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HUI WANG

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By

Hui Wang

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ABSTRACT

TRPV1 AND ITS REGULATION IN NORMAL AND HYPERTENSIVE RATS

By

Hui Wang

The transient receptor potential vanilloid type 1 (TRPV1) channel is a ligand-gated cation channel that can be activated by capsaicin, heat, protons and cytosolic lipids. TRPV1 is expressed primarily in sensory nerves and functions as a molecular integrator for multiple types of sensory input. Recent reports suggest that TRPV1 plays an important role in regulating renal blood flow, blood pressure and salt sensitivity in hypertension via release of vasoactive neuropeptides from sensory nerves.

Repeated applications of capsaicin (CAP) induce TRPV1 desensitization, which is reversed by ATP. In my studies the underlying signaling mechanism of TRPV1 resensitization by ATP was investigated in kidney-projecting sensory neurons of normal Wistar rats. Application of Fast Blue (FB) to the nerves surrounding the renal artery retrogradely labeled neurons in DRG of rats. Whole cell patch clamp recording was performed on FB-labeled neurons. CAP was used to activate TRPV1. Four types of kidney-projecting neurons were identified based on responses caused by CAP: 1) desensitizing, 2) non-desensitizing, 3) silent and 4) insensitive. Silent neurons responded to CAP only after treatment with extracellular ATP. ATP reversed desensitization in desensitizing neurons. Insensitive neurons never responded to CAP. Pharmacological studies using agonists and antagonists selective for subtypes of P2Y receptors and immunohistochemical studies showed that activation of $P2Y_2$ receptors modulates of TRPV1 activity on kidney-projecting sensory neurons. It was also shown that $P2Y_2$

receptors link to activation of protein kinase C which is responsible for ATP induced TRPV1 resensitization.

To determine the role of TRPV1 channels in development of salt-sensitive hypertension, Dahl salt-sensitive rats were studied. High salt diet significantly increased systolic blood pressure in DS/HS rats compared with DS/LS rats. CAP-induced TRPV1 currents were significantly decreased and the CAP half maximal effective concentration was significantly higher in neurons from DS/HS rats. The reversal effect of ATP on desensitized TRPV1 was enhanced in kidney-projecting neurons of DS/HS rats. The calcitonin gene-related peptide (CGRP)-positive sensory nerve fibers surrounding renal interlobar arteries were remarkably reduced in DS/HS rats.

These results indicate that extracellular ATP resensitizes TRPV1 on kidney-projecting sensory neurons via activation of $P2Y_2$ receptors and intracellular PKC pathway. $P2Y_2$ receptor modulation of TRPV1 function may be a mechanism of interaction between sympathetic and sensory nerves supplying the renal vasculature. ATP released from sympathetic nerves could modulate TRPV1 function in sensory nerves and therefore modulate release of vasodilators. Therefore vascular homeostasis of the kidney could be balanced by sympathetic nerves and sensory nerves. Studies in DS rats suggest that salt-induced impairment of TRPV1 function and disruption of CGRP-positive sensory nerves innervating kidney might be a novel mechanism contributing to salt-sensitivity of DS rats. Taken together, our studies reveal the importance of TRPV1-positive sensory nerves as a possible target for understanding development and treatment of salt-sensitive hypertension.

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1,4-di-[(3-isothiocyanato phenyl)-thioureido]butane	MRS 2578
2-chloro-N6-methyldeoxyadenosine 3 ,5 -biphosphate	MRS 2216
2-methylthio-ATP	2-Me-S-ATP
Adenosine-3 -phosphate-5 -phosphate	A3P5P
Adrenaline	EPI
Adrenergic receptor	AR
Angiotensin converting enzyme	ACE
Arginine vasopressin	AVP
Biphosphate nucleotide adenosine-2 -phosphate-5 -phosphate	A2P5P
Blood pressure	BP
Calcitonin gene-related peptide	CGRP
Capsaicin	CAP
Chelerythrine	CHT
Complementary DNA	cDNA
Cyclic AMP	cAMP
Dahl salt–sensitive	DS
Deoxycorticosterone acetate	DOCA
Diacyl glycerol	DAG
Dopamine	DA
Dorsal root ganglia	DRG
Endothelin	ET

Endothelium derived hyperpolarizing factor	EDHF
Fast Blue	FB
Forskolin	FSK
Glomerular filtration rate	GFR
Inactivation-no-afterpotential D	INAD
N6-methyldeoxyadenosine 3 ,5 -biphosphate	MRS 2179
Natriuretic peptide	ANP
Neurokinin A	NKA
Neurokinin B	NKB
Norepinephrine	NE
One-kidney wrap	1K-WRAP
Phorbol 12-myristate 13-actetate	PMA
Protein kinase A	PKA
Protein kinase C	PKC
Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid	PPADS
Renin-angiotensin system	RAS
Resiniferotoxin	RTX
Reverse transcriptase polymerase chain reaction	RT-PCR
Spontaneously hypertensive rat	SHR
Staurosporine	ST
Substance P	SP
Suramin	SUR
Transient receptor potential	TRP

Transient receptor potential vanilloid type 1	TRPV1
Transmembrane	TM
Trigeminal ganglia	TG
Tubuloglomerular feedback	TGF
Two-kidney, one-clip	2K1C
Tyrosin hydroxylase	TH
Uridine-5'-triphosphate	UTP
Vanilloid receptor 1	VR1

CHAPTER 1

GENERAL INTRODUCTION

1. TRP channel

1.1 General introduction of TRP channel

The transient receptor potential (TRP) channels are a superfamily of related channels, which are distinct from other groups of ion channels in displaying a broad diversity in ion selectivity, modes of activation, and physiological function. All TRP channels share the common features of six transmembrane domains with intracellular N- and C-termini, varying degrees of sequence similarity, and permeability to cations like sodium, calcium, magnesium and potassium (Montell 2005).

All TRP channels include six putative transmembrane domains. The fourth transmembrane domain lacks the complete set of positively charged residues necessary for the voltage sensor in many voltage-gated ion channels (Catterall 2000). The structure of a TRP channel has not been solved; however, the channels appear to form tetrameric assemblies, similar to the structure of voltage-dependent potassium channels (Kedei et al. 2001; Kuzhikandathil et al. 2001; Hoenderop et al. 2003). The TRP superfamily is composed of seven subfamilies: TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, and TRPV (Fig. 1). Six of the subfamilies include members that are conserved in organisms as divergent as worms, flies, and humans. The remaining subfamily, TRPN, contains members expressed in invertebrates and zebrafish (Ramsey et al. 2006). TRP channels are divided into two groups based on their sequence similarity. The group 1 TRP channels (TRPC, TRPV, TRPM, TRPN and TRPA) share substantial sequence identity in the transmembrane domains. The groups 2 TRPs (TRPP and TRPML) are only distantly related to the group 1 TRPs, owing to low sequence similarity and a large extracellular loop between the first and second transmembrane domains (Fig. 1).

TRP channels are important in sensory physiology. TRP channels are involved in many sensory modalities, including vision, taste, smell, hearing, mechano- and thermosensation. TRP channels also allow individual cells to sense changes in the local environment, such as alterations in fluid flow and mechanical stress.

The TRPV channel is one subfamily of the TRP superfamily. On the basis of structure and function, TRPV channels comprise four groups of mammalian TRPVs: TRPV1/TRPV2, TRPV3, TRPV4 and TRPV5/TRPV6 (Benham et al. 2002; Gunthorpe et al. 2002). The *C. elegans* Osm-9 and the *Drosophila* Nanchung (Nan) also belong to the TRPV family (Colbert et al. 1997; Kim et al. 2003). TRPV1, 2, 3, and 4 have a P_{Ca}/P_{Na} permeability ratio between 1 and 10 (Clapham 2003). TRPV5 and TRPV6 are less similar (22-24% identity) to other TRPVs. They are the only highly Ca^{2+} selective channels in the TRPV family ($P_{Ca}/P_{Na} > 100$), and are tightly regulated by $[Ca^{2+}]_i$ (Vennekens et al. 2000). TRPV1-4 are non-selective cation channels, which are thermosensitive, although TRPV1 and 4 can also be activated by numerous other stimuli. TRPV3, and to a lesser extend also TRPV2 and TRPV1, but not TRPV4, can be activated by 2-aminoethoxydiphenyl borate (2-APB), which, in contrast, blocks some TRPC and TRPM channels (Pedersen et al. 2005). All TRPV channels contain 3-5 NH_2-terminal ankyrin repeats responsible for receptor-protein interaction, which is important for the regulation of TRPV channels, such as multimerization and desensitization (Montell 2005; Lishko et al. 2007).

1.2 TRPV1 channel

TRPV1, also known as capsaicin or vanilloid receptor 1 (VR1), is the most thoroughly studied TRPV channel. TRPV1 was first cloned from rat dorsal root ganglia (DRG) using a functional screening strategy for isolating candidate complementary DNA (cDNA) clones (Caterina et al. 1997). This newly cloned cDNA was named vanilloid receptor type 1 (VR1). Then VR1 was identified as a member of TRP channel superfamily. So it was assigned the name as TRPV1 to denote this association. By now, TRPV1 has been cloned from human, guinea pig, rabbit, mouse and porcine tissues. In different species, TRPV1 shows different pharmacological profiles, including sensitivity to various agonists and antagonists (Pingle et al. 2007).

TRPV1 has a diverse tissue distribution. As a nociceptor, TRPV1 is mainly expressed in sensory nervous system. High levels of expression are observed in DRG, trigeminal ganglia (TG), and nodose ganglia (NG). TRPV1 is predominantly expressed in small- and medium-sized sensory neurons and corresponding C- and Aδ-sensory fibers (Helliwell et al. 1998; Michael et al. 1999). TRPV1 is also expressed in various brain regions including the hypothalamus, cerebellum, cerebral cortex, striatum, midbrain, olfactory bulb, pons, medulla, hippocampus, thalamus, and substantia nigra. TRPV1 expression is also detected in some non-neuronal tissues, such as keritinocytes of the epidermis, bladder urothelium, smooth muscles, glial cells, liver, polymorphonuclear granulocytes, mast cells, and macrophages (Tominaga et al. 2005).

A large number of TRPV1 agonists have been identified. The most commonly used agonist is capsaicin (CAP), which was initially identified in the nineteenth century as the pungent component of chilly peppers in the genus *Capsicum*. Later capsaicin was

identified as an acylamide derivative of homovanillic acid, 8-mehthyl-N-vanillyl-6-nonenamide. In addition to capsaicin, numerous other vanilloids, such as resiniferotoxin (RTX), olvanil and zingerone, as well as many non-vanilloids are TRPV1 agonists. Lipids, including several lipoxygenase products and the endocannabinoid anandamide, can also activate TRPV1 (Gunthorpe et al. 2002). Other stimuli such as moderate heat (≥ 43°C) or low pH (≤ 5.9) can also activate TRPV1. Because capsaicin and its analogues are lipophilic, it is quite possible that they pass through the cell membrane and act on binding sites present in the intracellular surface of TRPV1. Capsaicin binding sites in the cytosolic domain of TRPV1 have been demonstrated using a synthetic water-soluble capsaicin analogue (Jung et al. 1999). Several TRPV1 antagonists are available now, such as capsazepine, ruthenium red, A-425619, IBTU, SB-366791, and AMG 9810. Capsazepine is most widely used TRPV1 antagonist with the similar structure to capsaicin. It competes for the capsaicin-binding site on TRPV1, inhibits capsaicin-mediated channel activation, and can displace RTX from its binding site in radioligand-binding assays (Pingle et al. 2007).

Rat TRPV1 cDNA contains an open reading frame of 2,514 nucleotides. The TRPV1 subunit is a 95-kDa, 838 amino acids protein, consisting of six transmembrane (TM) domains, with a short pore-forming region between the fifth and sixth TM domains (Fig. 2). Structurally, TRPV1 consists of a long 400-amino-acid amino-terminus containing three ankyrin-repeat domains and a carboxy-terminus containing a TRP domain close to the sixth TM domain. Functional TRPV1 channels exist as homo- or heteromultimers. TRPV1 can form functional multimers, whereas homotetramer is the predominant form of functional TRPV1 channels (Kedei et al. 2001). Recently several

alternative splicing products of TRPV1 have been found. TRPV1α and TRPV1β are two variants discovered in mouse DRG containing 839 and 829 amino acids, respectively. TRPV1β is a dominant-negative regulator of TRPV1 responses, since it is not functional by itself, but inhibits TRPV1α function during coexpression (Wang et al. 2004). TRPV1b, a human TRPV1 splicing variant expressed in trigeminal ganglion neurons, is unresponsive to capsaicin or protons, but can be activated by high temperatures (> 47°C) (Lu et al. 2005). A rat TRPV1 splice variant was also found in taste-receptor cells. This TRPV1 variant is constitutively active in the absence of a ligand at 23°C and is not modulated by protons (Lyall et al. 2004).

1.3 Desensitization of TRPV1 channel

Upon activation by capsaicin TRPV1 desensitizes. This phenomenon can either occur rapidly during single application (fast desensitization) or desensitization may require repeated agonist applications (slow desensitization). Desensitization is blocked in Ca^{2+}-free conditions or by the use of intracellular Ca^{2+} chelators, so TRPV1 desensitization is Ca^{2+}-denpendent. The possible mechanism of Ca^{2+}-denpendent TRPV1 desensitization is regulated by phosphorylation/dephosphorylation of TRPV1. Inhibition of calcineurin, Ca^{2+}-activated phosphatase, reduces TRPV1 desensitization. Calcineurin activated Ca^{2+} influx induced by activation of TRPV1 activation might dephosphorylate TRPV1, linking desensitization to a dephosphorylation event (Docherty et al. 1996). Protein kinase A (PKA) is involved in TRPV1 resensitization. Ser 116 and Thr 370 residues of TRPV1 are responsible for the PKA-dependent reduction of TRPV1

slow desensitization (Mohapatra et al. 2003). Protein kinase C (PKC) is also involved in TRPV1 resensitization. Ser 502 and Ser 800 of TRPV1 are the targets of PKC (Mandadi et al. 2004) (Fig. 2). In addition, Ca^{2+} may signal via calmodulin which interacts with TRPV1 at amino- and carboxyl-terminal regions (positions 189-222 and 767-801). Disruption of calmodulin binding segments prevents Ca^{2+}-dependent TRPV1 fast desensitization (Rosenbaum et al. 2004).

Phosphorylation of TRPV1 by PKC can also induce channel activity at room temperature in a voltage-dependent manner (Premkumar et al. 2000). Moreover, PKC-mediated phosphorylation of TRPV1 not only potentiates capsaicin- or proton-evoked responses, but also reduces its temperature threshold such that receptors are active under physiological conditions (37°C). Calmodulin-dependent kinase II also phosphorylates TRPV1 at Ser-502 and Thr-704 and plays an important role in channel activation by capsaicin. Similarly, Src kinase positively regulates TRPV1 channel activity by tyrosine phosphorylation (Jin et al. 2004). In addition to phosphorylation, activity of TRPV1 may be regulated by N-glycosylation, which might be responsible for TRPV1 post-translational modification. Extracellular Asn 604 has been identified as the site for glycosylation of TRPV1 (Jahnel et al. 2001).

TRPV1 acts as a transducer of noxious thermal and chemical stimuli in nociceptive sensory neurons and is vital in mediating enhanced heat sensitivity during inflammation. Many inflammatory mediators, including nerve growth factor, prostaglandin, bradykinin, serotonin, ATP, lipoxygenase products, and adenosine regulate TRPV1 sensitization via corresponding intracellular signaling pathways. In addition, inflammation and ischemia are associated with tissue acidification, further

potentiating TRPV1 activity. Taken together, these factors increase TRPV1 responses and lower the temperature threshold for heat activation, so that the channel can be activated at normal body temperature. Modulation of TRPV1 expression and/or activity could form a promising target for pain control (Pingle et al. 2007).

1.4 Function of TRPV1 channel

Besides the major function as an integrator of various pain stimuli, TRPV1 also plays important roles in various physiological conditions. It is important in regulating normal urinary tract function. It has been shown that TRPV1 knockout animals have greater short-term voluntary urination and abnormal urodynamic responses, with an increase in the frequency of nonvoiding contractions, increased bladder capacity, and inefficient voiding (Birder et al. 2002). In the gastrointestinal tract, TRPV1 maintains mucosal homeostasis, and protect against mucosal injury by increasing blood flow, bicarbonate and mucus secretion. Inflammatory bowel disease is also associated with upregulation of TRPV1 in nerve fibers of colon (Yiangou et al. 2001). In central nervous system, it is possible that activation of TPV1 maintains a tonic control of glutamate neurotransmission and likely plays an important role in the functions associated with dopaminergic transmission, including motor activity (Marinelli et al. 2003). Recent studies demonstrate the important role of TPV1 in mediating airway hypersensitivity. Some inflammatory factor released from epithelial cells in airways can activate TRPV1 channels and induce TRPV1-mediated airway hypersensitivity during asthma (Jia et al. 2005). Recently a TRPV1 splice variant was found involved in amiloride-insenstive salt taste. All in all, TRPV1 not only contributes to inflammation and functions as sensor for

noxious stimulation, but also it plays a role in variety of other physiological functions in different tissues and organs.

1.5 Efferent function of TRPV1 positive sensory nerves

TRPV1 is a molecular integrator for multiple types of sensory input, particularly pain. As a nociceptor, activation of TRPV1 causes the propagation of sensory information back to the central nervous system. Although these nerves have traditionally been considered to sense noxious stimuli in the periphery and transmit the information centrally, TRPV1-positive sensory nerves also have a "sensory-efferent" function. Activation of TRPV1 leads to Ca^{2+}-dependent neuropeptide release from some primary afferent neurons (Maggi 1993). At least 12 different types of transmitters are present in CAP-sensitive sensory neurons. Some are neuropeptides that can be released from TRPV1-positive sensory nerve endings (Maggi 1993). Calcitonin gene-related peptide (CGRP) and substance P (SP) are potent vasodilators released from sensory nerves in response to TRPV1 activation.

2. Hypertension

Hypertension is the medical name for high blood pressure. It is defined as systolic blood pressure of 140 mmHg or higher, or diastolic blood pressure of 90 mmHg or higher, or both. In the US alone, there are over 65 million people who are hypertensive. According to recent estimates, about one in three U.S. adults has high blood pressure (American Heart Association). Hypertension is a serious problem because people with this condition have a higher risk for heart disease and other medical problems than people with normal

blood pressure. If left untreated, hypertension can lead to a number of medical conditions, including: arteriosclerosis, heart attack, heart failure, stroke, cardiac hypertrophy and kidney damage. There is no cure for primary hypertension, but blood pressure can almost always be reduced with the correct treatment. The major goal of treatment is to avoid the most serious complications of hypertension. In cases of secondary hypertension, one approach is to treat the medical condition that causes hypertension. Efforts may be made at the same time to reduce the patient's blood pressure.

There are two types of hypertension. One is called primary or essential hypertension. Another is secondary hypertension. No identified causes are found causing elevated blood pressure in essential hypertension. About 90-95% of hypertension is essential hypertension. Secondary hypertension is secondarily caused by known causes, such as renal disease, endocrine disorder, tumors, or other pathophysiological conditions.

2.1 Regulation of arterial blood pressure

Arterial blood pressure is determined by two major factors, cardiac output and total peripheral resistance. Cardiac output is defined as amount of blood pumped by heart per minute, which is determined by stroke volume and heart rate. Peripheral resistance is mainly determined by blood flow resistance caused by peripheral small arteries and arterioles.

2.2 Short-term regulation of blood pressure

Short-term regulation of arterial blood pressure occurs in seconds or hours. Any reduction in arterial blood pressure will elicit homeostatic reflexes to maintain the normal

blood pressure. At this condition, the cardiac output and/or total peripheral resistance will be adjusted accordingly to fulfill the requirement of regulation. The short-term regulation is mainly accomplished by baroreceptor reflexes. They usually utilize the changes of activities of autonomic nerves supplying heart, blood vessels and adrenal gland. The baroreflex is the primary mechanism for buffering of acute blood pressure fluctuations during physiological or pathophysiological conditions such as postural change, behavioral and physiological stress, or changes in blood volume.

The primary baroreceptors are arterial baroreceptors. They are located in two carotid sinuses and aortic arch attached to vagal and glossopharyngeal axons. Baroreceptors also exist in the cardiac atria and ventricles, called cardiopulmonary or "low-pressure" baroreceptors, which are innervated by vagus nerve. Nonarterial baroreceptors are located in the systemic veins, pulmonary vessels, and walls of heart. The primary integrating center for baroreflex is medullary cardiovascular center, which is located in the brainstem oblongata. There are two major parts in this center, a vasomotor area and a cardioinhibitory area. Then neurons in this center receive input from the various baroreceptors. This input determines the outflow from the center along neural pathways that project to the heart, arteries and veins. Increases in blood pressure and baroreceptor activity initiate enhanced parasympathetic activity, sympathetic inhibition, and decrease in heart rate and vascular resistance. All these activities are to oppose the rise in blood pressure. Conversely, decrease in blood pressure may reduce baroreceptor activity to initiate increased heart rate and vascular resistance. So the baroreflex provides a moment-to-moment negative feedback regulation that maintains the normal level of arterial blood pressure. In addition to responding to changes in blood pressure, increased

baroreceptor activity may inhibit sympathetic nerve activity and limit the release of vasopressin from pituitary gland and renin from kidney.

In chronic hypertension, baroreceptors are reset to still higher pressure. The resting level of baroreceptor activity returns to near "normal" level even if the high blood pressure exists. Also the sensitivity of baroreflex is decreased in response to elevated blood pressure. Structural changes induced by high blood pressure including decreased arterial compliance and cardiac hypertrophy contribute to the decreased sensitivity.

2.3 Long-term regulation of blood pressure

Long-term regulation of arterial blood pressure occurs in hours and days. Over the longer time, the baroreflexes become less important, and factors contributing to blood volume regulation play a dominant role in determining blood pressure. In long-term regulation, humoral controls mainly contribute to the homeostasis of the circulation. The targets for long-term regulation are blood vessels and kidney.

There are two classes of humoral factors influence the circulation. The first is vasoactive substances released in the blood, or in the proximity of vascular smooth muscle, which modulates vasomotor tone of arteries and veins, affecting blood pressure and the distribution of blood flow. The vasoactive substances include biogenic amines, such as epinephrine, serotonin, histamine, et al. Some peptides also belong to vasoactive substances, such as angiotensin II, arginine vasopressin, endothelin, kinin, CGRP, SP, et al. These substances elicit either vasoconstriction or vasodilation effect when acting on corresponding receptors on vascular smooth muscle cells. The second is nonvasoactive substances, which act on targets other than cardiovascular system, controlling the

effective circulating volume by modulating extracellular fluid volume. The nonvasoactive substances include aldosterone, arginine vasopressin, atrial natriuretic peptide, et al.

2.4 Neurohumoral factors involved in blood pressure regulation

Catecholamine. There are three endogenous catecholamines in human body, which are norepinephrine (NE), adrenaline (EPI), and dopamine (DA). All of them are synthesized from tyrosine, which exists in a variety of cell types, such as sympathetic neurons, adrenomedullary cells, and gastrointestinal parenchymal cells. EPI is a neurotransmitter of central nervous system, as well as of autonomic nervous system (Esler et al. 1995). A small portion of NE is released from adrenal gland. The majority of NE in the circulation is from sympathetic nerves innervating blood vessels (Silverberg et al. 1978). Dopamine is the precursor of NE and EPI. It is colocalized with them in various tissues. DA is also an important neurotransmitter in central nervous system. And it is involved in the peripheral nerve regulation as well (Bell 1987).

NE and EPI act on the adrenergic receptors (ARs) to mediate cellular responses. These hormones are secreted by adrenal gland and also released as neurotransmitters from adrenergic neurons within the central nervous system and postganglionic peripheral sympathetic neurons. Activation of cardiac AR increases heart rate and induces cardiac contraction. And activation of AR in vascular smooth muscle cells causes vasoconstriction and elevated blood pressure (Tsuru et al. 2002). In other cell types, such as liver and adipose cells, activation of AR triggers the liberation of glucose or fatty acids (Hoffman 2007).

DA is released from postganglionic sympathetic neurons, dopaminergic neurons, and nonchromaffin tissues, such as renal proximal tubules and gastrointestinal epithelial cells. The majority of circulating DA comes from kidney. DA modulates a variety of physiological conditions, including behavior, movement, nerve conduction, hormone synthesis and release, ion transport, vascular tone, and blood pressure regulation (Izzo et al. 2008).

Endothelin. Endothelins (ETs) are 21-amino acid peptides, which were originally discovered as products of vascular endothelial cells. They cause an extremely potent and long-lasting vasoconstriction in most vascular smooth muscle cells. Three types of ETs have been identified, which are ET-1, ET-2, and ET-3. The main ET secreted by endothelium is ET-1. It is the main isoform in human cardiovascular regulation (Davenport 2002). Two types of ET receptors have been cloned, ET_A and ET_B receptors (Sakurai et al. 1990). Endothelial ET-1 acts on ET_A and ET_B receptors to induce vasoconstriction, proliferation, smooth muscle cell proliferation and hypertrophy (Haynes et al. 1995). ET-1 may also act on endothelial ET_B receptors, inducing release of nitric oxide causing vasodilation effect (Rubanyi et al. 1994). In the heart, ET-1 acts on ET_A receptors in cardiomyocytes and fibroblasts. Overactivation of ET_A receptors causes cardiac fibrosis and microvascular remodeling, which has been reported in some hypertensive animal models (Karam et al. 1996). In kidney, ET receptors are mainly expressed in blood vessels and mesangial cells. ET_A receptors are predominantly expressed in kidney. But less ET_B receptors also plays an important role in some pathophysiological conditions. In renal distal tubules, activation of ET_B receptors

promotes sodium excretion. Several agents, such as angiotensin II, arginine vasopressin, thrombin, and shear stress, may trigger the release of ETs causing vasoconstriction of renal blood vessels locally.

Elevated ETs levels have been reported in some forms of human hypertension (Shichiri et al. 1990; Yoshibayashi et al. 1991). In patients with essential hypertension, an increased vasoconstrictor response to ET-1 was reported, indicating enhanced vascular endothelin vasoconstrictor activity (Cardillo et al. 1999). In addition, ET-1-induced vasoconstriction of glomerular arterioles causes a decrease in glomerular filtration rate (GFR), renal plasma flow, and sodium excretion, as well as a significant increase in renal vascular resistance (Lopez-Farre et al. 1989). Furthermore, in SHR rats less ET-1 is produced in the renal medulla. ET-1 mediates excretion of sodium and water through kidney is attenuated resulting in water/sodium retention and hypertension (Hughes et al. 1992).

Angiotensin. Renin-angiotensin system (RAS) has been viewed as a circulating endocrine system. Renin is produced by juxtaglomerular cells, which cleaves angiotensinogen in the liver to form angiotensin I. Angiotensin I is converted in pulmonary endothelium to the potent vasoconstrictor angiotensin II (Izzo et al. 2008). Besides vasoconstriction effect, angiotensin II stimulates the secretion of aldosterone from adrenal gland. There are two types of angiotensin receptors, AT_1 and AT_2 receptors, which have strong affinity for angiotensin II. Activation of AT_1 receptors triggers elevated intracellular calcium causing vasoconstriction. Angiotensin II also stimulates formation of reactive oxygen species, such as superoxide, which inactivates nitric oxide.

These actions promote vasoconstriction and vascular remodeling (Zhou et al. 2005). Binding of angiotensin II to AT_2 receptors stimulate the formation of bradykinin, followed by production of nitric oxide causing vasodilation. AT_2 receptor mediated vasodilation effect is predominant in kidney (Izzo et al. 2008).

CGRP. CGRP is a 37-amino acid neuropeptide derived from the tissue-specific splicing of the primary RNA transcript of calcitonin/CGRP gene, which is referred to as the α-CGRP gene (Amara et al. 1982). Calcitonin is produced mainly in the parafollicular cells of the thyroid, but CGRP synthesis occurs almost exclusively in the central and peripheral nervous systems. Another CGRP gene is called ß-CGRP gene, which also produces CGRP but not calcitonin in the central nervous system (Wimalawansa 1996). These two CGRP genes differ in their protein sequences by one and three amino acids respectively. But the biological activities of the two peptides are quite similar in most vascular beds.

CGRP is widely distributed in the central and peripheral nervous systems. It is primarily located in small and medium sized sensory neurons and nerve fibers, which is similar with the distribution of TRPV1. CGRP release is triggered by TRPV1 activation. Two CGRP receptors were discovered in the late 1980s: CGRP1 and CGRP2 (Dennis et al. 1989). Vasodilation caused by CGRP is mediated by the CGRP1 receptor. There are two possible mechanisms involved in CGRP-mediated vasodilation. The first one is endothelium-dependent relaxation. The mechanism occurs in most blood vessels. CGRP released from sensory nerve fibers acts on CGRP1 receptors, which is a G-protein coupled receptor. Activation of CGRP1 causes an increase of intracellular cAMP in the

endothelial cells that leads to the NO release, which causes relaxation of smooth muscle cells. The second mechanism is endothelium-independent as CGRP acts at CGRP1 receptors on smooth muscle cells to stimulate adenylate cyclase. The resulting increase in intracellular cAMP leads to K^+ channel phosphorylation causing K^+ channel opening and smooth muscle cell membrane depolarization and relaxation (Brain et al. 2004).

SP. SP is an 11-amino acid neuropeptide that is widely distributed in the nervous system. In sensory nerves, SP mediates pain, touch, and temperature. SP is also involved in many physiological activities including smooth muscle contraction and vasodilation (Holzer 1988). SP is a member of the tachykinin family. The three major mammalian tachykinins are SP, neurokinin A (NKA), and neurokinin B (NKB). SP and NKA are encoded by the preprotachykinin A gene, and NKB is encoded by the preprotachykinin B gene (Maggi 1995). SP is often released from the same sensory nerve terminals as CGRP. There are three subtypes of tachykinin receptors. They are NK-1, NK-2, and NK-3. SP mainly acts on NK-1 receptors, NKA for NK-2, and NKB for NK-3. But all three tachykinins have some affinity for all NK receptors if a high enough dose is given. Unlike CGRP, the vasodilation effect of SP is always endothelium dependent and mediated by NK-1 receptors localized in endothelial cells. SP released from sensory nerve fibers acts G-protein coupled NK-1 receptors causing release of NO from endothelial cells. NO relaxes smooth muscle cells (Maggi 1995).

Ohters. There are many other hormones that are important in blood pressure regulation. For example, atrial natriuretic peptide (ANP) is released from atrial myocytes

in response to stretch, which is a potent vasodilator. ANP also inhibits renin secretion and lowers tubuloglomerular feedback causing reduced renal blood flow and GFR. Arginine vasopressin (AVP), also known as antidiuretic hormone, is released from posterior pituitary gland. AVP increases water reabsorption through increasing the expression of water channel in renal collecting ducts. Also massive AVP release triggers the systemic vasoconstriction effect than contributes to a transient elevation of blood pressure. Nitric oxide (NO) is released from endothelial cells, which is a potent vasodilator. But it is not clear that if it plays an important role in systemic blood pressure regulation (Boron et al. 2003).

3. CGRP and SP in hypertension

CGRP and SP are often co-localized and co-released by sensory nerves. When TRPV1 positive nerve fiber or neurons are activated by capsaicin or endogenous TRPV1 agonists, they release CGRP and SP. As I mentioned above, CGRP and SP are potent vasodilator, the role of CGRP and SP in blood pressure regulation and development of hypertension has been widely investigated.

CGRP plays a compensatory depressor role to attenuate elevated blood pressure in deoxycorticosterone acetate (DOCA)-salt hypertension and in subtotal nephrectomy-salt (SN-salt) hypertension. In DOCA-salt hypertensive model, the rat undergoes a uninephrectomy followed by excess mineralocorticoid and salt treatment. In SN-salt model, the rat undergoes a uninephrectomy plus surgical removal of 66% of remaining kidney followed by excess salt treatment (Supowit et al. 1997; Supowit et al. 1998). The compensatory depressor effect of CGRP is mediated through increased neuronal

expression and peptide release in DOCA-salt hypertension, whereas in SN-salt hypertension, this depressor effect is mediated by the enhanced sensitivity of the vasculature to the vasodilator activity of CGRP. The same effect caused by SP also occurs in DOCA-salt and SN-salt hypertensive rats (Kohlmann et al. 1997). In spontaneously hypertensive rats (SHR), which is a genetic hypertensive animal model, neuronal expression of CGRP is decreased (Supowit et al. 1993). Also reduced levels of SP have been reported in essential hypertension in humans and in stroke-prone SHR (Mori et al. 1993). The decrease of neuronal expression of CGRP is also reported in Dahl-salt hypertension (Katki et al. 2001). Therefore, CGRP and SP in sensory nerves may contribute to blood pressure regulation and hypertension.

4. TRPV1 in cardiovascular function and hypertension

TRPV1 is involved in neuropathic pain, hyperesthesia, hyperalgesia, allodynia, and spontaneous burning pain (Nilius et al. 2005). As I mentioned above, there is a "sensory-efferent" function on TRPV1 positive sensory nerve fibers. So TRPV1 can regulate arterial tone. It was found that in small mesenteric resistance arteries, myogenic responses caused by increases in intraluminal pressure were blocked by capsazepine or by TRPV1 desensitization induced by capsaicin (Scotland et al. 2004). Recent evidence supports involvement of TRPV1 in control of blood flow and blood pressure via release of vasoactive neuropeptides from sensory nerves (Leung 1993; Zygmunt et al. 1999; Movahed et al. 2005). But these studies have not shown whether impairment of TRPV1-positive sensory nervous system is sufficient to produce hypertension. Further studies have investigated the mechanisms by which TRPV1 channels are linked to salt sensitive

hypertension. Several animal models have been used for defining the role of TRPV1-positive sensory nerves in the etiology of salt-sensitive hypertension. One model is the one-kidney wrap (1K-WRAP) hypertensive model. In this model, capsaicin treatment enhanced the development of hypertension compared with vehicle treated 1K-WRAP rats (Burg et al. 1994). A similar study was performed on DOCA-salt hypertensive rats. It was found that pretreatment with capsaicin caused a quicker onset and greater magnitude of hypertension (Manzini S 1998). In another hypertensive model, high-dose capsaicin (50 mg/kg) was administrated to newborn Wistar rats to destroy TRPV1-positive sensory nerves. Then high or normal sodium diet was given immediately after weaning. It was found that neonatal treatment with capsaicin led to elevation of blood pressure in rats fed a high sodium diet, but not in those fed a normal sodium diet. Urine volume and sodium excretion were lower in capsaicin -treated rats fed a high-salt diet compared with vehicle-treated rats fed a high-salt diet (Wang et al. 1998).

These results suggest that impairment or loss of capsaicin-sensitive (TRPV1-positive) sensory nerves contributes to the development of salt-sensitive hypertension and that impairment of TRPV1 function on sensory nerves might affect renal function and salt sensitivity when rats are salt-loaded. Dahl salt–sensitive (DS) rats have been used as a model of human salt–sensitive hypertension as salt load exaggerates the hypertension in this strain that is genetically predisposed to hypertension (Rapp et al. 1982). TRPV1 expression and function are impaired in DS rats, which render DS rats sensitive to salt load in terms of blood pressure regulation (Wang et al. 2006).

The above mentioned studies emphasize the important role of TRPV1 in salt-sensitivity and development of hypertension. However, to better understand TRPV1

regulation of salt-sensitivity and blood pressure, studies on kidney specific sensory neurons are needed. Because the kidney is the key organ regulating sodium and water balance, how sensory nerve activity regulates renal blood flow, GFR, and water transport should be investigated. The studies I have proposed will focus on sensory neurons innervating the kidney to find how TRPV1 channels contribute to regulation of renal function.

5. ATP as a modulator of sensory nerve function

Extracellular ATP plays a significant role in the regulation of many biological processes including neurotransmission in the peripheral and central nervous systems (Burnstock 2006). Extracellular ATP can also modulate cardiac function (Olsson et al. 1990), immune responses and pain sensation (Burnstock 1996). ATP exerts its effect by binding to either of two classes of receptor: P2X and P2Y receptors. P2X receptors are ligand-gated cation channels (Vial et al. 2004), while P2Y receptors are G-protein coupled receptors (Gordon 1986; Dubyak 1991). P2X and P2Y receptors are widely distributed in the nervous system (Neary et al. 1996; Moriyama et al. 2003) where ATP serves as a peripheral mediator for pain sensation (Hamilton et al. 2000). It is proposed that ATP released from different cell types may initiate pain responses by acting on P2 receptors on sensory nerve terminals. Further studies suggested that pain sensation evoked by ATP is predominantly mediated by P2X receptors expressed in nociceptive sensory neurons (Tsuda et al. 1999). Recent studies showed that nociceptive sensory neurons also express P2Y receptors, which may be involved in the potentiation of pain sensation. In the presence of extracellular ATP, the temperature threshold for TRPV1

activation was reduced from 42 to 35°C, such that normal body temperature is capable of activating TRPV1, which might cause pain sensation (Tominaga et al. 2001; Moriyama et al. 2003). There are two P2Y subtypes ($P2Y_1$ and $P2Y_2$) that might be involved in the ATP-induced potentiation of pain (Tominaga et al. 2001; Moriyama, Iida et al. 2003). The identity of the P2Y receptor subtype that contributes to potentiation of pain is controversial. ATP potentiates TRPV1 currents evoked by capsaicin or protons. This effect was mediated by $P2Y_1$ receptors coupled to activation of a protein kinase C (PKC)-dependent pathway (Tominaga et al. 2001). The direct phosphorylation of TRPV1 by PKC has been proven biochemically, and two serine residues as substrates for PKC-dependent phosphorylation have been identified (Fig.2) (Numazaki et al. 2002). These data suggest that phosphorylation by PKC changes the agonist sensitivity of TRPV1.

ATP is also a transmitter in the nervous system (Burnstock 2006). In the sympathetic nervous system, ATP is co-stored in synaptic vesicles with norepinephrine and neuropeptide Y (Pablo Huidobro-Toro et al. 2004). One of the roles of ATP as a co-transmitter from sympathetic nerves includes vascular constriction. ATP released from sympathetic nerves acts at P2X receptors on vascular smooth muscle cells to induce vasoconstriction (Burnstock et al. 2000). ATP may also act on P2Y receptors to initiate the synthesis of the endothelial-derived vasodilators, nitric oxide and arachidonate metabolites (Giaroni et al. 2002). The role of ATP in producing vasoconstriction *in vivo* depends on the tissue bed and species being examined. In general, it appears that ATP has its greatest effect in mediating vascular tone in tertiary branches of the mesenteric artery and afferent arterioles in the kidney (Gitterman et al. 2001; Inscho 2001). Autoregulation of afferent arterioles in the kidney have been attributed to locally released

ATP via activation of $P2X_1$ receptors (Inscho 2001). Purinergic transmission to renal arterioles is impaired in a model of hypertension induced by chronic angiotensin II infusion (Zhao et al. 2005). ATP can also modulate norepinephrine release and the release of endothelium derived hyperpolarizing factor (EDHF) via $P2Y_2$ receptors (Thapaliya et al. 1999). It was also reported that ATP released from sympathetic nerve endings may act on P2X receptors of sensory nerve endings, contributing to the initiation of pain (Burnstock 1996).

6. P2Y receptors in nervous system

Extracellular nucleotides bind to a family of membrane-associated P2 receptors. There are two principal subfamilies of P2 receptors. They are distinguished from each other based on their structure and function. P2X receptors are ligand-gated ion channels composed of three subunits, each has two transmembrane domains, and P2Y receptors belong to the superfamily of G protein-coupled receptors with seven transmembrane domains. Seven mammalian P2X receptors ($P2X_{1-7}$) and eight mammalian P2Y subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified (North et al. 1997; North 2002).

P2 receptors have a wide tissue distribution and in the central and peripheral nervous systems, both types of P2 receptors contribute to signaling between neurons and between neurons and glial cells. For example, activation of P2X or P2Y receptors expressed on glial cells triggers the increase of intracellular Ca^{2+} and leads to long-term changes such as proliferation or cell death (Neary et al. 1996; Weisman et al. 2005). Neurons also express both P2X and P2Y receptors. P2X receptors are mainly involved in

fast synaptic transmission, whereas P2Y receptors mediate slow synaptic transmission (Abbracchio et al. 2009).

Most of the known subtypes of P2Y receptors are expressed in central nervous system. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) revealed that $P2Y_1$ and $P2Y_{11}$ mRNA was higher expressed in human brain than other tissues. Only low to moderate levels of $P2Y_2$, $P2Y_4$ and $P2Y_6$ were detected in brain (Moore et al. 2001). The mRNA for all P2Y receptors except $P2Y_{11}$ and $P2Y_{14}$ has been detected in sympathetic neurons (Vartian et al. 2001; Lechner et al. 2004). In the intramural parasympathetic ganglia of the cat urinary bladder, the presence of $P2Y_{1,2,4,6,12}$ was revealed by immunohistochemistry (Ruan et al. 2006). In rat sensory neurons, only $P2Y_{1,2,4,6}$ expression was detected (Ruan et al. 2003).

The pharmacological characteristics of P2Y receptor subtypes are different. For $P2Y_1$ receptors of most species, the rank order of agonist potency is 2-MeSADP>2-MeSATP>ADP>ATP. For $P2Y_2$ receptors, ATP and UTP are equipotent agonists, and ADP, UDP, or 2-MeSATP has weak or no activity. $P2Y_4$ receptors are equally activated by ATP and UTP. UDP is the most potent agonist for $P2Y_6$ receptors, whereas ADP, ATP, and UTP are weak agonists. Human $P2Y_{11}$ receptors are activated at rank order ATP>2-MeSATP>ADP. For $P2Y_{12}$ receptors, 2-MeSATP is the most potent agonist compared with ADP and ATP. $P2Y_{13}$ receptors are activated by 2-MeSATP, ADP, and

ATP, but the rank order of agonist potency is different for human and rat receptors. $P2Y_{14}$ receptors are sensitive to various UDP sugars, but not to adenosine or uridine nucleotides (von Kugelgen 2006).

The most widely used P2 receptor antagonists, suramin and reactive blue 2, block not only several P2Y receptors, but also P2X receptors. $P2Y_1$ receptors are blocked by suramin, PPADS, and reactive blue 2. But these antagonists can also block P2X receptors. There are more selective $P2Y_1$ antagonists available. For instance, the biphosphate nucleotide adenosine-2'-phosphate-5'-phosphate (A2P5P) and adenosine-3'-phosphate-5'-phosphate (A3P5P) block $P2Y_1$ receptors. MRS 2179 and 2-chloro-N6-methyldeoxyadenosine 3',5'-biphosphate (MRS 2216) are selective and competitive antagonists of $P2Y_1$ receptors with nanomolar affinity. There is no selective $P2Y_2$ receptor antagonist available. Suramin blocks $P2Y_2$ receptors but not $P2Y_4$ receptors. $P2Y_6$ receptors are blocked selectively by 1,4-di-[(3-isothiocyanato phenyl)-thioureido]butane (MRS 2578). $P2Y_{12}$ receptors are blocked by antithrombotic drugs ticlopidine and clopidogrel. (von Kugelgen 2006).

7. The basis for an interaction between sensory and sympathetic nerves

We propose that ATP released from sympathetic nerve endings acts on P2Y receptors of sensory nerve endings. Activation of P2Y receptors may sensitize TRPV1 on sensory neurons innervating kidney, contributing to the release of vasoactive factors from sensory nerve endings. In order for this to occur there must be a close spatial

relationship between sympathetic and sensory nerve endings supplying the kidney. The distribution of perivascular nerves and their morphological relationships to the renal arterial tree has been studied in serially sectioned rat kidneys. It was reported that SP and CGRP immunoreactive axons are evenly dispersed throughout the more numerous noradrenergic axons that form perivascular nerve bundles (Ferguson et al. 1985; Gibbins et al. 1985). The demonstrated close spatial relationship between sensory and sympathetic axons in renal perivascular nerve bundles provides the morphological basis for the proposed interaction between sympathetic and sensory nerves.

Most functional studies of the neurohumoral control of vasculature have focused on the role of neurotransmitters released from sympathetic perivascular nerves. In recent years, several modulatory mechanisms have been recognized, including presynaptic inhibition or enhancement of neurotransmitter release, and postsynaptic neurotransmitter action (Mione et al. 1990). Recent data show that blood vessels are not only innervated by sympathetic, parasympathetic or sensory nerves, but also the interactions among these different nerves also play an important role in regulation of neurotransmitter release and vascular tone. Studies using cats and dogs have suggested that neurotransmitter released from sympathetic nerves can attenuate not only the vagal effect on the heart, but also the parasympathetic vasodilation effect in the nasal mucosa (Lacroix et al. 1994; Lacroix et al. 1994). A modulatory effect of sympathetic activity on sensory nerve-induced vascular reactions was discovered in rat dental pulp. It was found that neurotransmitter released from sympathetic nerves to dental pulp can inhibit the release of vasoactive and inflammatory mediators (Kerezoudis et al. 1993). It was also reported that SP released

from sensory nerves acting on sympathetic neurons produces noncholinergic slow-excitatory potentials within guinea pig sympathetic ganglia.

8. The kidney in blood pressure regulation and salt-sensitive hypertension

The kidneys are paired, bean-shaped organs lying behind the peritoneum on each side of vertebral column. Kidneys play important roles in physiological and pathophysiological conditions through several aspects. First, they filter metabolic products and toxins from blood and excrete them through urine excretion. Second, they play an important role to maintain homeostasis by regulating extracellular fluid status, electrolytes balance and acid-base balance. Third, they produce hormones, which are involved in erythrogenesis, calcium metabolism, and regulation of blood pressure.

There are two layers in the parenchyma of kidney, cortex and medulla. The cortex is composed of glomeruli, renal capillaries, convoluted renal tubules and connective tissue. The medulla lacks glomeruli and consists of parallel renal tubules and small blood vessels. About 20% of cardiac output goes through kidney. This high blood flow renders the kidney as an important organ for regulation of blood pressure. The renal circulation has a unique sequence of vascular organization: a high resistant arteriole (the afferent arteriole), followed by a high-pressure glomerular capillary network for filtration. After filtration network, blood flows into another high resistant arteriole (the efferent arteriole). The following low-pressure capillary network surrounding renal tubules (peritubular capillaries) takes up the fluid and electrolytes absorbed by renal tubules. The functional unit of kidney is the nephron. Each kidney consists of 800,000 to 1, 2000,000 nephrons. A nephron consists of a glomerulus and a tubule. The tubule is composed of proximal

tubule, Henle's loop, distal tubule, and colleting duct. The major function of nephron is filtering water, electrolytes and other substance through glomerulus, reabsorbing all of part of them by renal tubule and excreting the rest as urine from kidney. The nephron plays an important role in regulation of blood volume and blood pressure, elimination of wastes from body (Boron et al. 2003).

8.1 Autoregulation of kidney

Two mechanisms are involved in the autoregulation of kidney. One is tubuloglomerular feedback (TGF). Another is myogenic response of renal arterioles. An increase in distal tubule blood flow generates signals from macular densa cells in the wall of ascending limb of Henle's loop to the afferent arterioles to cause vasoconstriction, whereas decrease in flow causes afferent vasodilation (Braam et al. 1993). The efficiency of TGF mechanism is regulated by sodium intake and blood volume. High sodium intake and blood volume may decrease the sensitivity of TGF mechanism allowing more water and sodium excretion. The myogenic response is intrinsic characteristic of small blood vessels. An increased vascular extension by blood volume triggers elevated vascular tone and decreased vessel diameter. Preglomerular and afferent arterioles have stronger myogenic responses than postglomerular and efferent arterioles (Carmines et al. 1990). So during changes in arterial pressure, renal blood flow and GFR are autoregulated with high efficiency, which dependent on adjustment of resistance to flow through preglomerular arterioles. Although efferent resistance also can be regulated by other mechanisms, it does not significantly affect autoregulation. Through autoregulation, the

GFR, filtered sodium, and the intrarenal pressure are maintained stable in face of various external stimuli (Navar 1997).

8.2 Sodium reabsorption in renal tubules

Under normal conditions, less than 1% of filtered sodium is excreted through kidney. The most filtered sodium is reabsorbed by different mechanisms along renal tubules. The proximal tubules absorb 60% to 70% of filtered sodium from glomeruli. The reabsorption is accomplished by both active and passive transport mechanisms that reabsorb sodium and other solutes from the lumen into the lateral spaces and interstitial compartment. Along the proximal tubules, sodium is co-transported into the cells with glucose, amino acids, citrate, phosphate, and sulfate. Na-H exchanger also plays an important role in sodium reabsorption. Sodium transport in the thin descending and ascending limbs of Hene's loop is almost entirely passive and paracellular. In distal convoluted tubules, transport of sodium is mainly accomplished by Na/Cl contransporter on the apical cell membrane. In the connecting tubules, Na channels and Na/H exchangers are predominant for sodium reabsorption. Sodium transport in collecting ducts is mainly via Na channels on apical cell membrane (Boron et al. 2003).

8.3 Neurohumoral regulation of kidney

Sympathetic effects. The sympathetic regulation serves as the major mechanism for short-term regulation of blood pressure. Also it plays a long-term role by influencing sodium reabsorption and excretion. The direct effects of sympathetic nerve activity on kidney are to decrease sodium excretion caused by decreases in filtered load and

increases in tubular reabsorption (DiBona et al. 1997). Sympathetic activation induces vasoconstriction of both afferent and efferent arterioles causing decreased renal blood flow. The Na/H exchanger function is enhanced by sympathetic activation (Boron et al. 2003).

Renin-angiotensin system. This system serves as one of the most powerful regulators of blood pressure and sodium balance. Decreased blood volume or arterial pressure triggers the release of rennin from juxtaglomerular apparatus, which stimulates the formation of angiotensin II. Angiotensin II exerts short- and long-term actions, including vasoconstriction and stimulation of aldosterone release. Angiotensin II causes vasoconstriction of both afferent and efferent arterioles. The activity of Na/H exchanger on proximal tubules is also enhanced by angiotensin II causing increased sodium reabsorption. Angiotensin II may also enhance the sensitive of TGF mechanism (Braam et al. 1995). Aldosterone increases sodium reabsorption and potassium excretion in distal segments of nephron by binding to the cytoplasmic mineralocorticoid receptors. Aldosterone may increase the activity of Na channels on apical cell membrane, the expression of Na-K pump on basolateral membrane and mitochondrial ATP production in the cell (O'Neil 1990).

The effects of other hormones in kidney, such as ANP and AVP, have been discussed in previous section.

8.4 Kidney in hypertension

Despite extensive study of hypertension, the mechanisms responsible for development of essential hypertension remain unclear. The long term regulation of

arterial pressure is intimately linked to the ability of the kidneys to excrete sufficient sodium to maintain normal sodium balance, extracellular fluid volume (Navar 1997). So sodium balance plays an important part in the development of hypertension. Basic and clinical studies emphasize the crucial role of sodium in the regulation of blood pressure and implicate an abnormal sodium balance in hypertension development in animal models and humans (Kuller 1997; Bayorh et al. 1998).

In normal persons, an increased intake of sodium leads to appropriate adjustments in the activity of various humoral, neural, and paracrine mechanisms. These mechanisms alter systemic and renal hemodynamics and increase sodium excretion without increasing arterial blood pressure (Navar 1997). Normally acute elevations in arterial blood pressure produce natriuresis, whereas reductions in arterial blood pressure cause sodium retention (Navar et al. 1996). When renal function is normal and responsive to sodium regulatory mechanisms, sodium excretion rates are adjusted to match sodium intake. When the regulatory mechanisms are operated inappropriately, the kidney can not adjust sodium excretion rates at the challenge of high sodium intake. The results are sodium retention, expansion of extracellular fluid volume, and increased arterial blood pressure. When the impairments also include increased level of humoral or neural factors that directly cause vascular smooth muscle constriction, these effects increase peripheral vascular resistance and decrease vascular capacitance.

Extrinsic factors and intrarenal impairments can cause sodium retention. Many factors also exist that alter cardiac output, total peripheral resistance, and vascular capacitance. For the kidney, any pathological conditions that cause enhanced tubular sodium reabsorption or decreased glomerular filtering capacity could result in sodium

and water retention causing elevated arterial blood pressure. So understanding of normal mechanisms regulating sodium balance and renal homeostasis can provide the basis for a rational approach to the treatment of hypertension.

The kidney is the key determinant of the blood pressure response to salt (Rettig et al. 1995). Three major renal mechanisms may mediate the development of salt sensitive hypertension: 1) an increased pre-glomerular vascular resistance; 2) a decrease in whole kidney ultrafiltration, and 3) an increase in tubular sodium reabsorption (Guyton 1989). The renal hemodynamic response to changes in sodium intake has been studied in salt-sensitive and salt-resistant hypertensive patients. It was found that renal hemodynamic response to salt loading in salt-sensitive patients was characterized by a decrease in renal blood flow (Williams et al.1991; van Paassen et al. 1996).

9. The Dahl salt-sensitive (DS) model of hypertension

Habitual dietary salt intake has been shown to increase blood pressure in some people but not in others. High salt diet contributes to the development of hypertension in "salt sensitive" individuals (Sullivan 1991). It has been shown that salt sensitivity is predominantly determined by genetic factors. The incidence of hypertension is generally high er among salt-sensitive individuals than those salt-resistant individuals. In animal models of hypertension, especially inbred hypertensive rats, salt sensitivity has been a central issue and widely studied.

In the 1960s, Dahl and his colleagues developed DS rats as genetic model for salt-sensitive hypertension (Dahl et al. 1962). DS hypertensive rats have been widely investigated as one of the principal salt-sensitive hypertensive models and changes in

kidney function are the primary cause of hypertension in these rats. It was reported that increased blood pressure was associated with decreased renal blood flow without changes in plasma sodium, potassium, or aldosterone levels during high salt treatment in DS rats (Bayorh et al. 1998; Bayorh et al. 1999; Miyata et al. 1999). It was also reported that there were no changes in noradrenergic innervation or release of neurotransmitters in the mesenteric or renal vasculature of DS hypertensive rats (Kong et al. 1991). So reductions of renal blood flow in DS rats with high salt intake could contribute to hypertension development.

Controversial results reveal that rennin-angiotensin-aldosterone system is enhanced in DS hypertensive rats. And blockade of angiotensin receptor reduced elevated blood pressure in these rats (Zhu et al. 2009). Oxidative stress also plays an important role in DS hypertensive rats (Manning et al. 2003). Several chromosome regions containing quantitative trait loci for blood pressure have been identified in DS hypertensive rats including angiotensin converting enzyme (ACE) gene and 11β-hydroxylase gene (Deng 1998).

Recently studies demonstrate that TRPV1 also plays a role in development of DS hypertensive rats. TRPV1 function is impaired in the kidney of DS hypertensive rats, which contributes to the lower GFR and sodium/water excretion (Li et al. 2008). TRPV1 expression is reduced in mesenteric arteries, renal cortex, and renal medulla of DS hypertensive rats and CGRP levels are reduced in dorsal root ganglia (DRG) of the same animals (Katki et al. 2001; Wang et al. 2006).

10. Agonist and antagonist used in my studies

2-methylthio-ATP: 2-methylthio-ATP (2-Me-S-ATP) is a synthetic analog of ATP. The potency of 2-Me-S-ATP at P2X receptors is equal to ATP. For metabotropic P2Y receptors, 2-me-S-ATP predominantly activates $P2Y_1$ receptor. It has weak effect on $P2Y_6$ and $P2Y_{11}$ receptors, but no effect on $P2Y_2$ receptor.

Uridine-5'-triphosphate: Uridine-5'-triphosphate (UTP) is a pyrimidine nucleotide. It is an endogenous P2 receptor agonist, which also works as an energy source. UTP has no effect on P2X receptors. For P2Y receptors, UTP works as an agonist for $P2Y_2$, $P2Y_4$, $P2Y_{11}$ receptors, but not for $P2Y_1$ receptor.

N6-methyldeoxyadenosine 3',5'-biphosphate: N6-methyldeoxyadenosine 3',5'-biphosphate (MRS 2179) is a synthetic competitive $P2Y_1$ receptor antagonist.

Suramin: Suramin (SUR) is a broad P2 receptor antagonist. For ionotropic P2X receptors, suramin may block the effect of $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_{2/3}$ receptors. For metabotropic P2Y receptors, suramin blocks the effect of all P2Y receptors when higher concentration is applied (30-100μM) except for $P2Y_4$ receptor.

Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid: Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is also a broad P2 receptor antagonist. For

P2X receptors, PPADS may block the effect of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_{2/3}$, and $P2X_7$ receptors. For P2Y receptors, PPADS blocks the effect of $P2Y_1$, $P2Y_6$, and $P2Y_{13}$ receptors, but not $P2Y_2$ receptor.

Forskolin: Forskolin (FSK) is a compound produced by a plant called *Coleus forskohlii.* FSK is commonly used as a selective indirect agonist of PKA. FSK activates the enzyme adenylyl cyclase and increases the intracellular levels of cyclic AMP (cAMP). CAMP binds to specific location on regulatory unit of PKA causing release of catalytic subunit of PKA.

Phorbol 12-myristate 13-actetate: Phorbol 12-myristate 13-actetate (PMA) is a type of phorbol esters, which is derived from some plants family such as Euphorbiacaeae and Thymelaeacease. PMA mimic the effect of diacyl glycerol (DAG), which is the endogenous activator of PKC.

Chelerythrine: Chelerythrine (CHT) is a benzophenanthridine alkaloid produced by a plant called *chelidonium majus.* CHT is a potent PKC inhibitor, which binds to the catalytic domain of PKC inhibiting PKC translocation from cytosol to cell membrane.

Staurosporine: Staurosporine (ST) is produced by bacterium *Streptomyces staurosporeus.* ST has strong affinity to the ATP binding site of kinase. So it works as a

non-selective protein kinase inhibitor through preventing binding of ATP to protein kinase.

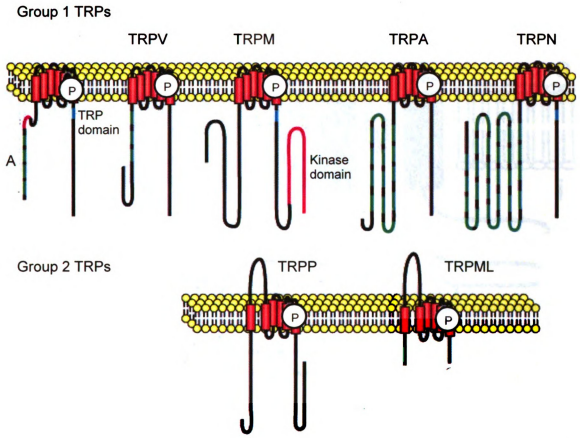
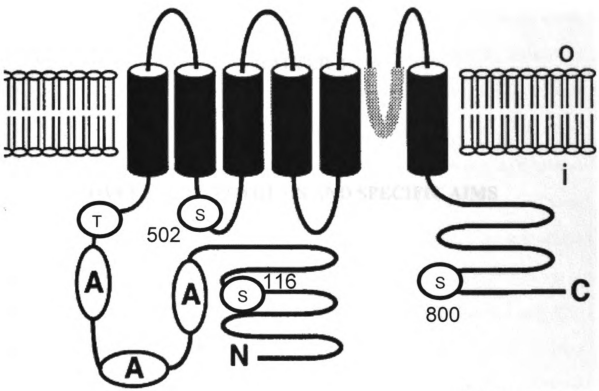


Fig. 1. The seven TRP subfamilies. Representatives of the five group 1 and two group 2 subfamilies are indicated at the top and bottom, respectively. Several domains are indicated: ankyrin repeats (A), protein kinase domain (TRPM6/7 only), transmembrane segments, and the TRP domains.



Szallasi, et al. 1999

Fig. 2. Schematic representation of TRPV1 channel. The TRPV1 subunit is a 95-kDa, 838 amino acids protein, consisting of six transmembrane (TM) domains, with a short pore-forming region between the fifth and sixth TM domains. Structurally, TRPV1 consists of a long 400-amino-acid amino-terminus containing three ankyrin repeats (A) domains and a carboxy-terminus. Positions of Serine (S) and Threonine (T) residues sensitive to PKA or PKC-dependent phosphorylation are shown.

CHAPTER 2

OVERALL HYPOTHESIS AND SPECIFIC AIMS

Overall hypothesis

TRPV1 channel is a ligand-gated cation channel expressed by sensory nerves. Besides the role in sensory physiology, TRPV1 also regulates blood pressure, renal blood flow and salt-sensitivity in hypertension via release of vasoactive peptide through "sensory-efferent" effect of TRPV1-positve sensory nerves. The goal of my studies is to investigate the properties of TRPV1 in kidney-projecting sensory neurons. ATP-induced TRPV1 resensitization and underlying intracellular mechanism are studied in these neurons. The major source of ATP for TRPV1 regulation might be from sympathetic nerves, which acts on both renal arterioles causing vasoconstriction and sensory nerves to enhance the release of vasodilators. I hypothesize that TRPV1 function and P2Y mediated facilitation of TRPV1 are impaired in DS hypertensive model. A novel mechanism contributing to the development of salt-sensitive hypertension might be revealed.

Specific aims

Specific aim 1: Establish the expression, electrophysiological and pharmacological properties of TRPV1 in kidney-projecting sensory neurons.

Aim 1a: Establish the expression of TRPV1 and CGRP in kidney-projecting sensory neurons.

Aim 1b: Investigate the distribution of sympathetic and sensory nerve fibers surrounding renal arteries.

Aim 1c: Establish the concentration-response curve for capsaicin (CAP) as an activator of TRPV1 on kidney-projecting sensory neurons.

Aim 1d: Characterize the distribution of silent, desensitizing and non-desensitizing kidney-projecting sensory neurons identified by different CAP-induced responses.

Specific aim 2: Characterize the P2Y receptor subtypes and signaling mechanism linking P2Y receptors to facilitation of TRPV1 function in sensory neurons innervating kidney.

Aim 2a: Establish the expression of TRPV1 and P2Y receptors on kidney-projecting sensory neurons.

Aim 2b: Identify the P2Y receptor subtypes responsible for ATP-induced TRPV1 resensitization on kidney-projecting sensory neurons.

Aim 2c: Investigate the intracellular signaling mechanism underlying P2Y receptor induced TRPV1 resensitization.

Specific aim 3: Study the function of TRPV1 on kidney-projecting sensory neurons during development of salt-sensitive hypertension.

Aim 3a: Compare CAP-induced TRPV1 currents between hypertensive and normotensive rats.

Aim 3b: Compare P2Y mediated facilitation of TRPV1 function in kidney-projecting sensory neurons between hypertensive and normotensive rats.

Aim 3c: Compare the proportion of silent, desensitizing and non-desensitizing kidney-projecting sensory neurons identified by different CAP-induced responses between hypertensive and normotensive rats.

CHAPTER 3

GENERAL METHODS

Animals

All animal use protocols were reviewed and approved by the All University Committee for Animal Use and Care at Michigan State University. Normal Wistar rats (male, 250-350 g, Charles River Laboratories, Portage, MI) were used in the study of ATP-mediated TRPV1 resensitization. All rats were provided with food and tap water *ad libitum* in a temperature-controlled room with a 12:12-hour light/dark cycle. For the studies of involvement of TRPV1 in salt-sensitive hypertension, male Dahl salt-sensitive (DS) rats (5 weeks old, Charles River Laboratories, Wilmington, MA) were used. DS rats were randomly divided into two groups (5-6 rat per group) and fed with either low-salt (DS/LS, 0.3% NaCl) or high-salt (DS/HS, 8% NaCl) diets (Harlan Teklad, Madison, WI) for 3 weeks. All rats were provided with tap water *ad libitum* in a temperature-controlled room with a 12:12-hour light/dark cycle. Systolic blood pressure was measured using tail-cuff method (HATTERAS blood pressure analysis system) one day before salt diet treatment and every seven days after treatment.

Retrograde labeling

Rats were anesthetized using isoflurane inhalation. A left flank incision was made. The renal nerve bundles were dissected free of the renal artery and surrounding tissue and crushed with the sharp end of a needle. A small sheet of parafilm was placed beneath usually 1-2 nerve bundles to avoid leakage of dye. Fast Blue (1 μl of a 2% solution in 2% acetic acid) (Polysciences, Warrington, PA) was applied to the nerve bundles for 0.5 h, after which the excess dye was blotted off, and the nerves were washed with sterile normal saline. The nerves were placed back on the surface of the renal artery.

Ticarcillin (1 ml) solution was applied locally for bacterial prophylaxis and the incision was closed with 3.0 silk suture. Tylenol (200mg/kg/day in drinking water) was given for 3 days after the surgery to relieve surgical pain.

Isolation of sensory neurons

Six days after surgery and FB labeling, rats were euthanized using a lethal injection of pentobarbital (50 mg/kg, i.p.). The left dorsal root ganglia (T10-L2) were dissected. Then they were moved to Hanks' balanced salt solution containing papain (25 μl/ml) (Worthington, Lakewood, NJ) and dithioerythritol (1 mg/ml) (Calbiochem, Los Angeles, CA) and incubated in a 37 °C shaking bath for 15 minutes. Ganglia were moved to Hanks' solution containing collagenase Type I (1 mg/ml) (Worthington, Lakewood, NJ) and trypsin inhibitor (0.75 mg/ml) (Sigma, St. Louis, MO) at 37 °C for 10 minutes. The ganglia were triturated using long neck fire-polished pipettes with sequentially smaller tip sizes. After trituration, enzyme activity was blocked by the addition of culture medium containing 4% fetal bovine serum and the suspension was centrifuged for 3 minutes at 900 *g*. The supernatant was discarded and 5 ml of culture medium was added before centrifuging again for 3 minutes. Finally, the supernatant was discarded and the pellet containing neurons was resuspended using culture medium. The neurons were plated on eight poly-L-lysine (50 μg/ml) coated round coverslips (~500 neurons/coverslip) and incubated at 37 °C in 5% CO_2 over night before electrophysiological studies.

Immunocytochemistry

Cultured DRG neurons on coverslip were fixed using 2% Zamboni's fixative solution for 20 minutes at room temperature. The neurons were washed with 0.1 M PBS and then incubated in PBS with blocking serum diluted in triton-X100 (0.2%) for 1 hour. The neurons were then incubated overnight at 4°C in diluted primary antibodies. Polyclonal goat anti-TRPV1 (1:200), polyclonal rabbit anti-$P2Y_1$ (1:400), polyclonal goat anti-$P2Y_2$ (1:200) (Santa Cruz, CA) or polyclonal rabbit anti-CGRP (1:400) (Abcam, Cambridge, MA) were used. The next day, the neurons were washed with PBS and incubated 1 hour at room temperature with diluted secondary antibodies conjugated with FITC or Cy^3 (Jackson ImmunoResearch, West Grove, PA). Neurons then were washed in PBS, mounted on slides, and examined using a Nikon TE2000-U inverted microscope. Photographs were taken using a SPOT Insight Color Mosaic camera (Mager Scientific, Inc.) with MetaImaging Series software. Controls with no primary antibodies were used to ensure that binding is specific.

Immunohistochemical

The renal lobar or interlobar artery was cleaned of surround connective tissue. Then the dissected arteries were fixed with 2% Zamboni's fixative solution over night at 4 °C. The next day, the tissue was washed with 0.1 M PBS and then incubated in PBS with blocking serum diluted in triton-X100 (1.0%) for 1 hour. Tissues were then incubated overnight at 4°C in diluted primary antibody solutions against markers of sympathetic nerves (mouse monoclonal anti-tyrosine hydroxylase, 1:200, Calbiochem,

Los Angeles, CA) and sensory nerves (goat polycolonal anti-CGRP, 1:1000, Abcam, Cambridge, MA). Next, tissues were washed with PBS three times and incubated for 1 hour at room temperature with diluted secondary antibodies conjugated with fluorescene isothiocyanate (FITC) or Cy^3 (Jackson ImmunoResearch, West Grove, PA). Tissues were washed again with PBS, and mounted on slides and examined using a Zeiss Pascal confocal laser-scanning microscope.

Whole cell voltage clamp

FB labeled sensory neurons were visualized using epifluorescence optics attached to an Olympus IX70 inverted microscope at UV excitation (360 nm). Membrane currents were recorded using the conventional whole-cell configuration of the patch-clamp technique (Hamill et al. 1981). Whole-cell recordings were made with 4-6 MΩ electrodes mounted on the head stage of an Axopatch 1D patch-clamp amplifier (Molecular Devices Inc., Sunnyvale, CA). The membrane potential was voltage-clamped at −70 mV and currents were filtered at 2 kHz and sampled at 5 kHz by an A/D converter (Digidata 1322A, Molecular Devices) in conjunction with a personal computer and stored on the hard disk of the computer. Membrane currents were expressed as current amplitude over cell capacitance (pA/pF) to normalize current amplitudes from neurons of different sizes. The standard pipette solution contained (mM): 122.5 K-Aspartate, 20 KCl, 1 $MgCl_2$, 2 MgATP, 10 HEPES and 10 EGTA (pH 7.3 with KOH). The external solution contained (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 12 Glucose and 10 HEPES (pH 7.4 with NaOH). Drugs were applied via a pipette with a tip diameter of 100 μm, positioned

within 100 μm of the cell body. Solution changes through the drug pipette were gated using solenoid controlled valves and drug solution changes occurred within 500 ms.

Statistical Analysis

Data are means ± standard error. Difference among groups were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test for multiple comparison. Differences were considered statistically significant at $P < 0.05$.

CHAPTER 4

$P2Y_2$ RECEPTORS MEDIATE ATP-INDUCED RESENSITIZATION OF TRPV1 EXPRESSED BY KIDNEY-PROJECTING SENSORY NEURONS

ABSTRACT

TRPV1 channel is a ligand-gated cation channel expressed by sensory nerves. TRPV1 regulates salt-sensitivity in hypertension and renal blood flow and blood pressure via release of vasoactive peptide from sensory nerves. My immunohistochemical studies showed that sympathetic nerves are closely aligned with sensory nerves in the renal artery. ATP from sympathetic nerves might act on P2Y receptors expressed by sensory nerves. I studied interactions between P2Y receptors and TRPV1 function on kidney-projecting sensory neurons. Application of Fast Blue (FB) to the nerves surrounding the renal artery retrogradely labeled neurons in DRG of rats. Whole cell patch clamp recording was performed on FB-labeled neurons maintained in primary culture. Capsaicin was used to activate TRPV1. Four types of kidney-projecting neurons were identified based on responses caused by capsaicin: desensitizing, non-desensitizing, silent, and insensitive. Silent neurons responded to capsaicin only after treatment with extracellular ATP. ATP reversed desensitization in desensitizing neurons. Insensitive neurons never responded to capsaicin. UTP, a $P2Y_2/P2Y_4$ receptor agonist, reversed capsaicin-induced TRPV1 desensitization. 2- Me-S-ATP, a $P2Y_1$ receptor agonist, did not change desensitization. MRS2179, a selective $P2Y_1$ antagonist, didn't block resensitization caused by ATP. PPADS, a non-selective P2 receptor antagonist (not for $P2Y_2$), didn't block resensitization caused by ATP. Suramin, a $P2Y_2$ receptor antagonist, blocked resensitization caused by UTP. Immunocytochemical studies showed that FB-labeled neurons co-expressed $P2Y_2$ receptors and TRPV1. I conclude that $P2Y_2$

receptors modulate TRPV1 activity on kidney-projecting sensory neurons. $P2Y_2$ receptor modulation of TRPV1 function may be a mechanism of interaction between sympathetic and sensory nerves supplying the renal vasculature.

INTRODUCTION

Mammalian TRP channels consist of seven related sub-families that are involved in a variety of physiological and pathophysiological functions (Montell 2005). The TRPV1 channel, a member of the TRPV sub-family, is a Ca^{2+}-permeable nonselective cation channel. TRPV1 is expressed predominantly on small- to medium-diameter DRG neurons and corresponding C- and Aδ-sensory fibers (Michael et al. 1999). TRPV1 is a molecular integrator for multiple types of sensory input, particularly pain. TRPV1 is activated or regulated by membrane depolarization, noxious heat, vanilloid and endocannabinoid compounds, extracellular protons, and inflammatory mediators (Nilius et al. 2005). Capsaicin is a hot chilli pepper-derived vanilloid compound that is a TRPV1 agonist used to study TRPV1 function (Ahern et al. 2005; Huang et al. 2008). One of the characteristics of TRPV1 is that prolonged or repeated applications of capsaicin induce receptor desensitization. Desensitization of TRPV1 is a Ca^{2+}-dependent process, which is inhibited or enhanced by TRPV1 phosphorylation or dephosphorylation (Docherty et al. 1996; Koplas et al. 1997; Liu et al. 2005). ATP is an important signaling molecule in the nervous system (Fredholm 1995). Extracellular ATP potentiates TRPV1 activity via metabotropic ATP P2Y receptors (Tominaga et al. 2001; Moriyama et al. 2003), but the involvement of P2Y receptors in modulation of TRPV1 desensitization has not been established.

Besides the sensory function, TRPV1-positive sensory neurons also have an efferent function. Activation of TRPV1 leads to Ca^{2+}-dependent peptide release from sensory nerve endings (Maggi 1993). At least 12 different types of transmitters are

present in capsaicin-sensitive sensory neurons. CGRP and SP are potent vasodilators released from sensory nerves in response to TRPV1 activation by capsaicin (Buck et al. 1986). Recent evidence indicates a role for TRPV1 in control of blood flow and blood pressure via release of vasoactive neuropeptides from sensory nerves (Wang 2005; Wang et al. 2006).

The kidney contributes to blood pressure regulation and salt sensitivity (Rettig et al. 1995). Recent studies showed that TRPV1-positive sensory nerves in the kidney enhance renal excretory function (Zhu et al. 2005). Impaired renal TRPV1 activity increases salt sensitivity and development of salt-sensitive hypertension (Li et al. 2008). Therefore, studies of TRPV1 and its regulation are important to our understanding of blood pressure regulation and salt-sensitive hypertension.

The kidney is innervated by both sympathetic and sensory nerves. The distribution of perivascular nerves and their morphological relationships to the renal arterial tree has been studied in serially sectioned rat kidneys. It was reported that SP and CGRP immunoreactive axons are evenly dispersed throughout the more numerous sympathetic nerve fibers that form perivascular nerve bundles (Ferguson et al. 1985; Gibbins et al. 1985). In the sympathetic nervous system, ATP is co-stored in synaptic vesicles with norepinephrine and neuropeptide Y (Fried et al. 1985). I hypothesize that ATP released from sympathetic nerve endings acts on the metabotropic P2Y receptors on sensory nerve endings in the kidney to facilitate the function of TRVP1. There are no techniques to study electrophysiological properties of receptors or channels on nerve endings. However, it is well accepted that electrophysiological studies of events in the soma can model events occurring at nerve endings.

Individual DRG contain sensory afferent neurons that supply multiple visceral organs (Kandel ER 2000; Lu et al. 2001; Bossowska 2002). Retrograde tracing techniques have been used to identify the sources of sensory afferent and sympathetic efferent nerves supplying the kidney in rats (Donovan et al. 1983; Gattone et al. 1986). Calcium channel function has been studied in sympathetic neurons supplying the kidney (Vari et al. 1999) but the functional properties of kidney-projecting sensory neurons have not been done. I used a retrograde labeling technique to identify kidney-projecting sensory neurons enabling us to study TRPV1 function in sensory neurons supplying the kidney.

RESULTS

Localization of kidney-projecting neurons in DRG. DRG neurons (T10-L2 segments) and nodose ganglion neurons were isolated for primary culture and FB-labeled sensory neurons were visualized under fluorescence microscope (Fig. 3). About 5% of the DRG neurons ipsilateral to the FB application side were FB-labeled but no FB-labeled neurons were detected in contralateral DRG. I did not detect FB labeled neurons in the nodose ganglia. Immunocytochemical studies revealed that TRPV1 and CGRP were co-localized in all FB-labeled, kidney-projecting DRG neurons (Fig. 4).

Alignment of sympathetic and sensory nerves supplying the renal lobar artery. Immunostaining of sensory nerve fibers and sympathetic nerve fibers surrounding renal lobar arteries was performed. I used CGRP as a marker of sensory nerve fibers and for sympathetic nerve fibers I used tyrosine hydroxylase (TH) as a marker. CGRP-immunoreactive nerve fibers formed a nerve network (Fig. 5A). The distribution pattern of TH-immunoreactive nerve fibers was similar to the sensory nerve fibers except that sympathetic nerve fibers were more numerous (Fig. 5B). Sensory and sympathetic nerve fibers were often found to be closely aligned with one another (Fig. 5C, D). These results suggest that transmitters release from one type of nerve fiber have the opportunity to modulate the function of adjacent nerve fibers. For example, ATP released by sympathetic nerve endings could act on receptors on sensory nerves to regulate their activity locally, or vice versa.

Capsaicin activates TRPV1. I first constructed capsaicin concentration-response curves (0.03-3 μM) for TRPV1 activation in kidney-projecting sensory neurons. There were two types of capsaicin responsive neurons. In the first type, repeated capsaicin

application didn't induce TRPV1 desensitization. The amplitude of capsaicin-induced TRPV1 currents increased in a concentration dependent manner (n=16, EC_{50}= 0.23 μM) (Fig. 6A). In the second type of neuron, TRPV1 desensitization occurred at higher capsaicin concentrations (n=9) (Fig. 6B). Further studies of TRPV1 function were done using capsaicin at a concentration of 1 μM as this was near the maximum of the concentration response curve and was the good concentration for inducing TTRPV1 desensitization.

TRPV1 and P2Y expression on kidney-projecting sensory neurons. Previous studies showed that extracellular ATP potentiates capsaicin-induced TRPV1 currents via an action at metabotropic $P2Y_1$ or $P2Y_2$ receptors (Tominaga et al. 2001; Moriyama et al. 2003). I speculated that ATP might also modulate TRPV1 desensitization-resensitization and this effect would be mediated by either $P2Y_1$ or $P2Y_2$ receptors. I first used immunocytochemical methods to identify P2Y receptors expressed by FB-labeled sensory neurons. I found that all FB labeled neurons co-expressed TRPV1, $P2Y_1$ and $P2Y_2$ receptors (Fig. 7).

$P2Y_2$ receptors mediate TRPV1 resensitization. I studied recovery of TRPV1 from desensitization in kidney-projecting sensory neurons using whole cell patch clamp recordings. The first application of capsaicin (1 μM) induced an inward TRPV1 current (n = 6). Then capsaicin was applied at 2 minute intervals to induce TRPV1 desensitization. TRPV1 currents evoked by the second and third CAP applications were significantly smaller than the first response (n=6, $P< 0.01$) (Fig. 8A,B). After TRPV1

was desensitized by repeated applications of capsaicin, neurons were treated with ATP (100 μM). The inward current caused by subsequent capsaicin application was not different from the initial response (n=6) (Fig. 8C,D).

To identify the ATP receptor subtypes responsible for ATP-induced TRPV1 resensitization, I first examined the effect of 2-Me-S-ATP. 2 Me-S-ATP is a $P2Y_1$ receptor agonist (von Kugelgen 2006). After 6 s pretreatment of desensitized neurons with 2 Me-S-ATP (100 μM), the capsaicin induced current was significantly smaller than the TRPV1 current caused by the first 100 μM application (n=6, $P< 0.01$) (Fig. 9A,B); 2-Me-S-ATP did not restore TRPV1 function. UTP is an agonist of $P2Y_2$ and $P2Y_4$ receptors (von Kugelgen 2006). I found that in neurons previously desensitized by capsaicin, UTP (50 μM), restored capsaicin-induced TRPV1 currents (n=6) (Fig. 9C, D).

MRS 2179 is a selective $P2Y_1$ receptor antagonist. After pretreatment with 10 μM MRS 2179 for 2 mins, ATP reversed TRPV1 desensitization. Capsaicin-induced currents were not different from initial response (n=6) (Fig. 10A, B). To further explore whether $P2Y_2$ or $P2Y_4$ receptor mediates TRPV1 resensitization induced by ATP, PPADS, a non-selective P2 receptor antagonist (not for $P2Y_2$), was tested. Desensitized neuron was pretreated with 10 μM PPADS, the reversal effect of ATP was not blocked by PPADS. The capsaicin-induced currents were not different from initial response (n=4) (Fig. 10C, D). Then suramin, a $P2Y_1/P2Y_2$ receptor antagonist, was tested. I pretreated desensitized neuron with 30 μM suramin for 10 s and UTP for 6s. Under these conditions, UTP didn't reverse TRPV1 desensitization. Capsaicin-induced inward currents were significantly

smaller than the current caused by the first capsaicin application (n=6, P< 0.01) (Fig. 11A,B). These experiments demonstrated that $P2Y_2$ receptors mediate ATP-induced TRPV1 resensitization.

Subtypes of kidney-projecting sensory nerves identified by capsaicin responses. My data showed that repeated applications of *capsaicin* cause TRPV1 desensitization which can be reversed by extracellular ATP in kidney-projecting sensory neurons. However, this interaction did not occur in all neurons studied. Four types of kidney-projecting sensory neurons could be discriminated based on the properties of capsaicin-induced inward currents. The first type of neuron exhibited a desensitizing capsaicin response (Fig. 12A); this response occurred in 66 of 189 neurons (35%) studied. The second type of neuron exhibited a non-desensitizing capsaicin response (Fig. 12B) in which repeated capsaicin applications didn't cause obvious TRPV1 desensitization. In these neurons, capsaicin was applied at least 6 times at 2 minute intervals and there was no decline in current amplitude. This type of response was detected in 55 of 189 neurons (29%). The third type of neuron exhibited a "silent" capsaicin-induced response (Fig. 12C). Capsaicin only caused an inward current after these neurons were pretreated with extracellular ATP; this response occurred in 11 of 189 neurons (6%). The fourth type was TRPV1 negative (Fig. 12D) in which capsaicin did not evoke an inward current even after ATP pretreatment; this group composed 30% of the 189 neurons studied. The proportion of different responses to CAP was summarized in chapter 5 (Table 1).

DISCUSSION

Retrograde labeling has been used previously to identify the sources of sensory nerves supplying the kidney in rats. It has been reported that the kidney in rats is supplied by sensory nerves predominately from ipsilateral DRG (Ferguson et al. 1986). My data are consistent with these findings as I only found FB-labeled neurons in ipsilateral DRG at the lower thoracic and upper lumbar spinal levels. However, I did not detect any FB-labeled neurons in contralateral DRG. Previous work also showed that nodose ganglion neurons also supply the kidney (Gattone et al. 1986). I did not detect FB-labeled neurons in either nodose ganglia. These differences could be due to the use of different retrograde tracing techniques or to the primary culture technique we have used which may have resulted in loss or death of neurons during the dispersion and protocol.

$P2Y_2$ receptors modulate TRPV1 function in kidney-projecting sensory neurons. The function of nociceptive pathways has been intensively studied (Caterina et al. 1997; Tominaga et al. 1998) and extracellular ATP modulates pain sensation either through ionotropic P2X receptors or metabotropic P2Y receptors expressed by sensory neurons (Sawynok et al. 1989; Moriyama et al. 2003). Activation of $P2Y_1$or $P2Y_2$ receptors potentiates capsaicin-induced TRPV1 currents, which contributes to hyperalgesia (Tominaga et al. 2001; Moriyama et al. 2003). Sensory neurons in the DRG are heterogenous and the target tissues or organs of those neurons modify the phenotype and function of sensory neurons (Lu et al. 2001). This heterogeneity makes it difficult to explore the characteristics of a receptor as the properties can vary among neurons

supplying different target tissues. For example some studies show that extracellular ATP potentiates TRPV1-mediated thermal sensation via an action at $P2Y_1$ receptors (Tominaga et al. 2001). However, others have found that $P2Y_2$ receptors mediate ATP-induced thermal hyperalgesia (Moriyama et al. 2003). Using a retrograde labeling technique, I identified a subpopulation of sensory neurons supplying the kidney. Therefore, I minimized variability that might occur across DRG neurons supplying different target tissues by studying only those neurons supplying the kidney.

My data show that brief pretreatment with ATP reversed TRPV1 desensitization in kidney-projecting sensory neurons. The work described above indicates that $P2Y_1$ or $P2Y_2$ receptors can resensitize TRPV1 to capsaicin or other stimuli. Buy which P2Y subtype is involved in TRPV1 resensitization in kidney-projecting neurons is unknown. My immunohistochemical data revealed that kidney-projecting sensory neurons expressed immunoreactivity for both $P2Y_1$ and $P2Y_2$ receptors. I next used a pharmacological approach to determine which P2Y receptor mediated TRPV1 resensitization. My agonist studies revealed that UTP, a $P2Y_2/P2Y_4$ agonist but not 2-Me-S-ATP, a $P2Y_1$ agonist, restored TRPV1 function after desensitization. A $P2Y_1$ antagonist (MRS 2179) and non-$P2Y_2$ antagonist (PPADS) didn't block reversal effect of ATP on desensitized TRPV1. Finally, I found that suramin, an antagonist at $P2Y_2$ but not $P2Y_4$ receptors blocked UTP. I conclude that $P2Y_2$ receptors link to modulation of TRPV1 in kidney-projecting sensory neurons. This response could occur via $P2Y_2$

linked activation of PKC which is known to reverse capsaicin-induced desensitization of TRPV1 via channel phosphorylation (Mandadi, Numazaki et al. 2004). If it this true, resensitization occurs within just a few seconds so $P2Y_2$ receptors and TRPV1 channels must be closely localized in the plasma membrane of kidney-projecting sensory neurons.

Capsaicin activates TRPV1 and one of the characteristic properties of TRPV1 is capsaicin-induced desensitization. Previous studies have shown that one of the factors affecting TRPV1 desensitization is capsaicin concentration (Szallasi and Blumberg 1999). My data in kidney-projecting sensory neurons also show that TRPV1 desensitization by capsaicin is concentration-dependent where concentrations of capsaicin, <1 μM produce little TRPV1 desensitization.

When I used the maximum concentration of capsaicin (1 μM), I found that TRPV1 occurs in several function states in kidney-projecting sensory neurons. Capsaicin did not cause an inward current in 30% of kidney-projecting neurons; it is likely that this latter group of cells does not express TRPV1. In 35% of kidney-projecting sensory neurons brief applications of the maximum concentration of capsaicin caused TRPV1 desensitization; this is the conventional capsaicin-induced response. In this group of neurons, TRPV1 function is regulated by its phosphorylation state as described above and by phosphatidylinositol 4,5-biphosphate (PIP_2) which can enhance or inhibit TRPV1 function (Liu et al. 2005). In 29% of kidney-projecting neurons, repeated applications of maximum concentrations of capsaicin produced stable amplitude inward currents. In these neurons, it is possible that the constitutive intracellular signaling activity is shifted towards a state that promotes maintained TRPV1 activity (high phosphorylation, low phosphatase activity, high PIP_2 levels) (Mandadi et al. 2004;

Mohapatra et al. 2005; Rohacs et al. 2008). In 6% of neurons capsaicin only elicited an inward current after ATP pretreatment suggesting that there may be a high constitutive phosphatase/phosphorylation ratio in these neurons. Studies in heterologous expression systems have shown that there are potential multiple intracellular signaling pathways by which TRPV1 function can be modulated (Cesare al. 1999; Bhave et al. 2002; Lazar et al. 2003). Multiple mechanisms might contribute to different response of TRPV1 upon repeated applications of capsaicin on kidney-projecting sensory neurons. It is also important to note that even brief applications (<6 s) were capable of either re-sensitizing TRPV1 in the first group of neuron or sensitizing TRPV1 to capsaicin. This result suggests that TRPV1 function in kidney-projecting sensory neurons is dynamic and its function can be modulated on a moment-to-moment basis.

TRPV1 is widely expressed in the nervous system functioning as a molecular integrator for multiple types of sensory input, particularly those that function in nociception. However, TRPV1-expressing sensory nerves can also have an efferent function. I showed that TRPV1 is expressed by sensory neurons supplying the renal artery and that these neurons co-express CGRP. This is similar to previous data showing that TRPV1 is expressed by sensory nerves supplying the vasculature and TRPV1 activation causes vasodilation through release of CGRP and SP from sensory nerves (Arulmani et al. 2004; Gupta et al. 2007). TRPV1 activation in the kidney increases GFR and sodium/water excretion (Li et al. 2008; Xie et al. 2008) and TRPV1 agonists prevent ischemia/reperfusion-induced renal injury (Ueda et al. 2008). TRPV1 in the kidney is also activated by high sodium levels and this leads to increased sodium excretion which prevents increases in blood pressure. This mechanism is compromised in DS rats and

likely contributes to the sodium dependent hypertension in this strain of rat (43). My data show that TRPV1 function in kidney-projecting sensory neurons is modulated by extracellular ATP and this modulation can occur rapidly (within a few seconds). ATP is a co-transmitter released from periarterial sympathetic nerves (Wier et al. 2009) and my data show that sensory nerves and sympathetic nerves are in close proximity at the adventitial surface of the renal lobar artery. ATP released from sympathetic nerves could modulate TRPV1 function on sensory nerves and therefore modulate release of vasodilator peptides. Impairment of this mechanism might compromise the response of the kidney to high salt intake and this impairment could contribute to salt-sensitive hypertension.

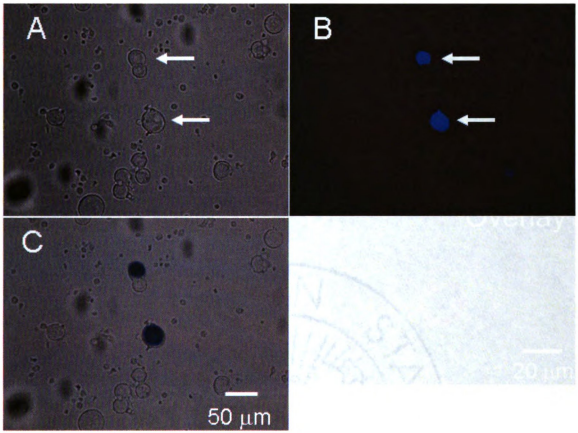


Fig. 3. Isolated DRG neurons retrogradely labeled by FB. *A*: DRG neurons under bright field illumination one week after FB application. *B*: FB labeled DRG neurons revealed using UV epifluorescence illumination, same field as (B). Arrows indicate FB labeled neurons. *C*: Overlay of A and B.

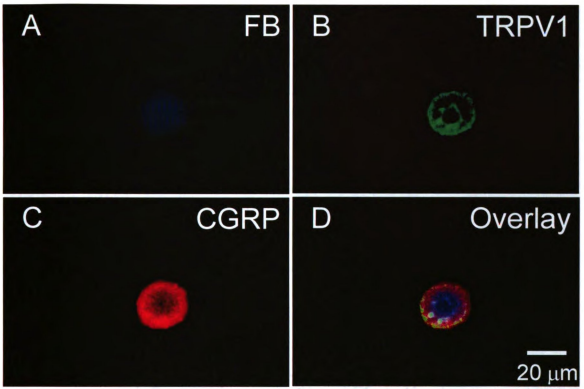


Fig. 4. Co-localization of TRPV1 and CGRP on kidney-projecting sensory neurons using fluorescence microscopy. *A*: Kidney-projecting sensory neuron labeled by FB (blue). *B*: TRPV1- immunostaining (green) on the same neuron. *C*: CGRP-immunostaining (red) on the same neuron. *D*: Overlay picture.

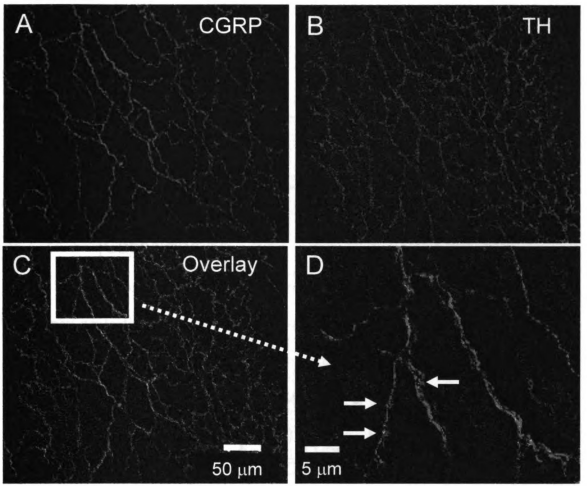


Fig. 5. CGRP- and TH-immunostaining shows close spatial relationship between sympathetic and sensory nerves surrounding renal lobar artery. *A*: Confocal image of CGRP immunostaining of sensory nerves associated with the renal lobar artery. *B*: TH immunostaining of sympathetic nerves associated with the same renal lobar artery. *C*: Overlay picture of (A) and (B). *D*: Magnified picture of the area indicated by the box in "C". Arrows show the close association of sensory and sympathetic nerve fibers.

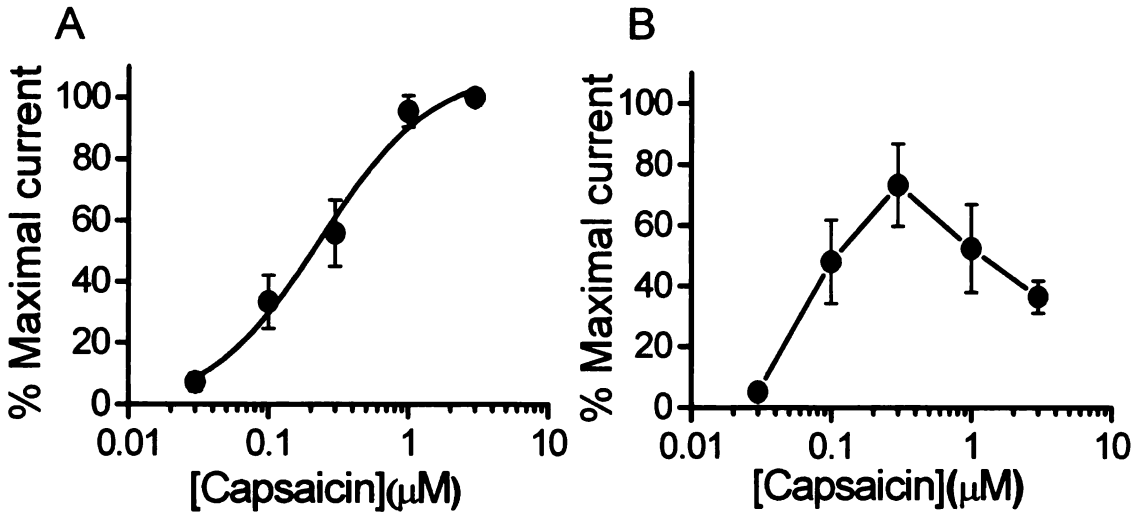


Fig. 6. Concentration response curves for capsaicin-induced activation of TRPV1 on kidney-projecting sensory neurons. *A*: Concentration response curve for neurons exhibiting non-desensitizing TRPV1 responses. *B*: Concentration response curve obtained from neurons exhibiting desensitizing TRPV1 responses. Desensitization was induced the CAP concentration was higher than 0.3μM. Data are mean ± s.e.m. of 16 (A) and 9 (B) neurons.

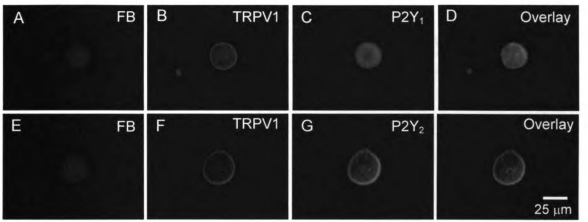


Fig. 7. Co-localization of TRPV1 and P2Y receptor subtypes on kidney-projecting sensory neurons. *A*: Kidney-projecting sensory neurons labeled by FB. *B*: TRPV1-immunostaining (green) in the same neurons shown in A. *C*: $P2Y_1$ receptor immunostaining in the same neuron shown in A and B. *D*: Overlay photomicrograph. *E*: Kidney-projecting sensory neurons labeled by FB. *F*: TRPV1-immunostaining (green) in the same neuron shown in E. *G*: $P2Y_2$ receptor immunostaining in the same neuron shown in E and F. *H*: Overlay photomicrograph.

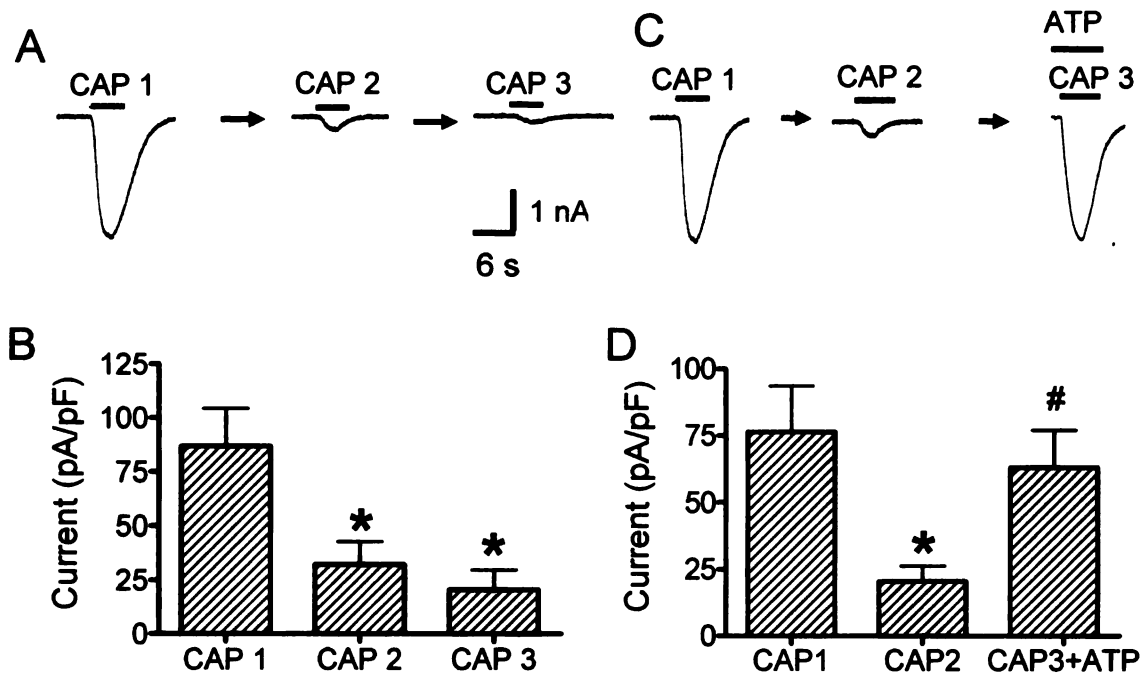


Fig. 8. Capsaicin-induced TRPV1 desensitization and reversal caused by ATP on kidney-projecting sensory neurons. *A*: Representative trace shows that repeated capsaicin applications induce TRPV1 desensitization. *B*: Mean data of experiments illustrated in "A". Data are mean ± s.e.m. (n=6 neurons). *C*: Representative trace shows that ATP (100 μM) reversed capsaicin-induced TRPV1 desensitization. *D*: Mean data of experiments illustrated in "C". Data are mean ± s.e.m. (n=7 neurons). CAP1, CAP2, and CAP3 represent the sequence of CAP applications *, $P < 0.01$ vs. first application of CAP. #, $P < 0.01$ vs. second application of CAP.

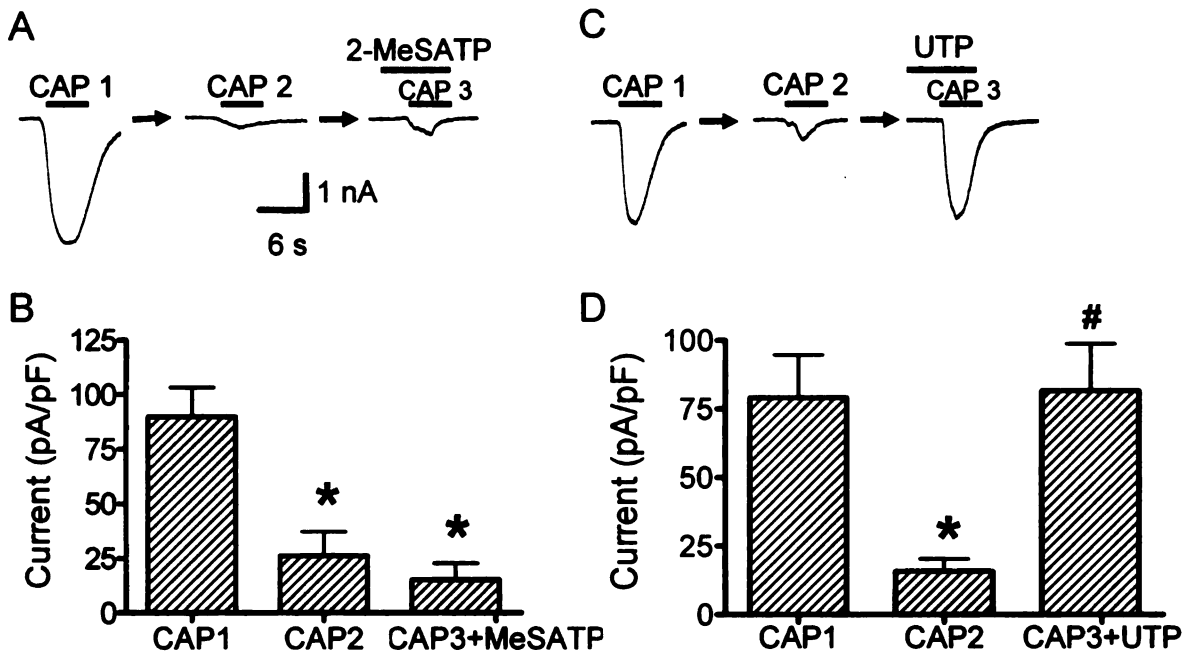


Fig. 9. ATP-induced TRPV1 resensitization is mediated by $P2Y_2$ receptors on kidney-projecting sensory neurons. *A*: Representative recordings showing that the $P2Y_1$ receptor agonist, 2 Me-S-ATP (100 μM) fails to reverse capsaicin-induced TRPV1 desensitization. *B*: Mean data of experiments illustrated in "A". Data are mean ± s.e.m. (n=6 neurons) *C*: Representative trace shows that UTP (50 μM) reverses capsaicin-induced TRPV1 desensitization. *D*: Mean data of experiments illustrated in "C". Data are mean ± s.e.m. (n=6 neurons). *, $P< 0.01$ vs. first application of capsaicin. #, $P< 0.01$ vs. second application of capsaicin.

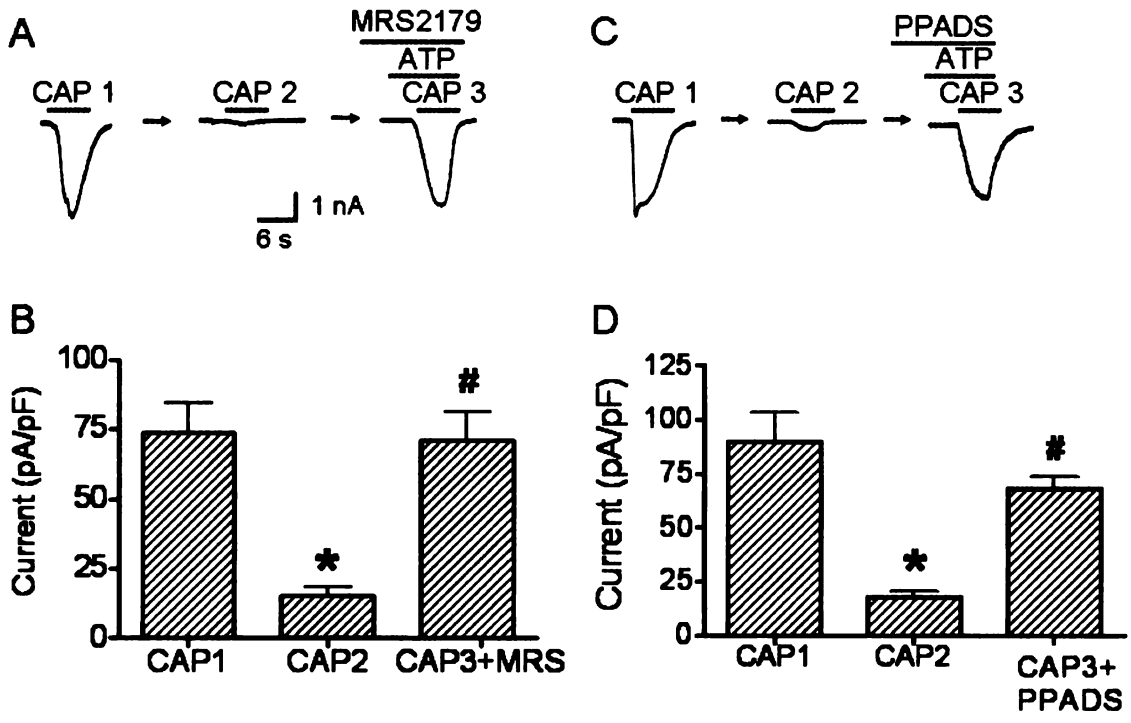


Fig. 10. ATP-induced TRPV1 resensitization is not blocked by $P2Y_1$ receptor or non-$P2Y_2$ receptor antagonist. *A*: Representative recordings showing that the $P2Y_1$ receptor antagonist, MRS 2179 (10 μM) doesn't block ATP-induced TRPV1 resensitization. *B*: Mean data of experiments illustrated in "A". Data are mean ± s.e.m. (n=6 neurons). C: Representative trace shows that PPADS (10 μM) doesn't block ATP-induced TRPV1 resensitization. *D*: Mean data of experiments illustrated in "C". Data are mean ± s.e.m. (n=4 neurons). *, $P< 0.01$ vs. first application of capsaicin. #, $P< 0.01$ vs. second application of capsaicin.

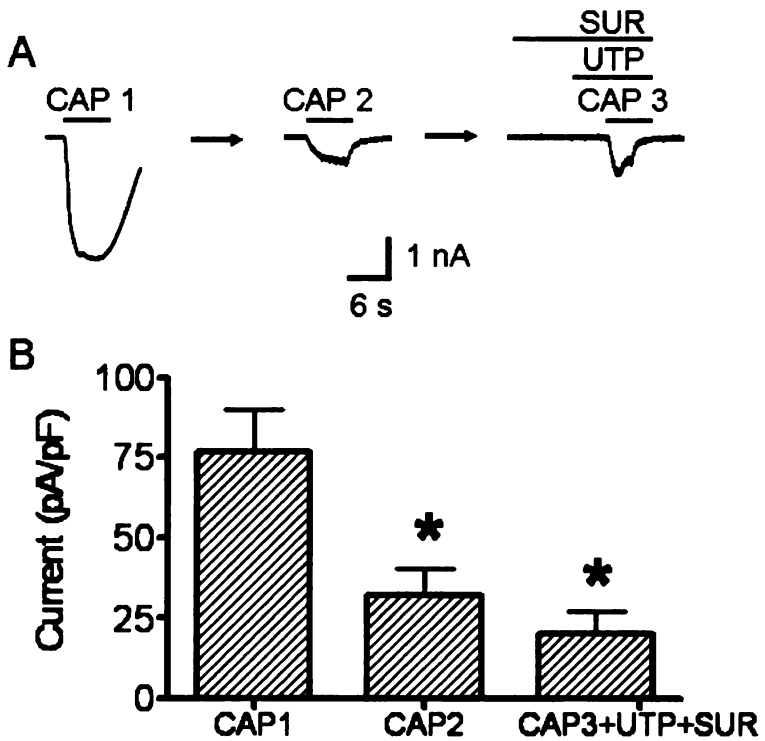


Fig. 11. The $P2Y_2$ receptor antagonists blocks UTP induced resensitization of TRPV1. *A*: Representative recording showing that suramin (SUR) (30 μM) blocks the resensitization of TRPV1 caused by UTP. *B*: Mean data of experiments illustrated in "A". Data are mean ± s.e.m. (n=6 neurons). *, $P< 0.01$ vs. first application of capsaicin.

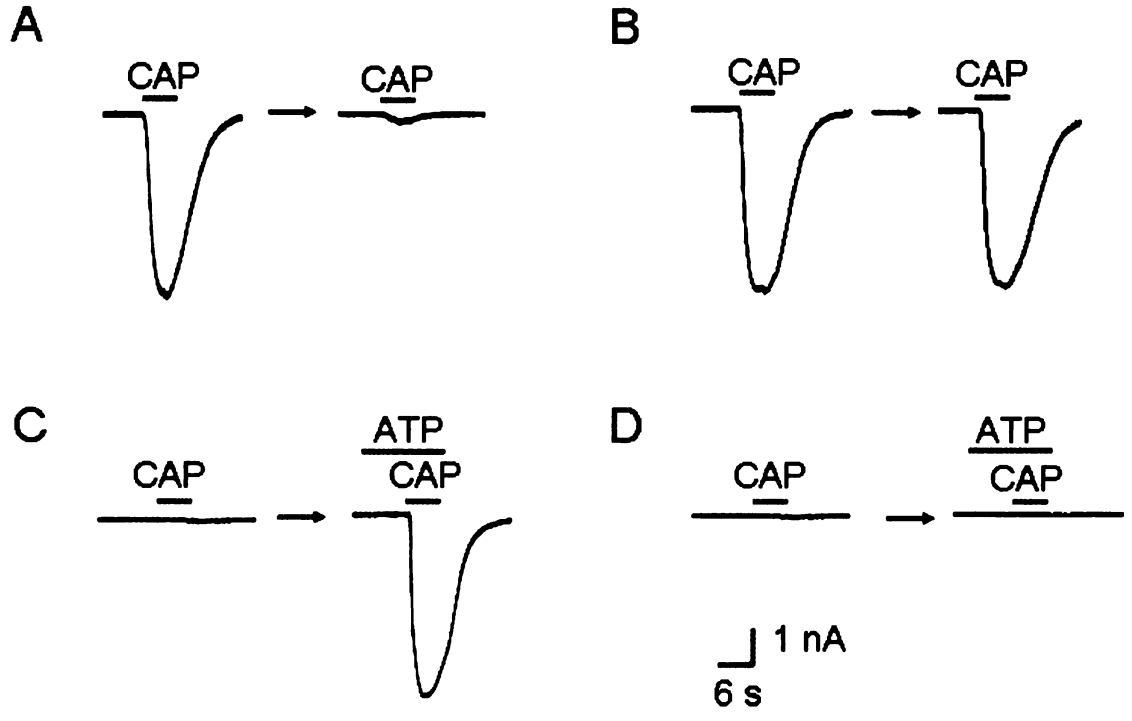


Fig. 12. There are 4 types of kidney-projecting sensory neurons based on capsaicin reactivity. Traces are representative recordings the different types of capsaicin (1 μM) responses. *A*: Desensitizing CAP response. Repeated applications of capsaicin cause TRPV1 desensitization. *B*: Non-desensitizing CAP response. In the second type of neuron, TRPV1 is not desensitized by repeated applications of capsaicin. *C*: Silent CAP response. The third type of neuron expresses silent TRPV1. Capsaicin doesn't induce a TRPV1 response until after pretreatment with ATP (100 μM). *D*: TRPV1-negative response. Capsaicin does not cause a TRPV1 response even after ATP pretreatment. Responses are representative of recordings obtained from 189 kidney-projecting sensory neurons.

CHAPTER 5

$P2Y_2$ RECEPTORS COUPLE TO RESENSITIZATION OF TRPV1 VIA ACTIVATION OF PKC IN KIDNEY-PROJECTING SENSORY NEURONS

ABSTRACT

TRPV1 channel is a ligand-gated cation channel expressed in sensory nerves. TRPV1 regulates salt-sensitivity, renal blood flow and blood pressure via release of CGRP and SP from sensory nerves supplying renal arteries. Sympathetic nerves are closely aligned with sensory nerves in the renal artery and sympathetic nerves release ATP that can act on $P2Y_2$ receptors expressed by sensory nerves. I used FB to retrogradely label kidney-projecting sensory neurons in DRG of rats. I studied the intracellular signaling pathway by which ATP, acting at $P2Y_2$ receptors, resensitizes TRPV1 in kidney-projecting sensory neurons. Whole cell patch clamp recordings were obtained from FB-labeled neurons maintained in primary culture. Capsaicin was used to activate TRPV1. UTP, acting at $P2Y_2$ receptors, reversed capsaicin-induced desensitization of TRPV1; this effect was blocked by the protein kinase inhibitor, ST (1 μM). PMA, a PKC activator, and FSK (50 μM), an indirect PKA activator, reversed capsaicin-induced TRPV1 desensitization. CHT (10 μM), a PKC inhibitor, blocked the effects of PMA and UTP, but not FSK. In some kidney-projecting neurons, capsaicin activated TRPV1 only after UTP pretreatment; this effect was also blocked by CHT. I conclude the interaction between $P2Y_2$ receptors and TRPV1 is mediated by PKC activation in kidney-projecting sensory neurons. $P2Y_2$ receptor modulation of TRPV1 function via PKC activation may be a mechanism of interaction between sympathetic and sensory nerves supplying the renal vasculature.

INTRODUCTION

TRPV1 channel is a Ca^{2+}-permeable non-selective cation channel, which belongs to the TRP superfamily of ion channels. TRPV1 is mainly expressed in CAP-sensitive sensory neurons and sensory nerve fibers (Michael et al. 1999). It has been demonstrated that TRPV1 serves as a molecular integrator for multiple types of sensory input induced by noxious stimulations, particularly pain. TRPV1 is activated or regulated by chemical or physical stimuli including membrane depolarization, noxious heat, vanilloid and endocannabinoid compounds, low pH, and inflammatory factors (Nilius et al. 2005). Capsaicin is derived from hot pepper, which is a potent TRPV1 agonist widely used to study the properties of TRPV1 channel (Ahern et al. 2005; Huang et al. 2008).

One of the characteristics of TRPV1 is that prolonged or repeated applications of capsaicin induce receptor desensitization. Desensitization of TRPV1 is a Ca^{2+}-dependent process, which is inhibited or enhanced by TRPV1 phosphorylation or dephosphorylation (Docherty et al. 1996; Koplas et al. 1997; Liu et al. 2005). PKA reverses TRPV1 desensitization via phosphorylation of Ser-116 and Thr-370 residues (Mohapatra et al. 2003) while PKC reverses TRPV1 desensitization via phosphorylation of Ser-502 and Ser-800 residues (Mandadi et al. 2004). My previous studies showed that extracellular ATP acts on $P2Y_2$ receptors to reverse desensitized TRPV1 in kidney-projecting sensory neurons. But the intracellular signaling pathway involved in $P2Y_2$ receptor and TRPV1 interaction in kidney-projecting sensory neurons has not been established.

Once TRPV1 is activated, the electrical signals are generated in these nerves and sent to central nervous system to form pain sensation. Meanwhile these signals may induce local Ca^{2+} influx in the nerve terminals leading to Ca^{2+}-dependent neuropeptide release from some primary afferent neurons (Maggi 1993). CGRP and SP are potent vasodilators released from sensory nerves in response to TRPV1 activation by capsaicin (Buck et al. 1986). Recent evidence indicates a role for TRPV1 in control of blood flow and blood pressure via release of vasoactive peptides from sensory nerves (Wang 2005; Wang et al. 2006). The kidney contributes to blood pressure regulation and salt sensitivity (Rettig et al. 1995). Recent data show that efferent activity of TRPV1 positive sensory nerves also plays an important role in the maintenance of renal homeostasis (Zhu et al. 2005; Xie et al. 2008). Therefore, studies of TRPV1 and its regulation are important to my understanding of blood pressure regulation and salt-sensitive hypertension.

Axons supplying multiple visceral organs project to DRG (Lu et al. 2001; Bossowska 2002). Retrograde labeling techniques have been widely used to trace the sources of afferent and efferent nerves innervating kidney in rats (Donovan et al. 1983; Gattone et al. 1986). Calcium channel function has been studied in sympathetic neurons supplying the kidney (Cesare et al. 1999) but the functional properties of kidney-projecting sensory neurons have not been established. My previous studies have showed that $P2Y_2$ receptors couple to TRPV1 resensitization by ATP in kidney-projecting sensory neurons. Here I studied the possible intracellular pathway responsible for TRPV1 resensitization by ATP and found that PKC activation mediates TRPV1 resensitization. A novel capsaicin-induced TRPV1 response is also mediated by intracellular PKC

activation. I used a retrograde labeling technique to identify kidney-projecting sensory neurons enabling us to study TRPV1 function in kidney-projecting sensory neurons.

RESULTS

Repeated applications of capsaicin (1 μM) desensitized TRPV1 in kidney-projecting neurons (Fig. 13A). After TRPV1 was desensitized by repeated capsaicin applications, neurons were treated with ATP (100 μM) applied from a flow tube positioned near the neuron. The inward current caused by subsequent capsaicin application was not different from the initial response (Fig. 13B). ATP acts at $P2Y_2$ receptors on kidney-projecting sensory neurons to cause TRPV1 resensitization. Here I studied the intracellular signaling pathway responsible for receptor interaction between $P2Y_2$ receptors and TRPV1 channels in kidney-projecting neurons.

A protein kinase mediates TRPV1 resensitization. The recovery of TRPV1 from desensitization in kidney-projecting sensory neurons was studied using whole cell patch clamp recordings. After TRPV1 was desensitized by a second application of capsaicin, neurons were treated with UTP (50 μM), which is an agonist of $P2Y_2$ and $P2Y_4$ receptors. I found that UTP restored capsaicin-induced TRPV1 currents in kidney-projecting sensory neurons (Fig. 14A, B). To further explore the possible intracellular signaling pathway involved in TRPV1 resensitization, a non-selective protein kinase inhibitor ST was tested. I pretreated capsaicin desensitized neurons with ST (1 μM) for 2 mins and UTP for 6s. Under these conditions, UTP failed to reverse TRPV1 desensitization. Capsaicin-induced inward currents were significantly smaller than the current caused by the first capsaicin application ($n=4$, $P< 0.01$) (Fig. 14C, D). These experiments demonstrated that activation of a protein kinase mediates $P2Y_2$-TRPV1

resensitization in kidney-projecting sensory neurons. To explore which protein kinase plays a role, PMA, a selective PKC activator, was tested. After 6s pretreatment of desensitized neurons with PMA (0.1 μM), the capsaicin-induced TRPV1 currents were restored to the control level (n=7) (Fig. 15A,B). To study if other protein kinases could also be involved in TRPV1 resensitization, I used an adenylyl cyclase activator FSK as an indirect activator of PKA. I found that FSK (50 μM) also restored the capsaicin-induced TRPV1 currents in kidney-projecting neurons (n=5) (Fig. 15C,D).

CHT blocks TRPV1 resensitization. CHT is a selective PKC inhibitor. In my studies, CHT was applied to the patch pipette solution via dialysis into intracellular side to study if ATP-induced TRPV1 resensitization is mediated by PKC. At in intracellular concentration of 5 μM, CHT did not block the effects of either PMA or FSK on TRPV1 resensitization (Fig. 16A, B). At 10 μM, CHT blocked the effects of PMA (Fig. 17A, B) but not FSK (Fig. 17C, D), while at 20 μM CHT blocked the effects of both PMA and FSK (Fig. 16C, D).

PKC involves in $P2Y_2$-mediated TRPV1 resensitization. I next identified the protein kinase that links $P2Y_2$ receptors to TRPV1 resensitization in kidney-projecting sensory neurons. CHT (10 μM) in pipette solution was applied via dialysis into the neuron. TRPV1 desensitization was induced by repeated applications of CAP and neurons were treated with UTP (10 μM) for 6 s. Under these conditions, UTP didn't reverse TRPV1 desensitization. Capsaicin-induced inward currents were significantly smaller than the current caused by the first capsaicin application (n=4, $P< 0.01$) (Fig. 18A,B). To further investigate which PKC isoform involves in $P2Y_2$-mediated TRPV1

resensitization, I tested εV1-2 (200 μM in the pipette solution), a selective PKC_ε inhibitor. I found that εV1-2 did not block the reversal effect of UTP on desensitized TRPV1 with intracellular application of εV1-2 for up to 14 minutes (Fig. 18C). These experiments demonstrate that $P2Y_2$-mediated TRPV1 resensitization is through the activation of a PKC isoform other than PKC_ε pathway in kidney-projecting sensory neurons.

Sensitization of silent CAP-induced TRPV1 response is mediated by PKC. My previous studies revealed that some kidney-projecting neurons did not respond to CAP unless they were pretreated with ATP or UTP; these were called silent neurons. I tested neurons with CHT (10 μM) in the pipette solution and confirmed that some neurons did not respond to capsaicin unless they were pretreated with UTP (50 μM). I allowed CHT to diffuse into the neuron for 8 minutes and retested capsaicin and UTP. Under these conditions, UTP was no longer able to resensitize TRPV1 and capsaicin-induced inward currents were significantly smaller than the control currents ($n=8$, $P<0.01$) (Fig. 19A, B). I also tested the effect of FSK on these silent neurons. I found that FSK also sensitized silent neurons ($n=7$) (Fig. 19C, D) but CHT dialysis failed to block resensitization caused by FSK on these kidney-projecting sensory neurons ($n=7$) (Fig. 19C, D). Therefore, I conclude that the resensitization of silent TRPV1 channels by UTP is mediated by PKC activation instead of PKA.

DISCUSSION

Retrograde labeling is a powerful technique to demonstrate anatomical and functional relationships between neuronal cells and targeted tissues or organs. Here I used FB as a retrograde tracer to label sensory neurons supplying kidney in order to study the intracellular signaling pathway linking $P2Y_2$ receptors to TRPV1 resensitization.

TRPV1 function contributes to nociceptive signaling (Caterina et al. 1997; Tominaga et al. 1998; Davis et al. 2000) and extracellular ATP also plays an important role in pain signaling via actions at ionotropic P2X receptors or metabotropic P2Y receptors (Sawynok et al. 1989; Moriyama et al. 2003). $P2Y_1$ and $P2Y_2$ receptors link to potentiation of capsaicin-induced TRPV1 currents, which might be a mechanism of hyperalgesia under some conditions (Tominaga et al. 2001; Moriyama et al. 2003). My previous studies demonstrate that ATP-induced TRPV1 resensitization is mediated by $P2Y_2$ receptors in kidney-projecting sensory neurons.

Studies in heterologous expression systems have shown that there are potential multiple intracellular signaling pathways by which TRPV1 function can be modulated (Cesare et al. 1999; Bhave et al. 2002; Lazar et al. 2003). The Ca^{2+}-dependent TRPV1 desensitization is modulated by an intracellular phosphorylation and dephosphorylation mechanism. Inhibition of Ca^{2+}-activated phosphatase reduces the TRPV1 desensitization (Docherty et al. 1996). PKA and PKC activation reverse TRPV1 desensitization (Mohapatra et al. 2003; Mandadi et al. 2004). But the linkage between $P2Y_2$ receptors

and a specific protein kinase in causing TRPV1 resensitization in primary sensory neurons has not been established.

My results show that ST, a non selective protein kinase inhibitor, blocks UTP induced resensitization of TRPV1. This result confirms that $P2Y_2$ receptors link to a protein kinase which likely phosphorylates TRPV1 to reverse capsaicin induced desensitization. PMA and FSK both mimicked the effects of UTP by resensitizing TRPV1 indicating that PKA and PKC dependent mechanisms were functional in kidney-projecting sensory neurons. To investigate which protein kinase is involved in $P2Y_2$-mediated TRPV1 resensitization, I dialyzed neurons with 10 μM CHT. I verified that at this concentration CHT was specific for PKC as it blocked the resensitizing effects of PMA but not FSK on TRPV1. CHT inhibited UTP-mediated TRPV1 resensitization and I conclude that $P2Y_2$-mediated TRPV1 resensitization in kidney-projecting sensory neurons is mediated by PKC. Two serine residues on TRPV1 are targets for phosphorylation by the PKC isoform, PKC_ε. Mutations of these residues disrupt the PMA-mediated TRPV1 potentiation when TRPV1 is expressed in HEK-293 cells (Numazaki et al. 2002). I tested a PKC_ε inhibitory peptide, εV1-2, and found that it did not block the effects of UTP on TRPV1 resensitization even though the peptide was applied intracellularly and for a longer period of time than was required for effective CHT intracellular dialysis. It is likely that PKC isoforms other than PKCε are involved in $P2Y_2$ receptor mediated TRPV1 resensitization in kidney-projecting neurons.

Silent nociceptive afferent nerves or silent nociceptors have been reported before. They are not usually activated by physiological stimuli. Pathophysiological

conditions, such as inflammation, sensitize the silent nociceptors which then respond to the stimuli associated with tissue injury (Michaelis et al. 1996). But the details about properties and regulatory mechanisms about these nociceptors have not been well demonstrated. My results show that some TRPV1 positive kidney-projecting neurons do not respond to capsaicin stimulation unless the neurons are pretreated with ATP or UTP. The sensitization effect of UTP on silent TRPV1 expressing neurons was inhibited by CHT so I conclude that the sensitization of silent TRPV1 by ATP or UTP is mediated by PKC.

TRPV1 is widely expressed in the nervous system functioning as a molecular integrator for multiple types of sensory input, particularly those that function in nociception. But the efferent function is also important in regulating vascular homeostasis and maintaining sodium/water balance through release of CGRP and SP from sensory innervating vasculature (Li et al. 2008; Xie et al. 2008). A recent study has shown that TRPV1 expression and function are reduced in DS hypertensive rats and this could contribute to the sodium dependent hypertension in this strain of rat (Wang et al. 2006). Sensory nerves and sympathetic nerves are in close proximity at the adventitial surface of the renal artery. So ATP, a co-neurotransmitter released from periarterial sympathetic nerves (Wier et al. 2009) might act on sensory nerves to regulate TRPV1 activity and therefore modulate release of vasodilator peptides. The activation of TPRV1 channel triggers the release of vasodilator from sensory nerve endings. The vasodilator release is temporarily attenuated once TRPV1 is desensitized. ATP released from closely aligned sympathetic nerve endings acts on $P2Y_2$ receptors to resensitize TRPV1 or sensitize originally silent TRPV1. So the vasodilator peptide release is initiated again via TRPV1

activation. Renal vascular homeostasis may maintained partly by cross-talk between sympathetic and sensory nerves. The discovery of intracellular PKC pathway for TRPV1 sensitization and resensitization mediate by $P2Y_2$ receptors in kidney-projecting sensory neurons might reveal the novel mechanism for the development of salt-sensitive hypertension. Impairment of the balance between sympathetic and sensory innervations or regulatory mechanism TRPV1 by ATP might compromise the response of the kidney to high salt intake and this impairment could contribute to salt-sensitive hypertension.

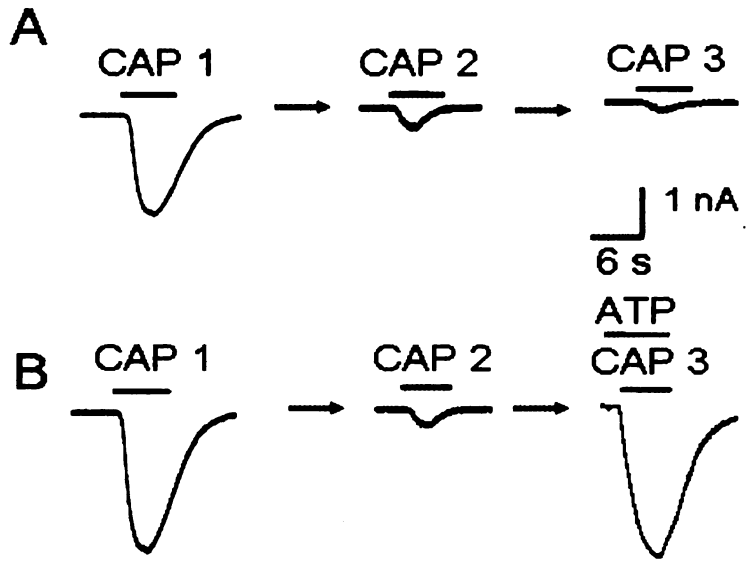


Fig. 13. Capsaicin-induced TRPV1 desensitization and reversal caused by ATP in kidney-projecting sensory neurons. A: Representative trace shows that repeated capsaicin (1 μM) applications induce TRPV1 desensitization. B: Representative trace shows that ATP (100 μM) reversed capsaicin-induced TRPV1 desensitization. CAP1, CAP2, and CAP3 represent the sequence of CAP applications.

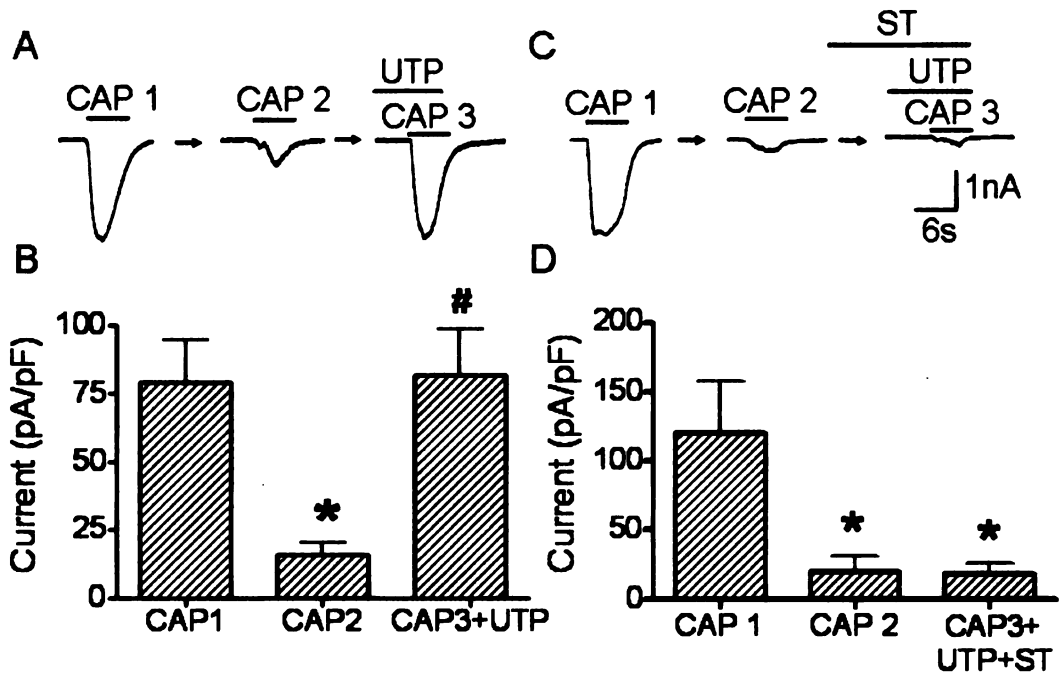


Fig. 14. UTP-induced TRPV1 resensitization is mediated by protein kinase in kidney-projecting sensory neurons. A: Representative trace shows that UTP (50 μM) reverses capsaicin-induced TRPV1 desensitization. B; Mean data of experiments illustrated in "A" (n=6 neurons). C: Representative trace shows that reversal effect of UTP was inhibited by pretreatment of staurosporin (ST) (1 μM) for 2 mins. D: Mean data of experiments illustrated in "B" (n=4 neurons). Data are mean ± s.e.m. *, $P< 0.01$ vs. first application of capsaicin. #, $P< 0.01$ vs. second application of capsaicin.

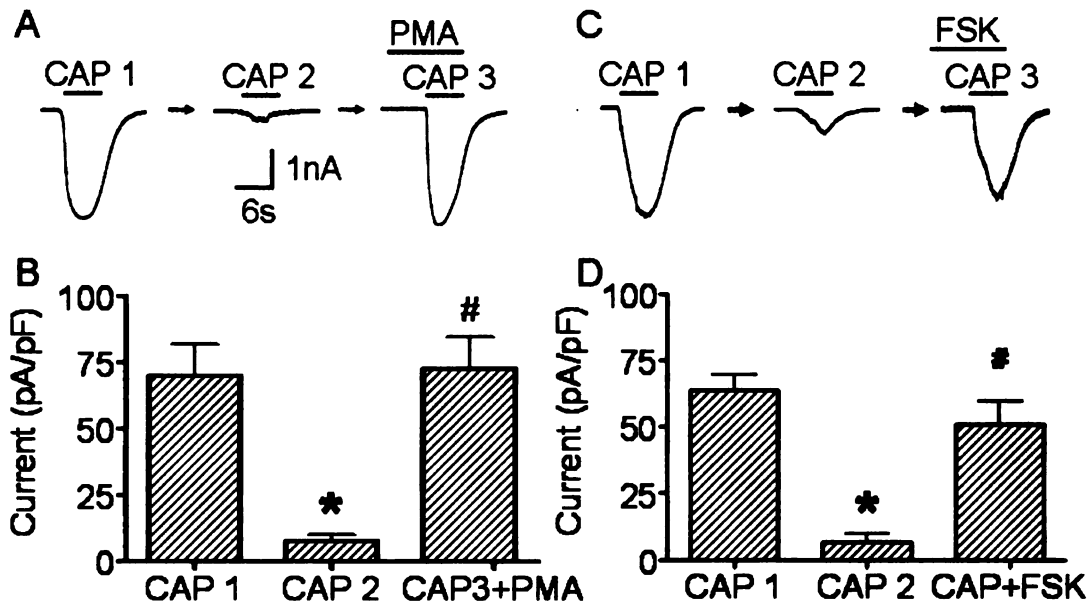


Fig. 15. PKC and PKA reverse TRPV1 desensitization in kidney-projecting sensory neurons. A: Representative trace shows that selective PKC activator PMA (0.1 μM) reverses capsaicin-induced TRPV1 desensitization. B: Mean data of experiments illustrated in "A" (n=7 neurons). C: Representative trace shows that indirect PKA activator FSK (50 μM) reverses capsaicin-induced TRPV1 desensitization. D: Mean data of experiments illustrated in "C" (n=5 neurons). Data are mean ± s.e.m.. *, $P< 0.01$ vs. first application of capsaicin. #, $P< 0.01$ vs. second application of capsaicin.

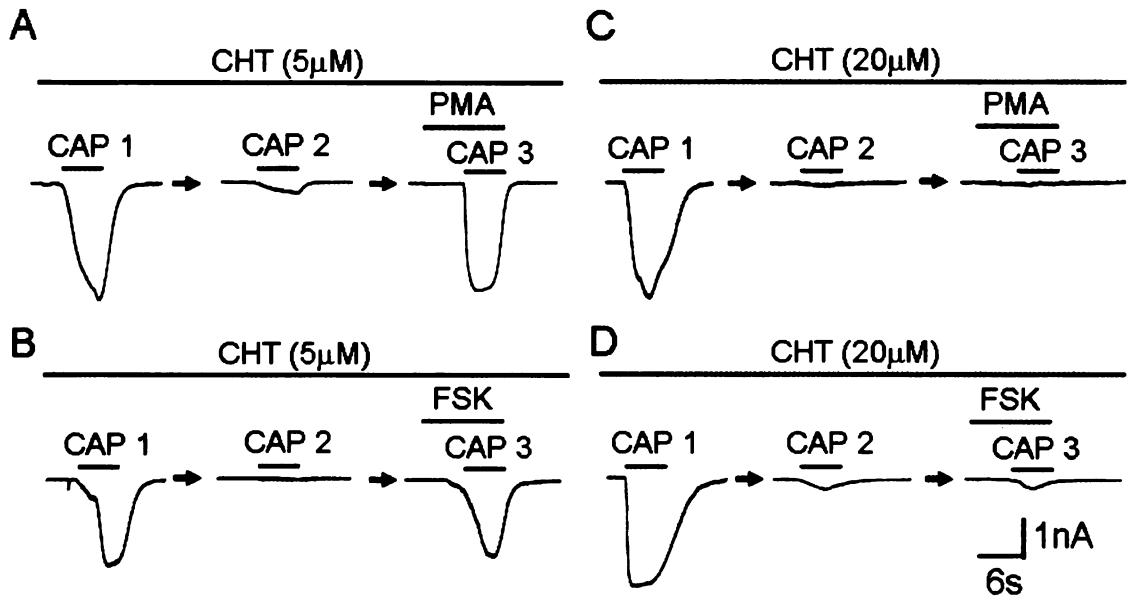


Fig. 16. Chelerythrine (CHT) as a PKC inhibitor in kidney-projecting sensory neurons. CHT in the pipette solution was applied via dialysis. A,B: Representative traces show that CHT (5 μM) fails to inhibit the resensitization of TRPV1 caused by PMA or FSK. C,D: Representative traces show that CHT (20 μM) inhibits resensitization of TRPV1 caused by PMA and FSK.

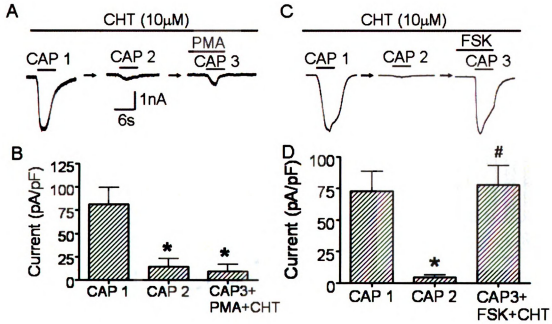


Fig. 17. Chelerythrine (CHT) is a selective PKC inhibitor at 10 μM in kidney-projecting sensory neurons. CHT was applied in pipette solution. A: Representative trace shows that CHT (10 μM) inhibits the resensitization of TRPV1 caused by PMA. B: Mean data of experiments illustrated in "A" (n=3 neurons). C: Representative trace shows that CHT (10 μM) doesn't inhibit the resensitization of TRPV1 caused by FSK. D: Mean data of experiments illustrated in "C" (n=5 neurons). Data are mean ± s.e.m. *, P< 0.01 vs. first application of capsaicin. #, P< 0.01 vs. second application of capsaicin.

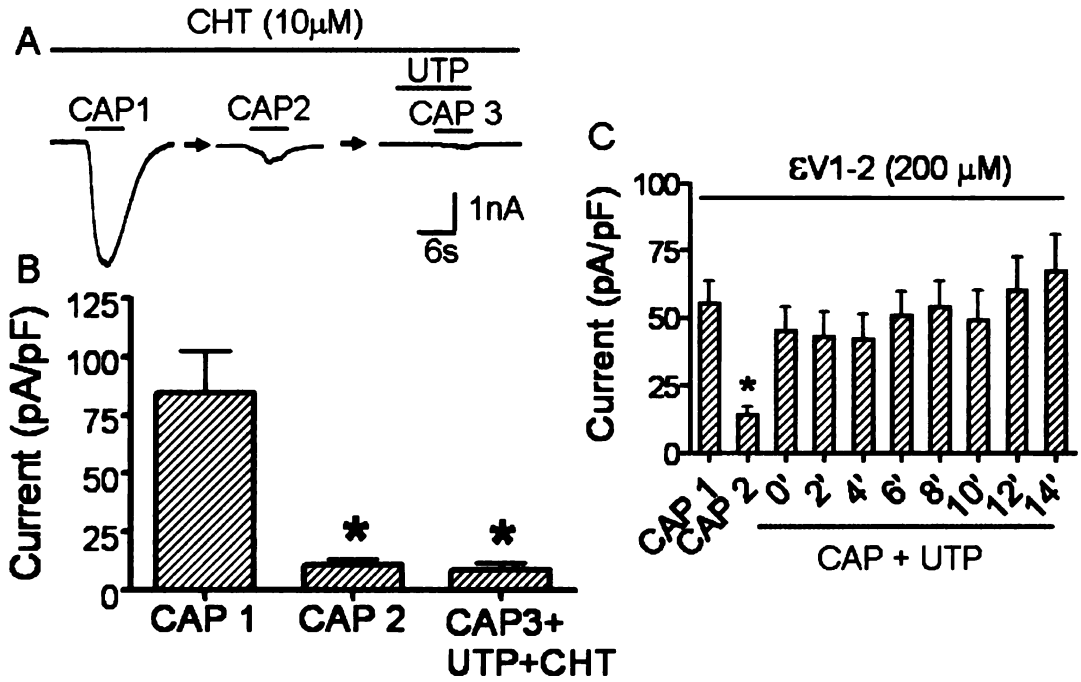


Fig. 18. UTP-induced TRPV1 resensitization is mediated by PKC activation in kidney-projecting sensory neurons. CHT was applied in pipette solution. A: Representative trace shows that CHT (10 μM) inhibits the resensitization of TRPV1 caused by UTP. B: Mean data of experiments illustrated in "A". Data are mean ± s.e.m. (n=4 neurons). C: A selective PKC_{ε} inhibitor εV1-2 (200 μM in pipette solution) fails to block the resensitization of TRPV1 caused by UTP. Capsaicin and UTP were applied repeatedly at 2 minute intervals. UTP restored TRPV1 sensitivity to capsaicin even after 14 minutes of intracellular dialysis with εV1-2. 0'-14' represent the time course of UTP and CAP treatment after desensitization of TRPV1. Data are mean ± s.e.m. (n=7 neurons). *, $P< 0.01$ vs. first application of capsaicin.

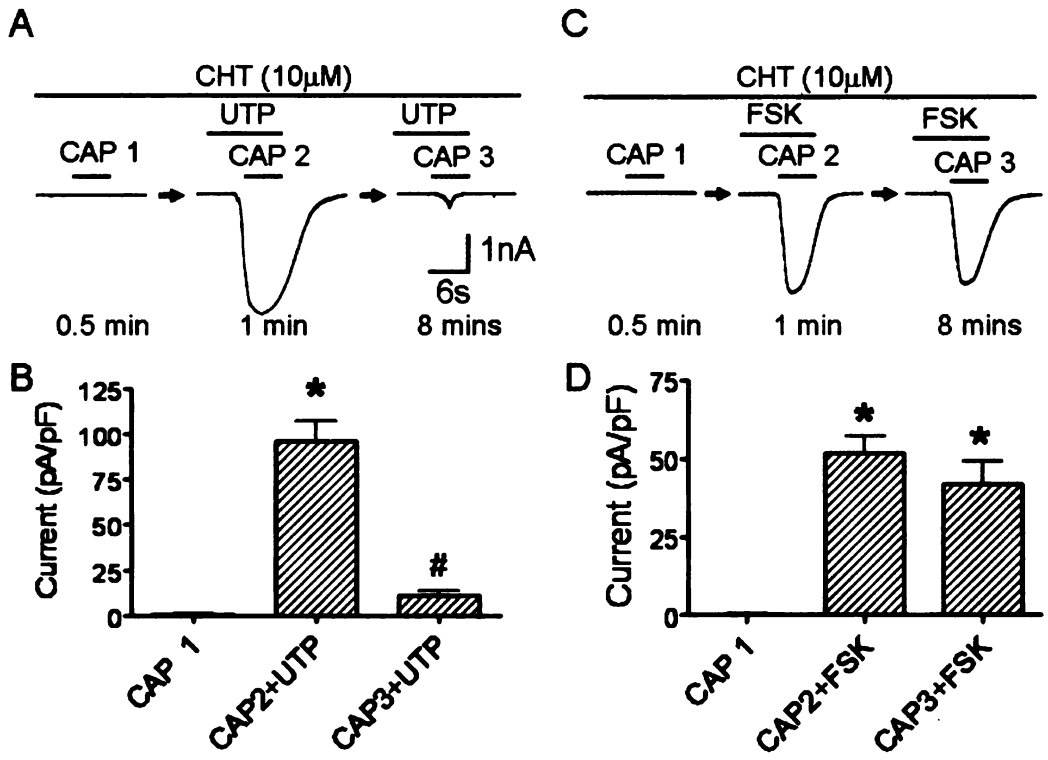


Fig. 19. The sensitization of silent CAP-induced TRPV1 response is mediated by PKC activation in kidney-projecting sensory neurons. A: Representative trace shows that silent TRPV1 is sensitized by the treatment of UTP. Sensitization effect of UTP is inhibited by CHT (10 μM) in pipette solution. B: Mean data of experiments in "A" (n=8 neurons). C: Representative trace shows that silent TRPV1 is sensitized by pretreatment with FSK. CHT (10 μM) in pipette solution did not inhibit TRPV1 sensitization caused by FSK. D: Mean data of experiments in "C" (n=7 neurons). CAT was applied within 0.5 min after establishing whole cell recording conditions. Capsaicin and UTP were applied 1 minute after the first capsaicin application. CAP and UTP were applied again 8 minutes after the first capsaicin application. This was sufficient time to allow for intracellular dialysis with CHT. Data are mean ± s.e.m. *, $P< 0.01$ vs. first application of capsaicin. #, $P< 0.01$ vs. second application of capsaicin.

CHAPTER 6

INVOLVEMENT OF TRPV1 IN DEVELOPMENT OF DAHL SALT-SENSITIVE HYPERTENSION

ABSTRACT

TRPV1 channel is a ligand-gated non-selective cation channel expressed primarily in sensory nerves of unmyelinated C-fibers or thinly myelinated A σ -fibers. TRPV1 contributes to regulation of salt-sensitivity, renal blood flow and blood pressure. To determine the role of TRPV1 channels in development of salt-sensitive hypertension, DS rats were fed high salt (DS/HS) or low salt diet (DS/LS) for 3 weeks. HS but not LS diet increased systolic blood pressure. Application of FB to the nerves surrounding the renal artery retrogradely labeled neurons in DRG of DS/HS and DS/LS rats. Capsaicin (1 μM)-induced TRPV1 currents were smaller in FB-labeled kidney-projecting sensory neurons of DS/HS rats and the capsaicin EC_{50} was higher in DS/HS rats compared with DS/LS rats. ATP-induced resensitization of TRPV1 after capsaicin desensitization was enhanced in kidney-projecting sensory neurons of DS/HS rats. CGRP-positive sensory nerve fibers were reduced in renal interlobar arteries from DS/HS rats. Immunostaining of TRPV1, CGRP and $P2Y_2$ receptors in kidney-projecting sensory neurons were similar between DS/LS and DS/HS rats. The proportions of kidney-projecting sensory neurons expressing desensitizing, non-desensitizing, silent, TRPV1 type responses upon capsaicin activation were not different in DS/LS and DS/HS rats. Enhancement of ATP-mediated TRPV1 resensitization in kidney-projecting sensory neurons may compensate for impaired TRPV1 function and decreased CGRP expression in kidney-projecting sensory neurons from DS/HS rats. Impaired function of TRV1 triggered by a high salt diet may contribute to the salt sensitivity and development of hypertension in salt sensitive individuals.

INTRODUCTION

Both clinical and experimental studies have shown that dietary salt intake is closely correlated with development and progression of hypertension (Haddy 2006). Hypertension is a multifactor disease, several mechanism have been reported to be involved in the development of hypertension including sympathetic nervous system, endothelin, RAS, ROS, et al (Yoshibayashi et al. 1991; Somova et al. 1999; Zhou et al. 2005; Zhu et al. 2009). Recently studies show that sensory nerves also play an important role in preventing high salt-induced elevation in blood pressure, suggesting that a defect in sensory function might contribute to development of salt-sensitive hypertension (Wang 2005).

TRPV1 channel is a ligand-gated nonselective cation channel expressed mainly in sensory nerves of C- and Aδ-sensory fibers and corresponding sensory neurons in DRG (Michael et al. 1999). TRPV1-positve sensory nerve fibers innervate multiple tissues including skin, heart, kidney, and blood vessels (Guo et al. 1999). As is a molecular integrator for multiple types of sensory input, TRPV1 is activated by multiple chemical or physical stimuli, such as CAP, noxious heat, endocannabinoid compounds, protons, and inflammatory mediators (Nilius et al. 2005). CAP is a vanilloid compound that is a potent TRPV1 agonist, which is widely used as a tool to study the function and property of TRPV1 channel (Ahern et al. 2005; Huang et al. 2008). Repeated applications of capsaicin induce TRPV1 desensitization. Desensitization of TRPV1 is a Ca^{2+}-dependent process, which is inhibited or enhanced by TRPV1 phosphorylation or dephosphorylation (Docherty et al. 1996; Koplas et al. 1997; Liu et al. 2005).

Activation of TRPV1 channel induces CGRP and SP release from sensory nerves,

which are potent vasodilators (Buck et al. 1986; Maggi 1993). TRPV1-positive sensory nerves play an important role in regulating salt sensitivity in hypertensive animal models via release of SP and CGRP (Wang 2005; Wang et al. 2006). DS rats are genetically predisposed to hypertension when exposed to high salt diets (Dahl et al. 1967; Deng 1998). Salt sensitivity is regulated partly by the kidney (Rettig et al. 1995) and activation of TRPV1-positive sensory nerves supplying the kidney enhances renal sodium excretion (Zhu et al. 2005). TRPV1 function is impaired in the kidney of DS hypertensive rats, which contributes to the lower GFR and sodium/water excretion (Li et al. 2008). TRPV1 expression is reduced in mesenteric arteries, renal cortex, and renal medulla of DS hypertensive rats and CGRP levels are reduced in DRG of the same animals (Katki et al. 2001; Wang et al. 2006). These data imply that impaired TRPV1 function in the kidney or renal sensory nerves contributes to salt-sensitive hypertension.

Retrograde tracing is a widely used technique for tracing the neurons innervating specific visceral organ in DRG, which contains sensory neurons from variable sources (Gattone et al. 1986; Kandel ER 2000; Lu et al. 2001; Bossowska 2002). In my studies, I used FB as retrograde tracer to identify sensory neurons innervating kidney in DRG. The properties and regulation of TRPV1 channel in kidney-projecting sensory neurons were studied. My previous studies show that extracellular ATP reverses TRPV1 desensitization via $P2Y_2$ receptor-PKC dependent mechanism in kidney-projecting sensory neurons. SP and CGRP release from sensory nerves might be controlled by this regulatory mechanism. Previous studies have shown that TRPV1 expression and function in vivo are impaired in DS hypertensive rats (Wang et al. 2006). However, the function of TRPV1 in individual DRG neurons in DS hypertensive rats has not been

studied. Furthermore, facilitation of TRPV1 function in kidney-projecting sensory neurons by extracellular ATP on kidney-projecting sensory neurons from hypertensive rats has not been studied. Here I used a retrograde labeling technique to identify kidney-projecting sensory neurons enabling us to define the function of TRPV1 and its regulation in sensory neurons supplying kidney in DRG of DS rats.

RESULTS

Blood pressure in LS and HS fed rats. Systolic blood pressure in DS/HS rats began to increase within 1 week after starting the HS diet and reached a plateau at 2-3 weeks. Systolic blood pressure did not change significantly over the 3 weeks of measurements in the DS/LS group (Fig. 20) At 3 weeks systolic blood pressure in DS/HS rats was 186 $\pm$ 6 mmHg (n=7) compared with134$\pm$ 9 mmHg) (n=5, $P<0.01$) in DS/LS rats.

TRPV1 function is reduced in kidney-projecting sensory neurons from DS/HS rat. To test whether TRPV1 function is impaired in DS/HS hypertensive rats I constructed capsaicin concentration-response curves (0.1 -10 μM) for activation of TRPV1-mediated inward currents. As shown in Fig. 21, the amplitude of capsaicin-induced TRPV1 currents increased in a concentration dependent manner in both DS/LS and DS/HS rats. At 1, 3, and 10 μM, the capsaicin induced TRPV1 currents were significantly higher in kidney-projecting sensory neurons (n=18) from DS/LS rats compared with neurons (n=12, $P<0.05$) from DS/HS rats. The EC_{50} value for capsaicin responses in neurons from DS/LS rats was 0.45$\pm$0.1 μM (n=18), which was significantly lower than EC_{50} value obtained in neurons from DS/HS rats (0.9$\pm$0.4 μM n=12, $P<0.05$).

ATP mediated TRPV1 resensitization. Extracellular ATP reverses TRPV1 desensitization on kidney-projecting sensory neurons. ATP-induced TRPV1 resensitization was evaluated in kidney-projecting sensory neurons using whole cell patch clamp recordings in both DS/LS and DS/HS rats. In this study, capsaicin was applied at 2 minute intervals to induce TRPV1 desensitization. After TRPV1 was desensitized by

repeated applications of capsaicin (1 μM), neurons were treated with ATP (100 μM) for 6 seconds and capsaicin was applied again. As shown previously in Wistar rats, desensitization of TRPV1 was reversed by extracellular ATP (Fig. 22). In the kidney-projecting sensory neurons of DS/HS rats, I found that the TRPV1 current recorded after ATP pretreatment was significantly greater than that induced by first application of capsaicin ($n=5$, $P<0.05$) (Fig. 22B, C). ATP reversed capsaicin induced desensitization of TRPV1 in neurons ($n=5$) from DS/LS rats but the current recorded after ATP application was similar in amplitude to the original TRPV1 current (Fig. 22A, C).

In some neurons, capsaicin only caused an inward current after pretreatment of the neurons with extracellular ATP. There was a trend towards an enhancement of ATP mediated TRPV1 sensitization of these "silent" neurons in DS/HS rats compared with DS/LS rats. However, that enhancement was not statistically different (Fig. 23).

Distribution of subtypes of kidney-projecting sensory neurons identified by capsaicin responses. My previous data showed that four types of kidney-projecting sensory neurons could be discriminated based on the properties of capsaicin-induced inward currents in normal Wistar rats. The first type of neuron exhibited a desensitizing capsaicin response (Fig. 22A). The second type of neuron exhibited a "silent" capsaicin-induced response, in which TRPV1 only responded to CAP after pretreatment with ATP (Fig. 23A). The third type of neuron exhibited a non-desensitizing capsaicin response in which repeated capsaicin applications didn't cause obvious TRPV1 desensitization. The fourth type was TRPV1-negative in which capsaicin did not evoke an inward current even after ATP pretreatment. I found that in DS/LS rats, 71% of neurons (31 of 44) were desensitizing, 18% (8 of 44) were "silent", and 11% (5 of 44) were TRPV1-negative. In

DS/HS rats, 62% of neurons (21 of 34) were desensitizing, 29% (10 of 34) were "silent, and 9% (3 of 34) were TRPV1-negative. Non-desensitizing responses were not detected in both groups (Table 1). The proportions of the neuronal subsets were not different between the two groups.

Expression of TRPV1 and CGRP in kidney-projecting sensory neurons. Immunocytochemical studies were performed on kidney-projecting sensory neurons. TRPV1 and CGRP were co-localized in all FB-labeled DRG neurons. TRPV1 was predominantly expressed on cell membrane while CGRP was mainly localized intracellularly. There were no obvious differences in TRPV1 or CGRP staining in neurons from DS/LS and DS/HS rats (Fig. 24).

TRPV1 and $P2Y_2$ receptor expression on kidney-projecting sensory neurons. My previous studies showed that extracellular ATP reverses capsaicin-induced TRPV1 desensitization via an action at metabotropic $P2Y_2$ receptors on kidney-projecting sensory neurons. I compared $P2Y_2$ receptor expression between DS/LS and DS/HS rats using immunocytochemical staining. I found that $P2Y_2$ receptors were expressed on the cell membrane of FB-labeled sensory neurons in both groups. And immunostaining was similar in neurons from DS/LS and DS/HS rats (Fig. 25).

Expression of CGRP in sensory nerve fibers. Immunostaining of the marker of sensory nerve fibers (CGRP) surrounding renal interlobar arteries was performed. The staining density of CGRP positive sensory nerve fibers was remarkably less in DS/HS rats after 3 weeks high salt diet compared with DS/LS rats fed with low salt diet (Fig.

26A, B). At high power resolution, the staining of CGRP positive sensory nerve fibers was remarkably stronger in DS/LS rats compared with DS/HS rats (Fig. 26C, D).

DISCUSSION

This study was designed to determine whether TRPV1 function was altered in kidney-projecting sensory neurons of DS hypertensive rats. TRPV1 plays an important role in blood pressure regulation and hypertension (Wang 2005). The efferent function of TRPV1 positive sensory nerve fibers regulates kidney function through the release of vasodilators such as CGRP and SP (Maggi 1993). Desensitization and resensitization of TRPV1 controls SP and CGRP release. This TRPV1-mediated vascular regulatory mechanism functions in renal arteries and arterioles. So it is important to determine whether TRPV1 function and its regulation by ATP are impaired in renal sensory nerves of hypertensive rats.

In vivo studies have shown that capsaicin-induced depressor effects are impaired in DS/HS rats (Wang et al. 2006). High salt diet induces a decrease in GFR and renal excretory function in DS rats, which is caused by dysfunction of TRPV1 in the kidney (Li et al. 2008). In this study, capsaicin-induced TRPV1 currents were reduced in kidney-projecting sensory neurons in DS/HS hypertensive rats.

My data showed that ATP-mediated TRPV1 resensitization was enhanced in kidney-projecting sensory neurons of DS/HS hypertensive rats compared to DS/LS normotensive rats. ATP is a neurotransmitter in the nervous system (Phillis et al. 1975; White 1988) And it is also released from sympathetic nerve endings acting on vascular smooth muscle cells to induce vasoconstriction (Burnstock et al. 2000). My previous studies have shown that sensory and sympathetic nerves are in close proximity at the adventitial surface of the renal arteries. ATP released from sympathetic nerves could facilitate TRPV1 function on closely aligned sensory nerve fibers in the renal vasculature.

TRPV1 activation plays a compensatory role in preventing salt-induced increases in blood pressure via release of SP or CGRP from sensory nerve endings in DS rats (Wang et al. 2006). Enhanced resensitization of TRPV1 by ATP in kidney-projecting sensory neurons might be a compensatory mechanism to increase vasodilator release from renal sensory nerve endings. Vasodilation would promote increased renal blood flow and sodium and water excretion in DS/HS rats. It is also possible that long-term HS treatment might exceed the limit of this compensatory mechanism resulting in elevated blood pressure.

CGRP plays a compensatory role to attenuate elevated blood pressure in several animal models of hypertension (Supowit et al. 1997; Supowit et al. 1998). Decreased availability of CGRP in sensory neurons or sensory nerve fibers might attenuate compensatory depressor effect of CGRP during the development of hypertension. CGRP staining in kidney-projecting sensory neurons was similar between DS/LS and DS/HS rats. However, the density and intensity of CGRP-positive sensory nerve fibers surrounding renal interlobar arteries was reduced in DS/HS hypertensive rats. Previous studies have shown that the same reduction in CGRP-positive sensory nerve fibers is not observed in deoxycorticosterone acetate (DOCA)-salt hypertensive rats with similar blood pressure (Wang et al. 2006). This suggests that elevated blood pressure alone may not cause the disruption or loss of sensory nerve fibers. It is possible that reduced CGRP-positive sensory nerve fibers are caused by decreased CGRP availability instead of the loss of sensory nerve fiber. Therefore less CGRP-positive sensory nerve fibers were visualized in immunohistochemical studies. The reduced availability of CGRP in renal

sensory nerve fiber might cause decreased CGRP release upon activation of TRPV1, which contributes to impaired renal function in DS/HS hypertensive rats.

CGRP levels are reduced in sensory neurons of DRG in DS/HS hypertensive rats compared with DS/LS rats (Wang et al. 2006). However my immunocytochemical studies showed that the patterns of CGRP staining were similar in kidney-projecting sensory neurons between DS/LS rats and DS/HS rats. Other more sensitive methods, such as single neuron PCR, might be required to detect the difference on synthesis of CGRP in this subpopulation of sensory neurons innervating kidney.

Four types of kidney-projecting sensory neurons have been identified based on the properties of capsaicin-induced inward currents in kidney-projecting sensory neurons of normal Wistar rats. It is possible that changes in the proportion of these neurons might contribute to disruption of CGRP release from TRPV1 positive renal sensory nerves in response to sodium loading. However my study showed that the distributions of these different types of neurons were similar between DS/LS rats and DS/HS rats. No non-desensitizing sensory neurons were detected in either group. And the proportion of TRPV1-negative neurons was much higher in normal Wistar rats compared with DS/LS and DS/HS rats. DS strain is derived from Sprague-Dawley rats instead of Wistar rats. Therefore these differences might be caused by strain differences between DS rats and Wistar rats. Finally it is possible that distribution of different kidney-projecting sensory neurons does not contribute to the development of high blood pressure in DS/HS hypertensive rats.

DS hypertensive rats have been widely used for studies of salt-sensitive hypertension. Rennin-angiotensin-aldosterone system, endothelin-1 and oxidative stress

all contribute to increased salt sensitivity in DS rats (Kassab et al. 1997; Manning et al. 2003; Zhu et al. 2009). My findings suggest that salt-induced impairment of TRPV1 function and reduced CGRP immunostaining in sensory nerves innervating kidney might be a novel mechanism contributing salt-sensitivity of DS rats. In my study, I used DS/LS rats as a control to determine the function of TRPV1- positive renal sensory nerves. As DS/LS and DS/HS rats shares the same genetic background, high salt loading to DS rats might trigger the impairment of TRPV1 function and reduced CGRP expression during the development of hypertension in DS rats. My results provide a novel mechanism contributing to the development of salt-sensitive hypertension. A new strategy for the treatment of hypertension targeting TRPV1 and its regulation might be revealed.

Table 1: Distribution of different CAP response (%)

	Wistar	DS/LS	DS/HS
Desensitizing	35	71	62
Non-desensitizing	29	none	none
Silent	6	18	29
TRPV1-negative	30	11	9

Table 1. Distribution of different responses of kidney-projecting sensory neurons to CAP (1 μM). 189 neurons were studied in Wistar rats. 44 neurons were studied in DS/LS rats. 34 neurons were studied in DS/HS rats.

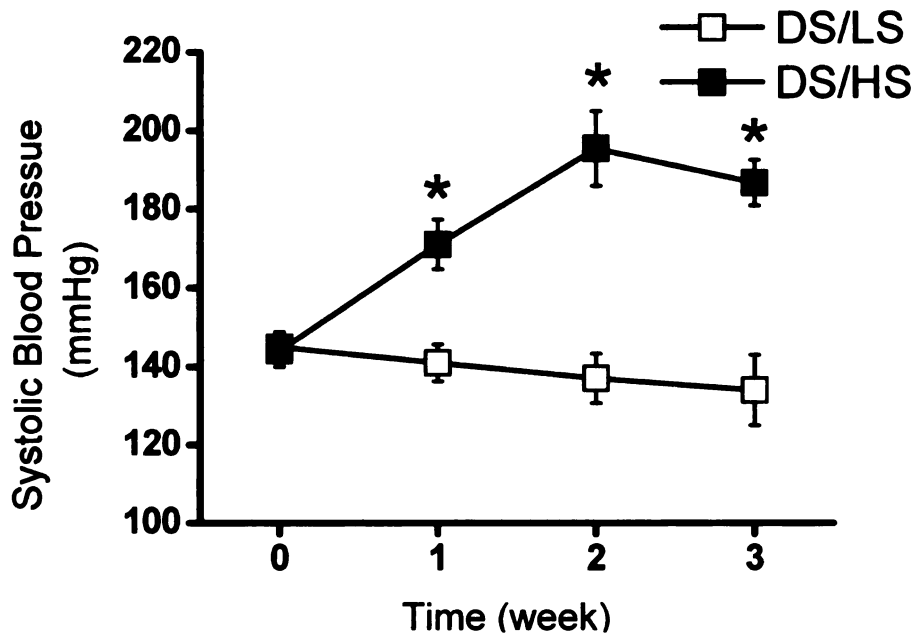


Fig. 20. Time course of increases in systolic blood pressure in DS/LS (n=5) rats and DS/HS rats (n=7) fed with either LS or HS diet for 3 weeks. Data are mean ± s.e.m. *, $P < 0.01$ vs. DS/LS rats.

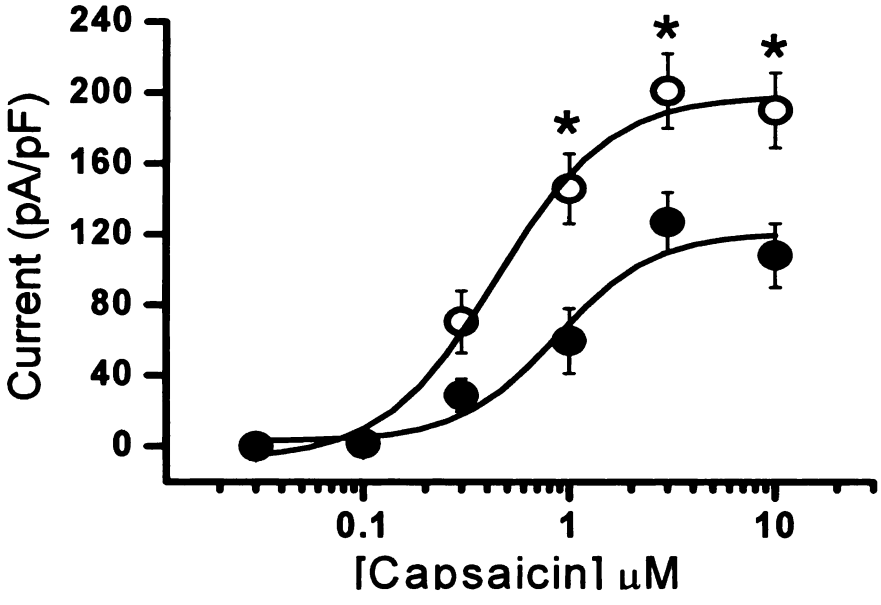


Fig. 21. Concentration-response curves for capsaicin-induced activation of TRPV1 on kidney-projecting sensory neurons in DS/LS and DS/HS rats fed with either low salt or high salt diet for 3 weeks. Data are mean ± s.e.m. of 18 neurons of DS/LS rats (open circle) and 12 neurons of DS/HS rats (closed circle). *, $P < 0.05$ vs. DS/HS rats.

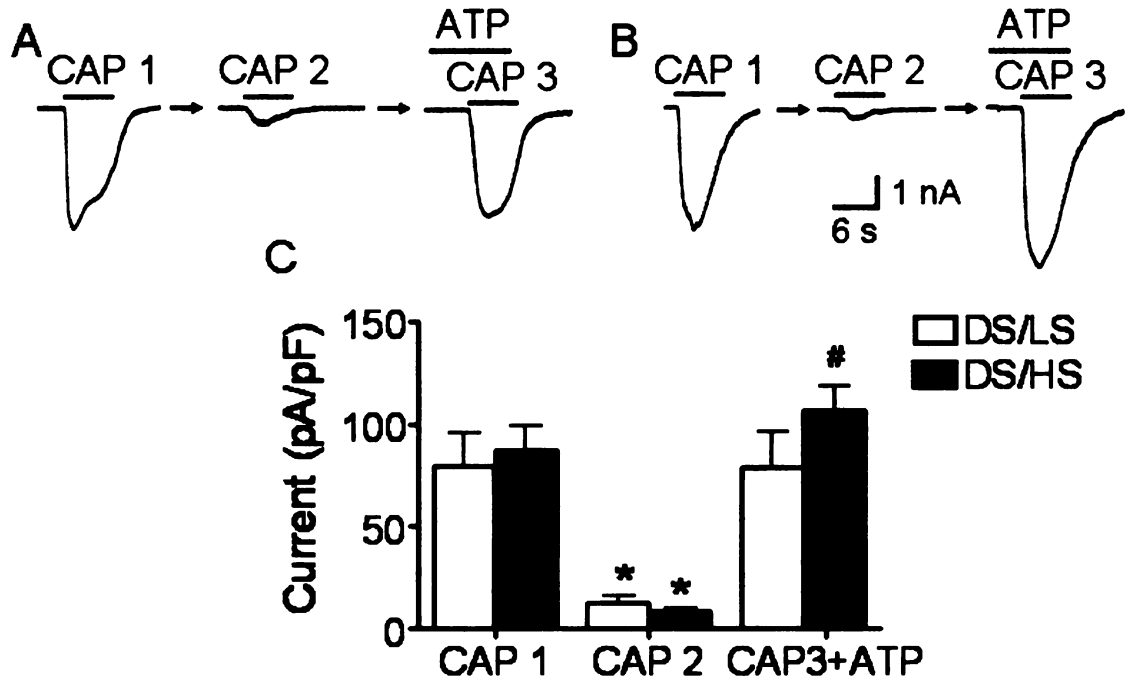


Fig. 22. ATP-induced TRPV1 resensitization on desensitizing neurons of kidney-projecting sensory neurons in DS/LS and DS/HS rats. *A*: Representative recordings showing that ATP reverses TRPV1 desensitization in DS/LS rats. *B*: Representative recordings showing that ATP reverses TRPV1 desensitization in DS/HS rats. *C*: Mean data of experiments illustrated in "A" and "B". Data are mean $\pm$ s.e.m. (n=5 neurons). *, $P < 0.01$ vs. first application of capsaicin in the same group. #, $P < 0.05$ vs. first application of capsaicin in the same group.

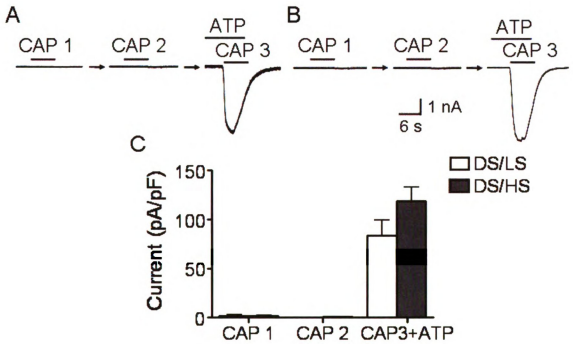


Fig. 23. ATP-induced TRPV1 resensitization on "silent" neurons of kidney-projecting sensory neurons in DS/LS and DS/HS rats. *A*: Representative recordings showing that ATP sensitizes TRPV1 in DS/LS rats. *B*: Representative recordings showing that ATP sensitizes TRPV1 in DS/HS rats. *C*: Mean data of experiments illustrated in "A" and "B". Data are mean $\pm$ s.e.m. (n=9 neurons in DS/LS rats, 5 neurons in DS/HS rats).

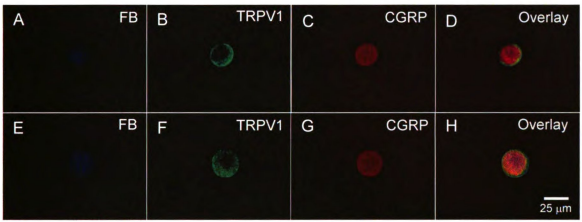


Fig. 24. Co-localization of TRPV1 and CGRP on kidney-projecting sensory neurons in DS/LS (A, B, C, D) and DS/HS (E, F, G, H) rats using fluorescence microscopy. *A, E*: Kidney-projecting sensory neuron labeled by FB (blue). *B, F*: TRPV1 immunostaining (green) on the same neurons shown in A and E. *C, G*: CGRP immunostaining (red) on the same neurons shown in A and E. *D, H*: Overlay picture.

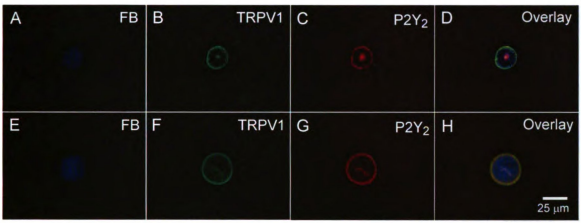


Fig. 25. Co-localization of TRPV1 and $P2Y_2$ receptors on kidney-projecting sensory neurons of DS/LS (A, B, C, D) and DS/HS (E, F, G, H) rats. *A*, *E*: Kidney-projecting sensory neurons labeled by FB (blue). *B*, *F*: TRPV1 immunostaining (green) on the same neurons shown in A and E. *C, G*: $P2Y_2$ receptor immunostaining (red) on the same neuron shown in A and E. *D, H*: Overlay pictures.

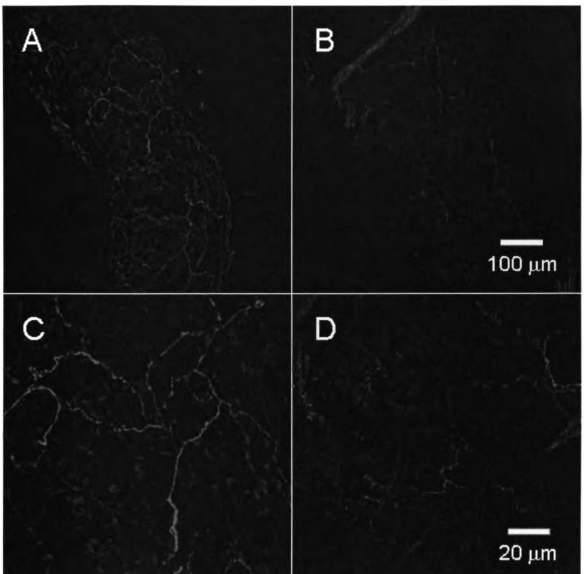


Fig. 26. Confocal microscopic images of CGRP immunostaining of sensory nerve fibers surrounding renal interlobar artery in DS/LS and DS/HS rats. *A*: CGRP immunostaining of sensory nerves associated with the renal artery in DS/LS rats. *B*: CGRP immunostaining of sensory nerves associated with the renal artery in DS/HS rats. *C*: High power image of A. *D*: High power image of B.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

TRPV1 is an ion channel expressed by sensory neurons and sensory nerve fibers whose contribution to pain pathology has been studied intensively (Caterina et al. 1997; Tominaga et al. 1998; Davis et al. 2000). Besides pain perception, TRPV1-positive nerve fibers also have a "sensory-efferent" function. Activation of TRPV1 induces release of vasodilator neuropeptides such as CGRP and SP (Maggi 1993). Recent data show that TRPV1 plays an important role in regulation of blood pressure and development of hypertension by activating the sensory-efferent release of vasodilator neuropeptides (Watson et al. 2002; Wang 2005).

TRPV1 and its involvement in normotensive and hypertensive animals have been studied. However, TRPV1 modulation by extracellular ATP on kidney-projecting sensory neurons has not been studied in normotensive or hypertensive animals. Here I used a retrograde labeling technique to identify kidney-projecting sensory neurons enabling us to define the function of TRPV1 and its regulation in sensory neurons supplying kidney in normal and salt-sensitive hypertensive rats. The results of my studies extend our knowledge of efferent function of sensory nerves, the regulation of TRPV1 channel, and the interaction between sympathetic and sensory nerves. These results are novel and important in understanding the characteristics of TRPV1 and its regulation in kidney-projecting sensory neurons.

There are several major conclusions could be drawn from the results of my studies.

1. ATP-induced TRPV1 resensitization is mediated by $P2Y_2$ receptors on kidney-projecting sensory neurons.
2. Facilitation of TRPV1 by ATP is mediated by intracellular PKC pathway in kidney-

projecting sensory neurons.

3. Four different CAP-induced responses can be detected in kidney-projecting sensory neurons.
4. Sympathetic and sensory nerve fibers are closely aligned surrounding renal arterioles. So it is possible that neurotransmitter released from sympathetic nerves may act on the sensory nerve fibers to modulate neuropeptide release from sensory nerves, and vice versa.
5. In DS hypertensive rats, the activity of TRPV1 on kidney-projecting sensory neurons is impaired in response to high salt loading.
6. High salt diet induces reduced CGRP immunoreactivity in sensory nerve fibers innervating kidney in DS rats.
7. ATP mediated TRPV1 resensitization is enhanced on kidney-projecting sensory neurons in response to high salt loading in DS rats.

P2Y receptor-mediated TRPV1 facilitation by ATP

The principal functions of neurons are to receive, modify, and transmit messages. The transmission of signals within one neuron or between different neurons depends on the electrical activity provided by ligand- and voltage-gated ion channels. So the changes in the responsiveness of a neuron must rely on the opening and closure of ion channels, and such effects can be mediated by G protein coupled receptors. For example, activation of $P2Y_2$ receptors inhibits both voltage-gated Ca^{2+} channel and K^+ channel in sympathetic neurons (Filippov et al. 1998).

Neuronal expressed P2Y receptors play important roles in regulation of synaptic transmission. The released neurotransmitters from presynaptic nerve terminals act on the corresponding receptors on postsynaptic membrane. The synaptic transmission could be modulated on either presynaptic side or postsynaptic side. P2Y receptors are expressed on both presynaptic and postsynaptic membranes in central and peripheral nervous system. However, in many cases, the P2Y subtypes involved in synaptic modulation are still not clearly demonstrated. In central nervous system, it has been reported that presynaptic P2Y receptors are involved in the inhibition of glutamate release in hippocampal neurons (Mendoza-Fernandez et al. 2000). In the medial habenula, presynaptic $P2Y_4$-like receptors are involved in the enhanced glutamate release, whereas $P2Y_2$-like receptors might be involved in inhibition of glutamate release (Price et al. 2003). It was also reported that postsynaptic activation of $P2Y_1$ receptor inhibits glutamate release in prefrontal and parietal cortex (Luthardt et al. 2003). In peripheral nervous system, the presynaptic P2 receptors have been reported to involve in the regulation of acetylcholine and norepinephrine release (Cunha et al. 2000). It has been reported that $P2Y_{12}$ or $P2Y_{13}$ receptors are involved in the autoinhibitory mechanism of neurotransmitter release from sympathetic neurons (Queiroz et al. 2003). In sensory neurons, $P2Y_1$ receptors are involved in the reduced glutamate release (Gerevich et al. 2004). It was also found that activation of $P2Y_1$ receptors increases the touch-induced action potentials in sensory nerve fibers (Nakamura et al. 1996). Furthermore, lines of evidence have shown that P2Y receptors are important in controlling the activities of

varieties of neuronal ion channels including voltage-gated calcium and potassium channels as well as ligand-gated ion channels (Hussl et al. 2006). So P2Y receptors play an important role in modulating neuronal excitability and synaptic transmission.

The function of TRPV1 mediated nociceptive pathways has been intensively studied (Caterina et al. 1997; Tominaga et al. 1998) and extracellular ATP modulates pain sensation either through ionotropic P2X receptors or metabotropic P2Y receptors expressed by sensory neurons (Sawynok et al. 1989; Moriyama et al. 2003). Extracellular ATP potentiates CAP-induced TRPV1 currents via activation of $P2Y_1$or $P2Y_2$ receptors (Tominaga et al. 2001; Moriyama et al. 2003). Which P2Y receptor subtype involves in TRPV1 resensitization has not been studied in kidney-projecting sensory neurons.

It has been reported that $P2Y_1$/$P2Y_2$ receptors are involved in potentiation of TRPV1 by ATP. So it is possible that resensitization effect of ATP on TRPV1 is also mediated by activation of $P2Y_1$ or $P2Y_2$ receptor on kidney-projecting sensory neurons. But I can not completely rule out the involvement of other P2Y receptors. Most of known P2Y receptor subtypes have been discovered in both central and peripheral nervous systems. Larger amount $P2Y_1$ and $P2Y_{11}$ mRNA levels have been detected in human brain compared with other tissue. But only low to moderate levels of $P2Y_2$, $P2Y_4$ and $P2Y_6$ are detected (Moore et al. 2001). $P2Y_{13}$ mRNA is also detected in cerebellum, hippocampus, substantia nigra, and thalamus (Communi et al. 2001). In rat sensory neurons, mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ has been detected (Ruan et al. 2003). The protein expressions of $P2Y_1$, $P2Y_2$ and $P2Y_4$ are found in sensory neurons. But only

the activities of $P2Y_1$ and $P2Y_2$ receptors have been reported on these neurons. The functions of other P2Y receptors expressed on sensory neurons are not clear (Hussl et al. 2006). So $P2Y_1$ and $P2Y_2$ receptors might be good candidates for studying the resensitization effect of ATP on TRPV1.

In my studies, repeated applications of CAP were performed on kidney-projecting sensory neurons to induce TRPV1 desensitization. Because no selective agonists or antagonists are available for P2Y receptors, alternative ways were applied in my studies to investigate the P2Y subtype involved in TRPV1 resensitization by ATP. Through pharmacological studies, I found that ATP resensitization effect on TRPV1 was mimicked by $P2Y_2$ receptor agonist, not by $P2Y_1$ agonist. And ATP-mediated resensitization was blocked by $P2Y_2$ antagonist, not by $P2Y_1$ antagonist. So I conclude that $P2Y_2$ receptors modulate TRPV1 activity on kidney-projecting sensory neurons.

To understand the mechanism underlying receptor interaction TRPV1 and $P2Y_2$ receptors, the intracellular signaling pathway was studied in kidney-projecting sensory neurons. My data showed that PKC activator reversed capsaicin-induced desensitization of TRPV1 as UTP. Both non-selective PKC inhibitor and selective PKC inhibitor blocked reversal effect of PMA and UTP. So I conclude that $P2Y_2$-mediated TRPV1 resensitization is caused by activation of intracellular PKC in kidney-projecting sensory neurons.

It has been well known that P2Y receptors play an important role in modulation of synaptic transmission. My studies broad the existing knowledge of function of $P2Y_2$

receptors in modulation of TRPV1 channels on kidney-projecting sensory neurons. The "sensory-efferent" function of TRPV1-positive sensory nerves is determined by the activation of TRPV1 channels. So the studies on regulatory mechanism of TPRV1 are important for understanding vasodilators release from TRPV1-positive sensory nerves. Once TRPV1 is desensitized, it can not be activated any more by subsequent application of TRPV1 agonist. This characteristic renders the TRPV1 unresponsive to noxious simulations at some pathophysiological conditions. But at physiological conditions, no noxious stimuli exist. Desensitization of TRPV1 attenuates the release of vasodilator release from TRPV1-positive sensory nerves. The resensitization effect of extracellular ATP may recover the release of vasodilators. So this ATP-mediated facilitation of TRPV1 function might play an important role in the modulation of "sensory-efferent" function of TRPV1-positive sensory nerves and the regulation of blood pressure.

Fast phosphorylation of TRPV1 by PKC

My results show that $P2Y_2$-mediated TRPV1 resensitization is caused by activation of intracellular PKC pathway in kidney-projecting sensory neurons. In my studies, several $P2Y_2$ and PKC agonist were tested to phosphorylate and resensitize TRPV1. And these agonists could resensitize TRPV1 within a few seconds. For intracellular PKC activation, several steps are involved including activation of G-protein coupled receptor, activation of PLC, dissociation of PIP_2, translocation of PKC, et al. To make it possible that resensitization occurs in a few seconds, the signaling components involved in these complicated steps must be very close to each other on the cell membrane or intracellular side.

How these signaling components are organized together for TRPV1 regulation is not clear. But it has been reported that many members of TRP superfamily exit in macromolecular assemblies composed of multiple signaling components. In Drosophila photoreceptor cell, a scaffolding protein called inactivation-no-afterpotential D (INAD) has been discovered. INAD binds directly to at least seven proteins, including TRPL, PLC, rhodopsin, PKC, calmodulin, and myosin, to form signaling complex named signalplex. These proteins function together in phototransduction (Shieh et al. 1996; Xu et al. 1998).

Mammalian TRPC channels seem also to be organized into macromolecular assemblies. It was reported that TRPC1 is localized to a subset of lipid rafts called caveolae, which might be a part of signaling complex. Lipid rafts are glycosphingolipid- and glycerol-enriched membrane microdomains that appear to organize certain transmembrane proteins together. Calveolin, a transmembrane cholesterol-binding protein that exists in caveolae, might serve as a scaffolding protein for binding of signaling components (Okamoto et al. 1998). TRPC1 is localized to calveolin-containing lipid rafts, in which association of IP_3 receptor and $G\alpha$ also exits (Lockwich et al. 2000). Other study has also shown that TRPC4 and TRPC5 bind to a macromolecular complex, which is similar to Drosophila signalplex (Tang et al. 2000).

The evidences above confirm the existence of macromolecular complex, which may facilitate the association of TRP channels with other intracellular signaling components. These protein complexes may be important for TRP channel localization, stability, and activity. It is also possible that they contribute to the speed and specificity in signaling transduction. Even though no evidence has been discovered for TRPV1

channel, it can not be excluded that a macromolecular complex also exits in TRPV1-positive kidney-projecting sensory neurons, which facilitate the intracellular signaling mechanism for rapid resensitization of TRPV1 by ATP.

TRPV1 and sympathetic nervous system

Sympathetic nervous system plays an important role in short-term and long-term blood pressure regulation (Boron et al. 2003). Sympathetic nerves innervating the splanchnic circulation are particularly important in blood pressure regulation (Kawasaki and Takenaga 2000). In the kidney, sympathetic nerves innervate afferent and efferent renal arterioles, proximal tubules, Henle's loop, and distal tubules (Boron et al. 2003) Therefore, sympathetic nerves play an important role in regulation of renal function including vascular hemokinetics, rennin release, and sodium/water excretion (Schrier 1974). Lines of evidence have shown that sympathetic nervous system is an initiating mechanism responsible for elevated arterial blood pressure in both animal models and human hypertension. In animal models of hypertension, such as spontaneous hypertensive rats, DOCA-salt hypertensive rats and Dahl salt-sensitive hypertensive rats, the increased sympathetic nerve activity has been detected, which may contribute to the development of hypertension through modulation of renal function (Oparil 1986; Somova et al. 1999).

The regulation of sympathetic nerve activity by sensory nervous system has been investigated. It has been reported that intravenous infusion of CGRP attenuates baroreceptor afferent nerve activity and enhances renal sympathetic efferent nerve activity in rabbit. And sinoaortic and vagal deafferentation abolished the effect of CGRP

(Okamoto et al. 1992). So it is possible that regulation of sympathetic nerve activity modulated by sensory nerves is mediated by baroreflex mechanism. The TRPV1 positive sensory neurons containing CGRP and SP send axons to both peripheral tissues and dorsal horn of spinal cord. These centrally projecting afferent sensory nerves synapse with intermediate cell column of the spinal cord, which contains the sympathetic preganglionic neurons. Therefore regulatory mechanism of sensory nerves on sympathetic nerve activity possibly occurs at the level of spinal cord. Other study has been shown that vasoconstriction induced by adrenergic nerve stimulation is potentiated by CAP-induced denervation of sensory nerves or by blockade of CGRP receptors in mesenteric vascular beds, which suggests that peripheral sensory nerves inhibit sympathetic nerve activity (Ralevic et al. 1995; Takenaga et al. 1999). It was also reported that genetic depletion of CGRP causes increased blood pressure and development of hypertension (Gangula et al. 2000) So TRPV1 may also plays a role in long-term regulation of blood pressure.

It has been found that pretreatment with subcutaneous CAP on newborn rat causes denervation of TRPV1-positive sensory nerves, which renders an adult rat salt-sensitivity and development of hypertension (Wang et al. 1998). In this hypertensive model, it was found that sympathectomy produced by administration of guanethidine attenuated development of salt-sensitive hypertension induced by sensory nerve denervation (Wang et al. 2001). This finding suggests that enhanced sympathetic nerve activity may contribute to the increase in blood pressure in CAP treated rats in response to high salt loading. And there might be a balance between antihypertensive effect of TRPV1-postive sensory nerves and prohypertensive effect of sympathetic nerves.

The evidences mentioned above demonstrate the relationship between sensory and sympathetic nervous system in regulation of blood pressure. But the underlying mechanisms still need to be elucidated. Because of desensitization of TRPV1 induced by prolonged or repeated agonist, the release of vasodilators from TRPV1-postive sensory nerves is attenuated even if the continuous existence of agonists. My immunohistochemical study showed that sympathetic nerves are closely aligned with sensory nerves on the renal lobar artery. This is the anatomical evidence for the interaction between sympathetic nerves and sensory nerves surrounding renal arteries. My studies demonstrate a novel mechanism that extracellular ATP resensitizes TRPV1 to facilitate vasodilators release. The major source of ATP might be from closely aligned sympathetic nerve fibers. ATP from sympathetic nerves causes vasoconstriction, whereas CGRP and SP from sensory nerves cause vasodilation. Therefore renal vascular homeostasis is possibly maintained by the cross-talk between sympathetic and sensory nerves at physiological conditions (Fig. 27).

In DS hypertensive rats, I tested the resensitization effect of ATP on TRPV1 in kidney-projecting sensory neurons from DS rats. I found that ATP-mediated TRPV1 resensitization was enhanced in response to high salt loading. This is possibly a compensatory effect in DS rats to counteract the elevated blood pressure via release of vasodilators from TRPV1-postive sensory nerves. The underlying mechanism for enhanced ATP resensitization effect is unknown, but several possibilities might be involved. First, the $P2Y_2$ receptor expression might be elevated in kidney-projecting sensory neurons, which causes over activation of intracellular PKC pathway to phosphorylate desensitized TRPV1. But I didn't detect any difference of $P2Y_2$ receptor

expression through immunocytochemical studies on kidney-projecting sensory neurons between DS/LS and DS/HS rats. More accurate quantitative studies, such as western blot or PCR, should be performed to reveal this possible difference. Second, the intracellular signaling pathway might be enhanced upon activation by ATP. I found that activation of PKC pathway is involved in the TRPV1 resensitization by ATP in kidney-projecting sensory neurons. Several factors are involved in PKC signaling pathway, such as G protein, PIP_2, PLC, et al. The overexpression of any these factors may cause increased phosphorylation and resensitization of TRPV1.

In my study, the enhanced ATP resensitization effect was found in isolated sensory neurons in DS hypertensive rats. The possible changes of ATP release from perivascular sympathetic nerves surrounding renal vasculature is not determined. ATP as a cotransmitter from sympathetic nerves plays an important role in blood pressure regulation. The role of ATP varies depending on tissue beds and species being examined. But in general, it appears that ATP has its greatest role in mediating vascular tone in small blood vessels like tertiary branches of mesenteric blood vessels and arterioles in kidney (Gitterman et al. 2001; Inscho 2001) . The role of ATP released from sympathetic nerves in development of hypertension has not been clearly demonstrated. Recent study has discovered that ATP-mediated purinergic neurotransmission to kidney arterioles is impaired in a model of hypertension induced by chronic angiotensin II infusion (Zhao et al. 2005). Another study reported that ATP-mediated purinergic neurotransmission is reduced surrounding mesenteric arteries of DOCA-salt hypertensive rats because of decreased ATP bioavailability in sympathetic nerves (Demel et al. 2008). These data highlight the potential importance of impaired purinergic regulation of arterial tone in the

development of hypertension. The purinergic neurotransmission of mesenteric or renal arteries in DS hypertensive rats has not been studied. I can not exclude the possibility that ATP release from sympathetic nerves innervating kidney is also reduced in DS hypertensive rats. If this happens, resensitization of TRPV1 will be attenuated in response to less ATP release from adjacent sympathetic nerves. Even though enhanced TRPV1 resensitization by exogenous applied ATP was discovered in kidney-projecting sensory neurons in DS hypertensive rats, the further studies on purinergic neurotransmission in this hypertensive model still need to be performed.

CGRP in DS hypertension

As I mentioned in introduction section, a few neurotransmitters are released from sensory nerve endings upon activation of TRPV1. CGRP and SP are potent vasodilators released among these neurotransmitters. The distribution of CGRP containing sensory neurons in DRG is much higher than SP containing sensory neurons in rats (Doutova et al. 1996). So CGRP is considered the prominent vasodilator in TRPV1-positice sensory nerves. Recently studies have shown that CGRP plays an important role in modulating total peripheral resistance of systemic circulation and development of hypertension (Deng et al. 2005).

Even though CGRP in hypertension has been widely studied, the role of CGRP in different models of hypertension is still controversial. The evidence has shown that plasma concentration of CGRP in patients with uncomplicated essential hypertension is decreased, which contributes to the development of hypertension (Portaluppi et al. 1992). However, in patients with hypertension secondary to phaeochromocytoma or primary

aldosteronism, plasma concentration is elevated. A marked decrease in mean arterial pressure and increase in plasma concentration of CGRP were observed in patients with adrenalectomy, which indicates that elevated CGRP might be a compensatory response to elevated blood pressure after surgery (Masuda et al. 1992).

In contrast to hypertension in humans, the role of CGRP in hypertensive animal models have also been studied. It has been reported that age-related decrease in neuronal expression of CGRP in SHR and decrease in DRG content of CGRP in DS hypertensive rats could contribute to the development and maintenance of hypertension (Supowit et al. 1993; Katki et al. 2001). In my studies, I found that staining of CGRP-positive sensory nerve fibers surround renal interlobar arteries was reduced in DS hypertensive rats. The reasons for the reduced CGRP staining are not known, but may be due to: (1) the synthesis of CGRP in kidney-projecting sensory neurons was reduced in response to high salt loading in DS rats; (2) compensatory release of CGRP counteracting elevated blood pressure caused depletion of CGRP in sensory nerves; (3) transportation of CGRP from sensory neuron soma to sensory nerve endings was inhibited in DS rats with high salt diet. Which mechanism is involved in the decreased CGRP expression is still not clear.

In other hypertensive animal models, controversial results about the role of CGRP are found. It was reported that acute administration of CGRP receptor antagonist CGRP8-37 potentiates the elevation of blood pressure in DOCA salt-sensitive hypertension and SN-salt hypertension. The synthesis and release of CGRP are increased in DOCA salt-sensitive hypertension, but not in SN-salt hypertension (Supowit et al. 1997; Supowit et al. 1998). Further studies indicate that in SN-salt hypertension vascular sensitivity to CGRP is enhanced to lower elevated blood pressure (Watson et al. 2002). In two-kidney,

one-clip (2K1C) hypertensive rats, in which one renal artery is restricted by clip, increased circulating CGRP and CGRP content in DRG are discovered (Deng et al. 2003). In these hypertensive animal models, it seems like the increase in blood pressure dose not reduce the synthesis and release of CGRP. The elevated CGRP content and release from sensory nerves seem to be secondary compensatory effect to depress elevated blood pressure in the development of hypertension.

The studies mentioned above suggest that CGRP plays an important role in the development of hypertension. But its function varies in different hypertensive animal models. In genetic hypertension, such as SHR and Dahl salt-sensitive rats, the decrease of CGRP content in DRG and plasma CGRP might be genetically determined. Lower availability of CGRP attenuates the vasodilation effect of TRPV1-positive sensory nerves in these models, which may contribute to the elevated blood pressure. In acquired hypertension, such as DOCA salt-sensitive, SN-salt, and 2K1C hypertensive rats, increased CGRP level or vascular sensitivity to CGRP seems to be secondary from elevated blood pressure. And CGRP plays a compensatory depressor role in these models (Fig. 28).

TRPV1 in DS hypertension

DS hypertensive rats have been widely investigated as one of the principle salt-sensitive hypertensive models. High salt loading triggers the development of hypertension in this strain that is genetically predisposed to salt sensitivity. The involvement of renin-angiotensin-aldosterone system, endothelin-1 and oxidative stress has been reported in DS hypertensive rats. (Kassab et al. 1997; Manning et al. 2003; Zhu

et al. 2009). A novel mechanism contributing to development of hypertension in hypertensive animal models has been discovered in recent years. As "sensory-efferent" function of TRPV1-positve sensory nerves, TRPV1 plays a compensatory role in preventing elevated blood pressure (Watson et al. 2002; Wang 2005). It is reported that TRPV1 function and expression is impaired in kidney contributing to decreased renal excretory function in DS hypertensive rats (Wang et al. 2006; Li et al. 2008). My data showed that the function of TRPV1 on kidney-projecting sensory neurons was impaired in DS hypertensive rats using whole cell patch clamp recording. My results indicate that high salt-induced impairment of TRPV1 function in sensory nerves innervating kidney might be a novel mechanism contributing to reduced renal excretory function and salt-sensitivity of DS rats.

In my studies, four different CAP-induced responses were discovered in kidney-projecting sensory neurons of normal Wistar rats. I proposed that these different functional states of TRPV1 might reflect the capabilities of the release of neuropeptides from TRPV1-positive sensory nerves. However, the distribution of these different types of kidney-projecting sensory neurons was not different in DS/HS hypertensive rats compared with DS/LS rats. So the distribution of these neurons might not contribute to the development of hypertension in DS rats.

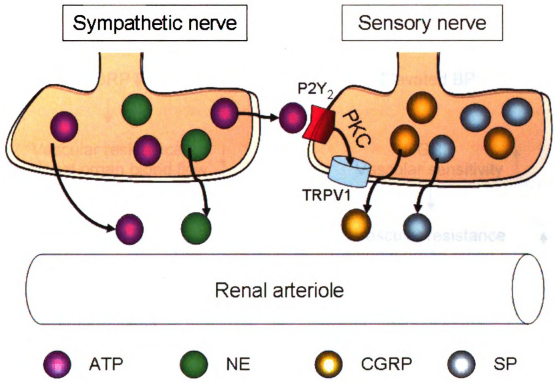


Fig. 27. Schematic representation of the mechanisms involved in the facilitation of TRPV1 activity by ATP released from sympathetic nerves via $P2Y_2$ receptors on sensory nerves. ATP release from sympathetic nerves either causes vasoconstriction directly or acts on $P2Y_2$ receptors on sensory nerves. Resensitization or sensitization effects of ATP on TRPV1 via PKC pathway facilitate vasodilators release from sensory nerves surrounding renal arterioles.

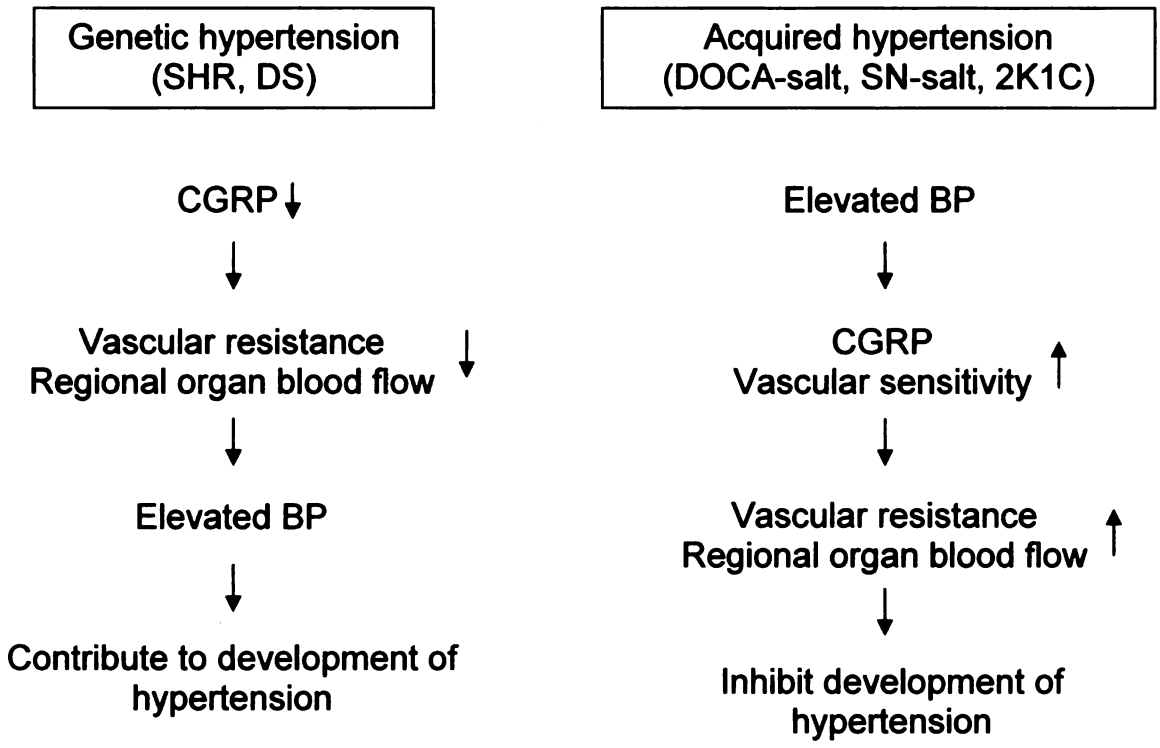


Fig. 28. Proposed role of CGRP in genetic and acquired hypertension.

Perspective

My studies first demonstrate the ATP-induced TRPV1 resensitization in kidney-projecting sensory neurons is mediated by activation of $P2Y_2$ receptors and intracellular PKC pathway. The possible source of extracellular ATP for TRPV1 resensitization might be from closely aligned sympathetic nerve fibers on renal artery. In the studies of DS rats, my findings suggest that salt-induced impairment of TRPV1 function or CGRP synthesis in renal sensory neurons or nerve fibers may be genetically predisposed in DS rats, which leads to withdrawing of the protective mechanisms in face of salt challenging. Hypertension is a multifactor disease. My studies might reveal a novel mechanism contributing to the development of salt-sensitive hypertension and may advance our knowledge about blood pressure and renal function regulation in hypertension research. A new strategy for the treatment of salt-sensitive hypertension targeting TRPV1 and its regulation might be revealed.

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