

# Suramin disrupts receptor–G protein coupling by blocking association of G protein $\alpha$ and $\beta\gamma$ subunits

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Running title: **Suramin blocks association of G protein  $\alpha$  and  $\beta\gamma$  subunits**

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**ABBREVIATIONS:** ANOVA, analysis of variance; BS<sup>3</sup>, bis(sulfosuccinimidyl) suberate; DSS, disuccinimidyl suberate; EGS, ethylene glycol bis(succinimidylsuccinate); G $\alpha$ , G protein  $\alpha$  subunit; G $\beta\gamma$ , G protein  $\beta\gamma$  subunits; GDP $\beta$ S, guanosine 5'-O-(2-thiodiphosphate); GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); NF007, 8-(3-nitrobenzamido)-1,3,5-naphthalenetrisulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VIP, vasoactive intestinal peptide

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## ABSTRACT

Most drugs target a receptor for a hormone or neurotransmitter. A newer strategy for drug development is to target a downstream signaling element, such as the G protein associated with a receptor. Suramin is considered a lead compound targeting this moiety. It inhibits binding of guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S) to G proteins and reduces agonist binding to G protein-coupled receptors. Suramin is thought to uncouple the G protein from its associated receptor, though there is no direct evidence for this mechanism. We have now examined the effect of suramin on G protein signaling for the vasoactive intestinal peptide (VIP) receptor in lung. The primary experimental strategy was a two-step cross-linking reaction that covalently captures the VIP–receptor–G protein ternary complex. Such cross-linking provided the first direct evidence that suramin physically disrupts receptor–G protein coupling. We investigated how this uncoupling relates to the inhibition of GTP $\gamma$ S binding. Suramin indiscriminately hindered the dissociation of various guanine nucleotides from the G protein, implying that its action is not allosteric. Further cross-linking studies suggested that suramin does not obstruct the receptor docking site directly, but appears to block the interface between G protein  $\alpha$  and  $\beta\gamma$  subunits. Observations with a purified system of recombinant G protein subunits without a receptor yielded direct evidence that suramin suppresses the association between these subunits. This action can explain how it both disrupts receptor–G protein coupling and inhibits guanine nucleotide release. The improved understanding of suramin's action advances the development of selective inhibitors of G protein signaling.

Many hormones and neurotransmitters exert their effects on a target cell through a receptor in the seven transmembrane domain superfamily. These receptors activate an associated G protein by triggering the binding of GTP in exchange for GDP (Cabrera-Vera et al., 2003). Many therapeutic agents in current use target receptors in this superfamily. However, an emerging strategy for drug development is to target the G protein itself. Suramin, a polysulfonated naphthylurea, is regarded as a lead compound for developing such drugs (Freissmuth et al., 1999). It is reported to inhibit the binding of guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S) to G proteins and to reduce the binding affinity of agonists for various G protein-coupled receptors (Huang et al., 1990; Beindl et al., 1996). Suramin is considered to uncouple the receptor from the G protein. There is, however, no direct evidence for such uncoupling. The important question of how suramin perturbs receptor-G protein coupling remains to be answered.

Few studies have examined the site on the G protein where suramin might act. Although it reduces the rate of GDP release from the G protein, this may be an indirect effect (Freissmuth et al., 1996). Suramin does not appear to interact with the receptor docking domain on the G $\alpha$  subunit, as binding of an antibody against this domain was unaffected. One suggestion is that suramin may interact with the binding site on G $\alpha$  for its downstream effector, such as adenylyl cyclase. This putative mechanism is based mainly on the observation that suramin interfered with the immunoprecipitation of G $\alpha$  by an antibody against a putative effector binding domain (Freissmuth et al., 1996). The question of how an interaction with this domain might perturb receptor-G protein coupling has not been addressed explicitly.

We have devised a multiple-step cross-linking strategy for direct visualization of the ternary complex formed by a peptide agonist, its receptor and the associated G protein (Kermode et al., 1992). The strategy has been validated in prior studies of the receptors for vasoactive intestinal peptide (VIP), a neurotransmitter and neuromodulator that is distributed widely in the peripheral and central nervous system (Said, 1991; Muller et al., 1995). VIP mediates its effects through VPAC receptors, which belong to the class II subfamily of G protein-coupled receptors (Harmar et al., 1998; Laburthe et al., 2002). The VPAC1 receptor is the predominant subtype in the periphery, with

a high density in lung (Ishihara et al., 1992). It is reported to couple to both  $G_s$  and  $G_{i3}$  in rat lung (Diehl et al., 1996). The cross-linking strategy is an invaluable tool to investigate the mechanism of action of agents, such as suramin, that perturb receptor–G protein coupling.

In this report, we have examined how suramin disrupts receptor–G protein coupling through application of our multiple-step cross-linking strategy, assays of  $GTP\gamma S$  binding, and studies in a purified system comprising recombinant G protein subunits. Our studies provide direct support for the concept that suramin uncouples the receptor from its G protein. They also shed considerable insight into how this uncoupling occurs.

## Materials and Methods

**Materials.**  $^{125}\text{I}$ -VIP (2200 Ci/mmol),  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  (1250 Ci/mmol) and  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  (6000 Ci/mmol) were purchased from NEN PerkinElmer (Boston, MA). Non-radioactive VIP was obtained from Bachem (Philadelphia, PA). The bifunctional cross-linking agents, biotinylation reagent and bicinchoninic acid protein assay kit were from Pierce Chemical Co. (Rockford, IL). 8-(3-Nitrobenzamido)-1,3,5-naphthalenetrisulfonic acid (NF007) was obtained from Calbiochem (San Diego, CA). Suramin and other chemicals were purchased from Sigma (St. Louis, MO). The recombinant rat G protein  $\alpha_{i3}$  and  $\beta_1\gamma_2$  subunits (both obtained from Calbiochem) were derived by baculoviral expression in Sf9 insect cells using the methods of Graber et al. (1992) and Kozasa and Gilman (1995), respectively; recombinant G protein subunits expressed in this manner have proved to be functionally equivalent to the native subunits in several assays.

**Isolation of rat lung plasma membranes.** Studies on animals were conducted in accord with the *Guide for the Care and Use of Laboratory Animals*, and the protocol was approved by the local Institutional Animal Care and Use Committee. A fraction enriched in plasma membranes was isolated from the lung of male Sprague–Dawley rats (150–175 g; Harlan, Indianapolis, IN) by differential centrifugation after homogenization in the presence of a cocktail of protease inhibitors, as described previously (Kermode et al., 1992). The protein concentration of the final membrane suspension was determined by the bicinchoninic acid method.

**GTP $\gamma$ S binding assay.** Binding of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  to the lung membranes was assessed by a modification of the procedure of Lazareno (1997). Briefly, rat lung membranes (5  $\mu\text{g}$  protein) were incubated at 30°C for the designated time with 1 nM  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  in 0.4 ml buffer containing 10  $\mu\text{M}$  GDP, 5 mM  $\text{MgSO}_4$ , 100 mM NaCl, 50 mM HEPES (pH 7.4). The reaction was stopped by addition of 3 ml ice-cold phosphate buffer (20 mM  $\text{NaPO}_4$ , pH 7.4) and filtration through Whatman GF/B membranes. The filters were washed three times, and bound  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  was assessed with a Tri-Carb 1.09 scintillation counter (PerkinElmer, Wellesley, MA).

**Cross-linking of VIP to its receptor and of the receptor to its associated G protein.**

Ternary complexes of VIP, its receptor and G protein were captured by the two-step covalent cross-linking procedure of Kermode et al. (1992). In brief, rat lung membranes (500  $\mu$ g protein) were incubated for 30 min at room temperature with 250 pM  $^{125}$ I-VIP in 0.5 ml phosphate-magnesium buffer (1 mM  $\text{MgSO}_4$ , 50 mM  $\text{NaPO}_4$ , pH 7.4). Unbound VIP was removed by centrifugation and resuspension. The membranes were then incubated for 30 min at room temperature with the first cross-linker, 5 mM disuccinimidyl suberate (DSS). The reaction was quenched by addition of 20 mM glycine. The membranes were washed by centrifugation, then resuspended in buffer with 1% (w/v) digitonin and different concentrations of suramin or NF007, and incubated for 30 min at 4°C. After centrifugation, the supernatant with the extracted membrane proteins was incubated for 30 min at room temperature with the second cross-linker, 5 mM bis(sulfosuccinimidyl) suberate ( $\text{BS}^3$ ). The sample was then treated for 20 min at room temperature with sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 1% 2-mercaptoethanol, 2% SDS, 0.001% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) before analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970). The dried gel was analyzed for  $^{125}$ I on a Molecular Dynamics phosphor imager (Amersham Biosciences, Piscataway, NJ) after a 24–48 h exposure. The integrated intensity (background corrected) for each relevant band in the gel image was determined using ImageQuant software.

A three-step cross-linking procedure was substituted for some studies (as indicated in the *Results*). In this case, a second cross-linking reaction was performed with 5 mM ethylene glycol bis(succinimidylsuccinate) (EGS) prior to the digitonin extraction;  $\text{BS}^3$  cross-linking followed this extraction, as in the two-step procedure. This three-step procedure provided greater flexibility. The second cross-linking reaction could be performed either in the presence of magnesium (1 mM  $\text{MgSO}_4$ ) or in its absence (with 1 mM EDTA), and the magnesium could be replenished before the third cross-linking step by adding  $\text{MgSO}_4$  (to yield 1 mM free  $\text{Mg}^{2+}$ ).

**GTPase assay.** The catalytic activity of the recombinant G protein  $\alpha_{i3}$  subunit was assessed by a modification of the GTPase assay procedure of Brandt and Ross (1985). Briefly, the  $\alpha_{i3}$  subunit (0.6 pmol) was incubated at room temperature for 10–40 min with 6 nM [ $\gamma$ - $^{32}$ P]GTP

in 10  $\mu$ l HEPES buffer (100 mM NaCl, 1 mM  $MgCl_2$ , 1 mM dithiothreitol, 40 mM HEPES, pH 7.5) supplemented with 0.3  $\mu$ M GDP. The reaction was stopped by addition of 0.5 ml ice-cold 10% (w/v) suspension of activated charcoal in 50 mM  $NaPO_4$  (pH 2.5). The charcoal and bound [ $\gamma$ - $^{32}P$ ]GTP were precipitated by centrifugation, and phosphate release was determined by measuring the  $^{32}P$  content of the supernatant fraction.

**Biotinylation of G protein  $\beta_1\gamma_2$  subunits.** Recombinant rat G protein  $\beta_1\gamma_2$  subunits (2–5  $\mu$ g) were biotinylated on available sulfhydryl groups by a 1 h incubation at room temperature with 1 mM (+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine in 100 mM NaCl, 1 mM EDTA, 50 mM  $NaPO_4$  (pH 6.8). The reaction was stopped by a 100-fold dilution in HEPES buffer, and the excess biotinylation reagent removed by repeated washing in a Microcon YM-10 centrifugal filter (Millipore, Billerica, MA). The final concentration of the  $\beta_1\gamma_2$  subunits was adjusted to 400 nM in HEPES buffer supplemented with 0.2% (w/v) bovine serum albumin.

**Assay of G protein heterotrimer formation in a purified system.** Biotinylated G protein  $\beta_1\gamma_2$  subunits (1.2 pmol) were immobilized on streptavidin-coated magnetic beads (10  $\mu$ g; Dynal Biotech, Oslo, Norway) by a 1 h incubation at 15°C. The  $G\beta\gamma$ -loaded beads were isolated using an MPC-S magnetic separator (Dynal Biotech) and washed twice with 100  $\mu$ l HEPES buffer. The washed beads were resuspended with various concentrations of suramin or NF007 in HEPES buffer supplemented with 0.4% (v/v) Lubrol PX and 40  $\mu$ M GDP. Recombinant rat G protein  $\alpha_{i3}$  subunit (1.2 pmol) was added to the  $G\beta\gamma$ -loaded beads and incubated for 1 h at 15°C to allow G protein heterotrimers to form. This association reaction was stopped by dilution with 100  $\mu$ l HEPES buffer before isolating and washing the magnetic beads. The captured G proteins were finally eluted by treatment with SDS sample buffer, resolved by SDS-PAGE and detected by silver staining (kit from Bio-Rad, Hercules, CA). The abundance of salient protein bands was assessed with a Molecular Dynamics laser-scanning densitometer and quantified by ImageQuant software.

**Statistical analysis.** Composite data from each series of experiments are presented as the mean value  $\pm$  S.E.M. The data were analyzed by one-way repeated-measures analysis of variance (ANOVA), using SigmaStat software (Systat). Subsequent multiple comparisons used Dunnett's

test, in the case of comparisons against a control group without suramin, or Student–Newman–Keuls test, when all pairwise comparisons were required. The multiple comparison procedure used is indicated in the *Results* section or the appropriate figure legend.

## Results

**Inhibition of GTP $\gamma$ S binding by suramin.** The propensity for a G protein to bind the non-hydrolyzable GTP analog GTP $\gamma$ S is a useful measure of its activation (Wieland and Jakobs, 1994). Suramin has previously been shown to inhibit both basal and agonist-stimulated GTP $\gamma$ S binding to plasma membrane extracts from several tissues (Beindl et al., 1996). We found a similar pattern of inhibition with a membrane fraction isolated from rat lung (Fig. 1). Suramin suppressed the basal GTP $\gamma$ S binding with an IC<sub>50</sub> of 3–10  $\mu$ M; a somewhat higher concentration (IC<sub>50</sub>  $\approx$  30  $\mu$ M) was required to suppress the increment in binding with VIP. Specific binding of GTP $\gamma$ S was inhibited completely by 1 mM suramin, as binding under these conditions was indistinguishable from the non-specific binding to a boiled membrane preparation (data not shown).

**Disruption of receptor–G protein coupling by suramin.** Suramin has been shown to reduce the binding affinity of various agonists for their receptors. This effect has served previously as the sole indicator for disruption of receptor–G protein coupling. We sought more direct evidence in the present study, using a cross-linking strategy to examine whether suramin perturbed the formation of VIP–receptor–G protein ternary complexes. A plasma membrane extract from a native tissue was preferred for these studies, because it provides a natural lipid environment and ensures a physiological stoichiometry between receptor and G protein. <sup>125</sup>I-VIP can be cross-linked to its receptor and associated G proteins in rat lung membranes by a two-step strategy using DSS and BS<sup>3</sup> (Fig. 2A, lane 1). We have previously shown the three covalently labeled bands arising with this strategy to constitute a VIP–receptor binary complex (58 kDa), a complete VIP–receptor–G protein ternary complex (184 kDa), and a complex of VIP, its receptor and G $\alpha$  (114 kDa) (Kermode et al., 1992). Only the 58 kDa band is seen when cross-linking is limited to a single step with DSS (see also Fig. 4, lane 1).

Suramin caused a concentration-dependent reduction in prevalence of the higher molecular weight bands (114 and 184 kDa) that result from two-step cross-linking (Fig. 2A, lanes 2–5). The 58 kDa band showed a matching increase in intensity. Quantitative analysis of the band intensities

confirmed this pattern (Fig. 2B). In contrast, the prevalence of all three bands was unaffected by NF007, an inactive analog that resembles a half moiety of suramin (Fig. 2A, lanes 6–8).

These observations provide the first direct evidence that suramin opposes formation of an agonist–receptor–G protein ternary complex and favors retention of the receptor in an inactive binary complex with the agonist alone. The relatively high concentration of suramin required for this action can be attributed to its lower potency at inhibiting an agonist-stimulated response together with the irreversible nature of cross-linking. As suramin was added after cross-linking  $^{125}\text{I}$ -VIP to the receptor, a reduction in binding affinity for VIP cannot explain its effect on the band pattern. The lack of effect of NF007 also precludes a direct inhibition of the cross-linking reaction.

**Kinetics of suramin inhibition of GTP $\gamma$ S binding.** The effect of suramin on the kinetics of GTP $\gamma$ S binding was next examined. Incubation of lung membranes with [ $^{35}\text{S}$ ]GTP $\gamma$ S in the absence of VIP resulted in a progressive increase in GTP $\gamma$ S binding over a 40 min period (Fig. 3A, closed circles). Gradual turnover of GDP on a heterotrimeric G protein under basal conditions governs this increase in GTP $\gamma$ S binding (Wieland and Jakobs, 1994). Suramin (1 mM) was added at different time points (0, 10, 20 or 30 min) during a 40 min incubation with [ $^{35}\text{S}$ ]GTP $\gamma$ S to examine its influence on this process. Binding of [ $^{35}\text{S}$ ]GTP $\gamma$ S at the end of the 40 min incubation period (Fig. 3A, open circles) was indistinguishable from that in other samples for which the incubation was terminated at the time of suramin addition ( $p > 0.1$ , ANOVA with Student–Newman–Keuls post-hoc test). Suramin stopped the progression of [ $^{35}\text{S}$ ]GTP $\gamma$ S binding abruptly whenever it was added. However, it did not cause [ $^{35}\text{S}$ ]GTP $\gamma$ S that was already bound to dissociate, despite its use at a maximal effective concentration. Other studies confirmed that binding of [ $^{35}\text{S}$ ]GTP $\gamma$ S remained constant at different time intervals after adding the suramin (data not shown).

Two different mechanisms could explain these findings. Suramin might inhibit GDP release from the G protein by an allosteric increase in its binding affinity, as suggested by Freissmuth et al. (1996). Alternatively, it might directly block access to the binding pocket by any guanine nucleotide. Studies with guanosine 5'-*O*-(2-thiodiphosphate) (GDP $\beta$ S) allowed these mechanisms to be discriminated. GDP $\beta$ S is a phosphorylation-resistant GDP analog that competes with GTP $\gamma$ S for the

guanine nucleotide binding site on a G protein. Suramin and GDP $\beta$ S (at 1 mM concentration) both abolished GTP $\gamma$ S binding when added simultaneously with the [ $^{35}$ S]GTP $\gamma$ S (Fig. 3A). When they were added after the [ $^{35}$ S]GTP $\gamma$ S had bound, however, only GDP $\beta$ S was able to reverse this binding (Fig. 3B). Incubation with GDP $\beta$ S for 20 min typically halved the binding of [ $^{35}$ S]GTP $\gamma$ S, implying that about half of the GTP $\gamma$ S dissociated from the G protein in a 20 min period. Suramin seemed to block this dissociation of GTP $\gamma$ S, as it was unable to lower the observed binding. When added in combination with GDP $\beta$ S, suramin did not enhance the ability of GDP $\beta$ S to reverse GTP $\gamma$ S binding; instead, it hindered the action of GDP $\beta$ S (Fig. 3B). These observations imply that suramin blocks the release of any guanine nucleotide from the G protein, rather than selectively increasing its affinity for GDP. Moreover, it does not function as a competitive inhibitor.

**Design of a cross-linking strategy to examine G $\alpha$ –G $\beta\gamma$  association.** The putative access route for guanine nucleotides to the binding pocket on G $\alpha$  largely overlaps its binding surface for G $\beta\gamma$  (Iiri et al., 1998). If suramin were to block this access route, it would be likely to perturb the association between G $\alpha$  and G $\beta\gamma$ . We thus devised a new cross-linking strategy that specifically examines the G $\alpha$ –G $\beta\gamma$  interaction.

We previously reported EGS to cross-link the VIP receptor and G protein in the membrane milieu (without digitonin extraction) (Kermode et al., 1992). In the presence of magnesium, EGS cross-linking in lung membranes resulted in a similar pattern of three cross-linked bands to BS<sup>3</sup> cross-linking after digitonin extraction (Fig. 4, lanes 2 and 4). If magnesium was absent during the second cross-linking reaction, however, we found only a trace of the 184 kDa band comprising the VIP–receptor–G $\alpha$ –G $\beta\gamma$  complex (lane 3). This pattern also arose with BS<sup>3</sup> cross-linking after digitonin extraction (see Fig. 5A, lane 1). Magnesium seems to facilitate cross-linking of G $\alpha$  to G $\beta\gamma$ . This phenomenon might be related to the ability of Mg<sup>2+</sup> to cause a conformational change in the switch I and switch II regions of G $\alpha$  (Coleman and Sprang, 1998). Though the absence of Mg<sup>2+</sup> at the second cross-linking step precluded cross-linking of G $\alpha$  to G $\beta\gamma$ , we found that the 184 kDa band (including both these moieties) could be recovered by a third cross-linking reaction after digitonin extraction and the replenishment of free Mg<sup>2+</sup> (Fig. 4, lane 5). This three-step cross-linking strategy

with  $Mg^{2+}$  depletion and replenishment is an invaluable tool to dissect different steps in the receptor–G protein activation cycle. It makes it possible to discriminate between receptor– $G\alpha$  and  $G\alpha$ – $G\beta\gamma$  interactions.

**Inhibition of  $G\alpha$ – $G\beta\gamma$  cross-linking by suramin.** Our initial studies with the two-step cross-linking strategy (with DSS and BS<sup>3</sup>) indicated that suramin was a more potent inhibitor of the 184 kDa cross-linked band than the 114 kDa band (Fig. 2B). Moreover, when  $Mg^{2+}$  was excluded at the second cross-linking step to focus on the receptor– $G\alpha$  interaction, suramin did not affect the prevalence of the 114 kDa cross-linked band that represents the VIP–receptor– $G\alpha$  complex (Fig. 5A, lanes 1–4, and Fig. 5B;  $p > 0.5$ , ANOVA). These data imply that suramin does not directly perturb interaction between the receptor and  $G\alpha$ . They thus support the concept that it might interfere with the association between  $G\alpha$  and  $G\beta\gamma$ .

The three-step cross-linking strategy gave further evidence of such an action. Suramin was added, in these studies, after receptor– $G\alpha$  cross-linking at the second step (EGS without  $Mg^{2+}$ ) but before  $G\alpha$ – $G\beta\gamma$  cross-linking at the third step (BS<sup>3</sup> with  $Mg^{2+}$ ). Suramin still caused a substantial reduction in the 184 kDa cross-linked band that comprises the VIP–receptor– $G\alpha$ – $G\beta\gamma$  complex (Fig. 5A, lanes 5–8, and Fig. 5C). This finding implies that suramin disrupts receptor–G protein coupling by perturbing  $G\alpha$ – $G\beta\gamma$  association.

**Direct evidence that suramin disrupts  $G\alpha$ – $G\beta\gamma$  association.** Further evidence for this mechanism was sought through studies on a purified recombinant G protein system comprising two widely distributed G protein subunits,  $G\alpha_{i3}$  and  $G\beta_1\gamma_2$ . The primary requirement for such studies was a method to distinguish the G protein heterotrimer from its dissociated subunits. As the recombinant  $G\beta_1\gamma_2$  selected for these studies had a hexahistidine tag on the  $\gamma_2$  subunit, our initial experimental strategy was to use this tag as a means to immobilize the  $G\beta\gamma$ , and thereby capture any associated  $G\alpha$ . This approach proved effective at such capture. Unfortunately, however, suramin caused the release of immobilized  $G\beta\gamma$  from a nickel or cobalt chelate, thereby precluding the use of this approach to examine its effect on  $G\alpha$ – $G\beta\gamma$  association. Similar problems were encountered when an antibody against the hexahistidine tag was used to immobilize the  $G\beta_1\gamma_2$  subunit.

We considered that suramin was less likely to interfere with immobilization through a biotin–avidin interaction owing to its high affinity. Such a strategy, however, required the biotinylation of either  $G\alpha_{i3}$  or  $G\beta_1\gamma_2$  without compromising its function. Previous studies have indicated that attachment of a fluorescent label to  $G\alpha$  may cause some impairment in its function, whereas the  $G\beta\gamma$  subunits retain their full function after attachment of a fluorophore to the sulfhydryl groups (Phillips and Cerione, 1991; Heithier et al., 1992; Kwon et al., 1993). We thus chose to attach a biotin moiety to sulfhydryl groups on the recombinant  $G\beta_1\gamma_2$  subunits (see *Methods* for details). Immobilization of the biotinylated  $G\beta_1\gamma_2$  on streptavidin-coated beads allowed us to document the formation of G protein heterotrimers through the capture of recombinant  $G\alpha_{i3}$  by the immobilized  $G\beta\gamma$ . A series of studies was conducted to validate this experimental approach (Fig. 6). The functional nature of the recombinant  $G\alpha$  was confirmed by measuring its catalytic activity; this myristoylated  $G\alpha_{i3}$  subunit caused the progressive release of phosphate from  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  (Fig. 6A). One of the primary functions of the  $G\beta\gamma$  subunits is the ability to associate with the  $G\alpha$  subunit. Efficient capture of the  $G\alpha$  subunit (Fig. 6B, lane 2) verified that the recombinant  $G\beta\gamma$  subunits were functional and that they retained their function after biotinylation. Moreover, there was negligible non-specific binding of  $G\alpha$  to the streptavidin-coated beads in the absence of the biotinylated  $G\beta\gamma$  subunits (lane 6). Preliminary studies with a single concentration of suramin (100  $\mu\text{M}$ ) indicated that this agent did not cause the release of any immobilized  $G\beta\gamma$  from the beads (lane 3). However, it completely abolished the capture of  $G\alpha$  by  $G\beta\gamma$ . In contrast, the capture of  $G\alpha$  was unaffected by the inactive suramin analog NF007 at a concentration of either 200  $\mu\text{M}$  (lane 4) or 2 mM (data not shown). Quantitative analysis of the complete series of validation studies with the streptavidin-coated beads confirmed these observations (Fig. 6C).

A further series of studies was conducted to ascertain the concentration dependence for this effect of suramin. Increasing concentrations of suramin (1–100  $\mu\text{M}$ ) progressively suppressed the capture of  $G\alpha$  by the immobilized  $G\beta\gamma$  (Fig. 7A). There was no effect on the recovery of the  $G\beta\gamma$  subunits. These data thus provide direct evidence that suramin inhibits the association between  $G\alpha$  and  $G\beta\gamma$  subunits. Quantitative analysis by laser-scanning densitometry indicated an  $\text{IC}_{50}$  of 1–5  $\mu\text{M}$

for this action of suramin (Fig. 7B).

## Discussion

Suramin exerts multiple biological effects through inhibition of several enzymes involved in signal transduction and antagonism of various receptors. It inhibits the function of G proteins (Freissmuth et al., 1999) and protein kinase C (Khaled et al., 1995), as well as antagonizing  $P_2$  purinoceptors (Voogd et al., 1993) and several growth factor receptors (Firsching et al., 1995). Despite these multiple actions, suramin is considered a lead compound for developing G protein selective drugs (Freissmuth et al., 1999). Although suramin itself does not discriminate among different G proteins, analogs with significant selectivity for a particular G protein have been described (Hohenegger et al., 1998). Suramin appears to be a direct G protein inhibitor, though its mechanism of action is poorly understood. Its ability to reduce agonist binding affinity provides indirect evidence that suramin disrupts receptor–G protein coupling. We have obtained the first direct evidence of such uncoupling by using a cross-linking strategy. Suramin caused a concentration-dependent decrease in prevalence of the VIP–receptor–G protein ternary complex with a reciprocal increase in the uncoupled VIP–receptor binary complex (Fig. 2).

Suramin also inhibits other aspects of G protein function (Freissmuth et al., 1996), such as the binding of GTP analogs. How these actions relate to the uncoupling effect is not clear. Uncoupling of a G protein from the receptor should reduce its binding of a GTP analog (Gilman, 1987). Such an effect is anticipated only in the presence of a receptor, yet suramin has also been shown to inhibit GTP $\gamma$ S binding to a recombinant G protein in the absence of a receptor (Hohenegger et al., 1998). Moreover, it suppresses both basal and agonist-stimulated GTP $\gamma$ S binding. Our observations indicate suramin to be much less effective at preventing cross-linking between the receptor and G $\alpha$  than between G $\alpha$  and G $\beta\gamma$  (Fig. 5). A prior study reported that it did not affect the binding of an antibody targeting the receptor docking domain of G $\alpha$  (Freissmuth et al., 1996). These findings, together, suggest that disruption of receptor–G protein coupling is unlikely to be the primary action of suramin.

Freissmuth et al. (1996) contend that suramin interacts with the effector binding domain on

G $\alpha$ . Their hypothesis is based on observations that it suppresses immunoprecipitation of G $\alpha_s$  by an antibody against this domain, and that purified adenylyl cyclase partially reverses suramin's inhibition of GTP $\gamma$ S binding to G $\alpha_s$ . This scheme requires an allosteric mechanism to explain suramin's effect on GTP $\gamma$ S binding. Its ability to slow GDP dissociation from G $\alpha$  (Freissmuth et al., 1996) and its higher binding affinity for GDP-bound than GTP-bound G $\alpha$  (Beindl et al., 1996) lend support to this mechanism. Nonetheless, this allosteric mechanism seems at variance with our current understanding of G protein–effector signaling. As activation of a G protein reveals the effector binding domain (Skiba et al., 1996; Hatley et al., 2003), it should be GTP, rather than GDP, that enhances exposure of this domain to suramin and increases its binding affinity.

We re-examined this issue by assessing the kinetics of suramin's action. When added during the process of GTP $\gamma$ S binding, suramin prevented any further binding of GTP $\gamma$ S without affecting the pre-existing binding (Fig. 3). In contrast, GDP $\beta$ S substantially reversed the pre-existing binding (Fig. 3B). As GDP $\beta$ S and GTP $\gamma$ S compete for the guanine nucleotide binding site, this reversal reflects the spontaneous dissociation of GTP $\gamma$ S from the G protein. The observations with suramin imply that it suppresses such spontaneous dissociation; it seems to freeze guanine nucleotide binding at its current state. Such an action can also explain why GDP $\beta$ S was less effective at reversing the binding of GTP $\gamma$ S when combined with suramin (Fig. 3B). In conjunction with the prior evidence that suramin inhibits GDP release (Freissmuth et al., 1996), our findings suggest that suramin blocks the release of any guanine nucleotide but does not perturb the conformation of the G protein. This concept is consistent with recent spectroscopic evidence that the conformation of transducin is unchanged when suramin uncouples it from rhodopsin (Lehmann et al., 2002). The inability of suramin to reverse the pre-existing binding of GTP $\gamma$ S (Fig. 3) echoes the previous finding that it is a much less effective inhibitor of G protein stimulated adenylyl cyclase activity when the G protein is pre-treated with GTP $\gamma$ S (Hohenegger et al., 1998). These data imply that suramin disrupts signal transduction at a step before guanine nucleotide exchange, and does not block signaling from G $\alpha$  to the effector. Suramin's action does not comply with the behavior expected for either a competitive or a non-competitive inhibitor. It does not compete for the same site on the G protein as the guanine

nucleotides, yet it can prevent binding of a guanine nucleotide. These unusual kinetic properties suggest that suramin is a unique form of inhibitor.

Suramin would be expected to stabilize the heterotrimeric form of the G protein if it were to enhance GDP binding (Sprang, 1997). If it were to disrupt receptor–G protein coupling directly, suramin would have caused similar reductions in intensity for the 114 kDa (VIP–receptor–G $\alpha$ ) and 184 kDa (VIP–receptor–G $\alpha$ –G $\beta\gamma$ ) bands in our two-step cross-linking experiments (Fig. 2). With such a mechanism, it would still have reduced the intensity of the 114 kDa band when Mg<sup>2+</sup> was excluded during the second cross-linking step to prevent formation of the 184 kDa band (Fig. 5A, lanes 1–4). However, it would not have affected the intensity of the 184 kDa band when the receptor–G $\alpha$  tandem was cross-linked before the suramin treatment in a three-step reaction (Fig. 5A, lanes 5–8). In every case, our experimental findings are diametrically opposed to the predictions for this mechanism (Fig. 2B and Fig. 5B,C). These observations argue strongly against a direct effect of suramin on receptor–G protein coupling and against an allosteric effect to enhance GDP binding to the G protein. They suggest, instead, that the primary action of suramin is to disrupt the association between G $\alpha$  and G $\beta\gamma$ . As G $\beta\gamma$  is required for receptor–G protein interaction (Sprang, 1997), such action also explains the impairment of receptor–G protein coupling. The spatial arrangement of the G protein (Iiri et al., 1998) also suggests that binding of suramin at the interface between G $\alpha$  and G $\beta\gamma$  could obstruct access to and from the guanine nucleotide binding pocket, and thereby prevent guanine nucleotide release without competing directly for its binding site.

Interference with G $\alpha$ –G $\beta\gamma$  association has not previously been proposed as the primary action of suramin. Nonetheless, prior studies have provided hints of such a mechanism. Suramin mimics the effect of GTP $\gamma$ S in causing receptor–G protein uncoupling and does not cause any greater uncoupling when combined with GTP $\gamma$ S (Hohenegger et al., 1998). These findings suggest that suramin may function in the same manner as GTP $\gamma$ S, which promotes the dissociation of G $\alpha$  from G $\beta\gamma$ . Suramin has been shown to reduce the affinity of the transducin  $\alpha$  subunit for the plasma membrane (Lehmann et al., 2002); this effect can be attributed to disruption of G $\alpha$ –G $\beta\gamma$  association, as G $\beta\gamma$  plays a major role in anchoring G $\alpha$  to the membrane (Sternweis, 1986; Kurstjens et al.,

1991). Moreover, a docking simulation has suggested that the binding site for suramin is in the vicinity of a conserved arginine residue (Arg<sup>201</sup>) in the switch II region of G $\alpha$  (Lehmann et al., 2002); this binding site may extend towards the N-terminus in the three-dimensional structure. The N-terminal region of G $\alpha$  is part of the contact site for G $\beta\gamma$  (Medkova et al., 2002). Mutation of this arginine residue impairs the ability of G $\alpha$  to sequester G $\beta\gamma$ , but not its ability to activate the effector (Farfel et al., 1996; Ho et al., 1999).

Our studies with a purified G protein system comprising recombinant  $\alpha_{i3}$  and  $\beta_1\gamma_2$  subunits gave direct evidence that suramin interferes with G $\alpha$ –G $\beta\gamma$  association. Suramin potently inhibited association between these two G protein subunits (Fig. 7), whereas its inactive analog NF007 had no effect (Fig. 6B,C). The IC<sub>50</sub> for suramin (1–5  $\mu$ M) in this simplified system was comparable both to its IC<sub>50</sub> for inhibition of basal GTP $\gamma$ S binding in the present study (Fig. 1) and to that reported in prior studies with purified G proteins (Freissmuth et al., 1996; Lehmann et al., 2002). We tested the disruptive effect of suramin on G $\alpha$ –G $\beta\gamma$  association only in a single purified G protein system. However, the high degree of conservation in amino acid residues at the G $\alpha$ –G $\beta\gamma$  interface among G proteins (Hildebrandt, 1997) suggests that it is a universal mechanism.

In conclusion, our studies have shown that the G $\alpha$ –G $\beta\gamma$  interface is the primary site of action for suramin. Perturbation of the association between G $\alpha$  and G $\beta\gamma$  can explain the effects observed in both the present study and most previous studies. Not only does this mechanism reconcile many prior discrepancies in interpreting the effects of suramin on G protein function, it further validates the use of suramin as a direct G protein inhibitor. Establishment of the precise inhibitory mechanism enhances its usefulness as a prototype for the development of G protein selective drugs.

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## Footnotes

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## Figure Legends

**Fig. 1.** Effect of suramin on basal and agonist-stimulated GTP $\gamma$ S binding. Rat lung membranes (5  $\mu$ g of protein) were incubated for 30 min at 30°C with 1 nM [ $^{35}$ S]GTP $\gamma$ S and different concentrations of suramin in the absence (open circles) or presence (closed circles) of 0.1  $\mu$ M VIP. Specific binding data are expressed as a percentage of the basal GTP $\gamma$ S binding in the absence of suramin. They are shown as the mean value  $\pm$  S.E.M. of data from five independent experiments. Data were analyzed by ANOVA with Student–Newman–Keuls post-hoc test: \*\* and \*\*\*, significantly different ( $p < 0.01$  and  $p < 0.001$ , respectively) from measurements in the absence of suramin; # and ##, significantly different ( $p < 0.05$  and  $p < 0.01$ , respectively) from measurements in the absence of VIP.

**Fig. 2.** Disruption of VIP receptor–G protein coupling by suramin. Rat lung membranes (500  $\mu$ g of protein) were incubated with 250 pM  $^{125}$ I–VIP for 30 min at room temperature to form a reversible  $^{125}$ I–VIP–receptor complex.  $^{125}$ I–VIP was cross-linked to the receptor with 5 mM DSS. Membrane proteins were extracted with 1% digitonin and treated without any agent (lane 1) or with either suramin (0.1–3 mM, as indicated; lanes 2–5) or NF007 (0.6–6 mM; lanes 6–8) before a second cross-linking reaction with 5 mM BS $^3$ . (Two-fold higher concentrations of NF007 were used, as it resembles a half moiety of suramin.) Samples were analyzed by SDS–PAGE (8% gels) and phosphor image analysis. A, gel image from a typical one of four such experiments. The prominent bands (arrows) in the absence of suramin represent VIP–receptor (58 kDa), VIP–receptor–G $\alpha$  (114 kDa) and VIP–receptor–G $\alpha$ –G $\beta\gamma$  (184 kDa) complexes, as depicted on the right. B, summary of intensity data from all four experiments (suramin inhibition only) for each cross-linked band: open bars, 58 kDa; hatched bars, 114 kDa; cross-hatched bars, 184 kDa. Data are normalized in terms of the intensity of the same band in the absence of suramin; they are shown as the mean value  $\pm$  S.E.M. Data were analyzed by ANOVA with Dunnett’s post-hoc test: \*\*, significantly different ( $p < 0.01$ ) from the corresponding band in the absence of suramin.

**Fig. 3.** Effect of suramin on guanine nucleotide exchange. A, the time course of [ $^{35}$ S]GTP $\gamma$ S binding to rat lung membranes was assessed under basal conditions (solid line and closed circles). The effect of suramin on the progression of [ $^{35}$ S]GTP $\gamma$ S binding was also examined; 1 mM suramin was added at various time intervals (0–30 min) after the [ $^{35}$ S]GTP $\gamma$ S and incubation continued for a total of 40 min (hatched lines and open circles). Binding of [ $^{35}$ S]GTP $\gamma$ S was also measured after 40 min incubation in the presence of 1 mM GDP $\beta$ S (open triangle). B, binding of [ $^{35}$ S]GTP $\gamma$ S was measured after 20 min incubation under basal conditions (open bar). Other samples (hatched bars) were incubated for 20 min with [ $^{35}$ S]GTP $\gamma$ S alone and a further 20 min in the presence of suramin (1 mM), GDP $\beta$ S (1 mM), or both suramin and GDP $\beta$ S. Data were analyzed by ANOVA with Student–Newman–Keuls post-hoc test: \*\* and \*\*\*, significantly different ( $p < 0.01$  and  $p < 0.001$ , respectively) from basal conditions; #, significantly different ( $p < 0.05$ ) from treatment with GDP $\beta$ S. A and B, the results in each panel summarize data from at least four such experiments. Data are expressed as a percentage of GTP $\gamma$ S binding after 40 min (A) or 20 min (B) incubation under basal conditions; they are shown as the mean value  $\pm$  S.E.M.

**Fig. 4.** Magnesium requirement for cross-linking between G $\alpha$  and G $\beta\gamma$ .  $^{125}$ I-VIP was cross-linked to the receptor on lung membranes with DSS (as in Fig. 2). Equal portions of the membranes were processed in five different ways. One portion was not cross-linked further (lane 1). Two portions were cross-linked a second time with 5 mM EGS in the presence of either 1 mM Mg $^{2+}$  (lane 2) or 1 mM EDTA (lane 3); the longer spacer arm in EGS allows cross-linking between the receptor and G protein in the membrane milieu. These three portions were then extracted with digitonin. A fourth portion was extracted with digitonin before cross-linking with 5 mM BS $^3$  in the presence of 1 mM Mg $^{2+}$  (lane 4). The last portion was cross-linked with 5 mM EGS in the presence of 1 mM EDTA, extracted with digitonin, adjusted to 1 mM free Mg $^{2+}$  and cross-linked again with 5 mM BS $^3$  (lane 5). All samples were finally analyzed by SDS-PAGE (8% gels) and phosphor image analysis. The results are from a typical one of two such experiments.

**Fig. 5.** Inhibition of  $G\alpha$ – $G\beta\gamma$  cross-linking by suramin. VIP–receptor–G protein complexes were cross-linked by either a two-step (lanes 1–4) or a three-step procedure (lanes 5–8). Both procedures first involved DSS cross-linking in lung membranes. In the two-step procedure, membrane proteins were extracted with digitonin then cross-linked a second time with BS<sup>3</sup> under  $Mg^{2+}$ -free conditions. In the three-step procedure, cross-linking with EGS under  $Mg^{2+}$ -free conditions preceded digitonin extraction and a third cross-linking reaction with BS<sup>3</sup> after the replenishment of free  $Mg^{2+}$ . With both procedures, different concentrations of suramin (0–3 mM, as indicated) were included in the digitonin extraction solution before the final cross-linking reaction with BS<sup>3</sup>. A, gel image from a typical one of four such experiments. B and C, summary of intensity data from all four experiments for each band with the two-step (B) and three-step (C) cross-linking procedures: open bars, 58 kDa band; hatched bars, 114 kDa; cross-hatched bars, 184 kDa. Data are normalized in terms of the intensity of the same band in the absence of suramin; they are shown as the mean value  $\pm$  S.E.M. (The 184 kDa band was too weak to quantify after two-step cross-linking.) Data were analyzed by ANOVA with Dunnett's post-hoc test: \*\*, significantly different ( $p < 0.01$ ) from the corresponding band in the absence of suramin.

**Fig. 6.** Verification of functional nature of recombinant G protein subunits and validation of procedure to assess  $G\alpha$ – $G\beta\gamma$  association. A, the time course for GTP hydrolysis by the recombinant G protein  $\alpha_{i3}$  subunit was assessed as an index of its catalytic activity.  $G\alpha_{i3}$  was incubated with 6 nM [ $\gamma$ -<sup>32</sup>P]GTP for 10, 20 or 40 min at room temperature in the absence (open circles) or presence (closed circles; non-specific hydrolysis) of 100  $\mu$ M non-radioactive GTP. Data have been corrected for free [<sup>32</sup>P]phosphate in the [ $\gamma$ -<sup>32</sup>P]GTP stock; they are shown as the mean value  $\pm$  S.E.M. from two independent experiments. Data were analyzed by ANOVA with Student–Newman–Keuls post-hoc test: \*\* and \*\*\*, significantly different ( $p < 0.01$  and  $p < 0.001$ , respectively) from non-specific hydrolysis. B and C, initial characterization of the capture of  $G\alpha$  by immobilized  $G\beta\gamma$ . Biotinylated G protein  $\beta_1\gamma_2$  subunits were immobilized on streptavidin-coated magnetic beads. G protein  $\alpha_{i3}$  subunits were incubated for 1 h at 15°C with the beads in the absence (lane 2) and presence of

100  $\mu$ M suramin (SUR; lane 3) or 200  $\mu$ M NF007 (NF; lane 4). Control analyses were performed on beads with immobilized  $G\beta_1\gamma_2$  alone (lane 5), and after incubation of  $G\alpha_{i3}$  with beads lacking the immobilized  $G\beta_1\gamma_2$  (lane 6). G protein subunits and heterotrimers captured on the beads were analyzed by SDS-PAGE and silver staining. B, gel image from a typical one of four such experiments. The  $\alpha_{i3}$  and  $\beta_1\gamma_2$  subunits (non-biotinylated) were also run directly on the gel (lane 1) as markers for the  $G\alpha$  (41 kDa) and  $G\beta$  (37 kDa) bands (arrows). (Note that biotinylation slightly retarded migration of the  $G\beta$  subunit, whereas the  $G\gamma$  subunit stained too weakly for detection.) C, summary of intensity data for the  $G\alpha$  (solid bars) and  $G\beta$  (open bars) bands from these four experiments. Data are normalized in terms of the intensity for the same band under basal conditions (lane 2 on gel); they are shown as the mean value  $\pm$  S.E.M. Data were analyzed by ANOVA with Dunnett's post-hoc test: \*\*, significantly different ( $p < 0.01$ ) from the corresponding band under basal conditions. There were no significant differences in intensity for the  $G\beta$  band ( $p > 0.05$ , ANOVA).

**Fig. 7.** Concentration dependence for disruption of  $G\alpha$ – $G\beta\gamma$  association by suramin. G protein  $\alpha_{i3}$  subunits were incubated for 1 h at 15°C with immobilized G protein  $\beta_1\gamma_2$  subunits (as in Fig. 6) in the absence (lane 2) or presence of various concentrations of suramin (1–100  $\mu$ M, as indicated; lanes 3–6). Captured G protein subunits and heterotrimers were analyzed by SDS-PAGE and silver staining. A, gel image from a typical one of four such experiments. The  $\alpha_{i3}$  and  $\beta_1\gamma_2$  subunits were also run directly on the gel (lane 1) as markers for the  $G\alpha$  (41 kDa) and  $G\beta$  (37 kDa) bands (arrows). B, summary of intensity data for the  $G\alpha$  (solid bars) and  $G\beta$  (open bars) bands from these four experiments. Data are normalized in terms of the intensity for the same band in the absence of suramin; they are shown as the mean value  $\pm$  S.E.M. Data were analyzed by ANOVA with Dunnett's post-hoc test: \*\*, significantly different ( $p < 0.01$ ) from the corresponding band in the absence of suramin. There were no significant differences in intensity for the  $G\beta$  band ( $p > 0.5$ , ANOVA).

FIG. 1.

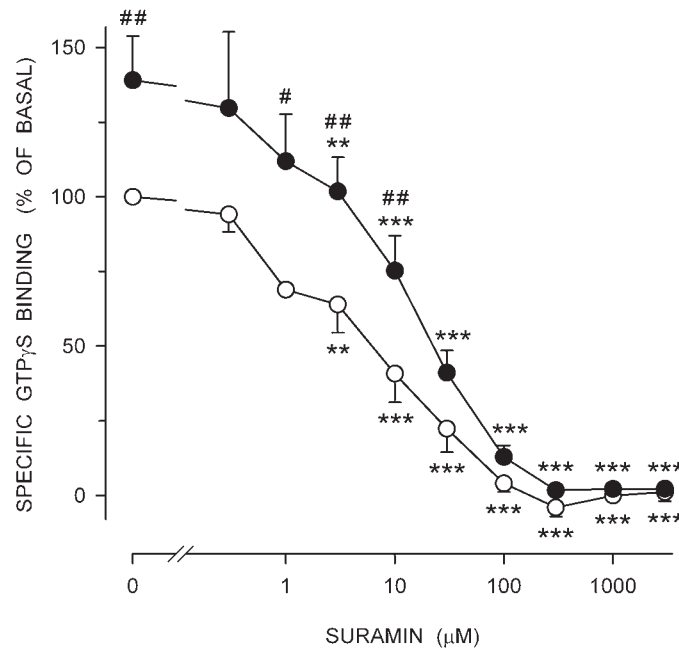


FIG. 2.

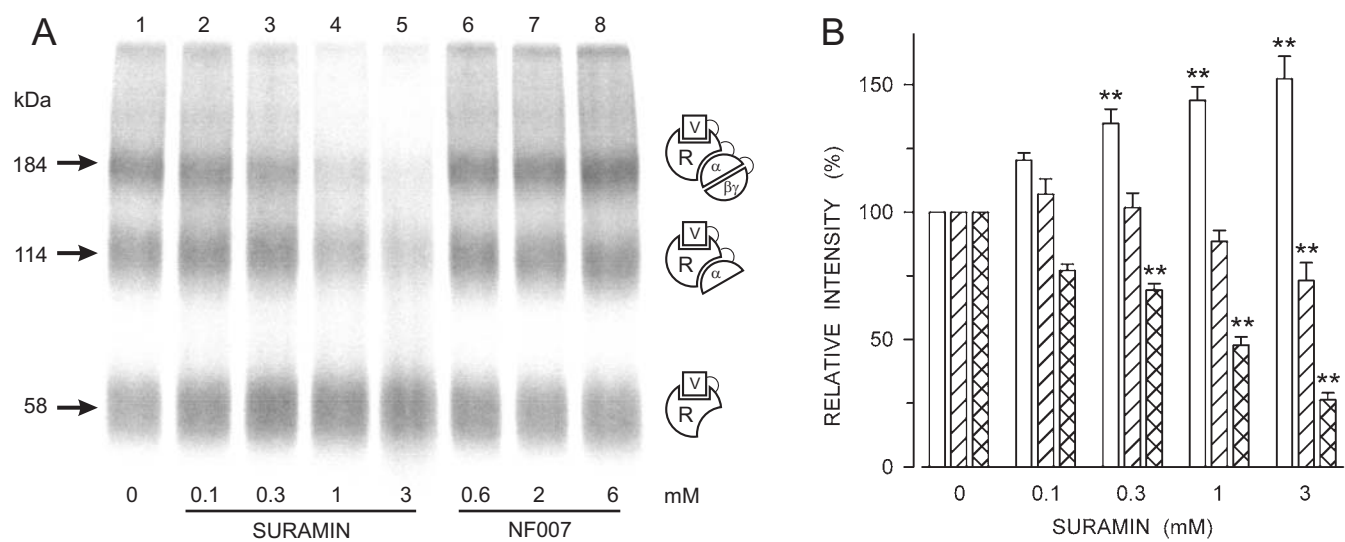


FIG. 3.

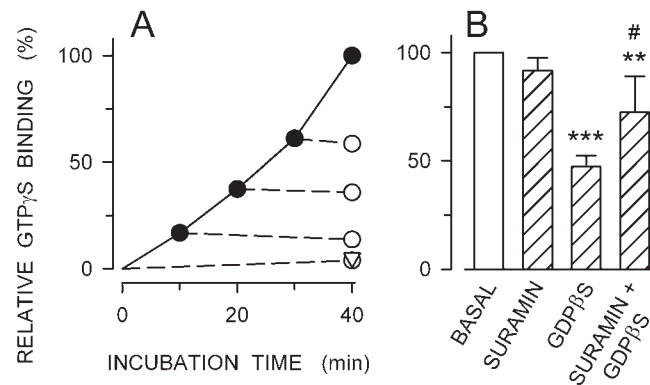


FIG. 4.

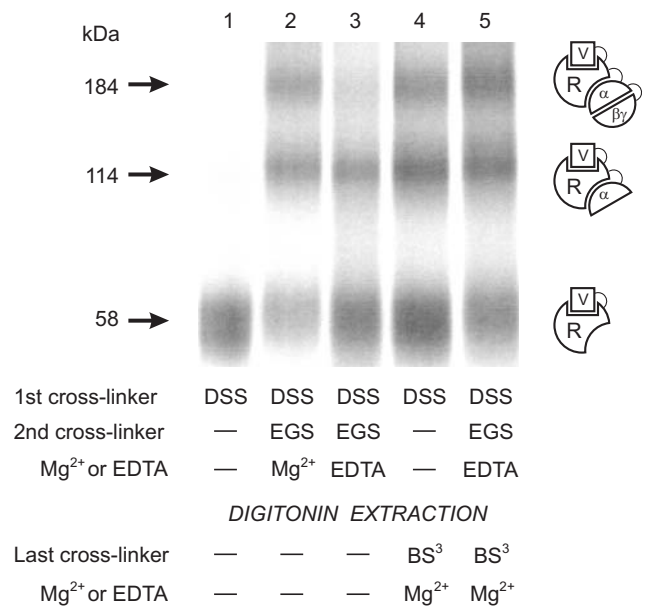


FIG. 5.

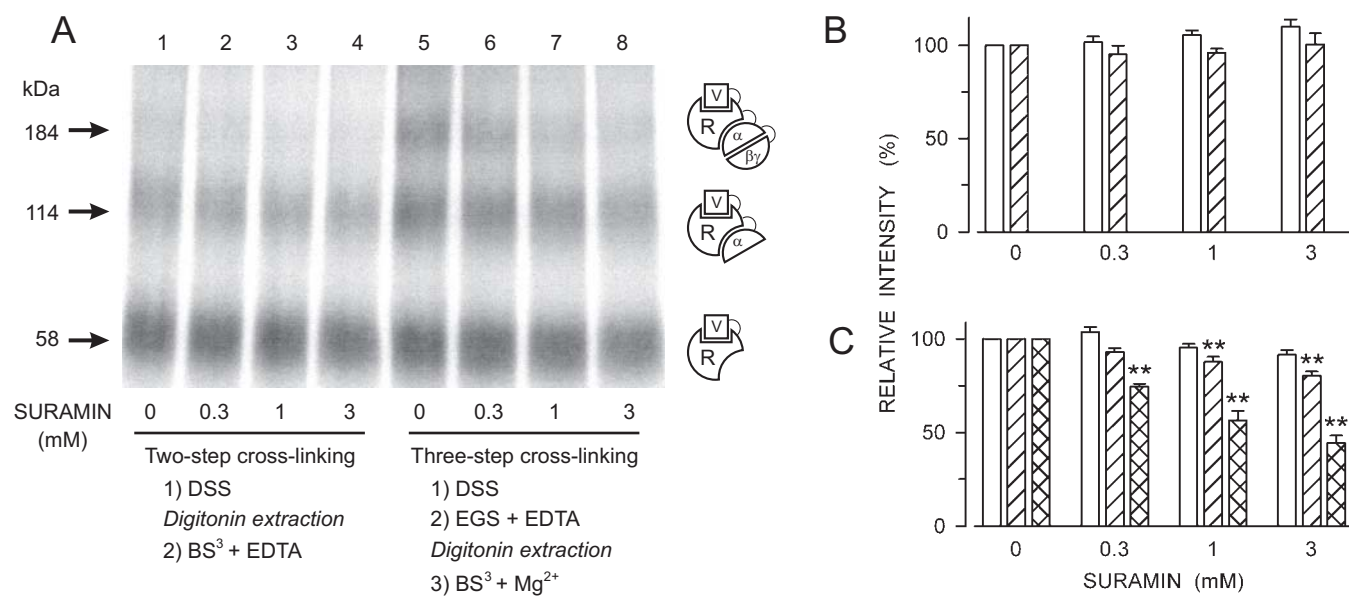


FIG. 6.

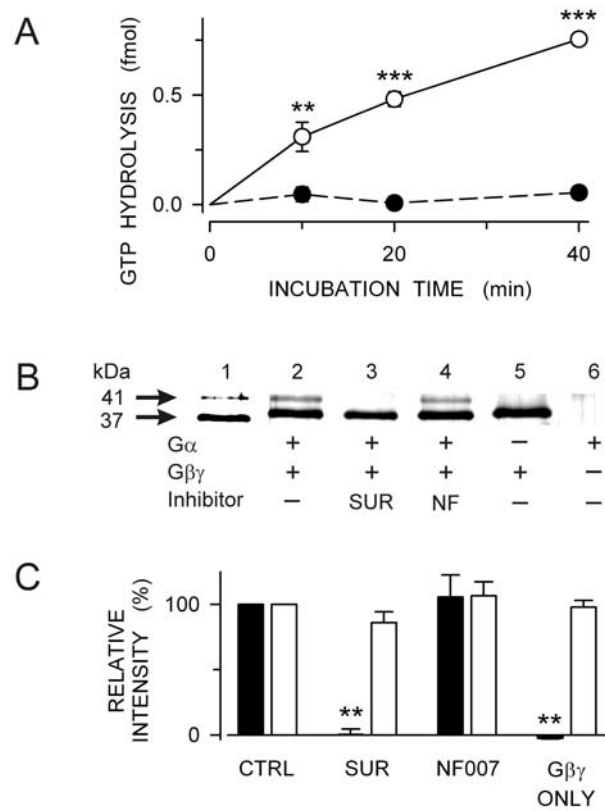


FIG. 7.

