Effects of a Prostaglandin I$_2$ Analog Iloprost on Cytoplasmic Ca$^{2+}$ Levels and Muscle Contraction in Isolated Guinea Pig Aorta

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Received September 18, 1995   Accepted April 17, 1996

ABSTRACT - In the isolated guinea pig aorta, the prostaglandin I$_2$ analog iloprost (0.01–10 μM) inhibited the contractions induced by the thromboxane A$_2$ analog U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin F$_{2\alpha}$; 30 nM) and prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$, 1 μM) in a concentration-dependent manner. In contrast, iloprost only partially inhibited the high K$^+$ (65.4 mM)-induced contraction. In the muscle stimulated with high K$^+$, verapamil (0.3 and 10 μM) inhibited [Ca$^{2+}$]$_i$ and muscle tension in parallel, whereas iloprost (1 μM) inhibited muscle tension with only a small decrease in [Ca$^{2+}$]$_i$. In the muscle stimulated with U46619 (30 nM), verapamil and iloprost decreased both [Ca$^{2+}$]$_i$ and muscle tension. However, as compared with the effect of verapamil, iloprost more strongly inhibited muscle tension than [Ca$^{2+}$]$_i$. The iloprost (0.1–1 μM)-induced relaxation was accompanied by a concentration-dependent increase in cAMP content. It was further demonstrated that inhibition of the U46619-contractions was augmented in the presence of cycloxygenase inhibitors, such as indomethacin (10 μM), ibuprofen (10 μM) and aspirin (10 μM). In contrast, the inhibition of PGF$_{2\alpha}$-induced contraction was not affected by indomethacin. Similarly, the inhibitory effect of forskolin on U46619-induced contractions, but not on PGF$_{2\alpha}$-induced contraction, was enhanced by indomethacin. These results suggest that iloprost inhibits vascular smooth muscle contraction by decreasing [Ca$^{2+}$]$_i$ and the Ca$^{2+}$ sensitivity of contractile elements through a cAMP-dependent mechanism. The results also suggest that in U46619-stimulated muscle, vasoactive prostaglandins that counterbalance the relaxing action of cAMP may be generated.

Keywords: Iloprost, Aorta (guinea pig), cAMP, Relaxation

Prostacyclin (prostaglandin I$_2$: PGI$_2$) and nitric oxide are endothelium-derived vasodilators that play an important role in the regulation of vascular tone. It has been reported that a stable PGI$_2$ analog iloprost inhibits vascular smooth muscle contractions in vitro (1–3), which may be mediated by an elevation of cAMP (4). In isolated vascular smooth muscles, it has been reported that forskolin, an activator of adenylate cyclase, inhibits smooth muscle contraction not only by decreasing the cytoplasmic Ca$^{2+}$ level ([Ca$^{2+}$]$_i$) but also by decreasing the Ca$^{2+}$-sensitivity of contractile elements (5). Similar results have been obtained with the membrane permeable analog of cAMP, dibutyryl cyclic AMP, in rat aorta (6) and with isoproterenol in porcine coronary artery (7). In tracheal (8), gastric (9) and intestinal smooth muscle (10), it has also been reported that activation of the receptor-coupled adenylate cyclase by agonists, such as isoproterenol, vasoactive intestinal peptide and calcitonin gene-related peptide, increases cAMP and inhibits smooth muscle contraction by either decreasing [Ca$^{2+}$]$_i$ or decreasing the Ca$^{2+}$-sensitivity of contractile elements. To understand the effect of PGI$_2$ on [Ca$^{2+}$]$_i$ and the Ca$^{2+}$ sensitivity of vascular smooth muscle, we have examined the effects of iloprost on [Ca$^{2+}$]$_i$ and muscle force in the isolated guinea pig aorta. Results indicated that iloprost increased cAMP and inhibited smooth muscle contraction by decreasing both [Ca$^{2+}$]$_i$ and the Ca$^{2+}$ sensitivity of contractile elements. We also found that a thromboxane A$_2$ analog may generate a vasoconstricting cycloxygenase product that counterbalances the relaxing effects of cAMP.
MATERIALS AND METHODS

Preparations, solutions and measurement of muscle tension

The thoracic aorta was isolated from male guinea pigs (150–200 g; Shiraishi Laboratory Animals, Tokyo), cut into spiral strips (2-mm-wide, 10-mm-long) and placed in physiological salt solution (PSS) containing: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 23.8 mM NaHCO₃, 0.01 mM ethylene diamine tetraacetic acid (EDTA) and 5.5 mM glucose. The endothelium was removed by gently rubbing the intimal surface with the flat face of forceps moistened with PSS. High K⁺ solution was made by substituting NaCl with equimolar KCl. These solutions were saturated with a 95% O₂-5% CO₂ mixture at 37°C and pH 7.4. Muscle tension was recorded isometrically with a force-displacement transducer. Each muscle strip was attached to a holder under a resting tension of 10 mN and then equilibrated for 60 to 90 min in a 10-ml muscle bath until the contractile response to high K⁺ solution (65.4 mM) became stable. The high K⁺ (65.4 mM)-induced contraction was considered to be the reference response (100%).

Simultaneous measurement of muscle tension and [Ca²⁺]i

[Ca²⁺], was measured as reported by Ozaki et al. (11) and Sato et al. (12) with the fluorescent Ca²⁺ indicator fura-PE3, which leaks out of the cells more slowly than fura-2. The use of fura-PE3 enabled us to measure [Ca²⁺], for hours without a significant decline of fluorescence (13). Muscle strips were treated with the acetoxyethyl ester of fura-PE3 (fura-PE3/AM, 10 μM) for 5–6 hr at room temperature. A noncytotoxic detergent, cremophor EL (0.02%), was added to increase the solubility of fura-PE3/AM. After loading, the muscle strip was washed with PSS at 37°C for 20 min to remove uncleaved fura-PE3/AM and was held horizontally in a temperature-controlled, 7-ml organ bath. One end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction. The muscle strip was illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The intensity of 500 nm fluorescence (F340 and F380) was measured by using a fluorimeter (CAF100; Jasco, Tokyo). The ratio of F340 to F380 was calculated as an indicator of [Ca²⁺]. The absolute Ca²⁺ concentration was not calculated in this experiment because the dissociation constant of the fluorescent indicator for Ca²⁺ in the cytosol may be different from that obtained in vitro (14, 15). Therefore, the ratio obtained in resting and high K⁺ (72.4 mM)-stimulated muscle was taken as 0 and 100%, respectively.

Measurement of cAMP content

cAMP content in guinea pig aorta was measured by enzyme immunoassay. After an incubation, muscle strips were frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. Trichloroacetic acid in the supernatant after centrifugation was removed by washing with water-saturated ether. cAMP was assayed by a competitive enzyme immunoassay with cAMP peroxidase conjugate.

Chemicals

The chemicals used were norepinephrine bitartrate (Wako Pure Chemicals, Osaka); PGF₂α, indomethacin, aspirin, ibuprofen, verapamil hydrochloride (Sigma Chemical, St. Louis, MO, USA); cremophor EL (Nacalai Tesque, Kyoto); cAMP enzyme immunoassay system (Amersham Japan, Tokyo) and fura-PE3/AM (Texas Fluorescence Laboratory, Austin, TX, USA). Iloprost and U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂α) were generous gifts from Eisai Co. (Tokyo) and Ono Pharmaceutical Co. (Osaka), respectively.

Statistics

The results of the experiments are expressed as the mean ± S.E.M. Student’s t-test or analysis of variance (ANOVA, when a comparison involved more than two groups) was used for statistical analyses of the data. A P value less than 0.05 was considered to be statistically significant.

RESULTS

In the isolated guinea pig aorta, high K⁺ (65.4 mM), PGF₂α (1 μM) and U46619 (30 nM) induced sustained contractions. Iloprost (1 nM–1 pM) was added after the contractions reached a steady level. Iloprost inhibited the contractions induced by PGF₂α and U46619 in a concentration-dependent manner (Fig. 1). The contraction induced by PGF₂α (1 μM) inhibited the high K⁺-induced contraction by 2.3±0.8% and 26.4±2.9%, respectively (n=4). On the other hand, iloprost showed only a small inhibitory effect on the high K⁺-induced contraction.

Figure 2 shows the changes in [Ca²⁺], and muscle tension induced by the addition of high K⁺ solution, U46619 or PGF₂α, in the fura-PE3 loaded guinea pig aorta. Addition of 72.7 mM KCl, 30 nM U46619 or 1 μM PGF₂α induced an initial transient increase followed by a sustained increase in [Ca²⁺], and sustained contraction. Iloprost (1 μM) inhibited the high K⁺-induced increase in [Ca²⁺], and contraction by 2.3±0.8% and 26.4±2.9%, respectively (n=4). On the other hand, iloprost inhibited in parallel the increases in [Ca²⁺], and contractions induced
Iloprost and PGF$_2\alpha$ inhibited the U46619-induced increases in $[\text{Ca}^{2+}]_i$ and contraction by 83.4±7.70% and 58.0±5.0%, respectively (n=4), and inhibited the PGF$_2\alpha$-induced increases in $[\text{Ca}^{2+}]_i$ and contraction by 97.8±1.40% and 100±0%, respectively (n=4).

We also examined the effect of verapamil (0.3 and 1 μM) on $[\text{Ca}^{2+}]_i$ and muscle tension stimulated by high K$^+$ (72.7 mM) or U46619 (30 nM). In the presence of high K$^+$, 0.3 μM verapamil inhibited $[\text{Ca}^{2+}]_i$ and muscle tension by 47.9±2.1% and 31.4±3.0% (n=4), respectively. Verapamil (1 μM) inhibited $[\text{Ca}^{2+}]_i$ and muscle tension by 77.6±13.9% and 73.7±9.0%, respectively (n=4). In the presence of U46619, 1 μM verapamil inhibited $[\text{Ca}^{2+}]_i$ and muscle tension by 76.2±6.8% and 16.2±0.5%, respectively.

Fig. 1. Concentration-response relationship for the inhibitory effects of iloprost on the contractions induced by high K$^+$ (65.4 mM, ○), PGF$_2\alpha$ (1 μM, □) and U46619 (30 nM, ▽) in the guinea pig aorta. Iloprost (1 nM to 1 μM) was cumulatively added after the contractions reached a steady state. Each point represents the mean of 4 experiments, and the S.E.M. is shown by a vertical bar.

Fig. 2. The effect of iloprost (1 μM) on $[\text{Ca}^{2+}]_i$ (upper trace) and muscle tension (lower trace) in the guinea pig aorta stimulated by high K$^+$ (72.7 mM) (A), U46619 (30 nM) (B) and PGF$_2\alpha$ (1 μM) (C). 100% represents the steady state $[\text{Ca}^{2+}]_i$ in the presence of 72.7 mM K$^+$. WO: washout.
respectively (n = 4).

Figure 3 summarizes the effect of iloprost and verapamil on [Ca^{2+}] and muscle tension in the presence of high K\(^+\) (72.7 mM) or U46619 (30 nM). After the [Ca^{2+}] and contraction reached maximum, iloprost (1 pM, Ilo) or verapamil (0.3 μM or 1 μM, Ver) was added. 100% represents the steady state [Ca^{2+}] in the presence of 72.7 mM K\(^+\). Each point represents the mean of 4 experiments, and the S.E.M. is shown by a vertical and horizontal bars.

The effect of forskolin on the U46619-induced contraction was also enhanced by indomethacin, while the effect on the PGF\(_{2α}\)-induced contraction was not (Fig. 6: A and B).

DISCUSSION

Iloprost inhibited the contractions induced by U46619, PGF\(_{2α}\) and KCl in the isolated guinea pig aorta. The order of the inhibitory potency of iloprost was PGF\(_{2α}\) >
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**Fig. 5.** Effect of indomethacin on the concentration response relationship for the inhibitory effect of iloprost on the contractions induced by U46619 (30 nM) (A) and PGF$_2$$_\alpha$ (1 nM) (B). Iloprost (1 nM - 1 µM) was cumulatively added after the contractions reached a steady state in the absence (●) or presence (○) of indomethacin (10 µM). Indomethacin was added 15 min before the addition of stimulants. Each point represents the mean of 4 to 5 experiments, and the S.E.M. is shown by a vertical bar. **: Significantly different from the control with $P<0.01$.

**Fig. 6.** Effect of indomethacin on the concentration response relationship for the inhibitory effect of forskolin on the contractions induced by U46619 (30 nM) (A) and PGF$_2$$_\alpha$ (1 nM) (B). Forskolin (1 nM - 1 µM) was cumulatively added after the contractions reached a steady state in the absence (●) or presence (○) of indomethacin (10 µM). Indomethacin was added 15 min before the addition of stimulants. Each point represents the mean of 4 experiments, and the S.E.M. is shown by a vertical bar. *, **: Significantly different from the control with $P<0.05$ and $P<0.01$, respectively.

**U46619 > KCl.** Measurement of [Ca$^{2+}$] showed that iloprost inhibited the high K$^+$-induced contraction with little effect on [Ca$^{2+}$]. These results suggest that iloprost inhibits the high K$^+$-induced contraction by decreasing the Ca$^{2+}$ sensitivity of contractile elements. U46619 induced a greater contraction than high K$^+$ at a given [Ca$^{2+}$], suggesting that U46619 increases the Ca$^{2+}$ sensitivity of contractile elements. In the presence of U46619, iloprost inhibited the contraction by decreasing the [Ca$^{2+}$]. In comparison to the effect of verapamil, iloprost inhibited muscle tension more strongly than [Ca$^{2+}$], suggesting that iloprost inhibits the U46619-induced contraction not only by decreasing the [Ca$^{2+}$], but also by decreasing the Ca$^{2+}$ sensitivity of contractile elements.
elements. Iloprost showed similar effects on [Ca$$^{2+}$$], and contraction stimulated by PGF$_{2\alpha}$.

Furthermore, the present study showed that iloprost increased the cAMP content in a concentration-dependent manner. The effects of iloprost are similar to those of forskolin and the membrane permeable cAMP analogues that inhibit contraction not only by decreasing [Ca$$^{2+}$$], but also by decreasing the Ca$$^{2+}$$ sensitivity of contractile elements (5, 6). From these results, we concluded that the inhibitory effect of iloprost is mediated by the increase in cAMP.

With regards to the mechanisms of cAMP-induced decrease in Ca$$^{2+}$$ sensitivity of contractile elements, Sellers and Adelstein (16) suggested that gizzard myosin light chain kinase is phosphorylated by protein kinase A and that this phosphorylation reduces the affinity of myosin light chain kinase for the Ca$$^{2+}$$/calmodulin complex. In contrast, Stull et al. (17) suggested that this mechanism may not be physiologically important in smooth muscle. Further studies are necessary to clarify the effect of cAMP on the Ca$$^{2+}$$ sensitivity of contractile elements.

There are several mechanisms for the relaxant action of cAMP. It has been reported that the open probability of Ca$$^{2+}$$-activated K$^+$ channels is increased by the catalytic subunit of protein kinase A in tracheal (18) and colonic smooth muscle cells (19). It is therefore possible that the increase in cAMP production in response to iloprost might activate K$^+$ channels via protein kinase A-mediated protein phosphorylation and hyperpolarize the membrane. Since high K$^+$ solution counteracts the hyperpolarizing effect of K$^+$ channel opening, this may be the reason why iloprost preferably inhibited the agonist-induced contractions over the high K$^+$-induced contraction.

Another explanation for the preferential inhibitory effect of iloprost on receptor agonists-mediated responses is the inhibition of receptor-mediated signal transduction resulting in decreasing of [Ca$$^{2+}$$], (20). Inhibition by forskolin and dibutylryl cAMP of phosphatidyl-inositol hydrolysis has been demonstrated in tracheal (21) and vascular smooth muscles (22). In addition, activation of protein kinase A by forskolin in canine colonic smooth muscle desensitizes muscarinic receptors and inhibits phosphatidil-inositol turnover (23). The decrease in [Ca$$^{2+}$$], may also be due to the activation of sarcoplasmatic Ca$$^{2+}$$ uptake induced by cAMP (24), but not to the inhibition of L type Ca$$^{2+}$$ channels (22).

In the presence of a cyclooxygenase inhibitor, indomethacin, the relaxing effect of iloprost on U46619-induced contraction was greatly enhanced. Other cyclooxygenase inhibitors, such as aspirin and ibuprofen, showed similar effects. Furthermore, the effects of forskolin on U46619-induced contraction were also augmented by indomethacin. These results suggest that, in the U46619-stimulated muscle, vasoactive prostaglandins may be produced that counterbalance the relaxing action of cAMP.

Acknowledgments

This work was partly supported by a Grant-in-Aid for Scientific Research from Ministry of Education, Science, Sports and Culture, Japan. We thank Eisai Co. and Ono Pharmaceutical Co. for supplying iloprost and U46619, respectively.

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