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An inositol 1,4,5-trisphosphate receptor-dependent cation entry pathway in DT40 B lymphocytes

Guillermo Vazquez, Barbara J.Wedel, Gary St J.Bird, Suresh K.Joseph[1] and James W.Putney,Jr[2]

Calcium Regulation Section, Laboratory of Signal Transduction, National Institute of Environmental Health Sciences—NIH, PO Box 12233, 111 TW Alexander Drive, Research Triangle Park, NC 27709 and [1]Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19103, USA

[2]Corresponding author
e-mail: putney@niehs.nih.gov

We examined the roles of inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R) in calcium signaling using DT40 B lymphocytes, and a variant lacking the three IP_3R isoforms (IP_3R-KO). In wild-type cells, B cell receptor (BCR) stimulation activates a cation entry route that exhibits significantly greater permeability to Ba^{2+} than does capacitative calcium entry. This cation entry is absent in IP_3R-KO cells. Expression of the type-3 IP_3R (IP_3R-3) in the IP_3R-KO cells rescued not only agonist-dependent release of intracellular Ca^{2+}, but also Ba^{2+} influx following receptor stimulation. Similar results were obtained with an IP_3R-3 mutant carrying a conservative point mutation in the selectivity filter region of the channel (D2477E); however, an IP_3R-3 mutant in which this same aspartate was replaced by alanine (D2477A) failed to restore either BCR-induced Ca^{2+} release or receptor-dependent Ba^{2+} entry. These results suggest that in DT40 B lymphocytes, BCR stimulation activates a novel cation entry across the plasma membrane that depends upon, or is mediated by, fully functional IP_3R.

Keywords: B lymphocyte/calcium channels/inositol trisphosphate receptor/phospholipase C/plasma membrane

Introduction

Activation of the phospholipase C pathway in non-excitable cells results in the generation of inositol 1,4,5-trisphosphate (IP_3), which stimulates the release of Ca^{2+} store in the endoplasmic reticulum (Berridge, 1993). This release of Ca^{2+} is generally associated with an increase in Ca^{2+} entry across the plasma membrane that can serve to replenish stores or to contribute to Ca^{2+}-dependent signaling. In the majority of instances, this entry of Ca^{2+} appears to be signaled through a poorly understood mechanism that is initiated by depletion of the Ca^{2+} stores (Putney, 1986), a process known as capacitative calcium entry (CCE) or store-operated calcium entry (Putney, 1997a).

Non-CCE mechanisms have been described in a variety of cell types (Fasolato *et al*., 1993; Byron and Taylor, 1995; Shuttleworth and Thompson, 1996; Carroll and Peralta, 1998; Broad *et al*., 1999). The mechanisms controlling Ca^{2+} channel activation in such instances are not known, but may involve other second messengers, for example arachidonic acid. One second messenger that has been considered as a signal for activating plasma membrane Ca^{2+} entry is IP_3 acting on plasma membrane IP_3 receptors (IP_3R). In fact, a number of studies have provided biochemical evidence for the presence of IP_3R in the plasma membrane of cells (Guillemette *et al*., 1988; Fujimoto *et al*., 1992; Khan *et al*., 1992a,b, 1996; Feng and Kraus-Friedmann, 1993; Bush *et al*., 1994; Mayrleitner *et al*., 1995; Quinton and Dean, 1996; El-Daher *et al*., 2000; Tanimura *et al*., 2000). However, there is little physiological evidence that these receptors play a significant role in Ca^{2+} signaling at the plasma membrane. The one notable exception is vertebrate and invertebrate olfactory neurons, for which there is considerable functional evidence for the presence of plasma membrane IP_3-gated channels (Cunningham *et al*., 1993; Fadool and Ache, 1994; Okada *et al*., 1994; Kashiwayanagi, 1996; Lischka *et al*., 1999; Munger *et al*., 2000). A role for the IP_3R type-3 (IP_3R-3) has been proposed as responsible for capacitative Ca^{2+} entry in some cell types (Putney, 1997b), but it is clear this cannot be the case in all cell types (Wang *et al*., 2001).

Both T and B lymphocytes have been shown, by immunostaining, to possess plasma membrane IP_3R, and these receptors have been suggested to play a role in apoptosis (Khan *et al*., 1992a,b, 1996). We have investigated the role of IP_3R in plasma membrane calcium fluxes, utilizing the DT40 B lymphocyte cell line. This is an excellent model for investigating the function of IP_3R in B cell signaling because of the availability of a mutant DT40 line lacking all three IP_3R isoforms (Sugawara *et al*., 1997). DT40 is a chicken B cell line expressing an IgM isotype B cell receptor (BCR) in the plasma membrane (Baba *et al*., 1985) that is linked to intracellular Ca^{2+} ($[Ca^{2+}]_i$) signaling through phospholipase Cγ (PLCγ) activation (Buerstedde and Takeda, 1991; Sugawara *et al*., 1997). PLCγ activation results in conversion of phosphatidylinositol 4,5-bisphosphate into IP_3, which then releases Ca^{2+} from thapsigargin-sensitive intracellular stores by interacting with specific IP_3R in the endoplasmic reticulum (Berridge, 1993). This release results in transient elevation of cytosolic calcium concentration and is followed by a more sustained Ca^{2+} entry from the outside, which is mediated, at least in part, by the store-operated, CCE pathway (Putney, 1986; Berridge, 1995; Parekh and Penner, 1997; Barritt, 1999; Sugawara *et al*., 1997). In the present work we demonstrate that there is also a non-capacitative pathway for calcium entry in DT40 B lymphocytes, activated through the BCR. This entry pathway, while not mediated by store depletion, requires a

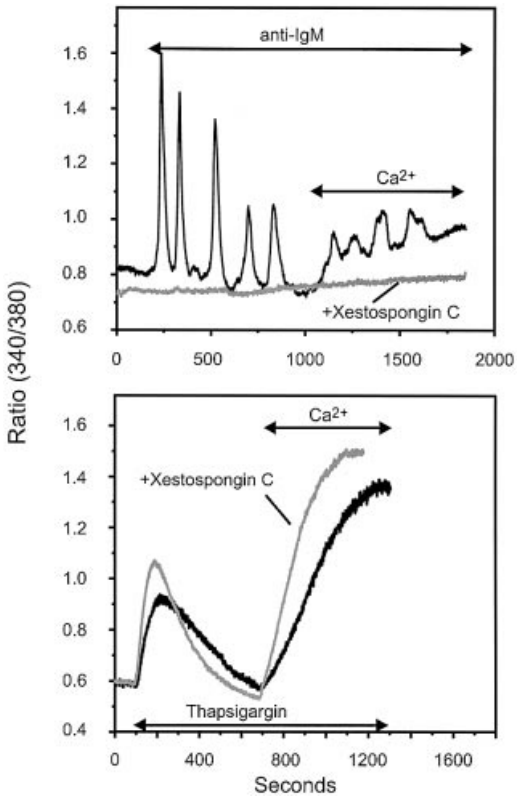

Fig. 1. The IP_3R inhibitor xestospongin C effectively suppresses both BCR-induced Ca^{2+} release and calcium entry in DT40 cells B lymphocytes, but does not affect thapsigargin-induced Ca^{2+} release or entry. DT40 cells were incubated for 45 min in the presence of 25 μM xestospongin C. Cells were then bathed in nominally Ca^{2+}-free medium, exposed to 5 μg/ml anti-IgM antibody (top) or 2 μM thapsigargin (bottom), as indicated. Ca^{2+} (1.5 mM) was added where indicated. In this experiment, relatively higher ratio values were obtained as compared with other experiments due to the use of a different Ca^{2+} measuring system; in this series (and for experiments in Figure 3), a photon counting system was used, while in the other series, an imaging system was used. Black trace: control (no xestospongin C); gray trace, 25 μM xestospongin C. Shown are representative traces from three independent experiments.

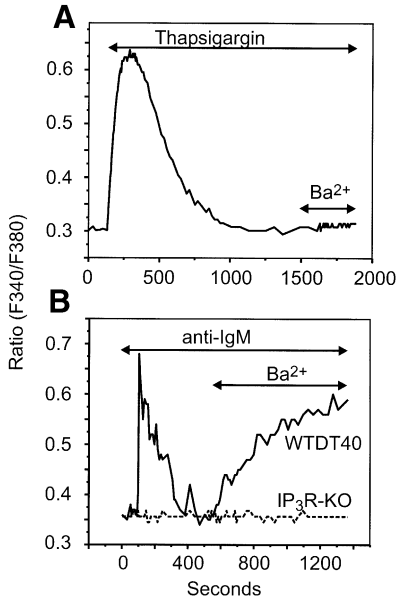

Fig. 2. BCR activation, but not store depletion, induces Ba^{2+} entry in DT40 B lymphocytes. (**A**) Fura-2-loaded WTDT40 cells were incubated in nominally Ca^{2+}-free medium and then exposed to 2 μM thapsigargin in order to deplete intracellular Ca^{2+} stores. After cytosolic Ca^{2+} returned to basal levels, Ba^{2+} (10 mM) was added to the medium. A representative trace from at least five independent experiments is shown. (**B**) Wild-type (WTDT40) or IP_3R-KO (broken line) DT40 cells were maintained in nominally Ca^{2+}-free medium, exposed to 5 μg/ml anti-IgM antibody, and then Ba^{2+} (10 mM) was added where indicated. Representative traces from at least five independent experiments are shown.

functional IP_3R. In light of previous work providing biochemical evidence for IP_3R in the plasma membrane of B cells and other cell types, we suggest that this entry of divalent cations may result from direct gating of plasma membrane IP_3R by IP_3.

Results

As shown in Figure 1, activation of the BCR in DT40 B lymphocytes, in the absence of extracellular Ca^{2+}, results in an oscillatory $[Ca^{2+}]_i$ signal, presumably due to cyclical release of Ca^{2+} from intracellular stores. In the current study, Ca^{2+} responses to BCR activation were observed in 60–70% of cells tested (n = 195). Restoration of extracellular Ca^{2+} to the responsive cells revealed a stimulated entry of Ca^{2+} in 80% of the BCR-responsive cells. Both the release and entry of Ca^{2+} appear to depend on IP_3R in some manner, because both phases of the response are lost in cells lacking IP_3R (Sugawara *et al*., 1997), and both phases are completely blocked by the potent membrane permeant IP_3R inhibitor, xestospongin C (Gafni *et al*., 1997; Figure 1, top). Release of Ca^{2+} by the SERCA inhibitor, thapsigargin, and the resulting CCE, were not inhibited by xestospongin C (Figure 1, bottom).

The function of IP_3R in the Ca^{2+} entry phase could be due to their role in the release of store Ca^{2+} and activation of store-operated channels, or alternatively there may be some other, distinct mechanism for regulation of Ca^{2+} by IP_3R. If the latter were the case, then one prediction is that BCR stimulation should activate an entry in addition to that, dependent solely on Ca^{2+} store depletion. Furthermore, this response should be absolutely dependent on the presence of IP_3R. This appears to be the case, as shown by results in Figure 2. In these experiments, we took advantage of the fact that under our experimental conditions, the store-operated Ca^{2+} entry pathway involves either no detectable Ba^{2+} entry or, in some experiments, a small Ba^{2+} entry (Vazquez *et al*., 2001; data not shown; Figure 2A). However, unlike thapsigargin-activated CCE, BCR stimulation results in a substantial entry of Ba^{2+} entry (Figure 2B). This clearly indicates that BCR-dependent cation entry involves not only store-operated channels, but also a population of somewhat less selective cation channels requiring signals other than, or in addition to, store depletion.

In experiments utilizing Ba^{2+} to assess entry, 102 of 127 anti-IgM responsive cells showed Ba^{2+} entry. Note that in a previous publication, Venkatachalam *et al*. (2001) failed to observe agonist-activated Ba^{2+} entry. This difference is most likely due to the fact that Venkatachalam and colleagues used 1 mM Ba^{2+}, while in our experiments the concentration of Ba^{2+} was 10 mM.

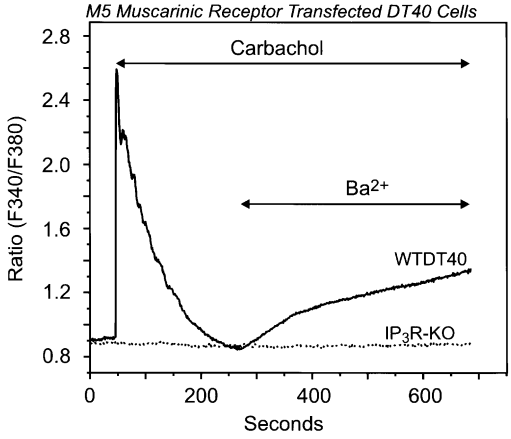

Fig. 3. Activation of the G-protein-coupled M5 muscarinic receptor results in stimulation of Ba^{2+} entry in wild-type but not in IP_3R-KO DT40 cells. Ba^{2+} influx was measured in single Fura-2-loaded wild-type (WTDT40) or IP_3R-KO (dotted line) DT40 cells transfected with the human M5 muscarinic receptor. The cells were maintained in nominally Ca^{2+}-free medium, exposed to 100 μM carbachol, and then Ba^{2+} (10 mM) was added where indicated. The relatively higher ratio values in this experiment are due to the use of a different Ca^{2+} measuring system; in this series, a photon counting system was used, while in the other series, an imaging system was used. Representative traces from three independent experiments are shown.

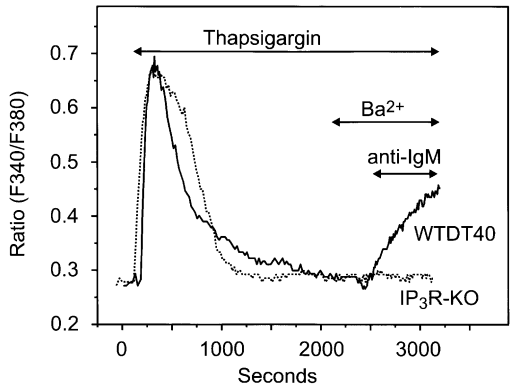

Fig. 4. BCR-dependent activation of Ba^{2+} entry in wild-type but not in IP_3R-KO DT40 cells. Fura-2-loaded wild-type (WTDT40) and IP_3R-KO DT40 cells were incubated in nominally Ca^{2+}-free medium and then exposed to 2 μM thapsigargin. After complete store depletion, Ba^{2+} (10 mM) was added to the medium where indicated, and 2 min later anti-IgM (5 μg/ml) was added. Representative traces from three independent experiments are shown.

The failure to observe cation entry in all anti-IgM-responsive cells could result from rapid desensitization of the receptor–phospholipase C pathway. In fact, desensitization of the B cell antigen receptor may occur with as few as 5% of the receptors occupied, resulting in blockade of the signaling pathways derived from receptor activation by a mechanism apparently involving a short-term action of PKC (Vilen *et al*., 1997). There was no detectable Ba^{2+} entry in those cells unable to respond to BCR crosslinking (68 of 68 cells). Importantly, the receptor-dependent Ba^{2+} entry was never observed in IP_3R knockout cells (IP_3R-KO, a variant lacking the three IP_3R isoforms, IP_3R-1, -2 and -3) (Figure 2B), indicating that the receptor-activated cation influx is dependent on the presence of IP_3R rather than depletion of Ca^{2+} stores.

In the remaining experiments, we took advantage of the apparent higher Ca^{2+} selectivity of the endogenous store-operated channels in order to assess BCR-dependent cation (Ba^{2+}) entry into the cells without contribution from receptor activated endogenous CCE channels (see for example Vazquez *et al*., 2001). In addition, as Ba^{2+} is a poor substrate for endoplasmic reticulum or plasma membrane calcium pumps (Vanderkooi and Martonosi, 1971; Yamaguchi *et al*., 1989) and thus is not readily cleared from the cytoplasm, it provides a reliable way to monitor unidirectional cation entry that avoids potential complications derived from changes in cellular Ca^{2+} metabolism or Ca^{2+}-dependent regulation of the cation channels (Byron and Taylor, 1995).

The failure of BCR stimulation to activate entry in IP_3R-KO cells indicates the importance of the IP_3R in this response, but does not rule out contributions of other elements of the BCR signaling pathway. Thus, we next transiently transfected DT40 cells with the human M5 muscarinic receptor, which would activate a different PLC, PLCβ, through a G-protein rather than tyrosine kinase-dependent mechanism. As shown in Figure 3, carbachol stimulation of the M5-expressing cells resulted in a rapid and transient cytosolic calcium rise (12 of 17 $EYFP^+$ cells), which rapidly declined to basal levels in ~2 min; addition of Ba^{2+} to the external medium resulted in significant cation entry, which was not observed in those cells unable to respond to muscarinic stimulation. Again, this receptor-dependent Ba^{2+} entry was never observed in IP_3R-KO cells transfected with the M5 receptor. These results indicate that the cation entry route following receptor activation was related to IP_3 production and IP_3R expression, but not linked to the type of receptor eliciting the signal.

We next considered the possibility that activation of Ba^{2+} entry following BCR crosslinking could be the consequence of activation of Ca^{2+}-activated non-selective cation channels due to the transient cytosolic Ca^{2+} rise that follows IP_3-mediated Ca^{2+} mobilization from inner stores. In order to demonstrate clearly the dependence of the Ba^{2+} entry on BCR stimulation, we utilized the following protocol. Both wild-type (WTDT40) and IP_3R-KO cells were treated with thapsigargin to discharge intracellular stores completely. Ba^{2+} was then added and, as shown previously, no detectable entry of Ba^{2+} occurred. Subsequently, anti-IgM was added, and in the WTDT40, but not in the IP_3R-KO cells, Ba^{2+} entry occurred almost immediately (Figure 4). Interestingly, with this protocol a greater proportion of the cells (67 of 71) exhibited significant cation entry upon BCR stimulation, probably due to the fact that Ba^{2+} entry is triggered almost as soon as the receptor pathway becomes activated, thus minimizing any possible desensitization of the receptor signaling route.

One interesting point that can be taken from the protocol shown in Figure 4 is that the delay in Ba^{2+} entry is noticeably briefer than that for release following BCR activation. The average delay for the increase in Ba^{2+} entry after anti-IgM addition from the experiments in Figure 4 was 18 ± 10 s, while the delay for release from various protocols varied from 40 to 60 s. This is perhaps not surprising, if the entry is due to IP_3 formed and acting at

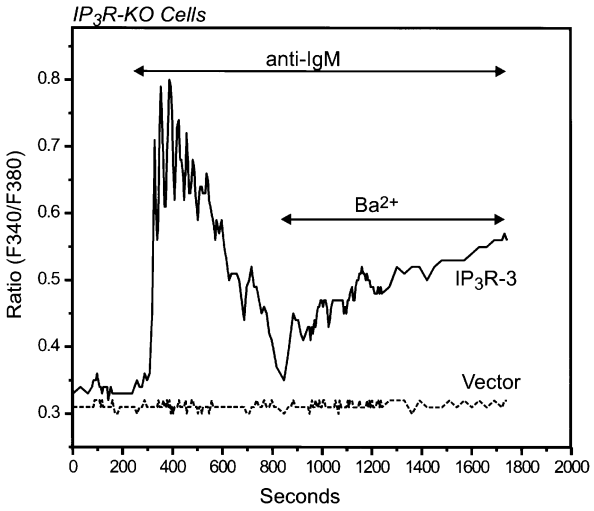

Fig. 5. Transient expression of an IP_3R restores agonist-induced Ba^{2+} entry in IP_3R-KO DT40 cells. Ba^{2+} influx was measured in Fura-2-loaded IP_3R-KO DT40 cells transfected with either the rat IP_3R-3 or its vector (Mock). The cells were maintained in nominally Ca^{2+}-free medium, exposed to 5 μg/ml anti-IgM antibody, and then Ba^{2+} (10 mM) was added where indicated. Representative traces from three independent experiments are shown.

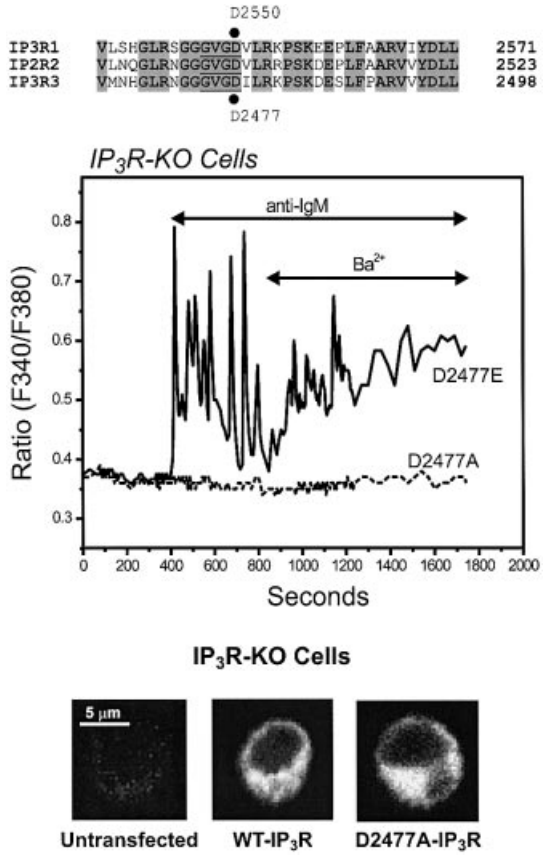

Fig. 6. Effect of single point mutations within the putative pore forming region of IP_3R-3 on restoration of agonist-induced Ba^{2+} entry in IP_3R-KO DT40 cells. The top of the figure shows an alignment of a region including the predicted pore of rat IP_3R-1–3 (the putative selectivity filter sequence is underlined). D2477 of rat IP_3R-3, which was mutated into glutamate (D2477E) or alanine (D2477A), corresponds to D2550 of rat IP_3R-1. In the experiment shown below, Ba^{2+} influx was measured in Fura-2-loaded IP_3R-KO DT40 cells transfected with either the D2477E (solid line) or the D2477A (broken line) mutants of the rat IP_3R-3 (see Materials and methods for details on the mutations). The cells were maintained in nominally Ca^{2+}-free medium, exposed to 5 μg/ml anti-IgM antibody, and then Ba^{2+} (10 mM) was added where indicated. Representative traces from three independent experiments are shown. At the bottom, immunolocalization of transiently expressed rat IP_3R-3 ($pCB6^+$ vector) in DT40 B cells is shown. IP_3R-KO cells untransfected, or transfected with the wild-type rat IP_3R-3 (WT-IP_3R), or the rat IP_3R-3 mutant D2477A (D2477A-IP_3R; see text for details) were incubated with a mouse anti-IP_3R-3 monoclonal antibody and then with the secondary Alexa Fluor 488-labeled anti-mouse IgG antibody. The fluorescence images were acquired with a Zeiss 410 confocal microscope.

the plasma membrane, while release results from an action of IP_3 at the endoplasmic reticulum.

To confirm that it is the IP_3R that is necessary for the BCR-dependent Ba^{2+} entry in DT40 cells, we attempted to restore this entry mechanism in IP_3R-KO cells by transient expression of the rat IP_3R-3. As predicted, stimulation of BCR signaling in IP_3R-3-expressing IP_3R-KO cells resulted not only in reappearance of the BCR-dependent, IP_3-mediated release of stored Ca^{2+}, but also in a significant Ba^{2+} entry following receptor activation (21 of 26 IgM-responsive $EYFP^+$ cells; Figure 5). Similarly, carbachol stimulation of IP_3R-KO cells transiently co-transfected with the M5 muscarinic receptor and the rat IP_3R-3 resulted in rapid release of calcium from stores followed by activation of the Ba^{2+} entry route in response to carbachol (data not shown). Neither the transient Ca^{2+} release nor the Ba^{2+} entry was detected in mock-transfected IP_3R-KO cells. These results indicate that in DT40 B lymphocytes, activation of the BCR signaling pathway results in generation of IP_3, which in turn activates not only the endoplasmic reticulum-located IP_3R that cause Ca^{2+} release from endogenous stores, but also a cation entry pathway in the plasma membrane strongly linked to IP_3R expression. Unlike the highly selective CRAC channels, the IP_3R is a relatively less selective cation channel more permeable to Ba^{2+} than to Ca^{2+} (Bezprozvanny and Ehrlich, 1994, 1995). Thus, the simplest explanation for this IP_3R-dependent entry is that it represents cation flux through plasma membrane IP_3R. Although this would appear to be the most straightforward explanation for the current observations, we cannot rule out an indirect regulation of a plasma membrane channel by IP_3R, as in the conformational coupling mechanism for TRP or store-operated channels (Irvine, 1990; Berridge, 1995; Kiselyov *et al*., 1998). In an attempt to resolve this issue we transiently expressed two different pore mutants of the IP_3R-3 into IP_3R-KO cells and tested their abilities to restore BCR stimulation of the Ba^{2+} entry pathway. It has recently been shown that the aspartic acid residue at position 2550 of the rat IP_3R-1 plays a critical role in cation permeation through the channel (Boehning and Joseph, 2000). This amino acid is located within a highly conserved sequence in the IP_3R (GVGD) that is homologous to the selectivity filter in potassium channels (Doyle *et al*., 1998; see diagram in Figure 6). Replacing this aspartate by alanine (D2550A) results in an IP_3R that is still able to bind IP_3 but devoid of Ca^{2+} release activity, i.e. rendering an impermeable channel. A conservative mutation in which the aspartate is replaced by a glutamic acid (D2550E) fully preserves the IP_3-induced Ca^{2+} release activity of the channel. These mutations do not

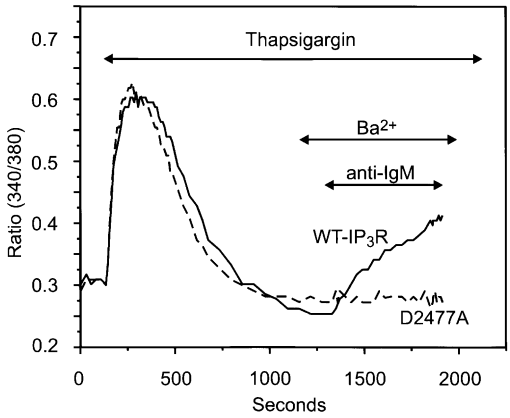

Fig. 7. The D2477A mutant of the rat IP_3R-3 does not restore BCR-dependent activation of Ba^{2+} entry in Ca^{2+}-depleted, IP_3R-KO DT40 cells. Fura-2-loaded IP_3R-KO DT40 cells transfected with either the wild type or the D2477A mutant of the rat IP_3R-3 were incubated in nominally Ca^{2+}-free medium and then exposed to 2 μM thapsigargin. After complete store depletion, Ba^{2+} (10 mM) was added to the medium where indicated, and 2 min later anti-IgM (5 μg/ml) was added. Solid line, wild-type IP_3R; dashed line, D2477A IP_3R. Representative traces from three independent experiments are shown.

alter the ability of the channels to express at high levels or to form oligomers (Boehning and Joseph, 2000). Unfortunately, we have not as yet successfully expressed the rat IP_3R-1 in DT40 IP_3R-KO cells. Thus, we engineered these same two point mutations in the equivalent aspartate residue at position 2477 in the rat IP_3R-3 (mutants D2477A and D2477E, respectively). As shown in Figure 6, the D2477A mutant, when expressed in IP_3R-KO cells, failed to restore either BCR-induced Ca^{2+} release or receptor-dependent Ba^{2+} entry. Heterologous expression of the D2477A IP_3R-3 mutant was confirmed by immunocytochemistry (Figure 6), and the extent of expression and localization of the mutant appeared to be identical to that of the wild-type IP_3R-3. Unfortunately, because of the small size of DT40 and the expression of IP_3R-3 on endoplasmic reticulum through the non-nuclear regions, we were not able to definitively localize the receptor to the plasma membrane by immunocytochemistry.

Because we cannot be certain that depletion of stores is not required for activation of this pathway, we also tested the D2477A mutant in a protocol in which Ca^{2+} stores were first depleted by thapsigargin; again, no BCR stimulation of Ba^{2+} entry was observed (Figure 7). The inability of this mutant to restore receptor-stimulated, IP_3-mediated release of Ca^{2+} from intracellular stores is consistent with the expectation that replacement of the D2477 within the predicted selectivity region of the channel by a non-negatively charged amino acid would abrogate cation permeation through the channel pore. However, the D2477E mutant, in which the negative charge is preserved by replacing aspartate with glutamate, fully retained the phenotype exhibited by IP_3R-KO cells transiently expressing the wild-type IP_3R-3. Mutant or truncated IP_3R lacking functional pores are known to be fully capable of activating plasma membrane channels through the conformational coupling mechanism (Kiselyov *et al*., 1998, 1999). Thus, if the BCR-activated Ba^{2+}-permeable pathway were mediated by plasma membrane cation channels conformationally coupled to IP_3R, then the D2477A IP_3R-3 mutant would be expected to gate the coupled Ba^{2+}-permeable receptor-activated channel in the plasma membrane despite its inability to release calcium from the stores. However, only the D2477E mutant, with intact gating and permeation properties, was able to restore both the release and the cation entry pathways driven by BCR stimulation.

Discussion

WTDT40 B lymphocytes co-express three subtypes of IP_3R: IP_3R-1, -2 and -3 (Sugawara *et al*., 1997), which functionally interact to produce the oscillatory Ca^{2+} pattern observed with BCR activation by antigen- or antibody-dependent crosslinking (Miyakawa *et al*., 1999). A variant of these cells has been generated in which the genes coding for the three IP_3R isoforms have been disrupted by homologous recombination (Sugawara *et al*., 1997). Such IP_3R-KO cells respond to PLC-coupled receptors with generation of IP_3, but they do not generate PLC-linked cytosolic Ca^{2+} signals. It has been shown previously that store-operated Ca^{2+} entry occurs normally in both wild-type and IP_3R-KO DT40 cells following passive depletion of endogenous stores with thapsigargin (Sugawara *et al*., 1997; Broad *et al*., 2001; Ma *et al*., 2001). This endogenous store-operated pathway is highly selective for Ca^{2+} and exhibits minimal permeability to Ba^{2+} or Sr^{2+} (Figure 2A; see also Vazquez *et al*., 2001; Venkatachalam *et al*., 2001). Consistent with this conclusion, which is based on fluorescence measurements, Prakriya and Lewis have recently demonstrated that the store-operated entry in DT40 involves the highly selective calcium current, I_{crac} (Prakriya and Lewis, 2001).

The results of the current study demonstrate the presence of a novel mechanism for BCR-activation of divalent cation entry across the plasma membrane of B cells that requires fully functional IP_3R, and apparently does not involve a conformational coupling mechanism. The most likely scenario is that in DT40 B lymphocytes, stimulation of the BCR-associated signaling machinery results in generation of IP_3, which, in addition to inducing calcium release from stores through activation of endoplasmic reticulum-located IP_3R, also activates plasma membrane located IP_3R. Ca^{2+} entry through this pathway may contribute to BCR-mediated Ca^{2+} signaling, or, possibly, cation fluxes may modify store-operated Ca^{2+} entry in other ways. For example, if substantial cation fluxes were to result in membrane depolarization, then store-operated entry might actually be inhibited. This would be consistent with the observation of Hashimoto *et al*. (2001), who, using a membrane potential-sensitive dye, observed membrane depolarization and inhibition of CCE by BCR activation in DT40 cells. Thus, it would be useful to demonstrate the presence of an agonist-activated membrane current with properties expected of an IP_3R. However, probably due to the small size of this current, we have not as yet succeeded in identifying a non-capacitative whole-cell current activated by agonist in either WTDT40 or IP_3R-3 transfected IP_3R-KO cells.

Although not the major focus of this study, the experiments involving transfection of IP_3R-KO cells

with IP_3R-3 revealed interesting patterns of Ca^{2+} signaling in the release phase as well. Miyakawa *et al*. (1999) generated different combinations of single IP_3R-KO DT40 cells and evaluated the contribution of each of the IP_3R subtypes normally expressed in these cells (IP_3R-1, -2 and -3) to the oscillatory Ca^{2+} pattern observed upon BCR activation. Interestingly, those studies suggested that only mutants expressing the IP_3R-2 exhibited BCR-dependent Ca^{2+} oscillations comparable to the wild-type cells, whereas mutants expressing either IP_3R-1 or IP_3R-3 responded with a monophasic Ca^{2+} transient. In the present studies, we observed that transient expression of the rat IP_3R-3 in IP_3R-KO DT40 cells is sufficient to restore an oscillatory pattern for the Ca^{2+} response to BCR stimulation (26 of 42 $EYFP^+$ cells). As differences in the expression level of the IP_3R isoforms may affect the Ca^{2+} signaling pattern, it is possible that transient overexpression of the IP_3R-3 could account for such a discrepancy. Our observations are also inconsistent with the suggestion from Hagar *et al*. (1998) that cells expressing predominantly type 3 IP_3R would not support Ca^{2+} oscillations due to a lack of Ca^{2+}-dependent inhibition of the channel. In this context, the ability of the IP_3R-KO cells transiently and solely expressing the rat IP_3R-3 to respond to BCR stimulation with clearly defined cytosolic Ca^{2+} oscillations could be taken as indirect evidence for biphasic regulation of the IP_3R-3 upon cytoplasmic Ca^{2+} concentration; however, it is also possible that cyclical modulation of PLC activity could underlie these oscillations.

As discussed above, a number of publications have raised the issue of plasma membrane IP_3R, but, with the exception of invertebrate olfactory neurons, the significance of these observations is unclear (Fasolato *et al*., 1994). Confusion undoubtedly arises because in some instances, IP_3 appears to be capable of regulating store-operated channels (Vaca and Kunze, 1995; Kiselyov *et al*., 1998; Zubov *et al*., 1999); this probably represents a conformational coupling mechanism, rather than the presence of plasma membrane IP_3R. In the current study, however, the use of Ba^{2+} as a surrogate for Ca^{2+}, as well as findings presented in Figures 3–5, clearly demonstrate that DT40 B cells express a novel entry pathway that is distinct from the store-operated pathway, does not appear to involve conformational coupling and is dependent on the presence of a cation-permeable IP_3R. Taken together with previous immunological evidence for the presence of IP_3R in the B cell plasma membrane (Khan *et al*., 1996), it seems likely that this IP_3R-dependent pathway reflects Ba^{2+} entry through plasma membrane IP_3R cation channels.

Non-CCE mechanisms have been described in a variety of cell types (Fasolato *et al*., 1993; Byron and Taylor, 1995; Shuttleworth and Thompson, 1996; Carroll and Peralta, 1998; Broad *et al*., 1999). One of the hallmarks of non-capacitative pathways is their greater permeability of Ca^{2+} surrogates, as compared with Ca^{2+} (Byron and Taylor, 1995). In at least two different cell types, the receptor-activated, non-capacitative Ca^{2+} entry can be activated by arachidonic acid (Shuttleworth, 1997; Broad *et al*., 1999), leading to speculation that a receptor-regulated phospholipase A_2 may be involved. However, in HEK293 cells, we previously found (Luo *et al*., 2001) that the non-capacitative Ca^{2+} entry activated by low concentrations of muscarinic agonists was inhibited by 2-aminoethoxydiphenyl borane, a compound that inhibits both store-operated channels, as well as IP_3R (Maruyama *et al*., 1997; Braun *et al*., 2001; Broad *et al*., 2001; Dobrydneva and Blackmore, 2001; Gregory *et al*., 2001; Iwasaki *et al*., 2001; Ma *et al*., 2001; Prakriya and Lewis, 2001). It is thus tempting to speculate that plasma membrane IP_3R may underlie, or at least contribute to, non-capacitative Ca^{2+} entry in cell types other than the B lymphocyte. This issue should be the focus of investigation in future studies in a variety of Ca^{2+}-regulated systems.

Materials and methods

Cell culture, transfection and measurement of intracellular calcium

The immortalized chicken B lymphocyte cell line, DT40 (RIKEN Cell Bank No. RCB1464), and its mutant variant lacking all three IP_3R types (RIKEN Cell Bank No. RCB1467) were kindly provided by Dr Tomohiro Kurosaki (Department of Molecular Genetics, Kansai Medical University). Cell culture and handling for intracellular calcium measurements using the calcium-sensitive dye Fura-2 were as described previously (Vazquez *et al*., 2001).

DT40 cells were transiently transfected by electroporation (300 V, 500 μF) with either the rat wild-type IP_3R-3 in $pCB6^+$ (provided by Dr Graeme Bell, University of Chicago, Chicago, IL), its mutants (see below) or vector alone (mock-transfected cells), along with pEYFP-C1 (Clontech, Palo Alto, CA) as a transfection marker. In some experiments, cells were transfected with the human M5 muscarinic receptor (kindly provided by Dr Lutz Birnbaumer, National Institutes of Environmental and Health Sciences, Research Triangle Park, NC); the corresponding cDNA was subcloned into an expression vector (lcf201, provided by Drs Jean-Marie Buerstedde/Hiroshi Arakawa, Heinrich-Pette-Institute, University of Hamburg, Germany) under the control of the chicken β-actin promoter for expression in DT40 cells. Cells were assayed 18–30 h post-transfection. Fluorescence measurements were performed on $EYFP^+$ cells selected by their green fluorescence when excited at 485 nm. The fluorescence intensity of multiple Fura-2-loaded DT40 cells was monitored with a CCD camera-based imaging system (Universal Imaging) mounted on a Zeiss Axiovert 35 inverted microscope equipped with a Zeiss 40× (1.3 NA) fluor objective. A Sutter Instruments filter changer enabled alternative excitation at 340 and 380 nm, while the emission fluorescence was monitored at 510 nm with a Paultek Imaging camera (model PC-20) equipped with a GenIISys intensifier (Dage-MTI, Inc.). The images of multiple cells collected at each excitation wavelength were subsequently processed using the MetaFluor software (Universal Imaging Corp., West Chester, PA) to provide ratio images. In some experiments, responses of single cells were analyzed by utilizing a photomultiplier-based system, as described previously (Vazquez *et al*., 2001) (for example, Figures 1 and 3). Cells transfected with vector alone (mock-transfected cells) were used in parallel as controls. All experiments were performed at room temperature. The data are expressed as a ratio of Fura-2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm (F340/F380).

Mutagenesis

The cDNA of rat IP_3R-3 (Blondel *et al*., 1993) in $pCB6^+$ (kindly provided by Dr Graeme Bell) was used as a template for mutagenesis. D2477A and D2477E point mutations were generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutations were verified by DNA sequencing.

Immunocytochemistry for IP_3R-3

DT40 cells attached to coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% NP-40 in phosphate-buffered saline. Non-specific sites were blocked with 3% bovine serum albumin in TBST (Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature for 30 min, and the cells were then incubated with anti-IP_3R-3 monoclonal antibody (Transduction Laboratories, Lexington, KY) at 1:500 dilution. Secondary antibody was the Alexa fluor 488-labeled goat anti-mouse IgG antisera. The fluorescence images were acquired with a Zeiss LSM410 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY) using an argon–krypton laser and excitation at 488 nm

through a 40× (1.2 NA, water immersion) objective lens (optical slice thickness 1.5 μm).

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