly found in fraction 8 (Fig. 2C and G), while the late endosome marker Rab 7 was mostly isolated in the more dense fractions 1–4 (Fig. 2D and H). Melittin treatment did not significantly alter the distribution of any of these marker proteins. Interestingly, there is considerable overlap in the distribution of the Rab molecules in the density gradient. Since this procedure only separates structures based on density, not cellular distribution, this experiment demonstrates the similar densities of the endosomal membranes containing the various Rab proteins.

3.2. Melittin alters colocalization of dopamine transporters with subcellular proteins

Density fractionation indicates that melittin alters dopamine transporter localization within cells, but it is impossible to determine accurately which subcellular organelle it is associated with due to the overlap of endosomal marker densities. To further clarify the melittin-induced changes in dopamine transporter redistribution, we utilized immunocytochemistry to more precisely localize the dopamine transporter during the internalization pathways. The PKC activator, PMA, was used as a comparison since it also causes dopamine transporter internalization (Daniels and Amara, 1999; Melikian and Buckley, 1999; Pristupa et al., 1998; Sorkina et al., 2003). Multiple methods verified the effect of PMA on transporter function and cellular localization. In agreement with others (Cervinski et al., 2005; Foster et al., 2008; Miranda et al., 2005; Sorkina et al., 2005; Vaughan et al., 1997) PMA treatment of HEK-dopamine transporter cells decreased transporter function as measured by rotating disk electrode voltametry, decreased protein levels as measured by [125 I]RTI-55 binding, and decreased cell surface protein as measured by biotinylation (data not shown).

Dual laser confocal microscopy was used to analyze the colocalization of the dopamine transporter with several cellular proteins. PMA induces transporter internalization through clathrin-dependant mechanisms (Daniels and Amara, 1999; Foster et al., 2008; Sorkina et al., 2005). Consistent with the previous reports, we observed that 30 min treatment with PMA (100 nM) causes a 3.3 fold increase in dopamine transporter colocalization with clathrin (data not shown). To determine whether melittin-induced dopamine transporter internalization was mediated by clathrin, cells were dual-labeled using antibodies for the dopamine transporter and for endogenous clathrin. The ratio of dopamine transporter colocalized with clathrin compared to total levels of dopamine transporter in each cell was used to detect trafficking changes (Fig. 3). Treatment with 1.3 μ M melittin for 30 min caused a significant increase in total dopamine transporter colocalized with clathrin (4.2 ± 0.4 fold increase over control, $P<0.01$), suggesting the transporter is internalized through clathrin coated pits. Regions of colocalization can be seen near the membrane (Fig. 3). To prevent the formation of the clathrin structure, cells were treated with 0.45 M sucrose

(Heuser and Anderson, 1989) for 15 min prior to melittin treatment. Neither sucrose alone, nor sucrose followed by melittin treatment, significantly altered dopamine transporter colocalization with clathrin compared to control cells ($P<0.05$, Fig. 3B).

Pretreatment with 1 μM cocaine for 45 min did not significantly alter clathrin co-localization as compared to control values, but pretreatment with cocaine for 15 min before and during the 30 min melittin treatment prevented the dopamine transporter colocalization with clathrin. This level of colocalization was significantly less than the effect of melittin treatment alone ($P<0.01$) and was comparable to control levels of colocalization. To determine if these effects of melittin were dependent on protein synthesis, cells were treated with the protein synthesis inhibitor cyclohexamide (350 μM) for 1 h. The cells were then either treated with melittin or vehicle for 30 min. Colocalization of the dopamine transporter with clathrin following treatment with cyclohexamide alone was not significantly different than control levels, and cyclohexamide treatment followed by melittin caused a significant increase in dopamine transporter colocalization with clathrin (3.5 ± 0.9 fold increase, $P<0.05$). This increase is not significantly different than melittin treatment alone ($P<0.05$) (Fig. 3B). These results indicate that melittin-induced internalization of the dopamine transporter (Keith et al., 2011) is mediated by clathrin-dependant mechanisms.

The destination of internalized dopamine transporter can be determined by increases in colocalization with proteins used as markers for different subcellular endosomal trafficking compartments. To further examine the trafficking pathways engaged by melittin treatment, confocal microscopy was used to measure dopamine transporter colocalization with endosomal markers (Fig. 4). Cells on glass coverslips were treated with melittin or vehicle, fixed, stained, and imaged as described above. Antibodies recognizing EEA1 and the small GTPase Rab 5A were used as markers for the early endosome, an antibody recognizing the small GTPase, Rab 7, was used as a late endosome marker, and an antibody recognizing the small GTPase Rab 11 was used as a marker for the recycling endosome (Pfeffer, 2001; Sonnichsen et al., 2000; Zerial and McBride, 2001). In polarized cells EEA1 does not colocalize with clathrin (Wilson et al., 2000). GTPases are involved in vesicle docking and tethering and are localized to distinct intracellular subdomains (Pfeffer, 2001). Previous studies have used colocalization or co-occurrence of EEA1, Rab 11, and Rab 5A to track dopamine transporter trafficking following PKC stimulation (Melikian and Buckley, 1999; Sorkina et al., 2003).

Melittin treatment (1.3 μM , 30 min) caused a significant increase in dopamine transporter colocalization with the early endosome marker Rab 5A (1.49 ± 0.2 fold change, $P<0.05$; Fig. 4E), while not affecting colocalization following 15 min (0.86 ± 0.2 fold change) or 60 min (1.0 ± 0.2 fold change) of treatment. Melittin did not significantly alter dopamine transporter colocalization with EEA1 following 15, 30, or 60 min of treatment (1.3 ± 0.42 , 0.8 ± 0.1 , 1.1 ± 0.33 fold change, respectively, $P<0.05$, Fig. 4F). However, melittin treatment (30 min) caused a significant reduction in dopamine transporter colocalization with the recycling endosome marker Rab 11 (0.5 ± 0.1 fold change, $P<0.05$, Fig. 4G) and a significant reduction in colocalization with the late endosome marker Rab 7 (0.4 ± 0.1 fold change, $P\pm0.001$, Fig. 4H). There were no significant changes in colocalization patterns following 15 or 60 min of treatment.

To directly compare these results, in the same experiments, with other known trafficking patterns and to verify the validity of this quantification method, cells were treated with the phorbol ester PMA. Activation of the PKC signaling pathway with PMA reduces dopamine transporter-mediated uptake by increasing internalization (Daniels and Amara, 1999; Melikian and Buckley, 1999; Sorkina et al., 2003). Treatment with 100 nM PMA for 30 min caused a significant increase in dopamine transporter colocalization with EEA1 (2.3 ± 0.2

fold increase over 0.1% DMSO control, $P<0.05$, Fig. 4F). The effects of PMA on dopamine transporter colocalization with Rab 5A (Fig. 4E) and Rab 11 (Fig. 4G) were not significant at any treatment time point. Interestingly, PMA treatment (60 min) caused a significant increase in dopamine transporter colocalization with the late endosome marker Rab 7 (2.3 ± 0.3 fold increase, $P<0.05$, Fig. 4H). Thus the effects of melittin on internalized dopamine transporter disposition differ substantially from those of PMA.

To compare the melittin results with an antagonist of the transporter, cells grown on coverslips were treated with 1 μ M cocaine or vehicle for 0, 15, 30, or 60 min. The fixed, permeabilized, and blocked cells were colabeled as described above. Cocaine treatment did not significantly alter dopamine transporter colocalization with any of the chosen markers ($P<0.05$, Fig. 4E–H). This is in agreement with our previous findings that cocaine treatment does not alter dopamine transporter internalization in these cells (Keith et al., 2011).

We additionally analyzed changes in colocalization by calculating the Pearson's correlation coefficient for each of the treatments. The Pearson's correlation coefficient determines the linear dependency between the two channels without being affected by intensity values (Adler and Parmryd, 2010). The Pearson's correlation analyses confirmed the significant increases and decreases in colocalization of dopamine transporter with the marker proteins. Additionally, we found no significant differences in the ratios of total green fluorescence to total red fluorescence between control and treated cells, indicating the changes in colocalization were not due to changes in protein expression or detection.

3.3. Melittin reduces cell surface biotinylation of dopamine transporter

The lack of a melittin effect, observed in both the density gradient and microscopy, following 60 min of treatment, in conjunction with our previous findings that 30 min of melittin treatment causes an increase in dopamine transporter internalization (Keith et al., 2011), suggest that the dopamine transporter is trafficked back to the membrane. To explore this possibility cells were treated with melittin for 60 min and proteins remaining on the membrane after treatment were biotinylated. Cell surface, biotinylated proteins were isolated by avidin purification and separated by SDS-PAGE. The level of cell surface dopamine transporter following 60 min of melittin treatment was not significantly different from controls ($96.5\pm 8.7\%$; $P<0.05$).

To inhibit protein synthesis, cells were treated with 350 μ M cyclohexamide for 1 h prior to 1 h of melittin treatment. Cyclohexamide did not significantly alter dopamine transporter membrane expression ($117.1\pm 11.0\%$ of control), nor did it alter dopamine transporter membrane expression following melittin treatment ($106.3\pm 9.0\%$ of control).

Bafilomycin inhibits recycling of endocytosed proteins, such as the transferrin receptor, to the membrane by inhibiting the vesicular H^+ ATPase (Crippa et al., 2006; Loder and Melikian, 2003). Cells were treated with 1 μ M bafilomycin for 30 min prior to melittin treatment. Bafilomycin alone did not significantly alter dopamine transporter membrane expression ($81.7\pm 10.9\%$ of control), but following melittin treatment the level of dopamine transporter on the membrane was significantly reduced to $45.4\pm 4.8\%$ of control ($P<0.01$, Fig. 5). These results support the hypothesis that the dopamine transporter is trafficked back to the plasma membrane following 60 min of melittin treatment.

4. Discussion

We reported previously that melittin interacts with the dopamine transporter to inhibit antagonist binding, and this interaction causes transporter internalization (Keith et al., 2011). In the current study we examined trafficking patterns of the internalized dopamine

transporter following melittin treatment. As assessed by confocal microscopy, melittin caused an increase in colocalization of dopamine transporter with endogenous clathrin (Fig. 3B), suggesting that the transporter is internalized through clathrin-coated pits, a process prevented by cocaine pretreatment. These data agree with our previous findings (Keith et al., 2011) using cell surface biotinylation to demonstrate that melittin-induced internalization of the dopamine transporter is blocked by pretreatment with cocaine. A similar method detected colocalization of the GABA transporter with GFP-labeled clathrin and demonstrated that the GABA transporter is also internalized through clathrin-coated pits (Deken et al., 2003). Interestingly, the initial clathrin-dependent process of dopamine transporter internalization following treatment with melittin is similar to that observed following PMA treatment. Direct injection of melittin into the brain can cause sensitization to subsequent psychostimulant treatment (Reid et al., 1996) and can induce axonal regeneration of noradrenergic neurons (Nakamura, 1993). These effects suggest the need to further investigate the mechanisms of melittin in an effort to understand various neuropsychiatric disorders and to develop appropriate neuro-interventions. Our studies provide the first investigations into these mechanisms and lay the foundation for further studies.

In our control cells, 6.2% of the total dopamine transporter colocalized with clathrin, which may represent constitutive recycling during basal cellular activity. Following 30 min of melittin treatment, internalized dopamine transporter was found in Rab 5A-containing endosomes (Fig. 4) and continued to be internalized by clathrin-coated pits. After 60 min of melittin treatment, the dopamine transporter level at the cell surface was similar to that in untreated cells (Fig. 5). Clathrin-coated buds associated with transferrin receptors form from early endosomes (Futter et al., 1998; Stoorvogel et al., 1996) and are distinct from vesicles originating at the plasma membrane. Thus, clathrin may play a role in recycling vesicles back to the plasma membrane as well as in endocytosis, supporting the possibility that the dopamine transporter internalized by melittin is recycled back to the membrane.

The dopamine transporter colocalized with clathrin represents internalizing transporter rather than newly synthesized transporter trafficking from the golgi complex, as the colocalized transporter is not decreased following treatment with cyclohexamide (Fig. 3B). Our method does not distinguish between clathrin-dependent endocytosis and clathrin-dependent exocytosis from a recycling pathway. In rat hippocampal neurons Rab 3 associates with vesicles recycling to the membrane, while Rab 5A is excluded from these recycling vesicles (Star et al., 2005). Thus, the dopamine transporter associated with Rab 5A-containing endosomes, here, likely represents only the endocytosed transporter and not transporter trafficking back to the membrane. It is surprising that the dopamine transporter was internalized following 30 min of melittin treatment but membrane-associated transporter was unchanged following 60 min of treatment, without being detected in the Rab 11-associated recycling endosome. The melittin-induced dopamine transporter recycling pathway in HEK cells may not utilize Rab 11-associated membranes, but instead cycle through membranes associated with another protein such as Rab 4. In HEK cells transfected with AT1 angiotensin receptor, agonist stimulation causes receptor internalization and then recycling to the cell surface through Rab 4-associated membranes distinct from Rab 11-associated membranes (Hunyady et al., 2002). Similarly, results of experiments with MDCK cells co-transfected with the transferrin and the polymeric immunoglobulin receptors suggest that most recycling to the basolateral surface occurs directly through early endosomes, rather than recycling endosomes (Sheff et al., 1999). Measurement of dopamine transporter colocalization with additional proteins, such as Rab 4, following melittin treatment may further elucidate this trafficking pathway and explain why the transporter did not colocalize with Rab 11 prior to recycling back to the membrane.

In these cells, the trafficking patterns initiated by melittin and PMA were significantly different. Although both PMA and melittin increased colocalization of dopamine transporter with clathrin, PMA initially increased colocalization of transporter with EEA1 and subsequently with the late endosome marker Rab 7, suggesting that the transporter may be trafficked for degradation under these conditions. We observed that melittin-induced internalization of the dopamine transporter was blocked by pretreatment with cocaine, and cocaine treatment did not alter transporter colocalization with any subcellular organelle markers (Fig. 4). Cocaine treatment does not alter transporter surface expression or the PMA-induced transporter internalization (Daniels and Amara, 1999), supporting the hypothesis that melittin affects different mechanisms than PMA and cocaine to alter dopamine transporter function and localization.

Use of the Optiprep equilibrium density gradient is instrumental in demonstrating the trafficking patterns of internalized dopamine transporter over the course of melittin treatment. Following 30 min of melittin treatment the distribution of the dopamine transporter through the density fractions indicated that the transporter associated with a density fraction that contained Rab 5A. This data supports the confocal colocalization data suggesting that the dopamine transporter was internalized to Rab 5A-associated endosomes or vesicles. Immunodetection of early endosome markers Rab 5A and EEA1 indicated that they were not always associated with the same fractions, but overlapped in the low-density fractions. The difference in distribution could explain the differences in dopamine transporter colocalization with these markers seen using confocal microscopy. Electron microscopy indicates that EEA1 partially colocalizes with Rab 5A and the transferrin receptor, but not with clathrin (Wilson et al., 2000), while Rab 5A does colocalize with clathrin (for review see (Zerial and McBride, 2001)). If other agents, such as sucrose, are used to generate a density gradient, the distribution of cellular organelles across fractions may differ as compared to those seen with the Optiprep gradient. Use of a 10–50% sucrose gradient resulted in more overlap between EEA1 and Rab 5A (Melikian and Buckley, 1999) than our Optiprep iodixanol gradient results.

Immunodetection of subcellular organelles in equilibrium density gradient fractions indicated that melittin treatment did not significantly alter distribution of the organelle markers (Fig. 2). Changes in colocalization of the dopamine transporter with each marker protein may represent altered distribution of the transporter rather than melittin-induced disruptions of the endogenous internalization pathways. Interestingly, there was considerable overlap in the distribution of Rab 5A and Rab 11 in the density gradient. Since this procedure only separated structures based on density, not cellular distribution, this experiment demonstrated the similar densities of the endosomal membranes containing the various Rab proteins.

Confocal microscopy demonstrated that 30 min of melittin treatment decreased dopamine transporter colocalization with the recycling endosome marker Rab 11 and the late endosome marker Rab 7 (Fig. 4), yet the density gradient experiments did not yield similar decreases (Fig. 2). This may represent different sensitivities between the two methods. Using a 5–20% Optiprep density gradient collected as 34 equal fractions, recycling (Rab 11) and early endosome (Rab 4) markers are separated, with Rab 11 in the lower density fraction (Sheff et al., 1999). Thus, it is possible that more density fractions would provide higher resolution of Rab 11 and Rab 5A and may further explain the differences seen in dopamine transporter colocalization with the markers that were analyzed using confocal microscopy.

Studies using subcellular fractionation demonstrate a PMA-activated and a constitutive dopamine transporter recycling pattern coinciding with transferrin receptor trafficking through recycling endosomes (Melikian and Buckley, 1999), while studies utilizing confocal

microscopy and fluorescence resonance energy transfer demonstrate PMA-induced transporter colocalization with early and recycling endosomes (Sorkina et al., 2003), or sequential colocalization with transferrin then, lysosomal markers (Daniels and Amara, 1999). Additionally, in cultured neurons dopamine transporter membrane localization was unaffected by PMA treatment (Eriksen et al., 2009). These variances may result from trafficking differences between cell types, tags on the dopamine transporter, or the differences in PMA concentration and treatment time. In our cells, the dopamine transporter trafficking pattern following melittin treatment was unlike that following PMA treatment (Fig. 4) and throughout 60 min of treatment. Thus, the effects of melittin on dopamine transporter function and trafficking are likely mediated through distinct mechanisms utilizing a pathway that does not coincide with the PMA-stimulated PKC pathway.

Melittin binding to the dopamine transporter causes internalization, but it is not known if melittin is removed from the transporter before reinsertion into the membrane. It is also unclear if the acidic environment of early endosomes causes melittin to disassociate from the transporter. If melittin dissociates, internalization may serve a protective function to remove dopamine transporter antagonists for proper cell function, which would be distinct from the cellular response to antagonists such as cocaine. This study does not address these questions, but an antibody generated against melittin, or a fluorescently-labeled melittin (Postupalenko et al., 2011) would facilitate its tracking relative to the dopamine transporter, endosomal markers, and other trafficking proteins. In addition, whether melittin causes internalization of only those dopamine transporters not associated with lipid rafts, as PMA does, remains a question for future studies (Foster et al., 2008). The unique, melittin-induced dopamine transporter trafficking pattern indicates another level of regulation for transporter function, and also the varied cellular responses possible with selected treatments.

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Fig. 1.

Melittin shifts dopamine transporter distribution in equilibrium density gradient. Cells were treated with melittin (open squares) or vehicle control (closed squares) for 30 min (A) or 60 min (B). The cells were gently homogenized, layered over an Optiprep density gradient, and centrifuged to equilibrium. Sixteen equal volume fractions were collected. Binding of [125 I]RTI-55 to the dopamine transporter was measured in each fraction. The specific binding in each fraction is represented as the percentage of total specific binding across all fractions. The results are the mean $\pm$ S.E.M from 5 independent experiments (*, $P < 0.05$; ANOVA followed by Bonferroni test).

Fig. 2.

Distribution of subcellular markers across equilibrium density gradient. Cells were treated with melittin (open squares) or vehicle control (closed squares) for 30 min (A)–(D) or 60 min (E)–(H). The cells were gently homogenized, layered over an Optiprep density gradient, and centrifuged to equilibrium. Sixteen equal volume fractions were collected with fraction 1 being the most dense. Protein samples from each fraction were isolated by SDS-PAGE. The immunoblots were detected with anti-Rab 5A (A) and (E), anti-EEA1 (B) and (F), anti-Rab 11 (C) and (G), or anti-Rab 7 (D) and (H) antibodies. Representative blots of each antibody are shown for control samples. Band optical density in each fraction was quantified and expressed as percent of total band intensity across all fractions. The results are the mean $\pm$ S.E.M from three to four experiments for each antibody.

Fig. 3.

Melittin increases dopamine transporter colocalization with clathrin. A. Cells were treated with vehicle control or melittin for 30 min. The cells were then fixed, permeabilized, and colabelled for dopamine transporter and endogenous clathrin. The immunoreactivity was imaged by confocal microscopy as described in the text. The representative fluorescent images show dopamine transporter (green) and endogenous clathrin (red). The colocalized image shows only those pixels containing both green and red fluorophores (pseudocolor yellow). The experiment was repeated 3 times with similar results using 9–18 independent images per condition. B. Cells grown on coverslips were treated with melittin (1.3 μ M) for 30 min, cocaine (1 μ M) for 45 min, cocaine for 15 min prior and during 30 min melittin, sucrose (0.45 M) for 45 min, sucrose 15 min prior and during 30 min melittin, cyclohexamide (cyclohex, 350 μ M) for 90 min, or cyclohexamide 60 min prior and during melittin treatment. The cells were then fixed, permeabilized, and colabeled for dopamine transporter and endogenous clathrin. Immunoreactivity was imaged using confocal microscopy. Colocalization was quantified as described in the text. The graph represents the changes in dopamine transporter immunoreactivity that is colocalized with clathrin immunoreactivity for each treatment condition. The results are presented as a fold change of colocalization in each treatment condition relative to colocalization in vehicle treated cells. The results are the means $\pm$ S.E.M of 3 independent experiments with 8–10 independent images per condition (**, $P < 0.01$; *, $P < 0.05$ ANOVA followed by Tukey's multiple comparison test). There were no significant differences in the ratios of total green fluorescence to total red fluorescence for any of the treatment conditions compared to control conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Melittin and PMA alter dopamine transporter colocalization with endosomal markers Cells grown on coverslips were treated with melittin, PMA, cocaine or vehicle control as indicated. Images demonstrate colabeling for transfected dopamine transporter and endogenous Rab 5A (A), EEA1 (B), Rab 11 (C), or Rab 7 (D). These representative images are of markers and times that were significant across multiple experiments. The fluorescent images show the immunodetection of each marker and the colocalized images show only those pixels that contain fluorophores for both dopamine transporter and the marker protein (pseudocolor yellow). The graphs show the fold change in dopamine transporter colocalization with each marker relative to colocalization in vehicle treated control cells at each treatment time. Cells were treated with melittin (solid bars), PMA (open bars), or cocaine (gray bars) for 15, 30, or 60 min. The cells were then fixed, permeabilized, and colabeled for dopamine transporter and endogenous Rab 5A (E), EEA1 (F), Rab 11 (G), or Rab 7 (H) as described in the text. The results are the means $\pm$ S.E.M from 3 experiments collecting 6–10 independent images per treatment (*, $P<0.05$; ANOVA followed by Bonferroni test). There were no significant differences in the ratios of total green fluorescence to total red fluorescence for any of the treatment conditions compared to untreated control conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

The dopamine transporter traffics to the membrane following 60 min of melittin treatment. HEK-D₂R-human dopamine transporter cells were treated with vehicle or melittin (1.3 μ M) for 60 min, cycloheximide (350 μ M) alone or for 60 min prior and during 60 min melittin treatment, or bafilomycin (1 μ M) alone or for 30 min prior and during 60 min melittin (1.3 μ M) treatment. Following treatment the cells were rapidly chilled and the media removed. The proteins remaining on the surface were biotinylated, isolated by avidin purification, and separated by SDS-PAGE. The immunoblot was detected with anti-human dopamine transporter antibody. The representative blot shows total surface biotinylated product for control and treated cells. Band optical density was quantified and expressed as a percent of vehicle treated control. Graph: Cell surface dopamine transporter expressed as means $\pm$ S.E.M (**, $P < 0.01$; ANOVA followed by Dunnetts's multiple comparisons test).