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Redox-Regulated Heterogeneous Thresholds for Ligand Recruitment among $InsP_3R$ Ca^{2+}-Release Channels

Horia Vais,[†] Adam P. Siebert,[†] Zhongming Ma,[†] Marisabel Fernández-Mongil,[†] J. Kevin Foskett,[†‡] and Don-On Daniel Mak[†*]

[†]Department of Physiology and [‡]Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

ABSTRACT To clarify the molecular mechanisms behind quantal Ca^{2+} release, the graded Ca^{2+} release from intracellular stores through inositol 1,4,5-trisphosphate receptor ($InsP_3R$) channels responding to incremental ligand stimulation, single-channel patch-clamp electrophysiology was used to continuously monitor the number and open probability of $InsP_3R$ channels in the same excised cytoplasmic-side-out nuclear membrane patches exposed alternately to optimal and suboptimal cytoplasmic ligand conditions. Progressively more channels were activated by more favorable conditions in patches from insect cells with only one $InsP_3R$ gene or from cells solely expressing one recombinant $InsP_3R$ isoform, demonstrating that channels with identical primary sequence have different ligand recruitment thresholds. Such heterogeneity was largely abrogated, in a fully reversible manner, by treatment of the channels with sulfhydryl reducing agents, suggesting that it was mostly regulated by different levels of posttranslational redox modifications of the channels. In contrast, sulfhydryl reduction had limited effects on channel open probability. Thus, sulfhydryl redox modification can regulate various aspects of intracellular Ca^{2+} signaling, including quantal Ca^{2+} release, by tuning ligand sensitivities of $InsP_3R$ channels. No intrinsic termination of channel activity with a timescale comparable to that for quantal Ca^{2+} release was observed under any steady ligand conditions, indicating that this process is unlikely to contribute.

INTRODUCTION

Ca^{2+} release through ubiquitous, endoplasmic-reticulum (ER)-localized inositol 1,4,5-trisphosphate ($InsP_3$) receptor ($InsP_3R$) channels is the building block of complex intracellular Ca^{2+} signals that control numerous physiological processes, including apoptosis, secretion, immune responses, and memory (1–3). Complex modulation of $InsP_3R$ channel activities, including biphasic regulation by cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and cooperative activation by $[InsP_3]$, gives rise to intricate Ca^{2+} signals including repetitive $[Ca^{2+}]_i$ oscillations with frequencies tuned to stimulation level, propagating $[Ca^{2+}]_i$ waves, and highly localized Ca^{2+} release events known as ‘‘blips’’ and ‘‘puffs,’’ as well as ‘‘quantal release’’—graded release of Ca^{2+} in response to incremental levels of extracellular stimulation or $[InsP_3]$ (2). Although $InsP_3R$-mediated quantal Ca^{2+} release has been observed in many studies in different cell types using Ca^{2+} flux assays or microfluorimetry, it remains unclear how such quantal release is generated in the presence of positive feedback activation of $InsP_3R$ by released Ca^{2+}. Several molecular mechanisms have been proposed to account for the phenomenon, including $InsP_3$ regulation of $InsP_3R$ channel activity and intrinsic termination of $InsP_3R$ activity leading to partial emptying of intracellular Ca^{2+} stores (4), regulation of $InsP_3R$ channel activity by $[Ca^{2+}]$ in the ER lumen (5), or the presence of channels with different $InsP_3$ sensitivities in discrete Ca^{2+} stores (5,6). Three $InsP_3R$ genes are expressed in vertebrate cells with alternatively spliced forms, and the isoforms can form homo- as well as heterotetrameric channels (3), suggesting that graded ligand sensitivities underlying quantal Ca^{2+} release may be mediated by the presence of a large diversity of $InsP_3R$ channel types within cells. Here, we have discovered that heterogeneous ligand sensitivities can be present in a uniform population of $InsP_3R$ channels. Cytoplasmic-side-out (cyto-out) nuclear membrane patches were obtained from insect cells expressing only a single $InsP_3R$ isoform, or from $InsP_3R$-deficient DT40 cells stably transfected with the cDNA of the rat type 3 $InsP_3R$ isoform (DT40-KO-r-$InsP_3R$-3 cells). In patches exposed alternately to optimal and suboptimal ligand concentrations, no rapid intrinsic termination of $InsP_3R$ channel activity was observed, whereas the number of channels activated depended on both $InsP_3$ and Ca^{2+} concentrations. These results indicate that heterogeneous sensitivity to ligand recruitment exists even within a homogeneous population of channels with identical primary sequence. Such heterogeneity is shown to be regulated largely by reversible sulfhydryl redox modifications of the channels.

MATERIALS AND METHODS

Cyto-out nuclear patch-clamp experiments with rapid bath solution perfusion

Generation and maintenance of DT40-KO-r-$InsP_3R$-3 cells and maintenance of Sf9 cells were described (7). Nuclear patch-clamp experiments were performed using nuclei isolated from Sf9 or DT40-KO-r-$InsP_3R$-3 cells as described (7). $InsP_3R$ current traces were acquired as described (4).

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*Correspondence: dmak@mail.med.upenn.edu

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The solutions perfusing excised nuclear membrane patches in the cyto-out configuration were rapidly switched, as described (8) (Fig. 1, *B–C*). Perfusion solutions contained 140 mM KCl, 10 mM HEPES (pH 7.3), various [$InsP_3$], 0.5 mM Ca^{2+} chelator (1,2-bis(*o*-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) for 40–560 nM [Ca^{2+}]$_i$, (2-hydroxyethyl) ethylenediaminetriacetate (HEDTA) for 2–3 μM [Ca^{2+}]$_i$) with various [$CaCl_2$] to give free [Ca^{2+}] as stated (7). The pipette solution contained 140 mM KCl, 10 mM HEPES (pH 7.3), 2 μM free [Ca^{2+}], and 10 μM $InsP_3$. Perfusion and pipette solutions for Sf9 nuclei contained 0.5 mM Na_2ATP, and those for DT40 nuclei contained 5 mM Na_2ATP to ensure that all $InsP_3R$ channels were saturated with ATP^{4-}. Free [Ca^{2+}]s in all experimental solutions were confirmed by fluorimetry.

Counting active $InsP_3R$ channels in an isolated membrane patch

From a patch-clamp current record, NP_o was evaluated as equal to either $(\langle i \rangle - i_c)/i_o$, where $\langle i \rangle$ is the mean current, i_c is the closed-channel background current, and i_o is the single-channel current; or $(\sum nT_n)/(\sum T_n)$, where T_n is the time during which the recorded current was at the *n*th level and *n* is from 0 (closed-channel current level) to N_A, the maximum number of open-channel current levels observed and, therefore, the number of active channels in the patch. T_n was determined by Qub software (9). Assuming that all observed channels were identical, the single-channel open probability is $P_o = NP_o/N_A$. To ensure that N_A observed when the patch was exposed to optimal [$InsP_3$] and [Ca^{2+}]$_i$ (N_{opt}) was accurate (with confidence level >99%), only records $>T_{99\%}(N_{opt}, NP_o/N_{opt})$ were analyzed. As a first-order estimate, assuming that all channels in a patch are identical and gate with simple, time-independent single-exponential kinetics,

$$T_{99\%}(N, P_o) = \frac{-\tau_o \log_e(0.01)}{(N+1)P_o^{(N+1)}} \exp\left[\frac{(N+1)\,T_{res}}{\tau_o}\right],$$

where T_{res} is the empirically determined temporal resolution of the acquisition system (0.2 ms) (10), and τ_o is the mean open-channel duration determined from single-channel on-nucleus current records analyzed using Qub software. N_A observed when the patch was exposed to suboptimal ligand concentrations (N_{sub}) was evaluated using only exposures with durations longer than a more conservative 99%-confidence duration: $T_{99\%}(N_{sub}, NP_o/N_{opt})$ derived assuming that all N_{opt} channels activated by optimal ligand conditions contributed to the NP_o measured. In Sf9 cells, expression level of the endogenous channels in the nuclear membrane was relatively low, with a mean N_{opt} of 3.8 ± 0.4, so that it was possible to determine N_A properly in ligand conditions in which P_o is as low as 0.2: $T_{99\%} = 80$ s for $N_{opt} = 4$ and $\tau_o \sim 40$ ms (10). In DT40-KO-r-$InsP_3R$-3 cells, expression level of the recombinant channels was significantly higher, with a mean N_{opt} of 10.8 ± 1.0, so it was generally possible to determine N_A for ligand conditions in which $P_o > 0.5$: $T_{99\%} = 20$ s for $N_{opt} = 10$ and $\tau_o \sim 10$ ms (observed in these experiments). Only patches with lower N_{opt} were used for determining N_A for $P_o < 0.5$. Although abrupt, irreversible channel inactivation was consistently observed for $InsP_3R$ from both Sf9 and DT40-KO-r-$InsP_3R$-3 cells during nuclear patch-clamp experiments in on-nucleus or excised luminal-side-out configurations, the rate of channel inactivation was significantly slower in excised cyto-out membrane patches, for unknown reason(s). Thus, long continuous current records of $InsP_3R$ channels with multiple exposures ($>T_{99\%}$) to various ligand conditions were regularly obtained for Sf9 $InsP_3R$ (>200 s) and $InsP_3R$ in DT40-KO-r-$InsP_3R$-3 cells (>10 min).

[Ca^{2+}]$_i$ imaging

DT40-KO-r-$InsP_3R$-3 cells were preexposed to culture medium with or without 10 mM dithiothreitol (DTT) for 2 h. Cells were loaded with fura-2 and imaged as described previously (11). Cells were sequentially exposed to perfusion solutions containing 0, 50 ng/ml, and 5 μg/ml anti-IgM antibody to stimulate $InsP_3R$-mediated Ca^{2+} release through the B-cell receptor

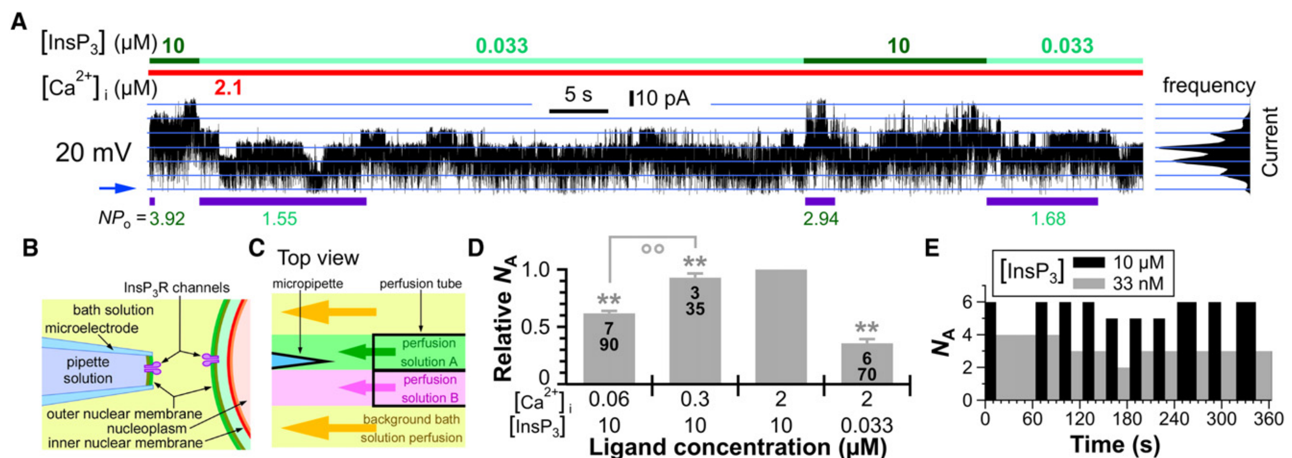

FIGURE 1 More Sf9 $InsP_3R$ channels were activated in the same nuclear membrane patch by more favorable ligand conditions. (*A*) $InsP_3R$ channel current trace from a cyto-out membrane patch excised from an isolated Sf9 nucleus containing many $InsP_3R$ channels with high P_o. In this and subsequent current traces, cytoplasmic ligand concentrations in perfusion solutions are indicated by color bars at top, blue arrow indicates the closed-channel current level, blue lines mark evenly spaced current levels derived from the current amplitude histogram shown on the right, and the applied transmembrane potential used is indicated. NP_o values during each exposure to various [$InsP_3$]s are tabulated below the current trace. $T_{99\%}$ durations evaluated for each exposure are indicated by the purple bars below the trace. (*B*) Schematic diagram showing the orientation of $InsP_3R$ channels in an isolated nucleus (*right*) and an excised cyto-out nuclear membrane (*left*). (*C*) Schematic diagram showing the arrangement of the rapid perfusion solution exchange system. The micropipette tip and excised cyto-out nuclear membrane were exposed alternately to perfusion solutions A or B by moving the perfusion tube so that the micropipette tip crossed the interface between the two perfusion solutions. (*D*) Plot of relative N_A for Sf9 $InsP_3R$ channels in various ligand conditions. In this and subsequent relative N_A or P_o plots, * and ** indicate statistically significant deviation from 1 ($p < 0.05$ and $p < 0.005$, respectively, by paired Student *t*-test); °° indicates statistically significant difference between values of relative N_A connected by the line ($p < 0.005$ by unpaired *t*-test); numbers tabulated in the bars are of membrane patches (*upper*) and solution switches (*lower*) used to determine the relative N_A values. Error bars indicate mean ± SE. (*E*) Plot of the number of $InsP_3R$ channels activated by various [$InsP_3$] conditions in the same membrane patch used in *A*.

pathway (12). In experiments using DTT-pretreated cells, dye-loading and perfusion solutions contained 10 mM DTT. The average $[Ca^{2+}]_i$ (with the baseline level subtracted) observed during exposure to one anti-IgM antibody concentration was used as a measure of the $InsP_3R$-mediated $[Ca^{2+}]_i$ signal intensity.

RESULTS

Graded recruitment by ligands of endogenous $InsP_3R$ channels in insect Sf9 cells

The number of channels activated (N_A) by different cytoplasmic ligand conditions in the same excised cyto-out nuclear membrane patch was monitored by rapid perfusion switching between optimal and suboptimal ligand concentrations (8) (Fig. 1, *B* and *C*). The relative N_A observed at a solution switch is the ratio of N_A in suboptimal ligand conditions to N_A in optimal conditions immediately before or after the solution switch. If ligand concentrations only affect channel P_o, then all activatable channels present in the patch should gate under both conditions, albeit with different P_o, and relative N_A should be 1. However, more $InsP_3R$ channels were consistently observed in optimal conditions (Fig. 1, *A* and *D*). Furthermore, there was a direct correlation between relative N_A and the level of ligand stimulation. For example, fewer channels were activated by 60 nM Ca^{2+} compared with the more favorable 300 nM in the same membrane patches (Fig. 1 *D*), even though both concentrations are suboptimal (10). To ensure that N_A was not underestimated due to reduced P_o in the suboptimal conditions, the patches were exposed to each solution for long periods ($>T_{99\%}$) before the next solution switch, enabling N_A to be determined with >99% confidence (see Materials and Methods). It is interesting that even though fewer $InsP_3R$ channels were activated by suboptimal ligand conditions, frequently the same number was activated (± 1) in sequential exposures to the same suboptimal conditions (Fig. 1, *A* and *E*), suggesting that the number of channels that can be activated is not determined by a stochastic process. These observations indicate that even within a population of Sf9 $InsP_3R$ channels in close physical proximity, there are heterogeneous sensitivities to ligand recruitment.

Graded ligand recruitment of homogeneous recombinant $InsP_3R$ channels

Although only one $InsP_3R$ gene has been identified in invertebrates, *Drosophila* and *Caenorhabditis elegans* $InsP_3R$ can exist as splice variants (3). Thus, variations in the primary sequence of the endogenous Sf9 $InsP_3R$ as a cause of the observed heterogeneous ligand sensitivities cannot be ruled out. To determine whether heterogeneous ligand sensitivities exist in a population of channels with identical primary sequences, we used a stably transfected cell line (DT40-KO-r-$InsP_3R$-3) derived from DT40-$InsP_3R$-KO cells (12) that have all three endogenous $InsP_3R$ genes knocked out, so that only recombinant rat type 3 $InsP_3R$ ($InsP_3R$-3) is expressed (7). All $InsP_3R$ channels in these cells must be homotetrameric, with subunits having the same primary sequence. Remarkably, graded activation by ligands of these homogeneous $InsP_3R$ channels was also observed, with relative N_A increasing gradually as ligand conditions became progressively more favorable (Fig. 2, *A* and *C* and Fig. S1 *A* in the Supporting Material). Also reminiscent of Sf9 channels, repeated exposures of patches to the same suboptimal conditions consistently activated the same number of channels (± 1). These observations demonstrate that a population of $InsP_3R$ channels with homogeneous primary sequence can exhibit intrinsic heterogeneous sensitivities to recruitment by $Ca^{2+}{}_i$ and $InsP_3$.

Heterogeneous ligand sensitivity is largely regulated by reversible redox modulation

The heterogeneous ligand sensitivities observed in a homogeneous population of $InsP_3R$ channels cannot be easily modeled by kinetic schemes involving various activation states. Thus, it seems more reasonable to assume that the homogeneous population of channels becomes heterogeneous due to posttranslational modifications. Regulation of $InsP_3R$ channel activity by phosphorylation, redox status, and protein interaction has been documented (3), although the possible roles of such regulation in ''increment detection'' (6,13,14), or graded recruitment of $InsP_3R$ by ligands, have not been studied. In preliminary studies, isolated DT40-KO-r-$InsP_3R$-3 nuclei treated with 0.7 unit/μl bovine intestinal alkaline phosphatase for 30 min at room temperature exhibited a ligand-dependent change in N_A similar to that seen with untreated nuclei. In contrast, the heterogeneous sensitivity was largely abrogated by exposure of isolated nuclei to DTT (3 mM for at least 60 min) (Fig. 2, *B* and *C*, and Fig. S1, *B* and *C*). Of note, the normal dependence of channel P_o on $[InsP_3]$ and $[Ca^{2+}]_i$ was unchanged after the channels were exposed to DTT, with P_o decreasing when $[Ca^{2+}]_i$ was reduced from optimal 2.3 μM to suboptimal 170 and 40 nM, and when $[InsP_3]$ was reduced from saturating 10 μM to subsaturating 3 and 1 μM (Fig. 2 *D*). This indicates that the effects of ligand concentrations on the number of channels activated and on P_o are independent. Similar results were obtained with another sulfhydryl reducing reagent, tris(2-carboxyethyl)phosphine (TCEP; 6 mM for at least 90 min) (Fig. 2, *C* and *D*, and Fig. S1 *D*), indicating that sulfhydryl reduction of $InsP_3R$ regulates these effects.

We attempted to observe acute effects of DTT on $InsP_3R$ channel N_A in continuous cyto-out nuclear patch-clamp experiments with pipette and perfusion solutions containing DTT using nuclei that had not been preexposed to DTT. However, the decrease in N_A normally observed at $[Ca^{2+}]_i$ reduction from 2 μM to 170 nM was not altered by 30 min exposure to DTT, even when [DTT] was raised to 20 mM

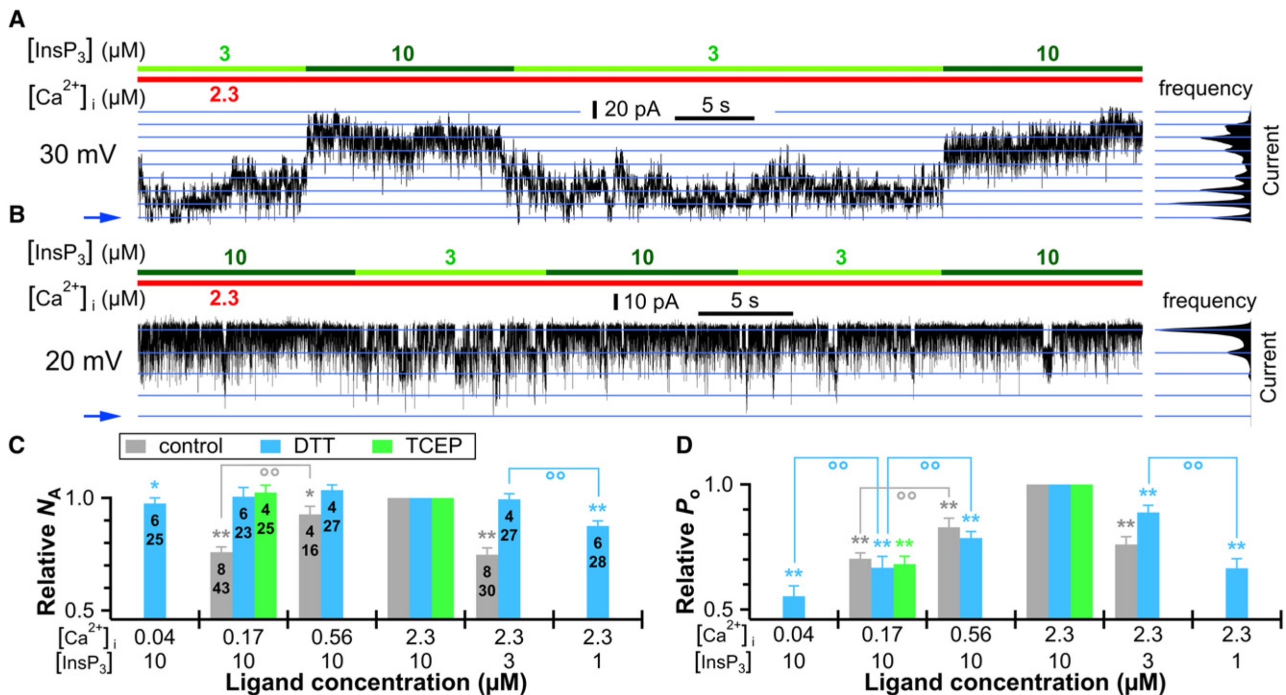

FIGURE 2 Effects of sulfhydryl reduction on graded ligand recruitment and activity level of homotetrameric recombinant rat type 3 $InsP_3R$ channels consisting of subunits with identical primary sequence expressed in DT40-KO-r-$InsP_3R$-3 cells. (*A* and *B*) Current trace of control (*A*) and DTT-treated (*B*) $InsP_3R$-3 channels alternately exposed to optimal and suboptimal [$InsP_3$] conditions. (*C* and *D*) Plots of relative N_A (*C*) and relative P_o (*D*) of control, DTT-treated (3 mM for >60 min) and TCEP-treated (6 mM for >90 min) $InsP_3R$-3 channels in various ligand conditions. Note that the ranges of the relative N_A and P_o axes are from 0.5 to 1 to emphasize the change in P_o and N_A in various ligand conditions. Convention for symbols *, ** and °° is the same as in Fig. 1.

for ~7 min. Because cyto-out nuclear patches rarely remained stable under constant perfusion for >20 min, we probed the duration of DTT exposure required to abrogate the heterogeneity in $InsP_3R$ $[Ca^{2+}]_i$ sensitivity by using nuclei first exposed to 3 mM DTT for ~20 min. Cyto-out membrane patches from these nuclei were then used in experiments with pipette and perfusion solutions containing DTT (3 mM). In two experiments, a change in N_A was observed at the beginning but was abrogated during the experiments. Total DTT exposure times that eliminated heterogeneity of channel $[Ca^{2+}]_i$ sensitivity were 37 and 55 min. Thus, in our in vitro experimental conditions, a long exposure to a reducing environment is required to substantially reduce the heterogeneity in $InsP_3R$ channel ligand sensitivity.

To determine the reversibility of the abrogation of heterogeneous $InsP_3R$ ligand sensitivities by sulfhydryl reducing reagents, DT40-KO-r-$InsP_3R$-3 nuclei were first incubated with 6 mM TCEP for 120 min, to substantially reduce the heterogeneity, and then exposed to H_2O_2 (5 mM for at least 60 min) before cyto-out nuclear patch-clamp experiments were performed. H_2O_2 treatment completely restored heterogeneous ligand sensitivities to the same levels observed in untreated channels (Fig. 3 *A* and Fig. S1 *E*). Relative P_o of the H_2O_2-treated channels was also very similar to those of untreated channels in all suboptimal ligand concentrations (Fig. 3 *D*). Similar effects on N_A were obtained with thimerosal (TMS) (Fig. 3 *A*), another oxidizing reagent that has been shown to modify $InsP_3R$-mediated Ca^{2+} release (15–19). However, TMS-treated $InsP_3R$ channels exhibited substantially lower P_o (0.38 ± 0.06) under optimal ligand conditions than either untreated (0.72 ± 0.02) or H_2O_2-treated channels (0.73 ± 0.01) (Fig. 3, *C* and *E*). Furthermore, whereas a negligible amount of channel run-down (decrease in N_A with time) was observed in cyto-out patch-clamp experiments using untreated or DTT-, TCEP-, or TCEP⇒H_2O_2-treated $InsP_3R$ channels, TMS-treated $InsP_3R$ channels inactivated rapidly, with a time constant of ~80 ± 40 s (N = 5), even though saturating $InsP_3$ was continuously present (Fig. S1 *F*). Thus, TMS has complex effects on the gating behaviors of $InsP_3R$ channels not observed with H_2O_2.

We performed a series of cyto-out patch-clamp experiments after various durations of H_2O_2 exposure to gauge the rate of restoration by H_2O_2 of ligand sensitivity heterogeneity for TCEP-treated channels. Whereas 45 min was the longest H_2O_2 exposure after which there was still no change in N_A when $[Ca^{2+}]_i$ was dropped from 2.3 μM to 170 nM, the change in N_A with $[Ca^{2+}]_i$ decrease was restored in all six experiments with H_2O_2 exposures >58 min. Thus, a long exposure to H_2O_2 was required to modify $InsP_3R$ channel ligand sensitivities under our in vitro experimental conditions.

None of the redox treatments significantly altered the number of $InsP_3R$ channels activated by optimal ligand

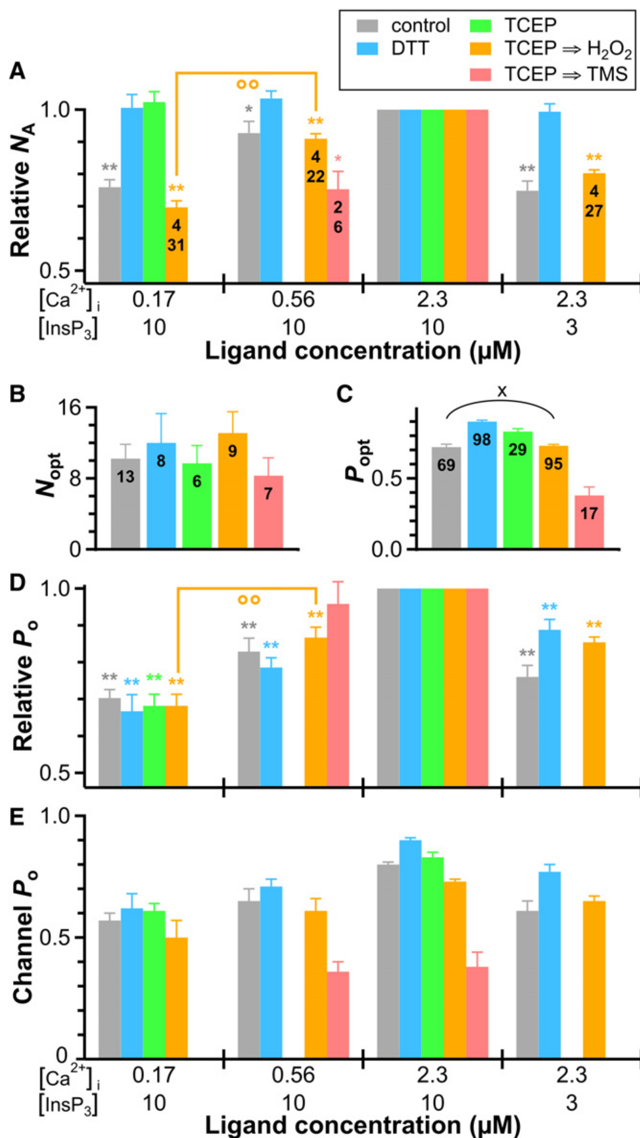

FIGURE 3 The effects of sulfhydryl reduction on relative N_A and P_o of $InsP_3R$-3 channels are reversible. (*A*) Relative N_A plot shows effects of exposure to H_2O_2 (5 mM for >60 min) and TMS (100 μM for >90 min) after TCEP treatment, in comparison with those of other redox treatments. Convention for symbols *, ** and °° is the same as in Fig. 1. (*B*) Plot of number of channels observed in optimal ligand conditions N_{opt} after various redox treatments. There is no significant difference between any of the N_{opt} values ($p > 0.05$ by one-way ANOVA test). Total numbers of cyto-out membrane patches used are tabulated. (C) Plot of optimal P_o (P_{opt}) observed after the redox treatments. All P_{opt} values are significantly different from one another ($p < 0.05$ by unequal variance t-test) except that for control channels and that for channels treated with TCEP and then H_2O_2, connected by the round bracket marked with ×. Total numbers of P_{opt} measurements performed are tabulated. (*E*) Plot of $InsP_3R$ channel P_o in different ligand conditions after various redox treatments. Note that the range of the channel P_o axis is 0 to 1.

conditions, N_{opt} (Fig. 3 *D*). Thus, regardless of their redox status, all functional $InsP_3R$ channels can be similarly activated by optimal ligand stimulation. In contrast, the number of channels activated by suboptimal ligand conditions is significantly increased by a reducing environment and decreased by an oxidizing one (Fig. 3 *A*). On the other hand, channel P_o was only modestly changed (~10%) by all redox reagents except TMS over a wide range of ligand concentrations (Fig. 3, *C–E*). Thus, the main effect of the redox status of $InsP_3R$ channel is to determine the number of channels that can be activated in suboptimal ligand conditions by modulating the sensitivities of the channel to activation by $InsP_3$ and $Ca^{2+}{}_i$.

Cellular redox potential modulates $InsP_3R$-mediated Ca^{2+} signaling

The physiological significance of the observed redox modulation of heterogeneous ligand sensitivities of $InsP_3R$ was evaluated by studying the effects of DTT treatment on $InsP_3R$-mediated Ca^{2+} signaling in intact DT40-KO-r-$InsP_3R$-3 cells. Cells were sequentially exposed to increasing concentrations of anti-IgM antibody (Ab) to stimulate $InsP_3R$-mediated Ca^{2+} release through the endogenous B-cell antigen receptor pathway (12). The average $[Ca^{2+}]_i$ above the basal level was used as a measure of Ca^{2+} signal intensity (Fig. 4). Although increasing concentrations of Ab elicited incrementally higher $[Ca^{2+}]_i$ signals in both control and DTT-treated cells, $[Ca^{2+}]_i$ signals in DTT-treated cells were consistently higher. For example, $[Ca^{2+}]_i$ signals in DTT-treated cells exposed to 50 ng/ml Ab were comparable to those in control cells exposed to saturating (5 μg/ml) [Ab]. In contrast, saturating [Ab] elicited similar $[Ca^{2+}]_i$ signals in DTT-treated and control cells. Of note, $[Ca^{2+}]_i$ signals were significantly elevated in DTT-treated cells even in the absence of Ab stimulation, and comparable to those in control cells exposed to subsaturating (50 ng/ml) [Ab]. Although $InsP_3R$-mediated Ca^{2+} release is only one of a number of mechanisms controlling $[Ca^{2+}]_i$ signaling, these effects of DTT are remarkably consistent with those expected based on the effects of redox modification on single $InsP_3R$ channel activity since sulfhydryl reduction of $InsP_3R$ channels increases the number of activated channels in response to suboptimal ligand stimulation, but does not affect the number of channels activated in response to strong stimulation. These results demonstrate that $InsP_3R$-mediated Ca^{2+} signaling can be modulated by the cellular redox potential, with possible isoform specificity, and they suggest that this occurs largely through regulation of the number of channels involved in Ca^{2+} release. Furthermore, these results also suggest that graded recruitment of $InsP_3R$ channels by incremental ligand stimulation plays a significant role in the regulation of Ca^{2+} signaling at the cellular level.

DISCUSSION

Graded recruitment of single $InsP_3R$ channels by incremental ligand activation

Graded recruitment of individual $InsP_3R$ channels by increasing levels of ligand stimulation was observed

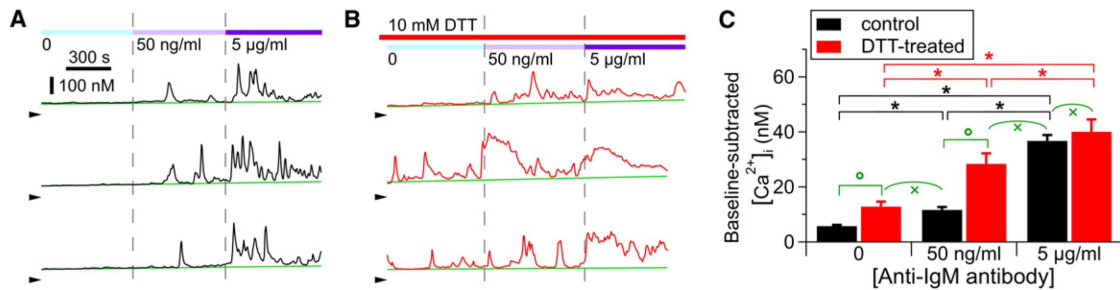

FIGURE 4 DTT treatment increases sensitivity of anti-IgM-induced $InsP_3R$-mediated $[Ca^{2+}]_i$ signals in DT40-KO-r-$InsP_3R$-3 cells. (*A* and *B*) $[Ca^{2+}]_i$ in control (*A*) and DTT-treated (*B*) DT40-KO-r-$InsP_3R$-3 cells exposed to various [anti-IgM antibody] levels. Color bars at top indicate [anti-IgM antibody] and [DTT] in perfusion solutions. Green lines mark baseline $[Ca^{2+}]_i$ levels. Arrowheads indicate $[Ca^{2+}]_i = 0$. (*C*) Plot of averaged Ca^{2+} signal levels observed in 310 control and 152 DTT-treated cells. * and ° indicate statistically significant differences between signal levels connected by the square bracket, with $p < 0.05$ by paired and unpaired *t*-test, respectively. × indicates no significant difference between mean signal levels connected by round bracket ($p > 0.05$ by unpaired *t*-test).

previously in a study of the endogenous channels in insect Sf9 cells using on-nucleus patch-clamp electrophysiology (10). Not only did the channel P_o increase in more favorable ligand conditions, as expected, but the mean number of active channels also increased, which was unexpected. However, technical issues limited confidence in the conclusion that only a subset of channels could respond to intermediate levels of ligand stimulation. The cytoplasmic solution inside the micropipette could not be changed (8), so the number of active channels could be monitored under just one set of ligand conditions in each experiment. Although factors that randomly affect the number of active channels recorded, like the size of the micropipette tip and the expression level of the $InsP_3R$, might cancel out over large numbers of experiments, systematic factors, like possible ligand-induced $InsP_3R$ channel clustering (20), could not be properly controlled for. In this study, graded recruitment of $InsP_3R$ channels was directly evaluated by recording the number of active channels in the same excised cyto-out membrane patches under optimal and suboptimal ligand conditions. For both endogenous $InsP_3R$ channels in insect Sf9 cells and recombinant rat $InsP_3R$-3 channels expressed in chicken DT40 B cells with no endogenous $InsP_3R$ background, the number of activated channels was observed to be a graded function of the strength of ligand stimulation.

The inability to activate all the channels in subsaturating $[InsP_3]$ cannot be accounted for by kinetic schemes involving channels in some ligand-bound sequestered state(s) being unable to open (10), because channels in such states cannot be activated without prior dissociation of bound ligand. In contrast, more $InsP_3R$ channels were activated when $[InsP_3]$ or $[Ca^{2+}]$ were rapidly stepped from subsaturating to saturating levels. In a similar way, kinetic schemes involving identical channels, as in the case of the recombinant $InsP_3R$-3-expressing cells, cannot account for the observation that the same number of channels remained unactivated in repeated steps to suboptimal ligand concentrations, suggesting that a nonstochastic process was involved. These observations demonstrate that multiple levels of sensitivity to ligand (both $InsP_3$ and $Ca^{2+}{}_i$) stimulation exist, even among a population of homotetrameric recombinant $InsP_3R$ channels consisting of subunits with identical primary sequences.

It is important to recognize that ligand recruitment of $InsP_3R$ channels is a different kind of ligand regulation of the channels that is unrelated to and independent of the ligand regulation of $InsP_3R$ channel P_o. This is most clearly demonstrated by the observation that as the heterogeneity in ligand sensitivity of the channels was abrogated by sulfhydryl reducing agents (Fig. 3 *A*), the channel P_o in suboptimal ligand conditions was nevertheless significantly lower than P_{opt} (Fig. 3 *E*). If the reducing treatment increases N_A at suboptimal ligand conditions by enhancing the capacity of the ligand to increase channel P_o, then the channel P_o observed at suboptimal ligand conditions should increase also.

A model that can account for our observations is illustrated in Fig. 5. In this model, a channel remains closed, with $P_o = 0$, when ligand (Ca^{2+} or $InsP_3$) concentrations are below a threshold ($[\text{ligand}]_{thres}$). When [ligand] increases above $[\text{ligand}]_{thres}$, the channel starts to gate, with P_o jumping from 0 to a finite value. Further increases in [ligand] raise channel P_o, in accordance with the ligand dependence of channel P_o observed in previous single-channel studies (3). Thus, $[\text{ligand}]_{thres}$ is a measure of the sensitivity of the channel to ligand recruitment, which is different from the ligand dependence of channel P_o. The graded recruitment of channels by increasing levels of ligand activation observed in this study implies that many degrees of ligand sensitivity (i.e., $[\text{ligand}]_{thres}$) exist even among homotetrameric channels, as shown in Fig. 5. Steady-state patch-clamp experiments measuring P_o of these channels at various [ligand]s will always observe active channels with identical channel P_o, detecting no apparent heterogeneity in the ligand regulation of channel P_o. However, in experiments in which the ligand concentration is changed, the number of active channels observed will change with [ligand] due to the heterogeneity in $[\text{ligand}]_{thres}$ among the channels.

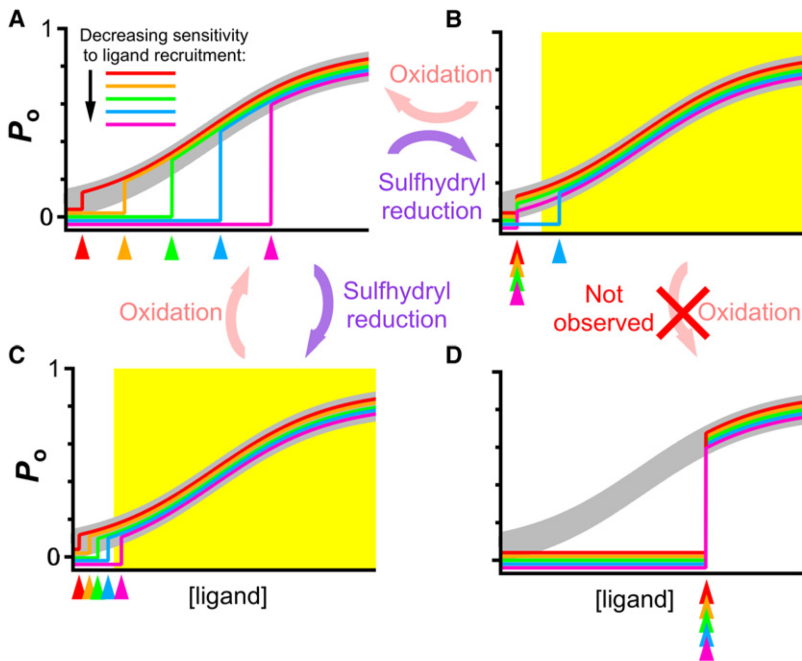

FIGURE 5 Model to account for graded ligand recruitment of $InsP_3R$ channels due to heterogeneous ligand sensitivities. (*A*) Plot of channel P_o versus [ligand] curves for $InsP_3R$ channels. Each color curve represents the ligand dependence of P_o for $InsP_3R$ channels with a different sensitivity to ligand recruitment. $[ligand]_{thres}$ values are indicated by colored arrowheads. The thick gray curve represents the homogeneous ligand dependence of channel P_o observed for all the channels. (*B* and *C*) Possible effects of sulfhydryl reduction treatment on sensitivities of $InsP_3R$ channels, homogenizing channel sensitivities (*B*) or sensitizing the channels (*C*). Range of physiologically relevant [ligand] is indicated by yellow region. (*D*) A possible effect of H_2O_2 treatment on $InsP_3R$ channel ligand sensitivities that was not observed in this study.

Sulfhydryl redox modification largely accounts for heterogeneous $InsP_3R$ ligand sensitivities

All three $InsP_3R$ isoforms contain tens of cysteine residues (3), which can be in different redox states (21). We found that the heterogeneity in sensitivity to $InsP_3$ and $Ca^{2+}{}_i$ activation of homotetrameric $InsP_3R$-3 channels consisting of subunits with identical primary sequence was mostly abrogated by treatment with sulfhydryl reducing agents (DTT or TCEP). That such effects were observed in excised nuclear membrane patches suggests that they were caused by modifications of the intrinsic sulfhydryl redox status of the channels. Although sulfhydryl reduction enabled more channels to become activated under suboptimal ligand conditions, it did not increase the total number of activatable channels (Fig. 3 *B*). Furthermore, sulfhydryl reduction only modulated channel P_o to a modest extent (~10%) in all ligand conditions (Fig. 3 *E*). Thus, the major effect of sulfhydryl reduction on single $InsP_3R$ channel gating behaviors is to enable them to be recruited into activity in less favorable conditions of $[InsP_3]$ and $[Ca^{2+}]_i$. In our model, this can be achieved either by homogenizing the sensitivity of the channels so that most channels have the same low $[ligand]_{thres}$ (Fig. 5 *B*) or by sensitizing the channels so that their $[ligand]_{thres}$, although still different, are mostly lower than the relevant physiological [ligand] range (Fig. 5 *C*). In both cases, heterogeneity in sensitivity of the channels to ligand recruitment observed within the physiological [ligand] range is reduced.

The effects of DTT or TCEP were completely reversed by H_2O_2. This is remarkable, because if sulfhydryl reduction changes the $[ligand]_{thres}$ of most channels to the same low value (Fig. 5 *B*), then prolonged treatment of the channels with H_2O_2 (as used in our protocol) might be expected to change the $[ligand]_{thres}$ of most/all channels to the same high value (Fig. 5 *D*). The observed restoration of channel heterogeneity by H_2O_2 suggests either that there is some intrinsic difference among the $InsP_3R$ channels that prevents H_2O_2 from changing the $[ligand]_{thres}$ of all of them to the same high value, or that sulfhydryl reduction sensitizes all the channels to ligand recruitment while retaining their differences in $[ligand]_{thres}$ (Fig. 5 *C*) and H_2O_2 restores them to their original, different ligand sensitivities (Fig. 5 *A*).

Besides H_2O_2, TMS (100 μM) also reversed the effects of sulfhydryl reduction, but it had additional effects of reducing P_o under optimal conditions and inducing rapid irreversible channel inactivation. This agrees with reports that high concentrations (>2 μM) of TMS, but not other biological oxidizing agents, irreversibly suppress $InsP_3R$-mediated Ca^{2+} release in various cell types (17,22,23). These TMS-specific effects may be due to reactions involving the mercury present in TMS (24).

Our observations that treatment with sulfhydryl reducing agents enables $InsP_3R$ channels to be recruited by less favorable $[Ca^{2+}]_i$ and $[InsP_3]$ conditions and enhances submaximally stimulated $InsP_3$-mediated Ca^{2+} release in DT40-KO-r-$InsP_3R$-3 cells disagree with observations from previous studies that $InsP_3R$-mediated Ca^{2+} signaling is potentiated by treatment with sulfhydryl oxidizing regents (e.g., oxidized glutathione, *tert*-butyl hydroperoxide, and low concentrations of TMS (15,16,18,23,25,26)) and inhibited by DTT (17,27). However, different sensitivity to TMS treatment has been observed for different $InsP_3R$ isoforms (18,23,28). Furthermore, enhancement of Ca^{2+} release by the sulfhydryl reducing reagent NADH (29) and by high concentrations (1 mM) of DTT, reduced glutathione, or

β-mercaptoethanol in the presence of inhibitory levels (1 mM) of TMS (17) have been reported. Thus, the variability in the observed effects of redox modifications of $InsP_3R$-mediated Ca^{2+} release, especially in intact or permeabilized cells, may be due to the complexity of the systems used. It may also suggest that $InsP_3R$ channels contain multiple functional redox-sensing thiol groups that mediate different effects on channel activity with different susceptibilities to modification by different redox reagents.

Effects of sulfhydryl redox modification of $InsP_3R$ channels on intracellular Ca^{2+} signaling

Our results suggest that reversible posttranslational redox modification of $InsP_3R$ channels can be a physiological mechanism that regulates intracellular Ca^{2+} signaling by fine-tuning the ligand sensitivities of the channels. This is supported by the strong correlations between the effects of DTT treatment on $InsP_3R$ ligand sensitivity and the intensity of $[Ca^{2+}]_i$ signals generated by extracellular stimuli in DT40-KO-r-$InsP_3R$-3 cells. DTT treatment only increased the number of channels activated by suboptimal ligand concentrations, and it substantially enhanced the intensity of Ca^{2+} signals only in cells stimulated by subsaturating agonist concentrations. In our in vitro experiments with isolated nuclei, prolonged (tens of minutes) exposures to relatively high concentrations of sulfhydryl redox reagents were required to significantly alter the ligand sensitivities of $InsP_3R$ channels. In contrast, much shorter (1- to 5-min) exposures to sulfhydryl redox reagents could alter $InsP_3R$-mediated Ca^{2+} release in permeabilized cells (16,26,27). The difference may be the result of loss in the isolated nuclei used in our experiments of soluble cytosolic oxidases and reductases that catalyze redox modifications of $InsP_3R$ in vivo. Our results suggest that the sulfhydryl redox state of the $InsP_3R$ may be quite stable naturally despite changes in the local redox potential until it comes into contact with an appropriate oxidoreductase to catalyze its redox modification. With specific localization of enzymes that regulate local cytoplasmic and ER luminal redox potentials (30) and of oxidoreductases that catalyze redox modifications of various thiol groups in the $InsP_3R$, the distribution of $InsP_3R$ channels with different ligand sensitivities could be highly compartmentalized. Such compartmentalization can play a significant role in determining the spatial characteristics of intracellular Ca^{2+} signals, like generating $InsP_3R$ channels with high $InsP_3$ sensitivity at highly localized discrete ''hot spots'' to release Ca^{2+} locally in ''blips'' under resting or low-stimulation conditions (31), and forming regions with $InsP_3R$ channels with lower $InsP_3$ and ${Ca^{2+}}_i$ sensitivities to constrain Ca^{2+} release to ''puffs'' at suboptimal stimulation conditions so that propagating Ca^{2+} waves will only be triggered when stimulation exceeds some critical level (32). Sulfhydryl redox modification of $InsP_3R$ channel ligand sensitivities provides an intricate means to allow the intracellular Ca^{2+} signaling pathway to be regulated by the cellular redox status. Future studies of the effects of redox modifications of $InsP_3R$ channels properties can shed light on how physiological processes regulating the oxidative state of cells can affect Ca^{2+} signaling and how pathologic conditions resulting in abnormal cellular redox status can lead to Ca^{2+} signaling dysregulation.

Graded $InsP_3R$ channel recruitment and quantal Ca^{2+} release

Our observations validate heterogeneous ligand sensitivities of $InsP_3R$ channels as a mechanism that can contribute to the phenomenon of $InsP_3R$-mediated quantal Ca^{2+} release (6). With $InsP_3R$ channels that have different $InsP_3$ sensitivities in different discrete intracellular Ca^{2+} stores, incremental $[InsP_3]$ can progressively empty stores with less sensitive $InsP_3R$ channels (5,6). Existence of ligand sensitivity heterogeneity among $InsP_3R$ channels containing subunits with identical primary sequence explains how quantal Ca^{2+} release can be generated by purified homotetrameric cerebellar $InsP_3R$ reconstituted into lipid vesicles (33,34). Our study suggests that discrete Ca^{2+} stores containing $InsP_3R$ channels with different $InsP_3$ sensitivities can be generated by different redox environments around the stores.

Graded recruitment of $InsP_3R$ by ligands can also increase the dynamic range of Ca^{2+} release in response to stimulation. The amount of Ca^{2+} released by a population of $InsP_3R$ channels is proportional to the activity (P_o) of individual channels and the number of channels activated (N_A). As shown previously by Ionescu et al. (10) and more clearly here, ligand regulation of $InsP_3R$ P_o and N_A are independent. When $[InsP_3]$ increases as a result of extracellular stimulation, more Ca^{2+} will be released from the ER Ca^{2+} store than one would expect from the increase in channel P_o alone because of the recruitment of more activated channels. Heterogeneous $[Ca^{2+}]_i$ sensitivities of the channels will in turn generate stronger positive feedback in Ca^{2+}-induced Ca^{2+} release because of the increase in both P_o and N_A generated by more favorable $[Ca^{2+}]_i$. Thus, graded recruitment of $InsP_3R$ channels by ligands can enhance the increase in Ca^{2+} release generated by higher $[InsP_3]$ and contribute to the transformation of localized Ca^{2+} release events (puffs) into propagating Ca^{2+} waves in response to more intense intracellular or extracellular stimulation (31,32,35).

Finally, it has been suggested that time-dependent intrinsic reduction or termination of $InsP_3R$ channel activity leading to partial emptying of Ca^{2+} stores can be a mechanism contributing to quantal Ca^{2+} release (36–39). However, within the timescale for termination of the fast phase of quantal Ca^{2+} release (~10 s) (6,13,14), no intrinsic time-dependent reduction in P_o or N_A has been observed for Sf9 channels after $[InsP_3]$ jumps from 0 to saturating (8) or subsaturating levels (33 nM; $n = 92$), or here in either Sf9 channels or recombinant channels in DT40-KO-r-$InsP_3R$-3

cells in various combinations of constant suboptimal ligand conditions. Abrupt channel inactivation in constant [$InsP_3$] has been observed in nuclear patch-clamp studies of endogenous channels from Sf9 cells (10) and type 1 $InsP_3R$ channels from *Xenopus* oocytes (38), but with long mean channel activity duration (20–30 s) even under suboptimal [$InsP_3$] and [Ca^{2+}]$_i$ conditions. The mean activity duration before abrupt channel inactivation was even longer for recombinant type 3 $InsP_3R$ channels from DT40-KO-r-$InsP_3R$-3 cells (120 s). Thus, intrinsic reduction or termination of $InsP_3R$ channel activity is unlikely to contribute significantly to $InsP_3R$-mediated quantal Ca^{2+} release.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00532-1.

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