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Hyposecretion of β-Adrenergically Induced Sweating in Cystic Fibrosis Heterozygotes

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ABSTRACT. In order to determine if expression of the cystic fibrosis gene can be detected in heterozygotes, we determined sweat responses induced by local stimulation with cholinergic and β-adrenergic agents for 20 heterozygotes, 19 age- and sex-matched controls, and five subjects with cystic fibrosis. Active sweat glands were counted and sweat droplets were collected in constant bore capillaries and measured optically. Each subject was tested two to six times. The central finding was that the sweat response of carriers was significantly lower than controls to β-adrenergic stimulation ($p = 0.0013$, two-tailed t test; $p < 0.02$, Mann-Whitney U), while cystic fibrosis homozygotes did not sweat at all. In contrast, the cholinergic sweat responses did not differ between carriers and controls. For both groups the correlation between cholinergic and β-adrenergic sweating was positive, but a linear regression of β-adrenergic sweat responses as a function of cholinergic sweat responses yielded slopes that were significantly different for the two groups. The ratio of β-adrenergic to cholinergic sweating was plotted for each subject; the mean ratio of the carriers was approximately half of the mean for the controls ($p = 0.0002$ using t test or $p < 0.002$ using the Mann-Whitney U). Our results confirm previous studies and provide new evidence that carriers have, on average, a β-adrenergically stimulated secretory response that is significantly reduced relative to the control response. (*Pediatr Res* 22: 271–276, 1987)

Abbreviation

CF, cystic fibrosis

Eccrine sweat glands are the most accessible organs affected by CF. They have thus played a key role in research aimed at identifying the basic defect. The high concentration of Na^+ and Cl^- in the sweat of CF homozygotes (1), which is the single most reliable indicator of the disease, results from impermeability to Cl^- in the reabsorptive duct (2, 3). Since Cl^- impermeability also occurs in respiratory tissue (4), it may be a direct consequence of the genetic abnormality that causes CF.

Until recently the secretory process of the sweat gland was thought to be normal in CF (5). Primary sweat of CF homozygotes has a normal ion content (6), and secretory rates of CF and control subjects are equivalent (3, 5, 7). However, while sweat rates in response to cholinergic stimulation are normal, CF homozygotes do not sweat at all in response to β-adrenergic agonists, even though cAMP levels within the sweat gland cells rise normally (8). These remarkable results suggest a highly specific defect in a distal stage of the excitation-secretion coupling mechanism that is engaged by β-adrenergic agonists in CF sweat glands.

Sato and Sato (8) also reported that CF *heterozygotes* sweat less than controls in response to β-adrenergic agonists, and although no test of significance was given, the response appeared to be substantially lower than control values. We consider it especially important to reassess the evidence that heterozygotes express a defect in β-adrenergic sweat secretion. No consistent physiological correlate of heterozygosity for the CF gene has ever been established. Even the most reliable physiological measures that distinguish between CF homozygotes and controls, namely sweat electrolyte levels (9) and nasal transmucosal potential differences (4, 10), do not detect differences between CF heterozygotes and controls. This may mean that the measured properties can be maintained in the normal range by decreased levels of a gene product, or it may mean that the quantity of the gene product is kept at normal levels within the heterozygotic cell by homeostatic regulatory mechanisms. If the latter is true, no test of cell proteins or the functions that depend on them will detect CF heterozygotes. Detection of a consistent heterozygote difference would rule out the latter possibility.

Although previous attempts to distinguish CF heterozygotes have been inconsistent, the β-adrenergic sweating results reported by Sato and Sato (8) provide a much stronger point of departure than results of any previous study. Their results uniquely combine two key features: they were obtained from exocrine tissue which is known to express the defective gene, and the differences between CF homozygotes and controls were absolute.

METHODS

Subjects. The 44 subjects comprised 20 parents of children with CF (obligate heterozygotes), 19 age- and sex-matched controls, and five subjects with CF (homozygotes, identified by the usual criteria). One control subject was of Asian ancestry and one was black; all other subjects were Caucasian. CF subjects were registered at the Cystic Fibrosis Center, Children's Hospital at Stanford. Lack of sweating to β-adrenergic agents by CF homozygotes was clearly established by Sato and Sato (8); since the effect is virtually absolute, we tested only five subjects with repeat tests of only one. (Additional characteristics of subjects are summarized in Table 1. Because many of these subjects were known to the investigators, it was not possible to do the study blind.) Subjects were tested January–June with each group represented throughout. Procedures were approved by the Stanford Medical Committee for the Use of Human Subjects in Research; informed consent was obtained from each subject.

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Table 1. *Subject characteristics*

	n	Age ± SEM	Asthma (*n*)	Atopy* (*n*)
Control males	11	37.7 ± 2.4	1	3
Heterozygous males	12	40.6 ± 2.5	1	2
Control females	8	38.2 ± 2.7	0	1
Heterozygous females	8	40.4 ± 4.2	1	5
CF males	4	31.8 ± 3.8	0	1
CF female	1	25.0	0	1

* Number of subjects who had previously been tested for allergies.

Drug delivery and sweat collections. Intradermal Stimulation by β-Agonists. We used modifications of methods reported previously (8). Sweating was induced on the volar surface of the forearm by an intradermal injection of 0.2 ml of a mixture of 8 × 10^{-5} M isoproterenol, 10^{-2} M theophylline (as aminophylline) and 1.4 × 10^{-4} M atropine. This combination of a β-agonist, phosphodiesterase inhibitor, and muscarinic cholinergic blocker was previously shown to elicit a pure β-adrenergic sweat response, as indicated by its total block by propranolol (1). We tested one control and one heterozygote with propranolol, and also obtained complete block of the response (data not shown). After the area was rubbed with Sylgard 184 (Dow Corning), a well was placed over the 7-mm wheal and water-saturated mineral oil immediately added. This procedure was performed on all heterozygotes and controls from two to six times (mean measures per subject = 3.9 ± 0.19 SEM for this and all subsequent numbers). In about one-fifth of the sessions, the above procedure was carried out in duplicate at separate sites on the forearm (see below).

Iontophoretic Stimulation by Cholinergic Agents. This procedure was adapted from a method previously used by Bijman and Quinton (3). A current of 200 μA was passed for 5 min through a solution of 2% acetylcholine and 0.1% methacholine in a pipette (tip diameter 1 mm) pressed against the volar surface of the forearm. An identical well was then fixed to the arm so the site of stimulation was barely out of the field of view, and water-saturated mineral oil was immediately applied. This procedure was performed on each subject from one to seven times (mean 3.3 ± 0.26).

The proximal-distal position of cholinergic and β-adrenergic stimulation was alternated for each session for each subject; when two β-agonist responses were measured, the cholinergic stimulation site was between them. Each stimulation site was at least 4 cm from any other.

Collection of Sweat. Before each collection the number of secreting sweat glands was counted in a 12.6 mm^2 area defined by a grid in a stereomicroscope at ×25. After counting, the droplets were drawn into constant bore (62 μm) capillaries with a hand-held mouth pipet; droplets from all secreting glands were collected together with no attempt to determine individual differences among glands. Volumes were determined by measuring the lengths of the fluid columns using a micrometer at ×12 (Fig. 1). Average volumes per gland are reported.

The protocols used to collect sweat differed significantly for the two kinds of stimulation. The protocols were designed to give the most reproducible results in each case, but they have the drawback that comparisons of sweat responses are strictly relative to our procedures: absolute sweat rates, including maximal rates, were not measured. For β-adrenergic stimulation, sweat was collected once, 40 min after stimulation, to maximize the amount of sweat obtained in each test (most sweating to β-adrenergic stimulation occurs in the first 20 min following an injection, and is virtually complete after 30 min) (8).

For cholinergic stimulation, sweat rates are sometimes so fast that fusion of sweat droplets from adjacent glands can occur, making accurate counts of active glands impossible. To minimize this problem, all sweat produced in the first 10 min by cholinergic stimulation was discarded; then glands were counted and sweat collected after each of the next two 5-min intervals. There was no systematic difference in sweat responses between the two collections.

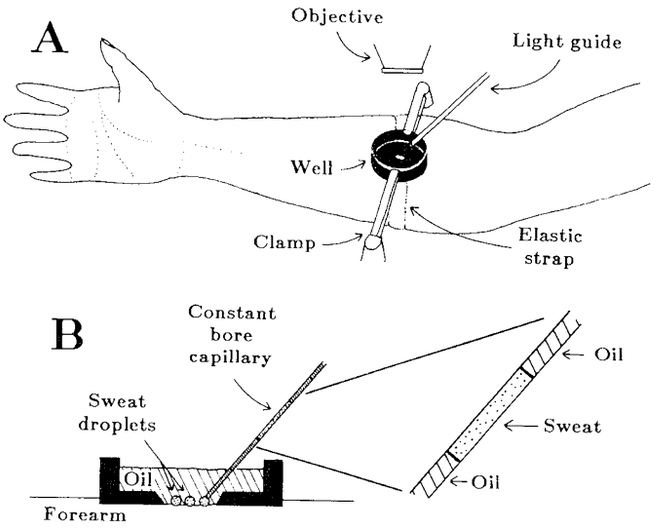

Fig. 1. Experimental arrangement. *A*, oil-filled plastic wells are secured to the arm with elastic straps, and then the arm and well are held securely to the table by a metal clamp, positioned so that the hole in the center of the well can be viewed through the microscope. Only one well is shown, but as many as three were attached, and then sequentially clamped for viewing and sweat collection. *B*, Schematic view of the method for collecting and measuring sweat droplets (4).

Estimation of number of tests required. In order to estimate the number of tests required to give a reasonably accurate estimate of each subject's β-adrenergic and cholinergic sweat responses, we initially tested 12 subjects on each of 5 separate days. An analysis of the results indicated that day-to-day variations in cholinergic and β-adrenergic responsiveness within subjects were uncorrelated and that just two tests of cholinergic responsiveness and three tests of β-adrenergic responsiveness were sufficient to give mean ratios for the heterozygote and control groups that were essentially equal to the mean ratios obtained from all five tests. (Mean results for each individual shifted by at most ± 6.5% of their original value.) Accordingly, most remaining subjects were given two cholinergic and three to four β-adrenergic tests. In summary, for each subject each of the following measures was obtained for (on average) three to four different tests: 1. number of glands in 12.6 mm^2 secreting in response to acetylcholine; 2. number of glands in 12.6 mm^2 secreting in response to isoproterenol; 3. volume of cholinergically induced sweat (as defined above); and, 4. volume of β-adrenergically induced sweat (as defined above).

These basic data were then analyzed in a variety of ways that are described in "Results." Significance was tested using both Student's *t* test and the nonparametric Mann-Whitney U test (11), which does not require that the data be normally distributed.

Adverse reactions. Two of the 44 subjects tested, both control males with strong allergic histories, experienced mild delayed hypersensitivity (48 h) skin reactions at the sites of the β-adrenergic injections. Both of these responded well to local application of corticosteroid cream. Several other subjects gave strong allergic histories but did not experience any side effects of the testing.

RESULTS

Sweating by heterozygotes in response to β-adrenergic stimulation is significantly reduced relative to a matched control sample. The central finding is that heterozygotes' sweat responses are significantly lower than control responses to β-adrenergic stimulation ($p = 0.0013$, two-tailed *t* test; $p < 0.02$, Mann-Whitney U), while homozygotes do not sweat at all (difference from

control group significant at $p < 0.0001$). For heterozygotes *versus* controls, a significant difference was maintained for males compared separately (Fig. 2, $p = 0.0034$), and for females ($p = 0.046$). In contrast, the cholinergic sweat responses did not differ between heterozygotes and controls. The cholinergic sweat responses of the CF males were significantly lower than controls ($p = 0.0092$), probably because of the reduced activity among the CF males, all of whom were hospitalized at the time of their tests. These findings agree with and extend the results of Sato and Sato (8).

Sources of variability. Responses to cholinergic agents vary greatly among individuals. Among the factors that contribute to variability in normal sweat responses are age, gender, activity level, race, and seasonal changes (12, 13). Previous data suggested that cholinergic and β-adrenergic sweat responses might be positively correlated (8). We therefore asked: To what extent are sweat responses to β-adrenergic agonists consistent for an individual, what is the distribution of sweat responses to the two kinds of stimulation within the heterozygote and control populations, and what is the relation between cholinergically induced and β-adrenergically induced sweat responses?

To assess these variables, a joint plot was made of the mean ± SEM of cholinergic and β-adrenergic sweat responses for each subject, and the linear regression of β-adrenergic sweating on cholinergic sweating was calculated for heterozygotes and controls. The results are shown in Figure 3. Each point in Figure 3 represents the mean value for an individual subject. The error bars show that most subjects were quite consistent, but there was large intersubject variability.

For both controls and heterozygotes the correlation between cholinergic and β-adrenergic sweating was positive; the correlation coefficients were 0.59 and 0.67, respectively. A linear regression of β-adrenergic sweating as a function of cholinergic sweating yielded slopes that were significantly different for the two groups: for controls the slope of β-adrenergic sweating as a function of cholinergic sweating was 0.187, while for heterozygotes the slope was only 0.064. This difference is significant, $F(1,35) = 25.6$, $p < 0.001$.

The shallow slope for heterozygotes means that the separation between heterozygotes and controls was much greater at the higher cholinergic sweat responses, and this in turn means that males, who typically sweat more to cholinergic stimuli than females, will usually be easier to differentiate with this test.

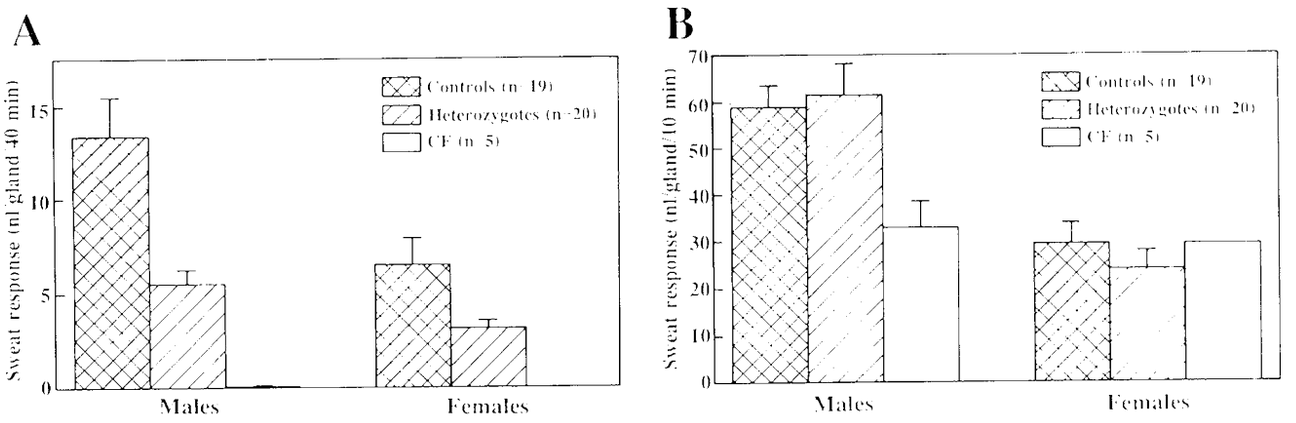

Fig. 2. *A*, mean sweat responses to β-adrenergic stimulation in a control group and in people heterozygous and homozygous for the CF gene, divided according to sex. *B*, mean values for cholinergic sweat responses for the same groups. *Error bars* show SEM.

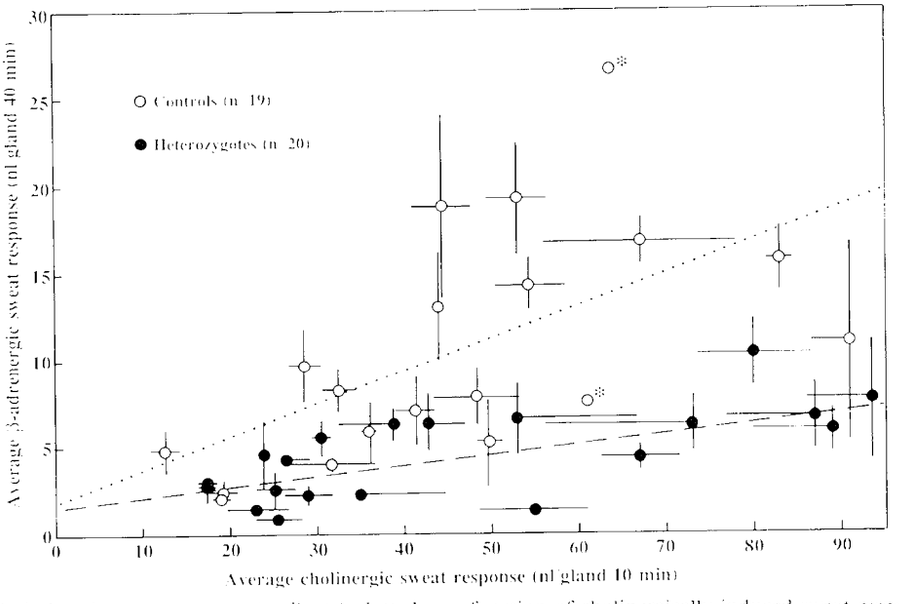

Fig. 3. β-adrenergically induced sweat responses (*ordinate*) plotted as a function of cholinergically induced sweat responses. Each *point* is the mean of two to six β-adrenergic tests and one to five cholinergic tests for a single individual; *error bars* show SEM. *Asterisks* indicate two individuals who experienced contact dermatitis and therefore had only one session consisting of one cholinergic and two β-adrenergic tests. Apparent lack of error bars in other cases indicates that SEMs were less than the diameter of the *symbol*. CF homozygotes are not shown on this graph. *Regression lines* shown for each group differ significantly (see text).

Given the positive correlation between cholinergically induced and β-adrenergically induced sweat responses, the distribution of β-adrenergic sweat responses within each group was compared by determining the ratio of β-adrenergic to cholinergic sweating for each subject, to help compensate for large individual differences in gland size and hence sweat responses. The ratio was determined by dividing the mean β-adrenergically induced sweat response by the mean cholinergically induced sweat response for each subject. The results are shown in Figure 4. Several features of these distributions are noteworthy. First, the mean β-adrenergic:cholinergic ratios were 0.23, 0.11, and 0.00 for controls, heterozygotes, and homozygotes, respectively. The heterozygote ratio differed significantly from both controls ($p = 0.002$) and homozygotes ($p = 0.000$). Second, the coefficients of variation were essentially equal (47.9% for heterozygotes *versus* 48.3% for controls). Third, the use of a ratio measure eliminated the sex difference that is apparent in Figure 2. Finally, there is a suggestion of bimodality in each distribution, or at least a clustering of points at the low end of each distribution, which might indicate that the nonparametric Mann-Whitney U is the more appropriate test.

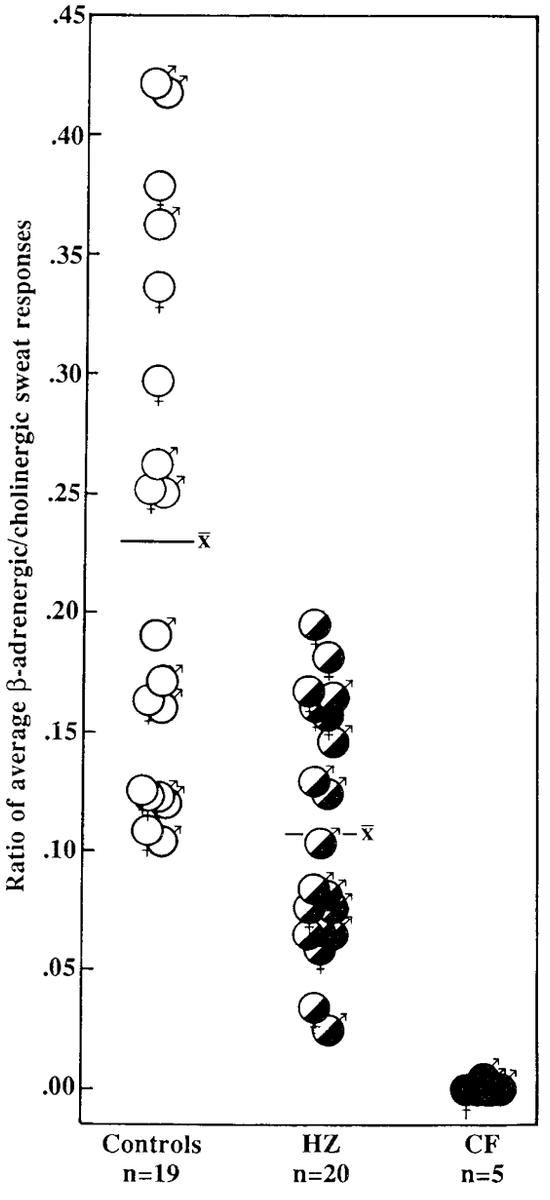

Fig. 4. Ratio of sweat responses to β-adrenergic *versus* cholinergic agonists for each individual, with gender indicated, grouped as controls, heterozygotes, and homozygotes. Each *point* was obtained by dividing the mean β-adrenergically induced sweat response for an individual by their mean cholinergically induced response.

Active gland counts. Cholinergic Stimulation. The number of glands secreting in the 12.6 mm^2 test area were counted for every test; this number was multiplied by 7.94 to give the number of glands per cm^2. Results are summarized in Table 2 and Fig. 5. The gland density seen with cholinergic stimulation in our experiments was similar to that previously reported by Kawahata (14) for upper extremities of Japanese males induced to sweat physiologically (mean 183 glands per cm^2 for four males aged 20–35). The number of glands induced to sweat with cholinergic stimulation did not differ between controls and heterozygotes.

Our results with cholinergic stimulation also confirmed an unusual sex difference noted in previous studies using thermal stimulation (15–17); men had significantly fewer active glands than women ($p = 0.007$). Also, there was an overall negative correlation of sweat rate with gland number ($r = -0.37$, $p < 0.05$).

For females, the mean number of glands per cm^2 was 152 and the mean sweat response was 26.9 nl/gland/10 min, to give a mean sweat response of 4.09 $\mu l/cm^2/10$ min, while for males the equivalent figures were 118 glands, 60.1 nl/gland/10 min, and 7.09 $\mu l/cm^2/10$ min.

β-Adrenergic Stimulation. The pattern of active gland counts *versus* sweat responses to β-adrenergic stimulation (Fig. 6) has a different form from the plot of cholinergically induced sweating. For β-adrenergic stimulation the correlation between sweat gland number and sweat response is positive ($r = 0.61$, $p < 0.01$). Furthermore, the sex difference that is so apparent with cholinergic stimulation is no longer seen, and, most importantly, heterozygotes have significantly fewer active glands (Table 2, $p = 0.0015$). The lower active gland counts could indicate that more glands are refractory to β-adrenergic stimulation in heterozygotes, or it could simply reflect the decreased secretory responses already documented.

Table 2. *Mean counts of active sweat glands in 12.6 mm^2 area (± SEM)*

	Cholinergic		β-adrenergic	
	Male	Female	Male	Female
Control	15.6 ± 1.0	20.4 ± 1.7	12.1 ± 1.2	12.7 ± 1.1
Heterozygotes	14.3 ± 0.7	18.0 ± 0.8	9.5 ± 0.5	8.3 ± 0.7*
CF	23.0 ± 2.3	22.0	0.2 ± 0.2†	0.5 ± 0.0

* $p = 0.008$ compared to control females.
† $p = 0.0000$ compared to control males.

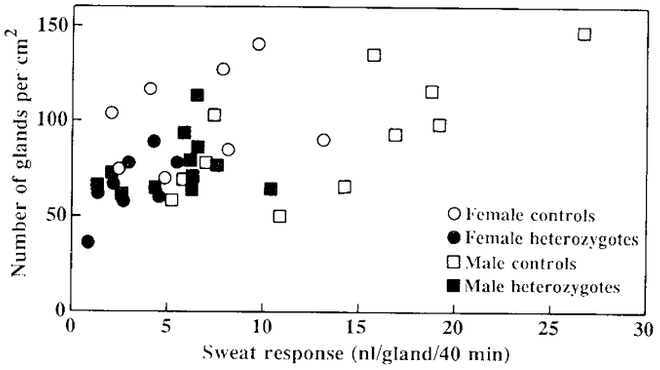

Fig. 5. Number of active glands per cm^2 after cholinergic stimulation, as a function of sweat response, for heterozygotes and controls. There was no significant difference between the groups, but females had significantly more active glands and lower sweat responses than males.

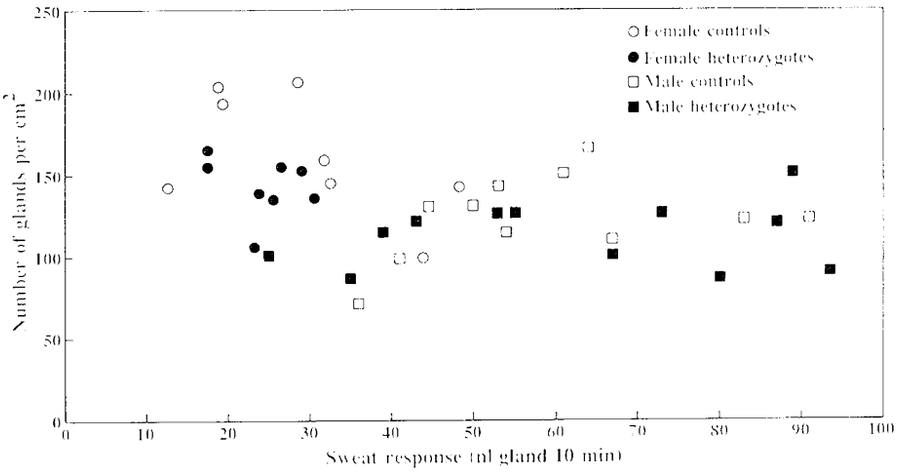

Fig. 6. Number of active glands per cm² after β-adrenergic stimulation, as a function of sweat response, for heterozygotes and controls. Heterozygotes had significantly fewer active glands than controls.

How can these patterns be explained? One possibility is that cholinergic stimulation causes detectable sweating in a large and consistent proportion of glands and so reveals intrinsic differences in gland density among individuals. If that were true, the greater gland density of females might reflect their generally smaller body size, since there is evidence that gland number is fixed early in life, so that lower gland densities are expected in larger individuals. In contrast, β-adrenergic stimulation, which evokes a smaller secretory response, might on that basis alone be expected to cause detectable sweating in a smaller proportion of glands. In such a circumstance, the proportion of secreting glands and the apparent gland density will both be increased by factors that increase the sweat rate, and this could produce the positive correlation between the apparent gland density and sweat response that was observed (Fig. 6).

DISCUSSION

Our results confirm those of Sato and Sato (1) and provide new evidence that heterozygotes have, on average, a β-adrenergically stimulated secretory response that is significantly reduced relative to the control response. These data require three facts to be interpreted: Why is β-adrenergic stimulated sweating absent in CF homozygotes and reduced in heterozygotes? Why is cholinergically stimulated sweating unaffected in CF? Why do reliable tests for CF homozygotes, namely sweat chloride levels and nasal potential difference measurements, generally fail to distinguish between heterozygotes and controls?

Our explanation for the absence of β-adrenergic-stimulated sweating in CF homozygotes and hyposecretion in heterozygotes begins with the general model of Cl^--mediated fluid secretion developed by Frizzell *et al.* (18) (Fig. 7) and since widely confirmed (19). Our hypothesis is that β-adrenergic-stimulated sweat secretion fails to occur in CF because apical Cl^- channels in the sweat gland cells fail to open in response to the rise of cytosolic cAMP (20). This hypothesis is based on the original findings of defective excitation-secretion coupling but normal cAMP response in β-adrenergic-stimulated sweating by Sato and Sato (1); on evidence for normal stimulation of cAMP in respiratory tissue from CF subjects (21–23); on the inability of forskolin or membrane-permeable cAMP analogs to gate chloride channels from CF tissue (20) and on growing evidence that the basic defect in CF involves cAMP-gated apical Cl^- channels (3, 5, 20, 24).

This explanation differs from prior interpretations of autonomic defects in CF homozygotes and heterozygotes. The reported differences include increased airway reactivity to methacholine, increased pupillary responsiveness to both cholinergic and α-adrenergic stimulation, and decreased cAMP levels in

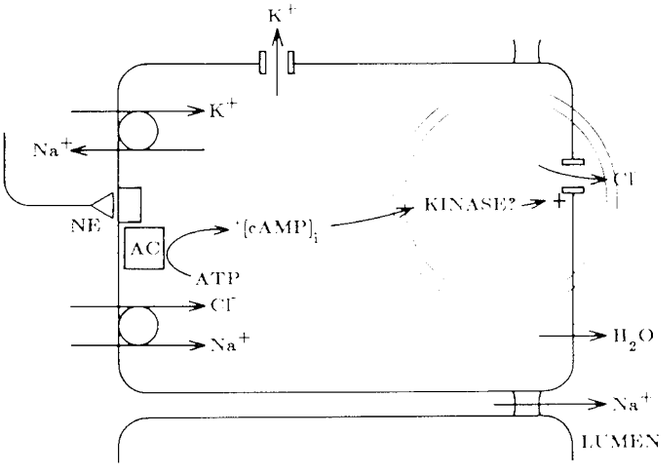

Fig. 7. Simplified schematic diagram of the excitation-secretion pathway for β-adrenergic-stimulated sweating and the site of the defect that affects sweat secretion of CF homozygotes and heterozygotes (1).

leukocytes stimulated with isoproterenol (25–29). The premise of most of this work was that a defect exists at some early stage of the response pathway, either the β-adrenergic receptors themselves (29) or receptor-cyclase coupling (28). Although we hypothesize that the reduced β-adrenergic sweating of heterozygotes is a result of the same post-cAMP defect that was first documented in homozygotes by Sato and Sato (1), we have not shown this directly, and in light of the persistent findings of reduced cAMP responses in leukocytes of heterozygotes (25–29), it will be important to determine cAMP responses to β-adrenergic stimulation in sweat glands of heterozygotes.

Why do cholinergic sweat rates remain normal in CF homozygotes? We think the evidence suggests that different mechanisms exist for sweating in response to cholinergic and β-adrenergic agents. It is clear that the early stages of excitation-secretion differ. Cholinergic sweating differs from β-adrenergic sweating in that it requires extracellular Ca^{2+} and is more copious (30). Cholinergic sweating might employ the basic model for secretion mentioned earlier, but with additional Ca^{2+}-activated pathways operating in parallel; *e.g.* the apical Cl^- channels might be gated by Ca^{2+} as well as by cAMP (20), or Ca^{2+} might gate a basolateral K^+ channel or stimulate a K^+ pump to hyperpolarize the cell and enhance the secretory response (31). However, we

think it is also possible that a different mechanism exists which does not rely on passive chloride flow from the cell.

If heterozygotes do express the CF gene, it remains to explain why tests of sweat chloride concentrations (9) and nasal potential differences (5, 10) have generally failed to distinguish between heterozygotes and controls. We do not yet have a quantitative model for fluid and electrolyte transport in these tissues (32, 33), and therefore we cannot predict the values expected for heterozygotes. However, general considerations suggest that most physiological measurements of heterozygotes will be near normal, because the gene product or the function it controls is usually present in excess, so that a 50% reduction is usually not rate limiting for the process being measured. The challenge is to move from generalizations to specifics: we recognize the importance of providing quantitative models of how decreased chloride permeability will affect higher order physiological processses in different systems, and are attempting to obtain the data needed for such models.

In summary, we propose that both the absence of sweating in CF homozygotes and the reduced sweating of CF heterozygotes reflect reduced levels of a gene product that is rate limiting for β-adrenergic-stimulated sweat secretion. The gene product is still unidentified, but salient possibilities are cAMP-gated chloride channels, or a molecule involved in gating those channels. Experiments with cells from heterozygotes should be especially informative in identifying the gene product. Increased availability is one advantage: CF heterozygotes are approximately 100 times more prevalent than CF homozygotes in the United States population. But beyond that, affected heterozygous cells should have distinct experimental advantages, since they can be viewed as fusion products of normal and homozygous cells.

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