

# Counting Functional Inositol 1,4,5-Trisphosphate Receptors into the Plasma Membrane<sup>\*S</sup>

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Inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R) within the endoplasmic reticulum mediate release of Ca<sup>2+</sup> from intracellular stores. Different channels usually mediate Ca<sup>2+</sup> entry across the plasma membrane. In B lymphocytes and a cell line derived from them (DT40 cells), very few functional IP<sub>3</sub>R (~2/cell) are invariably expressed in the plasma membrane, where they mediate about half the Ca<sup>2+</sup> entry evoked by activation of the B-cell receptor. We show that cells reliably count ~2 functional IP<sub>3</sub>R into the plasma membrane even when their conductance and ability to bind IP<sub>3</sub> are massively attenuated. We conclude that very small numbers of functional IP<sub>3</sub>R 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 (IP<sub>3</sub>R)<sup>2</sup> belong to a family of intracellular Ca<sup>2+</sup> channels that mediate release of Ca<sup>2+</sup> from the intracellular stores of most animal cells (1, 2). Most IP<sub>3</sub>R in most cells are expressed in the membranes of the endoplasmic reticulum (ER), but smaller numbers of IP<sub>3</sub>R may also be targeted to additional intracellular organelles, including secretory vesicles (3, 4), the Golgi apparatus (5), and the nucleoplasm (6). IP<sub>3</sub>R can also be expressed in the plasma membrane (PM) (7–9), and we recently demonstrated that in B lymphocytes these IP<sub>3</sub>R mediate about half the Ca<sup>2+</sup> 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 IP<sub>3</sub>R in the PM, which are nevertheless sufficient to contribute substantially to the Ca<sup>2+</sup> 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 Ca<sup>2+</sup>-activated K<sup>+</sup> 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 IP<sub>3</sub>R 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 IP<sub>3</sub>R into the PM in the absence of feedback signals.

## EXPERIMENTAL PROCEDURES

**Expression of IP<sub>3</sub>R in DT40 Cells**—DT40 cells were cultured at 37 °C in humidified air containing 5% CO<sub>2</sub> 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 IP<sub>3</sub>R1 without the S1 splice site (GenBank<sup>TM</sup> accession number JO5510). Constructs were verified by sequencing. Stable cell lines were selected (18) and IP<sub>3</sub>R1 expression levels measured using <sup>3</sup>H-IP<sub>3</sub> binding or Western blotting as reported (10).

**IP<sub>3</sub> Binding to the IP<sub>3</sub> Binding Core**—The 1–604 fragment of IP<sub>3</sub>R1 was amplified by PCR from full-length IP<sub>3</sub>R1 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 His<sub>6</sub>-tagged fusion proteins were expressed in *Escherichia coli* (19) and cleaved from their His<sub>6</sub> tags using thrombin. Equilibrium-competition binding assays using <sup>3</sup>H-IP<sub>3</sub> (0.7 nM) were performed and analyzed as reported previously (19).

**IP<sub>3</sub>-evoked Ca<sup>2+</sup> Release**—The ER of DT40 cells was loaded with a low affinity Ca<sup>2+</sup> indicator (Mag fluo-4), and IP<sub>3</sub>-evoked Ca<sup>2+</sup> 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 CaCl<sub>2</sub> (free [Ca<sup>2+</sup>] = 246 nM), pH 7.1, and pipette solution contained: 140 mM KCl, 10 mM Hepes, 100 μM BAPTA, 48.7 μM CaCl<sub>2</sub> (free [Ca<sup>2+</sup>] = 212 nM), 500 μM ATP, pH 7.1, and IP<sub>3</sub> (usually 10 μM). Most recordings were performed at negative potentials (–100 to –40 mV) to avoid contributions from voltage-gated K<sup>+</sup> channels.

## RESULTS AND DISCUSSION

**DT40 Cells Unfailingly Express Very Few Functional IP<sub>3</sub>R in the Plasma Membrane**—Fig. 1A shows the number of functional IP<sub>3</sub>R detected in whole-cell patch clamp recordings from

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<sup>S</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Methods and references, supplemental Figs. S1 and S2, and supplemental Table S1.

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<sup>2</sup> The abbreviations used are: IP<sub>3</sub>R, inositol 1,4,5-trisphosphate receptor(s); ER, endoplasmic reticulum; γ, single channel conductance; PM, plasma membrane; BS, bathing solution; P<sub>o</sub>, single channel open probability.

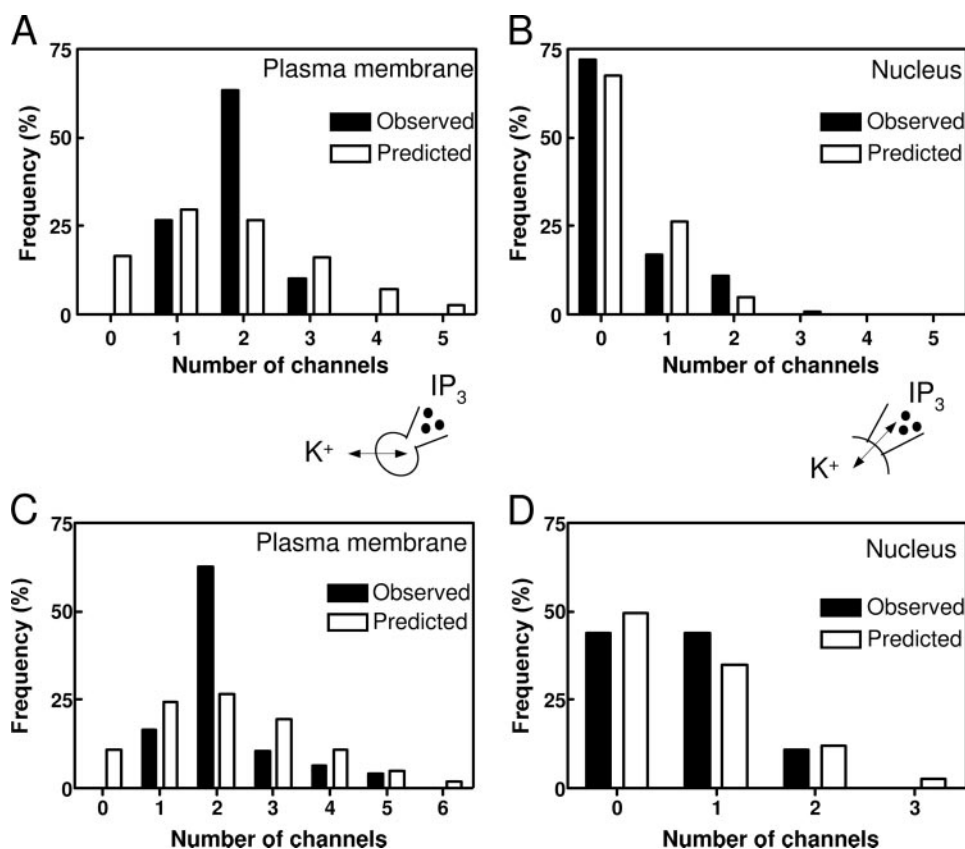


FIGURE 1. **Reliable expression of very few functional IP<sub>3</sub>R in the plasma membrane.** *A* and *B*, patch clamp recording from whole cells (*A*,  $n = 30$ ) or isolated patches from nuclear envelope (*B*,  $n = 18$ ) of wild-type DT40 cells was used to identify the number of functional IP<sub>3</sub>R during stimulation with 10  $\mu\text{M}$  IP<sub>3</sub>. 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 IP<sub>3</sub>R are randomly distributed within the membrane; the distribution of PM IP<sub>3</sub>R is unexpectedly concentrated around 2–3/cell with no cells lacking IP<sub>3</sub>R. *C* and *D*, similar analysis applied to DT40 cells expressing IP<sub>3</sub>R1 at  $\sim 24$  times the level of endogenous IP<sub>3</sub>R (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  $1.8 \pm 0.1$  functional IP<sub>3</sub>R in its PM ( $\sim 1$  IP<sub>3</sub>R/100  $\mu\text{m}^2$ ), and no cells (from  $>200$  recordings of native DT40 cells or those expressing recombinant IP<sub>3</sub>R) fail to express functional PM IP<sub>3</sub>R. Because DT40 cells allow expression of recombinant IP<sub>3</sub>R in cells uniquely lacking endogenous IP<sub>3</sub>R (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 IP<sub>3</sub>R in the PM (10).

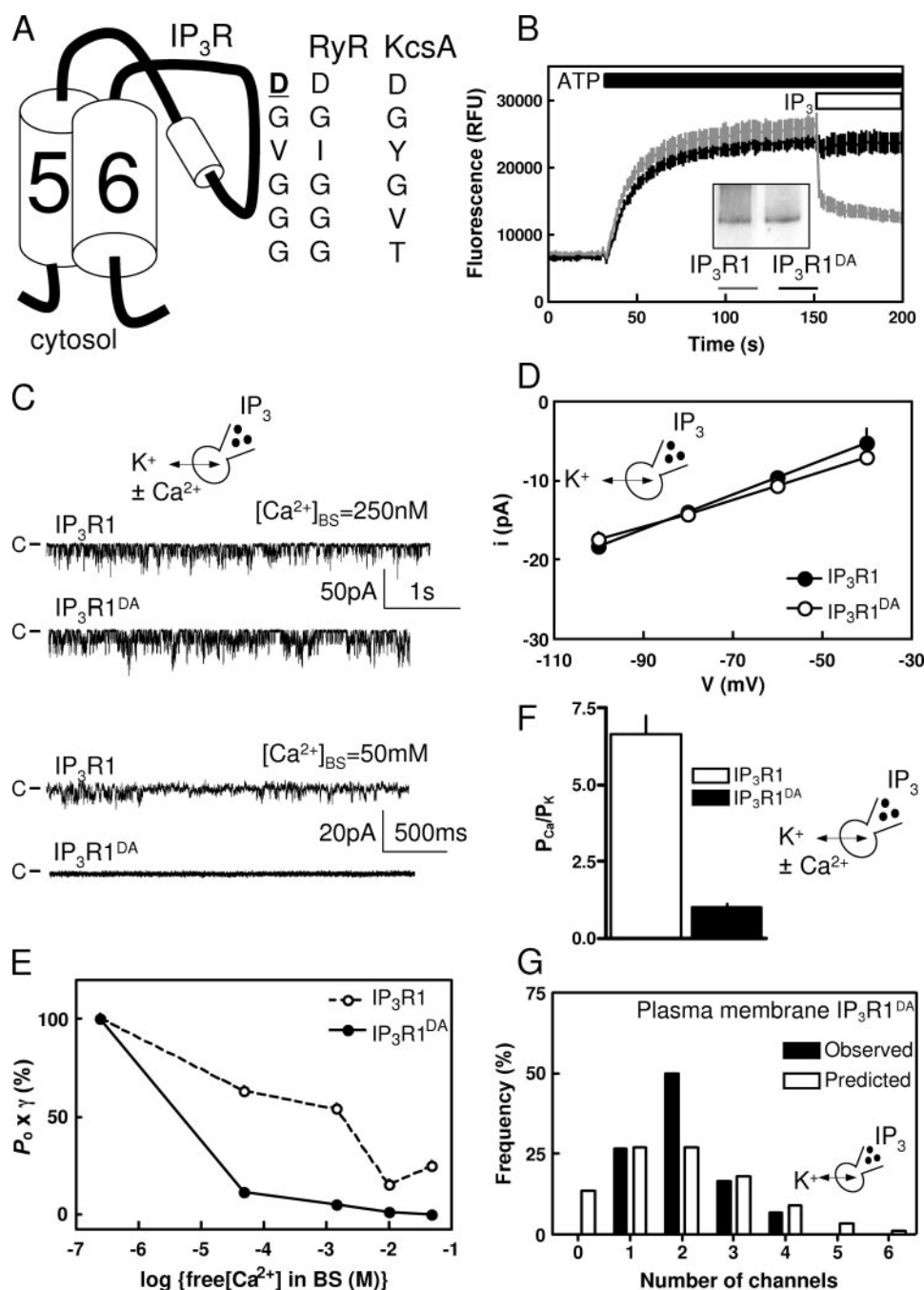
These results are surprising, first because channels are usually expressed at much higher densities (typically  $>100$  channels/ $\mu\text{m}^2$ , and often much higher) (12), and second because for channels expressed at such low density, their numbers should follow a Poisson distribution (see supplemental Methods). We would therefore expect to find many cells without PM IP<sub>3</sub>R (Fig. 1*A*). Indeed, using the same cells for patch clamp recording from the nuclear envelope, which is continuous with the ER, we found  $0.39 \pm 0.16$  IP<sub>3</sub>R in each patch (estimated recording area  $\sim 0.8 \mu\text{m}^2$ ,  $\sim 0.5$  IP<sub>3</sub>R/ $\mu\text{m}^2$ ). However, in the nucleus, there

are many recordings without IP<sub>3</sub>R and the numbers of IP<sub>3</sub>R/patch fit a Poisson distribution (Fig. 1*B*). When IP<sub>3</sub>R1 was expressed at a higher level ( $\sim 24$ -fold higher than native IP<sub>3</sub>R), we again detected only  $2.2 \pm 0.14$  IP<sub>3</sub>R in the PM; their expression in the nuclear envelope was increased (to  $0.7 \pm 0.24$ /patch), but again only for nuclear IP<sub>3</sub>R 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 IP<sub>3</sub>R into the PM. But under conditions similar to those used for recording from DT40 cells, we detected no IP<sub>3</sub>R 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 IP<sub>3</sub>R into the PM without failures and in the face of massively increased overexpression of intracellular IP<sub>3</sub>R?

*A Functional Channel Is Not Required for IP<sub>3</sub>R to be Counted into the Plasma Membrane—*Ca<sup>2+</sup> inhibits IP<sub>3</sub>R (2, 22). We had therefore considered whether the small numbers of IP<sub>3</sub>R detected within the PM might result from silencing of inactive channels by Ca<sup>2+</sup> passing through those that are open. How-

ever, we detect identical numbers of PM IP<sub>3</sub>R whether they are conducting K<sup>+</sup> (as shown in Figs. 1–3 and supplemental Fig. S1) or Ba<sup>2+</sup>, which does not inhibit IP<sub>3</sub>R (22), or Ca<sup>2+</sup>, which does inhibit (10). It is therefore unlikely that the small number of IP<sub>3</sub>R detected in the PM results from acute silencing of a larger population of IP<sub>3</sub>R by Ca<sup>2+</sup> passing through open channels. Instead, cells seem reliably to express  $\sim 2$  functional IP<sub>3</sub>R 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 Ca<sup>2+</sup> 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 IP<sub>3</sub>R might provide a feedback signal that allowed cells continuously to monitor the number of IP<sub>3</sub>R within the PM and so adjust their insertion, recycling, or activity to ensure stable expression of  $\sim 2$  functional IP<sub>3</sub>R/cell. Patch clamp recording is the only method sensitive enough to count so few IP<sub>3</sub>R (10). We had therefore to ensure that IP<sub>3</sub>R 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 IP<sub>3</sub>R (D2550A, IP<sub>3</sub>R1<sup>DA</sup>) (Fig. 2*A*) satisfied these requirements.



**FIGURE 2. Cells reliably count  $\text{Ca}^{2+}$ -impermeant IP<sub>3</sub>R into the plasma membrane.** *A*, predicted selectivity filter of IP<sub>3</sub>R compared with that of ryanodine receptor and the bacterial K<sup>+</sup> channel KcsA. Asp-2550 of the IP<sub>3</sub>R is highlighted. *B*, the intracellular stores of cells expressing only IP<sub>3</sub>R1<sup>DA</sup> fail to release  $\text{Ca}^{2+}$  in response to IP<sub>3</sub>. Results are means  $\pm$  S.E. from three experiments. The *inset* shows a typical immunoblot from the two cell lines (10  $\mu\text{g}$  of protein/lane) using an anti-peptide antiserum selective for IP<sub>3</sub>R1 (10). *C*, typical whole-cell recordings from DT40 cells expressing IP<sub>3</sub>R1 or IP<sub>3</sub>R1<sup>DA</sup> and with either 250 nM (*upper pair*) or 50 mM (*lower pair*) free  $\text{Ca}^{2+}$  in BS. *C* denotes the closed state. The traces shown in the *upper panel* are from cells with two (IP<sub>3</sub>R1) or three (IP<sub>3</sub>R1<sup>DA</sup>) functional IP<sub>3</sub>R in the PM. Summary data and additional examples of the effects of  $\text{Ca}^{2+}$  on IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>DA</sup> are shown in supplemental Fig. S2. *D*, current (*i*)-voltage (*V*) relationship for PM IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>DA</sup>, recorded under the same conditions as in *panel C*. *E* and *F*, effects of varying the  $[\text{Ca}^{2+}]$  of BS on the single channel properties of PM IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>DA</sup>. *G*, observed and predicted numbers of IP<sub>3</sub>-gated channels detected in the PM of DT40R1<sup>DA</sup> cells ( $n = 30$ ).

As with other "P-loop" cation channels, the selectivity filter of the tetrameric IP<sub>3</sub>R 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 IP<sub>3</sub>R (10, 25) and ryanodine receptors (26). We examined Asp-2550, which others have suggested contributes to a  $\text{Ca}^{2+}$ -binding site within the pore (25). By analogy with  $\text{Ca}^{2+}$ -selective channels (27, 28) and ryanodine receptors (26), we anticipated that disrupting this site might cause high affinity block by luminal (or extracellular)  $\text{Ca}^{2+}$ . IP<sub>3</sub> does not release  $\text{Ca}^{2+}$  from the intracellular stores of DT40 cells expressing IP<sub>3</sub>R1 with Asp-2550 mutated to Ala (DT40R1<sup>DA</sup>) (10) (Fig. 2*B*) nor does activation of the B-cell receptor stimulate  $\text{Ca}^{2+}$  entry (10). Such observations led us and others (25, 29) to conclude that IP<sub>3</sub>R1<sup>DA</sup> lacks a functional pore. Single channel analyses reveal instead that with very low extracellular  $\text{Ca}^{2+}$  and with K<sup>+</sup> as charge carrier, mutant and normal PM IP<sub>3</sub>R1 have indistinguishable properties (Fig. 2, *C* and *D* and Table 1). It is therefore possible to record the activity of IP<sub>3</sub>R1<sup>DA</sup> in the PM in the absence of extracellular  $\text{Ca}^{2+}$ . However, IP<sub>3</sub>R<sup>DA</sup> has lost its  $\text{Ca}^{2+}$  selectivity: with  $\text{Ca}^{2+}$  present in the bathing solution, its open probability ( $P_o$ ) and single channel conductance ( $\gamma$ ) are significantly decreased (Fig. 2*E* and Table 1) and  $\text{Ca}^{2+}$  blocks K<sup>+</sup> 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  $\text{Ca}^{2+}$  selectivity was reported for the more conservative D2550E mutant of IP<sub>3</sub>R1, although this mutation incompletely attenuated IP<sub>3</sub>-evoked  $\text{Ca}^{2+}$  release (25).

The combined effects of  $\text{Ca}^{2+}$  in culture medium ( $\sim 0.5$  mM) on decreasing  $P_o$ ,  $\gamma$ , and  $P_{\text{Ca}}/P_K$  in IP<sub>3</sub>R1<sup>DA</sup> (Fig. 2, *E* and *F*) ensure that its maximal ability to conduct  $\text{Ca}^{2+}$  across the PM is  $\sim 1\%$  that of normal IP<sub>3</sub>R (supplemental Fig. S2). Within the ER, where the free  $[\text{Ca}^{2+}]$  ( $\geq 50$   $\mu\text{M}$ ) (18) is lower than in culture medium, IP<sub>3</sub>R1<sup>DA</sup> would conduct  $\text{Ca}^{2+}$  at  $\sim 2\%$  that of normal IP<sub>3</sub>R (supplemental Fig. S2). These results demonstrate that throughout its normal life history within the ER, PM, or intervening organelles (5), the  $\text{Ca}^{2+}$  (or cation)-conducting activity of IP<sub>3</sub>R1<sup>DA</sup> is massively attenu-

TABLE 1

Single channel properties of IP<sub>3</sub>R1<sup>DA</sup>

Whole-cell patch clamp recording was used to determine the properties of IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>DA</sup> expressed in the PM of DT40 cells. Pipette solution included 10  $\mu$ M IP<sub>3</sub>, and the free [Ca<sup>2+</sup>] of the bathing solution (BS) was altered (as shown) by iso-osmotic replacement of K<sup>+</sup>. Results, means  $\pm$  S.E.,  $n \geq 4$ . Single channel open probability ( $P_o$ ) and single channel conductance ( $\gamma$ ) were determined in cells with a single PM IP<sub>3</sub>R.

[Ca <sup>2+</sup> ] in BS	IP <sub>3</sub> R1				IP <sub>3</sub> R1 <sup>DA</sup>			
	$P_o$	$\gamma$	IP <sub>3</sub> R/cell	$P_{Ca}/P_K$	$P_o$	$\gamma$	IP <sub>3</sub> 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 $\mu$ 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			

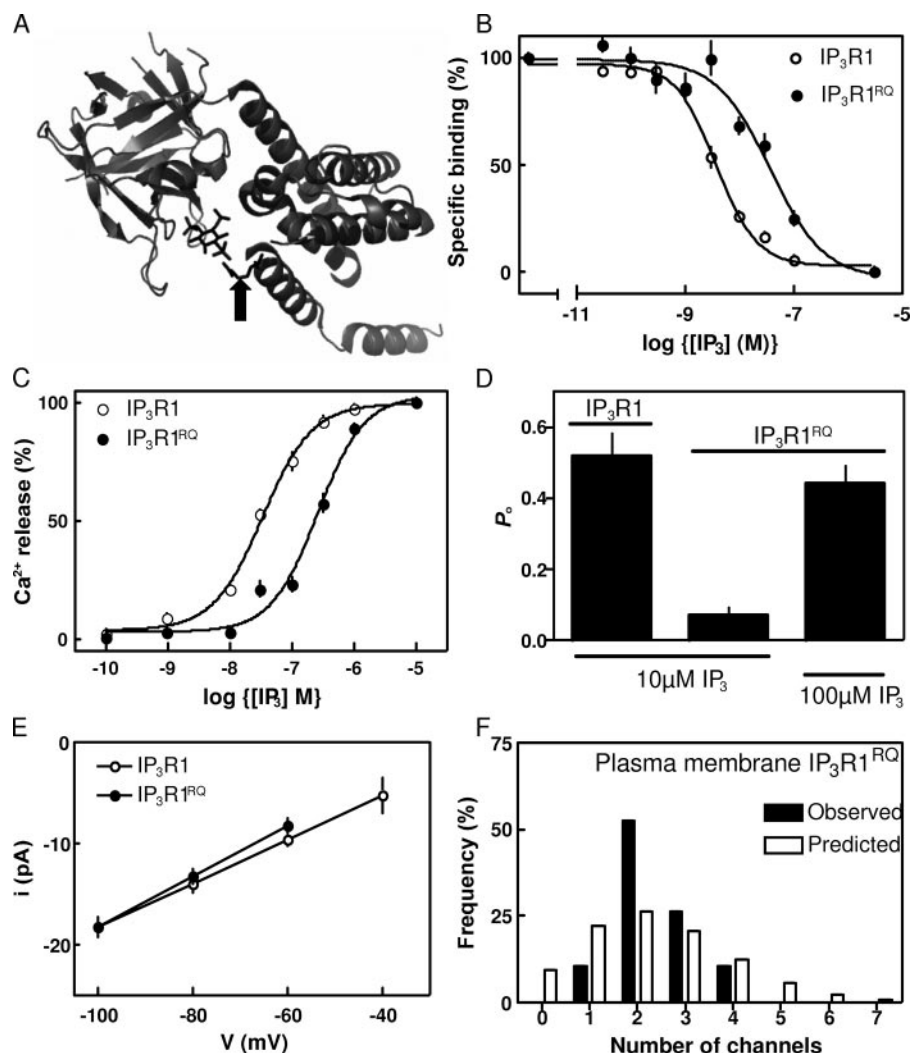


FIGURE 3. IP<sub>3</sub> binding is not required for IP<sub>3</sub>R to be counted into the plasma membrane. *A*, structure of the IP<sub>3</sub> binding core (35) with the interaction between Arg-568 and the 1-phosphate of IP<sub>3</sub> highlighted. *B*, displacement of <sup>3</sup>H-IP<sub>3</sub> from the N-terminal residues (1–604) of IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>RQ</sup> by the indicated concentrations of IP<sub>3</sub>, from which the equilibrium dissociation constant ( $K_D$ ) of the two fragments for IP<sub>3</sub> was determined to be  $3.57 \pm 0.3$  and  $32.2 \pm 7.0$  nM ( $n = 4$ ), respectively. *C*, concentration-dependent release of Ca<sup>2+</sup> by IP<sub>3</sub> in permeabilized DT40 cells expressing IP<sub>3</sub>R1 or IP<sub>3</sub>R1<sup>RQ</sup>. *D*,  $P_o$  for PM IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>RQ</sup> stimulated with 10 or 100  $\mu$ M IP<sub>3</sub>. *E*,  $i$ - $V$  relationship for PM IP<sub>3</sub>R1 and IP<sub>3</sub>R1<sup>RQ</sup>. *F*, observed and predicted numbers of IP<sub>3</sub>-gated channels detected in the PM of DT40R1<sup>RQ</sup> cells ( $n = 16$ ), determined by whole-cell recording with 100  $\mu$ M IP<sub>3</sub> in pipette solution. Results (B–E) show means  $\pm$  S.E.

ated relative to normal IP<sub>3</sub>R. However, cells reliably count just  $2.0 \pm 0.16$  functional IP<sub>3</sub>R1<sup>DA</sup> into the PM (Fig. 2G), just as they reliably count  $2.2 \pm 0.14$  normal IP<sub>3</sub>R1 into the PM. We conclude that feedback signals arising from Ca<sup>2+</sup> (or other cat-

ions) passing through IP<sub>3</sub>R are not required for cells reliably to express  $\sim 2$  functional IP<sub>3</sub>R in the PM.

**IP<sub>3</sub> Binding Is Not Required for IP<sub>3</sub>R to be Reliably Counted into the Plasma Membrane**—With the contentious exception of Ca<sup>2+</sup>-binding protein 1 (CaBP1) (31–34), IP<sub>3</sub> is the only physiological ligand known to initiate IP<sub>3</sub>R activation (1). It does so by binding to the IP<sub>3</sub> binding core (residues 224–604), within which Arg-568 and Lys-569 contact the 1-phosphate of IP<sub>3</sub> (35) (Fig. 3A). Mutation of one of these key residues (R568Q, IP<sub>3</sub>R1<sup>RQ</sup>) reduces the affinity of the IP<sub>3</sub>R for IP<sub>3</sub> by  $\sim 10$ -fold (Fig. 3B) without preventing a response to a supra-maximal concentration of IP<sub>3</sub> (Fig. 3, C and D). The expression level of IP<sub>3</sub>R1<sup>RQ</sup> was lower than that of IP<sub>3</sub>R1, such that the maximal IP<sub>3</sub>-evoked Ca<sup>2+</sup> release was reduced from  $83 \pm 2$  to  $51 \pm 3\%$ . However, the difference in expression did not affect the sensitivity of the intracellular stores to IP<sub>3</sub> because in both functional (Fig. 3C) and binding (Fig. 3B) assays IP<sub>3</sub>R1<sup>RQ</sup> was 10-fold less sensitive than IP<sub>3</sub>R1. Furthermore, our earlier work showed that very substantial changes in IP<sub>3</sub>R expression do not affect the number of functional IP<sub>3</sub>R expressed in the PM (10).

In whole-cell recordings from DT40 cells expressing IP<sub>3</sub>R1<sup>RQ</sup>, a normally maximal concentration of IP<sub>3</sub> (10  $\mu$ M) activated IP<sub>3</sub>R but with low  $P_o$ , and 100  $\mu$ M IP<sub>3</sub> increased  $P_o$  to the level typical of a maximally activated wild-type IP<sub>3</sub>R (Fig. 3D).

At the submaximal concentrations of IP<sub>3</sub> present within cells, IP<sub>3</sub>R1<sup>RQ</sup> would therefore be activated to only  $\sim 10\%$  of the level of normal IP<sub>3</sub>R. Nevertheless, we detected  $2.4 \pm 0.2$  functional IP<sub>3</sub>R<sup>RQ</sup> in the PM of each cell; they had the usual  $\gamma$  ( $204 \pm 19$

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

**Counting Proteins without Feedback**—DT40 cells entirely lacking IP<sub>3</sub>R survive and proliferate (21), yet the same cells when stably expressing IP<sub>3</sub>R, just as with native DT40 cells or B lymphocytes (10), reliably express ~2 functional IP<sub>3</sub>R in the PM. Such targeting is not programmed genetically because it occurs whether IP<sub>3</sub>R are expressed under the control of endogenous or heterologous promoters, nor does feedback monitoring of IP<sub>3</sub>R activity contribute to such reliable counting because similar numbers of functional IP<sub>3</sub>R are found at the PM when either IP<sub>3</sub> binding or channel activity are massively attenuated. The small number of IP<sub>3</sub>R invariably detected in the PM does not reflect the outcome of a selection pressure arising from a need for cells to have some PM IP<sub>3</sub>R for survival while avoiding the toxic consequences of having too many. We instead conclude that functional IP<sub>3</sub>R are reliably counted into the PM by a mechanism that does not require feedback signals from the active protein.

Most IP<sub>3</sub>R are expressed in the ER, where they mediate the Ca<sup>2+</sup> release evoked by receptors, like B-cell receptors in DT40 cells, that stimulate formation of IP<sub>3</sub>. But Ca<sup>2+</sup> entry across the PM is also important. In DT40 cells, store-operated Ca<sup>2+</sup> entry and PM IP<sub>3</sub>R contribute similarly to the Ca<sup>2+</sup> signals evoked by the B-cell receptor (10). We speculate that the small Ca<sup>2+</sup> flux through each of the thousands of I<sub>CRAC</sub> (Ca<sup>2+</sup>-release-activated current) channels that mediate store-operated Ca<sup>2+</sup> entry and the huge Ca<sup>2+</sup> flux through each of the very few PM IP<sub>3</sub>R may regulate different cellular responses (10).

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## Supplemental Data

### METHODS

*Rare, random insertion of IP<sub>3</sub>R into the PM should fit a Poisson distribution -* Very few IP<sub>3</sub>R (2-3) are selected for insertion into the PM from a very much larger pool of intracellular IP<sub>3</sub>R ( $\geq 10,000$  for DT40R1 cells) (10). Such rare events occurring independently are described by the Poisson distribution, from which (36):

$$P(n) = (e^{-\lambda} \lambda^n) / n!$$

where,  $P(n)$  = probability of finding  $n$  IP<sub>3</sub>R in the PM  
 $\lambda$  = mean number of IP<sub>3</sub>R in PM

The predicted distributions of PM IP<sub>3</sub>R shown in the figures were calculated from this formula.

*Reliably identifying the number of IP<sub>3</sub>R present -* The number ( $n$ ) of IP<sub>3</sub>R detected within either whole-cell or excised nuclear patch-clamp recordings was calculated by assuming  $n$  to be equal to the maximal number of simultaneous openings to the unitary current amplitude. But there are two potential problems:

1. The unitary current may correspond to a sub-conductance level, such that its multiples reflect the same IP<sub>3</sub>R opening to higher conductances; or
2. Individual openings ( $P_o$ ) may be so rare that simultaneous openings of all IP<sub>3</sub>Rs present are too unlikely ( $P_o^n$ ) to be detectable in recordings of attainable duration.

Neither problem applies to our analysis:

1. We never detected direct transitions between the different current levels for recordings in which  $n > 1$  (supplemental Fig. S1), and we have observed many records with only a single unitary current amplitude and others with  $> 2$  current amplitudes (Fig. 1A). Neither observation is consistent with the different current amplitudes reflecting transitions between sub-conductance states of a single IP<sub>3</sub>R.
2. The likelihood of detecting simultaneous openings of  $n$  channels, each with an independent probability of opening ( $P_o$ ), depends upon the mean duration of each open event ( $t_o$ ), the shortest opening events detectable under the recording conditions ( $T$ ), and the duration of the recording ( $D$ ). The number of multiples of the unitary current amplitude detected during a recording is likely ( $p < 0.01$ ) to report the total number of active channels if (37):

$$D > 5(\sigma_{n+1})$$

where,

$$\sigma_n = \{t_o / n(P_o)^n\} \{\exp(-nT/t_o)\}$$

For our whole-cell recordings (all with a maximal concentration of IP<sub>3</sub>):  $P_o = 0.4$ ,  $t_o \leq 30$  ms, and  $T = 0.5$  ms. From which, even with  $n = 5$  (the largest number of simultaneous openings detected in the PM),  $D \geq 6$  s would be sufficient to allow  $n$  to be estimated with  $p < 0.01$ . Our patch-clamp recordings always lasted longer than 45 s (15 s at 3 different holding potentials) and typically lasted 2-3 min. Only events lasting  $\geq 0.7$  ms were included in the analysis. Our methods therefore allow reliable counting of the

numbers of active IP<sub>3</sub>R within both the PM and nuclear envelope (similar analysis not shown).

#### SUPPLEMENTAL REFERENCES

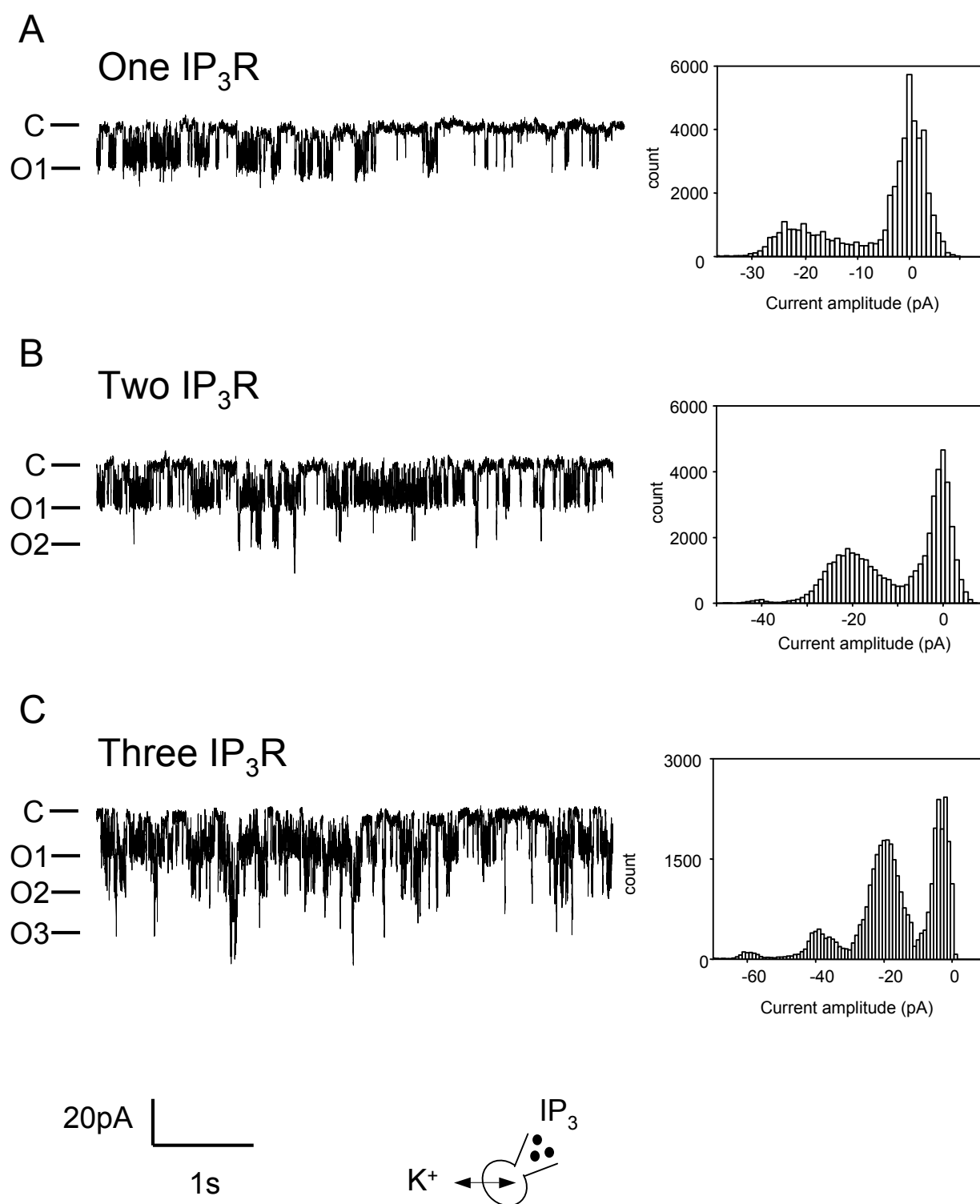
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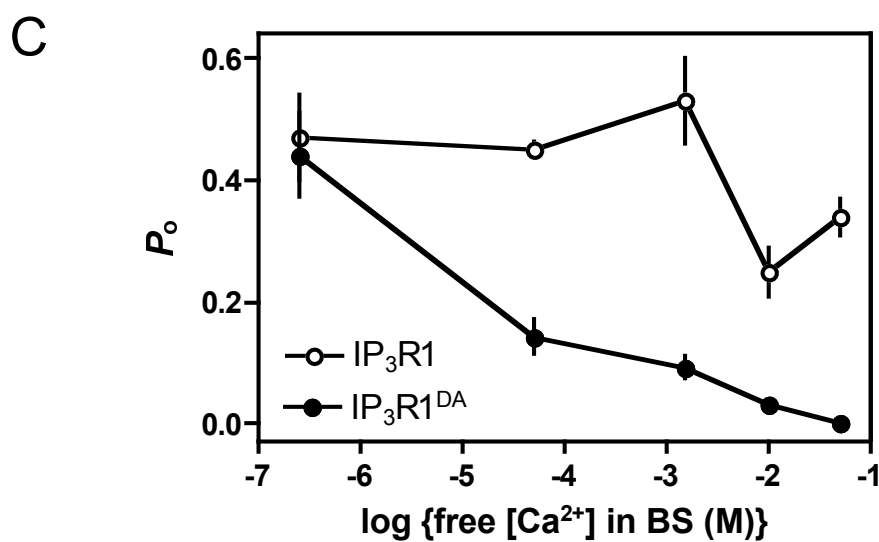
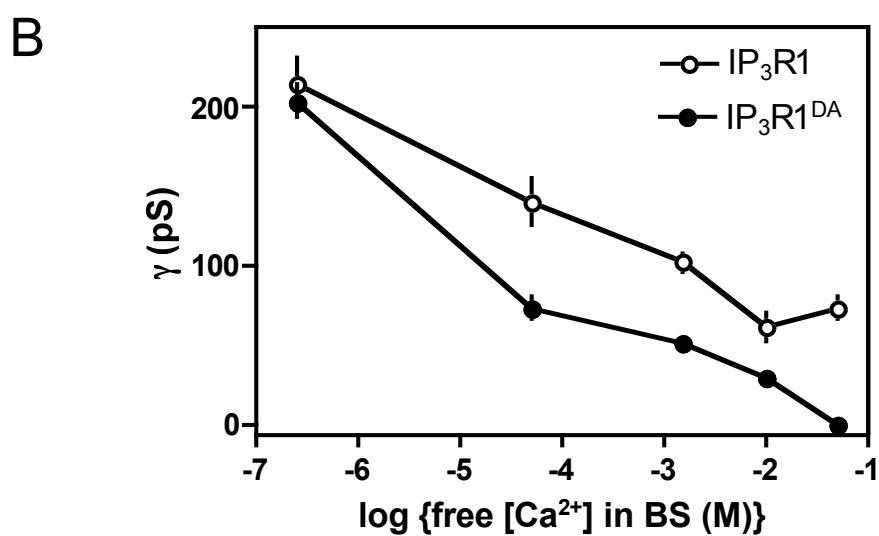
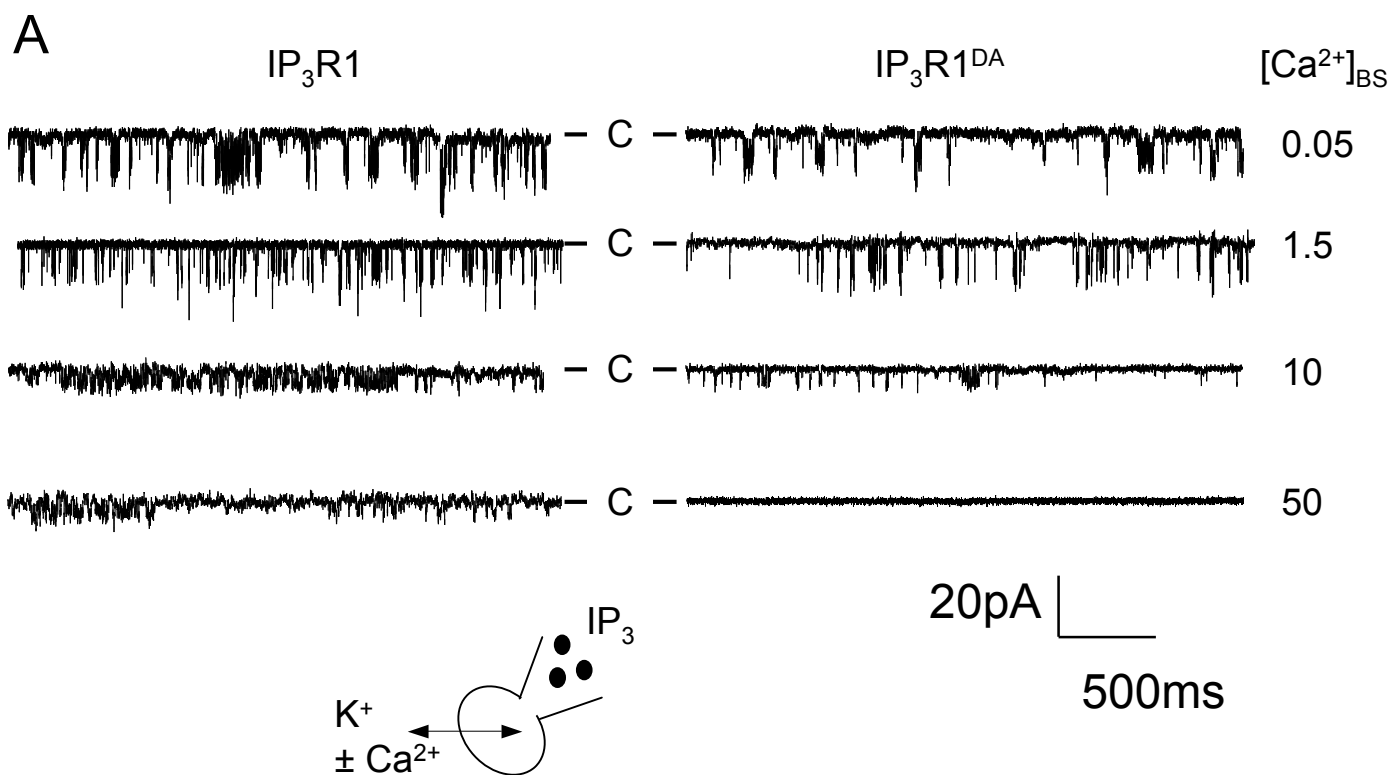
TABLE S1. Primers used for mutagenesis of 1-604 fragment of IP<sub>3</sub>R1

Construct	Primer
1-604	F 5'-AACGTCGACCTGGTTCCGCGTGGATCCATGTCTGACAAATGTCTAGT-3'
1-604	R 5'-CTGGAATTCTCACTTTCGGTTGTTGTGGAGCAGGGCAGTGATGGTGTGTC-3'
R568Q	F 5'-TCACAGCAAGACTACCAGAAGAACCAGGAGTAC-3'
R568Q	R 5'-GTACTCCTGGTTCCTCTGGTAGTCTTGCTGTGA-3'

FIGURE S1. **Unitary currents reflect openings of single IP<sub>3</sub>R.** A-C, Typical whole-cell recordings are shown for DT40IP<sub>3</sub>R1 cells in which multiples of 1 (A), 2 (B) or 3 (C) openings to the unitary current level were detected. The current-amplitude histograms from the recording (right) show that events are evenly spaced with a unitary current amplitude of ~20 pA at a holding potential of -100 mV. Inspection of the traces containing multiples of the unitary openings (B and C) fails to detect any direct transitions to the higher levels. These results suggest that the multiples of the unitary current level reflect opening of different IP<sub>3</sub>R, rather than multiple sub-conductance states of the same IP<sub>3</sub>R.

FIGURE S2. **K<sup>+</sup> currents through PM IP<sub>3</sub>R1<sup>DA</sup> are more sensitive than IP<sub>3</sub>R1 to blockade by Ca<sup>2+</sup>.** A, Typical whole-cell recordings (holding potential = 100 mV) from DT40 cells expressing IP<sub>3</sub>R1 or IP<sub>3</sub>R1<sup>DA</sup> with 10 μM IP<sub>3</sub> in PS and with the free [Ca<sup>2+</sup>] of BS (mM) varied as indicated. C denotes the closed state. B, Single channel slope-conductances (γ, measured at holding potentials between -100 and -40 mV) were determined for the two IP<sub>3</sub>R with the [Ca<sup>2+</sup>] of BS varied as shown. C, Effects of varying [Ca<sup>2+</sup>] of BS on P<sub>o</sub>. Results (B, C) are means ± SEM, n > 5. The summary data are shown in Fig. 2E.





## Counting Functional Inositol 1,4,5-Trisphosphate Receptors into the Plasma Membrane

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