Harmony and Discord in Endothelial Calcium Entry

To The Editor:

This letter addresses the recent report by Abdullaev et al.1 In 2006, the seminal work of Feske and colleagues2 identified membrane proteins that were named Orais, after the Greek keepers of Heaven’s gate: Harmony (Orai1), Justice (Orai2), and Peace (Orai3). The proteins emerged through a study of severe combined immune deficiency (SCID), which is caused by a defect in Ca\(^{2+}\) entry of T lymphocytes. A mutation in the Orai1 gene underlies the defect.

There has been particular interest in Orais because of their relationship with the widely occurring store-operated Ca\(^{2+}\)-entry (SOCE) phenomena, which reflect Ca\(^{2+}\)-permeable plasma membrane mechanisms that respond to the depletion status of endoplasmic reticular Ca\(^{2+}\) stores. In lymphocytes, the store-operated mechanism is commonly observed as a Ca\(^{2+}\)-selective and inwardly rectifying current, \(I_{\text{CRAC}}\). The mechanism is defective in SCID and rescued by expression of wild-type Orai1.2 In predicted structure Orais belong to the tetraspanin family3 rather than resembling known ion channels but mutations in Orais modify ion-selectivity, suggesting Orais generate ion permeation.4

Endothelial \(I_{\text{CRAC}}\)-Like Phenomenon

The search by Abdullaev et al1 for \(I_{\text{CRAC}}\) in cultured endothelial cells adds to an expanding view that the importance of Orai1 and \(I_{\text{CRAC}}\) is not restricted to the immune system. A striking feature of \(I_{\text{CRAC}}\) is its small amplitude, well below the size of most other whole-cell currents and close to the resolving power of whole-cell patch clamp. Abdullaev et al1 are to be commended for persisting when the current turned out to be at least 5 times smaller than the current of immune cells. Unfortunately, the current could only be convincingly shown in the absence of divalent cations, leaving open the possibility that Ca\(^{2+}\) shuts it down and, strictly, not enabling it to be described as \(I_{\text{CRAC}}\) (i.e., a Ca\(^{2+}\) current). Nevertheless, the current was lost when Orai1 was knocked down by RNA interference. Anionic current was also observed in the presence of a high concentration of extracellular Ca\(^{2+}\) but barely above background noise, making it difficult to define. Therefore, Abdullaev et al1 observed an \(I_{\text{CRAC}}\)-like phenomenon linked to Orai1, but its existence in the presence of Ca\(^{2+}\) is uncertain.

Although an \(I_{\text{CRAC}}\)-like phenomenon was observed using a common \(I_{\text{CRAC}}\) protocol,1 there is little direct evidence it depended on store depletion. All recordings of a clear \(I_{\text{CRAC}}\)-like signal used a patch pipette containing a high concentration of the Ca\(^{2+}\) chelator BAPTA. Although BAPTA depletes intracellular stores, it also lowers the global cytosolic Ca\(^{2+}\) concentration. Therefore, observation of current with BAPTA in the pipette is not proof that the current arose because of store depletion. Therefore, Abdullaev et al1 also used another method for store depletion (application of the SERCA inhibitor thapsigargin). However, the observed current had unexpected characteristics: it developed abruptly and then declined (unlike \(I_{\text{CRAC}}\) of immune cells), the current–voltage relationship was not like that of \(I_{\text{CRAC}}\), and gadolinium sensitivity was not demonstrated because the current declined before gadolinium was applied. Ca\(^{2+}\)-activated current could have been a confounding factor because the Ca\(^{2+}\)-free BAPTA available for buffering may have been relatively little, especially given the dialysis time required for such large cells.

TRPC Discord

Abdullaev et al1 addressed and ruled out contribution of canonical transient receptor potential (TRPC) proteins. However, previous studies of endothelial SOCE all suggested TRPCs contribute the underlying ion permeation pathway.5–9 In studies of human umbilical vein endothelial cells (HUVECs) (the same cell type used by Abdullaev et al1), store depletion evoked nonselective cationic current that was abolished by dominantly negative TRPC3 construct. In other studies of HUVECs (and other endothelial cells), store depletion evoked a similar nonselective cationic current that was enhanced by overexpressing TRPC1 and suppressed by antisense DNA or antibody targeting TRPC1. In other endothelial cells, a mutant form of TRPC4 inhibited Ca\(^{2+}\) current observed during store depletion. Current resembling \(I_{\text{CRAC}}\) was observed in endothelial cells from wild-type but not TRPC4 knockout mice.

The TRPC hypothesis is appealing because TRPCs are bona fide ion pore-forming proteins with Ca\(^{2+}\) permeability. However, TRPCs do not have the Ca\(^{2+}\) selectivity and inward rectification of \(I_{\text{CRAC}}\) and are activated by a multitude of factors,10 such that association with or dependence on store depletion could be a relatively minor aspect of their biology. Nevertheless, independent investigators have published data suggesting TRPCs are involved in endothelial SOCE, as well as associated \(I_{\text{CRAC}}\)-like or nonselective cationic currents. Resolutions to the apparent discord may come through answers to very specific questions, such as: (1) Do TRPCs activate when physiological concentrations of physiological agonists evoke Ca\(^{2+}\) release in physiological conditions? Probably yes. (2) Are TRPCs activated by only some experimental protocols designed to evoke store depletion? Probably yes. (3) When the only event is store depletion, do TRPCs respond? Although many investigators are keen to answer this question, it is currently impossible to be sure that store depletion is the only event. (4) Do protocols isolating \(I_{\text{CRAC}}\) shut down other mechanisms? Almost certainly yes. Abdullaev et al excluded TRPCs based on RNA interference experiments aimed at knocking down expression of TRPC1 or TRPC4,1 which are 2 of the 6 human TRPCs. Demonstration of effective knockdown is important in such a situation. Unfortunately, the size of the protein suggested to be TRPC1 was 20% larger than the predicted mass of TRPC1, overexpressed TRPC1 clone, or native TRPC1 validated in studies of TRPC1−/− mice. Although glycosylation of TRPC1 was suggested, supporting data were not provided and the only potential N-linked glycosylation site is weak. Whereas the band intensity was reduced by TRPC1 small interfering RNA, other bands labeled by the antibody were also decreased, as was the amount of β-actin.

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Although TRPC1 RNA interference affected cell proliferation, the Western blotting fails to confer confidence that the effect resulted from knockdown of TRPC1. The TRPC4 data, however, appear convincing.

What are we to concluded when previous studies provide data suggesting TRPCs contribute to SOCE of endothelial cells,\textsuperscript{5–9} whereas the data from the study by Abdullaev et al indicate no role? Abdullaev et al suggest that most previous studies relied on antibodies that yielded nonspecific effects, but the antibody data comprise only a small component of the endothelial cell evidence. Alternatively, could the details of the experimental protocol be crucial in determining whether an investigator observes a TRPC contribution? This would not be surprising. However, if so, where does the difference lie and does it matter for understanding the biology?

Because $I_{\text{CRAC}}$ is so small, particular conditions are needed to remove other, larger, unwanted currents. Might these conditions compromise the function of TRPCs? The Ca\textsuperscript{2+} concentration may be critical. Store depletion can lead to a rise in cytoplasmic Ca\textsuperscript{2+}, stimulating Ca\textsuperscript{2+}-activated ion channels. However, this is not what is usually meant by store-operated channels; instead, the channels sense only the depletion status of the stores. To exclude changes in Ca\textsuperscript{2+}, the experimenter must tightly buffer cytosolic Ca\textsuperscript{2+}. Ca\textsuperscript{2+}“add-back” experiments using a Ca\textsuperscript{2+} indicator dye do not meet this condition. Commonly $I_{\text{CRAC}}$ is studied with inclusion of a high concentration of BAPTA in the patch pipette, which helps to deplete the stores and buffer Ca\textsuperscript{2+} but also reduces cytosolic Ca\textsuperscript{2+} to subphysiological levels. Less commonly, $I_{\text{CRAC}}$ is evoked by a SERCA inhibitor during buffering of cytosolic Ca\textsuperscript{2+} at the physiological concentration. Almost always, 2 nonphysiological conditions are used in the extracellular medium: a high concentration of Ca\textsuperscript{2+} (10 to 20 mmol/L) or a DVF (divalent cation-free) solution. Such maneuvers aid observation of $I_{\text{CRAC}}$ but may divert investigators from TRPCs, which have complex Ca\textsuperscript{2+} dependencies and regulation.

An additional factor to consider is inositol 1,4,5-triphosphate (IP\textsubscript{3}). Notably, it was included in the patch pipette for the TRPC4\textsuperscript{4–7} studies\textsuperscript{4} but not in the studies by Abdullaev et al.\textsuperscript{1} TRPC channels couple to IP\textsubscript{3} receptors, and so involvement of TRPCs in SOCE may conceivably depend on a permissive concentration of IP\textsubscript{3}. However, nonselective cationic store-operated current has been observed in endothelial cells without IP\textsubscript{3} in the patch pipette\textsuperscript{5,8,9} and activation of TRPCs by other nonselective cationic store-operated current has been observed in endothelial cells without IP\textsubscript{3}. If permissive IP\textsubscript{3} is nevertheless required (or just important), we know that it would usually be present when agonists cause Ca\textsuperscript{2+} release. In contrast, physiological concentrations of agonists may evoke Ca\textsuperscript{2+} release without there being appreciable store depletion. Indeed, it is unclear whether cells experience store depletion of the type evoked by thapsigargin or intracellular BAPTA.

**Potential for Harmony**

If Orai1 does indeed confer a widespread $I_{\text{CRAC}}$ data, the data from the study by Abdullaev et al\textsuperscript{4} show us that the current may often be so small in physiological conditions that it is undetectable. Nevertheless, the Ca\textsuperscript{2+} entry may be sufficient to alter behavior of other ion channels, especially if they are physically coupled to Orai. Such close relationships between ion channels have precedence, for example, between voltage-gated Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, as well as different ligand-gated ion channels. Similarly, Orains and TRPCs may form complex molecular arrangements or webs. There is biochemical evidence for Orains interacting with TRPCs, either directly or via the auxiliary STIM1 protein, and knockdown of Orai1 can abolish current carried by overexpressed TRPC1.\textsuperscript{11–13} Perhaps Orains and TRPCs are separable by experimental conditions but often partners in physiology. There is much work to do, and the study by Abdullaev et al\textsuperscript{1} makes an important contribution to the campaign to solve these complex processes.

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