ARC Channels: A Novel Pathway for Receptor-Activated Calcium Entry

An increase in the cytosolic concentration of Ca^{2+} ions ([Ca^{2+}]i) is a major component of cellular signals generated by the actions of a variety of hormones and neurotransmitters acting on receptors coupled to phospholipase C (PLC). Such [Ca^{2+}]i signals invariably involve both an inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores and the activation of an enhanced entry of extracellular Ca^{2+}. Although the latter is often critical in achieving an effective sustained response from the cells, our understanding of the nature of the relevant Ca^{2+}-entry pathway and its regulation has lagged far behind the study of IP_3-mediated Ca^{2+} release, a fact due, as much as anything, to the technical difficulties involved in directly measuring the activities of the generally small conductances involved.

For much of the past 18 years, attention has focused on the so-called “capacitative” or store-operated mechanism of Ca^{2+} entry first defined by Putney (24, 25). In this, the two processes that make up PLC-dependent [Ca^{2+}]i, signaling are intimately and sequentially linked in that the emptying of agonist-sensitive stores alone is both necessary and sufficient to activate Ca^{2+} entry. The channels responsible for this entry are generically described as store-operated channels, the archetypal version of which are the Ca^{2+} release-activated Ca^{2+} channels (CRAC channels) first described in mast cells and Jurkat cells (8, 36). Despite extensive study, the molecular identity of these channels and the precise mechanism whereby depletion of the agonist-sensitive Ca^{2+} stores induces their activation have yet to be resolved (20, 23).

Nevertheless, the role of the CRAC channels and other similar store-operated Ca^{2+}-selective channels (SOC channels) in cellular Ca^{2+} signaling is clear. Entry through these channels determines the level of the sustained elevation in [Ca^{2+}]i observed following stimulation with high agonist concentrations and is responsible for the subsequent refilling of intracellular agonist-sensitive Ca^{2+} stores following termination of the signal. However, it is significant that activation of these channels has been shown to require a rather profound and sustained depletion of the intracellular stores (5, 19), although this issue remains controversial (7). In certain cell types (e.g., T lymphocytes), the limited size of the agonist-sensitive store means that such a profound depletion is readily achieved at all levels of stimulation, and in these cells it seems that CRAC channels provide the principal source of Ca^{2+} entry under all conditions (23). In many other cells, however, the oscillatory [Ca^{2+}]i, signals typically seen following stimulation at lower, more physiologically relevant agonist concentrations reflect only a transient and/or partial release of Ca^{2+} from the stores (21). As discussed above, the precise relationship between the extent of store depletion and the magnitude of the CRAC current that results remains controversial. In contrast, it is clear that the activation of CRAC channels is slow, generally taking several tens of seconds or even minutes to complete following a maximal depletion of the stores (5, 7, 36). Such behavior immediately raises questions as to whether store-operated entry could function effectively during the brief release events associated with such oscillatory signals. Nevertheless, the entry of Ca^{2+} is clearly increased during these types of responses, much as it is following maximal agonist stimulation. Subsequent direct examination of the entry of Ca^{2+} under these low levels of stimulation revealed features that were inconsistent with the known properties of store-operated entry (30), leading to a search for possible alternative agonist-activated routes of Ca^{2+} entry. A result of this was the discovery of a novel mode of entry whose activation was entirely independent of store depletion and was instead dependent on the receptor-mediated generation of arachidonic acid (28, 31). Such arachidonic acid-dependent, noncapacitative Ca^{2+} entry has since been reported in several different cell types (3, 6,
Moreover, the evidence indicated that this form of Ca$^{2+}$ entry was particularly active at low agonist concentrations when oscillatory [Ca$^{2+}$]$_i$ signals were generated. Here the principle role of the agonist-activated entry of Ca$^{2+}$ is not to contribute directly to the changes in [Ca$^{2+}$]$_i$, but rather to modulate the frequency of the [Ca$^{2+}$]$_i$ oscillations (10). A conductance underlying this novel Ca$^{2+}$ entry pathway was subsequently described, and the channels responsible were named arachidonate-regulated Ca$^{2+}$ (ARC) channels (11).

**Properties of ARC Channels**

Characterization of the properties of these novel channels using whole cell patch-clamp techniques has demonstrated that they are superficially similar to CRAC channels and the “CRAC-like” SOC channels seen in various cell types. Thus they display marked inward rectification, reversal potentials greater than +40 mV, high selectivity for Ca$^{2+}$, and inhibition by La$^{3+}$ (FIGURE 1A). Despite these similarities, further examination of the ARC channels shows that they possess certain unique biophysical and pharmacological features that clearly distinguish them from the classic CRAC channels as well as from the CRAC-like SOC channels found in the same cells. ARC channels and SOC channels are therefore entirely distinct but coexisting entities. This is perhaps most clearly demonstrated by the fact that the ARC channels can be readily activated in cells whose Ca$^{2+}$ stores have been maximally depleted (e.g., by treatment with thapsigargin) and that the macroscopic (whole cell) ARC currents and endogenous SOC currents are additive in the same cell (11). Unlike CRAC channels and the endogenous SOC channels, ARC channels do not show any fast inactivation and they are not inhibited by reductions in extracellular pH. Their sensitivity to the widely used blocker Gd$^{3+}$ is similar to that seen with various SOC channels, but they are insensitive to 2-APB (14). Like CRAC channels and voltage-

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**FIGURE 1. Basic features of currents through arachidonate-regulated Ca$^{2+}$-sensitive channels**

A: current-voltage relationship of arachidonate-regulated Ca$^{2+}$-sensitive (ARC) currents activated by exogenous arachidonic acid (8 μM) and their inhibition by La$^{3+}$ (50 μM). Data were obtained by application of 150-ms voltage ramps from –100 to +30 mV. B: inhibition of monovalent currents through ARC channels by external Ca$^{2+}$. ARC currents are inward current densities measured at –80 mV in the absence of external divalent cations (except as indicated). C and D: activation of ARC currents by carbachol (C) and typical [Ca$^{2+}$]$_i$ signals (D) generated in the same cells by the corresponding carbachol concentrations. ARC currents are inward current densities as measured at –80 mV [Ca$^{2+}$]$_i$ signals are presented as fluorescence ratios (405/485) of indo-1-loaded cells. Adapted from Refs. 11, 13, and 14, with permission.
gated Ca\(^{2+}\) channels, complete removal of external divalent cations permits the permeation of monovalent cations through ARC channels. However, the resulting macroscopic Na\(^{+}\):Ca\(^{2+}\) current ratio is some four times larger than that seen with CRAC channels (12). The marked inward rectification, very positive reversal potential, and sensitivity to inhibition by La\(^{3+}\) all suggest that the ARC channels are Ca\(^{2+}\) selective. Support for this comes from data showing that complete replacement of external Na\(^{+}\) with NMDG\(^{-}\) has no significant effect on the macroscopic current magnitude or the current-voltage relationship of the ARC channels. A more quantitative and readily comparable measure of Ca\(^{2+}\) selectivity can be estimated from the ability of external Ca\(^{2+}\) to inhibit the monovalent currents through the channels (FIGURE 1B), and such studies demonstrate that ARC channels are indeed highly Ca\(^{2+}\) selective; in fact, they are some 50 times more Ca\(^{2+}\) selective than CRAC channels (14).

Of course, the most fundamental difference is that activation of the ARC channels is entirely independent of store depletion and specifically dependent on arachidonic acid. Activation occurs at low concentrations of exogenously applied arachidonic acid (2–8 μM), which is an important point because at higher concentrations (>20 μM) large, highly nonspecific conductances are observed that are indicative of a breakdown in membrane integrity as a result of the detergent-like actions of the fatty acid at such concentrations. Other polyunsaturated fatty acids are comparatively poor activators of the channel, and saturated fatty acids and monounsaturated fatty acids are both ineffective (14). Significantly, activation by arachidonic acid is unaffected by inhibition of the lipoxygenase, cyclooxygenase, or p450 metabolic pathways, indicating an effect of the fatty acid itself. Moreover, experiments using arachidonylcoenzyme A, a charged membrane-impermeant analog of arachidonic acid, demonstrate that the activation of the channel is specifically via an action at the intracellular face of the membrane (14). Whether this involves a direct action of the fatty acid on the channel protein itself or an action on some intermediary molecule is, as yet, uncertain.

Conductances displaying all of the key features of these ARC channels have now been identified in several different cell types, including mammalian and avian cell lines as well as dissociated mouse and human parotid cells (14). However, it should be noted that other noncapacitative pathways likely exist in cells (2) and that ARC channels may not even be the only such pathway in which arachidonic acid is involved. For example, a relatively nonsclective cation-permeable conductance activated by arachidonic acid has been described in endothelial cells (22). In addition, arachidonic acid-mediated noncapacitative Ca\(^{2+}\) entry pathways that are apparently dependent on nitric oxide have been described in A7r5 cells (16) and in parotid acinar cells (34). However, the biophysical characteristics of the conductances underlying these nitric oxide-dependent responses were not determined, and the concentrations of arachidonic acid used were very high (45–50 μM). Given the diverse nonspecific effects induced in cells by such concentrations (see above), the physiological relevance of these findings remains uncertain.

**Reciprocal Regulation of ARC and SOC channels**

Critical to the proposition that it is the ARC channels that are responsible for the entry of Ca\(^{2+}\) during responses seen in many cells at low levels of stimulation is the demonstration that these channels are, indeed, activated by the same low concentrations of agonist. Studies using human embryonic kidney 293 cells stably transfected with the M3 muscarinic receptor (m3-HEK cells) demonstrated activation of ARC channels at carbachol concentrations as low as 0.2 μM, reaching a maximum at ~1 μM (13). This is precisely the range over which oscillatory Ca\(^{2+}\) signals are produced in the same cells (FIGURE 1, C AND D). Activation of the channels by these low concentrations of carbachol was entirely dependent on the generation of arachidonic acid, consistent with the demonstration that Ca\(^{2+}\) entry in intact cells (measured as Mn\(^{2+}\) quench) under the same conditions is similarly dependent on arachidonic acid generation (13). These data demonstrate that it is the ARC channels, and not the SOC channels, that provide the predominant route of receptor-activated Ca\(^{2+}\) entry at low agonist concentrations in these cells. A more surprising finding, however, was that similar determinations of Ca\(^{2+}\) entry at high (about maximal) agonist concentrations showed that this was predominantly via the SOC channels and that little or no obvious arachidonic acid-dependent component could be detected. This contradicts data from whole cell patch-clamp experiments in which the macroscopic currents through these two channels were clearly additive. This apparent loss of ARC channel activity at high agonist concentrations in intact cells could not be explained by any corresponding decline or loss of arachidonic acid generation that continues over this same concentration range. This raises the obvious question: why does Ca\(^{2+}\) entry via the ARC channels apparently disappear at high agonist concentrations? The answer came with the finding...
that the activity of the ARC channels is profoundly inhibited by sustained increases in [Ca^{2+}] (13). This inhibition is dependent on global cytosolic Ca^{2+} concentrations, rather than a local effect of the Ca^{2+} actually entering through the channels, and is maximal at [Ca^{2+}] above 250 nM, well within the range of sustained [Ca^{2+}] signals seen at the high agonist concentrations (FIGURE 2A). The net effect is that, at high agonist concentrations, the prolonged depletion of the intracellular Ca^{2+} stores and subsequent activation of the SOC channels results in a sustained elevated [Ca^{2+}] signal that, in turn, acts to inhibit the activity of the ARC channels. Additional studies showed that this Ca^{2+}-dependent inhibition of the ARC channels is a slow process, taking ~100 s to reach completion, and ARC channel activity is therefore unaffected by the brief transient increases in [Ca^{2+}], associated with oscillatory responses. In this way, the transition from oscillatory [Ca^{2+}] signals to a sustained [Ca^{2+}] signal is associated with a progressive switch in the predominant mode of Ca^{2+} entry from the ARC channels to the SOC channels, a phenomenon described as the “reciprocal regulation” of ARC and SOC channels (13).

Clearly, the key action underlying this reciprocal regulation is the Ca^{2+}-dependent inhibition of the ARC channels. Studies designed to explore the biochemical basis for this revealed that it depended on an action of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (15). Thus pharmacological inhibition of calcineurin activity using cyclosporin A or the FK506 analog ascomycin resulted in a complete reversal of the Ca^{2+}-dependent inhibition of the ARC channels (FIGURE 2B). This was confirmed by studies in which the COOH-terminal domain of the calcineurin-inhibitory protein CAIN was transfected into cells, again resulting in a complete loss of the Ca^{2+}-dependent inhibition of ARC currents (FIGURE 2C). Further studies using the calcineurin-dependent nuclear translocation of a transfected green fluorescent protein-tagged NFAT domain (FIGURE 2D) or an NFAT-driven luciferase reporter gene as assays of calcineurin activation showed that this only occurred at agonist concentrations that activate the SOC channels and produce sustained elevated [Ca^{2+}] signals (15). Agonist concentrations that generate oscillatory [Ca^{2+}] signals and specifically activate Ca^{2+} entry through the ARC channels fail to increase calcineurin activity. Thus calcineurin is the key mediator of the switch between the ARC channels and the SOC channels that underlies the reciprocal regulation of these entry pathways, and this is directly associated with the change from oscillatory to sustained Ca^{2+} signals as agonist concentrations increase. The result is that these two coexisting but entirely distinct Ca^{2+} entry channels each play a unique and nonoverlapping role in overall [Ca^{2+}] signaling, one modulating the frequency of oscillatory signals (ARC channels), the other determining the amplitude of sustained signals (SOC channels) (FIGURE 3).

**What Do ARC Channels Do?**

As already discussed, the main effect of Ca^{2+} entry via the ARC channels is to modulate the frequency of agonist-induced [Ca^{2+}] oscillations. Exactly how this is achieved, however, is not entirely clear. In some cell types, inhibition of Ca^{2+} entry results in...
an immediate cessation of oscillations (10, 27, 35), whereas in others oscillations can continue for some time in the absence of external Ca\(^{2+}\), although their frequency is markedly slowed (9, 26). These differences have led to some confusion in the literature concerning the actions of Ca\(^{2+}\) entry on such signals. This is resolved by the realization that the effect is strictly on the frequency of the induced oscillations and that the above examples merely reflect extremes in a range of essentially identical effects. Consistent with the entry of Ca\(^{2+}\) being independent of store depletion (i.e., noncapacitative) under these conditions, the effect of entering Ca\(^{2+}\) does not involve any obvious influence on the rate of refilling of intracellular stores between each oscillation (29). Instead, an effect on the release of stored Ca\(^{2+}\) was indicated, and the assumption was that this probably reflected an effect of entering Ca\(^{2+}\) acting as a coagonist, along with IP\(_3\), on the IP\(_3\) receptors. However, it now seems that this is probably, at best, an oversimplification. For example, in polarized epithelial cells (such as parotid and pancreatic acinar cells), oscillatory [Ca\(^{2+}\)]\(_i\) signals are initiated at specific “trigger zones” located in the apical region of the cell. Significantly, this phenomenon can be readily activated by local stimulation of appropriate receptors at the basal end of the cell (1). For these signals to be influenced by Ca\(^{2+}\) entry via the ARC channels, either Ca\(^{2+}\) would have to diffuse from basally activated ARC channels to the apical trigger zone or arachidonic acid would have to diffuse from its site of generation, presumably close to the basal receptors, to activate apically localized ARC channels. The limited diffusion ranges of both free Ca\(^{2+}\) and arachidonic acid in the cytosol make such mechanisms unlikely, suggesting that the actions of Ca\(^{2+}\) entering via the ARC channels in the modulation of [Ca\(^{2+}\)]\(_i\) oscillation frequency are indirect. This is clearly an area worthy of investigation.

In addition to effects on [Ca\(^{2+}\)]\(_i\) signals, the entry of Ca\(^{2+}\) via ARC channels may have additional, more direct effects on cellular responses. As already discussed, the phenomenon of “reciprocal regulation” results in the ARC channels and the SOC channels being differentially active over distinct ranges of agonist concentration. A potential consequence of this is that the targeting of specific subsets of effectors to the two channel types could result in the activation of different responses in the cell in a novel, uniquely agonist concentration-dependent manner. In this scenario, such direct effects will be a reflection of the specific and sele-
tive activation of these two channel types at different agonist concentrations. It has already been shown (4) that the certain Ca\(^{2+}\)-sensitive adenyl cyclases whose activities are specifically influenced by Ca\(^{2+}\) entry through SOC channels are unaffected by similar entry through the ARC channels (32). It seems reasonable to conceive that other unique targets may also exist that are specifically coupled to Ca\(^{2+}\) entry through ARC channels.

Therefore, the indication is that, although ARC channels and SOC channels coexist in cells, they are not simply alternative, mutually redundant routes of Ca\(^{2+}\) entry. As noted, each plays a unique and nonoverlapping role in Ca\(^{2+}\) signaling; they are separately activated at different levels of agonist stimulation and serve distinct functions in the overall control of Ca\(^{2+}\) signals. However, additional unique responses could result from the targeting of specific effectors to these channels. These responses would depend on agonist concentration but in a manner that is essentially independent of the nature and magnitude of the overall Ca\(^{2+}\) signal itself.

Are ARC Channels TRPs?

Key to obtaining a full understanding of the regulation and function of the ARC channels is the determination of their molecular identity. However, in the current absence of any obvious clues to facilitate the isolation of the protein, or the gene encoding it, this remains unknown. In recent years, much has been made of the idea of members of the TRPC family of proteins (17) as potential candidates for agonist-activated Ca\(^{2+}\)-entry channels in various cells. Could the ARC channels be a TRPC? On the basis of existing data, this would seem to be highly unlikely. The fundamental problem is that the biophysical properties of the ARC channels (especially their very high selectivity for Ca\(^{2+}\)) are entirely distinct from any of the known members of the TRPC family of proteins. A common response to this discrepancy is the proposition that heteromers of different TRPCs might form channels that possess properties (including Ca\(^{2+}\)-selectivity) distinct from their individual family members. However, none of the heteromeric channels studied so far have shown high selectivity for Ca\(^{2+}\), and this would seem to be critical for any argument proposing the TRPC proteins as potential candidates for ARC channels. In their general biophysical properties, ARC channels are most similar to TRPV5 and TRPV6, but again clear differences exist. For example, both TRPV5 and TRPV6 are inhibited by elevations in external Ca\(^{2+}\); they show a profound and relatively rapid Ca\(^{2+}\)-dependent inactivation, and their monovalent currents show a marked Mg\(^{2+}\)-dependent activation on pulsing to negative potentials, resulting in a unique negative slope for the monovalent current-voltage curve at these potentials (33). None of these properties are seen in ARC channels. Moreover, neither TRPV5 or TRPV6 is apparently activated by arachidonic acid (Bernd Nilius, personal communication).

In conclusion, although the molecular identity of the ARC channels remains unknown, it seems likely that they play a key role in providing a major route for the agonist-activated entry of Ca\(^{2+}\) at physiologically relevant levels of stimulation in many nonexcitable cells. The characterization of the properties, behavior, regulation, and roles of these novel channels is therefore of critical importance to our understanding of [Ca\(^{2+}\)]\(_i\) signals in these cells and may ultimately provide the key clues that will lead to their identification.

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References


