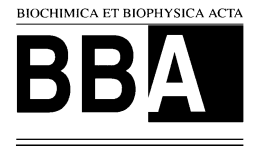




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## Modulation of $\text{Ca}^{2+}$ -dependent anion secretion by protein kinase C in normal and cystic fibrosis pancreatic duct cells

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### Abstract

The study investigated the role of protein kinase C (PKC) in the modulation of agonist-induced  $\text{Ca}^{2+}$ -dependent anion secretion by pancreatic duct cells. The short-circuit current ( $I_{\text{SC}}$ ) 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  $I_{\text{SC}}$  responses induced by the  $\text{Ca}^{2+}$ -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  $I_{\text{SC}}$  in CFPAC-1 cells and the ionomycin-induced  $I_{\text{SC}}$  in CAPAN-1 cells. An inactive form of PMA, 4 $\alpha$ D-phorbol 12,13-didecanote (4 $\alpha$ D), was found to exert insignificant effect on the agonist-induced  $I_{\text{SC}}$ , indicating a specific effect of PMA. Our data suggest a role of PKC in modulating agonist-induced  $\text{Ca}^{2+}$ -dependent anion secretion by pancreatic duct cells. Therapeutic strategy to augment  $\text{Ca}^{2+}$ -activated anion secretion by cystic fibrosis pancreatic duct cells may be achieved by inhibition or down-regulation of PKC. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** PMA;  $\text{Cl}^-$  secretion; Pancreatic duct; Cystic fibrosis; PKC

### 1. Introduction

The cAMP-dependent  $\text{Cl}^-$  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  $\text{Cl}^-$  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  $\text{Cl}^-$  secretion have been proposed as the basis for therapeutic intervention, and it has been demonstrated that the  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretion is intact in a number of CF tissues, including the pancreatic duct cells [4,5]. Previous studies [6,7] have also shown that  $\text{Ca}^{2+}$ -mobilizing agents, ATP, AII as well as the  $\text{Ca}^{2+}$  ionophore, ionomycin, stimulate anion secretion across CFPAC-1 cells, a human CF pancreatic duct cell line that displays the CF defect – lacking cAMP-dependent  $\text{Cl}^-$  channel activation [8].

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

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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  $\text{Cl}^-$  channel [21], the effects of PKC on  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  secretion [22–26], as well as volume-sensitive  $\text{Cl}^-$  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  $\text{Ca}^{2+}$ -dependent secretory response [8], to investigate the role of PKC in modulating  $\text{Ca}^{2+}$ -dependent pancreatic ductal secretion. We examined the effect of PKC on agonist-induced  $\text{Ca}^{2+}$ -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  $\text{Cl}^-$  channels which are crucial to pancreatic ductal  $\text{HCO}_3^-$  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 ( $I_{\text{SC}}$ ) measured. Our results indicated that PKC attenuates  $\text{Ca}^{2+}$ -dependent agonist-induced  $I_{\text{SC}}$  in both normal and CF cells and that augmentation of the agonist-induced  $I_{\text{SC}}$  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-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), staurosporine, cal-

phostin C, adenosine 5'-triphosphate (ATP), 4 $\alpha$ -phorbol 12,13-didecanote (4 $\alpha$ D), ionomycin, Hank's balanced salt solution (HBSS), sodium pyruvate and trypsin. Chelerythrine was purchased from Calbiochem Novabiochem (California, USA). Phorbol 12-myristate 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  $1.5\times 10^6$ /ml. A volume of 0.25 ml of the cell suspension was then plated onto each permeable support (area of  $0.45\text{ cm}^2$ ) floating on culture medium and incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ /95%  $\text{O}_2$  for 4–5 days before  $I_{\text{SC}}$  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;  $\text{MgSO}_4$ , 1.2;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{NaHCO}_3$ , 24.8;  $\text{CaCl}_2$ , 2.56; glucose, 11.1; with an osmolarity of 285 mOsm gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

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 4 $\alpha$ D 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  $\pm$  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 CFAC cells

AII (5  $\mu$ M), one of the Ca<sup>2+</sup>-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  $2.5 \pm 0.3$  to  $0.6 \pm 0.1$   $\mu$ A/cm<sup>2</sup> ( $P < 0.01$ ) 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  $\mu$ M), from  $2.5 \pm 0.3$  to  $5.4 \pm 0.4$   $\mu$ A/cm<sup>2</sup> ( $P < 0.01$ ) 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  $9.2 \pm 0.9$  ( $n = 6$ ) to  $2.7 \pm 0.5$   $\mu$ A/cm<sup>2</sup> ( $n = 4$ ,  $P < 0.01$ ) with a total reduc-

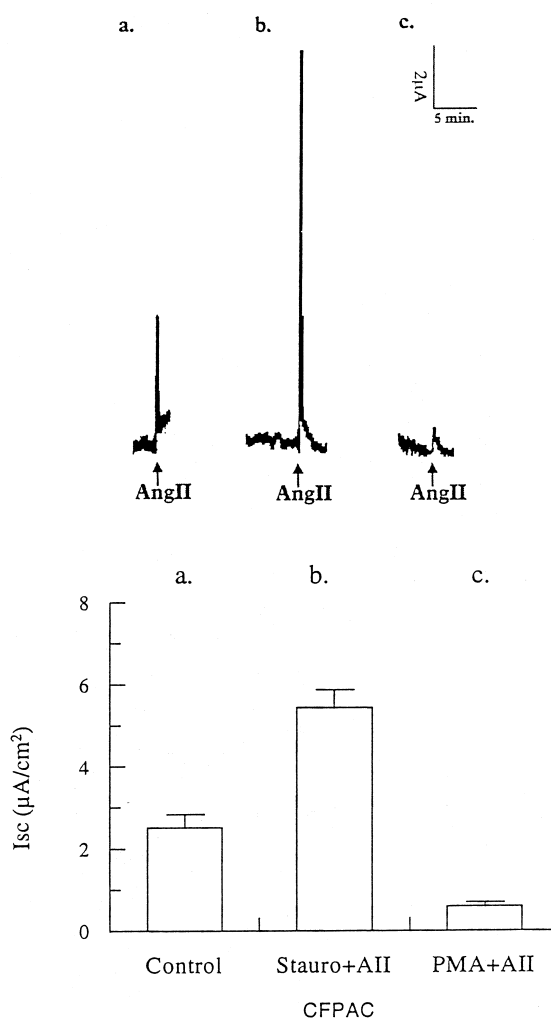


Fig. 1. Effect of PKC inhibitor and activator on the angiotensin II-stimulated  $I_{SC}$  in CFPAC-1 cells. Upper panel:  $I_{SC}$  response to AII (5  $\mu$ M) in control (a), staurosporine (10  $\mu$ M)-treated (b) and PMA (0.1  $\mu$ M)-treated cells (c). Staurosporine and PMA were added 5 min prior 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.

tion of 71% (Fig. 2). Calphostin C was found to potentiate the ATP-induced  $I_{SC}$  by 64%, from  $9.2 \pm 0.9$  to  $15.1 \pm 1.2$   $\mu$ A/cm<sup>2</sup> ( $n = 4$ ,  $P < 0.01$ , 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.

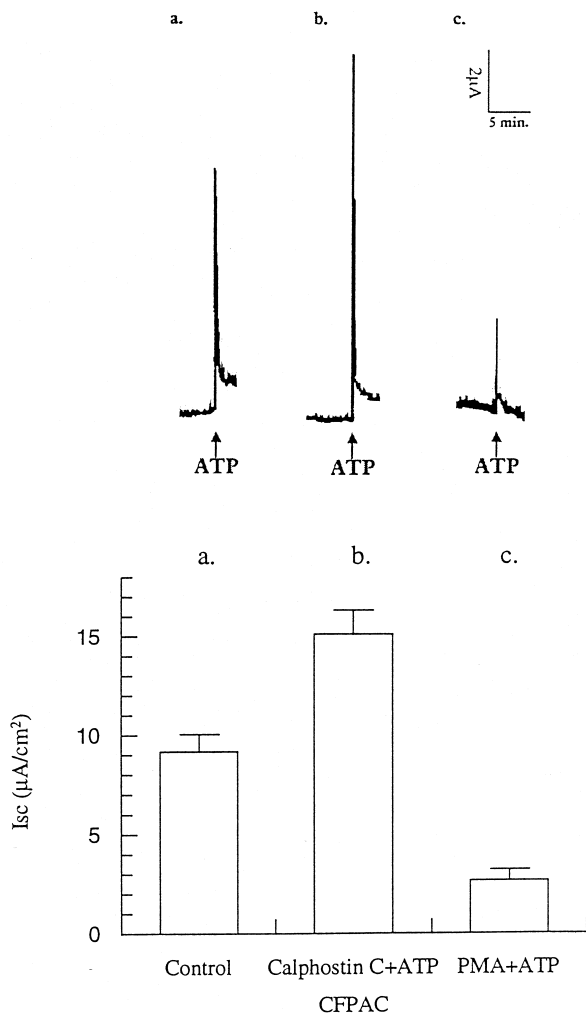


Fig. 2. Effect of PKC inhibitor and activator on the ATP-stimulated  $I_{sc}$  in CFPAC-1 cells. Upper panel:  $I_{sc}$  response to ATP (10  $\mu$ M) in control (a), calphostin C (10 nM)-treated (b) and PMA (0.1  $\mu$ M)-treated cells (c). Lower panel: summary of corresponding results. Calphostin C 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  $\mu$ M), 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  $14.2 \pm 1.3$  to  $1.3 \pm 0.4$  ( $n=7$ ,  $P<0.01$ , Fig. 4), while staurosporine enhanced it by 83%, from  $13.2 \pm 0.7$  ( $n=6$ ) to  $24.1 \pm 2.1$   $\mu$ A/ $cm^2$  ( $n=4$ ,  $P<0.01$ , Fig. 5). The specificity of PMA was tested

by examining the effect of its inactive analog,  $4\alpha$ D (1  $\mu$ M), on the ionomycin-induced  $I_{sc}$ . As shown in Fig. 6,  $4\alpha$ D exerted insignificant effect on the ionomycin-induced  $I_{sc}$ , excluding any non-specific effect of PMA.

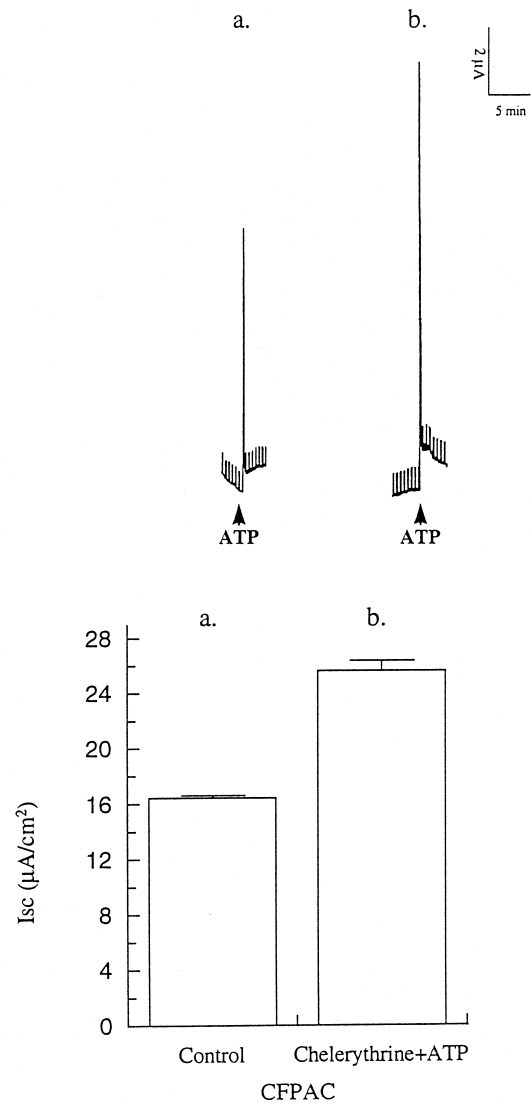


Fig. 3. Effect of chelerythrine on the ATP-stimulated  $I_{sc}$  in CFPAC-1 cells. Upper panel:  $I_{sc}$  response to ATP (10  $\mu$ M) in control (a) and chelerythrine (10 nM)-treated cells (b). Lower panel: summary of corresponding results. Chelerythrine is a newly developed specific inhibitor of 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.

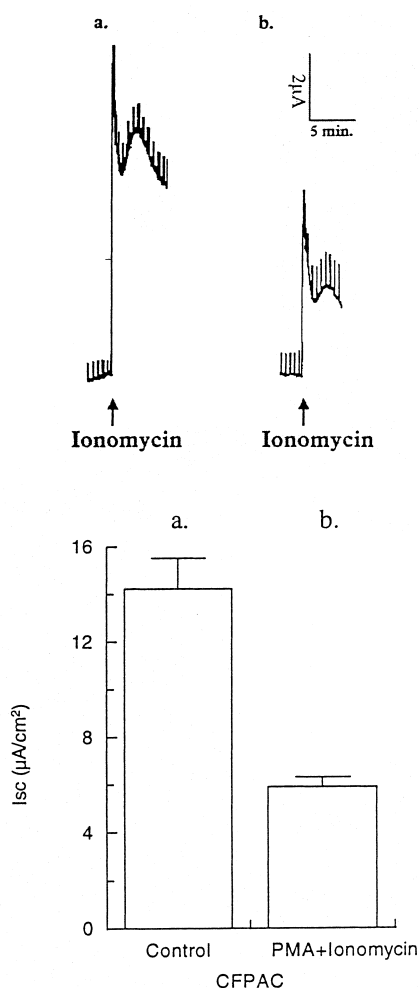


Fig. 4. Effect of PKC activator on the ionomycin-stimulated  $I_{sc}$  in CFPAC-1 cells. Upper panel:  $I_{sc}$  response to the  $Ca^{2+}$  ionophore, ionomycin (1  $\mu M$ ,  $n=7$ ) in control (a) and PMA (0.1  $\mu M$ ,  $n=9$ )-treated cells (b). Lower panel: summary of corresponding results.

### 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

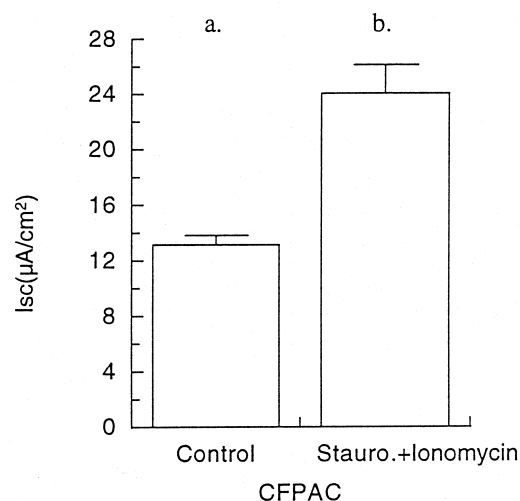


Fig. 5. Effect of PKC inhibitor on the ionomycin-stimulated  $I_{sc}$  in CFPAC-1 cells.  $I_{sc}$  response to the  $Ca^{2+}$  ionophore, ionomycin (1  $\mu M$ ) in control (a) and staurosporine (10  $\mu M$ )-treated cells (b).

the basal level in CAPAN-1 cells was significantly longer. An averaged magnitude of  $14.2 \pm 1.0 \mu A/cm^2$  ( $n=9$ ) was observed for the ATP-induced  $I_{sc}$  in CAPAN-1 cells, which could be increased by staurosporine to  $18.0 \pm 2.0 \mu A/cm^2$  ( $n=4$ ,  $P < 0.01$ , 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).

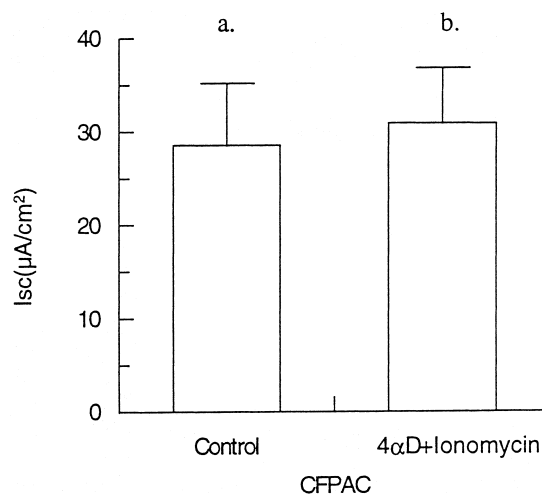


Fig. 6. Demonstration of PKC involvement in CFPAC-1 cells.  $I_{sc}$  response to the  $Ca^{2+}$  ionophore, ionomycin (1  $\mu M$ ,  $n=4$ ) in control (a) and 4 $\alpha$ D (1  $\mu M$ ,  $n=7$ )-treated cells (b). 4 $\alpha$ D is an inactive structural analog of PMA. The ineffectiveness of 4 $\alpha$ D 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  $16.8 \pm 2.3$  ( $n=6$ ) to  $25.8 \pm 1.1$   $\mu\text{A}/\text{cm}^2$  ( $n=7$ ,  $P < 0.01$ ) with an increase of 54%, but suppressed by PMA to  $10.6 \pm 1.9$   $\mu\text{A}/\text{cm}^2$  ( $n=7$ , Fig. 8) with a 37% reduction.

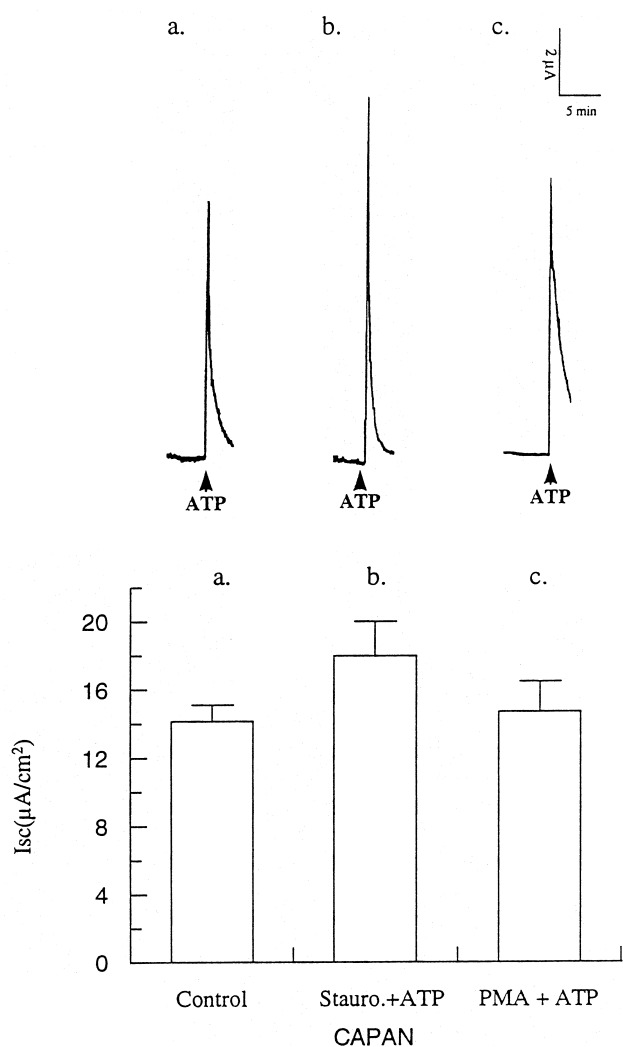


Fig. 7. Effect of PKC inhibitor and activator on the ATP-stimulated  $I_{SC}$  in normal pancreatic duct CAPAN-1 cells. Upper panel:  $I_{SC}$  response to ATP ( $10 \mu\text{M}$ ,  $n=6$ ) in control (a), staurosporine ( $20 \mu\text{M}$ ,  $n=7$ )-treated (b) and PMA ( $0.1 \mu\text{M}$ ,  $n=7$ )-treated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

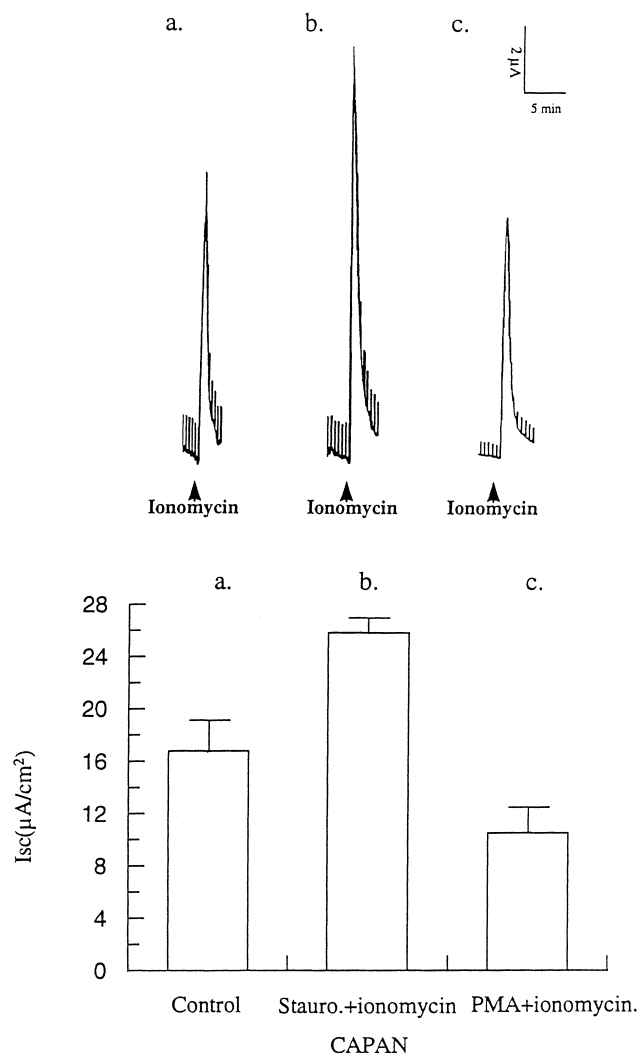


Fig. 8. Effect of PKC inhibitor and activator on the ionomycin-stimulated  $I_{SC}$  in normal pancreatic duct CAPAN-1 cells. Upper panel:  $I_{SC}$  response to the  $\text{Ca}^{2+}$  ionophore, ionomycin ( $1 \mu\text{M}$ ,  $n=9$ ) in control (a), staurosporine ( $20 \mu\text{M}$ ,  $n=4$ )-treated (b) and PMA ( $0.1 \mu\text{M}$ ,  $n=7$ )-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  $\text{Ca}^{2+}$ -dependent mechanism in these agonist-induced responses demonstrated [6,7].

The present study further indicates the presence a  $\text{Ca}^{2+}$ -dependent pathway in both normal and CF pancreatic cells; however, the agonist-induced  $I_{\text{SC}}$  responses in these two cell lines were different in a number of aspects. First, the ATP-induced  $I_{\text{SC}}$  response in CAPAN-1 cells lasted significantly longer than that observed in CF cells. Secondly, the ionomycin-induced  $I_{\text{SC}}$  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  $\text{Ca}^{2+}$ -dependent machinery, e.g. receptors, intracellular  $\text{Ca}^{2+}$  pools, in these cell lines may be altered to different extents through the process of transformation.

Despite the apparent differences in the  $\text{Ca}^{2+}$ -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  $I_{\text{SC}}$  responses in CFPAC-1 cells. On the other hand, inhibitor of PKC, staurosporine, enhances the  $I_{\text{SC}}$  responses. The involvement of PKC in modulating agonist-induced  $I_{\text{SC}}$  is indicated by the observations that the inhibitory effect of PMA on the agonist-induced  $I_{\text{SC}}$  was not observed using an inactive analog of PMA, 4 $\alpha$ D, 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  $\text{Ca}^{2+}$ -activated anion secretion is thus clearly demonstrated. Interestingly, similar inhibitory and enhancing effect on the ionomycin-induced  $I_{\text{SC}}$  was also observed with PKC activator and inhibitor, respectively, in CAPAN-1 cells which possess both the cAMP and  $\text{Ca}^{2+}$ -dependent pathways. These results suggest an inhibitory role of PKC in  $\text{Ca}^{2+}$ -dependent pancreatic ductal secretion. PKC could attenuate the  $\text{Ca}^{2+}$ -activated ductal secretion and thereby serve as a turn-off mechanism. This may be achieved by modulating intracellular  $\text{Ca}^{2+}$  store [37] or inactivating the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels [38] on which  $\text{Cl}^-$  secretion largely depends. In contrast to the previ-

ously observed defective regulation of  $\text{Cl}^-$  secretion by PKC in CF cells [10,13,14], the modulation of  $\text{Ca}^{2+}$ -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  $\text{P}_2$  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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ -dependent mechanism. The augmentation of the  $\text{Ca}^{2+}$ -dependent anion secretion in CF cells by inhibition of PKC may have therapeutic implication since the  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$ -dependent anion secretion is currently under investigation.

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