Effect of NYD-SP27 down-regulation on ATP-induced Ca\(^{2+}\)-dependent pancreatic duct anion secretion in cystic fibrosis cells

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Received 26 April 2006; revised 17 October 2006; accepted 16 November 2006

Abstract

Our previous study demonstrated that NYD-SP27 is a novel inhibitory PLC isoform expressed endogenously in human pancreas and upregulated in CFPAC-1 cells. The present study investigated the effect of NYD-SP27 down-regulation on the ATP-stimulated and Ca\(^{2+}\)-dependent pancreatic anion secretion by CFPAC-1 cell line using short-circuit current (\(I_{SC}\)) recording. NYD-SP27 antisense-transfected CFPAC-1 (AT-CF) cells exhibited a significantly higher basal transmembrane potential difference and current than those of empty vector-transfected CFPAC-1 (VT-CF) cells. Cl\(^{-}\)/C\(_0\) channel blocker, DPC or Glibenclamide (1 mM), and inhibitor of Na\(^{+}\)-K\(^{+}\)-Cl\(^{-}\)/C\(_0\) cotransporter, bumetanide (100 \(\mu\)M) significantly inhibited the basal current in AT-CF cells. The inhibitor of adenylate cyclase, MDL12330A (20 \(\mu\)M), and Ca\(^{2+}\)-dependent Cl\(^{-}\) channel (CaCC) blocker, DIDS (100 \(\mu\)M) also significantly reduced the basal current in AT-CF. Apical application of ATP (10 \(\mu\)M) stimulated a fast transient \(I_{SC}\) increase in VT-CF cells, but a more sustained rise with slower decline in AT-CF cells. Pretreatment with BAPTA-AM (50 \(\mu\)M) reduced the ATP-induced \(I_{SC}\) response in AT-CF cells by 77.9%. PMA (1 \(\mu\)M), a PKC activator, inhibited the ATP-stimulated current increase (the transient peak) in VT-CF cells, but had no effect on the AT-CF cells. However, PKC inhibitor, staurosporine (40 \(\mu\)M) could inhibit the ATP-induced \(I_{SC}\) response in AT-CF cells. The present results confirm the previously proposed inhibitory role of NYD-SP27 in the PLC pathway and demonstrate that the suppression of its expression could result in an enhancement of ATP-stimulated Ca\(^{2+}\) dependent pancreatic anion secretion.

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Keywords: NYD-SP27; Ca\(^{2+}\); PKC; CF; Short-circuit current

1. Introduction

Cystic fibrosis (CF) is a common lethal genetic disease caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR). It is characterized by defective secretion of chloride and bicarbonate ions in most exocrine glands and organs (Zhu et al., 2003). CFTR, a cAMP-dependent Cl\(^{-}\) channel, is activated by PKA and PKC, and gated by ATP hydrolysis (Riordan et al., 1989; Anderson et al., 1991; Tabcharani et al., 1991; Gadsby et al., 1995). A deletion of the phenylalanine amino acid at position 508 (F508) in the NBD\(_1\) (nucleotide-binding domain 1) can cause protein trafficking defect with most of the protein failing to progress to the Golgi apparatus and cell membrane (Welsh and Smith, 1993; Cheng et al., 1991).

Extracellular purines such as ATP and adenosine can activate P2Y receptors through the PLC signaling pathway, which cleaves PIP2 into DAG and IP\(_3\). DAG recruits PKC, a calcium-dependent kinase, which phosphorylates many other proteins, including CFTR, which lead to subsequent cellular responses. IP\(_3\) binds to receptors on the ER causing the release of Ca\(^{2+}\) into the cytosol, and the rise in [Ca\(^{2+}\)], triggers the subsequent responses of the cell (Bucheimer and Linden, 2004). Our previous studies have demonstrated that ATP stimulates anion...
secretion in pancreatic duct cells via Ca\(^{2+}\)-dependent pathway and that the Ca\(^{2+}\)-activated pancreatic anion secretion can be modulated by PKC (Cheng et al., 1999).

NYD-SP27, a novel inhibitory PLC isoform, is expressed endogenously in human pancreas and upregulated in CF. We have recently reported that inhibition of NYD-SP27 expression results in augmentation of PLC-coupled Ca\(^{2+}\) release and PKC activity leading to enhancement of 5F508-CFTR reached the plasma membrane and restoration of cAMP-activated pancreatic anion secretion in the CF cells (Zhu et al., 2003). However the effect of down regulation of NYD-SP27 on Ca\(^{2+}\)-dependent pancreatic anion secretion has not been investigated. The present study investigated the role of Ca\(^{2+}\) and PKC in ATP-stimulated pancreatic anion secretion in Vector-transfected and NYD-SP27 antisense-transfected CFPAC-1 cell line using the short-circuit current technique.

2. Materials and methods

2.1. Construction of vectors and transfection of CFPAC-1 cells

The antisense vector was generated by inserting a fragment (from 370 bp—615 bp with no homology to other PLC isoforms known) of NYD-SP27 into a pCI-neo vector in the reverse orientation. CFPAC-1 was purchased from American Type Culture Collection (Maryland, USA). They (2 × 10\(^5\)/well) were seeded into 6-well plates and transfected using Lipofectin Reagent (Gibco) according to the manufacturer’s instructions with antisense vectors, Iscove’s modified Dulbecco’s medium containing G418 (400 mg/ml) was used to select the stable transfected clones. The clones were further confirmed by semi-quantitative RT-PCR.

2.2. Cell culture

NYD-SP27 antisense-transfected CFPAC-1 (AT-CF) cells and control vector-transfected CFPAC-1 (VT-CF) cells were grown in Iscove’s modified Dulbecco’s medium containing G418 (400 μg/ml) was used to select the stable transfected clones. The clones were further confirmed by semi-quantitative RT-PCR.

2.3. Short circuit current measurement

The measurement of \(I_{SC}\) has been described previously (Zhu et al., 2003). AT-CF and VT-CF monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber. The monolayers were bathed with Krebs-Henseleit solution, bubbled with 95% O\(_2\) and 5% CO\(_2\) to maintain the pH of the solution at 7.4 and maintained at 37 °C. \(I_{SC}\) was measured by the Ag/AgCl reference electrodes (World Precision Instruction) connected to a preamplifier that was in turn connected to a voltage-clamp amplifier (World Precision Instruction, DVC-1000). In most of the experiments, the change in \(I_{SC}\) was defined as the maximal rise in \(I_{SC}\) or the total charge transfer (area under the \(I_{SC}\) response curve) following agonist stimulation and they were normalized as changes per unit area of the epithelial monolayer (\(µA/cm^2\) or \(µC/cm^2\)). Experiments were repeated in different batches of culture to ensure that the data were reproducible.

2.4. Solutions & chemicals

Hank’s balanced salt solution (HBSS) and fetal bovine serum was from Gibco Laboratories (New York, NY, USA). 4, 4-diisothiocyanostilbene-2, 2-disulfonic acid (DIDS), phorbol 12-myristate 13-acetate (PMA), and glibenclamide were from Sigma (St Louis, MO, USA). Dipyridamole-2, 2-dicarboxylic acid (DPC) was obtained from Riedel-de Haen Chemicals (Hanover, Germany). Calbiochem (San Diego, CA, USA) was the source for MDL-12330A (adenylate cyclase inhibitor), BAPTA-AM and staurosporine.

Krebs-Henseleit (K-H) solution had the following composition (mM): NaCl, 117; KCl, 4.5; CaCl\(_2\), 2.5; MgCl\(_2\), 1.2; NaHCO\(_3\), 24.8; KH\(_2\)PO\(_4\), 1.2; Glucose, 11.1. The solution was gassed with 95% O\(_2\) and 5% CO\(_2\) and kept the pH at 7.4.

2.5. Statistics

Results were expressed as mean ± standard error of mean (SEM). Comparisons between groups of data were made by either the Student’s t-tests (2-group comparison) or one-way ANOVA with Newman–Keuls post-hoc test (3-group comparison). A probability value of less than 0.05 (>95% confidence) was considered statistically significant.

3. Results

3.1. Basal transmembrane electrophysiological properties in AT-CF cells

Compared with empty vector-transfected CFPAC-1 (VT-CF) cells (\(n = 38\)), antisense-transfected CFPAC-1 cells (AT-CF) (\(n = 39\)) had a much higher basal transmembrane

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**Fig. 1.** Effect of NYD-SP27 down-regulation on basal potential difference (PD) (a); and basal \(I_{SC}\) (b). VT-CF: vector-transfected CFPAC-1 cells; AT-CF: NYD-SP27 antisense-transfected CFAC-1 cells.
potential difference and current (Fig. 1, $P < 0.001$). $\text{Cl}^-$ channel blockers, DPC or Glibenclamide (1 mM), and inhibitor of Na$^+$-K$^+$-Cl$^-$ cotransporter, bumetanide (100 $\mu$M) significantly inhibited the basal current in AT-CF cells from 20.74 ± 1.4 $\mu$A/cm$^2$ to 5.6 ± 0.79 and 8.78 ± 2.3 $\mu$A/cm$^2$, respectively (Fig. 2). The inhibitor of adenylate cyclase, MDL12330A (20 $\mu$M), and Ca$^{2+}$-dependent Cl$^-$ channel (CaCC) blocker, DIDS (100 $\mu$M, added to apical membrane) significantly reduced the basal current in AT-CF cells from 17.56 ± 1.16 $\mu$A/cm$^2$ to 10.58 ± 1.85 and 12.13 ± 1.55 $\mu$A/cm$^2$, respectively, which indicated that both cAMP- and Ca$^{2+}$-dependent Cl$^-$ channels were activated at the basal condition.

### 3.2. ATP-induced $I_{SC}$ increase in VT-CF and AT-CF cells

Fig. 3 shows the ATP-induced $I_{SC}$ responses in VT-CF and AT-CF cells. Apical application of ATP (10 $\mu$M) stimulated a fast transient $I_{SC}$ increase (29.7 ± 1.5 $\mu$A/cm$^2$) in VT-CF cells (Fig. 3a), which was similar to that previously observed in ATP-induced and Ca$^{2+}$-dependent $I_{SC}$ in non-transfected CFPAC-1 cells (Cheng et al., 1999). However, a more sustained rise with slower decline was observed in AT-CF cells (Fig. 3b) with the transient peak reduced, 12.7 ± 1.3 $\mu$A/cm$^2$, but the total charges transported, the area under curve (1413 ± 159 $\mu$C/cm$^2$), were approximately 4 times larger than that (473.3 ± 81 $\mu$C/cm$^2$) observed in VT-CF cells.

![Fig. 2. Effects of Cl$^-$ channel blockers and transporter inhibitor on basal current in NYD-SP27 down-regulated AT-CF cells. (a) Effect of Glibenclamide/DPC (Cl$^-$ channel blockers, 1 mM, $n = 11$) and Bumetanide (Na$^+$-K$^+$-Cl$^-$ cotransporter inhibitor, 100 $\mu$M, $n = 4$). (b) Effect of MDL12330A (adenylate cyclase inhibitor, 20 $\mu$M, $n = 5$), and DIDS (CaCC blocker, 100 $\mu$M, $n = 5$). *$P < 0.05$, ***$P < 0.001$.](image1)

![Fig. 3. ATP-induced $I_{SC}$ response in VT-CF and AT-CF cells. Representative ATP (10 $\mu$M)-induced $I_{SC}$ response in VT-CF (a) and AT-CF cells (b). Comparison of the ATP-induced transient peak of $I_{SC}$ (c) and the transferred total charges (d) between VT-CF ($n = 4$) and AT-CF cells ($n = 6$). **$P < 0.01$, ***$P < 0.001$.](image2)
3.3. Involvement of Ca\(^{2+}\) and PKC in the ATP-enhanced \(I_{SC}\) response

After pretreatment of AT-CF cells with BAPTA-AM (50 μM, \(n = 4\)) for 50 min to chelate intracellular Ca\(^{2+}\), the ATP-induced \(I_{SC}\) response was reduced by 77.9%, from 2750 ± 94.7 to 606.7 ± 214.3 μC/cm\(^2\) (Fig. 4). PMA (1 μM), a PKC activator, inhibited ATP-stimulated fast current peak from 29.7 ± 1.5 to 11.3 ± 2.3 μA/cm\(^2\) (Fig. 5a) and total charges transferred from 473.3 ± 81.0 to 124.4 ± 25.5 μC/cm\(^2\) (Fig. 5b) with a total reduction of more than 70% in VT-CF cells (\(n = 4\), \(P < 0.001\)). However, PMA had no effect on the ATP-induced \(I_{SC}\) response in AT-CF cells (\(n = 6\), Fig. 5a,b). In contrast, 32.5% of the ATP-induced \(I_{SC}\) in AT-CF cells was inhibited by PKC inhibitor, staurosporine (40 μM, \(n = 5\), Fig. 6), indicating significant involvement of PKC in the ATP-induced \(I_{SC}\) response in AT-CF cells.

4. Discussion

As demonstrated in the previous study (Zhu et al., 2003), NYD-SP27 appears to be a negative regulator of PLC-coupled PKC and Ca\(^{2+}\) activity, an action that has not been described for any of the PLC isoforms known to date, although a negative regulator of PLC-δ-type isoforms with demonstrated inhibitory effect on IP\(_3\) activity has been reported (Nagano et al., 1999). After suppression of NYD-SP27 by transfection of its antisense into human cystic-fibrosis pancreatic-duct cells, PLC-coupled calcium release and protein kinase C activity were shown to be increased, which resulted in the rescue of defective cAMP-dependent pancreatic duct anion secretion in cystic fibrosis cells (Zhu et al., 2003). However, the effect of NYD-SP27 down-regulation on the Ca\(^{2+}\)-dependent pancreatic duct anion secretion was not investigated. In this study, NYD-SP27 down-regulated AT-CF cells exhibit a higher basal anion secretion than that observed in VT-CF cells, which was shown to be inhibited by inhibitors either inhibiting the cAMP pathway (MDL123) or blocking the Ca\(^{2+}\)-activated Cl\(^{-}\) channel (DIDS), indicating that both pathways were enhanced by NYD-SP27 down-regulation even at basal condition.

Extracellular ATP has been shown to stimulate Ca\(^{2+}\)-dependent pancreatic duct anion secretion with a biphasic current kinetic (Chan et al., 1996) similar to that observed in the present study in VT-CF cells. VT-CF cells also exhibit similar sensitivity to PKC activator, PMA, with reduction in the ATP-induced \(I_{SC}\) response consistent with the previously demonstrated inhibition of the Ca\(^{2+}\)-dependent pancreatic anion secretion by activation of PKC (Cheng et al., 1999). These results suggest that transfection of empty vector in CF cells does not alter the secretory characteristic of the cells. However, transfection of NYD-SP27 antisense, or its expression down-regulation, appears to affect the ATP-induced \(I_{SC}\) response in AT-CF cells.

Firstly, the ATP-induced fast transient current peak in AT-CF cells is reduced as compared to the control VT-CF cells. This can be explained by the previously demonstrated...
enhancement of PKC activity due to down-regulation of NYD-SP27. The ATP-induced transient peak in pancreatic duct cells has been shown to be dependent on intracellular Ca\textsuperscript{2+}, which can be inhibited by PKC (Cheng et al., 1999). Since NYD-SP27 is a PLC inhibitor and its down-regulation has been shown to upregulate PKC (Zhu et al., 2003), the reduction in the ATP-induced transient peak in AT-CF cells may be a result from PKC enhancement. In fact, further administration of PKC activator, PMA, to AT-CF cells did not produce additional effect on the first peak (Fig. 5a), indicating that PKC activity in the AT-CF cells may have been maximally activated by down-regulation of NYD-SP27.

Secondly, the ATP-induced sustained current peak in NYD-SP27 down-regulated AT-CF cells is enhanced as compared to VT-CF controls (Fig. 3d). Our previous study has demonstrated that this NYD-SP27 down-regulation-induced peak is mediated by cAMP-dependent CFTR. In CF cells, mutation of CFTR results in the inability of CFTR to reach the plasma membrane. However, when NYD-SP27 is down-regulated, increased PLC-coupled Ca\textsuperscript{2+} release and PKC activity can enhance CFTR trafficking as well as CFTR activation, respectively. In this study, the ATP-induced sustained current in AT-CF cells can be significantly reduced by the treatment with BAPTA-AM, a Ca\textsuperscript{2+} chelator, and a PKC inhibitor, staurosporine (Fig. 4b), confirming the involvement of both Ca\textsuperscript{2+} and PKC in mediating the ATP-induced pancreatic anion secretion.

While the Ca\textsuperscript{2+} involvement is well known in the ATP-induced response (Chan et al., 1996), the role of PKC, which has been shown to be inhibitory in pancreatic anion secretion (Cheng et al., 1999), appears to be stimulatory in NYD-SP27 down-regulated CF cells probably due to PKC activation of CFTR whose trafficking to the plasma membrane has been previously shown to be enhanced by ATP in NYD-SP27 down-regulated CF cells (Zhu et al., 2003). In fact, long term treatment of the NYD-SP27 down-regulated CF cells with PKC activator, PMA, a treatment known to suppress CFTR expression, significantly reduced the ATP-induced response (data not shown), consistent with the involvement of CFTR. Thus, the involvement of PKC and CFTR, which is not observed in the empty vector-transfected CF cells, explains the alteration in the ATP-induced current kinetics in the NYD-SP27 down-regulated CF cells.

In summary, the alteration in the current kinetics of ATP-induced Ca\textsuperscript{2+}-dependent pancreatic anion secretion and its sensitivity to PKC inhibitor in the NYD-SP27 down-regulated CF cells indicate that NYD-SP27, as an inhibitory isoform of PLC, plays a role in regulating Ca\textsuperscript{2+} and PKC activity and thereby modulating pancreatic anion secretion. The ability of NYD-SP27 to modulate both cAMP-dependent, as previously demonstrated, and Ca\textsuperscript{2+}-dependent, as demonstrated here, pancreatic anion secretion suggests that it may be a new therapeutic target for the treatment of pancreatic insufficiency including cystic fibrosis.

Acknowledgements

This work was supported by the Strategic Program of The Chinese University of Hong Kong.

References


