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Biological Activities of Cystic Fibrosis Serum. IV. Stimulation of the Calcium Mediated K^+ Efflux from Rat Submandibular Gland Fragments

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Summary

Cystic fibrosis (CF) and heterozygote sera stimulate a significant K^+ efflux from rat submandibular gland fragments in the presence of 1 mM ouabain. This sensitive parameter can be maximally stimulated by as little as 0.5% CF serum and is inhibited by the calcium channel blocker D600 and EGTA. Specific receptor blockers propranolol, phenoxybenzamine or atropine do not inhibit the CF serum-stimulated K^+ efflux and agonists do not supramaximally stimulate K^+ efflux when added with serum. CF serum-induced K^+ efflux did not result in the leakage of lactic dehydrogenase into the bathing media nor did it mimic the action of the calcium ionophore A23187 when added in the presence of D600. In addition, ultrafiltrates of CF serum (less than 10,000 daltons) also stimulated K^+ efflux from rat submandibular gland tissue fragments.

Speculation

Cystic fibrosis serum factor(s) may be altering membrane permeability to calcium which results in the release of K^+ from rat submandibular gland fragments.

Cystic fibrosis (CF) is a genetic disease that is characterized by an exocrine gland dysfunction (13, 16). CF patients and obligate heterozygote individuals have a factor(s) in their serum that stimulates a ciliary dyskinesia in rabbit tracheal explants (10, 24–26). We have demonstrated that the ciliary dyskinesia and mucous secretion aspects of this reaction are calcium-dependent (2, 3-5). Other investigators have observed that the CF-serum-stimulated mucous secretion (11) and the increased ciliary beating frequency (26) are calcium-dependent. It was also reported that CF serum stimulates an increase in $^{45}Ca^{++}$ uptake in rabbit tracheal explants (5) and in human leukocytes (1). CF fibroblasts have an enlarged calcium pool (1, 14) although elevated calcium levels occur in some, but not all, exocrine secretions from CF patients (2, 9, 13, 15).

These observations led us to investigate whether CF factor(s) can stimulate known calcium-mediated secretory parameters in experimental exocrine gland models. This paper describes our quantitative observations concerning CF serum stimulation of the calcium-mediated potassium (K^+) efflux from rat submandibular tissue fragments in the presence 1 mM ouabain.

MATERIALS AND METHODS

After informed consent, venous blood was drawn from CF-affected patients (n = 25), obligate heterozygotes (n = 11) and control individuals (n = 10) at the CF Clinic, St. Vincent's Hospital and Medical Center. Between four and nine different sera were used in each experimental protocol. Medical histories excluded autoimmune and pulmonary diseases. The blood was allowed to clot at 4°C and after clot retraction, centrifuged at 850 X g for 10 min. The serum was collected in small aliquots and stored in plastic tubes at −70° until used.

Female Wistar rats were starved overnight, anesthetized with ether and sacrificed by cardiac puncture. Tissue fragments were minced on dental wax in a fortified Krebs Ringer bicarbonate (KRB) medium as previously described (18). The medium consists (in mM) 117 NaCl; 4 KCl; 1.2 $MgSO_4$; 25 $NaHCO_3$; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 10 inosine; 0.5 adenine; 5 beta-hydroxybutyric acid and 5 glucose. The medium was gassed with 95% O_2–5% CO_2 at 37°C. The incubation medium contained 1 mM ouabain (6, 7, 8, 17, 21, 22) except when indicated.

Tissue fragments approximately equal to one gland were incubated in 1 ml of the KRB medium with 10 μl aliquots taken at specific time intervals. At the end of the experiment, the medium was decanted and the tissue fragments blotted, weighed, homogenized in 2 ml KRB medium and centrifuged at 1200 X g for 10 min. Ten μl aliquots were added directly to 2 ml of the LiCl diluent. Sodium and potassium were determined on a Turner Model 500 flame photometer using LiCl as an internal standard. Percent K^+ efflux was calculated as the increase in K^+ concentration in the incubation medium at a specific experimental time divided by the K^+ concentration of the tissue fragments (6, 22). The % K^+ efflux = (Kt − Ko) Vm/(Kh − Kk) Vh x 100. Kt was the potassium concentration at a specific time interval; Ko was the potassium concentration at time zero; Kh was the potassium concentration of the homogenized tissue fragments; Kk was the potassium concentration of the incubation medium used to homogenize the fragments; Vm was the volume of incubation medium plus weight of tissue fragments; and Vh was the volume used to homogenize the tissue fragments plus weight of tissue fragments.

Lactic dehydrogenase (LDH) released into the incubation medium was measured as a parameter of cell viability. LDH was determined by following the changes in optical density at 340 nm per min. The assay medium consisted of 7.6 mM pyruvate, 0.2 mM NADH and a 20 μl aliquot of medium or supernatant of the tissue fragment homogenate. A unit of activity was defined as the change in optical density 340 nM per min per ml. Units of LDH released into the medium were expressed as a % of the total units found in the tissue fragments at zero time.

Serum samples were subjected to ultrafiltration through Amicon PM10 filters which exclude molecules greater than 10,000 daltons. Samples were filtered using nitrogen gas at 40 psi at 4°C.

Tissue fragments were prepared and incubated in parallel studies for ultrastructural analysis. Fragments were gently pipetted and placed in 3% glutaraldehyde 0.1 M cacodylate buffer at pH

7.4 for 2 h at 4°C. Fragments were washed, postfixed in 1% osmium tetroxide 0.1 M cacodylate buffer solution, washed, dehydrated, and embedded in epon 812 as previously described (6). Sections were cut on a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmsiskop 1A electron microscope.

RESULTS

Both cholinergic and α-adrenergic agonists stimulate a significant K^+ efflux from rat submandibular gland fragments that can also be stimulated to a lesser extent by ionophore A 23187 in the presence of 1 mM ouabain (6, 7, 17). The addition of control serum to the incubation medium in a final concentration of 15% did not stimulate a significant increase in basal K^+ efflux. However, the addition of 15% CF serum stimulated a significant K^+ efflux above basal in the presence of 1 mM ouabain (Table 1). The stimulation of K^+ efflux was also accomplished by the addition of 15% heterozygote serum.

The concentration of CF serum required to stimulate 50% and 0% of the biological activity (K^+ efflux) produced by the presence of 15% CF serum was investigated by addition of serial dilutions of CF serum to the incubation media in the presence of 1 mM ouabain. Concentrations of 10, 5, 1 and 0.5% CF serum from four different individuals stimulated K^+ efflux equivalent to that observed after the addition of 15% CF serum. Addition of 0.25% CF serum stimulated approximately 26% of the K^+ efflux that was produced by the addition of 15% CF serum, whereas 0.1% CF serum produced only basal levels of K^+ efflux from submandibular fragments. Serial dilutions of heterozygote serum ($n = 5$) produced similar results with 0.5% heterozygote serum inducing K^+ efflux equal to that stimulated by 15% heterozygote serum. Because small amounts of heterozygote and CF serum were required to stimulate K^+ efflux from rat submandibular gland fragments, we routinely used 5% serum in subsequent experiments. However, CF and heterozygote serum did not stimulate K^+ efflux with the same efficacy as acetylcholine or norepinephrine (Table 2). In addition, CF serum did not stimulate a significant K^+ efflux in the absence of the 1 mM ouabain (Table 2).

The calcium dependency of CF-serum-stimulated K^+ efflux from submandibular tissue fragments was probed by addition of the slow calcium channel blocker D600 (methoxyverapamil) or the chelator EGTA to the incubation medium. D600 (1 mM) or EGTA (2 mM) inhibited the K^+ efflux normally stimulated by 5% CF serum from submandibular gland fragments (Table 2). The dose response curve for D600 inhibition of the 5% CF serum-stimulated K^+ efflux indicated that 10^{-5} M D600 inhibited 50% of the serum activity, 10^{-6} M inhibited approximately 25% of the serum activity, whereas 10^{-7} M D600 did not inhibit the CF-serum-stimulated K^+ efflux from submandibular fragments (Fig. 1). D600 also reduced the basal rate of K^+ efflux in the presence and absence of CF as well as control serum. The calcium ionophore A23187 stimulated K^+ efflux at a similar efficacy as serum (Table 2). However, these two stimuli produced divergent results in the presence of 1 mM D600. CF serum activity was inhibited by D600. However, the addition of A23187 in the presence of 10^{-3} M D600 generated a supramaximal K^+ efflux from submandibular fragments (Table 2).

Table 1. *Effect of serum on K^+ efflux from rat submandibular gland fragments*[1]

Serum	$\frac{\text{Stimulated \% } K^+ \text{ efflux}}{\text{Basal \% } K^+ \text{ efflux}} \times 100$	*P* values
15% control serum	107 ± 9	
15% CF serum	195 ± 31	0.05
15% Heterozygote serum	178 ± 24	0.05

[1] Submandibular gland fragments were used for the determination of stimulated % K^+ efflux divided by basal % K^+ efflux × 100 after the addition of serum to a final concentration of 15%. Values are presented as the mean ± S.D. of four separate experiments after 20 min in the presence of 1 mM ouabain. Basal K^+ release after 20 min was 18 ± 2.10% (mean ± S.D. of the corresponding four experiments).

Table 2. *Effect of serum from normal and CF individuals on K^+ efflux from submandibular gland fragments*[1]

Treatment	Control	5% Control serum	5% CF serum
Blank	Basal	108 ± 6	168 ± 15[2]
20 μM acetylcholine	208 ± 5[3]	210 ± 21	216 ± 18
20 μM norepinephrine	218 ± 7[3]	220 ± 25[3]	210 ± 13
1 mM D600 (methoxyverapamil)	96 ± 6	97 ± 10	95 ± 16
2 mM EGTA	88 ± 8	104 ± 9	98 ± 09
20 μM Atropine	93 ± 7	92 ± 8	182 ± 6[3]
20 μM Phenoxybenzamine	91 ± 7	92 ± 8	180 ± 6[3]
20 μM Propranolol	98 ± 7	110 ± 6	170 ± 19[3]
D600 (methoxyverapamil) + Ionophore A23187	316 ± 11[3]		
Ionophore A23187 5 μg/μl	159 ± 29[2]		
Ethanol	102 ± 17		
Blank minus ouabain		88 ± 2	97 ± 4

[1] Values expressed in this table are mean ± S.D. of the stimulated % K^+ efflux divided by basal % K^+ efflux ×100 after 20 minutes. The results are derived from four to nine separate experiments.

[2] *P* value, 0.05.

[3] *P* value, 0.025.

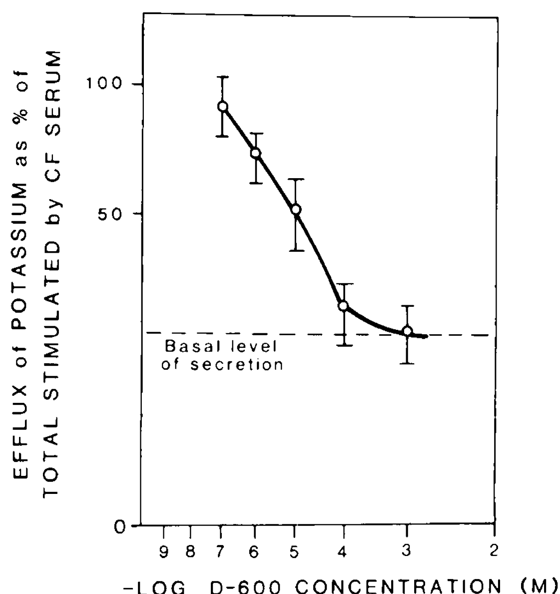

Fig. 1. Net K^+ efflux stimulated by 5% CF serum in the presence of various concentrations of D600 with 100% equal to the K^+ efflux induced by 5% CF serum without the addition of D600. The values represent the mean ± S.D. of three separate experiments.

The calcium-dependent K^+ efflux has been extensively studied in parotid and submandibular gland fragments (17–22). Potassium efflux from both systems is induced by neurohumoral agonists (Table 2) (7, 8, 17–21). Specific receptor antagonists were added to block alpha adrenergic, beta adrenergic and cholinergic receptors to determine whether the CF serum stimulation of K^+ efflux was produced by elevated levels of neurohumoral agonists in sera. Propranolol, phenoxybenzamine and atropine were added to the medium at a final concentration of 2×10^{-5} M (6–8, 17–21) in an attempt to inhibit serum-stimulated K^+ efflux. These antagonists did not inhibit the CF-serum-stimulated K^+ efflux (Table 2).

We also tried to determine if the addition of CF serum or control serum could produce a supramaximal stimulation when added together with 20 μM norepinephrine or acetylcholine. Neither CF nor control serum produced a supramaximal K^+ efflux in the presence of agonist (Table 2).

Ultrafiltrates of CF serum have been reported to have biologic

activity (10). Serum was passed through an Amicon PM10 filter at 4°C using nitrogen at 40 psi. These filters exclude molecules greater than 10,000 daltons. Ultrafiltrates of CF and control serum were added to the medium bathing rat submandibular gland fragments at a final volume of 5%. The ultrafiltrates of CF-sera-stimulated K^+ efflux at the same rate and magnitude as CF serum (Table 3), whereas control ultrafiltrates produced K^+ efflux only at basal levels.

Release of a cytoplasmic enzyme lactic dehydrogenase (LDH) into the incubation media as well as ultrastructural morphology were used as parameters of cell viability. LDH release into the incubation media was reported as a % of total tissue LDH. Leakage of LDH into the incubation media was between 2–3% of the total tissue LDH after 20 min of control incubation and did not fluctuate with the addition of either control or CF serum. Ultrastructural examination of tissue fragments after 20 min of incubation in control or CF serum did not indicate tissue damage. There was no membrane blebing, changes in mitochondrial density, cytoplasmic density, or swelling of the endoplasmic reticulum. However, after incubation with 5% CF serum, vacuoles did appear in golgi region of the acinar cells (Fig. 2a and b). These changes were similar to previous observations of vacuole formation after K^+ efflux due to stimulation with 20 μM acetylcholine or norepinephrine in submandibular gland and parotid fragments (6, 21).

DISCUSSION

CF and heterozygote serum and their ultrafiltrates clearly stimulated K^+ efflux from rat submandibular gland fragments. Very small concentrations of serum were required to stimulate K^+ efflux indicating that this parameter is sensitive for the detection of CF serum factors.

The CF-serum-stimulated K^+ efflux did not appear to be a function of cell damage. Potassium efflux was not accompanied by a release of the cytoplasmic enzyme, lactic dehydrogenase, into the incubation medium nor by the ultrastructural changes that are indicative of cell damage. Ultrastructural analysis did indicate the presence of cytoplasmic vacuoles confined to the acinar cells. These vacuoles were similar to vacuoles that have been described as the morphologic correlate of agonist-stimulated K^+ efflux in the parotid and submandibular gland fragments (6, 21). However, CF-serum-stimulated K^+ efflux was not the result of elevated levels of agonist in the serum, because it was not inhibited by the addition of the appropriate receptor antagonists.

CF serum is known to stimulate the ciliary dyskinesia reaction in rabbit tracheal epithelium. This reaction consists of an initial increased frequency in the ciliary beating pattern (25, 26) and a subsequent dyskinesia that is accompanied by mucous secretion (4, 10, 11, 16, 25). Our laboratory has observed a calcium dependence in the ciliary dyskinesia reaction accompanied by an increase in $^{45}Ca^{++}$ uptake (5). Others have observed a calcium dependence in the increased frequency of the ciliary beating pattern (26) and the mucous secretion (11) aspects of this reaction. Altered calcium metabolism has been studied extensively in relationship to CF. Several exocrine secretions, including saliva and tears have elevated calcium concentrations (2, 9, 13, 15). The submandibular gland saliva from CF patients is often turbid due to an increase in the calcium and protein concentrations which form a calcium-protein complex that can be reversed by calcium chelation (13, 15). However, several CF exocrine secretions, including sweat

Table 3. *Effects of ultrafiltrates on K^+ efflux from rat submandibular gland fragments*[1]

Treatment	$\frac{\text{Stimulated \% K}^+\text{ efflux}}{\text{Basal \% K}^+\text{ efflux}} \times 100$	*P* values
Blank		
PM10 control serum	99 ± 3	
PM10 CF serum	150 ± 10	0.025

[1] Values in this table represent the mean ± S.D. of stimulated % K^+ efflux above basal % K^+ efflux × 100 after 20 min in four different experiments. Serum from control and CF individuals were filtered through Amicon diaflo PM10 membranes at 40 psi and 0°C. Ultrafiltrates were added to a final concentration of 5%. The CF serum samples used in this experiment were from different donors than those used in Table 1.

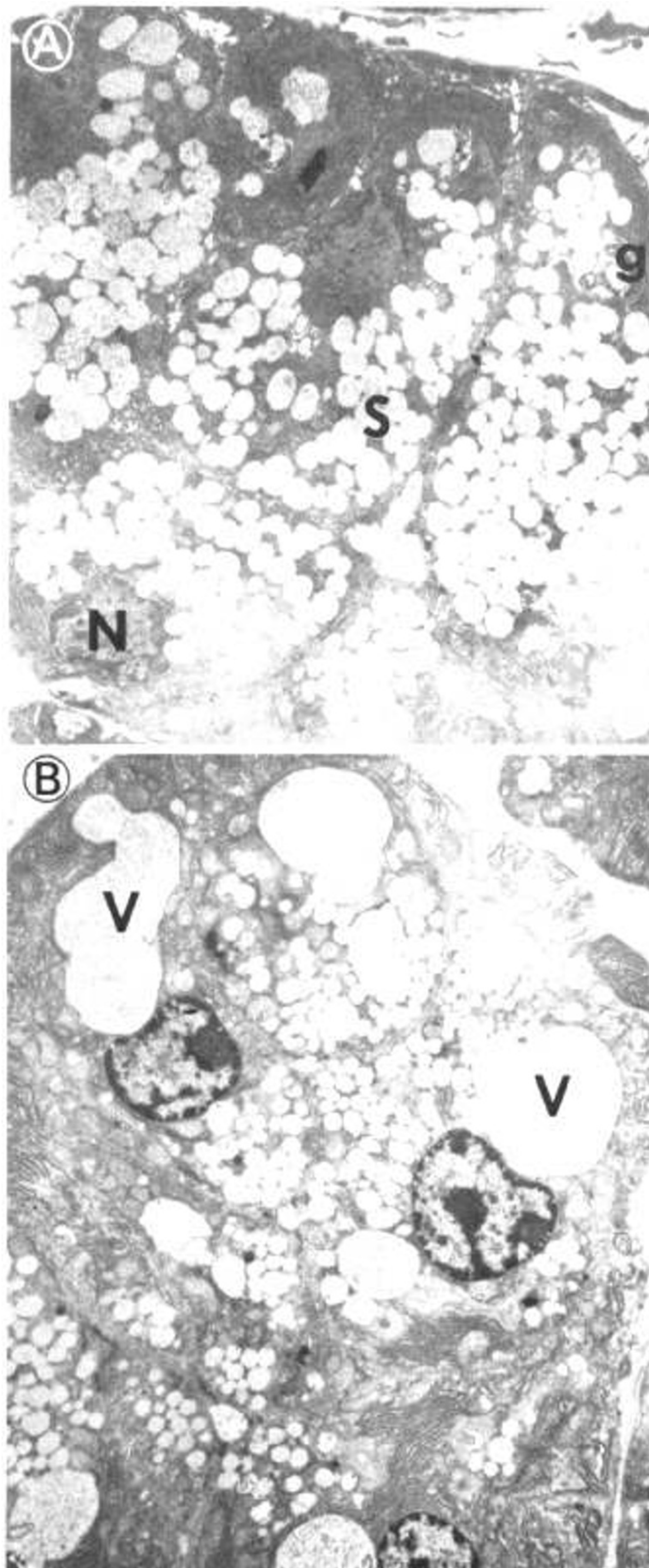

Fig. 2. *a*, Electron micrograph from rat submandibular gland fragments incubated for 20 min with 5% control serum. The acini are normal with no changes in nuclei (N), golgi apparatus (g) and secretory granules (S). (×4500). *b*, Electron micrograph from submandibular fragments incubated with 5% CF serum for 20 min. There are numerous vacuoles (V) in the golgi region of the acinar cells (×5100).

(12), do not have elevated Ca^{++} levels. A clinically altered systemic calcium metabolism does not appear to be the deficit in cystic fibrosis (12, 13) even though changes in calcium concentrations do appear in many CF secretions. Potassium efflux from submandibular and parotid gland fragments is calcium-dependent when stimulated by agonists (6–8, 17–21). The CF serum stimulation of K^+ efflux also appeared to be calcium dependent, inasmuch as it was effectively inhibited by both the slow calcium channel blocker D600 and the chelating agent EGTA. CF serum did not appear to act as an ionophore or calcium carrier in the membrane. This is evident by the divergent activities produced by CF sera and ionophore A23187 in the presence of D600. Both ionophore A23187 and CF serum produced K^+ efflux when added individually. D600 inhibited the CF-serum-generated K^+ efflux, whereas it potentiated an ionophore A23187-stimulated K^+ efflux. D600 blocks the calcium channel in excitable tissue. At these concentrations D600 is thought to block receptor activation but not to block calcium channel activation in nonexcitable tissues (19). However D600 does not block activation of all receptors such as the receptors for substance P (26). It still appears that calcium is required during the serum-stimulated K^+ efflux since addition of EGTA inhibited this activity. Calcium mobilization may be the final step or final common pathway in a series of membrane-related changes induced by CF serum or agonist which result in K^+ efflux.

Unexpectedly, we observed that ouabain is also required during serum-stimulated K^+ efflux but not during agonist-stimulated K^+ efflux. We initially included ouabain in our K^+ efflux protocol after we observed that the sustained phase of agonist-induced K^+ efflux was unmasked by ouabain (6). Subsequently, we sought to measure serum-stimulated K^+ efflux in the absence of ouabain and found that CF serum produced only basal levels of efflux. It is the sustained phase of agonist-stimulated K^+ efflux that is dependent on extracellular calcium (7, 18, 19). Another possibility is that the K^+ efflux stimulated by CF serum in the absence of ouabain may be of insufficient magnitude to alter the steady state between K^+ efflux and K^+ influx resulting from the activation of the Na^+-K^+ ATPase pump. Even in the presence of ouabain, the K^+ efflux produced by CF serum is of a smaller magnitude than that stimulated by acetylcholine. In addition, other leak-pump and exchange systems, resulting from the elevated cellular levels of sodium produced by ouabain, may be involved at some level in the CF serum stimulation of K^+ efflux (19, 20). These results are similar to the observation by Seale *et al.* (23), who did not observe a CF serum K^+ efflux from a dispersed acini preparation in the absence of ouabain.

We cannot directly extrapolate from our results to patient pathophysiology. However, these results indicate that CF serum may alter membrane permeability to calcium which, in turn, could produce biologic activity in some experimental models that are acutely exposed to this factor. In addition, because very small concentrations of CF serum are required, K^+ efflux, or possibly some other calcium-mediated secretory parameter, could be utilized as a sensitive assay in future isolation and characterization studies of CF factor from known sources of serum.

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