ORGANIC CATION TRANSPORTERS ARE DIFFERENTIALLY REGULATED BY INTRACELLULAR cAMP POOLS THAT ARE FURTHER MODULATED BY BICARBONATE-RESPONSIVE SOLUBLE ADENYLYL CYCLASE IN HEK 293 CELLS

BY

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS

FACULTY OF MEDICINE

UNIVERSITY OF MANITOBA

WINNIPEG, MANITOBA, CANADA

FEBRUARY 2004

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Organic Cation Transporters are Differentially Regulated by Intracellular cAMP Pools that are Further Modulated by Bicarbonate-Responsive Soluble Adenylyl Cyclase in HEK 293 Cells

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Alexander Mark Gerlyand

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

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ABSTRACT

Organic cation transporters (OCTs) are widely expressed among all tissues, and are highly localized in the kidney. Many drugs, body metabolites and environmental toxins exist physiologically as organic cations. Advances have been made in understanding the renal organic cation transport systems, but relatively little is known about their regulation. We have previously demonstrated that diabetes modulates these transporters. Cyclic AMP (cAMP) is an evolutionarily conserved regulator of metabolism. This study demonstrates that insulin and glucose can differentially modulate OCTs via a cAMP mechanism. These intracellular pools of cAMP may be further modulated by a bicarbonate-dependent soluble adenylyl cyclase (sAC) that may act within the cell as a putative metabolic sensor. **Methods**: Uptake experiments were performed using amantadine and TEA as cationic marker substrates in the absence/presence of bicarbonate (CT and KHS buffers respectively) on native and OCT transfected human embryonic kidney (HEK 293) cells. Cells were pre-incubated with varying glucose (0-25 mM), and insulin (0.8-20 mU/ml) concentrations, and with Br-cAMP (100 uM), dibutyryl cAMP (100 uM), and forskolin (10 uM). **Results**: We found that amantadine transport increased 72% and was dose related to added insulin regardless of buffer or glucose concentration. Amantadine energy-dependent uptake is maximal in the presence of bicarbonate, is inhibited 50% in the absence of bicarbonate ($p<0.01$), and 67% in the presence of elevated glucose concentration in the medium ($p<0.01$). TEA transport on the other hand is

decreased in OCT1 and OCT2 transfected cells in a dose dependent manner (up to 30% and 20 % respectively) regardless of glucose concentration, but dependent on buffer conditions. Acute treatment with insulin and cAMP analogues demonstrated synergistic activation of the amantadine transporter, and synergistic inhibition of OCT1 and OCT2. Glucose starvation increased amantadine transport by native HEK 293 cells, and decreased TEA transport in OCT1 and OCT2 transfected cells. **Conclusion:** These data indicate an important differential role for bicarbonate, insulin and glucose in the regulation of organic cation transport in the kidney, and suggest a direct link between metabolic activity, sAC, cAMP, and OCTs function.

ACKNOWLEDGEMENTS

Contemplation owes its origins to a Greek word contemplari, meaning to mark out a temple; knowing this, my contemplation about the past several years in my life has been a recognition of the profound impact of the all those who have graced my life. I believe that when we are born, we are born into this world as scientists. As children we spring forth into a world of the unknown, it is there where by the use of our intellect and experience we begin to organize endless curiosity into knowledge; some of it is comes from trial and error, other times events are serendipitous. I've been blessed with the opportunity to learn a tremendous amount about science and life; the two are truly intertwined. One lesson I hold dear, is that inherent, intense organization lies in the apparent chaos of the universe; from whose like man has been made a model. To quote an ancient proverb: "As above, so below." This organization of information (frozen matter/fluid energy, 1s and 0s) has a complexity that can only be described as "intelligence", and we, and the life on this planet are a microcosm within a macrocosm, and vice-versa. Such a notion is becoming increasingly and objectively supported by the systems-based mathematical concept of "self-organization" (Thank-you Steven Wolfram); this type of thinking, I believe, as do many others, heralds a new kind of science. Therefore, if my life and my work is to be its own glass bottle/capsule thrown into the oceans of knowledge and time, then I must thank all my "fellow travellers" that have co-habited my life: the role-models, the anti-role-models, and those who, whether they knew it or not, have helped me along the way. I have learned much from you all. Thank-you.

Much love goes to my mother for instilling in me a restless curiosity, a sense of humility, and an appreciation of nature and beauty; to my father the meteorologist, for instilling in me an appreciation of the sky, the art of argument, debate, and logic, with equal weight given to lessons in humour; and finally to my companion, Roberta, for her boundless support, her love, and for the time we've shared: the trials, the laughter, and the madness; my life has forever been enlivened. Thanks for being on my side.

A special thanks goes to my supervisor, Dr. Dan Sitar for his mentorship, his support and guidance (he believed in me when granting agencies and others had not). Sincere thanks, to the members of my committee, Dr. Grant Hatch and Dr. Don Smyth, for their support and timely advice (both are wonderful testaments to the art of pedagogy). I would also like to thank all the members, past and present, of the department, and especially my fellow graduate students: the serfs of science (Diane, Christy and Kristin), who made graduate life delightfully bitchable.

Lastly, I must thank Leggo™ and biomolecules (two brothers of form). I must also give a PLUR to the crew: Kevin, Beanie, Curty (DJ Buck-Naked), the girls at the G spot (Judgement Spice, BrandiFlakes, and Momma Bear), Tannis aka Tanroth, John "Ban-mon", Wayne Rainbow, Grandmaster Doug Lee PhD, Handsome Pete, the Feline Four (Madame Zu, Dexter, Obi, and Osirus), and the spirit of Da Vinci. It has been a regular "Alex in Wonderland". To all those scholars that have come before me: Thank-you for your sacrifices upon the "altar of knowledge". My advice to those who will arrive after me: Do not seek to follow in the footsteps of the wise; seek what they sought.

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ABBREVIATIONS

αKG^{2-}	Alpha ketoglutarate
8-Br-cAMP	8-bromoadenosine 3′,5′- cyclic adenosine monophospate
A	Amantadine
AC	Adenylyl Cyclase
AKAPs	A-kinase anchoring proteins
ASP^{+}	4-[4-(dimethylamino)styryl]-N-methylpyridinium
CA	Carbonic anhydrase
CaM	Ca^{2+} / calmodulin dependent kinase
cAMP	3′,5′- cyclic adenosine monophospate
cNGC	Cyclic nucleotide gated channels
CT	Cross Taggart
dibutyryl cAMP	N^{6},2′-O-Dibutyryladenosine 3′,5′- cyclic adenosine monophospate
DMEM	Dulbecco's Modified Eagle Medium
EPAC	Exchange protein activated by cAMP
GFR	Glomerular filtration rate
IRR	Insulin receptor-related receptor
KHS	Krebs-Henseleit Saline
MPP	Neurotoxin 1-methyl-4-phenylpyridinium
NLT	Novel liver-specific transport protein
NMN	N_1-Methylnicotinamide
NSAIDS	Nonsteroidal anti-inflammatory drugs
OA^{-}	Organic Anion

ABBREVIATIONS

OC^+	Organic Cation
OCTs	Organic cation transporters
PAH^-	Para-aminohippuric acid
PBS	Phospate buffered saline
pCO_2	Partial pressure of carbon dioxide
pHi	Intracellular pH
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A.
PKC	Protein kinase C
PKG	Protein kinase G
sAC	Soluble Adenylyl Cyclase
STZ	Streptozotocin
TbuMA	Tri-n-butylmethylammonium
TEA	Tetraethylammonium
tmAC	Transmembrane Adenylyl Cyclase

GENERAL INTRODUCTION

Foreword

Many organic compounds ranging from those of a xenobiotic nature, such as drugs, synthetic and natural environmental toxins, to endogenous metabolites (neurotransmitters, cofactors etc.) acquire a positive charge at physiological pH. During the course of evolution, transporter proteins have evolved to handle these structurally diverse compounds, either for detoxification purposes, cell-cell communication, or other yet undefined physiological purposes. The system of proteins responsible for these diverse functions has become known in scientific literature as the organic cation transport system. These transporters although highly localized in the kidney are also present throughout the body including the brain, liver and gut. Thus, understanding these transporters has translational benefit. During the past several decades, the mechanisms of the processes surrounding these transporters have been intensively studied, including the nature of the carriers, their interaction with substrates, and the forces driving transport. Recently, this knowledge base has been further extended by the molecular identification of the specific transport proteins involved. Understanding the interplay between transporters and regulatory elements involved in the organic cation transport system is the focus of current literature. The focus of this present dissertation lies in developing the understanding of that regulation. The information derived from such knowledge would be of great clinical significance in predicting interactions that result in the suppression of these processes. Such events could increase the exposure of the body to

potentially dangerous xenobiotics, thereby increasing the potential for developing an array of pathophysiological disorders. In addition, these interactions would become exacerbated in those with decreased renal function such as pediatric, the chronically ill, and geriatric populations. Compound those aforementioned concerns with the rise of global industrialization and the associated environmental changes induced in the atmosphere, water and soil, and understanding the regulatory elements behind the handling of xenobiotics becomes paramount.

I. Membrane Transport Processes

Membranes define the external boundary of all living cells and regulate the molecular traffic across that boundary; they divide the internal space into discrete compartments, allowing segregation of processes and components; they are central to both biological energy conservation and cell-to-cell communication. The cell membrane is the business end of the cell. Every cell must acquire from its surroundings the raw materials for biosynthesis and for energy production, and must release to its environment the byproducts of metabolism (Lehninger et al., 1993a). Passive diffusion, facilitated, and active protein-mediated transport are the three types of membrane transport processes that allow for movement of compounds through biological membranes. Since drug transport system(s), and specifically their regulation, form the central focus of the present dissertation,

the following section is dedicated to reviewing the basic principles of membrane transport.

I.1. Passive Diffusion

When two aqueous compartments containing unequal concentrations of a soluble compound or ion are separated by a permeable membrane, the solute moves spontaneously by random motion from a region of high concentration to a region of low concentration without direct expenditure of metabolic energy. In passive transport, the flux of the solute can be described by the following equation:

$$V=K_d(S_1-S_2) \quad (1)$$

where V is the transport rate of the solute, K_d is the diffusion constant, and S_1 and S_2 represent the substrate concentration on either side of the membrane (Christensen, 1975). K_d decreases with increasing molecular size, with decreasing lipid solubility, decreased temperature, increased membrane thickness, and with a decreased surface area available for diffusion. Moreover, small hydrophobic molecules will passively diffuse through the membrane at a higher rate than larger more hydrophilic molecules due to the lipid nature of membranes. It is further evident from the equation, that for uncharged molecules, the rate of diffusion will be directly proportional to the concentration gradient. When the substrate concentration on each side of the

membrane becomes equal, the net rate of diffusion becomes zero. Diffusion across the membrane will be initially linear when all solute (S) originates on one side of the membrane and the initial rate becomes directly proportional to the starting solute concentration. If the solute carries a net charge, however, both its concentration gradient and the electrical potential difference across the membrane (the membrane potential) influence its transport. The concentration gradient and the electrical gradient can be combined to calculate a net driving force, or electrochemical gradient, for each charged solute (Lehninger et al., 1993a).

I.2. Membrane Transport Proteins – Channels and Carriers

Cell membranes generally allow nonpolar molecules (ie CO_2 and O_2) to permeate by simple diffusion, with the exception of H_2O whose levels are regulated additionally by a family of aquaporin channel proteins (Schrier and Cadnapaphornchai, 2003). Cell membranes, however, also allow the passage of various polar molecules, such as ions, sugars, amino acids, nucleotides and many cell metabolites. Unaided, these processes occur across the membrane very slowly. However special membrane proteins have evolved which are responsible for the transfer of a specific ion or molecule or a group of closely related ions or molecules across cell membranes. These proteins, referred to as membrane transport proteins, occur in many forms and in all types of biological membranes. There are two classes of membrane proteins: channels and carriers; both form continuous protein pathways across the lipid bilayer. Whereas transport by channel proteins is always passive (down a

concentration gradient), transport by carrier proteins can be either active (up a concentation gradient) or passive (down a concentration gradient) (Lehninger et al., 1993a).

I.2.a. Channel Proteins

Channel proteins need not bind the solute. Instead, they form hydrophilic pores that extend across the lipid bilayer. Although they have similar kinetics to carrier proteins under non-saturating substrate concentrations, protein channels only allow for the passage of ions and/or ionized molecules of appropriate size and charge to pass through them and thereby cross the membrane. This type of flux is completely dependent on diffusion down concentration gradients. Not surprisingly, transport through channel proteins occurs at a very much faster rate than transport mediated by carrier proteins. For example Na^+ and K^+ channels allow the passage of their respective ions down an electrochemical gradient, and thus allow the cell to both control its membrane potential and to propagate neural responses (Berne and Levy, 1998)

I.2.b. Carrier Proteins

The systems discussed so far lead to equilibrium, not accumulation. Carrier proteins (also called carriers, permeases, or transporters) bind the specific solute to be transported and undergo a series of conformational changes in

order to transfer the bound solute across the membrane. The process by which a carrier protein transfers a solute molecule across the lipid bilayer resembles that of an enzyme-substrate reaction, where the carriers behave as specialized membrane-bound enzymes. Each type of carrier protein has one or more specific binding sites for its solute (substrate). When the carrier is saturated (that is, when all these binding sites are occupied), the rate of transport is maximal. This rate, referred to as Vmax, is characteristic of the specific carrier l(Lehninger et al., 1993a).

The movement of a substrate across the membrane using a carrier of limited capacity, proposed by Michaelis and Menten (1913), is given by equation (# 2). The derivation can be found in most biochemistry textbooks.

$$V=(Vmax.S)/(Km+S) \qquad (2)$$

This relationship describes a rectangular parabola, the Langmuir isotherm (Lehninger et al., 1993a), that exhibits saturation **(Fig I-1).** V is the measured rate of transport and S is the substrate concentration. Vmax is the maximal transport capacity of the system. It provides an indication of carrier density (number of carriers) active during the transport process. It is important to emphasize that no direct correlation exists between Vmax and the number of transporters present in the system. Km is the concentration of substrate that will produce half the maximal transport rate (ie. 1/2 Vmax). The value of Km gives an indication of the affinity of the carrier for the substrate.

Protein-mediated transport can be categorized as either facilitative diffusion or active transport. In facilitated diffusion, transport depends on the interaction of the solute with a specific protein in the membrane that facilitates the movement of the solute across the hydrophobic membrane. Facilitated transport is not linked to metabolic energy and can't move charged molecules against electrochemical gradients or uncharged molecules against chemical gradients. Examples of facilitated diffusion include uniport glucose or urea transporters (Alberts et al., 1994). Active transport also depends on the interaction of the solute with a specific protein in the membrane, but it requires metabolic energy to enable it to move compounds against concentration or electrochemical gradients. Active transport may be primary, secondary or tertiary (Alberts et al., 1994). A primary active transport process is directly coupled to a high-energy releasing reaction (such as hydrolysis of ATP). An example is the Na^+/K^+ ATPase, which uses the energy generated from the hydrolysis of ATP to pump Na^+ and K^+ ions against their chemical gradients (Alberts et al., 1994). Secondary and tertiary active transport generally use the potential energy stored in ionic gradients, which were originally achieved by energy expenditure, to drive transport of compounds against their electrochemical or chemical gradient. For example the movement of one chemical across the membrane, down its concentration gradient drives the transport of a second compound uphill against its concentration gradient. The direction of transport of the two substrates may be in the same direction (symport) or opposite direction (antiport) (Lehninger et al., 1993a). The Na^+/H^+ exchanger in the luminal membrane of proximal tubules is an example of a secondary active transport process. These proteins

use the energy stored in the Na^+ gradient (low intracellular concentration, high extracellular concentration), established by the Na^+/K^+ ATPase to reduce acidity by driving the extrusion of H^+ from the cell. The organic cation/H^+ exchanger in the luminal membrane of proximal tubules is an example of a tertiary active process that uses the energy stored in the H^+ gradient, established by the secondary Na^+/H^+ exchanger, to drive organic cation extrusion from the cell (Takano et al., 1984).

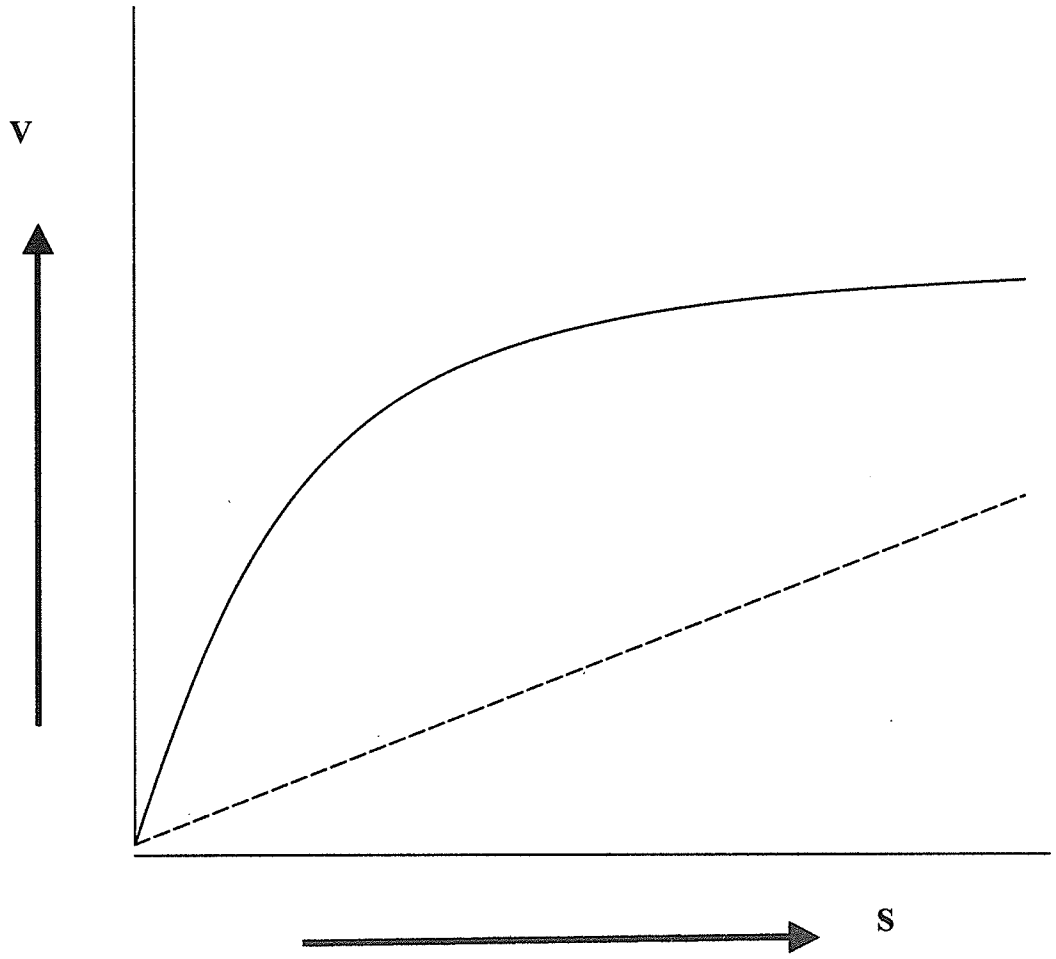

Figure I-1: Comparison between rate of transport (v) versus substrate concentration (S) for passive diffusion (dotted line) and protein-mediated transport (solid line) (Goralski, 1999).

II. Kidney Physiology and Renal Drug Transport

The functional unit of the kidney is the nephron. There exists approximately 1.2 million nephrons; these can conceptually be envisioned as hollow tubes composed of a single cell layer. There are three types of nephrons: superficial, midcortical and juxtamedullary nephrons (Tisher and Madsen, 1996). The basic components of all nephrons include a filtering component (the glomerulus) and a connecting tubular part. Blood arrives at the glomerulus, which acts as a molecular sieve for small molecules present in the plasma, a process known as ultrafiltration. The protein-free ultrafiltrate flows into the tubular part connected to the glomerulus by the Bowman's capsule. For the length of the nephron, the tubular component is formed of a monolayer of epithelial cells resting on a basement membrane. There is considerable heterogeneity among the different parts of the tubule. The tubule is grossly divided into four sections: The proximal tubule receives the ultrafiltrate, and it is this part of the nephron in which most of the reabsorptive processes for the filtrate components are present. Further flow of the ultrafiltrate will move it into the loop of Henle. The loop is divided in turn into descending (descends into the medulla), and ascending loops (which returns into the cortex). At the end of the ascending loop of Henle, the distal tubule begins, and the connecting and collecting ducts follow (Vander, 1995).

Within these gross divisions, there are further anatomical divisions of the proximal tubule. The first segment of the proximal tubule is convoluted, pars convoluta, and it is known as the S1 segment. The S2 segment follows and it lies between the pars convoluta and the S3 segment or pars recta, the part that connects the proximal tubule with the loop of Henle. There is extensive functional heterogeneity among these segments as well (Berne and Levy, 1998).

The tubule epithelia possess the ability to remove compounds from the ultrafiltrate and to return them to the circulation. This process is known as reabsorption. The opposite process is present also, whereby substrates present in the blood, which did not filter through the glomerulus, are actively transported into the lumen. This process is known as secretion. Whether the substrate appears in the urine, or not, will depend on the extent to which it is filtered, secreted, and reabsorbed (Vander, 1995).

It is through the processing of blood by removing and adding substances to it, that the kidney performs a variety of functions. These are summarized below:

1) Regulation of water and inorganic-ion balance
2) Removal of metabolic waste products from the blood and their excretion in the urine
3) Removal of foreign chemicals from the blood and their excretion in the urine

4) Gluconeogenesis

5) Secretion of hormones (ie. renin, erythropoietin)

Central to this dissertation is the kidney's ability to eliminate numerous potentially toxic xenobiotics, including drugs, toxins, and endogenous metabolites. Both exogenous and endogenous compounds can be metabolized to charged organic molecules within the body, to aid in their elimination. The kidney is responsible for most of the removal of these organic anions and cations, and specific transporters are present in the renal tubules to perform this task. However, it should be stressed that these systems are not specific for the kidney, and similar mechanisms are present in the liver (Despopoulos, 1966), choroid plexus (Barany, 1972), the ciliary body (Barany, 1972), and the thyroid (Hamilton, 1953). Thus, comprehending the transporters in the kidney will have translational benefit.

III. Renal Organic Anion and Cation Transport

The kidney plays a pivotal role in the elimination of numerous xenobiotics, including drugs, toxins, and endogenous compounds. Renal elimination involves glomerular filtration, tubular secretion, and tubular reabsorption. Of these, the secretion and reabsorption of drugs in renal tubules are essentially saturable processes, where plasma membrane located transport proteins mediate transepithelial transport. These high capacity

transport systems help to serve two functions for the kidney: prevention of the urinary loss of filtered nutrients, and facilitation of tubular secretion of xenobiotics that escape hepatic extraction processes. These transport systems responsible for renal tubular secretion of drugs have been classified into two categories, based on their preferential substrate selectivity, organic anion transporters (OATs) and the cation transport systems (OCTs) (Inui et al., 2000). In addition to these organic ion transporters, the adenosine 5'-triphosphate (ATP)-dependent primary active transporters such as P-glycoprotein (Fojo et al., 1987; Thiebaut et al., 1987) and the multidrug resistance-associated protein (MRP) family have been suggested to function as the drug efflux pumps in renal tubules (Kool et al., 1999; Schaub et al., 1999).

The mechanisms mediating tubular secretion have been intensively studied over the past several decades, and the features of organic anion and cation transport, the nature of carriers and their interaction with substrates, and the forces driving transport through basolateral and luminal membranes have been characterized (Roch-Ramel et al., 1992; Pritchard and Miller, 1993; Sica and Schoolwerth, 1996; Roch-Ramel and Diezi, 1997). This information has recently been extended by the identification, using molecular cloning techniques, of specific transport proteins, which mediate the translocation of organic anions and cations across cell membranes to result in net vectorial secretion into the tubular lumen (Sweet et al., 1997).

Understanding the regulation of these transport systems is now becoming the focus of current literature, and should prove to be of great clinical significance for several reasons. First, suppression of tubular secretion may increase the exposure of the body to potentially dangerous synthetic and natural xenobiotics. Second, stimulation of tubular transport may be useful for prevention or treatment of occupational diseases by elimination of environmental toxins. Third, pathophysiological conditions (e.g. diabetes) may alter those transport processes, and compromise therapy. Renal handling of most charged drugs is the result of the interaction with these transporters. Therefore, elucidating the physiological functions, substrate specificities, and regulatory mechanisms will lend itself to the individualization of future drug therapy.

III.1. Renal Organic Anion Transport

Organic anions are chemically heterogeous substances containing carbon backbones and negative charges at physiological pH. Numerous substances, such as environmental xenobiotics, drugs, plant, or animal toxins, and the metabolites of both exogenous and endogenous chemicals exist as organic anions at physiological pH **(Table I-1)**, and are actively secreted by renal tubular epithelia. Some of the more notable examples include cyclic nucleotides, prostaglandins, uric acid, β-lactam antibiotics, methotrexate, and nonsteroidal anti-inflammatory drugs (NSAIDS). Thus, the organic anion transport system plays a critical role in protecting against the potential toxic

effects of anionic compounds by mediating their excretion into urine (Dantzler et al., 1995).

Historically, the first reports of tubule secretion of organic anions were made by Marshall and associates who demonstrated that the anion dye, phenol red, stained the tubules of anaesthetized and conscious dogs (Marshall and Vickers, 1923; Marshall and Grafflin, 1928; Marshall, 1931), rat, and frog renal cortex (Edwards and Marshall, 1924). Subsequent reports, using other species (Chambers and Kempton, 1933), stop-flow (Malvin et al., 1958) and micropuncture techniques (Cortney et al., 1965), confirmed findings by Marshall and co-workers. Later studies showed that both a negative charge and a large hydrophobic region are necessary structural prerequisites for transport by this system (Taggart, 1958; Essig and Taggart, 1960; Knoefel et al., 1961; Despopoulos, 1965; Moller and Sheikh, 1983).

Further investigation into this system was unable to determine any specificity of transport. However, Smith et al. (1945) showed that organic anions were capable of inhibiting the transport of other organic anions. This suggested that they were all transported by a common system. Of the hippuric acid derivatives tested by Smith et al (1945), p-aminohippuric acid (PAH) was the most avidly secreted by this system. Subsequently, PAH has been used as a prototypical marker substrate for the organic anion transport system. This use stems from the fact that PAH is efficiently secreted; it undergoes limited metabolism and renal tubule reabsorption, and it is easily

determined chemically(Moller and Sheikh, 1983). The transport system for PAH has become known as the "classical organic anion transport system".

Studies in dog and rat using stop-flow and microperfusion techniques, have demonstrated that the proximal tubule is the primary site for organic anion secretion (Malvin et al., 1958; Cortney et al., 1965). Heterogeneity of PAH transport along the proximal tubules has been shown. It appears that secretion is higher in the *pars recta* (S2 and S3) than in the early convoluted (S1) segment of the tubule (Tanner and Isenberg, 1970). Later studies indicated that PAH secretion was greater in S2 segments compared to S1 and S3 segments (Woodhall et al., 1978). Regardless, all portions of the tubule secrete PAH and differences in transport activity reflect carrier density (Vmax) rather than differences in affinity (Km). At present, there have been no reports of distal tubular organic anion secretion.

III.1.a. Mechanism of Organic Anion Transport

The secretion of organic anions by renal tubules is mediated by the concerted function of three distinct transport steps, one at the peritubular basolateral membrane, another intracellularly, and lastly one at the luminal brush-border membrane of tubular cells **(Fig I-2)** (Pritchard and Miller, 1993; Roch-Ramel, 1998). Energy-dependent uphill uptake is first required for the basolateral entry step of the negatively charged anion in order to cross the electrical potential barrier represented by the basolateral membrane. Shimada, Moewes, and Burckhardt reported that uptake of PAH in isolated

basolateral membrane vesicles, was markedly enhanced in the presence of an outward α-ketoglutarate gradient (Shimada et al., 1987). This finding suggested that dicarboxylates are the physiological counterions for the uphill transport of organic anions. The outward α-ketoglutarate gradient is sustained not only by intracellular metabolic dicarboxylate generation, but also by the Na^+/α-ketoglutarate cotransport system driven by the inward Na^+ gradient (Pritchard, 1988), which is established by Na^+/K^+ ATPase **(Fig I-2, #1)**. Therefore, the basolateral transport of organic anions appears to be driven by indirect coupling to the Na^+ gradient through Na^+/α-ketoglutarate cotransport **(Fig I-2, #2)** and organic anion/dicarboxylate exchange **(Fig I-2, #3)** (Pritchard, 1995).

Once inside the cell, organic anions may be sequestered within vesicular proteins or they may bind to intracellular proteins **(Fig I-2, #4)** (Miller et al., 1993). However, very little is actually known about those intracellular events, except that they may affect accumulation and therefore secretion (Shuprisha et al., 2000).

Transport into the lumen is the final step and also a mediated process. In rat renal brush-border membrane vesicles, the uptake of PAH was stimulated by an inside-positive membrane potential created by K^+ and valinomycin (Martinez et al., 1990). In addition, PAH uptake was stimulated by PAH/PAH or PAH/Cl^- exchange, and the PAH/PAH exchange was insensitive to the membrane potential. The potential-stimulated PAH uptake was more sensitive to drugs such as furosemide and 4,4′-diisothiocyano-2,2′-

disulfonic stilbene than PAH/PAH exchange (Ohoka et al., 1993). These findings suggest that PAH is transported by two distinct transport systems in rat renal brush-border membranes: a potential-sensitive transport system **(Fig I-2, #5)** and an anion exchanger **(Fig I-2, #6)**. The anion exchanger is found to be expressed in urate-reabsorbing species such as the rat and the dog, and accepts various organic and inorganic anions (including PAH, urate, Cl^-, Br^-, HCO_3^-, and OH^-) as substrates (Guggino et al., 1983; Kahn et al., 1983). In addition to these proposed organic anion transport systems, functional studies using isolated perfused renal tubules have demonstrated the participation of multiple transport proteins (ie. P-glycoprotein, and MDR family) in secretion of organic anions at the brush border luminal membranes **(Fig I-2, #7)** (discussed later in this dissertation) (Roch-Ramel, 1998).

ENDOGENOUS COMPOUNDS	EXOGENOUS COMPOUNDS (XENOBIOTICS)
Bile Acids	**Antibiotics**
Cholate	Carbenecillin
Taurocholate	Sulfisoxazole
	Penicillin G
Cyclic nucleotides	
cAMP	**Diuretics**
cGMP	Acetazolamide
	Bumetanide
Metabolites	Furosemide
Hippurate	Metolazone
Oxalate	
Urate	**NSAIDS**
	Indomethacin
Prostaglandins	Phenylbutazone
PGE_2	Salicylate
Vitamins	**Miscellaneous**
Ascorbate	Methotrexate
Folate	PAH
	Phenol red
	Probenecid
	2,4-Dinitophenol

Table I-1: Endogenous and exogenous compounds that are organic anions. Information for this table was obtained from the following references, (Uwai et al., 1998; Apiwattanakul et al., 1999)

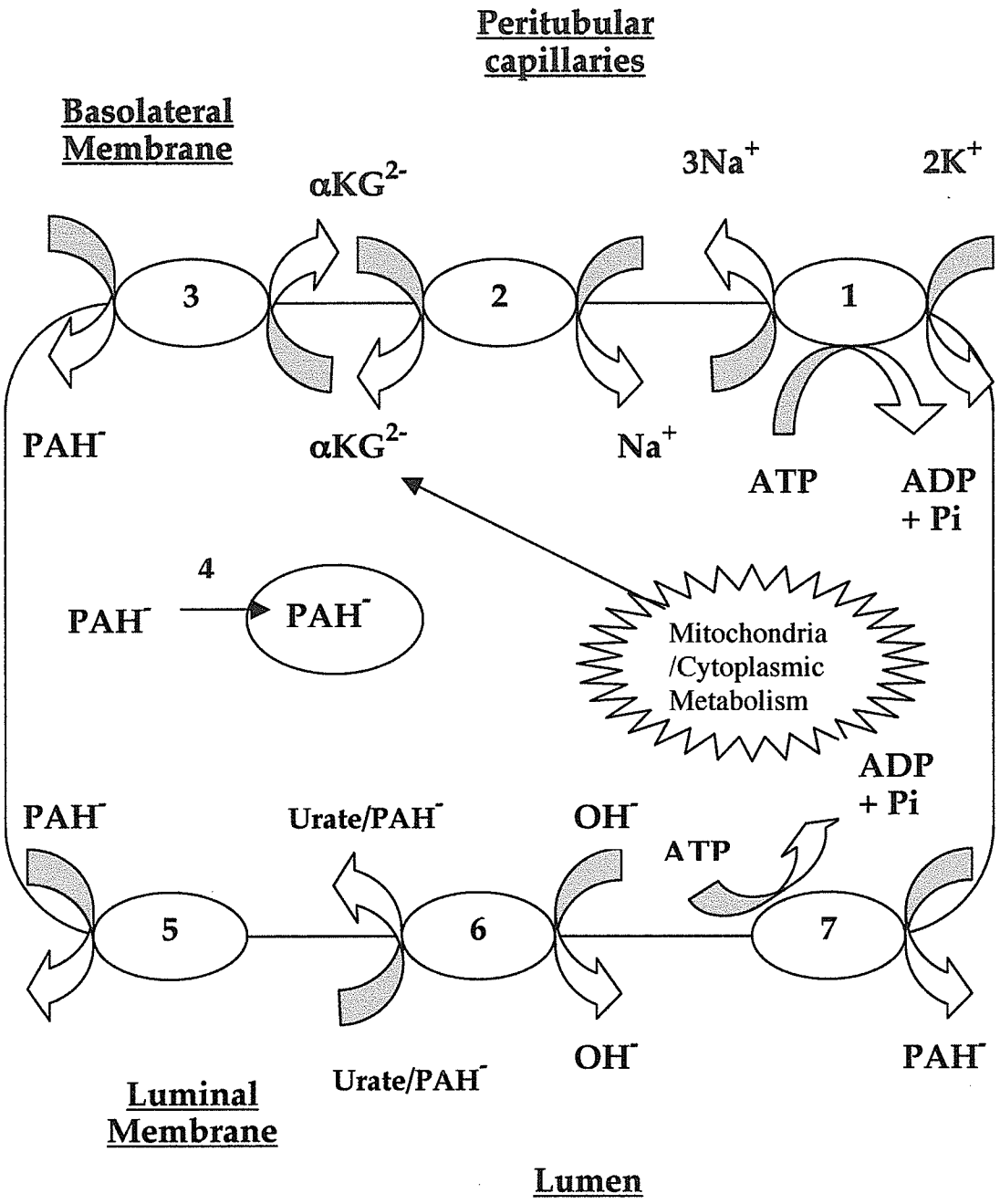

Figure I-2: Model of organic anion transport

III.2. Molecular Biology of Organic Anion Transport

III.2.a. The Organic Anion Transporter (OAT) Family

In 1997, two independent laboratories, Sekine et al. (1997) and Sweet et al. (1997) successfully isolated a 2.2 kb cDNA clone from rat kidney encoding the PAH /dicarboxylate exchanger protein by expression cloning with oocytes. This protein was designated as OAT1. Rat (r)OAT1 is comprised of 551 amino acids with 12 putative membrane-spanning domains. When expressd in Xenopus oocytes, both groups demonstrated saturable uptake of PAH with similar Km values. The uptake of PAH was independent of membrane potential, and was markedly enhanced in the presence of an outward gradient of dicarboxylate, indicating that OAT1 functions as an organic anion/dicarboxylate exchanger (Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997). rOAT1 had a wide substrate specificity for endogenous anions such as cyclic nucleotides, prostaglandins, uric acid, and structurallly diverse drugs such as β-lactam antibiotics, methotrexate, and nonsteroidal anti-inflammatory drugs (NSAIDS) (Uwai et al., 1998; Apiwattanakul et al., 1999). Sekine et al. (1997) further localized mRNA expression of OAT1 to the convoluted portion (S2) of the proximal tubule, which is consistent with the classical PAH model in rats (Tune et al., 1969).

Two cDNAs, human (h) OAT1-1 encoding a 556 amino acid protein and hOAT1-2 (PAHT, hROAT1) encoding a 550 amino acid protein were identified in the human kidney (Reid et al., 1998; Hosoyamada et al., 1999; Lu

et al., 1999). In contrast, hOAT1-2 was suggested to show a narrow substrate specificity, since prostaglandins and methotrexate were not transported (Lu et al., 1999). Alternative-splicing mechanisms could explain the differences seen in substrate specificity between the two hOAT1 isoforms.

Other members with significant homology to OAT1 have been identified **(TABLE I-2).**

Novel liver-specific transport protein (NLT), isolated as a liver-specific transporter from the rat (Simonson et al., 1994), shows a 42% homology with rOAT1. When expressed in oocytes, NLT mediated the uptake of organic anions such as salicylate, acetylsalicylate, prostaglandin E2, dicarboxylates, and PAH (Sekine et al., 1998). Expression of NLT mRNA was predominatly detected in the liver and to a lesser extent in the kidney (Simonson et al., 1994; Sekine et al., 1998). Thus, it has been renamed rOAT2. In contrast to OAT1, NLT/rOAT2 appears not to be an organic anion/dicarboxylate exchanger, and its driving force has not been identified.

A cDNA encoding another member of the multispecific organic anion transporter family, rOAT3, was isolated from rat brain by the RT-PCR cloning method (Kusuhara et al., 1999). rOAT3 mRNA appeared to be expressed in the liver, brain, kidney, and eye (Kusuhara et al., 1999). When expressed in oocytes, rOAT3 mediated the uptake of the anionic marker PAH, ochratoxin A, and estrone sulfate, and the cationic drug cimetidine. rOAT3 has been

suggested to participate in the excretion and/or detoxification of endogenous and exogenous organic anions, especially from the brain.

TRANSPORTER	SPECIES	TISSUE DISTRIBUTION	MEMBRANE LOCALIZATION	MAIN SUBSTRATES
OAT1	Human Rat Mouse (NKT) Winter flounder	Kidney>>> brain (rat)	Basolateral	PAH, α-KG, cAMP, cGMP, NSAIDS, methotrexate, β-lactam antibiotics, uric acid, prostaglandin E_2
OAT2	Human (NLT) Rat (NLT)	Liver>>> kidney (rat)		PAH, α-KG, salicylate, acetylsalicylate, prostaglandin E_2
OAT3	Human Rat	Liver> kidney> brain (rat)	Basolateral	PAH, ochratoxin A, estrone-3-sulfate, cimetidine
oatp1	Rat	Liver> kidney	Basolateral Apical	ouabain, taurocholic acid, cholic acid, leukotriene C_4
oatp2	Rat	Liver> brain> retina		Digoxin, ouabain, taurocholic acid, cholic acid, thyroxine, 3,5,3'-triiodo-L-thyronine
oatp3	Rat	Kidney>retina, liver		Taurocholic acid, thyroxine, 3,5,3'-triiodo-L-thyronine
OAT-K1	Rat	Kidney	Apical, Basolateral	Methotrexate, folic acid
OAT-K2	Rat	Kidney	Apical	Methotrexate, folic acid, taurocholic acid, prostaglandine E_2
OATP	Human (SLC21A 3)	Brain		taurocholic acid, cholic acid

Table I-2: Some characteristics of cloned organic anion transporters. (Bergwerk et al., 1996; Saito et al., 1996; Masuda et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997; Hosoyamada et al., 1999; Lu et al., 1999; Masuda et al., 1999)

III.2.b. The Organic Anion Transporting Polypeptide (oatp) Family

The oatp transporters are a family of Na^+ independent organic anion transporters that are about 670 amino acids in length and have 10 putative transmembrane domains. Presently, three members (oatp1, oatp2, and oatp3) of the oatp gene family have been isolated from rat tissues, cloned and studied in vitro (Jacquemin et al., 1994; Noe et al., 1997; Abe et al., 1998; Kakyo et al., 1999). Northern blot analysis demonstrated expression of oatp1 mRNA in rat liver, kidney, brain, lung, skeletal muscle, and proximal colon. Immunohistochemical examination of the liver revealed the sinusoidal plasma membrane localization of oatp1 (Bergwerk et al., 1996). In rat kidney, oatp1 was detected in renal brush-border membranes in the S3 segment of the proximal tubule of the outer medulla (Bergwerk et al., 1996). When expressed in Xenopus ooycytes, oatp1 mediates the uptake of several exogenous and endogenous organic acids (sulfobromophthalein, bile acids, estrogen conjugates), neutral steroids (aldosterone, dexamethasone, digoxin and ouabain) and some bulky cationic compounds (Jacquemin et al., 1994; Bossuyt et al., 1996; Kanai et al., 1996; Eckhardt et al., 1999). Given its liver location, oatp1 may serve as an apical exit pathway for endogenous neutral steroid molecules and anionic bile salts (Bossuyt et al., 1996).

Two other members of the oatp gene family, oatp2 and oatp3, were isolated from the rat brain and retina, respectively (Noe et al., 1997; Abe et al., 1998). Oocytes injected with synthetic RNA encoding oatp2 and oatp3 showed an uptake of taurocholate, thyroxine, and triiodothyronine in a

saturable manner. In addition, the oatp2-expressing oocytes stimulated the uptake of cardiac glycosides such as digoxin and ouabain. Oatp2 mRNA was found to be widely expressed in the central nervous system, specifically among neuronal cells as well as in the retina and liver. In contrast, oatp3 mRNA was expressed in the kidney and moderately expressed in the retina. It has been suggested that oatp2 and oatp3 are multifunctional transporters in the brain, retina, liver, and kidney. The membrane localization of both transporters has not yet been identified.

Saito et al. (1996) identified another member of the oatp gene family, designated OAT-K1. Rat OAT-K1 is comprised of 669 amino acids and shows 72% homology with rat oatp1. Northern hybridization indicated that it is exclusively expressed in the rat kidney. In rOAT-K1-transfected LLC-PK1 cells, the protein was localized in the basolateral membranes and mediated Na^+-independent uptake of methotrexate and folate, but not PAH, prostaglandin E2, or leukotriene C4 (Saito et al., 1996). By RT-PCR detection, OATK-1 mRNA was found predominately in superficial and juxtamedullary proximal straight tubules (Masuda et al., 1997). Another kidney-specific isoform of the oatp family, designated as OAT-K2 (Masuda et al., 1999), was isolated from rat kidney. OAT-K2 is comprised of 498 amino acids and shows 91% identity with rat OAT-K1. OAT-K2 mRNA was detected in proximal convoluted and straight tubules and cortical collecting ducts.

III.3. Renal Organic Cation Transport

Organic cations are generally carbon-backboned, nitrogen-containing primary, secondary, or tertiary amines or quaternary ammonium salts; all are weak bases, with one or more positive charges at physiological pH. Numerous substances are categorized as organic cations. These compounds can be subdivided into endogenous and exogenous compounds. The endogenous group consists of guanidine, choline, N^1-methylnicotinamide (NMN), and bioactive monoamines (dopamine, epinephrine, and histamine). The exogenous group consists of cationic drugs and toxins, which include antiviral agents, anticholinergic agents, H_2 receptor blockers, sympathomimetic agents, opioids, antiarrhythmic agents, α and β-adrenergic receptor blocking agents, diuretic agents, and ganglionic blocking agents (Rennick and Moe, 1960; Pilkington and Keyl, 1963; Acara and Rennick, 1972; Rennick, 1981; Weiner and Roth, 1981; McKinney and Speeg, 1982; Pritchard and Miller, 1993) **(Table I-3).**

Organic cation secretion in renal tubules was first demonstrated in excretion studies of tetraethylammonium (TEA) in dogs and humans (Rennick et al., 1947; Rennick et al., 1956; Rennick and Farah, 1956) and N^1-methylnicotinamide (NMN, a metabolite of vitamin B) in the chicken and dog (Beyer et al., 1950; Peters et al., 1955; Sperber, 1959). Both these compounds were inhibited by $cyanine_{863}$, suggesting that the transport process was carrier mediated. Moreover, both compounds appeared to describe a renal tubule secretion mechanism that was different from that of the prototypical organic

anion (PAH) secretion system, as evidenced by the inability of PAH to inhibit NMN and TEA renal excretion (Peters et al., 1955; Rennick and Farah, 1956). This renal tubule secretion mechanism has now become known as the "organic cation transport system", and TEA and NMN have gained the status of prototypical marker substrates for the characterization of renal tubule organic cation transport mechanisms, with TEA being used most frequently. TEA's use as a marker substrate, comes from the fact that it is efficiently secreted; it undergoes limited metabolism and renal tubule reabsorption; and it is easily determined chemically (Rennick and Farah, 1956).

Initial characterization of the renal tubule transport of organic cations had suggested that the proximal tubule is the location where organic cation secretion is primarily mediated (Rennick and Moe, 1960; Pilkington and Keyl, 1963). Since those original observations, organic cation transport activity has also been detected in distal tubules and collecting ducts (Hohage et al., 1994b; Miller et al., 1998). The existence of organic cation transport in distal tubules has been further corroborated by previous reports from our laboratory and by molecular biology studies (Wong et al., 1993; Escobar et al., 1994; Escobar et al., 1995; Escobar and Sitar, 1995; Gorboulev et al., 1997). Organic cation transport in the proximal tubule has demonstrated axial heterogeneity of secretion of certain organic cations in different segments of the tubule. For example, TEA and procainamide have been shown to be secreted primarily in the early segments of the proximal tubule (S1>S2>S3), while NMN is primarily secreted in the later portions of the proximal tubule (S1<S2=S3)

(McKinney and Speeg, 1982; Schali et al., 1983; Besseghir and Roch-Ramel, 1987). Reasons for the differences in secretory rate have not been determined.

ENDOGENOUS COMPOUNDS	EXOGENOUS COMPOUNDS (XENOBIOTICS)
Monoamine neurotransmitters	**Anticholinergic agonists**
Acetylcholine	Atropine
Dopamine	
Epinephrine	**Antiviral agents**
Histamine	Amantadine
5-hydroxytryptamine	
	Antibiotics
Metabolites	Trimethoprim
Choline	
Creatinine	**Antiarrhythmic agents**
NMN	Procainamide
	Quinidine
Vitamins	Verapamil
Riboflavin	
Thiamine	**β-adrenergic receptor blockers**
	Pindolol
	Nadolol
	Diuretics
	Amiloride
	Triamterene
	Ganglionic blocking agents
	TEA
	Opioids
	Morphine
	Meperidine
	Dihydromorphine
	Sympathiomimetic agents
	Ephedrine
	Isoproterenol
	Miscellaneous
	Cisplatin
	Mepiperphenidol
	Quinine

Table I-3: Endogenous and exogenous compounds that are organic cations. Information for this table was obtained from the following references, (Uwai et al., 1998; Apiwattanakul et al., 1999)

III.3.a. Amantadine model

Presently, the predominance of information regarding renal tubule organic cation transport system comes from studies which use tetraethylammonium (TEA) as the prototypical organic cation substrate (Inui et al., 2000; Berkhin and Humphreys, 2001).

However, the TEA model alone may not be sufficient to account for the renal tubule secretion pathways of all organic cations. One such exception, amantadine, is a clinically relevant organic cation drug that is central to this dissertation, and has been used by our laboratory to characterize a transport system unique from that of TEA. Amantadine is a pharmacological agent. It is used as prophylaxis against viral influenza A and for symptomatic relief of Parkinson's disease (Schawab et al., 1969; Parkes, 1974; Aoki et al., 1979; Oxford and Galbraith, 1980). It is an achiral, weak base (pKa 10.1) that is almost completely ionized at physiological pH. It is largely non-metabolized in the body and its excretion occurs mainly via the kidneys (Aoki et al., 1979). Past literature has reported that amantadine is actively accumulated by renal human and rat tissue (Wong et al., 1990; Wong et al., 1991; Wong et al., 1992a; Wong et al., 1992b; Wong et al., 1993). Amantadine is actively transported by the renal tubules and transport is bicarbonate and metabolic activity dependent (Escobar et al., 1994; Escobar et al., 1995; Escobar and Sitar, 1995). Past literature has demonstrated first that amantadine is eliminated clinically by the kidneys through renal tubule secretion, and secondly *in vivo* where

amantadine transport and accumulation occurs in proximal and distal tubules of male and female rats, with transport properties being heterogeneous within tubules, and between tubules (Wong et al., 1990; Wong et al., 1991; Wong et al., 1992a; Wong et al., 1992b; Escobar et al., 1994; Escobar et al., 1995; Escobar and Sitar, 1995; Escobar and Sitar, 1996; Goralski, 1999; Goralski and Sitar, 1999).

III.3.b. Mechanisms of Organic Cation Transport

Investigations into the mechanisms of the organic cation transport system have employed isolated renal tubules (Schali et al., 1983; Smith et al., 1988; Groves et al., 1994), stop-flow microperfusion of proximal tubule (David et al., 1995), and plasma membrane vesicles isolated from renal proximal tubules (Hsyu and Giacomini, 1987; Montrose-Rafizadeh et al., 1989; Pritchard and Miller, 1993). These studies suggested that secretion of cationic substances in renal tubules is operated effectively by the cooperative function of three distinct organic cation systems: one facilitated by the transmembrane electrical potential difference in the basolateral membranes **(Fig I-3, #1)**, another intracellularly **(Fig I-3, #2)**, and the other driven by the transmembrane H^+ gradient in the brush border membranes **(Fig I-3, #3)**. In addition, Na^+/K^+-ATPase generates favorable gradients for these transport processes to occur **(Figure I-3, #4)**.

Studies using isolated rat hepatocytes have suggested the existence of two different transport systems for organic cations: a type 1 system mediating uptake of small hydrophilic organic cations such as TEA, cimetidine, procainamide and guanidine, and a type 2 system mediating uptake of hydrophobic organic cations such as quinidine, quinine, d-tubocurarine, and vecuronium (Mol et al., 1988; Steen et al., 1992). Most substrates of the type 2 system have potent inhibitory effects on the type 1 system. Although the uptake of type 2 substrates has not been directly demonstrated in renal basolateral membranes, such transport systems could exist, as renal secretion of type 2 cations such as quinine and quinidine has been observed (Notterman et al., 1986). Therefore, the multispecific organic cation transport systems in renal basolateral membranes may comprise more than a single transporter protein. Consistent with these functional studies, several organic cation transporters have been identified at the molecular level (discussed later in this dissertation).

Once inside the cell, reports indicate that organic cations may undergo intracellular accumulation or protein binding **(Figure I-3, #2)** (Pritchard and Miller, 1993; Pritchard and Miller, 1996). The nature and extent of these interactions is presently undetermined.

Functional properties of the organic cation transport system across the renal brush-border membranes have been extensively investigated using isolated brush-border membrane vesicles (Takano et al., 1984; Sokol et al., 1985; Wright and Wunz, 1987; Miyamoto et al., 1989) and cultured renal

epithelial cell lines (Takami et al., 1998). A large body of data from these studies revealed that the transport system in these membranes is mediated by an electroneutral H^+/organic cation antiporter energized by transmembrane H^+ gradient, which can be sustained by the Na^+/H^+ exchanger **(Figure I-3, #5)** and/or H^+-ATPase **(Figure I-3, #6)**. The existence of an additional H^+/organic cation antiporter, which is more specific for guanidine, was demonstrated in the renal brush-border membranes of the rabbit (Miyamoto et al., 1989). This H^+/guanidine antiporter was not inhibited by TEA or NMN. Trans-stimulation (cation/cation exchange activity) experiments and uptake studies showed that the H^+/organic cation antiporter mediates the translocation of a wide range of cationic compounds with diverse structures, such as endogenous cations including NMN and choline, and drugs including TEA, cimetidine, procainamide, neurotoxin 1-methyl-4-phenylpyridinium (MPP), and amino-beta-lactam antibiotics (Inui et al., 1985; Wright and Wunz, 1987). Therefore, the brush-border membrane H^+/organic cation antiporter appears to be a multispecific transporter for type 1 organic cations as well as the basolateral transporter. The brush-border H^+/organic cation antiporter shows substrate specificity that is similar, but not identical, to the basolateral membrane organic cation transporter (Wright and Wunz, 1987). Large and more hydrophobic compounds belonging to the type 2 cations are not translocated by the H^+/organic cation antiporter. Transport activity for such hydrophobic drugs and cations of the renal brush-border membranes appears to be relatively small, and MDR1/P-glycoprotein **(Figure I-3, #7)** and/or other transport mechanisms could be involved in the efflux of such compounds.

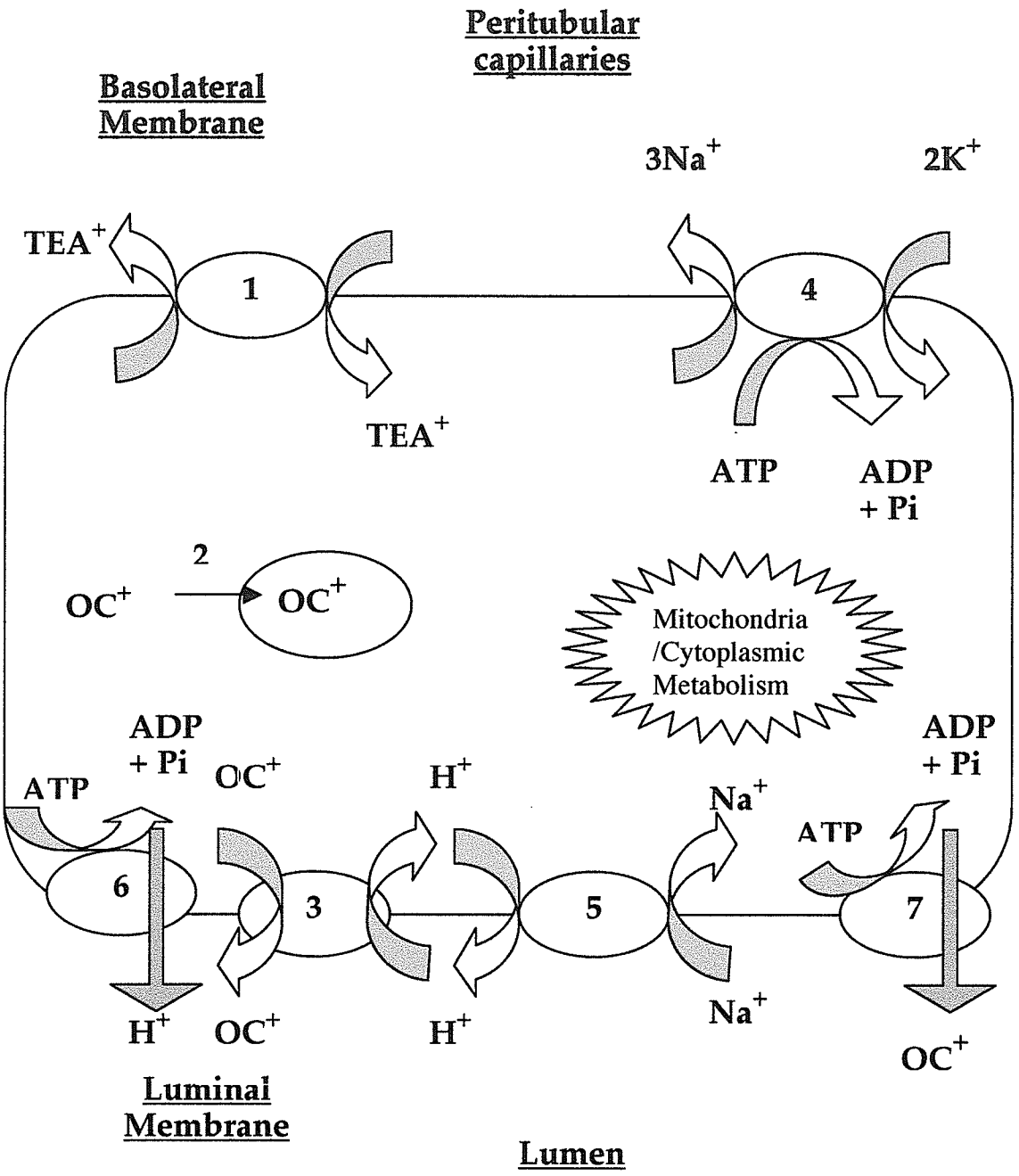

Figure I-3: Model of organic cation transport.

III.4. Molecular Biology of Organic Cation Transport

III.4.a. OCT family

In 1994, Gründemann et al identified the first member of the organic cation transporter family, designated as OCT1, from the rat kidney by expression cloning (Grundemann et al., 1994). Rat (r)OCT1 is comprised of 556 amino acids with 12 putative transmembrane domains. Northern blot analysis showed that rOCT1 mRNA was expressed in the liver, kidney, and intestine. In the kidney, rOCT1 mRNA was detected in proximal tubules, glomeruli, and in cortical collecting ducts, but not in distal tubules. By immunohistochemical analysis, rOCT1 was localized to the basolateral membranes of S1 and S2 segments of proximal renal tubules and the small intestine and liver. When expressed in oocytes, rOCT1 stimulated uptake of TEA, which was inhibited by diverse organic cations. Electrophysiological experiments using rOCT1-expressing oocytes under voltage-clamped conditions demonstrated that positive inward currents were induced when TEA, NMN, choline, dopamine, or MPP were added to the bath medium, indicating that rOCT1-mediated cation uptake is electrogenic (Busch et al., 1996). Thus OCT1 is a potential-driven uniporter.

Human hOCT1 is comprised of 554 amino acids and shows 78% homology with rOCT1. Its mRNA transcript was detected exclusively in the liver (Gorboulev et al., 1997). There are distinct species differences in tissue distribution and histochemical localization of OCT1. After expression in

oocytes, hOCT1 mediated the uptake of type 1 organic cations such as NMN, TEA, and MPP, suggesting that hOCT1 may participate primarily in hepatic excretion of organic cations in humans (Zhang et al., 1997). hOCT1-mediated MPP uptake was saturable and was sensitive to transmembrane potential. The type 2 hydrophobic cations such as vecuronium and decynium-22 as well as the type 1 hydrophilic cations such as TEA and NMN inhibited MPP uptake. hOCT1 has lower binding affinity for several cations such as decynium-22, tetrapentylammonium, quinine, and NMN than rOCT1, indicating species differences in substrate specificity (Zhang et al., 1997). The human genes of hOCT1 and hOCT2 (also named SLC22A1 and SLC22A2) have been localized in close proximity on chromosome 6q26 (Koehler et al., 1997).

Since OCT1 was cloned, other gene products with significant homology to it have been identified (**Table I-4**). Using hybridization techniques, Okuda et al. isolated a cDNA encoding OCT2 from rat kidney (Okuda et al., 1996). rOCT2 is comprised of 593 amino acids with 12 proposed putative transmembrane domains showing a 67% homology to rOCT1. On Northern hybridization and RT-PCR analysis, the rOCT2 mRNA transcript was detected predominantly in the kidney, at higher levels in the medulla than the cortex, but not in the liver, lung, or intestine. When rOCT2 was expressed in oocytes, uptake of TEA was suppressed by the replacement of Na^+ with K^+, suggesting that the uptake was membrane potential-dependent (Okuda et al., 1999). Acidification of the extracellular medium resulted in a decreased uptake of TEA, whereas the efflux of TEA

out of rOCT1- and rOCT2-expressing oocytes was not stimulated by the inward H+ gradient (Okuda et al., 1999). To compare the functional characteristics of rOCT1 and rOCT2, Urukami et al. established stable transfectants using Madin Darby Canine Kidney cells (Urakami et al., 1998). TEA uptake by both rOCT1 and rOCT2 transfectants grown on microporous membrane filters was markedly enhanced when TEA was added to the basolateral bath medium, but not to the apical medium. TEA uptake by both transfectants was decreased by acidifying the medium pH, suggesting that rOCT1- and rOCT2-mediated TEA transport was pH sensitive. Efflux of TEA out of the transfectants was unaffected or moderately inhibited by acidification of the medium. Structurally diverse organic cations, including the type 1 cations such as MPP, cimetidine, NMN, nicotine, and procainamide, and type 2 cations, such as quinine and quinidine, inhibited TEA uptake in the transfectants (Urakami et al., 1998). Inhibition experiments suggested that rOCT1 and rOCT2 had similar inhibitor binding affinities for many compounds, but showed moderate differences in inhibitor sensitivity for several compounds such as MPP, procainamide, dopamine, and testosterone by a factor of 2 to 3 (Urakami et al., 1998; Okuda et al., 1999). rOCT2 and hOCT2, which share 80% amino acid homology, have been shown to accept monoamine neurotransmitters such as dopamine, norepinephrine, epinephrine, 5-hydroxytryptamine, and amantadine as substrates (Busch et al., 1998; Kekuda et al., 1998). These findings raise the possibility that OCT2 plays a physiological role beyond redundent handling of bioactive monoamines (after more specific

monoamine transporters); thus OCT2 may be important in the modulation of neurotransmitter levels throughout the body.

A cDNA encoding an additional member of the OCT gene family, designated as OCT3, was isolated from rat placenta (Kekuda et al., 1998). rOCT3 is comprised of 551 amino acids with 12 putative transmembrane domains, and shows 48% homology to rOCT1. Northern blot analysis indicated that rOCT3 mRNA was detected most abundantly in the placenta and moderately in the intestine, heart, and brain. Expression of rOCT3 mRNA was comparatively low in the kidney and lung, and it was not detected in the liver. OCT3 mRNA expression has been detected in mouse proximal and distal tubules, but its assignment to the apical or basolateral membrane has not been determined (Wu et al., 2000). When expressed in HeLa cells and Xenopus oocytes, rOCT3 induced uptake of TEA and guanidine, which could be inhibited by MPP (Kekuda et al., 1998). Under voltage-clamped conditions, rOCT3-mediated TEA uptake evoked a potential-dependent inward current. The current induced by the TEA uptake was markedly influenced by extracellular pH. However, such pH dependence of TEA uptake by rOCT3-expressing oocytes could not be confirmed under voltage clamp conditions. Therefore, rOCT3 appears to be a potential-sensitive and pH gradient-independent organic cation transporter. Although the distribution and localization of rOCT3 in the kidney has not yet been determined, this transporter may also participate in the renal handling of a variety of organic cations.

TRANSPORTER	SPECIES	TISSUE DISTRIBUTION	MEMBRANE LOCALIZATION	MAIN SUBSTRATES
OCT1	Human Rabbit Rat Mouse	Liver, kidney>small intestine (rat)	Basolateral (rat)	TEA, NMN, choline, dopamine, MPP
OCT2	Human Pig Rat Mouse	Kidney>> brain (rat)		TEA, choline, dopamine, MPP, guanidine
OCT3	Human Rat Mouse	Placenta>> small intestine, heart, brain> kidney, lung (rat)		TEA, guanidine
OCTN1	Human Rat Mouse	Kidney, spleen, bone marrow, etc. ubiquitous (human)	Apical (human)	L-carnitine, quinidine, verapamil, TEA
OCTN2	Human Rat Mouse	Kidney, skeletal muscle, placenta , pancreas (human)	Apical (human)	L-carnitine, TEA

Table I-4: Some characteristics of cloned organic cation transporters. (Okuda et al., 1996; Gorboulev et al., 1997; Tamai et al., 1997; Zhang et al., 1997; Tamai et al., 1998; Wu et al., 1998; Zhang et al., 1998)

III.4.b. OCTN family

By their homology to OCT transporters, two additional members of the OCT gene family, named hOCTN1 (SLC22A4) (Tamai et al., 1997) and hOCTN2 (SLC22A5) (Tamai et al., 1998; Wu et al., 1998), have been identified (**Table I-4**). A cDNA encoding hOCTN1 was cloned from human fetal liver and encodes a 551 amino acid residue protein with 11 putative transmembrane domains and one nucleotide binding site motif (Tamai et al., 1997). hOCTN1 mRNA was found to be abundant in the kidney, trachea, bone marrow, fetal liver and several human cancer cell lines, but not in adult liver. OCTN1 was shown to be a H^+/organic cation exchanger. When expressed in HEK 293 cells, hOCTN1 mediated saturable and pH-dependent uptake of TEA with higher activity at neutral and alkaline than at acidic pH (Yabuuchi et al., 1999). In addition, the efflux of TEA out of the cells was pH-dependent, with an accelerated rate at acidic external medium pH. TEA uptake was not influenced by membrane potential, and hOCTN1-mediated TEA uptake was inhibited by other organic cations such as cimetidine, procainamide, quinidine, quinine, and verapamil. When expressed in oocytes, hOCTN1 stimulated uptake of quinidine, verapamil, and zwitterionic l-carnitine (Yabuuchi et al., 1999).

hOCTN2 was identified as a homologue of hOCTN1 from human kidney. hOCTN2 cDNA encodes a 557-amino acid residue protein with 76% homology to hOCTN1 (Tamai et al., 1998; Wu et al., 1998). hOCTN2 is

strongly expressed in the kidney, trachea, spleen, bone marrow, skeletal muscle, heart, and placenta in adult humans. When expressed in HEK 293 cells, hOCTN2 mediated the uptake of L-carnitine in a Na^+-dependent manner, whereas it mediated some minor uptake of TEA and guanidine (Tamai et al., 1998). The physiological function of hOCTN2 is suggested to be a high-affinity Na^+-carnitine cotransporter. Carnitine is a crucial co-factor for transporting fatty acids into the mitochondria and is crucial for fatty acid oxidation (Lehninger et al., 1993d).

Investigations into OCTN rat counterparts, indicate that rOCTN2 is a Na+-independent organic cation transporter as well as a Na+-dependent carnitine transporter, which is expressed in the heart, kidney, placenta, and brain of the rat (Wu et al., 1999). In rat kidney, rOCTN2 mRNA is predominantly expressed in the cortex, while there is very little expression in the medulla. In the cortical region, rOCTN2 mRNA was found in the proximal and distal tubules.

Several laboratories reported that primary systemic carnitine deficiency, which is an autosomal recessive disease characterized by low serum and intracellular concentrations of carnitine, is caused by mutations in the hOCTN2 gene (Nezu et al., 1999; Tang et al., 1999; Wang et al., 1999). Primary carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation caused by defective carnitine transport. This disease presents early in life with hypoketotic hypoglycemia or later in life with skeletal myopathy or cardiomyopathy. There have been two mutations reported

that result in amino acid substitution in OCTN2, P478L (hOCTN2) and L352R (mouse OCTN2) (Wu et al., 1999). These mutations in hOCTN2 cause complete loss of carnitine transport function. In contrast, only the M352R mutant appeared to be associated with complete loss of organic cation transport function, whereas the P478L mutant had higher organic cation transport activity than the wild-type transporter (Tang et al., 1999). These studies suggested that the binding sites for carnitine and organic cations in OCTN2 exhibit significant overlap but are not identical. Therefore, there may be clinical implications for pharmacotherapy in individual patients with primary carnitine deficiency if the mutations in OCTN2 also affect organic cation transport activity.

IV. (ATP) Binding Cassette Transporter Family and Di/Tri-peptide Transporters

Even though the focus of this dissertation is on organic ion transporters, several other types of transporters, namely the ATP binding cassette transporter family and the di/tri-peptide transporters, should be briefly discussed as they have been implicated in drug transport.

Specific transport proteins transfer organic compounds into tubular fluid. The best studied is the multidrug transporter P-glycoprotein. P-

glycoproteins are membrane-located proteins that possess ATPase activity. They belong to a superfamily of transport proteins that contain an adenosine 5'-triphosphate (ATP) binding cassette. Other family members include the multidrug resistance (MDR)-associated proteins Mrp1 and Mrp2 and the multispecific organic anion transporter (MOAT). They are often called MDR proteins because they hasten the removal of drugs from the cell interior and thus are associated with resistance of tumor cells to the action of these drugs (Gottesman and Pastan, 1988; Ambudkar et al., 1999). The physiological role of P-glycoproteins may be in the protection of the organism from xenobiotics (Gottesman and Pastan, 1988; Ambudkar et al., 1999). In proximal tubule epithelium, P-glycoprotein is normally expressed on the apical membrane of cells (Horio et al., 1989), where it can mediate transport into the tubular fluid. Numerous drugs are secreted into the tubular lumen through the P-glycoprotein pathway independent of, or in addition to, the organic anion and cation secretory pathways described. Mrp2 is also located in high concentration in the brush border of proximal tubular cells (Schaub et al., 1997) and shares many properties with P-glycoprotein. However, substrate specificities differ, with P-glycoprotein transporting primarily uncharged and cationic species, while Mrp2 transports conjugated anionic compounds (Keppler et al., 1998; Masereeuw et al., 2000). In addition, there is only approximately 25% amino acid homology between Mrp2 and P-glycoproteins (Keppler and Konig, 1997; Keppler et al., 1998).

Presently, two human di/tri-peptide transporters, hPepT1 and hPepT2 have been identified and functionally characterized. In the small intestine hPepT1 is exclusively expressed, whereas both PepT1 and PepT2 are expressed in the proximal tubule. The transport via di/tri-peptide transporters is proton-dependent, and the transporters thus belong to the Proton-dependent Oligopeptide Transporter (POT)-family. The transporters are not drug targets in the classical sense, but due to their uniquely broad substrate specificity; they have proved to be relevant to drug transport. Drug molecules such as oral active beta-lactam antibiotics, bestatin, prodrugs of acyclovir and gancyclovir have oral bioavailabilities, which largely are a result of their interaction with PepT1 (Nielsen and Brodin, 2003).

V. Regulation of Renal Tubular Transport of Organic Compounds

Understanding the regulation of these transport systems is now becoming the focus of current literature, and should prove to be medically significant for three reasons: First, suppression of tubular secretion may increase the exposure to potentially dangerous synthetic and natural xenobiotics. Second, stimulation of transport may be helpful for the prevention and treatment of occupational diseases. Third, pathophysiological conditions (e.g. diabetes) may affect OCT regulation. Elucidating the mechanisms controlling these transport processes will

represent a significant contribution to the existing medical body of knowledge, and will move the pharmaceutical field a step closer towards the individualization of drug therapy. Previous in vivo data regarding regulation is being reinterpreted in light of the molecular characterization and cloning of drug transporters. This advance has allowed for their examination in simplified systems, separating them from the involvement of other transporters, and other modulating regulatory factors present in *in vivo* preparations.

It should be noted that this dissertation focuses predominately on the molecular regulation of organic cation transport by specific transporters, namely rOCT1, rOCT2 and the amantadine transporter. Since organic cations have significant vascular effects, research progress in the field of organic cation renal transport has developed slowly. Previous studies examining renal tubular regulation have primarily focused on organic anion transport using PAH. Shared underlying components in both transport systems suggest the understanding of one may provide insight into the regulation of the other. It is for that reason that we will explore literature regarding the regulation of OATs in parallel to OCTs.

V.1. Neural, Endocrine and Metabolic Regulation of Organic Ion Transporters

Early studies utilizing renal denervation (to decrease renal nerve activity) or electro-physiological stimulation of renal nerves (to increase

nerve activity) indicated the possibility of regulating tubular secretion; however, subsequent pharmacologic studies are in some ways at odds with those studies. In the dog, it has been demonstrated that renal excretion of PAH is decreased after renal denervation (Szalay et al., 1977). Yet, denervation does not prevent the compensatory increase in tubular secretion following unilateral nephrectomy, indicating that the mechanism of this increase may not be neurally mediated but is perhaps due to humoral or hemodynamic factors (Vasilenko, 1963). Electrical stimulation of renal efferent nerves leads to an increase in the maximal transport of diodrast (an organic anion) (Lysov, 1968). Pharmacologic studies examining tubular transport vary. Results obtained using membrane vesicles from renal tubular cells suggest that epinephrine and norepinephrine enhanced PAH transport into basolateral membrane vesicles prepared from rat proximal tubules (Jensen and Berndt, 1988). Speculations that this effect was mediated by α_2 adrenoreceptors was reinforced by the use of clonidine, an α_2 adrenergic agonist, to produce an elevation of uptake into these vesicles (Jensen and Berndt, 1988). Confirmation of these findings is indicated in a study of dichlorophenoxyacetic acid transport in primary cultures of winter flounder proximal tubule cells. The α-adrenergic agonist oxymetazoline stimulated, and dopamine inhibited, secretion of this organic anion (Halpin and Renfro, 1996). In conflict with culture studies, animal studies suggest that there is no direct effect on tubular secretion of diodrast in dogs or rats after intravenous infusion of norepinephrine, isoproterenol (Brukhanov, 1977), or dopamine (Brukhanov, 1980). Diodrast transport changed only

when the glomerular filtration rate changed simultaneously. This lack of effect could be related to the complex hemodynamic changes that occur during intravenous infusion of these agents. Acetylcholine did not change the maximum secretion of PAH or diodrast after infusion into the renal artery in dogs, even though renal vasodilation likely occurred (Brukhanov, 1980). This finding suggests that renal nerve effects are also not solely due to hemodynamic changes, but include a neural component. Further studies may help clarify the nature of these interactions.

The physiological importance of endocrine glands, particularly anterior pituitary, thyroid, and gonads, in the regulation of tubular secretion has been well established by older studies. Hypophysectomy decreases the tubular transport of PAH in vivo and in vitro (Farah et al., 1956; Misanko et al., 1977). This effect may reflect deficiency of growth hormone because administration of growth hormone increased tubular transport in both hypophysectomized and intact animals (Farah et al., 1956). Tubular transport of organic substances is also reduced after thyroidectomy and is restored by thyroxine therapy (Farah et al., 1956). The effect of thyroid hormones on tubular secretion may be age-related. Hirsch and Hook found that administration of triiodothyronine (T3) to weanling rats for three or seven days caused an increase in PAH transport in renal cortical slices, while treatment of adult rats did not alter transport significantly (Hirsch and Hook, 1969). When added to renal slices in vitro, T3 inhibited PAH uptake. This report confirmed the earlier observation concerning thyroxine action in vitro (Nepumucheno and Little, 1964) and was considered to be the result

of competition with PAH, since the hormone is transported as an organic anion (Hirsch and Hook, 1969). Subsequent studies demonstrated that thyroid hormones increased tubular secretion in adult rats and rabbits without a change in glomerular filtration rate (GFR). Aurantin, which blocks the synthesis of mRNA and protein, prevented the stimulation of secretion by thyroid hormones (Berkhin and Galyuteva, 1974), suggesting that an effect on gene transcription likely occurs in addition to any competition for transport. Braunlich confirmed the increase in tubular secretion by thyroid hormones administered to rats of different ages (Braunlich, 1984; Braunlich, 1987); the effect was associated with an increase in protein synthesis in kidney tissue (Ortweiler et al., 1987). Recently, stimulation of PAH secretion by T3 has also been observed in frog kidneys (Bakhteeva, 1991). These studies are consistent with earlier organ ablation experiments demonstrating that thyroidectomy reduced tubular transport of organic substances (Farah et al., 1956).

Studies examining parathyroid hormone and its intracellular second messenger cAMP have observed a spectrum of effects. One study, using cAMP, its analogue dibutyryl c-AMP, and isoproteronol (an activator of adenyly cyclase) and theophylline (phosphodiesterase inhibitor) to increase intracellular cAMP, have resulted in depression of PAH uptake by rabbit kidney cortex slices (Podevin and Boumendil-Podevin, 1975). In contrast is a study indicating that parathyroid hormone and cAMP (10^{-4} mol/L) both increase PAH uptake by suspensions of rabbit renal cortical tubules, while a higher concentration of cAMP (10^{-3} mol/L) inhibits PAH uptake, probably

by a competitive mechanism (Kippen et al., 1976). At present these observations remain unresolved.

Steroid hormones have also been shown to influence organic ion transport. Single injections of hydrocortisone (cortisol) increase diodrast excretion in dogs and rabbits and cause an increase in uptake of this compound by rabbit renal cortical slices. However, prolonged administration of hydrocortisone has the opposite effect (Fomenko, 1969). Prednisolone and dexamethasone increase the excretion of PAH and its accumulation by renal slices in immature but not in adult rats, while triamcinolone is effective regardless of age (Braunlich et al., 1992). The administration of testosterone increases the accumulation of PAH by renal slices from female but not male rats (Huang and McIntosh, 1955). This stimulatory effect of testosterone on tubular secretion in female rats and gonadectomized male rats can be prevented by aurantin (Kleinman et al., 1966) and is therefore likely due to enhanced synthesis of RNA and proteins. Related to these observations are others demonstrating sex-related differences in tubular transport. Renal cortical slices from male rats accumulate PAH to a greater extent than those from female rats (Kleinman et al., 1966; Bowman and Hook, 1972). This observation is also true for the organic cation TEA (Bowman and Hook, 1972). Castration of male rats causes a reduction in tubular secretion, which is corrected by treatment with testosterone, while bilateral ovariectomy does not alter tubular secretion in female rats (Huang and McIntosh, 1955; Ferguson and Matthews, 1963; Kleinman et al., 1966). Testosterone treatment increases the number of

functional carriers for PAH in the kidney (Reyes et al., 1998). However, some xenobiotics are transported better by female animals; among these are perfluorooctanoic acid (Hanhijarvi et al., 1982), pentachloronitrobenzene (Smith and Francis, 1983), zenarestat (Tanaka et al., 1991), and nilvadipine (Terashita et al., 1995). Species differences also exist: The sex difference in the excretion of zenarestat is seen in mice and rats, but not in dogs or humans (Tanaka et al., 1991).

Insulin effects on organic ion transport have also been observed. Intravenous injection in dogs increased the Tm of diodrast while urine flow and excretion of sodium and potassium diminished without change in GFR (Nikitin, 1971). Similarly, infusion of insulin into a renal artery was accompanied by a unilateral increase in tubular secretion and decrease in urinary sodium and potassium excretion. In rabbits, insulin stimulated the accumulation of diodrast by renal cortical slices whether the hormone was injected into the animals or added to the incubation medium of the slices (Nikitin, 1971). The mechanism of this effect of insulin on organic ion transport has not been established.

The metabolic state of kidney tubules has recently become appreciated to modulate organic ion transport. In regards to organic anion transport, the production of intracellular αKG is particularly important especially for mammalian renal tubules, and can be affected by cellular bicarbonate/CO_2 levels. For example, in the case of rabbit tubules, the control level of PAH secretion is about five times as high when the tubules

are perfused and bathed in a bicarbonate/CO_2-buffered medium as when they are perfused and bathed with any other buffer system (e.g. HEPES, Tris, phosphate)(Dantzler et al., 1995). Similar results have also been shown with the organic cation amantadine. In fact, our laboratory has independently reported amantadine transport in the rat kidney model to be strongly dependent on the presence of bicarbonate in the buffer system (about five times higher compared to bicarbonate-free buffer)(Escobar et al., 1994; Escobar et al., 1995; Escobar and Sitar, 1995; Escobar and Sitar, 1996). The reason for organic ion transport modulations is unclear. Nonetheless, to the extent that the previously discussed hormones affect the metabolic status of cells, with respect to energy and CO_2 generation, their mechanism of action should also be re-evaluated in light of their effect on a cell's metabolic machinery.

In the last few years an increasing number of studies concerned with regulation of di/tri-peptide transporter capacity have appeared. Given their capacity to transport some drugs and their role in nutrient acquisition, a brief mention of their regulation will be made. Studies on receptor-mediated regulation has shown that both PepT1 and PepT2 is down-regulated by long-term exposure to epidermal growth factor (EGF) due to a decreased gene transcription. PepT1-mediated transport is up-regulated by certain substrates and in response to fasting and starvation at the level of increased gene transcription. PepT1-mediated transport is up-regulated by short-term exposure to receptor agonists such as EGF, insulin, leptin, and clonidine.

Overall, the regulation of di/tri-peptide transport may be contributed to by changes in apical proton-motive force, recruitment of di/tri-peptide transporters from vesicular storages, and changes in gene transcription/mRNA stability (Nielsen and Brodin, 2003).

In conclusion, hormonal regulation of tubular secretion of organic compounds takes place through at least two pathways, and putatively a third **(Fig I-4)**. One involves regulation through cytoplasmic action(s); this pathway may be the one used by hormones and transmitters interacting with receptors on the surface of the tubular epithelial cell. Another occurs through effects on nuclear transcription and may involve synthesis of new transporters and substrates important in tubular secretion; this pathway is likely the one by which steroid hormones and perhaps other regulators act. Lastly, the third pathway may be of a metabolically derived origin; its nature and how it interacts with the other two pathways is however unclear. Future research will likely expand knowledge of these regulatory pathways and no doubt uncover other pathways by which regulation of tubular secretion occurs.

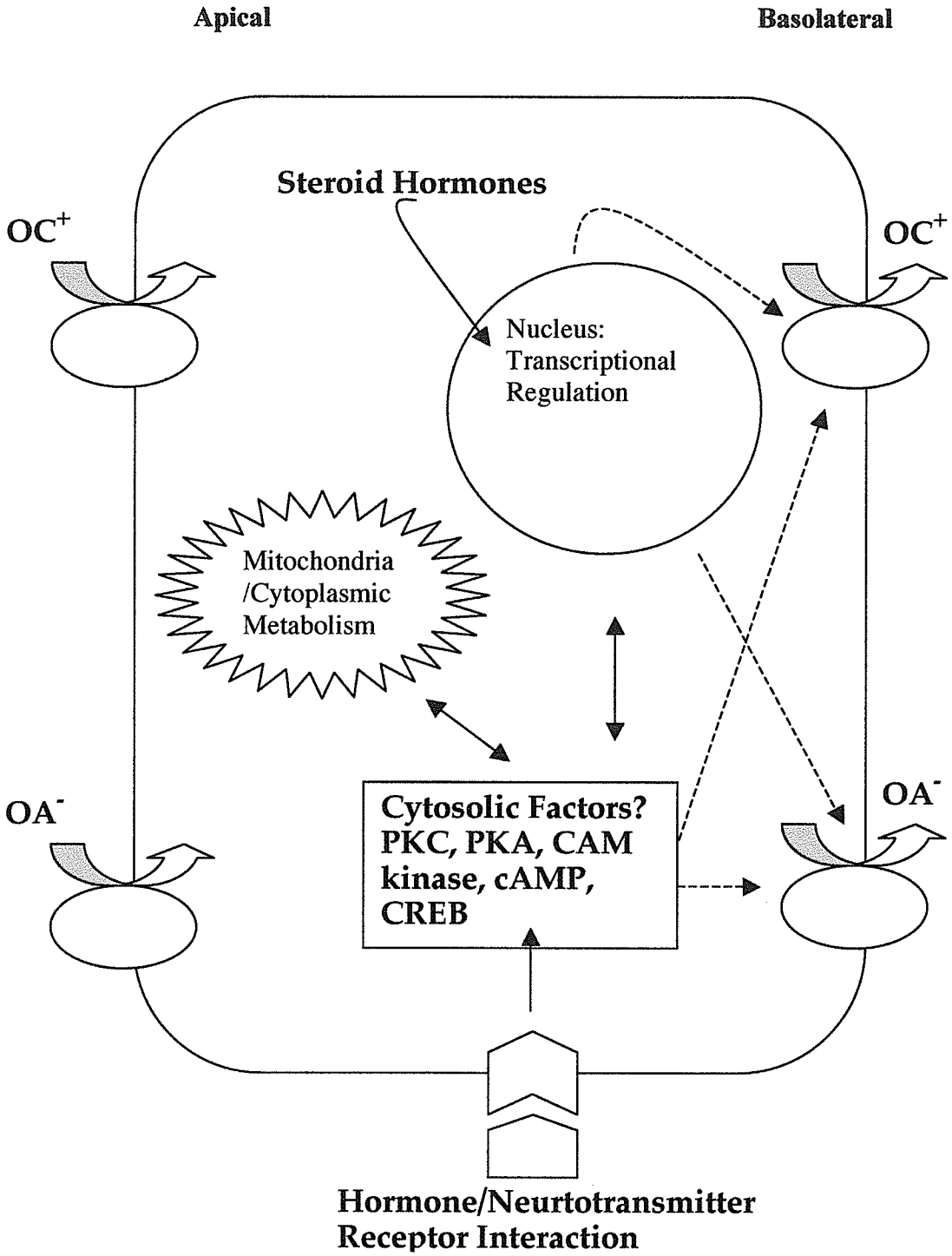

Figure I-4. Schematic diagram. Regulatory pathways affecting organic ion transport in renal tubular epithelial cells (Events are just as likely to affect the apical side).

V.2. Cellular Events Regulating Renal Organic Anion and Cation Transport

Little is known about the cellular events regulating organic anion and cation transport in kidney tubules. Yet, a common feature of the OCTs and OATs that has been cloned thus far, is the presence of several putative protein kinase phosphorylation sites (based on computer sequence analysis) for PKC, PKA and casein kinase II, in the intracellular loops, suggesting that their activity can be subjected to regulation (Saito et al., 1996; Sekine et al., 1997; Sweet et al., 1997; Zhang et al., 1997; Terashita et al., 1998; Zhang et al., 1998; Hosoyamada et al., 1999; Koepsell et al., 1999). Experimental studies indicate that activation of PKC with phorbol esters, or PKA by cAMP or forskolin inhibits uptake of compounds like PAH and TEA (Takano et al., 1996; Nagai et al., 1997; Miller et al., 1998; Terashita et al., 1998; Uwai et al., 1998; Lu et al., 1999). Transepithelial transport of the organic anion fluorescein by perfused rabbit tubules also was demonstrated to be inhibited by phorbol ester (a PKC activator); this inhibition was blocked by the PKC inhibitors staurosporine and bisindolylmaleimide (Shuprisha et al., 2000). Some studies link this effect of PKC activation to inhibition of entry into the cell across the basolateral membrane (Takano et al., 1996; Nagai et al., 1997; Miller et al., 1998; Terashita et al., 1998; Gekle et al., 1999), whereas another study has suggested that it could be mediated by PKC inhibition of Na^+,K^+-ATPase in the tubular cell, since transport was also inhibited by dopamine and stimulated by oxymetazoline (Halpin and Renfro, 1996). In contrast to this view of PKC activation inhibiting cellular uptake of organic ions are studies indicating that

phorbol esters actually stimulate uptake of TEA and PAH across the basolateral membrane of rabbit proximal tubular cells (Hohage et al., 1994a; Hohage et al., 1994b). Similar investigations into regulation of organic cation transport using isolated human proximal tubules obtained from human tumor nephrectomies showed that organic cation transport of ASP^+ is down-regulated when agonists stimulating PKA, PKC, or PKG are present (Pietig et al., 2001).

Once the first transporters were cloned, studies were performed to gain information on their individual properties by expressing them in Xenopus laevis oocytes or cell lines. At the molecular biological level, Mehrens et al. (2000) have shown that activation of PKC with subsequent phosphorylation of a serine residue of rOCT1 resulted in a stimulation of organic cation transport with an increase in substrate affinity, and that rOCT1 mediated organic cation transport was also activated by PKA and endogenously stimulated by tyrosine kinases (Mehrens et al., 2000). Regulation of hOCT3 by phosphoryation/dephosphorylation mechanisms was also shown (Martel et al., 2001b). hOCT3 was inactivated by phosphorylation especially by inhibition of MAP kinases, Ca^{2+}/calmodulin, phosphodiesterases or protein phosphatases. Recently, hOCT2 has been shown to be inhibited by phosphatidylinositol 3-kinase (PI3K) and PKA, and activated by CaM dependent signaling pathway(Cetinkaya et al., 2003).

Properties found for cloned transporters do not always match with the properties determined for the proximal tubule *in vivo* obtained by

micropuncture studies (Ullrich et al., 1991; Boom et al., 1992; Busch et al., 1996; Breidert et al., 1998). These differences may be due to the fact that additional transporters are involved in the *in vivo* situation or that transporter properties are further modified by physiological conditions *in vivo* under the influence of protein kinases. Care should be made in transferring results of organic ion transport in animal tubules and different expression systems to that of human proximal tubules. This point is especially important when considering the involvement of protein kinase regulation, and the history of species differences.

At present, there is no obvious way to reconcile these divergent observations. It may be that the effects of protein kinase activation on tubular secretion reflect different actions of the enzymes on Na^+,K^+-ATPase or on the transporters depending on oxygen availability and presumably the metabolic state of the tissue (Halpin and Renfro, 1996). The bulk of evidence indicates that PKC and PKA activation inhibits the transepithelial transport of organic compounds via an effect on basolateral transporters to reduce cellular uptake. As PKC is activated by numerous neural and humoral pathways, it seems likely that it represents a major intracellular regulatory mechanism for organic ion transport. PKA activation and its regulatory effects on organic ion transport, however requires further clarification. Future study of the regulation of transporter function will no doubt reveal new mechanisms that could be important in understanding the handling of xenobiotics by the body.

VI. Pathophysiological Regulation of Organic Cation Transport

The aim of the present dissertation is to discuss physiological, patho-physiological and drug-induced regulation of organic cation transport. An important component of that regulation, which hasn't been explored, is that of metabolism. Diabetes is a pathophysiological condition that is presently acknowledged to be a metabolic syndrome (Brownlee, 2001). For the purpose of this dissertation, it has served as a window into the mechanism behind the regulation of the function of OCTs, and will be discussed briefly in the following section.

VI.1. Diabetes

Diabetes mellitus, long considered a disease of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century (Zimmet, 1999). The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide (Amos et al., 1997; King et al., 1998). Pronounced changes in human environment, behaviour and lifestyle have accompanied globalization, and these have resulted in escalating rates of obesity and diabetes. There are two main forms of diabetes: Type 1 diabetes, which is due primarily to autoimmune-mediated destruction of pancreatic β-cell islets, resulting in absolute insulin deficiency; people with type 1 must take

exogenous insulin for survival to prevent the development of ketoacidosis; and Type 2 diabetes, which is characterized by insulin resistance and/or abnormal insulin secretion, either of which may predominate; people with type 2 diabetes are not dependent on exogenous insulin, but may require it for control of blood glucose levels if this is not achieved with diet alone or with oral hypoglycaemic agents (Reaven, 1988).

One of the major complications of diabetes is the microvascular degeneration of the kidney known as nephropathy. This condition is a major cause of morbidity and mortality in patients with diabetes mellitus. The nephropathy in both the insulin-dependent (Type 1) diabetes and the non-insulin-dependent (Type 2) forms of the disease is defined by persistent albuminuria, declining glomerular filtration rate and hypertension. Structural changes in the proximal tubule include basement membrane thickening and glycogen accumulation. Functional correlates, however, are generally lacking. The proximal tubule is distinguished by the remarkable variety and complexity of carrier-mediated transport processes that control the transepithelial distribution of inorganic ions and organic substrates between the luminal, intracellular and extracellular fluid compartments (Pritchard and Miller, 1996). Remarkably, little attention has been paid to the effect of diabetes on these essential processes.

Streptozotocin (STZ), an agent used to produce experimental diabetes in rodents, either by a single administration or by multiple low-dose administration, is taken to be a reliable model of induced autoimmune

diabetes in rodents (Type 1) (Herold et al., 1995). Indirect evidence that proximal tubular transport defects may indeed occur in diabetes is suggested by data from investigation of the so-called "protection" phenomenon seen in STZ-diabetic rats injected with nephrotoxic agents. This phenomenon refers to the remarkable resistance of these animals to the actions of a variety of tubular cell nephrotoxins, the best studied examples being gentamicin (Teixeira et al., 1982) , cephaloridine (Valentovic et al., 1989) and cisplatin (Scott et al., 1990). Subsequent experiments documented that injected gentamicin(Ramsammy et al., 1987), cephaloridine (Valentovic et al., 1989) and cisplatin (Cacini and Myre, 1985) had accumulated to a significantly lesser extent in the renal cortex of diabetic versus non-diabetic rats. The effect could not be explained by the diuretic action of glycosuria (Scott et al., 1990) or differences in bio-availability in diabetic versus non-diabetic rats (Sarangarajan and Cacini, 1996).

Alterations in membrane drug transport systems have been suggested as a possible explanation for the alterations in drug clearance seen in diabetes mellitus (Watkins and Dykstra, 1987). One study has presented evidence that *in vitro* cisplatin accumulation by renal cortex slices from non-diabetic rats involved both passive and active processes and that the active component was absent in slices from STZ-diabetic rats (Sarangarajan and Cacini, 1996). Recent investigations into the effect of early-stage diabetes mellitus on renal tubule transport of two cationic marker substrates amantadine (Goralski et al., 2001) and TEA (Grover et al., 2002) have specific impact on this dissertation. Both studies demonstrated altered transport in proximal tubules

of streptozotocin-induced diabetic rats compared to control. Vmax for amantadine transport was increased, while demonstrating no change in Km. On the other hand TEA accumulation in rat kidney cortical slices demonstrated a progressive decline in accumulation with increasing duration of diabetes. The accumulation of amantadine and TEA in both studies was energy-dependent and quinine-sensitive, indicating that the uptake was primarily mediated by the organic cation transport system. In both cases the observed transport perturbation was prevented by insulin treatment, indicating that the diabetic state itself is responsible. Presently, the exact mechanism by which diabetes mediates this impairment is unknown; its elucidation will represent a significant contribution to understanding OCT regulation and may influence decisions regarding therapeutic interventions.

STUDY OBJECTIVES AND BACKGROUND

Central Hypothesis

Organic cation transporters are differentially regulated depending on the specific transporters expressed, and by the presence of concurrent disease conditions.

Diabetes mellitus is a disorder of glucose homeostasis that is characterized by the elevation of both basal and postprandial blood glucose concentrations. All forms of diabetes are characterized by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology (ie end-stage renal disease) (Reaven, 1988). This dissertation has previously indicated that diabetes has been shown to modulate these transporters (Goralski et al., 2001; Grover et al., 2002). Large prospective clinical studies show a strong relationship between glycaemia, insulin and diabetic complications (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998). We speculated that understanding the disease in terms of these two key factors may help determine the mechanism for diabetes' effect on drug transport systems.

Therefore, for our first goal, we set out to examine the potential for insulin, glucose and bicarbonate to differentially regulate the function of OCTs in our HEK 293 cell model. Our findings suggested a basis for differential regulation of organic cation transport and indicated further studies into the underlying mechanism.

Our second goal is to demonstrate that insulin and glucose differentially modulates OCTs via a cAMP mechanism. The secondary messenger cyclic AMP (cAMP) is an evolutionarily conserved regulator of metabolism.

Our final goal is to demonstrate that these intracellular pools of cAMP may be further modulated by a bicarbonate-dependent soluble adenylyl cyclase (sAC) that may act within the cell as a putative metabolic sensor.

The sequence of the investigational studies presented herein is as follows:

I. Insulin and Organic Cation Transport

Insulin plays a central role in regulating mammalian metabolism. Insulin is a small protein that contains 51 amino acids arranged in two chains (A and B) linked by disulfide bridges. It is released from pancreatic B cells at a low basal rate and at a much higher stimulated rate in response to a variety of stimuli, e.g. vagal sitmulation, amino acids, and especially glucose. Insulin has important actions primarily on the liver, and secondarily on the kidney. These two organs serve as the two major sources of blood glucose supplying the brain during the fasting state (Vander, 1995). The two major sites of metabolism, the kidney and the liver (Madias, 1986), are also the two main organs that remove insulin from circulation (Vander, 1995). Insulin stimulates the enzymes involved in glycogen synthesis while inhibiting glycogenolytic and gluconeogenetic enzymes (Saltiel and Kahn, 2001). Several points led our group to examine insulin regulation of organic cation transport. First, the liver and kidney's cooperative ability to detoxify the body, and the presence of OCTs in both organs lends to the speculation of shared regulatory mechanisms, and specifically an undefined relationship relating to drug transport between insulin and the kidney. Second, insulin receptors are found in renal tubules, and insulin effects on organic ion transport have actually been observed. Intravenous insulin injection in dogs increased the Tm of diodrast (an organic anion) while urine flow and excretion of sodium and potassium diminished, without change in GFR (Nikitin, 1971). The

mechanism of this effect of insulin on organic ion transport has not been established. Lastly, transport perturbations of TEA in kidney cortex slices and amantadine uptake in renal rat tubule cell suspensions in STZ-induced diabetic rats were returned to normal by the addition of exogenous insulin (Goralski et al., 2001; Grover et al., 2002).

Based on these observations, the present studies were aimed at investigating more closely the role of insulin in regulating organic cation uptake. Our hypothesis was that insulin differentially regulates organic cation transport depending on the specific transporter expressed.

II. Glucose and Organic Cation Transport

Insulin is sometimes referred to as a storage hormone because it promotes anabolism while inhibiting the catabolism of carbohydrates, fatty acids and proteins. Regulation of this hormone is intimately connected to glucose levels and the energy demands of the body. Insulin increases glucose uptake in insulin-responsive tissues such as the kidney. Increases in intracellular glucose levels result in consequent alterations in cell-signalling and metabolism (Brownlee, 2001). Large prospective clinical studies demonstrate a strong relationship between glycaemia and the development of diabetic microvascular complications in both Type 1 and Type 2 diabetes (The Diabetes Control and Complications Trial Research Group, 1993; UK

Prospective Diabetes Study (UKPDS) Group, 1998). Early diabetic nephropathy is characterized by rapid growth of glomerular and tubular structures (Shankland and Scholey, 1995). It is now recognized that high glucose is the principal pathogenic factor triggering excessive growth, particularly hypertrophy, and accumulation of extracellular matrix (Glogowski et al., 1999). Prolonged exposure to elevated glucose or to recurrent changes in plasma glucose levels in the diabetic state may lead to altered acute responses and gene expression (Glogowski et al., 1999). The notion that normal physiological stimuli lead to abnormal responses in the diabetic milieu provides a potentially unifying mechanism linking factors that may contribute to altered OCT function. Our work not only focused on the early effects of high glucose, but also examined the effect of its absence during glucose starvation.

"Glycolysis was the first metabolic pathway to be elucidated. From the discovery by Eduard Buchner (in 1897) of fermentation in broken extracts of yeast cells until the clear recognition by Fritz Lipmann and Herman Kalckar (in 1941) of the metabolic role of high-energy compounds such as ATP in metabolism, the reactions of glycolysis were central to biochemical research" (Cited from (Lehninger et al., 1993b)). In fact, we are still elucidating the molecular mechanisms for the handling of cellular energy. Recently, there has been renewed interest in understanding glucose (nutritional) sensing and metabolism in eukaryotes (Johnston, 1999; Thevelein and de Winde, 1999; Dumortier et al., 2000; Rolland et al., 2000). Commercial interests by pharmaceutical companies looking at better tasting medications

and food sciences examining methods at taste modification (interestingly both utilize cAMP modulation in their process) (Ming et al., 1999; David, 2002), increased academic interest in diabetes research, and a freshly coined scientific division of "metabolomics", are all signs of renewal in a field that fundamentally looks at energy metabolism. A central convergence point among all of them is the cell's handling of glucose. Most catabolic processes converge on the citric acid cycle and on the electron transport chain, the end products of which are CO_2 and ATP. Our studies are generally aimed at examining a simple biochemistry tenet: a transporter's function is to transport nutrients in and waste out (Lehninger et al., 1993a). If these transporters mediate the transport of endogenous as well as exogenous metabolites, then in a state of nutrient or energy deficiency, metabolic regulation should shift transporters in the direction of nutrient acquisition. We were therefore curious of the effect of glucose manipulation on the function of OCTs in nutrient deficiency. Previous studies have generally concluded that OCTs and the amantadine transporter are energy-dependent (Steen et al., 1991; Ito et al., 1993; Escobar and Sitar, 1996; Yokogawa et al., 1999; Martel et al., 2000; Martel et al., 2001a). However there has been no direct examination, to the best of our knowledge, of the role of glucose directly. ATP generation by glycolysis among other catabolic processes and their respective interaction with electrochemical gradients are likely presumed to be the obvious source of a transporter's energy dependence. Nevertheless we were interested in clarifying organic cation transporters' specific response to glucose.

Based on observations that amantadine transport was increased while TEA transport decreased in STZ-induced diabetic rats, we speculated that the physiological stimuli of high glucose was differentially mediating these transport perturbations. Our hypothesis predicts that high extracellular glucose levels or the absence of glucose should differentially regulate TEA uptake by both rOCT1 and rOCT2-cells and amantadine uptake in native HEK 293 cells.

III. cAMP and Organic Cation Transport

Cells respond to their environment by taking cues from hormones or other chemical signals in the surrounding medium. The interaction of these extracellular chemical signals (first messengers) with receptors on the cell surface often leads to the production of second messengers inside the cell, which in turn lead to adaptive intracellular changes. Often, the second messenger is a nucleotide. One of the most common second messengers is the nucleotide adenosine 3',5'-cyclic monophosphate (cAMP), formed from ATP in a reaction catalyzed by adenylyl cyclase, an enzyme associated with the inner face of the plasma membrane.

cAMP is an evolutionary conserved regulator of metabolism. There is an intimate link between the regulation of cAMP levels in the cell and the levels of metabolism. Hyperglycemia results in increased intracellular ATP levels, due to increased glycolysis and oxidative phosphorylation. ATP is

converted by adenylyl cyclase (AC) to cAMP. Eukaryotic cells can use cAMP signalling for nutritional sensing. There are three mechanisms by which the yeast *Saccharomyces cerevisiae* responds to the presence of fermentable sugars in its medium, and all are thought to signal through cAMP (Johnston, 1999; Thevelein and de Winde, 1999; Dumortier et al., 2000; Rolland et al., 2000). cAMP signalling is mediated by multiple effector proteins, including exchange protein activated by cAMP (EPAC), cyclic nucleotide gated channels (cNGC) and protein kinase A (PKA). In sugar metabolism, cAMP, via PKA, controls the flux of metabolites through glycolysis, gluconeogenesis and the synthesis and breakdown of glycogen (Pilkis and Claus, 1991; Pilkis and Granner, 1992). A growing family of proteins called A-kinase anchoring proteins (AKAPs) tether PKA holoenzyme to various subcellular locations, including mitochondria, nuclear and plasma membranes, cytosol, and vesicles (Colledge and Scott, 1999). There are two isoforms of PKA, type I (PKA I) and type II (PKA II). Each contains the same overall domain organization, but differs in cAMP responsiveness and particular subcellular localization. The PKA I isoform is predominantly diffuse in the cytoplasm and is more sensitive to cAMP signaling (requiring lower levels of cAMP for kinase activation), whereas the PKA II isoform is more localized in cells and is less responsive to cAMP signaling (Doskeland et al., 1993; Skalhegg and Tasken, 2000; Feliciello et al., 2001). cAMP exerts its effects in animal cells mainly by activating the enzyme cyclic-AMP-dependent PKAs, which catalyze the transfer of the terminal phosphate group from ATP to specific serines, threonines, tyrosines and recently discovered histidines of selected proteins. The amino acids phosphorylated by PKA are marked by the

presence of two or more basic amino acids on their amino-terminal side. Covalent phosphorylation of the appropriate amino acids in turn regulates the activity of the target protein. Regulation of organic cation transport by PKA has been predicted and demonstrated in rOCT1 and rOCT2 (Grundemann et al., 1994; Grundemann et al., 1998; Mehrens et al., 2000).

Based on findings from the previous sections that insulin, glucose and starvation differentially regulate organic cation transporters, we decided to examine a putative candidate for its underlying mechanism of action as well as a very basic regulatory unit of metabolism – cAMP. Evidence from past studies implicated PKA modulation of organic cation transport. We speculated that the regulator of PKA, cAMP, could also regulate transport. In the present studies, we investigated the effect of altering intracellular cAMP alone and in conjunction with insulin on organic cation transport. Our hypothesis was that both insulin and cAMP should inhibit TEA uptake, and stimulate amantadine uptake. If the action of insulin on organic cation transport is mediated by cAMP, then in its presence the effects should be additive and inhibition/stimulation should be increased.

IV. Determination of tmAC and sAC Contributions to Intracellular cAMP Pools Affecting Organic Cation Transport

Investigation into the effect of cAMP and bicarbonate on OCT transporter function led us to examine the role of adenylyl cyclases, the cellular source of cAMP. Classical transmembrane adenylyl cyclases (tmACs) are membrane-bound, G-protein and forskolin sensitive. Recently, an additional source of cAMP in mammals was identified – the soluble adenylyl cyclase (sAC). sAC is molecularly and biochemically distinct from tmACs: sAC is insensitive to G-protein and to forskolin, and is soluble and particulate (indicating interaction at membrane and cytosolic sites) (Chen et al., 2000). RT-PCR analysis (Sinclair et al., 2000) and Western blotting using sAC-specific antisera (Chen et al., 2000) indicate sAC is expressed in virtually all tissues. Most importantly, it has been demonstrated that sAC is uniquely regulated by the bicarbonate ion (HCO_3^-). Regulation occurs via HCO_3^- itself, and not via carbon dioxide (CO_2) or a modulation of pH (Chen et al., 2000). Bicarbonate activated adenylyl cyclase activity had also been reported in fluid transporting tissues, including cortex and medulla of the kidney, ocular ciliary processes, corneal endothelium, and choroid plexus (Mittag et al., 1993).

Bicarbonate is the major buffering molecule by which plasma pH is controlled (Rennick, 1981). The central site of bicarbonate regulation is the proximal tubule of the kidney which is also the part of the nephron where excretion of organic cations occurs (Rennick and Farah, 1956). In previous reports, we have showed that bicarbonate is a modulating factor in the regulation of organic cation transport (Escobar et al., 1994; Goralski and Sitar, 1999). This bicarbonate effect is dependent on marker substrate (amantadine or TEA), cell system (Xenopus oocytes, HEK 293 cells, or rat renal proximal tubules), and transporter (rOCT1, rOCT2, or the unidentified amantadine transporter) (Goralski et al., 2002). Findings from our laboratory support bicarbonate modulation of rOCT1 and rOCT2 function in HEK 293 cells and Xenopus oocytes. However in proximal tubules only amantadine uptake was stimulated by bicarbonate (Goralski et al., 2002). Those findings in conjunction with our present studies indicate that there is an unidentified major bicarbonate-dependent component of amantadine basolateral membrane uptake in the renal proximal tubule.

Based on findings from the previous section that cAMP and the presence of bicarbonate together in the buffer can differentially modulate organic cation transport, we decided to pursue this unidentified bicarbonate-dependent component modulating organic cation transport. Speculations arose that sAC could be that component. Therefore, we decided to differentiate contribution of cAMP from tmACs and sAC to TEA and amantadine uptake using forskolin, and the presence/absence of bicarbonate in the buffer. Since our previous findings indicate that cAMP stimulates

amantadine uptake, while inhibiting TEA uptake in rOCT1 and rOCT2, we hypothesize that in the presence of bicarbonate, forskolin should strengthen the stimulation/inhibition of the uptake of organic cations through additive effects on intracellular cAMP pools.

MATERIALS AND METHODS

Cell Culture. Generation of the HEK 293 (human embryonic kidney cortex) cell line stably transfected with rOCT1 has been reported (Busch et al., 1996). HEK 293 cells expressing rOCT2 have been generated in the same way. Nontransfected HEK 293 cells (American Tissue Culture Collection, Manasas, VA) and HEK 293 cells stably transfected with rOCT1, rOCT2, (pRc-CMV; Invitrogen, Groningen, The Netherlands; cells provided by Dr. H. Koepsell, Germany) were grown to 90% confluency at 37°C in a 95% O_2/ 5%CO_2, humidified atmosphere in 175 ml culture flasks (Corning, New York, NY, USA) in low calcium, low glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 3.7 g/L $NaHCO_3$, 10% (v/v) foetal bovine serum (Gibco/Invitrogen, Grand Island, NY, USA), and geneticin (0.3 mg/ml) (Gibco/Invitrogen, Grand Island, NY, USA),. Monolayers were trypsinized with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and 0.25% Trypsin-EDTA (Gibco/Invitrogen, Grand Island, NY, USA). PBS contained: 2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 1.6 mM $NaHPO_4$-$7H_2O$ (pH 7.4). The flasks were then used to seed 75 ml culture flasks (Corning, New York, NY, USA) with 5 million cells in 10 ml medium (for long-term and short-term treatment experiments), and 175 ml flasks with 12.5 million cells in 25 ml medium (for time-course and glucose starvation experiments). The medium was changed daily. Cells for uptake studies were used on day 2-3 of growth when monolayers achieved 90% confluency.

Amantadine and tetraethylammonium (TEA) uptake assays. Uptake studies were performed with suspended cells in bicarbonate-free Cross Taggert (CT) buffer, or in bicarbonate-containing Krebs-Henseleit Saline (KHS) buffer. CT contained: 135 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 15 mM sodium phosphate buffer, 1.0 mM $CaCl_2$, 11mM glucose (pH 7.4). KHS contained: 125 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose (pH 7.4). For glucose starvation experiments, glucose (11mM) was exchanged for mannitol (6.7 mM) in the respective buffers, and equal osmolarity was confirmed using a microosmometer (μOSMETTE, Precision Systems Inc., Natick, Mass, USA). For all transport assays in KHS, the pH and pCO_2 levels of the buffer were adjusted by bubbling with O_2/CO_2 (95%/5%). For bicarbonate-free conditions (short incubations only), sealed flasks were used to maintain CT buffer under bicarbonate-free conditions. Viability of the cells was confirmed using Trypan blue staining. Before each assay, medium was removed from the cells; attached cells were washed with PBS and replaced with fresh buffer containing insulin (0.8-20 mU/ml), 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt (8-Br-cAMP) (100 μM) (Sigma, St. Louis, MO, USA), N^6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (dibutyryl cAMP) (Sigma, St. Louis, MO, USA) (100 μM), forskolin (Sigma, St. Louis, MO, USA) (1-10 μM) and staurosporine (Sigma, St. Louis, MO, USA) (6 nM) and allowed to incubate at 37°C in an incubator (Thermo Forma Series II - water jacketed) for 30 minutes for insulin and cAMP analogues, and 60 minutes for forskolin and staurosporine(Sigma, St. Louis, MO, USA).

Forskolin, and staurosporine were dissolved in dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO USA). Final DMSO concentration in CT or KHS buffer was 0.5% v/v. The control incubation contained 0.5% v/v DMSO in CT or KHS buffer solution. For long-term treatment of cells, appropriate reagents were prepared in fresh DMEM medium that was used to replace the original medium on subconfluent cultures 24 hours prior to the experiments.

Each flask was then rinsed with bicarbonate-free CT buffer or bicarbonate-containing KHS buffer (15 ml). Cells were detached by scraping in the presence of 15 ml of buffer followed by centrifugation (IEC-Model-Centra-4 centrifuge) at 1000 x g for 10 minutes. The pelleted cells were resuspended in 810 μL of buffer and placed in a water bath (25°C) with shaking (100 oscillations/minute) until ready for use. For glucose starvation and time course studies, the cells were resuspended in 3 ml of buffer. Cells, 90 μl, final protein content 4-6 mg/ml as measured by the Biuret assay (Gornall et al., 1949) were placed in microcentrifuge tubes in a water bath at 25°C with shaking (100 oscillations/minute). [^{14}C]TEA (10 μl, 20 μM final experiment concentration) or [^{3}H]amantadine (10 μM final experiment concentration) was added to the wall of the centrifuge tube. The transport reaction was started by vortex mixing and placed in a water bath (25°C) with shaking (100 oscillations/minute) for the appropriate time period. At the end of the time period, the reaction was stopped with 1 ml of ice-cold stopping buffer (10 μM quinine in KHS or CT). The tubes were then centrifuged for 1 minute at 13000 x g (Fisher, Model 235A) and the supernatant was discarded.

The cells were washed and centrifuged twice with 1 ml of the stopping buffer. Pellets were then dissolved in 200 μL of Triton X-100 (J.T. Baker Inc., Philipsburg, NJ, USA) (0.1% v/v), placed into scintillation vials containing 4 ml of Ready Safe scintillation fluid (Beckman Instruments Inc., Fullerton, CA) and counted in a Beckman model LS5801 scintillation counter.

Chemicals. [^{3}H] Amantadine (28 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). [^{14}C] TEA (55 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Unlabeled amantadine was obtained from DuPont Canada, Inc. (Mississauga, ON, Canada). All standard chemicals, forskolin, 8-Br-cAMP, dibutyryl-cAMP, staurosporine, quinine, and TEA were obtained in highest available purity from Sigma Chemical Co. (St. Louis, MO, USA). Human biosynthetic regular insulin (100 U/ml) was a gift from Novo Nordisk. All other chemicals were of the highest grade available from commercial suppliers.

Data Analysis. The ^{3}H and ^{14}C measurements from the liquid scintillation counter and the protein determinations for the amantadine and TEA uptake assays were used to calculate total uptake into the cells. Total uptake is reported as specific uptake (non-specific uptake subtracted) of amantadine or TEA by the HEK 293 cells in nmol/mg protein/10 minutes). In each experiment, triplicates were used for each treatment and the mean result was used. Data are shown for three to six independent experiments, as indicated by the "n" values. Specific uptake was calculated as the difference between

uptake of [^{3}H] amantadine and [^{14}C] TEA in the absence and presence of 10 μM quinine (the standard organic cation transporter inhibitor).

Data for ^{3}H amantadine and ^{14}C TEA uptake were analysed using a one-factor analysis of variance (ANOVA) followed by the Tukey post-hoc comparison among means (Prism 3, Graph Pad Software, San Diego, CA, USA). For glucose starvation experiments, the paired two-sided Student's t test was used to evaluate statistical significance of the effects. One-way ANOVA was used for all of the data to determine whether a buffer effect exists between the groups. The appropriate model analysis was used for all other comparisons. A probability level of 0.05 or less was used for statistical significance. Results were expressed as the mean $\pm$ S.E.M.

RESULTS

Time course of TEA uptake in rOCT1 or rOCT2-transfected, and amantadine uptake in native HEK 293 cells. Central to our studies was the discovery that native (untransfected), and empty vector transfected HEK 293 cells transported amantadine, but not TEA. OCT-transfected and native (non-transfected) HEK 293 cells provide us with a powerful model that allows us to examine the regulation of each transporter in a simplified system using TEA and amantadine as probes. Time courses were run examining total uptake of TEA in rOCT1- and rOCT2-transfected cells, and amantadine uptake in native HEK 293 cells in both bicarbonate-free (CT) buffer and bicarbonate-containing (KHS) buffer (Figure R-1, R-2 and R-3). A 10 minute incubation time for cationic marker substrates was used in all the experiments presented herein. While the initial slope represents cation uptake across the plasma membrane by the transporters, the maximal transport (reflected by the 10 minute incubation) is the sum of marker substrate uptake into the cells, exit of the cation from the cells, and intracellular compartmentalization. Since rOCT1 and 2 have been localized to the basolateral membrane and no native TEA transport mechanism exists in HEK 293 cells, we propose that the transport we observe is consistent with basolateral and intracellular events.

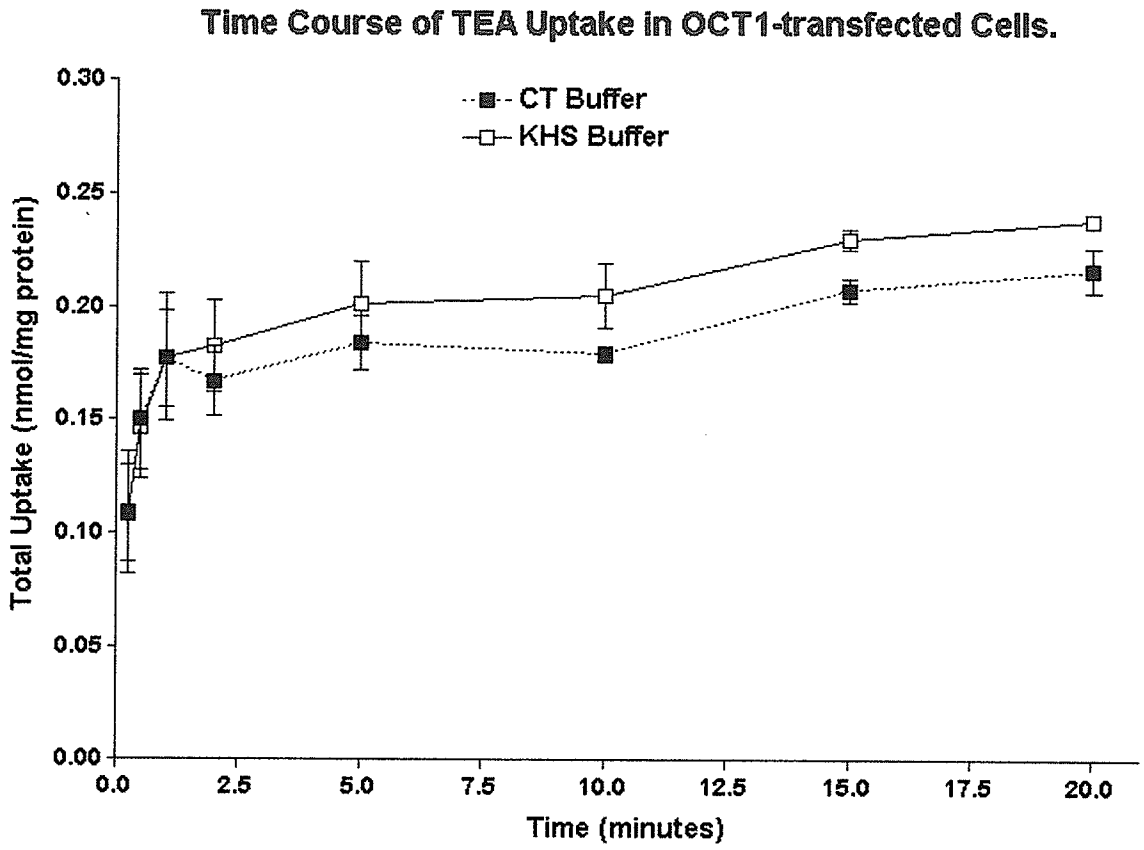

Figure R-1: Time course of TEA uptake in rOCT1 cells. Data represent the mean ± S.E. (n=3).

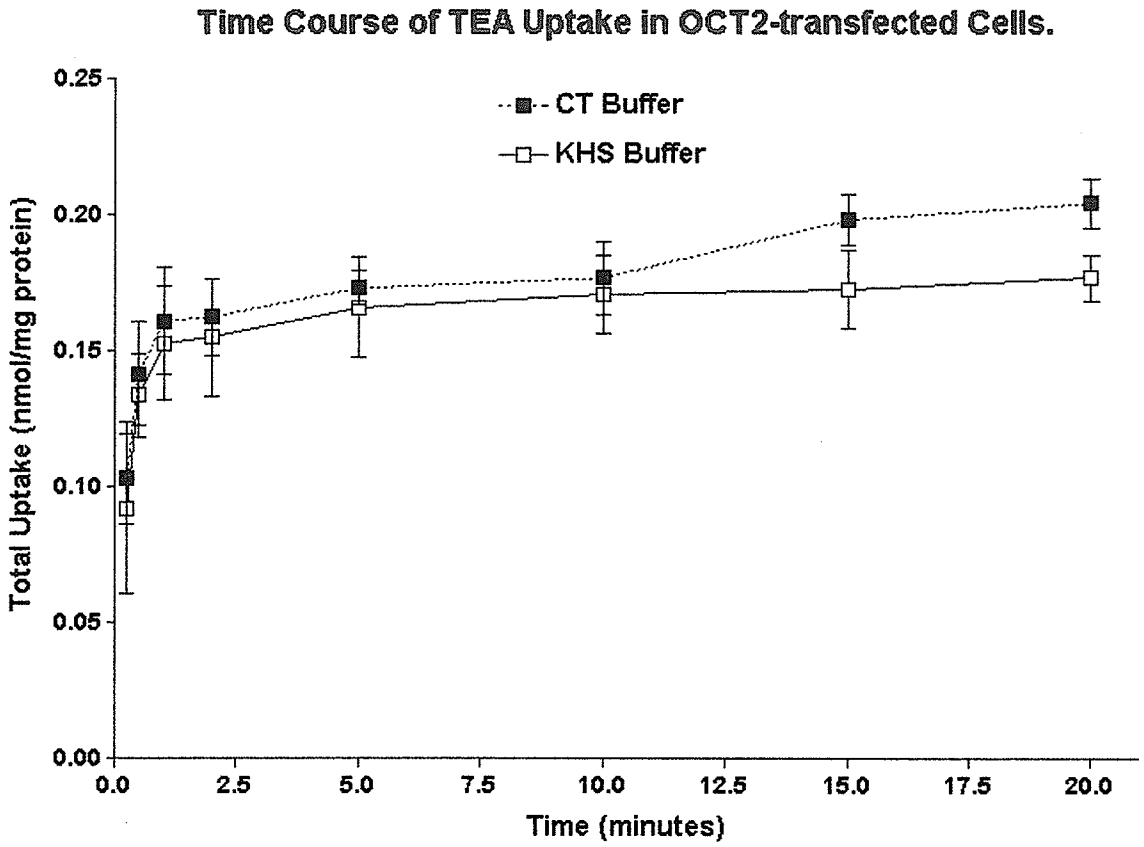

Figure R-2: Time course of TEA uptake in rOCT2 cells. Data represent the mean ± S.E. (n=3).

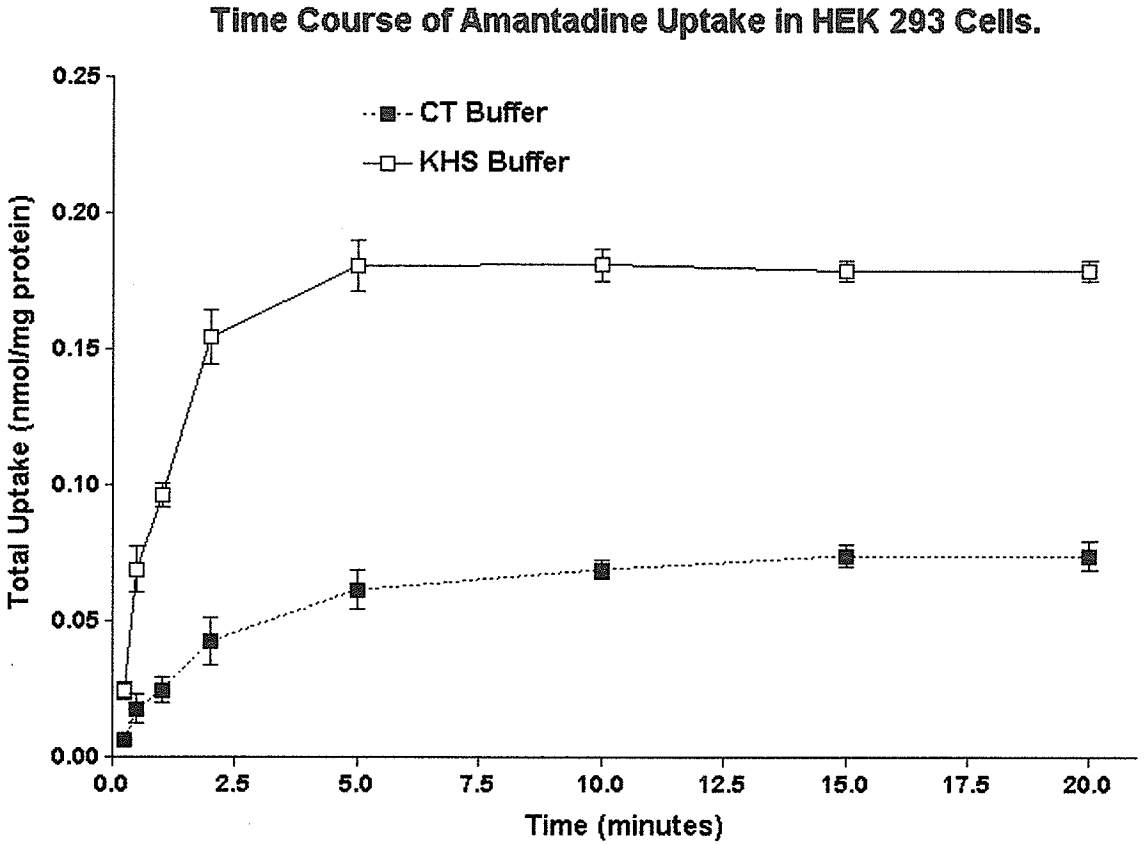

Figure R-3: Time course of amantadine uptake in HEK 293 cells. Data represent the mean ± S.E. (n=3).

24 Hour incubation with insulin results in differential regulatory trends of amantadine compared to the rOCT1 or rOCT2 transport system. We determined the concentration dependence of the exposure of insulin for 24 hours on organic cation transport of amantadine and TEA in native and transfected HEK 293 cells respectively. We have three treatment categories. (1) Cells incubated with insulin for 24 hours in low glucose (5.6 mM), and uptake was measured in the presence of bicarbonate (KHS buffer) (white bars), (2) cells incubated with insulin for 24 hours in low glucose (5.6 mM), and uptake measured in the absence of bicarbonate (CT buffer)(black bars), and (3) cells incubated with insulin for 24 hours in high glucose (25 mM), and transport measured in KHS (hatched bars) (Figure R-4, R-5 and R-6). No experiments were run for cells incubated in high glucose and whose transport was measured in the absence of bicarbonate (CT). Our experimental design's goal was to introduce glucose conditions as a second variable only for the purposes of first establishing a glycemic effect; later experiments would serve to clarify those interactions. In both rOCT1- (Figure R-4) and rOCT2- (Figure R-5) transfected cells, we observe that a 24 hour insulin pre-treatment results in decreased TEA transport compared to control among the rOCT1 treatment categories (29, 27, 31% respectively) and among those of rOCT2 (22, 27, 18% respectively). Insulin incubation decreased TEA transport significantly by rOCT1 (Figure R-4) at 10 mU/ml in all three treatment groups ($p<0.01$, one-factor ANOVA, Tukey's post-hoc comparison), and was unaffected by high glucose, or bicarbonate. In rOCT2-transfected cells (Figure R-5), long-term treatment (24 hours) with insulin appears qualitatively similar to rOCT1; TEA transport decreased (22, 27, 18% respectively) but was unable to achieve

significance. Increasing the statistical power of the experiment from a n(4) may have served to indicate otherwise. However, in native HEK 293 cells (Figure R-6) amantadine transport is regulated differently from cells transfected with rOCT1 and rOCT2. Treatment of native HEK 293 cells with insulin resulted in increased amantadine transport by 72% in a dose dependent manner regardless of glucose or buffer conditions ($p<0.01$, one-factor ANOVA). The proportional increase in amantadine transport by insulin was consistent among the different treatment groups, and independent to the effect of glucose and bicarbonate. Amantadine uptake was maximal in the presence of bicarbonate (Group 1), and was inhibited at least 50% in its absence (Group 2) ($p<0.01$, two-way ANOVA) in cells cultured in physiological levels of glucose (5.6 mM). Pre-treatment of native HEK 293 cells with hyperglycaemic levels of glucose (25 mM) (Group 3), alone and together with insulin, inhibited amantadine transport by 67% ($p<0.01$, two-way ANOVA) compared to Group 1, regardless of the presence of insulin. Since 20 mU/ml of insulin resulted in a marked effect for both the amantadine transporter (native HEK 293 cells) and rOCT1 containing cells, all further experiments were performed with this insulin concentration.

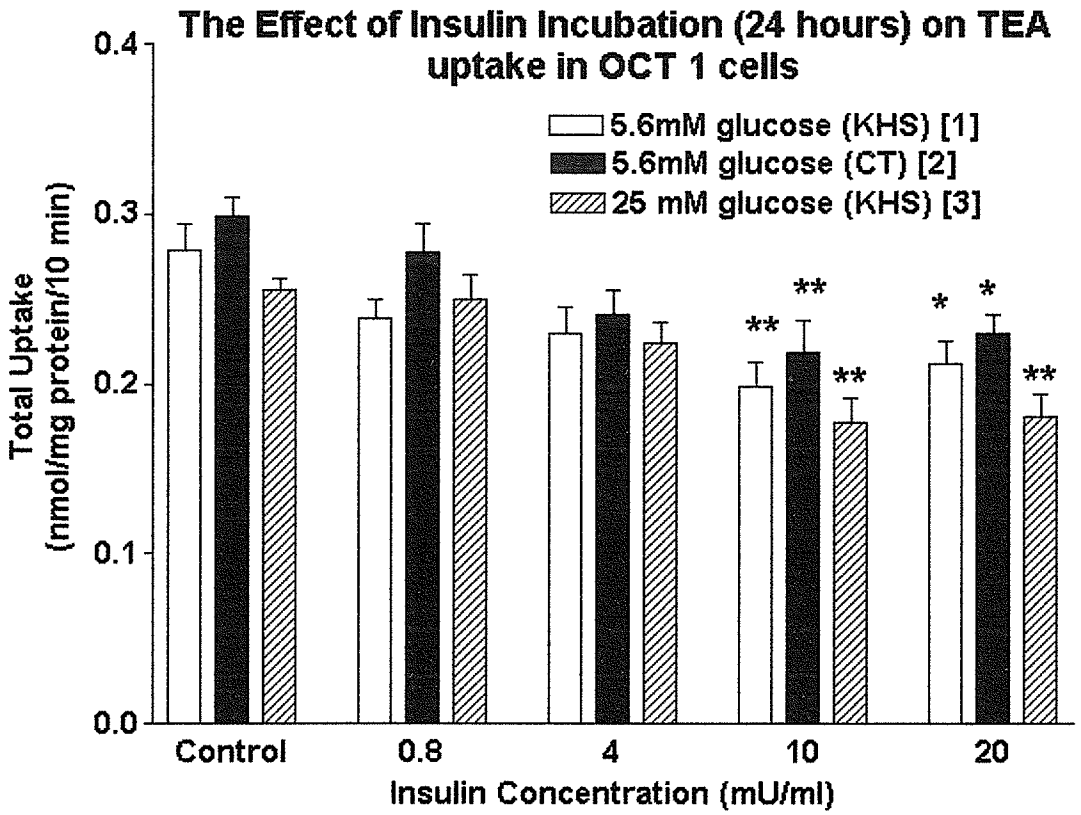

Figure R-4: The effect of insulin and high glucose pre-incubation (24 hours) on high capacity TEA uptake in rOCT1 cells. Data represent the mean ± S.E. (n=5). Asterisks indicate significant difference from control of respective treatment. (*P<0.05, ** P<0.01).

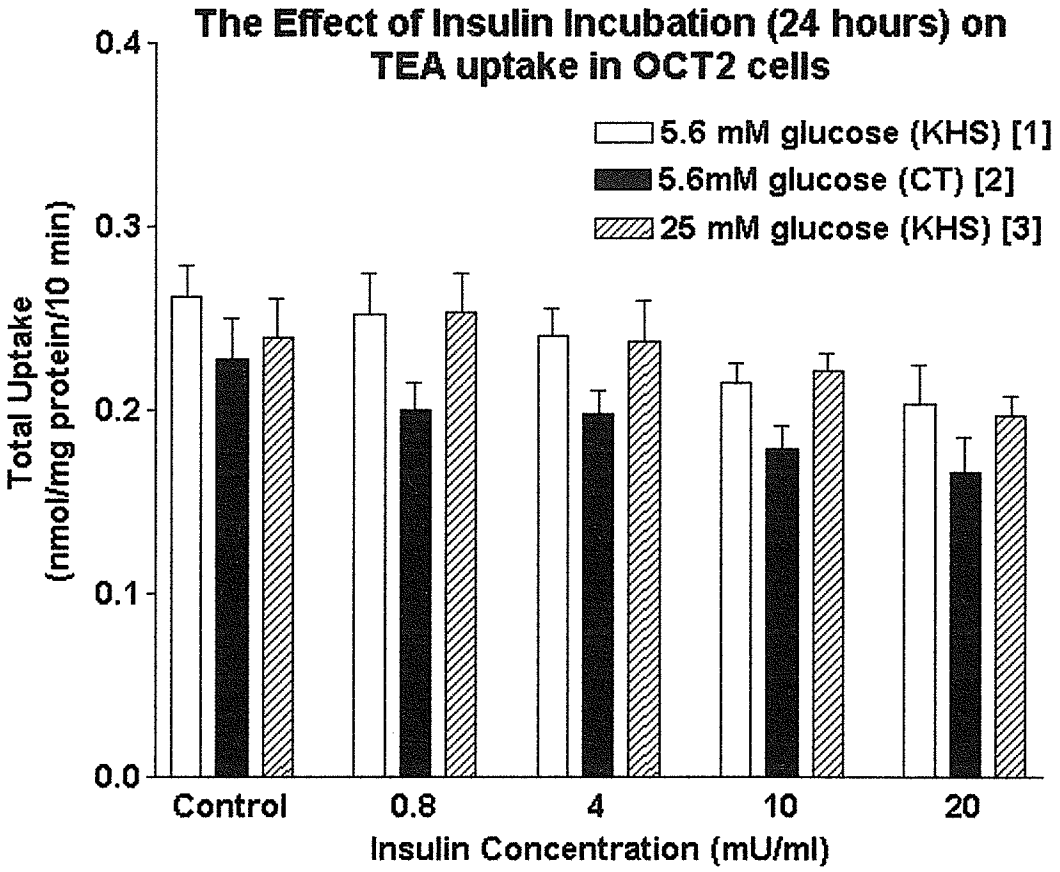

Figure R-5: The effect of insulin and high glucose pre- incubation (24 hours) on high capacity TEA uptake in rOCT2 cells. Data represent the mean ± S.E. (n=4).

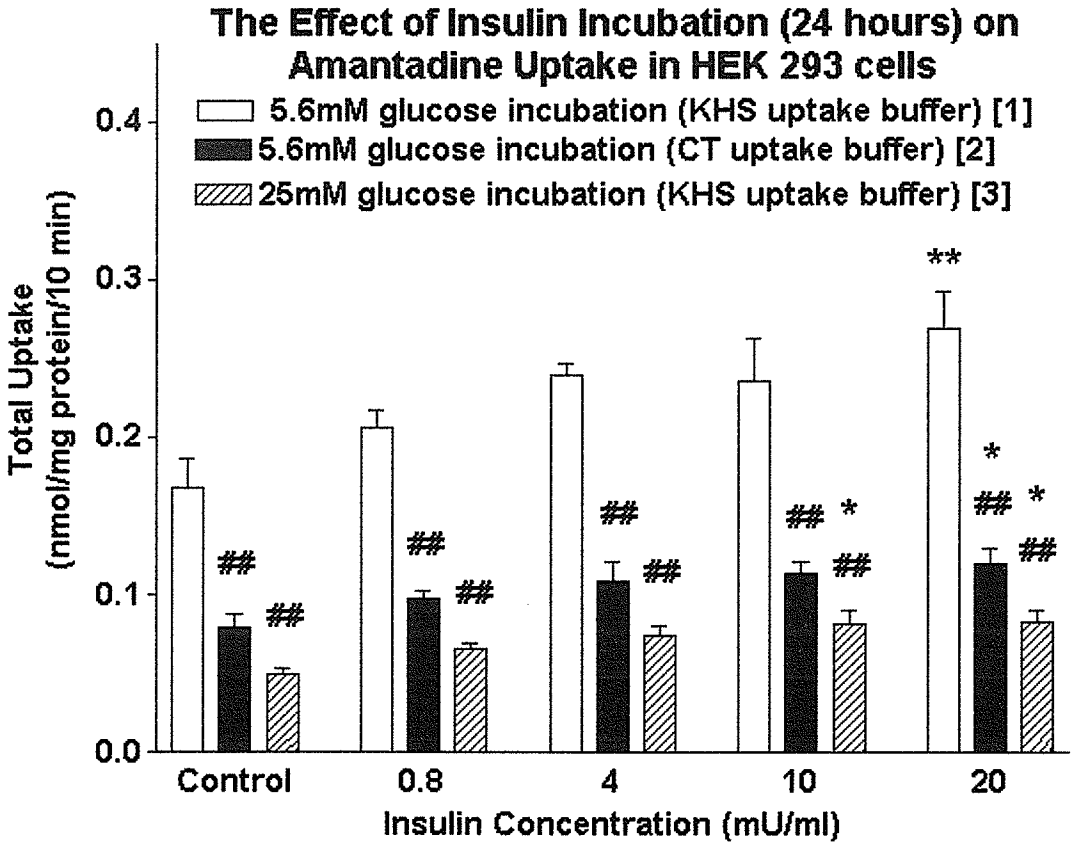

Figure R-6: The effect of insulin and high glucose pre-incubation (24 hours) on high capacity amantadine uptake in HEK 293 cells. Data represent the mean ± S.E. (n=4). Asterisks indicate significant difference from control of respective treatment. (*P<0.05, ** P<0.01). Number signs indicate significant difference between treatment groups compared to 5.6mM glucose KHS buffer treatment. (## P<0.01).

Acute glucose starvation provides initial evidence that acute cAMP/ATP levels may be modulating organic cation transport. Studies to determine the potential mechanism(s) of insulin's regulatory effect on OCTs, began by examining whether acute glucose levels in the medium modulate the levels of organic cation uptake. Preliminary experiments investigating the effect of incubating the cells in glucose levels of 2.8 – 25 mM (hypoglycaemic-hyperglycamic levels) acutely for 60 minutes was unable to demonstrate an effect on organic cation transport in any of the groups (data not shown). However, when we examined the effect of 30 minute acute glucose starvation (extreme hypoglycaemia) in CT and KHS buffers where glucose (11 mM) was replaced with osmotically equivalent mannitol (6.7 mM) compared to parallel control (glucose containing) buffers, an acute effect was demonstrated (Figure R-7, 8, 9). Viability studies using Trypan blue exclusion assay demonstrated cell viability of approximately 98% up to 3 hours in both the absence and presence of glucose in CT and KHS buffer (data not shown). In both rOCT1 and rOCT2 containing cells, we see a 50% reduction in TEA uptake compared to controls regardless of buffer treatment (Figure R-7, $p<0.05$, CT, $p<0.01$, KHS; Figure R-8, $p<0.001$, CT, $p<0.01$, KHS, respectively, paired Student t-test). The effect of 30 minute acute glucose starvation on amantadine transport in HEK 293 cells in CT and KHS buffers was also examined. Interestingly, we observed an increase in amantadine uptake in starved cells compared to control cells in both the absence and presence of bicarbonate (45% and 49% respectively) (Figure R-9, $p<0.05$, unpaired Student t-test). The effect of starvation in the presence of bicarbonate is more pronounced

compared to starvation in its absence (Figure R-9, $p<0.001$, unpaired Student t-test).

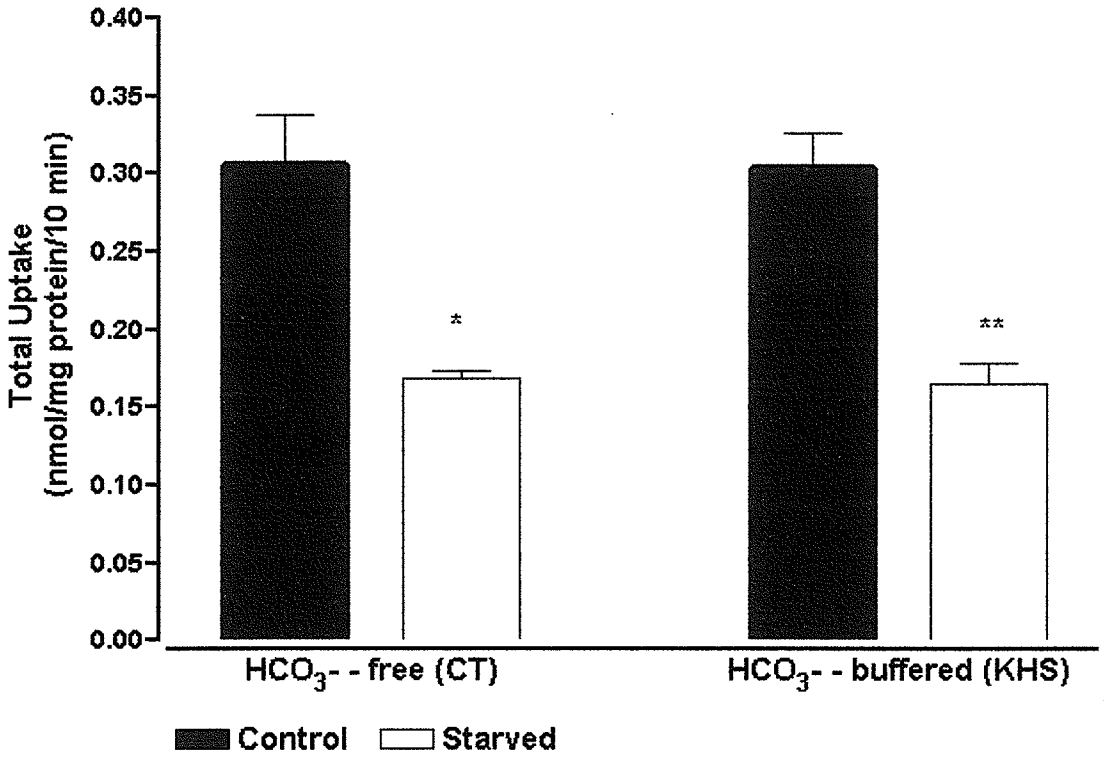

Figure R-7: The effect of acute glucose starvation (30 minutes) on high capacity TEA uptake in rOCT1 cells. Data represent the mean ± S.E. (n=4, CT; n=4, KHS). Asterisks indicate significant difference from control of respective treatment. (*P<0.05, ** P<0.01).

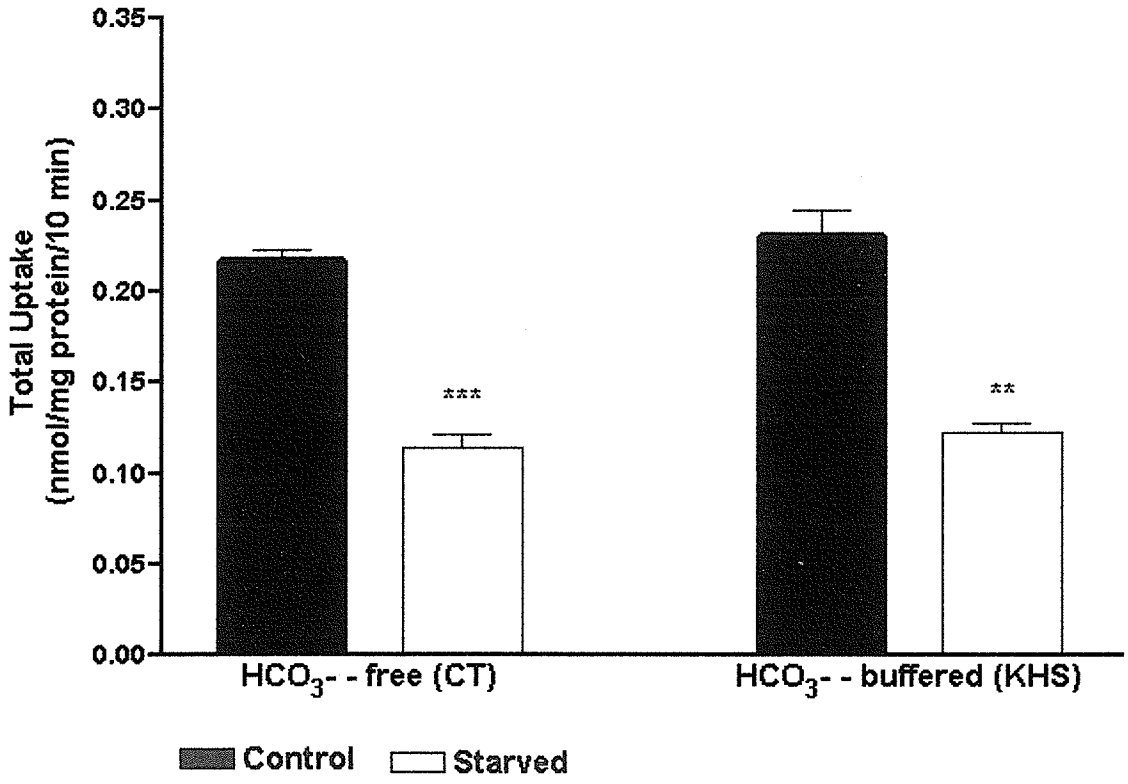

Figure R-8: The effect of acute glucose starvation (30 minutes) on high capacity TEA uptake in rOCT2 cells. Data represent the mean ± S.E. (n=4, CT; n=4, KHS). Asterisks indicate significant difference from control of respective treatment. (**P<0.01, *** P<0.001).

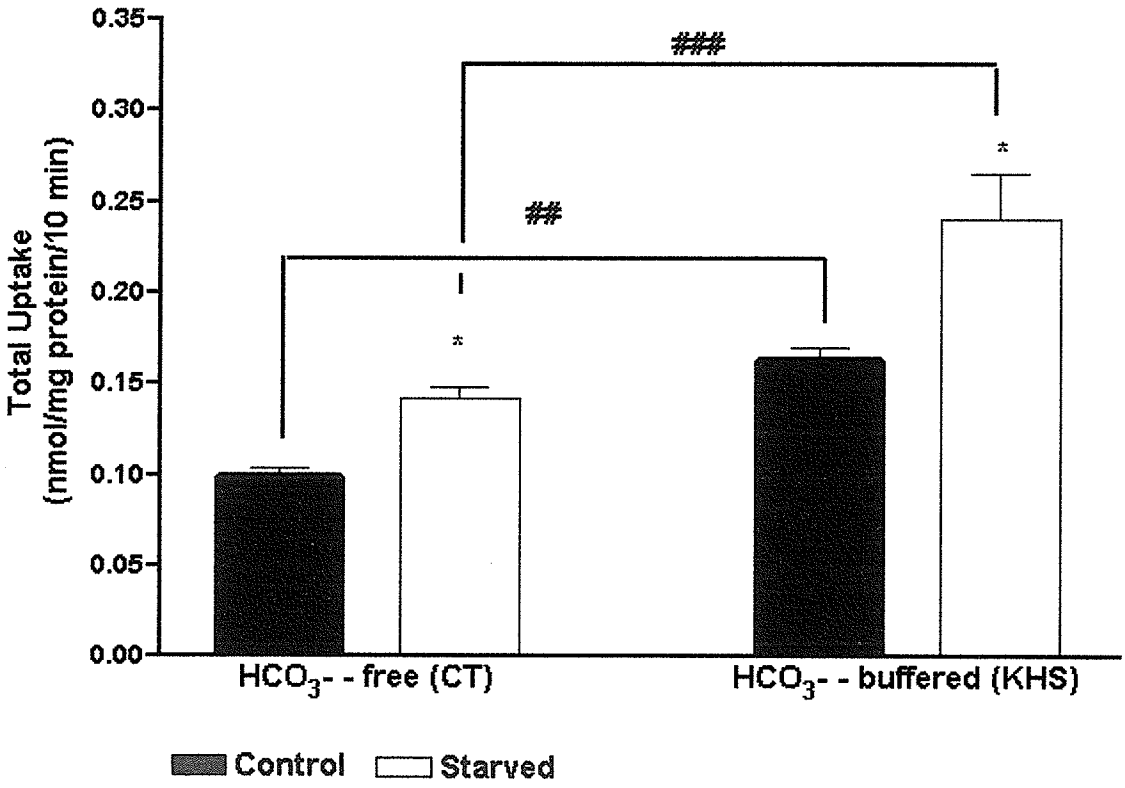

Figure R-9: The effect of acute glucose starvation (30 minutes) on high capacity amantadine uptake in HEK 293 cells. Data represent the mean ± S.E. (n=4, CT; n=5, KHS). Asterisks indicate significant difference from control of respective treatment. (*P<0.05). Number signs indicate significant difference between buffer treatment groups. (## P<0.01, ### P<0.001).

Acute incubations indicating that insulin is modulating organic cation transport through the metabolic signal cAMP. The effects of exposing cells to insulin (20 mU/ml), Br-cAMP or dibutyryl-c-AMP (100 μM, stable c-AMP analogues), and insulin and analogue together for 30 minutes before and not during incubation with ^{14}C-TEA or ^{3}H-amantadine were examined on uptake in the presence/absence of bicarbonate. Acute treatment of rOCT1 cells with Br-c-AMP and dibutyryl c-AMP (100 μM) (Figure R-10) results in a significant inhibition of uptake compared to control in CT buffer ($p<0.05$, $p<0.01$, respectively, ANOVA, Tukey's post hoc pairwise comparison) and in KHS buffer ($p<0.05$, $p<0.05$, respectively, ANOVA, Tukey's post hoc pairwise comparison). Inhibitory effects were additive when insulin is present in addition to Br-cAMP or dibutyryl-cAMP in both buffers ($p<0.01$, $p<0.001$, respectively,CT buffer; $p<0.001$, $p<0.001$, respectively, KHS buffer, ANOVA, Tukey's post hoc comparison). Assessment of differences in treatments shows that there is an effect of insulin in addition to Br-cAMP and dibutyryl-cAMP compared to either cAMP analogue alone in rOCT1-transfected cells alone in KHS (Figure R-10, $p<0.01$, ANOVA, Tukey's post hoc comparison). This effect was seen only in the presence of bicarbonate. In rOCT2-transfected cells, treatment with c-AMP analogues alone caused a marked decrease in uptake in KHS ($p<0.01$, ANOVA, Tukey's post hoc comparison); with insulin this effect was additive but once again only in the presence of bicarbonate ($p<0.001$, ANOVA, Tukey's post hoc comparison) (Figure R-11). One-way ANOVA of rOCT2 containing cells confirmed the presence of a buffer effect

between the two insulin treated groups ($p<0.05$, ANOVA, Tukey's post hoc pairwise comparison). Evidence of an effect of insulin and dibutyryl-cAMP compared to analogue alone was demonstrated in KHS ($p<0.05$, ANOVA, Tukey's post hoc comparison). Examination of the effect of insulin and increased c-AMP analogue levels on amantadine transport demonstrated an increase in amantadine uptake only when insulin and Br-cAMP or dibutyryl-cAMP is combined ($p<0.05$, $p<0.01$, respectively, CT buffer; $p<0.01$, $p<0.01$, respectively, KHS buffer, ANOVA, Tukey's post hoc comparison) (Figure R-12). One-way ANOVA of amantadine transport in native HEK 293 cells confirmed the presence of a buffer effect between all respective treatment groups ($p<0.05$ to $p<0.001$, ANOVA, Tukey's post hoc pairwise comparison). Treatment of the cells with insulin in addition to c-AMP analogue resulted in significant stimulation of uptake compared to c-AMP analogue alone (Figure R-12, $p<0.01$, ANOVA, Tukey's post hoc comparison). These results indicate that bicarbonate and cAMP analogues alone and in addition to insulin acutely modify OCT regulation. Evidence of additive effects among those components suggest a complementary mechanism of action that appears to be metabolic in origin; this signal may putatively involve the end product of catabolic metabolism, CO_2.

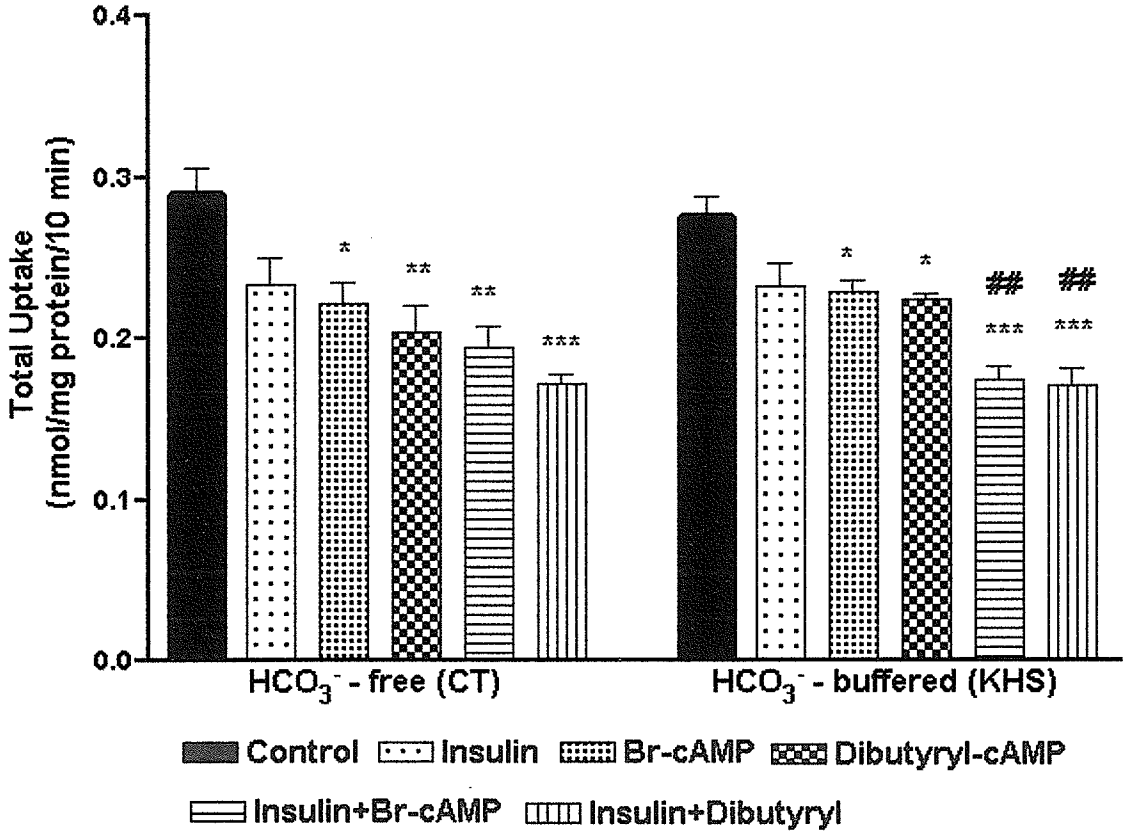

Figure R-10: The effect of acute treatment (30 minutes) of insulin (20 mU/ml) and cAMP analogues (100 uM) on high capacity TEA uptake in rOCT1 cells. Data represent the mean ± S.E. (n=4). Asterisks indicate significant difference from control (*P<0.05 **P<0.01,***P<0.001). Number signs indicate significant difference from respective cAMP analogue treatment (##P<0.01).

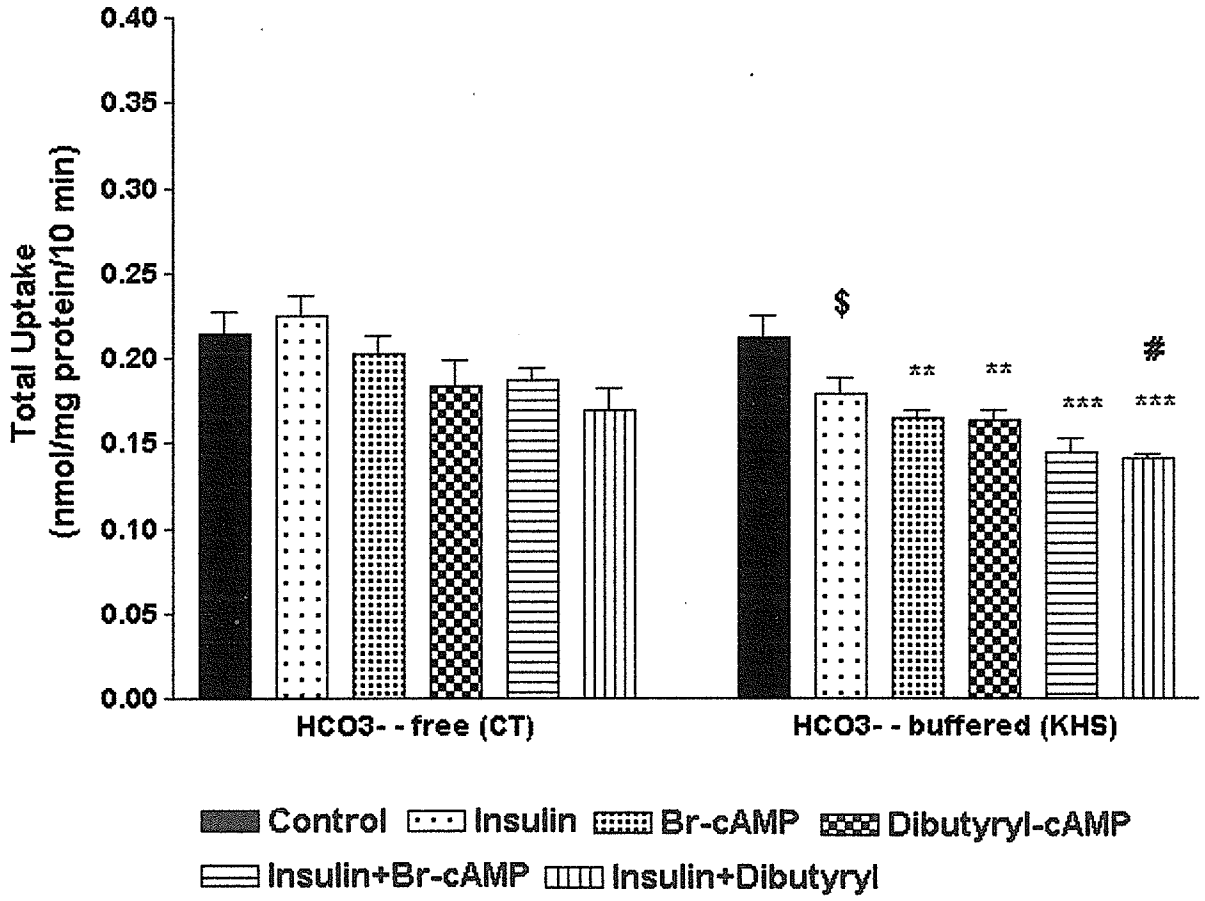

Figure R-11: The effect of acute treatment (30 minutes) of insulin (20 mU/ml) and cAMP analogues (100 uM) on high capacity TEA uptake in rOCT2 cells. Data represent the mean ± S.E. (n=5, CT; n=4, KHS). Asterisks indicate significant difference from control (**P<0.01,***P<0.001). Number signs indicate significant difference from respective cAMP analogue treatment (#P<0.05). Dollar signs indicate significant difference between buffer treatments ($P<0.05).

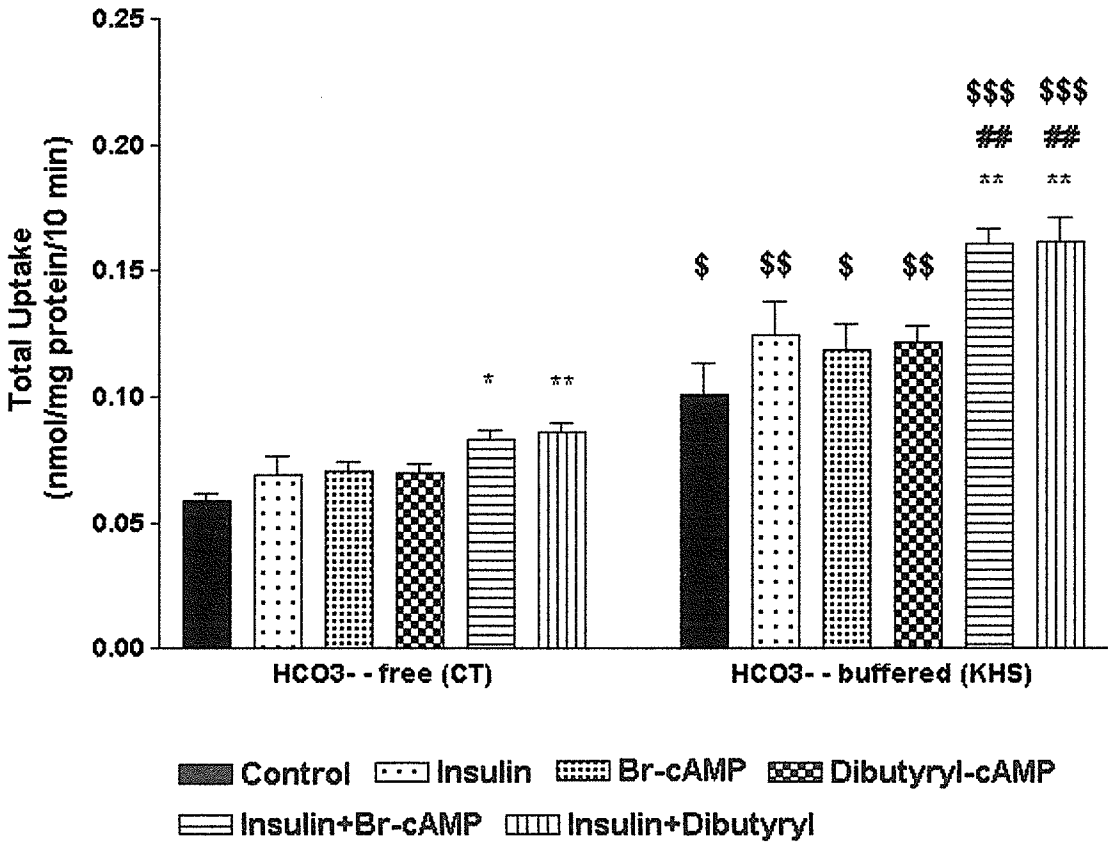

Figure R-12: The effect of acute treatment (30 minutes) of insulin (20 mU/ml) and cAMP analogues (100 uM) on high capacity amantadine uptake in HEK 293 cells. Data represent the mean ± S.E. (n=4, CT; n=5, KHS). Asterisks indicate significant difference from control (**P<0.01). Number signs indicate significant difference from respective cAMP analogue treatment (##P<0.01). Dollar signs indicate significant difference between buffer treatments ($P<0.05, $$P<0.01, $$$ P<0.001).

Determination of the contributions of intracellular cAMP pools from tmAC and sAC using forskolin and bicarbonate to organic cation transport. Investigation into the effect of bicarbonate on OCT transporter function led us to examine the role of tmACs and sACs. The contribution of intracellular cAMP from tmACs and sAC to the regulation of TEA and amantadine uptake was differentiated using forskolin (tmAC activator), and the presence/absence of bicarbonate (sAC activator) in the buffer (Chen et al., 2000). Forskolin's ability to increase cAMP levels have been documented in literature, as has its capacity to affect calcium currents, and phospholipid/Ca2+ dependent protein kinase (PKC) (Siddhanti et al., 1995). Possible cross talk between PKA, PKC and the calcium system such as that demonstrated in neuronal cells may be responsible for such an observed complexity (Kubota et al., 2003). Attention must be paid to separating PKA effects from PKC effects. The choice of a protein kinase inhibitor proved difficult in acknowledgement of evidenced crosstalk between PKA and PKC systems. The less specific protein kinase inhibitor staurosporine (6 nM) was chosen at a concentration that also has also been documented to inhibit PKA in addition to PKC (Tamaoki, 1990; Condrescu, 1999; Sielecki, 2000). An incubation time of 60 minutes was decided upon for forskolin. Lower end time points are associated with intense spikes, which are often examined electrophysiologically in conjunction with membrane channel activity and acute signalling events (Mittag et al., 1993; Siddhanti et al., 1995; Vincent and Brusciano, 2001). Since cAMP levels remain elevated in forskolin's presence (de Souza et al., 1983; Seamon and Daly, 1986), we were more interested in

later, more stabilized signalling activity, beyond those very acute events following cAMP elevation. Therefore 60 minutes, a later point in signalling activity was decided upon. This should allow stabilization of signalling in addition to allowing sufficient time for the uptake of staurosporine. Pre-incubation with 1 or 10 μM forskolin for 60 minutes were followed by uptake experiments examining [^{14}C]-TEA and [^{3}H]-amantadine uptake. In rOCT1 containing cells, we see a significant 30% reduction in TEA uptake in the presence of 10 μM of forskolin in CT buffer (Figure R-13, $p<0.001$, ANOVA, Tukey's post hoc comparison). In KHS buffer, rOCT1 containing cells demonstrated a significant 22% reduction in TEA uptake in the presence of 1 μM and 10 μM of forskolin (Figure R-13, $p<0.05$, $p<0.05$, respectively, ANOVA, Tukey's post hoc). Protein kinase inhibition by staurosporine alone and in addition to PKA activation by 10 μM forskolin resulted in significant inhibition of TEA uptake compared to control ($p<0.001$, both, ANOVA, Tukey's post hoc comparison), with no reportable differences between the two treatments in both buffers. In rOCT2 containing cells, we see a significant 19% reduction in TEA uptake by forskolin in CT buffer alone (Figure R-14, $p<0.05$, ANOVA, Tukey's post hoc comparison). Protein kinase inhibition by staurosporine alone and in addition to cAMP-dependent PKA activation by 10 μM forskolin resulted in significant inhibition in TEA uptake in both buffers ($p<0.001$, ANOVA, Tukey's post hoc comparison), with no reportable differences between the two treatments. One-way ANOVA confirmed the existence of a buffer effect between the 10 μM forskolin treatment groups ($p<0.05$, ANOVA, Tukey's post hoc pairwise comparison).

In contrast to the above findings, in nontransfected HEK 293 cells we see no effect of forskolin on amantadine uptake in the presence or absence of bicarbonate (Figure R-15). Staurosporine alone and in addition to forskolin resulted in the significant stimulation of amantadine uptake but only in CT buffer ($p<0.01$, ANOVA, Tukey's post hoc comparison). Further analysis of the data confirmed the existence of a buffer effect between respective treatment groups ($p<0.01$ to $p<0.001$, ANOVA, Tukey's post hoc pairwise comparison). These data indicate that the cAMP pools generated by tmAC stimulation with forskolin affect only rOCT1 and rOCT2 activity. The presence of bicarbonate in the buffer on the other hand, through its action on sAC, contributes to the cAMP pools modulating the portion of the organic cation transport system identified by the cationic marker amantadine, and not TEA.

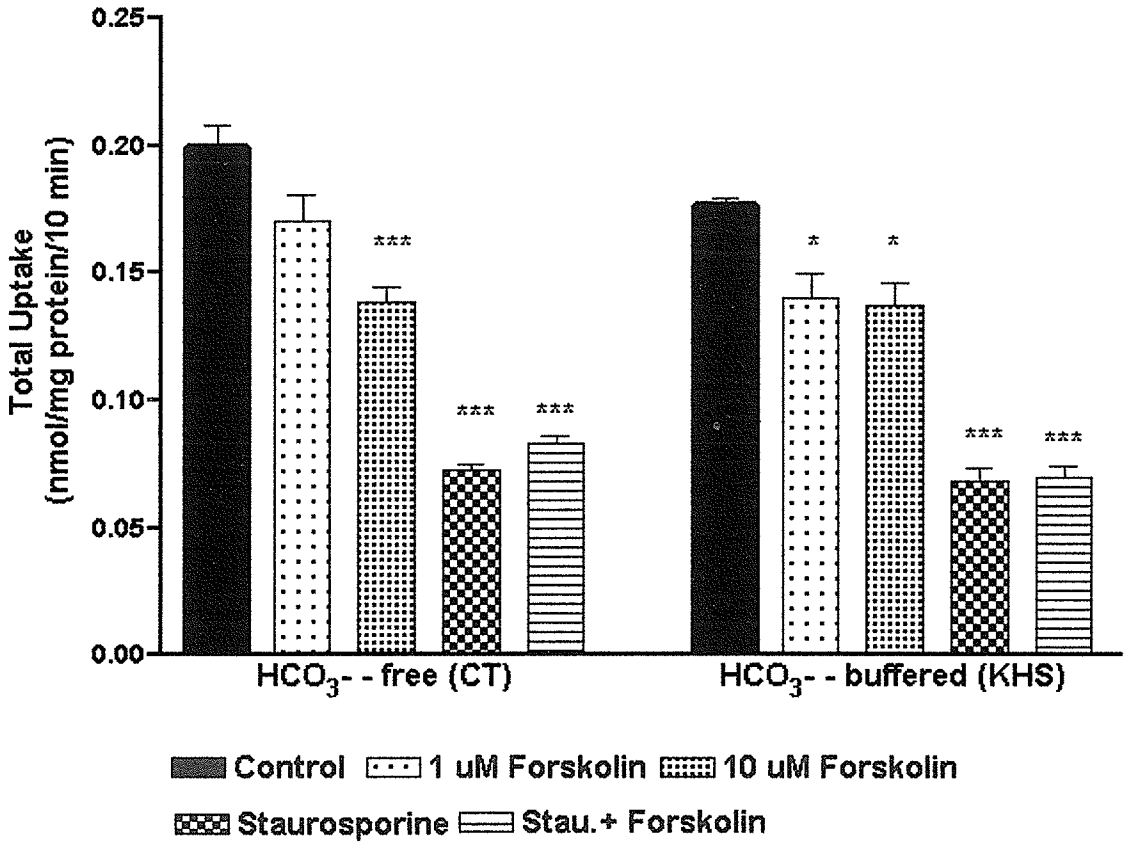

Figure R-13: The effect of acute (60 minutes) forskolin treatment on high capacity TEA uptake in rOCT1 cells. Data represent the mean ± S.E. (n=4, CT; n=4 KHS). Asterisks indicate significant difference from control (*P<0.05, ***P<0.001).

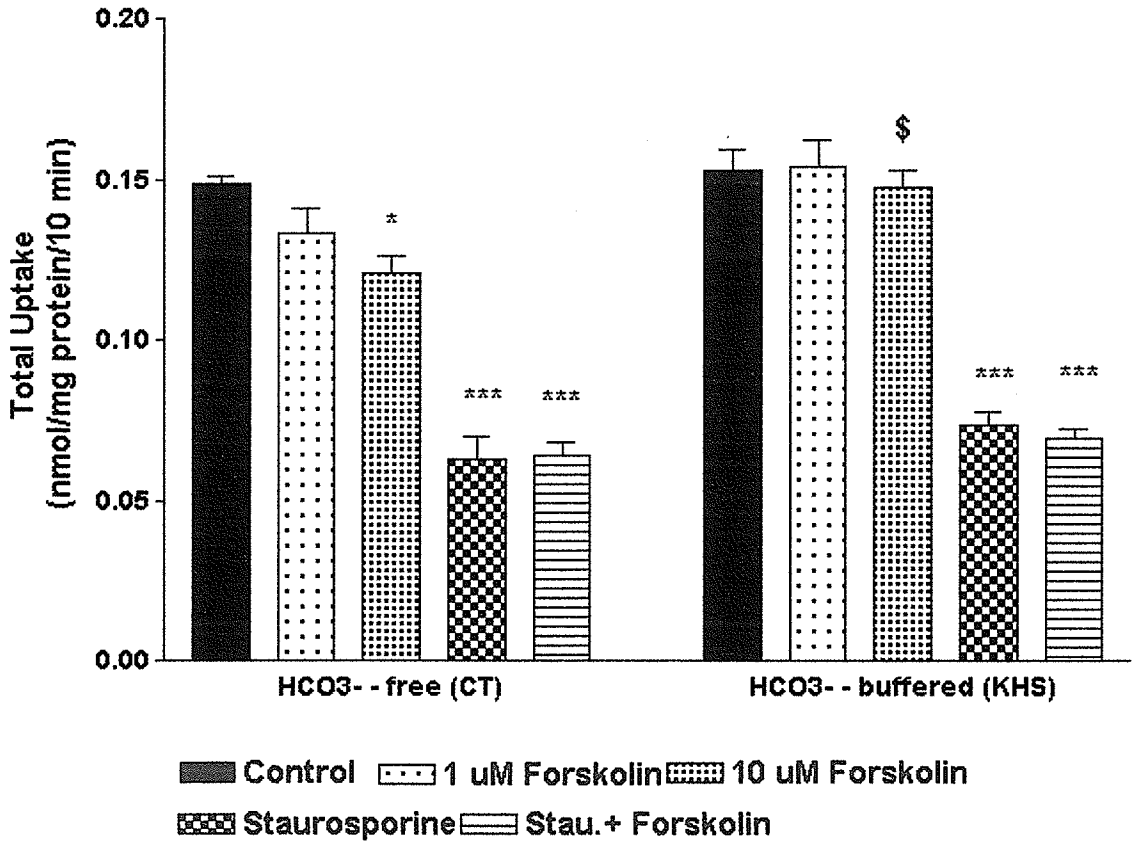

Figure R-14: The effect of acute (60 minutes) forskolin treatment on high capacity TEA uptake in rOCT2 cells. Data represent the mean ± S.E. (n=4, CT; n=4 KHS). Asterisks indicate significant difference from control (*P<0.05, ***P<0.001). Dollar signs indicate significant difference between buffer treatments ($ P<0.05).

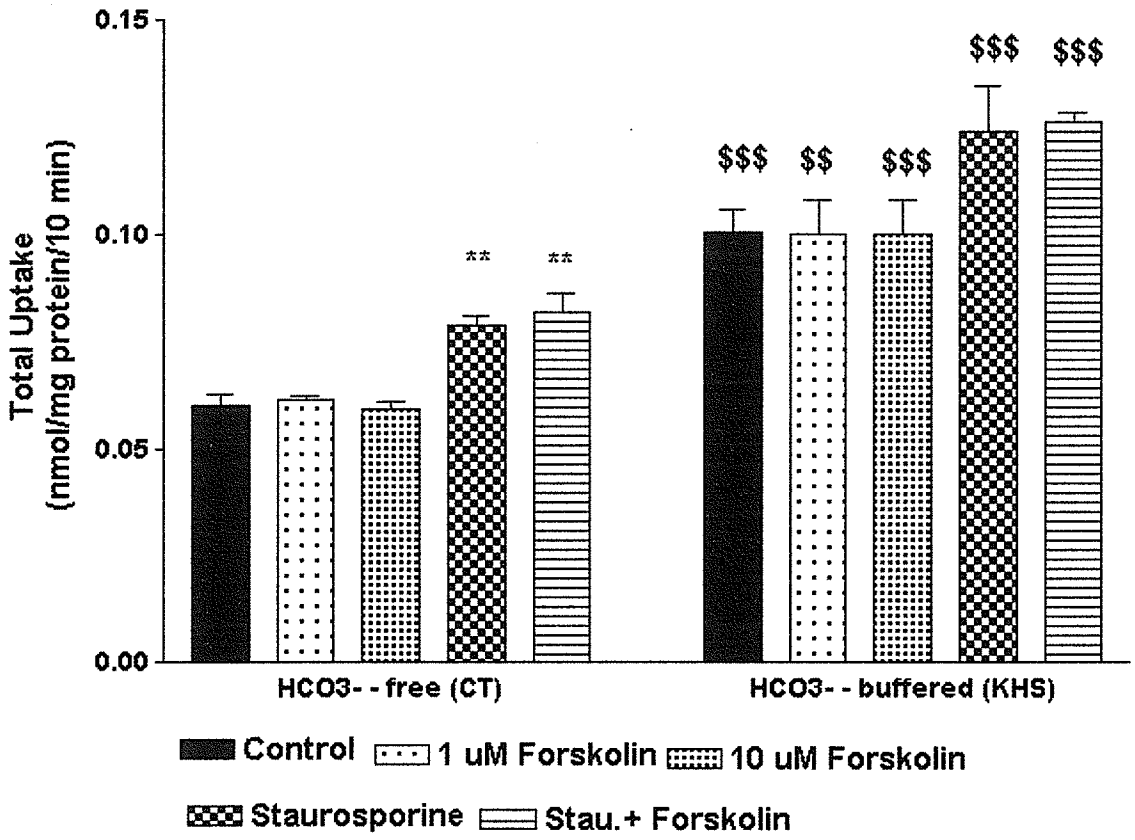

Figure R-15: The effect of acute (60 minutes) forskolin treatment on high capacity amantadine uptake in HEK 293 cells. Data represent the mean ± S.E. (n=4). Asterisks indicate significant difference from control (**P<0.01). Dollar signs indicate significant difference between buffer treatments ($P<0.05, $$P<0.01, $$$ P<0.001).

DISCUSSION

I. Methodological Considerations

Several methodological points concerning both the cell model and the experimental system will be examined in this section in order to clarify certain aspects of our protocol.

Central to our studies was the discovery that native (untransfected), and empty vector transfected HEK 293 cells transported amantadine, but not TEA. Speculations by our laboratory that the amantadine transporter was a unique organic cation transporter (Escobar et al., 1994; Goralski and Sitar, 1999; Goralski et al., 2002), from the cloned OCTs that the marker TEA identifies, have been further reinforced by the identification that this cell model transports amantadine and not TEA. OCT-transfected and native (non-transfected) HEK 293 cells provide us with a powerful model that allows us to examine the regulation of each transporter in a simplified system using TEA and amantadine as probes. Even though rat OCT transporters are used to transfect a human immortalized cell line, and recognizing that some species-associated discrepancies may result, an additional strength of this cell model lies in its human kidney origin, and its ability to provide a better approximation of clinically relevant human cell regulation compared to animal cell-lines previously used in other regulation studies.

A 10 minute incubation time for cationic marker substrates was used in all the experiments presented herein. While the initial slope represents cation uptake across the plasma membrane by the transporters, the maximal transport (reflected by the 10 minute incubation) is the sum of marker substrate uptake into the cells, exit of the cation from the cells, and intracellular compartmentalization. Since rOCT1 and 2 have been localized to the basolateral membrane and no native TEA transport mechanism exists in HEK 293 cells, we propose that the transport we observe is associated solely with basolateral and intracellular events. The key understanding being that we are also indirectly examining intracellular events as well. Previous studies by our laboratory used shorter incubation periods for TEA and amantadine, focusing on uptake across the membrane, rather than accumulation. Initial investigations into insulin's effect on organic transport using a shorter period (reflecting linear uptake across the basolateral membrane) was unable to demonstrate any effect. On the other hand, reportable and reproducible differences were observed with the 10 minute longer incubation time. Consequently, a 10 minute incubation period was used for further studies. An important consideration in longer uptake studies is metabolism of the probe. It should be noted that both amantadine and TEA are essentially unmetabolized by the body (Aoki et al., 1979; Rennick, 1981); there are some metabolites of amantadine, but they represent a very small proportion of an administered therapeutic dose, so any confusion in the results created by metabolism of the probe during the 10 minute period should not be a factor.

All potential modulators of regulation we studied (insulin, cAMP analogues, forskolin) were applied to attached plated cells because of the importance of cytoskeletal scaffolding in insulin signalling, and more specifically integrin/insulin-IGF1 crosstalk (Lebrun et al., 2000). The cells were then detached gently and used in suspensions during experimentation. Previous experiments by our laboratory examining the effect of insulin and diabetes on amantadine uptake in rat tubules used cell suspensions for uptake studies after diabetic induction in the animals. Significant results were achieved previously by this method (Goralski et al., 2001). Aside from the initial non-suspension agonist/inhibitor incubation period, experimental uptake conditions were those of a suspension model.

Concerns by our group over the integrity of the cells in all buffer conditions, including those of the glucose starvation experiments where glucose was replaced with an osmotically equivalent concentration of mannitol, were satisfied up to 3 hours (beyond any experimental incubation period reported in our data). A consistent 98%(# of live/# of total cells) viability of cells was confirmed by the Trypan blue dye exclusion assay. This confirmed the notion that the observed effects were not due to membrane destabilization associated with cell death.

Seeding the cells and careful monitoring of cell-growth allowed the protein concentration, and by inference cell-number, to be maintained qualitatively constant among experiments. Protein determination by the

biuret method was used (Gornall et al., 1949). This method is ideal for large quantities of protein, which were present throughout experimentation.

A point of clarification should be made as to why there was no examination of mRNA or protein expression of the transporters of interest in these studies, which would serve as molecular reinforcement of our findings. At present, the amantadine transporter remains unidentified. As a result, there would be no point of reference for comparing differential regulation among the transporters. Perhaps in the future, with the discovery of the identity of the amantadine transporter and easier availability of OCT-antibodies, experiments could be designed to corroborate our findings.

Potential limitations may still exist when the HEK 293 cell line is used for the above purposes. For example, this is a cell line with all of the limitations of cell lines in terms of alteration of function over time in cell culture. We therefore used cells only between the 10-20th passage. Finally, one last point of consideration lies in the fact that HEK 293 cells are reported to be derived from the transformation of a kidney cell of neuronal origin (Shaw et al., 2002). In such a case, the transport mechanisms observed in our model may be different from those occurring in the proximal and distal tubule cells that are primarily responsible for active drug secretion in the kidney. In the absence of any knowledge of a better human kidney cell model, this system provided us with the best approximation for studying

OCT regulation in the human kidney. Clearly, further studies with rat tubules or other cell models are indicated to correlate any findings.

II. Insulin and Organic Cation Transport

As described earlier, STZ-induced diabetic rats have been demonstrated to accumulate less TEA in kidney cortex slices compared to kidney tissue from control animals, while amantadine uptake in rat renal tubule cell suspensions showed the opposite trend by demonstrating increased amantadine uptake in diabetic cells (Goralski et al., 2001; Grover et al., 2002). Both transport perturbations were returned to normal by the addition of exogenous insulin. In this section, the focus is on reinforcing those aforementioned observations by establishing that insulin can modulate TEA and amantadine accumulation by differentially regulating organic cation transporters in the HEK 293 cell model. We also introduce plausible explanations for those observations, some of the associated obstacles, and we begin to postulate about a proposed mechanism.

In the present study, we determined the effect of 24 hour insulin treatment on OCT function in an HEK 293 cell model. We showed that the pre-incubation of cells with varying dosages of insulin resulted in the inhibition of TEA uptake in rOCT-1 and rOCT-2 transfected cells, up to 32% and 20% respectively from control, regardless of buffer or glucose conditions

(Figure R-4, R-5). In rOCT2-transfected cells, 24 hours of treatment with insulin appeared qualitatively similar to rOCT1 among the three treatment groups, but did not achieve significance. Further experiments beyond an n(4) might have helped to achieve significance.

In contrast to the regulation of TEA transport by cells transfected with rOCT1 and rOCT2, is amantadine transport in native HEK 293 cells. Treatment of native HEK 293 cells with insulin resulted in increased amantadine transport, up to 72% in a dose dependent manner, regardless of glucose or buffer conditions **(Figure R-6)**. The proportional insulin dose-related increase in amantadine transport suggests that the insulin effect is distinct, even to that of glucose. Of notable interest is our observation that the high glucose pre-treated group shows the lowest amantadine accumulation despite the presence of bicarbonate in the uptake buffer (KHS). This observation and its possible explanation will be expanded in the following sections.

Insulin plays a central role in regulating energy metabolism by stimulating cell growth and differentiation, promoting the storage of substrates in fat, liver, and muscle by stimulating lipogenesis, glycogen and protein synthesis, while inhibiting lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn, 2001). Changes such as the upregulation of glucose, peptide, and lipid transporters, reflecting the anabolic needs of the cell, occur in response to insulin both at the gene and protein level (Saltiel and Kahn, 2001; Nielsen and Brodin, 2003; Wilmsen et al., 2003). Evidence from

our studies now suggests that insulin may also play an important role in controlling organic cation accumulation. Our findings complement previous literature that established OCTs' capacity to transport important endogenous compounds (ie. vitamins, neurotransmitters, metabolites) (van Ginneken and Russel, 1989; Busch et al., 1998; Wang et al., 1999), and those that showed OCTs to be metabolic energy-dependent (Steen et al., 1991; Ito et al., 1993; Escobar and Sitar, 1996; Yokogawa et al., 1999; Martel et al., 2000; Martel et al., 2001a). It makes sense that their function should be subject to metabolic regulation, especially considering their role in detoxifying the body, redundant neurotransmitter transport function of serotonin and acetylcholine in the gut and placenta (ie. in addition to more selective serotonin and acetylcholine transporters)(Chen et al., 2001; Wessler et al., 2001), and putative vitamin and cofactor recycling. A possible reason for the opposite activity evidenced between rOCT1, rOCT2 and the amantadine transporter, is as a result of the stimulatory effects of insulin on metabolism, particulary its capacity to generate CO_2 and the resulting changes in the metabolic cAMP signal; this notion will be elaborated upon later in the discussion. However to the best of our knowledge, there have been no previous reports of insulin affecting organic cation transport in an *in vitro* cell model. A possible explanation for this observation may lie in the fact that past studies by groups either overlooked insulin or had likely focused on the linear transport rate (a typical experimental procedure for transport studies) that according to current theory serves as a model for the movement of organic cations across the basolateral membrane (Grundemann et al., 1994; Grundemann et al., 1998; Goralski and Sitar, 1999; Cetinkaya et al., 2003). A similar absence of effect

was observed by our examinations into insulin and initial transport rates (ie. 30 seconds, linear part of uptake). We were unable to show any insulin effect for the amantadine transporter, rOCT1 or rOCT2 (data not shown). But when we examined marker substrate accumulation in native and OCT-transfected HEK 293 cells at a time point of 10 minutes (maximal transport capacity, used by us in all our experimental studies), we began to differentiate significant reproducible effects of insulin on transport function. The key experimental variation may be that our chosen time point is likely incorporating intracellular events, such as alterations in protein trafficking, in addition to those events at the basolateral membrane.

Evidence by our studies for opposite regulatory trends in amantadine and TEA transport by insulin is consistent with previous reports involving the streptozotocin (STZ)-induced diabetic rat model (Goralski et al., 2001; Grover et al., 2002); keeping in mind that rOCT1 is the predominant basolateral OCT in the rat kidney (Grundemann et al., 1994). Interestingly, the experimental TEA incubation period chosen by the latter study in a kidney cortex slice model was 15 minutes, which is very close to the maximal transport capacity according to their own time course data; this period would also favour uptake and accumulation conditions similar to our experimental conditions. This notion supports our investigational decision to use maximal transport capacity as opposed to linear transport rate to demonstrate a regulatory effect for insulin.

However, one subtle point of clarification should be mentioned in regard to the comparison of our results to previous reports. If STZ-induced diabetes is considered to be a model for insulin-dependent Type 1 diabetes (Herold et al., 1995) (though even this categorization isn't quite accurate as these rats live without injected insulin), and TEA accumulation decreases in diabetic rats with transport perturbation returning to normal upon addition of exogenous insulin, then addition of insulin to normal cells should speculatively increase TEA accumulation; a similar argument could also exist for amantadine transport. But as diabetes is a complex disorder of energy-metabolism, one can only deduce from the STZ-induced diabetic rat studies that exogenous insulin corrects the underlying condition, preventing the alteration of drug transport function. Therefore in our studies, applying insulin to cells that are both "nondiabetic" and transformed (with an associated change in growth and metabolic profile) allows us only to establish the existence of a mechanism for insulin to change transporter function. The exact nature of insulin's effect on OCTs and possible diabetic implications will be developed in the following sections.

A fact in contention to our observations regarding the long-term effect of insulin, is that the lowest dose used in our study (800 μU/ml) is twenty times that of typically reported peak postprandial physiological plasma concentrations (40 μU/ml) (Shafrir, 1996). Precedent studies utilizing transformed cell-lines all commonly demonstrate biological effects using very high hormonal doses to demonstrate effect (Kanai et al., 1996; Shu et al.,

2001), perhaps in acknowledgement to a transformed cell's inherent chemical resistance. Our use of high hormone levels to establish effect appears justifiable. Nonetheless a certain concern arises when using such a high insulin concentration, namely cell death (for unknown reasons), which lead to a distortion in uptake. This scenario is conceivable but unlikely for three reasons. First, insulin is a growth factor that is known to generate signals for growth and survival. Second, trends are maintained throughout even at our lowest dose. Lastly, protein levels among the different dose-treated groups are consistent, thereby inferring the absence of cell death. Future experiments examining cytotoxicity could help to confirm the last point but are generally unwarranted.

The existence of a putative physiological process by which insulin differentially regulates OCTs merited investigation as to whether the underlying events were solely related to gene and protein expression, or whether an acute signalling component exists. When we preliminarily examined acute incubation periods with insulin for periods up to 3 hours, we were unable to demonstrate any significant effects (Data not shown). Similar results are seen by our data demonstrating the inability of 30 minute insulin incubation to inhibit TEA uptake in rOCT-1 **(Figure R-10)** and rOCT-2 **(Figure R-11)** transfected cells, or the stimulation of amantadine uptake **(Figure R-12)** in non-transfected HEK 293 cells in either buffer system (apart from the presence of qualitative trends in the data). From these findings and contrasting them to our 24 hour incubation studies, it seems that whatever changes are responsible for modulating organic cation accumulation are

compounded over time, and are likely to involve metabolic changes associated with glycolysis and energy status; this seems to indicate a good potential for the nucleotide ATP (central to reproduction and bioenergetic events) and its metabolite cAMP to provide the intermediate signal; however, clarification of these events first requires re-evaluation of insulin's mechanism of action.

Elucidation of the underlying mechanism of insulin's effect on the kidney and on organic cation transport is confused by the fact that what we are observing may not be as a result of a specific insulin receptor response, but rather part of a complex, still unraveled, insulin/insulin-like growth factor (IGF) signalling system. Insulin and insulin-like growth factor (IGF-I) have 50% sequence homology and regulate similar cellular functions (Hofmann et al., 1989). Their membrane receptors also share 84% homology in a tyrosine kinase domain essential to transmembrane signaling and may thus share common postreceptor paths. Type I insulin-like growth factor (IGF-1) promotes growth and differentiation in a variety of tissues (Baker et al., 1993). The IGF-1 pathway regulates renal growth and development, and has also been implicated in glomerular hypertrophy in pathophysiological conditions such as diabetic nephropathy (Grellier et al., 1996). These effects are largely mediated by the IGF-1 receptor, although IGF-1 can bind to the insulin receptor with low affinity (LeRoith et al., 1995). In addition to these effects, IGF-1 can mimic the metabolic actions of insulin to stimulate glucose and amino acid uptake, inhibit gluconeogenesis, and promote lipogenesis (Di Cola et al., 1997). Conversely, insulin can also bind to the IGF receptor (Patti

and Kahn, 1998). This diversity of functions is further complicated, or perhaps partially explained, by both receptors' ability to form functional hybrids (Patti and Kahn, 1998; Butler and LeRoith, 2001). Microarray data of our HEK 293 cell model confirms the presence of mRNA for both insulin and IGF receptors (Shaw et al., 2002). Analysis of mRNA expression levels indicate three-fold higher levels for the IGF-1 and IGF-2 receptor message compared to that of the insulin receptor message, but care should be taken as mRNA levels are not always indicative of levels of functional protein. Evidence exists to suggest that insulin receptors and IGF-1 receptors share a series of biological actions, while other effects are more unique. Studies in insulin and IGF-1 receptor knockout mice indicate that insulin generates metabolic action mainly through its receptor, while the IGF-I receptor would preferentially have an impact on growth and differentiation (Liu et al., 1993; Accili et al., 1996a; Accili et al., 1996b; Joshi et al., 1996). Another study obtained evidence that the two receptors can achieve a similar final biological endpoint by using different signalling pathways (Miele et al., 2000). At present, the involvement of these two signalling cascades in organic cation transport is unclear; include the fact that insulin and IGF-1 receptors can form functional hybrids, and the specificity of insulin's action in the course of these experiments becomes increasingly complicated.

Our inability to demonstrate an acute insulin effect ruled out primary signal transduction, and instead implicated secondary events as a possible cause for the observed differential regulation. We therefore shifted our focus to other possible candidates. Cross talk between the signalling pathways

activated by cAMP and the IGF tyrosine kinase receptor has been suggested by studies which show that cAMP agonists potentiate IGF-1 action both at the protein and the gene level (Cohick et al., 2000; Sugawara et al., 2000). On the other hand, insulin and cAMP have been suggested to have opposite regulatory effects on metabolism (Pilkis and Granner, 1992). Cyclic AMP has been generally shown to inhibit cell proliferation in cells; however more recently it has been shown to activate proliferation in the mitogenic action of thyrotropin on the thyroid cell (Dremier et al., 2002). Because our cells were used near confluency (90%) with the possibility of contact growth inhibition, metabolism rather than growth may arguably be the predominant signal.

Regardless of the specificity of the signal, investigation into the effect of insulin on organic cation transport, whether it was signalling or metabolic events mediated through insulin receptors, IGF-1, or both, revealed some interesting findings. To the best of our knowledge, the present study represents the first specific description of differential regulation of organic cation transport by insulin. Speculations that cellular metabolic dysregulation (and the associated changes in catabolism and CO_2 generation) is the cause of altered intracellular organic cation accumulation led us to pursue the underlying mechanism, which will be expanded in the following sections.

III. Glucose and Organic Cation Transport

In the previous section we proposed differential regulation of organic cation transport by insulin. We showed that long-term insulin treatment resulted in reduced uptake of TEA by OCT1, and enhanced uptake of amantadine by an unidentified amantadine transporter. We also proposed that metabolic dysregulation associated with energy status may be the cause of those observed changes in transport levels. As glucose is a core regulator of metabolism, and insulin is intimately connected to glucose levels, we decided to examine glucose's ability to affect organic cation transport in the case of its excess (hyperglycemia) and in its absence (glucose starvation). In the present section, we demonstrate that organic cation transport is differentially regulated by glucose and introduce possible mechanism(s) for those observations.

Plasma glucose generally, despite periods of feeding and fasting, remains in a narrow range between 4 and 7 mM in normal human individuals (World Health Organization, 1985). This tight control, mediated by insulin primarily, is governed by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. In the pathophysiological state of diabetes, loss of control over these processes can result in elevated glucose concentrations that reach levels well over 22 mM (Zimmet, 1995). A frequent adverse event associated with

diabetic treatment regimens that fundamentally include insulin and can additionally include sulphonylureas is hypoglycemia (glucose levels below 4 mM) (The Diabetes Control and Complications Trial Research Group, 1993). Thus in diabetic patients, glycaemic levels have the potential for wide fluctuations. Prolonged exposure to elevated glucose or to recurrent changes in plasma glucose levels in the diabetic state leads to altered acute responses and gene expression of numerous membrane and signalling proteins (Brownlee, 2001). Some of these alterations associated with diabetes may involve changes in drug transport systems.

Thus, we investigated our hypothesis that pathologically relevant levels of glucose alter organic cation transport as a possible explanation for the changes seen in diabetic rats. Our investigations into the effect of pre-treating rOCT1 and rOCT2-transfected HEK 293 cells with a high glucose concentration (25 mM) for a prolonged period of time (24 hours) indicate that treatment has no effect on TEA transport by either transporter **(Figure R-4, R-5 respectively)**. Parallel treatment of the cells with insulin in addition to high glucose concentration demonstrates that the decrease in rOCT1 activity mediated by insulin is not altered by high glucose concentration. This observation suggests that the changes in rOCT1 regulation induced by insulin are independent of the metabolic alterations induced by high-glucose concentration. rOCT2 on the other hand, is also not affected by high glucose concentration.

In contrast to rOCT1 and 2, our data indicated the presence of an inverse regulatory relationship between amantadine transport and glucose concentration **(Figure R-6)**. High glucose concentrations have a 67% inhibitory effect on amantadine accumulation (despite the presence of bicarbonate in the uptake buffer). Closer scrutiny of the data indicates that insulin's ability to modulate this effect is in addition to that of glucose. A key point to be aware of is that the proportional increase from control induced by insulin is consistent among treatment groups, regardless of glycaemic conditions. Therefore insulin's ability to modulate amantadine transport is independent of glucose concentration.

General biochemical dogma indicates that hyperglycemia results in increased intracellular ATP levels due to increased glycolysis and oxidative phosphorylation (Brownlee, 2001). Reasons for the observed effect of glucose on amantadine transport may be related with the energy status of the cell characterized by cellular ATP levels. Excessive catabolism results in the generation of high levels of ATP, which can then close ATP-dependent potassium channels (K^+_{ATP} channel) in endothelial cells (Nilius and Droogmans, 2001). Aggressive control of hyperglycaemia in patients can attenuate the development of chronic complications such as retinopathy and nephropathy (Moller, 2001). At present, therapy includes a spectrum of agents meant to enhance insulin release and action. One of these agents, sulphonylureas (ie glibenclamide), is an important component to a preventive regimen (Moller, 2001). Of specific interest to our argument is the fact that the mechanism of action of sulphonylureas is through the blocking of the

K^+_{ATP} channel (Moller, 2001). This notion reinforces the importance of the misregulation of the K^+_{ATP} channel in diabetes, and implicates it as a possible candidate through which ATP can affect organic cation transport.

Decreased outward potassium current associated with hyperglycaemic inhibition of the K^+_{ATP} channel can result in the depolarization of the cell. This effect in turn can result in the opening of voltage gated calcium channels (Nilius and Droogmans, 2001), which are known to be present in the HEK 293 cell model (Shaw et al., 2002). Work by our laboratory suggests that the amantadine transporter is inhibited by high levels of extracellular Ca^{2+} (5mM) (Escobar and Sitar, 1995), and by inference high levels of intracellular Ca^{2+}. Various pharmacological doctrines regarding amantadine's mechanism of action are at odds with one another: In regards to influenza A prophylaxis it is believed to affect endosomes; in regards to the nervous system (when used in the treatment of Parkinson's disease) one belief is that it is through Ca^{2+} antagonism by an unknown manner (Brenner, 2000); evidently, its mechanism of action is unclear. Acute hyperglycemia (60 minutes) causes a qualitative decrease in amantadine transport (data not shown). Prolonged hyperglycemia (24 hours), such as that demonstrated in **Figure R-6** would cause a chronic accumulation of Ca^{2+}. Therefore, it could be conceivable that the effect of hyperglycemia in modulating amantadine uptake is through altered Ca^{2+} levels, responding to K^+_{ATP} channel misregulation. The result would be decreased amantadine uptake because of the chronic presence of high levels of intracellular Ca^{2+}. In the absence of direct intracellular Ca^{2+}

measurements, it is uncertain as to the exact effects of high glucose on intracellular Ca^{2+} concentrations.

Pursuit into the underlying mechanism and speculations that ATP levels may be involved led us to investigate the effect of glucose starvation on organic cation transport. Reduced intracellular ATP content during ischemic/hypoxic conditions, such as that present in diabetic nephropathy, can be mimicked by intracellular dialysis with ATP-free solutions or application of extracellular glucose-free/NaCN solutions (Nilius and Droogmans, 2001). Under these conditions, increased whole cell and single-channel currents have been observed in endothelial cells from rat aorta and brain microvessels (Janigro et al., 1993). Lowering intracellular ATP or applying the K^+_{ATP} activator levcromakalim has also been shown to evoke unitary currents in rabbit aortic endothelial cells. These currents have additionally shown to be reversibly blocked by glibenclamide (a sulphonylurea) (Katnik and Adams, 1995; Katnik and Adams, 1997).

Mannitol is a biologically inert alcohol sugar used medically as an osmotic diuretic in the treatment of cerebral edema, acute glucoma and to promote the excretion of toxic substances (Brenner, 2000). By removing glucose (11 mM) from the buffer and replacing it with an osmotically equivalent concentration of mannitol (6.7 mM), we were able to create experimental glucose starvation conditions. Studies by our group examining whether acute glucose-free (starvation) conditions in the medium modulate

the levels of organic cation uptake indicate that an effect occurs at 30 minutes in CT and KHS buffers for all three transporters. In both rOCT1 and rOCT2 containing cells, we see a 50% reduction in TEA uptake upon starvation compared to parallel controls (glucose containing buffers) regardless of buffer treatment **(Figure R-7, R-8)**. The effect of 30 minute acute glucose starvation on amantadine transport in HEK 293 cells in CT and KHS buffers was also examined. Interestingly, we observe an increase in amantadine uptake in starved cells compared to control cells in both the absence and presence of bicarbonate. The effect of starvation on amantadine accumulation in the presence of bicarbonate is more pronounced compared to starvation in its absence **(Figure R-9)**. This effect and the explanation for it will be resolved in the next sections. From our findings, we may postulate that the effect of glucose starvation on organic cation transport is associated with the catabolic reduction of ATP generation, and the subsequent activation of the K^+_{ATP} channel currents. As ATP levels were not directly measured in this set of experiments, our observations instead serve as indirect evidence that energy level alterations are the principle cause of transport modulation.

The nature of those alterations are however unclear, and are further complicated by the discrepancy created by the absence of a high glucose effect on TEA uptake in contrast to glucose starvation in rOCT1 and rOCT2 cells. This finding either reduces the importance of ATP and hence the K^+_{ATP} channel to TEA transport, or requires us to re-evaluate our understanding of how metabolic events affect these transporters. In order to understand this

observation, we must reintroduce unclarified intracellular events as the possible explanation. An earlier study examining the effects of several metabolic inhibitors on the uptake of the organic cation tri-n-butylmethylammonium (TBuMA), a structural relative of TEA, in isolated rat liver mitochondria, isolated rat hepatocytes and isolated perfused rat livers concluded that a rapid decrease in cellular ATP was achieved within 3 min of inhibitor addition (Steen et al., 1993). The initial uptake rate of TBuMA was generally largely affected by these treatments. However fructose (10 mM) on the other hand, had no effect at all on the uptake rate of the cation despite decreasing cellular ATP to an extent comparable to that after treatment with metabolic inhibitors. In isolated perfused livers, preloaded with TBuMA, the addition of metabolic inhibitors valinomycin or carbonylcyanide-m-chlorophenyl-hydrazone leads to a marked backflux of the cations from the liver into the perfusion medium. This observation suggests strongly that a large part of the intracellular storage capacity is lost after metabolic inhibitor treatment. The group therefore hypothesized that the metabolic inhibitors affected the initial cellular uptake rate through either altered intracellular sequestration (e.g. mitochondria) or alternatively by direct effects on the plasma membrane rather than by decreasing cellular ATP.

Our demonstration that high glucose can affect amantadine transport, and that the absence/presence of glucose can affect rOCT1, rOCT2 and the amantadine transport system indicates a regulatory role for glucose in organic cation regulation. Whether this effect is related to ATP levels still remains to be confirmed. However a product of ATP, cAMP, a molecule

implicated in eukaryotic glucose-sensing machinery (Johnston, 1999; Thevelein and de Winde, 1999; Dumortier et al., 2000; Rolland et al., 2000) and metabolic regulation, is further explored as the possible mechanism in the next section.

IV. cAMP and Organic Cation Transport

In previous sections, we demonstrated that insulin and glucose modulate organic cation transport. We also implicated an undefined role for energy metabolism in that modulation. In the present section we attempt to demonstrate that cAMP, a conserved regulator of metabolism, may be differentially regulating organic cation transport. In addition, we propose that insulin, glucose and bicarbonate may be differentially regulating organic cation transport systems via cAMP-dependent PKA phosphorylation.

Classic biochemistry dogma states that glucose is stored in the body as glycogen, and that the balance between glycogen synthesis and breakdown is controlled by the hormones glucagon and insulin. These hormones, by regulating the levels of cAMP in their target tissues, determine the ratio of active to less active forms of glycogen phosphorylase and glycogen synthase (Lehninger et al., 1993c). Insulin's contribution to this control system lies in its capacity to reduce cAMP levels (Butcher et al., 1966; Butcher and Sutherland, 1967), thereby inhibiting cAMP-dependent PKA, which in turn stimulates glycogen synthase by promoting its dephosphorylation. The nature of that

interaction is not through any effect on phosphodiesterases, and has instead been suggested to affect the synthesis of endogenous cAMP levels (Blecher et al., 1968); thus, it's mechanism has never been clarified. Insulin is generally postulated, nonetheless, to cause a reduction in cAMP levels of target tissues. This connection, we now propose, is a metabolic one that links the end product of catabolism, CO_2, and cAMP

It is well established that the insulin receptor regulates intermediary metabolism and its organization in the cell; cAMP serves as an important signal in that regulation. Several previously mentioned factors however complicate our pursuit into insulin's underlying mechanism: For one, there is considerable homology among insulin, IGF and insulin receptor-related receptors (IRR) (Patti and Kahn, 1998); another factor is the capacity of all three receptors to form functional hybrids (Patti and Kahn, 1998; Butler and LeRoith, 2001); and last, is the ability of insulin and IGF hormones to interact with all three receptors and their hybrids (Patti and Kahn, 1998; Butler and LeRoith, 2001). Evidence exists to suggest that insulin receptors and IGF-1 receptors share a series of biological actions, while other effects are more unique. For example, IGF stimulation in contrast to insulin has been associated with increases in cAMP levels (Grellier et al., 1996). Yet, the addition of cAMP analogues alone have been shown to inhibit growth, decrease proliferation, increase differentiation, and induce apoptosis of cultured cells (Martin and Kowalchyk, 1981), suggesting a contrast to what would be expected given this second messenger's association with IGF action. Studies in insulin and IGF-1 receptor knockout mice indicate that insulin

generates a metabolic action mainly through its receptor, while the IGF-I receptor would preferentially have an impact on growth and differentiation (Liu et al., 1993; Accili et al., 1996a; Accili et al., 1996b; Joshi et al., 1996). Another study obtained evidence that the two receptors can achieve a similar final biological endpoint by using different signalling pathways (Miele et al., 2000). These differences are very likely due, at least in part, to different receptor expression patterns in distinct cell types.

Therefore, insulin's biological effects in our chosen kidney cell-model are unclear. Include the fact that these cells appear to be of a neuronal lineage derived from the kidney (Shaw et al., 2002), and one thereby introduces new concerns associated with neural cells and metabolism. Contrary to old assumptions, insulin's action in tissues not normally considered insulin sensitive, such as the brain and pancreatic B-cells, is becoming increasingly appreciated for its roles in glucose homeostasis (Bruning et al., 1998; Kulkarni et al., 1999). The nature of those interactions still requires clarification. Nonetheless evidence from our data, specifically the qualitative parallel increases/decreases in organic cation transport between insulin and cAMP analogues, and the existance of additive effects between the two, suggests that the hormone either acts to increase cAMP levels, or the two somehow complement one another's action in the HEK 293 cell model. This interpretation appears to agree with those aforementioned findings that indicate cAMP potentiates IGF responses both at the protein and at the gene level (Cohick et al., 2000; Sugawara et al., 2000). Numerous factors come into play in a cell's decision to shift its machinery towards a metabolically active

resting phase opposed to a growth phase. Our findings appear to indicate that our model system undergoes stimulation of metabolism rather than growth. Given that our cells were used near confluency (90%) with the possibility of contact growth inhibition, metabolism rather than growth may arguably predominate insulin/IGF signal transduction. In conclusion, we have evidence that indicates insulin's effect on transport in HEK 293 cells during experimental studies to be both metabolic events associated with the cell's energy status and linked to intracellular cAMP levels. Future experiments using fluorometric methods to measure cAMP levels may help to confirm these observations.

cAMP is an ubiquitous metabolic signal generated from the enzymatic action of adenylyl cyclases on ATP. It is therefore a second messenger signaling system that is intrinsically linked to energy metabolism, and apparently to the organic cation transport system as well. Our data indicate that the activities of organic cation transporters are modulated in response to elevations of intracellular cAMP. More importantly, this molecule may be the shared mechanism by which insulin, glucose and bicarbonate are regulating OCTs. Acute studies into the effects of membrane-permeable cAMP analogues Br-cAMP and dibutyryl-cAMP show that increasing intracellular levels by this method results in the significant inhibition of TEA uptake in rOCT1 cells in both buffers (**Figure R-10**) and in rOCT2 cells in KHS buffer alone (**Figure R-11**). No significant effect however is seen on amantadine transport in either buffer (**Figure R-12**). As was previously mentioned,

insulin alone is unable to significantly affect acute transport activity among the three transporters aside from qualitative observations. But when cAMP analogues are added in addition to insulin, we observe the additive effects of the metabolic signals that results in further inhibition of TEA uptake in rOCT1 in both buffers and in rOCT2 in KHS buffer alone, whereas amantadine transport increases significantly from control in both buffers and could even be described as synergism. Yet, its relative level in the presence of bicarbonate is consistently higher compared to its absence. Reasons for the observed OCT transport discrepancies between the two buffer systems may be explained in the events surrounding the disruption of cellular CO_2/HCO_3^- equilibrium.

Movement of organic cations inside cells, from the basolateral to the luminal side, may involve diffusion especially in mammalian tubules under physiological conditions. Under some circumstances in mammalian tubules, such as when the metabolic state is compromised, transport of organic cations may involve accumulation in some form of vesicles, which then move across the cells. Vesicle accumulation for organic anion fluorescein during a state of metabolic compromise induced by bicarbonate free buffer (Tris, HEPES or phosphate) in contrast to bicarbonate/CO_2 buffered medium has been reported (Dantzler et al., 1995). A similar situation can be conceived for organic cations. The observed discrepancy between insulin's inability to acutely alter organic cation transport and its ability long-term to alter organic cation transport may lie in secondary metabolic dysregulation induced in the cell, specifically in alterations in vesicle formation and trafficking. Moreover,

the presence of a long-term effect and the absence of an acute effect may suggest that alterations in metabolism and the generation of CO_2, as they require time to develop, may be at the cause of dysregulation, followed then by alterations in cAMP signalling. Of relevance to our argument is a previous study that showed that insulin increases pHi and CO_2 in a dose-dependent manner in rabbit tubules while constant extracellular pH conditions were maintained (Takahashi et al., 1996). As a result of the change in the evolution in CO_2, there would also be a change in the metabolic derived HCO_3^- signal because of insulin's activity. It must be borne to mind that pHi is a relationship among partial pressure of CO_2 (pCO_2) with water, concentrations of HCO_3^- and H^+, and is inherently tied to cellular metabolism **(Figure D-1)**. All cells regulate their intracellular pH (pHi) and often alter their behavior in response to changes in pH. The reaction catalyzed by CAs in response to energy metabolism is unique in that, with increasing pCO_2, it produces HCO_3, a base, and simultaneously lowers pHi (**Figure D-1.1**). By contrast, when metabolic processes generate a proton (pHi decrease) under constant pCO_2, HCO_3^- in the cell is depleted to restore pHi (**Figure D-1.3**). The opposite changes (decreasing pCO_2 (**Figure D-1.3**) or increasing pHi (**Figure D-1.4**)) also alter HCO_3^- , suggesting that any metabolic process modulating pHi, proton concentration or pCO_2 represents a putative modulating signal for sAC. Little is known about intracellular events surrounding organic cation transport, except that accumulation and protein binding have been reported (Pritchard and Miller, 1993). Movement of organic cations inside the cell, and across the luminal membrane into the lumen is clearly mediated in some fashion, but the nature of such mediated transport is not adequately

understood nor is its regulation. Thus, the degree of accumulation in intracellular compartments may not be a factor in transport under physiological conditions, but in a pathophysiological condition such as diabetes, it may prevail.

As we progress from a physiological to a molecular understanding of the role of bicarbonate in the regulation of organic cation transport, we begin to understand previous findings from our laboratory and their diabetic implications in a different light. The major determinant of the intracellular availability of bicarbonate is the metabolic status of the cell. In untreated or poorly regulated diabetes mellitus, a common complication is the development of ketoacidosis. This condition is characterized by metabolite abnormalities, two of which are low bicarbonate and high lactate levels (Foster and McGarry, 1983). Of particular interest is that these metabolite abnormalities are symptoms of disordered glucose metabolism. Normally, the final byproduct of the catabolism of glucose after shunting its products into the Krebs cycle and the electron transport chain is CO_2. This metabolically generated gas undergoes hydration to form carbonic acid, which then instantly dissociates into a proton and a bicarbonate anion. This is a naturally occurring event. However, this step is also catalyzed by the cytosolic enzyme carbonic anhydrase (CA). CA is ubiquitous, but is also abundant in the cytosol of proximal tubules. Evidence by our laboratory demonstrates that inhibition of CA with azetazolamide, thereby reducing intracellular CA-derived bicarbonate, had no effect on amantadine transport in proximal tubules (Escobar et al., 1994). A speculation by Goralski et al, (1999)

suggesting the possibility that bicarbonate could be metabolically derived now appears supported by our data. Previous findings that the bicarbonate-energy-dependent amantadine transporter is inhibited by high lactate levels(Escobar et al., 1995), can also be re-evaluated. If the final product of glycolysis -pyruvate - isn't shunted into the Krebs cycle and instead undergoes anaerobic metabolism, it then becomes the terminal electron acceptor of the electron transport chain and is reduced to lactate. Such an event occurs in the absence of O_2, or in the case of diabetes because of some other unknown trigger. Regardless of the cause, metabolically evolved CO_2 levels would fall and hence intracellular HCO_3^- levels. Additionally in static metabolism, outside pH fluctuations such as increasing extracellular H^+ (consistent with lactic acidosis) would require the consumption of intracellular HCO_3^- to stabilize intracellular pH. Intracellular CO_2 generation is a crucial component in pH regulation. Loss of HCO_3^- to stabilize pH would likely represent a feedback signal that disrupts glycolysis further upstream and hence CO_2 generation. Indeed, findings that the disruption of the CO_2/HCO_3^- equilibrium blocks glycolysis in muscle supports such a notion (Ko and Paradise, 1970a; Ko and Paradise, 1970b; Ko and Paradise, 1971b; Ko and Paradise, 1971a; Moore et al., 1979). In the circumstance of metabolic dyregulation, the alteration of the metabolically derived bicarbonate signal could be sufficient to affect organic cation transport rate.

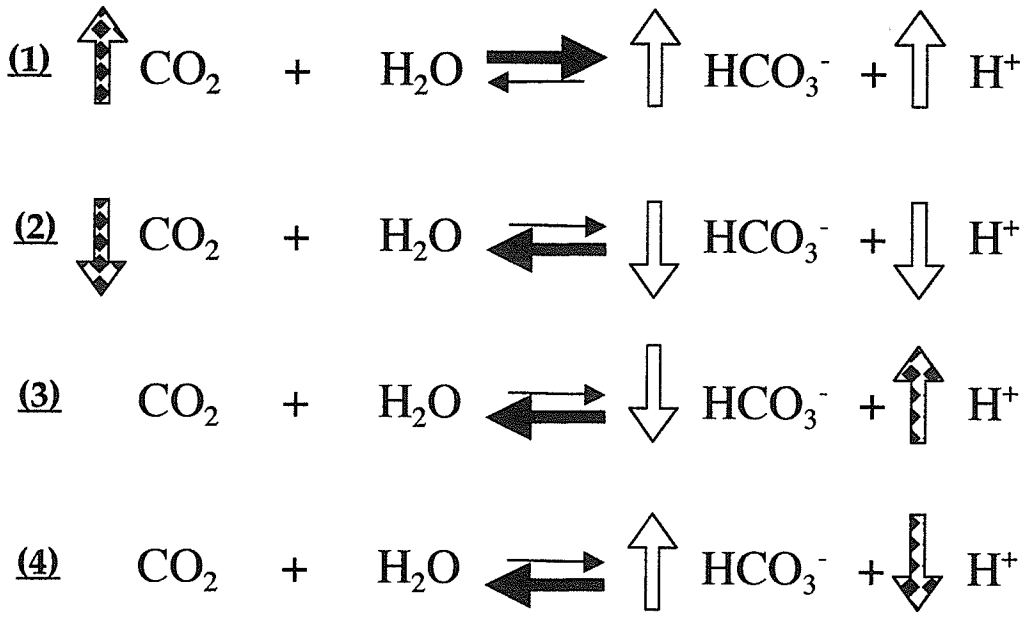

Figure D-1. The relationship between CO_2, pHi and HCO_3^-. (1) increase in pCO_2. (2) decrease in pCO_2. (3) decrease in pHi under constant pCO_2, and (4) increase in pHi under constant pCO_2. In each example, the component changed is the checkered arrow and the resulting changes are in white.

Adapted from (Zippin et al., 2001)

Effects of bicarbonate containing buffer modulating OCTs and speculations of undefined metabolic events have been indicated throughout this dissertation. We now propose that glucose, insulin (through their effects on CO_2 generation) and bicarbonate may be mediating those events on organic cation transport via a cAMP mechanism and through cAMP-dependent PKA phosphorylation. The discovery of two classes of adenylyl cyclases: the membrane located tmACs and the cytoplasmically located sACs, the latter being uniquely regulated by the bicarbonate ion (HCO_3^-), provide new possibilities in cAMP signaling within cells. These two sources of intracellular cAMP serve to generate localized signals through multiple effector proteins, including EPAC, cNGC and PKA. cAMP primarily exerts its metabolic effects in animal cells mainly by activating PKA. Regulation of organic cation transport by PKA has been predicted and demonstrated in rOCT1 and rOCT2 (Grundemann et al., 1994; Grundemann et al., 1998; Mehrens et al., 2000). The two identified isoforms of PKA have been indicated to display varying sensitivities to cAMP, with the predominantly cytosolic PKA I being more sensitive and the AKAP-organelle/membrane/cytoskeleton associated PKA II less responsive (Doskeland et al., 1993; Skalhegg and Tasken, 2000; Feliciello et al., 2001). Unravelling these complex signalling networks in the cell will require further determination of the functional importance of PKA isoform diversity and signalling through organized microdomains.

Interpretation of our data begins to indicate some of those aforementioned signalling complexities. From our data, we demonstrate that

rOCT1 inhibition of TEA uptake by insulin and cAMP occurs despite the presence of bicarbonate in the buffer; though in its presence there is some evidence of the strengthening of the additive effects of insulin-cAMP. rOCT2 on the other hand, is significantly inhibited both by cAMP analogues and insulin together only in the presence of bicarbonate. Examination of the data suggests that lower levels of cAMP are required to inhibit rOCT1, while higher levels are required to achieve rOCT2 inhibition. In the case of the amantadine transporter, we show that significance in stimulation is achieved only when cAMP analogue and insulin are added together in either buffer system, but is considerably enhanced in the presence of bicarbonate. Therefore, the sAC-bicarbonate-induced production of cAMP represents an additional mode of signal transduction that is best described as contributing to baseline of rOCT1, rOCT2 and amantadine transporter activity, as bicarbonate is always present *in vivo*. If one presumes that high internal levels of cAMP are the result of our cAMP/insulin/bicarbonate treatments, then one begins to speculate about the role of the different PKA isoforms in OCT regulation. For instance, it is plausible that the more sensitive PKA I is responsible for rOCT1 inhibition, while the less sensitive PKA II is regulating rOCT2 and the amantadine transporter. Perhaps with the development of isoform specific activators/inhibitors, future experiments will be able to differentiate their unique contribution to OCT signaling.

Recent experimental evidence supporting our insulin-cAMP-OCT proposal comes from the discovery that the activity of adenosine kinase, which catalyzes the phosphorylation of adenosine to AMP (a precursor to

ATP and cAMP), is significantly impaired in STZ-induced diabetic rat heart, liver and kidney tissues (Sakowicz and Pawelczyk, 2002). More importantly, is the finding that insulin corrects this impairment and its effect on the expression of adenosine kinase, and is not related to glucose level (Sakowicz and Pawelczyk, 2002). Under normal conditions, most of the adenosine formed in the cell is phosphorylated to AMP by adenosine kinase (Kroll et al., 1993). Intracellular concentration of adenosine would depend not only on metabolism of this compound, but also on its transport across the plasma membrane. The mammalian nucleoside transport system consists of two categories of uptake processes, namely equilibrative (ENT) and concentrative transport (CNT). RNase protection assays of STZ-induced diabetic rats demonstrated that mRNA levels of ENTs and CNTs in the kidney were altered in diabetic tissues (Pawelczyk et al., 2003). Given that many important physiological functions are attributed to adenosine and cAMP, sustained elevated levels of adenosine, inhibition of adenosine kinase, and subsequent reduction in AMP, ATP and cAMP may lead to the pathological changes observed in diabetic tissues. We only introduce this correlation between adenosine levels, insulin and diabetes as further evidence of a possible link among diabetes, cAMP and organic cation transport. Clearly, further investigations are indicated.

In summary, we've demonstrated that bicarbonate and cAMP analogues alone and in addition to insulin acutely modify OCT regulation. Insulin's effect on glucose metabolism, the generation of CO_2, HCO_3^- and hence sAC-derived cAMP contributes to the baseline cAMP signal. The

presence of additive effects in our data reinforces this complementary mechanism of action. The differential response of OCTs to that signal will be further unravelled in the next section.

V. Determination of tmAC and sAC Contributions to Intracellular cAMP Pools Affecting Organic Cation Transport

In previous reports, our laboratory established that bicarbonate is a modulating factor in amantadine transport (Escobar et al., 1994; Goralski and Sitar, 1999). Chronic and acute bicarbonate administration reduced the renal excretion of amantadine in humans (Geuens and Stephens, 1967). Similar effects of sodium bicarbonate loading on decreasing the renal clearance of other organic bases in rats and dogs have also been reported (Torretti et al., 1962; Weiner and Roth, 1981). Work by Ulrich et al (1991) showed a modestly higher uptake of NMN in the presence of bicarbonate in rat tubule cells using stop-flow capillary perfusion methods. This later report suggests the plausibility of bicarbonate modulation of NMN or TEA characterized organic cation systems. In the previous section, we proposed that the hormone insulin is mediating its effect through two intracellular sources of cAMP, tmAC and bicarbonate-responsive sAC, which in turn are responsible for regulating OCT activity. In this section we attempt to dissect contributions of bicarbonate-responsive sAC and tmAC to the intracellular cAMP pools regulating organic cation transport.

Contributions of cAMP from tmACs and sAC to TEA and amantadine uptake are differentiated in our studies by using forskolin, and the presence/absence of bicarbonate in the buffer. tmACs are G-protein and forskolin sensitive, while sAC is insensitive to G-protein and to forskolin, and is uniquely regulated by bicarbonate ion (HCO_3^-), and not via carbon dioxide (CO_2) or a modulation of pH (Chen et al., 2000). Forskolin is a membrane permeable labdane diterpenoid prepared from *Coleus forskohlii* roots, possessing antihypertensive, positive ionotropic, G protein, and tmAC activating properties (de Souza et al., 1983). Many of its biological effects are due to its activation of adenylate cyclase, the resulting increase in intracellular cAMP concentration, and subsequent PKA activation. An important consideration when examining the data, is that forskolin has also been shown to affect calcium currents, phospholipid/Ca2+ dependent protein kinase (PKC), and MAP kinase (Siddhanti et al., 1995). Evidence also indicates that low levels of PKA activation result in an increased extent and rate constants for IP_3-induced Ca^{2+} release, while at higher concentrations of PKA the extent and rate constants decrease (Dyer et al., 2003). Attention must be paid to separating PKA effects from PKC effects. Moreover substrates for kinases differ in different cell types, which is why their effects may vary depending on the target cell (Burns-Hamuro et al., 2003). Therefore, care should be taken in the interpretation of the signalling data. In the case of our HEK 293 model we were unsure of forskolin's non-specific effects.

When our group examined PKA stimulation using forskolin, the results paralleled our cAMP studies. rOCT1 and rOCT2 were generally inhibited but in a diminished capacity, while no effect was seen on amantadine transport in either buffer system. Our findings regarding forskolin are in keeping with the report that elevations of cAMP produced by this compound are traditionally lower than that found by using cAMP analogues (Seamon and Daly, 1986). Conceptually, this observation is understandable in terms of the limiting presence of substrate ATP required by tmAC to generate the necessary cAMP levels; this is direct contrast to the whole-cell elevations of the cAMP signal generated by its analogues, which unlike forskolin's activity are not limited by the presence of ATP. Consequently the reduced inhibitory effect of forskolin compared to cAMP analogue alone, as demonstrated by our data, is consistant with literature. Upon interpretation of our data, two novel aspects of OCT regulation become apparent: amantadine uptake was only increased in the presence of bicarbonate, and in the case of rOCT1 and rOCT2, bicarbonate seems to mute the inhibitory effect of forskolin, partially in the former and completely in the latter. Therefore, the data would suggest that sAC is both the primary contributor to amantadine transporter regulation and a possible source of OCT modulation. tmAC generated cAMP pools on the other hand appear to affect rOCT1 and rOCT2 exclusively.

In order to clarify signal transduction involved with OCT regulation, we used the protein kinase inhibitor staurosporine. The main intent of using it was to confirm PKA to be the site of action of forskolin generated cAMP

pools, and to eliminate the fear of nonspecific PKC activation further modulating transport function. When we examined the effect of staurosporine alone and in combination with 10 μM forskolin, we see that staurosporine blocked the effects of forskolin in rOCT1 and rOCT2 transfected cells in both buffers suggesting a role for PKA activation in the activity of forskolin. However care should be taken in the interpretation of the data, as staurosporine is not completely specific. In fact staurosporine is an antibiotic used traditionally in the inhibition of PKC, but has been shown to inhibit the PKA system as well (Bode et al., 1999). Even H-89 a more specific PKA inhibitor has been shown to affect calcium levels and PKC (Rosado et al., 2002). Possible cross talk between PKA, PKC and the calcium system such as that demonstrated in neuronal cells may be responsible for such an observed complexity (Kubota et al., 2003). Interestingly, sAC has also been shown to be modulated by calcium levels (Litvin et al., 2003); hence there is a synergism of signals (Ca^{2+} /HCO_3^-) leading to intracellular cAMP levels that in return can affect calcium, perhaps through cyclic-nucleotide gated channels and PKC. In our experimental situation, we took advantage of the nonspecific effects of staurosporine in an attempt to clarify signalling by removing calcium currents and PKC modulation from the picture. Future experiments should probably be performed using PKA inhibitor peptides to corroborate our PKA inhibition, and later with isoform specific inhibitors as they become available. Moreover, PKC activity and the possibility of bicarbonate modulating this signal should also be evaluated with respect to OCT function.

As for the definitive cause of the buffer effect on rOCT2, it eludes us at this time. We can propose several plausible explanations for the buffer effect. One may lie in the knowledge that various solvents, including ethanol, inhibit the activation of adenylate cyclase by forskolin (Huang et al., 1982). Presently the reason for the inhibitory action of solvents is unknown. Our forskolin mixtures were dissolved in DMSO, as is suggested by literature, to ensure its activity. Perhaps futher dilution in bicarbonate-containing buffer inactivates forskolin because of chemical solvent-solute interactions. Another possibility exists with the notion that certain compounds in solution alter cAMP baseline signaling. For instance, it has been shown that acute administration of ethanol increases intracellular cAMP levels in pancreatic, airway smooth muscle and other tissues by an unknown mechanism (Forget et al., 2003; Yamamoto et al., 2003; Yang et al., 2003). Conceptually one can envision a similar situation in the presence of bicarbonate, where its addition modulates forskolin-induced signal transduction in an undefined way. While it can give the appearance of inactivation, in reality we may be observing a masking effect through alterations of cAMP baseline signaling. Hypothetically, we may also be observing effectors' biphasic interpretation of two opposing cAMP signal pools. Older models suggesting diffusion of second messengers complicate selective activation of distal targets or generation of unique responses to distinct extracellular signals (Zippin et al., 2001). Diffusion-dependent models also necessitate conversion of greater amounts of ATP into cAMP to reach effective levels at distant targets. Cytosolic localization of sAC suggests a model whereby cAMP can signal in a complex consisting of both source and effector. How cAMP effectors interpret signals when intracellular signalling

pools combine (resulting in signal synergism or nullification) remains still unresolved.

Second, we can speculate about the functional importance of PKA isoform diversity and signalling through organized microdomains. Unravelling the complex signalling networks in the cell will require further determination of the role of those isoforms. Recent quantification methods, based on stimulation of cyclic nucleotide gated channels, indicate that local elevations of cAMP are far greater than the increases measured in whole cells (Rich et al., 2000). As was previously mentioned, elevations of cAMP-dependent PKA activation produced by forskolin are traditionally lower than those seen by using cAMP analogues. We already indicated that PKAI is more sensitive to cAMP, while PKAII is less sensitive. Both interact with the membrane. In contrast to the broad activation of cAMP activated effectors by the use of our cAMP analogues and bicarbonate, our forskolin-bicarbonate studies may hint at increased signal specificity. Perhaps, it is at this smaller, but more specific concentration generated by tmAC alone and in addition to sAC that cAMP microdomain(s), which allows specific activation of individual cAMP effectors, begin(s) to become differentiable at least in form.

Third, further complexity may lie at the level of protein phosphorylation. Effects of PKA stimulation on effector targets have indicated that some proteins show biphasic responses to activation by kinases. For example, the effects of PKA on type I isoform of IP_3 receptor has been shown to demonstrate a biphasic relationship dependent upon the

concentration of PKA used. At low concentrations of PKA, both the extent and rate constants of IP_3-induced Ca^{2+} release increased, while at higher concentrations the extent and rate constants decreased (Dyer et al., 2003). Structurally, the type I IP_3 possesses two sites for phosphorylation (S1589 and S1755) (Ferris et al., 1991), and these sites possess different potentials for phosphorylation, with S1755 requiring lower concentrations of PKA to become phosphorylated compared to S1589 (Dyer et al., 2003). The phosphorylation and biphosphorylation of histones at two sites has also become a recently appreciated level of information codification coined epigenetics (Jenuwein and Allis, 2001; He et al., 2003; Staynov et al., 2003). OCTs could also be regulated in a similar capacity. Computer structural analysis of potential phosphorylation sites by protein kinases indicate that rOCT2 possesses two PKA phosphorylation sites at S-334 and S-544 (Grundemann et al., 1998). While there are no reports for predicted phosphorylation sites of rOCT1 in the original publication (Grundemann et al., 1994), hOCT1, which possesses homology with its rat counterpart, is predicted to possess two PKA phosphorylation sites (Gorboulev et al., 1997). Thus, at low levels of cAMP, such as seen with forskolin treatment of rOCT2-containing cells in CT buffer, we see an inhibition of TEA uptake, while in the presence of bicarbonate (KHS buffer) we see the suppression of any inhibitory effect by forskolin. Patterns in phosphorylation may be at the root of this discrepancy.

Lastly, the role of calcium currents induced by forskolin and bicarbonate generated cAMP is unclear. hOCT 2 has been demonstrated to be

strongly regulated by calcium dependent kinases (CAM kinases) in an inhibitory capacity (Cetinkaya et al., 2003). As a result, we may speculate that there may be a further modulation of rOCT2 by CAM kinase; either activated by forskolin or bicarbonate, through cyclic nucleotide gated channels, or because of PKA phosphorylation.

Evidently, understanding the regulation of OCTs will require the refinement of tools used to study signalling, both phosphorylation site-specific antibodies to OCTs and isoform specific pharmacological agents. In addition, there must be an evolution of imaging techniques used to examine cAMP. Only then will we be able to begin to elucidate information codification among the cellular architecture. At present several fluorescent probes are available for cAMP, but all possess significant problems. FlCRhR, a holoenzyme constituted of PKA with fluorescein and rhodamine respectively labelled catalytic (C) and regulatory subunits (R), faithfully records cAMP changes, but ruptures the cell membrane upon introduction (Adams et al., 1991). The genetically encoded GFP-tagged probe based on the fusion of GFP-C and BFP-R subunits, requires equimolar concentrations of both to target to the same cellular region. Thus, it is unable to display a significant ratio change following an increase in cAMP concentration (Vincent and Brusciano, 2001). The ART probe, on the other hand, is not exactly a cAMP probe but reports the phosphorylation level of a peptide specifically phosphorylated by PKA (Nagai et al., 2000). Even though genetically encoded probes may represent a great potential for the future, sensitivites of

these and other cAMP probes need to be improved to address the subtle microdomain signaling that is becoming increasingly apparent.

In summary, our present studies reflect those previous observations that bicarbonate's major importance lies in the modulation of the portion of the organic cation system identified by the cationic marker amantadine, and not TEA; however, they also establish a basis for possible baseline modulation of rOCT1 and rOCT2 activity. Development of phosphorylation site-specific antibodies to OCTs may help future signal elucidation of OCT regulation. At present, our forskolin-bicarbonate studies suggest that the bicarbonate-dependent component that has previously been reported to modulate organic cation transport, specifically amantadine transport, is sAC.

GENERAL SUMMARY

A complete model of drug transport in the kidney, and specifically organic cation transport, will require the identification, localization and characterization of all transporters putatively involved; only then will we have the ability to begin to understand the full interplay between transporters and regulatory elements as they pertain physiologically and pathophysiologicaly to renal drug excretion. In our case, the absence of the molecular identification of the amantadine transporter(s) has prevented us from assigning its apical/basolateral polarity, or examining its levels of expression. Therefore, the model is far from complete. Yet, this dissertation is an important contribution toward the desired medical goal of individualizing drug therapy.

The origins of this dissertation owe themselves to observations of the misregulation of organic cation transport systems in the pathophysiological state of diabetes. We followed those observations into the realm of the physiological and later into that of the molecular in pursuit of a plausible mechanism. Our investigations into the mechanism for the diabetic regulation of OCTs resulted in findings that cAMP generated from membrane and cytosolic sources act as fundamental modulators of transporter function. In addition, metabolism and CO_2/HCO_3^- equilibrium, and insulin's effect on that relationship, are intrinsically linked to cAMP levels because of sAC. Evidence of acute changes is suggestive of rapid and important signal

transducing events. However, the effect(s) at the level of gene expression, and particularly at the cAMP response binding protein (CREB) are unclear.

An underlying concept that unifies the regulation of organic cation transport systems is that modulation of their function occurs to the extent that physiological and pathophysiological events are tied to cAMP levels in the cell. The increasingly apparent complexity of this microdomain signalling system is sure to be the source of these subtle regulatory variations. Nonetheless, they are still inherently rooted in a single basic regulatory unit - cAMP. If diabetes results in the decreased accumulation of organic cations such as TEA and a "protection phenomenon" associated with nephrotoxic drugs, then it is likely reflective of an altered energy-profile that in turn is affecting the kidney's ability to detoxify the body. On the other hand, the handling of drugs by the amantadine transporter is surprisingly enhanced. Correlation of our findings to a pathophysiological state deserves extrapolation. In the case of diabetes, there is a primary dysfunction in energy metabolism. Reduction in transluminal flux, or increased mitochondrial and vesicular sequestration of organic cations may be interconnected with intracellular accumulation; all of which may be unified by cAMP/ATP levels in the cell. Review of literature reveals that it is obvious that very little is understood about the luminal transport step or intracellular transport. Alterations in cAMP/ATP levels in diabetes may reduce P-glycoprotein function at the lumen, resulting in the reduction of efflux into the lumen. cAMP levels on the other hand may alter OCT function at the basolateral step. Disruption of metabolism, and additionally CO_2/HCO_3^-

equilibrium, and the cAMP associated signal may act to disturb those processes. The clinical relevance of these interactions deserves further investigation.

We are at the verge of understanding the role of drug transporters in endothelial/epithelial cells and to appreciate that these proteins may be important targets for the pharmacological modulation of fundamental cell functions. Indeed, we are at the very beginning of considering cell drug transporters as targets of novel therapeutic and nontherapeutic compounds to control drug entry and the driving force behind that entry. In addition, drug transporters might be the cause for cell dysfunction linked to human disorders. To catalyze a further understanding of cell physiology and pathophysiology, it is necessary to also include the expanding field of drug transporters expressed in endothelial/epithelial cells in the general interest of vascular cell biologists.

CONCLUDING REMARKS

In conclusion, I wish to propose a caveat, one cannot help but speculate about the putative linkage between the accumulation of dangerous synthetic and natural xenobiotics (including allergens) and the pronounced changes in human environment (specifically in reference to CO_2 gas emission), behaviour and lifestyle that has accompanied globalization. Could the modulation of the bicarbonate signal as a result of environmental toxins that act as metabolic inhibitors, or changes in pCO_2 levels in the global environment (with the corresponding changes in blood pH and cell pHi), have any association with the escalating rates of obesity, diabetes, childhood cancer, asthma and allergies? Moreover, could the effect of this signal be further exacerbated in pediatric patients possessing immature detoxification systems? Additional research on the effects of these factors on our ability to eliminate organic cations, anions and hydrophobic compounds by the kidneys of pediatric and geriatric populations would prove to be of great clinical insight in perhaps understanding the aetiology of such chronic diseases, whereas learning to stimulate these processes may become useful in their prevention and treatment.

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