Defective phosphorylation of a calmodulin-binding protein in cystic-fibrosis submandibular glands

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Calmodulin-binding proteins in fractions purified from human submandibular glands by calmodulin-Sepharose were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, in the absence of exogenous protein kinase. The major proteins phosphorylated had molecular masses of 45, 51 and 61 kDa. Phosphorylation was increased by activators of protein kinase C and inhibited by H-7. Phosphorylation of the 61 kDa band was markedly decreased in cystic-fibrosis submandibular glands.

INTRODUCTION

Cystic fibrosis (CF), a common lethal genetic disease of Caucasians, is characterized as an exocrinopathy involving disturbances in mucus secretion and electrolyte transport (Goodchild & Dodge, 1985; McPherson & Goodchild, 1988). A common abnormality has been demonstrated in CF epithelial tissues; thus protein secretion and Cl^- transport have been shown to be defective in response to stimuli which act primarily by increasing cyclic AMP levels (McPherson & Dormer, 1987, 1988). Evidence suggests that the abnormality in CF cells lies distal to cyclic AMP formation (McPherson & Dormer, 1987, 1988). The demonstration, using patch-clamp techniques, that apical-membrane Cl^- channels on airway epithelial cells are opened by protein kinase A in control cells, but not in CF cells (Schoumacher *et al.*, 1987; Li *et al.*, 1988), indicated a defect in phosphorylation of a key regulator protein involved in secretion. The present study was undertaken to examine the phosphorylation of calmodulin-binding proteins extracted from control and CF submandibular glands, since previous results (McPherson *et al.*, 1988a; Shori *et al.*, 1988) indicated that a protein which modulates the actions of calmodulin is altered in CF cells. In the present paper we show that a calmodulin-binding protein of molecular mass 61 kDa was phosphorylated to a much lesser degree in extracts from CF tissues as compared with controls.

MATERIALS AND METHODS

Tissues were obtained from submandibular glands removed from five control subjects (aged 4–55 years) either during operation for ductal calculi (two glands) or within 3 h of death (three glands) and from seven CF individuals (aged 15–44 years) within 3 h of death. The Ethical Committee of the University of Wales College of Medicine, Cardiff, has approved this work. Approx. 100 mg of tissue was homogenized in 1.5 ml of 0.3 M-sucrose/5 mM-dithiothreitol/1 mM-benzamidine/1 mM-phenylmethanesulphonyl fluoride/40 mM-imidazole/HCl,

pH 7.0 at 4 °C, with a motor-driven Teflon Potter-type homogenizer.

Purification of calmodulin-binding proteins

Homogenates were centrifuged at 10000 *g* for 15 min at 4 °C. Supernatants were mixed with DEAE-Sepharcel in 10 mM-imidazole/HCl, pH 7.0, containing 1 mM-benzamidine and 0.1 mM-dithiothreitol (imidazole buffer) + 4 mM-EGTA, for 90 min at 4 °C. After transfer to a column and washing with imidazole buffer + 1 mM-EGTA, proteins were eluted with the same buffer + 0.22 M-KCl. Calmodulin-Sepharose 4B (Pharmacia; 300 μg of coupled calmodulin; 0.3 ml) was mixed with eluates in the presence of 10 mM- CaCl_2 , for 90 min at 4 °C. After transfer to a column and washing with imidazole buffer containing 0.1 mM- CaCl_2 , calmodulin-binding proteins were eluted with imidazole buffer + 2 mM-EGTA + 0.15 M-KCl. Homogenate or purified calmodulin-binding proteins were solubilized, electrophoresed on SDS/polyacrylamide gels and transferred to nitrocellulose blots (Shori *et al.*, 1988). These were incubated either with ^{125}I -calmodulin and autoradiographed as previously described (Shori *et al.*, 1988) or with a 1:1000 dilution of affinity-purified antibodies raised against human calcineurin (kindly supplied by Dr. R. W. Wallace, University of Birmingham, AL, U.S.A.), followed by washing and incubation with a 1:100 dilution of goat anti-rabbit second antibody-gold conjugate (gift from Dr. J. A. Chandler, Biocell Research Laboratories, Cardiff, U.K.). After washing, blots were incubated in silver enhancing solution and Super Amfix, and dried.

Protein content of extracts was determined by the method of Lowry *et al.* (1951).

Phosphorylation of calmodulin-binding proteins

Purified calmodulin-binding protein fractions (20 μl) were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2 μCi , 2 mM; Amersham) in a final volume of 100 μl in 34 mM-imidazole/HCl buffer, pH 7.0, containing 110 mM-KCl, 40 mM-LiCl, 0.8 mM-EDTA, 4 mM- MgCl_2 , 0.4 mM-EGTA and 1 mM-benzamidine, for 5 min at 30 °C. In

Abbreviations used: CF, cystic fibrosis; PS, phosphatidylserine; DAG, 1,2-diocanoyl-*sn*-glycerol.

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some experiments (Table 1) extra additions were made: protein kinase A inhibitor (5-24-amide) was from Peninsula, St. Helens, Lancs., U.K., H-7 from Seikagaku Kogyo, and phosphatidylserine (PS) from Lipid Products, S. Nutfield, Redhill, Surrey, U.K. 1,2-Dioctanoyl-*sn*-glycerol (DAG) and cyclic AMP were from Sigma. Reactions were terminated by addition of trichloroacetic acid/phosphotungstic acid (final concns. 10% and 0.5%, w/v, respectively). Pellets were solubilized and proteins electrophoresed on SDS/9% (w/v) polyacrylamide gels; gels were stained with Coomassie Blue, dried and autoradiographed. The density of the main bands was quantified by laser-densitometric scanning (LKB soft-laser densitometer) and measurement of peak area.

RESULTS AND DISCUSSION

Fig. 1 shows the calmodulin-binding proteins present in submandibular homogenates (*a*) and purified calmodulin-binding protein fractions (*b*). The major calmodulin-binding protein band in homogenates ran at a molecular mass of 61 kDa (Fig. 1*a*) and was purified after elution from calmodulin-Sepharose (Fig. 1*b*). The degree of binding of the 61 kDa protein band to 125 I-calmodulin correlated with the amount of total protein applied to the gel, and no consistent differences were seen between control and CF extracts.

Fig. 2 shows that, under the conditions described in the Materials and methods section, several calmodulin-binding proteins were phosphorylated in both control and CF extracts. In control extracts three major phosphorylated bands ran at 45, 51 and 61 kDa. In CF extracts a similar pattern of phosphorylation was seen, except that the 61 kDa calmodulin-binding protein band was much less phosphorylated than in control extracts (Fig. 2). Quantification of densitometric scans of autoradiographs by measurement of peak area of the

61 kDa band gave $79.9 \pm 10.9 \text{ mm}^2$ ($n = 5$ control glands) and $9.4 \pm 1.1 \text{ mm}^2$ ($n = 7$ CF glands); $P < 0.001$ for difference from control. By contrast, although there was variability in phosphorylation patterns observed in different glands, no significant differences in peak area of other major phosphorylated bands (51 and 45 kDa) were seen in control and CF extracts (results not shown).

Table 1 shows results of investigations of the type of endogenous protein kinase present in the purified calmodulin-binding-protein fractions. Phosphorylation of the 45 and 61 kDa bands was unaffected by addition of protein kinase A inhibitor or cyclic AMP; however, cyclic AMP decreased phosphorylation of the 51 kDa band, which could represent the RII subunit of protein kinase A (Blumenthal *et al.*, 1986). Preliminary results showed that addition of purified catalytic subunit of protein kinase A (500 nM; gift from Dr. P. J. England, of Smith, Kline and French, Welwyn, Herts., U.K.) increased phosphorylation of 61 kDa protein in both control ($258.8 \pm 9.7\%$) and CF extracts ($385.7 \pm 56.9\%$) of endogenous phosphorylation ($n = 4$ observations from two glands).

Results (Table 1) showing stimulation of phosphorylation by PS/DAG/ Ca^{2+} and inhibition by H-7 suggest that protein kinase C is present in the calmodulin-binding-protein preparations. PS/DAG/ Ca^{2+} increased phosphorylation of the 61 kDa band to the same degree in control and CF tissues ($P < 0.05$ for difference from endogenous), and H-7 was as effective at decreasing this phosphorylation. Nevertheless H-7 (500 μM) did not decrease the phosphorylation in control tissues to that seen in CF.

Addition of trifluoperazine (100 μM) or Ca^{2+} /calmodulin (60 nM) did not significantly change the phosphorylation patterns. Phosphorylation of the 61 kDa protein was $125 \pm 58\%$ of basal ($n = 3$) in the presence of trifluoperazine and $162 \pm 65\%$ of basal

Fig. 1. Calmodulin-binding proteins in control and CF submandibular tissues: (*a*) homogenates; (*b*) purified calmodulin-binding proteins

Autoradiographs of Western blots incubated with 125 I-calmodulin in the presence [*a*(i), *b*] or absence [*a*(ii)] of Ca^{2+} as described in the text. Autoradiography was for 48 h (*a*) or 21 days (*b*). (*a*) Lanes 1, 3, 5, CF; lanes 2, 4, control (approx. 50 μg of protein applied to each lane) run on SDS/10%-acrylamide gels; Cn, calcineurin (bovine brain, Sigma; 22 ng applied). (*b*) Purified calmodulin-binding protein fractions run on SDS/9%-acrylamide gels: amount of protein applied to gel: lane 1, 3.6 μg ; lane 2, 4.5 μg ; lane 3, 2.7 μg ; lane 4, 4.7 μg . Lanes 1 and 2, fractions from CF glands; lanes 3 and 4, fractions from control glands; DF, dye front.

Fig. 2. Phosphorylation of calmodulin-binding proteins purified from control and CF submandibular glands

Autoradiographs of representative gels showing proteins phosphorylated in the presence of [γ - 32 P]ATP as described in the text. Autoradiography was for 170 h. Approx. 1 μ g of protein was applied to each lane. Lanes 1, 4, CF; lanes 2, 3, control; duplicate runs for each lane are shown.

Table 1. Actions of activators and inhibitors of specific protein kinases on endogenous protein phosphorylation

Calmodulin-binding proteins were purified from control and CF homogenates and phosphorylated as described in the Materials and methods section. Extra additions to the phosphorylation reaction (final volume 100 μ l) are shown. PS/DAG/ Ca^{2+} : PS and DAG were added as a sonicated suspension, each to a final concentration of 25 μ g/ml; 0.4 mM- CaCl_2 was also present. Values are expressed as a percentage of the corresponding molecular-mass peak area measured under basal phosphorylation conditions, for each gel run. Results are means $\pm$ S.E.M. for 6–10 observations from three control and four CF glands: * $P < 0.05$; ** $P < 0.02$ for difference from corresponding value from control glands, under the same phosphorylation conditions.

Molecular mass of band...	^{32}P content (% of basal)					
	45 kDa		51 kDa		61 kDa	
	Control	CF	Control	CF	Control	CF
Protein kinase A inhibitor (10 μ g/ml)	105.5 $\pm$ 12.4	81.9 $\pm$ 4.5	124.6 $\pm$ 14.3	91.7 $\pm$ 8.4	99.3 $\pm$ 13.0	89.6 $\pm$ 4.5
Cyclic AMP (10 μ M)	89.6 $\pm$ 12.2	105.1 $\pm$ 18.2	22.1 $\pm$ 3.2	28.2 $\pm$ 13.7	95.5 $\pm$ 11.0	104.6 $\pm$ 10.9
Protein kinase A inhibitor + cyclic AMP	93.6 $\pm$ 13.5	95.8 $\pm$ 10.0	8.6 $\pm$ 3.1	12.2 $\pm$ 4.8	91.9 $\pm$ 15.9	99.5 $\pm$ 19.5
H-7 (100 μ M)	17.1 $\pm$ 2.0	22.5 $\pm$ 5.4	70.7 $\pm$ 9.1	105.0 $\pm$ 29.8	47.5 $\pm$ 7.6	**106.1 $\pm$ 28.0
H-7 (500 μ M)	7.0 $\pm$ 1.7	12.3 $\pm$ 3.4	52.1 $\pm$ 11.3	71.4 $\pm$ 21.0	30.1 $\pm$ 6.7	*68.4 $\pm$ 15.2
PS/DAG/ Ca^{2+}	256.7 $\pm$ 26.7	277.7 $\pm$ 75.7	141.9 $\pm$ 21.9	157.9 $\pm$ 23.5	226.3 $\pm$ 30.3	195.7 $\pm$ 26.5
PS/DAG/ Ca^{2+} + H-7 (100 μ M)	43.2 $\pm$ 4.5	60.9 $\pm$ 12.8	92.9 $\pm$ 12.0	161.5 $\pm$ 41.2	70.6 $\pm$ 13.6	90.2 $\pm$ 30.5
PS/DAG/ Ca^{2+} + H-7 (500 μ M)	14.8 $\pm$ 1.1	11.1 $\pm$ 2.4	63.7 $\pm$ 3.2	59.3 $\pm$ 9.7	33.3 $\pm$ 5.7	35.1 $\pm$ 11.0

($n = 5$) in the presence of Ca^{2+} /calmodulin in control glands, indicating that endogenous phosphorylation did not result from the calmodulin-dependent form of protein kinase II.

The decreased phosphorylation of the 61 kDa calmodulin-binding protein in CF extracts is unlikely to be due to a decreased amount of the protein in CF extracts, since the total protein added to the phosphorylation reaction was the same in control and CF extracts, and because no differences were observed in the degree of ^{125}I -calmodulin binding to the 61 kDa band

(Fig. 1b). In agreement with the findings of others (Schoumacher *et al.*, 1987; Li *et al.*, 1988), it is unlikely that the difference is due to altered protein kinase A or C activity, since activators of protein kinase C or addition of protein kinase A gave a similar degree of stimulation in control and CF extracts (Table 1, and above). Thus it is likely either that the 61 kDa protein is an altered substrate for a protein kinase(s) or that there is an increased protein phosphatase activity directed towards this protein in the CF extracts. Fig. 3 shows that the 61 kDa calmodulin-binding protein cross-reacted with an

Fig. 3. Cross-reactivity of the 61 kDa protein with antiserum raised against calcineurin

Lanes 1–4, various concentrations (88, 176, 352, 704 ng) of bovine brain calcineurin (Sigma). The contaminating lower-molecular-mass cross-reacting band is probably a proteolytic fragment present in this preparation. Lane 5, calmodulin-binding proteins purified from a CF gland (39.6 μ g of protein applied); lane 6, calmodulin-binding proteins purified from a control gland (26.4 μ g of protein applied).

antiserum raised against human platelet calcineurin (gift from Dr. R. W. Wallace), and the intensity of staining correlated with the amount of total protein applied. Like the 61 kDa protein, calcineurin is a substrate for both protein kinase A and C (Lim Tung, 1986; Singh & Wang, 1986).

Defective phosphorylation of a calcineurin-like protein in CF submandibular glands might therefore be predicted to lead to an abnormally overactive phosphatase (Hashimoto *et al.*, 1988). This could explain the inability of protein kinases A and C to open Cl^- channels in CF respiratory epithelial cells (Schoumacher *et al.*, 1987; Li *et al.*, 1988, 1989; McPherson *et al.*, 1988b) and the apparent over-activity of cyclic AMP phosphodiesterase

in CF submandibular acinar cells (McPherson *et al.*, 1986).

The discovery of an alteration in phosphorylation of a calmodulin-dependent regulator protein in CF epithelial cells provides a link between observed disturbances in cyclic AMP and Ca^{2+} pathways for secretion (McPherson & Dormer, 1988; McPherson, 1988; McPherson *et al.*, 1988b); this should provide an indicator towards the basic biochemical defect and lead to potential site(s) of intervention to correct the secretory abnormality.

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