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Protein kinase A-dependent phosphorylation of B/K protein

Hemin Chin[1]*, Sung-Ho Choi[2,3]*, Yoon-Seong Jang[2], Sung-Min Cho[2], Ho-Shik Kim[2], Jeong-Hwa Lee[2], Seong-Whan Jeong[2], In-Kyung Kim[2], Grace J. Kim[4] and Oh-Joo Kwon[2,5]

[1]National Eye Institute, National Institutes of Health
Bethesda, MD 20892, USA
[2]Department of Biochemistry, College of Medicine
The Catholic University of Korea
Seoul 137-701, Korea
[3]Present address: R&D center, PetaGen Inc.
B199F Yonsei Engineering Research Complex
Seoul 120-140, Korea
[4]Princeton University, Princeton
NJ 08544, USA
[5]Corresponding author: Tel, 82-2-590-1179;
Fax, 82-2-596-4435; E-mail, ojkwon@catholic.ac.kr
*These authors contributed equally to this work.

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Abbreviations: CDS, coding sequence; DDAVP, (deamino-Cys[1], D-Arg[8])-vasopressin; ER, endoplasmic reticulum; GST, glutathione S-transferase; PKA, protein kinase A; SNAP, soluble NSF attachment protein; V1R, vasopressin receptor type 1; V2R, vasopressin receptor type 2

Abstract

We have previously isolated a novel protein "B/K" that contains two C2-like domains. Here, we report the isolatioin and mRNA distribution of a human B/K isoform, and protein kinase A (PKA)-dependent phosphorylation of the B/K protein. The 1.5 kb human B/K cDNA clone exhibits 89% and 97% identities with rat B/K in the sequences of nucleotide and amino acid, respectively. Human B/K isoform encodes a 474 amino acid protein and shows structural features similar to the rat counterpart including two C2 domains, three consensus sequences for PKA, absence of a transmembrane region, and conservation of the N-terminal cysteine cluster. On Northern and dot blot analyses, a 3.0 kb B/K transcript was abundantly present in human brain, kidney, and prostate. Among the brain regions, strong signals were observed in the frontal and temporal lobes, the hippocampus, the hypothalamus, the amygdala, the substantia nigra, and the pituitary. Recombinant B/K proteins containing three consensus sites for PKA was very efficiently phosphorylated *in vitro* by PKA catalytic subunit. B/K protein which was overexpressed in LLC-PK1 cells was also strongly phosphorylated *in vivo* by vasopressin analog DDAVP, and PKA-specific inhibitor H89 as well as type 2 vasopressin receptor antagonist specifically suppressed DDAVP-induced B/K phosphorylation. These results suggest that B/K proteins play a role as potential substrates for PKA in the area where they are expressed.

Keywords: calcium signaling; phosphorylation; protein kinase A; vasopressin

Introduction

Synaptic vesicle exocytosis is rapidly triggered by calcium influx into the presynaptic nerve terminals through the voltage-gated calcium channels (Atlas, 2001). The calcium concentration dependence suggests that the release of neurotransmitters requires the cooperative interactions of multiple calcium-binding sites and high concentration of calcium (Goda, 1997). Among the proteins that have been found in nerve terminals, synaptotagmin has been suggested as a primary candidate for calcium sensors in the release of neurotransmitters because of its location on synaptic vesicles and the presence of two C2 domains in its cytoplasmic region (Koh and Bellen, 2003).

The C2 domain was originally defined as a sequence motif in protein kinase C activated by calcium and phospholipids (Kikkawa *et al*., 1989), and the C2-like domain has been subsequently found in many other proteins. In neural tissues, multiple synaptotagmin isoforms (Sudhof, 2002), Rabphilin-3A (Shirataki *et al*., 1993), two Doc2 proteins (Orita *et al*., 1995), three Munc13 isoforms (Brose *et al*., 1995), N-copine (Nakayama *et al*., 1998), and Syt-like protein (Fukuda and Mikoshiba, 2001b) have been identified as proteins with two or more C2-like domains, and their roles in calcium-dependent exocytosis have been suggested. Recently, multiple C2 domain and transmembrane region proteins were newly classified as a novel family of evolutionarily conserved C2 domain proteins (Shin *et al*., 2005).

We have isolated a new protein which also contains two C2-like domains from rat brain (Kwon *et al*., 1996a). B/K is a 474 amino acid protein and is

predominantly expressed in the brain and kidney, hence the name. In its primary structure, B/K has several unique features. B/K protein can bind to the plasma membrane via the N-terminal cysteine cluster although it does not contain a transmembrane domain. Three negatively charged amino acids in the C2a domain that has been suggested to be necessary for calcium binding (Shao *et al*., 1996) are substituted. Moreover, B/K has three consensus sequences for protein kinase A (PKA). However, the role of B/K proteins attributed to these unique features still remains to be established. We recently reported the distribution pattern of B/K protein in the rat brain (Lee *et al*., 2001a) and the retina (Kwon *et al*., 2000), and found the induction of B/K protein expression in the vulnerable regions of the hippocampus by kainate-induced excitotoxic injury (Jang *et al*., 2004) as well as in the retina and kidney by ischemia-reperfusion (Ju *et al*., 2000; Lee *et al*., 2001b). These results suggest the possibility that B/K protein may play a role in some pathological conditions.

Here we report the cloning and tissue distribution of a human B/K isoform, and the PKA-dependent phosphorylation of B/K protein.

Materials and Methods

Cloning and sequencing of human B/K cDNA

Human B/K cDNA clones were isolated using GeneTrapper cDNA Positive Selection System (Life Technologies, Rockville, MD) according to the manufacturer's instruction. Briefly, the oligonucleotide primer BK-7-11 (5'-GGC GAA GAT GGC GTA CAT-3') was synthesized and biotinylated using biotin-14-dCTP. From a human brain cDNA library in pCMV-SPORT (Life Technologies), ten cDNA clones (hB/K-11 through hB/K-20) were isolated using the ^{32}P-labeled B/K cDNA probe. The 261 bp cDNA fragment corresponding to the nucleotide 266-526 of rat B/K (GenBank U30831) was used as a probe. Three positive clones (hB/K-11, -12, and -14) remained positive by Southern hybridization of purified cDNA with the same probe, and was sequenced. Among three clones, only hB/K-12 cDNA clone showed a high degree of identity in the nucleotide sequence with rat isoform. Using PCR primers from the sequences surrounding the unique region [HBK21S (5'-TGA GCG CGG GCG AAA ATG GCG TAC ATT C-3') and HBK472AS (5'-TTC CTG AAA TAG TCG TCG GGG TTA TAG G-3')], we prepared a new human B/K probe and isolated an additional cDNA from the human pituitary gland Marathon-Ready cDNA (Clontech, Palo Alto, CA), and subcloned it into the Zero Blunt PCR vector (Invitrogen, Carlsbad, CA).

Northern and dot blot analyses

Human RNA Master Blot and human brain Multiple Tissue Northern Blot II were purchased from Clontech. They were hybridized in ExpressHyb solution (Clontech) for three hours with the ^{32}P-labeled 261 bp B/K probe. The blot membranes were washed twice at room temperature with 2XSSC/0.05% SDS for 20 min each and subsequently with 0.1X SSC/0.1% SDS for 20 min at 50^{o}C, and exposed to Hyperfilm-Max (Amersham Pharmacia, Piscataway, NJ) at -70^{o}C for 3 days.

Preparation of GST-B/K fusion proteins

The rat B/K cDNA encoding whole coding region (BK-CDS) or the region containing all three putative PKA phosphorylation sites of B/K protein (BK-1528, corresponding to 1-175 amino acid of rat B/K protein) were amplified by polymerase chain reaction (PCR). The sense primer used for PCR was 5'-ATG GCG TAC ATC CAG TTG GAA CCA-3', and antisense primers were 5'-TCA GGT CAC CTC CAG CGA GGC CGG -3' for BK-CDS, and 5'-CGG TCA GAG AGT CTA CGT CAT CAA G-3' for BK-1528, respectively. The PCR products were subcloned into *Eco*RI site of pGEX-KG (pGEX-KG-BK-CDS and pGEX-KG-BK1526) and verified by DNA sequencing (Sanger *et al*., 1977). B/K protein which was fused with glutathione S-transferase (GST) was expressed in *Escherichia coli* DH5α (Invitrogen), and the cells were harvested and subjected to sonic dismembrator Model 300 (Fisher, Pittsburgh, PA). After centrifugation at 12,000 *g* for 15 min at 4^{o}C, aliquots were separated by 10% sodium dodesylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After gels were stained with 0.05% coomassie blue, target proteins were purified by electroelution from the gels using an electro-eluter model 422 (BioRad, Hercules, CA) according to the manufacturer's instruction. The purified protein was dialysed for 48 h against 50 mM Tris (pH 7.4) and the protein concentration was estimated by BCA protein assay reagent (Pierce, Rockford, IL).

In vitro phosphorylation of recombinant B/K proteins

In vitro phosphorylation of the recombinant B/K protein was performed as previously described (Izawa *et al*., 1996) with some modifications. Phosphorylation was carried out in 40 μl of phosphorylation buffer (50 mM Tris pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$) containing 0.1 μM phosphatase inhibitor calyculin, 20 μmoles of substrates (GST, GST-B/K proteins, and histone as a

control), and 10 U of PKA catalytic subunit (Sigma, St. Louis, MO). Reactions were initiated by adding 10 μl of phosphate donor mixture containing 0.1 mM ATP and 10 μCi of [γ^{32}P]ATP (Amersham Pharmacia), continued for the 40 min at 30°C, and terminated by adding 2.5 μl of 0.5 M EDTA. Ten microliters of the samples were subjected to SDS-PAGE and followed by protein staining and autoradiography.

Cell culture and transfection

LLC-PK1 cells, a porcine proximal tubule cell line, obtained from the American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5 mM glucose, 4 mM glutamine, 10 mM HEPES, and 100 U/100 mg of penicillin/streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C.

B/K expression constructs were prepared as follows. cDNA corresponding to the whole coding region of rat B/K was amplified with PCR and cloned into pCR3.1 vector in correct (pCR3.1-B/K-SE) or reverse (pCR3.1-B/K-AS) orientations.

LLC-PK1 cells were transfected with pCR3.1, pCR3.1-B/K-SE or pCR3.1-B/K-AS using lipofectamine (Life Technologies), and stably transfected cells were cultured in the presence of G418 and isolated using a cloning ring.

In vivo phosphorylation of B/K protein

Transfected LLC-PK1 cells (3×10^5 cells/well, 6 well plate) were precultured in phosphate-free medium for 0.5 h, and further incubated for 2 h in the presence of 100 μCi of ^{32}P-H_2PO_4 (Amersham Pharmacia). Type 2 vasopressin receptor (V2R)-mediated phosphorylation of B/K protein was initiated by treatment with 1 or 2 nM of a vasopressin analog (deamino-Cys^1,D-Arg^8)-vasopressin (DDAVP, Bachem, Bubendorf, Switzerland) for 15 min, and stopped by placing the plates on ice for 10 min. Phosphorylated B/K protein was quantified as follows. The cells were washed twice with cold phosphate-buffered saline, solubilized for 45 min with 1 ml of 20 mM Tris-Cl (pH7.5) containing 1% Triton X-100, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail (Boehringer Manheim, Mannheim, Germany), and 0.1 μM calyculin A (Sigma). After clearing the cell lysate by centrifuagation at 15,000 *g* for 30 min at 4°C, supernatants were preincubated with protein A-Sepharose CL-4B (Pierce) for 1 h and finally immunoprecipitated with 8 μg of affinity purified rabbit anti-rat B/K IgG (Lee *et al.*, 2001a). The immunoprecipitates were subject to

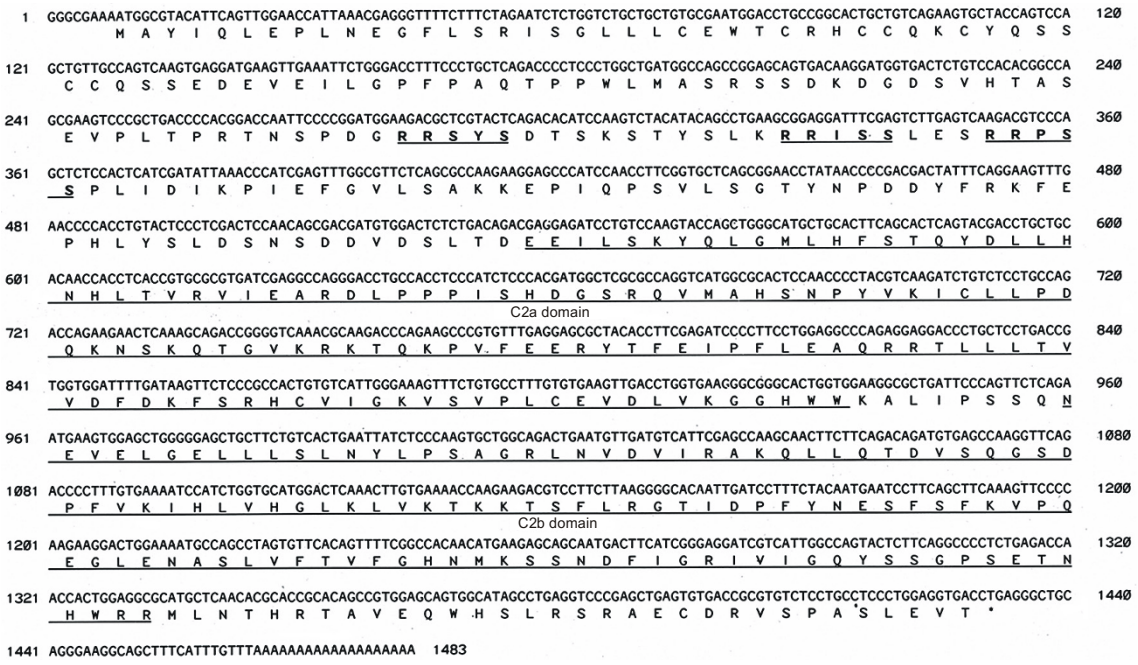

Figure 1. Nucleotide and deduced amino acid sequences of human B/K protein. The amino acid sequence is shown in a single-letter code below the nucleotide sequence, and the sequences are numbered on the left. Amino acid sequences corresponding to the C2a and C2b domains are underlined by solid lines. Putative protein kinase A phosphorylation sites of B/K are shown in boldface with underlines.

SDS-PAGE and followed by autoradiography. To examine the specificity of B/K protein phosphorylation to PKA, a PKA inhibitor (H-89, 40 μM, Sigma) was added 15 min prior to the addition of ^{32}P-H_2PO_4.. V2R specificity of the B/K protein phosphorylation was also evaluated by the pretreatement of a type 1 vasopressin receptor (V1R) antagonist [$d(CH_2)_5^1$,Tyr(Me)^2,Arg^8]-vasopressin (10 μM, Bachem) or a V2R antagonist [$d(CH_2)_5^1$,D-Ile^2,Ile^4,Arg^8,Ala-NH_2^9]-vasopressin (10 μM, Bachem) 5 min before DDAVP treatment.

Results

A human B/K cDNA (hB/K-12) which contained the 1.5 kb insert exhibited overall nucleotide sequence identity of approximately 90% with rat B/K, but differed significantly in its N-terminal region. To rule out the possibility that this discrepancy is due to a cloning artifact, we have isolated an additional cDNA clone from human pituitary cDNA using the primer pair derived from the N-terminal region of hB/K-12 cDNA, and confirmed the result (Figure 1). On the primary structure, the human isoform showed a high similarity to the rat B/K counterpart. First, both isoforms are 474 amino-acid proteins that share 89% and 97% identities in the sequences of nucleotide and amino acid, respectively (Figures 1 and 2A). Whereas overall identity of amino acid sequences between two isoforms is high, distinct sequences are present in the N-terminal region (corresponding to the amino acids 19-36 of rat B/K). Second, two putative calcium binding domains (C2a and C2b in Figure 1) are conserved and the amino acid identity between them reaches 98%. Third, all three B/K isoforms including the murine isoform show no transmembrane domain and instead the N-terminal cysteine cluster for membrane localization is completely conserved among them (Figure 2B) (Kwon *et al.*, 1996a; Fukuda and Mikoshiba, 2001a). Fourth, three consensus sequences for phosphorylaton by PKA are highly conserved except the substitution of one of the three serine residues for tyrosine in the first site of the human isoform (RRSSS to RRSYS) (Figure 2C). And finally, three of five potential calcium binding amino acids in C2a domain which are conserved in other proteins containing double C2-domains (Shao *et al.*, 1996) are also substituted in human isoform (Figure 2D).

On Northern blot (Figure 3A), a 3.0 kb B/K mRNA was highly expressed in the cerebral cortex, frontal and temporal lobes of the human brain regions. We further determined the distribution of B/K transcript in various human tissues by dot blot analysis (Figure 3B). In adult human tissues, B/K mRNA was expressed highly in the pituitary gland, kidney and prostate, and moderately in the thyroid gland. In the

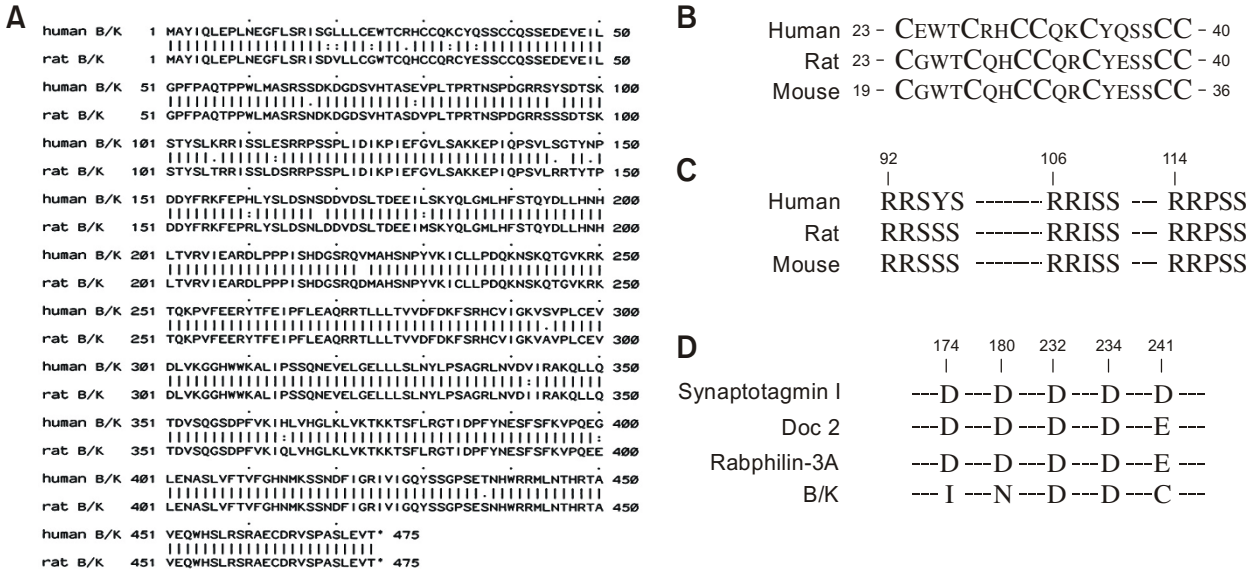

Figure 2. Sequence comparison of B/K proteins between the species. (A) The amino acid sequence of human B/K protein is aligned for maximal homology with rat B/K protein. Amino acids are indicated in a single-letter code. Identities between residues are expressed by a solid line (identical), two dots (high homology), one dot (low homology), or none (no homology). (B) A cysteine cluster which may play a role for membrane binding of B/K protein was conserved among mouse, rat and human B/K proteins. (C) Conservation of consensus sequences for PKA phosphorylation in three B/K protein isoforms. (D) Substitution of the amino acids necessary for calcium/phospholipids binding in the C2a domain of B/K protein. Amino acids of synaptotagmin I are numbered.

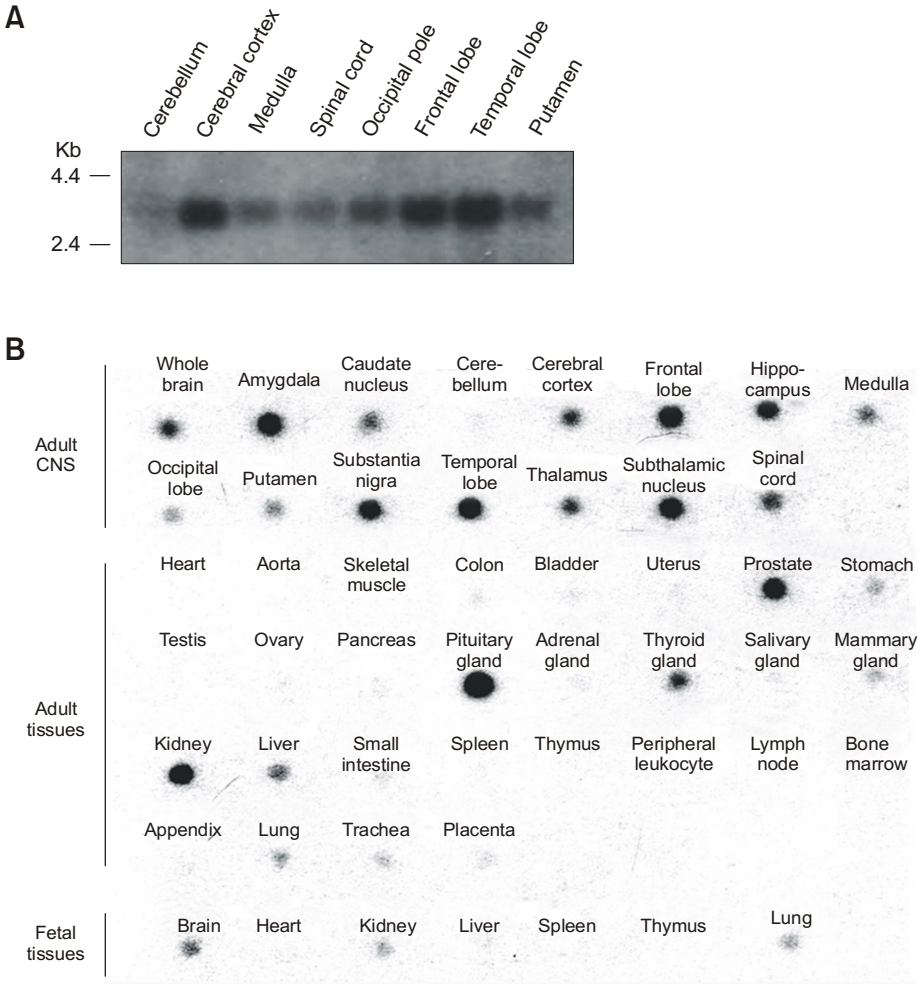

Figure 3. Distribution patterns of the human B/K transcript. Tissue distribution of the human B/K mRNA was examined using (A) human brain Multiple Tissue Northern Blot II (Clontech) and (B) human RNA Master Blot (Clontech). The blot membranes were hybridized with a ^{32}P-labeled 261 bp cDNA fragment of human B/K corresponding to the nucleotide 266-526, and autoradiographed as described in Materials and Methods. The size of the hybridized B/K transcript was 3.0 kb.

brain regions, the amygdala, cerebral cortex, frontal and temporal lobes, hippocampus, subthalamic nucleus, and substantia nigra showed significantly high levels of expressions. Cerebellum, medulla, putamen, occipital lobe and spinal cord showed no or very low level of B/K mRNA expression in both Northern and dot blot analyses. These results are consistent with our previous report showing the preference of B/K mRNA in a rostral portion of the rat brain (Kwon *et al.*, 1996a). In the fetal tissues, much lower level of expression of B/K mRNA was observed in the brain, kidney and lung.

Based on the conservation of three PKA phosphorylation sites in B/K isoforms, we examined the possibility of B/K proteins as substrates for PKA. For *in vitro* phosphorylation analysis, we produced two recombinant GST-fusion proteins which contain whole coding sequence (GST-B/K-CDS) and N-terminal region including three consensus sequences for PKA (GST-B/K-1528). As shown in Figure 4A, catalytic subunit of PKA successfully phosphorylated both recombinant proteins. *In vivo* phosphorylation of B/K protein by PKA was examined in LLC-PK1 cells, a porcine kidney cell line. LLC-PK1 cells express both V1R and V2R, but only the latter has been known to be coupled to cAMP-PKA signal transduction pathway, and therefore, vasopressin causes activation endogenous PKA within the cells. On Western and Northern blot analyses, B/K was expressed at very low level in LLC-PK1 cells (data not shown), and we transfected LLC-PK1 cells with the expression constructs for whole coding sequence in correct (pCR3.1-B/K-SE) or reverse (pCR-3.1-B/K-AS) orientations. B/K protein was successfully expressed and very efficiently phosphorylated *in vivo* by DDAVP, a vasopressin analog in the cells overexpressing pCR3.1-B/K-SE (Figure 4B *top*). To confirm the PKA-dependency of B/K phosphorylation, we examined the effect of PKA inhibitor and vasopressin receptor antagonsists on DDAVP-induced phosphorylation. Pretreatment of H-89, a selective and potent PKA inhibitor strongly

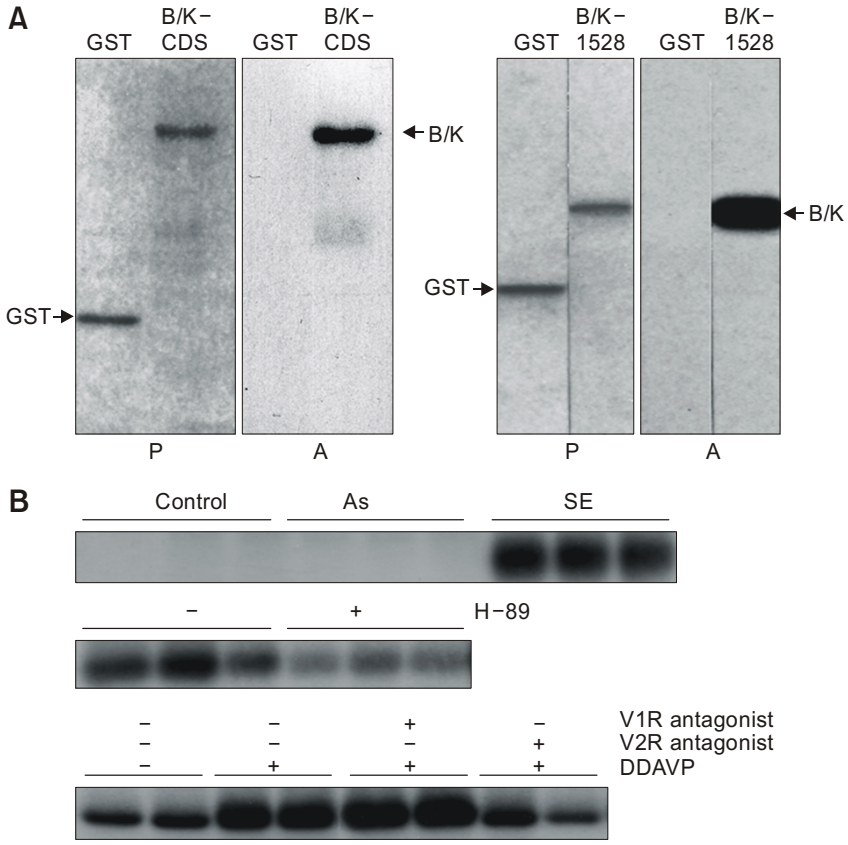

Figure 4. PKA-dependent phosphorylation of B/K protein. (A) *In vitro* phosphorylation. GST and recombinant rat GST-B/K fusion proteins corresponding to whole coding sequence (B/K-CDS) and to N-terminal region containing three PKA consensus sequences (B/K-1528) were expressed, isolated, and phosphorylated by the catalytic subunit of PKA as described in Materials and Methods. Ten microliters of reaction products were subjected to SDS-PAGE, and followed by protein staining with coommassie blue and autoradiography. P, protein staining; A, autoradiography. (B) *In vivo* phosphorylation. LLC-PK1 cells were transfected with pCR3.1 (Control), pCR3.1-B/K-AS (AS) or pCR3.1-B/K- SE (SE) constructs, and subjected to *in vivo* phosphorylation in the presence of vasopressin (DDAVP) as described in Materials and Methods (*top,* triplicated). DDAVP-induced phosphorylation of B/K protein was significantly reduced by a PKA-specific inhibitor (H-89) (*middle,* triplicated) and an antagonist of vasopressin receptor type 2 (V2R antagonist) but not by an antagonist of vasopressin receptor type 1 (V1R antagonist) (*bottom,* duplicated).

suppressed the phosphorylation of B/K protein (Figure 4B *middle*). Moreover, DDAVP-induced phosphorylation of B/K protein was selectively suppressed only by the antagonist to V2R (Figure 4B *bottom*).

Discussion

Constitutive PKA activity is necessary to maintain a large number of vesicles in the release-ready primed state, and the enhancement of regulated exocytosis by PKA has been observed in a wide variety of secretory cell types including neurons. Extensive analysis of the phosphorylation of exocytotic proteins has yielded many substrates of PKA such as synapsin (Fiumara *et al*., 2004), cysteine string protein (Evans and Morgan, 2003), syntaphilin (Boczan *et al*., 2004), SNAP-25 (Nagy *et al*., 2004), and Snapin (Thakur *et al*., 2004). However, physiological effects attributed to their phosphorylation as well as exact molecular mechanisms still remain unexplained. In this report, we suggest B/K proteins which are highly expressed in the nervous system as possible substrates for PKA.

Based on the presence or absence of the transmembrane region, proteins containing two C2-like domains present in nervous tissues can be divided into two groups, a family of synaptotagmins and double C2-domain proteins which include Rabphilin-3A, Doc2α and Doc2β, and B/K protein (Sudhof, 2002). Comparing the primary sequences of the double C2-domain family, B/K protein is the most unique member. It shows the lowest identity among the C2-domain protein family; particularly, 50 amino acids of C2a domain (I179 - N228) completely differ from other members (data not shown). In addition, B/K proteins conserved three putative optimal PKA phosphorylation sites (Figure 2C) and efficiently phosphorylated *in vitro* and *in vivo* by PKA (Figure 4), the most distinct feature of B/K proteins. To our knowledge, among double C2-domain proteins, only Rabphilin-3A which has one consensus sequence is the substrates for PKA (Numata *et al*., 1994). These findings strongly suggest unique roles of B/K proteins in PKA-mediated signaling pathways (Figure 4). Considering the partial overlap of the optimal consensus sequences between PKA (RRXS/TX) and calcium/calmodulin-dependent protein kinase II (RXXS/TX), it is also possible that B/K proteins may be phosphorylated by calcium/calmodulin-dependent kinase II.

Double C2-domain proteins does not have transmembrane domain. Instead, C2 domain may participate in calcium-dependent membrane phospholipid binding by negatively charged amino acids (D174, D180, D232, D234, D241 in synaptotagmin I) which are conserved in synaptotagmin I, Doc2, and Rabphilin 3A (Figure 2D) (Orita *et al*., 1995; Shao *et al*., 1996). For example, Doc2α and Rabphilin-3A were reported to bind membrane phospholipid (Chung *et al*., 1998) or enhance calcium-dependent exocytosis in PC12 cells (Komuro *et al*., 1996; Orita *et al*., 1996). However, in C2a domain of B/K protein, three conserved negatively charged amino acids are substituted for uncharged residues (D174I, D180N, and D241C) (Figure 2D), and moreover, the study showing the loss of calcium binding ability of synaptotagmin I by the missense mutation of single aspartic acid (D180N) in C2a domain (Li *et al*., 1995), the same substitution present in B/K, strongly indicates that B/K proteins may not bind to calcium and phospholipid. On the contrary, no substitution was observed in all seven mammalian Doc2s and Rabphilin-3As. Despite these results, perfect conservation of the N-terminal cysteine clusters in all B/K isoforms strongly indicates the possible interaction of B/K proteins with the membrane (Figure 2B, Fukuda and Mikoshiba, 2001a). Recently, calcium-independent roles of C2 domains as a protein interaction site have been reported. For example, interactions of synaptotagmins with AP-2 (Zhang *et al*., 1994), α-SNAP (Schiavo *et al*., 1995), polyinositolphosphates (Fukuda *et al*., 1994), and neurexins (Hata *et al*., 1993) occurs irrelevant to calcium through C2 domains. Doc2α also binds to munc18 in the absence of calcium (Verhage *et al*., 1997), and functions beyond calcium/phospholipid binding of synaptotagmin has been also suggested (Rickman and Davletov, 2003). Taken together, these findings suggest the possible interaction of B/K protein with the membrane or other synaptic proteins in a calcium-independent manner.

All three B/K proteins showed very high identity in amino acid sequence. Moreover, the pattern of B/K expression in human brain was highly similar to that in the rat brain except the very high level of B/K expression in human pituitary. Although we did not detect B/K mRNA expression in rat pituitary (Kwon *et al*., 1996a), results from PCR amplification of B/K from human pituitary cDNA (data not shown) as well as immunohistochemical studies showing high level of B/K expression in the rat pituitary gland (Lee *et al*., 2001a) are consistent with the result shown in Figure 3. Like rat counterpart, human B/K protein was highly expressed in the kidney (Figure 3). Moreover, *in vivo* phosphorylation experiments, B/K protein was very efficiently and specifically phosphorylated by vasopressin in a porcine kidney cell line LLC-PK1 (Figure 4) which has been used for studying PKA-dependent signal mechanisms such as the exocytosis of aquaporin-2 (Katsura *et al*., 1997). Our findings showing the type-2 vasopressin receptor-specific phosphorylation of B/K protein more clearly demonstrated the PKA-dependency of B/K protein phosphorylation. In fetal tissues, B/K protein was primarily detected in the brain, kidney and lung, but its expression level was relatively lower than that of the adult tissues, consistent with the previous findings from the *in situ* hybridization of the developing rat brain (Kwon *et al*., 1996b). Besides the brain and kidney, human B/K protein was also specifically expressed in various endocrine organs such as pituitary and thyroid glands (Figure 3). With the recent reports suggesting the role of SNARE proteins as common players for membrane trafficking such as neurotransmitter release and exocytosis (Hong, 2005), it is feasible that B/K protein may play a role in exocytosis in the tissues where it is expressed.

Although exact physiologic functions are not yet known, B/K protein expression is increased in some pathological conditions such as ischemia-reperfusion models (Ju *et al*., 2000; Lee *et al*., 2001b). We recently found that B/K protein expression was induced *in vivo* in the hippocampus by kainate, and *in vitro* by endoplasmic reticulum (ER) stress in PC12 cells (Jang *et al*., 2004). Recent reports suggesting the role of PKA in kainate-induced seizure (Rodriguez-Moreno and Sihra, 2004) or the importance of protein kinases in ER stress response (Kadowaki *et al*., 2004) support our hypothesis that B/K protein may be involved in the mechanisms related to phosphorylation.

In conclusion, our findings from three B/K isoforms suggest that B/K protein is an excellent substrate for PKA, and may play a role in calcium-independent and PKA-dependent processes. Further studies will be necessary to define the physiological role of B/K protein.

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