Quantification of signalling components and amplification in the β-adrenergic-receptor–adenylate cyclase pathway in isolated adult rat ventricular myocytes

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We have investigated the stoichiometric relationship of proteins involved in β-adrenergic-receptor-mediated signal transduction in isolated rat cardiac myocytes. These cells contain about 2.1×10^5 β-adrenergic receptors per cell, as determined by radioligand-binding assays. We have assessed the amount of $G_s\alpha$ present in myocyte membranes by immunoblotting using a purified glutathione S-transferase–$G_s\alpha$ fusion protein as a standard for quantification. By this method, we determined that cardiac myocytes contain about 35×10^6 and 12×10^6 molecules per cell of the 45 and 52 kDa forms of $G_s\alpha$, respectively. [^{3}H]Forskolin binding assays were used to assess the formation of high-affinity forskolin binding sites representing $G_s\alpha$–adenylate cyclase complexes occurring in response to $G_s\alpha$ activation. Quantification of the adenylate cyclase complexes was facilitated by the permeabilization of cells with saponin. The addition of isoprenaline (isoproterenol) and guanosine 5′-[γ-thio]trisphosphate to saponin-permeabilized myocytes results in the formation of 6×10^5 $G_s\alpha$–adenylate cyclase complexes. Taken together, the data presented here demonstrate that, in a physiologically relevant setting, G-protein is present in large stoichiometric excess relative to both receptor and effector. In addition, we show that, overall, only modest signal amplification occurs between receptor and adenylate cyclase. Thus adenylate cyclase (rather than G_s) is the component distal to receptor that limits agonist-mediated increases in cyclic AMP production. Although limited data are as yet available for other G-protein-regulated effectors, we hypothesize that the stoichiometry of signalling components and the extent of signal amplification described for the β-adrenergic response pathway will be applicable to other G-protein-coupled hormone receptor systems.

INTRODUCTION

The activation of adenylate cyclase by β-adrenergic-receptor agonists involves the interactions of β-agonist with receptor, receptor with G_s, and of $G_s\alpha$ with adenylate cyclase. The functioning of this pathway depends on both the affinities and the rates of these interactions, as well as the relative abundance of these components in the cell membrane. It is well known that changes in receptor affinity for agonist and in agonist concentration play an important role in receptor-mediated second-messenger production [1–3]. However, the extent to which the relative stoichiometry of receptor, G-protein and effector regulates signal transduction is not well understood. Previous work has demonstrated that, in the presence of β-adrenergic-receptor agonists, an excess of $G_s\alpha$, from several-fold to as high as 100-fold, can be activated in response to the activation of a single receptor molecule [4–7]. Because of this ability of a single receptor to activate many G-protein molecules, and considering the excess of $G_s\alpha$ relative to the amount of β-adrenergic receptor [8,9], one might expect that adenylate cyclase would in turn be present in equal or greater amounts than the $G_s\alpha$ protein to maintain this signal amplification. Although limited studies in continuous cell lines suggest that this is not the case [10–12], these cultured cell lines are often derived from transformed cells and may not accurately reflect the situation in a physiologically normal cell. Indeed, tumorigenic growth has been associated with elevated $G_s\alpha$ protein expression [13,14]. Information about the ratio between signalling proteins in native cells is lacking.

We have studied the stoichiometry of proteins involved in the β-adrenergic response of isolated adult rat cardiac myocytes. We have utilized quantitative immunoblots to quantify $G_s\alpha$ protein and [^{3}H]forskolin-binding assays to quantify $G_s\alpha$–adenylate cyclase complex formation. Forskolin-binding assays take advantage of the increased affinity of forskolin interaction with adenylate cyclase in the presence of $G_s\alpha$–GTP [10,12]. This assay therefore provides a measure of $G_s\alpha$–adenylate cyclase interaction that is independent of second-messenger production. Determination of the relative stoichiometry of proteins in the β-adrenergic-response pathway in cardiac myocytes is particularly important in that these cells represent a physiologically relevant setting and because the ratio of these proteins is thought to be of critical importance for normal cardiac function [14,15]. Indeed, recent evidence suggests that cardiac function may be regulated, in part, by altering the membrane content of various components of the β-adrenergic-receptor–adenylate cyclase signal-transduction pathway [15–17].

The current results demonstrate that $G_s\alpha$ protein is present in these cells in great excess relative to β-adrenergic receptor or adenylate cyclase. This suggests that either receptor and/or effector, but not $G_s\alpha$, is limiting for hormone-stimulated second-messenger generation. These results also support the idea that substantial signal amplification between $G_s\alpha$ and adenylate cyclase does not occur, in that β-agonist results in the activation of only a modestly greater number of adenylate cyclase molecules than of receptor.

MATERIALS AND METHODS

Materials

[^{3}H]Forskolin and horseradish peroxidase-coupled anti-rabbit IgG were purchased from NEN–DuPont. Isoprenaline (iso-

Abbreviations used: MEM, minimum essential medium; GTP[S], guanosine 5′-[γ-thio]trisphosphate; PVDF, poly(vinylidene difluoride); GST, glutathione S-transferase.

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proterenol) and saponin were from Sigma. Affinity-purified anti-$G_s\alpha$ antibody (RM) was kindly provided by Dr. A. Spiegel (NIH).

Isolation and permeabilization of cardiac myocytes

Ventricular myocytes from 250–300 g adult Sprague–Dawley rats were isolated by collagenase digestion as described previously [18]. Briefly, rat hearts were perfused at 32 °C for 45 min with minimum essential medium (MEM) containing 0.1 % collagenase (Boehringer-Mannheim), 0.1 % BSA and 25 μM Ca^{2+}. After removal of the atria, the ventricles were cut into pieces and myocytes dispersed by gentle agitation through a wide-bore serologic pipette. The cells were filtered through a nylon mesh and washed twice by centrifugation (50 *g*). The cell pellet was suspended in MEM containing 0.1 % BSA and Ca^{2+} (added slowly to a final concentration of 1 mM). The preparation typically provided (10–15) $\times 10^6$ myocytes per heart, with 80 % viability as assessed by rod-shaped morphology and exclusion of Trypan Blue.

For experiments involving permeabilized cells, isolated myocytes were pelleted by centrifugation (200 *g*) and resuspended in 'reverse' buffer (100 mM KCl, 20 mM NaCl, 1 mM NaH_2PO_4, 20 mM Hepes, 1 mM $MgSO_4$, pH 7.4) at a density of 5×10^5 cells/ml. Permeabilization was accomplished by addition of saponin (final concn. 20 μg/ml) to the cell suspension and incubation of cells at 37 °C for 10 min. Assessment by Trypan Blue uptake indicated that these conditions result in the permeabilization of 98 % of the cells. After incubation with saponin, cells were pelleted by centrifugation (5 min at 200 *g*) and resuspended at appropriate density in 'reverse' buffer.

Quantification of $G_s\alpha$

Isolated rat ventricular myocytes were pelleted by centrifugation; the media were aspirated, and cells were frozen and stored at −80 °C. Frozen cells were powdered with a stainless-steel mortar and pestle (also at −80 °C), suspended in buffer (10 mM Tris, 5 mM $MgSO_4$, 5 μg/ml pepstatin, 10 μg/ml benzamidine, 10 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.4), and homogenized in a glass/glass vessel. Crude cellular membranes were isolated by centrifugation (13300 *g*) for 10 min at 4 °C. Protein concentration was determined by the Bradford protein assay [19]. Proteins from three different cardiac myocyte membrane preparations were subjected to SDS/PAGE (10 % separating gel) and electroblotted on to a poly(vinylidene difluoride) (PVDF; Immobilon-P) membrane. A bacterially expressed fusion protein consisting of glutathione S-transferase (GST) fused to the C-terminus of $G_s\alpha$, purified on a glutathione affinity column, was used to generate a standard curve for quantification. Non-specific antibody binding was blocked by incubating membranes in 5 % BSA for 1 h at 25 °C, and $G_s\alpha$ was detected by using affinity-purified $G_s\alpha$-specific polyclonal antisera. Detection of primary antibody was accomplished by incubating membranes with horseradish peroxidase coupled to anti-rabbit IgG for 30 min at 25 °C. After the addition of chemiluminescent substrate (ECL, Amersham), blots were exposed to film for 0.5–3 min and band intensity was determined by densitometric analysis.

Cyclic AMP assays

Equilibrated suspensions of intact myocytes (2×10^5 cells/ml) were treated with agonists for 4 min at 32 °C, after which cold trichloroacetic acid (10 % final concn.) was added. The samples were centrifuged at 1200 *g* for 15 min and the trichloroacetic acid extracts were purified over Dowex AG50W X4 (200–400 mesh). Cyclic AMP content was determined by the protein-binding method of Gilman [20]. Acid-precipitable material was suspended in 0.4 M NaOH and the protein content determined by the method of Bradford [19]. Data are expressed as pmol of cyclic AMP (corrected for recovery over Dowex columns) per 2×10^5 cells.

[^{3}H]Forskolin-binding assays

[^{3}H]Forskolin-binding assays were conducted by a modification of published methods [10,12]. Permeabilized or intact cells (1×10^5) were incubated in 'reverse' buffer or Dulbecco's MEM supplemented with 20 mM Hepes (DMEH) respectively, with 20 nM [^{3}H]forskolin and the additions indicated in the Figure legends in a total volume of 0.5 ml for 10 min at 25 °C. Reactions were terminated by the addition of ice-cold wash buffer (50 mM Tris, 10 mM $MgCl_2$; pH 7.4) and the rapid filtration of cells on Whatman GF/C filters using a Brandel cell harvester. Filters were washed with 10 ml of wash buffer and radioactivity associated with cells was determined by scintillation counting of filters. Under these filtration conditions less than 5 % of bound forskolin dissociates in 5 min after dilution of cell suspensions; therefore, dissociation of bound forskolin in the 30–60 s wash period is negligible.

RESULTS

$G_s\alpha$ quantification

The amount of $G_s\alpha$ in cells has been quantified both by cholera-toxin-mediated ADP-ribosylation using labelled substrate and by quantitative immunoblotting [17,21–23]. Values obtained by toxin labelling are typically much lower than those obtained by immunoblots of G-protein in the same cells [17,23]. Whether this reflects a population of $G_s\alpha$ that is not a substrate for cholera toxin, or whether it is an inherent problem with this indirect method of quantification, is unclear. Therefore, we determined $G_s\alpha$ levels in cardiac myocytes by quantitative immunoblotting using an affinity-purified $G_s\alpha$-specific antibody and an affinity-purified GST–$G_s\alpha$ fusion protein (containing the epitope used to generate the anti-$G_s\alpha$ antibody) as a standard for quantification [17]. Membrane proteins prepared from three different ventricular myocyte isolations were separated by SDS/PAGE. Various amounts of the GST–$G_s\alpha$ protein were included to generate a standard curve. Myocyte membranes contain both 52 kDa and 45 kDa forms of $G_s\alpha$ in the ratio 1:3 (Figure 1a). $G_s\alpha$ protein concentration was determined by comparing the intensity of bands corresponding to $G_s\alpha$ in the membrane fractions with a standard curve generated from the GST–$G_s\alpha$ fusion protein. Using this procedure, we found that there were 13.7 ± 1.8 pmol/mg of membrane protein of the 52 kDa form of $G_s\alpha$ and 40.7 ± 7.5 pmol/mg of membrane protein of the 45 kDa form. These values correspond to approx. 12×10^6 and 35×10^6 molecules per cell respectively (1 mg of membrane protein $\approx 7.5 \times 10^5$ myocytes).

$G_s\alpha$-adenylate cyclase complex-formation

Since antibodies that are suitable for detecting adenylate cyclase protein are not available, two alternative methods have been used to assess the amount of adenylate cyclase present in cells. One method relies on enzyme activity; the other is based on forskolin binding [10,12,24]. Quantification using changes in enzyme activity is complicated by the complex regulatory mechanisms governing adenylate cyclase activity and second-messenger generation. Therefore we utilized [^{3}H]forskolin binding to

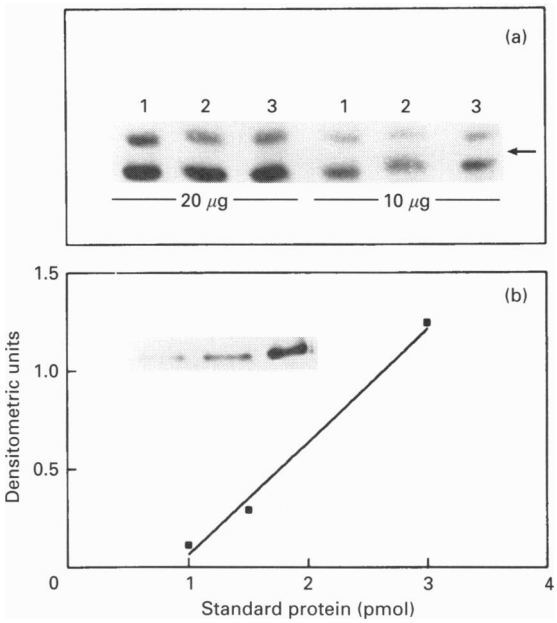

Figure 1 SDS/PAGE of $G_s\alpha$ proteins

Indicated amounts of myocyte membrane protein from three myocyte preparations (**a**) or affinity-purified GST–$G_s\alpha$ fusion-protein standard (**b**) were separated by SDS/PAGE (10% gel), electroblotted to a PVDF membrane, and probed with an affinity-purified $G_s\alpha$-specific primary antibody and horseradish peroxidase-coupled secondary antibody. Arrow indicates 48 kDa molecular-mass standard. $G_s\alpha$ was quantified by densitometric analysis of immunoblots. Panel (**b**) shows a representative standard curve generated by different amounts of GST–$G_s\alpha$ fusion-protein standard.

Table 1 Synergistic effect of isoprenaline and forskolin on cyclic AMP production in intact cardiac myocytes

Isolated myocytes (2×10^5/ml) were incubated for 4 min at 32 °C with the indicated agents. Cyclic AMP accumulation was assessed as described in the Materials and methods section. Data represent means ± S.E.M. of at least four experiments.

Addition	Cyclic AMP (pmol)
Buffer	8.6 ± 0.8
1 μM isoprenaline	20.5 ± 4.3
1 μM forskolin	12.7 ± 0.9
10 μM forskolin	23.5 ± 1.6
1 μM isoprenaline + 1 μM forskolin	202 ± 56
1 μM isoprenaline + 10 μM forskolin	659 ± 154

quantify adenylate cyclase. The synergistic enhancement of adenylate cyclase activity and formation of high-affinity forskolin-binding sites by either receptor- or cholera-toxin-induced activation of $G_s\alpha$ indicates that the interaction of forskolin with adenylate cyclase is greatly enhanced by functional $G_s\alpha$ [10,12,24–26]. As shown in Table 1, forskolin and isoprenaline each increased intracellular cyclic AMP in isolated myocytes. Importantly, when these agents were added together, cyclic AMP generation was up to 50 times greater than with either agent alone. This result is consistent with the $G_s\alpha$-dependent enhancement of forskolin–adenylate cyclase interaction observed in other cell systems.

Table 2 Effect of isoprenaline and cholera toxin on [^{3}H]forskolin binding to intact cardiac myocytes

Intact myocytes were preincubated with or without (control) 1 μg/ml cholera toxin at 32 °C for 4 h. Treated myocytes were subsequently incubated with 20 nM [^{3}H]forskolin and indicated agents for 10 min at 25 °C. Binding reactions were terminated by the rapid filtration of cells on Whatman GF/C paper and washing with ice-cold buffer. Radioactivity remaining associated with filters was determined by scintillation counting. Each reaction tube contained 1×10^5 myocytes. Data represent mean c.p.m. bound ± S.D. of quadruplicate determinations.

	[^{3}H]Forskolin bound	
Addition	Control	Cholera-toxin-treated
Buffer	1040 ± 139	1172 ± 93
10 μM isoprenaline	1202 ± 95	1104 ± 70
30 μM 1,9-dideoxyforskolin	405 ± 91	436 ± 30
10 μM forskolin	309 ± 48	422 ± 117

Accordingly, we predicted that activation of $G_s\alpha$ would also increase forskolin binding in cardiac myocytes. However, isoprenaline or cholera-toxin treatment of cells resulted in only slight stimulation of [^{3}H]forskolin binding relative to basal (Table 2). It should be noted that, under basal conditions, myocytes exhibited a substantial amount of specific [^{3}H]forskolin binding, which we initially thought represented the prior activation of $G_s\alpha$ and formation of $G_s\alpha$–adenylate cyclase complexes. However, 1,9-dideoxyforskolin, a forskolin analogue that does not activate adenylate cyclase ([27], and results not shown), completely inhibited this binding, thus suggesting that a substantial amount of bound [^{3}H]forskolin is associated with proteins other than adenylate cyclase. Importantly, 1,9-dideoxyforskolin at concentrations up to 30 μM did not inhibit cyclic AMP accumulation elicited by 1 μM forskolin (results not shown), indicating that 1,9-dideoxyforskolin was not competing for forskolin binding to adenylate cyclase. Proteins that are known to interact with forskolin and 1,9-dideoxyforskolin include ion channels and glucose transporters [27,28]. Thus the inability to detect the formation of $G_s\alpha$–adenylate cyclase complexes in intact myocytes using forskolin-binding assays may be attributed to the extensive interaction of forskolin with components other than adenylate cyclase.

As an alternative, we examined [^{3}H]forskolin binding to saponin-permeabilized cardiac myocytes in which $G_s\alpha$ was activated with isoprenaline and the non-hydrolysable GTP analogue, guanosine 5′-[γ-thio]trisphosphate (GTP[S]). Whereas 10 μM isoprenaline alone had no effect on forskolin binding relative to basal, the addition of 10 μM isoprenaline and GTP[S] greatly stimulated [^{3}H]forskolin binding (Figure 2). The binding of forskolin was dependent on the concentration of GTP[S], displaying an EC_{50} of 0.15 μM. This stimulation of forskolin binding to saponin-permeabilized myocytes reached a maximum by 5 min at 25 °C and was completely dissociated by 30 min after the addition of 10 μM unlabelled forskolin (results not shown).

To assess whether the extent of [^{3}H]forskolin binding to permeabilized cells represented the activation of a sub-set of $G_s\alpha$, intact myocytes were treated with cholera toxin and forskolin binding was examined after permeabilization. Although pretreatment of intact myocytes with 1 μg/ml cholera toxin (32 °C, 3 h) enhanced [^{3}H]forskolin binding to subsequently permeabilized cells (up to 35% above control), the amount of [^{3}H]forskolin bound to permeabilized cells in the presence of 10 μM isoprenaline and 30 μM GTP[S] was the same in control and

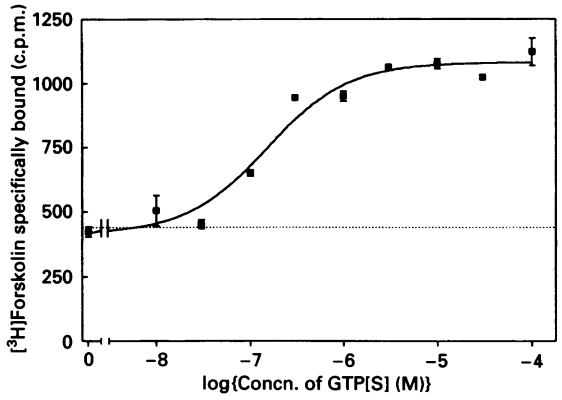

Figure 2 Dependence of [^{3}H]forskolin binding to saponin-permeabilized myocytes on guanine nucleotide and isoprenaline

Saponin-permeabilized cardiac myocytes (1×10^5) were incubated with 20 nM [^{3}H]forskolin for 10 min at 25 °C with various concentrations of GTP[S] in the presence of 10 μM isoprenaline. The dotted line represents binding in the absence of isoprenaline and nucleotide. The curve represents a mathematically modelled function describing the interaction of nucleotide with a single population of binding sites, $EC_{50} = 0.15$ μM. Data points are means ± S.E.M. of triplicate determinations from at least two independent experiments.

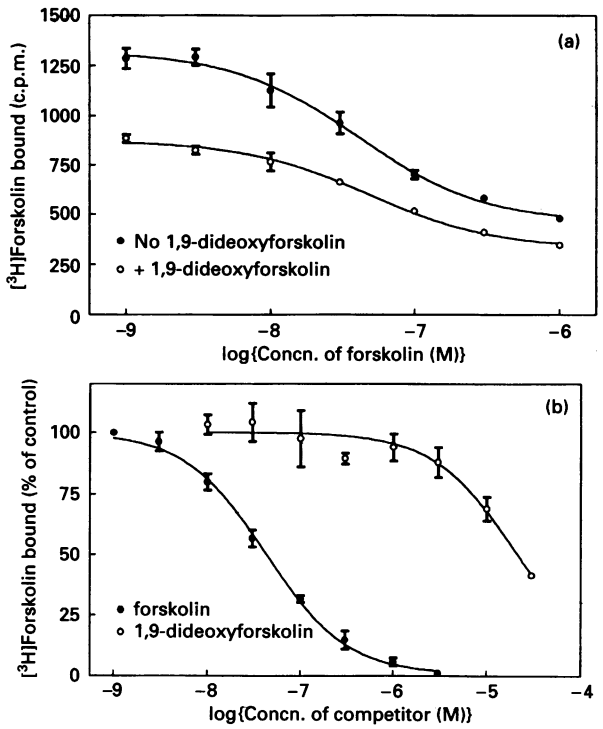

Figure 3 Inhibition by forskolin and 1,9-dideoxyforskolin of [^{3}H]forskolin binding to saponin-permeabilized myocytes

Saponin-permeabilized myocytes (1×10^5) were incubated for 10 min at 25 °C with 20 nM [^{3}H]forskolin, 10 μM isoprenaline, 30 μM GTP[S] and various concentrations of competititor. (**a**) Inhibition of [^{3}H]forskolin binding by forskolin in the presence (○) or absence (●) of 30 μM 1,9-dideoxyforskolin. Data points are means ± S.E.M. of triplicate determinations. (**b**) Inhibition of [^{3}H]forskolin binding by forskolin (●) or 1,9-dideoxyforskolin (○). Data points are means ± S.E.M. of triplicate determinations from three independent experiments. All curves represent mathematically modelled functions describing the inhibition of [^{3}H]forskolin binding to a single population of sites.

cholera-toxin-treated cells (832 ± 18 c.p.m. in cholera-toxin-treated versus 873 ± 112 c.p.m. in control). Thus addition of isoprenaline and GTP[S] to permeabilized cells maximally stimulates the formation of the $G_s\alpha$–adenylate cyclase complex.

The dependence of [^{3}H]forskolin association on isoprenaline and GTP[S] indicates that $G_s\alpha$ activation enhances forskolin interaction with adenylate cyclase and facilitates the quantification of $G_s\alpha$–adenylate cyclase complexes in cardiac myocytes. The reason for our ability to measure $G_s\alpha$-stimulated [^{3}H]forskolin binding in permeabilized, but not intact, myocytes is unclear, but appears to relate to the activity of saponin in lowering the binding of [^{3}H]forskolin to sites other than adenylate cyclase. Use of [^{3}H]forskolin-binding assays in saponin-permeabilized cells may facilitate quantification of adenylate cyclase complexes in other cell systems (results not shown).

Quantification of $G_s\alpha$–adenylate cyclase complexes

The specificity of [^{3}H]forskolin binding for adenylate cyclase was examined more closely in competition assays using unlabelled forskolin and 1,9-dideoxyforskolin. Figure 3(a) depicts the profile of forskolin competition for [^{3}H]forskolin binding. Mathematical modelling of the data indicates that forskolin binds to a single population of sites with an IC_{50} of about 43 nM (K_d = 30 nM). This value is consistent with that obtained for the high-affinity forskolin interaction with $G_s\alpha$–adenylate cyclase complexes in other cell types [10,12,24,28]. Including 30 μM 1,9-dideoxyforskolin decreased both basal and stimulated [^{3}H]forskolin binding proportionately without affecting the apparent affinity of [^{3}H]forskolin binding. This suggests that, although 1,9-dideoxyforskolin does not activate adenylate cyclase, it apparently can decrease the $B_{max.}$ for [^{3}H]forskolin binding by binding to $G_s\alpha$–adenylate cyclase complexes. Indeed, as shown in Figure 3(b), 1,9-dideoxyforskolin competes for stimulated [^{3}H]forskolin binding, albeit with low affinity (IC_{50} 9 μM). From the data in Figure 3(b), we calculated that 5.8 (± 0.95) $\times 10^5$ $G_s\alpha$–adenylate cyclase complexes per cell are formed in response to maximal activation of $G_s\alpha$ after incubation of permeabilized cells with isoprenaline and GTP[S]. This contrasts with the 35×10^6 and 12×10^6 molecules per myocyte of 45 kDa and 52 kDa forms of $G_s\alpha$ (the present paper) and 2.1×10^5 β-adrenergic receptors per myocyte, primarily of the β1 subtype, observed in radioligand binding studies [29,30].

DISCUSSION

An important concept in signal transduction is amplification, whereby one receptor promotes the activation of many effector molecules. The hypothesis that signal amplification results, in part, via interaction of a single receptor with multiple molecules of a particular G-protein is supported by numerous observations [4–9], including the large excess of G-proteins relative to receptor. The idea that amplification might also occur between G-proteins and effector molecules has not been well studied, but for hormonal systems is not well supported by the limited data currently available. In the instance of rhodopsin–transducin, a photon of light activates a single rhodopsin molecule, which, in turn, activates up to 500 transducin molecules and thereby a roughly equivalent number of phosphodiesterase molecules [31,32]. By contrast with the rhodopsin system, agonist binding to isolated cardiac myocytes (the present paper) and cultured cells [10,12] appears to activate a number of adenylate cyclase molecules that is only slightly greater than the number of receptors activated. Together, the data suggest that adenylate

Table 3 Stoichiometric relationship between molecules of the β-adrenergic response pathway

The number of β-receptors was calculated from radioligand binding assays [29,30]. The amount of $G_s\alpha$ represents the total of both 45 and 52 kDa forms detected by immunoblot. The quantity of adenylate cyclase was determined by using [^{3}H]forskolin-binding assays and represents the total number of adenylate cyclase molecules that interact with $G_s\alpha$.

Molecule	No. of molecules/cell	No. of molecules/ receptor number
β-Receptor	2.1×10^5	1
$G_s\alpha$	47×10^6	224
Adenylate cyclase	6×10^5	3

cyclase is limiting for maximum cyclic AMP production occurring in response to hormones and neurotransmitters. Taken further, it appears that altering the amount of effector would be a more efficient means of regulating second-messenger production than by altering the level of G-protein.

The stoichiometry of signal transduction components may also be important in determining the specificity of receptor–G-protein coupling. An early idea in G-protein-mediated signal transduction suggested that a receptor interacted with only one type of G-protein. Recent data demonstrate that this is not the case. In fact, many types of receptors have been shown to interact with multiple types of G-protein (e.g. [33–36]). For β-adrenergic receptors, interaction with G_i, as well as G_s, is suggested by the observation that the β-adrenergic-receptor agonist, [^{3}H]hydroxybenzyl-isoprenaline, binds with high affinity to membranes lacking $G_s\alpha$ (from the cyc$^-$ variant of S49 lymphoma cells). Because treatment of cyc$^-$ cells with pertussis toxin markedly decreases binding of the agonist, the β-adrenergic receptor appears to interact with G_i in the absence of G_s. Overall, these results suggest the possibility that receptor–G-protein specificity may, in part, be a function of the relative quantities of receptors and G-proteins.

As summarized in Table 3, by use of quantitative immunoblotting and [^{3}H]forskolin-binding assays we have determined that, whereas cardiac myocytes contain about 47×10^6 molecules of $G_s\alpha$ per cell, only 6×10^5 $G_s\alpha$–adenylate cyclase complexes per cell are formed in response to maximal $G_s\alpha$ activation. Since myocytes express multiple forms of adenylate cyclase (predominantly Type V and Type VI), the potential underestimation of the quantity of adenylate cyclase complexes resulting from differences in the $G_s\alpha$-sensitive forskolin interaction with these adenylate cyclase isoforms cannot be overlooked. However, several results suggest that this is not the case. First, there is a synergistic increase in cyclic AMP formation in these myocytes occurring in the presence of forskolin and isoprenaline, indicating the enhancement of forskolin–adenylate cyclase interaction by $G_s\alpha$. Second, S49 lymphoma cells, which express Type VI adenylate cyclase, display a similar $G_s\alpha$-sensitive forskolin-binding profile [10]. Third, $G_s\alpha$ binding to Types V and VI adenylate cyclase over-expressed in Sf9 cell membranes is enhanced in the presence of forskolin [37]. Fourth, as reported recently, Types II, V and VI adenylate cyclase demonstrate enhanced forskolin binding in the presence of $G_s\alpha$; Type I adenylate cyclase binds forskolin with high affinity even in the absence of $G_s\alpha$ [38]. Types IV and VII adenylate cyclase have been detected in heart tissue [39,40], but substantial quantities are not likely to be present in isolated myocytes, since Type IV mRNA is only detectable in myocardial preparations by polymerase chain reaction. Also, the activity of Type VII is increased by phorbol ester; but we do not observe an enhancement of cyclic AMP production by phorbol esters (results not shown). Therefore, it would seem unlikely that we have substantially underestimated the number of adenylate cyclase complexes present in cardiac myocytes.

Since previous data indicate that each cardiac myocyte has about 2.1×10^5 β-adrenergic receptors, overall there appears to be about a 3-fold amplification of signal between receptor and effector. In contrast, the amount of cyclic AMP produced in response to isoprenaline and forskolin treatment (Table 1) is in great excess relative to β-receptor. Therefore, amplification of the primary signal (hormone binding) observed by measuring cyclic AMP production arises primarily from the enzymic activity of adenylate cyclase, but not from the activation of a large number of adenylate cyclase molecules.

The biological utility of such a large excess of $G_s\alpha$ relative to receptor and effector is not obvious. One explanation is that $G_s\alpha$ couples to large quantities of many types of receptors (as yet unknown) and to effectors other than adenylate cyclase. To our knowledge, L-type Ca^{2+} and Na^+ channels are the only effectors other than adenylate cyclase shown to interact with cardiac $G_s\alpha$ [41,42]. In cardiac myocytes, adenylate cyclase, Na^+ channels and Ca^{2+} channels are present in similar amounts and together are much less abundant than $G_s\alpha$ (the present paper; [43–45]). Alternatively, although $G_s\alpha$ is in great stoichiometric excess relative to β-adrenergic receptors and adenylate cyclase, it may not be in functional excess. Potentially, (a) $G_s\alpha$ has a relatively low affinity for adenylate cyclase, such that activated $G_s\alpha$ must be present in great amounts in order to achieve high levels of intracellular cyclic AMP, (b) the relative abundance of $G_s\alpha$ ensures specific coupling of β-adrenergic receptor with adenylate cyclase, (c) excess $G_s\alpha$ may increase the rate and duration of the adenylate cyclase response, or (d) the $G_s\alpha$ interaction with receptor and effector may be physically constrained by compartmentation or by cytoskeletal associations, as has been recently postulated [46]. That $G_s\alpha$ is in functional excess is supported by the observation that myocardial membranes prepared from transgenic mice overexpressing $G_s\alpha$ protein demonstrated no difference in basal or stimulated (GTP + isoprenaline, NaF, or forskolin) adenylate cyclase activity relative to control myocardial membranes [47]. It will be important to assess receptor–effector coupling in cells where the expression of individual components of the β-adrenergic-receptor–adenylate cyclase signal-transduction pathway has been altered.

Overall, the results presented here demonstrate that in a physiological setting $G_s\alpha$ is in great excess relative to either receptor or effector. In addition, we show that substantial amplification of the primary signal (hormone binding) does not occur between receptor and effector, in that receptor occupancy results in the activation of only a modestly greater number of adenylate cyclase molecules than of receptors. The amplification of second messenger relative to β-adrenergic receptor seems to arise from the enzymic activity of adenylate cyclase. Thus it appears that effector (adenylate cyclase) is likely to be the most limiting factor for maximal hormone-mediated cyclic AMP production. We hypothesize that the stoichiometry observed for components of the β-adrenergic-receptor–adenylate cyclase system will be applicable to other G-protein-linked effector systems.

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