shifted toward higher or lower concentrations (Fig. 6); there is therefore no competition between ammoresinol and uncouplers for the binding capacity of the membrane.

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ACTIVITY OF ADENYLATE CYCLASE AND PHOSPHODIESTERASE IN NORMAL AND COLD-RESISTANT MOUSE L-FIBROBLASTS

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Mouse L-fibroblasts as well as the more rapidly growing, cold-resistant LC sublines derived therefrom display differences in the activity of enzymes degrading and synthesizing cyclic adenosine 3',5'-monophosphate. The cold-resistant subline shows a lower activity of phosphodiesterase and a higher one of adenylate cyclase. Adenylate cyclase of both sublines is very sensitive to activating effects of sodium fluoride and prostaglandin E_1. The basal enzyme activity is markedly increased after several hours of latency if the cultures are exposed to choleragen. The effect is probably not due to synthesis of new molecules of the enzyme protein in the cell membrane.

Tissue cultures are used for studying regulatory mechanisms at the cellular level. Repeated exposure of cultures of mouse L-fibroblasts to +4°C results in a cold-resistant subline[1] designated LC. The subline thus derived differs from the original culture mainly in an increased growth rate. Metabolic differences between the original cell population and the derived subline LC were examined by a number of authors. All the enzymes investigated so far display a markedly higher activity in the cold-resistant population[2-4].

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) is a mediator of a number of cell functions in eukaryotic cells. The present paper is devoted to the activity of adenylate cyclase which catalyzes the synthesis of cyclic AMP and, at the same time, the activity of the enzyme breaking down this nucleotide, *viz.* phosphodiesterase, both in normal L-fibroblasts and in the more rapidly growing cold-resistant LC subline. Furthermore, we attempted to establish whether the activity of adenylate cyclase in these cell populations can be increased by adding cholera toxin which is known to stimulate the activity of this enzyme in various tissues[5].

EXPERIMENTAL

Chemicals. Medium for tissue cultures TM Sevac II (Parker) and inactivated calf serum were prepared in the Institute of Sera and Vaccines in Prague. Highly purified cholera-exo-enterotoxin, choleragen, No 0572, was prepared[6] under contract for the National Institute of Allergy and Infectious Diseases, by Dr R. A. Finkelstein, University of Texas Southwsteern Medical School, Dallas, Texas. Prostaglandin E_1 was a kind gift from Dr E. Pike, Upjohn Co., Kalamazoo,

Michigan. Adenosine 5′-triphosphate (trisodium salt), 2-phosphoenolpyruvic acid (tricyclohexylammonium salt), pyruvate kinase from rabbit muscle (300 I.U. per ml, per 9·3 mg protein), myokinase from rabbit muscle (660 I.U. per mg protein) and the venom of *Ophiophagus hanah* were products of Calbiochem, Lucerne; cyclic adenosine 3′,5′-monophosphate was from Lachema, Brno; puromycin and actinomycin D were made by Serva. Neutral alumina for chromatographic adsorption analysis according to Brockmann (II) was a product of Reanal, Dowex 1, (AG 1—X2, 200—400 mesh, Cl form) was from Calbiochem. Adenosine 5′-triphosphate-[α-^{32}P] as sodium salt (500—3000 mCi/mmol), cyclic 3′,5′-adenosine monophosphate-[^{3}H] (24 Ci/mmol) were from the Radiochemical Centre, Amersham.

Cell cultivation. Monolayer cultures of L-fibroblasts were grown in the TM Sevac II medium containing 10% calf serum. The antibiotics were added at a concentration of 100 μg penicillin and 100 μg streptomycin per ml medium. During transfers the cells were released from the glass surface with a 0·2% solution of trypsin in an isotonic phosphate buffer. Three days after the last transfer the cultures were used for experiments. They were scraped off the glass with a rubber scraper.

Adenylate cyclase activity was measured as described before[7], using 10^{-4}M-ATP-[α-^{32}P] (1 μCi) The final volume of the incubation mixture was 50 μl and contained 25—35 μg enzyme protein[8]. The reaction was stopped by adding 1 ml of 0·05 M-HCl which contained 50 μg of nonradioactive cyclic AMP. The cyclic AMP-[^{32}P] formed was isolated[9] on a column of neutral alumina (4 . 0·6 cm). Before application to the column, the reaction mixture was boiled for 5 min and the volume of the sample applied was 1 ml. The cyclic nucleotide was eluted with 2·5 ml 0·1M-Tris-HCl buffer, pH 7·5. Recovery of absorbance was measured at 260 nm and radioactivity of ^{32}P was assayed by Cherenkov's radiation[10]. The enzyme activity was expressed in pmol cyclic AMP formed per 20 min per mg protein.

Phosphodiesterase activity was determined according to Brooker and coworkers[11] under conditions described before[7] in the presence of cyclic AMP-[^{3}H] and nonradioactive cyclic nucleotide as substrate. After 10 min of incubation at 30°C the reaction mixture was combined with the snake venom and incubated for further 10 min so as to convert the 5′-AMP-[^{3}H] formed to adenosine-[^{3}H]. The reaction was stopped by adding 1 ml Dowex 1—X2 with ethanol (1 : 1, v/v) which quenches the radioactivity present in the nucleotides. The radioactivity was measured by using naphthalene–dioxane scintillation liquid (SLD-31). The level of radioactivity was taken to correspond to the activity of phosphodiesterase present and was expressed as nmol cyclic AMP consumed in 20 min per mg of protein sample.

RESULTS

The activity of phosphodiesterase was determined in a crude homogenate of normal and cold-resistant fibroblasts in the presence of four different concentrations of cyclic AMP as substrate. The most pronounced difference in the enzyme activities of the two sublines examined was observed at the highest concentration of substrate (Fig. 1) when the activity of the control cells is higher than in the cold-resistant subline. As the substrate concentration decreased, the difference in the enzyme activities diminished until at 0·1 mM the phosphodiesterase activity was the same in both sublines.

Preliminary results[12] showed that the difference in the basal activity of adenylate cyclase in normal and cold-resistant L-cells is very small. Hence we examined the

effect of compounds influencing the activity of the enzyme in the two cell sublines. First of all, the activity of adenylate cyclase was assayed in the presence of sodium fluoride which increases the activity of the enzyme in all eukaryotic cells. In the present experiments, sodium fluoride brought about a several-fold increase of the activity of adenylate cyclase in both cell types. A striking stimulatory effect was also displayed by prostaglandin E_1 (PGE_1). Neither epinephrine nor glucagon was effective. A striking rise of activity was brought about by choleragen which is known to increase the basal activity of adenylate cyclase[13,14]. In contrast with the above-mentioned compounds, choleragen was added directly to the growing culture. After 5 min of exposure of the culture to the toxin, a fresh cultivation medium was added and the cells were cultivated further, usually for 24 h.

Fig. 2 summarizes the results documenting the effect of PGE_1 and sodium fluoride on adenylate cyclase in homogenates of control L-fibroblasts and of cultures exposed to the effect of choleragen. Sodium fluoride increased the activity of adenylate cyclase in control fibroblasts almost eight-fold, PGE_1 ($2{\cdot}5 \cdot 10^{-5}$M) more than six-fold. Cultures exposed to the effect of 1 μg choleragen had a basal activity three times higher than the control cells. Enzyme activity assayed in the presence of sodium fluoride was somewhat lower in cells exposed to the toxin as compared with the controls. The effect of prostaglandin was the same in control and in choleragen-treated cells.

In another series of experiments we examined the dependence of adenylate cyclase activity on choleragen concentration. Fig. 3 shows the rise of adenylate cyclase activity in cell homogenates, prepared from cultures to which increasing doses of choleragen were added. While in the presence of sodium fluoride the enzyme activity practically

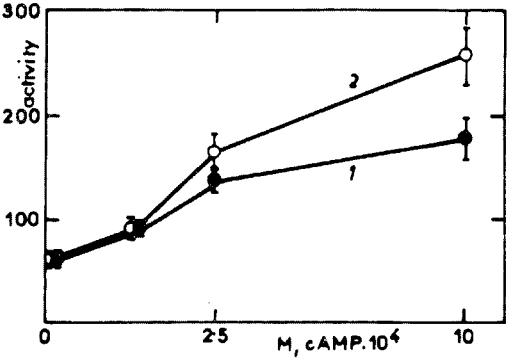

FIG. 1

Phosphodiesterase Activity in Normal and Cold-Resistant Mouse Fibroblasts

Cell homogenate (75—100 μg protein) was incubated for 20 min at 30°C in the presence of increasing concentrations of substrate. Each point is a mean from 5 estimations ± S.E. *1* LC cells; *2* L cells.

does not change as the concentration of choleragen is increased, the basal activity of adenylate cyclase increases already after the lowest dose of toxin applied (0·01 μg). When using 10 μg choleragen, the enzyme activity reaches values approaching those obtained in the presence of sodium fluoride.

Comparison of adenylate cyclase activity in normal and cold-resistant cells was performed both in the control group and in cells exposed to submaximal doses of choleragen (1 μg). The basal activity of adenylate cyclase in cold-resistant cells was insignificantly increased as compared with normal fibroblasts (Fig. 4) but the addition of sodium fluoride increased this difference to 40%. Exposure of the cultures to the effects of choleragen led in both sublines to a marked increase of basal activity which was again 30% higher in the cold-resistant subline. The activity of adenylate cyclase in the presence of sodium fluoride did not fundamentally differ in the cultures exposed to choleragen from the activity of the enzyme in control cultures.

Actinomycin D or puromycin brought about no substantial change in the basal activity of adenylate cyclase. The increase of basal enzyme activity caused by the choleragen was not affected by any of the inhibitors used (Table I). Likewise, the stimulatory effect of prostaglandin and sodium fluoride was not influenced by the presence of actinomycin D or puromycin either in control cultures or in cultures cultivated in the presence of choleragen. Changes in the activity of adenylate cyclase in cold-resistant cells corresponded to the enzyme activity in normal fibroblasts (not shown here).

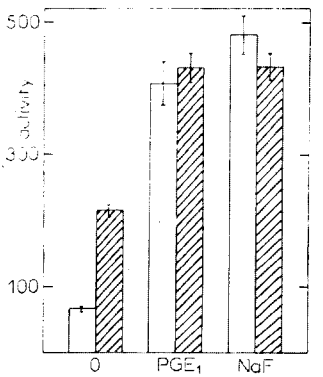

FIG. 2

Comparison of the Effect of Prostaglandin E_1 and Sodium Fluoride on Adenylate Cyclase Activity of Control L-Fibroblasts and Cultures Exposed to Choleragen

Monolayer cultures of L-fibroblasts were exposed for 5 min to choleragen (1 μg/ml) and, after discarding the toxin, cultivated for further 4 h in a fresh medium. The enzyme activity was measured in the cell homogenate in the absence of drugs in the presence of $2 . 10^{-5}$M-PGE_1 and of 10^{-2}M-NaF. The values represent averages from 7 estimations ±S.E. Blank columns, control cultures; hatched columns, cultures after exposure to choleragen.

DISCUSSION

Cyclic AMP plays an important role in the regulation of cell growth. With the exception of the stem cells of bone marrow where it accelerates growth, application of cyclic AMP usually retards growth and causes a morphological normalization of tumour cells[15]. It was found in the present experiments that the cold-resistant line of mouse L-fibroblasts, which also grows faster, has a lower activity of phosphodiesterase and a slightly higher activity of adenylate cyclase than cultures of normal fibroblasts. The findings might indicate a higher level of cyclic AMP in the cells of the cold-resistant subline.

When studying the differences in the activity of adenylate cyclase in L and LC cells we observed a pronounced activating effect of prostaglandin E_1. L-Fibroblasts and the cold-resistant LC subline derived therefrom represent another type of eukaryotic cells which is highly sensitive to the effect of choleragen. The reaction of the two cell populations to the toxin is analogous to that in previously studied tissues[13,14], *i.e.* the response of the enzyme to an addition of sodium fluoride does not change and it appears that we are dealing here merely with an increase of basal enzyme activity

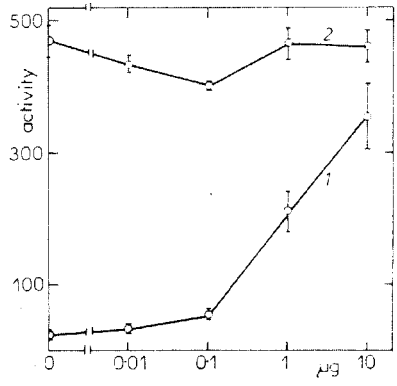

FIG. 3

Effect of Increasing Amounts of Choleragen on the Activity of Adenylate Cyclase in Cultures of Normal L-Fibroblasts

Equal amounts of L-cell suspension after trypsinization were transferred to 10 Roux bottles. After three days of cultivation, the individual bottles contained about 10^7 cells. After decanting the incubation medium, the cultures were covered either with 1 ml redistilled water or with 1 ml solution containing choleragen at concentrations of 0·01—10 μg/ml. After 5 min of treatment the toxin was removed and a fresh cultivation medium was added; the cultures were cultivated for further 24 h at 37°C. After this time, the cells were mechanically scraped off the glass and homogenized. The homogenate was assayed for the basal enzyme activity and for the activity in the presence of 10^{-2}M-NaF. The values shown are means from three estimations ±S.E. *1* Basal enzyme activity; *2* activity in the presence of sodium fluoride.

TABLE I

Effect of Actinomycin D and Puromycin on the Activity of Adenylate Cyclase Stimulated by Choleragen

Cell suspension after trypsinization was placed into Roux bottles so as to have 10^6 cells in each bottle. After 72 h of cultivation at 37°C, one-half of the bottles were exposed for 5 min to 1 μg choleragen. After removing the toxin, fresh medium was added and the cells were cultivated for further 4 h. Actinomycin D was added to the bottles to a concentration of 50 μg/ml medium; puromycin to a concentration of 500 μg/ml medium. The results represent means from 3 estimations ±S.D.

Addition	Exposure h	Adenylate cyclase pmol/20 min/mg
0	0	60·9 ± 2·5
Choleragen	1	79·9 ± 19·8
Choleragen	4	249·8 ± 24·3
Antinomycin D	4	89·5 ± 0·6
Choleragen + actinomycin D	4[a]	218·2 ± 12·5
Puromycin	3	73·3 ± 5·5
Choleragen + puromycin	4[b]	231·2 ± 11·4

[a] Actinomycin D was added together with the choleragen. [b] Puromycin was added 1 h after the choleragen.

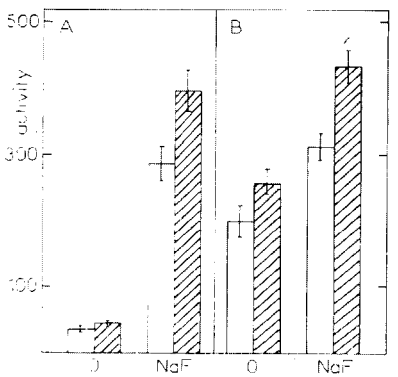

FIG. 4

Comparison of Adenylate Cyclase Activity in Normal and Cold-Resistant L-Fibroblasts

Cultures of normal and cold-resistant L-fibroblasts were exposed to 1 μg choleragen in 1 ml redistilled water and incubated in a fresh medium for further 24 h. Both groups were assayed for the basal activity of adenylate cyclase and for the enzyme activity in the presence of 10^{-2}M-NaF. The columns indicate the means of five experiments ±S.E. A Control cultures; B choleragen-treated cultures. Blank columns, L cells; hatched columns, LC cells.

without a more pronounced alteration of the PGE_1 effect. The high sensitivity of these cultures to cholera toxin might suggest it for application as a homogeneous material not only for the testing of the action of the choleragen but also for the analogously acting toxin from *Escherichia coli*[16].

The mechanism of action of cholera toxin is not yet clear. A several-hour latency period before the reaction sets in might indicate that new proteins are being synthesized during that time. The present experiments, with the use of relatively high doses of actinomycin D and of puromycin, indicate that choleragen neither markedly increases DNA synthesis nor affects the formation of the protein. The findings are in full agreement with the observation of Cuatrecasas[17] in adipose tissue. The possibility of translocation of membrane proteins represents one of the further possible explanations of the effect of choleragen. The problem is being now solved morphologically, by investigating the structure of normal and choleragen-treated cells.

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