Ca\textsuperscript{2+}-Channel Blockade in Rat Thoracic Aorta by Protopine Isolated from Corydalis Tubers

Feng-Nien Ko\textsuperscript{1}, Tian-Shung Wu\textsuperscript{2}, Sheng-Teh Lu\textsuperscript{3}, Yang-Chang Wu\textsuperscript{3}, Tur-Fu Huang\textsuperscript{1} and Che-Ming Teng\textsuperscript{1,*}

\textsuperscript{1}Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan
\textsuperscript{2}Department of Chemistry, National Cheng-Kung University, Tainan, Taiwan
\textsuperscript{3}School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan

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ABSTRACT—The pharmacological properties and mechanism of the action of protopine on isolated rat thoracic aorta were examined. It inhibited norepinephrine (NE, 3 \( \mu \)M)-induced tonic contraction in rat thoracic aorta in a concentration-dependent manner (25–100 \( \mu \)g/ml). The phasic contraction caused by NE was inhibited only by a high concentration of protopine (100 \( \mu \)g/ml). At the plateau of NE-induced tonic contraction, the addition of protopine also caused relaxation. This relaxing effect of protopine was not antagonized by indomethacin (20 \( \mu \)M) or methylene blue (50 \( \mu \)M), and it still existed in denuded rat aorta or in the presence of nifedipine (2–100 \( \mu \)M). Protopine also inhibited high potassium (60 mM)-induced, calcium-dependent (0.03–3 mM) contraction of rat aorta in a concentration-dependent manner. Neither cAMP nor cGMP level was changed by protopine. Both the formation of inositol monophosphate caused by NE and the phasic contraction induced by caffeine were also not affected by protopine. \(^{45}\text{Ca}^{2+}\) influx caused by either NE or K\textsuperscript{+} was inhibited by protopine concentration-dependently. It is concluded that protopine relaxed the rat thoracic aorta mainly by suppressing the Ca\textsuperscript{2+} influx through both voltage- and receptor-operated calcium channels.

\textit{Corydalis} tuber is a Chinese herb used as one of the traditional medicines for the treatment of thromboembolism. It has also been employed in the treatment of pain in the chest, stomach and abdomen; injuries due to impact, fractures, contusions and strains; pain due to blood stasis and abdominal pain in the postpartum period. The alcohol extract of the herb markedly dilated coronary vessels, decreased coronary resistance and increased blood flow of the isolated rabbit heart (1). Protopine (Fig. 1) is an active principle isolated from \textit{Corydalis} tuber, which possessed antiplatelet activity (2–4). Protopine also inhibited endotoxin-induced disseminated intravascular coagulation.

\textsuperscript{*}To whom correspondence should be addressed.
vascular coagulation in rats and the pulmonary thromboembolism in mice (2). In the preliminary test, we found that protopine also possessed a vasorelaxing action in rat thoracic aorta. In this paper, we demonstrated this vasorelaxing effect of protopine and also tried to elucidate its mechanisms of action.

MATERIALS AND METHODS

**Mechanical response**

Wistar rats of either sex weighing 250 to 300 g were killed by a blow to the head. The thoracic aorta was isolated, and excess fat and connective tissue were removed. Vessels were cut into rings of about 5 mm in length and mounted in organ baths containing 5 ml of Krebs solution of the following composition: 118.2 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$ and 11.7 mM glucose. The tissue bath solution was maintained at 37°C and bubbled with a 95% O$_2$ and 5% CO$_2$ mixture. Two stainless steel hooks were inserted into the aortic lumen, one was fixed while the other was connected to a transducer. Preparations were equilibrated in the Krebs solution for 90 min under an optimal tension of 1 g before specific experimental protocols were initiated. The solution was changed three times during this equilibration period. Contractions were recorded isometrically via a force-displacement transducer connected to a Gould polygraph (Model 2400). In some experiments, the endothelium was removed by rubbing with a cotton ball, and the failure of acetylcholine-induced relaxation was taken as an indicator that vessels were denuded successfully.

Contractile effects of calcium were studied in rings stabilized in high K$^+$ solution without Ca$^{2+}$. Calcium was then added from stock dilutions to obtain the desired concentrations, and the effect of Ca$^{2+}$ of each concentration was recorded. Maximal tension attained at 3 mM Ca$^{2+}$ was considered as 100%. High K$^+$ solution was prepared by substituting NaCl in the solution with equimolar KCl (60 mM).

**cAMP assay of rat aorta**

The content of cAMP was assayed on aortic rings as described by Kauffman et al. (5). After incubation of aortic rings in the Krebs solution containing IBMX (10 μM) for 5 min, DMSO, forskolin or protopine was added and incubation continued for another 2 min. At the end of the reaction, aortic rings were rapidly frozen in liquid nitrogen and stored at −80°C until they were homogenized in 0.5 ml of 10% trichloroacetic acid using a potter glass/glass homogenizer. The homogenate was centrifuged at 10,000 × g for 5 min, and the supernatant was removed. The supernatant was extracted with 4 × 3 volumes of ether, and then the cAMP content was assayed using RIA kits. The precipitate was used for protein assay (6). cAMP levels were expressed as pmol/mg protein.

**cGMP assay of rat aorta**

Aortic rings prepared as described above, but not put under tension, were placed in 1 ml of Krebs solution for 1 hr with 95% O$_2$ and 5% CO$_2$ at 37°C. After incubation of the rings with the DMSO (0.1%, control), sodium nitroprusside or protopine for 2 min, the reaction was stopped by immersing the tissue into liquid nitrogen and stored it at −80°C. The rings were thawed in 10% trichloroacetic acid and 4 mM EDTA. After homogenization with a potter glass/glass homogenizer for 2–3 min, the homogenate was centrifuged at 10,000 × g for 5 min. After the supernatant was extracted 4 times with ether, its cGMP content was assayed with an RIA kit (7). The precipitate was used for protein assay (6). cGMP levels were expressed as pmol/mg protein.

**Measurement of [3H]inositol monophosphate**

The same procedure as described by Hirata et al. (8) was employed. Briefly, rat thoracic aortae were exposed to Krebs solution containing 10 μCi/ml of myo-[2-3H]inositol for 3 hr and gassed with a 95% O$_2$ and 5% CO$_2$ mixture. Tissues were then transferred to tubes containing fresh Krebs solution with DMSO or protopine for 15 min, and then
saline or NE (3 μM) was added and incubation continued for another 15 min. LiCl (10 mM) was added 5 min before NE to inhibit inositol monophosphatase (9). Aortae were then frozen in liquid nitrogen and homogenized in 1.3 ml of 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was collected and trichloroacetic acid was removed by washing with 4 X 3 volumes of ether. The inositol monophosphate in the aqueous phase was analyzed by application of the sample to a 1-ml Dowex-1 ion-exchange column according to the method of Neylon and Summers (10). The pellets of the tissues were resuspended in 1.0 N NaOH and assayed for protein according to the method of Lowry et al. (6).

\[45\text{Ca}^{2+}\] influx

\[45\text{Ca}^{2+}\] influx was measured as described by Kaushik et al. (11). Aortic rings were placed in test tubes containing Krebs solution with 2 μCi/ml of \[45\text{Ca}^{2+}\] in the presence of DMSO (0.1%) or protopine and incubated for 15 min. NE (3 μM) or \(K^+\) (60 mM) was then added and incubation continued for another 15 min. After the incubation period, the tissues were transferred into test tubes containing 2 ml of ice-cold \(Ca^{2+}\)-free Krebs solution with 2 mM EGTA for 40 min to remove extracellular \[45\text{Ca}^{2+}\]. Tissues were then removed, lightly blotted with No. 5 Whatman filter paper, weighed and dissolved in 37% perchloric acid (0.1 ml) at 75°C. Radioactivity was counted by a liquid scintillation counter (Packard Model 2200 CA).

**Data analysis**

The concentrations of drugs were expressed as the final bath concentration. Results are expressed or plotted as the means ± S.E.M. Student’s t-test was used for statistical analyses; P values of less than 0.05 were considered to be significant.

**Materials**

Protopine (4,6,7,14-tetrahydro-5-methyl-bis-[1,3]benzodioxolo[4,5-c:5′,6′-glazeccin-13(5H)-one) is an alkaloid isolated from Corydalis tashiroi and Corydalis pallida as previously described by Lu et al. (12, 13). Norepinephrine, acetylcholine, 3-isobutyl-1-methylxanthine (IB MX), sodium nitroprusside, methylene blue, trichloroacetic acid, bovine serum albumin, EGTA, EDTA, Dowex-1 (100-200 mesh: χ8, chloride) resin, myo-inositol and forskolin were obtained from Sigma Chem. Co. \(^{45}\text{Ca}^{2+}\), myo-[\(2-^{3}\text{H}\)]inositol and cAMP and cGMP RIA kits were purchased from Amer sham. When drugs were dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1% and had no effect on the muscle contraction.

**RESULTS**

**Effects of protopine on norepinephrine-induced contractions**

Exposure of rat aorta to 3 μM of norepinephrine (NE) caused a phasic contraction followed by a tonic one that was maintained for at least 30 min. Acetylcholine (ACh, 3 μM) caused a relaxation (more than 70%), indicating that the endothelium in this preparation was intact. Pretreatment of protopine at 25 to 100 μg/ml for 15 min inhibited the NE-induced tonic contraction in a concentration-dependent manner with an IC50 of about 50 μg/ml. The phasic contraction of NE was inhibited significantly by protopine only at the concentration of 100 μg/ml (Fig. 2). After equilibrium in the \(Ca^{2+}\)-free Krebs solution for 5 min, NE (3 μM) caused a phasic contraction (40.0 ± 5.0% of the NE-induced contraction in normal Krebs solution) followed by a small sustained tonic contraction. Protopine (100 μg/ml) had no effect on this NE-induced phasic contraction in \(Ca^{2+}\)-free Krebs solution. When protopine was added at the state of tonic contraction (10 min after the exposure to NE), a relaxation was observed (Fig. 3A). This relaxing action of protopine was not blocked by either indomethacin (20 μM) or methylene blue (50 μM) which was added 3 min before protopine (data not shown). In the denuded rat aorta, the relaxing action of ACh
was completely abolished, while protopine still relaxed the aorta, although the potency was somewhat less than that in the intact aorta (Fig. 3B). By treating the denuded rat aorta with 2 μM nifedipine for 15 min, the high K⁺ (80 mM)-induced contraction was completely blocked (data not shown). Protopine relaxed the nifedipine (2 μM)-pretreated, NE-induced aortic contraction concentration-dependently (Fig. 4, b, c and d). Although the concentration of nifedipine was increased to 20 or 100 μM, the NE-induced tonic contraction was inhibited progressively; the remaining tension was still relaxed by protopine (Fig. 4, e and f).

**Effects of protopine on high K⁺-induced calcium-dependent contraction**

In Ca²⁺-free Krebs solution containing 60 mM K⁺, cumulative addition of Ca²⁺ (0.03 to 3 mM) caused a stepwise increase of the contraction in rat aorta. Protopine (10 to 50 μg/ml) inhibited this contraction in a concentration-dependent manner with an IC₅₀ of about 10 μg/ml for a calcium concentration of 1 mM (Fig. 5).

**Effects of protopine of caffeine-induced contraction**

After equilibrium in the Ca²⁺-free Krebs solution for 5 min, caffeine (10 mM) caused a rapid phasic contraction of rat aorta (0.40 ± 0.03 g, n = 4). Protopine (100 μg/ml) did not affect this contraction (0.39 ± 0.03 g, n = 4). Nifedipine (2 μM) also had no effect (0.38 ± 0.03 g, n = 4), while procaine (10 mM) completely abolished this caffeine-induced phasic contraction.
Fig. 4. Relaxing effect of protopine on NE-induced tonic contraction in the presence of nifedipine. After pretreatment of denuded rat aorta with DMSO (0.1%) or nifedipine (2 μM for b, c, d; 20 or 100 μM for e and f) for 15 min, NE (3 μM) was used to induce muscle contraction. DMSO (0.1%) or protopine was added 10 min after the exposure of aorta to NE to cause the relaxation. Acetylcholine (ACh, 3 μM) was used to determine whether the endothelium was intact or denuded.

Fig. 5. Effects of protopine on the contraction of a high K+-depolarized preparation of rat aorta. Aorta was preincubated with 0.1% DMSO (○) or protopine (10 μg/ml: ●, 25 μg/ml: △, 50 μg/ml: ▲) for 15 min, and then cumulative concentrations of Ca²⁺ (0.03–3 mM) were used to induce the contraction. Each point represents the means ± S.E.M. of 6 determinations. All control data points were significantly inhibited by various concentrations of protopine.

Effects of protopine on cAMP and cGMP formation

The cyclic nucleotide contents of the aorta were measured by radioimmunoassay. As shown in Table 1, sodium nitroprusside and forskolin elevated the cGMP and cAMP level in rat aorta, respectively. In the rat aorta, protopine did not exert any effect on the contents of these cyclic nucleotides.

Table 1. Effect of protopine on the cGMP and cAMP formations of rat aorta

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/mg protein)</th>
<th>cAMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.16 ± 0.20</td>
<td>2.12 ± 0.31</td>
</tr>
<tr>
<td>Sodium nitroprusside (10 μM)</td>
<td>6.93 ± 0.57***</td>
<td></td>
</tr>
<tr>
<td>Forskolin (10 μM)</td>
<td></td>
<td>26.60 ± 5.15***</td>
</tr>
<tr>
<td>Protopine (100 μg/ml)</td>
<td>2.64 ± 0.26</td>
<td>1.86 ± 0.16</td>
</tr>
</tbody>
</table>

After preincubation of aortic rings with (for cAMP) or without (for cGMP) IBMX (10 μM) for 5 min, DMSO (0.1%, control), sodium nitroprusside, forskolin or protopine was added for another 2 min, and then the reaction was stopped by immersing the tissue into liquid nitrogen. cGMP and cAMP contents in rat aorta were measured. Results are expressed as the means ± S.E.M. (n = 5–7). ***: P < 0.001 as compared with the respective control.
Effects of protopine on NE-induced inositol monophosphate formation

Exposure of rat aorta to 3 μM of NE for 15 min in the presence of 10 mM of LiCl elevated inositol monophosphate formation. Pretreatment of protopine (100 μg/ml) for 15 min did not inhibit the inositol monophosphate formation caused by NE (Table 2).

Table 2. Effect of protopine on the [3H]inositol monophosphate formation of rat thoracic aorta

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]inositol monophosphate (c.p.m./mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>1385 ± 156</td>
</tr>
<tr>
<td>NE (3 μM)</td>
<td>2755 ± 313*</td>
</tr>
<tr>
<td>Protopine (100 μg/ml) + NE</td>
<td>2681 ± 399*</td>
</tr>
</tbody>
</table>

[3H]inositol monophosphate formation of rat thoracic aorta was measured as described in “Methods”. Results are expressed as the means ± S.E.M. (n = 4). *: P < 0.05, compared with that of resting tissues.

Effects of protopine on 45Ca2+ influx induced by NE or high K+

In the presence of NE (3 μM), the 45Ca2+ influx was increased 38.9 ± 3.1% over the control rings. This increase of 45Ca2+ influx was inhibited by protopine (25 to 100 μg/ml) concentration-dependently (Fig. 6A). A similar inhibitory effect on high K+-induced 45Ca2+ influx was also shown in Fig. 6B. K+ (60 mM) caused a 52.1 ± 6.1% increase of 45Ca2+ influx over the control rings, and this increase was also concentration-dependently inhibited by protopine (10 to 50 μg/ml).

DISCUSSION

The present study demonstrated that protopine inhibited both the contractile responses to NE and high K+. When protopine was added during the tonic contraction induced by NE, it also caused relaxation. All these effects of protopine were concentration-dependent.

Vascular endothelium controls vascular tone by secreting both relaxant and contractile fac-
tors (14, 15). Endothelial cells respond to a variety of neurohumoral and physical stimuli to release endothelium-dependent vasodilators such as endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI₂). It is generally accepted that the released EDRF and PGI₂ may have important physiological roles as mediators of dilating activity in certain vessels. The relaxing action of protopine persisted in denuded or intact aorta in the presence of indomethacin (20 μM) or methylene blue (50 μM). Indomethacin is known to block the formation of PGI₂ while methylene blue is reported to inhibit the EDRF activation of guanylate cyclase (16). Thus, the vasorelaxation caused by protopine was independent of endothelium and not mediated by either EDRF or PGI₂.

Other important mediators for relaxing the vascular smooth muscle are cyclic nucleotides. The action of cGMP includes an increase in Ca²⁺ extrusion or sequestration (17, 18) and inhibition of Ca²⁺ uptake (19), contractile elements (20) or receptor-linked phosphoinositide breakdown (21, 22). cAMP can dilate the vascular smooth muscle either by phosphorylation of myosin light chain kinase (23, 24), by increasing calcium uptake by the sarcoplasmic reticulum (25), or by acting in other ways to reduce free cytosolic calcium (26). Because both cAMP and cGMP levels were not increased by protopine, this implies that the relaxing effects of protopine is not due to the formation of cyclic nucleotides.

High concentration of protopine (100 μg/ml) also inhibited the phasic contraction induced by NE (Fig. 2). It has been reported that activation of α₁-adrenergic receptors by NE stimulates phosphoinositides turnover to increase the concentration of inositol triphosphate and subsequently release the intracellular Ca²⁺ store to mediate the phasic contraction (27, 28). However, NE-induced inositol monophosphate formation was not affected by protopine, and this rules out that protopine may inhibit NE-induced phasic contraction through inhibition of NE-induced phosphoinositides hydrolysis. Protopine also had no effect on caffeine-induced phasic contraction. It is now generally accepted that caffeine can release intracellular Ca²⁺ in vascular smooth muscle; and there is an overlap between the IP₃- and caffeine-sensitive Ca²⁺ stores, although there are differences among the reported figures (29–31). Therefore, this result may imply that protopine does not inhibit the release of intracellular Ca²⁺ from the caffeine-sensitive store as well as the IP₃-sensitive Ca²⁺ store. However, the action mechanism(s) of protopine on the inhibition of NE-induced phasic contraction is not clear, but may act on the site(s) after Ca²⁺ was released.

High K⁺-induced, Ca²⁺-dependent contraction was suppressed by protopine in a concentration-dependent manner. It has been reported that high K⁺-induced contraction in smooth muscle is mediated by an increase in Ca²⁺ influx through voltage-operated Ca²⁺ channels (34). Since protopine inhibited Ca²⁺-dependent contraction and ⁴⁵Ca²⁺ influx in high K⁺ medium, it may be a blocker of voltage-operated Ca²⁺ channels. Maintenance of tension (tonic contraction) in response to NE results primarily from Ca²⁺ entry through receptor-operated Ca²⁺ channels with little requirement for Ca²⁺ entry through voltage-operated Ca²⁺ channels (32, 33). In our experiments, both NE-induced tonic contraction and ⁴⁵Ca²⁺ influx were inhibited by protopine (Figs. 2 and 6A). Furthermore, protopine still relaxed the NE-induced tonic tension concentration-dependently in the presence of nifedipine (2 μM), which completely blocked high K⁺ (80 mM)-induced contraction. This indicates that protopine may be a blocker of receptor-operated Ca²⁺ influx.

In rabbit aorta, the voltage- and receptor-operated Ca²⁺ channels are independent: the former is activated by membrane depolarization and is selectively blocked by organic Ca²⁺ channel blockers (e.g., verapamil, nifedipine and diltiazem), whereas the latter is activated by binding of agonist to their receptors and is selectively blocked by sodium nit-
roprusside (19, 35). However, these two types of Ca\textsuperscript{2+} channels are functionally not completely separated in rat aorta (32, 34), and voltage-operated Ca\textsuperscript{2+} channel blockers (eg., nitrendipine, nifedipine, verapamil) usually inhibited NE-induced contraction completely (36). Thus, in rat aorta, the contraction induced by either high K\textsuperscript{+} or NE was inhibited by protopine. Further experiments are needed to determine if protopine is a selective inhibitor of receptor- or voltage-operated Ca\textsuperscript{2+} channels in rabbit aorta.

It is concluded that the inhibitory effect of protopine on the contractile response caused by high K\textsuperscript{+} and NE in rat thoracic aorta is mainly due to inhibition of Ca\textsuperscript{2+} influx through both voltage- and receptor-operated Ca\textsuperscript{2+} channels.

Acknowledgement

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