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Possible regulation of CFTR-chloride channels by membrane-bound phosphatases in pancreatic duct cells

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We have studied CFTR-Cl^- channels in non-CF CAPAN-1 and in CFTR-transfected CFPAC-PLJ-CFTR-6 epithelial cells from human pancreas. Theophylline and IBMX induced the opening of cell-attached CFTR-Cl^- channels. Theophylline, IBMX and the alkaline phosphatase (AP) inhibitor levamisole enhanced the activity of excised channels and reduced by 70–75% the apical membrane-associated APs activity. Okadaic acid had no effect on APs and channel activities. A polyclonal anti-alkaline phosphatase antibody (which detected apical APs) reduced APs activity and activated quiescent excised chloride channels. These results suggest that CFTR channels may be regulated by membrane-bound phosphatases.

CFTR-chloride channel; Phosphatase; Pancreas

1. INTRODUCTION

Cystic fibrosis (CF) is a recessive disorder characterized by defective electrolyte transport in epithelial cells in several organs, including lungs and pancreas [1,2]. The recent identification that the CF transmembrane conductance regulator (CFTR), the protein product of the CF gene [3], is a phosphorylated low conductance chloride channel [4–8], has provided insight into the basic defect in this fatal disease. The CFTR-Cl^- channel activity is normally (i.e. in non-CF epithelial cells) controlled by cAMP-dependent phosphorylation [5–8] and dephosphorylation processes [5]. The CFTR-Cl^- channel is spontaneously silenced within a minute on excision [5,7]. This does not required the presence of exogenous phosphatases. This observation suggests that some phosphatases could remain functionally associated with the channel [5].

As an approach to this question, the present study focusses on the spatial and functional relationship that could exist between phosphatases and CFTR-Cl^- channels. On the basis of cytological, immunological and electrophysiological data, we show that the CFTR-Cl^- channel may be regulated by membrane-bound alkaline phosphatases in human pancreatic duct cells.

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PCR, polymerase chain reaction; AP, alkaline phosphatase; PKA, protein kinase A.

2. MATERIALS AND METHODS

2.1. *Cell culture*

The cell lines CAPAN-1, CFPAC-1, CFPAC-PLJ-6 and CFPAC-PLJ-CFTR-6 were cultured as previously described [9,10]. CFPAC cells and CFTR transfected cells were obtained from R. Frizzell.

2.2. *Polymerase chain reaction (PCR)*

RNA isolation was performed on cellular pellets from each cell type [11] (approximately 5×10^7 cells per pellet). Total RNA was resuspended in 20 μl sterile water and checked by agarose gel electrophoresis. Synthesis of total cDNA and cDNA-PCR amplification was performed using the Gene AMP RNA PCR kit (Perkin-Elmer Cetus). The oligonucleotides synthetic primers flanked the ΔF508 mutation: C16B (5′-GTTTTCCTGGATTATGCCTGGGCAC-3′) and C16D (5′-GTTGGCATGCTTTGATGACGCTTC-3′) [12]. The thermal cycle profile consisted of (i) denaturation for 60 s at 94°C, (ii) primer annealing for 45 s at 62°C and (iii) extension for 120 s at 72°C. The amplification was performed for 28 cycles. An additional elongation cycle was performed for 7 min. The amplification product (20 μl) was analyzed on polyacrylamide (10%) gel electrophoresis (overnight at 300 V) and visualized under UV after ethidium bromide staining.

2.3. *Membrane localization of CFTR*

The CFTR protein was visualized by indirect immunofluorescence using polyclonal antibodies (1/200) raised against the regulatory (R) region of the protein [13]. Antigen–antibody complexes were revealed with goat anti-rabbit gammaglobulins coupled to fluorescein isothiocyanate (1/80).

2.4. *Membrane localization of alkaline phosphatases (APs)*

Alkaline phosphatases were visualized by immunoperoxydase staining using purified polyclonal anti-alkaline phosphatases antibodies raised against the human placental AP. The antibodies recognized the placental and liver forms of the enzyme [10]. Cytoenzymatic reaction was performed as previously described [14].

2.5. *Alkaline phosphatase activity*

The alkaline phosphatase activity was measured spectrophotometrically at 410 nm after hydrolysis of *p*-nitrophenyl phosphate (5 mM) into *p*-nitrophenol [10]. Assays were carried out on 5- to 8 day-old CAPAN-1 cells.

2.6. *Patch-clamp*

Patch-clamp experiments [14] were performed on confluent cells using a LIST EPC-7 amplifier as reported elsewhere [9]. Voltage refers to the bath with respect to the patch pipette. Outward currents are displayed upward. Dashed lines give the zero-current baseline when channels are closed. For analysis, recordings were low pass filtered at 100–200 Hz. The pipette solution contained (in mM): 150 KCl (or 150 NaCl or 150 choline chloride), 2.5 $CaCl_2$, $MgCl_2$ and 5 Tris (pH 7.6). The NaCl saline was used to perfuse the cells. Theophylline, IBMX, levamisole (tetrahydro-phenylimidothiazole) and okadaic acid were added to the bath. The channel opening probability was calculated continuously and each point represented the value for 2s-sweeps. Inside-out excised patches were bathed in the above salines in which the Ca^{2+} content was reduced to 10–100 μM. Channel phosphorylation was achieved by adding cAMP-dependent protein kinase (PKA, from bovine heart, phosphorylating activity: 1.5 nM/mg protein), ATP (1 mM) and cAMP (1 mM) to the bath. Alkaline phosphatase from bovine intestinal mucosa (60 units/ml) was used to dephosphorylate PKA-activated CFTR channels as described in [5].

All chemicals were from Sigma except okaidaic acid which was from Calbiochem.

3. RESULTS AND DISCUSSION

The CFTR protein expressed in the pancreas [3] is specifically located in duct cells [15,17]. We used the following cell lines derived from human pancreatic adenocarcinomas of ductal origin: CAPAN-1 [9,10], CFPAC-1 [18], CFPAC-1 transfected [19] with either retroviral vectors alone (CFPAC-PLJ-6) or retroviral vectors incorporating the normal CFTR gene (CFPAC-PLJ-CFTR-6). Deletion of phenylalanine at position 508 in CFTR is the most common mutation in cystic fibrosis [3,12]. We used cDNA-PCR amplification and polyacrylamide gel electrophoresis to assess whether the CAPAN-1 and CFPAC-PLJ-CFTR-6 cells expressed the non-mutated CFTR mRNA. Results are shown in Fig. 1a. CAPAN-1 cells expressed the normal CFTR mRNA. As expected, CFPAC-PLJ-CFTR-6 cells expressed both normal and mutated mRNA, whereas CFPAC-1 and CFPAC-PLJ-6 expressed only the mutated mRNA. Immunofluorescence labelling with a polyclonal antibody raised against the regulatory region of CFTR showed that the protein was incorporated in the plasma membrane (Fig. 1b, small arrows) and was specifically located on the apical pole of CAPAN-1 cells (Fig. 1b, large arrows). These results showed for the first time, the expression and the apical localization of normal CFTR proteins in a human pancreatic duct cell line (CAPAN-1), which allowed the analysis of the CFTR protein to be performed in this cell line.

Single-channel currents were recorded from cell-attached and cell-free inside-out patches [14] performed on the apical membrane of the four cell lines. No spontaneous activity of cell-attached CFTR-like channels

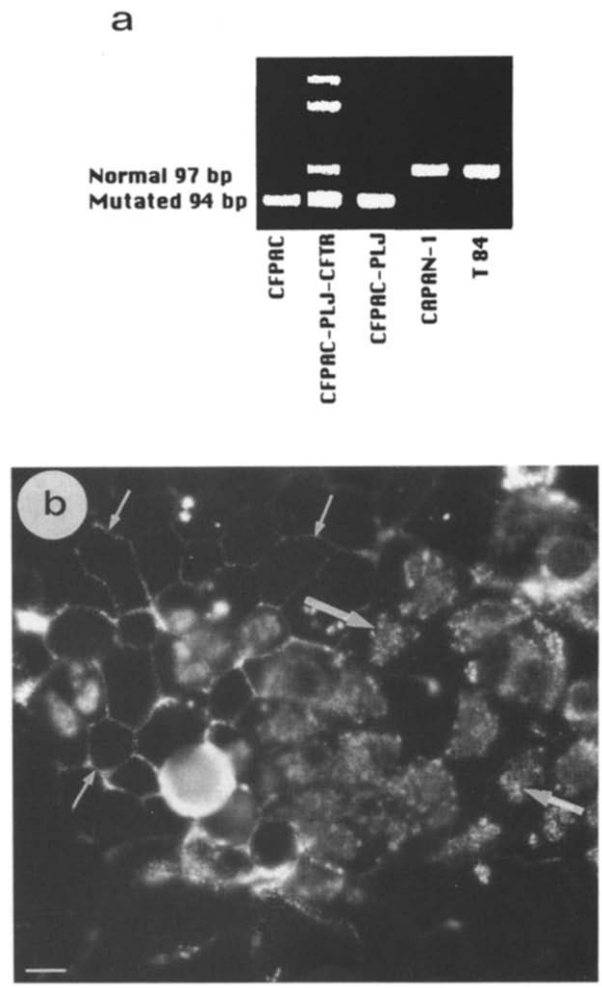

Fig. 1. Expression and localization of the CFTR protein in CAPAN-1 cells. (a) Polyacrylamide gel electrophoresis of normal and mutated (ΔF 508) CFTR transcripts by cDNA-PCR amplification. T84 cells were used as control. Additional bands in CFPAC-PLJ-CFTR-6 are heteroduplex forms due to competitive hybridization of mutant and normal trends toward the end of the PCR reaction. (b) Detection by immunofluorescence of the CFTR protein at the periphery (small arrows) of unpolarized cells and at the apex (large arrows) of polarized CAPAN-1 cells. The photograph was focused on the apex of the cells. Calibration bar: 10 μm.

was recorded in cells bathed in the physiological saline. When theophylline (1-3 dimethyl xanthine) was added to the bath, low conductance Cl^- channels were observed in CAPAN-1 and CFPAC-PLJ-CFTR-6 but not in CFPAC-1 and CFPAC-PLJ-6 (Fig. 2a–c). The channels had a linear *I–V* relationship. The current reversed direction at the cell resting potential with pipettes filled with either choline chloride (n = 8), NaCl (n = 8) and KCl (n = 28). This indicated that the channels were mainly selective for Cl^-. The unitary conductance was 9.8 ± 0.7 pS in CAPAN-1 (n = 29) and 9.6 ± 0.9 pS in CFPAC-PLJ-CFTR-6 (n = 15). The channel opening probability (0.58 ± 0.21; n = 26, Fig. 2d) was not voltage-dependent although it displayed fluctuations (from

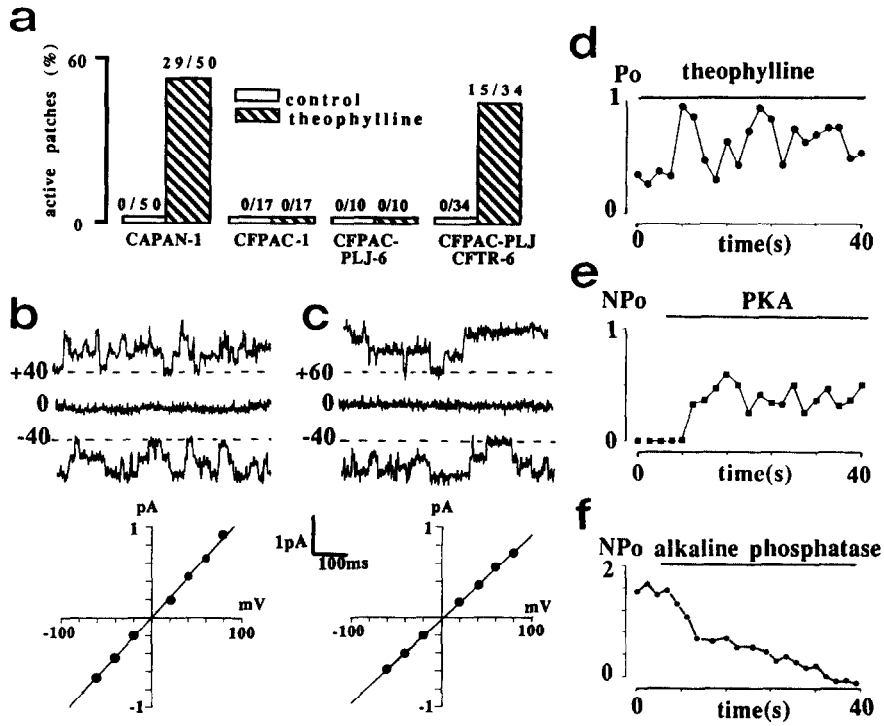

Fig. 2. Theophylline-induced low conductance Cl^- channels in CAPAN-1 and CFTR-transfected CFPAC cells. (a) Percentage of cell-attached patches containing active Cl^- channels in non-stimulated and theophylline-stimulated cells. (b) and (c) Cell-attached recordings of Cl^- channels induced in CAPAN-1 (b) and CFPAC-PLJ-CFTR-6 (c) by 1 mM theophylline and corresponding *I–V* relationships (fitted by linear regression). (d) Fluctuation in the opening probability (P_o) of cell-attached Cl^- channels in a theophylline-stimulated CAPAN-1 cell. (e) and (f) Inside-out patches excised from CAPAN-1 cells; (e) Protein kinase A (PKA) activation of quiescent Cl^- channels; (f) PKA-induced channels were blocked by adding alkaline phosphatase (60 units/ml).

0.28 to 0.94) that could result from up and down-regulatory processes. IBMX (3-isobutyl-1-methyl-xanthine, 1 mM) was also found to be effective (7/10) in inducing the 10-pS Cl^- channel in cell-attached patches. The channels were also induced in CAPAN-1 by the adenylate-cyclase activator forskolin ($n = 2$) as already reported for CFPAC-PLJ-CFTR-6 [20]. Since mutated CFTR channels were not activated in cell-attached patches, we focussed our study on normal non-mutated channels expressed in CAPCAN-1 and CFPAC-PLJ-CFTR-6 cells.

When excised in the inside-out configuration, theophylline-induced channels remained active for several minutes (CAPAN-1, $n = 29$; CFPAC-PLJ-CFTR-6, $n = 15$) provided that theophylline was present in the bath. Theophylline-free salines resulted in the deactivation of the channels. The excised channels had a linear *I–V* relationship (symmetrical KCl channels) and a unitary conductance of 9.7 ± 0.8 pS ($n = 44$). Gluconate and I^- substituted for Cl^- shifted the reversal potential to −45 and −18 mV, respectively. The permeability ratio $P_{gluc}/P_{Cl} = 0.12$ ($n = 3$) and $P_I/P_{Cl} = 0.47$ ($n = 4$) led to a permselectivity $Cl^- > I^- >$ gluconate.

Quiescent channels in cell-free patches could be reactivated (9/22) by adding cAMP-dependent protein kinase (PKA) together with the PKA activators Mg^{2+}, ATP and cAMP (Fig. 2e). The opening probability increased from nominally zero to 0.41 ± 0.11 ($n = 9$). No channel activation was observed with the PKA activators alone ($n = 22$), which indicated that excised channels were free from membrane-associated PKA. Consequently, in situ channel phosphorylation might involve cytosolic PKA [5–8]. PKA-activated excised channels were blocked (up to 90%) by adding alkaline phosphatase to the PKA-containing saline ($n = 6$) (Fig. 2f).

These results demonstrated that the theophylline-induced channels, controlled by PKA and phosphatases, had properties similar to those of the CFTR-channel expressed in various cells [5,7,13]. We thus concluded that the xanthine-activated 10-pS chloride channel was the CFTR-channel.

Theophylline and IBMX and widely used to inhibit cyclic nucleotide phosphodiesterases. They also inhibit alkaline phosphatases [21,22]. Since reactivation of quiescent excised channels by PKA was unsuccessful in 60% of the experiments, we hypothesized that membrane-bound phosphatases prevented the channels from being activated by PKA. The presence and localization of alkaline phosphatases in CAPAN-1 were detected by performing immunoperoxydase staining with a polyclonal anti-AP antibody and cytoenzymatic reaction combined with light and electron microscopic examination. Both methods showed that APs were present on the apical membrane of polarized CAPAN-1 cells (Fig. 3a

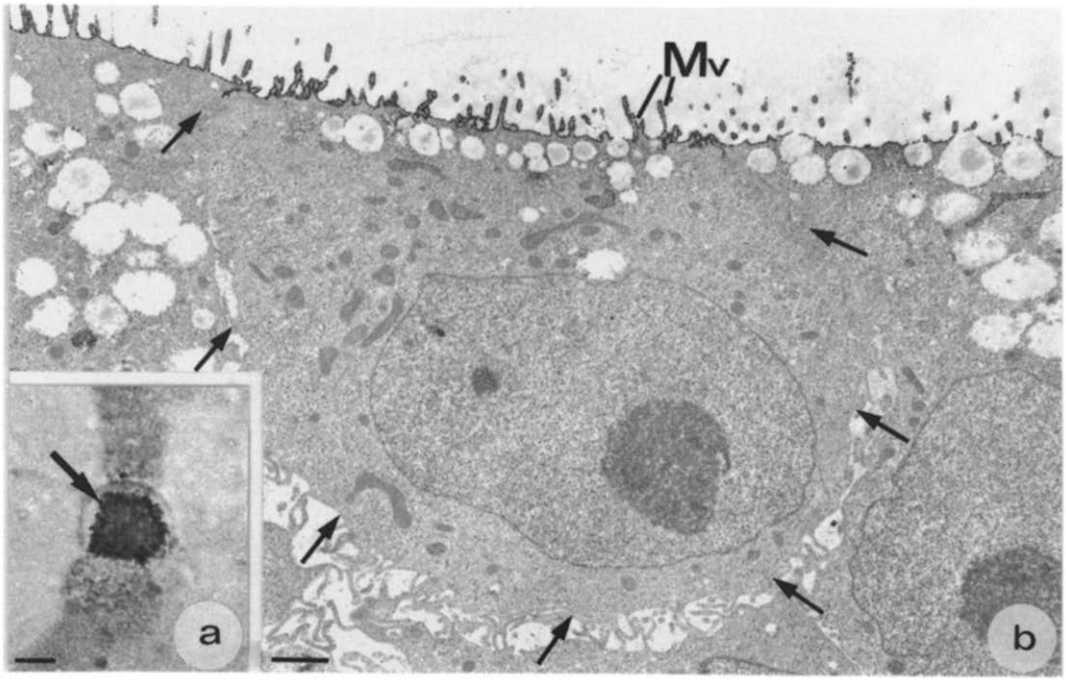

Fig. 3. Apical localization of alkaline phosphatases. (a) Immunoperoxydase staining of alkaline phosphatases on the cell apex (arrow) of a polarized cell. The microphotograph was focused on the cell apex. (b) Electron micrograph showing the apical localization of alkaline phosphatase activity by cytoenzymatic reaction in polarized CAPAN-1 cells. Note the positive reaction on the apical pole and on microvilli (Mv) and the absence of reaction on the basolateral membranes (arrows). Calibration bars: 5 μm (a) and 1 μm (b).

and b). No AP staining was observed on the basolateral membrane (Fig. 3b, arrows). A similar localization of APs was observed in CFPAC-1, CFPAC-PLJ-6 and CFPAC-PLJ-CFTR-6 cells (not shown). We then found that various substances that favoured the channel opening were strong inhibitors of APs. The CAPAN-1 AP activity was inhibited (by 60–80%) by theophylline, IBMX, levamisole (a specific inhibitor of AP) [23,24] and by the polyclonal antibody raised against AP (anti-AP antibody) (Fig. 4a). Levamisole enhanced threefold the PKA-induced current (n = 3, Fig. 4b). Since levamisole had no effect on PKA activity [24], its potentiating effect might have resulted from the blockade of endogenous AP. Similarly, theophylline (n = 3, Fig. 4c) enhanced the current induced by PKA alone by 330%. Okadaic acid (0.5–10 μM), an inhibitor of types 1 and 2A protein phosphatases but not of alkaline phosphatase [25], was ineffective in blocking the AP activity and in activating CFTR channels, which confirms previous results obtained CFTR-transfected cells [5,26].

These results led us to suppose that the membrane-bound alkaline phosphatase could regulate the CFTR-Cl^- channel. The polyclonal anti-AP antibody used for staining AP proteins was applied to cell-free patches. The antibody added to the PKA-containing bath induced a sustained irreversible increase in current in both CFPAC-PLJ-CFTR-6 (n = 2) (Fig. 5a) and CAPAN-1 (n = 5) (Fig. 5b). The pre-immune serum was ineffective in blocking the APs activity and in inducing channel openings, which suggested that the antibody effect on the channel resulted from the specific blockade of alkaline phosphatases. The antibody-induced openings of CFTR-channels were observed at both positive and negative potentials (Fig. 5c). The I–V relationship was linear and the current reversed direction at 0 mV. The channel conductance was 10 pS (Fig. 5d). Gluconate substituted to Cl^- shifted the reversal potential from 0 to −40 mV (n = 2). These properties were similar to those of the CFTR-channels described above.

Alkaline phosphatases hydrolyse a wide variety of phosphate monoesters and membrane phosphoproteins [27–29]. In this study, we show that the CFTR-Cl^- channel in the human pancreatic duct CAPAN-1 and CFPAC-PLJ-CFTR-6 cells is under the control of PKA and alkaline phosphatases. Similar regulatory mechanisms have been described following heterologous expression of the CFTR protein in CHO cells [5]. Since okadaic acid failed to enhance the activity of the CFTR-Cl^- channel in CHO [5], CAPAN-1 and CFPAC-PLJ-CFTR-6 (this study), we postulate that alkaline phosphatases but not types 1 or 2 protein phosphatases remained attached to the excised CFTR-Cl^- channels. We found that the inhibition of membrane-bound AP by theophylline, IBMX, levamisole and the anti-AP antibody, not only enhanced but also triggered the CFTR- Cl^- channel activity in cells that expressed the CFTR- channel either normally (CAPAN-1) or following correction by retrovirus-mediated CFTR gene transfer (CFPAC-PLJ-CFTR-6). These results are thus consistent with the hypothesis that the phosphatase ac-

a

	Theophylline (1mM)	IBMX (5mM)	Levamisole (5mM)	Anti-AP (1%)
Inhibition of AP activity (%)	70.5±4	70.45±3	76.73±8	63±2.2

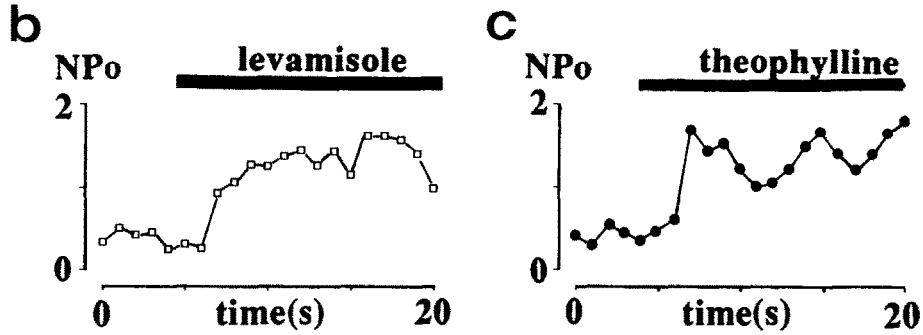

Fig. 4. Activation of excised low conductance Cl^- channels by factors that reduce the alkaline phosphatase (AP) activity. (a) Inhibition of AP activity by theophylline, IBMX, levamisole and the anti-AP antibody. (b) and (c) PKA-activated inside-out channels excised from CAPAN-1 cells. Levamisole (5 mM) and theophylline (1 mM) drastically increased the channel opening probability.

tivity is closely associated to the CFTR-Cl^- channel. They suggest also that the blockade of channel-associated APs as well as the activation of cytosolic PKA [5,7] and the presence of ATP [26] may be required to open the channel in pancreatic duct cells. The phosphatase activity would thus play a major role in the control of the channel. It has been suggested that exogenous APs reduced CFTR activity by consuming the bath ATP [8]. However, MgATP which is usually present in the bath saline is not a substrate for APs [24].

Alternatively, APs could hydrolyse the ATP molecules bound to the CFTR nucleotide binding domains. Additional data are needed to clear up this point.

The main question raised by our results concerns the respective location of the CFTR protein and APs. Both are found in the apical membrane; APs are anchored on

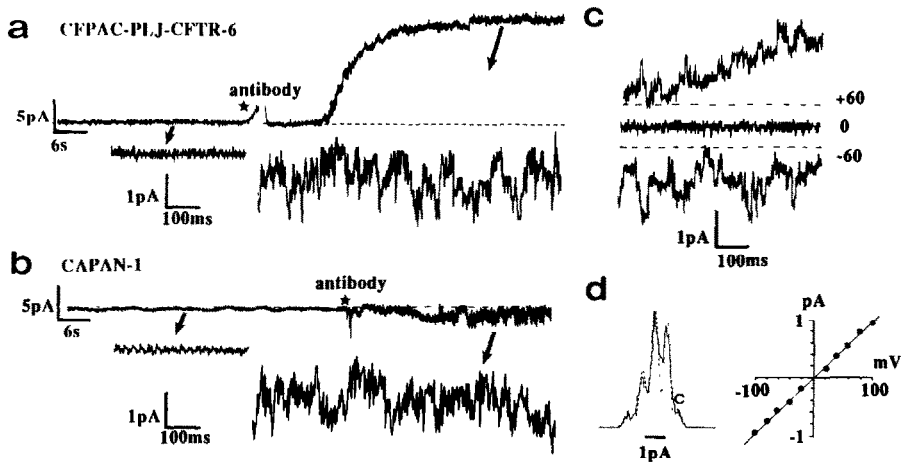

Fig. 5. The antibody raised against alkaline phosphatase activates quiescent CFTR-channels. (a) and (b) Excised inside-out patches bathed in PKA-containing saline. Patch potential: +60 mV in (a) and −60 mV in (b). Long-lasting recordings and expanded traces of anti-AP antibody (1/200)-induced CFTR-channels in CFPAC-PLJ-CFTR-6 (a) and CAPAN-1 (b) cells. The antibody was added at the time indicated by stars and current transients. Tests with the pre-immune serum were ineffective in inducing CFTR-channels ($n = 3$). (c) Details of the antibody-activated CAPAN-1 CFTR-channels recorded 8 min after their induction. (d) Amplitude histogram (−80 mV) and unitary current–voltage relationship of antibody-activated channels. The amplitude distribution was fitted to Gaussian curves predicted for 4 identical independent channels (opening probability: 0.56, c: closed stated). The unitary channel conductance was ≈ 10 pS.

the cell surface by glycosylated phosphatidylinositol and are active on the external medium [24] whereas the CFTR regulatory domains are cytosolic [26]. This seems to rule out a direct regulation of the CFTR channel by APs. However, the involvement of cytoplasmically oriented enzymes anchored with phosphatidylinositol has received little attention [30]. In CAPAN-1, functional APs have been detected in several intracellular structures: Golgi saccules, endoplasmic reticulum and microvesicles [10]. Our results suggest that part of the apically-located APs is cytoplasmically oriented, which could not be resolved with the cytological methods used here to detecting APs.

Theophylline has been shown to increase the pulmonary mucociliary clearance [31] and to significantly improve the pulmonary function in CF patients [32,33]. Although these observations are in line with our results, it remains to be evaluated whether drugs such as levamisole that reduce APs activity are able to favor the opening of mutated CFTR channels before considering any therapeutic use.

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