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STIM and Orai proteins and the non-capacitative ARC channels

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1. ABSTRACT

The ARC channel is a small conductance, highly Ca^{2+}-selective ion channel whose activation is specifically dependent on low concentrations of arachidonic acid acting at an intracellular site. They are widely distributed in diverse cell types where they provide an alternative, store-independent pathway for agonist-activated Ca^{2+} entry. Although biophysically similar to the store-operated CRAC channels, these two conductances function under distinct conditions of agonist stimulation, with the ARC channels providing the predominant route of Ca^{2+} entry during the oscillatory signals generated at low agonist concentrations. Despite these differences in function, like the CRAC channel, activation of the ARC channels is dependent on STIM1, but it is the pool of STIM1 that constitutively resides in the plasma membrane that is responsible. Similarly, both channels are formed by Orai proteins but, whilst the CRAC channel pore is a tetrameric assembly of Orai1 subunits, the ARC channel pore is formed by a heteropentameric assembly of three Orai1 subunits and two Orai3 subunits. There is increasing evidence that the activity of these channels plays a critical role a variety of different cellular activities.

2. IMPORTANCE AND ROLE OF STORE-INDEPENDENT CALCIUM ENTRY

Since the concept of capacitative, or store-operated, Ca^{2+} entry was first defined and characterized by Putney in the mid 1980's (1), the field of agonist-induced Ca^{2+} influx has been largely dominated by this mode of entry. Undoubtedly, evidence has demonstrated that such store-operated Ca^{2+} entry appears to be an almost universal feature of all cell types (2). However, whilst store-operated Ca^{2+} entry has been shown to play a critical role in agonist-induced responses in certain cells – most noticeably in hematopoietic cells such as lymphocytes and mast cells (3; 4) – direct evidence for its essential role in the responses of many other cell types is generally lacking. Instead, its contribution to such responses has often been assumed based simply on its demonstrated presence in such cells, rather than actually directly verified.

That this is something of a concern is emphasized by the fact that examination of many of the particular features of this mode of entry raise several apparent potential problems for an assumed universal role in Ca^{2+} signaling. For example, the prolonged depletion of

intracellular Ca^{2+} stores (most notably the ER) that would necessarily accompany a sustained store-operated Ca^{2+} entry signal might be expected to have significant consequences for various critical cellular activities that rely on adequate Ca^{2+} concentrations within the ER store – such as the correct folding and processing of newly synthesized proteins. In addition, the ability of simple increases in cytosolic Ca^{2+} to selectively induce unique specific responses at different agonist concentrations would be expected to require some form of finely graded response. However, at the individual cell level, studies have indicated that the agonist-induced activation of store-operated Ca^{2+} entry occurs in an essentially "all-or-none" manner (5; 6). Additional potential problems arise when the oscillatory Ca^{2+} responses that are generally considered to represent the more physiologically relevant signal, are considered. Here, evidence (e.g. in pancreatic acinar cells) has indicated that the extent of store depletion even at the peak of each oscillation is minimal – perhaps 2-3% at most (7). Clearly, this is unlikely to be adequate to induce any significant store-operated Ca^{2+} entry, yet a Ca^{2+} entry is demonstrably activated under these conditions and acts to modulate the frequency of the agonist-induced oscillations (8; 9; 10; 11; 12). In fact, recent evidence evaluating the detailed relationship between the extent of store depletion and the activation of STIM1 has only served to confirm this apparent contradiction. Here the data indicate that the oligomerization and mobilization of STIM1 to sites close to the plasma membrane that is an essential prerequisite for activation of the store-operated CRAC channels only occurs after Ca^{2+} concentrations within the ER have fallen by some 35-40% of their original value (13).

Finally, it is worth emphasizing that the activation of such store-operated pathways is, by definition, *exclusively* a result of a depletion of the ER Ca^{2+} stores. However, such depletion can often occur via means that are entirely unrelated to activation by an appropriate agonist. For example, depletion of ER Ca^{2+} can result from a reduced SERCA pump activity, general reductions in metabolic activity, or an increase in the rate of passive leak from the stores etc. Consequently, activation of store-operated Ca^{2+} entry under these circumstances would be expected to result in an inappropriate activation of the various downstream signaling events to which such entry is coupled. Given this, it is perhaps not unreasonable to propose that the principal function for such a store-operated entry of Ca^{2+} might be to simply provide a means to replenish any such "inadvertent" store depletion. In this way, store-operated entry of Ca^{2+} would act as a "biological safety net" for the cell, ensuring that the various cellular processes that rely on adequate levels of Ca^{2+} within the ER (as discussed above), and are critical for cell survival, are maintained.

Over the years, these and other similar considerations have led various groups to explore the possibility of alternative store-independent modes of agonist-activated Ca^{2+} entry. Apart from the relatively non-selective pathways involving the TRPC family of proteins (see the review by Singh *et al.* in this volume), the most extensively studied and thoroughly characterized highly Ca^{2+}-selective pathway is the Ca^{2+} entry pathway activated as a result of the agonist-induced generation of low levels of intracellular arachidonic acid (14; 15; 16; 17). Evidence for the existence of such a pathway is extensive, and includes the demonstration that exogenous administration of low concentrations of arachidonic acid (3-8 μM) induces a clear entry of Ca^{2+} without any detectable depletion of the intracellular stores, that the enzymatic machinery for the production of arachidonic acid is activated by low, physiologically relevant, concentrations of the appropriate agonists, and that pharmacological inhibition of the agonist-induced generation of arachidonic acid prevents the entry of Ca^{2+} associated with the same low concentrations of agonists (18; 19). One point to note here is that cells possess a range of different enzymatic pathways capable of coupling agonist-induced activation of various phospholipases and subsequent metabolism of membrane phospholipids, to the generation of intracellular arachidonic acid. These include direct generation of arachidonic acid from membrane glycerophospholipids in which arachidonic acid is the *sn*-2 substituent via members of the phospholipase A_2 family of enzymes, from phospholipase C-generated diacylglycerol via DAG lipase, and by the action of a phosphatidic acid phosphohydrolase on phosphatidic acid generated via a phospholipase D-dependent pathway. Which of these pathways will provide the predominant route for agonist-mediated generation of arachidonic acid will almost certainly depend on the nature of the agonist and, possibly, its concentration, along with the particular cell type involved. Consequently, the pharmacological inhibition of agonist-induced arachidonic acid generation can be an extremely complex issue. Even more complex is the plethora of bioactive downstream metabolites of arachidonic acid, collectively known as eicosanoids, which include multiple prostaglandins, thromboxanes, leukotrienes, and eicosatrienoic acids. Fortunately, subsequent experiments demonstrated that the specific action of arachidonic acid on Ca^{2+} entry represented an effect of the fatty acid itself, rather than any metabolite, in that it is unaffected by pharmacological inhibition of the enzymes responsible for the metabolism of arachidonic acid (18). Together, these data fulfill all the established essential requirements for the designation of arachidonic acid as a bone-fide second messenger for the activation of a unique agonist-induced, store-independent, pathway for Ca^{2+} entry.

3. PROPERTIES OF ARC CHANNELS

Following the demonstration of the above arachidonic acid activated Ca^{2+} entry pathway in the late 1990's, a search was initiated to identify the underlying conductance responsible. This eventually resulted in the characterization of a relatively small conductance, that was, at least superficially, very similar to the store-operated CRAC channel (20). Like the CRAC channel currents, the arachidonic acid-activated currents displayed all the features of a highly Ca^{2+}-selective conductance, including a markedly inwardly rectifying current-voltage relationship, a reversal potential of greater than +60 mV, and inhibition by La^{3+} (50 μM) and by Gd^{3+} (5 μM). Activation of this novel conductance was achieved at the same low concentrations

of exogenous arachidonic acid that had been shown to induce the entry of Ca^{2+} in the same cells (20; 21). Critically, it was shown that the currents through the arachidonic acid activated conductance and those through the co-existing "CRAC-like" store-operated channels, were strictly additive in the same cell, demonstrating that these two conductances represent entirely distinct channels (20). Consistent with this, additional studies revealed certain clear differences in their detailed biophysical and pharmacological properties. Thus, the arachidonic acid activated currents showed none of the obvious fast-inactivation during brief pulses to negative potentials that is a feature of the CRAC channels, they are insensitive to inhibition by reductions in the external pH, and are unaffected by the drug 2-aminoethoxyldiphenyl borate (2-APB) (20; 21). These novel channels were defined as ARC channels (for **A**rachidonate-**R**egulated **C**a^{2+} channels). Critically, studies involving the application of the low agonist concentrations that could be shown to result in the generation of oscillatory Ca^{2+} signals, revealed that such concentrations resulted in the selective activation of the ARC channels, and not the co-existing store-operated channels (22) (see later).

Further characterization of the activation of the ARC channels showed them to be highly selective for arachidonic acid, in that other polyunsaturated fatty acids, along with monounsaturated fatty acids and saturated fatty acids, were unable to induce significant activation (21). In addition, in contrast to certain members of the "canonical transient receptor potential" (TRPC) family of ion channels, diacylglycerol analogs at concentrations as high as 100 μM, were also without effect. Finally, experiments using arachidonyl coenzyme-A, a membrane-impermeant analog of arachidonic acid, demonstrated that the action of arachidonic acid on the channel was specifically via a site on the inner surface of the membrane (21). Activation of the channel could be demonstrated by the exogenous addition of arachidonic acid at concentrations as low as 2 μM, and increased more than 3-fold up to fatty acid concentrations of 8 μM, the highest concentration used. Importantly, such concentrations are likely to be within the physiologically relevant range for this fatty acid as it is known that most of the cytosolic enzymes involved in the metabolism of intracellular arachidonic acid have Km's in the same low micromolar range (23; 24; 25). However, it should be emphasized that the experimental importance of limiting the concentration of exogenously applied arachidonic acid to the low micromolar range cannot be overemphasized. The amphiphilic nature of arachidonic acid means that, at inappropriate concentrations, it can have profound effects on membrane fluidity and integrity. The latter generally result from the formation of micelles (typically at concentrations of around 10 μM and above) which can induce a variety of effects on membranes (26; 27), including the formation of lipid pores, that result in the indiscriminate passage of large, normally impermeant, ions (e.g. $NMDG^{+}$) across membranes. Whilst such effects are normally readily detectible in electrophysiological measurements, they may go undetected in the more commonly used assays involving fluorescence measurements of changes in cytosolic Ca^{2+} concentrations. Needless to say, at the arachidonic acid concentrations used in the above studies (< 8 μM), no such effects were seen.

Over the subsequent years, conductances displaying the above characteristic features of the ARC channels have been described in several common cell lines (HEK293, HeLa, RBL, COS cells) (21), in SY5Y neuroblastoma cells (28), and in K562 erythroleukemia cells (29), as well as in primary cells such as the exocrine secretory cells of the pancreatic and parotid acini (21), and the insulin-secreting beta cells in the islets of Langerhans (30). Clearly, like the store-operated Ca^{2+} entry channels, the store-independent ARC channels are widely expressed in a variety of different cell types.

4. STIM1 AND THE REGULATION OF ARC CHANNEL ACTIVITY

In the past 4-5 years, our understanding of the nature and regulation of agonist-activated Ca^{2+} entry pathways has been fundamentally transformed by the discoveries of two groups of proteins – the STIM (stromal interacting molecule) proteins (31; 32), and the Orai proteins (33). As detailed elsewhere in this volume, the STIM proteins, particularly STIM1, were identified as the elusive sensor(s) of the depletion of Ca^{2+} in the ER store, an activity that is mediated via a Ca^{2+}-binding EF-hand domain within the N-terminal part of molecule which lies in the lumen of the ER. Loss of Ca^{2+} from this domain as ER Ca^{2+} falls induces the oligomerization of the STIM1 molecules, which then move through the ER membrane to sites close to the plasma membrane (31; 34; 35). Here they interact with the CRAC channels, the pore of which is comprised of Orai1 subunits (36; 37; 38), to induce the opening of the channel. Not surprisingly, given the delay of more than ten years between the biophysical characterization of the CRAC channels and the identification of their molecular composition, these new findings resulted in a flood of reports detailing the underlying mechanisms involved. At the same time, the clear identification of STIM1 as the sensor of store-depletion that is critical for the activation of store-operated Ca^{2+} entry, led to the obvious expectation that this molecule would play no role in the action of the store-independent ARC channels. It therefore came as something of a surprise that depletion of cellular STIM1 protein levels by expression of an appropriate siRNA not only resulted in the inhibition of CRAC channel currents, but also inhibited ARC channel currents to a similar extent (39). In much the same way, overexpression of STIM1 induced a marked increase in ARC channel currents, as well the CRAC channel currents.

The explanation for these unexpected findings came with the realization that, although the identification of STIM1 as the critical sensor of store-depletion in the activation of the CRAC channels was an entirely novel finding, the protein had actually been first described almost nine years earlier (40), and subsequently definitively characterized as a plasma membrane protein in 2000 (41). In these studies, STIM1 (previously known as GOK or SIM) was shown to be a suppressor of cell growth in

various tumor cells and, consistent with its expression in the plasma membrane, to be involved in interactions with pre B lymphocytes in the bone marrow stroma. Given this, we wondered whether the STIM1 that was constitutively resident in the plasma membrane might be the relevant pool for the regulation of ARC channel activity. Biotinylation studies indicated that approximately 10-15% of the total cellular STIM1 was present in the plasma membrane in HEK293 cells under resting conditions. Addition of exogenous arachidonic acid to activate the ARC channels induced no obvious change in the size of this pool. The original studies on STIM1 had shown that the delivery and/or insertion of the molecule into the plasma membrane depended on *N*-linked glycosylation at two sites in the N-terminal region (N131, and N171) (42). However, the experiments described in this study involved the use of tunicamycin, which might have blocked surface expression of STIM1 by means other than an effect on glycosylation. Because of this, we chose to use an alternative, mutational approach to examine the effect of surface expression of STIM1 on ARC and CRAC channel activity. In this, we first knocked-down the endogenous STIM1 using a standard siRNA approach, and then essentially replaced this with an siRNA-resistant form of either the wild-type STIM1 (as a control), or an siRNA-resistant mutant STIM1 in which the two above *N*-linked glycosylation sites had been mutated to glutamines. The results obtained showed that expression of the glycosylation-mutant STIM1 resulted in a profound inhibition of measured ARC channel currents, whilst the co-existing CRAC channel currents were entirely unaffected (39). These data were further supported by the finding that, in intact cells, application of an antibody specific to a region within the extracellular N-terminal of STIM1 resulted in the inhibition of ARC channel currents without affecting the co-existing CRAC channel currents. It should be noted however that Spassova *et al.* (43) reported that CRAC channel activity was also susceptible to an externally applied STIM1 antibody. The reasons for the discrepancy between these two studies is unclear, but the experimental approaches did differ somewhat. In particular, in the latter study, the cells were kept at 4°C for up to 60 minutes immediately prior to evaluation of CRAC channel activity, which was then recorded as monovalent currents in the absence of external divalent cations, and higher concentrations of antibody (20 μg/ml versus 5 μg/ml) were used.

The above findings have several, potentially important, implications. First, they confirm that STIM1 should not be considered, or described, as an exclusively ER-resident protein – a pool of the total cellular STIM1 (approximately 10-25%, depending on cell type) is constitutively resident in the plasma membrane. The presence of the protein in this location is not apparently a result of cell stimulation – it is present even in resting cells (39). Secondly, STIM1 is essential for the activation of both the store-operated CRAC channels and store-independent ARC channels. Therefore, simple inhibition of Ca^{2+} entry induced by the knock-down or elimination of STIM1 cannot be used as a criteria to specifically ascribe such entry to a store-operated or CRAC channel-mediated process. Finally, the orientation of the STIM1 molecules resident in the plasma membrane is such that the N-terminal EF-hand will be exposed to the extracellular environment. Given that Ca^{2+} concentrations in this environment are typically in the 1-3 mM range, and are usually tightly regulated, leads to the conclusion that the Ca^{2+}-binding behavior of the EF-hand of STIM1, that is so critical to its role in regulation of the store-operated CRAC channels, is unlikely to play any role in the activation of the ARC channels. This immediately raises the question of how the STIM1 in the plasma membrane acts to determine or regulate the activation of the ARC channels by arachidonic acid. Does arachidonic acid initiate an interaction between STIM1 and the channel in a manner analogous to its action in activating the CRAC channels? Or is STIM1 constitutively associated with the channel in a pre-assembled complex that is sensitive to activation by arachidonic acid? As yet, there are no data providing any insight into these issues, but the new information on the molecular nature of the ARC channel (see below) may provide the tools to begin to address these questions.

5. ORAI PROTEINS AND THE MOLECULAR MAKE-UP OF ARC CHANNELS

As noted above, the second major breakthrough in our understanding of the molecular basis of agonist-activated Ca^{2+}-selective entry pathways came with the discovery of Orai1 and its identification as the pore-forming subunit of the CRAC channels. The finding that STIM1 was involved in the regulation of the activity of the store-independent ARC channels as well as the store-dependent CRAC channels as described above, together with the obvious biophysical similarities between the CRAC and ARC channels, raised the possibility that the Orai proteins might also be involved in the formation of the ARC channel pore. Indeed, the unique nature of many of the key biophysical characteristics that are shared by both ARC and CRAC channels would be consistent with the fact that the Orai proteins appear to share no obvious homology with any other known ion channel family.

Mammalian cells possess three distinct, but closely related, Orai proteins (33). Examination of arachidonic acid activated currents in HEK293 cells following co-transfection of STIM1 along with each of these revealed marked increases in currents on expression of either Orai1 or Orai3, but no increase with Orai2 (44). These data suggested that both Orai1 and Orai3 might be involved in the formation of the functional ARC channel. However, when expressed in a HEK293 cell line engineered to isogenically express a constant level of STIM1 ("STIM1-stable cells"), only expression of Orai1, and not Orai3, resulted in increased ARC channel (44). One possibility that could explain this apparent discrepancy was that Orai3 was constitutively present in excess in the STIM1-stable cell line. This was supported by data obtained in STIM1-stable cells stably expressing Orai1. Here, expression of Orai3 did induce a significant (~40%) increase in ARC channel currents, without affecting the corresponding CRAC channel currents. Together, these data indicated that, unlike the CRAC channel, both Orai1 and Orai3 were involved in the formation of functional

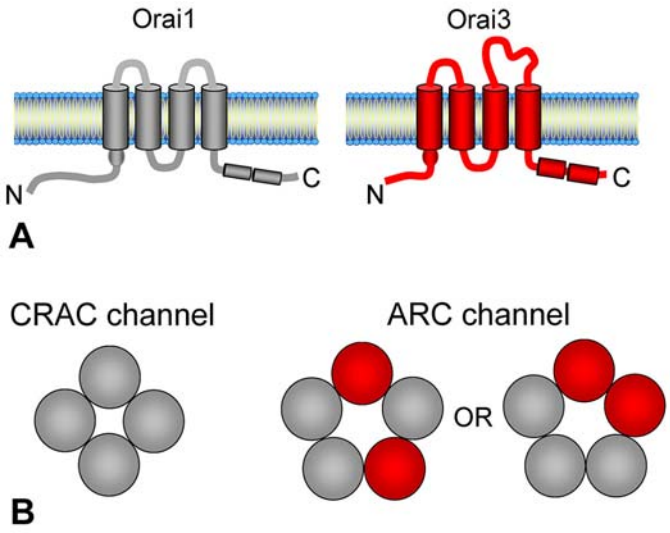

Figure 1. A. Diagrams comparing the basic structure of Orai1 and Orai3 proteins. Each has four transmembrane spanning domains, with intracellular N and C termini. Orai 3 has an extended extracellular loop between transmembrane domain 3 and 4. Also note the shorter (60 amino acid) N-terminal cytosolic domain in Orai3 that has been shown to play a critical role in determining the ARC channel's specific selectivity for activation by arachidonic acid. B. Diagrams illustrating the differences in the molecular stoichiometry between CRAC channels and ARC channels. The CRAC channel pore is formed from a tetramer of Orai1 subunits (grey) (46-50), whilst the functional ARC channel pore is formed from a pentameric assembly of three Orai1 subunits and two Orai3 subunits (red) (51). Two possible conformations of such a heteropentamer are possible, and expression of either of these as "preassembled" concatenated constructs (51) results in currents that display all the features of the endogenous ARC channels.

ARC channels. Confirmation of this was demonstrated by examining the effects of expression of a dominant-negative form of Orai3. Previous studies had shown that a glutamate-to-glutamine mutation at position 106 in Orai1 effectively eliminated both CRAC channel currents and the store-operated entry of Ca^{2+}, essentially acting as a dominant negative construct (45; 36; 46; 37). Expression of Orai3 bearing the equivalent mutation (E81Q) in the STIM1-stable cells, had no effect on the CRAC channel currents, but effectively eliminated the corresponding ARC channel currents (44). Even more dramatic was the effect of expression of the Orai3(E81Q) mutant in the STIM1-stable cells stably expressing Orai1. Here, compared to expression of the wild-type Orai3, expression of the dominant-negative Orai3 mutant reduced measured ARC channel currents by more than 98%, but had no significant effect on the corresponding CRAC channel currents. Based on the above, it was concluded that both Orai1 and Orai3 were essential components of the functional ARC channel.

Whilst the above data demonstrated the requirement of both Orai1 and Orai3 to form the functional ARC channel, the number and stoichiometry of these subunits within the channel was unclear. Previously, several studies utilizing a variety of approaches and techniques had demonstrated that the functional CRAC channel pore was comprised of a tetrameric arrangement of Orai1 subunits (47; 48; 49; 50; 51). It therefore seemed reasonable to propose that the ARC channel pore would be formed from a similar tetrameric assembly, but containing both Orai1 and Orai3 subunits. Using an approach we had previously developed to determine the CRAC channel stoichiometry, we generated series of concatenated Orai1/Orai3 tetramers comprising all possible number and arrangements of subunits and expressed these in the STIM1-stable cells. With the sole exception of a tetramer containing three Orai3 subunits, which gave only minimal currents, all of these heterotetrameric concatenated constructs resulted in increased arachidonic acid activated currents (52). Moreover, the induced currents all displayed the markedly inwardly rectifying current-voltage relationship and positive reversal potential typical of a highly Ca^{2+}-selective conductance. However, further studies revealed that expression of each of these Orai1/Orai3 tetrameric constructs also resulted in significantly increased store-operated, Ca^{2+}-selective CRAC-like currents (52). Because a defining property of the endogenous ARC channels is that they are entirely unaffected by store depletion, it was clear that each of these constructs failed to duplicate this critical behavior. Therefore, the conclusion was that none of these heterotetrameric Orai1/Orai3 assemblies could represent the true ARC channel.

Subsequently, as a result of an extensive examination of the effects of expression of a variety of different concatenated Orai1/Orai3 constructs with and without relevant wild-type or dominant negative Orai monomers, it was found that the minimal construct that resulted in increased arachidonic acid activated currents that duplicated the essential properties of the endogenous ARC channels were the heteropentameric assemblies of three Orai1 subunits and two Orai3 subunits in either a 31311 or a 31113 arrangement (52) (Figure 1). Critically,

although expression of these constructs resulted in 7-8-fold increases in the arachidonic acid activated currents, store-operated currents were not significantly changed from those recorded in untransfected cells. An important consideration in the application of this concatenated construct approach is to ensure that constructs form in the predicted manner – that is, without either the exclusion of any of the linked subunits, or the incorporation of additional subunits from the pool of endogenous Orai proteins. To this end, the validity of these expressed pentameric constructs was confirmed by demonstrating that the inclusion of a single dominant-negative Orai1 or Orai3 subunit within the concatenated construct was sufficient to completely eliminate the increased arachidonic acid activated currents. In contrast, co-expression of the pentamers with a dominant-negative Orai1 or Orai3 monomer failed to significantly affect the increased arachidonic acid activated currents, thereby excluding the possibility that the ARC channel was encoded by an even larger assembly (hexamer etc.) (52). Further experiments revealed that these heteropentameric constructs displayed identical current-voltage relationships to the native ARC channels, were equally sensitive to activation by the same low concentrations of exogenous arachidonic acid (2-8 μM), and were similarly sensitive to inhibition by La^{3+} (100 μM) and Gd^{3+} (5 μM). Finally, it was demonstrated that the arachidonic acid activated currents induced by expression of the constructs displayed the same unique dependence on the specific pool of STIM1 present in the plasma membrane for their activation, as revealed by their complete elimination on expression of the glycosylation-mutant STIM1 described above (52).

Given that several studies have established that the functional CRAC channel is formed by a tetramer of Orai1 subunits, the finding that ARC channel is formed as a pentamer was something of a surprise. In particular, the differences in overall structure between these two channels might be expected to have a significant impact on the biophysical properties of the resulting conductance yet, as detailed above, the basic features of the CRAC and ARC channels are very similar. Perhaps the most obvious feature that such differences in structure might be expected to influence would be their relative ion selectivity. However, as already discussed, data indicate that both ARC and CRAC channels possess comparable high selectivity to Ca^{2+} ions. In this case, perhaps any effect of the presumed difference in geometry of the channels is offset by the increase in the total number of interaction sites for the entering Ca^{2+} resulting from the presence of an additional Orai subunit in the ARC channels. However, in the absence of any information on the detailed molecular configuration of the channels, such suggestions are almost certainly oversimplistic.

The apparent unique requirement for Orai3 in the formation of the functional store-independent arachidonic acid dependent ARC channel immediately raises the question of how the Orai3 protein might act to impart such a requirement. Examination of the effects of expressing a range of different concatenated heteropentameric Orai1/Orai3 constructs, revealed that whilst a pentamer containing only a single Orai3 subunit was sensitive to activation by arachidonic acid, even larger (approximately 2-fold) currents were induced by depletion of intracellular Ca^{2+} stores (53). In other words, the resulting conductance was arachidonic acid *sensitive*, but not arachidonic acid *selective* in it's mode of activation. Only with the inclusion of a second Orai3 subunit (i.e. a pentamer containing three Orai1 subunits and two Orai3 subunits) did the resulting conductance become essentially exclusively selective to activation by arachidonic acid. To examine the molecular basis for this selectivity, experiments were performed based on the co-expression of a 3111 tetramer, along with either various Orai1/3 chimeric or deletion-mutant monomers, with the aim that these monomers would incorporate with the tetramer to form the appropriate "chimeric" pentamer (53). Importantly, these monomers were transfected at DNA concentrations that did not, on their own, induce any significant currents. In this way, the specific effects of the co-assembly of the monomers with the tetramer to form a pentamer, could be evaluated. The essential validity of this approach was confirmed by experiments where the 3111 tetramer was co-expressed with either a wild-type Orai1 monomer or the corresponding Orai3 monomer. In each case, the resulting increased currents displayed a distinct "activation selectivity profile" (i.e. store-operated versus arachidonic acid activated) that precisely mimicked that of the corresponding concatenated pentamer (i.e. the 31111 and 31113 pentamers, respectively). Using this approach it was found that co-expression of the 3111 tetramer with an chimeric Orai1 subunit containing just the 60-amino acid sequence that forms the cytosolic N-terminal region of Orai3, was sufficient to result in a conductance that was essentially exclusively activated by arachidonic acid (53). This was confirmed by including this chimeric subunit in a concatenated pentameric construct, expression of which resulted in the appearance of a increased current whose activation was again exclusively dependent on arachidonic acid. However, precisely how this domain acts to impart such selectivity for arachidonic acid remains unclear and will probably only be resolved when more information about the detailed mechanism of activation of the channel, along with the essential role of the pool of STIM1 in the plasma membrane, is revealed. Thus, the demonstration that the cytosolic N-terminal region of Orai3 is critical for the ARC channel's unique selectivity for activation by arachidonic acid does not necessarily indicate that this domain of Orai3 is the site where arachidonic acid acts to open the channel. For example, it is possible that this domain is essential for interactions between the channel and STIM1 in the plasma membrane. This interaction may be induced by arachidonic acid acting on STIM1, or STIM1 and the channel may exist as a preformed complex which, itself, is subject to activation by arachidonic acid (Figure 2).

Just as several important implications arise from the discovery that STIM1 was involved in the activation of both CRAC and ARC channels, the above demonstration of the heteropentameric Orai1/Orai3 assembly that represents the functional ARC channel raises certain important considerations. Not least of these is the fact that, as with STIM1, protocols describing the inhibition of Ca^{2+} entry

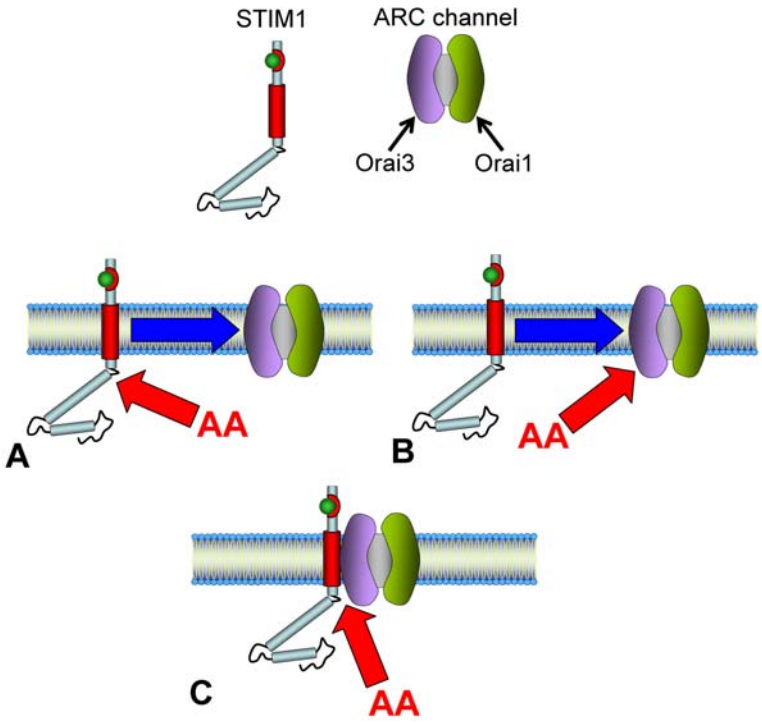

Figure 2. Possible modes of activation of the ARC channels by arachidonic acid. Plasma membrane-resident STIM1 and Orai3 both represent unique essential components for functional ARC channel activity. A. STIM1 resident in the plasma membrane may be the site of action of arachidonic acid (AA), which induces an interaction between the STIM1 and the ARC channel. B. The target for arachidonic acid may be the channel itself (possibly acting at the cytosolic N-terminus of Orai3), resulting in an interaction between the channel and STIM1 resident in the plasma membrane. C. Plasma membrane STIM1 and the ARC channel may pre-exist as a molecular complex even under resting conditions, and it is this complex that is the target for agonist-generated arachidonic acid.

resulting from the knock-down or elimination of Orai1 protein cannot be considered as adequate criteria to specifically ascribe such entry to a store-operated or CRAC channel-mediated process. Clearly, both CRAC channels and ARC channels would be equally sensitive to such procedures. On the other hand, specific knock-down of Orai3 might represent a strong, potentially specific, indicator for any action of the ARC channels. However, two recent studies have indicated that Orai3 is involved in an endogenous store-operated Ca^{2+} entry pathway in estrogen receptor-expressing breast cancer cell lines (e.g. MCF-7), suggesting that Orai3 may be involved in Ca^{2+}-selective conductances in addition to the ARC channels (54; 55). Moreover, in any such approach, it would be important to demonstrate that the knock-down of Orai3 protein levels does not induce any compensatory increase in the expression of the other Orai proteins. For example, given the demonstrated co-assembly of Orai1 and Orai3 subunits in the ARC channel, it would seem possible, or perhaps likely, that all the three members of this family have at least some level of affinity for each other. A compensatory increase in the level of expression of one or more of the different members might therefore result in a "forced" assembly of Orai subunits that does not occur under normal conditions. In such cases, the expression of the dominant-negative mutant of Orai3 might be a more appropriate approach.

In a similar way, the finding that the functional ARC channel is a heteromeric assembly of Orai subunits has potential implications for protocols involving the simple overexpression of Orai monomers in cells. For example, it raises the possibility that these monomers may assemble in any number of different ways, most likely in a manner that would be dependent on the relative level of expression and relative affinities of the various components. As such, whilst they may result in the formation of functional channels, their actual molecular composition may be one that does not occur under normal conditions. A possible example of this is indicated in experiments involving the overexpression of Orai3 monomers, which has been shown to result in the appearance of a novel store-operated conductance (56; 57; 58; 59). As such, these "Orai3 channels" are often considered as a new form of CRAC channel. However, many of the properties of these Orai3 channels – including a direct, store-independent and STIM1-independent activation of a non-selective cation current by the CRAC channel inhibitor 2-APB, etc. – would seem to indicate a very different conductance from the genuine native CRAC channels. Consequently, their description as a CRAC channel (which, by definition, is strictly a store-operated Ca2+-selective channel) would seem to be unnecessarily confusing.

Finally, the findings described above clearly demonstrate that we have a situation where the activity of the native ARC channels is absolutely dependent on a minimum of three different proteins – STIM1, Orai1, and Orai3 – the expression levels, and correct targeting etc. of any one of which may be rate limiting for channel formation and/or activity. In addition, at least two of these three molecules (STIM1 and Orai1) are also critical for CRAC channel activity, raising the possibility of a potential competition for these components between the two distinct Ca^{2+} entry conductances.

6. FUNCTIONAL ROLE OF ARC CHANNELS

Together, the findings that have been accumulated over the 15 or so years since the first demonstration of an arachidonic acid dependent Ca^{2+} entry have shown that such a pathway exists in a wide variety of different cell types, and provides an alternative or additional source of agonist-activated Ca^{2+} entry that is entirely independent of store depletion. Given the virtual universal presence of some form of store-operated Ca^{2+} entry pathway, the obvious question arises as to what role might this distinct Ca^{2+} entry pathway play in cell physiology – is it simply an essentially redundant alternative to the store-operated pathway, or does it serve some unique purpose that is not, or cannot be, performed by the store-operated pathway? Clearly the fact that these two pathways appear to routinely co-exist in the same cell would suggest that they might serve distinct functions. In this context, it is worth remembering that the search for an alternative store-independent pathway was largely initiated by the indication that certain features of the known store-operated pathways appeared incompatible with their operation during the oscillatory Ca^{2+} signals typically generated at low agonist concentrations (15). Despite this, support for the idea that CRAC channels do in fact provide the route for Ca^{2+} entry during such oscillatory responses remains strong. As noted by Putney and Bird (60) the best evidence for a role of CRAC channels in oscillations "would be the demonstration in an oscillating cell of a current with properties similar to the well-characterized calcium-release-activated current". However, as these authors point out, because "the amount of Ca^{2+} lost from the ER during each oscillation appears to be a very small fraction of stored Ca^{2+}" (7; 61), direct detection of such a conductance would likely be difficult (60). Consequently, with the exception of T-lymphocytes where the essential role of the CRAC channels in the signals underlying agonist-induced cell activation is clear (33; 62), evidence to date for the activity of CRAC channels during oscillatory responses is largely based on pharmacological approaches that inevitably suffer from questions regarding the specificity of the agents used and the appropriateness of the concentrations at which they were applied.

Using a potentially more specific molecular approach to this question, Wedel *et al.* (61) showed that RNAi knockdown of either STIM1 or Orai1 resulted in a significant reduction in oscillation frequency. Subsequently, Bird *et al.* (63), demonstrated that, at least in a proportion of cells examined, oligomerization and translocation of STIM1 can be detected during Ca^{2+} oscillations which, they suggested, indicated activation of CRAC channels under such conditions. However, consistent with other reports (13), this study also found that a "substantial depletion of intracellular stores" appears to be necessary for such translocation to occur. As the authors noted, this feature appeared to contradict the earlier demonstration that overall Ca^{2+} store depletion is minimal under such oscillatory conditions, as noted above (61). To reconcile these findings, it was proposed that the observed effect must reflect a highly localized substantial depletion of stored Ca^{2+}, consistent with the suggestion that the activation of the store-operated CRAC channels is under the preferential control of a small critical pool of the total ER Ca^{2+} store (63). Whilst this is an intriguing idea, it has to be noted that the data obtained are open to alternative interpretations. For example, with regard to the RNAi data of Wedel *et al.* (61), because we now know that both STIM1 and Orai1 are also essential for ARC channel activity (39; 44), these data can no longer be considered as exclusively indicating the involvement of CRAC channels. Moreover, whilst it is clear that the STIM1 puncta formation demonstrated by Bird *et al.* (63) is essential for activation of CRAC channels, it is unclear whether such a phenomenon is specifically indicative of such activation. Indeed, if such significant activation was occurring, it might be expected to be detectable by direct electrophysiological means. In this context, data obtained from studies using the rate of Mn^{2+} quench of intracellularly loaded fluorescent probes as a measure of the overall rate of Ca^{2+} entry in oscillating cells, indicate that it is only some 30% less than that seen after maximal depletion of the stores (22). This, in itself, would seem to suggest that an alternative pathway of Ca^{2+} entry might be operating during such oscillatory responses. Consequently a key finding was that direct electrophysiological recording of the conductances induced by application of appropriate agonists at concentrations that result in the generation of oscillatory Ca^{2+} signals in HEK293 cells, revealed a selective activation of the ARC channels and not the co-existing store-operated CRAC-like channels (22). As such, these data were consistent with earlier studies showing that the entry of Ca^{2+} associated with agonist-induced oscillatory Ca^{2+} signals in both exocrine avian nasal gland cells (18) and in HEK293 cells (19) was eliminated by inhibition of the agonist-activated generation of arachidonic acid. Subsequently, similar electrophysiological recordings of a specific activation of the ARC channels by low, physiologically relevant, agonist concentrations was demonstrated in the secretory acinar cells of the parotid salivary gland (64). Here, the selective activation of the ARC channels was made even more clear by the fact that, in these cells, the predominant store-operated conductance involves channels encoded by members of the TRPC family of proteins (65; 66). As such, these conductances display features typical of a relatively non-selective cation conductance, including an essentially linear or somewhat outwardly rectifying current-voltage relationship, with reversal potentials close to zero. Such features are clearly distinct from those of the highly Ca^{2+}-selective ARC channels. Essentially identical findings were found in pancreatic acinar cells (64), but here the immediate

physiological relevance of such a selective activation of the ARC channels can be demonstrated by the fact that the precise range of concentrations of cholecystokinin (CCK), a major physiological mediator of pancreatic secretion, that occur in the blood following ingestion of a meal are known (67). Application of CCK at these same concentrations exclusively results in the appearance of currents bearing all the features of ARC channels (64). Again, these can be definitively distinguished from the co-existing store-operated conductances in the same cell as, like the parotid cells, these are essentially non-selective cation conductances encoded by members of the TRPC family of proteins (68). Indeed, rather than playing a physiological role in the acinar cells, the activity of these store-operated conductances is typically associated with pathological conditions involving the premature activation of trypsin within the cells, and the resulting induction of pancreatitis (69). Together then, there is convincing evidence from diverse cell types, for a specific role of the ARC channels in providing the principal source of the agonist-activated entry of Ca^{2+} during oscillatory Ca^{2+} signals. Given that it has long been known that the rate of such entry plays an important role in determining the frequency of the Ca^{2+} oscillations (70), and that such frequency is a key determinant in the selective action of discrete downstream effectors in the cell (71; 72; 73; 74; 75), an important the role for the ARC channels in influencing the ultimate physiological responses in such cells would seem to be indicated.

As previously noted, the store-independent ARC channels and the store-operated CRAC channels have been shown to co-exist in the same cells and that, in such cells, maximal store-operated currents and arachidonic acid activated currents are strictly additive (20). However, studies using the rate of Mn^{2+} quench as a measure of overall Ca^{2+} influx in intact cells indicate that whilst the influx rate at low agonist concentrations is entirely dependent on the generation of arachidonic acid, influx at maximal agonist concentrations is entirely unaffected by pharmacological inhibition of such generation, and is exclusively dependent on the depletion of the intracellular stores (22). The obvious conclusion is that the ARC channels are not active at these higher agonist concentrations. However, this apparent loss of ARC channel activity is not a result of any decline in arachidonic acid generation (22). Instead, it was found that, in intact cells, the sustained elevation of cytosolic Ca^{2+} that results from activation of the store-operated CRAC channels, induces a calcineurin-mediated inhibition of the activity of the co-existing ARC channels in a process described as the "reciprocal regulation" of Ca^{2+} entry (76; 22). Of course in whole-cell patch clamp experiments intracellular Ca^{2+} concentrations are buffered by the pipette solution and, under these conditions, the two currents remain strictly additive.

More recently, as the presence of ARC channels is beginning to be examined in more cell types, additional specific roles are being revealed. For example, circulating fatty acids have long been known to have a significant enhancing effect on the glucose-stimulated release of insulin from the beta cells of the pancreas (77; 78; 79). Although the precise basis for this effect was unclear, and the observed effects appeared very dependent on whether acute or chronic exposure to the fatty acid was applied, it was shown to involve an increase in the entry of Ca^{2+} (80). A further complication arises from reports noting that the GPR40 receptor that is abundantly expressed in beta cells, is a $G_{q/11}$-protein coupled receptor that is activated by fatty acids, particularly arachidonic acid (81). This obviously raises the possibility that the reported effects of arachidonic acid on insulin secretion might involve an action at the GPR40 receptor, resulting in the generation of $InsP_3$ and the release of Ca^{2+} from intracellular stores. However, depletion of such stores by the SERCA-pump inhibitor thapsigargin had no effect on insulin secretion stimulated by either the muscarinic agonist carbachol, or the circulating peptide hormone CCK (82), suggesting that neither store-depletion, or the store-operated Ca^{2+} entry that would result, play any significant role in increasing insulin secretion. Instead, both carbachol and CCK have been shown to stimulate the generation of arachidonic acid in islet cells indicating that the fatty acid might be inducing a stimulation of insulin secretion via a more direct, intracellular action (83; 82). Consistent with this, the membranes of beta cells are known to contain unusually high levels of glycero-phospholipids with arachidonic acid as the *sn*-2 substituent (84), and several studies have demonstrated that the generation of arachidonic acid as a result of the hydrolysis of membrane phospholipids by members of the phospholipase A_2 group of enzymes participates in the control of glucose-induced insulin secretion (82; 85; 83). Moreover, pharmacological studies showed that these effects are via the arachidonic acid moiety per se, and not one of its metabolites (85; 86). It was further shown that the effect of this generation of arachidonic acid by the stimulators of insulin secretion was associated with increases in cytosolic Ca^{2+} and an enhanced entry of Ca^{2+} (80). In a recent study (30), it was shown that this arachidonic acid induced entry of Ca^{2+} could be induced by concentrations of exogenous arachidonic acid as low as 5 μM, and was associated with the development of a clear small-magnitude inward current at -70 mV. Critically, this effect was shown to be independent of the activity of voltage-gated Ca^{2+} channels, and was unaffected by 2-APB, an inhibitor of store-operated channels. Moreover, it was not mimicked by application of oleic acid, but was duplicated by the intracellular introduction of the relatively membrane impermeant arachidonic acid analog arachidonyl co-enzyme A. Together, these features all suggest that the arachidonic acid induced entry of Ca^{2+} in these cells reflects the activity of ARC channels. Based on these findings, the authors concluded that the ARC channels likely play a key role in the arachidonic acid dependent modulation of insulin secretion in the pancreatic beta cells (30).

Finally, very recent data have indicated the presence of a channel in vascular smooth muscle cells that displays essentially all of the unique features of the ARC channel (see the review by Trebak *et al.* in this volume). Interestingly, this channel in the smooth muscle appears to be specifically activated by the agonist thrombin, whilst

other agonists (e.g. platelet derived growth factor, PDGF) activate a store-operated entry pathway. This would seem to indicate potentially distinct roles for these two modes of Ca^{2+} entry in these cells.

7. CONCLUSIONS

Despite the predominance of the store-operated mode of Ca^{2+} entry in the current literature, and the fact that it appears to be an essentially ubiquitous feature of cells, there is now ample evidence demonstrating that a range of diverse cell types also express the store-independent, arachidonic acid activated, ARC channels. Moreover, the existing data suggest that these two separate pathways are activated under distinct conditions and circumstances, and function in unique individual ways to regulate agonist-activated cell activities. Perhaps, given the obvious importance of agonist-induced Ca^{2+} entry in influencing or determining the nature of such a wide range of critical cellular responses, it is not surprising that nature has evolved more than one pathway for achieving such entry. However an important, yet often overlooked, implication of this is that the simple demonstration that store-operated entry exists in a cell can no longer be used to conclude that this pathway alone is responsible for all processes dependent on agonist-induced Ca^{2+} entry. Unfortunately, whilst the methods for demonstrating the presence of a store-operated Ca^{2+} entry pathway are relatively straightforward (e.g. application of the SERCA pump inhibitor thapsigargin), demonstration of an arachidonic acid-dependent, store-independent pathway is often more problematic. Even the simple application of exogenous arachidonic acid to examine its ability to induce an entry of Ca^{2+} has many practical difficulties, as arachidonic acid is rapidly oxidized and denatured in air at room temperatures. In addition, the direct measurement of the currents through the ARC channels and the factors that affect them is fraught with the same problems as with the CRAC channels. Paramount among these is the fact that, typically, these are both very small conductances with whole-cell current densities of around 1 pA/pF or less at voltages of -80 mV. Accurate measurement of such small conductances necessitates obtaining and maintaining very high-resistant seals between the patch pipette and the cell membrane. Achieving these conditions in freshly isolated primary cells is particularly problematic. Indeed, this probably accounts for the fact that the number of different cell types where the ARC channel currents have been directly measured is not appreciably different from those in which similar direct measurements of CRAC channels have been made – despite the fact that CRAC channels were first characterized some 10 years prior to the first description of the ARC channels .

An additional very important point has become apparent as a result of the discoveries of the molecular nature of these agonist-induced Ca^{2+} entry pathways. This is the fact that, like the CRAC channels, both STIM1 and Orai1 are essential components in the regulation and formation, respectively, of the functional ARC channel. As a consequence of this finding, demonstrating that the knock-down or elimination of either of these proteins can no longer be used as a definitive indicator that the CRAC channel is involved. The same argument can be applied to protocols involving the expression of any appropriate dominant-negative version of either of these proteins. Hopefully, as more groups begin to look for alternative store-independent pathways of agonist-induced Ca^{2+} entry, and more cell types are examined, the importance of definitively distinguishing between such pathways and the co-existing store-operated pathways will become better appreciated. In a similar way, it would seem likely that such a widening of the appreciation of different modes of agonist-induced Ca^{2+} entry will probably reveal yet more unique functions, for both CRAC-mediated and ARC-mediated responses in different cell types.

Finally, the fact that Orai proteins have been shown to form the pore of both the CRAC channels and the ARC channels suggests that these two channel types can be considered to represent the first two members of an entirely new family of ion channels – the "Orai channels". Besides their molecular make-up, these channels share the unique biophysical features of being small, highly Ca^{2+}-selective conductances whose gating is voltage-independent. That both these channels are involved in providing independent pathways for the agonist-induced increases in Ca^{2+} entry is abundantly clear. However, it should always be remembered that our understanding of the role and functioning of the Orai proteins is barely four years old. Undoubtedly we still have a great deal to learn about how these novel proteins operate and interact in a variety of diverse cellular contexts.

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Abbreviations: 2-APB: 2-aminoethoxyldiphenyl borate, ARC channel: Arachidonate-regulated calcium channel, CCK: cholecystokinin, CRAC channel: Calcium release-activated calcium channel, ER: endoplasmic reticulum, SERCA pump: sarcoplasmic-endoplasmic reticulum calcium pump, STIM1: stromal interacting molecule 1, TRPC: transient receptor potential canonical

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