PERGAMON

Archives of Oral Biology 45 (2000) 569–575

Archives of Oral Biology

www.elsevier.com/locate/archoralbio

Effects of an anticariogenic casein phosphopeptide on calcium diffusion in streptococcal model dental plaques

R.K. Rose*

Division of Restorative Dentistry, The Dental School, Bristol, UK

Accepted 18 January 2000

Abstract

Casein phosphopeptides (CPP) stabilize amorphous calcium phosphate (ACP) and may be used to localize ACP in dental plaque, maintaining a state of supersaturation with respect to tooth enamel, reducing demineralization and enhancing remineralization. The aim here was to investigate these effects by measuring the effect of CPP–ACP on calcium diffusion in plaque. Using Dibdin's effusion system, calcium diffusion was measured in streptococcal model plaques. This demonstrated that by providing a large number of possible binding sites for calcium, 0.1% CPP–ACP reduces the calcium diffusion coefficient by about 65% at pH 7 and 35% at pH 5. Hence, CPP–ACP binds well to plaque, providing a large calcium reservoir within the plaque and slowing diffusion of free calcium. This is likely to restrict mineral loss during a cariogenic episode and provide a potential source of calcium for subsequent remineralization. Overall, once in place, CPP–ACP will restrict the caries process.

Keywords: Casein phosphopeptide; Calcium; Remineralization; Diffusion

1. Introduction

The use of topical fluoride has produced a large decrease in the incidence of dental caries, but there still remains a small portion of the population that is refractory to the use of oral hygeine products and there is evidence that fluoride alone cannot completely eradicate the disease. Milk and milk products such as cheese have been shown to have anticariogenic properties in human and animal models (Reynolds and Johnson, 1981; Rosen et al., 1984; FitzGerald, 1998). It has been suggested that the mechanism of this action is due to a direct chemical effect from a component of the cheese (Krobicka et al., 1987), or, more specifically, due to the phosphoprotein casein and calcium phosphate components (Harper et al., 1986). Casein phosphopeptides (CPP) have the ability to stabilize calcium phosphate in solution through binding amorphous calcium phosphate (ACP) with their multiple phosphoserine residues. This allows the formation of small CPP–ACP clusters, but without allowing growth to the critical size required for nucleation of crystal growth and subsequent precipitation of calcium phosphate (Holt et al., 1996; Holt, 1998). These CPP–ACP clusters are prepared by tryptic

Abbreviations: ACP, amorphous calcium phosphate; CCP, casein phosphopeptide.

* Corresponding author. Tel.: +44-117-928-4334; fax: +44-117-928-4778.

E-mail address: keith.rose@bristol.ac.uk (R.K. Rose).

0003-9969/00/$ - see front matter
PII: S0003-9969(00)00017-0

digestion of bovine casein phosphopeptide, with subsequent purification by ultracentrifugation (Gagnaire et al., 1996), or the more commercially viable technique of ultrafiltration (Reynolds, 1991). Extensive characterization of these preparations has taken place (Reynolds et al., 1994; Park et al., 1998; Park and Allen, 1998; Holt et al., 1998), and their calcium-binding properties have been examined (Meisel and Olieman, 1998; Park and Allen, 1998). From observations that there is an inverse relation between plaque calcium and caries incidence (Shaw et al., 1983; Margolis and Moreno, 1992), it follows that a product such as CPP–ACP, which can significantly enhance the availability of calcium in plaque, should have an anticaries protective effect, by suppressing demineralization, enhancing remineralization, or possibly a combination of both. Reynolds (1997) has demonstrated that CPP–ACP can remineralize subsurface lesions in human enamel and this is indeed the basis of the claim of his patent (Reynolds, 1991), although there is compelling evidence that these compounds can also inhibit the adhesion of cariogenic streptococci to the tooth surface (Schupbach et al., 1996).

All substances that are found in plaque fluid and are not derived from the bacterial components of plaque must diffuse into the biofilm, creating gradients between the outer and inner concentrations. Diffusion of any substance in plaque is controlled by three factors: the molecular-sieve effect that allows small molecules to diffuse through gaps that are impenetrable to larger ones; the molecular weight of the diffusing species, the square of which is inversely proportional to the diffusion coefficient; the binding characteristics of the diffusing species, which dictate how much is free to diffuse at a given time (Rose and Turner, 1998). For calcium diffusion in the CPP–ACP/Ca system, only the last two are of importance (assuming the CPP–ACP is already in place). At neutral pH, calcium diffusion is limited by the significant quantity of calcium which is bound, reducing the effective diffusion coefficient (D_e), and creating a measurable restricted effective diffusion coefficient ($_rD_e$) where $_rD_e = D_e/(R + 1)$ in which R is the ratio of bound to free calcium. It has been demonstrated that by providing a large number of potential binding sites for calcium, the calcium-binding groups on bacterial cell surfaces have significant effects on the calcium diffusion coefficient and this effect is maintained at lower pH, although overall diffusion is slightly faster (Rose and Dibdin, 1995; Rose et al., 1997). It is likely that by providing even extra calcium-binding sites, CPP–ACP will have measurable effects on calcium diffusion in plaque. Hence, the aim now was to elucidate the effect of CPP–ACP on calcium diffusion in plaque at different pH.

2. Materials and methods

2.1. Bacteria

Diffusion was studied in model plaques prepared from *Streptococcus mutans* R9, a strain isolated from a human carious lesion by P.D. Marsh. The organism was maintained on Todd–Hewitt agar plates (+0.4% glucose). For preparation of experimental cultures, colonies were inoculated into 20 ml Todd–Hewitt broth and grown anaerobically overnight at 37°C. A portion (1 ml) of this culture was inoculated into 400 ml of Todd–Hewitt broth and incubated for 17 h at 37°C. The culture was centrifuged at 9000 ***g*** for 20 min at 4°C and pellets were pooled and washed twice by resuspension in 10 ml chilled experimental buffer and centrifugation at 6000 ***g*** for 10 min at 4°C.

2.2. Effusion experiments

About 250 mg of the resulting pellet was transferred to a weighed Eppendorf tube and resuspended, for 30 min at 4°C, in 500 μl buffer, containing 185 kBq [^{3}H]inulin (Amersham International plc, Amersham, UK) and 76 kBq ^{45}Ca (Amersham) with variable amounts of unlabelled calcium carrier, and with CPP–ACP (kindly supplied by SmithKline Beecham, Weybridge, UK) at 0.1–1.0% by weight. For experiments at pH 7.0, the buffer used was 0.05 mol/l dipotassium PIPES, containing 0.1 mol/l D-glucose, while for experiments at pH 5.0, 0.1 mol/l MES containing 0.1 mol/l KCl and 0.1 mol/l D-glucose was used. After incubation, the cells were spun at 6000 ***g*** for 10 min. The supernatant was recovered and three 25-μl samples were taken for dual-channel scintillation counting (see below). These gave the tracer concentrations in equilibrium with the sample at the start of each effusion experiment ($[^3\text{Hinulin}]_0$ and $[^{45}\text{Ca}]_0$). About 25 mg of the sediment was transferred to 1 mm-deep effusion wells [see Rose et al. (1997)], spread evenly and weighed. This model plaque was then covered with a high-porosity Anotec alumina membrane support (Anodisc 13, 0.2-μm pore size; Whatman Ltd, Maidstone, UK), which was in turn held in place with a silicone rubber 'O' ring. The well assembly was placed into the effusion chamber, and effusion started by addition of 6 ml of tracer-free buffer containing the same concentration of calcium carrier and CPP–ACP as used above. Six chambers were filled and run concurrently. Samples (25 μl) were taken at intervals of 3 min to start with, increasing somewhat toward the end of the experiment — usually about 200 min. Samples were taken with a 25-μl glass syringe, which was rinsed three times in deionized water between samples. The samples were placed in polypropylene scintillation mini vials, mixed with 3.5 ml of scintillation fluid (LKB Optiphase

'Safe'; Fisons plc, Loughborough, UK), shaken twice and counted for 300 s (LKB 1217; Rackbeta LKB-Wallac, Milton Keynes, UK) with windows set for dual counting of the two isotopes. The three 25-μl samples of extracted incubating fluid described above were also counted at the same time.

2.3. Data analysis

2.3.1. Correction for sample size

As a volume of up to 1 ml (1/6 of the total sample volume) was removed during the experiments, a short BASIC program, ALEX, was used to allow for the lowered volume and so calculate corrected counts.

2.3.2. Calculation of the diffusion coefficient

Eq. (1) below is a saturating exponential, defining counts in the clearance solution, in which C_∞ is the asymptotic count, C_t is the volume-corrected count at time t and α is an exponential constant. It was fitted to each set of ^{45}Ca and [^{3}H]inulin effusion data using Fig.P/P.Fit software (Biosoft, Cambridge). The data were curve-fitted with a variable x-axis intercept (δ) to allow for the fact that the first 30% of effusion does not follow this equation (Dibdin, 1988)

$$C_t = C_\infty(1 - e^{-\alpha(t+\delta)}). \quad (1)$$

For an effusion system of the type used in this work, the exponential constant is related to the diffusion coefficient by:

$$_rD_e = \frac{4\alpha\ell}{\pi^2} \quad (2)$$

in which ℓ is the depth of the well (McNee et al., 1979; Dibdin, 1993). The effusion of a strongly bound divalent cation through a bacterial plaque would have a retarded effective diffusion coefficient [$_rD_e$ (Rose and Dibdin, 1995)] because of reversible adsorption at specific sites. $_rD_e$ is reduced from D_e (the effective diffusion coefficient) by:

$$_rD_e = \frac{D_e}{R+1} \quad (3)$$

in which R (Crank, 1975) represents the ratio of bound to free calcium, i.e.

$$R = \frac{[Ca]_b}{[Ca]_f} \quad (4)$$

where $[Ca]_f$ is, by definition, the same as in the bathing solution and R is a constant during the experiment if, as throughout our system, $[Ca]_{carrier} > > [^{45}Ca]$. $_rD_e$ asymptotically approaches D_e as the ratio of bound to free calcium tends towards zero. The point (k), at which $_rD_e = D_e/2$ (and $[Ca]_b = [Ca]_f$) may be used as a measure of the effect of [Ca] on the change in $_rD_e$. Bound and free calcium are related by the equation (Rose and Hogg, 1995):

$$[Ca]_b = \frac{C_{max}\,[Ca]_f}{K_d + [Ca]_f} \quad (5)$$

in which C_{max} is the binding capacity and K_d is the dissociation constant. Concentration therefore affects the rate at which a front moves through the plaque or film. Although binding is not linearly related to the free calcium concentration, the use of a chosen concentration of calcium 'carrier' in this work effectively ensured that the binding ratio, R, is constant with position and time. All differences were tested for significance by one-way ANOVA.

3. Results

Fig. 1 shows an example effusion profile for calcium with CPP–ACP and fluoride for comparison (from Rose and Turner, 1998). In this form of curve, the steepness is proportional to the diffusion coefficient (see Eqs. 1 and 2) and the height of the asymptote represents the amount of calcium released at equilibrium, which is a function of the amount present in the model plaque at the start of the diffusion. It may be clearly seen that, in this example, fluoride increases the rate of diffusion and the total amount available. CPP–ACP, in contrast, increases the amount but decreases the rate.

Figs. 2 and 3 show the effect of CPP–ACP and F on the restricted calcium diffusion coefficient at pH 7.0 and 5.0, respectively. Fluoride increases diffusion, whereas CPP–ACP reduces it. Note that all three curves converge on an asymptote at high [Ca] where the free Ca predominates but that the F curve reaches this at lower calcium concentration, the calcium-only curve next and then the CPP–ACP curve reaches the asymptotic value at high calcium concentration. At pH 5.0, the three convergences on the asymptote take place in the same order but at lower [Ca].

Figs. 4 and 5 show plots of diffusion coefficients for calcium against [CPP–ACP]. From this one can see that there is a significant reduction in the diffusion between 0 and 1 g/l [CPP–ACP] but addition of CPP–ACP beyond 1 g/l has no additional effect. At pH 5.0, the diffusion coefficients are greater, but once again, the maximum change occurs between 0 and 1 g/l CPP–ACP.

Table 1 shows the values of k and D_e derived from Figs. 2 and 3. Treatment with fluoride significantly reduces the value of k, indicating faster diffu-

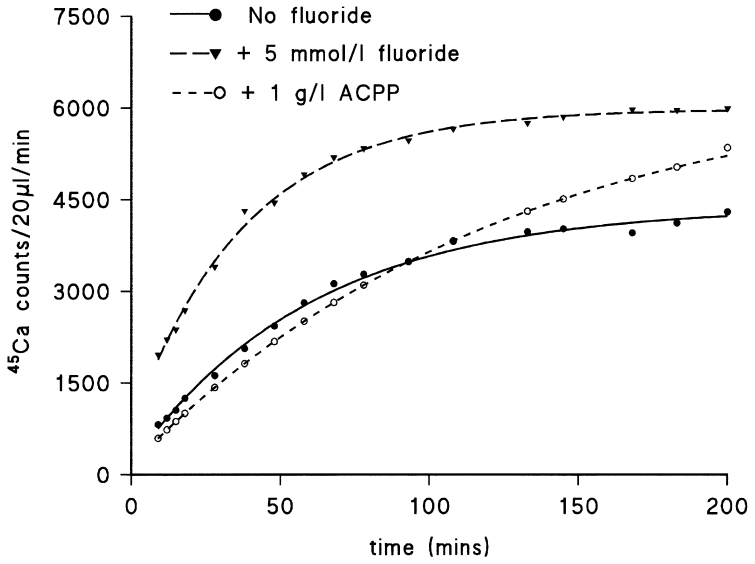

Fig. 1. Example effusion profile for calcium with CPP–ACP [and fluoride for comparison, from Rose and Turner (1998)]. Points are individual measurements from one effusion cell.

sion at physiological calcium concentration. Treatment with CPP–ACP has the opposite effect, raising the value of k significantly and indicating slower diffusion at physiological calcium concentration. In each case, no significant change was found in the value of D_e (found from the asymptotic values of the curves in Figs. 2 and 3, see Eq. 3). This shows that, as expected, any diffusion restriction brought about by binding can be overcome at unphysiologically high calcium concentrations.

4. Discussion

For diffusates such as calcium, Dibdin's effusion cell provides a reproducible method for measuring diffusion under a wide range of chosen conditions.

In milk, casein stabilizes the structure of the liquid in order for it to maintain its high calcium phosphate concentration without allowing precipitation. Three caseins are known (α,β,κ) with different functions and peptide sequences, but all contain

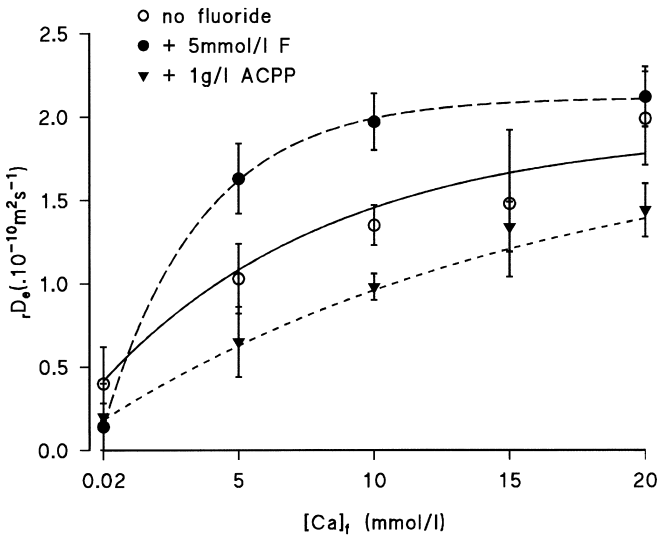

Fig. 2. The effect of CPP–ACP and F on the restricted effective calcium diffusion coefficient at pH 7.0. At physiological [Ca] fluoride increases diffusion, whereas CPP–ACP reduces it. Points are mean ± SD, $n = 6$.

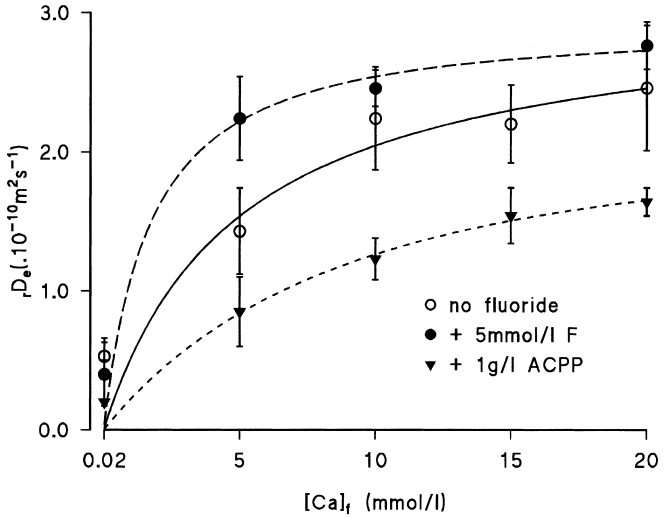

Fig. 3. The effect of CPP–ACP and F on the restricted effective calcium diffusion coefficient at pH 5.0. Points are mean ± SD, $n = 6$.

the phosphoseryl cluster sequence -ser(P)-ser(P)-ser(P)-glu-glu, clearly a region of high negative charge and a likely calcium-binding site. The major peptides produced by tryptic digestion are β(1–25) and α_{s1}(59–79), each of which contains one of the phosphoseryl cluster sequences (Reynolds, 1991). The product has been shown to contain ACP in the form $Ca_3(PO_4)_{1.87}(HPO_4)_{0.2}.xH_2O$ with the CPP/ACP ratio of $[\alpha_{s1}(59–79)(ACP)_8]_n$ where the predominant form has $n = 6$ (Reynolds, 1991).

Competitive-binding studies using equilibrium dialysis have shown that CPP–ACP and calcium compete for the same binding sites in dental plaque (Rose, 2000). From this work, it is estimated that the CPP–ACP binding affinity is about 0.55 g/l and the capacity is 0.16 g/g wet wt. By analogy with known calcium-binding affinities, this shows that roughly three calcium ions per CPP–ACP unit are involved in binding to bacterial cells and this corresponds to a calcium binding affinity of 0.3–0.5 mmol/l. At pH 5.0, there is a significant loss of binding sites due to neutralization by the increased proton concentration, reducing the bind-

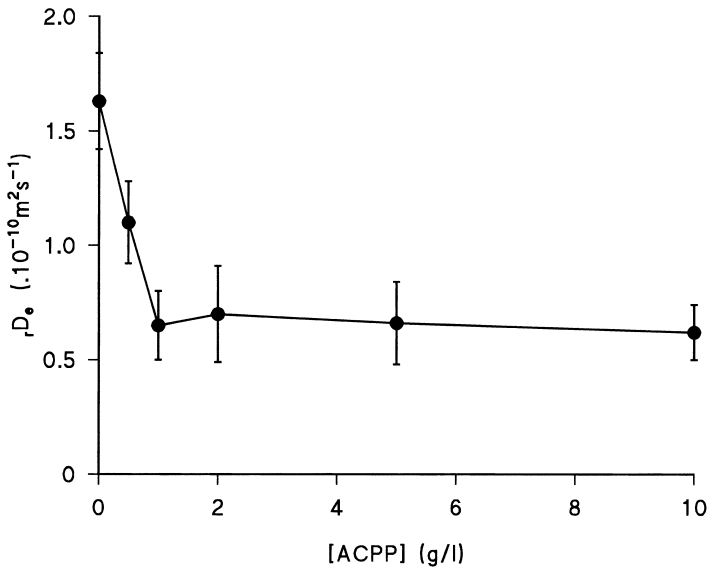

Fig. 4. Plot of the variation in restricted effective diffusion coefficient for calcium at 5 mmol/l [Ca] against [CPP–ACP], pH 7.0. Points are mean ± SD, $n = 6$.

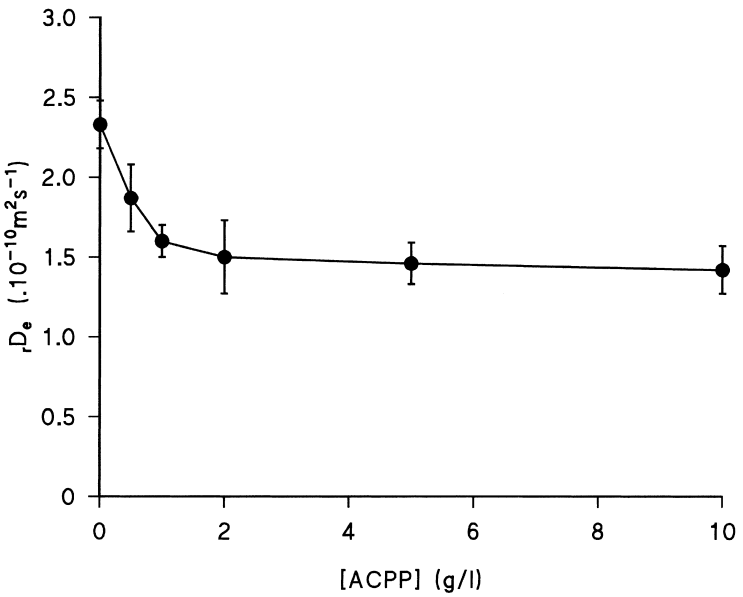

Fig. 5. Plot of the variation in restricted effective diffusion coefficients for calcium at 5 mmol/l [Ca] against [CPP–ACP], pH 5.0. Points are mean ± SD, $n = 6$.

ing capacity to 0.11 g/g wet wt cells with no measurable change in affinity (Rose, 2000).

The benefits of having such a large source of calcium and phosphate at or near the sites of possible demineralization are clear. There can be little doubt that, when present, CPP–ACP will release calcium and this is likely to inhibit demineralization, enhance remineralization, or possibly both. From this work, it is clear that CPP–ACP increases the number of potential calcium-binding sites thereby decreasing the calcium diffusion constant. This large effect will decrease the rate of calcium loss from plaque during a cariogenic attack, and although the diffusion coefficient is raised as binding sites are lost through neutralization at the lower pH typically found in plaque during a cariogenic episode, it remains considerably lower than it would be in the absence of CPP–ACP. However, the decreased diffusion rate will continue to occur as plaque returns to the resting pH value and then beyond that, slowing diffusion of salivary calcium into plaque. Once again, this slowing would tend to decrease the potential for remineralization were it not for the ability of CPP–ACP to provide much greater plaque calcium concentrations.

The potential effects of the presence of CPP–ACP in plaque (reduction in diffusion; inhibition of demineralization; increase in calcium binding; enhanced remineralization) are mirrored by similar effects from the single most useful anticaries agent, fluoride. Given that CPP–ACP can be incorporated into foodstuffs as well as therapeutic agents and demonstrates none of the adverse effects of fluoride overuse (fluorosis at moderate doses and toxicity at higher doses), it is possible that this product may become an important anticaries agent in the future.

In summary, CPP–ACP significantly reduces the rate of calcium diffusion in model dental plaques at neutral pH and pH typically found during a cariogenic challenge. Hence, application of CPP–ACP to plaque may reduce calcium loss during a low-pH episode and

Table 1
Variation in k and D_e with fluoride of CPP–ACP treatment at pH 7 and 5, derived from Figs. 2 and 3[a]

	No treatment		+5 mmol/l fluoride		+1% CPP–ACP	
	D_e ($\times 10^{10}$ m^2s^{-1})	k (mmol/l)	D_e ($\times 10^{10}$ m^2s^{-1})	k (mmol/l)	D_e ($\times 10^{10}$ m^2s^{-1})	k (mmol/l)
pH 7.0	2.2 ± 1.2	5.7 ± 1.9	2.3 ± 0.7	2.4 ± 0.4***	2.9 ± 1.5	12.3 ± 1.4***
pH 5.0	2.8 ± 0.6	4.1 ± 2.3	2.6 ± 0.5	2.1 ± 0.6***	3.0 ± 0.9	14.9 ± 2.2***

[a] All figures are in mmol/l, *** denotes significant differences between treated and untreated plaques at $p < 0.001$.

limit demineralization. The presence of CPP–ACP in plaque should, therefore, permit a rapid return to resting calcium concentrations and allow more immediate remineralization. Overall, once in place, CPP–ACP is likely to restrict the caries process.

Acknowledgements

This work was supported by SmithKline Beecham, UK.

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