Protein Kinase G Regulates Potassium Chloride Cotransporter-3
Expression in Primary Cultures of Rat Vascular Smooth Muscle Cells*

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K-Cl cotransport (KCC) is activated by nitric oxide donors and appears to be regulated by the cGMP signaling pathway. Expression of KCC mRNAs (KCC1–KCC4) in rat vascular smooth muscle cells (VSMCs) is unknown. We have reported the presence of KCC1 and KCC3 mRNAs in primary cultures of VSMCs by specific reverse transcription-polymerase chain reaction. KCC2 mRNA appeared at extremely low levels. KCC4 mRNA was undetectable. Semiquantitative reverse transcription-polymerase chain reaction revealed a 2:1 KCC1/KCC3 mRNA ratio in VSMCs. Depletion of protein kinase G (PKG)-1 from VSMCs did not change KCC3 mRNA expression. Analogous results were obtained with PKG-1-catalytic domain- and vector only-transfected VSMCs lacking endogenous PKG, suggesting no involvement of PKG-1 in the maintenance of basal KCC3 mRNA expression. However, 8-bromo-cGMP, a PKG stimulator, acutely increased KCC3 mRNA expression in a concentration- and time-dependent fashion; this effect was blocked by the PKG inhibitor KT5823 but not by actinomycin D. These findings show that VSMCs express mainly two mRNA isoforms, KCC1 and KCC3, and suggest that PKG participates post-transcriptionally in the acute KCC3 mRNA regulation. The role of KCC3 on cell volume and electrolyte homeostasis in response to PKG modulators remains to be determined.

PKG signaling pathway modulates the activity of ion transport where PKG plays an important role (3, 4).

PKG-1 in the maintenance of basal KCC3 mRNA expression. PKG signaling results in the activation of PKG-1 catalytic domain-transfected VSMCs: PKG+–, vector only-transfected VSMCs; 8-Br-cGMP, 8-bromo-cGMP; RT-PCR, reverse transcription-polymerase chain reaction.

K-Cl cotransport (KCC),1 the coupled movement of potassium and chloride, is involved in cell volume maintenance, and its activity is highly regulated (1). We recently found that KCC activity is present in VSMCs and that it is activated by NO donors, an effect prevented by inhibitors of the cGMP pathway, protein phosphatases and tyrosine kinases (1, 2). The cGMP/PKG signaling pathway modulates the activity of ion transport where PKG plays an important role (3, 4).

PKG-1 catalytic domain-transfected VSMCs: PKG+–, vector only-transfected VSMCs; 8-Br-cGMP, 8-bromo-cGMP; RT-PCR, reverse transcription-polymerase chain reaction.
and PKG—VSMCs. Furthermore, in all cell types, KCC3 mRNA was expressed at lower levels than KCC1 mRNA, and PKG appeared to be involved in the regulation of the KCC3 mRNA isoform. KCC1 mRNA regulation by the NO/cGMP-signaling pathway is the subject of a separate study.

EXPERIMENTAL PROCEDURES

Materials—The Renaissance NEL-100 immunochromeluminescence kit was from PerkinElmer Life Sciences. Primary rabbit polyclonal anti-human PKG-1-specific antibody, secondary horseradish peroxidase-conjugated mouse anti-rabbit IgG antibody, KT5823, and actin-mouse antibody were from Calbiochem. Dulbecco’s modified Eagle’s medium, tissue culture grade, and molecular biology reagents were purchased from Life Technologies, Inc. Total RNA extraction, Access RT-PCR kit, rat actin PCR primers, and 100-bp DNA ladder were from Promega Corp.

Primary Culture of Rat VSMCs—Primary cultures were obtained according to the protocols described previously (10) with some modifications. Briefly, aortas from Harlan Sprague-Dawley rats (150–200 g) sacrificed by asphyxiation in CO$_2$-saturated chambers were obtained from the Wright State University Animal Facilities. Aortas were excised and placed in a wash medium of Dulbecco’s modified Eagle’s medium with 20 mM HEPES, 5 mM amphotericin B, and 50 μg/ml gentamicin. The aortas were cleaned and placed in digestion medium (130 mM NaCl, 0.5 mM CaCl$_2$ type IV and 5 mM glucose) at 37 °C. The tunica adventitia was removed, and the medial layers were minced and further digested for 1–2 h in digestion medium containing 200 units/ml collagenase until a single cell suspension was obtained. Cells were washed twice in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics, 70 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, and 2.5 mM amphotericin B and plated in 6-well culture plates. Cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum as before in a controlled atmosphere of air-CO$_2$ (5%) at 37 °C. The culture medium was changed every 3–4 days until they reached confluence. The culture medium was changed every 3–4 days until they reached confluence. The culture medium was changed every 3–4 days until they reached confluence.

KCC mRNA Isoform Expression in VSMCs—Total RNA from normal, PKG–, and PKG+ VSMCs was obtained using standard procedures (22). Specific sets of primers for all KCC mRNA isoforms were synthesized according to the sequences previously published and used by others (14, 15, 17). These primers were used to obtain the first cDNA strand by RT, and the subsequent amplification of each KCC mRNA isoform present in VSMCs was done by PCR. Briefly, RT: 45 min at 48 °C; denaturation, 2 min at 94 °C; and extension, 1 min at 68 °C, during 40 cycles. The semiquantitative RT-PCR conditions were established in our laboratory to allow comparisons between the expression of KCC1, KCC3, and actin transcripts. Under these conditions, the efficiency of the RT-PCR reaction for each gene did not plateau, and the numbers of cycles used in these experiments were kept to a minimum (Fig. 2B, inset). The relative expression levels of KCC1 and KCC3 mRNA isoforms were determined by using 1.0 μg of total RNA as template and 30 cycles of PCR with the same thermal conditions as before. As control, we analyzed the expression of actin mRNA using specific rat primers, the same amount of total RNA as before, and 25 PCR cycles. These were optimal conditions for the semiquantitative analysis of VSMC KCC mRNAs, and the analysis was limited to the products generated only in the exponential phase of the amplification (Fig. 2B). Lipid metabolism and the PKG catalytic domain of PKG-1 or with the empty vector, as reported earlier (11). Transfected VSMCs were cultured in the same culture medium and conditions as above but in the presence of 10% CO$_2$ and 1 mM geneticin. Thus, we performed simple RT-PCR reactions with KCC isoform-specific primers to obtain some information about the different KCC mRNA isoforms in VSMCs. Furthermore, in all cell types, KCC3 and actin, and PKG, and actin, respectively. As a negative control for each set of primers, RT-PCR reactions were performed in the absence of reverse transcriptase and/or RNA. After RT-PCR, the content of each independent reaction tube was analyzed by 2% agarose gel electrophoresis. The bands (KCC1, 233 bp; KCC3, 663 bp; and actin, 285 bp) were visualized with ethidium bromide. All of the ethidium bromide-stained gels were depicted as an inverse image for clear results. The identities of rat KCC1 and KCC3 were confirmed by restriction enzyme digestion according to the sequences published and the expected DNA fragments obtained (data not shown).

Preparation of Total Cellular Protein Extract—VSMCs were rinsed twice with cold PBS and resuspended in cold lysis buffer containing 10 mM HEPES (pH 7.4), 1 mM MnCl$_2$, 10 mM MgCl$_2$, 0.1 mM EGTA, 0.5% Triton X-100, and protein phosphatase and protease inhibitors (40 μM β-glycerol phosphate, 1 mM Na$_3$VO$_4$, 0.1 mM PMSF, 10 μg/liter leupeptin, 10 μg/liter aprogen A, 1 mM Pefabloc). Total cellular protein extracts were obtained by successive passages through a syringe with needle and centrifuged at 13,000 × g (23). The protein content of the supernatant was determined by the BCA method (Pierce).

Expression of PKG-1 by Western Blot—Expression of PKG-1 (75 kDa) was assayed in protein extracts from normal VSMCs by Western blot using a specific PKG-1 antibody directed against the last 15 amino acids at the C terminus, according to published procedures (24). Briefly, protein extracts (50 μg) from normal VSMCs were diluted with 1 μl Tris-HCl buffer (pH 6.8), plus loading buffer and subjected to 10% PAGE (Mini-Protein II, electrophoresis cell, Bio-Rad) with 0.4% SDS, in parallel with prestained molecular weight markers (Amersham Pharmacia Biotech). Proteins were transferred to a nitrocellulose membrane at 4 °C during 70 min at 100 V in a Trans-Blot cell (Mini Trans-Blot, electrophoretic transfer cell, Bio-Rad). PKG-1 antibody was used in a 1:1000 dilution. Primary rabbit polyclonal horseradish peroxidase-conjugated secondary reagent. The horseradish peroxidase-reaction was developed by immunochromeluminescence.

Statistical Analysis—The analysis of multiple intergroup differences in each experiment was conducted by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. A p < 0.05 was used as the criterion of statistical significance. Except where indicated, all values were obtained from three independent experiments in which at least triplicate samples were assayed.

RESULTS

KCC mRNA Isoform Expression in VSMCs—Expression of the different KCC mRNA isoforms in VSMCs is unknown. Thus, we performed simple RT-PCR reactions with KCC isoform-specific primers to obtain some information about the expression of KCC mRNAs in these cells, as well as in PKG– and PKG+ VSMCs. Under normal conditions, KCC1 and KCC3 mRNA isoforms of the expected sizes were detected in the three VSMC types (Fig. 1A). To corroborate the ability of our KCC-specific primers to amplify each KCC isoform, we conducted simultaneously RT-PCR using total RNA extracted from rat kidney and brain, because it has been reported that these tissues express mainly KCC1 and KCC3 and all of the mRNA isoforms, respectively (Fig. 1B). We also detected a faint band of the size expected for the brain-specific KCC2 mRNA isoform in VSMCs under our experimental conditions. Furthermore, semiquantitative RT-PCR revealed that KCC1 mRNA expression was more abundant than KCC3 mRNA, both relative to actin mRNA expression (Fig. 2). In this study, only results for KCC3 mRNA regulation have been presented. Parallel studies involving KCC1 mRNA regulation will be reported separately.

KCC mRNA Expression in Normal VSMCs as a Function of Cellular Passage—To define the relationship between PKG and KCC3 mRNA expression in VSMCs, we performed semiquantitative RT-PCR in normal VSMCs as a function of cell passage, a well documented way to deplete gradually endogenous PKG-1 expression (7). Fig. 3A shows the semiquantitative RT-PCR results as well as the densitometric analysis normalized with respect to actin (Fig. 3B). The expression levels of PKG-1 protein in normal VSMCs were simultaneously examined by Western blot. Fig. 3C shows a representative Western blot in which the anti-PKG antibody detected a single major band with an apparent molecular mass of 75 kDa corresponding to the native endogenous PKG. These experiments demonstrate no correla-
tion between endogenous PKG and basal KCC3 mRNA expression levels. Furthermore, no significant differences were found between the relative expression levels of KCC3 mRNA in normal, PKG
1
, and PKG
2
 VSMCs (Fig. 4).

PKG Involvement in the Acute Regulation of KCC3 mRNA Expression—To establish a possible relationship between PKG activity and KCC3 mRNA expression, we incubated normal VSMCs at passage 0–2 with 8-Br-cGMP, a known stimulator of PKG activity. As shown in Fig. 5, KCC3 mRNA expression was acutely stimulated at the lowest cGMP analog concentration (0.1 mM) in normal VSMCs following the indicated exposure time to the drug (Fig. 5, A and B). At higher 8-Br-cGMP concentrations, the effect reached a plateau (Fig. 5, C and D). These findings suggest for the first time that PKG activity participates in acute regulation of KCC3 mRNA expression in normal VSMCs.

The opposite situation was found in the presence of KT5823, a known inhibitor of PKG actions. As shown in Fig. 6, A and B, KT5823 was able to inhibit the 8-Br-cGMP-induced effect on KCC3 mRNA expression in normal VSMCs. Furthermore, KT5823 per se had no effect on KCC3 mRNA expression under these experimental conditions (Fig. 6C).

Post-transcriptional Regulation of KCC3 mRNA Expression—Because the onset of the effect of 8-Br-cGMP was fast, we studied whether the changes observed in KCC3 mRNA expression were due to transcriptional regulation. To this end, we incubated normal VSMCs at passage 0–2 with 0.1 mM 8-Br-cGMP in the presence or absence of 10 μg/ml actinomycin D, an inhibitor of RNA polymerase II. As shown in Fig. 7, A and B, no major changes induced by actinomycin D in the presence of the cGMP analog were observed in our experimental conditions. This finding indicates that the transcriptional machinery is not needed for the 8-Br-cGMP-induced effect on KCC3 mRNA expression.

DISCUSSION

Analysis of rat normal, PKG−, and PKG + VSMC mRNAs by RT-PCR using specific sets of primers showed a specific expression pattern of KCC isoforms. KCC1 and KCC3 mRNAs were found to be abundantly expressed, with higher KCC1 mRNA levels relative to KCC3 mRNA in VSMCs. Similar results have also been shown by others in human umbilical vascular endothelial cells in which KCC1 mRNA was found at highest abundance with respect to KCC3 mRNA (15). KCC1 mRNA has been found to be expressed in all tissues tested so far (20). Northern blot analysis of KCC3 mRNA expression revealed a more tissue-restricted expression pattern than KCC1 mRNA (15). However, the KCC1 mRNA levels relative to KCC3 mRNA in the different tissues were not determined, and the reason for the selective VSM distribution of these two different KCC mRNAs remains unknown. We were unable to detect KCC4 mRNA, which suggests undetectable levels or absence of KCC4 mRNA in VSMCs under our experimental conditions. On the other hand, a faint band of the expected size for KCC2 mRNA was detected by RT-PCR in our cells. This implies that using Northern blot as the major criteria for tissue-specific distribution of
transcripts clearly needs further reconsideration.

The absence of correlation between KCC3 mRNA expression levels and PKG protein expression as a function of cell passage and the finding of a similar KCC3 mRNA expression level in normal, PKG<sup>2</sup>, and PKG<sup>1</sup> VSMCs suggest no major involvement of this protein kinase in the maintenance of basal KCC3 mRNA expression. Recently, it has been shown that basal expression levels of the mitogen-activated protein kinase remain without changes in normal VSMCs and in endogenous PKG-depleted VSMCs. However, the NO/cGMP signaling pathway increases mitogen-activated protein kinase activity only in normal VSMCs (24). Besides, the absence of differences between KCC3 mRNA expression levels in normal low and high passaged PKG-depleted VSMCs and in PKG<sup>−</sup> and PKG<sup>+</sup> VSMCs suggests a lack of PKG-dependent phosphorylation of putative proteins involved in KCC3 mRNA regulation.

Little information is available on how the cGMP-activated PKG-dependent signal transduction pathways may influence gene expression. Recently, NO-releasing agents and membrane-permeable analogs of cGMP at concentrations able to increase protein phosphorylation in VSMCs have been shown to activate transcription from activator protein-1 responsive

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**Fig. 2.** KCC1 and KCC3 mRNA expression levels in normal VSMCs. Semiquantitative RT-PCR analysis was performed using 1.0 μg of total RNA from VSMCs and the conditions described under “Experimental Procedures.” A, representative RT-PCR products separated in 2% agarose gel electrophoresis and stained with ethidium bromide showing the bands of the expected sizes, 233 (KCC1), 663 (KCC3), and 285 bp (Actin). B, bars (optical density in arbitrary units) represent the mean ± S.E. from three independent experiments, each done in triplicate. *p < 0.01 versus KCC1 mRNA. All of the results were normalized with respect to actin. Inset, exponential amplification of 1.0 μg of total RNA as a function of PCR cycles. The kinetics described for the three genes of interest are shown (KCC1, KCC3, and Actin).

**Fig. 3.** KCC3 mRNA expression levels in normal VSMCs as a function of cell passage. Semiquantitative RT-PCR analysis was performed using 1.0 μg of total RNA from normal VSMCs at different cell passage, as described under “Experimental Procedures.” A, representative RT-PCR products separated in 2% agarose gel electrophoresis and stained with ethidium bromide showing the bands of the expected sizes: 663 (KCC3) and 285 bp (Actin). B, densitometric analysis (optical density in arbitrary units) representing the mean ± S.E. from three independent experiments, each done in triplicate. All of the results were normalized with respect to actin. C, representative Western blot from a pool of two samples showing the disappearance of PKG-1 protein levels in VSMCs as a function of cell passage.
promoters or by other mechanisms in rodent fibroblast and epithelial cell lines (25–27). NO can also regulate RNA polymerase II-independent gene expression by altering mRNA stability (28–30).

The positive effect of 8-Br-cGMP on KCC3 mRNA expression suggests a direct participation of PKG as a modulator of KCC3 mRNA expression. The inhibitory actions of KT5823 on the 8-Br-cGMP-stimulated KCC3 mRNA expression levels in normal VSMCs occurred at concentrations that selectively block PKG-driven phosphorylations (24). Although the mechanisms of action of these drugs are still obscure, our findings suggest that the PKG pathway is important in KCC3 mRNA regulation. Besides, the fact that KT5823 has little or no effect on cAMP-dependent protein kinase (24) and that there is no effect of 8-Br-cGMP in VSMCs lacking endogenous PKG but with normal cAMP-dependent protein kinase (data not shown) suggest that cGMP is not cross-talking with the cAMP-activated protein kinase signaling pathways, as has been proposed for other effects of the nucleotide (24, 31, 32). However, cAMP and other signaling pathways should not be simply ignored in KCC regulation because both may target the same factors before converging in the same final effect (8). Further confirmation is needed to elucidate the molecular mechanisms involved in the regulation of the KCC3 gene expression in VSMCs.

Post-transcriptional modifications may also contribute to the induction of KCC3 mRNA expression in VSMCs. The inhibition of the transcriptional machinery during the exposure time of the cGMP analog by actinomycin D showed no effect on KCC3 mRNA expression. These data suggest that the cGMP/PKG signaling pathway leads to an increase in KCC3 mRNA expression by a post-transcriptional mechanism(s) in VSMCs. Several lines of evidence support the notion that NO is able to promote

**Fig. 4.** KCC3 mRNA expression in PKG−, normal, and PKG+ VSMCs. PKG−, PKG+, and normal VSMCs were cultured as described under “Experimental Procedures.” Total RNA was extracted, and KCC3 and actin mRNA expression levels were tested by RT-PCR using specific primers. A, representative RT-PCR products separated in 2% agarose gel electrophoresis and stained with ethidium bromide showing the bands of the expected sizes: 663 (KCC3) and 285 bp (Actin). B, densitometric analysis (optical density in arbitrary units as a % of Normal cells) representing the mean ± S.E. from three independent experiments, each done in triplicate. All of the results were normalized with respect to actin.

**Fig. 5.** Effect of 8-Br-cGMP on KCC3 mRNA expression levels in normal VSMCs. Cells were grown as described under “Experimental Procedures.” VSMCs were treated with 0.1 mM 8-Br-cGMP for the indicated periods of time or in the presence of variable amounts of the cGMP analog (0.1–1.0 mM) for 1 h. Total RNA from normal VSMCs treated with or without the cGMP analog was obtained and 1.0 µg each was subjected to semiquantitative RT-PCR analysis. A and C, semiquantitative RT-PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide to show the bands of the expected sizes: 663 (KCC3) and 285 bp (Actin). B and D, densitometric analysis (optical density in arbitrary units as a % of Control) representing the mean ± S.E. from three independent experiments, each done in triplicate; *, p < 0.001 versus control. All of the results were normalized with respect to the actin signal.
stabilization of different mRNAs against target endonucleolytic degradation in the absence of active transcription (28, 30). Additionally, it has been recently demonstrated that the fate of mRNAs are mostly controlled by interactions between specific mRNA-binding proteins and certain mRNA 5'- or 3'-untranslated regions (33). Thus, it will be of interest to identify the pertinent 5'- and/or 3'-untranslated control regions of the KCC3 mRNA and the PKG-regulated protein(s) that interact with them.

The fact that KT5823 inhibited the 8-Br-cGMP effect on KCC3 mRNA expression, that these effects cannot be reproduced in VSMCs lacking endogenous PKG (data not shown), and that there was a lack of effect of actinomycin D on the 8-Br-cGMP-induced KCC3 mRNA expression are consistent with the hypothesis that KCC3 gene expression is regulated by PKG at the post-transcriptional level in VSMCs. Together these data show for the first time a possible and direct role of PKG in the acute regulation of KCC3 mRNA expression in VSMCs.

REFERENCES

1. Lauf, P. K., and Adragna, N. C. (2000) Cell Physiol Biochem. 10, 341–354
2. Adragna, N. C., White, R. E., Orlov, S. N., and Lauf, P. K. (2000) Am. J. Physiol. 278, C381–C389
3. Hamet, P., Orlov, S. N., and Tremblay, J. (1995) Hypertension: Pathophysiol-
| Reference                                                                 | Year | Title                                                                 |
|--------------------------------------------------------------------------|------|----------------------------------------------------------------------|
| 1. White, R. F., Darkow, D. J., and Flav Lang, J. L. (1995)               | 1995 | Circ. Res. **77**, 936–942                                          |
| 2. Orstavik, S., Natrajan, V., Tasken, H., Jahnson, T., and Sandberg, M.  | 1997 | Genomics **42**, 311–318                                            |
| 3. Ruth, P., Pfeifer, A., Kamm, S., Klatt, P., Dostmann, W. R. G., and   | 1997 | Hofmann, F. (1997) J. Biol. Chem. **272**, 10522–10528             |
| 4. White, R. E., Darkow, D. J., and Flavo Lang, J. L. (1995)             | 1995 | Circ. Res. **77**, 936–942                                          |
| 5. Orstavik, S., Natarajan, V., Tasken, H., Jahnsen, T., and Sandberg, M. | 1997 | Genomics **42**, 311–318                                            |
| 6. Orstavik, S., Natarajan, V., Tasken, H., Jahnsen, T., and Sandberg, M. | 1997 | Genomics **42**, 311–318                                            |
| 7. Soff, G. A., Cornwell, T. L., Cundiff, D. L., Gately, S., and Lincoln, | 1997 | J. Biol. Chem. **274**, 8391–8404                                   |
| 8. Ruth, P., Pfeifer, A., Kamm, S., Klatt, P., Dostmann, W. R. G., and   | 1997 | Hofmann, F. (1997) J. Biol. Chem. **272**, 10522–10528             |
| 9. Soff, G. A., Cornwell, T. L., Cundiff, D. L., Gately, S., and Lincoln, | 1997 | J. Biol. Chem. **272**, 10522–10528               |
| 10. Boerth, N. J., Dey, N. B., Cornwell, T. L., and Lincoln, T. M. (1997) | 1997 | J. Vasc. Res. **34**, 245–259                                         |
| 11. Boerth, N. J., and Lincoln, T. M. (1994) FEBS Lett. **342**, 255–260    | 1994 |                                                                      |
| 12. Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Sausbier, M., Hirneijl,  | 1998 |                                                                      |
| 13. Payne, J. A., Stevenson, T. J., and Donaldson, L. F. (1996) J. Biol.   | 1996 |                                                                      |
| 14. Gillen, C. M., Brill, S., Payne, J. A., and Forbush, B., III (1996)   | 1996 | J. Biol. Chem. **271**, 16245–16252                                    |
| 15. Hiki, K., D’Andrea, R. J., Furze, J., Crawford, J., Woollatt, E., Sut- | 1999 |                                                                      |
| 16. Holzman, E. J., Kumar, S., Faaland, C. A., Warner, F., Logue, P. J.,   | 1997 |                                                                      |
| 17. Mount, D. B., Mercado, A., Song, L., Xu, J., George, A. L., Jr., Del-  | 1999 |                                                                      |
| 18. Pellegrino, C. M., Rybicki, A. C., Musto, S., Nagel, R. L., and Schwa- | 1999 |                                                                      |
| 19. Race, J. E., Makhlow, F. N., Logue, P. J., Wilson, P. H., Dunham, P.  | 1999 |                                                                      |
| 20. Su, W., Shumakler, B. E., Chernova, M. N., Stuart-Tilley, A. K., de  | 1999 |                                                                      |
| 21. Adraga, N. C., and Lauf, P. K. (1999) J. Membr. Biol. **166**, 157–167 | 1999 |                                                                      |
| 22. Chomczyanski, P., and Sacchi, A. (1987) Anal. Chem. **162**, 156–159    | 1987 |                                                                      |
| 23. Di Fulvio, M., Coleoni, A. H., Pelizzon, C. G., and Masini-Repiso, A.  | 2000 |                                                                      |
| 24. Komalavilas, P., Shah, P. K., Jo, H., and Lincoln, T. M. (1999) J. Biol. | 1999 |                                                                      |
| 25. Pilz, R. B., Suhasimi, M., Idress, S., Meinold, J. L., and Boss, G. R.  | 1995 |                                                                      |
| 26. Godi, T., Huvvar, I., Meineke, M., Lohmann, S. M., Illes, G. R., and Pilz, R. B. (1996) J. Biol. Chem. **271**, 16245–16252 |
| 27. Idriss, S. D., Godi, T., Casteel, D. E., Khairtonov, V. G., Pilz, R. B., and Boss, G. R. (1999) J. Biol. Chem. **274**, 9439–9493 |
| 28. Pantopoulos, K., and Hentze, M. W. (1999) Proc. Natl. Acad. Sci. U. S. A. **92**, 1267–1271 |
| 29. Filipov, G., Bloch, D. B., and Bloch, K. D. (1997) J. Clin. Investig. **100**, 942–948 |
| 30. Bouton, C., and Demple, B. (2000) J. Biol. Chem. **275**, 32688–32693 |
| 31. Hayden, M. A., and Nakayama, D. K. (1999) J. Surg. Res. **82**, 222–227 |
| 32. Lincoln, T. M., Komalavilas, P., Boerth, N. J., MacMillan-Crow, L. A., and Cornwell, T. L. (1999) Adv. Pharmacol. **43**, 305–312 |
| 33. Kaldy, P., Menotti, E., Moret, R., and Kuhn, L. C. (1999) EMBO J. **18**, 6073–6083 |
| 34. Di Fulvio, M., Lauf, P. K., and Adragna, N. C. (2001) FASEB J. **15**, 114 (abstr.) |
Protein kinase G regulates potassium chloride cotransporter-4 expression in primary cultures of rat vascular smooth muscle cells.

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The oligonucleotides used in this paper for KCC3 mRNA amplification in vascular smooth muscle cells (VSMCs) were designed based on sequences reported in Mount et al. (1). Because the KCC3 sequence described by Mount et al. in fact corresponds to KCC4 (see Ref. 1 below), the title of our paper (shown corrected above) as well as figure labels and their legends making reference to KCC3 mRNA expression or regulation in VSMCs should be changed to refer to KCC4 (potassium chloride cotransporter-4). Since, independently, we have shown the presence of KCC3a and KCC3b isoforms and their regulation by the NO/sGC/PKG/cGMp signaling cascade (2), this correction does not change the overall interpretation of the data, except that we now conclude that KCC3a, KCC3b, and KCC4 mRNAs are subjected to regulation by the signal transduction pathways originally proposed by us (2, 3).

REFERENCES
1. Mount, D. B., Mercado, A., Song, L., Xu, J., George, A. L., Jr., Delpire, E., and Gamba, G. (1999) J. Biol. Chem. 274, 16355–16362
2. Di Fulvio, M., Lauf, P. K., and Adragna, N. C. (2003) Nitric Oxide 9, 165–171
3. Di Fulvio, M., Lauf, P. K., Shah, S., and Adragna, N. C. (2003) Am. J. Physiol. 284, H1686–H1692

The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity.

Stéphane Mantard, Fokko Zandbergen, Esther van Straten, Walter Wahl, Folkert Kuipers, Michael Müller, and Sander Kersten

In Fig. 4D, the labels above the lanes were inadvertently switched. The left lanes are the wild-type mice, and the lanes on the right are FIAF-Tg mice.

Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death.

Sandy D. Westerheide, Tiara L. A. Kawahara, Kai Orton, and Richard I. Morimoto

The labeling at the top of Fig. 5 was inadvertently duplicated from Fig. 4. The correctly labeled Fig. 5 is shown below.

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding.

Shawn K. Milano, You-Me Kim, Frank P. Stefano, Jeffrey L. Benovic, and Charles Brenner

In the legend to Fig. 1, the latter part of the second to last sentence should read: “... where the negative and positive potential were set to $-5$ and $+5\, kT$ (where $k$ is the Boltzmann constant and $T$ is the absolute temperature).”

Co-activation of atrial natriuretic factor promoter by Tip60 and serum response factor.

Min-Su Kim, Xanthi Merlo, Catherine Wilson, and John Lough

Fig. 6C: The proteins recognized by the anti-FLAG antibody in the upper part of panel C were mislabeled. The left side should read “Tip60βΔc,” and the right side should read “Tip60αΔc.”