Nitric oxide (NO) is a gaseous signal mediator showing numerous important biological effects. NO has been shown in many instances to exercise its action via the protein S-nitrosylation mechanism, in which binding of NO to Cys residues regulates protein function independently of activation of soluble guanylate cyclase. The direct link between protein S-nitrosylation and functional modulation, however, has been demonstrated only in limited examples. Furthermore, although most proteins have more than one Cys residue, the mechanism by which a certain Cys becomes a specific target residue of S-nitrosylation is poorly understood. We have previously reported that NO regulates currents through the cardiac slowly activating delayed rectifier potassium channel (I_{Ks}) irrespective of soluble guanylate cyclase activation. Here we demonstrate using a biotin-switch assay that NO induced S-nitrosylation of the \( \alpha \)-subunit of the I_{Ks} channel, KCNQ1, at Cys445 in the C terminus. A redox motif flanking Cys445 and the interaction of KCNQ1 with calmodulin are required for preferential S-nitrosylation of Cys445. A patch clamp experiment shows that S-nitrosylation of Cys445 modulates the KCNQ1/KCNE1 channel function. Our data provide a molecular basis of NO-mediated regulation of the I_{Ks} channel. This novel regulatory mechanism of the I_{Ks} channel may play a role in previously demonstrated NO-mediated phenomenon in cardiac electrophysiology, including shortening in action potential duration in response to intracellular Ca^{2+} or sex hormones.

S-Nitrosylation is a nitric oxide (NO)\(^2\)-induced post-translational modification in which a cysteinyl thiol (R-SH) is converted to a nitrosothiol (1–3) and acts as a regulatory mechanism of various classes of proteins, including ion channels, such as the skeletal muscle type ryanodine receptor (ryanodine receptor type 1) channel (4, 5), the \( \lambda \)-methyl-aspartate receptor channel (6, 7), the cardiac L-type Ca\(^{2+} \) channel (8), and the cardiac Na\(^{+} \) channel (9). We have previously reported that NO derived from endothelial NO synthase activates ion currents through the cardiac slowly activating delayed rectifier potassium channel (\( \lambda \)_{Ks} \) composed of the pore-forming \( \alpha \)-subunit KCNQ1 and the auxiliary \( \beta \)-subunit KCNE1. The NO-dependent regulation of the I_{Ks} channel plays a pivotal role in regulation of cardiac membrane potential by intracellular Ca\(^{2+} \) (10) and by sex hormones (11–13). Because the NO-dependent I_{Ks} activation was inhibited by an inhibitor of soluble guanylate cyclase, 1H-(1,2,4)oxadiazolo[4,3-\( \alpha \)]quinoxlin-1-1 (ODQ), with only a limited magnitude but was robustly inhibited by a thiol-alkylation reagent, N-ethylmaleimide, and reversed by a reducing reagent, diithiothreitol, soluble guanylate cyclase-independent, the protein S-nitrosylation mechanism is posited to be mainly involved (14). However, the following issues remain to be addressed: (i) Is the I_{Ks} channel S-nitrosylated? (ii) If so, then what is the target of S-nitrosylation between the \( \alpha \)-subunit KCNQ1 and the \( \beta \)-subunit KCNE1? (iii) Among multiple Cys residues, which Cys is a target of S-nitrosylation? and (iv) How does NO specifically recognize the target Cys? In the present study, we used the biotin-switch assay and functional patch clamp experiment to answer these questions. Our data show that KCNQ1 is a target of S-nitrosylation, and the presence of a redox motif contributes to making the Cys at 445 in the C terminus of KCNQ1 a preferential target of S-nitrosylation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—To create GST fusion protein containing Cys34 and Cys122, Cys180, Cys381, and Cys445, or Cys642 of human KCNQ1, the partial cDNA fragments containing Cys34 and Cys122 (Met1–Tyr125), Cys180 (Thr167–Pro197), Cys381 and Cys445 (Val355–Leu374), or Cys642 (His620–Ser676) were amplified by PCR using the full-length human KCNQ1 as a template and were subcloned into the pGEM-T Easy vector (Promega, Madison, WI). BamHI-EcoRI-digested fragments were then subcloned into BamHI-EcoRI site of pGEX4T1 (Amersham Biosciences). The pairs of primers used were as follows: Met1–Tyr125 of KCNQ1: a sense strand, 5′-ggatcccccggccccagggccggag; an antisense strand, 5′-tcaGTTGGTAAACGAGAGCATT; Thr167–Pro197 of KCNQ1: a sense strand, 5′-ggatccAGCGGATACGTGGTCCGG; an antisense strand, 5′-tcaGGGTTTCCGGGCAAGCGG; Val355–Leu619 of
KCQ1: a sense strand, 5'-ggatccGCAGGCACCTCACTC; an antisense strand, 5'-tcaATGCTGGGCGATGTTTCC; and His442-See876 of KCQ1: a sense strand, 5'-ggatccACGGGTCGCAGACCCCC; an antisense strand, 5'-tcaGGACCCTCTCATCGGG. Lowercase characters in the sense primers indicate the BamHI recognition site, and those in the antisense primers indicate the stop codon. Replacements of His442 with Ala (H442A), Cys445 with Ala (C445A), Asp446 with Asn (D446N), and Asp446 with Glu (D446E) were made by using the QuikChange site-directed mutagenesis kit (Stratagene).

To introduce a V5 epitope (GLPIPNPLLGLDST-), the C-terminal KCQ1 fragment with V5-epitope tag sequence was made by PCR using a full-length human KCQ1 as a template, digested with MroI and BamHI, and subcloned into MroI-BamHI-digested KCQ1/pcDNA3.1(−) (KCQ1-V5). The pair of primers used was as follows: a sense strand, 5'-CATCGCTTCTGCCTCCTC; and an antisense strand, 5'-ggatccCGTAAGAATCGAGACCCCTCATCGGG. Lowercase characters in the antisense primers indicate the BamHI recognition site, and those in the antisense primers indicate the stop codon. Underlined uppercase characters indicate the sequence encoding the V5 epitope. To facilitate identification of cells in which CaM had been transfected in patch clamp experiments, we subcloned either CaM or CaM1234 with EGFP in the pIRES vector (Clontech) (EGFP-CaM/pIRES, EGFP-CaM1234/pIRES).

Preparation of GST Fusion Protein and Biotin-Switch Assay—GST fusion proteins were purified as described previously (15). Briefly, expression of GST fusion proteins in Escherichia coli strain BL21(DE3) (Takara) was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside overnight at 18–20 °C. Cells were harvested by centrifugation and were resuspended in MroI-BamHI-digested KCQ1/pcDNA3.1(−) (KCQ1-V5). The pair of primers used was as follows: a sense strand, 5'-CATCGCTTCTGCCTCCTC; and an antisense strand, 5'-ggatccCGTAAGAATCGAGACCCCTCATCGGG

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were incubated for 1 h at a room temperature. The beads were washed with HEN buffer containing 0.5% Triton X-100, and proteins were eluted by incubation with HEN buffer containing 100 mM β-mercaptoethanol at 37 °C for 20 min. Eluted samples were electrophoresed in SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and immunoblotted with a 1:4000-diluted polyclonal anti-KCNQ1 antibody (C-20) or a 1:2000-diluted polyclonal anti-KCNE1 antibody (N-16).

Patch Clamp Experiments—HEK 293 cells were transfected with one of EGFP/pIRES, EFGP-CaM/pIRES, or EFGP-CaM(A233)/pIRES, either of KCNQ1WT/pcDNA3.1 or KCNQ1(C445)/pcDNA3.1, and KCNE1/pcDNA3.1 using Lipo-jectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Expressed KCNQ1/KCNE1 currents were recorded with a whole cell patch clamp technique using an Axopatch 200B amplifier (Axon Instruments) as described previously (10). The pCLAMP software (version 8.0, Axon) was used to generate voltage-pulse protocols and to acquire and analyze data. KCNQ1/KCNE1 currents were elicited by 2-s test pulses (Vτ) to +60 mV followed by 2-s repolarization pulses to −40 mV from a holding potential (Vh) of −65 mV at 0.1 Hz. Peaks of tail current amplitude was measured during the repolarization pulse. All experiments were performed at a room temperature of 22 ± 2 °C. Pipette solution contained (in mM): 110 aspartic acid, 1 CaCl2, 1 MgCl2, 5 ATP potassium salt, 10 HEPES, 11 EGTA (pH 7.3 adjusted with KOH); in some experiments, 11 mM EGTA was replaced with equimolar Ca2+ at low concentrations. External (bath) solution was the Tyrode’s solution containing (in mM): 135 NaCl, 4.8 KCl, 2 CaCl2, 1.2 MgCl2, 5 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH).

Statistics—All numerical values are presented as mean ± S.E. Statistical significance was evaluated by an analysis of variance followed by a Bonferroni multiple comparison test. p < 0.05 was taken as the significance level.

RESULTS

S-Nitrosylation of KCNQ1 but Not KCNE1—We first examined if either or both of KCNQ1 or/and KCNE1 is S-nitrosylated in cultured cells. Human V5-tagged KCNQ1 or KCNE1 was expressed in HEK293 cells, immunoprecipitated with an anti-V5 antibody or an anti-KCNE1 antibody, and treated with an NO donor SNAP at a concentration of 0.5 mM. In the pilot experiments, we confirmed that transfection of HEK293 cells with V5-tagged KCNQ1 and KCNE1 yielded similar currents to those obtained by transfection with KCNQ1 and KCNE1. An immunopositive band was detected after avidin purification for KCNQ1 (lane 3, Fig. 2A). For KCNE1, although an immunopositive band was detected without application of a thiol-reacting reagent MMTS to block free thiol (R-SH) (lane 1, Fig. 2B), no such band was detected after blocking free thiol with MMTS (lane 2, Fig. 2B). We also found that exogenously applied SNAP induced S-nitrosylation of KCNQ1 of guinea pig ventricular myocytes (supplemental Fig. S1), which indicates that KCNQ1 is the target of S-nitrosylation also under a physiological condition.

Specific S-Nitrosylation of Cys445 of KCNQ1—We next attempted to pinpoint the target Cys residue for S-nitrosylation among nine Cys residues present in KCNQ1. Because Cys residues that are subject to S-nitrosylation have been shown in a number of studies to reside in a juxtamembrane zone (6, 17), we focused on six such Cys residues (Cys34, Cys122, Cys180, Cys381, Cys445, and Cys642) (closed circles in Fig. 3A). We attempted to create GST fusion proteins containing each Cys residue. In E. coli, although the GST fusion protein containing Cys180 (Thr167-Pro197) or Cys642 (His620-Ser670) became soluble, the GST fusion protein containing Cys34, Cys122, Cys381, or Cys445 alone was hardly soluble. Alternatively, we created and examined the GST fusion protein with Cys34 and Cys122 (Met1-Tyr125 of KCNQ1), and that with Cys381 and Cys445 (Val355-Leu619 of KCNQ1). Each of four GST fusion proteins was purified from E. coli, incubated with an NO donor, and subjected to the biotin-switch assay. S-Nitrosylation was detected only in the GST fusion protein with Cys381 and Cys445 (Val355-Leu619 of KCNQ1) (lane 3, Fig. 3B).

To examine if Cys381 or/and Cys445 were S-nitrosylated by an NO donor, we replaced Cys381 with Ala (C381A) or Cys445 with Ala (C445A) and carried out the biotin-switch assay. S-Nitrosylation remained detected for C381A mutant, but was completely eliminated for the C445A mutant (Fig. 3C). To further corroborate that Cys445 is the main target of S-nitrosylation, we expressed V5-tagged full-length KCNQ1 in which Cys445 had been replaced with Ala leaving remaining eight cysteines intact (KCNQ1(C445A)) in HEK293 cells. Cell lysates were treated with 0.5 mM SNAP and subjected to the biotin-switch assay. Application of an NO donor barely induced S-nitrosylation of KCNQ1(C445A) (lane 3, Fig. 3D), which verifies that Cys445 is the main target of S-nitrosylation.

Role of Redox Motif for Specific Cys445 S-Nitrosylation—Several mechanisms have previously been implicated for the site-specific S-nitrosylation in several proteins, which include the
presence of Cys residue in the hydrophobic milieu (18), flanking of the Cys residue by a redox (acid-base) motif (19, 20), allosteric effects by protein-protein interaction (4, 5), and compartmentalization of NO sources and targets (21, 22). The Kyte-Doolittle hydropathy profiling indicates that Cys445 does not reside in the hydrophobic milieu. Cys445, but none of the other eight cysteines in KCNQ1, is immediately followed by an acidic amino acid. Such a sequence is partially matched to the proposed consensus sequence for $\text{S}$-nitrosylation, (Lys/Arg/His)-Cys-(Asp/Glu), in which the presence of an acidic amino acid immediately after Cys is proposed to be especially critical (19).

To explore if an acidic amino acid, Asp446, has some integral roles for $\text{S}$-nitrosylation of Cys445, we made a mutant GST fusion protein of the KCNQ1 C terminus (Val355–Leu619) with replacement of Asp446 with Asn (D446N) leaving Cys445 intact, and performed $\text{in vitro}$ biotin-switch assay. An $\text{S}$-nitrosylated band was not detected (lane 3 in Fig. 4, A and B). When Asp446 was replaced with another acidic amino acid Glu (D446E), $\text{S}$-nitrosylation was clearly detected (lane 5 in Fig. 4A and column 2 in Fig. 4B), suggesting that $\text{S}$-nitrosylation of Cys445 requires a negative charge at KCNQ1 residue 446.

Because the basic amino acid closest to Cys445 is His442, we replaced His442 with Ala (H442A). We could still observe $\text{S}$-nitrosylation in the H442A mutant (lane 2 in Fig. 4A and column 4 in Fig. 4B). Collectively, the acidic amino acid Asp446, but not the basic amino acid His442, appears to be required for site-specific $\text{S}$-nitrosylation of Cys445.

CaM Is Required for $\text{S}$-Nitrosylation in Living Cells—To assess whether Cys445 also is the $\text{S}$-nitrosylation target and whether redox-motif is critical for $\text{S}$-nitrosylation of Cys445 also in living cells, biotin-switch assays were performed in lysates from HEK293 cells expressing V5-tagged full-length KCNQ1 WT or KCNQ1(C445A). The left panel is the representative data for immunoblotting with an anti-V5 antibody, and the right panel is the result of densitometric analysis. *, p < 0.05.
S-Nitrosylation of the KCNQ1 Channel

Western blot analysis indicates that CaM level is ~8 times higher in CaM-transfected cells than in CaM non-transfected cells (supplemental Fig. S2). In the presence of CaM, KCNQ1 WT was clearly S-nitrosylated (lane 2 in Fig. 5B), whereas KCNQ1(C445A) or KCNQ1(D446N) was not (lanes 3 and 4 in Fig. 5B). When we co-transfected CaM między 12, mutant with KCNQ1 WT, in which four Ca2+ binding motifs are disrupted (29), S-nitrosylation was not observed even in KCNQ1 WT (lane 2 in Fig. 5C). Thus, interaction with CaM in the Ca2+-bound form is critical for S-nitrosylation of KCNQ1 in living cells. We found using co-immunoprecipitation experiments that the interaction with CaM was intact for KCNQ1(D446N) (supplemental Fig. S3), indicating that the failure of an NO donor to S-nitrosylate KCNQ1(D446N) is due to the disruption of a redox motif but not to the disturbed interaction with CaM.

S-Nitrosylation on KCNQ1 Channel Function—We employed a patch clamp experiment to investigate functional consequences of S-nitrosylation of Cys445 of the KCNQ1 channel. HEK 293 cells expressing KCNQ1 and KCNE1 conduct slowly activating outward currents with very similar kinetics to those of Ik in cardiac myocytes (Fig. 6A). Without co-transfection of CaM, the amplitude of outward currents increased monotonically during the depolarization pulse (upper panel in Fig. 6A), whereas, with co-transfection of CaM, outward currents appeared to saturate at the later phase of the depolarization pulse (lower panel in Fig. 6A), in agreement with the previous reports that CaM induces inactivation of the KCNQ1/KCNE1 current (24, 25). With co-transfection of CaM, application of SNAP increased KCNQ1/KCNE1 current amplitudes in a dose-dependent manner (red traces in Fig. 6, A and B, second bar in Fig. 6C), whereas SNAP at 100 nM did not significantly enhance KCNQ1(C445A)/KCNE1 currents (third bar in Fig. 6C). S-nitrosoglutathione, a different class of NO donor, also enhanced KCNQ1/KCNE1 currents (data not shown). Even without application of SNAP (0 nM), Ik tail amplitude was slightly increased (first bar in Fig. 6B): this slight increase appeared to be due to the nonspecific effect of DMSO used as a vehicle, because in the time control experiment application of DMSO slightly enhanced KCNQ1/KCNE1 currents (data not shown).

Without co-transfection of CaM (fourth bar in Fig. 6C) or with co-transfection of CaM between 12, mutant (fifth bar in Fig. 6C), SNAP at 100 nM failed to enhance KCNQ1/KCNE1 currents. To examine the effects of intracellular Ca2+, we replaced 11 mM EGTA with equimolar BAPTA. In the presence of BAPTA, Ik amplitude suffered from significant rundown, which agrees with the finding that injection of BAPTA into oocytes (estimated intracellular concentration of 5–10 mM) reduced KCNQ1/KCNE1 currents by >50% (25). Although the BAPTA-induced rundown made the effects of SNAP difficult to assess, we could at least conclude that SNAP did not enhance Ik tail amplitude in the presence of BAPTA (sixth bar in Fig. 6C).

With co-transfection with wild-type CaM, SNAP slightly enhanced KCNQ1(C445A)/KCNE1 currents, although not significantly (third bar in Fig. 6C). We had previously reported that, although NO donor-induced enhancement of Ik was mostly inhibited by an alkylating reagent, N-ethylmaleimide, and reversed by a reducing reagent, dithiothreitol, some frac-
tion was inhibited by an inhibitor of soluble guanylate cyclase, ODQ (14). We tested the hypothesis if small SNAP-induced enhancement for KCNQ1(C445A)/KCNE1 currents is due to a minor contribution of soluble guanylate cyclase-dependent mechanism. In the presence of ODQ, SNAP failed to enhance KCNQ1(C445A)/KCNE1 currents (seventh bar in Fig. 6C), indicating that the SNAP-induced enhancement of KCNQ1(C445A)/KCNE1 currents is likely due to the cGc-dependent mechanism, but not due to the effects on redox-sensitive Cys other than Cys445.

**DISCUSSION**

Emerging evidence demonstrates that protein S-nitrosylation is an important NO-mediated regulatory mechanism of various classes of proteins (1–3), including ion channels (4–9). However, the direct link between protein S-nitrosylation and functional relevance has been proven only for limited examples. Furthermore, the mechanism underlying preferential S-nitrosylation of the target Cys is not fully understood. In the present study, we demonstrate that an NO donor induces S-nitrosylation at Cys445 in the C terminus of the pore-forming α-subunit KCNQ1. We provide convincing evidence to show that the redox motif flanking Cys445 is required for the site-specific S-nitrosylation of Cys445. S-Nitrosylation at Cys445 of the KCNQ1 channel functionally regulates the KCNQ1/KCNE1 complex channel.

Among nine Cys in KCNQ1, only Cys445 is immediately followed by the acidic amino residue, and the in vitro biotin-switch assay indicates that the presence of acidic amino acid at the 446th residue is required for S-nitrosylation of Cys445. His442 is the closest basic amino acid, but the replacement of His442 to a neutral amino acid Ala does not disrupt S-nitrosylation of Cys445. We are not sure if the presence of basic amino acids is not required or if other distal basic amino acids possibly located close to Cys445 in the three-dimensional structure are required. In living cells, an NO donor failed to S-nitrosylate WT KCNQ1 in the absence of CaM, and even in the presence of CaM, an NO donor failed to S-nitrosylate KCNQ1(D446N), implying that both of the redox motif flanking Cys445 and the presence of CaM are required. We have previously demonstrated that endothelial NO synthase and KCNQ1 co-localize in the caveolae fraction of guinea pig hearts (12), indicating the close proximity of the NO donor and the target protein. Thus, multiple factors orchestrate to make Cys445 of KCNQ1 a target of molecule-specific and site-specific S-nitrosylation. The presence of CaM is also a pre-requisite for S-nitrosylation of the skeletal muscle type ryanodine receptor type 1 (4, 5). It is suggested that the binding of Ca2+–bound CaM, but not apo-CaM, unmasks the target Cys residue or provide hydrophobic milieu (4, 5). CaM frequently acts at the level of electron transfer, such as that between the FAD reductase and heme domains to regulate NOS activity (30). Thus, it might be an alternative possibility that CaM acts as an electron transfer cofactor between an NO donor and Cys445 surrounded by the redox motif. Co-transfection of CaM is required for S-nitrosylation of KCNQ1 in living cells, but not in cell lysates, for which we have currently no clear explanation. It might be a potential explanation that to induce S-nitrosylation in the reducing intracellular milieu, the presence of CaM would be required. However, more work will clearly be needed to clarify the role of CaM for S-nitrosylation of KCNQ1.

An exogenously applied NO donor increased the amplitude of KCNQ1/KCNE1 channel currents. An NO donor failed to enhance KCNQ1(C445A)/KCNE1 currents or KCNQ1/KCNE1 currents without co-transfection of CaM or with co-transfection of CaM1234. Because each of the three experimental conditions dislocates S-nitrosylation of the KCNQ1 channel in the biotin-switch assay, these data serve as evidence for the direct link between S-nitrosylation of Cys445 and the activation of the KCNQ1/KCNE1 channel. We used a high concentration of SNAP (0.5 mM) to assess S-nitrosylation of KCNQ1, because a similar concentration (1 mM) of SNAP was used in the original biotin-switch assay report (16). However, this concentration was much higher than that (1–100 nM) used for patch clamp experiments, which would certainly raise a question on the causative linkage between S-nitrosylation and KCNQ1 current enhancement. We, therefore, examined the concentra-
tion dependence for KCNQ1 S-nitrosylation by SNAP: SNAP induced S-nitrosylation of KCNQ1 above 10 nM in a concentration-dependent manner (supplemental Fig. S3), suggesting that S-nitrosylation of the KCNQ1 channel and its activation occur within a similar dosage of SNAP.

The underlying mechanism for S-nitrosylation-induced activation of the KCNQ1 channel remains to be addressed. Biochemical, spectroscopic, and crystallographic analyses have recently provided the structure model for the C terminus of KCNQ1, which consists of four α-helices, helices A, B, C, and D, and an unstructured loop between helices A and B (31). Helices A and B provide binding sites for CaM, and the Cys445, the target of S-nitrosylation resides in the midst of the unstructured loop between helix A and B. Because CaM constitutively binds to both helix A and helix B (31), bridging of two helices by CaM might create a milieu for S-nitrosylation of Cys445 in the midst of the loop. Although functional assembly of KCNQ1 channels has been shown to require its interaction with CaM (24, 25), similar KCNQ1/KCNE1 channel currents were recorded in the absence and presence of CaM co-expression. Endogenous levels of CaM in HEK293 cells was about one-eighth of those in HEK293 cells (supplemental Fig. S2), which might be sufficient for functional assembly of KCNQ1 but not for KCNQ1 S-nitrosylation. Certainly, more work will be needed to decipher the role of CaM for S-nitrosylation and the mechanism linking S-nitrosylation of the KCNQ1 channel to its activation.

The S-nitrosylation of the KCNQ1 channel is the novel regulatory mechanism of this channel that may have a great impact on physiological regulation of cardiac electrical activity, because the I_{Ks} channel composed of KCNQ1 and KCNE1 has a crucial regulatory role in cardiac electrophysiology, including response to autonomic nervous stimulation (32) and adaptation to heart rate changes (33). In fact, we have previously depicted the role of NO-dependent regulation of the I_{Ks} channel in feedback regulation of Ca^{2+} homeostasis (9) and the gender difference in the life-threatening cardiac arrhythmias (10–13).

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REFERENCES

1. Hess, D. T., Matsumoto, A., Nudelman, R., and Stamler, J. S. (2001) Nat. Cell Biol. 3, E46–E49
2. Hess, D. T., Matsumoto, A., Kim, S.-O., Marshall, H. E., and Stamler, J. S. (2005) Nat. Rev. Mol. Cell Biol., 6, 150–166
3. Mitchell, D. A., and Marletta, M. A. (2005) Nat. Chem. Biol. 1, 154–158
4. Eu, J. P., Sun, J., Xu, L., Stamler, J. S., and Meissner, G. (2000) Cell 102, 499–509
5. Sun, J., Xin, C., Eu, J. P., Stamler, J. S., and Meissner, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11158–11162
6. Choi, Y. B., Tenneti, L., De, D. A., Ortiz, J., Bai, G., Chen, H. S., and Lipton, S. A. (2000) Nat. Neurosci. 3, 15–21
7. Lipton, A., Choi, Y. B., Takahashi, H., Zhang, D., Li, W., Godzik, A., and Bankston, L. A. (2002) Trends Neurosci. 25, 474–480
8. Sun, J., Morgan, M., Shen, R. F., Steenbergen, C., and Murphy, E. (2007) Circ. Res. 101, 1155–1163
9. Ueda, K., Valdivia, C., Medeiros-Domingo, A., tester, D. J., Vatta, M., Farrugia, G., Ackerman, M. J., and Makielski, J. C. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 9355–9360
10. Bai, C.-X., Namekata, I., Kurokawa, J., Tanaka, H., Shigenobu, K., and Furukawa, T. (2005) Circ. Res. 96, 64–72
11. Bai, C.-X., Kurokawa, J., Tamagawa, M., Nakaya, H., and Furukawa, T. (2005) Circulation 112, 1701–1710
12. Nakamura, H., Kurokawa, J., Bai, C.-X., Asada, K., Xu, J., Oren, R. V., Zhu, Z. I., Clancy, C. E., Isobe, M., and Furukawa, T. (2007) Circulation 116, 2913–2922
13. Kurokawa, J., Tamagawa, M., Harada, N., Honda, S., Bai, C.-X., Nakaya, H., and Furukawa, T. (2008) J. Physiol. 586, 2961–2973
14. Bai, C.-X., Takahashi, K., Masumuya, H., Sawanobori, T., and Furukawa, T. (2004) Br. J. Pharmacol. 142, 567–575
15. Dilly, K. W., Kurokawa, J., Terrenoine, C., Reiken, S., Lederer, W. J., Marks, A. R., and Kass, R. S. (2004) J. Biol. Chem. 279, 40778–40787
16. Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001) Nat. Cell Biol. 3, 193–197
17. Broillet, M. C. (2000) J. Biol. Chem. 275, 15135–15141
18. Nedospasov, A., Rafikov, R., Beda, N., and Nudler, E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13543–13548
19. Stamler, J. S., Toone, E. J., Lipton, S. A., and Suchler, N. J. (1997) Neuron 18, 691–696
20. Perez-Mato, I., Castro, C., Ruiz, F. A., Corrales, F. J., and Mato, J. M. (1999) J. Biol. Chem. 274, 17075–17079
21. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) Science 269, 1737–1740
22. Brennan, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Breed, D. S. (1996) Cell 84, 757–767
23. Yus-Najera, E., Santana-Castro, I., and Villarroel, A. (2002) J. Biol. Chem. 277, 28545–28553
24. Shamgar, L., Ma, L., Schmitt, N., Hainin, Y., Peretz, A., Wiener, R., Hirsch, J., Pongs, O., and Attali, B. (2006) Circ. Res. 98, 1055–1063
25. Ghosh, S., Nunziato, A., and Pitt, G. S. (2006) Circ. Res. 98, 1048–1054
26. Gamper, N., Li, Y., and Shapiro, M. S. (2005) Mol. Biol. Cell 16, 3538–3551
27. Tohse, N. (1990) J. Inorg. Biochem. 39, 41–50
28. Nitta, J., Furukawa, T., Marumo, F., Sawanobori, T., and Hiraoka, M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11630–11634
29. Bankston, L. A. (2002) Circ. Res. 90, 1155–1163
30. Li, H., and Poulos, T. L. (2005) J. Inorg. Biochem. 99, 293–305
31. Wiener, R., Hainin, Y., Shamgar, L., Fernández-Alonso, M. C., Martos, A., Chomsy-Hecht, O., Rivas, G., Attali, B., and Hirsch, J. A. (2008) J. Biol. Chem. 283, 5815–5830
32. Marx, S. O., Kurokawa, J., Reiken, S., Mota, H., Diaz, A., R., and Kass, R. S. (2002) Science 295, 496–499
33. Clancy, C. E., Kurokawa, J., Tateyama, M., Wehrens, X. H., and Kass, R. S. (2003) Annu. Rev. Pharmacol. Toxicol. 43, 441–461