Kinetic studies on insulin inhibition of fat cell adenylyl cyclase

Estudios cinéticos de la inhibición por insulina de la adenilil ciclasa de células adiposas

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Evidence obtained in these laboratories indicates that insulin is able to decrease adenylyl cyclase activity in membrane preparations from fat cells, liver, Neurospora, and fibroblasts (1-4). The hormonal effect only requires the presence of insulin, membranes and other components necessary for the assay of such enzyme activity.

Figure 1 shows the kinetics of the insulin effect on the cyclase activity associated to a fat cell membrane fraction purified by centrifugation on a sucrose gradient. In the absence of hormone, the activity tends to decline with increasing incubation time. In the presence of two different concentrations of insulin it is evident that after some period the hormone increases the rate of enzyme inactivation. Such period tends to decrease when the hormone concentration is increased. In addition, after the onset of the insulin effect, the extent of the inhibition is relatively independent of the insulin concentration in a range between 100 and 1000

μUnits per ml. This is shown in Figure 2, which compares the transient rates and the corresponding values for the inhibitions obtained at these two concentrations.

It is evident that kinetic analysis of adenyl cyclase associated to fat cell membranes is complicated by the fact that under most of the conditions explored, time courses are not linear. In order to simplify such analysis, two kinetic components have been defined. The first one, termed "initial" might indicates the influence of factors in rapid equilibrium with the enzyme system. The second one, termed "final", would reflect effects of factors affecting the relatively slow interconversions of the cyclase. In the following discussion "initial" and "final" rates will correspond to transient rates measured between the beginning and 1.5 minutes, and between 3 and 5 minutes of assay respectively.

The absolute magnitude of the insulin effect will be expressed as the difference between
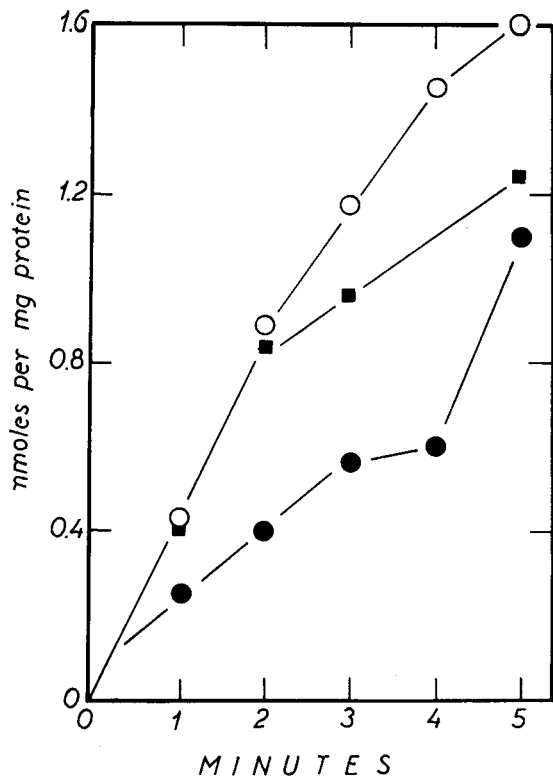
Fig. 1. Effect of insulin on adenylyl cyclase from a fat cell "pure membrane preparation". A fat cell suspension

(12), in Krebs-Ringer bicarbonate buffer containing 1% albumin, from 50 Sprague-Dawley rats (about 125 g body weight) was homogenized with two volumes of ice-cold 1 mM sodium bicarbonate solution using and all-glass 50 ml Dounce-type homogenizer (loose fitted pestle; five strokes). The homogenate was centrifuged for 5 minutes at 46,000 × *g*. The membrane pellet was resuspended in 2 ml of ice-cold 1 mM sodium bicarbonate solution. Aliquots of this suspension (about 0.6 ml) were loaded on the top of discontinuous sucrose gradients made as follows: plastic tubes (7/16 × 2 3/8") were succesively filled with 0.8 ml aliquots of the following sucrose solutions: 1.2 M, 0.86 M, 0.52 M, and 0.26 M. The tubes were centrifuged for 15 minutes at 58,000 r.p.m. using a SW60 Spinco rotor. The interphases between 1.2 M and 0.86 M sucrose were collected and further diluted with 1 mM sodium bicarbonate solution. This "pure membrane preparation" was used as enzyme source. The standard incubation mixture for adenylyl cyclase assay contained 50 mM Tris-HCl buffer, pH 7.5, 0.2 mM 3-isobutyl-l-methyl xanthine, 1 mM cyclic AMP, 2.5 mM $MnCl_2$, 0.5 mM $[\alpha^{-32}P]ATP$ (specific activity, 100 to 400 cpm per pmole), 2 mM phosphocreatine, 0.2 mg per ml creatine kinase, 0.1 mg per ml albumin and membranes (10 to 20 µg of protein). The total volume was 0.1 ml. Incubations were performed at 37°. Reactions were stopped as indicated by Rodbell (12). Cyclic AMP in the samples was purified according to the sequential column procedure (Dowex 50 and alumina) described by Solomon *et al.* (13).

Assays were done in the absence of insulin ((○) or in the presence of 125 µUnits/ml (■) or 1,250 µUnits /ml (●) insulin respectively.
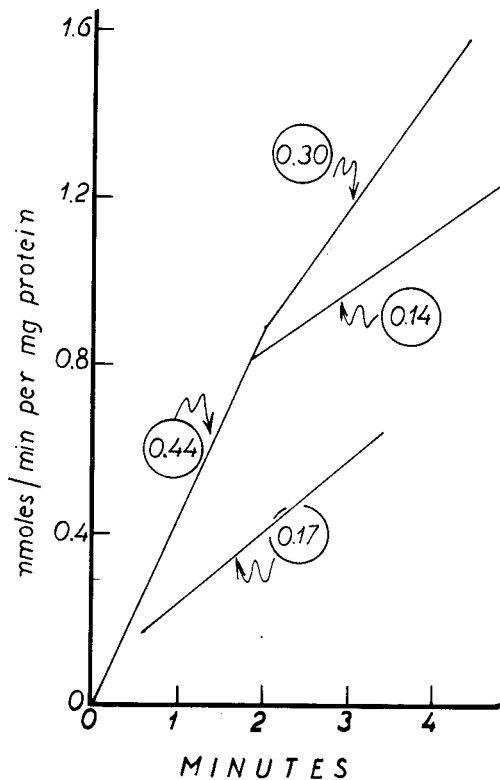
CONDITION	TRANSIENT RATES nmoles/min / mg pr.		INHIBITION by INSULIN %
	1st to 2nd min	2nd to 4th min	
BASAL	0.44	0.30	–
125 µU/ml insulin	–	0.14	53.3
1250 µU/ml insulin	0.17	–	61.4

Fig. 2. Effect of insulin on transient rates of adenylyl cyclase reaction catalyzed by a "pure membrane preparation". The slopes shown in this figure correspond to the time courses ilustrated in Figure 1.

the rate (initial or final) measured in the presence of the hormone and that measured in its absence. On the other hand, the relative extent of the effect will be indicated as the ratio between this difference and the rate measured in the absence of insulin.

The kinetics of adenylyl cyclase reaction were studied using ATP or adenyl-5'-yl imidodiphosphate [AMP-P(NH)P] as adenylyl donor, and Mn^{++} or Mg^{++} as divalent cation, in the presence or absence of insulin. The use of AMP-P(NH)P in these studies is justified by the fact that this ATP analog is a well known substrate for adenylyl cyclase (5). In addition, this nucleotide does not appear to participate in phosphorylation reactions as a phosphate donor and it is resistant to nucleotide phosphohydrolases (6, 7). Consequently this compound could be important for ascertaining whether the hydrolysis or transfer of the terminal phosphate group of ATP could be a requirement for the effect of insulin on cyclase.

I. *Kinetics of Adenylyl Cyclase Measured with ATP*

a) Basal Rates.

Figures 3 and 4 show plots of enzyme activity measured, as a function of ATP concentration, at a fixed concentration of Mn^{++} or Mg^{++} respectively. In the presence of Mn^{++}, initial and final rates resulted nearly equivalent in a range between 0.01 and 0.26 mM. At higher ATP concentrations, final rates were slightly lower than the initial ones.

In the presence of Mg^{++} initial rates resulted three to four times higher than the final ones, without any significant change of the corresponding V_i/V_F ratio between them. Such results could indicate that under conditions for cyclase assay the enzyme tends to inactivate. Such inactivation seems to be independent of the ATP concentration and strikingly enhanced by Mg^{++}. In addition activities measured in the presence of Mn^{++} were higher than those detected with Mg^{++}.

Figures 5 and 6 show plots of adenylyl cyclase activity measured at a fixed ATP concentration, varying that of divalent cation (Mn^{++} or Mg^{++} respectively) With Mn^{++}, final rates
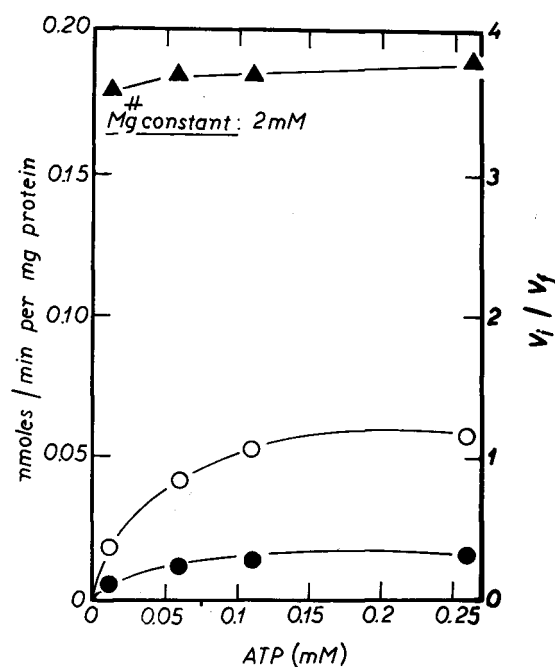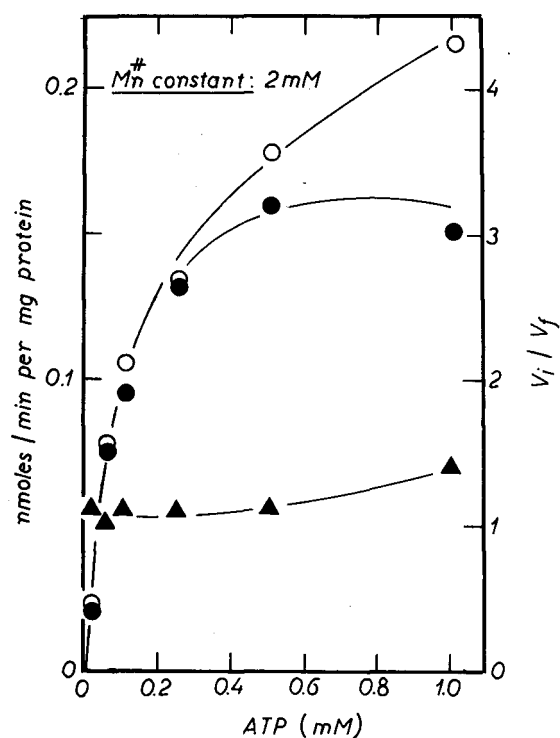
Figs. 3 and 4. Basal adenylyl cyclase activities measured, in the presence of 2 mM $MnCl_2$ (Figure 3) or 2 mM $MgCl_2$ (Figure 4), as a function of ATP concentration. O , initial rates; ●, final rates, ▲, ratio between initial and final rates (V_i/V_f). Other conditions were as indicated in the legend of Figure 1 and in the text.
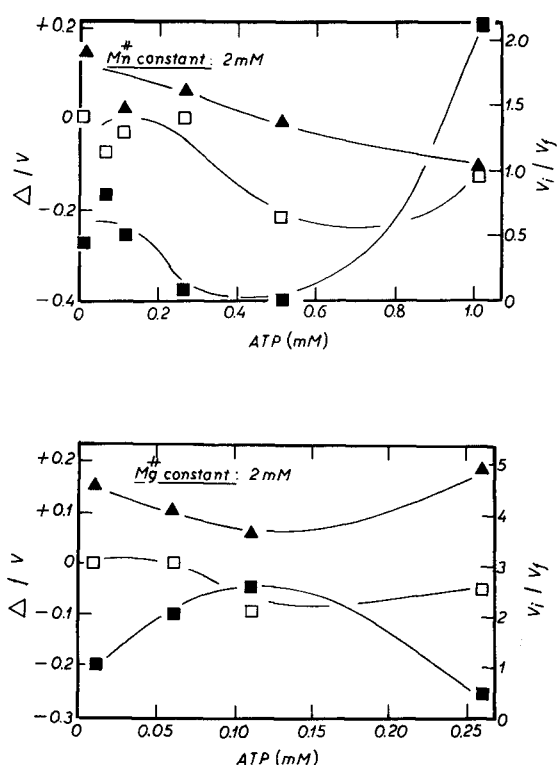
Figs. 5 and 6. Effect of insulin on adenylyl cyclase activities measured, in the presence of 2 mM MnCl₂ (Figure 5) or 2 mM MgCl₂ (Figure 6), as a function of ATP concentration. Δ/V ratio correspond to: □, [V_i(insulin) V_i(basal)]/ [V_i(basal)] and ■, [V_f(insulin) V_f(basal)]/[V_f(basal)] ▲ , V_i(insulin)/V_f (insulin) · Other conditions were as in Figures 1, 3 and 4.

resulted slightly lower than the initial ones, as it corresponds to a relatively high substrate concentration (0.5 mM; see Figure 3). The ratio between initial and final rates resulted insensitive to the divalent cation concentration.

With Mg^{++} the results were clearly different. In a range between 1.5 and 2.5 mM Mg^{++} final rates resulted three to four times lower that the initial ones. Higher or lower Mg^{++} concentrations gave velocity ratios similar to those observed with Mn^{++}

The interpretation of these results is difficult. In some range of Mg^{++} concentrations, enzyme inactivation was more evident than with Mn^{++}, indicating that Mg^{++} is more efficient than Mn^{++} in bringing about the cyclase inactivation. However the clear enhancement of final

velocities observed at 4 and 6 mM Mg^{++}, could suggest that at high concentrations Mg^{++} enables the expression of the putative "inactive" cyclase form. On the other hand, as above discussed, velocities resulted much higher with Mn^{++} than with Mg^{++}.
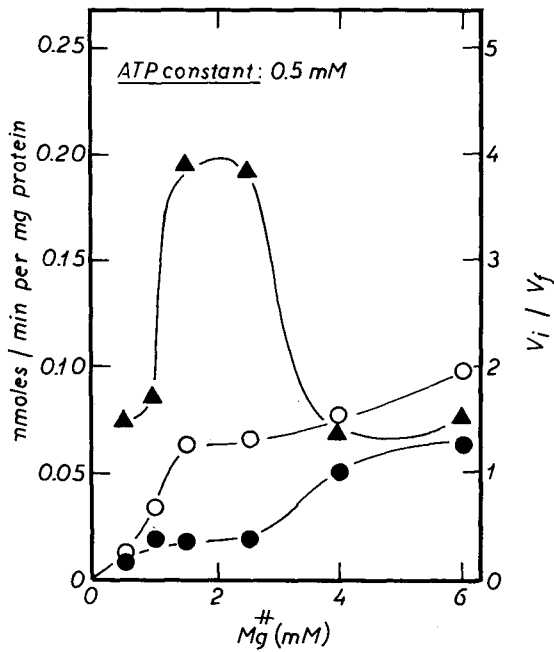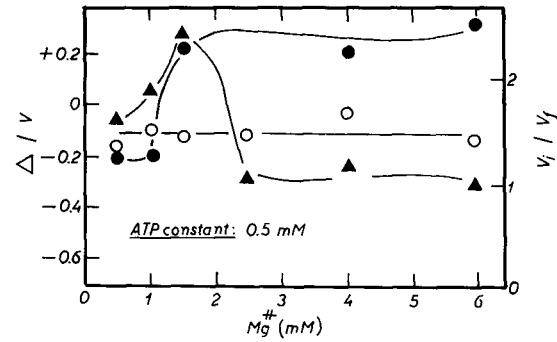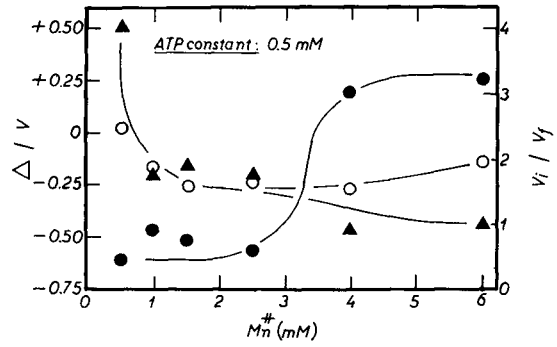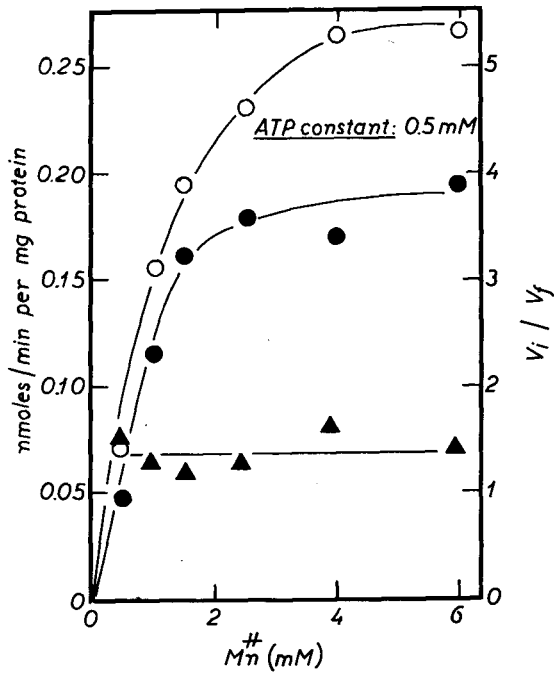
b) Effect of insulin

In the presence of 2 mM Mn^{++}, insulin effects were strikingly influenced by the ATP concentration. Maximal inhibitions were observed on final rates in the range between 0.26 and 0.5 mM and the effect was completely abolished at 1.0 mM ATP (Figure 7). On the other hand, insulin effects on initial rates were much less evident in the whole interval of concentrations explored.

When the assays were performed in the presence of Mg^{++} insulin effects were less marked than those observed with Mn^{++} in the range of ATP concentrations explored (Figure 8).

Evidence on a dual role or divalent cations on the control of adenylyl cyclase was observed in the experiments performed with variable concentrations of divalent cation. As shown in Figure 9, insulin lead to a clear decrease of the final velocity at Mn^{++} concentrations up to 2.5 mM. At higher concentrations the effect was completely reversed. Insulin effects on initial rates were less evident.

In the presence of Mg^{++}, the insulin dependent inactivation of adenylyl cyclase was small and only evident at the two lowest concentrations tested (Figure 10). Above such concentratios there was a recovery of final velocities slightly above the controls.

This evidence could indicate that in some way insulin is able to accelerate cyclase inactivation. The hormone effect on adenylyl cyclase is strikingly dependent on the composition of the reaction mixture. The inactivation is only observed clearly and reproducibly when Mn^{++} instead og Mg^{++} is included as the standard divalent cation. In this regard there appears to be a negative correlation between the magnitude of the insulin effect in the presence of Mn^{++} or Mg^{++} and the ability of the divalent cation to affect spontaneous inactivation of the cyclase. In addition it seems that a high divalent cation or ATP concentration might fulfil the

Figs. 9 and 10. Effect of insulin on adenylyl cyclase activities measured, in the presence of 0.5 mM ATP, as a function of $MnCl_2$ (Figure 9). or $MgCl_2$ (Figure 10) concentration. Other conditions and symbols were as in Figures 5 and 6.

requirements for the expression of the putative "inactive" cyclase form(s).

II. Kinetics of Adenylyl Cyclase Measured with AMP-P(NH)P

a) Basal Rates

Figures 11 and 12 show plots of initial and final rates as a function of Mn^{++} or Mg^{++} respectively; AMP-P(NH)P was fixed at 0.1 mM. In the presence of Mn^{++} the enzyme system showed a clear inactivation at the lowest divalent cation concentrations. With Mg^{++} the inactivation increased with increasing concentrations of the cation.

In terms of the specific activities detected with AMP-P(NHP it is evident that they resulted ten times lower that those observed

Figs. 7 and 8. Basal adenylyl cyclase activities measured in the presence of 0.5 mM ATP, as a function of $MnCl_2$ (Figure 7) or $MgCl_2$ (Figure 8) concentration. Other conditions and symbols were as indicate in Figures 3 and 4.
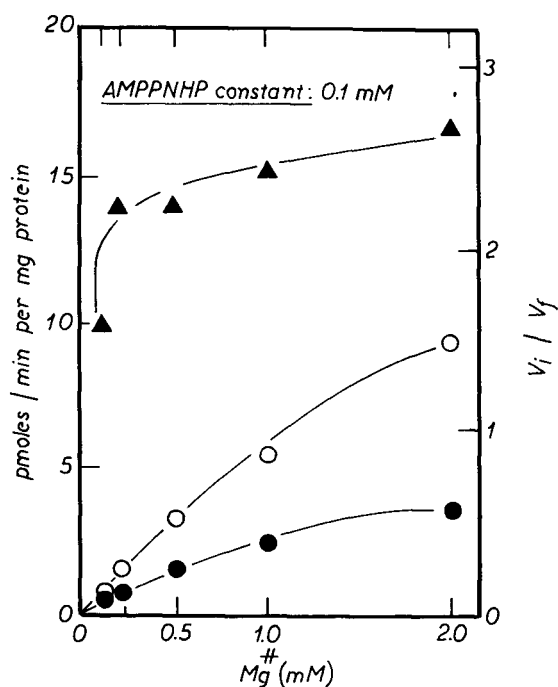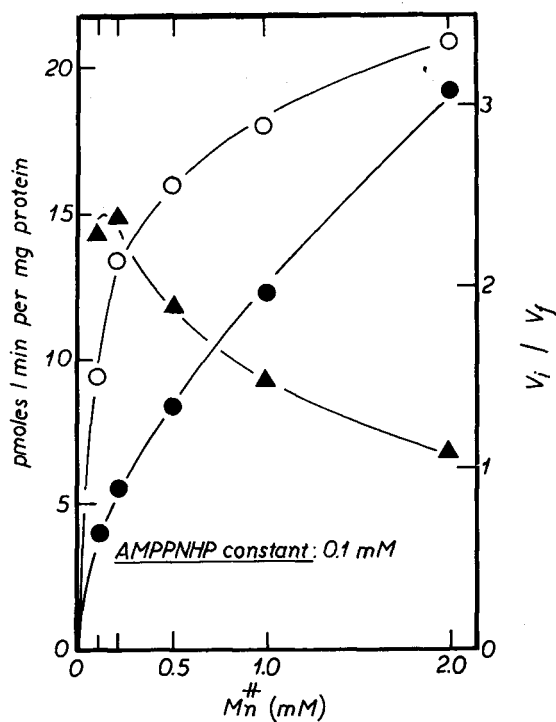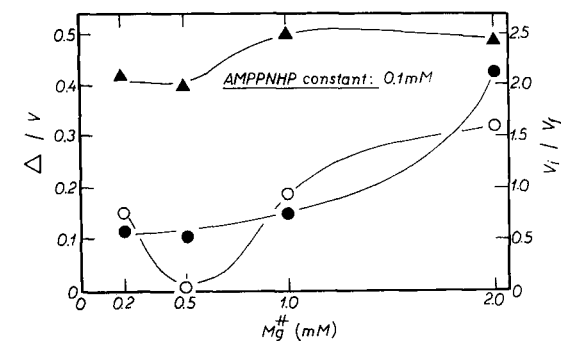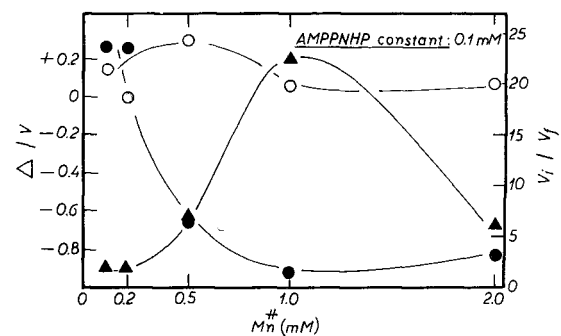
Figs. 11 and 12. Basal adenylyl cyclase activities measured, in the presence of 0.1 mM AMP-P(NH)P, as a function of $MnCl_2$ (Figure 11) or $MgCl_2$ (Figure 12) concentration. Standard components of adenylyl cyclase asay mixtures were: 50 mM Tris-HCl buffer, pH 7.4; 0.2 mM 3-isobutyl-l-methyl xanthine; 1 mM cyclic AMP; 01 mg per ml albumin, and membranes. Concentration of $[\alpha^{32}P]$AMP-P(NH)P (ICN; about 1×10^7 cpm per assay) and divalent cation was indicated in each Figure. Other conditions and symbols were as indicated in the legend of Figures 3 and 4.

b) Effect of insulin

In the presence of insulin, increasing concentrations of Mn^{++} lead to an increased degree of hormone-dependent inactivation of cyclase (Figure 13). The hormone did not influence initial rates. In addition the insulin-dependent inactivation was observed at Mn^{++} concentrations that did not determined *per se* a high inactivation of cyclase.

with ATP. This suggest that the nature of the bridge between β and γ phosphates might influence the rate of cyclase catalyzed reaction.

Figs. 13 and 14. Effect of insulin on adenylyl cyclase activities measured, in the presence of 0.5 mM ATP, as a function of $MnCl_2$ (Figure 13) or $MgCl_2$ (Figure 14) concentration. Other conditions and symbols were as in Figure 11, 12, 5 and 6.

When the results are compared with those found with ATP, it is clear that inactivation of cyclase is more efficient in the presence of the analog.

In the case of assays performed in the presence of Mg^{++} (Figure 14) insulin did not increase the rate of cyclase inactivation. On the contrary some activation was found at the level of both, initial and final component.

Data discussed in this paper could be amenable to a more rigurous analysis only after a best knowledge of the adenylyl cyclase system. However from these results and previous evidence reported by these and other laboratories (8-11), the following conclusions could be reasonably accepted:

1) The substrate of the cyclase reaction is the metal-ATP complex: $[MnATP]^{2-}$ or $[MgATP]^{2-}$. AMP-P(NH)P can replace ATP in the corresponding complexes but less efficiently.

2) Activities measured with Mn^{++} are higher than those found with Mg^{++}.

3) Free divalent cations (Mn^{++} or Mg^{++}) affect adenylyl cyclase activity in three, apparently different, ways:

a) Increasing the activity of catalytically "active form(s)";

b) Enabling the expression of putative "inactive form(s)";

c) Eliciting the conversion of cyclase to putative "inactive form(s)".

4) Conversion of "active" to "inactive" cyclase forms is more efficient in the presence of Mg^{++} than with Mn^{++}. With the latter divalent cation it is possible to increase such a conversion with insulin.

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