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PROPERTIES OF ADENYLATE CYCLASE IN MUCOSAL CELLS OF THE RABBIT ILEUM AND THE EFFECT OF CHOLERA TOXIN

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SUMMARY

A study of the properties of adenylate cyclase in mucosal epithelial cells of rabbit ileum has been performed under control conditions and after stimulation by cholera toxin. A 3–5 fold stimulation of enzyme activity occurred after treatment with cholera toxin. Optimal conditions for activity were attained with Mg^{2+} concentrations between 5 and 25 mM at a pH of 8.0–9.0. Linearity of reaction rate was achieved over 25 min at protein concentrations of the homogenate of up to 40 μg per 50 μl incubation mixture when an ATP regeneration system was employed. Mn^{2+} at $3 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ M could replace Mg^{2+} but was inhibitory at 5 mM. Ca^{2+} was inhibitory at all concentrations above 10^{-5} M. Michaelis–Menten plots were linear with regard to ATP concentrations under both sets of conditions, V was 28 pmoles·mg^{-1} protein·min^{-1} for control and 90 pmoles·mg^{-1} protein·min^{-1} for toxin-treated enzyme. K_m for both conditions were $4 \cdot 10^{-4}$ M. No evidence of cooperativity was detected. The mechanisms of activation by F^- and cholera toxin were presumed different from an analysis of experiments involving combinations of F^- and toxin and by a study of pyrophosphate inhibition. Isoproterenol at 10^{-5} M was stimulatory to the control enzyme, while no effect of epinephrine or propranolol was detected. In contrast, isoproterenol, epinephrine and propranolol were inhibitory to the enzyme after stimulation by cholera toxin.

INTRODUCTION

The diarrhea of cholera is due to the action of an entero–exotoxin on the small

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intestine[1]. In experimental animals, the application of toxin to the lumen of the small intestine results, after a latent period, in progressive fluid accumulation[1,2]. This will occur at any level of the small intestine and even brief exposure of the cells to toxin is sufficient to elicit the response[2,3].

It is now known that the toxin acts *via* stimulation of adenylate cyclase[4–9] and an increase in the intracellular concentration of adenosine 3′,5′-cyclic monophosphate (cyclic AMP)[10]. It is probable that in the production of fluid, cyclic AMP stimulates an anion transport system from blood to lumen in excess of the reabsorptive capacity of the intestine, and that water follows osmotically[11,12].

In this paper the characteristics of the enzyme under normal and toxin-stimulated conditions are described. Our purpose was to seek for differences induced in the enzyme by the action of cholera toxin in an attempt to obtain information relevant to its mechanism of action.

METHODS

New Zealand white rabbits (2–2.5 kg) were used. Laparotomy was performed under sodium pentobarbital anesthesia. The succus entericus was washed out of the portion of intestine chosen for study with Ringer's solution. Loops of 5–10 cm were tied off with double sutures so that all test and control loops had small spacer loops between them. Care was taken to maintain the blood supply to the loops intact. Rubber catheters were tied into one end of each loop to drain off excess fluid. Cholera toxin in Ringer's solution was injected into the loops as required. When the incubation was complete the loops were removed and placed in ice-cold Ringer's solution, cut longitudinally, and washed. They were then washed in 75 mM Tris buffer (pH 8.5) containing 12.5 mM $MgCl_2$, blotted on filter paper and the cells scraped off with glass slides. Homogenization was carried out in Dounce homogenizers in the Tris–$MgCl_2$ buffer. Protein content was estimated by the method of Lowry *et al.*[13] and the homogenate diluted to the required concentrations.

Adenylate cyclase activity was determined by the method of Krishna *et al.*[14]. Samples of the epithelial cell homogenate, usually containing 20–40 μg of protein per 20 μl were added to 30 μl of incubation mixture containing 1 μCi [α-^{32}P]ATP and incubated at 37 °C. Most incubations were of 20 min duration. The final composition of the incubation mixture was: 30 mM Tris–HCl buffer (pH 8.5), 0.1 mM cyclic AMP, 0.1 mM ATP, 5 mM $MgCl_2$, 5 mM phosphoenol pyruvate, 40 μg/ml pyruvate kinase and 20 μg/ml myokinase. Reaction blanks for each set of experiments were estimated simultaneously. The reaction was terminated by the addition of 1 ml of a solution containing 50 μg of cyclic AMP, 100 μg ATP and cyclic [^{3}H]AMP for calculation of recovery, followed by immersion of the reaction tube in boiling water for 5 min. The contents of the tubes were cooled and transferred to 0.6 cm × 4.0 cm chromatographic columns of AG 50W-X2 (200–400 mesh) resin. The columns were eluted with water and cyclic AMP collected in the 4th and 5th ml of effluent. This fraction was purified by two precipitations with 200 μl of 0.25 M $ZnSO_4$ and 200 μl of 0.25 M $Ba(OH)_2$, care being taken that the final pH was close to pH 7.5. After the second centrifugation the supernatant was transferred to scintillation vials and ^{3}H and ^{32}P estimated by double isotope counting in a liquid scintillation counter. Recovery of cyclic AMP was usually between 50–70%. The

amount of cyclic AMP found was calculated from the specific activity of the [α-^{32}P]-ATP in the incubation mixture and the amount of cyclic [α-^{32}P]AMP recovered *minus* the reaction blank. Results are expressed in pmoles of cyclic AMP·mg^{-1} protein·time^{-1}.

MATERIALS

Crude exotoxin of *Vibrio cholera* was the Wyeth-NIH lot 001. Purified cholera toxin (choleragen) was a gift from Dr Richard A. Finkelstein. [α-^{32}P]ATP (spec. act. 4–6 Ci/mmole) was obtained from International Chemical and Nuclear Corp., Irvine, Calif. Cyclic [^{3}H]AMP (spec. act. 12.7 Ci/mmole) was obtained from Schwarz Bioresearch, Orangeburg, N.Y. Ion-exchange resin AG 50 W-X2 (200–400 mesh), ATP (disodium salt·4H_2O), cyclic AMP, 2-phosphoenolpyruvate (tricyclohexammonium salt), pyruvate kinase from rabbit muscle (300 I.U./ml/9.3 mg protein) and myokinase from rabbit muscle (660 I.U./mg protein) were obtained from Calbiochem, Los Angeles, Calif.

RESULTS

(a) Effects of different concentrations of Mg^{2+} in the reaction mixture

Mg^{2+} is an essential cation for the activity of adenylate cyclase. Consequently, the effect of different concentrations of $MgCl_2$ were tested on the activity of the basal enzyme, cholera toxin-stimulated enzyme and F^--stimulated enzyme. For these studies the epithelial cells were washed, scraped off and homogenized in 75 mM Tris–HCl buffer without $MgCl_2$. $MgCl_2$ at different concentrations was then added directly to the reaction mixture. The results are shown in Fig. 1. Activity was low at 1 mM $MgCl_2$ and rose to maximum values between 5 and 25 mM. Higher concentrations were inhibitory and at 312 mM activity was abolished.

(b) Effect of pH

The pH for the incubation medium was varied with Tris buffer between 6.0 and 9.0. It was found that the pH optimum for intestinal adenylate cyclase activity was between 8.0 and 9.0 for the toxin-treated enzyme. Little activity was observed at pH 6.0 and 6.5 but increased sharply from 6.5 to 8.0. No difference in activity was seen between 8.0 and 9.0 in contrast to most adenylate cyclases studied thus far, which show a decrease in activity between pH 8.0 and 9.0. With the basal enzyme, low levels of activity were seen to rise from 6.5 to 7.0 and then remain constant to pH 9.0. The results are shown in Fig. 2.

(c) Activity of adenylate cyclase as a function of protein concentration and time of incubation

Homogenates of intestinal cells were prepared so that the reaction mixture contained different concentrations of protein from 20 to 80 μg. These were assayed for different time intervals from 10 to 25 min. The results of one such experiment are shown in Fig. 3 as pmoles cyclic AMP·mg^{-1} protein·time^{-1}. The enzyme activity was linear with time up to 25 min except for the assay in which the highest protein concentrations (74 and 79 μg) were used. It is clear, however, that the activity of the

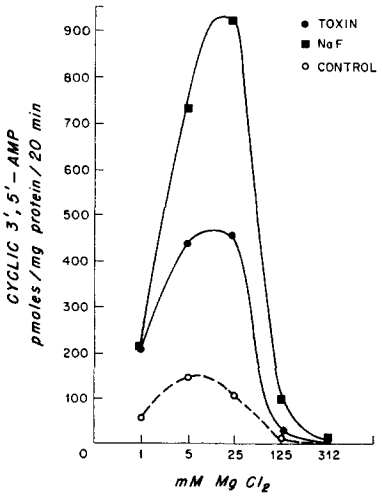

Fig. 1. Effect of different concentrations of Mg^{2+} on adenylate cyclase activity of control tissue (○---○), toxin-treated tissue (●—●), and F^--stimulated tissue (■—■). Results are expressed as pmoles cyclic AMP·mg^{-1} protein·20 min^{-1}.

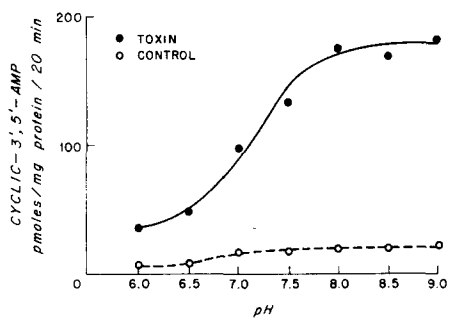

Fig. 2. Effect of pH on adenylate cyclase activity of control tissue (○---○), and toxin-treated tissue (●—●).

preparation per mg of protein does not show exact proportionality, and as the protein concentration is increased the enzyme activity is reduced. For this reason all comparative assays, *e.g.* of control and toxin treated intestinal loops, were used at concentrations of 20–40 μg and were carefully matched for protein content.

(d) Activity of adenylate cyclase as a function of the concentration of ATP

Adenylate cyclase activity was measured in homogenates from control and toxin treated mucosal epithelial cells at concentrations of ATP from 10^{-5} to $3 \cdot 10^{-3}$ M. Activity in the toxin treated tissue was at least 3-fold greater than in the control tissue at all concentrations tested, with no difference in the proportionality at high or low concentrations (see Fig. 4). Michaelis–Menten plots were linear with regard to ATP concentration under both sets of conditions (Fig. 5). V was 28 pmoles·mg^{-1} protein·min^{-1} for control and 90 pmoles·mg^{-1} protein·min^{-1} for toxin treated enzyme. K_m for both control and toxin stimulated enzymes was $4 \cdot 10^{-4}$ M. When

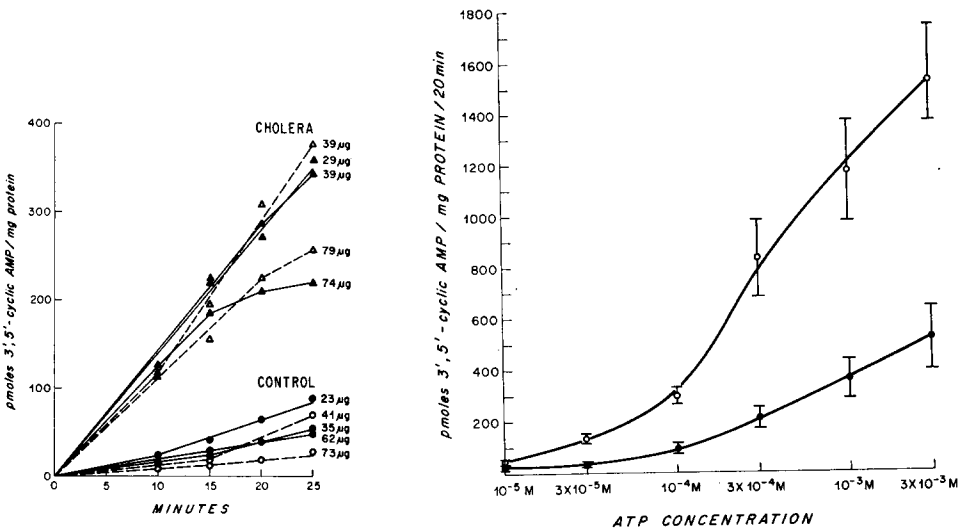

Fig. 3. Accumulation of cyclic AMP as a function of protein concentration of the homogenate and tissue. Linearity was achieved for 25 min at protein concentration of 20–40 μg per 50 μl incubation volume.

Fig. 4. Effect of different concentrations of ATP on reaction rate for control tissue (●—●) and toxin-treated tissue (○—○).

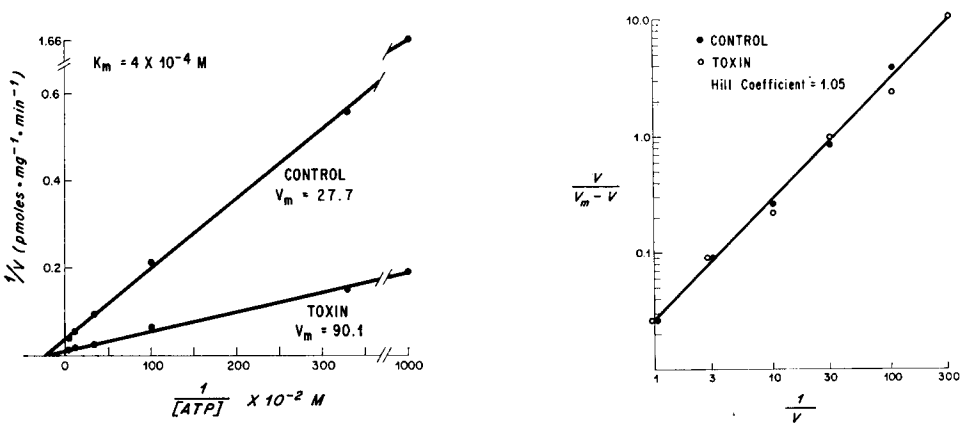

Fig. 5. Double reciprocal plot of the velocity of reaction and substrate concentration.

Fig. 6. Hill plot derived from the reaction velocities at different substrate concentration.

the results are expressed in terms of the Hill plot[15,16] a value of $n = 1.05$ for both control and toxin treated enzymes suggests no evidence of cooperativity in the stimulation of enzyme activity.

(e) Activity of adenylate cyclase as a function of Ca^{2+} concentration

No effect of Ca^{2+} was detected with concentrations at, or below, 10^{-5} M. Concentrations of Ca^{2+} above 10^{-5} M caused progressive inhibition of activity with marked inhibition at 10^{-3} M (Fig. 7).

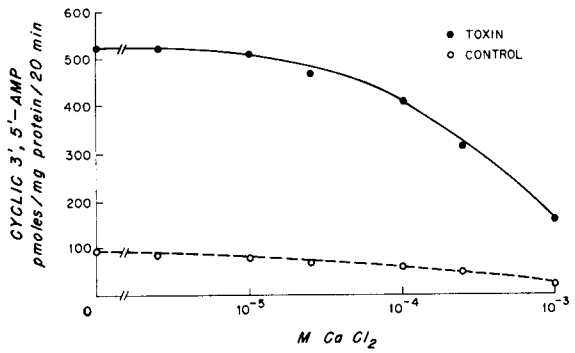

Fig. 7. Effect of different concentrations of Ca^{2+} on adenylate cyclase activity in control tissue (○---○), and toxin-treated tissue (●—●). Results expressed as pmoles cyclic AMP·mg^{-1} protein·20 min^{-1}.

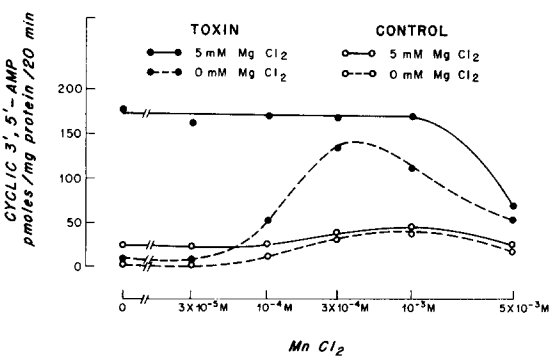

Fig. 8. Effect of different concentrations of Mn^{2+}, in the presence and absence of 5 mM Mg^{2+}, on control and toxin-treated tissue.

(f) Effects of Mn^{2+} on adenylate cyclase activity

In Fig. 8 are shown the effects of different concentrations of Mn^{2+} on homogenates of control and toxin treated intestinal cells in the presence and absence of Mg^{2+}. For the basal enzyme Mn^{2+} was stimulatory at $3 \cdot 10^{-4}$ and at $1 \cdot 10^{-3}$ M in the presence and absence of Mg^{2+}. For the toxin treated cell homogenate, Mn^{2+} at 5 mM was inhibitory in the presence of Mg^{2+}. In the absence of Mg^{2+}, Mn^{2+} was capable of stimulating enzyme activity between $1 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M, with peak activation at $3 \cdot 10^{-4}$ M. Indeed, at this concentration Mn^{2+} had almost the same activating ability as $5 \cdot 10^{-3}$ M Mg^{2+}.

(g) Stimulation of adenylate cyclase by F^- in homogenates of control and toxin treated cells

The activation of adenylate cyclase by 10^{-2} M NaF is shown in Table I. The absolute level of enzyme activity stimulated by F^- from tissue treated with a maximal amount of cholera toxin was significantly higher than the level of activity achieved in control tissues. The increment in activity caused by F^-, however, was significantly lower in the cholera treated tissue than the control. Thus, the effects

TABLE I

ACTIVITY OF ADENYLATE CYCLASE UNDER BASAL CONDITIONS AND AFTER STIMULATION WITH A MAXIMAL DOSE OF CHOLERA TOXIN IN THE PRESENCE AND ABSENCE OF NaF

Activity expressed as pmoles cyclic AMP·mg^{-1} protein·20 min^{-1}. NaF concentration, 10^{-2} M.

	Control	*Toxin*	*Δ ± S.E.*	*P*	*n*
Basal	96	396	300 ± 34	0.001	20
NaF	441	572	131 ± 46	0.01	20
NaF *minus* basal	345	176	−169 ± 37	0.001	20

of cholera toxin and F^- to stimulate adenylate cyclase are neither additive nor completely overlapping.

(h) Effect of pyrophosphate on adenylate cyclase activity

As pyrophosphate has been reported to inhibit the F^- activation of adenylate cyclase, but to have little effect upon the hormonally stimulated enzyme[17], the effect of pyrophosphate was studied on control, F^- and toxin stimulated adenylate cyclase activity. From the results in Table II it can be seen that pyrophosphate ($5 \cdot 10^{-4}$ M) had no effect on basal enzyme activity. The F^- response was reduced by 57% while the toxin treated enzyme activity was decreased by only 11%. There was a highly significant effect of pyrophosphate on F^- stimulation relative to toxin stimulation. This provides a second distinction between the actions of F^- and cholera toxin to stimulate adenylate cyclase.

TABLE II

ACTIVITY OF ADENYLATE CYCLASE UNDER BASAL, TOXIN-STIMULATED AND FLUORIDE-STIMULATED CONDITIONS IN THE PRESENCE AND ABSENCE OF PYROPHOSPHATE

Activity expressed as pmoles cyclic AMP·mg^{-1} protein·20 min^{-1}. Pyrophosphate concn $5 \cdot 10^{-4}$ M.

	Pyrophosphate		*Δ ± S.E.*	*% inhibition*	*P*	*n*
Control	*Absent*	*Present*				
Control	67	67	0 ± 3	0		6
Toxin	320	284	− 36 ± 14	11	< 0.05	6
NaF	344	146	−198 ± 66	57	< 0.05	6

(i) The effects of epinephrine, isoproterenol and propranolol on adenylate cyclase under control and cholera toxin stimulated conditions

Epinephrine and isoproterenol were tested at 10^{-7}–10^{-5} M for their effects on adenylate cyclase activity, as was the β blocking agent propranolol. The results obtained under control conditions are shown in Table III. Isoproterenol at 10^{-5} M produced a significant increase in activity, but only by 18%. With epinephrine and propranolol no effects were detected. In contrast to these results, all three agents were inhibitory to adenylate cyclase after stimulation by cholera toxin (see Table IV). Epinephrine at 10^{-6} M and isoproterenol at 10^{-6} and 10^{-5} M were slightly inhibitory (12%). Propranolol was inhibitory at all three concentrations tested (10^{-7}–10^{-5} M),

enzyme activity which may become maximal after as long as 5 or 6 h. Thus, the stimulation is gradual and prolonged. This pattern of activation might be achieved if the toxin is itself an enzyme which gradually changes the inhibitory membrane environment of adenylate cyclase.

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activated by cholera toxin. Thus the increased activity of intestinal adenylate cyclase after exposure to cholera toxin appears to be achieved without any obvious change in the characteristics of the enzyme.

In a previous paper[5] it was suggested that as the response of adenylate cyclase in rabbit intestine to cholera toxin and F^- was overlapping, the mechanisms of stimulation by these two agents might be similar. However, the response to F^- by adenylate cyclase under basal and toxin stimulated conditions has been reported to be different in rabbit[5] and human small intestine[6,7], on the one hand, and guinea pig small intestine[4] on the other. In guinea pig ileum the response to F^- in cholera treated tissue gave a higher level of activity than F^- in control tissue[4]. As our previous reports on rabbit and human tissue had shown no such difference, we repeated the rabbit study with a larger group of animals. The result of this latter study now agrees with the finding of Kimberg *et al.*[4] in that a significantly greater activity of the enzyme was achieved with F^- in the toxin treated tissue than in the control tissue. As the F^- stimulation was maximal in these studies, the mechanisms of activation by F^- and by cholera toxin are presumably different. As the increment of F^- activation was significantly lower in the toxin treated tissue than the control, then the two effects are neither additive nor completely overlapping. However, the conclusion from previous studies that cholera toxin is not causing the synthesis of new enzyme[4–7] still holds because there is no proportionality between the increase in activity due to cholera toxin and the increase in the F^- response. One further point which differentiates the stimulation by cholera toxin from the stimulation by F^- can be seen in the results with pyrophosphate. Birnbaumer and Rodbell[17] have reported that pyrophosphate inhibits the activation of adenylate cyclase by F^- while having little effect upon basal activity or stimulation by hormones. In our studies on rabbit ileum, it was found that pyrophosphate at a concentration of $5 \cdot 10^{-4}$ M inhibited the effect of F^- by 60% while only inhibiting the toxin stimulated enzyme by 10%. It had no effect upon basal enzyme activity. Another interesting theory to explain the mechanism of stimulation by cholera toxin has been proposed by Gorman and Bitensky[18]. In studies on mice injected with cholera toxin they found that adenylate cyclase in liver showed increased adenylate cyclase activity. Furthermore, when they investigated the response of liver homogenates to epinephrine and glucagon, they found that control tissue responded to both hormones while liver from injected animals responded only to glucagon. Thus, they suggested that cholera toxin might stimulate adenylate cyclase by use of the β-adrenergic stimulatory mechanism. With this in mind, we investigated the effects of β-adrenergic stimulators and a β-blocking agent. The basal enzyme activity in control tissue from rabbit ileum was stimulated slightly by high concentrations of isoproterenol (10^{-5} M). Epinephrine was not stimulatory. Thus, β-adrenergic stimulation in these cells seems to be of limited physiological importance. In the toxin-stimulated tissue, however, all three agents tested, namely epinephrine, isoproteronol and propranolol, were inhibitory. The reason for these unusual results is not clear and further studies are necessary.

It is clear that stimulation of adenylate cyclase by cholera toxin has some unusual features when compared with stimulation by hormones. For instance, a stimulation of enzyme activity by application of cholera toxin to homogenates of cells has not been demonstrated. It appears that the toxin has to be applied to the intact cell and that after a short latent period, there starts a progressive increase

enzyme activity which may become maximal after as long as 5 or 6 h. Thus, the stimulation is gradual and prolonged. This pattern of activation might be achieved if the toxin is itself an enzyme which gradually changes the inhibitory membrane environment of adenylate cyclase.

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