Effect of selective phosphodiesterase inhibitors on response of ovine pulmonary arteries to prostaglandin E₂

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Gao, Yuansheng, Jean-François Tolsa, Hai Shen, and J. Usha Raj. Effect of selective phosphodiesterase inhibitors on response of ovine pulmonary arteries to prostaglandin E₂. J. Appl. Physiol. 84(1): 13–18, 1998.—Several adenosine 3',5'-cyclic monophosphate (cAMP)-hydrolyzing phosphodiesterase isozymes are present in the pulmonary vasculature. The present study was designed to determine the effect of selective inhibitors of phosphodiesterase subtypes on prostaglandin E₂ (PGE₂)-induced relaxation of isolated fourth-generation pulmonary arteries of newborn lambs. PGE₂ and forskolin caused pulmonary arteries to relax and induced an increase in the intracellular cAMP content in the vessels. The relaxation and change in cAMP content were augmented by milrinone and rolipram, inhibitors of phosphodiesterase type 3 (PDE3) and type 4 (PDE4), respectively. The augmentation in relaxation and the increase in cAMP content caused by milrinone plus rolipram was greater than the sum of the responses caused by either of the inhibitors alone. 8-Methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine, an inhibitor of phosphodiesterase type 1, had no effect on relaxation and change in cAMP induced by PGE₂ and forskolin. Acetylcholine alone had no effect on cAMP content in the vessels but augmented the relaxation and the increase in cAMP induced by PGE₂ and forskolin in arteries with endothelium. This effect was not observed in arteries without endothelium or in arteries with endothelium treated with Nω-nitro-L-arginine. These results suggest that PDE3 and PDE4 are the primary enzymes hydrolyzing cAMP of pulmonary arteries of newborn lambs and that an inhibition of both PDE3 and PDE4 would result in a greater effect than that caused by inhibition of either one of the subtypes alone. Furthermore, endothelium-derived nitric oxide may enhance cAMP-mediated relaxation by inhibition of PDE3.

PERINATAL PULMONARY CIRCULATION; FORSKOLIN; MILRINONE; ROLIPRAM

A NUMBER OF VASODILATORS SUCH AS PROSTAGLANDIN E₂ (PGE₂), PROSTAGLANDIN I₂ (PGI₂), AND β-ADRENERGIC AGONISTS PLAY AN IMPORTANT ROLE IN PERINATAL PULMONARY CIRCULATION (14, 22, 33). THEY ACTIVATE ADENYLYL CYCLASE AND THEREBY INCREASE INTRACELLULAR ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (cAMP) CONTENT, WHICH RESULTS IN VASODILATION (8, 29). PDE IS HYDROLYZED BY PHOSPHODIESTERAS. INHIBITION OF PDE AUGMENTS CAMP-MEDIATED VASODILATION (2, 29).

PDE CONSISTS OF AT LEAST SEVEN DISTINCT ISOZYMES (2). IN THE PULMONARY VASCULARITY, THREE PDE ISOZYMES HAVE BEEN IDENTIFIED TO HYDROLYZE cAMP, namely, calcium/calmodulin-dependent PDE (PDE1), guanosine 3',5'-cyclic monophosphate (cGMP)-inhibitable PDE (PDE3), and cAMP-specific PDE (PDE4) (10, 30). Milrinone, a specific inhibitor of PDE3 (35), causes human pulmonary arteries to relax and reduces pulmonary hypertension (15, 30). Rolipram, a specific inhibitor of PDE4 (21), induces human pulmonary vessels to relax and reverses pulmonary vasoconstriction of isolated rabbit lungs (27, 30).

ALTHOUGH IT IS RECOGNIZED THAT MULTIPLE cAMP- HYDROLYTIC PDE ISOZYMES exist in pulmonary vasculature (10, 30), the relative contribution of the different PDE isozyms in modulating cAMP-dependent relaxation is not known. IN THE PRESENT STUDY, WE EXAMINE THE EFFECTS OF SELECTIVE PDE-SUBTYPE INHIBITORS ON CAMP-MEDIATED RESPONSE. OUR RESULTS SHOW THAT THE PDE3 AND PDE4 MAY BE THE PRIMARY cAMP-HYDROLYTIC ENZYMES IN PULMONARY ARTERIES OF NEWBORN LAMBS. WE USED PGE₂ AND FORSKOLIN TO INDUCE CAMP-MEDIATED RESPONSES. PGE₂ IS AN IMPORTANT VASODILATOR OF THE PERINATAL PULMONARY VASCULARITY THAT ELEVATES cAMP BY A RECEPTOR-COUPLED MECHANISM (8, 13, 33). Forskolin increases cAMP by directly stimulating adenyl cyclase (20).

WHEN TWO MAJOR cAMP-HYDROLYTIC PDES ARE PRESENT, IT IS LIKELY THAT, IF ONE OF THEM IS INHIBITED, THE OTHER MAY COMPENSATE IN THE HYDROLYSIS OF cAMP (2, 29). Therefore, we hypothesized that, if the two major cAMP-hydrolytic isozyms of pulmonary arteries of newborn lambs (PDE3 and PDE4) were inhibited, the ability of the vessels to hydrolyze cAMP would be greatly restricted. Consequently, the cAMP-mediated response could be greatly enhanced. Our results show that the augmentation of PGE₂- and forskolin-induced response of pulmonary arteries by the inhibition of PDE3 plus PDE4 was greater than the sum of responses caused by the inhibition of PDE3 and PDE4 separately.

AMONG THE cAMP-HYDROLYTIC PDE ISOZYMES, PDE3 CAN BE INHIBITED BY cGMP (2, 29). HENCE, AN INCREASE IN cGMP IN VASCULAR SMOOTH MUSCLE MAY AUGMENT CAMP-MEDIATED VASODILATION. SUCH A SYNERGISTIC INTERACTION BETWEEN cGMP AND cAMP HAS BEEN IMPlicated IN ISOLATED RAT AORTAS (11, 16, 23) AND IN PERFUSED RABBIT LUNGS (7). IN THE PRESENT STUDY, WE ELEVATED cGMP BY STIMULATING THE RELEASE OF ENDOTHELium-Derived Nitric Oxide (EDNO) WITH ACETYLCHOLINE (ACH) (14). AFTER ACH, PGE₂- and forskolin-induced relaxation and increase in cAMP content of pulmonary arteries of newborn lambs were augmented, suggesting that EDNO may augment cAMP-mediated vasodilation by the inhibition of PDE3.

MATERIALS AND METHODS

Lungs of 33 neonatal lambs (7–13 days old, either sex, Nebeker Ranch, Lancaster, CA) were used. Lambs were anesthetized with ketamine hydrochloride (30 mg/kg im) and killed with an overdose of pentobarbital sodium (100 mg/kg iv) (13, 14). Fourth-generation pulmonary arteries were dissected from the lungs and cut into rings. The outside diameters of the...
vessels were 2.0–2.5 mm. In some rings, the endothelium was removed by gently rubbing the luminal surface with the tips of a watchmaker’s forceps. Removal of the endothelium was confirmed by lack of relaxation to ACh (3 × 10⁻⁵ M) (13, 14).

Organ chamber study. Vessel rings were suspended in organ chambers filled with 10 ml of modified Krebs-Ringer bicarbonate solution [composition (in mM): 118.3 NaCl; 4.7 KCl; 2.5 CaCl₂; 1.2 MgSO₄; 1.2 KH₂PO₄; 25.0 NaHCO₃; and 11.1 glucose] maintained at 37 ± 0.5°C and aerated with 95% O₂-5% CO₂ (pH = 7.4). Each ring was suspended by two stirrups passed through the lumen. One stirrup was anchored to the bottom of the organ chamber; the other one was connected to a strain gauge (model FT03C, Grass Instrument, Quincy, MA) for the measurement of isometric force.

At the beginning of each experiment, vessel rings were brought to their optimal tension by stretching the tissues progressively by ~0.2-g increments until the contractile responses to 100 mM KCl were maximal. The optimal resting tension of vessels with endothelium (1.1 ± 0.1 g, n = 7) was not significantly different from that of vessels without endothelium (1.0 ± 0.1 g, n = 33; P > 0.05). One hour of equilibration was allowed after tissues were brought to their optimal tension.

Relaxation of pulmonary arteries to PGE2, and other vasodilators was determined during contraction to endothelin-1. To exclude the possible interference of endogenous prostanoids, all experiments were performed in the presence of indomethacin (10⁻⁵ M) (5, 13).

Radioimmunoassay of cAMP and cGMP. Rings of pulmonary arteries were incubated in modified Krebs-Ringer bicarbonate solution (37°C, 95% O₂-5% CO₂) in the presence and absence of different inhibitors of PDEs. To exclude the possible interference of endogenous prostanoids, experiments were performed in the presence of indomethacin (10⁻⁵ M) (5, 13). After 45 min of equilibration, PGE2, forskolin, or ACh was added. Vessel rings were rapidly frozen in liquid nitrogen at 2, 10, and 2 min after the administration of PGE2, forskolin, and ACh, respectively. They were then thawed in trichloroacetic acid (6%), followed by homogenization in a glass tube with a motor-driven Teflon pestle, sonicated for 5 s, and centrifuged (13,000 g for 15 min). The supernatant was extracted with four volumes of water-saturated diethyl ether and lyophilized. The lyophilized samples were resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2), and the contents of cAMP or cGMP were determined by using cAMP or cGMP kits (Biomedical Technologies, Stoughton, MA). The cyclic nucleotide content is expressed as picomoles per milligram protein of vessel homogenate. The protein content was determined by using the Bradford dye-binding procedure (4).

Drugs. The following drugs were used (unless otherwise specified, all were obtained from Sigma Chemical, St. Louis, MO): 8-bromoadenosine 3'5'-cyclic monophosphate, endothelin-1 (human, porcine; American Peptide Company, Sunnyvale, CA), forskolin, indomethacin, 8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine (8M-IBMX; Biomol, Plymouth Meeting, PA), milrinone, N⁷-nitro-L-arginine, PGE₂ (Cayman Chemical, Ann Arbor, MI), and rolipram (Biomol).

8M-IBMX, forskolin, milrinone, and rolipram were dissolved in dimethyl sulfoxide (final concentration in organ chamber: 1%). Dimethyl sulfoxide at this concentration did not affect contraction of pulmonary vessels to endothelin-1 or relaxation to PGE₂ (data not shown). Indomethacin (10⁻⁵ M) was prepared in equal molar Na₂CO₃. This concentration of Na₂CO₃ did not significantly affect the pH of the solution in the organ chamber. The other drugs were prepared by using distilled water. All inhibitors and antagonists were given at least 30 min to equilibrate before their effects were tested.

Data analyses. Contractions are expressed in grams. Relaxations are expressed as percent of contraction of tissues to endothelin-1. Data are means ± SE. When mean values of two vessel groups were compared, Student’s t-test for unpaired observations was used. When the mean values of the same group before and after stimulation were compared, Student’s t-test for paired observations was used. Comparison of mean values of more than two groups was performed with one-way analysis of variance test with Student-Newman-Keuls test for post hoc testing of multiple comparison. All these analyses were performed by using a commercially available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA). Statistical significance was accepted when the P value (two-tailed) was <0.05. In all experiments, n represents the number of animals studied for each protocol (14).

RESULTS

Organ chamber studies. Experiments were performed in pulmonary arteries without endothelium that were contracted with endothelin-1 (3 × 10⁻⁹ M). The increase in vessel tension after treatment was 1.85 ± 0.12 g (n = 33). In arteries treated with milrinone (10⁻⁴ M) or rolipram (10⁻⁴ M) [inhibitors of PDE3 and PDE4, respectively (21, 32, 34, 35)], endothelin-1 at 10⁻⁸ M was needed to raise tension to a level equal to that in control vessels. In arteries treated with milrinone (10⁻⁴ M) plus rolipram (10⁻⁴ M), endothelin-1 at 10⁻⁷ M was needed to raise tension to a level similar to that in control vessels. In some experiments with ACh, arteries with endothelium were used. The tension in these vessels was raised by 1.78 ± 0.15 g (n = 7) by using endothelin-1 at a concentration of 10⁻⁸ M, which was comparable to the tension in vessels without endothelium.

PGE₂ induced a concentration-dependent relaxation of pulmonary arteries. The relaxation was augmented to an equal extent by milrinone (10⁻⁴ M) and rolipram (10⁻⁴ M) (Fig. 1). 8M-IBMX (10⁻⁴ M), an inhibitor of PDE1 (36), had no significant effect on PGE₂-induced relaxation (Fig. 1).

![Fig. 1. Effect of 8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine (8M-IBMX; 10⁻⁴ M), milrinone (10⁻⁴ M), and rolipram (10⁻⁴ M) on relaxation of pulmonary arteries without endothelium to prostaglandin E₂ (PGE₂). Experiments were performed during contraction to endothelin-1. Change in tension was expressed as %contraction to endothelin-1. Data are means ± SE; n = 6 animals for each group. *Significant difference between control and milrinone or rolipram group (P < 0.05).](http://jap.physiology.org/DownloadedFrom)
was administrated together with ACh, the resultant
was significantly augmented by milrinone (10\(^{-4}\) M) induced minimal relaxation. However, the relaxation was significantly greater than the sum of the relaxation obtained in the presence of both milrinone and rolipram was significantly greater from vessels treated with milrinone or rolipram alone (P < 0.05).

Forskolin, a direct activator of adenyl cyclase (20), caused a moderate relaxation. When PGE2 or forskolin was administrated together with ACh, the resultant relaxation was greater than the sum of relaxation caused by either ACh or PGE2 alone or that caused by either ACh and forskolin alone (Fig. 4). Such a phenomenon was not observed in arteries without endothelium or in arteries with endothelium treated with N\(^{G}\)-nitro-L-arginine (10\(^{-4}\) M), an inhibitor of nitric oxide synthase (26) (Fig. 4).

Cyclic nucleotide content. Under basal conditions, the intracellular content of cAMP in pulmonary arteries without endothelium was 23.7 ± 3.5 pmol/mg protein (n = 15). In the presence of 8M-IBMX (10\(^{-4}\) M), milrinone (10\(^{-4}\) M), or rolipram (10\(^{-4}\) M) the cAMP content was 21.9 ± 3.0 pmol/mg protein (n = 5), 25.3 ± 2.6 pmol/mg protein (n = 7), and 24.6 ± 3.3 pmol/mg protein (n = 7), respectively. These values are not significantly different from control vessels (P > 0.05). In the presence of milrinone (10\(^{-4}\) M) plus rolipram (10\(^{-4}\) M), the cAMP content was 36.8 ± 3.1 pmol/mg protein (n = 7), which is significantly different from control vessels (P < 0.05).

PGE2 and forskolin induced a concentration-dependent increase in the intracellular cAMP content (Fig. 5). PGE2 and forskolin at lower concentrations (3 × 10\(^{-9}\) M and 3 × 10\(^{-8}\) M, respectively) had no significant effect on cAMP content of control vessels but increased the cAMP content of vessels treated with milrinone (10\(^{-4}\) M), rolipram (3 × 10\(^{-5}\) M), or milrinone (10\(^{-4}\) M) plus rolipram (10\(^{-4}\) M). The increase in cAMP content obtained with the combination of milrinone and rolipram was significantly greater than the sum of that with either inhibitor alone (Fig. 6).

In the presence and absence of 8M-IBMX (10\(^{-4}\) M), the increase in cAMP in response to PGE2 (3 × 10\(^{-8}\) M) was 4.9 ± 0.8 pmol/mg protein (n = 6) vs. 4.5 ± 0.9 pmol/mg protein (n = 6). In the presence and absence of 8M-IBMX (10\(^{-4}\) M), the increase in cAMP in response to forskolin (3 × 10\(^{-7}\) M) was 10.1 ± 1.7 pmol/mg protein (n = 6) vs. 12.1 ± 1.3 pmol/mg protein (n = 6). There is no significant difference between control and 8M-IBMX-treated vessels in the change in cAMP content to PGE2 and forskolin (P < 0.05).
ACh (3 × 10^{-5} M) had no effect on cAMP content of pulmonary arteries. However, in the presence of ACh, the increase in cAMP caused by PGE_2 (3 × 10^{-8} M) or forskolin (3 × 10^{-7} M) was significantly enhanced in vessels with endothelium but not in vessels without endothelium and not in vessels with endothelium treated with N^G-nitro-L-arginine (10^{-4} M) (Fig. 7). The cGMP content of pulmonary arteries with endothelium was 4.4 ± 1.7 pmol/mg protein (n = 7), which was significantly different from arteries with endothelium treated with N^G-nitro-L-arginine (10^{-4} M; 0.8 ± 0.3 pmol/mg protein, n = 7; P < 0.05) and arteries without endothelium (0.6 ± 0.3 pmol/mg protein, n = 7; P < 0.05). ACh (3 × 10^{-5} M) caused a significant increase in the intracellular content of cGMP of pulmonary arteries (15.1 ± 3.5 pmol/mg protein; n = 7, P < 0.05) but had no effect on cAMP content of arteries with endothelium treated with N^G-nitro-L-arginine (3 × 10^{-5} M; n = 7) and that of arteries without endothelium (n = 7).

DISCUSSION

In vascular smooth muscle, PGE_2 induces relaxation primarily by activating adenylyl cyclase and thus increasing the intracellular cAMP content (1, 8). Because cAMP is hydrolyzed by PDEs, the inhibition of these enzymes with selective inhibitors augments relaxation mediated by cAMP (29). In our study, both the amount of relaxation and the increase in cAMP content of pulmonary arteries induced by PGE_2 and forskolin [a direct activator of adenylyl cyclase (20)] were enhanced by milrinone and rolipram, selective PDE3 and PDE4 inhibitors, respectively (21, 32, 34, 35). However, 8M-IBMX, a PDE1 inhibitor (3, 36), had no significant effect on the PGE_2- and forskolin-induced responses. Hence, it seems that both PDE3 and PDE4 are important enzymes hydrolyzing cAMP in pulmonary arteries of newborn lambs, but PDE1 may not be playing a significant role in cAMP metabolism in these vessels. In the human pulmonary artery, PDE3 and PDE4 are the major enzymes hydrolyzing cAMP (30).

Although milrinone and rolipram (inhibitors of PDE3 and PDE4, respectively) significantly augmented the increase in cAMP content induced by PGE_2 and forskolin, these inhibitors did not affect the basal cAMP. A number of studies suggest that a change in cAMP content occurs in the subcellular locations (17, 19, 31). Thus, if an increase in cAMP is small (such as the change in basal content after PDE inhibitors), the increase in the basal cAMP content might not be detected with the current methods employed. However, such an increase in cAMP content might be sufficient to modify the response of the smooth muscle. This might explain the observation that pulmonary vessels treated with milrinone or rolipram required a higher concentration of endothelin to elicit the similar contraction in comparison with control vessels (10^{-8} vs. 3 × 10^{-9} M).
It is noted that, when milrinone plus rolipram were present, the basal cAMP content increased and even a higher concentration of endothelin was needed to elicit the similar contraction in comparison to vessels treated with milrinone or rolipram alone (10^{-7} vs. 10^{-8} M).

In our study, treatment with both PED3 and PDE4 inhibitors caused a greater relaxation and a greater increase in cAMP content of pulmonary arteries to PGE\(_2\) and forskolin than the sum of the responses induced by either of these inhibitors used alone. It is likely that, when one of the cAMP-hydrolyzing PDE isozymes is inhibited, the other one may compensate in the hydrolysis of cAMP (2, 29). When both PDE3 and PDE4 were inhibited, the ability of the vessels to degrade cAMP would be greatly restricted. Consequently, PGE\(_2\) and forskolin induced markedly greater relaxation and a greater increase in cAMP content when both PDE3 and PDE4 inhibitors were used together. Inhibitors of PDE3 and PDE4 cause isolated human pulmonary arteries to relax (28, 30). In an in vivo study, milrinone reduced pulmonary arterial pressure and pulmonary vascular resistance in neonates (6). In isolated rabbit lungs, rolipram reverses pulmonary vasoconstriction induced by platelet-activating factor (27). Because both PDE3 and PDE4 are active in pulmonary vessels, our present results suggest that a combined use of PDE3 and PDE4 inhibitors would result in a greater effect than the sum of the effect caused by use of PDE3 inhibitor or PDE4 inhibitor alone. Also, the combined use of different subtype PDE inhibitors may reduce the dose of each inhibitor required and thus reduce the side effects of these drugs.

In the present study, the concentrations of PGE\(_2\) and forskolin that induced similar degree of relaxation of pulmonary arteries did not increase cAMP content by a similar amount. Forskolin induced a greater increase in cAMP content. Such a phenomenon has also been reported in other types of smooth muscles. It is thought that this is related to the fact that there are multiple subcellular compartments of cAMP. Some of the cAMP elevated after stimulation with forskolin may be in subcellular compartments that are not accessible to the protein kinase A that is involved in vasodilation (25, 37).

The activity of PDE3 can be inhibited by cGMP (2, 29). Therefore, an increase in cGMP in vascular smooth muscle after stimulation with nitric oxide or nitrovasodilators may augment cAMP-mediated vasodilation in response to agents such as PGE\(_2\) and \(\beta\)-adrenergic agonists. In this study, we raised intracellular cGMP of pulmonary arteries with ACh. In pulmonary vessels of newborn lambs, the endothelium-dependent response induced by ACh was abolished by N\(^{-2}\)-nitro-L-arginine (14). Thus the endothelium-dependent response of pulmonary arteries of newborn lambs to ACh is likely to be mainly mediated by EDNO, and the increase in cAMP content after ACh is due to the release of EDNO (18, 24, 26). After pretreatment with ACh, the increase in cAMP in pulmonary arteries induced by PGE\(_2\) and forskolin was markedly augmented. ACh alone had no effect on cAMP content in the vessels. Hence, the augmented increase in cAMP as well as augmented relaxation to PGE\(_2\) and forskolin can be best explained by an inhibition of PDE3 by the endothelium-dependent increase in cGMP caused by ACh. Such a synergistic action between cGMP and cAMP has also been implied in isolated rat aortas (11, 16, 23) and in perfused rabbit lungs (7).

In perinatal lungs, both cGMP pathway and cAMP pathway play an important role in modulating the response of pulmonary vessels (12, 14, 22, 33). For instance, the production of EDNO and vasodilator prostaglandins (PGI\(_2\) and PGE\(_2\)) is stimulated by an increase in oxygen tension occurring after birth (9, 33). PGI\(_2\) and PGE\(_2\) cause vasodilation by activating adenylyl cyclase and elevating cAMP (8). By a cGMP-mediated inhibition of PDE3, EDNO may augment PGI\(_2\) and PGE\(_2\)-mediated vasodilation of perinatal lungs (1, 10, 29).

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18 PHOSPHODIESTERASES AND PULMONARY ARTERIES


