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6-KETO-PROSTAGLANDIN E_1-SENSITIVE ADENYLATE CYCLASE AND BINDING SITES IN MEMBRANES FROM PLATELETS AND CULTURED SMOOTH MUSCLE CELLS

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Abstract—6-keto-PGE_1 elicits the same biological effects as PGI_2 in human platelets and in rabbit aorta and mesenteric artery, being, however, less potent. We report here that 6-keto-PGE_1 dose-dependently stimulates adenylate cyclase activity in membranes of human platelets and cultured myocytes from rabbit aorta and mesenteric artery. The extent of stimulation of the enzyme by 6-keto-PGE_1 is the same as elicited by PGI_2, while the apparent affinity is lower than that of prostacyclin, both in platelets and in vascular smooth muscle cells. At the level of platelet membranes, 6-keto-PGE_1 interacts with the binding sites labelled by PGI_2. However, in platelets as well as in mesenteric artery myocytes, 6-keto-PGE_1 interacts with only one class of sites as demonstrated either by binding or by adenylate cyclase studies, whereas PGI_2 in the same conditions recognizes two different classes.

It has been recently suggested that prostacyclin (PGI_2), or its hydrolysis product 6-keto-prostaglandin (PG)$F_{1\alpha}$, can be converted into the chemically stable compound 6-keto-PGE_1 in liver [1, 2], kidney [3], and platelets [4–6]. The actual extent of the conversion of PGI_2 to 6-keto-PGE_1 is still debated [7, 8]; there is no doubt, however, that 6-keto-PGE_1 can mimic PGI_2 in eliciting a number of biological effects. In particular, 6-keto-PGE_1 has been demonstrated to be a potent vasodilator, especially of the mesenteric vascular bed [9, 10], an activator of cholesteryl ester hydrolase in aortic smooth muscle cells [11] and an inhibitor of platelet aggregation [1, 12, 13].

Since all the above-mentioned effects are supposed to be mediated by an increase in intracellular cAMP levels, we have investigated whether 6-keto-PGE_1 can activate adenylate cyclase (E.C. 4.6.1.1.) in membranes from vascular smooth muscle cells and platelets.

The similar pattern of biological action elicited by PGI_2 and 6-keto-PGE_1 might suggest that they share a common receptor. Since no direct evidence supporting this hypothesis has yet been reported, it was also decided to investigate whether 6-keto-PGE_1 interacts with the same binding sites as PGI_2.

MATERIALS AND METHODS

Materials. [8-^{14}C]ATP, [8-^{3}H]cAMP and [9-^{3}H] PGI_2 were from New England Nuclear, Boston, MA, U.S.A.; ATP, cAMP, GTP, creatine phosphate and creatine phosphokinase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Prostacyclin and 6-keto-PGE_1 were supplied by The Wellcome Research Laboratories, Beckenham, U.K. and the Upjohn Co., Kalamazoo, MI, U.S.A., respectively. The solutions of PGI_2, which was stored in ethanol at −20°, were freshly prepared immediately before use in 10 mM Tris–HCl buffer, pH 8. 6-keto-PGE_1 was dissolved in the same buffer.

The purity of [9-^{3}H]PGI_2 was checked by thin-layer chromatography on silica gel G with two different solvent systems (water-saturated ethyl acetate: formic acid, 50:1; ethyl acetate:trimethilpentane:methanol:acetic acid:water, 110:30:35:25:100, organic phase) and was greater than 95%.

Eagle's minimum essential medium F 11, fetal calf serum, trypsin–EDTA, penicillin (10,000 U/ml), streptomycin (10 mg/ml), tricine buffer (1 M) and non-essential amino acids (100 X) were purchased from Grand Island Biological Co., Madison, WI, U.S.A.; disposable culture flasks and petri dishes were from Corning Glass-works, Amedfield, MA, U.S.A.

Cell cultures. Male white New Zealand rabbits (2–3 kg) were used. Cultures of smooth muscle cells from intima-medial layer of rabbit aorta and mesenteric arteries were prepared according to the method of Ross [14], as previously described [15].

Preparation of membranes. Platelet concentrates (collected in citric acid/sodium citrate/sodium phosphate/dextrose) from 3–4 healthy male volunteers were pooled. A crude membrane preparation (pellet at 27,000 *g*) was prepared as described by Lombroso *et al.* [16].

Smooth muscle cell monolayers from rabbit aorta and mesenteric artery (used between the 8th and 14th passage) were washed in 50 mM Tris–HCl buffer (pH 7.4), harvested by scraping, pooled and the membrane preparation (pellet at 15,000 *g*) was obtained as described by Oliva *et al.* [15].

Adenylate cyclase assay. The standard assay mixture (final volume: 100 μl) contained: 10 mM Tris–HCl buffer (pH 8); 0.10 mM [8-^{14}C]ATP (50 dpm/pmol); 0.5 mM [8-^{3}H]cAMP (approximately 360 dpm/nmol); 2 mM $MgCl_2$; 2 mM creatine phos-

phate; 17 U/ml creatine phosphokinase; 10^{-5} M GTP and the indicated prostaglandins. The incubation, started with the addition of the membrane preparation (0.040–0.090, 0.10–0.20 and 0.06–0.10 mg protein/sample for platelet, aorta and mesenteric membranes, respectively), was carried out at 30° for 8 min. [8-^{3}H]cAMP was included in the assay mixture to permit correction for column loss and for the possible effect of phosphodiesterases [17] which in any case was almost negligible. [8-^{14}C,8-^{3}H]cAMP was isolated and detected according to Salomon *et al.* [18]. Protein concentration was determined according to Bradford [19].

Binding assay. Assays were carried out in a total volume of 250 μl, as described by Lombroso *et al.* [16]. The membrane preparation (350–750 μg protein/sample) was incubated at 30° for 5 min (unless otherwise indicated) in 10 mM Tris–HCl, pH 8, with 3×10^{-8} M [9-^{3}H]PGI_2 (10 Ci/mmole) and different concentrations of unlabelled 6-keto-PGE_1. Specific binding was considered to be that displaced by 10^{-4} M PGE_1 (which binds to the same sites as prostacyclin, see Lombroso *et al.* [16]). Specific binding was 40–60% of total binding. The incubation was terminated by filtration under vacuum on glass-fiber filters (Whatman GF/C) and a rapid washing with 2 × 3 ml of ice-cold 10 mM Tris–HCl, pH 8.

Analysis of data. The data were fitted by an iterative computer program (RECEPT) for nonlinear regression analysis [20] both to a one-site and a two-site model. The equations employed for the analysis of binding data were:

$$B = \frac{\text{Inhib.}_{max} \times [L]}{IC_{50} + [L]}, \text{ and}$$

$$B = \frac{\text{Inhib.}_{max1} \times [L]}{IC_{50_1} + [L]} + \frac{\text{Inhib.}_{max2} \times [L]}{IC_{50_2} + [L]}$$

(for the one-site and two-site model, respectively), where B = bound labelled ligand, Inhib._{max} = maximal %-inhibition, [L] = concentration of unlabelled ligand, IC_{50} = concentration of unlabelled ligand inhibiting 50% of binding.

The equations employed for the analysis of adenylate cyclase stimulation were:

$$\Delta V = \frac{\Delta V_{max} \times [L]}{K_{Dapp} + [L]};$$

$$\Delta V = \frac{\Delta V_{max1} \times [L]}{K_{D1app} + [L]} + \frac{\Delta V_{max2} \times [L]}{K_{D2app} + [L]}$$

(for the one-site and the two-site models, respectively), where ΔV = increase of enzyme activity over basal value, ΔV_{max} = maximal increase of enzyme activity, [L] = concentration of activating agent, K_{Dapp} = apparent dissociation constant. The two-site model was considered more appropriate when the correlation coefficient was higher than for the one-site model, and the improvement of goodness-of-fit was statistically significant on the basis of an F-test on the sums of squared errors. Data shown are means ± S.D. of three replicates from single experiments representative of at least two other ones.

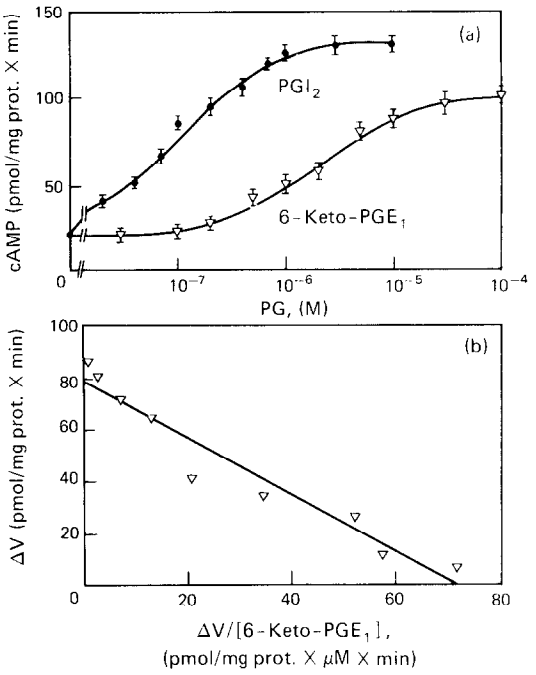

Fig. 1. Dose–response curves (a) and Eadie–Hofstee plot (b) for the activation of adenylate cyclase in human platelet membranes. ΔV represents the increase in adenylate cyclase activity elicited by 6-keto-PGE_1 above basal activity, which was 15.41 ± 1.44 pmol cAMP/mg protein × min.

RESULTS

Human platelets. As shown in Fig. 1a, 6-keto-PGE_1 is able to activate adenylate cyclase in a dose-dependent fashion in human platelet membranes. The maximal stimulation (6.6-fold) was attained at 3×10^{-5} M and in some, but not in all the platelet preparations it was slightly lower than that obtained with PGI_2. The concentration eliciting half-maximal stimulation ($EC_{50} \simeq 1.6 \times 10^{-6}$ M) was higher by approximately one order of magnitude than that of prostacyclin ($EC_{50} \simeq 1.1 \times 10^{-7}$ M). Computer analysis of the dose–response curve for 6-keto-PGE_1 indicated that the enzyme is activated through interaction of this PG with a single class of components, as suggested also by the Eadie–Hofstee plot (Fig. 1b) which is better fitted by a single straight line.

6-keto-PGE_1 competed with [^{3}H]PGI_2 for the binding sites. As shown in Fig. 2a, 6-keto-PGE_1 dose-dependently inhibited [^{3}H]PGI_2 specific binding, reaching the half-maximal value (IC_{50}) at 4.7×10^{-7} M. Inhibition of [^{3}H]PGI_2 binding was practically complete at 10^{-5} M 6-keto-PGE_1. The competition curve for unlabelled PGI_2 is shown for comparison. Computer analysis indicated that the competition curves, as well as the Eadie–Hofstee plot (modified Scatchard plot, Fig. 2b), are better fitted by the curve based on a one-site model.

Smooth muscle cells. In membranes of cultured smooth muscle cells from rabbit mesenteric artery (Fig. 3a) and aorta (Fig. 3b), 6-keto-PGE_1 dose-dependently stimulated adenylate cyclase. The

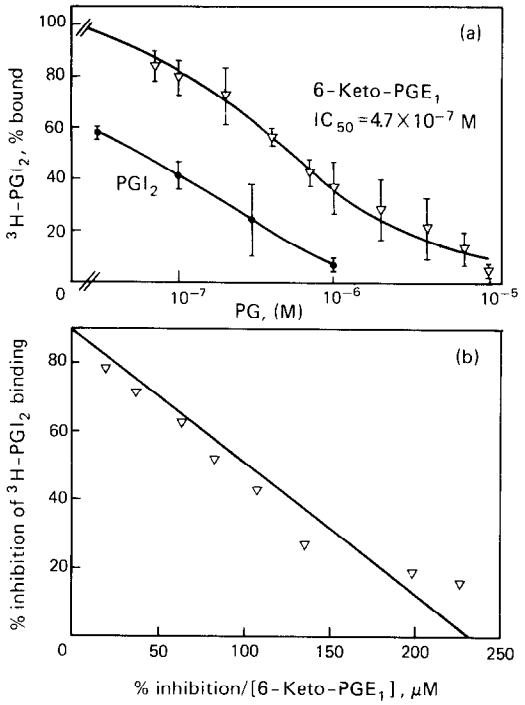

Fig. 2. Inhibition of [^{3}H]PGI_2 specific binding by 6-keto-PGE_1 and PGI_2 (a) and Eadie–Hofstee plot for the inhibition of [^{3}H]PGI_2 specific binding by 6-keto-PGE_1 (b) in human platelet membranes. 100% [^{3}H]PGI_2 specific binding was 0.49 ± 0.03 pmol/mg protein.

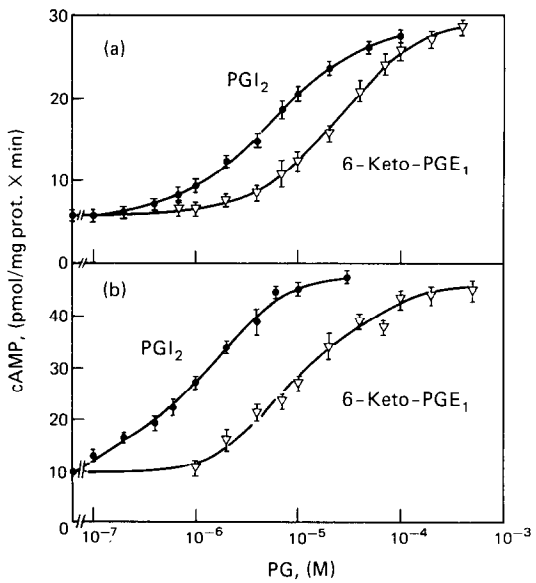

Fig. 3. Dose–response curves for the activation of adenylate cyclase by PGI_2 and 6-keto-PGE_1 in membranes from rabbit mesenteric artery (a) and aorta (b).

apparent affinity was lower for 6-keto-PGE_1 than for PGI_2. In fact, in mesenteric artery membranes, the EC_{50}s were 2.2×10^{-5} and 5×10^{-6} M for 6-keto-PGE_1 and PGI_2, respectively. The maximal stimulation attained was the same (4.6-fold) for the two PGs, and the curves were approximately parallel.

A similar pattern was obtained in membranes from rabbit aorta myocytes, and the EC_{50}s were 9 and 1.2×10^{-6} M for 6-keto-PGE_1 and PGI_2, respectively.

As in the case of platelet adenylate cyclase, also in smooth muscle cells the dose–response curves for 6-keto-PGE_1 are better fitted by the computer-generated curve based on the one-site model. This is in agreement with the linear Eadie–Hofstee plots obtained in cells from both blood vessels (Fig. 4a and b).

Direct binding studies were performed in membranes from smooth muscle cells; however, it was not possible to reveal any specific binding of [^{3}H]-PGI_2.

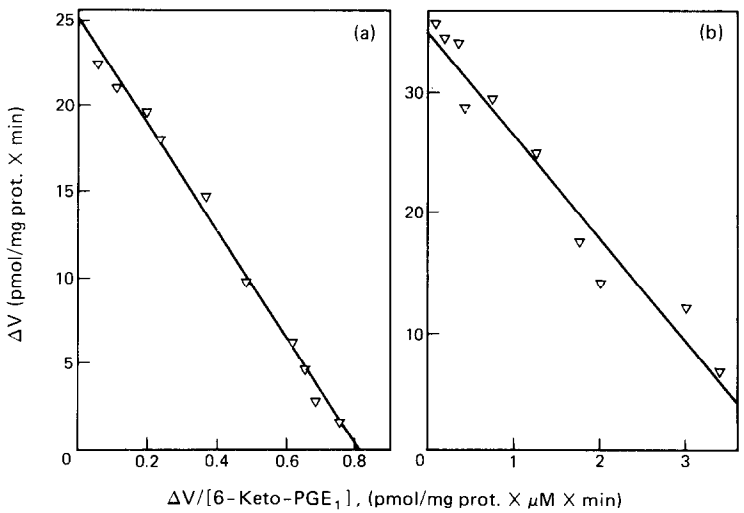

Fig. 4. Eadie–Hofstee plots for the activation of adenylate cyclase by 6-keto-PGE_1 in membranes from rabbit mesenteric artery (a) and aorta (b). ΔV represents the increase in adenylate cyclase activity elicited by 6-keto-PGE_1 above basal activity (6.08 ± 0.32 and 9.64 ± 0.43) pmol cAMP/mg protein × min in membranes from mesenteric artery and aorta, respectively).

DISCUSSION

We demonstrate here that 6-keto-PGE_1 stimulates adenylate cyclase activity in membranes of both human platelets and smooth muscle cells from rabbit aorta and mesenteric artery.

The enzyme activation seems to be mediated, at least in platelets, by the interaction of 6-keto-PGE_1 with the receptors specific for PGI_2. In fact, 6-keto-PGE_1 competes with [^{3}H]PGI_2 for its binding sites, with an IC_{50} ten-fold higher than that of PGI_2 [16]. Previous results obtained with various stable prostaglandins [16] suggest that the binding sites labelled by [^{3}H]PGI_2 might represent the receptors involved in the activation of adenylate cyclase. This hypothesis is supported also by the present finding that the potency ratios of 6-keto-PGE_1 and PGI_2 for the inhibition of [^{3}H]PGI_2 binding and for the activation of adenylate cyclase are almost identical. Thus, at the platelet level the biological action of 6-keto-PGE_1 is likely to be mediated by the prostacyclin receptors.

However, the two prostaglandins exhibit a different pattern of interaction with these binding sites. In fact, 6-keto-PGE_1 interacts with a single class of sites, and, consistently, the activation of adenylate cyclase seems to be brought about by an interaction of the prostaglandin with a single component. On the contrary, PGI_2 has been already demonstrated to recognize two classes of binding sites with different affinities [16, 21, 22] and to stimulate the enzyme through interaction with two components [16]. The fact that [^{3}H]PGI_2-binding is almost completely inhibited by 6-keto-PGE_1, suggests that the latter PG can probably interact with all the sites labelled by PGI_2, being however unable to discriminate between the two classes of sites.

We have not been able to set up a reliable binding assay for [^{3}H]PGI_2 in membranes from cultured myocytes. This failure might have been caused by the much lower apparent affinity that the PGs display in membranes from smooth muscle cells with respect to platelets (see also ref. [15]), as evaluated by the activation of adenylate cyclase. We have, therefore, no direct evidence of 6-keto-PGE_1 interacting with prostacyclin receptors. However, the fact that 6-keto-PGE_1 and PGI_2 stimulate adenylate cyclase activity to the same extent, and that their effects are not additive (data not shown), is consistent with the hypothesis that, in smooth muscle cells from both vessels, these two PGs might share identical receptors.

Should this be the case, then the hypothesis could be put forward that the mode of action of 6-keto-PGE_1 in membranes from rabbit mesenteric artery might be similar to that described for human platelets. In fact, computer analysis of the dose–response curves indicates that 6-keto-PGE_1 activates adenylate cyclase through interaction with a single component of the enzyme system, while PGI_2 seems to act through a higher- and a lower-affinity component in most of the preparations we have examined [15]. Thus, in mesenteric artery myocytes, as in platelets, 6-keto-PGE_1 would not be able to recognize the two classes of components as separate.

In conclusion, the fact that 6-keto-PGF_1 elicits the same biological effects as PGI_2 in human platelets, rabbit aorta and mesenteric artery, though being less potent, might be explained by the two PGs sharing the same mechanism of action (activation of adenylate cyclase), and possibly the same receptors.

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