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Properties of NO-activated guanylyl cyclases expressed in cells

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1 Physiological nitric oxide (NO) signal transduction occurs through activation of guanylyl cyclase (GC)-coupled receptors, resulting in cGMP accumulation. There are five possible receptors: four heterodimers ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha1\beta2$, $\alpha2\beta2$) and a presumed homodimer ($v\beta2$). The present study investigated the kinetic and pharmacological properties of all these putative receptors expressed in COS-7 (or HeLa) cells.

2 All exhibited NO-activated GC activity, that of $\alpha1\beta1$ and $\alpha2\beta1$ being much higher than that of the $\beta2$-containing heterodimers or $v\beta2$. All were highly sensitive NO detectors. Using clamped NO concentrations, EC_{50} values were 1 nM for $\alpha1\beta1$ and 2 nM for $\alpha2\beta1$. With $\alpha1\beta2$, $\alpha2\beta2$ and $v\beta2$, the EC_{50} was estimated to be lower, about 8 nM.

3 All the GCs displayed a marked desensitising profile of activity. Consistent with this property, the concentration–response curves were bell-shaped, particularly those of the $\beta2$ heterodimers and $v\beta2$.

4 Confocal microscopy of cells transfected with the fluorescently tagged $\beta2$ subunit suggested targeting to the endoplasmic reticulum through its isoprenylation sequence, but no associated particulate GC activity was detected.

5 The NO-stimulated GC activity of all heterodimers and $v\beta2$ was inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and, except for $v\beta2$, was enhanced by the allosteric activator YC-1.

6 It is concluded that all the four possible heterodimers, as well as the putative $v\beta2$ homodimer, can function as high-affinity GC-coupled NO receptors when expressed in cells. They exhibit differences in NO potency, maximal GC activity, desensitisation kinetics and possibly subcellular location but, except for $v\beta2$, cannot be differentiated using existing pharmacological agents.
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Abbreviations: DEA/NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; DETA/NO, (Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; EYFP, enhanced yellow fluorescent protein; GC, guanylyl cyclase; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; $NO_{GC}R$, guanylyl cyclase-coupled nitric oxide receptor; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

Introduction

Nitric oxide (NO) functions as a diffusible messenger in almost all tissues and exerts most of its physiological effects by binding to its guanylyl cyclase (GC)-coupled receptors ($NO_{GC}Rs$). The ligand-binding site on the receptors is a specialised haem group, the occupation of which results in conformational changes that trigger GC activity and so the generation of cGMP from GTP. The ensuing accumulation of cGMP then engages various downstream targets, including protein kinases, phosphodiesterases and ion channels to bring about changes in cell function, such as smooth muscle relaxation, platelet disaggregation and synaptic plasticity (Mayer, 2000).

$NO_{GC}Rs$ appear to exist predominantly as heterodimers comprising one α and one β subunit, with the haem prosthetic group being attached to a histidine residue on the β subunit (Denninger & Marletta, 1999; Koesling & Friebe, 2000). Two types of α and two β subunits have so far been identified, providing a possible four different isoforms ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha1\beta2$ and $\alpha2\beta2$). Only two have hitherto been shown to exist *in vivo* at the protein level: $\alpha1\beta1$, which is expressed widely and $\alpha2\beta1$, which was originally identified in human placenta but which has now also been found in rat brain where it associates with synaptic scaffold proteins (Russwurm *et al*., 2001). At the mRNA level, the $\alpha2$ subunit is widely expressed in the brain with a particular concentration in the cerebellum and hippocampus (Gibb & Garthwaite, 2001), two regions in which the NO-cGMP pathway contributes prominently to synaptic plasticity (Garthwaite, 2000). When studied as purified proteins, the $\alpha1\beta1$ and $\alpha2\beta1$ isoforms have appeared similar in terms of their maximal activity, sensitivity to the NO donor diethylamine/NO adduct (DEA/NO) and pharmacological characteristics (Russwurm *et al*., 1998). Measured in this way, however, the potency of DEA/NO is a poor indicator of sensitivity to NO (Bellamy *et al*., 2002) and so neither the absolute nor relative potencies of NO on the two receptors can be deduced from the results.

The $\beta2$ subunit has remained enigmatic. The cDNA was first identified in the kidney with lesser amounts in the liver (Yuen *et al*., 1990), but recently $\beta2$ mRNA was also detected in the brain (Gibb & Garthwaite, 2001). Several attempts to obtain NO-stimulated GC activity in cells transfected with $\beta2$ together with other subunits have apparently been unsuccessful (Denninger & Marletta, 1999; Koesling & Friebe, 2000),

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Table 1 Primers used to generate full-length GC cDNAs

PCR primers	*Sequence 5′–3′*	*Accession*
α1 (f)	GCAGGATCCAGGAACACC**ATG**TTCTGCAGG	M57405
α1 (r)	AACACAGAAACAATGGCGGCTC	
β1 (f)	GCAGTCGACGACACC**ATG**TACGGTTTTGTG	M22562
β1 (r)	GTTCTTTTTGCCCCACAAAGG	
α2 (f)	GGCGGATCCAGC**ATG**TCTCGCAGGAAGATTTCATC	AF109963
α2 (r)	GGCGGATCCATCTCAGAGGCTAGTTTCTCGGAG	
β2 (f)	GGAAGATCTGTGTCC**ATG**GAAGCCATTCTG	M57507
β2 (r)	GCGGGATCCTTCTCGTGATC**A**CAGCACCACAAC	
vβ2 (f)	GGGGGATCCACC**ATG**TATGGATTCATCAACACCTGC	AY004153
vβ2 (r)	CCAAGTGTCCGCAGCATCCTGTC	AY004153 M57507

Restriction sites are underlined and the ATG initiation codons are in bold. Disruption of the β2 isoprenylation consensus sequence was achieved by mutating the β2 stop codon to a glycine residue by altering the adenine indicated in bold to cytosine.

although there has been one report that the combination of α1 and β2 tagged with green fluorescent protein (GFP) was able to generate GMP when expressed transiently in COS-7 cells (Gupta *et al*., 1997). Recently, the role of the β2 subunit has been further complicated by the discovery of a variant β2 (vβ2), containing an additional 60 amino acids at the N terminus, adjacent to the haem-binding domain. It has been claimed that this protein functions as a homodimer and that it has a 10-fold higher affinity for NO than the α1β1 heterodimer (Koglin *et al*., 2001). This latter conclusion is in doubt because of the method used (Bellamy *et al*., 2002). Furthermore, the physiological relevance of the vβ2 subunit remains questionable because GC activity required a high concentration (4 mM) of Mn^{2+} (rather than the usual Mg^{2+}), which does not exist in cells.

As with any signalling molecule, it is important to understand how the different receptors function when they are expressed within cells, but for the known and putative NO_{GC}Rs this remains largely unexplored. In cells from a brain region (the cerebellum), several properties of the operative receptor (probably the α1β1 isoform; Gibb & Garthwaite, 2001) were different from those reported for the α1β1 and α2β1 isoforms in cell-free systems (Bellamy *et al*., 2000; Bellamy & Garthwaite, 2001). While some of these differences have now been reconciled (Bellamy *et al*., 2002), one property that remains peculiar to GC in its natural environment is that it desensitises rapidly following addition of NO (Bellamy *et al*., 2000; Bellamy & Garthwaite, 2001). In concert with varying phosphodiesterase activities, this profile of activity has been hypothesised to generate diverse cGMP signals, enabling the second messenger to engage different downstream targets in different cells. Whether desensitisation is peculiar to specific NO_{GC}Rs or is a general property of the receptor within cells has not been investigated. The aim of the present work was to analyse this and other properties of all the known and putative NO_{GC}Rs expressed in cells.

Methods

Materials

YC-1, DEA/NO and DETA/NO were from Alexis Biochemicals (Nottingham, U.K.); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and L-nitroarginine were from Tocris (Avonmouth, U.K.); 3-isobutyl-1-methylxanthine (IBMX) and haemoglobin were from Sigma-Aldrich (Dorset, U.K.).

RT–PCR and cloning of GC subunits

Total RNA was prepared from fresh lung (for α1 and β1 subunits) or fresh kidney tissue (for β2 and vβ2) from 8-day-old Wistar rats (either sex). Trizol reagent (Sigma-Aldrich) was used according to the manufacturer's instructions to isolate the RNA. For α2, rat kidney poly A^+ RNA was purchased from BD Biosciences (Oxford, U.K.). First-strand cDNA synthesis was performed using Thermoscript reverse transcriptase (Invitrogen Ltd, Paisley, U.K.) and 1–2 μg of total or poly A^+ RNA per reaction. Platinum *Pfx* proofreading DNA polymerase was subsequently used to generate the PCR products from 5% of the reverse transcription reaction. The primers used are listed in Table 1 and PCR was performed as follows: α1, 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 68°C for 2 min, followed by 10 min at 68°C; α2, 95°C for 5 min, 65°C for 1 min, 68°C for 3 min, followed by 40 cycles of 94°C for 30 s, 65°C for 30 s, 68°C for 2 min 15 s, followed by 10 min at 68°C; β1, 95°C for 5 min followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 2 min 10 s followed by 10 min at 68°C; β2, 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 2 min 10 s, followed by 10 min at 68°C; vβ2, 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 68°C for 30 s, followed by 5 min at 68°C. PCR products were cloned into the vector pCR2.1 using the TA-Cloning system (Invitrogen Ltd, Paisley, U.K.) and sequenced by MWG Biotech-UK, Ltd. Sequences were confirmed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Full-length subunit cDNAs were subcloned into an expression vector derived from pECFP-C1 (BD Biosciences, Oxford, U.K.), pCMV, in which the fluorophore encoding sequence was removed by digestion with *Age*I and *BrsG*I, end repaired with T4 DNA polymerase (InVitrogen Ltd, Paisley, U.K.) and ligated overnight with T4 DNA ligase (New England Biolabs UK Ltd, Hertfordshire, U.K.).

Generation of full-length vβ2 *subunit cDNA*

RT–PCR was used to amplify a 278 bp region from the amino terminus of the vβ2 mRNA. Full-length vβ2 cDNA was

generated by exploiting the unique amino terminal *Xmn*I restriction site common to both the $\beta 2$ and $v\beta 2$ cDNA. The $v\beta 2$ PCR product was digested with both *BamH*I and *Xmn*I and the resulting fragment spliced directly into the corresponding region of the pCMV/$\beta 2$ plasmid *via* the *Bgl*II and *Xmn*I sites.

Tagging the β2 subunit with enhanced yellow fluorescent protein (pEYFP)

RT–PCR was used to generate either full-length wild-type $\beta 2$ subunit cDNA or full-length mutant $\beta 2$ cDNA in which the carboxy terminal isoprenylation consensus sequence was ablated (see Table 1). Newly introduced *Bgl*II and *BamH*I restriction sites were used to introduce both cDNAs into the pEYFP vector in-frame, producing amino-terminally tagged $\beta 2$ subunits.

Transfections and tissue culture

COS-7 cells were maintained in Dulbecco's modified Eagles medium containing 10% foetal bovine serum and 100 U ml^{-1} of both penicillin and streptomycin (Invitrogen Ltd, Paisley, U.K.). Transfections were optimised and performed using Effectene Transfection Reagent (Qiagen U.K. Ltd, West Sussex, U.K.) according to the manufacturer's instructions. Transfected cells were left for 48 h prior to harvesting by trypsinisation. The transfection efficiency was routinely approximately 40%.

Determination of cGMP accumulation in transfected cells

For each experiment, cells from two or four 35 mm wells were pooled, spun at $1500 \times g$ for 5 min, the supernatant aspirated and the cells resuspended in 400 or 800 μl of incubation medium (NaCl 130 mM, KCl 3 mM, $MgSO_4$ 1.2 mM, Na_2HPO_4 1.2 mM, Tris HCl 15 mM, $CaCl_2$ 2 mM, glucose 11 mM, IBMX 1 mM, and L-nitroarginine 100 μM, pH 7.4). DEA/NO was freshly prepared in 10 mM NaOH prior to use. ODQ and YC-1 were prepared as concentrated stock solutions in DMSO, such that the final concentration of DMSO in each experiment did not exceed 1%. Cell suspensions were preincubated at 37°C for 10 min in the presence of IBMX, with or without YC-1 or ODQ, before addition of DEA/NO for the required time (see figure legends). At the end of the experiment, aliquots of cells (50 μl) were inactivated by boiling in 200 μl Tris-HCl 50 mM, EDTA 4 mM (pH 7.4) for 3 min. Levels of cGMP were determined by radioimmunoassay and protein levels by BCA assay (Perbio Science U.K. Ltd, Cheshire, U.K.). One set of control experiments used untransfected COS-7 cells, which showed no significant alteration in cGMP levels on exposure to DEA/NO (200 nM; 2 min), and no significant effect of YC-1 (30 μM) or ODQ (1 μM) in the absence or presence of DEA/NO. Controls for the activity of the heterodimers included transfecting COS-7 cells with cDNAs for each of the single subunits. No measurable increase in cGMP occurred on addition of DEA/NO (100–200 nM, 2 min exposure), indicating that the endogenous expression of any of the subunits is too low to contribute to the results.

Assay of soluble and particulate GC activity

Combinations of the GC subunits were cotransfected as described above. After 48 h, two wells were harvested and resuspended in 500 μl ice-cold lysis buffer (Tris-HCl, 10 mM; DTT, 1 mM) and then sonicated briefly (four 0.5 s pulses) on ice. Sonicated samples were then centrifuged at $100{,}000 \times g$ for 1 h at 4°C. The supernatant fraction was stored on ice while the pellet was carefully resuspended in 500 μl lysis buffer. Both fractions were assayed immediately for NO-activated GC activity as described (Bellamy *et al*., 2000).

Applying clamped NO concentrations

Clamped NO concentrations were achieved by allowing a dynamic equilibrium to exist between NO release from the donor DETA/NO and NO inactivation by haem in red blood cells (Bellamy *et al*., 2002). The red blood cells were resuspended at around 2 million cells ml^{-1} in incubation medium (as described above) with the addition of 0.5% bovine serum albumin (Sigma, Dorset, U.K.) and kept on ice. Before use, the red blood cells were warmed in a shaking water bath at 37°C for 1 min and then DETA/NO was added. After 2 min, when a steady-state NO concentration is established, COS-7 cells transfected with GC were added (80 μl ml^{-1}), with mixing, from a suspension (1 mg protein ml^{-1}) that had been preincubated for 10 min at 37°C in the presence of 1 mM IBMX and 0.5% bovine serum albumin. Aliquots of the mixture (50 or 100 μl) were withdrawn at various times and inactivated as above. In each experiment, the amplitude of the clamped NO concentration was measured (ISO-NOP, World Precision Instruments, Stevenage, U.K.) 2 min after addition of DETA/NO (100–500 μM).

Results

Responses of heterodimer combinations to DEA/NO

To investigate the comparative properties of $NO_{GC}R$ heterodimers in living cells, COS-7 cells were transfected with each of the four subunit combinations ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 1\beta 2$ and $\alpha 2\beta 2$) and exposed to DEA/NO which degrades with a half-life of 2.1 min at 37°C (pH 7.4), releasing the authentic NO radical. Receptor function was monitored by measuring the associated GC activity (i.e. the enzymatic activity of the NO-bound receptor). IBMX (1 mM) was present throughout to reduce cGMP breakdown by phosphodiesterases to negligible rates (see below) and L-nitroarginine was included to inhibit any NO synthase activity. Following a 2 min exposure, when the NO concentration is near its peak (Schmidt *et al*., 1997), cells transfected with all the four combinations generated cGMP. Concentration–response curves showed that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ both produced high-amplitude cGMP responses, peaking at 500–800 pmol mg protein^{-1} (Figure 1a,c). In addition, the two isoforms appeared similarly sensitive to DEA/NO, the EC_{50} values both being about 5 nM. The $\beta 2$ combinations were markedly less active, maximal cGMP accumulation being about 20 and 10 pmol mg protein^{-1} from $\alpha 1\beta 2$ and $\alpha 2\beta 2$, respectively. Moreover, sensitivity to DEA/NO was reduced compared with the corresponding $\beta 1$-containing subunits, the EC_{50} values both being about 20 nM (Figure 1b,d). The responses of $\alpha 2\beta 2$ and $\alpha 1\beta 2$ peaked at 100 nM DEA/NO, but

the curves were distinctly bell-shaped, such that cGMP accumulation became progressively less at higher concentrations (Figure 1b,d).

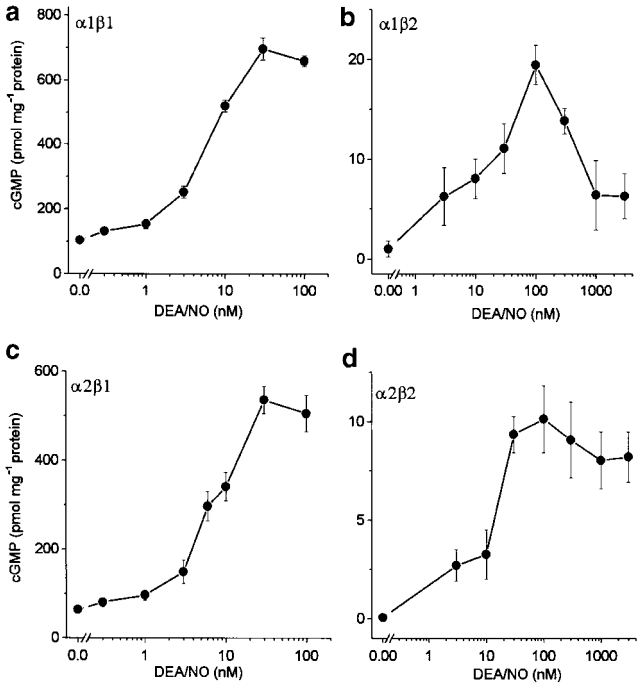

Figure 1 Sensitivity of the four possible heterodimeric GC-coupled NO receptors to DEA/NO. COS-7 cells cotransfected with different combinations of α and β subunits (as indicated) were exposed to different concentrations of DEA/NO for 2 min. Data are means $\pm$ s.e.m. ($n=8$).

The activation kinetics of the different heterodimer combinations was examined using DEA/NO concentrations yielding maximal cGMP responses so as to avoid the variation in GC activity over time occurring at submaximal concentrations as a result of the continuously rising NO concentrations. On addition of 100 nM DEA/NO to cells expressing both $\alpha1\beta1$ and $\alpha2\beta1$, cGMP rose rapidly over the first 20 s, but then the rate of accumulation slowed and a plateau developed after 60–90 s (Figure 2a,c). In order to extract the kinetic profile of GC activity from these data, it is necessary to know the rate of cGMP breakdown and, if required, correct for it (Bellamy *et al*., 2000). To do so, cells expressing $\alpha1\beta1$ were allowed to accumulate cGMP maximally by stimulating them with 100 nM DEA/NO for 2 min. Then 10 μM haemoglobin was added to remove free NO and cGMP followed over the next 2.5 min. The level of cGMP fell only slowly (e.g. it remained at $91\pm5\%$ of the prehaemoglobin level after 1 min and $83\pm3\%$ after 2 min; $n=3$), indicating that phosphodiesterase activity in the presence of 1 mM IBMX was negligible over the relevant time period. This being so, the profile of sGC activity can be extracted from the cGMP accumulation data simply by fitting the data with a suitable function and then differentiating. As in previous studies on brain cells (Bellamy *et al*., 2000), the data were well fitted by a hyperbolic function. The corresponding GC activity in the cells expressing $\alpha1\beta1$ and $\alpha2\beta1$ extracted in this way was essentially identical. It rose to a peak of over 20 pmol mg^{-1} protein s^{-1} and then sharply declined such that the activity was halved by 30 s and almost back to zero after 2 min (Figure 2a,c).

With the $\alpha1\beta2$ and $\alpha2\beta2$ subunit combinations, cGMP accumulated more gradually, but the rate still slowed after about 30 s. The corresponding GC profile indicated relatively

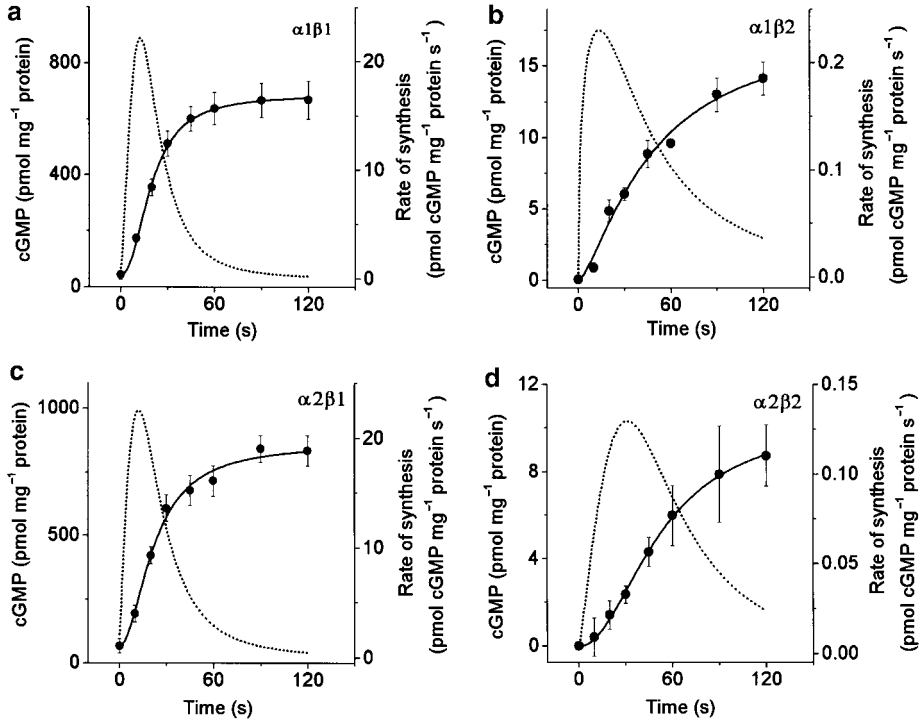

Figure 2 Activation kinetics of the four possible heterodimeric GC-coupled NO receptors on addition of DEA/NO. The cells were exposed to supramaximal concentrations of DEA/NO (100 nM for $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha2\beta2$, 200 nM for $\alpha1\beta2$) at $t=0$ and cGMP levels followed over time. Data are means $\pm$ s.e.m. ($n=6$). Solid lines are hyperbolic fits to the data which, when differentiated, provide the underlying profiles of GC activity (broken lines, right axes).

low peaks of activity, approximately 0.2 pmol mg^{-1} protein s^{-1} for $\alpha1\beta2$ and 0.1 pmol mg^{-1} protein s^{-1} for $\alpha2\beta2$. Thereafter, the activity fell more slowly than with the $\beta1$-containing isoforms, the halftimes being about 60 s (Figure 2b,d).

Sensitivity of the α1β1 and α2β1 isoforms to clamped NO concentrations

The responsiveness of the GCs to DEA/NO may be an unreliable predictor of their absolute and relative sensitivities to NO, because the NO concentration changes with time (Bellamy *et al*., 2002). To address this problem, experiments were carried out using constant, known NO concentrations. Clamped concentrations were achieved using a method whereby NO release from the slow donor DETA/NO is allowed to become balanced by NO inactivation by red blood cells (Bellamy *et al*., 2002). Cells were then added to the pre-equilibrated mixture, but only small volumes can be added without perturbing the equilibrium significantly. This meant that, for the resulting cGMP levels to be measurable, the experiments were restricted to cells expressing the more active $\alpha1\beta1$ and $\alpha2\beta1$ heterodimers. A detailed measurement of the time course of cGMP accumulation in cells expressing the $\alpha1\beta1$ combination and exposed to 10 nM NO (Figure 3a) showed the shape to be similar to that found when a supramaximal DEA/NO concentration (100 nM) was applied to cells containing $\alpha1\beta1$ or $\alpha2\beta1$ (Figure 2a, c). The GC kinetics extracted from the data using the clamped NO concentration was more rapid, however, with the peak activity occurring within 2 s, followed by desensitisation to 50% of the maximum by about 12 s (Figure 3a).

Steady-state concentration–response curves were obtained for each of the receptors using a 1 min exposure to NO (Figure 3b). Both curves were maximum at 10 nM NO but were bell-shaped, such that higher concentrations (30–70 nM) generated lower cGMP levels. The threshold NO concentration in both cases was about 0.5 nM. Fitting the rising portions of the curves to the Hill equation indicated that the $\alpha1\beta1$ receptor had an EC_{50} of 1.2 ± 0.1 nM and a Hill coefficient of 1.8 ± 0.3, and the $\alpha2\beta1$ receptor an EC_{50} of 2.2 ± 0.2 nM and a Hill coefficient of 1.2 ± 0.1.

Activity of the νβ2 subunit

Transfection of the $v\beta2$ subunit on its own into COS-7 cells resulted in cGMP accumulation in response to DEA/NO that was maximally about 13 pmol cGMP mg protein^{-1} and so was of comparable magnitude to the responses found with the $\beta2$ heterodimers (Figure 4a). Also, similar to these heterodimers, the concentration–response curve was bell-shaped and showed an EC_{50} of about 20 nM DEA/NO (Figure 4b). If the cells were cotransfected with $\alpha1$ or $\alpha2$, there was no discernable effect on $v\beta2$ activity (data not shown).

Analysis of the kinetics in $v\beta2$-transfected cells (Figure 4b) indicated a faster initial rise in cGMP than with the $\beta2$ heterodimers, with the plateau level being attained after about 30 s. Accordingly, the extracted GC profile rose sharply to peak at about 0.5 pmol mg protein^{-1} s^{-1} and then fell at a similar rate to that seen with the $\beta1$-containing isoforms.

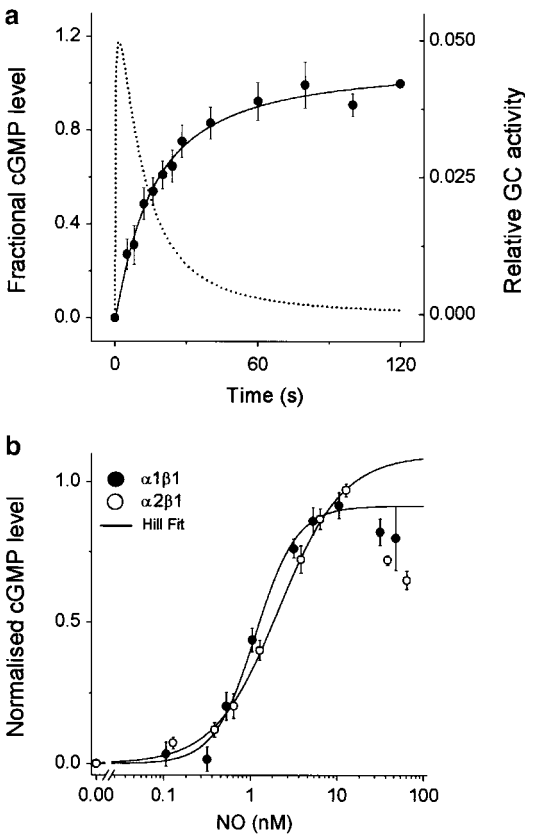

Figure 3 Kinetics of $\alpha1\beta1$ and $\alpha2\beta2$ NO_{GC}Rs exposed to clamped NO concentrations. In (a), COS-7 cells expressing the $\alpha1\beta1$ receptor were exposed to 10 nM NO at $t=0$ and then cGMP levels were followed over time. Each point is the mean$\pm$s.e.m. of data normalised to the cGMP level found after 120 s exposure, which in different experiments ranged from 400 to 700 pmol mg^{-1} protein ($n=6$). The solid line through the data is a fit to a hyperbolic function which, when differentiated, gives the indicated profile of GC activity (broken line; right axis). (b) Concentration–cGMP response curves for cells expressing the $\alpha1\beta1$ and $\alpha2\beta1$ receptors, as indicated. The cells were exposed to fixed NO concentrations for 1 min. The data are normalised to the maximum cGMP response observed in each experiment (range, 300–500 pmol mg^{-1} protein) and represent the means$\pm$s.e.m. ($n=6$–9). The solid lines are fits of the rising portions of the curves to the Hill equation.

Pharmacological properties

Two pharmacological tools are widely used to probe the involvement of NO_{GC}Rs in biological phenomena, the allosteric activator YC-1 (Friebe & Koesling, 1998), and the inhibitor ODQ (Garthwaite *et al*., 1995). Experiments were carried out to evaluate their activity towards the different GCs in cells. Under 'basal' conditions (no added NO), cGMP was highest (about 100 pmol mg protein^{-1}) in the cells expressing the $\alpha1\beta1$ and $\alpha2\beta1$ isoforms and exposure to YC-1 (30 μM) resulted in marked (five- to six-fold) increases, to levels normally attained on maximal NO stimulation (Figure 5a). Basal cGMP in the $\beta2$ combinations was about 10 pmol mg protein^{-1}. With $\alpha1\beta2$, YC-1 caused a two-fold elevation, whereas with $\alpha2\beta2$ no significant increase was observed. ODQ (1 μM) more than halved the basal level of cGMP in the cells expressing $\alpha1\beta1$ and $\alpha2\beta1$ but had no significant effect in cells containing $\alpha1\beta2$ or $\alpha2\beta2$ (Figure 5a).

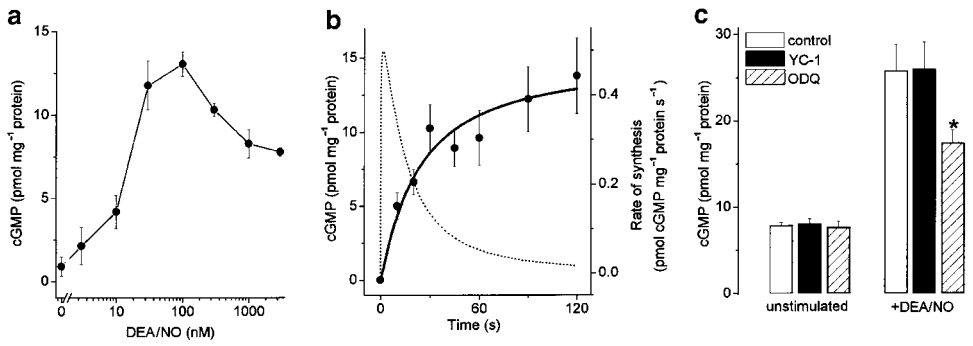

Figure 4 Properties of the $v\beta2$ subunit expressed in COS 7 cells. (a) The cells were exposed to different concentrations of DEA/NO for 2 min. Data are means ± s.e.m. ($n=8$). (b) Cells were exposed to supramaximal concentrations of DEA/NO (100 nM) at $t=0$ and cGMP levels followed over time. Data are means ± s.e.m. ($n=6$). The solid line is a hyperbolic fit to the data which, when differentiated, provides the underlying profile of GC activity (broken line; right axis). (c) cGMP levels in cells that were unstimulated (three left panels) or stimulated with 100 nM DEA/NO (three right panels) in the absence or presence of YC-1 (30 μM) or ODQ (1 μM) as indicated. The preincubation period with YC-1 or ODQ was 10 min. Results are means ± s.e.m. ($n=6$); $^*P<0.05$ versus DEA/NO alone (*t*-test).

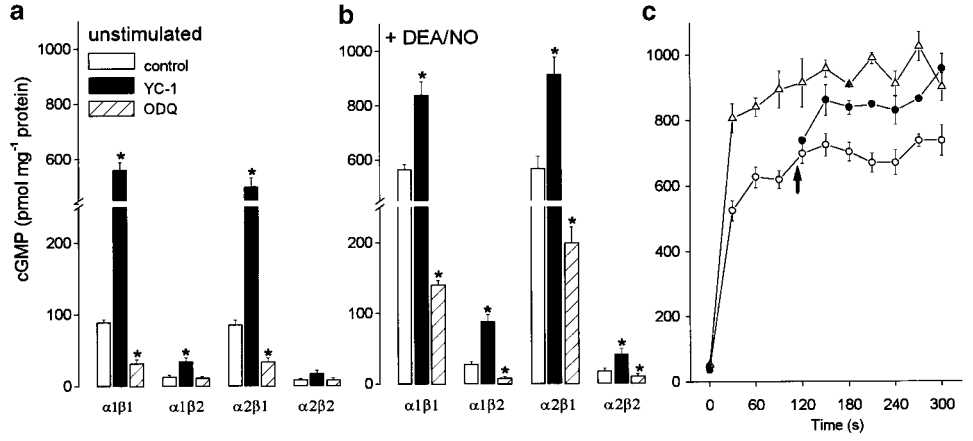

Figure 5 Pharmacological properties of the four possible heterodimeric GC-coupled NO receptors expressed in COS-7 cells. The data in (a) show the basal cGMP levels (open bars) and the effect of YC-1 (30 μM, filled bars) and ODQ (1 μM, hatched bars) in the absence of any added DEA/NO. $^*P<0.05$ versus basal cGMP levels. The data in (b) show the equivalent levels following exposure to supramaximal DEA/NO concentrations (100 nM for $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha2\beta2$, 200 nM for $\alpha1\beta2$). $^*P<0.05$ versus DEA/NO alone. In all cases, the cells were preincubated for 10 min with YC-1 or ODQ; they were then sampled to obtain the data in (a) and then DEA/NO was added and the cells inactivated 2 min later for the data in (b). Results are means ± s.e.m. ($n=6$); statistical significance was assessed by the Student's *t*-test. (c) Effect of YC-1 (100 μM) on cells expressing the $\alpha1\beta1$ receptor, added before ($t=-5$ s, open triangles) and after ($t=115$, arrow, filled circles) DEA/NO-induced desensitisation of GC activity, compared to control (open circles). Data are means ± s.e.m. ($n=6$).

YC-1 has only a very weak ability to activate GC in cells in the absence of NO (Schmidt *et al*., 2001; Bellamy & Garthwaite, 2002). The marked stimulatory effect of YC-1 and inhibitory effect of ODQ on cGMP in cells expressing $\alpha1\beta1$ and $\alpha2\beta1$ in the presence of an NO synthase inhibitor and in the absence of added NO indicated, nevertheless, that NO was present in active concentrations. To test this, haemoglobin (10 μM) was added to cells transfected with $\alpha1\beta1$. The basal cGMP was reduced by 65 ± 2% ($n=3$). Within the same experiments, ODQ (1 μM) reduced the levels 68 ± 1% ($n=3$) and no further reduction was observed with a combination of haemoglobin and ODQ (68 ± 1%; $n=3$).

Under conditions of maximal GC stimulation by DEA/NO, YC-1 caused a further 40–100% increase in cGMP accumulation in all heterodimer combinations (Figure 5b). In addition, in cells expressing the $\alpha1\beta1$ isoform, addition of YC-1 at a time when the receptor is maximally desensitised (after 2 min exposure to DEA/NO) resulted in an additional burst of cGMP formation (Figure 5c) indicating that, as with the native receptor (Bellamy & Garthwaite, 2002), YC-1 reverses GC desensitisation. ODQ inhibited the DEA/NO-stimulated cGMP accumulation in all combinations to 25–50% of control (Figure 5b).

Cells transfected with $v\beta2$ showed no change in basal cGMP levels following addition of either YC-1 or ODQ (Figure 4c) and, in the presence of 100 nM DEA/NO, both control cells and cells exposed to YC-1 generated the same (three-fold) increases in cGMP. Exposure to ODQ, however, reduced the NO-stimulated rise by about 50% (Figure 4c).

Subcellular location of the β2 subunit

Of the GC subunits, $\beta2$ and $v\beta2$ uniquely contain an isoprenylation consensus sequence (CVVL) at the carboxy terminal which, in principle, could target the enzyme to membranes (Yuen *et al*., 1990). In order to address this possibility, we tagged the amino terminal of the $\beta2$ subunit with EYFP and used confocal microscopy to study its

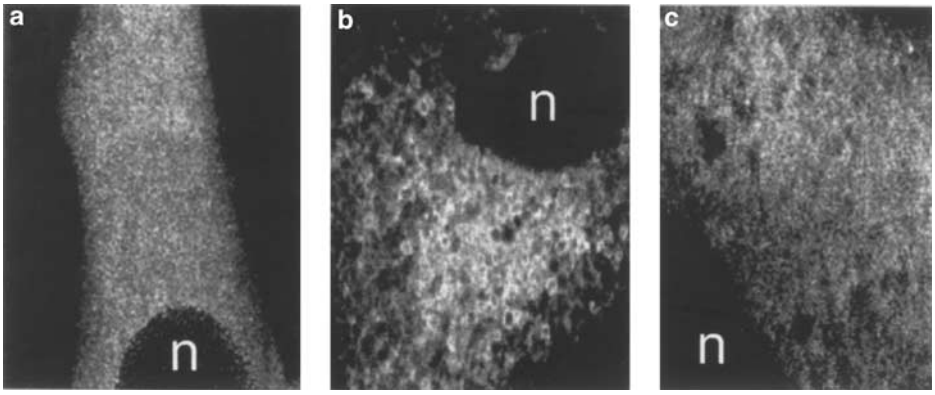

Figure 6 Confocal laser imaging of fluorescently tagged GC subunits in COS-7 cells. The panels show representative images of cells transfected with α1-EYFP/β1 (a), α1/EYFP-β2 (b) and α1/EYFP-β2 with a mutated isoprenylation sequence (c). Nuclei are labelled '*n*'.

Table 2 Partitioning of GC activity

	COS-7	*α1β1*	*α1β2*	*Mutated α1β2*	*α1 only*
Supernatant	37±1	31316±2813	457±16	434±11	62±1
Pellet	46±1	2879±205	66±1	64±3	27±1

Results are expressed as pmol cGMP mg^{-1} total protein. Samples were stimulated by exposure to 10 μM DEA/NO for 10 min at 37°C ($n=5$).

intracellular location when cotransfected with α1 into COS-7 cells. For comparison, the EYFP-tagged α1 was cotransfected with β1. The α1-EYFP/β1 cotransfected cells displayed an even fluorescence throughout the cytoplasm, with apparent exclusion from the nuclei (Figure 6a). In the α1/EYFP-β2 cotransfected cells, the nucleus was similarly nonfluorescent, but the protein appeared to be associated with a membranous complex within the cytoplasm (Figure 6b). To determine the role of the isoprenylation consensus sequence in this localisation, an EYFP-tagged mutant of β2 lacking this sequence was generated. Cotransfection of this mutated EYFP-β2 with α1 resulted in a loss of apparent association with intracellular membranes and, instead, a diffuse cytosolic distribution resembling that of α1/EYFP-β1 (Figure 6c). These localisation studies were all repeated in HeLa cells, with essentially identical results (data not shown), indicating that the findings with COS-7 cells are not cell specific.

When the cells transfected with α1β1 or α1β2 were homogenised and GC activity in supernatant and particulate fractions assayed, it appeared predominantly in the supernatant in both cases and no difference was observed whether the isoprenylation sequence in β2 was mutated or not (Table 2).

Discussion

That cells expressing the two established heterodimeric NO_{GC}Rs, α1β1 and α2β1, generated large cGMP responses was as predicted (Russwurm *et al*., 1998). More surprising was that the α1β2 and α2β2 combinations both possessed NO-stimulated GC activity, although their maximal activities were relatively low. In several tests, transfection of HeLa cells with α1 or α2 together with β2 did not result in measurable DEA/NO-evoked cGMP accumulation (B.J. Gibb & J. Garthwaite, unpublished observation), suggesting that cell-specific factors may be required for active β2 heterodimers. In this respect, it may be relevant that COS-7 cells are derived from the kidney, which contains the most abundant message for β2 (Yuen *et al*., 1990). Moreover, EYFP tagging indicated that the β2 subunit associated with an intracellular membranous complex by virtue of its isoprenylation sequence. This complex had the typical appearance of the endoplasmic reticulum in COS-7 cells (Shakur *et al*., 2001). Although no particulate activity attributable to membrane association through the isoprenylation sequence was detected in cell lysates, it may be that, as with cGMP-inhibited phosphodiesterase (PDE3) in these cells (Shakur *et al*., 2001), the association with the endoplasmic reticulum is weak and easily disrupted. In contrast to the apparent localisation of the β2 subunit, the α2β1 can associate with synaptic proteins in the brain (Russwurm *et al*., 2001) and α1β1 appears to attach to the cell membrane in response to a rise in Ca^{2+} (Zabel *et al*., 2002), so that all possible GC isoforms may be differentially localised in cells.

The absence of measurable GC activity of the *v*β2 protein in extracts of Sf9 cells, except in the presence of high Mn^{2+} concentrations or when the isoprenylation site was mutated (Koglin *et al*., 2001), questions its physiological significance. Our finding that it is active on its own in COS-7 cells suggests that it could form a functional homomer in the absence of Mn^{2+} (or mutation). The maximal cGMP response in cells expressing *v*β2 was comparable to that of the α1β2 and α2β2 heterodimers, indicating that they all possess similar GC activity. Although relatively low, the 10–20 pmol mg $protein^{-1}$ formed by *v*β2 or the β2 combinations corresponds to an average intracellular cGMP concentration in the low micromolar range (assuming 10 μl cell volume mg $protein^{-1}$). If applicable to normal cells, this is well in the range needed to activate cGMP-dependent protein kinases.

All GC subunits expressed in cells were highly sensitive NO detectors, the EC_{50} values for DEA/NO being about 5 nM (α1β1, α2β1) or 20 nM (α1β2, α2β2, *v*β2). These values are 1–2 orders of magnitude lower than those typically found for DEA/NO using standard GC assays on cell lysates or purified proteins (Russwurm *et al*., 1998; Koglin *et al*., 2001), but the EC_{50} values reported by these assays are poor indicators of the sensitivity of the receptors to NO (Bellamy *et al*., 2002). Using clamped NO concentrations, we found the EC_{50} values of α1β1 and α2β1 to be 1 and 2 nM respectively and the maximal

responses to occur at about 10 nM. These values are compatible with the data using DEA/NO once the profile of NO release is considered. Assuming that the stoichiometry of NO release is 1.6 (Griffiths & Garthwaite, 2001) and that NO is consumed by autoxidation (Schmidt *et al*., 1997), with 5 nM DEA/NO (approximately the EC_{50} for both heterodimers), the average NO concentration over the 2 min exposure is 2 nM; at the maximum for DEA/NO (30 nM), it is 13 nM. Thus, use of DEA/NO during the relatively short exposures can provide quite an accurate index of sensitivity to NO.

When constant NO concentrations are applied to the purified $\alpha1\beta1$ receptor, the EC_{50} (4 nM) is about three-fold higher than that found in cells expressing this isoform. This difference is predicted by GC desensitisation in cells truncating the concentration–response curve (Bellamy & Garthwaite, 2001). Furthermore, the apparent Hill slope in the cells expressing the $\alpha1\beta1$ isoform (1.8) was similar to that reported for the purified protein (Bellamy *et al*., 2002) suggesting that the receptor in cells also exhibits at least two NO-binding sites. Whether this also applies to the $\alpha2\beta1$ receptor (apparent Hill slope of 1.2) is ambiguous. Studies on the purified protein, where the complication of desensitisation is eliminated, are needed to evaluate this possibility.

All the recent evidence, therefore, converges to position the potency of NO for its $\beta1$-containing heterodimeric receptors in the very low nM range, which is far from the value of 250 nM (Stone & Marletta, 1996) that has generally been considered correct. The high sensitivity of the $\alpha1\beta1$ and $\alpha2\beta1$ receptors to NO is further attested by the finding that the 'basal' cGMP levels in COS-7 cells expressing these isoforms in the absence of endogenous or added NO were about 70% reduced by the NO scavenger haemoglobin or the GC inhibitor ODQ. Presumably, the environment was contributing biologically active concentrations of NO (Friebe *et al*., 1996) which, from the concentration–response curves (Figures 1a,c and 3b), would be in the 0.5–1 nM range. This source of NO is not usually considered to contribute to results obtained *in vitro* but, we suggest, should be in the future.

Concerning the $\beta2$-containing heterodimers and the presumed $v\beta2$ homomer, analysis of the data using DEA/NO (as above) suggests that NO is about four-fold less potent (EC_{50} ~8 nM) than on the $\beta1$ varieties. This contradicts the conclusion, made on the basis of the potency of DEA/NO in standard GC assays, that the $v\beta2$ homomer is 10-fold more sensitive to NO than the $\alpha1\beta1$ receptor (Koglin *et al*., 2001).

A prominent feature of native NO_{GC}Rs in cerebellar cells is that they desensitise rapidly (Bellamy *et al*., 2000; Bellamy & Garthwaite, 2001). This behaviour is typical of most neurotransmitter receptors but, in the case of NO_{GC}Rs, is not seen in cell-free preparations. It is apparently also not obvious in intact platelets under conditions where phosphodiesterases maintain cGMP at low levels (Mullershausen *et al*., 2001), possibly because cGMP itself regulates receptor desensitisation (Wykes *et al*., 2002). When expressed in COS-7 cells all the heterodimer combinations, and $v\beta2$ on its own, desensitised. With a clamped NO concentration (10 nM), the speed of desensitisation of the $\alpha1\beta1$ receptor was similar to that measured in the cerebellar cells (Bellamy & Garthwaite, 2001). With $\alpha1\beta2$, $\alpha2\beta2$, and to a lesser extent $v\beta2$, desensitisation appeared somewhat slower, but the lower potency of NO for these GCs means that the buildup of NO to threshold concentrations and above will necessarily be slower than with the $\beta1$ heterodimers, which may contribute to the slower apparent desensitisation kinetics. The bell-shaped concentration–response curves observed for all GCs (most obviously with $\alpha1\beta2$ and $v\beta2$) is characteristic of rapidly desensitising receptors (Raman & Trussell, 1992; Paternain *et al*., 1998). The finding that the $\beta2$-containing GCs desensitise similarly to the $\alpha1\beta1$ and $\alpha2\beta1$ isoforms, despite the absolute activities of the $\beta2$-containing GCs being at least 50-fold lower, adds to the already considerable evidence (Bellamy *et al*., 2000; Bellamy & Garthwaite, 2002) that desensitisation is not simply caused by depletion of GTP substrate (cf Mullershausen *et al*., 2001).

YC-1 and ODQ had their predicted effects on the $\alpha1\beta1$ and $\alpha2\beta1$ receptors, but neither agent had been tested previously on the $\beta2$ heterodimers or on the presumed $v\beta2$ monomer. Our finding that YC-1 enhanced the NO-stimulated activity of both heterodimers indicates that they are also equipped with a functional YC-1-binding site. As YC-1 synergises with NO (Friebe *et al*., 1998), the lesser (or not significant) effect of this compound on the corresponding basal cGMP levels, compared with the $\beta1$-containing receptors, can be explained by the $\beta2$ heterodimers being intrinsically less sensitive to NO (see above) such that the ambient (environmental) NO concentration is too low to have much effect even in the presence of YC-1. The inhibitor ODQ, which oxidises the GC haem group (Garthwaite *et al*., 1995; Schrammel *et al*., 1996), affected the $\beta2$-containing GCs similarly to the $\beta1$-containing GC isoforms, suggesting that this approach is unlikely to be useful in differentiating between different GCs. The only agent found to discriminate between GCs was YC-1, which failed to influence NO-stimulated GC activity in cells expressing $v\beta2$. This finding supports the proposal that the YC-1-binding site is located within the α subunits (Friebe *et al*., 1999; Koglin *et al*., 2002).

In conclusion, all the putative NO_{GC}Rs when expressed heterologously possess NO-stimulated GC activity and are extremely sensitive to this diffusible messenger. They can be split into two broad groups: the $\beta1$-containing heterodimeric receptors, which possess high GC activity and the lowest EC_{50}s for NO (1–2 nM), and the $\beta2$-containing GCs which have lower GC activity and higher EC_{50}s (about 8 nM). All the GCs share a strongly desensitising profile of activity and, apart from a lack of effect of YC-1 on the presumed $v\beta2$ monomer, are indistinguishable by existing pharmacological interventions. The expression of the $\beta2$ subunits *in vivo* requires investigation.

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