RELAXANT ACTIONS OF PGE₂ AND ITS DERIVATIVE (YPG-209) ON CANINE TRACHEAL SMOOTH MUSCLE AND THEIR ENHANCEMENT OF Ca⁺⁺ UPTAKE BY THE MICROSOMAL FRACTION

Kenichi TOMIOKA, Michio TERAJ and Hiroo MAENO

Department of Pharmacology and Biochemistry, Central Research Laboratories,
Yamanouchi Pharmaceutical Co., Ltd., Itabashi-ku, Tokyo 174, Japan

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Abstract—The spasmogenic action of tetraethylammonium on canine tracheal smooth muscle was dependent on the extracellular Ca⁺⁺ concentration. Acetylcholine, on the contrary, produced partial contraction for a certain period of time after removal of extracellular Ca⁺⁺, suggesting the release of Ca⁺⁺ from intracellular Ca⁺⁺ stores into the cytosol. PGE₂, 16(S)-methyl-20-methoxy-PGE₂ (YPG-209), verapamil, and dibutyryl cyclic AMP relaxed the acetylcholine-induced contraction of the trachea. Pretreatment with prostaglandins or dibutyryl cyclic AMP more efficiently suppressed acetylcholine-induced contraction, whereas verapamil blocked the spasmogenic action of tetraethylammonium in a relatively selective manner. The prostaglandins and dibutyryl cyclic AMP also inhibited the spasmogenic action of acetylcholine in a Ca⁺⁺-free medium. Influx of ⁴⁵Ca into tracheal smooth muscle which was produced by 83 mM KCl plus 20 mM tetraethylammonium was not significantly affected by prostaglandins as measured by the lanthanum method. In addition, PGE₂ increased ATP-dependent Ca⁺⁺ uptake by the microsomes from tracheal smooth muscle. These results suggest that prostaglandins induce relaxation of the airway smooth muscle mainly through affecting the intracellular Ca⁺⁺ movement such as by enhancement of sequestration of Ca⁺⁺ to the microsomes.

It is now well-known that prostaglandin Es are able to relax airway smooth muscle (1-4) and to antagonize bronchoconstriction which is caused by several chemical mediators (3-5). Furthermore, prostaglandins can inhibit release of the chemical mediator from mast cells (6, 7). However, the cellular mechanism for relaxation of airway smooth muscle by the prostaglandins is yet poorly understood. Contraction by tetraethylammonium of the airway smooth muscle such as that of bovine trachea requires extracellular Ca⁺⁺, whereas a contractile response to

acetylcholine depends on both intracellular and extracellular Ca⁺⁺ (8). Thus, the prostaglandin-induced relaxation is presumably caused either by decreasing the membrane permeability to Ca⁺⁺, by reducing the cytosolic release of intracellularly sequestered Ca⁺⁺, or by stimulating its sequestration.

We now report the effects of prostaglandins on tetraethylammonium- and acetylcholine-induced contraction of canine tracheal smooth muscle, cellular ⁴⁵Ca influx, and microsomal ⁴⁵Ca uptake in comparison with the effects of

verapamil and cyclic nucleotides.

MATERIALS AND METHODS

Materials: PGE₂, 16(S)-methyl-20-methoxy-PGE₂ (YPG-209) (9), and nicardipine hydrochloride (10) were synthesized in our laboratories. EGTA and ATP were purchased from Sigma. Dibutyryl cyclic AMP, cyclic AMP, and cyclic AMP phosphodiesterase were obtained from Boehringer-Mannheim/Yamanouchi. The following materials were also commercially obtained: ⁴⁵CaCl₂ (New England Nuclear), acetylcholine chloride (Daiichi), tetraethylammonium chloride (Wako Junyaku), l-isoproterenol hydrochloride (Nikken Kagaku), verapamil hydrochloride (Eisai), and lanthanum chloride (Koso Chemicals). PGE₂ and YPG-209 were dissolved in 1 ml of 99.5% ethyl alcohol and then diluted with 0.9% saline to give a final concentration of 3 mg/ml in 10% ethyl alcohol. This prostaglandin stock solution was kept frozen at -20°C in sealed ampoules until use, and at the time of the experiments, the stock was appropriately diluted with 0.9% saline. Other materials were dissolved in 0.9% saline.

Tracheal preparations: Mongrel dogs of either sex weighing 15 to 30 kg were anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg) and were killed by bleeding from the common carotid artery. The trachea was rapidly excised and placed in a Tyrode solution (pH 7.8) of the following composition in mM: NaCl 137, KCl 2.7, MgCl₂ 1.1, CaCl₂ 1.8, NaH₂PO₄ 0.42, NaHCO₃ 11.9, and glucose 5.5. During the bubbling of 95% O₂ and 5% CO₂, the pH of the Tyrode solution changed and was stabilized at pH 7.4. In some experiments involving lanthanum which forms insoluble precipitates with phosphate and bicarbonate in normal Tyrode solution, the trachea was placed in a pH 7.4 Tris-buffered physiological solution aerated with 100% O₂ of the

following composition in mM: NaCl 140.2, KCl 5.8, MgCl₂ 1.2, CaCl₂ 2.5, glucose 11.1, and Tris HCl 5.0. A single ring was cut off from the trachea, and then connective tissues, mucous membranes, and most of the cartilages were removed before attaching threads to the small pieces of cartilage remaining at each end of the tracheal muscle. Thereafter, tracheal strips were suspended with a tension of 1 g in an organ bath containing 10 ml of Tyrode solution aerated with a mixture of 95% O₂ and 5% CO₂ at 37°C. While the tracheal smooth muscle was incubated for 3–4 hr in the bath before addition of drugs, the Tyrode solution was replaced every 15 min and the tension was repeatedly readjusted to 1 g. The tracheal response was isometrically measured with a strain gauge (SB-1T Nihon Kohden) and recorded on a Reticorder (RJG-3006, Nihon Kohden).

Contractile response to tetraethylammonium or acetylcholine: After the contractile response was recorded in Tyrode solution containing pharmacologically equivalent concentrations of tetraethylammonium (5×10^{-3} M) or acetylcholine (1.7×10^{-7} M), the muscle preparations were fully relaxed by washing three times with normal Tyrode solution. The medium was then replaced by Ca⁺⁺-free Tyrode solution containing 100 μM EGTA. After 10 min (11), tetraethylammonium or acetylcholine was added to the bath. In order to determine if the contractility of the tissue could still be restored to the predrug-level, 1.8 mM Ca⁺⁺ was added at the end of the incubation.

Relaxation induced by prostaglandins and some other drugs: After a constant tone level was obtained by addition of 1.7×10^{-7} M acetylcholine to the bath fluid, the tracheal strips were exposed to various concentrations of the test compounds for 5 min. Relaxation was expressed as a percentage of acetylcholine-induced tension.

Effects of prostaglandins and some other drugs on contraction induced by tetraethylammonium or acetylcholine: Tracheal responses were recorded to 5×10^{-3} M tetraethylammonium or 1.7×10^{-7} M acetylcholine in Tyrode solution in the absence and then the presence of various concentrations of PGE₂, YPG-209, verapamil, or dibutyryl cyclic AMP. The tissue was incubated for 10 min with the compounds. The percentage inhibition was calculated by comparing the responses before and after addition of the test compounds. Influences of prostaglandins and dibutyryl cyclic AMP on the contraction induced by 1.7×10^{-7} M acetylcholine in the Ca⁺⁺-free Tyrode solution were also determined. In these cases, the tissues were incubated for 10 min (11) with the test compounds.

Isolation of tracheal smooth muscle: Trachea was placed in cold modified Tyrode solution (pH 7.4) of the following composition in mM: NaCl 137, KCl 2.7, MgCl₂ 1.1, CaCl₂ 1.0, NaH₂PO₄ 0.42, NaHCO₃ 11.9, and glucose 9.1. The dorsal tracheal smooth muscle layer was carefully separated from cartilages, connective tissues, and mucous membranes.

Determination of cellular ⁴⁵Ca uptake: Cellular ⁴⁵Ca uptake was determined by the lanthanum method (12) with some modification (13). Cleaned tracheal smooth muscle was cut transversely into blocks weighing about 50 mg. Following equilibration in HEPES-buffer A (140 mM NaCl, 2.68 mM KCl, 1.25 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 5 mM HEPES-Tris buffer, pH 7.4) at 37°C for 60 min, the preparations were preincubated with PGE₂, YPG-209, or verapamil for 10 min. The ⁴⁵Ca uptake was then initiated by transferring the tissue preparations to fresh HEPES-buffer A or HEPES-buffer B (40 mM NaCl, 83 mM KCl, 20 mM tetraethylammonium, 1 mM MgCl₂, 10 mM glucose, and 5 mM HEPES-

Tris buffer, pH 7.4) containing 1.25 mM CaCl₂ with 0.2 μ Ci ⁴⁵Ca/ml and continued for 30 min under various conditions. The uptake was terminated by addition of 15 mM lanthanum, and the preparations were exposed to the lanthanum for 5 min to completely block ⁴⁵Ca exchange between the inside and outside of the cells. The preparations were then transferred into HEPES-buffer A in which CaCl₂ was replaced by 15 mM lanthanum, and incubated further at 37°C for 60 min to expel the extracellularly bound ⁴⁵Ca. Throughout the experiments up to this stage of the incubation, all mixtures including the tracheal smooth muscles were continuously oxygenated with 100% O₂. The preparations were then blotted, weighed, and solubilized with 1 ml Soluene 350 (Packard) at 50°C for 3 or more hr in a shaking water bath. The radioactivity of ⁴⁵Ca was determined by a Packard Tri-Carb liquid scintillation spectrometer.

Microsomal preparation: The tracheal smooth muscle was homogenized with a Polytron homogenizer (Kinematica) in 9 volumes of 0.25 M sucrose containing 50 mM Tris-Maleate buffer, pH 7.4, and centrifuged at 10,000 $\times$ g for 20 min. The supernatant fluid was further centrifuged at 77,000 $\times$ g for 90 min, and the pellet was used as the microsomal fraction after resuspension in 50 mM Tris-Maleate buffer, pH 7.4. This preparation also contained plasma membranes and no more than 10% of the total mitochondria. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard.

Standard assay for microsomal Ca⁺⁺ uptake: Microsomal Ca⁺⁺ uptake was assayed by the method of Nishikori et al. (15) with a slight modification. The final reaction mixture contained 50 mM Tris-Maleate buffer, pH 7.4, 80 mM KCl, 2.5 mM Tris-oxalate, 0.2 mM ATP, 0.8 mM MgCl₂, 3 mM phosphoenolpyruvate, 1 unit of

pyruvate kinase (5 μ g protein), 0.125 mM CaCl_2 with 1 μCi ^{45}Ca , 0.156 mM EGTA, and microsomes (100–200 μ g protein) in a total volume of 0.2 ml. The concentration of free Ca^{++} in the reaction mixture was calculated to be 6 μM from the affinity constant of 5×10^{-5} M (16) between EGTA and Ca^{++} at pH 7.4. After preincubation for 1 min at 25°C, Ca^{++} uptake was started by addition of 30 μl $^{45}\text{CaCl}_2$ -EGTA buffer, pH 7.4, containing 50 mM Tris-Maleate, 0.83 mM CaCl_2 with 33.3 μCi ^{45}Ca /ml, and 1.04 mM EGTA and the reaction was carried out for 1 min. Thereafter, 2 ml of Ca^{++} -free buffer containing 50 mM Tris-Maleate buffer, pH 7.4, 80 mM KCl, 2.5 mM Tris-oxalate, 1.5 mM MgCl_2 , and 1 mM EGTA was added to the reaction mixture, and then the mixture was filtered through a HA Millipore filter disc (pore size 0.45 μm , Millipore Japan). The disk was quickly washed with the ice-cold Ca^{++} -free buffer. The radioactivity on the filter disc was counted with a Packard Tri-carb liquid scintillation spectrometer. Blank values of the Ca^{++} uptake were determined by using 2 min-boiled microsomes.

Statistics: The EC_{50} values were calculated by the Probit method. Statistical significance of the data was estimated using the Student's *t*-test.

RESULTS

Relaxation by prostaglandins and some relaxants: As shown in Fig. 1, PGE_2 and its derivative YPG-209 relaxed in a dose-dependent fashion the acetylcholine-contracted canine trachea with EC_{50} values (median effective concentrations required to result in 50% relaxation) of $(1.6 \pm 0.9) \times 10^{-6}$ M and $(4.7 \pm 1.8) \times 10^{-6}$ M, respectively. Verapamil, a calcium antagonist, also exhibited similar dose-dependent relaxation of acetylcholine-contracted trachea with an EC_{50} of $(3.5 \pm 0.6) \times 10^{-6}$ M. Isoproterenol, a stimulant of β -adrenergic receptors, also

Fig. 1. Relaxing effects of PGE_2 , YPG-209, verapamil, and dibutyl cyclic AMP (DibcAMP) on 1.7×10^{-7} M acetylcholine-contracted trachea. After a constant level of tone in response to acetylcholine was obtained, the tracheal strips were exposed to various concentrations of the test compounds for 5 min. The results represent the means $\pm$ S.E. of 5 experiments and are expressed as the percent relaxation of acetylcholine-induced tension.

relaxed the contracted trachea with an EC_{50} value of $(4.0 \pm 2.0) \times 10^{-6}$ M (not illustrated), similar to that for the prostaglandins. Of the drugs tested, dibutyl cyclic AMP was found to be the least potent with an EC_{50} of $(1.3 \pm 0.6) \times 10^{-3}$ M.

Effects of prostaglandins on contraction induced by tetraethylammonium or acetylcholine in Tyrode solution: In order to determine whether relaxation of the tracheal smooth muscle by prostaglandins is caused by reducing a Ca^{++} influx or by affecting the intracellular Ca^{++} movement, we first investigated possible modes for the actions of tetraethylammonium and acetylcholine on the contraction of trachea in the presence and absence of Ca^{++} in the incubation medium.

As shown in Fig. 2A, 5×10^{-3} M tetraethylammonium produced contraction of the tracheal smooth muscle in Tyrode solution after a short lag period (a few minutes). Following removal of tetraethylammonium, the tracheal smooth muscle was exposed to Ca^{++} -free Tyrode solution. Under the

Fig. 2. Spasmogenic action of tetraethylammonium (TEA) on canine tracheal smooth muscle in Tyrode solution in the presence and absence of Ca⁺⁺ (A). The developed tension (mean $\pm$ S.E. of 40 experiments) produced by 5×10^{-3} M tetraethylammonium in Tyrode solution was 1.53 ± 0.08 g. W indicates 3 times washing with Tyrode solution. Spasmogenic action of tetraethylammonium on canine tracheal smooth muscle in the Tris-buffered physiological solution in the presence and absence of lanthanum (La⁺⁺⁺) (B). W indicates 3 times washing with the Tris-buffered physiological solution.

Ca⁺⁺-free conditions, tetraethylammonium failed to produce contraction. However, the addition of 1.8 mM Ca⁺⁺ restored the contractile response to tetraethylammonium. The spasmogenic action of tetraethylammonium in the Tris-buffered physiological solution was completely blocked after a 20 min incubation with 5 mM lanthanum (Fig. 2B), an inhibitor of the transmembrane flux of Ca⁺⁺ (12), as in the case of bovine tracheal smooth muscle (8). These results indicate that the spasmogenic action of tetraethylammonium is dependent upon the presence of extracellular Ca⁺⁺.

When 1.7×10^{-7} M acetylcholine was used, the response of tracheal smooth muscle in Ca⁺⁺-free Tyrode solution was rather different from that to tetraethylammonium (Fig. 3A). The contractile response to acetylcholine 10 min after removal of Ca⁺⁺ was reduced to $38.6 \pm 2.3\%$ of the control. However, the full amplitude of contraction was restored after addition of 1.8 mM Ca⁺⁺ to the incubation medium. It was of interest that, in contrast to tetraethylammonium, acetylcholine still produced a partial contraction (22.2%) even in the presence of 5 mM lanthanum in the Tris-

Fig. 3. Spasmogenic action of acetylcholine (ACh) on canine tracheal smooth muscle in Tyrode solution in the presence and absence of Ca^{++} (A). The developed tension (mean $\pm$ S.E. of 40 experiments) produced by 1.7×10^{-7} M acetylcholine in Tyrode solution was 1.55 ± 0.09 g. W indicates 3 times washing with the Tyrode solution. Spasmogenic action of acetylcholine on canine tracheal smooth muscle in the Tris-buffered physiological solution in the presence and absence of lanthanum (La^{+++}) (B). W indicates 3 times washing with the Tris-buffered physiological solution.

buffered physiological solution (Fig. 3B) as in the case of bovine tracheal smooth muscle (8). These results suggest that the contraction of canine tracheal smooth muscle by acetylcholine is regulated by not only intracellular but also extracellular Ca^{++} .

Based on these data, the effect of pretreatment with prostaglandins was studied to see which agent-induced contraction was more altered by the prostaglandins. As shown in Fig. 4A and B, both PGE_2 and YPG-209 showed considerable selective inhibition of the spasmogenic action of acetylcholine in the presence of extracellular Ca^{++} . Dibutyryl cyclic AMP also inhibited the action of acetylcholine in a slightly more selective manner (Fig. 4D). On the contrary, verapamil

(Fig. 4C), an inhibitor of Ca^{++} influx (17), more specifically blocked the spasmogenic action of tetraethylammonium.

Effects of prostaglandins on acetylcholine-induced contraction in Ca^{++} -free Tyrode solution: Ten minutes after removal of Ca^{++} , 1.7×10^{-7} M acetylcholine produced contraction of the tracheal tissue by as much as $38.6 \pm 2.3\%$ of the control response in normal Tyrode solution as mentioned above. This contraction in the absence of Ca^{++} was blocked by prostaglandins in a dose-dependent fashion between 10^{-8} M and 10^{-4} M as depicted in Table 1. Dibutyryl cyclic AMP also inhibited the acetylcholine-induced contraction at concentrations above 10^{-4} M. The median effective concentrations required to result in 50% inhibition (EC_{50}) were 1.2×10^{-6} M, 1.9×10^{-6} M, and 5.1×10^{-4} M for PGE_2 , YPG-209, and dibutyryl cyclic AMP, respectively.

Effects of prostaglandins on cellular ^{45}Ca uptake: Since the pharmacological data shown above suggested that PGE_2 results in tracheal relaxation at least partly through an intracellular Ca^{++} movement, an experiment was attempted to see the effect of PGE_2 on ^{45}Ca influx by the tissue. ^{45}Ca uptakes by canine tracheal smooth muscle in HEPES-buffer A and B were about 100 and 135 nmol/g wet tissue, respectively, during a 30 min incubation. The addition of 15 mM lanthanum markedly reduced ^{45}Ca uptake to a level of 40 nmol/g wet tissue in either HEPES-buffer A or B. As shown in Table 2, the increase in ^{45}Ca influx by 83 mM KCl plus 20 mM tetraethylammonium (83 mM KCl plus 20 mM tetraethylammonium was used to produce a full depolarization) (8) was completely abolished by 10^{-5} M verapamil and inhibited by about 50% by 10^{-7} M verapamil (data is not shown), whereas ^{45}Ca uptake in the presence of 2.68 mM KCl was not significantly altered as reported with rabbit aorta (13, 18). In

Fig. 4. Inhibitory effects of PGE₂, YPG-209, verapamil, and dibutyryl cyclic AMP (DibcAMP) on the spasmogenic action of 5×10^{-3} M tetraethylammonium (TEA) or 1.7×10^{-7} M acetylcholine (ACh). Tracheal responses were recorded in Tyrode solution in the absence and then the presence of various concentrations of the test compounds. The tissue was incubated for 10 min with the test compounds. The results represent the means $\pm$ S.E. of 5 experiments and are expressed as the percent inhibition of the control response to each agonist.

contrast to verapamil, 10^{-5} M PGE₂ and 10^{-4} M YPG-209 were unable to affect significantly the cellular ⁴⁵Ca uptake in either HEPES-buffer A or B.

Effects of PGE₂ and cyclic AMP on microsomal Ca⁺⁺ uptake: Microsomal Ca⁺⁺ uptake was increased linearly up to at least 250 μ g of protein, and the reaction proceeded linearly for at least 1 min. In the absence of ATP and its regenerating system, Ca⁺⁺ uptake was about 1.6% of that at the maximal effective dose of ATP (1.6 mM), indicating

that the microsomal Ca⁺⁺ uptake completely depends on ATP under these conditions (Fig. 5). The apparent K_m value for ATP (the concentration required to produce half-maximal activation) was about 0.25 mM, and the maximum velocity (V_{max}) of Ca⁺⁺ uptake was about 1.4 nmol/mg protein/min.

Figure 6 shows the time course of microsomal Ca⁺⁺ uptake in the presence and absence of 3×10^{-5} M PGE₂ or 3×10^{-5} M cyclic AMP. PGE₂ increased by about two-fold the Ca⁺⁺ uptake, whereas stimulation of

Ca⁺⁺ uptake by cyclic AMP was about 50% during the linear phase of the reaction. However, the stimulatory effect of PGE₂ and cyclic AMP were strictly dependent upon the concentration of ATP as shown in Table 3. An increase in the concentration of ATP from 0.2 mM to 0.8 mM reduced markedly the stimulation of Ca⁺⁺ uptake by

Table 1. Dose-dependent effects of PGE₂, YPG-209, and dibutyl cyclic AMP (DibcAMP) on the spasmogenic action induced by acetylcholine in Ca⁺⁺-free Tyrode solution

Drug ^a	Concentration (M)	n	Contraction ^b (%)	Inhibition (%)
Control	—	5	38.6±2.3	—
PGE ₂	10 ⁻⁸	4	29.2±2.9*	22.5
	10 ⁻⁷	5	26.0±1.8**	32.6
	10 ⁻⁶	5	16.5±1.7**	57.6
	10 ⁻⁵	5	14.8±0.5**	61.7
	10 ⁻⁴	5	10.8±1.0**	72.0
YPG-209	10 ⁻⁸	4	30.3±1.4*	21.5
	10 ⁻⁷	5	25.0±1.4**	35.2
	10 ⁻⁶	4	22.0±0.9**	43.0
	10 ⁻⁵	5	14.5±0.3**	62.4
	10 ⁻⁴	5	10.8±1.2**	72.0
DibcAMP	10 ⁻⁴	5	27.9±2.1*	27.7
	10 ⁻³	5	20.1±4.7**	47.9
	10 ⁻²	5	1.8±0.5**	95.3

^a Drugs were added at the time of change from normal to Ca⁺⁺-free Tyrode solution. After 10 min, acetylcholine was added.

^b Contraction was expressed as the percent contraction of the control response in normal Tyrode solution.

* Significantly lower than the control (P<0.05).

** Significantly lower than the control (P<0.01).

Table 2. Effects of PGE₂, YPG-209, and verapamil on cellular ⁴⁵Ca uptake

Drug	Concentration (M)	⁴⁵ Ca uptake (nmol/g wet tissue/30 min)	
		2.68 mM KCl	83 mM KCl plus 20 mM TEA
Control	—	61.2±5.1 (6)	93.2±2.7 (6)
PGE ₂	10 ⁻⁵	56.3±3.7 (4)	79.3±8.5 (4)
YPG-209	10 ⁻⁴	69.9±11.5 (4)	82.8±6.1 (4)
Verapamil	10 ⁻⁵	62.4±6.8 (4)	46.8±2.3 (6)*

After tracheal smooth muscle preparations were preincubated for 10 min with PGE₂, YPG-209, or verapamil, ⁴⁵Ca uptake was initiated by transferring the tissue preparations to the fresh buffers containing 1.25 mM CaCl₂ with 0.2 μ Ci ⁴⁵Ca/ml and continued for 30 min under the various conditions. The uptake reaction was terminated by addition of 15 mM lanthanum and extracellularly bound ⁴⁵Ca was expelled in 15 mM lanthanum-containing Ca⁺⁺-free buffer for 60 min. Cellular ⁴⁵Ca uptake was defined as the difference in ⁴⁵Ca uptake between in the absence and presence of 15 mM lanthanum. The results represent the means±S.E. of several experiments as indicated in the parenthesis.

*Significantly lower than the control (P<0.05).

either PGE₂ or cyclic AMP. Table 4 shows the effect of various concentrations of PGE₂ and cyclic AMP on the Ca⁺⁺ uptake under the standard conditions. Both PGE₂ and cyclic AMP increased the Ca⁺⁺ uptake in a dose-dependent fashion. PGE₂ increased basal Ca⁺⁺ uptake at most two-fold with a K_a value (concentration required for half-

maximal stimulation) of 6×10^{-7} M. Addition of cyclic AMP did not result in further increase in the Ca⁺⁺ uptake in the presence of 3×10^{-5} M PGE₂. Cyclic AMP increased basal Ca⁺⁺ uptake at most 1.5 fold with a K_a value of about 10^{-6} M. Addition of 3×10^{-5} M nicardipine hydrochloride, a cyclic AMP phosphodiesterase inhibitor with an inhibition constant (K_i) of about 2×10^{-6} M (19), was unable to further increase the Ca⁺⁺ uptake in the presence of 3×10^{-5} M cyclic AMP. As shown in Table 5, the addition

Fig. 5. Effect of ATP on microsomal Ca⁺⁺ uptake. Freshly prepared microsomes (160 μ g of protein) were added to the incubation medium containing 50 mM Tris-Maleate, pH 7.4, 80 mM KCl, 2.5 mM Tris-oxalate, 3 mM phosphoenolpyruvate, 1 unit of pyruvate kinase, and 6 μ M Ca⁺⁺ (⁴⁵Ca/EGTA buffer containing 125 μ M CaCl₂) with a constant molar ratio of MgCl₂ to ATP (4 to 1). Incubations were carried out for 1 min at 25°C. The results represent the means $\pm$ S.E. of 4 experiments. The blank value of Ca⁺⁺ uptake was 0.14 ± 0.02 nmol/mg protein.

Fig. 6. Microsomal Ca⁺⁺ uptake in the absence (—●—) and presence of 3×10^{-5} PGE₂ (—○—) or 3×10^{-5} M cyclic AMP (—▲—). Incubations were carried out with 150 μ g microsomal protein under the standard conditions with 0.2 mM ATP and its regenerating system, except for varying the incubation time. The results represent the means $\pm$ S.E. of 6 experiments.

Table 3. Effect of ATP on stimulation of microsomal Ca⁺⁺ uptake by PGE₂ and cyclic AMP

Drug	Concentration (M)	Ca ⁺⁺ uptake (nmol/min/mg protein)	
		ATP 0.2 mM	ATP 0.8 mM
Control	—	0.57 ± 0.06	1.21 ± 0.30
PGE ₂	3×10^{-5}	$1.05 \pm 0.14^*$	1.34 ± 0.32
Cyclic AMP	3×10^{-5}	$0.82 \pm 0.08^*$	1.37 ± 0.28

Incubations were carried out with 160 μ g microsomal protein under the standard conditions except for varying ATP concentration and the presence of indicated drugs. The results represent the means $\pm$ S.E. of four experiments.

*Significantly higher than the control ($P < 0.05$).

Table 4. Effects of PGE₂ and cyclic AMP on microsomal Ca⁺⁺ uptake

Drug	Concentration (M)	Ca ⁺⁺ uptake (percent of control)
Control	—	100.0±4.3 ^a
PGE ₂	3×10 ⁻⁷	132.1±11.5*
	3×10 ⁻⁶	179.4±17.4**
	3×10 ⁻⁵	197.6±19.1*
PGE ₂	3×10 ⁻⁵	192.0±23.4*
+ Cyclic AMP	3×10 ⁻⁵	
Cyclic AMP	3×10 ⁻⁷	115.5±6.8
	3×10 ⁻⁶	131.4±6.7*
	3×10 ⁻⁵	148.2±13.7*
Cyclic AMP	3×10 ⁻⁵	148.7±12.4*
+ Nicardipine ^b	3×10 ⁻⁵	

Incubations were carried out with 160 µg microsomal protein under the standard conditions except for the presence of indicated drugs. The results represent the means±S.E. of four experiments.

^a The control value was 0.57 nmol/min/mg protein.

* Significantly higher than the control (P<0.05).

** Significantly higher than the control (P<0.01).

^b Nicardipine was formally referred to as YC-93 (10, 19).

Table 5. Effect of cyclic AMP phosphodiesterase on stimulation of microsomal Ca⁺⁺ uptake by PGE₂ and cyclic AMP

Drug	Concentration (M)	Ca ⁺⁺ uptake (nmol/min/mg protein)	
		Phosphodiesterase	
		Absence	Presence
Control	—	0.58±0.06	0.49±0.09
PGE ₂	3×10 ⁻⁵	1.12±0.17*	0.82±0.09*
Cyclic AMP	3×10 ⁻⁵	0.83±0.07*	0.41±0.06

Beef heart cyclic AMP phosphodiesterase (300 µg protein) was added to the reaction mixture containing microsomes (240 µg protein) 30 sec before preincubation at 25°C. The preincubation was started by addition of ATP and its regenerating system. Other conditions were as described under "Methods". The results represent the means±S.E. of four experiments.

*Significantly higher than the control (P<0.05).

of cyclic AMP phosphodiesterase to the reaction mixtures completely eliminated stimulation of the microsomal Ca⁺⁺ uptake by 3×10⁻⁵ M cyclic AMP, whereas the stimulation by 3×10⁻⁵ M PGE₂ was only slightly decreased, suggesting that a cyclic AMP-related mechanism is not apparently involved in the stimulation of Ca⁺⁺ uptake

by PGE₂.

DISCUSSION

In the present experiments, tetraethylammonium-induced contraction of canine tracheal smooth muscle is completely abolished either by removal of Ca⁺⁺ from the incubation medium or by the addition of

lanthanum, suggesting that the contraction is dependent upon extracellular Ca⁺⁺, as in the case of bovine tracheal smooth muscle (8). On the other hand, acetylcholine is able to produce a partial contraction either in a Ca⁺⁺-free solution or in the presence of lanthanum, suggesting that acetylcholine is able to mobilize Ca⁺⁺ from the intracellularly sequestered Ca⁺⁺ store. Thus, acetylcholine may induce contraction by concomitantly using both intracellular and extracellular Ca⁺⁺ as already suggested by Kirkpatrick (8).

Pretreatment with the prostaglandins or dibutyryl cyclic AMP resulted in more efficient suppression of the acetylcholine-induced contraction. This suggests that prostaglandins- and dibutyryl cyclic AMP-induced suppressions do not result from the inhibition of Ca⁺⁺ influx across the membrane but from the inhibition of the intracellular Ca⁺⁺ movement, since the contractile response to acetylcholine is inhibited only slightly by verapamil in concentrations sufficient to cause marked attenuation of contraction induced by tetraethylammonium. This is consistent with the biochemical data which demonstrate that the influx of ⁴⁵Ca in canine tracheal smooth muscle as measured by the lanthanum method (12) is not affected by prostaglandins. Van Breemen (20) has reported that RO 20-1724, a phosphodiesterase inhibitor, reduces the development of the tension by high K⁺ without affecting the high K⁺-induced net Ca⁺⁺ flux across the cell membrane in rabbit aorta. These results are consistent with the interpretation that Ca⁺⁺ entering the cells by high K⁺ stimulation is partly sequestered by the sarcoplasmic reticulum and mitochondria before it reaches the active sites on the myofilament. Thus it is likely that attenuation of the contractile response to tetraethylammonium by high concentrations of prostaglandins result from the stimulation of microsomal Ca⁺⁺ uptake. The prostaglandins and dibutyryl cyclic AMP

inhibit the spasmogenic action of acetylcholine in a Ca⁺⁺-free medium. This may be attributable to the stimulation of microsomal Ca⁺⁺ uptake. It must be emphasized, however, that stimulation of microsomal Ca⁺⁺ uptake by PGE₂ and cyclic AMP requires the optimal concentration of ATP (Table 3). It has been already reported that maximal stimulation of microsomal Ca⁺⁺ uptake by cyclic AMP occurs at ATP concentrations of 0.25 mM and 0.3 mM in rat uterus (15) and rabbit colon (21), respectively. Since the microsomal preparation contained adenylate cyclase activity, cyclic AMP endogenously formed when a higher concentration of ATP was added may mask its stimulating effect. A role of the prostaglandins appears to increase the affinity for ATP. The lack of cyclic AMP-induced stimulation of microsomal Ca⁺⁺ uptake in the trachea reported by Sands et al. (22, 23) may be attributed to utilization of 5 mM ATP in the incubation mixture. The present data suggest an at least partial involvement of a cyclic AMP system in the mechanism by which the prostaglandins relax airway smooth muscle. However, some data against cyclic AMP involvement are obtained in the prostaglandin action. For instance, cyclic AMP phosphodiesterase completely eliminates stimulation by cyclic AMP of microsomal Ca⁺⁺ uptake, but only slightly reduces the stimulation by PGE₂. Moreover, stimulation of Ca⁺⁺ uptake by PGE₂ is much greater than that by cyclic AMP.

Although theophylline has been believed to relax the airway smooth muscle through its ability to raise the intracellular level of cyclic AMP, Kolbeck et al. (24) have demonstrated recently that theophylline increases the sequestration of Ca⁺⁺ to the mitochondria of guinea-pig tracheal smooth muscle without affecting cyclic AMP and total tissue Ca⁺⁺ levels. Therefore, they suggest that cyclic AMP is not involved in

the relaxing effect of theophylline on airway smooth muscle.

In summary, PGE₂ and YPG-209 induce the relaxation of airway smooth muscle mainly through affecting the intracellular Ca⁺⁺ movement such as by enhancement of sequestration of Ca⁺⁺ to the microsomes.

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