Mechanisms of Vasodilation Induced by NKH477, a Water-Soluble Forskolin Derivative, in Smooth Muscle of the Porcine Coronary Artery

Jonaid Shafiq, Satoshi Suzuki, Takeo Itoh, and Hiroshi Kuriyama

To study the mechanism of vasodilation induced by 6-(3-dimethylaminopropionyl) forskolin (NKH477), a water-soluble forskolin derivative, its effects on the acetylcholine (ACh)–induced contraction of muscle strips of porcine coronary artery were examined. [Ca\(^{2+}\)]\(_i\), isometric force, and cellular concentrations of cAMP and inositol 1,4,5-trisphosphate were measured. NKH477 (0.1–1.0 \(\mu\)M), isoproterenol (0.01–0.1 \(\mu\)M), or forskolin (0.1–1.0 \(\mu\)M) increased cAMP and attenuated the contraction induced by 128 mM K\(^+\) or 10 \(\mu\)M ACh in a concentration-dependent manner. These agents, at concentrations up to 0.3 \(\mu\)M, did not change the amount of cGMP. NKH477 (0.1 \(\mu\)M) attenuated the contraction induced by 128 mM K\(^+\) without corresponding changes in the evoked [Ca\(^{2+}\)]\(_i\) responses. ACh (10 \(\mu\)M) produced a large phasic increase followed by a small tonic increase in [Ca\(^{2+}\)]\(_i\), and produced a sustained contraction. The ACh-induced phasic increase in [Ca\(^{2+}\)]\(_i\) but not the tonic increase, disappeared after application of 0.1 \(\mu\)M ionomycin. NKH477 (0.1 \(\mu\)M) attenuated both the increase in [Ca\(^{2+}\)]\(_i\), and the force induced by 10 \(\mu\)M ACh in muscle strips that were not treated with ionomycin and inhibited the ACh-induced contraction without corresponding changes in [Ca\(^{2+}\)]\(_i\) in ionomycin-treated muscle strips. These results suggest that NKH477 inhibits ACh-induced Ca\(^{2+}\) mobilization through its action on ionomycin-sensitive storage sites. In ionomycin-treated and 128 mM K\(^+\)–treated muscle strips, 0.1 \(\mu\)M NKH477 shifted the [Ca\(^{2+}\)]\(_i\)–force relation to the right in the presence or absence of 10 \(\mu\)M ACh. In 6-yl-skinned smooth muscle strips, 0.1 \(\mu\)M NKH477 shifted the pCa–force relation to the right but had no effects on Ca\(^{2+}\)-independent contraction. We conclude that in smooth muscle of porcine coronary artery, NKH477 inhibits ACh-induced contraction by both attenuating ACh-induced Ca\(^{2+}\) mobilization and reducing the sensitivity of the contractile machinery to Ca\(^{2+}\), possibly by activating cAMP-dependent mechanisms. (Circulation Research 1992;71:70–81)

**KEY WORDS** • cAMP • forskolin derivative • vascular smooth muscle • cellular Ca\(^{2+}\) concentration • contractile proteins

Isoproterenol inhibits agonist-stimulated smooth muscle contraction by increasing the intracellular concentration of cAMP through its action on \(\beta\)-adrenoceptors.\(^1\) Although forskolin directly activates adenylate cyclase and has been used to study the roles of cAMP in cellular functions in various cell types,\(^2\) another direct activator of adenylate cyclase, \(N,N\)-dimethyl-\(\beta\)-alanine[3R-(3\(\alpha\),4\(\alpha\),5\(\beta\),6\(\beta\),6\(\alpha\),10\(\alpha\),10\(\alpha\),10\(\beta\),10\(\beta\)]-5(acetyloxy)-3-ethenylodecahydro-10,10b-dihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-1H-naphthol[2,1-b]pyran-6-yl ester hydrochloride (NKH477), has been recently introduced.\(^3\) Unlike forskolin, this agent is relatively soluble in water and is potentially useful for long-term intravenous infusion in patients with congestive heart failure, especially when these patients are relatively insensitive to \(\beta\)-adrenoceptor stimulants or to inhibitors of phosphodiesterase.

Two hypotheses have been proposed to explain the mechanisms of vasodilation induced by agents that increase cAMP. One is that cAMP decreases [Ca\(^{2+}\)]\(_i\) and then produces vasodilation. This action is caused by the inhibition of Ca\(^{2+}\) influx,\(^4\)–6 by the activation of Ca\(^{2+}\) sequestration,\(^7\)–10 or by the activation of Ca\(^{2+}\) extrusion to the extracellular space.\(^11\) cAMP also inhibits agonist-induced increases in [Ca\(^{2+}\)]\(_i\), by inhibiting the synthesis of inositol 1,4,5-trisphosphate (IP\(_3\))\(^12\)–14 or by the phosphorylation of the IP\(_3\) receptor.\(^15\)\(^16\) The second hypothesis is that cAMP decreases the affinity of myosin light chain kinase for the Ca\(^{2+}\)-calmodulin complex through the phosphorylation of myosin light chain kinase and thus inhibits phosphorylation of myosin light chains, causing inhibition of agonist-induced contraction.\(^17\)\(^18\) It was reported that in ferret aorta, [Ca\(^{2+}\)]–force and [Ca\(^{2+}\)]–myosin phosphorylation curves (but not the phosphorylation–force curve) were shifted to the right by forskolin in the presence of high K\(^+\).\(^19\) This may support the above-mentioned hypothesis. However, it
was recently found that stimulation of cAMP formation results in relaxation, with different effects obtained with agonist-induced contractions versus high K+-induced contractions. Kamm and Stull also suggested that the phosphorylation of myosin light chain kinase by cAMP-dependent protein kinase would play only a minor role in the vasodilation induced by agents that increase cAMP under physiological conditions. Thus, the mechanisms of vasodilation induced by agents that increase cAMP have not yet been determined.

To explore these possibilities, we investigated the effects of N KH477 on the relation between \( [\text{Ca}^{2+}]_i \) and force and during agonist-induced stimulation of endothelium-denuded strips of porcine coronary artery smooth muscle. The relation between \( [\text{Ca}^{2+}]_i \), and force was also studied in depolarized smooth muscle strips in which \( \text{Ca}^{2+} \) storage sites were functionally removed with ionomycin. The effects of N KH477 on contractile proteins were studied by examining the \( \text{Ca}^{2+} \)-induced contraction of \( \beta \)-escin–skinned smooth muscle of the porcine coronary artery.

\[ \text{Materials and Methods} \]

\[ \text{Drugs} \]

Drugs used were fura-2, fura-2 AM, EGTA, PIPES, HEPES, and MOPS (Dojin, Japan); GTP, calmodulin, and \( \beta \)-escin (Sigma Chemical Co., St. Louis, Mo.); isoproterenol (Nacalai, Japan); ATP (sodium salt, Kojin, Japan); ionomycin (Calbiochem Corp., La Jolla, Calif.); adenosine 5'-O-(3-thiotriphosphate) (ATP\( \gamma \)S, Boehringer Mannheim-Yamanouchi, Japan); acetylcholine HCl (Daichi, Japan); and N KH477 (Nippon Kayaku Ltd., Japan). Forskolin was kindly provided by Nippon Kayaku.

\[ \text{Materials} \]

The left anterior descending branch of the coronary artery was excised at room temperature from fresh porcine hearts. Arteries in Krebs' solution were rinsed free of blood, and connective tissue was removed under a binocular microscope.

\[ \text{Recording of Mechanical Activity} \]

Circularly cut strips (length, 0.3–0.5 mm; width, 0.07 mm; thickness, 0.03–0.05 mm) of the porcine coronary artery were prepared, and endothelial cells were removed by gentle rubbing of the intimal surface with a small razor blade, as reported previously. Strips were then transferred to a small 0.5-ml chamber through which test solutions could be changed rapidly by injecting fresh solution at one end and simultaneously draining the other end with a water pump. Both ends of the preparation were fixed between pieces of Scotch double-sided tape with thin silk threads, and isometric force was recorded using a strain gauge transducer (U-gauge, Shinko Co., Tokyo). Resting force was adjusted to obtain a maximal contraction in 128 mM K+.

In the present experiments, removal of endothelium was not confirmed histologically; instead, functional removal of the endothelium in the strips was shown by the effect of 0.1 \( \mu \)M calcium ionophore A23187 on the tonic contraction induced by 0.3 \( \mu \)M acetylcholine (ACH), as described previously. All experiments were performed at room temperature (23–25°C).

\[ \text{Simultaneous Recording of Ca}^{2+} \text{ and Force} \]

To permit the simultaneous recording of \( [\text{Ca}^{2+}]_i \), and isometric force, fine circularly cut strips (length, 0.3–0.5 mm; width, 0.04–0.05 mm; thickness, 0.02–0.03 mm) were prepared. Endothelial cells were removed, as described above. The strips were transferred to a chamber of 0.3-ml volume and mounted horizontally on an inverted microscope (Diaphot TMD with special optics for epifluorescence, Nikon, Tokyo). Resting force was adjusted to obtain a maximal contraction in 128 mM K+.

To load fura-2 into smooth muscle cells of the strip, 1 \( \mu \)M acetoxyethyl ester of fura-2 (fura-2 AM) dissolved in dry dimethyl sulfoxide (1 mM stock solution) was applied for 1 hour in Krebs' solution at room temperature (23–25°C). The strip was moved to the center of the field, and a mask (0.04-mm square) was placed in an intermediate image plane of the microscope to reduce background fluorescence. The fura-2 fluorescence emission at 510 nm (passed through an interference filter centered at 510 nm with a full width at half transmission of 20 nm) was passed through the objective lens (×20 Fluo, Nikon) and collected with a photomultiplier tube (side-on type, model R928, Hamamatsu Photonics, Japan) via a dichroic mirror (model DM-400, Nikon), which was substituted for the photochamber in a Nikon Diaphot TMD microscope. Two alternative excitation wavelengths, 340 and 380 nm (each slit, 5 nm), were applied by a spectrofluorimeter (SPEX Industries, Inc., Edison, N.J.), and the data were analyzed using customized software (DM-3000CM, SPEX).

The ratio of the fura-2 fluorescence intensities excited at 340 and 380 nm was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) excited at 340 and 380 nm UV light was measured after application of a solution containing 50 \( \mu \)M ionomycin, 20 mM MnCl\( \text{2} \), 110 mM KCl, and 10 mM MOPS (pH 4.8) after the experiment. Mn\( \text{2}^{+} \) quenches the fura-2 fluorescence but does not affect the autofluorescence of the cells, the fluorescence of the acetoxyethyl ester, or the partial hydrolyzed products of fura-2 AM. Under these conditions, the background fluorescence intensity was 10–15% of the fura-2 signals in smooth muscle strips at either excitation wavelength. Cytosolic Ca\( \text{2}^{+} \) concentrations were calculated using the formula described by Grynkiewicz et al and in vitro calibration. The ratio of maximum fluorescence (F\( \text{max} \)) to minimum fluorescence (F\( \text{min} \)) was determined in the calibration solution after subtraction of background excited at either 340 or 380 nm, and the 380-nm signals of fura-2 were assumed to decrease by 15% in the cell because of the possible intracellular viscosity effects of the dye. The K\( \text{d} \) value for fura-2 was estimated to be 200 nM. To compare the effects of drugs in intact and skinned smooth muscle strips and to prevent the extrusion of fura-2, the present experiments were done at room temperature.

We also tried, unsuccessfully, to estimate \( [\text{Ca}^{2+}]_i \) using an internal calibration method in some strips. Briefly, the minimum fluorescence ratio (340/380) of the strip was obtained by application of Ca\( \text{2}^{+} \)-free solution containing 10 mM EGTA with 100 mM K+ and 10 mM MOPS at pH 8.5 followed by application of 50 \( \mu \)M ionomycin. Then, the solution containing 10 mM Ca\( \text{2}^{+} \)


with 100 mM K+ and 10 mM MOPS (pH 8.5) was applied to get the maximum fluorescence ratio of the strip. Fmax and Fmin were calculated after the subtraction of background fluorescence of the strip excited at either 340 or 380 nm. However, since the ratio of the Fmax was not constantly bigger than that of 128 mM K+ in the Krebs’ solution even in very thin strips, we could not use this method in the present experiment.

It has been suggested that, when fura-2 AM is used for loading fura-2 into the cells, problems in [Ca2+], measurement may arise because of a possible amount of fura-2 AM remaining in the cells, partially hydrolyzed fluorescent intermediates of fura-2 AM, a formation of bleach intermediates of fura-2, and the possible loading of fura-2 into the cellular organelle.29 More problems may arise in the tissue experiments than in the single-cell experiments. To avoid these possibilities as much as we can, we used a low concentration of fura-2 AM (1 μM), and the loading was done within 1 hour at room temperature using very small muscle strips, since a high concentration of fura-2 AM at a high temperature (32–37°C) would cause the heterogeneous distribution of fura-2 in some cell types.29 The time for application of the ultraviolet light was minimized (normally within 20–30 minutes for the total exposed time) since this may cause photobleached fluorescent derivatives of fura-2, as suggested previously.30 In the preliminary experiments we found that, before and after application of 1 μM fura-2 AM for 1 hour, 10 μM ACh could produce almost the same extent of contraction in the presence or absence of Ca2+, suggesting that fura-2 in the cells may not greatly influence this agonist-induced Ca2+ mobilization under the present conditions. Further, to get enough deesterification of fura-2 AM, we did some experiments using the muscle strips that were kept over 2 hours in the dark after removal of fura-2 AM. The effects of drugs used in the present experiments on [Ca2+]i, and force were identical compared with those observed in the strips kept for 1 hour after removal of fura-2 AM. However, we cannot deny the possibility that the calculated Ca2+ may not be accurate by the reasons described above.

**Experiment on Chemically Skinned Smooth Muscle Strips**

Chemically skinned smooth muscle strips were made using β-escin.32 The methods used to skin the muscle strips and the compositions of the solutions used have been described elsewhere.32–33 Ionomycin (1 μM) was present throughout the experiment to deplete intracellular Ca2+ stores.

**Assays for cAMP and cGMP**

Muscle strips of the porcine coronary artery were prepared as described above. NKH477, forskolin, or isoproterenol was applied for 3 minutes after the strips were equilibrated for 2 hours, and the reaction was halted by soaking the strips in ice-cold 8% trichloroacetic acid. The strips were then homogenized in the solution containing 8% trichloroacetic acid using a glass homogenizer. The homogenate was centrifuged, and the pellet was used for protein assay.34 The supernatant fraction was treated with water-saturated ether three times and assayed for cAMP and cGMP using radioimmunoassay kits from Amersham International.

**Measurement of IP3**

Endothelium-denuded strips of the porcine coronary artery were equilibrated for over 2 hours at 32°C in Krebs’ solution. After this, the strips were transferred to Ca2+-free Krebs’ solution containing 2 mM EGTA for 2 minutes, and then 10 μM ACh was applied for 10 seconds. NKH477, isoproterenol, or forskolin was given during a 3-minute pretreatment in Krebs’ solution, during a 2-minute pretreatment in Ca2+-free solution, and during the application of ACh. The reaction was stopped by the addition of a large amount of ice-cold 8% trichloroacetic acid, and the strips were homogenized. The homogenate was centrifuged, and the supernatant fraction was treated with water-saturated ether three times and assayed for IP3 using a radioimmunoassay kit from Amersham International. Care was taken to maintain the pH of the homogenate at 9.0–9.5 to optimize the binding of the binding protein to IP3. To minimize the loss of IP3, Teflon tubes were used instead of glassware after homogenization. The pellet was used for protein assay.34

**Solutions**

The ionic composition of the Krebs’ solution was as follows (mM): Na+ 137.4, K+ 5.9, Mg2+ 1.32, Ca2+ 2.6, HCO3− 15.5, H2PO4− 1.2, Cl− 134, and glucose 11.5. The concentration of K+ was modified by isosmotically replacing NaCl with KCl. Ca2+-free solution was made by substituting an equimolar concentration of MgCl2 for CaCl2 and adding 2 mM EGTA. The solutions were bubbled with 95% O2/5% CO2, and their pH was maintained at 7.3–7.4.

The calibration solution for the Ca2+ measurements in intact strips contained 11 mM EGTA, 110 mM KCl, 1 mM MgCl2, 2 μM fura-2, and 20 mM HEPES (pH 7.1) with or without 11 mM CaCl2.

For experiments on skinned muscle, the composition of the relaxing solution was 87 mM potassium methanesulfonate (KMs), 20 mM PIPES, 5.1 mM Mg(MS)2, 5.2 mM ATP, 10 mM phosphocreatine, 4 mM EGTA, and 1 μM ionomycin. Various Ca2+ concentrations were obtained by adding appropriate amounts of Ca(MS)2 to 4 mM EGTA, based on the calculation reported previously.30 The pH of the solution was adjusted to 7.1 at 25°C with KOH, and the ionic strength was standardized at 0.2 M by changing the amount of KMs added.

To get a Ca2+-independent contraction in the skinned strips, a solution containing ATPγS and 0.3 μM Ca2+ or a rigor-producing solution was used. The ATPγS solution was composed of (mM) HEPES 20, Mg(MS)2 2.6, ATPγS 2, and EGTA 4. The ionic strength of the solution was adjusted to 0.2 M by adding KMs. The free Ca2+ concentration was calculated to be 0.3 μM. The composition of the rigor-producing solution was (mM) KMs 130, Mg(MS)2 1, EGTA 4, and HEPES 2, and the pH was adjusted to 7.1 using KOH.

**Statistics**

The values measured were expressed as mean±SD. Differences between means within each experiment were evaluated by analysis of variance. If significant differences were demonstrated, Student’s paired or unpaired t test was used to determine which pairs of
FIGURE 1. Graph showing the concentration-dependent effects of isoproterenol (●), forskolin (□), and NKH477 (○) on the production of cAMP in smooth muscle strips of the porcine coronary artery. Each symbol represents the mean (n=5–10), with the SD shown by vertical bars. **Statistically significant difference (p<0.01) from the value obtained in the absence of isoproterenol, forskolin, or NKH477 (9.1±5.0 pmol/mg protein, n=10).

means were significantly different. Values of p<0.05 were considered significant.

Results

Effects of Isoproterenol, Forskolin, and NKH477 on Cellular Concentration of cAMP

Figure 1 shows the effects of isoproterenol, forskolin, and NKH477 on the amount of cAMP in smooth muscle strips of the porcine coronary artery. The amount of cAMP observed in the resting condition was 9.1±5.0 pmol/mg protein (n=10). Isoproterenol (0.01–1 μM), NKH477 (0.1–1 μM), and forskolin (0.1–1 μM) increased the amount of cAMP in a concentration-dependent manner (Figure 1). The maximum effect obtained by 1 μM NKH477 or 1 μM forskolin was significantly larger than that induced by 0.1 μM isoproterenol (p<0.05, n=8). The effect of 0.3 μM NKH477 was larger than that of 0.3 μM forskolin (p<0.01, n=8). None of the drugs up to 0.3 μM increased the cellular concentration of cGMP. The cellular concentrations of cGMP were 0.08±0.04 pmol/mg protein in the control condition, 0.08±0.05 pmol/mg protein in the presence of 0.3 μM isoproterenol, 0.09±0.03 pmol/mg protein in the presence of 0.3 μM forskolin, and 0.11±0.05 pmol/mg protein in the presence of 0.3 μM NKH477 (p>0.05, n=5–10).

Effects of Isoproterenol, Forskolin, and NKH477 on the Contraction Induced by 128 mM K⁺ or 10 μM ACh

Figure 2 shows the effects of isoproterenol, forskolin, and NKH477 on the contraction induced by 128 mM K⁺ (panel A) or 10 μM ACh (panel B) in smooth muscle strips of the porcine coronary artery. High K⁺ (128 mM) produced a phasic and a subsequent tonic contraction in the muscle strips, and the latter response was maintained over 1 hour. ACh (10 μM) produced a monotonc contraction that was maintained over 30 minutes. The maximum amplitude of contraction induced by 10 μM ACh was 1.8±0.3 times that induced by 128 mM K⁺ (n=8). Isoproterenol, forskolin, or NKH477 was used to pretreat the strips for 5 minutes and was present throughout the application of 128 mM K⁺ or 10 μM ACh. In the presence of 128 mM K⁺, all drugs inhibited both the phasic and subsequent tonic contractions, but the phasic contractions were less sensitive than the tonic contractions (see Figure 3 for NKH477). Since 10 μM ACh slowly developed the force (=2 minutes to reach steady-state force), the effects of isoproterenol, forskolin, and NKH477 were observed at 2 minutes after the application of 128 mM K⁺ or 10 μM ACh. Isoproterenol (0.01–0.1 μM), forskolin (0.01–1 μM), and NKH477 (0.01–1 μM) inhibited the contraction induced by 128 mM K⁺ or 10 μM ACh in a concentration-dependent manner. The concentrations that produced half relaxation (IC₅₀) of the high K⁺-induced contraction were 0.05±0.02 μM for isoproterenol, 0.15±0.10 μM for forskolin, and 0.08±0.03 μM for NKH477 (n=4). IC₅₀ values for the ACh-induced contraction were 0.05±0.02 μM for isoproterenol, 0.17±0.07 μM for forskolin, and 0.07±0.03 μM for NKH477 (n=4–7). The concentrations of isoproterenol, forskolin, and NKH477 necessary to inhibit the contraction induced by 128 mM K⁺ or 10 μM ACh seemed to be well correlated with their effects on cAMP production. The order of potency of the above three cAMP-increasing agents for inhibition of the contraction induced by high K⁺ or ACh was isoproterenol>NKH477>forskolin. These results suggest that NKH477, a forskolin derivative, is a more potent cAMP-increasing vasodilator than forskolin in smooth muscle strips of the porcine coronary artery.
Effects of NKH477 on Changes in $\left[Ca^{2+}\right]_{i}$ and Force Induced by 128 mM K$^+$ or 10 μM ACh

Figure 3 shows the effects of NKH477 on changes in $\left[Ca^{2+}\right]_{i}$ and in force induced by 128 mM K$^+$ or 10 μM ACh in a thin smooth muscle strip of the porcine coronary artery. High K$^+$ (128 mM) was first applied for 2 minutes and then rinsed for 3 minutes in Krebs' solution. ACh (10 μM) was then applied for 2 minutes to the same strips. The resting $\left[Ca^{2+}\right]_{i}$ and force in the muscle strips were 105.4±10.9 nM and 1.3±0.5 mg respectively (n=5). High K$^+$ (128 mM) produced a phasic and a subsequent tonic increase in $\left[Ca^{2+}\right]_{i}$, and in force. The maximum increase in $\left[Ca^{2+}\right]_{i}$ and force were 784.2±112.5 nM and 12.3±2.5 mg, respectively (n=4). ACh (10 μM) produced a large phasic increase followed by a small tonic increase in $\left[Ca^{2+}\right]_{i}$, and a sustained contraction. The ACh-induced phasic and the subsequently generated tonic (measured at 2 minutes after the application) increases in $\left[Ca^{2+}\right]_{i}$ were 600.8±71.2 and 143.0±13.8 nM, respectively (n=4). The maximum force induced by 10 μM ACh was larger than that induced by 128 mM K$^+$, although the ACh-induced tonic increase in $\left[Ca^{2+}\right]_{i}$, was much smaller than that induced by high K$^+$.

When 0.1 μM NKH477 was applied for 5 minutes before and during the application of 128 mM K$^+$ or 10 μM ACh, the resting $\left[Ca^{2+}\right]_{i}$, and force were slightly reduced. NKH477 (0.1 μM) inhibited the amplitude of contraction induced by 10 μM ACh and decreased both the phasic and the tonic increases in $\left[Ca^{2+}\right]_{i}$. By contrast, 0.1 μM NKH477 inhibited both the phasic and the subsequently generated tonic contractions induced by 128 mM K$^+$, with a slight increase in $\left[Ca^{2+}\right]_{i}$, (Figure 3 and Table 1).

Effects of NKH477 on Changes in $\left[Ca^{2+}\right]_{i}$ and Force Induced by 128 mM K$^+$ or 10 μM ACh in Ionomycin-Treated Muscle Strips

cAMP increases Ca$^{2+}$ uptake into intracellular storage sites in smooth muscle of guinea pig and rabbit mesenteric arteries.7,8 To eliminate the effects of NKH477 on intracellular Ca$^{2+}$ storage sites, 0.1 μM ionomycin was applied 60 minutes before and during the application of 128 mM K$^+$ or 10 μM ACh.

After application of 0.1 μM ionomycin, resting $\left[Ca^{2+}\right]_{i}$ slowly increased from 99.7±31.8 to 324.9±184.6 nM within 1 minute and then decayed to almost the steady $\left[Ca^{2+}\right]_{i}$ (level (159.4±63.8 nM) within 60 minutes. When 128 mM K$^+$ was applied 60 minutes after the application of 0.1 μM ionomycin, the phasic increase in $\left[Ca^{2+}\right]_{i}$, was slightly smaller, but the tonic increases in $\left[Ca^{2+}\right]_{i}$, and in force were slightly larger than those observed before the application of 0.1 μM ionomycin (Figure 4, Tables 1 and 2). In contrast, in the ionomycin-treated strips the phasic increase in $\left[Ca^{2+}\right]_{i}$, induced by 10 μM ACh completely disappeared, but the small tonic increase in $\left[Ca^{2+}\right]_{i}$, was not changed. The rate of decline of $\left[Ca^{2+}\right]_{i}$, after washout of 128 mM K$^+$ or 10 μM ACh by Krebs' solution was greatly reduced in ionomycin-treated strips. The rate of rise of force and the maximum force

### Table 1. Effects of 0.1 μM NKH477 on $\left[Ca^{2+}\right]_{i}$ and Force Induced by 128 mM K$^+$ or 10 μM Acetylcholine in Smooth Muscle Strips of the Porcine Coronary Artery

<table>
<thead>
<tr>
<th></th>
<th>Resting $\left[Ca^{2+}\right]_{i}$, (nM)</th>
<th>128 mM K$^+$</th>
<th>10 μM acetylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\left[Ca^{2+}\right]_{i}$, (nM)</td>
<td>Force</td>
<td>$\left[Ca^{2+}\right]_{i}$, (nM)</td>
</tr>
<tr>
<td>Control</td>
<td>105.4±10.9</td>
<td>784.2±112.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1 μM NKH477</td>
<td>94.1±14.6</td>
<td>850.8±741.1</td>
<td>0.61±0.07*</td>
</tr>
</tbody>
</table>

Values are mean±SD, n=4.
The maximum force induced by 128 mM K$^+$ was normalized as relative force of 1.0 in each strip.

*Significant difference (p<0.05) from the value obtained in the absence of 0.1 μM NKH477 when analyzed by a paired t test.
induced by 10 μM ACh was smaller in ionomycin-treated muscle strips than in strips observed before the application of ionomycin (Tables 1 and 2). NKH477 (0.1 μM) attenuated the contraction induced by 128 mM K⁺ or 10 μM ACh without corresponding changes in [Ca²⁺]. (Figure 4, Table 2).

Effects of NKH477 on [Ca²⁺] – Force Relations in Ionomycin-Treated Smooth Muscle Strips During Membrane Depolarization by 128 mM K⁺

To investigate the effects of NKH477 on [Ca²⁺] force relations, Ca²⁺-free solution containing 5.9 mM K⁺ and 2 mM EGTA was first applied for 1 minute to ionomycin-treated smooth muscle strips, and then Ca²⁺-free solution containing 128 mM K⁺ and 2 mM EGTA was applied. At this point, various concentrations of Ca²⁺ (0.16–2.6 mM) were cumulatively applied from low to high in the presence of 128 mM K⁺. After application of Ca²⁺-free solution with 5.9 mM K⁺ for 1 minute, the resting [Ca²⁺] and force slightly decreased to 97.6±33.2 nM and to 1.0±1.2 mg, respectively. Subsequently applied Ca²⁺-free solution with 128 mM K⁺ did not increase [Ca²⁺], and force. Figure 5 shows the effects of 0.1 μM NKH477 on the [Ca²⁺]–force relations at various concentrations of Ca²⁺ in solutions containing 128 mM K⁺ in ionomycin-treated muscle strips. NKH477 (0.1 μM) inhibited the contraction induced by all concentrations of Ca²⁺ without corresponding changes in the evoked [Ca²⁺].

Figure 6 shows the effects of 0.1 μM NKH477 on the [Ca²⁺] force relation in an ionomycin-treated muscle strip at various concentrations of Ca²⁺ in solutions containing 128 mM K⁺ and 10 μM ACh. ACh (10 μM) slightly enhanced the increase in [Ca²⁺], induced by Ca²⁺ (0.16–2.6 mM), especially at the lower concentrations of Ca²⁺. ACh (10 μM) shifted the [Ca²⁺] force relation to the left and increased the maximum amplitude of contraction induced by 2.6 mM Ca²⁺ (2.1±0.5 times the control, n=3). NKH477 (0.1 μM) shifted the [Ca²⁺] force relation to the right without effecting the maximum force induced by 2.6 mM Ca²⁺ in the presence of 128 mM K⁺ and 10 μM ACh.

Effects of NKH477 on Ca²⁺–Force Relations in β-Escin–Treated Skinned Smooth Muscle Strips

To investigate the action of 0.1 μM NKH477 on the contractile proteins more directly, the effects on Ca²⁺-induced contraction were observed in the presence of 1 μM ionomycin in β-escin–treated skinned smooth muscle strips. In the skinned strips, the minimum concentration of Ca²⁺ that produced the contraction was 0.3 μM, and the maximum contraction was obtained at 10

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**TABLE 2. Effects of 0.1 μM NKH477 on Tonic Increase in [Ca²⁺] and Force Induced by 128 mM K⁺ or 10 μM Acetylcholine in Ionomycin-Treated Smooth Muscle Strips of the Porcine Coronary Artery**

<table>
<thead>
<tr>
<th></th>
<th>Resting [Ca²⁺] (nM)</th>
<th>128 mM K⁺</th>
<th>10 μM ACh</th>
<th>Force</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Ca²⁺] (nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>159.4±63.8</td>
<td>320.8±15.9</td>
<td>0.83±0.29</td>
<td>193.1±38.1</td>
</tr>
<tr>
<td>0.1 μM NKH477</td>
<td>163.0±71.0</td>
<td>324.6±18.6</td>
<td>0.33±0.21*</td>
<td>197.8±46.1</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=4. Strips were pretreated with 0.1 μM ionomycin for 60 minutes before the application of 128 mM K⁺ or 10 μM acetylcholine. The maximum force induced by 128 mM K⁺ or 10 μM acetylcholine was expressed as relative force. The tonic force induced by 128 mM K⁺ before application of 0.1 μM ionomycin was normalized as relative force of 1.0 in each strip.

*Significant difference (p<0.05) from the value obtained in the absence of 0.1 μM NKH477 when analyzed by a paired t test.
μM Ca²⁺. Figure 7 shows the effects of 0.1 μM NKH477 on contraction induced by various concentrations of Ca²⁺ (0.1–10 μM) in the skinned strips. NKH477 (0.1 μM) shifted the pCa–force relation curve to the right and inhibited the maximum amplitude of contraction induced by 10 μM Ca²⁺. The concentration of Ca²⁺ required for half-maximum force (EC₅₀) was 0.8±0.1 μM in the control condition and 1.8±0.4 μM in the presence of 0.1 μM NKH477 (p<0.05, n=4).

Calmodulin shifts the pCa–force relation to the left in saponin-treated skinned smooth muscle of rabbit mesenteric artery. To study the effects of calmodulin on the inhibitory actions of NKH477 on Ca²⁺-induced contraction in β-escin–treated skinned smooth muscle of porcine coronary artery, the effects of 0.1 μM NKH477 on the contraction induced by various concentrations of Ca²⁺ (0.1–10 μM) were observed in the presence of 2 μM calmodulin. Calmodulin (2 μM) shifted the pCa–force relation to the left (EC₅₀=0.6±0.1 μM Ca²⁺, n=4) but had no effect on the contraction induced by 10 μM Ca²⁺ (1.06±0.04 times the control condition, n=4). In the presence of 2 μM calmodulin, 0.1 μM NKH477 shifted the relation to the right (EC₅₀=0.9±0.1 μM Ca²⁺, p<0.05, n=4) and inhibited the maximum contraction induced by 10 μM Ca²⁺. The extent to which the curve was shifted and the extent of inhibition of the maximum Ca²⁺-induced force in the presence of 2 μM calmodulin were almost the same as those observed in the absence of calmodulin (Figure 7).

To further investigate the mechanism of action of NKH477 on the contractile proteins, the effect of this drug on Ca²⁺-independent contraction was observed. The experimental procedure is indicated in Figure 8. After maximum contraction was induced by 10 μM Ca²⁺, the strip was rinsed with relaxing solution until it completely relaxed. After the application of rigor solution (Ca²⁺-free solution containing 4 mM EGTA and no MgATP) for 2 minutes, only a small contraction was evoked ("rigor contraction"). When 2 mM ATP+3% with 0.3 μM Ca²⁺ was applied for 10 minutes, force developed slowly. Subsequent application of the rigor solution halted the contraction at the level reached at the termination of the application of 2 mM ATP+3% with 0.3 μM Ca²⁺. Then, when a Ca²⁺-free solution containing 4 mM EGTA and 4 mM MgATP was applied, additional force rapidly developed, and the contraction was sustained at the same amplitude for over 30 minutes ("Ca²⁺-independent contraction"). The amplitude of this Ca²⁺-independent contraction was almost the same as that induced by 10 μM Ca²⁺. NKH477 (0.1 μM) did not modify the Ca²⁺-independent contraction (Figure 8A).

The effect of NKH477 on the rigor contraction was also observed. After the application of 10 μM Ca²⁺ to the strips, the solution was rinsed off using a Ca²⁺-free solution with no MgATP; thus, a rigor contraction was produced, as reported previously.33 NKH477 (0.1 μM) had no effect on this rigor contraction (Figure 8B), suggesting that NKH477 itself did not inhibit actin–myosin interactions directly.

ACh with GTP enhances the Ca²⁺-induced contraction in α-toxin–treated, chemically skinned smooth muscle strips.32,35,36 To investigate the mechanism of action of NKH477 on the Ca²⁺-induced contraction further, its effect on the contraction induced by Ca²⁺ (0.1–10 μM) was observed in the presence of ACh with GTP. ACh (10 μM) with GTP (3 μM) enhanced the contraction induced by any concentration of Ca²⁺ (0.1–10 μM) compared with the control condition. NKH477 (0.1 μM) shifted the relation to the right and inhibited the maximum contraction induced by 10 μM Ca²⁺ in the presence of 10 μM ACh with 3 μM GTP (Figure 9).

Effects of NKH477 on ACh-Induced IP₃, Production in Smooth Muscle Strips

To investigate the mechanisms of the vasodilation induced by NKH477 on ACh-induced contraction, the effects of 0.3 μM NKH477 on the synthesis of IP₃, induced by 10 μM ACh were examined in endothelium-
removed smooth muscle strips of the porcine coronary artery. ACh (10 \( \mu \)M) was applied for 2 minutes after application of Ca\(^{2+}\)-free solution containing 2 mM EGTA and 5.9 mM K\(^+\). Under these conditions, 10 \( \mu \)M ACh transiently increased the amount of IP\(_3\) at 10 seconds. The effect gradually decayed within 60 seconds. IP\(_3\) concentrations were 4.7±1.1 pmol/mg protein (n=3) before the application of 10 \( \mu \)M ACh and 9.1±1.6 pmol/mg protein (n=8) 10 seconds after the application of 10 \( \mu \)M ACh. The effects of NKH477 on the production of IP\(_3\) induced by 10 \( \mu \)M ACh were then measured. NKH477 (0.3 \( \mu \)M) had no effect on the synthesis of IP\(_3\) induced by 10 \( \mu \)M ACh after 10 seconds (10.2±1.8 pmol/mg protein in the presence of 0.3 \( \mu \)M NKH477, n=5). Forskolin (0.3 \( \mu \)M) or isoproterenol (0.1 \( \mu \)M) also had no effect on the synthesis of IP\(_3\) activated by 10 \( \mu \)M ACh (8.8±1.4 pmol/mg protein in the presence of 0.1 \( \mu \)M isoproterenol, n=4; and 8.7±2.7 pmol/mg protein in the presence of 0.3 \( \mu \)M forskolin, n=8).

**Discussion**

NKH477, a water-soluble forskolin derivative, activates adenylyl cyclase. The efficacy of this drug is approximately two times that of forskolin in membrane preparations of the guinea pig ventricle.\(^{37}\) NKH477 up to 0.1 mM did not affect the binding of \(^3\)HJCGP12177, a \( \beta \)-adrenoceptor ligand in the same preparation.\(^{38}\)

In the present experiments, NKH477 (>0.1 \( \mu \)M) increased cAMP and inhibited the contraction induced by high K\(^+\) or ACh in muscle strips of porcine coronary artery. These actions of NKH477 were more potent than those of forskolin. Further, NKH477 up to 0.3 \( \mu \)M did not increase cGMP even though 0.3 \( \mu \)M of the drug produced maximum vasodilation in the presence of high K\(^+\) or ACh. These results suggest that NKH477 produces vasodilation through a direct activation of adenylyl cyclase and that it is more potent than forskolin in smooth muscle of the porcine coronary artery.

In the present experiments, we found that cAMP was increased more by 1 \( \mu \)M NKH477 (13.8-fold) than by 0.1 \( \mu \)M NKH477, but this drug at 0.1 \( \mu \)M maximally inhibited the high K\(^-\) - or ACh-induced contraction. Isoproterenol (0.1 \( \mu \)M) increased cAMP to a lesser extent than did 1 \( \mu \)M NKH477 but inhibited the high K\(^-\) - or ACh-induced contraction to the same extent as did 1 \( \mu \)M NKH477 (the concentrations of cAMP were 156 pmol/mg protein in the presence of 0.1 \( \mu \)M isoproterenol and 718.2 pmol/mg protein in the presence of 1 \( \mu \)M NKH477). These results suggest that in smooth muscle of the porcine coronary artery, a relatively small (=20-fold) increase in cellular cAMP can cause maximal vasodilation by cAMP-increasing agents. However, the possibility remains that NKH477 may elicit relaxation by mechanisms not involving activation of adenylyl cyclase, as reported in the case of forskolin.\(^{39,40}\)
In the porcine coronary artery, ACh produces contractions with no change in membrane potential or membrane resistance. In the present experiments, ACh produced a large phaseic increase followed by a small tonic increase in [Ca\(^{2+}\)], and generated a sustained contraction. The ACh-induced phaseic increase in [Ca\(^{2+}\)] persisted in Ca\(^{2+}\)-free solution but disappeared in ionomycin-treated muscle strips in the presence or absence of Ca\(^{2+}\). In contrast, the ACh-induced tonic increase in [Ca\(^{2+}\)] persisted in ionomycin-treated muscle strips but was blocked in Ca\(^{2+}\)-free solution. These results suggest that the ACh-induced phaseic and tonic increases in [Ca\(^{2+}\)] are probably due to the release of Ca\(^{2+}\) from intracellular storage sites and the activation of Ca\(^{2+}\) influx, respectively.

The rate of decline of [Ca\(^{2+}\)] after washout of high K\(^+\) or ACh was greatly reduced in ionomycin-treated muscle strips of the porcine coronary artery. Ionomycin or A23187, at low concentrations, selectively acts on cellular Ca\(^{2+}\) storage sites to release Ca\(^{2+}\) and, at high concentrations (>1 \(\mu\)M), acts on the plasma membrane as a Ca\(^{2+}\) carrier in vascular smooth muscle. In the present experiments, 0.1 \(\mu\)M ionomycin was used and, at this concentration, completely inhibited the ACh-induced release of [Ca\(^{2+}\)], but slightly increased steady-state [Ca\(^{2+}\)] measured several minutes after application. These results suggest that the slow decline of the [Ca\(^{2+}\)] in ionomycin-treated muscle strips may be due to the loss of function of cellular Ca\(^{2+}\) storage sites, indicating that these storage sites may have an important role in determining the rate of decline of [Ca\(^{2+}\)] in smooth muscle cells of the porcine coronary artery.

ACh activates the synthesis of IP\(_3\), through the hydrolysis of phosphatidylinositol 4,5-diphosphate, and IP\(_3\) releases Ca\(^{2+}\) from the cellular storage sites in smooth muscle of the porcine coronary artery. Thus, IP\(_3\) is a possible second messenger for agonist-induced Ca\(^{2+}\) release in various cell types, including vascular smooth muscle. It has been found that elevation of cAMP...
reduces agonist-mediated inositol phosphate formation in platelets,12 neutrophils,13 and A10 (A10, ATCC CRL 1476)-cultured vascular smooth muscle cells.65 cAMP-dependent protein kinase reduces the ability of IP3 to release stored Ca2+ through the phosphorylation of the IP3 receptor in isolated vesicles from brain synaptosomes.15 cAMP also increases Ca2+ uptake into intracellular storage sites in saponin-skinned smooth muscle of guinea pig and rabbit mesenteric arteries.7,8,47 In the present experiments, NKH477 slightly inhibited ACh-induced release of [Ca2+.]. However, NKH477, forskolin, and isoproterenol did not modify ACh-induced IP3 production in muscle strips of the porcine coronary artery. These results suggest that NKH477 inhibits ACh-induced Ca2+ release but does not inhibit ACh-induced IP3 synthesis in smooth muscle of the porcine coronary artery. Further studies are required to clarify the sites of the action of agents that increase cAMP and thereby inhibit agonist-induced Ca2+ release.

NKH477 inhibited the ACh-induced tonic increase in [Ca2+]i, but this action of NKH477 completely disappeared in ionomycin-treated muscle strips. Since intracellular Ca2+ storage sites were functionally removed in ionomycin-treated muscle strips, these results suggest that NKH477 inhibits the ACh-induced tonic increase in [Ca2+]i through the activation of Ca2+ release into the storage sites, possibly through cAMP-dependent mechanisms.

In the present experiments, 128 mM K+ produced a phasic increase followed by a tonic increase in [Ca2+]i in smooth muscle strips of the porcine coronary artery. In Ca2+-free solution containing 2 mM EGTA, the increases in [Ca2+]i induced by 128 mM K+ completely disappeared. Ionomycin slightly inhibited the high K+-induced phasic, but not tonic, increase in [Ca2+]i. These results suggest that a Ca2+-induced Ca2+ release mechanism activated by voltage-dependent Ca2+ influx may have a role on the high K+-induced phasic increase in [Ca2+]i in smooth muscle of the porcine coronary artery. In smooth muscle cells of the guinea pig taenia coli, Fukumitsu et al.48 using the whole-cell patch-clamp method, reported that forskolin increases the amplitude of Ca2+ current. NKH477 (0.1 μM) slightly increased the phasic increase in [Ca2+]i induced by 128 mM K+ in the absence of ionomycin. These results suggest that cAMP may increase voltage-sensitive Ca2+ influxes or activate the Ca2+-induced Ca2+ release mechanism in smooth muscle of the porcine coronary artery.

When [Ca2+]i was measured using aequorin in muscle strips of the porcine coronary artery, agonists induced greater force for a given [Ca2+]i, compared with high K+-induced contractions at a steady-state level,49 as reported in other arteries.20,50 In the present experiments, the maximum amplitude of contraction induced by 10 μM ACh was 1.8 times that induced by 128 mM K+, although the ACh-induced tonic increase in [Ca2+]i was almost half that induced by 128 mM K+. Thus, although the fura-2 method used in the present experiments may include some unexpected artifacts (see “Materials and Methods”), the remarkable similarities of the present results and the previous results obtained by aequorin19,20,49,50 may give credibility to the [Ca2+]i measurements used in the present experiments.

In the present experiments, ACh shifted the [Ca2+]i-force relation to the left and increased the maximum amplitude of contraction induced by 2.6 mM Ca2+ in ionomycin-treated, membrane-depolarized muscle strips. It has been reported that α-toxin– or β-escin–skinned vascular smooth muscle, agonists increase the sensitivity of the contraction to Ca2+ and also enhance the maximum contraction induced by 10 μM Ca2+ in phasic smooth muscle.51 We partly confirmed this by demonstrating that ACh with GTP increased the amplitude of contraction induced by Ca2+ (0.1–10 μM) in β-escin–skinned smooth muscle of the porcine coronary artery. These results indicate that in smooth muscle of the porcine coronary artery, ACh increases the sensitivity of the contractile proteins to Ca2+ and produces larger contractions at a relatively low [Ca2+]i.

In β-escin–treated skinned smooth muscle strips of the porcine coronary artery, 0.3 μM Ca2+ was the threshold for producing a contraction; yet, in the intact strips, when the [Ca2+]i was reduced from the resting level (159 mM) to 98 mM after removal of extracellular Ca2+, the resting force decreased. Since calmodulin (our present results) or a high concentration of GTP (>5 μM, authors’ unpublished observations) increases the amplitude of Ca2+-induced contraction, these or other cytosolic components may leak out from the skinned cells. Further, we added 10 mM phosphocreatine in the skinned solution to maximize the ATP/ADP ratio in the skinned cells. Since high concentrations of phosphocreatine inhibit the Ca2+-activated force in skinned smooth muscle strips of the rabbit mesenteric artery,52 the solution used for the skinned strips may not be adequate to compare the sensitivity of contractile proteins to Ca2+ between intact and skinned strips. These possibilities warrant further investigation.

It is believed that phosphorylation of myosin light chain by Ca2+-calmodulin–dependent myosin light chain kinase is sufficient to regulate smooth muscle contraction, although the coexistence of thin-filament–linked regulation has not been ruled out.21,52–54 Sellers and
Adelstein\textsuperscript{55} reported that, after activation of cAMP-dependent protein kinase, myosin light chain kinase from gizzard smooth muscle is phosphorylated in vitro. They found that, in the absence of Ca\textsuperscript{2+}-calmodulin, two sites (A and B) of myosin light chain kinase are phosphorylated, whereas in the presence of Ca\textsuperscript{2+}-calmodulin, only one site (B) is phosphorylated. Phosphorylation of the A site decreases the affinity of myosin light chain kinase for Ca\textsuperscript{2+}-calmodulin.\textsuperscript{21,55} We previously found that in saponin-treated smooth muscle strips of the rabbit mesenteric artery, cAMP with cAMP-dependent protein kinase shifted the pCa–force relation curve to the right.\textsuperscript{47} In the present experiments, NKH477 shifted the pCa–force relation curve to the right and inhibited the maximum contraction induced by 10 \textmu M Ca\textsuperscript{2+} in \beta-escin–treated smooth muscle strips of the porcine coronary artery. Since NKH477 did not have any effect on Ca\textsuperscript{2+}-independent contraction in \beta-escin– and ATP/PS-treated smooth muscle, the inhibitory action of NKH477 on Ca\textsuperscript{2+}-induced contraction should relate to the level of phosphorylation of the myosin light chain.

It was recently found that the phosphorylation of myosin light chain kinase in site A is provoked by carbachol or high K\textsuperscript{+}, but not by isoproterenol, in bovine tracheal smooth muscle, suggesting that an increase in [Ca\textsuperscript{2+}]; (but not in cAMP) may be responsible for the A-site phosphorylation of myosin light chain kinase.\textsuperscript{56} In crude smooth muscle homogenate from swine carotid artery, high K\textsuperscript{+} (but not histamine) stimulation inhibits the [Ca\textsuperscript{2+}]; sensitivity of myosin light chain kinase.\textsuperscript{57} In the present experiments, NKH477 shifted the pCa–force relation to the right and inhibited the maximum contraction induced by 10 \textmu M Ca\textsuperscript{2+} in the presence or absence of 2 \textmu M calmodulin. Although it is unknown whether the A site of myosin light chain kinase in the skinned muscle is phosphorylated in the presence of NKH477 under our experimental conditions, these results suggest that the phosphorylation of myosin light chain kinase by cAMP-dependent protein kinase may not be the only mechanism for cAMP-induced suppression of Ca\textsuperscript{2+}-induced contraction in \beta-escin–skinned smooth muscle of the porcine coronary artery. Other mechanisms related to the phosphorylation of myosin light chain may also be involved.

In conclusion, NKH477 is a more potent drug than forskolin for increasing the cellular cAMP in smooth muscle of the porcine coronary artery. NKH477 attenuates ACh-induced Ca\textsuperscript{2+} mobilization through its actions on the ionomycin-sensitive Ca\textsuperscript{2+} storage site. However, this agent did not modify the ACh-induced synthesis of IP\textsubscript{3}. NKH477 decreased the sensitivity of the contractile proteins to Ca\textsuperscript{2+} in the presence of high K\textsuperscript{+} or ACh. Thus, it can be suggested that, in smooth muscle of the porcine coronary artery, NKH477 attenuates ACh-induced contraction by both attenuating ACh-induced Ca\textsuperscript{2+} mobilization and reducing the sensitivity of the contractile machinery to Ca\textsuperscript{2+}, possibly by activating cAMP-dependent mechanisms.

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