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Abnormal levels of 3′:5′-cyclic AMP in isoproterenol-stimulated fibroblasts from patients with cystic fibrosis

(genetic disease/human diploid fibroblasts/β-adrenergic agonists)

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ABSTRACT **To determine if the abnormalities of exocrine secretion characteristic of cystic fibrosis could be investigated *in vitro*, I studied the synthesis of 3′:5′-cyclic AMP after isoproterenol stimulation in skin fibroblasts derived from patients with cystic fibrosis and from normal individuals. Comparison of normal and cystic fibrosis cells showed that the latter had 2- to 5-fold greater levels of intracellular cyclic AMP after stimulation with isoproterenol. The difference between the strains was observed at every stage of the culture cycle and was specific to stimulation by β-adrenergic agonists. It could not be accounted for by different dose- or time-response curves nor by leakage of cyclic AMP into the medium. The increased sensitivity to catecholamines may reflect an intrinsic genetic property of cystic fibrosis cells, and it may be feasible to use this system to study the biochemical basis of the genetic defect in cystic fibrosis.**

Cystic fibrosis (CF) is the result of homozygosity for an autosomal gene, and there may be more than one genetic type (1). Skin fibroblast strains derived from patients have been used to study the biochemical defect in this disease. Danes and Bearn have defined two or three classes of CF fibroblast strains based on metachromatic staining (2). Several laboratories (3–6) have shown that the spent medium from such cultures contains a factor, the ciliary dyskinesia factor, which disrupts normal ciliary motion in several tissues (7, 8). Also, there have been reports that CF fibroblast strains and lymphoid cell lines may differ from normal ones with respect to the activity of lysosomal enzymes (9, 10), glycogen content (11), mucopolysaccharide metabolism (12), and some ultrastructural features (13). However, these results have not provided a clear-cut indication of the biochemical basis of the defect in CF.

An alternative approach has been to study the nature and control of exocrine secretions in CF patients. Chernick *et al.* (14) observed 2- to 5-fold increases in submaxillary amylase and ribonuclease and, more recently, Bloomfield and her coworkers have shown elevations in calcium, protein, and amylase in both submandibular (15) and parotid secretions (16). These results support the suggestion (17) that the CF phenotype may be due to hyperactivity of the autonomic nervous system, a hypothesis also supported by the observation that chronic treatment of rats with isoproterenol (18) or reserpine (19) causes morphologic and physiologic changes in parotid and submaxillary glands that resemble those seen in CF patients.

Strong evidence implicates 3′:5′-cyclic AMP (cAMP) as a crucial component of the secretory response of a wide variety of mammalian cells to hormonal stimulation (20, 21). Human diploid fibroblasts have been shown to possess the elements necessary for the control of cAMP metabolism and to possess cell surface receptors capable of recognizing certain agents that raise intracellular cAMP levels (22–27). Since such cells from CF patients are known to secrete the anticiliary factor (3–5), it seemed possible that they might also demonstrate an abnormality in the hormonal control of cAMP metabolism that might be relevant to the regulation of the secretory process in these cells. To examine this question, I have studied the effect of isoproterenol on intracellular cAMP levels and I report here the observation that cultured skin fibroblasts from patients with CF synthesize about 3-fold more cAMP after stimulation with isoproterenol than do cells from normal donors.

Abbreviations: CF, cystic fibrosis; cAMP, adenosine 3′:5′-cyclic monophosphate.

MATERIALS AND METHODS

Origin and Handling of Strains. The origins of the skin fibroblast strains used in this work are given in Table 1. Strains were started from skin biopsies as described by Goldstein and Littlefield (28). Cell culture was always done with α-medium (29), lacking antibiotics, ribosides, and deoxyribosides, and supplemented with 15% fetal calf serum. Strains were subcultured by trypsinization, frozen in culture medium with 10% glycerol, and stored in liquid nitrogen. Strains were found uncontaminated when tested for the presence of mycoplasma by microbial culture methods (P. Quinn, manuscript in preparation) at several different times during their growth.

Reagents. The chemicals used and their sources were: Tris·HCl, cAMP, L-isoproterenol·HCl, L-epinephrine bitartrate, DL-propanolol, and Hepes [(*N*-2-hydroxyl)ethylpiperazine-*N*′-2-ethanesulfonic acid] (Sigma); Dowex resins (Bio-Rad); [*G*-^{3}H]cAMP, 38 Ci/mmol (New England Nuclear), Millipore filters (Millipore Canada); Ro20-1724 (Dr. H. Sheppard, Hoffmann-La Roche); prostaglandin E_1 (Dr. J. E. Pike, The Upjohn Co.); all other chemicals (Fisher).

Measurement of cAMP in Cells and Medium. Cyclic AMP was measured using the method of Gilman (30). The inhibitor and binding protein were prepared as described in that paper. The latter had a specific binding activity of 0.042 pmol of cAMP bound per ng of protein. Standards were prepared fresh each day from frozen stocks of a solution of 1 mg/ml of cAMP. The [^{3}H]cAMP was purified through a Dowex 50-H^+ column before use (31). The binding assays were done in a total volume of 0.05 ml in 0.04 M sodium acetate buffer at pH 4.0 using 1 pmol of [^{3}H]cAMP, 4 ng of binding protein, 27 ng of inhibitor protein, and standards or unknowns, in each reaction tube. Cells for cAMP measurements were grown from 1:8 subcultures in 60-mm dishes and were stimulated with catecholamines according to the protocol used by Gilman and Minna (31). The phosphodiesterase inhibitor Ro20-1724 (32) was added as a solution in dimethylsulfoxide. The final concentration of the solvent (1%) does not affect intracellular cAMP levels. The cells were processed for cAMP assays as described by Gilman (30); the separated medium was processed (when necessary) according to Gilman and Minna (31). Recoveries of cAMP were monitored by adding a small amount of [^{3}H]cAMP at the beginning of the purifications and were generally 60–90%. Each sample was assayed at two different dilutions in triplicate and

Table 1. Source of strains

Strain	Age (years)	Sex	Site of biopsy	Reason for biopsy
Cystic fibrosis strains				
CF 22	6.5	M	Forearm	Donor*
CF 32	13	M	Forearm	Donor*
CF 35	12	M	Forearm	Donor*
CF 58	11	M	Forearm	Donor*
CF 59	13	M	Forearm	Donor*
Normal strains				
N 28	11	M	Forearm	Donor; cystinuria type II
N 54	22	M	Forearm	Donor
N 162	13	M	Thigh	Plastic surgery for burn
N 174	12	M	Neck	Plastic surgery for cosmetic reasons
N 412	11	M	Forearm	Donor†

* These donors were patients of Dr. D. N. Crozier, Director of the Cystic Fibrosis Clinic, Hospital for Sick Children, and had clinical findings consistent with the diagnosis of CF.
† Provided by Dr. S. Goldstein, McMaster University, Hamilton, Ontario.

gave proportional results. Cells from parallel plates were counted on a Celloscope counter (Particle Data) after trypsinization or used for measurements of total cell protein (33).

RESULTS

Preliminary experiments in which cells from patients with CF and from normal donors had been exposed to isoproterenol showed significant differences in the intracellular cAMP levels of these two cell strains. To compare properly the effect of isoproterenol on these cells, I explored various aspects of cAMP metabolism in more detail. In many of these experiments the phosphodiesterase inhibitor Ro20-1724 was used to increase the amount of intracellular cAMP accumulated during stimulation. In the absence of isoproterenol stimulation, the phosphodiesterase inhibitor had no effect on the intracellular cAMP levels of either normal or CF strains.

Effect of time of exposure to isoproterenol

Stimulation of fibroblasts with catecholamines results in rapid changes in intracellular cAMP levels (22–26). The accumulation of intracellular cAMP after isoproterenol addition was compared in two normal and two CF strains. Fig. 1 shows the data for strains N 162 and CF 22. No differences in the time course of cAMP accumulation were seen, even though the CF strain produced five times the amount of cAMP. Strains N 412 and CF 32 gave essentially the same results. Both types of strains had maximal cAMP levels at 15 min, and all subsequent experiments were done with 15-min incubations with isoproterenol. These results are similar to those reported with normal strains (23, 26).

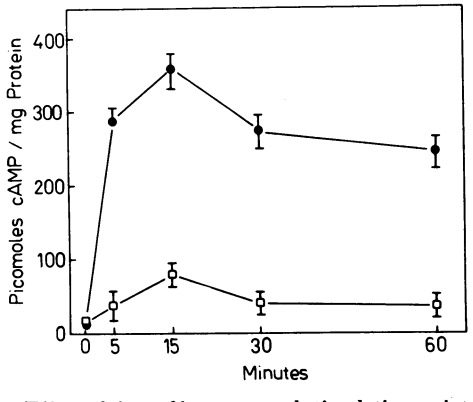

FIG. 1. Effect of time of isoproterenol stimulation on intracellular cAMP levels in normal and CF cells. Duplicate plates of strain N 162 (□) (25th generation, 1.0×10^6 cells, 0.48 mg of protein) and strain CF 22 (●) (25th generation, 0.90×10^6 cells, 0.48 mg of protein) were exposed to 10^{-6} M L-isoproterenol for the appropriate time in the presence of 7×10^{-4} M Ro20-1724, and then assayed for cAMP. In this and the next figure the bars represent the range of the measurements.

Effect of concentration of isoproterenol

The above experiments were done at high concentrations of isoproterenol (10^{-6} M). I also investigated the effect of isoproterenol concentration on intracellular cAMP levels to determine if the two types of cell strains had different hormone concentration response profiles. As is shown in Fig. 2, strains N 162 and CF 22 reached maximal levels at approximately 10^{-7} M isoproterenol and did not change at higher concentrations. The shape of the dose-response curve was similar for both strains, and the CF cells synthesize more cAMP at all isoproterenol concentrations measured. Strains N 54 and CF 58 behaved similarly. Other workers have observed similar dose-response curves with normal strains (26, 27).

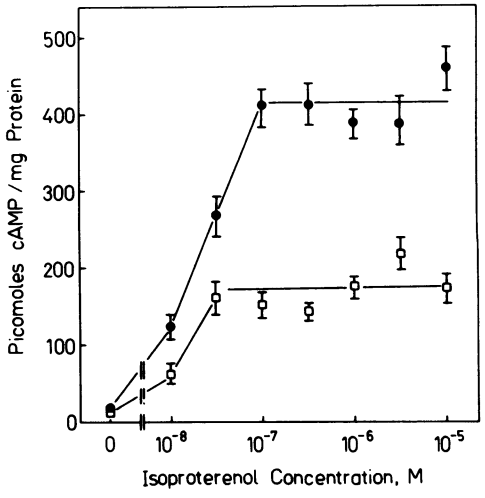

FIG. 2. Effect of isoproterenol concentration on the amount of intracellular cAMP in normal and CF cells. Duplicate plates of strain N 162 (□) (25th generation, 1.0×10^6 cells, 0.48 mg of protein) and strain CF 22 (●) (25th generation, 0.95×10^6 cells, 0.50 mg of protein) were exposed to the appropriate concentration of L-isoproterenol for 15 min in the presence of 7×10^{-4} M Ro20-1724 and then assayed for cAMP.

Table 2. Effect of isoproterenol on intracellular cAMP levels as a function of culture cycle

Strain	Time*	mg of protein/plate†	cAMP levels: Without inhibitor: cAMP‡	Mean ± SD	CF/N	P§	With inhibitor: cAMP‡	Mean ± SD	CF/N	P§
N 28		0.155	—				200			
								215 ± 30		
N 162		0.105	—	—			230			
	43								2.1	<0.01
CF 22		0.125	—		—	—	360			
								455 ± 115		
CF 32		0.125	—	—			550			
N 28		0.27	210				880			
				210 ± 10			—	880 ± 15		
N 162		—	—							
	90				2.5	<0.01			1.7	<0.02
CF 22		0.19	515				1600			
				535 ± 50				1480 ± 190		
CF 32		0.245	555				1360			
N 28		0.29	55				320			
				60 ± 15				375 ± 75		
N 162		0.24	65				430			
	163				1.9	<0.01			2.0	<0.001
CF 22		0.29	125				760			
				115 ± 15				745 ± 50		
CF 32		0.36	105				730			
N 28		0.51	30				265			
				30.5 ± 3.1				230 ± 40		
N 162		0.50	31				195			
	235				1.9	<0.001			2.6	<0.001
CF 22		0.55	59				565			
				57.5 ± 2.6				600 ± 50		
CF 32		0.66	56				635			

* Hours after subculture when cells were exposed to 10^{-5} M L-isoproterenol for 15 min in the presence or absence of 7×10^{-4} M Ro20-1724.
† Protein assays were done on triplicate plates at two dilutions with a standard error of less than 5%.
‡ cAMP was assayed at two dilutions on duplicate plates. Values are in pmol/mg of protein with a standard error of less than 10%.
§ *P* values were determined using Student's *t* test.

Comparisons of normal and CF strains

In comparing strains it was important to determine whether such comparisons depended on the time after subculture that cells were stimulated with isoproterenol (see ref. 26). Thus, the studies of intracellular cAMP levels consisted of two types of experiments. In the first experiment I compared two normal and two CF strains, matched for age and sex of the donors and for age in culture, throughout a culture cycle. The strains were subcultured at a 1:8 dilution and fed every 3 days. On four occasions the cells were stimulated with isoproterenol and the cAMP was measured in both cells and medium. Parallel plates were used for cell counts and protein determinations. All four strains grew at approximately the same rate and reached similar maximum cell densities (see Fig. 3). Table 2 shows the data on the intracellular cAMP levels found when the cells were incubated in the presence or absence of the phosphodiesterase inhibitor. As can be seen, at all stages of the growth cycle the CF strains synthesize more cAMP than the control strains, and significant differences were observed. The ratio of the average values of cAMP in CF cells to that in normal cells range from 1.7 to 2.6. The presence or absence of the phosphodiesterase inhibitor did not affect the relationship between the CF and control strains even though in the absence of the inhibitor the amounts of cAMP assayed were approximately five times lower. The amount of cAMP in the medium was less than 5% of the intracellular amount of both sets of strains, which suggests that

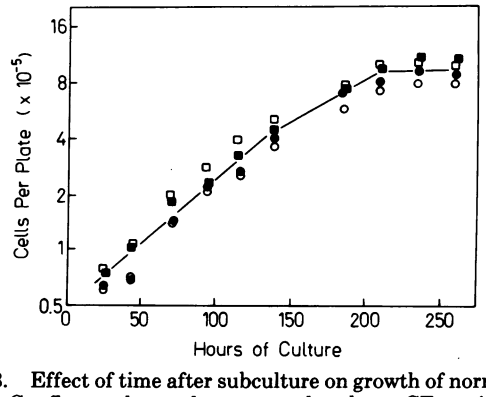

FIG. 3. Effect of time after subculture on growth of normal and CF cells. Confluent plates of two normal and two CF strains were subcultured 8-fold at time 0 and fed every 3 days. At the times indicated, duplicate plates of each strain were counted in an electronic cell counter after trypsinization. N 162 (○), N 28 (□), CF 22 (●), and CF 32 (■).

Table 3. Intracellular cAMP concentrations after isoproterenol stimulation of normal and cystic fibrosis strains

Strain	Generation no.	Doubling time (hr)	Cells/plate ($\times 10^{-6}$)	mg Protein/ 10^6 cells	Intracellular cAMP
CF 22	21	41	1.02	0.54	560 ± 50
CF 32	21	42	1.20	0.55	620 ± 60
CF 35	16	52	0.73	0.56	860 ± 90
CF 58	21	50	0.74	0.54	560 ± 50
CF 59	21	44	0.80	0.46	690 ± 40
Average ± SEM					650 ± 60
N 28	23	40	0.99	0.51	260 ± 10
N 54	21	50	0.86	0.57	140 ± 10
N 162	21	41	0.84	0.59	200 ± 30
N 174	16	58	0.73	0.57	210 ± 20
N 412	23	26	0.92	0.68	130 ± 10
Average ± SEM					190 ± 30

The number of cells per plate indicates the maximum number of cells achieved by the strain as well as the number of cells used for stimulation with 10^{-5} M L-isoproterenol for 15 min in the presence of 7×10^{-4} M Ro20-1724.

the increased effect of isoproterenol on CF cells is not due to differences in leakage of cAMP into the medium.

Having shown that the differences between CF and normal strains were observed at all time points, I then examined three other CF and three more normal strains. The data for these strains obtained when the cells were confluent, and the equivalent data from the strains used in the growth curve experiments, are summarized in Table 3. The five normal strains have intracellular cAMP levels in the range 130–260 pmol/mg of protein, whereas the five CF strains have levels between 560 and 860 pmol/mg of protein. The ratio of the average values is approximately 3, and the difference between the means of the two groups is significant ($P < 0.001$, degrees of freedom = 8). I conclude that for these strains there is a significant difference between the normal and CF cells with no overlap in the values observed.

Effect of *in vitro* age on isoproterenol-stimulated cAMP levels

It has been shown that human fibroblast strains close to the end of their lifespan *in vitro* are more sensitive to stimulation with epinephrine (24) or isoproterenol (26) than strains in the middle of their lifespan. Thus it was possible that the observations described above could be explained if the CF strains were senescent while the control strains were not. I therefore compared three normal and three CF strains throughout their lifetimes *in vitro*. Strains were subcultured in 1:8 splits from the seventh generation until they would no longer form a confluent monolayer. At several stages during this cultivation, confluent cells were stimulated with isoproterenol in experiments similar to those described in Tables 2 and 3. The data for representative experiments are shown in Table 4. The difference between normal and CF strains can be observed at the 11th and 17th generations, when all strains are more than 15 generations from senescence. Stimulation of strains N 162, N 412, and CF 35 near senescence results in higher cAMP levels relative to the measurements earlier in their lifespan. Strains N 54, CF 32, and CF 59 do not show elevations of cAMP near the end of their lifetimes *in vitro*.

Specificity of the action of isoproterenol

The cellular responses to stimulation by several drugs were compared in normal and CF strains to determine if the observed effect was specific to β-adrenergic agents. The shape of the time-response curve with these drugs was the same for both CF and control strains. Stimulation with epinephrine and isoproterenol, drugs that cause elevations in cAMP by virtue of interactions with the β-receptor, produced 400 and 700 pmol of cAMP per mg of protein, respectively, in strain CF 35 and 40 and 50 pmol/mg of protein in strain N 54. The effect of these two drugs on both sets of cells could be completely inhibited by propranolol, a specific inhibitor of β-agonists (34). Prostaglandin E_1 acts by mechanisms not involving the β-receptor (21), and treatment of cells for 60 min with 10^{-6} M prostaglandin E_1 resulted in similar intracellular cAMP levels in normal and CF cells (2100 and 2000 pmol/mg of protein, respectively). This result is consistent with the hypothesis that the differences in intracellular cAMP levels produced by stimulation with isoproterenol are manifestations of specific differences in the interaction of the cells with the drug.

Table 4. Effect of *in vitro* age on isoproterenol-stimulated intracellular cAMP levels*

Strain	Generation at time of assay				Lifetime†
	11	17	30	50	
N 54	140 ± 15	170 ± 15	150 ± 30	—	35
N 162	130 ± 10	150 ± 25	180 ± 20	410 ± 40	53
N 412	170 ± 15	130 ± 15	110 ± 20	590 ± 60	52
CF 32	460 ± 60	470 ± 60	490 ± 50	—	36
CF 35	840 ± 70	690 ± 70	1580 ± 220	—	35
CF 59	430 ± 60	430 ± 40	920 ± 120	670 ± 30	52

* Figures represent cAMP/mg of protein ± SEM after stimulation with 10^{-5} M L-isoproterenol for 15 min in the presence of 7×10^{-4} M Ro20-1724.

† Last generation at which a confluent monolayer was formed.

DISCUSSION

The stimulation of CF cells with isoproterenol results in higher intracellular cAMP levels than those seen in cells from control donors. This effect is specific to β-adrenergic agonists and cannot be attributed to different dose- or time-response curves or to leakage of cAMP into the medium. The difference between the two strains can be demonstrated throughout a culture cycle when the strains are carefully matched, even though the intracellular cAMP levels change with time after subculture. The differential effect of isoproterenol is also not due to an earlier senescence of CF strains. These results suggest that the difference is not due to a tissue culture artifact but to an intrinsic property of the cells.

The effect of isoproterenol on CF cells could be due to a difference in phosphodiesterase or adenylate cyclase activities. Some of our results argue against the former possibility. First, when CF and control strains were compared in the presence or absence of Ro20-1724, no differences in the ratio of intracellular cAMP were seen even though the amount of cAMP measured was about 5-fold different. Second, the effect was observed when cells were stimulated with catecholamines but not when prostaglandin E_1 was used. Measurements of phosphodiesterase activity on unstimulated cells have shown no differences between control and CF strains; however, to effectively rule out an involvement of this enzyme the experiments need to be repeated on isoproterenol-stimulated cells. It would seem, therefore, that the effect may be mediated by differences in catecholamine-sensitive adenylate cyclase, but I have not yet measured this activity in cell-free preparations.

The observation reported in this paper, that CF cells synthesize more cAMP than normal cells when stimulated with isoproterenol, is statistically significant, even though only a limited number of strains has been examined. More strains need to be studied to confirm that the phenomenon is characteristic of CF. This altered response of CF cells may be the primary biochemical defect or may be a secondary consequence of the genetic error. If it is the basic biochemical defect, it is either a small alteration or I may not have optimized the conditions to measure this biochemical manifestation of the gene defect. Conversely, the impact of this enhanced hormone responsiveness on cell or organ physiology could be envisioned as large enough to cause the CF phenotype. For example, it is known that cAMP is a mediator of the hormonal stimulation of secretion, and if hormonal stimulation results in abnormally prolonged elevations of intracellular cAMP, the end result could well be abnormalities in the control of secretion.

On the other hand, the basic genetic defect could involve some other cellular function, for example, that which results in the production of the ciliary dyskinesia factor, and the altered sensitivity to isoproterenol may be a secondary consequence. Even if this is the explanation for our results, the observation of altered isoproterenol sensitivity may still be crucial to an understanding of the phenotypic consequences of the CF mutation.

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