Membrane association of nitric oxide-sensitive guanylyl cyclase in cardiomyocytes

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Abstract

Objective: Although the importance of the cyclic GMP (cGMP) signaling pathway in cardiac myocytes is well established, little is known about its regulation. Ca\(^{2+}\)-dependent translocation of nitric oxide (NO) sensitive guanylyl cyclase (GC\(_{NO}\)) to the cell membrane has been recently proposed to play a role. The aim of this study was to determine the possible functional relevance of GC\(_{NO}\) bound to the cardiomyocyte membrane.

Methods: Cytosolic and particulate fractions of adult rat cardiomyocytes were isolated and blotted, and their GC\(_{NO}\) activity was assayed in parallel experiments.

Results: In untreated cardiomyocytes, approximately 30% of \(\beta_1\)-and \(\alpha_1\)-subunits of GC\(_{NO}\) and a similar proportion of GC\(_{NO}\) activity were found in the particulate fraction. The dependence of GC\(_{NO}\) activity on pH, Ca\(^{2+}\), GTP and NO donor concentrations was similar in particulate and cytosolic fractions. Treatment of cardiomyocytes with the ionophore A23187 caused GC\(_{NO}\) to translocate to the sarcolemma, increased GC\(_{NO}\) activity in this fraction, and potentiated NO-mediated cGMP synthesis. These effects appeared to be mediated by Ca\(^{2+}\)-dependent changes on the phosphorylation status of GC\(_{NO}\), since they were enhanced by the non-selective inhibitor staurosporine and by the selective inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinase KN-93. The effect of drugs increasing intracellular Ca\(^{2+}\) on cGMP synthesis was clearly correlated with their effects on membrane-associated GC\(_{NO}\) activity but not with their effects on cytosol-associated GC\(_{NO}\).

Conclusion: These results are the first evidence that 1) GC\(_{NO}\) is associated with the cell membrane in cardiomyocytes, 2) the regulation of membrane-associated GC\(_{NO}\) differs from that of cytosolic GC\(_{NO}\), and 3) membrane association may have a crucial role in determining the response of cells to NO.

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1. Introduction

Cyclic GMP (cGMP) modulates important physiological functions in the cardiovascular system as vasodilation, Ca\(^{2+}\) cycling, endothelium permeability, or myocardial contractility [1]. Abundant evidence indicates that cGMP can modulate cell death during ischemia-reperfusion [2–7] and cGMP has been described to mediate late preconditioning in conscious rabbits [8].

cGMP can be synthetized by two different types of guanylyl cyclases: a nitric oxide (NO)-sensitive guanylyl cyclase (GC\(_{NO}\)), generally known as cytosolic or soluble guanylyl cyclase, and guanylyl cyclases that are integral proteins in the plasmatic membrane of the cell and can be stimulated by natriuretic peptides. GC\(_{NO}\) is constituted of...
two subunits, α and β, and two different isoforms of the α subunit (α₁ and α₂) and of the β subunit (β₁ and β₂) have been described. The α₁β₁ heterodimer is predominantly found in the cardiovascular system, while α₂β₁ has been mainly found in brain.

Little is known about how GCₐ is modulated in vivo. Rapid desensitisation of the signal [9] and phosphorylation by different protein kinases, as protein kinase C (PKC) [11], cyclic AMP-dependent protein kinase (PKA) [10,11], and cGMP-dependent protein kinase (PKG) [12], have been described.

Recent studies have challenged the classical concept of an exclusive cytosolic location of GCₐ. The isoform α₂β₁ has been found tightly associated to the neuron membrane through a PSD-95 mediated interaction [13]. A histochecmical study suggested the presence of the more amply distributed heterodimer, α₁β₁, in the sarcolemmal region of skeletal muscle fibres [14]. This association to the particulate fraction of α₁ and β₁ subunits has been recently demonstrated in myocardial tissue, endothelial cells and platelets [15], and in these later cells activation with ADP or collagen has been correlated with enzyme translocation to the membrane [15]. However, the mechanism of the interaction of α₁β₁ with the membrane was unresolved. A recent study has described a protein complex formed by eNOS, Hsp90 and GCₐ in endothelial cells, and that bradykinin and vascular endothelial growth factor potentiate the formation of this complex [16]. The contribution of particulate GCₐ to the cell response to NO remained unknown.

In this study, we analyze how association of GCₐ to membrane affects its biochemical properties in cardiomyocytes, the functional relevance of this association, and its potential regulation by Ca²⁺.

2. Materials and methods

The animal protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Research Commission on Ethics of Hospital Vall d’Hebron.

2.1. Cardiomyocyte and platelet isolation

Cardiomyocytes were obtained from adult rat hearts as previously described [17]. At the end of the procedure, culture dishes contained >85% of quiescent rod-shaped cells. Rat washed platelets were isolated from venous blood collected with sodium citrate [18].

2.2. Intracellular cGMP synthesis

After treatment, cells were stimulated for 1 minute with SNAP 100 μM (unless otherwise indicated) in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) as inhibitor of cGMP degradation. cGMP was quantified by radioimmunoassay using acetylated [³H]cGMP [17].

2.3. Membrane and cytosolic fractions

Following incubation with the different agents, cardiomyocytes were homogenized with a straight wall grinder (Radnoti Glass Technology) in ice-cold buffer A [in mM: Tris·HCl 50 (pH 7.4), sucrose 250, EDTA 0.1, dithiotreitol 1, plus protease inhibitors [17], the protein kinase inhibitor staurosporin 1 × 10⁻³, and the Ser/Thr protein phosphatase inhibitors okadaic acid 1 × 10⁻³ and cypermethrin 5 × 10⁻⁴]. After clearing the homogenates by centrifugation at 1000 × g for 15 min, the particulate and cytosolic fractions were obtained by centrifugation (100,000 × g for 1 h). Membrane fractions were homogenized with buffer A plus 10% glycerol. Triton X-100 solubility of particulate GCₐ was assessed as described [19]. Treated platelets were centrifuged, resuspended in buffer A, frozen in liquid nitrogen, thawed slowly on ice and then homogenized with a straight wall grinder and ultracentrifuged as mentioned for cardiomyocytes.

2.4. Guanylyl cyclase activity

Guanylyl cyclase activity was determined [17] by measuring cGMP (radioimmunoassay) synthesized after incubating the particulate and cytosolic extracts with no-additions (basal) or 100 μM SNAP in assay buffer [final concentrations (in mM): Tris·HCl 50 (pH 7.4), EGTA 1, dithiotreitol 1, GTP 2.5, MgCl₂ 5, phosphocreatine 10, IBMX 1, plus creatine kinase (50 U/ml)] at 37 °C for 30 min. GCₐ activity, calculated by subtracting basal activity, was linear with respect to time in the assay period. To analyze pH dependence of GCₐ activity, Tris·HCl was substituted by 30 mM PIPES (pH 6.0–6.8) or 30 mM HEPES (pH 6.8–8.0). Free Ca²⁺ concentrations were calculated by a modification of the RECIPC program (S. Roberston, University of Cincinnati, 1981).

2.5. Western-blotting

Proteins were separated by electrophoresis on a 10% SDS gel and transferred onto nitrocellulose membrane (Hybond-ECL, Amersham). Membranes were incubated with rabbit polyclonal antibodies to β₁ (aminoacids 605–619; used at 1/2000 dilution; Sigma) and α₁ (aminoacids 673–690; 1/20,000; Sigma) subunits of soluble guanylyl cyclase. A goat anti-rabbit IgG horseradish peroxidase conjugated (1/50,000; Pierce) was used as secondary antibody. Specificity of the immunostaining was assessed by displacing the corresponding bands by incubating in the presence of their respective immunization peptides (synthesized by Sigma Genosys). Quantitative chemiluminescence detection was performed with SuperSignal West Dura Extended Substrate.
(Pierce) and a 16-bit cooled CCD camera system (LAS-3000, Fujifilm). Equal loading of the different samples was confirmed by Ponceau Red staining.

### 2.6. Modulation of the membrane association of GC\(_{NO}\)

The effect of increased cytosolic Ca\(^{2+}\) concentration on the association of GC\(_{NO}\) to the membrane fraction was investigated by incubating cells 1 min with A23187 or 5 min with thapsigargin. Then, cells were: a) stimulated with SNAP for determining NO-dependent cGMP synthesis, and b) homogenized for determining content of \(\alpha_1\) subunit and GC\(_{NO}\) activity in cytosolic and membrane fractions.

The potential role of changes in GC\(_{NO}\) phosphorylation in this effect was investigated by analyzing its modulation by the protein kinase inhibitors staurosporin, Gö-6976, H-89, and KN-93. These drugs were added to the incubation media 4 min before stimulation with A23187, and maintained during the time of incubation with the ionophore.

### 2.7. Detection of GC\(_{NO}\) phosphorylation in intact cells

Cardiomyocytes or platelets were incubated for 3 h in medium containing 0.1 mCi/ml \(^{32}\)Porthophosphate (Amersham), washed with ‘cold’ medium, incubated for 5 min with or without 1 \(\mu\)M staurosporin and stimulated for 1 min with 10 \(\mu\)M A23187 or with no drugs. At the end of the stimulation period, cells were homogenized as described, but including a phosphatase inhibitor cocktail (Sigma) in the homogenization medium. Homogenates were fractionated by centrifugation at 100,000 \(\times\)g, and the \(\beta_1\) subunit of the cytosolic and particulate fractions immunoprecipitated by incubation with Protein G-agarose beads (Amersham Biosciences) previously bound to 15 \(\mu\)g of anti-\(\beta_1\) antibody. Phosphorylation of the \(\beta_1\) subunit in the immunoprecipitates was assessed by Western-blotting and phosphor screen (Fuji Photo Film Co.) autoradiography with a red laser scanner (Typhoon 9400, Amersham Biosciences). Analysis of \(\alpha_1\) phosphorylation was not...
possible since the antibody used against this subunit did not significantly immunoprecipitate the protein.

2.8. Intracellular Ca\(^{2+}\)

Changes induced by the Ca\(^{2+}\) ionophore A23187 and by thapsigargin were monitored by ratio-fluorescence imaging in cardiomyocytes loaded with fura 2-acetoxymethyl ester (Molecular Probes) as previously described [20].

2.9. Data analysis and statistics

Differences between groups were evaluated by means of paired Student’s \(t\) test when appropriated or one-way analysis of the variance. Individual comparisons between groups were performed using the Student–Newman Keuls test. Values are expressed as mean ± SEM. Nonlinear fitting was performed using SigmaPlot (SPSS Inc.).

3. Results

3.1. Membrane association of GC\(_{NO}\) in cardiomyocytes and platelets

Cytosolic and particulate fractions (100,000 \(\times g\)) of adult rat cardiomyocytes were isolated and blotted. Staining with an antibody against the \(\beta_1\) subunit of the GC\(_{NO}\), demonstrated that 28 ± 5% of \(\beta_1\) (65.1 ± 0.2 kDa), \(n = 6\), was in the membrane fraction (Fig. 1 A). A similar proportion of the \(\alpha_1\) subunit (75.7 ± 2.9 kDa) seemed to be associated to the particulate fraction, but the limited sensitivity of the antibody against this subunit in cardiomyocytes precluded precise analysis (Fig. 1B). Immunization peptides displaced \(\beta_1\) and \(\alpha_1\) bands both in platelets and cardiomyocytes (Fig. 2A and B). However, as shown in Fig. 2, an additional band in the cytosolic fraction (of 110–115 kDa) and in the membrane fraction (of 85–90 kDa) of cardiomyocytes were also displaced. Since the identity of these other bands is unknown, they were not used for the quantification of the \(\beta_1\) content. Throughout the rest of the study only the antibody against the \(\beta_1\)-subunit was used for blot analysis.

Measurement of GC\(_{NO}\) activity showed that 33 ± 4% of the activity was associated to the particulate fraction (5.1 ± 2.2 and 2.5 ± 1.1 pmol/mg protein \(\times\) min in the cytosolic and membrane fractions, respectively, \(p = 6\)). For comparison with a cell model in which the association of GC\(_{NO}\) to the membrane had been previously described, [15,21,22] cytosolic and membrane fractions were obtained from rat platelets (Fig. 1B). According to densitometric analysis of blots, platelets contained 100–150 times more GC\(_{NO}\) than cardiomyocytes. Specific activity of membrane-associated GC\(_{NO}\), calculated by dividing GC\(_{NO}\) activity by the densitometry of \(\beta_1\) in this fraction (in arbitrary units), was similar in platelets and cardiomyocytes (Fig. 1C).

To rule-out an unspecific presence of GC\(_{NO}\) in the membrane fraction, cardiomyocyte homogenates were extensively diluted (15 times) in a homogeneization buffer.

![Fig. 2. \(\beta_1\) (panel A) and \(\alpha_1\) (panel B) immunoreactivity of 65.1 kDa (for the \(\beta_1\) band) and 75.7 kDa (for the \(\alpha_1\) band) were blocked by their respective immunization peptides in cytosolic and particulate fractions of platelets and cardiomyocytes. Other bands (110–115 kDa in the cytosolic fraction and 85–90 kDa in the particulate fraction) were also displaced by peptides. Membrane association of GC\(_{NO}\) in cardiomyocytes was not altered by diluting the homogenate in a KCl containing buffer, but was completely disrupted by the presence of detergent (panel C).](https://academic.oup.com/cardiovascres/article-abstract/68/1/65/288281)
medium containing 150 mM of KCl instead of sucrose, and were incubated in agitation at 4°C for 30 min before the 100,000 × g centrifugation. These manoeuvres had no effect on the observed membrane association of the β1 subunit (Fig. 2C). To examine the possible presence of GCNO in glycolipid-rich domains, part of the homogenates was incubated in a sucrose containing homogenization medium (standard medium) with 1% Triton X-100 (30 min at 4°C in agitation). After this incubation, no significant immunostaining for the β1 antibody persisted in the particulate fraction (Fig. 2C).

3.2. Biochemical differences between cytosolic and particulate GCNO activity

Dependence of GCNO activity on GTP-, SNAP-, H⁺-and Ca²⁺-concentrations was analyzed for GCNO associated to the cytosolic and membrane fractions. Significant, although

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Fig. 3. GCNO activity in the soluble (>) and membrane (•) fractions of cardiomyocytes assayed at different concentrations of GTP (panel A) or SNAP (panel B). P values refer to the differences in the EC₅₀. C, GCNO activity in the absence of GTP or SNAP for each fraction.

![Graphs](image5.png)

Fig. 4. Cytosolic (>) and membrane (•) associated GCNO activity assayed at different pH values (panel A) or Ca²⁺ concentrations (panel B). C, GCNO activity when no Ca²⁺ was added to the assay medium for each fraction.

Table 1

|               | Cytosol | Membrane |
|---------------|---------|----------|
|                | EC₅₀ (EC₅₀) μM | Hill coefficient | EC₅₀ (IC₅₀) μM | Hill coefficient |
| GTP           | 588.5±56.4 | 2.30±0.80 | 296.4±67.4 | 1.90±0.50 |
| SNAP          | 160.2±11.5 | 1.28±0.42 | 55.2±9.4 | 1.15±0.31 |
| Ca²⁺(high affinity) | 0.03±0.015 | 1.62±0.13 | 0.079±0.057 | 1.22±0.29 |
| Ca²⁺(low affinity) | 42.5±12.6 | 1.73±0.02 | 49.7±12.1 | 1.75±0.03 |

* Nonlinear regression analyses were performed fitting each individual experiment to the Hill Equation (one-component model, for GTP, and SNAP curves, or two-component model, for Ca²⁺).

b Values ± SEM of the effective concentrations of the compound yielding 50% of the response (stimulatory, EC₅₀, or inhibitory, IC₅₀) in n different experiments.

c,d Significant differences (P<0.05, c; P<0.01, d) between values in the particulate and the cytosolic fractions as determined by a two tailed, paired Student’s t test.
small, differences were found for the EC_{50} values for GTP (589 and 296 μM for the cytosolic and particulate activity, respectively; P < 0.05; Fig. 3A and Table 1) and for the NO donor SNAP (160 and 55 μM, respectively; P < 0.01; Fig. 3B and Table 1). In both cases, the concentration–response curves were biphasic, and the last value was excluded in the non-linear fitting. However, no differences were observed regarding the dependence on pH (Fig. 4A) or Ca^{2+} (Fig. 4B). GC NO activity was maximal at pH 7.4 for the particulate and cytosolic fractions (as previously described for the cytosolic enzyme), and the decrease in activity observed at basic or acidic pHs was identical for the two fractions. Ca^{2+} exerted a profound inhibitory effect in both cardiomyocyte fractions (no remnant GC NO activity was observed at 1 mM Ca^{2+}), that was best suited to a two-component model (IC_{50} values calculated for the high and low affinity effects are shown in Table 1).

3.3. Effect of increasing cytosolic Ca^{2+} concentration on membrane-associated GC NO

Since Ca^{2+} has been proposed to be a regulating factor of the GC NO association to membrane in platelets [15], the effects of the ionophore A23187 (10 μM) and thapsigargin (0.1 μM) on this association were analyzed. Both drugs increased cytosolic Ca^{2+} concentration, although according to clearly distinct patterns (Fig. 5). Addition of A23187 induced a rapid, marked and transient increase in the intracellular Ca^{2+}, while thapsigargin induced a small, but sustained increase. The effects of incubating cardiomyocytes with A23187 (for 1 min) or thapsigargin (for 5 min) on NO-dependent cell synthesis of cGMP, distribution of the β₁ subunit between the particulate and the cytosolic fraction, and GC NO activity in both fractions, were analyzed. Thapsigargin did not modify cGMP synthesis induced by 0.1 mM SNAP (Fig. 6A) nor the proportion of β₁ associated...
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applied).

amount of 

GCNO activity (panel C) measured in cytosolic and membrane fractions. The 
P
staurosporin plus A23187 (panel B) increased by 132% in this experiment 
membrane fractions assayed for 
stimulation (panel A), or cultures were homogenized and cytosolic and 
(A23: 10 
fraction (measured in the presence of Ca 2+ chelating agents; 
Membrane 

P<0 . 0 5 ; Fig. 6 B), and particulate GC NO activity was 

significant decrease of GC NO activity in the cytosolic 
associated to the membrane was found (161 


P<0.05 respect to the effect of A23187.

to membrane after homogeneization (Fig. 6B), but caused a 
significant decrease of GC NO activity in the cytosolic 

fraction (measured in the presence of Ca2+-chelating agents; 

45 ±11% of the activity in non-treated-cells, n = 6; P<0.05) 

without apparent changes in the activity associated to the 

particulate fraction (Fig. 6C).

On the other hand, in cardiomyocytes treated with 
A23187, cGMP synthesis in response to SNAP was enhanced 

(152 ±17%, n = 6; P<0.05; Fig. 6A), a slight increase in β1 

associated to the membrane was found (161±20%, n = 5; 

P<0.05; Fig. 6B), and particulate GC NO activity was 

increased more than fourfold (P<0.05) while cytosolic 

GC NO activity did not increase significantly (Fig. 6C).

Platelets were labelled with [32P]orthophosphate, stimulated with drugs, 

homogenized, and the 

extracts are shown.

3.4. Role of changes in phosphorylation status as mediators of the effects of increased cytosolic Ca2+ concentration

In cells treated with A23187, staurosporin at 1 μM (a 
concentration that inhibits Ca2+-calmodulin-dependent 
protein kinase or CaMK, myosin light chain kinase, 
PKC, PKA and PKG), potentiated the cGMP response to 

SNAP (165 ±21% of the ionophore effect, n = 3; P<0.05; 

Fig. 7A), and induced an increase in both β1 immunos-
taining (138±3%, n = 3; P<0.05; Fig. 7B) and GC NO 
activity (165 ±21%, n = 3; P<0.05; Fig. 7C) in the 
particulate fraction, without significant effects on cyto-

solic GC NO activity. In cells not treated with A23187, 
staurosporin did not significantly increase β1 immunos-
taining (results not shown) or GC NO activity in the 
membrane fraction (Fig. 7C). Gö-6976, an inhibitor of 
Ca2+-dependent PKC isozymes, at 1 μM had no significant effect on GC NO 
activation by A23187 on both 

cytosolic and membrane extracts (Table 2), and H-89, an 

inhibitor of PKA and PKG, at 10 μM inhibited the effect 
of A23187 on the cytosolic GC NO. Only KN-93, a 
selective CaMK inhibitor, at 30 μM had a potentiating 

Table 2

| Cytosol* | Membrane |
|----------|----------|
| Gö-6975 1 μM (Ca2+-dependent PKC isozymes) | 193±63b 88±26 |
| H-89 10 μM (PKA and PKG) | 23±2 135±40 |
| KN-93 30 μM (CaMK) | 75±18 266±50b |

* Values are means of the effect of the different inhibitors used±SEM expressed as percentage of the effect of A23187 on SNAP-activated GC NO in each individual experiment (n=3) and in the same fraction. 
** Inhibitors had no significant effect when added alone. 
*** Significantly different (P<0.05) from the NO-dependent response in the presence of A23187 alone in the same fraction as determined by Student’s t test (two tailed).

Fig. 7. Effect of staurosporin on the effect of A23187 on NO-dependent cGMP synthesis (panel A), and on β1 immunoreactivity (panel B) and GC NO activity (panel C) measured in cytosolic and membrane fractions. After treating cardiomyocytes without (Ct) or with staurosporin (Stau: 1 μM for 5 min) and then incubated in the absence (Ct) or presence of A23187 (A23: 10 μM for 1 min), cGMP synthesis was measured after SNAP 

stimulation (panel A), or cultures were homogenized and cytosolic and 

membrane fractions assayed for 

β1 immunoreactivity (panel B) and 
GC NO activity (panel C). GC NO activity was expressed as percentage of total activity in controls (cytosolic plus membrane-associated GC NO). The amount of β1 in the membrane fractions of cardiomyocytes treated with 

staurosporin plus A23187 (panel B) increased by 132% in this experiment 

respect to those cells receiving only A23187 (mean of 138±3%, n = 3; 

P<0.05). *P<0.05 respect to control cardiomyocytes (same cell fraction, if applied). **P<0.05 respect to the effect of A23187.
effect on particulate GC\textsubscript{NO} activity similar to that of staurosporin (Table 2). On the other hand, the phosphatase inhibitors cypermethrin at 0.05 μM (selective inhibitor of calcineurin) or okadaic acid at 1 μM (inhibitor of PP1 and PP2A) did not block the potentiating effect of A23187 on membrane GC\textsubscript{NO} activity; in fact, okadaic acid enhanced it (290±15% of the ionophore effect, \( n = 3; P < 0.05 \)).

We were not able to detect in vivo \(^{32}\)P-labelling of GC\textsubscript{NO} in cardiomyocytes neither under control nor after staurosporin plus A23187 treatment. In platelets, incubation with the ionophore A23187 in the presence of staurosporin decreased phosphorylation of the β\(_1\) subunit compared to non-treated cells (to about 25% of the initial value; Fig. 8). Stimulation with A23187 in platelets preincubated with okadaic acid had a similar effect (Fig. 8).

3.5. cGMP synthesis versus GC\textsubscript{NO} activity in cells fractions

The effects on cGMP synthesis of the different treatments assayed thorough the present manuscript were significantly correlated with their effects on membrane-associated GC\textsubscript{NO} activity, but not with the effects on GC\textsubscript{NO} activity in the cytosolic fraction (\( P < 0.001 \); Fig. 9).

![Graph](https://example.com/graph.png)

**Fig. 9.** The effects of treatments on cGMP synthesis in intact cells were not significantly correlated with the effects on GC\textsubscript{NO} activity in the cytosolic fraction (panel A), but were closely correlated with the effects on GC\textsubscript{NO} in the membrane fraction (panel B). Data of cGMP synthesis shown in previous figures (Figs. 6 and 7, A panels) were plotted versus GC\textsubscript{NO} activity in particulate and cytosolic fractions of cardiomyocytes for each treatment (Figs. 6 and 7, C panels). The different measurements were expressed as percentage of their value in control cells.

4. Discussion

This study provides the first direct evidence supporting the specific association of GC\textsubscript{NO} to the particulate fraction of cardiomyocytes. Membrane-associated GC\textsubscript{NO} activity showed similar concentration-dependence to GTP, NO donors, Ca\(^{2+}\) and pH than GC\textsubscript{NO} in the cytosolic location. As previously shown for the cytosolic GC\textsubscript{NO}, increasing Ca\(^{2+}\) concentration in the assay medium inhibited particulate GC\textsubscript{NO}. However, treating cardiomyocytes with the Ca\(^{2+}\) ionophore A23187 promoted translocation of GC\textsubscript{NO} to the membrane fraction, increased cGMP synthesis in response to stimulation of the cells with NO, and enhanced GC\textsubscript{NO} activity in this fraction when assayed in vitro. These effects were enhanced by staurosporin and the CaMK inhibitor KN-93. These results suggest that cytosolic Ca\(^{2+}\) concentration regulates the intracellular distribution of GC\textsubscript{NO}, and differentially regulates the activity associated to the membrane and the cytosolic fraction, probably through changes in its phosphorylation status. The observation that the effects of several treatments on NO-induced cGMP synthesis in cardiomyocytes closely correlate with their effects on GC\textsubscript{NO} activity in the particulate fraction, but not with their effects on cytosolic GC\textsubscript{NO}, suggests that membrane-associated GC\textsubscript{NO} largely determines NO-induced cGMP synthesis.

Although different data indicate that changes in GC\textsubscript{NO} activity greatly affect the function of the NO/cGMP pathway in several tissues [17,23–30], little information is available on the regulation of this enzyme. A previous study [15], demonstrated the presence of α\(_1\) and β\(_1\) subunits in the membrane fraction of rat myocardium, platelets and endothelial cells. This study described enzyme translocation to the membrane in activated platelets [15], and by means of in vitro experiments of GC\textsubscript{NO} association/dissociation to cell membranes suggested an important role of Ca\(^{2+}\). However, although GC\textsubscript{NO} in the particulate fraction of rat heart was responsive to NO, it was unclear whether GC\textsubscript{NO} associated to cell membrane was important in the response of the intact cell to NO.

We found that approximately 30% of the GC\textsubscript{NO} immunostaining for α\(_1\) and β\(_1\) and a similar proportion of GC\textsubscript{NO} activity were associated to the particulate fraction of cardiomyocytes. β\(_1\) association to membranes resisted extensive washing in a KCl buffer, that mimics the high intracellular potassium concentration. However, no apparent immunostaining persisted in the particulate fraction after washing in a Triton X-100 medium. This is similar to what was described by Zabel et al. [15] for the platelet particulate GC\textsubscript{NO}. Given the scarce immunostaining found in cardiomyocytes for GC\textsubscript{NO} subunits, we did not make any attempt to further analyze their subcellular location.

Membrane-associated GC\textsubscript{NO} and cytosolic GC\textsubscript{NO} showed similar biochemical characteristics. pH-dependence of GC\textsubscript{NO} was identical for the two locations and similar to that described previously [17,31]. Membrane GC\textsubscript{NO} had a
significantly lower EC_{50} value for GTP than the cytosolic, but the difference was small and its physiological relevance is doubtful. The difference in sensitivity to the NO donor SNAP was more clear and similar to that described previously in heart extracts [15]. However, a very recent study [21] has suggested that contaminating myoglobin in cytosolic extracts from heart tissue neutralizes a significant part of the NO released by NO donors. According to this, our results may underestimate GC_{NO} activity in the cytosol. Specific activity in cytosol could be thus higher than in the particulate fraction, as observed in platelets.

As previously described [32,33], in the present study Ca^{2+} inhibited cytosolic GC_{NO}. We found that membrane-bound GC_{NO} was also inhibited by Ca^{2+} and that in the two cell fractions GC_{NO} showed a biphasic pattern very similar to that recently described in GC_{NO} purified from bovine lung [34]. IC_{50} values calculated for the low affinity sites for Ca^{2+} were the same for cytosolic and particulate GC_{NO} and comparable with those previously reported [34], and IC_{50} values for the high affinity sites of GC_{NO} were also similar in both cell fractions. But, besides this inhibitory effect of Ca^{2+} when added to the assay medium (mediated by a direct binding of Ca^{2+} to GC_{NO}), we observed in this study effects of increasing cytosolic Ca^{2+} in intact cells that had not been previously described. These effects are persistent, and can be detected after cell homogenization in the presence of Ca^{2+} quelling agents. Importantly, the different agents used to increased cytosolic Ca^{2+} concentration have different effects, suggesting distinct roles for different levels of physiological concentrations of intracellular Ca^{2+} or for different subcellular location of these increases. The moderate and slow Ca^{2+} increase evoked by thapsigargin did not alter GC_{NO} activity associated to the particulate fraction, and inhibited GC_{NO} in the cytosolic fraction, while the more marked Ca^{2+} increase elicited by the Ca^{2+} ionophore A23187 increased several times GC_{NO} activity in the particulate fraction without significant effects on cytosolic GC_{NO}. In parallel with the increase in activity in the particulate fraction, A231287 increased the amount of β_{1} subunit associated to this fraction. This is similar to the translocation previously observed in activated platelets [15]. However, the change in quantity of β_{1} associated to the particulate fraction was much smaller than the change observed in GC_{NO} activity. A critical point is that for the different conditions assayed, cytosolic and membrane-associated GC_{NO} were found to respond differentially to increased cytosolic Ca^{2+}.

A potential explanation for the increase in specific activity of membrane-associated GC_{NO} induced by A23187 is that the increase in cytosolic Ca^{2+} concentration induced by the drug causes a modification in the phosphorylation status of the enzyme. Few studies have analyzed GC_{NO} regulation by phosphorylation with conflicting results. Some studies suggested that GC_{NO} phosphorylation increases its activity. Both in vitro phosphorylation by PKC and PKA [11] and in vivo phosphorylation by PKA [35] have been described to increase GC_{NO} activity, while dephosphorylation of the β_{1} subunit has been associated to a decrease in GC_{NO} activity [23]. A very recent study has described, in contrast, a decrease in GC_{NO} activity associated to an increase in GC_{NO} phosphorylation in response to PKG activation [12]. In the present study, the protein kinase inhibitors staurosporin and KN-93 activated membrane-associated GC_{NO} sinergically with A23187 suggesting that in cardiomyocytes intracellular Ca^{2+} potentiates GC_{NO} activity probably by promoting GC_{NO} dephosphorylation. Although, direct evidence of GC_{NO} dephosphorylation in response to A23187 could not be obtained, the evidence obtained in platelets supports this hypothesis. However, the fact that the protein phosphatase inhibitor okadaic acid also enhanced the response to A23187 suggest that regulation of GC_{NO} activity by phosphorylation may be complex. A protein phosphatase activated by phosphorylation, as found in chromaffin cells [23], or an additional regulatory site (in the α_{1} subunit or in some of regulatory proteins recently described: as Hsp90 [16], Hsp70 [36], or CCT [37]) that would increase GC_{NO} activity after phosphorylation could explain the results. To sum up, our observations suggest that the increase in cytosolic Ca^{2+} induced by A23187 has two opposite effects on NO-mediated cGMP synthesis: a direct inhibitory effect and an indirect stimulatory effect mediated by β_{1} dephosphorylation resulting in membrane-associated GC_{NO} activation.

The present study provides information that strongly suggests an important functional role of GC_{NO} localized in the membrane fraction of cardiomyocytes. Our results show that a profound inhibition of cytosolic GC_{NO} do not significantly affect the cell response to SNAP, while activation of the particulate fraction markedly increases it. As shown in Fig. 9, cGMP synthesis in the entire cell correlates well with changes in membrane GC_{NO} activity, but not with changes in cytosolic GC_{NO} activity. This is in agreement with recent results in bovine aortic endothelial cells, indicating a decrease cell-response to NO stimulation when the formation of a membrane-associated protein complex between eNOS, HSP90 and GC_{NO} is inhibited [16].

The association of a fraction of GC_{NO} to cell membrane in cardiomyocytes, the important role of membrane-associated GC_{NO} on the cell response to NO, and the fact that the regulation of its activity differs from that of the cytosolic enzyme, may be of great relevance for the better understanding of pathophysiological conditions in which the NO/cGMP-pathway is compromised, and in the design of new therapies for these conditions.

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