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Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice

[1]A.W. Cuthbert, Margaret E. Hickman, Lesley J. MacVinish, *M.J. Evans, *W.H. Colledge, *Rosemary Ratcliff, P.W. Seale & P.P.A. Humphrey

Department of Pharmacology, Glaxo Institute of Applied Pharmacology and *Wellcome/CRC Institute of Cancer and Developmental Biology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ

1 Guanylin, a 15 amino acid endogenous gut peptide, increased the short circuit current (SCC) in the epithelium of the mouse colon, but only when applied to the apical and not the basolateral surface.

2 By use of selective blockers of epithelial ion transport and modification of the bathing solution, it was concluded that guanylin increased electrogenic chloride secretion but also had a minor effect on electrogenic sodium absorption. In addition there were small residual currents which remained unresolved.

3 The threshold concentration of guanylin causing a SCC increase was less than 50 nM, but at concentrations 40 times greater no indication of a maximally effective concentration was found.

4 Two guanylin isomers with the same amino acid sequence but with the disulphide bridges joined in an alternate fashion showed no activity. Thus only guanylin with the greatest structural homology to heat stable enterotoxin (STa) showed biological activity.

5 The action of guanylin was virtually eliminated in colonic epithelia from transgenic cystic fibrosis (CF) mice. As these animals lack the chloride channel coded by the CF gene sequence, it is likely that the final effector process in murine colonic epithelia involves the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel.

6 Opportunistic infections of the gut generating STa lead to diarrhoeal conditions via an action of the toxin on apical guanylin receptors. Thus, as discussed, the CF heterozygote may have a genetic advantage in this circumstance.

Keywords: Guanylin; chloride secretion; sodium absorption; epithelia (colonic); cystic fibrosis; heat stable enterotoxin

Introduction

Guanylin is a recently discovered peptide of 15 amino acids isolated from the rat small intestine (Currie *et al.*, 1992). Since the mRNA for the peptide is most abundant in the colon (Weigand *et al.*, 1992) we decided to examine the effects of the peptide on the epithelium of this organ. The peptide is of interest because of structural homology with heat stable enterotoxin (STa) which is produced by *E. coli* and is responsible for some forms of secretory diarrhoea. The structure of guanylin in relation to the structure of STa is shown in Figure 1. Guanylin can displace radiolabelled STa from binding sites in cultured epithelial cells, two populations of binding sites being detectable (Forte *et al.*, 1993). The peptide causes electrogenic chloride secretion in monolayers of T_{84} epithelial cells and increases guanosine 3':5'-cyclic monophosphate (cyclic GMP) content. STa and presumably guanylin are considered to activate GC-C, a plasma membrane form of guanylyl cyclase (Wong & Garbers, 1992; Li & Goy, 1993). This paper gives details of the effects of guanylin on murine colonic epithelium, this species being chosen so that a comparison could be made of the effects of the peptide on both the normal epithelium and that from transgenic cystic fibrosis (CF) animals. It is known that intestinal epithelia from CF mice fail to show chloride secretion in response to agents which elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Ratcliff *et al.*, 1993). The CF gene codes for a protein, CFTR, which behaves as a small conductance epithelial chloride channel (for a review, see Cuthbert, 1992). It is this channel which fails to function properly in CF so that if chloride secretion is normal in CF tissues exposed to guanylin then the peptide might have some application in treatment. Alternatively if chloride secretion is deficient in CF colons treated with guanylin then it is probable that the final effector mechanism in the secretory process is the CFTR chloride channel. CF heterozygotes (carriers) producing fewer channel molecules may therefore withstand better the opportunistic infections by organisms generating STa. A second objective of this study was to examine the relative activities of the two other possible guanylin isomers which while possessing the same amino acid sequence differed in the way the disulphide bridges are joined.

[1] Author for correspondence.

Methods

Animals and tissues

Embryonic stem cells carrying a disruptive mutation in exon 10 of the cftr locus were injected into C57Bl/6 host blastocytsts to derive chimeric animals. Heterozygote F_1 animals were intercrossed to generate homozygous CF offspring, the genotypes of the F_2 offspring being ascertained by Southern blot analysis (Ratcliff *et al.*, 1993). CF and wild type mice were treated in identical fashion. Mice were killed by exposure to 100% CO_2 and the large intestine removed in its entirety and small lengths around 0.5 cm long were cut from the distal colon starting at about 1 cm from the anal end. Maximally four pieces were taken from mice weighing 25–30 g. Each piece was opened longitudinally and the muscle layers dissected away under a microscope. The tissues were mounted in Ussing chambers with a 20 mm^2 window using Parafilm washers to cushion the tissue. Transepithelial potentials were monitored by fine polythene tubes filled with Krebs Henseleit solution (KHS) which ended within a mm of the tissue surface. These tubes led, via a 3 M KCl solution and calomel cells to the input stage of a voltage clamp (WPI

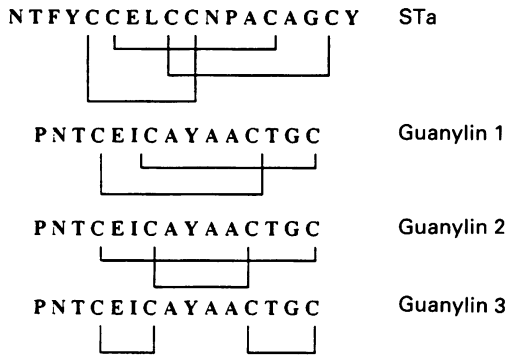

Figure 1 Structure of STa and three guanylin peptides showing the position of the disulphide bonds. The single letter amino acid code is used to define the peptide sequence.

Dual Voltage Clamp). Current was passed via Ag/AgCl electrodes and gel filled tubes containing 3 M KCl to current ports in the chambers far removed from the tissue. Each side of the tissue was bathed in 20 ml KHS solution maintained at 37°C and gassed with 95% O_2/5% CO_2.

In a few instances preparations were made from the murine ileal epithelium at its mid region. Exactly the same procedure was used as for the colon.

Solutions

The KHS solution used had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1. This solution had a pH of 7.4 when gassed with 95% O_2/5% CO_2 at 37°C. In some experiments modified bathing solutions were used. When chloride was replaced by gluconate the following changes were made: NaCl was replaced with Na gluconate, KCl and $CaCl_2$ with the sulphate salts except a ten fold excess of the calcium salt was used to maintain sufficient ionised calcium in the presence of gluconate (Boron & Boulpaep, 1983). When it was necessary to remove all permeable anions, the same substitutions were made as above except that NaCl and $NaHCO_3$ were replaced with Na isethionate. In this circumstance it was not necessary to add excess calcium. The pH of this solution was controlled with Tris buffer, 10 mM, pH 7.4 and the solution was gassed with pure O_2.

Preparation of peptides

The guanylin isomers were prepared by solid phase peptide synthesis using a Novabiochem Crystal continuous flow synthesizer and the Fmoc strategy. Pairs of cysteine residues were orthogonally protected with trityl (Trt) or acetamidomethyl (acm) protecting groups. Cleavage of the assembled sequences from the solid phase with trifluoroacetic acid (TFA) gave the bis-acm dithiol tetra cysteine peptides. Air oxidation of the thiol groups yielded the first disulphide bridge. Oxidation of the bis-acm disulphide with iodine in acetic acid formed the second disulphide bridge. Peptides were purified by h.p.l.c. using a Dynamax C_{18} column in acetronitrile-water containing 0.1% TFA, and were characterized by amino acid analysis and liquid secondary ion mass spectra (LSIMS).

Materials

Drugs were obtained from the following sources: niflumic acid and amiloride from Sigma Chemical Co., Poole, Dorset; frusemide from Hoechst, Hounslow and acetazolamide from Lederle Laboratories, Gosport, Hants. All other chemicals used were of reagent grade.

Results

Characteristics of the responses to guanylin

Guanylin caused a rapid increase in the SCC of mouse colon epithelium *in vitro* which was maintained as long as the peptide was present (up to 1 h). This action of the peptide was reversible, so that repeated responses could be obtained. In this study we allowed 90 min between consecutive applications of peptide to ensure all the peptide was removed and the epithelium had returned to a steady state. Under these conditions, a 50% reduction in the size of the responses was seen up to 4.5 h, but in some individual tissues no diminution occurred at all.

A series of experiments, all performed with guanylin 1 μM is shown in Figure 2 to illustrate the main characteristics of the responses to the peptide. Addition of the peptide to both surfaces of the epithelium produced no greater response than with apical application alone, while basolateral addition was without effect (Figure 2a). Amiloride, the epithelial sodium channel blocker, produced a minor reduction in SCC when applied during the plateau response to guanylin but produced no effect when added before the peptide. Frusemide, the NaK2Cl cotransport inhibitor, reduced the response to guanylin when added before the peptide and reduced the SCC

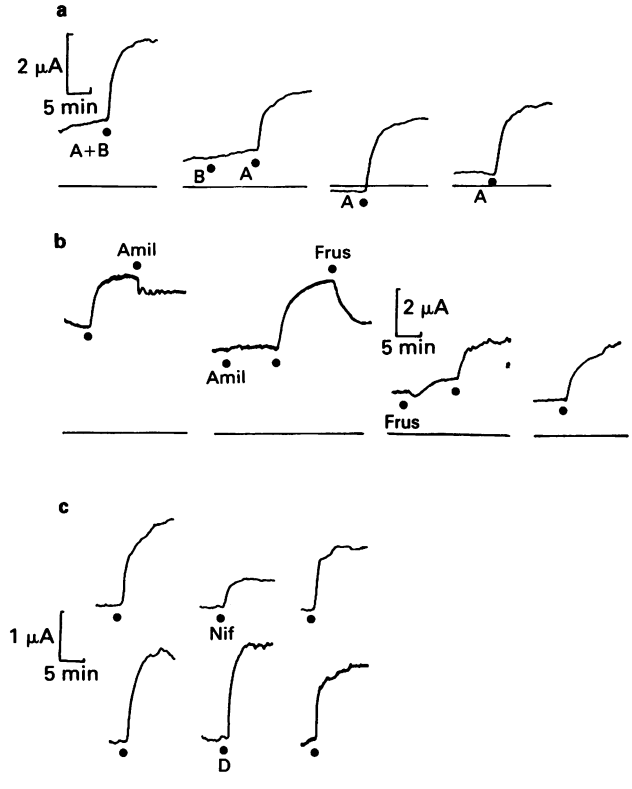

Figure 2 Effects of guanylin on SCC in mouse colon. At each unlabelled symbol guanylin, 1 μM, was added to the apical bathing solution of a mouse colonic epithelium, area 20 mm^2, with an interval of 90 min between each addition with washing between. In (a) and (b) the horizontal lines indicate the value of zero SCC. The mean basal SCC values for the two preparations shown in (c) were 1.9 μA (upper) and 0.5 μA (lower). In (a) the symbols A, B, A + B indicate when guanylin was added to the apical, basolateral or both solutions. In (b) amiloride (Amil), 100 μM, was added to the apical bathing solution at the times indicated. Similarly Frus indicates when frusemide, 1 mM, was applied basolaterally. In (c) the upper and lower sets of records are from two adjacent pieces of epithelium. In the response labelled Nif, niflumic acid 100 μM, was present in the apical bathing solution 15 min before guanylin was added, while D indicates when an equivalent amount of solvent (DMSO) had been added 15 min before the peptide.

when applied during the plateau phase of the response (Figure 2b). However, the response to frusemide was not impressive, a substantial residual current remaining. Finally, the chloride channel blocker, niflumic acid, attenuated the response to guanylin when applied apically, while the solvent for this agent was without effect on the response (Figure 2c). Niflumic acid had a minor effect on basal SCC, but no greater than the effect of an equivalent amount of dimethylsulphoxide (DMSO) in which it was dissolved. Niflumic acid also inhibited the effect of the known chloride secretagogue, lysylbradykinin, on the mouse colon epithelium.

Nature of the transported ions(s)

In two sets of experiments chloride ions were replaced by impermeable anions (gluconate and isethionate) either with or without simultaneous removal of bicarbonate. When chloride was substituted with gluconate in the continued presence of bicarbonate responses to guanylin, 1 μM, were unimpaired. Acetazolamide and amiloride both caused minor reductions of SCC during the plateau response to guanylin. However, amiloride had no effect on the basal current in the absence of guanylin (Figure 3a).

In the second series, both chloride and bicarbonate were replaced by isethionate and the bathing solution buffered to pH 7.4 with Tris. In this situation the responses to guanylin were reduced to about 30% of the control value in chloride containing solution. Nevertheless, there was still a distinct increase in current, which could not have been due to chloride secretion (Figure 3b).

In the four experiments illustrated in Figure 3, amiloride caused inhibition of the SCC indicating that some part, at least, of the response in chloride-free solutions was due to electrogenic sodium transport, since when amiloride was added before guanylin there was no rapid fall in SCC, indicating that sodium transport was not active in the absence of the peptide.

Further experiments were limited by the availability of the peptide but taking the findings of this and the previous section together it is possible to be definitive about the nature of the effects of guanylin on ion transport as discussed later.

Concentration-response relationship to guanylin

The maintenance of the plateau response in the presence of guanylin made it possible to determine a concentration-response relationship by cumulative addition to the apical bathing solution. A partial concentration-response relationship is given in Figure 4 with no indication that the maximal effect had been reached, indeed the curve is steepening at the maximum concentration used (2 μM). Lack of peptide prevented further additions at higher concentrations. The threshold concentration for the SCC effect was less than 50 nM.

Response of mouse ileal epithelium

As guanylin was first isolated from the rat small intestine (Currie *et al.*, 1992) it might be presumed to have some epithelial function at that site. Therefore guanylin, 1 μM, was applied apically, every 90 min to stripped ileal preparations. The results are shown in Figure 5 for three separate ileal preparations subjected to this protocol. Responsiveness declined considerably at the second exposure to the peptide but more importantly the responses to guanylin were not maintained, the SCC often returning to baseline values within a few minutes in the continued presence of the peptide (data not shown). We have not taken any steps in this study to prevent the degradation of the peptide when applied to tissues and it is possible that activity in the ileum may have been enhanced if such precautions had been taken. Nevertheless an endogenously released peptide would not have such protection physiologically, although nothing is yet

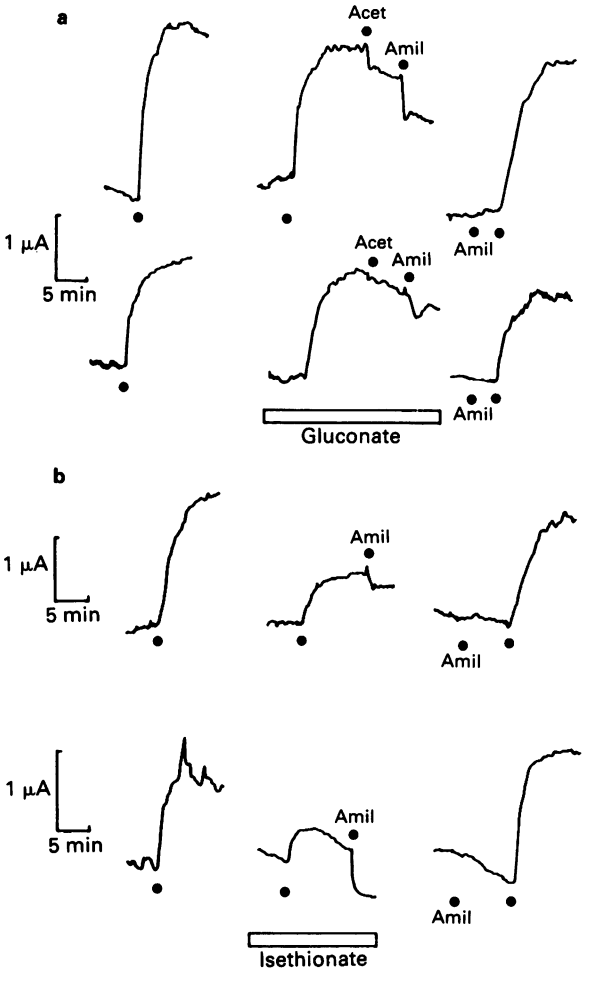

Figure 3 SCC responses in mouse colon epithelium to guanylin. At each unlabelled symbol guanylin, 1 μM, was added to the apical bathing solution. Responses were repeated at 90 min intervals. In (a) both preparations were from the same mouse while in (b) separate epithelia from two mice were used. During the middle response of each set of three responses the bathing solution was modified. In (a) chloride was replaced with gluconate with bicarbonate still present, while in (b) the chloride and bicarbonate were replaced by isethionate. Acet indicates when acetazolamide, 450 μM, was present in both bathing solutions, while Amil indicates the addition of amiloride, 100 μM, to the apical solution.

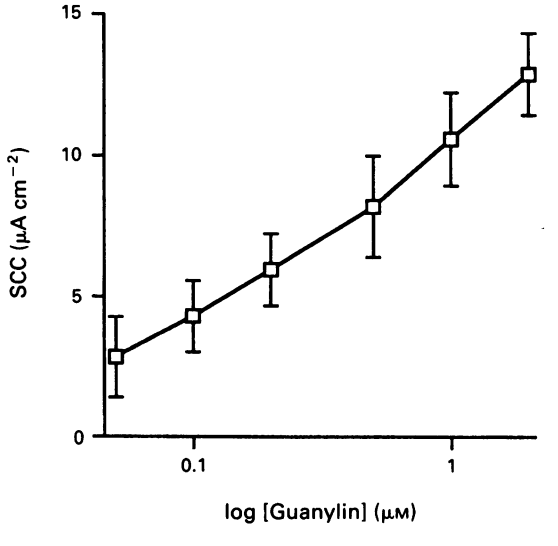

Figure 4 SCC increases in response to guanylin at different concentrations on murine colonic epithelia. Each point shows the mean value ± s.e.mean for three separate preparations.

known of its metabolism. It is evident that guanylin's activity is less impressive on the murine ileum than the colon.

Effects of guanylin in CF mouse colon epithelium

CF mouse colon epithelium was prepared in the same way as for normal colons and exposed to guanylin, 1 μM, on the apical side every 90 min with washing between each application. The responses of normal epithelia and those from CF transgenic mice were compared and the data are given in Figure 6. They show that there is a highly significant ($P < 0.005$, Student's t test) reduction in the response of CF tissues at all time points. In normal colons the mean value of the control responses was about 50% of its initial value at 4.5 h in this series, although as stated earlier some control tissues show no decrement in the responses to guanylin over 6 h. The residual responses in CF tissues were small making them difficult to investigate systematically. The initial approach was to examine if the residual guanylin response in CF colon was sensitive to amiloride, but this entailed a more detailed study of the mouse colon to amiloride. With a maximally effective concentration (100 μM) not all colons showed a response. In normal colons the amiloride-sensitive SCC was 4.8 ± 1.2 μA cm^{-2} (mean ± s.e.mean) (34 observations, 16 animals, 76% responders) whereas in CF colons the values were 3.5 ± 1.0 μA cm^{-2} (38 observations, 16 animals, 76% responders). To examine specifically the nature of guanylin/amiloride interactions in CF colons, four preparations were exposed to guanylin every 90 min for 4.5 h with washing in between. For two tissues, amiloride was present when guanylin was added, while in the other two it was present only after the response to guanylin had developed. The first two responses of each tissue are shown in Figure 7. In the top trace amiloride removed SCC equivalent to the whole of the current generated by guanylin, while the bottom trace with an identical format showed no response to amiloride. In the middle traces it is shown that amiloride sometimes affected the basal current whereas in others it had no effect. Thus guanylin can still cause a minor SCC increase even in the presence of amiloride in CF colons (Figure 6).

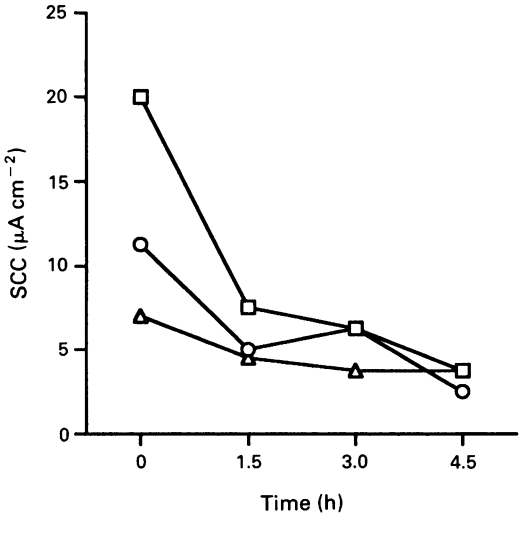

Figure 5 Illustrates the SCC responses of three separate murine ileal epithelial tissues (each 20 mm^2) to guanylin, 1 μM, applied every 90 min with washing between.

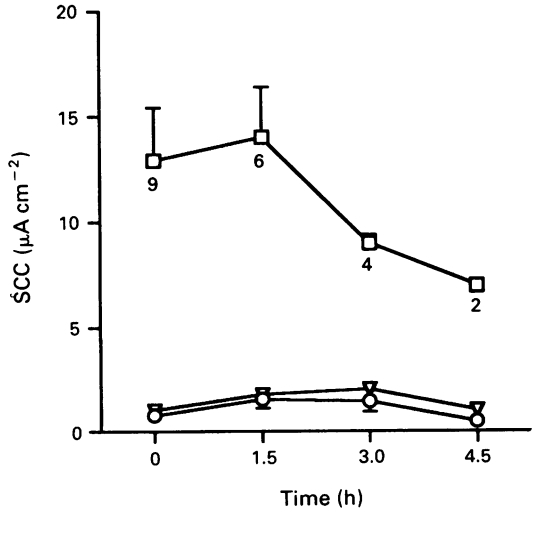

Figure 6 SCC responses in normal and CF murine colonic epithelia to guanylin, 1 μM, every 90 min with washing between; (□) normal tissues, with mean values ± s.e. and the number of observations for each value shown; (○) mean ± s.e. for CF tissues, each for five observations; (△) mean responses in two CF tissues in the presence of amiloride, 100 μM.

Activity of guanylin analogues

Guanylin isomers in which the amino-acid backbone was conserved but with different disulphide bridges were synthesized (see Figure 1). Throughout the study so far we have used only guanylin 1 (referred to throughout as guanylin), the isomer with the closest analogy to heat stable enterotoxin (STa). In two experiments, the relative activities of guanylin 2 and guanylin 3 were compared to that of guanylin. No activity of guanylin 2 or 3 was detected, either as an agonist or antagonist of the effects of guanylin, given the constraint that limited amounts of material were available.

Figure 8 gives an example of the type of experiment carried out, in this instance with guanylin 3. Paired colonic

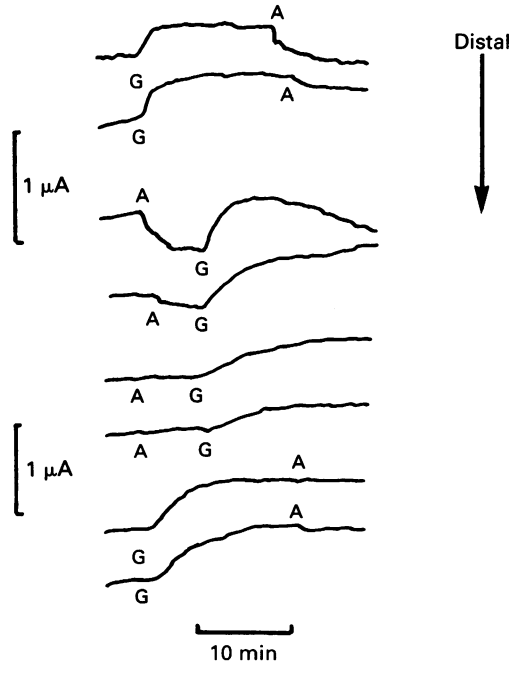

Figure 7 Responses to guanylin, 1 μM, and amiloride, 100 μM, both applied apically in four CF colonic epithelia all derived from the same mouse and designated by G and A respectively. Each pair of responses was obtained consecutively 90 min apart. The most distal tissue is represented by the upper pair of traces, the other three pieces were adjacent and consecutively more proximal. Note in some tissues amiloride was given before guanylin and in others afterwards and that the calibration for the lower two tissues is different from the upper pair.

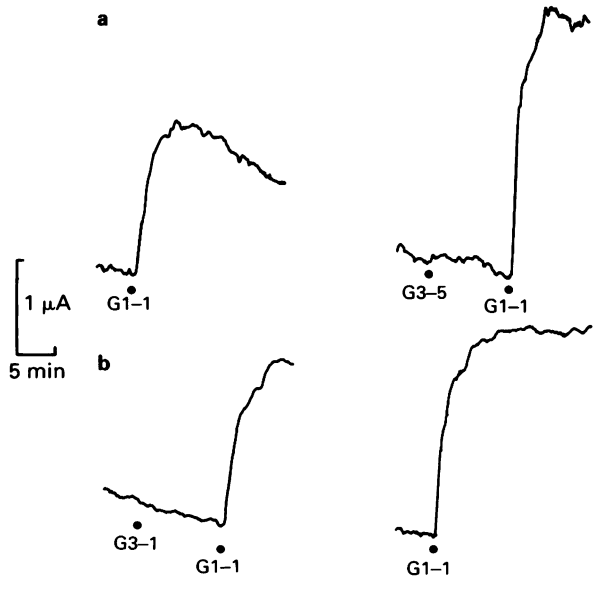

Figure 8 SCC responses to two guanylin analogues, guanylin (G1) and G3. The concentrations applied to the apical surface of colonic epithelia, in μM, are indicated by the symbol for the peptide used. Two adjacent pieces of epithelia were used. In one (a) G1 was applied first and then, 90 min later, G3 followed by G1. In the other (b) the tissue was exposed to G3 then G1 and 90 min later to G1. Note G3 neither affected SCC alone nor inhibited the action of G1.

preparations from adjacent sections of the gut were used in a crossover test. One preparation received guanylin and the other guanylin 3, then to the preparation that received guanylin 3 the active peptide was added 10 min later in the continued presence of the inactive peptide. After washing the tissues the protocol was repeated 90 min later but changing the tissue which was exposed first to the inactive peptide. It is apparent that guanylin 3 has no effect on SCC at a concentration of 5 μM, neither does it markedly affect the action of guanylin at this concentration. Similarly with guanylin 2 (1 μM) there was no indication that this peptide had either agonist or antagonist activity at the level of the guanylin receptor(s). While it was not possible to use significantly higher concentrations of guanylins 2 or 3, any action they might have is insignificant when given that the threshold for the guanylin effects is < 50 nM.

Discussion

The direction of the SCC responses to guanylin, the partial inhibition of the responses by frusemide and niflumic acid, indicating the involvement of the NaK2Cl-transporter and chloride channels respectively, suggest that the peptide increases chloride secretion in the mouse colon. The hypothesis confirms an expectation by analogy with the well known anion-secretory activity of heat stable enterotoxin (STa) (Field *et al.*, 1978) with which guanylin has a partial structural homology. STa, generated *in vivo* by opportunistic bacterial infections of the gut, acts only from the apical side of the epithelium, as does guanylin. Furthermore, T_{84} monolayers (a human tumour-derived cell line with the characteristics of colonic epithelial crypt cells) also show chloride secretion with guanylin but only when applied apically (Forte *et al.*, 1993). The mouse colon is distinctly more complex than T_{84} monolayers because in addition to anion secretory crypt cells the surface cells are presumed to be absorptive, by analogy with other species.

The mouse colon appears to have intermediate properties to that of the rabbit and the rat. The former always shows an amiloride sensitive SCC while the latter only demonstrates this after treatment with mineralocorticoids (Will *et al.*, 1981). Five examples are given (Figures 2 and 3) showing an effect of amiloride during the plateau response to guanylin, yet in each example the sodium transport blocker had no effect in the absence of the peptide, suggesting that the latter itself stimulated electrogenic sodium absorption. As with normal colons, only some CF preparations showed amiloride-sensitivity in the basal state. In the presence of amiloride, guanylin was still able to elicit a small SCC increase of a similar size to that seen in its absence. The small size of these currents make investigation difficult and furthermore, amiloride addition may polarize the epithelium in a way which alters the ionic gradient for other small, unresolved currents. In summary the evidence is that guanylin stimulates sodium absorption as well as chloride secretion in normal mouse colon but the evidence that the former is still present in CF colon is equivocal.

In the absence of chloride, but with bicarbonate present, the response to guanylin was not modified. Acetazolamide produced only a minor reduction in SCC in this situation, perhaps not an unexpected result since the solutions were gassed with 5% CO_2 and the hydration of CO_2 within the cells probably remained high. In some species (rabbit) the colonic epithelium can secrete bicarbonate, especially in the absence of chloride (Grasl & Turnheim, 1984) and it seems probable that the same is true for the mouse. Bicarbonate ions may be secreted through chloride channels or residual intracellular chloride may be recycled across the apical membrane in parallel with a chloride bicarbonate exchanger. When both chloride and bicarbonate were replaced by isethionate the response to guanylin was reduced to around 30% of the control values. Part of the residual response seems to be due to sodium absorption, but in some individual tissues there remained a significant current which is unexplained. It is notable that frusemide never gave complete inhibition of the guanylin SCC response, while it did so in T_{84} monolayers (Forte *et al.*, 1993) again indicating the presence of some unresolved current(s). This is what might be expected since T_{84} monolayers consist exclusively of crypt cells.

The location of guanylin receptors in gut epithelium is unknown but if they occur in both crypt and surface cells it may be that the chloride secretory effect arises in the latter, while the effect on sodium transport is mediated by an action on surface epithelial cells. In the rat GC-C mRNA is expressed in the crypt epithelium but to a lesser exent in surface epithelial cells, while guanylin mRNA is expressed strongly in surface cells and in the upper 20% of the crypts (Li & Goy, 1993). Nevertheless taking all the present evidence together it indicates that guanylin causes chloride secretion in the mouse colon and that under some conditions bicarbonate may be secreted in place of chloride. Also there is strong evidence that guanylin can cause electrogenic sodium absorption but these two transporting activities do not necessarily arise from stimulation of the same epithelial cells.

The inability of guanylins 2 and 3 to produce any discernible effect on SCC suggests that the restriction of the peptide conformation by the disulphide bridges as in guanylin and STa are crucial for biological activity.

Of particular interest is the finding that the action of guanylin is virtually eliminated in the colonic epithelium from CF transgenic mice. This indicates that agents acting via the guanylin receptor to increase cyclic GMP levels are not likely to be useful agents in the treatment of CF. However it is known that cGK (cyclic GMP-dependent protein kinase) can phosphorylate CFTR, the protein coded for by the CF gene, *in vitro*. Yet cyclic GMP is unable to increase chloride conductance in human cultured airway epithelium (Berger *et al.*, 1993). CFTR contains seven serines which can be phosphorylated but it is not necessary that all of these are modified in order to activate the chloride channel (Cheng *et al.*, 1991) so cGK may phosphorylate only non crucial sites.

There has a been a report that cyclic GMP stimulates chloride secretion in normal but not CF human intestinal epithelia but also that the intestine contains a different isoform of cGK, which may be capable of activating CFTR (de Jonge *et al.*, 1989; O'Loughlin *et al.*, 1991). CFTR not only acts as a chloride channel but its expression in cells has a permissive effect on other channels, for example the ORDIC (outwardly rectifying depolarization induced channel) channels fail to be activated by protein kinase A (PKA) in the absence of CFTR (Egan *et al.*, 1992). Thus there are three possible explanations of the failure of guanylin to activate chloride secretion in murine CF colon.

The first, and most likely, is the absence of CFTR which is normally activated by an intestinal form of cGK. Alternatively it is possible that cyclic GMP is able to activate PKA (Forte *et al.*, 1992) which in a native tissue would activate CFTR. Finally, but highly improbable, is the possibility that guanylin activates a non-CFTR channel which needs CFTR permissively to be activated.

There has been a great deal of speculation as to why the CF gene has not been eliminated by natural selection and the conclusion that there is a heterozygote advantage has been advanced (Hansson, 1988; Field & Semrad, 1993). In CF the CFTR chloride channel fails to be activated by cyclic AMP so that in diseases such as cholera, where the toxin activates adenylate cyclase, heterozygotes may show a lesser diarrhoeal response because less CFTR is expressed. A similar argument is applicable to the STa toxin which is considered to act upon apically located guanylin receptors. It should now be possible to test this hypothesis experimentally by comparing responses in wild type homozygotes with those of heterozygotes.

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