Published in final edited form as:

Messenger (Los Angel). 2015 June ; 4(1): 53–66. doi:10.1166/msr.2015.1040.

Preferential Coupling of the NAADP Pathway to Exocytosis in T-Cells

Lianne C. Davis*, Frances M. Platt, and Antony Galione*

Department of Pharmacology, University of Oxford, Oxford, OX1 3QT, UK

Abstract

A cytotoxic T-lymphocyte (CTL) kills an infected or tumorigenic cell by Ca^{2+} -dependent exocytosis of cytolytic granules at the immunological synapse formed between the two cells. However, these granules are more than reservoirs of secretory cytolytic proteins but may also serve as unique Ca^{2+} signaling hubs that autonomously generate their own signals for exocytosis. This review discusses a selective role for the Ca^{2+} -mobilizing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) and its molecular targets, two-pore channels (TPCs), in stimulating exocytosis. Given that TPCs reside on the exocytotic granules themselves, these vesicles generate as well as respond to NAADP-dependent Ca^{2+} signals, which may have wider implications for stimulus-secretion coupling, vesicular fusion, and patho-physiology.

Keywords

Ca^{2+} ; NAADP; Exocytosis; TPC; Lysosomes; Cytolytic Granule; CTL; T-Lymphocyte

The Secretory Lysosome

The lysosome is a highly specialized acidic intracellular organelle that mediates a complex set of functions in eukaryotic cells (Saftig and Klumperman, 2009). Lysosomes are the main degradative compartment within the vacuolar system of the cell, where endogenous and exogenous macromolecules derived from biosynthetic and endocytic pathways are degraded, in addition to the catabolism of cytosolic components that are obtained from the autophagic pathway (Saftig and Klumperman, 2009). However, many immune cell types have modified their lysosomal degradative compartment so that it also functions as their secretory compartment—termed a ‘secretory lysosome’ (Blott and Griffiths, 2002). Mechanisms which control the secretory aspects of the lysosome play a role in maintaining immunity to infection depending on the proteins that each cell type synthesizes, stores and secretes. For example, cytotoxic T-lymphocytes (CTLs) and natural killer cells recognize and destroy tumorigenic and virus-infected cells by exocytosing perforin and granzymes from cytolytic

*Authors to whom correspondence should be addressed. lianne.davis@pharm.ox.ac.uk, antony.galione@pharm.ox.ac.uk.

Since the acceptance of this manuscript, a paper has been published demonstrating that acidic stores are a primary source of Ca^{2+} for NAADP-mediated Ca^{2+} release in conventional naive T-cells (Ali et al., 2016).

Ali, R. A., Camick, C., Wiles, K., Walseth, T. F., Slama, J. T., Bhattacharya, S., Giovannucci, D. R., and Wall, K. A. (2016). Nicotinic acid adenine dinucleotide phosphate plays a critical role in naive and effector murine T cells but not natural regulatory T cells. *The Journal of Biological Chemistry*, Doi: 10.1074/jbc.M115.681833.

granules; antigen-presenting cells degrade and process endocytosed material in order to load antigen derived peptides onto MHC class II molecules; mast cells and basophils release histamine stored in their dense core granules upon recognition of IgE to initiate an inflammatory response (Blott and Griffiths, 2002).

As accords with their dual functionality, classical and secretory lysosomes share many biochemical features; the membrane of these organelles contains a set of highly glycosylated proteins (e.g., LAMPs: lysosomal-associated membrane proteins), a proton pump for the maintenance of an acidic luminal pH, and storage of mature acidic hydrolases in their lumen. As the name implies, the most obvious distinction is that secretory, but not classical lysosomes (with the notable exception of plasma membrane-repair mechanisms (Andrews, 2000; Rodriguez et al., 1997)), exocytose their content (Dell'Angelica et al., 2000) (Fig. 1). In CTLs there may be only one population of 'lysosomes' since all lysosomes appear to contain secretory proteins, i.e., the cytolytic granules. These granules possess proteins required for exocytosis, such as SNAREs, synaptotagmins and VAMPs, and also contain unique exocytotic machinery components such as the small molecular weight G-protein Rab27a (Blott and Griffiths, 2002; Griffiths, 2002; Haddad et al., 2001; Stinchcombe et al., 2001).

Exocytosis of Cytosolic Granules Requires Ca^{2+} Signaling

Cytolytic granules provide a simple switch for execution of dual lysosomal and secretory function modulated by pH; the luminal granule acidic pH (5.1–5.4) is optimal for the lysosomal hydrolases but inhibitory for lytic factors; exocytotic release of the lytic granule contents (perforins, granzymes, granulysin) into the more favourable neutral extracellular pH therefore activates the cytolytic proteins (Burkhardt et al., 1990).

It has long been known that elevations in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) trigger the exocytosis of 'secretory lysosomes,' in several different cell types (Camacho et al., 2008; Rodriguez et al., 1997; Xu et al., 2012a), indeed, mast cell degranulation represents one of the earliest described examples of a Ca^{2+} -dependent biological process in non-excitabile cells (Foreman et al., 1973; Kanno et al., 1973). Like regulated secretion events in other cell types, the exocytosis of cytolytic granules is highly orchestrated, involving several distinct steps dependent upon the $[\text{Ca}^{2+}]_i$. Signaling is initiated via the T-cell receptor (TCR) upon contact with antigen-MHC complexes on the target cell, which leads to a large biphasic increase in $[\text{Ca}^{2+}]_i$ due to an initial release of Ca^{2+} from intracellular stores followed by a sustained influx of extracellular Ca^{2+} (Esser et al., 1998; Gray et al., 1987). A complex structure called the immunological synapse forms at the interface between the CTL and the target and attendant $[\text{Ca}^{2+}]_i$ signals mobilize the cytolytic granules for degranulation (Berke, 1994; Griffiths, 1995; Lyubchenko et al., 2001). Cytolytic granules are anchored to microtubules and together with the CTL's microtubule organizing centre reorient towards the immunological synapse before granule contents are vectorially released only at the point of contact with the target (Burkhardt et al., 1993; Kuhn and Poenie, 2002; Kupfer and Dennert, 1984). The pore-forming activity of perforin allows proapoptotic granzymes to gain access to the cytoplasm of the target cell, where they cleave key substrates to initiate apoptotic cell death (Voskoboinik et al., 2006).

How do Cells Generate Different Ca^{2+} Signals?

Although increases in Ca^{2+} can drive various cellular events (including exocytosis and gene expression (Dolmetsch et al., 1998)), not all Ca^{2+} signals are equivalent and particular patterns of Ca^{2+} signals selectively couple to particular downstream responses. How do cells generate a varied palette of Ca^{2+} signals and, thereby, different downstream responses? By selecting a sub-population(s) of Ca^{2+} channels (be they in the plasma membrane or on intracellular Ca^{2+} stores) Ca^{2+} can be focally delivered to a set of proximal Ca^{2+} -decoding proteins without necessarily activating other, distal ones.

On intracellular Ca^{2+} stores there are three distinct ‘receptor’ Ca^{2+} -channel families, each of which uniquely recognizes its cognate second messenger ligand. The ER Ca^{2+} store uses inositol 1,4,5-trisphosphate (IP_3/IP_3 receptors (IP_3R)) and cyclic ADP-ribose (cADPR/ryanodine receptors (RyR)) whereas nicotinic acid adenine dinucleotide phosphate (NAADP) targets different channels and a different Ca^{2+} store: NAADP mobilizes acidic Ca^{2+} stores (Churchill et al., 2002) (a family of organelles including endosomes, lysosomes, lysosome-related organelles and secretory vesicles/lysosomes) probably by activating two-pore channels (TPCs) (albeit with some controversy (Morgan and Galione, 2014)). NAADP is the most potent Ca^{2+} -releasing agent known, ~1000 times higher affinity than IP_3 (Lee and Aarhus, 1995).

Any extracellular stimulus can therefore shape its Ca^{2+} response by ‘mixing and matching’ the second messenger complement to select different populations of Ca^{2+} release channels (Cancela et al., 2002) and deliver Ca^{2+} to subcellular loci with Ca^{2+} -sensitive targets e.g., plasma membrane ion channels, mitochondrial transporters. This signal compartmentation is important for maintaining the fidelity between an extracellular stimuli and its appropriate downstream response.

Indeed, T-cells exhibit a variety of different Ca^{2+} signatures that have physiological consequences. These Ca^{2+} signatures depend not only on the type of T-cell (naïve, cloned, hybridoma) but also its state of maturation, or the nature of the stimulus (the antigen-presenting cell or the antigen itself) (Chen et al., 2010; Lewis, 2001). Downstream, these different Ca^{2+} signatures selectively couple to the expression of different genes (Dolmetsch et al., 1998). In summary, in Ca^{2+} signaling not all sources (or patterns) of intracellular Ca^{2+} are equivalent and Ca^{2+} channels can differentially couple to cellular processes.

Mechanisms of Ca^{2+} Mobilization in CTLs

Although the requirement of an increase in $[\text{Ca}^{2+}]_i$ for CTL activation and degranulation has been known for some time (Imboden et al., 1985; Lancki et al., 1987), the molecular mechanisms by which TCR-mediated Ca^{2+} signaling occurs and the role of this process in stimulating cytolytic granule exocytosis are still incompletely understood. In terms of the sources of Ca^{2+} , it is clear that TCR-induced killing is dependent on both the release of intracellular Ca^{2+} and on Ca^{2+} influx (Zweifach, 2000).

The biphasic Ca^{2+} signal upon TCR activation is comprised of a small intracellular Ca^{2+} release phase followed by a larger Ca^{2+} influx phase; understandably, the predominant Ca^{2+}

influx has therefore garnered more attention in T cells (Dolmetsch and Lewis, 1994; Donnadieu et al., 1992; Lewis and Cahalan, 1989) and has been implicated in supporting granule exocytosis and target-cell killing (Maul-Pavicic et al., 2011).

The major source of Ca^{2+} influx in CTLs is store-operated Ca^{2+} entry where Ca^{2+} channels in the plasma membrane are opened by a signal from depleted ER Ca^{2+} stores (Hogan et al., 2010; Lewis, 2001). Briefly, TCR activation results in the stimulation of phospholipase C (PLC) and generation of IP_3 that releases Ca^{2+} from the ER; a fall in the luminal $[\text{Ca}^{2+}]$ is detected by STIM1 (the ER luminal Ca^{2+} sensor) which in turn forms a complex with and opens ORAI1 (a CRAC channel in the plasma membrane) that mediates Ca^{2+} influx (Feske, 2011; Hogan et al., 2010 and Fig. 4). As a refinement, Ca^{2+} entry through ORAI1 channels at the immunological synapse is modulated by closely apposed mitochondria (Kummerow et al., 2009; Quintana et al., 2007).

Ca^{2+} Release from Intracellular Stores

Due to the small size of the ER in T-cells, Ca^{2+} released from the store results in only a moderate and transient increase in $[\text{Ca}^{2+}]_i$. Although this Ca^{2+} store is somewhat limited in its capacity, it is nonetheless crucial because emptying of this store is the vital trigger to activate store-operated Ca^{2+} channels (Orai) (Feske, 2007; Joseph et al., 2014). Due to their being inextricably linked, it is practically difficult to assess the effect of Ca^{2+} influx in isolation from Ca^{2+} release; activation of Ca^{2+} influx without depleting the ER can now be effected with optogenetic Orai variants (Ishii et al., 2015; Pham et al., 2011) but this has not yet been applied to T-cells to the best of our knowledge. In summary, we conclude that cytosolic Ca^{2+} dynamics (ranging from isolated transients to sustained oscillations and elevated plateaus) are shaped by *both* the Ca^{2+} release from intracellular stores and extracellular Ca^{2+} influx in order to precisely control many T-cell functions, including, T-cell activation and proliferation (Dadsetan et al., 2008; Fanger et al., 1995; Feske et al., 2001; Le Deist et al., 1995).

All three Ca^{2+} mobilizing second messengers, IP_3 , cADPR and NAADP, have been implicated in the generation of Ca^{2+} signals following TCR stimulation by releasing Ca^{2+} from intracellular stores (Berg et al., 2000; Davis et al., 2012; Gasser et al., 2006; Guse et al., 1999; Imboden and Stobo, 1985). IP_3 is the most extensively studied of these and dates back to 1985: the initial phase of T-cell Ca^{2+} signaling has mainly been attributed to IP_3 because upon anti-CD3 TCR stimulation, IP_3 levels increased transiently, IP_3 released Ca^{2+} from stores, and the consequent emptying of the ER Ca^{2+} store triggered Ca^{2+} influx via ORAI1/STIM1 (Guse et al., 1995; Guse et al., 1993; Imboden and Stobo, 1985; Maul-Pavicic et al., 2011). Three isoforms of the IP_3R are expressed in T-cells, each with a characteristic sensitivity to activation by IP_3 and to allosteric regulation by Ca^{2+} (reviewed in (Taylor et al., 2009)).

Like IP_3 , cADPR also acts as a Ca^{2+} -mobilizing messenger in T-cells with levels increasing after anti-CD3 stimulation in Jurkat T-cells through activation of an ADP-ribosyl cyclase (Malavasi et al., 2008); injection of cADPR releases Ca^{2+} from stores through type 3 RyRs, and a cADPR antagonist inhibits TCR-induced Ca^{2+} release (Guse et al., 1999; Kunerth et

al., 2004). Interestingly, IP_3 and cADPR appear to interact functionally so that Ca^{2+} released from the ER via IP_3 receptors acts as a co-activating factor for the ryanodine receptor (Guse et al., 1999). More recently, both resting and stimulated cADPR levels in T-cells were shown to be influenced by modulating precursor substrate (β -NAD⁺) availability (Magnone et al., 2012).

NAADP is the most recent addition to the arsenal of Ca^{2+} mobilizing messengers in lymphocytes, with levels increasing in response to either TCR activation (T-cells) or to IL-8 (in Killer cells): following TCR stimulation, NAADP levels in T-cells are transiently elevated approximately eight-fold within 30 s and precede the global elevation of $[Ca^{2+}]_i$, consistent with NAADP evoking an initial local Ca^{2+} response (Gasser et al., 2006). A secondary smaller rise in NAADP levels was also detected ~5 min after TCR stimulation that was longer lasting (up to 20 min) (Gasser et al., 2006), which points to an additional role of NAADP during the Ca^{2+} plateau phase. Similarly, NAADP increases within seconds and remains sustained upon stimulation of Killer cells with IL-8 (Rah et al., 2010). However, the signal transduction pathway(s) that couples stimuli to NAADP synthesis is unclear (we cannot even agree on the enzymatic route (Palade, 2007), and several transduction pathways have been implicated in driving NAADP increases in various cell types and include Ca^{2+} itself (Morgan et al., 2013; Vasudevan et al., 2010) or cAMP (Rah et al., 2010). CD38 is a multifunctional enzyme that can synthesise cADPR or NAADP (Lee, 2000; Malavasi et al., 2008; Zhang et al., 2010), but its physiological role in NAADP signaling in immune cells is unclear (though CD38 has been linked in several immune contexts including HIV infection, leukaemia (Malavasi et al., 2008)). Physiologically, NAADP has been implicated in regulating T-cell motility and recruitment (Cordiglieri et al., 2010).

NAADP-Mediated Ca^{2+} Release from Intracellular Stores

Whilst a role for NAADP-mediated Ca^{2+} signaling has clearly been shown in T-cells (Davis et al., 2012; Gasser et al., 2006) the Ca^{2+} store and the molecular targets of NAADP have proven unclear and even controversial (Marchant and Patel, 2013; Morgan and Galione, 2014). NAADP appears to release Ca^{2+} from acidic (endolysosomal) Ca^{2+} stores via TPCs in various cell types but in a human Jurkat T-lymphoma cell line some have reported that NAADP activates directly RyR on the ER Ca^{2+} store (Dammermann and Guse, 2005; Langhorst et al., 2004; Steen et al., 2007; Wolf et al., 2015); similar conclusions have been drawn from studies in rat primary T-cells (Cordiglieri et al., 2010). Nonetheless, the more expected coupling of NAADP to acidic Ca^{2+} stores was inferred in primary mouse Killer (LAK) cells (Rah et al., 2010) so this need not be an immune cell peculiarity. We therefore recently sought to clarify these controversies by investigating the mode of action of NAADP in both a primary human CTL clone (ESO 4D8) and a Jurkat T-cell line.

NAADP: Which Ca^{2+} Store?

Using pharmacological tools, we found both pH-neutral ER as well as acidic Ca^{2+} stores in CTLs (Davis et al., 2012) but which couple to NAADP? In contrast to some Jurkat clones (Dammermann et al., 2009), the NAADP-induced Ca^{2+} oscillations of primary CTLs and a 1G4 Jurkat clone were inhibited by agents that abrogate acidic Ca^{2+} stores (bafilomycin A1,

nigericin, monensin, GPN) or by the NAADP antagonist, Ned-19 (Davis et al., 2012; Naylor et al., 2009). Since none of these agents affect IP₃- or cADPR-mediated Ca²⁺ release, the data are consistent with NAADP directly targetting acidic organelles in primary CTLs (ESO 4D8 clone) (Davis et al., 2012).

Although NAADP primarily mobilizes acidic Ca²⁺ stores, this Ca²⁺ release can, in many cell types, secondarily recruit Ca²⁺ channels on the ER. That is, NAADP conforms to the ‘trigger hypothesis’ or two-pool model whereby NAADP provides the initial ‘trigger’ Ca²⁺ from acidic stores that is subsequently amplified by Ca²⁺ release from the ER by virtue of the Ca²⁺-sensitivity of the IP₃R or RyR, so-called Ca²⁺-induced Ca²⁺ release (CICR) (Galione and Churchill, 2002; Patel et al., 2010). Obviously, this is a complicating factor because NAADP can potentially recruit multiple Ca²⁺ sources by direct and indirect means: first, acidic Ca²⁺ stores, then ER Ca²⁺ stores. In T-cells, we confirmed that this is likely to be the case because responses to NAADP were synergistically *enhanced* by co-stimulation with IP₃ but conversely *reduced* by inhibitors of ER Ca²⁺ stores. Although the interaction of the Ca²⁺ circuitry is a complicating factor, we favour the interpretation that T-cells adhere to the more usual paradigm of NAADP directly coupling to acidic stores (whereas ER Ca²⁺ release and SOCE are secondary consequences).

Which Channel is Activated by NAADP?

In a Jurkat T-cell line, NAADP was first suggested to stimulate Ca²⁺ release from the ER via RyR, not via secondary recruitment (CICR) but as a primary event (Cordiglieri et al., 2010; Dammermann and Guse, 2005; Langhorst et al., 2004; Steen et al., 2007; Wolf et al., 2015). This was not without precedent since others had proposed that NAADP activates RyRs (e.g., (Gerasimenko et al., 2003; Hohenegger et al., 2002; Mitchell et al., 2003)), although this would probably have to be via an accessory protein (Guse, 2012; Wagner et al., 2014).

Nonetheless, we provided multiple lines of evidence that NAADP does not elicit Ca²⁺ signals via RyR-mediated Ca²⁺ release from the ER (Davis et al., 2012), the most compelling of which are:

- (a) NAADP-induced Ca²⁺ responses are inhibited by the NAADP receptor antagonist Ned-19 in three different CTL clones: primary human CTL clone (ESO 4D8), Jurkat 1G4 and TALL-104 cells (Fig. 2 and (Davis et al., 2012));
- (b) RyRs are functionally undetectable in primary T-cells (Davis et al., 2012) and entirely absent in Jurkat 1G4 cells (as judged by RT-PCR (Davis et al., 2012)), even though NAADP responses are robust in both (Fig. 2 and (Davis et al., 2012)). The absence of RyRs is also consistent with other Jurkat clones (Bennett et al., 1996; Hosoi et al., 2001) and unequivocally indicates that RyRs cannot be the direct NAADP target in these cells.

If it is not RyRs, what is the channel target of NAADP? Although there is some current controversy (Guse, 2012; Marchant and Patel, 2013; Morgan and Galione, 2014; Yamaguchi et al., 2011), the most compelling candidate Ca²⁺ channel target for NAADP is the endo-lysosomal TPC family that recapitulates the properties of the ‘NAADP receptor’ (Morgan and Galione, 2014). Since Jurkat 1G4 cells provided an excellent null background for RyR,

we utilized these cells in order to determine whether TPCs are targeted by NAADP. Accordingly, in Jurkat 1G4 cells that express both human TPC1 and TPC2, TPC1/2 siRNA significantly reduced the Ca^{2+} signal evoked by NAADP compared to non-targeting siRNA and mock-transfected cells (Davis et al., 2012). This is consistent with NAADP acting via TPCs.

It must be noted that NAADP may not bind directly to its target channels but rather to an intermediary accessory protein: photoaffinity labelling of various cell extracts (including Jurkat T-lymphocytes) revealed a set of polypeptides (20–45 kDa) to which NAADP binds that are much smaller than either TPCs or RyRs (~83–95 and 250 kDa respectively) (Ishibashi et al., 2000; Lin-Moshier et al., 2012; Walseth et al., 2012a; Walseth et al., 2012b). Thus, the NAADP receptor is likely to be a multi-protein complex. Indeed, a separate but promiscuous NAADP-binding protein has been invoked as a possible way to rationalize NAADP regulation of ryanodine receptors reported in some Jurkat T-lymphocyte clones (Guse et al., 1999; Guse et al., 2013).

In summary, intracellular Ca^{2+} release in T-cells is complex and multiple intracellular Ca^{2+} channels are involved depending upon the cell source and include TPCs, RyRs and IP_3 receptors (Dadsetan et al., 2008; Davis et al., 2012; Guse et al., 1999; Guse et al., 1993; Imboden and Stobo, 1985; Steen et al., 2007). The differential expression of different channels and indeed different isoforms of any given channel, is thus likely to play a role in shaping the pattern of $[\text{Ca}^{2+}]_i$ responses (reviewed in (Lewis, 2001)).

What is the Role of NAADP-Mediated Ca^{2+} Signaling in CTL?

If NAADP can indeed mobilize acidic Ca^{2+} stores in CTLs, do they play any physiological role during TCR activation? Several pieces of evidence suggest they do. Upon engagement with antigen-loaded antigen presenting cells, primary CTLs manifest the expected two phases of Ca^{2+} increase (Davis et al., 2012) but, crucially, these TCR-induced Ca^{2+} signals were not only reduced by agents that target acidic Ca^{2+} stores (Bafilomycin A1, nigericin, monensin, GPN) but also by manoeuvres that inhibit the NAADP pathway (the antagonist Ned-19 or by desensitization by NAADP itself) (Davis et al., 2012). Complementing the pharmacology, siRNA knockdown of the NAADP targets, TPCs, similarly reduced TCR-induced Ca^{2+} signals. Together, these results reinforce that NAADP/acidic stores/TPCs are physiologically important for TCR-evoked Ca^{2+} signals.

Granule Translocation

An important consequence of T-cell Ca^{2+} signals is the translocation and exocytosis of the cytolytic granules. The fact that cytolytic granules are actually secretory lysosomes (and the only lysosomal compartment) raised the possibility that they are themselves the acidic Ca^{2+} stores actually targeted by NAADP. Consistent with this, both TPC1 and TPC2 co-localized with markers of the cytolytic granules (Granzyme B and LAMP-1)—with TPC2 showing the strongest correlation—which agrees with the identification of TPC2 in the proteome of the secretory lysosome membrane of the related Natural Killer cells (Casey et al., 2007). What are the consequences of this localization for T-cell function?

After TCR—induced Ca^{2+} signals, the cytolytic granules rapidly polarize along microtubules towards the immunological synapse before undergoing Ca^{2+} -dependent exocytosis at the plasma membrane (Berke, 1994; Bromley et al., 2001; Burkhardt et al., 1993; Griffiths, 1995; Kuhn and Poenie, 2002; Kupfer and Dennert, 1984; Lyubchenko et al., 2001; Monks et al., 1998). Since the TPC targets of NAADP appeared to be localized on the cytolytic granules themselves we tested whether TPC1 and TPC2 are recruited to the immunological synapse by visualizing YFP-tagged channels in live cells. Upon T-cell activation following contact with anti-CD3-coated coverslips, TPCs rapidly migrated from all parts of the cell and clustered at the immunological synapse formed at the contact point (Davis et al., 2012) (Fig. 3). This is the first report of a directed movement and clustering of TPCs upon agonist stimulation.

NAADP is a Selective Stimulus of Exocytosis

In view of the localization and migration of TPC1/TPC2 we tested whether NAADP itself is required for TCR-induced exocytosis. Up to this point, a link between Ca^{2+} influx and exocytosis had been well established (Lyubchenko et al., 2001), but a role for intracellular stores in driving secretion was less well understood.

Given that NAADP signaling contributed to TCR Ca^{2+} signals, we anticipated that NAADP would contribute to downstream exocytosis, but what we found was quite unexpected. First, we measured granzyme B secretion 15 minutes after antigen presentation, i.e., at an early time point. Intriguingly, inhibition of NAADP signaling with Ned-19 and bafilomycin A1 substantially inhibited granzyme B exocytosis and this translated into a reduction in target cell killing (Davis et al., 2012). However, what was unexpected was that this was not merely a trivial consequence of a reduced global Ca^{2+} signal because inhibiting IP_3Rs or Ca^{2+} influx (with 2 μM and 50 μM 2-APB, respectively) lowered the Ca^{2+} signal to similar levels and yet granzyme B secretion and cell killing were unaffected (Davis et al., 2012); this is broadly similar to previous observations with 2-APB (Maul-Pavicic et al., 2011). If *global* Ca^{2+} signals are a poor predictor of exocytosis, one way to rationalize this is that *local* Ca^{2+} signals generated by NAADP are more relevant for secretion than are the global Ca^{2+} signals. Can we find more evidence for this?

Because of the multiplicity of pathways recruited by TCR activation, we tested whether NAADP alone was sufficient to evoke exocytosis of cytolytic granules. It has long been known that TCR-induced exocytosis of cytolytic factors is Ca^{2+} -dependent (Lyubchenko et al., 2001) but, surprisingly, global Ca^{2+} signals (ER Ca^{2+} release and the store-operated Ca^{2+} -influx via STIM/Orai) are not, by themselves, sufficient stimuli for exocytosis; for reasons that are unclear, such Ca^{2+} signals can only evoke secretion if protein kinase C (PKC) is pharmacologically co-activated with phorbol esters (Berrebi et al., 1987; Haverstick and Gray, 1993; Lancki et al., 1987). In view of this, we were not optimistic that NAADP would induce exocytosis without phorbol esters.

Nevertheless, what was remarkable was that NAADP *per se* did evoke cytolytic granule exocytosis and without pharmacological kinase activation: NAADP stimulated exocytosis with a striking bell-shaped concentration-response curve (a characteristic of mammalian

NAADP-induced Ca^{2+} release (Aley et al., 2010; Berg et al., 2000; Davis et al., 2012; Johnson and Misler, 2002; Macgregor et al., 2007; Masgrau et al., 2003; Ruas et al., 2015)) and, this was inhibited by antagonists of NAADP-induced Ca^{2+} release (Ned-19, bafilomycin, desensitizing concentrations of NAADP) (Davis et al., 2012). In contrast, neither IP_3 nor ionomycin could evoke exocytosis (unless combined with phorbol esters to activate PKC/ERK (Grybko et al., 2007; Lancki et al., 1987)). Therefore, NAADP evokes the *only* Ca^{2+} signal that can drive exocytosis without requiring additional stimuli. Considering that the TPC targets of NAADP are located on the cytolytic granules themselves, it suggests that critical, local Ca^{2+} nanodomains around these channels stimulate granule exocytosis. In summary, we hypothesise that cytolytic granules are not only exocytotic vesicles but also acidic Ca^{2+} stores that are mobilized by NAADP via resident TPCs i.e., the vesicles provide their own Ca^{2+} for exocytosis (Fig. 4).

NAADP Versus Other Ca^{2+} Sources: A Unifying Hypothesis for T-Cells?

The uniqueness of NAADP (but not IP_3) in coupling efficiently to exocytosis reinforces the differential physiological roles of different second messengers and different sources of Ca^{2+} . However, how can we rationalize our NAADP hypothesis with two major themes in the literature:

- (a) the overwhelming evidence that Ca^{2+} entry is essential for exocytosis;
- (b) the permissive effect of phorbol esters?

Clearly, Ca^{2+} entry is a requisite for exocytosis, but the fact that it is not a sufficient stimulus is equally beyond doubt. There are several ways that Ca^{2+} entry could be incorporated into our hypothesis without compromising either model. A non-exhaustive list of suggestions are:

- (a) Ca^{2+} entry might be required to maintain the Ca^{2+} -filling state of the NAADP-sensitive store (cytolytic granules) and thereby prevent NAADP-dependent local Ca^{2+} signals from running down;
- (b) Ca^{2+} entry may facilitate TPC activation (a co-agonist with NAADP?) (Pitt et al., 2014);
- (c) acidic Ca^{2+} stores and the ER communicate via Ca^{2+} in both directions to mutually reinforce their respective signals (Morgan et al., 2013); Ca^{2+} entry refills and maintains the ER component which, in turn, could promote local Ca^{2+} release from cytolytic granules;
- (d) by analogy with Ca^{2+} entry supporting IP_3 synthesis (Balla et al., 1994; Wojcikiewicz et al., 1994), Ca^{2+} entry may be important for supporting NAADP synthesis (it is certainly Ca^{2+} -stimulated in some cells (Morgan et al., 2013; Vasudevan et al., 2008; Vasudevan et al., 2010)).

Secondly, what is the connection between Ca^{2+} , phorbol esters and exocytosis? As many authors have shown, mobilization of extracellular or ERCa^{2+} elicits robust global Ca^{2+} signals in T-cells that, by themselves, are surprisingly ineffective at driving exocytosis. However, the very same global Ca^{2+} signals can evoke exocytosis if PKC/ERK is activated

with phorbol esters (Grybko et al., 2007; Lancki et al., 1987). Why then should NAADP not require pharmacological kinase activation? Again, there are multiple potential explanations, of which we offer several candidates:

- (a) local NAADP Ca^{2+} signals naturally facilitate PKC/ERK activation (whereas other Ca^{2+} sources do not);
- (b) phorbol esters result in the phosphorylation of the exocytotic machinery, thereby sensitizing it to Ca^{2+} influx, i.e., normally the low Ca^{2+} -affinity of the machinery means that ER/influx alone cannot attain activating levels (whereas locally high NAADP-induced Ca^{2+} signals can reach the threshold);
- (c) phorbol esters promote NAADP synthesis (and/or confer sensitivity) in response to other Ca^{2+} sources i.e., phorbols mimic NAADP (but in a Ca^{2+} -dependent manner).

It will be invaluable to place NAADP in the context of other Ca^{2+} sources by disentangling these outstanding issues with the literature.

Advantages of Local NAADP Signals

Polarization of TPCs at the immunological synapse potentially provides an architectural explanation for the local Ca^{2+} domains that are known to be required for CTL lytic granule exocytosis (Lyubchenko et al., 2001). Because the single-channel conductance of TPCs is quite modest (Human TPC1 and TPC2 exhibit a Ca^{2+} conductance of 15–40 pS (Brailoiu et al., 2010; Pitt et al., 2010; Pitt et al., 2014)) the clustering of TPCs at the immunological synapse, could collectively favour localized domains of high $[\text{Ca}^{2+}]$ sufficient to activate exocytosis.

Moreover, TPCs join the list of other ion channels that also cluster at the immunological synapse, including the Orai1/STIM1 complex (Lioudyno et al., 2008), and two types of K^+ channels, the voltage-gated Kv1.3 and the Ca^{2+} -activated $\text{K}_{\text{Ca}3.1}$ (Nicolaou et al., 2007a; Nicolaou et al., 2007b; Panyi et al., 2004). It is conceivable that multiple channel families functionally interact to shape signaling at the synapse.

In addition to channels, Ca^{2+} -buffering systems are also recruited to the immunological synapse, including mitochondria (Quintana et al., 2007) and the plasma membrane Ca^{2+} -ATPase (Ritchie et al., 2012). It is tempting to speculate that NAADP provides local Ca^{2+} domains around the cytolytic granules that are shielded from these Ca^{2+} buffering systems (contrasting with ER Ca^{2+} release/ Ca^{2+} entry that are sensitive to them), potentially another reason why NAADP is a more efficient stimulus of exocytosis.

Furthermore, different types of vesicles at the immunological synapse may be quite important. The accumulation, docking and efficient cytolytic granule exocytosis at the immunological synapse in human CTLs is substantially enhanced by their tethering with CD3-containing endosomes by the SNARE protein, Vti 1b, (Qu et al., 2011). Whether TPC1 and TPC2 are localized in two different vesicles that are tethered together or in close proximity to each other is currently unknown, but it has been observed that TPC1 has a

broader disruption in the endocytic pathway, whereas TPC2 is predominantly lysosomal (Morgan et al., 2011). It is therefore possible that TPC1 and TPC2 residing in two closely interacting vesicles may act in concert to evoke cytolytic granule exocytosis; thus the relationship between different acidic vesicles, as well as different TPC isoforms is likely to play a role in shaping the localized Ca^{2+} signal essential for cytolytic granule release at the immunological synapse.

Acidic Ca^{2+} Stores in Immune Disease

Not only do acidic Ca^{2+} stores play a role in physiology, they are also emerging as players in patho-physiology (Morgan et al., 2011). Some lysosomal storage diseases (LSDs) such as Niemann-Pick Type C (NPC) and Mucopolipidosis type IV manifest aberrant Ca^{2+} signaling that profoundly impacts upon cellular physiology because Ca^{2+} is so inextricably linked with normal endo-lysosomal function. NPC manifests defective endo-lysosomal Ca^{2+} release—as judged by reduced NAADP-induced Ca^{2+} release or by reduced Ca^{2+} release through TRPML1 (Shen et al., 2012)—possibly as a consequence of defective Ca^{2+} filling of these vesicles (Lee et al., 2010; Lloyd-Evans et al., 2008; Speak et al., 2014; Visentin et al., 2013; Xu et al., 2012b).

A natural question that arises from this is whether such aberrant endo-lysosomal Ca^{2+} homeostasis has a deleterious effect upon immune cell function, and exocytosis/cell killing in particular (the potentially harmful consequences of inappropriate cytotoxic granule exocytosis have been reviewed (de Saint Basile et al., 2010)). Consistent with our hypothesis, we recently described a defect in cytolytic granule exocytosis in Natural Killer cells from Niemann Pick type C (NPC1-deficient) mice that is associated with reduced lysosomal Ca^{2+} (Speak et al., 2014). As in CTLs, therefore, acidic store Ca^{2+} appears to contribute to the Ca^{2+} release required for Natural Killer cell cytotoxicity. The notion that LSD patients and animal models of LSDs have altered immune cell function is a growing concept (Daly et al., 2000; Jeyakumar et al., 2003; Speak et al., 2014; Wu and Proia, 2004) and raises the possibility that the NAADP pathway may offer a novel site of therapeutic intervention to ameliorate aspects of these diseases.

Similarly, NAADP in T-cells has been implicated as a potential therapy for auto-immune disease (Cordiglieri et al., 2010). Autoaggressive T-cells that mediate inappropriate responses are activated by the NAADP pathway; consequently, NAADP antagonism repressed the autoimmune encephalomyelitis (Cordiglieri et al., 2010).

A General Role for Acidic Ca^{2+} Stores in Exocytosis

The importance of NAADP for exocytosis is not limited to the CTL or Natural Killer cells since it has been implicated in other cellular contexts: formation of the fertilization envelope in eggs (Churchill et al., 2002; Lim et al., 2001; McNeil et al., 2000), the acrosome reaction in sperm (Arndt et al., 2014; Vasudevan et al., 2010), insulin secretion from pancreatic β cells (Arredouani et al., 2015; Kim et al., 2008; Mitchell et al., 2003), exocytosis of von-Willebrand factor from endothelial cells (Esposito et al., 2011), translocation of vesicular

GLUT-4 transporters (Song et al., 2012) and neurotransmitter release at the frog neuromuscular junction (Brailoiu et al., 2003) are all prime examples.

These studies reinforce the concept that a variety of exocytotic vesicles contribute as well as respond to Ca^{2+} signals, i.e., they can themselves be Ca^{2+} stores that release Ca^{2+} (e.g., in response to NAADP). The idea that *intravesicular* Ca^{2+} could be involved in exocytotic processes is not a new concept (Borowitz, 1967) and has been bolstered by the fact that secretory vesicles can contain large amounts of total Ca^{2+} (20–95 mM), consistent with their additional role as dynamic Ca^{2+} stores (Bulenda and Gratzl, 1985; Clemente and Meldolesi, 1975; Fasolato et al., 1991; Gillot et al., 1991; Grinstein et al., 1983; Yoo et al., 2007).

Stronger evidence that secretory vesicles act as Ca^{2+} stores that release Ca^{2+} upon demand arose from the technology to monitor $[\text{Ca}^{2+}]$ around (or within) the vesicles. For example, Ca^{2+} release from secretory vesicles manifests as a local increase in the peri-vesicular space (Gerasimenko et al., 1996; Mitchell et al., 2001; Shen et al., 2012) mirrored by a fall in the luminal $[\text{Ca}^{2+}]$ (Borges et al., 2012; Camacho et al., 2008; Mitchell et al., 2003; Moreno et al., 2005). Needless to say, the cytolytic granules of CTLs or NK cells need to be monitored using similar approaches to confirm and understand how NAADP functions in exocytosis. However, the acidic lumen is, technically, a major confounding factor and Ca^{2+} reporters that are insensitive to the pH in these vesicles will first need to be engineered (Morgan et al., 2015).

One final thought is that exocytotic vesicles are not only Ca^{2+} stores but also, potentially, stores of Ca^{2+} -mobilizing second messengers themselves. This signaling paradigm requires

- (a) messenger-synthesising enzymes within the granule lumen and
- (b) transporters for substrate and product in the vesicle membrane.

Evidence for both has been found in egg exocytotic vesicles (Davis et al., 2008). The idea of secretory vesicles as autonomous signaling hubs (providing messenger, Ca^{2+} , channels and read-out) is an attractive one and demands investigation in T-cells. Indeed, CD38—an enzyme that can synthesise either NAADP or cADPR—polarizes to the immunological synapse in T-cells, one pool of which is vesicular (Munoz et al., 2008) which is consistent with this scheme.

Conclusion

Just as lysosomes now assume a multiplicity of roles, so too do lysosome-related organelles such as secretory lysosomes. In T-cells they are more than reservoirs of secretory cytolytic proteins but also may serve as unique Ca^{2+} signaling hubs that autonomously generate their own signals for exocytosis. The relationship between these vesicles, NAADP and TPCs and, in turn, how they interact with more established signaling pathways in immune cells opens up exciting avenues of future research in patho-physiology.

Acknowledgments

This work was supported by The Wellcome Trust. AG is a Wellcome Trust Senior Investigator. FMP is a Royal Society Wolfson Research Merit Award holder. We thank Dr. Anthony J. Morgan for critically reading this article and for helpful discussions.

Biographies

Lianne C. Davis conducted her doctoral studies in the Department of Pharmacology, University of Oxford, where she worked with Professor Antony Galione and Dr. John Parrington on the characterization of ADP-ribosyl cyclases (the enzymes which synthesize cADPR and NAADP). Following the completion of her Ph.D., Dr. Davis was awarded a Todd-Bird Junior Research Fellowship from New College, Oxford, focusing upon the roles of acidic (endo-lysosomal) organelles in Ca^{2+} signaling, which she held in the laboratories of Professor Antony Galione and Professor Frances Platt.

Frances M. Platt studied for her Ph.D. at the University of Bath in Animal Physiology. In 1989 she joined the Department of Biochemistry, University of Oxford. She was awarded a Lister Institute Senior Research Fellowship in 1996 then moved to the Department of Pharmacology, University of Oxford in 2006 and became Professor of Biochemistry and Pharmacology in 2008. Her main research interest is in sphingolipid lysosomal disorders. In 1999 she was awarded, with Dr. Terry Butters, the Alan Gordon Memorial Award from the UK Gaucher Association and the Horst Bickel Award for advances in metabolic disease therapy. She was elected a Fellow of the Academy of Medical Sciences in 2011 and is a trustee for Gordon Research Conferences.

Antony Galione was educated at Trinity College, Cambridge, and received a B.A. in Natural Sciences and a Ph.D. on the role of Ca^{2+} oscillations in cell activation in Michael Berridge's lab. After a short spell at UCL working on mammalian fertilisation with Michael Whitaker, he went to Johns Hopkins University as a Harkness Fellow studying the role of Ca^{2+} signals in early development. Since joining the Department of Pharmacology, University of Oxford, he has been successively a Beit Memorial Fellow, Wellcome Trust Career Development Fellow and Wellcome Trust Senior Fellow. He was elected to a Tutorial Fellowship at New College in 1998 and elected to the Professorship of Pharmacology at Oxford in 2006. He was Chairman of the Department from 2006–2015. He was elected as a Fellow of the Academy of Medical Sciences in 2010 for his contributions to the advancement of medical science. His primary work has been concerned with the mechanisms and roles of cADPR and NAADP as intracellular messengers.

References

- Aley PK, Mikolajczyk AM, Munz B, Churchill GC, Galione A, Berger F. Nicotinic acid adenine dinucleotide phosphate regulates skeletal muscle differentiation via action at two-pore channels. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:19927–19932. [PubMed: 21041635]
- Andrews NW. Regulated secretion of conventional lysosomes. *Trends in Cell Biology*. 2000; 10:316–321. [PubMed: 10884683]
- Arndt L, Castonguay J, Arlt E, Meyer D, Hassan S, Borth H, Zierler S, Wennemuth G, Breit A, Biel M, Wahl-Schott C, et al. NAADP and the two-pore channel protein 1 participate in the acrosome reaction in mammalian spermatozoa. *Molecular Biology of the Cell*. 2014; 25:948–964. [PubMed: 24451262]
- Arredouani A, Ruas M, Collins SC, Parkesh R, Clough F, Pillinger T, Coltart G, Rietdorf K, Royle A, Johnson P, Braun M, et al. Nicotinic acid adenine dinucleotide phosphate (NAADP) and endolysosomal two-pore channels modulate membrane excitability and stimulus-secretion coupling in mouse pancreatic beta cells. *The Journal of Biological Chemistry*. 2015; 290:21376–21392. [PubMed: 26152717]
- Balla T, Nakanishi S, Catt KJ. Cation sensitivity of inositol 1,4,5-trisphosphate production and metabolism in agonist-stimulated adrenal glomerulosa cells. *The Journal of Biological Chemistry*. 1994; 269:16101–16107. [PubMed: 7515876]
- Bennett DL, Cheek TR, Berridge MJ, De Smedt H, Parys JB, Missiaen L, Bootman MD. Expression and function of ryanodine receptors in nonexcitable cells. *The Journal of Biological Chemistry*. 1996; 271:6356–6362. [PubMed: 8626432]
- Berg I, Potter BV, Mayr GW, Guse AH. Nicotinic acid adenine dinucleotide phosphate (NAADP⁺) is an essential regulator of T-lymphocyte Ca²⁺-signaling. *The Journal of Cell Biology*. 2000; 150:581–588. [PubMed: 10931869]
- Berke G. The binding and lysis of target cells by cytotoxic lymphocytes: Molecular and cellular aspects. *Annual Review of Immunology*. 1994; 12:735–773.
- Berrebi G, Takayama H, Sitkovsky MV. Antigen-receptor interaction requirement for conjugate formation and lethal-hit triggering by cytotoxic T lymphocytes can be bypassed by protein kinase C activators and Ca²⁺ ionophores. *Proceedings of the National Academy of Sciences of the United States of America*. 1987; 84:1364–1368. [PubMed: 3103133]
- Blott EJ, Griffiths GM. Secretory lysosomes. *Nature Reviews Molecular Cell Biology*. 2002; 3:122–131. [PubMed: 11836514]
- Borges R, Dominguez N, Estevez-Herrera J, Pereda D, Machado JD. Vesicular Ca²⁺ mediates granule motion and exocytosis. *Cell Calcium*. 2012; 51:338–341. [PubMed: 22222091]
- Borowitz JL. Calcium binding by subcellular fractions of bovine adrenal medulla. *J Cell Physiol*. 1967; 69:311–319. [PubMed: 5584323]
- Brailoiu E, Patel S, Dun NJ. Modulation of spontaneous transmitter release from the frog neuromuscular junction by interacting intracellular Ca²⁺ stores: Critical role for nicotinic acid-adenine dinucleotide phosphate (NAADP). *Biochem J*. 2003; 373:313–318. [PubMed: 12749764]
- Brailoiu E, Rahman T, Churamani D, Prole DL, Brailoiu GC, Hooper R, Taylor CW, Patel S. An NAADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying Ca²⁺ signals. *The Journal of Biological Chemistry*. 2010; 285:38511–38516. [PubMed: 20880839]

- Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. The immunological synapse. *Annual Review of Immunology*. 2001; 19:375–396.
- Bulenda D, Gratzl M. Matrix free Ca^{2+} in isolated chromaffin vesicles. *Biochemistry*. 1985; 24:7760–7765. [PubMed: 3004565]
- Burkhardt JK, Hester S, Lapham CK, Argon Y. The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. *The Journal of Cell Biology*. 1990; 111:2327–2340. [PubMed: 2277062]
- Burkhardt JK, McIlvain JM Jr, Sheetz MP, Argon Y. Lytic granules from cytotoxic T cells exhibit kinesin-dependent motility on microtubules *in vitro*. *J Cell Sci*. 1993; 104(Pt 1):151–162. [PubMed: 8449993]
- Camacho M, Machado JD, Alvarez J, Borges R. Intravesicular calcium release mediates the motion and exocytosis of secretory organelles: A study with adrenal chromaffin cells. *The Journal of Biological Chemistry*. 2008; 283:22383–22389. [PubMed: 18562320]
- Cancela JM, Van Coppenolle F, Galione A, Tepikin AV, Petersen OH. Transformation of local Ca^{2+} spikes to global Ca^{2+} transients: The combinatorial roles of multiple Ca^{2+} releasing messengers. *The EMBO Journal*. 2002; 21:909–919. [PubMed: 11867519]
- Casey TM, Meade JL, Hewitt EW. Organelle proteomics: Identification of the exocytic machinery associated with the natural killer cell secretory lysosome. *Mol Cell Proteomics*. 2007; 6:767–780. [PubMed: 17272266]
- Chen JL, Morgan AJ, Stewart-Jones G, Shepherd D, Bossi G, Wooldridge L, Hutchinson SL, Sewell AK, Griffiths GM, van der Merwe PA, Jones EY, et al. Ca^{2+} release from the endoplasmic reticulum of NY-ESO-1-specific T cells is modulated by the affinity of TCR and by the use of the CD8 coreceptor. *J Immunol*. 2010; 184:1829–1839. [PubMed: 20053942]
- Churchill GC, Okada Y, Thomas JM, Genazzani AA, Patel S, Galione A. NAADP mobilizes Ca^{2+} from reserve granules, lysosome-related organelles, in sea urchin eggs. *Cell*. 2002; 111:703–708. [PubMed: 12464181]
- Clemente F, Meldolesi J. Calcium and pancreatic secretion. I. Subcellular distribution of calcium and magnesium in the exocrine pancreas of the guinea pig. *The Journal of Cell Biology*. 1975; 65:88–102. [PubMed: 165206]
- Cordiglieri C, Odoardi F, Zhang B, Nebel M, Kawakami N, Klinkert WE, Lodygin D, Luhder F, Breunig E, Schild D, Ulaganathan VK, et al. Nicotinic acid adenine dinucleotide phosphate-mediated calcium signalling in effector T cells regulates autoimmunity of the central nervous system. *Brain: A Journal of Neurology*. 2010; 133:1930–1943. [PubMed: 20519328]
- Dadsetan S, Zakharova L, Molinski TF, Fomina AF. Store-operated Ca^{2+} influx causes Ca^{2+} release from the intracellular Ca^{2+} channels that is required for T cell activation. *The Journal of Biological Chemistry*. 2008; 283:12512–12519. [PubMed: 18316371]
- Daly TM, Lorenz RG, Sands MS. Abnormal immune function *in vivo* in a murine model of lysosomal storage disease. *Pediatric Research*. 2000; 47:757–762. [PubMed: 10832733]
- Dammermann W, Guse AH. Functional ryanodine receptor expression is required for NAADP-mediated local Ca^{2+} signaling in T-lymphocytes. *The Journal of Biological Chemistry*. 2005; 280:21394–21399. [PubMed: 15774471]
- Dammermann W, Zhang B, Nebel M, Cordiglieri C, Odoardi F, Kirchberger T, Kawakami N, Dowden J, Schmid F, Dornmair K, Hohenegger M, et al. NAADP-mediated Ca^{2+} signaling via type 1 ryanodine receptor in T cells revealed by a synthetic NAADP antagonist. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:10678–10683. [PubMed: 19541638]
- Davis LC, Morgan AJ, Chen JL, Snead CM, Bloor-Young D, Shenderov E, Stanton-Humphreys MN, Conway SJ, Churchill GC, Parrington J, Cerundolo V. NAADP activates two-pore channels on T cell cytolytic granules to stimulate exocytosis and killing. *Current Biology: CB*. 2012; 22:2331–2337. [PubMed: 23177477]
- Davis LC, Morgan AJ, Ruas M, Wong JL, Graeff RM, Poustka AJ, Lee HC, Wessel GM, Parrington J, Galione A. Ca^{2+} signaling occurs via second messenger release from intraorganelle synthesis sites. *Current Biology: CB*. 2008; 18:1612–1618. [PubMed: 18951023]

- de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nature Reviews Immunology*. 2010; 10:568–579.
- Dell'Angelica EC, Mullins C, Caplan S, Bonifacio JS. Lysosome-related organelles. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*. 2000; 14:1265–1278. [PubMed: 10877819]
- Dolmetsch RE, Lewis RS. Signaling between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels generates $[\text{Ca}^{2+}]_i$ oscillations in T lymphocytes. *J Gen Physiol*. 1994; 103:365–388. [PubMed: 8195779]
- Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*. 1998; 392:933–936. [PubMed: 9582075]
- Donnadieu E, Bismuth G, Trautmann A. Calcium fluxes in T lymphocytes. *The Journal of Biological Chemistry*. 1992; 267:25864–25872. [PubMed: 1464601]
- Esposito B, Gambaro G, Lewis AM, Palombi F, D'Alessio A, Taylor LX, Genazzani AA, Ziparo E, Galione A, Churchill GC, Filippini A. NAADP links histamine H1 receptors to secretion of von Willebrand factor in human endothelial cells. *Blood*. 2011; 117:4968–4977. [PubMed: 21364192]
- Esser MT, Haverstick DM, Fuller CL, Gullo CA, Braciale VL. Ca^{2+} signaling modulates cytolytic T lymphocyte effector functions. *The Journal of Experimental Medicine*. 1998; 187:1057–1067. [PubMed: 9529322]
- Fanger CM, Hoth M, Crabtree GR, Lewis RS. Characterization of T cell mutants with defects in capacitative calcium entry: Genetic evidence for the physiological roles of CRAC channels. *The Journal of Cell Biology*. 1995; 131:655–667. [PubMed: 7593187]
- Fasolato C, Zottini M, Clementi E, Zacchetti D, Meldolesi J, Pozzan T. Intracellular Ca^{2+} pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release. *The Journal of Biological Chemistry*. 1991; 266:20159–20167. [PubMed: 1939077]
- Feske S. Calcium signalling in lymphocyte activation and disease. *Nature Reviews Immunology*. 2007; 7:690–702.
- Feske S. Immunodeficiency due to defects in store-operated calcium entry. *Annals of the New York Academy of Sciences*. 2011; 1238:74–90. [PubMed: 22129055]
- Feske S, Giltner J, Dolmetsch R, Staudt LM, Rao A. Gene regulation mediated by calcium signals in T lymphocytes. *Nature Immunology*. 2001; 2:316–324. [PubMed: 11276202]
- Foreman JC, Mongar JL, Gomperts BD. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature*. 1973; 245:249–251. [PubMed: 4126372]
- Galione A, Churchill GC. Interactions between calcium release pathways: Multiple messengers and multiple stores. *Cell Calcium*. 2002; 32:343–354. [PubMed: 12543094]
- Gasser A, Bruhn S, Guse AH. Second messenger function of nicotinic acid adenine dinucleotide phosphate revealed by an improved enzymatic cycling assay. *The Journal of Biological Chemistry*. 2006; 281:16906–16913. [PubMed: 16627475]
- Gerasimenko JV, Maruyama Y, Yano K, Dolman NJ, Tepikin AV, Petersen OH, Gerasimenko OV. NAADP mobilizes Ca^{2+} from a thapsigargin-sensitive store in the nuclear envelope by activating ryanodine receptors. *The Journal of Cell Biology*. 2003; 163:271–282. [PubMed: 14568993]
- Gerasimenko OV, Gerasimenko JV, Belan PV, Petersen OH. Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca^{2+} from single isolated pancreatic zymogen granules. *Cell*. 1996; 84:473–480. [PubMed: 8608601]
- Gillot I, Ciapa B, Payan P, Sardet C. The calcium content of cortical granules and the loss of calcium from sea urchin eggs at fertilization. *Developmental Biology*. 1991; 146:396–405. [PubMed: 1864463]
- Gray LS, Gnarr JR, Engelhard VH. Demonstration of a calcium influx in cytolytic T lymphocytes in response to target cell binding. *J Immunol*. 1987; 138:63–69. [PubMed: 3491161]
- Griffiths G. What's special about secretory lysosomes? *Seminars in Cell and Developmental Biology*. 2002; 13:279–284. [PubMed: 12243727]
- Griffiths GM. The cell biology of CTL killing. *Current Opinion in Immunology*. 1995; 7:343–348. [PubMed: 7546398]

- Grinstein S, Furuya W, Vander Meulen J, Hancock RG. The total and free concentrations of Ca^{2+} and Mg^{2+} inside platelet secretory granules. Measurements employing a novel double null point technique. *The Journal of Biological Chemistry*. 1983; 258:14774–14777. [PubMed: 6418738]
- Grybko MJ, Pores-Fernando AT, Wurth GA, Zweifach A. Protein kinase C activity is required for cytotoxic T cell lytic granule exocytosis, but the theta isoform does not play a preferential role. *J Leukoc Biol*. 2007; 81:509–519. [PubMed: 17077164]
- Guse AH. Linking NAADP to ion channel activity: A unifying hypothesis. *Science Signaling*. 2012; 5:pe18. [PubMed: 22534131]
- Guse AH, da Silva CP, Berg I, Skapenko AL, Weber K, Heyer P, Hohenegger M, Ashamu GA, Schulze-Koops H, Potter BV, Mayr GW. Regulation of calcium signalling in T lymphocytes by the second messenger cyclic ADP-ribose. *Nature*. 1999; 398:70–73. [PubMed: 10078531]
- Guse AH, Ernst IM, Fliegert R. NAADP signaling revisited. *Curr Top Med Chem*. 2013; 13:2978–2990. [PubMed: 24171770]
- Guse AH, Goldwisch A, Weber K, Mayr GW. Non-radioactive, isomer-specific inositol phosphate mass determinations: High-performance liquid chromatography-micro-metal-dye detection strongly improves speed and sensitivity of analyses from cells and micro-enzyme assays. *J Chromatogr B Biomed Appl*. 1995; 672:189–198. [PubMed: 8581124]
- Guse AH, Roth E, Emmrich F. Intracellular Ca^{2+} pools in Jurkat T-lymphocytes. *Biochem J*. 1993; 291:447–451. Pt 2. [PubMed: 8484725]
- Haddad EK, Wu X, Hammer JA, Henkart PA. Defective granule exocytosis in Rab27a-deficient lymphocytes from ashen mice. *The Journal of Cell Biology* (3rd). 2001; 152:835–842. [PubMed: 11266473]
- Haverstick DM, Gray LS. Increased intracellular Ca^{2+} induces Ca^{2+} influx in human T lymphocytes. *Molecular Biology of the Cell*. 1993; 4:173–184. [PubMed: 8443415]
- Hogan PG, Lewis RS, Rao A. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annual Review of Immunology*. 2010; 28:491–533.
- Hohenegger M, Suko J, Gscheidlinger R, Drobny H, Zidar A. Nicotinic acid-adenine dinucleotide phosphate activates the skeletal muscle ryanodine receptor. *Biochem J*. 2002; 367:423–431. [PubMed: 12102654]
- Hosoi E, Nishizaki C, Gallagher KL, Wyre HW, Matsuo Y, Sei Y. Expression of the ryanodine receptor isoforms in immune cells. *J Immunol*. 2001; 167:4887–4894. [PubMed: 11673493]
- Imboden JB, Stobo JD. Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *The Journal of Experimental Medicine*. 1985; 161:446–456. [PubMed: 3919143]
- Imboden JB, Weiss A, Stobo JD. The antigen receptor on a human T cell line initiates activation by increasing cytoplasmic free calcium. *J Immunol*. 1985; 134:663–665. [PubMed: 3871211]
- Ishibashi K, Suzuki M, Imai M. Molecular cloning of a novel form (two-repeat) protein related to voltage-gated sodium and calcium channels. *Biochem Biophys Res Commun*. 2000; 270:370–376. [PubMed: 10753632]
- Ishii T, Sato K, Kakumoto T, Miura S, Touhara K, Takeuchi S, Nakata T. Light generation of intracellular Ca^{2+} signals by a genetically encoded protein BACCS. *Nature Communications*. 2015; 6:8021.
- Jeyakumar M, Thomas R, Elliot-Smith E, Smith DA, van der Spoel AC, d'Azzo A, Perry VH, Butters TD, Dwek RA, Platt FM. Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis. *Brain: A Journal of Neurology*. 2003; 126:974–987. [PubMed: 12615653]
- Johnson JD, Misler S. Nicotinic acid-adenine dinucleotide phosphate-sensitive calcium stores initiate insulin signaling in human beta cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:14566–14571. [PubMed: 12381785]
- Joseph N, Reicher B, Barda-Saad M. The calcium feedback loop and T cell activation: how cytoskeleton networks control intracellular calcium flux. *Biochim Biophys Acta*. 2014; 1838:557–568. [PubMed: 23860253]

- Kanno T, Cochrane DE, Douglas WW. Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. *Can J Physiol Pharmacol.* 1973; 51:1001–1004. [PubMed: 4777235]
- Kim BJ, Park KH, Yim CY, Takasawa S, Okamoto H, Im MJ, Kim UH. Generation of nicotinic acid adenine dinucleotide phosphate and cyclic ADP-ribose by glucagon-like peptide-1 evokes Ca^{2+} signal that is essential for insulin secretion in mouse pancreatic islets. *Diabetes.* 2008; 57:868–878. [PubMed: 18184929]
- Kuhn JR, Poenie M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity.* 2002; 16:111–121. [PubMed: 11825570]
- Kummerow C, Junker C, Kruse K, Rieger H, Quintana A, Hoth M. The immunological synapse controls local and global calcium signals in T lymphocytes. *Immunological Reviews.* 2009; 231:132–147. [PubMed: 19754894]
- Kunert S, Langhorst MF, Schwarzmann N, Gu X, Huang L, Yang Z, Zhang L, Mills SJ, Zhang LH, Potter BV, Guse AH. Amplification and propagation of pacemaker Ca^{2+} signals by cyclic ADP-ribose and the type 3 ryanodine receptor in T cells. *J Cell Sci.* 2004; 117:2141–2149. [PubMed: 15054112]
- Kupfer A, Dennert G. Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. *J Immunol.* 1984; 133:2762–2766. [PubMed: 6384372]
- Langcki DW, Weiss A, Fitch FW. Requirements for triggering of lysis by cytolytic T lymphocyte clones. *J Immunol.* 1987; 138:3646–3653. [PubMed: 2953785]
- Langhorst MF, Schwarzmann N, Guse AH. Ca^{2+} release via ryanodine receptors and Ca^{2+} entry: Major mechanisms in NAADP-mediated Ca^{2+} signaling in T-lymphocytes. *Cell Signal.* 2004; 16:1283–1289. [PubMed: 15337527]
- Le Deist F, Hivroz C, Partiseti M, Thomas C, Buc HA, Oleastro M, Belohradsky B, Choquet D, Fischer A. A primary T-cell immunodeficiency associated with defective transmembrane calcium influx. *Blood.* 1995; 85:1053–1062. [PubMed: 7531512]
- Lee H, Lee JK, Min WK, Bae JH, He X, Schuchman EH, Bae JS, Jin HK. Bone marrow-derived mesenchymal stem cells prevent the loss of Niemann-Pick type C mouse Purkinje neurons by correcting sphingolipid metabolism and increasing sphingosine-1-phosphate. *Stem Cells.* 2010; 28:821–831. [PubMed: 20201063]
- Lee HC. Enzymatic functions and structures of CD38 and homologs. *Chemical Immunology.* 2000; 75:39–59. [PubMed: 10851778]
- Lee HC, Aarhus R. A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. *The Journal of Biological Chemistry.* 1995; 270:2152–2157. [PubMed: 7836444]
- Lewis RS. Calcium signaling mechanisms in T lymphocytes. *Annual Review of Immunology.* 2001; 19:497–521.
- Lewis RS, Cahalan MD. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} current in human leukemic T cells. *Cell Regul.* 1989; 1:99–112. [PubMed: 2519622]
- Lim D, Kyozuka K, Gragnaniello G, Carafoli E, Santella L. NAADP⁺ initiates the Ca^{2+} response during fertilization of starfish oocytes. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology.* 2001; 15:2257–2267. [PubMed: 11641253]
- Lin-Moshier Y, Walseth TF, Churamani D, Davidson SM, Slama JT, Hooper R, Brailoiu E, Patel S, Marchant JS. Photoaffinity labeling of nicotinic acid adenine dinucleotide phosphate (NAADP) targets in mammalian cells. *The Journal of Biological Chemistry.* 2012; 287:2296–2307. [PubMed: 22117075]
- Lioudyno MI, Kozak JA, Penna A, Safrina O, Zhang SL, Sen D, Roos J, Stauderman KA, Cahalan MD. Orail and STIM1 move to the immunological synapse and are up-regulated during T cell activation. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105:2011–2016. [PubMed: 18250319]
- Lloyd-Evans E, Morgan AJ, He X, Smith DA, Elliot-Smith E, Sillence DJ, Churchill GC, Schuchman EH, Galione A, Platt FM. Niemann-pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat Med.* 2008; 14:1247–1255. [PubMed: 18953351]

- Lyubchenko TA, Wurth GA, Zweifach A. Role of calcium influx in cytotoxic T lymphocyte lytic granule exocytosis during target cell killing. *Immunity*. 2001; 15:847–859. [PubMed: 11728345]
- Macgregor A, Yamasaki M, Rakovic S, Sanders L, Parkesh R, Churchill GC, Galione A, Terrar DA. NAADP controls cross-talk between distinct Ca^{2+} stores in the heart. *The Journal of Biological Chemistry*. 2007; 282:15302–15311. [PubMed: 17387177]
- Magnone M, Bauer I, Poggi A, Mannino E, Sturla L, Brini M, Zocchi E, De Flora A, Nencioni A, Bruzzone S. NAD^+ levels control Ca^{2+} store replenishment and mitogen-induced increase of cytosolic Ca^{2+} by Cyclic ADP-ribose-dependent TRPM2 channel gating in human T lymphocytes. *The Journal of Biological Chemistry*. 2012; 287:21067–21081. [PubMed: 22547068]
- Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, Vaisitti T, Aydin S. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiological Reviews*. 2008; 88:841–886. [PubMed: 18626062]
- Marchant JS, Patel S. Questioning regulation of two-pore channels by NAADP. *Messenger*. 2013; 2:113–119. [PubMed: 24829847]
- Masgrau R, Churchill GC, Morgan AJ, Ashcroft SJ, Galione A. NAADP: A new second messenger for glucose-induced Ca^{2+} responses in clonal pancreatic beta cells. *Current Biology: CB*. 2003; 13:247–251. [PubMed: 12573222]
- Maul-Pavicic A, Chiang SC, Rensing-Ehl A, Jessen B, Fauriat C, Wood SM, Sjoqvist S, Hufnagel M, Schulze I, Bass T, Schamel WW, et al. ORAI1-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:3324–3329. [PubMed: 21300876]
- McNeil PL, Vogel SS, Miyake K, Terasaki M. Patching plasma membrane disruptions with cytoplasmic membrane. *J Cell Sci*. 2000; 113(Pt 11):1891–1902. [PubMed: 10806100]
- Mitchell KJ, Lai FA, Rutter GA. Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca^{2+} release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). *The Journal of Biological Chemistry*. 2003; 278:11057–11064. [PubMed: 12538591]
- Mitchell KJ, Pinton P, Varadi A, Tacchetti C, Ainscow EK, Pozzan T, Rizzuto R, Rutter GA. Dense core secretory vesicles revealed as a dynamic Ca^{2+} store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimera. *The Journal of Cell Biology*. 2001; 155:41–51. [PubMed: 11571310]
- Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*. 1998; 395:82–86. [PubMed: 9738502]
- Moreno A, Lobaton CD, Santodomingo J, Vay L, Hernandez-SanMiguel E, Rizzuto R, Montero M, Alvarez J. Calcium dynamics in catecholamine-containing secretory vesicles. *Cell Calcium*. 2005; 37:555–564. [PubMed: 15862346]
- Morgan AJ, Davis LC, Galione A. Imaging approaches to measuring lysosomal calcium. *Methods Cell Biol*. 2015; 126:159–195. [PubMed: 25665446]
- Morgan AJ, Davis LC, Wagner SK, Lewis AM, Parrington J, Churchill GC, Galione A. Bidirectional Ca^{2+} signaling occurs between the endoplasmic reticulum and acidic organelles. *The Journal of Cell Biology*. 2013; 200:789–805. [PubMed: 23479744]
- Morgan AJ, Galione A. Two-pore channels (TPCs): Current controversies. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*. 2014; 36:173–183.
- Morgan AJ, Platt FM, Lloyd-Evans E, Galione A. J Molecular mechanisms of endolysosomal Ca^{2+} signalling in health and disease. *Biochem J*. 2011; 439:349–374. [PubMed: 21992097]
- Munoz P, Mittelbrunn M, de la Fuente H, Perez-Martinez M, Garcia-Perez A, Ariza-Veguillas A, Malavasi F, Zubiaur M, Sanchez-Madrid F, Sancho J. Antigen-induced clustering of surface CD38 and recruitment of intracellular CD38 to the immunologic synapse. *Blood*. 2008; 111:3653–3664. [PubMed: 18212246]
- Naylor E, Arredouani A, Vasudevan SR, Lewis AM, Parkesh R, Mizote A, Rosen D, Thomas JM, Izumi M, Ganesan A, Galione A, et al. Identification of a chemical probe for NAADP by virtual screening. *Nat Chem Biol*. 2009; 5:220–226. [PubMed: 19234453]

- Nicolaou SA, Neumeier L, Peng Y, Devor DC, Conforti L. The Ca^{2+} -activated K^{+} channel KCa3.1 compartmentalizes in the immunological synapse of human T lymphocytes. *Am J Physiol Cell Physiol*. 2007a; 292:C1431–1439. [PubMed: 17151145]
- Nicolaou SA, Szigligeti P, Neumeier L, Lee SM, Duncan HJ, Kant SK, Mongey AB, Filipovich AH, Conforti L. Altered dynamics of Kv1.3 channel compartmentalization in the immunological synapse in systemic lupus erythematosus. *J Immunol*. 2007b; 179:346–356. [PubMed: 17579055]
- Palade P. The hunt for an alternate way to generate NAADP. Focus on “NAADP as a second messenger: neither CD38 nor baseexchange reaction are necessary for in vivo generation of NAADP in myometrial cells.”. *Am J Physiol Cell Physiol*. 2007; 292:C4–7. [PubMed: 16899546]
- Panyi G, Vamosi G, Bacso Z, Bagdany M, Bodnar A, Varga Z, Gaspar R, Matyus L, Damjanovich S. Kv1.3 potassium channels are localized in the immunological synapse formed between cytotoxic and target cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:1285–1290. [PubMed: 14745040]
- Patel S, Marchant JS, Brailoiu E. Two-pore channels: Regulation by NAADP and customized roles in triggering calcium signals. *Cell Calcium*. 2010; 47:480–490. [PubMed: 20621760]
- Pham E, Mills E, Truong K. A synthetic photoactivated protein to generate local or global Ca^{2+} signals. *Chem Biol*. 2011; 18:880–890. [PubMed: 21802009]
- Pitt SJ, Funnell TM, Sitsapesan M, Venturi E, Rietdorf K, Ruas M, Ganesan A, Gosain R, Churchill GC, Zhu MX, Parrington J, et al. TPC2 is a novel NAADP-sensitive Ca^{2+} release channel, operating as a dual sensor of luminal pH and Ca^{2+} The *Journal of Biological Chemistry*. 2010; 285:35039–35046. [PubMed: 20720007]
- Pitt SJ, Lam AK, Rietdorf K, Galione A, Sitsapesan R. Reconstituted human TPC1 is a proton-permeable ion channel and is activated by NAADP or Ca^{2+} *Science Signaling*. 2014; 7:ra46. [PubMed: 24847115]
- Qu B, Pattu V, Junker C, Schwarz EC, Bhat SS, Kummerow C, Marshall M, Matti U, Neumann F, Pfreundschuh M, Becherer U, et al. Docking of lytic granules at the immunological synapse in human CTL requires Vti1b-dependent pairing with CD3 endosomes. *J Immunol*. 2011; 186:6894–6904. [PubMed: 21562157]
- Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, Hoth M. T cell activation requires mitochondrial translocation to the immunological synapse. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:14418–14423. [PubMed: 17726106]
- Rah SY, Mushtaq M, Nam TS, Kim SH, Kim UH. Generation of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate by CD38 for Ca^{2+} signaling in interleukin-8-treated lymphokine-activated killer cells. *The Journal of Biological Chemistry*. 2010; 285:21877–21887. [PubMed: 20442403]
- Ritchie MF, Samakai E, Soboloff J. STIM1 is required for attenuation of PMCA-mediated Ca^{2+} clearance during T-cell activation. *The EMBO Journal*. 2012; 31:1123–1133. [PubMed: 22246182]
- Rodriguez A, Webster P, Ortego J, Andrews NW. Lysosomes behave as Ca^{2+} -regulated exocytic vesicles in fibroblasts and epithelial cells. *The Journal of Cell Biology*. 1997; 137:93–104. [PubMed: 9105039]
- Ruas M, Davis LC, Chen CC, Morgan AJ, Chuang KT, Walseth TF, Grimm C, Garnham C, Powell T, Platt N, Platt FM, et al. Expression of Ca^{2+} -permeable two-pore channels rescues NAADP signalling in TPC-deficient cells. *The EMBO Journal*. 2015; 34:1743–1758. [PubMed: 25872774]
- Saftig P, Klumperman J. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nature Reviews Molecular Cell Biology*. 2009; 10:623–635. [PubMed: 19672277]
- Shen D, Wang X, Li X, Zhang X, Yao Z, Dibble S, Dong XP, Yu T, Lieberman AP, Showalter HD, Xu H. Lipid storage disorders block lysosomal trafficking by inhibiting a TRP channel and lysosomal calcium release. *Nature Communications*. 2012; 3:731.

- Song EK, Lee YR, Kim YR, Yeom JH, Yoo CH, Kim HK, Park HM, Kang HS, Kim JS, Kim UH, Han MK. NAADP mediates insulin-stimulated glucose uptake and insulin sensitization by PPARgamma in adipocytes. *Cell Reports*. 2012; 2:1607–1619. [PubMed: 23177620]
- Speak AO, Te Vuchte D, Davis LC, Morgan AJ, Smith DA, Yanjanin NM, Simmons L, Hartung R, Runz H, Mengel E, Beck M, et al. Altered distribution and function of natural killer cells in murine and human Niemann-Pick disease type C1. *Blood*. 2014; 123:51–60. [PubMed: 24235134]
- Steen M, Kirchberger T, Guse AH. NAADP mobilizes calcium from the endoplasmic reticular Ca^{2+} store in T-lymphocytes. *The Journal of Biological Chemistry*. 2007; 282:18864–18871. [PubMed: 17446167]
- Stinchcombe JC, Barral DC, Mules EH, Booth S, Hume AN, Machesky LM, Seabra MC, Griffiths GM. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *The Journal of Cell Biology*. 2001; 152:825–834. [PubMed: 11266472]
- Taylor CW, Rahman T, Tovey SC, Dedos SG, Taylor EJ, Velamakanni S. IP_3 receptors: Some lessons from DT40 cells. *Immunological Reviews*. 2009; 231:23–44. [PubMed: 19754888]
- Vasudevan SR, Galione A, Churchill GC. Sperm express a Ca^{2+} -regulated NAADP synthase. *Biochem J*. 2008; 411:63–70. [PubMed: 18215126]
- Vasudevan SR, Lewis AM, Chan JW, Machin CL, Sinha D, Galione A, Churchill GC. The calcium-mobilizing messenger nicotinic acid adenine dinucleotide phosphate participates in sperm activation by mediating the acrosome reaction. *The Journal of Biological Chemistry*. 2010; 285:18262–18269. [PubMed: 20400502]
- Visentin S, De Nuccio C, Bernardo A, Peponi R, Ferrante A, Minghetti L, Popoli P. The stimulation of adenosine A2A receptors ameliorates the pathological phenotype of fibroblasts from Niemann-Pick type C patients. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 2013; 33:15388–15393. [PubMed: 24068806]
- Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol*. 2006; 6:940–952. [PubMed: 17124515]
- Wagner LE 2nd, Groom LA, Dirksen RT, Yule DI. Characterization of ryanodine receptor type 1 single channel activity using “on-nucleus” patch clamp. *Cell Calcium*. 2014; 56:96–107. [PubMed: 24972488]
- Walseth TF, Lin-Moshier Y, Jain P, Ruas M, Parrington J, Galione A, Marchant JS, Slama JT. Photoaffinity labeling of high affinity nicotinic acid adenine dinucleotide phosphate (NAADP)-binding proteins in sea urchin egg. *The Journal of Biological Chemistry*. 2012a; 287:2308–2315. [PubMed: 22117077]
- Walseth TF, Lin-Moshier Y, Weber K, Marchant JS, Slama JT, Guse AH. Nicotinic acid adenine dinucleotide 2'-phosphate (NAADP) binding proteins in T-lymphocytes. *Messenger (Los Angel)*. 2012b; 1:86–94. [PubMed: 24829846]
- Wojcikiewicz RJ, Tobin AB, Nahorski SR. Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca^{2+} and by rapid desensitization. *J Neurochem*. 1994; 63:177–185. [PubMed: 8207426]
- Wolf IM, Diercks BP, Gattkowski E, Czarniak F, Kempinski J, Werner R, Schetelig D, Mittrucker HW, Schumacher V, von Osten M, Lodygin D, et al. Frontrunners of T cell activation: Initial, localized Ca^{2+} signals mediated by NAADP and the type 1 ryanodine receptor. *Science Signaling*. 2015; 8:ra102. [PubMed: 26462735]
- Wu YP, Proia RL. Deletion of macrophage-inflammatory protein 1 alpha retards neurodegeneration in Sandhoff disease mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:8425–8430. [PubMed: 15155903]
- Xu J, Toops KA, Diaz F, Carvajal-Gonzalez JM, Gravotta D, Mazzoni F, Schreiner F, Rodriguez-Boulan E, Lakkaraju A. Mechanism of polarized lysosome exocytosis in epithelial cells. *J Cell Sci*. 2012a; 125:5937–5943. [PubMed: 23038769]
- Xu M, Liu K, Swaroop M, Porter FD, Sidhu R, Firnkes S, Ory DS, Marugan JJ, Xiao J, Southall N, Pavan WJ, et al. Delta-tocopherol reduces lipid accumulation in Niemann-Pick type C1 and Wolman cholesterol storage disorders. *The Journal of Biological Chemistry*. 2012b; 287:39349–39360. [PubMed: 23035117]

- Yamaguchi S, Jha A, Li Q, Soyombo AA, Dickinson GD, Churamani D, Brailoiu E, Patel S, Muallem S. Transient receptor potential mucolipin 1 (TRPML1) and two-pore channels are functionally independent organellar ion channels. *The Journal of Biological Chemistry*. 2011; 286:22934–22942. [PubMed: 21540176]
- Yoo SH, Chu SY, Kim KD, Huh YH. Presence of secretogranin II and high-capacity, low-affinity Ca^{2+} storage role in nucleoplasmic Ca^{2+} store vesicles. *Biochemistry*. 2007; 46:14663–14671. [PubMed: 18020452]
- Zhang F, Xia M, Li PL. Lysosome-dependent Ca^{2+} release response to Fas activation in coronary arterial myocytes through NAADP: Evidence from CD38 gene knockouts. *Am J Physiol Cell Physiol*. 2010; 298:C1209–1216. [PubMed: 20200208]
- Zweifach A. Target-cell contact activates a highly selective capacitative calcium entry pathway in cytotoxic T lymphocytes. *The Journal of Cell Biology*. 2000; 148:603–614. [PubMed: 10662784]

Figure 1.

A comparison of classical lysosomal Ca^{2+} signaling with Ca^{2+} release from cytolytic granules. Simplistic cartoons of Ca^{2+} and H^+ homeostasis in acidic organelles: the $V-H^+$ -ATPase pumps H^+ into and acidifies the organelle lumen. Ca^{2+} is imported into the lumen by exploiting the energy of the H^+ gradient in some version of Ca^{2+}/H^+ exchange (CHX). Ca^{2+} is released through Ca^{2+} -permeable TPCs gated by NAADP. (A) Lysosomes are important acidic Ca^{2+} stores contributing to distal downstream global Ca^{2+} responses, such as muscle contraction and cell differentiation. (B) Cytolytic granules are essentially the lysosomes of the cytotoxic T-lymphocyte, but with the additional function of being 'secretory lysosomes': Granzyme B (GrB) and Perforin are exocytosed in response to Ca^{2+} release by NAADP in an 'autocrine' fashion (Davis et al., 2012).

Figure 2.

NAADP-induced Ca²⁺ release in Cytotoxic T-lymphocytes. NAADP evokes Ca²⁺ signals in three different cytotoxic T-lymphocytes: a primary human clone (ESO 4D8) and the Jurkat cell line (clone 1G4), used in (Davis et al., 2012) and also TALL-104 cells. (A) Single-cell [Ca²⁺]_i responses normalized to initial fluorescence (F/F₀) upon addition of cell-permeant NAADP/AM (2.5–10 μ M, indicated by arrow). (B) NAADP-elicited Ca²⁺ oscillations were blocked by the NAADP receptor antagonist *trans*-Ned-19 (+Ned, 10 μ M): ****P* < 0.001

versus control (NAADP). Maximal Ca^{2+} changes are expressed as the percentage of control; error bars are mean $\pm$ SEM of 83–152 cells.

Figure 3.

TPCs translocate to an immunological synapse. Confocal images of the 3D distribution of eYFP-tagged human TPC1 (green) expressed in Jurkat 1G4 cells labelled with FM4-64 (red) to delineate the plasma membrane (PM). (A) Under control conditions cells were adhered to glass coverslips coated with poly-L-lysine; TPC puncta were distributed throughout the cell. (B) However, when anti-CD3 was present at the contact surface, a pronounced translocation of TPC to the pseudo-immunological synapse (IS) was observed.

Figure 4.

Cytolytic granules respond to NAADP-induced Ca^{2+} signals to drive their own exocytosis. All three Ca^{2+} mobilizing second messengers, IP_3 , cADPR and NAADP, have been implicated in the generation of Ca^{2+} signals following T cell receptor (TCR) stimulation by a peptide-MHC on an antigen presenting cell (APC). Cytolytic granules (CG) are acidic Ca^{2+} stores that release Ca^{2+} when TPC Ca^{2+} -permeable channels are stimulated by NAADP. In our model, NAADP-induced Ca^{2+} release generates localised Ca^{2+} domains around the granules (in grey) that stimulates exocytosis of Perforin and Granzyme B (GrB). IP_3 and cADPR lead to the release of Ca^{2+} from the endoplasmic reticulum (ER) via IP_3Rs and RyR, respectively. Ca^{2+} depletion from the ER induces store-operated Ca^{2+} -influx via STIM/Orai. These global Ca^{2+} signals are not, by themselves sufficient stimuli for CG exocytosis, unless PKC/ERK are co-activated. The uniqueness of NAADP in evoking the only Ca^{2+} signal that can trigger exocytosis *per se*, reinforces the differential physiological roles of different Ca^{2+} -mobilizing second messengers and different sources of Ca^{2+} .