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Effect of modulation of protein kinase C on the cAMP-dependent chloride conductance in T84 cells

Maria Cristina Dechecchi[a], Rossella Rolfini[a], Anna Tamanini[a], Chiara Gamberi[b], Giorgio Berton[c] and Giulio Cabrini[a]

[a]*Centro Fibrosi Cistica, Ospedale Civile Maggiore,* [b]*Istituto Scienze Biologiche* and [c]*Istituto Patologia Generale of the University, Verona, Italy*

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The regulation of chloride conductance was investigated in the T84 human colon carcinoma cell line by the quenching of the fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolinium. The permeable cAMP analog 8-Br-cAMP (100 μM) and the calcium ionophore ionomycin (1 μM) activate a chloride conductance. A prolonged (4 h) preincubation of cells with phorbol 12-myristate 13-acetate (100 nM) or with the diacylglycerol analog 1-oleoyl-2-acetyl-glycerol (100 μM): (i) down-modulates to almost zero the protein kinase C activity in the membranes; (ii) inhibits the activation of the chloride conductance mediated by 8-Br-cAMP but not by calcium; (iii) reduces the mRNA without changing the expression of the protein product of the cystic fibrosis gene. The data suggest that PKC is essential for the activation of the cAMP-dependent chloride conductance in T84 cells.

Chloride transport; Protein kinase C; Protein kinase A; Cystic fibrosis; T84 cell

1. INTRODUCTION

Alterations of the cAMP-dependent chloride conductive pathway underlie the abnormal function of electrolyte transport in the epithelia of Cystic Fibrosis (CF) patients [1]. Recent studies demonstrated that CF is caused by mutations in the gene coding for a transmembrane protein denominated Cystic Fibrosis Transmembrane conductance Regulator, CFTR [2–4]. Transfection experiments [5–7] and the functional reconstitution into liposomes [8] demonstrated that CFTR is indeed a cAMP-dependent chloride transporter.

Recent data obtained in CHO cells transfected with the CFTR gene showed a potentiating effect of protein kinase C (PKC) on the protein kinase A (PKA)-dependent activation of CFTR [9], suggesting a synergism between converging kinase regulatory pathways on CFTR. These experiments are of interest since the R domain of CFTR, as predicted by the cDNA structure, shows both 9 putative consensus sequences for PKA and 7 for PKC dependent phosphorylation [3]. While four serines of the R domain have already been identified as the residues responsible for the PKA-dependent activation of CFTR [10], the understanding of the role of PKC and its possible cross-talk with PKA on this specific target deserves further investigation.

The human colon carcinoma cell line T84 has been shown to express a high level of CFTR [3] and a cAMP-dependent chloride conductance with the same biophysical characteristics of the channel generated after transfection of the CFTR gene in cells with undetectable endogenous CFTR mRNA [5,6]. We have therefore initiated studies on the role of PKC on the chloride conductance of T84 cells. The results reported in this paper show that, under conditions in which the membrane associated fraction of CFTR is down-modulated, cAMP fails to activate the chloride conductance. We suggest that PKC exerts not only a facilitating [9] but also a permissive role in the PKA dependent activation of CFTR.

Correspondence address: G. Cabrini, Centro Fibrosi Cistica, Ospedale Civile Maggiore, 37126 Verona, Italy. Fax: (39) (45) 830 1200.

2. MATERIALS AND METHODS

2.1. *Materials*

T84 human colon carcinoma cell line (passage 50–60) was from American Type Culture Collection (Rockville, MD), 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) from Molecular Probes (Eugene, OR), 1-oleoyl-2-acetyl-glycerol (OAG), ionomycin from Calbiochem (San Diego, CA), radioactive reagents from Amersham (Buckinghamshire, UK), all the other reagents from Sigma (St. Louis, MO).

1.2. *Chloride conductance by fluorescence microscopy*

Chloride conductance was measured by the anion-sensitive fluorescent probe SPQ [11]. Glass coverslips were mounted in a perfusion chamber, cells were washed with solution A (136 mM NaCl, 14 mM NA gluconate, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 KCl, 5 mM glucose, 5 mM HEPES-Tris, pH 7.4) and SPQ (5 mM) was loaded by transient permeabilization with a 1:1 mixture of solution A and double distilled water for 30 min at 37°C. After loading, cells were perfused with solution A for 20 min in order to remove extracellular SPQ and restore the cellular volume with an isotonic buffer. Cells were perfused for 20 min with solution A in which [Cl^-] was 93 mM and [I^-] was 50 mM.

After this partial substitution of intracellular chloride with iodide, SPQ fluorescence was measured at 25°C in cells perfused by a gravity perfusion system (3 ml/min) with solution A in the presence of 200 μM furosemide in response to different agonists. Fluorescence was collected with a Nikon TMD epifluorescence inverted microscope (Tokyo, Japan) interfaced with an SLM 8000C spectrofluorometer (Urbana, IL). Excitation light was at 360 ± 8 nm. Emitted light was filtered by an interference filter (443–459 nm) and detected by a R928S Hamamatsu photomultiplier tube (Middlesex, NJ).

2.3. *Protein kinase A and C assay*

Protein kinase C activity was measured after partial purification by DEAE-cellulose (DE52) column chromatography [12] and protein kinase A as described [13], using the synthetic heptapeptide as phosphate acceptor.

2.4. *Northern blot analysis*

Polyadenylated RNA has been extracted, electrophoresed and blotted as described [14]. CFTR mRNA was detected by hybridization with the radiolabeled probe C1-1/5 [3]. The filters were also hybridized with cDNA probes for β-actin and for heterogeneous nuclear ribonucleoprotein I (hnRNP type I – I_{55} kDa) [15] that were used to control for variation in sample loading and to normalize the CFTR mRNA expression. The extent of hybridization was quantitated with a 2202 Ultrascan laser densitometer (LKB, Gaithersburg, MD).

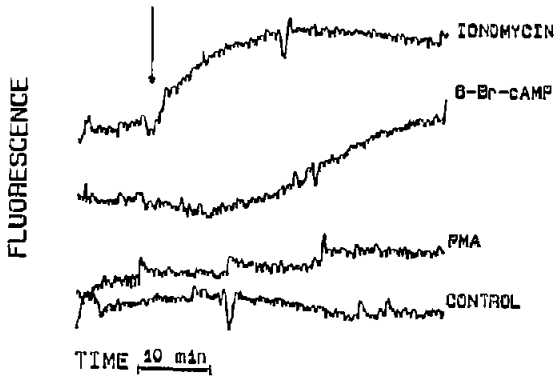
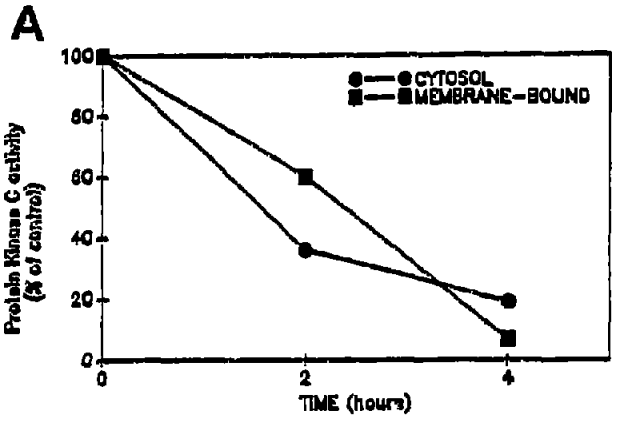
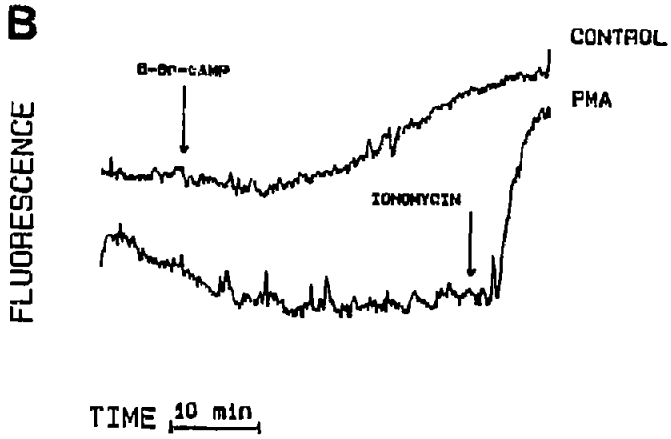
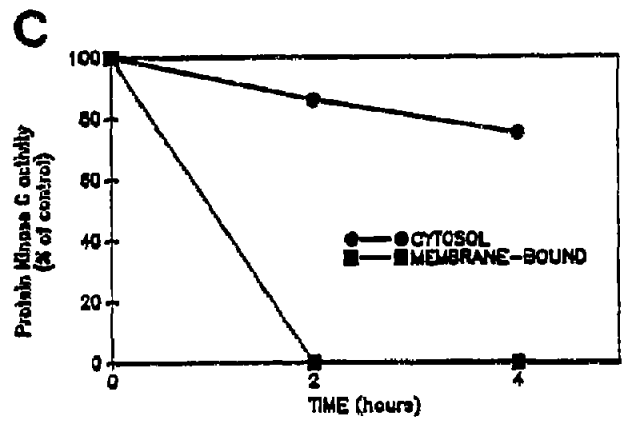


Fig. 1. Effect of calcium, 8-Br-cAMP and PMA on chloride conductance. Cells loaded with SPQ were perfused as described in section 2. 8-Br-cAMP (100 μM) with theophylline (1 mM), ionomycin (1 μM), PMA (100 nM) or dimethylsulfoxide (0.1%, control trace) were added at the time indicated by the arrow. The traces were displaced in the Y-direction to show the parallel time courses. The fluorescence value at time zero, the signal to autofluorescence ratios and the Y-axis amplifications of the 4 traces were strictly similar.







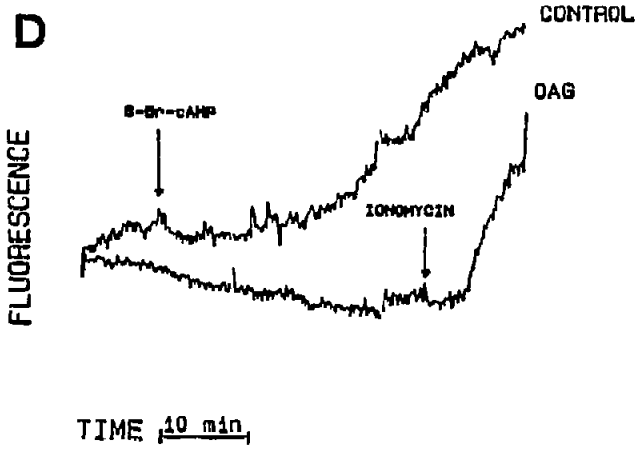


Fig. 2. Effect of preincubation with PMA or OAG on PKC activity and cAMP-dependent chloride conductance. 100 nM PMA or 100 μM OAG or dimethylsulfoxide (control) were added to cells at 37°C before enzyme activity measurement or fluorescence experiments. (A) PKC activity after 100 nM PMA. (B) Effect of preincubation with 100 nM PMA for 4 h on the chloride conductance. 1 μM ionomycin was added after 30 min of absence of response of 8-Br-cAMP. (C) PKC activity after 100 μM OAG. (D) Effect of preincubation with 100 μM OAG for 4 h on the chloride conductance.

2.5. *Expression of CFTR*

The following monoclonal antibodies were utilized. Mouse anti-human CFTR (IgG_1) [16] was kindly provided by Dr. S.H. Cheng (Genzyme, Framingham, MA). 5E9 anti transferrin receptor (IgG_1) [16] was from American Type Culture Collection (Rockville, Maryland). B66.6 anti CD4 antigen (IgG_1) [18] was kindly provided by Dr. G. Trinchieri (The Wistar Institute for Anatomy and Cell Biology, Philadelphia, PA). OKM.1 anti complement receptor type 3 — CR3 or CD11b (IgG_{2b}) [19] was kindly provided by Dr. P. Rao (Robert Wood Johnson Pharmaceutical Research Institute, Raritan, NJ). Monoclonal antibodies were used as ascites fluid (5E9) at 1:500 dilution or as purified preparations (α-CFTR, B66.6, OKM.1) at the final concentration of 2 μg/ml (100 ng/well). Cells grown to confluence in 96-well plates were incubated for different times with 100 nM PMA or 100 μM OAG and fixed with methanol (50 μl/well) for 10 min at −20°C. Primary antibodies were diluted in PBS containing 0.1% Tween 20, 2% bovine serum albumin (BSA) and 1% human and incubated for 90 min at 4°C. The secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse IgG) was at 1:2000 final dilution and incubated for 30 min at 4°C. Binding was detected by *o*-phenylenediamine and absorbance was read at 490 nm wavelength.

3. RESULTS AND DISCUSSION

T84 cells express three different chloride channels that are operated by cAMP, calcium and cell swelling, respectively [20]. Fig. 1 shows the results obtained with the fluorescent probe SPQ. The cAMP-operated chloride conductance is indicated by the increase of the fluorescent signal after the addition of the cAMP analog 8-Br-cAMP, that presents a lag time ranging from 10 to 15 min. The calcium dependent conductance activated by the addition of the calcium ionophore ionomycin (1 μM) shows much faster kinetics (<1 min), while the tumor promoter phorbol 12-myristate 13-acetate (PMA, 100 nM) does not open this conductive pathway, also according to previously reported data [21].

To reveal a possible role of PKC in the regulation of the cAMP-dependent chloride conductance, T84 cells were incubated with 100 nM PMA for 4 h at 37°C in order to deplete cells of PKC activity [22], as shown in Fig. 2A. Under these conditions, 8-Br-cAMP failed to elicit the chloride conductance (Fig. 2B). Parallel experiments demonstrated that protein kinase A activity was not affected by preincubation of PMA for 4 h (data not shown). Phorbol esters are known to influence the chloride secretion in different ways in human intestinal cells. Firstly, long term incubation with PMA (3 h, 500 nM) inhibited the K^+ channels in the HT-29.cl.19A cell line [23], thus exerting a secondary block on the different chloride conductive pathways. In our assay system this effect can be excluded since the calcium ionophore ionomycin activated a chloride conductance, as shown in Fig. 2B. Secondly, massive activation of protein kinase C (100 nM PMA from 20 to 30 min) exerted an inhibitory effect on the calcium-dependent chloride conductance in T84 cells [21]. On the other side, under our experimental conditions the membrane fraction of PKC is down-modulated and therefore it can not exert this inhibitory effect.

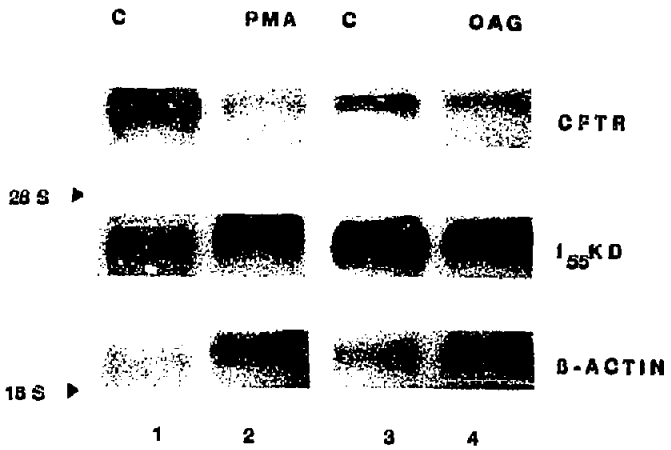


Fig. 3. Northern blot of CFTR mRNA after 4 h of incubation with 100 nM PMA (lane 2), 100 μM OAG (lane 4) or dimethylsulfoxide (0.1%) as control (lanes 1 and 3). CFTR is shown together with the controls of variation in sample loading (hnRNP type I – I_{55} kDa and β-actin). The position of the 28 S and 18 S rRNA bands are shown on the left.

Since there is no definite proof for PKC being the sole target of PMA [22], we also tested the diacylglycerol analog 1-oleoyl-2-acetyl-glycerol (OAG) trying to mimic a more physiological stimulation of the PKC pathway. As for PMA, the preincubation of T84 cells with 100 μM OAG for 4 h depleted PKC activity (Fig. 2C) and abolished the 8-Br-cAMP-dependent activation (Fig. 2D); also under these conditions the calcium-operated chloride conductance was observed.

Since PMA has been reported to down-modulate CFTR mRNA [24], the steady-state levels of CFTR mRNA were investigated after 4 h of incubation of T84 cells with 100 nM PMA or 100 μM OAG. Fig. 3 shows that PMA and OAG reduce CFTR mRNA at different extents, the former to an average 5.4% of the control levels (S.D.=1.5%, n=3) and the latter to 46.6% (S.D.=16.2%, n=2). CFTR protein expression was studied in parallel. Fig. 4 demonstrates that neither PMA nor OAG decreases the level of expression of CFTR up to 4 h of incubation. In the assay used for the experiments reported in Fig. 4 we did not detect the CFTR protein in cells that do not express CFTR mRNA like NIH 3T3 fibroblasts (data not shown). Fig. 4 also shows that the assay system utilized can recognize the presence of the transferrin receptor (5E9), while proteins that are selectively expressed by leukocytes (CD4, CD11b) are undetectable on T84 cells. From our data it seems likely that the inability of cAMP to activate chloride conductance in T84 cells, after treatment with PMA or OAG for 4 h, is due to down-modulation of PKC activity and not to the elimination of CFTR. In fact, the same effect of PMA is obtained with OAG, which reduces d CFTR mRNA only to approximately half of the control CFTR mRNA and, more importantly, CFTR protein is still present in both conditions.

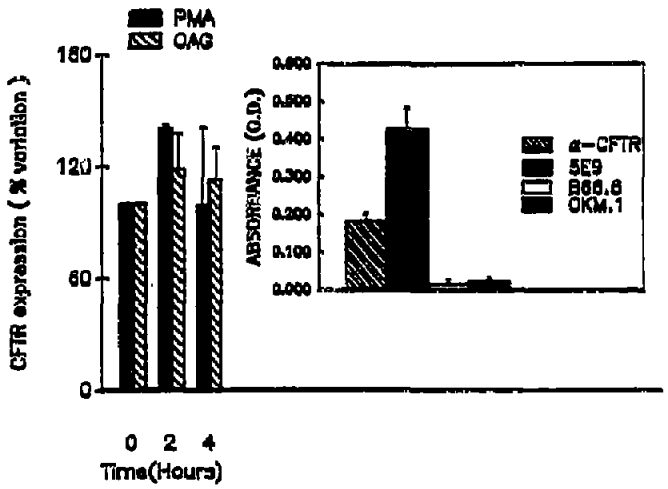


Fig. 4. Expression of CFTR protein after PMA and OAG. Cells incubated with 100 nM PMA or 100 μM OAG for the indicated time were assayed with the α-CFTR antibody as described in section 2. (Inset) Binding of the antibodies against CFTR (α-CFTR), transferrin receptor (5E9), CD4 antigen (B66.6) and the complement receptor type 3 (OKM.1) in the absence of PMA or OAG.

The present data seems to imply that a basal phosphorylation of some of the consensus sequences of CFTR is necessary for the activation of CFTR through PKA. Interestingly, experiments on the R domain of CFTR demonstrated at least 5 phosphorylation sites that are present in the basal state and do not coincide with PKA sites [10]. Whether these basal phosphorylation sites could be related to PKC consensus sequences needs further investigation at the light of the evidence we have presented for a possible cross-talk between PKC and PKA in regulating a chloride conductive pathway in T84 cells.

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