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Research Article

Arachidonic acid release by ionomycin and phorbol ester is similar in C127 epithelial cells expressing wild-type or mutated (ΔF508) cystic fibrosis transmembrane conductance regulator

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Abstract. The Ca^{2+} ionophore ionomycin induced cytosolic $[Ca^{2+}]_i$ elevation as well as strong activation of Cl^- efflux in mouse mammary epithelial cell lines expressing wild-type or mutated (deletion of phenylalaline 508) cystic fibrosis transmembrane conductance regulator (CFTR) or vector. Ionomycin-induced Cl^- efflux was abolished by the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, whereas both activators and inhibitors of phospholipase A_2 had no effect, indicating the involvement of Ca^{2+}-dependent Cl^- channels. Stimulation of arachidonic acid release by ionomycin and phorbol ester was not significantly different between wild-type or mutated cell lines, whereas vector-transfected cells exhibited a significant higher release, which was shown to be due to larger amount of immunoreactive cytosolic phospholipase A_2. These results indicate that phospholipase A_2 activity of C127 cells was not influenced by the presence of wild-type or mutated CFTR.

Key words. CFTR; arachidonic acid; phospholipase A_2; Ca^{2+}-dependent chloride efflux.

Cystic fibrosis (CF) is a severe genetic disease caused by mutations in a single gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [1, 2]. CFTR is an epithelial Cl^- channel regulated by cyclic-adenonine monophosphate (cAMP)-dependent protein kinase A [3–5]. The most common mutation of CF is deletion of phenylalanine 508 (ΔF508) in the CFTR. Proteins with this mutation remain incompletely processed in the endoplasmic reticulum and are not delivered at the plasma membrane [6].

It is not yet clear how a defect in Cl^- conductance could account for the numerous abnormalities of the CF phenotype. In addition of playing a role as Cl^- channel, recent data support the view that CFTR acts also a 'conductance regulator' (for a review see [7]), i.e. it can regulate the function of other channels, such as Na^+ channel [8] and outwardly rectifying chloride channel (ORCC) [9, 10], or of transporters, such as the anion exchanger [11]. Other studies showed that a few metabolic pathways might also be influenced by CFTR and its mutation. In particular, it was reported that the release of arachidonic acid in response to the agonist bradykinin was significantly increased in a tracheal epithelial cell line from a CF patient bearing the ΔF508 mutation, in comparison with control cells [12]. In immortalized tracheal cell lines from patients, it was sub-

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sequently shown that ΔF508 mutation induced the stimulation of phospholipase A_2 (PLA_2) by G_q-mediated receptors, in a protein kinase C (PKC)-independent and mitogen activated protein (MAP) kinase-dependent manner, whereas normal CFTR somehow inhibits this PLA_2 stimulation [13]. Similar results have been reported also by Miele et al., who compared arachidonic acid release in an intestinal cell line (T84), expressing wtCFTR, and in a cell line established from a CF patient with pancreatic adenocarcinoma (CFPAC-1 cells) and in CFPAC-1 cells transfected with wtCFTR [14].

In the present study we have determined the activation of Cl^- efflux in a model system of mouse mammary epithelial cell lines transfected with wtCFTR or with ΔF508CFTR or with vector alone. As expected, only C127wt cells responded to cAMP-elevating agents with stimulation of Cl^- efflux, whereas the other two cell lines were unresponsive. Addition of the Ca^{2+} ionophore ionomycin caused a strong stimulation of Cl^- efflux as well as $[Ca^{2+}]_i$ elevation. The role of wt and mutated CFTR expression on PLA_2 activity was determined by observing arachidonic acid release. The results obtained are in disagreement with those previously reported and indicate that the ability to release arachidonic acid was no different in cell expressing wt and mutated CFTR.

Materials and methods

Materials. SPQ, phorbol 12-myristate 13-acetate (PMA), ionomycin and arachidonyl trifluoromethyl ketone were from Calbiochem (San Diego, CA, USA), fura-2/AM from Molecular Probes (Eugene, OR), $Na^{36}Cl$ from Amersham (Stevenage, UK). Forskolin, IBMX and lysophosphatidic acid were purchased from Sigma (St. Louis, MO). [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid was from NEN Products (Boston, MA).

Cell culture. C127 mouse mammary epithelial cell lines stably transfected with wtCFTR (C127wt), ΔF508-CFTR (C127ΔF508) or vector (C127i) (a gift from Genzyme, Framingham, MA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FCS) (DMEM-FCS medium); C127wt and C127ΔF508 lines were grown in the same medium supplemented with 200 μg/ml geneticin (GIBCO) [15].

SPQ fluorescence measurement. Cl^- efflux was determined by following the fluorescence changes of the Cl^--sensitive indicator 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) [15]. Trypsinized cells (8×10^6 cells/ml) were resuspended in a medium containing 135 mM KCl, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM D-glucose, 10 mM Hepes (pH 7.4). Cell suspension was made hypotonic by dilution with water (~ 150 mOsm/kg), 10 mM SPQ was added and, after 5 min incubation with continuous stirring at room temperature, the cell suspension was returned to isotonicity. Cells, washed twice and resuspended with DMEM-FCS, were maintained in ice and in the dark until use. Entrapped SPQ leakage from cells was $< 5\%$/h at 4 °C and $< 10\%$/h at 37 °C. An aliquot of SPQ-loaded cells (3×10^5 cells) was centrifuged for 4 min at 3000 rpm with a 5415 Eppendorf centrifuge and resuspended with a Cl^--free saline solution containing 135 mM Na-gluconate, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM D-glucose, 10 mM Hepes and 1 mM Ca-gluconate (pH 7.4). K-gluconate solution contained 135 mM K-gluconate instead of Na-gluconate. SPQ fluorescence was measured in the cuvette compartment (37 °C) of a Jasko SP-770 spectrofluorimeter (Tokyo, Japan), with excitation and emission wavelengths of 320 and 445 nm, respectively [16]. In each experiment, the fluorescence values were normalized by considering as zero and 100% the fluorescence values determined at time $t = 0$, and after addition of 0.1% Triton X-100 to the cell suspension, respectively.

$^{36}Cl^-$ efflux. It was determined following the experimental procedure described in [17]. Briefly, 10^5 cells were seeded in 36-mm-diameter wells and allowed to grow for 2 days. For the experiments, cells were loaded with 5 μCi/ml $Na^{36}Cl$ for 2 h in a water bath at 37 °C. Cells were then washed, and $^{36}Cl^-$ efflux from cells was determined by sequentially removing aliquots of the extracellular solution and replacing them with fresh solution. At the end of the experiment, cells were dissolved in 0.2 M NaOH. Aliquots of cell lysate were collected for determination of radioactivity and protein content. The total radioactivity in the cells at zero time was calculated by summing the radioactivity in each aliquot of the efflux medium plus the radioactivity remaining in the cells. Data points follow a single exponential decay. The rate constant (k) of Cl^- efflux was determined in the time interval 0.25–3 min, according to a Marquardt-Levenberg algorithm.

$[Ca^{2+}]_i$ determination. Loading with fura-2/AM was performed as described in [18]. Briefly, trypsinized cells (4×10^6/ml) were incubated with 4 μM fura-2/AM for 30 min at 37 °C with continuous stirring in DMEM supplemented with 2 mg/ml bovine serum albumin (BSA) and 0.2 mM sulfinpyrazone (pH 7.4). Cells were then washed with DMEM and maintained in the dark until use. Aliquots were resuspended in a Ca^{2+}-free saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5.5 mM D-glucose, 0.2 mM sulfinpyrazone and 20 mM Na-Hepes (pH 7.4). Fluorescence was measured at 37 °C with a Jasko FP-770 spectrofluorimeter (Tokyo, Japan) at the excitation and emission wavelengths set at 340 and 510 nm, respectively. $[Ca^{2+}]_i$ was determined after cell lysis with 0.1% Triton X-100, as described in [19].

Assay for arachidonic acid release. Cells, grown in six-well multiwells, were labeled for 24 h with DMEM containing 10% FCS and 0.25 μCi/ml [^{3}H]-arachidonic acid, as described in [18], then washed three times with phosphate-buffered saline (PBS) at 37 °C. Radioactivity incorporated in cells was 50–60% of total label. Cells were incubated for 5 min with different agents in 1 ml of saline solution containing 0.2% BSA at 37 °C; the solution was aspirated and centrifuged for 20 s at 7000 rpm in an Eppendorf 5248 centrifuge to remove floating cells; and aliquots of the resulting supernatants were counted for radioactivity. Data are expressed as dpm released per well. The radioactivity incorporated in each well was determined in aliquots of cells lysed with 0.2 M NaOH.

Immunoblot analysis. Cells were stimulated as detailed in the legends of figures, washed twice with PBS and scraped off into lysis buffer containing 1% Triton X-100, 0.5 mM EDTA, 0.6 mM phenylmethylsulfonyl fluoride, 90 μg/ml apoprotein and 50 μg/ml leupeptin in PBS. Cell lysates were frozen to −80 °C and thawed five times, centrifuged at 30,000*g* for 30 min a 4 °C. Aliquots of the supernatants were used for protein determination with the Bradford method [20] and kept at −80 °C. Cell lysate (25 μg) was separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins in the gels were transferred onto nitrocellulose, washed and incubated for 1 h with 5% nonfat dry milk, then for 1 h with anti-cPLA$_2$ mouse monoclonal antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody solution was decanted, and the nitrocellulose was washed, incubated with the secondary antibody and developed using the Amersham ECL system. The light signal was detected and analyzed using a low-light-level imaging apparatus (LB981 Luminograph, EG&G Berthold, Bad Wildbab, Germany) based on a back-illuminated, cooled charge coupled device (CCD) camera. The detection system is connected to a PC for quantitative image analysis, and a sample dark box is provided to prevent contact with external light. Digital images of the light output were acquired at an optimized accumulation time of 1 min. The light emission from each protein band was quantified by defining fixed areas and counting the number of photon fluxes from within those areas (expressed as photons/s/area). The background light emission was measured inside areas of the same size placed in different parts of the membrane not corresponding to proteins. The background average value plus three times its standard deviation was defined as the threshold value above which the chemiluminescent signal was considered positive.

Statistical evaluation. All values are expressed as means ± SD, with the number of experiments in brackets. *P* is the level of significance on a Student *t*-test.

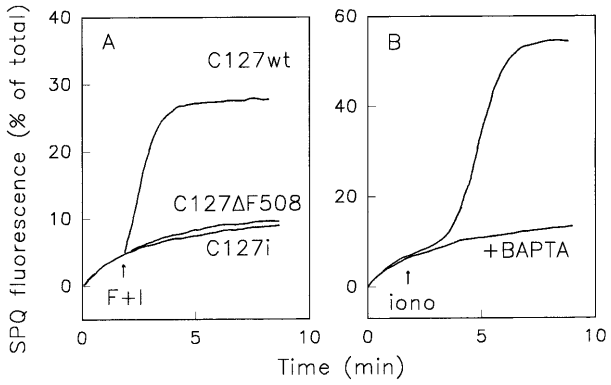

Figure 1. Effect of forskolin plus IBMX and ionomycin on Cl^- efflux from SPQ-loaded C127 cell lines. SPQ-loaded cells were resuspended in Cl^--free gluconate medium. At the arrows: (*A*) 10 μM forskolin plus 0.1 mM IBMX (F + I). (*B*) 1 μM ionomycin (iono) was added to C127wt cells. Where indicated, 50 μM BAPTA was added to cell suspension during SPQ loading. Fluorescence is expressed as percentage of total fluorescence, as described in 'Materials and methods'. The traces are representative of 4 to 12 determinations.

Results and discussion

Cl^- efflux was assessed with the halide-sensitive fluorescent dye SPQ. Figure 1A shows the time course of fluorescence change of SPQ-loaded mouse mammary epithelial cell, incubated in Cl^--free medium containing gluconate, to abolish the contribution of the anion exchanger [21]. The fluorescence signal became linear after approximately 1 min; the basal rate of increase of SPQ fluorescence was 0.76 ± 0.08 ΔF% min^{-1}, $n = 5$. The increase in SPQ fluorescence is indicative of Cl^- efflux from cells. Addition of 10 μM forskolin, an adenylate cyclase activator, plus 100 μM 3-isobutyl-1-methylxantine (IBMX), a phosphodiesterase inhibitor, induced a sharp change in the rate of Cl^- efflux in C127wt cells only (20.7 ± 5.1 ΔF% min^{-1}, $n = 6$), whereas it was completely ineffective in C127ΔF508 and C127i cells (0.86 ± 0.01 ΔF% min^{-1}, $n = 3$, and 0.77 ± 0.09, $n = 3$ ΔF% min^{-1}, respectively, shown in fig. 1A). This experiment was routinely carried out to test the functional state of CFTR in C127wt cells. These results are consistent with data previously reported in the same cell lines, showing that CFTR but not ΔF508 expression resulted in a cAMP-dependent stimulation of conductive Cl^- efflux [22].

Addition of the Ca^{2+} ionophore ionomycin (1 μM) caused a strong stimulation of Cl^- efflux (9.3 ± 1.8 ΔF% min^{-1}, $n = 6$) as reported in figure 1B. In contrast to cAMP-elevating agents, which caused an immediate stimulation of Cl^- efflux, a lag time variable of 20–60 s

Table 1. Effect of ionomycin on Cl^- efflux from C127 cell lines.

Cell lines	Rate constant of Cl^- efflux ($\Delta F\%\ min^{-1}$)		% of total fluorescence released*
	control	1 μM ionomycin	
C127I	0.75 ± 0.08 (8)	9.97 ± 2.61 (8)	54 ± 6 (8)
C127wt	0.76 ± 0.08 (8)	9.32 ± 1.82 (6)	51 ± 3 (6)
C127ΔF508	0.85 ± 0.12 (5)	9.12 ± 1.95 (6)	52 ± 3 (8)

The rate of Cl^- efflux was determined during 1–3 min after ionomycin addition. * The values are the extent of SPQ fluorescence increase induced by ionomycin, expressed as percentage of total SPQ fluorescence. Data are means ± SD. The values from each column were not significantly different.

preceded the onset of stimulation. Furthermore, the total amount of Cl^- released by ionomycin was significantly higher (approximately 50% of total fluorescence) than that caused by cAMP elevation (approximately 20–25% of total fluorescence). The values of the rate of Cl^- efflux were similar in Na-gluconate and K-gluconate saline solution (result not shown), i.e. under experimental conditions where K^+ conductance should not limit Cl^- efflux, suggesting the involvement of a Ca^{2+}-dependent Cl^- efflux pathway. As reported in table 1, both rate and extent of Cl^- efflux by ionomycin were similar in C127wt, C127ΔF508 and C127i cells.

When cells were loaded with SPQ in the presence of the Ca^{2+} chelator BAPTA, Cl^- efflux by ionomycin was completely abolished, as shown in figure 1B. From these results it can be concluded that the three C127 cell lines are endowed with Ca^{2+}-activated pathway for Cl^- efflux. However, it is not known whether activation of Cl^- efflux is due to a direct effect of Ca^{2+} on the Cl^- conductance pathway or, alternatively, to another mechanism strictly dependent on $[Ca^{2+}]_i$ elevation. It is well known that a cytosolic form of PLA_2 (cPLA$_2$) of 85 kDa can be rapidly activated by translocation to the membrane mediated by elevation of $[Ca^{2+}]_i$ and phosphorylation by PKC [23–25]. Accordingly, incubation with PMA plus ionomycin is generally used to obtain the maximal activation of PLA_2 activity. The addition of 100 nM PMA, a PKC activator (fig. 2A) or of arachidonyl trifluoromethyl ketone (AACOCF3, 10 μM), a specific cPLA$_2$ inhibitor [26] (fig. 2B), did not change the ionomycin-induced Cl^- efflux, indicating that the effect of Ca^{2+} ionophore does not seem to involve PLA_2 activation. This finding is further supported by the data reported in figure 2C, showing that the products of PLA_2 activity, arachidonic and lysophosphatidic acid (5 μM), failed to stimulate Cl^- efflux. Results comparable to those described in figure 2C were also obtained with measurements of $^{36}Cl^-$ efflux from $^{36}Cl^-$-loaded cells, where the rate constant of Cl^- efflux was $0.099 \pm 0.020\ min^{-1}$, $n = 5$, in control cells, $0.075 \pm 0.016\ min^{-1}$, $n = 4$, in the presence of

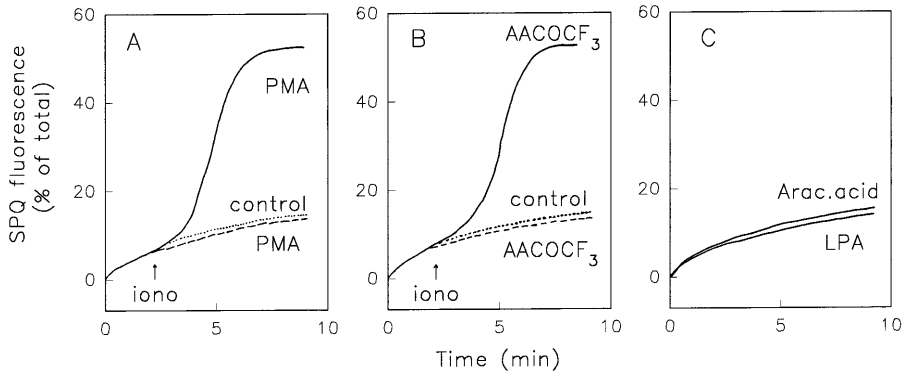

Figure 2. Effect of PMA, $AACOCF_3$, ionomycin, lysophosphatidic and arachidonic acid on Cl^- efflux. (*A*) SPQ-loaded C127i cells were resuspended in Na-gluconate medium (control, dotted line); 100 nM PMA was added at time zero (PMA, dashed line); at the arrow, 1 μM ionomycin (iono) was added in the presence of 100 nM PMA (solid line). (*B*) $AACOCF_3$ (10 μM) was preincubated with cells for 10 min before resuspension in Na-gluconate medium ($AACOCF_3$, dashed line); at the arrow; 1 μM ionomycin was added to cells preincubated with 10 μM $AACOCF_3$ (solid line); no addition (control, dotted line). (*C*) Arachidonic acid (Arac.acid) (5 μM) or lysophosphatidic acid (LPA) were added at time zero. Traces are representative of four to six determinations.

Table 2. $[Ca^{2+}]_i$ elevation by ionomycin.

Cell lines	Resting value	1 μM ionomycin
	nM	*Δ* nM
C127i	146 ± 22 (7)	606 ± 143 (7)
C127wt	125 ± 26 (4)	585 ± 82 (4)
C127ΔF508	102 ± 19 (9)	499 ± 98 (9)

Fura-2-loaded cells were incubated in a Ca^{2+}-free saline solution containing 0.1 mM EGTA. Data are means ± SD.

5 μM arachidonic acid and 0.105 ± 0.014 min^{-1}, $n = 4$, in the presence of 5 μM lysophosphatidic acid (not significantly different from control). These results suggest that the effect of ionomycin on Cl^- efflux is likely due to a direct effect of Ca^{2+} ions on Ca^{2+}-activated Cl^- channels.

Table 2 reports that the values of ionomycin-induced elevation of $[Ca^{2+}]_i$ were not significantly different in the three cell lines. It seems therefore that C127 cell lines represent a good model to assess whether *Δ*F508 mutation is associated with an intrinsic abnormality of Ca^{2+}-dependent arachidonic acid release. Incubation with 100 nM PMA alone failed to increase arachidonic acid release from C127i cells, whereas ionomycin (1 μM) caused a significant stimulation (three-fold over control, fig. 3), which was further increased after addition of both PMA and ionomycin (5.2-fold over control, fig. 3). No significant difference between C127wt and C127*Δ*F508 cell lines was observed, whereas C127i cells exhibited a significantly higher release of the fatty acid after stimulation with PMA plus ionomycin, as shown in figure 4.

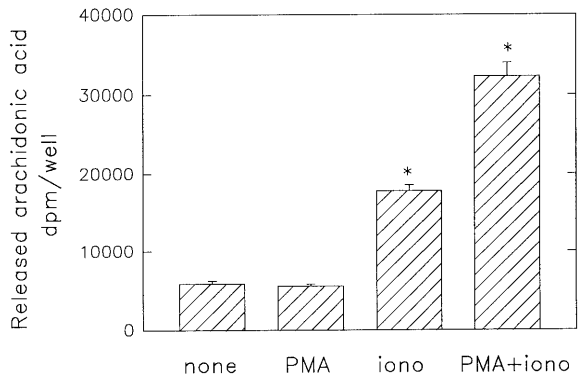

Figure 3. Effect of PMA and ionomycin on arachidonic acid release from C127i cells. Cells were labeled for 24 h with 0.5 μCi/ml [^{3}H]-arachidonic acid and treated as described in 'Materials and methods'. Where indicated, 100 nM PMA and 1 μM ionomycin were added. Data are means ± SD of three experiments performed in triplicate. * values are significantly different from control (none), $P < 0.001$.

Immunoblot experiments using anti-$cPLA_2$ antibody were carried out to assess the relative content of $cPLA_2$ in the three cell lines. The anti-$cPLA_2$ antibody revealed a single band (fig. 5A) of approximately 97 kDa. Careful quantitative determination of light emission showed that C127i cells have a larger amount of immunoreactive $cPLA_2$ than C127wt and C127*Δ*F508, which indeed showed a similar amount of immunoreactive protein (fig. 5B). This result is in agreement with the functional data of PLA_2 activity obtained with arachidonic acid release, reported in figure 4. These data indicate that expression of wt or mutated CFTR does not significantly modify PLA_2 activity in C127 cell lines.

These results are different from those reported by Bereguard et al., who showed an increase of $cPLA_2$ activity and immunoreactive $cPLA_2$ in response to bradykinin in cells with *Δ*F508 mutation, thus explaining the greater arachidonic acid release from these cells in comparison with cells expressing wtCFTR [13]. Accordingly, these authors suggested that the decreased amount of *Δ*F508CFTR at the plasma membrane, caused by a defective processing in the mutated protein [6], might induce various cellular responses, among which stimulation of pathways activating PLA_2. Conversely, CFTR normally processed to the plasma membrane was suggested to inhibit the production of lipid mediators in response to bradykinin [13]. It has been recently reported that $cPLA_2$ fused with a green fluorescent protein, translocated from the cytosol to the perinuclear region in response to increased $[Ca^{2+}]_i$ elevation caused by agonist or ionomycin [27]. Accordingly, a direct interaction between wtCFTR at the plasma membrane and PLA_2 appears unlikely, in disagreement with the conclusion proposed in [13]. The reason for the discrepancy between the results of the present study and those previously presented is at present unknown. One reason might be the different origin of cell lines, being C127 cells derived from mouse, whereas the other studies were performed in cells derived from human tissues [12–14]. Another possible reason might be related to the level of CFTR expression, taking into consideration that C127wt cells are a low-expression system [28], whereas T84 cells [14] have been reported to express a relatively high level of wtCFTR. In this respect, it is interesting that Lee et al. showed a small but significant increase (1.75-fold) in anion exchanger activity also in the absence of forskolin-induced PKA stimulation only in cells with a high level of wtCFTR expression [11].

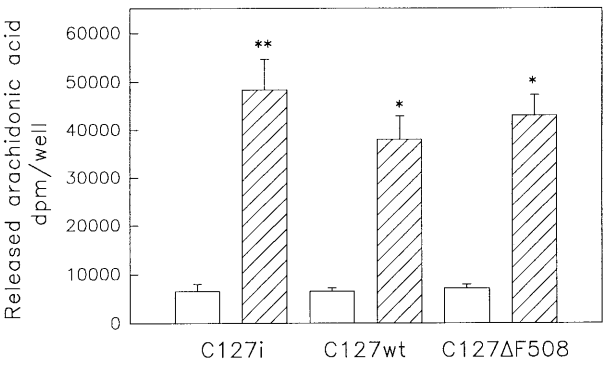

Figure 4. Effect of PMA plus ionomycin on the release of arachidonic acid from C127i, C127wt and C127ΔF508 cell lines. Experimental conditions as in figure 3. Open bars: control; striped bars: 100 nM PMA plus 1 μM ionomycin. Data are means ± SD of five to seven determinations. * values significantly different from control **, $P < 0.05$.

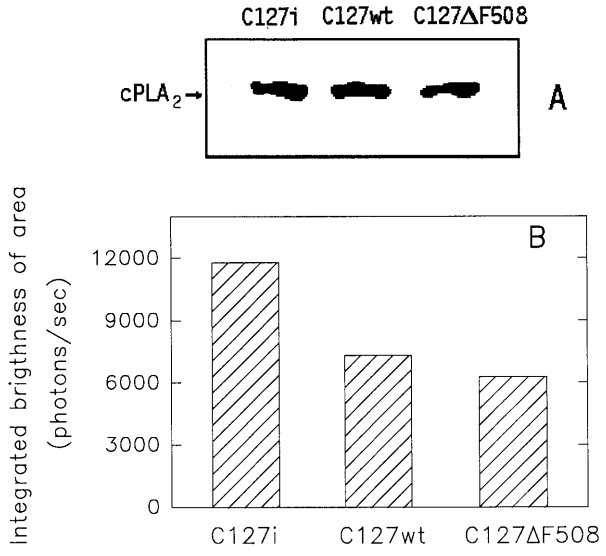

Figure 5. Immunoblot analysis of cytosolic PLA_2 in C127 cell lines. (*A*) Immunoblot analysis of $cPLA_2$ content in cell lysates of C127 cell lines. The blot is representative of three highly consistent experiments. (*B*) Quantitative detection of light emission from each protein band of $cPLA_2$ was performed with a low-light-level imaging apparatus, as described in 'Materials and methods'. Data are expressed as photons/s. The relative light emission of C127wt and C127ΔF508 cell lines was 63 ± 12%, $n = 3$, and 55 ± 10%, $n = 3$, respectively, of light emission of C127i cells, taken as 100 ± 4%, $n = 3$.

In conclusion, our data indicate that under conditions of similar immunoreactive $cPLA_2$ content, no significant difference in the activity of this enzyme was shown in cells expressing wt or mutated CFTR, suggesting the lack of a functional relationship between these two proteins, at least in C127 cells.

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