Original Paper

Regulation of Intracellular Calcium by
Carbon Monoxide in Human Bronchial
Epithelial Cells

Rui-Gang Zhang Chung-Yin Yip Wing-Hung Ko

School of Biomedical Sciences, The Chinese University of Hong Kong, N.T., Hong Kong, China

Key Words

Carbon monoxide • Airway epithelia • Purinergic receptor • Calcium signaling • Store-operated calcium entry • Soluble guanylyl cyclase

Abstract

Background/Aims: Carbon monoxide (CO) is an important autocrine/paracrine messenger involved in a variety of physiological and pathological processes. This study aimed to investigate the regulatory role of CO released by CO-releasing molecule-2 (CORM-2) in a P2Y receptor-mediated calcium-signaling pathway in the human bronchial epithelial cell line, 16HBE14o-. **Methods:** Intracellular calcium ($[Ca^{2+}]_i$) was measured by fura-2 microspectrofluorimetry. D-myo-inositol-1-phosphate (IP_1) levels and cGMP-dependent protein kinase activity (PKG) were also quantified. **Results:** The exogenous application of CORM-2 increased both intracellular Ca^{2+} and IP_1 , which are inhibited by U73122, a phospholipase C (PLC) inhibitor. In contrast, the $P2Y_2/P2Y_4$ receptor-mediated intracellular Ca^{2+} release and influx induced by UTP were inhibited in the presence of CORM-2. However, CORM-2 did not affect the store-operated Ca^{2+} entry (SOCE) induced by thapsigargin (Tg). Moreover, the inhibitory effect of CORM-2 on UTP-induced calcium increase could be attenuated by a soluble guanylyl cyclase (sGC) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), or a Protein Kinase G (PKG) inhibitor, KT5823, suggesting the involvement of sGC/PKG signaling in this process. **Conclusion:** CORM-2 serves a dual role in modulating $[Ca^{2+}]_i$ in 16HBE14o- cells. Thus, CO released by CORM-2 may act as a regulator of calcium homeostasis in human airway epithelia. These findings help further elucidate the function of CO in many physiological and pathological conditions.

Introduction

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Carbon monoxide (CO) is a potent gas molecule with a variety of physiological functions, including vasodilation, anti-inflammation, anti-oxidative stress, and neuromodulation [1]. CO is produced endogenously by the heme oxygenase (HO) enzymes HO-1, HO-2, and HO-3. HO-2 and HO-3 (only expressed in rats) are constitutively expressed, whereas HO-1 is induced by a wide range of stimuli [2, 3]. Both HO-1 and HO-2 are reportedly expressed in human airway

Wing-hung KO, PhD

School of Biomedical Sciences, The Chinese University of Hong Kong, Lo Kwee-Seong
Integrated Biomedical Sciences Building, CUHK, Shatin, Hong Kong, (China)
Tel. 852-39436781, Fax. 852-26035123, E-Mail whko@cuhk.edu.hk

epithelial cells [4]. The measurement of the rate of endogenous CO production in humans using blood carboxyhemoglobin (CO-Hb) analyses was first described by Coburn et al. and was found to be ~0.42 ml/hr [5]. Endogenously produced CO is sufficient to maintain a CO-Hb saturation level of 0.4–0.96% [6]. This CO-Hb level can be elevated to 5% or even higher in heavy smokers [7]. Although it has been demonstrated that CO is a potential therapeutic agent in different animal models of inflammatory disease [8, 9], increasing the CO-Hb level to 7% by CO inhalation had no anti-inflammatory effects in a systemic lipopolysaccharide-induced inflammation model in humans [10]. Therefore, further clinical experiments are required to investigate the safety and efficacy of CO inhalation as a treatment for human inflammatory lung diseases.

To provide safe and controlled delivery of CO into cells, CO-releasing molecules (CORMs) have been developed and tested over the past decade [11]. CORM-2, a lipid-soluble metal carbonyl complex tricarbonyl dichlororuthenium (II) dimer ($[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$), is commonly used experimentally to release CO and to elicit various biological activities such as a vasodilatory effect on rat aortic rings [12], an anti-inflammatory effect in murine macrophages [13, 14], and an anti-bacterial effect [15]. By measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin spectrophotometrically, it has been estimated that for each mole of CORM-2, approximately 0.7 moles of CO are rapidly released with a half-life of about 20 minutes [12].

P2Y receptors are expressed in virtually all epithelia, where they are activated by extracellular nucleotides such as ATP and UTP [16] and control the transport of fluid and electrolytes. Eight P2Y receptor subtypes have been defined. We previously reported four subtypes of P2Y receptors (P2Y₁, P2Y₂, P2Y₄, and P2Y₆) expressed in human bronchial epithelial cell line 16HBE14o-. 16HBE14o- cells are an immortalized cell line that was derived from the human bronchial surface epithelium [17]. These cells represent a good model for investigating transepithelial Cl⁻ secretion [18, 19], airway inflammation, and airway remodeling [20–22]. Our previous studies showed that activation of P2Y receptors regulated epithelial Cl⁻ secretion via both Ca²⁺- and cAMP-dependent pathways [23] and activated the PLC- D-myo-inositol 1, 4,5-triphosphate (IP₃)-Ca²⁺ signaling cascade in 16HBE14o- cells, which is also responsible for the secretion of at least two pro-inflammatory cytokines, interleukin (IL)-6 and IL-8, when the cells were challenged by cationic proteins [24]. Therefore, we sought to test whether CO would modulate the P2Y receptor-mediated calcium-signaling pathway.

Calcium ions (Ca²⁺) impact nearly every aspect of life; therefore, calcium signaling and Ca²⁺ levels are highly controlled [25]. Intracellular Ca²⁺ may come from either calcium release from intracellular Ca²⁺ stores, such as the endoplasmic reticulum (ER), and/or Ca²⁺ influx. In electrically non-excitabile cells, the major Ca²⁺ entry pathway is reported to be store-operated calcium entry (SOCE) [26]. However, there is substantial evidence that a non-store-operated calcium entry pathway, known as receptor-operated calcium entry (ROCE), may exist. Although ROCE is well studied in excitable cells, it also exists in non-excitabile cells [27, 28]. Therefore, it is possible that the activation of P2Y receptors could activate both SOCE and ROCE in human bronchial epithelia; thus, it would be interesting to test if CORM-2 has any effect on SOCE and/or ROCE. Although some reports suggest that CO maybe a regulator of ion channels [29], and that CO can inhibit T-type calcium channels in neuronal cells [30] and affect calcium signaling in pancreatic acinar cells [31], the regulatory effects of CO on intracellular calcium in human airway epithelia remain largely unknown. To our knowledge, this is the first study to examine the effect of CO released by CORM-2 on Ca²⁺ signaling and its interaction with the purinergic signaling pathway in human bronchial epithelial cells.

Materials and Methods

Cell culture

16HBE14o- cells were maintained in Minimum Essential Medium with Earle's salt supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) L-glutamine, 100 IU/ml penicillin, and 100 µg/ml

streptomycin, as previously described [23]. Cells were cultured in plastic flasks coated with fibronectin and collagen (BD Biosciences, San Jose, CA, USA) and were incubated in humidified 5% CO₂ air at 37°C. For the IP₁ assay, cells were seeded in a 96-well plate at the density of 2×10⁴/well. For calcium measurement, cells were seeded in a standard culture dish (35mm) with a coverslip.

Calcium measurement

Cells were incubated with fura-2 AM (3 μM) and pluronic F127 (1.6 μM) for 40 min at 37°C. Cells were then washed with Krebs-Henseleit (KH) solution and the coverslip was transferred to a closed perfusion chamber and mounted on the stage of an inverted microscope (Nikon ECLIPSE TE300, Japan) coupled to the PTI Ratio-Master fluorescence system (Photon Technology International, NJ, USA). The fluorescence intensity ratio (excitation at 340/380 nm; emission >510 nm) was collected. The data are shown quantitatively as a change in the Fura-2 fluorescence ratio (340/380 nm). The KH solution contained 117 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, and 11 mM D-glucose, pH 7.4, when mixed with 5% CO₂ and 95% O₂. The composition of the nominally Ca²⁺-free KH was the same as normal KH with the exception that there was no CaCl₂ in the solution.

IP₁ assay

Because the lifetime of IP₃ within the cell is very short before it is transformed into IP₂ and IP₁, IP₁ accumulation levels can be used to represent IP₃ levels in cells [32]. The IP₁ measurements were performed as recently described [32]. Agonist-induced IP₁ in LiCl-treated cells was quantified using the Cisbio IP-One kit (Cisbio Bioassays, Codolet, France) per the manufacturer's instructions. Light emitted at 615 and 665 nm was measured on a Spectra Max i3X Multimode Plate Reader (Molecular Devices, CA, USA) using the HTRF mode after excitation at 340 nm. The FRET ratios (665 nm/615 nm) were converted to concentrations of IP₁ using the provided standard.

PKG activity assay

Cells were grown in 35mm cell culture dishes (SPL Life sciences Co. Ltd, Korea). Cells were incubated in serum-free medium overnight after they reached confluence. Cells were then treated with either CORM-2 or RuCl₃, the negative control for CORM-2, for 15 min. KT5823 was applied 10 min before CORM-2 treatment. Protein was extracted using standard methods. Protein (50 μg) was added to each well for further assay. The PKG activity assay was performed using the Cylex cyclic GMP-dependent protein kinase assay kit (MBL, USA) as previously described [33].

Materials

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA), and all cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Data analyses

Data are presented as mean ± standard error (S.E.) and the values of *n* refer to the number of experiments in each group. Statistical comparisons were performed using either a Student's *t*-test or one-way ANOVA (followed by post hoc tests). A *p* < 0.05 was considered significant.

Results

CORM-2 caused an increase in [Ca²⁺]_i

Fig. 1A shows the effect of different concentrations of CORM-2 on the increase in [Ca²⁺]_i as monitored by the Fura-2 fluorescence ratio. CORM-2 was applied to the perfusate at time 0 min and was present throughout the entire experimental period. The application of CORM-2 to the 16HBE140- cells in the Ca²⁺-containing KH solution induced a slow increase in [Ca²⁺]_i. Increasing the concentration of CORM-2 caused a larger increase in [Ca²⁺]_i and shortened the time to reach peak [Ca²⁺]_i. The maximum increase in [Ca²⁺]_i was concentration-dependent, with an EC₅₀ of 52.7 ± 0.5 μM as shown in Fig. 1B. The inactive form of CORM-2, iCORM-2 (100 μM), had no significant effect (Fig. 1C). A slight increase in [Ca²⁺]_i was observed when the cells

Fig. 1. Characteristics of calcium increase induced by CORM-2. (A) 16HBE14o- cells were exposed to different concentrations of CORM-2 in Normal KH at time 0 min and the maximal increase in the Fura-2 fluorescence ratio was quantified. Similar results were obtained for at least three replicates. (B) Concentration-response curve for the change in the Fura-2 to CORM-2 ratio. (C) 16HBE14o- cells were stimulated at time 0 min with (i) CORM-2 (100 μ M) in Normal KH or nominally Ca^{2+} -free solution; (ii) inactive CORM-2 (iCORM-2) (100 μ M) in Normal KH solution; and (iii) CORM-2 (100 μ M) and U73122 (10 μ M) in Normal KH solution. Similar results were obtained for at least four replicates. (D) Statistical results for (C). Each column represents the mean $\pm$ S.E. ($n = 4$); * $p < 0.05$, one-way ANOVA with Dunnett's post hoc test. (E) 16HBE14o- cells were superfused with normal KH solution and then stimulated with CORM-2 and treated with Pyr-6 (10 μ M), 2-APB (10 μ M), or both after the CORM-2-induced calcium changes had reached peak levels. Inhibition was calculated as a percent relative to the maximal increase in the Fura-2 ratio induced by CORM-2 in the presence of Pyr-6, 2-APB, or both ($n = 3-5$).

were stimulated with 100 μ M CORM-2 in Ca^{2+} -free KH solution (Fig. 1C). The summarized data are shown in Fig. 1D. The results indicated that the calcium increase induced by 100 μ M CORM-2 was mainly due to Ca^{2+} entry from the extracellular medium.

To test whether calcium entry induced by CORM-2 is mediated by the emptying of Ca^{2+} stores by the PLC-IP₃ signaling pathway, the cells were stimulated with CORM-2 (100 μ M) at time 0 min in the presence of the PLC inhibitor U73122 (Fig. 1C). In the presence of U73122 (10 μ M), the CORM-2-induced Ca^{2+} increase was almost completely abolished (CORM-2, change in ratio = 2.18 ± 0.18 ; CORM-2 + U73122, change in ratio = 0.45 ± 0.23 ; $p < 0.05$, $n = 4$; Fig. 1D).

To study the involvement of different Ca^{2+} entry pathways in CORM-2-mediated calcium increase, two calcium entry blockers, N-[4-[3, 5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-3-fluoro-4-pyridinecarboxamide (Pyr6) and 2-Aminoethoxydiphenyl borate (2-

APB), were used. Pyr6 is a novel inhibitor of SOCE, which exhibited 37-fold higher inhibition of Orai1-mediated Ca^{2+} entry versus TRPC3-mediated Ca^{2+} entry [34]. 2-APB is widely used as an inhibitor of voltage-independent Ca^{2+} entry including SOCE [35] and TRP channel-mediated calcium entry [36]. In addition, the IP_3 receptor-mediated calcium release can also be suppressed by 2-APB [37]. Figure 1E shows the percent inhibition of the CORM-2 induced Fura-2 ratio by Pyr6 and 2-APB. The cells were stimulated with CORM-2 (100 μM) and Pyr6 (10 μM) and/or 2-APB (10 μM) was added once the CORM-2-induced calcium levels had reached the maximum level. When Pyr6 (10 μM) was added at the peak of the CORM-2-induced Ca^{2+} increase, it suppressed $34.0\% \pm 8.6\%$ (before Pyr6, change in ratio = 2.06 ± 0.20 , $n = 5$; after Pyr6, change in ratio = 1.43 ± 0.13 , $n = 3$) of the maximal calcium response (Fig. 1E). However, 2-APB alone largely reduced the CORM-2-induced Ca^{2+} increase (after 2-APB, change in ratio = 0.78 ± 0.07 , $n = 5$; % inhibition = $61.6\% \pm 6.0\%$). The % inhibition increased to $82.7\% \pm 1.6\%$ (after Pyr6 + 2-APB, change in ratio = 0.28 ± 0.06 , $n = 3$) when both Pyr6 and 2-APB were present. Taken together, these results indicate that CORM-2-induced calcium signaling involves the activation of the PLC/ IP_3 pathway and the involvement of the Pyr-6- or 2-APB-sensitive calcium entry pathway.

CORM-2 induced IP_3 production

Because the PLC inhibitor abolished the calcium increase induced by CORM-2, it is likely that CORM-2 could induce IP_3 production via the PLC/ IP_3 signaling cascade. To examine the activity of PLC, 16HBE14o- cells were stimulated with different concentrations of CORM-2 for 1 hr and IP_1 , a non-degradable stable product of IP_3 , was measured. Our results indicated that CORM-2 induced IP_1 formation in a concentration-dependent manner with an EC_{50} of 9.98 μM (Fig. 2A). For the positive control experiment, activation of $\text{P2Y}_2/\text{P2Y}_4$ receptor by UTP caused an increase in IP_1 formation, which could be inhibited by U73122 (Fig. 2B). Similarly, U73122 abolished IP_1 accumulation induced by CORM-2 (Fig. 2B), indicating that CORM-2 could activate the PLC/ IP_3 signaling cascade. iCORM-2 has no significant effect on IP_1 formation.

CORM-2 suppressed UTP-induced calcium increase

To test whether CORM-2 has an effect on G protein-coupled receptor-mediated Ca^{2+} signaling, the cells were stimulated with UTP (a $\text{P2Y}_2/\text{P2Y}_4$ receptor agonist) in the absence or presence of 30 μM CORM-2 (Fig. 3A). At this concentration, CORM-2 did not induce a significant increase in $[\text{Ca}^{2+}]_i$ as shown in Fig. 1A. CORM-2 suppressed the calcium increase induced by 5 μM UTP in a concentration-dependent manner, with an IC_{50} of 11.45 μM (Fig. 3B). We further studied whether CORM-2 inhibited the P2Y receptor-mediated calcium release and/or influx component in 16HBE14o- cells using an extracellular Ca^{2+} removal/re-addition protocol as previously described [38]. The control experimental protocol involved stimulating the cells with UTP in a Ca^{2+} -free solution to deplete the internal Ca^{2+} stores, after

Fig. 2. CORM-2 induced IP_1 formation depended on PLC activation. (A) IP_1 production was stimulated by different concentrations of CORM-2 for 1 hr. Each data point represents the mean $\pm$ S.E. for three to five experiments. (B) Summarized data showing the effect of U73122 (10 μM) on UTP- or CORM-2-stimulated IP_1 production. Each column represents the mean $\pm$ S.E. ($n = 3 - 5$; * $p < 0.05$ compared with control, One-way ANOVA with Dunnett's post hoc test).

which 2.0 mM Ca^{2+} was added to the perfusion solution to detect the Ca^{2+} influx (Fig. 3C). In another series of experiments, the same protocol was applied but the cells were perfused with different concentrations of CORM-2, as indicated, to determine its effect on UTP-induced calcium release and influx component. These results demonstrate that both calcium release (Fig. 3D) and influx (Fig. 3E) could be inhibited by CORM-2 in a concentration-dependent manner with an IC_{50} of 9.39 and 7.75 μM , respectively.

The inhibitory effect of CORM-2 on UTP-induced calcium influx was not due to the decreased calcium release

Although it is clear that both the calcium release and influx induced by UTP were inhibited by CORM-2, it is possible that the suppressed calcium influx was due to impaired calcium release and retention of Ca^{2+} in calcium stores. Therefore, a similar protocol as described in Fig. 3C was used, but CORM-2 was added after the UTP-induced calcium release from

Fig. 3. CORM-2 suppressed UTP-induced calcium increase. (A) 16HBE14o- cells were stimulated with UTP (5 μM) in the presence or absence of CORM-2 (30 μM). The arrow indicates where UTP was added ($n = 3-4$). (B) Summarized data showing the UTP-induced increases in $[\text{Ca}^{2+}]_i$ were inhibited by different concentrations of CORM-2. Each data point represents the mean $\pm$ S.E. ($n = 3-4$). (C) Representative recordings of Fura-2 fluorescence ratios showing the calcium release and calcium influx induced by UTP in the absence or presence of CORM-2. 16HBE14o- cells were superfused with nominally Ca^{2+} -free KH solution and then stimulated with UTP. Once $[\text{Ca}^{2+}]_i$ returned to basal levels, external Ca^{2+} was subsequently restored (2 mM) ($n = 5-7$). Summarized data demonstrate CORM-2 concentration-dependently inhibited both calcium release (D) and calcium influx (E). Each data point represents the mean $\pm$ S.E. for 5-7 experiments.

Fig. 4. The inhibitory effect of CORM-2 on calcium influx induced by UTP was calcium release-independent. (A) Representative recordings of Fura-2 fluorescence ratios showing the UTP-induced

calcium influx in the absence or presence of CORM-2 (30 μ M). A Ca^{2+} removal/addition protocol similar to that described in Fig. 3. was used. However, CORM-2 was added after the UTP-induced Ca^{2+} -release reached its peak ($n = 4$). (B) Summarized data showing the UTP-induced calcium influx was inhibited by different concentrations of CORM-2. Each data point represents the mean $\pm$ S.E. ($n = 4$).

internal stores. Fig. 4A shows that CORM-2 (30 μ M) was still capable of inhibiting calcium influx ($33.4\% \pm 14.9\%$ of control) despite no significant change in the calcium release component (control, change in ratio = 1.12 ± 0.13 ; CORM-2, change in ratio = 1.09 ± 0.12 ; $p > 0.05$, $n = 5$) upon UTP stimulation. In the absence of CORM-2 (control), the UTP-induced increase in the Fura-2 ratio associated with the calcium influx was 1.09 ± 0.16 . Fig. 4B shows that this inhibitory effect of CORM-2 on the UTP-induced calcium influx was concentration-dependent with an IC_{50} of 12.41 μ M (Fig. 4B).

CORM-2 had no inhibitory effect on SOCE

SOCE is assumed to be a major ubiquitous pathway for calcium entry in non-excitable cells, such as epithelial cells [26]. Thapsigargin (Tg), a specific ER Ca^{2+} -ATPase inhibitor [39], was used as a pharmacological tool to deplete the internal calcium store and to bypass the receptors and the subsequent signaling cascade to activate SOCE. In the absence of extracellular Ca^{2+} , Tg (1 μ M) induced the emptying of intracellular Ca^{2+} stores, and a calcium influx occurred upon restoring extracellular Ca^{2+} in the perfusate (Fig. 5A and C). We examined the effects of CORM-2 on SOCE by incubating the cells with CORM-2 throughout the entire experimental period (Fig. 5A) or after calcium stores had been depleted by Tg (Fig. 5C). CORM-2 did not affect calcium released by Tg (Fig. 5B). Calcium entry after store depletion was not significantly different between control and CORM-2-treated cells (Fig. 5B and D) in either protocol. Together with previous data, these results suggest that the P2Y receptor-induced calcium entry pathway inhibited by CORM-2 is different from the SOCE activated by Tg.

UTP-induced calcium influx was insensitive to SOCE inhibitor

To distinguish between SOCE and UTP-induced ROCE, Pyr6 and 2-APB were used to differentiate the two different calcium influx pathways. The cells were stimulated with UTP in Ca^{2+} -free solution to deplete the intracellular Ca^{2+} stores, followed by the addition of Ca^{2+} back into the perfusion solution in the continued presence of UTP. The non-specific calcium entry blocker, 2-APB, almost completely suppressed the P2Y receptor-mediated calcium entry (Fig. 6A, red trace) when compared to the control cells (black trace). The addition of 2 mM calcium to the perfusion solution induced only a minimal increase in calcium entry (control: change in ratio = 0.72 ± 0.09 , $n = 7$; + 2-APB, change in ratio = 0.07 ± 0.06 , $n = 3$, $p < 0.05$; % inhibition = $90.8\% \pm 6.1\%$). In contrast, Fig. 6B shows that the SOCE specific inhibitor, Pyr6, had no significant effect on the ROCE activated by UTP (+ Pyr6: change in ratio = 0.67 ± 0.22 , $n = 4$, $p > 0.05$). On the other hand, the SOCE activated by Tg was sensitive to both 2-APB (Fig. 6C) and Pyr 6 (Fig. 6D). The addition of 2-APB or Pyr6 to the cells when the Tg-induced calcium influx had reached a plateau caused a rapid decline in the Fura-2 ratio towards the

Fig. 5. CORM-2 did not suppress the Tg-induced calcium release and influx. Representative recordings of Fura-2 fluorescence ratios showing the effect of CORM-2 (100 μ M), either treated before (A) or after (C) the cells were stimulated with 1 μ M Tg. Summarized data showing the effects of CORM-2 on Tg-induced calcium release and influx (B) or influx alone (D). Each column represents the mean $\pm$ S.E. ($n = 4-8$; Student's t-test with Welch's correction).

Fig. 6. Different sensitivity of the UTP- and Tg-induced calcium influx to Pyr6 and 2-APB. A Ca^{2+} removal/addition protocol similar to that described in Fig. 3. was used. Representative recordings of Fura-2 fluorescence ratios showing the effects of 2-APB (A, C) or Pyr6 (B, D) on UTP (A, B) or Tg (C, D)-induced calcium influx (red trace) versus control without the addition of Pyr6 or 2-APB (black trace). Similar results were obtained for at least three to eight replicates.

pre-stimulated level. When compared with the Fura-2 ratio at the same time point when the calcium levels in the control cells reached a steady level, the percent inhibition of 2-APB and Pyr6 on the Tg-induced calcium influx was $83.4\% \pm 6.6\%$ (control; change in ratio = 2.41 ± 0.05 , $n = 8$; + 2-APB, change in ratio = 0.33 ± 0.18 , $n = 5$, $p < 0.05$) and $74.2\% \pm 7.7\%$ (+ Pyr6, change in ratio = 0.47 ± 0.14 , $n = 3$, $p < 0.05$), respectively. The data clearly demonstrate that UTP-induced calcium influx was different from typical SOCE. To examine whether UTP can affect the Tg-sensitive calcium stores, Tg was added after the UTP-induced calcium increase returned to basal level (Fig. 7A). An increase in calcium was observed, and the ratio change of Tg-induced calcium increase was not statistically different from that of the control (in the absence of UTP; Fig. 7B). These data indicate that 5 μ M UTP was not sufficient to deplete the Tg-sensitive intracellular calcium stores.

Fig. 7. UTP did not deplete the Tg-sensitive calcium stores. (A) Representative recording of Fura-2 fluorescence ratio showing the effect of Tg-induced calcium increase after the intracellular stores were released by UTP under Ca^{2+} -free condition. (B) Summarized data comparing the calcium increase induced

by Tg in the presence or absence of UTP. Each column represents the mean $\pm$ S.E. ($n = 4-6$; Student's t-test with Welch's correction).

Fig. 8. The inhibitory effect of CORM-2 on UTP-induced calcium increase was dependent on sGC/PKG pathway. (A) Representative recordings of Fura-2 fluorescence ratios showing the effects of 30 μM CORM-2 (blue trace), ODQ with CORM-2 (red trace) and KT5823 with CORM-2 (green trace) on UTP-induced calcium increase. The arrow indicates where UTP was added. (B) Summarized data showing the effects of ODQ or KT5823 on the inhibitory effect of CORM-2. (C) Effect of CORM-2 on PKG activity. Each column represents the mean $\pm$ S.E. ($n = 4-5$; * $p < 0.05$ compared with control, one-way ANOVA with Dunnett's post hoc test).

The inhibitory effect of CORM-2 on P2Y receptor-mediated calcium increase was sGC/PKG dependent

Carbon monoxide is known to activate the sGC/PKG pathway [1]. Therefore, we examined whether sGC/PKG is involved in the inhibitory effect of CORM-2 on P2Y receptor-mediated calcium signaling. Fig. 8A shows that CORM-2 suppressed UTP-induced calcium increase significantly (blue trace). However, this inhibitory effect was attenuated by 100 μM ODQ, a sGC inhibitor (red trace), or 5 μM KT5823, a PKG inhibitor (green trace). In the presence of CORM-2, UTP-induced calcium increase was only $34.5\% \pm 0.7\%$ compared to that of the control, and this percentage increased to $70.6\% \pm 6.4\%$ and $77.5\% \pm 7.7\%$ when ODQ or KT5823 was present, respectively (Fig. 8B). Treatment with CORM-2 led to increased PKG activity (1.47 ± 0.08 – fold vs. control), and this effect could be blocked by KT5823 (1.13 ± 0.04 – fold vs. control). The CORM-2 negative control, RuCl_3 , had no effect on PKG activity (Fig. 8C).

CORM-2 and UTP did not have any additive effect on IP_3 formation

Because UTP-induced calcium increase was dependent on the PLC/ IP_3 signaling pathway and because CORM-2 could also induce IP_1 formation, we examined whether UTP and CORM-

Fig. 9. Effect of CORM-2 on UTP-induced IP_1 accumulation. CORM-2 and UTP did not have any additive effect on IP_1 accumulation. Each column represents the mean $\pm$ S.E. ($n = 3-5$; One-way ANOVA with Dunnett's post hoc test).

2 had an additive effect on the formation of IP_1 . Fig. 9 shows that UTP (10 μ M) or CORM-2 (10 μ M) alone, induced an increase in IP_1 formation. However, in the presence of both CORM-2 (10 μ M) and UTP (10 μ M), IP_1 formation did not differ significantly from that of UTP or CORM-2 alone. Similarly, the IP_1 formation induced by CORM-2 (100 μ M) and UTP (10 μ M) was not statistically different from that of CORM-2 alone. These data suggest that there was no additive effect of the combination of both CORM-2 and UTP on IP_1 formation, suggesting that CORM-2 and UTP may share a common pathway to induce IP_3 formation.

Discussion

This study demonstrated, for the first time, the regulatory effect of CO released by CORM-2 on calcium homeostasis in 16HBE14o- human bronchial epithelial cells. Although we did not directly measure the actual CO concentration in the solution bathing the cells, Motterlini et al. performed a series of elegant experiments to demonstrate that the CORMs, including CORM-2, rapidly liberate CO to elicit vasodilatory effects in rat aortic rings by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin spectrophotometrically [12]. In this study, at higher concentrations (e.g., 100 μ M), CORM-2 could induce a slow, yet long-lasting, calcium increase in human bronchial epithelial cells. This effect appeared to be dependent on the CO released by CORM-2 because iCORM-2, which did not liberate CO, had no effect on the calcium levels. The time course is similar to what was previously observed in rat pancreatic acinar cells when 100 or 300 μ M of CORM-2 was applied to the cells [31]. However, in pancreatic cells, the $[Ca^{2+}]_i$ changes were found to be oscillatory at medium concentrations (0.4 – 0.5 mM) in the extracellular solution, and monophasic (1 mM) at the highest concentration, which is typical of pancreatic acini cells when stimulated with physiological secretagogues such as CCK-8 or acetylcholine [31]. The fact that calcium increase induced by CORM-2 could be abolished in cells superfused with Ca^{2+} -free KH solution or by pretreatment of the cells with PLC inhibitor U73122, suggests a joint participation of both intracellular calcium release and extracellular calcium entry. This is different from pancreatic cells in which the increase in $[Ca^{2+}]_i$ was predominately due to PLC/ IP_3 -triggered intracellular Ca^{2+} mobilization [31]. CORM-2 could induce IP_1 accumulation in a concentration-dependent manner, and it is interesting to note that 10 μ M CORM-2 almost induced the same amount of IP_1 production as that of 10 μ M UTP, indicating that CO released by CORM-2 may be an effective PLC activator as compared to the activation of a G protein-coupled receptor. Because activation of the PLC/ IP_3 pathway leads to intracellular calcium release, intracellular calcium stores of 16HBE14o- cells might be, at least partially, depleted upon CORM-2 treatment resulting in the activation of SOCE. Consistent with this hypothesis, both 2-APB and Pyr6 suppressed CORM-2-induced calcium increase (Fig. 1E). Pyr6 only partially suppressed the calcium influx, and the rest may be due to other calcium entry pathways, such as PLC/diacylglycerol (DAG) activated calcium influx [40, 41].

Although 100 μ M CORM-2 induced more IP_1 production than UTP, CORM-2 induced only a minor release of calcium in a calcium-free solution (Fig. 1C and D). One possibility is that CORM-2 induced the production of IP_3 in a relatively slow fashion and the newly produced

IP₃ was rapidly degraded. This is consistent with our findings of the CORM-2-induced calcium increase, in which more time was required to reach peak levels. Because the assay only allowed for the end-point measurement of IP₁ accumulation within a fixed time interval, it was not possible to describe the localization and dynamic changes of IP₃ levels in different subdomains within the cells. Therefore, it was not possible to correlate the changes in [IP₁] to that of intracellular Ca²⁺ release or subsequent changes in [Ca²⁺]_i. It is interesting to note that a recent report from Charles et al. demonstrated that real-time increases in IP₃ levels could be monitored using confocal microscopy in HeLa cells transfected with a fusion protein made of the Pleckstrin Homology (PH) domain of PLCδ and enhanced green fluorescent protein (eGFP) [42]. The fusion protein binds to phosphatidylinositol 4, 5-bisphosphate (PIP₂) and IP₃ and allows the monitoring of IP₃ localization after PIP₂ cleavage regardless of the PLC isoform [42]. Future studies involving this advanced approach will be required to unravel the underlying mechanisms.

This study also revealed an inhibitory effect of CORM-2 on P2Y receptor agonist-induced calcium signaling. As shown in Fig. 3 and 4, CORM-2 inhibited both calcium release and influx induced by UTP in a concentration-dependent manner. The inhibitory effect on calcium release was probably due to the fact that both agents share a common PLC pathway to induce IP₃ formation. CORM-2 also exerted a direct inhibitory effect on UTP-induced calcium influx, which was due to sGC/PKG signaling activated by CORM-2. As shown in Fig. 8, inhibition of sGC or PKG reversed the inhibitory action of CORM-2 on UTP-induced calcium signaling. The cGMP signaling pathway is known to participate in vasodilation, which can be blocked by ODQ or KT5823 [43]. Similar to nitric oxide, CO is considered a major component in the regulation of cardiovascular circulation [44] via activation of the cGMP signaling pathway [45, 46]. Together with these reports, our data further confirm that sGC, which localizes to the cell surface, is one of the proximal targets for CO [11].

Because the UTP-mediated Ca²⁺ influx was not sensitive to Pyr-6 (Fig. 6B), it is likely that CORM-2 could inhibit other calcium entry pathways, such as ROCE, which is activated by the P2Y receptor. Although it has been reported that CORM-2 inhibits L-type [47] and T-type [30] Ca²⁺ channels, there is currently no evidence that 16HBE14o- cells express these two types of voltage-gated Ca²⁺ channels.

The finding that the activation of SOCE by Tg was not affected by 100 μM CORM-2 (Fig. 5) was unexpected. In contrast, it has been shown that CO inhibits the capacitative calcium entry induced by Tg in human platelets [48]. CO also inhibits the calcium influx induced by ADP and thrombin, although it was not known whether the Ca²⁺ entry was mediated through SOCE or ROCE [48]. On the contrary, CO released by CORM-2 enhanced the Tg-induced capacitative calcium entry in SH-SY5Y cells, a human neuroblastoma cell line, possibly by inhibiting Ca²⁺ extrusion via plasma membrane Ca²⁺-ATPase through a nitric oxide-dependent increase in reactive oxygen species [49]. Therefore, CO causes diverse modulatory effects on the calcium entry pathway and the effect is probably cell-type specific.

To demonstrate the difference between SOCE and ROCE, 2-APB and Pyr6 were used. In the present study, Pyr6 only suppressed SOCE elicited by Tg, whereas 2-APB inhibited both the Tg- and UTP- induced calcium entry (Fig. 6). Activation of P2Y receptors by UTP would also deplete intracellular calcium stores by activating IP₃/calcium signaling, leading to the activation of SOCE. However, our data suggest that 5 μM UTP did not significantly deplete the Tg-sensitive calcium store, which is not sufficient to activate SOCE. As shown in Fig. 7, the ratio change of Tg-induced SOCE was not significantly different in the control cells or when the cells were previously treated with UTP. Moreover, the P2Y receptor-mediated calcium influx component only occurred in the continuous presence of UTP (data not shown), suggesting that store depletion alone might not be sufficient to activate calcium entry. The second possibility is that the UTP-releasable Ca²⁺ stores do not overlap with Tg-releasable calcium stores. Therefore, UTP was not able to activate SOCE as Tg. However, we have demonstrated previously that 10 μM ATP (P2Y₁/P2Y₂ agonist) can stimulate the oligomerization and translocation of stromal interaction molecule 1 (STIM1) near the plasma membrane [32]. Also, 10 μM ATP induced a significant increase in STIM1 puncta formation in

16HBE14o- cells [32]. Therefore, the most likely explanation is that UTP activates a calcium entry pathway that is truly distinct from SOCE. However, the detailed molecular mechanism underlying the inability of UTP to induce Pyr6-inhibitable SOCE remains unresolved.

Some studies suggest that TRP cation channels formed ROCE [50, 51]. Among the TRP channels, the TRPC family was indeed a perfect candidate to form ROCE; TRPC2, 3, 6, and 7 can be activated by DAG, a potent lipid messenger produced from PIP₂ by PLC activation, and activation of TRPC4 and 5 was PLC-dependent [52]. In mouse airway epithelial cells, TRPV4 participated in ROCE instead of SOCE [27], further indicating the possible involvement of TRP channels in ROCE. However, reports on the effects of CO on TRP channels are scarce. The molecular identity of ROCE activated by UTP remains unknown and requires further investigation.

In the future, it will be important to measure the real-time changes in CO levels inside living cells in the presence of CORMs and to correlate CO levels with their biological activities, although this still remains as an ongoing challenge. It has been reported that cellular CO levels could be measured using a selective fluorescent probe [53] or a genetically-encoded fluorescent probe containing a CO-sensing heme protein from *Rhodospirillum rubrum* [54].

Conclusion

CORM-2 had dual effects on intracellular calcium regulation in 16HBE14o- cells. At higher concentrations, CORM-2 stimulated the PLC/IP₃/calcium pathway to increase [Ca²⁺]_i, whereas at lower concentrations, CORM-2 inhibited calcium signaling induced by P2Y receptor agonists in an sGC/PKG-dependent manner. P2Y receptor agonists stimulated the release of two important pro-inflammatory cytokines, IL-6 and IL-8, and this process was calcium-dependent [7]. Therefore, CO might exert its anti-inflammatory effect in human bronchial epithelial cells by regulating the P2Y receptor-mediated calcium-signaling pathway. Thus, CO might act as an important regulator of calcium homeostasis in human airway epithelia.

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Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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