IDENTIFYING RYANODINE RECEPTOR MODULATORS: FROM HIGH-THROUGHPUT SCREENING TO SINGLE CHANNEL RECORDING

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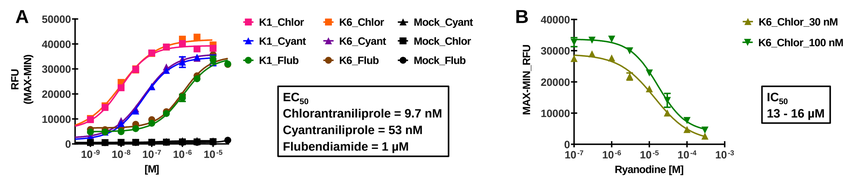
INNOVATES, ENABLES, PERFORMS

ABSTRACT

The ryanodine receptor Ca^{2+}-release channels (RyRs) are central to cytoplasmic Ca^{2+} signaling in particular in the skeletal muscle of both mammals and insects. Regarding the latter, several insecticides target RyRs but recent studies have shown that specific RyR point mutations can confer insecticide resistance. Consequently, research for new RyR modulators remains crucial for crop protection. The full-length coding sequence of the *Plutella xylostella* RyR was synthesized and cloned into suitable mammalian expression vector. We engineered HEK-293 expressing the insect target and functionally selected stable clones using Ca^{2+}-sensitive fluorescence. In order to exclude any endogenous non-specific response, a related mock clone was also generated in parallel. Stimulation of the cells to activate the RyR elicited a Ca^{2+} mobilization, which was detected as a fluorescence increase using a FLIPR-TETRA instrument. This RyR cell-based assay was validated with RyR ligands and putative blockers and further optimized to make it suitable for high throughput screening. Drug discovery programs, which may involve the screening of $>10^5$ compounds, requires a high-fidelity method of determining the effect of putative hits on the function of the target protein. Since RyR expression is targeted to internal membranes, patch-clamp of the nuclear membrane has previously been shown to be an effective method of isolating them for single-channel recording. We therefore set up this very challenging technique to measure the RyR activity single-channel properties (conductance, open probability -P_o- and dwell-time distribution) and to show the modulatory effect of Ca^{2+} on these above-mentioned parameters. From cell cloning to mode of action determination, we develop an assay funnel, optimized for hit sensitivity and specificity that is easily scalable to larger compound libraries.

FIGURE 1: *Plutella* RyR cell line characterization



The humanized version of the full-length coding sequence of the *Plutella Xylostella*, diamondback moth, ryanodine receptor (PxRyR) was cloned in the mammalian expression vector pcDNA3.1. The construct was then stably transfected into HEK-natClytin cells. The PxRyR stable clones, in parallel with the mock one, were selected.
Insecticidal ryanodine modulators such as chlorantraniliprole, cyantraniliprole and flubendiamide (IRAC Group 28) are potent on the HEK-natClytin/PxRyR cells with EC_{50}'s of 9.7nM, 53nM and 1µM, respectively, assayed by Ca^{2+}-sensitive fluorescence (Fluo-8, Screen Quest™ No-Wash Calcium Assay kit), figure 1A. Activation by chlorantraniliprole can be blocked by ryanodine with IC_{50} in the µM range (figure 1B).

FIGURE 2: *In-vitro* screen of small targeted library

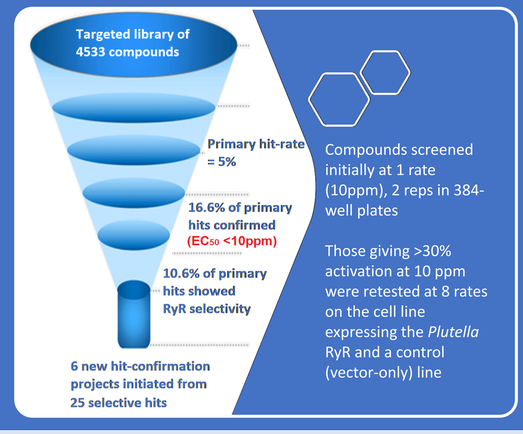
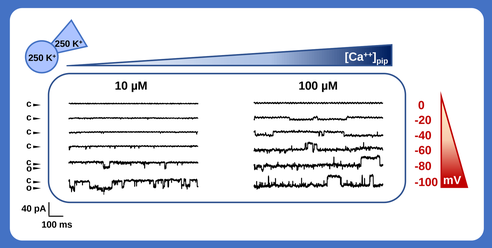


FIGURE 3: On-nucleus single-channel recordings



Nuclei isolated from HEK-natClytin/PxRyR were used to record the spontaneous open channel activities at different voltages, using the I/V protocol showed in material and methods. Single channel current traces illustrated in figure 3 show the different behaviors in the presence of increasing Ca^{2+} concentrations (0, 10, 100µM) in the pipette solution ($[Ca^{2+}]_{pip}$). RyR "open" and "closed" configuration are indicated by "O" (lower levels) and "C" (upper levels), respectively.
Single channel current traces do not show any RyR activity when calcium is completely omitted from the pipette solution (data not shown).
Experiments carried out using different concentrations of calcium (10 and 100µM $[Ca^{2+}]_{pip}$), suggest a dose-dependent increase in the opening events of RyR channels, without affecting the current amplitudes.
The traces clearly show that the higher the hyperpolarization, the higher: 1) open channel probability; 2) time spent in the open state; 3) current amplitude.

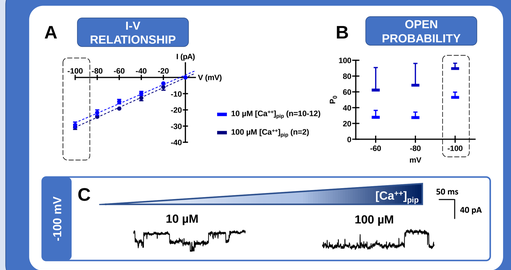


FIGURE 4: I/V relationship and open probability

Spontaneous open channel events were used to calculate the current amplitudes at different voltages (average values between the "open" and the "closed" channel configuration), using symmetrical K^+ in the bath and pipette solutions.
I/V relationships (A) were obtained by the fitting of data points using a linear regression equation, yielding the following channel conductance at -100mV: -29.32±1.83 pA (n=10-12); -30.55±1.54 pA (n=2), in the presence of 10, and 100µM $[Ca^{2+}]_{pip}$, respectively.
The open channel probability (P_0) represents the ratio between the total time spent in the open state and the total time of recording. P_0 was calculated during 500ms of recording at different voltages (-60, -80, -100mV), in the presence of increasing concentrations of Ca^{2+}. The graph shows the higher hyperpolarization steps induce an increase in the open channel probability.
The mean P_0 values, reported in the graph as mean ± SEM (B) are the following: -60mV, 27.7±8.6 % vs. 62.4±28.4 %; -80mV, 27.5±6.8 % vs. 68.5±27.5 %; -100mV, 53.0±6.8 % vs. 89.5±6.8 % in the presence of 10µM (n=10-12) and 100µM (n=2) $Ca^{2+}]_{pip}$, respectively.
The sample traces in the inset correspond to the -100mV step (C) and clearly show the difference in the current amplitude and in the P_0 at different Ca^{2+}concentrations.

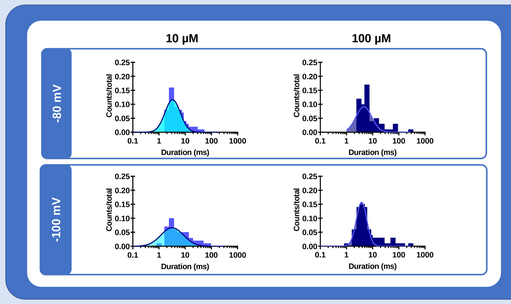
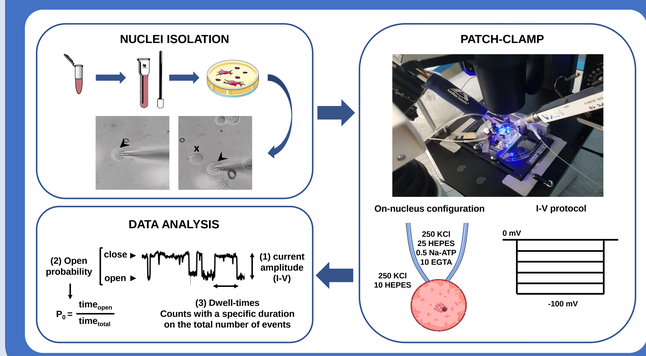


FIGURE 5: dwell-time analysis

Nuclei isolated from HEK-natClytin/PxRyR cells were analyzed in terms of kinetics properties, calculating the ratio of counts with a specific duration on the total number of events (dwell-times); RyR-evoked single channel events at different potentials (-80mV, -100mV) are reported in the dwell-time histograms in the absence and in the presence of two different Ca^{2+} concentrations (10, and 100µM $[Ca^{2+}]_{pip}$) on the patch solution.
Similar behaviors were observed using 10 and 100µM $[Ca^{2+}]_{pip}$, where most open event durations are included between 1 and 15ms.
In addition, we observed that more negative voltages evoke events with longer duration; in the presence of 10µM $[Ca^{2+}]_{pip}$ the 25.0% of events have a duration longer than 15ms at -80 mV; this percentage is increased with more hyperpolarizing potential (40.6% at -100mV). Similar results were obtained with 100µM $[Ca^{2+}]_{pip}$, where the 19.5, and 31.9% of events are longer than 15ms at -80, and -100mV, respectively.

MATERIAL AND METHODS



Nuclei isolation: HEK-natClytin/PxRyR were detached using trypsin and resuspended in the homogenization buffer. 1.5/2x10^6 cells were transferred to an ice-cold homogenizer and subject to an appropriate number of up-and-down strokes of the pestle. The homogenate was transferred to a 35mm petri dish ten minutes before starting the patch-clamp experiment. The preparation was used within 90-120min. Homogenization buffer (HB) contains (in mM); 250 sucrose, 150 KCl, 10 Tris-HCl, 1.4 β-mercaptoethanol (after pH adjustment); pH 7.3. A complete protease inhibitor table (Roche) was added after pH adjustment. Patch-clamp: Experiments were carried out in the "on-nucleus" configuration (analogous to the "on-cell" configuration in plasma membrane patching), at room temperature. Current-Voltage (I-V) protocol consists in 5 seconds steps recorded from -100mV to 0mV, with 20mV of delta, sampled at 20kHz and filtered at 5kHz, during the perfusion of bath solution. Data analysis: Parameters analyzed were; 1) current amplitude, 2) open probability (P_0), 3) dwell-time.

CONCLUSIONS

RyR modulators play a crucial role in crop protection. Axxam developed, validated, and optimized a RyR cell-based assay suitable for both high throughput screening and on-nucleus patch-clamp experiments. Single channel properties, in terms of conductance, open probability, and dwell-time, were analyzed in order to set the best performing conditions to use in a further compound profiling phase. Data collected during the on-nucleus patch-clamp experiments produced the following key points:

- In the absence of calcium, no channel activity was evoked;
- different concentrations of calcium added (10 and 100µM) to the patch solutions, induced a dose-dependent increase in the opening events of RyR channels;
- I-V relationships are comparable between 10 and 100µM, suggesting no difference in current amplitude and channel conductance;
- 100µM of calcium increases the channel activity (i.e. the time that RyR channels spend in the open state), if compared to 10µM.
- Dwell-time analysis show similar behaviors between 10 and 100 µM, where most open event durations are included between 1 and 15ms. In addition, the number of longer events is increased when more hyperpolarizing voltage steps are applied.

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Wagner LE II, Groom LA, Dirksen RT, Yule DI - **Characterization of Ryanodine Receptor Type 1 Single Channel Activity Using "On-Nucleus" Patch Clamp** - *Cell Calcium*. 2014 August; 56(2): 96–107. doi:10.1016/j.ceca.2014.05.004.