*Biochimica et Biophysica Acta*, 673 (1981) 46—54


BBA 29501

# PARATHYROID HORMONE AND ISOPROTERENOL STIMULATION OF ADENYLATE CYCLASE IN RAT OSTEOSARCOMA CLONAL CELLS

## HORMONE COMPETITION AND SITE HETEROGENEITY

SEVGI B. RODAN and GIDEON A. RODAN *

*Department of Oral Biology, University of Connecticut School of Dental Medicine, Health Center, Farmington CT 06032 (U.S.A.)*

(Received July 11th, 1980)

*Key words: Adenylate cyclase; Parathyroid hormone; Isoproterenol; Guanine nucleotide; Site heterogeneity; (Osteosarcoma cell)*

## Summary

A clonal cell line from rat osteosarcoma was found to possess parathyroid hormone and isoproterenol sensitive adenylate cyclase. This study examines the relationship between the two hormones and triphosphoguanine nucleotides with respect to enzyme activation. Concentration-dependence curves, analyzed by computer-aided curve fitting, revealed: (1) in the presence of 5 $\mu$M GTP there were two apparent affinities for parathyroid hormone ($K_m$ 9 and 98 nM) and isoproterenol ($K_m$ 72 and 340 nM); (2) and two affinities for guanosine-5'-($\beta,\gamma$-imido)triphosphate ($K_m$ 0.25 and 1.3 $\mu$M); (3) hormones and guanine nucleotides reciprocally shifted each other's concentration dependence curves to the high affinity sites; (4) parathyroid hormone and isoproterenol interacting with high affinity sites competed for the same adenylate cyclase; (5) parathyroid hormone and isoproterenol, acting on low affinity sites had additive effects and also stimulated adenylate cyclase in the absence of added guanine nucleotides. The findings are consistent with: (i) competition of parathyroid hormone and isoproterenol for the activation of the high (hormone) affinity complex containing: receptors, nucleotide subunit, triphosphoguanine nucleotide, catalytic unit and (ii) the apparent presence of receptor-nucleotide subunit-GDP-catalytic unit complexes with low hormone affinity which are stimulated by parathyroid hormone and isoproterenol separately.

* To whom correspondence and reprint requests should be addressed.
Abbreviations: buffer I, 10 mM Tris-HCl/1 mM dithiothreitol/0.5 mM EGTA, pH 7.8; NTP, nucleotide triphosphate

## Introduction

Osteoblast-like cells recently cloned in our laboratory from osteosarcoma were found to possess parathyroid hormone and isoproterenol sensitive adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) [1]. Catecholamine dependent adenylate cyclase activity has been described in many tissues [2] often along with adenylate cyclase stimulation by other tissue specific hormones [3—7]. Birnbaumer and Rodbell [8] have shown that different lipolytic hormones probably activate the same adenylate cyclase pool since their combined effects were less than additive. The same conclusion was reached regarding glucagon and epinephrine in cat liver [9] and heart muscle [10]. In turkey erythrocytes, catecholamines and adenosine were also shown, by kinetic studies, to compete for a common adenylate cyclase pool [11]. Bovine parathyroid hormone and isoproterenol, at saturating concentrations, had additive effects on isolated liver cell adenylate cyclase [12], suggesting action on separate enzyme moieties. This interpretation was complicated by the cellular heterogeneity of the tissue. We reexamined this question in an osteosarcoma clonal cell line. Another question addressed was a potential difference between low ('physiological') and high hormone concentrations, known from binding studies to act on apparently different sites [13—17]. Since hormone binding was shown to be affected by triphosphoguanine nucleotides, we also examined their effect on site heterogeneity for hormone activation of adenylate cyclase.

## Materials

Tissue culture dishes were purchased from Falcon Plastics, Oxnard, CA or Costar, Cambridge, MA; MCDB medium was obtained from Pacific Biologicals, Richmond, CA or prepared from modified F-12 powder (Formulation 78-5504) from Gibco, Grand Island, NY. Kanamycin, fetal calf serum and Hanks' balanced salt solution were also purchased from Gibco. Glucagon, (—)-alprenolol, (±)-propranolol and (—)-norepinephrine were products of Sigma Chemical Co., St. Louis, MO. Follicle stimulating hormone and thyroid stimulating hormone were kindly supplied by the National Institute of Arthritis and Metabolic Diseases, Pituitary hormone distributing program. Salmon calcitonin was a gift from Armour Pharmaceuticals, Kankakee, IL. All other materials were obtained as recently described [18].

### *Cultures*

Cell cultures were initiated and maintained as described previously [19]. The clonal cell line 2/3 was used in these experiments.

### *Preparation of particulate fractions*

The culture medium was removed by washing the culture plates twice with calcium/magnesium-free Hanks' balanced salt solution. The cells were scraped with a plastic spatula in 1 ml buffer I (10 mM Tris-HCl/1 mM dithiothreitol/0.5 mM EGTA, pH 7.8), homogenized on ice in a Dounce homogenizer with the tight fitting glass pestle A and centrifuged for 30 min at 47 000 × *g*. From the pellet a purer membrane fraction, floating on 1.155 g/l sucrose, was prep-

ared as described [18]. This fraction had 5-fold higher adenylate cyclase activity per mg protein but both had the same hormone responsiveness, with and without added GTP. The 47 000 × *g* pellet suspended in buffer I was used in subsequent experiments. This fraction, quick frozen in liquid nitrogen, was stable at −80°C for at least 6 months. Protein was determined by the method of Spector [20] using bovine serum albumin as the standard.

*Determination of adenylate cyclase activity*

Adenylate cyclase activity was assayed by the method of Salomon et al. [21]. The reaction mixture of 100 μl contained 6.2 U phosphocreatine kinase/5 mM phosphocreatine/25 mM Tris-HCl (pH 7.8)/1 mM dithiothreitol/1 mM cyclic AMP and about $2 \cdot 10^6$ cpm [$\alpha$-$^{32}$P]ATP. The concentration of $MgATP^{2-}$ was 0.2 mM and that of $Mg^{2+}$, added as $MgCl_2$ and calculated by solving the multiple equilibria equation [22], is given in the figure legends. The reaction was started by adding protein (5—12 μg) to assay mixture at 30°C and was stopped after 7 min, unless stated otherwise. When Gpp(NH)p was present, the particulate fraction was preincubated with the nucleotide for 5—7 min at 30°C prior to addition of assay mixture. The enzymatic activity was linear with time for at least 15 min at low and high $Mg^{2+}$ concentrations, low and high concentrations of hormone, low and high concentrations of Gpp(NH)p. Determinations were done in triplicate and each experiment was performed at least three times. The precision was 5% (mean coefficient of variation).

## Results

*Concentration dependence of PTH and (−)-isoproterenol stimulation of adenylate cyclase; site heterogeneity*

Adenylate cyclase activity was stimulated in a dose dependent manner by bovine parathyroid hormone and (−)-isoproterenol (Fig. 1). Eadie-Hofstee plots (Fig. 1 insert) revealed two apparent affinities for adenylate cyclase activation by either hormone. Numerical curve fitting [23] yielded the following $K_m$ values: 9.0 nM and 98.4 nM for bovine parathyroid hormone (at 5 μM GTP and 5 mM $Mg^{2+}$) and 72 nM and 341 nM for isoproterenol (at 5 μM GTP and 1 mM $Mg^{2+}$). In the absence of added GTP low affinity hormone stimulation was observed, starting at 50 nM bovine parathyroid hormone and 1.0 μM isoproterenol, to the extent of about 1.5-fold. Since the membranes were obtained from clonal cells we assumed that the bovine parathyroid hormone and catecholamine receptors were present in the same cells.

*Characterization of the catecholamine receptor*

The relative potency of catecholamine stimulation followed the pattern characteristic for β-catecholamine receptors. The $K_m$ values for (−)-isoproterenol (at 5 μM GTP and 5 mM $Mg^{2+}$) were 0.07 μM and 1.1 μM, and for (−)-epinephrine, 0.86 μM and 1.7 μM. (−)-Norepinephrine had a $K_m$ of 11 μM. (−)-Alprenolol inhibited (−)-isoproterenol stimulation with an apparent $K_i$ of 10 nM. Phentolamine had no effect on either basal or (−)-isoproterenol activated adenylate cyclase activity. (±)-Propranolol completely abolished adenylate cyclase stimulation produced by (−)-isoproterenol but had no effect on

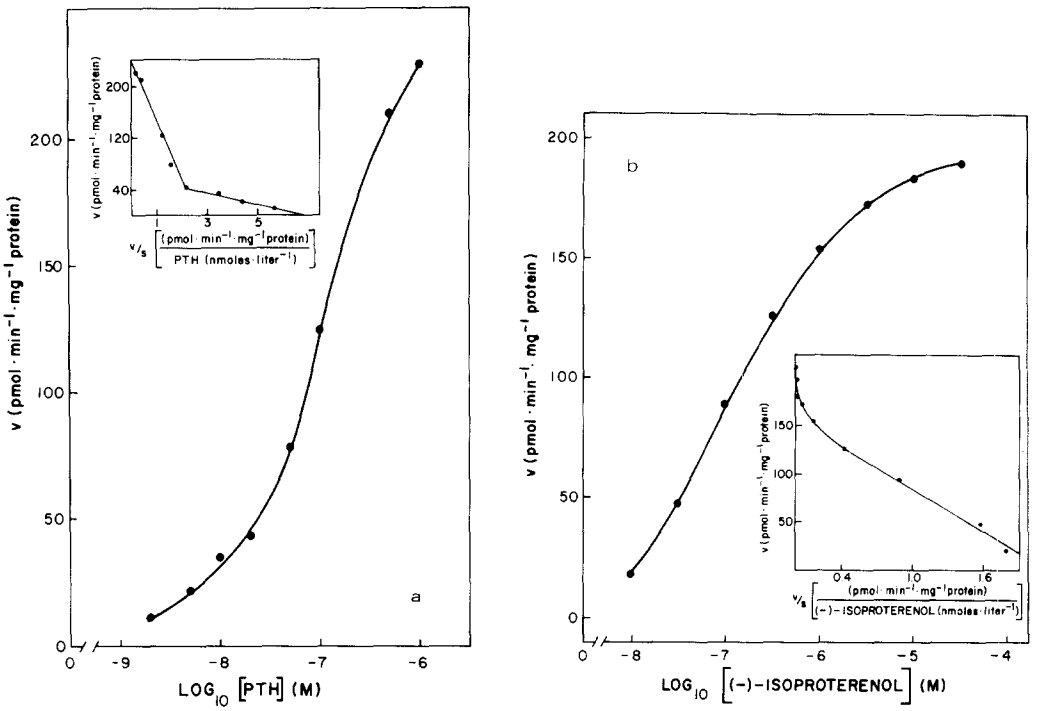


Fig. 1. Bovine parathyroid hormone, (PTH) and isoproterenol stimulation of adenylate cyclase; concentration-dependence curves. The concentration of $Mg^{2+}$ was 5 mM in panel (a) and 1 mM in panel (b). GTP was 5 $\mu$M. Values are the difference between means of triplicate determinations of hormone-stimulated minus basal activity. Inserts: Eadie-Hofstee plots of same data.

bovine parathyroid hormone stimulation (Table I), demonstrating the separate identity of the two receptors. Follicle stimulating hormone 2 $\mu$g/ml, thyroid stimulating hormone 2 $\mu$g/ml, glucagon 1 $\mu$M and calcitonin 0.1 $\mu$M did not stimulate adenylate cyclase.

TABLE I

EFFECT OF (±)-PROPRANOLOL ON (—)-ISOPROTERENOL AND BOVINE PARATHYROID HORMONE STIMULATED ADENYLATE CYCLASE

Adenylate cyclase was assayed on 14.4 $\mu$g protein for 10 min. $Mg^{2+}$ concentration was 1 mM and GTP 5 $\mu$M. The hormones and antagonist were present in the assay mixture when protein was added. Values are means ± (S.E.) of triplicate determinations. PTH, bovine parathyroid hormone.

| Additions to assay mixture | Adenylate cyclase activity (pmol · mg protein$^{-1}$ · min$^{-1}$) |
|---|---|
| None | 22.0 ± 0.7 |
| (±)-Propranolol (2 mM) | 23.3 ± 2.6 |
| (—)-Isoproterenol (100 $\mu$M) | 115.5 ± 3.3 |
| (—)-Isoproterenol (100 $\mu$M) + (±)-propranolol (2 mM) | 22.0 ± 1.3 |
| PTH (20 nM) | 53.6 ± 1.5 |
| PTH (20 nM) + (±)-propranolol (2 mM) | 50.8 ± 2.4 |

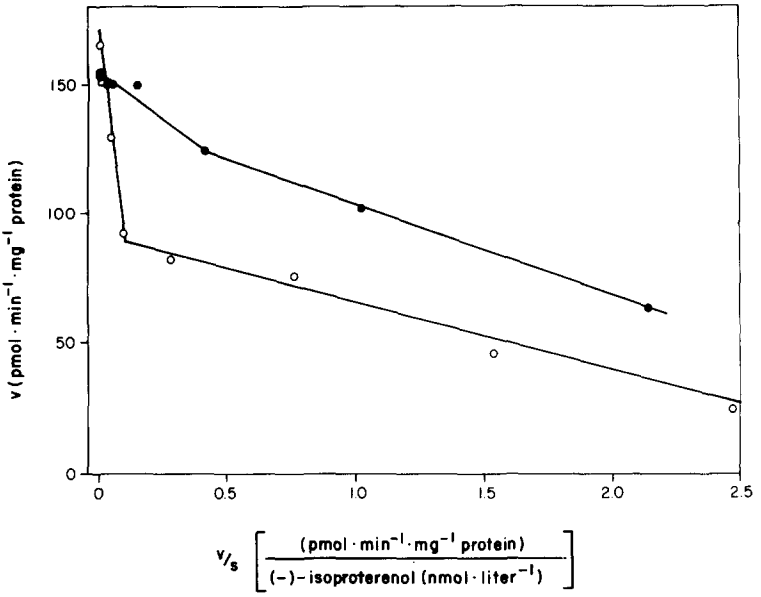


Fig. 2. The effect of Gpp(NH)p on (—)-isoproterenol site heterogeneity for adenylate cyclase stimulation, Eadie Hofstee plot. Adenylate cyclase was assayed on 8 μg protein for 7 min after 5 min incubation with 1 μM (○——○) or 50 μM (●——●) Gpp(NH)p. (—)-Isoproterenol at indicated concentrations was present in the assay mixture which was added to membranes. $Mg^{2+}$ concentration was 1 mM. Values are the difference between means of triplicate determinations of enzyme activity in the presence, minus absence of hormone.

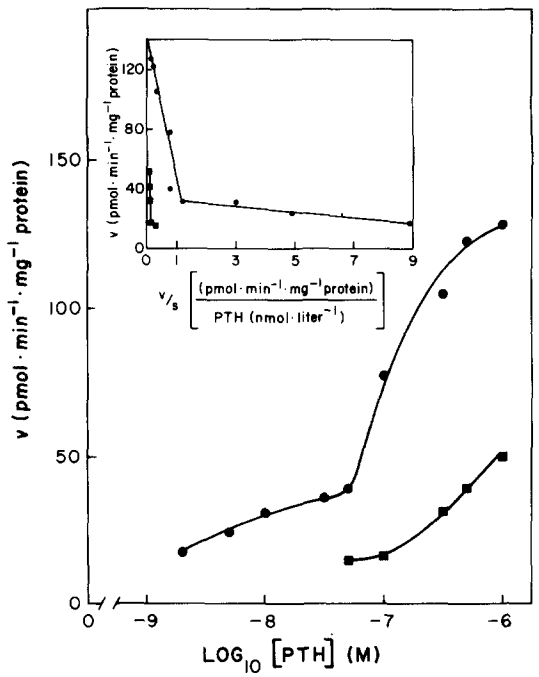


Fig. 3. The effect of Gpp(NH)p on bovine parathyroid hormone (PTH) site heterogeneity for adenylate cyclase stimulation. Adenylate cyclase was assayed on 6 μg protein for 7 min after 5 min incubation with 1 μM (■——■) and 50 μM (●——●) Gpp(NH)p. PTH at indicated concentrations was present in the assay mixture when added to the membranes. $Mg^{2+}$ concentration was 1 mM. Values are difference between means of triplicate determinations of enzyme activity in the presence minus absence of hormone. Insert: Eadie-Hofstee plot of same data.

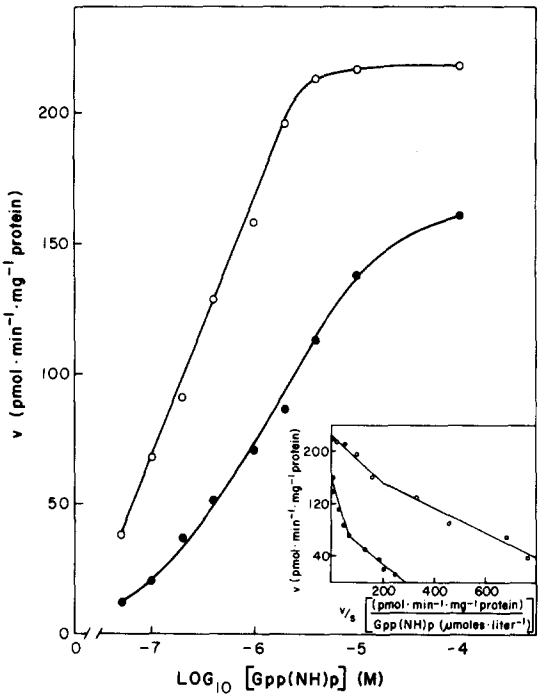


Fig. 4. Effect of bovine parathyroid hormone (PTH) on Gpp(NH)p site heterogeneity for adenylate cyclase stimulation. Adenylate cyclase was assayed on 9 μg protein for 10 min with (○———○) and without (●———●) 100 nM PTH following 5 min preincubation at indicated concentrations of Gpp(NH)p. $Mg^{2+}$ concentration was 5 mM and $MgATP^{2-}$ 0.2 mM. Values are the difference between means of triplicate determinations in the presence of Gpp(NH)p minus basal activity.

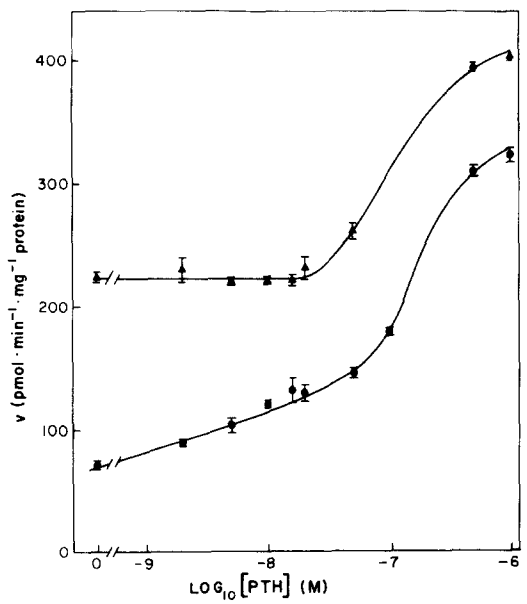


Fig. 5. PTH dose-response curve for adenylate cyclase stimulation in the presence (▲———▲) and absence (●———●) of 100 μM (—)-isoproterenol. Adenylate cyclase was assayed on 8 μg protein. $Mg^{2+}$ was concentration was 5 mM and GTP 5 μM. Values are means ±S.E. of triplicate determinations.

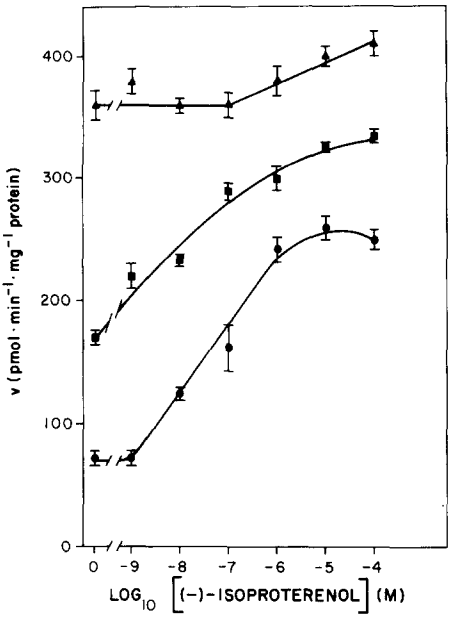


Fig. 6. (—)-Isoproterenol dose-response curve for adenylate cyclase stimulation in the presence of 0 (●———●), 5 (■———■) and 100 nM (▲———▲) bovine parathyroid hormone. Adenylate cyclase was assayed on 9 μg protein. $Mg^{2+}$ concentration was 5 mM and GTP 5 μM. Values are means ±S.E. of triplicate determinations.

*Mutual effects of hormone and Gpp(NH)p on apparent site heterogeneity for adenylate cyclase activation*

In the presence of 1 μM Gpp(NH)p there were two apparent (—)-isoproterenol sites with $K_m$ values of 24 nM and 764 nM, respectively (Fig. 2). At 50 μM Gpp(NH)p, the higher $K_m$ value was reduced to 68 nM, not significantly different from the lower $K_m$ value which remained unchanged (35 nM). Gpp(NH)p had similar effects of bovine parathyroid hormone activation, shifting stimulation towards the high affinity sites (Fig. 3).

The reciprocal effect of hormones on Gpp(NH)p site heterogeneity is shown in Fig. 4. Gpp(NH)p stimulation also exhibited two sites with $K_m$ values of 0.25 ± 0.04 μM and 1.3 ± 0.3 μM, respectively. At saturating bovine parathyroid hormone or isoproterenol concentrations both affinities were very close to the higher affinity: 0.22 ± 0.2 μM and 0.41 ± 0.05 μM, respectively. In the absence of hormone 64 ± 4% Gpp(NH)p stimulation occurred via high affinity sites. Thus hormones and guanine nucleotides seem to stimulate adenylate cyclase via high and low affinity sites and the presence of either, increases the affinity for the other stimulating agent.

*Competition of (—)-isoproterenol and bovine parathyroid hormone acting on 'high affinity' sites for a common adenylate cyclase pool*

Fig. 5 shows a bovine parathyroid hormone-adenylate cyclase dose response curve in the presence of saturating concentrations of (—)-isoproterenol. No bovine parathyroid hormone stimulation was observed until the bovine parathyroid hormone concentrations exceeded 20 nM. Fig. 6 shows the (—)-isopro-

terenol dose response curve in the presence of saturating concentrations of bovine parathyroid hormone. In both cases it appears that only high hormone concentrations interacting with the low affinity sites stimulated the adenylate cyclase in the presence of the other hormone. In other words, bovine parathyroid hormone and (—)-isoproterenol high affinity sites competed for the same adenylate cyclase pool.

## Discussion

Site heterogeneity has been repeatedly observed in hormone binding studies [13—17] but was documented only in few hormone adenylate cyclase activation studies [24—27]. The two affinity constants derived in this investigation are descriptive (rather than thermodynamic) parameters. They combine ligand-binding and coupling-efficiency, two possibly discreet processes which cannot be separated kinetically. There are three possible explanations for site heterogeneity: (1) negative cooperativity, which has not been supported by hormone displacement experiments in binding investigations [13—17]; (2) topographically separated receptors with different affinities (for binding and/or coupling) and (3) low and high affinity states of the receptors, analogous to the proposed catalytically active and inactive states of the enzyme [28]. Our findings are consistent with the latter hypothesis, in view of the shift in the ratio of high to low affinity sites in response to hormones and guanine nucleotides (Figs. 2 and 3). The mutual increase in ligand affinity would have a positive feedback effect on cyclic AMP accumulation during hormone stimulation. This kinetic pattern is often observed in live cells. The shift in affinity produced by GTP was not as extensive as that of the nonhydrolyzable analog. Since GTP complexes are in equilibrium with GDP complexes, the latter appear to have low affinity for hormone-adenylate cyclase stimulation. However, GDP occupancy does not seem to completely inactivate the enzyme (also reported in other systems [28,29]); or prevent hormone stimulation, albeit at high concentrations. Hormone activation was also observed in the presence of 300 $\mu$M 'non-phosphorylatable' guanosine-5'-*O*-(thiodiphosphate) trisodium salt (unpublished data).

The fact that only high affinity sites competed for enzyme activation, suggests that bovine parathyroid hormone competed with isoproterenol for the formation of the NTP high affinity (high activity) state. This is consistent either with larger NTP 'activated' complexes [30] containing receptors for both hormones, or with competition for 'collision coupling [11]. The synergistic effects of subthreshold concentrations of bovine parathyroid hormone and isoproterenol (Fig. 6) favor the 'large-complex' model. The smaller GDP (non-NTP)-complexes can be independently stimulated by high concentrations of either hormone.

The increased affinity for adenylate cyclase activation produced by NTP, in this and other studies [28,31] is in apparent contradiction to the effect of NTP on hormone binding [32,33]. Stadel et al. [ 16] showed two binding states for isoproterenol in frog and turkey erythrocyte membranes, which shifted to one homogeneous lower binding state in the presence of Gpp(NH)p. Abramowitz et al. [34] postulated two sites for nucleotides: one which

decreases receptor binding and one which increases adenylate cyclase coupling. Our data would not require separate guanine nucleotide effects if the high affinity receptor state is short lived [16] and decays to a low (binding) affinity state when activating adenylate cyclase.

From a physiological point of view only the high affinity sites are of significance, since hormone concentrations in vivo are not high enough to interact with low affinity sites. Competition of the two hormones for the same adenylate cyclase implies generation of the same cyclic AMP mediated biological responses. This offers a tool to examine the role of cyclic AMP as a second messenger in this system [35]. If the same relationship between catecholamines and peptide hormones exists in other tissues, epinephrine could act as a master hormone which mobilizes many cyclic AMP-mediated functions in the organism.

## Acknowledgement

This work was supported by U.S. Public Health Service Grant AM 17848.

## References

1 Majeska, R.J., Rodan, S.B. and Rodan, G.A. (1978) Exp. Cell Res. 111, 465–468
2 Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1968) Ann. Rev. Biochem. 37, 149–174
3 Bitensky, M.W., Russell, V. and Robertson, W. (1968) Biochem. Biophys. Res. Commun. 31, 706–712
4 Chase, L.R. and Aurbach, G.D. (1970) J. Biol. Chem. 245, 1520–1526
5 Rodan, S.B. and Rodan, G.A. (1974) J. Biol. Chem. 249, 3068–3074
6 Marcus, R. and Aurbach, G.D. (1969) Endocrinology 85, 801–810
7 Martin, T.J., Ingleton, P.M., Underwood, J.C.E., Michelangeli, V.P. and Hunt, N.H. (1976) Nature 260, 436–438
8 Birnbaumer, L. and Rodbell, M. (1969) J. Biol. Chem. 244, 3477–3482
9 Makman, M.H. and Sutherland, E.W. (1964) Endocrinology 75, 127–134
10 Levy, G.S. and Epstein, S.E. (1969) Cir. Res. 24, 151–156
11 Tolkovsky, A. and Levitzki, A. (1978) Biochemistry 17, 3811–3817
12 Moxley, M.A., Bell, N.H., Wagle, S.R., Allen, D.O. and Ashmore, J. (1974) Am. J. Physiol. 227, 1058–1061
13 Malbon, C.C. and Cabelli, R.J. (1978) Biochim. Biophys. Acta 544, 93–101
14 Giudicelli, Y., Agli, B. and Lacasa, D. (1979) FEBS Lett. 104, 431–436
15 Powell-Jones, C.J.J., Thomas, Jr., C.G. and Nayfeh, S.N. (1980) J. Biol. Chem. 255, 4001–4010
16 Stadel, J.M., DeLean, A. and Lefkowitz, R.J. (1980) J. Biol. Chem. 255, 1436–1441
17 Cabelli, R.J. and Malbon, C.C. (1979) J. Biol. Chem. 254, 8903–8908
18 Rodan, S.B., Egan, J.J., Golub, E.E. and Rodan, G.A. (1980) Biochem. J. 185, 617–627
19 Majeska, R.J., Rodan, S.B. and Rodan, G.A. (1980) Endocrinology, 107, 1494–1503
20 Spector, T. (1978) Anal. Biochem. 86, 142–146
21 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–549
22 Rodan, S.B., Golub, E.E., Egan, J.J. and Rodan, G.A. (1980) Biochem. J. 185, 629–637
23 Hooke, R. and Jeeves, T.A. (1961) J. Assoc. Comput. Mach. 8, 212–229
24 Winand, R.J. and Kohn, L.D. (1975) J. Biol. Chem. 250, 6522–6526
25 Oron, Y., Kellogg, J. and Larner, J. (1978) Molec. Pharmacol. 14, 1018–1030
26 Robberecht, P., Deschodt-Lankman, M., Camus, J.-C., Deneef, P., Lambert, M. and Christophe, J. (1979) FEBS Lett. 103, 229–233
27 Lucas, M., Hanoune, J. and Bockaert, J. (1978) Molec. Pharmacol. 14, 227–236
28 Iyengar, R., Abramowitz, J., Bordelon-Riser, M. and Birnbaumer, L. (1980) J. Biol. Chem. 255, 3558–3564
29 Kimura, N. and Nagata, N. (1977) J. Biol. Chem. 252, 3829–3835
30 Rodbell, M. (1980) Nature 284, 17–22
31 Schramm, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 2232–2237
32 Ross, E.M., Maguire, M.E., Sturgill, T.W., Biltonen, R.L. and Gilman, A.G. (1977) J. Biol. Chem. 252, 5761–5775
33 Lin, M.C., Nicosia, S., Lad, P.M. and Rodbell, M. (1977) J. Biol. Chem. 252, 2790–2792
34 Abramowitz, J., Iyengar, R. and Birnbaumer, L. (1979) Mol. Cell Endocrinol. 16, 129–146
35 Peck, W.A. (1979) Calcif. Tissue Res. 29, 1–4