S-Nitrosothiol Controls Gating and Conductance of the α1 Subunit of Class C L-type Ca2+ Channels*

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Modulation of smooth muscle, L-type Ca2+ channels (class C, Ca1.2b) by thionitrite S-nitrosoglutathione (GSNO) was investigated in the human embryonic kidney 293 expression system at the level of whole-cell and single-channel currents. Extracellular administration of GSNO (2 mM) rapidly reduced whole-cell Ba2+ currents through channels derived either by expression of α1C-b or by coexpression of α1C-b plus β2a and α2-6. The non-thiol nitric oxide (NO) donors 2,2-diethyl-1-nitroso-oxhydrazin (2 mM) and 3-morpholinosydnonimine-hydrochloride (2 mM), which elevated cellular cGMP levels to a similar extent as GSNO, failed to affect Ba2+ currents significantly. Intracellular administration of copper ions, which promote decomposition of the thionitrite, antagonized its inhibitory effect, and loading of cells with high concentrations of dithiothreitol (2 mM) prevented the effect of GSNO on α1C-b channels. Intracellular loading of cells with oxidized glutathione (2 mM) affected neither α1C-b channel function nor their modulation by GSNO. Analysis of single-channel behavior revealed that GSNO inhibited Ca2+ channels mainly by reducing open probability. The development of GSNO-induced inhibition was associated with the transient occurrence of a reduced conductance state of the channel. Our results demonstrate that GSNO modulates the α1 subunit of smooth muscle L-type Ca2+ channels by an intracellular mechanism that is independent of NO release and stimulation of guanylyl cyclase. We suggest α-S-nitrosation of intracellularly located sulfhydryl groups as an important determinant of Ca2+ channel gating and conductance.

The identification of nitric oxide (NO)† as the major endothelium-derived relaxing factor led to the discovery of a variety of NO-mediated signal transduction mechanisms (1–4). NO-mediated control of vascular functions involves the modulation of various ion transport systems including the high voltage-activated L-type Ca2+ channels (5–7). Both increases (5) and decreases (6, 7) of L-type Ca2+ current in response to NO donors have been reported. Cellular regulation of Ca2+ current was suggested to depend at least in part on NO-induced activation of guanylyl cyclase and subsequent modification of the phosphorylated state of channel proteins (8–11). However, S-nitrosothiols (thionitrites) were recently demonstrated to exert a potent inhibitory effect on class C, L-type Ca2+ channels, which was suggested to be independent of intracellular cGMP accumulation (12). Thus, a more direct modulation of L-type Ca2+ channels by NO donors appears likely. So far it is unclear whether the inhibition of Ca2+ channels by thionitrites requires the release of NO and how single-channel properties are affected by this mechanism of regulation. Modulation of Ca2+ channels by the S-nitrosothiol nitrosoglutathione (GSNO) appears to be of particular physiological interest, because GSNO is the most abundant endogenous thionitrite and has been suggested as a potential NO storage site or transport species (11, 13, 14). GSNO decomposes slowly to generate NO, a reaction which is catalyzed by traces of metal ions (14–17). Alternatively GSNO is able to modify protein thiol residues via nitrosation or glutathionylation reactions. In the present study we investigated whether Ca2+ channel inhibition by GSNO involves (i) decomposition of the thionitrite to yield NO, (ii) transnitrosation reactions, or (iii) the formation of mixed disulfides. Moreover, the changes in single Ca2+ channel function associated with GSNO-induced inhibition were characterized. Our results strongly suggest intracellular S-nitrosation as a mechanism involved in physiological control of Ca2+ channel function and demonstrate a unique modification of Ca2+ channel function by GSNO, involving joint alteration of gating and permeability of the pore-forming α1C subunit.

EXPERIMENTAL PROCEDURES

Cells—α1C-b channels were stably expressed in HEK293 cells (18). In some sets of experiments, cells were transfected to express β2a and α2-6 subunits†, in addition to the pore-forming subunit. Positively transfected cells were identified because of the expression of the human T-lymophocytic cell cluster of differentiation (CD8) as a marker gene (19). The cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium + 10% fetal calf serum and 0.25 mg/ml G418 (Sigma).

Electrophysiology—Whole-cell membrane currents were recorded with a bath solution containing (in mM) 40 BaCl2, 60 NaCl, 30 tetraethylammoniumchloride, 15 HEPES, 0.5 EDTA, pH adjusted to 7.4 with methane sulfonic acid. The pipette solution contained (in mM) 115 cesium methanesulfonate, 20 CsCl, 15 HEPES, 10 EGTA, pH 7.4 adjusted with N-methyl-β-glucamine.

For single-channel cell-attached recordings, the whole-cell bath solution was used as pipette solution. In experiments with single α1C-b

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‡The abbreviations used are: NO, nitric oxide; GSNO, S-nitrosoglutathione; HEK, human embryonic kidney; P0 (open probability), probability of a single channel to be found in an open state; P(D, availability), probability of a single channel to open during depolarization; SIN-1, 3-morpholinosydnonimine-hydrochloride; DEA/NO, 2,2-diethyl-1-nitroso-oxhydrazin; DTPA, diethyleneetriamine-pentaacetic acid; DTT, dithiothreitol.

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‡SPREMBL Q8NY47.
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**RESULTS**

Inhibition of a1C-b Currents by GSNO Is Independent of NO Release and Involves an Intracellular Site of Action—Fig. 1 compares the effects of GSNO, DEA/NO, and SIN-1 on whole-cell Ba\(^{2+}\) currents through a1C-b channels. Extracellular administration of GSNO (2 mM) rapidly inhibited the inward Ba\(^{2+}\) currents as illustrated in Fig. 1, A and B. The current to voltage relation was not affected by GSNO (not shown). GSNO was tested at concentrations between 50 \(\mu\)M and 2 mM, which inhibited a1C-b currents in the range of 13 \(\pm\) 5\% (50 \(\mu\)M; \(n = 3\)) to 38 \(\pm\) 6\% (2 mM; \(n = 17\)). Neither DEA/NO nor SIN-1 mimicked the action of GSNO. DEA/NO (2 mM) moderately promoted the Ba\(^{2+}\) current run-down (Fig. 1C), and SIN-1 (2 mM) failed to affect a1C-b currents (Fig. 1D). Similarly, channels comprised of a1C-b, \(\beta2a\), and a2-\(\delta\), corresponding to channel complexes expressed in cardiovascular tissues (21, 22), were rapidly inhibited by GSNO (\(n = 5\); see Fig. 2) but barely affected by DEA/NO or SIN-1 (data not shown).

Fig. 2. GSNO inhibits L-type Ca\(^{2+}\) channels comprised of a1C-b and the auxiliary subunits \(\beta2a\) and a2-\(\delta\). Left, time course of peak Ba\(^{2+}\) inward currents recorded at 30 mV. GSNO induces a partial inhibition of L-type current in cells expressing a1C-b/\(\beta2a\)/a2-\(\delta\). Right, individual current traces taken from the experiment shown on the left. Ba\(^{2+}\) inward currents were recorded during depolarizing voltage steps from \(-70\) to 30 mV before (1) and after (2) extracellular administration of GSNO.
reduced metal ions, in particular Cu\(^{+}\), were reported to catalyze decomposition of GSNO (15, 23, 24), both CuSO\(_4\) (50 \(\mu\)M) and the reductant DTT (500 \(\mu\)M) were included in the pipette solution. If decomposition of GSNO within the cell was essential to Ca\(^{2+}\) channel inhibition, loading of the cells with copper ions would be expected to enhance inhibition, otherwise this treatment should suppress the action of the thionitrite. Fig. 4A shows that the effects of GSNO (2 mM) on whole-cell Ba\(^{2+}\) currents were completely abolished in cells that were loaded with a pipette solution containing 50 \(\mu\)M CuSO\(_4\) plus DTT (500

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**FIG. 3.** Inhibition of whole-cell Ba\(^{2+}\) currents by GSNO is not mediated by cGMP. A, Ba\(^{2+}\) current inhibition by GSNO (2 mM; \(n = 17\)), DEA/NO (2 mM; \(n = 7\)), and SIN-1 (2 mM; \(n = 5\)). The asterisk indicates significant difference versus control (reference value). B, intracellular cGMP levels in the absence of NO donors (control) and in the presence of GSNO (2 mM; \(n = 3\)), DEA/NO (2 mM; \(n = 3\)), and SIN-1 (2 mM; \(n = 3\)).

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**FIG. 4.** Inhibition of whole-cell Ba\(^{2+}\) currents by GSNO is antagonized by intracellular administration of copper ions. Time courses of peak Ba\(^{2+}\) current recorded at 30 mV in the presence of 50 \(\mu\)M CuSO\(_4\)/500 \(\mu\)M DTT (A) and in the presence of 50 \(\mu\)M CuSO\(_4\)/500 \(\mu\)M DTT plus the metal chelators neocuproine (1 mM) and DTPA (50 \(\mu\)M) (B) in the pipette solution are shown. Extracellular application of 2 mM GSNO is indicated. The inset shows current recordings during single voltage steps before and after inhibition by GSNO. Scaling bars, 200 pA/200 ms. C, mean values \(\pm\) S.E. of GSNO-induced current inhibition in the absence or presence of CuSO\(_4\) and the metal chelators neocuproine plus DTPA (MC) in DTT containing pipette solution are given (\(n = 6–9\)). The asterisk indicates a statistically significant difference.

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**FIG. 5.** Inhibition of whole-cell Ba\(^{2+}\) currents by GSNO is antagonized by intracellular administration of DTT but not affected by GSSG. Time courses of peak Ba\(^{2+}\) current recorded at 30 mV in the presence of 2 mM DTT (A) and in the presence of 2 mM GSSG (B) in the pipette solution are shown. Extracellular application of 2 mM GSNO is indicated. C, mean values \(\pm\) S.E. of GSNO-induced current inhibition in the absence (\(n = 17\)) and presence of DTT (\(n = 6\)). The asterisk indicates a statistically significant difference. MC, metal chelators neocuproine plus DTPA.
To confirm that this inhibition is dependent on metal ions we performed experiments using the metal chelators neocuproine (50 μM) plus DTPA (50 μM) to remove catalytically active metal ions, in particular Cu. Addition of neocuproine plus DTPA recovered the inhibitory effects of GSNO to the extent observed in presence of DTT (500 μM) alone (Fig. 4, B and C). Thus the intracellular presence of metal ions significantly antagonized the inhibitory action of GSNO. Nonetheless, the inhibitory effects of GSNO observed in the presence of DTT (500 μM) plus metal chelators or in the presence of DTT (500 μM) alone were significantly blunted as compared with control (see Figs. 1 and 2), indicating that DTT by itself interferes with the action of the thionitrite.

Inhibition of α1C-b Currents by GSNO Involves Nitrosation of Critical Thiol Residues—An excess of DTT is expected to compete with cellular thiols in terms of transnitrosation or oxidation and is therefore expected to inhibit thionitrite effects that are based on these reactions. Indeed, GSNO-induced inhibition of whole-cell Ba2+ currents in cells dialyzed with 500 μM DTT plus metal chelators was clearly less pronounced (15 ± 4%; n = 6) as compared with control conditions (38 ± 6%; n = 17). Therefore we tested whether loading of the cells with high concentrations of DTT (2 mM) by itself is sufficient to prevent the action of GSNO. Fig. 5 illustrates that GSNO barely inhibits α1C-b currents in cells that were dialyzed with 2 mM DTT (n = 6). Prevention of GSNO effects by excess of DTT was not dependent on metal ions as metal chelators did not recover inhibitory effects of GSNO in the presence of 2 mM DTT. Inhibition of GSNO effects by intracellular DTT suggests an intracellular transnitrosation or glutathionylation reaction as the chemical basis of GSNO effects. To test whether oxidation of thiols and/or the formation of mixed disulfides can account for the effects of GSNO, we performed experiments in which the cells were loaded with 2 mM oxidized glutathione (GSSG). As shown in Fig. 5B, dialysis with GSSG failed to suppress α1C-b currents (n = 3) and did not affect the inhibitory action of GSNO. Thus GSNO-induced modulation of α1C-b channels is suggested to be based on the nitrosation of critical thiol residues.

Inhibitory Modulation by GSNO of Class C, L-type Ca2+ Channels Is Associated with a Reduction of Open Probability and the Occurrence of a Reduced Conductance State—Inhibition by GSNO of α1C-b channels was confirmed in single-channel recordings, using the cell-attached configuration (Fig.
Note that in this recording configuration, access of bath-applied GSNO to the channels in the patch requires transport across the cell membrane. Overall channel activity in cell-attached patches (mean patch current) decreased to values between 40 and 10% of control within a 2- to 10-min exposure to GSNO (2 mM), respectively. Prolonged exposure to GSNO resulted in complete loss of channel activity in most experiments. The inset in Fig. 6A shows that the control activity in cell-attached patches was fairly stable during prolonged perfusion with bath solution. Fig. 6B summarizes the inhibitory effects of GSNO observed in cell-attached experiments. A similar inhibition was observed in cells expressing α1C-b/β2α2-δ as illustrated in Fig. 7. Neither DEA/NO (2 mM) nor SIN-1 (2 mM) nor 8-Br-cGMP (2 mM) exerted a significant inhibitory modulation (data not shown). In an attempt to gain further information on Ca\(^{2+}\) channel modulation by GSNO, we analyzed the changes in single-channel gating properties induced by the thionitrite. Because most of the cell-attached patches contained more than one channel, we analyzed single-channel \(P_o\) and \(P_a\), as well as the number of active channels in the patches, by use of a recently developed method (20) that allows the analysis of multichannel data. GSNO inhibited α1C-b, as well as α1C-b/β2α2-δ, channels mainly by reduction of \(P_o\) (Fig. 8). In addition we observed a slight reduction of \(P_a\) from 0.48 ± 0.02 to 0.31 ± 0.03 for α1C-b, which was statistically significant, and from 0.39 ± 0.05 to 0.31 ± 0.06 for α1C-b/β2α2-δ channels. It is of note that the single-channel experiments with α1C-b were performed in the presence of S-Bay-K 8644 (1 μM) to prolong open times and enable analysis. The asterisks indicate statistically significant differences.

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FIG. 8. Inhibition of α1C-b and α1C-b/β2α2-δ currents by GSNO is because of a reduction of open probability of the channels. Mean \(P_o\) ± S.E. before (black columns) and 2–10 min after (white columns) extracellular administration of 2 mM GSNO are given for α1C-b (left; \(n = 5\)) and α1C-b/β2α2-δ (right; \(n = 6\)). S-Bay-K 8644 (1 μM) was used in experiments with the α1C-b subunit alone to prolong open times and enable analysis. The asterisks indicate statistically significant differences.

FIG. 9. GSNO-induced modulation of α1C-b channels is associated with changes in unitary conductance of α1C-b channels. A, upper panel, time course of mean patch currents recorded during depolarizing pulses from −70 to 0 mV before and after perfusion with 2 mM GSNO. Lower panel, individual traces of channel activity at the indicated time points. Scaling bars, 1 pA/200 ms. The asterisks indicate the subconductance levels. B, amplitude histograms derived from the experiment shown in A before and after administration of 2 mM GSNO. The asterisk indicates the subconductance level.

The results of the present study suggest that nitrosothiols such as GSNO affect central functions of the pore-forming α1 subunit of class C L-type Ca\(^{2+}\) channels, i.e. gating, as well as ion permeation, because of S-nitrosation of a critical thiol group.

Molecular Basis of L-type Ca\(^{2+}\) Channel Modification by GSNO—Three potential NO donors (GSNO, DEA/NO, and SIN-1) were compared for their effects on whole-cell Ba\(^{2+}\) currents in HEK293 cells stably transfected with the smooth muscle Ca\(^{2+}\) channel α1C-b subunit. All three compounds are well known to increase cGMP levels in various cellular systems (6, 10, 11, 25). Despite this principle similarity, the three compounds favor distinct chemical reactions that have been suggested to mediate (patho-) physiological effects of NO (26, 27). DEA/NO releases NO, which is readily available to stimulate soluble guanylyl cyclase, whereas SIN-1 decomposes to produce both NO and superoxide (28), two species that combine rapidly to form peroxynitrite, a well known nitrating and oxidizing species. It is unlikely that peroxynitrite contributes to the effects of GSNO in our whole-cell experiments, because
HEPES, which has recently been demonstrated to react with peroxynitrite to form NO donors (29), was used as a buffer system. GSNO, by contrast, not only decomposes slowly to release NO but is, in addition, able to react with protein thiol groups to form S-nitrosothiols or mixed disulfides (30). Consistent with the view that all three compounds release NO under certain conditions, they all elevated cGMP levels of HEK293 cells, with DEA/NO being most effective. In clear contrast to its superior action as a stimulator of soluble guanylyl cyclase, DEA/NO exerted rather moderate inhibitory effects on L-type Ca\(^{2+}\) channels, indicating that cGMP does not control \(\alpha_1\)C-b channels in the HEK293 expression system. The notion that the observed Ca\(^{2+}\) channel inhibition by GSNO does not involve cGMP was further corroborated by the observation that 8-Br-cGMP (2 mM) did not affect \(\alpha_1\)C-b channels in cell-attached experiments. These results are in line with a previous study demonstrating that inhibition of L-type Ca\(^{2+}\) channels by S-nitrosothiols is insensitive to inhibition of soluble guanylyl cyclase (12). Thus GSNO appears to exert a specific, cGMP-independent effect on L-type Ca\(^{2+}\) channels. To test whether this effect of GSNO is based on a direct interaction of the thionitrite with intracellular protein thiol residues or requires the release of NO, we studied its effect in cells loaded with copper ions to promote decomposition of GSNO (13, 14, 17, 23, 24) or excess of DTT, which competes with endogenous thiols for S-nitrosation and oxidation (31–33). Both conditions clearly blunted or even eliminated the action of GSNO, supporting the concept that Ca\(^{2+}\) channel modulation by GSNO involves a reaction of the thionitrite with intracellular sulfhydrils. Two types of reaction between GSNO and protein thiol residues were considered, transnitrosation and glutathionylation. Our observation that even excessive loading of cells with GSSG failed to affect channel function, which argued against glutathionylation as a mechanism of channel modulation. This conclusion is further supported by our previous experiments with the lipophilic oxidant tert-butyldihydroperoxide, which is known to induce oxidation of cellular thiols and formation of mixed disulfides but failed to affect \(\alpha_1\)C-b channels in the HEK293 expression system. Thus we suggest nitrosation of critical protein thiol groups as a mechanism of L-type Ca\(^{2+}\) channel regulation. The modest inhibitory effects observed with the NO donor DEA/NO may, therefore, be because of nitrosation of thiol groups, a reaction that was shown to occur at high concentrations of NO in the presence of oxygen (28).

It is tempting to speculate about the modification of a critical cysteine residue of the Ca\(^{2+}\) channel \(\alpha_1\) subunit itself as the regulatory principle mediating the effects of GSNO. Although modification of a yet unknown regulatory protein cannot be excluded, our experiments demonstrate that Ca\(^{2+}\) channel regulation by GSNO does not require expression of any of the known auxiliary subunits of the channel. Thus the \(\alpha_1\)C protein appears to be the most likely target for GSNO modulation. An intracellular localization of the regulatory thiol residue is suggested by the following lines of evidence: (i) the effects of GSNO were sensitive to intracellular administration of copper ions and DTT, and (ii) GSNO inhibited L-type Ca\(^{2+}\) channels not only in the whole-cell but also in the cell-attached configuration of the patch clamp technique. A truncated form of \(\alpha_1\)C that lacks the cytoplasmic C-terminal 438 amino acids including 13 cysteine residues was also sensitive to inhibition by GSNO. The sequence of this truncated \(\alpha_1\)C includes 11 cysteine residues that are potentially accessible from the cytoplasmic side and may be considered as candidates for a regulation by transnitrosation.

**Protein Thiol Nitrosation as a Unique Regulatory Mechanism That Affects both Gating and Conductance of L-type Ca\(^{2+}\)**

Our analysis of the inhibitory effect of GSNO on single-channel parameters revealed that the reduction of whole-cell currents was mainly because of suppression of single-channel open probability. In addition we observed a slightly reduced channel availability and number of active channels in the patches already within 5–10 min of exposure to GSNO, and channel activity was completely lost during prolonged (>10 min) exposure. A detailed inspection of the single-channel behavior during the development of GSNO inhibition uncovered a striking phenomenon. A defined and reproducible subconductance level of about 50% of the full unitary conductance was recorded in about 30% of the experiments. This conductance state was observed only transiently during the development of the GSNO-induced inhibition of channel activity. Thus we report here on a subconductance state of \(\alpha_1\)C-b channels that is induced by a potential (patho-)physiologic regulator of L-type Ca\(^{2+}\) channels. The transient nature of the GSNO-induced subconductance state precluded a more detailed analysis of this phenomenon. Nonetheless, our results strongly suggest that GSNO exerts a modulatory effect on both gating and permeation in the \(\alpha_1\)C-b protein. The observed GSNO-induced modulation of gating and conductance was independent of the expression of auxiliary \(\beta_2\)a and \(\alpha_2\)-\(\delta\) subunits and may well explain inhibitory effects of nitrosothiols on native cardiovascular Ca\(^{2+}\) channels (5, 34, 35).

In summary, we provide evidence for inhibitory modulation by GSNO of the principle pore-forming subunit of smooth muscle L-type Ca\(^{2+}\) channels by intracellular S-nitrosation. GSNO-mediated regulation of Ca\(^{2+}\) channels involves unique changes in the functional properties of the pore-forming \(\alpha_1\) subunit and may represent an important mechanism that links NO signaling to Ca\(^{2+}\) channel functions in the cardiovascular system.

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