Vol. 106, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1982 Pages 298-304

INHIBITION OF PROTEIN SYNTHESIS ON HOMOLOGOUS AND HETEROLOGOUS DESENSITIZATION IN THE RAT ADIPOCYTE

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Received March 30, 1982

Summary: Desensitization of lipolysis was induced in isolated rat adipocytes by incubation with isoproterenol 10^{-5}M or ACTH 250 mU/ml for two and three hours, respectively. Those cells desensitized with isoproterenol were restimulated with either isoproterenol 10^{-7}M or ACTH 6 mU/ml and those cells desensitized with ACTH were restimulated with isoproterenol 10^{-7}M. Lipolysis was quantitated by the release of cyclic AMP and glycerol. No effect on either homologous or heterologous desensitization was observed when either cycloheximide 2 µg/ml or puromycin 10^{-4}M was included in the incubation media during the induction of desensitization. These findings support the conclusion that protein synthesis plays no role in the desensitization of lipolysis in the isolated rat adipocyte.

Desensitization is a feature of the adenylate cyclase system (1, 2). The importance of new protein synthesis in this process remains uncertain. In some systems, dependence of desensitization on protein synthesis has been demonstrated (3, 4); while in others, protein synthesis inhibitors have had no effect (5, 6). In the present study, we have used the rat adipocyte to further investigate this phenomena. One reason for using the rat adipocyte is that activation of adenylate cyclase in this system provides several points at which desensitization can be quantitated. A second reason is that adenylate cyclase in the rat adipocyte is activated by several hormones (7), providing the opportunity to study the effects of protein synthesis inhibition on both heterologous as well as homologous desensitization.

MATERIALS AND METHODS

Chemicals: L-isoproterenol, fraction V bovine serum albumin (BSA #A-9647), lubrol-PX, cycloheximide, puromycin and all enzymes, except collagenase, were purchased from Sigma. The ACTH preparation used was Acthar from the Armour Corporation. Crude collagenase was obtained from Worthington; alumina from Merck. Cyclic AMP was determined using a Becton Dickenson kit. [1-^{14}C] leucine and [1-^{14}C]α-aminoisobutyric acid were purchased from New England

0006-291X/82/100298-07$01.00/0

Nuclear. Tritiated cyclic AMP used to measure recovery from our alumina columns was obtained from the Amersham Corporation. Buffer used for both collagenase digestion and all incubations consisted of 3% BSA, 124 mM NaCl, 4.8 mM KCl, 25 mM Tris and 1 mM each of KH_2PO_4, $MgSO_4$, $CaCl_2$ and glucose. The pH was 7.4.

Assay: Glycerol release was quantitated using a modification of the fluorometric method of Chernick (8). Samples for cyclic AMP determination were purified by passing them through alumina columns (9). The effluent was diluted 4:1 and cyclic AMP determined by radioimmunoassay using a modification (10) of the method of Steiner (11).

Adipocyte Preparation: 150 g male Sprague Dawley rats obtained from Charles River were sacrificed on the day of each experiment. The epididymal fat pads were dissected out and isolated adipocytes prepared by collagenase digestion as described by Rodbell (12). To quantitate the amount of fat used, fat was extracted and weighed using the method of Dole (13).

Protocol: All experiments were performed at 37°. The effects of protein synthesis inhibitors were assessed by incubating cells in C^{14} labeled leucine in buffer alone or in the presence or cycloheximide 2 µg/ml or puromycin 10^{-4}M. Incubates were sampled at two and three hours; these aliquots were washed once with 5 ml of saline and precipitated with 10% trichloroacetic acid. The supranatant was discarded and the precipitate solubilized with 1 ml of 18% lubrol-PX and added to scintillation vials and counted after the addition of 10 ml of hydrofluor. Controls were cells incubated with C^{14} labeled α-aminoisobutyric acid, a non-metabolizable amino acid and carried through the same procedure. Total counts obtained with these control incubations were subtracted from those obtained with labeled leucine, after appropriate adjustment for slightly differing specific activity of the two labeled amino acids.

Each desensitization experiment consisted of four aliquots of cells in buffer. To the first aliquot nothing was added; to the second, the ligand; and to the third and fourth, the ligand plus either cycloheximide (2 µg/ml) or puromycin (10^{-4}M). The ligands used were ACTH 250 mU/ml or ℓ-isoproterenol 10^{-5}M. After incubation for two hours with isoproterenol and three hours with ACTH, the cells were washed four times with fresh buffer. One ml aliquots from each of the four incubates were then restimulated with either 10^{-7}M isoproterenol or 6 mU/ml of ACTH. Five minutes after restimulation, samples were taken for measurement of cyclic AMP production and thirty minutes after stimulation, samples for determination of glycerol release. Aliquots sampled for cyclic AMP determination were immediately added to 1/10 volume 5M perchloric acid. 100 µl of chloroform was added and the mixture sonicated briefly; the supranatant was then applied to an alumina column. Aliquots for glycerol determination were also added immediately to 1/10 volume of 5 M perchloric acid. They were neutralized with KOH (1/2 ml of approximately 0.4 M) and placed in ice buckets to precipitate $KClO_3$. Supernatants were aspirated for determination of glycerol concentration.

To evaluate the possibility that incubation with cycloheximide or puromycin might themselves induce a blunted response, cells were incubated for two hours in either buffer alone, cycloheximide 2 µg/ml or puromycin 10^{-4}M and restimulated with the same concentration of isoproterenol (10^{-7}M) or ACTH (6 mU/ml) employed in the desensitization experiments. Sampling for cyclic AMP and glycerol was performed as previously described.

STATISTICS To determine statistical significance of the desensitization experiments, the two-tailed Dunnett's test was employed (14). The response of cells incubated with hormone was compared both to the response of cells incubated in buffer alone and with cells incubated with hormone plus a protein synthesis inhibitor. Results were considered significant if p was less than 0.05. To determine the significance of those control experiments performed to assess the effects of cycloheximide and puromycin on hormone-induced lipolysis and cyclic AMP generation, single factor analysis of variance was employed.

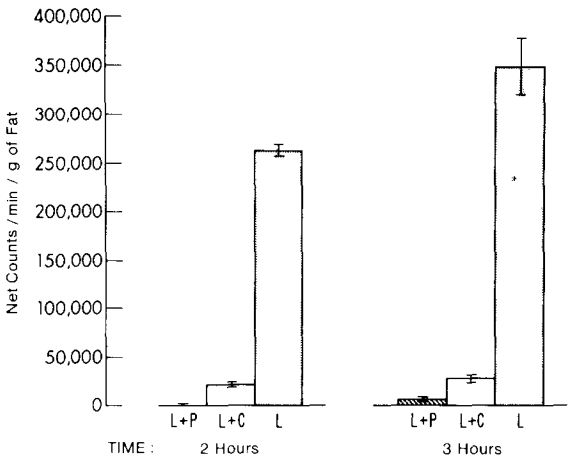


Fig. 1. Inhibition of protein synthesis by cycloheximide and puromycin. Cells were incubated at 37° with [1-^{14}C]L-leucine, 7.7 uCi/g of fat, and buffer alone (L), added cycloheximide 2 ug/ml (L+C), or added puromycin 10^{-4}M (L+P). Aliquots were sampled at two and three hours, washed with saline and precipitated with 10% trichloroacetic acid. Net counts were determined by incubating cells at 37° in the non-metabolized amino acid [1-^{14}C]α-aminoisobutyric acid and subtracting the counts obtained at two and three hours from those obtained when [1-^{14}C]L-leucine was used. Each point is the mean of three experiments ± S.E.

RESULTS

Inhibition of Protein Synthesis: Figure 1 demonstrates that puromycin (10^{-4}M) and cycloheximide (2 μg/ml) in the concentrations employed was effective in inhibiting protein synthesis. In the presence of puromycin, virtually no protein synthesis occurred at either two or three hours and with cycloheximide protein synthesis was inhibited by 92% at both times.

Effects of Cycloheximide and Puromycin on Hormone-Induced Glycerol Release and Cyclic AMP Generation: Tables I and II show that prior incubation of adipocytes with either cycloheximide or puromycin for two hours had no significant effect on glycerol release and cyclic AMP generation on subsequent restimulation by isoproterenol (10^{-7}M) or ACTH (6 mU/ml).

Effects of Cycloheximide and Puromycin on Homologous Desensitization: The stimulation of glycerol release in Figure 2A and cyclic AMP generation in Figure 2B by isoproterenol following incubation with buffer alone are both markedly suppressed when the initial incubation was with isoproterenol (10^{-5}M). Addition of either protein inhibitor, cycloheximide or puromycin, to the initial incubation with isoproterenol, did not change the response.

Table I. Effect of prior incubation of adipocytes in the presence of protein synthesis inhibitors on subsequent response to isoproterenol 10^{-7}M.

Initial Incubation	Buffer	Cycloheximide	Puromycin	p
n	3	3	3	
Glycerol[a] ± S.E.	11.48 ± .38	11.69 ± .34	11.69 ± .39	
Significance				> .25
n	3	3	3	
cAMP[b] ± S.E.	107.39 ± 15.2	120.44 ± .8.7	113.46 ± 17.6	
Significance				> .25

Table II. Effect of prior incubation of adipocytes in the presence of protein synthesis inhibitors on subsequent response to ACTH 6 mU/ml.

Initial Incubation	Buffer	Cycloheximide	Puromycin	p[c]
n	3	3	3	
Glycerol[a] ± S.E.	11.18 ± .47	11.32 ± .37	10.91 ± .26	
Significance				> .25
n	3	3	3	
cAMP[b] ± S.E.	86.59 ± 13.3	110.58 ± 17.5	108.20 ± 10.5	
Significance				> .25

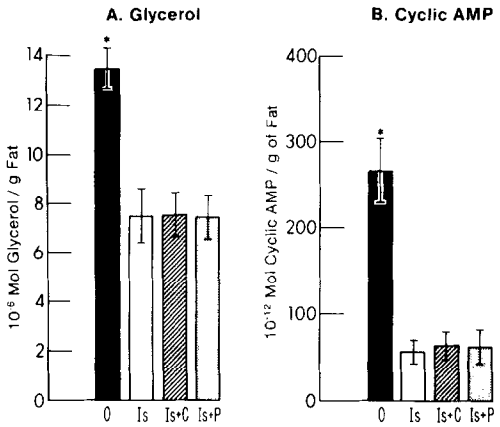
Adipocytes incubated for two hours in either buffer, cycloheximide 2 ug/ml and puromycin 10^{-4}M washed and then restimulated with either isoproterenol or ACTH.

[a] 10^{-6}mol glycerol/gram of fat

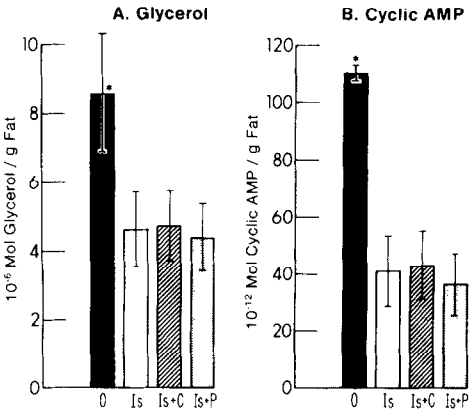
[b] 10^{-12}mol cyclic AMP/gram of fat

[c] Significance determined by single factor analysis of variance

Effects of Cycloheximide and Puromycin on Heterologous Desensitization: Figure 3 demonstrates that cycloheximide and puromycin had no effect on the induction of heterologous desensitization by isoproterenol. The first bar in Figures 3a and 3b depicts the stimulation of lipolysis and cyclic AMP generation, respectively, by ACTH in adipocytes incubated initially for two hours in buffer alone. The remaining bars demonstrate isoproterenol-induced desensitization of the lipolytic and cyclic AMP response of adipocytes to ACTH. Cells were incubated for two hours in isoproterenol (Is), isoproterenol and cycloheximide (Is+C), or isoproterenol and puromycin (Is+P) prior to restimulation



(2) * p<.01 for difference in response between cells incubated in buffer alone and cells incubated in isoproterenol.



(3) * p<.01 for difference in response between cells incubated in buffer alone and cells incubated in isoproterenol.

Fig. 2. Homologous desensitization with isoproterenol. Cells were incubated at 37° for two hours in either buffer alone (0), isoproterenol 10^{-5}M (Is), isoproterenol 10^{-5}M plus cycloheximide 2 ug/ml (Is+C) or isoproterenol 10^{-5}M plus puromycin 10^{-4}M (Is+P). Cyclic AMP response at five minutes and glycerol response at 30 minutes were determined after restimulation with isoproterenol 10^{-7}M. Height of each bar is the mean of three experiments ± S.E. P values calculated by the two-tailed Dunnett's test.

Fig. 3. Heterologous desensitization with isoproterenol. Cells were incubated at 37° for two hours in either buffer alone (0), isoproterenol 10^{-5}M (Is), isoproterenol 10^{-5}M plus cycloheximide 2 ug/ml (Is+C), or isoproterenol 10^{-5}M plus puromycin 10^{-4}M (Is+P). Cyclic AMP response at five minutes and glycerol response at 30 minutes were determined after restimulation with ACTH 6 mU/ml. Height of each bar is the mean of three experiments ± S.E. P values calculated by the two-tailed Dunnett's test.

with ACTH. The amount of desensitization seen was not significantly affected by the presence of inhibitors of protein synthesis during the induction of desensitization.

Figure 4 demonstrates that inhibition of protein synthesis had no effect on the induction of heterologous desensitization by ACTH. The decrease in lipolysis and cyclic AMP generation demonstrated in cells exposed to 250 mU/ml of ACTH for three hours prior to restimulation with isoproterenol was not effected by the inhibition of protein synthesis during the induction of desensitization.

DISCUSSION

Both heterologous and homologous desensitization of lipolysis have been demonstrated in the rat adipocyte (7). This process is time dependent and occurs maximally at two or more hours (7). We sought to determine in this study if protein synthesis plays a role in the time delay in desensitization

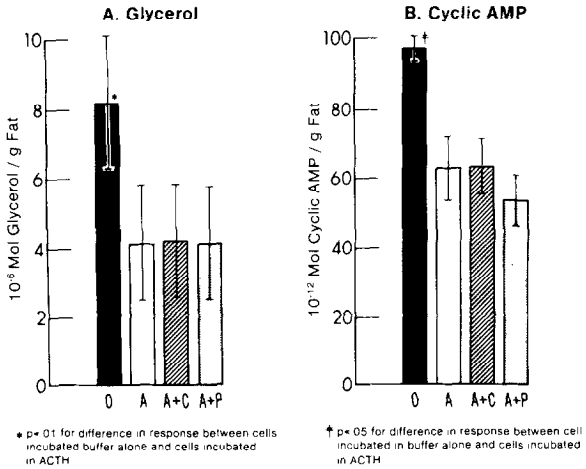


Fig. 4. Heterologous desensitization with ACTH. Cells were incubated at 37° for three hours in either buffer alone (0), isoproterenol 10^{-5}M (Is), isoproterenol 10^{-5}M plus cycloheximide 2 ug/ml (Is+C), or isoproterenol 10^{-5}M plus puromycin 10^{-4}M (Is+P). Cyclic AMP response at five minutes and glycerol response at 30 minutes were determined after restimulation with isoproterenol 10^{-7}M. Height of each bar is the mean of three experiments ± S.E. P values calculated by the two-tailed Dunnett's test.

of the isolated rat adipocyte with either isoproterenol or ACTH. The induction of both heterologous and homologous desensitization as assessed by both cyclic AMP and glycerol release was unaffected by the presence of the protein synthesis inhibitors. Incubation of cells with cycloheximide or puromycin alone was without effect on the subsequent exposure of the adipocytes to isoproterenol or ACTH and established that the observed decrease in hormone responsiveness was not due to a direct or toxic action of the protein inhibitors.

The effects of protein synthesis inhibition on heterologous desensitization have not been previously reported, and the present data with heterologous desensitization supports the findings with homologous desensitization. Other groups using a variety of cell systems have also studied protein synthesis inhibition on homologous desensitization. Some, like ourselves, have observed an inhibition while others have not. For example, cycloheximide (10^{-4}M) inhibits TSH-induced desensitization of cultured human thyroid cells (15), whereas an even larger concentration of cycloheximide (2×10^{-4}M) had no effect on isoproterenol-induced desensitization in C_6 glioma cells (16). At present, it is not possible to reconcile these differences; however, the ability to induce desensitization in cell membranes (17, 18), a preparation which is devoid of

protein synthetic capacity, suggests that protein synthesis is not an essential mechanism.

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