Down-regulation of Cystic Fibrosis Gene mRNA Transcript Levels and Induction of the Cystic Fibrosis Chloride Secretory Phenotype in Epithelial Cells by Phorbol Ester*

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To evaluate the hypothesis that phorbol myristate acetate (PMA) might modulate the expression of the cystic fibrosis (CF) gene in epithelial cells, we examined the effect of PMA on CF mRNA levels and regulation of Cl⁻ secretion. Strikingly, PMA down-regulated CF mRNA transcript numbers in a dose- and time-dependent manner. Importantly, in parallel with the reduction of CF mRNA levels, PMA-treated cells were unable to up-regulate Cl⁻ secretion in a normal fashion in response to forskolin, an effect which was also dose- and time-dependent. Thus, PMA is capable of down-regulating the expression of the CF gene and induces T84 cells to adopt the "CF phenotype" in regard to regulation of Cl⁻ ion transport.

Cystic fibrosis (CF),¹ the most common fatal hereditary disorder of Caucasians in North America, results in early death primarily due to clinical manifestations within the lung (1, 2). Several lines of evidence suggest that the pathophysiology of CF is associated with an inability of epithelial cells of affected organs to increase Cl⁻ secretion in response to activation of specific pathways regulated by increased intracellular cAMP concentration or protein kinases (3–16). Recent identification of the normal CF gene and its mutations, together with studies demonstrating that defective cAMP-regulated Cl⁻ secretion in epithelial cells derived from individuals with CF can be restored by in vitro transfer of a normal functional CF gene cDNA, have linked this "CF phenotype" to the cystic fibrosis transmembrane conductance regulator (CFTR), the putative 1480-amino acid CF gene polypeptide product (17–29). Analysis of sequences 5' to exon I of the CF gene has demonstrated multiple potential binding sites for the AP-1/c-Jun nuclear transcription factor, a factor known to be induced by phorbol myristate acetate (PMA) (30). Consistent with the presence of AP-1/c-Jun binding sites in the promoter, the addition of PMA to the colon carcinoma cell line T84 caused a suppression in the rate of transcription of the CF gene (31).

The T84 cell line, originally derived from a human colon carcinoma, has typical features of epithelial cells and is known to express the CF gene and to regulate apical membrane Cl⁻ secretion in a normal fashion in response to agents which increase intracellular cAMP (e.g. forskolin (32–35)). In this regard, we hypothesized that the PMA suppression of transcription of the CF gene in T84 cells might down-regulate expression of the CF gene at the mRNA level and ultimately at the functional level in terms of regulation of Cl⁻ secretion.

MATERIALS AND METHODS

T84 colon carcinoma cells (American Type Culture Collection, CCL 248) were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin, 5% fetal bovine serum, and then incubated with 40 nM PMA for 12 h. Total RNA was then purified and evaluated by Northern analysis (10 μg RNA/lane) using a 32P-labeled (36) CF cDNA probe (pTG4976; a 4.5-kb cDNA encompassing the entire CFTR protein coding sequence, constructed by standard methods from oligo(dT)-primed cDNA and PCR-amplified fragments of cDNA derived from human lung poly(A)⁺ RNA). As a control, a 32P-labeled γ-actin cDNA probe (pHyA-F1, provided by L. Kedes and P. Gunning, Stanford University) (37) was used. For PMA dose dependence and time course studies, following incubation with PMA, total RNA was extracted and mRNA transcript levels quantified by slot blot hybridization analysis and densitometry (36) using the CF and γ-actin probes.

To evaluate the Cl⁻ secretory phenotype of T84 cells before and at various times after PMA exposure, confluent monolayers of T84 cells were incubated alone or with PMA for various times, washed twice in bicarbonate-free Ringer's solution, and loaded with 36Cl⁻ (3 μCi/ml, 2–4 h, 37 °C). Bicarbonate-free rinsing solution alone or with 3 μM forskolin was added and 36Cl⁻ efflux was quantified at 25 °C by sequentially removing and replacing aliquots of fresh solution. The amount of radioactivity in the aliquots was quantified by liquid scintillation counting, and the data were expressed as the percentage of forskolin-induced 36Cl⁻ secreted from cells as a function of time. Because the unstimulated base-line 36Cl⁻ efflux was similar in controls and PMA-exposed cells at the various times evaluated (p > 0.3, all comparisons of base-line efflux in PMA-exposed to corresponding groups of unexposed cells), the base-line 36Cl⁻ efflux was subtracted from the forskolin-stimulated efflux.

To correlate forskolin-stimulated 36Cl⁻ efflux with CF mRNA levels, cells were exposed to PMA for various times at either 40 or 100 nM, CF mRNA levels were determined as described above, and in parallel, 36Cl⁻ efflux was measured alone or in response to forskolin stimulation. 36Cl⁻ efflux was determined as described above and expressed as the percentage of radioactivity remaining within cells. Rate constants for 36Cl⁻ efflux were determined by fitting to the equation Y = Ae⁻kt + B, where Y is the amount of radioactivity remaining within cells at time t, k is the rate constant, A is the rate constant for the 36Cl⁻ efflux.

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1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; kb, kilobase(s); PMA, phorbol myristate acetate.

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amount of radioactivity within cells initially, and $B$ represents the small amount of radioactivity remaining after washing prior to forskolin stimulation.

**RESULTS AND DISCUSSION**

As expected, Northern analysis of T84 total RNA demonstrated 6.5-kb CF gene mRNA transcripts (Fig. 1, lane 1) (18). Interestingly, following exposure to PMA (40 nM, 12 h), T84 cells markedly down-regulated levels of CF mRNA transcripts (lane 2). This effect on CF mRNA levels was not a nonspecific effect, as the levels of $\gamma$-actin mRNA transcripts were not decreased by PMA (lanes 3 and 4). Likewise PMA exposure did not decrease mRNA levels for another membrane transport protein Na$^+$-K$^+$-ATPase (see below). In contrast to the specific effect of PMA, analogs such as phorbol, phorbol 12,13-diacetate, and phorbol 12-myristate-13-acetate-4-O-methyl had no effect on T84 mRNA transcript levels (not shown).

Quantitative analysis of T84 cell mRNA transcript levels in response to PMA exposure demonstrated a dose-dependent reduction of CF mRNA levels (Fig. 2). After 12 h of continuous PMA exposure, PMA concentrations of 1 nM and below had no effect ($p > 0.8$), but at 10 nM, transcript levels were decreased ($p < 0.01$) with maximal down-regulation of CF mRNA levels at doses of 40 nM and greater ($p < 0.001$, Fig. 2A). In contrast, levels of $\gamma$-actin mRNA transcripts increased up to 3-fold at PMA concentrations of 40 nM (Fig. 2D) and 100 nM (not shown). Although the mechanism(s) by which PMA effects changes in CF mRNA levels are unknown, similar CF gene expression changes are capable of modulating expression from AP-1/c-Jun promoter elements in a variety of genes and cell types (30, 38). Evaluation of the time-dependent changes in T84 cell CF mRNA transcript levels following 40 nM PMA exposure demonstrated a minimal change at 1 h ($p > 0.6$), but thereafter a marked decline, reaching a nadir of $<$10% of resting levels after 8–12 h (Fig. 2C). CF mRNA transcript levels rose slowly thereafter and by 48 h were close to 70% of that of unexposed cells. Increased doses of PMA (100 nM) suppressed T84 cell CF mRNA levels for longer periods such that the CF mRNA levels were 17% of control at 24 h ($p = 0.002$) and 22% of control at 48 h ($p < 0.001$, not shown). In contrast to the time-dependent decline in CF transcript levels, 40 nM PMA caused an increase in $\gamma$-actin mRNA transcripts, peaking at 4–8 h, and thereafter declining to base-line levels (Fig. 2D; a similar pattern was observed at 100 nM PMA, not shown). As a further control, resting T84 cells contained the expected 4.5-kb mRNA transcripts for Na$^+$-K$^+$-ATPase (39), a cell-membrane-bound ion transporter protein (not shown). Quantitative evaluation of Na$^+$-K$^+$-ATPase mRNA transcript levels showed no significant change in response to 40 nM PMA exposure ($p > 0.16$; levels at 1, 4, 8, 12, 24, and 48 h of PMA exposure compared with unexposed controls, not shown).

Although the function(s) of CFTR are not completely understood, several lines of evidence have led to the general acceptance of the concept that CFTR contributes to the regulation of transmembrane Cl$^-$ secretion and that in certain epithelial cell types, the CF phenotype is associated with abnormal regulation of Cl$^-$ channels. This is based on the observations that: 1) the pathophysiologic consequences of CF result directly from mutations within the CF gene (17–27); 2) in vivo abnormal electrical potentials exist across

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$^2$ Data are presented as the mean ± S.E. All statistical comparisons were made using the Student’s $t$ test.
respiratory epithelial apical membranes in individuals with CF compared with normals (40); 3) abnormalities exist in Cl⁻ transport in intact respiratory epithelium, primary and transformed respiratory tract epithelial cell lines, and plasma membranes from respiratory epithelium of individuals with CF compared with normals (3, 5–8, 10–14); and 4) in vitro transfer of a normal functional CF gene cdna restores defective cAMP-regulated Cl⁻ secretion in CF epithelial cells (28, 29).

In this context, the observation that PMA decreases T84 cell CF mRNA levels leads to the question: are decreases in T84 CF mRNA levels associated with decreased responsiveness to cAMP-mediated Cl⁻ secretion, i.e. does PMA induce a pattern of regulation of Cl⁻ secretion characteristic of the CF phenotype? To examine this question, T84 cells were evaluated for responsiveness to forskolin-stimulated Cl⁻ secretion before and at various time intervals following continuous exposure to PMA (Fig. 3). Consistent with previous studies, resting T84 cells had a low basal rate of Cl⁻ secretion, as measured by ✓Cl⁻ efflux, and this basal secretion was rapidly and significantly increased by forskolin stimulation (32–35). An identical pattern was observed in T84 cells exposed to 40 nM PMA for only 1 h (Fig. 3A), demonstrating that under these conditions, regulation of Cl⁻ secretion was still responsive to forskolin. In marked contrast, after 24 h of continuous 40 nM PMA exposure, forskolin was unable to increase the rate of Cl⁻ secretion in a normal fashion (Fig. 3B). After 48 h of continuous 40 nM PMA exposure, forskolin-stimulated regulation of Cl⁻ secretion was again normal (Fig. 3C). Consistent with the dose-response effect of PMA on CF mRNA transcript levels (Fig. 2A), increasing the PMA dose (100 nM) further reduced the forskolin-stimulated Cl⁻ secretion in T84 cells to negligible levels at 24 h (Fig. 3D) and also at 48 h (not shown). Importantly, PMA had no effect on the base-line ✓Cl⁻ efflux in unstimulated cells at the PMA doses and exposure times evaluated (p > 0.3 all comparisons of PMA-exposed cells to unexposed controls).

Comparison of the rate constants for ✓Cl⁻ efflux in T84 cells with CF mRNA transcript levels modulated by exposure of the cells to PMA showed that when the mRNA level was ≥67% of control, the rate constant for forskolin-stimulated Cl⁻ secretion was normal (p > 0.9, comparison of PMA-exposed to unexposed controls; Fig. 4). However, when the CF mRNA level fell to 33% of control, the rate constant for the forskolin-stimulated component of Cl⁻ secretion was ≤5% of unexposed controls, p < 0.01. Thus, above a certain "threshold" level of CF mRNA transcripts, the T84 cell Cl⁻ secretory phenotype was normal, whereas below this threshold value, the forskolin-stimulated Cl⁻ secretion response was progressively depressed in parallel with CF mRNA levels, i.e. the CF "phenotype" in relation to Cl⁻ secretion can be established in T84 cells by sufficiently depressing CF mRNA transcript levels.

Cl⁻ uptake and secretion by T84 cells has been extensively studied and has been used to evaluate epithelial cell apical membrane Cl⁻ channel activity and its regulation by agents which increase intracellular cAMP levels (32–35). Cl⁻ uptake across the basolateral membrane in these cells is modulated by the combined action of three transport processes including Na⁺/K⁺-ATPase, the Na⁺/K⁺-Cl⁻ cotransporter, and K⁺ channels, whereas current-generating net Cl⁻ secretion occurs through apical membrane Cl⁻ channels down a favorable electrochemical gradient (32–35). That Cl⁻ secretion occurs

![Fig. 3. Evaluation of the Cl⁻ secretory phenotype in T84 cells before and at various times after PMA exposure. A, forskolin-stimulated ✓Cl⁻ efflux after 1 h of continuous 40 nM PMA exposure. B, forskolin-stimulated component of the efflux is shown for PMA-exposed (+PMA) or unexposed (control) cells. The data represent the mean of five experiments. B, similar to A, except PMA exposure was for 24 h. The data represent the mean of four experiments. C, similar to A, except PMA exposure was for 48 h. The data represent the mean of four experiments. D, similar to A, except that PMA concentration was increased to 100 nM and exposure was for 24 h. The data represent the mean of five experiments.](image)

![Fig. 4. Correlation of forskolin-stimulated ✓Cl⁻ efflux with CF mRNA transcript levels in T84 cells. The conditions used for each data point, the CF mRNA level, the ✓Cl⁻ efflux rate constants, and the number of Cl⁻ efflux experiments, respectively, were: PMA, 100 nM, 24 h, CF mRNA level 17 ± 3%, forskolin-stimulated ✓Cl⁻ efflux 0.151 ± 0.038 min⁻¹, unstimulated ✓Cl⁻ efflux 0.140 ± 0.026 min⁻¹, n = 6; PMA, 100 nM, 48 h, CF mRNA level 22 ± 1%, forskolin-stimulated ✓Cl⁻ efflux 0.160 ± 0.017 min⁻¹, n = 6; forskolin-stimulated ✓Cl⁻ efflux 0.160 ± 0.017 min⁻¹, n = 6; unstimulated ✓Cl⁻ efflux 0.135 ± 0.027 min⁻¹, n = 6; PMA, 40 nM, 24 h, CF mRNA level 33 ± 1%, forskolin-stimulated ✓Cl⁻ efflux 0.280 ± 0.010 min⁻¹, n = 4; unstimulated ✓Cl⁻ efflux 0.102 ± 0.008 min⁻¹, n = 4; PMA, 40 nM, 48 h, CF mRNA level 67 ± 3%, forskolin-stimulated ✓Cl⁻ efflux 0.438 ± 0.146 min⁻¹, unstimulated ✓Cl⁻ efflux 0.102 ± 0.008 min⁻¹, n = 4; PMA, 40 nM, 1 h, CF mRNA level 82 ± 7%, forskolin-stimulated ✓Cl⁻ efflux 0.428 ± 0.004 min⁻¹, n = 3; unstimulated ✓Cl⁻ efflux 0.325 ± 0.035 min⁻¹, n = 6; unexposed, CF mRNA level 100%, forskolin-stimulated ✓Cl⁻ efflux 0.427 ± 0.004 min⁻¹, n = 5; unstimulated ✓Cl⁻ efflux 0.108 ± 0.027 min⁻¹, n = 22.](image)
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through apical membrane Cl- channels is supported by Ussing chamber studies demonstrating vectorial Cl- transport and studies with Cl- channel blocking agents such as diphenyl carboxylate and 5-nitro-2-(3-phenylpropylamino)benzoic acid which block membrane Cl- channels in T84 and other cells (33, 35, 41) and which block the cAMP-mediated Cl- efflux in T84 cells (33, 35) and apical membrane patches from these cells (34).

In the context of these considerations, if CFTR must be present in sufficient amounts and functioning normally to permit activation of the Cl- channel by a cAMP-dependent process like phosphorylation, then decreased levels of CFTR would interfere with forskolin-stimulated Cl- secretion. Thus, one explanation for our observations is that PMA, by reducing CF mRNA transcript levels, and presumably CFTR as well, interferes in this activation pathway by producing a physiologic state with severely reduced levels of otherwise functional CFTR. It should be possible to test this hypothesis when a reliable quantitative CFTR-specific assay is available and its activity can be directly measured. An alternative explanation is that protein kinase C-mediated phosphorylation normally activates Cl- channels independent of CFTR and that PMA induces the observed pattern of altered regulation of Cl- secretion by down-regulating protein kinase C levels. This mechanism is unlikely, because it is now well established that cAMP-mediated Cl- secretion is clearly dependent on CFTR, and because PMA does not alter adenylate cyclase activity nor the activity of cAMP-dependent protein kinase A (or its sensitivity to cAMP (9)) which is fully capable of activating the Cl- channel in the absence of protein kinase C (11, 12). Furthermore, although there is evidence (for short periods at high intracellular Ca2+ levels), that protein kinase C can inhibit the outwardly rectifying Cl- channel (13), it is also known that the phorbol ester phorbol dibutyrate rapidly down-regulates protein kinase C activity in T84 and other cells (42-45), and thus, after 24 h of PMA treatment when forskolin-activated Cl- efflux is reduced, protein kinase C activity may be very low. Therefore, the effect of 24-h PMA exposure on T84 cell forskolin-stimulated Cl- efflux is unlikely to be a direct effect of protein kinase C on membrane Cl- channels but is likely a result of the reduced CFTR levels. It is also unlikely that PMA has a direct effect on the Cl- channel itself, because after 1 h of PMA exposure, T84 cells were able to respond to forskolin stimulation by increasing 36Cl- efflux normally (Fig. 3A). Theoretically, it is also possible that PMA might alter the capacity of T84 cells to secrete Cl- by affecting one of the transport processes that regulate Cl- uptake. For example, PMA might affect Na+-K+-ATPase which might then affect the cell membrane potential and/or Cl- uptake and accumulation within the cells. Although this might occur at the protein level, it does not at the level of gene expression, since Na+-K+-ATPase mRNA levels were unchanged by PMA exposure. Regardless of the exact mechanism, by definition, because PMA blocks the forskolin-stimulated (cAMP-induced) Cl- secretion in T84 cells, PMA induces the "CF Cl- secretory phenotype." These observations have a number of interesting implications for the control of expression of the CF gene and the relationship of the putative CF gene product to modulation of Cl- secretion. First, the observation that a decrease in CF mRNA levels by PMA is associated with induction of the CF Cl- secretory phenotype supports the concept of a direct link between abnormalities of the function and/or amount of the CF gene product and the anion secretory abnormalities observed in cells expressing two abnormal CF genes.

Second, in the context that in T84 cells there appears to be a threshold level of CF mRNA required for normalcy, and because of the similarities between T84 cells and other human epithelial cells in regard to the regulation of Cl- secretion, it seems reasonable to suspect that a threshold of expression of normal CF mRNA (and presumably also CFTR protein) exists in vivo as well. This is consistent with the knowledge that individuals heterozygous for normal and ΔF508 CF alleles have equal levels of normal and abnormal CF mRNA transcripts, yet have no clinically apparent disease (45). Furthermore, the concept of a threshold level of CF gene expression is also consistent with other biologic systems such as the human blood complement cascade where factor deficiencies cause coagulopathies when factor levels fall below certain critical values (e.g. factor IX has a threshold level of 10% (46)). Interestingly, recent findings indicate that CF gene mutations other than ΔF508 can result in a less severe clinical course (26, 27). This suggests there may be individuals in which decreased expression of a structurally normal (with respect to CFTR) CF gene or normal expression of a CFTR molecule with partial function results in mild disease because of partially diminished, but not absent, cAMP-mediated regulation of Cl- secretion. This expectation is also consistent with the analogy to the coagulation system in that for some factors (e.g. factor VIII), the severity of the coagulopathy correlates with the level of depression of the factor.

Third, although CFTR protein has not been directly characterized, the fact that the transient decrease in CF mRNA levels induced by 40 nM PMA exposure was paralleled by a similar transient decrease in forskolin-stimulated Cl- efflux suggests that the CFTR protein may have a half-life in the range of hours. This indirect estimate is consistent with recent data (47, 48) demonstrating, with pulse-chase studies, that the intracellular product of the normal CF cDNA in COS7 cells has a lifespan of hours.

Fourth, in the context that PMA is a potent "inflammatory" stimulus (49), it is conceivable that other endogenous inflammatory stimuli might also down-regulate CF gene expression in normal epithelium in a milieu of local inflammation, e.g. by down-regulating CF mRNA transcript levels, inflammatory stimuli might induce a "localized cystic fibrosis" in the affected region of an organ. Thus, it will be interesting to evaluate CF mRNA transcript levels in respiratory epithelial cells of individuals with inflammatory airway disorders such as chronic bronchitis.

Finally, this model of down-regulation of CF gene expression and induction of the CF epithelial cell Cl- secretory phenotype induced by PMA in cells with a normal CF gene and non-CF physiology may be useful to study the regulation of the CF gene expression and the precise function(s) of its gene product in health and disease.

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