Korean J Physiol Pharmacol
Vol 9: 333-339, December, 2005

PKA-Mediated Regulation of B/K Gene Transcription in PC12 Cells

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B/K protein is a novel protein containing double C2-like domains. We examined the specific signaling pathway that regulates the transcription of B/K in PC12 cells. When the cells were treated with forskolin (50 μM), B/K mRNA and protein levels were time-dependently decreased, reaching the lowest level at 3 or 4 hr, and thereafter returning to the control level. Chemicals such as dibutyryl-cAMP, cell-permeable cyclic AMP (cAMP) analogue and CGS21680, adenosine receptor A_{2A} agonist, also repressed the B/K transcription. However, 1,9-dideoxyforskolin did not show inhibitory effect on B/K transcription, suggesting direct involvement of cAMP in the forskolin-induced inhibition of B/K transcription. Effect of forskolin, dibutyryl cAMP and CGS21680 was significantly reduced in PKA-deficient PC12 cell line (PC12-123.7). One cAMP-response element (CRE)-like sequence (B/K CLS) was found in the promoter region of B/K DNA, and electrophoretic mobility shift assay indicated its binding to CREM and CREB. Forskolin significantly suppressed the promoter activity in CHO-K1 cells transfected with the constructs containing B/K CLS, but not with the construct in which B/K CLS was mutated (AC : TG). Taken together, we suggest that the transcription of B/K gene in PC12 cells may be regulated by PKA-dependent mechanism.

Key Words: B/K protein, Cyclic AMP, Protein kinase A, PC12 cells, Forskolin, cAMP response element

INTRODUCTION

B/K is a newly identified gene from rat brain and classified as a member of double C2 domain proteins (Südhof & Rizo, 1996). It encodes a protein consisting of 474 amino acids, and is predominantly expressed in the brain and kidney, hence the name designated (Kwon et al, 1996). Structurally, B/K protein has several unique features. First, it has two C2 domains similar to those of synaptotagmins which are a family of calcium sensor proteins present in synaptic vesicles (Brose et al, 1992). Second, there are three consensus phosphorylation sites for PKA (protein kinase A), and they are also conserved in human B/K (GenBank accession No. AF220560). Third, negatively charged amino acids, which are evolutionarily conserved and known to be critical for calcium binding (Shao et al, 1996), are substituted in both C2A and C2B domains of B/K. Finally, the C2B domain of B/K, on the contrary to synaptotagmins, showed no inositol 1, 3, 4, 5-tetrakisphosphate binding activity (Ibata et al, 1998).

Within rat central nervous system, B/K protein is prominently expressed in the neurosecretory areas such as the hypothalamic neurons, the circumventricular organs, and some endocrine cells of the adenohypophysis (Lee et al, 2001). In rat retina, B/K protein is expressed in most of ganglion cells, a few amacrine cells, and the retinal fibers of Müller cells (Kwon et al, 2000). In addition, B/K expression was elevated in some pathological conditions such as retinal ischemia (Ju et al, 2000) and kainate seizure (Jang et al, 2004). Despite of these unique structural features and specific expression patterns, exact functions of B/K remain to be established.

Earlier study showed that, after pharmacological stimulation with forskolin or membrane depolarization, synaptotagmin IV mRNA is up-regulated in PC12 cells (Vician et al, 1995). Synaptotagmin IV, a double C2 domain protein, showed the highest similarity to B/K; both are apparently induced in the Müller cells (B/K) or hippocampal pyramidal and granule cells (synaptotagmin IV and B/K) by transient ischemia or seizure (Tocco et al, 1996; Ju et al, 2000; Jang et al, 2004), respectively. Calcium binding amino acids in C2A domain are also substituted in B/K (D216I, D228N, E288C) and synaptotagmin IV (D244S) (Vician et al, 1995; Kwon et al, 1996), suggesting their role in calcium-independent mechanism.

Based on these findings, we examined the signaling pathways by which the transcription of B/K is regulated and found that the transcription of B/K gene in PC12 cells may be regulated by PKA-dependent mechanisms.

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ABBREVIATIONS: PKA, protein kinase A; cAMP, cyclic AMP; CRE, cAMP response element; CREB, CRE bimding protein; CLS, CRE-like sequence; ICER, inducible cAMP early repressor.

METHODS

Reagents

Forskolin, phorbol-12-myristate-13-acetate (PMA), dibutyryl-cyclic adenosine monophosphate (db-cAMP) and 1,9-dideoxyforskolin were purchased from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) and horse serum (HS) were from HyClone (Logan, UT, USA), Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium were from WelGene (Dae-Gu, Korea), and penicillin, streptomycin and Opti-MEM medium were from Life Technology (Grand Island, NY, USA). All other chemicals were molecular biology grade obtained from Sigma (St. Louis, MO, USA).

Cell culture

PC12 cells were purchased from American Type Culture Collection (Rockville, MD, USA), and PC12-123.7 cell line was a generous gift from Prof. K.-S. Kim (Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School, Belmont, MA, USA). Both cells were maintained in DMEM supplemented with 10% FBS, 5% HS, 100 U/μl of penicillin and 100 μg/l of streptomycin as described previously (Scheibe et al, 1991). CHO-K1 cells were cultivated in Ham's F-12 medium supplemented with 10% FBS, 100 U/μl penicillin and 100 μg/l streptomycin at 37°C in an environment containing 5% CO_2.

Northern blot analysis

Cells were treated with indicated concentrations of forskolin, KCl, PMA, ATP, 1,9-dideoxyforskolin, CGS21680, db-cAMP, and butyric acid for 3 hr. For the time-dependent effect of forskolin, cells were treated with 50 μM forskolin for the times indicated.

Total cellular RNA was prepared using RNA STAT-60 (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's instruction. Equal amount of total RNA from each time point was separated on 1% agarose gels containing formaldehyde, blotted onto nylon membranes (Boehringer Mannheim, Mannheim, Germany), cross-linked by UV irradiation, and hybridized with digoxygenin (DIG, Boehringer Mannheim)-labeled DNA probe which corresponded to the nucleotides 1-526 of B/K cDNA (Kwon et al, 1996). RNA bands reactive to the probe were detected chemiluminescently using alkaline phosphatase-conjugated anti-DIG Fab fragments (Boehringer Mannheim) and CDP star (Boehringer Mannheim). Ribosomal RNAs or rat β-actin were used for normalization of RNA loading.

Immunoblot analysis

Cells were treated with 50 μM forskolin for the indicated times, and solubilized in RIPA buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.1% sodium deoxycholate]. Fifty microgram each of protein was separated by 10% SDS- polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA), blocked with 5% skim milk in phosphate buffered saline (PBS) for 1 hr, and incubated with the rabbit anti- B/K polyclonal anti serum (1 : 5,000 dilution) (Lee et al, 2001) overnight at 4°C. To confirm equal loading and transfer of proteins, reprobing with mouse anti-synaptotagmin I monoclonal antibody (Santa Cruz, CA, USA) was performed as described previously (Lao et al, 2000). Briefly, the blot was stripped in erase buffer (62.5 mM Tris-Cl, pH 7.5, 20 mM dithiothreitol and 1% SDS) for 30 min at 50°C, and washed with Tris-buffered saline containing 0.1% Tween-20, and the immunoreactivity was analyzed as described above.

Cloning of mouse genomic B/K DNA and preparation of deletion mutants

In order to study the transcriptional regulation of mouse B/K, a genomic library of 129/SvJ mouse (Stratagene, La Jolla, CA, USA) was screened by standard methods using the 5' end fragment of the mouse B/K cDNA (Poppe et al, 1999). One clone with 11 kb size including 8 kb upstream region from the transcription start site was isolated, cloned into the pGEX-KG vector (Pharmacia, Piscataway, NJ, USA), and sequenced.

Deletion constructs of B/K genomic DNA containing 0.8, 1.2, 2.0, and 3.0 kb upstream regions from the transcription initiation site were generated by PCR, and each amplified DNAs were cloned into the pGL3-basic vector (Promega, Madison, WI, USA) using *Xho* I and *Kpn* I or *Sac* I restriction sites that were built into the PCR primers, and named pGL3-B/K-0.8, -1.2, -2.0 and -3.3 dependent on the distance from the transcription initiation site. The sequences of the PCR-generated portions of all constructs were verified by DNA sequencing.

Site-directed mutagenesis

Mutations of the CLS of B/K was introduced into pGL3-B/K-3.3 by overlap extension method (Mukhopadhyay and Roy, 1998). The primers used for mutagenesis were as follows (mutation sites are underlined): CRE_{mt} (+), 5'-CCGGAACCCAGTCTTGAAGTGTGCTCACAAACTCATC-3'; CRE_{mt} (−), 5'-GATGAGTTTGTGAGCACACTTCAAGACTGGGTTCCGG-3'; BglII primer1, 5'-GAAGATCTTCATCAAAAGGCCCCAGG-3'; R_{mt} primer1, 5'-AAGAGGGTACACAGAGTCCAGGGG-3'. Two sets of primary PCR reactions were run using the CRE_{mt} (+)/R_{mt} primer1 and the BglII primer1/CRE_{mt} (−) pairs, respectively. The secondary PCR reaction was carried out using the combined gel-purified primary PCR products as templates. PCRs were hot started at 95°C for 5 min and then continued for 28 cycles for 1 min each at 95, 58, and 72°C, with a final extension at 72°C for 7 min. The final PCR product was digested with *Bgl* II and *Bst*X I, and cloned back into the pGL3-B/K-3.3 vector to produce pGL3-B/K-3.3 CRE_{mt}. DNA sequences of all mutant constructs were confirmed by DNA sequencing.

Preparation of nuclear extracts

PC12 cells were harvested, washed in cold PBS, and incubated in two packed cell volumes of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.2 mM Na_3VO_4, 1 μg/μl leupeptin, 1 μg/μl aprotinin and 0.6 % Nonidet P-40] for 15 min on ice. The crude nuclei released by lysis were collected by centrifugation at 12,000 $\times$g for 1 min, rinsed once in buffer A, and resuspended in 2/3 packed cell volume of buffer B [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM

dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4, 1 μg/μl leupeptin and 1 μg/μl aprotinin]. After incubation on ice for 1 hr, nuclear fraction was clarified by centrifugation at 12,000 $\times$g for 5 min, and the supernatant was harvested as the nuclear protein extract. The protein concentration was determined with the bicinchronic acid protein assay reagent (Pierce), and the preparation was stored at $-70^{\circ}C$ until use.

Electrophoretic mobility shift assay

Double-stranded oligonucleotides representing B/K CLS was labeled using a fill-in reaction with [α-^{32}P]dCTP (50 μCi at 3,000 Ci/mmole, Perkin Elmer, Boston, MA, USA) and the Klenow fragment of *Escherichia coli* DNA polymerase I. For each reaction (20 μl), labeled DNA probe (40,000 cpm) was mixed with 2 μg of nuclear extracts and 1 μg of poly (dI-dC) in 10 mM HEPES buffer (pH 7.9) containing 10 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Reaction mixture was incubated on ice for 15 min and electrophoresed on 6% polyacrylamide gel in 0.5x Tris borate-EDTA buffer at room temperature, and the gels were dried for autoradiography. In a competition or supershift assay, an excess amount of unlabeled DNA or antibodies were added to the reaction mixture for 15 min or 1 hr, respectively, prior to the addition of the labeled oligonucleotide probes. Antibodies used for the supershift assay were rabbit polyclonal antibodies against rat phospho-CREB and mouse CREM (Upstate, Lake Placid, NY, USA).

Promoter analysis

CHO-K1 cells (5×10^4 cells per well in 24 well culture plate) were cultured overnight in complete Ham's F-12 media, and transfected for 3 hr in Opti-MEM media (Life Technology) with 1 μg of pGL3-B/K-0.8, -1.2, -2.0 and -3.3 or pGL3-B/K-3.3 CRE_{mt} using 1 μl of Lipofectamine and 3 μl Plus reagent (Invitrogen). After incubation in complete Ham's F-12 media for 24 hr, cells were treated with saline or forskolin (10 μM) for 3 hr prior to harvest.

For luciferase assay, cells were washed twice with Ca^{2+}- and Mg^{2+}- free PBS, lysed with 100 μl of cell culture lysis reagent (Promega) and mixed for 30 min at room temperature in a shaker. Luciferase activity was determined using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) with 80 μl of luciferase assay reagent (Promega) mixed with 20 μl of protein extract. β-galactosidase assay was performed in 96 well plates with 90 μl β-galactosidase assay reagent [1 μl 100$\times$$Mg^{2+}$ solution, 22 μl 1$\times$*o*-nitrophenyl-D-galactopyranoside and 57 μl 0.1 μM sodium phosphate buffer (pH 7.5)] and 10 μl of protein extract. Mixtures were incubated at $37^{\circ}C$ for 30 min, and 1 M Na_2CO_3 was then added to stop the reaction. To correct differences in transfection efficiency between the experiments, luciferase activity was normalized with β-galactosidase activity, using pGL3-basic vector as control. The experiments were performed in triplicates, and the data were analysed by Student *t* test.

RESULTS

Effect of signal transduction modulators on B/K mRNA level

In our preliminary study, we obtained that B/K was highly expressed in PC12 cells. Therefore, we first examined the change of B/K transcription in PC12 cells in response to some well-known modulators of signal transduction such as forskolin, KCl, PMA, A23187 and ATP (Fig. 1A). Northern blot analysis revealed that B/K mRNA level was apparently decreased by 3 hr-treatment of an adenylate cyclase activator (forskolin, 50 μM) and a depolarizing reagent (KCl, 50 mM). However, a protein kinase C activator (PMA, 50 ng/ml), a secretagogue (ATP, 50 ng/ml), or Ca^{2+} carrier (A23187, 2 μM) did not change the B/K mRNA (Fig. 1A). Forskolin time-dependently decreased the expression of B/K gene at both mRNA and protein levels (Fig. 1B). B/K mRNA level began to diminish from 2 hr after the treatment, reaching the lowest level at 3 or 4 hr and then slowly recovering to the control level (Fig. 1B, upper panel). On immunoblot analysis, forskolin was found also

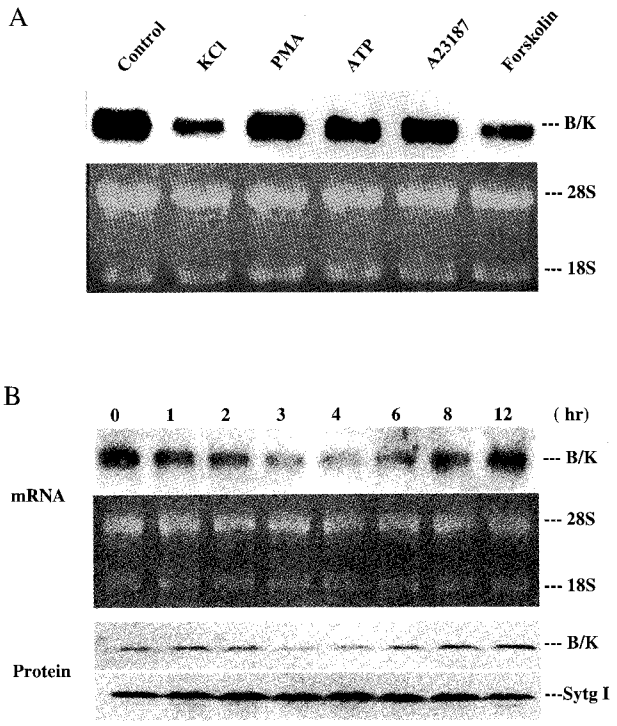

Fig. 1. Effect of signal transduction modulators on B/K mRNA level. (A) Inhibition of B/K transcription in PC12 cells by forskolin and KCl. PC12 cells were treated for 3 hr with saline (Control), KCl (50 mM), PMA (50 ng/ml), ATP (50 ng/ml), A23187 (10 μM) or forskolin (50 μM). Total RNAs were prepared, and Northern blot analysis was performed using B/K DNA probe as described in Materials and Methods. Ribosomal RNAs served as a loading control. (B) Temporal pattern of forskolin-induced inhibition of B/K expression. PC12 cells were treated with forskolin (50 μM) for indicated times, and the expression of B/K gene in mRNA (upper) and protein (lower) levels were examined by Northern and immunoblot analyses as described in Methods. Ribosomal RNAs and synaptotagmin I were used as loading controls. Sytg I, synaptotagmin I.

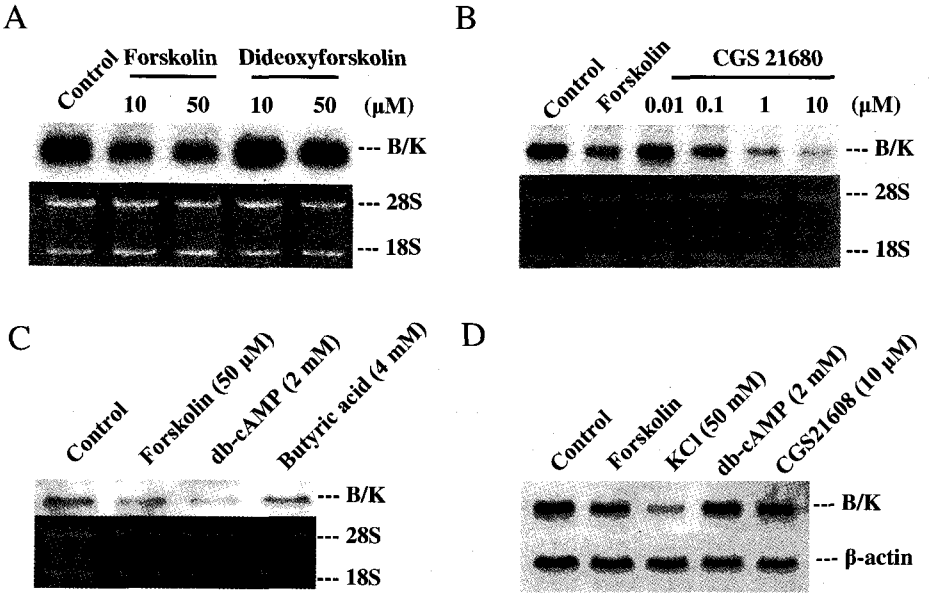

Fig. 2. Involvement of protein kinase A in the forskolin-induced suppression of B/K gene transcription. PC12 (A~C) or PC12-123.7 cells (D) were treated with forskolin, dideoxyforskolin (A), CGS21608 (B), db-cAMP or butyric acid (C), and KCl (D) at the indicated concentration for 3 hr. Saline was used as a vehicle (Control) in each experiment. Total RNAs were prepared, and Northern blot analyses were performed as described in Methods. Ribosomal RNAs and β-actin served as loading controls.

to reduce the expression of B/K protein with the temporal course similar to that of mRNA (Fig. 1B, lower). Expression of synaptotagmin I which has been known not to be change by forskolin in PC12 cells (Vician et al, 1995) was used as a control.

Involvement of cAMP and PKA in forskolin-induced suppression of B/K transcription

Physiological responses of forskolin are considered to be due to increased cAMP levels by activation of adenylate cyclase. However, several studies demonstrated the cAMP-independent activity of forskolin (Hoshi et al, 1988; Wagoner & Pallotta, 1988). Therefore, in order to elucidate the involvement of cAMP in forskolin-induced suppression of B/K transcription, we examined the effect of 1,9-dideoxyforskolin, CGS21680, and dibutyryl-cAMP (db-cAMP) (Fig. 2). 1,9-Dideoxyforskolin, an inactive analogue of forskolin without adenylate cyclase-activating activity, did not repress B/K transcription at 10 and 50 μM, concentrations at which forskolin potently inhibited the B/K transcription (Fig. 2A). An endogenous adenosine receptor A_{2A} agonist (CGS21680), which can also activate adenylate cyclase (Saitoh et al, 1994), inhibited B/K mRNA expression in a dose-dependent manner (Fig. 2B). Finally, 2 mM db- cAMP, a cell-permeable cAMP analogue, showed even more potent effect on B/K transcription than forskolin (Fig. 2C). After taken up into the cells, db-cAMP degrades into monobutyryl cAMP and butyrate, and some of the effects of db-cAMP can be attributed to the action of butyrate, causing actions other than mimicking endogenous cAMP (Nakamura et al, 1979). To rule out this possibility, we treated the cells with 4 mM butyrate, but it did not show any inhibitory effect on B/K transcription (Fig. 2C).

It is well established that cAMP can modulate transcription of many genes via the cAMP-dependent activation of PKA (for review, Montminy, 1997). We examined, therefore, the role of PKA in the forskolin-induced inhibition of B/K transcription using PKA-deficient PC12 cell derivative, PC12-123.7, which has been used for determining potential roles of PKA in various signaling pathways (Ginty et al, 1991; Tigyi et al, 1996). As shown in Fig. 2D, transcription of B/K in PC12-123.7 cells was not effectively reduced by forskolin, CGS21680 or db-cAMP (Fig. 2D).

Identification of CRE-like sequence in B/K DNA and promoter analysis

cAMP-response element (CRE)/CRE-binding protein (CREB)-mediated regulation of gene expression is a typical mechanism for PKA-induced control of transcription. To examine the presence of CRE sequence in B/K gene promoter region, we cloned mouse B/K genomic DNA, and found a B/K CRE-like sequence (CLS) (TGACGTCA), identical to the consensus CRE sequence (TGACCTCA) except one base substitution at the region spanning from $-3{,}165$ to $-3{,}158$, suggesting the involvement of B/K CLS in the PKA-mediated down- regulation of B/K transcription. Results from the promoter analysis in CHO-K1 cells, that were transiently transfected with four deletion constructs (pGL3-B/K-0.8, -1.2, -2.0 and -3.3), showed significant suppression of the promoter activity only with the construct containing B/K CLS (pGL3-B/K-3.3) (Fig. 3A). Moreover, forskolin did not suppress the promoter activity in the cells that were transfected with the construct (pGL3-B/K-3.3CRE_{mt}) in which B/K CLS was mutated to the form (AC : TG) that has been known to be unable to bind CREB (Saini et al, 2004) (Fig. 3B).

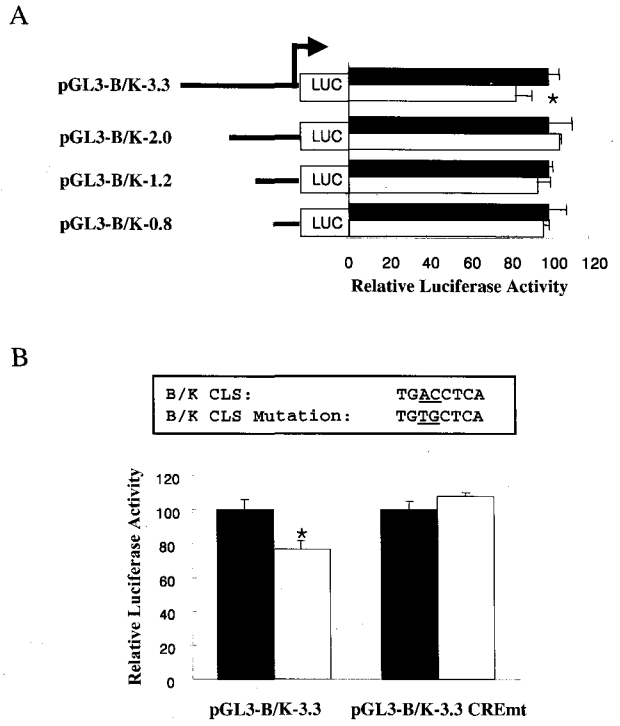

Fig. 3. Promoter Analysis. CHO-K1 cells were transiently transfected with pGL3-B/K deletion constructs as indicated (A) or with the constructs which contained wild type (pGL3-B/K-3.3) or mutated B/K CLS (pGL3-B/K-3.3 CRE_{mt}) (B). The cells were treated with saline (■) or forskolin (10 μM, □) for 3 hr, and luciferase activity was measured at 24 hr after the transfection as described in Methods. *$P<0.05$, compared with control.

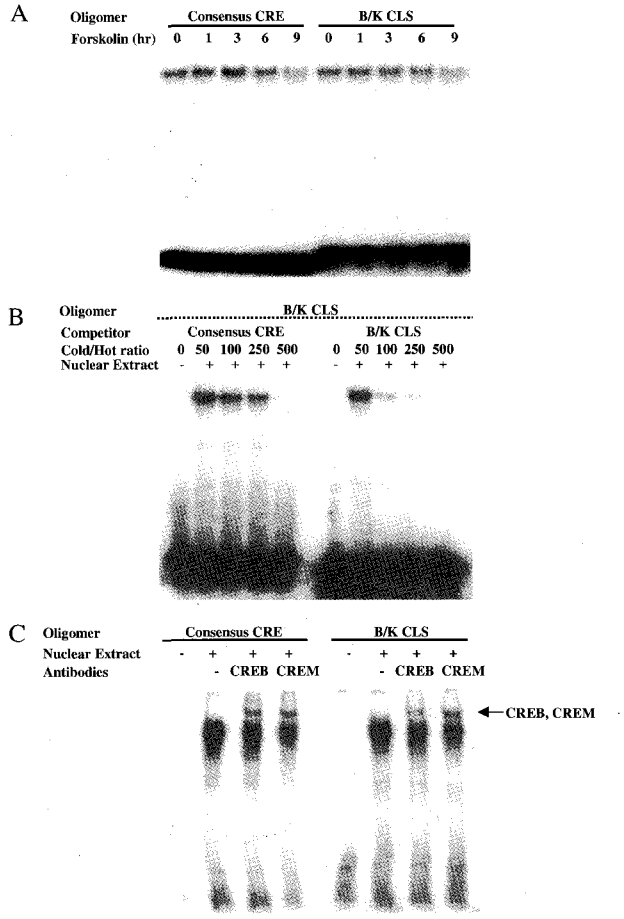

Fig. 4. Electrophoretic mobility shift assay. Nuclear extracts obtained from PC12 cells that had been treated with forskolin (10 μM) for the indicated times were incubated for 15 min with α-^{32}P-labeled CRE or B/K CLS oligonucleotide probes, electrophoresed on 6% agarose gel, and autoradiographed as described in Methods. For competition (B) and supershift (C) assays, nuclear extracts were preincubated for 15 min with indicated molar excess of unlabeled consensus CRE or B/K CLS (B), or for 1 hr with antibodies against phospho-CREB and CREM (C).

Electrophoretic mobility shift assay (EMSA)

Interaction of B/K CLS with the transcription factors involved in PKA-mediated signaling mechanism was determined by EMSA. Thus, radiolabeled double stranded CRE or B/K CLS oligonucleotide probes were incubated with nuclear extracts from PC12 cells which had been treated for 1, 3, 6, and 9 hr with forskolin. As shown in Fig. 4A, consensus CRE sequence was found to find to a protein from foskolin-stimulated PC12 cells in a time-dependent manner; the maximum binding at 3 hr, returning to the control level at 6 hr, and less than the control level 9 hr after the forskolin treatment. B/K CLS also showed similar time-dependent binding pattern, however, the binding level was much weaker than consensus CRE, suggesting the possibility that B/K CLS may bind to the transcription factor(s) which was weakly induced by forskolin. The competition assay, that was performed by preincubation of the reaction mixture for 15 min with an excess amount of unlabeled probes, showed specific binding of the nuclear extract to B/K CLS, and the binding affinity to B/K CLS was much higher than consensus CRE (Fig. 4B). Using antibodies against phospho-CREB and CREM, supershift assay was performed. Figure 4C clearly shows that both antibodies supershifted the bands in the control. However, the binding of anti-phospho-CREB antibody to B/K CLS-binding protein was much weaker than consensus CRE counterpart.

DISCUSSION

Transcriptional regulation by cAMP has been known to be mediated by the CREB/CRE modulator (CREM)/activator transcription factor (ATF) family of transcription factors. These factors are activated through protein phosphorylation primarily by PKA, and their subsequent binding to CRE in the promoter region regulates the transcription of cAMP-inducible genes (Montminy, 1997). In general, expression of genes containing CRE is up-regulated by forskolin through PKA-dependent mechanism. However, our results in the present experiments showed the cAMP- and PKA-dependent down-regulation of B/K gene transcription (Figs. 1 and 2).

Mechanism of cAMP-induced down-regulation of transcription is not fully understood yet. Inducible cAMP early repressor (ICER) has been suggested as a possible candidate molecule (Molina et al, 1993), and it is produced by

alternate transcriptional initiation from an intronic promoter of the CREM gene, and contains only the DNA binding domain without a transcriptional activation domain, thereby acting as a powerful transcriptional inhibitor through CRE (Stehle et al, 1993; Fitzgerald et al, 1996). ICER is mainly expressed in the neuroendocrine cells including PC12 and GH_3 cells (Molina et al, 1993), and induced by forskolin and db-cAMP in PC12 cells (Tinti et al, 1996; Monaco and Sassone-Corsi, 1997) by the mechanisms involving PKA-induced phosphorylation of CREB (Foulkes et al, 1996; Servillo et al, 1997). These reports lead us to speculate that ICER may be involved in forskolin-induced inhibition of B/K transcription. Furthermore, since the transcription of ICER is initiated by four CREs in the promoter region of ICER gene, it can down-regulate its own expression, an autoregulatory negative feedback loop. These characteristics of ICER may possibly explain the transient nature of down-regulation of B/K transcription. Consistent with our hypothesis, ICER in PC12 cells was time-dependently transcribed up to 2 hr after forskolin treatment and declined thereafter (Molina et al, 1993; Thommesen et al, 2000). To clearly ascertain the direct involvement of ICER in B/K gene transcription, further studies using ICER will be necessary.

In the present study, suppression of B/K transcription was also induced by potassium depolarization (KCl), implying the involvement of intracellular calcium increase. It is still controversial that KCl-induced depolarization can increase the level of cAMP in PC12 cells (Van Nguyen et al, 1990; Agnihotri et al, 1997), however, our result which showed significant suppression of B/K transcription by KCl in PKA-deficient PC12 cells (Fig. 2D) strongly indicates that cAMP and PKA are not involved in KCl-mediated processor in B/K transcription. These results indicate the presence of PKA-dependent (forskolin) and independent (KCl) pathways for the inhibition of B/K transcription in PC12 cells. CREB is also known to be activated and phosphorylated at the Ser^{133} in KCl-treated PC12 cells, as in PKA-induced phosphorylation by Ca^{2+}-calmodulin-dependent protein kinases (CaM kinases) I and II (Sheng et al, 1990, 1991). Several lines of evidences also suggest the possibility that the transcriptional regulatory signals induced by KCl and cAMP converge (Van Nguyen et al, 1990; Sheng et al, 1991; Krueger et al, 1999). Based on these findings, it is possible that the inhibition of B/K transcription in PC12 cells may be mediated by the activation of both PKA and CaM kinases through ICER/CRE mechanism.

It is also possible that mechanisms other than ICER/CRE are also involved in the regulation of B/K gene transcription. Histone deacetylases differentially regulate CREB target genes by contributing to either activation or cessation of transcription (Fass et al, 2003), and the restriction of CREB binding in the genome by DNA methylation-dependent mechanism has recently been reported (Zang et al, 2005). In addition, although B/K was not suppressed by inactive forskolin analogue (1,9-dideoxyforskolin) in PKA-deficient cells (PC-123.7), we still cannot exclude possible participation of PKA-independent mechanism in B/K gene expression. For example, forskolin suppresses insulin transcription in INS-1 cells through a PKA-independent mechanism that probably involves MAP kinase signaling (Ding et al, 2002). In this regards, Hansen et al, (2004) reported MAP kinase-mediated phosphorylation of CREB by KCl, which in our present study potently down-regulated B/K transcription by a PKA-independent mechanism.

To summarize, the data presented in this report demonstrated that the processes which involve PKA can down-regulate the B/K gene expression in PC12 cells. To understand the exact regulatory mechanism at the molecular level, we are in a process to clone and characterize ICER.

ACKNOWLEDGEMENT

This investigation was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A040042) (Oh-Joo Kwon).

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