Modification of phospholipase C-γ-induced Ca^{2+} signal generation by 2-aminoethoxydiphenyl borate

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The mechanisms by which Ca^{2+}-store-release channels and Ca^{2+}-entry channels are coupled to receptor activation are poorly understood. Modification of Ca^{2+} signals by 2-aminoethoxydiphenyl borate (2-APB), suggests the agent may target entry channels or the machinery controlling their activation. In DT40 B-cells and Jurkat T-cells, complete Ca^{2+} store release was induced by 2-APB (EC_{50} 10–20 μM). At 75 μM, 2-APB emptied stores completely in both lymphocyte lines, but had no such effect on other cells. In DT40 cells, 2-APB mimicked B-cell receptor (BCR) cross-linking, but no effect was observed in mutant DT40 lines devoid of inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) or phospholipase C-γ2 (PLC-γ2). Like the BCR, 2-APB activated transfected TRPC3 (canonical transient receptor potential) channels, which acted as sensors for PLC-γ2-generated diacylglycerol in DT40 cells. The action of 2-APB on $InsP_3Rs$ and TRPC3 channels was prevented by PLC-inhibition, and required PLC-γ2 catalytic activity. However, unlike BCR activation, no increased $InsP_3$ level could be measured in response to 2-APB. Also, calyculin A-induced cytoskeletal reorganization prevented 2-APB-induced $InsP_3R$ and TRPC3-channel activation, but not that induced by the BCR. 2-APB still activated TRPC3 channels in DT40 cells with fully depleted Ca^{2+} stores, indicating its action was not via Ca^{2+} release. Significantly, 2-APB-induced $InsP_3R$ and TRPC3 activation was prevented in DT40 knockout cells devoid of the BCR- and PLC-γ2-coupled adaptor/kinases, Syk, Lyn, Btk or BLNK. The results suggest that 2-APB activates Ca^{2+} signals in lymphocytes by initiating and enhancing coupling between components of the BCR–PLC-γ2 complex and both Ca^{2+}-entry and Ca^{2+}-release channels.

Key words: 2-aminoethoxydiphenyl borate, canonical transient receptor potential channel (TRPC channel), calcium signalling, inositol 1,4,5-trisphosphate receptor ($InsP_3R$), phospholipase C (PLC), protein kinase C (PKC).

INTRODUCTION

Cytosolic Ca^{2+} signals control a wide array of cellular functions ranging from short-term responses, such as contraction and secretion, to longer-term regulation of cell growth and proliferation [1]. Ca^{2+} signals generated in response to receptors are complex involving two closely coupled components: rapid, transient release of Ca^{2+} stored in the endoplasmic reticulum (ER), followed by slowly developing extracellular Ca^{2+} entry [1–4]. Receptors coupled to activation of either PLC (phospholipase C)-β or PLC-γ generate the two second messengers, $InsP_3$ (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). $InsP_3$ diffuses rapidly within the cytosol to interact with $InsP_3Rs$ ($InsP_3$ receptors) in the ER, which serve as Ca^{2+} channels, to release luminally stored Ca^{2+} and to generate the initial Ca^{2+} signal phase [1]. The resulting depletion of Ca^{2+} stored within the ER lumen serves as the primary trigger for a message, which is returned to the plasma membrane, resulting in the activation of store-operated channels (SOCs), which mediate capacitative Ca^{2+} entry [2–4]. The activation of SOCs is relatively slow (10–100 s) compared with the activation of channels such as voltage-sensitive Ca^{2+} channels [4]. This second Ca^{2+} entry phase of Ca^{2+} signals serves to mediate longer-term cytosolic Ca^{2+} elevations and provides a means to replenish intracellular stores [3,4]. In certain cell types, including haematopoietic cells, SOCs carry a highly Ca^{2+}-selective, non-voltage-dependent and inwardly rectifying current, termed the Ca^{2+}-release-activated Ca^{2+} current, or I_{CRAC} [2]. The mechanism of coupling of depleted ER Ca^{2+} stores to activate Ca^{2+} entry through SOCs remains a crucial, but unresolved, question.

In addition to SOCs, Ca^{2+}-entry channels can be activated by more direct receptor-induced mechanisms. Thus the other product of PLC activation, DAG, can activate certain members of the TRPC (canonical transient receptor potential) family of Ca^{2+}-conducting plasma membrane cation channels [4–7]. TRPC channels are expressed ubiquitously in vertebrate cells and are the products of seven genes encoding cation channels activated primarily in response to PLC-coupled receptors [4,8,9]. Evidence indicates that they can function as SOCs [3,4,10–12], and there are a number of studies suggesting that TRPC channels in the plasma membrane interact with intracellular $InsP_3Rs$, suggesting that they can receive information directly from Ca^{2+} stores [11,13–15]. However, there is also evidence that TRPC channels can function independently of stores [3,4,9,16]. In studies utilizing the triple $InsP_3R$-deficient variant of the DT40 chicken B-cell line ($InsP_3R^{-/-}$), SOCs operate identically with those in wild-type DT40 (DT40 WT) cells, suggesting that $InsP_3Rs$ are not essential for endogenous SOC activation [17–19]. Also, TRPC3 channels

Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borate; BCR, B-cell receptor; BLNK, B-cell linker; DAG, diacylglycerol; ER, endoplasmic reticulum; eYFP, enhanced yellow fluorescent protein; FBS, foetal bovine serum; fura-2/AM, fura-2 acetoxymethyl ester; I_{CRAC}, Ca^{2+}-release-activated Ca^{2+} current; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PH, pleckstrin homology; PIP_2, PtdIns(4,5)P_2; PIP_3, PtdIns(3,4,5)P_3; PKC, protein kinase C; PLC, phospholipase C; LIM, lipase-inactive mutant of PLC-γ2; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SOC, store-operated channel; TCR, T-cell receptor; TG, thapsigargin; TRPC, canonical transient receptor potential; WM, wortmannin; WT, wild-type.

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expressed in DT40 $InsP_3R^{-/-}$ cells can be activated by PLC-coupled receptors identically with TRPC3 channels expressed in DT40 WT cells [20,21]. Members of the closely related subgroup of TRPC3, TRPC6 and TRPC7 channels, can each be activated in response to DAG [5]. In DT40 cells, the activation of TRPC3 channels in response to BCR-induced PLC-γ stimulation is mimicked by exogenously added DAG [20,22], indicating that PLC-generated DAG is the activating agent. The DAG-mediated activation of TRPC channels is independent of DAG-induced PKC (protein kinase C) activation [5]. However, this stimulatory action of DAG is distinct from an opposing DAG-induced inhibition of TRPC channels, which, in contrast, is mediated by PKC [23].

The identity of the channels mediating Ca^{2+} entry in response to receptor activation and the mechanism by which emptied ER stores communicate information to activate Ca^{2+}-entry channels remain unknown. Contributing to this paucity of information has been a lack of pharmacological tools to modify the channels or their coupling mechanism. One agent receiving attention is the permeant $InsP_3R$ antagonist, 2-aminoethoxydiphenyl borate (2-APB), which, at 75 μM, fully blocks SOCs in most cells [4,14,17,21,24–29]. Originally, this action was interpreted as indicating a role of the $InsP_3R$ in SOC activation [14]. However, 2-APB still blocks SOCs operating in the DT40 $InsP_3{}^{-/-}$ cell line, indicating that the action of 2-APB to inhibit SOCs is not mediated by $InsP_3Rs$ [17,24]. Some researchers have suggested the action of 2-APB is directly upon the SOC channel [24,30]; however, others have interpreted its actions as targeting the coupling machinery to activate SOCs [21,31] as opposed to the channel itself. 2-APB has also been observed to have inhibitory actions on SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) pumping activity in cells [32–34], although such pump inhibition cannot explain inhibition of SOCs. In certain cell types (DT40 B-cells and Jurkat T-cells), the action of 2-APB on SOCs is biphasic, inducing a striking stimulatory action on store-operated Ca^{2+} entry and I_{CRAC} at lower concentrations (5–10 μM) as opposed to a strongly inhibitory action at higher levels [17,24]. We recently reported that in DT40 B-cells, 2-APB induces emptying of stores, which we ascribed to an effect on the $InsP_3R$, speculating that the $InsP_3R$ Ca^{2+}-release channel may be biphasically modified in a fashion perhaps analogous to its action on SOCs [21]. In the present paper, we reveal that this action of 2-APB is specific to lymphocytic cells and involves a site upstream from the $InsP_3R$. In B-cells, it appears that 2-APB may specifically target components mediating the coupling between the BCR–PLC-γ2 complex and Ca^{2+}-entry and Ca^{2+}-release channels.

EXPERIMENTAL

Culture of cells

The DT40 chicken B-cell lines, DT40 WT, triple $InsP_3R$ knockout (DT40 $InsP_3R^{-/-}$) and PLC-γ2 knockout (DT40 PLC-$\gamma2^{-/-}$) cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10 % (v/v) foetal bovine serum (FBS), penicillin, streptomycin and L-glutamine, as described previously [17,18,21]. Jurkat T-cells were grown in RPMI 1640 and 10 % (v/v) FBS under similar conditions.

Transfection of cells

DT40 cells (WT and mutant lines) were cultured overnight in RPMI 1640 with 10 % (v/v) FBS, harvested from plates by scraping, washed in reduced-serum Optimem (Life Technologies), then resuspended in Optimem at a final concentration of 10^7 cells/ml. A 12 μg sample of each of the plasmids to be transfected [containing either human TRPC3, rat PLC-γ2 or the H327F ($His^{327} \rightarrow Phe$) mutant of rat PLC-$\gamma$2, each in the pcDNA3.1 vector] were mixed with 5 μg of the marker DNA (eYFP; enhanced yellow fluorescent protein) and added to 0.5 ml transfection cuvettes with an electrode gap of 0.4 cm, followed by the addition of 0.5 ml of the cells in Optimem (10^7 cells/ml). After thorough mixing of cells and DNA, transfection was carried out using the Gene Pulser II electroporation system (Bio-Rad) at 350 mV, 960 μF and infinite resistance. The cells were then recovered in Optimem (no serum added) for 3 h and were then re-suspended in Optimem with 10 % (v/v) FBS and applied to coverslips. Cells were allowed to attach for 3 h before fura-2/AM (fura-2 acetoxymethyl ester) measurements were undertaken. The overall efficiency of transfection (eYFP-positive cells) was 10–20 %, as detected during fluorescent imaging. The methods were similar to those described previously [20,23,35,36].

Imaging of intracellular calcium in single transfected cells

DT40 cells or Jurkat T-cells grown on coverslips after transfection were placed in Hepes-buffered Kreb's medium [Solution A: 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.5 mM glucose, 0.1 % (w/v) BSA and 20 mM Hepes/KOH, pH 7.4] and loaded with fura-2/AM (2 μM) for 25 min at 20 °C. Cells were washed and dye was allowed to de-esterify for a minimum of 15 min at 20 °C. Approx. 95 % of the dye was confined to the cytoplasm as determined by the signal remaining after saponin permeabilization [37,38]. Cells on coverslips were place in 'cation-safe' medium free of sulphate and phosphate anions (107 mM NaCl, 7.2 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose and 20 mM Hepes/NaOH, pH 7.2), in the presence or absence of 1 mM $CaCl_2$, $SrCl_2$ or $BaCl_2$, as shown in the Figures [20]. Ca^{2+} measurements in single transfected and groups of untransfected cells were made using an InCyt dual-wavelength fluorescence imaging system (Intracellular Imaging, Cincinnati, OH, U.S.A.). Co-transfected eYFP served as the transfection marker and was detected at an excitation wavelength of 485 nm. Untransfected cells (those not expressing eYFP) were identified from the same field and served as control cells. After cell identification, fluorescence emission at 505 nm was monitored with excitation at 340 and 380 nm; intracellular bivalent cation (Ca^{2+}, Sr^{2+} or Ba^{2+}) measurements are shown as 340/380 nm ratios obtained from groups of single untransfected and transfected cells. Details of these bivalent cation measurements were described previously [14,17,20,29]. Resting Ca^{2+} levels in all the DT40 and Jurkat T-cell lines were similar, approx. 100–130 nM. All measurements shown are means of multiple single-cell Ca^{2+} traces, and the results are representative of a minimum of three and, in most cases, a larger number of independent experiments.

Measurement of $InsP_3$ levels

Cells (1×10^6 cell/ml) were incubated in Solution A [inositol-free 1 % (w/v) BSA] containing 2 μCi/ml *myo*-[^{3}H]inositol at 37 °C for 4 h. Cells were washed with Solution A containing 10 mM LiCl and incubated at room temperature (25 °C) for 10 min. Each sample contained 2.5×10^5 cells. Cells were stimulated by adding agents and incubated for appropriate times at 37 °C. The incubation was stopped and extracted by adding 0.8 ml of 0.4 M perchloric acid. The cell extracts were neutralized with 0.5 ml of 0.72 M KOH. [^{3}H]Inositol phosphates in the cell extracts were analysed by anion-exchange chromatography on

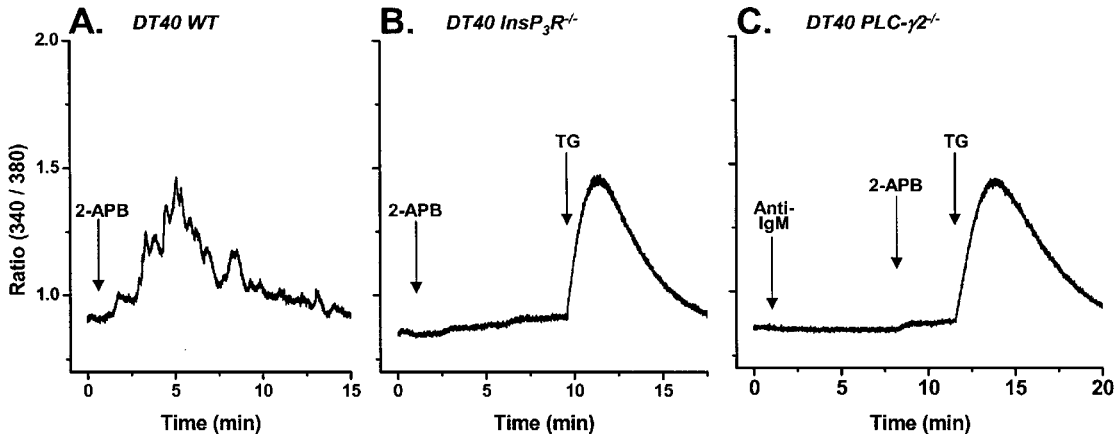

Figure 1 2-APB stimulates Ca^{2+} release in DT40 cells, which is dependent on both PLC-γ2 and $InsP_3R$

The experiments were conducted in nominally Ca^{2+}-free medium. (**A**) In the DT40 WT cells, addition of 75 μM 2-APB induced $InsP_3$-mediated Ca^{2+} release. (**B**) Using DT40 $InsP_3R^{-/-}$ cells, addition of 75 μM 2-APB failed to elicit any Ca^{2+} release, whereas subsequent addition of 2 μM TG caused a large irreversible depletion of Ca^{2+} stores. (**C**) With the DT40 PLC-$\gamma2^{-/-}$ cells, addition of either 3 μg/ml anti-IgM or 75 μM 2-APB did not cause release of Ca^{2+} whereas subsequent addition of 2 μM TG again caused a large release of Ca^{2+} from stores.

gravity-fed Bio-Rad AG 1-X8 (formate form) columns. Cell extracts were applied to the columns and were washed three times with 3 ml of 0.1 M formic acid. $InsP_3$ was eluted twice with 3 ml of 0.8 M ammonium formate/0.1 M formic acid. $InsP_3$-containing fractions were mixed with 6 ml of Aquasol II and were counted for radioactivity. Details of this method are described in [39].

Materials and miscellaneous procedures

Plasmid hTRPC3 cDNA was from Professor Craig Montell (Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.), PLC-γ2 and the H327F lipase-inactive mutant were from Dr Randen Patterson (Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.). eYFP cDNA was from Clontech. OAG (1-oleoyl-2-acetyl-*sn*-glycerol), U-73122 and wortmannin (WM) were from Calbiochem. AG 1-X8 (formate form, 200–400 mesh) anion-exchange resin was from Bio-Rad. *myo*-[^{3}H]Inositol was from NEN Life Science Products (Boston, MA, U.S.A.). EGTA was from Sigma. Thapsigargin (TG) was from LC Services (Woburn, MA, U.S.A.). Fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). Anti-chicken IgM (M-4 clone) was from Southern Biotechnology Associates (Birmingham, AL, U.S.A.). All the DT40 cell lines were kindly supplied by Dr Tomohiro Kurosaki (Department of Molecular Genetics, Kansai Medical University, Moriguchi, Japan). The Jurkat T-cell line (clone E6-1) was from the A.T.C.C. (Manassas, VA, U.S.A.).

RESULTS AND DISCUSSION

Whereas specific target molecules with which 2-APB may interact are unidentified, this amphipathic and reactive borane has provided some interesting modifications of Ca^{2+} signalling pathways. We previously determined that it can react with itself, MS analysis revealing the formation of a number of species, of which a non-polar hydrophobic dimer predominates [29]. This ten-membered ring formed by co-ordinate covalent N $\rightarrow$ B linkages is likely to be freely membrane-permeant and may be the predominant species that enters cells [29]. There is abundant evidence that 2-APB applied to cells inhibits store-operated Ca^{2+}-entry channels [4,14,21,24–28]. It is also clear that 2-APB can inhibit $InsP_3$Rs in many cell types [14,25,27,29,32,34,40]. However, it does not appear that the action of 2-APB on SOCs is mediated by the $InsP_3R$ [17,24,26,27]. At higher concentrations (>50 μM) 2-APB blocks SOC activity in most cell types. However, we noted that, in DT40 B-cells, SOCs are strongly activated by lower (5–10 μM) 2-APB levels [21], an effect also observed in Jurkat T-cells [24]. This biphasic action of 2-APB was not observed in other cell types. We also observed that in DT40 cells, 2-APB induces a rapid and pronounced release of Ca^{2+} from intracellular Ca^{2+} stores, which we suggested might reflect a permissive effect of 2-APB upon $InsP_3$ receptors allowing Ca^{2+} release from the ER [21]. However, the results in Figure 1 suggest that this interpretation may not be the case. The Ca^{2+}-releasing action of 2-APB in DT40 cells is shown in Figure 1(A). Using 2-APB at 75 μM (the concentration maximally blocking SOC activity), the agent induces a large increase in cytosolic Ca^{2+} when added to cells in the absence of extracellular Ca^{2+}. This increase is due to Ca^{2+} release from stores and is sufficient to entirely empty stores, since, as we revealed previously, blockade of SERCAs by the addition of 2 μM TG after 2-APB-induced release, results in no further Ca^{2+} release [21]. The Ca^{2+}-release response to 2-APB appears as a prolonged series of fluctuations in Ca^{2+}, quite different from the 'smooth' release response to TG (Figures 1B and 1C). Individual cells show 2-APB-induced Ca^{2+} release occurring after a latency period that varies from 1 to 5 min between cells. The average 2-APB response for many cells shown in Figure 1(A) is almost identical in appearance with that induced following BCR activation (see below). In contrast, the release in response to TG-induced Ca^{2+}-pump blockade is much more uniform between cells. The EC_{50} for the Ca^{2+}-releasing action of 2-APB in DT40 cells is in the 10–20 μM range (results not shown). Using the triple $InsP_3R$ knockout DT40 cell line in which all three $InsP_3R$ genes have been eliminated [18], the addition of 2-APB resulted in virtually no Ca^{2+} release and stores remained releasable with TG (Figure 1B). Although this indicated the Ca^{2+}-releasing action of 2-APB could be upon the $InsP_3$Rs, examination of the action of 2-APB using PLC-$\gamma2^{-/-}$ DT40 cells in which the PLC-γ2 gene had been eliminated [41] revealed a similar lack of Ca^{2+} release (Figure 1C). Unlike most other cell types

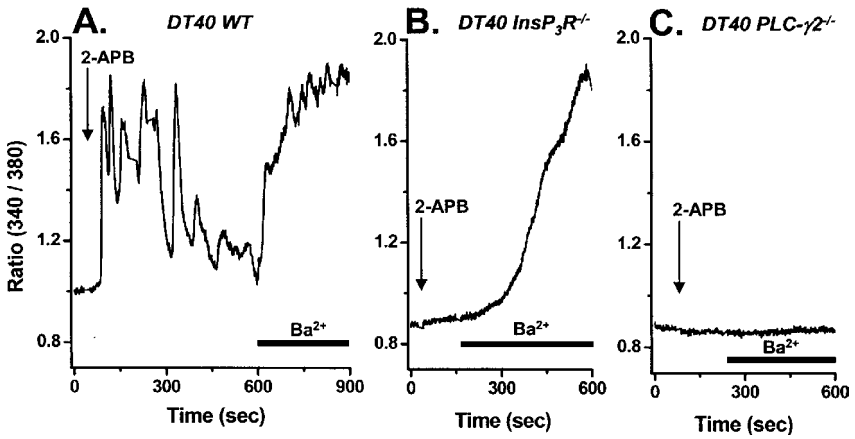

Figure 2 TRPC3 channels transfected into the DT40 cells are activated in response to the addition of 2-APB in an $InsP_3R$-independent and PLC-γ2-dependent fashion

The experiments were conducted in Ca^{2+}-free medium; horizontal bars indicate replacement of Ca^{2+}-free medium with medium containing 1 mM Ba^{2+}. (**A**) In the DT40 WT cells, addition of 75 μM 2-APB elicited an $InsP_3R$-mediated Ca^{2+}-release response. Subsequent addition of 1 mM Ba^{2+} caused TRPC3-mediated entry of Ba^{2+}. (**B**) As in (**A**), but in DT40 $InsP_3R^{-/-}$ cells in which there is no Ca^{2+} release, but a large TRPC3-mediated Ba^{2+} entry. (**C**) Same as in (**A**), but in the DT40 PLC-$\gamma2^{-/-}$ cells in which there is neither release nor entry.

that express both of the two PLC-γ isoforms (PLC-γ1 and PLC-γ2), B-cells express only PLC-γ2 [42]. DT40 cells are identical in this respect, and elimination of the PLC-γ2 gene results in the elimination of all PLC-γ activity [41]. Thus, in the DT40 PLC-$\gamma2^{-/-}$ cells, there is a complete loss of the BCR-induced Ca^{2+}-release response, which is mediated through PLC-γ2 [36,41]. In WT cells, addition of anti-IgM to cross-link and activate the BCR results in a large increase in cytosolic Ca^{2+} due to emptying of Ca^{2+} stores [20,23,36]. In PLC-$\gamma2^{-/-}$ cells, anti-IgM caused no change in cytosolic Ca^{2+} (Figure 1C). Subsequent addition of 75 μM 2-APB also resulted in no Ca^{2+} release, whereas addition of TG revealed that stores were still replete (Figure 1C). Thus the Ca^{2+}-response phenotype of PLC-$\gamma2^{-/-}$ cells with respect to 2-APB is the same as that of $InsP_3R^{-/-}$ cells. Our studies reveal that the PLC-$\gamma2^{-/-}$ cells have normal functional $InsP_3Rs$, as determined by transfection of cells with the G-protein-coupled muscarinic receptor which mediates $InsP_3$ production through the PLC-β enzyme and gives a Ca^{2+}-release response the same as WT cells [36].

These results suggest that the action of 2-APB is upstream from the $InsP_3R$, perhaps at the level of the PLC-γ2 enzyme. We sought to assess whether or not the PLC-γ2 enzyme was being activated by 2-APB by analysing the function of TRPC3 channels transiently transfected into DT40 cells. Thus we revealed previously that TRPC3 channels expressed in these cells respond to DAG, an effect which is independent of the presence or absence of $InsP_3Rs$ and independent of store-depletion [20]. Hence the TRPC3 channel is an effective 'sensor' of DAG production. As shown in Figure 2(A), using TRPC3-transfected DT40 cells, following Ca^{2+} release activated by 2-APB, the subsequent addition of 1 mM Ba^{2+} resulted in substantial Ba^{2+} entry. We have shown previously that there is no basal Ba^{2+} entry in these cells, nor is there any Ba^{2+} entry through SOCs, which are highly selective for Ca^{2+} [20,21,23]. Hence the Ba^{2+} entry observed is a clear manifestation of TRPC3-channel activation [20]. We also assessed the activation of TRPC3 channels transfected in $InsP_3R^{-/-}$ DT40 cells. As shown in Figure 2(B), in these cells, 2-APB addition resulted in no release of Ca^{2+}, yet there was a clear and large entry of Ba^{2+} following Ba^{2+} addition. In contrast, using TRPC3-transfected PLC-$\gamma2^{-/-}$ DT40 cells, there was neither Ca^{2+} release nor Ba^{2+} entry in response to 2-APB (Figure 2C), indicating that PLC-γ2 is required for the action of 2-APB on the TRPC3 channel. This result indicates that 2-APB does not have a direct stimulatory action on the TRPC3 channel. Moreover, the permissive effect of 2-APB on the TRPC3 channel is mediated only through PLC-γ and not through PLC-β, which, as we revealed previously, is clearly functional in DT40 cells [20,36]

The 2-APB-induced Ba^{2+} entry in TRPC3-transfected $InsP_3R^{-/-}$ cells (Figure 2B) appears to develop more slowly than the release of Ca^{2+} from stores following 2-APB addition. The kinetics of TRPC3-channel activation following 2-APB in WT cells is difficult to assess, since Ca^{2+} release continues for several minutes (Figure 2A). Using the $InsP_3R^{-/-}$ cells, we compared the kinetics of TRPC3-channel activation by 2-APB with that in response to BCR activation. For this, Ba^{2+} was added first, followed by addition of either 2-APB or anti-IgM antibody. The time of appearance and rate of TRPC3-mediated Ba^{2+}-entry in response to either agent was similar: in both cases, Ba^{2+} entry occurred after a delay of approx. 1–2 min (results not shown). A significant question is why 2-APB activates store-release and Ba^{2+} entry with different kinetics when both processes should be activated by products ($InsP_3$ and DAG respectively) of the same PLC-γ2-catalysed reaction. It is possible that the metabolism of $InsP_3$ and DAG occur at different rates. It is also possible that the threshold of sensitivity for $InsP_3$-induced Ca^{2+} release through $InsP_3Rs$ is below that of DAG-induced Ba^{2+} entry through TRPC3 channels. Thus small levels of $InsP_3$ may be sufficient to activate release, whereas larger levels of DAG may be required for activation of TRPC3 channels. We recently revealed that DAG has a dual role in controlling TRPC3 channels [23]. DAG-induced PKC activation blocks TRPC3 channels, whereas DAG has a strong permissive effect on the same channels. Hence it is possible that the competing actions of DAG result in a different time course of TRPC3-channel activation from that of $InsP_3$-mediated Ca^{2+} release.

The results indicate that the action of 2-APB is mediated by the PLC-γ enzyme in DT40 cells. We verified this further by analysis of pharmacological PLC-modifiers. Thus, in TRPC3-transfected DT40 WT cells, the PLC-inhibitor U-73122 [20] abolished the Ca^{2+}-release response to both 2-APB and anti-IgM (Figure 3A). Similarly, the response to 2-APB was blocked by pretreatment with WM, which at 10 μM inhibits PtdIns 4-kinase [20],

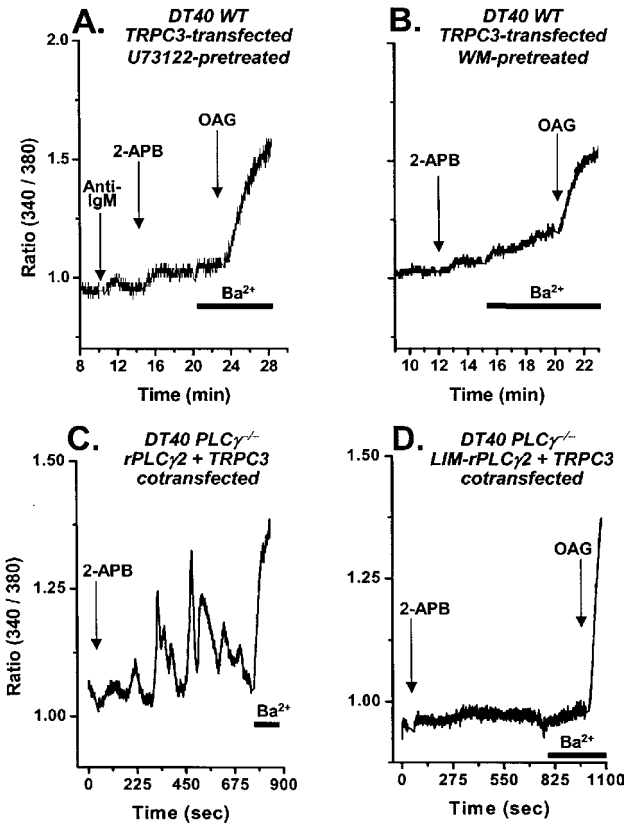

Figure 3 2-APB-mediated Ca^{2+} release and TRPC3 activation requires the lipase activity of PLC-γ2

Experiments were conducted in Ca^{2+}-free medium; horizontal bars indicate the replacement of Ca^{2+}-free medium with medium containing 1 mM Ba^{2+}. (**A**) In TRPC3-transfected DT40 WT cells, pre-treatment with 15 μM U-73122 for 5 min abolished Ca^{2+} release in response to either 3 μg/ml anti-IgM or 75 μM 2-APB, and TRPC3-mediated Ba^{2+} entry. Subsequent addition of 100 μM OAG activated TRPC3-mediated Ba^{2+} entry. (**B**) In TRPC3-transfected DT40 WT cells, pre-treatment with 10 μM WM for 30 min abolished Ca^{2+} release in response to 75 μM 2-APB, and TRPC3-mediated Ba^{2+} entry. Subsequent addition of 100 μM OAG activated TRPC3-mediated Ba^{2+} entry. (**C**) In DT40 PLC-$\gamma2^{-/-}$ cells transfected with rPLC-γ2 and TRPC3, there was recovery of both Ca^{2+} release upon addition of 75 μM 2-APB, and TRPC3-mediated Ba^{2+} entry upon addition 1 mM Ba^{2+}. (**D**) In DT40 PLC-$\gamma2^{-/-}$ cells transfected with LIM-rPLC-γ2 and TRPC3, there was neither Ca^{2+} release upon addition of 75 μM 2-APB nor TRPC3-mediated Ba^{2+} entry upon addition 1 mM Ba^{2+}. Subsequent addition of 100 μM OAG activated TRPC3-mediated Ba^{2+} entry.

and hence depletes the substrate for PLC, PIP_2 [PtdIns(4,5)P_2] (Figure 3B). In both experiments, TRPC3 channels were clearly still functional, since the addition of OAG induced Ba^{2+} entry. It was reported previously that U-73122 was toxic to DT40 cells and itself caused significant rises in intracellular Ca^{2+} [19]. However, we did not observe such effects of U-73122 in this or previous studies on DT40 cells [20], Ca^{2+} stores remaining unchanged in the presence of U-73122.

The involvement of PLC-γ in the activation of Ca^{2+} release and Ca^{2+} entry in response to 2-APB led us to question whether or not the enzymic function of PLC-γ is required for the mediation of these effects. Thus we recently revealed that PLC-γ1 and PLC-γ2 can play non-enzymic roles in mediating Ca^{2+} entry in response to receptor activation [36]. We investigated whether or not transfection of WT PLC-γ2 or a mutated lipase-inactive PLC-γ2 derivative [36] was able to mediate the actions of 2-APB. Using the DT40 PLC-$\gamma2^{-/-}$ cell line devoid of any PLC-γ activity, co-transfection with WT PLC-γ2, together with the TRPC3 channel, resulted in cells gaining back the $InsP_3$R-mediated Ca^{2+}-release

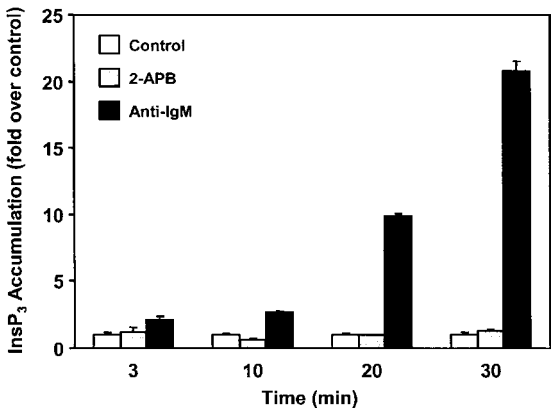

Figure 4 2-APB does not induce a global accumulation of $InsP_3$ in the DT40 cells

The accumulation of [^{3}H]$InsP_3$ was measured in DT40 cells either under control conditions (white), or with addition of 75 μM 2-APB-treated (light grey), or 3 μg/ml anti-IgM treated (dark grey). [^{3}H]$InsP_3$ accumulation was measured at the different time periods indicated. Results are the means $\pm$ S.E.M. for triplicate measurements.

response as well as the TRPC3-mediated Ba^{2+}-entry response (Figure 3C). In contrast, using the H327F PLC-γ2 mutant, which is devoid of lipase activity [36], there was no recovery of 2-APB-induced Ca^{2+} release from stores or TRPC3-mediated Ba^{2+} entry (Figure 3D). However, subsequent addition of OAG induced substantial Ba^{2+} entry, revealing the expression of functional TRPC3 channels as a positive control.

These results indicate that 2-APB has a functional effect similar to the activation of the BCR. Thus it appears that the enzymic activity of PLC-γ2 is required, leading to activation of responses that correspond to the products of PLC. An obvious further analysis was to directly assess the production of $InsP_3$ by measuring $InsP_3$ levels in DT40 cells. However, the results from such analysis were surprising, as shown in Figure 4. Thus we compared the levels of $InsP_3$ subsequent to activation of the BCR with anti-IgM with those following addition of 2-APB. Measurements were continued over a period of 30 min, since the Ca^{2+}-release response to either BCR cross-linking or 2-APB occur over a prolonged period of 10 min or more [21]. Whereas it was clear that BCR activation resulted in a substantial accumulation of $InsP_3$ over this time period, the results indicated no effect of 2-APB on $InsP_3$ levels. Indeed, even at the shortest period of measurement (3 min), there was a significant increase in $InsP_3$ levels following BCR-activation, but no indication of an increase in response to 2-APB.

This result is interesting, since it reveals a clear difference between the function of 2-APB and the BCR. One possible explanation is that 2-APB induces the formation of locally increased $InsP_3$ levels as opposed to more globally increased levels in the case of the BCR. Such local $InsP_3$ increases may be below the threshold of measurement. This interpretation may gain support from our previous work, which revealed that the Ca^{2+}-releasing action of 2-APB in DT40 cells was prevented by pre-treatment of cells with the phosphatase-inhibitor calyculin A [21]. Previously we revealed calyculin A to be a powerful inducer of F-actin reorganization and to induce the formation of a tight subcortical layer closely associated with the plasma membrane [14,35]. This physical rearrangement of the actin cytoskeleton prevents plasma membrane–ER interactions and was shown to completely prevent SOC activation in a number of cell types, including DT40 cells [14,21,35], indicating that close plasma membrane–ER association is required for SOC activation. In

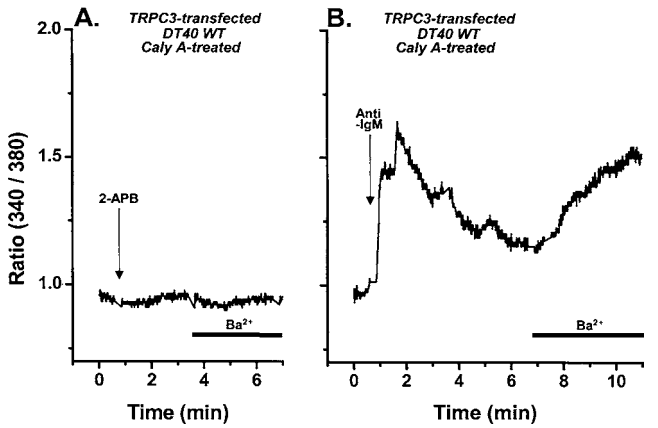

Figure 5 Uncoupling of plasma membrane–ER interactions with calyculin A blocks 2-APB-mediated Ca^{2+} release and TRPC3 activation, but does not block BCR-activation of Ca^{2+} release or TRPC3 activation

Standard conditions included Ca^{2+}-free medium; horizontal bars indicate replacement of Ca^{2+}-free medium with medium containing 1 mM Ba^{2+}. (**A**) In TRPC3-transfected DT40 WT cells, pre-treatment with 100 nM calyculin A for 30 min abolished Ca^{2+} release in response to 75 μM 2-APB, and TRPC3-mediated Ba^{2+} entry upon re-addition of 1 mM Ba^{2+}. (**B**) As in (**A**), but addition of 3 μg/ml anti-IgM which leads to normal Ca^{2+} release and TRPC3-mediated Ba^{2+} entry.

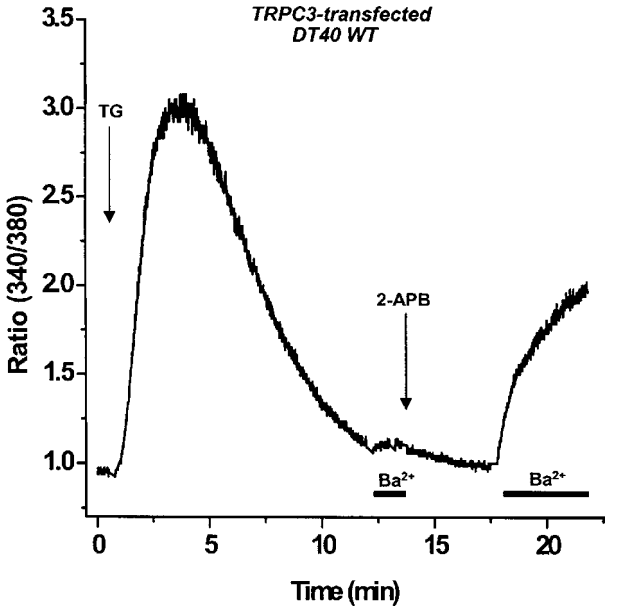

Figure 6 TRPC3 channels transfected into the DT40 cells are activated in response to the addition of 2-APB, but not by passive Ca^{2+} depletion following the addition of TG

Ca^{2+}-free medium was used throughout; horizontal bars indicate replacement medium containing 1 mM Ba^{2+}. In TRPC3-transfected DT40 WT cells, the addition of 2 μM TG led to an irreversible and complete depletion of Ca^{2+} stores. Subsequent addition of 1 mM Ba^{2+} did not elicit any TRPC3-mediated Ba^{2+} entry. The addition of 75 μM 2-APB did not cause Ca^{2+} release, since stores were empty; however, further addition of 3 mM Ba^{2+} after 2-APB revealed rapid TRPC3-mediated Ba^{2+} entry.

contrast, the calyculin A-induced actin rearrangement has no effect on basal cytosolic Ca^{2+}, the size of Ca^{2+} stores or the ability of PLC-coupled receptors to induce $InsP_3$-mediated release of Ca^{2+} from stores [14,35]. As shown in Figure 5(A), using TRPC3-transfected DT40 WT cells, a 30 min pre-treatment of cells with 100 nM calyculin A was able to completely prevent any 2-APB-induced release of Ca^{2+} from stores. In addition, this pre-treatment completely prevented the 2-APB-induced activation of Ba^{2+} entry through TRPC3 channels. In contrast, in the same cells under identical conditions, the action of IgM to induce Ca^{2+} release and Ba^{2+} entry was not altered by the calyculin A pre-treatment (Figure 5B). This is a significant result, since it reveals a further important difference between the action of 2-APB and the BCR. If a local action of $InsP_3$ was induced by 2-APB and was prevented by calyculin-induced plasma membrane–ER separation, we might expect that the activation of the TRPC3 channel would be unaffected by this treatment. Thus PLC-γ and TRPC3 are both in the plasma membrane, and any separation away from the ER would not be expected to prevent the activation of the TRPC3 channel, which is mediated by production of DAG in these cells [20]. On the other hand, the prevention of organization between ER and plasma membrane may also preclude the organization of components within the plasma membrane. Thus, considering the models of organized receptor–G-protein–PLC–actin complexes in the plasma membrane [4,6], the reorganization of actin itself and/or the removal of important ER-interacting species, may themselves result in dissociation and uncoupling of TRPC3 channels from the vicinity of the PLC-γ2.

We also considered another potential mechanism by which 2-APB might activate PLC-γ. Thus it was possible that a small release of Ca^{2+} from ER stores lying close to the plasma membrane might activate the enzyme. Supporting this hypothesis is information that 2-APB can inhibit SERCAs [33,34]. Moreover, we can observe in both $InsP_3^{-/-}$ and PLC-$\gamma 2^{-/-}$ DT40 cells a slight increase in cytosolic Ca^{2+} following the addition of 2-APB (see Figures 1A and 1C), which could result from a modest inhibition of SERCA activity. If local Ca^{2+} release from stores was the trigger for PLC-γ activation, then the action of calyculin A to prevent both $InsP_3$R-mediated Ca^{2+} release and TRPC3-mediated Ba^{2+} entry might be explained. We therefore undertook the experiment shown in Figure 6 in which the actions of 2-APB were examined after the complete emptying of stores with TG. In this experiment, DT40 cells transfected with the TRPC3 channel were treated first with TG. After the release of Ca^{2+} was complete and cytosolic Ca^{2+} had returned to the resting level, the transient addition of external Ba^{2+} revealed no activation of TRPC3 channels, indicating that there was no PLC-γ activation. After 2-APB addition, Ba^{2+} was re-added to reveal robust TRPC3-channel activation. Hence 2-APB had successfully induced PLC-γ activation under conditions in which Ca^{2+} stores had been entirely emptied and therefore without the possibility of release of Ca^{2+} from stores.

Our studies thus far indicated an action of 2-APB upon the PLC-γ2 enzyme. The question we addressed next was whether 2-APB targeted the PLC-γ2 enzyme itself or acted upon members of the complex of proteins that PLC-γ2 interacts with [43,44]. Thus the PLC-γ2 enzyme in B-cells is a central component of a large regulatory complex of kinases and adaptor proteins, as shown in Figure 7. The BCR is a membrane-tethered antibody comprising heavy and light chains, attached intimately to two invariant Ig-like proteins, Igα and Igβ. The latter have cytoplasmic domains containing tyrosine phosphorylation sites that are crucial to transducing information resulting from the primary signal, which is cross-linking of the BCR by antigen. Following cross-linking, two protein tyrosine kinases are activated: one is Syk, the other is the membrane-tethered Src-family protein tyrosine kinase, Lyn [43,44]. The Lyn kinase phosphorylates Igα and Igβ, resulting in Syk becoming tethered to the phosphorylated tyrosine residues on Igα and Igβ. Syk then phosphorylates a key adaptor protein, BLNK, which is crucial in associating with and activating

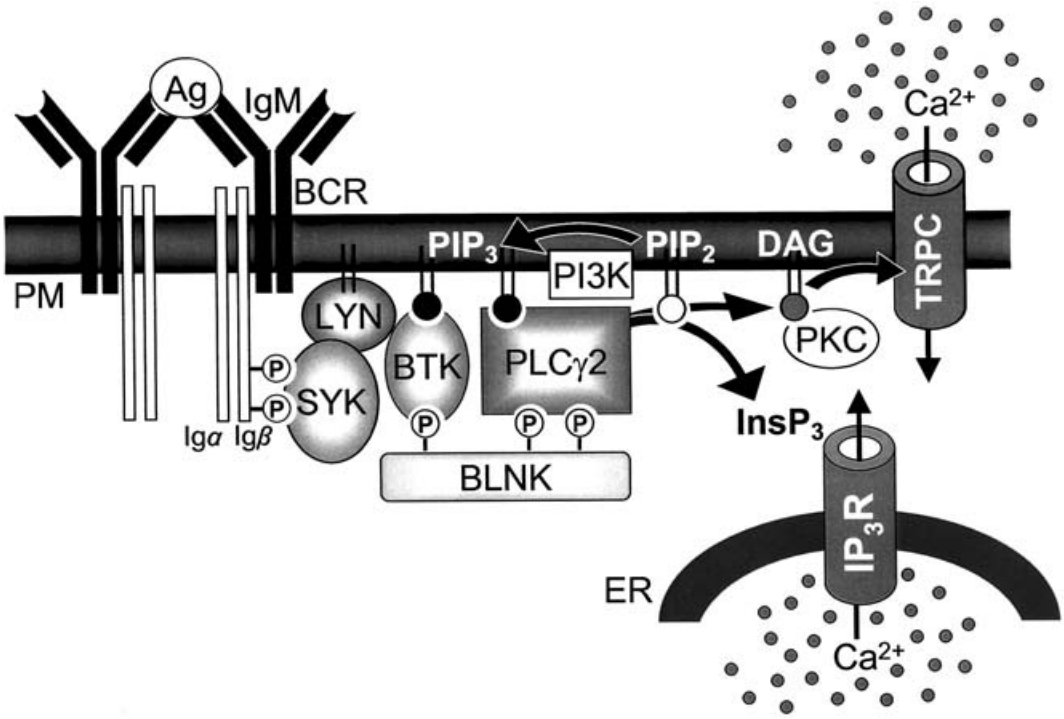

Figure 7 Model of the B-cell receptor complex components and coupling to Ca^{2+} release and Ca^{2+} entry

Details are given in the text. IP_3R, $InsP_3R$; PI3K, phosphoinositide 3-kinase.

the enzyme PLC-γ2. Syk also activates the Tec-related protein tyrosine kinase, Btk, and the enzyme PI3K (phosphoinositide 3-kinase) which converts PIP_2 into PIP_3 [PtdIns(3,4,5)P_3] in the plasma membrane. Proteins with PH (pleckstrin homology) domains avidly bind PIP_3 and bind the complex tightly to the membrane. Btk and PLC-γ2, and a further adaptor protein, Bam32, each contain PH domains and bind to PIP_3 to form a complex tightly associated with the membrane [43,44]. The end result of this series of events is activation of PLC-γ2 to produce $InsP_3$ and DAG.

We utilized a series of mutant DT40 cell lines in which genes for each of the proteins Lyn, Btk, BLNK (B-cell linker) and Syk had been eliminated by homologous recombination [43,45,46]. As shown in Figure 8(A), using $Lyn^{-/-}$, $Btk^{-/-}$, $BLNK^{-/-}$ or $Syk^{-/-}$ DT40 cells, the Ca^{2+}-releasing action of 2-APB was completely eliminated in each case. Thus the Ca^{2+}-response phenotype resulting from the elimination of any one of these proteins is the same as that of the PLC-$\gamma2^{-/-}$ cells. We considered whether perhaps the elimination of these proteins might have some indirect inhibitory effect on the function of the $InsP_3R$. Therefore we assessed the action of 2-APB on TRPC3 channels transfected in DT40 $Syk^{-/-}$ cells to determine whether or not DAG was being derived from PLC-γ2 activation. As shown in Figure 8(E), the addition of Ba^{2+} after 2-APB activation in $Syk^{-/-}$ cells resulted in no entry, whereas the presence of expressed TRPC3 channels was verified by the addition of OAG. This result suggests that the site of action of 2-APB may lie upstream from PLC-γ2 and may not necessarily be directly upon the PLC-γ2 enzyme. However, as depicted in Figure 7, PLC-γ2 is a key component linked within a closely coupled signalling complex involving each of the above kinase/adaptor proteins. Indeed, both PLC-γ1 and PLC-γ2 have important adaptor roles in a number of cell types and are shown to interact with and modify a variety of effector proteins [36,47–49]. In the case of B-cell signalling, the Syk, Lyn, Btk and BLNK proteins are each known to be essential components in the successful activation of PLC-γ2 in response to cross-linking of BCRs [43,44]. The elimination of any one of the four proteins gives rise to cells that are unable to couple BCR cross-linking to activation of PLC-γ2, thus the action of 2-APB may be upon any component of this complex.

Lastly, we considered the question of the specificity of the Ca^{2+}-releasing action of 2-APB among cell types. Thus we noted that other cell types (for example, HEK-293 cells, RBL (rat basophilic leukaemia) mast cells and A7r5 smooth muscle cells) did not give this response (results not shown). Since B-cells are unusual in utilizing PLC-γ2 in their Ca^{2+}-signalling pathway, it was possible that the action of 2-APB was specific to the PLC-γ2 complex. To address this, we examined the action of 2-APB in the Jurkat T-cell line. In these cells, the TCR (T-cell receptor) complex bears many structural and functional analogies with the BCR complex, and both the TCR and BCR signal Ca^{2+} through a cascade involving PLC-γ. However, in T-cells (including the Jurkat line), the receptor complex is coupled through PLC-γ1 as opposed to PLC-γ2 in B-cells [48,49]. Using Jurkat T-cells, we found 2-APB activated Ca^{2+} release virtually identically with its action in DT40 B-cells (results not shown). Indeed, the release from stores in Jurkat T-cells was sufficient that subsequent addition of TG caused no further release, exactly as we observed for DT40 cells [21]. Therefore, although the Ca^{2+}-releasing action of 2-APB does appear to be a property exclusive to lymphocytes, it is not specific to the PLC-γ2-coupled pathway. Although B-cells exclusively utilize the PLC-γ2 isoform, PLC-γ1 expressed in B-cells can substitute for PLC-γ2 and mediate essentially normal BCR-induced Ca^{2+} signals [42].

Thus, whereas the action of 2-APB is likely to be manifested upon the PLC-γ-coupled complexes in both cell types,

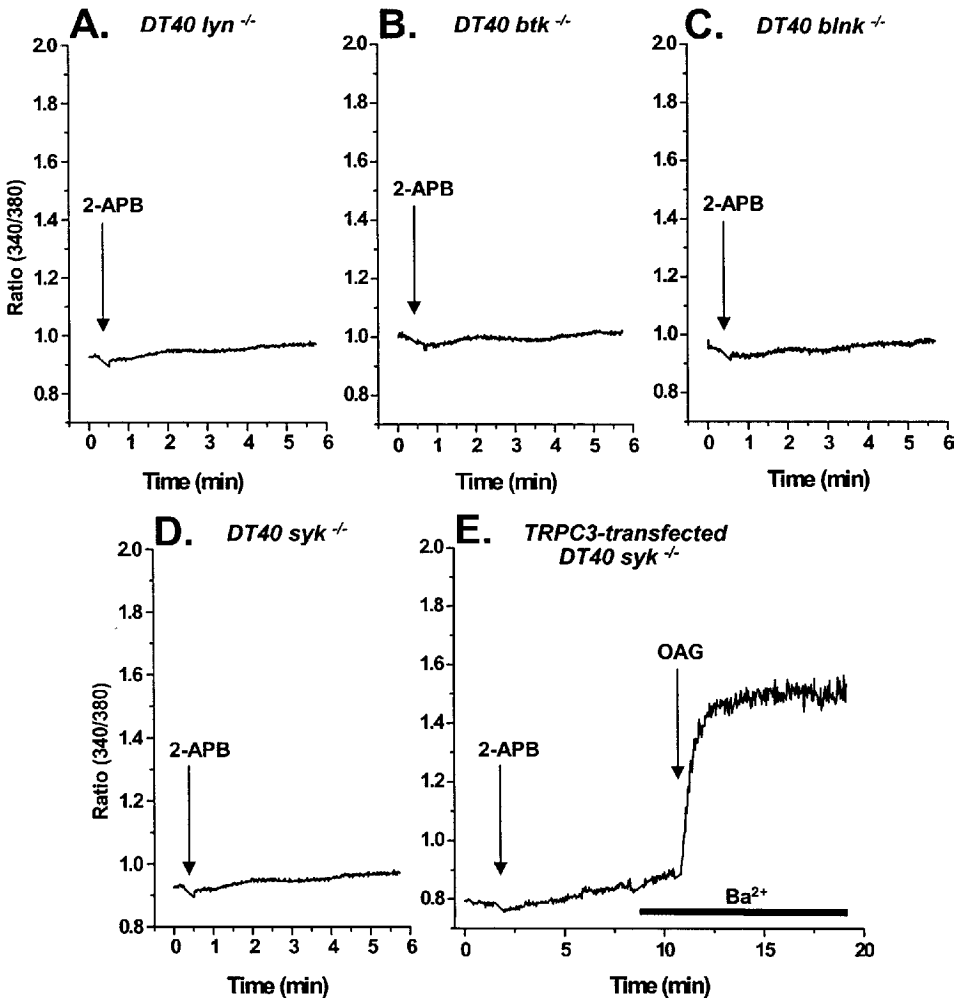

Figure 8 2-APB mediated Ca^{2+} release and TRPC3 activation is dependent upon the non-receptor tyrosine kinases and adaptor proteins involved in the BCR-induced cascade

The experiments were conducted in Ca^{2+}-free medium; horizontal bars indicate addition of 1 mM Ba^{2+}. (**A**) In the DT40 $Lyn^{-/-}$ cells, addition of 75 μM 2-APB induced no Ca^{2+} release. (**B**) As in (**A**), but in DT40 $Btk^{-/-}$ cells. (**C**) As in (**A**), but in DT40 $BLNK^{-/-}$ cells. (**D**) As in (**A**), but in DT40 $Syk^{-/-}$ cells. (**E**) In TRPC3-transfected DT40 $Syk^{-/-}$ cells, addition of 75 μM 2-APB induced neither Ca^{2+} release nor TRPC3-mediated Ba^{2+} entry; however, subsequent addition of 100 μM OAG led to TRPC3-mediated Ba^{2+} entry.

considering that each component in the two complexes is distinct, 2-APB is not necessarily directed specifically to a single component. Instead, it may more likely reflect a target involved in the coupling between either of the two complexes and the Ca^{2+}-signalling machinery. Although the effect of 2-APB is analogous to BCR activation through receptor cross-linking, we know that there are important differences between the action of 2-APB and BCR cross-linking with respect to the results on $InsP_3$ measurements and the effects of calyculin A, described above. Therefore it is clear that the action of 2-APB is not simply to cause cross-linking of the BCR as anti-IgM does. In previous work, we suggested that the action of 2-APB is upon the machinery that couples ER to the activation of SOCs [21]. This conclusion was also drawn from the action of 2-APB to modify TRPV6 channels [31], which can operate in a store-dependent mode when expressed in cells [31,50]. In the DT40 B-cell and Jurkat T-cell lines, 2-APB can induce a substantial augmentation in the activation of SOCs in response to store-emptying [21,24]. This augmentation of the coupling of SOCs is not observed in other cell types, which instead show only 2-APB-induced inhibition of SOCs. Thus we would suggest that the action of 2-APB is related to its ability to augment coupling between the ER and the plasma membrane. One possibility is that the induction of a closer proximity between the ER and plasma membrane is the basis for increased Ca^{2+} release by allowing $InsP_3Rs$ to respond to sub-threshold PLC-γ-generated $InsP_3$ levels [21]. This same reorganization may also result in a closer proximity between PLC-γ and TRPC3 channels, allowing a more efficient coupling to Ca^{2+} entry. Such a scheme derives support from the considerable recent evidence that signalling domains comprising receptors, G-proteins, PLC and Ca^{2+}-entry channels in the plasma membrane may become closely associated with ER domains containing Ca^{2+}-release channels [4,6]. Indeed, there is considerable evidence that ER $InsP_3Rs$ can interact directly with TRPC3 channels in the plasma membrane [4,11,13–15]. Moreover, we showed recently that the PLC-γ1 enzyme can interact directly with TRPC3 channels [36]. If such coupling were enhanced by the action of 2-APB, then our results suggest that the kinase/adaptor components of the BCR complex shown in Figure 7 may be an integral part of this association process. Finally, regardless of the mechanism by which 2-APB initiates and enhances Ca^{2+} release and Ca^{2+} entry in lymphocytes, 2-APB may serve as the prototype

of a class of agents able to induce activation of immune cell responses through triggering of BCR- or TCR-mediated coupling to the Ca^{2+}-signalling machinery. In this way, they may target a therapeutically important mechanism for controlling B- and T-cell function.

We thank Dr Tomohiro Kurosaki for providing DT40 cell lines and helpful advice, Dr Damian van Rossum and Dr Randen Patterson for invaluable feedback, and Dr Wen Xu and Mr Thomas Crumbacker for expert assistance. This work was supported by National Institutes of Health (NIH) grant HL55426 (to D. L. G.), and a studentship from the Combined Biochemistry Graduate Program, University of Maryland School of Medicine (to F. J.)

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Received 3 September 2003/8 October 2003; accepted 15 October 2003
Published as BJ Immediate Publication 15 October 2003, DOI 10.1042/BJ20031345