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STIM1/Orai1, I_{CRAC} and Endothelial SOC

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To the editor:

Store-operated calcium (Ca^{2+}) entry (SOCE) is an evolutionarily conserved pathway of regulated Ca^{2+} entry into cells whereby the depletion of Ca^{2+} from internal stores signals the activation of plasma membrane SOC channels[1, 2]. Non-selective canonical transient receptor potential (TRPC) channel proteins were investigated as potential components of SOC channels in mammals. However, the resulting literature offer no consensus among investigators and the role of TRPC in encoding SOC remains a contentious issue[2]. In our recent study[3] we challenged the role of TRPC in endothelial cell (EC) SOCE by showing evidence for I_{CRAC} in EC encoded by STIM1 and Orai1 independently of TRPC1 and TRPC4. In his letter[4], Beech pointed out the discrepancies between previously published data and ours and contended: i) *that although the biophysical properties of the I_{CRAC} recorded in divalent-free (DVF) solutions are convincing, its small size in the presence of extracellular Ca^{2+} and intracellular BAPTA makes its existence uncertain and that observation of current with BAPTA in the pipette is not proof that it is store-operated*; and ii) *we failed to provide confidence for the knockdown of TRPC1*. We reject these contentions and maintain that I_{CRAC} encoded by STIM1/Orai1 accounts for EC SOCE based on the following evidence:

1. It has been clearly established that I_{CRAC} can be activated to the same extent with any means that deplete the stores, these include: BAPTA or inositol-1,4,5-trisphosphate (IP_3) dialysis through the pipette; SERCA inhibitors or ionomycin applied to the bath; sensitizing IP_3 receptors to resting levels of IP_3 with thimerosal; and loading of stores with the membrane-permeable metal Ca^{2+} chelator TPEN[1, 2]. We maintain that all our recordings in which store depletion was elicited by either thapsigargin or BAPTA in 10mmol/L external Ca^{2+} consistently produced currents with similar sizes, displaying the biophysical and pharmacological characteristics of I_{CRAC} (total block by 1–10μmol/L gadolinium or 30μmol/L 2-APB).
2. HEK293 cells show almost undetectable endogenous I_{CRAC} in Ca^{2+}-containing solutions that is revealed only in DVF solutions[5], yet in these cells I_{CRAC} is largely responsible for SOCE and is encoded by Orai1[6]. Thorough comparisons of SOC currents in HEK293, RBL and EC by Fasolato and Nilius[7] failed to detect non-selective SOC currents in these cells and described I_{CRAC} currents in EC with similar size to ours. Further, estimations of Ca^{2+} entry in HUVEC in the presence of 10 mmol/L extracellular Ca^{2+} suggests that when entry through highly Ca^{2+} selective I_{CRAC} occurs (considering Ca^{2+} fraction of the current 100%; P_{Ca}/P_{Na} ~1000), around 1pA is needed to reach global μmol/L Ca^{2+} concentrations[8]. On the other hand, TRPC are *bona fide* non-selective channels where a major portion of

Disclosures
None

their currents is carried by sodium. There is no evidence that TRPC conduct Ca^{2+} under native conditions; the agonist-induced Ca^{2+} entry measured by Fura2 imaging when TRPC are expressed in HEK293 cells might correspond to an artifact of overexpression[9]. Beech[4] refers to the study by Freichel et al[10] describing I_{CRAC}-like currents in EC from wild-type mice but not in TRPC4 knockout mice but did not elaborate on how these Ca^{2+} selective currents resembling I_{CRAC} could be reconciled with either non-selective TRPC4 channels as their molecular correlates or non-selective "SOC" currents recorded by other groups. Based on Ca^{2+} imaging experiments using ectopic expression of Orai and TRPC in HEK293 cells, a recent report proposed an attractive model where Orai/TRPC complexes would mediate SOCE[11]. However, it is not clear how the ectopic expression in HEK293 cells relates to native conditions and the data reported would need to be confirmed under voltage-clamp conditions. While the results by Freichel et al on TRPC4 knockout mice are quite puzzling[10], this same group questioned a role for TRPC as a component of I_{CRAC} in a subsequent perspective[12].

3. As is the case with any scientific question, it is best to use a combination of experimental approaches. While patch clamp is the most direct and unambiguous method for studying SOC, when SOC currents appear very small, the advantage of fluorescent indicators over direct current measurements is their greater sensitivity to small Ca^{2+} changes. The use of several SOCE inhibitors at similar concentrations for patch clamp recordings and Fura2 imaging strengthens the results. We would like to point out that in our study i) SOCE measured with Fura2 imaging and I_{CRAC} have similar pharmacological profiles; ii) Most importantly, SOCE in response to thapsigargin and to agonist (thrombin) have similar pharmacological profiles and were both essentially abrogated upon STIM1 and Orai1 knockdown iii) In these cases, SOCE was rescued by STIM1 and Orai1 expression; and iv) SOCE in EC has one of the most peculiar characteristics of Orai1 (potentiation with 5μmol/L of 2-APB and block with 30–50μmol/L). Taken together, these results strongly argue that EC SOCE is mediated by I_{CRAC} encoded by STIM1/Orai1.

4. In our study, we ruled out the involvement of TRPC1 and TRPC4 in SOCE and I_{CRAC} using two independent siRNA sequences[3]. Beech[4] questioned the specificity of the TRPC1 antibody used in our study. However, the same anti-TRPC1 antibody was used in studies cited by the Beech letter, from groups that proposed TRPC1 as a SOC component[13]. In our study, two independent siRNA against TRPC1 significantly decreased the TRPC1 mRNA (figure7A) and reduced the TRPC1 protein band to the level of background; in figure 7B, we show a statistical analysis of the anti-TRPC1 Western blots where densitometry of TRPC1 bands relative to β-actin is depicted[3]. Beech argued that only a small fraction of previous studies relied on inhibitory antibodies as evidence for a role of TRPC in SOCE. We strongly disagree with this statement; to our knowledge there is no molecular evidence that store depletion activates TRPC currents when intracellular Ca^{2+} is buffered and only two studies reported attempts at TRPC knockdown in EC and both utilized the less specific technique of antisense RNA[14, 15]. In these studies, currents were recorded with low intracellular buffering (1.15mmol/L EGTA or 1mmol/L BAPTA)[14, 15] such that Ca^{2+}-activated TRPC-mediated currents could have been a confounding factor.

5. In the original siRNA screens identifying STIM1 and Orai1 as components of SOCE by 4 independent groups, none showed involvement of the 28 members of the larger TRP family[6]. Studies from the Gudermann group showed that smooth muscle isolated from aorta and cerebral arteries of TRPC1 knockout mice possess

SOCE currents that were comparable to those recorded in cells from wild-type mice[16]. Furthermore, the replenishment of Ca^{2+} stores is normal in central neurons from TRPC3 knockout mice and TRPC1/4/6 triple knockout mice[17], arguing that Ca^{2+} entry through TRPC channels is not required for refilling of internal stores.

In fact, the biophysical and pharmacological heterogeneity of the presumed non-selective "SOC" currents recorded by different groups is quite astonishing ([2]and references therein). Many of the discrepancies between different groups and controversies regarding TRPC encoding SOC channels may originate from differences in techniques, patch clamp protocols, and concentrations of pharmacological reagents used. Our study[3] used a combination of patch clamp and Fura2 imaging with two pharmacological inhibitors at similar concentrations in both techniques. We used molecular tools to knockdown in parallel in two different EC types several molecular candidates of the SOCE pathway (STIM1, Orai1, TRPC1 and TRPC4). Therefore, we believe our study provides significant insights into the mechanism of SOCE in EC. It is certainly conceivable that TRPCs in EC as well as smooth muscle carry out important signaling functions albeit through mechanisms not necessarily dependent on calcium store depletion.

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