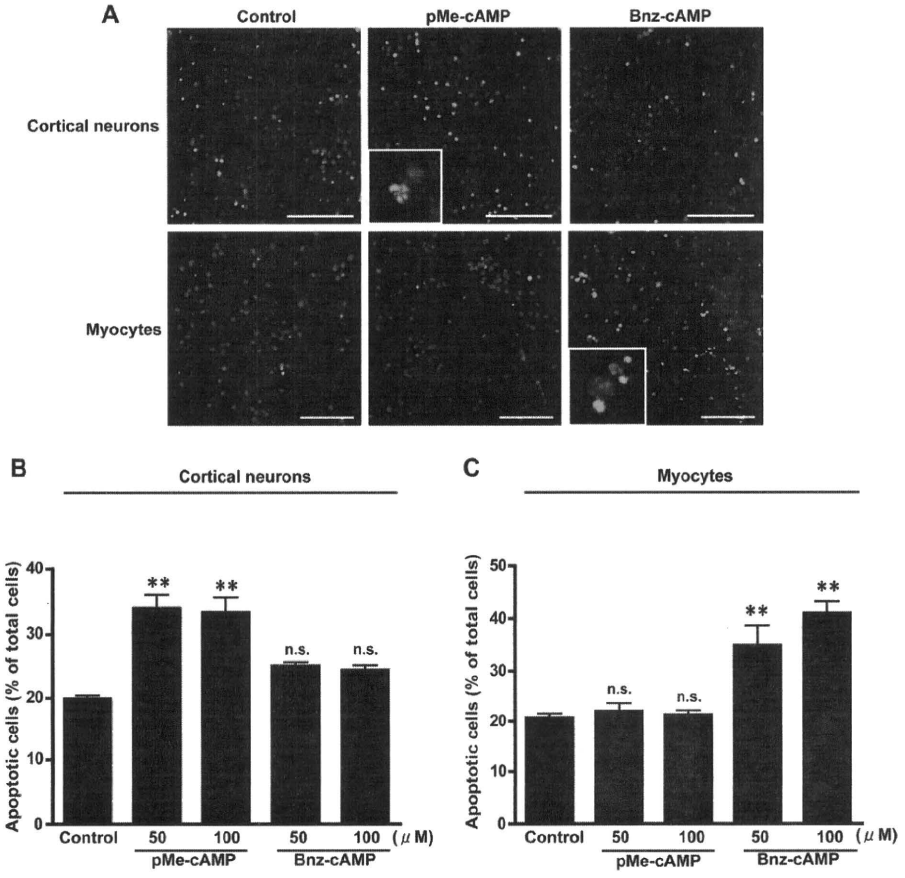

FIGURE 1. **Effects of activation of Epac and PKA on apoptosis in cortical neurons and cardiac myocytes.** *A*, apoptotic cells (*green*) in cortical neurons and myocytes were examined by means of TUNEL staining 48 h after treatment with pMe-cAMP (50 μM) or Bnz-cAMP (50 μM). Nuclei were stained with DAPI (*blue*). *Scale bar*, 100 μm. The *inset* is magnified five additional times. *B* and *C*, quantification of TUNEL-positive cells by counting nuclei in cortical neurons and cardiac myocytes is shown. The results are presented as percentages of the total cell number. $n = 6$ from 3 independent experiments. **, $p < 0.01$ *versus* control. *n.s.*, not significant.

Immunoprecipitation—Lysates from cells treated with pMe-cAMP or Bnz-cAMP for 24 h were incubated with 2 μg of anti-Bcl-2 or anti-Bim antibody overnight. Immune complexes were captured with protein G-Sepharose 4 Fast Flow (GE Healthcare). Beads were washed 3 times in the lysis buffer and boiled in an SDS sample buffer. Samples were subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA).

Adenovirus Construction—For construction of adenoviral vectors, full-length cDNA-encoding human Epac1, Epac2, and PKA α catalytic subunits were cloned into an adenoviral vector using an AdenoX adenovirus construction kit (Clontech, Mountain View, CA) (17). Human Epac1 and Epac2 cDNAs were kindly provided by Dr. J. L. Bos of University Medical Center, Utrecht, The Netherlands. Adenovirus-mediated transfection was performed using LacZ control. The cells were infected with adenoviruses at the indicated multiplicities of infection and used for their corresponding assays 24 h later.

Transfection of siRNA in Cortical Neurons—Silencing of Bim was carried out using Accell SMARTpool siRNAs (Dharmacon Inc., Lafayette, CO), each of which contains four siRNAs designed for use with the Bim gene (5′-CUGGCUUCCUUUACGUUUU-3′, 5′-CUAUGAAUUGUAGAAGUAU-3′, 5′-CGCUUAUUUAA-AUGUCUUA-3′, and 5′-UCAUAA-UUAAGGAUUUGUA-3′) according to the manufacturer's instructions. Briefly, 48 h after plating, cortical neurons were transfected with 1 μM siRNA and subsequently cultured in a neurobasal medium. The efficiency of the knockdown of the Bim protein was evaluated 72 h after transfection by Western blot analysis. Scrambled siRNAs for Bim-targeted siRNA (Dharmacon Inc.) were used as a negative control.

Terminal Deoxynucleotidyltransferase-mediated Biotin Nick End-labeling (TUNEL) Assay—In situ labeling of fragmented DNA in cultured cortical neurons and cardiac myocytes was performed using the DeadEnd™ fluorometric TUNEL system (Promega, Madison, WI) according to the manufacturer's instructions. Cells were incubated with the presence or absence of pMe-cAMP or Bnz-cAMP for 48 h, fixed with 4% paraformaldehyde for 25 min, and then incubated with 0.2% Triton X-100 for 5 min. The cells were equilibrated with a buffer consisting of 200 mM potassium cacodylate (pH 6.6), 25 mM Tris-HCl (pH 8.0), 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride at room temperature for 10 min followed by 60 min of incubation with a terminal deoxynucleotidyltransferase reaction buffer containing 100 μM dATP, 5 μM fluorescein-12-dUTP, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 40 μM terminal deoxynucleotidyltransferase enzyme at 37 °C. DNAs were stained with DAPI (4′, 6-diamidino-2 phenylindole). The percentage of the total cells that were TUNEL-positive was determined in a blinded manner. Approximately 2000–3000 cells in 10 randomly selected fields from each sample were counted. For detection of apoptosis in brain tissues from WT or Epac1 KO mice, deparaffinized tissue sections were treated with 20 μg/ml proteinase K and 50 mM EDTA in 100 mM Tris-HCl (pH 8.0). The sections were fixed with 4% paraformaldehyde for 15 min at room temperature and then subjected to the equilibration step in the procedures described above.

Analysis of DNA Fragmentation by Enzyme-linked Immunosorbent Assay—Histone-associated DNA fragments were quantified using the Cell Death Detection enzyme-linked immunosorbent assay kit (Roche Diagnostics) according to the manufacturer's instructions. After cortical neurons and cardiac myocytes were incubated in the presence or absence of pMe-cAMP or Bnz-cAMP for 48 h, they were gently washed with phosphate-buffered saline and incubated with a lysis buffer

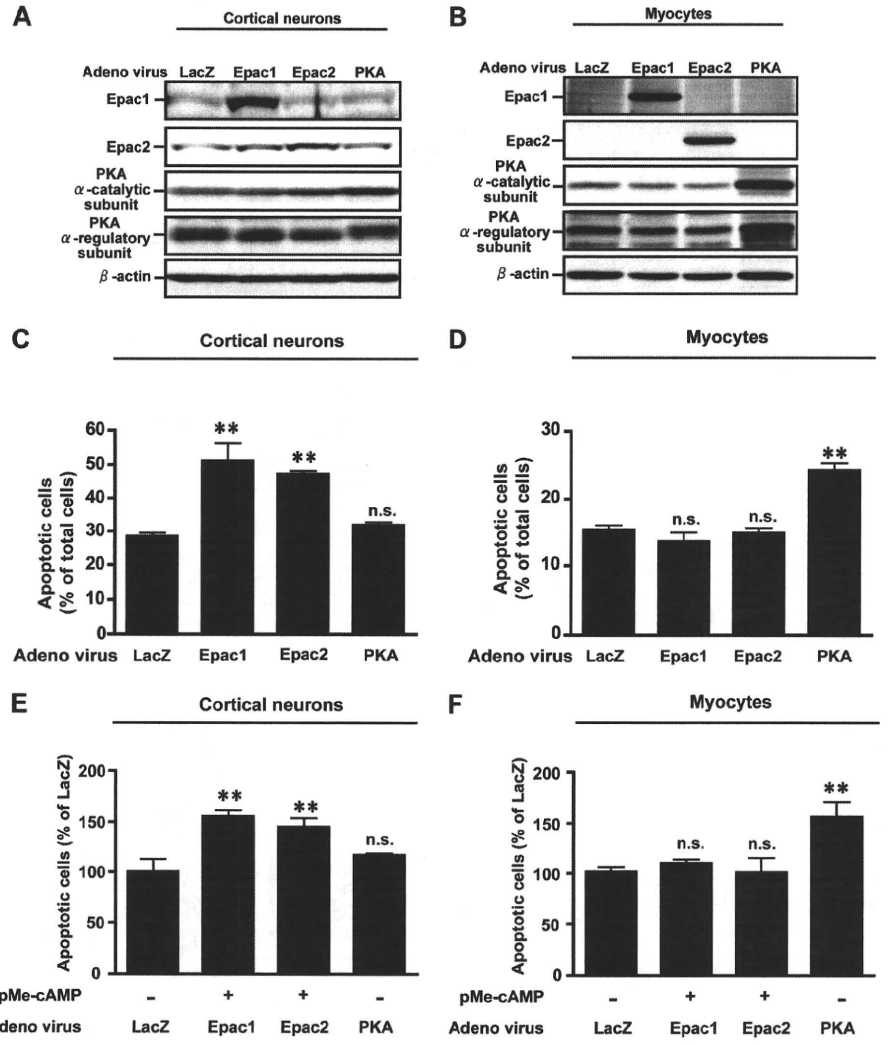

FIGURE 2. **Effects of overexpression of Epac and PKA on apoptosis in cortical neurons and cardiac myocytes.** *A*, shown are the representative immunoblots of cortical neurons or myocytes transfected with Epac1, Epac2, PKA α catalytic subunit, PKA regulatory subunit, and LacZ for 12 h (multiplicity of infection = 2). β-Actin served as an internal control. *C–F*, apoptosis was evaluated by means of TUNEL staining (*C* and *D*) and cell death detection enzyme-linked immunosorbent assay (*E* and *F*) 48 h after incubation with indicated adenovirus and an Epac-selective cAMP analog in cortical neurons and cardiac myocytes. The results are presented as percentages of the total cell number. $n = 4–8$ from 2 independent experiments. **, $p < 0.01$, *versus* LacZ control. *n.s.*, not significant.

(phosphate-buffered saline containing 10 mM EDTA (pH 7.2) and 0.1% Triton-X) for 1 h at 37 °C followed by vigorous shaking for 30 s. The cell lysates containing cytoplasmic histone-associated DNA fragments were applied to a streptavidin-coated microtiter plate. Subsequently, a mixture of biotin-labeled anti-histone antibody and peroxidase-conjugated anti-DNA antibody was added, and the resulting mixture was incubated with moderate shaking for 2 h. After unbound antibodies were removed by washing, the amount of nucleosomes was quantified based on the peroxidase retained in the immune complex. The activity of the peroxidase was determined photometrically using 2,2-azino-di-[3-ethylbenzthiazoline-sulfonate] as a substrate. The values from triplicate absorbance (at 405 nm) measurements were then averaged.

Mitochondrial Membrane Potential Analysis—Mitochondrial membrane potential of cortical neurons was quantified using a Mitocapture™ Mitochondrial Apoptosis Detection kit (BioVision Inc., Mountain View, CA) according to the manufacturer's instructions. Cortical neurons were incubated on a 12-mm glass coverslip in the presence or absence of pMe-cAMP or Bnz-cAMP for 48 h, then stained with Mitocapture reagent and incubated in DAPI to allow the visualization of all nuclei. The images were obtained using an inverted microscope (TE2000-E, Nikon, Japan). The red emission of the dye detected at 543 nm is due to a potential-dependent aggregation in the mitochondria reflecting normal membrane potential. Green fluorescence detected at 488 nm reflects the monomeric form of Mitocapture™, appearing in the cytosol after mitochondrial membrane depolarization. The percentage of the total cells representing apoptotic cells was determined in a blinded manner by counting ~1000–3000 cells in 10 randomly selected fields from each sample.

Rap1 Activation Assay—Rap1 activity was measured using the EZ-Detect RAP1 activation kit (Pierce) according to the manufacturer's instructions. Primary renal epithelial cells from WT and Epac1 KO mice were lysed 15 min after stimulation with pMe-cAMP (50 μM). Cell lysates were incubated with the Rap binding domain RalGDS-RBD fused to a glutathione *S*-transferase disk. After cells were washed several times, bound GTP-Rap1 was removed from the disk through boiling in an SDS sample buffer and analyzed by Western blotting using an anti-Rap1 antibody.

In Vivo Experiment and Tissue Preparation—3-Propionic acid (3-NP, Sigma) was prepared and administered as previously described (26). 3-NP was dissolved in saline, and the resulting solution was adjusted to pH 7.3–7.4 with 5 N NaOH. 3-NP (140 mg/kg/day) was the injected intraperitoneally into the animals once per day for 2 days. Twenty-four hours after the second injection, the mice were anesthetized with pentobarbital and transcardially injected with 10% paraformaldehyde/phosphate-buffered saline (pH 7.4). Brains were fixed in the same fixative solution overnight, immersed in 70% ethanol for 24 h, then embedded in paraffin. Sections 4 μm in thickness were subjected to TUNEL staining.

Statistical Analysis—All data are reported as the mean ± S.E. Comparisons between two groups were analyzed using Student's *t* test. For multiple groups, one-way analysis of variance

was used with a Bonferroni post-hoc test. $p < 0.05$ was considered to indicate significance.

RESULTS

Differential Effects of Epac on Apoptosis in Mouse Cortical Neurons and Myocytes—We first investigated whether the stimulation of Epac has similar effects on apoptosis in various kinds of post-mitotic cells using a primary culture of mouse cortical neurons and cardiac myocytes. Apoptosis was detected by means of TUNEL staining 48 h after treatment with pMe-cAMP or Bnz-cAMP, the Epac- and PKA-selective cAMP analogs, respectively (27). We observed strong TUNEL labeling of apoptotic bodies in cortical neurons treated with pMe-cAMP (Fig. 1*A*, *upper panels*, *inset*). In contrast, pMe-cAMP did not induce apoptosis in cardiac myocytes (Fig. 1*A*, *lower panels*). The number of TUNEL-positive cells was quantified, showing that stimulation of Epac significantly increased apoptosis in cortical neurons but not in cardiac myocytes (Fig. 1, *B* and *C*). Because a high Bax/Bcl-2 ratio is associated with greater vulnerability to apoptotic activation (28, 29), we quantified the protein expression of Bax/Bcl-2 to confirm our findings. Activation of Epac by pMe-cAMP increased the Bax/Bcl-2 ratio in cortical neurons but not in cardiac myocytes (supplemental Fig. 1, *A–D*).

It is already known that activation of cAMP/PKA signaling plays a protective role in neuronal cells but a deteriorative role in myocardial cells (30, 31). In accordance with previous reports, the PKA-selective cAMP analog Bnz-cAMP induced apoptosis in cardiac myocytes but not in cortical neurons, even at 100 μM (Fig. 1, *A–C*). These results suggest that the activation of Epac has different effects on apoptosis in neuronal cells and cardiac myocytes.

Effects of Overexpression of Epac on Apoptosis in Cortical Neurons and Cardiac Myocytes—To confirm that Epac was involved in neuronal cell death, we performed adenovirus-mediated gene transfer of Epac1 and Epac2 (as both isoforms of Epac (9) are known to be activated by pMe-cAMP), a PKA α catalytic subunit, or a LacZ control. Overexpression of Epac1, Epac2, PKA α catalytic subunit proteins, or PKA α regulatory subunit proteins in cortical neurons and cardiac myocytes 12 h after infection with each adenovirus is shown in Fig. 2, *A* and *B*. Endogenous protein expression of Epac1, Epac2, and PKA subunits was not significantly affected by any of the adenoviruses. We also confirmed that overexpression of PKA α catalytic subunit significantly increased PKA activity in cortical neurons (supplemental Fig. 2). Overexpression of Epac1 or Epac2, unlike LacZ, significantly increased the incidence of TUNEL-positive apoptotic cells in cortical neurons but not in cardiac myocytes (Fig. 2, *C* and *D*). In contrast, overexpression of PKA increased the number of TUNEL-positive cells in cardiac myocytes but not in cortical neurons. An enzyme-linked immunosorbent assay yielded results similar to those of TUNEL staining (Fig. 2, *E* and *F*), indicating that overexpression of Epac1 or Epac2 increased DNA fragmentation in cortical neurons but not in cardiac myocytes. Importantly, these results are different from those obtained through PKA overexpression, suggesting that, at least in part, Epac promotes neuronal, but not myocardial, apoptosis.

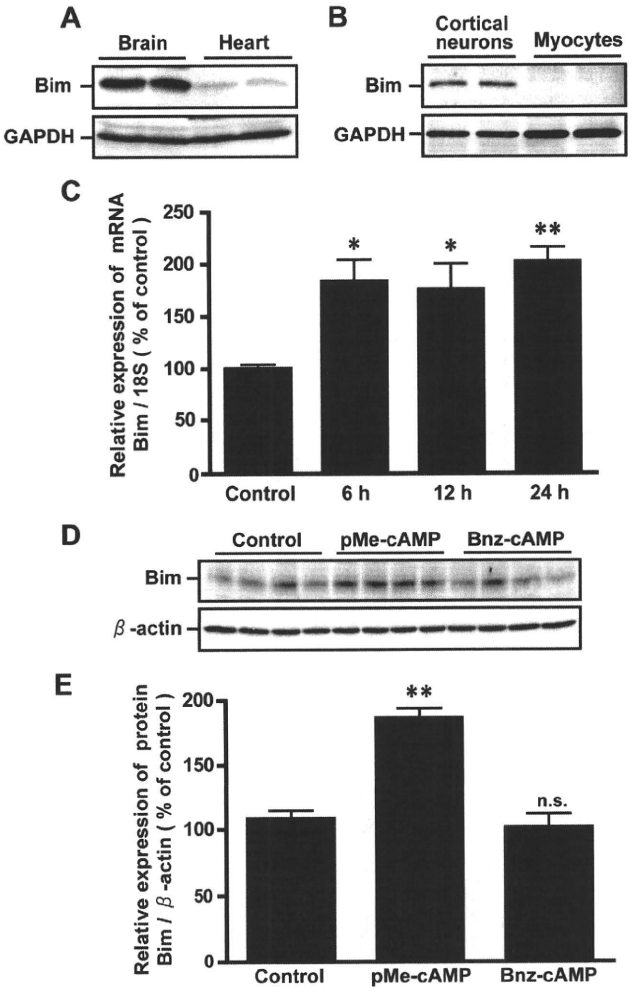

FIGURE 3. **Activation of Epac increased the expression of Bim mRNA and protein in cortical neurons.** *A* and *B*, shown is expression of endogenous Bim protein in brain and heart tissues (*A*) and cultured cortical cells and cardiac myocytes (*B*). *C*, the expression of Bim mRNA in cortical neurons was quantified using real-time RT-PCR. The data are normalized to 18 S ribosomal RNA. *D*, shown are representative immunoblots of Bim 24 h after the addition of pMe-cAMP (50 μM) or Bnz-cAMP (50 μM) in cortical neurons. β-Actin served as an internal control. *E*, shown is quantification of cAMP analog-induced Bim expression from three independent experiments. The results are presented as percentages of the amount of Bim expressed in the control experiment. $n = 6–8$, **, $p < 0.01$ *versus* control. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Epac Activation Increases Bim Expression—The Bcl-2 interacting member (Bim) is a sensor of apoptotic stress located upstream of the Bcl-2 family. Bim regulates Bcl-2 in the mitochondrial membrane, resulting in apoptosis (32, 33). Bim protein was highly expressed in brain tissue and cortical neurons (Fig. 3, *A* and *B*) but was expressed either slightly or not at all in heart tissue and cardiac myocytes, as previously described (34, 35). A previous paper suggested the involvement of cAMP/PKA signaling in regulating Bim expression in lymphoid cells (55). We, therefore, examined the change in the expression levels of Bim mRNA and protein in cortical neurons using pMe-cAMP. We found that pMe-cAMP increased expression of Bim mRNA and protein in a time-dependent manner (Fig. 3*C* and see Fig. 5, *C* and *F*). Furthermore, pMe-cAMP, but not Bnz-cAMP, signif-

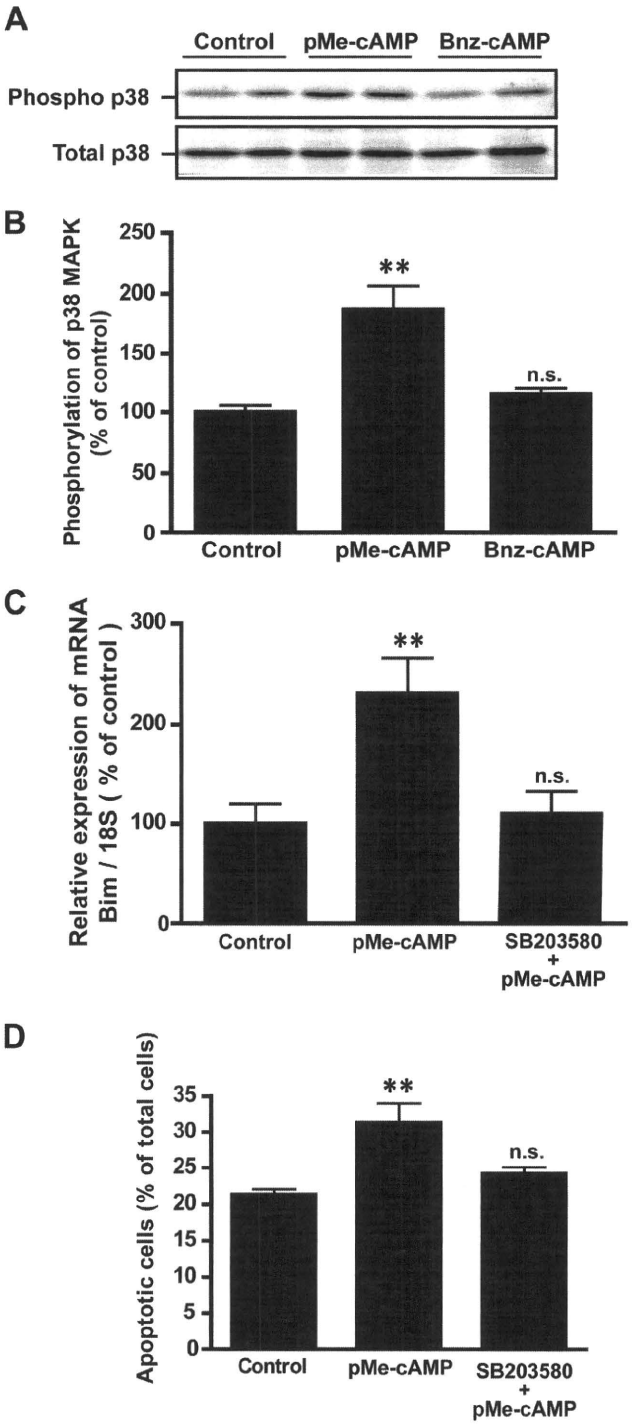

FIGURE 4. **Effect of p38 MAPK on Epac-induced Bim expression and apoptosis in cortical neurons.** *A* and *B*, shown are representative images and quantification of phosphorylation/total p38 protein in mouse cortical neurons treated with pMe-cAMP (50 μM) or Bnz-cAMP (50 μM) for 24 h. The results are presented as percentages of the amount of phosphorylation observed in the control experiment. $n = 6$ from 3 independent experiments. **, $p < 0.01$ *versus* control. *C*, the expression of Bim mRNA 24 h after the addition of pMe-cAMP (50 μM) or SB203580 (10 μM) plus pMe-cAMP (50 μM) in cortical neurons was quantified using real-time RT-PCR. The data are normalized to 18 S ribosomal RNA. $n = 6-8$; **, $p < 0.01$ *versus* control. *D*, apoptotic cells in cortical neurons and myocytes were examined by means of TUNEL staining 48 h after treatment with pMe-cAMP (50 μM) or SB203580 (10 μM) plus pMe-cAMP (50 μM). TUNEL-positive cells were quantified by counting nuclei in cortical neurons. The results are presented as percentages of the total cell number. $n = 4-6$; **, $p < 0.01$ *versus* control. *n.s.*, not significant.

icantly increased Bim protein in cortical neurons 24 h after treatment (Fig. 3,*D* and *E*). These results suggest that the stimulation of Epac increases Bim expression by enhancing transcription in cortical neurons, although the stimulation of PKA does not.

Inhibition of p38 MAPK Attenuates Epac-induced Bim Expression and Apoptosis—Three signal pathways have been implicated in regulating Bim protein expression: the JNK/c-Jun, cell cycle (Cdk4/E2F/Myb), and p38MAPK/FoxO pathways (36, 37). We next sought to determine which of these pathways plays the most important role. We found that an Epac-selective cAMP analog increased phosphorylation of p38 MAPK in cortical neurons, although a PKA-selective cAMP analog did not (Fig. 4, *A* and *B*). In contrast, there was no significant difference between Epac and PKA stimulation in terms of their effects on the phosphorylation of p44/42 MAPK, JNK1, or JNK2/3 (data not shown). Furthermore, SB203580, a p38 MAPK inhibitor, attenuated Epac-induced Bim expression and apoptosis (Fig. 4, *C* and *D*). These results suggest that Epac-induced neuronal apoptosis is mediated by the elevation of Bim expression via p38 MAPK.

Epac Activation Increases Interaction of Bim with Bcl-2—Bim is thought to exert its pro-apoptotic activity by binding to Bcl-2, thereby blocking the anti-apoptotic function of Bcl-2 (38). After demonstrating that stimulation of Epac increased Bim expression, we needed to confirm that the binding of Bim to Bcl-2 was likewise increased. We conducted pulldown assays using anti-Bcl-2 and anti-Bim antibodies after treatment with pMe-cAMP or Bnz-cAMP and found that the activation of Epac by pMe-cAMP significantly increased the amount of Bim associated with Bcl-2 in cortical neurons 24 h after the treatment (Fig. 5, *A* and *D*). Association of Bim with Bcl2 was also increased 10 h after Epac activation (Fig. 5, *B* and *E*), suggesting that association of Bim with Bcl2 was increased in accordance with increased Bim expression. Activation of PKA by Bnz-cAMP did not promote binding Bcl-2 with Bim even 24 h after treatment (Fig. 5, *H* and *I*).

Because it is already known that Bcl-2 regulates the mitochondrial pathway of apoptosis, we next explored whether pMe-cAMP induced apoptosis through the mitochondrial pathway in cortical neurons. Disruption of mitochondrial transmembrane potential is one of the earliest intracellular events, and such disruption occurs after induction of apoptosis via mitochondria (1). In apoptotic cells, the mitochondrial membrane potential is dissipated, and thus, the Mitocapture dye is dispersed in the cell as green fluorescent monomers detected at 488 nm. We found that Epac activation with pMe-cAMP (50 μM) promoted the disruption of mitochondrial transmembrane potential based on 488-nm-positive (green fluorescence) intensity in cortical neurons (Fig. 5, *J* and *K*). Taken together, these data suggest that stimulation of Epac promotes the binding of Bim to Bcl-2, leading to neuronal apoptosis via the mitochondrial pathway.

Epac-induced Neuronal Apoptosis Is Mediated by Bim—To further confirm the contribution of Bim to Epac-induced apo-

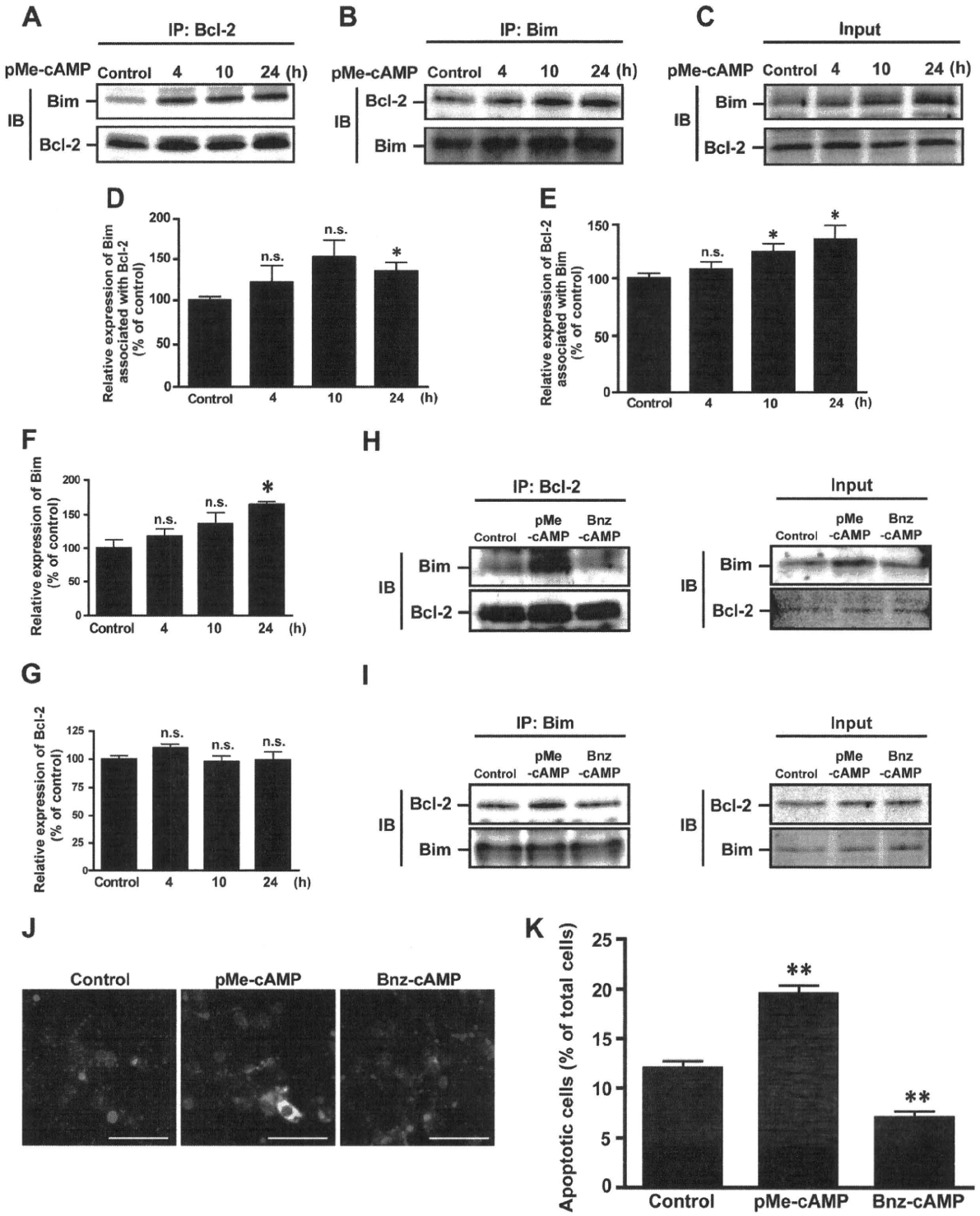

FIGURE 5. **Activation of Epac promoted interaction between Bcl-2 and Bim protein and decreased mitochondrial transmembrane potential in cortical neurons.** *A–C*, a representative immunoblot (*IB*) shows the interaction between Bcl-2 and Bim protein was increased by treatment with pMe-cAMP (50 μM) in a time-dependent manner. *IP*, immunoprecipitate. *D–G*, the pixel intensity of the bands obtained in each experiment was calculated. The results are presented as percentages of the intensity of the corresponding bands in the control experiment. $n = 5$ from 5 independent experiments. *n.s.*, not significant. **, $p < 0.05$ *versus* control. *H* and *I*, interaction of Bim and Bcl-2 in cortical neurons 24 h after incubation with pMe-cAMP (50 μM) or Bnz-cAMP (50 μM) was observed by means of immunoprecipitation with an antibody to Bim or Bcl-2. *J*, the changes in mitochondrial transmembrane potential were detected using the MitoCapture Apoptosis detection kit 48 h after the addition of pMe-cAMP (50 μM) or Bnz-cAMP (50 μM) in cortical neurons. Representative photomicrographs of cortical neurons stained with a cationic dye that fluoresces red in intact cells and green in apoptotic cells are shown. All nuclei were stained with DAPI (*blue*). *Scale bar*, 50 μm. *K*, apoptotic cells were counted, and their incidence was calculated. The results are presented as percentages of the total cell number. $n = 4$; **, $p < 0.01$ *versus* control.

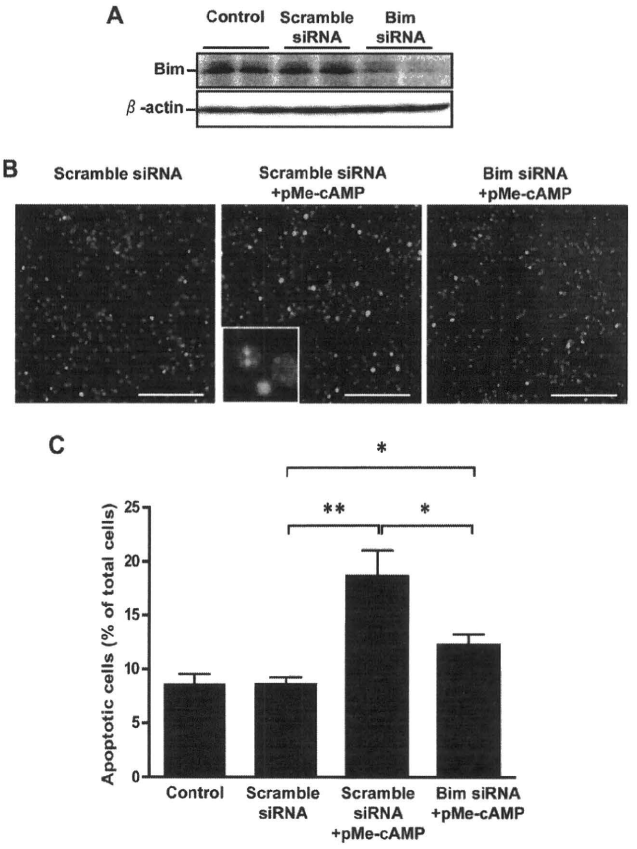

FIGURE 6. **Silencing of Bim attenuated Epac-induced apoptosis in cortical neurons.** *A*, shown is a representative immunoblot of Bim 72 h after transfection of Bim-targeted siRNA or negative siRNA control in cortical neurons. *B*, cortical neurons were transfected with indicated siRNA for 72 h, then treated with pMe-cAMP (50 μM) for 48 h. Apoptotic cells (*green*) were analyzed for TUNEL staining. All nuclei were stained with DAPI (*blue*). *Scale bar*, 100 μm. *C*, TUNEL-positive cells were quantified by counting nuclei in cortical neurons. The results are presented as percentages of the total cell number. $n = 4$; **, $p < 0.01$; *, $p < 0.05$ *versus* scramble siRNA. *n.s.*, not significant.

ptosis is in cortical neurons, we used Bim-targeted siRNA. Changes in Bim protein expression caused by the siRNAs are shown in Fig. 6*A*. When Bim was silenced, the effect of pMe-cAMP on the number of TUNEL-positive cells in cortical neurons became significantly smaller (Fig. 6, *B* and *C*), although Epac-induced apoptosis was not completely abolished. The evidence suggests that enhanced Bim expression via p38 MAPK appears to play an important role in Epac-induced apoptosis in neuronal cells.

Effects of Apoptotic Stimuli on Cortical Neurons of Epac1 KO Mice—Two isoforms of Epac, Epac1 and Epac2, have been previously identified (9) and are known to be activated by pMe-cAMP. In the present study we focused on the role of Epac1, because changes in Epac1 expression have been demonstrated in Alzheimer disease and in neuronal cells (13–15). It has, therefore, been tentatively proposed that Epac inactivation might play a protective role against neuronal apoptosis.

To test this theory, we generated Epac1 KO mice (see "Experimental Procedures" and Fig. 7*A*), which lacked Epac1 expression in neuronal cells as shown by Northern blot analysis (Fig. 7*D*). pMe-cAMP-induced Rap1 activation in Epac1 KO mice was significantly decreased in renal epithelial cells (Fig. 7*E*). Because renal epithelial cells do not express Epac2, this decrease in Rap1 activation most likely mirrors the impact of Epac1 deletion.

Induction of neuronal apoptosis has been well demonstrated in cortical neuronal cells using 3-NP (26, 39) or hydroxyl peroxide (40, 41). Using these pharmacological stressors, we examined whether the induction of apoptosis could be altered in cultured neuronal cells obtained from Epac1 KO mice. Apoptosis induced by hydroxyl peroxide or 3-NP, an irreversible inhibitor of mitochondria complex II, and detected through TUNEL staining was significantly decreased in neuronal cells from Epac1 KO mice (Fig. 8, *A* and *B*). Furthermore, both mRNA and protein expression levels of Bim remained significantly lower in cells from Epac1 KO mice than in those from WT mice (Fig. 8, *C–E*), suggesting that Epac1 deletion plays a protective role against neuronal stresses.

Deletion of Epac1 Attenuates 3-NP-induced Neuronal Apoptosis in Vivo—To examine the effect of Epac1 deletion *in vivo*, we administered 3-NP systemically to intact mice; this is a chemical and pathological way to induce mitochondrial and degenerative disorders *in vivo* (26, 39). We determined the number of apoptotic cells in cortical and striatal regions of Epac1 KO and WT mice through TUNEL staining. We found that 3-NP-induced apoptosis was significantly decreased in both the cortices and the striata of Epac1 KO mice *in vivo* (Fig. 9, *A*, *B*, *D*, and *E*). We confirmed that TUNEL-positive cells were stained with NeuN, a neuron-specific marker (supplemental Fig. 3). Furthermore, the number of cleaved caspase 3-positive cells was significantly increased in WT mice treated with 3-NP and was attenuated in Epac1 KO mice treated with 3-NP (Fig. 9, *A*, *C*, *D*, and *F*).

Taken together, these results reveal that Epac plays an important role in inducing neuronal, but not myocardial, apoptosis. More importantly, its role in this process is different from that of PKA. We found that neuronal apoptosis was, at least partially, mediated by Epac-Bim signaling and that Epac silencing had a protective role against apoptosis *in vivo*. Inhibition of Epac might be considered as a therapeutic strategy for the treatment of neurodegenerative diseases.

DISCUSSION

It is well known that cAMP signaling increases neuronal cell survival and decreases myocardial cell survival. We have demonstrated here that the activation of cAMP signaling does not protect neuronal cells when Epac is selectively activated. Rather, cAMP signaling increased apoptosis in neuronal cells when Epac1 was activated. In myocardial cells, however, Epac activation does not promote apoptosis. To our knowledge this is the first demonstration of the differential role of Epac in apoptosis in neuronal and myocardial cells, both of which are typical post-mitotic cells. The present study suggests that neuronal apoptosis is partly mediated by Epac through increased Bim expression and that the inhibition of Epac signaling plays a protective role in neuronal apoptosis *in vivo*.

The Roles of Epac and PKA in Apoptosis—The effect of cAMP signaling on cell death has been explored in multiple cell types, although most of these studies were conducted before Epac was

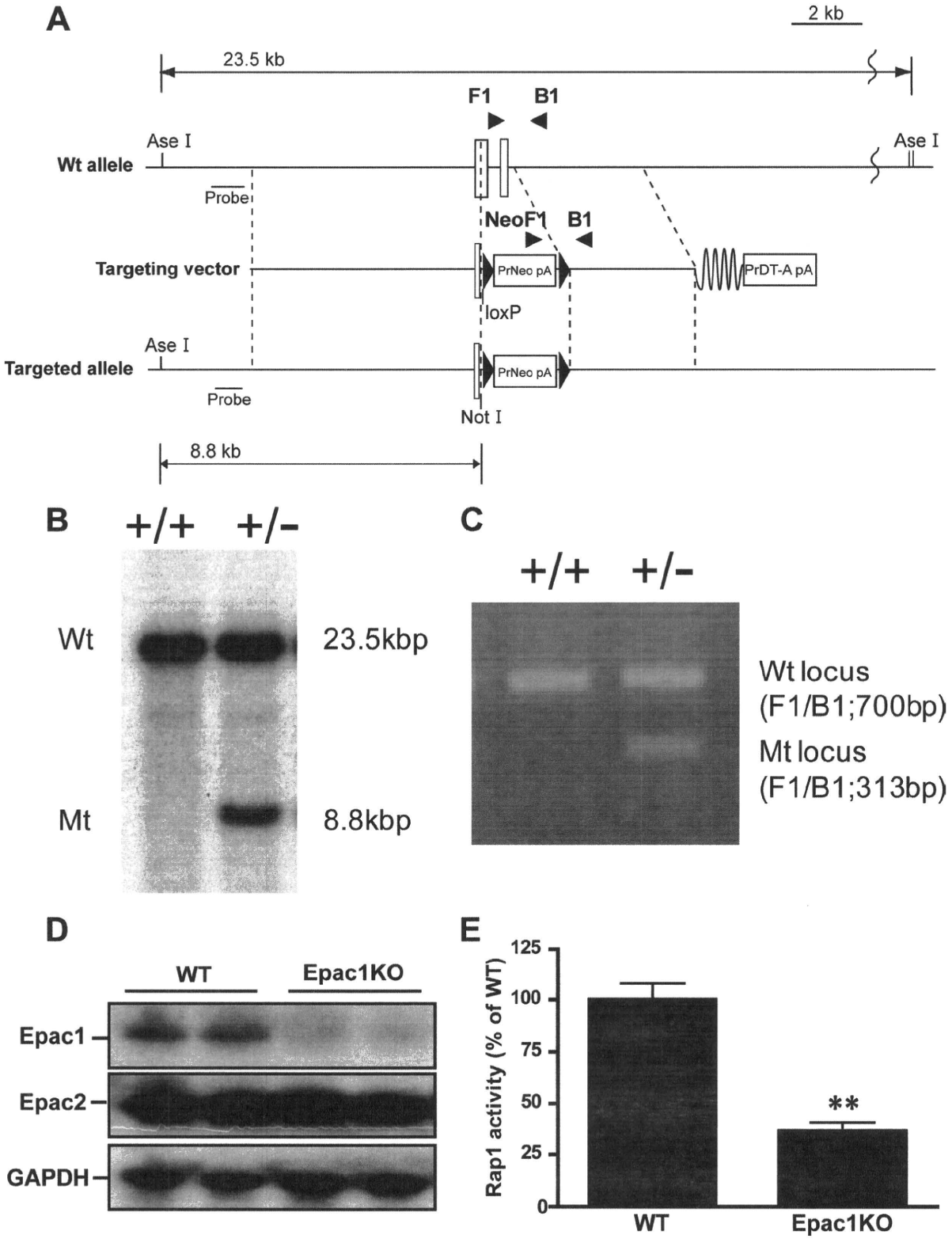

FIGURE 7. **Generation of Epac1 gene-targeted mice.** *A*, targeted disruption of the Epac1 gene is shown. The partial structure of the Epac1 gene (WT) and the resultant mutated allele (Epac1 KO) are shown. The positions of the phosphoglycerate kinase promoter neo cassette (*Neo*) and 5′-probe are indicated. *B*, shown is a Southern blot analysis of targeted embryonic stem cell (*ES*) clones. Genomic DNA from control TT2 ES cells and homologous targeted clones was digested with AseI and Not1 and hybridized with the probe as indicated in *A*. *C*, genotyping mice by PCR is shown. *D*, Northern blot analysis of Epac1, Epac2, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the brains of WT and Epac1 KO mice is shown. *E*, Rap1 activation of renal epithelial cells from WT and Epac1 KO mice 15 min after treatment with pMe-cAMP (50 μM) is shown. The data are normalized to total Rap1. $n = 4$; **, $p < 0.01$ *versus* WT mice.

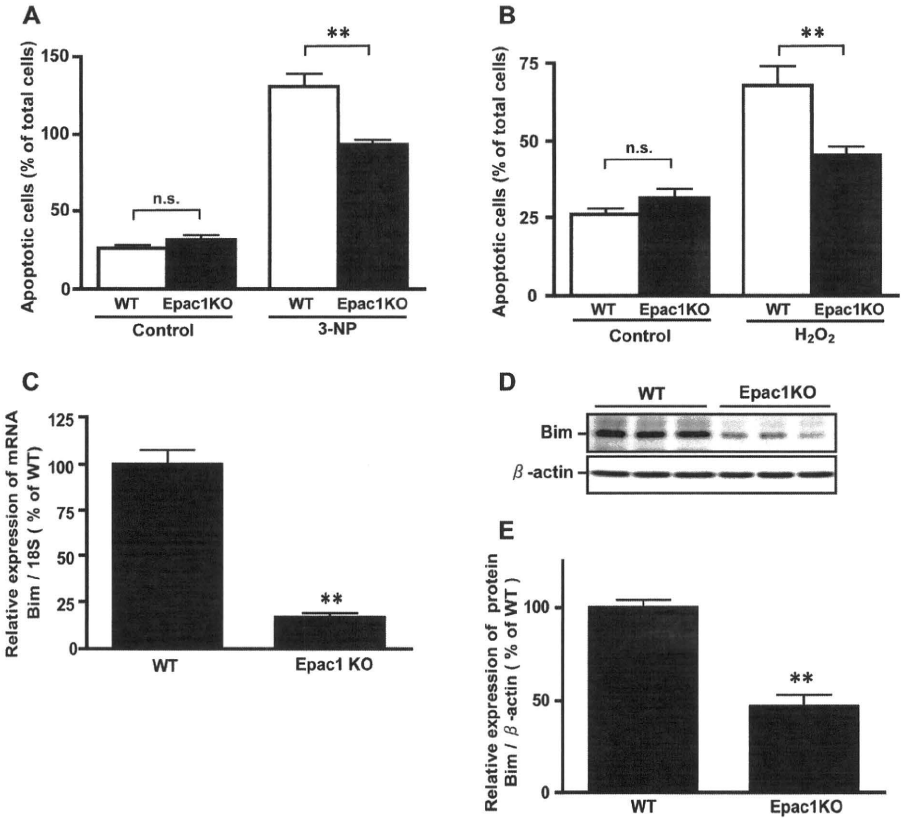

FIGURE 8. **The effect of 3-NP and hydroperoxide on apoptosis in cortical neurons from WT and Epac1 KO mice.** *A* and *B*, apoptosis was evaluated by means of TUNEL staining 48 h after the addition of the indicated reagents in cortical neurons from WT and Epac1 KO mice. The results are presented as percentages of the total cell number. *C*, the expression of endogenous Bim mRNA in cortical neurons from WT and Epac 1KO mice was quantified using real-time RT-PCR. The data are normalized to 18 S ribosomal RNA. $n = 6-8$; **, $p < 0.01$ *versus* WT. *D*, shown is a representative immunoblot of endogenous Bim protein expression in cortical neurons from WT and Epac1 KO mice. β-actin served as an internal control. *E*, the pixel intensity of the representative bands obtained in each experiment was calculated as described. $n = 4$; **, $p < 0.01$ *versus* WT mice. *n.s.*, not significant.

identified. In neuronal cells activation of cAMP/PKA signaling inhibited apoptosis induced by KCl in cerebella granule neurons (42) or by human immunodeficiency virus protein gp120 in the brain (43), promoting survival pathways in multiple neuronal cells (44, 45); these findings are in agreement with ours (supplemental Fig. 4). In cardiac myocytes, on the other hand, activation of cAMP signaling through such triggers as β-adrenergic receptor stimulation increased apoptosis (7, 8). In these studies the role of cAMP has been described primarily in terms of the activation of PKA.

Recently, several studies have suggested a contribution of either Epac alone or both Epac and PKA to apoptosis in restricted cell types including B-cell chronic lymphocytic leukemia (12), human leukocytes (11), immature B lymphoma cells (46), RINm5F β-cells (47), and H9c2 cells (48), showing that Epac and PKA play a protective role in apoptosis either alone and/or in concert in immune cells. However, the role of Epac in neuronal and myocardial apoptosis remains unknown despite the importance of cell death in tissues composed of post-mitotic cells. Our results show that stimulation and overexpression of Epac induces apoptosis in neurons but not in cardiac myocytes, implying that there are cell type-based differences in the effect of Epac activation on cell survival.

Epac-induced Apoptosis through Increased Bim Expression in Neuronal Cells—Our study demonstrated that Epac-induced apoptosis is mediated through the regulation of Bim, which acts on mitochondria as a pro-apoptotic factor, leading to disruption of the mitochondrial membrane potential. Bim binds to Bcl-2 and neutralizes its pro-survival function, resulting in apoptosis in multiple cell types (38, 49, 50). Bim is known to be expressed in neurons, hematopoietic cells, germ cells, lymphoid tissues, myeloid cells, and epithelial cells but not in cardiac myocytes, skeletal muscle, or neural-supporting cells, including glial, astrocytes, and oligodendrocytes (35). In agreement with these reports, our results show that Bim protein was highly expressed in primary culture of mouse cortical neurons but not in mouse cardiac myocytes. In cortical neurons we found that an Epac-selective cAMP analog increased Bim protein at the transcriptional level. When Bim was silenced, Epac-induced apoptosis was attenuated in neuronal cells. It should be noted that we were not able to exclude the possibility of off-target effects of the siRNAs because the rescue experiment that might exclude them is technically difficult. However, our results together with other data indicating that the suppression of the p38 MAPK pathway inhibits the elevation of Bim mRNA expression and Epac-induced apoptosis suggest that Epac-induced apoptosis is at least partly mediated by increased Bim expression. In fact, gene transfer of Bim to cardiac myocytes, which do not express Bim protein, induced apoptosis (supplemental Fig. 5). Taken together, the evidence strongly suggests that the expression of Bim is responsible for Epac-triggered apoptosis in neuronal cells, whereas Epac does not induce apoptosis in cardiac myocytes due to a lack of endogenous Bim expression. Further investigation is needed to identify the precise mechanism of Epac-induced transcriptional regulation of Bim in neuronal cells.

Changes in Epac Expression in Pathological/Physiological Conditions—Recent studies have indicated that the expression profile of Epac is altered during chronic degenerative inflammatory diseases. Epac1 mRNA, but not Epac2 mRNA, was increased in a mouse vascular injury model and was decreased in cardiac fibroblasts activated by transforming growth factor β (17, 23). Studies have reported the up-regulation of Epac1 mRNA and down-regulation of Epac2 mRNA in Alzheimer disease (13) and the up-regulation of Epac1 protein expression in inflamed rat neurons (14). These stud-

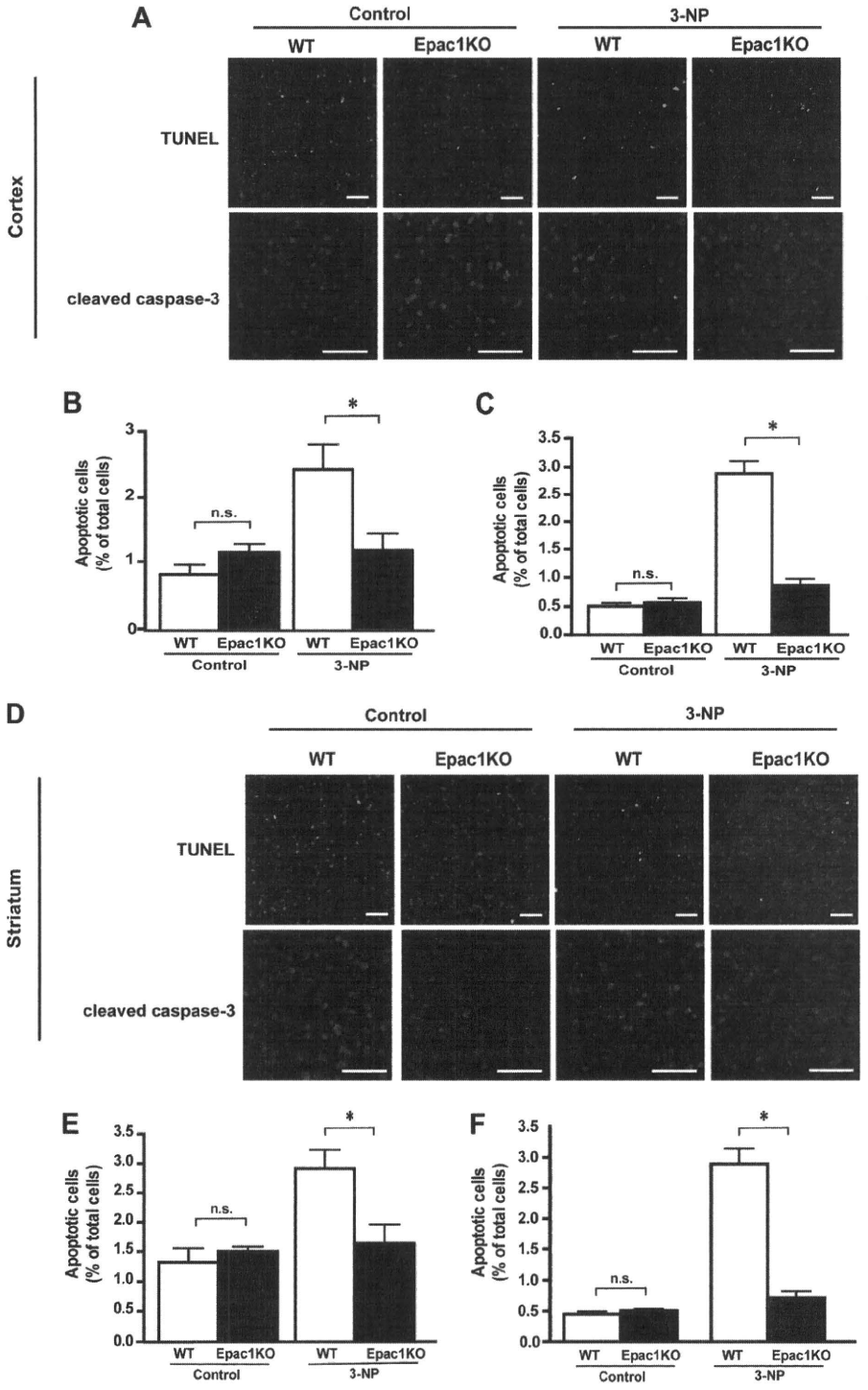

FIGURE 9. **Deletion of Epac1 suppressed 3-NP-induced brain cell apoptosis *in vivo*.** *A* and *D*, representative images of TUNEL staining and immunohistochemistry of cleaved caspase 3 of cortical and striatal sections from WT or Epac1 KO mice 24 h after injection of 3-NP. *Scale bar*, 100 μm. *B* and *E*, TUNEL-positive cells were quantified by counting nuclei in five randomly chosen fields. *C* and *F*, cleaved caspase 3-positive cells were quantified by counting nuclei in 5 randomly chosen fields. Deletion of Epac abrogated 3-NP-induced apoptosis in both the cortex and the striatum *in vivo*. The results are presented as percentages of the total cell number. $n = 5$ from 2 independent experiments. *, $p < 0.05$. *n.s.*, not significant.

ies indicate that the stoichiometry of Epac and especially that of Epac1 can be changed and selectively activated in disease conditions including neurodegenerative disorders.

Approximately half of all neurons in the nervous system undergo apoptosis during embryonic and early postnatal development (51), a period when Epac1 is highly expressed in the brain (15). Our results indicate that Epac1-induced neuronal apoptosis may be involved in the mechanisms underlying neuronal development. Nevertheless, Epac1 KO mice showed normal development up to at least 12 months of age, although no detailed assessment of their behavior, cognition, or learning memory has been made. Further studies using Epac2 KO mice and Epac1 and Epac2 double KO mice will need to be conducted given our observation that overexpression of Epac2 induced neuronal apoptosis *in vitro*.

The Effect of Epac1 Deletion on Apoptosis in Vivo—The mechanisms of neurological disorders such as Alzheimer disease, Huntington disease, and Parkinson disease are thought to stem from mitochondrial dysfunction (52). 3-NP, an irreversible inhibitor of the mitochondrial enzyme succinate dehydrogenase, is often administered systemically to treat these conditions and is considered to possess unique chemical and pharmacological traits that are accordingly considered in the generation of models of mitochondrial disorders and degenerative disorders (26, 39). The mechanisms of 3-NP toxicity are also thought to involve enhanced production of reactive oxygen species, including hydrogen peroxide, which can cause oxidative damage to DNA, lipids, and proteins (53). In the present study both 3-NP and hydrogen peroxide failed to induce apoptosis in cultured cortical neurons from Epac1 KO mice, and 3-NP-induced neuronal apoptosis was abolished in Epac1 KO mice *in vivo*. In contrast, there was no difference between Epac1 KO and WT mice in terms of 3-NP-induced apoptosis. Cardiac myocytes from Epac1 KO mice did not differ in their sensitivity to cAMP analogs (supplemental Figs. 6 and 7). Although this and our previous studies show that the inhibition of Epac1 protects 3-NP-mediated neuronal apoptosis *in vivo*

and *in vitro*, the relevance of Epac2 to this phenomenon needs to be examined in future studies. A recent study has demonstrated that Epac is involved in the secretion of an amyloid precursor protein, which has been known to induce apoptosis leading to Alzheimer disease (54). Together with our data, this indicates that selective inhibition of the Epac signal may prove useful as a therapeutic strategy in treating neurodegenerative diseases.

In conclusion, Epac induces neuronal apoptosis through increased Bim expression. Because disruption of Epac1 exerts a protective effect on neuronal apoptosis *in vivo*, inhibition of Epac may be a useful tactic in the treatment of neurodegenerative diseases.

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Review

Accessory proteins for heterotrimeric G-protein: Implication in the cardiovascular system

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Abstract

The G-protein signaling system plays an important role in controlling cellular responses to numerous hormones and neurotransmitters involved in homeostasis of the cardiovascular system. In addition to traditional determinants of G-protein signaling such as the G-protein-coupled receptor (GPCR), heterotrimeric G-proteins and effectors, accumulating data indicate the existence of entities that directly regulate the activation status of G-proteins independent of GPCR. To date, there have been a number of reports on accessory proteins that influence GDP dissociation, affect nucleotide exchange at the Gα subunit, alter subunit interactions within heterotrimeric Gαβγ independent of nucleotide exchange, or form complexes with Gα or Gβγ independent of the typical Gαβγ heterotrimer. Such proteins may provide an additional signal input to the G-protein signaling system in the absence of GPCR or may act as an alternative binding partner of G-protein subunits serving unknown roles of G-proteins in cells. Accumulating information suggests that accessory proteins for G-proteins are actually involved in the regulation of the signaling system to maintain homeostasis and the dynamic responses to physiological and pathological challenges. It is likely that alterations in signal processing may be achieved by the modulation of signal processing within the cell using accessory proteins for G-proteins. The loss of regulation of this system, leading to inappropriate activation or inactivation of G-protein signaling, is strongly implicated in various human diseases. In this review, we update current information and discuss different accessory proteins for heterotrimeric G-proteins in terms of their involvement in the regulation of the cardiovascular system. Such information may contribute to uncovering mechanisms underlying cardiovascular disease as well as the development of novel therapeutic approaches to human disease.

Keywords: Heterotrimeric G-protein; Signal transduction; Activator of G-protein signaling (AGS); Regulator of G-protein signaling (RGS); Cardiovascular disease

Contents

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1. Introduction

Signal processing via heterotrimeric G-proteins is one of the most widely used systems for information transfer across the cell membrane. This system is essential for maintaining tissue homeostasis, but is also involved in the development of cardiac hypertrophy [1], remodeling of the heart [1], ischemic preconditioning [2,3], and angiogenesis [4]. In addition, many of the therapeutic interventions are used in the management of cardiovascular disease targeting G-protein signaling systems via cell surface G-protein-coupled receptors (GPCRs).

In this system, seven transmembrane domain GPCRs are activated by extracellular stimuli, such as hormones and neurotransmitters, inducing a conformational change in the Gα subunit, and then catalyzing GDP release from Gα (Fig. 1). Binding of GTP to Gα destabilizes Gαβγ complex, leading to a structural rearrangement of Gα-GTP, Gβγ and the receptor. This is followed by Gα-GTP disassociation from the receptor and Gβγ. Both subunits, Gα-GTP and Gβγ, stimulate distinct downstream effector molecules including adenylyl cyclases, phospholipases, ion channels, and protein kinases [5–7]. The activation of the signaling pathway is terminated when Gα hydrolyzes GTP to GDP by its intrinsic guanosine triphosphatase (GTPase) activity; it then reassociates with Gβγ, thus completing the cycle. Reassociation of Gβγ with Gα-GDP terminates interactions of effector molecules. Gβγ facilitates the coupling of Gα to GPCR and also acts as a guanine nucleotide inhibitor (GDI) for Gα-GDP, slowing spontaneous exchange of GDP for GTP (Table 1).

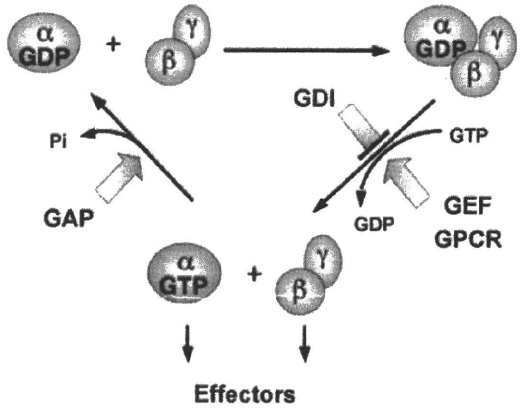

Fig. 1. Schematic diagram indicating G-protein activation/deactivation cycle and influences of Gα regulatory protein. GEF: guanine nucleotide exchange factor; GDI: guanine nucleotide dissociation inhibitor; GAP: GTPase-activating protein; GPCR; G-protein-coupled receptor.

Table 1
Abbreviations.

AGS, activator of G-protein signaling
ERK, extracellular signal-regulated kinase
GAP, GTPase-activating protein
GDI, guanine nucleotide dissociation inhibitor
GEF, guanine nucleotide exchange factor
GDP, guanosine-5′-diphosphate
GIRK, G-protein-activated inwardly rectifying potassium channel
GPCR, G-protein-coupled receptor
GPR, G-protein regulatory
GRK, G-protein-coupled receptor kinase
GTP, guanosine-5′-triphosphate
GTPγS, guanosine-5′-O-(3-thiotriphosphate)
HSP-90, heat shock protein 90
KATP, ATP-sensitive potassium
MAP, mitogen-activated protein
MEK, MAP/ERK kinase
NG-GPA, NG108-15 G-protein activator
NMDA, N-methyl-D-aspartate
Pcp2, Purkinje cell protein-2
PBP, phosphatidylethanolamine-binding protein
RACK1, receptor for activated C kinase 1
RGS, regulator of G-protein signaling
Ric, resistance to inhibitors of cholinesterase
RKIP, Raf-1 kinase inhibitor protein
siRNA, small interfering RNA
SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

These events are dynamically regulated to optimize signal specificity, maximize signal efficiency and integrate diverse stimuli. As a consequence of this central role in signal integration, the subtle adaptations or maladaptations that occur in response to both physiological and pathophysiological challenges can play a key role in the manner in which the effector cell responds to such challenges. Regulations of the G-protein system include the segregation of specific signaling molecules in cell microdomains, receptor phosphorylation and internalization, and crosstalk between signaling pathways. In addition to these, a number of proteins that regulate the basal activation state of G-proteins independently of cell surface GPCR have been discovered. Such accessory proteins influence the activation or deactivation of the Gα subunit and alter subunit interactions within heterotrimeric Gαβγ independent of nucleotide exchange, or form complexes with

Gα or Gβγ independent of the typical Gαβγ heterotrimer. Accessory proteins for G-proteins may provide an additional signal input to the G-protein signaling system in the absence of GPCR or may act as an alternative binding partner of G-protein subunits serving unknown roles of G-proteins in cells. In previous work, we evolved the observation of such accessory proteins and tuning of the signaling system concerned based on cell-specific differences [8,9] and the subsequent biochemical characterization of such differences [9–12]. We later addressed the impact of accessory proteins in terms of system adaptation of the GPCR system [13] and identified novel accessory proteins for G-proteins induced in the myocardium in response to transient ischemia [14].

An adaptation of the signaling system is critical for maintaining homeostasis and the dynamic responses to physiological and pathological challenges. It is likely that subtle alterations in signal processing may be achieved by discrete modulation of signal processing within the cell using accessory proteins for G-proteins. Current information suggests that accessory proteins for G-proteins are actually involved in the regulation of the signaling system for maintaining homeostasis and the dynamic responses to physiological and pathological challenges. The loss of regulation of this system, leading to inappropriate activation or inactivation of G-protein signaling, is strongly implicated in various human diseases.

In this review, we update current information and discuss various accessory proteins for heterotrimeric G-proteins in terms of their involvement in the regulation of the cardiovascular system (Table 2). Such information may contribute to uncovering the mechanisms underlying cardiovascular disease as well as the development of a novel therapeutic approach to human disease.

2. Guanine nucleotide exchange factors (GEFs) for Gα subunit

The first class of accessory proteins facilitates guanine nucleotide exchange of Gα subunits such as activated G-protein-coupled receptors. Proteins have been identified as nonreceptor GEFs that increase GTPγS binding to the Gα subunit similar to activated GPCR, and include GAP43 [15,16], NG-GPA [8,9], β-APP [17], presenilin I [18], AGS1 [19–23], PBP/RKIP [24–28], and Ric-8 [29]. Among 16 of the Gα genes identified [30,31], most of them preferentially activate the $G\alpha_i$ family; however, Ric-8A can also activate $G\alpha_q$ in vitro [29]. Although the regulation of GEF and its physiological relevance are not completely understood at present, its potential association with cardiovascular disease has been reported in several proteins (Table 2).

2.1. Activator of G-protein signaling 1 (AGS1, DexRas1, and RASD1)

AGS1 was initially discovered as dexametasone-inducible, ras-related cDNA (DexRas) in mouse corticotroph cells [21]. AGS1 is a member of the Ras family of monomeric GTPases which is most closely related to Rhes (60% amino acid identity), a Ras-related protein with unique insert regions [32,33]. Purified AGS1 directly binds to $G\alpha_i$, and increases GTPγS binding to purified $G\alpha_{i1}$ and $G\alpha_{i2}$ in vitro [22].

In mammalian cells, transfected AGS1 activates the Erk1/2 pathway without receptor stimulation [22]. AGS1 also inhibits cAMP accumulation in response to forskolin or a constitutive active $G\alpha_s$ [34], which is also consistent with the regulation of $G\alpha_i$ by AGS1, indicating the function of AGS1 as a direct Gα activator. However, when transiently co-transfected with a $G\alpha_i$-coupled receptor, AGS1 blocks the downstream signaling of the agonist-stimulated receptor, as well as the receptor-mediated heterologous sensitization of adenylyl cyclase [35].

In human tissues, AGS1 is highly enriched in heart, brain, liver, skeletal muscle and bone marrow, with lower expression in pancreas, kidney, thymus, lung and placenta [36,37]. AGS1 expression is induced by various stimuli related to stress. Moreover, AGS1 expression is up-regulated in cultured cells following glucocorticoid treatment [21] and in whole animals following severe blood loss [38], suggesting that AGS1 plays a role in mediating the stress response. The disruption of AGS1 in mice suggests its importance in the signaling of the NMDA-mediated circadian cycle [39].

A comprehensive concept of AGS1 function in the context of the whole signaling system is still being developed.

2.2. Growth-associated protein 43 (GAP43)

GAP43 enriched in the neuronal growth cone plays an important role during development and axonal regeneration. GAP43 increases the rate of GDP dissociation and GTPγS association to purified brain heterotrimer G-proteins and purified $G\alpha_o$ free of Gβγ [15,16]. In oocytes, GAP43 enhanced GPCR signaling in response to an agonist and was able to cause a transient current flow without receptor activation [40]. GAP43 is involved in the innervation of cardiomyocytes and left stellate ganglions [41]. The upregulation of GAP43 was reported in several experimental models, including myocardial infarction [41,42] and right ventricular hypertrophy [43].

2.3. Phosphatidylethanolamine-binding protein (PBP)/Raf kinase inhibitor protein (RKIP)

PBP or RKIP is a 23-kDa soluble protein that inhibits the activation of MEK [24,25] by RAF1 and also blocks GRK2 activity [26], suggesting a role in signal crosstalk in cells. PBP/RKIP facilitates binding of GTPγS to purified $G\alpha_{i1}$, and appears to enhance $G\alpha_{i/o}$-mediated signaling in vivo [28]. Lorenz et al. demonstrated that PKC activation following GPCR activation triggered RKIP dissociation from Raf-1 to associate with GRK2 [26]. Thus, the GPCR signal is enhanced both by removing an inhibitor from Raf-1 and by blocking receptor internalization via GRK2.

Table 2
Accessory proteins for heterotrimeric G-protein involved in the regulation of cardiovascular system.

Proteins	Function on G-protein	Association with cardiovascular system	References
Guanine nucleotide exchange factors (GEFs) for Gα subunit			
AGS1	GEF for $G\alpha_i$	Relative high level expression in human heart; up-regulated in response to biological stress	[19–23,35–39]
GAP-43	GEF for brain G-protein and $G\alpha_o$	Enriched in stellate ganglion; upregulation in myocardial infarction and right ventricular hypertrophy	[15,16,40–43]
PBP/RKIP	GEF for purified $G\alpha_{i1}$	Involved in β-adrenergic signaling and contractility of cardiomyocyte	[24–28]
Presenilin	GEF and GAP for $G\alpha_o$	Critical factor for cardiac development and familial DCM	[18,44–46]
Tubulin	Transactivate Gα subunit, interact with Gβγ subunit	Multiple roles including protein sorting; microtubule regulation	[47–52]
Guanine nucleotide dissociation inhibitors (GDIs) for Gα subunit			
AGS3	GDI for $G\alpha_{i/o}$, stabilize Gα-GDP complex	Short variant expressed in the heart; AGS3 null mice exhibited lower arterial pressure and an enhanced gain of the baroreceptor reflex	[13,32,33,55–56]
Caveolin	Inhibit GTPase activity and GTPγS binding to $G\alpha_o$ Interact with $G\alpha_{i2}$	Involved in various signaling system via its scaffolding function	[111–114]
GTPase-activating proteins (GAPs) for Gα subunit			
RGS2	GAP for $G\alpha_q \gg G\alpha_{i/o}$	Deletion of RGS2 caused strong hypertension in mice	[70,76–82]
RGS4	GAP for $G\alpha_{i/o}$, $G\alpha_q$	RGS4 overexpressing mice delayed hypertrophy in response to the pressure stress or $G\alpha_q$ pathway stimulation	[66,69,71–75]
Hax-1	Enhances $G\alpha_{13}$-mediated Rac activity	Inhibits apoptosis of adult cardiomyocyte through interaction with caspase-9	[115]
Accessory proteins interact with Gβγ subunits			
AGS8	Interact with Gβγ subunit	Identified in repetitive transient ischemia model of rat heart; increased expression in response to ischemia/hypoxia	[14,90]
Phosducin and phosducin-like protein	Interact with Gβγ subunit	Expression in the heart; overexpression of part of phosducin improved contractivity of failing heart	[102–110]
RACK1	Interact with Gβγ subunit	Suggested involvement in reduced contractility of senescent cardiomyocyte	[92–96]
Syntaxin1	Interact with Gβγ subunit	Modulate K^+ and Ca^{2+} channel activity; involvement in atrial natriuretic peptide release from cardiomyocyte	[97–101]
GRK2	Interact with Gβγ subunit	Regulation of cell surface GPCR	[116,117]
Others			
HSP-90	Alter $G\alpha_{12}$ signaling	Involved in multiple signaling cascades via chaperone function	[118]

A physiologic role for this mechanism was shown in cardiomyocytes in which the downregulation of RKIP restrains β-adrenergic signaling and contractile activity. Thus, the relative importance of these different actions of PBP/RKIP needs to be addressed.

2.4. Presenilin-1

Presenilin-1 is the gene responsible for the development of early-onset familial Alzheimer's disease and is involved in multiple cellular events [44]. The C-terminus of presenilin directly interacts with and enhances both GTPγS binding and GTP hydrolysis of $G\alpha_o$ [18]. Presenilin-1 is also expressed in the heart and is critical for cardiac development [45]. Li et al. reported familial DCM and heart failure associated with missense mutation of presenilin [46]. The role of the interaction of these proteins with G-proteins in the development of heart failure is not well understood at present.

2.5. Tubulin

Tubulin activates the Gα subunit by different mechanisms, and thus it increases GTP binding to Gα subunits by the transfer of GTP bound on tubulin to the GDP bound on

Gα [47–51]. Tubulin has also been reported to interact with GPCR and Gβγ [52]. From its functions, tubulin has been suggested to have multiple roles in trafficking, G-protein activation, and the control of microtubule dynamics [52]. Furthermore, tubulin and G-protein interaction may influence microtubule polymerization and alter cardiomyocyte architecture.

3. Guanine nucleotide dissociation inhibitors (GDIs) for Gα subunit

The second class of accessory protein is a group of guanine nucleotide dissociation inhibitors (GDIs). The majority of this subgroup of accessory proteins shares a common structural feature termed the G-protein regulatory (GPR) or GoLoco motif [32,33,53]. The GPR motif is a 20–25 amino acid cassette that serves as a docking site for $G\alpha_{i/o}$ and $G\alpha_t$. The interaction of GPR motifs with $G\alpha_{i/o}$ stabilizes the GDP-bound conformation of Gα and interferes with Gβγ for binding to $G\alpha_i$, which results in inhibition of Gα-mediated signaling and prolongation of Gβγ-mediated signaling. The GPR motif is found in AGS3 (GPSM1), LGN (GPSM2), AGS4 (GPSM3), Purkinje cell protein-2 (Pcp2)/L7, WAVE-1 and the GTPase-activating proteins RGS12, RGS14, RAP1GAPI (partial GPR motif), and Rap1GAPII [32,33,53]. The GPR proteins are also known as crucial players in asymmetric cell division during embryogenesis [54].

3.1. Activator of G-protein signaling 3 (AGS3)

AGS3 (GPSM1) is one of the proteins extensively studied in GDIs for Gα subunit. This protein was identified as a receptor-independent activator of G-protein signaling in a yeast-based functional screen of mammalian cDNAs. AGS3-LONG is a 650 amino acid protein containing 7 tetratricopeptide repeats as well as 4 GPR motifs, and is preferentially expressed in the brain. AGS3 has a short form enriched in the heart lacking the tetratricopeptide repeat motif domains and contains only three GPR motifs [55]. The impact of AGS3-SHORT in signal integration in cells was investigated in the sensitization of adenylyl cyclase. This sensitization is known as a transient enhancement of the activity of adenylyl cyclase following sustained stimulation of $G\alpha_i$-coupled receptors, and as an example of an adaptive response of cells [56,57]. The sensitization of $G\alpha_s$ regulation of adenylyl cyclase observed with sustained activation of $G\alpha_i$-coupled GPCR was blocked by the overexpression of heart-type AGS3 (AGS3-SHORT) in CHO cells [13]. This observation was associated with an increase in the stability of the $G\alpha_i$ subunit in the membrane. Interventions against AGS3 utilizing GPR-related components indicated that AGS3 also plays an important role in adaptive neuronal events associated with the drug-seeking behavior and locomotor sensitization following cocaine withdrawal [58,59]. These data suggested that AGS3 and GPR proteins influenced the adaptation process of the G-protein signaling system.

Recently, Lanier's group carried out an initial characterization of AGS3 null mice [60]. In these mice, the full-length AGS3 was disrupted, but a short variant form of AGS3 was still expressed in the heart. Interesting alterations were observed in the regulation of blood pressure and metabolic functions in AGS3 null mice. Thus, the mean arterial pressure was significantly lower in AGS3 null mice, and its diurnal variations were reduced. Moreover, AGS3 null mice showed enhanced gain of the baroreceptor reflex. Altered vascular control was also observed in terms of the suppression of recovery of arterial pressure following administration of sodium nitroprusside. In the analysis of metabolic homeostasis, AGS3 null mice exhibited a lean phenotype, reduced fat mass and increased nocturnal energy expenditure. The first observation of a genetically modified animal of AGS3 suggests the potential of AGS3 and perhaps other GPR proteins as a therapeutic target in human disease.

4. GTPase-activating proteins (GAPs) for Gα subunit

The majority of proteins of this group share 120–130 amino acids of the regulator of G-protein signaling (RGS) homology domain, which mediates the GTPase-accelerating activity at Gα subunits [61,62]. At least 30 mammalian proteins are known to share an RGS or RGS-like domain [63,64]. Most of the RGS proteins are GAPs for $G\alpha_{i/o}$ and $G\alpha_{q/11}$ family members with some exceptions for GAPs for either $G\alpha_{12/13}$ or $G\alpha_s$ family members [64,65]. The change in the expression of RGS proteins in human heart failure has also been reported. Mittmann et al. indicated an increase in RGS4 mRNA but no change in RGS2 or RGS3 in the myocardium from terminally failing human hearts with dilated or ischemic cardiomyopathy [66], whereas Owen et al. found an apparent upregulation of RGS3 and RGS4 proteins and mRNA in end-stage failing hearts of humans [67]. Takeishi et al. also identified an apparent decrease of RGS2 protein in human failing heart following treatment with left ventricular assist device [68]. Although these results which might reflect the background of the patients were not consistent, alterations in RGS2, RGS3 and RGS4 expression suggested their potential to alter cardiovascular pathophysiology. Among the RGS protein families, the physiological roles of RGS2 and RGS4 in the cardiovascular system were extensively studied in mice deficient for RGS2 [69] or overexpressing RGS4 [70].

4.1. RGS4

RGS4 has a GAP activity for $G\alpha_{i/o}$ and $G\alpha_q$ and appears to negatively regulate signaling events mediated by Gα subunits. Its association with schizophrenia has also been reported in some populations [71–73]. On the other hand, the role of RGS4 in cardiovascular function was previously examined utilizing transgenic mice overexpressing RGS4

[70]. The overexpression of RGS4 did not affect basal cardiac function or chronotropic response to dobutamine, but significantly reduced the ability of the heart to adapt to an increase in cardiac afterload induced by transverse aortic constriction. In cardiomyocytes, the overexpression of RGS4 inhibited phenylephrine- and endotherin-1-induced hypertrophy or contraction [66,74]. The co-overexpression of RGS4 and $G\alpha_q$ in transgenic mice delayed the $G\alpha_q$-mediated onset of cardiac hypertrophy, which exhibits a phenotype similar to human cardiac hypertrophy [75]. The increase of RGS4 in a failing heart may decrease contractile response; however, this increase reduces the adverse effect of $G\alpha_q$ signaling. At present, the overall direction toward protection of the heart by RGS4 remains controversial.

4.2. RGS2

RGS2 has unique binding and some forms of selectivity for $G\alpha_{q/11}$ rather than for $G\alpha_i$ [76,77]. It appears to attenuate signaling events mediated by $G\alpha_{q/11}$-coupled receptor, including vasocontraction and cardiac hypertrophy [69,76,78]. It has also been shown that $rgs2^{+/-}$ and $rgs2^{-/-}$ mice exhibit a similar and strong hypertensive phenotype, suggesting that both copies of the gene are essential for normal cardiovascular function. Further analysis suggested that this phenotype may be attributed to enhanced and/or prolonged vascular contractile responses to GPCR stimulation [69,79].

The overexpression of RGS2 in cardiomyocytes eliminated any increase in cell size and genetic markers of hypertrophy in response to α_1-adrenergic stimulation [80,81]. A recent study found RGS2 to be selectively downregulated during the early onset of cardiac hypertrophy with enhanced $G\alpha_{q/11}$ signaling [82]. SiRNA-mediated RGS2 knockdown increased phenylephrine- and endothelin-1-induced PLCβ stimulation and exacerbated the hypertrophic effect [82]. Interestingly, no overt changes were observed in the cardiac function of $rgs2^{-/-}$ mice up to 6 months of age [69]. These lines of evidence implied a beneficial effect of RGS2 in protecting the heart, although more studies are needed to clarify the role of RGS2 in hypertrophy-associated GPCR signals.

5. Accessory proteins interact with subunits of Gβγ

There are 5 Gβ and 12 Gγ subunits reported in humans and mice, offering potential of a large diversity of combinations of Gβγ dimers [6,30]. $G\beta_{1-4}$ share 82–92% homology with each other and $G\beta_5$ has approximately 50% divergent homology from others [6,30]. In contrast to the Gβ subunit, Gγ shares only 15–30% sequence identity within subunits [6,30]. The $G\beta_5$ subunit is unique since it can form a unique complex with RGS7 apart from the gamma subunit. Other RGS proteins containing the Gγ-like (GGL) domain, namely, RGS6, RGS7, RGS9, and RGS11 are also predicted to be able to assemble with $G\beta_5$ [83–85]. Although $G\beta_5$ is preferentially expressed in neuronal systems, a relatively higher expression in the heart was observed in humans than in mice [86]. However, the functional and/or physiological roles of this interesting complex are still not clear.

Gβ subunit and Gγ subunit are tightly associated and usually act as a heterodimer. It was once considered that the Gβγ subunit inhibited the guanine nucleotide exchange of Gα and terminated the G-protein signal by sequestrating Gα subunits. However, it is now known that Gβγ regulates many signaling molecules, including G-protein-activated inwardly rectifying potassium channel (GIRK), Ca channels, $PLC\beta_2$ and β_3, PLCε, and GRK2 [6,7]. Interestingly, mutational and structural studies have indicated that effector proteins and the Gα subunit share an overlapping interface on the surface of Gβγ [7,87]. The Gβ subunit does not have a catalytic site, and does not change its conformation when it dissociates from the Gα subunit on activation of a heterotrimer. Thus, the activation of Gβγ-mediated signaling is initiated by protein–protein interaction of Gβγ without sharing the interface for signal transduction.

Number of Gβγ-interacting proteins continues to increase. Because Gβγ-interacting proteins share a common region of the surface, they would cover up and inhibit the Gβγ signal to other molecules. Thus, the binding of Gβγ-interacting proteins to this sharing site is principally expected to exclude interaction of other proteins, resulting in the shutdown of Gβγ signaling as Gα does. Recently, Bonacci et al. have developed small molecules targeting the sharing interface of Gβγ and have also identified a compound selectively altering the Gβγ signaling pathway in cells [14,88].

5.1. Activator of G-protein signaling 8 (AGS8)

AGS8 was isolated utilizing a yeast-based functional screen expressing human Gα in place of the yeast Gα from a cDNA library generated from a rat model of repetitive transient ischemia of the heart [14]. In this model, rat hearts were subjected to repetitive transient ischemia by the inflation of an implanted pneumatic snare around the proximal left anterior descending coronary artery (40 s occlusion, 20 min interval, 8 h/day) that induced coronary collateral growth in 2 weeks [89].

AGS8 (GeneBank accession # DQ256268) encodes an uncharacterized 1730 amino acid protein (KIAA1866, FNDC1 in humans) with 4 fibronectin-type III domains, 3 at the N-terminus and 1 C-terminal [14]. The amount of AGS8 mRNA was increased in the ischemic area of left ventricle by 3.5-fold compared with the non-ischemic area; however, this increase was not induced in cardiac hypertrophy or heart failure, suggesting a specific role of AGS8 in the signaling processes underlying ischemia [14]. Incidentally, hypoxic treatment of adult ventricular cardiomyocytes up-regulated AGS8 mRNA by 8-fold. This increase was not observed in cultures of aortic endothelial cells, aortic vascular smooth muscle cells or cardiac fibroblasts. Protein interaction assays with GST-AGS8 and purified G-protein subunits or brain

lysates indicated direct binding of AGS8 to Gβγ [14]. Interestingly, subsequent studies suggested that the interaction of AGS8 with Gβγ occurred in a manner that did not alter the regulation of the effector PLC-β_2 by Gβγ. The AGS8 binding site on the Gβγ subunit appears to reside at the common interface of Gβ overlapping with PLC-β_2 and the Gα subunit [7,90]. This observation suggested, in line with expectations, that the mechanism of Gβγ interaction to other molecules might be more complex. An alternative mode of Gβγ interaction and the formation of signaling components are postulated [7,90].

AGS8 serves as an ischemia-inducible binding partner for Gβγ in cardiomyocytes providing an unexpected mode of signal input to the G-protein system. Such mechanisms of signal regulation may provide intrinsic cues for the adaptation of the heart to ischemia.

5.2. Receptor for activated C kinase 1 (RACK1)

Gβ belongs to a large family of WD40 repeat proteins [91] and interacts with other WD40 proteins, such as the receptor for activated C kinase 1 (RACK1) [92]. RACK1 shares 57% amino acid similarity with $G\beta_1$ lacking N-terminal extension for binding the Gγ subunit, and usually does not interact with the Gα subunit [93].

RACK1 inhibited the Gβγ-mediated activation of phospholipase $C\beta_2$ and adenylyl cyclase, whereas it showed no obvious effect on Gβγ-mediated chemotaxis, MAP kinase activation or Gα-mediated signaling. The association of Gβγ with RACK1 induces the translocation of RACK1 from cytosol to the plasma membrane. This has been suggested as a mechanism for recruiting PKC and other RACK1-binding proteins to the plasma membrane [93,94]. PKC recruitment to G-protein-coupled receptors by Gβγ/RACK1 can contribute to the efficient modulation of signaling by PKC. Furthermore, the role of the translocation of RACK1 on α_1-adrenergic-mediated myocardial contraction was investigated in senescent rat hearts. It was suggested that the decreased level of RACKs in the senescent myocardium was involved in an impairment of the translocation of PKCα and PKCε following α_1-adrenergic receptor stimulation, resulting in a defective α1-adrenergic-receptor-mediated contraction in aged rat hearts [95]. The influence of RACK1 was also investigated in H9C2 stably expressing RACK1. RACK1 influenced cell size, cell survival under oxidative stress, adhesion, and migration of H9C2 cells [96].

5.3. Syntaxin 1A

Syntaxin 1A is a protein of the presynaptic vesicle release complex. Syntaxin 1A has been shown to directly interact with the Gβγ subunit and to modulate N-type calcium channels expressed in tsA-201 (HEK) cells [97,98]. In freshly isolated cardiomyocytes, inhibition of sytaxin 1A on the ATP-sensitive potassium channel (KATP channel, Kir6.2/SUR2A) was also demonstrated, which may serve as a "brake" to temper the fluctuation of low pH-induced KATP channel opening that could induce fatal re-entrant arrhythmias [99]. Syntaxin 1 may also play a role in the secretion of atrial natriuretic peptide in adult cardiac myocytes as in the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) complex [100,101]. Although the roles of Gβγ in these functions are not fully identified, an interaction of syntaxin 1A with Gβγ has potentials to be involved.

5.4. Phosducin and phosducin-like protein

Phosducin is a 33-kDa cytosolic regulator of G-protein signaling, which was found in the retina and pineal gland as well as many other tissues including heart [102–104]. Phosducin-like protein, a protein sharing extensive amino acid sequence homology with phosducin, is also expressed in heart [105,106]. In the retina, phosducin interferes with the reassociation of $G\alpha_t$ and Gβγ following light-mediated G-protein activation. In an experimental system, phosducin and phosducin-like protein bound Gβγ [107–109] and effectively impeded Gβγ-mediated signaling. Although the overexpression of one part of phosducin improved the contractility of cardiomyocytes and a failing heart, its physiological relevance needs to be further determined [110].

6. Conclusions and perspective

A number of accessory proteins for heterotrimeric G-proteins have been identified in recent years. Each of them has unique selectivity for subunits and functions in the G-protein activation cycle or subunit association. Accumulating data indicates the involvement of these proteins in the regulation of the cardiovascular system. Also, the impact of several such proteins was compelling and has been characterized. However, the effects of many of these proteins on the cardiovascular system are described from their other functional properties rather than from their function on G-proteins.

The challenges facing this research field are to determine how these accessory proteins integrate into various signaling systems and to identify the role of the interaction of G-proteins to "other" functional properties of each accessory protein. Further precise information is expected to be elucidated on the basic mechanisms of accessory proteins on the activation of G-proteins as well as their role in G-protein-mediated signaling, especially in the face of physiological stress.

All these issues have important implications in relation to basic cell biology as well as to the understanding of the physiological response of the cardiovascular system to maintain homeostasis against stress. Further information will thus contribute to the development of novel therapeutics for human disease.

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