CYCLIC AMP INCREASES THE CONCENTRATION OF INSULIN RECEPTORS IN CULTURED FIBROBLASTS AND LYMPHOCYTES‡

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SUMMARY: Incubation of SV40 transformed fibroblasts with dibutyryl cyclic AMP, 8-bromo-cyclic AMP, or 1-methyl-3-isobutylxanthine (MIX), a phosphodiesterase inhibitor, produced a two-fold increase in insulin receptor concentration without an effect on receptor affinity. The increase was dose-dependent, was observed after 8 hrs of treatment, and reached a maximum level by 12 to 24 hours. Upon removal of the nucleotide, receptor number decreased towards basal level.

Incubation of cultured human lymphocytes (IM-9 line) with cyclic AMP derivatives or MIX also increased the number of insulin receptors without an alteration in receptor affinity. This effect was partially blocked by inhibition of protein synthesis and was independent of changes in cell cycle. The increase in insulin receptors was a specific response to cyclic AMP as the number of receptors for human growth hormone was unaltered. Incubation with 8-bromo-cyclic GMP did not alter the level of insulin binding.

INTRODUCTION: Recently we have described insulin receptors in normal and transformed mouse fibroblast cell lines (1). The level of ^{125}I-insulin binding was low in growing normal cells and rose when growth was arrested. The level of insulin binding was also low in a variety of transformed cells. These differences in ^{125}I-insulin binding were due to changes in the number of insulin receptors as opposed to an alteration in the affinity of the receptor for hormone (1). Since cell growth can be slowed and several other properties of the transformed cell can be partially reversed by cyclic AMP (2-4), the effect of the cyclic nucleotide on insulin receptors was investigated. To complement these studies, the role of cyclic AMP in controlling the number of insulin receptors in a line of human lymphocytes (IM-9) was also examined.

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Abbreviations: Bt$_2$cAMP, N^6-2'-O-dibutyryl adenosine 3', 5'-monophosphate; 8-Br-cAMP, 8-Bromo-adenosine 3',5'-monophosphate; 8-Br-cGMP, 8-Bromo-guanosine 3',5'-monophosphate; MIX, 1-methyl-3-isobutylxanthine; BSA, bovine serum albumin; PBS, phosphate buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediamine-tetracetic acid; hGH, human growth hormone.

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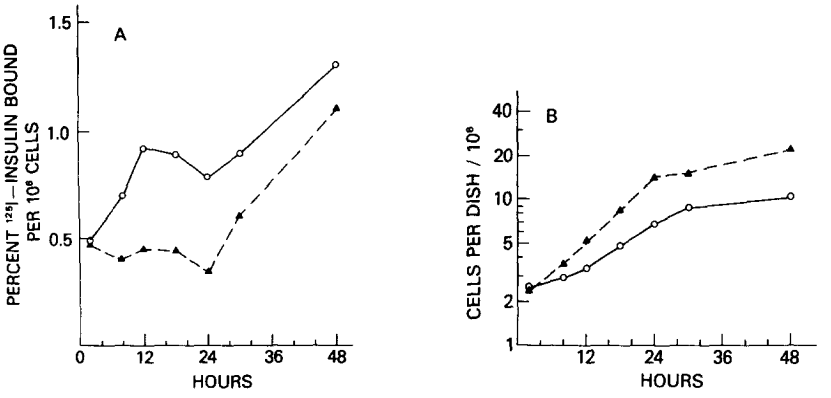
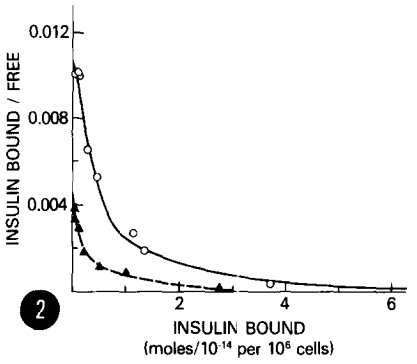


FIGURE 1: Time course of the Effect of Bt_2cAMP plus MIX on Insulin Binding to SVT_2 Fibroblasts. A and B: SVT_2 fibroblasts were cultured in the presence (0) or absence (▲) of Bt_2cAMP plus MIX (1 mM each). At the indicated time points the cells were detached from the dishes, counted, and the ^{125}I-insulin binding was measured (see Methods). Note that the first assay point is after 2 hrs. of culture. The ^{125}I-insulin was at 2×10^{-11} M. The cell concentration was 3.4 to 16×10^6 per ml for the controls and 3.4 to 14×10^6 per ml for the Bt_2cAMP plus MIX-treated cells.

MATERIALS AND METHODS: Porcine insulin (lot 7GU48L) was purchased from Elanco. Human growth hormone (1563-D) was generously supplied by Dr. A. E. Wilhelmi. Na^{125}I (carrier free) was purchased from Amersham/Searle, Bt_2cAMP, 8-Br-cAMP and 8-Br-cGMP from Sigma, MIX from Aldrich, and BSA (fraction V) from Armour. Bt_2cAMP was purified as described previously (5). All chemicals were of reagent grade.

SV40 transformed Balb 3T3 mouse fibroblasts (clone SVT_2) and cultured human lymphocytes (IM-9 line) were grown as described previously (1,6). The fibroblasts were grown in monolayer in the presence or absence of Bt_2cAMP plus MIX. For the binding studies, the fibroblasts were detached from the dishes by incubation with Ca^{+2} and Mg^{+2}-free Dulbecco's PBS (pH 7.4) and then sedimented by centrifugation (5 min at 250 x g). The cells were resuspended in assay buffer, sedimented (250 x g) and then resuspended in the same buffer at a final concentration of 2.6 to 20×10^6 cells per ml.

The lymphocytes were cultured with nucleotides for 18-24 hours after being split (1:4) in the late log phase of growth. Prior to the addition of nucleotide or MIX, the lymphocytes were centrifuged (800 x g) and resuspended in fresh culture medium. The cyclic nucleotides were added as aliquots to the cell suspension, while MIX was added by dissolving it in fresh culture medium just prior to cell resuspension. Following the appropriate length of preincubation, the lymphocytes were sedimented (10 min at 800 x g), resuspended in assay buffer, centrifuged (10 min at 800 x g), and then resuspended in the same buffer (2.5 to 17.5×10^6 cells/ml). The ^{125}I-insulin and the ^{125}I-hGH were prepared as described (7) at specific activities of 150 to 200 μCi/μg and 40 μCi/μg, respectively. The assays for measuring ^{125}I-insulin binding to fibroblasts (1) and lymphocytes (6) and for ^{125}I-hGH binding to lymphocytes (8) have been described elsewhere. In all experiments in which cells were cultured with cyclic nucleotide or MIX, the additions were continuously kept in the presence of the cells during the wash and assay procedures. Cell viability was determined at the conclusion of each experiment by the trypan blue exclusion method (9); in all experiments, 90% or more of the cells



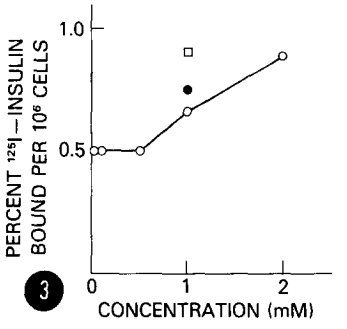


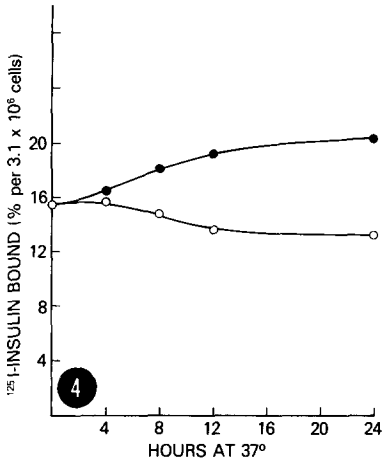
FIGURE 2: Scatchard Plots of Insulin Binding to SVT_2 Fibroblasts Cultured in the Presence or Absence of Bt_2cAMP Plus MIX. SVT_2 fibroblasts were cultured for 18 hr. in the presence (0) or absence (Δ) of Bt_2cAMP plus MIX (1 mM each). Following the incubation, the ^{125}I-insulin binding was measured. The ^{125}I-insulin was at 3 x 10^{-11} M, and the unlabeled insulin ranged from 0 to 1.7 x 10^{-7} M; the cell number was 3 x 10^6 per ml for the untreated cells and 2.3 x 10^6 per ml for the Bt_2cAMP plus MIX-treated cells.

FIGURE 3: Effect of Bt_2cAMP, MIX or 8-Br-cAMP on Insulin Binding to SVT_2 Fibroblasts. Binding of ^{125}I-insulin to SVT_2 fibroblasts was measured (see Methods) after the cells were cultured for 18 hrs. in the presence of the indicated concentration of either Bt_2cAMP (o), 8-Br-cAMP (•) or MIX (□). The ^{125}I-insulin was at 2 x 10^{-11} M and the cell number ranged from 2.1 to 4.2 x 10^6 cells per ml.

excluded the dye. Degradation of the unbound labeled insulin was assessed as described previously (1) and was negligible. The DNA content of the lymphocytes was measured by microfluorometric analysis (10).

RESULTS: SVT_2 fibroblasts cultured with 1 mM Bt_2cAMP and 1 mM MIX, a phosphodiesterase inhibitor, had higher levels of ^{125}I-insulin binding than did controls (Figure 1A). The effect of the cyclic nucleotide was not evident at 2 hrs., but appeared after 8 hrs. of incubation and was maximal between 12 and 24 hrs. After 24 hrs., the level of ^{125}I-insulin binding observed with controls increased spontaneously, and the relative effect of Bt_2cAMP decreased. The Bt_2cAMP-treated cells also grew at a slower rate than did control cells (Figure 1B). The binding data were analyzed according to the method of Scatchard (11), and the results were consistent with a change in receptor concentration with little or no change in receptor affinity (Figure 2). The effect of Bt_2cAMP was reversed when the SVT_2 fibroblasts that had been incubated with Bt_2cAMP were washed free of Bt_2cAMP and cultured without it. Within 6 hrs. after removal, the level of ^{125}I-insulin binding decreased towards basal levels.

The increase in the binding of ^{125}I-insulin to SVT_2 fibroblasts was greater with higher concentration of Bt_2cAMP (Figure 3). Increased insulin binding was



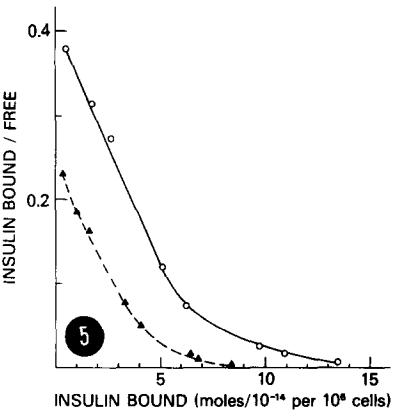


FIGURE 4: Time Course of the 8-Br-cAMP Effect on Insulin Binding to IM-9 Lymphocytes. The procedure employed was similar to Figure 2. The cell concentration was 3.1 x 10^6 per ml. o, no additions; •, 1 mM 8-Br-cAMP.

FIGURE 5: Scatchard Plots of Insulin Binding to IM-9 Lymphocytes Cultured in the Presence or Absence of Bt_2cAMP Plus MIX. The conditions were identical to those in Figure 2. The cell concentration during the binding assay was 4.6 x 10^6 cells per ml for the untreated cells (▲) and 3.5 x 10^6 cells per ml for the Bt_2cAMP-treated cells (o).

observed also by culturing cells with either 8-Br-cAMP or MIX alone.

Studies with IM-9 lymphocytes produced results similar to those observed with SVT_2 fibroblasts. Culturing IM-9 cells with 1 mM 8-Br-cAMP resulted in a similar time-dependent increase in insulin binding (Figure 4). The increased binding was due to an increase in receptor number (Figure 5). The level of ^{125}I-insulin binding per cell decreased in the untreated lymphocytes, which were in an exponential phase of growth; the decrease in binding may be related to the results reported for non-confluent fibroblasts (1). The increase in receptor number was specific for insulin receptors, as incubation of IM-9 lymphocytes with Bt_2cAMP plus MIX (1 mM each) resulted in an increase in insulin binding without an effect on ^{125}I-hGH binding (Table I). Incubation with 8-Br-cGMP at concentrations from 10^{-11} to 10^{-3} M for 18 hrs. was without effect on ^{125}I-insulin binding.

In most experiments with lymphocytes, Bt_2cAMP also inhibited cell division. This inhibition was not consistently related to the increase in insulin binding. A doubling of insulin binding was demonstrated without a measurable difference in cell number (Table II). Furthermore, analysis of the cell

TABLE I

ADDITIONS	Growth Hormone: $\frac{\text{Bound}}{\text{Free}}$ per 10^6 cells	Insulin: $\frac{\text{Bound}}{\text{Free}}$ per 10^6 cells	Cells: per flask
None	0.019	0.084	17.5×10^6
Bt_2cAMP + MIX	0.018	0.322	13×10^6

TABLE I: Effect of Bt_2cAMP Plus MIX on Binding of Insulin and Growth Hormone to Cultured Lymphocytes. IM-9 lymphocytes were cultured for 21 hrs. in the presence or absence of Bt_2cAMP plus MIX (1 mM each) and then the binding of ^{125}I-insulin or ^{125}I-hGH was measured (see Methods). Since the ratio of bound:free ^{125}I-hGH or ^{125}I-insulin was measured at very low levels of iodinated hormone; the value of bound:free is approximately equal to the product of the equilibrium constant and the total receptor concentration. ^{125}I-insulin was at 2×10^{-11} M, and the cell number was 3.7×10^6 per ml for the untreated cells and 3.3×10^6 per ml for the Bt_2cAMP plus MIX-treated cells. The ^{125}I-hGH was added at 10^{-10} M, and the cells were at a concentration of 14×10^6 per ml for the untreated cells and 10.4×10^6 per ml for the Bt_2cAMP plus MIX-treated cells.

TABLE II

	Percent of Cells in: G_1	S	G_2 + M	Insulin: $\frac{\text{Bound}}{\text{Free}}$ per 10^6 cells	Cells: per flask
Controls	55.2	13.3	31.5	0.16	10×10^6
Bt_2cAMP + MIX	61.9	14.6	23.5	0.30	10×10^6

TABLE II: Effect of Bt_2cAMP Plus MIX on the Cell Cycle of Cultured Lymphocytes. IM-9 lymphocytes were cultured for 19 hours in the presence or absence of Bt_2cAMP plus MIX (1 mM each). They were then sedimented and resuspended either in assay buffer for ^{125}I-insulin binding (see Methods) or in ethanol-PBS (25:75 v/v) for the DNA assay (see Methods). The ^{125}I-insulin concentration was 2×10^{-10} M and the cell number was at 2×10^6 per ml for both the untreated and the Bt_2cAMP plus MIX-treated cells. The cell number was at 2.7×10^6 per ml for the DNA assay.

cycle pattern of these Bt_2cAMP plus MIX-treated lymphocytes by microfluorometric analysis (10) showed only a minor increase in the percent of cells in G_1 (Table II).

The effect of cyclic AMP on insulin receptor number of IM-9 lymphocytes was partially blocked by treatment with 10^{-4} M cycloheximide. Incubation of

IM-9 cells with 10^{-4} M cycloheximide for 8 hrs. resulted in a 16% loss of insulin binding, presumably due to spontaneous receptor turnover. Incubation of cells with 1 mM MIX for 8 hrs. produced a 24% increase in receptor number. However, when cells were cultured with both MIX and cycloheximide for 8 hrs., the level of insulin binding was the same as untreated cells.

DISCUSSION: Pharmacological elevation of intracellular cyclic AMP induced a 2-fold or greater increase in the concentration of insulin receptors in both fibroblasts and lymphocytes. The increase in number of insulin receptors was a specific response to increased cyclic AMP levels, as all cyclic AMP derivatives, as well as MIX alone, were effective; 8-Br-cGMP did not have an effect on insulin receptor concentration. Moreover, cyclic AMP altered the level of insulin receptors without affecting the concentration of hGH receptors. Thus, the observed increase in insulin receptors does not appear to be merely related to a cell volume or cell surface change. The elevation in hormone receptors was reversed when the cells were transferred to medium free of these additions.

With the fibroblasts, the increase of insulin receptors had a lag period of at least 2 hrs., with the maximum effect observed within 12 to 24 hrs. These changes in the concentration of insulin receptors occurred after changes in cell morphology and adhesiveness (data not shown) (3,12). Past this time point, the relative increase in receptors induced by Bt_2cAMP became less important, as the untreated cells showed a spontaneous increase in the level of insulin binding. This appears to be related to the high cell densities obtained, since it has been already observed that even transformed cells increase their insulin binding as the cell density increases, although to a lesser degree than the normal cells (1). The intracellular concentration of cyclic AMP increases at confluence (4); however, the increase in receptor number observed at confluence (1) was 2-to 4-fold greater than the increase observed following preincubation with cyclic AMP. Thus, the spontaneous increase in receptor number observed at confluence can be accounted for only in part by the increased levels of cyclic AMP.

The increase in insulin binding produced by cyclic AMP was interpreted as a change in receptor concentration. However, our studies cannot eliminate the possibility of a simultaneous minor modification of receptor affinity. In fibroblasts, the increase of receptor number and the inhibition of growth rate were observed simultaneously. With the lymphocytes, the two phenomena were not constantly linked; the change in the number of receptors was observed without a modification in the rate of cell growth.

In conclusion, a pharmacological increase in intracellular cyclic AMP

concentrations caused a two-fold increase in the concentration of insulin receptors, with no major change in their affinity for the hormone. The response was specific for insulin receptors and was not observed with growth hormone receptors. These data indicate that the intracellular level of cyclic AMP could be one of the factors regulating the concentration of insulin receptors and that insulin has been found to lower cyclic AMP levels (13) could, in part, account for the fall in insulin receptors observed in insulin-treated cells (14).

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Note added in proof: De Pascuale, *et al.*, (1977) [*Proc. Natl. Acad. Sci., USA*, in press] have demonstrated that culturing Cloudman S-91 melanoma cells with dibutyryl cyclic AMP results in an increase in MSH receptor number.

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