Prostacyclin synthesis by vascular tissue

Effect of extracellular Ca^{2+}

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Prostacyclin (PGI_2) synthesis in vascular tissue is stimulated by free Ca^{2+} . Concentrations in excess of 4 mM result in a sustained release of PGI_2 , but not other prostanoids, from rat aorta. This effect arises by increased synthesis of PGI_2 and not through the inhibition of its metabolism. Physico-chemical examination of serum and plasma extract has indicated that much of the PGI_2 -stimulating activity reported for human serum derives from the presence of free Ca^{2+} .

INTRODUCTION

Prostacyclin (PGI_2) is a potent vasodilator and anti-aggregatory substance. It is believed to be important in the maintenance of vascular homeostasis in man by limiting thrombus extension at sites of vascular damage (Moncada & Vane, 1979). At neutral pH, PGI_2 is rapidly inactivated by hydrolysis to 6-oxoprostaglandin $\text{F}_{1\alpha}$ (6-oxo- $\text{PGF}_{1\alpha}$). This species, which is biologically inactive, is used as a quantitative marker of PGI_2 formation. It is unlikely that PGI_2 acts locally, as the circulating levels of 6-oxo- $\text{PGF}_{1\alpha}$ in healthy humans are too low ($< 5 \text{ pg/ml}$) for PGI_2 to function as a circulating hormone (Blair *et al.*, 1982). A number of substances have been shown to stimulate PGI_2 biosynthesis *in vitro*, including thrombin (Weksler *et al.*, 1978) and bradykinin (Hong, 1980), as well as endogenous factors in plasma (Remuzzi *et al.*, 1978). Human serum, obtained from plasma clotted through the intrinsic pathway, also stimulates PGI_2 formation (Ritter *et al.*, 1982). This bioactivity was associated with a heat-stable, diffusible 'serum factor'. Bioactivity was not recovered by recalcification of serum dialysis residue, and it was shown that the activity was not associated with other known PGI_2 -stimulating factors. Partial purification of this factor further characterized it as an anionic polar species (Chappell *et al.*, 1985). We report here that this activity is associated with free Ca^{2+} , with pH effects and citrate-complexing accounting for its apparently anomalous chromatographic and biological behaviour.

EXPERIMENTAL

Authentic 6-oxo- $\text{PGF}_{1\alpha}$ was a gift from Dr. John Pike (Upjohn Co. Kalamazoo, MI, U.S.A.). [^{14}C]Citrate and $^{45}\text{CaCl}_2$ were obtained from Amersham International, and [$9\text{-}^3\text{H}$] PGI_2 methyl ester from New England Nuclear. All other chemicals were of Analar grade (BDH Chemicals, Poole, Dorset, U.K.). PGI_2 was generated from the ester by saponification in $50 \mu\text{l}$ of $33 \mu\text{M}$ -NaOH in methanol/water (2:1, v/v) at 40°C for 3 h.

Blood from drug-free volunteers was collected into sodium citrate (0.31%) and the plasma separated by centrifugation (1000 g, 20 min). Half the plasma was

recalcified with 20 mM- CaCl_2 and allowed to clot at 37°C to obtain serum; the other half was used as the control. Active PGI_2 -stimulating factors were assayed on matched groups of fresh rat aortic rings, incubated with plasma or serum at 37°C for 1 h (Ritter *et al.*, 1982). Bioactivity was defined in terms of 6-oxo- $\text{PGF}_{1\alpha}$ accumulation in serum compared with that in the plasma control. 6-Oxo- $\text{PGF}_{1\alpha}$ was measured by radioimmunoassay (Orchard *et al.*, 1982), using antiserum kindly donated by Dr. L. Myatt (Royal Postgraduate Medical School, London W12 0HS, U.K.). The biologically active factor in serum was partially purified as reported previously, by dialysis, gel filtration (Sephadex G-15), anion-exchange adsorption at pH 8.5 and h.p.l.c. (Chappell *et al.*, 1985). H.p.l.c. of 6-oxo- $\text{PGF}_{1\alpha}$ and PGI_2 metabolites was undertaken on a Waters dual-pump system. Samples were chromatographed on a C2 reverse-phase column, elution being at 1 ml/min with 25% (v/v) acetonitrile in aq. 0.034 M- H_3PO_4 . Anion-exchange batch adsorption was carried out on Sephadex A-25 (Pharmacia) at pH 8.5 (50 mM- NH_4HCO_3); activity was eluted at pH 6 with 200 mM-pyridine/acetate buffer and aq. 5% (v/v) acetic acid. Radioactive isotopes were monitored by liquid-scintillation counting in 10 ml of Instagel (Packard Instruments). Free Ca^{2+} in solution was determined with a Calcium Selectrode (F2112 Ca^{2+} ; Radiometer, Copenhagen, Denmark) at constant ionic strength in KCl solution. Prostanoids from rat aortic rings were quantified as their methoxime *O*-trimethylsilyl ether pentafluorobenzyl ester derivatives by g.l.c. electron-capture negative-ion m.s. (Waddell *et al.*, 1984).

RESULTS AND DISCUSSION

The PGI_2 -stimulating behaviour of human serum had previously been shown to be associated with a low- M_r diffusible species (Ritter *et al.*, 1982) that is eluted in the salt region on gel filtration (Sephadex G-10 and G-15) and in the void volume on reverse-phase h.p.l.c. (Chappell *et al.*, 1985). Bioactivity was batch-adsorbed to anion-exchange resin in a pH-dependent manner (Fig. 1a). At pH 8.5 in NH_4HCO_3 buffer, all the bioactivity of dialysed human serum was adsorbed on to Sephadex

Fig. 1. Adsorption of PGI₂-stimulating activity on Sephadex A-25

(a) Adsorption of PGI₂-stimulating bioactivity of human serum after treatment with Sephadex A-25. Activity is indicated by the levels of 6-oxo-PGF_{1α} in serum (hatched bars) compared with the plasma control (open bars). At and above pH 8, bioactivity is adsorbed from solution; the 'basal' production of 6-oxo-PGF_{1α}, observed in the plasma control, arises through the mechanism trauma associated with preparation of the aortic rings. (b) Bioactivity, adsorbed on to Sephadex A-25 at pH 8.5, is eluted at and below pH 6. The starting concentrations of Ca²⁺ in partially purified serum and plasma were 20 mM and < 1 mM respectively.

A-25; activity was eluted from the gel at or below pH 6 in pyridine/acetate buffer (Fig. 1b). These data indicated the presence of anionic functions in the active species. Bioactivity was irreversibly adsorbed on to Sephadex C-25 over a wide range (results not shown).

Further chromatographic purification of this factor was hindered by precipitation of excess calcium. To facilitate purification, calcium was removed by precipitation with a 1.75 molar excess of sodium oxalate. However, this also resulted in complete loss of bioactivity from solution, implicating Ca²⁺ as a possible factor in the stimulatory process. Free Ca²⁺ was then measured in the diffusate of dialysed biologically active serum and inactive plasma; it was present at concentrations of 7–8 mM and < 1 mM respectively.

The effect of Ca²⁺ (7.2 mM) on 6-oxo-PGF_{1α} production by rat aortic rings was investigated. Ca²⁺ had no bioactivity below pH 7; however, at pH 7.4 and above, PGI₂ production was stimulated (Fig. 2). The bioactivity of Ca²⁺ was abolished in the presence of excess sodium oxalate or sodium citrate. The pH of many samples, previously assayed and stored at –20 °C, was determined. In all cases where bioactivity had been present, the solution was above pH 7.5. Further, a correlation was observed between bioactivity and free Ca²⁺ concentration. The threshold concentration at which stimulation was observed was about 1 mM-Ca²⁺, with stimulation consistently occurring at concentrations at and above 4 mM. Maximal stimulation occurred at around 25 mM-Ca²⁺ (cf. Fig. 2, where Ca²⁺ concentration is 7.2 mM). The full dose-response relationship requires further investigation. In a separate series of experiments, serum and plasma were dialysed. In each case, the dialysis residue was at pH 5 and the batch fluid (diffusate) at pH 9, explaining why recalcification of the dialysis residue failed to regenerate bioactivity (Ritter *et al.*, 1982).

In apparent contradiction to the ion-exchange behaviour of the PGI₂-stimulating factor (outlined in Fig. 1), the bioassay data indicated that bioactivity was intimately related to the free Ca²⁺ concentration. To resolve this anomaly, the chromatographic properties of Ca²⁺ were investigated. As expected, Ca²⁺ was eluted in the salt region on gel filtration, and in the void volume on reverse-phase h.p.l.c. Free Ca²⁺ (as ⁴⁵CaCl₂) did not adsorb to A-25 anion-exchange gel; indeed, it would have been most surprising if adsorption had occurred.

Fig. 2. pH profile of the PGI₂-stimulating activity of 7.2 mM-Ca²⁺ (+) and a low-Ca²⁺ control (–) in rat aortic rings incubated in Hepes buffers for 1 h at 37 °C

Stimulation occurs at pH 7.4 and above.

However, in the presence of citrate, 70% of the radiolabel was retained on the resin at pH 8.5 and was eluted at pH 6. In the presence of unlabelled CaCl₂ and [¹⁴C]citrate, radiolabel was only desorbed by 5% (v/v) acetic acid or formic acid at a much lower pH (< 2); unlabelled Ca²⁺ was eluted at pH 6 and was associated with all the bioactivity. Under the conditions of the experiment shown in Fig. 1, the Ca²⁺ concentration in serum supernatant was 20 mM and in plasma < 1 mM at pH 6 and 7. After adsorption at pH 8 and 9 the Ca²⁺ concentration in serum was estimated to be less than 1.1 mM on the basis of ⁴⁵Ca adsorption data.

The major PGI₂-stimulating properties of human serum can be accounted for by the presence of free Ca²⁺. The concentration of free Ca²⁺ in citrated plasma (< 1 mM) is insufficient to stimulate fresh rat aortic rings. Serum, from plasma clotted in the presence of Ca²⁺ and citrate, possesses sufficient free Ca²⁺ (6–8 mM) to stimulate PGI₂ release. Both ions are diffusible and are eluted together in the salt region on gel filtration and in the void volume on reverse-phase h.p.l.c. (leaving the free Ca²⁺ levels unchanged). On anion exchange, the 1:1 Ca²⁺–citrate monocation complex binds at pH 8.5, leaving insufficient free Ca²⁺ in solution (and thus removing bioactivity). At pH 6, Ca²⁺ and citrate dissociate, and calcium desorbs from the gel and bioactivity is recovered. The remaining citrate is not eluted until lower pH and is well separated from the free Ca²⁺.

There may be other factors, present at very low levels in serum, that effect PGI₂ production. For example, a species with weak PGI₂-stimulating activity was observed at the neutral position after paper electrophoresis at pH 6.5 of dialysed serum (Chappell *et al.*, 1985).

The stimulation of PGI₂ production by Ca²⁺ could arise either by increased biosynthesis or by inhibition of metabolism. [9-³H]PGI₂ was added to rat aortic-ring preparations in dialysed serum (containing 5–10 mM free Ca²⁺) and in a dialysed plasma (low Ca²⁺) control. After 1 h incubation, the tissue was removed and the supernatant subjected to reverse-phase h.p.l.c. Threefold stimulation of PGI₂ occurred with serum, although the ³H radioactivity profiles for serum and plasma (i.e. with or without Ca²⁺) were indistinguishable from each other and from that of authentic [9-³H] 6-oxo-PGF_{1α}, which was eluted at 8.2 ml. This demonstrates that extracellular metabolism of prostacyclin or its stable hydrolysis product, 5-oxo-PGF_{1α} had occurred. Thus stimulation occurs by increased PGI₂ synthesis, rather than decreased metabolism.

To determine the specificity of Ca²⁺ challenge on vascular tissue, a multi-prostanoid analysis was undertaken using g.l.c. electron-capture negative-ion m.s. (Waddell *et al.*, 1984). The data are summarized in Table 1. Whereas high-nanogram amounts of PGI₂ were produced, little PGE₂, PGD₂ or thromboxane A₂ were generated. There was no evidence for 6-oxo-PGE₁ or its 13,14-dihydro metabolite. The effect of Ca²⁺ on prostanoid biosynthesis by vascular tissue is very specific for PGI₂.

Ca²⁺ at concentrations only just greater than those

Table 1. Prostanoids generated by rat aortic rings in the presence of serum (+Ca²⁺) and plasma (–Ca²⁺)

Prostanoids (from three experiments) were analysed and quantified by electron-capture negative-ion m.s. as described by Waddell *et al.* (1984). A 4–8-fold stimulation of PGI₂ (measured as 6-oxo-PGF_{1α}) occurred with serum (+Ca²⁺, 5–10 mM) compared with plasma (–Ca²⁺, < 1 mM). Levels of the other prostanoids were much lower, with no evidence of stimulation. There was no evidence for the presence of 6-oxo-PGE₁ or its dihydro metabolite. Abbreviations used: TXB₂, thromboxane B₂; bd, below limit of detection (10 pg); ip, impurity peak masking prostanoid (estimated lower detection limit < 25 pg).

Prostanoid	Prostanoid (ng/mg of tissue)	
	Serum (+Ca ²⁺)	Plasma (–Ca ²⁺)
6-Oxo-PGF _{1α}	16.8	3.8
	15.8	3.2
	25.2	3.2
TXB ₂	bd	bd
	bd	0.53
	bd	0.42
PGE ₂	ip	ip
	ip	0.03
	ip	0.03
PGF _{2α}	0.26	0.80
	0.28	0.14
	0.31	0.15

found in man *in vivo* has a profound effect on the generation of PGI₂ by vascular tissue at physiological pH. The pharmacological consequence of this effect require further investigation.

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