Biochimica et Biophysica Acta, 972 (1988) 311–319
Elsevier

BBA 12358

Homologous and heterologous β-adrenergic desensitization in hepatocytes. Additivity and effect of pertussis toxin

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(Received 24 February 1988)
(Revised manuscript received 2 August 1988)

Key words: β-Adrenoceptor; Adenylate cyclase; Protein kinase C; Phorbol esters; Homologous–heterologous desensitization; Pertussis toxin

In hepatocytes obtained from hypothyroid rats, phorbol myristate acetate (PMA) and vasopressin diminished the accumulation of cyclic AMP and the stimulation of ureagenesis induced by isoprenaline or glucagon without altering significantly the accumulation of cyclic AMP induced by forskolin. Pretreatment with PMA markedly reduced the stimulation of ureagenesis and the accumulation of cyclic AMP induced by isoprenaline or glucagon. In membranes from cells pretreated with PMA, the stimulation of adenylate cyclase induced by isoprenaline + GTP, glucagon + GTP or by Gpp[NH]p were clearly diminished as compared to the control, whereas forskolin-stimulated activity was not affected. The data indicate heterologous desensitization of adenylate cyclase. It was also observed that the homologous (García-Sáinz J.A. and Michel, B. (1987) Biochem. J. 246, 331–336) and this heterologous β-adrenergic desensitizations were additive. Pertussis toxin treatment markedly reduced the heterologous desensitization of adenylate cyclase but not the homologous β-adrenergic desensitization. It is concluded that the homologous and heterologous desensitizations involve different mechanisms. The homologous desensitization seems to occur at the receptor level, whereas the heterologous probably involves the guanine nucleotide-binding regulatory protein, Ns.

Introduction

Desensitization is an adaptative process in which cell exposure to an agonist decreases its responsiveness to subsequent stimuli. Two basic types of desensitization have been described: homologous and heterologous. In the homologous desensitization, activation of a cell by an agonist decreases the subsequent response to the same agent; in the heterologous type, the responsiveness of the cell to agents chemically unrelated to the initial stimulus is decreased [1].

It has been shown that activation of protein kinase C blocks the α_1-adrenergic action in hepatocytes [2–8], and leads to α_1-adrenergic desensitization [9]. In the present communication it is shown that physiological or pharmacological stimulation of protein kinase C blocks the actions due to activation of adenylate cyclase-linked receptors (β-adrenoceptors and glucagon receptors) and leads to heterologous desensitization of adenylate cyclase. In addition, we have observed

Abbreviations: forskolin, 7β-acetoxy-8,12-epoxy-12,6β, 9α-trihydroxylabd-14-en-11-one; PMA, phorbol 12-myristate 13-acetate.

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that the homologous and heterologous types of β-adrenergic desensitization are additive and that the heterologous type is markedly reduced by pretreatment with pretussis toxin.

Materials and Methods

PMA, 6-*N*-propyl-2-thiouracil, methylisobutylxanthine, (−)-isoprenaline, urease, L-glutamine, L-ornithine, arginine-vasopressin, GTP, Gpp [NH]p, theophylline, ATP, phosphocreatine and creatine kinase were obtained from Sigma Chemical Co. Forskolin was obtained from Calbiochem. Glucagon was generously given by Eli Lilly. Cyclic [^{3}H]AMP (32 Ci/mmol) and [α-^{32}P]ATP (28.7 Ci/mmol) were obtained from New England Nuclear. Percoll was from Pharmacia. Pertussis toxin was purified by the method of Sekura et al. [10] from pertussis vaccine concentrates generously provided by the Institute of Hygiene (Mexico). Animals were injected with pertussis toxin (25 μg/100 g intraperitoneally) 3 days before the experiment was performed; this dose of toxin completely blocks the ability of angiotensin II to decrease glucagon-induced cyclic AMP accumulation. Female Wistar rats (approx. 200–250 g) fed ad libitum were used. Hypothyroidism was induced by giving the animals water containing 0.03% 6-*n*-propyl-2-thiouracil for 40–50 days and it was assessed by decreased weight gain, dryness of the fur and decreased levels of triiodothyronine [11].

Hepatocytes were isolated by the method of Berry and Friend [12]. Cells were incubated in 1 ml of Krebs-Ringer bicarbonate buffer under an atmosphere of 95% O_2/5% CO_2 (pH 7.4) at 37° C in a water-bath shaker. In the studies on ureagenesis, the medium was supplemented with 2 mM ornithine and 10 mM glutamine; after 60 min of incubation, urea was quantified in the supernatant by the method of Gutman and Bergmeyer [13]. Cyclic AMP accumulation was determined in cells plus medium by the method of Brown et al. [14]. The cyclic AMP determinations were performed in cells stimulated with the agents (isoprenaline, glucagon or forskolin) for 2 min and incubated during this period with 0.1 mM methylisobutylxanthine to inhibit phosphodiesterase activity.

Membranes were isolated from hepatocytes by a modification of the method of Loten and Redshaw-Loten [15]. In brief, 2–2.5 ml of packed cells were homogenized at 4° C with a Dounce homogenizer in 12.5 ml of 250 mM sucrose/10 mM Tris (pH 7.5). The homogenate was centrifuged at 1500 × *g* for 15 min; the pellet was resuspended in 12.5 ml of the same buffer, and 3.4 ml of Percoll and 0.5 ml of 2 M sucrose were added. This suspension was thoroughly mixed with the homogenizer and centrifuged at 35 000 × *g* for 20 min. The plasma membrane fraction was clearly visible at the top of the tube. This layer was removed and washed with 25 mM Tris/5 mM $MgCl_2$ (pH 7.5).

Adenylate cyclase activity was assayed in a mixture containing 25 mM Tris (pH 7.5)/0.4 mM ATP (containing (1–2) · 10^6 cpm of [α-^{32}P]ATP), 5 mM $MgCl_2$, 10 mM theophylline, 7.4 mg/ml phosphocreatine and 1 mg/ml creatine kinase. The reaction was initiated by the addition of membrane protein (about 150 μg) to a total volume of 0.1 ml and was carried out for 20 min at 30° C. Cyclic AMP was isolated as described by Salomon et al. [16]. Protein was quantified by the method of Lowry et al. [17] using bovine serum albumin as standard.

Results

Isoprenaline, a β-adrenergic agonist, clearly stimulated ureagenesis in hepatocytes obtained from hypothyroid rats; the maximal stimulation was of 30–40% over basal and the EC_{50} approx. 100 nM (Fig. 1). Similarly the beta-adrenergic agonist markedly stimulated the accumulation of cyclic AMP (EC_{50} 30 nM) (Fig. 1B). The stimulations of ureagenesis and cyclic AMP accumulation induced by isoprenaline were inhibited dose-dependently by the β-adrenergic antagonist, propranolol (data not shown).

PMA and vasopressin had no effect on basal ureagenesis in these hepatocytes. However, 100 nM PMA and 10 nM vasopressin clearly diminished (approx. 50%) the actions of isoprenaline (Fig. 1). Both vasopressin and PMA diminished the maximal stimulation of ureagenesis by isoprenaline without altering the EC_{50} for the agonist (Fig. 1A). Similarly, vasopressin and PMA di-

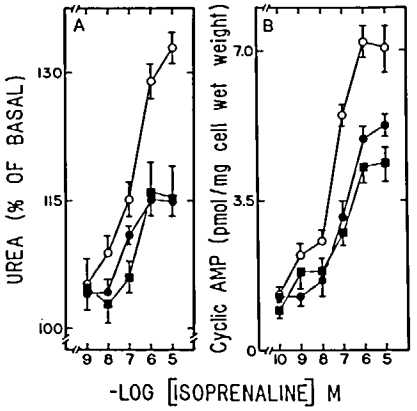


Fig. 1. Effect of PMA and vasopressin on the stimulation of ureagenesis and cyclic AMP accumulation by isoprenaline. Hepatocytes were incubated in the absence (○) or presence of 100 nM PMA (●) or 10 nM vasopressin (■) for 60 min; in the studies on ureagenesis (A) isoprenaline was present during the 60 min and the results are expressed as percentage of basal urea production, which was 40±2 nmol/mg cells (wet weight). In the studies on cyclic AMP accumulation, isoprenaline and 0.1 mM methylisobutylxanthine were present during the last 2 min of incubation. Plotted are the means, and vertical lines represent the S.E. of 6–12 determinations using different cell preparations.

minished (30–50%) the accumulation of cyclic AMP induced by the β-adrenergic agonist without altering its EC_{50} (Fig. 1B).

It has been previously shown, in hepatocytes obtained from euthyroid rats, that PMA decreases the cyclic AMP accumulation induced by glucagon [5,18,19]. Interestingly, in these cells PMA did not produce any significant change in the concentration-dependent activation of phosphorylase [5] and only minimally shifted to the right the concentration–response curve for the activation of ureagenesis by glucagon [19]. Surprisingly, in the present study using hepatocytes from hypothyroid animals, vasopressin and PMA markedly decreased both the cyclic AMP accumulation (approx. 40%) and also the stimulation of ureagenesis (approx. 70%) induced by glucagon (results not shown). The activation of adenylate cyclase by forskolin resulted in a marked stimulation of cyclic AMP accumulation which was not significantly altered by PMA (Fig. 2).

These data indicated that activation of protein kinase C blocks the actions of receptors coupled in an activatory fashion to adenylate cyclase. We next examined whether the activation of this protein kinase led to desensitization of the β-adrenergic action. For this purpose the cells were preincubated with or without PMA and then washed four times (by centrifugation and resuspension) with a least 10-times their volume of Krebs-Ringer bicarbonate buffer. After this pretreatment the cells were incubated for ureagenesis (60 min) or cyclic AMP accumulation (2 min).

Fig. 3 shows that preincubation of hepatocytes with 0.1 μM PMA for 15 min decreased the stimulation of ureagenesis (30–50%) and the accumulation of cyclic AMP (20–30%) induced by the subsequent stimulation with isoprenaline. Fig. 4 shows that this pretreatment desensitizes also the responsiveness to glucagon. As expected, the accumulation of cyclic AMP induced by forskolin was not affected by the pretreatment with PMA (results not shown, see Fig. 2).

These dose–response for the desensitization is presented in Fig. 5. It can be observed that PMA induced a dose-dependent desensitization of the

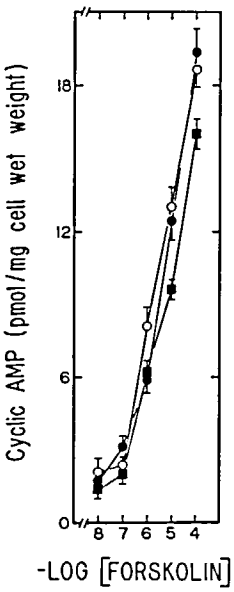


Fig. 2. Effect of PMA and vasopressin on the stimulation of cyclic AMP accumulation by forskolin. Hepatocytes were incubated in the absence (○) or presence of 100 nM PMA (●) or 10 nM vasopressin (■) for 60 min; forskolin and 0.1 mM methylisobutylxanthine were present during the last 2 min of incubation. Plotted are the means, and vertical lines represent the S.E. of 6–12 determinations using different cell preparations.

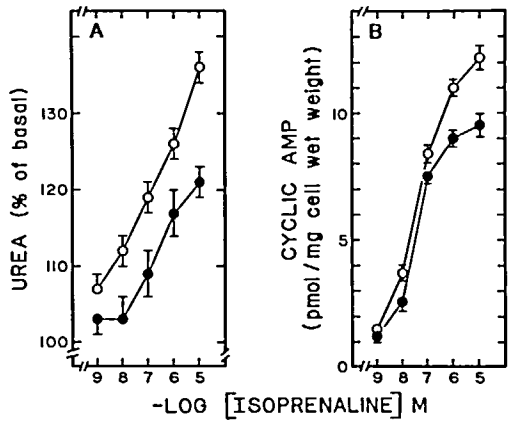


Fig. 3. Effect of preincubation with PMA on the stimulation by isoprenaline of ureagenesis and cyclic AMP accumulation. Hepatocytes were incubed for 15 min in the absence (○) or presence (●) of 0.1 μM PMA. The cells were extensively washed and then incubated as described in Materials and Methods, with different concentrations of isoprenaline. For the studies of ureagenesis (A) the results are expressed as percentage of basal urea production (40±3 and 34±3 nmol/mg cells (wet weight) in control and PMA-treated cells, respectively). In the studies on cyclic AMP accumulation (B) 0.1 mM methylisobutylxanthine was present during the 2 min of incubation with isoprenaline. Mean values are plotted, and vertical lines represent the S.E. for 6–8 different cell preparations.

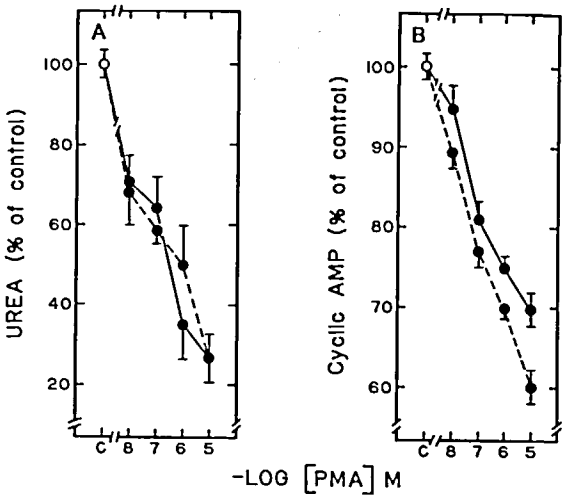


Fig. 5. Glucagon and β-adrenergic desensitizations as a function of PMA concentration during the preincubation. Hepatocytes were incubated for 15 min with the concentrations of PMA indicated. The cells were extensively washed and then challenged with 10 μM isoprenaline (solid lines) or 0.1 μM glucagon (broken lines). The metabolic effect on urea synthesis (panel A) and cyclic AMP accumulation (panel B) is shown; the data are expressed as percentages of the control response. The means are plotted and vertical lines represent the S.E. of triplicate determinations using 5–6 different cell preparations.

ureagenic responses to maximally effective concentrations of glucagon and isoprenaline (Fig. 5A); the desensitization in this metabolic parameter was as much as 70–80%. Similarly, the accumulation of cyclic AMP in response to glucagon or isoprenaline was markedly (up to 30–40%) and

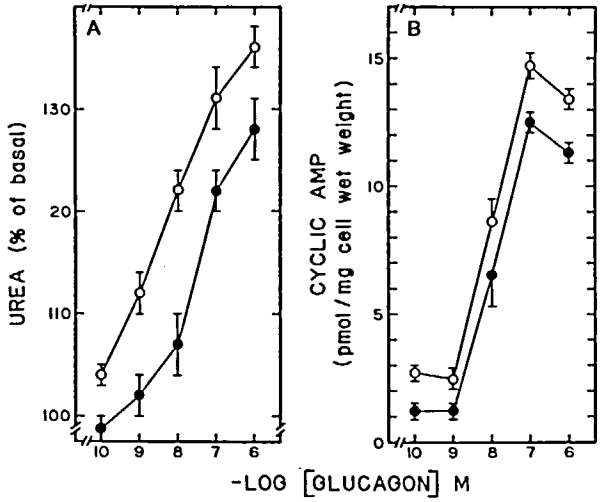


Fig. 4. Effect of preincubation with PMA on the effect of glucagon on ureagenesis and cyclic AMP accumulation. Hepatocytes were incubated for 15 min in the absence (○) or presence (●) of 0.1 μM PMA. The cells were extensively washed and then incubated as described in Materials and Methods with different concentrations of glucagon. Other indications as in Fig. 1.

TABLE I

DESENSITIZATION OF ADENYLATE CYCLASE ACTIVITY IN MEMBRANES FROM HEPATOCYTES PRETREATED WITH PMA, ISOPRENALINE, OR PMA PLUS ISOPRENALINE

Hepatocytes were preincubated with or without the various ligands at the concentration shown; membranes were prepared and adenylate cyclase assayed as described in Materials and Methods. Results are the means ± S.E. of triplicate determinations of 10–13 different membrane preparations.

Assay agent	Adenylate cyclase activity			
	control membrane specific activity (pmol/min per mg protein)	% of activity observed in control membrane		
		PMA 0.1 μM	isoprenaline 100 μM	isoprenaline 100 μM + PMA 0.1 μM
None	7.5 ± 0.67	124 ± 2	114 ± 1	93 ± 2
Isoprenaline 10 μM + 100 μ GTP	16.9 ± 1.0 (2.3 – fold)	82 ± 6 [a]	69 ± 5 [b]	56 ± 2 [c,d,e]
Glucagon 1 μM + 100 μM GTP	53.9 ± 4.6 (7.2 – fold)	82 ± 4 [a]	90 ± 4	75 ± 4 [a]
Gpp[NH]p 100μM	22.0 ± 2.0 (2.9 – fold)	69 ± 4 [b]	99 ± 6	79 ± 5 [a]
Forskolin 100 μM	63.8 ± 4.0 (8.5 – fold)	95 ± 4	112 ± 10	100 ± 8

[a] $P < 0.05$ vs. control.
[b] $P < 0.005$ vs. control.
[c] $P < 0.001$ vs. control.
[d] $P < 0.001$ vs. PMA pretreatment.
[e] $P < 0.025$ vs. Isoprenaline pretreatment.

dose-dependently decreased by the pretreatment with PMA (Fig. 5B). It should be mentioned that the dose-desensitization relationship was nearly identical for the action of both glucagon and isoprenaline, resulting in parallel curves.

The time-course for the desensitization is presented in Fig. 6. Cyclic AMP accumulation was used as parameter, since it evaluates an immediate (2 min) response to the agents. It was observed that the maximal desensitization took place after 30 min of preincubation with PMA; the $t_{1/2}$ was between 10 and 15 min. The time-courses of the desensitizations for isoprenaline and glucagon were very similar.

In order to localize the site affected by the pretreatment with PMA the activity of adenylate cyclase was studied in isolated membranes (Table I). In membranes from control cells (i.e., preincubated for 15 min without any agent) isoprenaline, (2-fold) glucagon (7-fold), Gpp[NH]p (3-fold and forskolin (8-fold) stimulated adenylate cyclase activity (Table I). In membranes from cells pretreated with PMA, forskolin-stimulated activity was nearly identical to that of the control, whereas glucagon-, isoprenaline- and Gpp[NH]p-stimulated activities were consistently desensitized 20–30% (Table I). The data suggest that treatment with PMA does not alter the function of the catalytic subunit of adenylate cyclase, but rather of the activatory guanine nucleotide-binding regulatory protein, Ns.

We previously showed that isoprenaline induces a rapid homologous desensitization in these cells [20]. Data confirming and extending this observation are presented in Tables I and II. It can be observed that pretreatment with isoprenaline selectively desensitizes the ability of isoproterenol to stimulate cyclic AMP accumulation in whole cells (Table II) or adenylate cyclase activity in membranes (Table I). We next studied what effect would produce a preincubation with both isoprenaline and PMA, i.e., inducing both homologous and heterologous β-adrenergic desensitizations. The data are presented for both cells (cyclic AMP accumulation, Table II) and membranes (adenylate cyclase activity, Table I). The

TABLE II

DESENSITIZATION OF ADENYLATE CYCLASE ACTIVITY IN HEPATOCYTES PRETREATED WITH PMA, ISOPRENALINE OR PMA PLUS ISOPRENALINE

Hepatocytes were preincubated for 15 min with or without the various ligands at the concentrations shown; after this, the cells were extensively washed and incubated for 2 min with 100 μM methylisobutylxanthine and the different assay agents shown. Results are the mean ± S.E. of triplicate determinations using 5–7 different cell preparations.

Assay agent	Cyclic AMP accumulation			
	control cells (pmol cAMP/mg cell)	% of accumulation observed in control cells		
		PMA 0.1 μM	isoprenaline 100 μM	isoprenaline 100 μM + PMA 0.1 μM
None	0.52 ± 0.05	108 ± 24	138 ± 13	127 ± 19
Isoprenaline 10 μM	9.66 ± 0.23	86 ± 2 [a]	69 ± 2 [a]	57 ± 3 [a,b,c]
Glucagon 100 nM	10.95 ± 0.38	81 ± 2 [a]	99 ± 2	81 ± 2 [a]

[a] $P < 0.001$ vs. control.
[b] $P < 0.001$ vs. PMA pretreatment.
[c] $P < 0.01$ vs. Isoprenaline pretreatment.

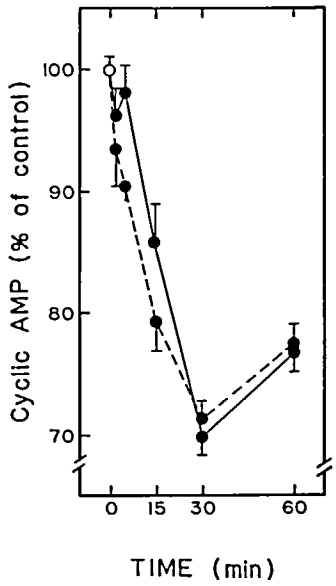
TABLE III

EFFECT OF PRETUSSIS TOXIN ON HOMOLOGOUS AND HETEROLOGOUS DESENSITIZATION

Rats were treated with pertussis toxin and hepatocytes isolated and preincubated with or without (control) the various ligands at the concentration shown. Cyclic AMP accumulation and adenylate cyclase activity were determined. Results are the means ± S.E. of triplicate determination of 7–8 different preparations. Other indications as in Table I and II.

Assay agent	Cyclic AMP accumulation		
	control cells (pmol cAMP/mg cell)	% of accumulation observed in control cells	
		PMA 0.1 μM pretreated	isoprenaline 100 μM pretreated
None	0.7 ± 0.1	164 ± 28	242 ± 28 [a]
Isoprenaline 10 μM	10.6 ± 0.3	91 ± 3	58 ± 2 [a]
Glucagon 100 nM	13.1 ± 0.3	98 ± 3	94 ± 2
Forskolin 100 μM	17.5 ± 0.4	107 ± 3	91 ± 2
Assay agent	Adenlyate cyclase activity		
	control membranes specific activity (pmol/min per mg protein)	% of activity observed in control membranes	
		PMA 0.1 μM pretreated	isoprenaline 100 μM pretreated
None	6.0 ± 0.53	98 ± 11	107 ± 7
Isoprenaline 10 μM + 100 μM GTP	15.9 ± 0.66 (2.7 fold)	94 ± 6	77 ± 5 [a]
Glucagon 1 μM + 100 μM	50.5 ± 4 (8.4 fold)	90 ± 5	92 ± 4
Gpp[NH]p 100 μM	18.7 ± 0.78 (3.1 fold)	99 ± 6	91 ± 2
Forskolin 100 μM	66.7 ± 6 (11 fold)	99 ± 3	101 ± 4

[a] $P < 0.001$.



g. 6. Time-course of the PMA-induced desensitization in patocytes. Hepatocytes were preincubated for the times indiled without (○) or with (●) 0.1 μM PMa; the cells were tensively washed and then rechallenged with 10 μM isopreline (solid lines) or 0.1 μM glucagon (broken lines) in the sence of 100 μM methylisobutylxanthine. The data are pressed as percentages of the control response. Mean values plotted and vertical lines represent the S.E. for triplicate determinations using five dirfferent cell preparations.

ıta clearly indicate that the two types of β-lrenergic desensitization are additive, i.e., in ıole cells, isoprenaline pretreatment induces a % β-adrenergic desensitization, PMA a 14% and ıth a 43% desensitization; in membranes, isopreıline induces a 31% desensitization, PMA and % and both a 44%. The Gpp[NH]p-stimulated lenylate cyclase activity was not altered by the ıprenaline pretreatment and was similarly densitized in membranes from cells treated with MA alone (31%) or with PMA plus isoprenaline 1%). The desensitization of glucagon action ıralleled that of Gpp[NH]p.

It has been shown that pertussis toxin blocks me desensitization processes [21,22]. Therefore, next examined the effect pertussis toxin. It was ıserved that the toxin treatment markedly reıced the heterologous desensitization of adenyte cyclase in both cells and membranes, but, in reement with our previous report [20], it did not fect the homologous β-adrenergic desensitizaın (Table III).

Discussion

Our present findings indicate that pharmacological (phorbol esters) or physiological (vasopressin) activation of protein kinase C markedly inhibits the action of agents acting through receptors coupled in an activatory fashion to adenylate cyclase such as isoprenaline and glucagon in hepatocytes obtained from hypothyroid rats.

We are surprised by the ability of PMA and vasopressin to inhibit the glucagon and isoprenaline-induced stimulations of ureagenesis. The data are particularly astonishing if we consider that the diminutions in glucagon-mediated accumulation of cyclic AMP induced by PMA are very similar in liver cells from euthyroid [17] and hypothyroid rats. However, it is far from clear yet what signal or amplifying factor(s) is (are) lacking in liver cells from hypothyroid rats that makes their metabolism more succeptible to the inhibitory action of PMA. Experiments are in progress to elucidate this point.

Phorbol esters inhibit the accumulation of cyclic AMP induced by glucagon or isoprenaline [5,18,19]. In the present paper we show that these activators or protein kinase C induce a clear heterologous desensitization of adenylate cyclase and that such desensitization has metabolic consequences. The desensitization affected to a similar extent, the actions of glucagon and isoprenaline, with similar time-courses and dose-dependencies. There was a good correlation between the data obtained in whole cells and those in membranes.

It should be mentioned that the concentrations of PMA required to induce β-adrenergic desensitization were higher than we had anticipated (i.e., 10^{-7} to 10^{-5} M) based on the ability of these agents to block the α_1-adrenergic action at somewhat lower concentration (10^{-8} to 10^{-6} M) [2–4]. It was recently reported that hormones that stimulate inositol phospholipid metabolism induce a rapid desensitization of glucagon-stimulated adenylate cyclase [23]. The data indicate that hormonal or pharmacological activation of protein kinase C induce heterologous adenylate cyclase desensitization.

A difference between our study and that of Murphy et al. [23] is that the time-course of the processes differs. In the study of Murphy et al.

[23] the desensitization was very rapid ($t_{1/2}$ 2–3 min) and was transient, returning to control or near-control activity after 20 min. In our study the rate of desensitization was slower and no such return to the nondesensitized state was observed. It is possible that the use of hypothyroid rats in our study may explain this difference. It is also possible that the transient desensitization observed by these authors [23] may reflect the transient breakdown of phosphatidylinositol bisphosphate induced by these hormones.

The site of the lesion in the heterologous desensitization seems to be at the level of the guanine nucleotide-binding regulatory protein, Ns, in accord with the data of Heyworth et al. [18] as evidenced by the altered Gpp[NH]p-stimulated adenylate cyclase activity. Surprisingly, in the study of Murphy et al. [23] little effect of the pretreatment with the calcium-mobilizing hormones on GTP- and NaF-stimulated adenylate cyclase activity was observed. The reason for this difference is at present unknown.

Another important finding in our study was the additivity of the homologous and heterologous β-adrenergic desensitizations. This additivity indicates that these processes operate through different pathways and possibly involve different sites of action. The homologous desensitization seems to take place at the β-adrenoceptor level (i.e., receptor modification and/or sequestration), whereas the heterologous desensitizations seem to occur at the level of Ns, as discussed above. In human astrocytoma cells and rat glioma cells it has also been observed that phorbol esters and β-adrenergic agonists mediate desensitization of adenylate cyclase by distinct mechanisms [24,25].

Pertussis toxin has been shown to block some [21,22] (but not all [20,26]) desensitization processes. In our study it was observed that the homologous β-adrenergic desensitization is not altered by pertussis toxin, as previously reported [20]. In contrast, the heterologous desensitization of adenylate cyclase was markedly reduced by pertussis toxin treatment. In hepatocytes, Heyworth et al. [21] have shown that pertussis toxin blocks glucagon desensitization. This emphasizes the similarities between such desensitization and the heterologous type presented here. However, the desensitizations induced by PMA and glucagon are reported to involve mutually exclusive processes [27].

The mechanism through which pertussis toxin blocks the heterologous desensitization is presently unknown. It has been reported that in MDCK cells glucagon induces a heterologous desensitization associated with increased labeling of Ni by pertussis-toxin-catalyzed ADP-ribosylation [28]. The possibility of an increased level or activity of Ni can not be ruled out. However, it seems unlikely that an increase in amount or activity of Ni may solely explain the results. Several data argue against such explanation. Firstly, under the conditions employed (membranes prepared in the absence of EGTA [29] and adenylate cyclase assayed in the absence of high concentrations of monovalent cation [29,30]) we obsrved little inhibition by guanine nucleotides of forskolin-stimulated activity (an index of Ni function). Furthermore, we would expect that an increase in Ni would also alter the effect of forskolin in whole cells, since it is known that Ni inhibits forskolin-activated adenylate cyclase activity. Further studies are aimed to localize and define the nature of the changes in the homologous and heterologous desensitization observed in this model.

Acknowledgements

This research was partially supported by Grants from CONACyT (PCEXCNA 040260), Secretaría de Salud and Fundación Miguel Alemán. The authors thank Dr. Jaime Martuscelli for his generous help. The skillful secretarial help of Ms. Guadalupe Ramírez is greatly appreciated.

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