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cAMP-Dependent Protein Kinase Responses of S49 Cells Are Reduced by Growth in Low Epinephrine Concentrations

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SUMMARY

S49 wild-type mouse lymphoma cells grown in 3 nM epinephrine are extensively desensitized. Cellular cAMP responses to subsequent challenges with 100 nM epinephrine are reduced by as much as 80–90%. In this report, we document that protein kinase activity ratios were also attenuated. For example, the activity ratios in naive cells were increased from 0.26 ± 0.02 to 0.72 ± 0.04 by incubation with 100 nM epinephrine for 2 min, whereas in cells grown in 3 nM epinephrine for 24 hr before the experiment they were 0.19 ± 0.02 and 0.29 ± 0.03. Attenuated protein kinase activity ratios were obvious at epinephrine challenge concentrations ranging from 10 to 1000 nM. Three kinds of experiments provided evidence that the reduced ratios in desensitized cells were secondary to diminished cAMP responses rather than to changes in the cAMP-dependent protein kinase itself. Firstly, when protein kinase activity ratios were plotted against cAMP levels in naive and desensitized cells, the points fell along a common line. Secondly, cell-free cAMP-dependent protein kinase preparations from naive or epinephrine-treated cells had similar activities in the presence of maximal exogenous cAMP and similar half-maximal cAMP concentrations. Finally, the levels of cAMP-binding proteins in extracts from naive and desensitized cells were essentially identical. We conclude that desensitization of S49 cells by very low levels of epinephrine significantly reduced cAMP-dependent protein kinase responses to much higher concentrations of the catecholamine.

Much has been learned about the process of desensitization as it affects cellular cAMP levels (1). However, there have been only a few studies on how the resulting attenuated cAMP levels affect cAPK and its substrates. Indeed, it seems unlikely that such effects could be seen in most of the systems used to study desensitization. The concentrations of agonist used were so high that, even though cellular cAMP might be much reduced, it would remain well above the level producing complete activation of the responding systems (2). This narrow range over which cAMP is rate limiting is noted almost everywhere. Even the activation of the cAPK in cell-free systems is limited to less than 1 order of magnitude of cAMP concentrations (2). Thus, there are examples of very significant desensitization at the level of cAMP accumulation with no change in the rate of the physiological processes instigated by it. For instance, the adenylate cyclase of lipocytes desensitizes very rapidly to epinephrine stimulation (3). However, even when cAMP levels have fallen far below the peak accumulation, maximal production and release of glycerol continues because the hormone-sensitive triglyceride lipase is fully activated. It follows then that merely a demonstration of desensitization at the level of cAMP accumulation cannot prove desensitization at the level of the cAPK or beyond.

We have reported that growth of S49 cells in 3 nM epinephrine for 24 hr produced profound attenuation of celluar cAMP responses to subsequent challenge with 100 nM epinephrine (4). This LTD was marked by decreased adenylate cyclase responses to epinephrine and increased cellular cAMP destruction. If LTD caused reduced responses to epinephrine challenges at the level of the cAPK, it might follow that even circulating levels of catecholamine could desensitize tissues in physiological responses. Additionally, even a straightforward demonstration of desensitization at the cAPK level would leave open the question of its exact origin. At a given epinephrine challenge concentration, desensitization might be manifest because of the reduced cAMP accumulation or it might be the result of a change in the quantity or status of the kinase itself.

A priori, we have no way of knowing which of the two above mechanisms is likely to be quantitatvely more important. Steinberg and Agard (5) reported that activation of protein kinase in S49 cells for extended periods of time led to decreased protein kinase activity. This was attributed to increased net degradation of both the regulatory and catalytic subunits in cells in which the cAPK was activated. A similar phenomenon has also been observed in Leydig cells (6). In addition, it has been shown

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ABBREVIATIONS: cAPK, cAMP-dependent protein kinase; LTD, long term desensitization; PKAR, protein kinase activity ratio; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SAD, sinoaortic denervation; MES, 2-[*N*-morpholino]ethanesulfonic acid; PGE_1, prostaglandin E_1.

that the level of phosphorylation of regulatory subunits differs in the kin⁻ S49 mutant from that found in wild-type cells. Clearly, 24 hr of treatment with 3 nM epinephrine might cause a change in the level of phosphorylation of the cAPK and, hence, a change in its activation parameters.

In the present work, we demonstrate that the PKAR is significantly attenuated during subsequent challenge as the result of treatment with 3 nM epinephrine for 24 hr. This attenuation was due not to changes in the protein kinase but was the result of desensitization at the level of cAMP.

Materials and Methods

Cell cultures. Wild-type S49 cells, clone 22, were grown in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, supplemented with 5% horse serum and antibiotics (7). The cultures, grown in roller bottles, were treated with epinephrine (Sigma Chemical Co.) or the carrier solution (1 mM thiourea, 0.1 mM ascorbate), as previously reported (4).

Sample preparation. During the experimental protocols, samples of the cell suspensions were removed and the cells were collected by centrifugation. The supernatant was removed by aspiration and the pellet was frozen immediately in liquid nitrogen. The cell pellet (containing 20×10^6 cells) was thawed in 1 ml of ice-cold homogenization buffer, which contained 50 mM Tris·HCl (pH 7.4), 250 mM sucrose, 3 mM $MgCl_2$, 4 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was refrozen in liquid nitrogen and thawed again. Microscopic examination of stained samples revealed over 90% cell lysis by this method. The cell lysate was centrifuged in a microcentrifuge for 25 sec at 14,000 rpm, and the cytoplasmic supernatant was immediately assayed for cAPK activity. Protein concentration was determined by the method of Lowry *et al.* (8).

Protein kinase activity. cAPK activity was assayed in a total volume of 50 μl containing 50 mM sodium acetate (pH 6.5), 10 mM $MgSO_4$, and 10 mM dithiothreitol, as previously described (9). Included in this incubation was 0.1 mM kemptide (purchased from Sigma) and 50 μM [γ-^{32}P]ATP (400,000 dpm, 150 dpm/pmol; purchased from ICN Radiochemicals). The reaction was initiated with the addition of 10 μl (25 μg of lysate protein) from the cytoplasmic fraction of the lysate and was incubated at 30° for 10 min. A 35-μl aliquot of the reaction mixture was spotted onto cellulose phosphate paper (Whatman P81) and washed three times in deionized water (10). After the filter paper was dried, it was placed in a scintillation vial with 10 ml of Betamax (Westchem) and counted in a Beckman scintillation counter.

The cAPK activity was measured in the presence and absence of 0.5 μM cAMP, to give a measurement of total activity and endogenously activated protein kinase activity, respectively. The PKAR was defined as the ratio of the activity in the absence of exogenously added cAMP to that in the presence of added cAMP (11).

Inhibition of cAPK activity with wiptide. Wiptide (Penninsula Laboratories), an 18-amino acid peptide inhibitor of cAPK, was dissolved in 10 mM MES (pH 6.8) containing 0.5 mg of bovine serum albumin, as reported (12). Cell lysates were mixed with wiptide and incubated on ice for 15 min. An aliquot of this lysate (corresponding to 25 μg of lysate protein) was assayed for protein kinase activity, as described above.

cAMP-binding protein assay. Cyclic AMP-binding proteins (regulatory subunits of cAPK) were assayed in the cytoplasmic fractions, as described (13). Assays were done in a final volume of 100 μl containing 50 mM sodium acetate, pH 4.0, 1 mM 2-mercaptoethanol, 50 μg of bovine serum albumin, and 1 μM cyclic [^{3}H]AMP (37 Ci/mmol, 20,000 dpm/pmol), and the reactions were initiated with the addition of the cytoplasmic fraction. Reactions were incubated on ice for 1 hr and then were diluted with 3 ml of 20 mM potassium phosphate buffer, pH 6.0 (14). Samples were filtered through Millipore filters (0.45 μm) and washed with phosphate buffer before the radioactivity on the filters was determined. Under the conditions of this assay, full occupancy of both cAMP sites would not be expected (15). However, the amount of bound cAMP should be a good index of the relative quantity of regulatory subunit present.

Measurement of cAMP accumulation. Cells were washed in serum-free HEPES-buffered Dulbecco's modified Eagle's medium at a population density of 20×10^6 cells/ml. They were incubated for 60 min at 37° in medium containing 10 μCi/ml [2,8^{3}H]adenine (specific activity, 16 Ci/mmol). Excess adenine was removed by washing and then there was a further incubation at 37° with fresh buffer for 30 min. Hormones or other agents were added and cells were incubated for the indicated times. Aliquots were removed and the cells were collected by centrifugation. The medium was removed and the reaction was stopped by addition of ice-cold 5% trichloroacetic acid with [^{14}C]ATP and [^{14}C]cAMP to monitor recovery. The trichloroacetic acid extracts were cleared of precipitated protein by centrifugation and were fractionated on Dowex 50 and alumina columns, as described previously (7). The cAMP concentration was expressed as percentage of conversion, defined as:

$$\% \text{ of conversion} = \frac{[^3\text{H}]\text{cAMP}}{[^3\text{H}]\text{cAMP} + [^3\text{H}]\text{AXP}}$$

where AXP was the radioactivity in those fractions containing ATP and ADP.

Results

Effect of growth of S49 cells in 3 nM epinephrine on protein kinase activation in response to 100 nM epinephrine. Cells grown in the presence or absence of 3 nM epinephrine for at least 24 hr were harvested, as described in Materials and Methods, and exposed to the thiourea and ascorbate vehicle or to 100 nM epinephrine for 2 min. Aliquots of the cells were then prepared for measurement of PKARs. As shown in Fig. 1, naive cells responded to 100 nM epinephrine with a very large increase of PKAR, whereas 100 nM epinephrine had no significant effect on the PKAR of cells grown in the presence of 3 nM epinephrine. Thus, growth in the low concentrations of epinephrine caused a very significant attenuation of the protein kinase response to short term incubation with 100 nM epinephrine. Protein kinase activities and responses to epinephrine in cells grown without thiourea and ascorbate were virtually indistinguishable from those grown in their presence (data not shown). When cells that had been grown in 3 nM epinephrine

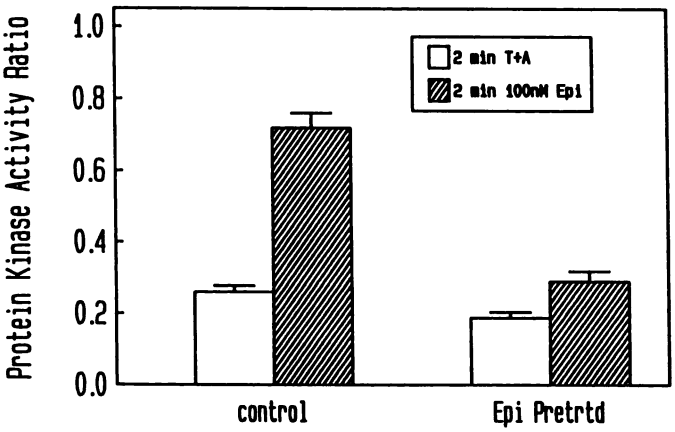

Fig. 1. Effect of pretreatment with 3 nM epinephrine on protein kinase activation in response to 100 nM epinephrine. The PKARs shown are in response to 2 min under basal conditions (□) or with 100 nM epinephrine (▨). The value of each *bar* is the mean and standard error of data from several experiments. Cells were pretreated >24 hr with 1 μM thiourea and 0.1 μM ascorbate (*control*) or with 3 nM epinephrine. (*Epi Pretrtd*).

were extensively washed in medium without the catecholamine and then grown for 1 day in the absence of the catecholamine, PKAR response to epinephrine (50 nM) were restored. We have previously reported partial restoration of S49 cAMP levels but were unable to maintain S49 cells so that cell division did not occur and so could not determine whether responses were regained through "resensitization" of the cAMP system or by the appearance of new cells.

Forskolin and PGE_1 also increased PKARs in S49 cells. Cells grown in 3 nM epinephrine had a PKAR of 0.20 in response to 30 nM PGE_1; the PKAR for control cells with the same PGE_1 treatment was 0.37. This was similar to the results reported previously when cAMP levels were measured (4). On the other hand, PKAR changes in response to 10 μM forskolin were not significantly altered by growth of the cells in 3 nM epinephrine.

Specificity of PKAR measurements. Over the years, considerable concern has been expressed about the accuracy of PKARs in reflecting what obtains in intact cells (16). We shared those reservations. However, some evidence suggesting that the methods employed here were valid was provided in experiments using wiptide, an inhibitor of the catalytic unit of the cAPK. Fig. 2 shows the effects of increasing concentrations of wiptide on the rates of ^{32}P incorporation measured in the standard assay. It may be observed from the data that 1) wiptide at concentrations from 1 to 10 μM had only minor effects on the activity of the kinase in unchallenged S49 cells and 2) at 10 μM wiptide the activities from all treatments were reduced to the same level. This argued that basal activity was not due solely to artifactual dissociation of the cAPK during the preparation of the extracts and that at least part of the basal values was derived from cAMP-independent kinases. Further, the figure shows that the large increases in kinase activity in response to the stimulatory agents were abolished by wiptide, suggesting that the assayed phosphorylation was carried out by cAPK.

Effect of varying challenge concentrations of epinephrine on the activation of cAPK in naive and desensitized S49 cells. Naive and desensitized S49 cells were challenged with concentrations of epinephrine ranging from 10 to 1000 nM for 2 min (Fig. 3). The PKARs ranged from 0.34 to 0.83 in the naive cells and from 0.26 to 0.41 in the desensitized cells. Intracellular cAMP levels in the same cells are presented in Fig. 3B. In the naive cells, the levels of cAMP were increased dramatically, in contrast to those in the 3 nM epinephrine-treated cells. Thus, there was a rough parallelism between the attenuated cAMP responses and the PKARs. In this and sone other experiments, it was observed that basal (i.e., unchallenged) PKAR was usually somewhat lower in the desensitized than in the naive cells. However, this difference was not consistent and may well have been due to assay variation. In any event, the apparently lower basal PKARs do not alter the significance of the changes induced by desensitization.

Effects of desensitization on the time courses of PKARs and cAMP levels in S49 cells. Untreated and 3 nM epinephrine-treated cells were challenged with 100 nM epinephrine, and the time courses of changes in intracellular cAMP levels and the activation of cAPKs were determined. Naive cells so challenged displayed a PKAR of 0.83 after 5 min of incubation. At longer times, the PKAR fell to about 0.6 and remained near that level throughout the 30 min of the experiment (Fig. 4A). In the desensitized cells, maximal activation of cAPK occurred at 2 min of treatment (PKAR = 0.38). Then the PKAR fell, reaching 0.16 by 30 min. Thus, during this 100 nM epinephrine challenge, the cAPK activation was significantly attenuated in desensitized cells. Accumulations of cAMP in these cells are shown in Fig. 4B. Just as with the PKARs, cAMP levels in 3 nM epinephrine-treated cells (as compared with naive cells) were much reduced. However, comparison of the protein kinase activation data with cAMP levels showed that, whereas the maximal activation of protein kinase occurred at 5 min in the naive cells, intracellular cAMP levels continued to increase throughout the experiment. We can only speculate that this anomalous decrease in the protein kinase activity may have been due to inactivation and/or degradation of free catalytic subunit molecules. Similar results were obtained when

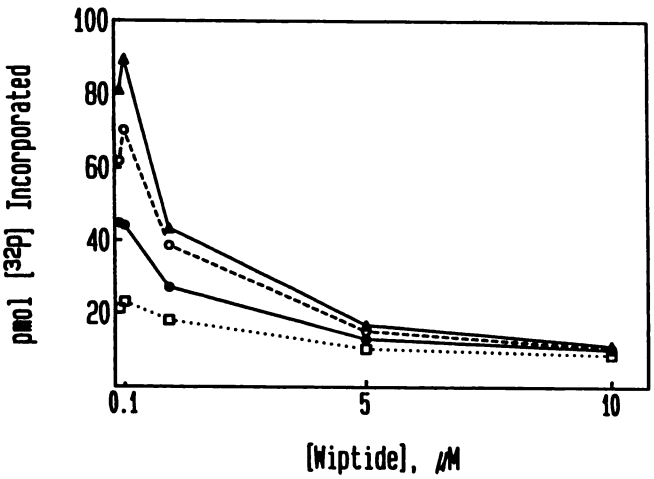

Fig. 2. Wiptide inhibition of cAPK activity. Wiptide was added to lysates 15 min before assay at the indicated concentrations. Lysates were assayed for cAPK activity, which had been activated by increased cAMP levels in response to 0.1 μM epinephrine (●), 1 μM epinephrine (○), 10 μM forskolin (▲), or basal conditions (□). The *Ordinate*, pmol of ^{32}P incorporated into the kemptide substrate.

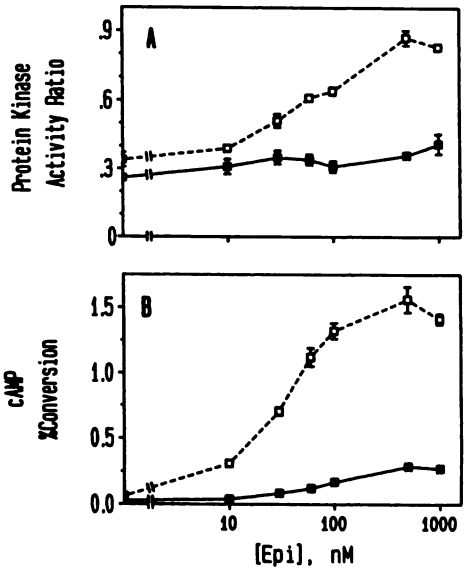

Fig. 3. Effect of pretreatment with 3 nM epinephrine on response to various epinephrine concentrations. Cells were pretreated with 3 nM epinephrine (■) or thiourea and ascorbate (□) for 4 days. Samples were challenged for 2 min at 37° with the indicated concentrations of epinephrine (*Epi*) and then assayed for cAPK activity (A) and cAMP (B). % *Conversion*, percentage of cellular ATP converted to cAMP.

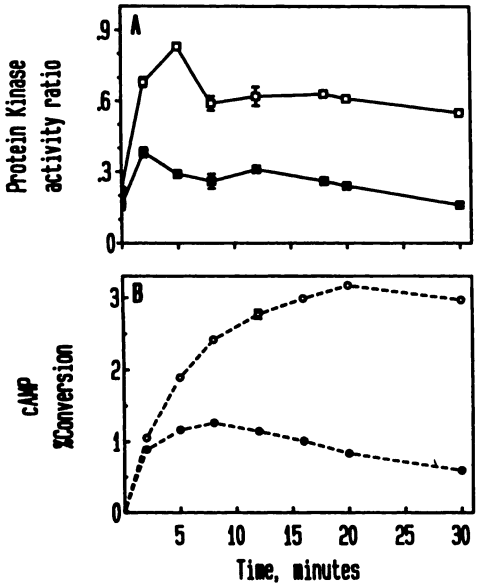

Fig. 4. Effect of pretreatment with 3 nM epinephrine on time course of response to 100 nM epinephrine. Cells were grown in the absence (*open symbols*) or presence (*closed symbols*) of 3 nM epinephrine for 2 days. A, Activation of cAPK in response to stimulation by 100 nM epinephrine; B, accumulated cAMP response. % *Conversion*, percentage of cellular ATP converted to cAMP.

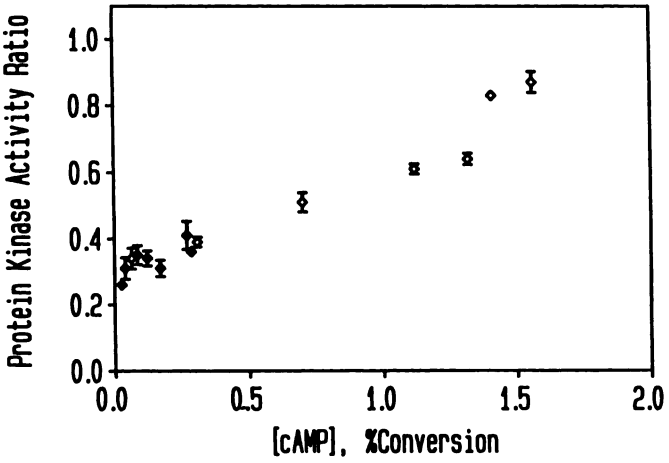

Fig. 5. Relationship of PKAR to cell cAMP. The cell cAMP concentration was altered by using various epinephrine concentrations and was measured as the percentage of cellular ATP converted to cAMP (% *Conversion*). The PKAR was determined for each epinephrine concentration and they are presented as the mean ± standard error. ◆, Data from cells pretreated with 3 nM epinephrine; ◇, data from control cells.

naive and desensitized cells were challenged with 30 nM epinephrine (data not shown).

Evidence that attenuated PKAR changes in desensitized cells are secondary to attenuation of cAMP responses. Indirect evidence in support of the hypothesis that growth in 3 nM epinephrine caused diminished PKAR through attenuation of cAMP responses rather than changes in the protein kinase per se is presented in Fig. 5. When PKARs and cAMP levels in cells from either desensitized or naive populations were plotted against one another, they were found to lie along a common line. Further, this comparison of cAMP levels and PKARs made possible estimates of the range of intracellular levels of cAMP required to produce activation of protein kinase in S49 wild-type cells. In these cells, 1% conversion of ATP corresponds to 20 μM cAMP (7). Thus, Fig. 5 shows that 16 μM cAMP is necessary for 50% activation of the kinase. More direct evidence for the primacy of the cAMP responses is presented in Fig. 6. Here we show that the activities of the protein kinases found in cell-free extracts incubated with and without exogenous cAMP were essentially identical. Further, the cAMP concentration-response patterns of these protein kinase activities in extracts from naive and desensitized cells were virtually indistinguishable (Fig. 7). In these cell-free systems, the half-maximal concentrations of cAMP appeared to be around 20 nM in both extracts. This figure was significantly lower than the half-maximal values found in intact cells and described above (16 μM). However, the concentration of cAPK in intact S49 cells has been estimated to be in the micromolar range (17). Because each cAPK binds four molecules of cAMP, it is not surprising that several micromolar cAMP might be required intracellularly for 50% activation. On the other hand, the measurements in the cell-free system were made at very low cAPK concentrations, and the system used in the cell-free

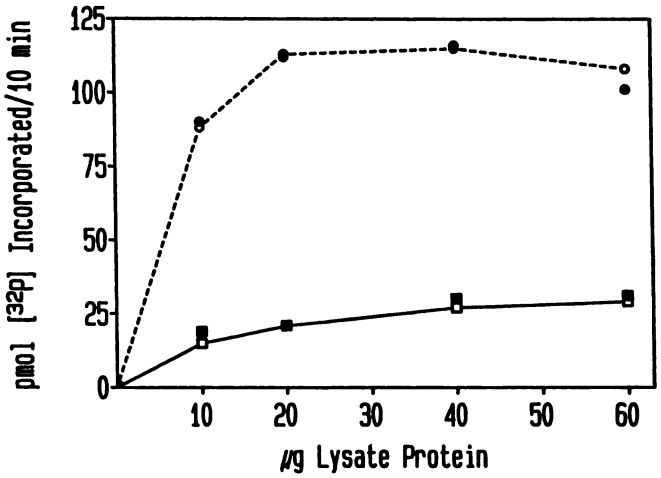

Fig. 6. Levels of cAPK. The protein kinase activity, measured as pmol of ^{32}P incorporated into substrate, was examined as a function of lysate protein. Protein kinase activity, both basal (*squares*) and with exogenous 0.5 μM cAMP (*circles*), is shown for lysates from cells pretreated without (*unfilled symbols*) and with (*filled symbols*) 3 nM epinephrine for 2 days.

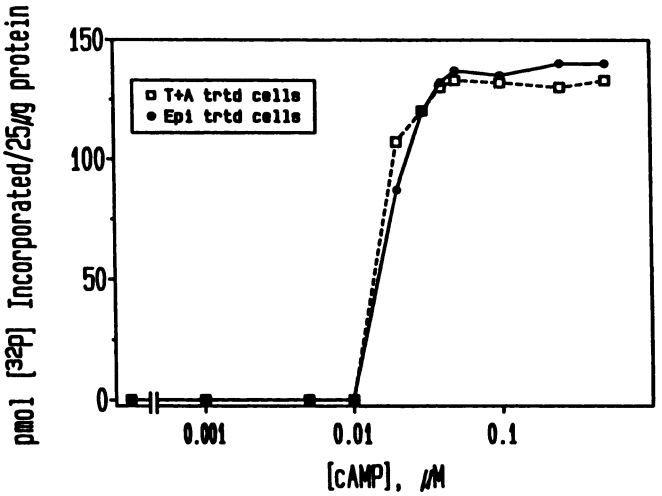

Fig. 7. Protein kinase activity in response to [cAMP]. Lysates from cells pretreated without (□) and with (●) 3 nM epinephrine for 2 days were assayed for protein kinase activity in response to exogenously added cAMP.

studies was optimized for sensitivity to cAMP. Thus, these values are not as unreasonable as they might seem. Finally, as shown in Fig. 8, the levels of cAMP-binding proteins in extracts from naive and desensitized cells were essentially identical.

Discussion

One matter of concern to us during these studies has been the fidelity with which the PKAR can measure the effects of desensitization on the activity of the cAPKs *in situ*. Corbin and his colleagues (11, 16) have provided an exhaustive list of potential pitfalls that can complicate the determination of the PKAR. We believe that we have controlled most if not all of these variables. More importantly, in the context of this report it was not essential that there be an exact quantitative identity between the immeasurable intracellular kinase ratio and the experimental PKAR but only that the PKAR reflect cellular cAPK activity. Obviously, it would be naive to assume that the allosteric effect of cAMP on the kinase in the intact cell would persist quantitatively throughout the assay. On the other hand, the reproducibility of our results (given that they were obtained using double-blinded protocols) provided significant levels of confidence that these PKAR determinations were valid *relative* measures of cellular cAPK activity. If one accepts that contention, then interpretation of the data becomes straightforward. That is, that the long term, or chronic, desensitization of cellular cAMP responses by physiological or near physiological levels of epinephrine has profound dampening effects on the responses of cellular cAPKs to challenges with higher concentrations of the catecholamine. Further, this reduced responsiveness of the kinases to epinephrine was attributable solely to attenuated cellular cAMP responses. Thus, we may conclude that the level of protein kinase activation required for the receptor desensitization, and produced by our 24-hr treatment with 3 nM epinephrine, is not sufficient to cause the protein kinase changes observed by Steinberg and Agard (5). In their experiments, Steinberg and Agard used 10 μM isoproteronol plus 50 μM 1-methyl-3-isobutylxanthine for up to 8 hr. These conditions caused a very significant reduction in the half-life for the cellular cAPK.

Is is generally assumed implicitly that desensitization is a biologically or physiologically useful feedback mechanism.

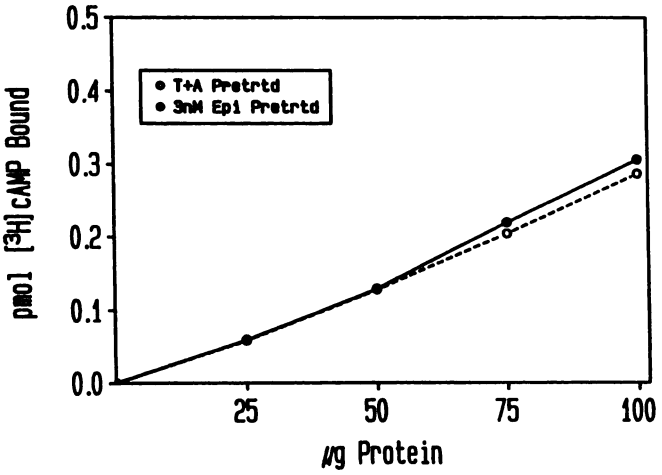

Fig. 8. cAMP-binding proteins in naive and desensitized cells. The binding of [^{3}H]cAMP by lysates prepared from cells pretreated without (○) and with (●) 3 nM epinephrine, with increasing amounts of lysate protein.

More specifically, it is assumed that it results in a modified response of the cell to the range of agonist concentrations to which it is normally subjected. In naive cells, we may assume that there is an exact set of correspondences between agonist concentration, cAMP accumulation, protein kinase activation, and phosphorylation of protein kinase substrates. From the physiological point of view, it is the state of these last proteins that is important. Thus, physiologically useful desensitization might in principle be attained by adjustment of the response at any stage of the agonist-cAMP-cAPK-phosphorylated protein cascade. In the present work, we have not investigated any part of the cascade beyond the protein kinase. However, we have shown that, as a result of LTD, there was no change in the properties or quantity of cAPK, the last common enzyme of whatever cAMP-dependent cascades exist within the cell.

Most desensitizing procedures described hitherto involve very extensive activation of adenylate cyclase and, hence, of protein kinase. The studies of Steinberg and Agard (5) suggest that in such cases the quantity of protein kinase in the cell should be reduced for some time after the desensitizing stimulation. Thus, we would hesitate to generalize these present findings to the processes where cells are treated with much greater concentrations of epinephrine. It will be interesting to see (if these drastic desensitizing treatments can be so applied) the degree to which desensitization at the physiological (as opposed to the adenylate cyclase) level depends on the desensitization of the cAPK system. Meanwhile, the demonstration in the present case that the cAPK is not involved greatly simplifies the task of understanding the state of cells under physiological conditions.

Studies by other groups have suggested that prolonged hormonal treatment can have effects manifested at the level of the cAPK. For example, Murray and Fletcher (18) ascribed the desensitization of Y-1 adrenal cells to adrenocorticotropic hormone (as measured by attenuated steroidogenic responses) to a modification of cAPK dissociation. Because they did not report measurements of cellular cAMP levels, that terminology might also be adequate for a mechanism similar to the one described here. Valet *et al.* (19) have made use of chronic SAD, which results in persistent elevated plasma catecholamine levels, to study desensitization *in vivo*. β_2 receptor levels were very significantly decreased in adipocyte membranes from SAD dogs, whereas β_1 receptor levels were unchanged. Interestingly, the lipolytic effects of epinephrine and other β agonists on adipocytes from SAD dogs were, relative to the controls, much reduced. The EC_{50} values were shifted rightward, and the maximal rates of glycerol production were much lower. Because the activation of the hormone-sensitive triglyceride lipase is believed to involve the cAPK (20, 21), it is tempting to speculate that attenuated kinase responses underlay the antilipolytic effect of the SAD procedure. Additional indirect evidence has been provided by Hoffman *et al.* (22), who have shown that in intact rats chronic infusion of phenylisopropyladenosine (an inhibitor of adenylate cyclase) led to increased cAMP responses and lipolysis in adipocytes prepared from such animals, as compared with controls. More recently, they have shown that the changes in the PKAR were in line with both the increased cellular cAMP and lipolytic responses (23).

This dampening of kinase activation by epinephrine could have profound physiological and pharmacological consequences on systems downstream in the cascade. Thus, the data pre-

sented here predict that chronic desensitization produced by epinephrine at physiological concentrations can and should have significant impact on subsequent cellular responses to hormonal challenges.

Acknowledgments

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