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Identification of a novel protein containing two C2 domains selectively expressed in the rat brain and kidney

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Abstract **We have isolated and characterized a rat brain cDNA clone which encodes a new protein of 474 amino acids in length which contains two C2 domains structurally homologous to those present in synaptotagmins. The overall amino acid identity in C2 domains between this protein and the synaptotagmins is 36–44%. This protein also contains 3 putative consensus sequences for phosphorylation by cAMP-dependent protein kinase. RNA blot hybridization revealed a 3.0 kb transcript abundantly expressed only in the rat brain and the kidney. Thus, we called this brain/kidney protein (B/K). In situ hybridization and Northern blot analyses showed that the B/K transcript was found in forebrain including the olfactory bulb, cerebral cortex, hippocampus, and hypothalamus. In the kidney, high levels of B/K transcript were expressed in the papillary region of the inner medulla, the inner stripe of the outer medulla and the cortex. The selective expression in forebrain and kidney suggests that B/K may be involved in similar cAMP-dependent processes at these very different sites.**

Key words: Synaptotagmin; Protein kinase A; Membrane trafficking; Vasopressin

1. Introduction

Synaptotagmins are a family of proteins which share a common five domain structure, including two C2 domains, C2a and C2b, homologous to the calcium-binding C2 domain of protein kinase C [1]. Approximately ten isoforms of synaptotagmin (Syt) have been identified [2–6] thus far in neuronal and non-neuronal tissues. The roles of Syts in calcium-dependent synaptic vesicle exocytosis are well-known (reviewed in [7,8]). However, Syts are also implicated in other membrane trafficking functions and mechanisms [8–12], and recently four Syt isoforms that do not bind calcium have been reported [2,4,6].

By homology cloning using probes based on synaptotagmin C2 domains, we isolated and characterized a novel protein from a rat hypothalamic cDNA library. This new protein had an unique pattern of expression in that its mRNA was present only in selected regions of brain and kidney. Hence, we have designated it as brain/kidney protein (B/K).

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank under accession number U30831.

Abbreviations: bp, base pair; kb, kilobase(s); RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISHH, in situ hybridization histochemistry; Syt, synaptotagmin; B/K, brain/kidney protein; CD, collecting duct of the kidney; V2R, V2 type vasopressin receptor; UT2, urea transporter 2.

2. Materials and methods

2.1. Cloning and sequencing of B/K

First strand cDNA was synthesized using 5 μg of total RNA isolated from rat supraoptic and paraventricular nuclei tissue punches from rat hypothalamus. The first strand cDNAs were amplified by polymerase chain reaction (PCR) in the presence of 10^{-6} M of tetramethyl ammonium chloride. The degenerate oligonucleotide primers for PCR were designed based on the nucleotide sequences of the regions corresponding to the C2a domains of rat Syt II and III. The sense and antisense primers used were SR-SE (5′-C(AC)G A(CT)C C(CT)T ACG T(CT)A A(AG)(AGC) T(GC)(CT) (AT)CC T-3′) AND SR-AS1 (5′-CC(CT) AAG T(CT)(GC) CA(AGC) AAG AAG AA(GT) T-3′), respectively. The PCR products were further amplified using the sense and antisense primers SR-SE and SR-AS2 (5′-(AG)(GC)(TC) (GC)(TC)A GGT A(AGC)(AC) (AG)(AG)(AC) (AG)(GC)T CCA-3′). The PCR condition was as follows: denaturation for 1 min at 94°C, annealing for 2 min, and extension for 2 min at 72°C. The annealing temperatures used for the initial and second amplification were 37°C and 45°C, respectively, and 40 cycles were used for each PCR amplification. The amplified products with the size between 300 and 500 bp were selected on 1.5% agarose gel electrophoresis, cloned into pCRII vector and sequenced using dideoxy chain termination reaction [13]. Sequencing analysis identified one clone (OJ102) which contained a 470 bp cDNA insert similar to the C2 domain of Syts. Rat hypothalamus and hippocampus cDNA libraries (3.8×10^5 and 5.0×10^5 plaques, respectively) were screened using a ^{32}P-labeled OJ102 cDNA insert as a probe. Positive clones, three from the hypothalamus cDNA library and six from the hippocampus cDNA library, were analyzed by Southern blot and restriction enzyme analyses, and the cDNA clones with the longest cDNA insert isolated from each library (pCR-40 and pCR-C5, respectively) were subjected to complete sequence analysis.

2.2. RNA preparation, RNA blot analysis, and RT-PCR

Total RNAs were prepared using a guanidinium thiocyanate-phenol-chloroform extraction method [14]. Poly(A$^+$)-enriched RNAs were prepared using immobilized oligo-dT beads. Poly(A$^+$) RNAs were electrophoresed on 1.5% agarose-formaldehyde gels, transferred to Nytran (Schleicher and Schuell, Keene, NH), and hybridized with ^{32}P-labeled cDNA probes, as described [15]. A 650 bp *Ava*I-digested fragment of B/K corresponding to the amino acid 1–176 was used as a probe (Fig. 1).

Total RNAs, prepared from whole kidney, inner medulla, outer medulla, and cortex of the rat kidney, were reverse-transcribed using Preamplification kit (GIBCO/BRL, Gaithersburg, MD). The resulting first strand cDNAs were amplified for 24 cycles of PCR (94°C for 1 min, 60°C for 1 min, 72°C for 2 min) with primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (for 24 cycles) or specific primers for B/K (for 27 cycles). The PCR products were separated on 0.8% agarose gel, visualized by ethidium bromide staining.

2.3. In Situ Hybridization Histochemistry (ISHH)

The brain and kidneys of adult male Sprague–Dawley rats were quickly removed, frozen on dry-ice and stored at −70°C until cutting. Serial tissue sections were cut either coronally or parasagittally (16 μm) on a cryostat, mounted onto subbed slides and processed for in situ hybridization histochemical studies. Frozen sections were fixed (30 min, 4% formaldehyde), rinsed in PBS (2 × 5 min) permeabilized with 0.3% Triton X-100/0.1 M Tris/0.05 M EDTA (30 min), rinsed in 0.1 M Tris/0.05 M EDTA (2 × 5 min), acetylated (10 min, 0.1 M triethanolamine hydrochloride-0.9% NaCl, pH 8.0 containing 0.25% acetic anhy-

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-87                                                GGCGGCCGTGCCACCTCGCCGGACCGGCGAGGGCAAAGGGGCTGTGGCGACGCATGCCCGGGCCGGAGTGAGTGAGCGCGGGCGAAG

  1 ATGGCGTACATCCAGTTGGAACCACTGAACGAGGGCTTTCTTTCCAGAATCTCTGATGTGCTGCTGTGTGGATGGACTTGTCAACACTGCTGTCAGAGGTGCTATGAGTCCAGCTGCTGC
  1 M  A  Y  I  Q  L  E  P  L  N  E  G  F  L  S  R  I  S  D  V  L  L  C  G  W  T  C  Q  H  C  C  Q  R  C  Y  E  S  S  C  C

121 CAGTCTAGTGAAGATGAAGTGGAGATCTTGGGACCTTTCCCTGCCCAGACTCCTCCATGGCTGATGGCCAGCCGGAGCAATGACAAGGATGGTGACTCTGTGCACACAGCCAGCGATGTT
 41 Q  S  S  E  D  E  V  E  I  L  G  P  F  P  A  Q  T  P  P  W  L  M  A  S  R  S  N  D  K  D  G  D  S  V  H  T  A  S  D  V

241 CCTTTGACCCCAAGGACCAACTCCCCTGATGGGAGACGCTCATCCTCAGACACATCCAAGTCCACATACAGCCTGACTCGGAGAATCTCCAGTCTGGATTCCCGGCGCCCCAGCTCCCCA
 81 P  L  T  P  R  T  N  S  P  D  G  R  R  S  S  S  D  T  S  K  S  T  Y  S  L  T  R  R  I  S  S  L  D  S  R  R  P  S  S  P

361 CTCATTGACATCAAACCTATTGAGTTCGGTGTTCTCAGTGCCAAGAAGGAGCCCATCCAGCCATCTGTGCTGCGCCGAACCTACACCCCTGATGACTACTTCAGGAAGTTTGAGCCCCGA
121 L  I  D  I  K  P  I  E  F  G  V  L  S  A  K  K  E  P  I  Q  P  S  V  L  R  R  T  Y  T  P  D  D  Y  F  R  K  F  E  P  R

481 CTTTACTCCCTTGACTCGAATCTTGATGAGTAGACTCTCTGACCGATGAGGAGATAATGTCCAAGTACCAGCTGGGCATGCTGCACTTCAGCACCCAGTATGACCTGCTGCACAACCAC
161 L  Y  S  L  D  S  N  L  D  D  V  D  S  L  T  D  E  E  I  M  S  K  Y  Q  L  G  M  L  H  F  S  T  Q  Y  D  L  L  H  N  H

601 CTCACCGTGAGGGTGATCGAGGCCCGGGACCTGCCGCCTCCAATCTCCCATGATGGCTCCCGCCAGGACATGGCCCATTCCAACCCCTACGTCAAGATCTGCCTCCTGCCTGACCAGAAG
201 L  T  V  R  V  I  E  A  R  D  L  P  P  P  I  S  H  D  G  S  R  Q  D  M  A  H  S  N  P  Y  V  K  I  C  L  L  P  D  Q  K

                                                                C2a Domain

721 AACTCCAAGCAGACGGGAGTCAAACGCAAGACCCAGAAGCCTGTGTTTGAGGAACGCTACACCTTCGAGATCCCCTTCCTAGAAGCCCAGAGGAGGACCCTGCTGCTCACTGTGGTGGAT
241 N  S  K  Q  T  G  V  K  R  K  T  Q  K  P  V  F  E  E  R  Y  T  F  E  I  P  F  L  E  A  Q  R  R  T  L  L  T  V  V  D

841 TTTGACAAATTCTCCCGCCACTGTGTGATTGGGAAAGTAGCGGTGCCTTTGTGTGAGGTGGACTTGGTGAAGGGCGGCCACTGGTGGAAAGCTCTGATCCCCAGCTCTCAGAATGAAGTG
281 F  D  K  F  S  R  H  C  V  I  G  K  V  A  V  P  L  C  E  V  D  L  V  K  G  G  H  W  W  K  A  L  I  P  S  S  Q  N  E  V

961 GAGCTGGGAGAGCTGCTGCTTTCTCTAAACTATCTCCCAAGTGCTGGCAGACTGAATGTGGACATCATTCGAGCCAAGCAACTCCTGCAGACAGATGTGAGCCAGGGTTCAGACCCATTT
321 E  L  G  E  L  L  L  S  L  N  Y  L  P  S  A  G  R  L  N  V  D  I  I  R  A  K  Q  L  L  Q  T  D  V  S  Q  G  S  D  P  F

1081 GTGAAAATCCAGCTGGTACACGGACTCAAGCTTGTGAAAACTAAGAAGACATCATTCTTACGAGGCACAATCGATCCTTTCTACAATGAATCCTTCAGCTTCAAAGTTCCCCAGGAAGAA
361 V  K  I  Q  L  V  H  G  L  K  L  V  K  T  K  K  T  S  F  L  R  G  T  I  D  P  F  Y  N  E  S  F  S  F  K  V  P  Q  E  E

                                                                C2b Domain

1201 CTAGAAAACGCCAGCCTAGTATTCACAGTGTTCGGTCACAACATGAAAAGCAGCAACGACTTCATCGGGAGGATCGTCATCGGGCAGTATTCCTCCGGCCCCTCGGAATCCAACCACTGG
401 L  E  N  A  S  L  V  F  T  V  F  G  H  N  M  K  S  S  N  D  F  I  G  R  I  V  I  G  Q  Y  S  S  G  P  S  E  S  N  H  W

1321 CGGCGAATGCTCAACACACACCGCACTGCTGTGGAACAGTGGCATAGCCTGAGGTCCCGGGCTGAGTGTGACCGAGTGTCTCCGGCCTCGCTGGAGGTGACCTGATCGCCATAAGGAAGG
441 R  R  M  L  N  T  H  R  T  A  V  E  Q  W  H  S  L  R  S  R  A  E  C  D  R  V  S  P  A  S  L  E  V  T  *

1441 CAACCTTCATTTGTTAAAAAAAGAGGAAAAAAATGTGTCACATAAATATTCAGCCCACACCTACGTATACTTTCACACATATGCACGTGCATACACAACGCCCTCTCTGTATCGAGAGCC
1561 AGCTGACAGTTGATCATGAAACAGCCCACCTCATTGAGGGAAGCTTGTCGAAGTTTCCAGACACTCCATTCATGTATAATCACAAGCCTGGTAGACTCTGCTGTGGGACTTACTAGCATT
1681 GCCTACTCTATCCACACTATGACCAAATGTACTTCGACCCTCAGGCCAGAGAAAGGAGGCCTTCCTAGGAAGAGCCAGAGTCAGTTTACCAAGAGTTCAGCTGTTATTTGGGGGGCTTGA
1801 TTCCCCACTTCAAAACCATATCTTCCAGGAAAGTTCTGAACAGGCCACTGTTAACTGGGGTCCCTTCTCCAACGGTGTAAGTACTCTTTATTTTCTCCTTAGCAAATATGATAAATAGAT
1921 TAGACCCTTTGGAGAGACCAAAGGGTAGCAAAAGACAGTAAGTTCGTGTCAGGAGCACACTCTCAGCTGCTCTGTCCTTGTGTTAACTTATTGCTGGGACTCCTTGTGGGTGTGTGTGTG
2041 TTATTGTGAGTGTGCCTCACGGGTAAGTACATGTGTGCCTGTTCATCCCATTAGACAGCAAGGCAGTAGCCAGGACCTCCATCCATGAAGCTCTCTTGCCTCTGCCTTTATCCAATGCA
2161 AGTTTACCCCATATCCTCTCACACATCAGAGACCCAACCTGAGGTAGTGATAGTTCACATATGTCCATAATAGGGTTTTGATAGAGCAGAGGTAACATGTCTGACTGAATTGTGGTGGTT
2281 ATAACCATTGGGGTTTGGCATGGGACGAGAATGGTACTACATCCCTCCACCATGACCTCTCCCCAAAACCAGACTCCAGTGAGCCTTCTGTTTCTGTGTGCACCACAGTCATGTCTCA
2401 TGTGCTAAGGCTTTAGTGTAGTTAGAATAGAGTTCCCTCACAGGATGCTTCTGTATCTTTCTCCACAGCGTATTGTCCTCTGTGATGGGGAGTTATCTGTTGTTGAAATAAAAGATTATT
2521 TATTTGTGACCC
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Fig. 1. Nucleotide and deduced amino acid sequence of rat B/K protein. The amino acid sequence is shown in single-letter code below the nucleotide sequence, and the sequences are numbered on the left. Amino acid sequences corresponding to the two C2 (C2a and C2b) domains are underlined by solid lines. Putative protein kinase A phosphorylation sites of B/K are indicated by double-underlines.

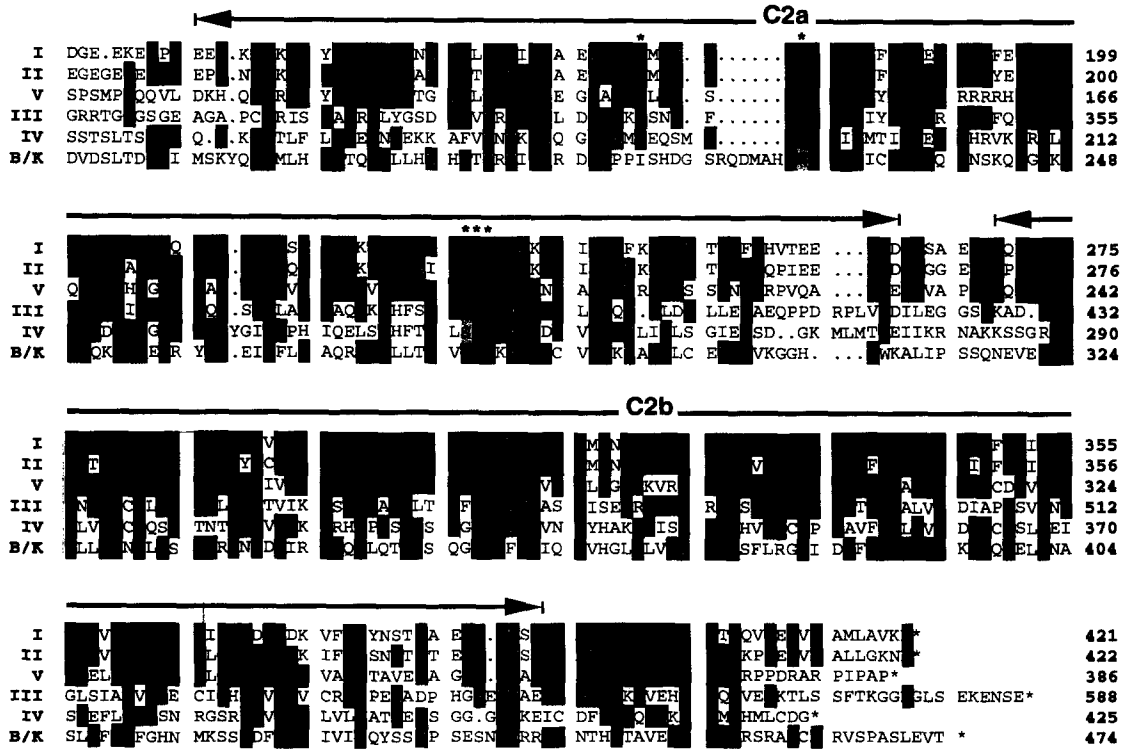

Fig. 2. Sequence comparison of C2 domains of rat B/K with rat and murine Syt isoforms. Alignment of amino acid sequences of C2 domains of B/K and five isoforms of the neuronal Syts (Syt I–IV, and V (5)) was generated by using the PileUp program. Amino acids are indicated in single-letter code. The identical residues present in 50% of the sequences are shown on a black background. Five conserved amino acids residues known to be implicated in calcium binding [17] are indicated by asterisks, and the substitutions of amino acids corresponding to Asp-178 and Asp-230 of Syt I which occur in Syt IV and B/K (see text) are shown with light gray shadings.

dride), rinsed in 2 × SSC (2 × 1.5 min), sequentially dehydrated in ethanol, delipidated in chloroform (4 min), rehydrated to 95% ethanol, air dried, and hybridized overnight at 55°C with [^{35}S]UTP-labeled riboprobes (1 × 10^6 cpm/100 μl) as described [16]. ^{35}S-labeled sense or antisense cRNA probes were synthesized from 1 μg of plasmids containing a fragment of cDNA corresponding to amino acids 1–176 of B/K and 3–113 of Syt II (accession no. M64488) subcloned between the bacteriophage SP6 and T7 polymerase promoters [16]. the slides were dehydrated, air dried and exposed to Hyperfilm β-max (Amersham) for 3 days, providing an overview of the regional distribution of B/K mRNA.

3. Results and discussion

Two overlapping rat brain cDNA clones encoding a novel protein whose composite sequence of 2.620 bp contained an open-reading frame of 474 amino acids with an M_r of 53,823 were isolated. The primary structure of this cDNA (Fig. 1) predicts a structure with two C2 domains, and data base searches using TFastA indicated that our clones had overall amino acid sequence identities in these C2 domains ranging between 36–44% when compared to synaptotagmins.

Several structural features of B/K are particularly notable. Of the five conserved amino acids in the C2a domain believed to be necessary for calcium binding ([17], see asterisks in Fig. 2), two are substituted by other amino acids in B/K. The substitution of the conserved amino acid corresponding to Asp-178 of Syt I with Asn in B/K suggests that B/K is not related to the calcium-dependent class of Syts [17]. In addition, unlike typical Syts, B/K has no clear transmembrane region. Another interesting feature is that B/K has three consensus sequences for cAMP-dependent protein kinase [18] (see double-underlined sequences in Fig. 1). Consistent with this, we found that bacterially expressed GST (glutathione S-transferase)-B/K fusion protein was phosphorylated in vitro by cAMP-dependent protein kinase (data not shown).

RNA blot analyses showed that the specific C-terminal coding region of our clone hybridized to a single 3.0 kb transcript (Fig. 3). This 3.0 transcript was highly expressed in the brain and kidney (Fig. 3A). B/K was expressed in forebrain including the olfactory bulb, hippocampus, frontal cortex, and hypothalamus (Fig. 3B), and throughout the kidney (Fig. 3C). The expression pattern of B/K transcripts in the brain and the kidney were examined at higher resolution by ISHH. In the rat brain (Fig. 4A), B/K transcripts were heavily expressed in the cortex (layers II, III, and VI), anterior olfactory nucleus, and hippocampus, and moderately in the substantia nigra and supraoptic nucleus. In the kidney, B/K transcripts were abundant in the papilla, the terminal portion of the inner medulla, and the inner stripe of the outer medulla where the labeling had a striated appearance (Fig. 4D). The sense probe of B/K (negative control) showed no hybridization signals in either the brain or the kidney (Fig. 4B,E). The positive control Syt II antisense probe (Fig. 4C) showed an expression pattern in the brain similar to that previously reported [19]. No specific hybridization was detected with the Syt II probe in the kidney (Fig. 4F), nor for Syt I, III, and V [5] (data not shown).

In the kidney, vasopressin regulates the permeability of the collecting duct (CD) to (i) water by the fusion of subapical cytoplasmic vesicles containing the water channel proteins, aquaporin-CD, into the apical membrane [20], and (ii) urea presumably by an increase in the urea transporter (UT2). The

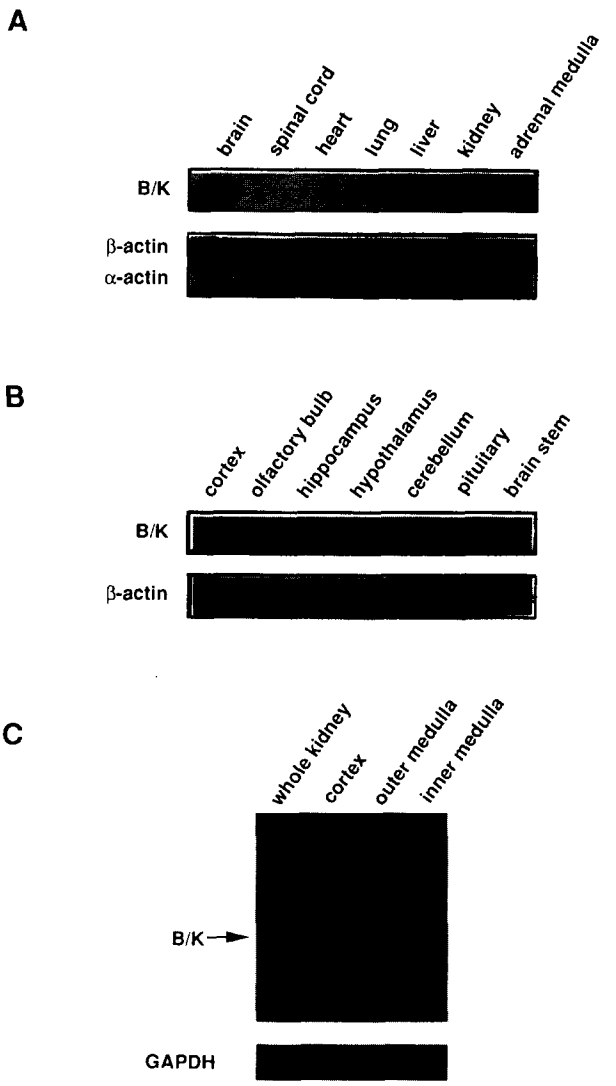

Fig. 3. RNA blot and RT-PCR analysis of B/K mRNAs shows selective expression in brain and kidney tissues. Northern blot analysis (A and B). Six micrograms of poly(A$^+$)-rich RNA per lane were analyzed on 1.3% agarose-formaldehyde gels as described in section 2. Expression of B/K mRNA was examined in rat tissues (A), and specific regions of rat brain (B). A ^{32}P-labeled *AvaI* fragment (650 bp) of B/K and an actin cDNA (that can hybridize with both α- and β-actin) were used as probes. For autoradiography the nylon membrane was exposed to X-ray film for 5 days (B/K) or 1 day (actin). The sizes of the hybridizing B/K transcripts are 3.0 kb. RT-PCR analysis (C). The first-strand cDNA reverse-transcribed using total RNAs from the whole kidney, cortex, inner medulla, and outer medulla were amplified with a specific B/K primers for 27 cycles and with a GAPDH primers for 24 cycles as an internal control. The PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. The identity of the PCR products for B/K was confirmed by Southern blot analysis using the 33-mer oligonucleotide probe of B/K (complementary to nucleotide residue 415–447) end labeled with ^{32}P (not shown).

mRNA expression pattern of B/K in the kidney (Figs. 3C and 4D) is intriguing as it overlaps with these two vasopressin-regulated proteins [21,22]. The vasopressin-mediated increases in water and urea permeability are known to be mediated by G-protein coupled V2-type vasopressin receptors (V2R) whose activation leads to an increase in cAMP and activation of pro-

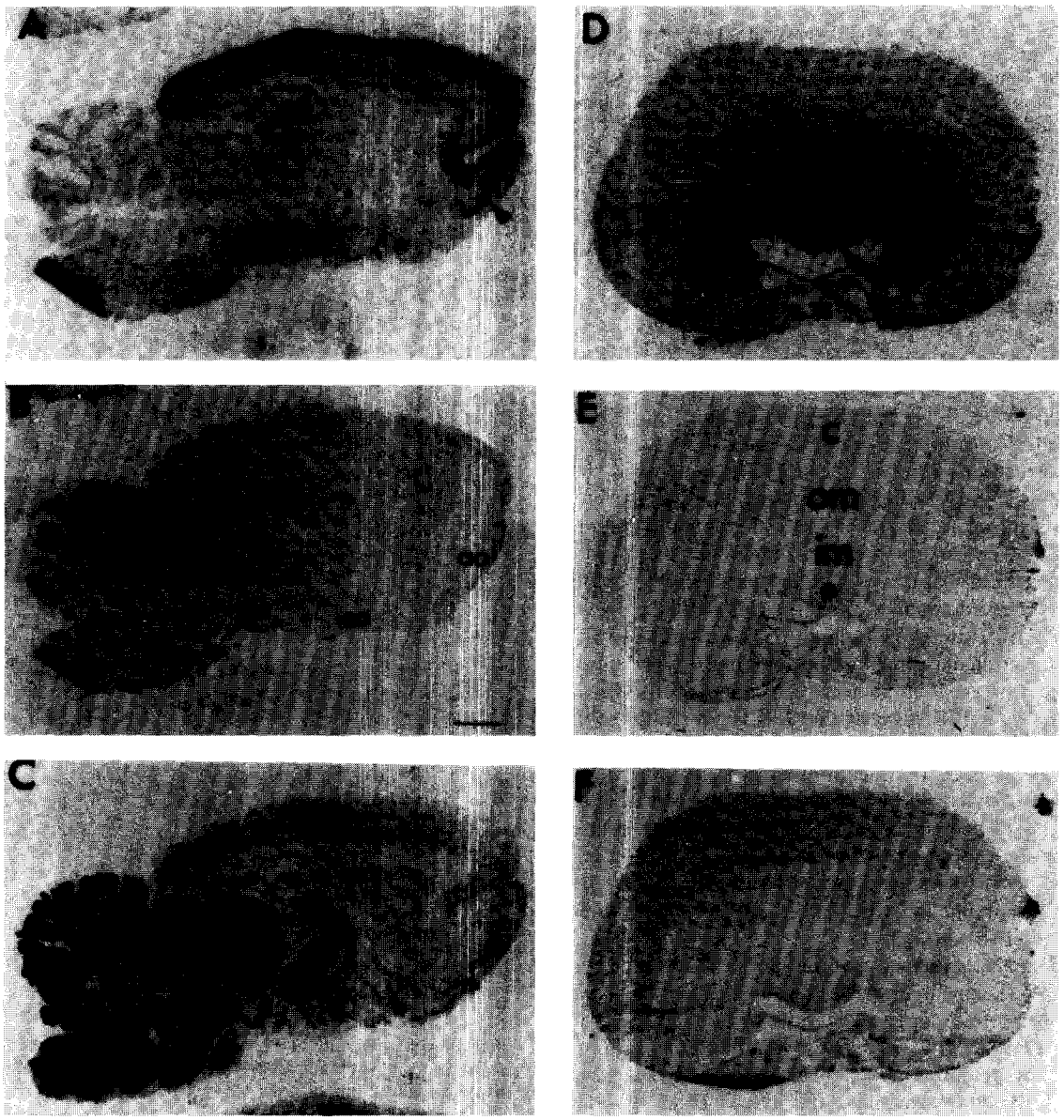

Fig. 4. Distribution of B/K mRNA in adult rat brain and kidney. Parasagittal sections of the rat brain (A–C) and kidney (D–F) were hybridized with ^{35}S-labeled antisense (A,D) or sense (B,E) B/K probes of Syt II antisense probes (C,F) and then exposed to film for 3 days. In the brain, B/K mRNA was heavily labeled in the anterior olfactory nucleus (large arrow head), hippocampus (large arrow), and less in substantia nigra (small arrow) and supraoptic nucleus (small arrow head). In the kidney, B/K mRNA was abundantly expressed in terminal region of the inner medullary region (long arrow) and the inner stripe of the outer medulla (short arrow). ao, anterior olfactory nucleus; c, cortex of the kidney; ce, cerebellum; co, cortex of the brain; h, hippocampus; ic, inferior colliculus; im, inner medulla of the kidney; om, outer medulla of the kidney; p, pons; sc, superior colliculus; sn, substantia nigra; so, supraoptic nucleus; *, papilla of the kidney. Bar means 2 mm.

tein kinase A. Recently, Kuwahara et al. reported that water permeability of aquaporin-CD was stimulated by cAMP-dependent phosphorylation, suggesting a possibility that cAMP-dependent phosphorylation might modulate the function of aquaporin-CD [23]. The overlapping expression patterns of V2R [24] and B/K mRNA (Fig. 4D) and our observation that B/K is a substrate for phosphorylation by cAMP-dependent protein kinase suggest that B/K could be involved in the V2R-linked effects of vasopressin on water and urea permeability. Whether B/K mediates similar functions in the kidney and the central nervous system remains to be determined. However, it is interesting that Jung et al. recently isolated an aquaporin isoform from the brain and showed that it is expressed in vasopressin-secretory neurons in supraoptic and paraventricular nuclei of hypothalamus [25].

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