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General anesthetic octanol and related compounds activate wild-type and delF508 cystic fibrosis chloride channels

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1 Cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is defective during cystic fibrosis (CF). Activators of the CFTR Cl^- channel may be useful for therapy of CF. Here, we demonstrate that a range of general anesthetics like normal-alkanols (*n*-alkanols) and related compounds can stimulate the Cl^- channel activity of wild-type CFTR and delF508-CFTR mutant.

2 The effects of *n*-alkanols like octanol on CFTR activity were measured by iodide (^{125}I) efflux and patch-clamp techniques on three distinct cellular models: (1) CFTR-expressing Chinese hamster ovary cells, (2) human airway Calu-3 epithelial cells and (3) human airway JME/CF15 epithelial cells which express the delF508-CFTR mutant.

3 Our data show for the first time that *n*-alkanols activate both wild-type CFTR and delF508-CFTR mutant. Octanol stimulated ^{125}I efflux in a dose-dependent manner in CFTR-expressing cells (wild-type and delF508) but not in cell lines lacking CFTR. ^{125}I efflux and Cl^- currents induced by octanol were blocked by glibenclamide but insensitive to 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, as expected for a CFTR Cl^- current.

4 CFTR activation by octanol was neither due to cell-to-cell uncoupling properties of octanol nor to an intracellular cAMP increase. CFTR activation by octanol requires phosphorylation by protein kinase-A (PKA) since it was prevented by H-89, a PKA inhibitor.

5 *n*-Alkanols chain length was an important determinant for channel activation, with rank order of potencies: 1-heptanol < 1-octanol < 2-octanol < 1-decanol. Our findings may be of valuable interest for developing novel therapeutic strategies for CF.
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Keywords: Cystic fibrosis; CFTR; delF508 mutant; chloride channels; Pharmacology; epithelial cells; *n*-alkanols; octanol

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; FSK, forskolin; α-GA, 18-α-glycyrrhetinic acid; glib, glibenclamide; GST, genistein; MPB, Benzo(c)quinolizinium compounds; *n*-alkanols, normal alkanols; PKA, protein kinase A; PKC, protein kinase C

Introduction

Cystic fibrosis (CF) is a fatal genetic disease characterized by abnormal ion transport across epithelia of exocrine glands that is caused by mutations in the *CF* gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Riordan *et al*., 1989). CFTR is well known as a cAMP-dependent protein kinase (PKA)-regulated Cl^- channel (Bear *et al*., 1992) located in the apical membrane of epithelial cells. Protein kinase C (PKC) also regulates CFTR channel activity but the mechanism involved is not yet well elucidated (Liedtke & Cole, 1998; Middleton & Harvey, 1998; Chappe *et al*., 2003).

The most common mutation in the *CF* gene is a three base pair deletion resulting in loss of phenylalanine residue at position 508 in the protein, delF508-CFTR (Riordan *et al*., 1989). This mutant protein is incorrectly processed and retained within the endoplasmic reticulum where it was degraded (Cheng *et al*., 1990). However, its overexpression in mammalian cells may allow delF508-CFTR to reach the plasma membrane where it exhibits a reduced Cl^- channel activity (Dalemans *et al*., 1991).

In addition to agents well known to increase cAMP level such as forskolin or xanthine derivatives (Chappe *et al*., 1998) and used to induce CFTR activation, several studies have focused their efforts to find novel more efficient and more specific molecules able to activate CFTR channels, for example, xanthine derivatives (Chappe *et al*., 1998) including CPX (Eidelman *et al*., 1992). Benzo(*c*)quinolizinium compounds (MPB) have been characterized as specific CFTR activators (Becq *et al*., 1999; Dérand *et al*., 2001; Dormer *et al*., 2001). In addition, the MPB-91 derivative has been shown to inhibit delF508-CFTR mutant degradation and to redirect delF508-CFTR to the plasma membrane (Dormer *et al*., 2001). CFTR can also be activated by the tyrosine kinase inhibitor genistein (GST) (Illek *et al*., 1995) and the K^+ channel activators benzimidazolone compounds NS004 and 1-EBIO (Gribkoff *et al*., 1994; Devor *et al*., 1996). However, the mode of activation and the specificity of these latter activators are still debated.

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n-Alkanols are known as anesthetic molecules and exert complex effects on biological membranes (see for reviews Goldstein, 1984; Weight, 1992; Dilger, 2002). A common feature of anesthetic action is the modulation of electrical signaling which is accomplished by altering membrane conductance through ion channels. *n*-Alkanols have been shown to regulate the activity of the γ-aminobutyric acid Cl^- receptor-channel ($GABA_A$) (Nakahiro *et al*., 1991; Marszalec *et al*., 1994; Narahashi *et al*., 1998), the nicotinic acetylcholine Na^+ receptor channel (Murrell *et al*., 1991; Wood *et al*., 1995), G protein-coupled inwardly rectifying potassium channels (Schmid *et al*., 1997; Lewohl *et al*., 1999) and the P2X purinoceptors (Weight *et al*., 1999). *n*-Alkanols have also been shown to relax airway smooth muscle by decreasing intracellular calcium concentration ($[Ca^{2+}]_i$) and the force produced for a given $[Ca^{2+}]_i$ during membrane receptor stimulation, and may be employed as bronchodilators (Sakihara *et al*., 2002). Mechanisms involved in *n*-alkanols-mediated effects are not elucidated. However, two general hypotheses are proposed to account for the *n*-alkanols effects on these membrane-associated proteins. The first one argues that *n*-alkanols bind directly to hydrophobic regions of specific proteins (Mascia *et al*., 2000). The second rather favors the view that *n*-alkanols alter physical properties of the membrane (Zavoico & Kutchai, 1980), necessary to the normal functioning of the membrane-associated protein. In addition, lipophilic agents, such as long-chain *n*-alkanols, have been involved in impairment of the conductance of gap junctions in various tissues and cells including epithelial cells (Weingart & Bukauskas, 1998).

Accordingly, the aim of the present study was to investigate the effects of *n*-alkanols on CFTR Cl^- channel activity. We report here, for the first time, that both wild-type CFTR and delF508-CFTR mutant channels are activated by *n*-alkanols as a function of the hydrophobicity of the alcohol molecules. Our data may represent a new approach toward treatment of CF disease.

Methods

Solutions and chemicals

Culture medium, serum, penicillin and streptomycin were purchased from BioMedia (Boussens, France), fura2-AM from Molecular Probes (Netherlands), ATP, 1-butanol, 1-hexanol, 1-heptanol, 1-octanol, 2-octanol, 1-decanol, 18-α-glycyrrhetinic acid (α-GA) and other chemicals from Sigma (St Louis, MI, U.S.A.). Forskolin (FSK) and fura2-AM were dissolved in DMSO. The final concentration of DMSO in the experiments was less than 0.1% and was found to have no significant effect on membrane current and iodide efflux. The concentrations of *n*-alkanols used in the present experiments were in a range from 0.1 to 10 mM as indicated and represented $v^{-1}v$ proportions of 0.001–0.1%.

Cell culture

Cell lines were grown in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C.

CHO cells stably transfected with pNUT vector containing wild-type CFTR (CHO-BQ1), were provided by J.R. Riordan and X.B. Chang, Scottsdale, AZ, USA (Chang *et al*., 1998). Cells were maintained in αMEM supplemented with 7.5% fetal calf serum (FCS), 2 mM glutamine, penicillin (50 IU ml^{-1}), streptomycin (50 $\mu g\,ml^{-1}$) and methotrexate (100 μM). CHO-K1 parental cells (American Type Culture Collection (ATCC) number CCL-61) were maintained in DMEM Ham's F-12 nutritive mix (1:1) supplemented by 7.5% FCS and 50 IU ml^{-1} penicillin plus 50 $\mu g\,ml^{-1}$ streptomycin.

Calu-3 (ATCC number HTB-55), an epithelial cell line of human pulmonary origin (Finkbeiner *et al*., 1993) was maintained in DMEM Ham's F-12 nutritive mix (1:1) supplemented by 10% FCS and 50 IU ml^{-1} penicillin plus 50 $\mu g\,ml^{-1}$ streptomycin.

The SV40 transformed nasal epithelial cell line JME/CF15 (Jefferson *et al*., 1990) from a patient with CF (homozygous for the deletion of Phe 508 (delF508) of CFTR) was cultured in DMEM/F-12 Ham (3:1) supplemented with defined concentrations of adenine (180 μM), insulin (5 $\mu g\,ml^{-1}$), transferrin (5 $\mu g\,ml^{-1}$), hydrocortisone (1.1 μM), tri-iodothyronine (2 nM), epinephrine (5.5 μM), epidermal growth factor (1.64 nM) and 10% FCS.

Measurement of $[Ca^{2+}]_i$ in CHO-BQ1 cells

Fura-2 fluorescence Ca^{2+} imaging was used to measure $[Ca^{2+}]_i$ as previously described (Marcet *et al*., 2003). Briefly, cells were cultured for 4 days on glass coverslips, and then loaded with 2.5 μM fura-2-AM for 1 h at 37°C in serum-free DMEM/F12 medium. After rinsing with modified Earle's salt solution (B medium) containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 10 HEPES-NaOH, pH 7.4, cells were placed into an open-topped microperfusion chamber and superfused with test solutions at 1 ml min^{-1}. Ratiometric fluorescence (340/380) was used to calculate $[Ca^{2+}]_i$ according to Grynkiewicz *et al*. (1985). For each experiment, 50 cells were individually analyzed and $[Ca^{2+}]_i$ was averaged and plotted *versus* time.

Measurement of intracellular cAMP in CHO-BQ1 cells

CHO-BQ1 cells grown for 4 days in 12-well plates were washed twice with 2 ml of B medium, then 0.5 ml of this buffer containing the drug to be tested were added to each well. After a 5 min incubation period at 37°C, the cells were permeabilized, and intracellular cAMP content was measured using the cAMP Biotrack Enzymeimmunoassay (EIA) system according to the manufacturer's instructions (Amersham Biosciences, U.K.). cAMP levels were expressed as pmol $well^{-1} \pm$ s.d.

Determination of iodide efflux

^{125}I efflux technique used to monitor CFTR activity in CHO cells was performed as previously described (Marcet *et al*., 2003). CHO cells were cultured for 4 days in 24-well culture plates. Before experiments, the culture medium was removed and CHO cells were washed twice with 500 μl of B medium (see above for composition). CHO cells were then loaded in B medium containing 1 μM KI and 0.5 μCi of Na $^{125}I\,ml^{-1}$ for 30 min. After loading, CHO cells were washed four times with 500 μl of iodide-free B medium. B medium (500 μl) was then added and removed sequentially to be counted every 30 s for 2.5 min. Agonists or antagonists to be tested were added at

time zero of the efflux. At the end of the efflux, intracellular ions were extracted by the addition of 1 ml trichloroacetic acid (7.5%) to the CHO cell layer. All samples were counted using a γ counter (Kontron). Precipitated proteins were solubilized in 0.1 N NaOH and quantified using Coomassie protein Assay Reagent (Pierce, U.S.A.). ^{125}I efflux analysis in CHO cells was realized as previously described (Marcet *et al*., 2003). Tracer contained in the cell layer at the onset of the efflux was calculated as the sum of samples and extract count. Efflux curves were constructed by plotting the percent of cell layer content (I%) remaining in the cell layer *versus* time. Rate constants (k, min^{-1}) from unstimulated or stimulated efflux were determined by fitting efflux curves to mono-exponential function $I\% = 100\exp(-kt)$ using linear regression of the neperian logarithm of the data. k is used in Results to calculate iodide accumulated in the medium *versus* time. We hypothesized that, in the presence of a stimulator, the efflux was the sum of two iodide effluxes occurring in parallel: a basal efflux and a stimulated efflux characterized by the rate constants k_b and k_s, respectively. The net total efflux was therefore described by $I_t\% = 100\exp(-k_t t)$ where k_t is the sum of k_b and k_s rate constants. Finally, k_s calculated as $k_t - k_b$ was used to established concentration–response relationships for agonists.

^{125}I efflux technique used to monitor CFTR activity in Calu-3 cells and JME/CF15 cells was performed as previously described (Dérand *et al*., 2001; Dormer *et al*., 2001). Calu-3 cells and JME/CF15 cells (Figures 5 and 6) grown in 24-well plates were washed twice with 2 ml of efflux medium containing (in mM): 137 NaCl, 4.4 KCl, 0.3 KH_2PO_4, 0.3 NaH_2PO_4, 4.2 $NaHCO_3$, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 5.5 glucose and 10 HEPES, (pH 7.5), then incubated in this efflux medium containing 1 μM KI (1 μCi $Na^{125}I$ ml^{-1}; NEN, Boston, MA, U.S.A.) for 1 h at 37°C. After washing with B medium, the same medium was sequentially added and removed each 1 min to be counted. This procedure was repeated every 1 min for 11 min. The first three aliquots were used to establish a stable baseline in efflux medium alone. Efflux medium containing the appropriate drug was used for the remaining aliquots. At the end of the experiment, the medium was recovered and cells were solubilized in 1 ml of NaOH (1 N). The radioactivity was determined using a γ counter (Cobra II, PerkinElmer Life Sciences, Courtaboeuf, France). ^{125}I efflux analysis in Calu-3 and JME/CF15 cells was calculated as follows (see also Dérand *et al*., 2001; Dormer *et al*., 2001). The fraction of initial intracellular ^{125}I lost during each time point was determined, and time-dependent rates of ^{125}I efflux were calculated from $\ln(^{125}I_{t1}/^{125}I_{t2})/(t_1 - t_2)$, where $^{125}I_t$ is the intracellular ^{125}I at time t, and t_1 and t_2 are successive time points (Venglarik *et al*., 1990). Curves illustrated in Figures 5 and 6 were constructed by plotting rates of ^{125}I *versus* time. Comparisons were based on maximal values for the time-dependent rates (peak rates), with exclusion of the points used to established the baseline.

FSK concentration–response curves were fitted using the hyperbolic equation and octanol concentration–response curves were fitted using the Hill equation (GraphPad Prism v3.0, GraphPad Software). Data are expressed as means ± s.e.m., and t-test was used to determine significance.

Whole-cell patch clamp recording in CHO cells

CHO cells were plated on 35 mm Petri dishes and cultured at 37°C in 5% CO_2 for 4 days before use. Currents were recorded using the patch-ruptured whole-cell variant of the patch clamp technique and measured with an Axopatch 200B amplifier and filtered at 2 kHz. The external solution consisted of (mM): 120 NaCl, 23 $NaHCO_3$, 3 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, 11 glucose (bubbled with a 95% O_2–5% CO_2 mixture, pH 7.4). The intracellular solution was (mM) 60 CsCl, 110 *N*-methyl-D-glucamine, 1 $MgCl_2$, 4 MgATP, 0.2 Na_3GTP, 11 EGTA, 0.5 $CaCl_2$, 10 HEPES (adjusted to pH 7.3 with HCl, 295 mOsmol l^{-1}). When filled with this internal solution pipette resistance was 5 MΩ. Cell membrane capacitance and series resistance compensations were applied (75–85%). The voltage–current relationships were determined using slow voltage ramps or step voltage protocols. Experiments were performed at room temperature and drugs were applied by using a gravity-fed perfusion system at 5–10 ml min^{-1}. Data were expressed as the mean ± s.e.m. and Student's t-test were used to determine the statistical significance and differences were considered significant if $P < 0.05$.

Results

n-*Alkanols activate CFTR channels in CHO-BQ1 cells*

We sought to determine whether 1-octanol and other *n*-alkanols could regulate CFTR activity in CFTR-expressing CHO-BQ1 cells. Figure 1a shows that 1-octanol alone induced a significant increase ($k_t = 0.8 \pm 0.1$ min^{-1}; $n = 12$) in ^{125}I efflux (t-test: $P < 0.001$) in comparison with basal ^{125}I efflux ($k_b = 0.2 \pm 0.01$ min^{-1}; $n = 12$). As a positive control we applied FSK (1 μM) and observed an enhancement of ^{125}I efflux rate ($k_t = 0.95 \pm 0.1$ min^{-1}; $n = 12$). In CHO-K1 cells, which do not express CFTR channels, 1-octanol (0.25–1 mM; k_t values: from 0.2 ± 0.01 to 0.25 ± 0.01 min^{-1}; $n = 12$), FSK (5 μM; $k_t = 0.25 \pm 0.02$ min^{-1}; $n = 12$) or FSK plus 1-octanol (with a maximum k_t value of 0.26 ± 0.03 min^{-1}; $n = 12$) did not enhance ^{125}I efflux above the basal efflux (Figure 1b and c).

To assess the involvement of CFTR in the 1-octanol-induced ^{125}I efflux we treated cells with glibenclamide (100 μM), which is commonly used to inhibit CFTR channel activity. Figure 1d shows that glibenclamide strongly decreased both the 1-octanol (1 mM; $n = 12$)- and the FSK (5 μM; $n = 12$)-induced ^{125}I efflux (t-test: $P < 0.001$), suggesting that the stimulation of ^{125}I efflux promoted by 1-octanol reflects CFTR activation. Moreover, the increase of ^{125}I efflux in response to either 1-octanol (0.1–5 mM) or FSK (0.1–5 μM) was found to be concentration-dependent with EC_{50} values of 0.6 ± 0.03 mM and 0.6 ± 0.16 μM, respectively (Figure 1e). The maximal CFTR activation was reached around a concentration of 1 mM of 1-octanol.

We then tested whether other alkanols could stimulate CFTR activity, beginning with 2-octanol. As shown in Figure 1f, 2-octanol (1 mM) induced CFTR activation, which was slightly, but significantly (t-test: $P < 0.01$), more efficient than that induced by 1-octanol (1 mM). *n*-Alkanols (1 mM each) with chain length less than that of 1-heptanol (e.g. ethanol, butanol and 1-hexanol) failed to activate CFTR whereas *n*-alkanols with chain length greater than that of 1-hexanol (e.g. 1-heptanol, 1-octanol, 2-octanol, 1-decanol) caused a significant activation of CFTR (t-test: $P < 0.001$). It is of interest to note that the stimulation of CFTR by *n*-alkanols increased as a function of the alcohol chain length,

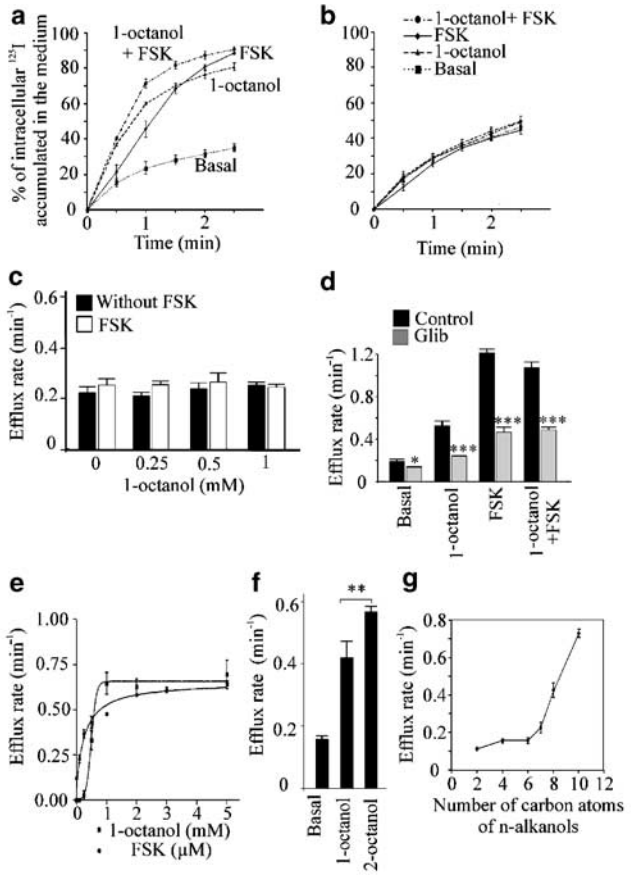

Figure 1 (a) Effect of 1-octanol (1 mM), FSK (1 μM) and 1-octanol (1 mM) plus FSK (1 μM) on stimulated ^{125}I efflux in CFTR-expressing CHO-BQ1 cells. ^{125}I efflux kinetics were measured in the absence or presence of FSK (1 μM) with or without 1-octanol (1 mM) as indicated in the figure. Results are expressed as % of intracellular ^{125}I accumulated in the medium *versus* time (min). Curves are exponential efflux rates for both basal and stimulated effluxes. (b) Absence of effect of 1-octanol (1 mM), FSK (5 μM) and 1-octanol (1 mM) plus FSK (5 μM), as indicated on the figure, on ^{125}I efflux *versus* time (min) in control CHO-K1 cells which do not express CFTR channel. Results are expressed as percent of intracellular ^{125}I accumulated in the medium. (c) Effect of increasing concentrations of 1-octanol (0–5 mM) with (open bars) or without FSK (5 μM) (black bars) on ^{125}I efflux rate (min^{-1}) in CHO-K1 cells, which do not express CFTR. (d) Effect of glibenclamide (glib; 100 μM) on 1-octanol (1 mM), FSK (5 μM)- and 1-octanol (1 mM) plus FSK (5 μM)-stimulated ^{125}I efflux rate (min^{-1}) in CFTR-expressing CHO-BQ1 cells. (e) Concentration-dependent activation of ^{125}I efflux for FSK or 1-octanol in CHO-BQ1 cells. The ^{125}I efflux rate (min^{-1}) was measured in the presence of increasing concentrations of FSK (black circle) or 1-octanol (black square). Curves were fitted using the hyperbolic equation described in Methods. (f) Comparison of effects of 2-octanol and 1-octanol on ^{125}I efflux rate (min^{-1}). (g) ^{125}I efflux rate (min^{-1}) plotted as a function of the number of carbon atoms of primary alcohols. Data are means ± s.d. for $n = 12$ experiments in each condition illustrated in every panel of the figure (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; *t*-test).

that is, the number of carbons in the alcohol molecule (Figure 1g). The order of potency of activation of CFTR by *n*-alkanols was found to be 1-heptanol < 1-octanol < 2-octano-1 < 1-decanol, suggesting that the potency of alcohols on CFTR activation increases with the increase in hydrophobicity of the molecules. Taken together, these results strongly suggest that *n*-alkanols activate CFTR-like ^{125}I efflux in CHO-BQ1 cells.

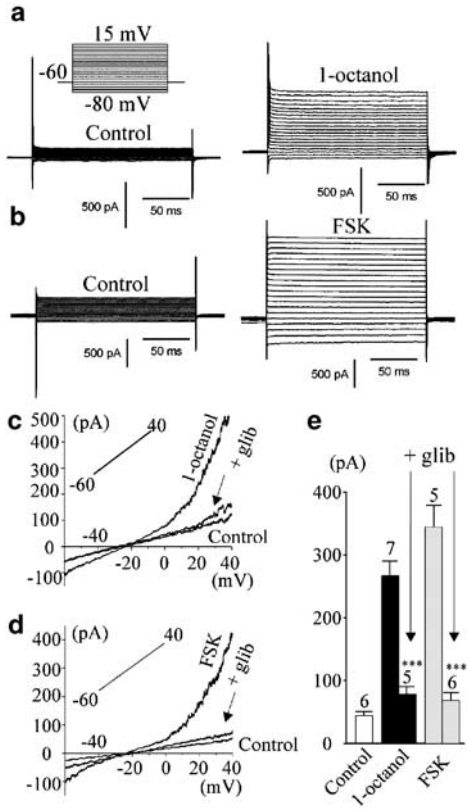

Figure 2 Activation of CFTR currents by 1-octanol in CHO-BQ1 cells. (a,b) Families of currents traces elicited by 150 ms test pulses from −80 to +15 mV in 5 mV increments in the absence (control) or presence of 1-octanol (1 mM, a) or FSK (1 μM, b). (c,d) Representative steady-state *I–V* relationships obtained using slow voltage ramps (as indicated in inset) in CHO-BQ1 cells in control conditions (unstimulated) and in the presence of 1-octanol (1 mM, c) or FSK (1 μM, d) with and without glibenclamide (100 μM). (e) Mean amplitude of glibenclamide-sensitive current measured at +20 mV in cells exposed to 1-octanol or FSK. Bars represent means ± s.e.m. for the number of cells indicated (*t*-test: ***, $P < 0.001$).

1-Octanol activates CFTR current in single cells

We further tested the effects of 1-octanol on CFTR activity in CHO-BQ1 cells using the whole-cell recording variant of the patch-clamp technique. The whole-cell current–voltage (*I–V*) relationships were studied both using step or ramp depolarizations and using intracellular and extracellular solutions giving a reversal potential for Cl^- of −20 mV (see Methods). Voltage pulses from a holding potential of −60 mV evoked very little current in CHO-BQ1 cells (Figure 2a). In contrast, 1-octanol, applied at 1 mM for 30 s, produced a five- to eight-fold increase in the whole-cell membrane conductance ($n = 6$) (Figure 2a and e). The current activated by 1-octanol reversed at −22 ± 2 mV (Figure 2c), showing that Cl^- was the major ion contributing to this current. Octanol-induced current was blocked by applying glibenclamide (100 μM) (Figure 2c and e) and was absent in CHO-K1 cells (data not shown). DIDS at 500 μM had no effects on the octanol-induced current (data not shown). Importantly, FSK produced currents that shared all the features of those induced by 1-octanol (compare Figure 2a and c with Figure 2b and d). These data show that general anesthetic octanol and related compounds represent a novel class of openers of the CFTR channel.

n-*Alkanols failed to promote an increase in intracellular cAMP level*

To test whether octanol was altering cAMP levels in CHO-BQ1 cells, thereby promoting CFTR activation, we measured the cAMP level in response to the application of different alcohols and – as positive control – of FSK. Figure 3a shows that 1-octanol (1.9 ± 0.5 pmol well^{-1}, $n=8$), 1-hexanol (1.5 ± 0.5 pmol well^{-1}, $n=8$) and ethanol (2.0 ± 0.5 pmol well^{-1}, $n=8$) (1 mM each) did not change significantly the intracellular cAMP levels as compared with basal cAMP levels (1.53 ± 0.9 pmol well^{-1}, $n=8$). As expected, FSK applied at 1 μM (10.7 ± 0.5 pmol well^{-1}, $n=8$) or 5 μM (56 ± 5.1 pmol well^{-1}, $n=8$) significantly increased cAMP levels (*t*-test: $P<0.001$). These results indicate that activation of CFTR by *n*-alkanols was not mediated by a cAMP increase.

Inhibition of PKA by H-89 pretreatment abolishes the effects of 1-octanol on CFTR activity

It is well established that CFTR is activated by PKA (Riordan *et al*., 1989; Bear *et al*., 1992). To examine whether the *n*-alkanols-induced CFTR activation requires phosphorylation by PKA, CHO-BQ1 cells were incubated for 30 min with the specific PKA inhibitor, H-89 (30 μM) (Chijiwa *et al*., 1990). Under these conditions, stimulation of 1-octanol- or FSK-induced ^{125}I efflux were totally inhibited (Figure 3b), suggesting that PKA activity was necessary to obtain activation of CFTR by 1-octanol.

Effects of PKC inhibition on octanol-induced CFTR activation

n-Alkanols such as 1-octanol are known to regulate activity of certain PKC isoforms (Slater *et al*., 1993; 1997). As it has been shown that CFTR channel activity could be regulated by PKC (Jia *et al*., 1997; Paradiso *et al*., 2001; Chappe *et al*., 2003), we then tested whether PKC can be involved in *n*-alkanol-induced CFTR activation. CHO-BQ1 cells were incubated for 30 min in the presence of bisindoylmaleimide (GF109203X; 100 nM) a specific PKC inhibitor (Toullec *et al*., 1991) prior to stimulation by 1-octanol (1 mM). Inhibition of PKC by GF109203X had no effect on basal CFTR activity and did not block the effect of 1-octanol on CFTR activity (Figure 3c).

1-Octanol and α-GA reduce the time course of ATP-induced Ca^{2+} response but α-GA had no effects on CFTR activity

Octanol and α-GA are often used as cell–cell uncoupling agents that inhibits gap junctions (Weingart & Bukauskas, 1998; Contreras *et al*., 2002). Gap junctions are known to be involved in intercellular spread of Ca^{2+} waves, and extracellular ATP has the ability to trigger this Ca^{2+} signaling by interacting with plasma membrane P2Y nucleotide receptor (see for review Evans & Martin, 2002). CHO cells possess a well-described P2Y2 nucleotide receptor signaling pathway, whose activation by ATP leads to a long lasting (~6 min) Ca^{2+} increase consisting of a fast release of Ca^{2+} from endoplasmic stores followed by a slowly decaying phase (Marcet *et al*., 2003). Using fura2-Ca^{2+} imaging, we found that octanol or α-GA produced a similar shortening of the

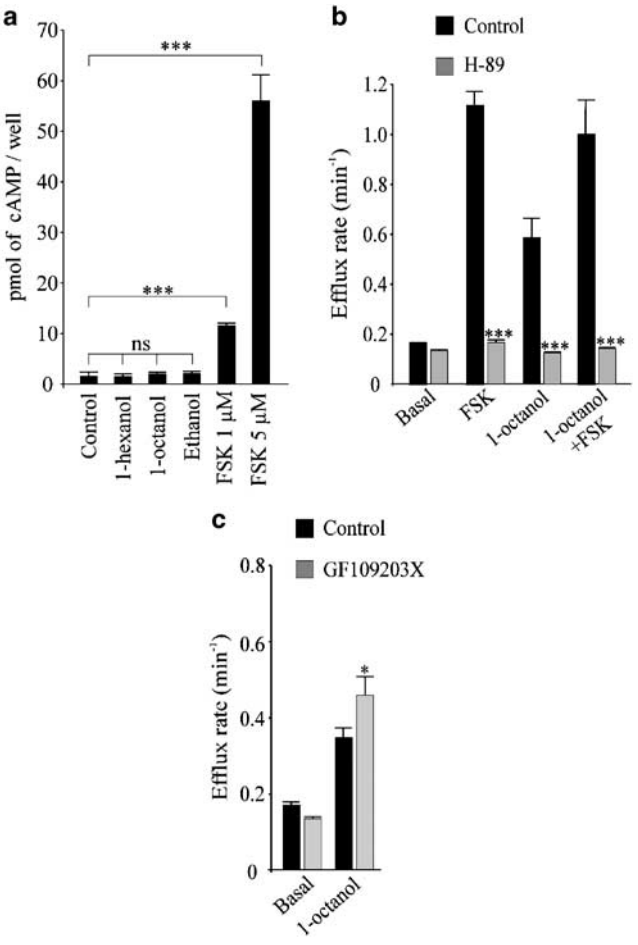

Figure 3 (a) Effect of *n*-alkanols on intracellular cAMP content, determined as described in Methods. cAMP content was measured in resting cells (control; $n=8$) or in the presence of ethanol (1 mM; $n=8$), 1-hexanol (1 mM; $n=8$) or 1-octanol (1 mM; $n=8$), FSK (1 μM; $n=8$) and FSK (5 μM; $n=8$). Data are expressed as pmol of cAMP per well and represent means ± s.e.m. for the number of experiments indicated (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns, not significant; *t*-test and one-way ANOVA test). (b) Effect of PKA inhibition by H-89 (30 μM, for 30 min) on 1-octanol (1 mM)-induced CFTR activation ($n=12$) in comparison with the effect observed on FSK (1 μM)-induced CFTR activation ($n=12$) and a combined application of FSK (1 μM) and 1-octanol (1 mM) ($n=12$). (c) Effect of the PKC inhibitor GF109203X (100 nM, for 30 min) on 1-octanol (1 mM)-induced CFTR activation ($n=12$) (***, $P<0.001$; *, $P<0.05$; *t*-test).

ATP-induced Ca^{2+} response (Figure 4a), suggesting that these agents prevented the spreading of ATP-evoked Ca^{2+} waves in interconnected CHO cells. Whether the cell–cell uncoupling property of octanol may be responsible for some part of the effects observed with the *n*-alkanols on CFTR activity was tested by comparing the effects of 1-octanol with those of α-GA. Figure 4b clearly shows that α-GA (10 and 100 μM) failed to mimic the effects of 1-octanol on CFTR activation. These data indicate that CFTR activation by octanol was not due to its cellular uncoupling properties.

Octanol activates endogenous CFTR in human airway Calu-3 epithelial cells

Calu-3 cells, a human airway epithelial cell line derived from a lung adenocarcinoma, form tight junctions and express CFTR

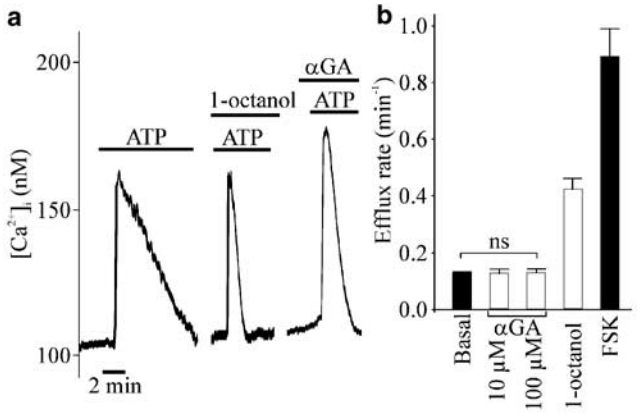

Figure 4 Effect of uncoupling agents on ATP-induced Ca^{2+} response and on CFTR activation in CHO-BQ1 cells. (a) Effect of the uncoupling agents 1-octanol (1 mM; $n=3$) and α-GA (10 μM; $n=3$) on the time course of the Ca^{2+} response induced by ATP (10 μM; $n=3$). (b) Effect of the uncoupling agent α-GA (10 and 100 μM) on ^{125}I efflux rate (min^{-1}) ($n=12$), in comparison with 1-octanol (1 mM; $n=12$) and FSK (5 μM; $n=12$).

channels which are the predominant pathway for cAMP-stimulated Cl^- secretion in these cells (Haws *et al*., 1994). Figure 5a and b shows that 1-octanol (1 mM; $n=8$) stimulated ^{125}I efflux in Calu-3 cells was not sensitive to DIDS (500 μM; $n=8$) but was strongly (*t*-test: $P<0.001$) inhibited by glibenclamide (100 μM; $n=8$), indicating an involvement of CFTR channels in response to 1-octanol stimulation. As observed in CHO-BQ1 cells, the ^{125}I efflux induced by 1-octanol (0.1–10 mM) was found to be concentration-dependent (EC_{50} value of 1.1 ± 0.15 mM) in Calu-3 cells. We observed that 1-octanol, at concentrations up to 0.1 mM, had additive effects on the FSK (1 μM)-induced CFTR activation (Figure 5c). In the presence of FSK (1 μM), the dose–response curve for 1-octanol was shifted leftwards and had an EC_{50} value of 0.5 ± 0.05 mM (Figure 5c and d). This indicates that CFTR activation by octanol was more efficient when CFTR channel was first phosphorylated by PKA.

Octanol activates delF508-CFTR Cl^- channels in human airway epithelial cells

Finally, we have studied the effect of 1-octanol on the delF508-CFTR mutant in JME/CF15 cells, which are human airway epithelial cells derived from CF patients homozygous delF508 (Jefferson *et al*., 1990). Functional CFTR channels are therefore absent of the plasma membrane in JME/CF15 cells (Jefferson *et al*., 1990). Stimulation by 1-octanol (1 mM; $n=8$) or FSK plus GST (10 and 30 μM, respectively; $n=8$) failed to activate any ^{125}I efflux (data not shown), indicating there was no functional CFTR channel at the cell surface and that neither 1-octanol nor FSK activate other Cl^- conductances in JME/CF15 cells. In cells pretreated with the MPB-91 (250 μM, 2 h), a benzo(*c*)quinolizinium compound that has been previously shown to inhibit degradation of delF508-CFTR and to redirect the channel at the cell surface (Dormer *et al*., 2001), 1-octanol ($n=8$) as well as FSK plus GST ($n=8$) were capable of activating ^{125}I efflux (Figure 6a and b). 1-Octanol-induced ^{125}I efflux was insensitive to DIDS (500 μM; $n=8$) but abolished by glibenclamide (100 μM; $n=8$) (Figure 6c). The activation of delF508-CFTR by 1-octanol amounted for about

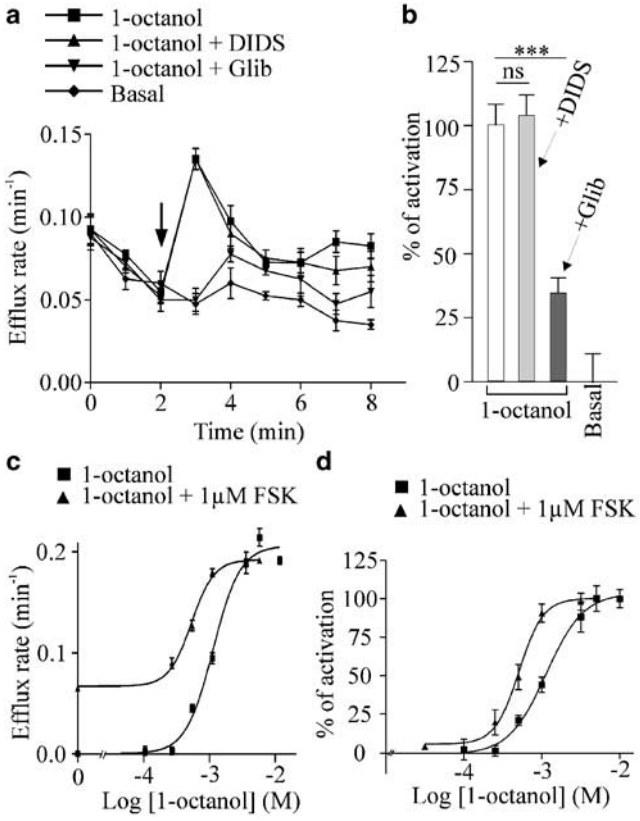

Figure 5 Effect of 1-octanol on CFTR activation in Calu-3 cells. (a) Effect of 1-octanol (1 mM) on ^{125}I efflux rate (min^{-1}) with or without glibenclamide (glib, 100 μM) or DIDS (500 μM). The arrow represents the time where drugs were added. (b) The effect of glibenclamide and DIDS were compared to the maximal response induced by 1-octanol alone and normalized to 100%. (c) Effect of increasing concentrations of 1-octanol in the presence (black triangle) or absence of FSK (1 μM) (black square) on ^{125}I efflux rate (min^{-1}). (d) Dose–response curves of 1-octanol in the presence or absence of FSK (1 μM), expressed as % of maximum activation obtained with 10 mM of 1-octanol. Data represent means $\pm$ s.e.m. for $n=8$ experiments for each condition (***, $P<0.001$; ns, not significant; *t*-test).

44 ± 6% of the maximal response obtained with a cocktail solution containing 10 μM FSK and 30 μM GST. Taken together, these results show that 1-octanol activates the delF508-CFTR mutant channel when this one was first redirected to the plasma membrane.

Discussion

In the present study, we demonstrate for the first time that *n*-alkanols activate wild-type CFTR as well as delF508-CFTR Cl^- channels in a cAMP-independent manner and therefore represent a novel class of CFTR chloride channel activators. Our findings may provide a preliminary basis for a novel pharmacological therapeutic strategy for CF disease.

n-*Alkanols selectively activate CFTR channels*

Our data demonstrate that *n*-alkanols activate CFTR channels with a rank order of potency of 1-heptanol < 1-octanol < 2-

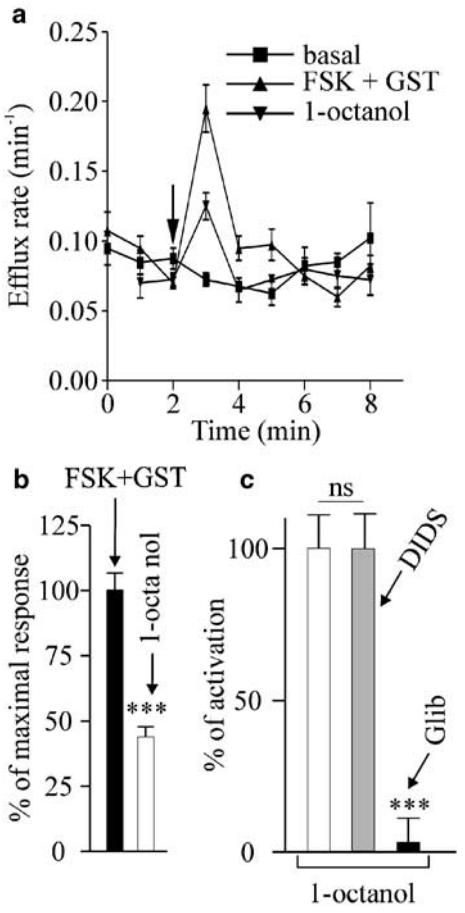

Figure 6 Effect of 1-octanol on delF508-CFTR activation in JME/CF15 cells. (a) Effect of 1-octanol (1 mM) or combined application of FSK 10 μM and GST 30 μM on ^{125}I efflux rate (min^{-1}). The arrow represents the time where drugs were added. (b) Effect of 1-octanol (1 mM) on delF508-CFTR activation, normalized to 100% from the maximal response obtained with combined application of FSK 10 μM and GST 30 μM. (c) Effect of glibenclamide (100 μM) or DIDS (500 μM) on the 1-octanol (1 mM)-induced CFTR activation, expressed as % of maximal response. Data represent means $\pm$ s.e.m. for $n=8$ experiments for each condition (***, $P<0.001$; ns, not significant; *t*-test).

octanol < 1-decanol. *n*-Alkanols had no effects on anion efflux in CHO-K1 cells, which do not express recombinant CFTR channels (Figure 1c), demonstrating that *n*-alkanols' effects depend on the presence of CFTR and were not due to unspecific effects on membrane properties such as fluidization (Zavoico & Kutchai, 1980). Consistently, *n*-alkanols stimulation was restricted to CFTR-expressing CHO cells and Calu-3 airway epithelial cells which endogenously express CFTR channels (Haws *et al*., 1994). The identity of CFTR channel as a target of octanol was further demonstrated by the finding that glibenclamide, which is commonly used to inhibit CFTR, abolished the octanol-induced responses. By contrast, DIDS, a blocker of various Cl^- channels, except CFTR when applied externally, had no effects on octanol-induced CFTR activation. Furthermore, we showed that 1-octanol was also capable of stimulating delF508-CFTR channel activity in the human airway epithelial JME/CF15 cells (delF508/delF508). This effect was only observed following treatment by MPB-91, a compound used to address delF508-CFTR at the cell surface (Dormer *et al*., 2001). Here again, glibenclamide application abolished 1-octanol-induced delF508-CFTR activity, while DIDS was without significant effects, indicating that the effects of octanol were strictly dependent upon CFTR.

Because octanol is often used to inhibit gap junctions (Weingart & Bukauskas, 1998; Contreras *et al*., 2002), we sought whether the effects of octanol on CFTR activity could be due to cell-to-cell uncoupling. We found that application of octanol markedly reduced the slow phase of the ATP-induced Ca^{2+} response mediated by P2Y2 receptors (Marcet *et al*., 2003). This effect was replicated by α-GA, another gap junction blocker. However, α-GA had no effect on CFTR activity, indicating that inhibition of gap junction and cellular uncoupling were not involved in octanol-induced CFTR activation.

Effect of octanol on CFTR activity requires PKA but not PKC activity

As CFTR is a cAMP-activated Cl^- channel (Riordan *et al*., 1989; Bear *et al*., 1992), we examined whether CFTR activation by octanol was mediated by an increase in intracellular cAMP level. We found that all the *n*-alkanols under study were without effects on the cAMP level, indicating that CFTR activation is independent of cAMP increase. On the other hand, H-89, a potent PKA inhibitor, strongly decreased octanol-induced CFTR activation, indicating that PKA activity was required to observe responses of octanol. This, together with the findings that activation of CFTR by octanol was enhanced by FSK and that octanol and FSK had additive effects, suggest that activation of CFTR by octanol requires prior phosphorylation by PKA and that octanol acts downstream to PKA, possibly directly on CFTR. Such a mechanism of regulation has been previously described for GST, which appears to activate directly CFTR channel independently of PKA in cardiac myocytes (Obayashi *et al*., 1999), but whose stimulatory effect was prevented by the H-89 PKA inhibitor.

Several works having reported that PKC activation may contribute to enhance CFTR activation (Jia *et al*., 1997; Chappe *et al*., 2003), we have examined a putative role for PKC in octanol-induced CFTR activation. Importantly, *n*-alkanols and particularly octanol have been shown to inhibit certain subtypes of PKC (Slater *et al*., 1993). However, depending on the subtype of PKC and experimental conditions such as the way by which PKC was stimulated, activation of PKC by *n*-alkanols could also be observed (Slater *et al*., 1997; Shen *et al*., 1999). Our results rule out an involvement of PKC in the effects of *n*-alkanols since treatment with GF109203X, a potent inhibitor of PKC, failed to block the octanol-induced CFTR activation.

Correlations between CFTR activation and potency of alcohols

Over the years, studies on ion channels have argued that *n*-alkanols affect protein functions by changing membrane properties (Zavoico & Kutchai 1980; Goldstein, 1984; Weight, 1992; Dilger, 2002) as well as by binding directly to hydrophobic pockets of the proteins (Li *et al*., 1994; Covarrubias *et al*., 1995; Mascia *et al*., 2000). In the present

work, we have first ruled out a nonspecific effect of octanol on membrane fluidity because no effect of octanol was observed in CFTR-lacking cells. In addition, we found that a short application ($\sim$30 s) of *n*-alkanols such as octanol was sufficient to enhance CFTR activity. Thus, we hypothesize that octanol binds to a site close to the external surface of CFTR channels, or penetrates the membrane to reach a site of action inside the membrane. Recently, it has been shown that *n*-alkanols could regulate ion channels activity, such as the $GABA_A$ ligand-gated Cl^- channel, by interacting directly with specific binding sites for alcohols on the receptor (Mascia *et al*., 2000). Such a mechanism has been previously proposed to be involved in *n*-alkanols-induced $GABA_A$ activation (Nakahiro *et al*., 1991). It is therefore plausible that a hydrophobic site could exist within the CFTR structure where the binding of alcohols modifies CFTR channel activity. This may be consistent with our observation that the potency of *n*-alkanols to enhance CFTR activity increases with the carbon chain length of molecules and with the increase in hydrophobicity of alcohols. Although octanol was not the most potent activator of CFTR among the many *n*-alkanols tested here, it presents the advantage of being previously used as therapeutic agent for treating acute pulmonary edema in human (Reich *et al*., 1953; Miller, 1973). Interestingly, a recent work has shown that CFTR channels play a key role in fluid absorption from the distal airspaces of the lung (Fang *et al*., 2002). The authors proposed a role of CFTR in the pathophysiology of pulmonary edema, based on the findings that the lack of functional CFTR in delF508 mice limits their capacity to remove alveolar edema (Fang *et al*., 2002). Our data suggest that therapeutic effects of octanol on acute pulmonary edema (Reich *et al*., 1953; Miller, 1973) may also involve octanol-induced CFTR activation.

Long-chain alcohols as new compounds for the development of therapeutics in CF?

Our findings that 1-octanol stimulates delF508-CFTR channels in the plasma membrane may provide an 'entrée' for molecular therapeutics in CF. Previous works have studied the use of octanol as antifoaming agent by aerosol to treat acute pulmonary edema in human (Reich *et al*., 1953; Miller, 1973). Inhalation of alcohol vapor for 30 min failed to significantly enhance systemic alcohol concentration arguing in favor of alcohol therapy by inhalation (Luisada *et al*., 1952). Other studies have demonstrated that no poisonous effects of octanol have been reported on workers commonly using octanol in various industries (Reich *et al*., 1953). Although the current knowledge in toxicity of octanol in human is still poorly documented, there is some recent information available concerning the risk and toxicity of 1-octanol (CAS No 111-87-5) according to the International Chemical Safety Cards (cf. http://www.itcilo.it/ and http://www.chemrest.com/ web sites). Octanol has not been shown either to be a potential carcinogen in humans or to be an extreme hazard. In agreement with this, a recent study on Wistar rats, carried out in accordance to the organization for economic cooperation and development, concluded of a no-observable-adverse-effect-level of subchronic inhalation of 2-ethylhexanol, a compound related to octanol (Klimisch *et al*., 1998). Although no significant increase in systemic alcohol concentration was observed following alcohol therapy by inhalation, the occurring of intolerance of such a long-term alcohol therapy on CF patients, whom 15% of them are more subjects to develop liver disorders (Davis *et al*., 1996), must be considered. In conclusion, octanol and related compounds, which can be used in aerosol therapy in combination with other drugs, may have interesting promise as lead compounds for development of therapeutics in the treatment of lung disease in CF.

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