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Changes in adenosine receptors during differentiation of 3T3-F442A cells to adipocytes

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Incubation of undifferentiated 3T3-F442A cells (preadipocytes) with 5'-*N*-ethylcarboxamidoadenosine (NECA) increases intracellular cyclic AMP in a dose-dependent manner. The effect of NECA is antagonized by 8-phenyltheophylline, but potentiated by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine, an inhibitor of cyclic AMP phosphodiesterase. Incubation of preadipocytes with (−)-N^6-(*R*-phenylisopropyl)adenosine (PIA) has no inhibitory effect on the basal concentration of cyclic AMP or on the stimulation of adenylate cyclase by isoprenaline or forskolin. Micromolar concentrations of PIA increase intracellular cyclic AMP, but with a lower potency than NECA. Similar findings are obtained with the non-differentiating cell line 3T3-C_2. Thus preadipocyte 3T3-F442A cells and 3T3-C_2 cells appear to express only stimulatory adenosine receptors. For some time after 3T3-F442A cells have differentiated to adipocytes, micromolar concentrations of NECA and PIA continue to increase cyclic AMP to a similar extent to that in preadipocytes, whereas nanomolar concentrations of PIA decrease the stimulatory effects of isoprenaline and forskolin on adenylate cyclase by 50%. However, several days after differentiation, the adipocytes gradually lose the major part of their positive response to NECA and reach a steady response to NECA 10 days after differentiation. The inhibition of adenylate cyclase caused by PIA remains constant for at least 2 weeks after differentiation. With membranes derived from the cells, the effects of NECA and PIA depend on GTP. These results indicate that, during the differentiation of 3T3-F442A cells to adipocytes, new inhibitory adenosine receptors are expressed, whereas the stimulatory receptors become attenuated.

INTRODUCTION

Adenosine and many of its analogues regulate the activity of adenylate cyclase. The external receptors that mediate inhibition (A_1) and stimulation (A_2) of adenylate cyclase possess different specificities towards adenosine analogues. For example, A_2 receptors show a greater affinity for NECA than for PIA, but the reverse is true for A_1 receptors (Londos & Wolff, 1977; Londos *et al.*, 1978, 1980). Interaction of adenosine with A_1 and A_2 receptors induces a wide range of physiological and biochemical effects (Cooper *et al.*, 1979; Belardinelli *et al.*, 1981; Bauman *et al.*, 1981; Dobson, 1983). Adenosine inhibits hormone-induced lipolysis of adipose tissue (Dole, 1961). Moreover, adenosine is released by adipocytes in amounts which inhibit cyclic AMP accumulation and lipolysis (Schwabe & Ebert, 1974; Schwabe *et al.*, 1973, 1975).

A_1 receptors were reported to be present in fat-cells by Fain & Malbon (1979). By inhibiting the effects of adenosine on A_1 receptors with pertussis toxin, Garcia-Sainz & Torner (1985) showed that A_2 receptors are also present in fat-cells.

In a process closely resembling the development of adipocytes, 3T3-F442A cells in culture can differentiate with high frequency into adipocytes (Green & Kehinde, 1976). During differentiation, the cells express enzymes involved in fatty acid and triacylglycerol synthesis and develop sensitivity to particular hormones (Rubin *et al.*, 1978). We have studied changes in adenosine receptors that occur in the course of differentiation of 3T3-F442A cells to adipocytes. Our results show that, during the early phase of differentiation, the cells still retain A_2 receptors, while new A_1 receptors are expressed. However, 2 weeks after differentiation, A_2 receptors have decreased substantially, but A_1 receptors remain high.

MATERIALS AND METHODS

Materials

NECA, ATP, cyclic AMP, phosphocreatine, insulin, isoprenaline (isoproterenol) and Oil Red O were purchased from Sigma Chemical Co. PIA, adenosine deaminase, GTP[S], myokinase and creatine kinase were obtained from Boehringer Mannheim. 8-Phenyltheophylline was from Calbiochem. Dulbecco's minimum essential medium, trypsin, calf serum and fetal-calf serum were from Gibco. Forskolin and Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine] were obtained from Behring Diagnostics. Goat anti-(cyclic AMP) antibody, normal goat serum and rabbit anti-goat IgG were purchased from Research Products International. Cyclic AMP 2'-*O*-succinyl([^{125}I]iodotyrosine methyl ester) and cyclic [2,8-^{3}H]AMP were obtained from New England Nuclear. Dowex 50W-X8 was obtained from Bio-Rad.

Cell culture

3T3-F442A and 3T3-C_2 cells (Green & Kehinde, 1976) were grown in Dulbecco's minimum essential medium supplemented with 10% (v/v) calf serum. Cells were

Abbreviations used: GTP[S], guanosine 5'-[γ-thio]triphosphate; NECA, 5'-*N*-ethylcarboxamidoadenosine; PIA, (−)-N^6-(*R*-phenylisopropyl)-adenosine.

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treated with trypsin, and 2×10^4 cells were incubated in 35 mm-diam. plastic Petri dishes in the presence of Dulbecco's minimum essential medium supplemented with 10% fetal-calf serum and 5 μg of insulin/ml, to promote the formation of adipocytes. Cultures were fed three times weekly. Confluence was attained approx. 5–6 days after incubation. Differentiation of about 95% of the cells was observed 14–15 days after plating. The percentage of differentiated cells per plate was estimated by counting cells containing and lacking fat droplets. The dye Oil Red O was used to identify cells containing fat droplets. The cells were trypsin-treated, fixed with 10% (v/v) formaldehyde, and counted for cells containing fat droplets. Measurements on preadipocytes and 3T3-C_2 cells were performed 4–5 days after plating the cells, whereas measurements on adipocytes were performed 14–16 days after plating of the cells, or as indicated in the Figure legends. The cells survived for about 4 weeks after plating when certain batches of fetal-calf serum were employed.

Preparation of membrane fraction

Cells were grown in 75 cm^2 flasks (1×10^5 cells/flask) as described above under 'Cell culture'. The cells were rinsed twice with phosphate-buffered saline (136 mM-NaCl, 8 mM-Na_2HPO_4, 2.6 mM-KCl, 1.4 mM-KH_2PO_4, pH 7.4) to remove the culture medium. The cells were then removed from the flasks with a rubber policeman in the presence of 3 ml of phosphate-buffered saline. The cells were centrifuged at 1000 *g* for 5 min, resuspended in 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-dithiothreitol, and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 5000 *g* for 10 min. The pellet was resuspended and homogenized in the same buffer with a glass/Teflon Potter/Elvehjem homogenizer. The resulting particulate fraction was used for assays of adenylate cyclase.

Determination of cyclic AMP

Cells were washed with Dulbecco's minimum essential medium at 37 °C. Unless otherwise indicated, experiments with the cells were conducted in 2 ml of Dulbecco's minimum essential medium supplemented with 3 units of adenosine deaminase/plate and 20 mM-Hepes/NaOH buffer, pH 7.4, and additions as indicated, at 37 °C for 5 min. The medium was then removed and the cells were washed with ice-cold phosphate-buffered saline. Ice-cold 0.1 M-HCl (0.5 ml) was then added, and the plate was kept on ice for 20 min. Cyclic [2,8-^{3}H]AMP (7000 d.p.m./tube) was added to the acid extract, which was then applied to a 1 ml column of Dowex 50W-X8 resin (H^+ form). The column was washed with 2 ml of water. Cyclic AMP was then eluted with 3 ml of water, and this eluate was freeze-dried and redissolved in 0.5 ml of 50 mM-sodium acetate buffer, pH 6.2. A portion of this sample was counted for ^{3}H radioactivity, so that the radioimmunoassay data could be corrected for recovery of cyclic AMP. A sample was acetylated and assayed in duplicate for cyclic AMP by radioimmunoassay as described by Brooker *et al.* (1979), except that the antigen–antibody complex was precipitated by adding normal goat serum and rabbit antiserum to goat IgG.

Adenylate cyclase determination

The assay contained 50 mM-glycylglycine, pH 7.5, 0.5 mM-ATP, 10 mM-$MgCl_2$, 5 units of adenosine deaminase/ml, 1 mM-dithiothreitol, 15 μM-Ro20-1724, and an ATP-regenerating system consisting of 2 mM-phosphocreatine, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 0.2 ml. The reaction mixture was preincubated for 2 min at 37 °C. The reaction was then started by adding particulate fraction equivalent to 50 μg of protein. Reactions were conducted at 37 °C for 10 min and were stopped by adding 0.3 ml of 0.1 M-HCl. The resulting mixture was then kept on ice for 20 min. The samples were centrifuged in a micro-centrifuge, and the supernatants were applied to Dowex 50W-X8 columns, and treated as described above under 'Determination of cyclic AMP'. Adenylate cyclase activity was assayed under conditions that were linear with respect to protein concentration and time of incubation.

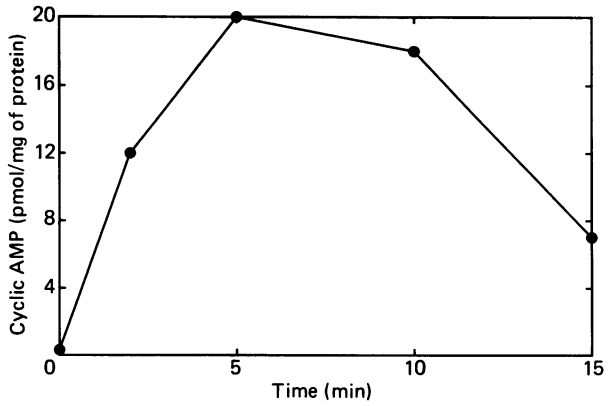

Fig. 1. Effect of NECA on cyclic AMP content of preadipocyte 3T3-F442A cells

The cells were incubated in the presence of 10 μM-NECA. For other details, see the Materials and methods section. Each point represents the mean of two determinations.

Protein determination

Precipitated protein remaining on the plates was dissolved in 1 ml of 0.4 M-NaOH at 100 °C for 10 min. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Incubation of preadipocyte 3T3-F442A cells with 10 μM-NECA caused a marked accumulation of cyclic AMP, which reached a peak after 5 min (Fig. 1). Longer incubations with NECA resulted in decreased cyclic AMP. A 5 min incubation time was therefore chosen for subsequent experiments. NECA caused a dose-dependent increase of cyclic AMP in preadipocyte 3T3-F442A cells (Fig. 2). The effect of NECA was antagonized by the adenosine-receptor antagonist 8-phenyltheophylline. It was potentiated by Ro20-1724, an inhibitor of cyclic AMP phosphodiesterase; for example, 15 μM-Ro20-1724 and 10 μM-NECA caused a 100-fold increase in cyclic AMP over basal values (Fig. 2). PIA at concentrations between 0.001 and 0.1 μM did not affect the cyclic AMP concentration significantly in preadipocytes (Fig. 3). However, 10 μM-PIA enhanced cyclic AMP accumulation, but with a lower potency than NECA (cf. Fig. 2). Cyclic AMP was increased with either forskolin (Seamon *et al.*, 1981; Seamon & Daly, 1981) or isoprenaline so

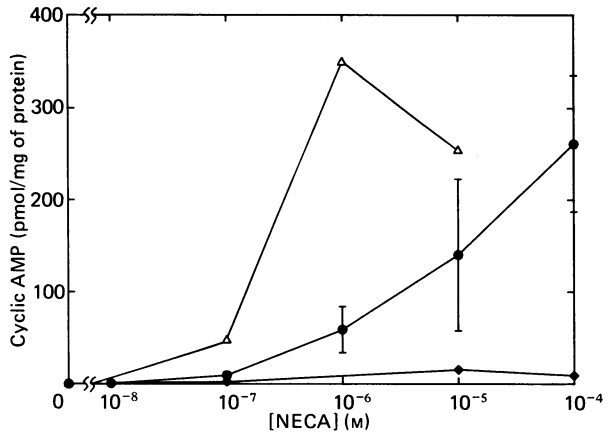

Fig. 2. Effect of NECA, Ro20-1724 and 8-phenyltheophylline on cyclic AMP content of preadipocyte 3T3-F442A cells

The incubation medium contained NECA as indicated and one of the following additions: ●, none; ◆, 20 μM-8-phenyltheophylline; △, 20 μM-Ro20-1724. For other details, see the Materials and methods section. Results are means ± S.D. of three experiments (● and ◆) or averages of two experiments (△). All S.D. values for ◆ were within the symbols.

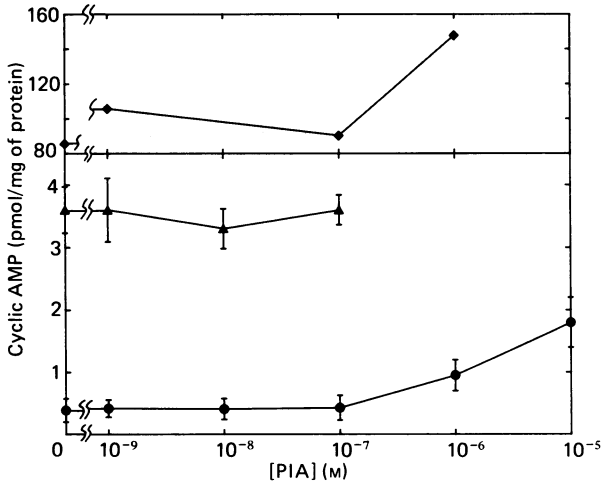

Fig. 3. Effect of PIA, isoprenaline and forskolin on cyclic AMP content of preadipocyte 3T3-F442A cells

The incubation medium contained PIA as indicated and one of the following additions: ●, none; ◆, 20 μM-forskolin; ▲, 1 μM-isoprenaline. For other details see the Materials and methods section. Results are means ± S.D. of three experiments (● and ▲) or averages of two experiments (◆).

that inhibition of adenylate cyclase could be detected. In preadipocytes, isoprenaline and forskolin increased cyclic AMP 10-fold and 160-fold respectively. Nanomolar concentrations of PIA had no effect on the cyclic AMP content of the preadipocytes incubated with forskolin or isoprenaline. PIA (1 μM) potentiated slightly the stimulatory effect of forskolin. These results show that only stimulatory adenosine receptors are expressed on undifferentiated 3T3-F442A cells.

In the early phase of differentiation of 3T3-F442A cells to adipocytes, NECA also increased cyclic AMP

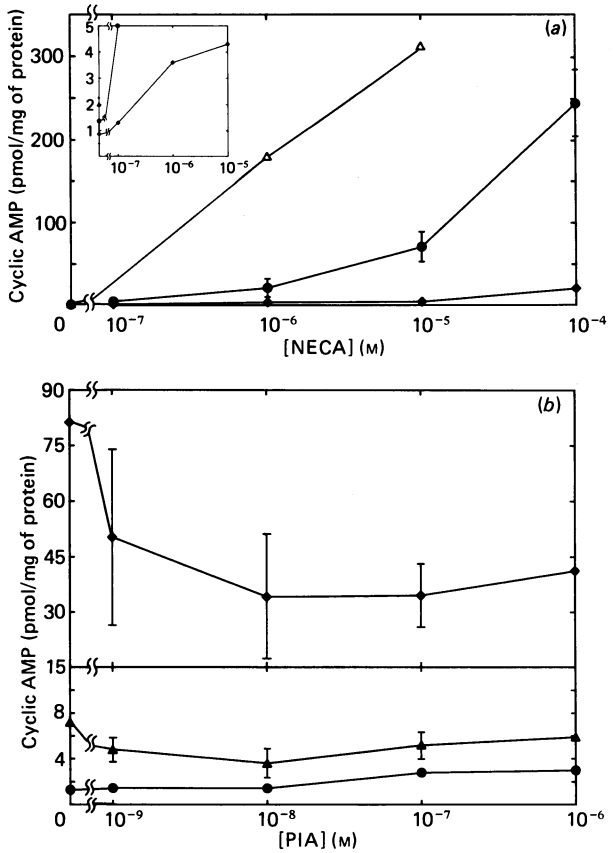

Fig. 4. Effect of NECA and PIA on cyclic AMP content of 3T3-F442A adipocytes

Cells were cultured for 16 days, at which point > 95% had differentiated to adipocytes. (*a*) The incubation medium contained NECA as indicated and the following additions: ●, none; ◆, 20 μM-8-phenyltheophylline; △, 20 μM-Ro20-1724. The insert shows an enlargement of values for ● and ◆ at low [cyclic AMP]. (*b*) The incubation medium contained PIA as indicated and the following additions: ●, none; ◆, 20 μM-forskolin; ▲, 1 μM-isoprenaline. For other details see the Materials and methods section. Results are means ± S.D. of three different experiments or averages of two experiments (where no error bars).

(Fig. 4*a*). The marked accumulation of cyclic AMP caused by NECA reached a peak after 5 min (results not shown). As with preadipocytes, the effect of NECA was antagonized by 8-phenyltheophylline and potentiated by Ro20-1724, indicating that the effect of NECA on cyclic AMP was not due to an effect on phosphodiesterase. Nanomolar concentrations of PIA decreased the stimulatory effects of forskolin and isoprenaline by 60 and 52% respectively (Fig. 4*b*). Micromolar concentrations of PIA doubled the concentration of cyclic AMP as in preadipocytes. These effects of NECA and PIA were observed in both the presence and the absence of insulin (results not shown).

Membranes were prepared from 3T3-F442A adipocytes and from the non-differentiating cell line 3T3-C_2. With both types of membrane, the stimulation of adenylate cyclase by NECA depended on GTP[S] (Table 1). The inhibition of adenylate cyclase by PIA occurred only in adipocytes and also depended on GTP[S]. These results indicate that guanine-nucleotide-regulatory pro-

Table 1. Stimulation of the effects of NECA and PIA by GTP[S]

Membranes were prepared from 3T3-C_2 cells 7 days after culturing and from 3T3-F442A cells 15-16 days after culturing, at which time > 95% of the latter had differentiated. Adenylate cyclase activity was determined as described in the Materials and methods section. Where indicated, 10 μM-GTP[S] was added to the assay medium. The data show means ± S.D. for four experiments.

Cell type	Additions	Adenylate cyclase activity (pmol of cyclic AMP/min per mg of protein) Basal	GTP[S]
3T3-C_2	None	0.35±0.10	4.8±0.5
	1 μM-NECA	0.75±0.22	20.3±3.2
	1 nM-PIA	0.42±0.06	4.4±0.5
3T3-F442A adipocytes	None	1.76±0.20	12.2±1.9
	1 μM-NECA	1.86±0.24	31.0±1.5
	1 nM-PIA	1.60±0.20	8.3±1.2

teins are required for coupling of A_2 and A_1 receptors to the catalytic moiety of adenylate cyclase, as has been demonstrated in other tissues (Wolff *et al.*, 1981).

The results reported so far show that before differentiation the cells expressed only A_2 receptors, and that immediately after differentiation they contained both A_1 and A_2 receptors. Since adipocytes isolated from fat tissue contain A_1 receptors (Fain & Malbon, 1979), the fate of A_2 receptors in 3T3-F442A cells was studied after their differentiation to adipocytes. Over 90% of the cells in culture differentiated within 14 days after plating in the presence of insulin and fetal-calf serum, and the cells remained about 98% differentiated for at least another 15 days (Fig. 5*a*). Although the 3T3-F442A cells differentiated almost completely to adipocytes within 14 days, they required longer to lose a major part of their response to NECA (Fig. 5*b*). After 24 days in culture, and 10 days after differentiating, the cells showed a steady response to NECA, i.e. 1 μM-NECA increased cyclic AMP about 10-fold. Thus the effect of NECA was lowered 100-fold compared with preadipocytes.

The inhibitory effect of PIA in 3T3-F442A cells was expressed after 14 days of culture in the presence of insulin and fetal-calf serum, and the extent of inhibition induced by PIA did not change significantly for at least 14 days (Fig. 5*c*). In non-differentiating 3T3-C_2 cells, 0.001–0.01 μM-PIA had no significant inhibitory effect on the increase in cyclic AMP caused by forskolin (results not shown). The stimulatory effect of NECA on cyclic AMP was not significantly altered for up to 25 days of culture (Fig. 6). These results suggest that the decrease in stimulation of adenylate cyclase of adipocytes by NECA was not due to the culture period by itself, and that it is related to the differentiation of the cells.

The present study demonstrates the occurrence of adenosine receptors before and 14 days after differentiation of 3T3-F442A cells to adipocytes. The expression of inhibitory adenosine receptors occurs during the early phase of differentiation of the fibroblasts (preadipocytes) to adipocytes. The decline of the stimulatory adenosine receptors in the newly differentiated cells is slow, possibly

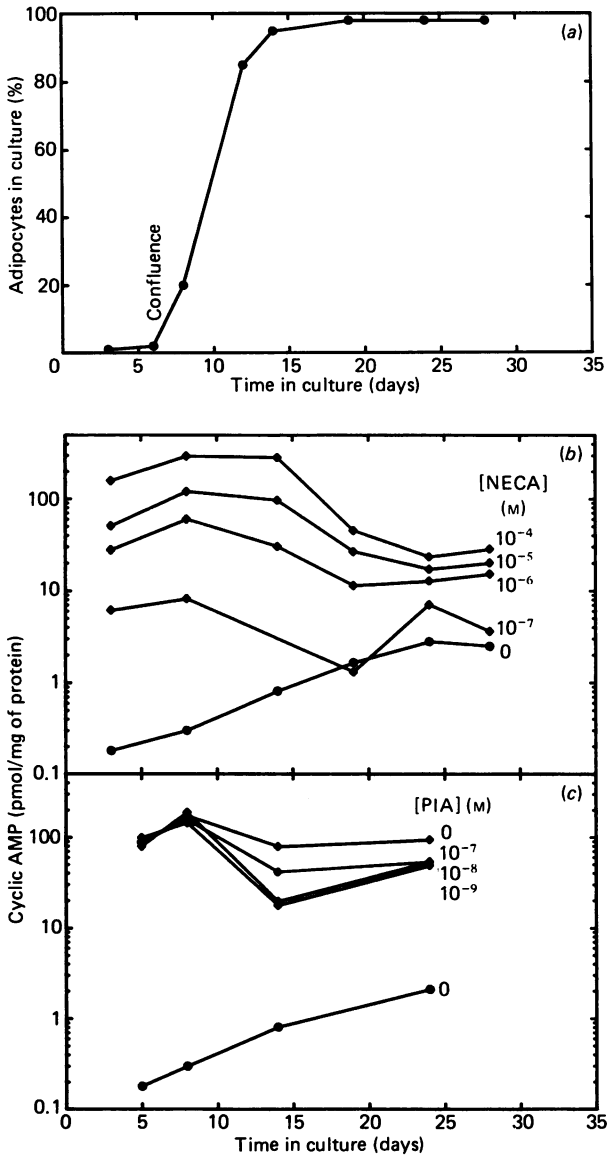

Fig. 5. Changes in cyclic AMP content during and after the differentiation of 3T3-F442A cells to adipocytes

Cells were cultured (2×10^4 cells/35 mm Petri dish) in the presence of insulin (5 μg/ml) and fetal-calf serum, as described in the Materials and methods section. The same batch of cells was used for the experiments described in (*a*), (*b*) and (*c*). (*a*) Percentage of adipocytes; (*b*) cyclic AMP formed in response to exposure to various concentrations of NECA at 37 °C for 5 min; (*c*) cyclic AMP formed in cells incubated at 37 °C for 5 min in the presence of (●) no additions or (◆) 20 μM-forskolin and PIA as indicated. Results are from a representative experiment.

because of a long lifetime of these proteins. The finding that NECA still has a stimulatory effect on adenylate cyclase in the adipocytes 10–14 days after differentiation, although to a lower extent than in preadipocytes, may be attributed to one or more of the following: (*a*) the small percentage of non-differentiated cells still found 2 weeks after differentiation (Fig. 5*a*) is responsible for at least part of the observed stimulatory effect of NECA on adenylate cyclase; (*b*) the adipocytes may develop A_2 receptors with a lower affinity for NECA than those

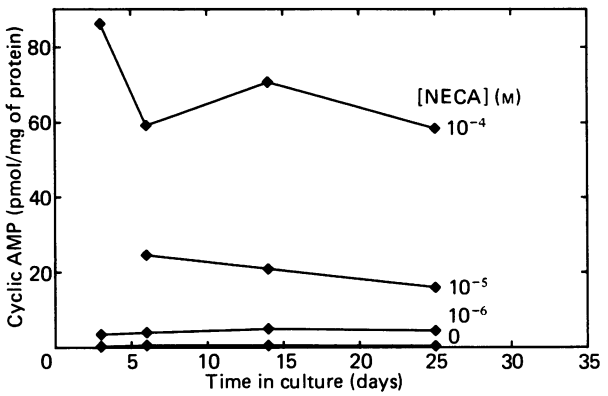

Fig. 6. Effect of NECA on cyclic AMP content of 3T3-C_2 cells as a function of time in culture

The cells were incubated in the presence of NECA as indicated at 37 °C for 5 min. For other details see the Materials and methods section. Results are representative of four separate experiments.

occurring in preadipocytes, but with an affinity still in the micromolar range; (*c*) the adipocytes may have preserved a small amount of preadipocyte-type A_2 receptors. The last two possibilities are in accord with the finding of Garcia-Sainz & Torner (1985), who demonstrated the existence of A_2 receptors in fat-cells.

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