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Activation of T lymphocytes by cross-linking of glycophospholipid-anchored Thy-1 mobilizes separate pools of intracellular second messengers to those induced by the antigen–receptor/CD3 complex

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SUMMARY

T cells can be activated, not only by the conventional (antigen–receptor/CD3 complex) route, but also by cross-linking any one of their lipid-anchored surface glycoproteins. We have compared early transmembrane signalling events mediated through CD3 with those mediated through Thy-1, a lipid-linked surface glycoprotein, on the human lymphoid cell line Jurkat and transfectants expressing higher levels of Thy-1. Cross-linking of Thy-1 causes immediate phosphatidylinositol (PI) turnover and an influx of extracellular Ca^{2+}, while releasing very little Ca^{2+} from intracellular stores. CD3 activation, on the other hand, causes PI turnover which releases intracellular Ca^{2+}, and only secondarily induces an influx of extracellular ions. The Thy-1 response is detectable at very low levels of surface Thy-1, and is not mimicked by enzymatic removal of lipid-linked proteins from the cell surface. The Thy-1-induced Ca^{2+} influx is more sensitive to L channel blockers than the CD3-mediated flux. These results indicate that the initial stages of Thy-1-mediated activation involve the rapid and extensive mobilization of the intracellular second messengers, PI and Ca^{2+}, by mechanisms separate to those activated by the antigen–receptor/CD3 complex.

INTRODUCTION

Cell-surface molecules which are anchored to the membrane via a glycophospholipid tail do not span the lipid bilayer. They could be expected, therefore, to interact only with molecules in the cell's environment, without mediating intracellular changes in response to such interactions. In fact, studies of these lipid-anchored molecules (e.g. Thy-1, Ly-6, Qa-2) on T lymphocytes suggest exactly the opposite: all, when cross-linked on the surface by antibodies, activate lymphocytes[1] and so clearly mediate transmembrane signalling, which profoundly alters the cell's metabolism. Moreover, this activation potential requires the glycophospholipid anchor, and is lost when a conventional polypeptide transmembrane anchor is substituted.[2]

This class of surface molecules is in other respects a very diverse group, with little in common apart from their membrane attachment and ability to activate lymphocytes.[3] Is, therefore, this property of activation physiologically significant, or is it a non-specific effect caused, for instance, by gross perturbation of the outer lipid leaflet affecting intracellular signalling pathways? Here we investigate the immediate effects of Thy-1-mediated activation on two intracellular second messenger systems, looking in particular at the kinetics, dose-dependency, and mechanism, in order to assess whether it could represent a primary transmembrane signalling pathway or be a secondary consequence of antigen cross-linking. We compare it at all stages with that of the well-studied mechanism of the antigen-dependent response mediated via the conventional transmembrane-spanning CD3 complex in the same cells.[4–6]

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MATERIALS AND METHODS

Cells and transfection

The J6 Jurkat subline was transfected by electroporation (240V, 10 msec) with 25 μg/ml of DNA (8·2 kb EcoR1 genomic Thy-1 fragment[7] cloned into PSV2neo, linearized at the Pvul site) per 3×10^6 cells in 0·75 ml. Stable transfectants were selected and cloned by limiting dilution in RPMI-1640 with 5% foetal calf serum (FCS) containing 1 mg/ml G418 (Sigma, Poole, Dorset, U.K.). These cells proved positive when tested for mycoplasma by standard bacteriological culture methods. The Thy-1-mediated Ca^{2+} influx experiments were particularly sensitive to the mycoplasma status of the cells, which was reduced but not eliminated by passage through G418-containing medium. The cells were therefore passaged through two cycles, each of 2

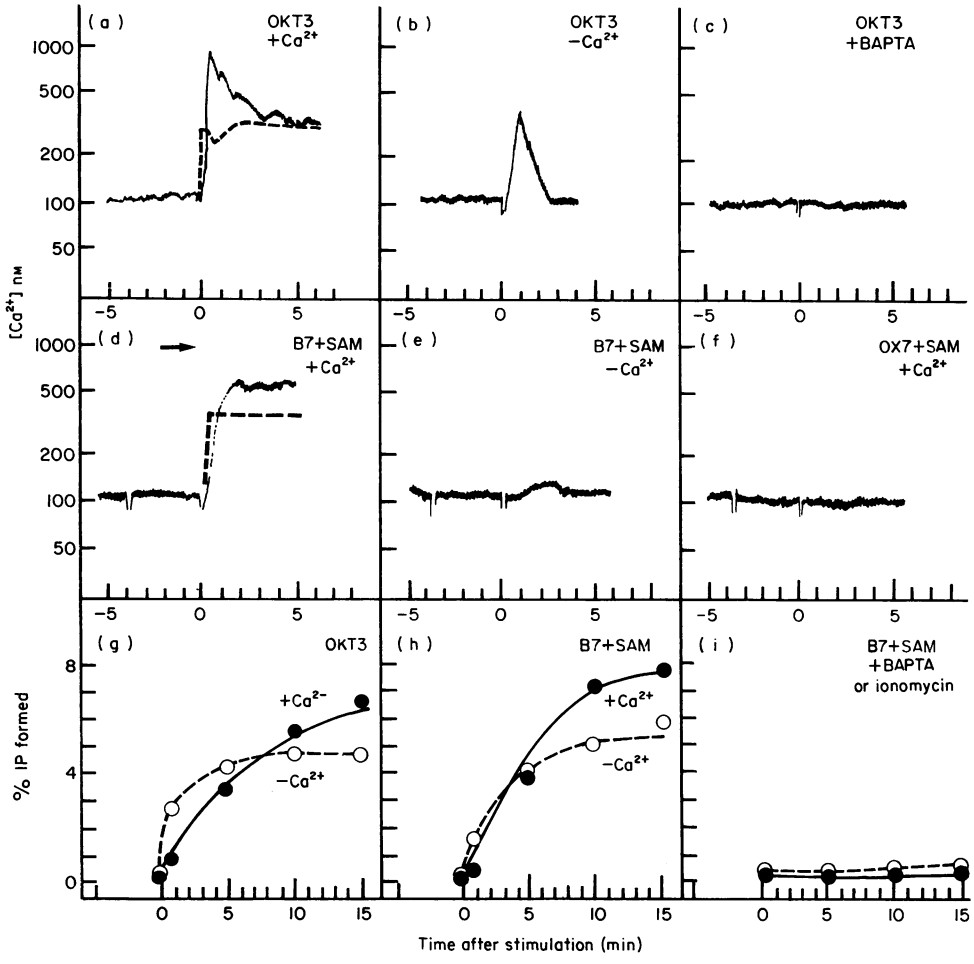

Figure 1. Ca^{2+} (a–f) and PI (g–i) responses mediated through CD3 by the OKT3 antibody (a–c, g) and through Thy-1 by the B7 antibody followed by sheep anti-mouse IgG (SAM) (d–f, h, i). The *x* axis shows elapsed time in minutes, the point of addition of the stimulatory antibody (OKT3 or SAM) being taken as 0. In the Ca^{2+} elevation studies the break in the trace shows the addition of antibody, in (d–f) the primary B7 antibody was added at −4 min. The dashed lines in (a, d) show the binding of ^{125}I-labelled OKT3 (a) or SAM (d) to the cells, done under the same conditions (10-second time-points assayed in triplicate, three separate determinations). The arrow in (d) shows the maximum Ca^{2+} level attained by OKT3 stimulation. + or $-Ca^{2+}$ refer to the presence or absence of this ion in the extracellular buffer; BAPTA is the intracellular Ca^{2+} buffering agent (○ in i); Ca^{2+} ionophore ionomycin (● in i) was added at 0 min, without any antibody addition.

weeks, in Mycoplasma Removal Agent (Flow, High Wycombe, Bucks, U.K.; 1:100 dilution in culture medium) and gentamycin (Flow; 200 μg/ml). Transfected lines additionally had G418 (1 mg/ml) present for this treatment. Subsequent tests for mycoplasma were always negative, and the results obtained with these cells are presented here.

Antibodies

The anti-human Thy-1 clone B7[8] was kindly provided by Dr Mary Ritter, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K. Cells were grown in serum-free culture in Nutridoma NS (Boehringer Mannheim, Mannheim, Germany) from which antibody was purified by a protein A column followed by gel filtration on a Waters 300SW column. OKT3 cells, producing antibody to human CD3 were obtained from the ATCC (Rockville, MD) collection, and antibody similarly purified. OX7 anti-Thy-1.1 IgG was used as a control. Sheep anti-mouse IgG (SAM) was immunoaffinity purified on a mouse IgG–Sepharose CL4B column, and then gel filtered as above. Fab antibody fragments were produced by digestion with immobilized papain (Pierce, Life Science Labs Ltd, Luton, U.K.), and the fragments not retained by the protein A column were gel filtered to obtain the Fab. Immunoglobulin concentration was estimated from the absorbance at 280 nm using an extinction coefficient of 1·38 in a 1-cm path. Radioiodination was done by a modified Chloramine T method.[9] For fluorescence detection, B7 IgG was directly coupled with fluorescein isothiocyanate (FITC); OKT3 and (in indirect fluorescence) B7 were detected using affinity-purified FITC-labelled rabbit $F(ab)'_2$ anti-mouse IgG.

The rate of binding of radioiodinated antibodies to the cells was determined by layering 100 μl of cells (2×10^5) over 0·5 ml of 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (containing Ca^{2+} and Mg^{2+} at 1·29 and 0·94 mM, respectively) in a 1·5 ml microfuge tube held at 37° in a water bath. Pre-warmed antibody was added (100 μl in 1% BSA/PBS: B7 added at 1·0 μg/ml, left for 3 min, then 100 μl of 8 μg/ml SAM (containing 5% ^{125}I-SAM to give a specific activity of 1·2 mCi/mg) added; OKT3 was added at 4 μg/ml (containing 20% ^{125}I-OKT3 to give a specific activity of 4·5 mCi/mg). After the appropriate incubation the tubes were centrifuged at 14,000

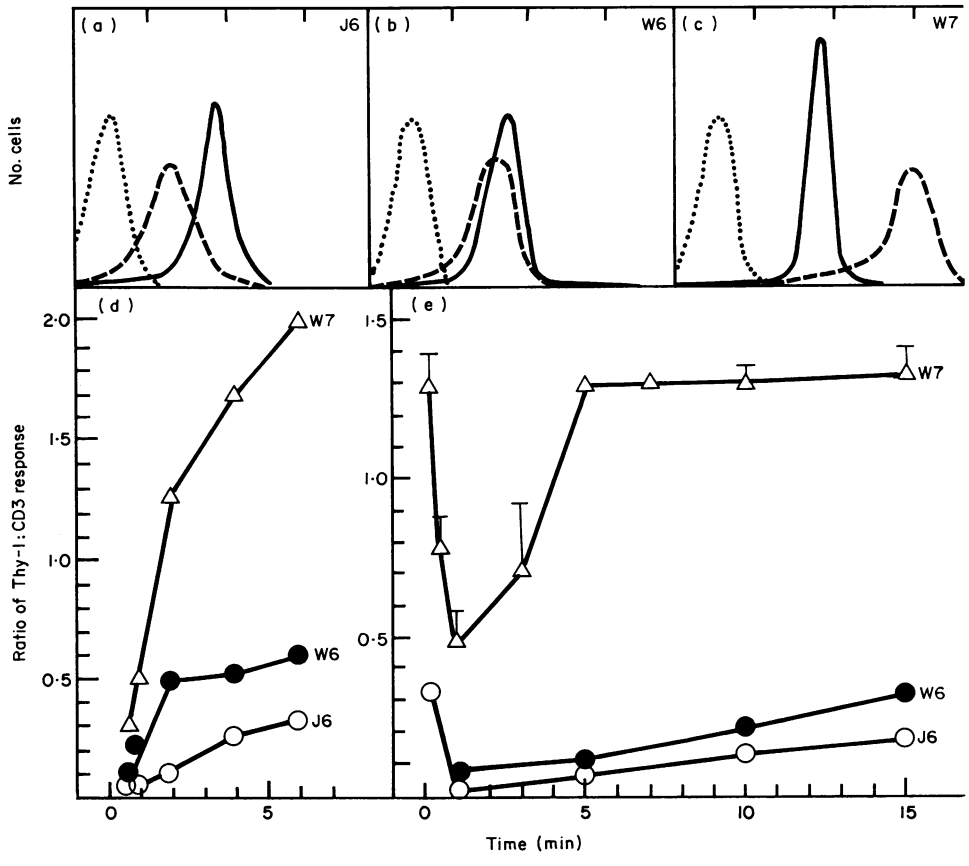

Figure 2. Jurkat lines with three different levels of Thy-1 (FACS profiles in a–c, dotted line is second antibody alone, straight line is OKT3 and dashed line B7, both with second antibody; $\log_{10}$ units of fluorescence intensity shown across top) showing different magnitudes of Ca^{2+} (d) and PI (e) response. For the latter two, at each time-point shown the Thy-1 response has been divided by the CD3 response to give the ratio shown. All points are the mean of triplicate determinations, for the W7 IP response three separate such experiments were done and the standard errors are shown where these fall outside the data point. To exemplify the real data on which the early points of (e) are based, for one of the three assays used the actual d.p.m. for 10, 30 and 60 seconds (mean ± SEM, three determinations) time-points were: for control (B7 without second antibody in this experiment; OX7 plus SAM, or SAM alone, gave similar results), 2850 ± 4, 2350 ± 25, and 2442 ± 59; for B7, 3977 ± 23, 4430 ± 63, and 5430 ± 60; for OKT3, 3622 ± 72, 4750 ± 59, and 6917 ± 186.

r.p.m. for 1 min. Pellets were washed once with 1 ml 1% BSA/PBS by centrifugation and then counted in a gamma counter. For controls, OX7 IgG was substituted for the B7, and ^{125}I-SAM for ^{125}I-OKT3. The amount of Thy-1 on W7 cells was determined by incubating increasing numbers of cells (10^5–10^6) with 10 μg/ml ^{125}I-Fab (3 mCi/mg) of B7 (or, as a control, OX7) for 60 min at 4°. The cells were washed twice with BSA/PBS as before, and antibody bound determined in the gamma counter. The number of antigenic sites was calculated from the range where antibody binding increased linearly with cell numbers. Relative levels of Thy-1 on the different cell lines were determined by quantitative absorption[9] and FACS analysis using a Becton-Dickinson FACStar Plus.

Ca^{2+} determinations

Cells were washed in PBS (containing Ca^{2+} and Mg^{2+} as before) supplemented with 1 : 10 volume of isotonic (0·32 M) sucrose and 1% BSA, then incubated at 2×10^6/ml for 40 min at 37° in the above plus 1 μM of the acetoxymethyl ester of Indo-1[10] (Molecular Probes Inc, Eugene, OR; stored at −70° as a 0·1 mM solution in dry dimethylsulphoxide). The cells were washed twice in the above buffer without dye, aliquoted into microfuge tubes on ice and assayed within 2 hr. Immediately before use they were pelleted by centrifugation and resuspended in the same volume of PBS supplemented only with 32 mM glucose at 37°. The same buffer (1·8 ml) was pre-equilibrated at 37° in a thermostated cuvette in a Perkin-Elmer Fluorescence Spectrophotometer MPF-4, 200 μl of cells added and kept until the fluorescence signal stabilized (about 1 min). B7 antibody (2 μl at 500 μg/ml) was added, followed after 3 min by SAM (2 μl at 2 mg/ml). For OKT3, a single addition of 4 μl at 1 mg/ml was used. Excitation and emission were at 340 and 395 nm, respectively, the intracellular Ca^{2+} was determined as described elsewhere.[11]

To remove extracellular Ca^{2+}, EGTA was added to the cuvette to 2·5 mM before adding cells. The Ca^{2+} L channel blockers diltiazem and nifedipine[12] were similarly added to the cuvette buffer before the cells. The intracellular Ca^{2+} buffer, BAPTA[13] (1,2-bis (o-aminophenoxyethane N,N,N′, N′-tetracetic acid, loaded at 1 μM in the acetyoxymethylester form

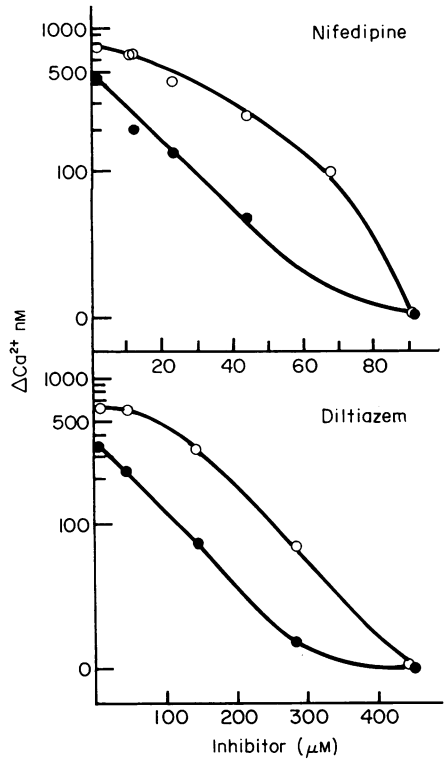

Figure 3. Dose–response curve of the L channel blockers nifedipine and diltiazem, acting on the elevation of intracellular Ca^{2+} measured 1 min after stimulation through Thy-1 (●) and CD3 (○).

(Molecular Probes) was loaded at the same time as Indo-1. Cells were incubated with cholera toxin (1 μg/ml; Sigma) for 3 hr, and pertussis toxin (1–150 ng/ml; Sigma) for 16 hr, immediately before loading with Indo-1; the toxins were kept at this concentration during washes and incubation in the cuvette. For treatment with phosphatidylinositol-specific phospholipase C (PI-PLC; purified from *B. thuringiensis*, obtained from Peninsula Laboratories, St Helens, Merseyside, U.K.), 90 mU were added to cells in the cuvette.

Inositol phosphate determination

Cells were labelled with myo-[^{3}H]-inositol (specific activity 80 Ci/mmole, 1 μCi/10^6 cells/ml) in myoinositol-free RPMI-1640 containing 10% dialysed FCS for 20 hr, then washed by centrifugation three times in RPMI-1640 with 5% FCS and 10 mM LiCl. They were incubated with antibodies (and various drugs) under the same conditions used for the Ca^{2+} experiments, except that 5% FCS/RPMI-1640, buffered at pH7·0 with 10 mM HEPES, was used as the buffer. The reaction was terminated by adding chloroform:methanol (1:2 v/v), and the inositol mono- to tetra-phosphates isolated as a single fraction on Dowex-1 columns using ammonium formate.[14] The results have been calculated by subtracting the values for the negative control, and expressing the experimental value as a percentage of the total myo-[^{3}H]-inositol incorporated by the cells. Points were done in triplicate, standard errors are not generally given as in almost all cases they fall within the point shown.

RESULTS

Ca^{2+} increase in response to Thy-1 and CD3 cross-linking

For these experiments the results obtained with the Jurkat-transfected cell line W7 are shown. This expresses a high level of human Thy-1: $4{\cdot}4 \times 10^6$ molecules per cell assessed by binding of ^{125}I-Fab of B7, or twice the level on human brain as assessed by quantitative absorption. The untransfected Jurkat cells had 1% and the W6-transfected line 3% of this level, as assessed by quantitative absorption and confirmed by FACS.

OKT3 antibody caused an immediate and substantial rise in intracellular Ca^{2+} (Fig. 1a, solid line), matching the rapid kinetics of binding of the antibody (Fig. 1a, dashed line). The Ca^{2+} response peaked at approximately 45 seconds, and then decayed over the next 3 min to a lower level which was maintained for at least 15 min. The antibody binding also showed a transient decrease at about 1 min (reproduced in three separate experiments). In extracellular Ca^{2+}-free medium (Fig. 1b), the initial Ca^{2+} increase was unaffected, although there was no long-term maintenance of an elevated level. Pre-loading the cells with the Ca^{2+} buffer BAPTA totally prevented the rise in intracellular Ca^{2+} (Fig. 1c).

Under the same conditions, the anti-Thy-1 monoclonal B7 antibody failed to cause any change in Ca^{2+} by itself, but on adding a second cross-linking antibody there was an immediate and substantial rise in intracellular Ca^{2+} which was maintained for at least 15 min (Fig. 1d, straight line). The kinetics of this response were matched by that of the binding of the secondary antibody (Fig. 1d, dashed line). The initial phase of this response was lacking in buffer free of Ca^{2+}, although a small late increase in intracellular Ca^{2+} was observed (Fig. 1e; this was $9{\cdot}2 \pm 0{\cdot}1$% the peak Ca^{2+} level induced when the cation was present in the extracellular medium, mean with SD for three determinations). With irrelevant primary antibodies (OX7 anti-Thy-1.1 is shown) no response was elicited (Fig. 1f).

Phosphatidylinositol (PI) turnover

Addition of OKT3 antibodies caused an immediate and sustained increase in PI turnover (Fig. 1g); in Ca^{2+}-free medium the initial phase was unaffected but turnover was not sustained (Fig. 1g). The anti-Thy-1 antibody again caused no effect by itself, but addition of SAM resulted in an immediate and sustained PI response, only the late phase of which was reduced in Ca^{2+}-free medium (Fig. 1h). Preloading the cells with the Ca^{2+}-buffer BAPTA blocked the Thy-1-induced PI response, and flooding the cells with Ca^{2+} using ionomycin (0·5 μg/ml) induced no elevation of inositol phosphate levels (Fig. 1i).

The parent Jurkat line J6 and transfectant line W6, both expressing similar levels of CD3 but much reduced levels of Thy-1 (Fig. 2a–c), gave Ca^{2+} and PI responses similar in their kinetics, but for Thy-1 much lower in their magnitude, than did line W7. In Fig. 2d, e, the Ca^{2+} and PI responses elicited by Thy-1 stimulation for the three cell lines are plotted, at various times after stimulation, as a proportion of the response produced in the same cells when stimulated through CD3 (i.e. the CD3 response is used as an internal standard for the cells). The rank order of Thy-1 responses (both Ca^{2+} and IP) by the cells matches their Thy-1 level. The Ca^{2+} responses are monotonic (Fig. 2d). They are initially low, as the CD3 response spikes, then increase because the CD3-stimulated Ca^{2+} levels decline,

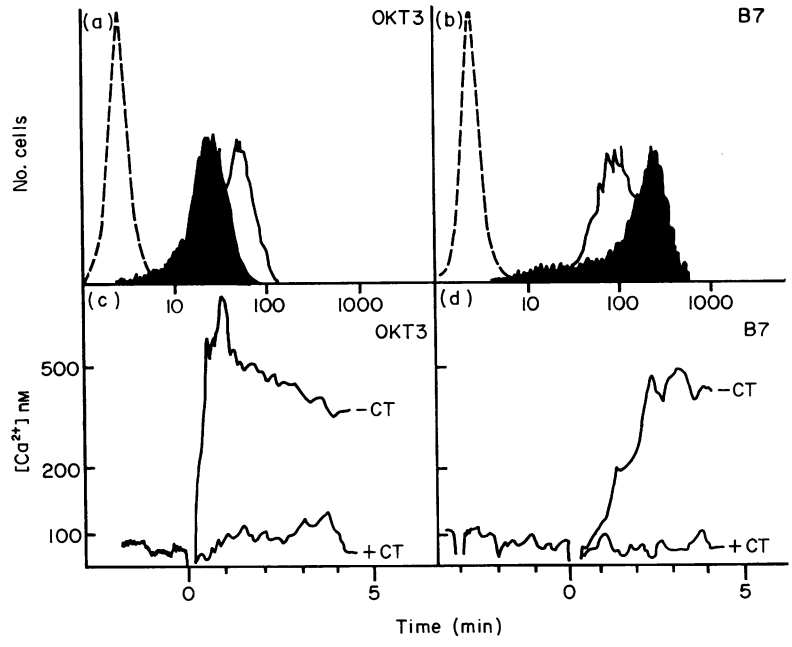

Figure 4. Effect of cholera toxin on the surface expression (a, b; FACS profile, fluorescence intensity plotted on *x* axis, dashed line shows cells with secondary antibody alone, open profile shows cells without toxin labelled with primary and secondary antibody, shaded profile shows labelling of cells treated with toxin) and Ca^{2+} response mediated through CD3 and Thy-1 (c, d), for W7 cells.

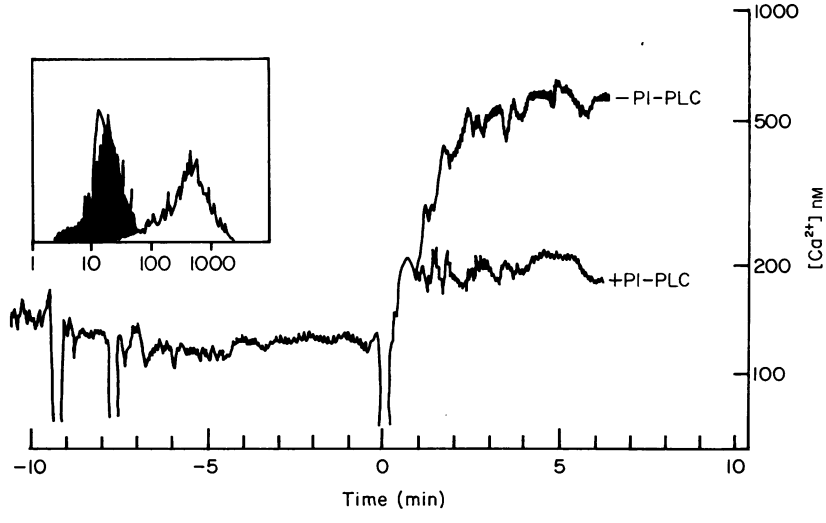

Figure 5. Effect of PI-specific phospholipase C on surface Thy-1 levels (FACS inset: open profile, W7 cells without enzyme; shaded profile, W7 after 10 min incubation with enzyme; open peak just to the left of the shaded one is that of W6 cells, without enzymatic treatment, which have 3% the Thy-1 level of W7) and Ca^{2+} (main figure). The first addition (−9·5 min) was of the enzyme, the second (−7·75 min) was of B7. FACS analysis showed that addition of B7 did not affect the rate of cleavage of Thy-1 by the enzyme.

whereas those stimulated by Thy-1 are more stable (c.f. Fig. 1a, d). The PI response is not monotonic (Fig. 2e). For W7, the first measurable response (at 10 seconds) is higher for Thy-1 than for CD3; the latter then dominates (30 seconds–3 min) but at longer times the Thy-1 response gains the ascendency. Although the magnitude of the Thy-1 PI response is lower, the same trend is visible in the parent J6 line (Fig. 1e).

Inhibitor studies

Antagonists of Ca^{2+} L-type channels inhibited the Ca^{2+} response mediated by both CD3 and Thy-1, although the Thy-1-mediated Ca^{2+} influx was more sensitive to inhibition than the CD3-mediated release of intracellular Ca^{2+} (Fig. 3).

Toxins which modulate G protein activity also affected the level of both Thy-1 and CD3 on the cell surface. With cholera toxin, the result was unambiguous: although it actually increased the surface level of Thy-1 (Fig. 4b) and decreased the level of CD3 (Fig. 4a), it completely inhibited the Ca^{2+} response mediated by both (Fig. 4c, d) and similarly the PI responses (not shown). Pertussis toxin reduced the level of Thy-1 by approximately 50%, when used at 150 ng/ml, as assessed by FACS (not shown). It reduced the Ca^{2+} response in a dose-dependent fashion to reach a maximal 60% inhibition at 150 ng/ml

(residual 40% Ca^{2+} influx remained at concentrations as high as 1·5 μg/ml). Partial inhibition of PI turnover was also found.

Phosphatidylinositol-specific phospholipase C treatment

This enzyme efficiently removed human Thy-1 from the surface of both the wild-type and transfected Jurkat cells (Fig. 5, inset). Cells so treated had a reduced Ca^{2+} and PI response commensurate with the reduction in surface Thy-1 (Fig. 5; only Ca^{2+} response shown). However, treatment of the cells with only the phospholipase C caused no detectable rise in intracellular Ca^{2+} (Fig. 5) or PI turnover (not shown).

DISCUSSION

Previous studies of immediate transmembrane signalling events mediated by Thy-1 have monitored only intracellular Ca^{2+}, and found a response occurring more than a minute after the addition of antibodies.[15-17] An effect of Thy-1 antibodies on the amplitude of a low-threshold transient Ca^{2+} current has been reported on neurons,[18] occurring in this case with a latency of about 15 min. This contrasts with the virtually instantaneous rise in intracellular Ca^{2+} seen after lymphocyte activation through the conventional (T-cell receptor complex) route, as seen for instance after application of antibodies to CD3,[4,6,15,16] and lends credence to suggestions that Thy-1 signalling might be secondary to relatively slow, and potentially non-specific, processes such as internalization of cross-linked molecule.[19]

The sluggish Thy-1 responses reported could reflect the kinetics of the antibodies used, which were not assessed. However, we initially found delays of about 50 seconds between addition of the second, cross-linking antibody and Ca^{2+} influx, which turned out to be due to a combination of factors. The first was mycoplasma contamination, so often endemic in human cell lines, which even at low levels markedly depressed the kinetics and extent of Thy-1-mediated Ca^{2+} influx. It was also important to use fresh cells—their responses declined within 1–2 hr. Finally, having pure monoclonal antibody (i.e. not contaminated with calf Ig from culture or mouse IgG from ascites) helped, presumably because irrelevant primary Ig bound to secondary anti-Ig and so inhibited cross-linking of surface antigen. We find the Thy-1-mediated rise in intracellular Ca^{2+} occurs almost as fast as the CD3-mediated rise; increased PI turnover, not previously reported for Thy-1 activation, is even faster over the first 10 seconds for Thy-1 than for CD3 (Fig. 2e).

In CD3 activation, production of IP3 causes the release of Ca^{2+} from intracellular stores, augmented thereafter by an influx of Ca^{2+}.[4,6] Thy-1-mediated elevation of Ca^{2+} and inositol phosphates proceeds by a different mechanism. The initial Ca^{2+} response is an influx, abolished when Ca^{2+} is removed from the external medium. It is more sensitive to nifedipine and diltiazem than the intracellular Ca^{2+} channel activated by CD3. The initial phase of Thy-1-mediated PI turnover is actually enhanced slightly in the absence of extracellular Ca^{2+} (Fig. 1h), yet leads to minimal release of Ca^{2+} from intracellular pools (Fig. 1e). The fact that similar levels of IP stimulation can in one case (OKT3) cause substantial intracellular Ca^{2+} release, and in the other (Thy-1) cause very little release of the same ion, suggests there must be differences in either the individual inositol phosphates stimulated, or in their intracellular location, in the two cases. It has previously been shown that different proteins are phosphorylated in response to Thy-1 and CD3 stimulation,[20] further demonstrating that different intracellular mechanisms are activated by the two signalling pathways.

How Thy-1, which only spans half the lipid bilayer, can mediate such efficient transmembrane signalling remains unexplained. Enzymatic cleavage of the protein by a specific phospholipase C to generate phosphotidylinositol is ruled out since the direct action of such an enzyme elevates neither intracellular Ca^{2+} nor PI turnover. Internalization of cross-linked Thy-1 would seem to be precluded, since this occurs with much slower kinetics (2% per hour,[19] equivalent to 15 molecules of Thy-1 internalized per minute for J6 cells). Interaction with some transmembrane molecule seems the most likely mechanism. This cannot be CD3, since it activates a different Ca^{2+} flux (this study) and the Thy-1-mediated Ca^{2+} response is found with CD3-negative Jurkat mutants.[17] However, CD3 is involved in downstream events in Thy-1 (and Ly-6, another PI-anchored surface protein) activation, since proliferation and lymphokine production is not found in these variants.[17,21-23] A feed-back loop involving CD3 might be biologically important to restrict this form of lymphocyte activation to augmenting the response of cells already stimulated by antigen. Certainly, similar G proteins appear to be used in both mechanisms of activation, since cholera toxin (which causes continuous activation of G_S and so sustained elevation of cAMP) inhibited both,[5,24] and pertussis toxin neither (beyond its effect on levels of surface antigen).[25]

Thus this study demonstrates that the transmembrane signalling initiated by cross-linking Thy-1 has the kinetics, magnitude and sensitivity to Thy-1 levels suggestive of a primary signalling mechanism, in which inositol phosphate generation feeds back on the surface membrane to open L type Ca^{2+} channels. If this mimics a physiological process, it implies there is a receptor for Thy-1 which cross-links more than two molecules of the glycoprotein to initiate this route of intracellular signalling. Two known features of Thy-1 would be explained by such a mechanism: such a quarternary (or higher) order molecular reaction would require high levels of Thy-1 to proceed with reasonable kinetics; and the kinetics would be enhanced if the mobility of Thy-1 was high, as is achieved by its insertion into the lipid bilayer by a glycophospholipid tail.[26]

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REFERENCES

1. Presky D.H., Low M.G. & Shevach E.M. (1990) Role of phosphotidylinositol-anchored proteins in T cell activation. *J. Immunol.* **144,** 860.
2. Robinson P.J., Millrain M., Antoniou J., Simpson E. & Mellor A.L. (1989) A glycophospholipid anchor is required for Qa-2-mediated T cell activation. *Nature (Lond.)*, **342,** 85.
3. Ferguson M.A.J. & Williams A.F. (1988) Cell-surface anchoring of proteins via glycosylphosphatidylinositol structures. *Ann. Rev. Biochem.* **57,** 285.
4. Imboden J.B. & Stobo J.D. (1985) Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor

complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. exp. Med.* **161,** 446.

5. IMBODEN J.B., SHOBACK D.M., PATTISON G.M. & STOBO J.D. (1986) Cholera toxin inhibits the T-cell antigen receptor-mediated increases in inositol trisphosphate and cytoplasmic free calcium. *Proc. natl. Acad. Sci. U.S.A.* **83,** 5673.
6. IMBODEN J.B. & WEISS A. (1987) The T cell antigen receptor regulates sustained increases in cytoplasmic free Ca^{2+} through extracellular Ca^{2+} influx and ongoing intracellular Ca^{2+} mobilization. *Biochem. J.* **247,** 695.
7. VIDAL M., MORRIS R., GROSVELD F. & SPANOPOULOU E. (1990) Tissue-specific control elements of the Thy-1 gene. *EMBO J.* **9,** 830.
8. MIYATA T., ISOBE K., DAWSON R., RITTER M.A., INAGI R., ODA O., TAGUCHI R. *et al.* (1990) Determination of the molecular nature and cellular localization of Thy-1 in human renal tissue. *Immunology,* **69,** 391.
9. MORRIS R.J. & RAISMAN G. (1983) Estimation of Thy-1 in cryostat sections of nervous tissue. *J. Neurochem.* **40,** 637.
10. GRIENKIEWICZ G., POENIE M. & TSIEN R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260,** 3440.
11. BIJSTERBOSCH M.J., RIGLEY K.P. & KLAUS G.G.B. (1986) Cross-linking of surface immunoglobulin on B lymphocytes induces both intracellular Ca^{2+} release and Ca^{2+} influx: analysis with Indo-1. *Biochem. Biophys. Res. Comm.* **137,** 500.
12. MILLER R.J. (1987) Multiple calcium channels and neuronal function. *Science,* **235,** 46.
13. TSIEN R.Y. (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry,* **19,** 2396.
14. BIJSTERBOSCH M.K., MEADE C.J., TURNER G.A. & KLAUS G.G.B. (1985) B lymphocyte receptors and polyphosphoinositide degradation. *Cell,* **41,** 999.
15. KROCZEK R.A., GUNTER K.C., SELIGMANN B. & SHEVACH E.M. (1986) Induction of T cell activation by monoclonal anti-Thy-1 antibodies. *J. Immunol.* **136,** 4379.
16. KROCZEK R.A., GUNTER K.C., GERMAIN R..N. & SHEVACH E.M. (1986) Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. *Nature (Lond.),* **322,** 181.
17. GUNTER K.C., GERMAIN R.N., KROCZEK R.A., SAITO T., YOKOYAMA W.M., CHAN C., WEISS A. & SHEVACH E.M. (1987) Thy-1-mediated T-cell activation requires co-expression of CD3/Ti complex. *Nature (Lond.),* **326,** 505.
18. SALEH M., LANG R.J. & BARTLETT P.F. (1988) Thy-1-mediated regulation of a low-threshold transient calcium current in cultured sensory neurons. *Proc. natl. Acad. Sci. U.S.A.* **85,** 4543.
19. BAMEZAI A., GOLDMACHER V., REISER H. & ROCK K.L. (1989) Internalization of phosphotidylinositol-anchored lymphocyte proteins. I. Documentation and potential significance for T cell stimulation. *J. Immunol.* **143,** 3107.
20. PEYRON J.-F., PONT S., PIERRES M. & FEHLMANN M. (1988) T cell activation: distinct pathways involve phosphorylation of different cellular proteins. *Eur. J. Immunol.* **18,** 1139.
21. GUIMEZANES A., BUFERNE M., PONT S., PIERRES M. & SCHMITT-VERHULST A.-M. (1988) Interactions between the Thy-1 and T-cell antigen receptor pathways in the activation of cytotoxic T cells: evidence from synergistic effects, loss variants, and anti-CD8 antibody-mediated inhibition. *Cell. Immunol.* **113,** 435.
22. SUSSMAN J.L., SAITO T., SHEVACH E.M., GERMAIN R.N. & ASHWELL J.D. (1988) Thy-1 and Ly-6-mediated lymphokine production and growth inhibition of a T cell hybridoma require co-expression of the T cell antigen receptor complex. *J. Immunol.* **140,** 2520.
23. BAMEZAI A., REISER H. & ROCK K.L. (1988) T cell receptor/CD3 negative variants are unresponsive to stimulation through the Ly-6 encoded molecule, TAP. *J. Immunol.* **141,** 1423.
24. SOMMERMEYER H., SCHWINZER R., KAEVER V., WESSEL K., SCHMIDT R.E., BEHL B., WONIGEIT K., SZAMEL M. & RESCH K. (1989) Cholera toxin modulates the T cell antigen receptor/CD3 complex but not the CD2 molecule and inhibits signaling via both receptor structures in the human T cell lymphoma Jurkat. *Eur. J. Immunol.* **19,** 2387.
25. GRAY L.S., HUBER K.S., GRAY M.C., HEWLETT E.L. & ENGELHARD V.H. (1989) Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. *J. Immunol.* **142,** 1631–1638.
26. ISHIHARA A., HOU Y. & JACOBSON K. (1987) The Thy-1 antigen exhibits rapid lateral diffusion in the plasma membrane of rodent lymphoid cells and fibroblasts. *Proc. natl. Acad. Sci. U.S.A.* **84,** 1290.