

TOPICAL REVIEW

The secret life of CFTR as a calcium-activated chloride channel

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Abstract cAMP-stimulated anion conductance is defective in cystic fibrosis (CF). The regulatory domain of CFTR, the anion channel protein encoded by the CF gene, possesses an unusually high density of consensus sequences for phosphorylation by protein kinase A (14 in a stretch of <200 amino acids). Thus it is not surprising that CFTR is viewed primarily as a cAMP-stimulated anion channel, and most studies have focused on this mode of activation. However, there is growing evidence that CFTR also responds to Ca²⁺-mobilizing secretagogues and contributes substantially to cholinergic and purinergic responses in native tissues. G protein-coupled receptors that signal through G_{αq} can stimulate CFTR channels by activating Ca²⁺-dependent adenylyl cyclase and tyrosine kinases, and also by inhibiting protein phosphatase type 2A. Here we review evidence for these novel mechanisms of CFTR activation and discuss how they may help explain previous observations.

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Introduction

Fluid secretion into the airway lumen is driven by Cl⁻ transport, which is mediated by at least two types of anion channels that respond to different stimuli. Secretagogues that elevate cAMP such as vasoactive intestinal peptide, adrenaline (epinephrine), adenosine and serine proteases stimulate secretion by causing protein kinase A (PKA)-mediated phosphorylation of the anion channel CFTR (cystic fibrosis transmembrane conductance regulator). By contrast, secretagogues that mobilize Ca²⁺ from endoplasmic reticulum (ER) stores such as acetylcholine and ATP induce secretion by stimulating Ca²⁺-activated Cl⁻ channels (CaCCs) such

as the recently identified TMEM16A (reviewed by Frizzell & Hanrahan, 2012).

This paradigm of separate anion conductances activated by cAMP and Ca²⁺ has provided a framework for understanding epithelial transport since early studies suggested that cAMP-dependent secretion is defective in CF whereas Ca²⁺-activated secretion is preserved (Sato & Sato, 1984; Anderson & Welsh, 1991). It also fits with the classical scheme for signalling by G protein-coupled receptors (GPCRs), which utilize cAMP and Ca²⁺ as second messengers for G_s- and G_{q/11}-coupled receptors, respectively. The importance of cAMP/PKA is further suggested by the exceptionally large number of consensus sequences for PKA phosphorylation on the regulatory (R)

Arnaud Billet (left) received his Doctorate degree in cellular and molecular biology from the Université de Poitiers (France) for studies of CFTR structure–function. He currently works as a Postdoctoral Fellow in the laboratory of John Hanrahan within the Cystic Fibrosis Translational Research centre (CFTRc) at McGill University (Canada). **John W. Hanrahan** (right) became interested in epithelial Cl⁻ transport during his Doctoral work at the University of British Columbia. He has studied CFTR channels in epithelial cells since 1989 and in various expression systems since 1990.



domain of CFTR. Early functional studies of CFTR using inside-out membrane patches and planar lipid bilayers revealed that the catalytic subunit of PKA + MgATP can activate CFTR channels whereas exposure to Ca^{2+} either alone, or in combination with calmodulin, has no effect, in contrast to the CaCCs characterized previously in exocrine glands (Evans & Marty, 1986).

Convergent cAMP and Ca^{2+} signalling by muscarinic agonists

Despite all the evidence for two separate anion conductances that respond specifically to cAMP or Ca^{2+} , some studies indicate overlap between these pathways. For example in pancreatic duct cells, Ca^{2+} signals were shown to stimulate CFTR-dependent HCO_3^- secretion (Namkung *et al.* 2003). The results were interpreted in the context of apical anion exchange, but Ca^{2+} may also have stimulated CFTR conductance, which is thought to mediate substantial HCO_3^- efflux in guinea pig and mouse pancreatic ducts (Ishiguro *et al.* 2009). Moreover, Ca^{2+} -stimulated anion secretion may not be as well preserved in CF as has been widely assumed. Carbachol-stimulated HCO_3^- secretion is reduced by 75–100% in the intestine of *cfr* null mice (Hogan *et al.* 1997; Seidler *et al.* 1997). In submucosal glands from human airways, the Ca^{2+} -mobilizing muscarinic agonist pilocarpine stimulates robust fluid secretion, and this response is reduced by ~60% in CF glands. The apparent decrease of 60% is even more striking when one considers that it is probably an underestimate, and that the rates would have been reduced more if normalized per unit area of epithelium to correct for 2- to 3-fold hypertrophy of CF glands. It is not immediately obvious why pilocarpine-induced secretion should be compromised in CF submucosal glands if it occurs via CaCCs, yet it is a consistent observation in glands from CF piglet trachea (Joo *et al.* 2010) and nasal turbinate (Cho *et al.* 2011), and tracheal xenografts (Sun *et al.* 2010). Pig glands exposed to the inhibitor CFTR_{inh}-172 (Thiagarajah *et al.* 2004) also have diminished responses to cholinergic agonists. Thus in some tissues, the premise that muscarinic (i.e. Ca^{2+} -activated) secretion is preserved in CF does not hold up well under scrutiny.

Serous cells express both CFTR and CaCCs, and respond to VIP and muscarinic stimuli. Consequently they can generate responses that are synergistic at several levels. One synergy occurs when cAMP enhances Ca^{2+} release from intracellular stores by stimulating PKA phosphorylation of the inositol trisphosphate (IP_3) receptor. The IP_3 receptor normally interacts with a protein called IRBIT (IP_3 receptor-binding protein released with IP_3), which inhibits IP_3 receptor channel function and is released

when the receptor binds IP_3 (Ando *et al.* 2003). After dissociating from the endoplasmic reticulum, IRBIT can interact with several acid–base transporters and regulate their activity at the plasma membrane, including the sodium bicarbonate cotransporter NBCe1-B and CFTR (Yang *et al.* 2011). IRBIT can mediate synergism between cAMP and Ca^{2+} because its release from the endoplasmic reticulum is enhanced by PKA phosphorylation of the IP_3 receptor (Park *et al.* 2013). Conversely, carbachol can amplify the response to VIP by stimulating Ca^{2+} -activated high conductance and intermediate conductance K^+ channels, thereby hyperpolarizing the cell membrane and increasing the driving force for apical anion efflux. cAMP and Ca^{2+} signalling during transepithelial transport has been discussed in a recent review article (Kunzelmann & Mehta, 2013), which emphasizes the upregulation of Ca^{2+} signalling in CF cells (Antigny *et al.* 2007; Balghi *et al.* 2011; Martins *et al.* 2011) and functional interactions between CFTR and the Ca^{2+} -activated Cl^- channel TMEM16A (Kunzelmann *et al.* 2012).

Cross-talk and synergy between VIP and cholinergic signalling have been appreciated since 1982 (Schultzberg *et al.* 1982) and are clearly important, but they do not explain why cholinergic responses are 60% smaller in CF glands than in non-CF glands. In this topical review we argue that CFTR itself mediates a substantial fraction of the ‘CaCC conductance’ during muscarinic stimulation of airway submucosal glands and perhaps some other tissues, and that CFTR activation results from Ca^{2+} -dependent phosphorylation by both PKA and tyrosine kinases. A contribution of CFTR to the cholinergic response of airway submucosal glands has already been suggested (Cho *et al.* 2011), and carbachol regulation of CFTR has recently been demonstrated directly by co-expressing CFTR together with the type 3 muscarinic receptor (M3R) in baby hamster kidney (BHK) cells (Billet *et al.* 2013). Remarkably, CFTR currents stimulated by carbachol in this heterologous expression system were about 40% larger than those induced by forskolin.

Cholinergic stimulation of airway epithelial cells is mediated by the M3R and, to a lesser extent, the M1 receptor. The M3R signals through $G_{\alpha q/11}$ to phospholipase C, which generates IP_3 and mobilizes Ca^{2+} from intracellular stores (Berridge, 2012) (see www.cellsignallingbiology.org). Although CFTR is not stimulated directly by Ca^{2+} , several adenylyl cyclase isoforms are Ca^{2+} activated (types 1, 8 and perhaps 3; Halls & Cooper, 2011) and thus could couple the phosphorylation of CFTR by PKA to muscarinic Ca^{2+} responses. This regulation may involve a localized pool of cAMP near the plasma membrane (Monterisi *et al.* 2012), which is isolated from the bulk cytoplasm by phosphodiesterases 4D (Barnes *et al.* 2005) and/or 3A (Penmatsu *et al.* 2010).

Tyrosine phosphorylation contributes to muscarinic stimulation of CFTR

Activation of CFTR by carbachol-induced elevation of cAMP was recently demonstrated in BHK cells that coexpress CFTR and M3R (Billet *et al.* 2013). However, to our surprise, only about half of the cholinergic response could be attributed to Ca^{2+} activation of adenylyl cyclase; $\sim 50\%$ of CFTR's response to carbachol persisted when PKA was inhibited by Rp-cAMPS. Similar results were obtained using a CFTR mutant that is unresponsive to forskolin due to removal of 15 PKA consensus sequences (Seibert *et al.* 1999). Thus about half of CFTR's response to carbachol does not involve activation of adenylyl cyclase by Ca^{2+} , yet the CFTR response to carbachol was clearly Ca^{2+} dependent since it was strongly inhibited by buffering intracellular Ca^{2+} with BAPTA-AM (Fig. 1). This raises the question, 'What Ca^{2+} -dependent pathway mediates CFTR activation by carbachol when the response of CFTR to PKA is blocked?'

Exposing excised patches to the tyrosine kinase Src increases fast CFTR channel gating and open probability (Fischer & Machen, 1996). Although the upstream stimulus for Src was not identified, many GPCRs are now known to recruit and activate Src. Therefore we examined the possible role of Src during muscarinic stimulation of CFTR and found that Src Inhibitor-1 (Inh-1) abolished the PKA-independent component of muscarinic stimulation (Billet *et al.* 2013). To explain how a brief Ca^{2+} transient during carbachol exposure might activate Src, we also investigated Pyk2, a Ca^{2+} -activated, proline-rich tyrosine kinase related to focal adhesion kinase (FAK). Pyk2 forms a complex with Src thereby facilitating their reciprocal phosphorylation and activation. Pyk2 has already been implicated in the regulation of CFTR (Liang *et al.* 2011) as has FAK itself (Marshall *et al.* 2009). Interestingly, like Src Inh-1, the specific Pyk2 inhibitor tyrphostin A9 abolished the PKA-independent component of CFTR stimulation by carbachol. These and other results led us to propose that Ca^{2+} activates CFTR, at least in part, through activation of the Pyk2/Src complex and tyrosine phosphorylation (Fig. 2).

This tyrosine kinase pathway suggests yet another mode of cross-talk between the Ca^{2+} and cAMP signalling pathways through the activity of a protein phosphatase. Protein phosphatase type 2A (PP2A) is known to interact with, and dephosphorylate, CFTR (Hwang *et al.* 1994; Luo *et al.* 1998; Thelin *et al.* 2005) and is strongly inhibited when phosphorylated by Src at residue Y307 (Chen *et al.* 1992). Thus Ca^{2+} -dependent activation of Src (presumably through its association with Pyk2) can enhance channel activity by inhibiting the dephosphorylation of PKA sites on CFTR. In support of this idea, Src Inh-1 causes a ~ 2 -fold greater inhibition of carbachol-stimulated current when tested alone than

when tested with calyculin A (a specific inhibitor of PP2A) present to mitigate its action on the phosphatase. These results indicate that Src normally suppresses PP2A activity, and when this inhibition is partially relieved by Src Inh-1 the dephosphorylation of PKA sites is enhanced. Calyculin A would prevent the upregulation of PP2A activity by Src Inh-1, thereby reducing the relative inhibition of the carbachol response by Src Inh-1. Exposing CFTR to protein kinase C (PKC) enhances subsequent activation of the channel by PKA, and since some conventional PKC isoforms are Ca^{2+} dependent, this suggests yet another mechanism by which Ca^{2+} could regulate CFTR. A CFTR mutant lacking nine predicted PKC sites was partially activated by M3 muscarinic receptors, apparently through tyrosine phosphorylation

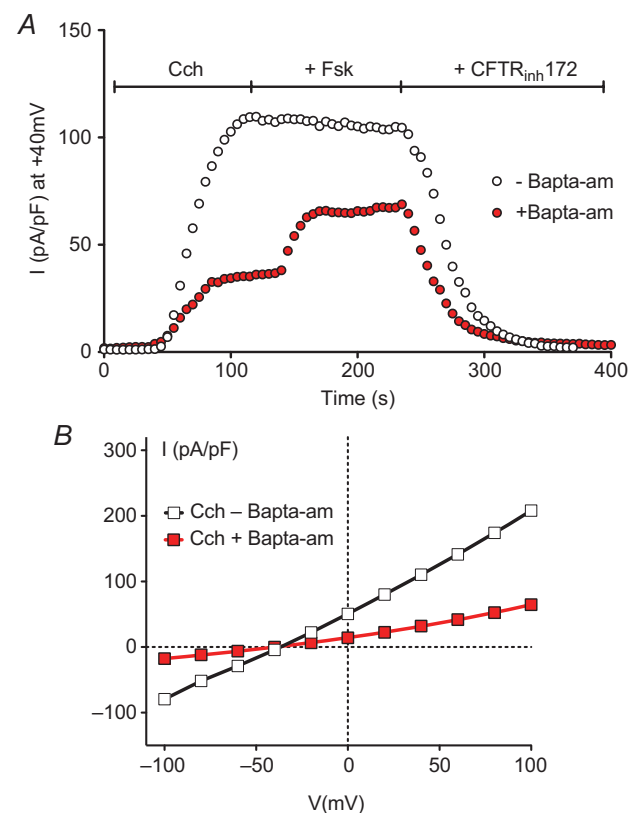


Figure 1. Time course and current-voltage relations of CFTR activation by carbachol and their dependence on intracellular Ca^{2+}

A, representative whole cell currents measured in BHK cells made to co-express the muscarinic type 3 receptor and CFTR. Cells were exposed sequentially to carbachol (Cch), forskolin (Fsk) and CFTR_{inh}172, each at $10 \mu\text{mol l}^{-1}$. Experiments were performed with or without 60 min preincubation with the membrane-permeant Ca^{2+} buffer BAPTA-AM ($500 \mu\text{mol l}^{-1}$) in the bath and pipette solutions. Note that stimulation was more robust with carbachol than with forskolin, that BAPTA-AM inhibited the Cch response $>60\%$, and the CFTR inhibitor CFTR_{inh}172 virtually abolished the response to Cch + Fsk. B, current-voltage relationship for currents measured in A during the plateau phase of stimulation.

since the response was abolished by tyrosine kinase inhibitors (Billet *et al.* 2013). Although the 'priming' effect of PKC is not required for activation of CFTR by tyrosine phosphorylation, further studies are needed to establish whether Ca^{2+} -dependent PKC activity enhances the component of the muscarinic response that is mediated by PKA.

In summary, CFTR can operate as a Ca^{2+} -activated Cl^- channel and respond to cholinergic stimulation. There is evidence for at least three Ca^{2+} -dependent mechanisms of CFTR activation: (1) upregulation of adenylyl cyclase by Ca^{2+} leading to conventional PKA-mediated phosphorylation of CFTR, (2) stimulation of Pyk2/Src, which inhibits PP2A and thus increases serine phosphorylation on CFTR, and (3) direct tyrosine phosphorylation of CFTR by the Pyk2/Src complex.

Basal CFTR activity in epithelial cells: possible role of Ca^{2+} entry

Calcium mobilization is necessary for CFTR activation during muscarinic stimulation, but does Ca^{2+} also influence basal CFTR activity? This was explored using unstimulated Calu-3 monolayers, which have substantial anion secretion ($\sim 1 \mu\text{equiv cm}^{-2} \text{h}^{-1}$) that is abolished by the CFTR inhibitor GlyH101. Basal anion secretion was inhibited 68–100% by the adenylyl cyclase inhibitor MDL-12330A and 68–88% by the PKA inhibitor Rp-cAMPS (Shan *et al.* 2012). Those pharmacological experiments suggested that low-level phosphorylation by PKA maintains a basal rate of anion secretion. Since basal anion secretion was strongly inhibited (65–90%) by 2-APB, an antagonist of store-operated Ca^{2+} entry, we proposed that slow leakage into resting cells stimulates Ca^{2+} -dependent adenylyl cyclase, raising [cAMP] locally and leading to partial PKA activation of CFTR (Fig. 2). Thus CFTR may contribute directly to Ca^{2+} -activated Cl^- conductance even in the absence

of secretagogue. Moreover Ca^{2+} signalling is regulated by CFTR, which suppresses Ca^{2+} release from intracellular stores (Antigny *et al.* 2007) and reduces Ca^{2+} influx through its modulation of store-operated Ca^{2+} channels (ORAI1; Balghi *et al.* 2011). CFTR normally inhibits the insertion of ORAI1 channels and suppresses Ca^{2+} entry by $\sim 50\%$; however, this downregulation of Ca^{2+} entry is lost in CF cells, resulting in exaggerated Ca^{2+} influx during stimulation by secretagogues that cause depletion of Ca^{2+} stores. In summary, although there is growing evidence that Ca^{2+} signalling and CFTR function are interdependent, the precise mechanisms remain to be determined. Ca^{2+} clearly mediates most of the CFTR response to muscarinic stimulation and probably contributes to tonic CFTR activity in some cell types.

Conclusions

Further studies of the CaCC activity of CFTR should clarify CFTR's role in anion secretion and other cellular processes. It has been a longstanding puzzle that loss of CFTR function in airway submucosal glands should have such dire consequences when the more robust CaCC response to carbachol is preserved. This becomes more understandable if CFTR contributes significantly to both Ca^{2+} - and cAMP-mediated secretagogues, although we note that the CaCC function of CFTR depends on cell type and may not occur in all tissues. For example, the response of salivary secretion to cholinergic stimulation is not affected noticeably in *cfr* null mice (Best & Quinton, 2005), suggesting it is mediated by bona fide CaCCs. Further studies should examine whether these differences in CFTR regulation arise at the level of receptors or downstream signalling. If other CaCCs are present in large numbers, they may dominate responses to moderate stimuli. Also, the CaCC activity of CFTR may be obscured during maximal stimulation if apical anion conductance is not the rate-limiting step for secretion.

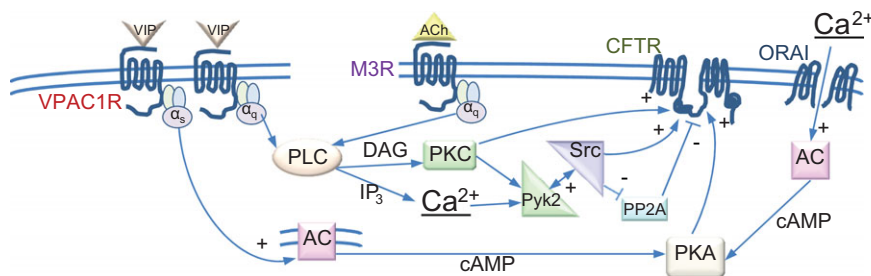


Figure 2. Simplified scheme for Ca^{2+} activation of CFTR via PKA and tyrosine kinases

VIP receptors couple strongly to $G_{\alpha s}$ and weakly to $G_{\alpha q}$, which stimulate adenylyl cyclase (AC) and phospholipase C (PLC), respectively. The muscarinic type 3 receptor (M3R) couples strongly to $G_{\alpha q}$. cAMP activates CFTR channels by means of the canonical PKA pathway. Diacylglycerol and inositol trisphosphate (IP_3) activate protein kinase C (PKC) and mobilize Ca^{2+} , which in turn activate the Pyk2/Src complex. Src stimulates CFTR activity by phosphorylating it directly and also by inhibiting its dephosphorylation through inactivation of protein phosphatase type 2A (PP2A). Under basal conditions, constitutive Ca^{2+} entry through store-operated Ca^{2+} channels partially activates adenylyl cyclase and induces tonic CFTR activity.

The identification of TMEM16A (also known as ANO1, DOG1, ORAOV2 and TAOS2) as a CaCC has been an exciting development in the field. Its role in airway and intestinal epithelia has been questioned based on studies with the new TMEM16A inhibitor T16A_{inh}-A01, which is a potent blocker of TMEM16A but has little effect on CaCC in those tissues (Namkung *et al.* 2011). This discrepancy may reflect the expression of alternatively spliced forms of TMEM16A, which differ in their functional properties and are expressed in a tissue-specific manner (Ferrera *et al.* 2009), or perhaps the presence of other members of the anoctamin family, which may differ in their sensitivity to pharmacological inhibitors. It will be interesting to see if residual CFTR activity also contributes to the T16A_{inh}-A01-insensitive component in airway and intestinal epithelia. This would be important because pharmacological stimulation of Ca²⁺-activated Cl⁻ conductance has been proposed as a therapeutic strategy for bypassing defective CFTR, but would not succeed if CFTR itself contributed a substantial fraction of the CaCC activity. Finally, elevated cytokine release by CF epithelial cells has been reported by many groups and can be suppressed by expressing an anion conductance even in the absence of secretagogue (Veit *et al.* 2012). The paradox that CFTR must be functional to suppress cytokine release, yet does not need to be activated by a secretagogue, might be explained if Ca²⁺ entry caused tonic CFTR activation as observed in Calu-3 (Shan *et al.* 2012). While there is little doubt that the CFTR channel functions (albeit indirectly) as a CaCC, we are just beginning to appreciate the complex relationship between intracellular Ca²⁺ signalling and CFTR-mediated secretion.

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Additional information

Competing interests

None declared.

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