The Interaction of Melittin with Membrane Fatty Acids and the Dopamine Transporter

by

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CERTIFICATE OF APPROVAL

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In loving memory of

Joanne Jean Keith

Table of Contents

List of Tables and Figures

Abbreviations

AA	arachidonic acid
ATPase	adenosine triphosphatase
cAMP	adenosine 3',5'-cycl ic monophosphate
CHO	chinese hamster ovary
COX	cyclooxygenases
DA	dopamine
DAG	diacylglycerol
DAT	dopamine transporter
D2R	dopamine D2 receptor
EAAT	excitatory amino acid transporter
EEA1	early endosome antigen 1
GLC	gas-liquid chromatography
G-protein	heterotrimeric guanosine triphosphate binding protein
GTP	guanosine triphosphate
HEK	human embryonic kidney 293
HPETE	hydroperoxy-eicosatetraenoic acid
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
6-OHDA	6-hydroxydopamine
PAF	platelet-activating factor
PBS	phosphate buffered saline
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin
TBS	tris buffered saline
TH	tyrosine hydroxylase
VTA	ventral tegmental area

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Abstract

The dopamine (DA) system in the brain modulates events such as movement and motivational control, and rapid removal of released DA from the synapse is important to enable spatial and temporal coordination of neurotransmission. The primary means of removing DA from the synapse is through the high affinity DA transporter (DAT), and recent evidence has begun to establish regulation of the DAT as a key point in regulating DA neurotransmission. Studies addressing DAT regulation are essential to understanding the neurobiological changes associated with psychostimulant drug addiction, as cocaine and amphetamine use are known to alter DAT function and membrane expression.

There are many cell signaling pathways that may be involved in DAT regulation, including protein kinase C, nitric oxide, mitogen activated protein kinase, and arachidonic acid (AA)-associated pathways. The primary goal of this research was to characterize the effects of activation of the AA cascade on the regulation of the DAT. AA is a 20 carbon, polyunsaturated fatty acid that is found in most cell types and has a role in a variety of signaling functions, including initiating long term potentiation, activating ion channels, and mediating tissue inflammation and pain response, depending on the particular site of action.

Phospholipase (PL) A2 activation causes release of AA from membrane phospholipids. We examined the pharmacological action of the purported

PLA2 stimulator melittin and how it affected DAT function (aim 1) and trafficking (aim 2). The experiments were performed in human embryonic kidney-293 (HEK) cells stably expressing the human form of the DAT and the long form of the DA D2 receptor (D2), representing a simplified model of a dopaminergic cell.

We used the purported PLA2 stimulator, melittin, to examine the effects of endogenous AA signaling on DAT function and trafficking. Melittin inhibited DAT function, and subsequent experiments measuring binding of a cocaine analogue to the DAT demonstrated a direct interaction of melittin with the transporter to inhibit antagonist binding. Additionally, measurements of membrane-incorporated fatty acid content demonstrated a melittin-induced release of AA, but phospholipase inhibitors did not prevent the release. We also found that melittin interacts directly with other membrane spanning proteins, including DA receptors. These results suggest that melittin does not function as a specific PLA2 stimulator in HEK cells.

To examine the effect of melittin on DAT membrane expression and trafficking we used biotinylation, confocal microscopy, and equilibrium density gradient centrifugation. While melittin did not act in the expected manner, the direct interaction of melittin with the DAT did cause an increase in transporter internalization, as pretreating the cells with cocaine blocked this effect. We used confocal microscopy to measure colocalization of the DAT with markers of subcellular organelles involved in endocytic membrane

trafficking and recycling, thus determining the effect of melittin on DAT membrane expression and trafficking. Melittin treatment caused an increase in DAT colocalization with the early endosome marker RAB 5A, and caused a decrease in colocalization with the late endosome marker RAB 7 and the recycling endosome marker RAB 11. We also treated cells with a phorbol ester that is known to activate protein kinase C and cause DAT internalization. By comparison, phorbol ester treatment increased DAT colocalization with the early endosome marker EEA1 (early endosome antigen 1) after 30 min of treatment, and increased DAT colocalization with RAB 7 after 60 min of treatment. Cocaine treatment did not affect DAT trafficking in these cells. Experiments using density gradient centrifugation confirm that melittin treatment causes the DAT to become associated with a density fraction containing the early endosome marker RAB 5A after 30 min of treatment. Interestingly, after 60 min of melittin treatment the DAT is recycled back to the cell membrane. Thus, melittin acts through multiple mechanisms to regulate cellular activity, including release of membrane-incorporated fatty acids and direct interaction with the DAT. Future experiments examining the regulation of AA signaling by the DA D2 receptor will be instrumental in understanding the multitude of signaling pathways originating from receptor activity, and how this affects global dopaminergic system function.

Introduction

The dopamine (DA) system in the brain is involved in mediating events such as movement, emotion, food intake, positive reinforcement and motivational control. Abnormalities in dopaminergic neurotransmission are involved in many pathological conditions such as attention deficit hyperactivity disorder (Solanto 2002), Parkinson's disease (Steece-Collier et al. 2002), and schizophrenia (Seeman 1987). A thorough understanding of how DA neurotransmission works on a system, cellular, and molecular level is important for developing treatments and enabling prevention of these disorders. This dissertation addresses some of the cellular signaling pathways involved in regulating DA neurotransmission, and discusses them in the context of schizophrenia and psychostimulant drug abuse.

Dopamine: system and cells. DA neurons are found in a variety of regions of the central nervous system, including the olfactory bulb, the nucleus tractus solitarius, and the arcuate and periventricular nuclei of the hypothalamus (Cooper et al. 2003). Studies concerning disregulation of the DAergic system in schizophrenia, chronic psychostimulant drug abuse, and Parkinson's disease predominantly examine the DA cells that originate in the ventral tegmental area (VTA) and the substantia nigra pars compacta (Epstein et al. 1999;Pierce and Kalivas 1997a;Hornykiewicz 2001). The neurons of the VTA project to the cerebral cortex, forming the mesocortical DA system. VTA neurons also project to the limbic areas, including the

hippocampus, amygdala, nucleus accumbens and limbic cortex, thus forming the mesolimbic DA system. Neurons of the substantia nigra project to the striatum to form the nigrostriatal DA system (Figure 1-1). All of these DA neurons synthesize DA from the amino acid precursor L-tyrosine, with L-

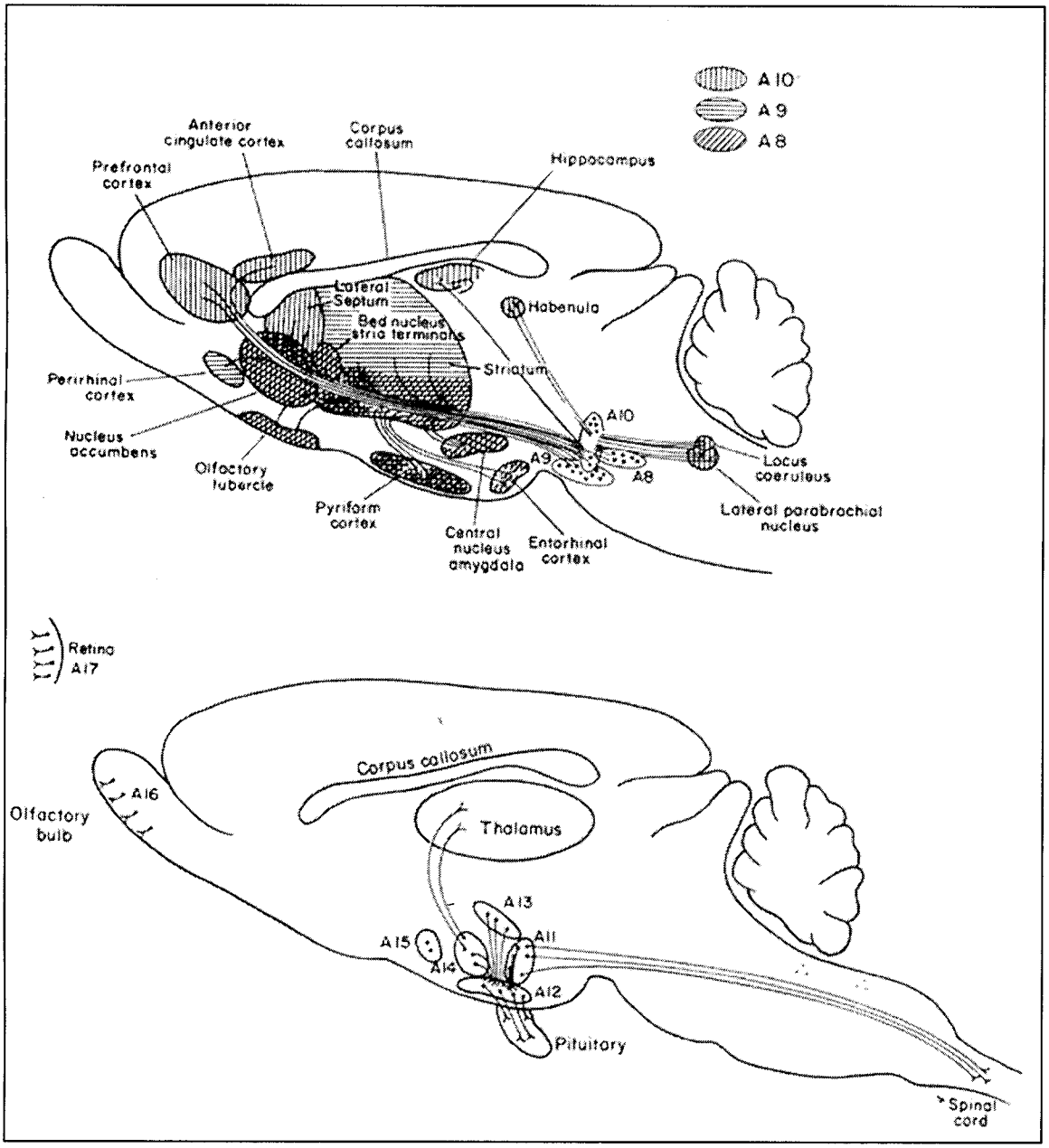


Figure 1-1: Dopaminergic projections in the brain
Cooper 2003

DOPA as an intermediate product. The vesicular monoamine transporter (VMAT2) then packages DA in to vesicles in the neuronal axon terminals. (Cooper et al. 2003).

DA neurotransmission can be regulation at multiple sites. The enzyme tyrosine hydroxylase (TH) converts L-tyrosine to L-DOPA, and this reaction is the rate-limiting step in the production of DA. Nerve terminal depolarization stimulates TH activity. TH activity is attenuated by end product inhibition by DA and by activation of autoreceptors. Autoreceptor activation also inhibits DA release (Cooper et al. 2003).

DA that is released into the synapse mediates its effects by acting on guanosine triphosphate (GTP) binding protein (G-protein)-coupled DA receptors. DA receptors are located on postsynaptic cellular membranes and presynaptic terminals, and are categorized as either D1-like or D2-like, based on their function and pharmacological profile. The D1-like receptors (D1 and D5) are coupled to stimulatory G-proteins to activate adenylyl cyclase and increase levels of cyclic adenosine monophosphate (cAMP). The D2-like receptors (D2, D3, and D4) are coupled to inhibitory G-proteins to inhibit adenylyl cyclase and decrease levels of cAMP. G-proteins derive energy from GTP hydrolysis to translate a plasma membrane receptor signal into an intracellular signaling cascade. Pertussis toxin (PTX), which inhibits G-proteins in the G_i/G_o families, inhibits D2 receptor signaling (Cooper et al. 2003;Missale et al. 1998). PTX ADP-ribosylates this specific type of G-

protein, which prevents GTP hydrolysis and thus prevents signal activation. This use of PTX serves as a valuable tool to distinguish D1- and D2-like receptors, and also to inhibit signaling through the G_i/G_o-proteins (Hertting et al. 1990;Neve et al. 2004).

THE DOPAMINE TRANSPORTER

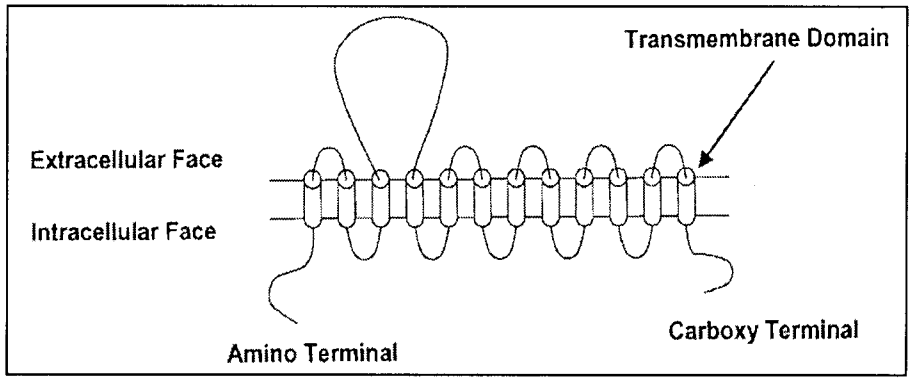
In order for the postsynaptic cell to recognize discrete DA signals from the presynaptic cell, the released DA must be quickly removed from the synaptic space. One method of inactivating released DA is to metabolize it into inactive products. Catechol-O-methyltransferse and monoamine oxidase metabolize DA that is released into the synaptic cleft, and an intracellular monoamine oxidase metabolizes DA that is taken up into the nerve terminal (Cooper et al. 2003). In addition to inactivation of DA by metabolism, the primary mechanism for clearing released DA from the extra-synaptic space is re-uptake into the presynaptic neuron through the high affinity dopamine transporter (DAT) (Wightman and Zimmerman 1990;Parsons and Justice, Jr. 1994). DA that is taken up into the cell can be recycled into vesicles for later release.

The DAT is a Na^+/Cl^- - dependent transporter expressed endogenously only in DA neurons (Bannon MJ 1998). This makes the DAT rather unique as most proteins expressed in DA neurons, such as DA receptors and ion channels, are expressed in multiple cell types. Additional members of the

Na^+/Cl^- - dependent transporter family include transporters for norepinephrine, serotonin, glycine and γ-aminobutyric acid (Zahniser and Doolen 2001). The Na^+/K^+ adenosine triphosphatase (ATPase) transports Na^+ ions out of cells and transports K^+ ions into cells to generate an ion gradient across the cellular membrane. The DAT utilizes this gradient to transport DA into the cell by simultaneously transporting Na^+ and Cl^- down their respective concentration gradients (Cooper et al. 2003).

The DAT has been cloned and sequenced from several species, including human (Eshleman et al. 1995;Giros et al. 1992), mouse (Wu and Gu 1999), rat (Kilty et al. 1991), rhesus monkey (Miller et al. 2001), cow (Usdin et al. 1991), zebra fish (Holzschuh et al. 2001), fruit fly (Porzgen et al. 2001), nematodes (Jayanthi et al. 1998), and a moth (Gallant et al. 2003). The human DAT has 620 amino acids, but the exact spatial arrangement of the amino acids is not known, as there is no crystallographic data available. Hydropathy analysis of the amino acid sequence suggests that the DAT contains twelve putative hydrophobic membrane-spanning domains,

Figure 1-2: Dopamine transporter structure
Volz and Schenk 2005



alternating intracellular and extracellular loops, intracellular N- and C-termini, multiple N- glycosylation sites and putative protein kinase C (PKC) and PKA cytoplasmic phosphorylation sites (Figure 1-2) (Kilty et al. 1991;Amara and Kuhar 1993;Volz and Schenk 2005). Immunohistochemical evidence indicates localization of membrane-expressed DAT on presynaptic terminals is primarily outside of the synapse, thus inactivating DA that has diffused from the synapse (Hersch et al. 1997).

A gene knockout mouse model has been developed that has a homozygous deletion of the DAT gene, and thus does not express the DAT. These mice are viable and develop normally, but they exhibit slow clearance of synaptically released DA, are hyperactive, and have elevated levels of synaptic DA. Microdialysis measurements indicate that DA remains in the synapse 100 times longer in these animals compared to wild type animals. Additionally, administration of psychostimulants does not further increase locomotion or alter dopamine clearance rates in these animals (Giros et al. 1996).

Regulation by drugs of abuse. The DAT is the primary site of action of psychostimulants, such as cocaine and amphetamines, (Ritz et al. 1987), and much of our knowledge of transporter function and regulation has come from studies of psychostimulants and their mechanisms of action on the brain. Psychostimulants are not selective for the DAT, as they have varying degrees of affinity for norepinepherine and serotonin transporters as well

(Eshleman et al. 1999). Psychostimulants acutely block uptake of DA, norepinepherine, and serotonin causing prolonged and accentuated activation of postsynaptic receptors. The increase in dopaminergic neurotransmission in limbic brain regions is thought to mediate the rewarding and addictive properties of psychostimulants (Koob and Bloom 1988). Psychostimulants that act on transporters can be generally distinguished as either transporter blockers or transporter substrates. Blockers, such as cocaine, interact directly with transporters to inhibit uptake of the endogenous neurotransmitter, while substrates, such as amphetamine, are translocated through the transporter in a manner similar to translocation of the endogenous neurotransmitter (Elliott and Beveridge 2005).

Additionally, there are compounds that interact directly with the DAT and block uptake of DA through the DAT, but they are not classified as psychostimulants. This includes drugs such as GBR12909. This compound binds with a high affinity to the DAT, does not cause a significant increase in DA levels in the synapse, and blocks the rise in synaptic DA caused by cocaine (Rothman et al. 1989).

Many studies have explored the effects of DAT substrates on transporter function and cellular expression. Amphetamine is a substrate of the DAT, and as such it is translocated from the synapse into the nerve terminal. Amphetamine can compete with DA for uptake through the DAT, thus "blocking" DA uptake. Additionally, amphetamine can stimulate release

of intracellular DA (Fischer and Cho 1979;Pierce and Kalivas 1997b). Treatment with the substrates DA or amphetamine causes a decrease in uptake of DA through the DAT and decreases DAT cell surface expression in HEK (Saunders et al. 2000), porcine aortic endothelial (Sorkina et al. 2003), and N2A mouse neuroblastoma neuronal (Little et al. 2002) cells transfected with the DAT. The substrate-induced decrease in DAT function and surface expression also occurs in *Xenopus laevis* oocytes transfected with the DAT (Gulley et al. 2002). Pretreating the cells with PKC inhibitors blocks this decrease in DAT surface expression (Gulley et al. 2002), indicating that intracellular phosphorylation of proteins is necessary for substrate-induced internalization of transporters. Saunders et al (2000) transfected HEK cells with a DAT that was tagged with a FLAG epitope (DYKDDDDK peptide sequence). Using confocal microscopy they were able to visually detect the substrate–induced internalization of the DAT to intracellular regions. Transfecting the cells with a dominant negative (inactive) form of dynamin prevented this internalization. Dynamin is a GTPase that is involved in pinching off newly formed vesicles. The dominant negative form is not capable of binding GTP, but it does interact with endogenous endocytosis machinery to compete with endogenous dynamin and disable internalization mechanisms. Pretreating the cells with transporter inhibitors also prevents this internalization. The authors propose that amphetamine decreases the capacity for DA uptake by increasing endocytosis of cell surface DAT. While

pretreating the cells with transporter inhibitors prevents this endocytosis, it is not yet determined if substrate interaction with the DAT is the cause for internalization, or if translocation of the substrate into the cytosol is necessary.

Cocaine acts as an antagonist and reversibly binds to the DAT, thus preventing uptake of DA (Ritz et al. 1987). It is uncertain whether cocaine and substrates have identical (Reith et al. 1992) or separate (Johnson et al. 1992) recognition sites on the DAT. The effects of cocaine exposure on DAT surface expression are unclear, as studies from postmortem human tissue and drug treated animals have produced variable results (Benmansour et al. 1992;Hitri et al. 1994;Hitri et al. 1996;Hurd and Herkenham 1993;Koff et al. 1994;Pilotte et al. 1996;Wilson et al. 1994;Wilson et al. 1996). The variations may be due to the differences in dose, length of administration, or withdrawal time. In a neuroblastoma-derived cell line transfected with the DAT, exposure to cocaine (1 μM) causes an increase in DA uptake and an increase in transporter surface expression following 12 and 24 hours of treatment (Little et al. 2002;Zhang et al. 1998). This increase in transporter function is also demonstrated in tranfected HEK cells following a shorter exposure time to a higher concentration of cocaine (10 μM, 10 min) (Daws et al. 2002). The increase in transporter surface expression may represent a compensatory response to inhibition of transport function.

Regulation by protein kinase C. Protein phosphorylation can also regulate DAT activity. PKC is a phospholipid-dependant serine/threonine kinase, and there are at least 12 isoforms of this kinase (Way et al. 2000). The DAT contains potential phosphorylation sites on its N-terminal region, and activation of PKC causes phosphorylation of the DAT (Vaughan et al. 1997;Huff et al. 1997). Furthermore, activation of PKC by phorbol esters is known to inhibit DAT function. This occurs by a reduction in maximal transport velocity and a reduction in cell surface DAT binding sites (Zhu et al. 1997;Pristupa et al. 1998;Huff et al. 1997). This may be due to increased transporter internalization, which can be blocked by PKC inhibitors (Daniels and Amara 1999;Melikian and Buckley 1999;Chang et al. 2001). Interestingly, deletion of PKC consensus sites prevents phosphorylation of the DAT, but the DAT continues to transport substrate normally and become internalized following PKC stimulation (Chang et al. 2001;Granas et al. 2003), suggesting that additional phosphoproteins are involved in PKC-induced DAT trafficking. Dynamic membrane regulation of the DAT is proving to be an integral component of DA neurotransmission regulation. Further analysis of the many signals that alter DAT membrane expression will be necessary to complete a "model" of the fine regulation of synaptic DA.

The exact location of internalized DAT following PKC stimulation seems to vary across studies. Daniels and Amara (1999) studied DAT trafficking in Madin-Darby canine kidney cells transfected with a green

fluorescent protein-labeled DAT and used confocal microscopy to visually detect internalized transporter. Stimulation of PKC with phorbol 12-myristate 13-acetate (PMA) causes the DAT to rapidly internalize with as little as 5 minutes of treatment, and during this time the transporter is colocalized with transferrin, presumably representing the early sorting endosome. Following 20 minutes of treatment the DAT becomes associated with lysosomes, and within 1-2 hours of PMA treatment the entire pool of internalized transporter is degraded. This internalization is a dynamin-dependant mechanism and pretreating the cells with cocaine to block the DAT does not prevent this endocytosis. The inability of cocaine to prevent the PMA-induced internalization suggests this process is not dependant on the DAT being in a particular conformation. Specifically, the DAT does not need to be in a functional conformation to be internalized through PKC stimulation. A separate study, in PC12 cells transfected with an unlabeled DAT (no green fluorescent tag), demonstrates that PMA treatment causes the DAT to traffic through recycling endosomes, possibly for reinsertion into the plasma membrane (Melikian and Buckley 1999). Thus the reported differences in DAT trafficking pathways following PKC stimulation may result from the use of fluorescent proteins fused to the transporter, differences in treatment times, differences in PMA concentrations, or differences in the detection methods used.

Understanding DAT regulation is crucial for understanding and treating problems of substance abuse. Chronic cocaine use may increase the number of DAT binding sites and DAT-mediated transport (Letchworth et al. 2001;Little et al. 1993;Little et al. 1999;Mash et al. 2002), and withdrawal results in significantly lower DAT binding and function (Volkow et al. 1997). Furthermore, the lowered uptake levels following withdrawal correlate with an increase in depressive symptoms and craving, suggesting a role of DAT function in withdrawal symptoms (Volkow et al. 1997).

DAT function and regulation are also clinically relevant to the pathologies of many neuropsychiatric conditions. For example the DA hypothesis of schizophrenia suggests that over activity in dopaminergic neurotransmission is responsible for schizophrenic psychoses (Meltzer 1987). Reports that the density of the DAT is decreased in postmortem striatal brain tissue of schizophrenics support this hypothesis (Dean and Hussain 2001).

D2 receptors and dopamine transporter regulation. There are many ways in which DAT function can be regulated acutely, including protein kinase C activation (Zhang and Reith 1996;Zhu et al. 1997) and nitric oxide production (Pogun et al. 1994). DAT function is also regulated *via* activation of the presynaptic DA D2 autoreceptor (Meiergerd et al. 1993;Cass et al. 1991). DA receptors that are expressed on the presynaptic neuron are classified as autoreceptors. DA that is released by the neuron terminals on which they are located stimulates these receptors. They are D2-like

receptors. There is considerable evidence demonstrating that acute antagonism (of less than 10 min) of the D2 receptor decreases DAT mediated reuptake of synaptically released DA, but D1 receptor antagonists do not affect DAT uptake (Cass and Gerhardt 1994;Rothblat and Schneider 1997). In contrast Meiergerd and coworkers (1993) demonstrated that acute stimulation of D2 receptors in striatal tissue increases uptake of DA. Furthermore, a knockout mouse model lacking the D2 receptor demonstrates decreased DA clearance in the striatum, and application of D2 antagonists to these mice does not further reduce clearance (Dickinson et al. 1999). These data suggest that the transporter is activated *via* feed back mechanisms through the D2 autoreceptor to remove released DA from the synapse and limit stimulation of postsynaptic receptors.

D2 receptor-mediated regulation of DAT is relevant to studies of substance abuse, as chronic antagonism of the D2 receptor inhibits the increase in DAT-mediated uptake that occurs with repeated administration of cocaine (Parsons et al. 1993). Additional studies using D2 receptors and the DAT expressed in Xenopus oocytes demonstrate that short-term (3 min) activation of the receptor increases [^{3}H]DA uptake and increases cell surface binding of the cocaine analog [^{3}H]WIN35,428. A PTX-sensitive G(i/o) protein mediates this effect (Mayfield and Zahniser 2001). D2 receptors also modulate cocaine-induced synaptic DA release (Rouge-Pont et al. 2002) and sensitization (Beyer and Steketee 2002). A thorough understanding of how

DAT and DA receptors are affected by, and mediate the effects of, drugs of abuse is critically important to developing effective treatments, while minimizing negative side effects.

The complete signaling pathways between the D2 receptor and the DAT are not understood. D2 receptors couple to multiple signaling pathways including inhibition of adenylyl cyclase (Onali et al. 1985) and PKC (Giambalvo and Wagner 1994), and activation of mitogen-activated protein kinase (MAPK) (Luo et al. 1998;Choi et al. 1999), G-protein coupled inwardly rectifying potassium channels (Werner et al. 1996), and phospholipase A2 (PLA2) (Vial and Piomelli 1995). Each of these pathways needs to be examined in detail to fully determine the contributions of D2 receptor activation on cell function and DAT regulation.

Phospholipase signaling pathways

Phospholipase A2. D2 receptors are coupled to G-proteins that activate PLA2. Activation of D2 receptors expressed in Chinese hamster ovary (CHO) cells hydrolyzes membrane phospholipids to cause a release of platelet-activating factor (PAF) and arachidonic acid (AA) (Piomelli et al. 1991;Vial and Piomelli 1995). PAF is also a bioactive phospholipid and contributes to mechanisms of synaptic plasticity by enhancing glutamate release and acting as a retrograde messenger in long-term potentiation (Bazan et al. 1993;Wieraszko et al. 1993). PLA2 is actually a family of heterogenous lipases that catalyze the hydrolytic cleavage of

glycerophospholipids at the sn-2 position, generating free fatty acids and lysophospholipid (Piomelli 1994). Members of the PLA2 family are divided into two groups based on subcellular distribution, molecular structure, and phospholipid selectivity: low molecular weight (12-18 kDa) secretory PLA2 and high molecular weight cytosolic PLA2 (Glaser et al. 1993;Mayer and Marshall 1993). Cytosolic PLA2 can be further categorized into calcium-dependent (cPLA2, 85-110 kDa) and calcium-independent (iPLA2, 39 kDa) forms. Cytosolic PLA2 has no sequence homology to secretory PLA2 (Sharp et al. 1991). It is cPLA2 that selectively hydrolyzes AA from phospholipids and that can be stimulated by increases in free Ca^{2+} levels following receptor activation, thus suggesting a mechanism for receptor-dependent AA release (Clark et al. 1991). iPLA2 mediates slow lipid remodeling as a "housekeeping" enzyme (Winstead et al. 2000).

Use of quantitative polymerase chain reaction to determine the relative levels of cPLA2 messenger ribonucleic acid in various human and mouse tissues demonstrated expression in brain, heart, spleen, lung, kidney, liver, testis, white blood cells, and platelets (Sharp and White 1993). These findings indicate a wide expression of cPLA2 throughout the body with varying expression levels in each cell type. To add to the complexity of PLA2 signaling pathways, a variety of mitogens and cytokines, such as interleukin 1, tumor necrosis factor, and macrophage colony-stimulating factor, as well as ATP and lipopolysaccharide are known to stimulate cPLA2 activity,

suggesting a variety of roles in many types of cellular processes (for review see Clark et al. 1995).

A number of specific receptor-dependant activities can stimulate cPLA2 activity. These include serotonin activation of the $5HT_{2A}$ receptor (Kurrasch-Orbaugh et al. 2003) and glutamate activation of metabotropic GLUR1 receptors (Aramori and Nakanishi 1992). Additionally, DA causes release of AA in CHO cells transfected with D2 receptors (Kanterman et al. 1991;Vial and Piomelli 1995), and D2 receptor stimulation enhances AA release in primary cultures of rat striatal neurons (Schinelli et al. 1994). Both of these DA stimulated effects requires the concomitant treatment with a Ca^{2+} ionophore to increase intracellular calcium, suggesting that DA receptors act to further amplify PLA2 activity once properly activated. Yet, subsequent studies have demonstrated D2 receptor-mediated activation of PLA2 in transfected CHO cells without the application of a Ca^{2+} ionophore (Nilsson et al. 1998;Hellstrand et al. 2002). Thus the precise role of D2 receptors in PLA2 activation is not yet determined.

Activation of cPLA2 requires translocation of the enzyme from the cytosol to the membrane, and this translocation is a Ca^{2+}-dependant process (Clark et al. 1991). Experiments using the outer segments of retinal photoreceptor rod cells demonstrate that a G-protein, specifically the $\beta\gamma$ subunit, mediates the activation of PLA2. PTX inhibits this activation, and the non-hydrolyzable GTP analogue GTPγs stimulates this activation

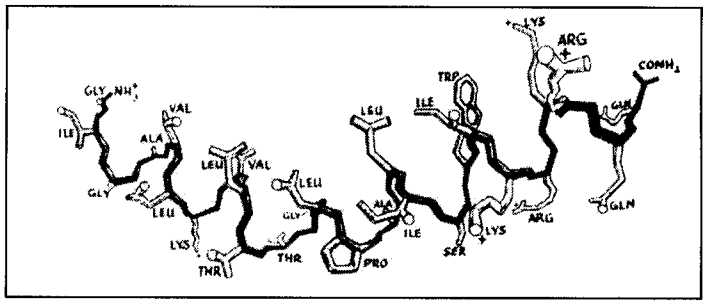
(Jelsema and Axelrod 1987). GTPγs also stimulates AA release in other cells, such as neutrophils (Nakashima et al. 1988) and platelets (Silk et al. 1989). Thus multiple converging signals are necessary for activation of cPLA2.

An additional converging signal that is necessary for cPLA2 activation is direct phosphorylation. Phosphorylation by MAPK stimulates cPLA2 enzymatic activity (Lin et al. 1993). Although cPLA2 also contains potential phosphorylation sites for PKC and PKA, phosphorylation by these kinases causes little or no change in cPLA2 activity (Lin et al. 1993;Nemenoff et al. 1993). Work in cell lysates suggests phosphorylation by MAPK is predominately responsible for cPLA2 activation (Lin et al. 1992), and it is necessary for full activation of cPLA2 (Lin et al. 1993). Although G-proteins are known to couple membrane receptor activation to cPLA2, there is no evidence of a direct interaction between G-proteins and cPLA2. Work in CHO cells demonstrates that receptor activation of MAPK is sensitive to PTX (Winitz et al. 1994), and PTX prevents phosphorylation of cPLA2 (Clark et al. 1995). Clark et al (1995) propose a model in which a G-protein regulates the MAPK cascade which leads to phosphorylation and activation of cPLA2, rather than a model in which a G-protein interacts with cPLA2 directly. In agreement with this hypothesis, experimental results using immunocytochemistry and subcellular fractionation indicates that cPLA2 colocalizes with MAPK, including ERK1, ERK2, p85, and p38, in arachidonate-rich lipid domains in U937 cells (Yu et al. 1998). Thus cPLA2

has many levels of regulation that must produce a coordinated action to stimulate activation. The presence of many converging signals suggests the biological necessity to prevent wayward activation of this enzyme.

Melittin. Throughout the literature melittin is typically used as a PLA2 activator. Unfortunately, melittin is not a specific pharmacological stimulator of PLA2. Melittin is a 26 amino acid amphiphilic peptide isolated from the venom of the honeybee, *Apis mellifera* (Figure 1-3). Melittin activates AA signaling pathways in neurons (Geddis et al. 2004;Muzzio et al. 2001) and in multiple cell lines including rat PC12, and mouse L1210 cells (Palomba et al. 2004;Lee et al. 2001), possibly through PLA2 stimulation. Yet, studies have begun to suggest that the effects of melittin on cellular function and fatty acid release are not specifically through PLA2 activation (Lee et al. 2001). Melittin has been used to examine the effects of PLA2 signaling on DA cell function (Reid et al. 1996;Zhang and Reith 1996), and the mechanism of action has been presumed to be release of AA. The precise mechanism of melittin action needs to be established to accurately interpret the previously

Figure 1-3: The primary amino acid structure of melittin
Dempsey 1990



mentioned findings. This dissertation directly addresses the effects of melittin on membrane fatty acids, DA receptors, and the DAT.

Arachidonic acid. AA is stored in the plasma membrane of cells, and is conjugated to the glycerol backbone of phospholipids. PLA2 can release AA from a variety of membrane phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositides, and phosphatidlyserine (Axelrod 1990), but it has a preference for phosphatidylcholine (Nalefski et al. 1998). This preference may play a role in targeting the enzyme to the proper membrane region. PLC can hydrolyze phosphoinositides or phosphatidylcholine to produce diacylglycerol (DAG), which can be further metabolized by DAG lipase and monoacylglycerol lipase to produce free AA. Additionally, hydrolysis by PLD produces phosphatidic acid, which can also be converted into DAG (Piomelli D 1995). Thus free AA is produced directly by PLA2 and indirectly by PLC and PLD (Figure 1-4). The mechanisms involved in maintaining the level of free and membrane-

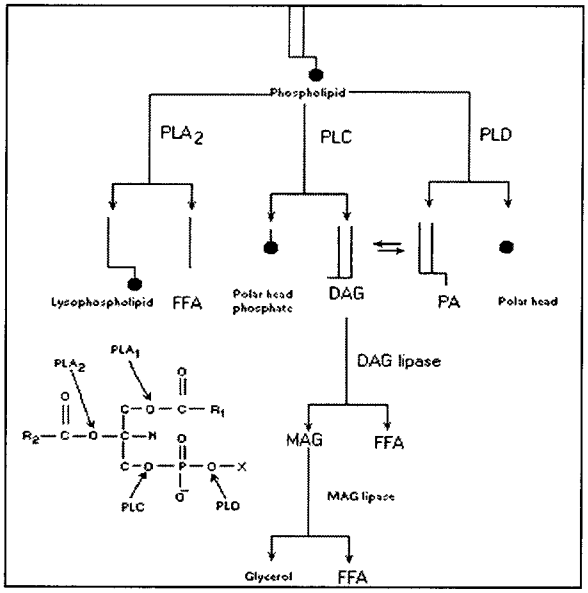
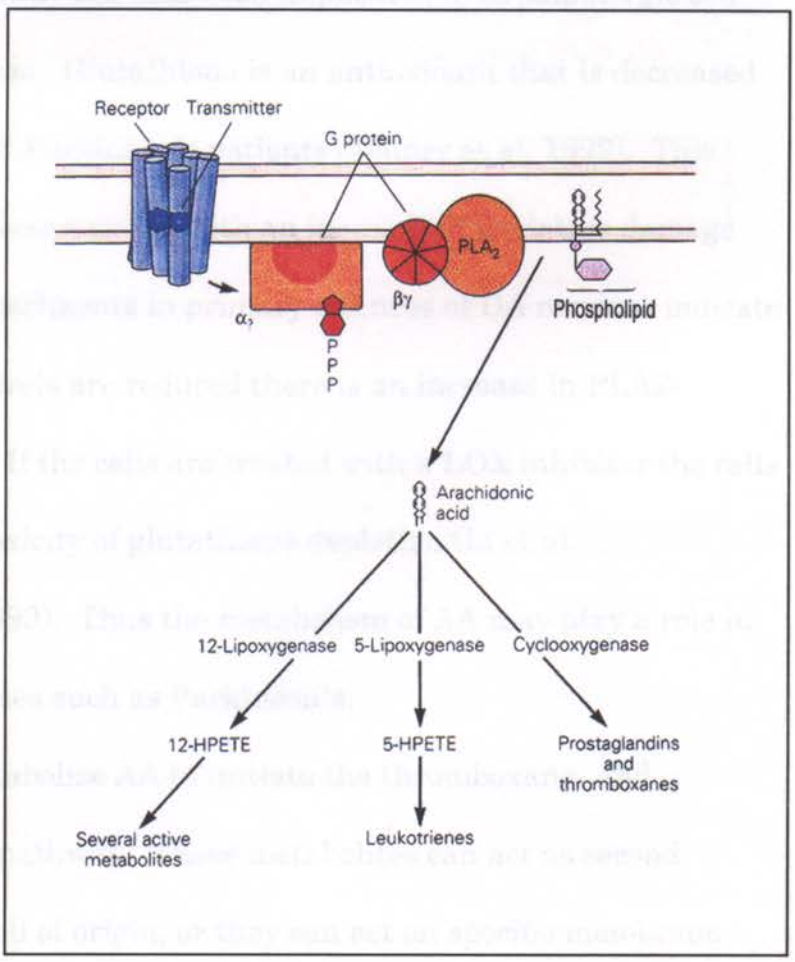


Figure 1-4: Generation of free fatty acids by phospholipases.
PL, phospholipase; DAG, diacylglycerol; FFA, free fatty acids; PA, phosphatidic acid; MAG, monoacylglycerol
Piomelli 1995

incorporated AA in cells is well reviewed by R.F. Irvine (1982). Briefly, in resting cells the level of free AA is relatively low, compared to other fatty acids. This level of free AA is maintained by the enzymes arachidonoyl-CoA synthetase, which catalyzes the formation of arachidonoyl-CoA in the cytoplasm, and an acyl transferase enzyme that transfers arachidonate from arachidonoyl-CoA to the membrane phospholipids.

Arachidonic acid metabolism. Once AA is released from the membrane, enzymes such as cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 can metabolize AA to produce numerous bioactive compounds, such as prostaglandins, leukotrienes, lipoxins, and thromboxanes, that function as second messengers in multiple signaling cascades

Figure 1-5: Arachidonic acid release and metabolism
Kandel et al. 2000



(Figure 1-5). AA liberation is the rate limiting step in the formation of these metabolites (Smith et al. 2000;Capdevila et al. 1981;Fitzpatrick and Murphy 1988;Thompson et al. 2000;Piomelli D 1995).

Effects of arachidonic acid metabolites on cellular function.

The family of LOX enzymes catalyzes the oxygenation of AA to form hydroperoxy-eicosatetraenoic acid (HPETE). HPETEs are the initial step in an extensive network of metabolic products. Some lipoxygenase-generated compounds such as 12-HPETE, 5-HPETE, and 15-HPETE are implicated in cell death (Canals et al. 2003) and altered Ca^{2+} conductance (Roudbaraki et al. 1996). The LOX pathway has also been implicated in dopaminergic cell loss in Parkinson's disease. Glutathione is an antioxidant that is decreased in the substantia nigra of Parkinson's patients (Jenner et al. 1992). This decrease in glutathione is associated with an increase in oxidative damage and toxicity to cells. Experiments in primary cultures of DA neurons indicate that when glutathione levels are reduced there is an increase in PLA2-mediated release of AA. If the cells are treated with a LOX inhibitor the cells are protected from the toxicity of glutathione depletion (Li et al. 1997;Mytilineou et al. 1999). Thus the metabolism of AA may play a role in neurodegenerative diseases such as Parkinson's.

COX enzymes metabolize AA to initiate the thromboxane- and prostaglandin-signaling pathway. These metabolites can act as second messengers within the cell of origin, or they can act on specific membrane

receptors on neighboring cells (Piomelli 1993). COX metabolic products can act on specific receptors to alter neurotransmitter release. Activation of the prostaglandin EP3 receptor expressed in PC12 cells causes inhibition of DA release, and this is mediated by PTX-sensitive and –insensitive mechanisms (Nakamura et al. 1998). Additionally, a subpopulation of schizophrenic patients demonstrates a diminished response to prostaglandins in platelets caused by decreased receptor sensitivity (Kanof et al. 1987). The authors of this study surmise that if this phenomenon also occurs in schizophrenic brains, then the patients may demonstrate decreased ability of prostaglandins to inhibit DA neurotransmission and thus cause exacerbated psychotic symptoms.

Direct effects of arachidonic acid on proteins. AA can directly alter protein function. Ordway et al (1989) used electrophysiological patch-clamp techniques to isolate potassium channels from smooth muscle cells. When AA is applied to the extracelluar surface, in an "outside-out" patch, or the cytosolic surface, in an "inside-out" patch, it activates the potassium channels. This activation is also seen when the saturated fatty acid myristic acid is applied. These experiments were performed without the inclusion of calcium and nucleotides such as ATP and GTP. All of these conditions indicate that fatty acids stimulate potassium channel activation, possibly through a fatty acid binding domain, or by effects of the fatty acids on the lipid membrane. Phosphorylation-, calcium-, or AA metabolite-dependant

mechanisms do not mediate this activation. A direct effect of AA on ion channels has been demonstrated with similar results in a variety of cellular preparations and ion channels (Kim and Clapham 1989;Takenaka et al. 1988;Hwang et al. 1990;Anderson and Welsh 1990;Zheng et al. 2005;Yang et al. 2005;Barrett et al. 2001;Liu et al. 2001).

While studies have not definitively demonstrated whether fatty acids act directly through protein-lipid interactions, or act by altering general properties of membrane fluidity, it is evident that membrane lipid composition and fatty acid signaling are important for proper function and regulation of membrane imbedded proteins, such as neurotransmitter receptors and transporters. In purified synaptosomes from rat striatum, AA dose-dependently increases spontaneous release of DA and decreases DA synthesis and uptake. AA directly mediates this effect rather than a metabolite. Additionally the AA effect is reduced by PKC inhibitors, but not by PKA inhibitors (L'hirondel et al. 1995). Electrophysiological recordings from *Xenopus* oocytes expressing the human DAT indicate that AA, in short treatments (2 min), acts directly to stimulate a cocaine-sensitive cation conductance that is distinct from current associated with substrate transport. Other fatty acids also generate this conductance (Ingram and Amara 2000). Studies using C6 rat glioma cells transfected with the DAT demonstrate preincubations with AA for short times (15-30 min) can stimulate DA uptake, while longer incubations (45-60 min) can inhibit uptake

in these cells. This study also demonstrates that melittin, LOX inhibitors, and lipid reacylation inhibitors (block acyltransferases that reesterify fatty acids into lipids) added to cells prior to the assay cause a decrease in [^{3}H]DA uptake (Zhang and Reith 1996). Studies of anandamide provide additional significance for the role of AA signaling in DAT function. Anandamide is an endogenous cannabinoid derived from AA (Devane et al. 1992), and both anandamide and AA inhibit uptake of the neurotransmitters DA and serotonin in rat neocortical synaptosomes (Steffens and Feuerstein 2004).

The effects of AA on glutamate uptake by excitatory amino acid transporters (EAAT) also demonstrate the role of lipids in regulating protein function. AA inhibits uptake of glutamate into astrocytes and synaptosomes from rat cerebral cortex (Volterra et al. 1994;Volterra et al. 1992) and alters proton conductance in the rat glutamate transporter EAAT4 (Fairman et al. 1998;Tzingounis et al. 1998). The effect of AA on glutamate transporters appears to be specific to the particular transporter subtype. Application of AA inhibits the human EAAT1 glutamate transporter, which is expressed throughout the brain and cerebellum, yet AA treatment increases glutamate transport through the EAAT2 transporter, expressed in midbrain and forebrain regions (Zerangue et al. 1995).

Further evidence to indicate that lipids affect protein function comes from studies of the phospholipid membrane components ceramide and cholesterol. Ceramide is derived from membrane-incorporated

sphingomyelin, and direct application of this lipid reversibly inhibits DAT function, possibly by decreasing the transporter's affinity for DA (Riddle et al. 2003). Depleting membrane cholesterol inhibits serotonin transporter function by decreasing its affinity for serotonin and by decreasing its maximal transport rate. Replenishing the membrane cholesterol levels reverses the inhibition of transporter function (Scanlon et al. 2001). Thus there seem to be important roles for lipids in regulating neurotransmitter transporters and dopaminergic cell function, and the specific effects of AA- and PLA2-mediated signaling need to be further characterized to fully understand this expanding field of knowledge.

Clinical implications of phospholipase A2 signaling in the dopaminergic system. PLA2 activity is linked to many neurological disorders such as dyslexia (MacDonell et al. 2000), Parkinson's disease (Ross et al. 2001), and schizophrenia (Horrobin et al. 1994). In clinical experiments, platelet and serum levels of PLA2 are elevated in drug-naive schizophrenic patients (Gattaz et al. 1995;Ross et al. 1997). Additional clinical studies report an accelerated metabolism of membrane phospholipids measured by [^{31}P]spectroscopy in drug-naïve schizophrenic patients (Pettegrew et al. 1991). Further studies specifically examining PLA2 activity in schizophrenia show increased activity of iPLA2 in temporal cortex and decreased activity of cPLA2 in prefrontal and temporal cortex and putamen (Ross et al. 1999). Experiments measuring the activity of PLA2 in rats

demonstrate that chronic treatment with the D2 antagonist haloperidol causes a down regulation of PLA2 mediated AA release in the mesocorticolimbic and nigrostriatal dopaminergic systems, while there are no significant changes in PLA2 activity in other brain regions (Myers et al. 2001;Trzeciak et al. 1995). In animal models of Parkinson's disease rats were injected with the DA neurotoxins 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to destroy DA neurons in the substantia nigra. Prior treatment with the PLA2 inhibitor quinacrine is protective against this toxin-induced neurodegeneration (Tariq et al. 2001). These findings demonstrate the clinical relevance of PLA2. Further examination of the effects of PLA2 activity on DA transport will be instrumental to more fully understand these complicated neurological and psychiatric conditions.

PLA2 also appears to play a role in drug addiction. Dopaminergic neurotransmission is involved in development and expression of sensitization to repeated administration of psychostimulants (Kalivas and Stewart 1991). This sensitization is seen as an increased behavioral response to repeated stimulant administration and increased self-administration in rats (Horger et al. 1992;Piazza et al. 1990;Robinson and Becker 1986;Robinson and Berridge 1993). Calcium and many calcium-dependent proteins are essential in synaptic modifications (Calabresi et al. 1994;Mayford et al. 1996), and Ca^{2+} conductance is involved in expression of behavioral sensitization to

psychostimulants (Martin-Iverson and Reimer 1994;Karler et al. 1991). This Ca^{2+} conductance implicates Ca^{2+}-activated PLA2 in psychostimulant sensitization; indeed Reid et al (Reid et al. 1996) demonstrated that inhibition of PLA2 dose-dependently blocks locomotor and stereotypic behavioral sensitization to cocaine and amphetamine that occurs following a withdraw period after chronic treatment. Additionally, PLA2 activators, locally injected in the ventral tegmental area of rats, induce psychostimulant sensitization (Reid et al. 1996), and COX inhibitors prevent the development of cocaine sensitization (Reid et al. 2002). Measurements of PLA2 activity in the post mortem brains of chronic psychostimulant drug abusers found decreased levels of cPLA2 in the putamen (Ross et al. 2002), and this effect was replicated in rats chronically treated with cocaine (Ross and Turenne 2002). The authors suggest this down regulation may be a compensatory reaction to excess DA stimulation of PLA2 through DA receptors. Experiments to measure the activity of PLA2 and prostaglandin production following withdrawal from chronic psychostimulants would help define the progression of PLA2 activity through drug craving, sensitization, and possible relapse. This evidence suggests the importance of examining the AA pathway and its role in modulating DA transmission. Experiments that quantify or visually localize DAT following altered PLA2 activity have not been reported in detail.

Specific aims

The primary goals of this dissertation are to characterize the effects of the AA cascade on the function and regulation of the DAT. The signaling cascades outlined in this dissertation are most relevant to studies of regulation of DA neurons, but the experiments were performed in HEK-293 cells stably transfected with the human form of the DAT and the DA D2 receptor. With any cellular model there are obvious considerations about the ability of the chosen cell line to emulate the function of a neuron. Unfortunately, few studies do comparative studies exploring protein regulation in HEK cells and neurons. The HEK-293 cell line was developed in 1977 (Graham et al. 1977), and since then has been used extensively as a model for kidney cell function and also as a vehicle for studying transfected proteins. A recent study has begun to shine new light on HEK-293 cells as a model system. A serendipitous scientific control experiment lead to the discovery that HEK-293 cells express the neurofilament subunit NF-M, which is a protein characteristic of neuronal cell lines. Microarray analysis was then used to analyze the types of proteins expressed in these cells, and immunocytochemistry was used to verify the expression of selected proteins. This study revealed the presence of other neuron specific proteins, such as NF-L, α-internexin, neuron-specific enolase, and neuron-specific clathrin. HEK-293 cells also express potassium channels, voltage-gated calcium channels, and the neuron-specific adhesion molecule NB-3 (Shaw et al. 2002).

The authors of this study also relate a number of previous studies that demonstrate neuronal-like properties of HEK-293 cells (Daaka et al. 1997;van Koppen et al. 1996;Schachter et al. 1997;Lin et al. 1999;Anderson et al. 1995), and suggest that the cell line may have been transformed from a rare neuronal lineage cell present in the human kidney. Thus HEK-293 cells are proving to be a rather valid model for neuronal cell function. Primary cultures of DA neurons could also be used to provide a physiologically and clinically relevant model of the endogenous cellular mechanisms affecting the AA signaling cascade and DAT function. Future experiments could be performed in cultured DA neurons to parallel the studies outlined in this dissertation.

Throughout these experiments I used the 26 amino acid peptide melittin, which is the most frequently used pharmacological stimulator of PLA2. Previous experiments have demonstrated a melittin-induced decrease in DA uptake through the DAT in transfected C6 rat glioma cells (Zhang and Reith 1996). Based on these experiments and on the use of melittin as a PLA2 stimulator throughout scientific literature, **I hypothesize that melittin inhibits DAT function and cellular localization through activation of PLA2 and initiation of AA signaling cascades**. The experiments in this dissertation further define the interaction of melittin with the DAT.

Specific aim 1: To determine the pharmacological mechanisms of melittin effects on the DAT. Based on the effect of PLA2 activation and AA on DAT function, I hypothesized that melittin would inhibit DAT function through stimulation of PLA2. The effect of melittin on DAT function was measured by radioligand uptake and binding studies. The effect of melittin on phospholipase activation and fatty acid release was determined by gas liquid chromatography and [^{3}H]AA release assays.

Specific aim 2: Characterize the trafficking pathway of internalized DAT. Melittin treatment caused internalization of the DAT. Colocalization of the transporter with endogenous protein markers of endosomes and endocytosis pathways was quantified. Confocal microscopy and equilibrium density gradient separation were used to determine the pathway and time course of internalized DAT.

Chapter II.

Melittin stimulates fatty acid release through non-phospholipase-mediated mechanisms and interacts directly with the dopamine transporter and other membrane spanning proteins

Abstract

Phospholipase (PL) A2 releases the fatty acid arachidonic acid (AA) from membrane phospholipids. We used the purported PLA2 stimulator, melittin, to examine the effects of endogenous AA signaling on dopamine (DA) transporter (DAT) function and trafficking. In HEK-293 cells stably transfected with the DAT, melittin reduced uptake of [^{3}H]DA. Additionally, measurements of fatty acid content following treatment demonstrated a melittin-induced release of membrane-incorporated AA, but inhibitors of PLC, PLD, and PLA2 did not prevent the release. Subsequent experiments measuring [^{125}I]RTI-55 binding to the DAT demonstrated a direct interaction of melittin with the transporter to inhibit antagonist binding. This effect was not specific to the DAT, as [^{3}H]spiperone binding to the recombinant DA D2 receptor was also inhibited by melittin treatment. Finally, melittin stimulated an increase in internalization of the DAT, and this effect was blocked by pretreatment with cocaine. Thus, melittin acts through multiple mechanisms to regulate cellular activity, including release of membrane-incorporated fatty acids and direct interaction with the DAT.

Introduction

The AA cascade is a complex biological signaling pathway that is found in most cell types. AA is a fatty acid that is released from cell membranes and is able to not only act on proteins within a cell by binding directly to them (Ordway et al. 1989) but also by second messenger actions of AA metabolites (Miller et al. 1992). AA can be metabolized by lipoxygenases and cyclooxygenases to form bioactive compounds such as leukotrienes, prostaglandins, hydroxyeicosatetraenoic acids, and many other bioactive metabolites (for review see Piomelli D 1995). Some lipoxygenase- and cyclooxygenase-generated compounds such as 12-hydroperoxyeicosatetraenoic acid (HPETE), 5-HPETE, 15-HPETE, and prostaglandin E2, are implicated in cell death (Canals et al. 2003), altered Ca^{2+} conductance (Roudbaraki et al. 1996), postsynaptic membrane excitability, and long-term synaptic plasticity (Bazan 2003), respectively.

Free AA is generated by PLA2-mediated hydrolysis of membrane phospholipids. Altered PLA2 activity is implicated in many neurological disorders such as dyslexia (MacDonell et al. 2000), Parkinson's disease (Ross et al. 2001) and schizophrenia (Horrobin et al. 1994). Additionally PLA2 activators can induce (Reid et al. 1996), while inhibitors of PLA2 (Reid et al. 1996) and of AA metabolism (Reid et al. 2002) can inhibit psychostimulant sensitization. Considering that hyperactivity of the DA system may be a

component of the etiology of schizophrenia, and the DAT is the primary mechanism of clearing released DA from the extra-synaptic space (Parsons and Justice, Jr. 1994;Wightman and Zimmerman 1990) and is the site of action of psychostimulants such as cocaine and amphetamines (Ritz and Kuhar 1989), understanding the effects of the AA signaling cascade on the transporter could be crucial to understanding symptoms of addiction and other neuropsychiatric disorders.

In purified synaptosomes from rat striatum, AA dose-dependently increases spontaneous release of DA and decreases DA synthesis and uptake. This effect is mediated by AA itself rather than by a metabolite (L'hirondel et al. 1995). AA also inhibits uptake of glutamate (Volterra et al. 1994;Volterra et al. 1992) and alters proton conductance by the rat glutamate transporter, EAAT4 (Fairman et al. 1998;Tzingounis et al. 1998). Electrophysiological recordings from *Xenopus* oocytes expressing the human (h)DAT indicate that AA acts directly to stimulate a cocaine-sensitive cation conductance that is distinct from current associated with substrate transport, and the effect can be generated by other fatty acids as well (Ingram and Amara 2000).

PLA2, which can be stimulated by G-protein coupled DA D2 receptors (D2R), hydrolyzes membrane phospholipids to cause a release of platelet-activating factor and AA (Vial and Piomelli 1995;Piomelli et al. 1991). In bovine cerebellar cells AA can activate PKC (Shearman et al. 1989), yet

Zhang et al (Zhang and Reith 1996) have shown that AA effects on DAT function are not mediated by PKC. Thus, the mechanisms mediating AA's effects on the DAT are not clear.

Melittin is a 26 amino acid amphiphilic peptide isolated from the venom of the honeybee, *Apis mellifera*. Melittin activates AA signaling pathways in neurons (Geddis et al. 2004;Muzzio et al. 2001) and in multiple cell lines including rat PC12, and mouse L1210 cells (Palomba et al. 2004;Lee et al. 2001), possibly through PLA2 stimulation. Additionally melittin acts through non-PLA2-mediated mechanisms to release fatty acids and alter cell function (Lee et al. 2001). While melittin has been used to examine the effects of PLA2 signaling on DA cell function (Reid et al. 1996;Zhang and Reith 1996), the mechanism of action has been presumed to be release of AA. The potential contributions of extra-AA effects of melittin to DA cell function have not been explored.

In this study we used HEK cells stably transfected with the DAT to examine the effects of melittin on DAT function and trafficking. Here, we demonstrate that treatment with melittin resulted in a decrease in DAT function. Additionally melittin directly inhibited antagonist binding and substrate uptake by the DAT. Melittin caused the release of membrane incorporated AA, but this effect was not mediated by phospholipases in these cells. Although melittin did not act through the expected mechanisms, it did

stimulate internalization of the DAT, and this effect was blocked by cocaine pretreatment, corroborating the direct interaction of melittin with the DAT.

EXPERIMENTAL PROCEDURES

Materials

Melittin was purchased from Sigma-Aldrich (St. Louis, MO USA). The radioligands [^{3}H]DA (59 Ci/mmol), [^{3}H]AA (60 Ci/mmol), and [^{3}H]spiperone (73 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ USA), and [^{125}I]RTI-55 (2200 Ci/mmol) was purchased from Perkin Elmer (Wellesley, MA USA). Rat anti-hDAT antibody was purchased from Chemicon (Temecula, CA USA) and goat anti-rat secondary conjugated to alkaline phosphate was purchased from Santa Cruz (Santa Cruz, CA USA). Other reagents were purchased from commercial sources (Sigma Aldrich, St Louis, MO USA)

Stable Cell Line Transfection

The cDNAs were cloned and human embryonic kidney-293 (HEK) cells were co-transfected with the cDNAs for the hDAT (in pcDNA1), the long form of the D2R (in pcDNA1) and the antibiotic resistance vector pBabepuro at stoichiometries of 7:7:1 using the calcium phosphate precipitation method (Chen and Okayama 1988). The cloning and characterization of the hDAT and D2R cDNAs were described previously (Eshleman et al. 1995;Neve et al.

1989). Cells were selected for resistance to puromycin (2 g/ml), and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, 2 g/ml puromycin, in a humidified 10% CO_2 incubator at 37°C

[^{3}H]spiperone binding

Cells were grown to confluence on 10 cm tissue culture plates then lysed with buffer (2 mM HEPES, 1 mM EDTA), centrifuged at 31,000g for 20 min, re-suspended and homogenized with a polytron (setting 6 for 10 seconds) in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6). [^{3}H]Spiperone binding assays were performed as previously described (Neve et al. 1989). Briefly, cell homogenates were incubated with the radioligand (0.0125nM to 0.4nM) for 60 min at 37°C. The assay was performed in TBS plus bovine serum albumin (BSA; 1 mg/100 mL) in a total volume of 1 mL. Nonspecific binding was determined in the presence of 2 μM butaclamol. The assay was terminated by filtration through Wallac filtermat A filters (Wellesley, 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 Wallac 1205 betaplate scintillation counter. Experiments were conducted with duplicate determinations at each radioligand concentration.

[^{125}I]RTI-55 binding

Experiments were performed as previously described (Eshleman et al. 1999). Briefly, cells were grown to confluence on 15 cm tissue culture plates. The cells were rinsed with Ca^{2+}, Mg^{2+} -free phosphate buffered saline (PBS; 0.1 M H_2PO4, 150 mM NaCl), and lysis buffer was added. Cells were then removed from the plate, centrifuged for 20 min at 30,000g, and the pellet was homogenized in 5 ml of 0.32 M sucrose with a Polytron homogenizer for 5 s. Membrane aliquots (10-30 μg of protein) were added to each assay, which resulted in binding of <10% of the total radioactivity added. Assays were performed in duplicate in Krebs-Hepes buffer (25 mM HEPES, 122 mM NaCl, 5 mM KCL, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 M pargyline, 100 M tropolone, 2 mg glucose/ml, 1 mM ascorbic acid, pH 7.4), in a final volume of 250 μl. [^{125}I]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 M mazindol. The assay was terminated and radiation quantified as described above. For the saturation binding experiments [^{125}I]RTI-55 was diluted with unlabeled ligand at concentrations ranging from 0.04-10 nM.

[^{3}H]DA uptake

For attached cell assays, cells were plated in 24-well tissue culture plates coated with poly-D-lysine (1 mg/ml in H_20). Cells were incubated at

37°C with drugs in Krebs-Hepes buffer in a total volume of 500 L. The uptake assay was initiated by addition of 50 nM [^{3}H]DA at 37°C. Nonspecific uptake was determined in the presence of 10 M mazindol, added 5 min before the radioligand. Uptake continued for 10 min and was terminated by washing the cells twice with ice-cold buffer. Radioactivity was extracted by rupturing the cellular membranes with 3% trichloroacetic acid for 30 min. Extracted radioactivity was measured using a Beckman LS 3801 scintillation counter (Fullerton, CA USA). Cells from one well of each assay plate were used to determine protein concentrations using the BCA protein assay kit (Pierce, Rockford, IL USA).

Fatty acid analysis by gas-liquid chromatography (GLC)

Cells grown on tissue culture plates were treated with drug in PBS at 37°C and the reaction was stopped by rapidly cooling the cells on ice. Cells were then rinsed with fatty-acid-free bovine serum albumin (0.1mg/ml in PBS) followed by a rinse in PBS. The cells were scraped from the plates and centrifuged at 200xg for 10 min. The pellets were retained for analysis. Fatty acid extraction and quantification was performed by the Lipid Atherosclerosis Laboratory at Oregon Health & Science University using the chloroform-methanol procedure described previously (Rapp et al. 1983) with some modifications. The cell extracts were mixed with chloroform:methanol (2:1), centrifuged, and the top layer removed. Free cholesteryl ester was

saponified with ethanol and KOH at 37°C for 60 min. The cholesterol was extracted with hexane. Acidifying the aqueous phase and re-extracting with hexane recovered the cholesteryl ester fatty acids. The fatty acids were methylated with boron trifluoride-methanol and percents of total fatty acid were determined by gas-liquid chromatography using a 30 meter Supelco SP 2330 fused silica capillary column (run at 190°) attached to a Perkin-Elmer AutoSystem chromatograph and a Perkin-Elmer Turbochrome 41 integrator. Fatty acid standards (Supelco, Inc., Bellefonte, PA USA) were analyzed in each experiment. The 17-carbon fatty acid (C17:0) was added to each sample to determine μg amounts of each fatty acid analyzed based on relative abundance to this standard. Levels of C17:0 are extremely low in naïve cells.

[^{3}H]AA release

Cells were grown on poly-D-lysine coated, 24-well tissue culture plates and incubated with 1nM [^{3}H]AA (106,000 DPM / well) for 18-24 hrs. DMEM supplemented with 0.2% fatty acid free BSA was used for all rinses and assay conditions. Cells were rinsed twice to remove unincorporated [^{3}H]AA. The reaction was performed in a total volume of 500 L. The medium was removed and centrifuged at 12,000x g for 1 min. Released [^{3}H]AA in 400 μl of supernatant was measured using a Beckman LS 3801 scintillation counter. Trypsin-EDTA was added to each well to remove cells remaining on the plates. This sample was also counted to measure incorporated (non-released)

[^{3}H]AA. The value for non-released [^{3}H]AA was combined with the value for released [^{3}H]AA to determine the total amount of [^{3}H]AA incorporated into cellular membranes in each well. Cells from one well of each assay plate were used to determine protein concentrations using the BCA protein assay kit (Pierce). Experiments were conducted in triplicate for each time point or drug concentration.

Biotinylation of membrane proteins

To measure DAT that was internalized following drug treatment, cells were incubated with 0.3 mg/ml NHS-SS-biotin (Pierce) 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 incubated with drug or vehicle in DMEM at 37°C. Removing the media and rapidly cooling the plates on ice halted the reaction. Remaining cell surface biotin was stripped with glutathione (150 mM glutathione, 150 mM NaCl, pH 8.75). Cells were then rinsed with 50 mM iodoactemide in PBS to neutralize the glutathione, and rinsed again with CHAPS buffer (1% CHAPS, 25mM Tris, pH 7.4, 150 mM NaCl), and lysed using a glass–glass homogenizer. The samples were centrifuged at 14,000xg, and the supernatants were reserved. The protein concentration of each sample was determined using the BCA Assay kit (Pierce). Biotinylated proteins were isolated from nonbiotinylated proteins by incubation with ImmunoPure™ Immobilized streptavidin (Pierce) for 40 min at 4°C with

gentle agitation. Proteins were eluted from the streptavidin beads with 30 μl of Laemmli sample buffer (Sigma) and constant mixing for 20 min. Samples were separated by SDS-PAGE and transferred to PVDF membranes for western blotting and DAT detection with anti-DAT antibody (Chemicon). DAT immunoreactivity was quantified by densitometry on a Typhoon phosphorimager using ImageQuant software (Molecular Dynamics; Sunnyvale CA USA).

Data Analysis

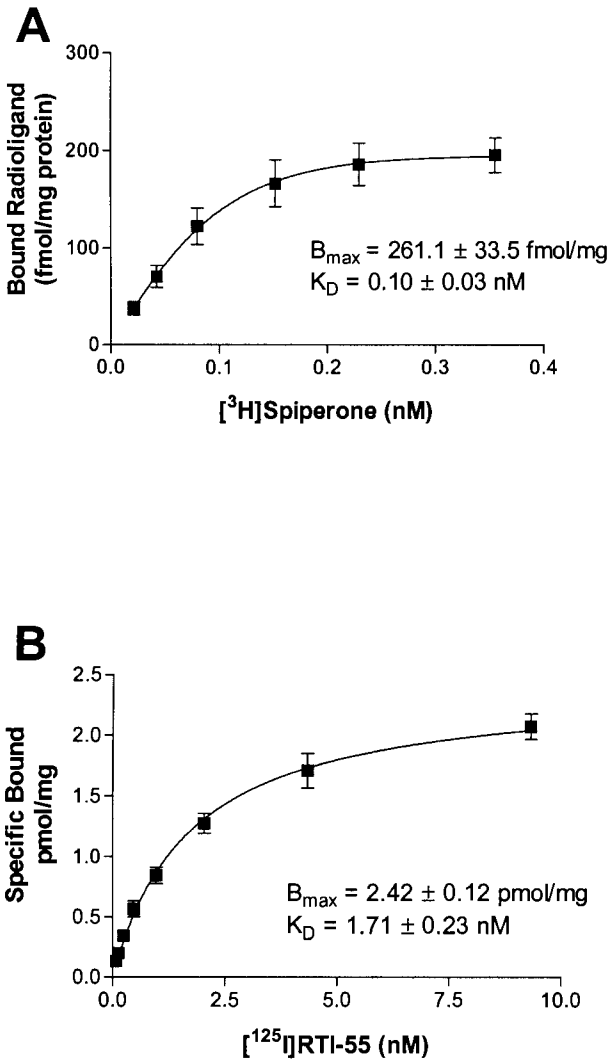
Dose-response curves were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA USA). Results were analyzed using one-way ANOVA followed by Tukey' s multiple comparison test.

RESULTS

Characterization of transfected HEK cells.

To produce a cell model for examining the effects of melittin on DAT trafficking and regulation, HEK cells were stably transfected with the hDAT and the DA D2R. Saturation binding experiments to characterize recombinant D2R expression were conducted using [^{3}H]spiperone concentrations of 0.0125nM to 0.4nM. The B_{max} for radioligand binding was 261.1 ± 33.5 fmol/mg with a K_D of 0.10 ± 0.03 nM (Fig 2-1A). Saturation

Fig 2-1. Characterization of stable transfections in HEK cells. Saturation binding experiments were performed as described in the text. 1A, [^{3}H]spiperone binding to the D2R is a composite of 5 independent experiments and [^{125}I]RTI-55 binding to the DAT, 1B, is a composite of 6 independent experiments, each conducted with duplicate determinations.



binding of the recombinant DAT was determined in the presence of 40 pM [^{125}I]RTI-55 diluted with unlabeled ligand. The B_{max} of the binding site was 2.42 ± 0.12 pmol/mg and the K_D was 1.71 ± 0.23 nM (Fig 2-1B) in the same cells.

Melittin treatment decreases DAT function.

To determine the effects of PLA2 activation on DAT function, HEK-D2-hDAT cells were incubated with melittin for 10-120 min (Table 1) followed by incubation with 50 nM [^{3}H]DA for 10 min. Functional activity of the DAT was quantified by measuring the [^{3}H]DA taken up by the cells. Melittin treatment times longer than 5 min reduced uptake almost completely. The IC_{50} concentration for melittin's effects was 1.3 μM at the 30 min time point. This concentration and treatment time were used in subsequent assays. Melittin was significantly less potent following a 5 min treatment, as compared to any other time point (one-way ANOVA, $p<0.001$). To eliminate possible effects of feedback by the D2R on DA uptake, butaclamol (100 nM) was added to the cells 10 min before uptake was initiated. There was no difference in [^{3}H]DA uptake between butaclamol-treated and untreated cells during this time period (Fig 2-2).

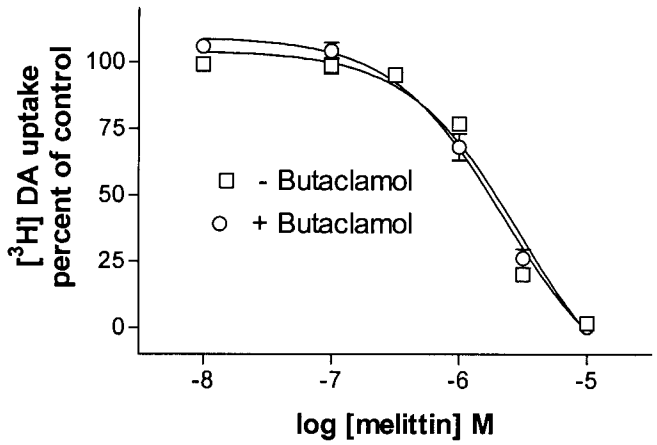
These findings agree with previous reports demonstrating a melittin-induced decrease in DAT-mediated uptake of DA following 15 min of

Table 1
Potency of melittin on inhibition of DAT function

Time	[^{3}H]DA uptake IC_{50} (μM)
5 min	4.2 ±0.1
10 min	0.82 ± 0.01
15 min	1.76 ± 0.1
30 min	1.3 ± 0.14
60 min	0.8 ± 0.1
120 min	1.79 ± 0.12

Cells were treated with melittin for the times indicated, followed by the addition of 50 nM [^{3}H]DA. Uptake continued for 10 min. Washes with ice-cold buffer terminated the assay, and the cells were ruptured to release the radioactivity. Data represent mean ± SEM of three independent experiments, each conducted with triplicate determinations. The effects of treatment for 5 min is significantly different than all longer times ($p<0.001$, one-way ANOVA), but treatment times from 10-120 min do not differ significantly ($p>0.05$, one-way ANOVA).

Fig 2-2. Melittin causes a dose dependent decrease in DAT-mediated uptake. HEK-D2-hDAT cells were treated with varying doses of melittin for 30 min, rinsed, and the uptake assay was initiated by the addition of 50 nM [^{3}H]DA at 37°C. Experiments to block the D2R were conducted in parallel using identical melittin treatments followed by the inclusion of butaclamol (100 nM) 10 min before the addition of [^{3}H]DA. Results are presented as a percent of vehicle treated control cells. The curves are mean ± SEM of three independent experiments, each conducted with triplicate determinations.



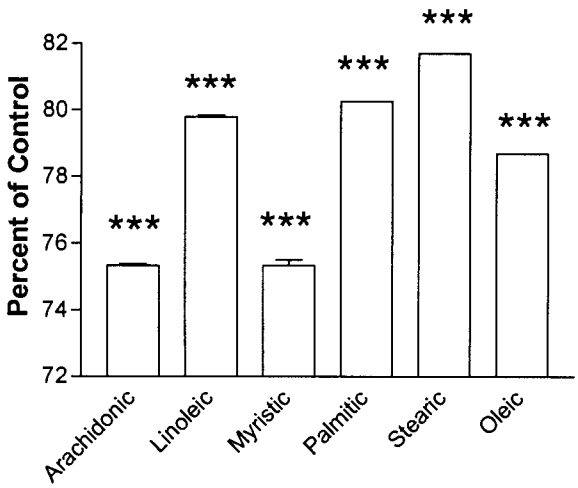
treatment of rat C6 glioma cells transfected with the DAT. The reported decrease in uptake coincided with a decrease in binding of the cocaine analogue WIN 35,428 to the DAT in whole, attached cells (Zhang and Reith 1996). The goal of the present study was to further characterize this effect and determine the precise mechanism of melittin's action.

Melittin releases arachidonic acid from cellular membranes.

To examine the ability of melittin to activate PLA2, the levels of fatty acids remaining in cellular membranes following treatments were quantified by GLC. Cells were treated with melittin (1.3 μM) for 30 min then rinsed with fatty acid-free BSA in PBS to bind and remove any free, released AA. Following treatment, the membrane bound AA was 75.33 ± 0.06% of vehicle-treated levels (Fig 2-3). There was also a 20% reduction in the AA precursor linoleic acid. Both values are significant ($p<0.001$) reductions. In addition to these expected reductions, there were also reductions in the saturated fatty acids myristic, palmitic, and stearic acid of 24%, 20%, and 18% respectively, and a 21% reduction in the unsaturated fatty acid oleic acid. These results demonstrate a melittin-induced release of AA. Release of the other fatty acids suggests additional actions of melittin on membrane lipids, not specific to PLA2 stimulation.

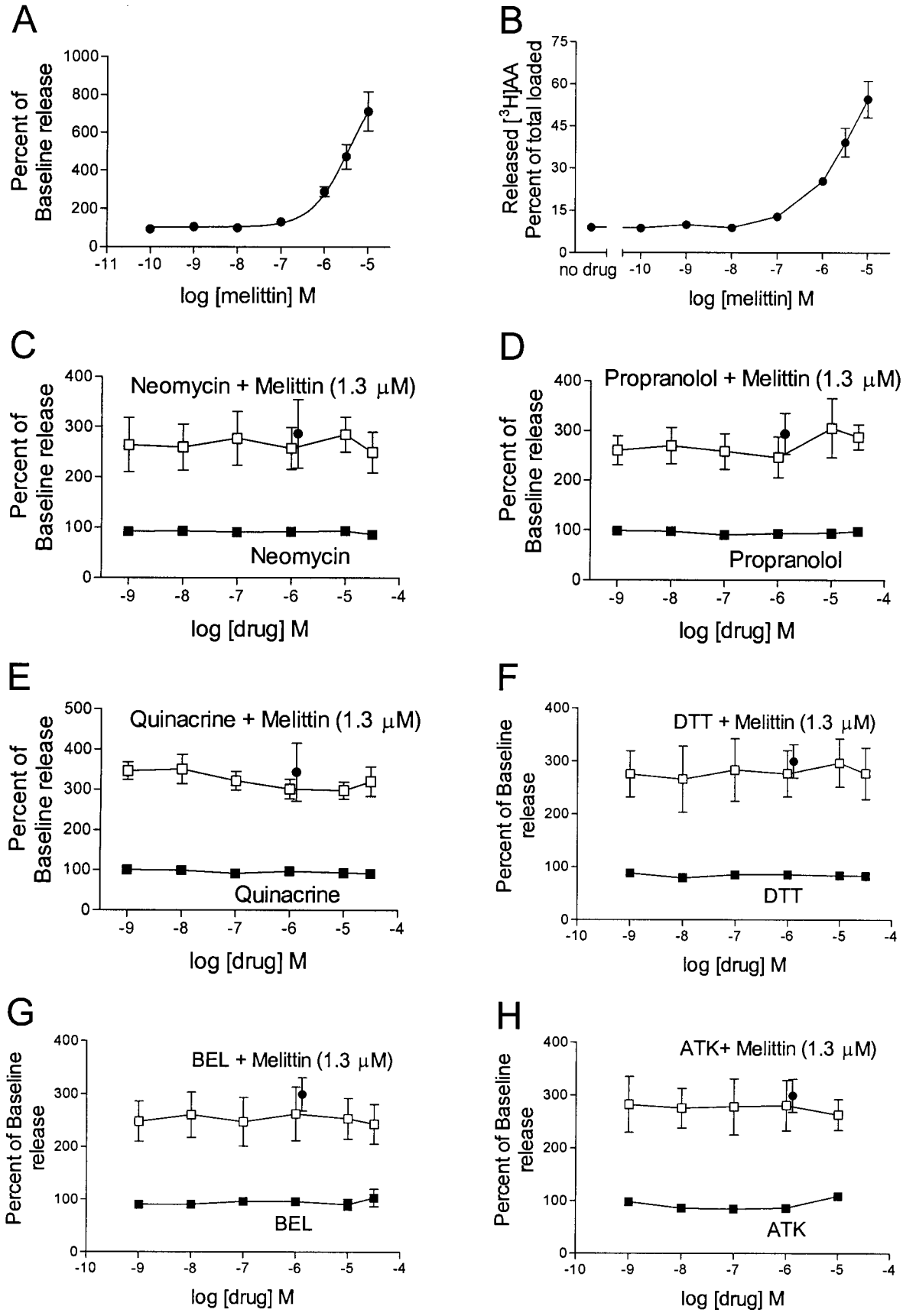
While melittin is typically utilized to stimulate PLA2, recent studies have revealed that melittin simultaneously stimulates multiple

Fig 2-3. Melittin releases AA from cell membranes. Cells were treated with melittin (1.3 μM) for 30 min and fatty acids remaining in the membrane after treatment were isolated for GLC analysis as described in the text. Levels of fatty acids remaining after treatment are presented as a percent of vehicle treated controls. Data are mean ± SEM of three independent experiments (***, $p<0.001$ by Student's t test).



phospholipases in mouse L1210 cells (Lee et al. 2001). To determine the specific phospholipase stimulated by melittin in transfected HEK cells, [^{3}H]AA was incorporated into lipid membranes, the cells were treated with melittin and inhibitors of PLC, PLD, or PLA2, and the amount of [^{3}H]AA released following treatment was quantified. Initially, cells were treated with melittin for 30 min, demonstrating a dose-dependent release of [^{3}H]AA. Following treatment with 1.3 μM melittin the amount of released [^{3}H]AA was 324.1 ± 34.2% of baseline release (Fig 2-4A), 28.4 ± 2.5% of total incorporated [^{3}H]AA (Fig 2-4B), and 77.4 ± 8.0 fmol [^{3}H]AA /mg of protein (data not shown). These data using preloaded AA correlate with GLC quantification of endogenous AA released following melittin treatment. AA can be released directly in a single reaction by PLA2. While not releasing AA directly, PLC and PLD generate free fatty acids that contain arachidonate and action by subsequent enzymes can produce free AA (for review see Piomelli D 1995). To determine if these phospholipases were involved in [^{3}H]AA release, cells were preloaded with [^{3}H]AA and treated for 60 min with phospholipase inhibitors alone or in combination with 1.3 μM melittin during the final 30 min of treatment. Treatment with neomycin, a PLC inhibitor (Carney et al. 1985), did not prevent melittin-induced [^{3}H]AA release (Fig 2-4C). While propranolol is often used as an antagonist of β-adrenergic receptors (Mehvar and Brocks 2001) it has also been used to inhibit PLD pathway-mediated release of AA in a variety of cells, including stimulated aortic cells

Fig 2-4. Melittin-stimulated release of AA is through non-phospholipase-mediated mechanisms. HEK-D2-hDAT cells were incubated with [^{3}H]AA for 18-20 hrs. Stimulated [^{3}H]AA release was analyzed as described in the text. Cells were treated with melittin for 30 min and the percent of baseline release (A) and the percent of total cell incorporated [^{3}H]AA released (B) were quantified. To determine the ability of melittin to stimulate phospholipases, cells were treated with neomycin (C), propanolol (D), quinacrine (E), DTT (F), BEL (G), or ATK (H) for 60 min either individually (closed squares) or with the addition of a single dose of melittin (1.3 μM, open squares) during the final 30 min of the treatment. The single closed circle in graphs C-H represents the effect of melittin alone (1.3 μM). Released [^{3}H]AA is presented as a percent of untreated baseline release levels. Data are the mean ± SEM of three experiments, each conducted with triplicate determinations.


A
Percent of Baseline release
1000
800
600
400
200
0
-11 -10 -9 -8 -7 -6 -5
log [melittin] M
B
Released [3H]AA Percent of total loaded
75
60
45
30
15
0
no drug -10 -9 -8 -7 -6 -5
log [melittin] M
C
Neomycin + Melittin (1.3 μM)
Percent of Baseline release
400
300
200
100
0
Neomycin
-9 -8 -7 -6 -5 -4
log [drug] M
D
Propranolol + Melittin (1.3 μM)
Percent of Baseline release
400
300
200
100
0
Propranolol
-9 -8 -7 -6 -5 -4
log [drug] M
E
Quinacrine + Melittin (1.3 μM)
Percent of Baseline release
500
400
300
200
100
0
Quinacrine
-9 -8 -7 -6 -5 -4
log [drug] M
F
DTT + Melittin (1.3 μM)
Percent of Baseline release
400
300
200
100
0
DTT
-10 -9 -8 -7 -6 -5 -4
log [drug] M
G
BEL + Melittin (1.3 μM)
Percent of Baseline release
400
300
200
100
0
BEL
-10 -9 -8 -7 -6 -5 -4
log [drug] M
H
ATK+ Melittin (1.3 μM)
Percent of Baseline release
400
300
200
100
0
ATK
-10 -9 -8 -7 -6 -5 -4
log [drug] M

(Shinoda et al. 1997), epithelial keratinocytes (Lefkowitz and Smith 2002), and myocytes (Albert et al. 2005). Treatment with propranolol did not inhibit melittin-induced [^{3}H]AA release (Fig 2-4D). Surprisingly the general PLA2 inhibitor quinacrine (Demuth et al. 2005) was also without effect on melittin-induced [^{3}H]AA release (Fig 2-4E). We additionally used inhibitors of specific subtypes of PLA2 to determine if these enzymes were stimulated by melittin to cause release of AA. PLA2 can be divided into extracellular and intracellular types. The extracellular type is a secreted enzyme (sPLA2) that does not exhibit preference for particular fatty acids, contains disulfide bonds, and is sensitive to reducing agents, such as dithiothreitol (DTT). The intracellular types can be further divided into Ca^{2+}-dependant type (cPLA2), requiring 0.1-1 μM of free Ca^{2+} for activation, and Ca^{2+}-independant (iPLA2) type (for review see Dennis 1994;Glaser et al. 1993). To inhibit sPLA2 cells were treated with the reducing agent DTT prior to the application of melittin. This agent was unable to inhibit the melittin-induced release of [^{3}H]AA (Fig 2-4F). The iPLA2 subtype is selectively inhibited by 4-bromoenol lactone (BEL) (Yang et al. 1999), and application of this inhibitor did not inhibit the effect of melittin (Fig 2-4G). Arachidonyl trifluoromethyl ketone (ATK) is a potent and selective inhibitor of cPLA2 (Street et al. 1993), yet this compound was also unable to inhibit melittin-induced release of [^{3}H]AA in these cells (Fig 2-4H).

Melittin decreases transporter function by binding to the DAT.

To examine possible mechanisms for how melittin inhibits DAT-mediated uptake, binding of the cocaine analogue [^{125}I]RTI-55 to the transporter was examined. Membranes from homogenized cells were treated with varying concentrations of melittin (Fig 2-5A) in the presence of 40 pM [^{125}I]RTI-55. The IC_{50} for melittin inhibition of [^{125}I]RTI-55 binding was 0.88 μM, and the Hill slope was -1.75 ± 0.21. For saturation equilibrium binding, cell membranes were incubated with 40 pM [^{125}I]RTI-55 and varying concentrations of non-radiolabeled ligand. Concurrent addition of melittin (1.3 μM) (Fig 2-5B) to the [^{125}I]RTI-55 binding assay significantly reduced the B_{max} from 2.49 ± 0.14 to 1.78 ± 0.13 pmol/mg ($p<0.001$). The K_D for [^{125}I]RTI-55 binding was 1.48 ± 0.23 nM and the K_D for [^{125}I]RTI-55 binding in the presence of melittin was 1.62 ± 0.33 nM. These were not significantly different, suggesting that melittin acts like a noncompetitive inhibitor of RTI-55 binding to the DAT. [^{125}I]RTI-55 binding in the presence of a greater concentration of melittin (10 μM) further reduced the B_{max} to 1.43 ± 0.48 pmol/mg while the K_D (4.44 ± 3.17nM) was not significantly altered, as compared to untreated cells (Table 2).

Studies examining the effects of membrane binding peptides on Ca^{2+} ATPase in the sarcoplasmic reticulum found that melittin inhibits ATPase activity by reducing the rotational mobility of the enzyme. Melittin also causes a 25% reduction in membrane surface lipid hydrocarbon chain

Fig 2-5. Melittin binds to the dopamine transporter. To generate dose-response binding curves for melittin (A) HEK-D2-hDAT cells were treated with varying concentrations of melittin in the presence of 40 pM [^{125}I] RTI-55 as described in the text. Equilibrium saturation binding experiments (B) were also conducted. The graph depicts [^{125}I]RTI-55 binding in the absence of melittin (closed squares), and [^{125}I] RTI-55 binding in the presence of 1.3μM (open circles) or 10μM (open squares) melittin. Data are the mean ± SEM of 4-6 independent experiments (***,$p<0.001$ by two-way ANOVA followed by Bonferroni post test). The role of temperature in melittin's effects on RTI-55 equilibrium saturation binding is shown in panel (C). The curves are averages of 4 independent experiments. [^{125}I] RTI-55 binding at 4°C (closed square) and at room temperature (open square), and [^{125}I] RTI-55 binding in the presence of melittin (1.3μM) at 4°C (closed circle) and room temperature (open circle) are depicted (*,$p<0.05$, by two-way ANOVA followed by Bonferroni post-test comparing treated to vehicle treated samples). The effect of melittin on [^{3}H]spiperone binding to the D2R was performed as described in the text (D) (*, $p<0.05$, ANOVA followed by Student's t test).

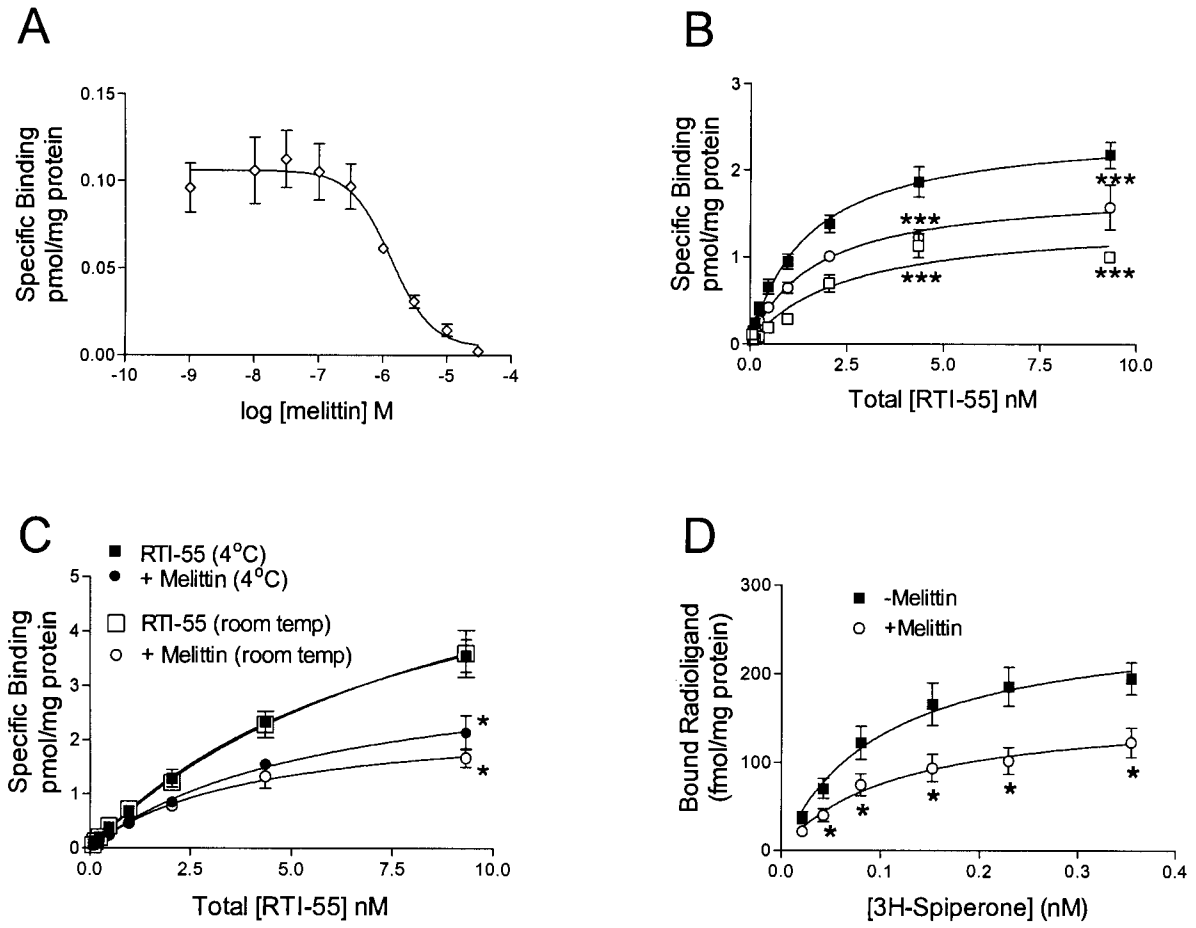


Table 2
Effects of melittin on RTI-55 saturation binding.

Treatment	[^{125}I]RTI-55 B_{max} (pmol/mg)	[^{125}I]RTI-55 K_D (nM)
None	2.49 ± 0.14	1.48 ± 0.23
Melittin 1.3 μM	1.78 ± 0.13	1.62 ± 0.33
Melittin 10 μM	1.42 ± 0.16	2.38 ± 0.7

Homogenized cell membranes were incubated for 90 min with 40 pM [^{125}I]RTI-55, varying concentrations of non-radiolabeled ligand, and melittin as indicated. Nonspecific binding was determined in the presence of 10 M mazindol. The assay was terminated by filtration. Data are mean ± SEM from at least 3 experiments.

mobility (Mahaney and Thomas 1991). Additionally melittin stabilizes Ca^{2+} ATPase in a position that has decreased affinity for its substrate (Voss et al. 1995). This mechanism may explain the effects of melittin on RTI-55 binding in DAT-expressing cells. To examine the effects of membrane fluidity on the ability of melittin to inhibit antagonist binding to the DAT, RTI-55 binding experiments were conducted at 4°C. Parallel experiments were conducted at room temperature and at 4°C in the presence of melittin (1.3μM). The experiments continued for 16 hrs to allow both temperature conditions to come to equilibrium. All samples contained protease inhibitors to prevent protein degradation at room temperature. For experiments conducted at 4°C, all reagents, drugs, and membranes were chilled on ice before the assays were initiated. Melittin treatment significantly reduced the B_{max} from 6.7 ± 0.8 to 2.9 ± 0.3 pmol/mg at 4°C and from 7.2 ± 1.3 to 2.4 ± 1.0 pmol/mg at room temperature ($p<0.05$) (Fig 2-5C). Melittin did not significantly shift the binding affinity (K_D 8.3 ± 1.8 to 4.5 ± 0.9 nM at 4°C and 9.4 ± 2.8 to 4.0 ± 1.0 at room temperature, $p>0.05$). Additionally, RTI-55 binding in the absence of melittin at 4°C was not significantly different from binding at room temperature, and binding in the presence of melittin at 4°C was not significantly different from RTI-55 binding in the presence of melittin at room temperature ($p>0.05$). Thus, membrane fluidity does not appear to play a role in melittin's effect on the DAT.

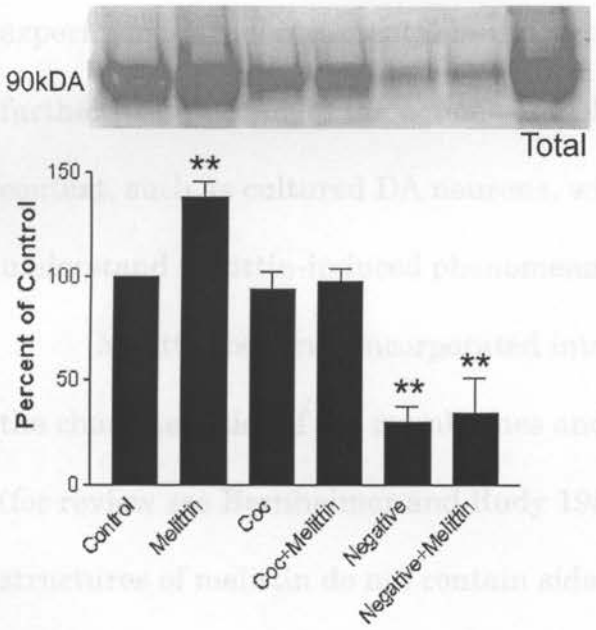
Additional studies were conducted in these cells to determine whether melittin interacts with the DA D2R (Fig 2-5D). Saturation binding experiments were conducted using [^{3}H]spiperone concentrations of 0.0125nM to 0.4nM alone, or in the presence of 1.3 μM melittin. Melittin caused a significant reduction in the B_{max} from 261.1 ± 33.5 fmol/mg to 158.5 ± 26.0 fmol/mg ($p<0.05$). Melittin did not significantly shift the binding affinity (K_D 0.10 ± 0.03 to 0.11 ± 0.05 nM, $p>0.05$). Thus melittin acts like a noncompetitive inhibitor of spiperone binding to the DA D2 receptor, and so the effect of melittin on radioligand binding is not specific to the DAT.

Melittin increases DAT internalization.

The decrease in DAT function may result entirely from melittin binding to the transporter, and subsequent inhibition of uptake. However activation of AA signaling by melittin may simultaneously contribute to changes in DAT function, or melittin may directly alter cell surface transporter expression. Previous studies have demonstrated that the decrease in DAT function following stimulation of PKC is due to an increase in transporter internalization in PC12 cells (Melikian and Buckley 1999) and MDCK cells (Daniels and Amara 1999) transfected with the DAT. Although the melittin-induced decrease in DAT function is not prevented with a PKC inhibitor (Zhang and Reith 1996), we wanted to determine whether there was an increase in transporter internalization due to melittin treatment. To

address this question cell surface biotinylation was performed. Extracellular proteins were labeled with a reducible form of biotin prior to treatments. Cell surface biotin remaining following treatment was cleaved. Thus biotin-labeled proteins internalized by treatment were protected from the reducing agent. Cells were treated with melittin (1.3 μM) or vehicle for 30 min at 37°C. The internalized proteins were isolated by avidin purification and separated by SDS-PAGE. Melittin-induced internalization was significantly ($p<0.01$) increased to 137.9 ± 7.6% of baseline levels (Fig 2-6). Interestingly, this effect was blocked by 15 min pretreatment with 1 μM cocaine (97.8 ± 6.2% of control), while cocaine treatment alone did not alter DAT trafficking (93.9 ± 8.4% of control). As a control, cells were biotinylated at 4°C, rinsed with cold glycine, and placed on ice while in DMEM. These cells demonstrate a non-permissive trafficking condition. Additionally, these cells demonstrate the removal of the cell surface biotin with the glutathione buffer. This condition reduced DAT internalization to 30.4 ± 7.2% of control and 34.3 ± 16.5% of control in the presence of melittin. Thus melittin not only binds directly to the transporter, but it also causes an increase in DAT internalization.

Fig 2-6. Melittin increases DAT internalization. HEK-D2-hDAT cells were biotinylated and then treated with vehicle or melittin (1.3 μM) for 30 min, cocaine (1 μM) for 45 min, or cocaine (1 μM) 15 min prior and during 30 min melittin (1.3 μM) treatment. Untreated, biotinylated negative control cells remained at 4°C for 30 min. Following treatment the cells were rapidly chilled and the media removed. The remaining cell surface biotin was stripped and the internalized DAT was isolated by avidin purification and separated by SDS-PAGE. The immunoblot was detected with anti-hDAT antibody. The representative blot shows biotinylated, internalized product for control and treated cells. Total biotinylated sample is also shown. Band optical density was quantified and expressed as a percent of vehicle treated control. Graph: DAT internalization expressed as means ± SEM from 3-6 experiments (**, $p < 0.01$ by Student's t test).



DISCUSSION

We generated a stable HEK cell line that expresses the recombinant hDAT, and the recombinant D2R (to be used in future experiments examining the receptor's modulation of PLA2 signaling). While DAT function and trafficking have been examined following PMA stimulation of PKC, few studies have explored the role of PLA2 stimulated AA in regulating the DAT. We used the purported PLA2 stimulator melittin throughout these experiments, and found that it not only stimulated the release of membrane-incorporated AA, but also acted directly on the DAT. While these experiments are instrumental in the initial investigation of melittin's effects, further examination of the actions of melittin in an appropriate physiological context, such as cultured DA neurons, will be beneficial to precisely understand melittin-induced phenomena.

Melittin becomes incorporated into outer cell membranes and alters the characteristics of the membranes and of the proteins embedded within (for review see Bernheimer and Rudy 1986). The primary and tertiary structures of melittin do not contain side chain structures that resemble DA. Based on structural information and the melittin-induced shift in the B_{max} and Hill slope for [^{125}I]RTI-55 binding without a change in K_D (Fig 2-5B & C), it is likely that melittin did not bind directly to the [^{125}I]RTI-55 recognition site. Instead, it acted like a non-competitive inhibitor, likely binding to a

region of the transporter that alters the conformation of the DAT making it unable to bind RTI-55. This designation of melittin as a non-competitive inhibitor is in a phenomenological sense analogous to enzyme kinetics. Non-competitive inhibitors bind to both the free receptor and to the receptor-ligand complex without altering the affinity of the ligand binding (Limbird 2005). The RTI-55 binding assay does not distinguish between the DAT-RTI-55 complex and the DAT-RTI-55-melittin complex.

Melittin's inhibition of [^{125}I]RTI-55 binding to the DAT could be due to nonspecific effects such as altered membrane structure. RTI-55 binds to the DAT while the membranes are at 4^{o}C, and the maximal binding is not significantly different from binding at room temperature (Fig 2-5C). Thus it is likely that the state of the membrane does not alter the ability of direct antagonists, such as RTI-55, to specifically bind to the DAT, and that melittin's effect on RTI-55 binding is not due to disruption of membrane structure. Melittin's influence on membrane fluidity has been studied in other cell systems. The effects of melittin on sarcoplasmic reticulum membrane fluidity are comparable to the effects on the membrane of decreasing the temperature by 5^{o}C. Melittin also decreases the rotational mobility of fluid lipids and of restricted lipids that immediately surround the proteins, and reduces the rotational ability of integral Ca^{2+} ATPase (Mahaney et al. 1992). In the current experiments melittin did not appear to alter

membrane fluidity, as its effects on RTI-55 binding to the DAT at 4°C and at room temperature did not differ (Fig 2-5C).

The inhibition of [^{125}I]RTI-55 binding to the DAT could have resulted from melittin-induced changes in DAT-associated proteins that prevent proper conformation of the transporter necessary for ligand binding. These experiments were performed in homogenized cells where additional proteins interacting with the DAT in a whole cell, may not be available in this homogenate. RTI-55 was able to bind to the DAT in these experiments indicating that additional accessory proteins are not necessary for proper ligand binding in this assay. Further more, homogenized cells were cooled to 4°C prior to the addition of melittin (Fig 2-5C), thus preventing further changes in membrane or protein conformations. The effect of melittin on RTI-55 binding to the DAT remains at 4°C, further indicating that conformational changes induced by additional accessory proteins are not necessary for ligand binding to the DAT.

While melittin interacted directly with the DAT, the inhibition of radioligand binding was not specific to the DAT. It also interacted with the DA D2R (Fig 2-5D), as a noncompetitive inhibitor of [^{3}H]spiperone binding, suggesting that melittin may interact nonspecifically with membrane-incorporated proteins. Thus melittin appears to act in a previously unreported pharmacological manner. This may serve as a confounding variable for other experiments that use melittin to stimulate PLA2.

Furthermore, melittin binds to a variety of cellular proteins including calmodulin (Maulet and Cox 1983), bovine mitochondrial F1-ATPase (Gledhill and Walker 2005) and to the ubiquitous transcription factor NF-κB (Park et al. 2004). While melittin has been used as a tool to examine the peptide binding properties of the previously mentioned proteins, this is the first examination of melittin's effects on cell surface membrane-incorporated proteins and how protein surface expression is altered following exposure.

Melittin treatment caused a strong reduction in uptake of [^{3}H]DA that occurs within 5-10 min of treatment (Table 1). The rapidity of the response may suggest that melittin is affecting the DAT through a disruption of membrane integrity or loss of ionic gradients necessary for DA transport. DAT antagonists, such as cocaine and mazindol, specifically interact with the DAT to inhibit uptake of [^{3}H]DA following a 1 min preincubation, and this effect is not significantly different from preincubation times of 30 min (Eshleman et al. 1999). Thus the rapid inhibition of DAT function does not exclude the possibility of a direct effect of melittin on the transporter. Additional experiments, such as electrophysiological recordings, can be done to determine whether melittin affects Na+ or Cl- gradients across cellular membranes. The effect of melittin on DAT-mediated uptake of [^{3}H]DA, together with the effect on binding of RTI-55, suggests a direct interaction of melittin with the transporter.

The observed decrease in cellular uptake of [^{3}H]DA *via* the DAT following melittin treatment was not necessarily the sole result of AA release, but may also have been caused by melittin preventing the binding of ligand to the transporter. These two mechanisms may also explain the disparities between the effects of direct application of AA and of melittin stimulated PLA2 activity. Zhang and Reith (1996) demonstrated that short periods (15-30min) of exogenous AA treatment cause an increase in DAT-mediated uptake of [^{3}H]DA while longer treatment times (45-60 min) cause an increase in uptake at low concentrations and a decrease in uptake at higher AA concentrations, while melittin treatment causes a decrease in uptake at all concentrations tested. Thus, melittin influences cell function in a complex manner represented by at least two distinct mechanisms: direct action on the DAT and release of fatty acids. One of the primary goals in this study was to more fully explore the pharmacological actions of melittin in an attempt to explain the disparities between the effects of melittin and the previously reported effects of AA.

Melittin not only stimulates PLA2 activity, but also PLC and PLD activity in L1210 cells. The effects of melittin on fatty acid release are completely blocked only when the cells are simultaneously pretreated with inhibitors of all three phospholipases, with PLA2 activity accounting for about 10% of the total effect (Lee et al. 2001). However, we found that melittin stimulated the release of AA from cellular membranes (Fig 2-3 & 2-

4), but this effect was not blocked by treatment with inhibitors of PLC, PLD, or PLA2. Thus, melittin did not act as a specific pharmacological stimulator of phospholipases. This effect may be particular to the chosen cell type. PLA2 inhibitors successfully block melittin-induced release of AA in rabbit proximal tubule cells (Han et al. 2002), but quinacrine is unable to inhibit melittin-induced release of AA in rat Leydig cells (Ronco et al. 2002). These differences may result from varying expression of phospholipases across cell types. At the very least, while some effects of melittin on cellular activity may be mediated through the AA signaling pathway, they may not be directly or exclusively produced by PLA2. Additional experiments utilizing siRNA to interfere with PLC, PLD, and PLA2 transcription may be useful to confirm that melittin does not stimulate these enzymes.

Since phospholipase inhibitors did not block melittin-stimulated AA release, there may be some aspect of the peptide that interacts with the membrane to cause release of fatty acids. The 14 amino acid peptide mastoparan, found in wasp venom, is used to stimulate GTP binding regulatory proteins (Higashijima et al. 1988). Interestingly, recent studies demonstrate that mastoparan does not actually stimulate these signaling proteins directly, but rather disrupts the membrane lipid rafts where many receptors and second messenger proteins are localized, thus releasing these proteins and lipids to the cytoplasm (Sugama et al. 2005). Mastoparan also releases AA from PC12 cells (Nakamura et al. 2004). Experiments using

nuclear magnetic resonance measurements demonstrate an interaction between melittin and ganglioside components of lipid rafts (Chatterjee and Mukhopadhyay 2002). Thus, it is possible that melittin stimulates fatty acid release and activates signaling pathways by direct interactions with the membrane.

Previous studies using rotating disk electrode voltammetry to study the effects of direct application of AA on DAT-mediated uptake demonstrate an AA-induced decrease in DA uptake. Additionally the cis-unsaturated fatty acids oleic and linoleic acid inhibit DAT mediated uptake, although to a lesser degree (Chen et al. 2003). These results suggest that there may be a property common to fatty acids that alter DAT function, rather than activity by a specific AA metabolite. It should also be noted that the other fatty acids released by melittin (Fig 2-3) could have contributed to the decrease in DAT-mediated uptake that is described in the current findings. While no studies have specifically explored the effects of AA on DAT trafficking, C6 cells expressing the hDAT demonstrate a decrease in B_{max} with no change in K_D following exposure to AA in attached, whole cell preparations (Zhang and Reith 1996), suggesting transporter internalization, although the mechanism has not been determined. It is possible that direct actions of AA on the DAT initiate trafficking pathways. AA ethyl ester, a non-metabolizable AA analogue, can be incorporated into the cell membrane, but does not mimic the effects of AA on DAT function (Chen et al. 2003;Ingram and Amara 2000).

This result suggests that AA acts from within the lipid phase of the membrane, but not by simply altering the lipid microenvironment surrounding membrane-spanning proteins. Rather this may involve a specific fatty acid binding domain on the DAT or on an interacting protein. While it is possible that AA released by melittin treatment was directly affecting the function and trafficking of the DAT in this current study, the data suggests that direct interaction of melittin with the DAT caused internalization, as cocaine pretreatment completely prevented melittin-induced internalization (Fig 2-6).

Although melittin directly affected radioligand binding to the DAT, the inhibition of DA uptake (Fig 2-2) could also be the result of a decrease in surface expression of the transporter (Fig 2-6). Simply blocking the DAT with cocaine did not alter transporter surface expression, yet cocaine pretreatment prevented the melittin-induced internalization (Fig 2-5). This may be due to a steric hindrance of the DAT caused by the interaction with cocaine that prevents melittin's interaction with the transporter. Previous studies using HEK cells transfected with the DAT found that cocaine (10 μM, 10 min) causes an increase in DAT surface expression (Daws et al. 2002). This may be due to the difference in drug concentration or cells used, but regardless, melittin does not affect DAT surface expression the way that cocaine does. It is not known whether cocaine also blocks the effects of AA applied to the DAT. Previous reports using PMA to induce DAT

internalization found that cocaine treatment does not alter transporter surface expression, nor does cocaine pretreatment alter the PMA effect (Daniels and Amara 1999). Thus it is likely melittin was operating through a different mechanism than PMA.

It is possible that melittin alters DAT conformation to expose additional lysine residues to biotin, thus the increase in immunodetected DAT would represent an increase in overall biotinylation rather than an increase in endocytosis (Fig 2-6). Excess, unbound biotin was removed prior to melittin treatment. If melittin did alter DAT conformation there would not be additional biotin available to bind to the newly exposed sites. Additionally, the biotin remaining on the surface of the cells was removed following melittin treatment, so only internalized biotinylated DAT was detected. The negative control samples that were maintained at 4°C while treated with melittin did not cause an increase DAT internalization. Thus melittin does not inherently increase DAT biotinylation. Melittin continued to interact directly with the DAT at 4°C (Fig 2-5C), but the induced internalization was prevented at this temperature. While melittin apparently did not act through the previously described mechanism, it did cause DAT internalization independent of phospholipase activation.

Interestingly, PLA2 activity may play a role in psychostimulant sensitization. Direct injections of melittin into rat ventral tegmental area cause sensitization, seen as increased locomotor activity, stereotypy, and DA

release in the nucleus accumbens, to subsequent cocaine administration. This sensitization is blocked by pretreatment with the PLA2 inhibitor quinacrine (Reid et al. 1996). Thus, the influence of phospholipase activity and AA signaling on DA system functioning may be important for understanding stimulant addiction.

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Chapter III.

Melittin initiates dopamine transporter trafficking pathways that differ from phorbol ester's and cocaine's effects

Abstract

Phospholipase A2 (PLA2) releases the fatty acid arachidonic acid (AA) from membrane phospholipids. We used the purported PLA2 stimulator melittin to examine the effects of endogenous AA signaling on dopamine (DA) transporter (DAT) function and trafficking. We previously reported that melittin interacts directly with the DAT to inhibit function and cause internalization of the transporter in HEK-293 cells stably transfected with the human DAT. In the current study melittin treatment caused an increase in DAT internalization as demonstrated by an increase in DAT colocalization with clathrin using confocal microscopy. This increase in colocalization was blocked by pretreatment with cocaine. To determine the intracellular location of the internalized DAT we measured DAT colocalization with markers of intracellular organelles. Melittin treatment caused an increase in DAT colocalization with the early endosome marker Rab 5A, and caused a decrease in colocalization with the late endosome marker Rab 7 and the recycling endosome marker Rab 11. By comparison, phorbol ester treatment increased DAT colocalization with early endosome antigen 1 (EEA1) after 30 min of treatment, and increased DAT colocalization with Rab 7 after 60 min of treatment. Cocaine treatment does not affect DAT trafficking in these cells. Experiments using density gradient centrifugation confirm that melittin treatment causes the DAT to become associated with a density fraction

containing Rab 5A. Following 60 min of melittin treatment the DAT is trafficked back to the membrane.

Introduction

The DA system in the brain is involved in mediating events such as movement and motivational control, and removal of released DA from the synapse is crucial for spatial and temporal coordination of neurotransmission. The high affinity DAT is the primary means of removing released DA from the synapse (Wightman and Zimmerman 1990;Parsons and Justice, Jr. 1994). The DAT contains twelve membrane-spanning domains, multiple N- glycosylation sites and putative protein kinase C (PKC) and PKA cytoplasmic phosphorylation sites (Amara and Kuhar 1993). The role of the DAT in modulating DA disposition is evident as DAT knockout mice exhibit slow clearance of synaptically released DA and are hyperactive (Giros et al. 1996). Additionally the DAT is a primary site of action of psychostimulants such as cocaine and amphetamine (for review see Zahniser and Doolen 2001).

Considering the contribution of the DAT to DA signal modulation, it is not surprising that DAT surface expression is highly regulated through protein kinase-induced and constitutive trafficking pathways. The use of homologous cells to express the DAT has been instrumental in exploring DAT trafficking. Treatment with phorbol 12-myristate 13-acetate (PMA) to activate PKC causes an increase in transporter internalization in MDCK (Daniels and Amara 1999), Sf9, COS-7 (Pristupa et al. 1998), PC12 (Melikian

and Buckley 1999), and PAE (Sorkina et al. 2003) cells transfected with the DAT. The location and destination of the internalized DAT varies in many of these studies and this may be due to differences in treatment conditions, cell types, or visual detection tags fused to the transporters. A role for mitogen activated protein kinase in regulating DAT function and surface expression in transfected cells and in striatal preparations is also beginning to emerge (Moron et al. 2003). Application of substrates, such as amphetamine and DA, to cells transfected with the DAT and in rat striatal synaptosomes also causes a decrease in DAT function caused by a decrease in transporter surface expression (Saunders et al. 2000;Chi and Reith 2003). Additionally, a model of constitutive DAT membrane trafficking is developing, which indicates a unique pathway for DAT regulation that is clathrin-dependant (Sorkina et al. 2005) and may be distinct from transferrin receptor trafficking patterns (Loder and Melikian 2003).

While treatments with PMA and methamphetamine do increase phosphorylation levels on the N-terminal region of the DAT (Vaughan et al. 1997) (Cervinski et al. 2005), it seems that direct phosphorylation of the transporter is not necessary to mediate the changes in DAT surface expression or function (Cervinski et al. 2005;Chang et al. 2001). Thus there are likely multiple interacting cellular components or pathways responsible for regulating DAT surface expression and function.

Though numerous studies have explored DAT trafficking following PKC activation and exposure to substrates, few studies have explored transporter trafficking following activation of PLA2 or stimulation with melittin. Free AA is generated by PLA2, which hydrolyzes membrane phospholipids to release fatty acids such as AA and platelet-activating factor (Piomelli et al. 1991;Vial and Piomelli 1995). AA dose-dependently increases spontaneous release of DA and decreases DA synthesis and uptake in purified synaptosomes from rat striatum (L'hirondel et al. 1995). AA also stimulates a cocaine-sensitive cation conductance in the DAT that is not associated with substrate transport (Ingram and Amara 2000). Similarly, AA alters glutamate transporter function by inhibiting uptake (Volterra et al. 1994) and inducing a proton conductance in the rat EAAT4 glutamate transporter (Fairman et al. 1998).

Melittin, a 26 amino acid amphiphilic peptide, is isolated from the venom of the honeybee, *Apis mellifera*. Previous reports have used melittin to stimulate PLA2 and cause a release of AA in neurons (Geddis et al. 2004;Muzzio et al. 2001) and in multiple cell lines, including human U937, rat PC12, and mouse L1210 (Palomba et al. 2004;Tommasini et al. 2002;Lee et al. 2001). In contrast to these findings, we previously reported that while melittin does cause release of AA in our model cell line, this release is not mediated by activation of phospholipases. We also discovered that melittin interacts directly with the DAT to inhibit antagonist binding. These

previously uncharacterized properties of melittin additionally stimulate internalization of the transporter, and this internalization is mediated by melittin's direct interaction with the DAT, as cocaine pretreatment prevents the melittin-induced internalization.

In this study we used HEK cells stably transfected with the DAT to examine the effects of melittin on DAT trafficking. Melittin treatment stimulates DAT endocytosis and trafficking through a clathrin-mediated and Rab 5A associated mechanism. In addition, melittin induces DAT trafficking patterns disparate from the effect of PKC- and cocaine-activated pathways.

EXPERIMENTAL PROCEDURES

Materials

Melittin and Optiprep were purchased from Sigma-Aldrich (St Louis, MO USA). Antibodies used include rat anti-DAT 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 (Santa Cruz, CA USA), 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 Molecular Probes (Eugene, OR USA).

Stably transfected cell line

The cDNAs were cloned and human embryonic kidney-293 (HEK) cells were co-transfected with the cDNAs for the human DAT (hDAT) (in pcDNA1), the long form of the D2R (in pcDNA1) and the antibiotic resistance vector pBabepuro at stoichiometries of 7:7:1 using the calcium phosphate precipitation method. The cloning and characterization of the DAT and D2 receptor cDNAs were described previously (Eshleman et al. 1995;Neve et al. 1989). Cells were selected for resistance to puromycin (2 g/ml). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.05 U penicillin/streptomycin, 2 g/ml puromycin, in a humidified 10% CO_2 incubator at 37°C

Immunocytochemistry

Cells were treated with drugs or vehicle as indicated at 37°C, fixed in 4% paraformaldahyde for 10 min at 37°C, and then washed three times in 1 ml PBS (136 mM NaCl, 25 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$) for 5 min at room temperature. Non-specific reaction sites were blocked and the cells were permeabilized in PBS with 4% goat serum or donkey serum, 1% BSA, and 0.2%Triton-X 100 for 1 hour.

Primary antibody was added in PBS with 0.25% BSA, 0.2% Triton-X 100 and the preparation was incubated for 1 hour. The antibodies were used at the following dilutions: rat anti-DAT, 1:250; goat anti-clathrin, 1:500; goat anti-Rab 7, 1:200; mouse anti Rab 11, 1:200; mouse anti-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 made in PBS with 1% goat or donkey serum and cells on coverslips were incubated at room temperature for 1 hour. Coverslips were mounted on glass microscope slides using Prolong Antifade kit (Molecular Probes, Eugene, OR USA), and cells were imaged using a laser-scanning confocal Leica TCS SP microscope with 100x objective. Consecutive sections were sequentially scanned alternating between 488 and 568 nm lasers, and sections were imaged at 300 μm intervals.

Colocalization

The colocalization of DAT 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 gray-scale values. The gray scale of fluorescently unsaturated images was proportional to the amount of flourophore 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. 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 DAT and marker proteins in control and treated cells.

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. They were 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 900xg for 10 min at 4°C. The supernatant was then centrifuged at 12,000xg for 20 min at 4°C. The resulting pellet was resuspended in HM buffer and layered above the prepared Optiprep layers and centrifuged at 90,000xg for 3 hr 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 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).

[^{125}I]RTI-55 binding

Experiments were performed as previously described (Eshleman et al. 1999) with some modifications. Total specific binding of [^{125}I]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 $MgSO_4$, 2.5 mM $CaCl_2$, 1 M pargyline, 100 M tropolone, 2 mg glucose/ml, 1 mM ascorbic acid, pH 7.4), in a final volume of 250 µl. [^{125}I]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 M mazindol. The assay was terminated by filtration through Wallac filtermat A filters (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 Wallac 1205 betaplate scintillation counter.

Biotinylation of membrane proteins

To measure the DAT that was remaining on the surface following treatment, cells were treated with drugs or vehicle in DMEM at 37°C. Removing the media and rapidly cooling the plates on ice halted the reaction.

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, 25mM Tris, pH 7.4, 150 mM NaCl) and lysed using a glass–glass homogenizer. The samples were centrifuged at 14,000xg, and the supernatants were reserved. The protein concentration of each sample was determined using the BCA Assay kit (Pierce, Rockford, IL). Biotinylated proteins were isolated from nonbiotinylated proteins by incubation with ImmunoPure™ Immobilized streptavidin (Pierce, Rockford, IL) for 40 min at 4°C with gentle agitation. 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-DAT antibody (Chemicon, Temecula, CA). DAT immunoreactivity was quantified by densitometry on a Typhoon phosphorimager using ImageQuant software (Molecular Dynamics; Sunnyvale CA).

RESULTS

We reported previously that treatment with melittin (1.3 µM, 30 min, 37°C) causes an increase in internalization of the DAT in HEK-D2R-DAT

cells. This internalization is completely blocked by pretreating the cells with cocaine prior to melittin application. Additionally, cocaine treatment did not alter transporter surface expression, thus while melittin and cocaine both interact directly with the transporter, they appear to act through distinct mechanisms. In this study our goal was to characterize the internalization pathway initiated by melittin treatment. We used HEK cells that were stably transfected with the DAT and DA D2R.

Inhibition of DAT 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 DAT, confocal microscopy was used to examine changes in DAT colocalization with clathrin following melittin treatment. Cells plated on glass coverslips were treated, fixed, and blocked to prevent nonspecific binding. Cells were then dual-labeled using antibodies for the DAT and for endogenous clathrin. The ratio of DAT colocalized with clathrin compared to total levels of DAT in each cell was used to detect trafficking changes (Fig 3-1 & 3-2). Under control conditions, 6.2 ± 0.8% of the total DAT was colocalized with clathrin. Treatment with 1.3μM melittin for 30 min caused a significant increase ($p<0.01$) to 26.1 ± 2.6% of the total DAT colocalized with clathrin, suggesting the transporter is internalized through clathrin coated pits. The regions of colocalization can be seen near the membrane. To prevent the formation of

Fig 3-1. DAT colocalization with clathrin. Cells were treated with vehicle (A-D) or melittin (E-H) for 30 min. The cells were then fixed, permeabilized, and colabelled for DAT and endogenous clathrin. The immunoreactivity was imaged by confocal microscopy as described in the text. The representative fluorescent images show DAT (green, A&E) and endogenous clathrin (red, B&F). The merged image (DAT + clathrin, C&G) shows co-localization (yellow). The merged and subtracted image shows only those pixels containing both green and red fluorophores (pseudocolor yellow, D&H). The experiment was repeated 3 times with similar results.

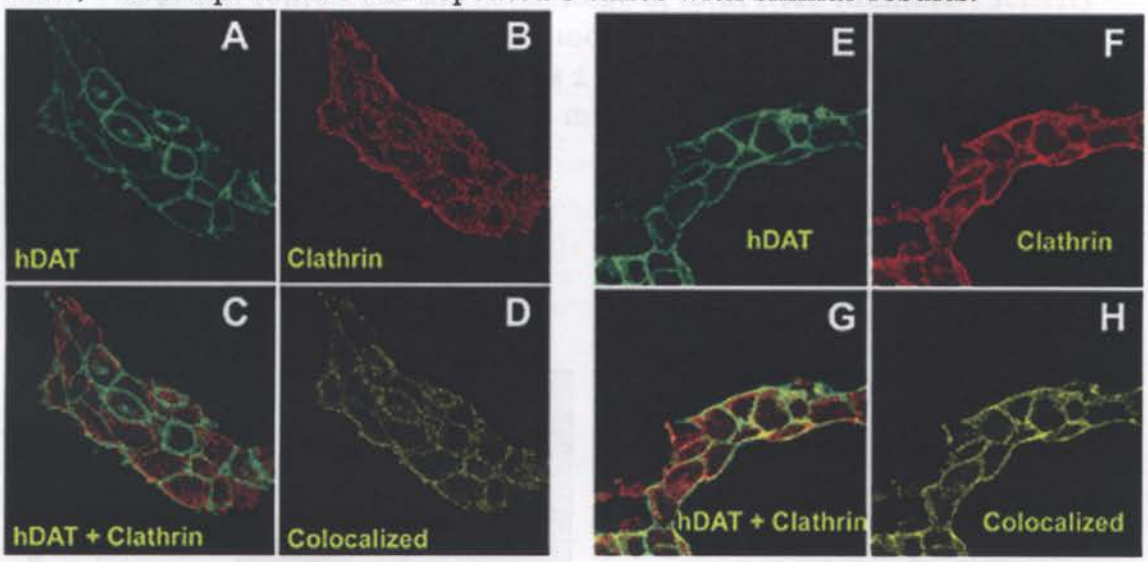
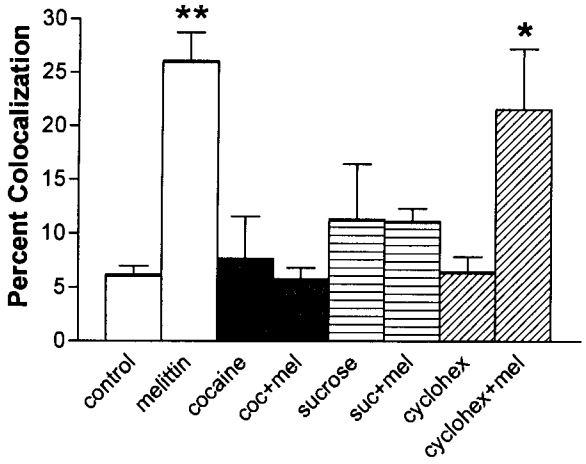


Fig 3-2. Melittin increases DAT colocalization with clathrin. Cells grown on coverslips were treated with melittin (1.3 μM) for 30 min, cocaine (1 μM) for 45 min, cocaine for 15min prior and during 30 min melittin, sucrose (0.45 M) for 45 min, sucrose 15 min prior and during 30 min melittin, cyclohexamide (350 μM) for 90 min, or cyclohexamide 60 min prior and during melittin treatment. The cells were then fixed, permeabilized, and colabeled for DAT and endogenous clathrin. Immunoreactivity was imaged using confocal microscopy. Colocalization was quantified as described in the text. The graph represents the percentage of total DAT immunoreactivity that is colocalized with clathrin immunoreactivity for each treatment condition. The results are the means ± SEM of 3 independent experiments (**, $p < 0.01$; *, $p < 0.05$ by Dunnett's multiple comparison test).



the clathrin structure, cells were treated with 0.45 M sucrose (Heuser and Anderson 1989) for 15 min prior to melittin treatment. Sucrose alone caused 11.3 ± 5.1% of the DAT to be colocalized with clathrin and sucrose and melittin together caused 11.1 ± 1.2% colocalization. Neither condition was significantly different from control treatment ($p>0.05$). (Fig. 3-2)

Pretreating the cells with 1 μM cocaine for 45 min did not significantly alter clathrin co-localization (7.7 ± 3.4%) as compared to control values, but treating the cells with cocaine for 15 min before and during the 30 min melittin treatment prevented the DAT colocalization with clathrin (5.8 ± 1.0%). This was significantly less than melittin treatment alone (26.1%; $p<0.01$) and comparable to control levels. To determine if these effects of melittin are dependent on protein synthesis, cells were treated with 350 μM cyclohexamide for 1 hour. The cells were then either treated with melittin or vehicle for 30 min. Colocalization of the DAT with clathrin following treatment with cyclohexamide alone was 6.5 ± 1.4%, which was not significantly different than control levels ($p>0.05$), and cyclohexamide treatment followed by melittin caused 21.6 ± 5.7% of the DAT to be colocalized with clathrin. This is not significantly different than melittin treatment alone ($p>0.05$) (Fig 3-2). Application of only the secondary antibodies did not produce detectable immunostaining.

To further examine the trafficking pathways engaged by melittin treatment, confocal microscopy was used to measure DAT colocalization with

endosomal markers (Fig 3-3). The destination of internalized DAT can be determined by increases in colocalization with proteins used as markers for different subcellular endosomal trafficking compartments. 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 a marker 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. Distinct compartments of the endosomal pathway express different Rab GTPases on their outer surfaces (for review see Pfeffer 2001). EEA1 is localized to early endosomes, but unlike Rab 5, never to the plasma membrane (Zerial and McBride 2001;Sonnichsen et al. 2000). Additionally, 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 DAT trafficking following PKC stimulation (Sorkina et al. 2003;Melikian and Buckley 1999).

Melittin treatment (1.3 μM, 30 min) caused a significant increase in DAT colocalization with Rab 5A from 6.6 ± 0.5% to 14.4 ± 2.5% ($p<0.05$; Fig 3-4A), while not affecting colocalization following 15 min (6.0 ± 1.0%) or 60 min (7.0 ± 1.3%) of treatment. Under control conditions 8.5 ± 0.9% of the DAT

Fig 3-3. DAT colocalization with selected markers after stimulation in HEK cells. Cells grown on coverslips were treated with melittin, PMA, or vehicle control as indicated. Fixed and permeabilized cells were colabeled for transfected hDAT and endogenous Rab 5A (A), EEA1 (B), Rab 11 (C), or Rab 7 (D). The representative fluorescent images show the immunodetection of each marker and the colocalized images show only those pixels that contain fluorophores for both DAT and the marker protein (pseudocolor yellow). The experiments were repeated 6-10 times with similar results.

Fig 3-3

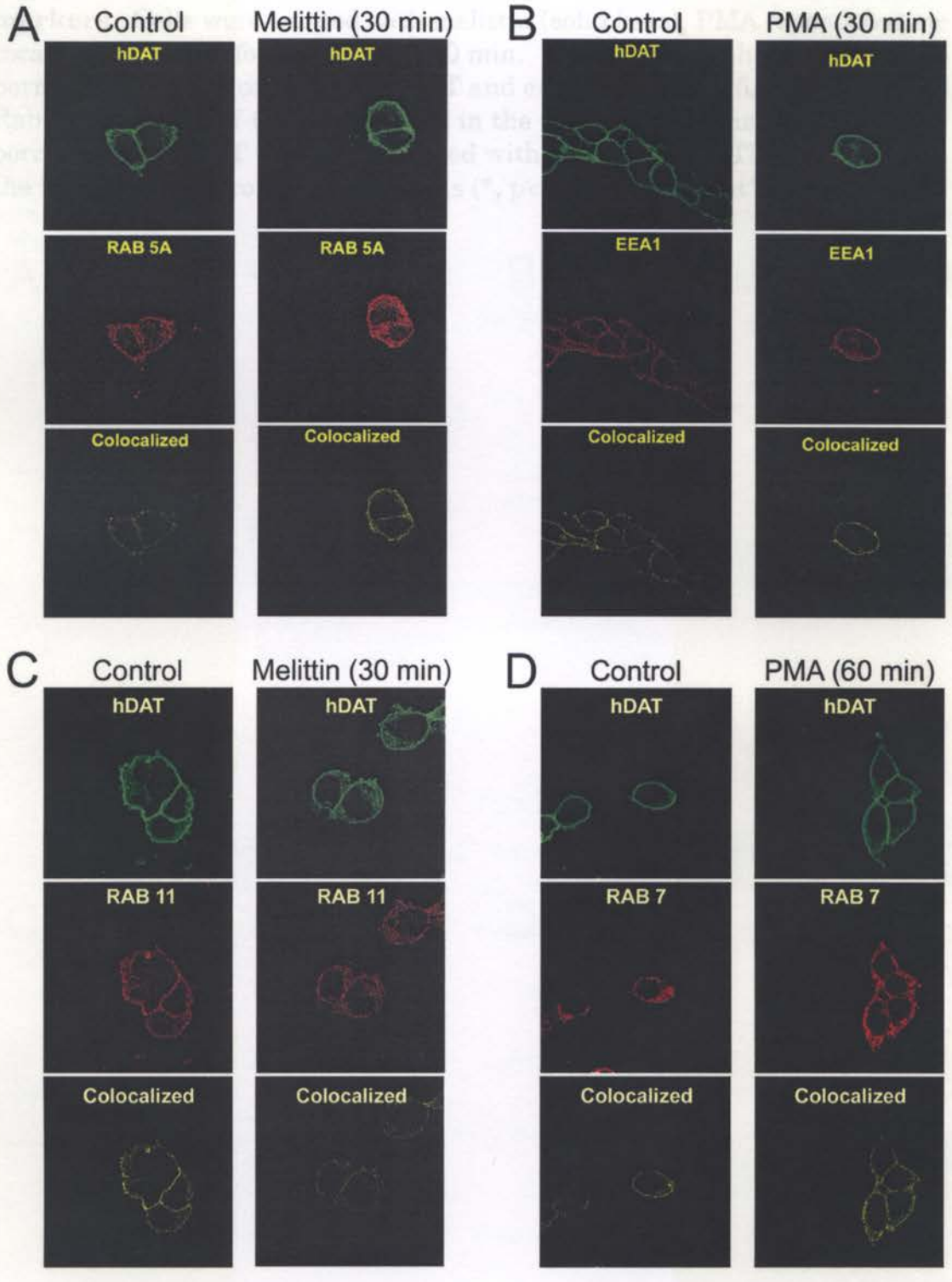
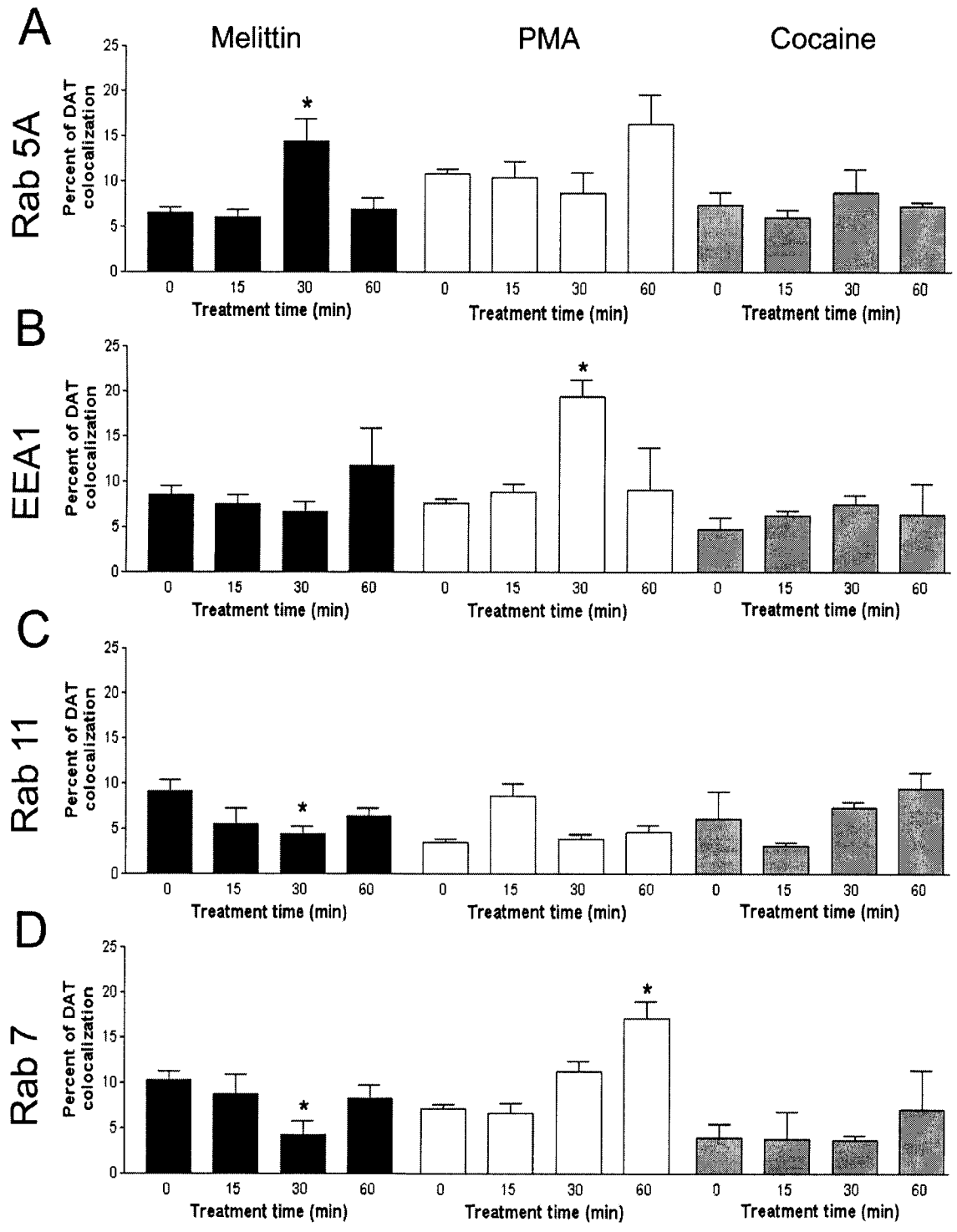

A
Control
Melittin (30 min)
hDAT
RAB 5A
Colocalized
B
Control
PMA (30 min)
hDAT
EEA1
Colocalized
C
Control
Melittin (30 min)
hDAT
RAB 11
Colocalized
D
Control
PMA (60 min)
hDAT
RAB 7
Colocalized

Fig 3-4. Melittin and PMA alter DAT colocalization with endosomal markers. Cells were treated with melittin (solid bars), PMA (open bars), or cocaine (gray bars) for 0, 15, 30, or 60 min. The cells were then fixed, permeabilized, and colabeled for DAT and endogenous Rab 5A (A), EEA1 (B), Rab 11 (C), or Rab 7 (D) as described in the text. The graphs show the percent of total DAT that is colocalized with each marker. The results are the means ± SEM from 3 experiments (*, $p<0.05$; by Student's t test).

Fig 3-4


A
Melittin
PMA
Cocaine
Rab 5A
B
EEA1
C
Rab 11
D
Rab 7
Percent of DAT colocalization
Treatment time (min)
0
15
30
60
*

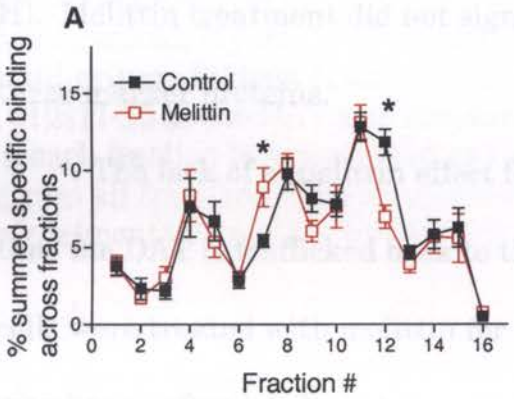
was colocalized with the early endosome marker EEA1. Melittin did not significantly alter DAT colocalization with EEA1 following 15, 30, or 60 min of treatment (7.6 ± 1.0%, 6.7 ± 1.0%, 16.6 ± 4.4% respectively, $p>0.05$) (Fig 3-4B). Melittin treatment did cause a significant reduction in DAT colocalization with the recycling endosome marker Rab 11 from 9.1 ± 1.2% to 4.4 ± 0.9% ($p<0.05$) (Fig 3-4C) and a significant reduction in colocalization with the late endosome marker Rab 7 from 10.3 ± 0.9% to 4.3 ± 1.4% ($p<0.001$) (Fig 3-4D). There were no significant changes in colocalization patterns following 15 or 60 min of treatment.

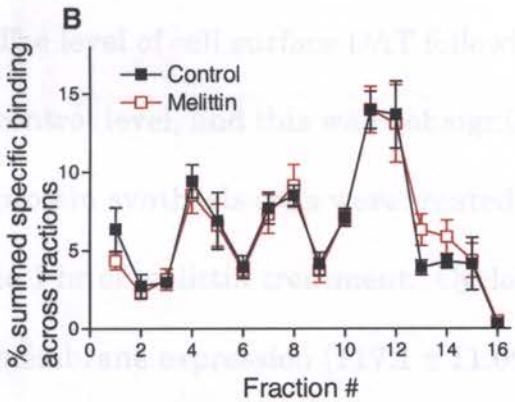
To compare these results with other known trafficking patterns and to verify the validity of this quantification method, cells were treated with the phorbol ester PMA. Activation of the PKC signaling pathway with PMA reduces DAT-mediated uptake by increasing internalization (Melikian and Buckley 1999;Sorkina et al. 2003;Daniels and Amara 1999). Treatment with 100 nM PMA for 30 min caused a significant increase ($p<0.05$) in DAT colocalization with EEA1, from 7.5 ± 0.5% (0.1% DMSO) to 19.3 ± 1.8% (Fig 3-4B). The effects of PMA on DAT colocalization with Rab 5A (Fig 3-4A) and RAB 11 (Fig 3-4C) were not significant at any treatment time. Interestingly, PMA treatment caused an increase in DAT colocalization with the late endosome marker Rab 7 with a significant change ($p<0.05$) from 7.2 ± 0.5 % to 17.1 ± 1.9% following 60 min of treatment (Fig 3-4D).

To compare the melittin results with those of a compound known to bind to the transporter, cells grown on coverslips were treated with 1 μM cocaine or vehicle for 0, 15, 30, or 60 min. The fixed, permeabilized, and blocked cells were also colabeled as described above. Cocaine treatment did not significantly alter DAT colocalization with any of the chosen markers ($p>0.05$) (Fig 3-4A-D).

To verify the results found using confocal microscopy we used equilibrium density gradient centrifugation to determine which subcellular organelle the DAT associates with following melittin treatment. Cells were treated with melittin (1.3 μM) or vehicle control and then gently homogenized. The cell lysates were separated into 16 fractions using Optiprep density gradient centrifugation. Binding of the cocaine analogue [^{125}I]RTI-55 in each fraction was used to determine the location of the DAT and immunoblotting was used to determine which fractions contained 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 3-5A). Following 60 min of melittin treatment (1.3 μM) there were no detectable changes, compared to vehicle treated control cells, in DAT association with equilibrium density gradient fractions (Fig 3-5B). Using the Optiprep samples to determine the location of the subcellular organelle marker proteins indicates that fraction 7 was associated with Rab 5A

Fig 3-5. Melittin shifts DAT 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 DAT 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 ± SEM from 5 independent experiments (*, $p<0.05$; by Student's t test).



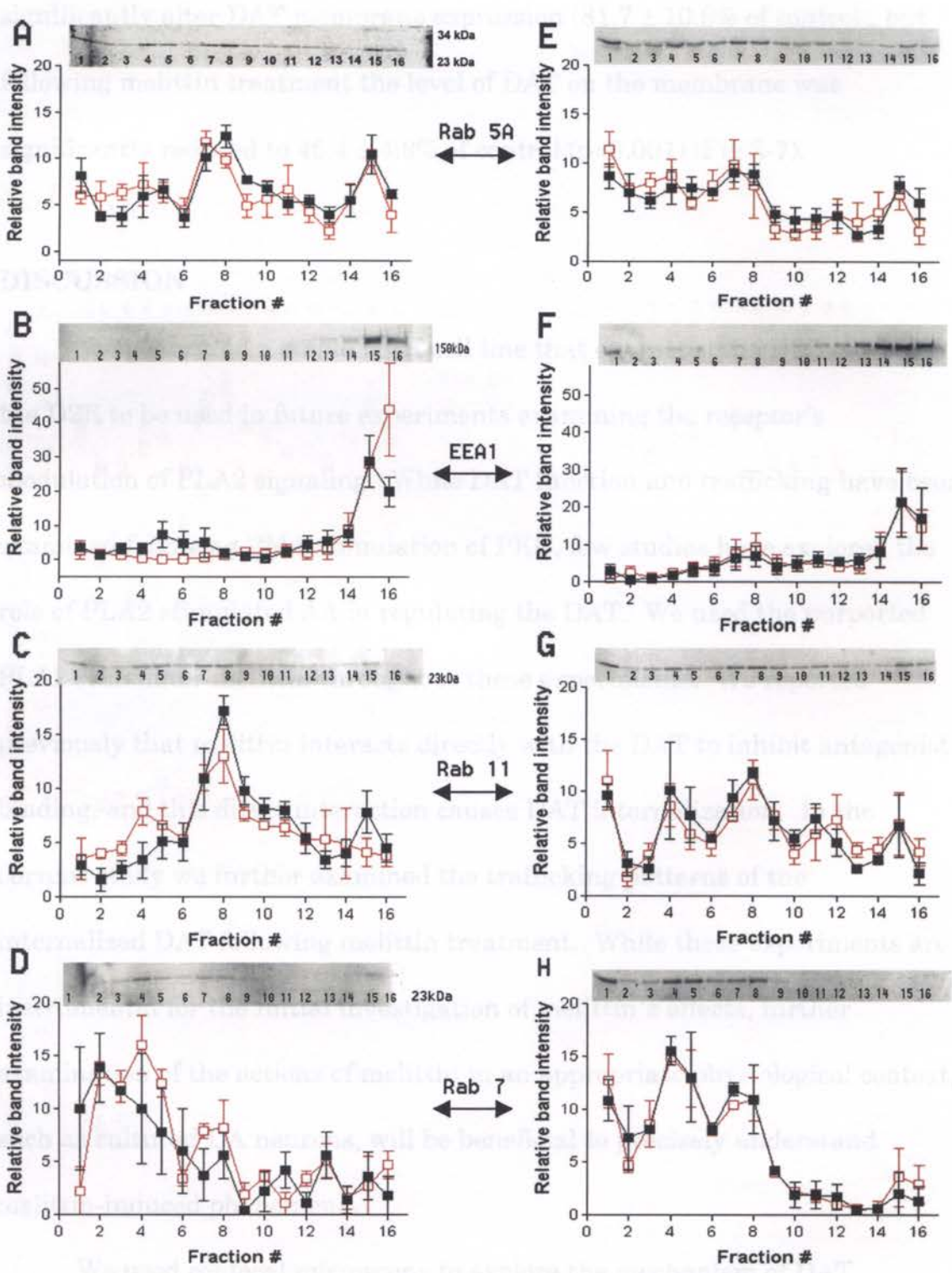


immunoreactivity (Fig 3-6A). The distribution of the early endosome markers Rab 5A and EEA1 only overlaps in the least dense fractions 15 and 16 (Fig 3-6A, B, E, & F). The recycling endosome marker Rab 11 is predominately found in fraction 8 (Fig 3-6C &G), while the late endosome marker Rab 7 is mostly isolated in the more dense fractions 1-4 (Fig 3-6 D & H). Melittin treatment did not significantly alter the distribution of any of these marker proteins.

The lack of a melittin effect following 60 min of treatment may suggest that the DAT 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 DAT following melittin treatment was 96.5 ± 8.7% of control level, and this was not significantly different ($p>0.05$). To inhibit protein synthesis cells were treated with 350 μM cyclohexamide for 1hr prior to 1 hr of melittin treatment. Cyclohexamide did not significantly alter DAT membrane expression (117.1 ± 11.0% of control), nor did it alter DAT membrane expression following melittin treatment (106.3 ± 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 μM bafilomycin for 30 min prior to melittin treatment. Bafilomycin alone did not

Fig 3-6. 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. The high density region of the gradient is on the left. Protein samples from each fraction were isolated by SDS-PAGE. The immunoblots were detected with anti-Rab 5A (A&E), anti-EEA1 (B&F), anti-Rab 11 (C&G), or anti-Rab 7 (D&H) antibodies. Representative blots of each antibody are shown. Band optical density in each fraction was quantified and expressed as percent of total band intensity across all fractions. The results are the mean ± SEM from 3-4 experiments for each antibody.

Fig 3-6


A
34 kDa
23 kDa
Rab 5A
E
B
150kDa
EEA1
F
C
23kDa
Rab 11
G
D
23kDa
Rab 7
H
Relative band intensity
Fraction #

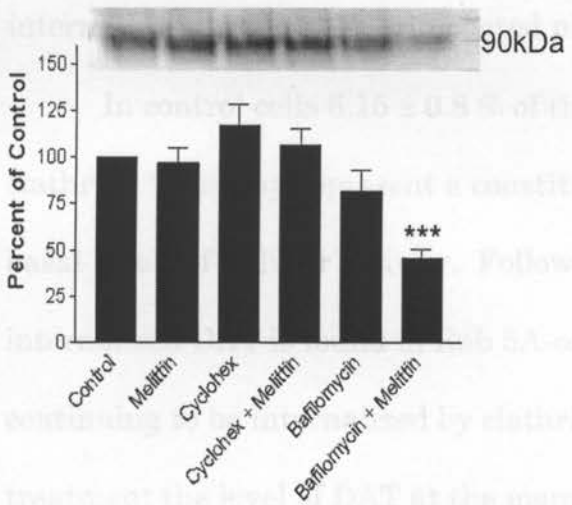
significantly alter DAT membrane expression (81.7 ± 10.9% of control), but following melittin treatment the level of DAT on the membrane was significantly reduced to 45.4 ± 4.8% of control ($p<0.001$) (Fig 3-7).

DISCUSSION

We generated a stable HEK cell line that expresses the DAT, and also the D2R to be used in future experiments examining the receptor's modulation of PLA2 signaling. While DAT function and trafficking have been examined following PMA stimulation of PKC, few studies have explored the role of PLA2 stimulated AA in regulating the DAT. We used the purported PLA2 stimulator melittin throughout these experiments. We reported previously that melittin interacts directly with the DAT to inhibit antagonist binding, and this direct interaction causes DAT internalization. In the current study we further examined the trafficking patterns of the internalized DAT following melittin treatment. While these experiments are instrumental for the initial investigation of melittin's effects, further examination of the actions of melittin in an appropriate physiological context, such as cultured DA neurons, will be beneficial to precisely understand melittin-induced phenomena.

We used confocal microscopy to explore the mechanism of DAT internalization. Melittin caused an increase in the total amount of DAT that

Fig 3-7. The DAT traffics to the membrane following 60 min of melittin treatment. HEK-D2R-hDAT cells were treated with vehicle or melittin (1.3 μM) for 60 min, cycloheximide (350 μM) alone or for 60 min prior and during 60 min melittin treatment, or bafilomycin (1 μM) alone or for 30 min prior and during 60 min melittin (1.3 μM) 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-hDAT 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 DAT expressed as means ± SEM (***,$p<0.001$; by Student's t test).



was colocalized with endogenous clathrin (Fig 3-2). This data suggest that the DAT is internalized through clathrin-coated pits and the internalization is prevented by cocaine pretreatment. This data is in agreement with our previous findings using cell surface biotinylation to indicate that melittin-induced internalization of the DAT is blocked by pretreatment with cocaine. A similar method has been used to detect colocalization of the GABA transporter with GFP-labeled clathrin to determine that it is also internalized through clathrin-coated pits (Deken et al. 2003).

In control cells 6.15 ± 0.8 % of the total DAT was colocalized with clathrin. This may represent a constitutive DAT recycling pathway during basal levels of cellular activity. Following 30 min of melittin treatment, internalized DAT is found in Rab 5A-containing endosomes (Fig 3-4) and is continuing to be internalized by clathrin-coated pits. After 60 min of melittin treatment the level of DAT at the membrane was similar to that in untreated cells (Fig 3-7). Additionally, clathrin coated buds associated with transferrin receptors have been reported to form from early endosomes (Stoorvogel et al. 1996;Futter et al. 1998). These budding vesicles are distinct from vesicles originating at the plasma membrane. Thus, it is likely that clathrin plays a role in recycling vesicles back to the plasma membrane as well as in endocytosis. This strengthens the possibility that the DAT that is internalized by melittin is recycled back to the membrane.

The time required for clathrin cages to form and then dissociate from vesicles is relatively fast (a half decay constant of 45 sec) (Mueller et al. 2004), while the dissociation stage alone is rapid enough to make detection of a clathrin-coated vesicle in the cytoplasm difficult with electron microscopy (Brodin et al. 2000). Thus it is unlikely that the confocal images of labeled clathrin are representative of mature budded vesicles, but more plausibly of clathrin coated pits initially forming from the membrane. Accordingly the colocalized staining appears to be near the cell surface (Fig 3-1).

The DAT colocalizing with clathrin represents internalizing DAT rather than newly synthesized DAT trafficking from the golgi complex, as it is not decreased following treatment with cyclohexamide (Fig 3-2). This method does not distinguish between clathrin-dependant endocytosis and clathrin-dependant exocytosis from a recycling pathway. In rat hippocampal neurons Rab 3 was shown to be associated with vesicles recycling to the membrane, while Rab 5A was excluded from these recycling vesicles (Star et al. 2005). Thus, in our experiments, the DAT associated with Rab 5A containing endosomes likely represents only the endocytosed DAT and not DAT that is trafficking back to the membrane. It is surprising that the DAT is internalized following 30 min of melittin treatment and is at the membrane again following 60 min of treatment, without being detected in the Rab 11 associated recycling endosome. It is possible that the melittin-induced DAT recycling pathway in HEK cells does not utilize Rab 11 associated

membranes, but may cycle through membranes associated with another protein such as Rab 4. In HEK cells transfected with the AT1 angiotensin receptor, agonist stimulation causes the receptor to internalize and then recycle back to the cell surface through Rab 4 associated membranes, that are distinct from Rab 11 associated membranes (Hunyady et al. 2002). Similarly, MDCK cells transfected with the transferrin receptor and the polymeric immunoglobulin receptor demonstrate that the majority of recycling to the basolateral surface occurs directly through early endosomes, rather than recycling endosomes (Sheff et al. 1999). Measures of DAT colocalization with additional proteins, such as Rab 4, following melittin treatment may further illustrate this trafficking pathway and explain why the DAT does not colocalize with Rab 11 prior to recycling back to the membrane.

The trafficking patterns initiated by melittin are unlike those induced by PMA in these cells. PMA caused the DAT to colocalize with EEA1 following 30 min of treatment and then colocalize with the late endosome marker Rab 7 following 60 min of treatment. This suggests that the DAT may be trafficked for degradation following exposure to PMA in these cells. We have reported that melittin-induced internalization of the DAT is blocked by pretreatment with cocaine, yet cocaine treatment does not alter DAT colocalization with any of the subcellular organelle markers. Previous reports using PMA to induce DAT internalization found that cocaine treatment does not alter transporter surface expression, nor does cocaine

pretreatment alter the PMA effect (Daniels and Amara 1999). This is further evidence to suggest that melittin is likely operating through a different mechanism than PMA and cocaine to alter DAT function and localization.

Use of the Optiprep equilibrium density gradient is instrumental to further clarify the trafficking patterns of internalized DAT. Following 30 min of melittin treatment the distribution of the DAT through the density fractions is altered, indicating the DAT becomes associated with a density fraction that also is associated with Rab 5A. This data correlates with the confocal colocalization data suggesting the DAT is internalized to Rab 5A associated endosomes or vesicles. The immunodetection of the early endosome markers Rab 5A and EEA1 indicates that these markers do not completely associate in the same fractions. There is overlap in the low-density fractions 15 and 16. This difference in distribution explains the differences in DAT colocalization with these markers seen using confocal microscopy. Examination of EEA1 localization using 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 are used to generate a density graidient, such as sucrose, the distribution of cellular organelles across fractions may be different than those seen with Optiprep. Use of a 10-50% sucrose gradient found more overlap

between EEA1 and Rab 5A (Melikian and Buckley 1999) than what we found with the Optiprep iodixanol gradient.

Immunodetection of subcellular organelles in each equilibrium density gradient fraction indicates that melittin treatment does not significantly alter distribution of the organelle markers (Fig 3-6). This suggests that the changes in colocalization of the DAT with each of the marker proteins represents altered distribution of the DAT rather than disruptions of the endogenous internalization pathways. Additionally, electron microscopy could be used to precisely examine the location of the DAT in relation to specific subcellular compartments. This would allow visual distinction between clathrin coated pits and vesicles. There is considerable overlap in the distribution of Rab 5A and Rab 11 in the density gradient. This procedure only separates structures based on density, not cellular distribution. This experiment demonstrates the similar densities of Rab proteins and endosomes.

It is interesting that the confocal microscopy analysis demonstrated a melittin-induced decrease in DAT colocalization with the recycling endosome marker Rab 11 and the late endosome marker Rab 7 following 30 min of treatment (Fig 3-4), yet the density gradient experiments do not yield similar decreases (Fig 3-6). This may represent different sensitivities between the two methods. The same antibodies were used for both confocal microscopy and western blot detection and, unfortunately, cell types lacking these

endosome markers are not available to utilize as negative controls. Examination of recycling (Rab 11) and early endosome (Rab 4) marker separation by 5-20% Optiprep density gradients indicates that if 34 equal fractions are obtained, the two markers can be separated into fractions 20 and 23 respectively, with the recycling endosome peaking in the lower density fraction (Sheff et al. 1999). It is likely that a greater number of obtained fractions would provide higher resolution isolation of Rab 11 and Rab 5A in the current experiments and may further explain the differences seen in DAT colocalization with the markers in confocal microscopy.

Studies using subcellular fractionation demonstrate a PMA-activated and a constitutive DAT 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 a PMA induced DAT colocalization with early endosomes and recycling endosomes (Sorkina et al. 2003), or colocalization with transferrin followed by colocalization with lysosomal markers (Daniels and Amara 1999). These variances may result from trafficking differences between cell types, fluorescent tags on the DAT altering the normal trafficking pathways, or the differences in PMA concentration and treatment time. In our cells, the DAT trafficking pattern following melittin treatment was unlike that following PMA treatment (Fig 3-4). Differences in induced trafficking patterns between melittin and PMA remain throughout the 60

min of treatment. Thus, the effects of melittin on DAT function and trafficking are likely mediated through a pathway that does not coincide with the more often studied PMA-stimulated PKC path. Thus melittin acts through distinct mechanisms to affect DAT function and trafficking.

If melittin binding to the DAT causes internalization, is melittin removed from the transporter before it is reinserted into the membrane? Does the acidic environment of the early endosome cause melittin to become disassociated from the DAT? If so, this internalization may serve a protective function to remove DAT antagonist for proper cell function. This would be rather distinct from the cellular response to antagonist such as cocaine. This study does not address these questions, but it would be interesting to use an antibody raised against melittin to follow its movement in relation to the DAT, endosomal markers, and other trafficking proteins. This unique, melittin-induced DAT trafficking pattern indicates another level of regulation for DAT function, and also the varied cellular responses possible with treatments.

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Chapter IV.

Discussion

Recent research has transformed the conceptualization of the DAT from a static membrane protein to a dynamically regulated and integral component of DA neurotransmission. The DAT plays a critical role in maintaining DA homeostasis and modulating DA synaptic transmission. This dissertation was initially designed to determine how PLA2 activation and AA signaling affect DAT function and localization. This regulation represents a possible signaling cascade originating from presynaptic DA D2-like receptor activation. Endogenous PLA2 activity is typically stimulated with the small peptide melittin. Melittin is isolated from honeybee venom, and the precise mechanism of action is not known. Based on the previously published uses of melittin, I hypothesized that melittin inhibits DAT function and cellular localization by activation of PLA2 and initiation of AA signaling cascades. Experiments in this dissertation were designed to address the effects of melittin on DAT function and trafficking.

The interaction of melittin with the phospholipid membrane.

As presented in this dissertation, there some newly presented difficulties pertaining to the use of melittin. The structure of melittin and the nature of its interactions with lipids may explain some of the limitations to the use of melittin. These limitations are based on the orientation of

melittin in the membrane and the concentration of melittin. Many experiments have examined the effects of melittin on isolated, experimentally formed lipid membranes. Melittin forms α-helices that become incorporated into lipid membranes, and the site of incorporation depends on the ionic charge of the phospholipids constituents. If the membrane is composed of negatively charged phospholipids, melittin becomes incorporated near the glycerol residues of the membrane. If the membrane is composed of zwitterionic phospholipids, melittin become incorporated into the aqueous-lipid interface. Insertion at either location causes decreased membrane fluidity (Ladokhin and White 1999);(Sheynis et al. 2003).

Concentration of melittin also influences its orientation in the membrane. In a membrane composed exclusively of 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, high concentrations of melittin (melittin: lipid molar ratio > 1:25) cause it to be orientated parallel to the lipid tails of the membrane, with four helices coming together to form a small pore-like structure. At lower concentrations of melittin (melittin: lipid molar ratio < 1:25), the peptide helix is orientated perpendicular to the fatty acid tails of the membrane (parallel to the plane of the membrane) (Figure 4-1). In this position a portion of the peptide is exposed to the aqueous environment and a portion of the molecule is exposed to the lipid environment (Chen et al. 2003a). Due to the amphiphilic nature of melittin, it is capable of interacting with amino acid residues in multiple locations. Melittin can interact with

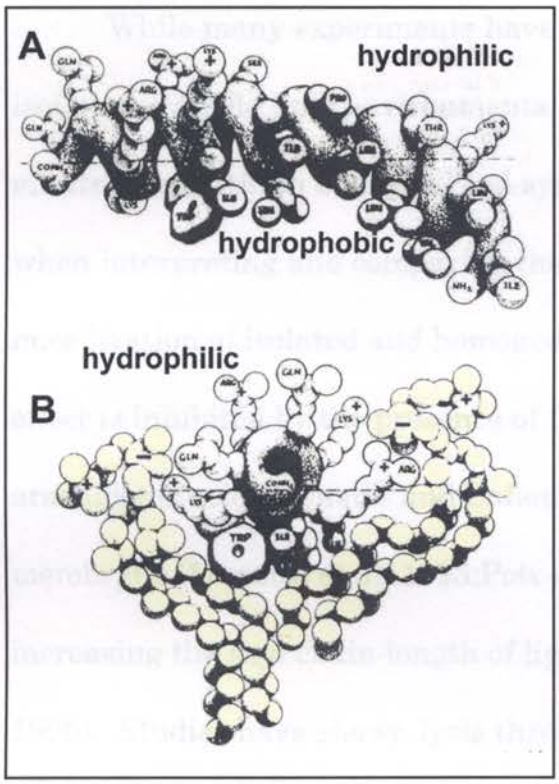


Figure 4-1: Interaction of melittin with phospholipids in bilayers.

A. Melittin forms an α-helix peptide structure with hydrophilic amino acids oriented in the opposite direction of hydrophobic residues. **B**. Phospholipids of the membrane are represented in yellow. The polar phosphate head groups are indicated by charges (+/-). The hydrophilic residues of melittin interact with the polar regions of the phospholipids. The hydrophobic residues of melittin interact with the non-polar lipid tail regions of the phospholipids.

Dempsey 1990

amino acids on the extracellular surface of proteins. Additionally, as the hydrophobic residues of melittin become incorporated into the phospholipid membrane it has access to regions of membrane-incorporated proteins that are typically protected within the phospholipid bilayer. Thus melittin may be interacting with exposed, extracellular regions of the DAT or with hydrophobic regions of the DAT within the cellular membrane. Site directed mutagenesis of the DAT could be used to determine which regions of the DAT are necessary for the interaction of melittin with the DAT. This information would be useful to understand the nature of melittin's interaction with proteins, and may also reveal regulation sites on the DAT that could be used to develop therapeutic compounds.

While many experiments have explored the effects of melittin on isolated or single lipid environments, few have studied the direct membrane effects of melittin in biological cell systems. Caution should be exercised when interpreting and comparing these results. Melittin induces micellization of isolated and homogenous lipid bilayer membranes, but this effect is inhibited by the presence of fatty acids such as linoleic, oleic, and arachidonic acid (Monette and Lafleur 1996), and by cholesterol in the membrane (Monette et al. 1993;Pott and Dufourc 1995), and also by increasing the acyl chain length of lipids in the membrane (Faucon et al. 1995). Studies have shown lysis through micellization does not occur at melittin: lipid molar ratios below 1:20. While our GLC experiments did not measure the total amount of lipid present in these cells, the ratio of just the select fatty acids measured, to the quantity of melittin used in the current experiments (1.3 μM) was less than 1:20. Presumably the ratio of total lipid to melittin would be substantially below this ratio. Thus cell lysis would not be occurring in the current experiments.

The nature of melittin's interaction with lipids.

The direct interaction of melittin with membrane phospholipids may explain the effects of melittin on membrane fatty acid release presented in this dissertation. To confirm that melittin was releasing AA, gas liquid chromatography was performed to measure the amount of AA remaining in

cellular membranes following melittin treatment. Melittin did release endogenous AA from the cells, but the analysis also indicated that melittin released other fatty acids; including oleic, linoleic, myristic, and palmitic acids; that are not known to be release by PLA2 (Figure 2-3). This may have resulted from melittin stimulating additional phospholipases such as PLC and PLD. Further investigation demonstrated that inhibitors of PLA2, PLC, and PLD were unable to prevent melittin-mediated release of AA from these cells (figure 2-4). These experiments indicate that melittin acts through mechanisms other than specific PLA2 stimulation in HEK cells.

It is typically presumed that PLA2 stimulation mediates the effects of melittin. In neuronal cultures melittin seems to affect filopodia growth through PLA2 stimulation. Developing neurons treated with PLA2 inhibitors demonstrate enhanced filopodia growth. Melittin pretreatment blocked this effect, while melittin alone has no effect on filopodia growth. Additionally, direct application of AA can block the effects of PLA2 inhibitors (Geddis et al. 2004). In L1210 cells melittin stimulates PLA2, PLC, and PLD simultaneously (Lee et al. 2001). In proximal tubule cells, PLA2 inhibitors significantly block melittin-induced release of AA (Han et al. 2002). Yet, PLA2 inhibitors do not block melittin-induced release of AA in Leydig cells (Ronco et al. 2002), similar to the results presented in this dissertation. In studies of insulin release from rat pancreatic islets, melittin dose-dependently releases AA and insulin, but a variety of PLA2 inhibitors do not

block this release (Metz 1986). Thus melittin's effect on PLA2 may be cell type specific. As a key component of any experimental design using melittin, the mechanism of melittin's action needs to be established for the particular cells employed in the study. Accurate experimental conclusions cannot be deduced unless the effects of melittin are known.

It is possible that melittin interacts directly with the membrane in HEK cells to release fatty acids. It has recently been presented that the peptide mastoparan, isolated from wasp venom, stimulates GTP binding regulatory proteins not by stimulating these signaling proteins directly, but rather by disrupting the membrane lipid rafts where many receptors and second messenger proteins are localized. This direct interaction with the phospholipid membrane releases these proteins and lipids to the cytoplasm (Sugama et al. 2005). Mastoparan also releases AA from PC12 cells (Nakamura et al. 2004). Experiments using nuclear magnetic resonance measurements demonstrate an interaction between melittin and ganglioside components of lipid rafts (Chatterjee and Mukhopadhyay 2002). It is not known whether mastoparan and melittin possess phospholipase activity or if the interaction of these peptides with membranes destabilizes the phospholipids structures.

Effects of AA on the DAT and cell function.

Direct application of AA affects the function of neurotransmitter transporters (Ingram and Amara 2000;Zerangue et al. 1995). Short treatments (2 min) with AA does not affect DAT-mediated uptake of DA, but it does stimulate a cation conductance that is associated with the DAT and not associated with DA transport (Ingram and Amara 2000). Longer treatment times with AA do inhibit DA uptake through the DAT (Chen et al. 2003b). Thus the effects of AA on DAT function and conformation are time- and concentration-dependant. A direct interaction of AA with the transporter likely mediates these effects, as other unsaturated fatty acids can also produce these effects with a rank order of potency corresponding to the degree of unsaturation. Studies exploring the effect of AA and other fatty acids on transporter function have revealed this channel-like property of the DAT. Further experiments are needed to understand the physiological relevance and significance of this induced current. Based on these experiments it seems that in a DA neuronal model AA may serve to enhance depolarization of the cell through the DAT, while simultaneously inhibiting uptake and extending the DA signal in the synapse.

Direct application of AA also affects the function of other membrane-incorporated proteins. AA enhances a calcium-activated potassium current in myoctyes (Zheng et al. 2005), inhibits presynaptic sodium channels in striatal neurons (Fraser et al. 1993), and potentiates glutamate N-methyl-D-aspartate receptor currents (Miller et al. 1992). In rat superior cervical

ganglion neurons a brief, extracellular exposure of AA enhances current flux through the N-type calcium channel, yet intracellular administration of AA inhibits these calcium channels (Liu et al. 2001;Barrett et al. 2001). These findings demonstrate the complex nature of AA signaling where site of action and specific protein affected influences whether AA enhances or inhibits neuronal excitability. Many of these studies determined that the effects of AA on ion channels were also produced with other fatty acids, suggesting that there is a direct interaction of these lipids with many cellular proteins.

Melittin was initially used to stimulate PLA2 activity. Melittin caused the release of AA and other fatty acids; such as linoleic, oleic, myristic, and palmitic acids; in HEK cells (Figure 2-3). It is possible that these lipids are affecting many cellular proteins, including the DAT, in these experiments. We discovered that melittin interacts directly with the transporter to inhibit binding of the cocaine analogue RTI-55 to the DAT. Unfortunately, as a result, the direct interaction of melittin with the transporter likely masks the effects of melittin-released lipids on DAT function. The previous experiments examining the effects of AA on transporter and ion channel function have all relied on application of exogenous AA. Studies that use endogenous AA release, in physiologically accurate concentrations, need to be performed to determine the effects of AA on DAT function.

Treatment with AA stimulates the release of multiple neurotransmitters. AA dose-dependently increases spontaneous release of

DA in synaptosomes from rat striatum (L'hirondel et al. 1995). AA enhances stimulated and basal release of the neurotransmitters gamma-aminobutyric acid (Cunha and Ribeiro 1999) and acetylcholine (Almeida et al. 1999) from hippocampal glutamatergic synapses. AA, rather than a metabolite, directly mediates these effects. PLA2 is expressed in a variety of tissues throughout the body, including brain, heart, spleen, lung, kidney, liver, testis, white blood cells, and platelets (Sharp and White 1993). This expression suggests the presence AA signaling in each of these regions. AA is also a major constituent of neuronal membranes and alterations in membrane AA content is related to multiple neurodegenerative disorders (for review see (Farooqui et al. 2000). Based on the effects of AA on neurotransmitters and the wide expression of PLA2, it is evident that AA signaling is a pervasive mechanism for modulating cell function and neurotransmission.

The membrane phospholipids hypothesis of schizophrenia implicates improper membrane structure and AA signaling as a component of this disorder. Subsets of schizophrenic patients demonstrate decreased levels of AA in platelets (Gattaz et al. 1995), red blood cells (Glen et al. 1994), and postmortem brain tissue (Yao et al. 2000). This decrease may be due to increased PLA2 activity as the rate of membrane AA turnover is increased in schizophrenic patients (Demisch et al. 1987). This altered lipid environment may prevent proper function of membrane incorporated proteins, such as DA receptor and the DAT. These patients may also have decreased levels of AA

available for signaling functions. These alterations could significantly affect neurotransmission and may cause some of the schizophrenic behavioral symptoms. Thus AA act directly on proteins to alter cell function and it globally affects cell function by altering membrane fluidity.

Ideally, a specific pharmacological stimulator of PLA2 should be developed to allow more precise examination of the signaling pathways between the DA D2 receptor and the DAT. Stimulating the receptor activates multiple signaling pathways and it is difficult, and likely not possible, to tease out the effects of each individual path. Until a new pharmacological tool is developed, it may be of some interest to repeat many of the experiments outlined in this dissertation using direct application of AA. One limitation of this is in the nature of fatty acids and their effect on membrane dynamics. In DAT transfected oocytes voltage-clamp experiments were performed to measure the currents induced by direct application of AA. When high concentrations of AA (100 μM) were applied, an inward current was detected that never attained steady-state. The authors suggest this may result from the formation of micelles due to the high fatty acid concentration disrupting the membrane dynamics (Ingram and Amara 2000). As a result all experiments were performed with lower concentrations and for rather short times (2 min). An additional study exploring the effects of exogenous AA on DAT function found that short treatment times (15 & 30 min) of 5-160 μM AA cause an increase in DAT mediated uptake of DA. With longer

treatment times (45 & 60 min) there is an increase in DA uptake at lower concentrations, but above 40 μM there is a decrease in DA uptake (Zhang and Reith 1996). The Trypane Blue exclusion test to determine cell viability demonstrates that 160 μM of AA, and other fatty acids, allows only 30% of cells to survive, but 80 μM of AA does not significantly reduce the cell survival rate (Chen et al. 2003b). Considering the effects of AA on cell membranes at high concentrations (>80 μM), these treatment conditions may have allowed DA that was taken up into the cytoplasm to leak out through ruptured membranes. This would erroneously appear to be a decrease in uptake.

There are a number of uncertainties concerning AA's and melittin's mechanisms of action, so ultimately it is difficult to definitively determine which effects in many published studies are specific and which are unforeseen properties of the pharmacological agents. The experiments in this dissertation have made it poignantly (at times painfully) clear that a researcher cannot make assumptions about the drugs and reagents used in research. A drug's true mechanism of action must be satisfactorily proven prior to use.

A specific PLA2 stimulator needs to be developed to successfully understand the effects of AA signaling pathways. A PLA2 activating protein (PLAP) has been isolated from mammalian cells (Clark et al. 1987). PLAP activates PLA2 and not PLC, but the precise mechanism of action is not yet

known. Direct application of purified PLAP to cell membranes causes release of AA, and PLA2 inhibitors prevent this release (Pilane and LaBelle 2004). The experiments outlined in this dissertation could be repeated with PLAP rather than melittin. Studies would need to confirm that this protein does not activate other phospholipases in the chosen cell line, and does not interact directly with receptors or transporters. PLAP may provide a method for direct stimulation of PLA2 while avoiding the use of melittin.

The nature of melittin's interaction with proteins.

Based on the effect of PLA2 activation and AA on DAT function, I hypothesized that melittin would inhibit DAT function through stimulation of PLA2. The effect of melittin on DAT function was measured by uptake of the substrate [^{3}H]DA into HEK cells transfected with the DAT and the DA D2 receptor (Figure 2-2). In agreement with previous reports, melittin did inhibit DAT mediated uptake of DA. Further analysis revealed that melittin, unexpectedly, interacted directly with the DAT to inhibit binding of the cocaine analogue RTI-55. Melittin also inhibited binding of the D2 receptor antagonist [^{3}H]spiperone in these same cells (Figure 2-5). These surprising findings revealed that melittin was interacting with membrane spanning proteins in a previously unreported manner.

The experiments in this dissertation demonstrated a direct interaction of melittin with the DAT and the D2 receptor to inhibit antagonist binding.

It is possible that melittin universally binds to proteins to inhibit proper function. Yet Yu et al (Yu et al. 1993) examined the effects of AA and PLA2 activation on glucose transporter function in mouse cerebral cortical astrocytes. Direct application of AA dose-dependently increases glucose uptake, and this effect is replicated with other unsaturated fatty acids, such as linolenic acid, but not with saturated fatty acids. Application of melittin causes a release of AA in these cells. Melittin also causes an increase in glucose uptake, similar to the effect of AA application. Thus, it is possible that melittin does not directly bind to the glucose transporter. This would indicate that the direct interaction of melittin with the DAT and the D2 receptor is not a nonspecific effect that applies to all membrane incorporated proteins. If melittin does interact directly with the glucose transporter, this interaction does not inhibit substrate uptake, and could suggest that melittin binding to a transporter could also alter the conformation of the protein to stimulate substrate uptake. No experiments have been reported concerning melittin binding to a glucose transporter, but this would provide an interesting contribution to understanding how melittin directly affects protein function.

Interestingly, the direct interaction of melittin with the DAT also caused internalization of the transporter. In this dissertation I examined the intracellular trafficking pathways of the internalized transporter. Colocalization of the transporter with endogenous protein markers of

endosomes and endocytosis pathways was quantified. Confocal microscopy and equilibrium density gradient separation were used to determine the pathway and time course of internalized DAT.

A variety of compounds and cell signaling pathways, including transporter substrates and blockers, and PKC, influence DAT membrane expression. Experiments in this dissertation address the ability of melittin to alter DAT surface expression and examine the endocytic pathways employed in this internalization. Biotinylation experiments indicated that melittin treatment caused internalization of the DAT following 30 min of treatment. Treating the cells with cocaine prior to application of melittin prevented this internalization (Figure 2-6). Blockade by cocaine suggests that the direct interaction of melittin with the DAT is the impetus for internalization. It is also possible that the DAT needs to be in a particular conformation for internalization. Cocaine binding to the DAT may alter the transporter's structure in a manner that prevents melittin-induced internalization. This structural inhibition may be directly due to an inappropriate conformation of the DAT, or it may prevent accessory proteins from interacting with the DAT. Studies using PMA to induce DAT internalization argue against this possibility. PMA treatment stimulates PKC, and PKC activation causes internalization of the DAT. Pretreating the cells with cocaine does not prevent the PMA-induced decrease in DAT function or cell surface expression (Daniels and Amara 1999). This suggests that PMA-induced internalization

of the DAT is not dependant on the transporter being in a particular conformation. This information also indicates that cocaine binding to the DAT does not alter the conformation of the transporter in a manner that inhibits interaction with proteins necessary for endocytosis.

It is also possible that fatty acids released by melittin cause internalization of the transporter. Free AA, or another released lipid, may interact directly with the DAT. No studies have been done without melittin to specifically stimulate release of endogenous AA and measure internalization of transporters. In attached, whole cell preparations, direct application of AA to C6 cells expressing the hDAT causes a decrease in the maximal binding of a cocaine analogue with no change in K_D (Zhang and Reith 1996). This result suggests that the transporter is being internalized, although the mechanism has not been determined. It would be interesting to see if cocaine pretreatment inhibits DAT internalization that is caused by exogenous AA. This experiment would help to tease out the effects of melittin.

As a future experiment, a dose-response effect for melittin-induced internalization could be determined. Using varying doses of melittin may demonstrate that there is a critical concentration of melittin that is necessary to cause DAT internalization. This would be an interesting result that would reflect the ability of cells to accommodate the functional loss of a certain number of transporters. Alternatively, there may be a graded response of

DAT internalization following increasing concentrations of melittin. It would be interesting to apply increasing concentrations of melittin and compare the effects on DAT internalization with the effects on AA release.

These experiments indicate some of the limitations of immunocytochemistry colocalization measurements. The confocal microscopy data is subjective to the conditions used across the experiments, such as antibody concentration, imaging gain, and image analysis programs. While the absolute values may be variable across different laboratories, the relative changes should be similar. Thus it is not necessary to assume that under control conditions nearly 30% of the total DAT is located in an endosome (as determined by the zero timepoint in Figure 3-4). The use of other experimental methods, such as electron microscopy and density gradient separation, following melittin treatment may provide a more accurate measure of DAT localization with each of the endosomal markers used in this study.

The trafficking experiments rely on the specificity of the antibodies. An excellent negative control experiment would be to expose cells that do not express one of the Rab proteins, to the antibody for that particular protein. An excellent positive control experiment would be to label cells that only express one of the Rab proteins with each of the antibodies. Unfortunately, those types of cells do not exist. If purified samples of Rab 5A, 7, and 11 were available, these proteins could be run through SDS-PAGE and the western

blot could be detected with each of the antibodies. This positive control would indicate the specificity of each of these antibodies. Cells in the current experiments were also incubated with only the secondary antibodies. This control confirmed that the images were not nonspecific staining due to secondary antibodies.

Rab proteins are frequently used to label distinct endosomal compartments, but there are some limitations to this application. A431 cells transfected with fluorescently tagged Rabs show overlap of markers for early and recycling endosomes, using confocal microscopy. Electron microscopy further resolves early and recycling endosomes as distinct regions of a continuous endosomal membrane (Sonnichsen et al. 2000). This may result from the temporary fusion of endosome compartments as early endosomes transition into recycling and late endosomes. Thus the limits of resolution of confocal microscopy may cause the colocalization of the DAT with each of the Rab proteins to be deceptively high due to overlap. Additionally, examination of recycling and early endosome separation by 5-20% Optiprep density gradients indicates that if 34 equal fractions are obtained, the two markers can be separated into fractions 20 and 23 respectively, with the recycling endosome peaking in the lower density fraction (Sheff et al. 1999). Thus, even the Optiprep separation requires a high degree of resolution to distinguish certain endosome markers. These previous studies demonstrate the limitations of each of these experimental methods. Future experiments

using electron microscopy would be beneficial to identify the subcellular localization of internalized DAT.

The direct interaction of melittin with the DAT causes internalization. What is the physiological reason for this? When cocaine binding directly to the extracellular domain of the DAT inhibits transporter function, cells tend to respond by increasing membrane surface expression of the transporter to compensate for the loss of uptake (Little et al. 2002;Daws et al. 2002). Why is the response to melittin different? Melittin-induced changes in DAT conformation may be distinctly different from cocaine-induced changes. Melittin is a peptide and binding to surface proteins may activate a mechanism to internalize the protein so that the foreign peptide can be removed and a "clean" and funct ional transporter can be reinserted into the membrane. The endocytosed DAT traffics through the early sorting endosome, but the DAT does not traffic to lysosomes to be degraded, but rather is recycled to the membrane (Figure 3-4).

Studies of the effects of ligand interactions with epidermal growth factor receptors (EGFR) and receptor trafficking may provide insight into melittin's interaction with the DAT. The early endosome is an acidic environment (pH 6). Treatment of EGFR with ligands that readily dissociated from the receptor at pH 6 enable the receptor to traffic through recycling endosomes to be reinserted into the membrane. Treatment with ligands that do not dissociate from the receptor in the early endosome cause

the receptor to be trafficked to lysosomes for degradation (French et al. 1995). Since melittin treatment causes the internalized DAT to recycle back to the membrane, rather than to the late endosome, this may suggest that the lower pH of the early endosme causes melittin to dissociate from the DAT. What would then become of the free melittin?

An interesting experiment to examine DAT-melittin association would be to use an antibody raised against melittin to track its location throughout treatment. Co-labeling of the DAT, early endosome marker proteins, or lysosome marker proteins along with melittin labeling would provide a visual map of where melittin was in relation to these proteins. This experiment could answer a number of questions. Is melittin internalized with the DAT? Does melittin dissociate from the DAT in the endosome or does it remain attached as the DAT recycles to the membrane? Does melittin get degraded inside the cell or does it maintain its structural integrity? If melittin is not degraded inside the cell, does it become associated with intracellular proteins to alter other aspects of cellular function? If this were the case, then melittin would appear to be more like an invading molecule, and no studies have been done to determine cell function or survival following prolonged treatment times with melittin.

Fluorescent protein labeled Rabs could also be used to differentiate melittin-induced trafficking pathways. Cells could be co-transfected with cyan fluorescent protein labeled-DAT and yellow fluorescent protein labeled-

Rab 5A, 4, 7, or 11. This co-expression would enable imaging of live cells following melittin treatment. DAT colocalization with these markers could be determined as a continuous progression in individual cells.

Additional experiments could be performed to determine the effects of melittin on DA D2 receptor function. Melittin inhibits binding of spiperone to the D2 receptor (Figure 2-5), but it is not known if this also inhibits receptor signaling. D2 receptors couple through inhibitory G-proteins to inhibit adenylyl cyclase production of cAMP. To determine the effect of melittin on D2 receptor activity, cells can be treated with melittin prior to application of receptor agonist, such as quinpirole, and cAMP production can be quantified. Similar biotinylation, confocal microscopy, and equilibrium density gradient centrifugation experiments as outlined in this project can also be performed. This will establish if melittin's interaction with the D2 receptor causes internalization of the receptor. It may prove that the effect of melittin on DAT trafficking is not unique to the transporter, but is a broad response to foreign peptides.

Additional experiments can be performed to examine the effects of melittin on proteins that interact with the DAT. The DAT exists as a regulated complex with a variety of proteins. These include the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein syntaxin 1A (Lee et al. 2004), protein phosphatase 2A (PP2A) (Bauman et al. 2000), the focal adhesion protein Hic-5 (Carneiro et al. 2002),

and the PDZ domain-containing protein PICK1 (Torres et al. 2001). Bauman et al (Bauman et al. 2000) demonstrated that PP2A also coimmunoprecipitates with the serotonin transporter in transfected HEK cells and in rat midbrain synaptosomes. The phosphatase inhibitor okadaic acid destabilizes this association and reduces uptake of serotonin through the transporter. Stimulation of PKC with PMA also destabilizes the transporter-PP2A association. Similar studies could be performed to determine if melittin treatment destabilizes the association of the DAT with PP2A. Protein phosphatases dephosphorylate many cellular proteins, including the DAT, and prevent DAT internalization (Vaughan et al. 1997). This experiment would provide further insight into the mechanism of melittin-induced internalization.

Diversity of cellular responses to stimuli.

Cells demonstrate a variety of responses to regulate membrane proteins following stimulation. These responses include recycling of G-protein coupled receptors to the membrane following internalization (Tsao et al. 2001), ubiquitin-mediated degradation of cytokine receptors (Rakesh and Agrawal 2005), and constitutive recycling of transferrin receptors (Lok and Loh 1998). Cells also have a variety of mechanisms to regulate the DAT. Specifically, the DAT constitutively recycles to the membrane in transfected PC12 cells (Loder and Melikian 2003), DAT membrane expression is

increased following prolonged cocaine treatment (Little et al. 2002), and the DAT is either recycled or degraded following stimulation of PKC (Melikian and Buckley 1999;Daniels and Amara 1999). The variety of mechanisms utilized by cells to regulate transporters can be seen in the internalization pathways. Additionally, this dissertation demonstrates the ability of a single cell type to use different mechanisms to regulate DAT membrane expression following melittin and PMA treatment.

In HEK cells melittin treatment caused an increase in DAT colocalization with clathrin (Figure 3-2). This result suggests that melittin-induced internalization of the DAT in transfected HEK cells is through a clathrin-mediated mechanism. Previous experiments have demonstrated that clathrin-dependant mechanisms mediate PMA-induced internalization of the DAT in transfected cells (Saunders et al. 2000;Daniels and Amara 1999;Sorkina et al. 2005). Clathrin- and dynamin-dependant internalization is involved in regulating membrane expression of a variety of cellular membrane proteins including DA D2 receptors (Kim et al. 2001), the CB1 cannabinoid receptor (Hsieh et al. 1999), and the serotonin 5HT2A receptor (Hanley and Hensler 2002).

While cells often use clathrin-dependant internalization methods to regulate membrane proteins, additional methods for the organization and endocytosis of cell surface proteins are available. Lipid rafts are microdomains of the plasma membrane composed of cholesterol and

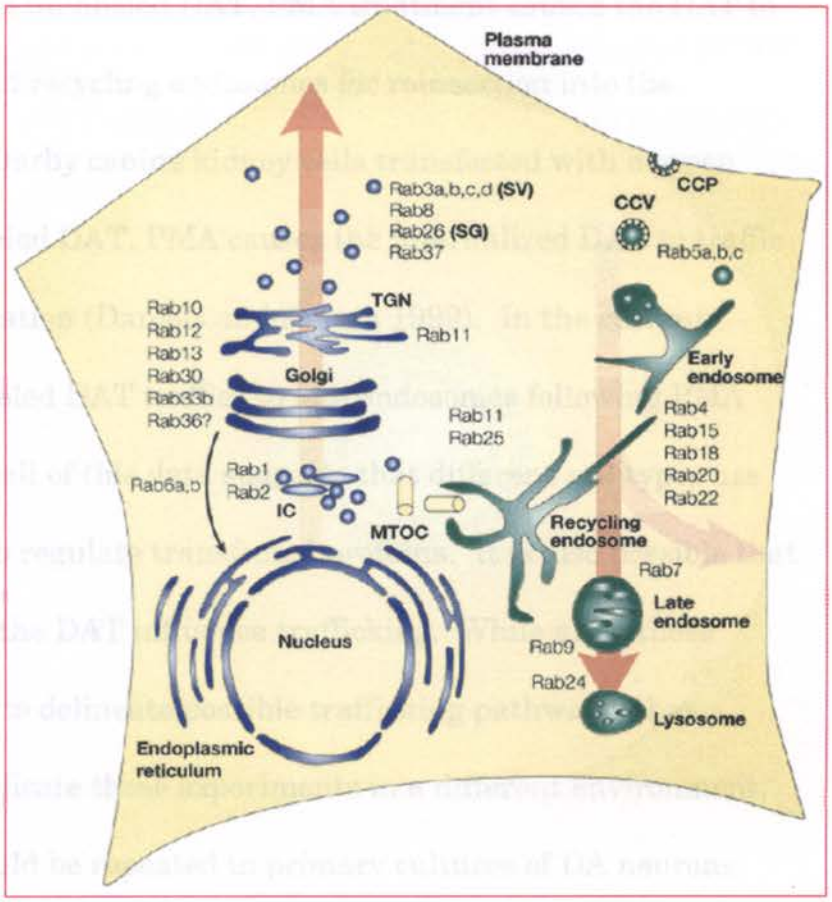
glycosphingolipids. These raft domains may play a role in protein trafficking and signal transduction. Specific proteins and lipids interact directly with raft anchoring proteins to localize components of signaling pathways, such as receptors and G-proteins into regions that are primed for signal transduction (for review see (Le Roy and Wrana 2005). Currently no studies have determined if the DAT is localized in lipid raft domains. The high affinity norepinephrine transporter (NET) removes the neurotransmitter norepinephrine from the synaptic space, and the NET has been isolated to lipid raft domains (Jayanthi et al. 2004). Similar to the DAT, stimulation of PKC with PMA causes an increase in NET internalization. Interestingly, expressing the dominant negative form of dynamin does not prevent this internalization, but treatment with filipin, which disrupts the cholesterol structures in raft domains and prevents lipid raft-mediated endocytosis, does prevent NET internalization (Jayanthi et al. 2004). Thus the NET is not internalized through the classical clathrin-mediated mechanisms. Examination of the association of the DAT with lipid raft domains demonstrates that treatment with filipin to disrupt lipid raft-dependant endocytosis does not prevent PMA induced internalization of the DAT (Sorkina et al. 2005). Thus, while PMA similarly regulates the functions of these neurotransmitter transporters, the mechanism of regulation is not precisely the same. This is an indication as to the many mechanisms employed by cells to regulate synaptic function.

I also examined the intracellular trafficking pathway of the DAT following endocytosis. Melittin treatment causes the DAT to traffic through early endosomes following 30 min of treatment. At this time point, there is also a decrease in DAT colocalization with markers of the recycling and late endosomes. At basal conditions there is some level of DAT that is associated with each of these endosomes. Melittin may inhibit the DAT from entering into these trafficking pathways. Alternatively, melittin may inhibit DAT colocalization with RAB 11-associated endosomal regions. This does not exclude the DAT from trafficking through the recycling endosome, as some recycling proteins are associated with Rab 4 and not Rab 11 (as discussed in Chapter 3).

Rab 11 is typically used as a marker for the recycling endosome, but it is also associated with vesicle transport from the golgi to the plasma membrane (Figure 4-2) (for review see (Zerial and McBride 2001). Thus this marker is not exclusive to endosomal regions. It is then possible that the decrease in colocalization of the DAT with Rab 11 following 30 min of treatment represents a decrease in DAT synthesis and transport to the cell membrane. Treatment with cyclohexamide inhibits proteins synthesis and thus inhibits trafficking of proteins from the trans-golgi network to the cell membrane. Cyclohexamide treatment alone did not alter DAT colocalization with clathrin (Figure 3-2). This result indicates that within a 30 min time window, there is little detectable DAT synthesis. Based on these results I

would hypothesize that, following 30 min of melittin treatment, the Rab 11 that is colocalized with the DAT represents recycling endosome-associated, not trans-golgi-associated, Rab 11. Additional experiments could be performed to measure DAT colocalization with Rab 4 following melittin treatment. This may decipher whether the DAT is excluded from the recycling endosome or if the recycling endosome marker was not appropriate.

Figure 4-2: Intracellular locations of Rab proteins. The figure depicts Rab proteins associated with endocytosis, exocytosis, and vesicle transport.
(CCV, clathrin coated vesicle; CCP, clathrin coated pit; SV, synaptic vesicle; SG, secretory granules; TGN, trans-golgi network; MTOC, microtubule-organizing center)
Zerial & McBride 2001



The experiments in this dissertation demonstrate the different mechanisms utilized by HEK cells to regulate DAT trafficking following melittin and PMA treatment. In HEK cells, melittin-induced trafficking of the DAT is unlike

PMA-induced trafficking of the DAT. Melittin causes the DAT to be internalized through early endosomes and subsequently recycled to the membrane. PMA treatment causes the DAT to be internalized through early endosomes and subsequently trafficked to late endosomes (Figure 3-4). Many studies have explored the trafficking patterns of internalized DAT following PMA treatment, yet there is not a consensus about the precise location of internalized DAT. In porcine aortic endothelial cells transfected with fluorescent protein labeled DAT, PMA caused the DAT to traffic through early endosomes and recycling endosomes (Sorkina et al. 2003). These results suggest the internalized DAT is recycled to the membrane. In PC12 cells transfected with an unlabeled DAT, PMA treatment causes the DAT to traffic through early and recycling endosomes for reinsertion into the membrane. In Madin-Darby canine kidney cells transfected with a green fluorescent protein-labeled DAT, PMA causes the internalized DAT to traffic to lysosomes for degradation (Daniels and Amara 1999). In the current experiments, the unlabeled DAT traffics to late endosomes following PMA treatment. Combining all of this data suggests that different cell types use different mechanisms to regulate transfected proteins. It is also possible that the fluorescent tags on the DAT influence trafficking. While all of these experiments are useful to delineate possible trafficking pathways, they indicate the need to replicate these experiments in a different environment. These experiments should be repeated in primary cultures of DA neurons.

Experiments in DA neurons would allow detection of endogenous DAT, without the confounding variables of fluorescent protein labels and model cell systems. The current experiments ultimately demonstrate that HEK cells utilize different mechanisms for sorting PMA-internalized DAT as compared to melittin-internalized DAT.

Clinical importance.

As with any biomedical research project, it is important to consider the clinical importance of these findings. What is the medical importance of the effects of melittin on the DAT? A review of the literature reveals that there are no reported cases of a human or animal being stung by a bee directly in the brain. Of course anything is possible, but the experiments in this dissertation used melittin, isolated from honeybee venom, as a tool to stimulate PLA2 mediated release of AA. In my studies I discovered that melittin does not act through the mechanisms that I would have predicted based on the published literature. Yet, I did find that the interaction of melittin with the DAT provides an interesting new method to study transporter trafficking, internalization, and recycling pathways. While melittin-induced alterations of the DAT may not prove to be the most valid model, it does demonstrate the variety of options available within a cell to respond to both typical and non-typical stimuli. Dynamic regulation of DAT

membrane expression is an important mechanism for cells to modulate synaptic strength and neurotransmission.

Genetic analysis of polymorphisms indicates that alterations in the DAT gene may play a role in the development of many neurological and psychiatric conditions. A genetic polymorphism in the gene coding for the DAT may be involved in the development of psychosis or dyskinesias in Parkinson's disease patients who have been treated with L–dopa (Kaiser et al. 2003). Additionally, alterations in the DAT gene may be involved in the development of bipolar disorder and may be used as a genetic screen for this condition (Waldman et al. 1997;Horschitz et al. 2005). The high concordance rate for the development of attention deficit hyperactivity disorder (ADHD) in monozygotic (81%) and dizygotic (27%) twins suggest there is a genetic factor associated with this disorder, and a polymorphism of the DAT has been linked to ADHD (Gill et al. 1997;Cook, Jr. et al. 1995). This is not surprising since many of the medications used to treat ADHD are compounds, such as methylphenidate, bupropion, and amphetamine, that block uptake of DA through the DAT (Eshleman et al. 1999). While there are no genetic correlations to suggest a polymorphism of the DAT gene is associated with development of schizophrenia or psychostimulant drug abuse, both of these conditions result in alterations in the function of the transporter (Dean and Hussain 2001;Letchworth et al. 2001;Volkow et al. 1997). Based on the clinical implications of DAT function and regulation, it is evident that a

thorough understanding of transporter function is necessary to treat and prevent many neurological and psychological disorders.

Summary of conclusions.

In this dissertation the interaction of the purported PLA2 stimulator melittin with the DAT was investigated. Melittin stimulated the release of membrane-incorporated fatty acids, including arachidonic, linoleic, oleic, myristic, and palmitic acids. We were unable to inhibit this fatty acid release with inhibitors of PLA2, PLC, or PLD. These findings indicate that melittin released fatty acids through non-phospholipase mediated mechanisms in HEK cells. Melittin inhibited DAT-mediated uptake of DA and interacted directly with the transporter to inhibit binding of a cocaine analogue (Figure 4-3). Melittin also interacted directly with the DA D2 receptor to inhibit binding of the antagonist spiperone (Figure 4-3). The direct interaction of melittin caused the DAT to internalize through clathrin-coated pits and associate with early endosomes following 30 min of treatment. Following 60 min of treatment the DAT was recycled to the plasma membrane (Figure 4-3). Melittin-induced internalization of the DAT was unlike PMA-induced internalization of the DAT in HEK cells. Treatment with PMA for 30 min caused the DAT to internalize to early endosomes. Following 60 min of PMA treatment the DAT traffics to late endosomes for degradation. Thus melittin acts through multiple mechanisms, including direct interaction with proteins

and release of fatty acids, to alter cell function. Additionally, HEK cells utilize multiple trafficking patterns to sort internalized DAT following various treatments. Thus melittin is a novel tool for exploring membrane protein trafficking patterns. We do not know if this trafficking response is unique to melittin directly interacting with the DAT, or if other molecules can interact with the DAT to cause internalization and recycling. Additionally, we do not know the effects of specifically stimulating PLA2, without melittin, on DAT function and localization.

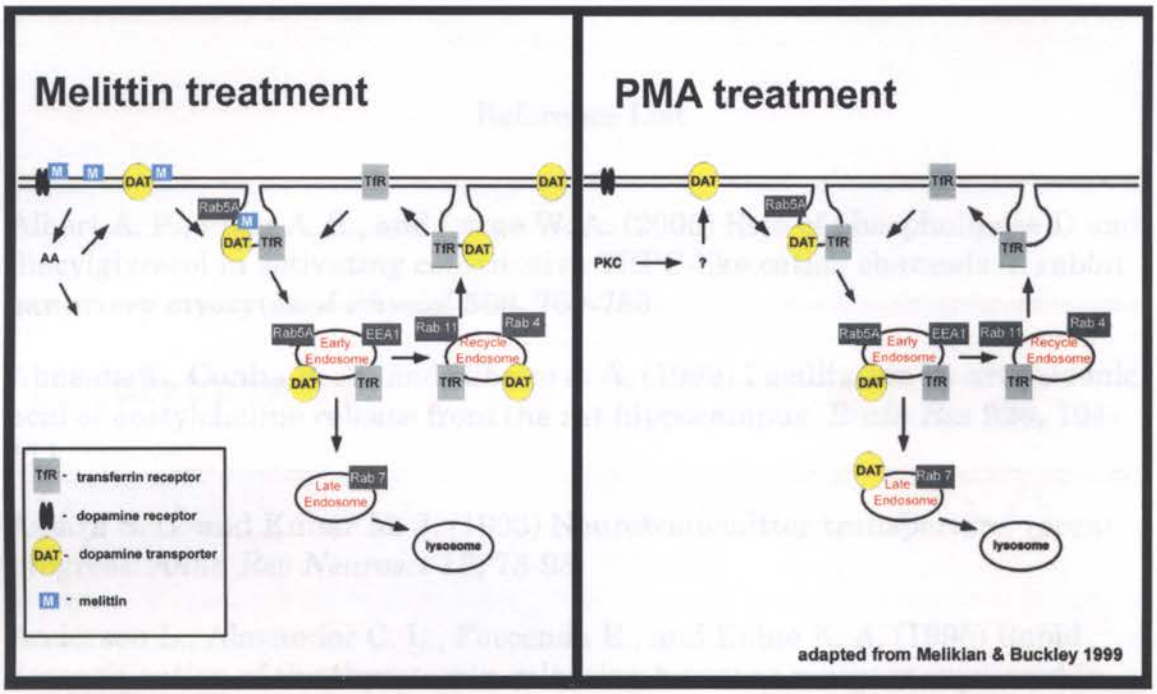


Figure 4-3: Melittin- and PMA-induced internalization of the DAT. Melittin interacts with DA D2 receptors, membrane phospholipids, and the DAT. Melittin causes the DAT to internalize through clathrin-coated pits. Internalized DAT traffics through early endosomes and, following prolonged treatment, is reinserted into the membrane. PMA stimulates PKC, which causes endocytosis of the DAT. The internalized DAT is trafficked through early endosomes and then through late endosomes. Constitutive trafficking of the transferrin receptor is shown for comparison.

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