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Phorbol ester-induced protein kinase C translocation and lysosomal enzyme release in normal and cystic fibrosis fibroblasts

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The ability of the tumor-promoting phorbol ester 4β-phorbol 12β-myristate 13α-acetate (PMA) to induce protein kinase C (PKC) translocation and lysosomal enzyme release was examined in skin fibroblasts from normal subjects and from patients with cystic fibrosis (CF). As compared to normal fibroblasts, those CF exhibited: (i) an increased sensitivity to the effect of PMA on the disappearance of PKC from cytosolic fractions as well as a greater and earlier recovery, in the membrane fraction, of the PKC activity lost in the cytosolic fraction; (ii) an earlier response to PMA for its effect on β-*N*-acetylglucosaminidase release. In contrast, the inactive phorbol ester 4α-phorbol 12,13-didecanoate (4αPDD) proved ineffective in inducing PKC translocation and β-*N*-acetylglucosaminidase release in both normal and CF fibroblasts. The data suggest a defect in the regulation of PKC activity in CF fibroblasts, which may lead to altered secretion.

Protein kinase C; Lysosomal enzyme release; Phorbol ester; Cystic fibrosis; (Skin fibroblast)

1. INTRODUCTION

Cystic fibrosis is an autosomal-recessive disease which is characterized as an exocrinopathy with disturbances in mucus secretion and electrolyte transport and which mainly affects epithelial cells. However, it has recently been shown that skin fibroblasts also exhibit the most common defect associated with this disease, i.e. decreased permeability to Cl^- [1], thus validating the use of this cell type as a model in investigations related to CF.

Here, we have compared, in skin fibroblasts from normal subjects and CF patients, the ability of PKC, an enzyme which has been shown to play a key role in secretory processes [2], to be exogenously activated. Taking advantage of the property of PMA of directly activating PKC by substituting for endogenous diacylglycerol, we thus evaluated in fibroblasts from both groups the ability of the phorbol ester to induce: (i) the translocation of PKC from cytosolic to membrane fractions, a process presumably associated with the activation of the enzyme [3,4]; and (ii) the release of β-*N*-acetylglucosaminidase, a lysosomal glycoprotein abundantly secreted in the extracellular space.

Our results provide clear evidence that, in response to PMA, the CF fibroblasts (as compared to normal ones) exhibit: (i) increased sensitivity with respect to the disappearance of PKC from the cytosolic fraction, with no modification of the maximal responsiveness; (ii) greater and earlier recovery, in the membrane fraction, of the enzyme activity lost in the cytosolic fraction; (iii) earlier release of β-*N*-acetylglucosaminidase. Taken together, these data demonstrate impairment of

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Abbreviations: BSA, bovine serum albumin; CF, cystic fibrosis; DMEM, Dulbecco's modified Eagle's medium; MUB, methylumbelliferyl; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, 4β-phorbol 12β-myristate 13α-acetate; 4αPDD, 4α-phorbol 12,13-didecanoate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine

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the responses of CF fibroblasts to the phorbol ester and suggest a defect in the regulation of PKC activity in these patients, leading to altered secretion.

2. MATERIALS AND METHODS

2.1. *Materials*

PMA, 4αPDD, PMSF, PS, 1,2-diolein, ATP, histone (type III-S) and MUB-2-acetamido-2-deoxy-β-D-glucopyranoside were purchased from Sigma (USA). DMEM was obtained from Flow Labs, DEAE-cellulose (DE-52) was from Pharmacia. [γ-^{32}P]ATP (30 Ci/mmol) was from New England Nuclear (Boston, MA).

2.2. *Cell culture and fractionation*

Cultures of human fibroblasts were established from skin biopsies performed on 5 control subjects and 3 CF patients. Cells were grown to confluence in DMEM supplemented with 10% (v/v) newborn calf serum, penicillin (250 mU/ml), streptomycin (250 μg/ml) and glutamine (5 mM). 48 h prior to use in an experiment, cells were serum-deprived. Immediately before the experiment, cells were washed 5 times with serum-free DMEM and then incubated in this medium at 37°C, in either the absence or presence of PMA or 4α PDD which were prepared and used under the conditions described in [5]. Treatments were stopped by decanting the media and rinsing monolayers 5 times with ice-cold PBS. Cells were then scraped with a rubber policeman, suspended in 2 ml ice-cold buffer A [20 mM Tris-HCl (pH 7.5) at 4°C, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 50 mM β-mercaptoethanol] and subsequently homogenized via 20 strokes of a tight-fitting Teflon homogenizer. The homogenates were centrifuged at 105000 × g. Supernates (cytosols) were decanted and the pellets suspended in 1 ml buffer A containing 0.2 mM PMSF and 0.1% Triton X-100. After 60 min at 4°C, the solubilized membranes were centrifuged as described above. As a purification step, cytosolic and membrane-solubilized fractions corresponding to 200 μg protein were applied to 0.5-ml columns of DE-52 cellulose which had been equilibrated in 5 ml buffer B [20 mM Tris (pH 7.4), 50 mM β-mercaptoethanol, 1 mM EGTA, 1 mM EDTA]. The columns were rinsed with 2 vols buffer B and then stepwise eluted with 2 column volumes of buffer B containing 0.05, 0.15 and 0.5 M KCl. PKC activity was recovered in the 0.05 M KCl fraction. Protein was determined by the Bradford dye method, using Bio-Rad reagent and BSA as the standard.

2.3. *PKC assay*

PKC activity was determined by slight modifications of previous methods [6,7]. The complete reaction mixture (0.25 ml) contained 5 μmol Tris-HCl (pH 7.5), 2.5 μmol Mg acetate, 25 nmol [γ-^{32}P]ATP, 20 μg PS, 5 μg 1,2-diolein, 125 nmol $CaCl_2$ (in excess of chelator concentration) and 50 μg histone III-S. Basal activity (Ca^{2+}/PS-independent kinase activity) was determined by incubation of samples in the absence of PS, diolein and Ca^{2+}. Reactions were initiated by addition of the cellular extracts (10 μg protein) and terminated after 5 min at 30°C as in [8]. PKC activity is expressed as the activity (pmol ^{32}P transferred to histone III-S/min per mg protein) measured in the presence of Ca^{2+}, PS and diolein minus the basal activity.

2.4. β-N-*Acetylglucosaminidase release*

Confluent serum-deprived cells were incubated at 37°C in DMEM without or with PMA or 4αPDD for different time periods. At the end of each time period, the medium was assayed for basal and phorbol ester-induced β-*N*-acetylglucosaminidase release. The enzyme activity was measured fluorometrically, using MUB-2-acetamido-2-deoxy-β-D-glucopyranoside (4 mM suspended in 0.1 M citrate buffer, pH 4.6) as described [9].

2.5. *Statistical analysis*

Where indicated, the differences between the mean values were evaluated by Student's t-test.

3. RESULTS AND DISCUSSION

Fig.1 shows the dose-response curves for the effect of PMA on the disappearance of cytosolic PKC activity in normal and CF fibroblasts. In normal fibroblasts, PMA at 0.16–16 nM had no effect on cytosolic PKC activity and a level of 160 nM was required to decrease the enzyme activity by 45%. Similar results were reported by Halsey et al. [10] for 3T3 fibroblasts. In contrast, in CF fibroblasts, a linear decrease in cytosolic PKC activity was observed over the range 0.16–16 nM, which reached 54% at 16 nM. Moreover, at 160 nM PMA, the decrease (72%) in PKC cytosolic activity of cells from CF subjects was 1.6-fold that elicited in the normal ones (45%). Thus, in CF fibroblasts, the dose-response curve for the effect of PMA on cytosolic PKC depletion exhibited a marked leftward shift as compared to that observed for normal cells which was superimposable to that reported in [10]. However, as shown in fig.1, no appreciable difference was noted between control and CF fibroblasts when considering the maximal decreases in cytosolic PKC activity elicited by PMA (75 and 81%, respectively). On the other hand, the inactive phorbol ester 4αPDD (1.6 μM) proved ineffective in inducing the disappearance of cytosolic PKC (9 and 7% decreases in control and CF fibroblasts, respectively), which provides assessment of the specificity of the effect of PMA. Taken together, the present results clearly indicate that CF fibroblasts exhibit greater sensitivity than the normal ones to the effect of PMA on the disappearance of cytosolic PKC, without any modifica-

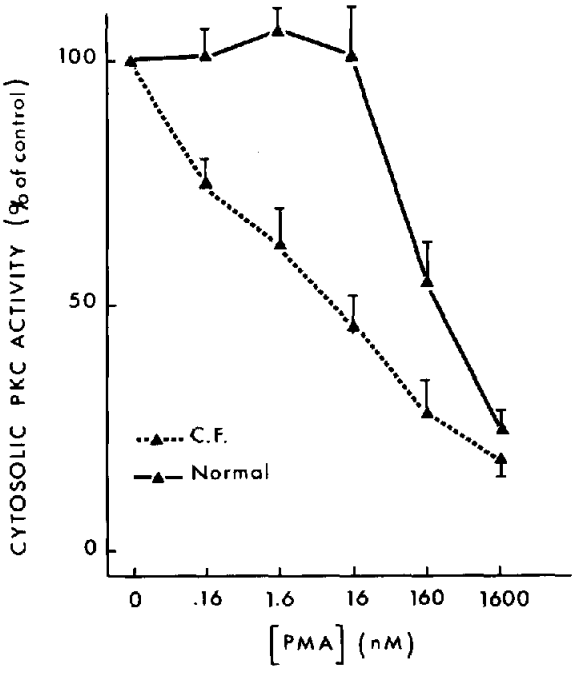

Fig.1. Dose-response curves for the effect of PMA on the disappearance of PKC activity from cytosolic fractions of normal and CF fibroblasts. Fibroblasts from normal and CF subjects were exposed for 6 min to the indicated concentrations of PMA. After cells had been rinsed 5 times with ice-cold PBS, cytosolic extracts were prepared and assayed for PKC activity as described in section 2. Results are expressed as percentage of control PKC activity (100%) which amounted to 156 ± 14 and 163 ± 9 pmol ^{32}P transferred to histone III-S/min per mg protein for normal and CF fibroblasts, respectively. Each point represents the mean ± SE of quadruplicate determinations. Data are from one representative experiment out of a series of 3 experiments.

tion of their maximal responsiveness to the phorbol ester.

Fig.2 shows the time course of PKC translocation from cytosolic to membrane fractions in normal (fig.2A) and CF (fig.2B) fibroblasts maximally stimulated by PMA. In both cell lines, the time course of the effect of PMA on the disappearance of cytosolic PKC activity was roughly similar: the phorbol ester rapidly depleted the enzyme activity with a maximum observed at 6 min in cytosols from either normal or CF fibroblasts. By contrast, both cell lines markedly differed in the ability of their membrane fractions to recover the PKC activity lost in the cytosolic fractions. Thus, in normal fibroblasts, the initial (measured at zero time) PKC activity of the membrane fractions remained unchanged for the first 2 min, whereas for those of CF, the initial membrane PKC activity exhibited a marked increase (217% over basal value) after only 30 s exposure to PMA, which was the earliest time tested. Moreover, whereas after 2 min exposure to PMA, the PKC activity was slowly recovered in the membrane fraction of normal fibroblasts and exhibited, after 6 min, a maximal increase of 102% over the basal value, in CF fibroblasts, the membrane-bound PKC activity rapidly increased up to a maximum (also at 6 min) of 541% over the basal value. Such a difference between normal and CF fibroblasts is accounted for by the data presented in fig.2C: after 6 min exposure of CF cells to PMA, their membrane fraction exhibited a selective increase in PKC activity, without any modification of the basal kinase activity (Ca^{2+}/PS-independent kinase). In contrast, in the membrane fraction from normal cells, phorbol ester treatment produced, along with an increase in PKC activity, a 2.1-fold increase ($p < 0.001$) in Ca^{2+}/PS-independent activity. A similar observation has been reported for other systems [10,11]. This suggests that the PKC activity recovered in the membrane fraction of normal cells is progressively converted to some extent into a Ca^{2+}/PS-independent kinase, presumably through a proteolytic step. Of interest in this regard is the finding reported in [12,13] that, once associated with the membrane, PKC becomes susceptible to proteolysis, a process which is now believed to be the first step in the enzyme down-regulation [14,15]. In any case, the present results provide further evidence for a clear-cut difference in the responses exhibited by CF and normal fibroblasts to the effect of PMA on the subcellular redistribution of PKC.

Since PMA has been shown to induce, most probably through PKC activation, lysosomal enzyme release [16], we next compared the effect of the phorbol ester on this process in normal and CF fibroblasts. We thus measured in both cell lines the time courses of PMA-induced release of *β-N*-acetylglucosaminidase. In these experiments, the release of enzyme was measured in parallel at each time point in either the absence (basal values) or presence of PMA, the results being expressed as percentage increase over corresponding basal

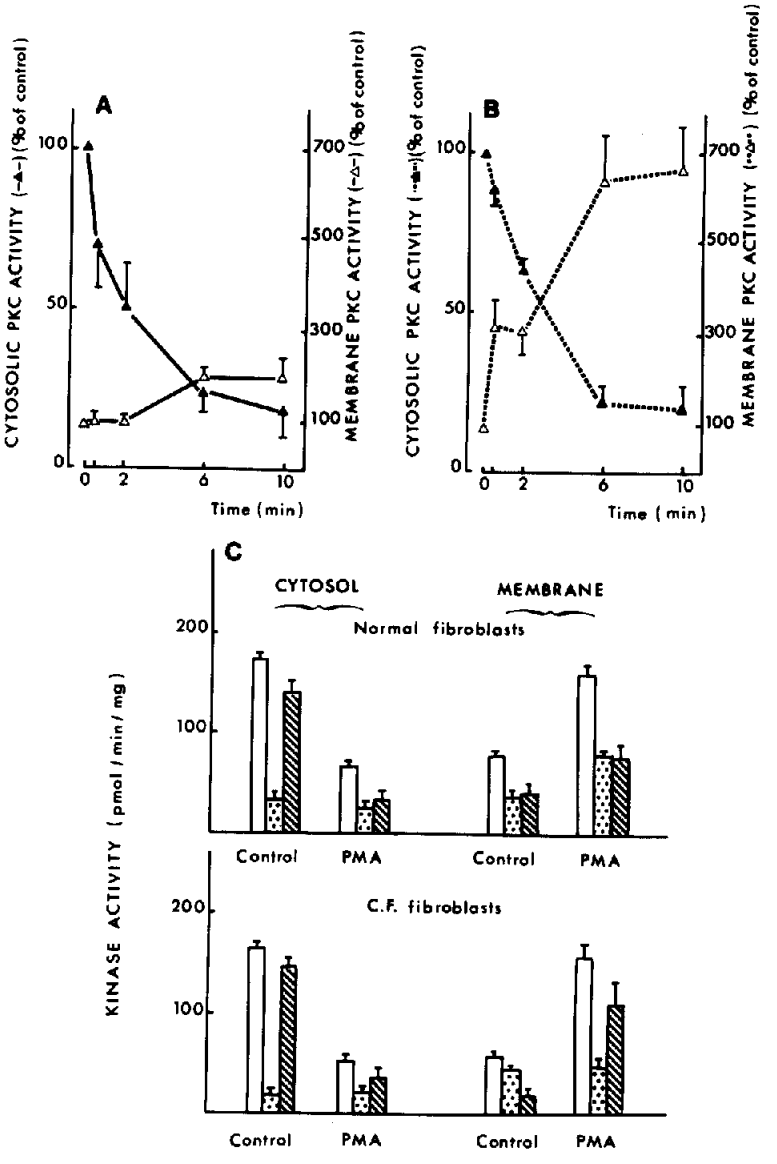

Fig.2. Time course of the effect of PMA on the subcellular PKC redistribution in normal and CF fibroblasts. Fibroblasts from normal (A) and CF (B) subjects were exposed to 1.6 μM PMA for the indicated times. After cells had been rinsed 5 times with ice-cold PBS, cytosolic and membrane fractions were prepared and assayed for PKC activity as described in section 2. 100% values of cytosolic and membrane PKC activity are given in panel C which details the calculation of the enzyme activity at time zero (control, i.e. 100%) and after 6 min exposure to PMA. Each bar represents the kinase activity measured in the presence (open bars) or absence (dotted bars) of Ca^{2+}, PS and 1,2-diolein. The hatched bar refers to the difference between the two conditions, i.e. PKC activity. Data are means ± SE of 3 (CF) or 5 (normal) experiments performed in quadruplicate.

values. This methodological approach was designed to obviate the incidence of spontaneous secretion of β-*N*-acetylglucosaminidase which has been shown to increase linearly as a function of time [17]. As shown in fig.3, PMA-induced β-*N*-acetylglucosaminidase release occurred earlier in CF fibroblasts than in the normal ones. After only 2 min exposure of CF fibroblasts to PMA, a 75%

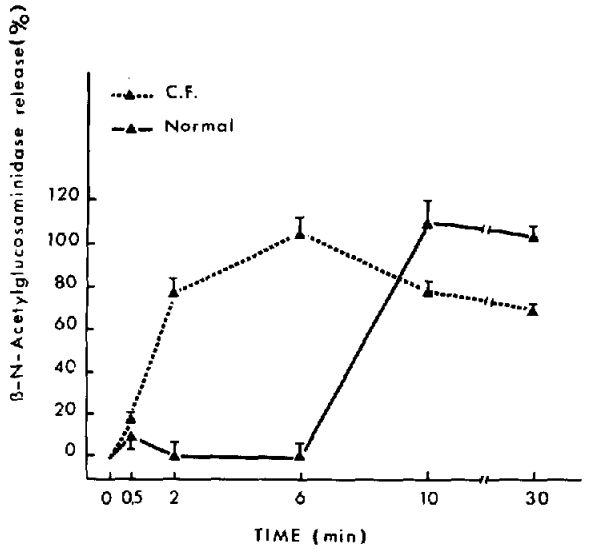

Fig.3. Time course of the effect of PMA on the release of β-*N*-acetylglucosaminidase by normal and CF fibroblasts. Fibroblasts from normal and CF subjects were incubated at 37°C in the absence or presence of 0.16 μM PMA. At the indicated times, the amount of β-*N*-acetylglucosaminidase released into the medium was measured as described in section 2. Results are expressed as the percent increase induced by PMA over the respective basal values (i.e. in the absence of PMA) which were determined in parallel at each time point. These basal values ranged from 12 to 19 nmol/h per ml medium for both normal and CF fibroblasts. Each point is the mean ± SE of 3 determinations. Data are from a representative experiment which was repeated twice.

increase in enzyme release was observed which remained roughly constant throughout the time period studied. By contrast, in normal fibroblasts, a latency period of 6 min was observed before an effect of PMA could be seen. Nevertheless, the PMA-induced increase in β-*N*-aceteylglucosaminidase release that was observed 10 min after normal cells were exposed to PMA (110 ± 11% over basal level) was slightly but not significantly higher than that measured in the CF ones (78 ± 4% over basal level) after the same time period. On the other hand, at any time point considered, 4αPDD (1.6 μM) failed to elicit significant modification of β-*N*-acetylglucosaminidase release in either normal or CF fibroblasts (109 ± 10 and 102 ± 8% of respective basal values). This finding, which agrees with results reported elsewhere [16] provides an assessment of the specificity of the effect of PMA on lysosomal enzyme release. Thus, our data show that, in CF fibroblasts, the PMA-induced release of β-*N*-acetylglucosaminidase was more rapid but not greater than that elicited in normal cells. This observation is consistent with the finding that the membrane-bound PKC, i.e. the active form of the enzyme, increased much more rapidly in CF fibroblasts than in normal cells. Also, the fact that no effect of PMA on β-*N*-acetylglucosaminidase release could be observed in normal cells before a 6 min exposure to the phorbol ester most probably reflects the slow appearance of membrane-bound PKC in these cells. Thus, the effects of PMA on the processes of PKC redistribution and lysosomal enzyme release appeared to be related, in both normal and CF fibroblasts.

In conclusion, we report here for the first time that CF fibroblasts behave quite differently from the normal cells with respect to their ability to respond to PMA in its effect on PKC translocation as well as lysosomal enzyme release. Our data suggest a defect in the regulation of PKC in CF fibroblasts, leading to altered release reactions.

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