Role of lipid rafts in the interaction between hTRPC1, Orai1 and STIM1

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Abbreviations: SOCE, store-operated Ca\(^{2+}\) entry; TRP, transient receptor potential; TRPC, canonical transient receptor potential; \([\text{Ca}^{2+}]_c\), cytosolic free Ca\(^{2+}\) concentration; PM, plasma membrane; TG, thapsigargin

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Store-operated Ca\(^{2+}\) entry (SOCE) is a mechanism regulated by the filling state of the intracellular Ca\(^{2+}\) stores that requires the participation of the Ca\(^{2+}\) sensor STIM1, which communicates the Ca\(^{2+}\) content of the stores to the plasma membrane Ca\(^{2+}\)-permeable channels. We have recently reported that Orai1 mediates the communication between STIM1 and the Ca\(^{2+}\) channel hTRPC1. This event is important to confer hTRPC1 store depletion sensitivity, thus supporting the functional role of the STIM1-Orai1-hTRPC1 complex in the activation of SOCE. Here we have explored the relevance of lipid rafts in the formation of the STIM1-Orai1-hTRPC1 complex and the activation of SOCE. Disturbance of lipid raft domains, using methyl-\(\beta\)-cyclodextrin, reduces the interaction between endogenously expressed Orai1 and both STIM1 and hTRPC1 upon depletion of the intracellular Ca\(^{2+}\) stores and attenuates thapsigargin-evoked Ca\(^{2+}\) entry. These findings suggest that TRPC1, Orai1 and STIM1 form a heteromultimer associated with lipid raft domains and regulated by the intracellular Ca\(^{2+}\) stores.

Store-operated Ca\(^{2+}\) entry (SOCE) is a mechanism for Ca\(^{2+}\) influx through the plasma membrane (PM) activated by a decrease in the Ca\(^{2+}\) content of the intracellular stores, although the precise mechanism underlying the activation of SOCE and the nature of the channels involved are not fully understood. The communication of the filling state of the stores to the PM has recently been attributed to the intraluminal Ca\(^{2+}\) sensor STIM1, while the conduction of stores-operated currents, named I\(_{\text{SOC}}\), that occur through poorly selective cation channels have been described in different cell types. Orai1, a relatively small (301 amino acid) protein with four predicted transmembrane domains and cytosolic N- and C-terminal tails, has been suggested to form the pore of the channel mediating I\(_{\text{CRAC}}\). Moreover, the cation channels formed by transient receptor potential (TRP) proteins have been shown to contain STIM1-mediated store depletion sensitivity to canonical TRP proteins (TRPC), thus suggesting a common molecular basis for the channels conducting I\(_{\text{CRAC}}\) and I\(_{\text{SOC}}\).

In human platelets we have shown that STIM1 is involved in the activation of SOCE and that electrotransjection of human platelets with anti-Orai1 antibody, directed toward the C-terminal region that mediates the interaction with STIM1, blocked the interaction between STIM1 and hTRPC1, an effect that was mimicked by stabilization of an actin cortical barrier using jasplakinolide. When the interaction hTRPC1-STIM1 was prevented hTRPC1 was no longer involved in SOCE; thus supporting the functional role of the STIM1-Orai1-hTRPC1 complex in the activation of SOCE.

hTRPC1 subunits have been reported to localize in heteromultimeric complexes associated with platelet lipid raft domains; in these domains hTRPC1 might interact with other Ca\(^{2+}\)-handling proteins. The specific cholesterol-binding reagent, methyl-\(\beta\)-cyclodextrin is frequently used to acutely deplete cells of cholesterol and disturb lipid raft domains. Extending our studies further, here we have investigated the involvement of lipid rafts in the association between hTRPC1, Orai1 and STIM1 by looking for co-immunoprecipitation from platelet lysates, as previously described. Immuno precipitation and subsequent SDS-PAGE and Western blotting were conducted using platelets incubated at 37°C for 30 min with 10 mM methyl-\(\beta\)-cyclodextrin, or the vehicle (DMSO) as control, in a medium containing 200 \(\mu\)M Ca\(^{2+}\). Cells were then stimulated in the absence of extracellular Ca\(^{2+}\) (250 \(\mu\)M EGTA was added to the medium) for 10 s with the inhibitor of the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) thapsigargin (TG; 200 nM) to induce depletion of the intracellular stores in platelets. After immunoprecipitation with anti-hTRPC1, Western blotting revealed the presence of Orai1 (Fig. 1A) and STIM1 (Fig. 1B) in samples from resting platelets. The specificity of the hTRPC1 antibody was tested with the anti-TRPC1...
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antibody T1E3, a specific tool in the investigation of mammalian TRPC1 proteins. We found that treatment with TG increased the association between hTRPC1 and both Orai1 (Fig. 1A) and STIM1 (Fig. 1B; p < 0.05 Student's t-test; n = 6). Western blotting of the same membranes with hTRPC1 confirmed similar protein content in all lanes (Fig. 1, lower). Preincubation of human platelets with 10 mM methyl-β-cyclodextrin for 30 min resulted in attenuation of TG-stimulated interaction between hTRPC1, Orai1 and STIM1 in human platelets (Fig. 1; p < 0.05 Student's t-test; n = 6), without having any effect on the interaction between these proteins in resting cells.

We have further investigated whether platelet treatment with methyl-β-cyclodextrin might alter SOCE. To assess this issue, platelets were incubated for 30 min at 37°C with 10 mM methyl-β-cyclodextrin or the vehicle, as control, in a medium containing 200 μM CaCl2 to avoid depletion of the stores at this stage. At the time of experiment 250 μM EGTA was added to perform the studies in a Ca2+-free medium and cytosolic free Ca2+ concentration ([Ca2+]c) was determined as described previously. Cells were then treated with 200 nM TG to induce depletion of the intracellular Ca2+ stores. As shown in Figure 2A, treatment of control cells with TG resulted in a prolonged elevation of [Ca2+]c, due to leakage of Ca2+ from intracellular stores (the integral for 3 min of the rise in [Ca2+]c after the addition of TG taking a sample every second was 32272 ± 3212 nM-s; Fig. 2B). The subsequent addition of Ca2+ (1 mM) to the external medium induced a sustained increase in [Ca2+]c, indicative of SOCE (the integral of the rise in [Ca2+]c after the addition of CaCl2 was 265369 ± 14330 nM·s; Fig. 2C). Preincubation with methyl-β-cyclodextrin was unable to alter TG-evoked Ca2+ release from the intracellular stores (Fig. 2A and B); however, this treatment significantly reduced TG-induced SOCE by 27% (the integral of the rise in [Ca2+]c after the addition of CaCl2 in the presence of methyl-β-cyclodextrin was 195171 ± 20638 nM·s; Fig. 2C; p < 0.05).

Our findings indicate that lipid raft domains are important for the formation of heteromultimeric complexes involving the Ca2+ sensor STIM1 and the plasma membrane Ca2+ permeable channels Orai1 and hTRPC1, which has been demonstrated to be important for the mode of activation of hTRPC1 subunits, as well as for the activation of SOCE. The regulation of the assembly and activation of the channels involved in the conduction of SOCE, as well as other Ca2+
Handling proteins is of broad interest, and our observations shed new light in the mechanisms of activation of store-operated channels and their possible location in specific plasma membrane domains.

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