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(54) Title: G PROTEIN-RELATED KINASE MUTANTS IN ESSENTIAL HYPERTENSION

(57) Abstract

The present invention provides diagnostic tests in which to identify individuals predisposed to essential hypertension, and the genetic, cellular and biochemical tools in which to carry out these tests. The present invention also provides methods of screening substances for anti-hypertensive properties in which to facilitate drug discovery for antihypertensive agents. A wide variety of tools are provided for these purposes as well. The present invention further provides compositions and methods for normalizing sodium transport in kidney cells of individuals having essential hypertension.

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G PROTEIN-RELATED KINASE MUTANTS IN ESSENTIAL HYPERTENSION

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Technical Field

The present invention relates to essential hypertension, and more particularly to the use of genetic markers in diagnostic and therapeutic approaches to this disease.

Background Art

Essential hypertension, or high blood pressure of unknown cause, is a disease that affects 25-30% of Caucasians in The United States. Left untreated, hypertension leads to heart disease, stroke, myocardial infarction, and end-stage kidney disease. Since hypertension patients do not generally feel sick, it is often undiagnosed and left untreated until end organ failure has begun. Thus hypertension is the leading cause of cardiovascular morbidity and mortality in humans. Many hypertensives are salt sensitive in that a high salt diet will cause an elevation in blood pressure or exacerbate an already elevated blood pressure. Finding a measure for the propensity to develop high blood pressure could have a significant impact on reducing cardiovascular disease.

It has been estimated that genetic factors account for 30-40% of blood pressure variability in humans (Ward R. (1990). In Hypertension: Pathophysiology, Diagnosis and Management, Laragh JH. And Brenner BM eds., (Raven Press, Ltd., New York, NY), pp 81-100.) However, other estimates have suggested that genetic heritability of hypertension may be as high as 80% with 40% accounted for by one major gene (Cavalli Sporza LL., Boomer WF. In The Genetics of Human Population (1971), (WH Freeman Co., South San Francisco, CA) pp 534-536.) The single major gene could effect blood pressure to such a significant extent that it would dominate many other genes that play a minor role in blood pressure control.

The central role of the kidneys in the genesis and maintenance of hypertension has been well established. When normal kidneys are transplanted into

hypertensive rats, their blood pressure is normalized. On the other hand, when kidneys from hypertensive rats are transplanted into normotensive rats, they develop hypertension. Thus hypertension seems to follow the kidneys. It is also known that most human genetic forms of hypertension are associated with enhanced reabsorption of sodium in the kidney. Although there are many hormonal systems that regulate renal sodium excretion and blood pressure, the renal paracrine function of dopamine is well established as an important mechanism in long-term blood pressure regulation. The increased avidity of the renal proximal tubule for sodium in hypertension may be caused by defective renal paracrine action of dopamine. Dopamine causes a decrease in sodium reabsorption. Thus a defect in the action of dopamine would lead to an increase in sodium reabsorption and hypertension.

Dopamine exerts its actions via a class of cell surface receptors that belong to the rhodopsin-like family of G protein coupled receptors; these receptors have in common 7 transmembrane domains. The dopamine receptors in the CNS and some endocrine organs are grouped into two major classes, the D1-like and the D2-like receptors. In the kidney and other organs outside the CNS, the D1-like receptors have been called DA1 receptors while the D2-like receptors have been called DA2 receptors. These distinctions are probably no longer necessary since no dopamine receptor is expressed exclusively inside or outside the CNS. However, there is differential regulation of the D1 receptor in neural and renal tissue. The two exons of the D1 receptor gene are transcribed in neural tissue while only the second exon is transcribed in renal tissue. The differential expression of the short and long D1 transcript may be due to tissue-specific expression of an activator protein driving transcription from a promoter at the 5' non-coding region of the D1 receptor gene. Each of the D2-like dopamine receptor subtypes has several isoforms. However, no particular isoform is specifically expressed in peripheral tissues. See, Jose, et. al., Pharmac. Ther. 80:149-182 (1998).

Two D1-like receptors are expressed in mammals: the D1 and D5 receptors which are known as D1A and D1B in rodents, respectively. Two additional D1-like receptors, D1C and D1D, are expressed in non-mammalian species. The D1-like receptors are linked to stimulation of adenylyl cyclase. The D1A receptor also stimulates phospholipase C activity, but this is secondary to

stimulation of adenylyl cyclase. There seems to be a D1-like receptor, that is, as yet uncloned, linked to phospholipase C (PLC), through a pertussis toxin insensitive G-protein, Gq, that is distinct from the D1 and D5 receptor (Jose et al., Pharmacol Ther 80:149-182 (1998). Three D2-like receptors are expressed in mammals: the D2, D3, and D4 receptors. The D2-like receptors are linked to inhibition of adenylyl cyclase and Ca2+ channels. The D2-like receptors also stimulate K+ channels although the D2 and D3 receptors have been reported to decrease voltage dependent potassium current in NG108-15 cells. Both the D2 and D3 receptors present in presynaptic nerves may also serve to decrease the release of both dopamine and norepinephrine.

All the mammalian dopamine receptors, initially cloned from the brain, have been found to be expressed in the kidney and urinary tract. Dopamine receptor subtypes are differentially expressed along the renal vasculature, the glomerulus, and the renal tubule where they regulate renal hemodynamics and electrolyte and water transport as well as renin secretion. Exogenous dopamine, at low doses, decreases renal vascular resistance and increases renal blood flow but with variable effects on glomerular filtration rate. Additional renal effects include an increase in solute and water excretion caused by hemodynamic and tubular mechanisms. The ability of renal proximal tubules to produce dopamine and the presence of receptors in these tubules suggest that dopamine can act in an autocrine or paracrine fashion. Endogenous renal dopamine increases solute and water excretion by actions at several nephron segments (proximal tubule, medullary thick ascending limb of Henle (mTAL), cortical collectingduct (CCD)). The magnitude of the inhibitory effect of dopamine on each nephron segment is modest but the multiple sites of action along the nephron cause impressive increases in solute and water excretion. The renal effects of dopamine are most apparent under conditions of solute (e.g. sodium, phosphate) or protein load. D1-like receptors, probably of the D1 subtype, vasodilate the kidney, inhibit sodium transport in proximal tubules by inhibition of sodium/hydrogen exchanger activity at the luminal membrane and sodium/potassium ATPase activity at the basolateral membrane. D1-like receptors also decrease sodium transport in the mTAL and in the CCD. The major functional D1-like receptor in the kidney is the D1 receptor. Presynaptic D2-like receptors

are also vasodilatory. Postsynaptic D2-like receptors, by themselves, stimulate renal proximal sodium transport and inhibit the action of vasopressin at the CCD. However, in concert with D1-like receptors, postsynaptic D2-like receptors may act synergistically to inhibit sodium transport in the renal proximal tubule. The major D2-like receptor in the proximal tubule is the D3 receptor while the major D2-like receptor in the CCD is the D4 receptor. The ability of postsynaptic D2-like receptors, probably of the D3 subtype, to inhibit renin secretion may counteract the stimulatory effect of D1-like receptors on renin secretion and contribute to their synergistic action to increase sodium excretion in sodium replete states (Jose et al., supra).

In conclusion, although many years of intensive effort have revealed much about the etiology of essential hypertension, a single major gene that controls blood pressure has not been found. Thus the discovery of a major gene associated with blood pressure regulation would be important for understanding the mechanisms causing essential hypertension and lead to important new diagnostics and therapeutics.

Summary of the Invention

Kinases are enzymes that catalyze the addition of a phosphate group onto proteins. G protein-coupled receptor kinases (GRKs) are a family of protein kinases that phosphorylate G protein-coupled receptor proteins on serine and threonine residues. GRKs, along with other proteins called arrestins, mediate homologous desentisitization of hormonal responses. See, *Premont, et al.*, FASEB J. 9:175-182 (1995). Six GRKs have been identified, i.e., GRK1-GRK6. See, *Premont, et al., supra.*; *Palczewski*, Protein Sci. 3:1355-1361 (1994); and *Inglese, et al.*, J. Biol. Chem. 268:23735-23738 (1993). GRK4 had been the least well understood member of the GRK family. *Premont et al.*, J. Biol. Chem. 271:6403-6410 (1996), determined its presence substantially in testis, and thus is the least distributed of any GRK except GRK1. Although the Premont publication acknowledges that it was not known as to which specific type of testis cell expressed GRK4, it speculates that GRK4 could bind to any one of a number of receptors, including the LH/CG receptor, the gonadotropin-releasing hormone receptor, and follicle-stimulating hormone receptor and a variety of olfactory

receptors. Later, *Gros*, J. Clin. Invest. 99(9):2087-2093 (1997), implicated GRK2 activity in reduced adenylyl cyclase activation in lymphocytes from hypertensive individuals. Gros also observed that the increase in GRK activity was associated exclusively with an increase in GRK2 expression, and that the activity of other GRKs was not altered.

Applicants have made several important discoveries. First, GRK4 isoform expression occurs to a significant extent in the kidney, and specifically in renal proximal tubule and cortical collecting duct cells. Second, Applicants discovered that several known polymorphic forms of GRK4, and three more previously unknown polymorphs, are prevalent in hypertensive individuals. Third, the D1 receptor/adenylyl cyclase coupling defect in renal proximal tubule cells known associated with essential hypertensive individuals is associated with but not limited to hyperphosphorylation of the D1 receptor.

Commercial embodiments of Applicants' invention fall into three primary areas, namely diagnostics, drug discovery and therapy. Accordingly, a first aspect of the present invention is directed to methods for identifying individuals predisposed to essential hypertension. The methods can be conducted using a sample of kidney cells that express a D1 receptor and GRK4, isolated from the individual, wherein the cells are assayed to determine the extent of post-translational modification of the D1 receptor, such as phosphorylation or palmitoylation, wherein a change in the post-translational modification of the receptor relative to cells isolated from a normotensive individual is indicative of predisposition to essential hypertension. Alternatively, a nucleic acid sample is isolated from the individual in order to analyze a GRK gene or fragment thereof to detect GRK4 associated with essential hypertension. Specific mutants that applicants have identified as being associated with essential hypertension include the following: R65L, A142V, A486V, the two double mutants R65L, A486V, and R65L, A142V, and the triple mutant R65L, A142V, A486V. Identifying yet other mutant GRK4s associated with essential hypertension can be conducted simply by analyzing GRK4s genes isolated from individuals diagnosed with essential hypertension, and analyzing the sequence of the GRK4 gene. The applicants further

demonstrated that expression of these GRK4s in non-renal cells cause these non-renal cells to fail to "properly" to normally transduce a dopaminergic signal.

A related aspect of the present invention is directed to isolated and purified nucleic acids encoding a GRK4 protein having an R65L, A142V double mutation, an R65L, A486V double mutation, or an R65L, A142V, A486V triple mutation. Oligonucleotides which specifically hybridize to GRK4 gene fragments containing the aforementioned mutations are also disclosed. Further disclosed are oligonucleotide primers, or primer pairs, which hybridize to fragments of the GRK4 gene containing a mutation associated with essential hypertension. Preferred primers which specifically hybridize to exon 3, 5, 8, 14 or 16 of a GRK4 gene and which is useful in amplifying DNA sequences including nucleotides 431-503 (exon 3), 594-697 (exon 5), 857-995 (exon 8), 1662-1798 (exon 14) or 1937-1991 (exon 16) of the GRK4 gene.

Another aspect of the present invention is related to various systems in which to test substances for anti-hypertensive activity by their ability to effect a change in GRK4 conformation and/or activity. These systems range from complexes between a GRK4 protein, e.g., wild-type or an isoform or mutant that is associated with essential hypertension, and an agent that causes a conformational change of the GRK4 protein upon interaction with an anti-hypertensive agent to be detected, to reconstituted systems containing GRK4 and a GRK4 substrate. Any system in which the interaction between GRK4 and a GRK4 substrate can be measured can be used to screen for potential anti-hypertensive agents. Thus, the systems range from cell-like parts such as an artificial membrane, e.g., lipid miscelle, to whole cells. Preferred whole cells include cells transfected with a D1 receptor gene (or a functional fragment thereof) and a wild-type or mutant GRK4 gene, and immortalized human proximal tubule cells. Changes in GRK4 activity that occur in these various systems can be detected by measuring pertubations in cell activity such as any second messenger component or endpoint such as (but not limited to) cAMP generated by adenylyl cyclase, G protein activity, sodium transporter or pump activity, and post-translational modifications such as phosphorylation and palmitoylation. *In vivo* systems such as transgenic animals containing a transgene encoding a GRK4 protein associated with essential hypertension, wherein the

transgene is expressed in renal cells to cause the transgenic animal to exhibit a state of essential hypertension, are also disclosed.

Yet another aspect of the present invention is directed to methods for decreasing sodium transport (increasing natriuresis) in renal proximal tubule cells *in vitro* or *in vivo*. The basic objectives of these therapeutic applications are to change GRK4 activity. One preferred method involves administration of an agent or agents that reduce or prevent expression of the GRK4s in renal cells of the hypertensive individual. GRK4 mRNA or DNA can be attacked with oligonucleotides such as antisense RNA or dominant negative mutants that prevent transcription or translation. Ribozymes that cleave GRK4 mRNA or pre-mRNA are also useful. Other therapeutic applications include drugs that alter e.g., inhibit or enhance, the activity of GRK4 (either inhibition or stimulation).

Without being bound by any particular theory of operation, Applicants believe that a renal defect is responsible for a certain portion of hypertension in human subjects, and that the GRK4 mutation either causes among other things, a direct or indirect ligand independent serine-hyperphosphorylation of the D1 receptor, resulting in its uncoupling from the G protein/effector complex. The result is that the natriuretic effect of dopamine is compromised and the kidney is unable to properly balance sodium and water, leading to sodium retention and elevated blood pressure. More specifically, renal proximal tubules obtained from human hypertensive subjects, but not from normotensive subjects, demonstrate a defective coupling of the dopamine D1 receptor with adenylyl cyclase. The defective coupling is associated with a ligand-independent phosphorylation of the D1 receptor. Applicants have discovered at least six mutated genes in G protein related kinase type 4 (GRK4), that regulate ligand-independent phosphorylation of the D1 receptor in hypertensive patients.

Brief Description of the Drawings

Fig. 1 is a graph that shows that a D1-like agonist stimulates GRK activity in renal proximal tubule cells from hypertensive but not from normotensive subjects;

Fig. 2 is a graph that show that prevention of the expression of GRK4 restores to normal values the ability of renal proximal tubule cells from hypertensive subjects to increase cAMP production caused by a D1-like agonist;

Fig. 3 is a graph that shows that the phosphorylation of D1 receptor in the resting proximal tubule cell which is greater in hypertensive subjects than in normotensive subjects does not respond to D1-like agonist stimulation. The phosphorylation of the D1 receptor can be abrogated if GRK4 expression is prevented;

Fig. 4 is a graph that shows an increase in GRK4gamma/ð expression in renal proximal tubules in response to D1-like agonist stimulation in hypertensive but not in normotensive subjects; and

Fig. 5 is a graph that shows that mutations of GRK4gamma decrease the ability of the D1 receptor to respond to D1-like agonist stimulation in Chinese hamster ovary cells made to hyper express GRK4gamma and D1 receptor.

Best Mode of Carrying Out Invention

The structure of the human GRK4 gene transcript undergoes extensive alternative splicing to generate four distinct forms of GRK4 mRNA that encode four forms of the GRK4 protein. The alternative splicing occurs at the amino- and/or carboxyl-terminal regions of GRK4, giving rise to the four isoforms.

GRK4 is originally reported in *Ambrose, et al.*, Hum. Mol. Genet. 1:697-703 (1993), and then more extensively characterized in *Premont et al.*, J. Biol. Chem. 271(11):6403-6410 (1996). Premont reports that GRK4 is highly abundant in testis only, GRK4 mRNA being present to a small extent in brain and skeletal muscle. The GRK4 gene, exclusive of promoter regions, spans approximately 75 kilobases (kDa), and is composed of 16 exons. The longest form of GRK4, with intact amino- and carboxyl-terminal alternative exon sequences, has been designated GRK4alpha. The deduced protein sequence contains 578 amino acids, with a predicted molecular mass of 66.5 kDa. The next shorter form, GRK4beta, lacks only the amino-terminal alternative exon, which is composed of codons, and thus contains 546 amino acids having a molecular mass of 62.kDa. GRK4gamma is the isoform lacking only the carboxyl-terminal alternative exon, which is 46 codons. Thus, this isoform contains 532 amino acids, and has a

predicted molecular mass of 61.2 kDa. GRK4gamma was formally called GRK4A. See *Sallese et al.*, Biochem. Biophys. Res. Commun. 199:848-854 (1994). GRK4delta contains 500 amino acids with a predicted molecular mass of 57.6kDA, and is the shortest isoform. It lacks both alternative exons. GRKdelta was originally designated IT11 and GRK4B. See *Sallese et al., supra.*, and *Ambrose, et al., supra.* More recently, two additional isoforms have been discovered, namely: GRK4epsilon which lacks exons 13 and 15,contains 466 amino acids with a predicted molecular mass of 53.6 kDa, and GRK4zeta which lacks exons 2, 13 and 15, contains 434 amino acids with a predicted molecular mass of 49.9 kDa.

Five single nucleotide polymorphisms of GRK4 are also known, namely: R65L (CGT to CTT); A142V (GCC to GTC); V247I (GTA to ATA); A486V (GCG to GTG) and D562G (GAC to GGC). See *Premont, et al., supra.* Applicants have discovered that the R65L, A142V and the A486V polymorphisms are associated with essential hypertension. Applicants have also discovered three additional polymorphisms prevalent in hypertensive individuals, namely: the double mutants R65L, A142V and R65L, A486V; and the triple mutant R65L, A142V, A486V. Table 1 shows the amino acid and corresponding nucleotide sequences of the six GRK4 isoforms. Amino acids and corresponding nucleotides that are changed in the polymorphs associated with essential hypertension are shown in bold.. The sequences of the 5' untranslated regions of the epsilon and Zeta isoforms are not shown.

Table 1

MELENIVANS	LLLKARQGGY	GKKSGRSKKW	KEILTLPPVS	QCSELRHSIE	50	**GRK4α**
MELENIVANS	LLLKARQ----	----------	----------	----------E		**GRK4β**
MELENIVANS	LLLKARQGGY	GKKSGRSKKW	KEILTLPPVS	QCSELRHSIE		**GRK4γ**
MELENIVANS	LLLKARQ----	----------	----------	----------E		**GRK4δ**
MELENIVANS	LLLKARQGGY	GKKSGRSKKW	KEILTLPPVS	QCSELRHSIE		**GRK4ε**
MELENIVANS	LLLKARQ----	----------	----------	----------E		**GRK4ζ**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD	100	**GRK4α**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD		**GRK4β**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD		**GRK4γ**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD		**GRK4δ**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD		**GRK4ε**
KDYSSLCDKQ	PIGR**R**LFRQF	CDTKPTLKRH	IEFLDAVAEY	EVADDEDRSD		**GRK4ζ**
CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	K**A**FEECTRVA	150	**GRK4α**
CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	K**A**FEECTRVA		**GRK4β**
CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	K**A**FEECTRVA		**GRK4γ**
CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	K**A**FEECTRVA		**GRK4δ**
CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	K**A**FEECTRVA		**GRK4ε**

CGLSILDRFF	NDKLAAPLPE	IPPDVVTECR	LGLKEENPSK	KAFEECTRVA		GRK4ζ
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE	200	GRK4α
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE		GRK4β
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE		GRK4γ
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE		GRK4δ
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE		GRK4ε
HNYLRGEPFE	EYQESSYFSQ	FLQWKWLERQ	PVTKNTFRHY	RVLGKGGFGE		GRK4ζ
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL	250	GRK4α
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL		GRK4β
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL		GRK4γ
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL		GRK4δ
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL		GRK4ε
VCACQVRATG	KMYACKKLQK	KRIKKRKGEA	MALNEKRILE	KVQSRFVVSL		GRK4ζ
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL	300	GRK4α
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL		GRK4β
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL		GRK4γ
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL		GRK4δ
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL		GRK4ε
AYAYETKDAL	CLVLTIMNGG	DLKFHIYNLG	NPGFDEQRAV	FYAAELCCGL		GRK4ζ
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV	350	GRK4α
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV		GRK4β
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV		GRK4γ
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV		GRK4δ
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV		GRK4ε
EDLQRERIVY	RDLKPENILL	DDRGHIRISD	LGLATEIPEG	QRVRGRVGTV		GRK4ζ
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ	400	GRK4α
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ		GRK4β
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ		GRK4γ
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ		GRK4δ
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ		GRK4ε
GYMAPEVVNN	EKYTFSPDWW	GLGCLIYEMI	QGHSPFKKYK	EKVKWEEVDQ		GRK4ζ
RIKNDTEEYS	EKFSEDAKSI	CRMLLTKNPS	KRLGCRGEGA	AGVKQHPVFK	450	GRK4α
RIKNDTEEYS	EKFSEDAKSI	CRMLLTKNPS	KRLGCRGEGA	AGVKQHPVFK		GRK4β
RIKNDTEEYS	EKFSEDAKSI	CRMLLTKNPS	KRLGCRGEGA	AGVKQHPVFK		GRK4γ
RIKNDTEEYS	EKFSEDAKSI	CRMLLTKNPS	KRLGCRGEGA	AGVKQHPVFK		GRK4δ
RIKNDTEEYS	EKFSEDAKSI	CRM----------	----------	----------		GRK4ε
RIKNDTEEYS	EKFSEDAKSI	CRM----------	----------	----------		GRK4ζ
DINFRRLEAN	MLEPPFCPDP	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY	500	GRK4α
DINFRRLEAN	MLEPPFCPDP	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY		GRK4β
DINFRRLEAN	MLEPPFCPDP	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY		GRK4γ
DINFRRLEAN	MLEPPFCPDP	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY		GRK4δ
----------	----------P	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY		GRK4ε
----------	----------P	HAVYCKDVLD	IEQFSAVKGI	YLDTADEDFY		GRK4ζ
ARFATGCVSI	PWQNEMIESG	CFKDINKSES	EEALPLDLDK	NIHTPVSRPN	550	GRK4α
ARFATGCVSI	PWQNEMIESG	CFKDINKSES	EEALPLDLDK	NIHTPVSRPN		GRK4β
ARFATGCVSI	PWQNE---------	----------	----------	----------		GRK4γ
ARFATGCVSI	PWQNE---------	----------	----------	----------		GRK4δ
ARFATGCVSI	PWQNE---------	----------	----------	----------		GRK4ε
ARFATGCVSI	PWQNE---------	----------	----------	----------		GRK4ζ
RGFFYRLFRR	GGCLTMVPSE	KEVEPKQC			578	GRK4α
RGFFYRLFRR	GGCLTMVPSE	KEVEPKQC			556	GRK4β
----------	-GCLTMVPSE	KEVEPKQC			532	GRK4γ
----------	-GCLTMVPSE	KEVEPKQC			510	GRK4δ

---------- -GCLTMVPSE KEVEPKQC 466 GRK4ε
---------- -GCLTMVPSE KEVEPKQC 434 GRK4ζ

Note: The bolded letters indicate the change in amino acid associated with hypertension R to L (argnine to leucine), A to V (alanine to valine), and A to V (alanine to valine).

Nucleotide sequence:

1 gcagccgccg cggtcgggct gccccctccc ctcgccccga ccgctcccct gctggtgagg **GRK4α**
gcagccgccg cggtcgggct gccccctccc ctcgccccga ccgctcccct gctggtgagg **GRK4β**
gcagccgccg cggtcgggct gccccctccc ctcgccccga ccgctcccct gctggtgagg **GRK4γ**
gcagccgccg cggtcgggct gccccctccc ctcgccccga ccgctcccct gctggtgagg **GRK4δ**

61 gcctgcgcag gcggcggcgg cggcgccctt ggtggcagtg gtggcggcgg agcagcctcc **GRK4α**
gcctgcgcag gcggcggcgg cggcgccctt ggtggcagtg gtggcggcgg agcagcctcc **GRK4β**
gcctgcgcag gcggcggcgg cggcgccctt ggtggcagtg gtggcggcgg agcagcctcc **GRK4γ**
gcctgcgcag gcggcggcgg cggcgccctt ggtggcagtg gtggcggcgg agcagcctcc **GRK4δ**

121 cgggatcgtg tctggagctc gaggagaggg tagtgcccgg cgagctatgc acgggggcgg **GRK4α**
cgggatcgtg tctggagctc gaggagaggg tagtgcccgg cgagctatgc acgggggcgg **GRK4β**
cgggatcgtg tctggagctc gaggagaggg tagtgcccgg cgagctatgc acgggggcgg **GRK4γ**
cgggatcgtg tctggagctc gaggagaggg tagtgcccgg cgagctatgc acgggggcgg **GRK4δ**

181 cggcgtctcc tcctgttccg cctcctcagt ctcctcggtc tcgcagaatc cgccggcggc **GRK4α**
cggcgtctcc tcctgttccg cctcctcagt ctcctcggtc tcgcagaatc cgccggcggc **GRK4β**
cggcgtctcc tcctgttccg cctcctcagt ctcctcggtc tcgcagaatc cgccggcggc **GRK4γ**
cggcgtctcc tcctgttccg cctcctcagt ctcctcggtc tcgcagaatc cgccggcggc **GRK4δ**
exon 1

241 ggcggcgcca ggac**atg**gag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4α**
ggcggcgcca ggac**atg**gag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4β**
ggcggcgcca ggac**atg**gag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4γ**
ggcggcgcca ggac**atg**gag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4δ**
atggag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4ε**
atggag ctcgagaaca tcgtggccaa ctcgctgctg ctgaaagcgc **GRK4ζ**
exon 2

301 gtcaaggagg atatggcaaa aaaagtggtc gtagtaaaaa atggaaggag atactgacac **GRK4α**
gtcaa-------- ---------- ---------- ---------- ---------- ---------- **GRK4β**
gtcaaggagg atatggcaaa aaaagtggtc gtagtaaaaa atggaaggag atactgacac **GRK4γ**
gtcaa-------- ---------- ---------- ---------- ---------- ---------- **GRK4δ**
gtcaaggagg atatggcaaa aaaagtggtc gtagtaaaaa atggaaggag atactgacac **GRK4ε**
gtcaa-------- ---------- ---------- ---------- ---------- ---------- **GRK4ζ**
exon 3

361 tgcctcctgt cagccagtgc agtgagctta gacattccat tgaaaaggat tatagcagtc **GRK4α**
---------- ---------- ---------- ----------gaaaaggat tatagcagtc **GRK4β**
tgcctcctgt cagccagtgc agtgagctta gacattccat tgaaaaggat tatagcagtc **GRK4γ**
---------- ---------- ---------- ----------gaaaaggat tatagcagtc **GRK4δ**
tgcctcctgt cagccagtgc agtgagctta gacattccat tgaaaaggat tatagcagtc **GRK4ε**
---------- ---------- ---------- ----------gaaaaggat tatagcagtc **GRK4ζ**

421 tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4α**
tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4β**
tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4γ**

tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4δ**
tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4ε**
tttgtgacaa gcaaccgata ggaagacgtc tcttcaggca gttctgtgat accaaaccca **GRK4ζ**

exon 4

481 ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4α**
ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4β**
ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4γ**
ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4δ**
ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4ε**
ctctaaagag gcacattgaa ttcttggatg cagtggcaga atatgaagtt gccgatgatg **GRK4ζ**

exon 5

541 aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4α**
aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4β**
aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4γ**
aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4δ**
aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4ε**
aggaccgaag tgattgtgga ctgtcaatct tagatagatt cttcaatgat aagttggcag **GRK4ζ**

601 ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4α**
ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4β**
ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4γ**
ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4δ**
ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4ε**
ccccttacc agaaatacct ccagatgttg tgacagaatg tagattggga ctgaaggagg **GRK4ζ**

exon 6

661 agaacccttc caaaaaagcc tttgaggaat gtactag agt tgcccataac tacctaagag **GRK4α**
agaacccttc caaaaaagcc tttgaggaat gtactagagt tgcccataac tacctaagag **GRK4β**
agaacccttc caaaaaagcc tttgaggaat gtactagagt tgcccataac tacctaagag **GRK4γ**
agaacccttc caaaaaagcc tttgaggaat gtactagagt tgcccataac tacctaagag **GRK4δ**
agaacccttc caaaaaagcc tttgaggaat gtactagagt tgcccataac tacctaagag **GRK4ε**
agaacccttc caaaaaagcc tttgaggaat gtactagagt tgcccataac tacctaagag **GRK4ζ**

721 gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4α**
gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4β**
gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4γ**
gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4δ**
gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4ε**
gggaaccatt tgaagaatac caagaaagct catattttc tcagtttta caatggaaat **GRK4ζ**

exon 7

781 ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4α**
ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4β**
ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4γ**
ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4δ**
ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4ε**
ggctggaaag gcaacccgta acaaagaaca catttagaca ttacagagtt ctaggaaaag **GRK4ζ**

exon 8

841 gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4α**
gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4β**
gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4γ**
gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4δ**

gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4ε**
gcggatttgg agaggtttgc gcctgtcaag tgcgagccac aggaaaaatg tatgcctgca **GRK4ζ**

901 aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4α**
aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4β**
aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4γ**
aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4δ**
aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4ε**
aaaagctaca aaaaaaaaga ataaagaaga ggaaaggtga agctatggct ctaaatgaga **GRK4ζ**

exon 9

961 aaagaattct ggagaaagtg caaagtagat tcgtagttag tttagcctac gcttatgaaa **GRK4α**
aaagaattct ggagaaagtg caaagtagat tcgtagttag tttagcctac gcttatgaaa **GRK4β**
aaagaattct ggagaaagtg caaagtagat tcgtagttag tttagcctac gcttatgaaa **GRK4γ**
aaagaattct ggagaaagtg caaagtagat tcgtagttag tttagcctac gcttatgaaa **GRK4δ**
aaagaattct ggagaaagtg caaagtagat tcgtagttag tttagcctac gcttatgaaa **GRK4ε**
aaagaattct ggagaaagtg caaagtagat tcatagttag tttagcctac gcttatgaaa **GRK4ζ**

1021 ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4α**
ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4β**
ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4γ**
ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4δ**
ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4ε**
ccaaagatgc cttgtgcttg gtgctcacca ttatgaatgg aggggatttg aagtttcaca **GRK4ζ**

1081 tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4α**
tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4β**
tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4γ**
tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4δ**
tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4ε**
tttacaacct gggcaatccc ggctttgatg agcagagagc cgttttctat gctgcagagc **GRK4ζ**

exon 10

1141 tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4α**
tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4β**
tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4γ**
tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4δ**
tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4ε**
tgtgttgcgg cttggaagat ttacagaggg aaagaattgt atacagagac ttgaagcctg **GRK4ζ**

exon 11

1201 agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4α**
agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4β**
agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4γ**
agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4δ**
agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4ε**
agaatattct ccttgatgat cgtggacaca tccggatttc agacctcggt ttggccacag **GRK4ζ**

exon 12

1261 agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4α**
agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4β**
agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4γ**
agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4δ**
agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4ε**
agatcccaga aggacagagg gttcgaggaa gagttggaac agtcggctac atggcacctg **GRK4ζ**

1321 aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4α**
aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4β**
aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4γ**
aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4δ**
aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4ε**
aagttgtcaa taatgaaaag tatacgttta gtcccgattg gtggggactt ggctgtctga **GRK4ζ**

1381 tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4α**
tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4β**
tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4γ**
tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4δ**
tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4ε**
tctatgaaat gattcaggga cattctccat tcaaaaaata caaagagaaa gtcaaatggg **GRK4ζ**

1441 aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4α**
aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4β**
aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4γ**
aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4δ**
aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4ε**
aggaggtcga tcaaagaatc aagaatgata ccgaggagta ttctgagaag ttttcagagg **GRK4ζ**

exon 13

1501 atgccaaatc tatctgcagg atgttactca ccaagaatcc aagcaagcgg ctgggctgca **GRK4α**
atgccaaatc tatctgcagg atgttactca ccaagaatcc aagcaagcgg ctgggctgca **GRK4β**
atgccaaatc tatctgcagg atgttactca ccaagaatcc aagcaagcgg ctgggctgca **GRK4γ**
atgccaaatc tatctgcagg atgttactca ccaagaatcc aagcaagcgg ctgggctgca **GRK4δ**
atgccaaatc tatctgcagg atg--------- ---------- ---------- ---------- **GRK4ε**
atgccaaatc tatctgcagg atg--------- ---------- ---------- ---------- **GRK4ζ**

1561 ggggcgaggg agcggctggg gtgaagcagc accccgtgtt caaggacatc aacttcagga **GRK4α**
ggggcgaggg agcggctggg gtgaagcagc accccgtgtt caaggacatc aacttcagga **GRK4β**
ggggcgaggg agcggctggg gtgaagcagc accccgtgtt caaggacatc aacttcagga **GRK4γ**
ggggcgaggg agcggctggg gtgaagcagc accccgtgtt caaggacatc aacttcagga **GRK4δ**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ε**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ζ**

exon 14

1621 ggctggaggc aaacatgctg gagccccctt tctgtcctga tcctcatgcc gtttactgta **GRK4α**
ggctggaggc aaacatgctg gagccccctt tctgtcctga tcctcatgcc gtttactgta **GRK4β**
ggctggaggc aaacatgctg gagccccctt tctgtcctga tcctcatgcc gtttactgta **GRK4γ**
ggctggaggc aaacatgctg gagccccctt tctgtcctga tcctcatgcc gtttactgta **GRK4δ**
---------- ---------- ---------- ---------- -cctcatgcc gtttactgta **GRK4ε**
---------- ---------- ---------- ---------- -cctcatgcc gtttactgta **GRK4ζ**

1681 aggacgtcct ggatatcgag cagttctcgg cggtgaaagg gatctacctg gacaccgcag GRK4"ð
aggacgtcct ggatatcgag cagttctcgg cggtgaaagg gatctacctg gacaccgcag GRK4$ð
aggacgtcct ggatatcgag cagttctcgg cggtgaaagg gatctacctg gacaccgcag GRK4(ð
aggacgtcct ggatatcgag cagttctcgg cggtgaaagg gatctacctg gacaccgcag GRK4*ð
---------- ---------- ---------- ---------- -cctcatgcc gtttactgta GRK4,ð
---------- ---------- ---------- ---------- -cctcatgcc gtttactgta GRK4.ð

exon 15

1741 atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatgaga GRK4"ð
atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatgaga GRK4$ð
atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatga-- GRK4(ð

atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatga_ - GRK4*ð
atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatga-- GRK4,ð
atgaagactt ctatgctcgg tttgctaccg ggtgtgtctc catcccctgg cagaatga-- GRK4.ð

1801 tgatcgaatc cgggtgtttc aaagacatca acaaaagtga aagtgaggaa gctttgccat **GRK4α**
tgatcgaatc cgggtgtttc aaagacatca acaaaagtga aagtgaggaa gctttgccat **GRK4β**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4γ**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4δ**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ε**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ζ**

1861 tagatctaga caagaacata cataccccgg tttccagacc aaacagaggc ttcttctata **GRK4α**
tagatctaga caagaacata cataccccgg tttccagacc aaacagaggc ttcttctata **GRK4β**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4γ**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4δ**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ε**
---------- ---------- ---------- ---------- ---------- ---------- **GRK4ζ**

exon 16

1921 gactcttcag aagagggggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4α**
gactcttcag aagagggggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4β**
---------- ----------gggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4γ**
---------- ----------gggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4δ**
---------- ----------gggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4ε**
---------- ----------gggc tgcctgacca tggtccccag tgagaaggaa gtggaaccca **GRK4ζ**

1981 agcaatgctg agcaccccgg tgcggaccac agagcagacc ctggcgccag gaaggagcat **GRK4α**
agcaatgctg agcaccccgg tgcggaccac agagcagacc ctggcgccag gaaggagcat **GRK4β**
agcaatgctg agcaccccgg tgcggaccac agagcagacc ctggcgccag gaaggagcat **GRK4γ**
agcaatgctg agcaccccgg tgcggaccac agagcagacc ctggcgccag gaaggagcat **GRK4δ**
agcaatgctg a **GRK4ε**
agcaatgctg a **GRK4ζ**

2041 gtgttagcgt ctcgtcccac ctggaattgt aataaataca tctaaataaa acatgccttg **GRK4α**
gtgttagcgt ctcgtcccac ctggaattgt aataaataca tctaaataaa acatgccttg **GRK4β**
gtgttagcgt ctcgtcccac ctggaattgt aataaataca tctaaataaa acatgccttg **GRK4γ**
gtgttagcgt ctcgtcccac ctggaattgt aataaataca tctaaataaa acatgccttg **GRK4δ**
GRK4ε
GRK4ζ

2101 ggagtgtaca gac **GRK4α** (1857 bp, 16 exons)
ggagtgtaca gac **GRK4β** (1761 bp, 15 exons, no exon 2)
ggagtgtaca gac **GRK4γ** (ð (1719 bp, 15 exons, no exon 15)
ggagtgtaca gac **GRK4δ** (1623 bp, 14 exons, no exon 2 & 15)
GRK4ε (1581 bp, 14 exons, no exon 13 & 15)
GRK4ζ (1487 bp, 13 exons, no exon 2, 13, & 15)

Note:

The bolded **atg** represents the start of translation.

The bolded and shaded nucleotides represent the polymorphic sites associated with hypertension g to t (exon 3), c to t (exon 5), and c to t (exon 14)

The exons are depicted by an underline and a double underline.

The nucleotides at 1989 to 1981 represent as stop codon.

A first aspect of Applicants' invention is directed to methods of screening individuals at risk for or who are susceptible or predisposed to essential hypertension. Essential hypertension is defined as hypertension of unknown etiology. Unlike some hypertensive diseases which have been fully characterized, there had been no known cause for essential hypertension. The identification of the association or relationship between the GRK4 gene, its basic functions and interaction with the D1 receptor, and essential hypertension allows for the screening of individuals to determine if they have a genetic basis for their measured high blood pressure or a predisposition to this disease if they present with a normal blood pressure. In the case of patients present with normal blood pressure (there are a variety of conditions that lead to false low blood pressure readings), but who also have clinical evidence for hypertension (such as end organ disease), the genetic screen for hypertensive mutations can be used to confirm the presence of hypertension. Thus, the individuals who are identified as predisposed to essential hypertension can then have their blood pressure more closely monitored and be treated, such as by way of diet modification, at an earlier time in the course of the disease.

. One such diagnostic method entails isolating kidney cells having a D1 receptor and which express GRK4, from the individual. Kidney cells useful for conducting this method include renal proximal tubule cells and cortical collecting duct cells. They may be conveniently obtained from urine samples. The extent of the post-translational modification of the D1 receptor in the cells is then measured. A change in post-translational modification of the D1 receptor relative to cells isolated from a normotensive individual is believed to be caused by a change in GRK4 activity, and in turn is indicative of predisposition to essential hypertension. Several post-translational events may occur within such cells, including palmitoylation and phosphorylation. The D1 receptor in such cells isolated from a hypertensive individual exhibit what is known as hyperphosphorylation. By this term, it is meant that the amount of D1 receptors with attached phosphorus molecule is increased. Post-translational modifications can be detected and

measured in accordance with standard techniques, such as immunoprecipitation of the D1 receptor with a D1 receptor antibody and immunoblotted against phosphoserine antibody, or labelling the cells with radioactive palmitic acid and immunoprecipitation with with a D1 receptor antibody (Ng et al., Eur. J. Pharmacol. *267*:7-19 (1994)).

Another such method entails obtaining a nucleic acid sample, e.g., DNA or RNA, from an individual and analyzing the nucleic acid sequence of the GRK4 gene of the individual for a mutation, whereby the presence of the mutation is indicative of predisposition of the individual to essential hypertension. The nucleic acid sample can be obtained from any cell type because GRK4 DNA is ubiquitous. The extraction of DNA from blood is a particularly suitable source. Referring to GRK4α numbering, preferred GRK4 mutants that are identified in this method include Arg→Leu at amino acid residue 65 (R65L), Ala→Val at amino acid residue 142 (A142V), Ala→Val at amino acid residue 486 (A486V), the double mutant R65L, A142V and R65L, A486V, and the triple mutant R65L, A142V, A486V. GRK4 alleles may be screened for mutations associated with essential hypertension directly or following cloning. Cloning can be connected using conventional techniques, e.g., by digesting genomic DNA into appropriate fragment sizes, and ligating the resulting fragments into a vector. On the other hand, polymerase chain reactions (PCRs) may be performed with primers for specific exons, e.g., exons 3, 5, 8, 14 and 16, of the GRK4 gene. Examples of such primers are set forth in Table 2. PCR can be formed on any sequence of the wild-type or mutant GRK4. PCR can also be performed on the GRK4 mRNA. Thus, those skilled in the art will appreciate that primers or primer pairs for the amplification of GRK4 alleles may be designed based on either nucleotide sequences identical in all isoforms and polymorphisms (as shown in Table 1), or they may be based on sequences that include the specific nucleotide substitution that results in the activating mutation. Other primers useful in practicing this aspect of the invention will amplify a DNA sequence including nucleotides 431-503 (exon 3), nucleotides 594-697 (exon 5), nucleotides 857-995 (exon 8), nucleotides 1662-1798 (exon 14), and nucleotides 1937-1991 (exon 16).

Table 2

Sequences of GRK4 primers (5' to 3')*.

Exon	Direction	Sequence
3	Forward	33 - AAAAGGATTATAGCAGTCTTTGTGACAA - 60
	Reverse	118 - CACTGCATCCAAGAATTCAATGTGCCTC - 143
5	Forward	35 - CTAATGGTTATGTATTTGGTT - 55
	Reverse	183 - ATGCAGGGCTCAGCATGA - 200
8	Forward	92 - AGGTGGACATAAACCTCC - 109
	Reverse	292 - CAAACAATGCACAGTGAAG - 309
14	Forward	65 - CCTCATGCCGTTTACTGTAAGGACGTCC - 92
	Reverse	176 - CTCATTCTGCCAGGGGATGGAGACACAC - 203
16	Forward	90 - GCATCAGCCGTGTGCCT - 106
	Reverse	297 - GTGCAGAAGGTCTGTACA - 314

*** GenBank Accession #U33153 to U33168**

The GRK4 alleles are tested for the presence of nucleic acid sequence different from the normal alleles by determining the nucleotide sequence of the cloned allele or amplified fragment and comparing it to the nucleotide sequence of the normal allele. Other known methods offer a more complete, yet somewhat indirect test for confirming the presence of an activating allele. These methods include single-stranded confirmation analysis, (SSCA), denaturing gradient gel electrophoresis (DGGE), RNase protection assays, allele-specific oligonucleotides (ASOs), the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein, and allele-specific PCR. These methods are disclosed in *Orita et al.*, Proc. Nat. Acad. Sci. USA *86*:2766-2770 (1989); *Sheffield et al.*, Proc. Nat. Acad. Sci. USA *86*:232-236 (1989); *Finkelstein et al.*, Genomics 7:167-172 (1990), and *Kinszler et al.*, Science *251*:1366-1370 (1991); *Conner et al.*, Proc. Nat. Acad. Sci. USA *80*:278-282 (1983); *Modrich*, Ann. Rev. Genet. *25*:229-253 (1991); and *Rano & Kidd*, Nucl. Acids Res. *17*:8392 (1989), respectively. For allele-specific PCR, primers are used which hybridize at their 3' ends to a particular GRK4 mutation. If the GRK4 mutation is not present, an amplication product is not detected. Detection of amplification product may be conducted by Amplification Refractory Mutation System (ARMS), as disclosed in EPA0332435.

In the first three methods (SSCA, DGGE and RNase protection assay), a new electorphoertic band appears. SSCA detects a band which migrates differentially because the sequence change causes a difference in single-strand

intramolecular base pairing. RNase protection involves cleavage of the mutant polynucleotide into two or more smaller fragments. DGGE detects differences in migration rates of mutant sequences compared to wild-type sequences, using a denaturing gradient gel. In an allele-specific oligonucleotide assay, an oligonucleotide is designed which detects a specific sequence, and the assay is performed by detecting the presence or absence of a hybridization signal. In the mutS assay, the protein binds only to sequences that contain a nucleotide mismatch in a heteroduplex between mutant and wild-type sequences.

Mismatches, according to the present invention, are hybridized nucleic acid duplexes in which the two strands are not 100% complementary. Lack of total homology may be due to deletions, insertions, inversions or substitutions. Mismatch detection can be used to detect point mutations in the gene or in its mRNA product. While these techniques are less sensitive than sequencing, they are simpler to perform on a large number of samples. An example of a mismatch cleavage technique is the RNase protection method. In the practice of the present invention, the method involves the use of a labeled riboprobe which is complementary to the human wild-type GRK4 gene coding sequence. The riboprobe and either mRNA or DNA isolated from the tumor tissue are annealed (hybridized) together and subsequently digested with the enzyme RNase A which is able to detect some mismatches in a duplex RNA structure. If a mismatch is detected by RNase A, it cleaves at the site of the mismatch. Thus, when the annealed RNA preparation is seperated on an electrophoretic gel matrix, if a mismatch has been detected and cleaved by RNase A, an RNA product will be seen which is smaller than the full length duplex RNA for the riboprobe and the mRNA or DNA. The riboprobe need not be the full length of the GRK4 mRNA or gene but can be a segment of either. If the riboprobe comprises only a segment of the GRK4 mRNA or gene, it will be desirable to use a number of these probes to screen the whole mRNA sequence for mismatches.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., *Cotton et al.*, (1988), Proc. Natl. Acad.Sci. USA 85:4397; *Shenk et al.*, (1975), Proc. Natl. Acad. Sci. USA 72:989; and *Novack et al.*, (1986), Proc. Natl. Acad. Sci. USA 83:586.

Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., *Cariello*, (1988), Human Genetics 42:726. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR before hybridization. Changes in DNA of the GRK4 gene can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the GRK4 gene which have been amplified by use of PCR may also be screened using allele-specific probes. These probes are nucleic acid oligomers, each of which contains a region of the GRK4 gene sequence harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the GRK4 gene sequence. By use of a battery of such allele-specific probes, PCR amplification products can be screened to identify the presence of a previously identified mutation in the GRK4 gene. Hybridization of allele-specific probes with amplified GRK4 sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the DNA sample as in the allele-specific probe. Examples of such allele-specific probes are set forth in Table 3.

Table 3: Sequences of *GRK4* allele specific oligonucleotides (5' to 3').

Nucleotide	Direction	Sequence
G448	Reverse	CCTGAAGAGACGTCTTCCTA
448T	Reverse	CCTGAAGAGAAGTCTTCCTA
C679	Forward	CCAAAAAAGCCTTTGAGGA
679T	Forward	CCAAAAAAGTCTTTGAGGA
G993	Forward	AGTAGATTCGTAGTAAGTG
993A	Forward	AGTAGATTCATAGTAAGTG
C1711	Forward	AGTTCTCGGCGGTGAAAGG
1711T	Forward	AGTTCTCGGTGGTGAAAGG
A1801	Forward	TGTTGTAGGACTGCCTGA
1801G	Forward	TGTTGTAGGGCTGCCTGA

*based on GRK4□, GenBank Accession # U33054

Mutations falling outside the coding region of GRK4 can be detected by examining the non-coding regions, such as introns and regulatory sequences near or within the GRK4 gene. An early indication that mutations in noncoding regions are important may come from Northern blot experiments that reveal messenger RNA molecules or abnormal size or abundance in hypertensive patients as compared to control individuals.

Alternation of GRK4 mRNA expression can be detected by any techniques known in the art. These include Northern blot analysis, PCR amplification and RNase protection. Diminished mRNA expression indicates an alteration of the wild-type GRK4 gene. Alteration of wild-type GRK4 genes can also be detected by screening for alteration of wild-type angiotensinogen. For example, monoclonal antibodies immunoreactive with GRK4 can be used to screen a tissue. Lack of cognate antigen would indicate a GRK4 gene mutation. Antibodies specific for products of mutant alleles could also be used to detect mutant GRK4 gene product. Such immunological assays can be done in any convenient formats known in the art. These include Western blots, immunohistochemical assays and ELISA assays. Any means for detecting an altered GRK4 can be used to detect alteration of wild-type GRK4 genes. Finding a mutant GRK4 gene product indicates alteration of a wild-type GRK4 gene.

Applicants speculate that GRK4 mutants other than the aforementioned six GRK4 mutants are associated with essential hypertension. Such mutants can be identified *in vitro* by measuring their ability to cause a D1 receptor-containing cell into which they are introduced not to transduce a dopaminergic signal. By this phrase, it is meant that the dopamine receptor fails to activate G protein subunits or fails to produce cytoplasmic second messengers that are needed to inhibit sodium transporters. Failure to transduce a dopaminergic is manifested in among other things, a D1 receptor/adenylyl cyclase (AC) or G protein coupling defect, and the post-translational modifications of the type described above. These phenomena can be measured by measuring the ability of dopamine or its agonists to stimulate: (a) adenylyl cyclase activity or cAMP production or activate protein

kinase A, (b) phospholipase C activity or activate protein kinase C, (c) phospholipase A2 activity, and (d) G-protein activity or inhibit sodium transport proteins such as the sodium/hydrogen exchanger or sodium/potassium ATPase.

Other GRK4s associated with essential hypertension can be identified by simply by sequencing a GRK4 gene obtained or cloned from an individual having essential hypertension.

Wild-type GRK4s or GRK4s associated with essential hypertension may be incorporated into a variety of systems in which to screen large numbers of different types of substances for anti-hypertensive activity. In general, any system that contains GRK4 and a GRK4 substrate, and from which GRK4 conformation or activity (and changes therein) can be measured, may be used in order to screen substances for anti-hypertensive activity. Thus, in the broadest sense of this aspect of the present invention, whole cells are not required. The system may be artificial in nature and housed within a lipid miscelle, for example. See, *Hammond et al.*, Nature *327*:730-732 (1987), for a discussion of cell-free systems in whch to study molecular interactions. Whole cells are preferred, though, as is the D1 receptor, or a functional fragment thereof, as the GRK4 substrate. By the term "functional fragment, it is meant any part of the receptor, which is phosphorylated, palmitoylated or post translationally modified by other means *in vitro*. A preferred method according to the present invention entails the use of cells transformed with a GRK4 nucleic acid. In general, a large variety of cell types can be used including mammalian, bacterial and insect cells. Mammalian cell lines such as Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) fibroblast (LTK) cells, MDCK and LLCPK cells are preferred. CHO cells are more preferred because they are expected to perform similarly to proximal tubule cells *in vivo*. Transforming cells with the GRK4 and D1 receptor nucleic acids may be conducted in accordance with standard procedures. See, e.g., *Sambrook et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), and *Ausubel et al.*, Current Protocols in Molecular Biology, Wiley & Sons (1994).

In a more preferred embodiment of this aspect of Applicants' invention, the method is conducted using immortalized renal proximal tubule cells

prepared using tubule cells isolated from a normotensive or hypertensive animal such as a human. In general, tubule cells are isolated from the kidney by cutting the cortex into small sections (e.g., 1 mm^3) and placing them on a suitable growth surface of a container (e.g., collagen-coated T-flasks.) After attachment is allowed by inverting the containers (e.g., for about 30 minutes at room temperature), the containers are righted and appropriate medium is added. Preferred medium is Dulbecco's Minimal Essential and F-12 medium with added substances (wt/ml): insulin (5 micrograms), transferrin (5 micrograms), selenium (5 nanograms), hydrocortisone (36 nanograms), triiodothyronine (4 picograms), and epidermal growth factor (19 nanograms). The tissues are incubated, left undisturbed for about three days at 37°C in 95% air, 5% CO_2. See, *Detrisac, et al.*, Kidney Int. 25:383-390 (1984). Alternatively, the pieces of cortex can be digested with collagenase progressively sieved at 212 and 140 micrometer and concentrated over a 40 micrometer sieve prior to culturing. See, *Courjault-Gautier et al.*, J. Am. Soc. Nephrol. 5:1949-1963 (1994). By the term "immortalized" it is meant that the cells grow indefinitely in culture. The isolated renal proximal tubule cells may be immortalized by infecting them with a retro-virus such as SV40 virus, et al., SV40tsA mutant virus and then obtaining outgrowing cells about 7-8 weeks after infection. These cells offer the advantage of more closely mimicking the *in vivo* environment in which the GRK4 protein functions. The immortalized cells from hypertensive subjects offer an almost limitless supply of cells that can be used to screen agents for anti-hypertensive activity.

Substances or agents possessing putative anti-hypertensive properties may be identified by determining a change in GRK4 confomation or activity upon addition of the substance or agent to the GRK4 system. GRK4 activity may be determined indirectly, such as by measuring adenylate kinase activity, or directly such as by measuring the extent of phosphorylation of a phosphorylatable substrate added to the culture. Any GRK4 activating or inactivating mutants, e.g., mutants or polymorphisms of GRK4 that lead to an increase in GRK4 activity or a decrease in GRK4 activity, respectively, are of interest. The alteration in GRK4 activity can lead to alteration in the function of G protein-coupled receptors exemplified by the D1 receptor. GRK4 may regulate the

function of other proteins involved in essential hypertension such as the renin-angiotensin system, kallikrein-kinins, endothelins, atrial and brain natriuretic peptide, nitric oxide, serotonin, vasopressin, calcium sensing receptor, and epithelial sodium channel.

Another type of screening agent involves a complex between a GRK4 protein, e.g., wild-type or an isoform or mutant that is associated with essential hypertension, and an agent that causes a conformational change of the GRK4 protein upon interaction with an anti-hypertensive agent to be detected. The choice of the complexing agent depends upon the method in which conformational analysis is conducted. Such analysis may be conduced by spectrophometry, fluorescence, nuclear magnetic resonance, evanescent wave technology and atomic force microscopy.

Yet another type of screening agent and protocol involves the use of a transgenic animal model of essential hypertension, wherein the animal expresses a transgenic nucleic acid encoding a wild-type GRK4 or a mutant GRK4 of the present invention. The expression of the mutant GRK4 manifests a phenotype which is characterized by hypertension and a decreased ability of the animal to excrete an acute or chronic sodium load. The transgenic models can also be used to test for the effects of dietary manipulation such as high calcium, high potassium and high magnesium that have been shown to lower blood pressure, on GRK4 expression and activity. Clearly, any animal with an excretory system can be used as a model of essential hypertension. Rodents such as mice are preferred.

The transgenic animal can be created in accordance with techniques known in the art. Applicable techniques for preparing transgenic animals are well known in the art. Any method can be used which provides for stable, inheritable, expressible incorporation of the transgene within the nuclear DNA of an animal. These transgenic animals are constructed using standard methods known in the art as set forth, for example, in U.S. Patent Nos. 4,873,191; 5,849,578; 5,731,489; 5,614,396; 5,487,992; 5,464,764; 5,387,742; 5,347,075; 5,298,422; 5,288,846; 5,221,778; 5,175,384; 5,175,383; 4,873,191; and 4,736,866, as well as Burke and Olson, *Methods in Enzymology 194*: 251-270 (1991), Capecchi, *Science 244*:1288-1292 (1989), Davies et al., *Nucleic Acids Research 20(11)*:2693-2698 (1992),

Dickinson et al., *Human Molecular Genetics 2(8)*:1299-1302 (1993), Huxley et al., *Genomics* 9:742-750 (1991), Jakobovits et al. *Nature* 362:255-261 (1993), Lamb et al., *Nature Genetics 5*:22-29 (1993), Pearson and Choi, *Proc. Natl. Scad. Sci. 90*:10578-10582 (1993), Rothstein, *Methods in Enzymology 194*:281-301 (1991), Schedl et al., *Nature 362*:258-261 (1993)], and Strauss et al., *Science* 259:1904-1907 (1993). Further, published international patent applications WO 94/23049, WO 93/14200, WO 94/06908 and WO 94/28123 provide further relevant teachings in these regards.

Any technique known in the art may be used to introduce a target gene transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P. C. and Wagner, T. E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA *82*:6148-6152 (1985)); gene targeting in embryonic stem cells (Thompson et al., Cell *56*:313-321 (1989)); electroporation of embryos (Lo, Mol. Cell. Biol. *3*:1803-1814 (1983)); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell *57*:717-723 (1989)). See Gordon, Transgenic Animals, Intl. Rev. Cytol. *115*:171-229 (1989), for a general review on these techniques.

The present invention provides for transgenic animals that carry the GRK4 transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teachings of Lasko et al., Proc. Natl. Acad. Sci. USA *89*:6232-6236 (1992). Those skilled in the art will appreciate that the regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest. When it is desired that the target gene transgene be integrated into the chromosomal site of the endogenous target gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous target gene of interest are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the

nucleotide sequence of the endogenous target gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene of interest in only that cell type, by following, for example, the teaching of Gu et al., Science *265*:103-106 (1994). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant target gene and protein may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR. Samples of target gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the target gene transgene gene product of interest.

The target gene transgenic animals that express target gene mRNA or target gene transgene peptide (detected immunocytochemically, using antibodies directed against the target gene product's epitopes) at easily detectable levels should then be further evaluated to identify those animals which display characteristic symptoms of essential hypertension.

In a preferred embodiment, the GRK4 transgene is inserted into an appropriate vector, inoperable association with a tetracycline sensitive promoter, and then introduced into embryonic stem (ES) cells. The ES cells are then reintroduced by microinjection of the genetically altered ES cells into host blastocysts or by morulae co-culture. Founder animals are obtained and animals homozygous for the GRK4 transgene are then used. See, *Thompson, et al.*, Am. J. Physiol. 269:E793-E803 (1995).

Therapeutic modalities entail targeting GRK4 activity to increase natriuresis or otherwise approach normalcy with respect to a proper balance of sodium and water. For example, GRK4 expression can be prevented by targeting at the RNA level or the DNA level by administering a drug that changes expression of

GRK4 in kidney cells. Such drugs are preferably oligonucleotide molecules such as antisense oligonucleotides, dominant negative mutant DNA molecules, and ribozymes that reduce or prevent GRK4 expression by binding GRK4 mRNA, pre-mRNA, or GRK4 DNA. The administration of antisense oligonucleotides to a hypertensive individual can be conducted in accordance with the formulations and vehicles described in U.S. Patent Nos. 5,856,099; 5,856,103; 5,783,683; 5,840,708; and 5,591,600; 5,849,903; 5,135,917; 5,098,890; and 5,087,617. Antisense technology, now well known in the art, is also described in *Uhlmann et al., Chem. Rev.* 90:543-584 (1990); *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression* (Cohen, ed. 1989); *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, CRC press (Saghir Akhtar, ed. 1995); and Stein, C.A., and Cohen, Jack S., "Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review," *Cancer Research,* 48:2659-2668 (1988).

Synthetic antisense oligonucleotides should be of sufficient length to hybridize to the target nucleotide sequence and exert the desired effect, e.g., blocking translation of an mRNA molecule. It is advantageous, however, to use relatively smaller oligonucleotides because they are likely to be more efficiently taken up by cells *in vivo*, such that a greater number of antisense oligonucleotides are delivered to the location of the target mRNA. Preferably, antisense oligonucleotides should be at least 15 nucleotides long, and preferably 20 nucleotides in length, to achieve adequate specificity. Preferred antisense oligonucleotides are (5' CAC GAT GTT CTC GAG CTC CAT 3', complementary to bases 255-275 and 5' CTC CAT GTC CTG GCG CCG 3' complementary to bases 243-260.

Small oligonucleotides such as those described above are highly susceptible to degradation by assorted nucleases. Moreover, such molecules are may be unable to enter cells because of insufficient membrane permeability. For these reasons, practitioners skilled in the art generally synthesize oligonucleotides that are modified in various ways to increase stability and membrane permeability. The use of modified antisense oligonucleotides is preferred in the present invention. The term "antisense oligonucleotide analog" refers to such modified oligonucleotides, as discussed hereinbelow.

The oligonucleotides of the invention are conveniently synthesized using solid phase synthesis of known methodology, and are designed to be complementary to and/or specifically hybridizable with the preselected sequence of the target GRK4 DNA or RNA encoding the sequences disclosed herein. Nucleic acid synthesizers are commercially available and their use is understood by persons of ordinary skill in the art as being effective in generating any desired oligonucleotide of reasonable length.

Ribozymes, e.g., of the hammerhead or haripin types, that catalyze the degradation of GRK4 mRNA or pre-mRNA can be designed and prepared in accordance with standard procedures. See, e.g., U.S. Patent No. 5,856,463 (and publications cited therein), for detailed teachings on methods of designing, making and formulating ribozymes for therapeutic uses.

GRK4 activity can also be targeted by administering agents such as pharmacologic antagonists or blockers that change (e.g., inhibit or enhance) catalytic activity, e.g., phosphorylating or non-phosphorylating action, of the fully or partially expressed GRK4 protein by acting directly upon the protein. Other therapeutic action entails direct binding of GRK4 protein with peptidic agentsAll of these methods and agents result in a normalization of D1 receptor/AC coupling in kidney cells that express GRK4, and as a result, decreased sodium transport in renal proximal tubule cells.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting as to the scope of the invention described herein, unless otherwise specified.

EXAMPLES

TISSUE CULTURE

Human kidneys were obtained as fresh surgical specimens from patients who had unilateral nephrectomy due to renal carcinoma. The patient records of the subjects were reviewed and classified into those with either normal blood pressure (n=9) or essential hypertension (n=14). Subjects with systolic blood pressures less than 140 mm Hg and diastolic blood pressures less than 90 mm Hg were considered normotensive. Subjects with systolic blood pressures equal to or

greater than 140 mm Hg or diastolic blood pressures equal to or greater than 90 mm Hg and/or on antihypertensive medications were considered hypertensive.

Cultures of renal proximal tubule cells from histologically-verified normal kidney sections (5 x 10^5 cells/well in 24 well plastic plates coated with 0.075% Type I collagen) were incubated at 37°C in 95% O_2/5% CO_2 and grown in a serum-free medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham's F12 medium supplemented with selenium (5 ng/ml), insulin (5µg/ml), transferrin (5µg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml)(5). When sub-confluent (90-95%), the cells were sub-cultured (passages 6-8) for use in experimental protocols using trypsin-EDTA (0.05%, 0.02%). The culture conditions are conducive for growth of human renal proximal tubules that retain characteristics of renal proximal tubule cells, *Sanada, H. et al.*, J. Invest. Med. 45:277A (1997).

LIGHT MICROSCOPIC IMMUNOHISTOCHEMISTRY

Immunohistochemistry of kidney tissues and cells in culture fixed in HISTOCHOICE was performed as described *Sanada, H. et al., supra.* Affinity-column purified polyclonal human D_1 receptor antibodies were raised against a synthetic peptide sequence GSGETQPFC (amino acids 299-307). See, *Sanada, H. et al., supra.* Two commercially available GRK4 isoform antibodies were used (Santa Cruz Biotechnology, Inc, Santa Cruz, CA); one GRK4 antibody recognized both the α and β isoform, while another recognized both the αβ isoform. The specificity of these antibodies has been previously reported *Sanada, H. et al., supra* and *Guyton A.C.* Circulatory Physiology III, Arterial Pressure and Hypertension, W.B. Saunders Co., Philadelphia, PA (1980).

Immunohistochemistry studies have shown *GRK4*α/β and *GRK4*α/δ isoform expression only in renal proximal and distal convoluted tubules (not in loops of Henle, cortical or medullary collecting ducts, glomeruli or renal arterial vessels). *GRK4*α/δ was found in both luminal and basolateral membranes while *GRK*α/β was found in the luminal membrane only. There were no differences in the renal expression of these two *GRK4* isoforms between hypertensive and

normotensive subjects (not shown). The expression of *GRK4α/β* and *GRK4α/δ* persisted in renal proximal tubule cells in culture (photographs not shown).

DETERMINATION OF GRK ACTIVITY

GRK activity was measured according to Benovic, Methods Enzymol. *200*:351-362 (1991). Renal proximal tubular extracts were prepared by homogenization in ice-cold lysis buffer containing (in mM): 25 Tris-HCl, pH 7.5, 5 EDTA, and 5 EGTA with leupeptin (10 μg/ml), aprotinin (20 μg/ml), and 1 PMSF. The crude homogenate was centrifuged at 30,000g for 30 min. The pellet was extracted by 200 mM NaCl on ice for 30 min and centrifuged at 30,000g for 30 min. The supernatant was used for all GRK assays and immunoblotting. Twenty μg of protein extract was incubated with rhodopsin-enriched rod outer segments in assay buffer with 10 mM $MgCl_2$ and 0.1 mM ATP (containing γ^{32}P-ATP). After incubation in white light for 15 min at room temperature, the reaction was stopped with ice-cold lysis buffer and centrifuged at 13,000g for 15 min. The pellet was resuspended in Laemmli buffer and subjected to 12% SDS-PAGE. The gels were subjected to autoradiography, and the phosphorylated rhodopsin was quantified using both densitometry and radioactive counting of the excised bands at the appropriate size . GRK activity was also measured in the presence or absence of a GRK isoform antibody.

Fig. 1 shows that the D_1-like agonist, fenoldopam, had no effect on GRK activity, assessed by the phosphorylation of rhodopsin, in renal proximal tubule cells from normotensive subjects. These data suggest that GRKs that can use rhodopsin as a substrate (i.e., *GRK2, GRK3, GRK4α, GRK5, GRK6*) are not involved in the desensitization of the D_1 receptor in renal proximal tubule cells when blood pressure is normal. It was also found that D_1 receptor and *GRK4* expressions in renal proximal tubule cells in culture were similar in hypertensive and normotensive subjects (data not shown). In renal proximal tubule cells from hypertensive subjects, however, fenoldopam increased GRK activity. Moreover, basal GRK activity in renal proximal tubule cells was greater in hypertensive than in normotensive subjects. These studies suggest an aberrant function of GRK in renal proximal tubules in hypertension. The increase in GRK activity produced by

fenoldopam (in hypertension) was blocked by antibodies to *GRK2*, *GRK3*, and *GRK4*α/δ (data not shown), indicating that activation of one or all of these GRKs may be involved in the fenoldopam-mediated increase in GRK activity. Tiberi. *et al.*, J. Biol. Chem. 271:3771-3778 (1996). However, the ubiquitous expression of *GRK2* and *GRK3* is at odds with the recognized pre-eminence of the kidney in the pathogenesis of both rodent and human essential hypertension. Guyton, W.B. Saunders Co. Phil., PA (1980); *Guidi et al.*, J. Am. Soc. Nephrol. 7:1131-1138 (1996). No difference was found in the sequence of the coding region of *GRK2* between hypertensive and normotensive human subjects (data not shown). This finding suggests that the increase in *GRK2* activity in lymphocytes of hypertensive patients (*Gros et al.*, J. Clin. Invest. 99:2087-2093 (1997)) is secondary to the high blood pressure, as has been suggested for the increase in *GRK5* activity and expression in rodents with genetic and induced hypertension. *Ishizaka et al.*, J. Biol. Chem. 272:32482-32488 (1997).

DETERMINATION OF cAMP ACCUMULATION

The cells were washed twice with Dulbecco's phosphate buffered saline (D-PBS), after which 1 mM 3-isobutyl-1-methyl-xanthine was added to each well. The cells were incubated at 37°C for 30 minutes with or without drugs: dopamine and the D_1-like receptor agonist, fenoldopam, the D_1-like receptor antagonist, SCH23390 (Research Biochemicals International, Natick, MA), and forskolin (Sigma Chemical Co., St. Louis, MO). Then, the cells were washed twice with D-PBS and frozen at -80°C and the cells were further lysed with 0.1N HCl. cAMP concentration was measured by radioimmunoassay. *Sanada, H. et al.*, *supra.*, and *Kinoshita, S. J.* Clin. Invest. 84:1849-1856 (1989). Protein concentration was measured with the BCA protein assay kit (Pierce Chem. Co., Rockford, IL).

To determine whether an increase in *GRK4* activity was responsible for the uncoupling of the D_1 receptor in renal proximal tubule cells in hypertension, the effect of D_1-like agonist stimulation on cAMP accumulation after inhibition of the translation of *GRK4* by antisense oligonucleotides was studied. Figure 2 shows that the D_1-like agonist, fenoldopam, increased cAMP accumulation to a greater extent in renal proximal tubule cells from normotensive than from hypertensive

subjects. Neither sense/scrambled nor antisense *GRK4* oligonucleotides affected basal or forskolin-stimulated cAMP production. Compared with fenoldopam alone, neither sense nor scrambled *GRK4* oligonucleotides significantly affected cAMP accumulation in either group. However, antisense *GRK4* oligonucleotides enhanced the ability of fenoldopam to stimulate cAMP accumulation in cells from hypertensive subjects (but not from normotensive subjects) such that the values approximated those observed in cells from normotensive subjects treated with fenoldopam.

IMMUNOPRECIPITATION

Proximal tubule cells were incubated with vehicle, fenoldopam, sense, scrambled or antisense propyne/phosphorothioate oligonucleotides (5 nM) as described above. The membranes were lyzed with ice cold lysis buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The lysates were incubated with IgG-purified anti D_1 receptor antibody on ice for 1 hr and protein-A agarose for 12 hrs with rocking at 4°C. The proteins separated by SDS-polyacryl-amide gel electrophoresis were electrophoretically transferred onto nitrocellulose membranes. The transblot sheets blocked with 5-10% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20 were incubated with diluted affinity-purified polyclonal anti-phosphoserine antibody (Zymed Lab, San Francisco, CA); *Sanada, H. et al., supra.* In some cases, the cells were labeled with ^{32}P and immunoprecipitated with anti D_1 receptor antibody. The autoradiograms and immunoblots, visualized with ECL system (Amersham, Arlington Heights, Il) were quantified by densitometry *Sanada, H. et al., supra.*

The next study was directed to whether the differential effects of antisense GRK4 oligonucleotides extended to the phosphorylation of the D_1 receptor. Fig. 3 shows that the basal levels of serine-phosphorylated D_1 receptor in renal proximal tubule cells were greater in hypertensive than in normotensive subjects and correlated with the increased basal levels of GRK activity in hypertensive subjects (as shown in Fig. 1). Fenoldopam increased the quantity of serine-phosphorylated D_1 receptor in normotensive but not in hypertensive subjects

in agreement with our previous report *Sanada, H. et al., supra*. Neither sense nor scrambled GRK4 oligonucleotides affected the phosphorylation of the D_1 receptor in fenoldopam-treated cells in either group of subjects. In contrast, *GRK4* antisense treatment almost completely abolished the phosphorylation of the D_1 receptor in fenoldopam-treated renal proximal tubule cells from hypertensive subjects to levels that are lower than basal values. *GRK4* antisense treatment also decreased the phosphorylation of the D_1 receptor in fenoldopam-treated renal proximal tubule cells from normotensive subjects but the values remained above baseline levels. The almost complete suppression of the phosphorylation of the D_1 receptor by antisense oligonucleotides to *GRK4* in renal proximal tubules in hypertensive subjects suggests that the major GRK involved in the phosphorylation and desensitization of the D_1 receptor in hypertension is *GRK4* and not other GRKs that may be expressed in this nephron segment.

GENOTYPING

Based upon the initial observations that the incidence of homozygous GRK4 gene variants is about 60% in hypertensive subjects and 16% in the general population, power analysis (power of 0.8, α of 0.05, and effect of 45%) indicated a sample size of 14-21 per group to detect any significant differences between groups. For this reason, DNA from peripheral blood of additional 18 hypertensive and 11 normotensive subjects were obtained. All volunteers were examined and their medical records were reviewed by at least two investigators. Subjects were classified as normotensive if they had no history of hypertension, no clinical evidence of underlying hypertension, were taking no antihypertensive medications, were not receiving vasodilator therapy or other drugs that could affect blood pressure, and had sitting systolic blood pressures less than 140 mm Hg and diastolic blood pressures less than 90 mm Hg on their three most recent clinic visits. Patients with hypertension had significant and sustained elevations in blood pressures (greater than 160 mm Hg systolic and 95 mm Hg diastolic) on at least three separate occasions. All hypertensive subjects (DNA from kidney, n=14, DNA from peripheral blood, n=18) were at least 20 years old. To obviate the problem inherent in the late onset of essential hypertension in some individuals, all

normotensive subjects (DNA from kidney, n=9, DNA from peripheral blood, n=11) were at least 45 years old.

Genomic DNA was extracted (salting out method) from renal proximal tubule cells in culture and kidney tissues or peripheral blood leukocytes of random. Exons of *GRK4* containing polymorphic nucleotides were amplified with primers listed in Table 2. Each 20 µl reaction mixture contained 1XPCR buffer, 0.2 mM each dNTP, 1.25 mM $MgCl_2$, 0.2 µM each primer, 0.5 unit Taq DNA polymerase and 50 ng genomic DNA. The reaction mixture was denatured at 94°C for 5 min, followed by 30 cycles of 30 sec of denaturation at 94°C, 30 sec of reannealment at 55°C, and 30 sec of extension at 72°C. The PCR was completed by a final extension at 72° for 5 min. Two µl of PCR product were spotted onto a Biodyne B+ membrane. Dot blots were prepared for each of the following wild type and variant allele specific oligonucleotide probes (Table 4). Probe labeling, membrane preparation, hybridization, and washing conditions were those of published procedures. See *Wong et al.*, Clin. Chem. 43:1857-1861 (1997). The nucleotide at position 1801 in 250 random subjects was invariant (G). It was also also found that the frequency of the polymorphic nucleotide at position 993 was not different between hypertensive and normotensive subjects. Therefore, only the results of the studies of 3 polymorphic sites at positions 448, 679, and 1711 (Table 4) are presented. The sequences of the cDNA were determined by the Sanger dideoxy chain termination method.

Table 4. GRK4 variants in normotensive and hypertensive subjects.

Phenotype	**Genotype**		
	Homozygous R65L	Homozygous A142V	Homozygous A486V
Hypertensive (n=32)	6	11	4
Normotensive (n=20)	1	0	0

Genotype was determined by dot blot analysis using allele specific oligonucleotides. Four hypertensive subjects were homozygous at two sites (amino acid position 65 and 142). The frequency of homozygous variants at R65L,

A142V, and/or A486V in hypertensive subjects (53%, 17 of 32) was significantly different from that noted in normotensive subjects (5%, 1 of 20) ($\chi 2$ = 10.56, P=0.0012). The frequency of homozygous variant A142V was also significantly different ($\chi 2$ = 6.78, P=0.0092) between hypertensive (34%, 11 of 32) and normotensive subjects (0%, 0 of 20).

Sequencing of *GRK4* cDNA from human kidneys and subsequent genotyping of 5 polymorphic sites in DNA from the kidney and peripheral white blood cells revealed that 3 variants: nucleotide 448, CGT to CTT (amino acid R65L), nucleotide 679, GCC to GTC (amino acid A142V), and nucleotide 1711, GCG to GTG (amino acid A486V) (autoradiograph not shown) occurred more frequently in hypertensive than in normotensive subjects (Table 4). The frequency of homozygous variations at R65L, A142V, and/or A486V in hypertensive subjects (53%, 17 of 32) was significantly different from that noted in normotensive subjects (5%, 1 of 20) (χ^2 = 10.56, P=0.0012) (Table 4) and different from those found in a random population of 50 adult subjects (χ^2=10.99, P=0.0009). In this random population with unknown blood pressure, 16% were homozygous at R65L and/or A486V and 50% were heterozygous at either R65L or A486V; the 16% frequency of homozygous alleles is close to the incidence of essential hypertension *Lifton, R. P.* Science 272:676-680 (1996). The homozygous variation at *GRK4* A142V, by itself, was also more frequent in hypertensive (34%, 11 of 32) than in normotensive subjects (0%, 0 of 20), (χ^2 = 6.78, P=0.0092).

GRK4α is the only GRK4 isoform that has been reported to phosphorylate rhodopsin (Sallese et al., J. Biol. Chem. *272*:10188-10195 91997)), but in our studies, D_1 agonist stimulation with fenoldopam failed to increase GRK activity in renal proximal tubule cells from normotensive subjects (Figure 1). Therefore, it was concluded that *GRK4α* is not involved in the desensitization of the D_1 receptor. The belief is that a *GRK4* isoform that does not normally phosphorylate rhodopsin (e.g. *GRK4γ*) (Premont et al., J. Biol. Chem. *271*:6403-6410 (1996); Sallese et al., *supra.*; and Virlon et al., Endocrinol. *139*:2784-2795 (1998)) may have become activated in hypertension. Indeed, it was found that the D_1-like agonist-mediated increase in GRK activity was associated with an increase

in membranous expression of *GRK4α/δ* in renal proximal tubule cells from hypertensive but not from normotensive subjects (Figure 4).

TRANSFECTION AND CELL CULTURE

The rat D_1 *(rD1) or* human D_1 *(hD1)* receptor cDNA was subcloned in the expression vector pPUR (Clontech, Palo Alto, CA) or pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA), respectively, between *EcoR1*and *Xba*I sites. The resulting constructs were used to stably transfect CHO cells expressing the *p*Tet-Off regulator plasmid (Clontech, Palo Alto, CA) using calcium phosphate. See Yamaguchi et al., Mol. Pharmacol. *49*:373-378 (1996). GRK4γ and GRK4δ cDNAs, obtained from RT/PCR of mRNA from human kidney cortex were subcloned into a *p*Tet-Off response plasmid *(pTRE-rD1 or pTRE-hD1* and *pTK-Hyg* mixed in a 20:1 ratio, respectively) (Clontech, Palo Alto, CA).

To determine if the variations in the *GRK4α* gene have any functional consequences, the effect of D_1-like agonist on cAMP production in Chinese hamster ovary (CHO) cells transfected with both the D_1 receptor and wild type or variant *GRK4α* cDNA was studied. *GRK4δ* was used for comparison. The dose response curve in CHO cells in the absence of *GRK4α* was similar to those noted with HEK-293 cells, a cell with low endogenous GRK activity. Premont et al., *supra*. The expression of wild type *GRK4α* decreased the ability of the D_1 agonist to stimulate cAMP production (Figure 5). However, the inhibition of the D_1 agonist action became even greater with the *GRK4α* variants R65L and/or A486V. The effect of wild type or variant *GRK4α* was not due to differences in the quantity of the expression of either the D_1 receptor or *GRK4α* (data not shown). Wild type *GRK4α* or its variants did not affect the ability of forskolin to stimulate cAMP accumulation indicating specificity of the interaction of *GRK4α* with the D_1 receptor. The action of fenoldopam was selective for the D_1 receptor since the fenoldopam effect was blocked by the D_1-like antagonist SCH23390 (data not shown). In other studies, there was no effect of wild type *GRK4δ* on D_1-like agonist-mediated cAMP accumulation (data not shown) compared to the desensitization of the D_1 receptor induced by the wild type *GRK4α*. The functional studies in renal proximal tubule cells and the expression studies in CHO cells

suggest that an increased activity of *GRK4α* is responsible for the decreased ability of D_1 receptor ligands to couple to effector enzymes and ion transport proteins in hypertension. In turn, the desensitization of the D_1 receptor in renal proximal tubules in hypertension may lead to a decreased ability of the kidney to eliminate a sodium chloride load. The failure of the kidney to excrete sodium chloride is thought to be crucial in the development of hypertension. *Guyton, A.C.*, Circulatory Physiology III, Arterial Pressure and Hypertension, W.B. Saunders Co., Philadelphia, PA (1980); Guidi et al., J. Am. Soc. Nephrol. *7*:1131-1138 (1996). Indeed, genes that regulate renal sodium transport have been shown to be important in the regulation of blood pressure. *Lifton, R.P.* Science *272*:676-680 (1996) and Karet & Lifton, Recent Prog. Horm. Res. *52*:263-276 (1997).

To determine if the infusion of a substance or agent into a living being that caused the reduction in GRK4 activity could serve as an antihypertensive therapeutic, furthr experiments were conducted in the spontaneously hypertensvie rat (SHR). Six male rats, 4 weeks of age, weighing 100 g were subjected to a left uninephrectomy and then allowed two weeks to recover from surgery. After recovery, a 30 day osmotic minipump equipped with a single outlet catheter was filled with either phosphorotioate/propyne-modified antisense GRK4 oligonucleotide (5 nM, one microliter/hr) or scrambled GRK4 oligonucleotide and then was implanted into the renal cortex of the remaining left kidney. The outlet of the catheter was inserted approximately 1mm deep into the renal cortex of the remaining kidney and secured with Superglue. The rats were then allowed to recover from surgery and daily measurements were made for blood pressure and urine output (volume and electrolytes). After 30 days, the rats were sacrificed and their remaining kidney was used for Western blot analysis of GRK4.Our studies demonstrated that blood pressure was reduced in rats treated with antisense oligonucleotide to GRK4 (n=3) when compared to rats treated with scrambled GRK4 oligonucleotide (n=3). Furthermore, it was demonstrated by Western blot analysis that antisense oligonucleotides reduced the expression of renal GRK4.

In conclusion, the examples demonstrate a D_1 receptor/adenylyl cyclase coupling defect in renal proximal tubule cells from subjects with essential hypertension. Increased GRK activity in renal proximal tubule cells in human

essential hypertension is due to activating missense variations of *GRK4*, an effect that was reproduced in a transfected cell model. Moreover, preventing the translation of *GRK4* normalized the coupling of the D_1 receptor to adenylyl cyclase in hypertension. Again, without intending to be bound by any particular theory of operation, Applicants believe that the homozygous amino acid variations cause a ligand independent serine-phosphorylation of the D_1 receptor which results in its uncoupling from the G protein/effector complex. The desensitization of the D_1 receptor in the renal proximal tubule may be the cause of the compromised natriuretic effect of dopamine that eventually leads to sodium retention and hypertension. These conclusions are supported by the results of experiments described above demonstrating that intrarenal infusion of Spontaneous Hypertensive Rats with antisense oligonucleotides to GRK4 results in an intrarenal reduction in the concentration of GRK4 and lowering of their mean arterial blood pressure. Thus, substances or agents that alter the concentration or activity of GRK4 represent a novel class of antihypertensive medications.

A nephron segment-specific defective coupling between the dopamine D1A receptor and the G protein/effector enzyme complex may be a cause of the renal sodium retention in spontaneously hypertensive rats (SHR). The decreased ability of exogenous and renal endogenous dopamine to inhibit sodium transport in renal proximal tubules co-segregates with hypertension in F2 crosses of SHR and its normotensive control, the Wistar-Kyoto (WKY) rat. Similar defects were found in the Dahl salt-sensitive rat and more importantly, in humans with essential hypertension. Thus, primary cultures of renal proximal tubules cells from hypertensive humans have a defective coupling of a renal D1-like receptor to adenylyl cyclase (AC), similar to the coupling defect found in hypertensive rodents. These in vitro data are in agreement with in vivo studies demonstrating a defective D1-like receptor from the G protein/effector enzyme complex is not due to homologous or heterologous desensitization, receptor down-regulation, G protein or effector enzyme "defects" or a mutation in the primary sequence of the D1-like receptors. Rather, the uncoupling of the D1-like receptor is due to a ligand-independent hyper-phosphorylation of the D-1 receptor (the major D1-like receptor

in the kidney) due to homozygous mutations of GRK4 isoform with limited organ and nephron expression.

Industrial Applicability

The diagnostic tests of the present invention will screen individuals to identify those predisposed to essential hypertension. Genetic, cellular and biochemical tools in which to carry out these tests are also provided. The present invention also provides for several tools and methods for conducting drug discovery and identify substances with anti-hypertensive activity or properties. The compositions and methods for normalizing sodium transport in kidney cells of individuals having essential hypertension provide means to treat this disease.

All patent and non-patent publications cited in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Claims:

1. An isolated and purified nucleic acid encoding a GRK4 protein having an R65L, A142V mutation, an R65L, A486V double mutation, or an R65L, A142V, A486V triple mutation.

2. An oligonucleotide which specifically hybridizes to a GRK4 gene having a sequence that encodes an R65L mutation, an A142V mutation, an A486V mutation, an R65L, A142V double mutation, an R65L, A486 double mutation or an R65L, A142V, A486V triple mutation.

3.. An oligonucleotide primer which hybridizes to exon 3, 5, 8, 14 or 16 of a GRK4 gene, and is useful in amplifying a DNA sequence including nucleotides 431 to 503 (exon 3), 594 to 697 (exon 5), 857-995 (exon 8), 1662 to 1798 (exon 14), and 1937 to 1991 (exon 16) of said gene.

4. A method of identifying individuals predisposed to essential hypertension, comprising:

obtaining kidney cells having a D1 receptor and expressing GRK4 from said individual; and

assaying said cells to determine extent of post-translational modification of said D1 receptor, wherein a change in post-translational modification of said D1 receptor relative to extent of post-translational modification of a D1 receptor in kidney cells having a D1 receptor and expressing GRK4 isolated from a normotensive individual, is indicative of a predisposition to essential hypertension

5. The method of claim 4, wherein said cells are assayed for the extent of palmitoylation of said D1 receptor.

6. The method of claim 4, wherein said cells are assayed for the extent of phosphorylation of said D1 receptor.

7. The method of claim 4, wherein said cells are assayed for hyperphosphorylation of said D1 receptor.

8. The method of claim 4, wherein said kidney cells are renal proximal tubule cells or cortical duct collecting cells.

9. A method of identifying individuals predisposed to essential hypertension, comprising:

obtaining a nucleic acid sample from an individual; and

analyzing a nucleic acid encoding GRK4, or a fragment thereof, from said sample for a mutation of GRK4 that causes a cell in which GRK4 is expressed not to transduce a dopaminergic signal; wherein said mutation of GRK4 is indicative of a predisposition to essential hypertension.

10. The method of claim 9, wherein said mutation of GRK4 causes a D1 receptor/adenylyl cyclase coupling defect in said cells that express said mutation of GRK4.

11. The method of claim 9, wherein said mutation of GRK4 causes a D1 receptor/G protein coupling defect in said cells that express said mutation of GRK4.

12. The method of claim 9, wherein said nucleic acid sample is a DNA sample.

13. The method of claim 9, wherein said nucleic acid sample is an RNA sample.

14. The method of claim 9, wherein said nucleic acid sample is a genomic DNA sample.

15. The method of claim 9, wherein said nucleic acid sample is a cDNA sample.

16. The method of claim 9, wherein a fragment of the nucleic acid encoding GRK4 is analyzed.

17. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation R65L.

18. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation A142V.

19. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation A486V.

20. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation R65L, A486V.

21. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation R65L, A142V.

22. The method of claim 9, wherein the GRK4 nucleic acid is analyzed for the mutation R65L, A142V, A486V.

23. The method of claim 9, wherein said detecting step is conducted by PCR.

24. A method for detecting a mutation in GRK4 associated with essential hypertension, comprising:

obtaining a nucleic acid sample from a hypertensive individual; and

sequencing a gene encoding GRK4 from said sample.

25. A reconstituted system that measures GRK activity, comprising GRK4 and a GRK4 substrate.

26. The reconstituted system of claim 25, wherein said GRK4 substrate is a D1 receptor or a functional fragment thereof.

27. The reconstituted system of claim 26, which is a whole cell that expresses said GRK4 and said GRK4 substrate.

28. The reconstituted system of claim 27, wherein said whole cell is a Chinese hamster ovary cell transfected with a first heterologous gene encoding a D1 receptor and a second heterologous gene encoding a GRK4 protein associated with hypertension.

29. The reconstituted system of claim 25, wherein said GRK4 protein is associated with essential hypertension.

30. A complex between a GRK4 protein associated with hypertension and an agent which provides a detectable conformational change in said GRK4 protein upon interaction with a substance being analzyed for anti-hypertensive activity.

31. An immortalized human proximal tubular cell.

32. An isolated and purified renal proximal tubular cell obtained from a hypertensive human.

33. The isolated and purified renal proximal tubular cell of claim 32, which is immortalized.

34. A transgenic animal, comprising a diploid genome comprising a transgene encoding a GRK4 protein which is expressed in renal cells to produce said GRK4 protein, and wherein expression of said transgene causes

said transgenic animal to exhibit a state of essential hypertension compared to a normotensive animal whose renal cells do not express said GRK4 protein.

35. The transgenic animal of claim 34, wherein said renal cells have a decreased abilitiy to reject sodium compared to a normotensive animal whose renal cells do not express said GRK4 protein.

36. The transgenic animal of claim 34, which is a rodent.

37. The transgenic animal of claim 34, which is a mouse.

38. A method of identifying putative anti-hypertensive agents, comprising:

adding at least one candidate agent to the reconstituted system of claim 25; and

detecting GRK4 activity, wherein a change in said activity is indicative of a putative anti-hypertensive substance.

39. The method of claim 38, wherein said step of detecting GRK4 activity comprises measuring adenylate cyclase activity.

40. The method of claim 38, wherein said step of detecting GRK4 activity comprises adding a substrate to which phosphate can be added, and a phosphate source to said culture, and measuring phosphorylation of said substrate.

41. A method of identifying putative anti-hypertensive agents, comprising:

contacting at least one candidate agent with the complex of claim 30, and detecting whether a conformational change in said GRK4 occurs, wherein a conformational change is indicative of putative anti-hypertensive activity.

42. The method of claim 41, wherein said detecting is conducted by spectrophotometry, fluorescence, nuclear magnetic resonance, evanescent wave technology or atomic force microscopy.

43. A method of identifying putative anti-hypertensive agents, comprising:

adding at least one candidate agent to a culture of immortalized kidney cells that express a D1 receptor and GRK4 isolated from a hypertensive animal; and

detecting a change in transduction of a dopaminergic signal in said cells, wherein a change in transduction of a dopaminergic signal is indicative of putative anti-hypertensive activity.

44. A method of identifying putative anti-hypertensive agents, comprising:

comparing electrolyte output of a first transgenic animal of claim 34 administered said agent, and a second transgenic animal of claim 34 not administered said agent, whereby a putative anti-hypertensive agent is identified by increased electrolyte output of said first transgenic animal as compared to said second transgenic animal.

45. A method of increasing natriuresis, comprising administering to an essential hypertensive individual a drug that interacts with GRK4 so as to increase natriuresis in said individual.

46. The method of claim 45, wherein said drug changes expression of GRK4 in kidney cells of said hypertensive individual.

47. The method of claim 45, wherein said drug comprises antisense RNA that binds GRK4 mRNA or DNA.

48. The method of claim 45, wherein said drug comprises a ribozyme that cleaves GRK4 mRNA or pre-mRNA.

49. The method of claim 45, wherein said drug comprises a dominant negative mutant DNA molecule.

50. The method of claim 45, wherein said drug binds GRK4 protein.

51. An oligonucleotide which specifically hybridizes to GRK4 mRNA *in vitro* or *in vivo*.

52. The oligonucleotide of claim 51, which is an antisense RNA molecule.

53. The oligonucleotide of claim 51, which is a dominant negative mutant DNA molecule.

54. A ribozyme that cleaves GRK4 mRNA or pre-mRNA.

Figure 1

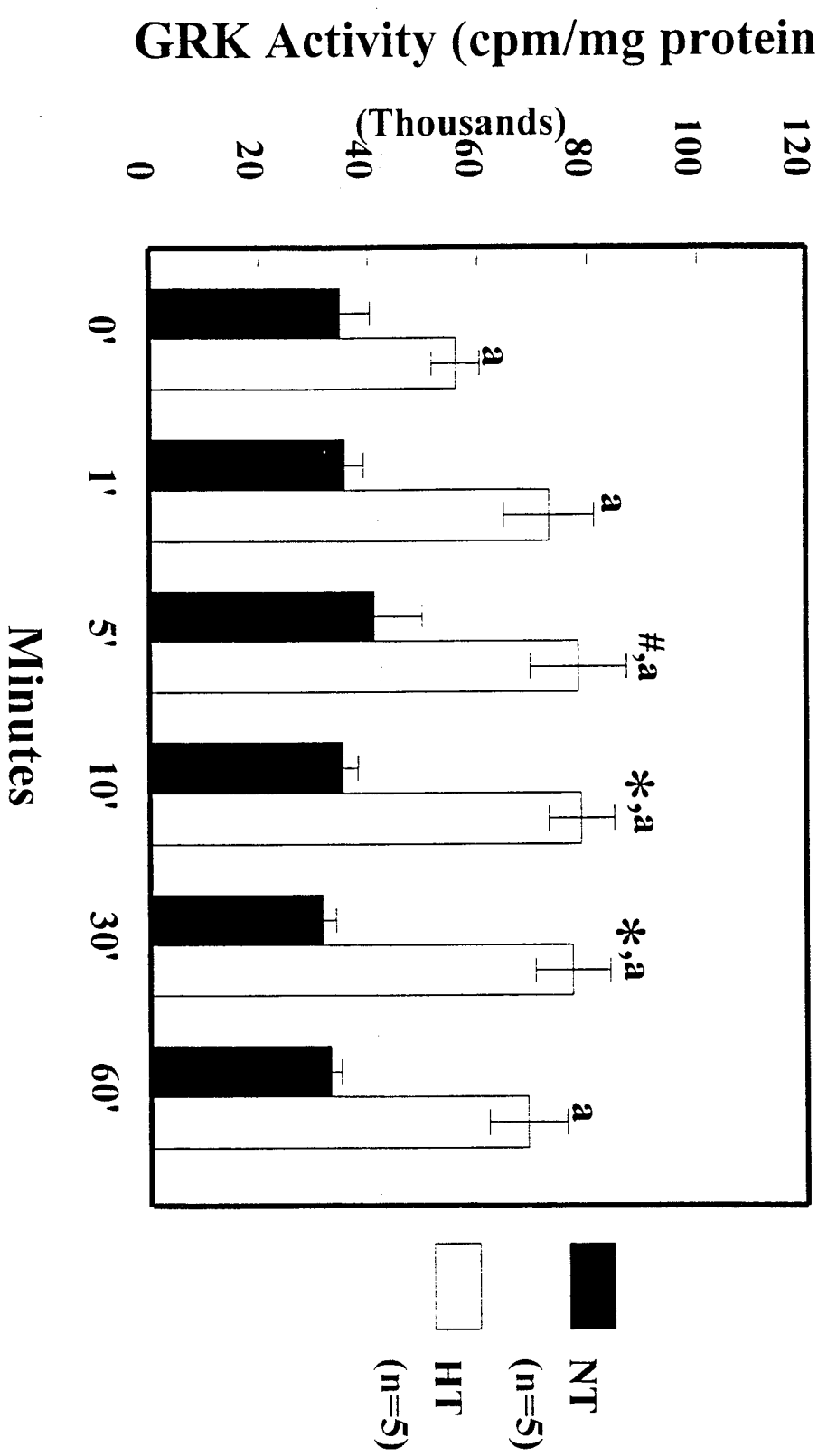

GRK Activity (cpm/mg protein)
(Thousands)
0
20
40
60
80
100
120
Minutes
0'
1'
5'
10'
30'
60'
a
a
#,a
*,a
*,a
a
NT (n=5)
HT (n=5)

Figure 2

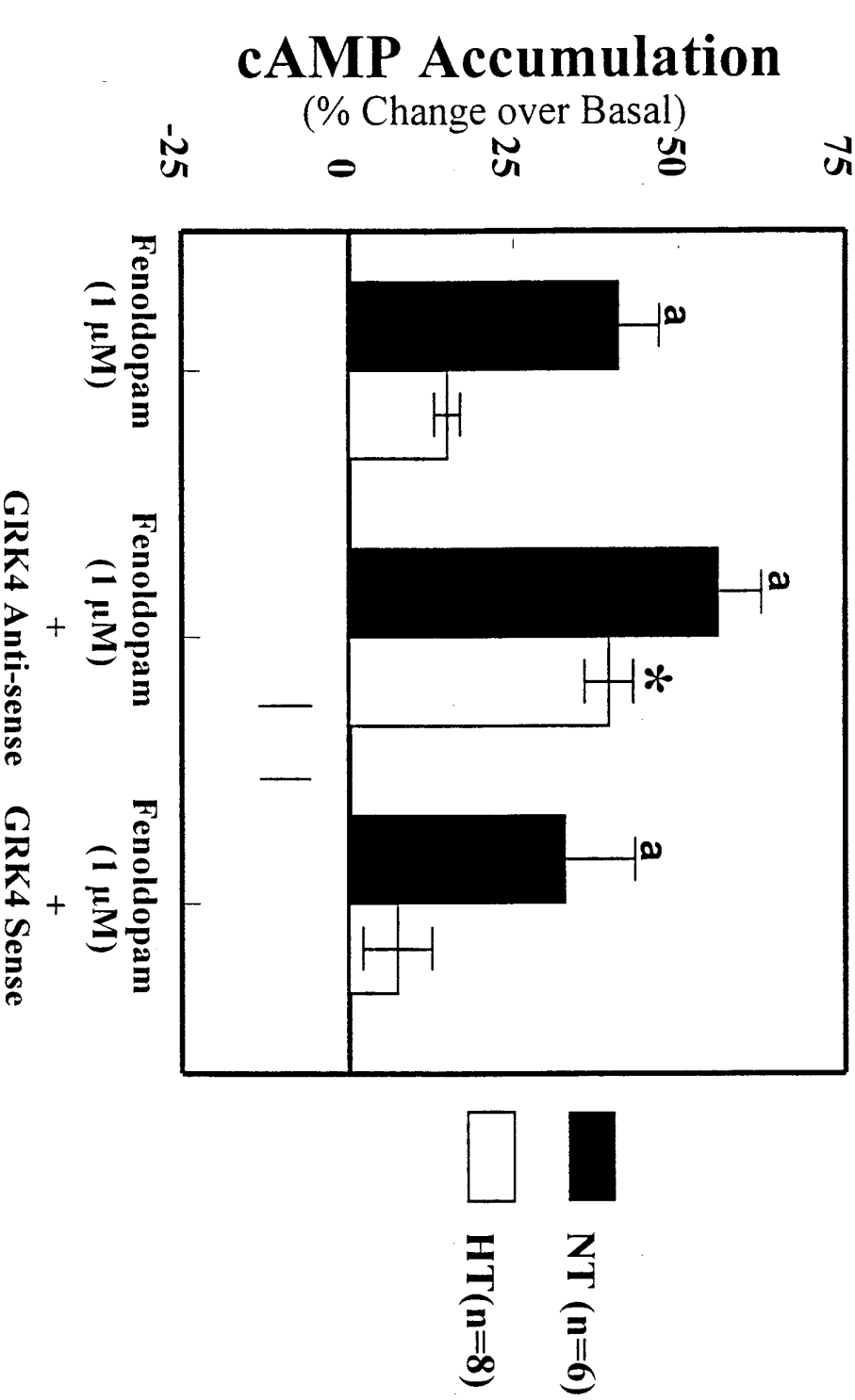

cAMP Accumulation
(% Change over Basal)
-25
0
25
50
75
Fenoldopam (1 μM)
Fenoldopam (1 μM) + GRK4 Anti-sense
Fenoldopam (1 μM) + GRK4 Sense
a
a
*
a
NT (n=6)
HT(n=8)

Figure 3

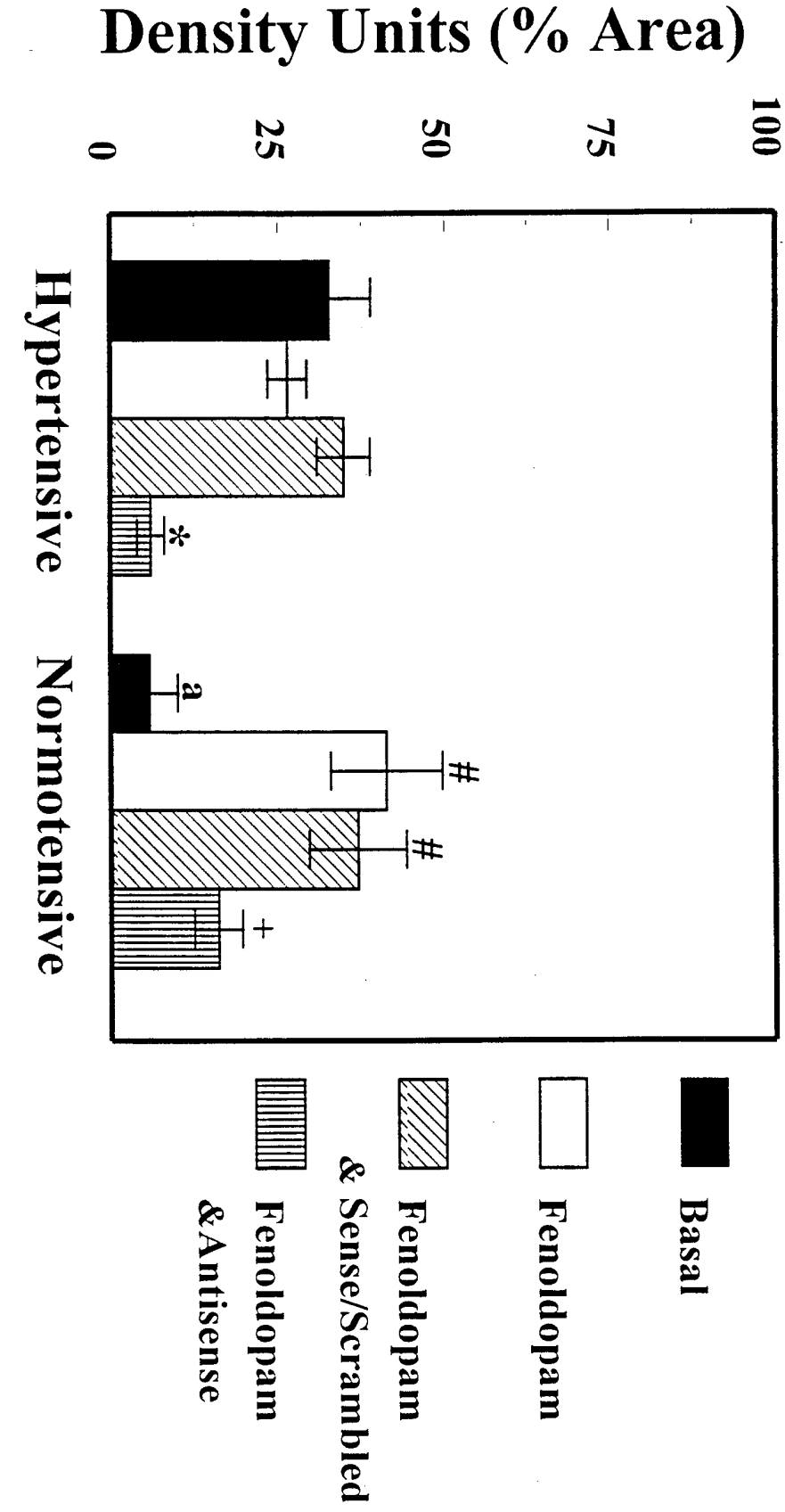

Density Units (% Area)
0
25
50
75
100
Hypertensive
Normotensive
*
a
#
#
+
Basal
Fenoldopam
Fenoldopam & Sense/Scrambled
Fenoldopam &Antisense

Figure 4

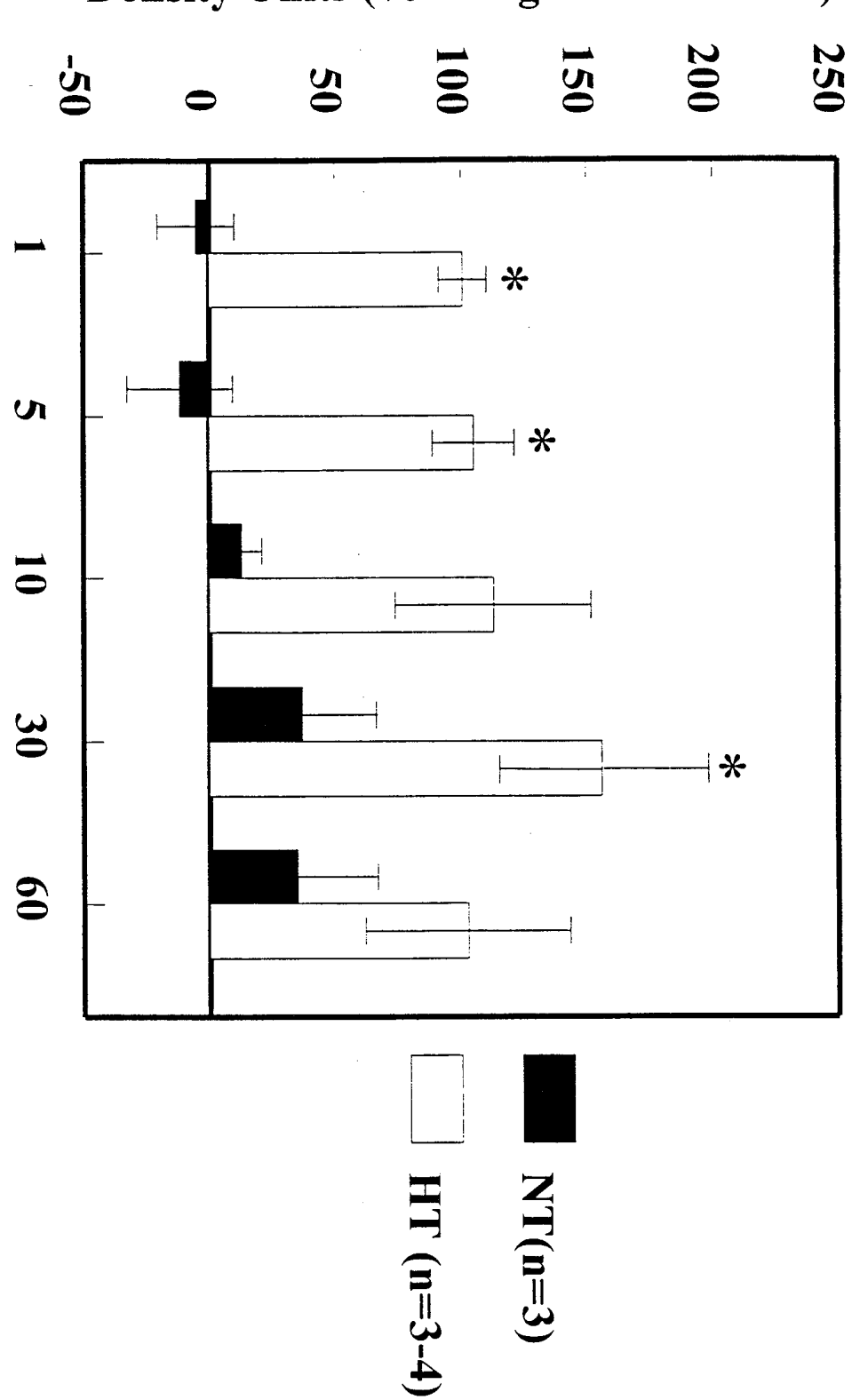
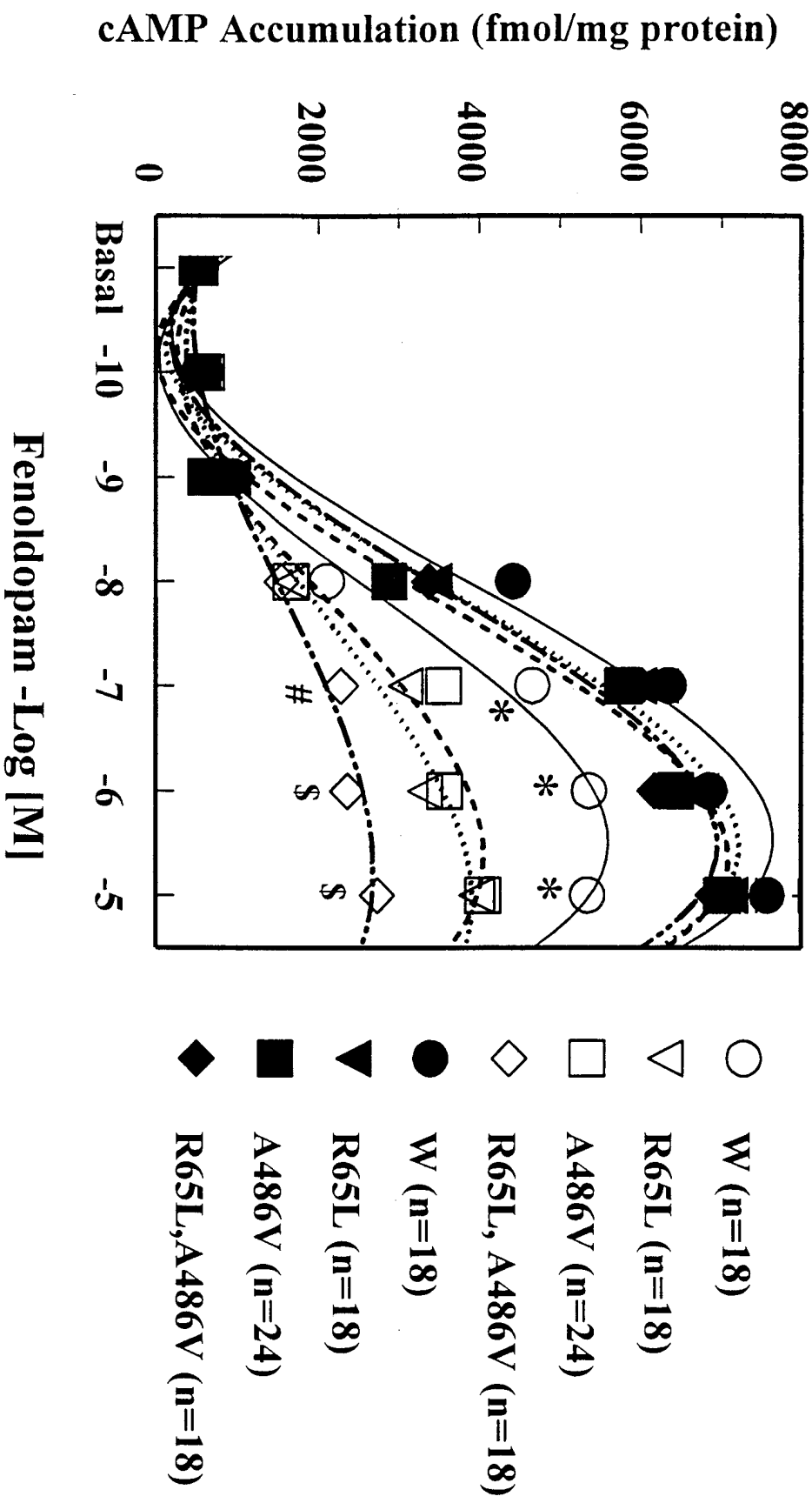

Density Units (%change from control)
-50
0
50
100
150
200
250
1
5
10
30
60
Minutes
*
*
*
NT(n=3)
HT (n=3-4)

Figure 5


cAMP Accumulation (fmol/mg protein)
0
2000
4000
6000
8000
Basal
-10
-9
-8
-7
-6
-5
Fenoldopam -Log [M]
*
*
*
#
$
$
W (n=18)
R65L (n=18)
A486V (n=24)
R65L, A486V (n=18)
W (n=18)
R65L (n=18)
A486V (n=24)
R65L,A486V (n=18)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00663

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/85, 15/86, 15/11, 15/00
US CL : 435/6, 325; 800/13, 14, 18; 536/23.1, 23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 325; 800/13, 14, 18; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MENARD et al. Members of the G protein-coupled receptor kinase family that phosphorylate the beta-2-adrenergic receptor facilitate sequestration. Biochemistry. 1996, Vol. 35, No. 13, pages 4155-4160, entire document.	1-54
Y	LOUDON et al. Altered activity of palmitoylation-deficient and isoprenylated forms of the G protein-coupled receptor kinase GRK6. J. Biol. Chem. 24 October 1997, Vol. 272, No. 43, pages 27422-27427, entire document.	1-54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 FEBRUARY 1999	Date of mailing of the international search report 01 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE-MARIE BAKER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00663

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PREMONT et al. Characterization of the G protein-coupled receptor kinase GRK4: Identification of four splice variants. J. Biol. Chem. 15 March 1996, Vol. 271, No. 11, pages 6403-6410, entire document.	1-54
Y	ALBRECHT et al. Role of the D1A dopamine receptor in the pathogenesis of genetic hypertension. J. Clin. Invest. May 1996, Vol. 97, No. 10, pages 2283-2288, entire document.	1-54
Y	OHBU et al. Dopamine-1 receptors in the proximal convoluted tubule of Dahl rats: defective coupling to adenylate cyclase. American J. of Physiology. 1995, Vol. 268, pages R231-R235, entire document.	1-54
Y	EISNER et al. Dopamine and diltiazem-induced natriuresis in the spontaneously hypertensive rat. Am. J. Physiol. 1997, Vol. 273, pages R317-R323, entire document.	1-54
Y	JIN et al. Dipeptide-induced Cl- secretion in proximal tubule cells. Am. J. Physiol. 1997, Vol. 273, pages C1623-C1631, entire document.	1-54
Y	WOOST et al. Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats. Kidney International. 1996, Vol. 50, pages 125-134, entire document.	1-54
Y	CHEN et al. Receptors in proximal tubular epithelial cells for tubulointerstitial nephritis antigen. Kidney International. 1996, Vol. 49, pages 153-157, entire document.	31-33
Y	RACUSEN et al. Renal proximal tubular epithelium from patients with nephropathic cystinosis: Immortalized cell lines as in vitro model systems. Kidney International. 1995, Vol. 48, pages 536-543, entire document.	31-33
Y	RYAN et al. HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. Kidney International. 1994, Vol. 45, pages 48-57, entire document.	31-33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00663

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS
DIALOG (file: medicine)
search terms: GRK4, renal, kidney, hypertens?, muta?, proximal tubul?, immortal?, D1 receptor