Effects on the Rabbit Coronary Artery of LP-805, a New Type of Releaser of Endothelium-Derived Relaxing Factor and a K\(^+\) Channel Opener

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In the rabbit epicardial coronary artery, 8-tert-butyl-6,7-dihydropyrolo[3,2-e][5-methy]pyrazolo[1,5-a]pyrimidine-3-carbonitrile (LP-805) hyperpolarized the muscle membrane in both proximal (diameter, 1.1-1.2 mm) and distal (diameter, 0.1-0.2 mm) regions of intact (+E) tissue, in which endothelium is present, and endothelium-denuded (-E) tissue. LP-805-induced hyperpolarization was inhibited by glibenclamide. In -E tissues in both regions, acetylcholine (ACH, >0.1 \(\mu\)M) depolarized the membrane, and LP-805 inhibited the depolarization. However, in +E tissues, ACh (>0.1 \(\mu\)M) transiently hyperpolarized the membrane that was not modified by glibenclamide (10 \(\mu\)M), charybdotoxin (100 nM), and \(\mathrm{N}^\circ\)-nitro-L-arginine (L-NNa, 100 \(\mu\)M). In -E tissues of both regions, LP-805 consistently inhibited the 10 \(\mu\)M ACh-induced contraction (IC\(_{50}\), 2.8 \(\mu\)M), and 10 \(\mu\)M glibenclamide shifted this concentration-response curve to the right (IC\(_{50}\), 20 \(\mu\)M). In +E tissues, LP-805 more potently inhibited the ACh-induced contraction (IC\(_{50}\), 0.3 \(\mu\)M), and this inhibition was prevented by L-NNa (100 \(\mu\)M) but not by indomethacin or glibenclamide (10 \(\mu\)M). In -E and +E tissues of both regions, LP-805 depolarized the high K\(^+\)—induced depolarization (<20 mM) and relaxed the tissues precontracted by high K\(^+\) (<30 mM); these electrical and mechanical effects of LP-805 were prevented by glibenclamide (10 \(\mu\)M) in +E tissues. In +E tissues, the K\(^+\)—induced contraction (<30 mM) was more strongly inhibited than in -E tissues, but after treatment with L-NNa, LP-805 relaxed -E and +E tissues precontracted to the same extent in the presence of high K\(^+\). LP-805 (10 \(\mu\)M) did not inhibit the Ca\(^{2+}\)—induced contraction in skinned muscle tissues but did slightly inhibit the ACh-induced contraction in Ca\(^{2+}\)-free solution containing 2 mM EGTA. Thus, LP-805 has a potent releasing action on endothelium-derived relaxing factor and also the potential to open the glibenclamide-sensitive K\(^+\) channel. These events would account for the dilation of the rabbit coronary artery exposed to LP-805. (Circulation Research 1992;71:859–869)

KEY WORDS • endothelium-derived relaxing factor • endothelium-derived hyperpolarizing factor • K\(^+\) channel opener • glibenclamide-sensitive K\(^+\) channel • coronary artery • vasorelaxation

A newly synthesized agent with vasodilatory effects, 8-tert-butyl-6,7-dihydropyrolo[3,2-e]5-methylpyrazolo[1,5-a]pyrimidine-3-carbonitrile (LP-805) decreases systemic blood pressure and increases coronary blood flow. The effect of LP-805 on blood flow was similar to that induced by diltiazem and three times more potent than that of nicardipine, with no effect on heart rate. Our preliminary observations suggested that LP-805 has a hyperpolarizing action on resting membrane.

In vascular smooth muscle, hyperpolarization of the membrane can be induced by various metabolic products and drugs. K\(^+\) channel openers such as cromakalim, nicardipine, pinacidil, and others hyperpolarize the resting membrane as well as the membrane depolarized by agonists or high K\(^+\) (<20 mM), and vasodilation occurs. This hyperpolarization is blocked by glibenclamide, an ATP-sensitive K\(^+\) channel blocker, in cardiac and smooth muscle cells and also in pancreatic B cells. Endogenous substances such as neurotransmitters, autacoids, and others can release endothelium-derived relaxing factor (EDRF). It has been reported that nitric oxide (NO), a key substance in the action of EDRF and synthesized from L-arginine, increases the amount of cGMP of the cytosol in smooth muscles and hyperpolarizes vascular smooth muscles. It was also reported that NO released from nonadrenergic, noncholinergic inhibitory nerve terminals relaxed tissues precontracted by agonists in intestinal smooth muscle cells. Endothelial cells release not only EDRF (NO) but also prostaglandin I\(_2\) (PGI\(_2\)) and endothelium-derived hyperpolarizing factor (EDHF). These two released substances also hyperpolarize the smooth muscle membrane. PGI\(_2\) enhances the synthesis of cAMP. In several tissues, cAMP has been shown to increase the open probability of Ca\(^{2+}\)-dependent K\(^+\) channels. The mechanism by which LP-805 produces hyperpolar-
ization and the role that EDHF, EDRF, or prostaglan-
dins plays in these events is still unclear.
We attempted to clarify the vasodilating action of
LP-805 by examining changes in the membrane and
mechanical properties of the rabbit coronary artery in
the presence and absence of endothelium, using micro-
electrode and tension recording methods.

Materials and Methods

Preparations
Male albino rabbits (Nippon White, 1.8–2.2 kg) were
anesthetized with 40 mg/kg i.v. sodium pentobarbital
(Pitman & Moor, New Jersey) and exsanguinated. The
epicardial coronary artery was isolated (outer diameter,
=1–1.2 mm excised from the proximal region and
=0.1–0.2 mm excised from the distal region of the left
anterior descending branch), and connective tissues
were carefully removed in a dissecting chamber using
a binocular microscope. To preserve the endothelial cells,
contact with the internal surface of the vessels was
avoided. For microelectrode experiments, isolated
preparations were cut in a circular direction (=3–4 mm
thick, an open-ring preparation). To record the tension
from intact (+E) and skinned (–E) muscle tissues, very
fine muscle strips (100–120 μm in width and 500 μM in
length) were prepared from the proximal region of the
coronary artery in the presence or absence of endothe-

dial cells, respectively. To obtain endothelium-denuded
(–E) tissues, endothelial cells were gently rubbed off
using the round surface of an insect pin, and the
complete removal of endothelial cells was verified by
application of 1 μM A23187 (see Reference 22 for
details).

Electrical-Response Recording Procedures
The electrical responses (membrane potential and
action potential generated from single smooth muscle
cells) were recorded using a glass capillary microelec-
trode (Hilgenberg Glass GmgH, FRG) filled with 3M
KCl and with a tip resistance of 40–80 MΩ. The tissue
was pinned onto a rubber plate in a chamber (1.5-ml
bath volume) and superfused with modified Krebs’
solution (32°C) at a flow rate of 3 ml/min. Measure-
ments of the membrane potential were made repeti-
tively during 3–4 hours after a 90-minute equilibration
period. The electrode was inserted into the smooth
muscle cells from the adventitial side of the vessel.

Mechanical-Response Recording Procedures
Muscle strips were transferred to a chamber (capa-
city, 0.9 ml) filled with modified Krebs’ solution and
mounted horizontally by securing both ends with a thin
soft thread. One end of the thread was connected to a
transducer (strain U-gauge, Shinko, Tokyo) and the
other to an anchorage point within the recording cham-
ber. The strip was stretched to =1.1 times the resting
length to maintain resting tension (≈10 μN). Solutions of
modified ionic composition (or containing drugs)
were added to the chamber during pumped removal of
solution already present. The test solution could be
changed within a few seconds. During the K⁺-induced
contractions, guanethidine (3 μM) and tetrodotoxin
(0.1 μM) were added to the high K⁺ solution to prevent
the release of neurotransmitters.

Skinned tissues were prepared using saponin (25
μg/ml for 25 minutes) according to methods previously
described. Briefly, after a K⁺-induced contraction (128
mM K⁺ with 10 μM acetylcholine [ACh]) of an intact
muscle had been recorded, the bathing solution was
replaced with relaxing solution. The preparation was
then left for 25 minutes in relaxing solution containing
25 μg/ml saponin and washed again with the relaxing
solution. To prevent deterioration of the Ca²⁺ sensitiv-
ity of contractile proteins, 0.2 mM calmodulin (Calbio-
chem-Behring) was present throughout the experi-
ment. Various concentrations of drugs were applied
after the Ca²⁺-induced contraction had reached a
steady level.

Solutions
The modified Krebs’ solution was of the following
ionic composition (mM): Na⁺ 137, K⁺ 5.9, Ca²⁺ 2.6,
Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134.4, and
glucose 11.5. High K⁺ solution was prepared by re-
placing NaCl with KCl isosmotically. Ca²⁺-free solution
was prepared by replacing CaCl₂ with MgCl₂ and adding
EGTA (Dojin, Japan). Solutions were bubbled with
97% O₂–3% CO₂, and the pH was adjusted to 7.4.

For experiments on skinned muscles, the following
relaxing solution was used (mM): potassium methane-
sulfonate (KMs) 110, Mg-KMs 2, EGTA 4, and PIPES
20 (Dojin). The pH was adjusted to 7.1 with KOH at
26°C. The ionic strength was adjusted to 0.17 M with
KMs. Solutions containing various concentrations of
Ca²⁺ were prepared by adding approximate amounts
of calcium methanesulfonate to the relaxing solution.
The precise methods used for calculating free ionic
centrations and binding constants have been described
by Itoh et al.

Drugs
The chemicals used in the experiments were LP-805
(provided by Pola Cosmetic Ltd. and Nippon Lederle
Ltd., Tokyo); ACh, histamine, 5-hydroxytryptamine
(5-HT, serotonin), indomethacin, glibenclamide, tetro-
dotoxin, atropine sulfate, and guanethidine (all from
Sigma Chemical Co., St. Louis, Mo.); prostaglandin F₁₂,
(PGF₂α, Ono, Osaka, Japan); N⁶-nitro-L-arginine
(L-NNA) and charybdotoxin (Protein Research
Foundation, Osaka, Japan); and saponin (ICN Biomedicals,
Inc., Costa Mesa, Calif.).

All solutions were freshly prepared before each
experiment. Water double-distilled in glass was used
in this study.

Statistics
The concentration of drug that was required to
produce the half-maximum inhibition was determined
as an IC₅₀ value. The measured values were expressed
as mean±SD of a number (n) of observations. The signif-
icance of differences between means was assessed
by Student’s t test for paired or unpaired values as
appropriate or by the Cochran-Cox test. Values of p<0.05
were considered statistically significant.

Results
Effects of LP-805 on Vascular Smooth Muscle
Membrane Potentials
Effects of LP-805 with various concentrations of K⁺. In
the presence or absence of endothelial cells, smooth
muscle tissues prepared from distal and proximal regions of rabbit coronary artery were electrically and mechanically quiescent. In -E tissues, the resting membrane potential in the proximal region (-58.1±2.0 mV, n=50 impalements, 10 different preparations) was less polarized than that of the distal region (-68.5±1.9 mV, n=50, 10 preparations). When the membrane potential was measured from +E tissues of both regions, it was -60.2±1.8 mV for the proximal region and -69.8±1.8 mV for the distal region (n=20, three preparations). In +E tissues, the membrane potential measured from both regions was slightly more polarized (p<0.05) than those measured from -E tissues.

LP-805 hyperpolarized the smooth muscle membrane of -E tissues in a concentration-dependent manner. In -E tissues, the minimum concentration of LP-805 required to produce hyperpolarization was 0.3 μM, and the maximum hyperpolarization occurred with application of 3 μM LP-805 (-76.1±2.3 mV, n=20, five preparations in the proximal region; -76.9±2.1 mV, n=20, five preparations in the distal region). Figure 1 shows the effects of LP-805 on smooth muscle cell membranes prepared from -E tissues in distal and proximal regions in the presence or absence of glibenclamide. The half-maximum hyperpolarization was induced by 1 μM LP-805 in both regions. These LP-805-induced hyperpolarizations (>1 μM) were prevented by a 5-minute pretreatment with 10 μM glibenclamide. When various concentrations of glibenclamide (0.5–10 μM) were included in the bath, a high concentration of glibenclamide (10 μM) slightly depolarized the membrane in the distal region (from -68.9±1.9 to -64.7±1.8 mV, n=9, three preparations, p>0.05) but not in the proximal region (from -58.1±2.1 to -56.7±1.9 mV, n=10, three preparations, p<0.05). On the other hand, in the distal region, the 10 μM LP-805–induced hyperpolarization was not modified by charybdotoxin, an inhibitor of the calcium-dependent high-conductance K+ channel24,25; i.e., the membrane potential was -67.1±1.7 mV in the control (resting) condition and -68.4±1.5 mV in the presence of 100 nM charybdotoxin (n=8, two preparations), and the membrane potential was -76.3±1.6 and -75.2±1.8 mV, respectively, in the presence of 10 μM LP-805 with 100 nM charybdotoxin (n=8, two preparations). With long exposure of tissues to LP-805, the amplitude of the hyperpolarization gradually attenuated over 10–15 minutes but was not abolished. When the tissue was subsequently rinsed with Krebs’ solution, the membrane gradually repolarized to the resting level but with a slow time course; i.e., in experiments using 1 μM LP-805, more than 20 minutes was needed to restore the membrane potential.

Figure 2 shows the effects of 10 μM LP-805 on the membrane potential recorded in the presence of various concentrations of K’ in -E tissues prepared from the proximal (panel A) and distal (panel B) regions of the rabbit coronary artery. In low K’ solutions (<5.9 mM), LP-805 consistently hyperpolarized the membrane. Smooth muscle preparations from the distal and proximal regions differed in their responses to low K’ solutions and to LP-805. In the -E tissues prepared from the distal region, reducing the concentration of K’ in the Krebs’ solution caused a consistent and proportional hyperpolarization of the membrane down to 1.2 mM K’; further reduction of K’ caused no further hyperpolarization of the membrane (Figure 2). On the other hand, in -E tissues prepared from the proximal region, reducing the concentration of K’ did not change the membrane potential. When LP-805 was applied in the presence of low concentrations of K’, the membrane of smooth muscles in both regions was hyperpolarized, and it was more strongly hyperpolarized in the proximal than in the distal region. The maximum hyperpolarization induced by 10 μM LP-805 was observed in 1.2 mM KCl solution in the distal region (-101±3 mV, n=6, three preparations), but the maximum hyperpolarization (-88.0±1.0 mV, n=7, three preparations) was observed in 2.4 mM K’ in the proximal region (Figure 2). In high K’ solutions (>20 mM K’), LP-805 did not modify the K’-induced depolarization.

When glibenclamide (10 μM) was applied in both regions, the membrane was consistently depolarized in low K’ solution (<5.9 mM) in the distal region but not in the proximal region. With simultaneous applications of LP-805 and glibenclamide, the 10 μM LP-805–induced hyperpolarization was no more observed in tissues from both regions in the presence of various concentrations of K’, and the membrane potentials...
observed before and during application of LP-805 with glibenclamide in both regions remained unchanged from the control values in any given concentrations of K⁺ (<20 mM) (Figure 2).

These results suggest that the relation between membrane potential and K⁺ differs significantly in the proximal versus the distal coronary artery in low K⁺ solutions (>5.9 mM) and that the response to glibenclamide in low K⁺ solution also differs. However, since LP-805 no longer hyperpolarizes cells in either region when added simultaneously with glibenclamide, it suggests that this agent increases the permeability of glibenclamide-sensitive K⁺ channels. In this way LP-805 resembles the actions of other K⁺ channel openers such as nicorandil, pinacidil, and cromakalim, which have been studied previously.

Effects of LP-805 on membrane potential in the presence of acetylcholine. In −E tissues prepared from the proximal region, ACh depolarized the membrane in a concentration-dependent manner (0.1 µM). A single action potential was often superimposed upon the depolarization (Figure 3A). LP-805 reduced the amplitude of the ACh-induced depolarization. When the membrane was maximally hyperpolarized by 10 µM LP-805, ACh (2 µM) did not depolarize the membrane (Figure 3A), but during the gradual repolarization of the membrane after removal of 10 µM LP-805, the depolarization to ACh recovered as the membrane potential returned to the resting level (Figure 3B).

In +E tissues prepared from the same proximal region, ACh (2 µM) transiently hyperpolarized the membrane from the resting membrane potential of −60.1±2.1 mV (n=10, five preparations) to −69.5±3.1 mV (n=10, five preparations). After the application of LP-805 (10 µM), ACh produced only a small hyperpolarization, the amplitude of which depended on the level of the membrane potential (Figure 4A). This transient hyperpolarization induced by ACh remained unchanged by a 12–15-minute pretreatment with 10 µM glibenclamide (Figure 4B, to −70.8±1.3 mV, n=10, three preparations) or with 100 µM L-NNA (Figure 4C, to 70.3±1.4 mV, n=10, three preparations).

Effects of LP-805 on the Mechanical Response Evoked in Intact and Skinned Muscle Tissues

Effects of LP-805 on the ACh-induced contraction. In +E tissues prepared from either proximal or distal regions of the rabbit coronary artery, application of 10

![Figure 2](http://circres.ahajournals.org/)

**FIGURE 2.** Graphs showing effects of LP-805 (10 µM), glibenclamide (Glib., 10 µM), or simultaneous application of LP-805 (10 µM) with glibenclamide (10 µM) on membrane potentials recorded in various concentrations of K⁺ in smooth muscle cells prepared from endothelium-denuded tissue of proximal (panel A) and distal (panel B) regions. Values are mean±SD; n=6–20 penetrations.

![Figure 3](http://circres.ahajournals.org/)

**FIGURE 3.** Tracings showing the effects of LP-805 (10 µM) on the acetylcholine (ACh)–induced depolarization in endothelium-denuded tissues prepared from the proximal region. Panel A: Before, during, and after the application of LP-805 (10 µM), 2 mM ACh was applied. Panel B: After application of LP-805 (10 µM), ACh (2 µM) was repetitively applied at certain time intervals.
followed by a slowly developed contraction (Figure 5b) in the proximal region. In many +E tissues, oscillatory twitchlike contractions were superimposed on the redeveloped contraction (Figure 5a). However, in −E tissues prepared from either region, 10 μM ACh produced a maintained tonic contraction in a concentration-dependent manner (e.g., see Figure 5b) in the proximal region. In +E tissues prepared from the proximal region, pretreatment with 100 μM L-NNA for 10 minutes attenuated the transient relaxation that occurred after application of 10 μM ACh, so that a tonic contraction with an enhanced amplitude was maintained (1.3±0.1 times control, n=5; see Figure 5b).

In tissues prepared from the proximal region, when LP-805 was applied during the redeveloped sustained contraction evoked by 10 μM ACh, both +E and −E tissues relaxed in a concentration-dependent manner but with different potencies. As shown in Figures 5a and 5d, 0.01 μM LP-805 slightly and 1 μM LP-805 completely relaxed the precontracted +E tissues. On the other hand, in −E tissues, the minimum concentration of LP-805 required to relax the tissue precontracted by 10 μM ACh was 1 μM, and to completely relax the tissue, 10 μM LP-805 was required. Therefore, LP-805 has a more potent inhibitory action on the ACh-induced contraction in +E tissues than in −E tissues. Figure 6 summarizes the effects of LP-805 on the ACh-induced contraction in −E and +E tissues prepared from the proximal region. The amplitude of contraction evoked by 10 μM ACh recorded in the presence or absence of endothelial cells was normalized as a relative tension of 1.0 in each case. Under treatment with 100 μM L-NNA
in \( +E \) tissues prepared from the proximal region, only 0.6 \( \mu M \) ACh was needed to produce the same amplitude of contraction as that evoked by 10 \( \mu M \) ACh in untreated \(+E\) tissues (1.09±0.12 times control amplitude, \( n=7 \) preparations, Figures 5F and 6). Since actions of vasorelaxing agents depended on the amplitude of contraction, the effects of LP-805 on the contraction evoked by 0.6 \( \mu M \) ACh (this value was also normalized as a relative tension of 1.0) in the presence of L-NNA in \(+E\) tissues were also investigated.

Removal of the endothelium produced a 10-fold shift in the \( IC_{50} \) value for LP-805 (0.27 \( \mu M \) for \(+E\) tissues versus 2.8 \( \mu M \) for \(-E\) tissues, \( n=5 \) preparations, Figure 6A). The LP-805-induced relaxation in \(+E\) tissues was not modified by pretreatment with 10 \( \mu M \) indomethacin for 60 minutes or with 10 \( \mu M \) glibenclamide for 10 minutes (Figure 6B). On the other hand, when \(+E\) tissues were pretreated with 100 \( \mu M \) L-NNA for 20 minutes, the \( IC_{50} \) for LP-805 in tissues contracted with 10 \( \mu M \) ACh shifted from 0.28 to 2.8 \( \mu M \). A similar \( IC_{50} \) value was obtained in the presence of L-NNA in tissues contracted with 0.6 \( \mu M \) ACh (\( IC_{50} \) value of 1.18 \( \mu M \), \( n=5 \) preparations, Figure 6A). Thus, the inhibitory action of LP-805 on \(+E\) tissues treated with L-NNA is the same as that observed on untreated \(-E\) tissues. In \(+E\) tissues prepared from the proximal region, 10 \( \mu M \) glibenclamide and 10 \( \mu M \) indomethacin had no effect on the LP-805-induced relaxation (\( IC_{50} \) of 0.27 \( \mu M \) in the presence of either agent, Figure 6B). In contrast, in \(-E\) tissues when 10 \( \mu M \) glibenclamide was given as a pretreatment for 10 minutes before application of ACh, the LP-805-induced relaxation was inhibited, and the \( IC_{50} \) shifted from 2.8 to 20 \( \mu M \).

After the removal of 1 or 3 \( \mu M \) LP-805 in \(+E\) tissues or 10 \( \mu M \) LP-805 in \(-E\) tissues, contraction reappeared in the presence of ACh. However, the latency for the redevelopment of contraction was much longer in \(+E\) tissues (4.95±1.63 minutes, \( n=5 \) preparations, Figures 5A and 5D) than in \(-E\) tissues (only a few seconds, \( n=9 \), Figures 5B and 5C). When 100 \( \mu M \) L-NNA was given as a pretreatment to the \(+E\) tissues, the latency for the redeveloped ACh-induced contraction after washout of LP-805 was reduced from 4.95 to 0.36±0.80 minutes (\( n=7 \) preparations). Much the same responses induced by LP-805 were also recorded from the \(+E\) and \(-E\) tissue prepared from the distal region.

Effects of LP-805 on the histamine-, 5-HT, or PGF\(_{2\alpha}\)-induced contraction. In the proximal region of the coronary artery (both in \(+E\) and \(-E\) tissues), histamine, 5-HT, or PGF\(_{2\alpha}\) (10 \( \mu M \)) provoked a contraction without generation of oscillation, and the amplitude was much smaller than that evoked by ACh (10 \( \mu M \)). LP-805 (>0.03 \( \mu M \)) consistently inhibited the above agonist-induced contraction in \(+E\) tissues. However, because the agonist-induced contraction induced by these agents in \(+E\) tissues was small, the effect of LP-805 could not be statistically determined. In \(-E\) tissues and also in the presence of L-NNA (100 \( \mu M \)) in \(+E\) tissues, the amplitude of contractions evoked by these agents was markedly enhanced, as observed with application of ACh. In \(+E\) tissues in the presence of L-NNA (100 \( \mu M \)), LP-805 inhibited the contraction evoked by histamine in a concentration-dependent manner (the \( IC_{50} \) values for histamine and PGF\(_{2\alpha}\) were 0.1 and 0.7 \( \mu M \), respectively; five preparations), and in \(-E\) tissues, these \( IC_{50} \) values were 0.5 and 7 \( \mu M \).
respectively (five preparations). In -E tissues, glibenclamide (10 μM) significantly reduced the LP-805-induced relaxation in both the histamine- or PGF$_{2\alpha}$-induced contractions (the IC$_{50}$ values of LP-805 for histamine- and PGF$_{2\alpha}$-induced contractions in the presence of glibenclamide were 3 and 25 μM, respectively; five preparations). Since 5-HT produced a transient contraction in the -E and in +E tissues in the presence of L-NNA, the effect of LP-805 was not measured.

These data indicate that inhibitory potencies of LP-805 for the contraction induced by individual vasoconstrictor agents differ, but this agent consistently inhibits the contraction, and this relaxing action of LP-805 is inhibited by glibenclamide in the same manner as observed in the case of the ACh-induced contraction.

**Effects of LP-805 on the K$^+$-induced contraction.**

In both +E and -E tissues, high concentrations of K$^+$ (up to 128 mM) produced a phasic and subsequently a tonic contraction, and 77 mM K$^+$ produced the maximum amplitude of contraction. Since more than 3 μM LP-805 itself produced a transient contraction, to assess the effects of LP-805 on the K$^+$-induced contraction, the amplitude of the tonic contraction was used.

Figure 7 shows the effects of LP-805 on the K$^+$-induced tonic contraction in both -E and +E tissues. In -E tissues, as shown in Figure 7A, LP-805 inhibited the contraction evoked by less than 30 mM K$^+$ in a concentration-dependent manner but not the contraction evoked by higher concentrations of K$^+$ (39–128 mM K$^+$). When the effects of LP-805 on the K$^+$-induced contraction were observed in both -E and +E tissues (20 mM K$^+$ in Figure 7B and 77 mM K$^+$ in Figure 7C), LP-805 relaxed to a greater extent the 20 mM K$^+$-induced contraction than it did the 77 mM K$^+$-induced contraction. In 20 mM K$^+$ in +E tissues, after application of 100 μM L-NNA, the LP-805-induced inhibition of the K$^+$-induced contraction was smaller than that observed in the absence of L-NNA. The concentration-inhibition curve, determined from experiments in the presence of L-NNA, as shown in Figure 7B in +E tissues, followed exactly the same pattern as that obtained from -E tissues. Furthermore, in -E tissues the LP-805-induced relaxation was markedly suppressed after application of 10 μM glibenclamide (Figure 7B). When 100 μM L-NNA and 10 μM glibenclamide were previously applied, relaxation of the 20 mM K$^+$-induced contraction in +E tissues produced almost the same curve as that obtained after application of 10 μM glibenclamide in -E tissues (not shown). In the presence of 77 mM K$^+$, as shown in Figure 7C, LP-805 (up to 1 μM) did not inhibit the K$^+$-induced contraction, although with higher concentrations of LP-805 (up to 10 μM), a slight inhibition was noted in +E but not -E tissues.

It would thus appear that LP-805 inhibits the 20 mM K$^+$-induced contraction by hyperpolarization of the membrane and in part by the release of EDRF; however, the concentration of LP-805 needed is much higher than that required to inhibit the ACh-induced contraction. In 77 mM K$^+$, the small relaxation induced by LP-805 is presumably due to a release of EDRF.

**Effects of LP-805 on the contraction evoked by released Ca$^{2+}$ in intact tissues and Ca$^{2+}$ in skinned muscle tissues.**

In Ca$^{2+}$-free solution containing 2 mM EGTA, 10 μM ACh or 10 mM caffeine produced only a transient contraction, attributed to the Ca$^{2+}$ released mainly from the sarcomplasmic reticulum. To investigate the action of LP-805 on the Ca$^{2+}$ store site, effects of this agent were observed on the ACh- or caffeine-induced contraction in 2.6 mM Ca$^{2+}$ and in Ca$^{2+}$-free solution containing 2 mM EGTA. In -E tissues prepared from the proximal region, LP-805 (10 μM) inhibited the ACh (10 μM)– or caffeine (10 mM)–induced contraction in Ca$^{2+}$-free solution (Figure 8, n=5, p>0.05). However, when LP-
Figure 8. Panels A and B: Tracings showing the effects of LP-805 (3 and 10 μM) on the contractions induced by acetylcholine (ACh, 10 μM; panel A) and caffeine (10 mM; panel B) in Ca²⁺-free solution containing 2 mM EGTA recorded from endothelium-denuded [E(-)] tissues of the proximal region. Panel C: Concentration-response curves for the LP-805-induced inhibition of the ACh- and caffeine-induced contraction in Ca²⁺-free solution. Values are mean ± SD; n = 5–7 preparations.

805 (>3 μM in both +E and −E tissues) was applied in Krebs’ solution (2.6 mM Ca²⁺), there was a transient and sharply developed concentration-dependent contraction, but this did not occur in the case of Ca²⁺-free solution (up to 30 μM LP-805, Figure 9). To facilitate a comparison with the amplitude of contraction evoked by LP-805, the 128 mM K⁺–induced contraction was normalized as 1.0 (Figure 9B). Therefore, the contraction evoked by LP-805 may not be due to release of Ca²⁺ from the SR. Since 30 μM LP-805 did not depolarize the membrane (Figure 3), the presumed Ca²⁺ influx may not be related to activation of the voltage-dependent Ca²⁺ channel.

Before skimming the −E tissue prepared from the proximal region, 128 mM KCl and 10 μM ACh were simultaneously applied to produce a maximum contraction, and then the tissue was treated with saponin in relaxing solution (see “Materials and Methods”). The minimum concentration of Ca²⁺ needed to produce contraction was 0.1 μM, and the maximum amplitude of contraction was evoked by 3 μM Ca²⁺. When 1 or 10 μM Ca²⁺ was applied, a monophasic contraction of sustained amplitude occurred. The 1 μM Ca²⁺–induced contraction was consistently larger than that evoked by high K⁺ with ACh in the intact tissue. After the amplitude of contraction had reached a steady level, various concentrations of LP-805 (0.3–10 μM) were cumulatively applied (Figure 10). LP-805 (up to 10 μM) did not modify the contraction evoked by either 1 μM Ca²⁺ (half-maximum contraction) or 10 μM Ca²⁺ (maximum contraction). This implies that LP-805 has no apparent effect on the phosphorylation of contractile proteins induced through the Ca²⁺-calmodulin–myosin light chain kinase 20-kd protein of the myosin light chain complex.

Discussion

The present experiments provided evidence that LP-805 has two significant actions on the rabbit coronary artery: 1) LP-805 relaxes tissues precontracted by ACh through a release of EDRF from endothelial cells. 2) LP-805 hyperpolarizes the smooth muscle membrane by activating the glibenclamide-sensitive K⁺ channel but not the charybdotoxin-sensitive K⁺ channel in the presence or absence of endothelial cells. However, it has no effect on the release of Ca²⁺ from the sarcoplasmic reticulum or on the Ca²⁺-induced contraction in
skinned muscle tissues. In +E tissues, the potency of the hyperpolarizing action of LP-805 was one fourth that of its relaxing action, as estimated from the finding that the concentration of the half-maximum hyperpolarization (1 μM) was four times the concentration required to cause half relaxation of precontracted tissues (IC50 of 0.28 μM). Therefore, the main action of LP-805 with respect to vasodilation may be due to release of EDRF from endothelial cells. Responses of smooth muscle membranes prepared from distal and proximal regions seem to differ, as estimated from the actions of LP-805 in various concentrations of K+.

**EDRF-Releasing Actions of LP-805**

In +E tissues, ACh produced a transient hyperpolarization, slightly depolarized the membrane, and produced an initial phasic, followed by a redeveloped, contraction. On the other hand, in −E tissues, ACh concentration-dependently depolarized the membrane and produced a monophasic sustained contraction with an enhanced amplitude compared with that noted in +E tissues. In −E tissues, the ACh-induced depolarization was blocked by LP-805. However, the inhibition of the ACh-induced depolarization by LP-805 was prevented by glibenclamide. We did not investigate the underlying ionic mechanisms of the ACh-induced depolarization, which may include activation of a nonsel ective cation channel (mainly activations of Na+ current32) or inhibition of a K+ current (i.e., the M current33).

It has been postulated that ACh releases both EDRF6 and EDHF27–19 from endothelial cells. Komori and Suzuki18 reported that oxotremorine, a muscarinic stimulant, released only EDRF and not EDHF and thatpirenzepine inhibited EDHF more potently than EDRF. Therefore, they postulated that EDRF and EDHF may be released through activation of the m2 and m3 receptors, respectively. An essential component of the relaxing action of EDRF is thought to be NO, a compound formed by the oxidation of one guanidino-nitrogen atom (N5) of L-arginine.7,6,21,34,35 In our experiments, LP-805 relaxed the +E tissue precontracted by ACh, and this action was markedly inhibited by pretreatment with L-NNA, a potent inhibitor of cytosolic nitric oxide synthesis from L-arginine.36 In +E tissues, the ACh-induced transient hyperpolarization was not modified by L-NNA or by glibenclamide, and in −E tissues, ACh solely depolarized the membrane. Therefore, the transient hyperpolarization induced by ACh required the presence of endothelial cells, independent of EDRF. It has been reported that NO synthesized from L-arginine produces a hyperpolarization in vascu-
ously released from the endothelial cells and diminishes the amplitude of the ACh-induced contraction, and 2) after the application of ACh, endothelial cells release both EDRF and EDHF but not PGI₂. Furthermore, LP-805 hyperpolarized the membrane to the same extent in -E and +E tissues, and the LP-805–induced hyperpolarization was blocked by glibenclamide with no change in the amplitude of the ACh-induced hyperpolarization. Thus, LP-805 may release EDRF but not EDHF or PGI₂.

**Actions of LP-805 as a K⁺ Channel Opener**

LP-805 hyperpolarized the membrane, and this action was prevented by glibenclamide but not by charybdotoxin. Since charybdotoxin blocks the Ca²⁺-sensitive high-conductance K⁺ channel, the hyperpolarization induced by LP-805 may not be related to the high-conductance K⁺ channel but may be due to activation of the glibenclamide-sensitive K⁺ channel opening. K⁺ channel openers are classified into various subtypes based on their chemical structures: 1) cromakalim (BRL 34915, benzopryan nucleus–containing structure) and its related derivatives, 2) pinacidil (thiourea- or guanidine-containing structure), 3) nicorandil (pyridine nucleus–containing structure), 4) minoxidil sulfate (pyridine or triazine nucleus–containing structure), 5) diazoxide (benzothiazi diazine nucleus–containing structure), 6) thioformamide (thioformamide nucleus–containing structure), and 7) dihydropyridine derivative [(+)-niguldipine] and their own derivatives. These basic K⁺ channel openers activate the ATP- and glibenclamide-sensitive K⁺ channel of smooth muscles, as measured using the voltage- and patch-clamp procedures. However, the chemical structure of LP-805 bears no relation to that of the above K⁺ channel openers. In -E tissues, the potency of LP-805 as a K⁺ channel opener to relax the tissue precontracted by ACh through hyperpolarization of the membrane seems to be much the same as that of cromakalim or pinacidil. In the rabbit coronary artery, the LP-805–induced half-maximum hyperpolarization occurred with 1 μM, but such measurements using cromakalim or pinacidil were not described. On the other hand, in the porcine coronary artery, the concentration of LP-805 required to produce the hyperpolarization was the same as that observed for pinacidil (1 μM, authors’ unpublished observations).

To determine whether the action of LP-805 was dependent on the kind of agonist used to induced contraction, additional experiments were undertaken with 5-HT, histamine, and PGF₂α. Histamine has been reported to release both EDRF and EDHF, whereas PGF₂α releases only EDRF; 5-HT also releases EDRF, but whether 5-HT releases EDHF has not been examined. The response to these agonists resembled responses to ACh in that removal of the endothelium (or application of L-NNA) significantly increased the amplitude of contraction. In the absence of endothelium, or with L-NNA present in +E tissues, LP-805 consistently produced relaxation, regardless of which spasmodogenic agent was tested. Although small differences in potency to LP-805 were observed between agonists, in each case the vasodilatation elicited with LP-805 was substantially reduced in the presence of glibenclamide. These results indicate that the action of LP-805 on smooth muscle are not restricted to contractions elicited with ACh but rather are more generally applicable to a variety of spasmodogenic agents. Indeed LP-805 not only inhibits the contractions evoked with agonist but also antagonizes contractions evoked with concentrations of K⁺ up to 30 mM.

LP-805 (<3 μM) did not modify the ACh- and caffeine-induced contraction in Ca²⁺-free solution nor the 1–10 μM Ca²⁺–induced contraction in skinned muscle tissues prepared using saponin. These results suggest that LP-805 does not act directly on the contractile machinery.

In conclusion, LP-805 indirectly relaxes smooth muscle tissue precontracted with vasoconstrictive agents and high K⁺ (<30 mM) indirectly via a release of EDRF from endothelial cells and also directly hyperpolarizes smooth muscle membranes by activating the glibenclamide-sensitive K⁺ channel. These actions of LP-805 would account for the dilation of the rabbit coronary artery.

**References**

Effects on the rabbit coronary artery of LP-805, a new type of releaser of endothelium-derived relaxing factor and a K+ channel opener.
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