RHO 蛋白在受體活化的平滑肌興奮-收縮連結中角色的探討 (II)

The role of Rho protein in receptor-mediated excitation-contraction coupling of Smooth muscle (II)

計畫編號：NSC89-2320-B-006-041
執行期限：87年08月01日至89年07月31日
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一、中文摘要

小分子 GTP 結合蛋白 RhoA 在非肌肉細胞內胞骨架的重組與平滑肌細胞的收縮扮演重要的角色。本實驗室先前的實驗成功地以細胞膜電擊通透法將小分子蛋白 (分子量 20-25KDa) 送入大白鼠尾動脈平滑肌束內並顯示 RhoA 蛋白與由α1-腎上腺素受體活化時引起的血管平滑肌興奮-收縮連結過程有關。但是 electroporation 的效率重現性並不高，因此本研究主旨在尋找最合適的 electroporation 條件，並設法由昆蟲細胞表現及分離 RhoA V14 與 RhoA N19 的突變蛋白。其中突變種的 RhoA cDNA 已成功轉殖入 pIZ/V5-His 載體中，並經核酸定序證實送入的基因是正確的。相對而言，electroporation 的部分進行得並不順利。經過反覆測試，我們確定最佳的 electroporation 條件為 400v/cm，125µF。利用此條件，我們將 GTPγS，GDPβS 及 C3 酵素負載入尾動脈肌束，結果發現 GTPγS 可增加收縮力，而 GDPβS 及 C3 則無效用，顯示目前使用的條件負載蛋白的效率仍無法令人滿意。儘管如此，我們仍然進行一系列的實驗以探討 GTPγS 對收縮力的作用及其相關的機轉。首先，GTPγS 負載的濃度 (由 0.1 至 1.0mM) 與其引發的收縮力呈正相關。L 型及受體聯結的鈣離子通道之抑制劑 nifedipine 及 SK&F 96365 均可抑制 GTPγS 引發的收縮力，但以 SK&F 96365 的效果較佳，另一個膜電位差活化的鈣離子通道抑制劑 verapamil 則完全無效。Tyrosine kinase 及 protein kinase C 的抑制劑 (tyrphostin B46 及 Ro-31-8220) 並不影響 GTPγS 的收縮，但 myosin light chain kinase (MLCK) 的抑制劑 ML-9 則具抑制作用。這些實驗結果顯示 GTPγS 可能經由活化受體聯結的鈣離子通道以增加細胞內鈣離子濃度，進而活化 MLCK，引發收縮。

關鍵詞：小分子 G 蛋白 RhoA、興奮-收縮連結過程、血管平滑肌、細胞膜電擊通透法

Abstract

The small GTP binding protein RhoA has been shown to be involved in the cytoskeletal organization of non-muscle cells and the contraction of smooth muscle cells (SMC). In previous experiments, we applied the electroporation method to introduce small proteins (Mr of 20-25 kDa) into rat tail artery strips and found that in intact vascular smooth muscle RhoA is involved in the excitation-contraction coupling pathways stimulated by α1-adrenoceptor agonist. The effectiveness of electroporation method was not highly reproducible, however. The present study was aimed to define the optimal conditions for electroporation to introduce proteins into arterial smooth muscle and to express & isolate two RhoA mutant proteins, RhoA V14 and RhoA N19, from insect cells. The cloning of both rhoA mutant cDNA into pIZ/V5-His vector has been completed. Nucleotide sequencing results confirmed the genes constructed being correct. The experiments on electroporation did not go well, however. Following extensive testing, the optimal conditions for electroporation functions was set at 400 V/cm and 125 µF. We have applied this condition to examine the effects of various molecules, including GTP □ S, GDP □ S and ADP ribosyltransferase C3. While the electroporation of GTP □ S increased tension of RTA strips, the electroporation of GDP □ S...
and C3 exoenzyme had no effect. These results indicated that the efficiency of electroporation under this condition was not quite satisfactory. Nonetheless, we examined dose-effects of GTP $\gamma$S and factors mediating GTP $\gamma$S-increased tension. GTP $\gamma$S increased tension in a concentration-dependent manner between 0.1 and 1.0 mM. Both nifedipine & SKF 96365 inhibited GTP $\gamma$S-increased tension with SKF 96365 being more effective while verapamil was not effective. Tyrosine kinase inhibitor Tyrophostin B46 and protein kinase C inhibitor Ro-31-8220 both had no effect while myosin light chain kinase inhibitor ML-9 was effective in inhibiting GTP $\gamma$S-increased tension. These results suggest that GTP $\gamma$S mainly activate receptor-operated calcium channels to increase $[\text{Ca}^{2+}]_i$ which subsequently activates myosin light chain kinase and force.

**Keywords:** small G protein RhoA, excitation-contraction coupling, vascular smooth muscle, electroporation

二、結果與討論

**Materials and Methods**

*Tissue preparations.* Tail arteries were removed from male Sprague-Dawley rats anesthetized with sodium pentobarbitol (50 mg/kg body weight). The vessel was dissected in a Sylgard 184-based dissecting dish filled with oxygenated physiological saline solution (PSS) at room temperature. The PSS was composed of the following (in mM): 120 NaCl, 5.9 KCl, 11.5 glucose, 25 NaHCO$_3$, 1.2 MgCl$_2$, 1.2 NaH$_2$PO$_4$, and 2.5 CaCl$_2$, and was oxygenated with 95% O$_2$-5% CO$_2$ with a resulting pH of 7.4 at 37 °C. The endothelium was removed by gently rubbing the interior surface of the artery. Helical strips, 1 mm wide and 5-6 mm long, was placed in a bath with one end held in a clamp and the opposite end attached to a force transducer. Tail artery strips were gradually stretched to the length for maximal force production, which is ~1.7-fold the resting length in rat tail artery strips, and equilibrated for 60-90 min at 37 °C. The stability of tail artery strips was tested by challenging each strip with 51 mM KCl for three consecutive times. The cumulative concentration response curves for phenylephrine (3x10$^{-7}$ M, 10$^{-6}$ M and 10$^{-5}$ M) can then be constructed both before and after electroporation of experimental molecules.

**Electroporation procedures.** In order to introduce different molecules into tail artery strips without jeopardizing the contractile functions of smooth muscle cells, the contractile responses towards 51 mM KCl or 10$^{-5}$ M phenylephrine before and after electroporation were compared to indicate the function of smooth muscle cells. The aequorin light signals and total aequorin luminescence collected following cell lysis were taken as an indicator for the amount of molecules being introduced. Prior to electroporation, tail artery strips were placed in Ca$^{2+}$-free PSS containing 1 mM EGTA for
10 min to remove extracellular Ca\(^{2+}\). Electroporation were carried out in an AlCl\(_3\)-coated gene pulser cuvette with a 0.4 cm electrode gap. The solution for performing electroporation contains 0.1 mM EGTA, 20 mM N-tris(hydroxymethyl) methyl-2-aminomethanesulfonic acid (TES, pH 7.4), 5 mM Na\(_2\)ATP, 120 mM KCl, 10 mM MgCl\(_2\), and 5% sucrose and is prechilled to 2 °C before use. The electrical pulses were applied 4 times with a BioRad Gene Pulsar II electroporator. To obtain a time constant approximating 3 msec, a range of electroporation conditions were tested. Following the electroporation, the concentration of Ca\(^{2+}\) in the bathing medium was gradually raised to 2.5 mM. Arterial strips were equilibrated at 37 °C for 2 h before further experiments were conducted.

**Results and Discussion**

*Evaluation of electroporation conditions.* The optimal conditions of the electroporation procedure were re-evaluated. Capacitance and the number of pulse were both tested with the electric field setting at 400 V/cm. While the contractile force towards 10\(^{-5}\) M phenylephrine decreased 16% after electroporation at 125 µF with 4 pulses, contractile force decreased 38% after electroporation at 150 µF with 4 pulses. Electroporation conducted with more than 4 pulses was found to decrease contractile forces. Therefore, we concluded that electroporation at 400 V/cm, 125 µF with 4 pulses causes less damage for rat tail artery strips.

We next examined the loading efficiency under the selected conditions. Among three molecules tested, GTP \(\gamma\)S increased tension of RTA strips whereas GDP \(\beta\)S and C3 exoenzyme had no effect. These results indicated that the efficiency of electroporation under this condition was not quite satisfactory. Collagenase digestion was tested to examine whether loosening extracellular matrix might improve loading efficiency. Collagenase digestion at 2 or 5 U/ml for 20 min at 37 °C did not increase the loading efficiency of GDP \(\beta\)S whereas 10 U/ml collagenase treatment decreased steady-state force stimulated by agonists.

*Characterization of GTP \(\gamma\)S-increased tension.* Because electroporation of GTP \(\gamma\)S was found to increase tension in RTA strips, we examined concentration effects of GTP \(\gamma\)S on contractile force. GTP \(\gamma\)S between 0.1 and 1.0 mM was found to increase contractile force in a concentration-dependent manner (Table 1). We next examined the effects of verapamil (1-100 µM), tyrphostin B46 (10 & 50 µM) and Ro-
31-6220 (0.1 & 1 μM) on GTP-γ-S-increased tension. None of these inhibitors were effective, suggesting that voltage-gated calcium channels, tyrosine kinase and protein kinase C activation did not mediate GTP-γ-S-increased tension. In contrast, a dihydropyridine type calcium channel blocker nifedipine (1 μM) inhibited 45% of tension induced by 0.3 mM GTP-γ-S. SK & F 96365, a receptor-operated calcium channel blocker, was more effective in inhibiting GTP-γ-S-increased tension with 10 μM SK & F inhibiting ~85% of steady-state tension (Table 2). Myosin light chain kinase inhibitor ML-9 at 30 μM inhibited 70% of contractile force (Table 3). These results clearly indicated that GTP-γ-S is likely to activate receptor-operated calcium channels to increase [Ca^{2+}]_{i}, resulting in the activation of myosin light chain kinase and hence contractile tension.

Table 1. Concentration-dependent effects of GTP-γ-S on rat tail artery (RTA) strips. GTP-γ-S was electroporated into RTA strips at 400 V/cm, 125 μF and 20 ohm with 4 pulses. Data are presented as mean±S.E.M. (n=3-5).

<table>
<thead>
<tr>
<th>GTP-γ-S</th>
<th>0.10 mM</th>
<th>0.30 mM</th>
<th>1.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension(g)</td>
<td>0.32±0.04</td>
<td>0.50±0.04</td>
<td>1.04±0.09</td>
</tr>
</tbody>
</table>

Table 2. The effects of ML-9 on GTP-γ-S-induced tension. After the electroporation of GTP-γ-S into RTA strips, ML-9 was added into solution C and maintained at constant concentration during the addition of Ca^{2+}. Data was presented as mean±s.e.m. (n=3-5).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ML9 (10μM)</th>
<th>ML9 (30μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension</td>
<td>0.47±0.04</td>
<td>0.20±0.05</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>Washout</td>
<td>n.d.</td>
<td>0.48±0.08</td>
<td>0.52±0.13</td>
</tr>
</tbody>
</table>

Table 3. The effects of SK&F 96365 on GTP-γ-S-induced tension. After the electroporation of GTP-γ-S into RTA strips, SK&F 96365 was added into solution C and maintained at constant concentration during the addition of Ca^{2+}. Data was presented as mean±s.e.m. (n=3-5).