Application Report

I$_{\text{CRAC}}$ on QPatch

Successful I$_{\text{CRAC}}$ measurements on QPatch using various approaches such as passive depletion or with IP3, ionomycin or thapsigargin to activate the I$_{\text{CRAC}}$ current.

Summary
Here we measure I$_{\text{CRAC}}$ from RBL-2H3 cells on QPatch and the current display the same biophysical characteristics as mentioned in the literature. We use a variety of approaches to activate the current; 1) Activation by passive depletion, 2) Activation with IP3, 3) Activation with ionomycin, 4) Activation with thapsigargin and we can conclude that QPatch is suitable for I$_{\text{CRAC}}$ measurements.

Introduction
Rat basophilic leukaemia (RBL) cells endogenously express calcium-release-activated-calcium (CRAC) channels (1). CRAC channels are activated by depletion of intracellular calcium stores, probably via the involvement of STIM-1 (stromal interaction molecule) sensing the depletion of the stores and travelling to the cell membrane activating the channel (2, 3). Several approaches can be used to deplete calcium stores ultimately leading to activation of I$_{\text{CRAC}}$ and most of these have been tried on QPatch. This document describes the results of these experiments and discusses the best conditions for studying I$_{\text{CRAC}}$ with QPatch.

Results and discussion
I$_{\text{CRAC}}$ is characterized by its inward rectifying I-V property and its very small conductance. Figure 1 shows raw data plots from the QPatch Assay software showing the sweep response before and after induction of I$_{\text{CRAC}}$.

Fig. 1 : Top: Inactivated RBL-2H3 and middle: fully activated I$_{\text{CRAC}}$ (passive depletion with 10 mM EGTA). Middle/bottom: Leak subtracted I$_{\text{crac}}$, the data from A and B where exported to Origin where the inactivated sweep was subtracted from the activated. Sweep cursors set at -80 mV and +95 mV.
As mentioned, there are several ways that depletion of Ca\(^{2+}\)-stores can be achieved, but commonly it is important to buffer Ca\(^{2+}\) in the pipette solution with a high concentration of a Ca\(^{2+}\)-chelator present in the pipette solution (i.e. BAPTA or EGTA).

**Activation of I\(_{\text{CRAC}}\) by passive depletion**

Passive depletion of internal Ca\(^{2+}\)-stores takes advantage of the constant leakage of Ca\(^{2+}\) from the endoplasmatic reticulum (ER). The Ca\(^{2+}\) is chelated by EGTA or BAPTA and will thus not be available to return to the ER via the Ca\(^{2+}\)-ATPase in the ER membrane. This approach is called passive depletion and is characterized by an initial delay followed by a relatively slow activation of I\(_{\text{CRAC}}\) (Figure 2). This agrees with what has been reported with manual patch clamp (4).

**Activation of I\(_{\text{CRAC}}\) with ionomycin**

The Ca\(^{2+}\)-ionophore ionomycin can also be used to deplete the Ca\(^{2+}\) stores, since it will make the ER membrane Ca\(^{2+}\)-permeable. A typical example of an experiment with activation by externally added ionomycin is shown in Figure 4.

**Activation of I\(_{\text{CRAC}}\) with thapsigargin**

The last approach that has been used on QPatch is to use the SERCA blocker thapsigargin. Using this approach thapsigargin blocks reuptake of Ca\(^{2+}\) and depletes the Ca\(^{2+}\)-stores thereby activating I\(_{\text{CRAC}}\). A typical example is shown in Figure 5.

As shown here there are different methods of activating I\(_{\text{CRAC}}\) and they are all working well on QPatch. Most commonly at Sophion Bioscience we use IP3 in combination with BAPTA. For measurements where a baseline is needed thapsigargin and EGTA has been used.

With the thapsigargin approach be aware that sometimes the current will activate due to passive depletion even without the presence of thapsigargin giving an I/t plot as shown in Figure 6. If this is unwanted it can be circumvented by adding thapsigargin just after break-in – due to the short delay for the pipette movement, there will be 5-10 measurements with non-stimulated I\(_{\text{CRAC}}\).
Fig. 6: Activation of I_{CRAC} by passive depletion in a thapsigargin assay.

Inactivation of I_{CRAC}

Sometimes I_{CRAC} is seen to inactivate after activation. This is most often seen when using passive depletion with EGTA. A typical experiment with inactivation is shown in Figure 7.

IC_{50} determination for YM-58483

YM-58483 (3,5-Bis(trifluoromethyl)pyrazole derivative (BTP2) inhibits Ca^{2+} influx by the concerted actions of store-operated Ca^{2+} channels and Ca^{2+}-activated cation channels (6). Figure 9 shows the I_{CRAC} current raw data, where 30 µM of YM-58483 were applied.

The reason for the inactivation is at least partly due to refilling of the Ca^{2+}-stores since I_{CRAC} can be restored with thapsigargin (Figure 8). But there is also evidence for a more direct interaction between the the Ca^{2+} running into the cell and the channel itself resulting in a rapid inactivation of I_{CRAC}. The problem can be solved by using BAPTA instead of EGTA as the chelator. BAPTA chelates Ca^{2+} faster thus hindering binding of Ca^{2+} to the channel and reuptake into ER of Ca^{2+} running into the cell via the CRAC channel (5).

Figure 10 shows a typical current versus time plot (I-T) for 6 increasing concentrations of YM-58483 on different cells. The highest concentration was 30 µM, testing in a 3-fold dilution. Due to the low current level of I_{CRAC} the compound was applied at one concentration per cell and the data was normalized subsequently. The green vertical bars indicate the steady-state current and the points used to calculate the IC_{50}-value in the Hill fit. The corresponding Hill Fit is shown in Figure 10.
Fig. 10: IT plot showing six applications of YM-58483.

Fig. 11: Hill fit for YM-58483 compound tests.

Fig. 12: $I_{CRAC}$ and MIC. The outward current was primarily seen when a phosphate based external solution was used.

Using the Ringer’s solution significantly reduces the presence of MIC current. Since MIC is inhibited by internal Mg$^{2+}$ with an $IC_{50}$ of about 0.5 mM (7) increasing the concentration in the internal solution might also be a possible option. A possible explanation for this could be that Ca$^{2+}$ had a tendency to precipitate in the phosphate based ringer after freezing and thawing. As it can be seen in Figure 13 the MIC current is greatly activated in the absence of divalent cations. Since MIC is strongly outward rectifying it will not severely affect the $I_{CRAC}$ measurements when the $I_{CRAC}$ is analyzed at -80 mV. An example is shown in Figure 13.

The $IC_{50}$ value of YM-58483 was determined to 586.82 nM, n=3.

Mg$^{2+}$-inhibited cation current (MIC)
It can be sometimes seen that at positive voltages there is an outward current. This component is not $I_{CRAC}$ and is thought to be endogenous outward rectifying MIC current (or MagNuM for magnesium-nucleotide-regulated metal cation channel) (Figure 12).

Fig. 13: Green line is MIC measured at +95 mV and the blue is $I_{CRAC}$ at -80 mV. The experiment type is passive depletion with EGTA followed by removal of extracellular Ca$^{2+}$ in the compound period which stops the Ca$^{2+}$ conductance of the CRAC channels but transiently stimulates MIC dramatically. There is no correlation between the two cursors.
I_{CRAC} in RBL-1

RBL-1 cells also express CRAC channels. Compared to RBL-2H3 cells, however, I_{CRAC} Current from RBL-1 cells was difficult to activate to a recordable size. We have speculated that the expression of CRAC channels in RBL-1 cells is lower compared to the level in RBL-2H3 cells. We therefore suggest that the RBL-1 cells are not suitable for QPatch for I_{CRAC} measurements. It should be noted that gigasealing as well as whole-cell establishment was very easily performed with the RBL-1 cells by QPatch.

For the experiments presented here many of the compounds have their effect inside the cell and therefore longer periods with compound application have been used. In general, the length of compound application must be balanced with the fact that the CRAC channel inactivates after a certain period and the assay needs to be adjusted accordingly.

Conclusion

In this report it is shown that measurements of I_{CRAC} from RBL-2H3 cells are possible with QPatch and that the current has the biophysical characteristics as described from the literature. We have shown a variety of approaches to activate the I_{CRAC} Current; 1) Activation by passive depletion, 2) Activation with IP3, 3) Activation with ionomycin, 4) Activation with thapsigargin. Each of the assay types were easily performed with the QPatch. Gigasealing and establishment of the whole-cell configuration was easily obtained with the cell line, and the success rate for the completed experiments is >50%. We therefore conclude that QPatch is suitable for I_{CRAC} measurements.

Methods

Cells: RBL-2H3 cells from ATCC were grown according to the SOP from Sophion Bioscience.

Protocols: For these experiments the whole-cell suction protocol used resembles the one normally used for CHO cell lines (i.e. high pressure for positioning the cell, gigasealing and for rupturing the membrane for the whole cell configuration). The CRAC channel has an extremely small unitary conductance estimated from noise analysis to be ~10 fS under physiological conditions (8). The resulting whole cell current (I_{CRAC}) is also very small (2.5-3 pA/pF at -80 mV) so it is of pivotal importance that the seal resistance is very high (>1 Gohm) to get good recordings.

The voltage-protocol used is a 100 ms ramp going from -100 to +100 mV with an interval of either 3 or 6 seconds. The cell is held at 0 mV between the sweeps. The I/t plots are made with the QPatch assay software by inserting a cursor at -80 mV and plotting the current as a function of time (see Figure 1). The application protocol is mainly dependent on the compounds the investigator wants to test and the method for activation of the channel.

References: