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Review

Role of calcineurin biosignaling in cell secretion and the possible regulatory mechanisms

Hani Alothaid [a,*], Mohammed S.K. Aldughaim [b], Saad Saeed Alamri [b], Jehan Saad M. Alrahimi [c], Saeedah Hamdan Al-Jadani [a]

[a] Department of Basic Sciences, Faculty of Applied Medical Sciences, Al-Baha University, Al-Baha, Saudi Arabia
[b] Research Center, King Fahad Medical City, Riyadh, Saudi Arabia
[c] Department of Biological Sciences, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Cyclic adenosine monophosphate (cAMP) and calcium ions (Ca^{2+}) are two chemical molecules that play a central role in the stimulus-dependent secretion processes within cells. Ca^{2+} acts as the basal signaling molecule responsible to initiate cell secretion. cAMP primarily acts as an intracellular second messenger in a myriad of cellular processes by activating cAMP-dependent protein kinases through association with such kinases in order to mediate post-translational phosphorylation of those protein targets. Put succinctly, both Ca^{2+} and cAMP act by associating or activating other proteins to ensure successful secretion. Calcineurin is one such protein regulated by Ca^{2+}; its action depends on the intracellular levels of Ca^{2+}. Being a phosphatase, calcineurin dephosphorylate and other proteins, as is the case with most other phosphatases, such as protein phosphatase 2A (PP2A), PP2C, and protein phosphatase-1 (PP1), will likely be activated by phosphorylation. Via this process, calcineurin is able to affect different intracellular signaling with clinical importance, some of which has been the basis for development of different calcineurin inhibitors. In this review, the cAMP-dependent calcineurin bio-signaling, protein-protein interactions and their physiological implications as well as regulatory signaling within the context of cellular secretion are explored.

Contents

Abbreviations: cAMP, cyclic adenosine monophosphate; PP, protein phosphatase; RyR, ryanodine receptor; CREB, cAMP-responsive element-binding protein; NFAT, nuclear factor of activated T-cells; ROS, reactive oxygen species; PKA, protein kinase A; Cn, calcineurin.

* Corresponding author at: Department of Basic Sciences, Faculty of Applied Medical Sciences, Al-Baha University, Al-Baha 1988, Saudi Arabia.
E-mail addresses: halothaid@bu.edu.sa (H. Alothaid), maldughaim@kfmc.med.sa (M.S.K. Aldughaim), salamri@kfmc.med.sa (S.S. Alamri), jalrehimi@kau.edu.sa (J.S.M. Alrahimi), saljadaani@bu.edu.sa (S.H. Al-Jadani).

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1. Background

Homeostatic processes in multicellular organisms rely on the release and receipt of a collection of chemical signals such as those involving neurotransmitters and hormones that, in turn, make up the bulk of the signals synthesized and released from different secretory cells. Cyclic adenosine monophosphate (cAMP) and calcium ions (Ca^{2+}) are two such chemical molecules that play a central role in the stimulus-dependent secretion processes within cells. Ca^{2+} acts as the basal signaling molecule responsible to initiate cell secretion (Kallenberg, 2000) in a number of secretory cells. However, its activity is often coupled with cAMP activities. cAMP can either modulate Ca^{2+} release for a cellular function or directly interact with other molecules to activate cellular secretion via Ca^{2+} in a dependent or independent manner (Ozaki et al., 2000). For instance, in cardiac myocytes, cAMP modulates Ca^{2+} release to mediate phosphorylation of ryanodine receptor (RyR) through its RyR calcium/CaM kinase motif (Pereira et al., 2007). cAMP-responsive element-binding protein (CREB) is a transcription factor through which Ca^{2+} and cAMP regulate transcription of several genes. It is noteworthy that Ca^{2+} and cAMP can facilitate phosphorylation of CREB independently in different pathways in order to control the transcription of specific genes (Grewal et al., 2000).

However, cAMP primarily acts as an intracellular second messenger in a myriad of cellular processes by activating cAMP-dependent protein kinases through association with such kinases in order to mediate post-translational phosphorylation of those protein targets. Put succinctly, both Ca^{2+} and cAMP act by associating or activating other proteins to ensure successful secretion. Calcineurin is one such protein regulated by Ca^{2+}; its action depends on the intracellular levels of Ca^{2+}. Being a phosphatase, calcineurin dephosphorylate and other proteins, as is the case with most other phosphatases, such as protein phosphatase 2A (PP2A), PP2C, and protein phosphatase-1 (PP1), will likely be activated by phosphorylation to effect specific phenotypes (Qian et al., 2011; Wigington et al., 2020). Such activities of calcineurin have been the rationale for development of calcineurin inhibitors with significant clinical significance such as in immunosuppression for organ transplant, dermatology and oral disease treatment (Al Johani, Hegarty, Porter, & Fedele, 2009; Farouk & Rein, 2020; Gutfreund et al., 2013). This review discusses the cAMP-dependent calcineurin bio-signaling, the regulation of such signaling, and existing insights into calcineurin regulation in the context of cell secretion. The review also discusses the Ca^{2+} and cAMP-dependent calcineurin bio-signaling within the context of cell secretion.

2. Calcineurin structure-function relationship

Calcineurin (Cn), a calcium-dependent calmodulin associating serine/threonine protein phosphatase, is a key player in several signaling processes such as in secretory, neuronal, muscle, and immune cells. Calcineurin thus mediates signaling events that are driven by Ca^{2+} signals to effect T-cellular processes like the regulation of gene transcription [reviewed in (Aramburu et al., 2000)]. Calcineurin, also known as protein phosphatase 2B (PP2B), mediates its cellular function by interacting with several other factors that act as substrates either in cell compartments or in the cytosol. Structurally, calcineurin has two subunits, 60 kDa enzymatic/catalytic A subunit (CnA) and 19 kDa regulatory B subunit (CnB). CnA contains a catalytic site at the N-terminal (residues 20–340), a binding segment for CnB (residues 349–372) and binding site for CaM C (residues 390–414), a second messenger whose function is modulated by Ca^{2+}, as well as an auto inhibitory domain (residues 469–486) that blocks the catalytic activity of calcineurin after becoming bound. CnB however, is the regulatory component of the protein and strongly interacts with CnA. A projection of 5 turn α-helix away from the catalytic site is the binding segment for CnB, such that the binding of CnB at the site serves to stabilize the helical structure (Jin and Harrison, 2002).

The crystal structure of CnA reveals that it has a central β-sandwich structure wedged in between two β-sheets, while CnB has four Ca^{2+} binding EF-hands. From the EF-hands, Ca^{2+} binds to a high (C-terminal) and low affinity (N-terminal) sites, both of which play crucial roles in CnB activities. The high affinity site at the C-terminus helps impart stability to Cn heterodimeric structure, whereas the low affinity N-terminal site acts as the regulatory site by acting as Ca^{2+} sensor towards elevated or depleted levels of Ca^{2+}. Binding of CaM with four bound Ca^{2+} ions to its site on CnA located between the CnB segment and the auto-inhibitory site leads to formation of an α-helical structure followed by stiffening of the structure to pull the auto-inhibitory site away from the catalytic site (Jin and Harrison, 2002).

3. Regulation of calcineurin

The known major mechanism by which calcineurin is regulated depends upon the intracellular level of Ca^{2+}. As such, calcineurin remain in an inactive form unable to bind CaM Ca in a resting cell where Ca^{2+} concentration is low. In a physiological state at high Ca^{2+} concentration, Ca^{2+} binds to CaM causing a conformational change that favors the interaction between CaM and calcineurin at the site of CnA. This interaction activates its phosphatase activity

in an interaction that is subsequently broken following a decline in Ca^{2+} concentration (Klee et al., 1998).

Calcineurin activity is also regulated by lipidation, such as myristoylation, and interaction with phospholipids. Myristoylation of CnB is a conserved structure–function phenomenon from yeast to humans. This process curtails calcineurin phosphatase activities via calcium signals in the cell (Connolly and Kingsbury, 2012). On the other hand, phospholipids bind to CnB subunit to enhance triptic digestion of calcineurin (Politino and King, 1990). In an *in vitro* study that involved the usage of yeast, the destruction of the phosphatase activity that initiates activation of calcineurin demonstrated the possible role of myristoylation of the CnB subunit in negatively regulating calcineurin activity (Connolly and Kingsbury, 2012). In a study using yeast cells that express *N*-myristoyltransferase at low levels, introduction of mutation to the myristoylation consensus on CnB resulted in three-fold increase in basal activity of non myristoylated-calcineurin, which caused reduced association of calcineurin with the cell membrane as compared to cells that express the wild type CnB, even after being treated with an external Ca^{2+} source. When the Ca^{2+} binding domain of calcineurin was mutated, the increase in basal calcineurin phosphatase was abrogated. As such, disruption of the CnB myristoylation sequence seems to enhance the sensitivity of calcineurin to Ca^{2+}, thus enhancing its phosphatase activity. This demonstrates that myristoylation of calcineurin may indeed serve as a form of negative regulation of calcineurin.

3.1. Phosphorylation-dependent regulation of calcineurin and other ser/thr phosphatases

Apart from Ca^{2+}-dependent regulation of calcineurin, phosphorylation is another crucial post translational regulation of calcineurin activities. Monitoring the release of ^{32}P from trypsin-digested and cyanogen bromide-treated peptides from ^{32}P-calcineurin revealed evidence for phosphorylation-mediated regulation of calcineurin. In addition, the information obtained from direct sequence determination by Hashimoto and Soderling (1989) showed that the phosphorylation sequence was -Arg-Val-Phe-Ser(PO_4)-Val-Leu-Arg-, which is similar to CaM-kinase (Arg-X-X-Ser/ Thr-) phosphorylation consensus at the C-terminus of calcineurin CaM-binding domain. A more recent study of *Aspergillus fumigatus* (Juvvadi et al., 2013) found that calcineurin is phosphorylated at a Serine-Proline rich region that is located between the CnA domains, the CnB-binding helix and the CaM-binding domain. This region contains four clustered serine residues (S406, S408, S410 and S413) that are phosphorylated as depicted in the mutation where mutation in any of the serine residues lead to block in phosphorylation, which is required for virulence in the fungus. Although this region is absent in humans, it still hints at the possibility of a therapeutic target in human diseases caused by *Aspergillus fumigatus*. Whether these sequence motifs are phosphorylated by cAMP/PKA or another known kinase is yet to be determined, thus giving one of the rationales behind the study.

4. Physiological and biological roles of calcineurin

Some of the biological roles of calcineurin have been well-studied using genetics by means deleting either of the calcineurin subunits and studying possible phenotypes. In other instances, the use of immunosuppressants, such as FK506 and cyclosporin A (CsA), both of which inhibit calcineurin in particular, have proven to be resourceful in studying its function (Farouk and Rein, 2020). However, the stimulation or activation of calcineurin by a kinase and the biological effect continues to be elusive. Ubiquitously expressed in mammalian cells, calcineurin is found with the highest expression level in the brain (Baumgartel and Mansuy, 2012). Numerous roles played by calcineurin have been identified, some of which include cardiac function and apoptosis. The role of calcineurin in the activation of T-cells via interaction with nuclear factor of activated T-cells (NFAT) is of more clinical importance. This mechanism was utilized in developing tacrolimus and cyclosporine, immunosuppressants that were used during organ transplantation in order to prevent activation of NFAT-dependent activation of T-cells via inhibition of calcineurin (Lee et al., 2013).

4.1. Calcineurin and cardiac function

Calcineurin with its major target protein, NFAT, have been implicated in the pathogenesis of cardiac hypertrophy and in transducing signals required for cardiac morphogenesis. The development of cardiac hypertrophy and myocardial infarction in transgenic mice overexpressing calcineurin and the perturbation of this condition upon treatment with CsA (Molkentin et al., 1998) is one of the earliest pieces of evidence in support of calcineurin in cardiovascular role. Several studies have demonstrated high levels of calcineurin in knockout mice prior to the development of cardiac hypertrophy (Pillai et al., 2012; Sussman et al., 1999). A study conducted by Sussman et al. (1999) found that the transgenic mice model of hypertrophy, which overexpresses tropomodulin, expresses a high level of calcineurin prior to the development of the hypertrophic phenotype. In addition, Pillai et al. (2012) showed in Nampt knock out mice that high expression of calcineurin is associated with heightened activation of mitogen activated protein (MAP) kinases such as p38, janus kinase (JNK1), and Erk from cardiomyocytes extracted from the mice after treatment with Nampt. Nampt is a protein that plays an important role in the development of diseases such as myocardial infarction, obesity, and diabetes (Pillai et al., 2013). These studies and several others have thus highlighted calcineurin as a possible target for treating cardiac dysfunctions.

In contrast to the above, calcineurin also plays a positive role in the development of cardiovascular tissues. Yang et al. (2014) used tissue-specific deletion of calcineurin B1 subunit (*CnB1*) in the embryonic epicardium of *CnB1*-null mice to postulate that calcineurin-NFAT signaling is required to direct the development of smooth muscle cells. In the mice model, the smooth muscle cells after deletion of *CnB1* developed cardiac dysfunction and failed to mature with reduced exercise capacity. Based on the above evidence, it seems that calcineurin plays a role in both the development and dysfunction of cardiac tissues and cardiovascular processes, which requires a specific level of expression and a delicate balance in the concentration of the protein at any given point in time for normal processes to occur.

4.2. Calcineurin and apoptosis

In cells such as in T- and B-lymphocytes and neuronal cells where calcineurin is abundantly expressed, the protein has been demonstrated to play a key role in apoptosis. In lymphocytes, calcineurin interaction with NFAT induce Fas ligand interaction with its receptor, resulting in apoptosis after T-cell receptor ligation. In neuronal cells, calcineurin and NFAT play the same role demonstrated by Jayanthi et al. (2005) in an attempt to analyze the pathway involved in methamphetamine-induced neuronal cell death. Methamphetamine was found to induce an increase in calcineurin expression and shuttling of NFAT from the cytosol into the nucleus of the methamphetamine-treated cells, thereby further inducing the interaction between Fas ligand and its receptor, causing cell death.

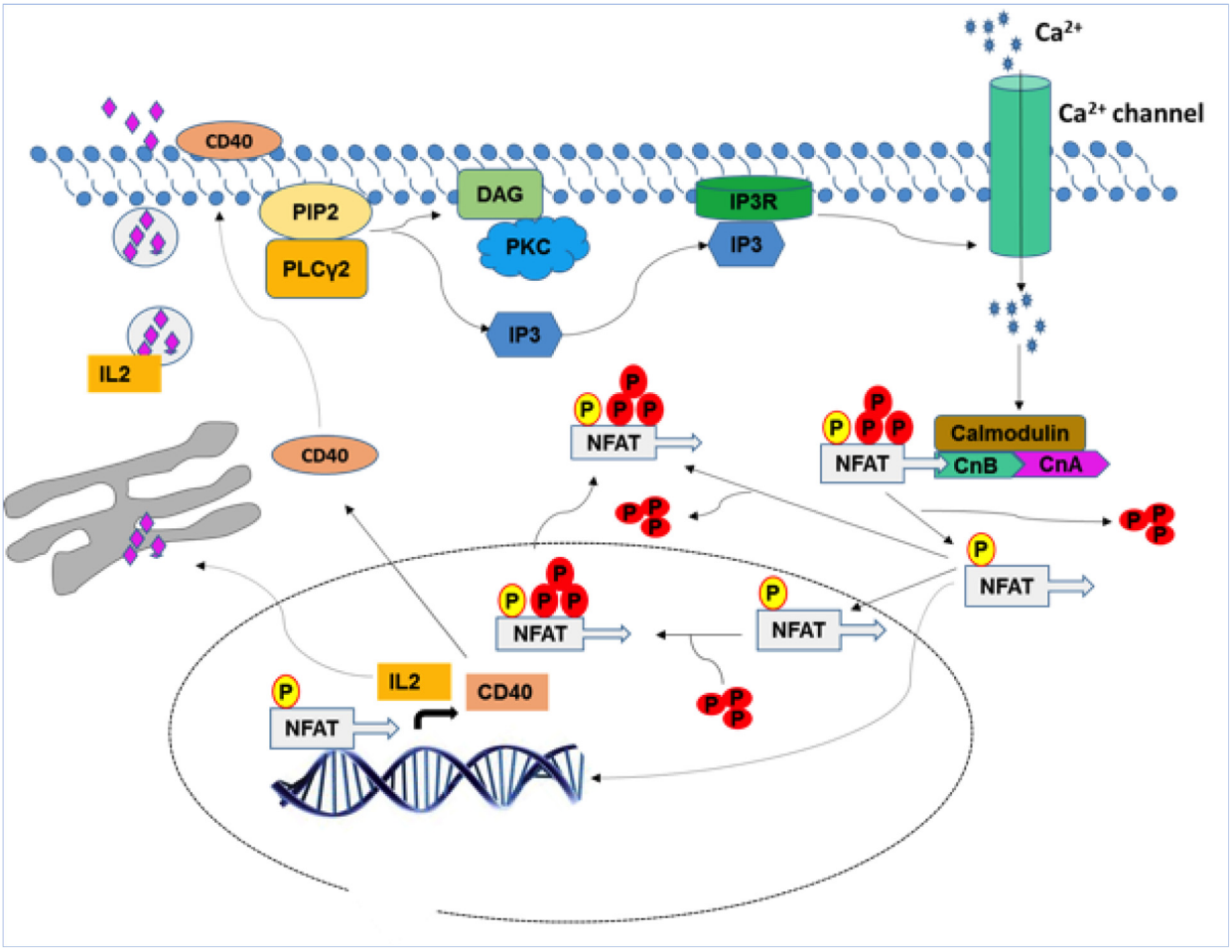

Fig. 1. A calcineurin-NFAT pathway for expression of cytokines and cell membrane proteins. After contacting pathogenic antigens, immune cells, such as dendritic cells, release cytokines such as IL2. Antigens, such as LPS trigger Ca^{2+}, influx into the cell through activation of PLC to breakdown PIP into DAG and IP3. Ca^{2+} released after binding of IP3 to its membrane receptor activates CaM and subsequently, calcineurin interaction with NFAT in order to facilitate its translocation into the nucleus for the transcription of cytokines and membrane proteins.

Proline oxidase is an enzyme that generates reactive oxygen species (ROS) when activated by p53 to sensitize the cells to stress, thus mediating apoptosis. In lung, colon, renal and ovarian carcinoma cells, p53 induces proline oxidase generation of ROS to mediate activation of calcineurin and NFAT in a manner that is ameliorated by treatment with Ca^{2+} channel blocker, calcineurin inhibitors, and ROS scavenger (Rivera and Maxwell, 2005). This indicates that proline oxidase mediates apoptosis by generating ROS that oxidizes calcium into Ca^{2+}, activating calcineurin. This calcineurin activation results in NFAT dephosphorylation, which mediates transcription of apoptotic factors such as TNF-related apoptosis-inducing ligand (TRAIL), leading to apoptosis (Liu et al., 2006).

4.3. Calcineurin and NFAT signaling

As a phosphatase, calcineurin dephosphorylates a wide range of proteins that serve as its substrate, one of which is the NFAT family of proteins 1–4 (NFAT1 to NFAT4). NFAT proteins are a family of transcription factors regulated by Ca^{2+} and its signaling pathway involving calcineurin, and this family assumes significance in several biological processes, such as in cardiovascular development and apoptosis (as elucidated above), and in neurodegenerative, cardiac, inflammatory bowel, and immune diseases (Hudry et al., 2012; Liu et al., 2012, 2011). Calcineurin regulates intracellular translocation of NFAT from the cytosol into the nucleus to facilitate cellular processes such as cell proliferation, differentiation, and growth. In addition, NFAT serves as a transcriptional factor for several cytokines and surface proteins, such as IL-2, IL-4, IFNγ and CD-40, by either binding to DNA dimers or as a co-transcriptional factor upon response to extracellular cues (e.g. activation of cell surface receptors) such as toll-like receptors by pathogenic antigens (e.g. bacterial or fungal lipopolysaccharide) (see Fig. 1).

Calcineurin seem to be the only phosphatase that dephosphorylates NFAT proteins. In lymphocytes, the classic calcineurin-NFAT pathway has been described in detail (Bueno et al., 2002). NFAT exists in a highly phosphorylated state in resting T-cells and is found in the cytosol. Phospholipase Cγ (PLCγ) is activated upon engagement of the T-cell receptors by an antigen, thus hydrolyzing phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce diacylglycerol and inositol-1,4,5-trisphosphate (IP3). IP3 subsequently binds to receptors on endoplasmic reticulum, which leads to the release of Ca^{2+} into the cytoplasm. This, in turn, triggers the release of more Ca^{2+} from Ca^{2+} channels activated by the initial Ca^{2+} release. This increase in the intracellular level of Ca^{2+} activates calcineurin for dephosphorylation of NFAT at a serine residue of its regulatory region (Okamura et al., 2000). Following this, NFAT is translocated into the nucleus where it engages other transcriptional factors, such as AP1 and IL2, in order to facilitate the transcription of specific genes (see Fig. 1).

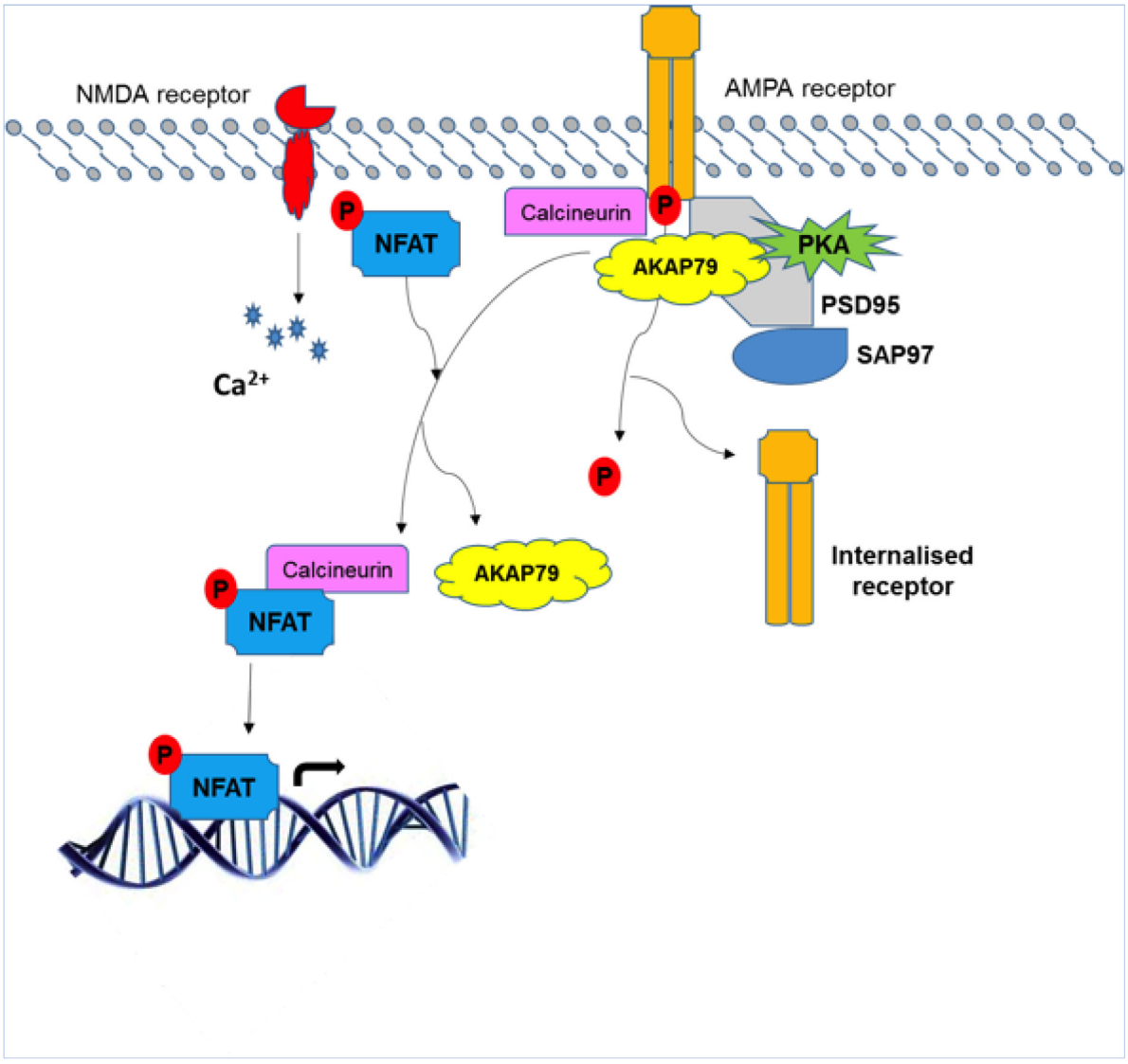

Fig. 2. AKAP79-mediated calcium release during neuronal transmission. AKAP79 targets AMPA receptor to PKA for activation as well as calcineurin to the complex of synaptic protein scaffolds, such as PSD95 and SAP97, thus allowing the NMDA receptor to permit the influx of calcium ions into the cell. AKAP79 also targets calcineurin to NFAT to facilitate nuclear transmission of NFAT by calcineurin.

5. Calcineurin structural interactions

5.1. NFAT

Site-directed mutagenesis has been conducted to determine the amino acid sequence required for NFAT-calcineurin interaction (Aramburu et al., 1998). Synthetic peptides that contain NFAT1 docking sequence PRIEIT, along with another different sequence containing PVIVIT, both of the PxIxIT motif present at the N-terminus, were found to compete with NFAT binding to calcineurin, with the former having more than 50% affinity for calcineurin binding.

In addition to the PxIxIT motif, the LxVP motif located at the C-terminal of NFAT regulatory domain have also been determined to influence calcineurin interaction (Wiedemann et al., 2004). The LxVP motif serves as a blockage to calcineurin phosphatase activity against phosphoRII of type IIα regulatory subunit (RIIα) in protein kinase A (PKA), which is a preferred substrate for calcineurin. Introduction of mutations into the LxVP motif into yeast calcineurin led to reduced interaction of calcineurin with many of its substrates, one of which is RCAN1 (Rodriguez et al., 2009). In particular, immunosuppressants-immunophilin complexes have been found to inhibit the interaction of calcineurin by targeting LxVP sites (Rodriguez et al., 2009). Both the PxIxIT and LxVP motifs are found in several other proteins that bind to calcineurin in addition to NFAT protein family members, thus making it a probable general binding motif for regulation of calcineurin.

5.2. Calcineurin and AKAP79

Human A-kinase anchoring protein 79 (AKAP79) is an anchor molecule, which targets calcineurin, PKA and PKC to certain cellular localizations, such as at contiguous locations to sites of calcium entry, like the *N*-methyl-D-aspartate (NMDA) receptor and the L-type Ca^{2+} channel. AKAP79 exhibits a bidirectional regulation mode for the control of Ca^{2+} signals. Firstly, AKAP79 targets PKA to the L-type Ca^{2+} channel to mediate the enhancement of Ca^{2+} current. During the second control phase, AKAP79 associates with both calcineurin and PKA, which leads to calcineurin dephosphorylating the L-type Ca^{2+} channel, thus attenuating Ca^{2+} current and

inhibiting channel phosphorylation (see Fig. 2) (Oliveria et al., 2007). In turn, this suppresses the activities of PKA on the channel. As observed in all calcineurin-associating proteins, the AKAP79 anchoring sequence, PIAIIIT, used for binding or anchoring calcineurin, is also similar to the NFAT-binding sequence and is found in the same N-terminal. Notably, bound calcineurin anchored on AKAP79 is inhibited, as AKAP79 fragments containing the calcineurin motif successfully block calcineurin-dependent cardiac myocyte hypertrophic growth (Dell'Acqua et al., 2002; Taigen et al., 2000). This is because PIAIIIT competes with PxIxIT of NFAT on calcineurin for docking, thus suggesting that AKAP79 may also function as a negative regulator of calcineurin as both fragments of and full-length AKAP79 inhibit the calcineurin phenotype. This, in turn, gives the impression that AKAP79 renders calcineurin inactive. However, a recent study showed that the recruitment of calcineurin by AKAP79 enhances NFAT signaling through a strong recruitment of calcineurin and subsequent efficient targeted release that allows NFAT activation (Li et al., 2012). In this instance, AKAP79 is capable of serving as a competitive inhibitor for calcineurin interaction with AMPA receptor; for instance, in preventing release of calcineurin for activation of NFAT (see Fig. 2).

6. cAMP-Dependent calcineurin signaling in cell secretion

6.1. cAMP-dependent protein kinase A (PKA) and its regulation

cAMP, a second messenger in cellular signaling, is an enzymatic product of adenylate cyclase, activated by a series of extracellular ligands for the activation of PKA, cyclic nucleotide-regulated ion channels, and Epac (for exchange proteins that are directly activated by cAMP) (Bachmann et al., 2013; Erdogdu et al., 2013; Yu et al., 2013). PKA phosphorylates cytoplasmic and nuclear substrates in processes that are crucial for performing different cellular functions, including metabolism, ion channel activity, synaptic signal transmission, cell differentiation, growth, tissue development, and diseases (Bachmann et al., 2013; Erdogdu et al., 2013; Yu et al., 2013). PKA is considered as the most crucial effector of cAMP activities, even though other proteins, such as the GTP-Exchange Factors (GEFs), are utilized by cAMP, independent of PKA (Cabrera and Ungermann, 2013). PKA comprises two subunits: regulatory and catalytic subunits. For the purpose of optimal catalytic activity, the catalytic subunit of PKA is first phosphorylated by phosphoinositide-dependent protein kinase at a specific threonine residue. In this manner, the activity is switched on and maintained by reduced turnover of the catalytic subunit caused by the phosphorylation (Moore et al., 2002). Subsequently, the catalytic subunit is regulated by its interaction with the regulatory subunit; a receptor for cAMP that also sequesters the catalytic subunit as inactive holoenzyme. cAMP binding to the regulatory subunit of PKA induces dissociation of PKA holoenzyme into the regulatory and catalytic subunits, which is followed by phosphorylation of specific intracellular and extracellular PKA substrates by their catalytic subunit, thus ensuring the initiation of several biological and cellular processes.

6.2. PKA and CFTR interaction in cell secretion

PKA-AKAP interactions regulate several other ion channels; one of which is the cystic fibrosis transmembrane conductance regulator (CFTR). This is a chloride ion channel in which mutation causes cystic fibrosis. CFTR is an epithelial ion channel that is activated by cAMP/PKA for the movement of chloride and bicarbonate ions across the epithelial membrane to aid fluid secretion (Hwang and Kirk, 2013). In doing so, the epithelial membrane strikes a balance between secretion of ions across the membrane and reabsorption of fluids back in through the membrane. This ensures that the epithelial cells provide a protective barrier function against foreign pathogens and ions, and allow normal respiration function of the respiratory system. As that happens, the epithelial layer, with the aid of the ciliary cells on the apical side, drives secreted fluid, mucus, and bacterial cells that are captured within the mucous out of the respiratory tract to prevent infection.

The regulatory domain of CFTR is considered its inhibitor and is released by PKA phosphorylation of CFTR. Nearly two thousand mutations have been identified in the CFTR gene, with many of these mutations occurring in the regulatory domain of CFTR that is responsible for interaction with PKA, thus indicating the inability of CFTR to be phosphorylated as a cause of such mutations, resulting in inactivity (Chappe et al., 2005). One important step

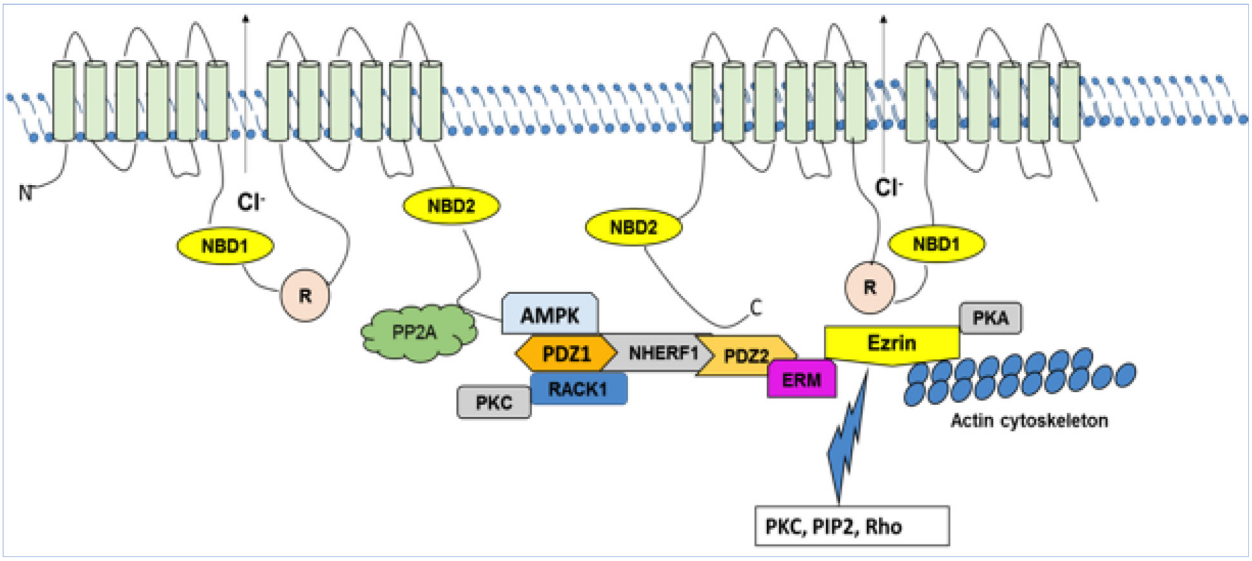

Fig. 3. Regulation of CFTR for chloride transport. Different proteins interact either directly or indirectly with CFTR so as to either inhibit or enhance the activities of the channel. Activation of PP2A causes the phosphatase to dephosphorylate AMP kinase (AMPK), thus activating it. Activated AMPK phosphorylate PDZ1, which activates it to facilitates its interaction with NHEFR1. This, in turn, binds to PDZ2 for anchoring a second CFTR channel by Ezrin. Therefore, this process allows for membrane dimerization of CFTR. ERM, ezrin, radixin, moesin binding domain; NBD, nucleotide-binding domain; R, regulatory domain of CFTR.

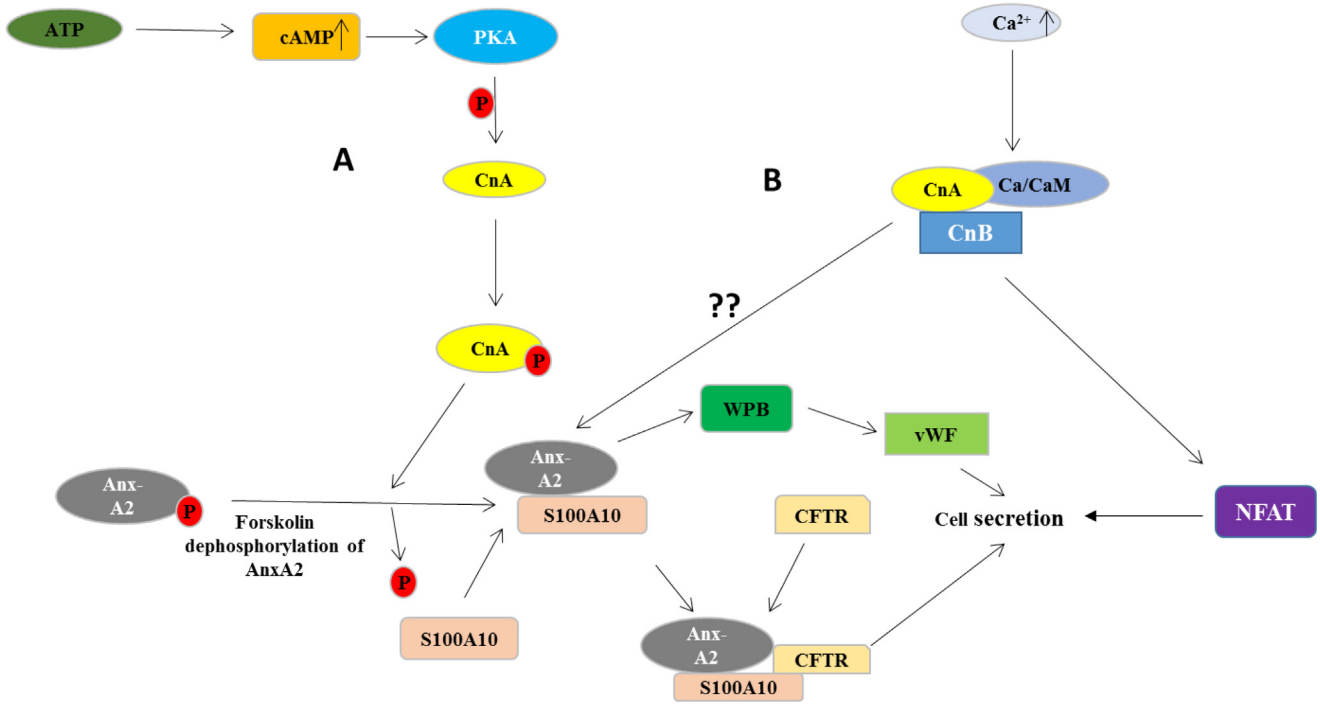

Fig. 4. Calcineurin activation by cAMP/PKA and interaction with CaM for cell secretion. (A) Activation of a CnA-like phosphatase activity by cAMP/PKA has been shown to enhance the release of von Willibrand factor (vWF) from Weibel-Palade bodies (WPB). The complex formed between S100A10 and Annexin A2 (AnxA2) by dephosphorylation of AnxA2 affects this. Notably, this cAMP/PKA activity is likely to work through the activation of CnA phosphatase in regulating AnxA2 and S100A10 complex formation. In the past, S100A10 and AnxA2 have also been shown to aid cell secretion interaction by interacting with CFTR. (B) Ca^{2+}/CaM also activates CnA in a different pathway regulated by Ca^{2+} levels. These pathways (cAMP/PKA- and CaM-dependent activation of CnA) may all be working in concert in order to aid cell secretion.

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001 MAGASELGTGPGAAGGDGDDSLYPIAVLIDELRNEDVQLRLNSIKKLSTIALALGVERTRSELLPF
067 LTDTIYDEDEVLLALAEQLGNFTGLVGGPDFAHCLLPPLENLATVEETVVRDKAVESLRQISQEHT
133 PVALEAYFVPLVKRLASGDWFTSRTSACGLFSVCYPRASNAVKAEIRQQFRSLCSDDTPMVRRAAA
200 SKLGEFAKVLELDSVKSEIVPLFTSLASDEQDSVRLLAVEACVSIAQLLSQDDLETLVMPTLRQAA
266 EDKSWRVRYMVADRFSELQKAMGPKITLNDLIPAFQNLLKDCEAEVRAAAAHKVKELGENLPIEDR
332 ETIIMNQILPYIKELVSDTNQHVKSALASVIMGLSTILGKENTIEHLLPLFLAQLKDECPDVRLNI
398 ISNLDCVNEVIGIRQLSQSLLPAIVELAEDAKWRVRLAIIEYMPLLAGQLGVEFFDEKLNSLCMAW
464 LVDHVYAIREAATNNLMKLVQKFGTEWAQNTIVPKVLVMANDPNYLHRMTTLFCINALSEACGQEI
530 TTKQMLPIVLKMAGDQVANVRFNVAKSLQKIGPILDTNALQGEVKPVLQKLGQDEDMDVKYFAQEA
597 ISVLALA

XXXX Myristoylation site
XXXX cAMP/cGMP Protein dependent kinase phosphorylation site
XXXX PKC phosphorylation site
XXXX CK2 phosphorylation site
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Fig. 5. Amino acid sequence of CnA and possible regulatory sequences. A prosite scan of sequence of CnA was performed at http://prosite.expasy.org/. CK2 and PKC phosphorylation sites are possible for either serine or threonine residues. The PKA phosphorylation site is highlighted in purple as the cAMP protein kinase site. It is also possible to regulate PKA activities through CK2, which has been shown by Rebholz et al. (2009). Thus, the activation of CnA by PKA could either be direct or indirect though the involvement of another protein kinase.

in determining CFTR function is the targeting of PKA to CFTR for phosphorylation by the interaction between PKA, Na^+/H^+ exchanger regulatory factor isoforms NHERF1 or NHERF2 and AKAP78 (ezrin), the last two of which play regulatory and structural roles in the stabilization and regulation of specific plasma membrane-bound proteins. Ezrin acts as a molecular switch, which when interacts with NHEFR1 so as to bind more tightly with CFTR after its activation by PKC, PIP_2, and Rho GTPases. This causes dimerization of CFTR and formation of a microdomain where PKA efficiently activates CFTR (see Fig. 3) (Sun et al., 2000).

7. cAMP-Dependent calcineurin signaling: a new direction in CFTR-mediated cell secretion

CFTR is able to implement ion transport and facilitate fluid secretion by dimerizing and associating with other membrane proteins. Annexin A2 is one of these proteins. It is a soluble protein belonging to the Annexin family of proteins that bind membrane phospholipids in a Ca^{2+} dependent manner (Borthwick et al., 2007). A cAMP/PKA/CnA-dependent multiprotein complex involving Annexin A2 and a calcium binding protein, S100A10, forms a

functional complex with CFTR in such a manner that regulates the channel function in airway epithelial cells. Additionally, dephosphorylation of Annexin A2 is required for the formation and stabilization of the complex between Annexin A2 and S100A10. A cAMP-PKA activated CnA-like phosphatase has also undertaken this dephosphorylation.

Similarly, cAMP/PKA/CnA has been shown to regulate the activities of the transient receptor potential vanilloid type 6 channel (TRPV6) to form a complex with Annexin A2 and S100A10 in a Ca^{2+} independent manner (Borthwick et al., 2008). Inhibition of both cAMP/PKA and calcineurin resulted in more Ca^{2+} uptake in the Caco2 cell line. Activation of a CnA-like phosphatase activity by cAMP/PKA also enhances the release of von Willibrand factor (vWF) and induces exocytosis of Weibel-Palade bodies (WPB) (Brandherm et al., 2013). Since this release is tightly regulated by phosphorylation/dephosphoryla tion on a specific Annexin A2 serine residue, it acts as a switch for the control of vWF release (see Fig. 4). In addition, Annexin A2-S100A2 complex has also been shown to influence forskolin-induced release of vWF from WPB in response to a surge in Ca^{2+} levels (Liu et al., 2015). Forskolin triggers dephosphorylation of Annexin A2 by a CnA-like phosphatase in such a manner that a complex of Annexin A2 and S100A2 is stabilized to promote the release of vWF, thus indicating the strong regulation of Ca^{2+} release and cAMP/PKA phosphorylation of the Annexin A2 complex. Although the formation of a membrane multi-protein complex involving CFTR in facilitating secretion has implicated a CnA like phosphatase, it is currently unknown where the phosphorylation site of PKA is on calcineurin for its activation and the effect of such phosphorylation on CnA functions with respect to its regulation by Ca^{2+}/CaM. However, a cAMP-dependent protein kinase phosphorylation site has been forecasted using the prosite scan online tool (see Fig. 5).

The implication of calcineurin regulation of cell secretion is also evident in the study by Clunes et al. (2012), where it was found that cigarette smoke influences epithelia hydration through reduction in epithelial cell CFTR activities. This reduced CFTR activity was found to be as a result of internalization of CFTR into the detergent-resistant fraction of the cell, and this was linked to Ca^{2+} signaling, impairing cell membrane transport of ions through CFTR. In a recent study, Patel et al. (2019) later found that increased intracellular Ca^{2+} level resulted in reduced conductance through cell surface CFTR, with a consequent reduction in CFTR expression. Interestingly, the impairment of CFTR conductance was found to be directly influenced by Ca^{2+} activation of calcineurin phosphatase activities on CFTR. Taken together, these findings suggest that calcineurin phosphatase activities are associated with Ca^{2+} activation of CnA phosphatase activities to modulate cell secretion.

8. Conclusion

As discussed in this review, calcineurin plays crucial role in cell secretion and other biological processes. These cellular functions have been employed for medical purposes, such as in the development of immunosuppressants [e.g. cyclosporine A (CsA)] used in organ transplantation. Calcineurin biosignaling does not seem to play a crucial role in the innate immune system. However, as discussed above, it seems to play a key role in cell secretion, as exemplified in cystic fibrosis. However, in this disease state, calcineurin regulation seems to be lacking, with little evidence pointing towards cAMP/PKA-dependent regulation. If properly investigated, this may create a new pathway for the treatment of cystic fibrosis and other associated diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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