assessed by addition of 300-fold molar excess of radioinert 17β-estradiol. Free [^{3}H]17β-estradiol was removed by incubation with Chardex (contains 5% activated charcoal and 0.5% dextran dissolved in phosphate-buffered saline) for 10 min at 4°C and centrifugation for 3 min at 15,000*g*. The bound [^{3}H]17β-estradiol was measured by scintillation counting. The data shown are representative of at least three independent experiments (D. M. Klotz, J. A. McLachlan, S. F. Arnold, in preparation).
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32. Ishikawa cells grown in a 35-mm plate were transfected with 2 μg of pERE-luc (containing two copies of the vitellogenin estrogen response element linked to the luciferase gene), 1 μg of a control β-Gal plasmid, and 20 ng of hER in a mammalian expression vector with 10 μl of lipofectamine (GIBCO BRL) for 5 hours. The cells were treated with vehicle, estradiol, or 2′,4′,6′-trichloro-4-biphenylol or 2′,3′,4′,5′-tetrachloro-4-biphenylol (or both) in Dulbecco's modified Eagle's medium with 5% charcoal-stripped fetal bovine serum for 18 hours. All chemicals were dissolved in DMSO and added to the media so that the concentration of DMSO did not exceed 1%. The cells were collected by incubation with lysis buffer (Analytical Luminescence, Ann Arbor, MI) for 15 min at 25°C, the protein concentration determined with the Bio-Rad protein assay reagent, and the β-Gal activity measured as described (*18*). The amount of extract used in the luciferase assay was normalized for protein and β-Gal activity. The luciferase assay was performed in the Monolight 2010 as recommended by the manufacturer (Analytical Luminescence).
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Regulation of the Inositol 1,4,5-Trisphosphate Receptor by Tyrosine Phosphorylation

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Tyrosine kinases indirectly raise intracellular calcium concentration ($[Ca^{2+}]_i$) by activating phospholipases that generate inositol 1,4,5-trisphosphate (IP_3). IP_3 activates the IP_3 receptor (IP_3R), an intracellular calcium release channel on the endoplasmic reticulum. T cell receptor stimulation triggered a physical association between the nonreceptor protein tyrosine kinase Fyn and the IP_3R, which induced tyrosine phosphorylation of the IP_3R. Fyn activated an IP_3-gated calcium channel in vitro, and tyrosine phosphorylation of the IP_3R during T cell activation was reduced in thymocytes from *fyn*$^{-/-}$ mice. Thus, activation of the IP_3R by tyrosine phosphorylation may play a role in regulating $[Ca^{2+}]_i$.

The IP_3R forms the IP_3-gated Ca^{2+} release channel on the endoplasmic reticulum in many cell types, including neurons (*1*) and T cells (*2*). The human type 1 IP_3R (IP_3R1) is a 308-kD polypeptide that contains two potential tyrosine phosphorylation sites, at residues 482 (Glu-Asp-Leu-Val-Tyr) and 2617 (Asp-Ser-Thr-Glu-Tyr) (*2*). Amino acid 482 is adjacent to the IP_3-binding site (*3*), and amino acid 2617 is near the COOH-terminus (*4*).

The IP_3R is autophosphorylated on serine and is phosphorylated by protein kinases A, C, and G and by Ca^{2+}-calmodulin–dependent kinase II (CaMkII) in vitro (*5*). Members of the Src family of nonreceptor protein tyrosine kinases function in signal transduction in the brain (*6*) as well as in T cells (*7*, *8*). To determine whether tyrosine phosphorylation of the IP_3R occurred, we immunoprecipitated the Ca^{2+} release channel with an IP_3R1-specific antibody (anti-IP_3R1) from both brain and T lymphocytes and subjected it to kinase assays with two nonreceptor protein tyrosine kinases, Src and Fyn. Both kinases induced tyrosine phosphorylation of the brain IP_3R (Fig. 1A). Similar results were obtained with canine, rabbit, and murine brain microsomes (*9*) and with purified IP_3R (Fig. 1A), which suggests that the kinase acts on the channel directly, not through an intermediary molecule.

Fyn and Src induced tyrosine phosphorylation of the IP_3R from unstimulated Jurkat cells (Fig. 1B) in vitro. Tyrosine phosphorylation of the IP_3R from unactivated Jurkat cells by Fyn was greater than that of the IP_3R from cells activated by incubation with monoclonal antibody to CD3 (CD3 mAb) (Fig. 1B). This result indicated that Fyn induces tyrosine phosphorylation of the IP_3R during T cell receptor (TCR) stimulation, and therefore that exogenous Fyn does not induce further tyrosine phosphorylation of the IP_3R in vitro.

To determine whether tyrosine phosphorylation of the IP_3R occurred in vivo, we prepared anti-phosphotyrosine immunoblots of IP_3R immunoprecipitated from unstimulated and from TCR-stimulated Jurkat cells. IP_3R was detected in both activated and unactivated cells, but tyrosine-phosphorylated IP_3R was detected only in TCR-stimulated cells (Fig. 1C). This finding indicated that tyrosine phosphorylation of the IP_3R occurs in intact cells.

During T cell activation, Fyn physically associates with the TCR (*10*) and the IP_3R cocaps with the TCR (*11*). To determine whether Fyn physically associated with the IP_3R during T cell activation, we used anti-IP_3R1 or Fyn mAb to immuno-

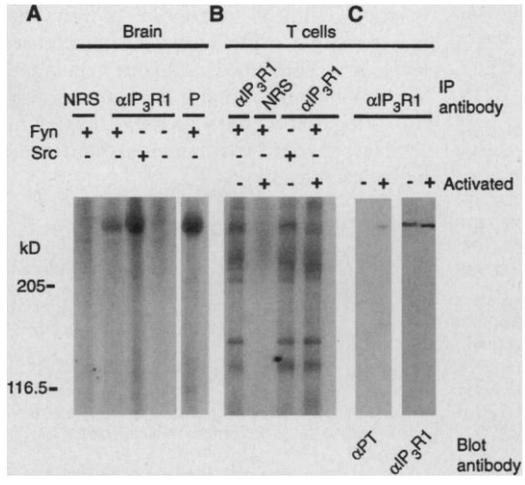

Fig. 1. Tyrosine phosphorylation of the ~300-kD IP_3R in brain and T lymphocytes (Jurkat) by Src and Fyn. IP_3R was immunoprecipitated from canine brain microsomes (**A**) and from lysates of unactivated Jurkat cells or cells activated with CD3 mAb (**B**). Immunoprecipitated proteins or purified IP_3R (P) were used in kinase assays with or without exogenous Fyn or Src (*21*). Preimmune serum (NRS) or affinity-purified anti-IP_3R1 (αIP_3R1) were used for immunoprecipitations (*21*). (**C**) Immunoprecipitated IP_3R from activated or unactivated Jurkat cells was immunoblotted with anti-phosphotyrosine (αPT) (4G10) and then, after stripping, with anti-IP_3R1 as described (*2*).

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precipitate proteins from lysates of activated and unactivated Jurkat cells or murine thymocytes. IP_3R and Fyn coimmunoprecipitated from activated Jurkat cells and from thymocytes, but not from unactivated cells (Fig. 2, A, B, and D). No Fyn was detected in immunoprecipitates prepared with anti-IP_3R1 from lysates of IP_3R1-deficient Jurkat cells (*12*) that had been stimulated with CD3 mAb (Fig. 2C), and no CD3 mAb was detected by immunoblot in lysates from TCR-stimulated Jurkat cells (9). Thus, immunoprecipitation of Fyn by anti-IP_3R1 was dependent on the presence of IP_3R1 in the cell and did not result from contamination with the CD3 mAb that was used to activate the cells.

To determine whether tyrosine phosphorylation of IP_3R had a functional effect on the channel, we made single-channel measurements on IP_3-gated channels in cerebellar microsomes fused to planar lipid bilayers (Fig. 3). The IP_3-gated channel was activated by IP_3 (2 to 5 μM) and had a Ca^{2+} conductance of 54 ± 3 pS (with 53 mM Ca^{2+} in the trans chamber at 0 mV), with a mean open time (τ_1) of 2.6 ms (all channel openings were used to calculate conductance and τ). The channel was blocked by heparin (50 μg/ml), consistent with the behavior of IP_3-gated Ca^{2+} channels. These properties were comparable to those reported for the cerebellar IP_3-gated channel (*13*). The single-channel conductance of 54 ± 3 pS represented the full conductance of the channel. We also observed subconductance states, although >90% of channel openings were to the full conductance, as previously reported (*14*, *15*).

Addition of Fyn (3 U/ml, where 3 U ≈ 1 μg of Fyn that transfers 1 fmol of PO_4^{2-} per minute to the substrate) increased the average open probability (p_o) of the channel to 3.5 times that of untreated channels ($P < 0.001$, $n = 5$, Fig. 3A) but did not alter the conductance or the mean open time (Fig. 3B). The increased p_o of the IP_3R occurred after addition of the same amount of Fyn that caused tyrosine phosphorylation of the channel (Fig. 1A). Heparin (50 μg/ml) blocked the channel after activation by Fyn plus IP_3 (Fig. 3A). Fyn added before IP_3 did not activate channels, which indicated that the IP_3R must first be activated by IP_3 before it can be modulated by Fyn ($n = 3$, Fig. 3C). Fyn that had been boiled for 5 min did not activate the IP_3-gated Ca^{2+} channel ($n = 3$), and addition of the kinase inhibitor genistein (1 μM) before Fyn blocked the effect of the kinase ($n = 3$). When Mg-dependent adenosine triphosphate (MgATP) was omitted from the bilayer chamber, Fyn had no effect on the IP_3-gated channel ($n = 3$, Fig. 3D), which suggested that the kinase activity of the enzyme is required for its effects on the p_o of the channel. Addition of Fyn also increased the p_o of the purified IP_3R ($n = 3$, Fig. 3E), which suggested that this activation results from direct phosphorylation of the channel itself rather than from an

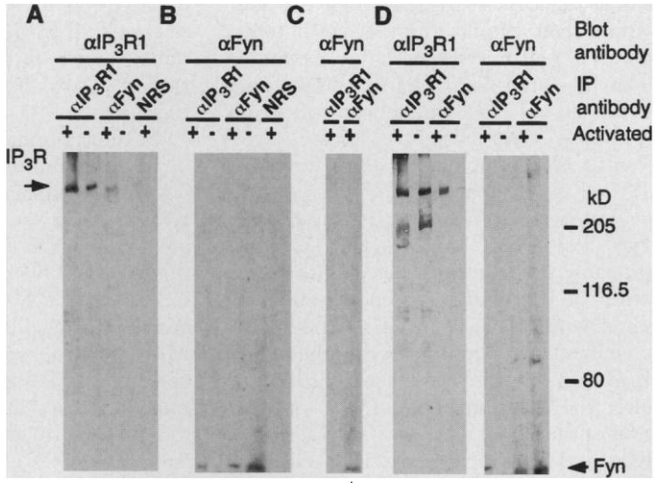

Fig. 2. Physical association between Fyn and IP_3R1 in activated T lymphocytes. IP_3R and Fyn were immunoprecipitated from lysates of unactivated or activated Jurkat cells (*21*), fractionated by SDS-PAGE (6% gels), and transferred to nitrocellulose as described (*2*). Identical immunoblots were probed with anti-IP_3R1 (αIP_3R1) (**A**) or with Fyn mAb (αFyn) (**B**) and detected with chemiluminescence. (**C**) IP_3R and Fyn were immunoprecipitated from lysates of activated IP_3R1-deficient Jurkat cells (*12*) and immunoblotted with anti-IP_3R1 or Fyn mAb. (**D**) IP_3R and Fyn were immunoprecipitated from unactivated and activated wild-type murine thymocytes and immunoblotted with anti-IP_3R1 or Fyn mAb.

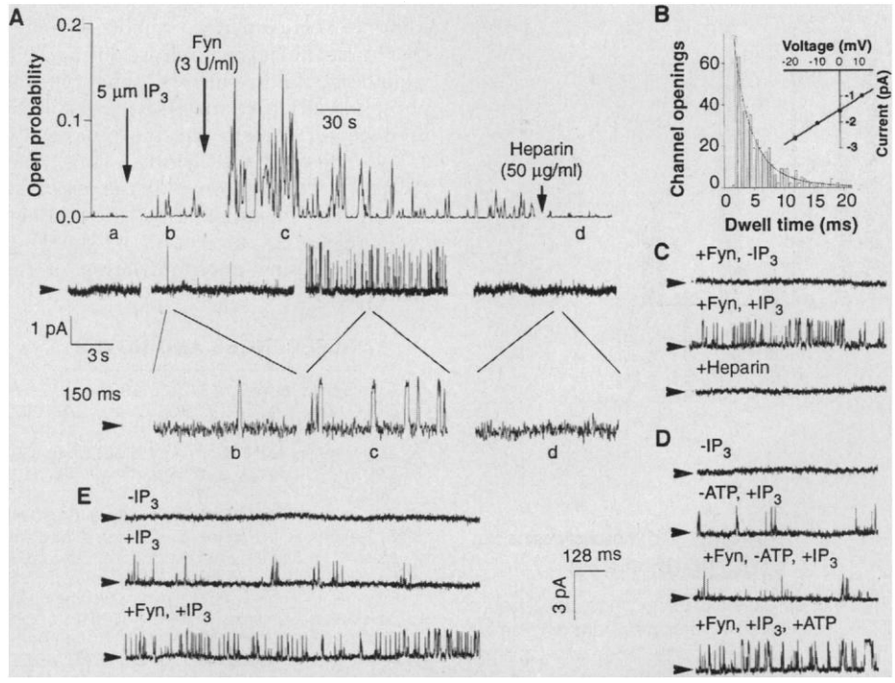

Fig. 3. Activation of the IP_3-gated Ca^{2+} channel by Fyn. (**A**) Upper panel: Open probability (p_o) plotted against time for the IP_3-gated Ca^{2+} channel in a planar lipid bilayer. Downward arrows indicate the addition of reagents to the cis or cytoplasmic chamber. Lower panel: Tracings show representative channel openings at the indicated time points [(a) to (d)] in compressed and expanded time scales, taken from one continuous recording of currents passing through a single channel. The free [Ca^{2+}] was 750 nM in the cis chamber. (**B**) Open-time distribution of the IP_3-gated Ca^{2+} channel after incubation with Fyn. Fitting with two exponentials yielded mean open times of $\tau_1 = 2.6$ ms and $\tau_2 = 6.2$ ms, and the single-channel conductance was 56 ± 4 pS; these values are comparable to those for the unphosphorylated channel. (**C**) Requirement of IP_3 for the effects of Fyn on the p_o of the channel. (**D**) Requirement of MgATP for the effects of Fyn (3 U/ml). (**E**) Activation of purified IP_3R by Fyn (3 U/ml). Breaks in the top tracing are points where stirring artifacts occurred during addition of reagents. Tracings in (A), (C), (D), and (E) were recorded from separate experiments. Arrowheads indicate the closed state of the channel; channel openings are in the upward direction (*22*).

intermediary molecule. ATP activates the IP_3-gated channel (*16*); however, the increase in p_o after addition of Fyn was apparently not caused by ATP, because in each experiment the p_o of the channel was stable for at least 3 min before the addition of Fyn. Similar results were obtained when T cell microsomes were examined. The IP_3-gated Ca^{2+} channel from Jurkat cells had a Ca^{2+} conductance of 50 pS, with τ = 3 ms and p_o = 2%; addition of Fyn (3 U/ml) increased the p_o from 2 to 12% ($P < 0.01$, $n = 3$) (*17*).

The decrease in the activating effect of Fyn over several minutes (Fig. 3A) was probably not the result of dephosphorylation of the channel because under the same conditions as those in the bilayer chamber, no dephosphorylation of the IP_3R was detected within the first 5 min after the addition of kinase (9). Activation of the IP_3R by Fyn was observed when $[Ca^{2+}]_i$ was as high as 750 nM (Fig. 3A). Maximal activation of the IP_3R occurs when $[Ca^{2+}]_i \approx 300$ nM, and at $[Ca^{2+}]_i$ = 750 nM the channel activity is reduced

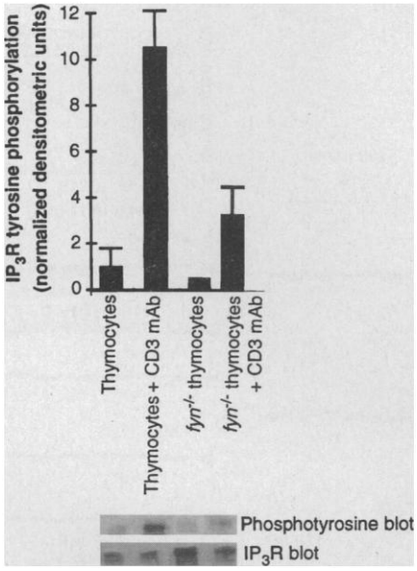

Fig. 4. Decreased tyrosine phosphorylation of IP_3R in activated thymocytes from $fyn^{-/-}$ mice. Thymocytes were isolated from wild-type and $fyn^{-/-}$ mice. Proteins immunoprecipitated with anti-IP_3R1 from lysates of unactivated or activated cells were immunoblotted with anti-phosphotyrosine and then, after stripping, with anti-IP_3R1 (*21*). Upper panel: Amount of tyrosine-phosphorylated IP_3R as determined by densitometric scanning of the band that comigrated with IP_3R. Results are presented as densitometric units normalized to the value for unstimulated control thymocytes. Error bars represent SEM. Lower panel: Representative immunoblots of IP_3R1 immunoprecipitated from unactivated and activated wild-type and $fyn^{-/-}$ thymocytes blotted with anti-IP_3R1 or anti-phosphotyrosine.

(*14*). The observation that tyrosine-phosphorylated IP_3R has increased activity (increased p_o) at higher $[Ca^{2+}]_i$ suggests that tyrosine phosphorylation could have the effect of shifting the Ca^{2+} dependence for inactivation to higher values of $[Ca^{2+}]_i$. Such a shift might permit the channel to remain open during the sustained elevation of $[Ca^{2+}]_i$ associated with T cell activation.

Several nonreceptor protein tyrosine kinases, including Fyn, have important roles in T cell signaling (*8*). Inhibition of tyrosine kinase activity blocks TCR-mediated signal transduction and alters Ca^{2+} fluxes (*18*). Overexpression of $p59^{FynT}$ in immature $CD4^+CD8^+$ thymocytes increases intracellular Ca^{2+} release (*7*), whereas thymocytes from $fyn^{-/-}$ mice have defective TCR signaling with reduced intracellular Ca^{2+} release (*19*, *20*). Relative to wild-type thymocytes, thymocytes from $fyn^{-/-}$ mice (*19*) exhibited one-third as much tyrosine phosphorylation of the IP_3R during TCR stimulation with CD3 mAb (Fig. 4). Taken together, these findings suggest that reduced tyrosine phosphorylation of the IP_3R in thymocytes from $fyn^{-/-}$ mice may contribute to defective intracellular Ca^{2+} release during TCR stimulation. Our results show that tyrosine phosphorylation of the IP_3R, probably by nonreceptor protein tyrosine kinases, increases the p_o of the channel. Thus, nonreceptor protein tyrosine kinases appear to be capable of modulating intracellular Ca^{2+} release by activation of the IP_3R through tyrosine phosphorylation of the channel.

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21. Human Jurkat T lymphocytes were maintained in RPMI 1640 media supplemented with fetal bovine serum (10%). Cells were stimulated by cross-linking with CD3 mAb (IgG2a, a mouse mAb to human CD3) for 3 min before collection, washed five times, then harvested and lysed as described (*2*, *12*). For immunoprecipitations, preimmune serum, anti-IP_3R1, or rabbit antibody to Fyn was added at dilutions of 1:500, 1:500, and 1:50, respectively. Samples were incubated for 2 hours on ice, 75 μl of protein A–Sepharose was added, and the mixture was incubated for 1 hour at 4°C with gentle agitation, followed by centrifugation at 4000*g* for 15 min. Immunoprecipitates were washed five times with lysis buffer (*2*) and once with a kinase buffer [20 mM Hepes (pH 7.5), 1 mM dithiothreitol, and 10 mM $MnCl_2$], and were incubated at room temperature for 10 min with 100 μM ATP, 10 μCi [γ-^{32}P]ATP (3000 μCi/mmol), and either Src or Fyn (3 U; Upstate Biotechnology, Lake Placid, NY) in 25-μl reactions. Kinase reactions were stopped by adding Laemmli sample buffer, and proteins were fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 6% gels, which were then dried and autoradiographed. For immunoblots, anti-IP_3R1 was used at 1:10,000 dilution and a Fyn mAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 dilution. Anti-IP_3R1 recognizes the COOH-terminal 10 amino acids of IP_3R1 that are unique among IP_3Rs (*2*, *12*). Relative amounts of tyrosine-phosphorylated IP_3R were estimated by densitometric scanning of gels with Adobe Photoshop 3.0 and Image 1.44 software.
22. Planar lipid bilayers were formed across a hole (diameter 0.05 to 0.3 mm) separating cis and trans chambers, with dioleoylphosphatidylethanolamine and dioleoylphosphatidylethanolserine (molar ratio 3:1) in *n*-decane (20 mg of lipids per milliliter). Cerebellar microsomes, isolated as described [K. A. Stauderman, G. D. Harris, W. Lovenberg, *Biochem. J.* **255**, 677 (1988)], or IP_3R, purified by sucrose density gradient centrifugation [S. R. Hingorani and W. S. Agnew, *Methods Enzymol.* **207**, 573 (1992)] and incorporated into liposomes, was added to the cis chamber. Ryanodine receptor–Ca^{2+} release channels were blocked by addition of 5 to 20 μM ruthenium red to the cis chamber. IP_3 (2 to 5 μM) was applied to the cis chamber. Ca^{2+} current was detected with an Axopatch 200 amplifier, recorded on digital audio tapes, and evaluated with Axopatch pClamp6 as described [A.-M. B. Brillantes *et al.*, *Cell* **77**, 513 (1994)]. The cis chamber contained 440 nM free Ca^{2+} (unless otherwise noted), 0.5 mM MgATP, and 250 mM Hepes (pH 7.35). The trans chamber contained 53 mM $Ca(OH)_2$ and 250 mM Hepes (pH 7.35). Calculations of p_o were based on samplings obtained for 2 min before and after addition of each reagent.

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