European Journal of Pharmacology 815 (2017) 33–41

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

CO-independent modification of K^+ channels by tricarbonyldichlororuthenium(II) dimer (CORM-2)

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ARTICLE INFO

Chemical compounds studied in this article:
CORM-2 (PubChem CID: 10951331)
CORM-3 (PubChem CID: 91826084)

Keywords:
Potassium channel
K^+ channel
Patch clamp
Carbon monoxide
Hemeoxygenase
Metal carbonyl

ABSTRACT

Although toxic when inhaled in high concentrations, the gas carbon monoxide (CO) is endogenously produced in mammals, and various beneficial effects are reported. For potential medicinal applications and studying the molecular processes underlying the pharmacological action of CO, so-called CO-releasing molecules (CORMs), such as tricabonyldichlororuthenium(II) dimer (CORM-2), have been developed and widely used. Yet, it is not readily discriminated whether an observed effect of a CORM is caused by the released CO gas, the CORM itself, or any of its intermediate or final breakdown products. Focusing on Ca^{2+}- and voltage-dependent K^+ channels ($K_{Ca}1.1$) and voltage-gated K^+ channels (Kv1.5, Kv11.1) relevant for cardiac safety pharmacology, we demonstrate that, in most cases, the functional impacts of CORM-2 on these channels are not mediated by CO. Instead, when dissolved in aqueous solutions, CORM-2 has the propensity of forming $Ru(CO)_2$ adducts, preferentially to histidine residues, as demonstrated with synthetic peptides using mass-spectrometry analysis. For $K_{Ca}1.1$ channels we show that H365 and H394 in the cytosolic gating ring structure are affected by CORM-2. For Kv11.1 channels (hERG1) the extracellularly accessible histidines H578 and H587 are CORM-2 targets. The strong CO-independent action of CORM-2 on Kv11.1 and Kv1.5 channels can be completely abolished when CORM-2 is applied in the presence of an excess of free histidine or human serum albumin; cysteine and methionine are further potential targets. Off-site effects similar to those reported here for CORM-2 are found for CORM-3, another ruthenium-based CORM, but are diminished when using iron-based CORM-S1 and absent for manganese-based CORM-EDE1.

1. Introduction

Carbon monoxide (CO) is an odorless toxic gas, typically generated during incomplete combustion of organic matter. However, CO is also endogenously produced in mammals (Tenhunen et al., 1968) and affects many processes such as vascular tone regulation (Coburn, 1979) and synaptic transmission (Zhuo et al., 1993). Therefore, CO may also serve as a physiological gaseous messenger with therapeutic potential. This aspect spurred the development of CO-releasing molecules (CORMs) to be used as drugs, circumventing complications during therapeutic inhalation of CO gas.

Among several different CORMs synthesized, tricarbonyldichlororuthenium(II) dimer (CORM-2; Motterlini et al., 2002), most likely because of its commercial availability, has been used extensively in in-vitro studies. Compared with application of CO itself, CORMs are safer and easier to use in experimental settings; however, a drawback of using CORMs is the potential problem of eliciting molecular reactions that are unrelated to CO itself but originate from other by-products. Unfortunately, such CORM-mediated side effects have not been studied systematically.

Studies utilizing CORMs have implicated numerous molecular effectors of CO (reviewed in e.g. Gullotta et al., 2012; Wegiel et al., 2013). For example, it is generally accepted that activation of large-conductance, Ca^{2+}- and voltage-activated K^+ ($K_{Ca}1.1$) channels contributes to the CO-dependent vasorelaxation (Wang et al., 1997; Williams et al., 2004). However, vasorelaxation induced by CO gas and CORM-2 apparently involves different molecular mechanisms (Decaluwé et al., 2012). Furthermore, CO-mediated activation of $K_{Ca}1.1$ channels in human umbilical vein endothelial cells is not mimicked by CORM-2 (Dong et al., 2008).

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http://dx.doi.org/10.1016/j.ejphar.2017.10.006
Received 19 June 2017; Received in revised form 26 September 2017; Accepted 4 October 2017
Available online 05 October 2017

The tetrameric $K_{Ca}1.1$ channels are composed of a transmembrane central pore domain surrounded by four voltage-sensing domains, similar to voltage-gated K^+ (Kv) channels. Two large cytosolic C-terminal domains (RCK1 and RCK2), which are absent in Kv channels, form a gating ring structure. The channel open probability is controlled by transmembrane voltage and the conformation of the gating ring, which changes upon binding of intracellular Ca^{2+} (Hoshi et al., 2013) or a plethora of molecules, among them possibly CO (Hou et al., 2009). Activating impacts of CO gas or several CORMs have been reported, but the underlying molecular mechanisms are still under debate. Proposed molecular determinants for CO effects on $K_{Ca}1.1$ include extracellular histidines (Wang and Wu, 1997), channel-bound heme (Jaggar et al., 2005), H365 and H394 within RCK1 (Hou et al., 2008b), and C911 within RCK2 (Williams et al., 2008; Telezhkin et al., 2011).

Here we analyzed the mechanism by which CORM-2 – as compared to CO gas – affects $K_{Ca}1.1$, Kv11.1 (hERG1) and Kv1.5 channels. We present generally applicable experimental strategies for identifying and avoiding side effects originating from CORM-2 and related CO-releasing compounds.

2. Materials and methods

2.1. Expression plasmids and mutagenesis

Wild-type human K^+ channels used in this study were: $K_{Ca}1.1$, (hSlo1, KCNMA1, U11058), Kv1.5 (KCNA5, P22460), Kv10.1 (hEAG1, KCNH1, AJ0013668), Kv11.1 (hERG1, KCNH2, NM_000238), and Kv11.3 (hERG3, KCNH7, NP_150375). Mutations were introduced by overlap extension PCR (Expand High Fidelity, Roche, Mannheim, Germany), verified by DNA sequencing.

2.2. Cell culture

HEK 293T cells (DSMZ, Braunschweig, Germany) were maintained in DMEM/F-12 (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. Cells were trypsinized, diluted with culture medium, and seeded on 12-mm glass coverslips. Patch-clamp experiments were performed 2–3 days after plating. Cells were transfected with the respective plasmids using the Rotifect® (Roth, Karlsruhe, Germany) transfection reagent. CD8-encoding plasmids (10–20% of total DNA) were co-transfected to allow identification of transfected cells using anti-CD8-coated beads (Dynabeads, Invitrogen, Karlsruhe, Germany).

2.3. Electrophysiological measurements

Whole-cell and inside-out voltage-clamp experiments were performed as described previously (Gessner et al., 2010, 2012). Briefly, patch pipettes with resistances of 1.0–2.5 MΩ were used. The series resistance was compensated for by more than 70% to minimize voltage errors. Patch-clamp amplifier EPC9 or EPC10 was operated with PatchMaster software (both HEKA Elektronik, Lambrecht, Germany). Leak and capacitive currents were corrected with a p/*n* method with a leak holding voltage of −110 mV. Currents were low-pass filtered at 5 kHz and sampled at a rate of 25 kHz. All experiments were performed at 21–23 °C.

Internal solutions contained (mM): 140 KCl, 10 EGTA, 10 HEPES (pH 7.4 with KOH). For whole-cell recordings the external (bath) solution consisted of (mM): 146 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH). To allow buffering of pH between 6.5 and 8.5, MES or TRIS was used instead of HEPES when appropriate. For inside-out recordings the external (pipette) solution composed of (in mM): 140 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (pH 7.4 with KOH).

Solutions containing CO gas (1 mM) were prepared on the day of experiments by bubbling the solution for > 20 min with CO gas and stored in gas-tight rubber-sealed glass vials. CORM-2 and CORM-3 aliquots were dissolved in DMSO (50 or 100 mM) vigorously mixed for 5 s and immediately diluted in the bath to the final concentration, resulting in a maximal final DMSO concentration of 0.1%. CORM-S1 and CORM-EDE1 were dissolved in DMSO (50 mM), aliquoted and stored at −20 °C in the dark for up to 2 months, a period during which no significant loss of CO-releasing activity was observed in a standard myoglobin assay. For CORM structures see Supplementary Fig. 1. CO release from CORM-S1 and CORM-EDE1 was triggered by illumination of the solution in the focus of a 40x objective with blue light from a 100-W HBO mercury lamp (450–490 nm). CORM-2 was from Sigma-Aldrich (Darmstadt, Germany); iCORM-2, CORM-3, CORM-S1 and CORM-EDE1 were synthesized according to protocols published previously (Motterlini et al., 2002; Johnson et al., 2007; Kretschmer et al., 2011; Mede et al., 2016).

2.4. Peptide synthesis

Solid-phase peptide synthesis was carried out on Rink amide MBHA resin (0.53 mmol/g, Iris) applying a standard Fmoc (9-fluorenylmethyloxycarbonyl) protocol with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) and hydroxybenzotriazole (HOBt) as coupling agents. Synthesis was performed using an automated peptide synthesizer EPS221 (Intavis, Cologne, Germany). Peptide cleavage was carried out using 1 ml of the mixture: 75 mg phenol, 25 µl ethanedithiol, 50 µl thioanisol, 50 µl water in 1 ml trifluoroacetic acid (TFA) per 100 mg of resin. The mixture was gently shaken for 3 h, and then the peptide was filtered off the resin and precipitated in cold diethyl ether. After several washing steps using diethyl ether, the crude peptides were purified by semi-preparative RP-HPLC on a Shimadzu LC-8A system equipped with a C18 column (Knauer Eurospher 100) using 0.1% TFA in water (eluent A) and 0.1% TFA in 90% acetonitrile/water (eluent B) as elution system. Aliquots were prepared, lyophilized and stored at −20 °C.

Peptides used:

Kv11.1 wild type H−GNMEQPHMDSRIGWLHNLGDQI−NH_2 (1)

Kv11.1 H578D H−GNMEQPDMDSRIGWLHNLGDQI−NH_2 (2)

Kv11.1 H587Y H−GNMEQPHMDSRIGWLYNLGDQI−NH_2 (3)

Kv11.1 H578D: H587Y H−GNMEQPDMDSRIGWLYNLGDQI−NH_2 (4)

Control−His H−AAAAHAAAA−NH_2 (5)

Control−Ala H−AAAAAAAAA−NH_2 (6)

Control−Cys H−AAAACAAAA−NH_2 (7)

2.5. Sample preparation

Peptides or Ac-His-NHMe were dissolved in ddH_2O and immediately mixed with freshly prepared CORM-2 (in ddH_2O) solutions at different ratios (4:1 and 1:1). Mixtures were incubated at room temperature for different durations (15 min or 12 h), centrifuged and the supernatant was injected into the LC-ESI-MS system micrOTOF-Q III (Bruker Daltonics GmbH, Bremen, Germany) equipped with a C18 column (EC100/2 Nucleoshell RP18 Gravity 2.7 µm column, Macherey-Nagel, Düren, Germany), and detected at 220 nm. The column temperature was 25 °C. Analysis of the MS data was performed using Bruker Compass Data Analysis 4.1 software.

2.6. Amino acid analysis

Peptide concentration was determined by amino acid analysis after hydrolyzing about 500 µg peptide in 6 N HCl in an oxygen-free