

Counting Functional Inositol 1,4,5-Trisphosphate Receptors into the Plasma Membrane^{*S}

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Inositol 1,4,5-trisphosphate receptors (IP₃R) within the endoplasmic reticulum mediate release of Ca²⁺ from intracellular stores. Different channels usually mediate Ca²⁺ entry across the plasma membrane. In B lymphocytes and a cell line derived from them (DT40 cells), very few functional IP₃R (~2/cell) are invariably expressed in the plasma membrane, where they mediate about half the Ca²⁺ entry evoked by activation of the B-cell receptor. We show that cells reliably count ~2 functional IP₃R into the plasma membrane even when their conductance and ability to bind IP₃ are massively attenuated. We conclude that very small numbers of functional IP₃R can be reliably counted into a specific membrane compartment in the absence of feedback signals from the active protein.

Inositol 1,4,5-trisphosphate receptors (IP₃R)² belong to a family of intracellular Ca²⁺ channels that mediate release of Ca²⁺ from the intracellular stores of most animal cells (1, 2). Most IP₃R in most cells are expressed in the membranes of the endoplasmic reticulum (ER), but smaller numbers of IP₃R may also be targeted to additional intracellular organelles, including secretory vesicles (3, 4), the Golgi apparatus (5), and the nucleoplasm (6). IP₃R can also be expressed in the plasma membrane (PM) (7–9), and we recently demonstrated that in B lymphocytes these IP₃R mediate about half the Ca²⁺ entry evoked by activation of the B-cell receptor (10). Remarkably, both native mouse B lymphocytes and avian DT40 cells, which are derived from B lymphocytes (11), reliably express just two or three functional IP₃R in the PM, which are nevertheless sufficient to contribute substantially to the Ca²⁺ signals evoked by a physiological stimulus (10).

Most ion channels (12), indeed most proteins (13), are expressed in cells in sufficiently large numbers (typically thousands/cell) that it is easy to envisage how their expression levels can be regulated by appropriate feedback signals (14): the law of large numbers provides stability (15). However, some ion channels are expressed at the PM in much smaller numbers: two or three ryanodine receptor-like channels in portal vein myocytes

(16) and ~10 Ca²⁺-activated K⁺ channels (IKCa1) in a resting T cell (17), for example. For such rare events as these and the expression of a very small number of IP₃R in the PM (10), the law of large numbers cannot apply and the stability must be provided by additional regulatory mechanisms. Here we show that cells can reliably count very small numbers of IP₃R into the PM in the absence of feedback signals.

EXPERIMENTAL PROCEDURES

Expression of IP₃R in DT40 Cells—DT40 cells were cultured at 37 °C in humidified air containing 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, 2 mM L-glutamine, and 10 μM 2-mercaptoethanol. QuikChange II XL (Stratagene) was used to introduce point mutations into rat IP₃R1 without the S1 splice site (GenBankTM accession number J05510). Constructs were verified by sequencing. Stable cell lines were selected (18) and IP₃R1 expression levels measured using ³H-IP₃ binding or Western blotting as reported (10).

IP₃ Binding to the IP₃ Binding Core—The 1–604 fragment of IP₃R1 was amplified by PCR from full-length IP₃R1 lacking the S1 splice region and ligated as a SalI/EcoRI fragment into the pTrcHis A vector at the XhoI/EcoRI sites. Mutagenesis used the QuikChange II XL site-directed mutagenesis kit and the primers listed in supplemental Table S1. The His₆-tagged fusion proteins were expressed in *Escherichia coli* (19) and cleaved from their His₆ tags using thrombin. Equilibrium-competition binding assays using ³H-IP₃ (0.7 nM) were performed and analyzed as reported previously (19).

IP₃-evoked Ca²⁺ Release—The ER of DT40 cells was loaded with a low affinity Ca²⁺ indicator (Mag fluo-4), and IP₃-evoked Ca²⁺ release was measured from the saponin-permeabilized cells using a FlexStation as previously reported (20).

Single Channel Recording—Single channel currents were recorded in the whole-cell configuration or from excised patches of nuclear envelope using the patch clamp technique exactly as reported (10). Except where indicated otherwise, bath solution (BS) contained 140 mM KCl, 10 mM Hepes, 500 μM BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), 270 μM CaCl₂ (free [Ca²⁺] = 246 nM), pH 7.1, and pipette solution contained: 140 mM KCl, 10 mM Hepes, 100 μM BAPTA, 48.7 μM CaCl₂ (free [Ca²⁺] = 212 nM), 500 μM ATP, pH 7.1, and IP₃ (usually 10 μM). Most recordings were performed at negative potentials (–100 to –40 mV) to avoid contributions from voltage-gated K⁺ channels.

RESULTS AND DISCUSSION

DT40 Cells Unfailingly Express Very Few Functional IP₃R in the Plasma Membrane—Fig. 1A shows the number of functional IP₃R detected in whole-cell patch clamp recordings from

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² The abbreviations used are: IP₃R, inositol 1,4,5-trisphosphate receptor(s); ER, endoplasmic reticulum; γ, single channel conductance; PM, plasma membrane; BS, bathing solution; P_o, single channel open probability.

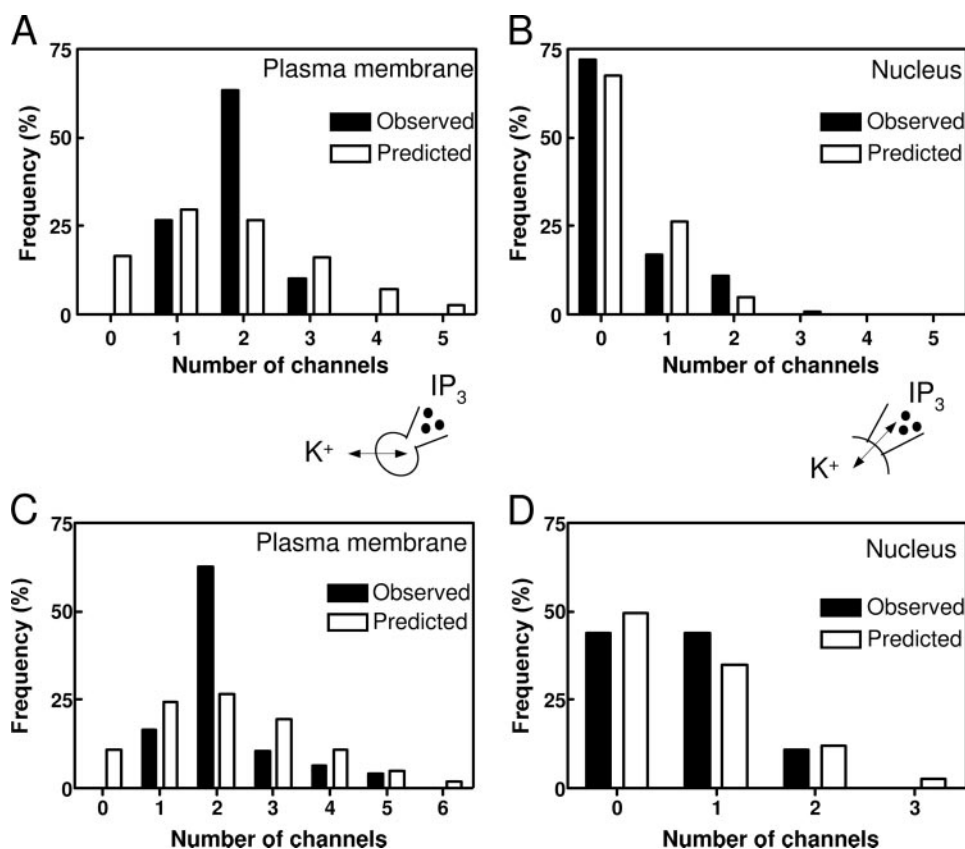


FIGURE 1. **Reliable expression of very few functional IP₃R in the plasma membrane.** A and B, patch clamp recording from whole cells (A, $n = 30$) or isolated patches from nuclear envelope (B, $n = 18$) of wild-type DT40 cells was used to identify the number of functional IP₃R during stimulation with $10 \mu\text{M}$ IP₃. The observed results are compared with those predicted from the average number of channels detected if their distribution followed a Poisson distribution (see supplemental Methods and supplemental Fig. S1). Only nuclear IP₃R are randomly distributed within the membrane; the distribution of PM IP₃R is unexpectedly concentrated around 2–3/cell with no cells lacking IP₃R. C and D, similar analysis applied to DT40 cells expressing IP₃R1 at ~ 24 times the level of endogenous IP₃R (10).

native DT40 cells, which are an avian pre-B lymphocyte cell line (11). The relatively high open probability (P_o) and duration of the recordings allow the number of functional channels to be confidently determined from the maximal number of simultaneous openings to the unitary current amplitude (see supplemental Methods and supplemental Fig. S1). Each DT40 cell expresses an average of only 1.8 ± 0.1 functional IP₃R in its PM (~ 1 IP₃R/ $100 \mu\text{m}^2$), and no cells (from >200 recordings of native DT40 cells or those expressing recombinant IP₃R) fail to express functional PM IP₃R. Because DT40 cells allow expression of recombinant IP₃R in cells uniquely lacking endogenous IP₃R (DT40KO cells) (21), we pursued our studies in these cells. Previous work had shown that native mouse B-cells also reliably express just 2–3 functional IP₃R in the PM (10).

These results are surprising, first because channels are usually expressed at much higher densities (typically >100 channels/ μm^2 , and often much higher) (12), and second because for channels expressed at such low density, their numbers should follow a Poisson distribution (see supplemental Methods). We would therefore expect to find many cells without PM IP₃R (Fig. 1A). Indeed, using the same cells for patch clamp recording from the nuclear envelope, which is continuous with the ER, we found 0.39 ± 0.16 IP₃R in each patch (estimated recording area $\sim 0.8 \mu\text{m}^2$, ~ 0.5 IP₃R/ μm^2). However, in the nucleus, there

are many recordings without IP₃R and the numbers of IP₃R/patch fit a Poisson distribution (Fig. 1B). When IP₃R1 was expressed at a higher level (~ 24 -fold higher than native IP₃R), we again detected only 2.2 ± 0.14 IP₃R in the PM; their expression in the nuclear envelope was increased (to 0.7 ± 0.24 /patch), but again only for nuclear IP₃R was the distribution fitted by a Poisson distribution (Fig. 1, C and D). We have not extensively explored whether other cell types also count small numbers of IP₃R into the PM. But under conditions similar to those used for recording from DT40 cells, we detected no IP₃R in the PM of Jurkat cells ($n = 15$, not shown), which are derived from T cells. How do DT40 cells reliably count such small numbers of functional IP₃R into the PM without failures and in the face of massively increased overexpression of intracellular IP₃R?

*A Functional Channel Is Not Required for IP₃R to be Counted into the Plasma Membrane—*Ca²⁺ inhibits IP₃R (2, 22). We had therefore considered whether the small numbers of IP₃R detected within the PM might result from silencing of inactive channels by Ca²⁺ passing through those that are open. How-

ever, we detect identical numbers of PM IP₃R whether they are conducting K⁺ (as shown in Figs. 1–3 and supplemental Fig. S1) or Ba²⁺, which does not inhibit IP₃R (22), or Ca²⁺, which does inhibit (10). It is therefore unlikely that the small number of IP₃R detected in the PM results from acute silencing of a larger population of IP₃R by Ca²⁺ passing through open channels. Instead, cells seem reliably to express ~ 2 functional IP₃R proteins in the PM of each cell.

Stability and reliability in biological systems are often provided by feedback mechanisms (14). Such activity-dependent regulation is, for example, important in determining, via intracellular Ca²⁺ signals, the expression of inhibitory and excitatory neurotransmitter systems in developing neurons and their synaptic organization (23, 24). We therefore assessed whether ions passing through the IP₃R might provide a feedback signal that allowed cells continuously to monitor the number of IP₃R within the PM and so adjust their insertion, recycling, or activity to ensure stable expression of ~ 2 functional IP₃R/cell. Patch clamp recording is the only method sensitive enough to count so few IP₃R (10). We had therefore to ensure that IP₃R were functionally inactive throughout their natural life cycle and then unmask their latent activity during patch clamp recording to measure expression levels. A mutation within the pore of the IP₃R (D2550A, IP₃R1^{DA}) (Fig. 2A) satisfied these requirements.

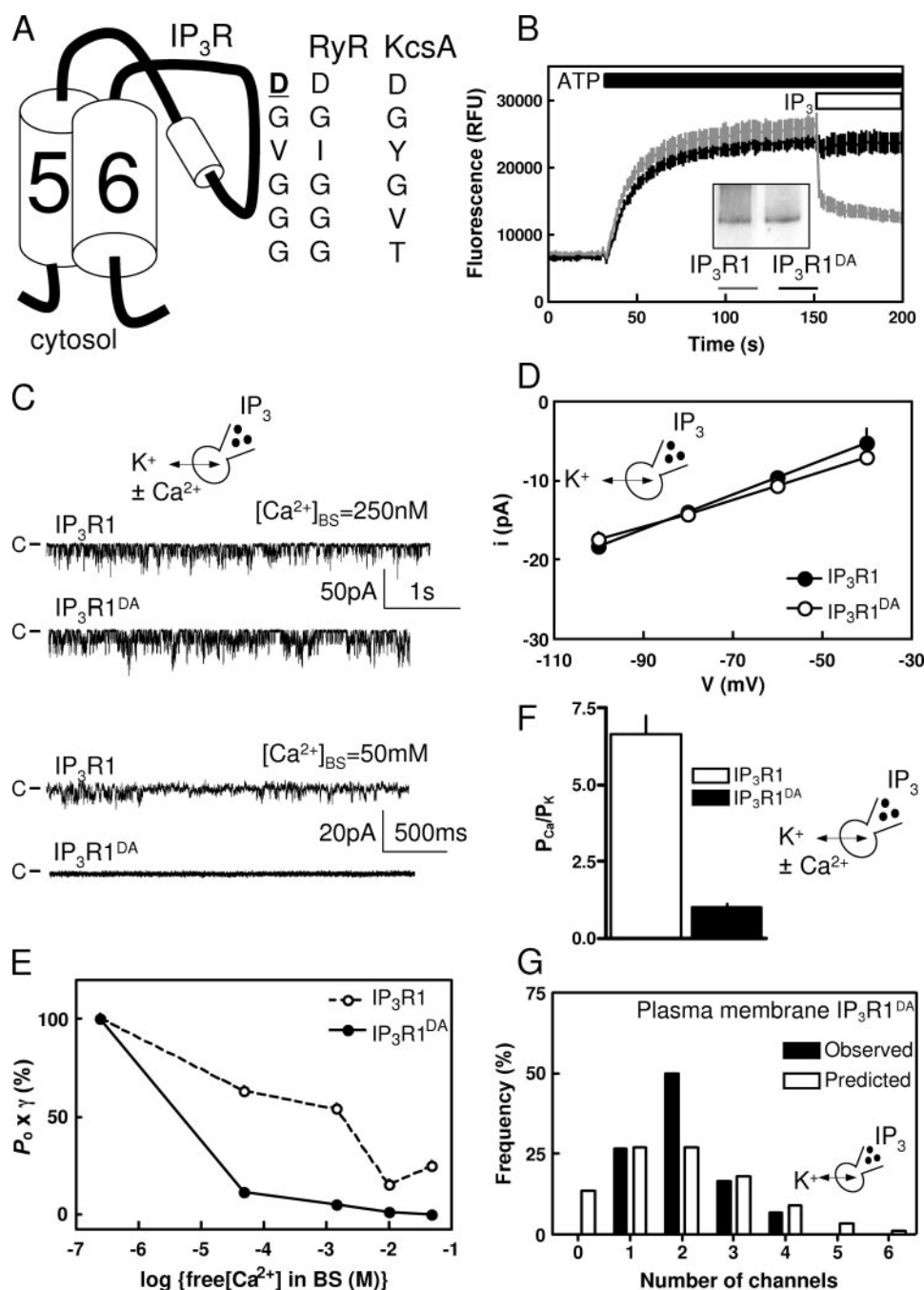


FIGURE 2. Cells reliably count Ca²⁺-impermeant IP₃R into the plasma membrane. *A*, predicted selectivity filter of IP₃R compared with that of ryanodine receptor and the bacterial K⁺ channel KcsA. Asp-2550 of the IP₃R is highlighted. *B*, the intracellular stores of cells expressing only IP₃R1^{DA} fail to release Ca²⁺ in response to IP₃. Results are means \pm S.E. from three experiments. The *inset* shows a typical immunoblot from the two cell lines (10 μ g of protein/lane) using an anti-peptide antiserum selective for IP₃R1 (10). *C*, typical whole-cell recordings from DT40 cells expressing IP₃R1 or IP₃R1^{DA} and with either 250 nM (*upper pair*) or 50 mM (*lower pair*) free Ca²⁺ in BS. *C* denotes the closed state. The traces shown in the *upper panel* are from cells with two (IP₃R1) or three (IP₃R1^{DA}) functional IP₃R in the PM. Summary data and additional examples of the effects of Ca²⁺ on IP₃R1 and IP₃R1^{DA} are shown in supplemental Fig. S2. *D*, current (*i*)-voltage (*V*) relationship for PM IP₃R1 and IP₃R1^{DA}, recorded under the same conditions as in *panel C*. *E* and *F*, effects of varying the [Ca²⁺] of BS on the single channel properties of PM IP₃R1 and IP₃R1^{DA}. *G*, observed and predicted numbers of IP₃-gated channels detected in the PM of DT40R1^{DA} cells (*n* = 30).

As with other "P-loop" cation channels, the selectivity filter of the tetrameric IP₃R is thought to be formed by a conserved sequence (GGVGD) within the luminal loop linking the last pair of transmembrane domains (TMD 5 and 6) of each subunit (Fig. 2*A*). Mutations within this region affect the conductance

or cation selectivity of IP₃R (10, 25) and ryanodine receptors (26). We examined Asp-2550, which others have suggested contributes to a Ca²⁺-binding site within the pore (25). By analogy with Ca²⁺-selective channels (27, 28) and ryanodine receptors (26), we anticipated that disrupting this site might cause high affinity block by luminal (or extracellular) Ca²⁺. IP₃ does not release Ca²⁺ from the intracellular stores of DT40 cells expressing IP₃R1 with Asp-2550 mutated to Ala (DT40R1^{DA}) (10) (Fig. 2*B*) nor does activation of the B-cell receptor stimulate Ca²⁺ entry (10). Such observations led us and others (25, 29) to conclude that IP₃R1^{DA} lacks a functional pore. Single channel analyses reveal instead that with very low extracellular Ca²⁺ and with K⁺ as charge carrier, mutant and normal PM IP₃R1 have indistinguishable properties (Fig. 2, *C* and *D* and Table 1). It is therefore possible to record the activity of IP₃R1^{DA} in the PM in the absence of extracellular Ca²⁺. However, IP₃R^{DA} has lost its Ca²⁺ selectivity: with Ca²⁺ present in the bathing solution, its open probability (*P*_o) and single channel conductance (γ) are significantly decreased (Fig. 2*E* and Table 1) and Ca²⁺ blocks K⁺ permeation more completely and with increased affinity (supplemental Fig. S2). The analogous mutation in type 1 ryanodine receptor (D4899Q) has similar effects (30), and a loss of Ca²⁺ selectivity was reported for the more conservative D2550E mutant of IP₃R1, although this mutation incompletely attenuated IP₃-evoked Ca²⁺ release (25).

The combined effects of Ca²⁺ in culture medium (\sim 0.5 mM) on decreasing *P*_o, γ , and *P*_{Ca}/*P*_K in IP₃R1^{DA} (Fig. 2, *E* and *F*) ensure that its maximal ability to conduct Ca²⁺ across the PM is \sim 1% that of normal IP₃R (supplemental Fig. S2). Within the ER, where the free [Ca²⁺] (\geq 50 μ M) (18) is lower than in culture medium, IP₃R1^{DA} would conduct Ca²⁺ at \sim 2% that of normal IP₃R (supplemental Fig. S2). These results demonstrate that throughout its normal life history within the ER, PM, or intervening organelles (5), the Ca²⁺ (or cation)-conducting activity of IP₃R1^{DA} is massively attenu-

TABLE 1

Single channel properties of IP₃R1^{DA}

Whole-cell patch clamp recording was used to determine the properties of IP₃R1 and IP₃R1^{DA} expressed in the PM of DT40 cells. Pipette solution included 10 μ M IP₃, and the free [Ca²⁺] of the bathing solution (BS) was altered (as shown) by iso-osmotic replacement of K⁺. Results, means \pm S.E., $n \geq 4$. Single channel open probability (P_o) and single channel conductance (γ) were determined in cells with a single PM IP₃R.

[Ca ²⁺] in BS	IP ₃ R1				IP ₃ R1 ^{DA}			
	P_o	γ	IP ₃ R/cell	P_{Ca}/P_K	P_o	γ	IP ₃ R/cell	P_{Ca}/P_K
		picoSiemens				picoSiemens		
250 nM	0.47 \pm 0.07	214 \pm 17	1.8 \pm 0.1		0.44 \pm 0.07	203 \pm 14	2.0 \pm 0.2	
50 μ M	0.45 \pm 0.001	140 \pm 15	1.7 \pm 0.3		0.14 \pm 0.03	73 \pm 8	2.0 \pm 0.3	
1.5 mM	0.53 \pm 0.07	102 \pm 6	2.2 \pm 0.3	5.76	0.09 \pm 0.02	51 \pm 3	1.7 \pm 0.2	0.85
10 mM	0.25 \pm 0.04	61 \pm 9	1.9 \pm 0.3	7.77	0.03 \pm 0.005	29 \pm 2	1.8 \pm 0.4	1.13
50 mM	0.34 \pm 0.03	73 \pm 7	1.9 \pm 0.2	6.44	0			

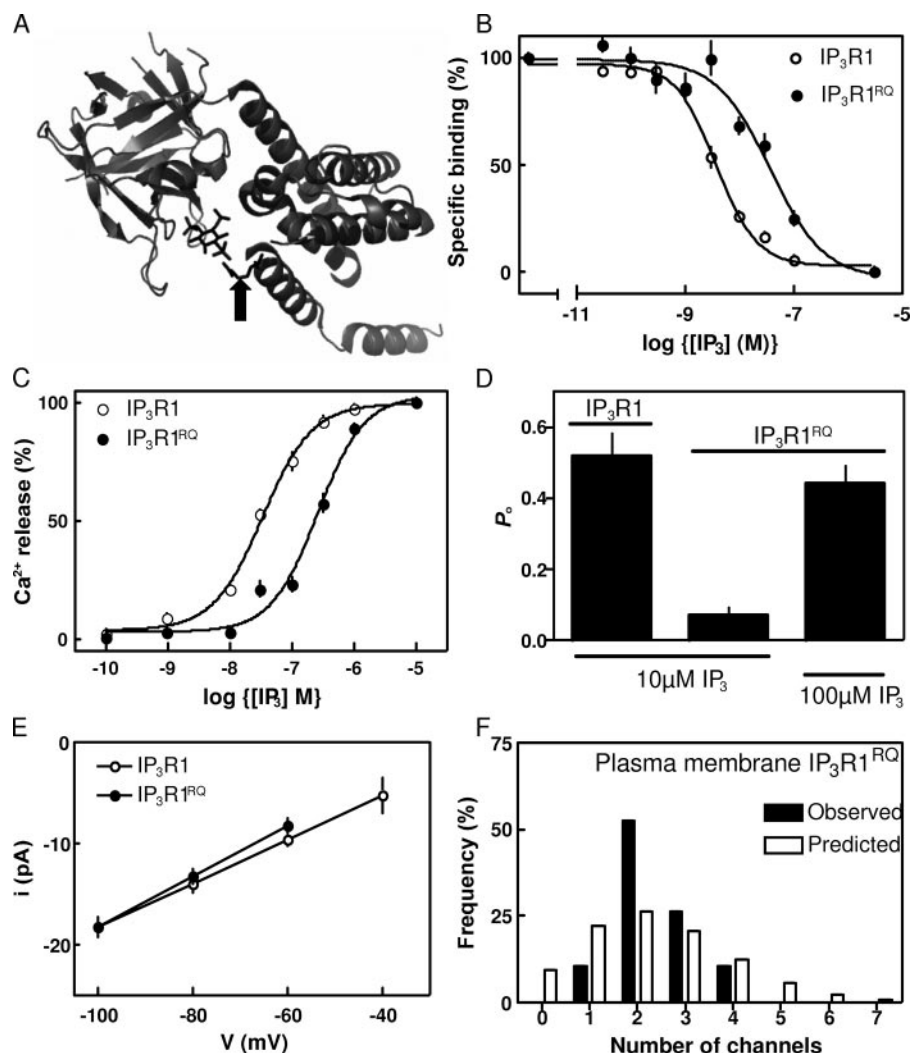


FIGURE 3. IP₃ binding is not required for IP₃R to be counted into the plasma membrane. *A*, structure of the IP₃ binding core (35) with the interaction between Arg-568 and the 1-phosphate of IP₃ highlighted. *B*, displacement of ³H-IP₃ from the N-terminal residues (1–604) of IP₃R1 and IP₃R1^{RQ} by the indicated concentrations of IP₃, from which the equilibrium dissociation constant (K_D) of the two fragments for IP₃ was determined to be 3.57 ± 0.3 and 32.2 ± 7.0 nM ($n = 4$), respectively. *C*, concentration-dependent release of Ca²⁺ by IP₃ in permeabilized DT40 cells expressing IP₃R1 or IP₃R1^{RQ}. *D*, P_o for PM IP₃R1 and IP₃R1^{RQ} stimulated with 10 or 100 μ M IP₃. *E*, i - V relationship for PM IP₃R1 and IP₃R1^{RQ}. *F*, observed and predicted numbers of IP₃-gated channels detected in the PM of DT40R1^{RQ} cells ($n = 16$), determined by whole-cell recording with 100 μ M IP₃ in pipette solution. Results (B–E) show means \pm S.E.

ated relative to normal IP₃R. However, cells reliably count just 2.0 ± 0.16 functional IP₃R1^{DA} into the PM (Fig. 2G), just as they reliably count 2.2 ± 0.14 normal IP₃R1 into the PM. We conclude that feedback signals arising from Ca²⁺ (or other cat-

ions) passing through IP₃R are not required for cells reliably to express ~ 2 functional IP₃R in the PM.

IP₃ Binding Is Not Required for IP₃R to be Reliably Counted into the Plasma Membrane—With the contentious exception of Ca²⁺-binding protein 1 (CaBP1) (31–34), IP₃ is the only physiological ligand known to initiate IP₃R activation (1). It does so by binding to the IP₃ binding core (residues 224–604), within which Arg-568 and Lys-569 contact the 1-phosphate of IP₃ (35) (Fig. 3A). Mutation of one of these key residues (R568Q, IP₃R1^{RQ}) reduces the affinity of the IP₃R for IP₃ by ~ 10 -fold (Fig. 3B) without preventing a response to a supra-maximal concentration of IP₃ (Fig. 3, C and D). The expression level of IP₃R1^{RQ} was lower than that of IP₃R1, such that the maximal IP₃-evoked Ca²⁺ release was reduced from 83 ± 2 to $51 \pm 3\%$. However, the difference in expression did not affect the sensitivity of the intracellular stores to IP₃ because in both functional (Fig. 3C) and binding (Fig. 3B) assays IP₃R1^{RQ} was 10-fold less sensitive than IP₃R1. Furthermore, our earlier work showed that very substantial changes in IP₃R expression do not affect the number of functional IP₃R expressed in the PM (10).

In whole-cell recordings from DT40 cells expressing IP₃R1^{RQ}, a normally maximal concentration of IP₃ (10 μ M) activated IP₃R but with low P_o , and 100 μ M IP₃ increased P_o to the level typical of a maximally activated wild-type IP₃R (Fig. 3D).

At the submaximal concentrations of IP₃ present within cells, IP₃R1^{RQ} would therefore be activated to only $\sim 10\%$ of the level of normal IP₃R. Nevertheless, we detected 2.4 ± 0.2 functional IP₃R^{RQ} in the PM of each cell; they had the usual γ (204 ± 19

picosiemens) (Fig. 3E), and as with wild-type IP₃R, their distribution was inconsistent with a Poisson distribution (Fig. 3F). We conclude that even when binding of endogenous IP₃ is reduced by ~90%, cells continue reliably to express very few functional IP₃R in the PM (Fig. 3F).

Counting Proteins without Feedback—DT40 cells entirely lacking IP₃R survive and proliferate (21), yet the same cells when stably expressing IP₃R, just as with native DT40 cells or B lymphocytes (10), reliably express ~2 functional IP₃R in the PM. Such targeting is not programmed genetically because it occurs whether IP₃R are expressed under the control of endogenous or heterologous promoters, nor does feedback monitoring of IP₃R activity contribute to such reliable counting because similar numbers of functional IP₃R are found at the PM when either IP₃ binding or channel activity are massively attenuated. The small number of IP₃R invariably detected in the PM does not reflect the outcome of a selection pressure arising from a need for cells to have some PM IP₃R for survival while avoiding the toxic consequences of having too many. We instead conclude that functional IP₃R are reliably counted into the PM by a mechanism that does not require feedback signals from the active protein.

Most IP₃R are expressed in the ER, where they mediate the Ca²⁺ release evoked by receptors, like B-cell receptors in DT40 cells, that stimulate formation of IP₃. But Ca²⁺ entry across the PM is also important. In DT40 cells, store-operated Ca²⁺ entry and PM IP₃R contribute similarly to the Ca²⁺ signals evoked by the B-cell receptor (10). We speculate that the small Ca²⁺ flux through each of the thousands of I_{CRAC} (Ca²⁺-release-activated current) channels that mediate store-operated Ca²⁺ entry and the huge Ca²⁺ flux through each of the very few PM IP₃R may regulate different cellular responses (10).

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