

Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor–intrinsic costimulation to class-switched B cells

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The improved antibody responses of class-switched memory B cells depend on enhanced signaling from their B cell antigen receptors (BCRs). However, BCRs on both naive and antigen-experienced B cells use the canonical immunoglobulin-associated α and β -protein signaling subunits. Here we identified a BCR isotype-specific signal-amplification mechanism. Whereas immunoglobulin M (IgM)-containing BCRs initiated intracellular signals exclusively through immunoglobulin-associated α - and β -proteins, IgG- and IgE-containing BCRs also used a conserved tyrosine residue in the cytoplasmic segments of immunoglobulin heavy chains. When phosphorylated, this tyrosine recruited the adaptor Grb2, resulting in sustained protein kinase activation and prolonged generation of second messengers, which together culminated in enhanced B cell proliferation. Hence, membrane-bound IgG and IgE exert antigen recognition as well as costimulatory functions, thereby rendering memory B cells less dependent on T cell help.

Distinct immunoglobulin isotypes can be expressed on the B cell surface as integral parts of the B cell antigen receptor (BCR) and/or can be secreted as soluble effector molecules after differentiation of activated B cells into plasma cells¹. The primary humoral immune response is characterized by secretion of immunoglobulin M (IgM) antibodies. Secondary encounter with the same antigen results in the production of other immunoglobulin isotypes owing to irreversible genetic rearrangements at the productively rearranged immunoglobulin heavy-chain allele². This process, referred to as ‘class-switch recombination’, preserves the antigen specificity but exchanges the constant region to equip soluble immunoglobulin with specific immune effector mechanisms needed for clearance of the particular antigen. In addition, the membrane-bound isoforms of IgG and IgE are equipped with cytoplasmic domains that have been linked to the establishment of memory B cell responses^{3–5}. A hallmark of memory B cells is their ability to undergo rapid activation and differentiation into antibody-secreting plasma cells after antigen recall, even in the absence of costimulation by helper T cells^{6,7}. In marked contrast, naive IgM- and IgD-expressing B cells require costimulatory signals induced by accessory receptors, in addition to BCR signals, for differentiation into plasma cells.

The canonical BCR signaling elements of naive and class-switched B cells are immunoglobulin-associated α - and β -proteins (Ig- α (CD79a) and Ig- β (CD79b)). These proteins form a disulfide-linked heterodimer that is noncovalently associated at a stoichiometry of 1:1

with membrane-bound immunoglobulin (mIg) heavy chains of all isotypes: μ m, δ m, γ m, ϵ m and α m^{8,9}. The cytoplasmic domains of Ig- α and Ig- β each contain a single immunoreceptor tyrosine-based activation motif (ITAM)¹⁰, which after dual phosphorylation recruits and activates the spleen tyrosine kinase Syk¹¹. Activated Syk phosphorylates the adaptor protein SLP-65 (also called BLNK or BASH), which controls several ‘downstream’ signaling cascades, including activation of phospholipase C- γ 2 (PLC- γ 2), followed by generation of inositol triphosphate (InsP₃) and mobilization of the key second messenger Ca²⁺ (ref. 12). SLP-65 can also be directly recruited to the activated BCR by virtue of a non-ITAM phosphorylated tyrosine (phosphotyrosine) residue in Ig- α , which is particularly important for T cell-independent B cell activation^{13,14}. These signaling events are initiated ‘downstream’ of all BCR classes. Nonetheless, IgG and IgE immune responses in the mouse seem to require BCR signaling functions executed by the cytoplasmic domains of mIgG^{3,4,15,16} and mIgE⁵, respectively. Mice lacking the cytoplasmic domain of either mIgG1 or mIgE show severely impaired secondary immune responses of the respective immunoglobulin isotype. However, the molecular mechanisms underlying these isotype-specific BCR signals remain obscure.

Here we demonstrate that mIgG- and mIgE-containing BCRs boosted B cell activation through phosphorylation of an evolutionary conserved tyrosine residue in the mIg cytoplasmic tails and subsequent complex formation with the cytoplasmic adaptor Grb2

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a

	K	V	K	W	I	F	S	S	V	D	L	K	Q	T	I	P	D	Y	R	N	M	I	G	Q	G	A
γ1m human																										
γ2m human																										
γ3m human																										
γ4m human																										
γ1m mouse																										
γ2am mouse																										
γ2bm mouse																										
γ3m mouse																										
γ3m rat																										
γ1m camel																										
γ1m cow																										
γ1m dog																										
γ1m pig																										

b

	IP: α-IgG		
	WT	YF	
α-IgG (min):	0	3	(kDa)
Syk			83
γ2am			62
	α-p-Tyr (100)		
γ2am			62
	α-γ2a		

c

	IP: α-IgG				
	Red		N.Red		
	0	3	3	3	
α-IgG (min):	0	3 <td>3<td>3</td><td>(kDa)</td></td>	3 <td>3</td> <td>(kDa)</td>	3	(kDa)
lgG2am					175
γ2am					83
					62
	α-p-Tyr (100)				
lgG2am					175
γ2am					83
					62
	α-γ2a				

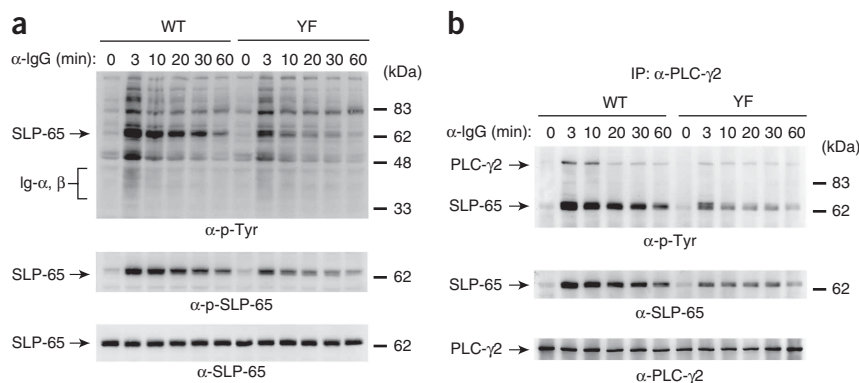


Figure 2 ITT phosphorylation of $\gamma 2am$ improves the activation of BCR-proximal signal effectors. (a) Immunoblot analysis of lysates of Ramos transfectants expressing wild-type or YF (ITT-mutant) $\gamma 2am$ as part of their BCR, left untreated (0) or stimulated for various times (above lanes) with anti-IgG, probed with anti-phosphotyrosine (top), anti-phospho-SLP-65 (middle) or anti-SLP-65 (bottom). (b) Immunoblot analysis of the phosphorylation of immunoprecipitated PLC- $\gamma 2$ in the cells in **a**, probed with anti-phosphotyrosine (top). Middle, immunoblot analysis of the binding of PLC- $\gamma 2$ to SLP-65, detected by anti-SLP-65; bottom, confirmation of equal loading by immunoblot analysis with anti-PLC- $\gamma 2$. Right margins, relative molecular masses of marker proteins. Data are representative of three (a) or two (b) independent experiments.

wild-type or YF IgG-BCRs responded to anti-IgG treatment with higher or moderate Ca^{2+} flux, respectively; this difference was determined mainly by the influx of Ca^{2+} across the plasma membrane and thus recapitulated the distinct Ca^{2+} -mobilization profiles observed in our mIgG-expressing Ramos transfectants (Fig. 3d). In line with published results from IgG-mutant mice^{15,16}, these experiments collectively establish that the wild-type IgG-BCR amplifies Ca^{2+} mobilization. Here we ascribe this boosting effect to phosphorylation of the ITT in γm immunoglobulin heavy chains.

ITT function distinguishes IgG-BCR from IgM-BCR signaling

In addition to the ITT phosphorylation motif, other conserved amino acids in the γm cytoplasmic domain (Fig. 1a) might also contribute to mIgG-specific augmented Ca^{2+} flux. To test that possibility, we extended our analysis to Ramos transfectants expressing a truncated $\gamma 2am$ variant that lacks the carboxy-terminal 25 amino acids ($\Delta tail$) and therefore contains a cytoplasmic tail identical to that of μm

BCRs and IgG-BCRs are accommodated solely in the γm cytoplasmic tail. To examine whether those observations could be explained by ITT phosphorylation, we recapitulated that approach in the K46 B cell system, established for studying mechanistic details of BCR-proximal signaling events^{22–24}. We generated K46 B cell transfectants expressing chimeric μ - $\gamma 2am$ proteins composed of the extracellular domains of μ and the transmembrane and cytoplasmic segments of wild-type, YF or $\Delta tail$ $\gamma 2am$. In association with the appropriate $\lambda 1$ light chain, the heavy-chain variants formed a chimeric BCR with specificity for the hapten NIP^{25,26}. The various chimeric receptors were present in equal amounts on the cell surface (Supplementary Fig. 4). Next we stimulated the transfectants with NIP coupled to poly-lysine or with anti- μ . Consistent with our results obtained with Ramos and primary B cells, the ITT⁺ BCR chimeras responded to both modes of stimulation by inducing much stronger Ca^{2+} flux than did the YF or $\Delta tail$ BCRs (Fig. 4c,d). Stimulation of the endogenous IgG-BCRs of the K46 clones demonstrated their equal Ca^{2+} mobilization capacity (Fig. 4e).

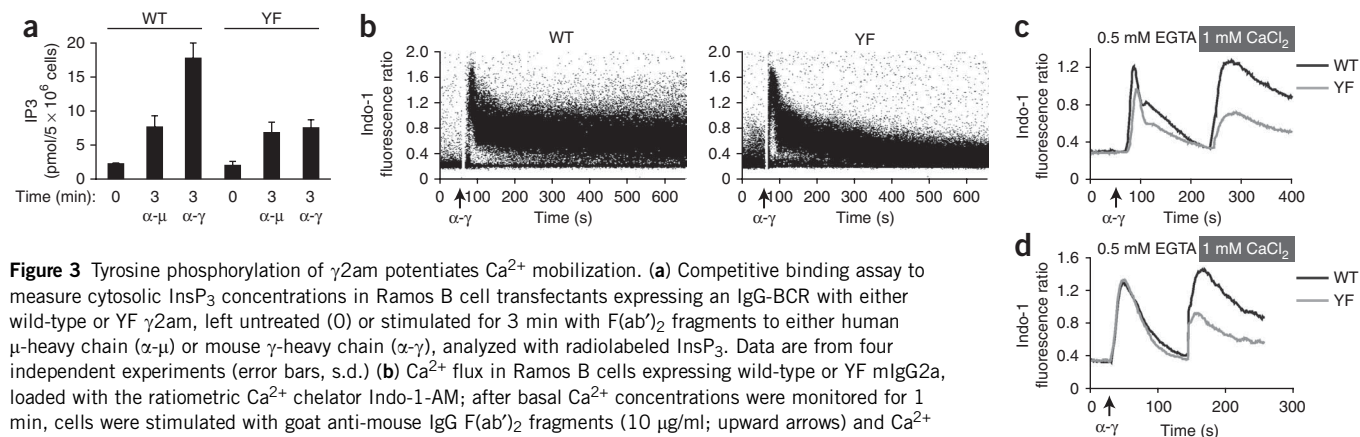


Figure 3 Tyrosine phosphorylation of $\gamma 2am$ potentiates Ca^{2+} mobilization. (a) Competitive binding assay to measure cytosolic $InsP_3$ concentrations in Ramos B cell transfectants expressing an IgG-BCR with either wild-type or YF $\gamma 2am$, left untreated (0) or stimulated for 3 min with $F(ab')_2$ fragments to either human μ -heavy chain (α - μ) or mouse γ -heavy chain (α - γ), analyzed with radiolabeled $InsP_3$. Data are from four independent experiments (error bars, s.d.). (b) Ca^{2+} flux in Ramos B cells expressing wild-type or YF mIgG2a, loaded with the ratiometric Ca^{2+} chelator Indo-1-AM; after basal Ca^{2+} concentrations were monitored for 1 min, cells were stimulated with goat anti-mouse IgG $F(ab')_2$ fragments (10 $\mu g/ml$; upward arrows) and Ca^{2+} flux was monitored for another 10 min in the presence of 1 mM extracellular $CaCl_2$. Data are representative of five experiments with five independent clones for each receptor. (c) Flow cytometry analysis of Ca^{2+} mobilization from intracellular stores in the Ramos transfectants in **b**, loaded with Indo-1-AM and after anti-IgG stimulation (upward arrow), measured for 3 min in the presence of 0.5 mM EGTA (absence of extracellular Ca^{2+}). Influx of Ca^{2+} across the plasma membrane was analyzed by adjustment of the extracellular $CaCl_2$ concentration to 1 mM. Data are representative of three independent experiments. (d) Analysis of Ca^{2+} mobilization as described in **c** in primary mouse splenic B cells transduced to express wild-type or YF $\gamma 2am$. Data are representative of three independent experiments.

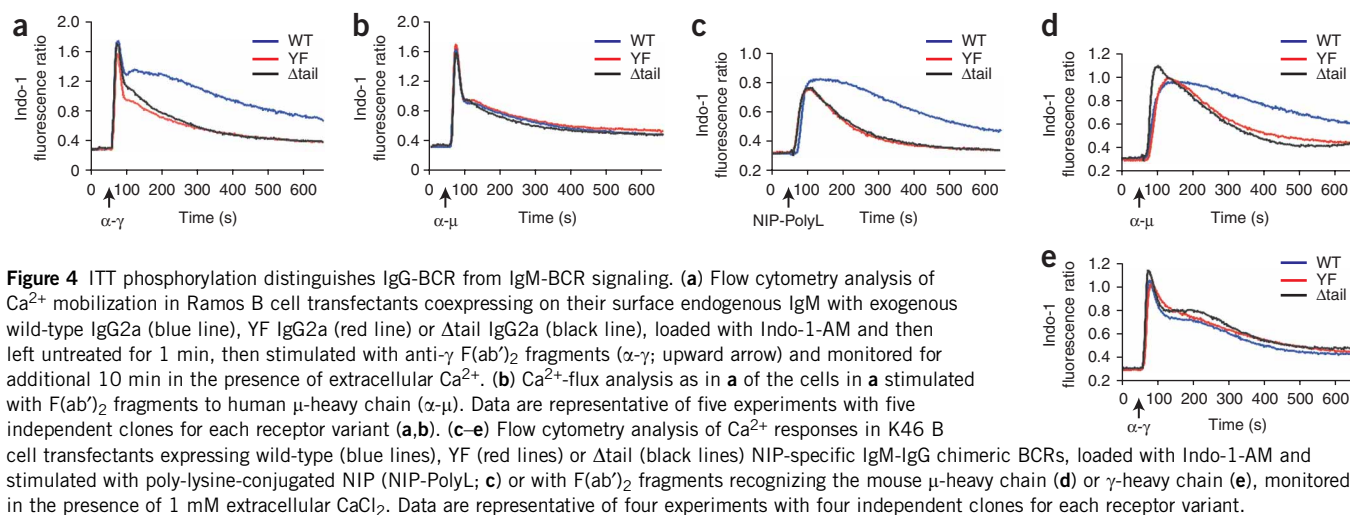


Figure 4 ITT phosphorylation distinguishes IgG-BCR from IgM-BCR signaling. **(a)** Flow cytometry analysis of Ca^{2+} mobilization in Ramos B cell transfectants coexpressing on their surface endogenous IgM with exogenous wild-type IgG2a (blue line), YF IgG2a (red line) or Δ tail IgG2a (black line), loaded with Indo-1-AM and then left untreated for 1 min, then stimulated with anti- γ F(ab')_2 fragments (α - γ ; upward arrow) and monitored for additional 10 min in the presence of extracellular Ca^{2+} . **(b)** Ca^{2+} -flux analysis as in **a** of the cells in **a** stimulated with F(ab')_2 fragments to human μ -heavy chain (α - μ). Data are representative of five experiments with five independent clones for each receptor variant (**a,b**). **(c–e)** Flow cytometry analysis of Ca^{2+} responses in K46 B cell transfectants expressing wild-type (blue lines), YF (red lines) or Δ tail (black lines) NIP-specific IgM-IgG chimeric BCRs, loaded with Indo-1-AM and stimulated with poly-lysine-conjugated NIP (NIP-PolyL; **c**) or with F(ab')_2 fragments recognizing the mouse μ -heavy chain (**d**) or γ -heavy chain (**e**), monitored in the presence of 1 mM extracellular CaCl_2 . Data are representative of four experiments with four independent clones for each receptor variant.

Collectively our data identify ITT phosphorylation of $\gamma 2\text{am}$ as a signal-amplification mechanism that distinguishes IgG2a-BCR signaling from that of the IgM-BCR in different stimulation conditions and in B cells from different species. As the ITT and surrounding amino acids are highly conserved among γ m cytoplasmic tails (**Fig. 1a**), we postulate that inducible ITT phosphorylation provides the molecular mechanism for the enhanced signaling capacity of all mIgG subtypes demonstrated before by various genetically engineered mouse model systems^{3,4,15,16}.

ITT boosts B cell proliferation via Grb2

To search for 'downstream' signal effector proteins of phosphorylated ITT, we used biotinylated peptides that constituted all combinations of phosphorylated and nonphosphorylated amino acid sequences of Ig- α , Ig- β or $\gamma 2\text{am}$. We used these peptides to affinity purify binding partners from Ramos B cell lysates. Only one protein with an apparent molecular mass of approximately 25 kilodaltons was detected

by silver staining as binding to $\gamma 2\text{am}$ in a phosphorylation-dependent way (**Supplementary Fig. 5**). We identified this protein by direct immunoblot analysis as the adaptor protein Grb2 (**Fig. 5a**). Its interaction with the activated IgG-BCR seemed to be highly specific, as we detected almost no binding of Grb2 to Ig- α - or Ig- β -derived peptides, which conversely purified their known binding partners Syk or SLP-65 (**Fig. 5a**) as reported before¹³. We detected no substantial binding between the phospho- $\gamma 2\text{am}$ peptide and the p85 subunit of PI(3)K by immunoblot analysis (data not shown). Furthermore, the different Ca^{2+} -signaling abilities of wild-type and mutant IgG-BCRs remained evident even in the absence of PI(3)K activity (**Supplementary Fig. 6**). Together, the last findings suggest that PI(3)K is not a major ITT effector protein.

We further confirmed the specificity of the phosphorylation-dependent interaction between mIgG and Grb2 by affinity purification with a glutathione S-transferase (GST) fusion protein encompassing the Grb2 SH2 domain. This GST-Grb2 SH2 fusion protein purified

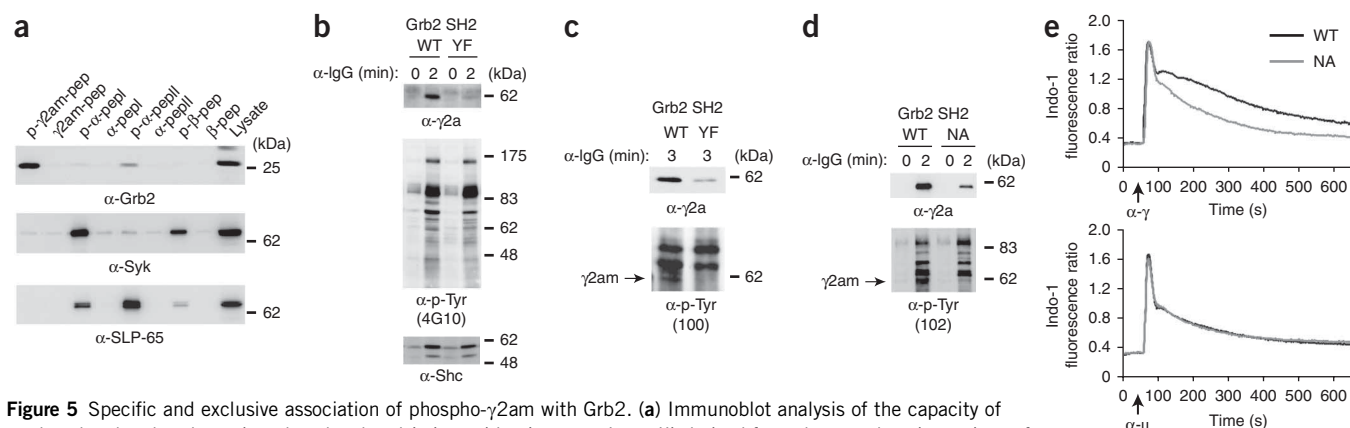
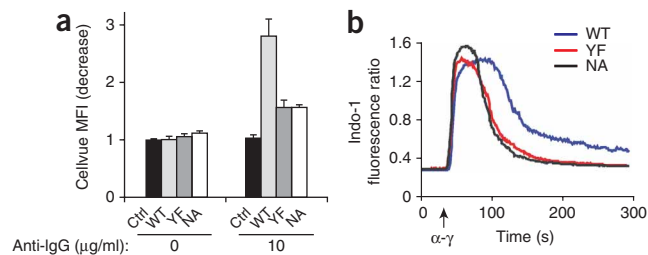


Figure 5 Specific and exclusive association of phospho- $\gamma 2\text{am}$ with Grb2. **(a)** Immunoblot analysis of the capacity of unphosphorylated and tyrosine-phosphorylated (p-) peptides (pep, pepI, pepII) derived from the cytoplasmic portions of Ig- α , Ig- β or $\gamma 2\text{am}$ (above lanes) to associate with Grb2, Syk or SLP-65 in resting Ramos cells, assessed in affinity-purified material from lysates. **(b)** Immunoblot analysis of affinity-purified proteins from lysates of Ramos B cell transfectants expressing wild-type or YF $\gamma 2\text{am}$, left untreated (0) or stimulated through their BCR for 2 min (2); proteins from lysates affinity purified with a recombinant GST-Grb2 SH2 fusion protein were analyzed with anti- $\gamma 2\text{a}$, anti-phosphotyrosine (mAb 4G10) or anti-Shc. **(c)** Immunoblot analysis of retrovirally transduced primary splenic B cells expressing wild-type or YF $\gamma 2\text{am}$, stimulated for 3 min with anti-IgG F(ab')_2 fragments and assessed as described in **b** except that mAb 100 (anti-phosphotyrosine) was used instead of 4G10. **(d)** Immunoblot analysis of Ramos B cell transfectants expressing wild-type or NA mutant $\gamma 2\text{am}$, assessed as described in **b** except that mAb 102 (anti-phosphotyrosine) was used. **(e)** Ca^{2+} responses in Ramos B cell transfectants that express, in addition to endogenous μ m, wild-type or NA $\gamma 2\text{am}$, stimulated with anti- γ or anti- μ F(ab')_2 fragments (upward arrows), monitored by flow cytometry in the presence of 1 mM extracellular CaCl_2 . Right margins (**a–d**), relative molecular masses of marker proteins. Data are representative of three (**a,c,d**) or five (**b**) independent experiments or five experiments with five independent clones (**e**).

Figure 6 Grb2 recruitment is critical for the IgG-BCR-induced proliferative burst. **(a)** Division of primary splenic B cells obtained from 8-week-old C57/BL6 mice, then retrovirally transduced to express wild-type or mutant (YF or NA) IgG2a along with internal ribosomal entry site-driven eGFP, or eGFP alone (control (Ctrl)); plasma membranes were then stained with CellVue Claret and stained cells were incubated for 96 h in the absence (0) or presence (10) of 10 $\mu\text{g}/\text{ml}$ of goat anti-mouse IgG F(ab')₂ fragments and the median fluorescence intensity (MFI) values for CellVue were recorded by flow cytometry for eGFP⁺ transductants; results are normalized to those for eGFP⁺ B cells from the same wells and BCR-induced proliferation is presented as the decrease in CellVue MFI. This eGFP-based gating of transductants excludes the use of CFSE as proliferation marker, as both dyes emit light of similar wavelengths. Data are representative of five independent experiments (error bars, s.d. of quadruplicate samples). **(b)** Intracellular Ca²⁺ mobilization in the cells in **a** after stimulation with anti-IgG F(ab')₂ fragments. Data are representative of two independent experiments.



phospho- $\gamma 2\text{am}$ from lysates of activated Ramos transfectants but not from unstimulated cells or from cells expressing $\gamma 2\text{am}$ with a mutant ITT (**Fig. 5b**). Reprobing of the blot with antibodies to phosphotyrosine and the known Grb2 ligand Shc confirmed the functionality of the GST-Grb2 SH2 fusion protein (**Fig. 5b**). We used the same experimental setup to assess the ITT phosphorylation of mIgG2a in primary B cells. For this, we retrovirally expressed wild-type or YF (ITT-mutant) IgG2a in mouse splenic B cells. We stimulated these cells and subjected their lysates to affinity purification with GST-Grb2 SH2. Wild-type but not YF $\gamma 2\text{am}$ was easily detected in the purified material (**Fig. 5c**). The YF ITT substitution in $\gamma 2\text{am}$ did not compromise the purification of Grb2-SH2 ligands other than $\gamma 2\text{am}$, which again demonstrated the integrity of the fusion protein and successful stimulation of the cells. Additional *in vitro* binding studies confirmed that binding between phospho- $\gamma 2\text{am}$ and Grb2 was direct (**Supplementary Fig. 7**). Collectively these data suggest SH2-mediated Grb2 recruitment as the effector mechanism that distinguishes activated IgG-BCR IgM-BCR signaling from that of the IgM-BCR. Moreover, Grb2 seems to be the exclusive binding partner of phosphorylated ITT.

We addressed the functional importance of the phospho- $\gamma 2\text{am}$ -Grb2 interaction by mutational analysis. We replaced the asparagine (Asn) residue of the Tyr-Arg-Asn-Met motif with alanine (Ala), thereby damaging the consensus Grb2-binding site but retaining ITT phosphorylation and potential binding to p85 of PI(3)K (the latter requires only the methionine in the phosphotyrosine +3 position). Wild-type and Asn-to-Ala (NA) IgG-BCRs were expressed in Ramos B cell transfectants in equal amounts and both mIgG variants were phosphorylated equally well (**Supplementary Fig. 8**).

Figure 7 The IgE-BCR uses ITT-mediated signal amplification.

(a) Comparison of γm and ϵm cytoplasmic tail sequences. Dashes indicate conserved amino acid residues; box outlines the Asp-Tyr-X-Asn (DYXN) motif. **(b)** Immunoblot analysis of immunoprecipitated $\gamma 2\text{a}-\epsilon\text{m}$ in proteins from lysates of Ramos B cell transfectants expressing a chimeric IgG-E-BCR containing a wild-type or YF ϵm tail, left untreated (0) or stimulated with anti-IgG for 2 min (2), probed with anti-phosphotyrosine (mAb 102) followed by horseradish peroxidase-linked anti-IgG1 as a secondary antibody (top). Below, blot reprobed with anti-IgG2a to confirm equal loading. **(c)** Immunoblot analysis of affinity-purified proteins from lysates of the cells in **b**, left untreated (0) or stimulated through the chimeric IgG-IgE-BCR (2); lysate proteins isolated by affinity purification with GST-Grb2 SH2 were analyzed with anti- $\gamma 2\text{a}$ or anti-phosphotyrosine. **(d)** Ca²⁺ mobilization from intracellular stores in the Ramos transfectants in **b**, loaded with Indo-1-AM and stimulated (upward arrows) with anti-IgG ($\alpha\text{-}\gamma$; left) or anti-IgM ($\alpha\text{-}\mu$; right), measured by flow cytometry for 3 min in the presence of 0.5 mM EGTA. Influx of Ca²⁺ across the plasma membrane was analyzed by adjustment of the extracellular CaCl₂ concentration to 1 mM. Right margins (**b,c**), relative molecular masses of marker proteins. Data are representative of three (**b,d**) or five (**c**) independent experiments.

As expected, the NA mutant of mIgG bound the Grb2 SH2 domain only weakly (**Fig. 5d**). Notably, the NA amino acid exchange resulted in much less anti-IgG-induced Ca²⁺ mobilization compared with the sustained response of cells expressing a wild-type IgG-BCR (**Fig. 5e**). Both transfectants had equal Ca²⁺-signaling capacity, as confirmed by stimulation of the endogenously expressed IgM-BCR (**Fig. 5e**). This genetic approach showed that ITT phosphorylation itself is not sufficient to enhance signaling of the IgG-BCR but instead that this requires subsequent binding to Grb2 but not PI(3)K or any other known Ca²⁺-promoting factor.

A key feature of secondary antibody responses is rapid reactivation of class-switched B cells. This process is promoted by the capacity of the IgG-BCR to trigger a so-called 'proliferative burst' after antigen recall in the mouse⁴. To test whether the burst-enhancing function of the IgG-BCR could be ascribed to the phosphorylated ITT and concomitant Grb2 recruitment, we retrovirally expressed wild-type and mutant mIgG2a in primary splenic B cells and monitored their proliferation after IgG-BCR stimulation with the far red-emitting, plasma membrane-intercalating dye CellVue Claret, which becomes diluted as cells divide. Efficient retroviral transduction required an initial activation step with mitogenic lipopolysaccharide. Hence, the transduced B cells already showed robust proliferation in the absence of BCR ligation (**Supplementary Fig. 9**). However, whereas activation of the wild-type IgG-BCR further increased proliferation of lipopolysaccharide-pretreated B cells, proliferation was only marginally augmented after

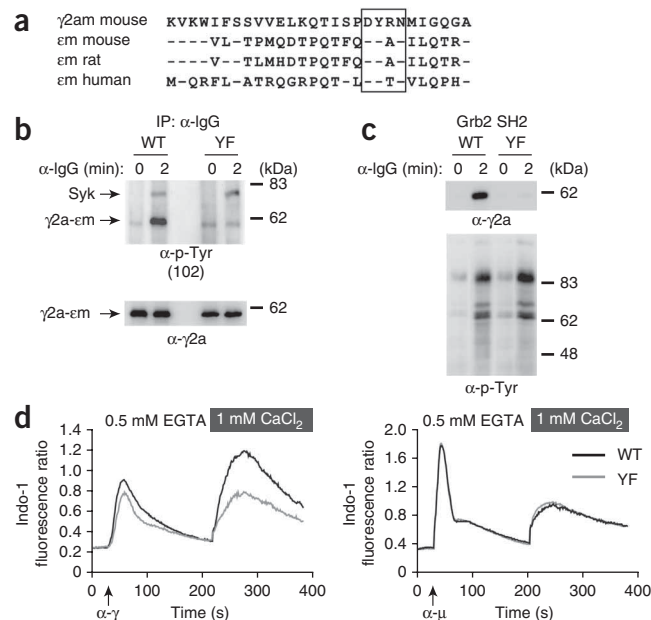
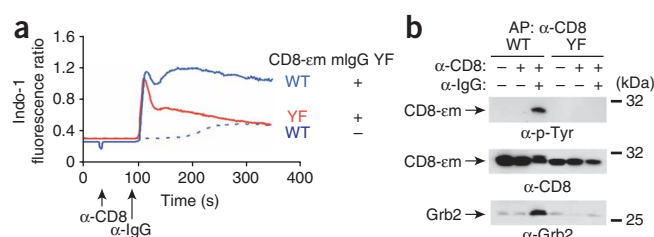


Figure 8 ITT signal amplification relies on BCR integration. (a) Ca^{2+} mobilization in Ramos transfectants expressing YF IgG2a-BCR together with wild-type CD8- ϵ m (solid blue line) or YF CD8- ϵ m (red curve) or expressing the wild-type CD8- ϵ m chimera alone (dashed blue line), pretreated for 1 min with anti-CD8 (mAb MEM31) and then stimulated with goat anti-mouse IgG F(ab')₂ fragments (10 $\mu\text{g}/\text{ml}$), monitored in the presence of 1 mM extracellular CaCl_2 . Data are representative of four experiments with at least four independent clones for each construct with similar cell surface expression. (b) Immunoblot analysis of affinity-purified (AP) CD8- ϵ m fusion protein from the cells in a, left untreated or stimulated for 3 min with biotinylated anti-CD8 alone or then additionally stimulated for 5 min with anti-IgG; CD8- ϵ m affinity-purified with streptavidin-sepharose was analyzed with anti-phosphotyrosine (top), anti-Grb2 (bottom) or anti-CD8 (middle). Right margin, relative molecular masses of marker proteins. Data are representative of two independent experiments.



ligation of YF or NA mutant IgG-BCRs (Fig. 6a). Consistent with results obtained with our Ramos B cell transfectants, YF or NA mutant IgG-BCRs induced lower Ca^{2+} responses in primary B cells (Fig. 6b). These results identify ITT-mediated incorporation of the Grb2 adaptor into the BCR signalosome as being mandatory for the burst-enhancing function of mIgG.

ITT potentiates signaling from IgE-BCRs

Like mIgG, mIgE carries a cytoplasmic segment associated with efficient B cell activation *in vivo*⁵. The amino acid sequence conservation between the γ m and ϵ m cytoplasmic tails is weak except for the ITT in an Asp-Tyr-X-Asn motif (where 'X' is any amino acid; Fig. 7a). Consequently, we tested the signal-amplification capacity of the ϵ m tail segment and in particular that of its ITT. We generated Ramos B cell transfectants that expressed chimeric IgG-IgE-BCRs containing either a wild-type or YF ITT-mutant form of ϵ m. After BCR stimulation, the wild-type but not the YF ϵ m underwent tyrosine phosphorylation and concomitant association with Grb2 (Fig. 7b,c). YF ϵ m induced lower Ca^{2+} responses in Ramos transfectants and primary B cells than did wild-type ϵ m (Fig. 7d and Supplementary Fig. 10). Thus, the ITT of ϵ m undergoes phosphorylation-dependent association with Grb2 to increase Ca^{2+} signaling from the IgE-BCR independently of other parts of mIgE. In addition, these findings suggest that the ITT motif is an amplification unit that functions in the context of very different amino acid environments (for example, γ m and ϵ m).

ITT-mediated costimulation requires BCR integration

Having established an active contribution of mIgG and mIgE to BCR-controlled Ca^{2+} mobilization and proliferative expansion, we sought to determine whether the underlying ITT represents an autonomous B cell signaling module or whether its signaling capacity relies on close association with the ITAM-controlled BCR signal-initiation 'machinery'. We therefore removed the ϵ m tail from the BCR and expressed it in Ramos B cells as a separate entity fused to CD8. The transfectants also expressed the YF IgG2a-BCR, which, like the IgM-BCR, failed to directly recruit Grb2 and induce Ca^{2+} augmentation (Fig. 3). This setup allowed us to activate the IgG-BCR mutant and the CD8- ϵ m fusion proteins individually or in combination (Supplementary Fig. 11) and thereby to test the idea of BCR-intrinsic costimulation of Ca^{2+} mobilization. We first treated cells with anti-CD8 and analyzed them for 1 min, during which no Ca^{2+} mobilization could be detected (Fig. 8a). Subsequently, we co-crosslinked the CD8- ϵ m molecule with the ITT-inactivated IgG-BCR by addition of anti-IgG F(ab')₂ fragments that recognized both anti-CD8 as well as the IgG-BCR. This treatment induced a strong and long-lasting Ca^{2+} response in cells expressing wild-type CD8- ϵ m but only a transient response in cells expressing YF CD8- ϵ m (Fig. 8a). Ramos cells

expressing the CD8- ϵ m wild-type chimera in the absence of mIgG2a showed a weak and delayed increase in intracellular Ca^{2+} concentrations (Fig. 8a). These experiments showed that cocrosslinking of the CD8- ϵ m chimera with the YF IgG-BCR mimics the sustained Ca^{2+} signal of wild-type IgE-BCRs or IgG-BCRs. This finding further supports our conclusion that ITT-mediated signal amplification works in the context of different polypeptides. However, these results indicate that for efficient signal amplification, the costimulatory relay needs to be physically incorporated into the BCR platform. This need for BCR integration for signaling is explained by the finding that only after BCR cocrosslinking did the wild-type CD8- ϵ m chimera undergo inducible phosphorylation and show binding to Grb2 (Fig. 8b).

DISCUSSION

Here we have elucidated the molecular mechanism that distinguishes antigen-induced signaling by the IgM-BCR from that induced by BCRs containing IgG or IgE in naive and class-switched B cells, respectively. Biochemical and mutational analyses combined with the generation of chimeric receptors showed that a sustained intracellular Ca^{2+} response as well as the burst-enhancing function of the mIgG and mIgE cytoplasmic segments is accomplished by inducible phosphorylation of the evolutionary conserved ITT and subsequent recruitment of the adaptor protein Grb2. These signaling processes occurred after stimulation of the BCR with antibodies as well as with antigen and were observed in various B cell lines of mouse and human origin as well as in primary B lymphocytes from the mouse. Our data establish that in contrast to the present dogma, tyrosine phosphorylation of the activated BCR is not confined to the ITAM-carrying Ig- α and Ig- β subunits but extends to the ITT in mIgG and mIgE. ITT phosphorylation increases the number of tyrosine-based signaling motifs in the IgG-BCR and the IgE-BCR to seven (two ITAM phosphotyrosines per Ig- α and Ig- β , one non-ITAM phosphotyrosine in Ig- α and two phosphotyrosines per mIgG or mIgE).

The ITT, especially that of mIgG, and its 'downstream' signaling properties are similar to those described for the well known phosphorylation-acceptor site Tyr-Met-Asn-Met of CD28, a costimulatory molecule key for activation of naive but not memory T cells²⁷. Phosphorylated CD28 has two putative binding partners, p85 of PI(3)K and Grb2, but only association with the latter promotes Ca^{2+} signaling and synthesis of interleukin 2 after CD28 activation^{28–30}. Likewise, Grb2 but not PI(3)K seems to be the main 'downstream' effector of phosphorylated ITT. That conclusion is supported first by the exquisite specificity of phosphorylated ITT for Grb2 demonstrated by our peptide-binding studies. In line with that finding, a published peptide library screen for highly specific SH2-domain ligands has shown that the SH2 domain of Grb2 but not that of p85 tolerates positively charged amino acid residues at the position +1 to the phosphotyrosine³¹, an amino acid arrangement

that is strongly conserved in γ -ITTs. Second, more direct evidence for the intimate functional connection between phosphorylated ITT and Grb2 is provided by the diminished proliferation and Ca^{2+} response of cells expressing the mIgG mutant with the Asn-to-Ala substitution, which impaired Grb2 recruitment but preserved ITT phosphorylation and the critical methionine of the potential p85-binding motif. Notably, the CD28-related inducible costimulator ICOS, which has a cytoplasmic Tyr-Met-Phe-Met motif, is unable to stimulate interleukin 2 production in T cells unless the phenylalanine in the phosphorylation site is replaced with asparagine to enable Grb2 binding³². Another notable similarity between CD28- and ITT-mediated costimulation is their dependence on the presence of a functional antigen receptor complex. Although the details of CD28 signal transduction are still elusive, it seems that CD28 couples to protein tyrosine kinases only indirectly through the T cell antigen receptor²⁹. Likewise, the isolated CD8- ϵ m chimera was phosphorylated only after cocrosslinking with the BCR, and vigorous 'super-crosslinking' was needed to partially overcome the need for BCR integration in our *in vitro* model system. *In vivo*, the unique process of immunoglobulin class switching directly integrates the costimulatory ITT signaling module into the BCR. The resulting BCR-intrinsic Ca^{2+} -amplification capacity may render IgG- and IgE-switched memory B cells less dependent on the accessory signals needed for primary activation of naive IgM^+ B cells^{6,7}.

The fact that transient versus sustained Ca^{2+} fluxing in lymphocytes can act as key determinant of overall immune cell function is again demonstrated by CD28-mediated signal transduction. The disastrous cytokine storm observed during a phase I clinical trial with the monoclonal CD28-specific antibody TGN1412 (ref. 33) has been attributed to the ability of this antibody to induce long-lasting Ca^{2+} entry across the T cell membrane³⁴. Stimulation of CD28 with 'superagonistic' antibodies can partially substitute for the presence of the T cell antigen receptor³⁴, which is again reminiscent of the responsiveness of our CD8- ϵ m chimera after vigorous 'super-crosslinking' in the absence of mIgG. Thus, the small Tyr-X-Asn-Met phosphorylation motif in CD28 and in BCRs containing IgG or IgE seems to be a powerful costimulatory module that controls the effector phase of T lymphocytes and B lymphocytes, respectively.

Several mechanisms are possible for how ITT-Grb2 complex formation improves the Ca^{2+} response. Given that Grb2 constitutively interacts in an SH3 domain-dependent way with SLP-65 (refs. 35,36), its recruitment to phosphorylated ITT might stabilize the Ca^{2+} -initiation complex at the activated BCR. Additionally, the reported feedback-inhibition loop of Grb2 (refs. 37,38) is locked when Grb2 is anchored to the ITT. It is also likely that phosphorylation of the ITT and its interaction with Grb2 directly affects the 'open' conformation of activated Ig- α -Ig- β , which is indeed more stable in the IgG-BCR than the IgM-BCR³⁹. These potential Grb2 effector mechanisms are not necessarily mutually exclusive but may operate simultaneously to produce higher concentrations of InsP_3 and diacylglycerol, which eventually increase the influx of Ca^{2+} across the plasma membrane via the endoplasmic reticulum Ca^{2+} sensor STIM1 (refs. 40–42), InsP_3 receptors in the plasma membrane^{43,44} or members of the TRP family of Ca^{2+} channels^{45,46}. These events may also be of particular importance for the homeostatic maintenance of memory B cells, which has been shown to rely on PLC- γ 2 expression⁴⁷. Notably, in addition to its involvement in Ca^{2+} signaling, BCR-associated Grb2 may also provide a direct link to activation of the GTPase Ras–mitogen-activated protein kinase pathway, which is pivotal in the IgG memory B cell response⁴⁸. Hence, ITT phosphorylation and recruitment of Grb2 may control multiple signaling pathways, which act in concert to improve the

establishment of secondary immune responses by class-switched B cells. Additional experiments are needed to delineate the complete BCR-Grb2 network. The results of our study, however, provide a molecular basis for the costimulatory function of the γ m and ϵ m cytoplasmic segments during B cell responses in the mouse^{3–5,15,16}. Notably, the dual function of mIg in antigen recognition and signaling described in our study confirms the final postulate of Paul Ehrlich's 'side-chain theory', in which membrane-bound antibodies (antitoxins) actively contribute to cellular activation and differentiation⁴⁹.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession code. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org/>): A001088.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

N.E. designed, did and supervised experiments and wrote the paper; L.M.K. designed and did experiments; C.H., J.L., S.G. and V.S. did experiments; T.T. provided essential reagents; and J.W. supervised the project and wrote the paper.

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ONLINE METHODS

Antibodies and reagents. Goat anti-immunoglobulin F(ab')₂ fragments and antibodies for BCR stimulation and immunoprecipitation were from Jackson ImmunoResearch (Dianova) and Southern Biotech (Biosol), respectively. Anti-phosphotyrosine (mAb 4G10) and anti-Grb2 (mAb 3F2) were from Upstate (Biomol). Anti-phosphotyrosine (mAb 100 and mAb 102) and anti-phospho-SLP-65 (mAb Tyr96) were from Cell Signaling Technology (NEB). Anti-SLP-65 was from BABCo. Anti-PLC-γ2 (Q-20) and anti-Syk (4D10) were from Santa Cruz. Anti-CD8 (MEM-31)⁵⁰ was provided by V. Horejsi. Biotinylated peptides were produced by Eurogentec. Peptide sequences were as follows: γ2am, biotin-NH-KQTISPDPYRNMVIGQ-COOH; α-pepI, biotin-NH-ENLYEGLNLDDCSM YEDISR-COOH; α-pepII, biotin-NH-CSMYEDISRLQGTQYQDVGN-COOH; β-pep, biotin-NH-DHTYEGLNIDQTATYEDIVTL-COOH. *In vitro* stimulation of B cells, the GST-Grb2 SH2 fusion protein, affinity purification of proteins from 1% (vol/vol) Nonidet P-40 lysates and immunoblot analysis have been described¹³.

Cells and expression vectors. The expression vectors for mouse γ2am immunoglobulin heavy-chain variants are based on pSV/γ2am-neo (provided by M. Reth) and were transfected into the human Burkitt lymphoma cell line Ramos by Amaxa technology. Clones were selected in the presence of the aminoglycoside G418 (2 mg/ml) and were maintained in RPMI medium plus 10% (vol/vol) FCS. The cDNA for mouse γ2am was amplified by RT-PCR from Ramos transfectants. The γ2am-ε chimera was generated by overlap-extension PCR. Mutations were introduced by PCR and were verified by sequencing (oligonucleotide sequences, **Supplementary Methods**). Each cDNA for retroviral transfection was ligated into MIGR1. The cDNA encoding chimeric IgM-IgG specific for NIP (4-hydroxy-5-iodo-3-nitrophenylacetyl) was ligated into pABESpuroII and was transfected into K46 cells expressing a NIP-specific λ1 light chain. K46 cells were selected in the presence of puromycin (2.5 μg/ml) or G418 (500 μg/ml). The CD8-εm chimera was generated by ligation of the PCR-amplified coding sequence of the mouse εm cytoplasmic tail to the coding sequence of the human CD8α extracellular and transmembrane domains (provided by B. Schraven). The resultant CD8-εm fusion construct as well as the YF mutant were sequenced and ligated into pcDNA3 (Invitrogen) equipped with a puromycin-resistance cassette (pcDNA3puro). Ramos transfectants were selected with puromycin (0.5 μg/ml).

Tyrosine-phosphorylation analysis of endogenous mIgG. K46 or IIA1.6 cells (5 × 10⁷ cells per ml) were prechilled on ice or were prewarmed for 5 min at 37 °C. Subsequently, biotinylated goat anti-mouse IgG (10 μg/ml; Southern-Biotech) was added to 1 ml of the cell suspension. Prechilled cells were incubated for 1.5 min on ice (unstimulated), and prewarmed cells were incubated for 3 min at 37 °C (stimulated) before they were quickly spun down and washed once with 1 ml ice-cold PBS. Cells were then lysed for 10 min on ice followed by 3 min at 37 °C with lysis buffer containing 0.5% (vol/vol) Nonidet P-40, 0.5% (vol/vol) Triton X-100, 0.25% (wt/vol) sodium deoxycholate and 0.05% (wt/vol) SDS supplemented with phosphatase and protease inhibitors.

Insoluble material was pelleted for 5 min at 20,000g and 4 °C and clear supernatants were used for affinity purification with 30 μl streptavidin sepharose (GE Healthcare) as the matrix. After 1 h of incubation with mild agitation at 4 °C, the matrix was washed extensively with lysis buffer before bound proteins were released by boiling in Laemmli buffer with or without β-mercaptoethanol. After being blotted onto nitrocellulose, tyrosine-phosphorylated proteins were detected with anti-phosphotyrosine (mAb 100 or mAb 102; Cell Signaling Technology-NEB).

Retroviral transduction of primary mouse splenic B cells. Splenocyte samples from 8-week-old C57/BL6 mice were depleted of CD43⁺ cells with anti-CD43 microbeads according to the manufacturer's instructions (Miltenyi). The remaining cells, which were over 90% positive for CD19 and IgM, were incubated for 24 h with lipopolysaccharide (10 μg/ml; Sigma) in RPMI-1640 medium supplemented with 10% (vol/vol) FCS and 50 μM β-mercaptoethanol. Subsequently, 2 × 10⁶ cells were infected with retrovirus-containing supernatants from Plat-E cells that had been transfected previously with MIGR1-containing cDNA, which allowed internal ribosomal entry site-driven coexpression of eGFP with either wild-type or mutant immunoglobulin chains. Transduction efficiencies usually reached 50–70%. At 24 h after infection, cells were loaded with Indo-1-AM for Ca²⁺-flux analysis or were used to monitor BCR-induced proliferation.

Proliferation analysis of retrovirus-transduced cells. At 24 h after retroviral infection, cells were stained with the plasma membrane-intercalating dye CellVue Claret (Sigma) and were seeded in 96-well plates at a density of 2 × 10⁵ cells per 200 μl medium. After stimulation for 96 h with goat anti-mouse IgG F(ab')₂ fragments (10 μg/ml), the CellVue MFI of eGFP⁺ cells was normalized to that of eGFP[−] cells from the same wells. The decrease in fluorescence intensity served as measure of proliferation.

Analysis of Ca²⁺ mobilization. For analysis of intracellular Ca²⁺ concentration, cells were loaded for 30 min at 30 °C with 1 μM Indo-1-AM (Molecular Probes) in RPMI medium containing 5% (vol/vol) FCS and 0.015% (vol/vol) Pluronic F127 (Molecular Probes) under mild agitation. Subsequently, cells were washed with and resuspended in Kreb's Ringer solution composed of 10 mM HEPES, pH 7.0, 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 10 mM glucose. After basal Ca²⁺ concentrations were monitored, cells were stimulated with various reagents. The fluorescence ratio of Ca²⁺-bound Indo-1 (405 nm) to Ca²⁺-unbound Indo-1 (530 nm) was monitored on an LSR II cytometer (Becton Dickinson). InsP₃ in 5 × 10⁶ cells was measured with the D-myo-Inositol 1,4,5-Trisphosphate (IP₃) - [³H] Biotrak Assay system according to the manufacturer's instructions (GE Healthcare).

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