β -Adrenergic-receptor-mediated dissociation and membrane release of the G_s protein in S49 lymphoma-cell membranes

Dependence on Mg^{2+} and GTP

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We reported [Ransnäs, Svoboda, Jasper & Insel (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7900–7903] that in intact S49 lymphoma cells the β -adrenergic-receptor agonist isoprenaline dissociates the stimulatory guanine-nucleotide-binding protein, G_s , into its α_s and $\beta\gamma$ subunits, leading to redistribution of α_s from plasma membranes to the cytoplasm. In the present studies we investigated the kinetics of G_s dissociation and membrane release in plasma membranes from S49 lymphoma cells. We analysed cholate extracts of membranes for α_s levels by a competitive e.l.i.s.a. with a polyclonal antibody that selectively recognizes monomeric α_s and we assayed supernatant fractions using both competitive e.l.i.s.a. and immunoblotting. The plasma membranes contained 19.3 ± 1.4 pmol of α_s /mg of membrane protein and lacked significant dissociation of G_s and activity of adenylate cyclase in the absence of guanine nucleotides. Mg^{2+} ions were obligatorily required for isoprenaline-induced dissociation of G_s in plasma membranes and for membrane release of α_s . At a physiological concentration of free Mg^{2+} ions (100 μ M), 100 μ M-GTP induced a slow first-order ($k = 0.038 \pm 0.004$ min⁻¹) dissociation of 17.8 ± 1.2 pmol of G_s /mg of membrane protein. A substantial increase in the dissociation rate of G_s was achieved by addition of 1 μ M-isoprenaline and 100 μ M-GTP; 18.4 ± 0.9 pmol of G_s /mg of membrane protein was dissociated, with a k of 1.49 ± 0.22 min⁻¹. The effect of isoprenaline on the dissociation rate and on membrane release of G_s was completely blocked by the β -adrenergic receptor antagonist propranolol. The concentration–response relationship for isoprenaline-induced dissociation during the first 1 min after addition of hormone yielded a $k_{act.}$ of 16 ± 5 nM, whereas the $k_{act.}$ for isoprenaline-induced membrane release was 10 nM. We conclude that release of α_s from plasma membranes is likely to accompany G_s -subunit dissociation and constitutes a potentially important facet of G_s action.

INTRODUCTION

Guanine-nucleotide-binding proteins (G-proteins) serve as membrane-associated signal transducers, coupling activation of receptors to regulation of intracellular effectors [1–5]. G-proteins are comprised of three subunits, termed α , β and γ . The α -subunits confer the individual characteristics of G-protein, whereas the $\beta\gamma$ -dimers, which are similar among the various G-proteins, are believed to be important for membrane association of the G-proteins [1–4]. One of the most extensively studied systems is β -adrenergic-receptor-activated adenylate cyclase. In reconstitution systems, agonist-liganded β -adrenergic receptors appear to activate G_s by increasing the dissociation of G_s -bound GDP, leading to an exchange of GDP for GTP [6,7] and to dissociate the α_s -subunit from the $\beta\gamma$ -dimer. The inherent GTPase activity of the α -subunit terminates the stimulation of the catalytic subunit of adenylate cyclase (by α_s -GTP). In experiments with purified G_o and G_s the bivalent cation Mg^{2+} has been shown to have multiple effects on G-protein activation and nucleotide exchange [8–11]. In such experiments, the GTP-liganded α -subunit seems to bind Mg^{2+} with high affinity, which leads to an enhanced intensity of the intrinsic fluorescence of the α_s -subunit, suggesting a conformational change [8]. Increasing Mg^{2+} concentrations antagonize the inhibitory influence of the $\beta\gamma$ -subunit on GDP release from α_s , which is believed to constitute the rate-limiting step for hormone-induced activation of G_s . In addition, whereas Mg^{2+} is required for the inherent

GTPase activity in GTP-liganded α -subunits, the other potentially deactivating reaction, nucleotide dissociation from dissociated α -subunits, is inhibited by this bivalent cation [2,10]. Experiments using intact cells [12,13] indicate that activation of G_s by agonists can promote a GDP/GTP exchange cycle and can dissociate G_s into its subunits, such that the α_s -subunits can be released from the plasma membrane to the cytosol.

By employing an anti- G_s antibody that selectively recognizes monomeric α_s , we have shown that hormonal activation in S49 lymphoma-cell membranes occurs through subunit dissociation of G_s [11]. We and others (e.g. [12–15]) have indicated the importance of subunit dissociation and subcellular redistribution of G_s . In the present report we have characterized the kinetics of G_s dissociation, and we show that hormone-induced G_s dissociation is dependent on Mg^{2+} and GTP and is accompanied by release of α_s -subunits from the cell membrane.

MATERIALS AND METHODS

Antibody preparation and purification

Antibodies GS-1 and GS-2 were generated in New Zealand White rabbits to a peptide conjugated to BSA, as described previously [11,16,17]. The amino acid sequence of the peptide corresponds to amino acids 28–42 in α_s . Rabbit sera were affinity-purified and characterized by immunoblots, as described in [16]. Antibodies denoted GS-1 recognize α_s as both monomeric and heterotrimeric [11,16], whereas antibodies termed GS-2

Abbreviations used G_s , stimulatory guanine nucleotide regulatory protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; TEN buffer, 20 mM-Tris/HCl/25 mM-NaCl/1 mM-EDTA; EC_{50} or $k_{act.}$, concentration of agonist producing 50% of maximal response.

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selectively detect monomeric α_s [11]; neither of the purified antibodies recognizes G_i , G_o , β -subunits or any other S49 lymphoma-cell membrane proteins [11,16,17].

The same peptide but without prior coupling to BSA was utilized to produce another antiserum against the α_s -subunit. The antiserum was partially purified by incubation with a cyc⁻ cell homogenate, lacking α_s , overnight at 4 °C. The mixture was centrifuged at 100 000 *g* for 1 h at 4 °C and the supernatant was further utilized as antiserum LR4. In other experiments, immunoblotting with LR4 was performed with antiserum partially purified by incubation with a cyc⁻ homogenate and then diluted 1:100. A volume of membrane pellet (60 μ g/well) of cholate-extracted membrane or a volume of supernatant that contained the equivalent volume of membrane protein from which the cholate extract was isolated was electrophoresed, transferred to nitrocellulose, and then immunoblotted overnight at 4 °C. Detection of bound antibody was accomplished by use of ¹²⁵I-Protein A. In some experiments the 45 kDa bands were removed for γ -radiation counting.

Competitive e.l.i.s.a.

Details of the validation of the e.l.i.s.a. are described elsewhere [15–17]. Briefly, sodium cholate extracts of incubated membranes or purified G_s [18] were incubated for 1 h at room temperature with either antibody GS-1 or GS-2 [15]. The incubation mixture was then applied to microtitre wells precoated with the peptide utilized for generating the antibodies. Antibody bound to the wells was determined by employing a goat anti-rabbit IgG antibody coupled to peroxidase, and colour development at 490 nm was measured. By comparing inhibition of antibody binding to peptide-coated microtitre wells by samples and by purified G_s , absolute concentrations of $\alpha_s + \alpha_s\beta\gamma$ or free α_s could be calculated, depending on whether the respective antibody GS-1 or GS-2 was used.

Membrane preparation, incubation and extraction

Wild-type S49 lymphoma cells were grown in suspension culture at $(1-2) \times 10^6$ cells/ml in Dulbecco's modified Eagle's medium and 10% (v/v) heat-inactivated horse serum. Purified S49-cell plasma membranes were prepared as described [19] by nitrogen cavitation and sucrose-gradient centrifugation. Membranes (15–60 μ g of protein per sample) were incubated for the indicated time at 37 °C in the absence or presence of various additions in TEN buffer (20 mM-Tris/HCl/25 mM-NaCl/1 mM-EDTA; pH 7.6). The incubation was stopped by addition of 1 vol. of ice-cold TEN buffer (pH 8.0) either with or without 2% sodium cholate. In the former case the suspension was mixed while kept on ice for 1 h, spun at 15 000 *g* for 20 min to remove insoluble proteins, and the supernatant was diluted with 4 vol. of 1.25% Tween-20 in TEN buffer and incubated with 1:600 or 1:2000 dilution of GS-1 or GS-2 respectively. In the latter case, the incubation mixture was further diluted 2-fold with TEN and centrifuged for 1 h at 100 000 *g* at 4 °C, and pellet and supernatant were then extracted and analysed separately by use of antibody GS-1. Free Mg^{2+} concentrations were calculated as described in [9]. Non-linear regression analysis was done as described in [20].

RESULTS

In previous reports, we showed that the β -adrenergic agonist isoprenaline (isoproterenol) promotes dissociation of G_s into its component subunits in S49 lymphoma-cell membranes and releases monomeric α_s subunits from the plasma membranes to the cytoplasm in intact S49 cells [12,15,21]. In the present paper we investigated the kinetics of G_s dissociation and release of α_s -

subunits from S49-cell membranes. The dissociation of G_s was monitored directly by utilizing an antibody that selectively detects the dissociated α -subunit of G_s . This anti-peptide antibody (GS-2) has previously been characterized and shown to recognize only monomeric α_s and not other G-proteins or heterotrimeric G_s [11]. Total levels of G_s ($\alpha_s\beta\gamma + \alpha_s$) were quantified in two ways, either by utilizing antibody GS-1 [16], which detects G_s (as opposed to other G-proteins) but does not distinguish between heterotrimeric or monomeric α_s , or by employing antibody GS-2 after dissociation of G_s by 20 μ M- $AlCl_3$, 10 mM- $MgCl_2$ and 10 mM- NaF . Release of monomeric α_s from the membranes *in vitro* was monitored after separation of the incubation mixture from the incubated membranes by centrifugation at 100 000 *g* for 1 h, by either using antibody GS-2 in the competitive e.l.i.s.a. or by immunoblot technique utilizing antibody LR4. On the basis of the fact that hydrophobic heterotrimeric G_s is not water-soluble, α_s released into the incubation mixture is monomeric [11]. The sucrose-gradient-

Fig. 1. Hormone- and guanine-nucleotide-dependent subunit dissociation of G_s in S49-cell membranes

In (a), purified membranes [16] were incubated with 1 μ M-(—)-isoprenaline (ISO) and 100 μ M-GTP ($\square$), 1 μ M-isoprenaline + 100 μ M-GTP + 10 μ M-propranolol ($\blacksquare$), 100 μ M-GTP ($\circ$) or 100 μ M-GDP ($\triangle$) in 20 mM-Tris/HCl (pH 7.6)/1 mM-EDTA/25 mM-NaCl/1.09 mM- $MgCl_2$ for the indicated time periods. The concentration of free Mg^{2+} was calculated to be 100 μ M [8]. The incubations were stopped by addition of an equal volume of 2% sodium cholate in TEN buffer and snap-frozen in liquid N_2 . Samples were assayed for dissociation as described in the Materials and methods section. Three independent experiments were run in quadruplicate and the results are expressed as means $\pm$ 1 s.d. In (b), influence of the dose of isoprenaline (1 nM, $\triangle$; 5 nM, $\blacksquare$; 10 nM, $\circ$; 50 nM, $\diamond$; 100 nM, $\square$) on the rate of G_s -subunit dissociation was determined in the presence of 100 μ M-GTP[S] and 100 μ M free Mg^{2+} . Purified membranes were incubated as in (a) with a fixed concentration of free Mg^{2+} (100 μ M) and various concentrations of (—)-isoprenaline and 100 μ M-GTP[S]. Experiments were analysed in quadruplicate and the results are expressed as means $\pm$ 1 s.d.

Fig. 2. Release of α_s from S49-cell membranes by isoprenaline and GTP or GDP

In (a), incubation of S49-cell membranes was performed exactly the same as in Fig. 1(a) but included 1 μM -isoprenaline (ISO) + 100 μM -GTP (top) or 100 μM -GDP (bottom). α_s was then determined separately in membranes and supernatant fractions as described in the Materials and methods section by using antibody GS-1 in an e.l.i.s.a. The data shown were obtained in two different experiments, each time point run in quadruplicate. In (b), immunoblot assay was performed by using antibody LR4 and preparations of pellet and supernatant after activation of G_s by various concentrations of isoprenaline for 20 min in the presence of 100 μM -GTP and 100 μM free Mg^{2+} . In (c), the concentration-response for isoprenaline-induced activation during 20 min in the presence of 100 μM -GTP and 100 μM free Mg^{2+} is shown for pellet (○) and supernatant (●) analysed separately in an immunoblot assay using antiserum LR4.

purified S49 lymphoma-cell membranes did not display adenylate cyclase activity in the absence of exogenously added guanine nucleotides ([19]; results not shown).

The experiments shown in Fig. 1(a) demonstrate that 100 μM -GTP, but not GDP, induced a slow dissociation of G_s; this process appeared to be first-order, with a rate of dissociation of $0.038 \pm 0.004 \text{ min}^{-1}$. Similar results were obtained at GTP concentrations between 50 and 200 μM (results not shown). Thus, in spite of GTPases that may be active in the membranes, at these concentrations sufficient GTP is available to allow continuing dissociation of G_s over the time course of these studies. The apparent rate of G_s dissociation was enhanced by addition of 1 μM -isoprenaline ($k = 1.49 \pm 0.22 \text{ min}^{-1}$), and this enhancement by isoprenaline was blocked by the β -adrenergic-receptor antagonist propranolol. The increase in the apparent rate of dissociation, as determined during the first 1 min after addition of isoprenaline, was concentration-dependent (Fig. 1b), with an EC_{50} ($k_{\text{activation}}$) of $16 \pm 5 \text{ nM}$. This concentration-dependence is similar to that previously observed for activation of adenylate

cyclase in S49-cell plasma membranes or of cyclic AMP accumulation in intact S49 cells [19,22]. These experiments were undertaken at a calculated free Mg^{2+} concentration of 100 μM , which is the physiological concentration of free Mg^{2+} in S49 lymphoma cells [23]. The maximal fraction of dissociated G_s achieved by guanine nucleotides and hormone or by guanine nucleotides alone was 18.4 ± 0.9 and $17.8 \pm 1.2 \text{ pmol/mg}$ of membrane protein respectively, and constituted $\geq 90\%$ of all G_s present ($19.3 \pm 1.4 \text{ pmol/mg}$ of membrane protein, as determined by antibody GS-1).

Experiments utilizing release of monomeric α_s from the plasma membranes (Fig. 2a, top) demonstrated that increases in monomeric α_s were accompanied by a corresponding decrease in heterotrimeric G_s, thus verifying that activation of G_s did not significantly alter the total level of protein recognizable by the antibodies. This release of α_s did not occur in the presence of GDP (Fig. 2a, bottom). Immunoblot experiments utilizing antibody LR4 (Fig. 2b) were undertaken to provide data independent of antibody GS-2 for the release of α_s from the membranes. The

Fig. 3. Basal membrane levels of dissociated α_s .

Purified S49 lymphoma-cell membranes were incubated for 10 min at various concentrations of MgCl_2 in TEN buffer, pH 7.6. The incubation was terminated by snap-freezing in liquid nitrogen. Samples were thawed, and an equal volume of 2% sodium cholate in TEN was added (a), or the sample were diluted 3-fold in TEN alone (b). In (a) monomeric α_s was determined by using antibody GS-2, and in (b) membranes were separated from the incubation mixture by centrifugation as described in the Materials and methods section. (a) shows the membrane concentration of dissociated G_s , whereas (b) demonstrates the distribution of total α_s between the membrane pellet (■) and supernatant (□). Results are expressed as means $\pm$ S.D. from two separate experiments analysed in quadruplicate.

Fig. 4. Influence of the concentration of free Mg^{2+} (100 μM , □; 1 mM, ●; 10 mM, ◇) on the dissociation of G_s by 100 μM -GTP[S] in the absence of hormone

Each experiment was analysed in quadruplicate, and results are shown as means $\pm$ 1 S.D.

EC_{50} ($k_{\text{activation}}$) for isoprenaline-induced release of α_s from the membranes (Fig. 2c) is 20 nM, i.e. a similar concentration-dependence as for dissociation of G_s .

Fig. 5. Influence of the free Mg^{2+} concentration on the rate of G_s -subunit dissociation by 1 μM -isoprenaline and 100 μM -GTP[S] (a) or 100 μM -GTP (b)

Purified membranes [14] were incubated as in Fig. 1 at different free Mg^{2+} concentrations; 1 nM (△), 10 nM (■), 100 nM (○), 1 μM (◆) and 10 μM (□). The degree of dissociation of G_s achieved after 1 min of hormone incubation with GTP[S] (□) or GTP (○) as percentage of maximal dissociation is shown in (c).

We found that Mg^{2+} exerted a major influence on both the 'basal' levels of monomeric α_s in plasma membranes and the rate of subunit dissociation of G_s . As previously reported [11], S49 lymphoma-cell membranes contain a considerable concentration of dissociated α_s in the apparent absence of hormonal stimulation and guanine nucleotides. This 'basal' level of monomeric α_s was found to depend on free $[\text{Mg}^{2+}]$ (Fig. 3a) and reached 4.5 ± 0.7 pmol of α_s /mg of membrane protein at 100 μM free Mg^{2+} . The experiments shown in Fig. 3(b) were undertaken to rule out the possibility that Mg^{2+} might change the interaction between antibodies that we utilized and G_s . In these studies, the amount of α_s was separately determined in membranes and incubation media after incubation with various concentrations of free Mg^{2+}

for 10 min at 37 °C. The results demonstrate that the increase in amount of monomeric α_s in the incubation media observed with increasing $[Mg^{2+}]$ was accompanied by a corresponding decrease of heterotrimeric G_s in the membranes themselves. Changing the buffer system to a phosphate or Hepes-based buffer (instead of Tris) or eliminating NaCl from the buffer did not significantly alter the findings (results not shown).

When we assessed the role of $[Mg^{2+}]$ on guanine nucleotide (GTP[S])-stimulated dissociation of G_s, a somewhat different result was observed. Although $[Mg^{2+}]$ increased the rate of dissociation of G_s by GTP[S] (Fig. 4), the concentrations of free Mg^{2+} that produced this effect were higher than the more physiological levels that regulated 'basal' levels of dissociated α_s . High $[Mg^{2+}]$ appeared to decrease an initial lag phase observed in dissociation of G_s by GTP[S].

By contrast with the effects of Mg^{2+} on GTP[S]-induced dissociation of G_s, the almost 40-fold stimulation in dissociation rate of G_s by isoprenaline was completely dependent on the Mg^{2+} concentration. Fig. 5(a) demonstrates the effect of varying the free Mg^{2+} concentration in the presence of 100 μM -GTP[S] on isoprenaline-stimulated dissociation of G_s. Fig. 5(b) demonstrates a similar set of experiments but with 100 μM -GTP instead of GTP[S]. The presence of Mg^{2+} was found to be an absolute requirement for isoprenaline-induced G_s dissociation in the presence of GTP, whereas a small dissociation still occurred even without addition of Mg^{2+} when the non-hydrolysable GTP analogue GTP[S] was utilized. The maximal degree of isoprenaline-stimulated G_s dissociation was similar at $[Mg^{2+}] \geq 10$ nM whether GTP or GTP[S] was included in the reaction mixture. However, a difference in the apparent rate of activation existed at every Mg^{2+} concentration tested. Experiments in which the concentration of GTP was varied between 50 and 200 μM yielded results similar to those shown in Fig. 5(b). Non-linear regression analysis [17] of initial rates of apparent dissociation of G_s using a single-component exponential equation (Fig. 5c) indicated that the apparent k_{act} for $[Mg^{2+}]$ was 23 μM in promoting isoprenaline-induced dissociation of G_s in the presence of GTP[S]. When GTP rather than GTP[S] was included, high (> 100 μM) $[Mg^{2+}]$ seemed to influence the apparent rate of dissociation of G_s in a negative manner. Although this might represent an inhibitory effect of high concentration of Mg^{2+} on the dissociation reaction itself, a more likely explanation may be the ability of these high $[Mg^{2+}]$ to promote interaction of G_s with other G-proteins such as G_i.

DISCUSSION

Although a variety of different models have been proposed for the regulation of adenylate cyclase activity by receptors, and even though G_s-subunit dissociation was suggested as the mechanism for G_s activation (see, e.g., refs. [2,11,24]), this was not demonstrated to occur in cell membranes until recently [15,16]. In addition, experiments utilizing intact cells have demonstrated that β -adrenergic-receptor-agonist-induced activation of G_s involves not only dissociation but also release of monomeric α_s from the plasma membrane to the cytoplasm [12]. In the present work, we have attempted to define kinetics of guanine-nucleotide- and hormone-induced dissociation of G_s and to monitor the hormone-induced release of α_s from cell membranes. Because of the necessity to centrifuge samples in order to prepare supernatant and membrane pellet fractions, it is difficult to know if membrane dissociation and release of G_s occur simultaneously. Nevertheless, our results strongly suggest that the activation of G_s by hormone leads to release of α_s from the membranes (Figs. 2a–2c), as judged by a similar concentration-dependence of isoprenaline.

Whether the observed membrane release constitutes a mechanism of regulation of non-plasma-membrane-bound effectors or a deactivating process is unclear.

We previously noted that under 'basal' conditions about 20–25 % of G_s appeared to be monomeric α_s in membranes from S49 lymphoma cells [15]. The present findings (Fig. 3a) emphasize that this concentration of monomeric α_s probably results from the concentration of Mg^{2+} (2 μM) utilized in those earlier experiments. Thus one role of Mg^{2+} appears to be to facilitate dissociation of G_s by endogenous nucleotides, hormones, or factors present in S49 cells and membranes. Further support for this conclusion is that a high level of dissociated α_s was not observed under 'basal' conditions for membranes derived from *unc* S49 cells (see Fig. 3 in [11]), cells that possess a mutation near the C-terminus of α_s in a region that interacts with hormone receptors [25].

A second role of Mg^{2+} appears to be the facilitation of guanine-nucleotide-mediated dissociation of G_s (Fig. 4). This effect, however, is a relatively modest one, at least in the case of GTP[S], and seems to involve relatively high, non-physiological, concentrations of Mg^{2+} .

The most impressive action of Mg^{2+} is its facilitation of β -agonist-promoted dissociation of G_s (Figs. 1a, 5a and 5b). The data indicate, in fact, that Mg^{2+} is obligatorily required for the dissociation of G_s by hormone plus guanine nucleotide in plasma membranes. This effect, which is compatible with previous results obtained with purified G-proteins, also probably explains, at least in part, the important role that Mg^{2+} exerts in hormonal stimulation of adenylate cyclase [11]. It is also attractive to speculate that the site at which Mg^{2+} exerts this action in facilitation of dissociation of G_s is the same site on α_s at which Mg^{2+} promotes formation of the high-affinity binding state between receptors and G-proteins [11].

When tested at physiological $[Mg^{2+}]$, both guanine nucleotides and β -adrenergic agonist were able to dissociate more than 90 % of all the G_s present in S49-cell membranes. There are two important implications of these results. Firstly, the findings re-emphasize the striking catalytic capacity of β -adrenergic receptor in these membrane preparations. For example, at 100 nM free $[Mg^{2+}]$, a concentration at which only a small quantity of G_s is dissociated in the absence of hormone or guanine nucleotide (Fig. 3a), about 20 pmol of G_s/mg of protein is dissociated by isoprenaline and guanine nucleotides (Figs. 5a and 5b). Given that the content of β -adrenergic receptors in these preparations is 121.7 ± 14.3 fmol/mg of protein (results not shown), each receptor would appear to have the capability of activating upwards of 200 molecules of G_s.

Secondly, previous evidence has suggested that only a limited fraction (< 20 %) of isolated purified G-proteins activated by GTP in phospholipid vesicles possess bound GTP [6]. The ability of guanine nucleotides plus β -agonist to promote a high degree of dissociation of G_s suggests that a substantial fraction of monomeric α_s may exist in a state not liganded with GTP, i.e. the degree of dissociation of G_s is not equivalent to the degree of activation, thus contributing to the difference between the kinetics of activation of adenylate cyclase and of dissociation of G_s. If, in fact, the monomeric molecules of α_s contain GDP as well as GTP, the results would suggest that GTP hydrolysis alone may be necessary, but not sufficient, to promote re-association of α_s to the $\alpha_s\beta\gamma$ in native cell membranes.

Thus, in contrast with findings in reconstitution systems, hormone-induced activation of G_s may involve an hitherto unrecognized mechanism in G_s activation, namely membrane release of α_s . This mechanism correlates with the dissociation cycle and must be taken into consideration not only when correlating G_s activation with stimulation of adenylate cyclase

but also in discussions of which effector systems might be regulated by α_s . In addition, evidence for membrane release of α_s implies that cytoplasmic factors may influence α_s action.

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