The Chemical Modification of K\(_{\text{Ca}}\) Channels by Carbon Monoxide in Vascular Smooth Muscle Cells

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The chemical modification of big conductance calcium-activated potassium (K\(_{\text{Ca}}\)) channels in rat tail artery smooth muscle cells by carbon monoxide (CO) was investigated using the cell-free single channel recording technique. Exposure of the internal surface of cell membranes to diethyl pyrocarbonate (DEPC) neither affected the characteristics of K\(_{\text{Ca}}\) channels nor modified the stimulatory effect of CO on K\(_{\text{Ca}}\) channels. However, when DEPC was applied to the external surface of cell membranes, the open probability of K\(_{\text{Ca}}\) channels was reduced. The pH and concentration dependence of the effect of DEPC indicated the specific modification of histidine residues. Kinetic analysis suggested that one externally located histidine residue was modified by DEPC. Treatment of the external surface of cell membranes with DEPC abolished the CO-induced increase in the open probability of K\(_{\text{Ca}}\) channels. Likewise, the presence of CO partially protected K\(_{\text{Ca}}\) channels from inhibition by DEPC. Moreover, photooxidation of the histidine residue located on the external membrane surface abolished the CO-induced activation of K\(_{\text{Ca}}\) channels. Our study demonstrates that the CO-induced increase in the open probability of K\(_{\text{Ca}}\) channels may rely specifically on the structure and topological locations of histidine residues.

The presence of both inducible and constitutive forms of heme oxygenase, which cleave the heme ring to form carbon monoxide (CO),\(^1\) has been demonstrated in vascular smooth muscle cells (1). Direct measurement of the endogenous production of CO from vascular smooth muscle cells has also recently been realized (2). Our previous study (3), as well as those of others (4–6), demonstrated the regulatory function of CO in various types of vascular tissues. For example, CO relaxed precontracted rat tail artery strips in a concentration-dependent manner (3). This effect of CO was mediated partially by a cGMP signaling pathway and partially by calcium-activated K (K\(_{\text{Ca}}\)) channels. Using the single channel recording patch-clamp technique, we found that CO increased the open probability (NP\(_{\text{O}}\)) of K\(_{\text{Ca}}\) channels.\(^2\) This effect of CO may be caused by a direct interaction between CO and K\(_{\text{Ca}}\) channels as the activities of the cGMP pathway and G proteins are not involved in the activation of K\(_{\text{Ca}}\) channels by CO. To date, the mechanism by which CO directly affects K\(_{\text{Ca}}\) channels remains unclear.

The electrical properties of K\(_{\text{Ca}}\) channels are determined by different amino acid residues that are constitutive components of the channel protein. The primary structure of K\(_{\text{Ca}}\) channels in several cell types, excluding vascular smooth muscle cells, is known, but the functional roles of various amino acids in the gating and conducting of K\(_{\text{Ca}}\) channels are still in question. Limited studies show that the modification of one or more amino acid residues may significantly change the conductance and/or NP\(_{\text{O}}\) of K\(_{\text{Ca}}\) channels (7–9). Both synthetic chemical reagents and some simple biological active molecules such as nitric oxide (7) and hydrogen peroxide (10) can specifically react with certain amino acid residues, thus affecting the functions of ion channels. CO is a biologically active molecule. A direct reaction between CO and certain amino acid residues may significantly affect the function of K\(_{\text{Ca}}\) channels. To test this hypothesis, we used chemical reagents to modify selectively certain amino acid residues of K\(_{\text{Ca}}\) channels. Subsequently, we tested the effect of CO on chemically modified K\(_{\text{Ca}}\) channels. Our results showed that histidine residues participated in channel gating of K\(_{\text{Ca}}\) channels. CO may specifically react with one histidine residue localized in the extracellular domain of K\(_{\text{Ca}}\) channels in vascular smooth muscle cells.

MATERIALS AND METHODS

Preparation of Single Smooth Muscle Cells—Single smooth muscle cells were isolated and identified as described previously (11). Briefly, rat tail arteries were isolated and connective tissues removed. The vessel was cut open longitudinally and enzymatically digested with collagenase/dispase, elastase, and collagenase in a stepwise manner. Dispersed cells were plated in 35-mm Petri dishes and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in a CO\(_2\) incubator at 37 °C. The cells were used 8–36 h after isolation (11).

Single Channel Recording—The inside-out and outside-out configurations of the patch-clamp technique were used to record single K\(_{\text{Ca}}\) channel currents. Pipettes with a resistance of 6–8 megoohms were used, and the seal resistance was usually greater than 10 gigohms. Membrane patches with no more than three channels were used for these experiments. Single channel currents were filtered at 2 KHz (8-pole Bessel, −3 dB) and recorded with a 5-μs sampling interval in a gap-free mode. For each concentration of tested agents, at least 60 s of channel activities were directly recorded onto the hard disk of a computer. The NP\(_{\text{O}}\), i.e. the fraction of time during which the channels are open within the total observation period with N representing the number of single channels in one patch (12), and single channel conductance were determined from all-point amplitude histograms using Fetchan and Patstat programs (Axon Instruments, Inc.). The NP\(_{\text{O}}\) for consecutive 10-s intervals was plotted as histograms to show the changes in channel activity with time. The mean NP\(_{\text{O}}\) during 2–5-min recordings was calculated in some cases to show the major changes in channel activity following different treatments. Patches with unstable NP\(_{\text{O}}\) over time were excluded from further analysis. Experiments that compared NP\(_{\text{O}}\) and/or single channel conductance before and after different treatments

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1 The abbreviations used are: CO, carbon monoxide; K\(_{\text{Ca}}\) channel, calcium-activated potassium channel; NP\(_{\text{O}}\), open probability; DEPC, diethyl pyrocarbonate.

2 R. Wang, L. Wu, and Z. Z. Wang, unpublished observation.
were usually conducted on the same patches. A current level greater than 50% of the amplitude of the unit channel current was considered to reflect a channel opening. The outside surface of membrane patches was bathed in a solution containing (in mM): 145 KCl, 10 HEPES, and 10 glucose. The inside surface of membrane patches was exposed to a solution containing (in mM): 145 KCl, 10 HEPES, 1.2 MgCl₂, 10 glucose, 1 EGTA, and different amounts of CaCl₂ to reach the desired final free Ca²⁺ concentrations.

Chemicals and Data—To prepare the CO solution, 20 ml of stock solution in a sealed glass tube was bubbled with a stream of CO (Canadian Liquid Air Ltd.) for 20 min under a pressure of 100 kilopascals at 0°C. One μl of this CO-saturated solution contained 30 ng of the gas (6). The stock solution of CO was prepared freshly before each experiment and then diluted immediately to the desired concentration with the bath solution.

Diethyl pyrocarbonate (DEPC) was diluted fresh with anhydrous ethanol prior to each experiment and added directly to the bath solution at pH 7.0 to superfuse the membrane patches for 5 min unless otherwise indicated. In photooxidation experiments, the cells were bathed in phosphate-buffered saline (pH 7.4) containing 1 μg/ml rose bengal. The Petri dishes with attached cells were placed in an ice chamber 20 cm underneath a 200-watt white bulb lamp for 20 min. After the illumination, the dye-containing bath solution was replaced with a dye-free bath solution for the patch-clamp experiments.

In all chemical modification experiments, the pH of reaction solutions was adjusted with the hydroxide of the major cation. Each experiment was bracketed by a control conducted at the same pH. A short period of exposure of membrane patches to the pH values used in chemical modification experiments did not yield sustained changes in the behavior of KCa channels. Unless specified, chemicals were obtained from Sigma. Osmalities of all recording solutions were adjusted to 290 mosm. All electrophysiological experiments were carried out at room temperature.

The data were expressed as means ± S.E. and analyzed using Student’s t test or analysis of variance in conjunction with the Newman-Keul test where applicable. Group differences were considered statistically significant at the level of p < 0.05.

RESULTS

In rat tail artery smooth muscle cells, a big conductance KCa channel was identified. With symmetric KCl (145 mM) on both sides of the patch membrane, single channel conductance was linearly related to membrane potentials over the range of −100 to +60 mV with no evidence of rectification. CO increased the channel activity in a concentration-dependent manner (3–30 μM) in both outside-out and inside-out patches. Although single channel conductance of KCa channels was not modified by CO (not shown), the NPo was increased significantly, and multiple channel openings were often elicited in the presence of CO.

Effects of DEPC on the NPo of KCa Channels—In inside-out patches (n = 3), bath application of DEPC (0.5 mM) neither affected the single channel conductance and NPo of KCa channels nor modified the stimulatory effect of 30 μM CO on the NPo in the same patches (Fig. 1). In contrast, bath application of DEPC to outside-out patches (n = 3) reduced the NPo of single KCa channels without affecting the current amplitude. This inhibitory effect of DEPC, if specific for histidine residues, should be a function of pH, since DEPC reacts only with the unprotonated imidazole ring. Hence, the effects of DEPC at different pH levels on the NPo of KCa channels were further investigated (Fig. 2A). At pH 6.3, a 46% inhibition of the NPo of KCa channels by DEPC was observed. At pH 5.2, the NPo of KCa channels was only slightly decreased by DEPC treatment (6%).

Kinetics of DEPC Effect—A kinetic analysis of the effect of DEPC on KCa channels is shown in Fig. 2, B and C. The decrease in the NPo of KCa channels was eminent 1 min after the DEPC application, and a 50% decrease of NPo was observed 4 min after the DEPC treatment. The decrease in the NPo of KCa channels by DEPC was also concentration-dependent from 0.1 to 2 mM and followed pseudo-first order kinetics. The reaction order obtained from the slope of the double logarithmic plot (Fig. 2C) was 1.0, indicating that one histidyl residue/channel protein might be involved in the modifying effect of DEPC (13, 14).

Interaction between CO and DEPC—The DEPC treatment abolished the CO-induced increase in the NPo of single KCa channels in four outside-out membrane patches (see one example in Fig. 3). In two other outside-out patches, CO was applied first. After washing out CO, the patches were exposed to DEPC followed by a reexposure to CO. The first application of CO increased the NPo of KCa channels, but the second application failed to do so (Fig. 4A). To examine further the specific interaction of DEPC and CO on histidine residues, the DEPC solution was maintained for 12 h at room temperature to inactivate DEPC spontaneously since the half-life of DEPC in an aqueous solution is less than 10 min at room temperature and pH 7 (15). This inactivated DEPC had no effect on KCa channels, and subsequently applied CO was still capable of increasing the NPo of KCa channels in the same patch (Fig. 4B). It was extremely difficult to reverse the effect of DEPC on KCa channels, at least over the time frame of our experiments (16). We have tried to apply hydroxylamine to remove DEPC from imidazoles. Since the membrane patches usually could not tolerate the high concentration of hydroxylamine (20 mM) for more than 30 min, we were unable to observe the recovery of KCa channel activity except in one outside-out patch, which lasted for more than 60 min. In that patch, the CO-induced modification of single KCa channel currents was recovered (Fig. 4C). An interaction between CO and DEPC on histidine residue was also demonstrated by the CO-induced protection of KCa channels from inhibition by DEPC. Fig. 5 shows that the presence of CO significantly inhibited the effect of DEPC on the NPo of KCa channels.

Photooxidation of KCa Channels—To confirm further the involvement of histidine residues in the modifying effect of CO on KCa channels, the cells were exposed to illuminated rose bengal, a treatment specifically modifying histidine residues on the external surface of cell membranes. Fig. 6 shows that in membrane patches isolated from photooxidized cells, the stimulatory effect of 30 μM CO on the NPo of KCa channels was abolished. However, CO still significantly increased the NPo of KCa channels in outside-out patches isolated from cells that were either preincubated with rose bengal in the absence of
light (Fig. 6) or exposed to illumination in the absence of the dye for 15 min (not shown). These results rule out possible nonspecific damage of KCa channels induced by nonilluminated dye or by photoinactivation of the KCa channels.

DISCUSSION

KCa channels are gated by voltage and calcium and have various conductances and pharmacological sensitivities. The big conductance KCa channels have been identified in many types of vascular smooth muscle cells (17–20). The functioning of these KCa channels controls membrane potential and affects vascular activity. The activation of KCa channels by CO may significantly affect vascular tone under physiological and pathophysiological conditions. Therefore, it is of great importance to understand the molecular mechanism underlying the direct effect of CO on KCa channels.

The big conductance KCa channels are composed of two noncovalently linked subunits: the pore-forming α subunit and the β subunit, which influences the electrophysiological behavior of KCa channel complexes (21). The amino acid sequences and topography of KCa channels in nonvascular smooth muscle cells have been known for years (22). To date, knowledge of the molecular structure of KCa channels, especially the α subunit, in vascular smooth muscle cells is lacking. In the present study,
we modified selectively certain amino acid residues of K\textsubscript{Ca} channel protein to probe the structure-function relationship of K\textsubscript{Ca} channels in rat tail artery smooth muscle cells. Since CO changed the NPo, but not the conductance, of K\textsubscript{Ca} channels, we speculate that the gating mechanism is modified by CO with the permeation of ions through the channel unchanged. An extrapolation of this speculation is that amino acid residues outside the pore-forming region of K\textsubscript{Ca} channel protein would be essential in mediating the effect of CO.

Using the chemical reagents and protocols described in this paper, we show that the direct effect of CO on K\textsubscript{Ca} channels is most likely the result of the interaction of CO and a histidine residue that is located on the external surface of K\textsubscript{Ca} channels in rat tail artery smooth muscle cells. Several lines of evidence support our conclusion. First, DEPC decreased the NPo of K\textsubscript{Ca} channels and abolished the effect of CO on K\textsubscript{Ca} channels. The DEPC-induced modification of histidine residues involves substitution at one of the nitrogen positions on the imidazole ring. Hydroxylamine reverses DEPC effects by removing DEPC from histidines (16). De Biasi et al. (23) found that the histidine-specific DEPC produced exaggerated blockade of the mutated potassium channel compared with the wild type. Using the cell-attached single channel recording technique, Bouzat et al. (24) showed that DEPC reduced the open time of an acetylcholine-activated channel in the cloned muscle cell line BC3H-1. Christensen and Hida (25) reported that DEPC reduced a kainate-induced current but that sulfhydryl-specific reagents were ineffective, indicating that histidine residues localized on the external membrane surface may be important in regulating channel gating. In our studies, the carbethoxylation reaction was carried out in outside-out and inside-out patches. The modification of K\textsubscript{Ca} channels was observed only when the external surface of the membrane patch was exposed to DEPC. The histidine residue in question may, therefore, be situated on the external surface of the membrane, possibly near or stretched to the “pore” of channels, thus providing a functional surface for the regulation of channel gating. Second, after DEPC was removed from the modified histidine residue by hydroxylamine, the stimulatory effect of CO on K\textsubscript{Ca} channels was recovered. Third, the kinetics of the DEPC-induced inhibition of the NPo of K\textsubscript{Ca} channels suggests the carbethoxylation of only one histidyl residue in the external surface of the cell membrane. Fourth, pH dependence for the inhibition of the NPo by DEPC is consistent with titration of the imidazole ring of a histidine residue, which has a pK\textsubscript{a} value between 6.4 and 7.5 in most proteins (26, 27). At a lower pH, protonation of the histidine makes the imidazole ring less reactive (25). Thus, DEPC is less effective in reducing the NPo of K\textsubscript{Ca} channels at pH 5.2 than at pH 6.3 or 7.4. Fifth, our photooxidation experiments provided additional proof for the specific involvement of histidine residue in the effect of CO. It is not feasible to isolate selectively the external surface of cell membrane for classical protein chemistry assay to detect the DEPC-induced changes in histidine residues. Therefore, photooxidation with rose bengal was chosen as an alternative means to modify histidine residues (28–30). After the cells were exposed to illuminated rose bengal, only those histidine residues located on the external surface of the cell membrane were presumably modified since rose bengal, like DEPC but with an even greater molecular mass, will not penetrate cell membranes to act on intracellular histidines (30). Similar to the effect of DEPC, photooxidation abolished the stimulatory effect of CO on the NPo of K\textsubscript{Ca} channels. Individually, photooxidation or DEPC treatment may not be fully specific for histidine residues. However, histidine (externally located) seems to be the only amino acid that clearly reacts with low concentrations of both DEPC and rose bengal (28). Finally, we showed that the presence of CO partially protected K\textsubscript{Ca} channels from inhibition by DEPC, suggesting that CO and DEPC may act on the same histidyl residue.

Since CO is membrane-permeable, a direct effect of CO on K\textsubscript{Ca} channels could be interpreted as the result of the modification of either membrane proteins or membrane lipids. However, membrane proteins seem much more likely to be modified than membrane lipids because the effect of CO on K\textsubscript{Ca} channels was not altered unless a specific amino acid residue, histidine, was modified. It is also worth noting that there were obvious differences between the effects of CO and DEPC on K\textsubscript{Ca} channels. DEPC decreased the NPo of K\textsubscript{Ca} channels in a relatively irreversible manner because this reagent is involved in the covalent modification of histidine. On the other hand, CO increased the NPo in a reversible fashion probably because of a relatively weak reaction between CO and imidazole group of histidine via hydrogen bonds. A similar mechanism is believed
to be important for the formation of heme-CO complex in which the distal histidine residue (His-64) in myoglobin (31) or histidine 25 in heme oxygenase (32) is involved.

Despite many efforts over decades of investigation, the ionic mechanisms involved in CO-mediated vasoactivity remain elusive. From this study, the direct interaction of CO with KCa channel proteins has been established. Given that the chemical reagents tested in this study did not diffuse readily through the cell membrane and were applied directly to the bath solutions in outside-out or inside-out patch recordings, our results provide an indication regarding the topological location of the CO-sensitive histidine residues. The modification of ion channel proteins using specific chemical reagents can be a valuable tool for identifying amino acid groups important for the functioning of the channels. However, the chemical identity and topography of the specific amino acid residues of KCa channels modified by chemical reagents, including CO, cannot be determined with certainty from our experiments and must await isolation, sequencing, and determination of the three-dimensional structure of the KCa channel protein in vascular smooth muscle cells.

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