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Factors influencing the corneal permeability of prostaglandin $F_{2\alpha}$ and its isopropyl ester in vitro

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Summary

The corneal uptake and permeability of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester were determined in vitro with a perfusion apparatus using pig cornea. A de-epithelization of the cornea increased the permeability of $PGF_{2\alpha}$, whereas the transport of $PGF_{2\alpha}$ isopropyl ester was decreased. Similar results were obtained in the presence of benzalkonium chloride (0.01%). $PGF_{2\alpha}$ isopropyl ester was hydrolyzed during its permeation and only $PGF_{2\alpha}$ was detected on the endothelial side. The hydrolysis occurred mainly in the epithelium. $PGF_{2\alpha}$ isopropyl ester was more stable to butyrylcholinesterase than $PGF_{2\alpha}$ methyl and benzyl ester. Incubation with cornea homogenate gave the same result. The cholinesterase inhibitor synstigmine methyl sulphate and benzalkonium chloride reduced the hydrolytic activity of the cornea homogenate. Leucine aminopeptidase and alkaline phosphatase activities were observed in the homogenate. These enzymes had no effect on the esters.

Introduction

It has been suggested that topical ocular administration of some prostaglandins (PG) can be the therapy of choice for patients with primary open angle glaucoma. Administration of $PGF_{2\alpha}$ and PGE_2 as eye drop solutions lower the intraocular pressure (IOP) in both healthy (Camras et al., 1977; Camras and Bito, 1981; Stern and Bito, 1981) and glaucomatous animals (Camras and Bito, 1981; Horowitz et al., 1986). An IOP-decreasing effect of $PGF_{2\alpha}$ has even been demonstrated in healthy human volunteers (Giuffré, 1985).

$PGF_{2\alpha}$ esters have been shown to be more potent than the free acid in lowering the IOP (Bito, 1984; Bito et al., 1986). In vitro experiments, with isolated pig cornea, have revealed that $PGF_{2\alpha}$ methyl and benzyl esters act as prodrugs with increased corneal uptake (Camber et al., 1986). The esters are hydrolyzed to $PGF_{2\alpha}$ in the cornea.

The purpose of this study was to investigate some factors influencing the corneal uptake and permeability of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester in an in vitro model. Also, the stability of $PGF_{2\alpha}$ isopropyl ester against enzymatic corneal hydrolysis was compared with other $PGF_{2\alpha}$ esters.

Materials and Methods

The entire eyeglobes of pigs (both male and female of the Yorkshire × Swedish Landrase), aged

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6–7 months, were excised at the local slaughter house (Farmek AB, Uppsala) and transported to the laboratory packed in ice. Immediately after arrival at the laboratory the corneas were excised with a 2-mm ring of the sclera. Some corneas were also de-epithelized, and they were all positioned in the perfusion apparatus within 1 h of death of the animals.

$PGF_{2\alpha}$ was purchased from Chinoin, Hungary, and [9-^{3}H]$PGF_{2\alpha}$ (548 GBq/mmol) from Amersham International plc, Amersham, U.K. $PGF_{2\alpha}$ isopropyl ester, [9-^{3}H]$PGF_{2\alpha}$ isopropyl ester (21 GBq/mmol), [9-^{3}H]$PGF_{2\alpha}$ methyl ester (857 MBq/mmol), and [9-^{3}H]$PGF_{2\alpha}$ benzyl ester (389 MBq/mmol) were obtained from Pharmacia AB, Sweden. The radiochemical purity was checked by thin-layer chromatography (TLC). The chromatograms were run in 3 different systems and analyzed with a Berthold radiochromatogram scanner (LB 2723). The radiochemical purity of the esters was higher than 97% and less than 0.5% was free $PGF_{2\alpha}$. Butyrylcholinesterase, 130 units/mg solid (obtained from horse serum, EC 3.1.1.8), alkaline phosphatase, 1.7 units/mg solid (obtained from calf intestine, type 1, EC 3.1.3.1), leucine aminopeptidase (EC 3.4.11.1), leucine-*p*-nitroanilid, and *P*-nitrophenyl phosphate were purchased from Sigma Chemicals, U.S.A. Synstigmine methyl sulphate (Neostigmin), 2.5 mg/ml was bought from Leo AB, Sweden. Casein (casein according to Hammarsten) was purchased from Merck, F.R.G. All other chemicals used were of analytical grade.

Radioactivity measurements

The radioactivity measurements were performed in a 1214 Rackbeta liquid scintillation counter, Wallac Oy, Finland. The liquid samples were mixed with a scintillation cocktail (PCS II, Amersham or Quickszint 2000, Zensser Analytic, F.R.G.), and adapted to darkness and temperature for at least 12 h before counting. The efficiency was generally higher than 40%. The metabolic conversion of $PGF_{2\alpha}$ esters was determined by TLC using precoated TLC plates, Silica Gel 60, F-254 Merck, and diethyl ether-methanol (10 : 2) as the mobile phase.

Corneal permeability in vitro

The corneal permeability of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester was studied in a perfusion apparatus (Camber et al., 1986). In some experiments benzalkonium chloride (BAC) in concentrations up to 0.01% was added to the epithelial solution in order to simulate a pharmaceutical eye drop solution. The apparent permeability coefficients (P_{app}, cm/s) and the partition coefficient (PC) between octanol and water for the compounds were determined as described previously (Camber, 1985; Camber et al., 1986).

The hydrolysis of $PGF_{2\alpha}$ isopropyl ester after 4 h of perfusion was studied in both the donor and the receiving compartments of the perfusion apparatus. When the perfusion experiments were finished, the remaining scleras of the corneas were removed. The trimmed corneas were placed in scintillation vials and weighed. After solubilization with a tissue solubilizer (Soluene-350, Packard) the resulting solutions were treated with isopropanol and hydrogen peroxide before the scintillation cocktail, Dimilume-30, Packard, was added.

Hydrolysis of $PGF_{2\alpha}$ methyl, benzyl and isopropyl esters by butyrylcholinesterase

Vials, each containing butyrylcholinesterase, 28.9 μg in 0.5 ml tris (hydroxymethyl)aminomethane 0.05 M, pH 7.4 (TRIS) were placed in a water bath, 37°C. Synstigmine methyl sulphate and BAC were added to some vials. After incubation for 15 min, $PGF_{2\alpha}$ methyl, benzyl or isopropyl ester (18.6 nmol) were added. 10 and 40 min after addition, the degree of hydrolysis was determined. In control experiments, the esters were incubated in TRIS without enzyme.

Preparation for corneal hydrolysis

Fresh corneas were cut into pieces and homogenized in ice-cold TRIS (1 cornea to 2 ml solution) with a Potter-Elvehjelm homogenizer. The homogenate was centrifuged at 4°C for 10 min at 3000 rpm in a Sorvall RT 6000, Dupont. The protein content in the resulting supernatant (cornea homogenate) was determined according to Bradford (1976). Possible proteolytic activity was investigated with casein as the substrate (Kunitz, 1947)

Leucine aminopeptidase activity was studied with leucine-*p*-nitroanilid as the substrate (Oettegen and Taylor, 1985). Alkaline phosphatase activity was studied using *p*-nitrophenyl phosphate (Worthington, 1978).

The corneal hydrolysis of the esters was investigated: 0.5 ml cornea homogenate was mixed with synstigmine methyl sulphate (1.25 mg) or BAC (0.3 mg). These mixtures were preincubated for 15 min at 37°C before the $PGF_{2\alpha}$ esters (37.2 nmol) were added. The hydrolysis of the esters was determined after 5, 20 and 40 min.

Results

Corneal permeability and uptake

The partition coefficient for $PGF_{2\alpha}$ isopropyl ester in octanol–water was determined to log 3.40. The P_{app} for $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester as determined in the perfusion apparatus are shown in Table 1. The increase of radioactivity in the receiving compartment was linear with time for the sample interval 80–240 min. $PGF_{2\alpha}$ isopropyl ester penetrated the normal cornea more than 200 times faster than $PGF_{2\alpha}$. The results also showed that the permeability rate for $PGF_{2\alpha}$ was lowest with intact cornea and increased when the donor solution contained 0.01% BAC. A further increase

TABLE 1

Permeability of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester across excised pig cornea

Compound	Perfusion condition	Permeability coefficient ($cm\ S^{-1}$) $\times 10^6$, mean ± S.E.M. $n = 6$
$PGF_{2\alpha}$	intact cornea	0.13 ± 0.03
$PGF_{2\alpha}$	0.01% BAC	0.93 ± 0.43
$PGF_{2\alpha}$	deepithelized cornea	8.23 ± 0.92
$PGF_{2\alpha}$ isopropyl ester	intact cornea	29.43 ± 1.25
$PGF_{2\alpha}$ isopropyl ester	0.01% BAC	15.91 ± 1.36
$PGF_{2\alpha}$ isopropyl ester	deepithelized cornea	3.25 ± 0.57

The initial concentration of the compounds in these experiments was 18.6 μM.

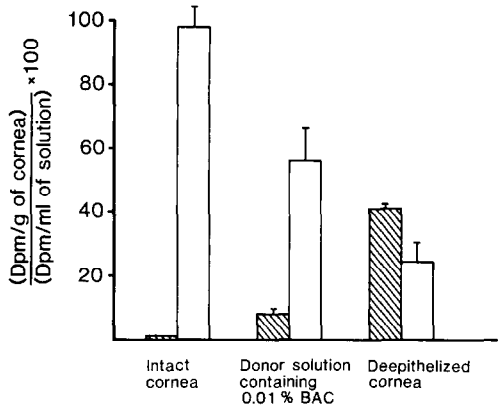

Fig. 1. Uptake of radioactivity in cornea at 3 different perfusion conditions. The initial concentration for $PGF_{2\alpha}$ (▨) and $PGF_{2\alpha}$ isopropyl ester (□) on the epithelial side was 18.6 μM. The bars show the mean and the range from 3 experiments.

was obtained with de-epithelized cornea. $PGF_{2\alpha}$ isopropyl ester showed the opposite result.

After 4 h of perfusion, only $PGF_{2\alpha}$ could be detected on the receiving side of the apparatus. Analysis of the donor solution revealed that 50–65% of the ester had been hydrolyzed when the corneal epithelium was present. In experiments where the corneal epithelium was removed, the ester was hydrolyzed to about 35–40%.

Uptake of $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester by the cornea after 4 h perfusion is demonstrated in Fig. 1. A correlation is seen between the corneal permeability for the $PGF_{2\alpha}$ compounds and the radioactivity content in the tissue. The amount of radioactivity in the cornea after perfusion with $PGF_{2\alpha}$ isopropyl ester decreased in the following order; intact cornea > intact cornea with donor solution containing 0.01% BAC > de-epithelized cornea. The opposite result was obtained with $PGF_{2\alpha}$.

Butyrylcholinesterase-mediated hydrolysis of $PGF_{2\alpha}$ esters

The susceptibility of $PGF_{2\alpha}$ methyl, benzyl and isopropyl esters to butyrylcholinesterase was investigated and compared (Table 2). The isopropyl ester is the most stable ester and in this experiment no hydrolysis was detected. However, at a higher enzyme concentration the isopropyl ester was susceptible to the enzyme. Addition of syn-

TABLE 2

Butyrylcholinesterase-mediated hydrolysis of $PGF_{2\alpha}$ isopropyl, methyl and benzyl ester

	% hydrolyzed	
	10 min	40 min
$PGF_{2\alpha}$ isopropyl ester	0	0
$PGF_{2\alpha}$ methyl ester	15	32
$PGF_{2\alpha}$ benzyl ester	100	100

stigmine methyl sulphate and BAC (0.005–0.01%) inhibited the butyrylcholinesterase. When the concentration of BAC was reduced to 0.001% no influence on the hydrolysis was seen.

Corneal hydrolysis of $PGF_{2\alpha}$ esters

Incubation of the 3 esters with cornea homogenate showed that the stability of the esters decreased in the following order; isopropyl > benzyl > methyl (Fig. 2a). Addition of cholinesterase inhibitor (synstigmine methyl sulphate) reduced the hydrolytic rate (Fig. 2b). Addition of BAC to a concentration of 0.01% in the homogenate mixtures led to a total inhibition of hydrolysis of $PGF_{2\alpha}$ benzyl and isopropyl esters and a significantly lower hydrolysis rate of the methyl ester.

The protein content of the cornea homogenate was 1.3 mg/ml. No unspecific proteolytic activity could be detected. The leucine aminopeptidase activity was determined to be 12.3 units/mg protein. A low alkaline phosphatase activity was detected. Incubation of the PG esters with leucine aminopeptidase or alkaline phosphatase did not affect the compounds: the esters were stable for more than 40 min.

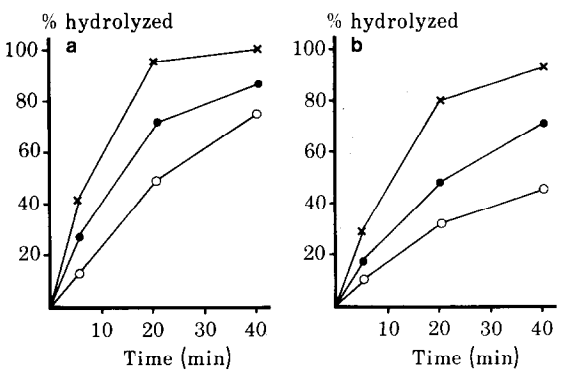

Fig. 2. Typical plots of hydrolysis of $PGF_{2\alpha}$ methyl ester (×), $PGF_{2\alpha}$ benzyl ester (●) and $PGF_{2\alpha}$ isopropyl ester (○) mediated by cornea homogenate. The following incubations were done: cornea homogenate (a), and cornea homogenate with synstigmine methyl sulphate (b).

Discussion

These studies clearly indicate that the lipophilic corneal epithelium acts as a barrier to $PGF_{2\alpha}$ while removal of the epithelium decreased the permeability of the $PGF_{2\alpha}$ isopropyl ester.

When the corneal epithelium was removed, the diffusion rate for $PGF_{2\alpha}$ isopropyl ester decreased 9- to 10-fold. The permeability of the free acid, on the other hand, increased more than 60-fold. A contributing factor to this permeability change is the difference in partition coefficient between octanol and water for $PGF_{2\alpha}$ and $PGF_{2\alpha}$ isopropyl ester, log 1.10 (Camber et al., 1986) and log 3.40, respectively. Removal of the epithelium enhances the permeability of water-soluble substances (Hull et al., 1974) such as $PGF_{2\alpha}$, whereas a lipid soluble prodrug like $PGF_{2\alpha}$ isopropyl ester was retarded. However, it should be noted that with the epithelium removed, the thickness and the weight of the corneas were increased, owing to hydration of the stroma.

On the endothelial side in the perfusion apparatus no ester could be detected, only $PGF_{2\alpha}$ was seen. An earlier report (Camber et al., 1986) from this laboratory, dealing with $PGF_{2\alpha}$ methyl and benzyl esters, revealed the same result. In that study both TLC and GC-MS were used. In the donor compartment about 50–65% of $PGF_{2\alpha}$ isopropyl ester was hydrolyzed, after 4 h of perfusion, when using intact cornea. The ester was probably hydrolyzed in the epithelium and the free acid refluxed to the donor solution. When the corneal epithelium was removed the recovery of intact ester was increased. This finding further strengthens the theory that the main site of hydrolysis is the epithelium.

Benzalkonium chloride (BAC) is often used as a preservative in eye drop solutions and it is also well known that BAC increases the corneal per-

meability of a variety of compounds e.g. small ions (Burstein and Klyce, 1977), fluorescein (Green and Tonjum, 1971; Burstein, 1984), inulin (Keller et al., 1980) and prednisolone phosphate (Green and Downs, 1974). A solution containing 0.01% BAC, to simulate a presumptive formulation, increased the corneal permeability for $PGF_{2\alpha}$ 10-fold, whereas $PGF_{2\alpha}$ isopropyl ester decreased it by 50%. Obviously, BAC acts as a permeability enhancer for hydrophilic substances. This effect of BAC is related to its adverse effects on the epithelium, resulting in cell damage and separation of the superficial epithelial cell layers (Pfister and Burstein, 1976).

Consequently, hydrophilic substances like $PGF_{2\alpha}$ penetrate the epithelium more easily, resulting in a higher permeability coefficient. It is known that quaternary ammonium salts inhibit butyrylcholinesterase (Augustinsson, 1960). As a consequence BAC would reduce the hydrolysis of the $PGF_{2\alpha}$ esters in cornea homogenate. This was shown to occur for the normal eye drop concentrations of BAC. One can assume that $PGF_{2\alpha}$ and BAC form an ion pair. A higher PC value (octanol–water) was obtained when BAC was present. The permeability properties for this (possible) ion pair were investigated using de-epithelized cornea in the perfusion apparatus. No difference in P_{app} or in R_f value for the compound in the receiving solution was observed, irrespective of whether BAC was present or not.

Butyrylcholinesterase has earlier been shown to hydrolyze $PGF_{2\alpha}$ methyl and benzyl ester (Camber et al., 1986). In this study the susceptibility to enzymatic hydrolysis of $PGF_{2\alpha}$ methyl, benzyl and isopropyl esters was compared. Incubation of the esters with butyrylcholinesterase or cornea homogenate indicated that the isopropyl ester resists enzymatic hydrolysis considerably better than the two other $PGF_{2\alpha}$ esters. Benzyl ester was much more stable in cornea homogenate than in butyrylcholinesterase solution while the opposite was seen with the methyl ester. A plausible explanation might be the existence of other hydrolytic enzymes in the cornea with better affinity for the methyl ester.

There are probably other enzymes beside butyrylcholinesterase that have hydrolytic activity and are not inhibited by synstigmine methyl sulphate. This is clearly shown in Fig. 2, which shows a reduction of the hydrolysis but no total inhibition.

A brief enzymatic assay of cornea homogenate revealed no proteolytic activity when using casein as substrate. This finding is contrary to the results of Cejková and Lojda (1986), who found protease activity in cornea. A plausible explanation might be that the method used (casein) is not sufficiently sensitive to detect low amounts of proteases. However, leucine aminopeptidase and alkaline phosphatase activities were observed in the homogenate, as is also found in the literature (Stratford and Lee 1985; Cejková and Bolková, 1977).

From this study it is clear that the corneal epithelium functions as a barrier for hydrophilic drugs and as the site of activation of prodrugs such as $PGF_{2\alpha}$ esters. It is also evident that agents like BAC can be contraindicated in formulations where the drug compound is dependent on an intact epithelium for its conversion to a pharmacologically active drug. An agent or vehicle which increases the corneal contact time, without destroying the epithelium, would be optimal for ophthalmic prodrugs.

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