Effect of transmembrane Ca^{2+} gradient on G_s function

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Abstract **G_s and adenylate cyclase from bovine brain cortices were co-reconstituted into asolectin liposomes with or without 1000-fold transmembrane Ca^{2+} gradient. Obtained results showed that G_s activities of both binding GTPγS and stimulating adenylate cyclase were the highest in proteoliposomes, with a transmembrane Ca^{2+} gradient similar to the physiological situation and the lowest while the transmembrane Ca^{2+} gradient was in the inverse direction. Such a difference could be diminished following the dissipation of the transmembrane Ca^{2+} gradient by A23187. Time-resolved fluorescence anisotropy of diphenylhexatriene (DPH) has been used to compare the physical state of phospholipids among those proteoliposomes. It is suggested that a proper transmembrane Ca^{2+} gradient is essential for higher membrane fluidity, which may favor G_s function with higher GTP-binding activity and stimulation of adenylate cyclase.**

Key words: Transmembrane Ca^{2+} gradient; Stimulatory GTP-binding protein; G_s; Adenylate cyclase; Lipid fluidity

1. Introduction

The cytosolic free $[Ca^{2+}]$ in most cells is around 10^{-7}–10^{-6} M, whereas the extracellular $[Ca^{2+}]$ is about 10^{-3}M. This results in a 1000–10,000 fold transmembrane Ca^{2+} gradient. The maintenance of such a concentration gradient has been proved to be of vital importance in cell function [1]. Although a transmembrane Ca^{2+} gradient exists across the plasma membrane, little attention has been paid to the effect of such a Ca^{2+} gradient on the structure and function of membrane proteins. In recent years the focus of our laboratory has been on the effect of the transmembrane Ca^{2+} gradient on the interaction of membrane lipids and proteins. By membrane resolution and reconstitution, we have found that a proper transmembrane Ca^{2+} gradient is essential for the optimal fluidity of the phospholipid bilayer, favoring the formation of a suitable conformation of the reconstituted adenylate cyclase with higher enzyme activity [2–4]. As stimulatory GTP-binding protein (G_s), a member of the G protein superfamily, mediates the stimulation of adenylate cyclase by a variety of hormones [5], a study of the modulation of the transmembrane Ca^{2+} gradient on the function of G_s is of great significance for both exploration of the effect of the transmembrane Ca^{2+} gradient on the function of membrane-bound proteins and elucidation of molecular mechanisms of signal transduction.

In the present study, the effect of a transmembrane Ca^{2+} gradient on G_s function and its relationship to the physical state of membrane phospholipids were investigated. This was achieved by co-reconstitution of the pure G_s and adenylate cyclase prepared from bovine brain cortices into asolectin liposomes with or without a 1000-fold transmembrane Ca^{2+} gradient. G_s activity of binding GTPγS and stimulating adenylate cyclase in four types of proteoliposomes were compared. In addition, the physical state differences of phospholipids in these vesicles were also detected by nanosecond time-resolved fluorescence anisotropy using DPH as a probe.

2. Materials and methods

2.1. Materials

Fresh bovine brains were purchased from Dahongmen Slaughterhouse in Beijing, China. ATP was from Fluke and Sepharose 4B from Pharmacia. Octyl glucoside, GTP, GTPγS, forskolin, diphenylhexatriene (DPH), A23187, and asolectin were all from Sigma. Dithiothreitol (DTT) and sodium cholate were obtained from SERVA. [^{35}S]GTPγS was purchased from Dupont Co. [^{3}H]cAMP was provided by Chinese Academy of Atomic Energy.

2.2. Membrane preparation

Cell membranes from bovine brain cortices were prepared as described by Sternweis and Robishaw [6].

2.3. Purification of G_s and adenylate cyclase from bovine brain cortices

G_s and adenylate cyclase were isolated as previously described [7]. The basal activity of the partly purified adenylate cyclase was 300–400 pmol cAMP/min/mg and could be markedly stimulated by forskolin or Mn^{2+}, but not by GTPγS or F^-. This indicates that adenylate cyclase used for the reconstitution was completely resolved from G_s. The purity of G_s was judged by SDS-PAGE which showed two protein bands of 45 and 36 kDa [7].

2.4. Incorporation of G_s and adenylate cyclase into asolectin liposomes

1.25 nmol of adenylate cyclase and 1.25 nmol of G_s were incubated with sonicated asolectin (35 mg in buffer A: 10 mM Tris-HCl, 1 mM DTT, 100 mM NaCl, 1 μM $[Ca^{2+}]$, pH 7.4; or buffer B, identical to buffer A except that 1 μM $[Ca^{2+}]$ was replaced by 1 mM $[Ca^{2+}]$) and 0.85% octyl glucoside for 30 min at 0°C. Removal of the detergent in the preparation was accomplished by Sepharose 4B gel-filtration. The elutes were centrifuged at 250,000 × *g* for 90 min and the protein–lipid pellets were resuspended [7]. Four types of proteoliposomes containing G_s and adenylate cyclase with different transmembrane Ca^{2+} gradients were reconstituted separately as follows:

Type a: inside higher Ca^{2+}-containing (1 μM $[Ca^{2+}]$ outside and 1 mM $[Ca^{2+}]$ inside, similar to physiological situation) proteoliposomes (the active center of enzyme exposed to outside) (Lca+−): the eluate in buffer B was centrifuged and the pellets were resuspended in buffer A.

Type b: two-side higher Ca^{2+}-containing (1 mM $[Ca^{2+}]$, similar to extracellular $[Ca^{2+}]$) proteoliposomes (Lca++): the pellets obtained to prepare type a proteoliposomes were resuspended in buffer B.

Type c: two-side lower Ca^{2+}-containing (1 μM $[Ca^{2+}]$, similar to cytosolic $[Ca^{2+}]$) proteoliposomes (Lca−−): the eluate in buffer A was centrifuged and the pellets were suspended in the same buffer.

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Abbreviations: G_s, stimulatory GTP-binding protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DPH, diphenylhexatriene; $[Ca^{2+}]$, Ca^{2+} concentration; ATP, adenosine triphosphate; GTP, guanosine triphosphate; DTT, dithiothreitol; cAMP, cyclic adenosine monophosphate; SDS, sodium dodecyl sulfate; kDa, kilodalton; Tris, *N*-tris(hydroxymethyl)aminomethane.

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Type d: outside higher Ca^{2+}-containing (1 mM [Ca^{2+}] outside and 1 μM [Ca^{2+}] inside, inverse to physiological situation) proteoliposomes (Lca−+): the pellets obtained to prepare type c proteoliposomes were suspended in buffer B.

2.5. [^{35}S]GTPγS binding assay

^{35}S-Labeled GTPγS binding assay was carried out as described in [8].

2.6. Determination of adenylate cyclase activity

G_s-stimulated adenylate cyclase activity was assayed as previously described [7] in the presence of 10^{-4} M GTPγS.

2.7. Time-resolved fluorescence measurement

Time-resolved fluorescence data were obtained on the nanosecond fluorescence system of Edinburgh Model 299T. The flashlamp operating conditions were: repetition rate 40 kHz, electrode separation 0.7 mm, pressure 0.5 B hydrogen, high voltage 7 kV. The spectrometer subsystem is configured in a T-geometry with hydrogen flashlamp source, excitation and analysis monochromators, sample chamber and single photon detection system. For anisotropy measurements the parallel ($I_\parallel(t)$) and perpendicular ($I_\perp(t)$) components of the fluorescence decay were collected alternately into two 1024-channel memories. The measurement data were stored in a Multi-Channel Analyzer(MCA) to build up a histogram of the fluorescence decay, and analyzed by software FLA-900S which is based on a Marquardt minimization of nonlinear, least-squares fit. Goodness of fit was assessed by a reduced chi-squared (χ^2) of close to unity and total random residuals using standard methods. The anisotropy is given by:

$$r(t) = \frac{I_\parallel(t) - I_\perp(t)}{I_\parallel(t) + 2GI_\perp(t)}$$

The anisotropy decay data were characterized by analysis of a sum of exponentials plus an r_∞ term [9]:

$$r(t) = r_\infty + (r_0 - r_\infty)\ \Sigma\alpha_i e^{-t/\tau i}$$

where τ is the rotational correlation time, and r_0 is the anisotropy before any rotation takes place ($t = 0$).

Proteoliposomes were labeled by adding 5 μl DPH (2 mM in tetrahydofuran). The ratio of probe-to-lipid was 1:500. The mixture was incubated at 30°C for 30 min. Fluorescence measurements were carried out at 30°C, the wavelengths of excitation and emission were 360 and 430 nm, respectively.

3. Results and discussion

3.1. A proper transmembrane Ca^{2+} gradient is essential for G_s function.

We have reported [2–4] that the transmembrane Ca^{2+} gradient plays an active role in regulating the activity of reconstituted adenylate cyclase. It was suggested that a proper transmembrane Ca^{2+} gradient is essential for the optimal fluidity of the phospholipid bilayer and suitable conformation of adenylate cyclase with higher activity. It is well known that adenylate cyclase is a component of the signal transduction system and

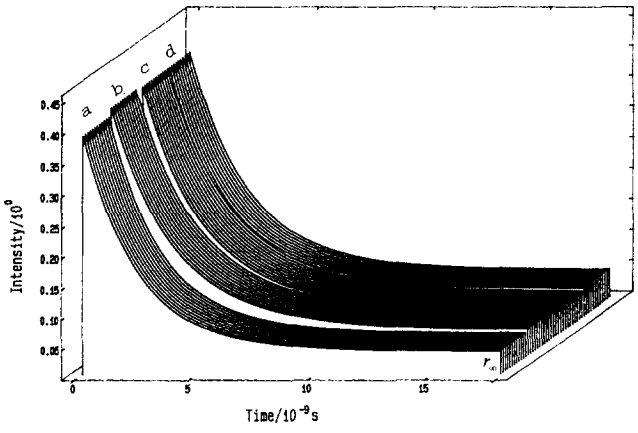

Fig.1 The three-dimensional depiction of time-resolved fluorescence anisotropy decays of DPH in proteoliposomes with or without a transmembrane Ca^{2+} gradient. Curve a, Lca+−; curve b, Lca++; curve c, Lca−−; curve d, Lca−+.

is activated by G_s protein. It would be interesting to understand whether a transmembrane Ca^{2+} gradient also plays a role in modulating the G_s function of stimulating adenylate cyclase activity. The data in Table 1 clearly show that G_s activities of both binding GTPγS and stimulating adenylate cyclase activity were the highest in proteoliposomes type a (Lca+−, lower [Ca^{2+}] outside), which is similar to the physiological situation, and the lowest in proteoliposomes type d (Lca−+) with an inverse transmembrane Ca^{2+} gradient (higher [Ca^{2+}] outside). Proteoliposomes without a transmembrane Ca^{2+} gradient (Lca++ or Lca−−) exhibited intermediate activities. It may be suggested from these results that a proper transmembrane Ca^{2+} gradient is important for G_s to exert its physiological function. This idea is further supported by the finding that dissipation of the transmembrane Ca^{2+} gradient by the ionophore A23187 leads to a decrease in G_s function in proteoliposomes type a and an increase in proteoliposomes type d (Table 1). The topology of G_s is different from that of adenylate cyclase; the latter is a transmembrane protein while the former is associated with the cytoplasmic surface of plasma membrane [5]. It may be deduced that the transmembrane Ca^{2+} gradient not only affects the activities of transmembrane proteins but also regulates the function of membrane proteins, such as G_s, partially embedded in the cytoplasmic surface of the plasma membrane.

3.2. The transmembrane Ca^{2+} gradient regulates G_s function by altering membrane fluidity

The time-resolved fluorescence anisotropy was analyzed using the mathematical models as described in section 2. The

Table 1
Effect of transmembrane Ca^{2+} gradient on G_s activities of binding [^{35}S]GTP$_\gamma$S and stimulating adenylate cyclase

Samples	G_s activity of binding [^{35}S]GTP$_\gamma$S (nmol/mg)		G_s-stimulated adenylate cyclase activity (nmol cAMP/min/mg)	
	−A23187	+A23187*	−A23187	+A23187*
Lca+−	3.318 ± 0.433	2.058 ± 0.307	3.740 ± 0.356	1.808 ± 0.190
Lca++	1.638 ± 0.374	1.722 ± 0.378	1.636 ± 0.170	1.743 ± 0.201
Lca−−	1.896 ± 0.323	1.806 ± 0.332	1.824 ± 0.158	1.689 ± 0.183
Lca−+	0.882 ± 0.197	1.680 ± 0.189	0.864 ± 0.117	1.584 ± 0.159

Each value in Table 1 represent the mean ± S.D. of 5 experiments
*Proteoliposomes were pretreated with A23187 (10 μg/ml) for 10 min at 0°C before assay.

reduced χ^2 and analysis of residuals (data not shown) indicated that the data were well described by the mono-exponential decay law plus a constant (r_∞) following extracting the scattered light component. Fig. 1 shows the three-dimensional depiction of anisotropy decays in those proteoliposomes. The initial slope of the four curves is about the same, showing no significant differences of rotational correlation time among these proteoliposomes. However, the anisotropy at long times (r_∞) changes in the order: a < b ≅ c < d, indicating that a physiological transmembrane Ca^{2+} gradient produces a lower order of lipids while an inverse Ca^{2+} gradient leads to a higher order of the lipids in the proteoliposomes as rod-like linear probes like DPH exhibit a wobbling motion confined to a double hard-cone. This motion has been proven to be sensitive to the packing density of the lipids and is related to the order parameter ($S = (r_\infty/r_0)$, where r_0 is the maximum anisotropy) [10–12]. Also, with all the membrane systems studied so far, it has been found that in the case of DPH, increasing the protein-to-lipid or cholesterol-to-lipid ratio in reconstituted systems has invariably led to an increase in r_∞ (order) without much effect on rotational correlation time [9,13,14]. For these consideration, it seems reasonable that the extent of the restricted motion of the acyl chains, which is a measure of the order of the anisotropic environment, is the most important parameter characterizing lipid fluidity of the membranes with DPH as the probe. In other words, in the case of DPH, lipid fluidity is inversely related to the order parameter. A higher order parameter correlates with lower membrane fluidity and vice versa. The present study clearly shows that the order parameter (S) of those proteoliposomes calculated from Fig. 1 increased in the order: a(0.328 ± 0.0049) < b (0.341 ± 0.0055) ≅ c(0.338 ± 0.0061) < d(0.352 ± 0.0050). The difference between proteoliposomes type a and d was significant ($P < 0.001$). It may indicate that membrane fluidity was higher in proteoliposomes type a (Lca+−) with a transmembrane Ca^{2+} gradient similar to the physiological situation than that in proteoliposomes type d (Lca−+) with a transmembrane Ca^{2+} gradient in the inverse direction. Most recently, a preliminary result in our laboratory has identified differences of G_s conformation among these four kinds of proteoliposomes using acrylodan as a probe (unpublished results). In accordance with these observations, our results may suggest that a proper transmembrane Ca^{2+} gradient is of crucial importance for optimal membrane fluidity, thus maintaining a suitable conformation of G_s with higher activities of binding GTP and stimulating adenylate cyclase.

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