Modulation of Mammalian Inositol 1,4,5-Trisphosphate Receptor Isoforms by Calcium: A Role of Calcium Sensor Region

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ABSTRACT  In the accompanying article, we compared main functional properties of the three mammalian inositol 1,4,5-trisphosphate receptors (InsP₃R) isoforms. In this article we focused on modulation of mammalian InsP₃R isoforms by cytosolic Ca²⁺. We found that: 1) when recorded in the presence of 2 μM InsP₃ and 0.5 mM ATP all three mammalian InsP₃R isoforms displayed bell-shaped Ca²⁺ dependence in physiological range of Ca²⁺ concentrations (pCa 8–5); 2) in the same experimental conditions InsP₃R3 is most sensitive to modulation by Ca²⁺ (peak at 107 nM Ca²⁺), followed by InsP₃R2 (peak at 154 nM Ca²⁺), and then by InsP₃R1 (peak at 257 nM Ca²⁺); 3) increase in ATP concentration to 5 mM had no significant effect of Ca²⁺ dependence of InsP₃R1 and InsP₃R2; 4) increase in ATP concentration to 5 mM converted Ca²⁺ dependence of InsP₃R3 from “narrow” shape to “square” shape; 5) ATP-induced change in the shape of InsP₃R3 Ca²⁺ dependence was mainly due to an >200-fold reduction in the apparent affinity of the Ca²⁺-inhibitory site; 6) the apparent Ca²⁺ affinity of the Ca²⁺ sensor region (Cas) determined in biochemical experiments is equal to 0.23 μM Ca²⁺ for RT1-Cas, 0.16 μM Ca²⁺ for RT2-Cas, and 0.10 μM Ca²⁺ for RT3-Cas; and 7) Ca²⁺ sensitivity of InsP₃R1 and InsP₃R3 isoforms recorded in the presence of 2 μM InsP₃ and 0.5 mM ATP or 2 μM InsP₃ and 5 mM ATP can be exchanged by swapping their Cas regions. Obtained results provide novel information about functional properties of mammalian InsP₃R isoforms and support the importance of the Ca²⁺ sensor region (Cas) in determining the sensitivity of InsP₃R isoforms to modulation by Ca²⁺.

INTRODUCTION

The inositol (1,4,5)-trisphosphate receptor (InsP₃R) is an intracellular calcium (Ca²⁺) release channel that plays a key role in Ca²⁺ signaling in cells (Berridge, 1993). Three mammalian InsP₃R isoforms—InsP₃R type 1 (InsP₃R1), InsP₃R type 2 (InsP₃R2), and InsP₃R type 3 (InsP₃R3)—are expressed in mammals (Furuichi et al., 1994), each with the unique expression pattern (Taylor et al., 1999). Modulation of InsP₃R by cytosolic Ca²⁺ is one of the most fundamental InsP₃R properties responsible for complex spatiotemporal aspects of Ca²⁺ signaling (Berridge, 1993). In the accompanying article (Tu et al., 2005), we used the planar lipid bilayer reconstitution technique to compare main functional properties (conductance, gating, InsP₃ sensitivity, and modulation by ATP) of the recombinant rat InsP₃R1, InsP₃R2, and InsP₃R3 expressed in Sf9 cells by baculoviral infection. In this article, we used the planar lipid bilayer reconstitution technique and biochemical experiments to compare modulation of mammalian InsP₃R isoforms by cytosolic Ca²⁺. In our previous studies, we identified a putative Ca²⁺ sensor region (Cas) within a modulatory domain of InsP₃R1 (Miyakawa et al., 2001; Tu et al., 2003). Here we show that the observed differences in Ca²⁺ sensitivity between mammalian InsP₃R isoforms can be explained by isoform-specific differences in affinities of the Cas region for Ca²⁺. Our results provide information about Ca²⁺ modulation of three mammalian InsP₃R isoforms and further support a role of the Ca²⁺ sensor region (Cas) in the InsP₃R modulation by Ca²⁺.

MATERIAL AND METHODS

Generation of recombinant baculoviruses

The baculoviruses expressing rat InsP₃R1 (RT1) and rat InsP₃R3 (RT3) have been previously described (Maes et al., 2000; Tu et al., 2002). The generation of baculovirus encoding rat InsP₃R2 (RT2) is described in the accompanying article (Tu et al., 2005). The RT1-V-3 and RT3-V-1 chimeric constructs in pFastBac1 vector (Invitrogen, Carlsbad, CA) were generated by PCR-mediated gene fusion and verified by sequencing. In RT1-V-3 construct amino acids M1835-F2242 of InsP₃R1 were replaced with amino acids M1835-F2242 of InsP₃R3; in RT3-V-1 construct amino acids M1835-F2242 of InsP₃R3 were replaced with amino acids E1932-L2312 of InsP₃R1. The RT1-V-3 and RT3-V-1 baculoviruses were generated and amplified using Bac-to-Bac system according to the manufacturer’s (Invitrogen) instructions. Expression of RT1-V-3 and RT3-V-1 proteins in Sf9 cells was confirmed by Western blotting with rabbit polyclonal anti-InsP₃R1 antibody T443 described previously (Kaznacheyeva et al., 1998) and the affinity purified rabbit polyclonal anti-InsP₃R3 antibody (IB7124-AP) described in the accompanying article (Tu et al., 2005).

Expression of InsP₃R in Sf9 cells and planar lipid bilayer experiments

RT1, RT2, RT3 isoforms, RT1-V-3, and RT3-V-1 chimeras were expressed in Sf9 cells and reconstituted into planar lipid bilayers as described in the accompanying article (Tu et al., 2005). Single-channel analysis of currents supported by RT1-V-3 and RT3-V-1 chimeras was performed as described for RT1, RT2, and RT3 isoforms in the accompanying article (Tu et al., 2005). Ca²⁺ dependence of InsP₃R isoforms and chimeras was determined.
as described in Bezprozvanny et al. (1991) by consecutive additions CaCl2 to the cis (cytosolic) chamber from the concentrated 20 mM CaCl2 stock with at least 30 s stirring of solutions in both chambers. Calcium concentration in 20 mM CaCl2 stock solution was verified by atomic absorption spectroscopy (Galbraith Laboratories, Knoxville, TN). Free Ca2+ concentration in the cis chamber was controlled in the range from 10 nM Ca2+ (pCa 8) to 100 μM Ca2+ (pCa 4) by the mixture of 1 mM EGTA, 1 mM HEDTA, and variable concentrations of CaCl2 and calculated by using a program described in Fabiato (1988). Evidence for the presence of multiple channels in the bilayer (multiple open levels) was obtained in the majority of the experiments. The single-channel open probability (Po) was estimated from multichannel records and normalized to the maximum Po observed in the same experiment as described for InsP3R and ATP-dependence experiments in the accompanying article (Tu et al., 2003). Obtained data from several experiments with each InsP3R isoform or chimera were averaged together for presentation and fitting as described for InsP3R and ATP-dependence experiments in the accompanying article (Tu et al., 2005).

To obtain parameters of Ca2+ dependence the normalized and averaged data were fit by the “bell-shaped equation”:

\[ P(Ca^{2+}) = \frac{4P_{max}[Ca^{2+}]^n}{(k_{Ca} + [Ca^{2+}]^m)(k_{Ca}^n + [Ca^{2+}]^n)}, \]

modified from Bezprozvanny et al. (1991), where \( P_m \) is a parameter proportional to the maximal normalized \( Po \) value, \( n \) is the Hill coefficient, \( k_{Ca} \) is the apparent affinity of the Ca2+-activating site, and \( k_{Ca}^n \) is the apparent affinity of the Ca2+-inhibitory site. As explained in Tu et al. (2003), parameter \( P_m \) is equal to maximal normalized \( Po \) only in the case when \( k_{Ca} = k_{Ca}^n \). If \( k_{Ca} \neq k_{Ca}^n \), parameter \( Po \) is proportional (and higher) than maximal \( Po \).

To obtain parameters of Ca2+ dependence of InsP3R3 and RT1-V-3 at 5 mM ATP, the normalized and averaged data were fit by the “biphasic Hill equation”:

\[ P(Ca^{2+}) = \frac{P_{max}}{(1 + (k_{Ca}/[Ca^{2+}]^m))(1 + ([Ca^{2+}]^m/k_{Ca}^n))}, \]

modified from Mak et al. (1998), where \( P_{max} \) is a parameter proportional to the maximal normalized \( Po \) value, \( Ha \) is the Hill coefficient of the activation phase, \( k_{Ca} \) is the apparent affinity of the Ca2+-activating site, \( H^i \) is the Hill coefficient of the inhibitory phase, and \( k_{Ca}^n \) is the apparent affinity of the Ca2+-inhibitory site.

The fits using Eqs. 1 and 2 were generated using least-squares routine (Sigma Plot 2001, Jandel Scientific) and the quality of the fit was evaluated from the coefficient of determination (R2). The standard errors of resulting parameters were obtained as the estimates of the uncertainties in the values of regression coefficients obtained as a result of the fitting procedure (Sigma Plot 2001, Jandel Scientific).

**RESULTS**

**Modulation of mammalian InsP3R isoforms by Ca2+**

In the accompanying article (Tu et al., 2005), we compared main functional properties (conductance, gating, InsP3 sensitivity, and modulation by ATP) of the recombinant rat InsP3R1, InsP3R2, and InsP3R3 expressed in Sf9 cells by baculoviral infection and reconstituted into planar lipid bilayers. Modulation of InsP3R by cytosolic Ca2+ is one of the most fundamental InsP3R properties responsible for complex spatiotemporal aspects of Ca2+ signaling (Berridge, 1993). Does cytosolic Ca2+ affect InsP3R function in an isofrom-specific manner? To answer this question we determined Ca2+ dependence of recombinant InsP3R1, InsP3R2, and InsP3R3 in the presence of 2 μM InsP3 and 0.5 mM ATP. Because most of the experiments resulted in multichannel bilayers, the Po values in each experiment were normalized to the maximal Po in the same experiment as described in Materials and Methods, and the normalized data from different experiments with each InsP3R isoform were averaged together for presentation and analysis. In agreement with our previous findings (Nosyreva et al., 2002; Tu...
et al., 2002, 2003), InsP3R1 expressed in Sf9 cells (RT1) display bell-shaped Ca$^{2+}$ dependence with the peak at 257 nM Ca$^{2+}$ (Fig. 1, open circles). Fit to the RT1 Ca$^{2+}$-dependence using Eq. 1 (Fig. 1, curve; $R^2 = 0.99$) yielded apparent affinities of activating (K$_{a2}$) and inhibitory (K$_{i2}$) sites equal to 0.17 ± 0.03 μM Ca$^{2+}$ and 0.37 ± 0.01 μM Ca$^{2+}$, respectively (Table 1). Similar to RT1, RT2 and RT3 also displayed bell-shaped Ca$^{2+}$ dependence in the physiological range of Ca$^{2+}$ concentrations (pCa 8–5). The peak of bell-shaped Ca$^{2+}$ dependence was located at 154 nM Ca$^{2+}$ for RT2 (Fig. 1, solid triangles), and at 107 nM Ca$^{2+}$ for RT3 (Fig. 1, solid circles). Fit to the RT2 and RT3 Ca$^{2+}$-dependence using Eq. 1 (Fig. 1, curves; $R^2 = 0.98$ for RT2 and $R^2 = 0.94$ for RT3) yielded the apparent affinities of the activating and inhibitory sites equal to 0.15 ± 0.04 μM Ca$^{2+}$ and 0.16 ± 0.04 μM Ca$^{2+}$ for RT2, and 0.06 ± 0.03 μM Ca$^{2+}$ and 0.17 ± 0.02 μM Ca$^{2+}$ for RT3, respectively (Table 1).

As described in the accompanying article (Tu et al., 2005), 5 mM concentration of ATP is required to maximally activate RT3 channels. Thus, in the next series of experiments we determined Ca$^{2+}$-dependence of RT3 in the presence of 2 μM InsP3 and 5 mM ATP. We found that in the presence of 5 mM ATP, RT3 displayed a “square-shaped” Ca$^{2+}$ dependence with the maximal channel activity observed in the range of Ca$^{2+}$ concentrations between 0.1 μM Ca$^{2+}$ and 10 μM Ca$^{2+}$ (pCa 7–5) (Fig. 2, solid circles). The “square-shaped” Ca$^{2+}$ dependence observed in this condition is similar to “square-shaped” Ca$^{2+}$ dependence described for Xenopus InsP3R (InsP3R1) (Mak et al., 1998) and rat InsP3R3 expressed in Xenopus oocytes (Mak et al., 2001b). Thus, to fit these data we used “biphasic Hill equation” (Eq. 2) adapted from Mak et al. (1998). Fit to RT3 Ca$^{2+}$-dependence data using Eq. 2 (Fig. 2, thick curve; $R^2 = 0.93$) yielded apparent affinities of activating and inhibitory sites equal to 0.029 ± 0.004 μM Ca$^{2+}$ and 37 ± 6 μM Ca$^{2+}$ (Table 1). Thus, increase in ATP concentration to 5 mM had only a twofold effect on the apparent affinity of the Ca$^{2+}$-activating site of InsP3R3, but resulted in a >200-fold reduction in the apparent affinity of the Ca$^{2+}$-inhibitory site of InsP3R3 (Table 1). In contrast to InsP3R3 (Fig. 2), we found that the Ca$^{2+}$ dependence of InsP3R1 and InsP3R2 was not significantly different at 0.5 mM ATP and at 5 mM ATP (data not shown).

**Ca$^{2+}$ binding to InsP3R Ca$^{2+}$ sensor**

Different Ca$^{2+}$ sensitivity of InsP3R1, InsP3R2, and InsP3R3 has been observed in our experiments performed at 2 μM InsP3 and 0.5 mM ATP (Fig. 1; Table 1). We previously proposed that the putative Ca$^{2+}$ sensor region (Cas) in the coupling domain of InsP3R1 is responsible for InsP3R1 modulation by Ca$^{2+}$ (Miyakawa et al., 2001; Tu et al., 2003). If “Ca$^{2+}$ sensor hypothesis” is correct, then the differences in Ca$^{2+}$ sensitivity between InsP3R isoforms should correlate with the differences in Ca$^{2+}$ affinities of corresponding Cas regions. To test this prediction, we expressed in bacteria and purified RT1-Cas (E1932-R2270 of rat InsP3R1), RT2-Cas (E1884-R2224 of rat InsP3R2), and RT3-Cas (M1835-R2199 of rat InsP3R3) proteins (Fig. 3 A). Similar yield of RT-Cas proteins was obtained as a result of our expression and purification procedure for each InsP3R isoform (Fig. 3 B).

Sequence alignment of RT1-Cas, RT2-Cas, and RT3-Cas regions shows a high degree of sequence conservation (Fig. 4). The E2100 glutamate residue that we previously identified to be critical for InsP3R1 modulation by Ca$^{2+}$ (Miyakawa et al., 2001; Tu et al., 2003) is conserved in InsP3R2 (E2053) and InsP3R3 (E2004) sequences (Fig. 4, arrow). In the previous study (Tu et al., 2003), we utilized intrinsic tryptophan fluorescence assay to compare Ca$^{2+}$ binding affinity of RT1-Cas wild type and E2100 mutants. The W2255 and W2267 residues of InsP3R1 are likely to be quenched in a Ca$^{2+}$-dependent manner similar to our previous findings with RT1-Cas (Tu et al., 2003).

To test this hypothesis, we collected emission spectra (excitation at 280 nm) of RT1-Cas, RT2-Cas, and RT3-Cas proteins at different Ca$^{2+}$ concentrations (pCa 9.4–2.0, buffered by 1 mM EGTA and 1 mM HEDTA). We found that the position of the emission peak ($\lambda_{max}$) remained constant at 332 nm for all three RT-Cas proteins at all Ca$^{2+}$ concentrations (Fig. 5, A–C). However, a systematic and saturable change in the intensity of the intrinsic fluorescent signal was observed for all three RT-Cas proteins as a function of Ca$^{2+}$ (Fig. 5, A–C). To compare the data

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**FIGURE 1** Ca$^{2+}$ dependence of mammalian InsP3R isoforms. The single-channel open probability ($Po$) for each InsP3R isoform was measured as a function of cytosolic Ca$^{2+}$ concentrations from 10 nM to 5 μM Ca$^{2+}$ on the cis (cytoplasmic) side of the membrane in the presence of 2 μM InsP3 and 0.5 mM Na$_2$ATP. The normalized and averaged data (see Materials and Methods) at each Ca$^{2+}$ concentration are shown as means ± SE ($n = 3$) for RT1 (C), RT2 (A), RT3 (B). These data were fitted by Eq. 1 (see Materials and Methods). The parameters of the best fit (curves) are in Table 1.
from different experiments, the observed changes in RT1-Cas, RT2-Cas, and RT3-Cas peak fluorescence intensity ($\Delta F$) were corrected for dilution (see Materials and Methods), normalized to the maximal change in the peak fluorescence intensity ($\Delta F_{\text{max}}$), averaged, and plotted against Ca$^{2+}$ concentration (Fig. 6). By fitting the obtained results using Eq. 3 (see Materials and Methods) at each Ca$^{2+}$ concentration (Fig. 6; Table 1). Obtained results are in quantitative agreement with the sensitivity of InsP3R isoforms to Ca$^{2+}$ concentration (Table 1). For all three RT-Cas regions, the Hill coefficient ($n_{\text{Hill}}$) determined in Ca$^{2+}$-binding experiments was in the range 0.4–0.5 (Fig. 6; Table 1). As discussed previously for RT1-Cas (Tu et al., 2003), an apparent negative cooperativity in association of RT-Cas regions with Ca$^{2+}$ may be due to multimerization of recombinant proteins during our measurements or due to the presence of multiple Ca$^{2+}$ binding sites within the RT-Cas regions.

**Functional analysis of InsP3R Ca$^{2+}$ sensor swap chimeras**

Is Cas region alone sufficient to determine InsP3R sensitivity to Ca$^{2+}$? To answer this question and to further test the “Ca$^{2+}$ sensor hypothesis” (Miyakawa et al., 2001; Tu et al., 2003), we generated chimeric RT1-V-3 and RT3-V-1 baculoviruses by swapping Cas-containing regions between InsP3R1 and InsP3R3 (Fig. 7 A). The regions swapped in RT1-V-3 and RT3-V-1 chimeras had the same aminoterminal boundary (E1932 in InsP3R1 and M1835 in InsP3R3) as the soluble RT1-Cas and RT3-Cas constructs expressed in bacteria (see Fig. 4). To simplify the construction, the swapped regions were 43 amino acids longer on carboxy-termini than the soluble RT1-Cas and RT3-Cas constructs (to the middle of the second predicted transmembrane domain, L2312 in InsP3R1 and F2242 of InsP3R3) (see Fig. 4). As discussed in the accompanying article (Tu et al., 2005), coupling domain of InsP3R1 contains high-affinity (ATPA) and low-affinity (ATPB) ATP-binding sites (Fig. 7 A). In contrast, coupling domain of InsP3R3 contains only low-affinity (ATPB) ATP-binding site (Fig. 7 A). Cas regions of InsP3R1 and InsP3R3 include corresponding ATPB sites in their sequence (Fig. 4). Thus, RT1-V-3 chimera contains the high-affinity ATPA site from InsP3R1 and the low-affinity ATPB site from InsP3R3 (Fig. 7 A). In contrast, RT3-V-1 chimera contains only the low-affinity ATPB site from InsP3R1 (Fig. 7 A). As discussed in the accompanying article (Tu et al., 2005), InsP3R1 and InsP3R3 differ dramatically in their sensitivity to ATP modulation. Thus, swapping Cas regions may affect not only Ca$^{2+}$, but also ATP dependence of parental constructs. Expression of RT1-V-3 and RT3-V-1 proteins in Sf9 cells was confirmed by Western blotting with anti-InsP3R1 T443 antibodies (Fig. 7 B) and anti-InsP3R3 affinity purified IB7124 antibodies (Fig. 7 C). The epitopes for T443 antibodies (Kaznacheyeva et al., 1998) and IB7124 anti-

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**TABLE 1 Ca$^{2+}$ dependence of mammalian InsP3R isoforms and Ca$^{2+}$ sensor swap chimeras**

<table>
<thead>
<tr>
<th>InsP3R</th>
<th>ATP (nM)</th>
<th>Activation $K_Ca$ (µM)</th>
<th>Inhibition $k_{ca}$ (µM)</th>
<th>Hill coefficient $n_{\text{Hill}}$</th>
<th>Peak Ca$^{2+}$ (nM)</th>
<th>Ca$^{2+}$ binding $K_Ca$ (µM)</th>
<th>Hill coefficient $n_{\text{Hill}}$</th>
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<tr>
<td>RT1</td>
<td>0.5</td>
<td>0.17 ± 0.03</td>
<td>0.37 ± 0.01</td>
<td>1.23</td>
<td>257</td>
<td>0.23 ± 0.04</td>
<td>0.47</td>
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<tr>
<td>RT2</td>
<td>0.5</td>
<td>0.15 ± 0.04</td>
<td>0.16 ± 0.04</td>
<td>2.05</td>
<td>154</td>
<td>0.16 ± 0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>RT3</td>
<td>5</td>
<td>0.029 ± 0.004</td>
<td>0.17 ± 0.02</td>
<td>1.17 (Hi)</td>
<td>100–10,000</td>
<td>0.10 ± 0.04</td>
<td>0.39</td>
</tr>
<tr>
<td>RT1-V-3</td>
<td>5</td>
<td>0.04 ± 0.003</td>
<td>0.16 ± 0.01</td>
<td>2.17</td>
<td>100</td>
<td>RT3</td>
<td>RT3</td>
</tr>
<tr>
<td>RT3-V-1</td>
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<td>0.24 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>1.65</td>
<td>239</td>
<td>RT1</td>
<td>RT1</td>
</tr>
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</table>

Entries in the “Ca$^{2+}$ binding” columns for RT1-V-3 and RT3-V-1 chimeras are used to indicate the “parental” InsP3R isoform for the Cas region in each chimera. Both $H_u$ and $H_i$ values (see Eq. 2) are entered in the $n_{\text{Hill}}$ column for RT3 Ca$^{2+}$ dependence at 5 mM ATP.

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![FIGURE 2](image)  
**FIGURE 2** Ca$^{2+}$ dependence of InsP3R3 isoform at 5 mM ATP. The single-channel open probability ($P_o$) for InsP3R3 (RT3) was measured as a function of cytosolic Ca$^{2+}$ concentrations from 10 nM to 100 µM Ca$^{2+}$ on the cis (cytoplasmic) side of the membrane in the presence of 2 µM InsP3 and 5 mM Na$_2$ATP. The normalized and averaged data (see Materials and Methods) at each Ca$^{2+}$ concentration are shown as means ± SE ($n \geq 3$). These data were fit by Eq. 2 (see Materials and Methods). The parameters of the best fit (thick line) are in Table 1. The fit to the RT3 Ca$^{2+}$ dependence in the presence of 2 µM InsP3 and 0.5 mM Na$_2$ATP (thin line) is from Fig 1.
bodies (Tu et al., 2005) are located at the carboxy-terminal ends of InsP$_3$R$_1$ and InsP$_3$R$_3$ sequences, which are not affected by swapping Cas-containing domains in RT1-V-3 and RT3-V-1 chimeras (Fig. 7A).

Recombinant RT1-V-3 and RT3-V-1 chimeric proteins formed functional InsP$_3$-gated channels in planar lipid bilayers when recorded in standard recording conditions (pCa 6.7, 0.5 mM ATP, 2 μM InsP$_3$) (Figs. 8A and 9A). The Gaussian fit to the amplitude histogram of currents supported by RT1-V-3 chimera (Fig. 8B) resulted in the average unitary current amplitude of RT1-V-3 channels equal to 1.86 ± 0.06 pA (n = 3) and the mean Po of RT1-V-3 channels equal to 20 ± 4% (n = 3). The open and closed dwell time distributions of RT1-V-3 channels could be fit with a single exponential function (Fig. 8, C and D) that yielded the mean open time of RT1-V-3 channels equal to 7.8 ± 0.9 ms (n = 3) and the mean closed time of RT1-V-3 channels equal to 10 ± 1 ms (n = 3). The Gaussian fit to the amplitude histogram of currents supported by RT3-V-1 chimera (Fig. 9B) yielded the average unitary current amplitude equal to
1.87 ± 0.06 pA (n = 3) and the mean \( Po \) equal to 8 ± 3% (n = 3). The open dwell time and closed dwell time distributions of RT3-V-1 currents were fit by a single exponential function (Fig. 9, C and D), resulting in the mean open time of RT1-V-3 channels equal to 8 ± 1 ms (n = 3) and the mean closed time equal to 83 ± 5 (n = 3). Thus, conductance and gating properties of RT1-V-3 and RT3-V-1 chimeric channels are similar to conductance and gating properties of wild-type InsP3R1 and InsP3R3 channels described in the accompanying article (Tu et al., 2005). Thus, we have not induced gross abnormalities in InsP3R gating and conductance properties by swapping Cas regions between InsP3R1 and InsP3R3.

We reasoned that low open probability of RT3-V-1 channels when compared with RT1-V-3 channels in standard recording conditions may be due to lower sensitivity of these channels to ATP modulation, as described for RT3 channels relative to RT1 channels in the accompanying article (Tu et al., 2005). To test this hypothesis, we compared channel activity of RT1-V-3 and RT3-V-1 chimeras in the presence of 2 μM InsP3 at pCa 6.7 in the absence of ATP, at 0.5 mM ATP, and at 5 mM ATP (Fig. 10). We found that both RT1-V-3 and RT3-V-1 channels display very low levels of activity in the absence of ATP (\( Po = 3 \pm 1\% \) (n = 3) for RT1-V-3 and \( Po = 2 \pm 1\% \) (n = 2) for RT3-V-1) (Fig. 10, A and B, top traces). Addition of 0.5 mM ATP potentiated activity of RT1-V-3 channels to \( Po = 20 \pm 4\% \) (n = 3) (Fig. 10 A, middle trace). The \( Po \) of RT3-V-1 channels at 0.5 mM ATP was much lower at 8 ± 3\% (n = 3) (Fig. 10 B, middle trace). However, addition of 5 mM ATP resulted in maximal level of activity of both RT1-V-3 (\( Po = 23 \pm 5\% \) (n = 3)) and RT3-V-1 (\( Po = 25 \pm 7\% \) (n = 2)) channels (Fig. 10, A and B, bottom traces). Comparison with results in the accompanying article (Tu et al., 2005) clearly shows that RT1-V-3 channels are modulated by ATP similar to RT1 channels, and RT3-V-1 channels are modulated by ATP similar to RT3 channels. Thus, the main reason for differences in ATP sensitivity of the RT1 and RT3 channels is related to the presence or absence of the high-affinity ATPA site (Fig. 7 A), in agreement with our previous conclusions based on the functional analysis of InsP3R1-opt mutant (Tu et al., 2002).

In the presence of 2 μM InsP3 and 0.5 mM ATP both InsP3R1 and InsP3R3 display narrow bell-shaped Ca\(^{2+}\)
Cas sequence (Miyakawa et al., 2001; Tu et al., 2003) and the correspond-

baculoviruses were analyzed by Western blotting with anti-InsP3R1 (RT1, RT3, RT1-V-3, and RT3-V-1 constructs. In RT1-V-3 construct the polyclonal antibody T443 (protein was loaded on the gel.

FIGURE 7 Expression of Ca2+ sensor swap chimeras. (A) The diagram of RT1, RT3, RT1-V-3, and RT3-V-1 constructs. In RT1-V-3 construct the putative InsP3R1 Cas region (E1932-L2312) was replaced by the Cas region of InsP3R3 (M1835-F2242; shaded). In RT3-V-1 construct the putative InsP3R3 Cas region (M1835-F2242) was replaced by the Cas region of InsP3R1 (E1932-L2312; open). Locations of E2100 residue in the InsP3R1 Cas sequence (Miya kawa et al., 2001; Tu et al., 2003) and the corresponding E2004 residue in the InsP3R3 sequence are shown. Also shown are locations of ATPA (177GGGGGPG1780 in InsP3R1) and ATPB (1911GGGLLLG1901 in InsP3R1 and 1991GGGLLLG1925 in InsP3R3) sites (Maes et al., 2001) and the epitopes for T443 (anti-InsP3R1) (Kaznacheyeva et al., 1998) and IB7124 (anti-InsP3R3) (Tu et al., 2005) polyclonal antibodies. (B and C) The microsomes isolated from noninfected S9 cells (S9), and from S9 cells infected with RT1, RT3, RT1-V-3, and RT3-V-3 baculoviruses were analyzed by Western blotting with anti-InsP3R1 polyclonal antibody T443 (B) and anti-InsP3R3 affinity purified polyclonal antibody IB7124-AP (C). For each microsomal preparation, 10 μg of total protein was loaded on the gel.

dependence of the peak at 257 nM Ca2+ for InsP3R1 and 107 nM Ca2+ for InsP3R3. To investigate the effects of Cas region swap on sensitivity of InsP3R to Ca2+, we determined Ca2+ dependence of RT1-V-3 and RT3-V-3 channels in the same recording conditions. Because most of the experiments resulted in multichannel bilayers, the Po values in each experiment were normalized to the maximal Po in the same experiment as described in Materials and Methods and the normalized data from different experiments with each chimera were averaged together for presentation and analysis. We found that the RT1-V-3 chimera displayed bell-shaped Ca2+ dependence that closely overlaps with the RT3 Ca2+ dependence with the peak at 100 nM Ca2+ (Fig. 11 A, solid circles). Fit to the Ca2+ dependence of RT1-V-3 channels using Eq. 1 (Fig. 11 A, smooth thick curve; R2 = 0.98) yielded the apparent affinities of activating and inhibitory Ca2+-binding sites equal to 0.06 ± 0.02 and 0.16 ± 0.01 μM Ca2+ (Table 1), identical to the values obtained for the RT3 channels. The difference between RT1-V-3 and RT3 Ca2+-dependence curves was due to differ-

ces in apparent Hill coefficients of Ca2+ modulation (n = 2.17 for RT1-V-3 and n = 1.46 for RT3; Table 1).

In contrast to RT1-V-3, the RT3-V-1 chimera displayed bell-shaped Ca2+ dependence that closely overlaps with the RT1 Ca2+ dependence with the peak at 239 nM Ca2+ (Fig. 11 B). Fit to the Ca2+ dependence of RT3-V-1 channels using Eq. 1 (Fig. 11 B, smooth thick curve; R2 = 0.99) yielded the apparent affinities of activating and inhibitory Ca2+-binding sites equal to 0.24 ± 0.01 and 0.25 ± 0.02 μM Ca2+ (Table 1), close to the values obtained for the RT1 channels. Similar to RT1-V-3 chimera, the difference between RT3-V-1 and RT1 Ca2+-dependence curves was largely due to differences in apparent Hill coefficients of Ca2+ modulation (n = 1.65 for RT3-V-1 and n = 1.23 for RT1; Table 1). The results obtained with RT1-V-3 and RT3-V-1 chimeras (Fig. 11) indicated that swapping Cas regions between InsP3R1 and InsP3R3 was sufficient to exchange sensitivities to modulation by Ca2+ between these two mammalian InsP3R isoforms.

In the presence of 5 mM ATP, the RT3 channels display “square-shaped” Ca2+ dependence (Fig. 2). In the next series of experiments we compared Ca2+ dependence of RT1-V-3 and RT3-V-1 chimeras in the presence of 2 μM InsP3 and 5 mM ATP. We found that in the presence of 5 mM ATP, RT1-V-3 chimera (Fig. 12, open circles) displayed a “square-shaped” Ca2+ dependence very similar to Ca2+ dependence of RT3 channels, whereas RT3-V-1 chimera (Fig. 12, triangles) displayed “narrow” Ca2+ dependence. Fit to the Ca2+ dependence of RT1-V-3 channels using Eq. 2 (Fig. 12, smooth thick curve; R2 = 0.98) yielded the apparent affinities of activating and inhibitory Ca2+-binding sites equal to 0.040 ± 0.003 and 33 ± 5 μM Ca2+ (Table 1), close to the values obtained for the RT3 channels at 5 mM ATP. Fit to the Ca2+ dependence of RT3-V-1 channels using Eq. 1 (Fig. 12, smooth thick curve; R2 = 0.96) yielded the apparent affinities of activating and inhibitory Ca2+-binding sites equal to 0.28 ± 0.03 and 0.31 ± 0.04 μM Ca2+ (Table 1), similar to the values obtained for the RT1 channels.

DISCUSSION

Ca2+ dependence of mammalian InsP3R isoforms

In standard recording conditions (2 μM InsP3 and 0.5 mM ATP) all three mammalian InsP3R displayed narrow bell-shaped Ca2+ dependence within a physiological range of Ca2+ concentrations (pCa 8–5) (Fig. 1; Table 1). In an independent study we demonstrated that Drosophila melanogaster InsP3R displays similar narrow bell-shaped Ca2+ dependence when reconstituted into planar lipid bilayers and analyzed in standard recording conditions (Srikanth et al., 2004). Thus, bell-shaped Ca2+ dependence of InsP3R appears to be a fundamental and evolutionary conserved feature of all InsP3R. This conclusion is in contrast with
Ca²⁺ Sensitivity of InsP₃R Isoforms

FIGURE 8 Functional properties of RT1-V-3 chimera. (A) Single-channel records of RT1-V-3 channels in planar lipid bilayers. The experiments were performed at pCa 6.7 in the presence of 0.5 mM ATP (control, top trace). Addition of 2 µM InsP₃ to the cis (cytoplasmic) side activates RT1-V-3 channels (middle trace). Current traces at the expanded timescale are also shown (bottom trace). (B–D) Analysis of the single-channel records of RT1-V-3 channels was performed and analyzed as described for InsP₃R1 in the accompanying article (Tu et al., 2005). The Gaussian peak corresponding to an open state of RT1-V-3 was centered at 1.89 pA, had σ = 0.56 pA, and area 28%. Open time distribution (C; number of events = 2200) was fitted with a single exponential function (curve) that yielded mean open time τₒ = 8.4 ms. Closed time distribution (D; number of events = 2200) was fitted with a single exponential function (curve) that yielded mean closed time τₜ₉ = 9.8 ms. The data from the same experiment with a single active channel in the bilayer were used for panels A–D.

some previous bilayer studies of InsP₃R2 (Ramos-Franco et al., 2000, 1998) and InsP₃R3 (Hagar et al., 1998). However, bell-shaped Ca²⁺ regulation of InsP₃R3 observed in our experiments is consistent with Ca²⁺ flux measurements in RIN-5F (Swatton et al., 1999) and 16HBE14o-bronchial mucosal cells (Missiaen et al., 1998) and with nuclear patch recordings of recombinant InsP₃R3 expressed in Xenopus oocytes (Mak et al., 2001b). The reasons for these discrepancies are not clear. The InsP₃R1 contain a high-affinity Ca²⁺/calmodulin (CaM)-binding site in the coupling domain, which is not conserved in the InsP₃R3 sequence (Yamada et al., 1995). The fact that InsP₃R3 display bell-shaped Ca²⁺ dependence despite the absence of a Ca²⁺/CaM-binding site further supports the notion that association with CaM at this Ca²⁺/CaM binding site does not play a role in biphasic modulation of InsP₃R by Ca²⁺ (Nossyrev et al., 2002; Zhang and Joseph, 2001) (but see Michikawa et al., 1999). With all three InsP₃R isoforms displaying bell-shaped Ca²⁺ dependence (Fig. 1), how can we explain isoform-specific Ca²⁺ oscillation profiles observed in the study with DT40 cells (Miyakawa et al., 1999)? We would like to suggest that the unique ability of InsP₃R2 to support robust Ca²⁺ oscillations observed in the study of Miyakawa et al. (1999) results from higher affinity of InsP₃R2 for InsP₃ when compared to InsP₃R1 and InsP₃R3 isoforms (Miyakawa et al., 1999; Sudhof et al., 1991; Tu et al., 2005). We would like to suggest that ligation of B-cell receptors (BCR) with anti-BCR antibody in the study with DT40 cells (Miyakawa et al., 1999) resulted in long-lasting InsP₃ elevation above the threshold of InsP₃R2 activation, close to the threshold of InsP₃R1 activation, and below the threshold of InsP₃R3 activation.

The narrow shape of InsP₃R1 bell-shaped Ca²⁺ dependence in our planar lipid bilayer experiments (Fig. 1 and
Nosyreva et al., 2002; Tu et al., 2002, 2003) is different from the “square” shape described for *Xenopus* InsP$_3$R (xInsP$_3$R) (InsP$_3$R1 homolog; Kume et al., 1993) in nuclear patch experiments by Mak et al. (1998), but it is consistent with the earlier nuclear patch experiments by Stehno-Bittel et al. (1995). Also, the maximal $P_o$ of InsP$_3$R1 in our experiments (Tu et al., 2005) and in experiments by Stehno-Bittel et al. (1995) is in the range of 20–30%, whereas $P_o$ of xInsP$_3$R in experiments of Mak et al. (1998) reaches 80%. What is an explanation of these differences? And more importantly, what behavior more closely reflects InsP$_3$R1 function in vivo? The $P_o$ of InsP$_3$R1 in vivo is unknown. However, all Ca$^{2+}$ flux measurements performed with permeabilized cells (Iino, 1990), isolated brain microsomes (Finch et al., 1991), and *Xenopus* oocytes (Parker and Ivorra, 1990; Yao and Parker, 1992) are consistent with the “narrow” shape of InsP$_3$R1 Ca$^{2+}$ dependence. In all of these experiments InsP$_3$-induced Ca$^{2+}$ release was completely blocked by Ca$^{2+}$ concentrations in the 5–10 μM range, whereas Ca$^{2+}$ concentrations in the 50–100 μM range were required to inhibit xInsP$_3$R and InsP$_3$R3 in nuclear patch recordings of Mak et al. (1998). Therefore, we concluded that “narrow” bell-shaped Ca$^{2+}$ dependence observed in our experiments (Fig. 1 and Nosyreva et al., 2002; Tu et al., 2002, 2003) more closely reflects the physiological behavior of InsP$_3$R1 and InsP$_3$R3 than the “square” Ca$^{2+}$ dependence of xInsP$_3$R reported by Mak et al. (1998).

A recently published mathematical modeling study (Fraiman and Dawson, 2004) offers a potential explanation to the different behavior of InsP$_3$R1 observed in planar lipid bilayer experiments (Bezprozvanny et al., 1991 and Fig. 1) and in nuclear patch recordings (Mak et al., 1998). Fraiman and
Dawson (2004) were able to explain most of the differences in results obtained in the planar lipid bilayer and nuclear patches recordings of InsP3R1 activity by introducing an additional intraluminal Ca2+ modulatory site. These authors were able to explain “square” Ca2+ dependence and high Po of xInsP3R in nuclear patch recordings of Mak et al. (1998) by the fact that these experiments were performed using monovalent cations to carry current in the absence of Ca2+ or other divalent cations. In contrast, the planar lipid bilayer recordings are performed using 50 mM Ca2+ (Bezprozvanny et al., 1991) or Ba2+ (Fig. 1 and Nosyreva et al., 2002; Tu et al., 2002, 2003) as a current carrier. In the previous study we compared gating of InsP3R1 with 50 mM Ca2+, Ba2+, Sr2+, and Mg2+ as current carriers and analyzed modulation of InsP3R1 by intraluminal Ca2+ levels (Bezprozvanny and Ehrlich, 1994). As discussed by Fraiman and Dawson (2004), the results from Bezprozvanny and Ehrlich (1994) are mostly consistent with the model proposed in their article. Thus, we concluded that “square” Ca2+ dependence and high Po of xInsP3R in nuclear patch recordings of Mak et al. (1998) most likely results from using divalent-free recording conditions as discussed above for InsP3R1.

Interestingly, the “narrow” Ca2+ dependence of InsP3R3 in standard recording conditions (2 μM InsP3 and 0.5 mM ATP) (Fig. 1) was converted to “square-shaped” Ca2+ dependence when recordings were performed in the presence of 2 μM InsP3 and 5 mM ATP (Fig. 2). The ATP-induced change in the shape of Ca2+ dependence was a unique feature of InsP3R3, as Ca2+ dependence of InsP3R1 remained “narrow” in the presence of 5 mM ATP (data not shown). Fit to “narrow” (Fig. 1) and “square” (Fig. 2)

FIGURE 10 ATP sensitivity of Ca2+ sensor swap chimeras. Representative current records of RT1-V-3 (A) and RT3-V-1 (B) channels in the bilayers in the presence of 2 μM InsP3 and pCa 6.7 at concentrations of Na2ATP as indicated on the cis (cytoplasmic) side of the membrane. The recordings from the same experiment are shown on each panel. Similar results were obtained in at least three experiments with each chimera.
Ca\textsuperscript{2+} -dependence curves of InsP\textsubscript{3}R indicated that an increase in ATP concentration from 0.5 to 5 mM resulted in a twofold increase in the apparent affinity of the Ca\textsuperscript{2+} -activating site of InsP\textsubscript{3}R and a 200-fold reduction in the apparent affinity of the Ca\textsuperscript{2+} -inhibitory site of InsP\textsubscript{3}R (Table 1). In experiments of Mak et al. (2001a) with InsP\textsubscript{3}R expressed in Xenopus oocytes an increase in ATP concentration from 0 to 0.5 mM resulted in a 10-fold increase in the affinity of the Ca\textsuperscript{2+} -activating site and a threefold increase in the affinity of the Ca\textsuperscript{2+} -inhibitory site. Unfortunately, the inhibitory phase of InsP\textsubscript{3}R Ca\textsuperscript{2+} dependence was not analyzed at ATP concentrations >0.5 mM by Mak et al. (2001a), so it is not clear if the differences between our studies and the Mak et al. studies are due to a different range of ATP concentrations compared (0.5 and 5 mM in our study and 0 and 0.5 mM in the Mak et al., 2001a study) or because of the use of divalent-free recording conditions as discussed above. Notably, Mak et al. (1998) earlier reported that the increase in InsP\textsubscript{3} concentration from 20 nM to 10 \textmu M leads to a 280-fold reduction in the apparent affinity of the Ca\textsuperscript{2+} -inhibitory site of xInsP\textsubscript{3}R. In our hands the affinity of the InsP\textsubscript{3}R Ca\textsuperscript{2+} -inhibitory site is affected in a very similar way by an increase in ATP concentration (Figs. 1 and 2; Table 1).

The role of InsP\textsubscript{3}R Ca\textsuperscript{2+} sensor

When modulation of different InsP\textsubscript{3}R isoforms by Ca\textsuperscript{2+} was compared side-by-side in standard recording conditions (2 \mu M InsP\textsubscript{3} and 0.5 mM ATP), we discovered that InsP\textsubscript{3}R3 are most sensitive to modulation by Ca\textsuperscript{2+} (peak at 107 nM Ca\textsuperscript{2+}), followed by InsP\textsubscript{3}R2 (peak at 154 nM Ca\textsuperscript{2+}), and then by InsP\textsubscript{3}R1 (peak at 257 nM Ca\textsuperscript{2+}) (Fig. 1; Table 1). High sensitivity to activation by Ca\textsuperscript{2+} observed for InsP\textsubscript{3}R2 is consistent with the previous Ca\textsuperscript{2+} flux studies in DT40 cells (Miyakawa et al., 1999) and with the single-channel recordings of native and recombinant InsP\textsubscript{3}R2 (Ramos-Franco et al., 2000, 1998). High sensitivity to activation by Ca\textsuperscript{2+} observed for InsP\textsubscript{3}R3 is also consistent with previous Ca\textsuperscript{2+} flux studies in DT40 cells (Miyakawa et al., 1999) and with single-channel recordings of recombinant InsP\textsubscript{3}R3 expressed in Xenopus oocytes (Mak et al., 2001b).

In the previous studies (Miyakawa et al., 2001; Tu et al., 2003) we identified a putative Ca\textsuperscript{2+} sensor (Cas) region in the InsP\textsubscript{3}R1 sequence. The sequence of Cas region is highly conserved between InsP\textsubscript{3}R isoforms (Fig. 4), including a conserved glutamate residue (E2100 in InsP\textsubscript{3}R1), the importance of which we established previously (Miyakawa et al., 2001; Tu et al., 2003). Are small variations in Cas sequence (Fig. 4) sufficient to explain different Ca\textsuperscript{2+} sensitivity of InsP\textsubscript{3}R isoforms? To answer this question, we expressed RT1-Cas, RT2-Cas, and RT3-Cas proteins in
bacteria (Fig. 3) and performed a series of intrinsic tryptophan fluorescence measurements at different Ca\(^{2+}\) concentrations by following a method described previously for RT1-Cas (Tu et al., 2003). Importantly, the W2255 and W2267 residues of InsP3R1 present within the RT1-Cas region (Fig. 4, open arrows) are conserved in InsP3R2 and InsP3R3. For all three RT-Cas proteins, the intensity of the intrinsic tryptophan fluorescence signal was quenched as a function of Ca\(^{2+}\) (Fig. 5), consistent with Ca\(^{2+}\)-induced conformational change. By fitting the obtained results we determined that the RT1-Cas apparent affinity for Ca\(^{2+}\) (\(K_{Ca}\)) is equal to 0.23 \(\mu\)M, RT2-Cas apparent affinity for Ca\(^{2+}\) is equal to 0.16 \(\mu\)M, and RT3-Cas apparent affinity for Ca\(^{2+}\) is equal to 0.10 \(\mu\)M (Fig. 6; Table 1). Thus, small variations in Cas sequence between different InsP3R isoforms (Fig. 4) do appear to be sufficient to result in different affinities for Ca\(^{2+}\) (Fig. 6). Future mutagenesis of the RT-Cas region or solution of the RT-Cas structure will be required to identify structural determinants that form a Ca\(^{2+}\)-binding site within the RT-Cas region.

Ca\(^{2+}\) binding affinities of isolated RT-Cas regions are in quantitative agreement with the sensitivity of corresponding InsP3R isoforms to the Ca\(^{2+}\) modulation determined in planar lipid bilayer experiments (Table 1). Are different RT-Cas affinities for Ca\(^{2+}\) sufficient to explain different Ca\(^{2+}\) sensitivity of InsP3R isoforms? To answer this question, we swapped Cas regions between the InsP3R1 and InsP3R3 isoforms and generated baculoviruses encoding RT1-V-3 and RT3-V-1 chimeras (Fig. 7). Single-channel analysis revealed that conductance and gating properties of RT1-V-3 and RT3-V-1 chimeras are similar to the properties of “parental channels” (Figs. 8 and 9). Thus, swapping InsP3R1 and InsP3R3 Cas regions did not induce gross abnormalities in InsP3R gating and conductance properties. The low-affinity ATP-binding site (ATPB) is contained within the Cas region (Fig. 4). Thus, RT1-V-3 chimera contains the ATPA site from InsP3R1 and the ATPB site from InsP3R3 (Fig. 7 A), and RT3-V-1 chimera contains the ATPB site from InsP3R1 (Fig. 7 A). Consistent with the predominant role played by the ATPA site in modulating InsP3R1 gating by ATP (Maes et al., 2001; Tu et al., 2002), RT1-V-3 chimera displayed ATP sensitivity similar to InsP3R1 (Fig. 10 A) and RT3-V-1 chimera displayed ATP sensitivity similar to InsP3R3 (Fig. 10 B).

Consistent with “Ca\(^{2+}\) sensor hypothesis,” we found that swapping Cas regions was sufficient to exchange Ca\(^{2+}\) sensitivities of InsP3R1 and InsP3R3 isoforms (Fig. 11, A and B; Table 1). Interestingly, both activating and inhibitory parts of bell-shaped Ca\(^{2+}\) dependence were affected by Cas domain swap in our experiments with RT1-V-3 and RT3-V-1 chimeras (Fig. 11, A and B). These results are consistent with our previous analysis of RT1-E2100 point mutants (Tu et al., 2003). As discussed in Tu et al. (2003), these data can be explained if Cas region forms a part of both Ca\(^{2+}\)-activating and Ca\(^{2+}\)-inhibitory sites, or by sequential Ca\(^{2+}\)-binding to activating and inhibitory sites of the InsP3R. In our experiments we found that the Ca\(^{2+}\) sensitivity of RT1-V-3 chimera closely resembles Ca\(^{2+}\) sensitivity of RT3 (Fig. 11 A), and the Ca\(^{2+}\) sensitivity of RT3-V-1 chimera closely matches Ca\(^{2+}\) sensitivity of RT1 (Fig. 11 B). Moreover, at 5 mM ATP RT1-V-3 chimera displayed “square-shaped” Ca\(^{2+}\) dependence similar to RT3, whereas RT3-V-1 chimera displayed “narrow” Ca\(^{2+}\) dependence similar to RT1 (Fig. 12). These results indicate that the features responsible for ATP-dependent transition from “narrow” to “square” Ca\(^{2+}\) dependence are uniquely encoded within RT3-Cas sequence.

Our results with RT1-V-3 and RT3-V-1 chimeras are in contrast to recent analysis of InsP3R1/2 and InsP3R2/1 coupling domain swap chimeras (Ramos et al., 2003). In the study of Ramos et al. (2003) Ca\(^{2+}\) sensitivity of 1-2-1 and 2-1-2 chimeras was largely lost and did not resemble Ca\(^{2+}\) sensitivity of either “parental” InsP3R (type 1 or type 2). Most likely explanation for the differences between our results and the results of Ramos et al. (2003) is related to the choice of boundaries for “domain swap”. In our RT1-V-3 and RT3-V-1 constructs we choose domain swap boundaries (E1932 in InsP3R1 and M1835 in InsP3R3, Figs. 4 and 7 A) from the limited trypsin digestion pattern of InsP3R1 (Yoshikawa et al., 1999), which also corresponds to the region of sequence divergence between InsP3R isoforms. In contrast, Ramos et al. (2003) choose the most conserved regions of the InsP3R1 and InsP3R2 sequences as boundaries for the coupling domain swap in 1-2-1 and 2-1-2 chimeras. Indeed, we obtained functional results similar to that of Ramos et al. (2003) with several other InsP3R3/1 chimeric constructs with domain swap boundaries chosen in conserved regions of InsP3R1 and InsP3R3 sequences (data not shown).

**Common and unique properties of mammalian InsP3R isoforms**

In summary, data presented in this and the accompanying article (Tu et al., 2005) as well as the majority of the previously published reports indicate that all three mammalian InsP3R isoforms share common gating and conductance properties and display bell-shaped sensitivity to Ca\(^{2+}\) in a physiological range of Ca\(^{2+}\) concentrations (pCa 8–5). When compared to each other, the InsP3R1 is a medium InsP3-affinity, high ATP-affinity (no cooperativity), and low Ca\(^{2+}\)-affinity isoform; the InsP3R2 is a high InsP3-affinity, ATP-independent, medium Ca\(^{2+}\)-affinity isoform; the InsP3R3 is a low InsP3-affinity, low ATP-affinity (high cooperativity), and high Ca\(^{2+}\)-affinity isoform. Interestingly, saturation of the ATP binding site of InsP3R3 (but not InsP3R1) reduces apparent affinity of the Ca\(^{2+}\)-inhibitory site by 200-fold and converts “narrow” bell-shaped Ca\(^{2+}\) dependence of InsP3R3 (Fig. 1) to “square” Ca\(^{2+}\) dependence (Fig. 2). It appears that high affinity of InsP3R2 for...
InsP₃ is encoded within the amino-terminal ligand-binding domain (Sudhof et al., 1991), high affinity of InsP₃R₁ for ATP is due to the high-affinity ATPA binding site in the InsP₃R₁ sequence (Fig. 10; also Maes et al., 2001; Tu et al., 2002), and the differences in Ca²⁺ sensitivity are encoded within a sequence of Ca²⁺ sensor (Cas) region (Figs. 4, 6, 11, and 12). These conclusions will be useful for understanding the mechanisms of InsP₃R function and for analysis of Ca²⁺ signals supported by various InsP₃R subtypes expressed in cells and tissues.

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