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Identification and Pharmacological Characterization of Prokineticin 2β as a Selective Ligand for Prokineticin Receptor 1

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Running Title: The multiple signaling pathways of prokineticin receptors

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ABBREVIATION: PK, prokineticin; PKR, prokineticin receptor; GPCR, G-protein coupled receptor; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; FLIPR, fluorescence imaging plate reader; CPRG, chlorophenol red-β-D-galactopyranoside; DMEM, Dulbecco's Modified Eagle's Medium; BSA, bovine serum albumin; PBS, phosphate buffered saline; CRE, cAMP responsive element; PTX, pertussis toxin; bp, base pair.

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ABSTRACT

Prokineticins 1 and 2 (PK1 and PK2) have been recently identified from humans and other mammals and play multiple functional roles. PK proteins are ligands for two G-protein coupled receptors, PK receptor 1 (PKR1) and PK receptor 2 (PKR2). Here we report the molecular cloning and pharmacological characterization of an alternatively spliced product of the PK2 gene encoding 21 additional amino acids compared with PK2, designated PK2L (for PK2 Long form). PK2L mRNA is broadly expressed, as is PK2. However, PK2L mRNA expression is lower in brain, undetectable in kidney, and much higher in lung and spleen than that of PK2. We expressed PK2L in mammalian cells and characterized the resulting peptide in comparison with PK1 and PK2. Biochemical characterization indicates that secreted PK2L protein is processed into a smaller peptide by proteolytic cleavage. We designate this smaller form of peptide as PK2β. Co-expression of furin with PK2L significantly increased the PK2β processing efficiency. Functional studies showed that PK1, PK2 and PK2β stimulate intracellular Ca^{2+} response in PKR1 expressing cells with similar potencies. However, the PK2β stimulus of Ca^{2+} responses in PKR2 expressing cells is at least 10-fold less potent than that of PK1 or PK2. Differences in receptor selectivity combined with differential tissue expression patterns suggest PK2 and PK2β might have different functions *in vivo*. PKRs have been reported to couple to G_q and G_i proteins. In this report, we show that PKs not only stimulate Ca^{2+} mobilization, but also induce cAMP accumulation in PKR expressing cells.

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Introduction

Recently two cysteine-rich peptides prokineticin 1 (PK1) and prokineticin 2 (PK2) have been identified and shown to stimulate gastrointestinal (GI) smooth muscle contractions (Li et. al., 2001). PK1, also known as endocrine gland vascular endothelial growth factor (EG-VEGF), stimulates endocrine glands cell proliferation/migration and promotes angiogenesis in the mouse ovary (LeCouter et. al, 2001). PK2, or mammalian Bv8, is believed to affect behavioral circadian rhythms in the suprachiasmatic nucleus (SCN) and promote angiogenesis in the testis (Cheng et al., 2002; Lecouter et al., 2003). PK1 and PK2 are highly homologous to each other as well as to mamba intestinal protein (MIT) (Schweitz et al., 1990, 1999) and a frog skin secreted protein, Bv8. Bv8 is a potent stimulator of GI smooth muscle contractions (Mollay et al, 1999) and stimulates the sensitization of peripheral nociceptors (Negri et al., 2002). PKs bind and activate two closely related G-protein coupled receptors, prokineticin receptor 1 (PKR1) and 2 (PKR2), which are 87% identical by sequence (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). PKs stimulate Ca^{2+} mobilization in PKR expressing cells, presumably through a receptor/G_q protein interaction (Lin et al., 2002a; Masuda et al., 2002; Soga et al., 2002). Pertussis toxin (PTX) inhibits PK1 induced mitogen-activated protein kinase (MAPK) signaling (Lin et al., 2002b), suggesting that PKRs may also couple to G_i proteins.

The non-selectivity of PK1 and PK2 *vs* their receptors (PKR1 and PKR2) *in vitro* raises the question of which ligand(s) activate(s) which receptor(s) *in vivo*. To investigate the

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ligand/receptor relationship of PK1, PK2 and their receptors, we systematically analyzed PK1, PK2, PKR1, and PKR2 mRNA expression in different human tissues and found two splice forms of PK2 mRNA. We isolated the cDNA for the alternatively spliced PK2 mRNA, designated PK2L, which encodes 21 additional amino acids compared with PK2. In this paper, we report that the expression of PK2L results in the production of a short form of the peptide, which we refer to as PK2β. Functional characterization of PK2β in comparison with PK1 and PK2 indicates that PK2β displays strong receptor selectivity for PKR1 over PKR2. In addition, signal transduction studies showed that PKs induce cAMP accumulation in PKR expressing cells, indicating that PKRs are also coupled to G_s proteins.

Materials and Methods

cDNA Cloning of PKR1 and PKR2. The cDNA coding regions for both PKR1 and PKR2 were amplified by polymerase chain reaction (PCR) from human fetal brain cDNA (Clontech, Palo Alto, CA). The primers used for PKR1 were P1: 5' ACG TGA ATT CGC CAC CAT GGA GAC CAC CAT GGG GTT CAT G 3', and P2: 5' ACG TAG CGG CCG CTT ATT TTA GTC TGA TGC AGT CCA CCT C3'. The primers used for PKR2 were P3: 5' ACG CGA ATT CGC CAC CAT GGC AGC CCA GAA TGG AAA CAC 3', and P4: 5' ACG CAT GCG GCC GCG TCA CTT CAG CCT GAT ACA GTC CAC 3'. The PCR conditions were 94ºC for 40 s, 65ºC for 40 s and 72ºC for 3 min (40 cycles). Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was used for all PCR

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reactions. The PCR products were cloned into pCIneo (Promega, Madison, WI) vector and the insert regions were sequenced using an automated DNA sequencer (ABI, Foster City, CA).

Expression and Purification of Prokineticins. Human PK1 mature peptide coding region was PCR amplified from human fetal brain cDNA (Clontech) using two primers P5: 5' TCA TCA CGA ATT CGA TGA CGA CGA TAA GGC TGT GAT CAC AGG GGC CTG TGA GCG GGA TG 3', and P6: 5' ACG ATA GGA TCC CTA AAA ATT GAT GTT CTT CAA GTC CAT G 3'. Human PK2 mature peptide and PK2L pro-peptide coding regions were PCR amplified from human fetal brain cDNA (Clontech) using two primers P7: 5' CAT CAC GAA TTC GAT GAC GAC GAT AAG GCC GTG ATC ACC GGG GCT TGT GAC AAG 3' and P8: 5' ACG ATA GGA TCC TTA CTT TTG GGC TAA ACA AAT AAA TCG 3'. The PCR conditions were 94ºC for 40 s, 65ºC for 40 s and 72ºC for 1 min (40 cycles). The PCR products for PK1, PK2, and PK2L were cloned into a modified pCMV-sport1 (Invitrogen) expression vector, which encodes an alpha peptide signal sequence followed by a FLAG tag (Liu et al., 2003). The PK cDNAs were cloned in-frame after the FLAG coding sequence and the insert regions were sequenced to confirm the identities. The resulting expression vectors encode fusion proteins with a mammalian secreted protein signal peptide followed by a FLAG peptide, an enterokinase cleavage site, and the relevant PKs without their natural signal peptide sequences. The PK1, PK2 and PK2L expressing plasmids were transfected into COS-7 cells using LipofectAmine (Invitrogen). Three days after transfection, the cell culture supernatants were collected and run through ANTI-FLAG M2 agarose (Sigma, St. Louis,

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MO) affinity columns, respectively. The columns were washed with phosphate-buffered saline (PBS) and eluted with 0.1 mM Glycine-HCl, pH 3.0. The eluted protein fractions were immediately neutralized with 1 M Tris-HCl, pH 8.0 and cleaved with enterokinase (Novagen, Madison, WI). The cleaved proteins, which are free of the FLAG tag, were then further purified by reverse phase HPLC using a C4 column (Vydac, Hisperia, CA).

Western Blot. The recombinant PK protein expression was monitored by Western Blot. Briefly, in a 1.5 ml tube, 20 µl of ANTI-FLAG M2 agarose beads slurry were added to 1 ml of cell culture media from COS-7 cells expressing either PK1, PK2, PK2L , co-expressing PK2L and furin, or medium from control COS-7 cells. At the same time, corresponding cell samples were lysed with lysis buffer (100 mM Tris-HCl PH 8.0, 150 mM NaCl, 1% Triton X-100, 1% protease inhibitor cocktail, Sigma) and mixed with the ANTI-FLAG beads. The tubes were incubated at 4ºC on a rocking platform overnight. The beads were centrifuged and washed twice with ice cold TBST (50 mM Tris-HCl PH 7.5, 150 mM NaCl, 0.05% Tween 20). The immuno-precipitated proteins were run onto a 4-20% SDS-PAGE gel under reducing conditions and transferred onto a PVDF membrane (Invitrogen). The membrane was blotted first with ANTI-FLAG M2 antibody (Sigma) and then with goat anti-mouse IgG (horseradish peroxidase conjugated, Sigma). The western blot membrane was then developed with an Amersham ECL kit and imaged by an x-ray film.

Expression, purification, and iodination of C-terminal FLAG-tagged PK2. C-terminal FLAG-tagged PK2 (PK2-f) was constructed as described (Soga et al., 2002).

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Two primers P9: 5' ATC GAG AAT TCG CCA CCA TGA GGA GCC TGT GCT GCG CCC 3' and P10: 5'ACC TGA GGA TCC CTA CTT ATC GTC GTC ATC CTT ATA ATC CTT TTG GGC TAA ACA 3' were used to amplified human whole brain cDNA (Clontech). The PCR-amplified PK2-f was cloned into a mammalian expression vector pCMV-sport1 (Invitrogen). The resulting clones were sequenced to confirm the identities and transfected into COS-7 cells using LipofectAmine (Invitrogen). Three days after transfection, the cell culture supernatant was collected and run through an ANTI-FLAG M2 agarose (Sigma) affinity column. The column was washed with PBS and eluted with 0.1 mM Glycine HCl, pH 3.0. The eluted protein fraction was immediately neutralized with 1 M Tris-HCl, pH 8.0 and then further purified by reverse phase HPLC using a C4 column (Vydac). The purified recombinant PK2-f protein was iodinated using Iodogen reagent (Pierce, Rockford, IL) and ^{125}I-NaI (PerkinElmer, Boston, MA) as described by Pierce. The iodinated PK2-f was purified by a G-50 (Amersham Pharmacia Biotech) gel filtration column.

Radioligand Binding Assays. PKR1 and PKR2 in the expression vector pCIneo (Promega) were transfected into COS-7 cells using LipofectAmine (Invitrogen). Two days after transfection, cells were detached from the culture dishes with 10 mM EDTA in PBS, washed with Dulbecco's Modified Eagle's Medium (DMEM) and seeded in 96-well opaque polylysine-coated plates (BD Biosciences, San Jose, CA) at a density of 50,000 cell per well. Two hours after the seeding, competition binding assays were carried out in the 96-well plates at presence of 100 pM ^{125}I-labeled PK2-f and various concentrations of unlabeled PK1, PK2 or PK2β as competitors. The binding assays were performed in

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DMEM plus 50 mM HEPES, pH 7.2 and 1% bovine serum albumin in a final volume of 100 µl. The binding assays were incubated at room temperature for 1 hour. The binding buffer was aspirated and the cells in 96-well plates were washed three times with ice cold PBS. Microscint-40 (Packard, Meriden, CT) was added to each well (50 µl/well) and the plates were counted on a micro-scintillation counter (Topcount NTX, Packard).

Intracellular Ca^{2+} Mobilization Assays. PKR1 or PKR2 expression construct, either alone or in co-transfection with a chimeric G-protein, Gqi5 (Conklin et al., 1993), were transfected into HEK293 cells using LipofectAmine (Invitrogen). Two days after transfection, cells were detached using PBS containing 10 mM EDTA and seeded in poly-D-lysine coated 96-well black wall tissue culture plates (BD Biosciences). Ligand stimulated Ca^{2+} mobilization was assayed using Fluo-3 Ca^{2+} dye (TEF Labs, Austin, TX) in a FLIPR (Molecular Devices, Sunnyvale, CA) as described previously (Liu et. al. 2001b).

PK stimulation of cAMP accumulation in PKR expressing cells. PK stimulated cAMP accumulation assays were performed using SK-N-MC/β-gal cells (Liu et al, 2001a) stably expressing PKR1 or PKR2. SK-N-MC/β-gal cells harbor a β-galactosidase reporter gene under the control of the cAMP responsive element (CRE). The stable cell lines were created under selection with 400 mg/L G418 (Sigma, St. Louis, MO) following the transfection of PKR1 or PKR2 expression vectors. Increase of the intracellular cAMP concentration is associated with the activation of the CRE promoter, which leads to

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higher β-galactosidase expression whose activity is measured using chlorophenol red-β-D-galactopyranoside (CPRG) as the substrate. Cells were seeded in 96-well tissue culture plates, stimulated with different concentrations of PK1, PK2 or PK2β. Intracelluar cAMP concentrations were indirectly measured by assaying the β-galactosidase activities in the cells as described (Liu et. al. 2001a).

In a different experiment, PKR1 or PKR2 in the expression vectors were co-transfected with a G_s protein expression plasmid into HEK293 cells (ATCC, Manassas, VA) using LipofectAmine (Invitrogen). Human Gs protein long form complete coding region (Bray et al., 1986) was cloned into a mammalian expression vector pcDNA 3.1 (Invitrogen). Two days after transfection, the cells were detached with 10 mM EDTA in PBS, resuspended in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) media, and then plated on 96-well plates at a density of 50,000 cells per well. Two hours after the seeding, cells culture medium was replaced with DMEM/F12 containing 2 mM isobutylmethylxanthine (Sigma) and incubated for 30 min. Different concentrations of PK1, PK2 or PK2β were added to the cells, which were incubated for additional for 30 min in a final volume of 200 μl/well. The reactions were stopped and cAMP was extracted by adding 20 μl of 0.5 N HCl to each well. Cell extracts were assayed for cAMP concentrations using the cAMP [^{125}I] FlashPlate Assay kit (PerkinElmer) as described by the manufacturer.

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RT-PCR detection of PK2L mRNA expression in different human tissues. Eleven cDNA pools (BD Biosciences) from various human tissues were analyzed for expression of PK1, PK2, PK2L, PKR1 and PKR2 mRNA using PCR amplification method. The PCR primers used in the reactions were P7 and P8 as described above for PK2 and PK2L; P11 (5'ACG TAA GAA TTC GCC ACC ATG AGA GGT GCC ACG CGA GTC TCA3') and P12 (5'ACG TAA GAA TTC CTA AAA ATT GAT GTT CTT CAA GTC CAT GGA3') for PK1; P13 (5'CAA CTT CAG CTA CAG CGA CTA TGA TAT GCC TTT GG3') and P14 (5'GAC GAG GAC CGT CTC GGT GGT GAA GTA GGC GGA AG3') PKR1, and P15 (5'TCT CCT TTA ACT TCA GTT ATG GTG ATT ATG ACC TC3') and P16 (5'CGA TGG GAT GGC AAT GAG AAT GGA CAC CAT CCA GA3') for PKR2. All of the PCR reactions were performed using Platinum *Taq* DNA polymerase (Invitrogen) at the conditions of 94 ^{o}C 40 sec, 65 ^{o}C 30 sec, and 72 ^{o}C 1 min for 40 cycles. The PCR products were run on 2% agarose gels, transferred onto nitrocellulose membranes and hybridized with ^{32}P-labeled oligo probes specific for PK1 (5'ACC TGT CCT TGC TTG CCC AAC CTG CTG TGC TCC AGG TTC3'), PK2 and PK2β (5'TGG GCA AAC TGG GAG ACA GCT GCC ATC CAC TGA CTC GTA3'), PKR1 (5'CTG ATT GCC TTG GTG TGG ACG GTG TCC ATC CTG ATC GCC ATC C3'), and PKR2 (5'CGG ATG AAT TAT CAA ACG GCC TCC TTC CTG ATC GCC TTG G3'), respectively. PCR detection of human β–actin gene expression was used for all tissue as a control for the quality of the cDNAs. The primers for PCR detection of human β-actin mRNA expression were 5'GAG AAG AGC TAC GAG CTG CCT GAC GGC CAG GTC 3' and 5'AAG GGT GTA ACG CAA CTA AGT CAT AGT CCG CCT A 3'.

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Results and Discussion

Identification of the PK2L cDNA and molecular characterization of PK2L mRNA tissue expression pattern

In the course of characterizing the PK2 mRNA tissue expression profile using RT-PCR, we identified a PCR fragment with slightly larger size than the predicted PK2 PCR product. Molecular cloning and DNA sequencing of that PCR product indicated that it has a 63-base pair (bp) insertion in the coding region of PK2, which resulted in a protein 21 residues longer (Fig. 1A) and is designated as PK2L. A Genbank search indicated that our sequence encodes a protein that is identical to a protein sequence in NCBI protein database (Genbank Accession Number: Q9HC23) published by Wechselberger and colleagues. (1999). Using a similar method, we have also isolated a rat PK2L cDNA from a rat lung cDNA pool. The complete cDNA sequences for human and rat PK2L have been submitted to Genbank (Genbank Accession Number: AY349131; AY348322). Comparison of the deduced protein sequences indicate the rat and mouse (Genbank Accession Number: NP_056583) PK2L proteins are essentially identical and are highly related to human PK2L with about 90% amino acid identity (Fig. 1B). A DNA sequence comparison of human PK2, PK2L cDNA and PK2 genomic DNA shows that the PK2 gene contains a putative exon region (63 bp) that is used by PK2L mRNA but not by PK2 mRNA (Fig. 1C), indicating that PK2L mRNA is an alternatively spliced isoform from the PK2 gene. The 63 bp insertion in PK2L mRNA leads to a 21-amino acid insertion between Lys^{47} and Val^{48} of the mature PK2 protein. A very similar splice variant was also found in the rat and has been reported in the mouse (Wecheselberger et al., 1999), suggesting that the function of PK2L is conserved among species.

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We have analyzed the mRNA expression profile of PK2L in parallel with that of PK1, PK2, PKR1 and PKR2 in 11 different human tissues using a RT-PCR method. As shown in Fig. 2, our results indicate that each of them has its own unique expression pattern. PK1 mRNA is found predominantly expressed in the placenta while PK2 mRNA is found in all tissues. PK2L mRNA was detected in most tissues tested and expression was found to be highest in the lung and spleen, barely detected in the brain and not detectable in the kidney where PK2 mRNA was detected. PKR1 mRNA was detected in the brain, lung, liver, spleen, and mammary gland. PKR2 mRNA had a very dominant expression in the brain with lower levels of expression in the spleen and mammary gland.

The mRNA expression analysis of PK2L indicates that the PK2L mRNA expression pattern is different from that of PK2, suggesting that the protein encoded by PK2L may function differently. The relatively abundant PK2L mRNA expression in the lung and spleen, where PKR1 mRNA is also expressed, suggests that PK2L may participate some immune functions through activation of PKR1. A chemo-attractive effect of PKs has been shown for adrenal cortical capillary endothelial (ACE) cells expressing PKR (LeCouter et al., 2003). It will be interesting to see whether immune cells express PKR and chemoattract in response to PKs.

Expression, purification, and biochemical characterization of PKs

We have expressed PK1, PK2 and PK2L as secreted fusion proteins with a N-terminal FLAG tag in COS-7 cells. The secreted fusion proteins in cell culture supernatants were

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purified using ANTI-FLAG M2 affinity columns. The affinity-purified proteins were cleaved with enterokinase, and further purified by reverse phase HPLC. The HPLC-purified proteins were greater than 98% pure. The sizes of PK1 (10 kDa) and PK2 (9 kDa) agreed with our prediction. However, the size (6 - 7 kDa) of the purified protein from COS-7 cells expressing PK2L is much smaller than what was predicted (11.5kDa) according to the PK2L cDNA. Since the PK2L coding region encodes 21 additional amino acids compared with PK2, we expected that PK2L should had a higher MW than PK2. However, the purified protein from PK2L transfected cells culture medium has a MW smaller than PK2, strongly suggesting that there is a pro-protein cleavage process for PK2L protein. Therefore we designate the full-length protein as PK2L and the cleaved protein as PK2β. Western Blot analysis of the PK2L expressing cell lysate and cell culture medium indicated that FLAG-PK2L was made in the cells as predicted (13.5 kDa), which is bigger than FLAG-PK1 (11.7 kDa) and FLAG-PK2 (10.8 kDa) (Fig. 3 A). Although trace amounts of FLAG-PK2L are detected in the culture medium, the majority of FLAG-PK2L present in the conditioned medium is processed into a smaller form (8 – 9 kDa), namely FLAG-PK2β (Fig. 3A). Based on the size of the cleaved PK2β, the protease cleavage site is predicted to be in the stretch of 21 additional amino acids present in PK2L. Protein sequence analysis of PK2L indicates that, in the 21 amino acid insert region, there exist two putative furin cleavage sites (Arg-Arg-Lys-Arg^{60} and Arg-Ser-Lys-Arg^{65}), which fit the Arg-X-Lys-Arg or Arg-X-Arg-Arg motif for furin cleavage sites (Steiner et al., 1992, Nakayama, 1997). Similar furin cleavage sites are also present in mouse and rat PK2L but are absent in PK1 and PK2 peptides. Since furin is expressed

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by many different cells including COS-7 cells (Yanagita et al., 1993), PK2L is probably cleaved by endogenous furin prior to secretion from COS-7 cells. Co-expression of furin facilitates the cleavage process (Fig.3A), which supports our theory. The doublet bands of the FLAG-PK2β that appeared in the western blot suggested the differential processing of the PK2β occured at the two different furin cleavage sites. The lower band of the mature PK2β was further purified by reverse phase HPLC and used for pharmacological characterization. Since the basic 21-amino acid insert in PK2L could be a substrate for many different serine proteases including furin and other pro-hormone convertases, what proteases are involved in the maturation of PK2β under the natural conditions remains to be further studied.

PK2β selectively activates PKR1

PK1 and PK2 have been reported to stimulate Ca^{2+} mobilization in PKR expressing cells (Lin et. al., 2002a; Soga et. al., 2002). We compared PK1, PK2 and PK2β stimulation of Ca^{2+} mobilization in PKR expressing cells. Our results showed that PK1, PK2 and PK2β stimulate Ca^{2+} mobilization in PKR1 expressing HEK293 cells at nanomolar concentrations. Unlike PK1 and PK2, which have high potency for both receptors, PK2β only shows high potency for PKR1. The EC_{50} values for all Ca^{2+} assays are summarized in Table 1. The recombinant PK2L protein has been expressed in bacteria and was shown to be active for both PK receptors with similar receptor selectivity to that of PK2β although with significantly lower potency (with an EC_{50} value ~ 500 nM for PKR1 and an EC_{50} value > 1000 nM for PKR2) (Bullock et al., 2004). It seems that the

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cleavage and removal of the 21 amino acids (mostly basic) are important for full activity of PK2β. We also tested N-terminally FLAG-tagged PKs as agonists for PKRs in the Ca^{2+} assay and found that they are inactive, suggesting that the exposure of the natural N-termini of PKs is critical for the agonist function of those peptides. Modifications of the N-terminus of PK1 described in a recent report (Bullock et al., 2004) abolishes the agonist activity of PK1, indicating the N-terminus of PK play an important role in the ligand receptor interaction. In contrast, tagging the C-terminus of PK does not affect the ligand activity significantly. PK2-FLAG, reported in this study and by Soga and colleagues (2002), binds and activates both PKR1 and PKR2 at high affinity.

The expression and functional characterization of PK2β strongly suggest that PKs have two domains. The N-terminal domain alone, amino acids 1 – 47 of PK1 or PK2 mature peptides, already posses both receptor binding and activation abilities. The function of the C-terminal domain (amino acids 48-86 of PK1 or 48-81 of PK2) remains to be studied but we speculate it may have functions such as maintaining structural stability or interacting with the extracellular matrix to form ligand gradients for chemotaxies, which has been reported for adrenal cortical capillary endothelial cells (LeCouter et al., 2003).

Natural disulfide bond assignments are important for organic peptide synthesis and future mutagenesis of peptides for detailed study of the structure/activity relationships of peptides. PKs posses 10 Cys residues, which presumably form 5 pairs of disulfide bonds. It is very difficult to assign the correct disulfide bridges between the 10 Cys residues. The results that we demonstrated by expression of PK2β, which dissected the PK peptides

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into two physical domains, indicate that the 6 Cys residues in the N-terminal domains (amino acids 1-47) form 3 pair of disulfide bonds while the 4 Cys residues in the C-terminal domains (amino acids 48-86 for PK1 and 48-81 for PK2) form 2 pairs of disulfide bonds. The results presented in this report will help detailed structural study of PK in the future.

Prokineticins bind PKR1 and PKR2 with different affinities.

To further characterize PK2β pharmacologically, we investigated the binding properties of PK2β to PKR1 or PKR2 in comparison with that of PK1 and PK2. We attempted to use human PK1 labeled with ^{125}I at Tyr^{75} as the radioligand in binding assays. However, we obtained very little specific binding using either PKR1 or PKR2 expressing cells. Since PK2 dose not have a Tyr, we expressed the C-terminal FLAG-tagged PK2, which has been reported to bind prokineticin receptors with high affinity (Soga et. al., 2002). PK2-FLAG has a Tyr in the FLAG-tag, and can be labeled with ^{125}I. Our results showed that ^{125}I-PK2-FLAG binds PKR1 and PKR2 with high affinities, producing an average signal to noise ratio of 8:1 in the binding assays, and was therefore used as the tracer in the binding assay. COS-7 cells transiently expressing PKR1 and PKR2 were used in competition binding assays. The results indicate that PK2 showed the highest affinity for both PKR1 and PKR2. PK1 demonstrated moderately high affinities for both PKRs. PK2β demonstrated a moderately high affinity for PKR1 but showed very low affinity for PKR2. The ligand rank order of potency for PKR1 is PK2 > PK2β ≅ PK1. The ligand

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rank order of potency for PKR2 is PK2 > PK1 >> PK2β. The IC_{50} values of PK1, PK2, and PK2β for PKR1 and PKR2 are listed in Table 2.

In the Ca^{2+} assay, PK1 demonstrated similar potency to that of PK2 for both PKRs. The binding assay revealed that compared to PK2, PK1 has significantly lower affinity for both receptors. The lower affinity of PK1 may explain the reduced specific binding observed when using ^{125}I-PK1 as the radioligand. Comparing the results from the Ca^{2+} assay and binding assay revealed significant differences between the EC_{50} values and IC_{50} values. The difference between the IC_{50} (from the binding assay) and EC_{50} (from the Ca^{2+} assay) values could be a result of the differences in the assay mechanisms. While IC_{50} values from the binding assay reflect the affinity (or K_d) of ligand/receptor interactions that reach equilibrium, The EC_{50} values from the Ca^{2+} assay may only represent the association rate of the ligand/receptor interactions. In the Ca^{2+} assay, the assay readout is measured seconds after the addition of ligand, which is, in most cases, long before the equilibrium is reached. Ligands with faster association rates (not necessarily higher K_d or affinity value) therefore tend to show higher potency in the Ca^{2+} assays.

Prokineticin receptors are coupled to multiple signal transduction pathways.

It has been reported that PTX inhibits PK stimulated MAP kinase signaling (Lin et al., 2002b), suggesting that PKR activates MAP kinase through activation of G_i-related proteins. A phenomenon we observed in the Ca^{2+} mobilization assays is that the maximum ligand stimulated Ca^{2+} mobilization in PKR2 expressing cells was consistently significantly lower than that in PKR1 expressing cells. Our observation is consistent with

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what has been reported (Lin et. al., 2002a). However, when PKR2 was co-expressed with a chimeric G protein (G_{qi5}), which shifts receptor/G_i coupling to Ca^{2+} mobilization signaling (Conklin et al., 1993), the maximum ligand stimulated Ca^{2+} mobilization in PKR2 expressing cells is dramatically increased to approximately the same level of that from PKR1 expressing cells. This suggests that PKR2 may also be coupled with G_i related G-proteins, in agreement with the earlier report by Lin et al (2002b). The Ca^{2+} mobilization in PKR1 expressing cells is not significantly affected by co-expression of G_{qi5}.

To further investigate the signal transduction pathways used by PKR1 and PKR2, we examined the effects of the PKs on the stimulation of cAMP accumulation in PKR1 and PKR2 expressing cells. We established PKR1 and PKR2 cell lines in SK-N-MC cells harboring a β-galactosidase gene under the control of a CRE promoter. In the host cells, increased cAMP concentration activated the CRE promoter, which led to increased β-galactosidase expression whose enzyme activity was measured using CPRG as the substrate. Our results indicate that PK1, PK2, and PK2β stimulated β-galactosidase activity in PKR expressing cells in a dose-dependent manner. Without PKR expression, SK-N-MC cells showed no response to PKs. The EC_{50} values for PKs to stimulate β-galactosidase activity in PKR expressing cells are shown in Table 3.

To confirm our observation, we measured the cAMP accumulation in HEK293 cells transiently expressing PKR1 or PKR2. Our results indicate that PK stimulates cAMP accumulation in PKR expressing cells specifically. HEK293 cells without PKR

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expression did not respond to PK stimulation. The ligand stimulated cAMP accumulation is significantly increased if the G_s protein is co-expressed with PKRs (Fig. 4). In the cAMP accumulation assays using HEK293 cell expressing G_s and PKR1 or PKR2, does response curves have been generated and the ligand rank order of potency for PK1, PK2, and PK2β to either PKR1 or PKR2 expressing HEK293 cells are similar to those from SK-N-MC cells expressing PKRs. The EC_{50} values for PKs in stimulation of cAMP accumulation in PKR1 or PKR2 expressing HEK293 cells are also shown in Table 3.

Some G-protein coupled receptors have been shown to interact with different G-proteins, including G_q, G_i and G_s proteins (Chabre et al., 1994; Liu et al., 2002). PKs stimulate Ca^{2+} mobilization in PKR expressing cells (Lin et. al., 2002a; Sago et. al., 2002), suggesting that PKRs are coupled with G_q proteins. The fact that PK induced activation of MAPK is PTX-sensitive (Lin et al., 2002b) suggests that PKR may be also coupled with G_i proteins. This hypothesis is supported by our results, which showed that the co-expression of G_{qi5} with PKR2 increases PK stimulated Ca^{2+} response in PKR2 expressing cells. Our results showing PK stimulation of cAMP accumulation in PKR expressing cells indicate that PKRs are capable of coupling to G_s proteins. These different lines of evidence strongly suggest that PKRs can couple to different G-proteins.

Conclusions

We have identified and characterized PK2β as a selective ligand for PKR1. Our results, in addition to adding new knowledge to the PK/PKR ligand/receptor system, provided a

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potential tool for *in vivo* functional study of PKR1. Also in this report, our results indicate that PK peptides posses two domains with the receptor binding and activation domain located at the N-terminus. The results from the recombinant expression of PK2β in mammalian cells also physically dissected PK peptides in two segments, which provide useful information for the future study of the structure-activity relationships of PK peptides. Functional studies indicate that, in addition to Ca^{2+} mobilization, PKs also stimulate cAMP accumulation in PKR expressing cells. G_{qi5} enhances Ca^{2+} response in PKR2 expressing cells suggesting possible PKR/G_i coupling. Our results in combination with previous reports (Lin et al., 2002a, b; Masuda et al., 2002; Soga et al., 2002) strongly suggest that PKRs are capable of coupling to multiple G-proteins. PKs are multi-functional peptides. Different natural cells expressing PKRs may have different G-protein expression patterns and hence respond to PK differently thus allowing those cells to perform different physiological functions in respond to the same ligand stimulation.

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Figure Legends

Fig. 1. A. Amino acid sequence comparison between human PK2 and PK2L (without signal peptide). The 21 additional amino acids in PK2L are highlighted in bold letters. The furin recognition sequences are underlined. The potential furin cutting sites are indicated by arrows. B. Amino acid sequence comparison between human and rat/mouse PK2L. Rat and mouse PK2L are identical. The putative furin recognition sequences are underlined. C. The gene structure of PK2 and the differential exon usage by PK2 and PK2L mRNA. The numbers indicate the nucleotide positions in PK2 and PK2L coding regions respectively. ATG and STP represent the translation start and stop codons, respectively.

Fig. 2. The mRNA expression profiles of PKs and PKRs. RT-PCR products for PKR1, PKR2, PK1, PK2 and PK2L mRNA samples were run in agarose gels, transferred to nitrocellulose membrane, and hybridized with specific probes respectively. RT-PCR detection of human β-actin mRNA expression was used as an internal control.

Fig. 3. A. Western blot analysis of recombinant PKs expressed in COS-7 cells. N-terminal FLAG-tagged PK1, PK2 and PK2L either from cell conditioned media or from cell lysates were analyzed by Western Blot using anti-FLAG M2 antibody. **B.** Schematic diagram for PK2, PK2L and PK2β. The 21 amino acid insertion in PK2L is shown in hatched box. The putative furin cleavage sites are indicated with arrows.

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Fig. 4. Stimulation of cAMP accumulation in HEK 293 cells expressing PKR1 or PKR2. HEK293 cells were either mock tranfected or transfected by PKR1, PKR2, Gs or co-transfection of Gs with PKR1 or PKR2. The transfected cells were stimulated either with 1 μM of PK2 or buffer as the control. The accumulated cAMP was measured using cAMP [^{125}I] FlashPlate Assay kit.

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Tables

Table 1

EC_{50}[a] values for PK1, PK2 and PK2β to stimulate Ca^{2+} mobilization in PKR expressing HEK293 cells

	PKR1	PKR2
PK1	1.1 ± 0.4	7.7 ± 2.1
PK2	0.8 ± 0.23	3.6 ± 1.6
PK2β	1.5 ± 0.56	80 ± 9.7

[a]EC_{50} values were expressed in nM (mean ± S.E.) from triplicate experiments

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Table 2

Comparison of IC_{50}[a] values of PK1, PK2 and PK2β on PKR1 and PKR2

	PKR1	PKR2
PK1	27.6 ± 8.2	52.2 ± 16.4
PK2	4.5 ± 0.8	6.4 ± 1.3
PK2β	34.6 ± 13.5	> 1,000

[a]IC_{50} values were expressed as nM (mean ± S.E.) from triplicate experiments in radioligand competition binding assays.

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Table 3

EC_{50}[a] values for PK1, PK2 and PK2β to stimulate cAMP accumulation in PKR expressing cells.

	SK-N-MC/β-gal Cells[b]		HEK293 Cells[c]	
	PKR1	PKR2	PKR1	PKR2
PK1	8.34 ± 2.3	20 ± 4.2	16.8 ± 3.4	60 ± 6.5
PK2	1.27 ± 0.4	12.1 ± 2.8	3.17 ± 1.6	41 ± 5.3
PK2β	11.5 ± 0.9	> 1,000	23.4 ± 4.4	> 1,000

[a]EC_{50} values were expressed as nM (mean ± S.E.) of triplicate experiments.

[b]Stable expressing cells.

[c]Transient expressing cells.

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Figure 1

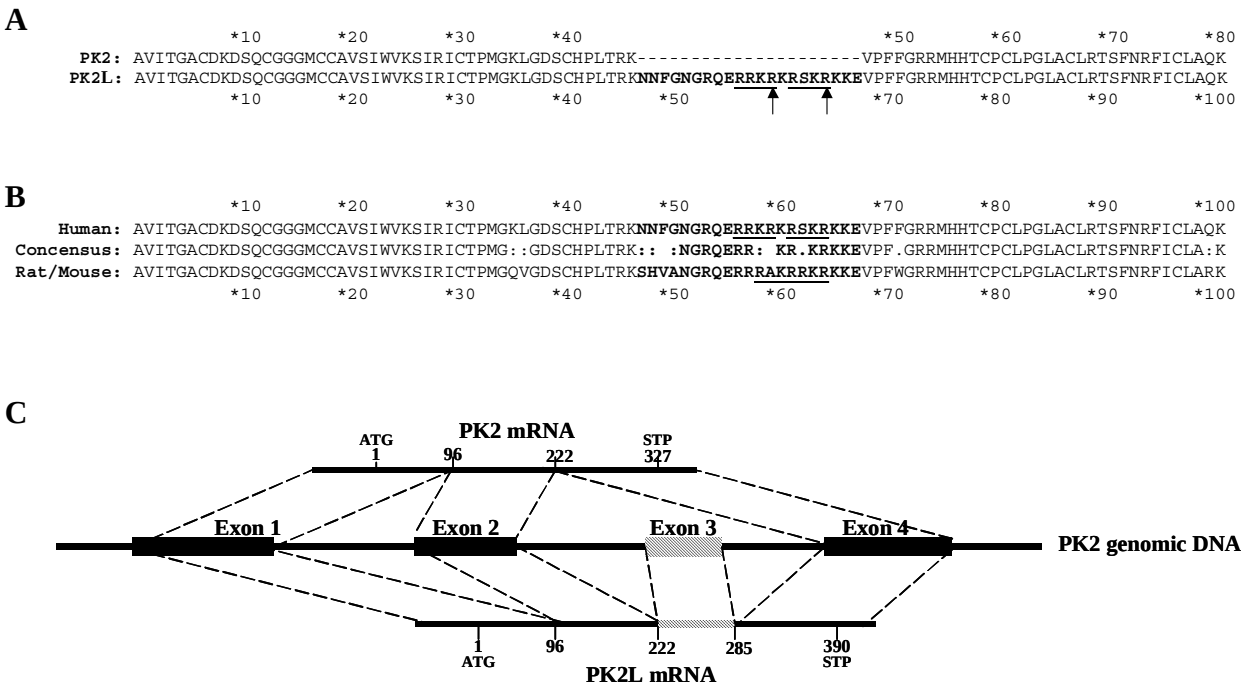

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Figure 2

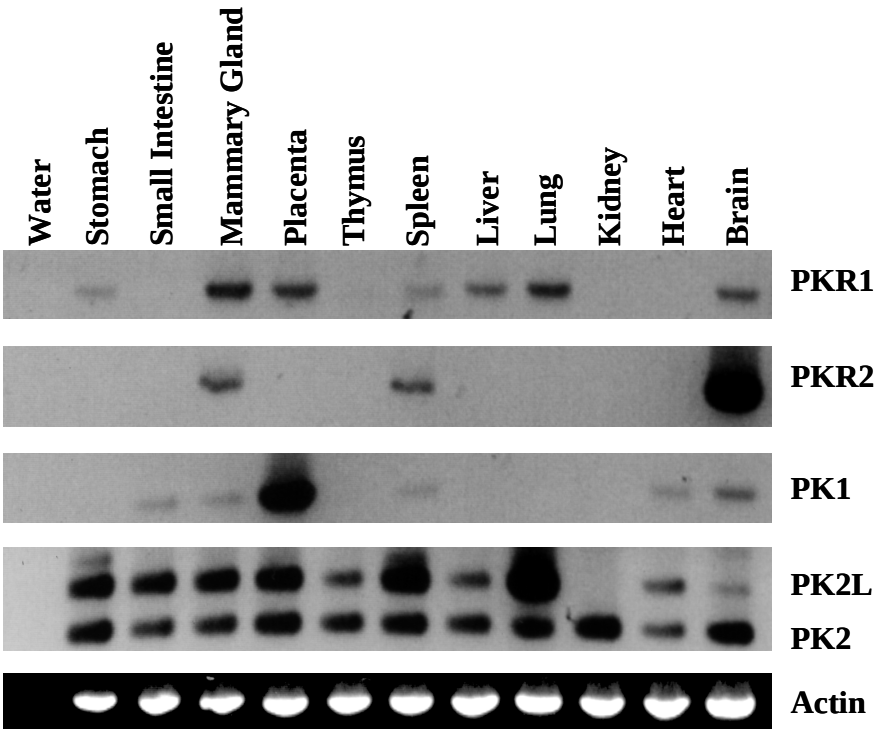

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Figure 3

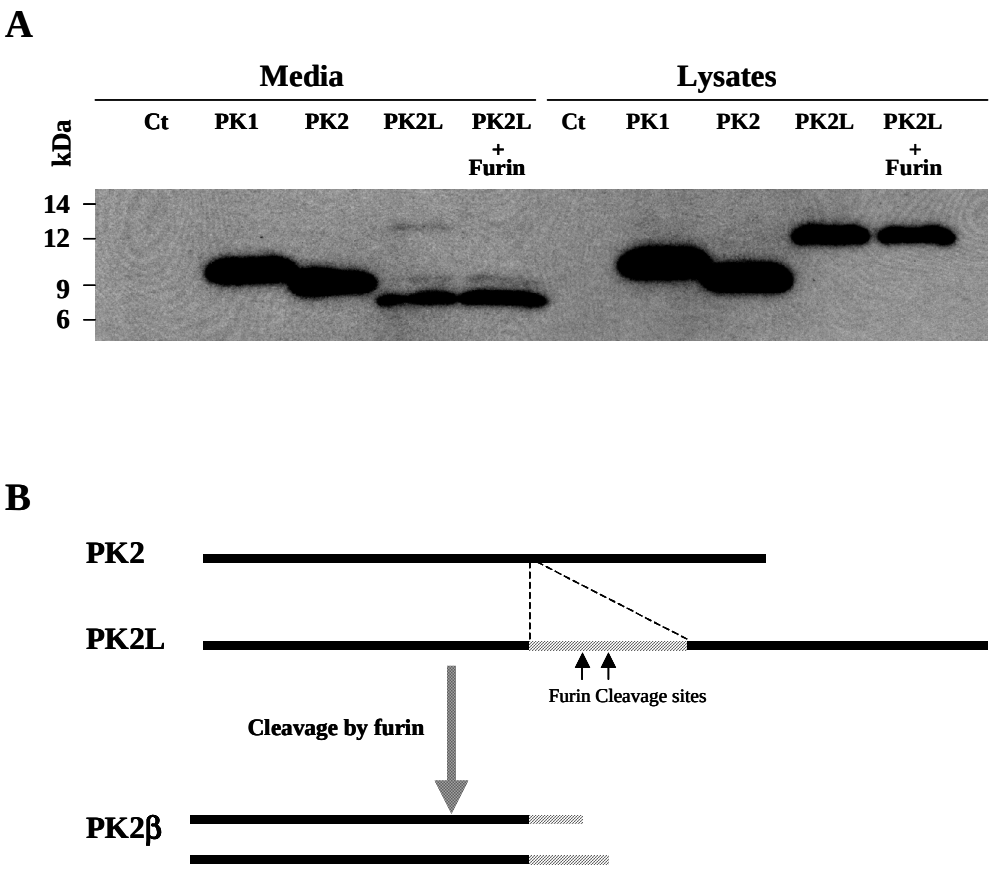

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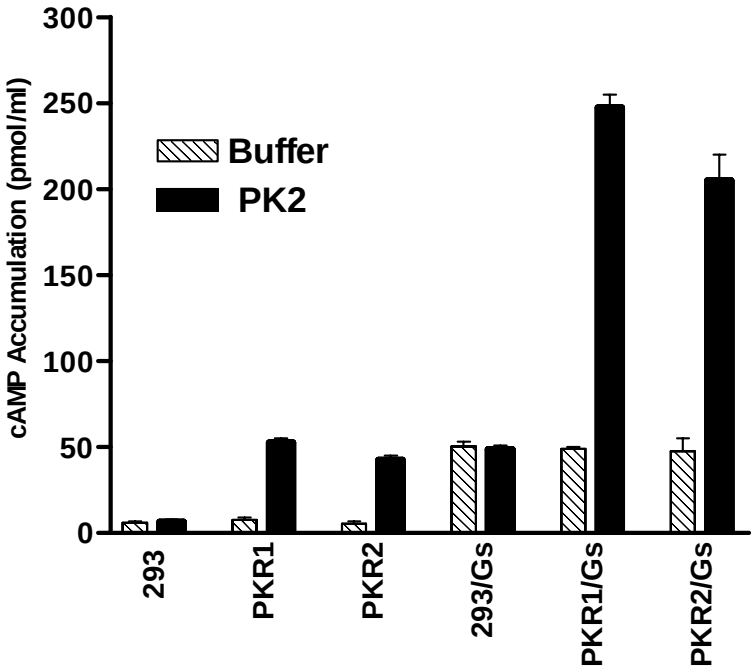
Figure 4



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