Effects of protopine on intracellular calcium and the PKC activity of rat aorta smooth muscle

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Abstract: We have previously shown that the vasodilator effect of protopine (Pro) on rabbit aorta is related to the elevations of cAMP and cGMP. In the present study, the vasodilator mechanisms of Pro were further explored by recording the isotonic contraction of the rat aortic strips, detecting directly the intracellular free Ca^{2+} concentration ([Ca^{2+}]_i) with Fura-2/AM loaded vascular smooth muscle cells (VSMCs) of rat aorta, and determining the activity of protein kinase C (PKC) in rat aortic tissue with radioactive isotope γ-32P-ATP-catalyzing assay. By recording the aortic strips contraction induced by noradrenaline (NA) and high potassium (K^+), Pro shifted nonparallelly the concentration-response curves of NA and high K^+ to right, in which the maximal response was depressed in the presence of Pro (30 and 100 µmol/L), and the values of pD_2' were 3.70±0.25 and 3.97±0.15 for NA and high K^+, respectively. In the Fura-2/AM loaded VSMCs, Pro (50 and 100 µmol/L) could not produce any significant change on the resting [Ca^{2+}]_i, but significantly decreased the [Ca^{2+}]_i elevated by NA and high K^+. Pro (30 and 100 µmol/L) had no significant effect on the activity of the cytosolic and membrane PKC in the aortic strips inpretreated by NA. However, in the aortic strips pretreated by NA, the activity of membrane PKC was significantly increased and the activity of cytosolic PKC tended to be decreased by Pro, while the activity of total PKC did not change. These results suggest that Pro seems to promote the translocation of PKC from the cytosol to the membrane in the presence of NA, its vasodilator effect may be the comprehensive result of its decreasing effect on the [Ca^{2+}]_i and the increasing effect on cAMP and cGMP, as well as its influence on the PKC.

Key words: protopine; intracellular free calcium; rat; vascular smooth muscle cells; protein kinase C

普罗托品对大鼠胸主动脉血管平滑肌细胞内游离钙浓度和蛋白激酶C活性的影响

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摘要: 本实验室先前的研究已证实, 普罗托品(protopine, Pro)舒张家兔主动脉的作用, 可能与其增加血管平滑肌细胞内cAMP和cGMP水平有关。为了深入探讨Pro的扩血管作用机制, 实验采用等张收缩记录大鼠离体血管条张力, 利用Fura-2/AM负载的大鼠胸主动脉血管条直接测定细胞内游离Ca^{2+}浓度([Ca^{2+}]_i), 并应用同位素γ-32P-ATP催化活性法测定蛋白激酶C(PKC)活性等方法, 分别观察了Pro的相关效应。结果表明, Pro (30和100 µmol/L)明显降低去甲肾上腺素(NA)和高钾所致的动脉条收缩幅度, 使二者的量效曲线呈非平行右移, 最大反应压低; pD_2'值分别为3.7±0.25和3.97±0.15; Pro (50和100 µmol/L)对静息状态下的[Ca^{2+}]_i没有任何影响, 但对NA和高钾引起的[Ca^{2+}]_i升高均有明显抑制作用; Pro (30和100 µmol/L)对未经NA处理血管条的胞浆和胞膜PKC活性均无明显影响; 但在NA预处理的血管条, Pro使NA所升高的胞浆内PKC的活性趋于降低, 而明显升高胞膜PKC的活性, 对PKC的总活性无明显影响。结果提示, 在有NA存在的情况下, Pro似能促使PKC从胞浆向细胞膜转移, 其扩血管效应似为降Ca^{2+}作用, 升高cAMP和cGMP的作用及其对PKC影响等几方面的综合结果。

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Protopine (tetrahydro-5-methyl bis-[1,3]benzdioxide-[4,5-C:5',6]-azecin-13(5H)-one, Pro) is an alkaloid present in different plants such as *Fumaria indica*, *Corydalis thyrsiflora* and *Corydalis meifolia* [1]. Pro has been shown to exhibit a number of pharmacological activities, such as the relaxing effect on the vascular smooth muscle [2], the inhibiting effect on rabbit blood platelet aggregation [3], and the decreasing effect on the blood pressure of the renal hypertension rats [4]. Recently, the studies in our laboratory showed that the vasodilator mechanism of Pro might be related to its increasing effect on the intracellular levels of cAMP and cGMP [2], and to its decreasing effect on the intracellular free Ca$^{2+}$ ([Ca$^{2+}$]i) of vascular smooth muscle [5]. However, the decreasing effect on [Ca$^{2+}$]i of Pro was only observed indirectly in the muscle tension experiment. It is necessary to confirm this effect with more direct and accurate method. Furthermore, protein kinase C (PKC) is known to influence contraction in vascular smooth muscle cells (VSMC) [6,7], but no information concerning the effect of Pro on PKC activity has been found. In order to further clarify the mechanism of the vasodilator effect of Pro, we detected directly the [Ca$^{2+}$]i with Fura-2/AM loaded vascular smooth muscle cell of rat aorta, and determined the PKC activity in rat aorta tissue.

1 MATERIALS AND METHODS

1.1 Materials

Male Wistar rats weighing (200±20) g were provided by the Animal Center of Institute of Field Surgery, Third Military Medical University; Pro with a purity of 98%, was provided by the Key Laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences; Fura-2 acetoxymethyl/ester (Fura-2/AM) purchased from the Sigma Chemical Co., was dissolved in dimethyl sulfoxide (DMSO); Protein kinase C assay kit was obtained from Transbio Corporation; $\gamma^{32}P$-ATP (specific activity is 18.5 TBq/mmol, concentration is 1.85 MBq/5 µl) was purchased from Beijing Furui Biological Produce Ltd.; GF-109203X (an inhibitor of PKC) was purchased from the ALEXS Chemical Company and dissolved in methanol.

1.2 Methods

1.2.1 Measurements of muscle tension [8,9]

The animal was killed by decapitation and the thoracic aorta was carefully removed. After the connective tissue was cleared, the blood vessel was cut into helicoid strips (about 0.3 cm×2 cm) in ice-cold (4 °C) Krebs solution (in mmol/L: NaCl 118, KCl 4.7, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, MgSO$_4$ 1.18, NaHCO$_3$ 25, glucose 11.1, pH 7.4). The endothelium was removed carefully, and then the strip was placed in a Krebs solution-filled 20 ml organ bath at 37 °C. The solution was bubbled continuously with 95% O$_2$ and 5% CO$_2$ mixture. Isometric tension was recorded under an initial tension of 1 g by force-displacement transducers on XWTD-204 homeostasis recorder (Dahua Instrument Factory, Shanghai). Each strip was allowed to equilibrate for 90 min before experiment.

Cumulative concentration-response curves for NA (1 nmol/L~100 µmol/L) and high K$^+$ (10~250 mmol/L) were carried out in Krebs solution. The typical contractive traces of the aortic strips of rat, induced by NA or high K$^+$ in the absence of Pro, were recorded and taken as control. The aortic strips were then washed with Krebs solution repeatedly and equilibrated in Krebs solution for 90 min until the contractive traces came back to the resting level. After Pro (30 or 100 µmol/L) was added to the organ bath 10 min, the same cumulative concentration of NA- or high K$^+$-challenges were performed, the concentration-response curves were obtained respectively. The values of pD$_2$' (−log$_{10}$ IC$_{50}$) were calculated according to described method [10].

1.2.2 Measurements of [Ca$^{2+}$]i in cultured vascular smooth muscle cells (VSMCs) of rat aorta

**Cell culture** Rat aortic smooth muscle cells were isolated from the thoracic aorta of rats using the explanting technique [11]. Briefly, after removal of the endothelium and adventitia of rat aorta, the aortic pieces (1~2 mm$^3$) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% bovine serum (the Shanghai Second Medical University), penicillin G 100 U/ml, streptomycin 100 µg/ml at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. After 2 weeks, cells that had migrated out of the explants (tissue pieces) were removed by trypsinization and successively subcultured. The purity and identity of cells were examined by immunostaining with antibody specific against smooth muscle cell $\alpha$-actin. Cells from passages 3 to 5 were used for the experiments.

**Measurements of [Ca$^{2+}$]i** The cultured VSMCs, harvested with 0.06% trypsin, were centrifuged at 150 g for

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5 min at room temperature. The supernatant was removed and the cells were washed twice in Hanks solution (in mmol/L: NaCl 137.0, CaCl\(_2\) 1.3, KCl 5.4, MgSO\(_4\) 0.8, Na\(_2\)HPO\(_4\) 0.38, KH\(_2\)PO\(_4\) 0.44, NaHCO\(_3\) 4.2, sucrose 5.6, plus BSA 0.2%; pH 7.4). The cells were then incubated in Hanks solution containing 3 µmol/L Fura-2/AM for 40 min at 37 °C and then were washed twice to remove extracellular dye. The final cell number was adjusted to 1×10\(^6\) cells/ml.

The fluorescence intensity at 480 nm was monitored by an RF-5000 (Shimadzu Company, Japan) dual-wavelength spectrofluorometer. The ratio (R) of the fluorescence signals at 340/380 (nm) was calculated automatically. \( R_{\text{max}} \) and \( R_{\text{min}} \) values were determined by the addition of 10 µl 10% TritonX-100 (final concentration 0.1%), and 10 µl 500 mmol/L EGTA (final concentration 5 mmol/L), respectively. The resting [Ca\(^{2+}\)]i levels as well as peak [Ca\(^{2+}\)]i evoked by 10% TritonX-100 (final concentration 0.1%), and 10 µl 500 mmol/L EGTA (final concentration 5 mmol/L), respectively. This method of [Ca\(^{2+}\)]i measurement is similar to that described elsewhere[12].

The aorta strips from each group described previously were picked out from liquid nitrogen, dissected and homogenized in homogenization buffer (in mmol/L: ethylene diamine tetra-acetic acid (EDTA) 2, EGTA 10, Tris-HCl 20, pH 7.5, sucrose 250) by 1:5 (W/V). The homogenate was centrifuged at 21 000 r/min for 2 h at 4 °C. The resulting supernatant was diluted (as necessary) with homogenization buffer and used as the cytosolic fraction in the PKC activity assays. The remaining pellet was suspended in the 1% Triton X-100 solution (Triton X-100 5 ml, 100 mmol/L EGTA 25 ml, 100 mmol/L EDTA 10 ml in 500 ml solution). After sonicated 4 times for 15 s, the sample was incubated in 1% Triton X-100 solution over night at 4 °C. Then the sample was centrifuged at 21 000 r/min for 2 h at 4 °C. The resulting supernatant was diluted with homogenization buffer and used as the membrane fraction. The protein extraction procedure was in accordance with what described by Shangtao [13]. The protein concentration in both cytosolic and membrane fractions were determined according to Lowry method. Protein fractions were kept at 4 °C and assayed for PKC activity on the same day.

**PKC activity assays** In non-NA-pretreated group, the 4 µg protein samples extracted from cytosolic and cell membrane were preincubated by Pro (30 and 100 µmol/L) and GF-109203X (5 µmol/L, an inhibitor of PKC) for 20 min at room temperature. The assay of PKC activity was then performed according to the method described by protein kinase C assay kit from Transbio Corporation, by which the activity of all Ca\(^{2+}\)/phospholipid dependent PKC was determined in the samples. Reaction was performed at 37 °C for 5 min and terminated by adding termination buffer of PKC assay kit[14]. The radioactive wash was disposed according to the regulations established by the Nuclear Regulatory Commission. The filter membranes were dried at 60 °C baker and analyzed in a scintillation counter (Beckman Co., USA).

In NA-pretreated group, the assay of PKC activity was directly performed with NA-pretreated cytosolic or cell membrane fractions. The method was same as above.
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mentioned.

1.3 Statistical analysis
All results are expressed as mean±SEM. Statistical analysis was performed by analysis of variance and t-test. *P < 0.05 were considered to reflect a significant difference.

2 RESULTS

2.1 Effects of Pro on the contraction induced by NA and high K⁺ in rat aorta strips
In the aorta strips incubated in Pro-free solution, NA and high K⁺ caused graded and sustained contractions with their concentration increasing cumulatively. After the aorta strip tension came back to the base line by repeated washes, an addition of Pro 30 or 100 µmol/L did not change the resting tension of the strip, but inhibited to some extent the strip contraction induced by following NA- and high K⁺-challenges, as shown in Fig.1 and 2. Pro shifted nonparallelly the concentration-response curves of NA and high K⁺ to right, the maximum responses were depressed by (43.6±8.5)% for NA-contraction and by (46.9±4.6)% for high K⁺-contraction in the presence of Pro 100 µmol/L. The values of pD2 were 3.70±0.25 and 3.97±0.15 for NA and high K⁺, respectively. (Fig.3, 4)

2.2 Effects of Pro on [Ca²⁺], in cultured VSMC
As shown in Fig.5, in the Fura-2/AM loaded VSMCs, high K⁺ (50 mmol/L) markedly increased the [Ca²⁺], from (177.2±10.3) nmol/L to (301.7±18.0) nmol/L (*P<0.01), addition of Pro (50 and 100 µmol/L) did not influence the
resting [Ca^{2+}], but the [Ca^{2+}] increased by high K+ tended to decrease in the presence of Pro 50 µmol/L, and was significantly decreased to (224.3±16.4) nmol/L by Pro 100 µmol/L \((P<0.05)\). As the positive control drug, the Ca^{2+}-channel blocker verapamil (10 µmol/L) could also markedly decrease the [Ca^{2+}], elevated by high K+ to (199.3±4.5) nmol/L \((P<0.01)\). Similar results were found in the test for observing the effect of Pro on the [Ca^{2+}] changed by NA. In this test, NA (1 µmol/L) significantly increased the [Ca^{2+}], from (182.0±12.6) nmol/L to (312.6±31.8) nmol/L \((P<0.01)\). Addition of Pro (50 and 100 µmol/L) did not change the resting [Ca^{2+}], but significantly decreased the [Ca^{2+}], elevated by NA to (281.5±17.6) nmol/L \((P<0.05)\) and (246.6±12.1) nmol/L \((P<0.01)\), respectively. The data are further shown in Fig.6.

2.3 Effect of Pro on the PKC activities in the VSMCs

In the non-NA-pretreated aorta strips, GF-109203X (5 µmol/L), a PKC inhibitor used as a positive control, decreased significantly the activities of the cytosolic and total PKC \((P<0.05)\), however, Pro (30 and 100 µmol/L) did not produce any significant change in the cytosolic, membrane and total PKC activities (Table 1). On the other hand, in the NA-pretreated aorta strips, NA (10 µmol/L) increased the cytosolic and total PKC activities, Pro (30 and 100 µmol/L) tended to reduce the cytosolic PKC activity elevated by NA, and increased significantly the membrane PKC activity \((P<0.05)\), while total PKC activity did not change (Table 2).

### Table 1. Effect of Pro on the activity of PKC in non-NA-pretreated aorta strips

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosolic PKC</td>
</tr>
<tr>
<td>Control</td>
<td>15.90±3.23</td>
</tr>
<tr>
<td>Pro 3×10^{-5}</td>
<td>15.32±2.88</td>
</tr>
<tr>
<td>Pro 1×10^{-4}</td>
<td>12.30±2.90</td>
</tr>
<tr>
<td>GF-109203X</td>
<td>9.48±4.70</td>
</tr>
</tbody>
</table>

\(^{*}P<0.05\) vs control. Values are mean±SEM of six experiments.

### Table 2. Effect of Pro on the activities of PKC in the NA-pretreated aorta strips

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>43.73±18.58</td>
</tr>
<tr>
<td>NA+Pro 3×10^{-5}</td>
<td>34.80±24.98</td>
</tr>
<tr>
<td>NA+Pro 1×10^{-4}</td>
<td>27.63±26.74</td>
</tr>
</tbody>
</table>

\(^{*}P<0.05\), \(^{**}P<0.01\) vs NA; Values are mean±SEM of six experiments.

3 DISCUSSION

Recent studies in our laboratory demonstrated that Pro has approximate inhibition effects on rabbit aorta strips contractions induced by phenylephrine (PE) and high K+ \cite{2}, on the basis of our study concerning the decreasing effect on blood pressure of Pro in renal hypertension rats\cite{4}, we further investigated the effect of Pro on rat aorta strips \textit{in vitro}. The results showed that Pro inhibited the NA- and high K+-contractions with a non-competitive antagonism manner, the \(P^{D_{2}}\) values of Pro for inhibiting NA- and high K+-contractions were similar. These results were consistent with that found in rabbit aorta experiment\cite{2}. More studies should be done for further clarifying the mechanisms of the vasodilator effect of Pro.

[Ca^{2+}], in VSMCs has been known to play an important role in vascular resistance\cite{15}. Our experiment for determining the [Ca^{2+}], with Fura-2/AM loaded VSMC confirmed the previous findings observed indirectly in muscle tension experiment that Pro decreased the [Ca^{2+}], elevated by NA\cite{3}. Because the test was carried out in the normal...
Ca\textsuperscript{2+} circumstance, the decreasing effect on [Ca\textsuperscript{2+}], of Pro might only reflect its comprehensive inhibiting effects on NA-induced Ca\textsuperscript{2+}-release and Ca\textsuperscript{2+}-influx. Through the experiments for determining [Ca\textsuperscript{2+}], and muscle tension in the present study, it was found that no change of Pro in the resting [Ca\textsuperscript{2+}] was consistent with its effect on the resting tension, and its inhibiting effects on the [Ca\textsuperscript{2+}] elevated by NA and high K\textsuperscript{+} were also consistent with its effects on NA- and high K\textsuperscript{+}-contractions. This consistency in the effects of Pro on [Ca\textsuperscript{2+}], and muscle tension suggest that the vasodilator effect of Pro may be related to its decreasing effect on [Ca\textsuperscript{2+}].

In recent years attention has been paid to the participation of PKC in the mechanisms of contraction in vascular smooth muscle. The pathways through which PKC mediates contraction fall into those involving alteration in [Ca\textsuperscript{2+}][6,7], and those independent of changes in [Ca\textsuperscript{2+}] and/or myosin light chain phosphorylation[13]. Hence, we investigated the role of PKC in the vasodilator effect of Pro in rat aorta tissues. In this test two administering procedures were designed according to the different aims. To observe whether Pro has direct inhibitory effect on the PKC activity (non-NA-pretreated group), we added Pro into the reaction system after termination of the production of PKC but not in the period of incubating the aorta tissues with Krebs solution. On the other hand, in order to observe whether Pro has effect on the changes of PKC activity induced by NA (NA-pretreated group), an agonist has been shown to activate PKC indirectly[19], we added Pro and NA into the reaction system in the incubating period. Furthermore, some drugs have been reported to promote the translocation of PKC to the cell membrane[20,21]. To observe the effect of Pro on the distribution of PKC, we investigated the influences of Pro on the PKC activity differently in the plasma membrane and cytosol.

The fact that Pro did not influence on the resting PKC activity of the aorta tissues (non-NA-pretreated group) suggests that Pro may not be a PKC inhibitor. However, in NA-pretreated group, the cell membrane PKC activity was significantly increased and the cytosolic PKC activity tended to be decreased by Pro, while the total activity did not change. The results indicate that in the presence of NA, Pro seems to promote the translocation of PKC from the cytosol to the membrane in rat aorta smooth muscle cells. Because the plasma membrane has been considered as an active pool of PKC for vascular smooth muscle contraction[22], the PKC translocation elicited by Pro seems in conflict with its vasodilator effect. All above mention together combining our previous report concerning the effect of Pro on the intracellular cAMP and cGMP levels[23], we suggest that the vasodilator mechanism of Pro seems to be related to the comprehensive results of its decreasing effects on [Ca\textsuperscript{2+}], and the increasing effects on cAMP and cGMP, as well as its influence on the PKC.

REFERENCES


