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CARBON MONOXIDE RELAXES ILEAL SMOOTH MUSCLE THROUGH ACTIVATION OF GUANYLATE CYCLASE

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Abstract—The reported relaxing effect of CO on various smooth muscle tissues could also be found in guinea pig ileal strips. The effect was pronounced after precontraction with 10–100 nM acetylcholine and rather small with KCl. Based on the photoreversibility of the CO-dependent relaxation, a photochemical action spectrum was established which showed a maximum at around 422 nm. This definitely rules out the participation of a cytochrome P450 dependent process as postulated for the CO induced relaxation of lamb ductus arteriosus. With regard to the potency of KCN and antimycin A to relax ileal smooth muscle, the involvement of respiratory chain inhibition was reinvestigated, but no indication for such a mechanism could be obtained. In analogy to the mechanism of CO-inhibition of platelet activation we found that CO about doubles cGMP levels in guinea pig ileal strips. This is similar to NO which also leads to effective relaxation. We propose that CO can be considered and experimentally used as a convenient activator of soluble G-cyclase in smooth muscle and platelets.

A better insight into the regulation of smooth muscle tone and activity is of considerable pathophysiological significance and all relaxation processes are of potential interest in therapeutical approaches towards hypertension, ischemia or heart failure. With this aspect, the relaxation by carbon monoxide of the smooth muscle of the ductus arteriosus [1, 2], the blockade of pulmonary hypoxic constriction [3], the vasodilation by CO in the aorta [4, 5] and the isolated perfused heart [6, 7], or the decrease in pulmonary vascular resistance [8] are interesting observations. Their physiological and biochemical background is, however, less clear, although two main hypotheses have been proposed:

(1) the blockade of the respiratory chain with the subsequent fall in ATP which is required for contraction [1] and
(2) the inhibition of a cytochrome P450 dependent monooxygenase reaction that normally would generate a contracting substance [2]. An arachidonic acid derivative was considered as the most likely chemical nature of such a contracting agent [9].

Indeed, these two mechanisms represent obvious biochemical processes in which the binding of CO to a reduced hemoprotein provides a basis for the relaxation of smooth muscle.

Recently, however, we have reported that inhibition of platelet aggregation by CO is accompanied by an increase in cGMP [10] and we could show that the partially purified guanylate cyclase from human platelets is stimulated in the presence of CO [11] and even shows a spectral shift in its heme-containing regulatory subunit [12].

Having in mind the well-established role of cGMP in smooth muscle relaxation [13], we would like to add a third hypothesis for the CO induced relaxation of smooth muscle, which consists in binding of CO to smooth muscle guanylate cyclase and a stimulation of the enzyme analogous to that observed with nitric oxide [14].

All effects of CO in biochemical systems can be related to the formation of reduced heme–CO complexes and, therefore, share the property of being light-reversible. In accordance with this, previous investigators could establish the photoreversibility of the CO-dependent relaxation and even could obtain a photochemical action spectrum but the results and hence their interpretation came out different. Fay and Jöbsis [15] reported a maximum of photoreversibility between 420 and 425 nm and found this in accordance with a photodissociation of a cytochrome a_3–CO complex, whereas Coceani *et al.* [16] reported a maximum of 450 nm which strongly supported their previous hypothesis of a cytochrome P450 involvement.

It is the purpose of the present investigation to make use of the light reversibility and by this elegant technique to obtain evidence for a cGMP dependent process as a basis for the relaxation of smooth muscle.

MATERIALS AND METHODS

Chemicals. Cyclic GMP and HEPES were from Boehringer, (Mannheim, F.R.G.), ScGMP-TME [^{125}J] (guanosine 3′5′-cyclic phosphoric acid), [^{125}J]-2′-0-succinyl (iodotyrosine methyl ester) was from New England Nuclear, (Dreieich, F.R.G.), sodium nitroprusside and acetic acid hydride were from Aldrich Chemical Co., (Steinheim, F.R.G.), carbon monoxide, oxygen and nitrogen were from Sauerstoffwerk (Friedrichshafen, F.R.G.). Protein reagents were from Pierce Europe B.V., (Oud Beijerland, The Netherlands), succinate and acetylcholine were from the Sigma Chemical Co., (F.R.G.). All other chemicals were of analytical grade and purchased from Merck, Darmstadt,

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(F.R.G.) or from Riedel de Haen, (Seelze, F.R.G.). Antibodies against cyclic GMP were kindly provided by Dr Hertting, Freiburg, F.R.G.

Isotonic contraction measurements. Segments of the ileum of white male guinea pigs were used for measurements of contraction. Ileal rings 7–9 mm long were mounted vertically in 5 mL water-jacketed organ baths maintained at 37°. One of the open ends was connected to a steel hook, the other to the force transducer. An *N*-2-hydroxyethylpiperazine - *N'*-2-ethanesulfonic acid (HEPES) buffered Tyrode solution of the following composition was used: 148 mM NaCl, 2.68 mM KCl, 0.9 mM $CaCl_2$, 1.05 mM $MgCl_2$, 5.55 mM Glucose, 5 mM HEPES pH 7.4. In experiments with KCl as the contracting agent, the content of the organ bath was exchanged with a buffer containing 40 mM KCl and a correspondingly reduced NaCl concentration. The solutions were gassed with either 100% oxygen or the indicated gas mixtures by means of a peristaltic pump connected to gas-tight bags. Gas mixtures were prepared by injection of appropriate volumes of the corresponding gasses into the evacuated gas bags using a 1500 mL Hamilton gas syringe. Contractions were recorded isotonically with a strain gauge transducer (TF6V5iso) connected with a DC-AMP-Filter Unit (W. Fleck Mainz, F.R.G.) and pen recorder (Servogor 130, BBC). To measure the relaxing effect of carbon monoxide, the segments were precontracted with the respective agent. When a stable contraction was obtained, the gassing was switched from oxygen or air to carbon monoxide. To express the degree of relaxation, the stable contraction was taken as 0% relaxation, with the baseline as 100%. Some of the preparations did not yield stable contractions but relaxed with a slow time course. In these cases the CO mediated relaxation was corrected by the relaxation in the absence of carbon monoxide. After long lasting contractions, the tissue was allowed to recover, which was tested by the reproducibility of 30 sec contractions performed at the beginning of the experiment.

Determination of cyclic GMP levels. Pieces of ileum, 3–4 mm long were incubated in Erlenmeyer flasks in a shaking waterbath at 37°. The Tyrode–HEPES buffer used was pregassed for 10 min with the required gas mixture after degassing under vacuum. The flasks were equipped with pierced rubber caps which maintained the gas supply through capillary tubes during the incubation. After 10 min, the pieces were quickly removed and frozen by immersion in an acetone–dry ice mixture (2–3 sec). Storage until cGMP analysis occurred at −80° and never exceeded one week. To extract the cyclic GMP the frozen tissue was ground in a mortar cooled with liquid nitrogen. The resulting powder was transferred to 12% TCA and after 10 min on ice the protein precipitated by centrifugation (10 min at 10,000 *g* and 4°). After extraction with diethylether (4×) and an acetylation step to increase the sensitivity of the assay, the supernatant was used for cyclic GMP determinations by radioimmunoassay as described [17]. The cross reactivity of the antiserum with cAMP was 0.01%. Counting and calculation of the calibration curve was performed by a Philips Gamma Counter PW 4800 with integrated Single Board Computer PW 4801. The pellet was redissolved in 1N NaOH and the protein concentration was determined as described [18].

Photochemical action spectrum. To obtain the photochemical action spectrum a 5 mL organ bath was mounted on an optical bank and illuminated with a 150 W xenon lamp (adapted from a Perkin Elmer MPF3 Fluorimeter). The light was focussed on the organ with a lens. Illumination was started with the opening of a shutter. Monochromatic light could be produced by insertion of interference filters (Schott, Mainz, F.R.G.) between lamp and lens. The filters used were of the wavelengths 405, 411, 422, 431, 440, 450, 460, 470, 490 and 509 nm and had a bandwidth of 8 nm. The specification of the wavelength and the bandwidth of the interference filters have been verified using an Aminco DW-2 spectrophotometer. To correct the action spectrum for the characteristics of the lamp and the various filters used, the energy emitted by each combination was measured using a compensated thermopile (Kipp and Zonen). The values obtained together with the contractile amplitudes were used to calculate the action spectrum.

Measurement of mitochondrial oxygen consumption. To measure the rate of mitochondrial oxygen consumption the organ bath mounted on the optical bank as described above was replaced by a thermostatted cuvette holder. The decrease in oxygen content was determined by a Clark oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Rat liver mitochondria prepared by differential centrifugation as described [19] were added to a mixture of CO-gassed and air-equilibrated buffer of the following composition: 0.25 M sucrose, 0.1 mM ethyleneglycolbis (aminoethylether) tetra - acetate (EGTA), 10 mM HEPES, 4.5 mM succinate, 3.6 mM $MgCl_2$, 3.6 mM Na_2HPO_4 and 4.5 mM glucose. Incubations took place in 3 mL optical cuvettes at 37° under constant stirring with an oxygen partial pressure between 35 and 20 mm Hg.

Statistics. The data collected represent mean ± SE. The difference between results was compared by Student's *t*-test for paired and unpaired values.

RESULTS

In order to select a suitable smooth muscle preparation for the CO induced relaxation, we tested human uterus smooth muscle (B. Brüne, V. Ullrich and H. P. Zahradnik, unpublished), the bovine coronary artery (V. Ullrich and K. Schrör, unpublished) and guinea pig ileum. Since the latter organ gave the most consistent results, it was used for our investigations.

In order to eliminate possible effects of oxygen, the gassing with CO was performed with 20% oxygen present to match the aerobic incubations with sodium nitroprusside (SNP) and blockers of respiration. Both acetylcholine (0.1 μM) and 40 mM KCl caused a contraction of the ileal strips. Some preparations with a subsequent decline in contractile force were eliminated from the experiments. When the gassing was switched to a mixture of 80% CO and 20% O_2, the contractions produced by 40 mM KCl were reduced by 5.4 ± 3.8%. In ACh precontracted strips

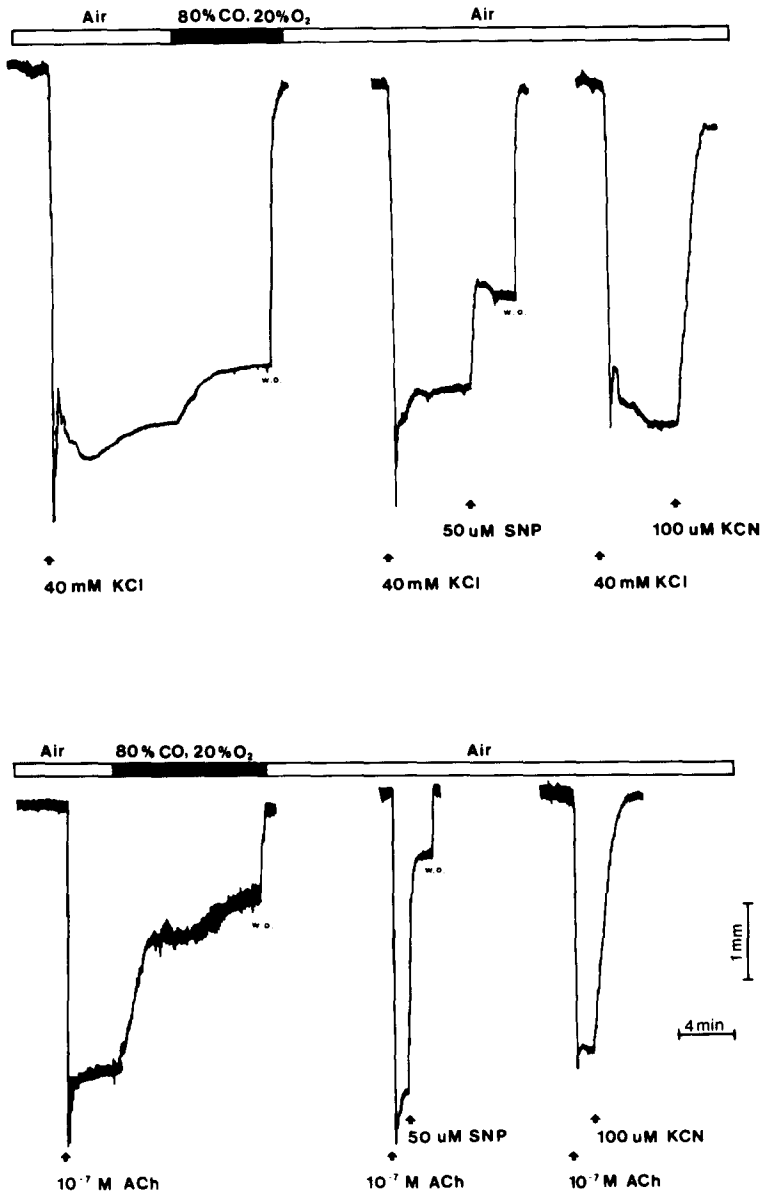

Fig. 1. Effects of CO, SNP and KCN on the guinea pig ileum precontracted with ACh or KCl. ACh, SNP and KCN have been added at the times indicated (arrow) from freshly prepared stock solutions to obtain the final concentrations indicated in the graph. Contractions by KCl have been produced by exchange of the buffer against a buffer containing 40 mM KCl and correspondingly reduced NaCl concentrations. Relaxations by CO have been achieved by changing the gassing of the precontracted organ from air to a mixture of 80% CO and 20% O_2. The time intervals between individual experiments shown in the graph do not represent the original time intervals. w.o., organ bath wash.

the relaxation by CO could vary between 20 and 85% with an average of 69 ± 15% (N = 16).

Addition of 50 μM SNP, a potent vasodilator which releases NO and thus stimulates soluble G-cyclase, reduces the tension evoked with 0.1 μM ACh by 82 ± 7% (N = 10). Segments precontracted with KCl showed only a relaxation of 28 ± 7% (N = 13) under these conditions. Increasing the concentrations of SNP up to 1 mM had no further relaxing effect (Fig. 1).

To investigate the effects of inhibitors of respiration on the contractile state, we determined the response of ileal segments precontracted with ACh or KCl to the addition of KCN or antimycin A. One hundred micromoles of KCN relaxed the ileum precontracted with 0.1 μM ACh by 73 ± 28% (N = 11) and the ileum precontracted with 40 mM KCl by 58 ± 20% (N = 7). After repeated washings of the organ, the tissue recovered completely from the KCN treatment. Addition of antimycin A in a concentration of 1.5 μg/mL caused complete relaxation of the ACh and KCl contracted ileum (not shown). Repeated washings could not restore the initial sensitivity of the organ.

By reducing the ACh concentration to 10 nM an almost complete relaxation with the 80% CO gas mixture could be obtained. These conditions were

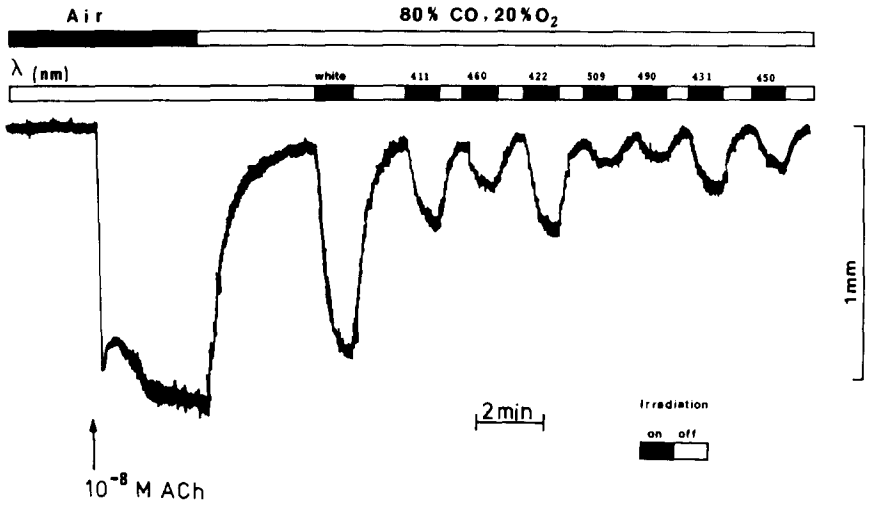

Fig. 2. Effects of light of different wavelengths on the CO relaxed guinea pig ileum precontracted with ACh. The segment was precontracted by the addition of ACh at a final concentration of 10^{-8} M and subsequently relaxed by a change of the gassing from air to a mixture of 80% CO and 20% O_2. Monochromatic light was produced with a xenon lamp equipped with interference filters. For quantitative analysis, the contractile amplitudes have been recorded with greater amplification than shown.

used in the irradiation experiments in which white light from a 150 W xenon lamp caused a more than 80% reversibility and smaller effects were observed with light of filter-selected wavelengths (Fig. 2).

When in these experiments the light energies at the different wavelengths were standardized on equal energies using a compensated thermopile, a photochemical action spectrum was obtained. The maximum effects were seen at 422 nm which therefore was set as 100%. Compared to this wavelength, the neighbouring wavelength at 411 and 430 nm showed a lower efficiency which was statistically highly significant (Fig. 3).

The exact relative responses were 71 ± 10% at 411 nm, 42 ± 12% at 431 nm, 28 ± 11% at 440 nm, 21 ± 6% at 450 nm, 13 ± 5% at 460 nm, 16 ± 12% at 490 nm and 18 ± 10% at 509 nm (N = 4). In a control experiment, the light intensities were not corrected but kept constant by means of a diaphragm. The same profile was obtained in this experiment.

The absorption maximum of the G-cyclase-CO complex was reported to be at 422 nm [20]. This spectrum is shown by the dashed line in Fig. 3. The corresponding CO complex of cytochrome oxidase is at 430 nm [21] with a photochemical action spectrum also around 429–430 nm [22–24]. Since the nominal band width of the filters used in our irradiation experiments was 8 nm, it seemed desirable to exclude possible errors originating from the optical equipment or from the intensity calculations. This was done by comparing the effects of the 422 and 431 nm on the CO-inhibited respiration of liver mitochondria. Setting the oxygen uptake in air saturated buffer as 100%, CO caused a reduction to 46 ± 3.6% (N = 5). Illumination with 431 nm increased the respiration activity in the presence of CO by 65 ± 14.8% (N = 11) which is significantly higher ($P < 0.005$) than the increase of 39 ± 16% under illumination with 422 nm. By this internal control, we can rule out a major role of mitochondrial respiratory chain inhibition in the relaxation process by CO.

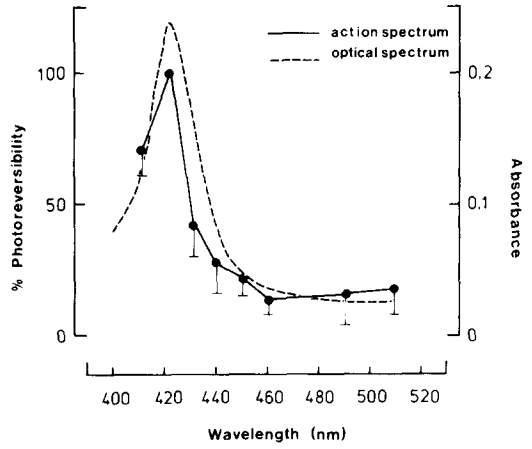

Fig. 3. Photochemical action spectrum of the light induced recontraction of the CO relaxed guinea pig ileum precontracted with ACh. The action spectrum was calculated from the contractile amplitudes produced by illumination (as shown in Fig. 2) after correction for the same energy. The maximal effect which occurred in all experiments at 422 nm has been set as 100%. The relative energy of light at the various wavelengths has been measured using a compensated thermopile. Data represent the means ± SE of four experiments. The dashed line represents the CO-binding spectrum of purified guanylate cyclase from bovine lung [20].

Since the photochemical action spectrum excludes a participation of a cytochrome P450-dependent process, we favoured an activation of soluble G-cyclase which must result in increased cGMP levels in the ileal strips. The values obtained from the cGMP measurements are shown in Table 1. A clear increase by about 100% could be verified after CO treatment. SNP is more effective which is in agreement with the rapid relaxation seen in Fig. 1. Interestingly, at 50 μM SNP, the increase in cGMP can be further stimulated by CO, whereas at 1 mM SNP saturation seemed to have occurred.

Table 1. Cyclic GMP levels in the guinea pig ileal segments

Gassing	Cyclic GMP (pmol/mg) 100% O_2	80% N_2, 20% O_2	80% CO, 20% O_2
SNP			
—	0.87 ± 0.26 (13)	0.81 ± 0.35 (7)	1.73 ± 0.46 (11)*
0.05 mM	4.7 ± 1.8 (12)	7.8 ± 3.2 (9)	16.6 ± 3.6 (9)*
1 mM	6.6 ± 1.3 (5)	10.4 ± 1.1 (3)	11.2 ± 3.1 (5)*

Assays were incubated for 10 min in Erlenmeyer flasks under the conditions indicated. The concentration of cGMP has been measured by radioimmunoassay. Data represent the means ± SE. Numbers in brackets give the number of experiments performed.
* $P < 0.01$ compared with the values under 100% oxygen.

DISCUSSION

Carbon monoxide not only causes a relaxation of the ductus arteriosus and has similar effects on the vascular system of heart and lung, but according to our results also relaxes the precontracted ileum. From the two mechanisms found in literature to explain this effect, we would like to definitely exclude the participation of a cytochrome P450 system. The photochemical action spectrum leaves no room for a cytochrome P450 mediated formation of a constricting substance as postulated by Coceani and Olley [9] from experiments on lamb ductus arteriosus. This tissue may be different from the guinea pig ileum, but a reinvestigation of the action spectrum in this tissue may be helpful to explain this discrepancy.

The involvement of the respiratory chain was more difficult to rule out since the Soret absorption of the CO-complex of cytochrome oxidase at 430 nm is close to our observed maximum at around 422 nm. The relaxations seen with KCN and antimycin A prove that blocking of respiration can indeed be an efficient mechanism, but several observations are not in favour of this possibility. First, the inhibition of respiration by 80% CO and 20% O_2 can certainly not be very high, since for beef heart at a CO/O_2 ratio of 3.3 cytochrome oxidase was blocked by only 25% [25]. When corrected for the solubilities in water, our experiment would yield a ratio of 3.08 for the CO/O_2 ratio. It was also reported that ventilation of the isolated perfused lung with a gas mixture containing CO and O_2 at a ratio of 4 did not affect the redox status of the tissue [26]. Coburn showed that CO can relax aorta smooth muscle pretreated with NaCN despite a complete inhibition of oxygen uptake under these conditions [5]. There was also a difference in our experiments between the relaxation by KCN and CO in smooth muscle precontracted by depolarization or by acetylcholine which also favours a different mechanism of action between CO and KCN. Direct proof for a different hemoprotein as a target of the CO effect came from the comparison of the two critical wavelengths 422 and 430 nm. From this, we favour our hypothesis that guanylate cyclase is activated by CO and this gains support from an actual increase of the cGMP level by about 100%. A similar increase of cGMP we observed in guinea pig urinary bladder [27] and in platelets where CO leads to a dramatic desensitation of the agonist induced aggregatory response [10]. Our hypothesis is further supported by recent findings of Ramos *et al.* [28]. They found a significant increase of the cGMP level in cultured smooth muscle cells from rat aorta after treatment with carbon monoxide.

An association between vascular smooth muscle relaxation and an elevation of the cellular cGMP concentration has been demonstrated in numerous studies. For guinea pig ileum longitudinal smooth muscle a relaxation by nitroglycerol was found which was correlated with an accumulation of cGMP. 8-Bromo-cGMP, the lipophilic cGMP analog, together with SNP as the most potent stimulator of G-cyclase cause relaxation of the precontracted longitudinal smooth muscle [29]. However, in nonvascular smooth muscles a strict correlation between cGMP and relaxation is not generally valid, since in several studies a dissociation between both parameters has been reported [30, 31].

One of the major consequences of a cGMP increase in smooth muscle cells is a reduction in the intracellular Ca-levels [32]. It has been demonstrated that a cGMP-dependent protein kinase is involved in this process [33]. Possible mechanisms include direct or indirect activation of plasmalemmal Ca-ATPase [34, 35], increased sequestration of Ca^{2+} into the sarcoplasmic reticulum [36], and inhibition of G-protein activation and coupling to phospholipase C [37].

In isolated aortic rings CO has been found to reduce intracellular Ca, but the levels of cyclic nucleotides were not measured in this study [38].

In myocytes from urinary bladder CO reduces the Ca^{2+}-activated potassium current after depolarization in a light reversible manner and increases cGMP [27].

Unfortunately, little exact knowledge is available about the activation of G-cyclase by NO or CO. Since CO must bind to a ferrous hemoprotein, we have correlated this binding with an allosteric effect on the G-cyclase active site. NO is also a tight binding ligand to hemoproteins and thus could act in the same manner. Analogous to hemoglobin, a high spin–low spin transition could be involved as a trigger for the conformational change.

If this mechanism applies, it would be the first case where CO does not competitively inhibit the addition of the O_2 molecule to a ferrous hemoprotein. We have at last tried to establish a role of dioxygen as an effector molecule for G-cyclase but were unsuccessful. The diminished activity of G-cyclase under 100% oxygen when stimulated by SNP could well be due to a trapping of NO released from SNP.

In summary, our results have rendered the two

previous explanations for the CO-induced smooth muscle relaxation unlikely. Instead, the data show an increase in cGMP which in smooth muscle and also in platelets has been correlated with a lowering of Ca-levels or a desensitisation of the cell response to agonists. Thus CO can be regarded as a model for the action of NO with the advantage over NO that its handling is experimentally easier, its binding to hemoproteins is theoretically clearer and by applying the technique of the photochemical action spectrum gives high specificity is obtained. After the discovery of EDRF as NO, the CO model may have considerable advantage in the study of the role of soluble G-cyclase.

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