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Age-Related Decrease of Protein Kinase G Activation in Vascular Smooth Muscle Cells

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Protein kinase G-I (PKG-I) activation is essential for vascular relaxation; however, its quantitative analysis in intact cells has been difficult. To overcome this difficulty, a monoclonal antibody, VASP-16C2, was recently developed that detects phosphorylated serine residue 239 of vasodilator-stimulated phosphoprotein (VASP), a substrate of PKG-I. In this study, we used this antibody to examine (i) possible functional differences between the α and β isoforms of PKG-I, (ii) ability of cAMP to activate PKG-I, as compared to cGMP, the principal PKG-I-activating cyclic nucleotide, and (iii) time course and levels of PKG-I activation in vascular smooth muscle cells (VSMC) of young and old rats. We created COS-7 cell clones that overexpressed PKG-Iα or PKG-Iβ, treated them with cAMP or cGMP, and analyzed their cell lysates for reactivity with VASP-16C2. The results showed that PKG-Iα phosphorylated VASP at a higher level than PKG-Iβ, and cAMP was slightly weaker than cGMP in PKG-I activation. VSMC of young rats responded to cAMP or cGMP stimulation in a dose-dependent manner with increasing levels of PKG-I activation. The response was detected within 10 min and continued for at least 24 h. In contrast, VSMC of old rats showed no PKG-I activation during the first hour of cAMP or cGMP stimulation and, at 24 h these cells showed only low-level PKG-I activation. We propose that the reduced PKG-I activation may explain why vascular relaxation is decreased in older individuals.

Key Words: **PKG; vascular smooth muscle; VASP; aging; cAMP; cGMP.**

The cyclic nucleotides cAMP and cGMP are intracellular messengers capable of altering cellular functions by the activation of protein kinases PKA and PKG. In mammals, protein kinase G (PKG) exists as two major forms, PKG-I and PKG-II, which are encoded by two separate genes [1, 2]. Through alternative splicing, PKG-I is expressed in two isoforms, PKG-Iα and PKG-Iβ, that differ structurally only in the N-terminal (3). PKG-Iα mRNA is most abundant in lung and placenta, whereas PKG-Iβ in urinary bladder, uterus, adrenal gland, and fallopian tube (4). Functionally, PKG-Iα and PKG-Iβ have no known differences, except that the isoform-specific domain of the β form, but not the α form, has been shown to possess transcriptional activities when artificially fused to the DNA-binding domain of the yeast transcriptional activator GAL4 (5).

Many cardiovascular disorders are treated with vasodilators that cause elevated concentrations of cAMP or cGMP. It is generally accepted that cAMP and cGMP cause vascular smooth muscle relaxation by activating PKA and PKG, respectively. However, over the last few years, several studies have indicated that activation of PKG by cAMP could play a key role in cAMP-induced vasodilation (6–11). In contrast, although cross-activation of PKA by cGMP has been shown to mediate nitric oxide-dependent inhibition of aortic smooth muscle cell proliferation (12) and cGMP-stimulated intestinal chloride transport (13), this pathway has not been found to cause vasodilation.

It is well known that aging is a risk factor in the pathogenesis of several cardiovascular diseases such as hypertension, coronary artery disease, heart failure, and atherosclerosis. In older individuals, the endothelium-dependent relaxation of blood vessels is reduced, possibly due to decreased endothelial synthesis or release of nitric oxide (14, 15). β-adrenoceptor-mediated relaxation of vascular smooth muscle is also known to decrease with aging (14). In addition, VSMC isolated from older rats express less contractile proteins than VSMC from younger rats (16). Despite these advances, our understanding of possible mechanisms underlying the age-related changes in vascular tone regulation is far from being complete. Because PKG-I activation is a critical step in the signaling cascade leading to vascular relaxation, we wished to examine in this study whether it is affected by aging.

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Quantitative analysis of PKG-I activation in intact cells has been difficult due to the low expression level of PKG-I in most cell types and the scarcity of specific PKG-I substrates. To overcome these difficulties, Smolenski *et al.* (17) have recently developed a method that relies on the ability of a monoclonal antibody, VASP-16C2, to detect the phosphorylation of the S239 residue of vasodilator-stimulated phosphoprotein (VASP). VASP is expressed in most cell types and is a substrate for PKG-I, which preferentially phosphorylates the S239 residue, but could also phosphorylate the S157 and T278 residues of VASP. VASP is also a substrate for PKA, which preferentially phosphorylates the S157 residue but could also phosphorylate S239 and T278.

We wished to exploit the potential of VASP-16C2 in the quantitative analysis of PKG-I activation. Our strategy for testing its specificity was to examine its reactivity in PKG-I-null COS-7 fibroblasts versus COS-7 cells that are engineered to express PKG-I. Treating these cells with cAMP would also provide firm evidence whether cAMP is able to induce PKG-I activation. Finally, comparing the reactivity of VASP-16C2 in VSMC of young and old rats could possibly provide an answer whether there are age-related changes in PKG-I activation.

MATERIALS AND METHODS

Cell culture. Monkey fibroblast cell line COS-7 was purchased from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Rat aorta smooth muscle cells were isolated and cultured as previously described (18, 19).

Antibodies. Polyclonal antibodies against PKA (product number KAP-PK001E) and against PKG-I (product number KAP-PK005) were purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Polyclonal antibody (M4, Catalog No. 0010-10) against vasodilator-stimulated phosphoprotein (VASP) was purchased from ImmunoGlobe Antikoerpertechnik GmbH & Co., (Groβostheim, Germany). Monoclonal antibody VASP-16C2 was purchased from Nano-Tools Antikoerpertechnik GmbH & Co. (Teningen, Germany).

Isolation of full-length cDNA encoding PKG-Iβ. RNA was isolated from human corpus cavernosum as described previously (20). Five micrograms of this RNA was reverse-transcribed (RT), with a PKG-I-specific oligonucleotide primer (PKG-Iβ-D, 5′- GCAGGTAA-GAGGAATTCA**TTA**GA) that is complimentary, except for the two underlined nucleotides, to a region spanning the termination codon (boldface letters in the primer sequence) of PKG-Iβ mRNA. The RT product was subjected to PCR using the Advantage-GC PCR kit (Clontech Laboratories, Inc., Palo Alto, CA). The PCR primers were PKG-Iβ-D and PKG-Iβ-U (5′- GCAGCCCGGGGGAGC**ATG**), the latter of which is homologous, except for the underlined nucleotide, to a region spanning the initiation codon (boldface letters) of PKG-Iβ mRNA. The reaction was run in DNA Engine (MJ Research, Inc., Waltham, MA) with setting of 94°C, 10 s; 53°C, 10 s; 72°C, 2 min for 5 cycles and 94°C, 10 s; 58°C, 10 s; 72°C, 2 min for 35 cycles. The PCR product, expected to be 2 kb in length, was verified by agarose gel electrophoresis, purified with QIAquick PCR Purification kit (Qiagene, Valencia, CA), and digested with *Eco*RI and *Xma*I restriction enzymes (the recognition sites were incorporated into PKG-Iβ-D and PKG-Iβ-U primers, respectively). The digested DNA was cloned in pBluescript plasmid (Stratagene Inc., La Jolla, CA) and transformed into *Escherichia coli* strain DH5α (Life Technologies, Inc., Rockville, MD). Two clones were fully sequenced and each was found to have a PCR-generated mutation in different parts of the cDNA. The mutations were eliminated by swapping DNA fragments of the two clones at a unique *Nde*I restriction site.

Construction of PKG-Iα and PKG-Iβ expression plasmids. The above full-length PKG-Iβ cDNA was cloned as a *Bam*HI/*Eco*RI fragment into mammalian expression plasmid pcDNA3 (Invitrogen Corp., Carlsbad, CA), which contains the CMV promoter to drive the expression of the cloned cDNA and the neomycin-resistance gene for the selection of stably transfected cells. PKG-Iα-expression plasmid was established by transferring a full-length bovine PKG-Iα cDNA from pMT3 vector (21) to pcDNA3. The PKG-Iα-containing pMT3 plasmid was kindly provided by Drs. Firestein and Bredt of the University of California at San Francisco.

Establishment of PKG-I-expressing COS-7 clones. COS-7 cells were seeded at approximately 2×10^5 cells in a 60-mm dish. The next day, 2 μg of pcDNA3-PKG-Iα or pcDNA3-PKG-Iβ plasmid DNA was transfected into the cultured cells by the Superfectin method (Qiagene, Valencia, CA). Two days later, the transfected cells were reseeded at approximately 2×10^5 cells in a 10-cm dish in medium containing 0.5 mg/ml of geneticin. One week later, the geneticin-selected cells were processed for single-cell cloning using standard limiting dilution protocol. Clonal cells were expanded and confirmed for PKG expression by Western blotting using a PKG-I-specific antibody.

Treatment of cells with cAMP and cGMP. Cells were seeded at 4×10^5 in each well of 6-well culture plate. Twenty-four hours later, cell culture medium was removed and the cells were rinsed twice with PBS. Fresh medium containing 0, 1, or 5 mM of db-cAMP or db-cGMP was added to the cells. At different intervals (10 min, 30 min, 1 h, and 24 h), the cells were lysed in a buffer containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1× PBS. After removing insoluble materials by centrifugation, the cell lysates were measured for protein concentration by the BCA method (Pierce Chemical Company, Rockford, IL).

Western blot analysis. Cell lysates each containing 25 μg of cellular protein were electrophoresed in 7.5% SDS–PAGE and then transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. The membrane was then washed with 25 ml of TBS (100 mM NaCl, 0.1% and 10 mM Tris–HCl, pH 7.5) for 5 min at room temperature and incubated in 25 ml of Blocking Buffer (TBS plus 0.1% Tween 20 and 5% nonfat milk) overnight at 4°C. To detect the protein of interest, the membrane was incubated with appropriate primary antibody (diluted 1:1000) in 2 ml of Blocking Buffer for 1 h at room temperature, washed three times for 5 min each with 15 ml of TBST (TBS plus 0.1% Tween 20), incubated with HRP-conjugated secondary antibody (1:15,000) in 15 ml of Blocking Buffer with gentle agitation for 1 h at room temperature, and finally washed three times for 5 min each with 15 ml of TBST. The membrane was then subject to ECL (Amersham Life Sciences Inc., Arlington Heights, IL) for the detection of the specific antigen. Before the membrane was used for the detection of another antigen, it was stripped in 62.5 mM Tris–HCl, pH 6.7, 2% SDS, 10 mM 2-mercaptoethanol at 55–C for 30 min and then washed four times in 1× TBS.

RESULTS

Establishment of PKG-I-Expressing COS-7 Cells

We wished to establish PKG-I-expressing cells for the following reasons. First, it has been shown that the best method to measure intracellular PKG-I activation

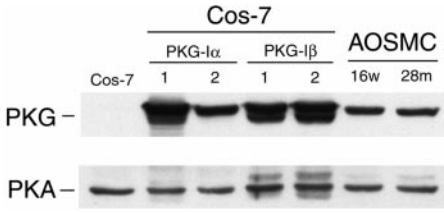

FIG. 1. Expression of PKG-I and PKA in Cos-7, Cos-7-PKG-Iα (clones 1 and 2), Cos-7-PKG-Iβ (clones 1 and 2), and rat aorta smooth muscle cells (AOSMC) (16w = 16-week-old rats; 28m = 28-month-old rats).

is through the use of VASP-16C2 antibody, which is capable of detecting phosphorylation of VASP at serine residue 239 (22). However, because PKA can also phosphorylate VASP at serine residue 239, albeit at a lesser degree than PKG, a comparison between PKG-I-null and PKG-I-expressing COS-7 cells (which express PKA endogenously) in the level of cGMP-induced VASP phosphorylation would further validate the usefulness of the VASP-16C2 antibody. Second, by treating PKG-I-expressing COS-7 cells with cAMP, it would allow us to assess whether cAMP could activate PKG-I. Third, comparison between PKG-Iα- and PKG-Iβ-expressing cells in their responses to cAMP and cGMP would allow us to assess whether there are functional differences between these two PKG-I isoforms.

Thus, we obtained a full-length PKG-Iα cDNA, constructed an expression plasmid, and established 2 PKG-Iα-expressing cell lines (Fig. 1). We also established 2 PKG-Iβ-expressing cell lines (Fig. 1) by isolating a full-length PKG-Iβ cDNA, linking the cDNA to an expression plasmid, and transfecting the plasmid into COS-7 cells. These clones expressed higher levels of PKG-I than aorta smooth muscle cells (AOSMC), while their PKA expression were at essentially equal levels (Fig. 1).

Detection of Phosphorylated VASP in PKG-I-Expressing COS-7 Cells

VASP is one of the few established PKG substrates. It contains three phosphorylation sites specific for PKA and PKG: the S157 and S239 residues are preferentially phosphorylated by PKA and PKG, respectively, while the T278 residue is a weaker phosphorylation site for both PKA and PKG. Phosphorylation of S157 leads to a marked shift in the apparent molecular mass of VASP in SDS–PAGE from 46 to 50 kDa. Phosphorylation of either the S239 or the T278 residue, however, does not cause changes in mobility of VASP in SDS–PAGE (23).

It should be noted that the above-mentioned selectivity of PKA and PKG for the phosphorylation sites on VASP was determined *in vitro*, in which purified PKA and PKG were used. In this study, we showed that treatment of COS-7 cells, which expressed PKA (Fig. 1), with cAMP or cGMP did not result in the detection of phosphorylated VASP-S239 (Fig. 2). In contrast, treatment of COS-7-PKG-Iα or COS-7-PKG-Iβ cells with cAMP or cGMP produced such phosphorylation (Fig. 2). In repeated experiments, we consistently observed higher levels of phosphorylated VASP-S239 in PKG-Iα-expressing cells than in PKG-Iβ-expressing cells. The significance of this difference is not known.

Activation of PKG-I by cAMP and cGMP in VSMC

Having determined that the VASP-16C2 antibody was capable of detecting PKG-I activation in intact cells and that the endogenously expressed PKA did not interfere with such detection, we then examined how VSMC responded to cAMP and cGMP stimulations. Using VSMC that were isolated from the aorta of 16-week-old rats, we found that cAMP and cGMP were very similar in all aspects of PKG-I activation (Fig. 3). Both nucleotides activated PKG-I to a higher level at higher concentration (5 mM vs 1 mM) and the activation was quick (within 10 min) and lasted for at least 24 h (Fig. 3).

Effect of Age on PKG-I Activation

It is well known that age is a risk factor for cardiovascular diseases. We hypothesized that age may negatively affect PKG-I activation, thereby contributing to poor vasodilation in older individuals. We examined this possibility with VSMC isolated from the aorta of 28-month-old rats. While these cells expressed PKA, PKG-I, and VASP at levels similar to those seen in VSMC of 16-week-old rats, they did not show any PKG-I activation when stimulated by cAMP or cGMP for up to 1 h (Fig. 4). At 24 h after cAMP or cGMP stimulation, these cells showed PKG-I activation at levels that were still much lower than those seen in VSMC of 16-week-old rats (Fig. 4). At this point, PKG-I

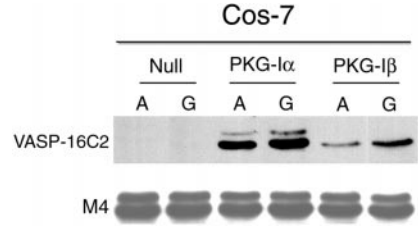

FIG. 2. Identification of PKG-I activation. Cos-7, Cos-7-PKG-Iα (clone 1), Cos-7-PKG-Iβ (clone 1) were treated with 5 mM of db-cAMP (A) or db-cGMP (G) for 24 h. Their cellular proteins were then subjected to Western blot analysis with antibodies VASP-16C2 and M4. VASP-16C2 detects VASP phosphorylated at serine 239; M4 detects VASP with or without phosphorylation. VASP is normally detected as two electrophoretic species that migrate to approximately 46- and 50-kDa positions, respectively. The 50-kDa band represents VASP phosphorylated at S157.

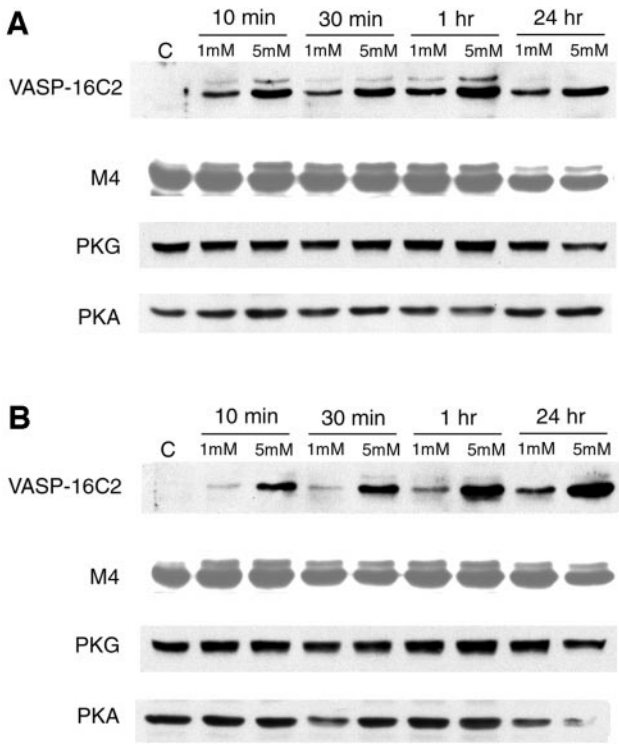

FIG. 3. Effects of cAMP (A) and cGMP (B) on VASP, PKG, and PKA in young rat cells. VSMC of 16-week-old rats were untreated (C) or treated with 1 or 5 mM of db-cAMP or db-cGMP for 10 min, 30 min, 1 h, and 24 h, and then subjected to ECL detection for phosphorylated VASP (with antibody VASP-16C2), VASP (with antibody M4), PKG, and PKA.

appeared to be expressed in lower levels, particularly when treated with 5 mM of cGMP (Fig. 4). Down-regulation of PKG-I expression by prolonged treatment with cGMP has previously been observed in VSMC (24).

DISCUSSION

Quantitative analysis of PKG activation in intact cells has been very difficult because the expression of PKG is much less than that of its closest functional homolog, PKA. To help solve this problem, we created clonal cells that overexpressed PKG-I. We then took advantage of the availability of a monoclonal antibody, VASP-16C2, whose detection of phosphorylated VASP at serine 239 is considered the best and most quantitative method for the measurement of PKG-I activation. We showed that the antibody detected phosphorylated VASP in cGMP- or cAMP-treated PKG-I-expressing COS-7 cells but not in untreated cells or in the parental COS-7 cells, treated or not treated with cyclic nucleotides. These results indicated that (i) indeed, the VASP-16C2 antibody was able to detect PKG-I activation, (ii) the endogenous PKA in COS-7 cells did not interfere with the detection of the activated PKG-I, and (iii) cAMP, like cGMP, was able to activate PKG-I, thus providing the most direct evidence that cAMP can activate PKG-I.

Although VSMC expressed much less PKG-I than the PKG-I-overexpressing COS-7 cells, they still responded to cAMP or cGMP treatment with the expression of phosphorylated VASP (at serine 239), an indication of PKG-I activation. The response, when examined in VSMC of young adult rats, was dependent on the concentration of cAMP or cGMP, but was only slightly affected by the duration of treatment (between 10 min to 24 h). In VSMC of old rats, which expressed PKA, PKG-I, and VASP at similar levels as in VSMC of young adult rats, cAMP or cGMP failed to activate PKG-I to a detectable level during the first hour of treatment. To the best of our knowledge, this is the first report that old age was a negative factor in PKG-I activation.

In summary, the present study demonstrated that the VASP-16C2 antibody was capable of detecting PKG-I activation in intact cells and that such detection was not interfered by the endogenously expressed PKA. We also provided direct evidence that both cAMP and cGMP could induce PKG-I activation in PKG-I-overexpressing COS-7 cells and in VSMC. More importantly, we have identified that PKG-I was much less

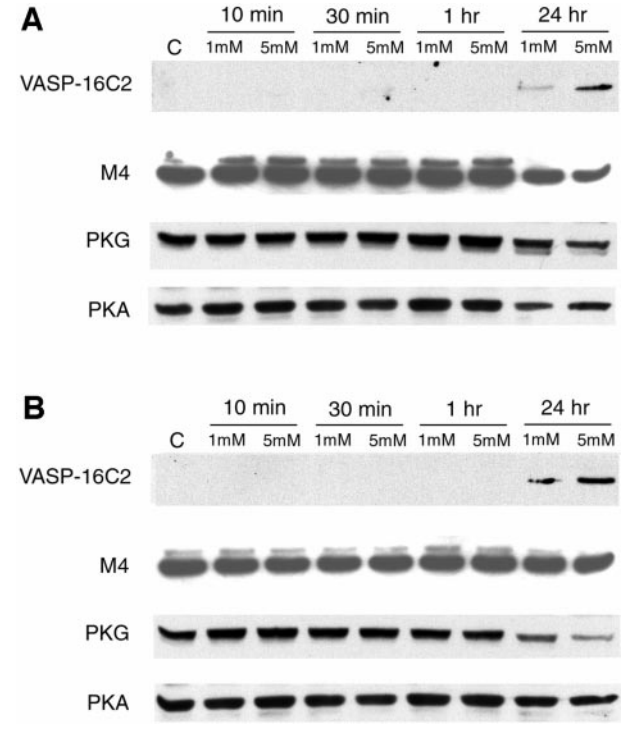

FIG. 4. Effects of cGMP (A) and cAMP (B) on VASP, PKG, and PKA in old rat cells. CSMC of 28-month-old rats were untreated (C) or treated with 1 or 5 mM of db-cAMP or db-cGMP for 10 min, 30 min, 1 h, and 24 h, and then subjected to ECL detection for phosphorylated VASP (with antibody VASP-16C2), VASP (with antibody M4), PKG, and PKA.

activated in VSMC of old rats than in VSMC of young rats. We suggest that the reduced PKG-I activation may explain why vascular relaxation is decreased with aging.

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