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Determining steps in the regulatory GTPase cycle of rat pancreatic adenylate cyclase

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The time course of activation and deactivation and the degree of activation at steady state $[E_a]/[E_{tot}]$ of adenylate cyclase, in semi-purified rat pancreatic plasma membranes, were compatible with a simple two-state model with three rate constants, so that $[E_a]/[E_{tot}] = k_{+1}/(k_{+1}+k_2+k_{-1})$.

The hormone CCK-8 increased k_{+1} with GTP in a dose-dependent manner, from 0.2 to 10.9 min^{-1}; k_{-1} increased from 0.01 to 0.3 min^{-1}, i.e. in proportion, but k_2 was unaltered at 7 min^{-1}, so that $[E_a]/[E_{tot}]$ increased 15-fold, from 4 to 61%.

A similar activation was obtained after cholera toxin pretreatment but by a different mechanism. The toxin pretreatment exerted a major inhibitory effect on the value of k_2 and on the corresponding GTPase activity. A pretreatment at the high cholera toxin concentration (30 μg/ml) exerted two additional effects that became evident when p[NH]ppG rather than GTP was used as activating nucleotide: (*a*) a relatively large increase in k_{-1} from an unmeasurably low control value to 0.3 min^{-1}, and (*b*) a four-fold increase in the p[NH]ppG activation rate, k_{+1}. This contrasted with the action of CCK-8, which increased k_{-1} and k_{+1} in proportion.

Introduction

The adenylate cyclase activity of rat pancreatic plasma membranes is stimulated, in the presence of GTP or a GTP analogue, by two families of gastrointestinal peptides that are best represented by cholecystokinin–pancreozymin (CCK) and secretin (Svoboda *et al.* 1976; Svoboda *et al.* 1978*a*, *b*; Christophe *et al.* 1980), respectively. In this pancreatic system as well as in other adenylate cyclase systems, the guanine nucleotide-binding regulatory site(s) (called here the N component(s)) facilitate(s) the interaction between subunits (Svoboda *et al.* 1978*a*; Schlegel *et al.* 1979; Iyengar & Birnbaumer 1979; Iyengar *et al.* 1979; Martin *et al.* 1979) and

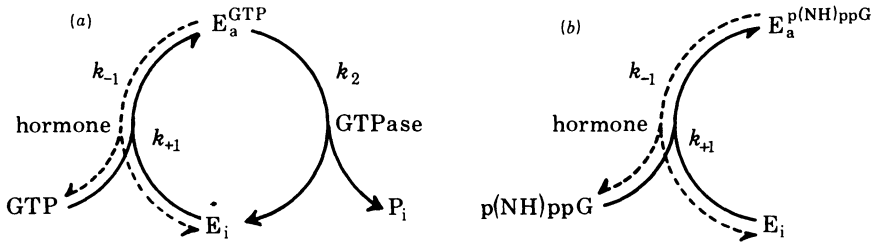

Figure 1. A two-state model comparing the dynamic equilibrium of pancreatic adenylate cyclase between inactive (E_i) and active (E_a) states, in the presence of either GTP (*a*) or p[NH]ppG or GTPγS (*b*). Addition of the hormone CCK-8 increases both k_{+1} and k_{-1} owing to the decrease of the activation energy (Svoboda & Christophe 1979), presumably by 'opening' guanine nucleotide regulatory sites and allowing easier access as well as departure of the activating nucleotide (Cassel & Selinger 1978; Svoboda & Christophe 1979).

activate(s) the catalytic subunit when occupied with GTP, p[NH]ppG† or GTPγS (Svoboda *et al.* 1978*a*; Rendell *et al.* 1977; Cassel & Selinger 1977, 1978). Further, the hydrolysis of GTP may represent the major turn-off mechanism of adenylate cyclase activity (Rendell *et al.* 1977; Cassel & Selinger 1977, 1978). Indeed, we demonstrated 2 years ago the existence of a specific hormone-dependent GTPase of low K_m in rat pancreatic plasma membranes (Lambert *et al.* 1979).

The two-state model of activation–deactivation and its experimental approach

The intricate interaction of hormone or cholera toxin or both with guanine nucleotides in an adenylate cyclase system can be studied by examining the kinetics of the intact system or the chemical properties of isolated and recombined subunits. In the present study, the interaction of the hormone CCK-8 and of a cholera toxin pretreatment with guanine nucleotides was examined on semi-purified rat pancreatic plasma membranes, and the values of the kinetic constants of activation and deactivation with GTP, p[NH]ppG and GTPγS were determined. To interrupt activation, we took advantage of GDPβS and of Bt_2 c-GMP as specific inhibitors of, respectively, GTP (Eckstein *et al.* 1979; Cassel *et al.* 1979; Svoboda *et al.* 1980) and CCK-8 (Robberecht *et al.* 1980). The alterations of kinetic parameters were correlated with the steady-state concentration of activated adenylate cyclase and the activity of specific GTPase observed at equilibrium.

The experimental data were tested against a two-state model (figure 1) in which the concentration of the activated enzyme increases when the activation rate increases, when the deactivation rates decrease, or both. This relation involves a dynamic equilibrium of pancreatic adenylate cyclase between inactive (E_i) and active (E_a) states.

In the presence of GTP (figure 1*a*), the determining step of activation is considered to be a pseudo-monomolecular process (rate constant k_{+1}). The GTP-activated enzyme is deactivated by either the hydrolysis of GTP by a GTPase closely associated to the regulatory site (rate constant k_2) or the dissociation of the intact nucleotide (rate constant k_{-1}). The total deactivation rate constant measured experimentally (k_{off}) includes both processes. GTP alone is a weak activator of pancreatic adenylate cyclase (table 1) (Svoboda *et al.* 1978*b*) because k_{+1} is small in the absence of hormone compared with k_{off}.

When a stable nucleotide is used (figure 1*b*), such as p[NH]ppG or GTPγS which cannot be hydrolysed by the GTPase, this nucleotide proves to be a better activator than GTP since $k_2 = 0$ and $k_{+1} > k_{-1}$.

The present model (Svoboda *et al.* 1981) is characterized, therefore, by three rate constants: k_{+1}, k_2, and k_{-1}. A simpler model of activation–deactivation, with only two rate constants (k_{+1} and k_2) (Blume & Foster 1976; Cassel *et al.* 1977), has been supported experimentally for the β-adrenergic-sensitive adenylate cyclase of turkey erythrocyte. Birnbaumer and his coworkers (Birnbaumer *et al.* 1980*a*, *b*; Iyengar *et al.* 1980) have recently suggested that the enzyme in hepatic plasma membranes may be active not only when occupied by GTP, but also when occupied by GDP, or when free of nucleotide. This is apparently not so in adenylate cyclase systems from rat pancreas or turkey erythrocyte, where enzyme activity is vanishingly low in

† The abbreviations used are: GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); p[NH]ppG, guanosine 5′-*O*-(2-3-imido)triphosphate; Bt_2 c-GMP, dibutyryl cyclic GMP; CCK-8, C-terminal octapeptide of cholecystokinin–pancreozymin.

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the absence of added nucleotide (Svoboda *et al.* 1978*b*; Tolkovsky & Levitzki 1978) or in the presence of GDPβS (table 1), and regardless of whether hormones are present or absent (Eckstein *et al.* 1979; Svoboda *et al.* 1980).

To test the present model, the effects of the hormone CCK-8 and of a cholera toxin pretreatment on the degree of adenylate cyclase activation at steady state and on the three rate constants k_{+1}, k_2 and k_{-1} were considered successively to examine whether the relation implied by the model, i.e. $[E_a]/[E_{tot}] = k_{+1}/(k_{+1}+k_2+k_{-1})$, was valid under all conditions.

TABLE 1. EFFECT OF CHOLERA TOXIN PRETREATMENT ON THE EFFICACY OF FOUR ACTIVATORS ON RAT PANCREATIC ADENYLATE CYCLASE ACTIVITY AT EQUILIBRIUM

(Membranes were pretreated in human erythrocyte cytosol, in the absence or presence of cholera toxin at 0.5 μg/ml or 30 μg/ml, as previously described (Svoboda *et al.* 1980). Membrane proteins (5–10 μg) were incubated for 7 min at 37 °C in 60 μl of an adenylate cyclase assay medium enriched with saturating concentrations of the listed activators. Mean activities ± s.e. from seven membrane preparations are expressed as a percentage of maximal activities attained with 1 μM GTPγS and 0.3 μM CCK-8.)

	cholera toxin concentration during membrane pretreatment/(μg/ml)		
activators added	0	0.5	30.0
1 μM GTPγS + 0.3 μM CCK-8	100	100	100
1 μM GDPβS (basal)	1 ± 1	1 ± 1	6 ± 2
10 μM GTP	4 ± 1	20 ± 4	51 ± 5
10 μM GTP + 0.3 μM CCK-8	61 ± 5	66 ± 4	79 ± 3
10 μM p[NH]ppG + 0.3 μM CCK-8	98 ± 3	89 ± 3	59 ± 4

Effects of CCK-8 and cholera toxin on the degree of adenylate cyclase activation at steady state

In table 1, maximal adenylate cyclase activity was that attained at equilibrium with 1 μM GTPγS and 0.3 μM CCK-8 and served as a reference. Compared with these values, the basal activity in native membranes was almost zero when tested in the presence of 1 μM GDPβS (to inhibit any endogenous GTP-like material). Adenylate cyclase was only weakly activated with GTP alone. A saturating 0.3 μM concentration of CCK-8 allowed a 15-fold increase in GTP fractional activation: from 4 to 61%. When membranes were pretreated with 0.5 and 30 μg/ml cholera toxin, the fractional steady-state activation attained with GTP increased from 4% in untreated membranes to 20 and 51% respectively.

Because the concentration of GTP-activated adenylate cyclase was in dynamic equilibrium between activation and deactivation rates, the increased GTP efficacy with CCK-8 or after pretreatment with cholera toxin was, conceivably, produced by an increased activation rate constant k_{+1}, a decreased deactivation rate constant k_{off}, or both.

Effects of CCK-8 and cholera toxin on the true rate constants k_{+1}, k_2 and k_{-1}

(*a*) *The rate constant of activation, k_{+1}*

k_{+1} is one of the three components of k_{obs}, which is the sum of k_{+1}, k_2 and k_{-1}. With the use of p[NH]ppG, a nucleotide that cannot be hydrolysed, the contribution of k_2 to k_{obs} is zero *a priori*. Furthermore, k_{-1} is very small compared with k_{+1} (see below), so that k_{obs} reflects mostly k_{+1} with p[NH]ppG. In practice, k_{+1} with p[NH]ppG was tested accurately by a two-step incubation method separating the activation of adenylate cyclase from the assay of the degree of activation. The time course of adenylate cyclase activation was followed by transferring at

indicated times aliquots to tubes containing [α-^{32}P]ATP and the p[NH]ppG inhibitor GDPβS. The reaction in each aliquot was stopped after 2 min and the cyclic [^{32}P]AMP formed was assayed.

To understand first the significance of k_{+1}, the pancreatic system was activated at increasing concentrations of p[NH]ppG. The time course of adenylate cyclase activation, at the three concentrations of p[NH]ppG tested (figure 2*a*), can be converted into a semilogarithmic plot of the time variation of activity (figure 2*b*) whose slope gives the value for $k_{+1} = k_{obs}$. Three

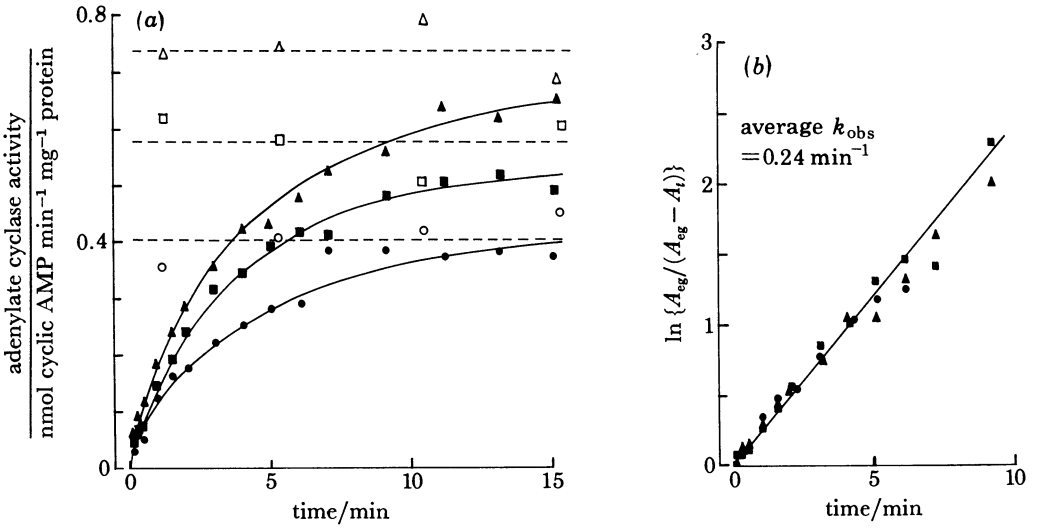

FIGURE 2. The p[NH]ppG activation rate constant, k_{obs}, of native rat pancreatic adenylate cyclase is independent of p[NH]ppG concentration. The experiment consisted of two incubations allowing, successively, the activation of adenylate cyclase and the determination of its activity. Activation with p[NH]ppG at 0.75 μM (●), 2.5 μM (■) and 7.5 μM (▲) was conducted at 37 °C in an adenylate cyclase assay medium lacking [α-^{32}P]ATP. The time course of activation was followed by transferring aliquots to tubes containing GTP and [α-^{32}P]ATP. The reaction in each aliquot was stopped after 3 min and the cyclic [^{32}P]AMP formed was isolated and assayed. The activity of p[NH]PPG-activated adenylate cyclase shown in (*a*) as a function of time is represented in (*b*) as a semilogarithmic plot of the time variation of $A_{eq}/(A_{eq}-A_t)$, where A_{eq} is activity at equilibrium and A_t is activity at time t.

The absence of enzyme degradation under these conditions was shown by the capacity of adenylate cyclase to remain maximally activated at the three p[NH]ppG concentrations tested (0.75 μM (○), 2.5 μM (□) and 7.5 μM (△)). After $1\frac{1}{4}$, $5\frac{1}{4}$, $11\frac{1}{4}$ and $15\frac{1}{4}$ min of activating incubation, aliquots were transferred and incubated with 0.3 μM CCK-8, 0.3 μM secretin and [α-^{32}P]ATP. There was no decay of maximal adenylate cyclase activation (---) during the $15\frac{1}{4}$ min incubation period at all p[NH]ppG concentrations tested. The activities at equilibrium, obtained in the presence of the peptide hormones, depended, on the other hand, on p[NH]ppG concentration and were very close to those extrapolated at equilibrium from curves of p[NH]ppG activation.

conclusions may be drawn. (1) The relatively large k_{+1} (0.24 min^{-1}) explains why the pancreatic system, unlike the turkey erythrocyte system, can be activated with p[NH]ppG alone. (2) The fact that this value for k_{+1} was independent of p[NH]ppG concentration (figure 2*b*) indicated that the activation process was pseudomonomolecular. This regulatory step, allowing easier access of the activating nucleotide, can be visualized as an 'opening' of the regulatory site (Cassel & Selinger 1977, 1978; Svoboda & Christophe 1979) and must be slow compared with the bimolecular binding of p[NH]ppG to its receptor. (3) The increasing degree of activation attained at equilibrium (i.e. after 15 min) with increasing p[NH]ppG concentration (figure 2*a*) obviously reflected a higher proportion of E_{tot} converted to E_a.

The value for $k_{+1} = k_{obs}$ with p[NH]ppG increased markedly and in a dose-dependent manner with CCK-8 (figure 3): there was, for instance, a six-fold increase with 5 nM CCK-8, and at CCK-8 concentrations of 50 nM or more the activation process was too rapid to allow an accurate determination of k_{+1}. An average 55-fold increase of this rate, from 0.2 to 10.9 min^{-1}, with 300 nM CCK-8 concentration was, however, derived indirectly from the efficacy of CCK-8 activation at equilibrium and from k_{off} (table 2 documents the calculated k_{+1} obtained with one plasma membrane preparation). This large effect of a saturating

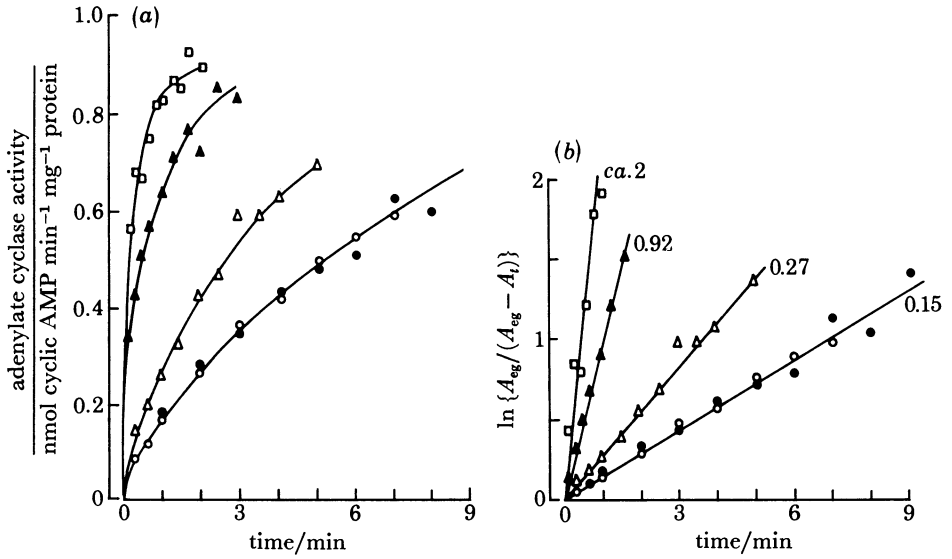

FIGURE 3. Effect of CCK-8 on the p[NH]ppG activation rate constant, k_{obs}, of native rat pancreatic plasma membranes. The activation rate constant was determined by the two-step incubation method described in figure 2: pancreatic membranes were incubated in an adenylate cyclase assay medium lacking [α-^{32}P]ATP and containing, at final concentration, 2.5 μM p[NH]ppG alone (●) or with CCK-8 at 0.05 nM (○), 0.5 nM (△), 5 nM (▲) or 50 nM (□). At indicated times, aliquots were transferred and incubated with 0.3 mM GDPβS, 0.7 mM Bt_2 c-GMP and [α-^{32}P]ATP. The numbers against lines plotted in (*b*) are k_{obs}/min^{-1}.

concentration of CCK-8 was shown by Svoboda & Christophe (1979) to reflect a change in configuration (an 'opening') of the system, whereby the hormone reduces the activation energy of p[NH]ppG activation by 100 kJ/mol.

The value for $k_{+1} = k_{obs}$ was not greatly affected in pancreatic membranes pretreated at the low (0.5 μg/ml) cholera toxin concentration (figure 4), but the situation was different in membranes pretreated with cholera toxin at 30 μg/ml: k_{obs} then increased to 1.1 min^{-1} but the k_{-1} was no longer negligible (0.3 min^{-1}: table 3, middle column) and must be subtracted from k_{obs} to obtain $k_{+1} = 0.8\ min^{-1}$. When taking this into account, it then appears that pretreating the adenylate cyclase system at the high cholera toxin concentration increased k_{+1} from 0.2 to 0.8 min^{-1}, i.e. fourfold.

The evidence collected so far is summarized in figure 5. In figure 5*a* the degree of activation is expressed as a percentage of the maximal value. In figure 5*b* the absolute values for the rate constant k_{+1} are compared with those for k_2 and k_{-1}. Control data are in black, those obtained with CCK-8 at a saturating concentration are stippled, and those observed after pretreatment at a high (30 μg/ml) or a low (0.5 μg/ml) cholera toxin concentration are hatched. It is clear

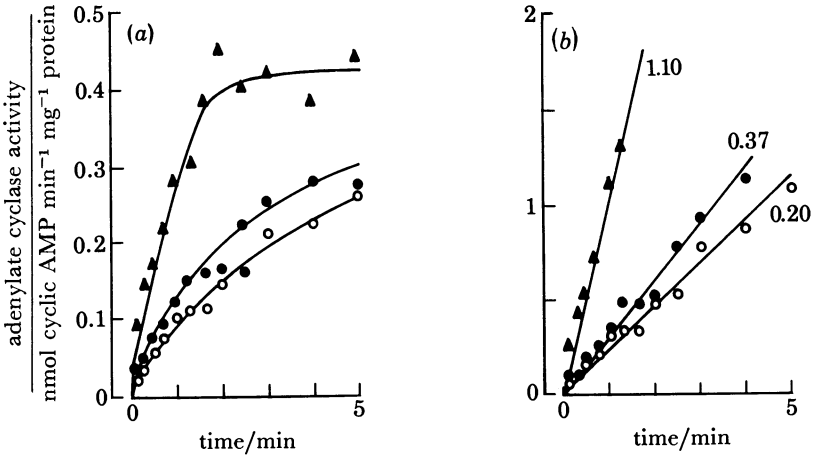

FIGURE 4. Effect of cholera toxin pretreatment on the p[NH]ppG activation rate constant, k_{obs}, of rat pancreatic adenylate cyclase. The activation constant was determined as described in figure 2; p[NH]ppG at 2.5 μM was used as activator and GDPβS at 0.3 mM was used to stop the activation process. The assay time of adenylate cyclase activity was 2 min. The experiments were conducted with control membranes (○) or membranes pretreated with cholera toxin at 0.5 μg/ml (●) or 30 μg/ml (▲). The numbers against lines plotted in (*b*) are k_{obs}/min^{-1}.

TABLE 2. EFFECT OF INCREASING CONCENTRATION OF CCK-8 ON THE FRACTION OF MAXIMAL ACTIVITY AT STEADY STATE AND ON THE EXPERIMENTAL AND CALCULATED RATE CONSTANT OF ACTIVATION OF PANCREATIC ADENYLATE CYCLASE

CCK-8 concentration	(*a*) 10 μM GTP-activated adenylate cyclase: activity	(*a*) 10 μM GTP-activated adenylate cyclase: fraction of maximal activity	k_{obs} of p[NH]ppG activation	(*b*) calculated k_{+1} of GTP activation
nM	pmol min^{-1} mg^{-1} protein		min^{-1}	min^{-1}
0	19	0.023	0.15	0.16
0.05	20	0.024	0.15	0.17
0.5	25	0.035	0.27	0.25
5	71	0.125	0.92	1.00
50	292	0.370	> 2.00	4.1
500	570	0.690	n.d.	15.6

(*a*) The activity of adenylate cyclase stimulated with 10 μM GTP (first column), is expressed in the second column as a fraction of maximal enzyme activity attained with 500 nM CCK-8 and 10 μM p[NH]ppG. This maximal activity was 840 pmol cyclic AMP formed per minute per milligram of protein.

(*b*) Derived from $[E_a]/[E_{tot}]$ (in the second column) and from the experimental value for k_{off} of 7 min^{-1}, which is independent of the presence of CCK-8:

$$k_{+1} = \frac{k_{off}[E_a]/[E_{tot}]}{1-[E_a]/[E_{tot}]}.$$

that the large degree of activation obtained with CCK-8 (stippled in figure 5*a*) can be explained largely by a 55-fold increase in k_{+1}, while the similar degree of activation obtained after pretreatment at the high cholera toxin concentration cannot be accounted for by that significant, yet too modest, four-fold increase in k_{+1}.

(*b*) *The rate constant of hydrolysis of GTP, k_2*

To obtain a more comprehensive view of the underlying mechanisms, it was therefore necessary to take into account k_2 and k_{-1}, the two last rate constants of the system, which together compose k_{off}.

Figure 6 illustrates how the effect of CCK-8 on the k_{off} of adenylate cyclase was tested in membranes preactivated with GTP and CCK-8. This determination required two series of three

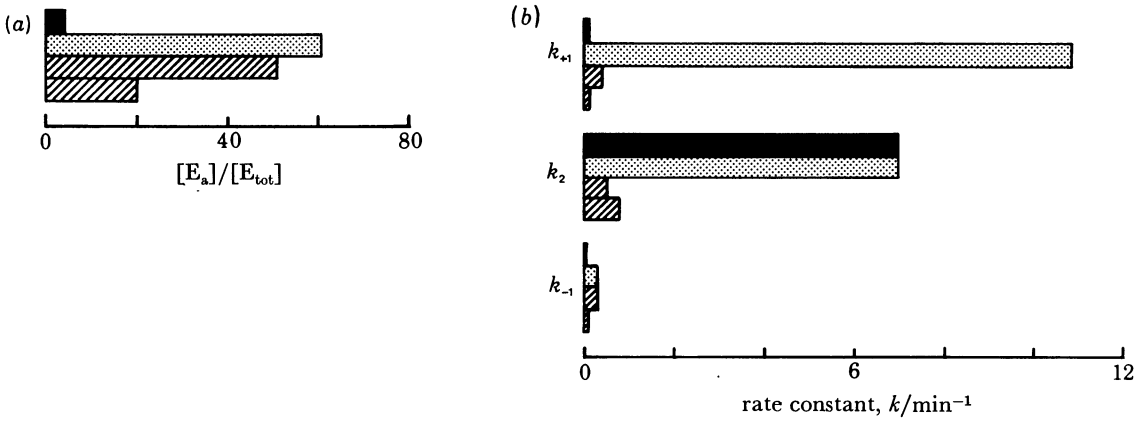

FIGURE 5. Average effects of CCK-8 and of a cholera toxin pretreatment on (*a*) the degree of GTP activation at steady state ($[E_a]/[E_{tot}]$) and (*b*) the three rate constants k_{+1}, k_2 and k_{-1}. For a definition of the parameters and a visual representation of the two-state model, see figures 1 and 8. Black bars, control; stippled bars, CCK-8; hatched bars, cholera toxin at 30 (upper) and 0.5 (lower) μg/ml.

TABLE 3. STIMULATORY EFFECT OF CCK-8 ON THE DEACTIVATION RATE CONSTANT, k_{off}, OF ACTIVATED ADENYLATE CYCLASE, PRETREATED OR NOT WITH CHOLERA TOXIN

(The values for k_{off} are expressed as min^{-1} (mean ± s.e., number of experiments in parentheses).)

	(*a*)		(*b*)		(*c*)	
activators...	0.25 μM GTP ($n = 4$) +50 nM CCK-8		0.25 μM p[NH]ppG ($n = 3$) +50 nM CCK-8		0.025 μM GTPγS ($n = 2$) +50 nM CCK-8	
inhibitor(s)...	GDPβS + Bt_2 c-GMP	GDPβS	GDPβS + Bt_2 c-GMP	GDPβS	GDPβS + Bt_2 c-GMP	GDPβS
control membranes	7 ± 1	7 ± 1	0.05	0.3 ± 0.1	0	0
0.5 μg/ml cholera toxin pretreated membranes	0.8 ± 0.2	1.1 ± 0.2	0.05	0.2	n.d.	n.d.
30 μg/ml cholera toxin pretreated membranes	0.5 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0	0.05

(*a*) With GTP, $k_{off} = k_2 + k_{-1}$ (see figure 1). (*b*), (*c*) With p[NH]ppG and GTPγS, $k_2 = 0$ and $k_{off} = k_{-1}$.

tubes. In tube I, in each series, membranes were preactivated with 0.25 μM GTP and 0.05 μM CCK-8. After 1.5 min the preactivation was stopped with either 0.9 mM GDPβS alone (figure 6*a*) or GDPβS and 2 mM Bt_2 c-GMP used in combination (figure 6*b*). [α-^{32}P]ATP was also added and the formation of cyclic [α-^{32}P]AMP was followed for 60 s. In tube II, the enzyme was not inhibited during the second step after the preactivation period. The value for k_{off} was derived graphically from the difference between curves II and I, according to Cassel *et al.*

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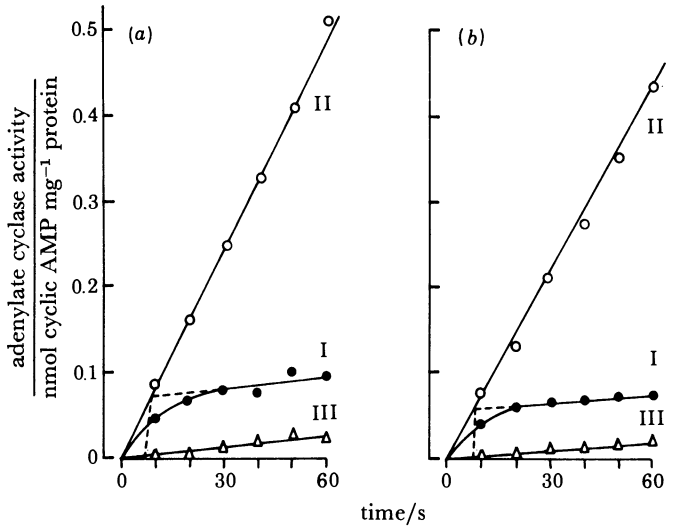

FIGURE 6. Comparison of the values of deactivation rate, k_{off}. observed in native rat pancreatic plasma membranes after addition, at final concentration, of 0.1 mM GDPβS (*a*) or 0.1 mM GDPβS and 0.67 mM Bt_2 c-GMP (*b*). Native membranes were preactivated for $1\frac{1}{2}$ min at 37 °C, in the presence of 0.05 μM CCK-8 and 0.25 μM GTP, in an adenylate cyclase assay medium lacking [α-^{32}P]ATP. [α-^{32}P]ATP with or without inhibitor(s) was then added. The control uninhibited activity was determined in the absence of GDPβS and Bt_2 c-GMP (curve II, ○). The decay of GTP+CCK-8 activation was measured when GDPβS without (*a*) or with (*b*) Bt_2 c-GMP was added together with [α-^{32}P]ATP (curve I, ●). The residual activity of the inhibited enzyme was tested with both inhibitors already present in the preincubation medium (curve III, △). Whenever used, GDPβS was present at a final concentration of 0.1 mM and Bt_2 c-GMP at 0.67 mM. At indicated times, aliquots were removed, added to the stopping solution, and cyclic [^{32}P]AMP formed was isolated and assayed. The value for k_{off} was determined by the graphical method of Cassel *et al.* (1977), and in both (*a*) and (*b*) is *ca.* 7 min^{-1}, as indicated by the broken lines.

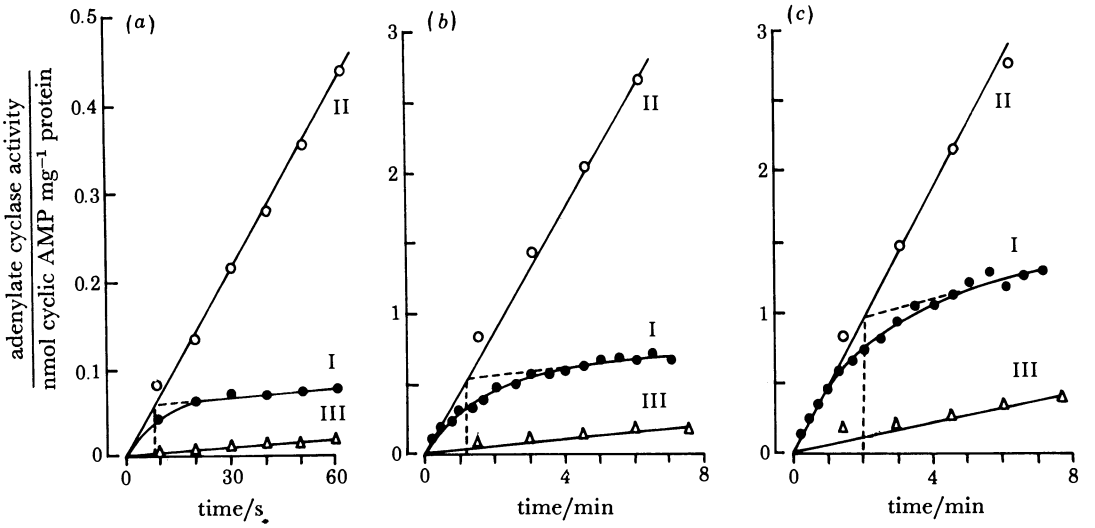

FIGURE 7. Effect of cholera toxin pretreatment (*a*, 0 μg/ml; *b*, 0.5 μg/ml; *c*, 30 μg/ml) on the deactivation rate constant k_{off} of (GTP+CCK-8)-activated rat pancreatic adenylate cyclase. The experiments consisted of two successive incubations. Adenylate cyclase was activated by preincubating membranes for $1\frac{1}{2}$ min at 37 °C in an adenylate cyclase assay medium containing, at final concentration, 0.25 μM GTP and 0.05 μM CCK-8 but lacking [α-^{32}P]ATP. The formation of cyclic [^{32}P]AMP was then allowed by adding [α-^{32}P]ATP (curve II). For the inhibited activities illustrated in curves I and III, the experimental conditions were those described in figure 6*b*. Values for k_{off} (broken lines) are: (*a*) 7 min^{-1}; (*b*) 0.8 min^{-1}; (*c*) 0.5 min^{-1}.

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(1977). The content of tube III served as a control, the enzyme being inhibited during the first step. Figure 6 shows clearly that the inhibition of CCK-8 action with Bt_2 c-GMP was unable to modify k_{off}, indicating that CCK-8 by itself was without significant effect on k_{off}.

The effects of cholera toxin on this parameter were quite different (figure 7). Here, deactivation was again followed for 60 s in untreated membranes (figure 7*a*) but for as much as 8 min in membranes pretreated with cholera toxin membranes (figure 7*b*, *c*), because the rate of deactivation was markedly reduced: the high k_{off} of 7 min^{-1} in native adenylate cyclase was reduced to 0.8 and 0.5 min^{-1} after pretreatment with 0.5 and 30 g/ml cholera toxin respectively. Table 3 and figure 5 also illustrate this major, 9–14-fold decrease in k_2.

TABLE 4. EFFECT OF CHOLERA TOXIN PRETREATMENT ON GTPASE ACTIVITIES AND THE CORRESPONDING ADENYLATE CYCLASE ACTIVITY

(GTPase activity was assayed as described in Lambert *et al.* (1979) on control membranes or cholera toxin (30 μg/ml) pretreated membranes. Non-specific residual GTPase activity was determined in the presence of 30 μM GTP; basal specific GTPase activity was that measured in the presence of 0.25 μM GTP minus non-specific residual GTPase activity; CCK-8-dependent GTPase activity was the increment of activity due to 0.1 μM CCK-8 added to the medium containing 0.25 μM GTP. Adenylate cyclase was assayed in the presence of 0.25 μM GTP and 0.1 μM CCK-8 (mean ± s.e., $n = 5$).)

	additions		
	GTP	CCK-8	
GTPase activity	μM	μM	percentage of control
non-specific	30	0	91 ± 4
basal specific	0.25	0	93 ± 9
CCK-8-dependent	0.25	100	56 ± 4
adenylate cyclase activity	0.25	100	171 ± 18

This effect of cholera toxin corresponded to a reduction in GTPase activity. Pancreatic plasma membranes have a hormone-dependent low K_m GTPase stimulated by CCK-8 and the two parent peptides caerulein and pentagastrin (Lambert *et al.* 1979). When GTPase activity was assayed with [γ-^{32}P]GTP, this CCK-8-dependent activity, tested at a low GTP concentration (0.25 μM), must be differentiated from (1) a non-specific residual NTPase activity that resisted the inhibitory effect of 0.5 mM ATPγS and can be tested at a high GTP concentration (30 μM), and (2) from a CCK-8-dependent (basal) low K_m GTPase activity, estimated by difference. Table 4 shows that a pretreatment with cholera toxin (at 30 μg/ml) reduced the CCK-dependent GTPase activity to 56% of control but had no effect on the two other GTPase activities.

(*c*) *The rate constant of dissociation of the intact nucleotide*, k_{-1}

Concerning the third rate constant, k_{-1}, our data strongly suggested its contribution to the operation of the model. This rate constant reflects the release of the intact nucleotide (figure 1) and its value corresponds to the experimental value of k_{off} when the k_2 component of k_{off} is zero, i.e. when the nucleotide cannot be hydrolysed. This was observed under two circumstances: (*a*) when GTP was applied to membranes pretreated with cholera toxin, and (*b*) when a stable nucleotide was applied to native membranes.

Under these conditions, CCK-8 was able to increase k_{off} moderately. This was shown in plasma membranes pretreated with cholera toxin and activated with GTP and CCK-8 (table 3, left columns), as well as in native membranes activated with p[NH]ppG and CCK-8 (table 3,

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middle columns). It can be inferred, by difference, that CCK-8 inhibition decreased the k_{off} value by 0.2–0.4 min^{-1}. Thus, CCK-8 increased k_{-1}. The absolute magnitude of this effect of CCK-8 was moderate. It is clear that this effect would be difficult to demonstrate when GTP instead of p[NH]ppG is used on native membranes, considering the high total k_{off} (7 min^{-1}) that is then recorded (figure 6). The relative extent of this effect was, nevertheless, rather large. Indeed, CCK-8 did not modify the degree of activation attained with p[NH]ppG at equilibrium and increased k_{+1} markedly, with the result that the hormone increased k_{-1} on average from 0.01 to 0.3 min^{-1}, i.e. 30-fold.

TABLE 5. COMPARISON OF THE VALUES OF GTP FRACTIONAL ACTIVATION MEASURED DIRECTLY AT EQUILIBRIUM OR DERIVED FROM KINETIC CONSTANTS

(Mean ± s.e., $n = 3$.)

membrane pretreatment	(*a*) measured GTP fractional activation	k_{+1} with p[NH]ppG (min^{-1})	k_{off} with GTP (min^{-1})	(*b*) calculated GTP fractional activation
control	0.03 ± 0.01	0.20 ± 0.03	7	0.03
0.5 μg/ml cholera toxin	0.25 ± 0.03	0.37 ± 0.11	0.8 ± 0.2	0.32
30 μg/ml cholera toxin	0.56 ± 0.04	0.80 ± 0.10	0.5 ± 0.1	0.62

(*a*) The observed GTP fractional activation was measured as the ratio of the activity in the presence of GTP alone to the activity in the presence of saturating concentrations of GTPγS and CCK-8.

(*b*) The calculation of GTP fractional activation was based on the formula

$$\frac{[E_a]}{[E_{tot}]} = \frac{k_{+1}}{k_{+1}+k_{off}}.$$

Apart from CCK-8, a cholera toxin pretreatment also increased the value of k_{-1} with p[NH]ppG but not with GTPγS. Table 3 shows that in control membranes, k_{off} ($= k_{-1}$) with p[NH]ppG (middle columns) and GTPγS (right columns) was so low that it could not be determined. This of course illustrates the persistence of p[NH]ppG and GTPγS activation in untreated membranes (Svoboda *et al.* 1978*a*). With p[NH]ppG, k_{off} remained minimal after a pretreatment with cholera toxin at 0.5 μg/ml but increased to 0.3 min^{-1} after a pretreatment with cholera toxin at 30 μg/ml. In contrast to p[NH]ppG activation, that produced by GTPγS persisted after pretreatment at the high concentration of cholera toxin which reflected a higher affinity of GTPγS than p[NH]ppG for the regulatory site, even when the latter was ADP-ribosylated by cholera toxin.

CONCLUSIONS

From the present data as summarized in figure 5, four conclusions can be drawn. The first three conclusions are formulated in the abstract. Figure 8 is a schematic representation of the distinct modes of action of CCK-8 and of a cholera toxin pretreatment. The last conclusion is that the theoretical requirements of this model were supported experimentally. For instance, the measured values of $[E_a]/[E_{tot}]$, defining GTP efficacy at equilibrium (table 5, column *a*) compared reasonably well with the calculated values of the same parameter (in column *b*) that were estimated from the experimental values for k_{+1} (with p[NH]ppG) and k_{off} (with GTP) that included k_2 and k_1 (table 5, middle columns).

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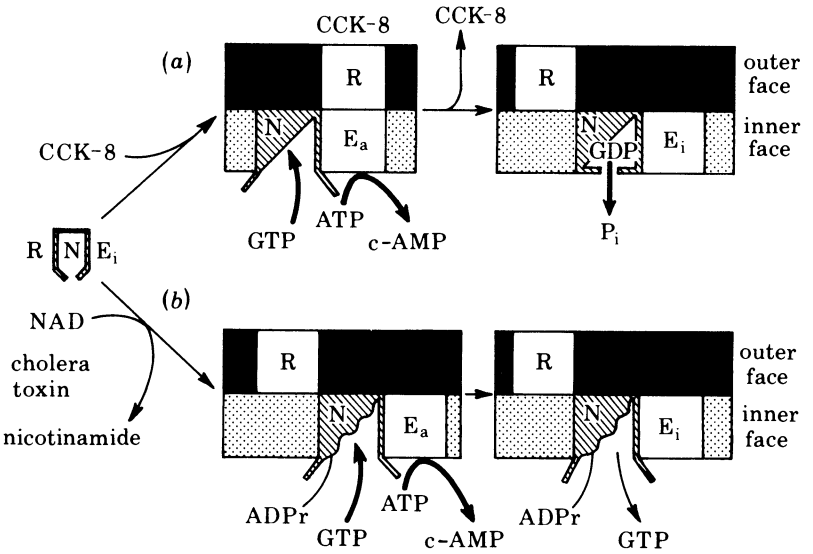

FIGURE 8. A diagrammatic representation of the postulated action of CCK-8 (*a*) and of a cholera toxin pretreatment (*b*) on the activation–deactivation cycle of pancreatic adenylate cyclase. R, CCK-8 receptor; E, catalytic subunit in the active (a) or inactive (i) state; N, guanine nucleotide regulatory site(s) in the 'open' or 'closed' configuration; c-AMP, cyclic AMP; ADPr, ADP–ribose.

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