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Regulation of Cyclic AMP Metabolism in the Rat Erythrocyte During Chronic β-Adrenergic Stimulation. Evidence for Calmodulin-Mediated Alteration of Membrane-Bound Phosphodiesterase Activity

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Summary. The regulation of cyclic AMP metabolism in the rat erythrocyte has been investigated during chronic exposure to the β agonist isoproterenol. A triphasic response is observed: 1) an acute increase in cyclic AMP to levels four- to fivefold greater than basal, maximal by 1 minute (Phase I); 2) a gradual decline in cAMP content to levels near basal during the next 15–20 minutes (Phase II) and a second sustained rise in cAMP, maximal by 60 minutes, to a concentration greater than that observed during the first minute (Phase III). Extensively washed Phase II and Phase III cells are refractory to a second challenge by isoproterenol. In phosphodiesterase-inhibited intact Phase II and III cells adenylate cyclase activity is maximally activated. Isoproterenol has no effect on soluble phosphodiesterase activity but increases membrane-bound phosphodiesterase activity 3- and 2.2-fold in Phase II and Phase III cells, respectively. The activation of this membrane-bound enzyme activity appears to be mediated by the calcium-dependent regulatory protein, calmodulin, because 1) the amount of exogenous calmodulin required to achieve half-maximal activation of membrane-bound phosphodiesterase is 3.7, 2.0, and 1.2 μg in control, Phase III and Phase II membranes, respectively; and 2) there is less calmodulin in membrane-free lysates prepared from Phase II cells than control cells. These data support the idea that the major mechanism regulating cAMP content in the rat erythrocyte during chronic isoproterenol stimulation is the membrane-bound phosphodiesterase and that there is a translocation of calmodulin from the cytoplasm to the membrane during hormone stimulation.

In many cells the interaction of catecholamines with β-adrenergic receptors results in the stimulation of the membrane-bound adenylate cyclase (E.C. 4.6.1.1.) and a concomitant, rapid accumulation of intracellular cyclic adenosine 3′,5′ monophosphate (cAMP). In most systems cAMP levels then decline, despite the continued presence of the hormone. This decline has been attributed primarily to alterations in adenylate cyclase activity because plasma membranes isolated from chronically stimulated cells are characterized by a reduction in both hormone-stimulated adenylate cyclase activity, and the number of specific β-adrenergic binding sites (Mukherjee, Caron & Lefkowitz, 1975; Brown, Fedak, Woodward, Aurbach & Rodbard, 1976; Cuatrecasas & Hollenberg, 1976; Lefkowitz & Williams, 1977, 1978). This phenomenon is termed "down regulation" and has been the subject of intense investigation. It is also clear that in a variety of cellular systems where cyclic AMP has been shown to be a second messenger in hormone action an interrelated and interdependent role for calcium ion has also been demonstrated (Rasmussen, 1970; Rasmussen & Goodman, 1977). In particular, a calcium-dependent control of phosphodiesterase activity has been identified (Teo & Wang, 1973; Lin, Liu & Cheung, 1975). However, the precise role of this calcium-dependent activity in modulating cellular response to hormonal stimuli has not been well-characterized in any intact cellular system.

Information regarding the control of cAMP metabolism during chronic hormone stimulation has come largely from studies using isolated membranes and has focused on adenylate cyclase regulation. However, the interpretation of results derived from studies employing isolated membranes with regard to cyclic AMP metabolism in intact cells is subject to limitations. Cell lysis and membrane isolation can produce reorientation and redistribution of membrane components, and the incubation medium used

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to study the activities of isolated membranes is only a rough approximation of the *in situ* membrane microenvironment. Additionally, most studies have neglected the potential role of the cAMP catabolic enzyme phosphodiesterase in the control of cellular cyclic AMP metabolism although the activity of this enzyme(s) has been shown to change after certain hormonal manipulations (Wells & Hardman, 1977).

In the present study we have investigated both the synthesis and degradation of cyclic AMP in the rat erythrocyte during chronic β-adrenergic stimulation. This specific system was chosen because unlike the human erythrocyte, the rat erythrocyte possesses a β-adrenergic receptor coupled to adenylate cyclase (Sheppard & Burghardt, 1969); and unlike the nucleated frog erythrocyte it contains an active intracellular phosphodiesterase (Gardner, Aurbach, Spiegel & Brown, 1976). Additionally, the rat erythrocyte is incapable of protein synthesis. Consequently, any changes in cyclic AMP metabolism must be due to the activation and/or inactivation of preexisting enzymes and proteins rather than alterations in the synthesis of new protein(s). In this study experiments were carried out in an effort to define the changes in activities of both components of the cAMP control systems in determining the changes in cAMP metabolism after hormonal stimulation.

Our results show that during chronic β-adrenergic stimulation in the rat erythrocyte there is a calcium-modulated triphasic response with regard to cellular cyclic AMP content: an initial rise, then a rapid fall and finally a sustained increase in cyclic AMP content. Data are presented which support the concept that during chronic β-adrenergic stimulation a membrane-bound phosphodiesterase is activated by redistribution of a calcium-dependent regulatory protein (calmodulin-CDR) from the cytoplasm to the cell membranes.

Materials and Methods

Cell Preparation and Incubation

Heparinized blood was obtained by cardiac puncture from etherized female Sprague Dawley rats (150–200 g). The blood was centrifuged at room temperature and the plasma and buffy coat were aspirated. Erythrocytes were washed three times in buffer A (in 145 mM: NaCl; 5 KCl; 1 $MgSO_4$; 1 $CaCl_2$; 1 NaH_2PO_4; 10 Tris; 10 glucose; and 2.5 μM sodium metabisulfite, pH 7.4).

After the final wash, erythrocytes were resuspended in Buffer A to a 40% packed cell volume and incubated with shaking at 37 °C in a Dulbecco water bath. This particular packed cell volume was chosen for incubation because it approximates the *in vivo* hematocrit of the rat. The incubation flasks were wrapped with aluminum foil to prevent photolysis of the catecholamines. Fresh β-adrenergic agonists were prepared daily in Buffer A and added at the appropriate concentrations to the diluted cell suspension. To determine cAMP content, cells plus medium (100 μliters) were added directly to 1 ml boiling water and boiled for 3 min. The tubes were removed, cooled, centrifuged, and a 100-μliter aliquot of the supernatant was analyzed for cAMP content by radioimmunoassay (Steiner, Parker & Kipnis, 1972). cAMP content is expressed per liter of packed cells based on the measured packed cell volume in the final suspension. Erythrocytes were calcium-depleted by incubation in calcium-free buffer A containing 0.1 mM EGTA and 0.3 μM A23187 for 1 h. Ionophore (A23187) was removed by washing cells in calcium-free buffer containing 5 g/liter fatty acid-free bovine serum albumin, followed by three washed in buffer without albumin (Sarkadi, Szasz & Gardos, 1976).

Membrane Preparation

After initial incubation for varying times in the presence of isoproterenol, cells were washed three times in 0.155 M Tris, pH 7.6 and cooled on ice. One milliliter of packed cells was lysed by the rapid addition and mixing of 40 ml of ice-cold 5 mM sodium phosphate buffer, pH 8.0. The lysate was centrifuged at $27{,}000 \times g$ for 10 min at 4 °C. The supernatant was aspirated and the pellet washed twice in 30 ml sodium phosphate buffer to reduce hemoglobin contamination.

Adenylate Cyclase Activity in Membranes

Adenylate cyclase activity was measured in membranes using a variation of the method of Sahyoun, Hollenberg, Bennet and Cuatrecases (1977). Briefly, 40 μliters of membrane suspension were added to 60 μliters of a solution containing 750 μM ATP; 35 mM $MgCl_2$; 50 mM theophylline; 35 mM phosphoenolpyruvate; 100 μg/ml pyruvate kinase; 5 μCi [α^{32}P]ATP; 0.1 mM GTP; and 250 mM Tris/HCl; pH 8.0. The mixture was incubated for 10 min at 37 °C in a shaking water bath and the assay was terminated by the addition of 1 ml of a solution containing 3 mM cAMP, 15,000 cpm [^{3}H]cAMP, 10 mM Tris/HCl, pH 8.0 and boiled for 3 min. The tubes were cooled and 0.6 g of neutral alumina (Sigma Chemical Co.) was added. The tubes were mixed, centrifuged, and 500 μliters of the supernatant were removed for radioactivity determination in a liquid scintillation counter using a double-channel technique. Using this technique the amount of cAMP in blanks was routinely less than 10% of the amount of cyclic AMP at the earliest time point of experimental incubations. The recovery of [^{3}H]cAMP was used to calculate the production of [^{32}P]cAMP.

Adenylate Cyclase Activity in Intact Cells

Cells were suspended in Buffer A and at time zero the phosphodiesterase inhibitor Ro 20-1724 (Sheppard & Wiggan, 1970) was added (10 μM final concentration). Ro 20-1724 was prepared as a 100X solution in 95% ethanol. Carrier ethanol was added to all control incubations. Aliquots (100 μliters) were removed and added to 1 ml of boiling water, boiled for 3 min, cooled and centrifuged. The cAMP content was determined on the supernatant by radioimmunoassay (Steiner et al., 1972).

Phosphodiesterase Activity

Phosphodiesterase activity was measured during the method of Thompson and Appleman (1971). Briefly, membranes or lysate were incubated at 30 °C in 400 μliters total volume of a buffer that contained 200,000 cpm [^{3}H]cyclic AMP, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3.75 mM mercaptoethanol, 40 mM Tris-HCl, pH 8.0; and various concentrations of cAMP. At the conclusion of the 10-min incubation, the tubes were boiled for 1 min, cooled, and 100 μliters of snake venum (Ophagus Hannah; 1 mg/ml, Sigma Chemical Co.) were added. The tubes were incubated at 30 °C for an additional

10 min, and cooled. One milliliter of a slurry of Dowex AG1-X2 resin (1 volume resin/2 volumes water) was added and the tubes were mixed vigorously and incubated on ice for 15 min. After centrifugation, an aliquot of the supernatant was counted in a liquid scintillation counter.

Calcium-Dependent Regulatory Protein Preparations

Outdated human blood was obtained from a blood bank. Erythrocytes were separated from plasma and buffy coat by centrifugation and the erythrocytes were washed three times in 0.155 M Tris/HCl, pH 7.6. Cells were lysed by a 1:1 dilution into 0.1 mM EGTA, 10 mM Tris/HCl, pH 7.6. The lysate was stirred in a boiling water bath for 8 min, cooled, and centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was adjusted to pH 6.1 with glacial acetic acid. DEAE cellulose (Whatman) (25 g/liter of supernatant) was added and the suspension was stirred for 1 hr at room temperature. The resin was filtered and washed with 10 mM imidazole, pH 6.1, until the eluant appeared colorless. The resin was then poured into a 2.5 × 40 cm column and a linear NaCl gradient (0 to 0.6 M) in 10 mM imidazole, pH 6.1 applied. CDR activity was localized by the ability of specific fractions to activate rat erythrocyte phosphodiesterase. CDR eluted between 0.3–0.4 M NaCl. These fractions were pooled, concentrated by ultrafiltration and dialyzed against 10 mM imidazole, pH 6.1. When this preparation was examined by SDS polyacrylamide electrophoresis there was only a single protein band in the 15- to 17,000 dalton region. Bovine brain calcium-dependent regulatory protein and phosphodiesterase were prepared from freshly frozen calf brains according to the procedure described by Wang and Desai (1977).

Ca^{++} ATPase Activity

Ca^{++} ATPase activity of isolated membrane was measured in a buffer containing (in mM): 3 ATP; 4 $MgCl_2$; 0.1 $CaCl_2$; 0.2 ouabain; and 70 Tris/HCl, pH 7.2. Mg^{++} ATPase activity was measured in a buffer containing 3 ATP; 4 $MgCl_2$; 0.2 EGTA; 0.2 ouabain; and 70 Tris/HCl, pH 7.2. Membranes (75 μliters) were added to 400 μliters of each of the above solutions containing 200,000 cpm of [^{32}P]ATP (specific activity, 3300 Ci/mmol). The mixture was incubated at 37 °C and the reaction was terminated by the addition of 500 μliters of 5% Norit A in 20 mM sodium phosphate. 1% HCl and the suspension was vortexed. After centrifugation, an aliquot of the supernatant was counted in a liquid scintillation counter. Ca^{++} ATPase activity was determined by subtracting the activity in the absence of calcium (Mg^{++} ATPase) from the activity in the presence of calcium.

Protein was determined by the method of Lowry, Rosebrough Farr and Randall (1951).

Materials

Catecholamines and nucleotides were purchased from Sigma Chemical Company. Radioimmunoassay kits were purchased from New England Nuclear. [^{32}P]ATP was purchased from Amersham Searle. A23187 was a gift of Dr. Robert Hamill, Eli Lilly Company; and Ro 20-1724 a gift of Dr. W.E. Scott, Hoffman-LaRoche.

Results

Chronic Exposure of Rat Erythrocytes to β-Adrenergic Agents

The time course of cAMP production in intact rat erythrocytes during continuous exposure to isoproterenol (10 μM) is shown in Fig. 1. Unlike the biphasic response observed in other systems, the rat erythrocyte exhibits a triphasic response during chronic isoproterenol stimulation: 1) Phase I, an acute rise in cAMP (three- to fourfold above basal, maximum by one minute); 2) Phase II, a gradual decline in [cAMP] to levels near basal over the next 15–20 min; and 3) Phase III, a second, sustained rise in [cAMP] which reaches a plateau by 60 min, at levels greater than those attained during acute stimulation, and remained elevated for up to 4 hr. Exogenous calcium is not required for this triphasic response; 10 μM isoproterenol produced identical time courses of cAMP accumulation in medium containing either 1 mM $CaCl_2$ or no added calcium with 0.1 M EGTA. However, when cells were depleted of calcium by preincubation in a medium containing EGTA plus 0.3 μM A23187 and then washed free of ionophore, addition of isoproterenol produced a strikingly different response. These calcium-depleted cells no longer exhibited a triphasic response. A monophasic threefold rise in [cAMP] that was sustained throughout the entire 70 min of incubation was observed (Fig. 1). The maximal increase in [cAMP] in these calcium-depleted cells was less than that seen in Phase II, calcium-replete cells. Also, if at any time during chronic hormone

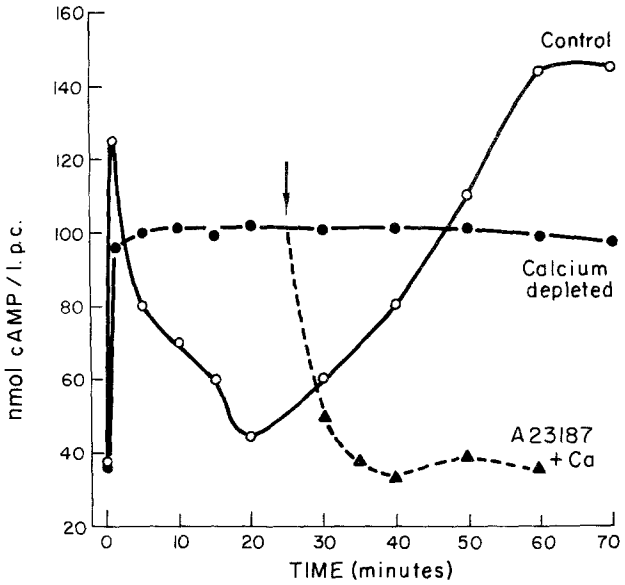

Fig. 1. The time course of the change in cyclic AMP concentration during continuous exposure to isoproterenol (10 μM) in control (○—○) and calcium-depleted (●—●) rat erythrocytes. Cells were calcium-depleted by incubation in 0.1 mM EGTA and 0.3 μM A23187 as described in Materials and Methods. Both control and calcium depleted cells were incubated at a 40% packed cell volume. Aliquots (100 μliters) were removed at the indicated times for cyclic AMP analysis. At the time indicated by the arrow 0.3 μM A23187 and $CaCl_2$ 1 mM (final concentrations) were added to a separate flask containing calcium-depleted cells (▲---▲)

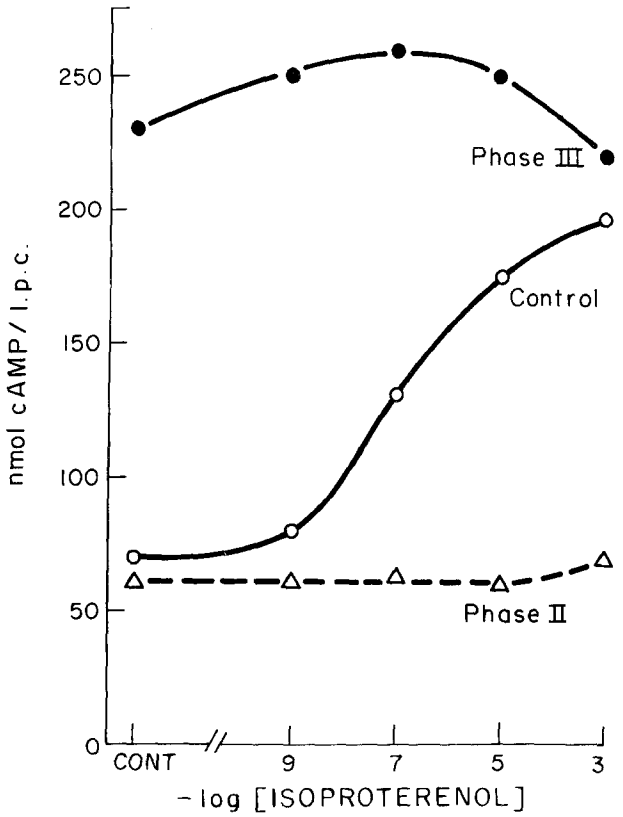

Fig. 2. The effect of isoproterenol rechallenge on cellular cyclic AMP accumulation in control (○—○), Phase II (△--△) and Phase III (●—●) rat erythrocytes. Washed erythrocytes were incubated at a 40% packed cell volume. Isoproterenol (10 μM) was added and the incubation allowed to continue for 10 min (Phase II) or 60 min (Phase III). Control cells were incubated for 60 min in the absence of hormone. At the appropriate times cells were centrifuged and washed three times in buffer. Cells were then resuspended at a 40% packed cell volume and treated with isoproterenol at the indicated concentrations. After 1 min 100-μliter aliquots of the cell suspensions were added to 1.0 ml of boiling water for cyclic AMP determination

exposure of the calcium-depleted cells, the calcium ionophore A23187 was added in the presence of extracellular calcium, there was a rapid decline in [cAMP] concentration to basal values.

Washed Phase II and III erythrocytes were examined to determine whether readdition of hormone would induce a rise in cAMP (Fig. 2). Cells were first exposed to isoproterenol for 10 min (Phase II) or 60 min (Phase III), washed to remove hormone and then rechallenged with increasing doses of isoproterenol. In washed Phase II cells the level of cAMP did not differ from control cells, and readdition of isoproterenol elicited no change in the level of cAMP (Fig. 2). In washed Phase III cells cyclic AMP content remained elevated at levels comparable to those observed before rewashing even after the cells had been washed 3 times. Readdition of isoproterenol over the entire dose range produced only a slight further increase in cAMP content.

Mechanism Regulating the Triphasic Response

Because the rat erythrocyte is incapable of protein synthesis we reasoned that the fluctuations in cAMP content observed during chronic exposure to isoproterenol must be due to changes in the rate of either cyclic AMP synthesis and/or degradation catalyzed by preexisting enzyme(s). Experiments were consequently designed to measure the relative contributions of adenylate cyclase and phosphodiesterase in determining the [cAMP] during Phases I, II, and III.

Adenylate Cyclase

To determine adenylate cyclase activity in membranes from control and Phase III cells, erythrocytes were incubated with or without 10 μM isoproterenol for 60 min. Then the cells were washed free of hormone and membranes prepared as described above. These membranes were then incubated in a buffer containing [α^{32}P]ATP, unlabeled ATP, 100 μM GTP, an ATP regenerating system, and varying concentrations of isoproterenol. In the absence of isoproterenol the amount of [^{32}P]cAMP produced by membranes prepared from Phase III cells was fivefold higher than that produced by comparable amounts of control membranes. If guanine triphosphate was omitted from the incubation medium isoproterenol produced only a slight stimulation in either type of membrane. It should be noted that the amount of [^{32}P]cAMP generated by control membranes stimulated by the highest doses of isoproterenol was greater than that produced by unstimulated Phase III membranes. On the other hand, at the highest concentrations of hormone Phase III membranes converted approximately 3 times as much [^{32}P]ATP to [^{32}P]cAMP as control membranes. These results indicate that the adenylate cyclase in isolated membranes from Phase III cells remained partially activated. However, the ability of isoproterenol to further activate the enzyme in isolated membranes did not correlate with the observation that intact washed Phase III cells were refractory to a second stimulation with isoproterenol (Fig. 2).

Since intracellular [cAMP] is a function of both synthesis and degradation, adenylate cyclase activity can be determined in the intact cell by measuring cAMP accumulation after inhibition of phosphodiesterase. To accomplish this the phosphosphodiesterase inhibitor Ro 20-1724 (Sheppard & Wiggan, 1970) was used. The approximate rate of cAMP production was calculated as the amount of cAMP produced per minute during the linear portion of the incubation. In

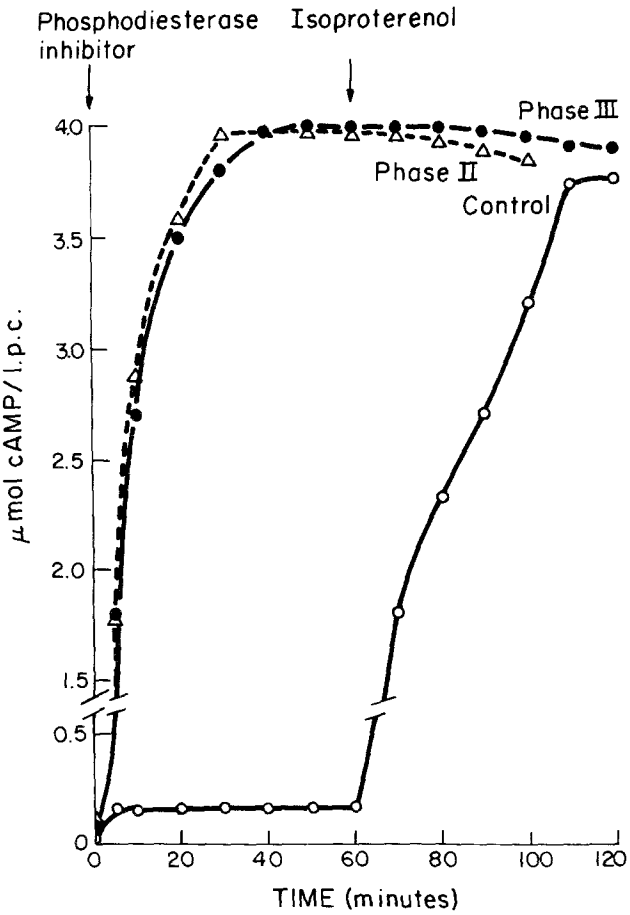

Fig. 3. Adenylate cyclase activity in intact control (○—○), Phase II (△---△), and Phase III (●—●) cells. Washed rat erythrocytes were incubated at a 40% packed cell volume. Isoproterenol (10 μM) was added and the incubation allowed to continue. After 10 min (Phase II) and 60 min (Phase III) aliquots of cells were washed 3 times in buffer and resuspended at a 40% packed cell volume. Phosphodiesterase inhibitor (Ro 20–10 μM) was added and at the times indicated 100-μliter aliquots of media were removed for cyclic AMP determination. Note difference in scale from previous figures

this calculation the amount of cAMP produced before the cells attained a linear cAMP production rate was not considered. The addition of 10 μM Ro 20-1724 to control cells caused the cAMP to rise at the rate of approximately 20 nmol/liter packed cells/min (Fig. 3), indicating that the adenylate cyclase is not very active in the unstimulated state. A new steady state was reached within 10 min after addition of inhibitor, indicating that not all the phosphodiesterase was inhibited. The addition of 10 μM isoproterenol to these Ro 20-1724-treated cells increased cAMP production to approximately 200 nmol/liter packed cells/min. After about 20-min exposure to isoproterenol [cAMP] reached a steady-state level 18-fold higher than before hormone addition.

The [cAMP] in washed Phase III cells was about fourfold higher than in control cells before Ro 20-1724 addition. Phosphodiesterase inhibition of Phase III cells produced an immediate increase in [cAMP] to levels about 20-fold higher than zero time. The intracellular [cAMP] reached a new steady state about 20 min after the addition of inhibitor. Addition of isoproterenol to phosphodiesterase-inhibited Phase III cells did not induce any further increase in cAMP production.

An identical procedure was carried out using washed Phase II cells (Fig. 3). Addition of 10 μM Ro 20-1724 caused the intracellular cAMP to rise rapidly. After about 20 min a new steady-state level 65-fold higher than basal was reached. The readdition of isoproterenol to phosphodiesterase-inhibited Phase II cells did not elicit any further increase in cAMP. The large, rapid rise in [cAMP] in Phase II cells was particularly surprising since, in the nonphosphodiesterase-inhibited Phase II cells, cAMP levels are near basal or pretreatment values (compare with Fig. 1). It is noteworthy that the initial rate of increase in [cAMP] was similar in Phase II and Phase III cells, approximately 170 nmol/liter packed cells/min.

Phosphodiesterase

The results of experiments exploying the phosphodiesterase inhibitor in intact cells suggested that phosphodiesterase is the major regulator of cAMP levels once the rat erythrocyte is hormonally activated. Consequently, both soluble and particulate enzymes were examined for alterations in activity. Soluble and membrane-bound phosphodiesterase was prepared by hypotonic lysis of control, Phase II, and Phase III erythrocytes. The cells were lysed by a 1:100 dilution into 5 mM sodium phosphate, pH 8.0, and the membranes were prepared by centrifugation at 27,000 × *g* for 30 min at 4 °C. The membrane pellet was resuspended in buffer and the kinetic behavior of the enzyme analyzed. The K_m and V_{max} were determined using linear regression analysis of Lineweaver-Burke plots. Substrate concentrations from 0.01 to 500 μM were employed (Table 1). The K_m of the soluble enzyme from the control cells was 1.33 ± 0.07 μM, a value similar to that reported from other laboratories (Sheppard & Wiggan, 1970; Patterson, Hardman & Sutherland, 1976). No change in this kinetic parameter is observed when enzyme from Phase II or Phase III cells is studied. Additionally the V_{max} of the soluble phosphodiesterase is not altered by hromone treatment, nor was it altered by direct *in vitro* addition of CDR and/or calcium.

The Lineweaver-Burke plots of the data obtained when the membrane-bound phosphodiesterase (MPDE) is examined are shown in Fig. 4 and the

Table 1. Kinetic parameters determined for the soluble and membrane-bound phosphodiesterase activity in control, Phase II and Phase III rat erythrocytes[a]

	Control	Phase II	Phase III
	Soluble phosphodiesterase		
K_m (μM)	1.33±0.07	1.33±0.03	1.30±0.06
V_{max} (nmol/10 min/mg prot)	4.65±0.07	4.72±0.05	4.77±0.03
	Membrane-bound phosphodiesterase		
K_m (μM)	2.48±0.11	6.43±0.11	5.85±0.08
V_{max} (nmol/10 min/mg prot)	4.27±0.03	12.52±0.08	9.80±0.04

[a] The results presented are the mean ± SEM of three experiments

kinetic parameters derived from these plots are shown in Table 1. In Phase II and Phase III both the K_m and the V_{max} of MPDE are increased. in each of three separate experiments total phosphodiesterase activity in the lysate increased (Phase II–47.0±6.5% over control and Phase III–21.5±4.7%). This increase in phosphodiesterase activity is due to an activation of MPDE and not a redistribution of soluble phosphodiesterase since the soluble activity did not change after hormone treatment. MPDE represents 15.7±0.7, 41.2±0.7 and 30.0±0.6% of the total phosphodiesterase activity in control, Phase II and Phase III cells, respectively, when the fraction of total phosphodiesterase activity contributed by MPDE activity is calculated by subtracting soluble activity from total activity present in the cell lysate and dividing this number by total activity. The observed effect of isoproterenol on MPDE required intact cells, since direct addition of hormone to isolated control membranes did not alter MPDE activity.

Mechanism of MPDE Activation

As shown in Fig. 1 calcium depletion of intact cells alters the triphasic response to chronic isoproterenol exposure. A series of experiments was then carried out to determine the role of intracellular calcium in

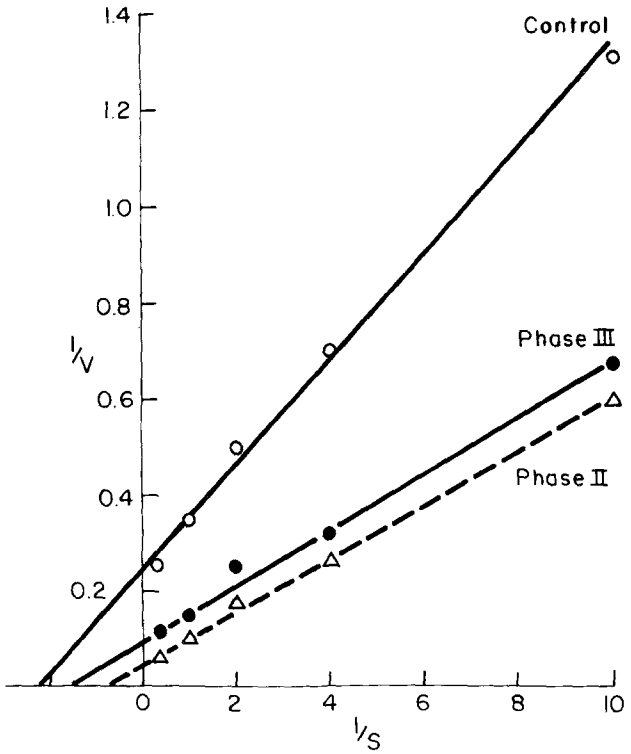

Fig. 4. Lineweaver-Burke plots of phosphodiesterase activity in membranes prepared from control (○—○), Phase II (△---△), and Phase III (●—●) cells. After appropriate incubation with isoproterenol (10 μM) membranes were prepared and phosphodiesterase activity determined in the presence of 0.01 to 500 μM cyclic AMP

the activation of MPDE during chronic isoproterenol exposure (Table 2). MPDE was assayed at three substrate concentrations. At each substrate concentration Phase III MPDE activity is increased as compared to control MPDE activity. This activity can also be increased by raising intracellular calcium in intact control cells by incubating cells with A23187. Conversely, depletion of intracellular calcium prevents the activation of MPDE brought about by chronic (1 hr) isoproterenol treatment.

In many tissues phosphodiesterase is activated by a heat-stable calcium-dependent regulator protein,

Table 2. Membrane-bound phosphodiesterase activity in control, Phase III, ionophore-treated control, calcium-depleted control and calcium-depleted isoproterenol-treated rat erythrocytes[a]

Substrate concentration (μM cAMP)	Enzyme activity (pmol/10 min/mg prot)				
	Control	Phase III	Ionophore-control	Calcium-depleted control	Calcium-depleted isoproterenol-treated
50	187.0±8.5	308.3±4.4	284.0±3.5	183.0±1.7	177.3±2.7
10	88.7±4.7	137.3±2.3	133.0±2.1	86.3±2.4	88.0±0.2
1	13.7±0.9	24.7±0.3	22.0±0.6	12.0±0.6	10.6±0.3

[a] The results presented are the mean ± SEM of three separate experiments

Table 3. Effect of Exogenous CDR addition upon the kinetic parameters of the membrane-bound phosphodiesterase in control, Phase II and Phase III rat erythrocytes[a]

	K_D (μM)	V_{max} (nmol/10 min/mg prot)
Control	2.48±0.11	4.27±0.03
Control+CDR	5.65±0.45	29.94±0.18
Phase II	6.43±0.11	12.52±0.08
Phase II+CDR	11.80±0.10	31.10±0.13
Phase III	5.25±0.08	9.80±0.03
Phase III+CDR	11.45±0.25	31.17±0.10

[a] The results presented are the mean±SEM of three separate experiments

calmodulin (Wells & Hardman, 1977). Experiments were undertaken to assess the role of this protein in MPDE activating during the triphasic response. A kinetic examination of the effect of purified bovine brain CDR showed that addition of maximal amounts of CDR increased both the V_{max} and the K_m of the phosphodiesterase in membranes isolated from control, Phase II, or Phase III cells (Table 3).

The ability of exogenous CDR to activate MPDE suggested that redistribution of intracellulr CDR might be part of the mechanism of MPDE activation in the cell. This hypothesis was tested in two separate experiments. First, the amount of an exogenous CDR preparation necessary to half-maximally activate MPDE prepared from control, Phase II, and Phase III cells was compared under standardized assay conditions (Fig. 5). Control membranes, required 3.7 μg; Phase II membranes 1.2 μg; and Phase III membranes 2.0 μg of protein, respectively. In all cases saturating amounts of the CDR preparation activated MPDE to the same maximal activity. In the second

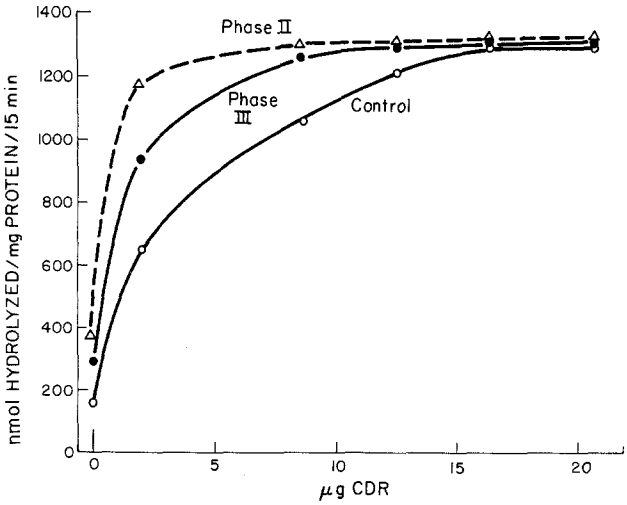

Fig. 5. The effect of increasing amounts of human erythrocyte CDR on MPDE activity in membranes prepared from control (○——○), Phase II (△---△) and Phase III (●——●) cells. Cells were initially incubated with isoproterenol (10 μM) and membranes prepared as described in Materials and Methods. Various amounts of CDR were added to 100-μliter suspensions of membranes and phosphodiesterase activity was determined using 5 μM cyclic AMP as substrate

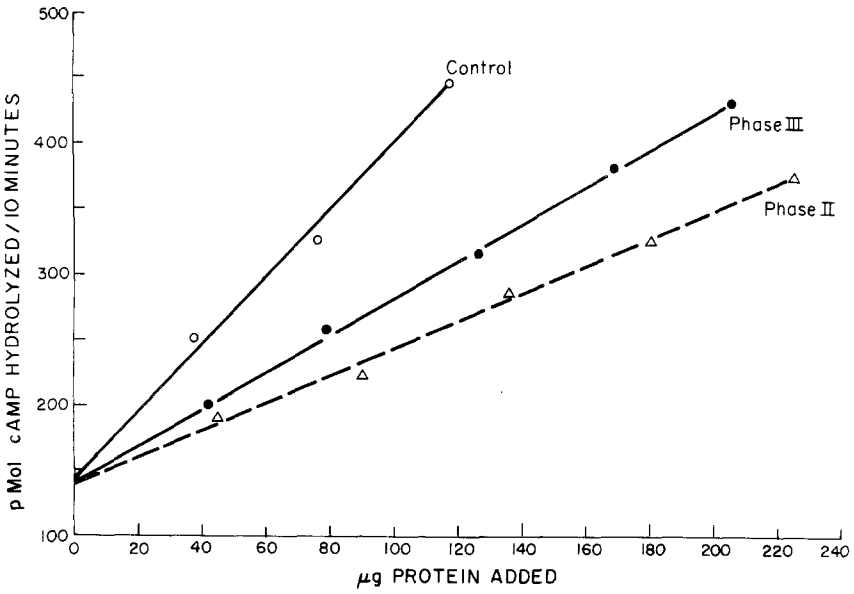

Fig. 6. The effect of increasing amounts of heat-treated rat erythrocyte cytoplasm derived from control (○——○), Phase II (△---△) and Phase III (●——●) on the activation of CDR-free bovine brain phosphodiesterase. Washed control, Phase II and Phase III erythrocytes were lysed in ice-cold 5 mM sodium phosphate, pH 8.0. After centrifugation (27,000× *g*, 30 min) an aliquot of the resulting supernatant was treated for 3 min in boiling water, colled and then centrifuged. Increasing amounts of the heat-treated boviene brain phosphodiesterase and enzyme activity determined using 25 μM cyclic AMP as substrate. Protein added was determined based on the hemoglobin content of the lysate prior to heat treatment

experiment the amount of soluble CDR activity remaining in the rat erythrocyte cytoplasm after the various phases of isoproterenol stimulation was determined. Washed control, Phase II, and Phase III cells were lysed by a 1:100 dilution in 5 mM sodium phosphate buffer, pH 8.0. The membranes were removed by centrifugation. An aliquot of each supernatant was boiled for 3 min, cooled on ice, and centrifuged. The amount of CDR activity in the clear, heat-treated supernatant was determined by measuring the ability of comparable aliquots to activate CDR-free bovine brain phosphodiesterase. The activation produced by increasing amounts of supernatant prepared from the three cell types is depicted in Fig. 6. The activity was greater in control cells and least in Phase II cells. The results of these two experiments suggest that following isoproterenol treatment there is a translocation of CDR from the cytoplasm to the membrane.

The association of CDR with the plasma membrane has also been shown to enhance Ca^{++} ATPase activity in the human erythrocyte membrane (Bond & Clough, 1973; Gospinath & Vincenzi, 1977; Jarrett & Penniston, 1977, 1978). This ATPase activity has assayed in control, Phase II and Phase III cells. As shown in Fig. 7 Ca^{++} ATPase activity was identical in control and Phase II membranes. Phase III membranes, however, had twice the Ca^{++} ATPase found either in control or Phase II membranes.

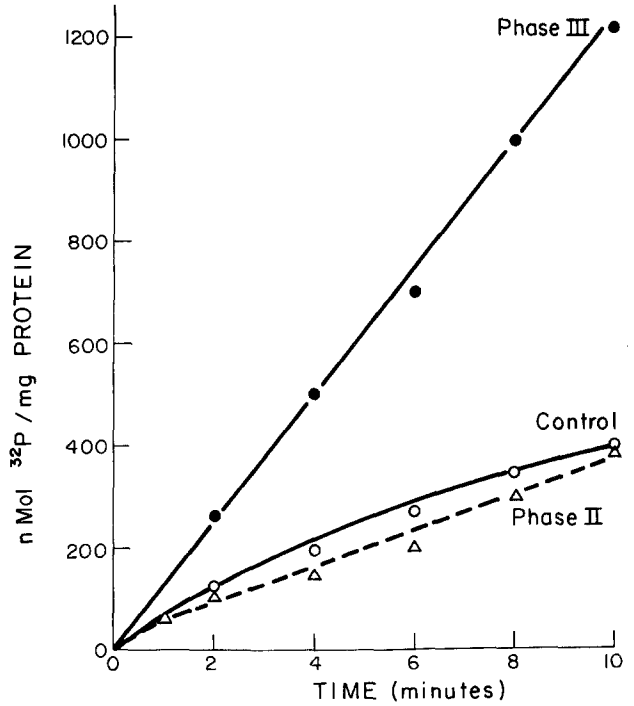

Fig. 7. Ca^{++} ATPase activity in membranes prepared from control (○—○), Phase II (△---△) and Phase III cells (●—●). Washed rat erythrocytes were incubated with isoproterenol for 10 (Phase II) or 60 min (Phase III). Membranes were then prepared and Ca^{++} ATPase assayed as described in Materials and Methods

Discussion

During chronic β-adrenergic stimulation changes in cellular cAMP content are observed. It is assumed that these changes reflect alterations in the activity of catecholamine-specific components of the adenylate cyclase system. These alterations are thought to result from a decrease in receptor number and/or the coupling efficiency of the β-adrenergic receptor to the adenylate cyclase. However, most studies have failed to examine the possibility that catecholamine-induced changes in cAMP degradation resulting from alterations in the activity of MPDE may also be an important component of the intracellular regulation of cAMP concentration during chronic hormone exposure. Our results indicate that MPDE activity is a dominant factor regulating cAMP content in the hormone-activated rat erythrocyte. During continuous stimulation with isoproterenol, the [cAMP] in this cell rises acutely (Phase I), declines (Phase II), and then rises for a second time, reaching a new steady-state level after 60 min (Phase III) (Fig. 1). Phases I and II are comparable to the pattern of intracellular cAMP accumulation observed in other cells during chronic β-adrenergic stimulation. However, the second sustained rise in [cAMP] during Phase III has not been described in other systems.

In order to identify the mechanism(s) regulating the triphasic response, both adenylate cyclase and phosphodiesterase activities were assayed. We found that the use of isolated membranes to measure adenylate cyclase activity was unsatisfactory in this system. Membranes isolated from Phase III cells were stimulated by the addition of isoproterenol, whereas washed intact Phase III cells were refractory to a second challenge with isoproterenol (Fig. 2). However, this apparent difference could be due to alterations in the respective membrane-bound cyclases during membrane isolation. Thus, a procedure was developed to assay the enzyme in the intact cell.

Adenylate cyclase activity was measured in intact cells by inhibiting phosphodiesterase activity with Ro 20-1724. Using this technique the adenylate cyclase was assayed in its physiological environment so that alterations induced by membrane preparation were avoided. In the absence of isoproterenol, phosphodiesterase inhibition caused the cAMP content to rise about fourfold. A new steady state was reached within 10 min, indicating that phosphodiesterase inhibition was incomplete. There is an apparent lag in the production of cAMP after Ro 20-1724 addition. We attribute this lag to the fact that the uptake and inhibitory effect of Ro 20-1724 are not immediate. When these Ro 20-1724 pretreated cells were challeneged with isoproterenol, the rate of cAMP production in-

creased immediately and reached a new steady state within 15–20 min at levels 70-fold greater than basal (Fig. 3). The magnitude of this increase is surprising since in the absence of Ro 20-1724, isoproterenol elicits only a three- to fourfold increase in cAMP content. Thus, there is an enormous capacity for both the synthesis and degradation of cAMP in the rat erythrocyte. When Ro 20-1724 was added to washed, intact Phase II or III cells, the cAMP content rose and reached a new steady state within 15–20 min. Further addition of isoproterenol did not cause a further increase in cAMP production in Ro 20-1724-treated Phase II or III cells (Fig. 4). It should be noted that the triphasic response observed in the primary response to isorpoterenol (Fig. 1) is abolished by the inclusion of the phosphodiesterase inhibitor Ro 20-1724.

Three important conclusions arise from these data. First, the adenylate cyclase is activated to the same extent in Phase I, Phase II, and Phase III cells. The rate of cAMP production in phosphodiesterase-inhibited Phase II and III cells was approximately the same as in Ro 20-1724-pretreated control cells challenged with isoproterenol (Phase I). Second, the activated cyclase in washed intact Phase II and III cells does not require receptor occupation by isoproterenol as shown by the fact that addition of Ro 20-1724 to extensively washed Phase II or III cells results in an immediate rapid increase in cyclic AMP accumulation and readdition of isoproterenol to these Phase II or III cells results in no further increase in cyclic AMP (Fig. 3). This state resembles the "persistently activated" cyclase observed in isolated membranes when nonhydrolyzable GTP analogs are substituted for GTP (Bilezikian & Aurbach, 1974; Pfeuffer & Helmreich, 1975; Lefkowitz, Mullikin & Caron, 1976). Third, phosphodiesterase activity is a major determinant of cAMP content in hormone-activated rat erythrocytes. Inclusion of Ro 20-1724 caused a very large rise in cAMP content in the isoproterenol-stimulated erythrocyte, much greater than the three- to fourfold increase seen in the absence of inhibitor. These results indicate that the dominant factor determining cAMP content during the triphasic response, once the cell has been activated by hormone, is the activity of the phosphodiesterase and not that of the adenylate cyclase.

When phosphodiesterase activity was measured in lysates from control, Phase II, and Phase III cells, it was found that there was more total activity in lysates from hormone-treated cells than from control cells. Subsequent analysis of particulate and soluble activities demonstrated that isoproterenol stimulation of intact cells increased the activity of the membrane bound, but not the soluble enzyme (Table 1). MPDE activity represented 40% of the total activity in Phase II cells and 30% in Phase III cells. In unstimulated control cells only 15% of the total activity was contributed by the particulate enzyme. The increase in MPDE activity in Phase II and III was not due to phosphodiesterase redistribution since the soluble activity was identical in all three preparations. Furthermore, isoproterenol-induced MPDE activation required the intact cell. Addition of isoproterenol directly to isolated membranes did not alter MPDE activity (data not shown). Kinetic analysis of MPDE activity in membranes from Phase II and III cells showed that the V_{max} was elevated threefold in Phase II and 2.2-fold in Phase III relative to control (Table 1). There was also an increase in the K_m of the enzyme in both these phases.

There have been several reports of changes in the activity of the particulate phosphodiesterase in response to hormones or to elevated substrate levels in intact cells. In some cultured cell lines the increase in activity is due to the synthesis of new enzyme molecules (Bourne, Tompkins & Dion, 1973; Schwartz & Passonneau, 1974). In the adipocyte insulin activation of a particulate phosphodiesterase is independent of protein synthesis (Manganiello & Vaughan, 1973; Zinman & Hollenberg, 1974; Correje, Auclair & Nunej, 1976). Although the mechanism of activation has not been identified as yet, it appears that CDR is not involved.

Exogenous CDR did not activate the soluble enzyme from these rat cells, but did activate the membrane-bound phosphodiesterase. Saturating concentrations of a CDR preparation produce a sevenfold increase in the V_{max} and a twofold increase in the K_m of the enzyme (Table 3). The mechanism for the observed CDR-mediated alteration of the MPDE is not clear. Most studies have shown that CDR lowers the K_m of the phosphodiesterase for substrate (Wolff & Brostrom, 1979), and it is not clear why in the present study CDR increases the K_m of the enzyme for cyclic AMP. To our knowledge this is the first demonstration that CDR activates a particulate phosphodiesterase localized in the plasma membrane. Teshima and Kakiuchi (1976) found that CDR stimulated cyclic nucleotide hydrolysis in membranes from rat brain, but these fractions were enriched for mitochondrial and microsomal marker enzymes. It has been reported (Sheppard & Tsien, 1975) that calcium activates the soluble phosphodiesterase in the rat erythrocyte. This activation is thought to involve some component associated with the membrane. Consequently, it is possible that CDR activates this factor rather than the phosphodiesterase directly.

The observation that exogenous CDR could stimulate MPDE activity in membranes does not establish

that this is the mechanism of MPDE activation *in situ*. However, the results of two experiments stronlgy support this hypothesis. First, the amount of an exogenous CDR preparation needed to half-maximally activate MPDE was 1.2 μg in Phase II membranes, 2.0 μg in Phase III membranes, and 3.7 μg in control membranes (Fig. 5). Second, the apparent activity of cytosolic CDR was inversely related to the extent of MPDE activation (Fig. 6). These data thus suggest that isoproterenol causes a translocation of CDR from the cytoplasm to the membrane.

There have been two reports that cAMP promotes CDR redistribution. Uzunov, Revuelta and Costa (1975) reported that reserpine-stimulated cAMP production preceded an increase in cytosolic CDR in the adrenal medulla. Gnegy, Uzunov and Costa (1976) found that cAMP-dependent phosphorylation of membranes from rat brain or adrenal medulla caused the release of membrane-bound CDR. Neither group was able to demonstrate that the plasma membrane was involved in the redistribution of CDR. In the present study, however, a role for the plasma membrane in the redistribution of intracellular CDR is clearly indicated. Elevated cAMP and/or the direct interaction of hormone with receptor causes the CDR to associate with the plasma membrane.

The results of experiments using calcium-depleted cells also support the hypothesis that CDR is involved in the regulation of MPDE activity in the intact cell. Calcium-depleted cells no longer exhibited Phase II during a continuous stimulation with isoproterenol (Fig. 1). Moreover, isoproterenol treatment did not elevate MPDE activity in calcium-depleted cells, indicating that increased [cAMP] alone is insufficient to activate MPDE (Table 2).

In membranes isolated from Phase III cells the activity of the Ca^{++} ATPase was twice that in membranes from control or Phase II cells (Fig. 7). Although the *in situ* mechanism of Ca^{++} ATPase activation was not identified in these studies, it was found that the enzyme was activated by CDR, in agreement with reports from other groups (Bond & Clough, 1973; Gospinath & Vincenzi, 1977; Jarrett & Penniston, 1977, 1978). What is of particular interest is the finding that the Ca^{++} ATPase activity was increased in Phase III but not in Phase II cells whereas the MPDE was increased in membranes of cells in both phases. Thus, even though the activities of both membrane-bound enzymes appears to be brought about by a CDR-dependent process, the time course of their respective activations is different. It is noteworthy that Steer and Levitski (1975) have reported that isoproterenol addition to the membrane of turkey erythrocytes leads to a release of membrane calcium. If this occurs in the red cell it would suggest a possible mechanism for the initial activation of MPDE by the hormone.

These data suggest the following model. The binding of isoproterenol to its receptor results in the release of membrane-bound calcium and the activation of adenylate cyclase (Phase I). The activation of the cyclase leads to the rapid increase in cAMP content of the cell (Phase 1). The increase in cytosolic calcium allows the association of calcium with cytosolic CDR. This activated CDR then binds to MPDE, leading to an increase in rate of cAMP hydrolysis and hence a fall in [cAMP] (Phase II). Activation of the Ca^{++} ATPase by mechanisms as yet unidentified, but probably also involving CDR and occurring more slowly, leads to a lowering of the cytosolic calcium concentration. This causes, in turn, the dissociation of a portion of the membrane-bound CDR from MPDE leading to a decrease in rate of cAMP hydrolysis and the second rise in cAMP content (Phase III) to a new steady-state level determined by the combined activities of the adenylate cyclase and the phosphodiesterase.

From the point of view of our understanding of cellular control mechanisms these data indicate that even in the simplest of cellular systems displaying a hormone-sensitive adenylate cyclase, there is an intimate relationship between calcium and its receptor protein, calmodulin, and the metabolism of cAMP.

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