Vascular smooth muscle contractile state is regulated by intracellular calcium levels. Nitric oxide causes vascular relaxation by stimulating production of cyclic GMP, which activates type I cGMP-dependent protein kinase (PKGI) in vascular smooth muscle cells (VSMC), inhibiting agonist-induced intracellular Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_i\)). The relative roles of the two PKGI isoforms, PKGI\(\alpha\) and PKGI\(\beta\), in cyclic GMP-mediated inhibition of [Ca\(^{2+}\)]\(_i\), in VSMCs are unclear. Here we have investigated the ability of PKGI isoforms to inhibit [Ca\(^{2+}\)]\(_i\), in response to VSMC activation. Stable Chinese hamster ovary cell lines expressing PKGI\(\alpha\) or PKGI\(\beta\) were created, and the ability of PKGI isoforms to inhibit [Ca\(^{2+}\)]\(_i\), in response to thrombin receptor stimulation was examined. In Chinese hamster ovary cells stably expressing PKGI\(\alpha\) or PKGI\(\beta\), 8-Br-cGMP activation suppressed [Ca\(^{2+}\)]\(_i\), by thrombin receptor activation peptide (TRAP) by 98 ± 1 versus 42 ± 5%, respectively (p < 0.0002). Immunoblots of cultured human VSMC cells from multiple sites using PKGI\(\alpha\)- and PKGI\(\beta\)-specific antibodies showed PKGI\(\alpha\) is the predominant VSMC PKGI isoform. [Ca\(^{2+}\)]\(_i\), following thrombin receptor stimulation was examined in the absence or presence of cyclic GMP in human coronary VSMC cells (Co403). 8-Br-cGMP significantly inhibited TRAP-induced [Ca\(^{2+}\)]\(_i\), in Co403, causing a 4-fold increase in the EC\(_{50}\) for [Ca\(^{2+}\)]\(_i\). In the absence of 8-Br-cGMP, suppression of PKGI\(\alpha\) levels by RNA interference (RNAi) led to a significantly greater TRAP-stimulated rise in [Ca\(^{2+}\)]\(_i\), as compared with control RNAi-treated Co403 cells. In the presence of 8-Br-cGMP, the suppression of PKGI\(\alpha\) expression by RNAi led to the complete loss of cGMP-mediated inhibition of [Ca\(^{2+}\)]\(_i\). Adenoviral overexpression of PKGI\(\beta\) in Co403 cells was unable to alter TRAP-stimulated Ca\(^{2+}\) mobilization either before or after suppression of PKGI\(\alpha\) expression by RNAi. These results support that PKGI\(\alpha\) is the principal cGMP-dependent protein kinase isoform mediating inhibition of VSMC activation by the nitric oxide/cyclic GMP pathway.

The cGMP-dependent protein kinase type I (PKGI)\(^2\) mediates vascular smooth muscle cell (VSMC) relaxation via the nitric oxide/cyclic GMP pathway. One of the PKGI-dependent mechanisms of VSMC relaxation involves the inhibition of Ca\(^{2+}\) mobilization (reviewed in Refs. 1, 2). Smooth muscle contraction begins upon receptor-mediated generation of inositol 1,4,5-triphosphate (IP\(_3\)), which releases intracellular stores of Ca\(^{2+}\) from the sarcoplasmic reticulum and is followed by an influx of extracellular Ca\(^{2+}\) via voltage-gated Ca\(^{2+}\) channels (1, 3, 4). A rise in intracellular Ca\(^{2+}\) activates the Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase, which phosphorylates the myosin light chain, activating the myosin ATPase and actomyosin cross-bridge cycling, leading to an increase in tension (1). The ability of PKGI to oppose agonist-mediated Ca\(^{2+}\) mobilization is well established (5–9), but the relative roles of the PKGI isoforms PKGI\(\alpha\) and PKGI\(\beta\) are poorly understood.

Many signaling events involved in Ca\(^{2+}\) mobilization are regulated by PKGI. The phosphorylation of the thromboxane receptor by PKGI desensitizes signaling by this receptor in a manner analogous to G-protein-coupled receptor kinases (10, 11). Some of these studies were done in human platelets, which express predominantly PKGI\(\beta\) (12), suggesting that PKGI\(\beta\) can phosphorylate and attenuate signaling by the thromboxane receptor (11). The phosphorylation and activation of the regulator of G-protein signaling 2 (RGS2) by PKGI\(\beta\) terminates thrombin receptor signaling by increasing the GTPase activity of Goq (13). One report suggests PKGI can phosphorylate and inhibit the activation of PLC\(\beta3\) (14). Voltage-gated Ca\(^{2+}\) channels regulate Ca\(^{2+}\) entry, and a primary regulator of membrane potential in VSMCs is the large conductance Ca\(^{2+}\)-activated K\(^+\) channel (BKCa\(^{2+}\)) (15). The activation of BKCa\(^{2+}\) by Ca\(^{2+}\) sparks (reviewed in Ref. 16) or PKGI\(\beta\) (17) hyperpolarizes the cell, leading to decreased Ca\(^{2+}\) entry and cellular relaxation. Ca\(^{2+}\) efflux from IP\(_3\)-sensitive intracellular stores also is inhibited by PKGI\(\beta\)-mediated phosphorylation of the IP\(_3\) receptor-associated cyclic GMP kinase substrate (IRAG) in a complex of sarcoplasmic reticulum membrane proteins, including PKGI\(\beta\), IRAG, and the IP\(_3\) receptor (18–21). The presence of IRAG is essential in the cyclic GMP-dependent inhibition of Ca\(^{2+}\) signaling in colonic SMCs, suggesting that PKGI\(\beta\) is a principal regulator of intracellular Ca\(^{2+}\) levels in these cells (19). However, in VSMCs derived from PKGI knock-out mice blood vessels, the transfection of PKGI\(\alpha\), but not PKGI\(\beta\), decreases noradrenaline-induced Ca\(^{2+}\) mobilization (9).

Our laboratory has shown that PKGI\(\alpha\) binds to and phosphorylates RGS2 to terminate PAR-1 thrombin receptor signaling (13), and others have shown that the stable expression of PKGI\(\alpha\) in CHO cells inhibits thrombin-mediated Ca\(^{2+}\) mobilization in the presence of 8-Br-cGMP (22). However, the role of PKGI\(\beta\) was not explored in either of these studies. The relative roles of the two PKGI isoforms, PKGI\(\alpha\) and PKGI\(\beta\), in cyclic GMP-mediated inhibition of Ca\(^{2+}\) transients in VSMCs therefore remain unresolved. In this study, we investigated the relative abilities of PKGI isoforms to inhibit a rise in Ca\(^{2+}\) in response to thrombin receptor-activating peptide (TRAP) in cells that lack PKGI.
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(CHO cells) and in human coronary artery VSMCs that express predominantly PKG1α (Co403 cells). We provide evidence in these studies that PKG1α is the predominant PKG1 isoform mediating inhibition of VSMC activation.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides and the thrombin receptor-activating peptide SFLLRN were synthesized and purified by the Tufts University Core Facility (Boston, MA). Lysophosphatidic acid and platelet-derived growth factor-BB were from Sigma. The thromboxane analog U46619 was from Calbiochem. The rabbit polyclonal anti-PKG1 antiserum was from Stressgen (Victoria, B.C, Canada), and the goat polyclonal anti-PKG1α antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Purified PKG1α was from Promega (Madison, WI). The LacZ adenovirus (23) and PKG1α and FLAG-PKG1β adenoviruses (24) were kind gifts from B. Berk (University of Rochester) and K. Bloch (Harvard Medical School), respectively. The Re-Blot Plus Western blot recycling kit was from Chemicon International (Temecula, CA).

Cell Culture—Chinese hamster ovary cells (CHO-K1) were purchased from the American Type Culture Collection and propagated in F12 medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) (Invitrogen). Immortalized human coronary artery smooth muscle cells (Co403) were developed in our laboratory by the explant method and characterized by both morphology and immunohistochemical studies of the expression of smooth muscle cell-specific α-actin. The cells were immortalized by retroviral constructs containing the E6 and E7 human papillomavirus proteins as we have reported previously (25). These cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) (Invitrogen). CHO cells and Co403 used in this study were from passages 8–12 and 9–14, respectively.

Antibodies—Isoform-specific antibodies against PKG1α and PKG1β were raised in rabbits using glutathione S-transferase-fused antigens containing the PKG1α-specific amino acids 1–59 or the PKG1β-specific residues 1–90. Antibodies were immunoaffinity purified from antisera. Briefly, PKG1α 2–58, PKG1β 2–33, and PKG1β 43–74 peptides were synthesized and high performance liquid chromatography-purified by the Tufts University Core Facility and covalently coupled to CNBr-activated Sepharose (Amersham Biosciences). The antigen-containing columns were washed with 10 mM Tris, pH 7.5, and streptomycin (100 µg/ml) (Invitrogen). CHO cells were stably transfected with PKG1α (CHO-PKG1α) and PKG1β (CHO-PKG1β) cDNA constructs. PKG1α and PKG1β cDNA constructs were kindly provided by J. Pierandrei (Carnegie Mellon University) and D. Williams (University of North Carolina), respectively.

FIGURE 1. Ca2+ mobilization studies in CHO cells expressing PKG1α or PKG1β. A, stable expression of PKG1 isoforms in CHO cells. Lysates (20 µg of total protein) from control CHO cells (CHO-WT) and CHO cells stably expressing either PKG1α (CHO-PKG1α) or PKG1β (CHO-PKG1β) were analyzed by Western blot. PKG1α, purified PKG1α control (10 ng). The immunoblot is representative of two experiments. B, representative single-cell Ca2+ transient induced by 5 µM TRAP from CHO-WT, CHO-PKG1α, or CHO-PKG1β cells in the absence (solid lines) or presence of 8-Br-cGMP (dashed lines). C, mean intracellular calcium mobilized (AUC ± S.E.) in CHO-WT, CHO-PKG1α, or CHO-PKG1β cells in the absence (open bars) or presence (solid bars) of 8-Br-cGMP (*, p < 0.002 versus control; **, p < 0.002 versus CHO-WT; ***, p < 0.002 versus CHO-PKG1α in the presence of 8-Br-cGMP, n = 3–4).
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20 bed volumes of 500 mM NaCl, 10 mM Tris, pH 7.5. Antibodies were eluted with 10 bed volumes of 100 mM glycine, pH 2.5, and fractions were collected in tubes containing 1 bed volume of 1 M Tris, pH 8.0. Fractions containing antibody were determined by UV spectrophotometry and SDS-PAGE followed by Coomassie staining and were pooled and applied to a PD-10 desalting column (Amersham Biosciences) to elute the antibodies into phosphate-buffered saline. The resulting antibodies were concentrated to ~1 mg/ml using a 30-kDa centrifugal concentrator (Millipore; Billerica, MA) and preserved in 0.02% sodium azide. A titration of purified PKGα and lystate from CHO cells transfected with PKGβ was run on a gel to confirm isoform-specific recognition by immunobeads conjugated to nitrocellulose. Membranes were stripped and reprobed with a common PKG antibody to determine the relative sensitivities of the isoform-specific antibodies.

RESULTS

Effect of PKGα and PKGβ on TRAP-induced Ca2+ Transients in CHO Cells—CHO cell lines stably expressing PKGα or PKGβ were created and used to study the effect of cyclic GMP on intracellular Ca2+ mobilization in response to thrombin receptor activation. The CHO cell lines expressed comparable levels of the two PKGI isoforms, PKGα and PKGβ (764 and 810 pg/μg total protein, respectively) (Fig. 1A). TRAP, a hexapeptide agonist of the PAR-1 thrombin receptor, was used to activate CHO-PKGα and CHO-PKGβ cells in the absence or presence of 8-Br-cGMP (Sigma) for 20–40 min. The Ca2+ transient was recorded in the light path of a Nikon Eclipse TE2000-U microscope equipped with a xenon light source. Recordings were made with an excitation wavelength of 340 or 380 nm and an emission wavelength of 500 nm; changes in the 340/380 ratio were assessed in response to contractile agonist treatment as reported previously (26, 27). Vasorelaxors were used at a concentration corresponding to the EC_{50} unless otherwise indicated.

RNA Interference and Adenovirus Infection—PKGα-specific oligonucleotides were designed by Dharmacon using the SMARTselection algorithm and corresponded to a pool of four 21-base pair oligonucleotides including bp 26–44, 110–128, 202–220, and 232–250 of PKGI cDNA. Three other PKGI-specific small interfering RNA (siRNA) target sequences corresponding to bp 116–134, 248–266, and 257–275 of PKGI cDNA and control scrambled double stranded-RNA, S’-ATTCAGCTCACAATTG-3’, were also synthesized by Dharmacon.

FIGURE 2. Expression of PKGI isoforms in human coronary artery SMCs (CASMCs). The expression of PKGI isoforms in lysates from Co403 cells (40 μg of cell protein/lane) by immunoblotting using isoform-specific PKGI antibodies. PKGα control, 10 ng of purified PKGα; PKGβ control, 5 μg of CHO-PKGβ cell lystate. One of three similar experiment is shown.

Cells were seeded in 6-well plates at a density of 1 × 10^{5} cells/well and transfected 2 days later with a final total concentration of 100 μM double-stranded RNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol as reported previously (28). Forty-eight hours later, cells were either infected with an adenovirus construct at multiplicity of infection of 500 (LacZ) or 300 (PKGα and FLAG-PKGβ) or washed and placed in fresh medium. The cells were split into a 12-well plate for immunoblotting and LabTek chamber slides for Ca2+ imaging 72 h after siRNA treatment. After the cells adhered to the plate and chamber slides, they were arrested in serum-free Dulbecco’s modified Eagle’s medium, and assays were conducted the next day. To determine silencing efficiency, cells were lysed in lysis buffer (250 mM NaCl, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate, 20 mM Tris, pH 7.5), and the protein concentration in the soluble fraction was determined by Bradford assay (Bio-Rad; Hercules, CA). 20 μg of each lystate was applied to a 7.5% gel, subjected to SDS-PAGE, and transferred to nitrocellulose. Membranes were immunoblotted using the indicated antibodies.

CHO Stable Cell Lines—The backbone vectors of the PKGα and PKGβ constructs were linearized by cleavage with Scal and were used to transfect subconfluent CHO cells using Polyfect (Qiagen; Valencia, CA). Stably transfected cell clones were selected in F12 medium containing 500 μg/ml of geneticin and supplemented with 10% fetal bovine serum. Two different cell clones expressing equivalent levels of PKGα (CHO-PKGα) and PKGβ (CHO-PKGβ) were chosen for experiments. Control cells (CHO-WT) were grown in F12 medium supplemented with 10% fetal bovine serum.

Measurements of Intracellular Ca2+—Cells were seeded into 4-well LabTek (Nalge Nunc; Rochester, NY) chambered cover glass slides or on coverslips to be mounted into a chamber and grown for 1 day until they reached a density of 50–70% confluency. CHO and Co403 cells were serum deprived for 32 h and, respectively. Cells were loaded for 30 min with 2.5 μM fura-2-AM (Molecular Probes; Carlsbad, CA) premixed with 0.02% pluronic F127 (Molecular Probes) in a modified Ringer’s buffer (140 mM NaCl, 6.6 mM KCl, 10 mM glucose, 2 mM CaCl2, 1.8 mM MgSO4, and 5 mM HEPES, pH 7.4), washed with phosphate-buffered saline, and treated with modified Ringer’s buffer in the presence or absence of 1 or 5 mM 8-Br-cGMP (Sigma) for 20–40 min. The chamber slides were placed in the light path of a Nikon Eclipse TE2000-U microscope equipped with a xenon light source. Recordings were made with an excitation wavelength of 340 or 380 nm and an emission wavelength of 500 nm; changes in the 340/380 ratio were assessed in response to contractile agonist treatment as reported previously (26, 27). Vasorelaxors were used at a concentration corresponding to the EC_{50} unless otherwise indicated.

RNA Interference and Adenovirus Infection—PKGα-specific oligonucleotides were designed by Dharmacon using the SMARTselection algorithm and corresponded to a pool of four 21-base pair oligonucleotides including bp 26–44, 110–128, 202–220, and 232–250 of PKGI cDNA. Three other PKGI-specific small interfering RNA (siRNA) target sequences corresponding to bp 116–134, 248–266, and 257–275 of PKGI cDNA and control scrambled double stranded-RNA, S’-AAATTCTATCAGCTCACAATTG-3’, were also synthesized by Dharmacon.
PKG\( \alpha \) Inhibition of VSMC Activation

Effect of PKG\( \alpha \) and PKG\( \beta \) on TRAP-induced Ca\(^{2+} \) Transients in Co403 Cells—Fourteen vascular smooth muscle cells from human aorta (two lines), internal mammary (two lines), carotid (one line), iliac (two lines), coronary (three lines), radial (two lines), and pulmonary (one line) arteries were screened for their levels of expression of PKG\( \alpha \) and PKG\( \beta \) using purified, isofrom-specific antibodies. In thirteen of the human VSMC lines examined, PKG\( \alpha \) was the predominant isoform detected (densitometric level from 55 to 96%). In the three human coronary artery SMC lines tested, PKG\( \alpha \) accounted for >95% total PKG in two of the lines and 65% of the total PKGI in the third. In one cell line (carotid artery), more PKG\( \beta \) than PKGI\( \alpha \) was detected (35% PKGI\( \alpha \)). Because the expression of PKGI decreases in VSMCs after passaging (29), prior to initiating calcium studies candidate cell lines also were screened for the stability of PKGI isoform expression after passaging. Carotid artery SMCs had variable PKGI expression, resistance to siRNA treatment, and inconsistent Ca\(^{2+} \) responses to vasoconstrictors, making them a poor model in which to test the relative roles of the PKGI isoforms in Ca\(^{2+} \) handling.

Three other cell lines (aortic, iliac, and radial artery) presented similar issues. Co403 human coronary artery smooth muscle cells were chosen for further study because they are physiologically relevant, expressed stable levels of PKGI in all passages tested, expressed predominantly PKGI\( \alpha \) (densitometric levels, 96% PKGI\( \alpha \), 4% PKGI\( \beta \); Fig. 2), were sensitive to siRNA treatment, and respond well to vasoconstrictors in single-cell Ca\(^{2+} \) assays.

Single Co403 cells were screened for a Ca\(^{2+} \) response to TRAP, thromboxane analog (U46619), lysophosphatidic acid, and platelet-derived growth factor-BB, induced a Ca\(^{2+} \) transient that was not affected by 8-Br-cGMP (Fig. 3A). The response of Co403 cells to TRAP was studied further because these cells respond robustly to TRAP and the role of PKGI\( \alpha \), but not PKGI\( \beta \), has been tested in thrombin receptor activation (13, 22). TRAP induced a rapid rise in Ca\(^{2+} \) that gradually returned to resting levels after about 2 min (Fig. 3B). Pretreatment with the PKGI activator 8-Br-cGMP consistently inhibited the magnitude of Ca\(^{2+} \) response at 3 \( \mu \)M TRAP (25 ± 6% inhibition, \( n = 8; p < 0.02 \) versus control; Fig. 3B). Pretreatment of Co403 cells with 8-Br-cGMP led to a right shift in the dose-response curve for TRAP activation and a 4-fold increase in the EC\(_{50} \) for TRAP-induced Ca\(^{2+} \) mobilization to 8 \( \mu \)M (Fig. 3C).

Effect of Suppression of PKGI\( \alpha \) Expression on TRAP-induced Ca\(^{2+} \) Transients in Co403 Cells—PKGI\( \alpha \) expression in Co403 cells was reduced using siRNA directed against PKGI\( \alpha \), which in all studies shown suppressed the expression of PKGI\( \alpha \) by >85% (Fig. 4A). The effect of PKGI\( \alpha \) suppression in Co403 cells on TRAP-mediated Ca\(^{2+} \) transients was studied next in the presence and absence of 8-Br-cGMP. In the absence of 8-Br-cGMP, the magnitude of the TRAP-induced Ca\(^{2+} \) transients in Co403 cells was not significantly different between mock treated cells and cells treated with control siRNA (Fig. 4B). The total Ca\(^{2+} \) response following decreased PKGI\( \alpha \) expression by PKGI\( \alpha \) siRNA treatment was significantly greater than controls (by 164 ± 19%, \( n = 3; p < 0.05 \); Fig. 4C, open bars). The TRAP-induced Ca\(^{2+} \) responses were studied next in the presence of 8-Br-cGMP in mock, control siRNA, and PKGI\( \alpha \) siRNA-treated cells (Fig. 4). In the presence of 8-Br-
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**FIGURE 4.** TRAP-induced Ca\(^{2+}\) transients in the absence or presence of 8-Br-cGMP in Co403 cells following suppression of PKG\(\alpha\) by siRNA. A representative PKG\(\alpha\) antibody immunoblot of cell lysates from human coronary artery SMCs treated with Lipofectamine alone (mock), control siRNA, or PKG\(\alpha\)-specific siRNA. PKG\(\alpha\), 10 ng of recombinant PKG\(\alpha\). B, representative single-cell TRAP-induced (3 \(\mu\)M) Ca\(^{2+}\) transients from mock (control), scrambled siRNA, or PKG\(\alpha\) siRNA-treated cells in the absence (solid lines) or presence (dashed lines) of 8-Br-cGMP. C, calcium mobilization data in the absence (open bars) or presence (solid bars) of 8-Br-cGMP from \(n = 3–6\) experiments (mean AUC \(\pm\) S.E.; *, \(p < 0.05\) versus the absence of 8-Br-cGMP; **, \(p < 0.01\) versus mock and control cells in the absence of 8-Br-cGMP; ***, \(p < 0.003\) versus mock and control cells in the presence of 8-Br-cGMP).

In the absence of a cyclic GMP donor (compare Fig. 4). In mock and siRNA treatments (mean AUC \(\pm\) S.E.; *, \(p < 0.05\) versus the absence of 8-Br-cGMP; **, \(p < 0.01\) versus mock and control cells in the absence of 8-Br-cGMP; ***, \(p < 0.003\) versus mock and control cells in the presence of 8-Br-cGMP).

cGMP, the total Ca\(^{2+}\) response in PKG\(\alpha\) siRNA-treated cells was 215 \(\pm\) 24% greater than controls (\(n = 3\), \(p < 0.02\); Fig. 4C) and reached a level similar to the level of control cells treated with TRAP in the absence of a cyclic GMP donor (compare Fig. 4). In mock and siRNA control treated cells, the duration of the Ca\(^{2+}\) response was significantly reduced similarly with 8-Br-cGMP pretreatment by \(\sim 25\%\) (Fig. 4B). By contrast, there was no effect on the duration of TRAP-induced Ca\(^{2+}\) transient in the presence of 8-Br-cGMP in human coronary artery SMCs treated with siRNA against PKG\(\alpha\) (Fig. 4B). 8-Br-cGMP inhibited TRAP-induced Ca\(^{2+}\) release in mock, control siRNA, and PKG\(\alpha\) siRNA-treated cells by 53 \(\pm\) 7, 49 \(\pm\) 13, and 33 \(\pm\) 8%, respectively (PKG\(\alpha\) siRNA versus mock control, \(p < 0.01\), \(n = 3–6\); Fig. 4C). These data show that a decrease in the level of expression of PKG\(\alpha\) in human coronary artery SMCs affects TRAP-mediated Ca\(^{2+}\) stimulation by 1) increasing the absolute magnitude of the TRAP response in the absence of 8-Br-cGMP, and 2) reducing the inhibitory effect of 8-Br-GMP on TRAP-stimulated cells.

Effect of PKG\(\alpha\) or PKG\(\beta\) Overexpression on TRAP-induced Ca\(^{2+}\) Transients in Co403 Cells—Co403 cells express predominantly PKG\(\alpha\) (>95%; Fig. 2). To test further the effect of PKGI isoform expression levels on TRAP-induced Ca\(^{2+}\) mobilization, Co403 cells were infected with adenoviral constructs expressing LacZ (control), PKG\(\alpha\), or PKG\(\beta\) (23, 24). Western blot analyses confirmed increased expression of each PKGI isoform (Fig. 5A). TRAP-induced Ca\(^{2+}\) transients in Co403 cells overexpressing PKGI\(\alpha\) or PKGI\(\beta\) were studied next. Overexpression of either of the PKGI isoforms in Co403 cells did not significantly alter the magnitude of TRAP-induced Ca\(^{2+}\) transients as compared with controls (Fig. 5B). Pretreatment of Co403 cells overexpressing either PKGI\(\alpha\) or PKGI\(\beta\) with 8-Br-cGMP significantly reduced the total Ca\(^{2+}\) mobilization by TRAP in comparison with control adenovirus-infected cells (by 52 \(\pm\) 13%, \(n = 4\), \(p < 0.05\) and 57 \(\pm\) 12%, \(n = 4\), \(p < 0.03\), respectively) (Fig. 5C). The ability of PKGI\(\beta\) to inhibit TRAP signaling in VSMCs was examined by simultaneously suppressing the expression of PKGI\(\alpha\) with siRNA and overexpressing PKGI\(\beta\) with an adenoviral construct (Fig. 6). This approach resulted in a final ratio of PKGI\(\beta\) to PKGI\(\alpha\) protein of about 10:1 in comparison with native Co403 cells (Fig. 6A). As in the experiments above, siRNA reduction of PKGI\(\alpha\) led to an increase in the total Ca\(^{2+}\) mobilization by TRAP (by 140 \(\pm\) 4%, \(n = 5\), \(p < 0.003\); Fig. 6C), and this response was not altered by the overexpression of PKGI\(\beta\) (Fig. 6C). SiRNA reduction of PKGI\(\alpha\) again led to a significant decrease in the ability of cyclic GMP to inhibit the TRAP-stimulated rise in Ca\(^{2+}\) (siRNA control versus PKGI\(\alpha\) siRNA + Ad control, \(p < 0.003\), \(n = 5\)), and this also was unaffected by overexpression of PKGI\(\beta\) (Fig. 6C).
failed to reestablish the expression of PKGIα. In conclusion, overexpression of PKGIβ did not alter cGMP-mediated inhibition of Ca^{2+} mobilization in Co403 cells.

**DISCUSSION**

Our data support a model in human coronary artery VSMC and in CHO cells in which PKGIα plays the predominant role in mediating the inhibitory effects of cyclic GMP on intracellular Ca^{2+} mobilization in response to thrombin receptor activation. Ca^{2+} mobilization is determined by a balance between intracellular signaling events that release Ca^{2+} into and sequester Ca^{2+} from the cytoplasm. The balance between these processes may differ in CHO and Co403 cells, because PKGIα activation completely inhibits Ca^{2+} signaling in CHO cells, but only partially in native Co403 cells, and PKGIβ alone has an inhibitory effect in CHO cells, but not in Co403 cells. It is also possible that PKGI isoforms act on distinct, cell-specific targets to oppose TRAP-induced activation in ways that differ between CHO cells and Co403 cells. Alternatively, PKGI isozymes may regulate the same targets in CHO and Co403 cells, but the inhibitory extent or the mechanism of Ca^{2+} mobilization by TRAP may differ in these cells such that PKGI isoforms have a greater inhibitory effect in CHO cells compared with Co403 cells.

G-protein-coupled receptor activation by TRAP, U4, and lysophosphatidic acid is inhibited by 8-Br-cGMP in human coronary artery SMCs. By contrast, Ca^{2+} mobilization by the receptor tyrosine kinase agonist, platelet-derived growth factor-BB, is not affected by 8-Br-cGMP in these cells. This suggests that 8-Br-cGMP inhibits a mechanism specific to G-protein-coupled receptor, but not receptor tyrosine kinase, activation in Co403 cells. The data also show that reduction in the level of PKGIα in human coronary artery SMCs leads to an increase in the agonist-stimulated Ca^{2+} response even in the absence of cyclic GMP, supporting that PKGIα (but not PKGIβ) has a basal inhibitory role on signaling in these cells. A previous report has shown that basal inhibition of PKGI with the peptide inhibitor DT-2 causes pressurized cerebral arteries to constrict beyond their resting diameter in the absence of a nitric oxide or cyclic GMP donor, which is also consistent with the model that basal activity of PKGI contributes to the resting tone of blood vessels (30). Overexpression of PKGIβ after suppression of PKGIα expression by RNA interference fails to rescue the Ca^{2+} response of Co403 cells to TRAP, further supporting that the basal activation of PKGIα, but not PKGIβ, is critical in regulating the extent of receptor-activated Ca^{2+} mobilization. The suppression of PKGIα expression significantly inhibits, but does not abolish, the 8-Br-cGMP-
dependent inhibition of Co403 activation by TRAP. This persistent effect of 8-Br-cGMP may be due to residual PKGI expression or to the effects of other cGMP effectors, including cGMP-gated channels, cGMP-regulated phosphodiesterases, or activation of protein kinase A. However, it is unlikely that these effects are due to PKGI, because the overexpression of PKGI in the relative absence of PKGI did not significantly alter the 8-Br-cGMP-mediated inhibition of TRAP-induced Ca\textsuperscript{2+} mobilization.

There are conflicting data about the roles of PKGI isoforms in mediating cyclic GMP-dependent relaxation in VSMC. There are several possible explanations for these discrepancies in the literature. VSMC from different vascular beds may express different subsets of proteins involved in PKGI-dependent vasorelaxation and Ca\textsuperscript{2+} handling. The relative roles of the PKGI isozymes may also be different with different agonists and the signaling pathways they enlist. Earlier pharmacological studies support that PKGI mediates the cyclic GMP-dependent relaxation of pig coronary arteries (35). In PKGI knock-out VSMCs, the transfection of PKGI, but not PKGI\beta, decreased noradrenaline-induced Ca\textsuperscript{2+} mobilization, supporting the importance of PKGI in VSMC (9). PKGI\alpha and PKGI\beta act on different targets and therefore may work in series to have an additive inhibitory effect. The leucine zipper domains of PKGI\alpha and PKGI\beta differ and are important for subcellular targeting of both kinases (13, 18, 31–34). Our laboratory has shown previously that the leucine zipper domain of PKGI\alpha mediates its interaction with two important proteins that regulate the contractile state of VSMCs, RGS2 and myosin phosphatase (13, 32, 33). The PKGI\alpha/RGS2 pathway limits upstream Gq signaling by increasing the GTPase activity of Gq (13). Because Co403 cells express predominantly PKGI\alpha, a PKGI\alpha-RGS2 mechanism is consistent with the observed 8-Br-cGMP-dependent inhibition of G-protein-coupled receptor, but not receptor tyrosine kinase, activation and with our previous studies (13).

Others have shown that the leucine zipper domain of PKGI\beta targets it to IRAG, a protein that regulates the release of Ca\textsuperscript{2+} from downstream IP\textsubscript{3}-sensitive stores (18, 34). However, VSMCs isolated from IRAG null mice have a markedly greater noradrenaline-induced Ca\textsuperscript{2+} response compared with WT mice in both the absence and the presence of a cyclic GMP donor (20), suggesting that IRAG has a role in Ca\textsuperscript{2+} signaling independent of cGMP. Furthermore, the mutation of IRAG in
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these studies decreases the concentration of PKGlβ and mutant IRAG in the aorta (20) and may also alter the amount of other proteins involved in Ca2+ handling in these cells, which may include PKGlα substrates. A PKGlα/RGS2 mechanism, by acting proximally and limiting the amplification of TRAP signaling, might be required to detect downstream effects of PKGlβ/IRAG, even in the absence of a cyclic GMP donor, in Co403 cells. The overexpression of PKGlα in these cells increases the amount of PKGlα available to act on its target(s), and this may simply elicit a similar response when PKGlβ is overexpressed in the presence of endogenous PKGlα.

In summary, our data show that PKGlα plays the predominant role in mediating the inhibitory effects of cyclic GMP on Ca2+ mobilization by TRAP in human coronary artery SMCs and CHO cells. Additional studies of specific isozyme function in conditional mouse models will be necessary to further dissect the physiologic significance of the two PKGl isoforms. We are also currently testing vascular regulation in mice containing a targeted mutation in the leucine zipper amino-terminal domain of PKGlα, and we are developing SMC-specific PKGlα knockout mice. These models will be important tools to further our understanding of the relative roles of PKGl isoforms in blood pressure regulation and VSMC proliferation.

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