Calcium- and CaMKII-dependent Chloride Secretion Induced by the Microsomal Ca^{2+}-ATPase Inhibitor 2,5-Di-(*tert*-butyl)-1,4-hydroquinone in Cystic Fibrosis Pancreatic Epithelial Cells

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Abstract

Microsomal Ca^{2+}-ATPase inhibitors such as thapsigargin (THG), cyclopiazonic acid (CPA) and 2,5-di-(*tert*-butyl)-1,4-hydroquinone (DBHQ) have been shown to inhibit Ca^{2+} reuptake by the intracellular stores and increase cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). DBHQ is a commercially available non-toxic synthetic compound chemically unrelated to THG and CPA. In this study, we tested the feasibility of utilizing DBHQ to improve Cl^- secretion via the Ca^{2+}-dependent pathway, in the cystic fibrosis (CF)-derived pancreatic epithelial cell line CFPAC-1. DBHQ stimulated ^{125}I efflux and mobilized intracellular free Ca^{2+} in a dose-dependent manner. The maximal effects were seen at concentrations of 25–50 μM. DBHQ (25 μM) caused a short-term rise in $[Ca^{2+}]_i$ in the absence of ambient Ca^{2+}, and a sustained elevation of $[Ca^{2+}]_i$ in cell monolayers bathed in the efflux solution (1.2 mM Ca^{2+}), which was largely attenuated by Ni^{2+} (5 mM). Bath-application of DBHQ induced an outwardly-rectifying whole-cell Cl^- current, which was abolished by pipette addition of BAPTA (5 mM) or CaMK [273–302] (20 μM), an inhibitory peptide of multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Pretreatment of monolayers of CFPAC-1 cells with DBHQ for 4–5 min significantly increased the Ca^{2+}-independent or autonomous activity of CaMKII assayed in the cell homogenates. Thus, DBHQ appears to enhance Cl^- channel activity via a Ca^{2+}-dependent mechanism involving CaMKII. Pretreatment of CFPAC-1 cells with up to 50 μM DBHQ for 6 h did not cause any detectable change in cell viability and did not significantly affect the cell proliferation rate. These results suggest that appropriate selective microsomal Ca^{2+}-ATPase inhibitors may be therapeutically useful in improving Cl^- secretion in CF epithelial cells. (*J. Clin. Invest.* 1995. 96:1794–1801) Key words: chloride channel • intracellular calcium • fura-2 • patch-clamp • CFPAC-1

Introduction

Cystic fibrosis (CF),[1] the most common lethal inherited disease among Caucasians, is caused by mutations in the gene encoding for the CF transmembrane conductance regulator (CFTR) Cl^- channel and characterized by defective salt and water secretion by epithelia, which normally can be activated by a cAMP-dependent pathway (for reviews see references 1 and 2). The aberrant Cl^- secretion, accompanied by increased Na^+ absorption, alters the volume and composition of the epithelial surface liquid and leads to complex pathophysiological manifestations, including electrolyte and fluid imbalance, abnormal mucous secretion, bacterial infection, and chronic inflammation (for reviews see references 1 and 3). Pharmacological strategies aimed at conquering the CF defect in Cl^- secretion have in recent years focused on discovering new agents that would stimulate epithelial Cl^- secretion through an alternate cAMP-independent mechanism (for review see reference 4). A number of purinoceptor agonists have recently been shown to enhance Cl^- secretion in CF epithelial cells (5–8). P_2 purinoceptor agonists ATP and UTP, in particular, have been tested in clinical trails (9).

Purinoceptor agonists apparently activate the plasma membrane Cl^- channels in CF epithelia primarily via a preserved Ca^{2+}-dependent signaling pathway (5, 7, 8) involving G proteins and phospholipase C (7). These agents induce a transient rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in both normal and CF-derived epithelial cells (5, 7, 8). Activation of membrane Cl^- channels by such Ca^{2+}-mobilizing agonists, however, tends to be limited by a seemingly concomitantly evoked inhibitory mechanism, which may be mediated by other intracellular messengers such as diacylglycerol (DAG) (10) and inositol tetrakisphosphate ($InsP_4$) (11). Thus, it would be of further therapeutic advantage to identify and develop pharmacological agents of favorable toxicity profiles, which can raise $[Ca^{2+}]_i$ without receptor activation and inositol phosphate production.

We have sought to investigate the feasibility of using a microsomal Ca^{2+}-ATPase inhibitor to increase $[Ca^{2+}]_i$ and

Part of this work has been presented in abstract form in *Pediatr. Pulmonol.* 10 (Suppl.):207, 1994.

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Received for publication 10 November 1994 and accepted in revised form 15 June 1995.

J. Clin. Invest.

0021-9738/95/10/1794/08 $2.00
Volume 96, October 1995, 1794–1801

1. *Abbreviations used in this paper:* AC-2, autocamtide; BAPTA, 1,2-*bis*(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CaMKII, multifunctional Ca^{2+}/calmodulin-dependent protein kinase; CF, cystic fibrosis; CPA, cyclopiazonic acid; DBHQ, 2,5-di-(*tert*-butyl)-1,4-hydroquinone; $InsP_4$, inositol tetrakisphosphate; THG, thapsigargin; V_m, membrane potential.

stimulate Cl^- secretion in CF epithelial cells. Thapsigargin (THG), cyclopiazonic acid (CPA), and 2,5-di-(*tert*-butyl)-1,4-hydroquinone (DBHQ) are specific microsomal Ca^{2+}-ATPase inhibitors which have been shown to initiate Ca^{2+} release from intracellular stores in a variety of cells (12–15). Depletion of Ca^{2+} from the microsomal storage organelles in turn induces plasmalemmal Ca^{2+} influx by a "capacitative" mechanism (16, 17), leading to sustained elevation of $[Ca^{2+}]_i$. Because of their toxic effects, neither THG or CPA can be considered for therapeutic application. THG, a naturally occurring sesquiterpene lactone, is a tumor promoter (18); CPA (*Penicillium cyclopinium*) is a neurotoxin. The third Ca^{2+}-ATPase inhibitor, DBHQ, which is also referred to as 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone, is a synthetic compound structurally unrelated to THG and CPA. DBHQ is commercially available and has no known toxic effects. In this study we show that DBHQ is effective in promoting Cl^- secretion in CF epithelial cells via a Ca^{2+}-dependent mechanism involving multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Agent(s) of this new class thus could be potentially therapeutically useful in treatment of CF.

Methods

Materials. Synthetic peptide CaMK [273–302] was kindly provided by Dr. Howard Schulman (Stanford University). DBHQ was purchased from Aldrich (Milwaukee, WI). Fura-2-AM, BAPTA (tetracesium salt), and pluronic F127 were from Molecular Probes, Inc. (Eugene, OR). Calmodulin was from Ocean Biologics (Edmonds, WA). AC-2 was from Peninsula Laboratories (Belmont, CA). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. CFPAC-1 cells were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in Iscove's Modified Delbecco's Medium supplemented with 10% fetal calf serum (GIBCO BRL, Grand Island, NY), 100 U/ml penicillin and 0.2 mg/ml streptomycin (Biofluids, Rockville, MD). The culture medium was replenished 2–3 times per week. Cells were used at passages 20–27.

Anion efflux assay. ^{125}I efflux experiments were carried out as described (8, 19). Briefly, cells were plated on 22 × 22-mm square plastic coverslips (VWR Scientific, San Francisco, CA) and studied at 90–100% confluency ~ 1 wk after seeding. The efflux solution contained (in mM): 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 10 glucose, and 10 Hepes (pH 7.4). Cells were first loaded with ^{125}I in efflux solution containing 20 μCi/ml ^{125}I for ~ 2 h in a water bath gassed with 100% O_2 at 37°C. Extracellular ^{125}I was eliminated by rapidly rinsing the cell monolayer on coverslip three times with efflux solution for a cumulative time of 1 min. The efflux experiment was then carried out by sequentially transferring the cell monolayer/coverslip at 1-min interval through a series of cell culture dishes (Costar, Cambridge, MA) each containing 3 ml efflux solution, at room temperature. ^{125}I effluxed into each dish was counted individually in a γ-radiation counter (LKB, Gaithersburg, MD). Efflux data is normalized as percent efflux per min (8, 19) and presented as mean±SE. Sample sizes are given as n = number of cell monolayers studied.

Fluorescence measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined as described (8). Briefly, cells were plated on 9 × 22-mm rectangular glass coverslips (Wheaton, Milville, NJ) and studied at 90–100% confluency ~ 1 wk after seeding. Cells were incubated in efflux solution containing 5–10 μM fura-2-AM and 0.05% (wt/vol) pluronic F127 for 20–30 min at 37°C and rinsed with dye-free efflux solution. The cell monolayer/coverslip was then mounted vertically in an acrylic cuvette containing 2 ml efflux solution and studied in a spectrofluorimeter (SLM-AMINCO, Urbana, IL) at ~ 30°C. The cell monolayer/coverslip was held at an angle ~ 60 degrees from the incident light. The excitation wavelength was altered between 340 and 380 nm every 0.2 s and emission fluorescence monitored at 510±10 nm. Agents were added into the cuvette during experiment by means of a Hamilton syringe. $[Ca^{2+}]_i$ was quantitated as described by Grynkiewicz et al. (20).

Electrophysiology. Whole-cell patch-clamp experiments were performed in single CFPAC-1 cells grown on glass coverslips 1–2 d after seeding. Cells/coverslip were placed in a 1-ml acrylic chamber on the stage of a Zeiss IM inverted microscope and bathed in a solution containing, in mM, 170 Tris-Cl, 1 $MgCl_2$, 2.5 $CaCl_2$, 15 glucose, and 10 Hepes (pH 7.4; ~ 330 mosm/kg) at 25–30°C. The pipette solution contained, in mM, 140 $CsCl_2$, 2 $MgCl_2$, 0.01 EGTA, 2 MgATP, 10 glucose, and 5 HEPES (pH 7.35; ~ 300 mosm/kg). The bath solution was made ~ 30 mosm/kg hypertonic compared with the pipette solution to prevent hypotonicity-induced Cl^- current (21). Micropipettes were made as described by Hamill et al. (22) and had a tip resistance of 2–3 MΩ. Whole-cell currents were recorded with an Axopatch amplifier (Axon Instruments, Foster City, CA). The resting membrane potential (V_m) was held at −70 mV. To examine the current-voltage (I-V) relation of recorded Cl^- currents, V_m was sequentially altered from −100 to 100 mV in 50 mV steps (8, 19, 23). Voltage-clamp protocols were run with an aid of a Tecmar 12-bit A/D-D/A converter and an IBM-AT computer. Signals, filtered at 1 kHz, were displayed on a strip-chart recorder and stored on floppy disks. Data were analyzed by means of pClamp, version 5.5 (Axon Instruments). Sample sizes are presented as n = number of cells studied.

CaM kinase activity assay. CaMKII Activity assay was carried out essentially as described elsewhere (24, 25). Briefly, CFPAC-1 cells were plated in 35-mm tissue-culture dishes (Becton Dicknson and Company, Lincoln Park, NJ) and studied at 90–100% confluency 5 d after seeding. The cell monolayers were bathed in efflux solution and stimulated by application of DBHQ (25 μM) for 4–5 min at 37°C. Stimulation was stopped by aspiration of the bathing solution and addition of cold homogenization buffer (10 mM Tris-HCl, 2 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 0.4 mM sodium molybdate, 2 mM dithiothreitol, and 10 μg/ml leupeptin; pH 7.5) at ~ 1 ml/10^6 cells. Cells were then disrupted by sonication for 15 s at 4°C, using a sonic cell dismembrator (Fisher Scientific, Pittsburgh, PA). Assay buffers contained 50 mM Pipes, 10 mM magnesium acetate, 10 μg/ml calmodulin, 10 μM autocamtide-2 (AC-2, a selective substrate of CaMKII) (26), 50 μM [γ-^{32}P]ATP (1 Ci/mmol), and either 1 mM $CaCl_2$ (for Ca^{2+}-stimulated maximal activity) or 2 mM EGTA (for Ca^{2+}-independent or autonomous activity). CaMKII activity in cell homogenates was assayed in triplicate by phosphorylation of AC-2; reactions were carried out for 30 s at 30°C. Background signal was determined in background control assays performed in an AC-2-omitted assay buffer containing EGTA but no Ca^{2+}. The Ca^{2+}-independent or autonomous activity of CaMKII is presented as the percentage of phosphorylation of AC-2 in the absence of Ca^{2+} with respect to its phosphorylation in the presence of Ca^{2+} (26, 27).

Trypan blue exclusion and cell proliferation assay. For the trypan blue exclusion assay, CFPAC-1 cells were grown on 16 × 8-mm rectangular tissue culture chambers (Nunc, Naperville, IL) and studied at 95–100% confluency. The cell culture medium was aspirated, replaced with fresh culture medium or culture medium plus desired agent, and incubated for 6 h in the cell culture incubator. Subsequently, the cell monolayers were rinsed with phosphate buffer solution (PBS) and incubated with 0.4% trypan blue (GIBCO BRL) for 5 min at room temperature. The cell monolayers were rinsed again with PBS and examined by light microscopy. Cells failed to exclude trypan blue and stained in dark blue resulting from trypan blue "invasion" are considered to be of compromised viability.

For the cell proliferation assay, CFPAC-1 cells were cultured on the 36-mm wells of the tissue culture–treated 6-well clusters (Costar, Cambridge, MA) and studied at ~ 30% confluency. The cell culture medium was freshly replaced. 2 h later, the cell cultures were, in groups of six wells each, exposed to 0 (control), 25 and 50 μM DBHQ respectively for 6 h. The cells were subsequently thoroughly washed and

replenished with fresh culture medium and grown for another 36 h. At the end of the experiment, the cells from each well were trypsinized and the number of cells in the well was counted by phase hemacytometry. The cell counts between different experimental groups were statistically compared by Student's unpaired *t* test.

Results

Effect of DBHQ on ^{125}I efflux rate. CF is characterized by defective regulation of the CFTR Cl^- channel by cAMP-dependent protein kinase and protein kinase C (for reviews see references 1, 2, and 27). To test whether DBHQ could stimulate epithelial Cl^- secretion via an alternate, cAMP-independent mechanism bypassing the CF defect, we initially examined the effect of DBHQ on ^{125}I efflux in cultured monolayers of CFPAC-1 cells. CFPAC-1 is a permanent cell line derived from the pancreatic carcinoma of a CF patient, is homozygous for ΔF508, the most common CF mutation, and has been shown to be non-responsive to cAMP stimulation (28). ^{125}I was used in the efflux assay because iodine is transported selectively through the Cl^- conductive pathway by epithelia (29). As shown in Fig. 1 *A*, bath-application of DBHQ enhanced ^{125}I efflux in a dose-dependent manner. The effect of DBHQ on ^{125}I efflux is summarized in Fig. 1 *B*. Among the concentrations tested, DBHQ seemed to induce a maximal stimulation at 25–50 μM. Because of its apparently limited solubility in aqueous solutions, the effect of DBHQ at doses of > 50 μM was not examined.

Effect of DBHQ on $[Ca^{2+}]_i$. Accumulating data have been presented suggesting that a Ca^{2+}-dependent pathway controlling Cl^- secretion is preserved in CF epithelia. Since DBHQ induces $[Ca^{2+}]_i$ rise in other cells (12–15), we examined whether DBHQ also stimulates Cl^- flux in CFPAC-1 cells by mobilizing intracellular free Ca^{2+}. As shown in Fig. 2 *A*, application of DBHQ caused a sustained increase in $[Ca^{2+}]_i$ in a dose-dependent manner.

When bathed in Ca^{2+}-omitted solution, addition of DBHQ induced a relatively short-term rise in $[Ca^{2+}]_i$ (see the lower trace in Fig. 2 *B*), suggesting that DBHQ is also effective in inducing Ca^{2+} release from intracellular stores in CFPAC-1. By comparing the time-course of $[Ca^{2+}]_i$ obtained in the absence and presence of external Ca^{2+} (1.2 mM, the efflux solution) respectively (see Fig. 2 *B*), it seems clear that a concurrent transmembrane Ca^{2+} influx was also induced upon the addition of DBHQ, which accounts for a large part of the total increase in $[Ca^{2+}]_i$ and is required for maintaining the sustained elevation of $[Ca^{2+}]_i$. As shown in Fig 2 *C*, the DBHQ-induced increase in $[Ca^{2+}]_i$ is largely diminished in the presence of Ni^{2+} (5 mM), a known Ca^{2+} channel inhibitor (30). The effect of DBHQ on $[Ca^{2+}]_i$ is summarized in Table I.

Activation of membrane Cl^- channels by DBHQ. Fig. 3 *A* shows a time-course of the whole-cell Cl^- currents recorded before and after application of DBHQ (25 μM). DBHQ elicited an outwardly-rectifying depolarization-activated whole-cell current characteristic of Ca^{2+}-stimulated membrane Cl^- current (Fig. 3, *B* and *C*). The recorded currents should be predominantly due to fluxes of Cl^- ions because Na^+ and K^+ were omitted in the bath and pipette solutions and Cs^+, used to substitute for pipette K^+, is known to block K^+ channels. The reversal potential was near 0 mV (Fig. 3 *C*), in good agreement to the predicted Nernst potential for Cl^- passive diffusion (−5 mV).

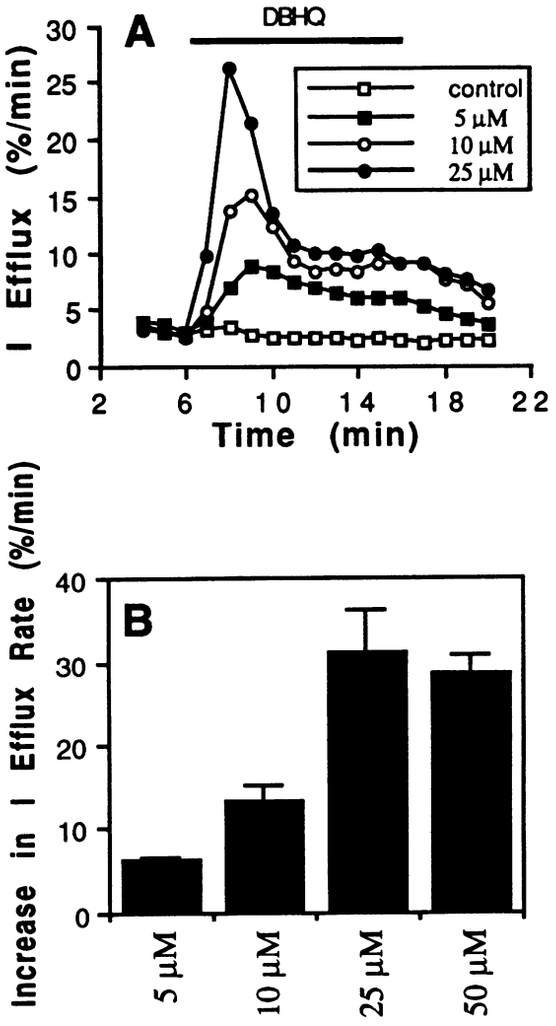

Figure 1. Effect of DBHQ on ^{125}I efflux in cultured monolayers of CFPAC-1 cells. (*A*) DBHQ stimulated ^{125}I efflux in a dose-dependent manner. DBHQ was applied where denoted by the horizontal bar at concentrations indicated. Results shown were obtained from paired experiments performed in cell monolayers cultured from the same passage under identical experimental conditions on the same day. (*B*) Summary of effect of DBHQ on ^{125}I efflux. Values represent the mean±SE (n = 3–6 cell monolayers).

To further support the notion that DBHQ enhances Cl^- secretion via a Ca^{2+}-dependent mechanism, the effect of DBHQ was re-examined in CFPAC-1 cells internally perfused with 5 mM BAPTA, a Ca^{2+} chelator. The presence of BAPTA completely abolished DBHQ stimulation (see Fig. 3, *D* and *F*).

CaMKII has been suggested to mediate Ca^{2+}-stimulated Cl^- secretion in secretory epithelia (8, 23, 31) and in human T lymphocytes (32). To test whether DBHQ stimulation is transmitted via the same signaling pathway involving Ca^{2+} and CaMKII, additional whole-cell patch-clamp experiments were performed in cells dialyzed with a selective inhibitory peptide of CaMKII (a synthetic peptide containing the autoinhibitory region of CaMK), CaMK [273–302] (20 μM), which inhibits brain CaMK activity at 1 μM (33). As shown in Fig. 3, *E* and *F*, when CaMKII was inhibited by CaMK [273–302] introduced into the cell, DBHQ stimulation was largely disrupted.

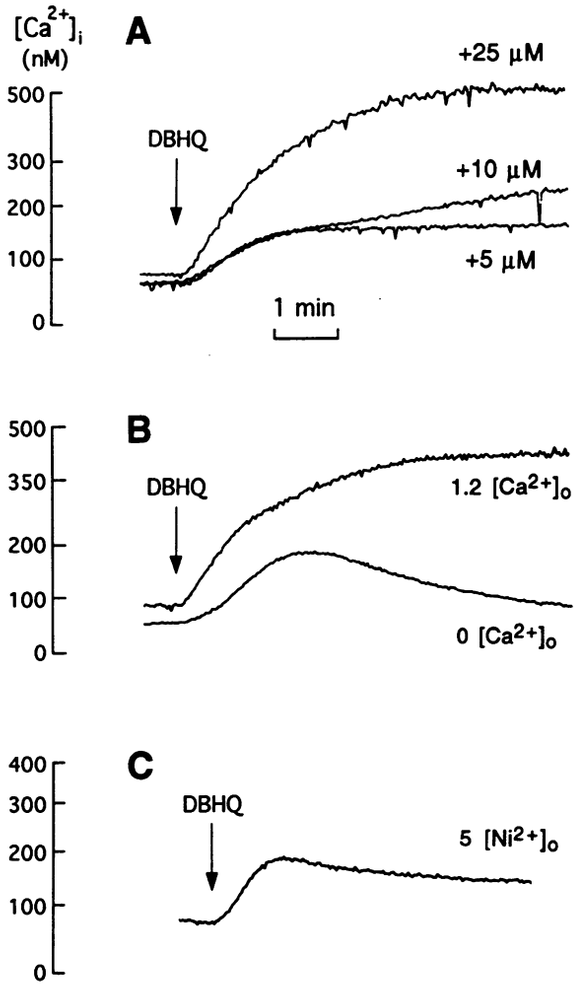

Figure 2. Effect of DBHQ on $[Ca^{2+}]_i$. (*A*) Concentration-dependence of DBHQ-induced increase in $[Ca^{2+}]_i$. DBHQ was added where indicated by the arrow at the concentrations specified. (*B*) Effect of addition of DBHQ (25 μM) on $[Ca^{2+}]_i$ in the absence (*lower trace*) and presence (1.2 mM; *upper trace*) of ambient Ca^{2+}. Results shown in the lower trace is representative of three experiments. (*C*) Effect of addition of DBHQ on $[Ca^{2+}]_i$ in the presence of ambient Ca^{2+} (1.2 mM) plus Ni^{2+} (5 mM). Results shown in *A* and *B* were each obtained from paired experiments performed under identical conditions on the same day.

Effect of DBHQ on Ca^{2+}-independent or autonomous activity of CaMKII. Although CaMKII is believed to mediate the effect of intracellular free calcium to stimulate epithelial Cl^- secretion, its activation by stimuli that raise $[Ca^{2+}]_i$ has not been shown in epithelia. CaMKII, upon activation by Ca^{2+}/calmodulin, autophosphorylates its autoinhibitory domain (on Thr^{286}) and becomes partially Ca^{2+}-independent or autonomous (for review see reference 34). The activated and autophosphorylated CaMK manifests an enhanced phosphorylation capacity on its substrates in the absence of Ca^{2+} and calmodulin. We examined the effect of DBHQ on autonomous CaMK activity in CFPAC-1 cells. DBHQ (25 μM) was applied to CFPAC-1 cell monolayers bathed in efflux solution for 4–5 min, the length of time needed for DBHQ to raise $[Ca^{2+}]_i$ to its plateau. As shown in Fig. 4, DBHQ stimulation resulted in an increase in the autonomous activity of CaMKII ($P < 0.05$).

Table I. Effect of DBHQ on $[Ca^{2+}]_i$ of CFPAC-1 Cells

	$[Ca^{2+}]_i$, nM			
Conc.	Baseline	+DBHQ*	Δ	*n*
μM				
5	67.5±3.5	225.8±49.5	158.2±46.3‡	4
10	64.6±4.7	281.3±36.9	216.8±37.5§	7
25	69.1±5.4	426.4±38.4	357.3±37.0‖	7
25 (+5 mM N_i)	68.9±2.0	145.2±10.2	76.3±8.2‡	3

Measurements were performed in the ^{125}I efflux solution (see Methods). *Values of $[Ca^{2+}]_i$ are taken 5–8 min after the addition of DBHQ when $[Ca^{2+}]_i$ approaches to a new steady-state level. ‡Different from the corresponding baseline $[Ca^{2+}]_i$ by Student's paired *t* test with $P < 0.05$. §Different from the corresponding baseline $[Ca^{2+}]_i$ with $P < 0.01$. ‖Different from the corresponding baseline $[Ca^{2+}]_i$ with $P < 0.001$. Values represent the mean±SE. *n*, number of cell monolayers studied.

Effect of DBHQ on viability and proliferation rate of CFPAC-1 cells. To examine whether DBHQ has any toxic or cell-damaging effect on CFPAC-1, trypan blue exclusion assay was performed in cells pretreated with 50 μM DBHQ for 6 h. Nonpretreated cells (control) and cells pretreated with the Ca^{2+} ionophore ionomycin, which also "nonselectively" raises $[Ca^{2+}]_i$, were also studied and compared with the cells pretreated with DBHQ. As shown in Fig. 5, DBHQ pretreatment (*B*) resulted in neither discernible cellular uptake of trypan blue nor morphological change of the cells, as compared with the cells in the control group (*A*). By contrast, pretreatment with 1 μM ionomycin led to "invasion" of trypan blue into many of the CFPAC-1 cells (see the "dark" cells stained by trypan blue in Fig. 5 *C*). After the pretreatment of 10 μM ionomycin, a substantial amount of cells detached from the culture well and a majority of the cells remaining attached was stained by trypan blue (Fig. 5 *D*), indicating severely decreased cell viability. These results demonstrate that DBHQ exerts mild, if any, toxicity to these epithelial cells.

In the cell proliferation experiment, 36 h after drug/control pretreatment, CFPAC-1 cells proliferated to $5.2+0.3 \times 10^5$ (control), $4.8+0.2 \times 10^5$ (25 μM DBHQ) and $4.5+0.2 \times 10^5$ (50 μM DBHQ) cells per well, respectively ($n = 6$ wells for each group). The cells from all the three groups grew from the original ~ 30% confluency to 60–70% confluency during the 36-h experimental period. There was no statistically significant difference in cell counts between the DBHQ-pretreated groups and the control group. Also, the DBHQ-pretreated cells remained able to grow to confluency. Thus, DBHQ did not seem to appreciably affect the cell proliferation.

Discussion

Chloride secretion by CF epithelia is defectively regulated by cAMP but remains inducible by a parallel Ca^{2+}-dependent signaling pathway (for reviews see references 1 and 2). We have shown that Cl^- conductance in CF-derived epithelial cells fails to respond to cAMP stimulation but can be activated by the Ca^{2+} ionophore ionomycin (23). Purinoceptor agonists such as ATP, UTP, and adenosine, which elevate intracellular Ca^{2+},

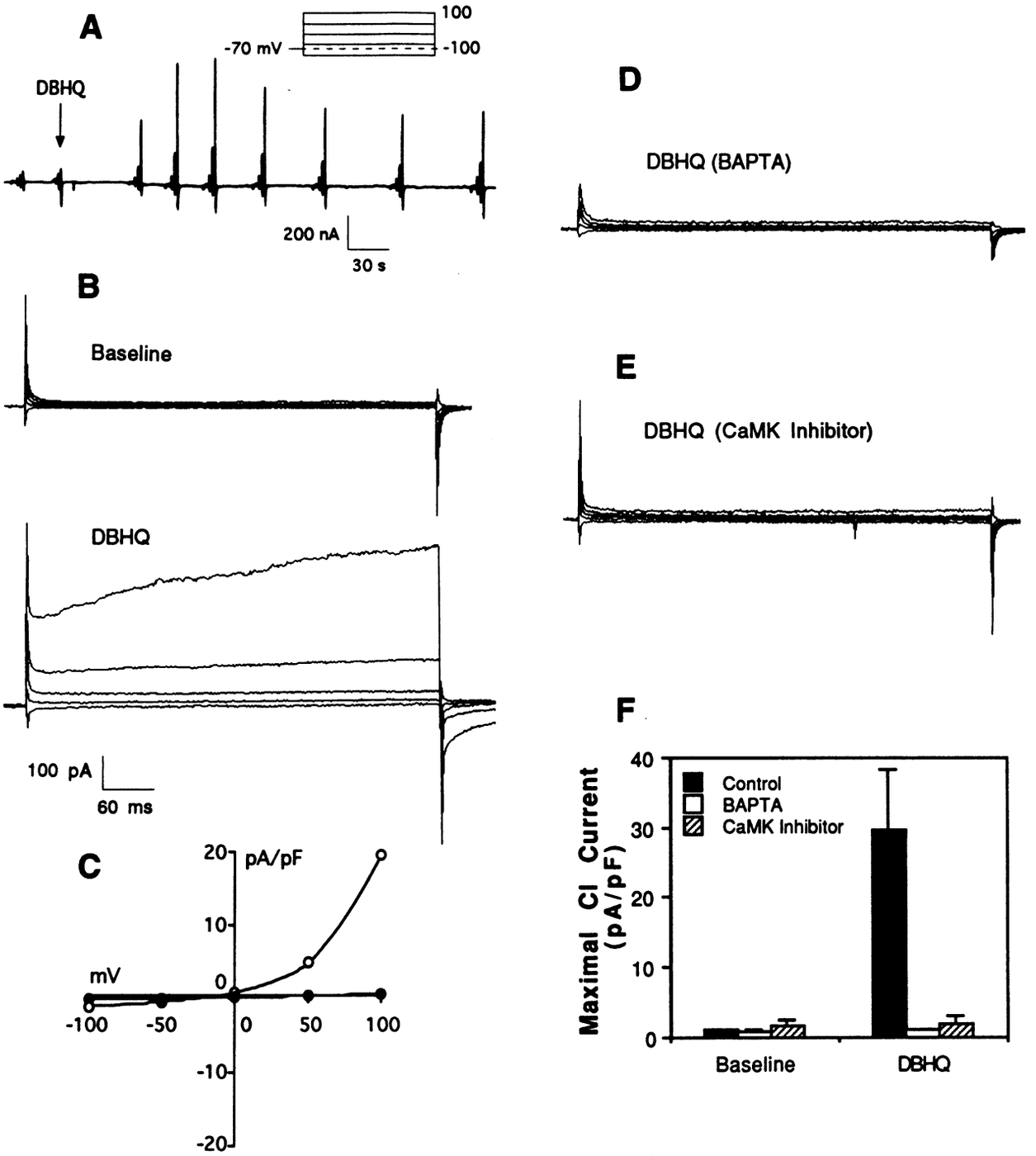

Figure 3. Effect of DBHQ on whole-cell Cl^- currents. (*A*) Time-course of the whole-cell Cl^- currents recorded before and after addition of DBHQ (25 μM). The resting V_m was held at −70 mV. The current spikes were elicited using the voltage-clamping protocol shown in the inset (see Methods). (*B*) Representative whole-cell Cl^- current recorded at baseline (above) and after application (below) of DBHQ. (*C*) I-V relations of whole-cell Cl^- currents recorded at baseline (*closed circles*) and after addition (*open circles*) of DBHQ. Data were averaged over the final 50 ms of the current pulses and normalized by dividing by cell capacitance, an index of cell surface area (8, 19, 23). (*D*) Representative whole-cell Cl^- current recorded after stimulation of DBHQ in the presence of BAPTA (5 mM). BAPTA was introduced into the cell interior from the recording pipette. (*E*) Representative whole-cell Cl^- current recorded after stimulation of DBHQ in cells internally perfused with CaMK [273–302] (20 μM), a selective inhibitory peptide of CaMK. (*F*) Baseline outward Cl^- current and maximal outward Cl^- current (recorded at +100 mV) induced by DBHQ in absence of inhibitor (*Control*; n = 10), in the presence of BAPTA (*BAPTA*; n = 7), and in the presence of CaMK [273–302] (*CaMK Inhibitor*; n = 4), respectively.

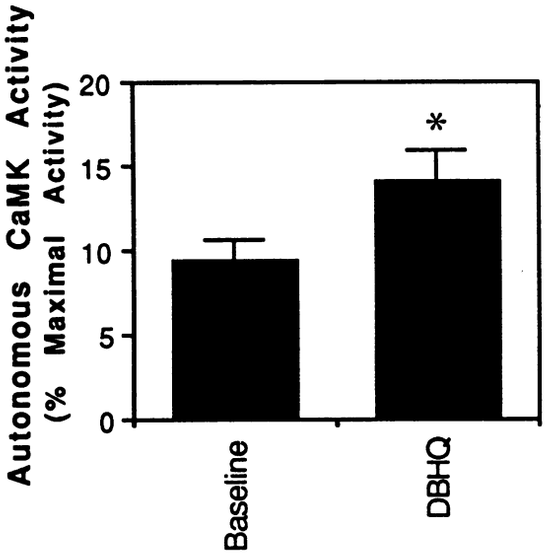

Figure 4. Effect of DBHQ on autonomous (Ca^{2+}-independent) CaMK activity. Autonomous CaMK activities were determined before (*Baseline*) and after stimulation of DBHQ (25 μM) for 4–5 min (*DBHQ*), respectively. Maximal Ca^{2+}/calmodulin-activated CaMK activity is not affected by DBHQ and defined as 100%. Values are mean±SE of six experiments performed in each group. $*P < 0.05$ compared with the baseline autonomous CaMK activity by Student's unpaired *t* test.

have recently been shown to enhance Cl^- secretion in CF epithelial cells (5, 7, 8). The receptor-mediated stimulation, however, seems to be limited by the brevity of the $[Ca^{2+}]_i$ rise induced and by other concurrently evoked cellular events. Agonist-evoked phosphatidylinositol (PI) turnover has been suggested to uncouple membrane Cl^- conductance from the elevated $[Ca^{2+}]_i$ (13), which is likely to be effected by other second messengers such as DAG (10) and $InsP_4$ (11). In contrast to most membrane receptor agonists, DBHQ causes a sustained rise in $[Ca^{2+}]_i$ in CF epithelial cells. DBHQ however, like THG and CPA, does not induce PI turnover (36). Thus, the action of DBHQ seems to effect a more prolonged and substantial activation of membrane Cl^- conductance.

Anion efflux and patch-clamp experiments show that DBHQ promotes Cl^- secretion in CF epithelial cells. This is consistent with the short-circuit current (I_{sc}) data reported by other groups showing that THG enhances I_{sc} in monolayers of colonic epithelial cells T_{84} (13) and HCA-7 (37). DBHQ stimulated ^{125}I efflux in CFPAC-1 in a dose-dependent manner. The maximal effect was seen at 25–50 μM, comparable to the concentration needed to maximally mobilize $[Ca^{2+}]_i$ in rat T lymphocytes (38).

DBHQ apparently stimulates Cl^- secretion via a Ca^{2+}-dependent pathway. Application of DBHQ raises $[Ca^{2+}]_i$ and induces an outwardly-rectifying whole-cell current characteristic of Ca^{2+}-stimulated Cl^- current. Prevention of $[Ca^{2+}]_i$ rise by pipette addition of BAPTA disrupted the DBHQ stimulation of Cl^- currents. DBHQ-induced anion efflux is at least in part due to Ca^{2+}-dependent activation of membrane Cl^- conductance. We have shown recently that P_1 purinoceptor agonists are capable of stimulating ^{125}I efflux in CF epithelial cells when the V_m is "clamped" to near 0 mV with elevated $[K^+]_o$ (8). Also, anion efflux would be accelerated by Ca^{2+}-stimulated K^+ conductance, which hyperpolarizes the plasma membrane and thus increases the driving force for Cl^- conductive exit. The DBHQ-induced whole-cell currents, however, may result primarily from activation of membrane Cl^- channels. In the patch-clamp experiments performed, K^+ was omitted in both the bath and pipette solutions and Cs^+, a K^+ channel blocker, was present in the pipette solution; this should virtually eliminate the membrane K^+ conductance.

CaMKII has been suggested to take part in the Ca^{2+}-dependent pathway which regulates epithelial Cl^- secretion (8, 23, 31). Abolition of DBHQ-elicited Cl^- currents by the selective inhibitory peptide of CaMKII, CaMK [273–302], suggests that DBHQ exerts its effect via the same signaling pathway. CaMKII (γ_B isoform) has been identified in colonic and airway epithelial cells (39) and is believed to activate a Ca^{2+}-dependent Cl^- channel by phosphorylation (35). Purified rat brain CaMK has been shown to directly activate membrane Cl^- channels in excised patches of T lymphocytes (32). Pretreatment with DBHQ enhanced the autonomous activity of CaMKII in CFPAC-1 homogenates. The DBHQ-induced increase in CaMKII autonomy, although seems modest, is greater than that measured in hippocampal slices pretreated for 5 min with either the Ca^{2+} ionophore A23187 or with KCl-induced depolarization, both of which cause large increases in $[Ca^{2+}]_i$ and induce activation/phosphorylation of CaMKII (40). Our data shown herein is the first evidence that CaMKII can be activated by an agent that increases $[Ca^{2+}]_i$ in epithelial cells. This result, combined with the abolition of DBHQ stimulation by CaMK [273–302], strongly suggests that CaMKII is responsible for transmitting the signal carried by intracellular free Ca^{2+}, which leads to activation of membrane Cl^- currents.

Activation of Cl^- secretion by the microsomal Ca^{2+}-ATPase inhibitor DBHQ in CF epithelial cells suggests a possible new pharmacological approach to CF therapy. DBHQ has no known toxicity and thus may be usable for this application. Although some DBHQ analogs, such as 2-phenyl-1,4-benzoquinone, a metabolite of the citrus fruit fungicide *o*-phenylphenol (OPP), have been shown to have a weak DNA-damaging activity in rats, such an effect is not detected in other DBHQ analogs like OPP itself and phenylhydroquinone (41). Our trypan blue exclusion experiment shows that pretreatment of CFPAC-1 monolayers with up to 50 μM DBHQ (which is almost the highest possible concentration we could test, due to its solubility limitation) for 6 h does not cause any discernible damage to the epithelial cells. These results demonstrate that DBHQ exerts only mild, if any, cytotoxic effect in vitro. It was reported recently (42) that DBHQ and THG can inhibit the proliferation of DDT_1MF-2 cells, a smooth muscle cell line derived from leiomyosarcoma of the ductus deferens of Syrian hamster. A striking distinction was found between these two inhibitors with respect to their reversibility of actions. Even after a 24-h continuous pretreatment , the effect of DBHQ on cell proliferation was fully recoverable, whereas the effect of THG was not. The THG-induced inhibition however could be reversed in the presence of a relatively high serum level (20%). In the present study, we show that subconfluent cultures of CFPAC-1 cells pretreated with DBHQ for 6 h remain able to proliferate at a comparable rate as the unpretreated cells, and are able to grow to confluency. Thus, it seems that, so long as not applied constantly and continuously, a microsomal Ca^{2+}-ATPase inhibitor with desired reversibility of action, like DBHQ, should not

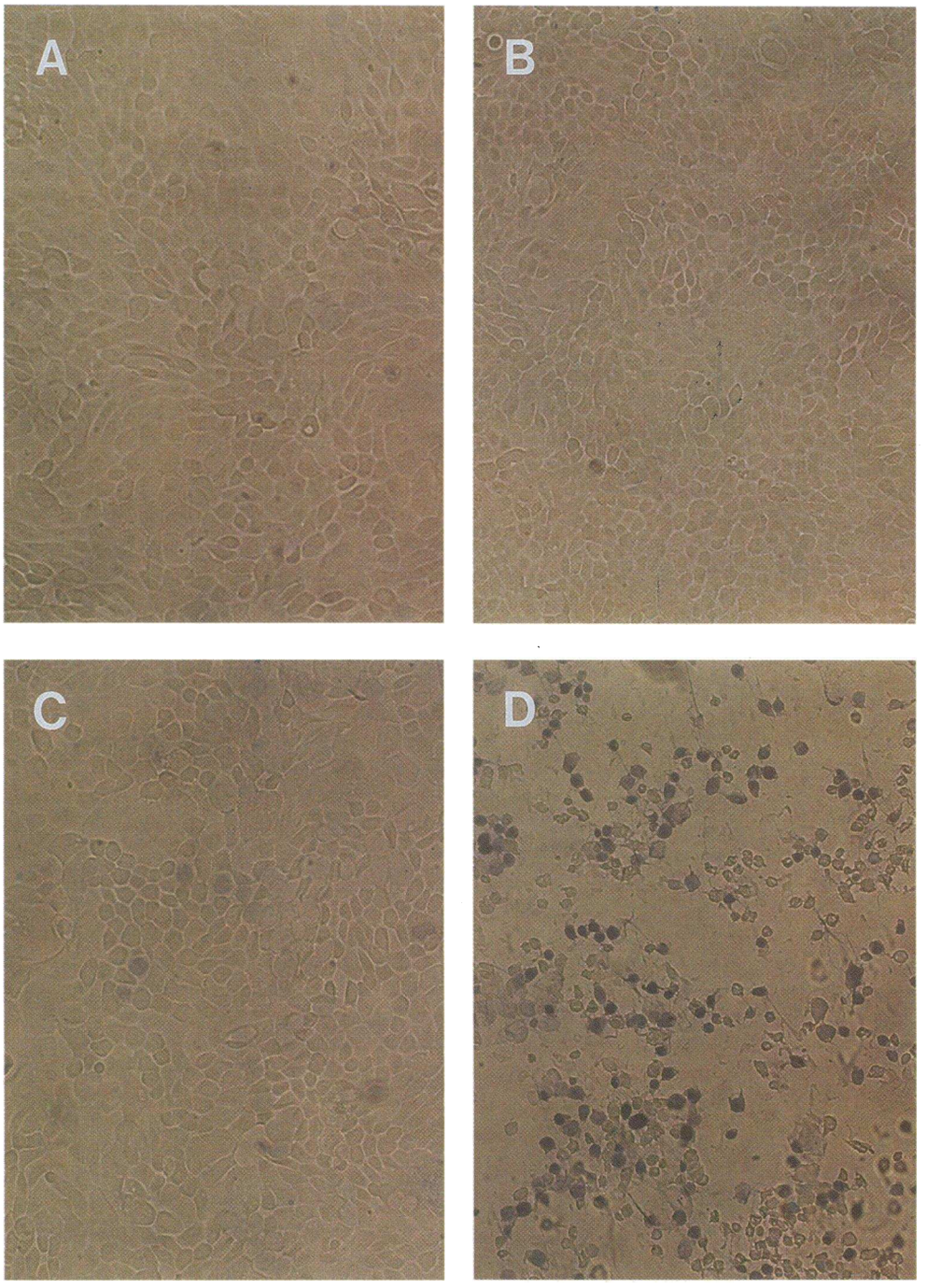

Figure 5. Effect of DBHQ and of ionomycin on the viability of cultured CFPAC-1 cells as assessed by the trypan blue exclusion assay. Shown are photographs of the control cell monolayer (no drug pretreatment; *A*), and cell monolayers pretreated for 6 h with 50 μM DBHQ (*B*), 1 μM ionomycin (*C*), and 10 μM ionomycin (*D*), respectively. Photographs are ×345.

substantially affect cell proliferation. As the experimental facilities and expertise required for conducting in vivo experimentation are so far unavailable in the authors' laboratory, whether DBHQ would exert any adverse effect in vivo has not yet been tested. Likewise, the long-term side-effect of DBHQ, if any, remains unknown and needs to be carefully examined. It is also noteworthy that the three currently commonly used Ca^{2+}-ATPase inhibitors, i.e., THG, CPA and DBHQ, are not structurally related. This implies that there may be opportunities to exploit additional microsomal Ca^{2+}-ATPase inhibitors with further improved toxicity profiles. Appropriate specific microsomal Ca^{2+}-ATPase inhibitors thus are likely to be therapeutically useful for improving epithelial Cl^- secretion in CF.

Acknowledgments

We wish to thank Dr. Howard Schulman for kindly providing us the synthetic peptide CaMK [273–302], and Dr. John Wagner and Dr. Melanie MacNicol for advice and helpful discussions.

This work was supported by National Institutes of Health grant DK-41324 and a grant from the Cystic Fibrosis Foundation (to P. Gardner). K. Kouyama was supported by a fellowship from the Cystic Fibrosis

Foundation. P. Gardner is a recipient of the Burroughs Wellcome Faculty Scholar Award in Clinical Pharmacology.

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