Application Report:

Icrac

Measured on QPatch

CRAC channels are activated by depletion of intracellular calcium stores, probably via the involvement of STIM-1 sensing depletion of the stores and travelling to the cell membrane activating the channel. Several approaches can be used to deplete calcium stores ultimately leading to activation of Icrac and most of these have been tried on the QPatch. Here we describe the results of these experiments and discuss the best conditions for studying Icrac with the QPatch.

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Introduction

Rat basophilic leukaemia (RBL) cells endogenously express calcium-release-activated-calcium (CRAC) channels (Hoth and Penner, 1993). 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 (Zhang et al, 2005; Vig et al, 2006). Several approaches can be used to deplete calcium stores ultimately leading to activation of Icrac and most of these have been tried on the QPatch. This document describes the results of these experiments and discusses the best conditions for studying Icrac with the QPatch.

Materials & Methods

Cells: RBL-2H3 cells were bought from ATCC (www.ATCC.org) and grown according to the SOP from Sophion Bioscience.

Solutions: The intracellular solution contained (in mM): 145 Glutamate, 8 NaCl, 1 MgCl2, 10 HEPES, 10 BAPTA (or EGTA). pH was adjusted to 7.2 with CsOH and osmolarity was adjusted with sucrose to 320 mOsm.

When IP3 was used as the activator 20 uM was added to the intracellular solution on the day of experiments.

The extracellular solution contained (in mM): 140 NaCl, 2.8 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 CsCl, 10 HEPES, 10 mM glucose. pH was adjusted with NaOH to 7.4 and osmolarity was adjusted with sucrose to 350 mOsm.

Blockers, 2APB, SKF-96365 and YM-58483 (3,5-Bis(trifluoromethyl)pyrazole derivative (BTP2) were dissolved in the extracellular solution.

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 (Zweifach and Lewis, 1993). The resulting whole cell current (Icrac) 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. With the RBL-2H3 cells gigaseals > 1Gohm are easily established and the success rate for completed experiments should be over 50 % (see Figure 1).

Pos.	Primed	Cell att...	Giga...	Whol...	R chip [MΩ]	R seal [MΩ]	R whole-cell...	WC duratio...	Completed ...
A1	✓				2.48	0	0	0	0
A2	✓	✓	✓	✓	2.6	7895.5	4305.9	855	1
B1	✓				2.49	0	0	0	0
B2	✓	✓	✓	✓	2.51	3223.7	10378.8	868	1
C1	✓	✓	✓	✓	2.44	3000.5	30000	879	1
C2	✓	✓			2.88	70	0	0	0
D1	✓	✓	✓	✓	2.48	6036.5	2584.7	903	1
D2	✓	✓	✓	✓	2.54	17533.6	3702.6	889	1
E1	✓	✓	✓	✓	2.67	4622.4	3026.4	889	1
E2	✓	✓			2.57	80.4	0	0	0
F1	✓	✓	✓	✓	2.57	30000	30000	903	1
F2	✓	✓	✓	✓	2.44	2362	1454.7	913	1
G1	✓	✓			2.45	86.5	0	0	0
G2	✓	✓	✓	✓	2.47	3379.2	4739.1	845	1
H1	✓	✓			2.49	59.6	0	0	0
H2	✓	✓	✓	✓	2.55	598.6	2835.5	908	1
Total	16	14	10	10					10
Succes...	100 %	88 %	63 %	63 %					

Pos.	Primed	Cell att...	Giga...	Whol...	R chip [MΩ]	R seal [MΩ]	R whole-cell...	WC duratio...	Completed ...
A1	✓	✓	✓	✓	2.21	30000	15408.3	1082	1
A2	✓	✓	✓	✓	2.1	18137.3	19387.3	1096	1
B1	✓	✓	✓	✓	2.04	19097.9	3443.2	1104	1
B2	✓	✓	✓	✓	2.25	145.2	437.7	1167	1
C1	✓	✓	✓	✓	2.22	21453.1	12082.2	1127	1
C2	✓	✓	✓	✓	2.22	8967.4	3578.5	1135	1
D1	✓				2.51	0	0	0	0
D2	✓	✓	✓	✓	2.01	17898.2	17533.6	1136	1
E1	✓	✓	✓	✓	2.19	77359.3	13497.7	1159	1
E2	✓	✓	✓		2.03	31.5	16.1	0	0
F1	✓	✓	✓	✓	2.16	11333.6	26213.4	1136	1
F2	✓	✓	✓	✓	1.98	6507.2	5500.4	1168	1
G1	✓	✓	✓	✓	2.07	9831.6	9295.6	1188	1
G2	✓	✓	✓	✓	2	2675.4	2267.8	1169	1
H1	✓	✓	✓	✓	2.02	30000	15924.8	1198	1
H2	✓	✓	✓	✓	1.97	11381.7	9277	1199	1
Total	16	15	15	14					14
Succes...	100 %	94 %	94 %	88 %					

Figure 1. Two examples of QPlate overviews from RBL-2H3 cells. As it can be seen the seal resistances are primarily > 1 Gohm.

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 2).

The application protocol is mainly dependent on the compounds the investigator wants to test and the method for activation of the channel.

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 has to be balanced with the fact that the CRAC channel inactivates after a certain period and the assay needs to be adjusted accordingly. Inactivation of Icrac will be discussed later.

Results & Discussion

Icrac is characterized by its inward rectifying I-V property, and its very small conductance. Figure 2 shows raw data plots from the QPatch Assay software showing the sweep response before and after induction of Icrac.

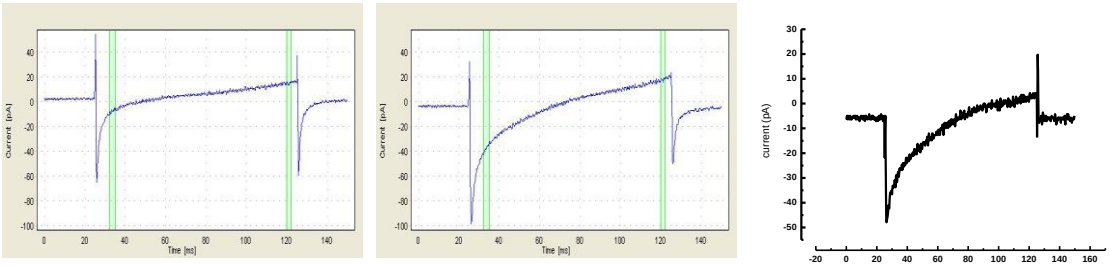

Figure 2. Left: Inactivated RBL-2H3 and middle: fully activated Icrac (passive depletion with 10 mM EGTA). Right: Leak subtracted Icrac, the data from A and B where exported to Origin where the inactivated sweep was subtracted from the activated. Sweep cursors set at -80 mV and +95 mV.

As mentioned there are several ways that depletion of Ca2+-stores can be achieved, but commonly it is important to buffer Ca2+ in the pipette solution with a high concentration of a Ca2+ chelator present in the pipette solution (i.e., BAPTA or EGTA).

Activation of I_{crac} by passive depletion

Passive depletion of internal Ca2+-stores takes advantage of the constant leakage of Ca2+ from the endoplasmatic reticulum (ER). The Ca2+ is chelated by EGTA or BAPTA and will thus not be available to return to the ER via the Ca2+-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 Icrac (see Figure 3). This is in agreement with what has been reported with manual patch clamp (Fierro et al, 1999).

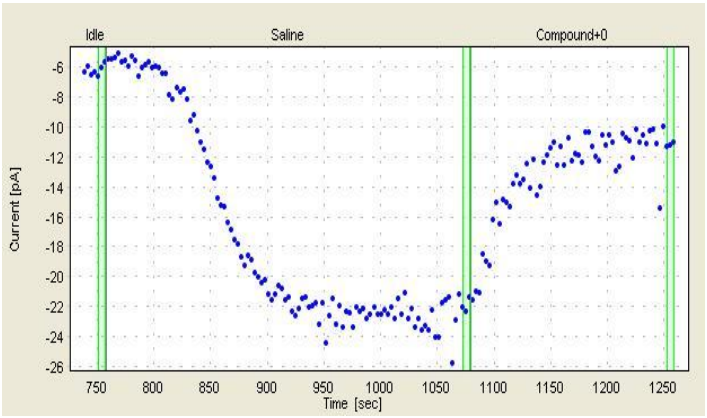

Figure 3. Typical time course of Icrac activation with passive depletion (10 mM EGTA), followed by 30 uM addition of 2-APB.

Activation of I_{crac} with IP3

One approach to actively deplete the Ca2+-stores is to have inositol trisphosphate (IP3) (20 µM) in the pipette solution. This triggers IP3-specific receptors in the ER membrane to release Ca2+ from the stores (see Figure 4).

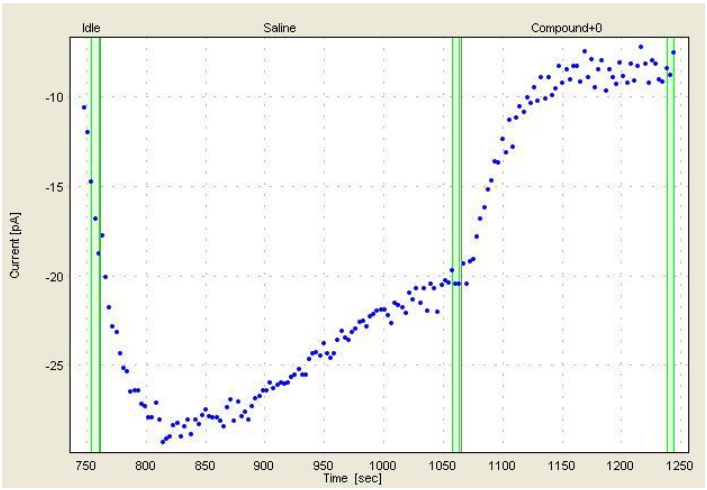

Figure 4. Typical activation of Icrac with 20 uM IP3 in the pipette again 30 uM 2APB was added to block the current. Note the lack of the delay phase and the faster activation of the current.

Activation of I_{crac} with ionomycin

The Ca2+ ionophore ionomycin can also be used to deplete the Ca2+ stores, since it will make the ER membrane Ca2+-permeable. A typical example of an experiment with activation by externally added ionomycin is shown in Figure 5.

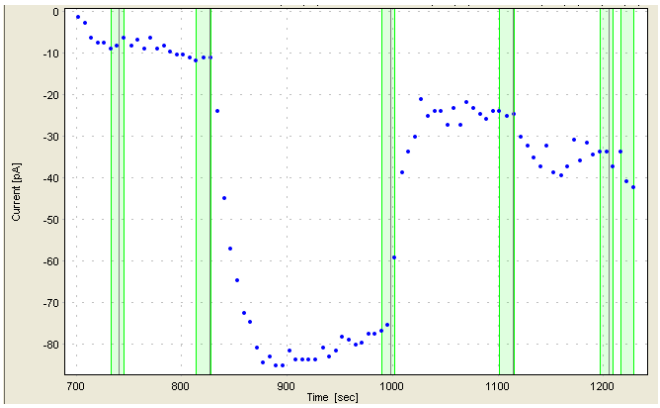

Figure 5. Icrac activation with 4 µM ionomycin. The current was blocked with 100 µM the Icrac blocker SKF 96365.

Activation of I_{crac} with thapsigargin

The last approach that has been used on the QPatch is to use the SERCA blocker thapsigargin. Using this approach thapsigargin blocks reuptake of Ca2+ and depletes the Ca2+-stores thereby activating Icrac. A typical example is shown in Figure 6.

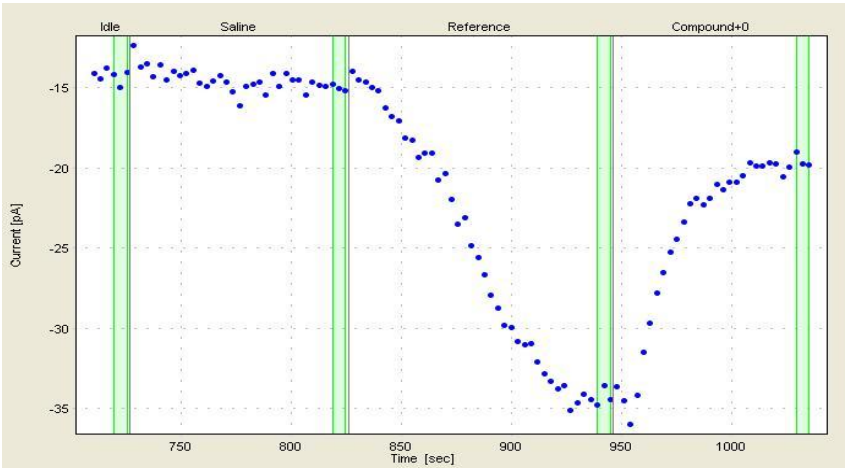

Figure 6. Icrac activation with 2 µM thapsigargin extracellularly applied. The current was blocked with 30 uM 2APB.

As shown here there are different methods of activating Icrac and they are all working well on the QPatch. Most commonly at Sophion Bioscience we use IP3 in combination with BAPTA. For measurements where a base line 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 7. 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 Icrac.

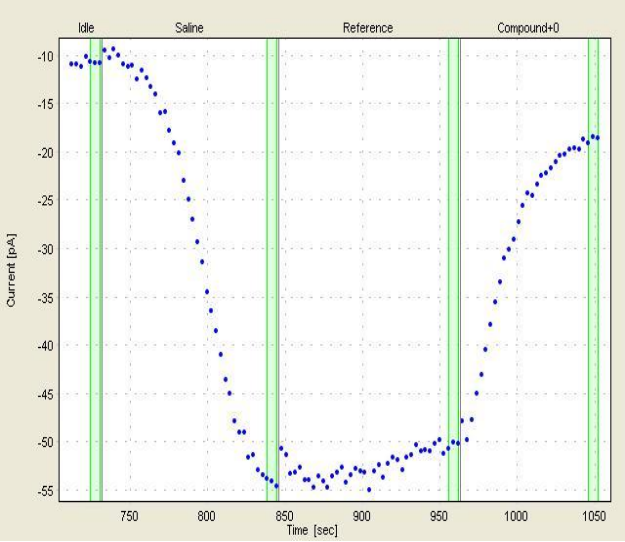

Figure 7. Activation of Icrac by passive depletion in a thapsigargin assay.

Inactivation of I_{crac}

Sometimes Icrac is seen to inactivate after activation. This is most often seen when using passive depletion with EGTA. A typical experiment with inactivation is shown below in Figure 8.

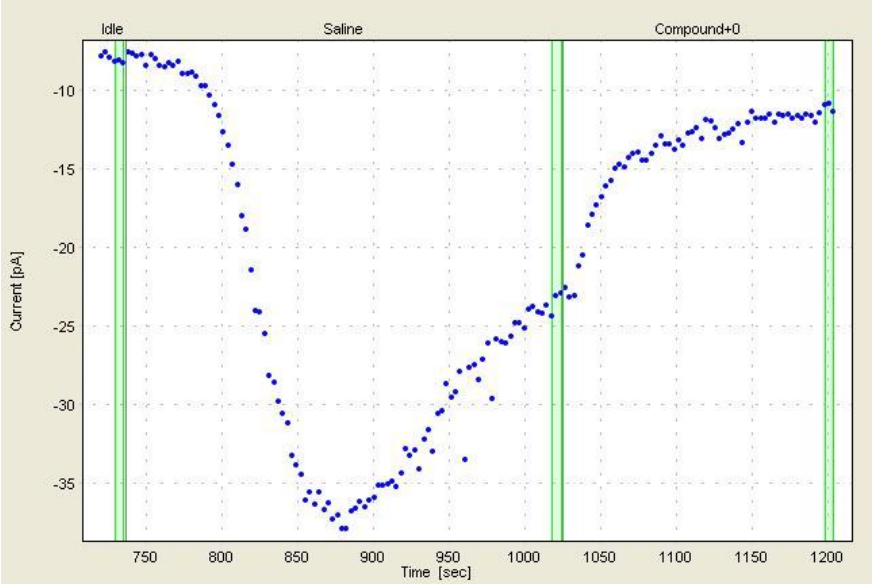

Figure 8. Rapid inactivation of Icrac activated with EGTA.

The reason for the inactivation is at least partly due to refilling of the Ca^{2+}-stores since Icrac can be restored with thapsigargin (See Figure 9). 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 Icrac. 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 also reuptake into ER of Ca^{2+} running into the cell via the CRAC channel (Zweifach and Lewis, 1995).

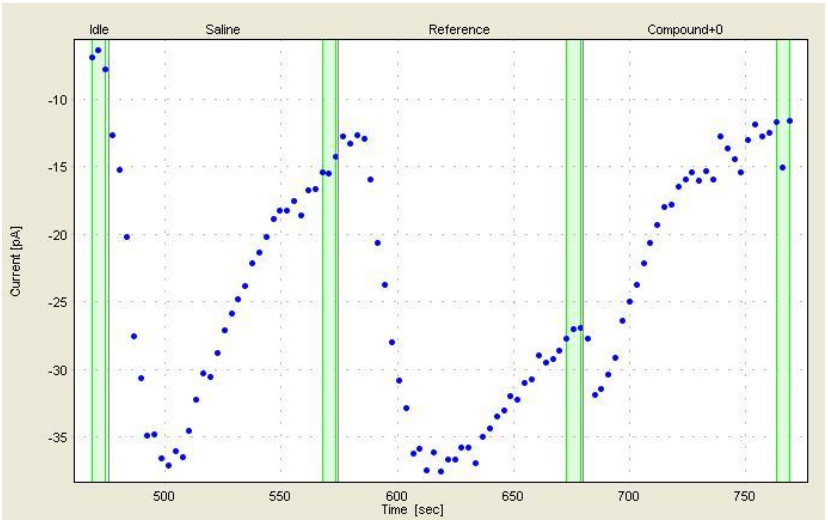

Figure 9. Icrac activated with passive depletion with EGTA. Icrac rapidly inactivates but reactivates when thapsigargin is added in the reference period. The current is blocked with 30 µM 2APB.

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 (Takezawa et al, 2006). Figure 10 shows the I_{CRAC} current raw data, where 30 µM of YM-58483 were applied.

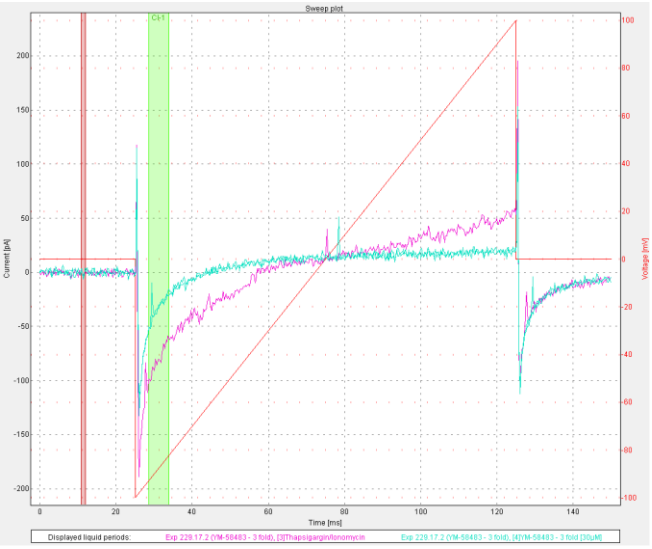

Figure 10. Raw data trace showing activation with thapsigargin and ionomycin (pink trace) and block with 30 µM YM-5843 (blue trace).

Figure 11 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 ICRAC 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 11.

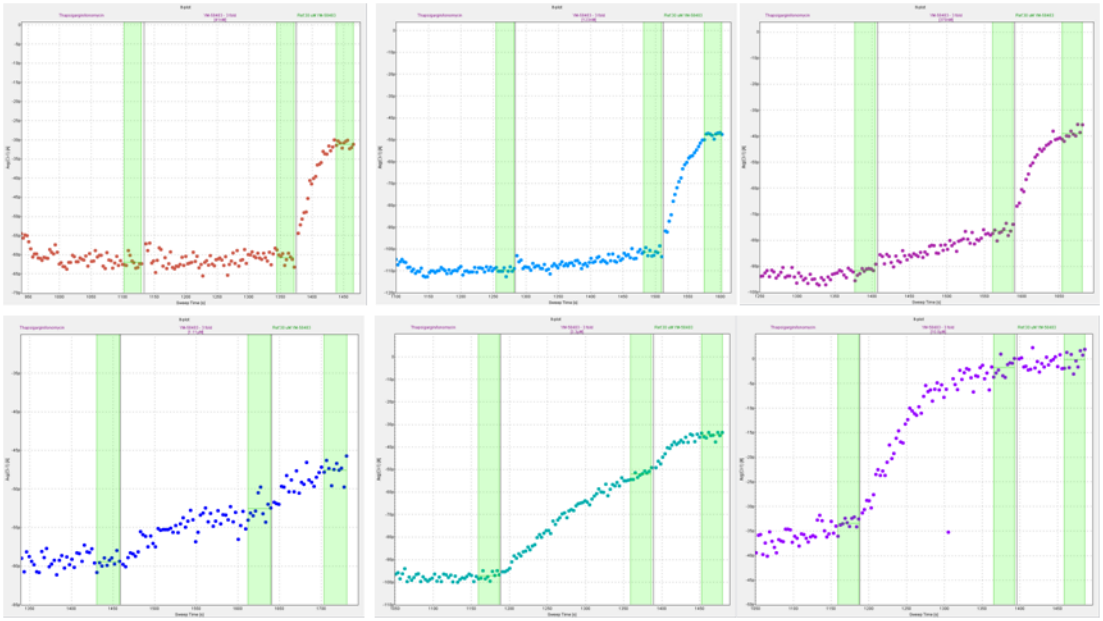

Figure 11. IT plot showing six applications of YM-58483.

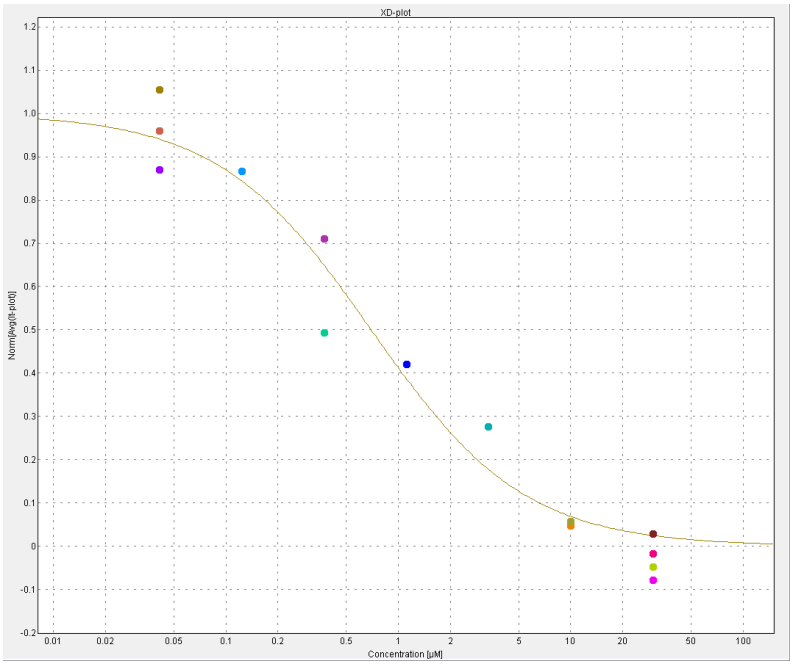

Figure 12. Hill fit for YM-58483 compound tests.

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 Icrac and is thought to be endogenous outward rectifying MIC current (or MagNuM for magnesium-nucleotide-regulated metal cation channel) (see Figure 10)

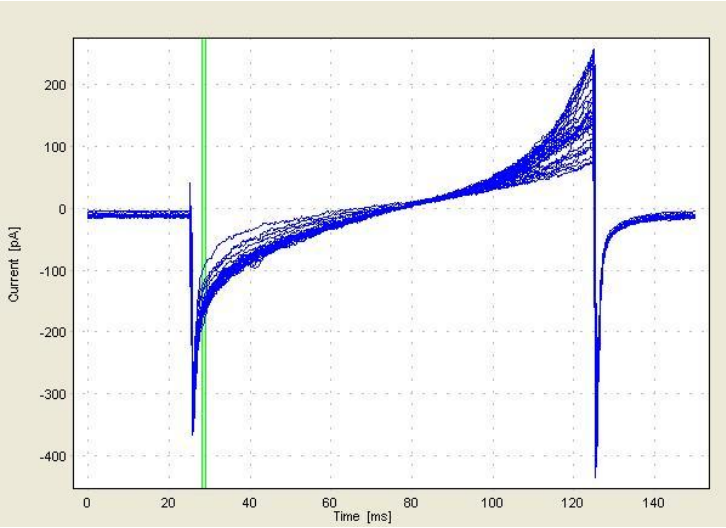

Figure 13. I_{crac} and MIC. The outward current was primarily seen when a phosphate based external solution was used

Using the ringer solutions described in the method section[1] significantly reduces the presence of MIC current, see Figure 2 for a typical example. Since MIC is inhibited by internal Mg2+ with an IC50 of about 0.5 mM (Clapham, 2002) increasing the concentration in the internal solution might also be a possible option. A possible explanation for this could be that Ca2+ had a tendency to precipitate in the phosphate based ringer after freezing and rethawing. As it can be seen in Figure 11 the MIC current is greatly activated in the absence of divalent cations. Since MIC is strongly outward rectifying it will not severely affect the Icrac measurements when the Icrac is analysed at -80 mV. An example is shown below in Figure 11.

[1] Some experiments were done with a phosphate based ringer: Hanks with 10 mM $CaCl_2$ and 5 mM $MgCl_2$.

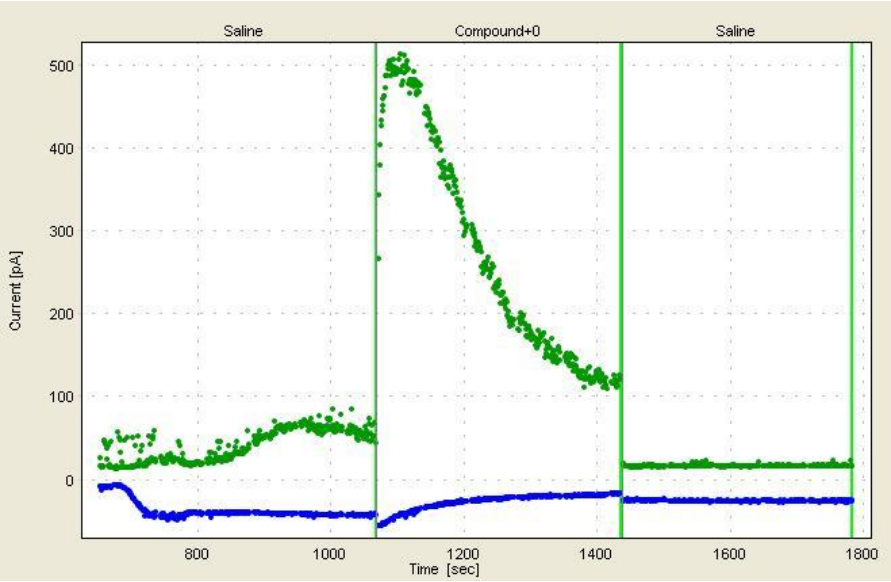

Figure 14. Green line is MIC measured at +95 mV and the blue is Icrac at -80 mV. The experiment type is passive depletion with EGTA followed by removal of extracellular Ca2+ in the compound period which stops the Ca2+ conductance of the CRAC channels but transiently stimulates MIC dramatically. There 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, Icrac 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 Icrac measurements. It should be noted that gigasealing as well as whole-cell establishment was very easily performed with the RBL-1 cells by QPatch.

References

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Zweifach A, Lewis RS:Rapid Inactivation of depletion-activated calcuim current (Icrac) due to local calcium Feedback, Journal of General Phyiology, 1995, 209-226.

Conclusion

In this report it is shown that measurements of Icrac from RBL-2H3 cells are possible with the QPatch and that the current has the biophysical characteristics as described from the literature. We have shown a variety of approaches to activate the Icrac 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 the QPatch is suitable for Icrac measurements.