

SPECIAL REPORT

Membrane-association and the sensitivity of guanylyl cyclase-coupled receptors to nitric oxide

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Nitric oxide (NO) signal transduction occurs through guanylyl cyclase-coupled receptors, which exist in both cytosolic and membranous locations. It has recently been reported from experiments using heart tissue that the membrane-associated receptor has enhanced sensitivity to NO. Owing to its potential importance, we tested this finding using a method of applying NO in known, constant concentrations. The results showed that the concentration–response curves for receptor activation in cytosolic and membrane preparations of two different tissues (cerebellum and platelets) were indistinguishable. In all cases, half-maximal activation required about 1 nM NO and the curves had Hill coefficients of close to 1. The differential sensitivity reported for the heart is attributed to NO being scavenged by myoglobin in the cytosol, but not in the membrane fraction.

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Abbreviations: CPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DEA/NO, diethylamine/NO adduct; GC, guanylyl cyclase

Introduction Nitric oxide (NO) functions as a signalling molecule in almost all tissues of the body and regulates diverse processes, including blood vessel dilatation, platelet function, and the induction of long-term changes in the strength of central synaptic transmission (Moncada *et al.*, 1991; Garthwaite & Boulton, 1995). The established mechanism for physiological NO signal transduction in these and other processes is through guanylyl cyclase (GC)-linked receptors. The resulting increase in intracellular cGMP can engage multiple downstream targets, including kinases, ion channels, and phosphodiesterases, to elicit various biological effects. The known GC-coupled receptors are $\alpha\beta$ -heterodimers of which two isoforms, $\alpha1\beta1$ and $\alpha2\beta1$, are widely expressed at the mRNA and protein levels (Friebe & Koesling, 2003). So far, the two isoforms have appeared functionally and pharmacologically similar, whether studied in intact cells or in cell lysates (Friebe & Koesling, 2003; Gibb *et al.*, 2003; Griffiths *et al.*, 2003). However, complexity might arise from differences in subcellular location. In this respect, early data showed that NO-evoked GC activity was present in varying amounts in the soluble (cytosolic) and insoluble (particulate) fractions of several tissues (Arnold *et al.*, 1977) and recent evidence indicates that the $\alpha1\beta1$ receptor translocates to membranes in response to a Ca^{2+} signal (Zabel *et al.*, 2002), whereas, in the brain, the $\alpha2\beta1$ isoform is targeted to membrane-linked synaptic scaffold proteins (Russwurm *et al.*, 2001).

From a study using heart tissue, an association with membranes was reported to sensitise the receptors to NO (Zabel *et al.*, 2002). If correct, this conclusion might be important because it could provide a device for modulating cellular responsiveness to NO and/or for concentrating

NO signal transduction into discrete subcellular domains analogous to those mediating conventional synaptic transmission. The result was based on differences in the concentrations of the NO donor, diethylamine/NO adduct (DEA/NO) needed to evoke GC activity in cytosol and membrane fractions. DEA/NO degrades to release NO with a half-life of 2.1 min (37°C). The amplitude, shape, and duration of the resulting NO concentration profile, however, is dependent on several factors, including pH and the rate of consumption of NO. Without rigorous checks, therefore, a shift in the DEA/NO concentration–response curve is difficult to interpret (Bellamy *et al.*, 2002). Accordingly we have evaluated the veracity of the result using a method for delivering NO in known, fixed concentrations (Griffiths *et al.*, 2003).

Methods *Tissue preparation* Adult female Sprague–Dawley rats (Charles River, Margate, U.K.) were anaesthetised with pentobarbitone (60 mg kg⁻¹, i.p.) as approved by the British Home Office and the local ethics committee. The animals were perfused through the left ventricle with PBS until the returning buffer ran clear. The cerebella and/or hearts were swiftly removed and immersed in ice-cold PBS. Adhering connective tissue and visible vasculature were discarded under a dissecting microscope and the tissues were then cut into 1 mm cubes using a McIlwain tissue chopper, before being frozen on dry ice and homogenised (while frozen) using a stainless-steel mortar and pestle, both precooled in dry ice. The heart tissue was resuspended at 3–4 hearts per 5 ml in ice-cold buffer A, which contained (mM): NaCl (137), MgCl₂ (0.5), NaH₂PO₄ (0.55), KCl (2.7), HEPES (25), glucose (5.6) and a protease inhibitor cocktail (complete mini EDTA-free; Roche, East Sussex, U.K.), pH 7.4. The cerebellar tissue was resuspended (at 3 per 5 ml) in buffer as before (Bellamy *et al.*, 2000), except that the DTT concentration was reduced 10-fold to avoid problems

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of NO consumption (Bellamy *et al.*, 2002). The preparations were then centrifuged for 5 min at 500 g to remove cellular debris.

To obtain platelets, whole blood was collected from four adult Sprague–Dawley rats into acid citrate dextrose solution (12.5%) and centrifuged at 300 g for 10 min. The platelet-rich plasma was withdrawn and recentrifuged to eliminate residual red and white blood cells. The supernatant was centrifuged for 10 min at 2000 g and the platelet pellet was resuspended in buffer A at a final concentration of 1 mg protein ml⁻¹. The platelets were lysed by addition of an equal volume of ice-cold distilled water containing the protease inhibitor cocktail and 0.2 mM DTT, followed by sonication. The lysate was kept on ice.

Approximately 3 ml of each homogenate was centrifuged for 1 h at 100,000 g at 4°C. The supernatants (cytosols) were removed and kept on ice and the pellets (crude membrane fractions) were resuspended in the corresponding homogenisation buffers to a fifth to a tenth of the original homogenate volume. The protein concentrations were measured using the bicinchoninic acid method; for platelets and cerebellum, these values were measured in samples prepared in parallel but lacking DTT (which interferes with the protein assay).

Measurement of GC activity The activity of the GC-coupled receptors in the different preparations was measured by exposing them to constant concentrations of NO, using a new method (Griffiths *et al.*, 2003) in which NO release from a NONOate donor, in this case spermine/NO adduct (Alexis Biochemicals, Nottingham, U.K.), is balanced by NO consumption by a chemical scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (CPTIO; Alexis Biochemicals). Aliquots of the tissue fractions were diluted 1:10 into an assay buffer containing 50 mM Tris-HCl, 3 mM MgCl₂, 0.1 mM EGTA, 0.01 mM DTT, 0.05% BSA, 1 mM GTP, 0.2 mg ml⁻¹ creatine kinase, 5 mM creatine phosphate, 1 mM 3-isobutyl-1-methylxanthine, 200 μM CPTIO, 1000 U ml⁻¹ superoxide dismutase, and 300 μM uric acid (pH 7.4 at 37°C). Spermine/NO adduct was added from concentrated stock solutions (in 10 mM NaOH) to achieve varying steady-state NO concentrations (achieved within about 1 s) and, 2 min later, 100 μl of the reaction mix was removed and inactivated by addition to 200 μl of boiling hypotonic buffer (50 mM Tris HCl, 4 mM EDTA). cGMP was measured using radioimmunoassay. Results are given as the mean cGMP levels from 'n' independent estimations ± s.e.m. Each concentration-response curve was fitted to the Hill equation (in the Origin™ version 6.1; Aston Scientific Ltd, Stoke Mandeville, U.K.) to obtain values of the EC₅₀ and Hill coefficient. Averaged data were analysed for significance using the unpaired Student's *t*-test (two-tailed). To scrutinise the results obtained by Zabel *et al.* (2002), the GC reaction mixture was changed to the one they used (with 1000 U ml⁻¹ superoxide dismutase as an additional ingredient) and DEA/NO was used as the donor. The methods otherwise were as detailed above, except that the tissue fractions were diluted 1:100 into the reaction mixture and aliquots were sampled for cGMP determination at 1 min intervals for a total of 10 min.

NO measurement NO concentrations were recorded in buffer A (1 ml) supplemented with 1000 U ml⁻¹ superoxide dismutase

and incubated in a sealed, stirred vessel (37°C) equipped with an NO electrode (Iso-NO, World Precision Instruments, Stevenage, U.K.).

Results We initially selected tissue from a brain region (the cerebellum) in which the NO-cGMP pathway is highly expressed (Garthwaite & Boulton, 1995). NO elicited GC activity in both cytosolic and membrane fractions of the cerebellum, with the former dominating (Figure 1a). Concentration-response curves were constructed using NO delivered in known, constant concentrations (Griffiths *et al.*, 2003). In both fractions, the threshold NO concentration required to stimulate GC activity was 50–100 pM and the maximum response occurred at about 10 nM (Figure 1a). The concentrations giving half-maximal activity (the EC₅₀ values) were very similar (0.94 ± 0.09 nM in the cytosol and 1.09 ± 0.15 nM in the membranes, *n* = 4; *P* > 0.4), as were the Hill slopes (1.1 ± 0.1 and 0.9 ± 0.1; *P* > 0.2). Accordingly, when normalised, the two curves were superimposable (Figure 1b).

In case the cerebellum behaved unusually, the experiments were repeated using blood platelets, a well-known target for NO (Schwarz *et al.*, 2001). The concentration-response curves for cytosol and membrane fractions were again closely similar (Figure 2a,b). The respective EC₅₀ values (0.90 ± 0.12 nM and 1.23 ± 0.04 nM; *n* = 3–4) were not significantly different from each other (*P* > 0.07), nor from the corresponding values in the cerebellum (*P* > 0.7 and 0.4). The Hill coefficients (1.2 ± 0.2 and 1.1 ± 0.03) were also no different from each other (*P* > 0.6) or from the cerebellar values (*P* > 0.3 and 0.1).

The experiments leading to the general conclusion that membrane association sensitises the receptors to NO were conducted on the rat heart (Zabel *et al.*, 2002). In view of the above negative findings, we began to investigate the possibility that the phenomenon might be peculiar to this organ. However, it soon became evident that such an undertaking would be compromised. A homogenate of three rat hearts (previously perfused thoroughly *in situ* to wash out red blood cells) in 5 ml buffer was used to prepare concentrated membrane and cytosol fractions. The membrane fraction was

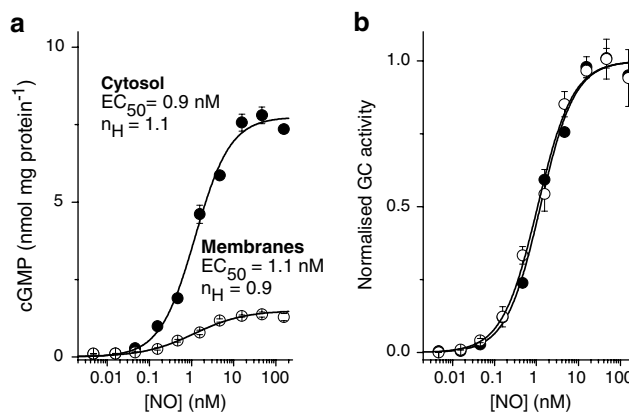


Figure 1 Comparison of the sensitivity of GC-coupled NO receptors in membrane and cytosol fractions of rat cerebellum. (a) Raw concentration-cGMP response curves for NO in cytosol and membrane fractions; *n* = 3–4. The solid lines fit the data to the Hill equation; *n*_H signifies the Hill coefficient. (b) The same data normalised to the maximal GC activity in each case (derived from the Hill fits). Protein concentrations were 140 μg ml⁻¹ (cytosol) and 290 μg ml⁻¹ (membranes).

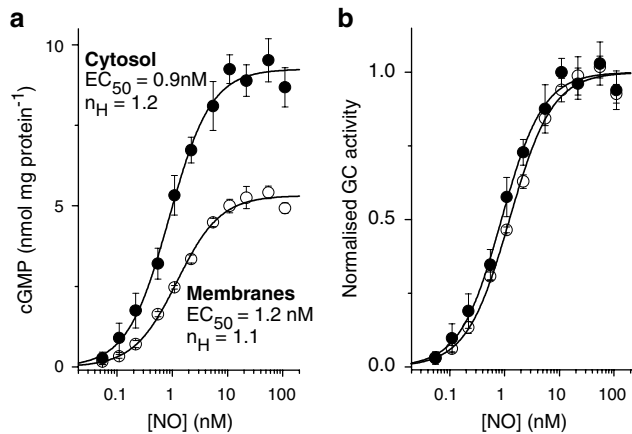


Figure 2 Comparison of the sensitivity of GC-coupled NO receptors in membrane and cytosol fractions of rat platelets. (a) Raw concentration–cGMP response curves for NO in cytosol and membrane fractions; $n=4$. The solid lines fit the data to the Hill equation; n_H signifies the Hill coefficient. (b) The same data normalised to the maximal GC activity in each case (derived from the Hill fits). Protein concentrations were $13 \mu\text{g ml}^{-1}$ (cytosol) and $40 \mu\text{g ml}^{-1}$ (membranes).

its usual milky-white colour, but the cytosol was bright red-pink due to the presence of myoglobin, a cytosolic protein expressed in sufficient abundance to give the heart its gross colour (Garry *et al.*, 1998). NO reacts very rapidly with myoglobin, forming nitrate and metmyoglobin (Eich *et al.*, 1996); consequently, the presence of significant quantities of myoglobin in the cytosol will reduce the amount of NO available for activating GC-coupled receptors. Hence, more NO would have to be added to match the degree of receptor activation taking place in the absence of myoglobin.

In order to evaluate this simple explanation for the results of Zabel *et al.* (2002), recordings were first made of the NO concentration profiles following addition of DEA/NO, the donor used to supply NO in the previous experiments. The DEA/NO concentration selected ($0.5 \mu\text{M}$) was one evoking approximately half-maximal activation of GC-coupled NO receptors in the heart cytosol preparations during a 10 min incubation (Zabel *et al.*, 2002). In buffer, the NO concentration rose to a peak of approximately 400 nM after about 3 min (Figure 3). Unfortunately, the dilution of heart cytosol used in the study of Zabel *et al.* was not disclosed, but when DEA/NO was added to a 1:100 dilution of the cytosol (giving a final protein concentration equivalent to that used for assaying GC activity in the cerebellar cytosol), NO was undetectable for about 2 min. Afterwards, the concentration rose to reach a plateau about three-fold lower than in buffer. On the other hand, when the test was repeated on the membrane fraction (similarly diluted), the NO profile was the same as in the buffer.

The method of assaying GC activity used by Zabel *et al.* was then applied to the heart fractions, except that the activity was followed at 1 min intervals for 10 min rather than at only a single time point (10 min). With a maximal DEA/NO concentration ($10 \mu\text{M}$; Zabel *et al.*, 2002), cGMP accumulated fairly linearly over the 10 min period in both fractions, with the GC activity being more than 10-fold higher in the cytosol than in the membranes (Figure 4). In the membranes, DEA/NO concentrations spanning the EC_{50} value reported by Zabel *et al.*

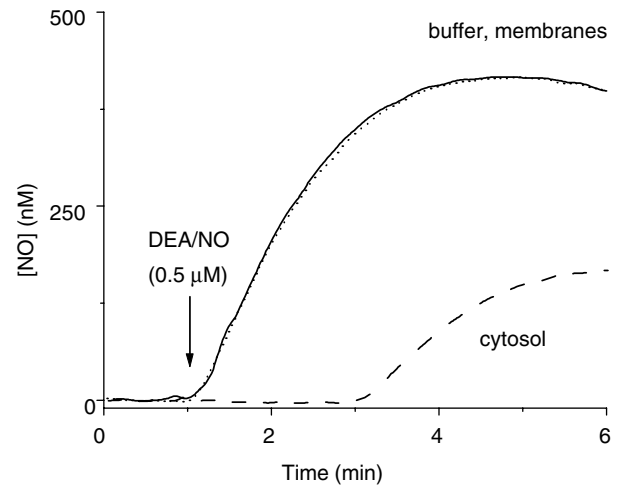


Figure 3 Consumption of NO by heart cytosol. Data are recordings of the NO concentration profiles over time resulting from addition of DEA/NO ($0.5 \mu\text{M}$; arrow) in buffer alone (solid line), or buffer containing the membrane fraction (dotted line) or the cytosolic fraction (dashed line) from rat heart. The traces are means of two runs and were smoothed by adjacent averaging (10 s bins). Protein concentrations were $130 \mu\text{g ml}^{-1}$ (cytosol) and $50 \mu\text{g ml}^{-1}$ (membranes).

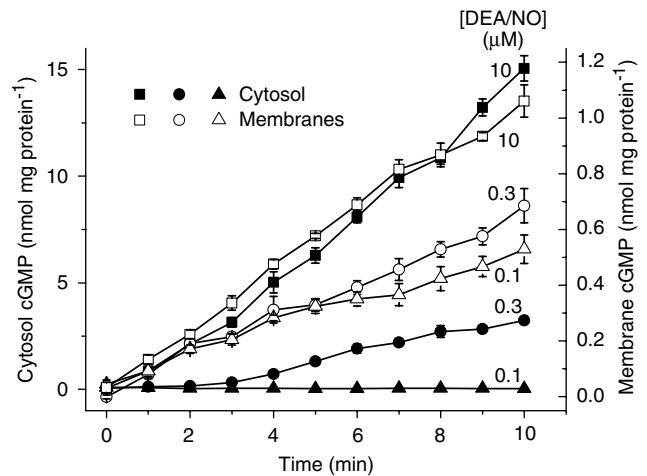


Figure 4 DEA/NO-induced cGMP accumulation in heart membranes and cytosol. The data show cGMP levels over time in response to addition of DEA/NO at the indicated concentrations to membranes or cytosol from rat heart; $n=4$. Protein concentrations were $86 \mu\text{g ml}^{-1}$ (cytosol) and $66 \mu\text{g ml}^{-1}$ (membranes).

(2002), namely 0.1 and $0.3 \mu\text{M}$, gave cGMP levels after 10 min that were, respectively, about 50 and 70% of the maximum. In the cytosol, the corresponding levels at this time were lowered to about 0 and 22%. These relative values are all closely similar to those reported by Zabel *et al.* (although, unfortunately, they did not disclose the absolute values). Inspection of earlier time points at the $0.3 \mu\text{M}$ concentration, however, showed that, in the membranes, there was near-maximal GC activity over the first 2 min (when the rate of NO release is highest), followed by a decline. In contrast, in the cytosol, there was no measurable GC activity for the same initial period, with a slow rise occurring afterwards. Measurement of the NO concentration under the same conditions gave the

same result as before (Figure 3): on addition of DEA/NO ($0.3 \mu\text{M}$), NO rose immediately in the diluted membranes but was undetectable for about 2 min in the diluted cytosol (results not illustrated).

Discussion From the two different tissues examined in detail, we conclude that there is no discernable alteration in NO sensitivity of membrane-associated *versus* cytosolic GC-coupled receptors and that previous evidence that the two differ is spurious because, in the tissue examined (heart), the exposures to NO in the two fractions were different.

The concentration of myoglobin in the heart is about $200 \mu\text{moles (kg wet weight)}^{-1}$ (Godecke *et al.*, 1999) from which it can be estimated that the diluted cytosol used in our experiments would contain the protein in the upper 100 nM range. At the concentration of DEA/NO tested in Figure 3 ($0.5 \mu\text{M}$), NO would be released at an initial rate of about 2.8 nM s^{-1} (assuming a half-life of 2.1 min and 1 mole of NO released per mole of DEA/NO; Schmidt *et al.*, 1997). To scavenge NO for the period observed (2 min) would require 250 nM myoglobin, a concentration well within the range predicted to be present, even allowing for some of the protein being in its oxidised, unreactive, form (metmyoglobin). A similar period of NO consumption was observed at a lower DEA/NO concentration ($0.3 \mu\text{M}$) in another experiment (the one whose cGMP results are reported in Figure 4), which is explained by the protein concentration also being lower (see legends to Figures 3 and 4): assuming a proportionate

reduction in myoglobin concentration (to 165 nM) it can be calculated that NO released from $0.3 \mu\text{M}$ DEA/NO would be grounded for 2 min, as was observed. The same myoglobin concentration would consume all NO releasable from $0.1 \mu\text{M}$ DEA/NO.

The presence of such a powerful NO scavenger means, of course, that the receptor cannot become activated until the scavenger is exhausted, which explains why, in the heart cytosol, there was no GC activity for 2 min following addition of $0.3 \mu\text{M}$ DEA/NO and no activity at all during a 10 min exposure to $0.1 \mu\text{M}$ DEA/NO whereas, with no scavenger (in the heart membranes), these same concentrations were sufficient to evoke half-maximal, or greater, net GC activity. Unfortunately, the problem imposed by myoglobin in the heart cytosol would also apply to the other NO delivery method used here for the cerebellum and platelets (NO reacts with myoglobin 1000-fold faster than with CPTIO); a method for removing myoglobin is needed to conduct the experiment meaningfully.

Rather than regulate their sensitivity to NO, the subcellular distribution of the GC-coupled NO receptors may reflect a physical compartmentation of the signal transduction cascade, although the possibility that functional differences exist when the proteins are in their normal cellular environment *in vivo* cannot be excluded.

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