Mechanism of glucagon activation of adenylate cyclase in the presence of Mn^{2+}

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For a variety of ligand states, adenylate cyclase activity in the presence of Mn^{2+} was greater than with Mg^{2+}. Trypsin treatment of intact hepatocytes, under conditions which destroy cell surface glucagon receptors, led to a first order loss of glucagon-stimulated adenylate cyclase activity in isolated membranes assayed in the presence of Mn^{2+} whether or not GTP (100 μM) was present in the assays. Arrhenius plots of basal activity exhibited a break at around 22°C, those with NaF were linear and those with glucagon ± GTP (100 μM) were biphasic with a break at around 28°C. It is suggested that Mn^{2+} perturbs the coupling interaction between the glucagon receptor and catalytic unit of adenylate cyclase at the level of the guanine nucleotide regulatory protein. This appears to take the form of Mn^{2+} preventing GTP from initiating glucagon's activation of adenylate cyclase through a collision coupling mechanism.

Adenylate cyclase *Glucagon* *Mn^{2+}* *Guanine nucleotide* *Receptor coupling*

1. INTRODUCTION

Adenylate cyclase catalyses the conversion of ATP to cyclic AMP. This reaction is usually taken to use Mg^{2+}, both as a co-substrate with ATP and also as a regulator, binding to a distinct divalent cation site [1–8]. Recently, there has been considerable interest expressed in the very different effects exerted when Mn^{2+} replaces Mg^{2+} in the assay for adenylate cyclase.

Hormone-stimulated adenylate cyclases consist of at least 3 distinct components: a catalytic unit responsible for cyclic AMP production; a receptor specific for a particular hormone; and one or more guanine nucleotide regulatory components. The guanine nucleotide regulatory proteins appear to regulate the coupling interaction between the receptor and catalytic unit [9–12]. There is now considerable evidence consistent with the hypothesis that hormonal activation of adenylate cyclase by glucagon [11,13,14], β-agonists [12,15,16] and neurohypophyseal hormone [17] is achieved via productive collisions, within the plane of the membrane, between these various components. In liver, 3 independent methods (receptor depletion [14]; Arrhenius plot analysis [14]; irradiation inactivation [13]), have been used to support this contention.

Here, we examine the effect of replacing Mg^{2+} with Mn^{2+}, in the adenylate cyclase assay, on the activity of the enzyme in liver plasma membranes and its mode of coupling to the glucagon receptor.

2. MATERIALS AND METHODS

Glucagon was a kind gift from Dr W.W. Bromer of Eli Lilly and Co (Indianapolis IN). Phosphocreatine, creatine phosphokinase, trypsin (type II from bovine pancreas) and trypsin inhibitor (type 1-S from soya bean) were from Sigma (St Louis MO). Cyclic AMP, ATP, GTP (Na salt),

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p[NH]ppG (guanosine 5'-[$\beta\gamma$-imido]triphosphate), triethanolamine–HCl and collagenase were from Boehringer (Mannheim). All other chemicals were of AR quality from BDH Chemicals (London).

Adenylate cyclase was assayed in a cocktail at pH 7.4 containing final concentrations of 25 mM triethanolamine–HCl, 1 mM EDTA, 1 mM theophylline, 1.5 mM ATP and an ATP-regenerating system of 7.4 mg creatine phosphate/ml and 1 mg creatine kinase/ml. These contained either 5 mM $MgSO_4$ or the stated concentration of $MnSO_4$. When $MnSO_4$ was used it was added to the reaction mixture immediately prior to initiating the reaction with membranes. Cyclic AMP produced was determined as in [18]. In all cases, initial rates were taken from linear timecourses of cyclic AMP production at 30°C. Arrhenius plots were constructed and data analysed by a fitting procedure detailed in [19]. Linearity of cyclic AMP production was ensured over the entire temperature range examined.

Plasma membranes were purified from male Sprague–Dawley rats as in [18,20]. Hepatocytes were also prepared from such animals and shown to be intact and viable [14]. As described in [14], cells were pre-incubated before treatment with trypsin (0.2 mg/ml) and incubated at 37°C. At appropriate time intervals samples were taken and quenched by immediate mixing with ice-cold stopping buffer containing excess trypsin inhibitor (details in [14]). The cells were then broken and a membrane fraction prepared for assay [14,21].

Protein was determined by a modified microbiuret method [19].

3. RESULTS

In the absence of either Mn^{2+} or Mg^{2+} there was no observable adenylate cyclase activity, irrespective of the stimulating ligand employed. However, low [Mn^{2+}] led to the expression of ligand-stimulated adenylate cyclase activity with half-maximal activations occurring at around 1–2 mM Mn^{2+} under the assay conditions employed. [Mn^{2+}] at >10 mM led to a decrease in the adenylate cyclase activity. This is similar to that reported in [6,7]. For the rest of this study we employed 5 mM Mn^{2+} in all assays where Mn^{2+} was to be added. Table 1 compares the specific activities and degree of activation elicited by various ligands when either Mg^{2+} or Mn^{2+} was added to assays.

Intact hepatocytes were incubated with trypsin (see [14]) for various time intervals at 37°C before quenching the reaction with ice-cold trypsin inhibitor and assay of adenylate cyclase in a broken membrane fraction prepared from these cells. In [14], we demonstrated that under these conditions trypsin does not cause cell breakage and merely acts on the external surface of the cell plasma membrane. There was no loss of basal, fluoride- or guanine-nucleotide-stimulated activity but there was a first-order loss of glucagon receptors [14]. In agreement with our studies using Mg^{2+} (5 mM) in adenylate cyclase assays, we observed a first-order loss of glucagon-stimulated adenylate cyclase activity. The loss of glucagon + GTP (100 μM)-stimulated activity was markedly non-linear (fig.1). However, if Mn^{2+} (5 mM) replaced Mg^{2+} in these assays then there was apparently a simple

Table 1

Adenylate cyclase activity in hepatocyte membranes

Divalent cation / Ligand	Mg^{2+} pmol.min^{-1}.mg protein^{-1} (-fold stimulation over basal)	Mn^{2+} pmol.min^{-1}.mg protein^{-1} (-fold stimulation over basal)	Ratio of activities: Mn^{2+}/Mg^{2+}
Basal	0.74 ± 0.25 (× 1)	3.95 ± 0.43 (×1)	5.3
GTP (100 μM)	1.48 ± 0.21 (× 2)	3.29 ± 0.38 (×0.8)	2.2
NaF (15 mM)	7.70 ± 0.31 (×10.4)	21.6 ± 3.3 (×5.5)	2.8
Glucagon (1 μM)	11.1 ± 1.1 (×15)	14.9 ± 1.5 (×3.8)	1.3
Glucagon + GTP	15.7 ± 0.5 (×21)	30.0 ± 0.6 (×7.6)	1.9

Assays were all carried out at 30°C for 10 min (see [18]); errors are SD; $n = 3$

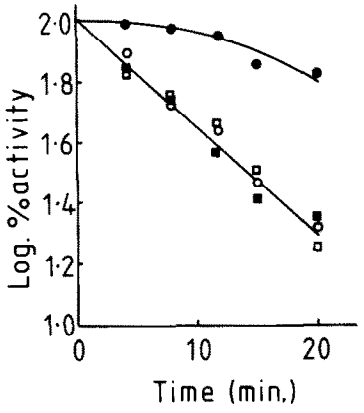

Fig.1. Glucagon-stimulated adenylate cyclase activity in trypsin-treated hepatocytes. Assays were carried out on glucagon (10^{-6} M)-stimulated (○,□) and glucagon + GTP (100 μM)-stimulated (●,■) adenylate cyclase activity in either the presence of 5 mM Mg^{2+} (●,○) or 5 mM Mn^{2+} (■,□). As detailed in [14], intact hepatocytes were treated for various time intervals with trypsin at 37°C. The reaction was terminated by adding excess trypsin inhibitor and cooling on ice. A membrane fraction was then assayed. As found in [14] there was no loss in basal, NaF or p[NH]ppG-stimulated adenylate cyclase activities over this entire period. Furthermore, receptor loss as followed by specific ^{125}I-glucagon binding occurred exponentially with a half-life that corresponded to that observed for the glucagon-stimulated adenylate cyclase activity, in the absence of added GTP and with Mg^{2+} (see [14]). This experiment shows data from a typical experiment repeated with 3 different cell preparations. In each case assays were performed with Mg^{2+} as well as with Mn^{2+} so as to act as an internal control [14].

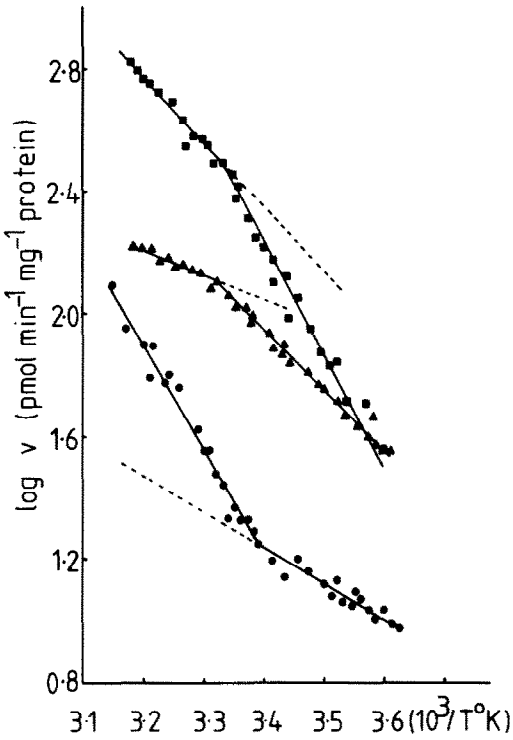

Fig.2. Arrhenius plots of adenylate cyclase activity in rat liver plasma membranes. Adenylate cyclase assays were carried out in the presence of 10^{-6} M glucagon (▲), glucagon + 100 μM GTP (■) and under basal conditions (●) in the presence of 5 mM Mn^{2+}. Purified rat liver plasma membranes were used [18] and linearity of cyclic AMP production was observed at all temperatures. Data were treated as in [19]. This shows a typical result.

first-order loss of glucagon-stimulated activity whether or not GTP (100 μM) was present (fig.1).

Table 2 summarizes the forms of the Arrhenius plots for the various ligand-stimulated states of adenylate cyclase, when Mn^{2+} (5 mM) was present in the assays. These bear comparison with those we obtained using Mg^{2+} (5 mM) in the assays with two exceptions: the basal and glucagon + GTP (100 μM)-stimulated states (fig.2). Arrhenius plots of basal adenylate cyclase activity were biphasic with a break occurring at around 22°C (fig.2) when Mn^{2+} was used. A break at this temperature occurs with Mg^{2+} too, although in that case activation energies were lower above the break than below [22]. The converse appears to be true for Mn^{2+} (fig.2). In the case of the glucagon + GTP (100 μM)-stimulated state, Arrhenius plots of this activity are clearly linear when Mg^{2+} was in the assay [14]. However, if Mn^{2+} replaced Mg^{2+}, then a biphasic plot resulted with a well-defined break at around 28°C (fig.3). In the absence of added GTP (100 μM), Arrhenius plots of glucagon-stimulated activity were biphasic, exhibiting a break at around 28°C with either Mn^{2+} (fig.2) or Mg^{2+} [14,18,22] in the assays.

4. DISCUSSION

As has been shown for a variety of adenylate cyclase systems [6,7,10], Mn^{2+} yields a basal adenylate cyclase activity that is profoundly greater than that observed with Mg^{2+} (table 1). We observed that in the presence of Mn^{2+} (5 mM) a variety of ligands, including glucagon are still able

to elicit activation of the enzyme. However, the degree of stimulation of activity, over that of basal, was severely reduced. In each case the specific activity expressed in the presence of Mn^{2+} was around 2-fold higher than when Mg^{2+} was used (table 1). This effect was magnified, however, in the basal condition which was some 5-fold higher with Mn^{2+} (table 1).

We [14], and others [13], have suggested that glucagon, in the presence of exogenous GTP (100 μM) can activate adenylate cyclase through a collision coupling mechanism as proposed in [15] for the β-agonist activation of turkey erythrocyte adenylate cyclase. This mechanism bears analogy with those proposed by others for β-agonist [12] and neurohypophyseal hormone [17] activation of adenylate cyclase. Such a mechanism allows for multiple catalytic units to be activated by a single occupied receptor. Hence, under these conditions, hormone-stimulated activity is relatively insensitive to receptor loss [14]. In contrast, the addition of glucagon alone appears to promote a mobile receptor mechanism of coupling [13,14,23]. Here, the receptor is presumed to form a transmembrane complex with an activated catalytic unit and regulatory protein (see [14]). In the absence of stimulating hormone, both of these mechanisms assume that the receptor, guanine nucleotide coupling protein and catalytic unit of adenylate cyclase migrate as independent entities in the plane of the bilayer. Only in the presence of hormone do they interact physically to elicit activation of adenylate cyclase. The basic difference in these mechanisms is that under conditions where activation via a mobile receptor model occurs, then these 3 interacting entities form a stable, activated, transmembrane complex. However, when activation occurs via a collision coupling mechanism, this complex breaks down to release a transiently activated catalytic unit [13–15,23].

Under conditions where a mobile receptor mechanism is obeyed, the selective depletion of glucagon receptors, achieved by trypsin treatment of intact hepatocytes, causes a proportional loss in the glucagon-stimulated adenylate cyclase activity of membranes derived from these cells [14]. Using such a procedure we observed identical first-order kinetics for loss of both glucagon binding and glucagon-stimulated adenylate cyclase activity [14]. However, by merely raising the GTP concentrations in such assays a remarkably different result was apparent. In this instance the glucagon (+ GTP)-stimulated activity was markedly insensitive to receptor loss [14] and the decay kinetics were distinctly non-linear ([14], fig.1). This is precisely what is expected for a collision coupling mechanism [15] where one occupied receptor leads to the production of a number of activated catalytic units.

In marked contrast to these observations, carrying out identical experiments, but just replacing Mg^{2+} in the assays with Mn^{2+}, we observed no effect of GTP on the sensitivity of glucagon-stimulated adenylate cyclase to receptor loss (fig.1). In both instances decay curves were clearly first-order and paralleled the loss in activity observed for glucagon-stimulated adenylate cyclase activity in the presence of Mg^{2+} (fig.1) implying that a mobile receptor mechanism was being obeyed. GTP, however, still exerts an activating effect on the glucagon-stimulated activity in the presence of Mn^{2+} (table 1). We suggest then, that Mn^{2+} perturbs the coupling between the receptor and catalytic unit, such as to prevent glucagon-activating adenylate cyclase through a collision coupling mechanism at elevated GTP concentrations.

To gain support for this contention, we analysed Arrhenius plots of various states of the enzyme with Mn^{2+} present in the assays. We have presented considerable evidence, based upon a variety of techniques (detailed in [14,22,24,25]), which is consistent with the notion that the liver plasma membrane exhibits a lipid phase separation occurring at around 28°C in the external half of the bilayer, whereas no such lipid phase separation occurs in the inner half of the bilayer. Both the guanine nucleotide regulatory protein (N_s) and catalytic unit of adenylate cyclase have limited hydrophobic surfaces [10,26] and are considered to have their functional globular regions restricted to the inner half of the bilayer [22,24,25]. Thus Arrhenius plots of NaF- or guanine nucleotide-stimulated activity are linear as they do not sense the lipid phase separation occurring in the external half of the bilayer. In contrast, under conditions where a mobile receptor mechanism is obeyed then we would expect the transmembrane complex formed with the glucagon receptor to experience the lipid environment of the external half of the

Table 2

Arrhenius plots of adenylate cyclase activity

Ligand	Form of plot	Break point (T_t °C)	Activation energy (kJ.mol^{-1})	
			$T>T_t$	$T<T_t$
Basal	Biphasic	21.8 ± 0.4	62.6 ± 1.4	22.3 ± 4.2
GTP	Linear	–	51.9 ± 3.4	
Fluoride	Linear	–	45.2 ± 12.3	
Glucagon	Biphasic	27.7 ± 2.6	20.4 ± 6.3	38.7 ± 2.9
Glucagon + GTP	Biphasic	28.8 ± 1.5	46.0 ± 3.9	75.1 ± 16.1

In all instances assays contained 5 mM Mn^{2+} rather than Mg^{2+}. Data were obtained over 2–42°C with determinations made at between 30–35 different temperatures. Data were analysed and break points determined by a least squares minimalisation procedure from [19]. All plots were carried out with 3 preparations and the figures shown are ± SD

bilayer. Under these conditions it does and Arrhenius plots exhibit a well-defined break at around 28°C when glucagon is added without high exogenous [GTP] [14,18]. However, under collision coupling conditions the steady state activity of adenylate cyclase is expressed by activated catalytic units which are not associated with the receptor. Thus Arrhenius plots of glucagon-stimulated adenylate cyclase in the presence of high GTP concentrations are linear [14]. However, if Mn^{2+} replaced Mg^{2+} in these assays, then Arrhenius plots of the glucagon-stimulated activity were biphasic, exhibiting a well-defined break at around 28°C, irrespective of whether or not GTP was added to the assays (fig.2; table 2). These data suggest that, under both conditions, a transmembrane complex is formed with the receptor, and regulatory protein, conveying sensitivity to the lipid phase separation, in the external half of the bilayer, upon the catalytic unit. This supports our contention that, unlike with Mg^{2+}, Mn^{2+} prevents glucagon activating the enzyme via a collision coupling mechanism at elevated [GTP].

In a number of systems Mn^{2+} has been shown to exert potent effects on adenylate cyclase at the level of guanine nucleotide regulation [1,3,5–7]. It has even been suggested that the guanine nucleotide regulatory protein exhibits a distinct, divalent cation, regulatory site to which free Mn^{2+}/Mg^{2+} can bind [6]. Mn^{2+} appears to be able to uncouple ACTH- [3] and β-agonist [6,27] stimulated adenylate cyclase. Indeed, at low concentrations it has been shown to selectively uncouple regulation of adenylate cyclase by inhibitory hormones [5]. These studies appear to support our observations of a perturbation at the level of coupling mediated by guanine nucleotides.

Mn^{2+}, however, also altered the form of the basal Arrhenius plot (fig.3) from that observed using Mg^{2+} [22]. Arrhenius plots of basal adenylate cyclase activity exhibit a break at around 20–22°C in liver and other systems (see [22,28]). This is apparently a protein-mediated event rather than being due to the lipid environment of the enzyme [22,28]. Here we show that for basal activity Mn^{2+} yielded a plot with activation energies which were greater above the break than below (fig.3) whereas the converse was due when Mg^{2+} [22] replaced Mn^{2+} in the assay. The ground state enzyme may exist in two states [29]. Presumably, the biphasic nature of the Arrhenius plot reflects the equilibrium between these two states and the rather different activation energies expressed in each case. Mn^{2+}, acting either on the guanine-nucleotide regulatory protein or directly on the catalytic unit, might then alter the conformation of the enzyme such that at $\geq$20°C an activated enzyme exhibiting a high activation energy predominates. Indeed, observations that the solubilized enzyme [2] and the enzyme from *cyc*$^-$ cells, which lack a stimulatory guanine-nucleotide regulatory protein [10], exhibit activity with Mn^{2+} but very little with Mg^{2+}, might suggest that Mn^{2+} can alter the conformation and hence activity state of the enzyme.

Clearly Mn^{2+} exerts potent effects on adenylate cyclase and is a useful tool for probing the regulation of this enzyme by guanine-nucleotide regulatory protein.

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