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Biochimica et Biophysica Acta 1418 (1999) 31–38

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Modulation of Ca^{2+}-dependent anion secretion by protein kinase C in normal and cystic fibrosis pancreatic duct cells

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Received 28 September 1998; received in revised form 6 January 1999; accepted 22 January 1999

Abstract

The study investigated the role of protein kinase C (PKC) in the modulation of agonist-induced Ca^{2+}-dependent anion secretion by pancreatic duct cells. The short-circuit current (I_{SC}) technique was used to examine the effect of PKC activation and inhibition on subsequent ATP, angiotensin II and ionomycin-activated anion secretion by normal (CAPAN-1) and cystic fibrosis (CFPAC-1) pancreatic duct cells. The I_{SC} responses induced by the Ca^{2+}-mobilizing agents, which had been previously shown to be attributed to anion secretion, were enhanced in both CAPAN-1 and CFPAC-1 cells by PKC inhibitors, staurosporine, calphostin C or chelerythrine. On the contrary, a PKC activator, phorbol 12-myristate 13-acetate (PMA), was found to suppress the agonist-induced I_{SC} in CFPAC-1 cells and the ionomycin-induced I_{SC} in CAPAN-1 cells. An inactive form of PMA, 4αD-phorbol 12,13-didecanote (4αD), was found to exert insignificant effect on the agonist-induced I_{SC}, indicating a specific effect of PMA. Our data suggest a role of PKC in modulating agonist-induced Ca^{2+}-dependent anion secretion by pancreatic duct cells. Therapeutic strategy to augment Ca^{2+}-activated anion secretion by cystic fibrosis pancreatic duct cells may be achieved by inhibition or down-regulation of PKC.

Keywords: PMA; Cl^- secretion; Pancreatic duct; Cystic fibrosis; PKC

1. Introduction

The cAMP-dependent Cl^- secretion is defective in cystic fibrosis (CF), a common lethal genetic disease affecting 1 in 2500 of the Caucasian population [1]. Impaired bicarbonate secretion, as well as Cl^- secretion, underlies the defective fluid secretory response in the cystic fibrosis pancreas [2,3]. Obstruction of ducts by inspissated secretion followed by tissue destruction appears to be the cause of some diseases of the pancreas, including endocrine pancreatic failure and diabetes. Alternative activation pathways of Cl^- secretion have been proposed as the basis for therapeutic intervention, and it has been demonstrated that the Ca^{2+}-dependent Cl^- secretion is intact in a number of CF tissues, including the pancreatic duct cells [4,5]. Previous studies [6,7] have also shown that Ca^{2+}-mobilizing agents, ATP, AII as well as the Ca^{2+} ionophore, ionomycin, stimulate anion secretion across CFPAC-1 cells, a human CF pancreatic duct cell line that displays the CF defect – lacking cAMP-dependent Cl^- channel activation [8].

Protein kinase C (PKC) has been implicated in regulating Cl^- secretion in a number of epithelia [9–16]. However, the role of PKC in modulating epi-

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PII: S0005-2736(99)00011-5

thelial functions or responses has been controversial largely due to its diverse effects on numerous regulatory pathways. While PKC is believed to play a role in regulating cystic fibrosis transmembrane conductance regulator [15,17–20], which has been shown to be a cAMP-activated Cl^- channel [21], the effects of PKC on Ca^{2+}-activated Cl^- secretion [22–26], as well as volume-sensitive Cl^- current [27,28], have been noted. The existence of different regulatory pathways in epithelial cells presents a difficulty in elucidating the precise role of PKC in modulating individual regulatory pathway.

The present study has made use of a cell line displaying the CF phenotype, with defective cAMP-regulated but intact Ca^{2+}-dependent secretory response [8], to investigate the role of PKC in modulating Ca^{2+}-dependent pancreatic ductal secretion. We examined the effect of PKC on agonist-induced Ca^{2+}-dependent anion secretion by CFPAC-1 cells and compared it to that by a normal pancreatic cell line CAPAN-1 which has been shown to conserve most of the properties of ductal epithelial cells [29,30] and possess apical cAMP-dependent Cl^- channels which are crucial to pancreatic ductal HCO_3^- secretion [31,32]. Pancreatic cells were grown on semi-permeable membranes and mounted in Ussing chambers. Electrogenic anion secretion was reflected by the short-circuit current (I_{SC}) measured. Our results indicated that PKC attenuates Ca^{2+}-dependent agonist-induced I_{SC} in both normal and CF cells and that augmentation of the agonist-induced I_{SC} in CF cells by PKC inhibitors may have therapeutic potentials.

2. Materials and methods

2.1. Materials

Hank's balanced salt solution (HBSS) was purchased from Sigma (St. Louis, MO, USA). Iscove's modified Dulbecco's medium, RPMI 1640 medium and fetal bovine serum (FBS), trypsin-EDTA were supplied by Gibco (New York, USA).

The following drugs were supplied by Sigma (St. Louis, MO, USA): *N*-methyl-D-glucamine (NMDG), calcium gluconate, *N*-2-hydroxethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), staurosporine, calphostin C, adenosine 5′-triphosphate (ATP), 4αD-phorbol 12,13-didecanote (4αD), ionomycin, Hank's balanced salt solution (HBSS), sodium pyruvate and trypsin. Chelerythrine was purchased from Calbiochem Novabiochem (California, USA). Phorbol 12-myrisate 13-acetate (PMA) was from RBI (Natick, MA, USA).

2.2. Cell culture

Normal and CF pancreatic duct cell lines, CAPAN-1 and CFPAC-1, were purchased from American Type Culture Collection (Maryland, USA). Culture procedure for CFPAC-1 cells, grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS, has been described previously [6]. CAPAN-1 cells were grown in RPMI 1640 medium with 15% FBS. When cells were disassembled from the culture flask, 0.25% trypsin-EDTA was added with extra care to avoid striking on cell layer directly. Quickly afterwards, less than 1 min, most of the trypsin was removed leaving about 0.5 ml in the flask which was then incubated for 2–3 min. Cells were then resuspended in serum-containing medium with gentle pipetting of the cells to break up the cell aggregations. The suspension was then transferred into a centrifuge tube for spinning at $800\times g$ for 5 min to remove any trypsin left. Supernatant was discarded and the cells were resuspended with desirable volume of medium to make up to a final cell concentration of 1.5×10^6/ml. A volume of 0.25 ml of the cell suspension was then plated onto each permeable support (area of 0.45 cm^2) floating on culture medium and incubated at 37°C with 5% CO_2/95% O_2 for 4–5 days before I_{SC} experiments.

2.3. Short-circuit current measurement

The basic principles of the short-circuit current experiments performed in the present study was the same as previously described [33]. Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber and bathed in Krebs–Henseleit (K–H) solutions with following composition (mM): NaCl, 117; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24.8; $CaCl_2$, 2.56; glucose, 11.1; with an osmolarity of 285 mOsm gassed with 95% O_2 and 5% CO_2.

All the electrodes were connected to the voltage–current clamp amplifier (DVC-1000, World Precision Instrument, Sarasota, USA). The signal output from the amplifier was the I_{SC} measured and was recorded on-line by the use of chart-recorder (Kipp and Zonen, Delft, Netherlands). A 0.1-mV voltage pulse was applied intermittently across the epithelium and the transepithelial conductance was calculated from the corresponding current changes.

PMA, ionomycin and staurosporine, chelerythrine and 4αD were dissolved in DMSO. To eliminate the solvent effect, control experiments were performed with equal amounts of DMSO added.

2.4. Statistical analysis

Results are expressed as mean ± S.E.M. Comparisons between groups of data were carried out using Student's unpaired *t*-test. A *P*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of PKC activator and inhibitor on agonist-induced I_{SC} in CF cells

AII (5 μM), one of the Ca^{2+}-mobilizing agonists, induced a transient rise in I_{SC} in CFPAC-1 cells (Fig. 1, upper panel). Its stimulating effect was suppressed by a PKC activator, PMA (100 nM), from the control value of 2.5 ± 0.3 to 0.6 ± 0.1 μA/cm^2 ($P < 0.01$) with a total reduction of 76.5% ($n = 8$, Fig. 1, upper panel). On the contrary, the AII-induced I_{SC} was increased by a PKC inhibitor, staurosporine (10 μM), from 2.5 ± 0.3 to 5.4 ± 0.4 μA/cm^2 ($P < 0.01$) with a total increase of 116.9% ($n = 7$, Fig. 1, upper panel). To eliminate the possibility of non-specific effect of staurosporine, the effect of another potent PKC inhibitor, calphostin C [34], was also examined. Similar enhancing effect on the AII-induced I_{SC} was observed (not shown), further indicating the involvement of PKC. Fig. 1 (lower panel) shows the summary of the effects of PMA and staurosporine.

Similar to its inhibitory effect on the AII-induced I_{SC}, PMA was also found to suppress the ATP-induced I_{SC} in CFPAC-1 cells, from 9.2 ± 0.9 ($n = 6$) to 2.7 ± 0.5 μA/cm^2 ($n = 4$, $P < 0.01$) with a total reduction of 71% (Fig. 2). Calphostin C was found to potentiate the ATP-induced I_{SC} by 64%, from 9.2 ± 0.9 to 15.1 ± 1.2 μA/cm^2 ($n = 4$, $P < 0.01$, Fig. 2). A similar potentiating effect on the ATP-induced I_{SC} with a 56% increase was also observed for chelerythrine, another potent and specific PKC inhibitor [35] as shown in Fig. 3.

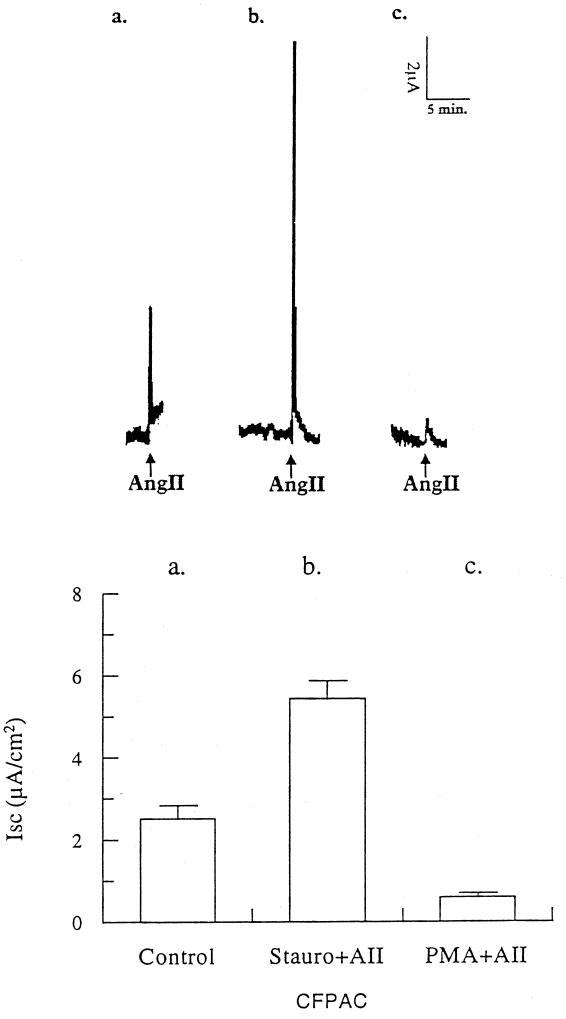

Fig. 1. Effect of PKC inhibitor and activator on the angiotensin II-stimulated I_{SC} in CFPAC-1 cells. Upper panel: I_{SC} response to AII (5 μM) in control (a), staurosporine (10 μM)-treated (b) and PMA (0.1 μM)-treated cells (c). Staurosporine and PMA were added 5 min prior to addition of AII (the same for other figures). Lower panel: summary of corresponding results. Staurosporine and PMA are PKC inhibitor and activator, respectively. Potentiating effect was observed with staurosporine while inhibitory effect with PMA. Each *P*-value was obtained when compared to the control. Number of experiments for each column ranges from five to eight.

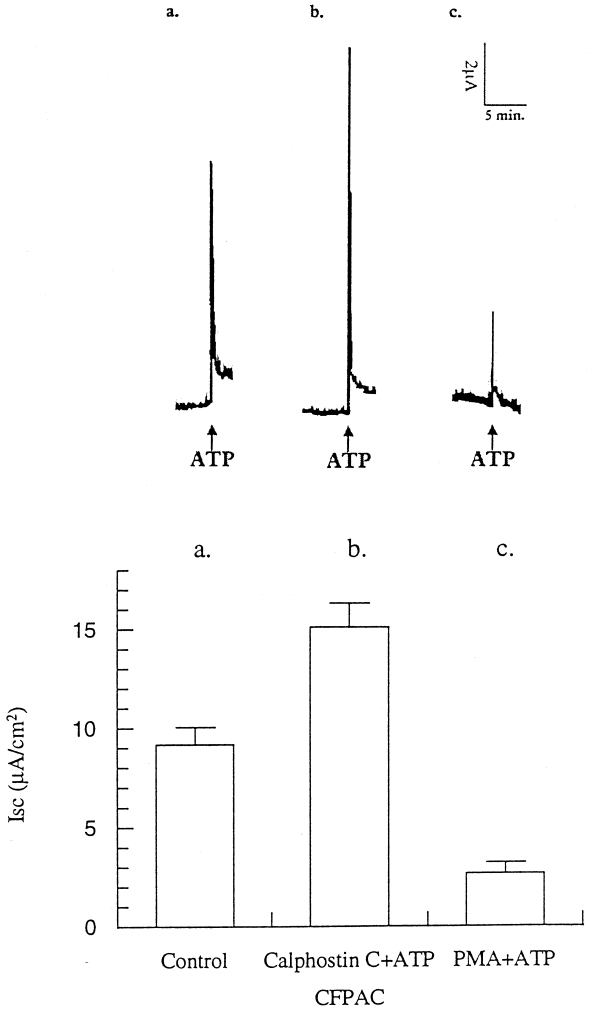

Fig. 2. Effect of PKC inhibitor and activator on the ATP-stimulated I_{SC} in CFPAC-1 cells. Upper panel: I_{SC} response to ATP (10 μM) in control (a), calphostin C (10 nM)-treated (b) and PMA (0.1 μM)-treated cells (c). Lower panel: summary of corresponding results. Calphostin C is another PKC inhibitor. Number of experiments ranges from four to six.

To confirm a role of PKC in modulating the Ca^{2+}-dependent anion secretion, the effects of PMA and staurosporine on the I_{SC} induced by a Ca^{2+} ionophore, ionomycin (10 μM), were also examined. In contrast to AII and ATP, ionomycin induced a biphasic I_{SC} response with a transient increase followed by a more sustained increase (Fig. 4). PMA was found to suppress the ionomycin-induced I_{SC} by 91%, from 14.2 ± 1.3 to 1.3 ± 0.4 ($n=7$, $P<0.01$, Fig. 4), while staurosporine enhanced it by 83%, from 13.2 ± 0.7 ($n=6$) to 24.1 ± 2.1 μA/cm^2 ($n=4$, $P<0.01$, Fig. 5). The specificity of PMA was tested by examining the effect of its inactive analog, 4αD (1 μM), on the ionomycin-induced I_{SC}. As shown in Fig. 6, 4αD exerted insignificant effect on the ionomycin-induced I_{SC}, excluding any non-specific effect of PMA.

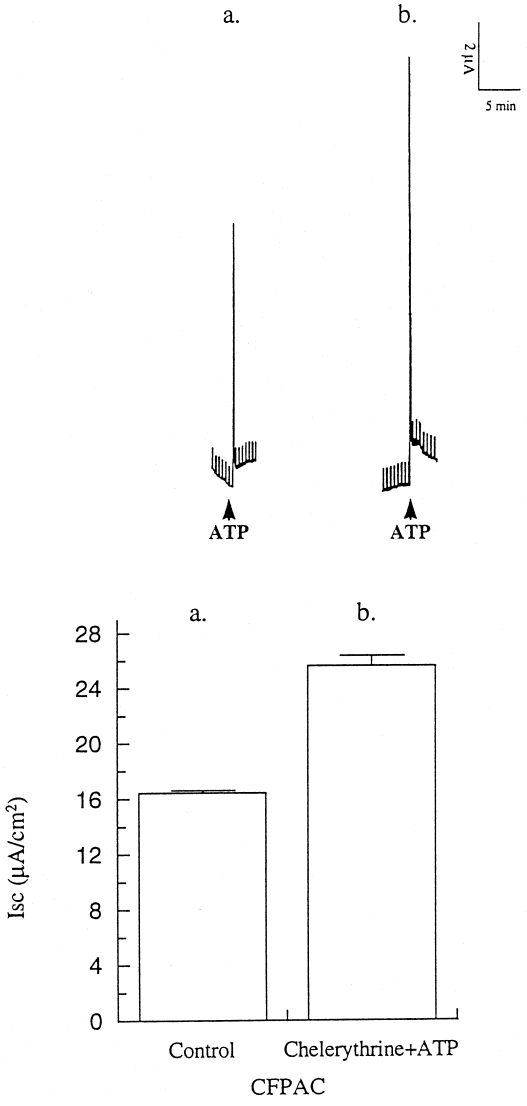

Fig. 3. Effect of chelerythrine on the ATP-stimulated I_{SC} in CFPAC-1 cells. Upper panel: I_{SC} response to ATP (10 μM) in control (a) and chelerythrine (10 nM)-treated cells (b). Lower panel: summary of corresponding results. Chelerythrine is a newly developed specific inhibitor of PKC. The potentiation of ATP-activated I_{SC} by chelerythrine mimicked the response in the presence of staurosporine or calphostin C, suggesting that inhibition of PKC indeed enhances Ca^{2+}-activated anion secretion. Data were obtained from six to eight individual experiments.

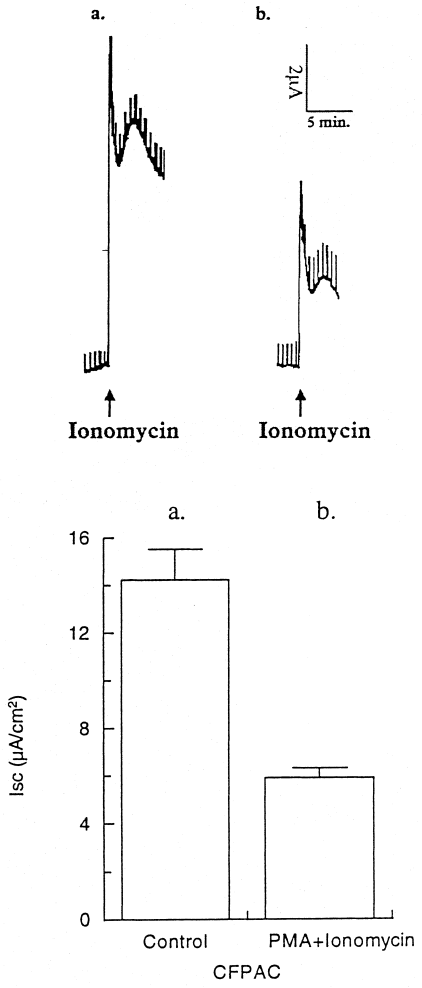

Fig. 4. Effect of PKC activator on the ionomycin-stimulated I_{SC} in CFPAC-1 cells. Upper panel: I_{SC} response to the Ca^{2+} ionophore, ionomycin (1 μM, $n=7$) in control (a) and PMA (0.1 μM, $n=9$)-treated cells (b). Lower panel: summary of corresponding results.

3.2. *Effects of PKC activator and inhibitor on agonist-induced I_{SC} in CAPAN-1 cells*

The effects of PMA and staurosporine on agonist-induced I_{SC} were also examined in normal pancreatic duct cells, CAPAN-1. AII was found to be ineffective in eliciting any I_{SC} response in these cells and, therefore, we were unable to assess the effect of PKC on this agonist-induced I_{SC}. On the contrary, ATP elicited a I_{SC} response in CAPAN-1 cells similar to that observed in CFPAC-1 cells in that they both were transient in nature (Fig. 7, upper panel). However, the time for the ATP-activated current to return to

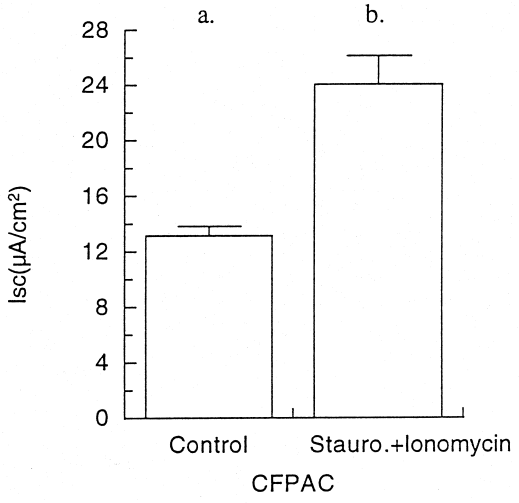

Fig. 5. Effect of PKC inhibitor on the ionomycin-stimulated I_{SC} in CFPAC-1 cells. I_{SC} response to the Ca^{2+} ionophore, ionomycin (1 μM) in control (a) and staurosporine (10 μM)-treated cells (b).

the basal level in CAPAN-1 cells was significantly longer. An averaged magnitude of 14.2 ± 1.0 μA/cm^2 ($n=9$) was observed for the ATP-induced I_{SC} in CAPAN-1 cells, which could be increased by staurosporine to 18.0 ± 2.0 μA/cm^{-2} ($n=4$, $P<0.01$, Fig. 7, lower panel) with a total increase of 27% ($n=4$). However, the effect of PMA on the ATP-induced I_{SC} was found to be insignificant in CAPAN-1 cells (Fig. 7).

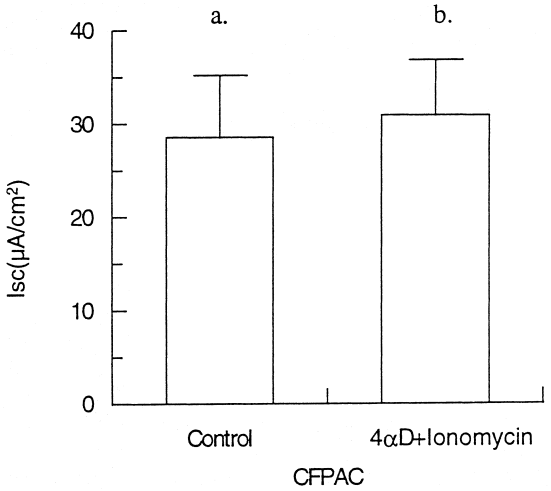

Fig. 6. Demonstration of PKC involvement in CFPAC-1 cells. I_{SC} response to the Ca^{2+} ionophore, ionomycin (1 μM, $n=4$) in control (a) and 4αD (1 μM, $n=7$)-treated cells (b). 4αD is an inactive structural analog of PMA. The ineffectiveness of 4αD on the ionomycin-activated I_{SC} suggests that the effect of PMA is specifically on PKC.

In contrast to the ionomycin-induced biphasic response observed in CFPAC-1 cells, the ionomycin-induced I_{SC} response in CAPAN-1 cells was transient with a time course similar to that elicited by ATP in CAPAN-1 cells (Fig. 8, upper panel). The ionomycin-induced I_{SC} in CAPAN-1 cells was enhanced by staurosporine, from 16.8 ± 2.3 ($n=6$) to 25.8 ± 1.1 μA/cm^2 ($n=7$, $P<0.01$) with an increase of 54%, but suppressed by PMA to 10.6 ± 1.9 μA/cm^2 ($n=7$, Fig. 8) with a 37% reduction.

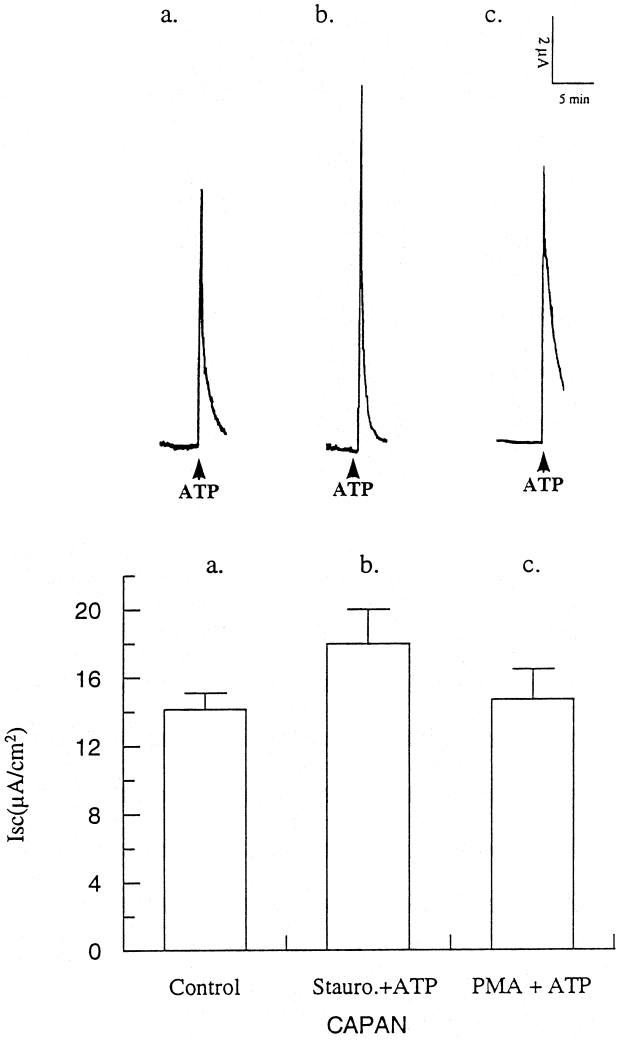

Fig. 7. Effect of PKC inhibitor and activator on the ATP-stimulated I_{SC} in normal pancreatic duct CAPAN-1 cells. Upper panel: I_{SC} response to ATP (10 μM, $n=6$) in control (a), staurosporine (20 μM, $n=7$)-treated (b) and PMA (0.1 μM, $n=7$)-treated cells (c). Lower panel: summary of corresponding results.

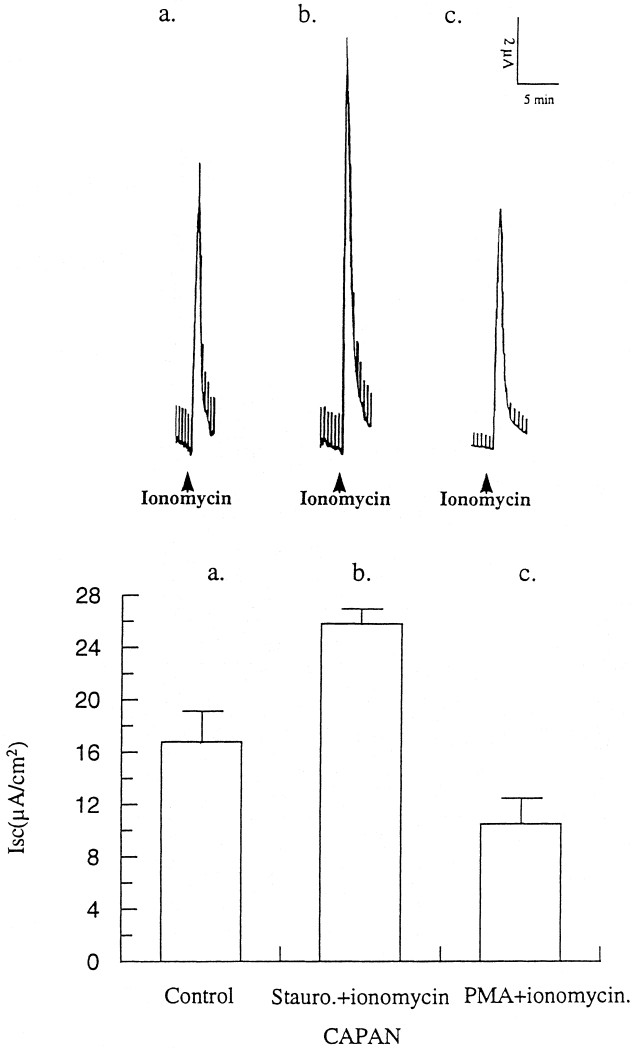

Fig. 8. Effect of PKC inhibitor and activator on the ionomycin-stimulated I_{SC} in normal pancreatic duct CAPAN-1 cells. Upper panel: I_{SC} response to the Ca^{2+} ionophore, ionomycin (1 μM, $n=9$) in control (a), staurosporine (20 μM, $n=4$)-treated (b) and PMA (0.1 μM, $n=7$)-treated cells (c). Lower panel: summary of corresponding results.

4. Discussion

As described in our previous studies [6,36], both CFPAC-1 and CAPAN-1 cells exhibit a very small basal current which was not altered significantly by different agents examined. Therefore, the present study focused on the agonist-induced I_{SC}. The effect of AII, ATP as well as ionomycin on anion secretion by CFPAC-1 cells has been studied previously and the involvement of Ca^{2+}-dependent mechanism in these agonist-induced responses demonstrated [6,7].

The present study further indicates the presence a Ca^{2+}-dependent pathway in both normal and CF pancreatic cells; however, the agonist-induced I_{SC} responses in these two cell lines were different in a number of aspects. First, the ATP-induced I_{SC} response in CAPAN-1 cells lasted significantly longer than that observed in CF cells. Secondly, the ionomycin-induced I_{SC} response in CF cells was biphasic, while only the transient response was observed in CAPAN-1 cells. Finally, the AII-induced response was only observed in CF but not CAPAN-1 cells. However, it is difficult to assess whether these differences truly reflect the differences between normal and CF pancreatic cells. It is possible that the Ca^{2+}-dependent machinery, e.g. receptors, intracellular Ca^{2+} pools, in these cell lines may be altered to different extents through the process of transformation.

Despite the apparent differences in the Ca^{2+}-dependent responses in the two cell lines, a role of PKC in modulating these responses has been demonstrated in the present study. It is shown that PKC activator, PMA, exerts an inhibitory effect on these agonist-induced I_{SC} responses in CFPAC-1 cells. On the other hand, inhibitor of PKC, staurosporine, enhances the I_{SC} responses. The involvement of PKC in modulating agonist-induced I_{SC} is indicated by the observations that the inhibitory effect of PMA on the agonist-induced I_{SC} was not observed using an inactive analog of PMA, 4αD, excluding the non-specific action of PMA. The enhancing effect of staurosporine was also mimicked by specific PKC inhibitors, calphostin C and chelerythrine, further indicating the involvement of PKC. Since the cAMP-dependent pathway is defective in CFPAC-1 cells, a role of PKC in modulating the Ca^{2+}-activated anion secretion is thus clearly demonstrated. Interestingly, similar inhibitory and enhancing effect on the ionomycin-induced I_{SC} was also observed with PKC activator and inhibitor, respectively, in CAPAN-1 cells which possess both the cAMP and Ca^{2+}-dependent pathways. These results suggest an inhibitory role of PKC in Ca^{2+}-dependent pancreatic ductal secretion. PKC could attenuate the Ca^{2+}-activated ductal secretion and thereby serve as a turn-off mechanism. This may be achieved by modulating intracellular Ca^{2+} store [37] or inactivating the Ca^{2+}-activated K^+ channels [38] on which Cl^- secretion largely depends. In contrast to the previously observed defective regulation of Cl^- secretion by PKC in CF cells [10,13,14], the modulation of Ca^{2+}-dependent anion secretion by PKC appears to operate normally in CF pancreatic duct cells.

The presently observed modulatory effect of PKC on AII and ATP-stimulated anion secretion may be of physiological significance since these agonists have been implicated in the regulation of pancreatic ductal secretion. Several key components of the renin–angiotensin system (RAS) has been demonstrated in the canine pancreas [39]. Immunohistochemical localization and distribution of AII as well as its receptors in the pancreas of rodents have been reported [40,41]. Our previous studies have also demonstrated the presence of AII receptor subtypes as well as the effect of AII on exocrine secretion in CFPAC-1 cells [7]. Taken together, pancreatic ductal secretion may be regulated by a local tissue RAS. On the other hand, release of ATP and/or ADP to activate P_2 purinoceptors has been implicated in pancreatic islets to increase the magnitude of insulin response to glucose stimulation [42]. It is possible that ATP released by the acinar cells may also influence ductal cell function. A recently elucidated autocrine mechanism of ATP in regulating anion secretion in numerous epithelial tissues [43] further supports a role of ATP in the regulation of pancreatic ductal secretion [6]. The fact that AII and ATP-stimulated anion secretion are modulated by PKC suggests that PKC may be important in the fine-tuning of Ca^{2+}-dependent pancreatic ductal secretion. The potentiating effect exerted by PKC inhibitors on the agonist-induced anion secretion suggests that PKC may be tonically activated in the duct cells or that it may be partially activated by AII and ATP, most likely via a Ca^{2+}-dependent mechanism. The augmentation of the Ca^{2+}-dependent anion secretion in CF cells by inhibition of PKC may have therapeutic implication since the Ca^{2+}-dependent pathway has been proposed as an alternative pathway for circumventing the defective cAMP-dependent anion secretion in CF cells. A recent clinical trial on CF patients using Ca^{2+} mobilizing agent, uridine 5′-triphosphate, has been conducted and shown to be effective [44].

In summary, the present study has demonstrated a role of PKC in modulating the Ca^{2+}-dependent anion secretion in pancreatic duct cells. Augmentation of anion secretion in CF cells could be achieved

by inhibition or down-regulation of PKC. The signaling mechanism involved in mediating the effect of PKC on Ca^{2+}-dependent anion secretion is currently under investigation.

Acknowledgements

The work was supported by the RGC of Hong Kong and New Asia College of the Chinese University of Hong Kong.

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