Amino Acids (1996) 11: 247–255

Amino
Acids

Changes in neutral amino acid efflux and membrane potential associated with the expression of CFTR protein

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Accepted February 20, 1996

Summary. The expression of wild type CFTR facilitates the efflux of neutral amino acids (Rotoli et al., Biochem. Biophys. Res. Commun. 204: 653–658, 1994); as a result, after an extensive depletion of intracellular amino acid pool obtained through an incubation in saline solution, the intracellular leucine levels were lower in murine C127 cells transfected with the wild type CF gene (C127 CFTRw/t) than in cells transfected with either mutant CF (C127 CFTRΔF508 cells) or mock vector only. No change in amino acid efflux was detected when C127 CFTRw/t and C127 CFTRΔF508 cells were studied under conditions known to activate protein kinase A. Upon an incubation in Cl^- free medium, a permeant analogue of cAMP caused a marked cell depolarization of C127 CFTRw/t cells but not of C127 CFTRΔF508 cells, thus showing a functional expression of CFTR protein in the former cell line. However, we found that, upon a Cl^- free incubation and in the absence of exogenous cAMP, C127 CFTRw/t cells developed a marked hyperpolarization that was not detected in C127 CFTRΔF508 cells. It is concluded that the expression of normal CFTR accelerates amino acid efflux and enhances cell hyperpolarization in Cl^- free media; both these effects appear to be independent from PKA stimulation of CFTR.

Keywords: Leucine – Arginine – C127 cells

Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; C127 CFTRw/t, C127i cells expressing CFTR wild type; C127 CFTRΔF508, C127i cells expressing CFTR bearing ΔF508 mutation; C127 mock, C127i cells transfected with the mock vector; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, Dulbecco's modified Eagle medium; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; PKA, protein kinase A

Introduction

It is now well established that CFTR protein is a cAMP-activated chloride channel expressed in a variety of secreting epithelia (Collins, 1992; Tabcharani et al., 1993; Welsh and Smith, 1993). However, an enlarging body of experimental data points to additive functions of the protein.

For example, CFTR exhibits several structural features of ABC (ATP Binding Cassette) transporters, a family of related proteins, widely distributed in prokaryotic and eukaryotic cells, that are involved in *trans*-membrane fluxes of organic solutes (Ames Ferro-Luzzi, 1986; Riordan, 1992; Higgins, 1992). One of mammalian ABC transporters, glycoprotein P, has been described to work both as a primary active transporter for organic substrates and as a chloride channel (Valverde et al., 1992; Gill et al., 1992).

Evidence has been obtained that supports the hypothesis that CFTR can modulate other Cl^- channels (Schwiebert et al., 1994); data have been also published indicating that ATP can be transported by CFTR protein and, once at the extracellular side of the membrane, can modulate the channel function either of CFTR itself (Reisin et al., 1994) or of other Cl^- channels (Cantiello et al., 1994; Schwiebert et al., 1995).

Other authors have found that the expression of CFTR in *Xaenopus* oocytes can elicit Cl^- currents but also raise membrane permeability to water and small organic osmolytes (Hasegawa et al., 1992).

These data give considerable support to the view that CFTR protein is a multifunctional protein and prompted us to look for an involvement of the protein in the transmembrane fluxes of other organic solutes, such as the amino acids.

We found that the expression of normal, but not of mutant CFTR, is associated to different efflux rates of neutral amino acids (Rotoli et al., 1994). In order to ascertain the relationships between this effect and the channel function of CFTR, we have studied the behavior of the same cell lines in Cl^- free media, under conditions in which the Cl^- fluxes through the channel are easily detectable in intact cells.

Materials and methods

Materials

Fetal bovine serum (FBS) was from Gibco. Growth medium (Dulbecco's modified Eagle medium, DMEM) was from Sigma. Geneticin was from Boehringer-Mannheim. Radiolabelled amino acids were obtained from New England Nuclear. Sigma was the source of unlabelled amino acids and other chemicals.

Cell lines

The lines C127 CFTRw/t, C127 CFTRΔF508, and C127 mock, obtained from transfection of C127i mouse cells with, respectively, wild type, mutated CF gene or vector only (Denning et al., 1992) were a kind gift of Genzyme Corporation. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 200μg/ml

geneticin. It has been recently demonstrated, with molecular and functional approaches, that C127 CFTRw/t and C127 CFTRΔF508 cells express normal and mutated CFTR, respectively (Dechecchi et al., 1993), while the mock line does not express the protein (unpublished results).

Efflux of amino acids

Efflux of leucine was performed by sequentially removing aliquots of the extracellular solution and replacing them with fresh solution as described for $^{36}Cl^-$ efflux (Lin and Gruenstein, 1987) with modifications. Briefly, cells were loaded with 20μCi/ml of ^{3}H-amino acid for 1 hour. After this period cells were washed twice with Earle's Balanced Salt Solution (EBSS) or with a modified EBSS in which gluconate substituted chloride in sodium and potassium salts. The efflux of the labelled amino acid was measured in EBSS supplemented with 3% dialyzed FBS at intervals of 30 sec up to six minutes. The experiment was carried on at 37°C. Data of amino acid efflux are expressed as percentage of radioactivity remaining in cells. Initial efflux was calculated from the derivative at time zero of the single exponential function.

$$y = A \cdot e^{-kx} + c \quad \text{(Eqn 1)}$$

and expressed as $\% \cdot min^{-1}$.

Membrane potential and intracellular leucine pool

The transmembrane gradient of L-arginine was employed as a probe of membrane potential (Bussolati et al., 1987, 1989). The cationic amino acid was preaccumulated over a 60-min period; its redistribution was then followed for the indicated times. Intracellular leucine pool was labelled by incubation in the presence of the labelled amino acid (0.81 mM in DMEM, 0.01 mM in EBSS; 2μCi/ml) for 60 min.

For both determinations the experiments were terminated with three ice cold washes in 300 mM urea, cells were extracted in ethanol, and radioactivity was counted in a liquid scintillation spectrometer.

Results

Figure 1 shows that no significant difference was detected among the intracellular leucine pools of the three C127 cell lines maintained in complete DMEM; on the contrary, after a 6h depletion in EBSS the intracellular pool of leucine was significantly smaller in C127 CFTRw/t than in either C127 CFTRΔF508 or C127 mock cells. These differences are due to changes in the leucine efflux rates measured in the three cell lines (Rotoli et al., 1994; Rotoli et al., unpublished results).

The relationships between Cl^- fluxes mediated by CFTR and changes in amino acid efflux were studied determining leucine efflux upon activation of protein kinase A (PKA), an experimental condition that causes the stimulation of Cl^- fluxes through CFTR channel. To this purpose, during the last 10 minutes of leucine pool labelling C127 CFTRw/t cells were treated with both a cell permeant cAMP analogue (100μM 8-Br-cAMP) and the phosphodiesterase inhibitor theophyilline (1 mM), two compounds that activate PKA. The efflux of the labelled amino acid was then followed both in

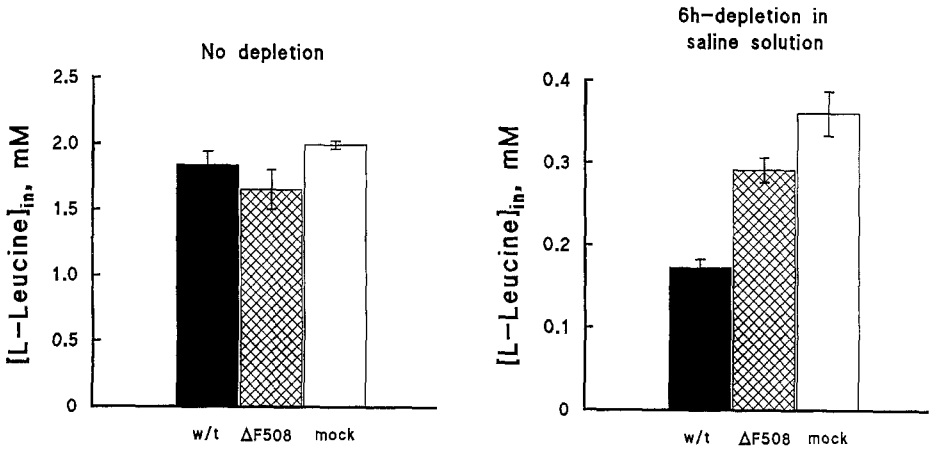

Fig. 1. Determination of intracellular L-leucine pool in C127 CFTRw/t, C127 CFTRΔF508 and C127mock cells. *Left panel.* C127 CFTRw/t (solid bars), C127 CFTRΔF508 (crossed bars) and C127mock cells (empty bars) were incubated for 60 min in DMEM in the presence of [^{3}H]-leucine (0.8 mM; 2 μCi/ml). After this period the medium was removed and the intracellular content of the amino acid was measured. *Right panel.* Cells were incubated for 6 hours in EBSS supplemented with 10% dialyzed FBS. The incubation was prolonged for 60 min in the same medium in the presence of [^{3}H]-leucine (0.01 mM; 2 μCi/ml). Data represent means (± S.D.) of three independent determinations

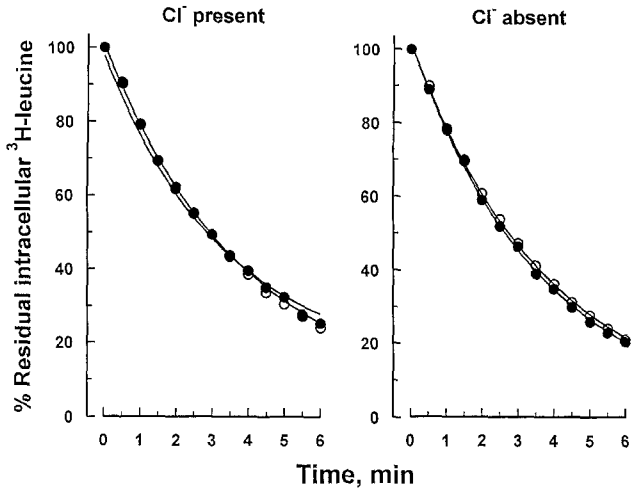

Fig. 2. Effect of 8-Br-cAMP and theophylline on the efflux of L-leucine in C127 CFTRw/t cells. C127 CFTRw/t cells were incubated for 50 min in DMEM in the presence of [^{3}H]-leucine (0.8 mM; 20 μCi/ml). After this period the incubation was prolonged for 10 min in the absence (open symbols) or in the presence (closed symbols) of theophylline (1 mM) and 8-Br-cAMP (100 μM). Cells were then washed twice in EBSS solution or in a modified Cl^--free EBSS and efflux assay was performed, as described in Methods, in the same solutions. Data are expressed as the percentage of radioactivity determined at the beginning of the assay that was found associated to cells at the indicated times of efflux. Each series of points represents sequential determinations in a single well; efflux curves were obtained in parallel in a representative experiment. Lines are computer-drawn, best-fit non linear regressions to Eqn. 1 (see Methods)

control EBSS and in Cl^--free EBSS, a medium usually employed in Cl^- efflux experiments. Under both conditions, PKA activation and, hence, the stimulation of Cl^- fluxes through CFTR were without any significant effect on leucine efflux rates (Fig. 2).

The functional status of CFTR in our cell models was checked taking advantage of the effects of CFTR channel opening on the membrane potential in Cl^--free media (Lukacs et al., 1993). The transmembrane gradient of the cationic amino acid L-arginine was employed as a non invasive probe of the membrane potential (Bussolati et al., 1987). The employment of the cationic amino acid as a non invasive probe for E_m in C127 cells was validated through an experiment (Fig. 3) in which arginine distribution ratio was measured over a range of $[K^+]_{out}$ from 10 to 100 mM. Under control conditions arginine distribution was poorly dependent on $[K^+]_{out}$ in C127 CFTRw/t cells; on the contrary, in the presence of the potassium ionophore valinomycin a marked dependence of arginine distribution on $[K^+]_{out}$ was observed. These results indicate that, as in other cell models (Bussolati et al., 1987, 1989), L-arginine can be employed as a probe of membrane potential in C127 cells.

Briefly, the approach employed (Lukacs et al., 1993) is based on the fact that, in the absence of extracellular Cl^-, CFTR channel opening produces a net efflux of Cl^- that leads to an easily detectable cell depolarization. Upon an incubation of 15 min in a Cl^--free solution the addition of 8-Br-cAMP caused, as expected, a readily detectable depolarization in C127 cells expressing w/t CFTR (Fig. 4). No change in membrane potential was seen either in C127 cells that express the mutant CFTR (Fig. 4) or in mock cells (not shown). It was,

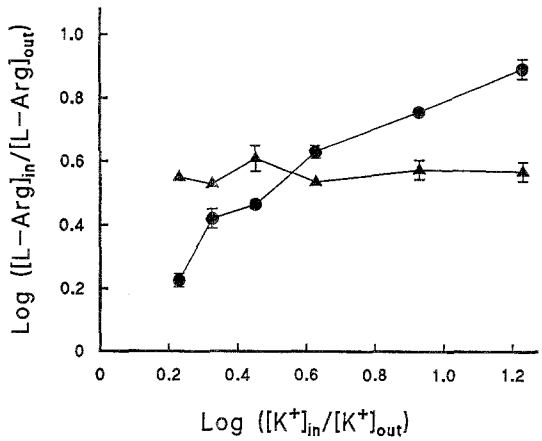

Fig. 3. Dependence of L-arginine distribution ratio ($[L\text{-}Arg]_{in}/[L\text{-}Arg]_{out}$) on the K^+ distribution ratio ($[K^+]_{in}/[K^+]_{out}$ in C127 CFTRw/t cells: effect of valinomycin. C127 CFTRw/t cells were preincubated 4 hours in EBSS supplemented with 10% dialyzed FBS. After this period cells were washed twice with a modified Cl^--free EBSS, in which $[K^+]_{out}$ ranged from 10 to 100 mM; cells were then incubated for 1 hour in the same solution employed for washes, supplemented with 3% dialyzed FBS and labeled L-arginine (0.02 mM, 1 μCi/ml), in the presence (●) or in the absence (▲) of valinomycin (100 μM). Data represent means(± S.D.) of three independent determinations

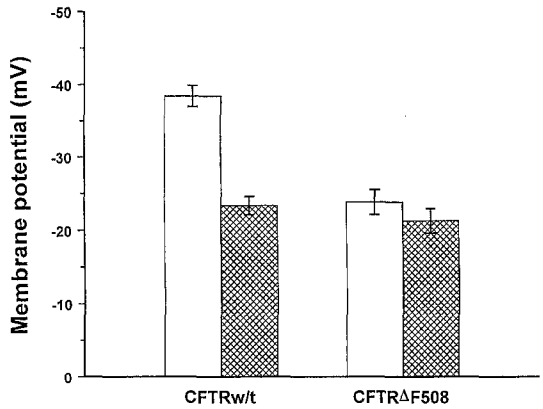

Fig. 4. Effect of cAMP-stimulated Cl^- efflux on the membrane potential of C127 cells. C127 CFTRw/t and C127 CFTRΔF508 cells were incubated for 50 min in EBSS in the presence of labeled L-arginine (0.02 mM; 0.5 μCi/ml). After this period the incubation was prolonged for 10 min in the absence (open bars) or in the presence (cross-hatched bars) of 8-Br-cAMP (100 μM) in a Cl^--free EBSS. Cells were then washed with $MgCl_2$ 0.1 M and the intracellular content of [^{3}H]L-arginine was determined. Values of membrane potential were calculated from L-arginine distribution ratio (see Methods). Data represent means (± S.D.) of three independent determinations

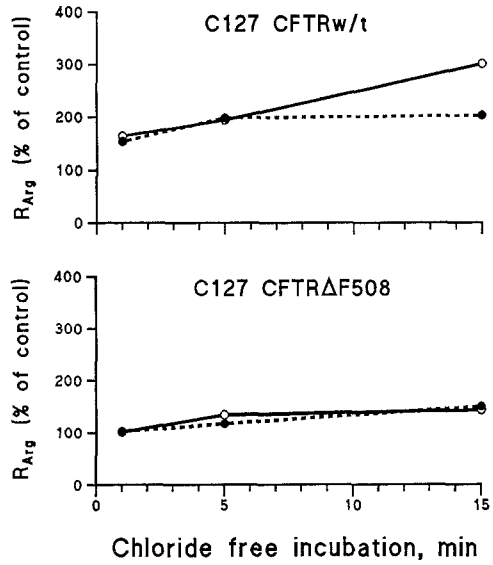

Fig. 5. Determination of L-arginine distribution ratio in C127 CFTRw/t and C127 CFTRΔF508 cells during an incubation in Cl^--free saline solution: effect of 8-Br-cAMP. C127 CFTRw/t and C127 CFTRΔF508 cells were incubated for 50 min in EBSS in the presence of labeled L-arginine (0.02 mM; 0.5 μCi/ml). At the end of this period (time 0) cells were washed twice in a modified Cl^--free EBSS and incubated in the same solution supplemented with [^{3}H]-L-arginine, at the same concentration and specific activity, in the absence (open symbols) or in the presence (solid symbols) of 8-Br-cAMP (100 μM). Intracellular water was measured in the same cells through the distribution space of ^{14}C-urea. The transmembrane distribution ratio of L-arginine was then calculated at the indicated times. Data represent means (± S.D.) of three independent determinations

however, evident that in the absence of 8-Br-cAMP C127 CFTRw/t cells were markedly hyperpolarized compared with C127 CFTRΔF508 cells. A time course of arginine distribution ratio in the two cell lines (Fig. 5) indicated that during 15 min of Cl^--free incubation C127 CFTRw/t cells underwent a progressive hyperpolarization that was completely suppressed in the presence of 8-Br-cAMP; on the contrary, under the same experimental condition, the membrane potential of C127 CFTRΔF508 cells remained substantially stable.

Discussion

The results presented here definitely confirm that the expression of wild type CFTR protein is associated with an accelerated efflux of L-leucine. The stimulation of efflux upon expression of wild type CFTR is not restricted to L-leucine but it is also detected for other amino acids with similar structural characteristics (see Rotoli et al., 1994).

Attempts to correlate the acceleration of amino acid efflux with the channel function of CFTR by studying the effects of PKA activation have failed; treatment with a permeant cAMP-analogue and theophylline (a device commonly employed to raise intracellular cAMP levels) does not alter the efflux rates of neutral amino acids at all. These results suggest that CFTR stimulates amino acid efflux independently of its function as a cAMP-stimulated chloride channel.

In this contribution an heterologous expression model is employed, the mouse cell line C127, in which no endogenous expression of mouse CFTR protein is detected. However, the possibility that some peculiar feature of this cell model somehow interferes with the functional expression of the human CFTR gene has been excluded by the experiment shown in Fig. 4. A clearcut depolarization is observed in Cl^- free medium upon cAMP treatment in cells that express wild type CFTR but not in C127 CFTRΔF508 cells; this result demonstrates that CFTR is not only expressed but also works normally as a PKA-activated Cl^- channel in the transfected C127 cell line employed here.

Moreover, the same experiment yielded another significant result: upon Cl^--free incubation cells that express normal CFTR are indeed significantly hyperpolarized compared with cells that express a mutant form of the protein. A time course of arginine distribution ratio (a probe of membrane potential validated also in the cell model employed here, see Fig. 3) has indicated that a progressive change in membrane potential is detected in the cells transfected with the wild type CFTR during an incubation in Cl^- free medium in the absence of exogenous cAMP. This result points to the existence of other cAMP-independent action(s) of CFTR. The hyperpolarization is by no means explained by a possible, partial activation of CFTR Cl^- channel, since, in this case, cells expressing w/t CFTR should be depolarized, and not hyperpolarized, compared to C127 CFTRΔF508 cells.

Other reports have suggested that CFTR is a multifunctional protein, playing roles other than cAMP-regulated chloride channel (Hasegawa et al.,

1992; Barasch et al., 1991; Gabriel et al., 1993; Stutts et al., 1995; Schwiebert et al., 1994, 1995; Cantiello et al., 1994). Moreover, selected examples of CFTR sensitivity to regulatory mechanisms independent from PKA activation have been described (Dechecchi et al., 1994; Cantiello et al., 1994; Prat et al., 1995) that could resemble the stimulation of amino acid efflux and the change in membrane potential oserved here. Therefore, the results presented here could constitute the basis for future research on alternative function(s) of CFTR protein.

Acknowledgements

We thank Else K. Hoffmann for helpful comments and suggestions. Financial support of Telethon- Italy (grant no. E.320) is gratefully acknowledged.

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Received January 15, 1996

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Hersteller: Satz und Umbruch: Best-Set Typesetter Ltd., Hong Kong; Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. – Printed in Austria.