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cAMP and Ca²⁺ signaling in secretory epithelia: Crosstalk and Synergism

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Abstract

The Ca²⁺ and cAMP/PKA pathways are the primary signaling systems in secretory epithelia that control virtually all secretory gland functions. Interaction and crosstalk in Ca²⁺ and cAMP signaling occur at multiple levels to control and tune the activity of each other. Physiologically, Ca²⁺ and cAMP signaling operate at 5–10% of maximal strength, but synergize to generate the maximal response. Although synergistic action of the Ca²⁺ and cAMP signaling is the common mode of signaling and has been known for many years, we know very little of the molecular mechanism and mediators of the synergism. In this review, we discuss crosstalk between the Ca²⁺ and cAMP signaling and the function of IRBIT (IP₃ receptors binding protein release with IP₃) as a third messenger that mediates the synergistic action of the Ca²⁺ and cAMP signaling.

Introduction

Ca²⁺ and cAMP signaling are pleiotropic primary second messengers that regulate all secretory epithelia functions and their over-activation is associated with many epithelial diseases. The Ca²⁺ and cAMP signaling pathways interact on numerous levels. They regulate the activity of each other to determine the intensity of their response and cooperate to determine the physiological response by integration of their stimulatory/inhibitory activities. Mutual regulation of the Ca²⁺ and cAMP signals is referred to as crosstalk, while integration of their effects can result in an additive or synergistic physiological response.

cAMP is a second messenger regulated by synthesis and breakdown. The cAMP signal is determined by the balance between the activities of adenylyl cyclases (ACs) that synthesize cAMP from ATP and the phosphodiesterases (PDEs) that hydrolyze the cAMP to 5'-AMP. The ACs are coded by 9 genes with several splice variants, several of which are ubiquitous

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while others show cell specific expression patterns. The plasma membrane ACs are regulated by G-protein-coupled receptors (GPCRs). In general, the Gs-coupled receptors activate and the Gi/o-coupled receptors inhibit the ACs through their interaction with G α s and G α i/o, respectively. However, several ACs can be regulated by G $\beta\gamma$ released from Gi/o [1]. A comprehensive and careful recent discussion of the properties and function of the ACs can be found in [2]. Another type of AC is the soluble AC (sAC) that is specifically activated by HCO $_3^-$. sAC is found throughout the cytosol, in the nucleus and mitochondria [3], and its role in generation of cAMP within the mitochondria has been demonstrated recently [4, 5]. The properties and functions of the sAC are discussed in [3]. An important aspect of the ACs is their compartmentalization to generate cAMP microdomains. This is achieved mostly by interaction of the ACs with the anchoring scaffolds A-kinase anchoring proteins (AKAPs). Several AKAPs recruit ACs to microdomains in close proximity to Ca $^{2+}$ signaling proteins, such as ryanodine receptors, L-type Ca $^{2+}$ channels and SERCA pumps. In turn, the activity of several ACs is regulated by [Ca $^{2+}$] $_i$ (see below). The role of the AKAPs is not discussed here but interested readers can consult a recent scholarly review on this topic [6].

The PDEs are encoded by 21 genes and are grouped into 11 families, with many isoforms in each family resulting in more than 60 PDEs. The families are grouped based on sequence homology, substrate specificity, kinetic properties, regulation, and pharmacological properties. PDE1, 2, and 3 hydrolyze both cAMP and cGMP at similar efficiency. PDE4 and 8 hydrolyze only cAMP, while PDE5 and 9 hydrolyze only cGMP. As with the ACs, the PDEs show cell specific and highly compartmentalized expression. Thus, the PDEs control the level of cAMP and also limit its diffusion to generate cAMP pools and microdomains to control specific cell functions. Several PDEs are localized in close proximity to Ca $^{2+}$ signaling proteins to affect their regulation by cAMP and cGMP. In turn, the activity of several PDEs is regulated by [Ca $^{2+}$] $_i$ (see below). Comprehensive discussion of the PDEs is given in [7, 8].

Cytoplasmic Ca $^{2+}$ ([Ca $^{2+}$] $_i$) is regulated by transport across cellular membranes by pumps and channels. The pumps generate large Ca $^{2+}$ gradients across the plasma membrane (PMCA) [9], the membrane of the endoplasmic reticulum (ER) (SERCA) [10] and the membrane of the Golgi apparatus (SPCA) [11]. Ca $^{2+}$ gradients are also maintained by intracellular organelles, in particular the endolysosomal system that depends on the organellar pH gradient [12] by an unknown transporter. Ca $^{2+}$ enters the cytosol by activation of Ca $^{2+}$ channels, primarily the IP $_3$ receptors (IP $_3$ Rs) in the ER and the Ca $^{2+}$ influx channels Orai1 and TRPC channels in the plasma membrane [13].

The receptor-evoked Ca $^{2+}$ signals involve activation of GPCRs or tyrosine-kinase dependent receptors that activate phospholipase C and generate diacylglycerol and IP $_3$. IP $_3$ activates the IP $_3$ Rs, releasing Ca $^{2+}$ primarily from the ER [14]. In secretory cells the IP $_3$ Rs are clustered at the apical pole [15–17]. Activation of several receptors generates additional Ca $^{2+}$ -mobilizing second messengers such as cADP ribose (cADPR) and NAADP [18]. cADPR is believed to activate the ryanodine receptors (RyRs) [19], while NAADP releases Ca $^{2+}$ from the acidic endolysosomal system by activation of the TPC channels [20]. In secretory cells Ca $^{2+}$ release by NAADP appears to function as a trigger that sensitizes the

IP₃-mediated Ca²⁺ release [21]. Ca²⁺ release from the ER activates the store-operated Ca²⁺ influx channels in the plasma membrane; the Orai [22] and TRPC channels [13] that are activated by the ER Ca²⁺ sensor STIM1 [23]. The rise in [Ca²⁺]_i activates cytosolic Ca²⁺ clearance mechanisms [24]. Part of the Ca²⁺ is incorporated by the mitochondria through the mitochondrial Ca²⁺ channel MCU [25, 26] and part reenters the ER through the SERCA pump, while most of the Ca²⁺ is extruded out of the cytosol by PMCA [13, 27]. At weak cell stimulation these events are repeated resulting in Ca²⁺ oscillations, while at intense stimulation a pump-leak steady-state is achieved at elevated [Ca²⁺]_i. Upon termination of the stimulus, the SERCA refills the stores with Ca²⁺ and the PMCA restores the resting [Ca²⁺]_i. Most of the Ca²⁺ transporters participating in the Ca²⁺ signal are regulated by the cAMP/PKA system (see below).

Regulation of cAMP/PKA signaling by Ca²⁺

Crosstalk between the Ca²⁺ and cAMP signaling occurs at the level of regulation of both ACs and PDEs by Ca²⁺ in signaling hubs and microdomains. In general, AC1, AC3 and AC8 are activated, whereas AC5 and AC6 are inhibited by a physiological increase in [Ca²⁺]_i [2, 28]. Ca²⁺ can also regulate ACs indirectly. For example, AC3 that is activated by Ca²⁺/calmodulin is inhibited by the Ca²⁺/calmodulin protein kinase CaMKII [29]. Other links between Ca²⁺ and cAMP/PKA are found in regulation of ACs by PKC and most prominently by Gβγ (evaluated and discussed in [2]).

Physical compartmentalization of the ACs leads to functional compartmentalization and oscillations in cAMP concentration. At the first level, the Ca²⁺-regulated AC1, AC5, AC6 and AC8 are targeted to plasma membrane rafts, whereas the Ca²⁺-independent AC2 and AC7 are excluded from the raft domains [30]. The more precise and specific compartmentalization by AKAPs places several ACs in proximity to Ca²⁺ transport proteins. Stimulation of AC8 by Ca²⁺ release from the ER was suggested [31]. In excitable cells the Ca²⁺-regulated ACs are activated by Ca²⁺ entry through the voltage-activated Ca²⁺ channels [32, 33]. A more extensively studied form of regulation is the regulation of ACs by Ca²⁺ influx through the store-operated Ca²⁺ channels. The topic has been explored mainly by the group of Cooper et al., [2]. Formation of a cAMP/PKA complex is mediated by AKAPs that recruit them to plasma membrane rafts where they interact with the cytoskeleton, which stabilizes the complexes [34]. In the rafts AC8 (and perhaps other ACs) interact directly with the pore forming SOC channel Orai1, where the ACs and Ca²⁺ influx through Orai 1 regulate the activity of each other [35].

A novel and very interesting form of regulation related to Ca²⁺ signaling proteins but does not involve changes in [Ca²⁺]_i is the recently reported regulation of AC3 by the ER Ca²⁺ sensor STIM1 directly, which was named store-operated cAMP signaling (SOcAMPS) [36]. This form of regulation requires depletion of ER Ca²⁺ and clustering of STIM1 at plasma membrane microdomains, but it is independent of Orai1, STIM2 or Ca²⁺ influx [36]. SOcAMPS appears to be cell specific with variable activity in different cell types and can involve other ACs [37, 38].

The sAC functions as HCO_3^- -activated, Ca^{2+} regulated adenylyl cyclase [3]. sAC is found mostly associated with microtubules, the nucleus and mitochondrial matrix [3]. Two recent studies showed that cAMP cannot enter the mitochondria from the cytosol, but rather it is generated by HCO_3^- stimulation of the mitochondrial sAC [4, 5]. Significantly, in the mitochondria sAC can be activated by an increase in matrix Ca^{2+} . The cAMP generated by the sAC upregulates ATP synthesis probably by activating PKA resident in the mitochondrial matrix [4].

Ca^{2+} also regulates the activity of several PDEs. The Ca^{2+} -dependent PDEs were studied to a lesser extent than the Ca^{2+} -dependent ACs [7]. Members of the PDE1 family have two N terminus calmodulin binding domains and are regulated by Ca^{2+} /calmodulin, which increases the V_{max} of PDE1 [39]. Ca^{2+} /calmodulin can increase the activity of the PDEs by as much as 10 folds [40]. Ca^{2+} /calmodulin relieve autoinhibition of the PDEs [41]. The affinity of the PDEs to Ca^{2+} /calmodulin is regulated by PKA and CaMKII phosphorylation [7]. The specific function of members of the family and their interaction with $[\text{Ca}^{2+}]_i$ likely contribute to the generation of cAMP microdomain. This is exemplified well in a recent study that examined the response of two PDE1 isoforms to Ca^{2+} influx through SOC channels [42]. Clearly, this topic deserves further probing. Several relationships between the Ca^{2+} and the cAMP signaling pathways are illustrated in Fig. 1.

Regulation of Ca^{2+} signaling by cAMP/PKA

The cAMP/PKA pathway shapes the Ca^{2+} signal in many cell types, including secretory cells, myocytes and neurons. Such regulation was demonstrated in pancreatic [43] and parotid acini [44], blowfly salivary glands [45], hepatocytes [46], airway epithelial cells [47], cardiac [48] and skeletal myocytes [49], and neurons [50]. Regulation of the Ca^{2+} signal involves the regulation of all Ca^{2+} transporting pathways by PKA-mediated phosphorylation that commonly activates the transporters. This is illustrated in Fig. 1.

Regulation of the Ca^{2+} channels IP_3Rs and RyRs has been studied most extensively out of all Ca^{2+} transporting pathways (reviewed in [51–53]). S1589 and S1755 of the $\text{IP}_3\text{R1}$ are phosphorylated by PKA [51, 54], while phosphorylation of S1755 was sufficient to increase the activity of the neuronal IP_3Rs and phosphorylation of either site of the peripheral $\text{IP}_3\text{R1}$ increased the response of the receptors to IP_3 [55]. S937 is phosphorylated by PKA in $\text{IP}_3\text{R2}$, which increases the activity of the prominent IP_3R present in exocrine cells [56]. Phosphorylation by PKA increased the open probability of $\text{IP}_3\text{R1}$ at submaximal IP_3 concentrations [51], suggesting that the phosphorylation increases the affinity of the receptors to IP_3 . In this respect, $\text{IP}_3\text{R1}$ activity is inhibited by IRBIT (IP_3 receptors binding protein released with IP_3) [30, 57], where IRBIT competes with IP_3 for interaction with the IP_3 binding pocket of the receptor [57]. We have shown recently that PKA phosphorylation of $\text{IP}_3\text{R1}$ reduces interaction of $\text{IP}_3\text{R1}$ with IRBIT by facilitating IRBIT release from the IP_3Rs by IP_3 [58]. Together, these findings suggest that phosphorylation by PKA increases the function of the IP_3Rs at least in part by reciprocally modulating the interaction of the IP_3Rs with IRBIT and IP_3 .

PKA phosphorylates RyR1 on S2843 and RyR2 on S2030, S2809 to increase Ca^{2+} permeability of the receptors [52]. The physiological significance of the phosphorylation is most extensively studied in the heart [48]. Sympathetic input to activate the cardiac β adrenergic receptors increases phosphorylation of cardiac RyR2 to increase Ca^{2+} release from the SR that results in stronger and faster contraction [59]. Under pathological conditions and stress RyR1 and in particular RyR2 were suggested to be hyperphosphorylated by PKA to cause SR Ca^{2+} leak and reduce muscle contraction [48]. However, this is not a consistent finding and phosphorylation by other kinases has also been implicated [52]. TPC2 that mediates the NAADP-induced Ca^{2+} release from endolysosomes [49] was shown to be phosphorylated by the mTORC1 complex [60], P38 and JNK kinase (Jha and Muallem to be published elsewhere). It will be interesting to test whether TPC2 and TPC1 are also regulated by PKA.

During receptor stimulation most Ca^{2+} enters the cytosol by Ca^{2+} influx through the plasma membrane localized SOCs Orai1 and TRPC channels [13]. In excitable cells Ca^{2+} influx during EC coupling in muscles and exocytosis in neurons is mediated by various isoforms of the voltage activated Ca^{2+} channels (VACC). Large body of literature discusses regulation of VACC, particularly the increased activation of the L type Ca^{2+} channels by PKA [61]. Phosphorylation of the VACC is aided by recruitment of PKA to the channels by AKAP15 and GPCRs that activate ACs like the β adrenergic receptors. PKA phosphorylates multiple PKA consensus serine residues that vary among VACC isoforms (see [61] for details).

Substantial indirect evidence supports regulation of several TRP and TRPC channels by cAMP/PKA signaling. TRPC4 and TRPC5 are inhibited by activation of endogenous G proteins with $\text{GTP}\gamma\text{S}$ due to PKA-dependent phosphorylation of TRPC5 S794 and S796 and unidentified residues in TRPC4 [62]. TRPC4 and TRPC5 are also activated directly by selective $\text{G}\alpha_i$ subunits when activated by $\text{GTP}\gamma\text{S}$ or by stimulation of the muscarinic M2 receptors [63]. Phosphorylation of TRPC6 on S28 by PKA [64] and phosphorylation by cGMP/PKG on T70 and S322 [65] were reported to inhibit the channel. On the other hand, TRPC6 is indirectly activated by PKA that acts on ERK1/2 and, in turn, phosphorylates TRPC6 on S281 [66]. Finally, activation of the cAMP/PKA pathway was reported to affect expression of TRPC1 and TRPC3 [67] and translocation of TRPC3 [68] and TRPC5 [69] to the plasma membrane. At present there is no direct evidence for regulation of the Orai1/STIM1-mediated Ca^{2+} influx or current by the cAMP/PKA pathway. One study reported activation of the CRAC current by infusing glial cells with cAMP that was inhibited by inhibition of PKA [70]. Evidently, additional studies are needed to evaluate potential regulation of the Orai1/STIM1 channel by PKA.

The cAMP/PKA pathway critically regulates the Ca^{2+} pathways mediating $[\text{Ca}^{2+}]_i$ clearance. The SERCA2 pump is regulated by phospholamban (PLN), a small phosphoprotein. This topic is reviewed in [71]. PLN is a single-pass transmembrane protein with several N-terminal phosphorylation sites, S16, S10 and T17. S16 is phosphorylated by PKA, S10 by PKC and T17 by CaMKII. PLN is expressed mainly in myocytes and its expression is restricted to the longitudinal region of the SR, where it forms a complex with SERCA2, PKA, CaMKII and the phosphatases PP1 and PP2B. In the resting state, the unphosphorylated PLN interacts with SERCA2 and inhibits its activity. Stimulation of β

adrenergic receptors to activate PKA phosphorylates PLN on S16 to dissociate between SERCA2/PLN, resulting in activation of Ca^{2+} pumping to facilitate clearance of $[\text{Ca}^{2+}]_i$ [71].

Most Ca^{2+} clearance during cell stimulation and at the end of the stimulation period is mediated by the plasma membrane Ca^{2+} ATPase pump (PMCA) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) isoforms that are particularly active in cardiac myocytes, neurons and the kidney. The main exchanger isoform is NCX1 that has several splice variants [72]. Several studies suggested that the cardiac NCX can be phosphorylated by PKA on sites located in the large intracellular loop, which increases the activity of the NCX [73]. However, this issue remains controversial and it is not clear whether or not cell stimulation with β adrenergic or other Gs-coupled receptors exert their effects on the NCX directly or indirectly [74, 75] or whether PKA phosphorylates NCX1 [76].

Regulation of PMCA by cAMP/PKA signaling is well established [24]. The PMCAs are coded by four genes with numerous splice variants, with PMCA1 and 4 expressed more widely, including in secretory cells [77], and PMCA2 and 3 are expressed mostly in neuronal tissues [9]. The main activator of PMCA is calmodulin, which increases the apparent affinity of the pump for Ca^{2+} and increases the pump rate [78]. Regulation of PMCA by calmodulin and PKA is isoform specific, where PMCA1 is phosphorylated by PKA better than PMCA2 and PMCA4 [79]. PKA phosphorylation potentiates the activity of PMCA in a Ca^{2+} -dependent manner, likely by activation of one of the Ca^{2+} -dependent ACs [80]. An interesting and unique arrangement exists in secretory cells, where PMCA1 is expressed mostly in the basolateral membrane with some expression at the luminal membrane, while PMCA4 is expressed exclusively in the luminal membrane [77]. In these cells, PKA specifically activated the luminal PMCA while phosphorylating only PMCA1 [81]. This is likely due to recruitment of PKA catalytic subunit to the luminal membrane to complex with PMCA1 at this membrane.

The relationships between Ca^{2+} transporting proteins and cAMP/PKA signaling are illustrated in Fig. 1. It is clear that the two signaling systems interact extensively at multiple levels to regulate each other's activity. These interactions allow generation of intricate and domain specific responses to determine the location and strength of physiological responses. A more detailed quantitative evaluation of each regulatory interaction between the two signaling pathways is needed in order to use an integrative system approach to evaluate the output signal generated by activation of the two pathways. Development of techniques for simultaneous measurement of Ca^{2+} and cAMP [82] should aid in addressing this challenge.

Synergistic action of the cAMP/PKA and Ca^{2+} signaling

The Ca^{2+} and cAMP signaling systems are the primary regulators of virtually all physiological function. The Ca^{2+} and cAMP signaling can act additively, but most often synergize to generate the final response. Among other advantages, synergism is very economical and greatly buffers the signaling inputs to increase response fidelity. Synergism is an excellent means to generate highly compartmentalized response and then control its spread/diffusion. Most importantly, strong and uncontrolled stimulation of signaling

pathways is highly toxic. Synergism drastically reduces the risk of toxicity due to an over-stimulation of signaling pathways. Synergism between Ca^{2+} and cAMP signaling is widespread and can affect gene regulation [83], neuronal [84], muscle [85], renal [86] and endocrine [87, 88] functions, just to name a few. In secretory epithelia the Ca^{2+} and cAMP signaling synergize to regulate their major function of protein and fluid and electrolyte secretion. Thus, synergism between Ca^{2+} and cAMP signaling regulates gastric pepsinogen [89] and acid secretion [90], exocytosis by pancreatic acini [91], catecholamine secretion [92], mucus secretion by the airway [93, 94], ciliary beat frequency [95], activation of K^{+} and Cl^{-} channels by salivary acinar cells [96], intestinal [97] and airway fluid secretion [98, 99]. In fact, hormonal synergism has been known for more than 80 years (see references in [100]), yet we do not know much about the molecular mechanism of synergism. This began to change with the finding of the role of IRBIT (IP₃ receptors binding protein released with IP₃) in secretory ducts fluid and HCO_3^{-} secretion [58]. Below, we briefly describe the mechanism of ductal HCO_3^{-} secretion, its regulation by the WNK/SPAK and IRBIT/PP1 pathways and how it was used to discover the role of IRBIT in the synergy between the Ca^{2+} and cAMP signaling pathways. Further details can be found in our recent reviews [101–103].

Ductal fluid and HCO_3^{-} secretion involves HCO_3^{-} influx across the basolateral membrane and HCO_3^{-} exit across the luminal membrane. Under physiological conditions most basolateral HCO_3^{-} influx is mediated by Na^{+} - HCO_3^{-} co-transporter that was first identified in rat pancreatic duct [104] and was later documented in the guinea pig [105, 106] and salivary gland ducts [107]. The secretory cells Na^{+} - HCO_3^{-} transporter isoform is the electrogenic NBCe1-B that mediates 1Na^{+} - 2HCO_3^{-} transport [108], resulting in accumulation of cytoplasmic HCO_3^{-} and a net influx of osmolytes. Ductal HCO_3^{-} exit across the luminal membrane is mediated by the coordinated activity of the Cl^{-} channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR) and the Cl^{-} / HCO_3^{-} exchanger slc26a6 [101]. The main function of CFTR is to recirculate the Cl^{-} across the luminal membrane to maintain the activity of slc26a6, although CFTR has finite HCO_3^{-} permeability [109–111] and CFTR-mediated HCO_3^{-} flux becomes important at the distal portion of the ducts [101]. The bulk of HCO_3^{-} secretion is mediated by electrogenic slc26a6. Slc26a6 functions as 1Cl^{-} / 2HCO_3^{-} exchanger in the duct luminal membrane [112–114] to mediate HCO_3^{-} secretion and net solute efflux needed for fluid secretion by the duct. CFTR and slc26a6 regulate the function of each other through interaction of the CFTR R domain and the slc26a6 STAS domain [115].

The activity of NBCe1-B, slc26a6 and CFTR is regulated by the WNK/SPAK and IRBIT/PP1 pathways. The With-No lysine (K) Kinases (WNKs) are members of the MAP kinase superfamily [116] and the SPAK/OSR1 are members of the sterile 20-like kinase superfamily [117]. The major role of the WNKs and SPAK/OSR1 is the regulation of Na^{+} , K^{+} , Cl^{-} , HCO_3^{-} and Ca^{2+} transporters associated with hypertension, by determining their surface expression and/or activity [116, 118, 119]. In many epithelia, the WNKs and SPAK/OSR1 function in the same pathway, where the WNKs scaffold and phosphorylate the SPAK/OSR1 to activate them. This is the case in secretory glands where the WNKs scaffold and recruit SPAK to the transporters to reduce their surface expression and the activity of NBCe1-B [120], Slc26a6 [121] and CFTR [120, 122]. IRBIT competes with IP₃ for binding

to the IP₃ binding site of the IP₃Rs [30] and also activates NBCe1-B [58, 123, 124], CFTR [124] and Slc26a6 [58]. IRBIT activates the transporters by two mechanisms. First, IRBIT recruits the phosphatase PP1 that dephosphorylates the sites phosphorylated by SPAK and thus reverses increases surface expression of the transporters [120, 124]. Then IRBIT removes autoinhibition of the transporters to increase their transport rate [125].

Studying the regulation of slc26a6 by IRBIT led to the discovery of the function of IRBIT as a third messenger that mediates synergistic activation of the Ca²⁺ and cAMP signaling pathways [58]. Expression of recombinant slc26a6 revealed that it is fully activated by maximal stimulation of Gq-coupled receptors and the stimulation required production of IP₃ but not an increase in [Ca²⁺]_i. Nearly maximal activation of slc26a6 is also achieved by concomitant and minimal stimulation of Ca²⁺ and cAMP signaling. Both forms of stimulation markedly increased interaction of slc26a6 with IRBIT. Similar findings were made with CFTR, except that maximal stimulation of CFTR was by maximal stimulation of the cAMP/PKA system [58]. Notably, these findings and the role of IRBIT in the synergistic activation of slc26a6 and CFTR were extended *in vivo* as well. The example traces and summary columns in Fig. 2a show that stimulation of salivary gland ducts with low concentrations of the Ca²⁺ mobilizing carbachol or the cAMP increasing forskolin only minimally activate ductal Cl⁻/HCO₃⁻ exchange, while activation with low concentrations of both carbachol and forskolin markedly activated the exchange. Most of the Cl⁻/HCO₃⁻ exchange in the duct is mediated by slc26a6 [58]. Significantly, knockout of IRBIT eliminated the synergistic activation of the Cl⁻/HCO₃⁻ exchange (Fig. 2a), while only modestly inhibiting Cl⁻/HCO₃⁻ exchange activated by maximal stimulation with carbachol [58]. Fig. 2b shows similar findings with CFTR, in which knockout of IRBIT eliminated the synergistic activation of the native CFTR by minimal but concomitant stimulation with carbachol and forskolin.

Finding the central role of IRBIT in synergizing Ca²⁺ and cAMP signaling raised the question of how IRBIT does so. IRBIT was discovered as a protein that binds to the purified IP₃ receptors and is released by IP₃ [30]. To determine if this is the case also in intact cells, we determined the interaction between IRBIT and IP₃R1 in resting cells and cells stimulated with high or low concentrations of IP₃-generating agonist and with or without low concentration of forskolin. Fig. 3a shows that partial stimulation of the cAMP/PKA system markedly facilitated dissociation of the IP₃Rs-IRBIT complex by low concentration of IP₃ generating agonist. The effect of IP₃Rs stimulation by PKA was further demonstrated by investigating the role of PKA-dependent phosphorylation of IP₃Rs in the dissociation of the IP₃Rs-IRBIT complexes by IP₃. PKA phosphorylate IP₃R1 at S1589 and S1755. Mutation of these sites to the phosphomimetic glutamates (IP₃Rs(SS/EE) or the non-phosphorylatable alanines (IP₃Rs(SS/AA) showed that IP₃Rs(SS/EE) facilitated release of IRBIT from the IP₃Rs, while IP₃Rs(SS/AA) retarded release of IRBIT from the IP₃Rs by low concentration of IP₃ (Fig. 3b). The physiological significance of PKA phosphorylation of IP₃Rs was established by expressing IP₃Rs(SS/AA) in the ducts. Notably, expression of IP₃Rs(SS/AA) eliminated the synergistic activation of ductal slc26a6 (Fig. 3c) and CFTR (Fig. 3d) by the Ca²⁺ and cAMP signaling pathways. Finally, and most significantly, Fig. 4 shows that

knockout of IRBIT eliminated the synergistic stimulation of ductal fluid secretion by the physiological co-activation of the Ca^{2+} and PKA pathway.

The available findings suggest that IRBIT is a central component of the mechanism mediating the synergism between the PKA and Ca^{2+} signaling pathways by functioning as a third messenger that translocates between the IP_3 Rs and target proteins. Another central component is the PKA phosphorylation of IP_3 Rs. For this, the IP_3 Rs, IRBIT, PKA and the effector proteins have to be assembled into complexes in micro or nanodomains to allow efficient shuttling of IRBIT. In resting cells when cellular IP_3 levels are low, IRBIT is bound to the IP_3 Rs, where the IP_3 Rs in effect function to buffer the availability of free IRBIT. Cell stimulation with Gs- and Gq-coupled receptors generates IP_3 and activates PKA. PKA phosphorylates the IP_3 Rs to increase their affinity for IP_3 and at the same time reduces their affinity for IRBIT. This leads to dissociation of IRBIT from the IP_3 receptors and translocation to effector proteins. By translocating from the IP_3 Rs in the ER to effector proteins either in intracellular organelles and/or the plasma membrane IRBIT functions as a third messenger that transmit the information of the second messengers cAMP and IP_3 . At the same time IRBIT integrates and synergizes the activity of the PKA and Ca^{2+} signaling system, providing a molecular mechanism for the synergistic action between them.

So far, the synergism has been demonstrated for the function of the salivary gland and pancreatic ducts. It will be of particular interest to determine whether IRBIT-mediated synergism operates in other physiological functions regulated by the cAMP and Ca^{2+} signaling pathways. These include fluid secretion by salivary gland acinar cells in which cAMP augment IP_3 -mediated Ca^{2+} release [126] and K^+ and Cl^- currents [96]; fluid secretion by airway serous cells that is synergistically stimulated by cAMP and Ca^{2+} increasing receptors (Lee and Foskett, this issue of Cell Calcium). In these specific cases, fluid secretion depends on the activity of the luminal Cl^- channel ANO1 and the basolateral NKCC1 and the K^+ channels MaxiK (KCNMA1) and IK1 (KCNN4). If IRBIT is involved in these and similar forms of epithelial fluid and electrolyte transport, it is possible that IRBIT also regulates the activity of one or several of these ion transporters.

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Fig. 1.
Interactions in the Ca^{2+} and cAMP signaling pathways

Fig. 2. IRBIT mediates the synergistic activation of native slc26a6 and CFTR by the Ca²⁺ and cAMP signaling pathways

Panel (a): Cl⁻/HCO₃⁻ exchange activity was estimated from the change in intracellular pH (pH_{in}) in response to removal and re-addition of external Cl⁻. pH_{in} was measured in isolated duct fragments from the submandibular glands of wild-type (green and blue traces and columns) and IRBIT^{-/-} mice (red trace and column). Panel (b): Ductal CFTR activity was measured as a forskolin-stimulated CFTR_{inh172}-inhibited Cl⁻/NO₃⁻ exchange in wild-type (red and blue traces and columns) and IRBIT^{-/-} (green and magenta traces and columns) ducts. The results were taken from [58].

Fig. 3. PKA-mediated phosphorylation of IP₃R1 facilitates dissociation of IP₃Rs-IRBIT complexes by IP₃

Panel (a) show that IRBIT and IP₃R1 expressed in intact HeLa cells interact and the complexes are dissociated by maximal stimulation of the Gq-coupled P2Y₂ receptors with 10 μ M ATP or by the concomitant stimulation with low concentrations of ATP and forskolin, but not by stimulation with low concentrations of either ATP or forskolin. Panel (b) shows that the IP₃Rs mutants resists (IP₃R1(AA)) and facilitates (IP₃R1(EE)) dissociation of the IRBIT-IP₃R1 complexes. Panels (c, d) show the expression of the mutant IP₃R1(AA) in the duct eliminates the synergistic activation of ductal Cl⁻/HCO₃⁻ exchange (c) and CFTR (d) by low concentrations of carbachol and forskolin. The results were taken from [58].

Fig. 4. IRBIT mediates the synergistic activation of ductal fluid secretion by the PKA and Ca^{2+} signaling pathways

Panels (a, b) show that IRBIT is required for synergistic activation of ductal fluid secretion. Fluid secretion in pancreatic ducts from wild-type (a) and IRBIT^{-/-} (b) mice was measured in sealed ducts in HCO_3^- -buffered media and stimulated with 5 or 0.1 μ M forskolin, 1 μ M carbachol or both 0.1 μ M forskolin and 1 μ M carbachol. The results were taken from [58].