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$InsP_3R$ channel gating altered by clustering?

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The inositol trisphosphate receptor ($InsP_3R$) forms a calcium channel that resides in the membrane of the endoplasmic reticulum and is activated by inositol trisphosphate ($InsP_3$). $InsP_3$ is a phosphorylated monosaccharide that is generated via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), a phospholipid that is located in the plasma membrane, and activation of the $InsP_3R$ is involved in a broad range of biological processes, including cell division, apoptosis and development. Rahman *et al.*[1,2] reported that exposure to low concentrations of $InsP_3$ induces rapid clustering of $InsP_3R$ Ca^{2+} release channels normally randomly distributed in endoplasmic reticulum/outer nuclear membranes. Importantly, clustered channels gate differently from lone channels. Using similar protocols, we observed $InsP_3R$ channel clustering without exposure to $InsP_3$ (Fig. 1a), as we found in other systems[3–5] with protocols designed to avoid $InsP_3$ pre-exposure. More significantly, we find that clustering has no effect on $InsP_3R$ channel gating. For this reason, we believe that $InsP_3$-induced channel clustering and modification of channel gating by clustering may not be universal phenomena.

Rahman *et al.*[1,2] reported that in sub-optimal cytoplasmic free Ca^{2+} concentrations ($[Ca^{2+}]_i$), clustered recombinant rat type 3 $InsP_3R$ ($InsP_3R3$) channels expressed in $InsP_3R$-deficient DT40-KO cells gated identically and independently, but with lower open probability (P_o) than lone channels, regardless of cluster size. In contrast, clustered channels had the same P_o as lone channels in optimal ligand conditions, but gated with positive cooperativity. If broadly observed, these surprising findings have important implications for understanding $InsP_3$-mediated Ca^{2+} signals, and for quantitative analyses in single-channel $InsP_3R$ electrophysiology.

To verify these observations, we examined the same $InsP_3R3$ channels in the same DT40-KO cells using similar protocols and ligand conditions. Specifically, we used 5 mM (same as Rahman *et al.*[1]) and 0.5 mM (more physiological) cytoplasmic free $[ATP^{4-}]$ ($[ATP]_f$). Records with ≤4 active channels were analysed with the same algorithm[1]. In addition, we similarly analysed nuclear patch-clamp records previously acquired under comparable ligand conditions for recombinant rat $InsP_3R3$ expressed in *Xenopus* oocytes[6], endogenous *Xenopus* type 1 $InsP_3R$ ($InsP_3R1$) in oocytes[7] and endogenous insect $InsP_3R$ in Sf9 cells[5]. For all channels examined in these various systems, we detected no statistical difference ($P > 0.05$, *t*-test) between P_o in single- versus multi-channel patches in saturating $[InsP_3]$ and sub-optimal $[Ca^{2+}]_i$ (Fig. 1b), or in sub-saturating $[InsP_3]$ and optimal $[Ca^{2+}]_i$ (Fig. 1c). Furthermore, in two-channel records, similar channel gating patterns were detected in all

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$[Ca^{2+}]_i$ (Fig. 2), with only a small fraction exhibiting positive cooperativity. Thus, our extensive data set reveals no effect of clustering on $InsP_3R$ channel gating in all ligand conditions.

In constant ligand conditions, we consistently observed abrupt, stochastic, irreversible inactivation of $InsP_3R$ in on-nucleus or excised luminal-side-out nuclear patches, with mean activity durations of ~40 s for oocyte $InsP_3R$ (ref. 3), ~100 s for Sf9 $InsP_3R$ (ref. 5) and ~140 s for $InsP_3R$ from DT40-KO cells, whereas Rahman *et al*. reported no such inactivation[1]. Importantly, we analysed only current records long enough for the number of active channels to be counted with >99% confidence[1,5,6,8]. Because finite time elapsed between pipettes making contact with the outer nuclear membrane and gigaohm seal formation (< 5 s for oocyte and Sf9 nuclei, ~10 s for DT40 nuclei), apparent single-channel patches possibly included a fraction (~11–26%) that actually contained multiple channels in which all but one channel inactivated before gigaohm seal formation. Yet, the mean number of active channels (N_A) we observed for $InsP_3R3$ in DT40 nuclear patches (1.36 ± 0.12 for 211 patches) is similar to that reported in Rahman *et al*.[1], suggesting that inactivation did not substantially impair our ability to count channels in these patches. We did detect larger N_A (10.8 ± 1)[8] in outside-out nuclear patches, which probably have significantly larger membrane areas and were isolated using a different technique, and therefore are not an appropriate comparison to illustrate either the variability in $InsP_3R$ expression level in DT40-KO cells or the effect of inactivation on N_A detected.

The P_o distributions that we observed in apparent single-channel and true multi-channel patches were similar, with no indication that two populations of channels with different P_o exist. Furthermore, mean P_o of our true multi-channel patches is comparable to the lone-channel P_o observed by Rahman *et al*.[1] (Fig. 1b). Thus, our conclusion that clustering does not affect $InsP_3R$ channel gating is not compromised by the irreversible inactivation of $InsP_3R$ channels.

We have no clear explanation for the discrepancies between our observations and those reported by Rahman *et al*.[1]. However, neither $InsP_3$-induced $InsP_3R$ clustering nor its modification of $InsP_3R$ gating are consistently observed for $InsP_3R$ expressed in DT40-KO and other cells. In contrast, channel clustering before $InsP_3$ exposure was observed in all cell systems investigated without effect on channel gating[3–5]. Thus, we suggest that $InsP_3$-induced channel clustering and modification of channel gating by clustering may not be universal phenomena.

METHODS SUMMARY

Single-channel P_o for a sufficiently long[5] current record with N_A active channels was evaluated as $=\sum_{i=0}^{N_A}[iP_i]/N_A$, where P_i, the probability that i channels were active simultaneously in the record, was determined by the same method as Rahman *et al*.[1]. The channel gating pattern for a two-channel current record was determined from P_i. If P_i are similar to the expected binomial values, $\left[2!\,P_o{}^i(1-P_o)^{2-i}\right]/[i!\,(2-i)!]$ for i = 0,1,2 ($P > 0.05$ by χ^2-test), the channels gated independently with similar P_o. Otherwise, if the cooperativity index, $(P_2+P_1/2)^2 - P_2$, is >0, they gated with different P_o, with or without negative cooperativity. If $(P_2+P_1/2)^2 - P_2 < 0$, they gated with positive cooperativity[9].

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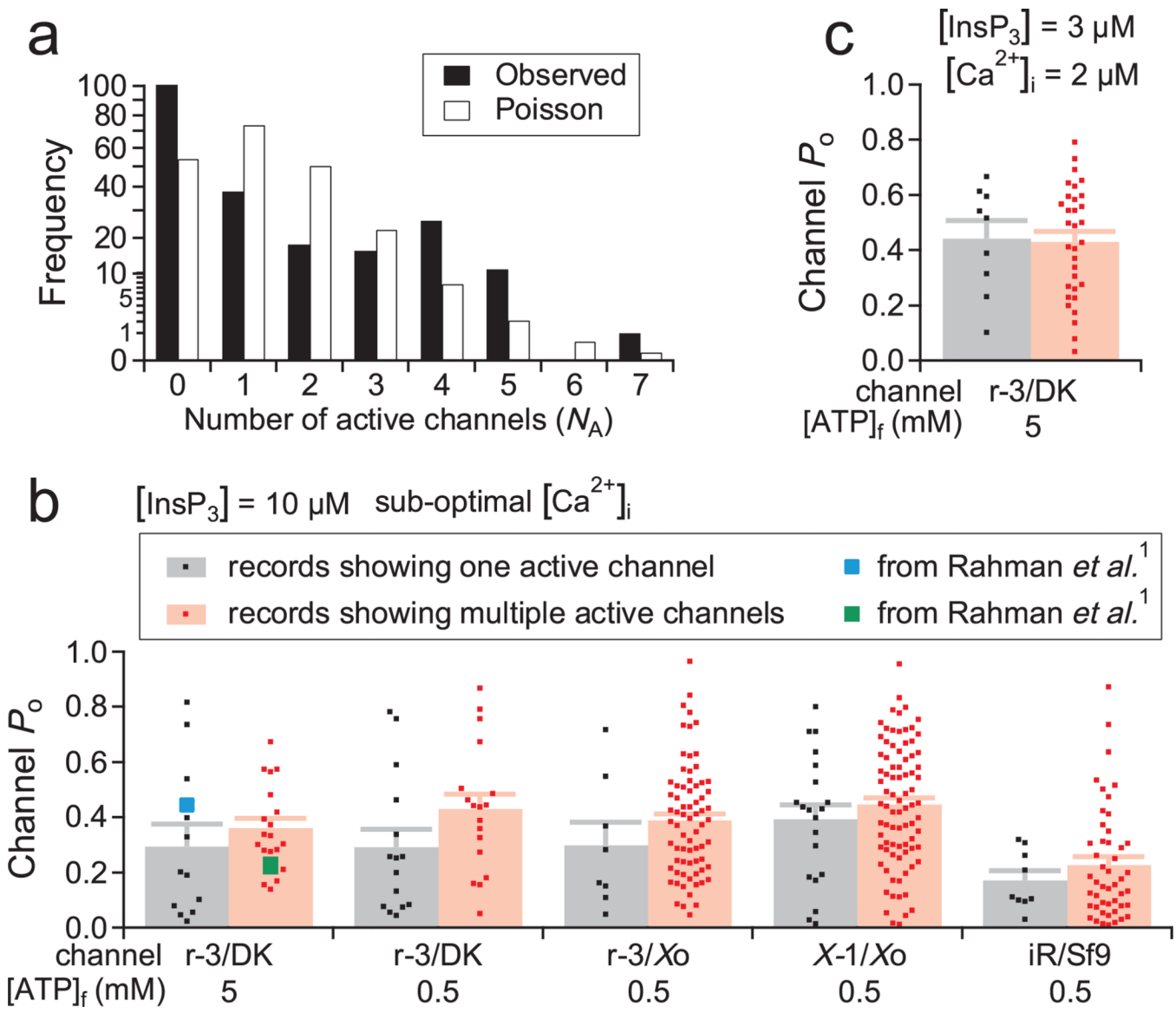

Figure 1. InsP$_3$R channels are clustered before exposure to InsP$_3$, with gating properties unaltered by clustering

a, N_A in nuclear membrane patches with no pre-exposure to InsP$_3$ obtained from InsP$_3$R3 expressing DT40-KO cells. Note nonlinear square-root scale for frequency axis. **b**, P_o observed under saturating [InsP$_3$] and sub-optimal $[Ca^{2+}]_i$ in multi- and apparent single-channel current records for recombinant rat InsP$_3$R3 (r-3) channels expressed in DT40-KO (DK) cells or *Xenopus* oocytes (*X*o), endogenous *Xenopus* InsP$_3$R1 (*X*-1) channels from *Xenopus* oocytes (*X*o), and endogenous insect InsP$_3$R (iR) channels from Sf9 cells. Concentrations of free ATP^{4-} ($[ATP]_f$) in the pipette solutions used are indicated. Mean P_o with s.e.m. (as error bars) and P_o for individual current records are shown, together with mean P_o from Rahman *et al.*[1] **c**, P_o of r-3 channels in DK cells in optimal $[Ca^{2+}]_i$ and sub-saturating [InsP$_3$], ligand conditions not investigated in Rahman *et al.*[1]. Same symbols as in **b** are used.

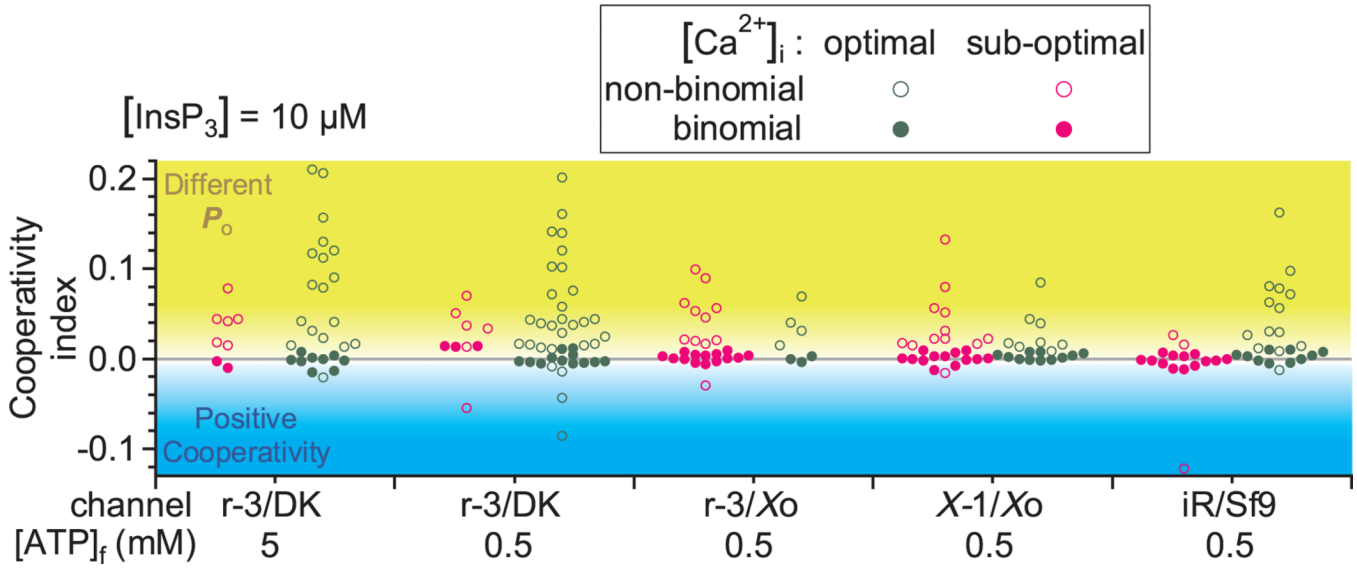

Figure 2. Distribution of cooperativity index for two-channel current records of different $InsP_3R$ channels in various systems in optimal and sub-optimal $[Ca^{2+}]_i$

Filled and open circles represent records with two channels exhibiting identical and independent, or non-binomial gating, respectively. Non-binomial records with cooperativity index, $(P_2+P_1/2)^2-P_2$, significantly greater than 0 (in yellow shaded region) had two channels gating with different P_o, and those with cooperativity index significantly smaller than 0 (in blue shaded region) had two channels gating with positive cooperativity. The cooperativity indices have no correlation with the durations of the current records (data not shown) and therefore are unlikely to be significantly affected by current record durations limited by channel inactivation.