ENHANCED ADENOSINE 3':5' - MONOPHOSPHATE RESPONSE TO BETA-ADRENERGIC STIMULATION IN CYSTIC FIBROSIS FIBROBLASTS AFTER REMOVAL OF CONDITIONED MEDIUM

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SUMMARY

Skin fibroblasts derived from 6 patients with cystic fibrosis (CF), 1-6 months old, and from 6 age matched donors were investigated for their ability to accumulate cyclic adenosine 3':5'-monophosphate (c-AMP) in response to isoproterenol and prostaglandin E₁ (PGE₁) using strictly defined culture conditions. In order to obtain, as far as possible, constant protein content and cell number, cultures were synchronized in the early G₁ phase of the cell cycle by growing them in serum free medium before adding stimulating drugs. There were no statistically significant differences both in basal c-AMP or after incubation with theophylline alone. When cultures were thoroughly washed prior to stimulation, c-AMP accumulation in response to isoproterenol was consistently higher ($p < 0.001$) in CF than in normal fibroblasts, whereas response to PGE₁ did not differ significantly. This difference in response cannot be attributed to differences in dose- or time-response curves, or to differential escape of cAMP into the culture medium. Returning the conditioned media (CM) to the cultures after the washing procedure, or omitting the washing procedure altogether, normalized the cAMP response of CF cells. These data indicate that CF fibroblasts "delete" or "add to" the conditioned medium a substance when washed out of the cultures leave the cells hypersensitized to β -adrenergic stimulation.

SPECULATION

The enhanced cAMP response to isoproterenol stimulation of CF-cells is most likely related to the washing off of a soluble substance which is secreted or deleted into the culture medium in excess of normals. This "factor" could exert its effect in the manner of an antagonist regulating catecholamine responsiveness as a part of a negative feed back loop.

Water, electrolyte and macromolecular secretions of exocrine cells are controlled by the action of neurotransmitters of the autonomic nervous system (ANS) (11). Since in CF the disturbance of the exocrine secretions is one of the main symptoms, ANS regulated functions have repeatedly been investigated. Clinical observations in CF patients have demonstrated abnormal pupillary reactions (28) and a clearing of the abnormal turbidity of the saliva after guanethidine treatment, which is an antiadrenergic agent (9). Furthermore a paradoxical discordance between the pulmonary response of the CF-patients to isoproterenol and the response to oral theophylline has been shown (30). In rats, chronically treated with isoproterenol or reserpine, morphological and secretory changes of the salivary glands were reported that strongly resembled the ones observed in CF (31, 21, 22, 23). All these observations lend support to the concept that the primary defect in CF could be linked to a derangement of the ANS in the patients. Adrenergic neurotransmitters stimulate their target cells through specific binding to α and β -receptor sites on the surface of the cells. As a consequence of the binding to β -receptors, cAMP is formed from ATP by the activation of the enzyme adenylate cyclase present in the plasma membranes of the cells. The synthesis of cAMP secondarily induces a chain of biochemical events of the cell to the external stimulus (e.g.) the secretion of amylase in the salivary glands (7) or the sodium and the bicarbonate transport in the exocrine pancreas (8). The presence of β -receptors and adenylate cyclase has been demonstrated in many eucariotic cells, including cultured human diploid fibroblasts. Therefore it seems likely that a genetic disturbance of this mechanism would also be expressed in cultured fibroblasts. On the cellular level in vitro the measurement of cAMP formation after β -adrenergic stimuli might be as relevant as the measurement of the final cellular response induced by the cAMP in vivo.

Hitherto, studies on cAMP responses to isoproterenol stimulation with cultured fibroblasts from CF patients have yielded contradictory results (5, 6, 10). It is difficult, however, to compare the results of the different studies since many factors can modify the cyclic nucleotide synthesis in cultured cells. Culture conditions such as passage number (14), cell density (16), time after subculture (20), serum concentration (19) and stage of the cell cycle (25) have been shown to influence significantly the response of the cAMP system. We therefore reinvestigated cAMP response to β -adrenergic stimuli in cultured fibroblasts from CF-patients and from normal controls using strictly defined culture conditions and examined the contribution of the conditioned culture medium to this system.

MATERIAL AND METHODS MEDIA, CHEMICALS AND HORMONES

Dulbecco's modification of Eagle's medium (D-MEM), Eagle's minimal essential medium (MEM) and trypsin were obtained from Gibco. Penicillin was a product of Novo. All experiments employed serum of the same lot (Seromed batch C-808595), 1-isoproterenol hydrochloride, 1-epinephrine bitartrate, 1-norepinephrine, dl-propranolol hydrochloride, theophylline and Dowex-resins were purchased from Sigma. PGE₁ was a generous gift of Dr. J.E. Pike (Upjohn Co.). Isoproterenol solutions containing ascorbic acid as antioxidant were prepared immediately before use and kept in the dark. PGE₁ was prepared at 10⁻³ M stock in absolute ethanol. At concentrations equivalent to that added with PGE₁ ethanol did not affect cAMP content.

CELL CULTURE

Skin biopsies from CF children (aged between 1/12 and 6/12 yr) were obtained from unrelated patients who had clinically confirmed CF with positive sweat tests. Control fibroblast strains were derived from age matched donors without personal or family history of CF (Table 1). Stock cultures were maintained for up to two weeks in 250 ml plastic culture flasks (Falcon) containing 12 ml growth medium consisting of Eagle's MEM supplemented with penicillin (100 units/ml), 1% (v/v) non-essential amino acids and 10% (v/v) fetal calf serum. The nutrient medium was renewed every 3 to 4 days and the cells were maintained at 37°C in a humidified 95% air - 5% CO₂ atmosphere.

EXPERIMENTAL INCUBATION PROCEDURE

Confluent monolayers of cells from stock cultures, matched for passage numbers, were harvested by incubation with 0.25% trypsin, and samples of each cell-line were counted on a Coulter-Counter equipped with a 100 μ m aperture, using electronic parameters that gave values identical to hemocytometer values. Fibroblasts from different strains were diluted to similar concentrations, plated on 60 mm dishes in 4 ml Eagle's MEM containing 10% fetal calf serum at a density of 15 x 10⁵ cells/cm² and grown for 24 hr. Thereafter growth was arrested by a cell synchronization procedure based on serum deprivation (27): Cultures were shifted from regular growth medium to serum-free D-MEM supplemented with 0.25% bovine serum albumin (BSA) but without antibiotics and maintained in this medium for 4 days. Except where noted, the fibroblasts were carefully washed three times with 4 ml serum-free D-MEM before addition of drugs and then incubated in a fresh 4 ml volume of this same medium together with appropriate drugs for 10 minutes at 37°C. Basal cAMP concentrations were analysed in cultures run in parallel.

ASSAY OF CYCLIC AMP

Incubations were terminated by the rapid removal of the medium, by rinsing with ice-cold PBS solution and addition of 2 ml of ice-cold ethanol-0.2N HCl. Cells were collected by scraping the cultures with a rubber-policeman and the acid extract was stored overnight at -20°C. After centrifugation at 1 000 g for 10 min to remove cellular debris, the supernatant fluids were dried in vacuo and assayed for cAMP in duplicate by the protein-binding method of Tovey et al. (32). Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as standard. In selected experiments cAMP in samples of incubation media was analyzed using a purification procedure as described by Kelly (16). Specificity of the assay was supported by disappearance of the binding activity in the extracts after phosphodiesterase digestion.

STATISTICAL ANALYSIS OF DATA

Because of the large variation in stimulated cAMP values, which was much larger between different experiments than within a single experiment, experiments were planned and performed with pairs of matched CF and normal cell-lines within the same experiment. The data were expressed as the absolute values and the differences between the two types of cells were evaluated by the paired-t-test.

RESULTS

STANDARDIZATION OF THE CULTURE CONDITIONS

In tissue culture c-AMP response to catecholamines is thought to be influenced by the degree of cell to cell contact (16). As shown in Fig. 1 we found an inverse relationship between the cell density and the c-AMP response to isoproterenol, which appears to be more pronounced in CF cells than in normal cells. In order to keep the variation of the cell density as small as possible we deprived the cultures of serum which is reported to result in growth arrest and synchronization of the cells in the early G₁ phase of the cell cycle (27). The effect of withdrawal of serum on the growth of normal and CF fibroblasts is shown in Fig. 2. In serum-free medium the cells cease to grow but remain healthy and viable for more than a week. In both phenotypes studied, incor-

poration of radiolabelled thymidine by growth arrested cells at day 4 was only 5-8 % of the controls growing in 10 % fetal calf serum (results not shown). Application of this technique made it possible that in individual experiments the protein content of the cultures varied only within a 10 % range. Between different experiments however, it proved difficult to keep the protein content standardized (Range: 0.13 - 0.29 mg / culture).

BASAL CYCLIC AMP-VALUES

Measurements of basal c-AMP values performed at the end of the synchronization period (day 4) revealed no significant differences ($p > 0.3$) between the normal and CF strains (range: 2.16 - 8.13 pmoles/mg of protein). Incubation of cells for 10 min with $5 \cdot 10^{-3}$ M theophylline alone only slightly increased the basal c-AMP concentrations (range: 3.12 - 10.12 pmoles/mg of protein). Again no statistically significant difference was found between the two types of strains ($p > 0.2$).

STIMULATED CYCLIC AMP VALUES

KINETICS OF THE C-AMP RESPONSE TO STIMULATION

The c-AMP synthesis in response to increasing concentrations of isoproterenol is shown in Fig. 3 A. Stimulation was maximal with 10^{-6} M isoproterenol. Although the shape of the dose-response curve was similar for normal and CF cells, the magnitude of response was less in the normal strain.

With increasing periods of incubation with isoproterenol c-AMP concentrations reached a plateau within 5 min in both normal and CF fibroblasts (Fig. 3B). In the presence of theophylline these levels are well maintained throughout the period of observation. The c-AMP synthesis in response to PGE₁ stimulation was similar in shape but up to 10 times higher than that seen with isoproterenol stimulation, reaching a maximal level at 10^{-6} M PGE₁ after a 10 min incubation. Thus, for all subsequent experiments an incubation period of 10 min duration was chosen. In the incubation medium of both types of strains the amount of c-AMP was less than 10 % after isoproterenol and less than 5 % of total c-AMP after PGE₁ stimulation. Thus, at the indicated time the extracellular compartment did not contribute significantly to the entire response of the cells.

c-AMP ACCUMULATION IN RESPONSE TO ISOPROTERENOL AND TO PROSTAGLANDIN E₁

A marked difference between the paired normal and CF strains became apparent in their ability to augment the intracellular level of c-AMP in response to isoproterenol stimulation (table 2). Except for one fibroblast strain (18 out of 20 pairs), CF fibroblasts generated larger amounts of c-AMP per mg of protein than the corresponding normal strain ($p < 0.001$). Calculating the data in terms of stimulation ratio (ratio of total c-AMP accumulated to basal c-AMP) instead of absolute values did not alter this result. In order to check the reproducibility of the results some pairs of normal and CF fibroblasts were compared in two or three different experiments. As is shown in Fig. 4 the percentage difference between a given pair of normal/CF was quite reproducible. In contrast to those obtained with isoproterenol the paired differences of the PGE₁ stimulated values revealed random distribution with no statistically significant differences (Table 3, Fig. 4).

INFLUENCES OF CONDITIONED MEDIUM ON RESPONSIVENESS TO ISOPROTERENOL

Several abnormalities have been found in conditioned media of CF fibroblasts (2, 4). Therefore, in order to investigate whether an abnormal extracellular "factor" secreted by the cells could account for the observed difference in adrenergic responsiveness, defined variations in culture conditions have been introduced (Fig. 5). In cultures that were treated as in an earlier experiments c-AMP accumulation was enhanced in CF (condition A). If, however, the 4 days conditioned medium was reintroduced instead of fresh medium (condition B) the increased c-AMP response of CF-cultures was practically normalized. The same reversal of response to normal could be observed in cultures where isoproterenol was added directly to the conditioned medium omitting the washing procedure altogether (condition C). The reduction of cAMP response in the presence of conditioned medium (condition A/B, A/C) in CF strains was highly significant ($p < 0.001$) whereas in normal strains a small reduction of the cAMP response was found which reaches statistical significance only in A/C ($p < 0.05$).

DISCUSSION

Under experimental conditions the measurement of cAMP synthesis provides a reliable and direct measure of β -adrenergic stimulation. However, in tissue culture the results between different strains can only be meaningfully compared if variables influencing β -adrenergic responses are kept as constant as possible. Studies in the intact human organism and in isolated human organs have established alterations in responsiveness to catecholamines (33) as well as reduced β -adrenergic receptor concentrations with increasing age (29). Thus, if donor fibroblasts preserve their typical pattern of β -receptors throughout the period of cultivation, the age of donor fibroblasts could possibly influence adre-

nergic responsiveness of cultured cells. Cultured fibroblasts appear to be more sensitive to catecholamines with increasing number of passages (14). Therefore in this study all fibroblast strains were from donors of about the same age and were used within the same passage number. Growth rates of CF fibroblasts have been reported to be different from normals (1) which could lead to differences in cell density and the stage of the cell cycle. Both parameters have been reported to influence hormone stimulated cAMP response of cultured cells. cAMP responses were found to be minimal at the time of mitosis, reaching a maximal value in the early G₁ phase of the cell cycle (25). Further, there is clear evidence to suggest an inverse relationship between the magnitude of the response of fibroblasts to catecholamines and the cell population density (16). In order to overcome these growth related influences on cAMP metabolism, growth of the cells was arrested in the early G₁ phase of the cell cycle by a shift to serum free medium, prior to stimulation. This procedure allowed the cells to recover from the trypsin treatment, avoided any possible influence of fetal calf serum and yielded cultures with stable basal cAMP values and, as far as possible with constant and comparable cell number and protein content.

Our results indicate that under standard culture conditions CF fibroblasts generate markedly more cAMP in response to isoproterenol than the control cells. Since all experiments were performed under steady state conditions giving stable, maximal c-AMP responses (Fig. 3) this statistically highly significant difference could not be attributed to possible differences in the time- or dose-response. As evidenced from c-AMP contents in the culture media, a differential c-AMP escape into extracellular compartment can also be dismissed as a possible explanation for the results. Since the results were all obtained in the presence of theophylline at a concentration ($5 \cdot 10^{-3}$), which has been shown to suppress the activity of nucleotide-phosphodiesterase (3), a differential degradation of c-AMP can be ruled out. The increased c-AMP response to adrenergic stimulation being measured in CF cells might reflect either modification in the number (or accessibility) of β -receptor sites coupled to adenylate cyclase or modification in the catalytic activity of the enzyme. Therefore c-AMP was measured under basal conditions and after stimulation with PGE₁, which is assumed to activate the same adenylate cyclase by binding to a separate and distinct receptor (26). Since no significant differences were found under these conditions it is suggested that the observed differences were generated at the level of β -receptor or at the level of the β -receptor-adenylate cyclase coupling mechanism. Enhanced c-AMP response to isoproterenol in CF fibroblasts could only be observed if the cultures had been extensively washed prior to stimulation. The increased response reverted to normal in the presence of conditioned medium (CM) either by returning the CM or by omitting the washing procedure. Therefore these results raise the possibility that CM of CF fibroblasts may contain a substance in excess of normals which masks an altered responsiveness to isoproterenol in the manner of an antagonist.

In this respect it may be interesting to note that human fibroblasts have the ability for a self-regulation of catecholamine responsiveness. Repeated stimulation of fibroblasts with β -adrenergic agonists leads to a rapid decrease in the cellular response to the drug with a slow recovery within 24 hrs (12), probably reflecting a loss of β -receptors (24). In contrast β -adrenergic antagonists themselves do not cause desensitization but removal from contact with the cells may produce a hypersensitivity state (13, 17). Another system of self-regulation was described in rat adipocytes where the formation and release of a soluble antagonist was postulated to account for hormone unresponsiveness following repeated exposure to epinephrine (15). In this system sensitivity to the hormone could be restored immediately by washing the cells with new medium. The existence of a similar mechanism in fibroblasts would offer an intriguing explanation for our results and for some of the conflicting results reported in respect of c-AMP metabolism in cultured CF-fibroblasts. Further experiments are being initiated to characterize more precisely the mechanism and the susceptibility to adrenergic desensitization and resensitization of CF strains.

In conclusion we speculate that CF-fibroblasts delete or secrete into the medium a substance which can be washed out of the cultures and promotes a hypersensitization of CF cells to β -adrenergic stimulation.

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Table 1
Cell strains

Strain	Donor age	Sex	Site of biopsy	Reason for biopsy	Passage number*
CF ₁	3/12	f	interscapular	Donor	5 - 11
CF ₂	6/12	m	interscapular	Donor	5 - 11
CF ₃	3/12	f	interscapular	Donor	5 - 11
CF ₄	4/12	f	interscapular	Donor	5 - 11
CF ₅	6/12	m	interscapular	Donor	5 - 11
CF ₆	1/12	f	interscapular	Donor	5 - 11
N ₁	4/12	m	interscapular	Cardiomyopathie	5 - 11
N ₂	1/12	m	interscapular	Confirmation of normal prenatal diagnosis	5 - 11
N ₃	5/12	m	inguinal	Surgery	5 - 11
N ₄	1/12	f	interscapular	Cardiomyopathie	5 - 11
N ₅	1/12	f	inguinal	Surgery	5 - 11
N ₆	6/12	f	inguinal	Surgery	5 - 11

*For the experiments 1-6 (Table 2) cells from passage 5, for all other experiments cells from passage 10 and 11 were used.

Table 2

Effects of isoproterenol and of PGE₁ on cyclic AMP accumulation in normal and CF-fibroblasts (picomoles per mg of protein)

Trial Nr.	Pair	Isoproterenol (10 ⁻⁶ M)			Prostaglandin E ₁ (10 ⁻⁶ M)		
		CF	N	Δ ¹	CF	N	Δ ²
1	CF ₁ -N ₁	103	214	-111	671	1100	-429
	CF ₂ -N ₂	322	218	+104	1204	474	+730
2	CF ₃ -N ₃	415	146	+269	1518	1060	+458
	CF ₄ -N ₄	325	212	+113	469	984	-515
3	CF ₅ -N ₅	86	53	+ 33	1215	1077	+138
	CF ₆ -N ₆	107	75	+ 32	536	823	-287
4	CF ₁ -N ₁	32	35	- 3	825	1217	-392
	CF ₂ -N ₂	50	39	+ 11	1183	605	+578
5	CF ₃ -N ₃	298	116	+182	1286	677	+609
	CF ₄ -N ₄	174	85	+ 89	996	1923	-927
6	CF ₅ -N ₅	189	88	+101	1100	826	+274
	CF ₆ -N ₆	129	102	+ 27	930	1292	-362
7	CF ₄ -N ₁	312	245	+ 67	1 p < 0.001 2 N.S.		
	CF ₅ -N ₃	294	205	+ 89			
8	CF ₃ -N ₃	553	225	+328			
	CF ₂ -N ₆	446	186	+260			
9	CF ₃ -N ₁	444	196	+248			
	CF ₆ -N ₂	272	152	+120			
10	CF ₆ -N ₆	203	138	+ 65			
11	CF ₃ -N ₄	343	254	+ 89			

Legend: Data are given as mean values (SD range: ± 4 - 20 %) from three cultures in response to the indicated concentrations of hormones. Incubation were for 10 min. in presence of theophylline.

Figure 1

c-AMP formation in CF and normal fibroblasts: relationship to cell-density and maximal response to isoproterenol

Legend: Cells were seeded at indicated initial cell densities and grown as described under experimental incubation procedure. Incubations with isoproterenol (10⁻⁶M) were for 10 min. in presence of 5.10⁻³M theophylline. Values are means ± standard deviation from three cultures.

Figure 2

Effect of serum-deprivation on cell number in a normal and a CF-cell-strain

Legend: Cells were seeded at a initial density of 15 000/cm² in MEM. After 24 hr of incubation, part of the dishes were shifted from regular growth medium to serum-free medium supplemented with 0.25 % BSA. Cell number was determined at 24 hr intervals for cultures in 10 % FCS (○△) and for cultures shifted to serum-free medium (●▲).

Figure 3

c-AMP accumulation of normal (○) and of CF cell strains (●) as a function of concentration (A) and length of exposure to isoproterenol (B)

Legend: Incubations with isoproterenol (10⁻⁶M) were performed as indicated in the text in the presence of theophylline (5.10⁻³M). Values are means ± standard deviation from three dishes.

Figure 5

Cultures were stimulated for 10 min with isoproterenol, which was added in a small volume (0.2 ml) to give a final concentration of 10⁻⁶M. Within the same experiments 3 different culture conditions were compared:

- Legend: A) 4 days conditioned medium was removed and preserved, than the cultures were washed twice and isoproterenol was added together with fresh medium.
- B) Other cultures were treated identically with the one exception that their own conditioned medium instead of fresh medium was added together with isoproterenol.
- C) To additional cultures isoproterenol was added directly to the 4 days conditioned medium, without any washing procedures.

Data are from 3 experiments. Symbols indicate mean values from three cultures. The line between the symbols represents the paired results.

Figure 4

Isoproterenol and PGE₁ stimulated c-AMP values in CF fibroblasts
 Legend: c-AMP increase or decrease was expressed as the percentual difference in relation to the paired normal values. The absolute values are given in Table 2. Incubations with hormones (10⁻⁶M) were carried out for 10 min. in presence of theophylline (5.10⁻³M) as indicated under experimental incubation procedure.