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

BY J. CHRISTOPHE, M. SVOBODA AND M. LAMBERT

Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, Boulevard de Waterloo 115, B-1000 Brussels, Belgium

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}] = \frac{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. 1978a, 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. 1978a; Schlegel et al. 1979; Iyengar & Birnbaumer 1979; Iyengar et al. 1979; Martin et al. 1979) and

![Figure 1](http://rstb.royalsocietypublishing.org/)
activate(s) the catalytic subunit when occupied with GTP, p[NH]ppG or GTPγS (Svoboda et al. 1978a; 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 1a), 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 $k_3$). 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. 1978b) because $k_{+1}$ is small in the absence of hormone compared with $k_{off}$.

When a stable nucleotide is used (figure 1b), 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. 1980a, 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. 1978b; 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>
<thead>
<tr>
<th>activators added</th>
<th>cholera toxin concentration during membrane pretreatment/(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>1 µM GTPyS + 0.3 µM CCK-8</td>
<td>100</td>
</tr>
<tr>
<td>1 µM GDPβS (basal)</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>10 µM GTP</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>10 µM GTP + 0.3 µM CCK-8</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>10 µM p[NH]ppG + 0.3 µM CCK-8</td>
<td>98 ± 3</td>
</tr>
</tbody>
</table>

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 GTPyS 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-32P]ATP and the p[NH]ppG inhibitor GDP. The reaction in each aliquot was stopped after 2 min and the cyclic [32P]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 2a), can be converted into a semilogarithmic plot of the time variation of activity (figure 2b) whose slope gives the value for $k_{+1} = k_{\text{obs}}$. Three

![Figure 2](http://rstb.royalsocietypublishing.org/)

**Figure 2.** The p[NH]ppG activation rate constant, $k_{\text{obs}}$, 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-32P]ATP. The time course of activation was followed by transferring aliquots to tubes containing GTP and [α-32P]ATP. The reaction in each aliquot was stopped after 3 min and the cyclic [32P]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 $A_{\text{eq}}/(A_{\text{eq}} - A_t)$, where $A_{\text{eq}}$ 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 14, 51, 113 and 155 min of activating incubation, aliquots were transferred and incubated with 0.3 μM CCK-8, 0.3 μM secretin and [α-32P]ATP. There was no decay of maximal adenylate cyclase activation (-----) during the 155 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 2b) 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 2a) obviously reflected a higher proportion of $E_{\text{tot}}$ converted to $E_a$. 

on January 5, 2018
The value for $k_{+1} = k_{\text{obs}}$ 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 $k_{\text{off}}$ (table 2 documents the calculated $k_{+1}$ obtained with one plasma membrane preparation). This large effect of a saturating 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 $k_{+1} = k_{\text{obs}}$ 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: $k_{\text{obs}}$ 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 $k_{\text{obs}}$ 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 5a the degree of activation is expressed as a percentage of the maximal value. In figure 5b 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 3. Effect of CCK-8 on the p[NH]ppG activation rate constant, $k_{\text{obs}}$, 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 $[\alpha-32P]ATP$ 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 Bt2 c-GMP and $[\alpha-32P]ATP$. The numbers against lines plotted in (b) are $k_{\text{obs}}$/min$^{-1}$.](http://rstb.royalsocietypublishing.org/)
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 GDP[S] 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

<table>
<thead>
<tr>
<th>CCK-8 concentration (nM)</th>
<th>10 μM GTP-activated adenylate cyclase</th>
<th>$k_{obs}$ of p[NH]ppG activation</th>
<th>calculated $k_{+1}$ of GTP activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>activity (pmol min$^{-1}$ mg$^{-1}$)</td>
<td>fraction of maximal activity</td>
<td>min$^{-1}$</td>
</tr>
<tr>
<td>0</td>
<td>19</td>
<td>0.025</td>
<td>0.15</td>
</tr>
<tr>
<td>0.05</td>
<td>20</td>
<td>0.024</td>
<td>0.15</td>
</tr>
<tr>
<td>0.5</td>
<td>25</td>
<td>0.035</td>
<td>0.27</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>0.125</td>
<td>0.92</td>
</tr>
<tr>
<td>50</td>
<td>292</td>
<td>0.370</td>
<td>&gt; 2.00</td>
</tr>
<tr>
<td>500</td>
<td>570</td>
<td>0.690</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

(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 5a) 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 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 GDP or GDPyS and 2 mM Bt2 c-GMP used in combination (figure 6b). [α-32P]ATP was also added and the formation of cyclic [α-32P]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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**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).)

<table>
<thead>
<tr>
<th>activators...</th>
<th>$0.25 \mu M$ GTP ($n = 4$)</th>
<th>$0.25 \mu M$ p[NH]ppG ($n = 3$)</th>
<th>$0.025 \mu M$ GTPyS ($n = 2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>inhibitor(s)...</td>
<td>GDPβS + GDPβS</td>
<td>GDPβS + GDPβS</td>
<td>GDPβS + GDPβS</td>
</tr>
<tr>
<td>control membranes</td>
<td>7 ± 1</td>
<td>7 ± 1</td>
<td>0.05</td>
</tr>
<tr>
<td>0.5 μg/ml cholera toxin</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>pretreated membranes</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>30 μg/ml cholera toxin</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>pretreated membranes</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
</table>

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

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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 GDPPS (a) or 0.1 mM GDPPS and 0.67 mM Bt$_2$c-GMP (b). Native membranes were preactivated for 15 min at 37 °C, in the presence of 0.05 pM CCK-8 and 0.25 pM GTP, in an adenylate cyclase assay medium lacking [$\alpha$-32P]ATP. [$\alpha$-32P]ATP with or without inhibitor(s) was then added. The control uninhibited activity was determined in the absence of GDPPS and Bt$_2$c-GMP (curve II, o). The decay of GTP + CCK-8 activation was measured when GDPPS without (a) or with (b) Bt$_2$c-GMP was added together with [$\alpha$-32P]ATP (curve I, •). The residual activity of the inhibited enzyme was tested with both inhibitors already present in the preincubation medium (curve III, a). Whenever used, GDPPS 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 [32P]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 15 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 [$\alpha$-32P]ATP. The formation of cyclic [32P]AMP was then allowed by adding [$\alpha$-32P]ATP (curve II). For the inhibited activities illustrated in curves I and III, the experimental conditions were those described in figure 6b. 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 Bt2 c-GMP was unable to modify \( k_{\text{off}} \), indicating that CCK-8 by itself was without significant effect on \( k_{\text{off}} \).

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 7a) but for as much as 8 min in membranes pretreated with cholera toxin membranes (figure 7b, c), because the rate of deactivation was markedly reduced: the high \( k_{\text{off}} \) 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 \( \mu \)g/ml) pretreated membranes. Non-specific residual GTPase activity was determined in the presence of 30 \( \mu \)M GTP; basal specific GTPase activity was that measured in the presence of 0.25 \( \mu \)M GTP minus non-specific residual GTPase activity; CCK-8-dependent GTPase activity was the increment of activity due to 0.1 \( \mu \)M CCK-8 added to the medium containing 0.25 \( \mu \)M GTP. Adenylate cyclase was assayed in the presence of 0.25 \( \mu \)M GTP and 0.1 \( \mu \)M CCK-8 (mean ± s.e., \( n = 5 \)).)

<table>
<thead>
<tr>
<th>additions</th>
<th>GTP</th>
<th>CCK-8</th>
<th>percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTPase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-specific</td>
<td>30</td>
<td>0</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>basal specific</td>
<td>0.25</td>
<td>0</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>CCK-8-dependent</td>
<td>0.25</td>
<td>100</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>adenylate cyclase activity</td>
<td>0.25</td>
<td>100</td>
<td>171 ± 18</td>
</tr>
</tbody>
</table>

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 \([\gamma-^{32P}]\)GTP, this CCK-8-dependent activity, tested at a low GTP concentration (0.25 \( \mu \)M), must be differentiated from (1) a non-specific residual NTPase activity that resisted the inhibitory effect of 0.5 mM ATP\( \gamma \)S and can be tested at a high GTP concentration (30 \( \mu \)M), 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 \( \mu \)g/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 \( k_{\text{off}} \) when the \( k_2 \) component of \( k_{\text{off}} \) 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 \( k_{\text{off}} \) 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,
middle columns). It can be inferred, by difference, that CCK-8 inhibition decreased the $k_{\text{off}}$ value by 0.2–0.4 min$^{-1}$. Thus, CCK-8 increased $k_+$. 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 $k_{\text{off}}$ (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 $k_+$ markedly, with the result that the hormone increased 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**

<table>
<thead>
<tr>
<th>membrane pretreatment</th>
<th>measured GTP fractional activation</th>
<th>$k_+$ with p[NH]ppG min$^{-1}$</th>
<th>$k_{\text{off}}$ with GTP min$^{-1}$</th>
<th>calculated GTP fractional activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.03 ±0.01</td>
<td>0.20 ±0.03</td>
<td>7</td>
<td>0.03</td>
</tr>
<tr>
<td>0.5 µg/ml cholera toxin</td>
<td>0.25 ±0.04</td>
<td>0.37 ±0.11</td>
<td>0.8 ±0.2</td>
<td>0.32</td>
</tr>
<tr>
<td>30 µg/ml cholera toxin</td>
<td>0.56 ±0.04</td>
<td>0.80 ±0.10</td>
<td>0.5 ±0.1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

(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

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

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, $k_{\text{off}}$ (= $k_{-1}$) 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. 1978a). With p[NH]ppG, $k_{\text{off}}$ 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 $[E_a]/[E_{\text{tot}}]$, 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 $k_+$ (with p[NH]ppG) and $k_{\text{off}}$ (with GTP) that included $k_2$ and $k_1$ (table 5, middle columns).
PANCREATIC ADENYLATE CYCLASE ACTIVITY

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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References (Christophe et al.)


