Mechanisms of Flunarizine-Induced Vasodilation in the Rabbit Mesenteric Artery

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The vasodilating effects of flunarizine on smooth muscle strips of rabbit mesenteric artery have been investigated and compared with those of nifedipine. Flunarizine (30–300 nM) dose-dependently inhibited Ca\(^{2+}\)-induced contractions in Ca\(^{2+}\)-free solution containing 100 mM K\(^+\). Double reciprocal analysis showed that this inhibition was either competitive at low concentrations (30–100 nM; nifedipine-like) or noncompetitive at high concentrations (0.3–1 μM). The latter seemed to be partly related to an inhibition of contractile proteins as estimated from Ca\(^{2+}\)-induced contractions in saponin-treated chemically skinned muscle strips. In contrast to the actions of nifedipine, flunarizine inhibited norepinephrine (NE)-induced contractions more than those induced by high K\(^+\), and at 0.3 μM, this agent totally blocked NE-induced contraction. Flunarizine also inhibited NE-induced contraction in Ca\(^{2+}\)-free solution containing 2 mM EGTA. In Ca\(^{2+}\)-free solution, NE rapidly hydrolyzed phosphatidylinositol 4,5-bisphosphate (PI-P\(_2\)) and produced phosphatidic acid (PA). Flunarizine (30 and 300 nM), but not nifedipine (100 nM), inhibited NE-induced hydrolysis of PI-P\(_2\) and production of PA. However, flunarizine (100 nM) did not modify the contraction induced by 10 μM inositol 1,4,5-trisphosphate in chemically skinned muscle strips. It is concluded that flunarizine inhibits both voltage-dependent (nifedipine-like) and receptor-operated Ca\(^{2+}\) influx induced by NE and also inhibits NE-induced Ca\(^{2+}\) release from intracellular stores due to inhibition of the hydrolysis of PI-P\(_2\).

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Flunarizine (a difluoro derivative of the diphenylpiperazine, cinnarizine) is a potent inhibitor of peripheral vasconstriction. Holmes et al concluded that flunarizine-induced vasodilation was due to the inhibition of Ca\(^{2+}\) influx evoked by various stimulants. However, the actions of this drug on norepinephrine (NE)-induced contraction in arterial tissues seem to be different from those of dihydropyridine derivatives like nifedipine and nisoldipine. For example, flunarizine greatly reduces NE contractions in vascular tissues, while nifedipine only partly inhibits this activity.

In the rabbit mesenteric artery, NE produces a contraction due to activation of both voltage-dependent (nifedipine-sensitive) and receptor-operated (nifedipine-resistant) Ca\(^{2+}\) influx and release of Ca\(^{2+}\) from intracellular storage sites. In preliminary experiments using rabbit mesenteric artery, flunarizine (but not nifedipine) blocked the NE-induced contraction. This suggests that in this tissue, flunarizine may also act at sites different from those indicated previously. The recent work of Hashimoto et al in rabbit mesenteric artery indicates that NE-mediated contractions are generated by Ca\(^{2+}\) release from intracellular stores following the production of inositol 1,4,5-trisphosphate (InsP\(_3\)). However, the effects of flunarizine on the breakdown of phosphoinositides induced by NE have not yet been examined.

In the present study, the effects of flunarizine and nifedipine were observed on NE- and high K\(^+\)-induced contractions in the presence or absence of Ca\(^{2+}\) in intact muscle strips. The effects of these agents on Ca\(^{2+}\)- and InsP\(_3\)-induced contractions in saponin-skinned muscle strips were also investigated with their effects on phosphatidylinositol 4,5-bisphosphate (PI-P\(_2\)) hydrolysis evoked by NE. This was undertaken to characterize the actions of flunarizine on NE-induced mechanical responses in the rabbit mesenteric artery and to indicate any differences between the actions of this diphenylpiperazine and those of nifedipine.

Materials and Methods

Preparation

Male albino rabbits (2.0–2.5 kg) were given sodium pentobarbital (40 mg/kg i.v.) and exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with Krebs solution, and the mesenteric artery was carefully excised under a binocular microscope. Arterial segments 0.2–0.3 mm in diameter were used for the experiments, and thin circular strips (0.3–0.5 mm long, 0.05–0.08 mm wide, and 0.02–0.03 mm thick) were prepared. The diameters of these strips were measured under an inverted microscope. In all experiments, the endothelium was carefully removed using small knives made from razor blades as indicated previously.

Recording of Mechanical Activity

Mechanical responses of intact and chemically skinned muscle strips were measured by attaching the strips to a strain gauge (UL-2 type, Shinko Co., Tokyo)
in a 0.6-ml chamber. The solution was changed by perfusing rapidly from one end and aspirating simultaneously with a water pump from the other end. To suppress sympathetic nerve activity, 0.3 μM tetrodotoxin (TTX) and 3 μM guanethidine were present in the Krebs solution throughout the experiments.

**Ca**<sup>2+</sup>-Induced Contractions in Intact Muscle Strips

Two basic procedures were used to study the effects of flunarizine or nifedipine in **Ca**<sup>2+</sup>-free conditions or in **Ca**<sup>2+</sup>-induced contractions: 1) some muscle strips were depolarized for 60 minutes by application of 100 mM K<sup>+</sup> in **Ca**<sup>2+</sup>-free Krebs solution containing 2 mM EGTA. In this solution, 2.6 mM **Ca**<sup>2+</sup> produced a maximum contraction; further addition of **Ca**<sup>2+</sup> resulted in a contraction of reduced amplitude. 2) Some strips were depolarized using a HEPES-buffered solution (see "Solutions"). Under these conditions, 10 mM **Ca**<sup>2+</sup> produced a maximal contraction that was also generated by a **Ca**<sup>2+</sup> concentration up to 50 mM. Such a procedure was ideal for the examination of the effects of flunarizine on **Ca**<sup>2+</sup> concentration-effect curves. Therefore, the effects on **Ca**<sup>2+</sup>-induced contractions were observed in **Ca**<sup>2+</sup>-free solution containing 100 mM K<sup>+</sup> buffered with HEPES.

**Chemically Skinned Muscle Strips**

Chemically skinned muscle strips were obtained by exposing them to saponin (25 μg/ml) for 20 minutes in relaxing solution. The tension-p**Ca** relation was obtained by cumulative application of increasing **Ca**<sup>2+</sup> concentrations in a stepwise manner. Flunarizine or nifedipine was applied for 20–30 minutes before and during application of **Ca**<sup>2+</sup>. To prevent deterioration of **Ca**<sup>2+</sup> contractions, 0.1 μM calmodulin was present throughout the experiments.

To observe the effects of flunarizine or nifedipine on **Ca**<sup>2+</sup> release from intracellular stores, 10 μM InsP<sub>3</sub> or 25 mM caffeine was applied in relaxing solution containing a low concentration of EGTA (0.5 mM) after 1 minute of application of 0.3 μM **Ca**<sup>2+</sup> buffered with 4 mM EGTA as previously described.

**Assays of Phosphatidylinositol 5-Monophosphate (PI-P), Phosphatidylinositol 4,5-Bisphosphate (PI-P<sub>2</sub>), and Phosphatidic Acid (PA)**

The amounts of PI-P, PI-P<sub>2</sub>, and PA in muscle strips of the rabbit mesenteric artery were measured before and after application of 10 μM NE in **Ca**<sup>2+</sup>-free solution containing 2 mM EGTA. Muscle strips (0.2–0.3 mm in outer diameter and 3–4 cm long) were labelled in outer diameter and 3-4 cm long were labelled in phosphate-free, HEPES-buffered solution containing 40 μCi/ml of <sup>32</sup>Pi (specific activity, 30–40 Ci/mmol; Japan Atomic Energy Research Inst.) at 37°C for 1.5 hours. The strips were washed three times with the above solution without <sup>32</sup>Pi and incubated for 1.5 hours in the presence or absence of flunarizine (30 or 300 nM). NE was applied in the presence or absence of flunarizine for 30 seconds after 2 minutes of removal of **Ca**<sup>2+</sup>. Nifedipine (100 nM) was applied in the dark to the tissue during the final 5 minutes of incubation after application of <sup>32</sup>Pi and was also present during application of NE. The reaction was halted by adding a solvent containing chloroform, methanol, and concentrated hydrogen chloride (100: 200: 2, vol/vol), and strips were then homogenized in a glass homogenizer. Crude phospholipid extracts in the solvent were chromatographed on Silica Gel 60 plates (Merck, Darmstadt, Federal Republic of Germany), according to Billah and Lapetina. The plates were then autoradiographed using Sakura x-ray film for 12–15 hours. The fractions corresponding to PI-P, PI-P<sub>2</sub>, and PA on the plates were cut out and counted for radioactivity in a liquid scintillation counter.

**Solutions**

The ionic concentrations of the Krebs solutions were as follows (mM): Na<sup>+</sup> 137.4, K<sup>+</sup> 5.9, Mg<sup>2+</sup> 1.2, **Ca**<sup>2+</sup> 2.6, HCO<sub>3</sub> - 15.5, H<sub>2</sub>PO<sub>4</sub> - 1.2, Cl<sup>-</sup> 134.4, glucose 11.4. The high K<sup>+</sup> solution was prepared by replacing sodium chloride with potassium chloride, isoosmotically. The solution was bubbled with 97% O<sub>2</sub>-3% CO<sub>2</sub>, and the pH of the solution was adjusted to 7.4. In **Ca**<sup>2+</sup>-free solutions, CaCl<sub>2</sub> was replaced with MgCl<sub>2</sub>, and 2 mM EGTA was added.

To prepare the solution containing various concentrations of **Ca**<sup>2+</sup>, HEPES buffer was used. The composition of HEPES buffer solution was as follows (mM): NaCl 45, K methanesulfonate (KMs) 99, KH<sub>2</sub>PO<sub>4</sub> 1, Mg(MS) 1, HEPES 5, glucose 5.6, and ionic concentrations of the Krebs solutions were as follows (mM): Na<sup>+</sup> 137.4, K<sup>+</sup> 5.9, Mg<sup>2+</sup> 1.2, **Ca**<sup>2+</sup> 2.6, HCO<sub>3</sub> - 15.5, H<sub>2</sub>PO<sub>4</sub> - 1.2, Cl<sup>-</sup> 134.4, glucose 11.4. The high K<sup>+</sup> solution was prepared by replacing sodium chloride with potassium chloride, isoosmotically. The solution was bubbled with 97% O<sub>2</sub>-3% CO<sub>2</sub>, and the pH of the solution was adjusted to 7.4. In **Ca**<sup>2+</sup>-free solutions, CaCl<sub>2</sub> was replaced with MgCl<sub>2</sub>, and 2 mM EGTA was added. The strips were degassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> and the pH of the solution was adjusted to 7.4 (adjusted with Tris).

In skinned muscles, the following relaxing solution was used (mM): KMs 114, Tris maleate 20, Mg(MS) 5.1, adenosine 5'-triphosphate (ATP) 5.2, and EGTA 4. Various **Ca**<sup>2+</sup> concentrations were prepared by adding appropriate amounts of Ca(MS)<sub>2</sub> to 4 mM EGTA. The binding constants used in this experiment have been previously reported.

**Drugs**

The chemicals used were 1-norepinephrine (NE) and tetrodotoxin (TTX) (Sigma Chemical Co., St. Louis, Mo.), guanethidine (Tokyo Kasei Kogyo, Tokyo), caffeine (Wako Pure Chemical Industries, Osaka, Japan), ethylenglycol-bis-(β-aminoethylether)-N,N',N''-tetraacetic acid (EGTA) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Dozin Laboratory, Kumamoto, Japan), nifedipine (Bayer Pharmaceutical Co., Basel, Switzerland), flunarizine dihydrochloride (1-bis(4-fluorophenyl)methyl-4-(3-phenyl-2-propenyl)piperazinedihydrochloride; Janssen Pharmaceutica, Beerse, Belgium). Nifedipine (0.7 mM) was originally dissolved in 15% ethyl alcohol and 15% polyethylene glycol 400. To avoid adverse solvent effects, this agent was used in a concentration below 0.3 μM (the concentration of ethyl alcohol was below 0.01%). To prevent inactivation of nifedipine by exposure to daylight, the experiments were carried out...
under a sodium lamp or conditions of subdued light. The highest grade of reagents was used for assay of PI-P, PI-P₂, and PA as previously indicated.¹ Calmodulin was prepared from dog brain.¹¹ Inositol 1,4,5-trisphosphate (InsP₃) was kindly provided by Dr. M. Hirata, Faculty of Dentistry, Kyushu University, Fukuoka, Japan.

Statistics
The results were expressed as mean ± SD (n = number of preparations; only one preparation was taken from each animal). The statistical significance was assessed using Student’s t test (paired and unpaired) or analysis of variance. Significance level was p < 0.05.

Results
Effects of Flunarizine on Mechanical Responses Evoked by High K+ in Intact Muscle Strips
As flunarizine-induced vasodilation appeared with a gradual onset and was of prolonged duration,³ we initially studied the time-dependent effects of flunarizine on contractions evoked by high K+. Figure 1 shows the effects of three different concentrations of flunarizine (0.1, 0.3, or 1 μM) on contractions evoked by 100 mM K+ in the rabbit mesenteric artery. These K+-induced contractions were evoked at 7-minute intervals and comprised a phasic followed by a tonic response (Figure 1A). Flunarizine (1 μM) gradually reduced the amplitudes of both mechanical components, and over 30 minutes these reduced amplitudes reached a steady level in all concentrations of flunarizine. When the tissue was rinsed with Krebs solution, the amplitudes of the contraction were gradually restored. In high concentrations of flunarizine (0.3 and 1 μM), however, the contraction did not completely recover even after 90 minutes of superfusion with Krebs solution (Figure 1B).

In contrast to the actions of flunarizine, nifedipine (100 nM) inhibited K+-induced contractions within 5 minutes when it was applied using the same protocol and when applied on tissue excised from the same branch of the artery (0.13 ± 0.05 times the control, n = 5). The time-course of inhibition by verapamil (10 μM) was the same as that exhibited by nifedipine.⁴,¹² These observations suggest that flunarizine requires approximately 30 minutes to exert its full inhibitory action. Therefore, flunarizine was applied to the tissue 30 minutes or more before and also during application of stimulants.

Figure 2 shows the effects of flunarizine on the phasic (maximal) contractions evoked by various concentrations of K+. The phasic contraction evoked by 144 mM K+ in the absence of flunarizine was normalized as 1.0. With application of 30 nM flunarizine, the contraction evoked by high K+ (50–144 mM) was consistently inhibited (p < 0.05) in a concentration-dependent manner.

To study further the action of flunarizine on voltage-dependent Ca²⁺ influx, its effects on contractions evoked by various concentrations of Ca²⁺ were observed in muscle strips depolarized using Ca²⁺-free 100 mM K⁺ solutions. Flunarizine (10 and 30 nM) consistently inhibited such Ca²⁺-induced contractions. The dose-response relation for Ca²⁺ shifted to the right in
a parallel manner in the presence of flunarizine (10 and 30 nM) as suggested by the double reciprocal plots in which lines met at 0 on the y axis (Figure 3A and 3B). At concentrations of 0.3 and 1 µM, the flunarizine-induced inhibition of Ca²⁺ contraction was largely of a noncompetitive nature (Figure 3C). Thus, flunarizine, competitively or noncompetitively, inhibited voltage-dependent Ca²⁺ influx in a concentration-dependent manner.

Effects of Flunarizine on Ca²⁺-Induced Contractions in Chemically Skinned Muscle Strips

Since high concentrations of flunarizine (0.3–1.0 µM) inhibited the Ca²⁺-induced contraction in a noncompetitive manner in intact muscle strips, the effects of this agent on the contractile proteins of vascular smooth muscles were studied. After skinning the tissue, three different concentrations of Ca²⁺ (0.3, 1, and 10 µM) were cumulatively applied in the presence of 0.1 µM calmodulin. The amplitude of the contraction evoked by 0.3 or 1 µM Ca²⁺ in skinned muscle strips was almost the same as the amplitude of the tonic or phasic contraction, respectively, evoked by 128 mM K⁺. When 1 µM flunarizine was applied before and during application of Ca²⁺, the contraction evoked by 0.3 µM Ca²⁺, but not by higher concentrations of Ca²⁺ (1 and 10 µM), was inhibited. However, flunarizine (<0.1 µM) and nifedipine (0.1 µM) had no effect on the Ca²⁺-induced contraction. These results indicate that high concentrations of flunarizine (1 µM) directly inhibit the contractile proteins of the vascular cells.

Effects of Flunarizine or Nifedipine on NE-Induced Contractions

The effect of flunarizine on the contraction evoked by 10 µM NE was compared with that on the contraction evoked by 40 mM K⁺. In the rabbit mesenteric artery, 40 mM K⁺ and 10 µM NE produced approximately the same amplitude of contraction; that is, when the maximum amplitude of contraction evoked by 128 mM K⁺ was normalized as 1.0, the maximum amplitude of contraction evoked by 10 µM NE was 0.53 ± 0.05, n = 5 and that evoked by 40 mM K⁺ was

![Figure 3](image-url)

**Figure 3.** Effects of flunarizine on tonic contractions evoked by various concentrations of Ca²⁺ in Ca²⁺-free solution containing 100 mM K⁺ in smooth muscle strips of rabbit mesenteric artery. A, the amplitude of the tonic contraction evoked by 10 mM Ca²⁺ in the absence of flunarizine was normalized as 1.0. Various concentrations of Ca²⁺ (0.3, 1, and 10 µM) were applied for 5 minutes at 10-minute intervals. Flunarizine was applied 60 minutes before application of Ca²⁺ (see "Results"). Each point represents mean value of 3 preparations. Curves were fitted by eye. B, double reciprocal plots of the tonic contractions evoked by various concentrations of Ca²⁺ in the presence or absence of flunarizine (10 and 30 nM). C, the same plots as described in B, but the concentrations of flunarizine were 300 nM and 1.0 µM.
0.51 ± 0.4, n = 5. Flunarizine (30 and 100 nM) inhibited the NE-induced phasic and tonic contractions more than the K⁺-induced ones, and at a concentration of 0.3 μM, this agent blocked the NE- and the K⁺-induced contractions.

Figure 4B shows the dose-dependent action of flunarizine on the contraction evoked by 10 μM NE or 40 mM K⁺. The maximum amplitude of contraction evoked by either agent in the absence of flunarizine was normalized as 1.0. The ID₉₀ value for the NE- or the K⁺-induced contraction was 27 ± 3 nM (n = 5) or 57 ± 5 nM (n = 5), respectively. In contrast to flunarizine, nifedipine (0.1 μM and 0.3 μM) suppressed the contraction evoked by 40 mM K⁺ more than that of 10 μM NE (Figure 5).

Concentration-response experiments using NE were performed in the presence of 0.3 μM propranolol to prevent β-receptor activation by NE both before and after application of flunarizine. These were performed because in this tissue, high concentrations of NE (1 μM and more) activate both α- and β-receptors.¹³

Flunarizine (3 nM) inhibited the contraction evoked by low concentrations of NE (0.1–1 μM) but did not affect the contraction evoked by high concentrations of NE (3–30 μM). In the concentration range 3–30 nM, this agent dose-dependently inhibited the NE-induced contraction with a rightward shift of the concentration-response curve. Analysis of the results obtained from three different preparations was carried out using linear regression analysis in which the values of log (dose ratio - 1) were plotted against the log molar concentration of flunarizine. The results indicate that flunarizine does not act on α₁-adrenergic receptors in a competitive manner (the slope of the regression line was 0.78 ± 0.12, which significantly differed from 1; p < 0.05).

**Effects of Flunarizine on NE- and Caffeine-Induced Contractions in Ca²⁺-Free Solution**

In the rabbit mesenteric artery, NE, but not high K⁺, produces contraction due to release of Ca²⁺ from intracellular stores in Ca²⁺-free solution containing 2 mM EGTA. Caffeine also produces contraction due to release of Ca²⁺ from intracellular stores that are closely related to or are the same as the NE-sensitive ones.⁸,¹⁴

To clarify the effects of flunarizine on NE-induced contraction in more detail, NE- or caffeine-induced contractions were evoked in Ca²⁺-free solution containing 2 mM EGTA. NE (10 μM) was applied for 2 minutes following 2 minutes of removal of Ca²⁺. Flunarizine was applied for 90 minutes before the above procedures were carried out (Figure 6). Flunarizine significantly inhibited the NE-induced contraction at 30 nM (0.45 ± 0.13 times the control, n = 5, p < 0.05), and at 300 nM, it blocked this contraction (0.03 ± 0.02 times, n = 5). Flunarizine had little effect on the contraction evoked by 10 mM caffeine (0.96 ± 0.05 times, n = 5, p > 0.05). In contrast, nifedipine (100 and 300 nM) inhibited neither the 10
Effects of Flunarizine on InsP$_3$- and Caffeine-Induced Contractions in Chemically Skinned Muscle Strips

To study the effects of flunarizine on the release of Ca$^{2+}$ from intracellular stores more directly, its effects on InsP$_3$- and caffeine-induced contractions were observed in chemically skinned muscle strips. After skinning, 0.3 μM Ca$^{2+}$ buffered with 4 mM EGTA was applied for 1 minute, and the tissue was rinsed with relaxing solution containing 4 or 0.5 mM EGTA. Subsequently, 25 mM caffeine or 10 μM InsP$_3$ was applied to evoke contraction in the relaxing solution containing 0.5 mM EGTA. When 0.1 μM flunarizine was added to the relaxing solution for 30 minutes before, during, or after application of Ca$^{2+}$, the contraction evoked by caffeine or InsP$_3$ was not affected. Since InsP$_3$ is thought to be a second messenger involved in NE-induced release of Ca$^{2+}$ from stores, these results suggest that flunarizine does not act on the final step of the Ca$^{2+}$-releasing mechanisms activated by NE.

Effects of Flunarizine on the Amounts of PI-P, PI-P$_2$, and PA

To search for the action site of flunarizine, its effects on changes in the amounts of phosphoinositides evoked by NE in Ca$^{2+}$-free solution were observed in rabbit mesenteric arterial strips (Figure 7). When 10 μM NE was applied to the tissue for 15 seconds 2 minutes after removal of Ca$^{2+}$, the amount of PI-P was reduced to 0.88 ± 0.08 times the control ($n = 5$; the control value of 131,098 ± 15,147 cpm/mg protein, $n = 5$, was normalized as 1.0). After 30 seconds, the amount of PI-P was reduced to 0.84 ± 0.04 times the control ($n = 5$), and after 2 minutes, the amount of PI-P was 0.82...
± 0.03 times the control (n = 5). When the amount of PA was measured 15 seconds after application of NE, it had increased to 1.52 ± 0.17 times the control. After 30 seconds, it had increased to 1.71 ± 0.18, and after 2 minutes, it was 1.67 ± 0.17 times the control (n = 5; the control value of 15,761 ± 1,615 cpm/mg protein, n = 5, was normalized as 1.0). The amount of PI-P measured 30 seconds after application of 10 μM NE was 1.02 ± 0.10 times the control (the control value of 33,268 ± 5,593 cpm/mg protein was normalized as 1.0, n = 5).

When tissues were treated with 300 nM flunarizine for 90 minutes, the amounts of PI-P, PI-P₂, and PA were not changed (1.04 ± 0.04 times the control for PI-P, 0.96 ± 0.03 for PI-P₂, and 1.03 ± 0.04 for PA). Figure 8 shows the effects of 30 nM and 300 nM flunarizine and 100 nM nifedipine on the amounts of PI-P, PI-P₂, and PA after exposure to 10 μM NE in Ca²⁺-free solution. NE was applied for 30 seconds 2 minutes after removal of Ca²⁺. Flunarizine (30 nM) significantly inhibited the breakdown of PI-P, and the synthesis of PA (p < 0.05), but in the presence of 300 nM flunarizine, the hydrolysis of PI-P induced by NE was blocked. Nifedipine (100 nM) had no effect on the amounts of PI-P₂ and PA in the presence of NE.

Discussion

Flunarizine is known to possess some of the properties of a Ca²⁺ channel blocker like nifedipine. It inhibits the Ca²⁺ spike evoked by an outward current pulse in the presence of TEA and also that associated with certain excitatory junctional potentials. Furthermore, flunarizine inhibits the Ca²⁺ influx provoked by high K⁺-induced depolarization. However, the present results obtained from a comparative study of the actions of flunarizine and nifedipine on contractions evoked by NE and high K⁺ indicate that flunarizine possesses some special inhibitory characteristics.

First, the inhibitory effects of flunarizine on the contraction evoked by high K⁺ or NE developed slowly and required more than 30 minutes to reach a maximum inhibitory value. In contrast, nifedipine produced maximum inhibition within 5 minutes, a time dependency also characteristic of verapamil and dilatazam. Since the preparations used in the present experiments were very small and thin (see "Materials and Methods"), the time required for diffusion of flunarizine was minimal. Therefore, the slow onset of flunarizine action did not result from the slow diffusion of this agent but may have been due to a specific characteristic that remains to be clarified. Second, flunarizine had more profound effects on the contraction evoked by NE than on that produced by high K⁺. Furthermore, the NE-induced contraction was blocked by flunarizine (100 nM) in either the presence or absence of Ca²⁺, which suggests that flunarizine may inhibit both Ca²⁺ influx and Ca²⁺ release from those intracellular stores activated by NE.

The K⁺-induced contraction was composed of phasic and tonic components in the smooth muscle strips of the rabbit mesenteric artery. The former component is a transient phenomenon and represents the voltage-dependent influx of Ca²⁺ across the cell membrane and release of Ca²⁺ from storage sites. The latter process reflects the sum of the Ca²⁺ influx, release, reuptake of Ca²⁺ at storage sites, and extrusion of Ca²⁺ from the cell. As a consequence, the tonic contraction is relatively well sustained.

Flunarizine inhibited both the phasic and tonic contractions evoked by high K⁺, and the latter component was more responsive to this agent than the former. Estimates made from double reciprocal plots of the tonic contraction evoked by Ca²⁺ suggest either a competitive inhibitory mechanism at low concentrations (10 and 30 nM) or a noncompetitive process at higher concentrations (0.3-1.0 μM). The present results suggest that this noncompetitive inhibitory process does not occur at the receptor level but may be caused by a direct inhibitory action on the contractile proteins in arterial cells. In chemically skinned muscle strips, the amplitude of the contraction evoked by 0.3 μM Ca²⁺ was larger than that of the tonic contraction evoked by 128 mM K⁺ in intact tissues, and flunarizine (1 μM) inhibited approximately 50% of this Ca²⁺-induced contraction in chemically skinned muscle strips. If this is so, the actions of flunarizine on voltage-dependent Ca²⁺ influx may be similar to those of dihydropyridine derivatives as estimated from the mechanical responses in the rabbit mesenteric artery. However, Spedding suggested that the site of action
of flunarizine may be different from that of nifedipine in K⁺-depolarized smooth muscle strips of the guinea pig taenia coli. This hypothesis was supported by radioligand-binding studies in which the diphenylalkylamines were less potent inhibitors of H-nitrendipidine binding than the dihydropyridines. Therefore, it remains to be determined whether flunarizine and nifedipine act at the same site on smooth muscle cells of the rabbit mesenteric artery.

In the rabbit mesenteric artery, exogenously applied NE (1–10 μM) depolarized the membrane and produced an initial phasic and a later tonic contraction. The latter ceased in Ca²⁺-free solution, but the NE-induced phasic contraction could still be evoked. Thus, in this tissue, NE produces a phasic contraction as a result of the release of Ca²⁺ from intracellular stores, while the tonic contraction probably results from an interplay between increases in Ca²⁺ influx and Ca²⁺ release from stores.

Nifedipine had no effect on the NE-induced phasic contraction in the presence or absence of Ca²⁺ and on the contractile proteins in chemically skinned muscle strips, but it partly inhibited the NE-induced tonic contraction. Kamnura et al. reported that nifedipine also had no effect on Ca²⁺ accumulation in and release from the NE-sensitive stores or on α₁-adrenoceptor-operated Ca²⁺ influx. However, it did inhibit voltage-dependent Ca²⁺ influx resulting from depolarization of the membrane following activation of the α₁-adrenoceptor. The results from the present study indicate that nifedipine acts on the voltage-dependent Ca²⁺ channel at the myoplasmic membrane of smooth muscle cells of the rabbit mesenteric artery. In contrast, flunarizine (10 and 30 nM) noncompetitively inhibited the NE-induced contraction and at a concentration of 100 nM, this agent totally blocked the effects of NE without changing the Ca²⁺ sensitivity of the contractile proteins. Godfraind and Miller reported that in rat aorta, NE-dependent Ca²⁺ influx was blocked by flunarizine, but a small, statistically significant residual NE-dependent Ca²⁺ influx was apparent in the presence of nifedipine. These results suggest that in contrast to the actions of nifedipine, flunarizine may inhibit both the receptor-operated and voltage-dependent Ca²⁺ influx evoked by NE.

Godfraind and Dieu reported that in rat aorta, NE-dependent Ca²⁺ efflux was relatively insensitive to flunarizine. This finding is inconsistent with our results that indicate flunarizine may inhibit NE-induced Ca²⁺ release from the stores. However, it is known that flunarizine exhibits both organ and regional specificity; the NE-induced contraction of the rat mesenteric artery is more sensitive than that of rat aorta to the inhibitory action of flunarizine. In rabbit mesenteric artery, the degree of reduction of NE-induced contractions by this agent was inversely related to the outer diameter of the vessel. Godfraind and Miller suggested that differences in vessel sensitivity to the action of flunarizine could be accounted for by differences in the affinity of the Ca²⁺-channels for this agent or by different receptor-response coupling characteristics. Such factors may help to explain why flunarizine acts differently on NE-induced contractions in these tissues.

In the present experiments, we did not examine whether flunarizine inhibited the binding of NE to the α₁-adrenoceptor. However, Nagao et al. showed that flunarizine (1 μM) modified neither the resting membrane potential nor the depolarization produced by activation of the α₁-adrenoceptor but did inhibit NE-induced contractions. It thus seems likely that flunarizine inhibits such contractions through a mechanism other than inhibition of NE binding to the α₁-adrenoceptor.

In saponin-skinned muscle strips, InsP₃ releases Ca²⁺ and produces a contraction due to the release of Ca²⁺ from intracellular stores. In the present experiments, NE rapidly reduced the amount of PI-3-P and generated InsP₃. Flunarizine modified neither InsP₃-induced nor caffeine-induced contractions. However, flunarizine did reduce the hydrolysis of PI-3-P, evoked by NE in a dose-dependent manner. Thus, it may be concluded that flunarizine inhibits the hydrolysis of PI-3-P, evoked by NE, and as a result, it reduces the amount of generated InsP₃, and inhibits NE-induced mechanical responses in Ca²⁺-free solution.

NE-induced contractions in Ca²⁺-free solution are also inhibited by some Ca²⁺ antagonists in arterial tissue. Saida and van Breemen found that diltiazem (1–100 μM) inhibits the NE-induced contraction in Ca²⁺-free solution. Further, Nishimura et al. reported that the specific binding of H-prazosin was inhibited by verapamil and diltiazem in membrane preparations from porcine aorta. These results suggest that these compounds, like flunarizine, also inhibit both the NE-induced Ca²⁺ influx and Ca²⁺ release from the stores. However, the concentrations of these drugs required to inhibit NE-induced Ca²⁺ release from intracellular stores or the binding of H-prazosin are higher than those necessary for inhibition of voltage-dependent Ca²⁺ influx. Therefore, the vasodilator features of these compounds seem to be different from those of flunarizine. In support of this hypothesis, Van Nueten et al. and Granger et al. have pointed out marked pharmacologic differences between flunarizine and nifedipine or verapamil. Moreover, Spedding suggested that the term "Ca²⁺-antagonist" may encompass at least two classes of drug. Thus, on the basis of the present results and those of previous workers, flunarizine must be differentiated from verapamil, diltiazem, and the dihydropyridines.

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