

A substantial proportion of cardiac G_s is not associated with the plasma membrane

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Received 19 July 1991; revised version received 28 October 1991

The precise interactions between the subunits of G_s (α_s , β , γ) and the plasma membrane remain to be established. If α_s is associated loosely with the inner membrane, is labile during activation, or is always present to some extent in the cytoplasm, then it should fractionate to the supernatant of a high-speed centrifugation. We identified abundant α_s (52–66% of total cellular) in the supernatant fraction of right atrial and left ventricular membrane preparations of porcine heart as shown by two distinct measures of α_s (immunoblotting and ADP ribosylation by cholera toxin). However, functional assays utilizing reconstitution of cardiac α_s with *eye*⁻ S49 membranes revealed that the supernatant fraction contained ~16% of total cellular α_s activity. The α_s present in the supernatant fraction did not result from contamination by sarcolemmal membrane fragments. We conclude that traditional methods for quantifying α_s which utilize only detergent extracts from high-speed pellets do not account for a sizable proportion of total cellular α_s , but that the majority of this population of cardiac α_s may not be functional, at least with respect to adenylyl cyclase activation.

G-protein: Signal transduction; Myocardium; *eye*⁻ reconstitution; Immunoblotting; Cholera toxin; ADP-ribosylation

1. INTRODUCTION

The stimulatory guanine nucleotide binding protein (G_s) is comprised of three subunits: α , β and γ . The stimulatory α subunit (α_s) transduces the activation signal between the G_s -linked receptors and adenylyl cyclase. The structural organization of α_s (associated, heterotrimeric form; or free, monomeric form) depends upon its functional state (GDP- and GTP-bound forms, respectively), and may reflect distinct modes of association of α_s with the plasma membrane. In the GDP-bound form, α_s appears to be bound tightly to the $\beta\gamma$ dimer which may anchor the α subunit to the plasma membrane [1]. Stimulation by agonists of receptors that are coupled to G_s induces exchange of GDP by GTP on α_s , which promotes dissociation of the GTP-liganded α_s from $\beta\gamma$ [2]. The subsequent interactions of the activated α_s monomer are not precisely known. In cardiac

tissue, association of activated α_s with the catalytic subunit of adenylyl cyclase generates cAMP, which leads to the cascade of intracellular events that enhance cardiac inotropy and chronotropy.

Recent reports have shown that activated G_s , or the α_s subunit alone, can regulate cardiac calcium channels both directly and indirectly (through activation of cAMP-dependent protein kinase) [3]. Moreover, three splice variants of α_s were shown to stimulate both adenylyl cyclase and calcium channels [4]. Cardiac β -adrenergic receptors also can inhibit Na^+ channels through G_s by both cytoplasmic (indirect) and membrane-delimited (direct) pathways [5]. Hence, G_s may act on at least three effectors in the heart *via* plasma membrane-associated, non-plasma membrane-associated, or combinations of the two: adenylyl cyclase, calcium channels, and sodium channels.

A variety of hormone and neurotransmitter receptors in heart [6,7] and other tissues [8,9] have been identified in intracellular pools associated with light vesicle fractions. Existence of cytosolic pools of G_i [10,11] and G_o [12] have also been suggested. We hypothesized that cardiac G_s might have two forms: one firmly associated with the plasma membrane, and one present in the cytosol. If α_s was associated loosely with the sarcolemma, was labile during activation, or was always present to some extent in the cytoplasm, then it should fractionate to the supernatant of a high-speed centrifugation. We provide evidence for such fractionation in the current studies.

Abbreviations: G-protein, guanine nucleotide-binding protein; G_s , the G-protein that mediates stimulation of adenylyl cyclase (EC 4.6.1.1) α_s , the α subunit of G_s ; $\beta\gamma$, the β and γ subunit dimer of G proteins; K^+ -pNPPase, K^+ -stimulated *p*-nitrophenyl phosphatase.

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2. EXPERIMENTAL

2.1. Materials

[32 P] α ATP (30 Ci/mmol), [32 P]NAD (800 Ci/mmol), rabbit anti- α , subunit antibodies, and [125 I]protein A (8.81 μ Ci/ μ g): DuPont/New England Nuclear (NEN, Boston MA, USA); [3 H]cAMP (30 Ci/mmol): Amersham, UK; sodium cholate: Calbiochem (La Jolla, CA, USA); electrophoresis and SDS-PAGE reagents: Bio-Rad (Richmond, CA, USA); cholera toxin and pertussis toxin: List Biological Laboratories (Campbell, CA, USA); all other reagents: Sigma (St. Louis, MO, USA).

2.2. Animals, tissue collection and preparation

Five pigs (*sus scrofa*, 33 \pm 23 kg) were used. Transmural samples of right atrial (RA) and left ventricular (LV) free wall were rinsed free of blood and stored at -80°C . Samples were powdered in a stainless steel mortar and pestle. Three ml of phosphate buffer (10 mM KH_2PO_4 , 5 mM $\text{MgCl}_2 \cdot \text{H}_2\text{O}$, 5 mM EDTA-Na, 1 mM EGTA, 50 KIU/ml (kallikrein inhibitory units) aprotinin, pH 7.5) were added to 40 mg of powdered sample and glass/glass homogenized (Kontes, Vineland, NJ, USA) on ice for \sim 1 min (30 strokes), as previously described [13,14]. No extraction of contractile proteins was employed unless indicated. Each sample then was centrifuged (45 000 \times g, 20 min, 4°C), and supernatant (SUP) and pellet (PEL) fractions obtained. PEL fractions were resuspended up to 1.5 ml in the same buffer, and an aliquot then was agitated with 1% purified sodium cholate for 60 min on ice. This cholate-treated pellet fraction then was centrifuged (20 000 \times g, 30 min, 4°C), and the resulting supernatant fraction, termed cholate extract (EXT), and pellet fraction (remnant) were obtained. Cardiac α_s was stabilized with 0.1 % Lubrol PX in the SUP and EXT fractions. Volume, protein concentration [15], and extraction yield were calculated. Assays were performed on the indicated number of animals at least twice on the same preparations on different experimental days. In some experiments, fresh RA and LV tissue was homogenized (2 \times 15 s bursts on a Tekmar Tissumizer, 20 500 rpm, 4°C) and analyzed side-by-side with the frozen preparation technique described above.

2.3. Immunoblotting of cardiac α_s and β subunits of G_i

Assessment of the α_s subunit of G_i was conducted using immunoblotting with rabbit anti- α_s subunit antibodies. Supernatant and cholate-extracted protein (60 μ g) were electrophoresed, treated with diluted anti- α_s peptide antibody (1:600), and autoradiographed [16]. The 45 kDa band then was removed for gamma counting. Assessment of the β subunit of the cardiac G-protein in SUP and PEL was determined as above by Western blotting with rabbit anti- β subunit antibodies kindly provided by Dr. G.M. Bokoch. Identified 35/36 kDa bands were removed for gamma counting.

2.4. Cholera toxin- and pertussis toxin-catalyzed ADP-ribosylation

Assessment of G_s and G_i by (cholera toxin and pertussis toxin, respectively) catalyzed ADP-ribosylation was conducted on SUP and EXT fractions [17]; the NAD concentration was increased to 5 μ M to obtain maximal labeling. In quantifying cardiac α_s , the 45 kDa bands were cut out and liquid scintillation counting was employed. The 52 kDa bands were observed inconsistently and only in the EXT, and thus were not included in calculations of total cellular activity. In quantifying G_i, the doublet at 40 kDa and the thin band at 39 kDa were cut out and counted as described above. Because the free α_i monomer cannot be ADP-ribosylated, purified $\beta\gamma$ was added to SUP and PEL fractions to detect monomeric α_i . Purified $\beta\gamma$ from porcine brain was kindly provided by Dr. T. Katada. Additional experiments using cholera toxin labelling of fresh right atrium and left ventricle, with and without ADP ribosylation factor (ARF) derived from mouse lymphoma S49 cys⁻ (G_i-deficient), with and without 1% sodium cholate were performed to control for the possible effects of sample storage, ARF deprivation, and detergent extraction.

2.5. Reconstitution of cys⁻ membranes by cardiac α_s

SUP and EXT fractions were used to quantify α_s activity of RA and

LV samples. The capacity of these fractions to reconstitute α_s -mediated (fluoride-stimulated) production of cAMP in membranes from α_s -deficient S49 murine lymphoma cells (94.15.1, cys⁻) served as a functional assay for α_s [18]. Preliminary studies showed cAMP production to be proportional to the amount of SUP and EXT added, and that the rate of cAMP synthesis remained linear with time for 40 min. Total activity of the tissue is expressed in pmol cAMP produced/mg wet weight.

2.6. Assays of sarcolemmal membrane markers

To determine if G_s activity in SUP resulted from plasma membrane fragments rather than free α_s , K⁺-pNPPase specific activity [19] and forskolin-stimulated (100 μ M) adenylyl cyclase specific activity [20] were used as markers for sarcolemmal membrane.

2.7. Mechanical release of cardiac α_s

To assess if tissue disruption might cause release of α_s from the sarcolemma, the effects of 30, 60, or 120 s of polytron homogenization (4°C , setting no. 5, Brinkman Instruments, Luzern Switzerland) on the release of α_s was determined by immunoblotting and by cholera toxin-catalyzed ADP-ribosylation.

3. RESULTS

3.1. Immunoblots

The first 5 lanes of Fig. 1A shows that in autoradiographs of immunoblots, increasing amounts of purified α_s (45 kDa species, kindly provided by Drs. M.E. Linder and A.G. Gilman) were detected with the anti- α_s antibody. An exemplary autoradiograph of EXT and SUP fractions from 6 porcine left ventricles are also shown in Fig. 1A (lanes 6–11 and 12–17, respectively). With 60 μ g of protein applied to each lane, the amount of α_s in the EXT was greater than that in the SUP. However, since all fraction volumes and protein concentrations were rigorously recorded for preparations used in this study, after calculation of total activity in a fraction, both the greater volume and protein content in the SUP fraction yielded a greater increase in total activity than in the EXT fraction. The sum of the total activity of these fractions when the entire PEL fraction was extracted was termed total tissue activity.

Results from immunoblotting studies that were quantified by gamma counting (Fig. 2A) showed that in RA, 52 \pm 14% of total tissue activity was distributed to SUP, and 48 \pm 14% to EXT. The SUP from LV contained 54 \pm 7%, and EXT contained 47 \pm 7% of total tissue α_s activity. LV total tissue activity was 2.4-fold higher than RA total tissue activity (LV: 4391 \pm 993 cpm/mg wet weight; RA: 1,799 \pm 840 cpm/mg wet weight, $P=0.004$). Immunoblots of fresh and frozen preparations from the right atrium and left ventricle probed side-by-side showed similar bands in both supernatant and pellet fractions.

3.2. Cholera toxin-catalyzed ADP ribosylation

Fig. 1B shows an exemplary autoradiograph of cholera toxin-catalyzed ADP ribosylation of the different fractions from the left ventricle. The 45 kDa band is seen in all fractions, most prominently in the EXT.

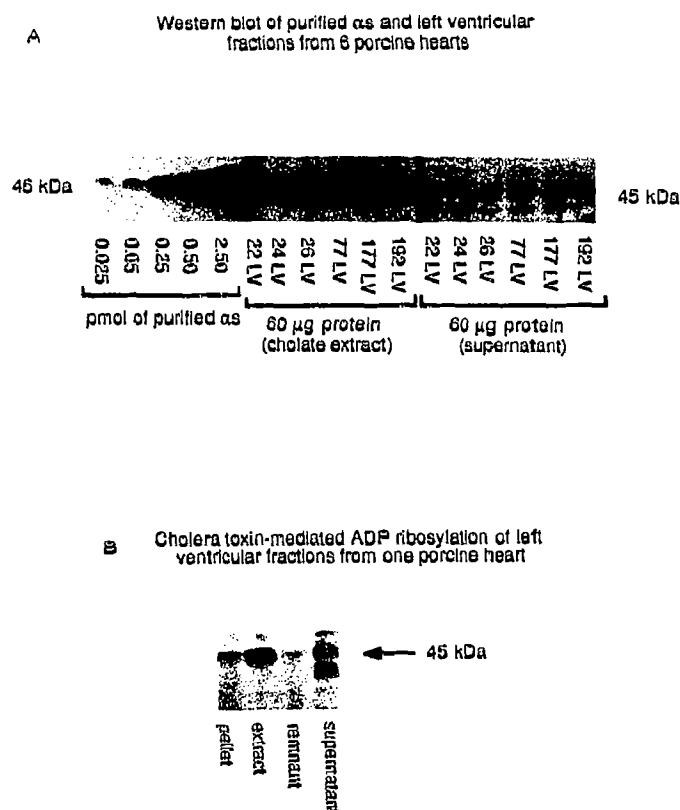


Fig. 1. Autoradiographs of prepared fractions of porcine left ventricle. (A) Immunoblot of purified α_s (45 kDa) and left ventricular (LV) fractions from 6 porcine hearts. Lanes 1–5 have indicated amounts of purified α_s added per well. Lanes 6–11 have 60 μ g of cholate extracted protein (EXT) added per well. Lanes 12–17 have 60 μ g of supernatant protein (SUP) added per well. (B) Autoradiograph of cholera toxin-mediated ADP ribosylation of four prepared fractions (pellet, cholate extract, remnant, supernatant) from one porcine left ventricle. Each lane was loaded with 16 μ g of protein from the indicated fractions. See text for fraction definitions.

However, after calculations taking into account the fraction volumes and protein content as described above, total tissue activity is distributed as seen in Fig. 2B. Results from quantitative ribosylation studies (Fig. 2B) showed that in RA, 61 \pm 10% of the total tissue activity was distributed to SUP, and 39 \pm 10% to EXT. The SUP from LV contained 66 \pm 6%, and EXT contained 34 \pm 6% of total tissue α_s activity. Again, LV total tissue activity was more than 2.5-fold higher than RA (LV: 26.3 \pm 5.3 fmol/mg wet weight; RA: 10.4 \pm 2.7 fmol/mg wet weight, $P=0.0003$).

We performed additional experiments using cholera toxin labelling of fresh right atrium and left ventricle, with and without ARF derived from mouse lymphoma S49 cyc^- (G_s -deficient), with and without 1% sodium cholate additions, and found identical results as reported above, with no effect of adding ARF, detergent, or using fresh tissue.

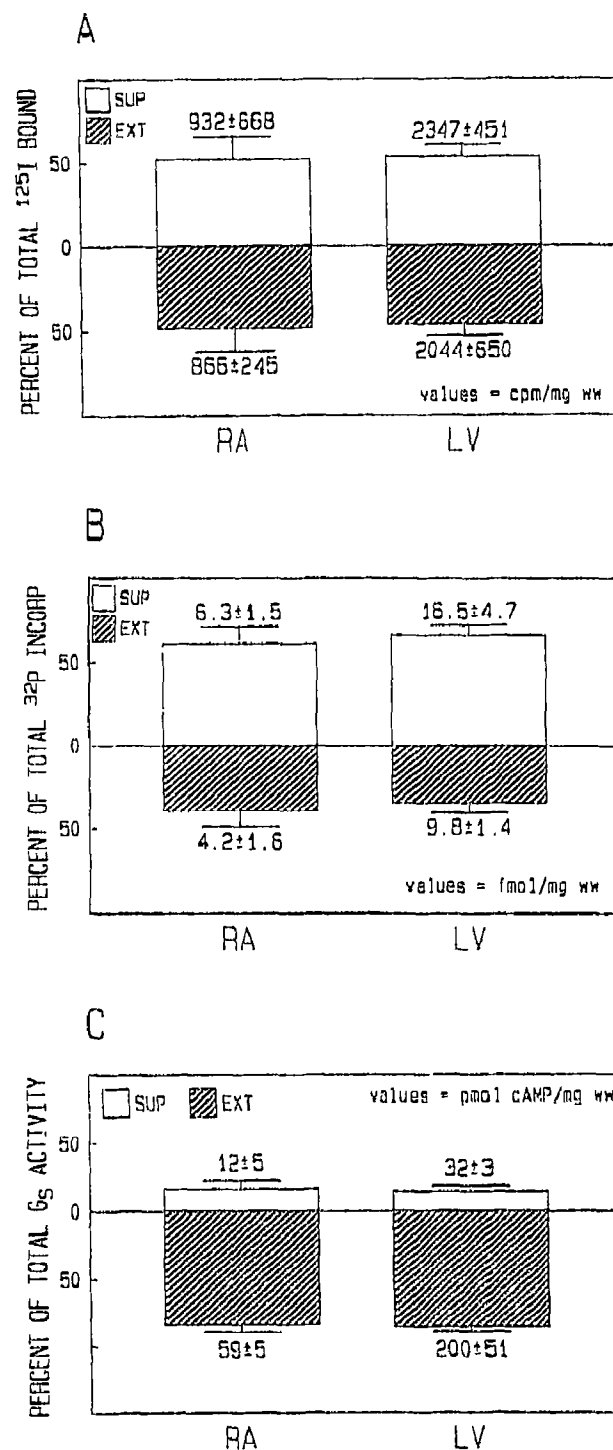


Fig. 2. Assessment of G_s activity in right atrial (RA) and left ventricular (LV) tissue by three methods. The ordinate is percent of total activity. Absolute activities are expressed in indicated units above or below each bar for the respective tissues and fractions. (A) Results of quantitative immunoblots utilizing rabbit anti- α_s antibodies and 125 I-protein A in supernatant (SUP) and cholate extract (EXT) fractions. (B) Results of quantitative cholera toxin-catalyzed ADP-ribosylation in supernatant SUP and EXT fractions. (C) Results of reconstitution assays utilizing S49 cyc^- membranes with 10 mM NaF stimulation in the SUP and EXT fractions.

3.3. Reconstitution

Fig. 2C shows the results of reconstitution studies on SUP and EXT fractions from RA and LV. In RA, $17 \pm 6\%$ of the total tissue activity was found in SUP, and $83 \pm 6\%$ in the EXT. Similar distributions were found in the LV (SUP: $15 \pm 4\%$; EXT: $85 \pm 4\%$). It is noteworthy that LV had 3-fold more α_s activity than the RA (LV: 232 ± 51 pmol cAMP produced/mg wet weight; RA: 71 ± 8 pmol cAMP produced/mg wet weight; $P < 0.0001$). In addition, when SUP, or sequential dilutions of SUP supplemented with heat-inactivated SUP (95°C , 5 min) were reconstituted under the same conditions, a linear response of cAMP production was obtained ($r=0.93$, $n=4$, data not shown). Reconstitution in cyc membranes was not affected by 1% cholate treatment of SUP fractions.

3.4. Assays of sarcolemmal membrane markers

Fig. 3 shows that in LV, the specific activity of K^+ -pNPPase in SUP was only 9% of the total activity, demonstrating that supernatant α_s activity cannot be explained by membrane fragment contamination. Forskolin-stimulated adenylyl cyclase specific activity confirmed these findings with only 3% of total adenylyl cyclase activity present in supernatant. Additionally, the pertussis toxin-catalyzed labeling of the SUP was very low compared to the PEL in both heart chambers, indicating only minimal contamination by membrane fragments in this preparation. Moreover, detection of β -subunit by Western blots with purified β -subunit antibodies in SUP was minimal compared to PEL fractions (data not shown).

3.5. Mechanical release of cardiac G_s

When the quantity of α_s in SUP and PEL fractions of LV tissues was assessed for total α_s content by both immunoblotting and cholera toxin-catalyzed ADP-ribosylation, we found that increased tissue disruption (30, 60 or 120 s of polytron homogenization) did not yield increased release of α_s to supernatant (data not shown), suggesting that the above findings cannot be explained by mechanical disruption alone. We are un-

able to determine whether 'basal' levels of α_s release result from mechanical disruption; there may be a vulnerable or loosely-associated population of α_s that accounts for this distribution. Nevertheless, we show this to be an important population of α_s when trying to quantify total α_s , and speculate that it may be a biologically important fraction. We employed the present sedimentation conditions to match those routinely reported in the literature, but we also found that the α_s activity in SUP was similar whether $45\,000 \times g$ or $180\,000 \times g$ centrifugations were employed.

Contractile protein extractions with high ionic strength solutions are used commonly when studying cardiac tissue in an attempt to obtain sarcolemmal membranes of higher purity for biochemical analyses [13,14]. We found similar amounts of α_s activity in SUP fractions whether or not crude cardiac membranes were extracted with 0.5 M KCl, implying that supernatant α_s is not due to the labile release of α_s from the cytoplasmic face of the plasma membrane. If initially a low-speed centrifugation ($1250 \times g$) was employed to pellet contractile proteins and nuclei from crude homogenate [21], followed by $45\,000 \times g$ centrifugation of the SUP, we found a loss of 72% of total tissue α_s activity (assessed by ADP-ribosylation) to the routinely discarded pellet of the $1250 \times g$ centrifugation. In addition, after 1% cholate extraction of PEL and subsequent centrifugation, about 36% of total α_s activity remained in the routinely discarded remnant fraction, a consistent finding whether or not an initial low-speed centrifugation was employed (data not shown).

4. DISCUSSION

When we assessed α_s by immunoblotting and cholera toxin-catalyzed ADP ribosylation, we found that a sizable proportion (52–66%) of total tissue α_s is discarded in the SUP fractions from routine preparations of myocardial tissue. This α_s activity does not result from membrane contamination of the supernatant during tissue preparation, and is not affected by either high salt treatment or initial low-speed centrifugation of a crude cardiac homogenate. Conversely, reconstitution of cardiac α_s with cyc⁻ S49 cell membranes yielded a much smaller proportion of functional α_s activity to the SUP (16%), where the majority of biologically active α_s was present in the EXT (84%).

The current findings have both methodological and, potentially, broad biological implications. Because a substantial proportion of cardiac α_s protein is discarded during routine membrane preparations, our results call into question the meaning of reported changes in α_s from studies in which only a fraction of total α_s may have been measured. Future studies will need to provide more detailed quantitative accounting of α_s .

The biological implications of our findings are important, for they suggest that α_s may not only be dedicated

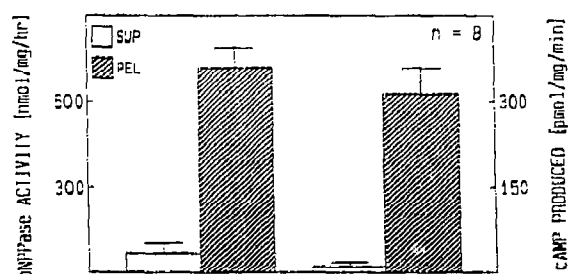


Fig. 3. Specific activity of two sarcolemmal membrane markers used to determine membrane content of supernatant (SUP) and pellet (PEL) fractions of left ventricle (LV). K^+ -stimulated p-NPPase specific activity (nmol/mg protein/hour, left panel) and forskolin-stimulated adenylyl cyclase specific activity (pmol/mg protein/min, right panel).

to plasma membrane-associated interactions, but may also have as yet unknown functions within the cell. The biological activity of this large population of α_s as a stimulator of adenylyl cyclase may be low as assessed by reconstitution (Fig. 2C), implying that the SUP may lack an essential cofactor for adenylyl cyclase activation, or that there has been a covalent or proteolytic modification of α_s such that effector coupling or activation is diminished even though mobility in SDS-PAGE is unaltered. We do not know if this α_s population is functional as a stimulator of other effectors. Using the reconstitution data to assess the function of the α_s in SUP and EXT fractions of the LV, and data from cholera toxin-mediated ADP ribosylation to quantify pmols of α_s in the two fractions, we calculate that the turnover rate in the EXT is more than 14-fold greater than that in the SUP (594 ± 120 vs 39 ± 2 pmol cAMP/min/pmol α_s ; $P < 0.001$).

In theory, the origin of the α_s population in the SUP fraction might be from intracellular pools associated with structures where the protein is synthesized, modified, and transported to the plasma membrane [23,24]. However, the high proportion of α_s we detect in the supernatant fractions, whether functional or not, is unlikely to be fully accounted for by those pools, and is not accounted for by plasma membrane contamination (Fig. 3). Alternatively, α_s in the supernatant fraction may represent a population of α_s normally present in the cytosol and associated with light vesicle fractions [24,25], pinocytotic vesicles [10], or may be a consequence of disengagement of membrane-bound G_s from the sarcolemma [5,26]. Perhaps during activation by hormones or neurotransmitters, α_s dissociates from the cytoplasmic face of the plasma membrane and becomes soluble in the cytoplasm, available to interact with membrane-bound effectors such as enzymes or ion channels [3-5], with intracellular organelles [22,23,27], or to remain in equilibrium as a labile pool of α_s [5,10,24,26].

We have provided evidence for two pools of G_s in the heart: plasma membrane-associated and non-plasma membrane-associated. Data suggesting non-plasma membrane-associated pools of α_s have been reported for several culture cell lines [11,22,24], but previous results have not shown that such pools exist in tissues *in vivo*. However, previous data have indicated that the heart and other cell types possess G_s in substantial excess of receptors. Moreover, G_s may interact with more than one effector [4,28], so that this supernatant population of α_s may integrate the input from several receptors, and may be a branch point for regulation of multiple effectors in response to a single signal. Since individual receptors are catalytic in activation of multiple G_s molecules [4,14,25], the current findings are consistent with the intriguing speculation that activation of G_s by agonists can program α_s molecules to perform multiple functions in target cells [27].

Acknowledgements: These studies were supported by grants from PHS HL07444-10 and Fellowship Award from the California Affiliate of AHA no. 90-69 (D.A.R.); Fellowship Award from the California Affiliate of AHA no. 90-77 and the Japan Heart Foundation (K.U.); Centre National de la Recherche Scientifique and NATO Fellowship (D.L.); Grant HL17682 (P.A.I.); VAMC Career Development and Merit Awards and AHA Grant-California Affiliate no. S121-89 (H.K.H.). The authors wish to thank Drs. M.E. Linder and A.G. Gilman for providing purified α_s , Dr. T. Katada for providing purified $\beta\gamma$ and Dr. G.M. Bokoch for providing purified β -subunit antibodies.

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