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Dopamine coordinates the effect of natriuretic and antinatriuretic factors

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The cells of our body are sustained in a fluid which salt content resembles that of the primordial sea where we assume we had our origin. At that time, several billion years ago, it is believed that the sea water contained one third of the salt found in present day sea water and that this resembles our extracellular milieu. *Strauss, 1957*

Till min älskade son Adrian

Abstract:

Living organisms are dependent on a precise regulation of water and sodium. The stability of the internal environment is maintained through a series of feedback mechanisms, in response to changes within the organism as well as to changes in the external environment. Many organs in the body participate in sodium- and water turn over, but the kidney is the only organ in the body that excretes or retains sodium and water in a regulated fashion. Salt retention is a risk factor in the development of hypertension which may lead to renal insufficiency, heart failure and cerebrovascular catastrophes. The traditional view has been that hypertension is caused by an excess of factors that produce vasoconstriction and sodium retention. This hypothesis has been modified after reports showing that a low availability of vasodilative, natriuretic factors also predisposes to hypertension. The precision by which sodium balance is regulated suggests an intricate interaction between modulatory factors released from intra- and extrarenal sources. Intrarenally produced dopamine has a central role in this interactive network. Dopamine acts as an autocrine and paracrine factor to inhibit the activity of renal tubular Na^+, K^+-ATPase as well as of a number of tubular sodium influx pathways. Other natriuretic factors activate the renal dopamine system via a heterologous recruitment of dopamine-1 like (D1R) to the plasma membrane, whereas dopamine counteracts the effect of antinatriuretic factors via unknown mechanisms. Prolactin regulates fluid transport across the plasma membrane by unknown mechanisms. Prolactin interacts with dopamine in various tissues. Here we report that prolactin induced a dramatic nine fold increase in urinary sodium excretion associated with a decrease in renal proximal tubular Na^+, K^+-ATPase activity. These effects were abolished by a D1R antagonist. We found that prolactin signals via similar pathways as D1R in the renal proximal tubules, including protein kinas A, protein kinase C and PI3 kinase activation and that prolactin induced a heterologous recruitment of D1R to the plasma membrane. These results suggest that the renal dopamine system has a permissive role for prolactin. Dopamine acting on the D1 family of receptors, and angiotensin II, acting on AT1 receptors, exert opposite effects on sodium excretion. Recent studies have shown that the AT1 receptor and the D1 receptor form a dimer, where they act as a unit of opposites. Here we report that the power of the AT1 receptor and D1 receptor interaction was increased by the AT1 receptor antagonist losartan**.** Losartan caused significant increase of the plasma membrane expression of D1 receptors. We conclude that the effect of losartan bound AT1 receptors on D1 receptor plasma membrane expression can be attributed to the function of the AT1 receptor-D1 receptor heterodimer. Taken together these results indicate that losartan will, by binding to the AT1 receptor, exert allosteric effects on its protomer, the D1 receptor, resulting in activation of D1 receptor signaling. Allosteric modulation within a heterodimer, where a structural modification in one protomer will affect the structure and function of the other protomer, has been intensively studied in the past decade, and is generally considered to be an important indirect mechanism for control of receptor function. To test the concept of allosteric interaction between the losartan bound AT1 receptor and the D1 receptors in an in vivo model, we compared the antihypertensive effects of losartan alone and with co-treatment of losartan and a D1 receptor antagonist in rats with experimental hypertension. We found that addition of a D1 receptor antagonist significantly attenuated the antihypertensive effect of losartan. Not only G protein coupled receptors but also a classic tyrosine receptor, the prolactin receptor, exert its salt-regulating effect by heterologous recruitment and associated activation of renal D1 receptors. The finding that an AT1R antagonist can activate D1R signaling and that this effect is dependent on AT1 receptor and D1 receptor interaction is a novel finding and has potential pharmacologic implications.

List of original papers

I. Ibarra F, Crambert S, Eklöf AC, Lundquist A, Hansell P, Holtbäck U.
Prolactin, a natriuretic hormone, interacting with the renal dopamine system
Kidney International 68: 1700-1707, 2005.

II. Crambert S, Sjöberg A, Eklöf A-C, Ibarra and Holtbäck U.
Prolactin and dopamine 1 like receptor interaction in renal proximal tubular cells
Am J Physiol Renal Physiol. 2010 Jul;299(1):F49-54. Epub 2010 May 12.

III. Crambert S, Li D, Scott L, Eklöf AC, Zelenin S, Di Ciano L, Ibarra F, Aperia A
Losartan binding to angiotensin AT1 receptors confers allosteric modification of dopamine D1 receptors (manus)

List of Abbreviations

AADC	aromatic amino acid carboxylase
ACE	Angiotensin converting enzyme
ADH	Anti diuretic hormone
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
AT1R	Angiotensin 1 receptor
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
COMT	catechol-O-methyl-transferase
D1R	Dopamine 1 receptor
ECV	Extra cellular volume
GFR	Glomerular filtration rate
GPCR	G protein coupled receptor
GTP	Guanosine triphosphate
ICV	Intra cellular volume
JAK 2	Janus kinase 2
K^+	Potassium
MAO	Mono amino oxidase
Na^+	Sodium
PKA	Protein kinase A
PKC	Protein kinase C
RGS	Regulator of G protein signaling
STAT	Signal transducers and activators of transcription
SHR	Spontaneous hypertensive rat

Introduction

The primary role of the kidney is to keep constancy of the intra and extracellular milieu in the body. Whereas the glomerular filtration rate is rather constant within one individual from day to day the rate of tubular transport is very carefully regulated to adjust for changes in intake, needs and losses.

The precision by which sodium balance is regulated suggests an intricate interaction between modulatory factors released from intra- and extrarenal sources. Intrarenally produced dopamine has a central role in this interactive network. Dopamine acts as an autocrine and paracrine factor to inhibit the activity of Na^+, K^+-ATPase as well as of a number of sodium influx pathways.

Studies have shown that dopamine coordinates the effects of salt regulating hormones and peptides. Other natriuretic factors activate the renal dopamine system via a heterologous recruitment of dopamine-1 like receptors (D1R) to the plasma membrane, whereas dopamine counteracts the effect of antinatriuretic factors via unknown mechanisms.

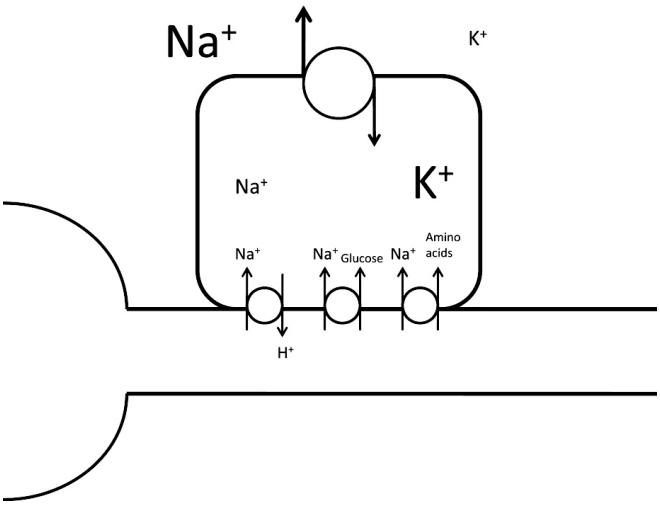
An inability to increase the renal dopamine tonus in situations of sodium retention and/or a defect in the dopamine signaling system would require an extensive adaptation of other salt regulating systems to maintain sodium balance and blood pressure normal.

Renal physiology

Sodium and water homeostasis

The volume and composition of body fluids are of outmost importance to a living organism. The stability of the internal environment is maintained through a series of feedback mechanisms, in response to changes within the organism as well as to changes in the external environment.

Our bodies are dependent on a precise regulation of water and sodium. The intake and excretion have to be precise to maintain the body homeostasis. In an adult human, approximately 60 % of the total body weight consists of water. This water is divided into an intracellular volume (ICV) of 2/3 and an extracellular volume (ECV) of 1/3. The content of electrolytes in these two compartments is very different. The major intracellular cation is potassium (K^+) and the major extracellular cation is sodium (Na^+).



Sodium homeostasis determines the volume of ICV and ECV. An ICV sodium deficit results in cell shrinkage, and opposite, ICV sodium retention leads to cell swelling and ECV sodium deficiency. ECV salt retention is a risk factor in the development of hypertension which may lead to renal insufficiency, heart failure and cerebrovascular ischemia. The internal milieu is dependent on the ability of the kidneys to retain or excrete water and sodium, and the kidneys ability to adjust this to the daily intake.

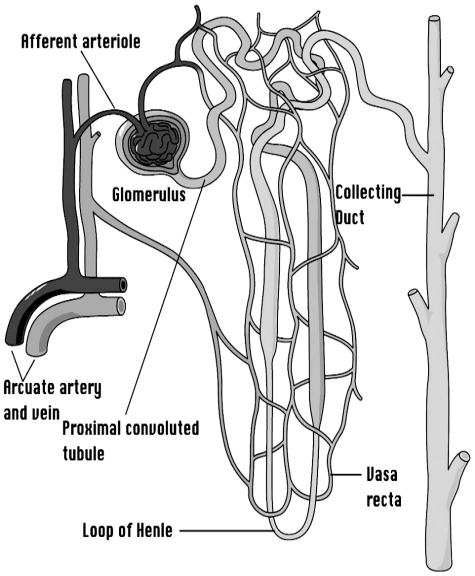
This distribution of Na^+ and K^+, which is essential for life in all organisms, is maintained by, Na^+, K^+ -ATPase, which is expressed in all mammalian cells. Ultimately, Na^+, K^+ -ATPase is

responsible for a cell assuming not only its basic functions such as regulation of cell volume, intracellular ion homeostasis and cellular uptake of nutrition's like glucose and essential amino acids, but also its differentiated functions like the propagation of nerve impulses, muscle contractions, and renal reabsorption of sodium.

Tubular reabsorption involves transport of substances across the tubular epithelial cell. Transporting epithelial cells are polarized i.e. have an apical and a basolateral plasma membrane. These membranes differ primarily with respect to the transporters they contain. Na^+, K^+-ATPase, the active transporter that carries out sodium from the cell, is exclusively found in basolateral membrane, whereas other transporters, that allow sodium to enter the cell from the tubular lumen, are found in the apical cell membrane.

Renal function

The human adult kidneys receive approximately 1.2 liters of blood per minute, equal to 20-25% of the cardiac output. The blood enters the renal nephron via the afferent arterioli and approximately 20 % is filtered in the Bowman capsule. The remaining blood continues into the efferent arterioli, which are surrounding the tubular structure.



The formation of urine begins with the filtration of plasma water and solutes from the capillaries into Bowmans space, a process known as glomerular filtration. Approximately 180 liters of water and 1.5 kg of salt forms the primary urine per day. These quantities become

even more astonishing when one considers that the total amount of body water is approximately 42 L and the pool of readily exchangeable sodium is approximately 100 g.

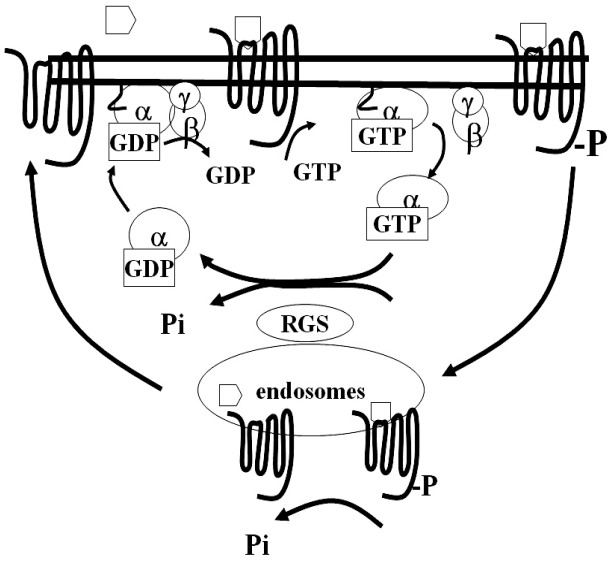
Reabsorption and excretion of water and solutes are performed by the renal tubules. The first part of the renal tubules can be divided into two parts; the proximal convoluted tubules and the proximal straight tubules. The major part of the filtrate, approximately 67% is reabsorbed in the proximal tubules. Water, salts and organic compounds such as glucose and amino acids are reabsorbed in this segment. This reabsorption is iso-osmotic. From the proximal tubule the primary urine enters the descending loop of Henle which is permeable to water but completely impermeable to sodium. This results in a water flow out from the descending limb as the filtrate descends deeper into the hypertonic interstitium of the renal medulla. Longer descending limbs allow more water to flow out and make the filtrate more hypertonic than the shorter limbs do. The filtrate enters the ascending loop of Henle, which extend from the medulla back to the renal cortex. The ascending loop is impermeable to water but actively pump sodium ions out of the filtrate and into the interstitium. This is the driving force for the movement of water from the descending limb. The filtrate is then passed to the distal tubule in the renal cortex where potassium is excreted and the fine tuned regulation of salt balance occurs. The distal convoluted tubules pass the filtrate into the collective ducts which extend from the renal cortex deep into the medulla. The capacity to concentrate and dilute the urine is located in the collecting duct. The collecting ducts are normally impermeable to water. In the presence of anti diuretic hormone (ADH), water channels (aquaporins) are transported to the plasma membrane and water is reabsorbed *(Nielsen et al. 1993, Christensen et al. 2000)*.

The final urine enters the renal papilla, the renal pelvis, the ureter and finally the urine bladder. From approximately 180 liters of primary urine 99% is reabsorbed to the body fluids and approximately 1.5 liter is excreted as urine.

G-protein coupled receptors

To respond to environmental changes, cells must transfer information concerning their environment across the impermeable plasma membranes. One solution to this problem involves three proteins: a receptor that detects information outside the cell, an effector molecule that alters the intracellular environment and a guanine nucleotide regulatory protein (G protein) which couples the receptor to the effector molecule. Many hormones, including dopamine and angiotensin II, exert their effect via activation of G-protein coupled receptors (GPCR).

Structurally the GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane α-helixes that span the membrane in an anti-clock wise manner and finally a cytoplasmic C-terminus.



The G proteins are heterotrimers, composed of three distinct subunits; α (39-46 kDa), β (37 kDa) and γ (8 kDa). In the basal state, G proteins exists as heterotrimers with GDP bound to the α-subunit. Upon agonist binding to the GPCR the receptor promotes a conformational change and dissociates from GDP and binds to GTP. This will induce a dissociation of the G-protein to an α and β/γ-subunit, both capable of signalling and activating effector molecules. The α-subunit has an intrinsic GTPase activity, which will finally terminate the signaling of the α-subunit, however, this activity can be enhanced by a family of proteins called regulators of G protein signaling (RGS). When signaling is terminated the α-subunit is again bound to GDP. The GDP bound form of the α subunit has high affinity for β, γ, and reassociation of α-GDP with β, γ returns the system to the basal state *(Hepler et al. 1992)*.

The Gα is divided into four types; $G\alpha_{s/olf}$, $G\alpha_{i}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$. The $G\alpha_{s/olf}$ stimulates adenylate cyclase and enhances the rate of cAMP synthesis from ATP. $G\alpha_{i}$ have the opposite effect, inhibiting adenylate cyclase and thereby cAMP formation. $G\alpha_{q/11}$ stimulates phospholipase C, and $G\alpha_{12/13}$ is connected to Rho signaling.

Although it is well documented that a monomeric GPCR activates heterotrimeric G-proteins, their function as monomers has been challenged by the discovery that numerous GPCRs form homo and/or hetero-oligomeres *(Javitch JA, 2004)*.

Dopamine

Dopamine receptors

Dopamine receptors belong to the rhodopsin family Class A of GPCRs *(Schiöth et al. 2005)*. Dopamine signaling is mediated by five GPCRs in mammals, divided into two groups based upon sequence homology, different G protein coupling and thereby different signaling pathways and pharmacological profiles. The D1 receptor group consists of the D1 and the D5 receptor. The D2 receptor group consists of the D2, D3 and D4 receptors. The D1 like receptors couple to $G\alpha_s$ and $G\alpha_{olf}$, activating adenelyl cyclase. The D2 receptor has two isoforms, a shorter form $D2_{short}$ (415 a.a) and a longer form $D2_{long}$ (444 a.a). D2, D3 and D4 are all coupled to inhibition of adenylyl cyclase *(Jose et al. 1992)*. Within the kidney the D1 receptor group has been localized to most tubular segments *(Felder et al. 1989, Amenta et al. 1990, Takemoto et al. 1991)*, whereas the D2 like receptors have been identified in cortex and inner medulla *(Jose et al. 1992)*. D1 like and D2 like receptors are present in the apical as well as in the basolateral plasma membranes.

Dopamine in the kidney

The synthesis of dopamine was first reported in 1910, and for the next 30 years the research was primarily cardiovascular comparisons of the action of dopamine with other amines *(Goldberg LI, 1972)*.

Dopamine was recognized in the early 1970s to increase sodium excretion. It was soon revealed that dopamine had a natriuretic effect independent of the glomerular filtration rate (GFR) *(McGrath et al. 1985)*. This effect is most pronounced during high salt diet. Ball et al showed that the urinary excretion of dopamine correlates directly with the urinary excretion of sodium *(Ball et al. 1978)*. At the end of the 1980s it was shown that dopamine regulates tubular sodium transport by decreasing the activity of tubular Na^+, K^+-ATPase in a dose dependent and reversible manner in intact cells *(Aperia et al. 1987)*.

Dopamine is produced in renal tubular cells and the renal precursor to dopamine is filtered L-dopa which enters the renal tubular cell via a sodium coupled transporter (*Baines et al. 1980, Hayassi et al. 1990, Soares-da-Silva et al. 1994)*. L-dopa is converted to dopamine by aromatic amino acid decarboxylase (AADC), which has been localized almost exclusively to the proximal tubules *(Hagege et al. 1985, Bertorello et al. 1988)*. Treatment with a kidney-specific L-dopa analogue (glu-dopa), results in an increased urinary sodium excretion associated with a decreased proximal tubular Na^+, K^+-ATPase activity *(Eklöf et al 1997)* as well as a decrease in the activity of the proximal tubular Na^+, H^+-antiporter, located in the apical plasma membrane *(Felder et al. 1993)*.

Dopamine is metabolized via deamination by mono amino oxidase (MAO) and via methylation by catechol-O-methyl-transferase (COMT) *(Kopin 1985)*. Both these enzymes are expressed in the kidney *(Fernandes & Soares-da-Silva 1992)*. Treatment with the COMT inhibitor Nitecapone results in increased urinary sodium excretion associated with decreased proximal tubular Na^+, K^+-ATPase activity. Thus, the natriuretic effect of the renal dopamine system is highly dependent on its capacity to be regulated in the renal tubular cell.

Dopamine appears to have a key role in the interactive regulation of renal tubular sodium transport, and the signal transduction of dopamine mediated effects on Na^+, K^+-ATPase activity is complex. In the rat proximal tubule, D1-like receptor signaling is referred to be functional either alone *(Baines et al. 1992, Chen et al. 1993)* or in conjugation with D2-like receptors *(Bertorello et al. 1990, Satoh et al. 1993)*. The D1 receptor is generating downstream messengers by activating adenelyl cyclase, thereby generating cAMP from ATP *(Nakajima et al. 1977)*. cAMP further activates protein kinase A (PKA) *(Bertorello et al. 1991)* which leads to a recruitment of intracellular D1-like receptors to the plasma membrane, where they become physiologically active *(Brismar et al. 1998)*. This D1-like receptor recruitment is a signal for PKC activation and translocation to the plasma membrane *(Kruse et al. 2003)*. The concentration of intracellular Ca^{2+} modulates the response of rat renal Na^+, K^+-ATPase to PKA and PKC activation *(Cheng et al. 1999)*. The D1 receptor is also known to couple to phospholipase C (PLC) *(Felder et al. 1989)*. The activation of PLC leads to regulation and expression of different forms of PKC. The effect of PKC is known to differ between normotensive and hypertensive objects *(Yao et al. 1998)*. The effects of noradrenalin, nitric oxide and atrial natriuretic peptide (ANP) are abolished by a D1 receptor antagonist *(Brismar et al. 2002, Venkatakrishnan et al. 2000, Holtbäck et al. 2000)*. Interestingly, β adrenergic agonist as well as ANP has been shown to recruit renal D1R to the plasma membrane.

Prolactin

Prolactin

Prolactin is a polypeptide hormone predominantly synthesized and secreted from lactotroph cells in the anterior pituitary gland. Prolactin is present in all vertebrates, and is therefore in evolutionary terms considered as a very ancient hormone. Genes encoding for prolactin, growth hormone and placental lactogen evolved from a common ancestral gene by gene duplication *(Niall et al. 1971)*. When comparing the coding gene sequences of prolactin and growth hormone in the same species, a postulation of 392 million years ago was set for the segregation of these two hormones *(Cooke et al. 1981)*. Prolactin is best known as the hormone that elicits lactation in mammals. However, it has been found to be highly versatile and can be found in a broad spectrum of functions such as metabolism, reproductive and parenteral behavior, immunoregulation, angiogenesis and osmoregulation. The name

prolactin is chosen from its lactating function *(Riddle et al. 1933)*. The transcription of the prolactin gene is regulated by two independent promoter regions *(Berwaer et al. 1991, 1994)*. Prolactin is transcribed as a prohormone consisting of 227 amino acids, among these 28 constitute a signaling peptide function. The mature hormone is composed of 199 amino acids in humans, and 197 amino acids in rodents *(Sinha, 1995)*. The mature form of prolactin has a molecular mass of approximately 23 kDa, but several other sizes have been described. Posttranslational modifications of prolactin are also processed in both the anterior pituitary gland and in plasma; these include dimerization, polymerization, phosphorylation, glycosylation, sulfation and deamidation *(Freeman et al. 2000)*.

Prolactin receptors

The prolactin receptor is not a GPCR but a single bound cytokine class 1 receptor. The receptor consists of an extracellular, a transmembrane and an intracellular domain. Several different isoforms of prolactin receptors have been described; these isoforms are the result of different promoters as well as alternative splicing *(Hu et al. 1991)*. The three major membrane bound isoforms described in rats are the short (291 aa), intermediate (393 aa) and long (591 aa) isoforms *(Freeman et al. 2000)*. The extracellular domain is identical in all three forms and consists of approximately 200 amino acids that can further be divided into two domains of 100 aa each *(Bole-Feysot et al. 1998)*. The intracellular domain of the receptor differs in length between these isoforms. Soluble forms of prolactin binding proteins have also been found in mammary epithelial cells and in milk *(Berthon et al. 1987, Postel-Vinay et al. 1991)*. Prolactin receptors have been found in many different tissues, including the kidney *(Bole-Feysot et al. 1998, Mountjoy et al. 1980)*

Prolactin receptor signaling

The activation of the prolactin receptor is initiated by binding of the ligand to the extra cellular domain of the receptor. The ligand is expressing two binding epitopes for the receptor, which further results in binding of another receptor molecule, resulting in a dimerization and a functional homodimeric complex. The membrane proximal intracellular part of the receptor is constitutively connected to a tyrosine kinase, Janus kinase 2 (Jak2). Activation of Jak2 is initiated upon receptor dimerisation, resulting in a transphosphorylation within the complex *(Ferrag et al. 1998)*. This results in phosphorylation of tyrosine residues within the receptor itself *(Rui et al. 1992)*. All active forms of the prolactin receptors are transphosphorylated via Jak2 *(Goupille et al. 1997)*. There are several classical signaling pathways induced by the prolactin receptor: The Jak/STAT pathway, where Jak proteins activate cytoplasmic transcription factors called “signal transducers and activators of transcription” (STAT) named for their ability to couple ligand binding to gene expression *(Ihle*

et al. 1994). The Ras/Raf/MAPK pathway *(Piccoletti et al. 1994)* which transduces extracellular signals to the cell nucleus and thereby activating genes specific for cell growth, division, differentiation, cell cycle regulation, tissue repair, integrin signaling and cell migration. Jak2 activates Ca^{2+} sensitive K^+ channels *(Prevarskaya et al. 1995)*, as well as Src and Fyn *(Berlanga et al. 1995, al-Sakkaf et al. 1997)*, which phosphorylates PI3 kinase *(Berlanga et al. 1997, Ratovondrahona et al. 1998)*.

Prolactin regulates fluid and electrolyte transport across the plasma membrane. Prolactin decreases Na^+ and increases K^+ transport in mammary epithelial cells *(Falconer et al. 1983, 1975)* Prolactin also regulates fluid transport across amniotic and intestinal epithelial cells *(Manku et al. 1975, Ramseydh et al. 1972)*.

It is well known that prolactin interacts with dopamine in various tissues. Dopamine acts as a prolactin inhibitory factor in hypothalamus *(Dickley et al. 1975, Ben-Jonathan et al. 2001).* Dopamine agonists are clinically used to inhibit lactation. Several studies, albeit with conflicting results, have shown that prolactin regulates ion and fluid transport in renal tissue *(Alder et al. 1986, Roberts et al. 1998, Morrissey et al. 2001, Lucci et al. 1975, Mills et al. 1983, Stier et al. 1984)*.

Angiotensin

Angiotensin

In response to low blood pressure, salt depletion or sympathetic nerve stimulation the proteolytic enzyme renin is formed in the kidney. Renin has no direct effect on blood pressure, but converts circulating angiotensinogen, primarily produced in the liver, to angiotensin I. The angiotensin converting enzyme (ACE), expressed in all arteries, further converts circulating angiotensin I to the potent compound angiotensin II. Angiotensin II has a key role in regulation of cardiovascular homeostasis, blood volume and vascular resistance. Thus, angiotensin II is produced in lung, proximal tubular cells, brain, heart and blood vessels *(Harris et al. 1985, Mitchell et al. 1988, Cogan 1990, Ingelfinger et al. 1990)*.

Angiotensin was discovered by two independent research groups in the 1930´s; Dr Houssay group at the University of Buenos Aires, Argentina and Dr Page group at Eli Lilly Research laboratories in Indianapolis, USA *(Fasciolo et al. 1938, Page 1935)*. Angiotensin II is an endocrine, paracrine, autocrine and intracrine hormone. Angiotensin II constricts the arterioles and raise blood pressure, it promotes release of aldosterone from the adrenal gland, which in turn acts in the renal distal tubules and promotes sodium retention and potassium excretion. Angiotensin II increases the water content in the body via two mechanisms: 1) It is a dipsogen, activating the thirst centre in the hypothalamus, and 2) it

stimulates ADH release from the posterior pituitary gland, resulting in renal water reabsorption.

The angiotensin receptor was identified by Lin and Goodfriend in 1970 *(Lin & Goodfriend, 1970)*. The angiotensin receptors belong to the super family of G-protein coupled receptors and are further divided into AT1, AT2, AT3 and AT4. The AT1 receptor is the most elucidated of these four receptors and mediates the majority of all known physiological actions of angiotensin II.

AT1 signals via several cytoplasmic signaling pathways and interacts with multiple heterotrimeric G-proteins including $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{12}$ and $G\alpha_{13}$. Activation of $G\alpha_{q/11}$ activates phospholipase C and increases cytosolic Ca^{2+} concentrations, thereby increasing protein kinase C activity. $G\alpha_{i/o}$ inhibits adenylyl cyclase and cAMP production. AT1 also activates various intracellular protein kinases including PKC *(Higuchi et al. 2007)*. The AT1 receptor, like other G-protein coupled receptors, has been proposed to undergo spontaneous isomerization, each associated with separate stages of receptor activation and regulation *(Thomas et al. 2000)*.

Angiotensin in the kidneys

Angiotensin II is a potent regulator of sodium balance in the proximal tubule, predominantly in the short S1 segment where approximately 20% of filtered sodium is reabsorbed *(Cogan 1990, Liu et al. 1987)*. Angiotensin II is known to have a biphasic effect on tubular sodium reabsorption. Low concentrations, in the picomolar range (10^{-12}M), increase sodium, bicarbonate, and fluid reabsorption *(Schuster et al. 1984, Liu et al. 1989)*, while higher concentrations induce natriuresis *(Navar 1987, Harris 1977)*. Angiotensin II is also known to have a biphasic effect on proximal tubular Na^+, K^+-ATPase activity. Picomolar concentrations of the hormone increase, whereas micromolar concentrations decrease Na^+, K^+-ATPase activity *(Aperia et al. 1994)*. However, under physiological conditions only picomolar concentrations of angiotensin II are produced *(Féraille et al. 2001, Efendiev et al. 2003)*.

Interactive regulation of renal salt balance

In 1997 J. C. Skou received the Nobel prize in chemistry for the discovery of Na^+, K^+-ATPase *(Skou, 1957)*. The main function of Na^+, K^+-ATPase is to pump three intracellular sodium ions (Na^+) out of the cell in exchange for two extracellular potassium ions (K^+) at the expense of ATP hydrolysis. The intracellular sodium concentration is normally in the 5-20 mM range, and Na^+, K^+-ATPase activity is stimulated by an increase in intracellular sodium. Na^+, K^+-ATPase maximum velocity (V_{max}) is achived at 60-100 mM Na^+. Thus, Na^+, K^+-ATPase works well below the V_{max} in intact cells. The renal cells have very high abundance of Na^+, K^+-

ATPase and approximately 80% of renal oxygene consumption is used by Na^+, K^+-ATPase to transport Na^+ and K^+.

Na^+, K^+-ATPase which consists of three subunits, α, β and γ, is located in the basolateral plasma membrane. The α-subunit is recognized as the catalytic subunit, binding sodium and ATP in the cytoplasmic domain. The binding sites for potassium and the inhibiting compounds, digitalis glycosides, are located in the extracellular domain *(Féraille et al. 2001)*.

Na^+, K^+-ATPase creates an electrochemical gradient across the plasma membrane which allows sodium to enter the cell at the apical plasma membrane via Na^+ coupled anti-porters and co-transporters and via Na^+ channels.

The precision by which sodium balance is regulated suggests a precise interaction between factors released from intra- and extra renal sources. Intra renal produced dopamine and angiotensin II act as autocrine and paracrine factors regulating the activity of Na^+,K^+-ATPase and the sodium influx pathways. The anti natriuretic effects of angiotensin II as well as of noradrenalin acting on α-adrenercic receptors are opposed by dopamine. Short term exposure of a D1R agonist results in internalization of A1R and a complete abolishment of A1R signaling, while exposure with angiotensin II results in internalization of the D1R and abolishment of D1 receptor signaling *(Khan et al. 2008)*. The effect of atrial natriuretic peptide, nitric oxide and noradrenaline acting on β-adrenergic receptors (β-AR), are dependent on an intact renal dopamine system *(Holtbäck et al. 2000, Venkatakrishnan et al. 2000, Brismar et al. 2002)*. During inhibition of the intrarenal dopamine synthesis or in the presence of D1R antagonist these natriuretic factors have no effect on renal salt balance regulation. These effects are explained by heterologous D1R recruitment to the plasma membrane.

Taken together, dopamine coordinates the effects of antinatriuretic and natriuretic factors. Intact renal dopamine and angiotensin systems are of major importance for the maintenance of sodium homeostasis and normal blood pressure.

Blood pressure

Blood pressure is the product of cardiac output and the vascular resistance. It is highest when the heart contracts; the systolic pressure, and lowest between contraction; the diastolic pressure. Thus blood pressure increases due to an increase in the cardiac output, an increase in vascular resistance, or a combination of the two.

Hypertension is the single leading risk factor predisposing to heart attack, cerebral stroke and renal failure. Identified causes to high blood pressure are referred to as secondary hypertension and include renal, endocrine, neurological diseases and vascular malformations. A small group of patients has also been found to have mutations in tubular

transporters or in proteins regulating tubular sodium transport *(Wilson et al. 2003)*, however, most patients with hypertension (> 90%) have high blood pressure of unknown causes i.e. essential hypertension.

Arterial blood pressure is regulated by several interrelated systems. Neural regulators of blood pressure act very fast to correct acute abnormalities in blood pressure, but lose their effects within hours to days. Hormonal induced vasoconstriction by norepinehrine-epinephrine provides moderately rapid control of arterial blood pressure. The main long-term regulator of blood pressure is the sodium transport system in the kidney.

Guyton described in 1987 the relationship between blood pressure (BP) and sodium excretion as the pressure–natriuresis curve *(Guyton 1987)*. Increase in BP causes increased natriuresis in order to return the BP to normal. However, impairments in the pressure–natriuresis relationship cause a rightward shift in the curve necessitating increased BP in order to restore the blood volume.

The traditional view has been that hypertension is caused by an excess of factors that produce vasoconstriction and sodium retention. The two most obvious candidates have been noradrenalin and angiotensin II. This hypothesis has been modified after reports showing that a low availability of vasodilative, natriuretic factors also predisposes to hypertension. Several lines of evidence suggest that dopamine has a role in the pathophysiology of hypertension. Clinical studies in patients with essential hypertension have shown dysregulations in dopamine formation, attenuated natriuretic response to dopamine and a defective D1 receptor coupling *(Harvey et al. 1984, Sanada et al. 1999)*. Experimental evidence for the role of dopamine in hypertension is also present. In rat strains genetically predisposed to develop hypertension, similar defects in the renal dopamine system are already present before the onset of the increased blood pressure *(Kinoshita et al. 1989, Chen et al. 1993)*. Furthermore, mice with mutations in any sub class of the dopamine receptors or in dopamine signaling proteins develop hypertension *(Albrecht et al. 1996, Asico et al. 1998, Eklöf et al. 2001)*.

A more likely hypothesis to explain hypertension is therefore that high blood pressure is caused by an altered balance between vasoactive, antinatriuretic factors and vasodilative, natriuretic factors.

Aims of the thesis

The aim of this study was to investigate by which mechanisms the salt regulating peptide hormones prolactin and angiotensin II interact with the renal dopamine system.

1. Does prolactin interact with the renal dopamine system? If so, what is the effect and by which mechanisms is this interaction occurring?

2. Do the D1 receptor and the AT1 receptor allosterically modulate one another, and if so, does angiotensin act as modulator of D1 receptor signaling.

Experimental procedures

For detailed description of all materials and methods used in this study, I would like to refer to the original papers enclosed. Here, is a general outline of the methods used.

Selection of animals

In these studies three strains of rats were used; 1) Sprague-Dawley, an albino rat strain extensively used in medical research, 2) The spontanous hypertensive rat (SHR), in which the development of hypertension has been associated with a reduced activity in the renal dopamine system *(Kawabe et al. 1978, Jose et al. 1996, Zeng et al. 2007)*, and 3) the Wistar-Kyoto rat, which is the normotensive control rat to SHR. Animals 6-8 weeks of age, corresponds to young adult humans, were used. Animals 3 weeks of age were used for preparations of renal proximal tubular cells. All experiments were performed according to Uppsala University and the Karolinska Institutet regulations concerning care and use of laboratory animals and approved by the Stockholm North ethical evaluation board for animal research. The studies performed in Argentina followed the Faculty of Medicine, University of Buenos Aires regulations concerning care and use of laboratory animals, and were approved by the Comité Institucional Para el Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Faculty of Medicine, University of Buenos Aires.

Study of renal function by inulin clearance

To study the effect of prolactin on renal function in vivo, rats were treated intravenously with luteotropic hormone isolated from sheep pituitary glands.

Experimental procedure This study was performed on male Sprague-Dawley rats 40-50 days of age. Both jugular veins were catheterized for infusion as well as the left femoral artery for measurements of mean arterial blood pressure and blood sampling. The urinary bladder was catheterized through a supra pubic incision for urine collection to measure urine volume, osmolality as well as sodium and potassium concentrations. To determine the GFR a ringer solution containing [3H]-methoxy-inulin was administered, initially as a bolus injection containing 4 µCi followed by a continous infusion of 2 µCi per 100g body weight.

The experiment was divided into 6 collection periods of 20 minutes each; the first two as control sampling periods (C1 and C2), and the following four as experimental sampling periods (E1-E4).

Rats were divided into three groups. Group 1 (prolactin): animals received an intravenous bolus injection of 0.3 IU prolactin followed by continuous infusion of 3 IU/hour/kg body weight during the four experimental sampling periods. Group 2 (vehicle): infusions and samplings were identical to the prolactin treated animals except that prolactin was omitted. Group 3 (prolactin and D1R antagonist): animals received the same prolactin treatment as group 1, but the rats were also treated with a D1R antagonist. The D1R antagonist SCH 23390 was first given as a bolus injection of 30µg/kg bw, followed by a continuous infusion of 30µg/hour/kg body weight during the whole experimental time, including stabilization (45 minutes after surgery) and control periods. Similar doses of prolactin have been reported to increase urinary sodium excretion in Long-Evans rats *(Ali et al. 1991)*.The dose of SCH 23390 has previously been shown to completely inhibit the effect of dopamine on urinary sodium excretion *(Eklöf et al. 1997, Hansell et al. 1991)*.

The urine volumes were measured gravimetrically, sodium and potassium concentrations were determined by flame photometry and osmolality was estimated from the depression of the freezing point. The amount of [3H]-methoxy-inulin in samples of plasma and urine was detected using a liquid scintillation counter, and GFR was estimated from the clearance (Ci) of inulin according to the equation: Ci=(Ui x V)/Pi, where Ui and Pi are the urinary and plasma concentrations of inulin, and V is the urine flow rate.

Blood pressure recording in rats with experimental hypertension

The interaction between D1R and AT1R was studied in an experimental model of renal hypertension using the AT1R antagonist losartan and the D1R antagonist SCH 23390.

Experimental procedure Rats were made hypertensive by a surgical constriction of the aorta, proximal to the renal artery *(Eklöf et al. 1990)*. Male Sprague-Dawley rats, 40 day old were subjected to ether anesthesia in an ad hoc chamber. Abdominal aorta, proximal to renal arteries was visualized through abdominal left flank incision. The vagus nerve was separated and a silk ligature wet in glycerol was placed around the aorta. A probe was placed on the aorta and the ligature was tied around it. Once the tight was secured, the probe was removed. The procedure lasted for less than 30 seconds. The kidney turned pale during ligation and regained normal color once the probe was removed. Ether was used to maintain a correct anesthesia during the surgical procedure.

One week after surgery the animals were divided into two groups: Group 1 (control) drinking regular water and group 2 (losartan) receiving 20 mg losartan per kg/day in their drinking water. At the beginning of third week: seven of the losartan treated animals started to receive co-treatment with the D1R antagonist SCH 23390, 1 mg per kg/day, administered sub cutaneous in two doses per day, continuously for four days: Group 3 (losartan + SCH).

After four days, rats were anesthetized with thiopental ip, placed on a heated stage at 37^{o}C, weighted and catheters were inserted into the carotid and right femoral arteries for recording of mean arterial pressure using a Statham transducer. The depth of anesthesia was controlled by the lack of response to stimulation of posterior limbs and visual observation of a stable and regular breathing.

Studying Na^+, K^+-ATPase activity by ouabain sensitive adenosine triphosphate hydrolysis

To study the effect of prolactin, dopamine and their signaling pathways on renal salt balance studies on proximal tubular Na^+, K^+-ATPase activity was performed. The Na^+, K^+-ATPase activity was determined as ouabain sensitive ATP hydrolysis in microdissected proximal tubular segments. This is a well established method in our research group. The following factors are known during the experimental procedure; the amount of added cold ATP and radioactive ^{32}P-ATP, the length of the proximal tubules and the concentration of intracellular sodium. The results are presented as pmol of ^{32}Pi hydrolyzed per mm of tubule per hour.

To exclude ATP hydrolysis originating from other ATP-ases, one part of the preparations were incubated with the Na^+, K^+-ATPase inhibitor ouabain. Measurements of ouabain incubated tubules were always performed in parallel to the experimental tubules. For each group per experiment five to eight tubular segments were measured. The result was calculated as the difference between the mean value for total ATPase and ouabain-insensitive ATPase activity, and given as absolute values.

Experimental procedure The experimental kidney was perfused in vivo with a solution containing 0.05% collagenase and 0.1 % serum albumin. The kidney was then removed and cut along the corticopapially axis into small pyramids. These preparations were incubated for 20 minutes in a solution containing 0.05% collagenase to disrupt the supporting collagen surrounding the tubules. The mitochondrial respiration in the preparations was optimized by adding butyrate and oxygen during the incubation period. After incubation, the tissue was rinsed in a solution containing the same composition as the perfusion solution except that collagenase, calcium and serum albumin were omitted. Single proximal tubular segments were then manually dissected from the outer renal cortex using a stereomicroscope, fine needles and mouth-pipetting. Length determinations of tubules were performed by transferring an individual tubule to a bacteriological glass slide to be photographed. The photographs were then used for tubular length determination. Continuously, segments within the range of 0.4 to 1.1 mm were further used for experiments. Tubules were stored

on ice until the dissection and photographing procedure was completed, for a maximum of 60 minutes.

To study the dose dependent effect of PRL on Na^+, K^+-ATPase activity doses between 1 ng to 1 μg was used. Since a maximal response was found at 1 μg/ml this concentration was further used in studies of the signaling pathways. Previous studies have shown a dose dependent effect of dopamine in the range between 10^{-7}M to 10^{-4}M, with a maximal response at 10^{-4} M at 30 min incubation *(Aperia et al. 1987)*. In this study dopamine was used at the concentration of 10^{-5} M. Inhibitors of the intra renal dopamine production; benserazide (10^{-5}M) and carbidopa ($5x10^{-4}$M) were added to the microdissected tubules in the dissection solution 30 min before treatment. Dopamine receptor agonists SCH 23390 (10^{-6}M) and raclopride (10^{-6}M) were added 10 minutes before treatment. Concentrations of these drugs were selected on the basis of previous studies *(Soares-da-Silva et al. 1998, Grenader et al. 1991, Holtbäck et al. 1999, Edwards et al. 2001)*. The PKA inhibitor: H89 (3 x 10^{-5}M), PKC inhibitor: bis indolyl maleic acid (BIM) (10^{-6}M), PI 3-kinase inhibitor: wortmannin (10^{-4}M) and JAK 2 inhibitor: AG490 (10^{-5}M) were used to study the signaling pathways, concentrations of these compounds were based on previous studies, and were added 10 min before treatment with prolactin. Subthreshold doses of prolactin 1 ng per ml and dopamine (1 nM) were used to study synergism. Tubular segments were treated at indicated times in room temperature, either in microdissection solution (controle tubules) or with indicated compounds (experimental tubules).

Tris-ATP, and γ-[^{32}P]-ATP were added to the tubules, which were made permeable by fast freezing and thawing, giving ATP and sodium free access into the cell. Tubules were incubated for 15 minutes at 37°C. Active charcoal was used to remove un-hydrolyzed ATP from the tubules. The tubules were then rinsed in a precise amount of phosphate buffer on top of a Millipore filter, allowing hydrolyzed Pi^{32} to pass and be collected. The radioactivity was counted using a liquid scintillation β-counter.

Studying protein expression, localization and interaction using western blot, immunoprecipitation, biotinylation and subcellular fractionation

Western blot is an anti-body based technique used to detect specific proteins in a given sample of tissue homogenate or extract. Electrophoresis is used to separate denaturated or native proteins by the length of the polypeptide (denaturated condition) or by the 3 dimensional structure of the protein (native/non-denaurating conditions). The western blot technique is for many protocols the final step by which the result is received, this also apply to the co-immunoprecipitation, biotinylation and subcellular fractionation techniques used in this study.

Immunoprecipitation is a technique where a specific protein is separated (precipitated) from a tissue homogenate using an antibody that specifically binds the protein of interest. The antibody should recognize an epitope on the protein in the native conformation, which is important to consider when selecting antibodies for this purpose. The antibody is further attached to a solid substrate; this was performed by using sepharose-G. The protein-antibody-complex was then possible to precipitate from the rest of the homogenate using centrifugation. To minimize unspecific binding in the homogenate to sepharose-G, a pre-clearing step was performed. The homogenate was incubated with sepharose-G for one hour and the sepharose containing unspecifically bound proteins was then discarded. In order to show specificity of the antibody used, Ig G of the same species as the precipitating antibody was treated in the same way as the rest of the experimental samples. Whole tissue homogenate was always added as a control.

Co-immunoprecipitating is using the above technique, and is designed to detect a protein connectet to the precipitated protein. This technique has been used in several parts of this study to demonstrate interaction between proteins such as; Na^+, K^+-ATPase/PI 3-kinase and the D1 receptor/AT1 receptor.

Experimental procedure Rat renal cortical slices were treated with indicated drugs and incubation times. Treatment was stopped by aspirating the treatment buffer and slices were immediately frozen. Slices were thawed and homogenized in a ripa buffer (pH 7.4) containing protease inhibitors to prevent protein degradation. The resulting homogenates were centrifuged for a short time at low speed to get rid of cell debris. Protein concentrations in the resulting supernatants were adjusted to equal amount between samples. The homogenates were then subjected to a clearing-step with sepharose-G for one hour, followed by the precipitating antibody for another hour at 4^{o}C using end to end turning. The homogenate-antibody mixtures were further subjected to sepharose-G over night using end to end turning at 4^{o}C. Proteins were eluted with Laemmli buffer, heated to 59-70°C for 15 min, and resolved by Western blot.

Biotinylation was used to detect changes in dopamine receptor abundance at the cell surface in intact proximal tubular cells and HEK 293 cells. Biotin binds covalently to all exposed proteins, which enables separation of these proteins from cytosolic proteins. The binding is rapid and unlikely to disturb the natural function of the molecule due to the small size of the biotin molecule (244 Da). The biotinylated proteins are further bound to streptavidin and separated by centrifugation. The bond between biotin and the protein is resistant to heat, pH and proteolysis, which enables the capture of biotinylated molecules in a wide variety of environments *(Hermanson et al. 1994)*.

Experimental procedures Proximal tubular cells were treated in the presence and the absence of prolactin at a final concentration of 1µg/ml for 10 minutes at 37°C. Surface membrane proteins were biotinylated by exposing cells to EZ-linked Sulfo-NHS-SS Biotin at a final concentration of 1 mg/ml in PBS at 4°C for 2 hours. Cells were rinsed, lysed and centrifuged at 14,000 x g for 10 min at 4°C. The supernatants were adjusted to equal protein amounts between samples. Biotinylated proteins were captured with streptavidin-agarose beads overnight at 4°C using end-to-end mixing. Proteins were eluted with Laemmli buffer, heated to 70°C for 15 min, and resolved by Western blot.

To study the effect of losartan in renal proximal tubular cells or in HEK 293 cells double transfected with D1R and AT1R, biotinylation techniques were performed. Cells were treated in the presence and the absence of losartan (10^{-5}M) in a Krebs buffer for 20 min. Cell surface proteins were biotinylated using EZ linked Sulfo-NHS-SS Biotin in PBS, at a final concentration of 1 mg/ml at 4°C for 2 hours, during gently shaking. Cells were rinsed and homogenized in a buffer containing sucrose (320 mM). Homogenates were centrifuged at 800 x g for 5 min, and supernatants were collected. Protein concentrations were adjusted to equal amount. Supernatants were subjected to centrifugation at 10.000 x g for 20 min. The resulting pellets were resuspended and proteins were adjusted to equal concentration. Biotinylated proteins were captured with streptavidin-agarose beads for 2 hours at 4°C using end to end turning. Proteins were recovered by centrifugation, washed and eluted with Laemmli sample buffer, heated to 70°C for 15 minutes and resolved by Western blot.

Subcellular fractionation Dopamine receptor abundance was studied in the presence and the absence of prolactin at a final concentration of 1µg/ml using subcellular fractionation techniques. The most commonly used method to break cells for preparation of a membrane fraction is to homogenize them in an isotonic buffer containing sucrose. Membrane proteins are relatively stable while they are embedded in the membrane, and differences in lipid and protein composition allow them to be separated from other molecular components and organelles *(Lin et al. 2009)*. Separation of the plasma membrane and cytosolic proteins were performed by centrifugation. The presence of dopamine receptors were examined in the plasma membrane fraction (Na^+, K^+-ATPase enriched) and cytosolic fraction (Na^+, K^+-ATPase non enriched) in the presence and the absence of prolactin.

Experimental procedure Renal cortical slices were incubated with prolactin (1µg/ml). The treatment was stopped by a rapid freezing procedure on dry ice. The renal slices were homogenized in a sucrose based buffer (250 mM) containing protease inhibitors, followed by a short low speed centrifugation to remove cell debris and whole cells. The supernatant was further subjected to centrifugation at 20.000 x g for 30 minutes. The resultant pellet, considered as the plasma membrane fraction and the resultant supernatant were adjusted to equal protein amounts and eluted with Laemmli buffer and resolved by western blot.

Studying receptor protein localization and movement using live cell imaging

Live cell imaging was used to study to movement of D1 receptors and AT1 receptors in the presence and absence of losartan.

A fluorophore is a molecule which will absorb energy of a specific wavelength and re-emit energy at a different specific wavelength. The protein of interest and the fluorescent molecule are conjugated in a construct which makes detection of the re-emitted signal from the protein-fluorescent molecule possible. Two different fluorophores were used in this study; Venus and CFP. To detect the signals from the constructs a Zeiss LSM 510 laser scanning confocal microscope was used. A confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate *out of focus* signal. Since the majority of the light from sample fluoresence is blocked at the pinhole, long exposure times are often required. Increasing illumination increases the rate at which the fluorophores bleach, limiting the duration of the experiment. It is therefore generally preferable to use the minimum possible illumination intensity to avoid bleaching *(Yuste & Konnerth, Cold spring harbor laboratory press. 2005)*.

Experimental procedure Full length constructs of D1R and AT1R were made; pD1R-CFP, pD1R-Venus, pAT1aR-CFP and pDEST-rAT1aR-Venus. A mutated form of D1R within the C-terminus was also performed. This form has serins at positions 397 and 398 mutated into alanins by cDNA site directed mutagenesis technique (U.S.E. Mutagenesis kit). The amino acids S397/398 are known to be of critical importance to interaction between D1R and AT1R *(Khan et al. 2008)*. Structure of all constructs was verified using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Sprague-Dawley rats, 3 weeks old, were used for preparation of primary cultures of renal proximal tubular cells. Cells were cultured in supplemented Dulbecco's modified Eagle's medium (Gibco 31600) containing 10% FBS, on 25 mm glass cover slips for 48h in 5 % CO_2 at 37°C. HEK 293a cells, passage 3-10 were cultured for 24-48 hours in Dulbecco´s modified eagle medium (Gibco 41966) at an approximate humidity of 95–98% with 5% CO_2.

All transfection protocols were performed according to the manufacturer's suggestion, and all double transfections were performed at 1:1 ratio. Transfection of HEK293a was performed using Exgene 500, which is based on internalization of nucleic acids by endosomes *(Kopatz et al. 2004)*. Renal proximal tubular cells were transfected using Lipofectamine 2000 which alters the plasma membrane, allowing nucleic acids to cross into the cytoplasm. Cells were cultured an additional 24h following transfection before subjected to live cell imaging.

Live cell imaging was performed using a Zeiss LSM 510 laser scanning confocal microscope, with a 40x (1.2 NA, water) objective. Emission was induced with laser lines at 514 nm and 485 nm. Detection was performed using LP 530 and BP 47-500 filters for Venus and CFP labels, respectively. Cells with low to medium strong fluorescence were selected for further studies. Images were collected before treatment, after 5 and 10 minutes or 20 and 30 minutes of treatment. For each time point at least 16 cells were analyzed. The membrane to cytosol ratio was calculated by dividing the average intensity of an area in the region of the plasma membrane with the average intensity of an identical area of the cytosolic region subjacent to the chosen region of the plasma membrane. Images were analyzed by using the imaging software Image J (National Institutes of Health).

Measurement of cAMP

Cyclic adenosine monophosphate (cAMP) is synthesized from ATP by adenelyl cyclase. Adenelyl cyclase is located at the inner side of the plasma membrane where it responds to Gα proteins, either stimulatory (Gαs) or inhibitory (Gαi). D1R is known to be positively coupled to GαS which activates adenelyl cyclase and enhances cAMP production. AT1R is known to be negatively coupled to adenelyl cyclase via Gαi which results in a reduction of cAMP. Measurement of cAMP was performed by using an enzymatic immunoassay. This method uses quantitative determination of cAMP via a polyclonal antibody which induces a color change in the samples. Measurement of the optical density was then performed to calculate the concentration of cAMP compared to a standard series performed at the same time. The magnitude of cAMP accumulation in intact cells depends upon the rates of both the synthesis and the break down and it has previously been reported that the half-life for cAMP in lymphoma cells is approximately 8 minutes *(Barber et al. 1988)*. To prevent break down of accumulated cAMP, the phosphdiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added during treatment *(Essayan, 2001)*.

Experimental procedure Sprague-Dawley rats, 3 weeks old, were used for preparation of primary cultures of renal proximal tubular cells. Cells were cultured in supplemented Dulbecco's modified Eagle's medium containing 10% FBS for 48h in 5 % CO_2 at 37°C. HEK 293a cells, passage 3-10 were cultured for 24-48 hours in Dulbecco´s modified eagle medium at an approximate humidity of 95–98% with 5% CO_2.

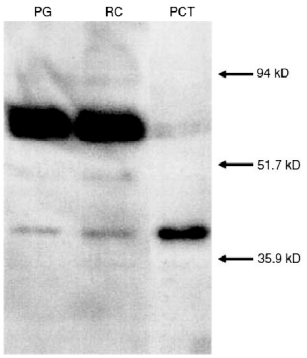
Double-transfection of HEK293a was performed using the AT1 receptor and D1 receptor. A double transfection of the AT1 receptor and the mutant form of D1 receptor (S397A/S398A) was also performed. Single transfections were made using the AT1 receptor, D1 receptor or the mutant D1 receptor.

To deplete the cellular environment from growth factors and hormones, cells were exposed to a serum free medium two hours before treatment. Cells were exposed to losartan (10^{-5} M), the D1 receptor agonist SKF-38393 (10^{-5}M) or vehicle in an oxygenated buffer containing 3-isobutyl-1-methylxanthine (IBMX) (0.5 µM) for 20 minutes at 37°C. After treatment, cells were rinsed with PBS and lysed in HCl (0.1 M) and protein concentration was measured. cAMP was determined using the Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs) according to manufacturers protocol.

Results

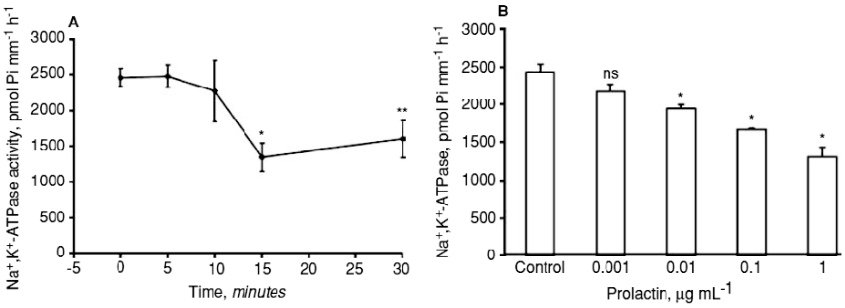
Prolactin receptors in rat renal proximal tubules

The prolactin receptor has been found as several isoforms, originating from alternative sites of transcription and different gene splicing. In rodents one long and three short isoforms have previously been described *(Bole-Feysot et al. 1998, Ali et al. 1991)*. We confirmed the presence of prolactin receptors in renal cortical slices and micro dissected renal proximal tubules by western blot. Two forms of prolactin receptors were detected, one long form of 70 kDa and one shorter form of 40 kDa. These isoforms correspond to the previously described long and medium short isoforms in rodents. Pituitary gland was used as a positive control.



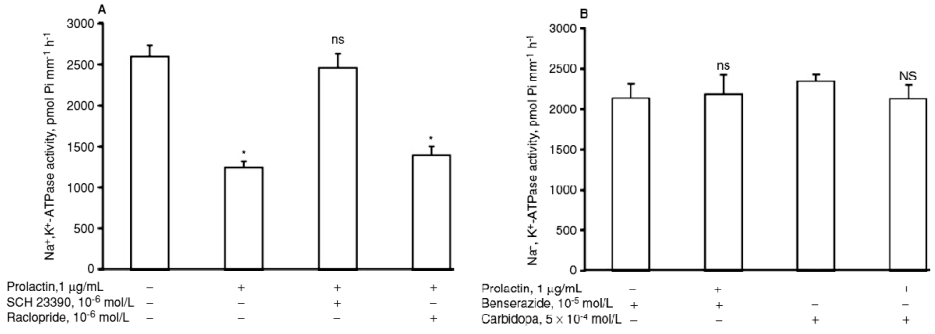
Prolactin dose dependently decreases renal proximal tubular Na^+, K^+-ATPase activity

Prolactin induced a time-dependent inhibition of proximal tubular Na^+, K^+-ATPase activity with a maximal inhibitory effect at 15 minutes incubation. Prolactin decreased Na^+, K^+-ATPase activity both when the enzyme was assayed with saturating Na^+ concentrations under V_{max} conditions (Na^+ 70 mM) (control, 2598 ± 135; prolactin, 1 µg per ml, 1206 ± 95 pmol Pi/mm/hour) ($P < 0.05$) and when a lower nonsaturating Na^+ concentration of 20 mM was used (control, 1674 ± 116; prolactin, 1 µg/mL, 1421 ± 142 pmol Pi/mm/hour) ($P < 0.05$). The inhibitory effect of prolactin during V_{max} conditions was dose-dependent with a maximal effect of 48% of control at 1 µg prolactin per ml and with a threshold effect between 1 and 10 ng per ml.



Prolactin is dependent on the D1 receptor and the presence of dopamine

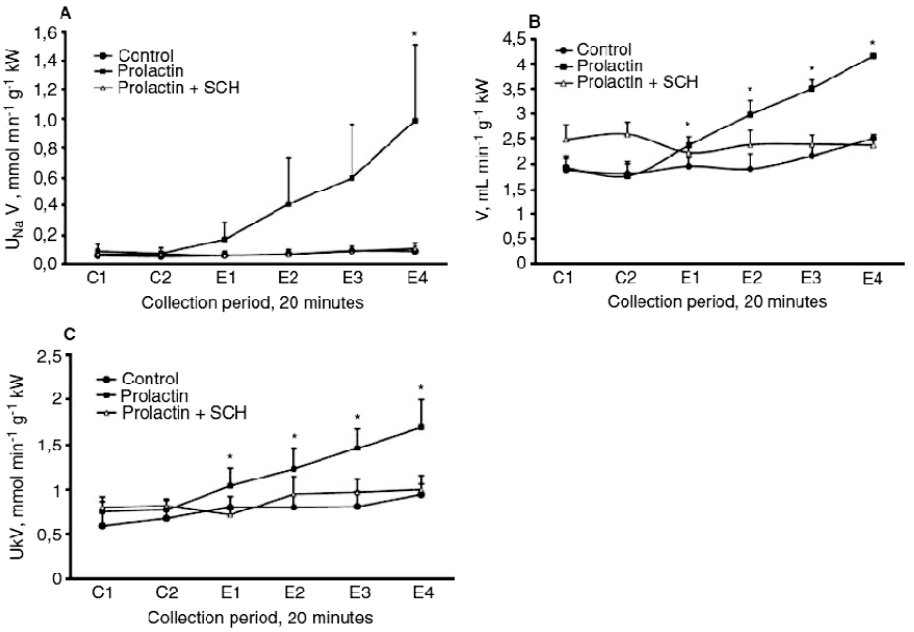
To study a possible interaction between prolactin and the renal dopamine system two dopamine receptor antagonists were used; for the dopamine D1 receptor (SCH 23390, 10^{-6} M) and for the dopamine D2 receptor (raclopride, 10^{-6} M). In tubules pretreated with the D2R antagonist, prolactin still inhibited Na^+, K^+-ATPase activity. In contrast, when tubules were preincubated with the dopamine D1 receptor antagonist the inhibitory effect of prolactin on Na^+, K^+-ATPase activity was completely abolished. This result suggested an interaction between prolactin and the renal D1 receptors.



We continued by examine the ability of prolactin to reduce Na^+, K^+-ATPase activity during inhibition of renal dopamine production. Two different inhibitors of the intrarenal dopamine production were used. Renal slices were preincubated with the aromatic amino acid carboxylase (AADC) inhibitors; benserazide (10^{-5} M) or carbidopa ($5x10^{-4}$ M) before treatment with prolactin. We found that prolactin was unable to decrease Na^+, K^+-ATPase activity during inhibition of AADC. From these experiments we concluded that the ability of prolactin to reduce Na^+, K^+-ATPase activity is dependent both on the dopamine D1 receptor and the access to intrarenal dopamine.

Effects of prolactin on renal function

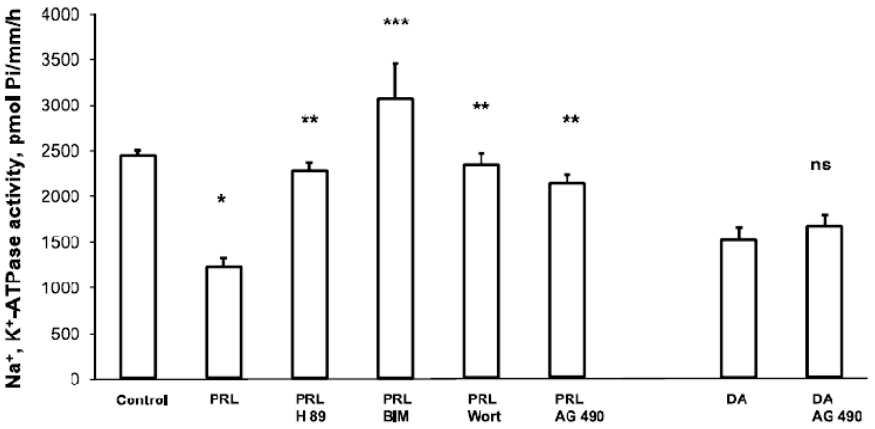
The effect of prolactin on renal function *in vivo* was studied by inulin clearance. Rats were treated intravenously with luteotropic hormone isolated from sheep pituitary glands. Prolactin induced an increase in urinary sodium excretion in every rat. The response was variable with a 9.1 ± 3.8-fold increase in urinary sodium excretion. This effect was associated with an approximate twofold increase in urinary flow rate and urinary potassium excretion. All these effects were abolished by the D1 receptor antagonist SCH. Prolactin had no significant effect on urinary osmolality, GFR, mean arterial blood pressure, or hematocrit.



The achieved plasma prolactin concentration during the present experiments can be estimated to 400 ng per ml based on data of *(Stier et al. 1984)* where the rats received the same doses of prolactin and the plasma levels were measured. This approximates the elevated plasma prolactin levels found during estrus and lactation *(Hardman JE et al. 2001)*. Assuming that the native hormone, rat prolactin, is as potent as ovine prolactin, the levels achieved in the current experiments may be consistent with physiologic levels under such conditions. The same levels of prolactin can also be seen during antipsychotic treatment. During chlorpromazine therapy, serum prolactin concentration increases to approximately 400 ng per ml *(Rang HP et al. 1999)*. These data imply that the results may be relevant in physiologic conditions in females and during antipsychotic treatment in both men and women. The D1 receptor antagonist treated rats showed a small increase in urine osmolality. The constant hematocrit indicates the presence of euvolemic conditions throughout the experiment.

Signaling pathways

The dopamine-induced decrease in proximal tubular Na^+, K^+-ATPase activity is mediated by PKA-and PKC dependent phosphorylation of Na^+, K^+-ATPase *(Bertorello et al. 1989, Fisone et al. 1994)*. The effect of prolactin (1 µg/ml) was completely abolished by a specific inhibitor of PKA, H89 (3×10^{-5} M), as well as by a specific inhibitor of PKC, bisindolyl maleic acid (BIM; 10^{-6} M). When added alone, neither H89 nor BIM had any significant effect on the Na^+, K^+-ATPase activity. The PI 3-K inhibitor wortmannin (10^{-5} M) abolished prolactin-induced decrease in proximal tubular Na^+, K^+-ATPase activity. Wortmannin added alone had no effect on Na^+, K^+-ATPase activity. The prolactin receptor activates Janus kinase 2 (JAK-2), which is specifically inhibited by AG-490 *(Bole-Feysot et al. 1998, Freeman et al. 2001)*. AG-490 (10^{-5} M) significantly reduced the prolactin induced decrease in proximal tubular Na^+, K^+-ATPase activity. AG-490 had no effect on the dopamine-induced decrease in Na^+, K^+-ATPase activity or the basal Na^+, K^+-ATPase activity when added alone. Ouabain-insensitive Na^+, K^+-ATPase activity was not affected by any drugs added.



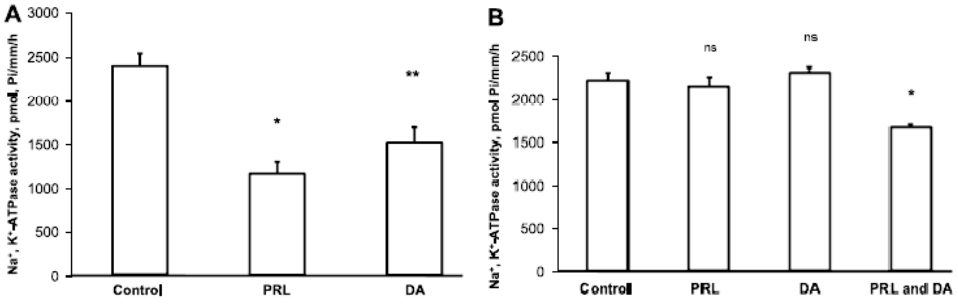
Dopamine, via PKC, phosphorylates serine-23 at the N-terminus of the rat α-subunit of Na^+, K^+-ATPase *(Bertorello et al. 1989, Feschenko et al. 1997)*. The PKC phosphorylation of Na^+, K^+-ATPase was visualized using a monoclonal antibody which detects the dephosphorylated form of Ser-23 in the Na^+, K^+-ATPase α-subunit *(Feschenko & Sweadner 1997)*. In isolated renal cortical slices, prolactin (1 µg/ml) reduced the amount of dephosphorylated Na^+, K^+-ATPase without any effect on the total amount of Na^+, K^+-ATPase, resulting in a significantly reduced ratio between dephosphorylated and total Na^+, K^+-ATPase compared to control (control: 0.93 ± 0.17; PRL 0.27 ± 0.08; $P<0.05$, $n=7$). We have previously used the same

antibody to demonstrate that dopamine has a similar effect and reduces the amount of dephosphorylated Na^+, K^+-ATPase *(Ibarra et al. 2002)*.

Dopamine induces co-immunoprecipitation between the phosphoinositide-3 kinase (PI 3-K) p85 α-subunit and Na^+, K^+-ATPase in renal proximal tubules *(Chibalin et al. 1998, Yudowski et al. 2000)*. In the presence of prolactin (1 µg/ml), the PI 3-K p85 α-subunit co-immunoprecipitated with the Na^+, K^+-ATPase α-subunit. This effect was abolished by the D1-like receptor antagonist SCH 23390.

Synergistic effects between dopamine and prolactin

Dopamine and prolactin dose dependently decrease proximal tubular Na^+, K^+-ATPase activity. Maximal effects are seen at 10^{-5} M dopamine and 1 µg/ml prolactin. Prolactin (1 µg/ml) decreased proximal tubular Na^+, K^+-ATPase activity to 48 ± 3% of control, whereas dopamine (10^{-5} M) decreased proximal tubular Na^+, K^+-ATPase activity to 63 ± 4% of control (*P*= 0.0005). Subthreshold doses of prolactin (1 ng/ml) and dopamine (5 x 10^{-8} M) had no effect added separately, but added together they significantly decreased proximal tubular Na^+, K^+-ATPase activity.



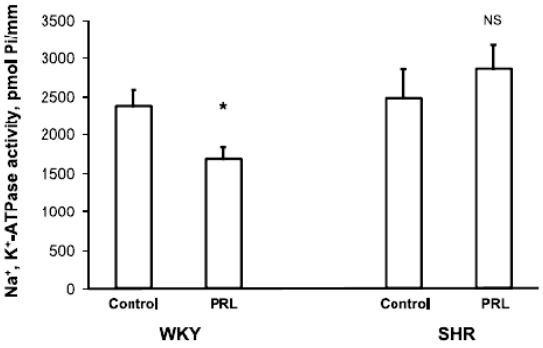
Dopamine 1 like receptors are recruited to the plasma membrane in response to prolactin

Permissive and synergistic effects can be explained by heterologous receptor recruitment to the plasma membrane, where the receptors become physiologically active. Two complementary techniques were used to examine whether prolactin was able to recruit D1 receptors to the plasma membrane: subcellular fractionation of the outer renal cortex and cell surface biotinylation, using primary cultured proximal tubular cells. Na^+, K^+-ATPase is generally used as a plasma membrane marker. We confirmed that our subcellular fractionation resulted in Na^+, K^+-ATPase -enriched and -nonenriched fractions. In the presence of prolactin, the D1-like receptor abundance was increased in the Na^+, K^+-ATPase -

enriched fraction and reduced in the Na^+, K^+-ATPase-nonenriched fraction (ratio Na^+, K^+-ATPase enriched/nonenriched, control: 0.73 ± 0.14, PRL: 1.54 ± 0.02). Biotinylation confirmed that prolactin significantly increased the amount of D1-like receptors inserted into the plasma membrane.

Spontaneous hypertensive rats

The hypertensive rat strain SHR has a blunted response to dopamine. This effect can be attributed to decreased D1-like receptor responsiveness *(Kinoshita et al. 1989, Zeng et al. 2007)*. We found that the response to prolactin on Na^+, K^+-ATPase activity was blunted in SHR.

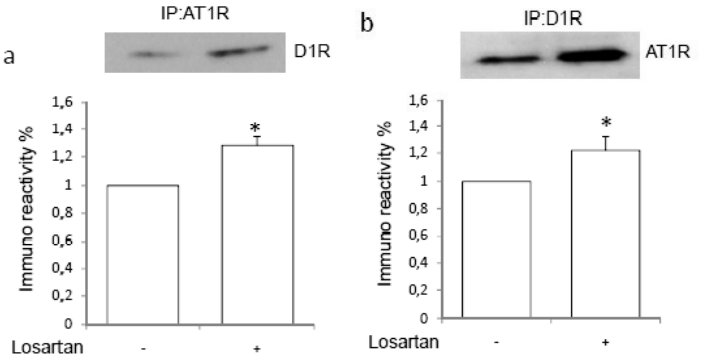


Prolactin signaling pathways appear to be identical to dopamine signaling pathways in proximal tubular cells, including PKA activation, PKC phosphorylation of the α-subunit of Na^+, K^+-ATPase and increased ability to co-immunoprecipitate the α-subunit of Na^+, K^+-ATPase and PI 3-kinase, and that this interaction is mediated vi the D1-like receptor.

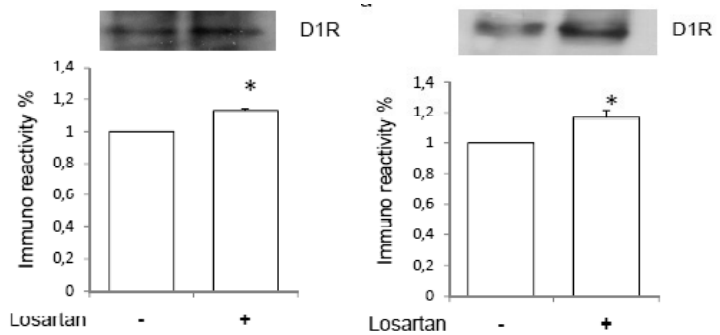
AT1 receptor and D1 receptor interaction

To test the role of losartan in the interaction between the AT1 receptor and D1 receptor, we first performed co-immunoprecipitation studies, using the outer 250 µm layer of renal cortical slices from 40 days old rats. The slices were incubated with losartan (10^{-5}M) or vehicle for 15 minutes. Lysates from these slices were immunoprecipitated with a D1 receptor antibody or an AT1 receptor antibody respectively and prepared for Western blot. We found, in line with what we have previously reported *(Khan et al. 2008)*, that the D1 receptor is co-immunoprecipitated with the AT1 receptor, and vice versa. Exposure to losartan significantly increased the power of interaction between the receptors. Co-

immunoprecipitaion of the AT1 receptor and detection of the D1 receptor resulted in a 28% increase in D1 receptors (n=4) (*p < 0.05 vs control). In the opposite direction co immunoprecipitation of D1 receptor and detection of the AT1 receptor resulted in a 22% increase in AT1 receptors (n=5) (*p < 0.01 vs control).



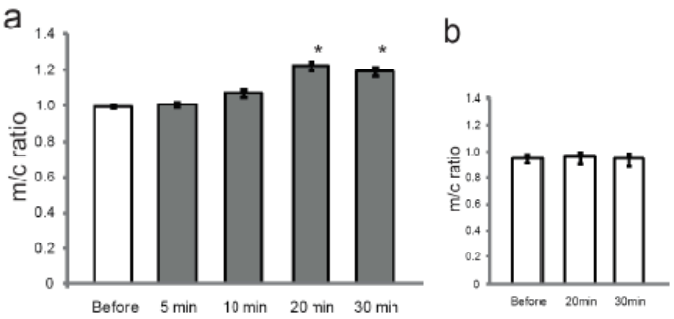
Next we examined whether losartan-bound AT1 receptors had an effect on the subcellular distribution of D1 receptors. We first performed cell surface biotinylation studies and found that losartan (10^{-5}M) significantly increased the abundance of biotinylated D1 receptors in the plasma membrane in proximal tubular cells by 13% (n=4) (*p < 0.05 vs control). A similar effect was observed in HEK293a cells double transfected with the D1 receptor and AT1 receptor by 17% (n=4) (*p < 0.05 vs control).



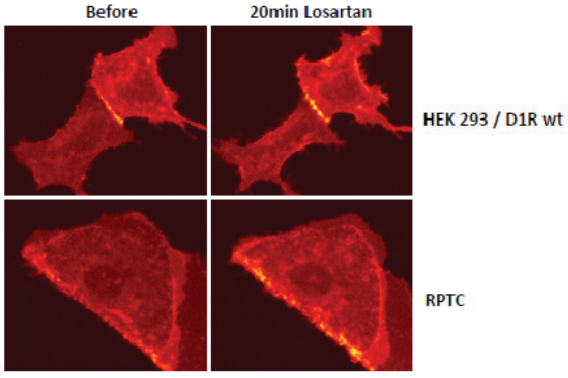
Live cell imaging

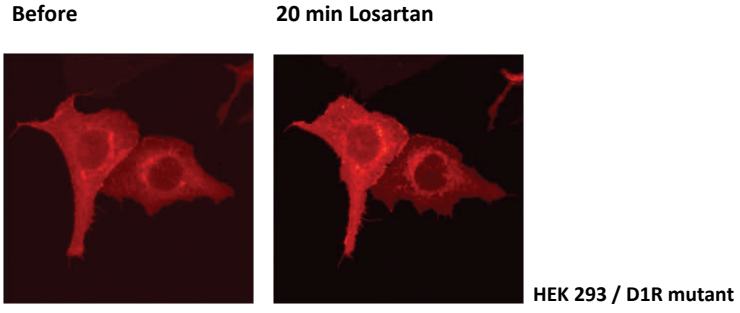
Next we studied the subcellular distribution of D1 receptors and AT1 receptors by live cell imaging techniques during losartan treatment in renal proximal tubular cells and HEK cells. Cells were transfected with the wild type D1 receptor and AT1 receptor. Images were collected before and at 5, 10, 20 and 30 minutes of exposure to losartan. We found that

losartan induced a time dependent increase in D1 receptor expression at the plasma membrane, with a significant maximal effect after 20 minutes exposure to losartan. Vehicle had no effect on D1 receptor localization.



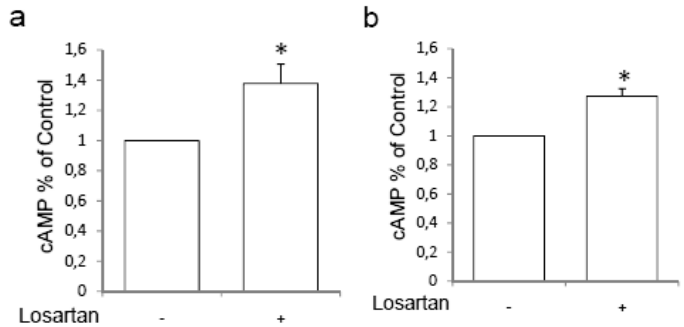
To examine whether this effect was dependent on a physical interaction between the lostartan bound AT1 receptor and the D1 receptor, cells were transfected with wild type AT1 receptor and mutant (S397A/S398A) D1 receptor. Losartan had no effect on D1 receptor localization in cells expressing the mutant D1 receptor.





cAMP generation

To test whether losartan-bound AT1 receptor had an allosteric effect on the D1 receptor, resulting in D1 receptor activation, we recorded the effect of losartan on cAMP generation in proximal tubular cells, which express relatively high levels of endogenous AT1 receptors and D1 receptors. We found that losartan significantly increased cAMP in these cells by 38% (n=7) (*p < 0.01 vs control). Next we recorded the effect of losartan on cAMP generation in HEK 293a cells, double transfected with the AT1 receptor and the D1 receptor. Losartan significantly increased cAMP in these cells by 27% (n=10) (*p < 0.001 vs control). Losartan did not have any effect on cAMP in cells single transfected with the AT1 receptor or D1 receptor alone.

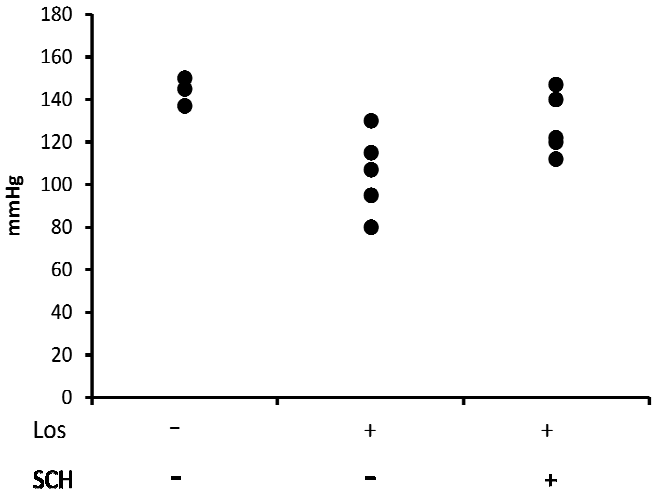


To examine whether the cAMP generating effect of losartan required physical interaction between the AT1 receptor and the D1 receptor, cells were transfected with wild type AT1 receptor and mutant D1 receptor (S397A/S398A). Losartan did not have any effect on cAMP production in HEK293a cells transfected with the AT1 receptor and the D1 receptor mutant. To confirm the functionality of the mutant D1 receptor, transfection of HEK cells with mutant D1 receptor alone was performed. Treatment with the D1 receptor agonist SKF-38393 (10^{-5}M) resulted in a significant increase of cAMP in these cells.

Losartan enhances D1 receptor effects in vivo

Hypertension was generated by performing a surgical procedure that leads to aortic coarctation proximal to the renal artery. In the control group (n=3) 14 days after the surgical procedure an increased blood pressure proximal to the coarctation was recorded (carotid) 144 ±3,8 mm Hg, and distal to the coarctation (femoral) by 106,7 ±14,8 mm Hg. In rats treated with losartan (20 mg/kg/day) (n=5) day 7 to 14 after the surgical procedure, the pressure proximal to the coarctation was 105 ±9 mmHg. Addition of a D1 receptor antagonist significantly attenuated the antihypertensive effect of losartan. In rats treated with losartan (20 mg/kg/day) day 7 to 14 after surgery and the D1 receptor antagonist SCH

23390 (1 mg/kg/day) day 10 to 14 after surgery (n=6), the pressure proximal to the coarctation was 126 ±6 mmHg. Treatment had relatively little effect on the pressure distal to the coarctation.



Discussion

Dopamine is well established as an intrarenal natriuretic hormone acting via inhibition of renal sodium transporters *(Aperia et al. 1987, Hansell et al. 1991, Felder et al. 1990, Eklöf et al. 1997)*. Many natriuretic factors, including atrial natriuretic peptide, and nitric oxide exert their effect via the renal dopamine system *(Holtbäck et al. 1999, Hansell et al. 1987, Costa et al. 2002)*. Prolactin has been shown to regulate fluid transport across cells in several different tissues, but relatively little information is available on its effects on renal ion transport and renal function. Several studies have shown that prolactin either induces an antidiuretic and antinatriuretic response *(Morrissey et al. 2001, Lucci et al. 1975, Mills et al. 1983, Stier et al. 1984)* or a diuretic and natriuretic response *(Alder et al. 1986, Roberts JR 1998, Morrissey et al. 2001)*. These discrepancies can be explained by contamination with vasopressin in the pituitary extract, different doses, differences in rat strains, or differences in the state of hydration prior to injection. During euvolemic conditions prolactin was found to have a pronounced natriuretic and diuretic response associated with a dose-dependent inhibition of proximal tubule Na^+, K^+-ATPase activity. Na^+, K^+-ATPase inhibition did not only occur during V_{max} conditions for Na^+, K^+-ATPase, but also when the enzyme was assayed with Na^+ 20 mmol/L, which is the approximate normal intracellular Na^+ concentration in renal proximal tubular cells. The effects of prolactin, both the in vivo and the in vitro effects, were abolished by a D1R antagonist. The L-dopa level in micro dissected proximal tubular segments is high enough to produce dopamine *(Ibarra et al. 1996)*.

We found that prolactin signaling pathways appear to be similar to dopamine signaling pathways in the proximal tubule. Subthreshold doses of prolactin and dopamine had a synergistic effect, and prolactin induced a heterologous recruitment of D1-like receptors from the interior of the cell into the plasma membrane. Dopamine mediates both PKA- and PKC-dependent phosphorylation of the catalytic α-subunit of Na^+, K^+-ATPase *(Bertorello et al. 1989, Fisone et al. 1994, Gomes et al. 2002)*. PKA-induced phosphorylation of Ser-943 enhances PKC-induced inhibition of Na^+, K^+-ATPase activity *(Cheng et al. 1997)*. In addition, dopamine-induced inhibition of Na^+, K^+-ATPase includes an activation of PI 3-K and a dimerization with Na^+, K^+-ATPase *(Chibalin et al. 1998, Yudowski et al. 2000)*. Besides JAK-2 activation, prolactin and dopamine seem to signal via similar pathways in the renal proximal tubule. Whether one or more of the JAK-2-phosphorylated proteins participate in D1-like receptor recruitment to the plasma membrane is an interesting topic for further studies.

In vivo treatment of Sprague-Dawley rats with prolactin resulted in a dramatic nine fold increase in urinary sodium excretion, without any effect on glomerular filtration rate or blood pressure. This natriuretic response was associated with kaliuresis, suggesting that the proximal tubule is the major site of action of prolactin. Prolactin treatment had no significant effects on GFR or blood pressure. We found that the renal effects of prolactin, including natriuresis, diuresis, kaliuresis, and Na^+, K^+-ATPase inhibition, were abolished by a D1

receptor antagonist. We also found that the response to prolactin on Na^+, K^+-ATPase activity was blunted in spontaneous hypertensive rats. The lack of prolactin effect in spontaneous hypertensive rats emphasizes the role of prolactin interacting with dopamine but does not rule out a possible defect of the prolactin receptor in these rats. This question deserves further investigations.

Taken together, the results suggest that the renal dopamine system has a permissive role for prolactin. The natriuretic potency of prolactin is in the order, or just above that for atrial natriuretic peptide *(Hansell et al. 1987)*.

The AT1 receptor plays a role for blood pressure regulation at all levels. Activation of the AT1 receptor in the brain (limbic, hypothalamic and brainstem) increases arterial blood pressure and plays a role for the central blood pressure control *(Buckley et al. 1963, Rettig et al. 1987)*.

Dopamine acting on the D1 family of receptors, and angiotensin II, acting on AT1 receptors, exert opposite effects on sodium excretion *(Zeng & Jose, 2011)*. Recent studies have shown that the AT1 receptor and the D1 receptor form a dimer, where they act as a unit of opposites *(Khan et al. 2008)*. The results from the co-immunoprecipitation studies were compatible with the notion that the D1 receptor and the AT1 receptor form a heterodimer.

Interestingly, agonist and antagonist binding to the AT1 receptor had opposite effects on the AT1 receptor-D1 receptor heteromeric complex. Results from a previous study have indicated that the power of interaction between the AT1 receptor and D1 receptor is decreased by angiotensin. Here we report that the power of the AT1 receptor and D1 receptor interaction is increased by the AT1 receptor antagonist losartan**.**

Losartan caused a small but significant increase of the plasma membrane expression of D1 receptors. In a previous study we identified two amino acids in the C-terminus tail of the D1 receptor, serine 397 and serine 398, to be of critical importance for D1 receptor and AT1 receptor interaction. Here we used transfected HEK cells to test whether the AT1 receptor might increase the surface expression of mutant D1 receptors that lacked the motif of importance for the interaction with the AT1 receptor. Surface expression of D1 receptors was not increased in these cells and we conclude that the effect of losartan-bound AT1 receptors on D1 receptor plasma membrane expression can be attributed to the function of the AT1 receptor-D1 receptor heterodimer. The losartan triggered increase in plasma membrane expression of the D1 receptor was confirmed in the live cell studies. This methodological approach allowed us to perform a time course recording, which showed that the increase in D1 receptor plasma membrane expression was not immediate, but appeared to reach a plateau after 20-30 min. This time schedule is more compatible with a reduced rate of D1 receptor internalization than with an increased rate of D1 receptor recruitment

triggered by losartan. The finding that losartan failed to increase plasma membrane expression of the mutant D1 receptor, that does not form a heterodimer with the AT1 receptor, supports the notion that losartan-bound AT1 receptor attenuates the internalization and recycling of the D1 receptor.

It is previously reported that the AT1 receptor activation by angiotensin II uncouples the D1 receptor from its signaling pathway in rat renal cells, and that activation of the D1 receptor uncouples AT1 receptor signaling. Since the D1 receptor is positively and the AT1 receptor is negatively coupled to adenylyl cyclase, we used the generation of cAMP as an index of D1 receptor activation. Losartan increased cAMP in cells expressing both AT1 receptor and wild type D1 receptor, but did not do so in cells expressing the AT1 receptor and the mutant D1 receptor, or in cells expressing the AT1 receptor or D1 receptor alone.

Taken together these results indicate that losartan will, by binding to the AT1 receptor, exert allosteric effects on its protomer, the D1 receptor, resulting in activation of D1 receptor signaling. Allosteric modulation within a heterodimer, where a structural modification in one protomer will affect the structure and function of the other protomer, has been intensively studied in the past decade, and is generally considered to be an important indirect mechanism for control of receptor function. Allosteric modulation among heterodimeric GPCRs, whereby agonist actions at one member of a heterodimer influences functional coupling of the other protomer, has been demonstrated for many GPCRSs, including the D1, D2 and D3 dopamine receptors, but, to the best of our knowledge, the action of an antagonist on one member of a heterodimer has not previously been reported to influence the functional coupling of the other protomer.

To test the concept of allosteric interaction between the losartan bound AT1 receptor and the D1 receptor in an in vivo model, we compared the antihypertensive effects of losartan alone with co-treatment of losartan and a D1 receptor antagonist in rats with experimental hypertension. We found that addition of a D1 receptor antagonist significantly attenuated the antihypertensive effect of losartan.

Future perspective

Many natriuretic factors, including atrial natriuretic peptide and nitric oxide exert their effect via the renal dopamine system. Heterologus receptor recruitment is one explanation for this effect. We used two complementary techniques, subcellular fractionation and biotinylation to examine the effect of prolactin in plasma membrane expression of the D1 receptor. We found that prolactin significantly increased D1 receptors inserted into the plasma membrane. The basal level of prolactin is elevated during pregnancy and after delivery, and gradually declines and return to near normal levels after 3 to 4 months post

delivery. Premature delivery is frequently associated with inadequate milk production *(Hill et al. 1999)*. Prolactin induces transcription of several different genes, both by positive and negative regulation. Is this transcription altered or enhanced in infants receiving a different amount of prolactin, and if so, should prolactin be a factor in consideration when producing baby formula. The infant does not have the ability to regulate sodium excretion or preservation as older children and adults, by having a blunted response to hormonal regulation. Speculatively - the maturation of this ability may be affected by an early exposure or lack of exposure to prolactin during the infant period, and predispose to different transcriptional levels of genes important in regulation of blood pressure.

Is there a tissue specific role of AT1 receptor-D1 receptor interaction? It is generally believed that while circulating angiotensin II levels exert direct effects on vascular resistance, the circulating levels of dopamine are too low to play a physiological or patho-physiological role. The current study was performed on renal tubular cells that play a central role in the regulation of salt balance, and indirectly, in blood pressure regulation. Will the results from this study be applicable for other tissues? Thus it should be of importance to examine the role of the AT1 receptor- D1 receptor complex in other cells of importance for cardio-vascular function and blood pressure regulation, such as the cardiomyocyte.

It is noteworthy that the interaction between the AT1 receptor and the D1 receptor was similar in the primitive HEK cell line and the renal proximal tubular cells, indicating that the AT1 receptor and D1 receptor allosterism does not require the presence of additional highly specific proteins.

The receptor heteromer concept is now widely accepted and it was recently postulated that more knowledge about the functional significance of receptor heterodimers will "revolutionize basic tenets of pharmacology and take drug development to a new level of specificity and efficacy" *(Ferré et al. 2009)*.

To test the concept of allosteric interaction between losartan bound AT1 receptors and D1 receptors in an in vivo model, we compared the antihypertensive effects of losartan alone with co-treatment of losartan and a D1 receptor antagonist in rats with experimental hypertension. The results provide proof of principle of a functionally important allosteric interaction between AT1R and D1R and imply that attempts should be made to develop low-dose combination therapy of AT1 receptor antagonists and D1 receptor agonists in the treatment of hypertension.

Conclusion

Prolactin is a potent natriuretic peptide. This effect is associated with an inhibition of proximal tubular Na^+, K^+-ATPase activity. The renal effects of prolactin are abolished during inhibition of the renal dopamine synthesis as well as by a D1 receptor antagonist. We conclude that dopamine has a central role in the interactive regulation of salt homeostasis. Not only G protein coupled receptors but also a classical tyrosine receptor, the prolactin receptor, exerts its salt-regulating effect by heterologous recruitment and an associated activation of renal D1 receptors. Hypothetically, heterologous receptor recruitment may be one explanation for cross talk between signaling pathways mediated by tyrosine kinase receptors and G protein-coupled receptors. The current study confirms the concept that the AT1 receptor and the D1 receptor form a heteromeric signaling complex and identifies losartan, an AT1 receptor antagonist and widely used antihypertensive drug, as an allosteric modifier of this complex. The finding that an AT1 receptor antagonist can activate D1 receptor signaling and that this effect is dependent on AT1 receptor and D1 receptor interaction is novel and has potential pharmacologic implications.

In these studies we have confirmed the central role of the renal dopamine system in the regulation of sodium balance. Prolactin induces a natriuretic effect that act via the renal dopamine system by heterologous recruitment of D1 receptors to the plasma membrane. Effects among dopamine D1 receptors and angiotensin II AT1 receptors are explained by the antagonist bound AT1 receptor exerting an allosteric effect on its protomer, the dopamine D1 receptor, resulting in D1 receptor activation and D1 receptor signaling.

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References

- Albrecht FE, Drago J, Felder RA, Printz MP, Eisner GM, Robillard JE, Sibley DR, Westphal HJ, Jose PA. (1996) Role of the D1A dopamine receptor in the pathogenesis of genetic hypertension. J Clin Invest. May 15;97(10):2283-8.
- Alder RA, Herzberg VL, Brinck-Johnsen T, Sokol W (1986): Increased water excretion in hyperprolactinemic rats. *Endocrinology* 118:1519–1522
- Ali S, Pellegrini I, Kelly PA (1991): A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. *J Biol Chem* 266:20110–20117
- Al-Sakkaf KA, Dobson PR, Brown BL (1997). Prolactin induced tyrosine phosphorylation of p59fyn may mediate phosphatidylinositol 3-kinase activation in Nb2 cells. J Mol Endocrinol. Dec;19(3):347-50.
- Amenta F, Ricci A (1990). Autoradiographic localization of dopamine DA-1 receptors in the rat renal vasculature using [3H]-SCH 23390 as a ligand. J Auton Pharmacol. Dec;10(6):373-83.
- Aperia A, Bertorello A, Seri I (1987). Dopamine causes inhibition of Na+-K+-ATPase activity in rat proximal convoluted tubule segments. Am J Physiol. Jan;252(1 Pt 2):F39-45.
- Aperia A, Holtbäck U, Syrén ML, Svensson LB, Fryckstedt J, Greengard P (1994). Activation/deactivation of renal Na+,K(+)-ATPase: a final common pathway for regulation of natriuresis. FASEB J. Apr 1;8(6):436-9.
- Asico LD, Ladines C, Fuchs S, Accili D, Carey RM, Semeraro C, Pocchiari F, Felder RA, Eisner GM, Jose PA (1998). Disruption of the dopamine D3 receptor gene produces renin-dependent hypertension. J Clin Invest. Aug 1;102(3):493-8.
- Ball SG, Lee MR, Oates NS (1978). The effect of inorganic salts on renal tissue dopamine levels in the rat. Br J Pharmacol. Jun;63(2):343P.
- Baines AD, Chan W (1980). Production of urine free dopamine from DOPA; a micropuncture study. Life Sci. Jan 28;26(4):253-9.
- Baines AD, Drangova R (1984). Dopamine production by the isolated perfused rat kidney. Can J Physiol Pharmacol. Mar;62(3):272-6.
- Baines AD, Drangova R, Hatcher C (1985). Dopamine production by isolated glomeruli and tubules from rat kidneys. Can J Physiol Pharmacol. Feb;63(2):155-8.
- Baines AD, Ho P, Drangova R (1992). Proximal tubular dopamine production regulates basolateral Na-K-ATPase. Am J Physiol. Apr;262(4 Pt 2):F566-71.
- Barber R, Butcher RW (1988). cAMP turnover in intact cells. Methods Enzymol. 159:50-60
- Ben-Jonathan N, Hnasko R (2001). Dopamine as a prolactin (PRL) inhibitor. *Endocrine Rev* 22:724–763
- Berlanga JJ, Fresno Vara JA, Martín-Pérez J, García-Ruiz JP (1995). Prolactin receptor is associated with c-src kinase in rat liver. Mol Endocrinol. Nov;9(11):1461-7.

- Berlanga JJ, Gualillo O, Buteau H, Applanat M, Kelly PA, Edery M (1997). Prolactin activates tyrosyl phosphorylation of insulin receptor substrate 1 and phosphatidylinositol-3-OH kinase. J Biol Chem. Jan 24;272(4):2050-2.
- Berthon P, Kelly PA, Djiane J. (1987) Water-soluble prolactin receptors from porcine mammary gland. Proc Soc Exp Biol Med. Mar;184(3):300-6.
- Bertorello A, Hökfelt T, Goldstein M, Aperia A. (1988) Proximal tubule Na+-K+-ATPase activity is inhibited during high-salt diet: evidence for DA-mediated effect. Am J Physiol. Jun;254(6 Pt 2):F795-801.
- Bertorello A, Aperia A. Na+-K+-ATPase is an effector protein for protein kinase C in renal proximal tubule cells. (1989) Am J Physiol. Feb;256(2 Pt 2):F370-3.
- Bertorello A, Aperia A. (1990) Inhibition of proximal tubule Na(+)-K(+)-ATPase activity requires simultaneous activation of DA1 and DA2 receptors. Am J Physiol. Dec;259(6 Pt 2):F924-8.
- Bertorello AM, Aperia A, Walaas SI, Nairn AC, Greengard P. (1991) Phosphorylation of the catalytic subunit of Na+,K(+)-ATPase inhibits the activity of the enzyme. Proc Natl Acad Sci U S A. Dec 15;88(24):11359-62.
- Berwaer M et al. (1991) Multihormonal regulation of the human prolactin gene expression from 5000 bp of its upstream sequence. Mol Cell Endocrinol. Sep;80(1-3):53-64.
- Berwaer M, Martial JA, Davis JR. (1994) Characterization of an up-stream promoter directing extrapituitary expression of the human prolactin gene. Mol Endocrinol. May;8(5):635-42.
- Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev. Jun;19(3):225-68. Review.
- Brismar H, Asghar, M., Carey, R.M., Greengard, P., Aperia, A. (1998) Dopamine-induced recruitment of dopamine D1 receptors to the plasma membrane. Proc Natl Acad Sci USA 95 , 5573 5578.
- Brismar H, Agrèn M, Holtbäck U. (2002) beta-Adrenoceptor agonist sensitizes the dopamine-1 receptor in renal tubular cells. Acta Physiol Scand. Aug;175(4):333-40.
- Buckley JP, Bickerton RK, Halliday RP, Kato H. (1963) Central effects of peptides on the cardiovascular system. Ann N Y Acad Sci. Feb 4;104:299-311.
- Chen C, Beach RE, Lokhandwala MF (1993). Dopamine fails to inhibit renal tubular sodium pump in hypertensive rats. Hypertension. Mar;21(3):364-72.
- Cheng XJ, Höög JO, Nairn AC, Greengard P, Aperia A. (1997) Regulation of rat Na, K-ATPase activity by PKC is modulated by state of phosphorylation of Ser-943 by PKA. *Am J Physiol Cell Physiol* 273: C1981–C1986.
- Cheng SX, Aizman O, Nairn AC, Greengard P, Aperia A. (1999) [Ca2+]i determines the effects of protein kinases A and C on activity of rat renal Na+,K+-ATPase. J Physiol. Jul 1;518 (Pt 1):37-46.

- Chen C, Lokhandwala MF. (1993) Inhibition of Na+,K(+)-ATPase in rat renal proximal tubules by dopamine involved DA-1 receptor activation. Naunyn Schmiedebergs Arch Pharmacol. Mar;347(3):289-95.
- Chibalin AV, Zierath JR, Katz AI, Berggren PO, Bertorello AM. (1998) Phosphatidylinositol 3-kinase-mediated endocytosis of renal Na+, K+-ATPase alpha subunit in response to dopamine. Mol Biol Cell. May;9(5):1209-20.
- Christensen BM, Zelenina M, Aperia A, Nielsen S. (2000) Localization and regulation of PKA-phosphorylated AQP2 in response to V(2)-receptor agonist/antagonist treatment. Am J Physiol Renal Physiol. Jan;278(1):F29-42.
- Cogan MG. (1990) Angiotensin II: a powerful controller of sodium transport in the early proximal tubule. Hypertension. May;15(5):451-8. Review.
- Cooke NE, Coit D, Shine J, Baxter JD, Martial JA. (1981) Human prolactin. cDNA structural analysis and evolutionary comparisons. J Biol Chem. Apr 25;256(8):4007-16
- Costa M De L, Loria A, Marchetti M, *et al*: (2002) Effects of dopamine and nitric oxide on arterial pressure and renal function in volume expansion. *Clin Exp Pharmacol Physiol* 29:772–776,
- Dickley RP, Stone SC. (1975) Drugs that affect the breast and lactation. *Clin Obstet Gynecol* 18:95–111,
- Edwards RM, Brooks DP. (2001) Dopamine inhibits vasopressin action in the rat inner medullary collecting duct via alpha(2)-adrenoceptors. *J Pharmacol Exp Ther* 298:1001–1006
- Efendiev R, Budu CE, Cinelli AR, Bertorello AM, Pedemonte CH. (2003) Intracellular Na+ regulates dopamine and angiotensin II receptors availability at the plasma membrane and their cellular responses in renal epithelia. J Biol Chem. Aug 1;278(31):28719-26.
- Eklöf AC, Aperia A. (1990) Renal hypertension following aortic constriction is abolished by angiotensin II converting enzyme inhibitor, but not by low-salt diet. Acta Physiol Scand. Jul;139(3):435-40
- Eklöf AC, Holtbäck U, Sundelöf M, Chen S, Aperia A. (1997) Inhibition of COMT induces dopamine-dependent natriuresis and inhibition of proximal tubular Na+,K+-ATPase. Kidney Int. Sep;52(3):742-7.
- Eklöf AC, Holtbäck U, Svennilson J, Fienberg A, Greengard P, Aperia A. (2001) Increased blood pressure and loss of anp-induced natriuresis in mice lacking DARPP-32 gene. Clin Exp Hypertens. Aug;23(6):449-60.
- Essayan DM. (2001) Cyclic nucleotide phosphodiesterases. J Allergy Clin Immunol. Nov;108(5):671-80. Review.
- Falconer IR, Rowe JM. (1975) Possible mechanism for action of prolactin on mammary cell sodium transport. *Nature* 256:327–328,
- Falconer IR, Langely JV, Vacek AT. (1983) Effect of prolactin on 86Rb+ uptake, potassium content and [G-3H] ouabain binding of lactating rabbit mammary tissue. *J Physiol (Lond)* 334:1–17,

- Fasciolo JC, Houssay BA, Taquini AC. (1938) The blood-pressure raising secretion of the ischaemic kidney. J Physiol. Dec 14;94(3):281-93.
- Felder CC, Blecher M, Jose PA. (1989) Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. J Biol Chem. May 25;264(15):8739-45.
- Felder CC, Campbell T, Albrecht F, Jose PA: (1990) Dopamine inhibits Na, H-exchanger activity in renal BBMV by stimulation of adenylate cyclase. *Am J Physiol* 259:F297–F303,
- Felder CC, Albrecht FE, Campbell T, Eisner GM, Jose PA. (1993) cAMP-independent, G protein-linked inhibition of Na+/H+ exchange in renal brush border by D1 dopamine agonists. Am J Physiol. Jun;264(6 Pt 2):F1032-7.
- Féraille E, Doucet (2001) A Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: hormonal control. Physiol Rev. Jan;81(1):345-418.
- Fernandes MH, Soares-da-Silva P. (1992) Type A and B monoamine oxidase activities in the human and rat kidney. Acta Physiol Scand. Aug;145(4):363-7
- Ferrag F, Pezet A, Chiarenza A, Buteau H, Nelson BH, Goffin V, Kelly PA. (1998) Homodimerization of IL-2 receptor beta chain is necessary and sufficient to activate Jak2 and downstream signaling pathways. FEBS Lett. Jan 2;421(1):32-6.
- Ferré S, Baler R, Bouvier M, Caron MG, Devi LA, Durroux T, Fuxe K, George SR, Javitch JA, Lohse MJ, Mackie K, Milligan G, Pfleger KD, Pin JP, Volkow ND, Waldhoer M, Woods AS, Franco R. (2009) Building a new conceptual framework for receptor heteromers. Nat Chem Biol. Mar;5(3):131-4
- Feschenko MS, Sweadner KJ. (1997) Phosphorylation of Na,K-ATPase by protein kinase C at Ser18 occurs in intact cells but does not result in direct inhibition of ATP hydrolysis. J Biol Chem. Jul 11;272(28):17726-33.
- Fisone G, Cheng SX, Nairn AC, Czernik AJ, Hemmings HC Jr, Höög JO, Bertorello AM, Kaiser R, Bergman T, Jörnvall H, et al. (1994) Identification of the phosphorylation site for cAMP-dependent protein kinase on Na+,K(+)-ATPase and effects of site-directed mutagenesis. J Biol Chem. Mar 25;269(12):9368-73.
- Freeman ME, Kanyicska B, Lerant A, Nagy G. (2000) Prolactin: structure, function, and regulation of secretion. Physiol Rev. Oct;80(4):1523-631.
- Goldberg LI. (1972) Cardiovascular and renal actions of dopamine: potential clinical applications. Pharmacol Rev. Mar;24(1):1-29.
- Gomes P, Soares-da-Silva P. (2002) Role of cAMP-PKA-PLC signaling cascade on dopamine-induced PKC-mediated inhibition of renal Na_-K_-ATPase activity. *Am J Physiol Renal Physiol* 282: F1084–F1096.
- Goupille O, Daniel N, Bignon C, Jolivet G, Djiane J. (1997) Prolactin signal transduction to milk protein genes: carboxy-terminal part of the prolactin receptor and its tyrosine phosphorylation are not obligatory for JAK2 and STAT5 activation. Mol Cell Endocrinol. Mar 28;127(2):155-69.

- Grenader A, Healy DP: (1991) Locally formed dopamine stimulates cAMP accumulation in LLC-PK1 cells via a DA1 dopamine receptor. *Am J Physiol* 260:F906–F912,
- Guyton AC. (1987) Renal function curve--a key to understanding the pathogenesis of hypertension. Hypertension. Jul;10 (1):1-6.
- de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. (2000) International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev. Sep;52(3):415-72. Review.
- Hagege J, Richet G. (1985) Proximal tubule dopamine histofluorescence in renal slices incubated with L-dopa. Kidney Int. Jan;27(1):3-8.
- Hansell P, Fasching A, Sjöquist M, *et al*: (1987) The dopamine receptor antagonist haloperidol blocks natriuretic but not hypotensive effects of the atrial natriuretic factor. *Acta Physiol Scand*130:401–407,
- Hansell P, Ulfendahl HR: (1987) Effects of atrial natriuretic peptide (ANP) during converting enzyme inhibition. *Acta Physiol Scand* 130: 393–399,
- Hansell P, Fasching A: (1991)The effect of dopamine receptor blockade on natriuresis is dependent on the degree of hypervolemia. *Kidney Int* 39:253–258,
- Hardman JE, Limbird LE, Gilman AG: (2001) Hormones and hormone antagonists, prolactin, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 10th ed., New York, McGraw-Hill, pp 1549–1551.
- Harris PJ, Young JA. (1977) Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in the rat kidney. Pflugers Arch. Jan 17;367(3):295-7.
- Harris PJ, Navar LG. (1985) Tubular transport responses to angiotensin. Am J Physiol. May;248(5 Pt 2):F621-30. Review.
- Harvey JN, Casson IF, Clayden AD, Cope GF, Perkins CM, Lee MR. (1984) A paradoxical fall in urine dopamine output when patients with essential hypertension are given added dietary salt. Clin Sci (Lond). Jul;67(1):83-8.
- Hayashi M, Yamaji Y, Kitajima W, Saruta T. (1990) Aromatic L-amino acid decarboxylase activity along the rat nephron. Am J Physiol. Jan;258(1 Pt 2):F28-33.
- Hepler JR, Gilman AG. (1992) G proteins. Trends Biochem Sci. Oct;17(10):383-7.
- Hermanson GT, Mattson GR, See SG, Seely SA. (1994) Preparation and use of biotin-binding supports on a biosupport medium. Am Biotechnol Lab. Sep;12(10):86, 88.
- Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. (2007) Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. Clin Sci (Lond). Apr;112(8):417-28. Review
- Hill PD, Aldag JC, Zinaman M, Chatterton RT Jr. (2007) Comparison of milk output between breasts in pump-dependent mothers. J Hum Lact. Nov;23(4):333-7
- Holtbäck U, Brismar H, Di Bona GF, *et al*: (1999) Receptor recruitment: Amechanism for interaction between G protein-coupled receptors. *Proc Natl Acad Sci USA* 96:7271–7275,

- Holtbäck U, Kruse MS, Brismar H, Aperia A. (2000) Intrarenal dopamine coordinates the effect of antinatriuretic and natriuretic factors. Acta Physiol Scand. Jan;168(1):215-8. Review.
- Hu ZZ, Dufau ML. (1991) Multiple and differential regulation of ovarian prolactin receptor messenger RNAs and their expression. Biochem Biophys Res Commun. Nov 27;181(1):219-25.
- Ibarra F, Aguirre J, Nowicki S, Barontini M, Arrizurieta EE, Armando I. (1996) Demethylation of 3-*O*-methyldopa in the kidney: a possible source for dopamine in urine. *Am J Physiol Renal Fluid Electrolyte Physiol* 270: F862–F868,.
- Ibarra FR, Cheng SX, Agrén M, Svensson LB, Aizman O, Aperia A. (2002) Intracellular sodium modulates the state of protein kinase C phosphorylation of rat proximal tubule Na+,K+-ATPase. Acta Physiol Scand. Jun;175(2):165-71
- Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvennoinen O. (1994) Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem Sci. May;19(5):222-7.
- Ingelfinger JR, Schunkert H, Ellison KE, Pivor M, Zuo WM, Pratt R, Dzau VJ. (1990) Intrarenal angiotensinogen: localization and regulation. Pediatr Nephrol. Jul;4(4):424-8. Review.
- Javitch JA. (2004) The ants go marching two by two: oligomeric structure of G-protein-coupled receptors. Mol Pharmacol. Nov;66(5):1077-82. Epub 2004 Aug 19.
- Jose PA, Raymond JR, Bates MD, Aperia A, Felder RA, Carey RM. (1992) The renal dopamine receptors. J Am Soc Nephrol. Feb;2(8):1265-78.
- Jose PA, Eisner GM, Drago J, Carey RM, Felder RA. (1996) Dopamine receptor signaling defects in spontaneous hypertension. Am J Hypertens. Apr;9(4 Pt 1):400-5. Review.
- Kawabe K, Watanabe TX, Shiono K, Sokabe H. (1978) Influence on blood pressure of renal isografts between spontaneously hypertensive and normotensive rats, utilizing the F1 hybrids. Jpn Heart J. Nov;19(6):886-94.
- Khan F, Spicarová Z, Zelenin S, Holtbäck U, Scott L, Aperia A. (2008) Negative reciprocity between angiotensin II type 1 and dopamine D1 receptors in rat renal proximal tubule cells. Am J Physiol Renal Physiol. Oct;295(4):F1110-6. Epub 2008 Aug 13
- Kinoshita S, Sidhu A, Felder RA. (1989) Defective dopamine-1 receptor adenylate cyclase coupling in the proximal convoluted tubule from the spontaneously hypertensive rat. J Clin Invest. Dec;84(6):1849-56.
- Kopatz I, Remy JS, Behr JP. (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. J Gene Med. Jul;6(7):769-76.
- Kopin IJ. (1985) Catecholamine metabolism: basic aspects and clinical significance. Pharmacol Rev. Dec;37(4):333-64. Review.

- Kruse MS, Adachi S, Scott L, Holtbäck U, Greengard P, Aperia A, Brismar H. *(1995)* Recruitment of renal dopamine 1 receptors requires an intact microtubulin network. *Am J Physiol 268:F1185–F1197*
- Lucci MS, Bengeleh H, Solomon S: (1975) Suppressive action of prolactin on renal response to volume expansion. *Am J Physiol* 229:81–85
- Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, Häussinger D. (1998) Functional significance of cell volume regulatory mechanisms. Physiol Rev. 78:247-306
- Lin SH, Guidotti G. (2009) Purification of membrane proteins. Methods Enzymol.;463:619-29. Review.
- Lin SY, Goodfriend TL. (1970) Angiotensin receptors. Am J Physiol. May;218(5):1319-28.
- Liu FY, Cogan MG. (1987) Angiotensin II: a potent regulator of acidification in the rat early proximal convoluted tubule. J Clin Invest. Jul;80(1):272-5.
- Liu FY, Cogan MG. (1989) Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. J Clin Invest. Jul;84(1):83-91.
- Manku MS, Mtabaji JB, Horrobin DF. (1975) Effect of cortisol, prolactin and ADH on the amniotic membrane. *Nature* 258:78–80,
- McGrath B, Bode K, Luxford A, Howden B, Jablonski P. (1985) Effects of dopamine on renal function in the rat isolated perfused kidney. Clin Exp Pharmacol Physiol. Jul-Aug;12(4):343-52.
- Mills DE, Buckman MT, Peake GT: (1983) Mineralocorticoid modulation of prolactin effect on renal solute excretion in the rat. *Endocrinology* 112:823–828,
- Mitchell KD, Navar LG. (1988) Enhanced tubuloglomerular feedback during peritubular infusions of angiotensins I and II. Am J Physiol. Sep;255(3 Pt 2):F383-90.
- Morrissey SE, Newth T, Rees R, *et al*: (2001) Renal effects of recombinant prolactin in anaesthetized rats. *Eur J Endocrinol* 145:65–71,
- Mountjoy K, Cowden EA, Dobbie JW, Ratcliffe JG: (1980) Prolactin receptors in the rat kidney. *J Endocrinol* 87:47–54,
- Nakajima T, Naitoh F, Kuruma I. (1977) Dopamine-sensitive adenylate cyclase in the rat kidney particulate preparation. Eur J Pharmacol. Jan 21;41(2):163-9.
- Navar LG, Carmines PK, Huang WC, Mitchell KD. (1987) The tubular effects of angiotensin II. Kidney Int Suppl. May;20:S81-8. Review.
- Niall HD, Hogan ML, Sauer R, Rosenblum IY, Greenwood FC. (1971) Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene reduplication. Proc Natl Acad Sci U S A. Apr;68(4):866-70.
- Nielsen S, DiGiovanni SR, Christensen EI, Knepper MA, Harris HW. (1993) Cellular and subcellular immunolocalization of vasopressin-regulated water channel in rat kidney. Proc Natl Acad Sci U S A. Dec 15;90(24):11663-7

- Page IH. (1935) Pressor substances from the body fluids of man in health and disease. J Exp Med. Jan 1;61(1):67-96
- Piccoletti R, Maroni P, Bendinelli P, Bernelli-Zazzera A. (1994) Rapid stimulation of mitogen-activated protein kinase of rat liver by prolactin. Biochem J. Oct 15;303 (Pt 2):429-33.
- Postel-Vinay MC, Belair L, Kayser C, Kelly PA, Djiane J. (1991) Identification of prolactin and growth hormone binding proteins in rabbit milk. Proc Natl Acad Sci U S A. Aug 1;88(15):6687-90.
- Prevarskaya NB, Skryma RN, Vacher P, Daniel N, Djiane J, Dufy B. (1995) Role of tyrosine phosphorylation in potassium channel activation. Functional association with prolactin receptor and JAK2 tyrosine kinase. J Biol Chem. Oct 13;270(41):24292-9.
- Ramseydh, Bernha. (1972) Stimulation by ovine prolactin of fluid transfer in everted sacs of rat small intestine. *J Endocrinol* 53:453–459,
- Rang HP, Dale MM, Ritter JM: (1999) The central nervous system, in *Pharmacology*, 4th ed., Edinburgh, Churchill Livingstone, , pp 483–542 (464–647).
- Ratovondrahona D, Fournier B, Odessa MF, Dufy B. (1998) Prolactin stimulation of phosphoinositide metabolism in CHO cells stably expressing the PRL receptor. Biochem Biophys Res Commun. Feb 4;243(1):127-30.
- Rettig R, Ganten D, Lang RE, Unger T. (1987) The renin-angiotensin system in the central control of blood pressure. Eur Heart J. May;8 Suppl B:129-32. Review.
- Riddle et al. (1933) The preparation, identification and assay of prolactin – A hormone of the anterior pituitary. Am J Physiol 105: p.191–216.
- Roberts JR: (1998) The effect of acute or chronic administration of prolactin on renal function in fetal chickens. *J Comp Physiol [B]* 168:25–31,
- Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL. (1992) Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. J Biol Chem. Nov 25;267(33):24076-81.
- Sanada H, Jose PA, Hazen-Martin D, Yu PY, Xu J, Bruns DE, Phipps J, Carey RM, Felder RA. (1999) Dopamine-1 receptor coupling defect in renal proximal tubule cells in hypertension. Hypertension. Apr;33(4):1036-42.
- Satoh T, Cohen HT, Katz AI. (1993) Different mechanisms of renal Na-K-ATPase regulation by protein kinases in proximal and distal nephron. Am J Physiol. Sep;265(3 Pt 2):F399-405.
- Schiöth HB, Fredriksson R. (2005) The GRAFS classification system of G-protein coupled receptors in comparative perspective. Gen Comp Endocrinol. May 15;142(1-2):94-101. Epub 2005 Feb 5.
- Schuster VL, Kokko JP, Jacobson HR. (1984) Angiotensin II directly stimulates sodium transport in rabbit proximal convoluted tubules. J Clin Invest. Feb;73(2):507-15.
- Selvaraj NG, Omi E, Gibori G, Rao MC: (2000) Janus kinase 2 (JAK2) regulates prolactin-mediated chloride transport in mouse mammary epithelial cells through

tyrosine phosphorylation of Na, K, 2Cl cotransporter. *Molecular Endocrin* 14:2054–2065,

- Sinha YN, (1995) Structural variants of prolactin: occurrence and physiological significance.Endocr Rev. Jun;16(3):354-69.
- Skou JC. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim Biophys Acta. Feb;23(2):394-401.
- Soares-Da-Silva O, Serrao MP, Vieiral-Coelho MA: (1998) Apical and basolateral uptake and intracellular fate of dopamine precursor Ldopa in LLC-PK1 cells. *Am J Physiol* 274:F243–F251,
- Soares-da-Silva P, Fernandes MH, Pinto-do-O PC. (1994) Cell inward transport of L-DOPA and 3-O-methyl-L-DOPA in rat renal tubules. Br J Pharmacol. Jun;112(2):611-5.
- Stier CT Jr, Cowden EA, Friesen HG, Allison ME. (1984) Prolactin and the rat kidney: a clearance and micropuncture study. Endocrinology. Jul;115(1):362-7.
- Takemoto F, Satoh T, Cohen HT, Katz AI. (1991) Localization of dopamine-1 receptors along the microdissected rat nephron. Pflugers Arch. Oct;419(3-4):243-8.
- Thomas WG, Qian H, Chang CS, Karnik S. (2000) Agonist-induced phosphorylation of the angiotensin II (AT(1A)) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. J Biol Chem. Jan 28;275(4):2893-900.
- Venkatakrishnan U, Chen C, Lokhandwala MF. (2000) The role of intrarenal nitric oxide in the natriuretic response to dopamine-receptor activation. Clin Exp Hypertens. Apr;22(3):309-24.
- Wilson FH, Kahle KT, Sabath E, Lalioti MD, Rapson AK, Hoover RS, Hebert SC, Gamba G, Lifton RP. (2003) Molecular pathogenesis of inherited hypertension with hyperkalemia: the Na-Cl cotransporter is inhibited by wild-type but not mutant WNK4. Proc Natl Acad Sci U S A. Jan 21;100(2):680-4
- Yao LP, Li XX, Yu PY, Xu J, Asico LD, Jose PA. (1998) Dopamine D1 receptor and protein kinase C isoforms in spontaneously hypertensive rats. Hypertension. Dec;32(6):1049-53.
- Yudowski GA, Efendiev R, Pedemonte CH, Katz AI, Berggren PO, Bertorello AM. (2000) Phosphoinositide-3 kinase binds to a proline-rich motif in the Na+, K+-ATPase alpha subunit and regulates its trafficking. Proc Natl Acad Sci U S A. Jun 6;97(12):6556-61.
- Yuste R and Konnerth A. (2005) Imaging in Neuroscience and Development, a laboratory manual. Cold spring harbor laboratory press, New York.
- Zeng C, Zhang M, Asico LD, Eisner GM, Jose PA. (2007) The dopaminergic system in hypertension. Clin Sci (Lond). Jun;112(12):583-97. Review.
- Zeng C, Jose PA. (2011) Dopamine receptors: important antihypertensive counterbalance against hypertensive factors. Hypertension. Jan;57(1):11-7. Epub 2010 Nov 22. Review.