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RESEARCH PAPER

Carbon monoxide is a rapid modulator of recombinant and native $P2X_2$ ligand-gated ion channels

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Background and purpose: Carbon monoxide (CO) is a potent modulator of a wide variety of physiological processes, including sensory signal transduction. Many afferent sensory pathways are dependent upon purinergic neurotransmission, but direct modulation of the P2X purinoceptors by this important, endogenously produced gas has never been investigated.
Experimental approach: Whole-cell patch-clamp experiments were used to measure ATP-elicited currents in human embryonic kidney 293 cells heterologously expressing $P2X_2$, $P2X_3$, $P2X_{2/3}$ and $P2X_4$ receptors and in rat pheochromocytoma (PC12) cells known to express native $P2X_2$ receptors. Modulation was investigated using solutions containing CO gas and the CO donor molecule, tricarbonyldichlororuthenium (II) dimer (CORM-2).
Key results: CO was a potent and selective modulator of native $P2X_2$ receptors, and these effects were mimicked by a CO donor (CORM-2). Neither pre-incubation with 8-bromoguanosine-3′,5′-cyclomonophosphate nor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (a potent blocker of soluble guanylyl cyclase) affected the ability of the CO donor to enhance the ATP-evoked $P2X_2$ currents. The CO donor caused a small, but significant inhibition of currents evoked by $P2X_{2/3}$ and $P2X_4$ receptors, but was without effect on $P2X_3$ receptors.
Conclusions and implications: These data provided an explanation for how CO might regulate sensory neuronal traffic in physiological reflexes such as systemic oxygen sensing but also showed that CO could be used as a selective pharmacological tool to assess the involvement of homomeric $P2X_2$ receptors in physiological systems.
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Abbreviations: 8Br-cGMP, 8-bromoguanosine-3′,5′-cyclomonophosphate; CORM-2, tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$); DMSO, dimethyl sulphoxide; HEK, human embryonic kidney 293; αβmeATP, α-,β-methyleneATP; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PC12, rat pheochromocytoma cells; Product control, $RuCl_2(DMSO)_4$; sGC, soluble guanylyl cyclase

Introduction

Physiologically, carbon monoxide (CO) and biliverdin are generated within cells from the hydrolysis of haem. This reaction, which has the strict requirement for molecular oxygen (O_2) and nicotinamide adenine dinucleotide phosphate, is catalysed by at least one of the three known haem oxygenase enzymes (see Maines and Gibbs, 2005). CO and biliverdin, which is rapidly metabolized to bilirubin by biliverdin reductase, are potent modulators of a diverse array of physiological processes (see Ryter *et al.*, 2006; Mann and Motterlini, 2007) including, for example, transcriptional activation (Shelver *et al.*, 1997), cardiovascular function (Wang *et al.*, 1997; Jaggar *et al.*, 2002; Motterlini *et al.*, 2002), O_2-sensing (Prabhakar *et al.*, 1995; Williams *et al.*, 2004) and neuronal modulation (Baranano and Snyder, 2001). Central to many of these processes, especially to the O_2-sensing by the carotid body and other peripheral sensory transduction mechanisms, is the activation of purinergic ionotropic P2X receptors (see Khakh and North, 2006; Burnstock, 2007).

P2X receptors (nomenclature follows Alexander *et al.*, 2008) are ligand-gated ion channels which are opened by the binding of extracellular ATP (Brake *et al.*, 1994; Valera *et al.*, 1994). Thus far, seven individual mammalian and several other non-mammalian (Agboh *et al.*, 2004; Fountain *et al.*, 2007) receptor subunits have been cloned. P2X receptor subunits are able to form both homo- and hetero-trimers (Lewis *et al.*, 1995). $P2X_2$ and $P2X_3$ receptors are often co-expressed,

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where they are also able to form heteromeric $P2X_{2/3}$ receptors (Lewis *et al.*, 1995). Recent studies on mice deficient in $P2X_2$ and $P2X_3$ subunits have highlighted the important role that these receptors play in signal transduction in many sensory reflexes such as O_2-sensing in the carotid body (Rong *et al.*, 2003), detection of bladder distension (Cockayne *et al.*, 2005) and taste (Finger *et al.*, 2005). However, distinguishing between the involvement of $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors in physiological studies is difficult in genetically unmodified organisms. In electrophysiological studies of native cells *in vitro*, the situation is slightly improved, because the ATP analogue α,β-methyleneATP (αβmeATP) can be used to activate both $P2X_3$ and $P2X_{2/3}$ receptors (Lewis *et al.*, 1995) and distinguishing between these two receptor assemblies is then possible on the basis of their desensitization rates. However, the involvement of $P2X_2$ homomeric receptors can only be differentiated from that of $P2X_{2/3}$ receptors by the use of non-specific pharmacological tools such as trinitrophenyl-ATP (Gever *et al.*, 2006).

Here, we demonstrate that CO, a well-established gas transmitter/second messenger is a potent activator of $P2X_2$ currents, is a modest inhibitor of $P2X_{2/3}$ and $P2X_4$ currents, and has no effect on $P2X_3$ currents. This observation represents the first time that an endogenously produced gas has been shown to regulate P2X receptors. It also provides a mechanism to explain how CO may regulate purinergic sensory transduction via direct modulation of $P2X_2$ receptors and a possible basis for identifying the involvement of homomeric $P2X_2$ receptors in physiology.

Methods

Expression systems

Four stable human embryonic kidney 293 (HEK) cell lines were used in this study (kindly provided by Prof. RA North, The University of Manchester, UK), each expressing different purinergic P2X receptors. The lines expressed either: (i) the homomeric rat $P2X_2$ ($P2X_2$) receptor, originally cloned from rat pheochromocytoma (PC12) cells (Brake *et al.*, 1994); (ii) the homomeric human $P2X_3$ ($P2X_3$) receptor, originally cloned from urinary bladder smooth muscle (Valera *et al.*, 1995); (iii) rat $P2X_2$ and $P2X_3$ receptors, co-expressed using a bicistronic vector, thus giving rise to heteromeric rat $P2X_{2/3}$ ($P2X_{2/3}$) receptor channels (Lewis *et al.*, 1995; Kawashima *et al.*, 1998); or (iv) the homomeric human $P2X_4$ ($P2X_4$) receptor, originally cloned from human brain (Garcia-Guzman *et al.*, 1997). All lines were maintained in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin G, 100 $\mu g \cdot mL^{-1}$ streptomycin, 250 $ng \cdot mL^{-1}$ amphotericin B and 150 $\mu g \cdot mL^{-1}$ Geneticin (all purchased from Invitrogen, Paisley, Strathclyde, UK) in a humidified incubator gassed with 5% CO_2/95% air. Cells were passaged every 3–4 days using 2.5 $g \cdot L^{-1}$ trypsin dissolved in Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (Sigma-Aldrich, Poole, Dorset, UK). For electrophysiological experiments, stably transfected HEK cells were plated onto glass coverslips and cultured at 37°C for 18–36 h before recording.

PC12 cells were grown in suspension culture in Roswell Park Memorial Institute medium, supplemented with 5% fetal calf serum, 10% horse serum, 2 mM L-glutamine, 10 $U \cdot mL^{-1}$ penicillin G and 10 $U \cdot mL^{-1}$ streptomycin, in a humidified incubator gassed with 5% CO_2/95% air. Cells were passaged every 3–4 days. For electrophysiological experiments, PC12 cells were plated onto poly-L-lysine (100 $\mu g \cdot mL^{-1}$) coated glass coverslips and cultured at 37°C for 18–36 h before recording.

Electrophysiology

Whole-cell patch-clamp recordings were made at ambient room temperature. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) and had resistances of 4–6 MΩ when filled with a pipette solution which contained (in mM) 117 KCl, 10 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 2 Na_2.ATP and 11 HEPES, with the pH adjusted to 7.2 with KOH. The bath solution contained (in mM) 135 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 5 HEPES and 10 glucose with the pH adjusted to 7.4 with NaOH. Stocks of ATP, α-,β-methyleneATP (αβmeATP), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 8-bromoguanosine-3′,5′-cyclomonophosphate (8Br-cGMP) (all from Sigma-Aldrich, Poole, Dorset, UK) were dissolved daily in bath solution to the concentrations defined in the text and figure legends.

Current recordings were made at a holding potential of –60 mV using an Axon Instruments Axopatch Multiclamp 700A amplifier and Digidata 1322A A/D interface (Molecular Devices, Sunnyvale, CA, USA). Whole-cell capacitance was measured and compensated for using the Multiclamp auto compensation feature.

CO gas (99.5%) (BOC gases, Guilford, UK) was bubbled through the bath solution, under positive pressure for at least 2 h. This solution was assumed to be saturated with CO (0.93 mM) and was diluted as indicated before applied to cells. The saturated solution of CO had a pH of 7.45. Working solutions of CO gas were prepared freshly every 3 h. The well-characterized CO donor molecule, tricarbonyldichlororuthenium (II) dimer $[Ru(CO_3)Cl_2]_2$ – also known as CORM-2, (Sigma-Aldrich; Mann and Motterlini, 2007) was also used. This CO donor was made up at 100 mM in dimethyl sulphoxide (DMSO) and stored frozen for up to 1 month. Working solutions containing CORM-2 were made up daily in extracellular bath solution at the concentrations described in the text and figures. CORM-2 (30 μM) solution had a pH of 7.41. Another ruthenium compound, $RuCl_2(DMSO)_4$, was used as a control for CORM-2 and was synthesized as described in detail elsewhere (Williams *et al.*, 2004); this control was also made up daily and is called 'Product control' throughout the results. All compounds were applied to cells using a rapid perfusion system (RSC-160, Biologic, Claix, France). This system allowed solution exchange times in the range 20–100 ms.

Data analysis

Data are reported as means ± SEM values and analysed with the pCLAMP 9.0 suite of software. Statistical comparisons of means were made with Student's paired *t*-test. Concentration-response curves for ATP and for CORM-2 were each fitted with

the Hill equation using an iterative fitting routine in Microcal Origin 6.0. In all cases, the figures show mean ± SEM, although concentration-response curves were fitted to the whole data set.

Results

Solutions of 10 μM ATP containing 30% CO gas evoked significantly larger currents through recombinant $P2X_2$ receptors than 10 μM ATP alone. Co-application of 30% CO gas solutions resulted in an increase of currents in 9/11 cells from 1049 ± 219 to 1183 ± 240 pA ($P < 0.02$, $n = 9$), a mean increase to 123 ± 4% of control (Figure 1A,B); the kinetics of CO action on $P2X_2$ receptors could be observed by co-applying CO gas solutions within a long ATP application. Sequential co-application of ATP and CO solutions produced a mean increase in $P2X_2$ currents which was similar to that seen when ATP and CO were co-applied simultaneously (130 ± 12% $n = 5$). The largest increase that was observed is shown in Figure 1C, in order to illustrate clearly the rapidity of action of CO. The maximal effect of CO solutions was observed within 500 ms of its application. The wash out of the effect of CO gas solutions was similarly rapid with currents returning to pre-CO levels within 500 ms (Figure 1C).

A comparable effect was observed when the ruthenium-based CO donor, CORM-2 (Motterlini *et al.*, 2002) was applied. When co-applied within a long ATP application, CORM-2 also evoked a potentiation of currents. Compared with solutions of CO gas, the kinetics of CORM-2 were much slower, with maximal effects observed within about 8–10 s of application. Washout of the donor effect was also slow and indistinguishable from receptor desensitization (Figure 2A). The persistence of effect following washout encouraged us to measure the effects of pre-application of the CO donor immediately prior to ATP application. Figure 2B shows the effect of increasing CORM-2 pre-application times, and shows maximal potentiation around 10–12 s, similar to that observed when CORM-2 was co-applied. Pre-application of CO gas in the same way had no effect on ATP-evoked currents (data not shown), confirming the faster kinetic properties of CO gas compared with those of CORM-2. Pre-application for 10 s of 30 μM CORM-2, resulted in a significant increase in currents evoked by 10 μM ATP at $P2X_2$ receptors. Thus, a 10 s pre-application of 30 μM CORM-2 before stimulation with 10 μM ATP resulted in an increase in current density in 70/78 cells from 70.6 ± 9.48 pA·pF^{-1} to 104 ± 12.4 pA·pF^{-1} ($P < 0.001$, $n = 70$) which was reversible within 90 s, to 80.6 ± 11.5 pA·pF^{-1} ($P < 0.001$, $n = 70$) and reproducible (Figure 2C). Application of the CO donor also caused a secondary effect on $P2X_2$ receptors, namely, slowing the rate at which the ligand-gated ionic current returned to baseline when ATP was removed (shown in Figure 2D, left). This effect was observed at all stimulating ATP concentrations. Neither the potentiation of peak current nor the apparent change in ATP off-rate was observed with pre-application of 30 μM of the product control (Figure 2C,D, right).

Use of the CO donor, CORM-2, allowed more accurate quantification of CO concentration than the use of CO gas, and full concentration-response data were obtained using 10 s pre-application of CORM-2 and stimulation of $P2X_2$ with 10 μM ATP (Figure 3A). Significant augmentation of the ATP-evoked current was observed with 10–30 μM CORM-2, with maximal augmentation of 121 ± 5% at 30 μM CORM-2 ($n = 5$) and no potentiation observed with 300 μM CORM-2, at which point this CO donor molecule is no longer fully soluble. Fitting the Hill equation to data over the range 0.3–100 μM CORM-2 gave an apparent EC_{50} of 3.1 ± 0.6 μM and Hill coefficient of 1.8 ± 0.4 ($n = 3$). Using a maximal concentration of CORM-2 (30 μM), a concentration-response curve to ATP was also obtained. Figure 3B shows that 30 μM CORM-2 significantly augmented $P2X_2$ currents at sub-EC_{50} ATP concentrations (1–10 μM) ($P < 0.001$; $n \geq 12$ for each

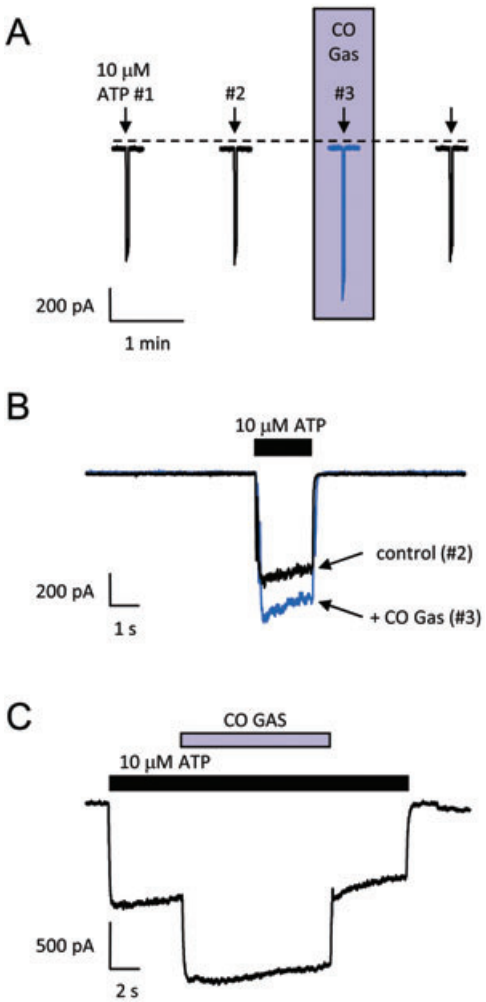


Figure 1 Enhancement by carbon monoxide (CO) of currents through recombinant $P2X_2$ receptors. (A) Typical continuous time-course of currents evoked by repeated exposure of $P2X_2$ receptors to 2 s pulses of 10 μM ATP or 10 μM ATP in a 30% CO saturated solution (shaded box). ATP applications were at 90 s intervals and are indicated by the downward arrows. In this and all subsequent figures, the human embryonic kidney 293 cells were voltage-clamped at –60 mV and the dotted lines represent the zero current levels. (B) Superimposed currents shown on a fast time-base taken from the continuous current record shown in (A) to illustrate the effects of 30% CO gas co-application with ATP (10 μM, 2 s – black bar). (C) Typical current trace evoked by 10 μM ATP where 90% saturated CO gas solution was co-applied during a 20 s exposure to 10 μM ATP. Application protocol is shown by the bars above the trace.

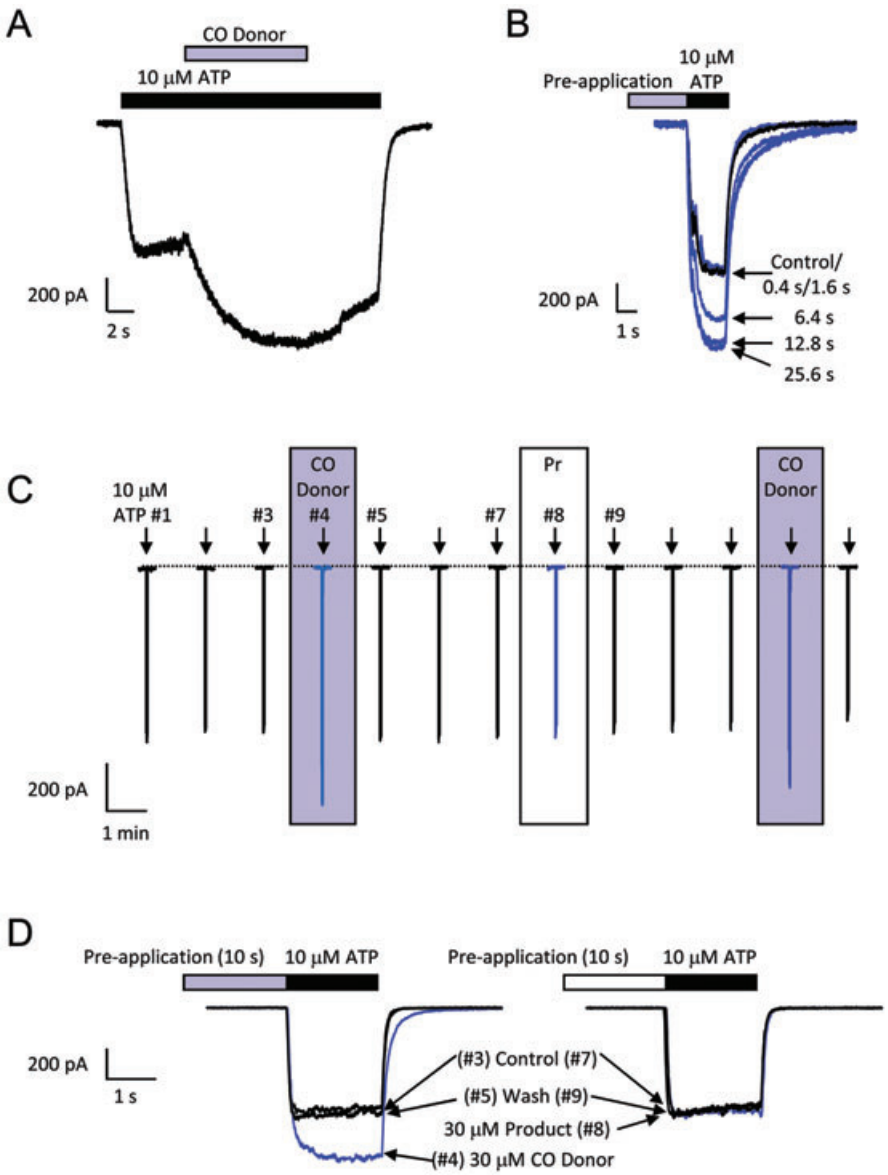


Figure 2 Enhancement by a carbon monoxide (CO) donor tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$) (CORM-2) of currents through recombinant $P2X_2$ receptors. (A) Typical current trace evoked by 10 μM ATP where 30 μM CORM-2 (CO donor) was co-applied during a 20 s exposure to ATP. Application protocol is shown by the bars above the trace. (B) Typical current showing effect of pre-application of CORM-2 (30 μM) for between 0.4 and 25.6 s before a 2 s exposure to 10 μM ATP. Application protocol is shown by bars above the traces; the CO donor was pre-applied for different durations as indicated. (C) Continuous time-course of currents evoked by repeated exposure of $P2X_2$ receptors to 2 s pulses of 10 μM ATP. ATP was applied at the points indicated by the arrows. Before each application of ATP, there was a 10 s pre-application of control solution, 30 μM CORM-2 (CO donor) or 30 μM Product control (Pr). ATP applications were 90 s apart. Scale bars apply to all traces within the panel. (D) Superimposed currents shown on a fast time-base taken from the continuous current record shown in (C) to illustrate the effects of pre-application of 30 μM CORM-2 (CO donor; left panel) or 30 μM Product control (Pr; right panel) before the 2 s application of 10 μM ATP. The current traces were recorded following 10 s pre-application of extracellular solution (Control, records #3 and #7), CO donor (30 μM CORM-2, record #4), Product control (30 μM Product, record #8) and extracellular solution following wash-out of CORM-2 or Product (Wash, records #5 and 9). Scale bar applies to both panels. Application details are shown by bars above the records.

concentration). At higher ATP concentrations (from 100–1000 μM), the CO donor caused a small, but significant ($P < 0.05$; $n \geq 7$ for each concentration) decrease in ATP-evoked currents (Figure 3B). As a consequence, the EC_{50} for ATP was only modestly, but significantly, changed by pre-application of the CO donor (EC_{50} in the absence of CORM-2 was 16.1 ± 1.9 μM whilst EC_{50} in the presence of CORM-2 was 12.5 ± 1.64 μM; $P < 0.01$; $n = 5$ separate, paired concentration-response curves – Figure 3C). Furthermore, the mean Hill coefficient was also significantly reduced from 1.79 ± 0.22 to 1.45 ± 0.2 ($P < 0.01$).

The effect of pre-incubation with CORM-2 was also examined at other P2X receptors stably expressed in HEK cells (Figure 4A–C). At all concentrations of ATP (10 μM shown in Figure 4A), a small, but significant inhibition of $P2X_4$ receptor-evoked currents was observed upon pre-incubation

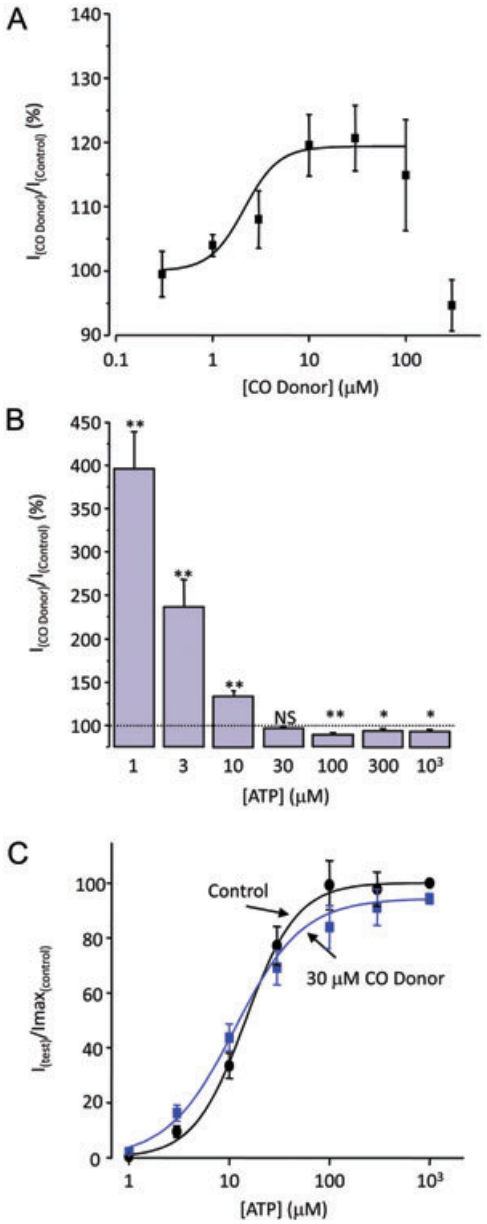


Figure 3 Concentration-response curves for carbon monoxide (CO) donor modulation of peak currents through $P2X_2$ receptors. (A) Mean (±SEM) concentration-response for the effects of tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$) (CORM-2) (CO donor) on currents evoked by 10 μM ATP. Enhancement or inhibition was expressed as a percentage and was calculated as ATP-evoked current following 10 s pre-application of CORM-2 [$I_{(CO\ Donor)}$] divided by the ATP-evoked current in the absence of CO donor pre-application [$I_{(control)}$] × 100. The Hill equation was fitted to enhancements observed, up to 100 μM CORM-2, in order to calculate an apparent EC_{50} (n = 6). (B) Bar graph of the mean (±SEM) enhancement/inhibition by 30 μM CORM-2 of currents evoked by 2 s pulses of ATP at the concentrations shown below each bar. Enhancement/inhibition was expressed as a percentage and was calculated as ATP-evoked current following 10 s pre-application of 30 μM CORM-2 [$I_{(CO\ Donor)}$] divided by the ATP-evoked current in the absence of CORM-2 pre-application [$I_{(control)}$] × 100. **P < 0.01, *P < 0.05, NS = not significant; current densities in presence versus absence of CORM-2 pre-application (n = 7–13 for each ATP concentration). (C) Mean (±SEM), paired concentration-response curves in the presence and absence of CORM-2 pre-application. The responses from each individual cell [$I_{(Test)}$], in the absence and presence of CO donor pre-incubation, were normalized such that the response to 1 mM ATP in the absence of the CO donor [$Imax_{(Control)}$] was considered to be 100% (n = 5).

of the CO donor for 10 s (peak current density evoked by 10 μM ATP was reduced from 6.51 ± 1.27 to 5.39 ± 1.26 pA·pF^{-1}, n = 9, P < 0.001). At $P2X_3$ receptors (Figure 4B), currents evoked by sub-EC_{50} ATP concentrations (0.1 or 0.3 μM) were not significantly different in the absence of or following the pre-application (10 s) of 30 μM CORM-2. For instance, at 0.3 μM, peak current following CORM-2 application was 99.6 ± 6.7% of control, n = 3, P > 0.1. Similarly, currents mediated by $P2X_{2/3}$ receptors co-expressed in another HEK stable cell line were not augmented by pre-application of CORM-2 (Figure 4C). Thus, at all concentrations of αβmeATP (0.3–300 μM; only 1 μM shown in Figure 4C), pre-application of 30 μM CORM-2 did not increase peak currents. Rather, there was a small but significant decrease; at 1 μM αβmeATP, peak current following CORM-2 was 82 ± 7% of control, n = 5, P < 0.02. In order to test the possibility that the lack of augmentation by CORM-2 of $P2X_{2/3}$ receptor currents was the result of using αβmeATP as the agonist, the same agonist was employed to stimulate $P2X_2$ receptors (Figure 4D). Using a sub-EC_{50} concentration of αβmeATP (300 μM), which activates $P2X_2$ receptors, the CO donor was still able to evoke a large and significant augmentation of $P2X_2$ currents to 284 ± 26% of control (P < 0.05; n = 8). This enhancement was similar in magnitude to that observed when low concentrations of ATP were employed (Figure 3B), which strengthens the notion that $P2X_{2/3}$ receptors are not enhanced by CO. Interestingly, although CORM-2 still enhanced the peak current that was evoked by αβmeATP at the $P2X_2$ receptor, it did not alter the apparent off-rate following the rapid removal of agonist (Figure 4D, right panels).

Importantly, the ability of CO to enhance $P2X_2$ currents was mirrored in cells which natively express $P2X_2$ receptors (PC12 cells – Figure 4E). Indeed, the pattern of modulation by the CO donor on these native $P2X_2$ receptors was remarkably similar to that observed using $P2X_2$ HEK cells. In 6/7 cells, 30 μM CORM-2 was able to enhance significantly the currents evoked by 10 μM ATP (to 160 ± 17%, P < 0.025, n = 6). Where full concentration-response curves could be generated, augmentation by CO was apparent at ATP concentrations up to and including 10 μM. At higher concentrations of ATP (30–1000 μM), modest inhibition or no effect was observed (see Figure 4E left panel; only 3–30 μM ATP ± CORM-2 is shown).

One well-defined mechanism by which CO may exert its actions is via the activation of soluble guanylyl cyclase (sGC) (Stone and Marletta, 1994). To test whether CO was exerting its effects on $P2X_2$ receptors via this mechanism, cells were treated with 100 μM 8Br-cGMP, a membrane-permeable guanosine-3′,5′-cyclic monophosphate (cGMP) derivative that would be expected to mimic effects of sGC activation. Bath application for 18 min had no effect on ATP-evoked currents, and did not affect the ability of the CO donor to augment the action of 10 μM ATP (Figure 5A, peak current 166 ± 19% of control, n = 3). Furthermore, following treatment with 8Br-cGMP, the augmentation of ATP-evoked

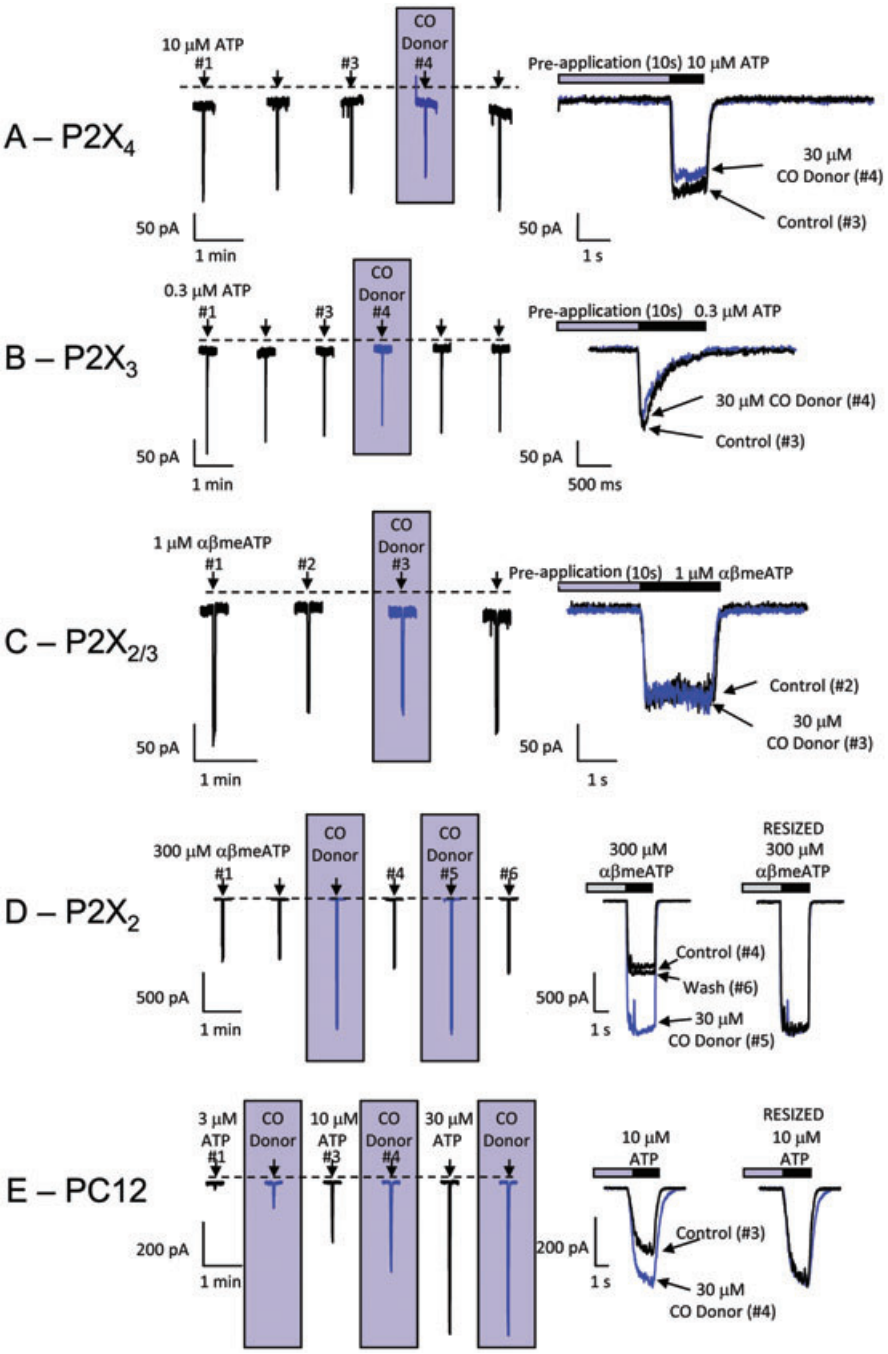
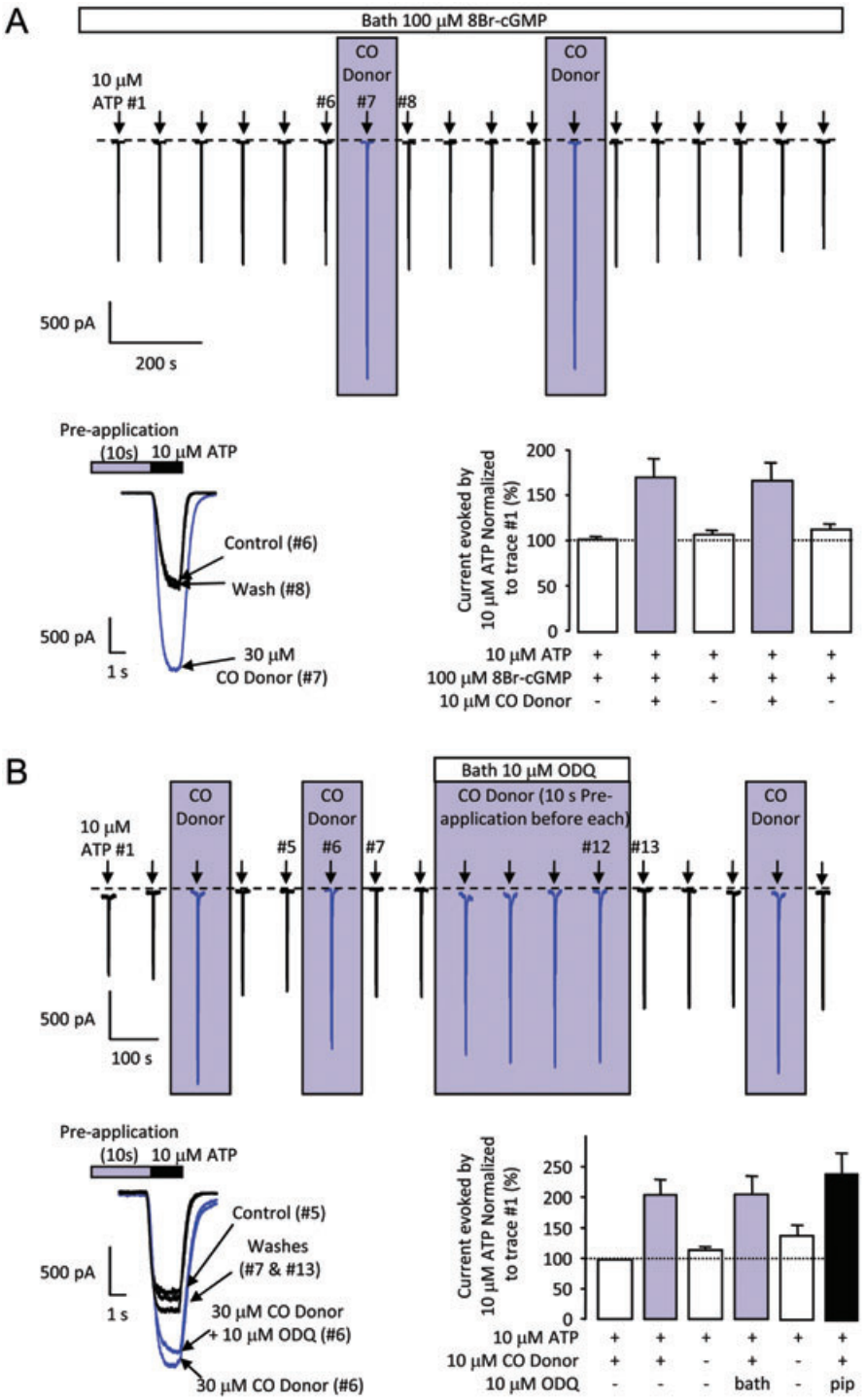


Figure 4 Receptor and agonist-specificity of modulation by carbon monoxide (CO) of currents through recombinant and native $P2X_2$ receptors. Typical traces, shown as continuous (left) and on fast time-base (right), currents recorded from human embryonic kidney 293 cells expressing $P2X_4$ (A) $P2X_3$ (B), $P2X_{2/3}$ (C), $P2X_2$ (D) receptors and PC12 cells which natively express $P2X_2$ receptors (E). Currents were evoked by 2 s pulses of 10 μM ATP (A) 0.3 μM ATP (B), 1 μM αβmeATP (C), 300 μM αβmeATP (D) and 3–30 μM ATP (E), as indicted by the bars above the traces. For each application of agonist, there was a 10 s pre-application of control solution or 30 μM tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$) (CO donor). Agonist applications were 90 s apart. In panels D and E, traces on the far right are rescaled to highlight the effect of the CO donor on the apparent off-rate. Scale bars apply only to the unscaled traces.

currents by CORM-2 was still reversible and reproducible. The effect of ODQ, a specific inhibitor of sGC (IC_{50} ~ 20 nM) (Garthwaite *et al*., 1995), was also examined (Figure 5B). ODQ (10 μM) applied either to the bath or included in the intracellular pipette solution did not diminish the ability of CORM-2 to enhance currents evoked by 10 μM ATP by 206 ± 29% (n = 5) and 236 ± 39% (n = 6) respectively.

Discussion and conclusions

This study shows that the gaseous transmitter and second messenger, CO, is a potent modulator of recombinant and native $P2X_2$ homomeric receptors. The modulation of $P2X_2$ peak current response by CO was: (i) rapid and reversible; (ii) dependent upon the agonist concentration; and (iii)



independent of the channel open state (Figures 1 and 2). Peak current augmentation by the CO donor, CORM-2, was observed only at sub-EC_{50} ATP concentrations, while at high ATP concentrations, CORM-2 was weakly inhibitory. The CO donor caused peak current augmentation in a dose-dependant manner with an apparent EC_{50} of approximately 3 μM, equating to 1.5–2 μM CO (Motterlini *et al.*, 2002). This makes $P2X_2$ receptors the most sensitive known target of reversible CO modulation.

In addition to its ability to augment peak $P2X_2$ currents at sub-EC_{50} agonist concentrations, CORM-2 evoked a slowing of the apparent off-rate following ATP removal (Figure 2D). This change in apparent off-rate occurred at all activating concentrations of ATP, even when CO did not augment peak current (data not shown), suggesting that these two actions of CORM-2 are independent. This notion is supported by observations that the CO donor did not alter the off-rate when αβmeATP was the agonist at $P2X_2$ receptors and is, therefore,

Figure 5 Effect of manipulating the carbon monoxide (CO)-soluble guanylate cyclase axis. (A) Typical continuous time-course of currents evoked by repeated exposure of $P2X_2$ receptors to 2 s pulses of 10 μM ATP. ATP was applied at the points indicated by the arrows (top of panel). 100 μM 8-bromoguanosine-3′,5′-cyclomonophosphate (8Br-cGMP) was applied continuously to the bath for 18 min before the first application of ATP shown and throughout the remainder of the experiment. For each application of ATP, there was a 10 s pre-application of control solution (no boxes) or 30 μM tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$) (CORM-2) (CO donor). ATP pulses were 90 s apart. Scale bars apply to all traces within the panel. In the lower left of the panel are shown three superimposed currents on a fast time-base taken from the continuous current record shown in the upper panel to exemplify the effects of pre-application of 30 μM CORM-2 in the presence of 100 μM 8Br-cGMP. The current traces were recorded following pre-application of extracellular solution (Control, record #6), CORM-2 (30 μM CO donor, record #7) and extracellular solution following wash-out of CORM-2 (Wash, record #8). Application details are shown by the bars above the records. The bar graph in the lower right of this panel shows the mean (±SEM) enhancement by 30 μM CORM-2 of currents evoked by 2 s pulses of 10 μM ATP under the conditions defined below each bar. Enhancement is expressed as a percentage and was calculated as ATP-evoked current of each record divided by ATP-evoked current of record #1. (B) Typical continuous time-course of currents evoked by repeated exposure of $P2X_2$ receptors to 2 s pulses of 10 μM ATP. ATP was applied at the points indicated by the arrows (top of panel). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM) was applied continuously to the bath for 270 s during the recording of records #8 to #12. For each application of ATP, there was a 10 s pre-application of control solution or 30 μM CORM-2 (CO Donor). ATP pulses were 90 s apart. Scale bars apply to all traces within the panel. In the lower left of the panel are shown five superimposed currents on a fast time-base taken from the continuous current record shown in the upper panel to illustrate the effects of pre-application of 30 μM CO donor in the presence of 10 μM ODQ. The current traces were recorded in the absence of ODQ following pre-application of extracellular solution (Control, record #5), CORM-2 (30 μM CO donor, record #6) and extracellular solution following wash-out of CO donor (Wash, record #7) or in the presence of 10 μM ODQ following pre-application of CORM-2 (30 μM CO donor, record #12), and extracellular solution following wash-out of CO donor (Wash, records #13). Application details are shown by the bars above the records. The bar graph in the lower right of this panel shows the mean (±SEM) enhancement by 30 μM CORM-2 of currents evoked by 2 s pulses of ATP under the conditions defined below each bar. Enhancement is expressed as a percentage and was calculated as ATP-evoked current of each record divided by the ATP-evoked current of record #1. Note that the filled bar represents the mean data from a separate set of experiments in which the pipette solution contained 10 μM ODQ.

agonist-specific. Although many factors, including dissociation of agonist and channel gating are involved in the current decay, given the agonist-specific nature of the effect, one possibility may be that the CO donor may be altering the rate at which ATP dissociates from the receptor.

Activation of $P2X_2$ receptors by CO appeared to be receptor-specific, as homomeric $P2X_4$ and heteromeric $P2X_{2/3}$ receptors were weakly inhibited and $P2X_3$ receptors were insensitive to pre-application of the CO donor (Figure 4). The $P2X_{2/3}$ receptor, which is activated by αβmeATP, is known to comprise two $P2X_3$ subunits and a single $P2X_2$ subunit (Jiang *et al.*, 2003; Wilkinson *et al.*, 2006). To investigate whether the lack of potentiation by CO at $P2X_{2/3}$ receptors was not merely due to the use of this different ligand, we tested αβmeATP at the $P2X_2$ receptor homomer. With αβmeATP as agonist, CO was still able to augment the peak current through $P2X_2$ receptors (Figure 4D). This implies that the lack of potentiation at $P2X_{2/3}$ receptors was not due to the use of αβmeATP as agonist, but is more likely to be an intrinsic property of this heteromeric channel. Finally, and perhaps most importantly, the effects of the CO donor were reproduced in cells natively expressing $P2X_2$, namely PC12 cells (Figure 4E) (Brake *et al.*, 1994).

At present, almost all actions of CO have been attributed to CO binding either to haem proteins or haem-binding proteins (see Desmard *et al.*, 2007). These include haemoglobin, myoglobin, nitric oxide synthase, cytochromes and sGC. Of these proteins, sGC appears, almost unanimously, to be a target for CO-dependent regulation of second messenger systems. For this reason, two independent methods of testing for the involvement of sGC in modulation of $P2X_2$ receptors were employed. Firstly, 8Br-cGMP, a membrane-permeable analogue of cGMP (the downstream product of sGC turnover), was applied to the cells. Secondly, a well-established inhibitor of sGC, ODQ (Garthwaite *et al.*, 1995), was applied both to the external and internal cellular compartments. Neither manoeuvre affected the augmentation of $P2X_2$ currents (both peak current and apparent off-rate) by CORM-2 which was observed at sub-EC_{50} concentrations of ATP (Figure 5). These data show that CO does not modulate $P2X_2$ receptors via sGC. Interestingly, such sGC-independent effects of CO have been reported only rarely, but thus far, all reported examples have been on ion channels (Wang and Wu, 1997; Liu *et al.*, 1999; Williams *et al.*, 2004; 2008; Scragg *et al.*, 2008). There are a number of potential mechanisms which may explain the action of CO on $P2X_2$ receptors. The rapidity of the effects of solutions of CO gas suggests a direct binding of CO to the channel, channel associated haem or a channel protein partner. To date, the former two models have been suggested for CO activation of large-conductance BK_{Ca} potassium channels (Jaggar *et al.*, 2005; Williams *et al.*, 2008). No information is currently available regarding whether haem can bind selectively to $P2X_2$ channels, but this seems unlikely given a lack of a consensus haem binding pocket [CXXCH – (Tang *et al.*, 2003)] in the receptor's primary sequence. Therefore, the possibility that CO may bind to the channel directly or via a protein partner of $P2X_2$ receptors both remain plausible.

Although there are no specific antagonists for $P2X_4$ receptors, their involvement can be identified by the use of ivermectin (Khakh *et al.*, 1999). However, distinguishing the involvement of $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors in specific physiological responses is difficult because of a dearth of subunit-specific pharmacological tools, other than acute hypoxia which indirectly, but selectively targets $P2X_2$ receptors (Mason *et al.*, 2004). This is important because $P2X_2$ subunits are often expressed alongside other P2X receptor subunits, particularly $P2X_3$ (Lewis *et al.*, 1995). At present, investigators tend to rely upon the combined use of several less specific agents and manoeuvres (see Evans, 2008); $P2X_{2/3}$ receptors are easily distinguished by their non-desensitizing response to low micromolar concentrations of αβmeATP. On the other hand, $P2X_3$ receptors are recognized by their rapidly desensitizing responses following application of low

micromolar concentrations of αβmeATP. However, responses of $P2X_2$ homomeric receptors are not easily identified. Thus, although $P2X_2$ receptor responses are potentiated by low micromolar zinc (Wildman *et al*., 1998), $P2X_{2/3}$ receptors are also weakly potentiated (Liu *et al*., 2001) and $P2X_3$ receptors demonstrate a mixed response to zinc (Wildman *et al*., 1999). Likewise, although $P2X_2$ receptors are potentiated, while $P2X_3$ receptors are inhibited by acidification (Stoop *et al*., 1997), the hetero-trimeric $P2X_{2/3}$ channel is also potentiated by extracellular protons (Liu *et al*., 2001). Although these approaches have been particularly helpful in ascribing particular responses to specific subunit combinations, there is clearly a need for a positive discriminator of homomeric $P2X_2$ receptors. The data described herein show that CO represents such an agent. CO is easy to apply in the form of a CO donor molecule, such as CORM-2, and its effects are rapid in onset and fully reversible.

CO is emerging as an important regulator of numerous physiological processes and the observation that it selectively augments $P2X_2$ receptors suggests that it may be involved in the physiological regulation of sensory signal transduction. Such a possibility is fully supported in several systems where $P2X_2$ receptors and haem oxygenase are already known to be co-expressed. For example, O_2-sensing by the carotid body is dependent upon $P2X_2$ receptor activation at the afferent carotid sinus nerve (Zhang *et al*., 2000; Rong *et al*., 2003) and can be either positively (Barbe *et al*., 2002) or negatively (Prabhakar *et al*., 1995) regulated by CO. Such bimodal regulation might be a function of the ATP concentration at the glomus cell/petrosal ganglion 'synapse', which is dependent upon the degree of glomus cell excitation/degree of hypoxia.

In several circumstances, even gene knock-out has produced data which leave the specific contribution of $P2X_2$ homomeric receptors either unresolved or controversial (Rong *et al*., 2003; Cockayne *et al*., 2005; Finger *et al*., 2005). Use of low concentrations of a CO donor would be helpful in resolving such controversies in studies employing either gene knock-out or wild-type animals. Furthermore, we would suggest that local endogenous release of CO, or its exogenous application using CO donor molecules, will modulate many other purinergic systems hitherto not yet shown unequivocally to be dependent upon activation of homomeric $P2X_2$ receptors.

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