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Studies on the gastrointestinal absorption of phosphocitrate, a powerful controller of hydroxyapatite formation

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Summary

In situ gastrointestinal perfusion and a chemical induced subcutaneous calcergy model were used to examine the oral bioavailability of the potent calcification inhibitor, phosphocitrate (PC). Formation of calcergic plaques was observed to decrease by approx. 34% when 450 mg PC/kg body weight per day was given to rats by gavage. An equivalent response was observed when only 10 mg PC/kg per day was given intraperitoneally thus indicating reduced bioavailability of the compound by the oral route. Luminal metabolism of PC did not contribute significantly to its overall poor oral bioavailability. Data derived from perfusion of in situ isolated intestinal segments with PC concentrations from 5 to 20 mM indicated luminal absorption by a passive transport process. Studies with radiolabeled PC confirmed the presence of small amounts of [^{14}C]PC and [^{14}C]citrate in portal blood. It was concluded that the membrane transport characteristics for PC were limiting transfer and that a possible future improvement might arise through the incorporation of a lipophilic moiety into the molecule.

Introduction

Pathological calcification is a key process in several degenerative disorders leading to speculation that treatment with anticalcifying agents may be beneficial. The clinical potential of several types of compounds including calcium channel blockers (Kjeldsen and Stender, 1989), bisphosphonates (Fleisch, 1983), and various calcium chelators (Block and Emmons, 1990) has been extensively reported. The modes of action of the various groups can be quite different. Calcium channel blockers modulate the entry of calcium into cells through selective or non-selective means and can hence prevent the toxic effects of excessive calcium accumulation (Fleckenstein et al., 1990). In addition, the blockers are generally more wide ranging in their effects encompassing anti-hypertensive action and the modulation of several tissue and hormone functions (Struyker-Boudier et al., 1990).

Unlike the calcium channel blockers, many other inhibitor types, such as the bisphosphonate class of compounds, directly restrict hydroxyapatite formation through interaction with the growing crystal structure. Bisphosphonates also

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have cellular effects and unfortunately, when given at high, non-pharmacologic doses, often elicit undesirable side effects including potentially deleterous effects on bone (Netelenbos et al., 1991) and renal tissue (Cal and Daley-Yates, 1990). There is still a need for a compound which can act as a direct inhibitor of calcification without secondary effects on other tissues.

One compound not yet trialled clinically but possessing characteristics required for effective inhibition of calcification is phosphocitrate (PC; 1,2,3-propanetricarboxylic acid-2-phosphonooxy). The compound is known to occur naturally (Williams and Sallis, 1981) and with the availability of synthetic PC (Tew et al., 1980; Williams and Sallis, 1980), extensive information on the chemistry and biological responses has evolved from wide ranging fields of investigation. The transformation of amorphous calcium phosphate to the hard crystalline form is powerfully inhibited by PC (Tew et al., 1980; Williams and Sallis, 1982). Hence, its potential to restrict the progression of calcific diseases is apparent. In this context, a multifunctional role for PC as a therapeutic agent is emerging as the compound has already proved efficacious in in vivo models of urolithiasis (Sallis et al., 1988), atherosclerosis (Ward et al., 1987) and osteoarthritis (Cheung et al., 1990). The unusual chemistry of the molecule with high negative charge/size ratio and inherent stereochemistry is believed primarily responsible for its actions (Ward et al., 1987).

Whilst small animal trials with PC have been most encouraging, its eventual role as a clinically useful drug relies upon the development of an acceptable presentation form of the compound. To date the intraperitoneal route has been preferred for PC therapy in small animals. Long term administration of a drug by this route however has obvious shortcomings. In a clinical setting oral administration is often preferable but to date this route has not been explored with PC.

The present studies then were designed to examine the bioavailability of PC when administered by the oral route. For this purpose PC was given by gavage to rats and its effectiveness to prevent the formation of chemically induced calcergic plaques determined. Additionally, in situ perfusion of isolated gut segments with PC was used to generate information on the compound's absorption characteristics.

Materials and Methods

Materials

Adult male Hooded Wistar rats (200–250 g) were maintained on normal laboratory chow. Food was withdrawn 16 h prior to perfusion experiments. With the exception of PC, all chemicals were purchased commercially and were of analytical grade. ^{14}C-labeled PC (specific activity, 1.37 mCi/mmol) and unlabeled PC (Mol. Wt 450) were synthesized and characterized by previously published methods (Tew et al., 1980; Williams and Sallis, 1980). Enzymes were products of Boehringer Mannheim (Australia).

Calcergy experiments

Subcutaneous calcergy was induced in rats according to the method of Doyle et al. (1979). A freshly prepared $KMnO_4$ solution (200 μl of 1 mg/ml) was injected subcutaneously in two positions either side of the spine in the interscapular region. In inhibitor trials, PC was administered dissolved in 0.9% saline and given daily for 9 days either by gavage (100 and 450 mg/kg body wt per day) or i.p. injection (2.5–100 mg/kg body wt per day). Control rats received an equivalent volume of 0.9% saline. After 10 days, the rats were killed, the plaques excised and pressed dry on tissue paper, freeze clamped and weighed. Frozen samples (20 mg) were digested in an oxidising mixture comprising perchloric acid (70% w/v, 200 μl) and hydrogen peroxide (30% w/v, 400 μl) by heating at 80°C for 2 h. After cooling, aliquots of digest were analysed for calcium (atomic absorption spectroscopy) and inorganic phosphate (Saheki et al., 1985). All data were compared using the single tailed Student's *t*-test.

Cannulation of the portal vein

Cannulations were performed in a similar manner to that described by Davison (1989). Fasted rats were anaesthetised by i.p. injection of sodium pentobarbital (stock solution 60 mg/ml;

220 μl/200 g rat) and positioned dorsally on a 37°C heating pad. A mid-line incision was made and the portal vein was exposed by clearing the surrounding adventitious tissue. The vein was held away from the body wall with forceps and two loose ligatures applied such that it became engorged with blood. The vein was then punctured with a 19 gauge hypodermic needle and a heparinized bevelled cannula (PE50, i.d. 0.50 mm, o.d. 1.00 mm) immediately inserted in a caudal direction with subsequent release of the ligatures. The cannula, fitted with a small metal plug at the free end, was sutured to the abdominal wall, remaining in position without ligation to the vein. Blood (200–300 μl) was withdrawn into a heparinized syringe. Prior to sampling, heparin was withdrawn from the cannula and later replaced.

Cannulation of the intestine

The intestine was cannulated immediately following the portal vein procedure using the technique described by Doluisio et al. (1969). A 30 cm section of intestine was isolated and small incisions made at the proximal and distal ends. Two L-shaped glass cannula were then inserted and secured with silk ligatures. Cannulation of a duodenal/jejunal segment started approx. 5 cm posterior to the pyloric sphincter whilst cannulation of the ileum ended approx. 5 cm above the caecum. Following the procedure, the intestines were returned to the abdominal cavity leaving the cannulae exposed and glass hypodermic syringes (20 ml) were connected using latex tubing (i.d. 1/32 inch, o.d. 1/8 inch). Finally, a warm saline gauze pad was applied to the abdomen and covered with thin plastic.

Intestinal perfusions

The perfusion protocol was also according to the method of Doluisio et al. (1969). Solutions were introduced into the intestine via the glass cannulae and samples were taken by briefly withdrawing the perfusate into one of the syringes. The volume was recorded and the perfusate immediately returned to the intestinal segment. Initially the intestine was perfused with 0.9% saline (5 min) followed by glucose supplemented Krebs Henseleit buffer (KHB; 15 min) before introducing the test concentration of PC dissolved in KHB. An aliquot of perfusate was immediately taken and this was defined as time zero. Perfusion samples (50 μl) were then collected at timed intervals. All buffers were maintained at 37°C and equilibrated with 5% CO_2/95% O_2 prior to use.

Digestion and counting of radioactivity in whole blood samples

Blood samples (300 μl) were digested by heating at 50–60°C for 1–2 h in perchloric acid (70% w/v, 200 μl) and hydrogen peroxide (30% w/v, 400 μl) in glass scintillation vials. After cooling, a scintillation cocktail (Fricke, 1975) was added and the mixture counted.

Treatment of blood samples for chromatography

Blood samples were processed at 4°C. An aliquot (300 μl) was added to ice-cold perchloric acid (1 M, 600 μl) and mixed vigorously. After 2 min, the mixture was centrifuged (2 min, 8000 × *g*) and the supernatant transferred to a separate tube. Potassium carbonate (2 M) was added dropwise until neutrality was indicated (1 drop of phenol red, 0.02%). The mixture was again centrifuged (2 min, 8000 × *g*) and the supernatant retained for lyophilization. The neutralization step was considered useful as it minimized interference by perchlorate during the later analysis of samples.

Chromatographic analysis of blood extracts

Lyophilizates were reconstituted in a minimum volume of water and applied to Whatman No. 1 chromatography paper as a thin band 10 cm long. Several applications were required and the paper was developed (2.5 h) by descending chromatography in a solvent system comprising *n*-propanol/water/glacial acetic acid (4:1:1, v/v). When dry the chromatograms were cut into 1.0 cm strips parallel to the application band. Each strip was cut into small pieces and packed into a 2.5 ml syringe for extraction with 1 M HCl (0.5 ml, ×2). The radioactive eluants were recovered by centrifugation, at low speed, directly into scintillation vials for counting.

Analysis of PC and citrate

Analysis of PC and citrate in lumen samples was performed using a coupled enzyme assay as previously described (Williams and Sallis, 1980). Total citrate (citrate originating from hydrolysed PC plus free citrate) was determined by first preincubating samples with alkaline phosphatase (37.5 units, 1 h, 37°C). Malate dehydrogenase, NADH and citrate lyase were then added and the conversion of NADH to NAD^+ followed fluorimetrically. Free citrate was determined by omitting the preincubation step.

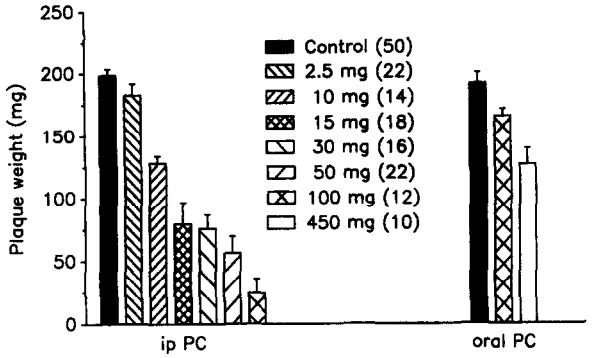

Fig. 1. The effect of intraperitoneal vs oral PC on plaque weight. Experimental conditions are described in the text. Data represent mean ± SE. *n* values (in parentheses) refer to the number of plaques.

Results and Discussion

Information concerning the oral bioavailability of PC was sought initially from in vivo studies relating PC dose to the inhibition of subcutaneous calcergic plaque formation. Plaque weight was used as the measured indicator as it appeared to accurately reflect responses to inhibitor administration. Determination of calcium and phosphate concentrations within plaques from representative treatment groups of rats given graded doses of PC was initially made. Confirmation was gained that total calcium and phosphate present was directly related to plaque weight (Table 1). The relatively constant Ca/P_i ratio also indicated the presence of hydroxyapatite.

Fig. 1 compares the ability of oral vs i.p. administration of PC to reduce plaque weight. With the exception of the minimum dose tested (2.5 mg/kg per day: $0.05 < p < 0.10$), a highly significant dose dependent inhibition of plaque weight was recorded with an 88% reduction at 100 mg/kg per day ($p < 0.0005$). Of interest, some animals were even noted to have a complete absence of plaque material at the highest PC dose tested. In contrast, the oral route, where PC was given by gavage, proved to be much less effective. At the highest dose (450 mg/kg per day) only 34% inhibition ($p < 0.0005$) was observed, corresponding in efficacy to an i.p. dose of 10 mg/kg per day. The data then suggests poor bioavailability of PC by the oral route.

It is recognized that the gut transport of small molecules can be affected by numerous biological factors such as gastrointestinal motility, gastric emptying and the rate of blood flow to the intestine (Lin and Chien, 1985). In the present instance however, it is more likely to be the physicochemical nature of PC which is impeding its passage. Highly charged water soluble molecules are known to pass only slowly across the gut (Schanker, 1971). Hence the strong negative charge of PC at physiological pH values (Ward et al., 1987) could be an important factor responsible for its inefficient absorption.

Perfusion of isolated in vivo gut segments with labeled and unlabeled PC was pursued in an attempt to gain additional information. Fig. 2 is a composite graph indicating the disappearance of PC from the intestinal lumen and the appearance

TABLE 1

Plaque weight, calcium and phosphate content and the Ca/P_i ratio in response to variable doses of phosphocitrate

PC [a] treatment	Plaque weight (mg)	Calcium (μmol/mg plaque)	Phosphate (μmol/mg plaque)	Ca/P_i
100 (6)	24	0.93 ± 0.32	0.61 ± 0.21	1.52
50 (9)	69	0.76 ± 0.12	0.54 ± 0.09	1.41
15 (15)	111	0.82 ± 0.18	0.56 ± 0.12	1.46
5 (12)	160	0.97 ± 0.11	0.64 ± 0.07	1.52
0 (9)	198	0.89 ± 0.10	0.61 ± 0.05	1.46

Results are mean ± SD.

[a] PC (mg/kg per day) was injected i.p. on days 1–9; animal numbers in parentheses.

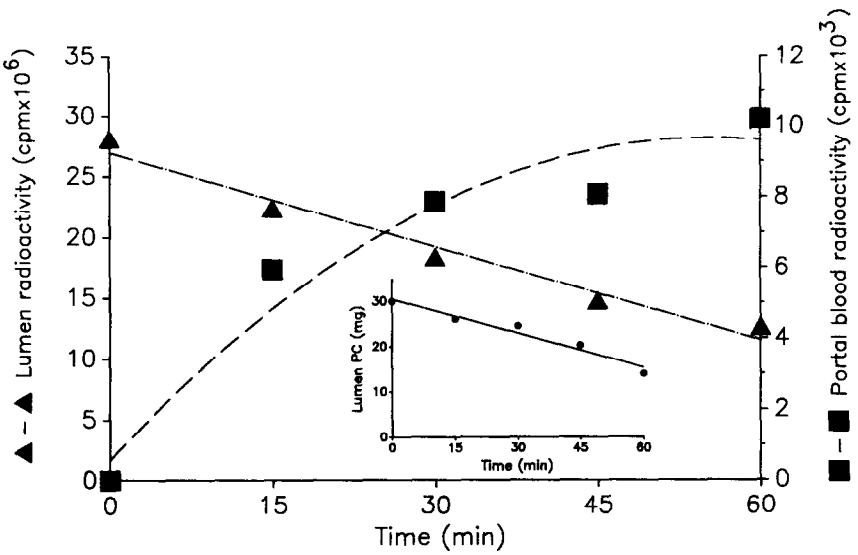

Fig. 2. Lumen radioactivity and appearance of radioactivity in portal blood with time following perfusion of an intestinal segment with [^{14}C]PC. Duodenal segments were isolated in situ and perfused with 5 mM [^{14}C]PC. Each data point represents the mean from four individual experiments. The inset indicates the amount of PC remaining in the lumen at each time point.

of radioactivity in the portal vein following perfusion with 5 mM [^{14}C]PC. A reduction in lumen PC content over the time scale of the perfusion was observed, with an asymptotic increase in radiolabel detected in the portal vein. At any one time though, the level of radioactivity was very low. A lack of high specific activity [^{14}C]PC and ultrasensitive analytical systems for detecting PC limited the experimental approach that could be taken to gain data for pharmacokinetic analysis. However, radiochromatography of portal blood samples during intestinal perfusion with [^{14}C]PC did provide useful information. As revealed in Fig. 3, a 60 min radiochromatogram showed definitively that PC was present in portal blood. This supports the in vivo findings that indeed some PC, when given by gavage, can be absorbed and have an influence at a pathological site of calcification. In addition though, the chromatogram indicated the presence of peaks corresponding to citrate and other unknown products. Clearly, some metabolism of PC was occurring either in the lumen or mucosal cells themselves.

The possibility that the site within the intestinal tract might influence the extent of PC metabolism or absorption was investigated by comparing the absorption profiles obtained from two different regions of the gastrointestinal tract. Fig. 4 shows the profiles from a duodenal/jejunal segment versus an ileal segment. The amount of PC remaining in the upper segment was significantly less than that observed for the ileum. Over the same time, free citrate increased slightly and was higher in the proximal segment compared to the distal portion of the small intestine.

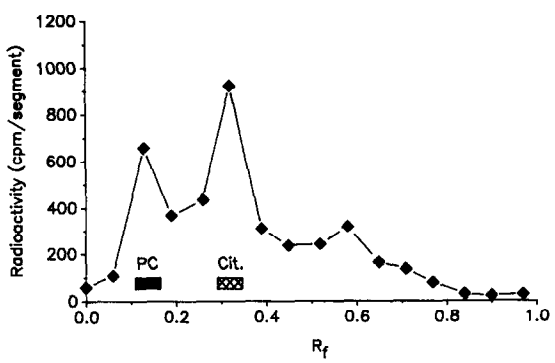

Fig. 3. Radiochromatographic profile of a portal vein sample. Portal blood was removed at 60 min, extracted, chromatographed and counted as described in the text. For comparative purposes the R_f values of PC and citrate standards are indicated as bars. Although representing a single experiment and single time point, the characteristics of the graph are typical of similar profiles observed at other time points.

The levels of citrate observed in the perfused segments were found to be low and did not indicate significant breakdown of PC. However, it is difficult to accurately quantitate events in relation to luminal citrate concentrations as the rates of metabolism, absorption and re-secretion of citrate are unknown. Luminal citrate might well have been derived from the metabolism of PC. In this respect, a catalytic role for alkaline phosphatase, an enzyme having its highest intestinal activity in the proximal small intestine (Weiser et al., 1986), was considered. However, this scenario is unlikely to be a major factor as the characteristics of this enzyme, which has a highly constrained active site (Vincent et al., 1992), do not favour PC as a substrate. Further, in other work, we have demonstrated that in the presence of strong alkaline phosphatase activity the anticalcifying activity of PC is still retained (Shankar et al., 1984). A more likely explanation for the appearance of some free citrate is that an intramolecular process (Bender and Lawler, 1963), involving carboxylic acid catalyzed hydrolysis of the phosphate is occurring. In any case, it should perhaps be pointed out that in all studies to date where PC has been shown to inhibit calcification, citrate itself has never been able to mimic similar responses (Williams and Sallis, 1982; Sallis et al., 1988). The data then, would suggest that significant luminal metabolism of PC does not occur leading to the conclusion that it is the membrane transporting events which are restrictive.

In experiments (Fig. 5) where the upper part of the small intestine was perfused with varying

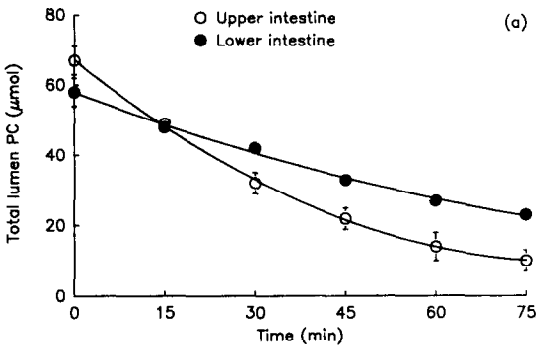

Fig. 4. Comparison of a duodenal/jejunal vs an ileal segment of intestine for residual lumen PC and citrate with time. PC (5 mM) was originally present in the lumen of an in situ isolated segment as described in the text. Panel (a) represents the disappearance of PC and (b) the appearance of citrate. Data represent mean ± SE ($n = 5$). Where not visible, error bars are within the symbol.

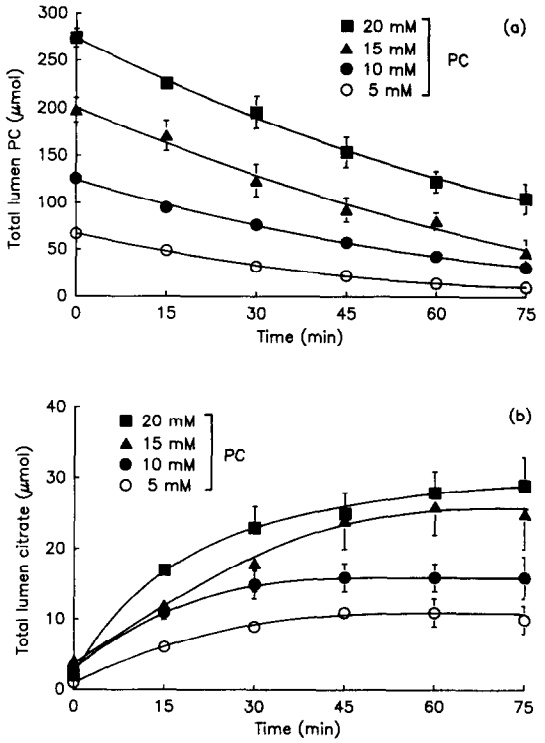

Fig. 5. Disappearance of lumen PC and appearance of citrate with time as a function of initial perfusate PC. Segments were isolated in situ and were perfused with the concentrations of PC indicated. Panel (a) represents the disappearance of PC and (b) the appearance of citrate. Data represent mean ± SE ($n = 6$ for 5 and 20 mM; $n = 5$ for 10 and 15 mM). Where not visible, error bars are within the symbol.

concentrations of PC (5–20 mM), profiles gave some insights into the mechanism of the transport process. At all concentrations the nature of the plots indicated first order kinetics characteristic of passive transport. Further treatment of the data (not shown here) revealed that the PC content of the lumen decreased log linearly with time for each perfusion concentration ($r > 0.98$ for all plots) confirming a passive rather than a facilitated process. Of interest, a carrier protein has been described for citrate in several tissues (Wright et al., 1980; Claeys and Azzi, 1989; Wolffram et al., 1992) but the rigid substrate requirements described for one of the proteins (Cheema-Dhadli et al., 1980) would undoubtedly exclude PC. Clearly, the phosphate moiety can strongly influence the transport process.

The goal of the present studies was to establish whether oral presentation of PC was efficient in respect to delivering an inhibitory concentration of the compound to a pathological calcifying site. Whilst absorption clearly does not appear to be efficient, it is apparent that when given at a high enough dose, PC can still actively inhibit. One future approach to improving the efficiency of absorption might be to attempt modification of the basic molecule. For example, the attachment of a suitable lipophilic moiety through ester formation should enhance membrane penetration and in addition, limit the overall charge of the molecule. The specific requirement for such a prodrug would be the need to ensure biodegradability for the release of the active molecule. The development of a PC prodrug would undoubtedly be beneficial from an oral delivery standpoint and could well lend itself to providing a sustained release form, thus permitting constant therapeutic levels in circulation. Another approach might be to open up tight junctions to allow a greater flow of PC. These ideas are now being considered.

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