Mechanisms for the emergence of catecholamine-sensitive adenylate cyclase and β-adrenergic receptors in cultured hepatocytes

Dependence on protein and RNA synthesis and suppression by isoproterenol

M. Refsnes, D. Sandnes, Ø. Melien, T.E. Sand, S. Jacobsen and T. Christoffersen

Department of Pharmacology, University of Oslo, PO Box 1057, Blindern, Oslo 3, Norway

Received 19 October 1983

Adult male rat hepatocytes, which normally respond poorly to β-adrenergic agents, acquire such responsiveness during primary monolayer culture. We here show that the rise in catecholamine-sensitive adenylate cyclase activity in hepatocytes in vitro is closely paralleled by an increase in the ability to bind the β-adrenoceptor ligand [^{125}I]cyanopindolol. The emergence of β-adrenergic responsiveness did not require cell attachment or serum. Addition of dexamethasone, insulin, thyroxine or dihydrotestosterone to the cultures, singly or in combination, did not prevent the augmented β-adrenergic responsiveness. The increase in catecholamine-sensitive adenylate cyclase activity and [^{125}I]cyanopindolol binding could be blocked by cycloheximide or actinomycin D. Exposure of the cultures to isoproterenol at 3-hourly intervals led to a dose-dependent suppression of the rise in isoproterenol-responsive adenylate cyclase and prevented the increase in β-adrenoceptor binding.

β-Adrenergic receptor *Adenylate cyclase* *Actinomycin D*
Cycloheximide *Rat hepatocyte* *Primary culture*

1. INTRODUCTION

In liver or freshly isolated hepatocytes from normal adult male rats, the adrenergic regulation of carbohydrate metabolism is mainly an α-type rather than a β-type effect [1–5], and the cyclic AMP system is relatively insensitive to catecholamines [3,5–8]. However, a number of physiological and pathological conditions have been recognized, including ontogenetic immaturity [7–9], glucocorticoid deficiency [10–12], experimental diabetes [13], hypothyreosis [14], estrogen influence [10,15], regeneration [16], cholestasis [5,17] and preneoplasia [6,8,18], in which the β-adrenergic mechanism via the adenylate cyclase is operational in the liver.

Recently it was demonstrated that when hepatocytes are kept in primary monolayer culture, a shift occurs from a predominantly α-adrenergic to a β-adrenergic control of glycogen phosphorylase [19]. Concurrently, the cells acquire a strongly increased cyclic AMP [8,19–21] and adenylate cyclase [8,21] response to catecholamines. We have investigated some of the mechanisms underlying this increase in β-adrenergic responsiveness. Our studies showed that the rise in catecholamine-sensitive adenylate cyclase activity in cultured hepatocytes was

Abbreviations: MIX, methylisobutylxanthine; ICYP, iodocyanopindolol; CHX, cycloheximide; Act. D, actinomycin D

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00 © 1983 Federation of European Biochemical Societies

associated with an increase in the number of β-adrenergic receptors, did not require cell attachment, and could be blocked by inhibitors of protein and RNA synthesis or by exposure of the cultures to the β-agonist isoproterenol. Preliminary reports have appeared [22,23].

After this work was completed an independent study was published which similarly provides evidence that the acquisition of increased β-adrenergic response during primary culture of hepatocytes is due to new synthesis of β-adrenergic receptors [24].

2. MATERIALS AND METHODS

2.1. *Materials*

(±)-[^{125}I]Cyanopindolol (2200 Ci/mmol) was from New England Nuclear (Boston). (−)Propranolol hydrochloride was a gift from Imperial Chemical Industries (London). The sources of other chemicals and radioisotopes have been described [25].

2.2. *Hepatocyte isolation and culture*

Male Wistar rats, 180–220 g, were fed ad libitum. Hepatocytes were isolated as in [26]. For culturing [27], the hepatocytes were washed twice in Dulbecco's Modified Eagle's Medium and seeded in the same medium (5–6 × 10^5 cells/ml) supplemented with 15% horse serum, 2.5% fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and other additions as indicated, using Costar culture flasks of varying size with 0.12 ml medium/cm^2.

2.3. *Assay of cyclic AMP and adenylate cyclase activity*

The cyclic AMP response in intact suspended or monolayer cells was measured [25] after washing, addition of Krebs-Ringer Hepes buffer, equilibration for 45 min at 37°C, and incubation for 60 s with the agonist to be tested in the presence of 0.5 mM methylisobutylxanthine.

For assay of adenylate cyclase activity, the cells were washed and lysed by hypotonic treatment for 2 min followed by freezing/thawing as in [25]. Incubations were carried out with ~100 μg lysate protein in 100 μl total volume at 0.2 mM ATP (containing ~10^6 cpm [α^{32}P]ATP). Other details have been described [25]. The labelled cyclic AMP formed was purified on Dowex-50/alumina double columns [28].

2.4. *β-Adrenergic binding*

β-Adrenoceptor binding was assessed by incubating washed particles with (±)-[^{125}I]cyanopindolol [29]. The cells were lysed as described above, the lysates were suspended in 50 mM Tris (pH 7.5), 30 mM KCl, 4 mM $MgCl_2$, and particles were prepared by centrifuging at 40000 × *g* for 20 min, washing and respinning. Samples (35–100 μg particle protein) were incubated at 37°C for 90 min in 50 mM Tris (pH 7.4) and 10 mM $MgCl_2$ in 200 μl total volume with varying concentrations of [^{125}I]cyanopindolol and unlabelled ligands. Bound ligand was separated from the free fraction by filtration through Whatman GF/C filters. Specific binding was defined as the difference in the amount of bound radioactive ligand obtained in the absence and in the presence of 0.3 μM (−)propranolol.

3. RESULTS

3.1. *Increased catecholamine-responsive adenylate cyclase activity in cultured hepatocytes; β_2-characteristics*

Hepatocytes in primary culture acquire an increased catecholamine-sensitive adenylate cyclase activity [8,21]. Fig.1 shows dose–response curves for isoproterenol on the adenylate cyclase in hepatocytes assayed immediately after isolation and after 48 h in monolayer culture. The order of potency of adrenergic agents (isoproterenol ⩾ epinephrine ≫ norepinephrine, fig.1, inset) indicated that the strong response evolving in vitro was of the β_2-type.

3.2. *Cell attachment or serum not required*

In order to decide if the increased response of the hepatocytes in monolayer culture to β-adrenergic agents was a result of the attachment of the cells to the substratum (which conceivably might affect the adenylate cyclase through changes in the cytoskeletal organization [30]) we carried out parallel experiments (table 1) on cells that were inoculated into culture flasks and either allowed to form monolayers or prevented from plating by continuous low-speed rotatory shaking. After 7 h there was a substantial and equal increase in the

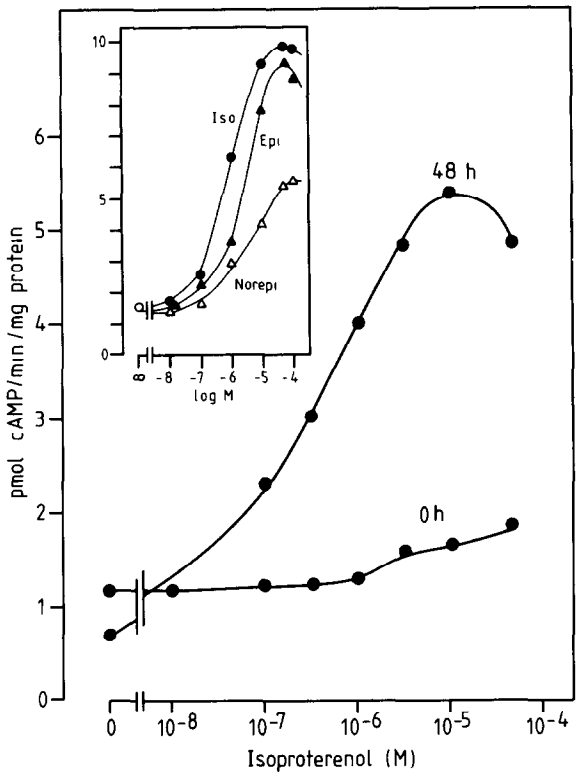


Fig.1. Catecholamine-responsive adenylate cyclase in cultured hepatocytes. Dose–response curve for isoproterenol (with 100 μM GTP) on the enzyme in rat hepatocytes immediately after isolation (0 h) and after 48 h in monolayer culture. Mean of duplicate measurements from one typical experiment. Inset: Relative effect of isoproterenol (—●—), epinephrine (—▲—) and norepinephrine (—△—) on the adenylate cyclase activity in hepatocytes cultured for 24 h. (In the experiment shown in the inset the cells were seeded with 0.4 μM insulin plus 0.25 μM dexamethasone.)

isoproterenol responsiveness in both the suspended and the plated cells, in terms of adenylate cyclase activity (not shown) as well as cyclic AMP response (table 1). It thus appears that the increased β-adrenergic responsiveness acquired by the liver cells in vitro is not attachment-dependent.

The increased isoproterenol response, measured as ability to accumulate cyclic AMP (table 2) or adenylate cyclase activity (not shown) was obtained also in monolayers that had been cultured without serum.

3.3. *Failure of insulin, dexamethasone, thyroxine and testosterone to suppress the rise in catecholamine response of the adenylate cyclase*

Enhanced β-adrenergic response has been found after adrenalectomy [10–12], in hypothyreosis [14] and in diabetes [13], and androgen administration in vivo lowered the hepatic epinephrine-sensitive adenylate cyclase activity [10]. It was thus conceivable that deprival of hormonal factors in the in vitro triggered the emergence of the β-adrenergic responsiveness. Table 3 shows that insulin, dexamethasone, thyroxine and dihydrotestosterone, singly or in combination, did not suppress the development of a highly isoproterenol-sensitive adenylate cyclase activity in the cultured hepatocytes. (Insulin and dexamethasone improve the plating efficiency and viability of the hepatocytes [21]. Therefore, these hormones were routinely used in the subsequent experiments.)

3.4. *Parallel increment in β-adrenoceptor binding and catecholamine-sensitive adenylate cyclase activity*

Table 1

Cyclic AMP response to isoproterenol and glucagon in hepatocyte suspensions and monolayers

Conditions	No stimulator	Isoproterenol (50 μM)	Glucagon (1.4 μM)
	pmol Cyclic AMP/mg protein[a]		
Suspensions (freshly isolated)	3.0 ± 1.1	4.1 ± 1.2	92.9 ± 4.7
Monolayers (7 h in vitro)	2.5 ± 0.6	26.4 ± 1.7	39.0 ± 0.7
Suspensions[b] (7 h in vitro)	1.4 ± 0.6	32.0 ± 0.7	45.5 ± 0.7

[a] Incubation for 60 s in the presence of 0.5 mM MIX. Mean ± SEM of results from 3 culture flasks
[b] Cells inoculated into culture flasks were prevented from plating by continuous rotatory shaking

Table 2

Cyclic AMP response in hepatocyte monolayers in the presence and absence of serum

Conditions	No stimulator	Isoproterenol (50 μM)	Glucagon (1.4 μM)
	pmol Cyclic AMP/mg protein[a]		
Freshly isolated cells	4.0 ± 1.2	8.0 ± 2.1	79.2 ± 9.1
Monolayers with serum (24 h)[b]	8.0 ± 1.9	93.7 ± 11.5	79.1 ± 8.2
Monolayers without serum (24 h)	7.5 ± 1.9	63.6 ± 7.0	70.6 ± 14.2

[a] Incubation for 60 s in the presence of 0.5 mM MIX. Mean ± SEM of results from 3 culture flasks
[b] 15% horse plus 2.5% fetal calf serum

Table 3

Effect of hormone pretreatment of cultured hepatocytes on isoproterenol-responsive adenylate cyclase

Conditions	Basal activity	Isoproterenol (50 μM)
	pmol Cyclic AMP · mg protein^{-1} · min^{-1}	
Freshly isolated hepatocytes	1.7 ± 0.5	2.4 ± 0.9
Hepatocyte monolayers[b]		
No addition	1.4 ± 0.4	7.0 ± 0.8
Dexamethasone (0.25 μM)	1.9 ± 0.4	9.1 ± 0.6
Insulin (0.4 μM)	1.9 ± 0.3	8.7 ± 0.3
Dexamethasone + insulin	1.9 ± 0.5	8.8 ± 0.8
Thyroxine (1 μM)	1.8 ± 0.4	7.1 ± 0.5
Dihydrotestosterone (10 μg/ml)	2.6 ± 0.1	7.1 ± 0.6
Dexamethasone + insulin + thyroxine + dihydrotestosterone	3.3 ± 0.4	8.7 ± 1.3

[a] Adenylate cyclase was assayed with 100 μM GTP. Mean ± SEM of determinations on 4 separate cell preparations for the freshly isolated cells, or from 3–4 separate monolayer cultures for each type of in vitro treatment
[b] The agents indicated were added at the time of plating. The cells were harvested for assay at 20 h

Incubation of washed hepatocyte 40000 × g particles with [^{125}I]cyanopindolol revealed an increased ability of the cultured cells to bind this β-adrenoceptor ligand (fig.2). After 24 h of culture the apparent number of β-adrenergic receptors had increased from 1.9 ± 0.9 fmol/mg crude particle protein (n = 4) to 25.2 ± 3.1 fmol/mg (n = 4). The experiments also demonstrated a close correlation, temporally and quantitatively, between the progressive increase in [^{125}I]cyanopindolol binding and the rise in isoproterenol-sensitive adenylate cyclase activity, indicating that the emerging β-adrenergic responsiveness of these cells in culture is a result of the appearance of β-adrenoceptors.

3.5. *Inhibitory effects of cycloheximide and actinomycin D*

The increase in both β-adrenergic adenylate cyclase activation and number of β-receptors apparently was dependent on protein synthesis, since

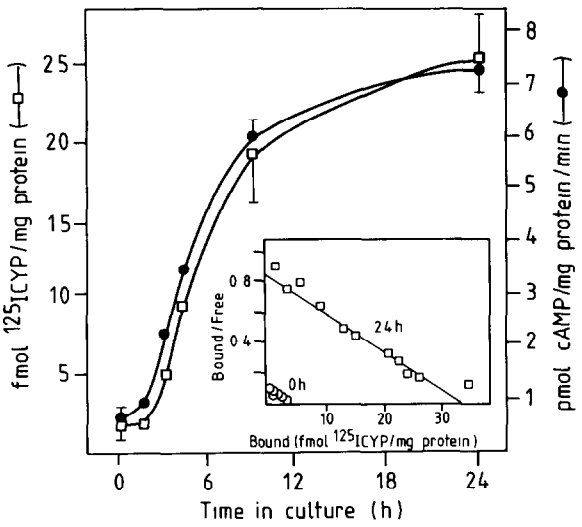


Fig.2. Time course of the increase in isoproterenol-sensitive adenylate cyclase (—●—) and β-adrenoceptor binding (—□—) in primary hepatocyte cultures. The cells were cultured in the presence of dexamethasone (0.25 μM) plus insulin (0.4 μM). The adenylate cyclase values represent the activation above basal activity (range 1.1–2.0 pmol·mg^{-1}·min^{-1}) produced by isoproterenol (50 μM). GTP was present (100 μM). β-Adrenoceptor binding was assessed in incubations of hepatocyte particles with 45 pM [^{125}I]cyanopindolol. The results are the means ± SEM (brackets) of measurements on 3–6 cultures at 0, 9 and 24 h after plating, and the means of duplicate flasks at the other time points. Inset: Scatchard plot of the binding data for [^{125}I]cyanopindolol from one of the experiments, comparing 0 h and 24 h of culturing.

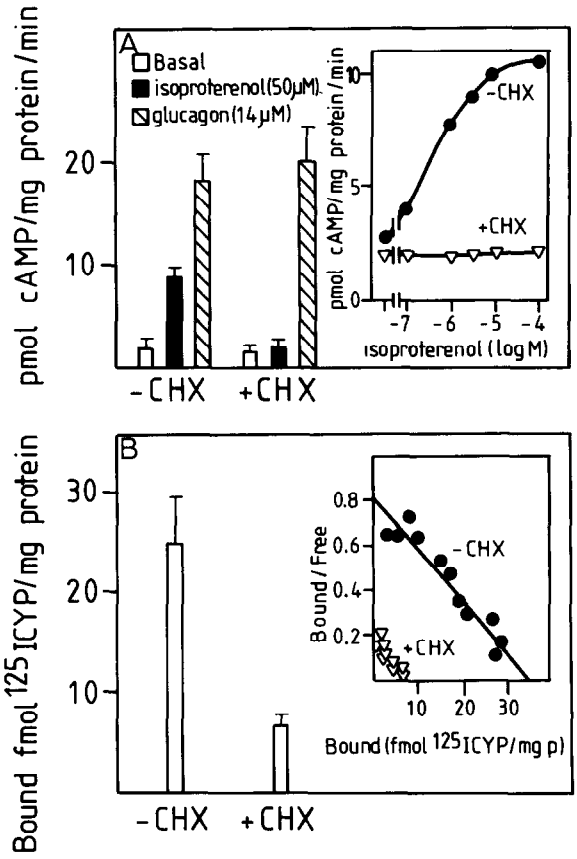


Fig.3. Effect of cycloheximide on isoproterenol-sensitive adenylate cyclase activity and β-adrenoceptor binding in cultured hepatocytes. Cycloheximide (CHX, 5 μg/ml) was added at 0 h. The cells were cultured for 24 h in the presence of dexamethasone (0.25 μM) and insulin (0.4 μM). (A) Adenylate cyclase activity. Inset in A: Dose–response curves for isoproterenol. (B) Number of β-adrenoceptors, based on binding experiments with [^{125}I]cyanopindolol at various concentrations. Inset in B: Scatchard plot. The data represent the means ± SEM (brackets) of results from 4 experiments; the insets show one experiment.

it was blocked almost completely by the addition of cycloheximide to the cultures at the time of seeding (fig.3). The increase was also sensitive to actinomycin D (fig.4), indicating a mechanism requiring gene transcription.

Neither cycloheximide nor actinomycin D treatment reduced the glucagon responsiveness of the hepatocytes (fig.3,4). Thus, the inhibition by these agents of the increase in β-adrenoceptor binding and development of highly catecholamine-sensitive adenylate cyclase activity was selective, and not merely a result of a general deterioration of plasma membrane function or ability to form cyclic AMP.

In view of the reported α-adrenergic, and slightly β-adrenergic, effect of cycloheximide [31] it was of significance that this agent did not in our experiments influence the hepatocyte adenylate cyclase activity when added directly to the assay tubes (not shown). It is thus unlikely that the blockade of the increase in catecholamine response and β-receptor number in cells pretreated with cycloheximide was due to a direct interaction with the receptor, rather than inhibition of protein synthesis. This conclusion is further corroborated by the fact that actinomycin D was also inhibitory.

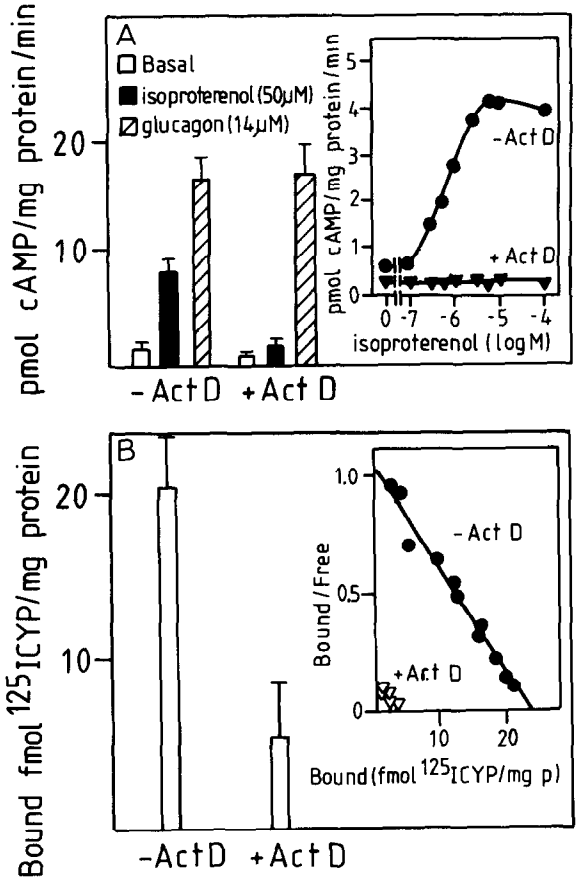


Fig.4. Effect of actinomycin D on isoproterenol-sensitive adenylate cyclase activity and β-adrenoceptor binding in cultured hepatocytes. Actinomycin D (Act.D, 0.2 μg/ml) was added at 0 h. The cells were cultured for 9 h in the presence of dexamethasone (0.25 μM) and insulin (0.4 μM). (A) Adenylate cyclase activity. (B) Number of β-adrenoceptors (see fig.3B). Inset in B: Scatchard plot. The data represent the means ± SEM (brackets) of results from 4 experiments; the insets show one experiment.

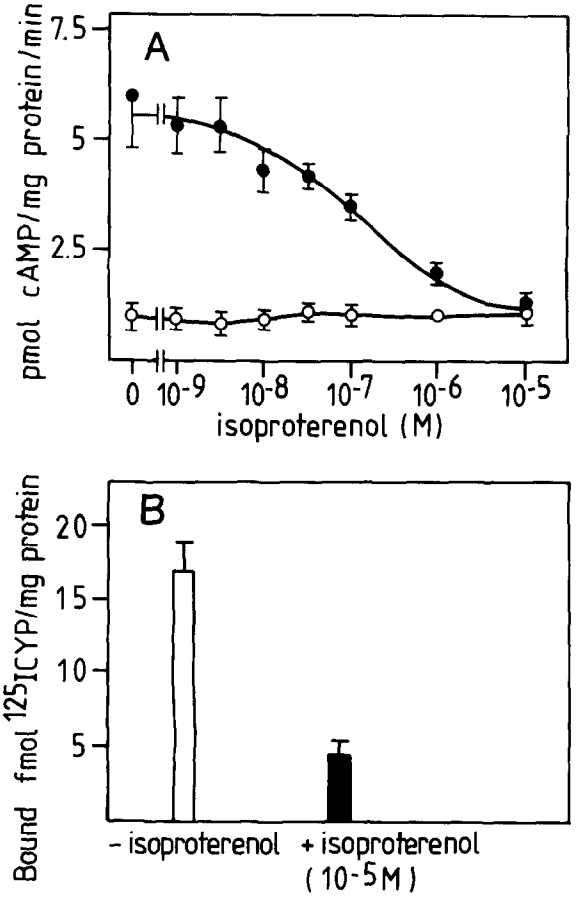


Fig.5. Effect of isoproterenol-exposure on isoproterenol-sensitive adenylate cyclase activity (A) and β-adrenoceptor binding (B) in primary hepatocyte monolayer cultures. Isoproterenol (concentrations as indicated) was added to the cultures at 0, 3 and 6 h after the seeding. Dexamethasone (0.25 μM) and insulin (0.4 μM) was present. At 9 h the cells were washed 5 times with 0.9% NaCl and harvested. Adenylate cyclase was assayed in the presence of 100 μM GTP, with (—●—) or without (—○—) 50 μM isoproterenol. The β-adrenoceptor binding was determined with 45 pM [^{125}I]cyanopindolol. The data represent the means ± SEM (brackets) of measurements on 3 cultures. 3 additional experiments yielded similar results.

3.6. *Suppression by isoproterenol*

Repeated additions of isoproterenol to the cultures (at 3-hourly intervals) prevented the rise in β-adrenergic binding and response (fig.5). The suppressing effect of this agonist on the subsequent activity of isoproterenol-sensitive adenylate cyclase was dose-dependent in the range of $\leqslant 10^{-8}$–10^{-6} M. Glucagon-sensitive cyclase activity was not affected (not shown).

4. DISCUSSION

The present results indicate that the augmented β-adrenergic responsiveness acquired by adult male rat hepatocytes in primary culture [8,19–21] evolves through gene activation and de novo synthesis of β-adrenoceptors (or, theoretically,

another protein factor required for receptor accessibility or binding function). The close correlation between the progressive increase in the activity of isoproterenol-sensitive adenylate cyclase and binding of [^{125}I]cyanopindolol (fig.2) further suggests that the availability of functional receptors is rate-limiting for the β-adrenergic response in these cells.

Although it is not yet clear why the up-regulation of β-adrenergic mechanism is elicited when the hepatocytes are kept in culture, our results show that it is not a consequence of the formation of attached monolayers and is not induced by any constituent of serum. One obvious possibility is that the β-adrenergic binding and effect increases in vitro due to release of the cells from a down-regulated state imposed upon them in vivo by endogeneous catecholamines. That this is at least a contributing factor seems likely in view of the dose-dependent suppression of isoproterenol-sensitive adenylate cyclase exerted by this agonist when added repeatedly to the cells during the culturing (fig.5). It is hard to compare directly these experiments (where isoproterenol was added at 3-hourly intervals) with the in vivo situation. However, the suppression started at low concentrations of isoproterenol (between 10^{-9} and 10^{-8} M).

In vivo the β-adrenergic responsiveness of the liver appears to be subject to a complex regulation [5–18]. Primary hepatocyte cultures may provide clues to a better understanding of the concerted actions of the factors that govern the response of liver cells to β-adrenergic activation. Thus, it may be possible to examine if agents that lower the hepatic β-response in vivo, such as glucocorticoids, insulin and thyroid hormones, but which did not in our experiments counteract the increase in catecholamine-sensitive adenylate cyclase (table 3), facilitate the agonist-induced desensitization of the β-response. This hypothesis is currently being tested in our laboratory, as part of a more systematic study of the mechanisms involved in up- and down-regulation of the β-adrenergic responsiveness of the hepatocytes.

ACKNOWLEDGEMENTS

M.R. is a research fellow of the Norwegian Cancer Society. The work was also supported by grants from The Norwegian Society for Fighting Cancer. We thank Ellen Johanne Johansen, Turid Veggan and Maisen Lied for excellent technical assistance.

REFERENCES

[1] Sherline, P., Lynch, A. and Glinsmann, W.H. (1972) Endocrinology 91, 680–690.
[2] Tolbert, M.E.M., Butcher, F.R. and Fain, J.N. (1973) J. Biol. Chem. 248, 5686–5692.
[3] Exton, J.H. and Harper, S.C. (1975) Adv. Cyclic Nucl. Res. 5, 519–532.
[4] Aggerbeck, M., Guellaen, G. and Hanoune, J. (1980) Biochem. Pharmacol. 29, 643–645.
[5] Schmelck, P.H. and Hanoune, J. (1980) Mol. Cell. Biochem. 33, 35–48.
[6] Boyd, H., Louis, C.J. and Martin, T.J. (1974) Cancer Res. 34, 1720–1725.
[7] Christoffersen, T., Mørland, J., Osnes, J.B. and Øye, I. (1973) Biochim. Biophys. Acta 313, 338–349.
[8] Christoffersen, T., Refsnes, M., Sand, T., Melien, Ø., Brønstad, G., Sandnes, D., Sager, G. and Jacobsen, S. (1983) in: Regulation of Target Cell Responsiveness (Aakvaag, A. et al. eds) Plenum, New York, in press.
[9] Blair, J.B., James, M.E. and Foster, J.L. (1979) J. Biol. Chem. 254, 7579–7584.
[10] Bitensky, M.W., Russel, V. and Blanco, M. (1970) Endocrinology 86, 154–158.
[11] Leray, F., Chambaut, A.M., Perrenoud, M.L. and Hanoune, J. (1973) Eur. J. Biochem. 38, 185–192.
[12] Wolfe, B.B., Harden, T.K. and Molinoff, P.B. (1976) Proc. Natl. Acad. Sci. USA 73, 1343–1347.
[13] Bitensky, M.W., Gorman, R.E. and Neufeld, A.H. (1972) Endocrinology 90, 1331–1335.
[14] Malbon, C.C. (1980) J. Biol. Chem. 255, 8692–8699.
[15] Studer, R.K. and Borle, A.B. (1982) J. Biol. Chem. 257, 7987–7993.
[16] Brønstad, G.O. and Christoffersen, T. (1980) FEBS Lett. 120, 89–93.
[17] Schmelck, P.H., Billon, M.C., Munnich, A., Geynet, P., Houssin, D. and Hanoune, J. (1979) FEBS Lett. 107, 259–263.
[18] Christoffersen, T., Brønstad, G.O., Walstad, P. and Øye, I. (1974) Biochim. Biophys. Acta 372, 291–303.
[19] Okajima, F. and Ui, M. (1982) Arch. Biochem. Biophys. 213, 658–668.
[20] Pariza, M.W., Butcher, F.R., Becker, J.E. and Potter, V.R. (1977) Proc. Natl. Acad. Sci. USA 74, 234–237.

[21] Christoffersen, T., Refsnes, M., Brønstad, G.O., Østby, E., Huse, J., Haffner, F., Sand, T.E., Hunt, N.N. and Sonne, O. (1983) Eur. J. Biochem., in press.
[22] Christoffersen, T., Refsnes, M., Melien, Ø. and Sand, T. (1982) Acta Pharmacol. Toxicol. 51, suppl.II, 14.
[23] Refsnes, M., Sandnes, D., Melien, Ø., Sand, T.E., Jacobsen, S. and Christoffersen, T. (1983) Adv. Cyclic Nucl. Res. 17, 22.
[24] Nakamura, T., Tomomura, A., Noda, C., Shimoji, M. and Ichiara, A. (1983) J. Biol. Chem. 258, 9283–9289.
[25] Brønstad, G.O. and Christoffersen, T. (1981) Eur. J. Biochem. 117, 369–374.
[26] Christoffersen, T. and Berg, T. (1974) Biochim. Biophys. Acta 338, 408–417.
[27] Brønstad, G.O., Sand, T.E. and Christoffersen, T. (1983) Biochim. Biophys. Acta 763, 58–63.
[28] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[29] Engel, G., Hoyer, D., Berthold, R. and Wagner, H. (1981) Arch. Pharmacol. 317, 277–285.
[30] Zor, U. (1983) Endocrine Reviews 4, 1–21.
[31] Huerta-Bahena, J., Villalebes-Molina, R. and Garcia-Saina, A. (1982) Life Sci. 30, 1757–1762.