Relationships between the degree of cross-linking of surface immunoglobulin and the associated inositol 1,4,5-trisphosphate and Ca^{2+} signals in human B cells

Fiona M. McCONNELL,*†§ Stephen B. SHEARS,† Peter J. L. LANE,*‡ Mark S. SCHEIBEL* and Edward A. CLARK*

*Regional Primate Research Center, University of Washington, Seattle, WA 98195, and †LCMP, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

Cross-linking of surface immunoglobulin (Ig) receptors on human B cells leads to the activation of a tyrosine kinase. The activated tyrosine kinase subsequently phosphorylates a number of substrates, including phospholipase C- γ . This enzyme breaks down phosphoinositol bisphosphate to form two intracellular messengers, diacylglycerol and inositol 1,4,5-trisphosphate, leading to the activation of protein kinase C and the release of intracellular Ca^{2+} respectively. We have used h.p.l.c. and flow cytometry to measure accurately the inositol phosphate turnover and Ca^{2+} release in anti-Ig-stimulated human B cells. In particular, we have examined the effect of dose of the cross-linking antibody on the two responses. The identity of putative messenger inositol phosphates has been verified by structural analysis, and the amounts of both inositol phosphates and Ca^{2+} present have been quantified. In the Ramos Burkitt lymphoma, which is very sensitive to stimulus through its Ig receptors, both inositol phosphate production and Ca^{2+} release were found to be related to the dose of anti-Ig antibody applied. This suggests that phospholipase C-mediated signal transduction in human B cells converts the degree of cross-linking of the immunoglobulin receptor quantitatively into intracellular signals.

INTRODUCTION

Receptor ligation of surface immunoglobulin (sIg) receptors in mammalian B cells leads to the activation of phospholipase C (PLC) (Cambier & Ransom, 1987; Klaus *et al.*, 1987). Several authors have implicated classical heterotrimeric G-proteins in the signal transduction pathway (Gold *et al.*, 1987; Harnett & Klaus, 1988; Monroe & Haldar, 1989). The best evidence supporting this contention is the GTP-dependence of phosphoinositide hydrolysis following immunoglobulin cross-linking. Although members of several classes of G-protein interact with PLC (Simon *et al.*, 1991), a specific heterotrimeric G-protein has not yet been identified as the link between sIg and PLC activation.

On the other hand, several studies now indicate that receptor ligation of immunoglobulin receptor leads to the activation of receptor-linked tyrosine kinase(s) (Gold *et al.*, 1990; Lane *et al.*, 1990a). At least two B-lymphocyte-specific tyrosine kinases have recently been identified, *blk* (Dymecki *et al.*, 1990) and *lyn*, which has been suggested to be associated with IgM at the cell surface (Yamanashi *et al.*, 1991). In B cells, tyrosine phosphorylation events are essential for the sIg-mediated activation of PLC (Lane *et al.*, 1990a). The GTP-binding proteins of the *ras* family seem to be involved in tyrosine kinase-mediated signal transduction, and this may explain the GTP-dependence of phosphoinositide hydrolysis in B cells. In other systems, the tyrosine phosphorylation of PLC- γ is required for its activation (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989). PLC catalyses the breakdown of $\text{PtdIns}(4,5)\text{P}_2$, releasing diacylglycerol, which activates protein kinase C (Nishizuka, 1984), and $\text{Ins}(1,4,5)\text{P}_3$, which elevates the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Berridge & Irvine, 1984). The release of $\text{Ins}(1,4,5)\text{P}_3$ in B lymphocytes entrains a series of Ca^{2+} oscillations (Wilson *et al.*, 1987). It has been suggested that the frequency of Ca^{2+} oscillations encodes the strength of the stimulus (Berridge, 1989).

In the work reported here, we looked for relationships between the strength of the cross-linking of Ig at the cell surface, the production of $\text{Ins}(1,4,5)\text{P}_3$ and inositol phosphates derived from it, and the release of intracellular Ca^{2+} . In addition, we have tried to assess the sensitivity of this and other methods for detecting inositol phosphates, with particular regard to claims that the Ca^{2+} response can be dissociated from PtdIns turnover (Monroe *et al.*, 1989).

Studies of lymphocytes have frequently analysed changes in inositol phosphate levels using gravity-fed anion-exchange columns (Brunswick *et al.*, 1989a; Rigley *et al.*, 1989), which separate inositol phosphates according to the number of phosphate groups on the inositol ring. Since the positions of the phosphate groups are not identified, it is impossible to separate the Ca^{2+} -releasing messenger [$\text{Ins}(1,4,5)\text{P}_3$] from other isomers which may be present in large amounts. Detection of inositol phosphates can be enhanced with lithium salts, which block the dephosphorylation of $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$ (Shears, 1989a), but which have the disadvantage of attenuating the levels of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ non-specifically (Kennedy *et al.*, 1989). Lithium salts (Brunswick *et al.*, 1989b; Rigley *et al.*, 1989), and the pooling of inositol mono-, bis-, and tris-phosphate fractions to give a figure for total inositol phosphate production (Harnett *et al.*, 1989), have been used to enhance the sensitivity of the inositol phosphate assay. These approaches suffice when all that is required is the implication of PLC in the response concerned, but are inadequate, for example, if the concentrations of inositol phosphates are of interest, or for the separation of messenger molecules from other metabolites. To meet such requirements, we have used h.p.l.c. analysis to separate the aqueous inositol phosphates of radiolabelled human B cells stimulated in the absence of lithium, and have confirmed the identifications of isomers of primary interest by structural analysis. We have used normal resting human B cells and two

Abbreviations used: sIg, surface immunoglobulin; PLC, phospholipase C; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; mAb, monoclonal antibody.

‡ Present address: Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland.

§ To whom correspondence should be addressed.

human B cell lines, all of which can be stimulated using antibodies to sIg. Cell lines afford the further advantage of enabling equilibrium labelling of inositol-containing compounds, so that reasonable estimates of their concentrations can be derived. For the Ramos Burkitt lymphoma, which is very sensitive to anti-IgM stimulus, we have quantified the relationships between antibody dose, inositol phosphate production and Ca^{2+} release. Experiments performed with anti-Ig-stimulated dense tonsillar cells confirmed that the inositol phosphate and Ca^{2+} responses observed in the two cell lines have similar counterparts in normal cells. The data are discussed with regard to the regulation by B lymphocytes of their responsiveness to antigen.

MATERIALS AND METHODS

Preparation of lymphoid cells

Tonsillar B cells were isolated as described elsewhere (Clark & Shu, 1987), and were greater than 95% CD20⁺. They were separated into different density fractions on discontinuous Percoll (Pharmacia) gradients of 55%, 45% and 35%. Cells which pelleted below 55% Percoll were used for these studies. Although the same experimental protocol was used for all tonsillar preparations, the history of the tonsils was beyond our control, and variation between experiments is to be expected. The results are therefore given in full, rather than as averages or a single representative experiment. The Ramos Burkitt's lymphoma line and the B lymphoblastoid line T5-1 were kindly provided by Dr. D. Pious and Dr. E. Mellins (University of Washington, Seattle, WA, U.S.A.).

Antibodies

A polyclonal affinity-purified F(ab')₂ goat anti-(human μ -chain) specific serum (Jackson Immunoresearch Labs, Avondale, PA, U.S.A.) was used routinely to cross-link sIgM receptors. 2C3, an IgG1 monoclonal antibody (mAb) specific for IgM (Clark *et al.*, 1986), was occasionally used for comparison, but did not elicit such good responses. For the anti-IgD tests, the mAb δ -TA4.1 [an IgG mAb (Kubagawa *et al.*, 1982)] was preferred for its consistency; the polyclonal F(ab')₂ fragment FA-04 (EY Labs, San Mateo, CA, U.S.A.) gave similar results.

Cell water volume determinations

Intracellular water volume was determined by a double isotope method (Stanton *et al.*, 1986). $^3\text{H}_2\text{O}$ was used as a marker for both intracellular and extracellular water, and [^{14}C]inulin was used to trace trapped extracellular water. The difference between the two was taken to be the intracellular water volume. Triplicate measurements with T5-1 and Ramos cells gave average water volumes per cell of 0.453 ± 0.018 and 0.417 ± 0.020 pl respectively.

Analysis of $[\text{Ca}^{2+}]_i$ mobilization

The method used has been described in detail (Rabinovitch *et al.*, 1986). Cells were loaded with Indo-1 by incubation with its acetoxymethyl ester (Molecular Probes, Junction City, OR, U.S.A.) at $1 \mu\text{M}$ concentration for 45 min (37°C). Cells were washed once and kept at room temperature in the dark until analysis was performed with on a FACStar Plus (Becton Dickinson, San José, CA, U.S.A.). An argon laser was used for u.v. excitation. Violet fluorescent emission was detected at 383–407 nm and the blue emission was detected at 480–520 nm; the ratio of Indo-1 violet to blue fluorescence was digitally calculated for each individual cell and displayed as a function of elapsed time. The entire analysis was performed at 37°C . Aliquots of 5×10^6 cells were used for each experiment with a typical flow rate of 400 cells/s. The ratio of Indo-1 violet to blue

was calibrated against $[\text{Ca}^{2+}]_i$ as described previously (Rabinovitch *et al.*, 1986).

Measurement of inositol phosphates

The Ramos Burkitt's line and T5-1 cells were labelled for 96–120 h (5–6 doublings) and 36–70 h (1.5–2.5 doublings) respectively with RPMI 1640 culture medium containing 12–47 mg of inositol/litre and 2–5 μCi of [^3H]inositol per 10^8 cells. Loading of dense tonsillar cells was performed overnight (18 h) at 37°C and 5% CO_2 in serum-free RPMI 1640 culture medium containing 5 mg of inositol/litre and 20 μCi of [^3H]inositol/ml per 10^7 cells. Prior to experimentation, cells were harvested, washed twice and resuspended in normal RPMI 1640 medium (47 mg of inositol/litre after addition of serum) and brought to 37°C in a water bath. The appropriate dose of anti-IgM was added to cells at a cell density of approx. 3×10^7 (cell lines) or 1.4×10^8 per ml of medium (tonsillar cells). Samples of 300 μl were taken at times up to 600 s and quenched in 0.5 ml of cold 20% trichloroacetic acid with added phytate (sodium hexaphosphate; Sigma; 250 $\mu\text{g}/\text{ml}$) and phytate hydrolysate (25 μg of phosphate/ml) (Wreggett *et al.*, 1987). Cell lysates were spun (700 g, 20 min) and the pellet was washed with 0.5 ml of trichloroacetic acid. The combined supernatants were washed five times with 2 ml of hydrated ether. Ether was allowed to evaporate from the final aqueous phase, to which 100 μl of 50 mM-EDTA was added. Samples were stored at -20°C for later analysis by h.p.l.c.

H.p.l.c. analysis of aqueous cell extracts was performed using a 10 cm Partisphere 5 SAX column with pre-column and guard cartridge (Whatman, Clifton, NJ, U.S.A.). Eluted ^3H -labelled compounds were identified by comparison with elution times of authentic tritiated standards from Amersham (Arlington Heights, IL, U.S.A.) [Ins1P, Ins(1,4) P_2 , Ins(1,4,5) P_3 , and Ins(1,3,4,5) P_4] or New England Nuclear (Boston, MA, U.S.A.) [Ins(1,3,4) P_3]. The tritiated standards were added quantitatively to [^3H]inositol-labelled cell lysates after addition of trichloroacetic acid, and gradients were calibrated with internal nucleotide standards (AMP, ADP, ATP and adenosine tetraphosphate; Sigma). The gradient used elution buffers of ultrapure water and monobasic ammonium phosphate (Sigma) at pH 3.8, and usually separated at least 15 inositol-containing compounds (fewer for dense tonsillar cells). The gradient commenced as 100% water, advancing to 0.05 M-ammonium phosphate over 25 min. Ammonium phosphate concentrations were then held at 0.2 M for 10 min, increased from 0.54 M to 0.56 M over 30 min, held at 0.8 M for 15 min and then at 1.6 M for 10 min, and finally maintained at 2.0 M for 15 min to terminate the gradient. Fractions of 0.5 min were collected and radioactivity was counted by liquid scintillation counting with correction for quench.

Identification of inositol phosphates by co-elution

H.p.l.c. analysis of the aqueous extracts of [^3H]inositol-labelled lymphocytes routinely provided a chromatogram containing at least 10 inositol phosphate peaks. Fig. 1(a) is a typical chromatogram with the elution times of authentic inositol phosphate isomers indicated on it. Peak elution times varied within the life of a column by no more than three fractions. Ins(1,4,5) P_3 and Ins(1,3,4) P_3 were sometimes, but not always, accompanied by one or two unidentified Ins P_3 isomers. Ins P_4 appeared as one peak for tonsillar cells and the Ramos line, but frequently as two peaks separated by 3 min in T5-1 samples (see Fig. 1a).

Groups of fractions identified as Ins(1,4) P_2 , Ins(1,3,4) P_3 , Ins(1,4,5) P_3 , and Ins(1,3,4,5) P_4 were combined after subtraction of background. [^3H]inositol for each peak was expressed as d.p.m./ 10^6 cells; for graphical representation of the Ramos data, which were obtained from two separate experiments (Fig. 3), the values were normalized to a total (all inositol-containing com-

Fig. 1. H.p.l.c. analysis of inositol phosphates

(a) Representative h.p.l.c. chromatogram (from an anti-Ig-stimulated T5-1 cell preparation as used for the analyses shown in Fig. 5). Retention times of standard inositol phosphate compounds are indicated at the top of the Figure. The standards are: a, $\text{Ins}1\text{P}$; b, $\text{Ins}(1,4)\text{P}_2$; c, $\text{Ins}(3,4)\text{P}_2^*$; d, $\text{Ins}(4,5)\text{P}_2^*$; e, $\text{Ins}(1,3,4)\text{P}_3$; f, $\text{Ins}(1,4,5)\text{P}_3$; g, $\text{Ins}(1,3,4,5)\text{P}_4$. Those marked * were prepared in S.B.S.'s lab; all others were procured commercially (see the Materials and methods section). Using the retention times of the standards, the peaks of radioactivity occurring in the cell extracts were identified as follows: 1, $\text{Ins}1\text{P}$; 2, $\text{Ins}(1,4)\text{P}_2$; 3, $\text{Ins}(3,4)\text{P}_3$; 4, $\text{Ins}(1,3,4)\text{P}_3$; 5, $\text{Ins}(1,4,5)\text{P}_3$; 6, unidentified $\text{Ins}P_3$; 7, $\text{Ins}P_4$. In separate preparations, peaks 4, 5, 6 and 7 were used for determination of isomer identities (see b). (b) Relevant section of the chromatograms from control (—) and anti-IgD-stimulated (---) T5-1 cells showing the peaks used for the structural identifications of $\text{Ins}P_3$ and $\text{Ins}P_4$ isomers. The putative identifications before analysis were: 4, $\text{Ins}(1,3,4)\text{P}_3$; 5, $\text{Ins}(1,4,5)\text{P}_3$; 6, unidentified $\text{Ins}P_3$; 7, $\text{Ins}P_4$.

pounds) of 40000 d.p.m./ 10^6 cells. Confidence limits on the values obtained for individual peaks, derived from the statistics of radioisotope detection, depended on the number of counts in the peak and the number of fractions over which they were collected. As a rule, $\text{Ins}P_3$ peaks were the smallest and had 95% confidence limits of approx. 12%; for all other peaks, the 95% confidence limits were within 10% of the reported value. In comparing samples, therefore, differences of < 15% were considered insignificant.

Preparation of putative $\text{Ins}P_3$ and $\text{Ins}P_4$ peaks from T5-1 cells for structural analysis

T5-1 cells were loaded in serum-free, inositol-free RPMI 1640 with 5 mg of added inositol/litre and 5 μCi of $[\text{^3H}]$ inositol/ml for 24 h. Harvested cells were washed and resuspended in normal RPMI 1640 at a density of 10^8 cells/ml. Samples of 0.5 ml of cell suspension were extracted in trichloroacetic acid as usual, with (stimulated) or without (control) addition of 10 μg of anti-IgD/ml 3 min prior to extraction. Replicate samples (10) were analysed by h.p.l.c. as described, and 100 μl portions of the resulting 5 ml fractions were counted for radioactivity by liquid scintillation chromatography. The fractions making up peaks corresponding by co-elution to authentic ^3H -labelled $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$, an unidentified $\text{Ins}P_3$, and $\text{Ins}P_4$ (peaks 4, 5, 6, and 7 in Fig. 1) were combined and desalted as detailed elsewhere (Shears *et al.*, 1988; Shears, 1989b).

Structural identification of $\text{Ins}P_3$ and $\text{Ins}P_4$ isomers

Under appropriate conditions, periodate will oxidize inositol phosphates only between adjacent hydroxyl groups; after subsequent reduction and dephosphorylation, identification of the ensuing polyol is indicative of the structure of the original inositol phosphate (Kirk *et al.*, 1990). The conditions we used for such experiments and the h.p.l.c. separation of polyols were as described in Menniti *et al.* (1990). A polyol dehydrogenase was used to distinguish the D- and L-enantiomers of iditol (Menniti *et al.*, 1990). $\text{Ins}(1,4,5)\text{P}_3$ was also assayed by its dephosphorylation using the stereospecific $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase (Cooke *et al.*, 1989). Putative $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ and an internal standard of $[\text{^32P}]\text{Ins}(1,4,5)\text{P}_3$ were incubated with purified 5-phosphatase as described (Menniti *et al.*, 1990) to give 80% hydrolysis. The reaction products were separated on gravity-fed ion-exchange columns (Kirk *et al.*, 1990). Inositol phosphates were also determined by an analysis of the inositol monophosphates that were produced upon alkaline hydrolysis (Menniti *et al.*, 1990). Note that this technique does not distinguish between $\text{Ins}1\text{P}$ and $\text{Ins}3\text{P}$, nor between $\text{Ins}4\text{P}$ and $\text{Ins}6\text{P}$, which are both enantiomeric pairs of monophosphates.

The identification of the inositol tetrakisphosphate isomers is described in detail elsewhere (McConnell *et al.*, 1991).

RESULTS

Structural analysis of inositol phosphates

Inositol trisphosphates. Two $\text{Ins}P_3$ peaks were always observed, corresponding to the elution positions of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ (Fig. 1). On some occasions, a third minor $\text{Ins}P_3$ was observed, although it only appeared upon stimulation; when this was detected, it represented 10–15% of $\text{Ins}(1,4,5)\text{P}_3$. Following periodate oxidation, reduction and dephosphorylation, the resultant polyol was identified as glucitol (83% of recovered ^3H). This peak also failed to yield any $\text{Ins}1\text{P}/\text{Ins}3\text{P}$ upon alkaline hydrolysis (results not shown). Thus (see Kirk *et al.*, 1990), this peak can only be $\text{Ins}(2,4,5)\text{P}_3$ or its enantiomer, $\text{Ins}(2,5,6)\text{P}_3$. The most likely alternative is $\text{Ins}(2,4,5)\text{P}_3$, which is formed as an artifact of acid-quench procedures from $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$, in turn a minor product of PLC (Wong *et al.*, 1988).

The second-eluting of the inositol trisphosphate peaks (peak 5 in Fig. 1) was initially identified as $\text{Ins}(1,4,5)\text{P}_3$ by its co-elution with a standard. This identification was confirmed using a purified $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase to analyse the material from one preparation of stimulated cells in which $\text{Ins}(1,4,5)\text{P}_3$ was separated from $\text{Ins}(2,4,5)\text{P}_3$, and also a preparation of control cells [which do not contain any $\text{Ins}(2,4,5)\text{P}_3$ (see above)]. In these experiments, the putative $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ was hydrolysed by the 5-phosphatase at the same rate and to the same extent as an

Fig. 2. Surface expression of IgM and IgD in T5-1 cells

T5-1 cells were stained indirectly with either δ -TA4.1 or 2C3 (see the Materials and methods section), followed by a second step of sheep anti mouse-fluorescein (Jackson Immunoresearch Labs, Avondale, PA, U.S.A.), and analysed for fluorescence intensity using a FACScan (Becton-Dickinson). The scale covers four log cycles, so that a 64 channel increase represents a 10-fold enhancement of intensity.

internal standard of [^3H]Ins(1,4,5) P_3 . Furthermore, in another experiment with material isolated from stimulated cells, the putative Ins(1,4,5) P_3 yielded iditol upon periodate oxidation, reduction and dephosphorylation (95% of recovered ^3H). The iditol was further shown to be entirely the D-enantiomer using a stereoselective polyol dehydrogenase (results not shown, see the

Table 1. Estimated concentrations of Ins(1,4,5) P_3 in Ramos and T5-1 cell lines

Results were derived from the ^3H activity measured in the peaks of interest (d.p.m./ 10^6 cells) and the specific radioactivities of loading media, assuming equilibrium labelling. Cell water volumes were 0.417 pl (Ramos) and 0.453 pl (T5-1). For Ramos, the stimulated level is from the 10 s time point. The data are those used in Fig. 3. The 60 $\mu\text{g}/\text{ml}$ values are representative of five similar experiments with anti-Ig doses of approx. 50 $\mu\text{g}/\text{ml}$ (1–2 $\mu\text{g}/10^6$ cells) and are published elsewhere (White *et al.*, 1991), whilst the lower dose figures were from a single experiment. For T5-1 cells the stimulated Ins(1,4,5) P_3 level is the peak level measured, which occurred at 180 s.

Cells	[IgM] ($\mu\text{g}/\text{ml}$)	Ins(1,4,5) P_3 levels			
		(d.p.m./ 10^6 cells)		(nM)	
		Resting	Stimulated	Resting	Stimulated
Ramos	0.7	21	26	750	929
	7	21	51	750	1822
	60	13	141	630	6831
T5-1	110	32	49	1704	2609

Materials and methods section). The latter result would only arise if Ins(1,4,5) P_3 and/or Ins(1,4,6) P_3 were the original Ins P_3 isomers. Since Ins(1,4,6) P_3 is not a substrate for the 5-phosphatase (Stephens *et al.*, 1989), yet all the material in this peak was attacked by this enzyme, the Ins P_3 was unequivocally identified as Ins(1,4,5) P_3 .

Peak 4 (Fig. 1) was originally believed to be Ins(1,3,4) P_3 on the basis of its elution with a standard of this isomer. However,

Fig. 3. Time course of changes in the levels of (a) Ins(1,4,5) P_3 , (b) Ins P_3 , (c) Ins(1,3,4) P_3 and (d) Ins(1,4) P_2 following stimulation of Ramos cells with different doses of anti-Ig antibodies

[^3H]Inositol-loaded Ramos cells were stimulated with different doses of anti-IgM antibody (●, 60 $\mu\text{g}/\text{ml}$; ◆, 7 $\mu\text{g}/\text{ml}$; ■, 0.7 $\mu\text{g}/\text{ml}$), quenched at times up to 5 min, and analysed by h.p.l.c. Inositol phosphates were identified by co-elution with genuine standards (see the Materials and methods section). The 60 $\mu\text{g}/\text{ml}$ data used here for comparison with the lower dose values have been published elsewhere (White *et al.*, 1991). These graphs are representative of five similar experiments with anti-Ig doses of approx. 50 $\mu\text{g}/\text{ml}$ (1–2 $\mu\text{g}/10^6$ cells); the lower dose figures were from a single experiment. Total d.p.m. (all inositol-containing compounds) per 10^6 cells were 32352 (60 $\mu\text{g}/\text{ml}$) and 43841 (0.7 and 7 $\mu\text{g}/\text{ml}$); for direct comparison of the two experiments, values have been normalized to a total d.p.m./ 10^6 cells of 40000.

Fig. 4. Population Ca^{2+} release in cells stimulated with anti-IgM and anti-IgD antibodies

Arrows indicate addition of agonist. Similar results have been obtained on 2–6 separate occasions. (a) Ramos Burkitt lymphoma: peak Ca^{2+} concentrations, calculated from the Indo ratios [see the Materials and methods section, and Rabinovitch *et al.* (1986)] were as follows: 0.1 $\mu\text{g}/\text{ml}$ ($\blacklozenge$), 375 nM; 0.5 $\mu\text{g}/\text{ml}$ ($\blacktriangle$), 704 nM; 1 $\mu\text{g}/\text{ml}$ ($\bullet$), 1046 nM; 10 $\mu\text{g}/\text{ml}$ ($\blacksquare$), 2170 nM. (b) T5-1 cells: $\blacksquare$, IgD (10 $\mu\text{g}/\text{ml}$); $\bullet$, IgM (10 $\mu\text{g}/\text{ml}$); $\blacktriangle$, IgM (1 $\mu\text{g}/\text{ml}$). (c) Dense tonsillar cells; anti-IgM ($\blacksquare$, 10 $\mu\text{g}/\text{ml}$; $\bullet$, 1 $\mu\text{g}/\text{ml}$; $\blacktriangle$, 0.5 $\mu\text{g}/\text{ml}$; $\blacklozenge$, 0.1 $\mu\text{g}/\text{ml}$). (d) Dense tonsillar cells; anti-IgD ($\blacksquare$, 10 $\mu\text{g}/\text{ml}$; $\bullet$, 1 $\mu\text{g}/\text{ml}$).

some preliminary experiments indicated that this material was heterogeneous in nature. First, the material from stimulated cells was subjected to alkaline hydrolysis, which causes phosphate elimination without phosphate migration. In these experiments about 8% ($n = 2$) of the monophosphate was $\text{Ins}2\text{P}$, indicative of the original $\text{Ins}P_3$ containing isomer(s) with a phosphate in the 2 position (results not shown). Unfortunately, the yield of $\text{Ins}2\text{P}$ cannot be interpreted quantitatively (Menniti *et al.*, 1990). Second, when the $\text{Ins}P_3$ was treated with periodate, followed by reduction and dephosphorylation, alritol [which $\text{Ins}(1,3,4)P_3$ would yield] amounted to only 33% of the ^3H recovered from material originating in control cells, and only 85% of material from stimulated cells (results not shown). The other ^3H -labelled polyols were arabitol [indicative of $\text{Ins}(1,2,6)P_3$ and/or $\text{Ins}(2,3,4)P_3$] and adonitol [indicative of $\text{Ins}(1,2,3)P_3$]. The heterogeneous nature of this peak prevented us from routinely quantifying the response of $\text{Ins}(1,3,4)P_3$ to receptor activation, and is likely to explain the slight shift in the elution time observed in stimulated cells (Fig. 1b).

Inositol tetrakisphosphates. In some experiments with anti-IgD-stimulated T5-1 cells, the $\text{Ins}P_4$ fraction eluted as two incompletely resolved peaks (e.g. peak 7 in Fig. 1a). Both of these peaks were found to contain $\text{Ins}(1,3,4,5)P_4$, mixed with $\text{Ins}(1,3,4,6)P_4$ in the earlier-eluting peak and with $\text{Ins}(3,4,5,6)P_4$ in the later-eluting peak (results not shown). In a further experiment with control and anti-IgD-stimulated T5-1 cells, a single $\text{Ins}P_4$ peak was observed (Fig. 1b). A detailed structural analysis of this material (McConnell *et al.*, 1991) revealed that,

for the control sample, $\text{Ins}(1,3,4,5)P_4$ [the putative messenger molecule (Changya *et al.*, 1989)] constituted 9% of total $\text{Ins}P_4$. $\text{Ins}(1,3,4,6)P_4$ and $\text{Ins}(3,4,5,6)P_4$ comprised 16% and 75% respectively.

After 10 min of stimulation with anti-IgD mAb ($n = 2$), $\text{Ins}(1,3,4,5)P_4$ made up 26% of the peak, representing a 5-fold increase compared with its starting level. $\text{Ins}(1,3,4,6)P_4$ increased 3.5-fold, bringing it up to 30% of the total, while there was little change in the level of $\text{Ins}(1,4,5,6)P_4/\text{Ins}(3,4,5,6)P_4$ (now 44% of the total counts in the peak). The extent of the increase in $\text{Ins}(1,3,4,5)P_4$ is therefore masked by the presence of substantial amounts of other isomers in the $\text{Ins}P_4$ peak.

Concentrations of inositol phosphates

Estimates of the intracellular concentration of the $\text{Ins}(1,4,5)P_3$ messenger in Ramos and T5-1 cells are given in Table 1. The resting level of $\text{Ins}(1,4,5)P_3$ calculated for Ramos cells was approx. 750 nM, while that found in T5-1 cells was more than twice as high. Structural analysis confirmed that the putative $\text{Ins}(1,4,5)P_3$ peak was entirely $\text{Ins}(1,4,5)P_3$ (see above).

Surface expression of immunoglobulin on Ramos and T5-1 cells

Normal resting B lymphocytes are poor subjects for the analysis of inositol phosphate turnover, since their metabolic inertness limits the incorporation of [^3H]inositol into their inositol phospholipids. In addition, the expression of sIg on normal human B cells is heterogeneous; most express approx. $(5\text{--}10) \times 10^4$ sIgM molecules, whilst sIgD appears on only a

Table 2. Changes in $\text{Ins}(1,4)P_2$, $\text{Ins}(1,3,4)P_3$, $\text{Ins}(1,4,5)P_3$ and $\text{Ins}P_4$ in ^3H -labelled dense tonsillar cells following anti-Ig stimulusValues are d.p.m./ 10^6 cells, corrected to the mean (total d.p.m./ 10^6 cells) for each experiment.

Trial	Isomer	Time (s)...	Inositol phosphate (d.p.m./ 10^6 cells)					
			0	10	20	30	40	60
1. IgM (150 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		5.1	7.9				
	$\text{Ins}(1,3,4)P_3$		0	0				
	$\text{Ins}(1,4,5)P_3$		1.4	3.9				
	$\text{Ins}P_4$		1.5	1.8				
1. IgD (150 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		5.1	6.6				
	$\text{Ins}(1,3,4)P_3$		0	1.6				
	$\text{Ins}(1,4,5)P_3$		1.4	2.3				
	$\text{Ins}P_4$		1.5	0				
2. IgM (150 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		0.5		2.3		2.6	
	$\text{Ins}(1,3,4)P_3$		0		0.8		2.7	
	$\text{Ins}(1,4,5)P_3$		0		1.7		0.8	
	$\text{Ins}P_4$		0		1.9		3.4	
2. IgD (150 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		0		3.1		2.8	
	$\text{Ins}(1,3,4)P_3$		0		1.5		2.3	
	$\text{Ins}(1,4,5)P_3$		0		1.1		0	
	$\text{Ins}P_4$		0		3.7		3.9	
3. IgM (50 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		7.0			11.6		
	$\text{Ins}(1,3,4)P_3$		0			0		
	$\text{Ins}(1,4,5)P_3$		6.6			5.3		
	$\text{Ins}P_4$		0			0		
4. IgM (50 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		10.8					12.1
	$\text{Ins}(1,3,4)P_3$		0					0
	$\text{Ins}(1,4,5)P_3$		1.8					0
	$\text{Ins}P_4$		0					1.4
5. IgM (50 $\mu\text{g}/\text{ml}$)	$\text{Ins}(1,4)P_2$		27.8					33.0
	$\text{Ins}(1,3,4)P_3$		0					0
	$\text{Ins}(1,4,5)P_3$		11.6					7.0
	$\text{Ins}P_4$		1.3					5.2

fraction of dense tonsillar cells (Ledbetter & Clark, 1986). Dose-response relationships are therefore better studied in human B cell lines, which are readily loaded to equilibrium with radiolabelled [^3H]inositol, and are more uniform in both their expression of surface Ig and their response to anti-Ig.

In vitro, the sIg receptor does not transduce a signal through the PLC pathway unless it is cross-linked, since Fab fragments of anti-Ig antibodies do not affect phosphatidylinositol turnover (Coggeshall & Cambier, 1984). Since the surface Ig expression in a cell line is constant, for an antibody of choice that is physically capable of cross-linking sIg, changes in antibody dose should correspond to changes in the degree of Ig cross-linking at the cell surface.

The Ramos line expresses 3–4 times as many IgM molecules as do dense tonsillar B cells or the Epstein–Barr virus-transformed line T5-1, for which the surface expressions are comparable (Clark *et al.*, 1986; Lane *et al.*, 1990b). T5-1 cells also express IgD weakly, at about one-twentieth the level of its sIgM expression (Fig. 2).

Dose-response in Ramos cells

Fig. 3 demonstrates the dose-dependency of the phosphoinositide response in the Ramos Burkitt lymphoma. The level of the Ca^{2+} -releasing messenger $\text{Ins}(1,4,5)P_3$, which increases substantially in the first 10 s after stimulus, returns to baseline by 30 s. In contrast, the sustained (3 and 5 min) levels of $\text{Ins}(1,4,5)P_3$ metabolites [identified in Fig. 3 as $\text{Ins}P_4$, $\text{Ins}(1,4)P_2$ and $\text{Ins}(1,3,4)P_3$] indicate that $\text{PtdIns}(4,5)P_2$ breakdown is continuing, even though an elevation in the $\text{Ins}(1,4,5)P_3$ level can no longer be measured. The attenuation of the $\text{Ins}(1,4,5)P_3$ signal after 30 s

may be due to one of the following. (i) Changes in the activity of the two enzymes removing $\text{Ins}(1,4,5)P_3$, i.e. $\text{Ins}(1,4,5)P_3$ 5-phosphatase and $\text{Ins}(1,4,5)P_3$ 3-kinase (Shears, 1989a). The 3-kinase in particular is activated by the increased intracellular Ca^{2+} concentration (Shears, 1989a), and the amounts of $\text{Ins}(1,4)P_2$ produced following anti-Ig stimulus indicate that the activity of the 5-phosphatase is also increased beyond its basal level (Fig. 3d). (ii) A decrease in PLC activity towards $\text{PtdIns}(4,5)P_2$ after the early seconds of the response, for example due to feedback inhibition from protein kinase C (Berridge *et al.*, 1988).

The Ramos line is characteristically extremely sensitive to stimulus through sIg. A concentration of 10 μg of anti-Ig/ml raises the $[\text{Ca}^{2+}]_i$ in nearly 100 % of cells from its resting level of 130 nM to over 2 μM within 15 s (Fig. 4).

Responses in T5-1 and dense tonsillar cells

There is a 1.3–2-fold accumulation of $\text{Ins}(1,4,5)P_3$ following engagement of the IgM receptor in normal (dense tonsillar) B cells (Table 2) and in the T5-1 line (Fig. 5), using large doses of anti-Ig Ab (50–150 $\mu\text{g}/\text{ml}$). This compares to a 7-fold increase in Ramos cells under similar conditions. The difference between Ramos and the other B cells may be a consequence of the degree of surface expression of sIgM, which would imply that sIg expression could provide a means of regulating responsiveness to sIg-mediated stimuli.

Difference in strength of responses to IgM and IgD stimuli

The T5-1 line expresses IgD at approx. one-twentieth of the level at which it expresses sIgM (Fig. 2). Nonetheless, the stimulation of T5-1 cells using an anti-IgD mAb produced a Ca^{2+}

Fig. 5. Time course of changes in the levels of $\text{Ins}(1,4,5)\text{P}_3$ and metabolites, following stimulation with anti-IgM and anti-IgD (both $110 \mu\text{g/ml}$) in T5-1 cells

Total d.p.m. (all inositol-containing compounds) per 10^6 cells was 94647. Anti-IgM (■) and anti-IgD (▲) were used. The data shown are representative of two similar experiments. (a) $\text{Ins}(1,4,5)\text{P}_3$, (b) InsP_4 , (c) $\text{Ins}(1,4)\text{P}_2$, (d) the peak co-eluting with standard $\text{Ins}(1,3,4)\text{P}_3$. See the Results section for explanation of likely contaminants in this peak.

release in excess of that elicited by an anti-IgM stimulus (Fig. 4). The fact that this Ca^{2+} release occurs when changes in the level of $\text{Ins}(1,4,5)\text{P}_3$ are undetectable (Fig. 5) highlights the difference in sensitivity between the two assays (see the Discussion section), and raises the possibility that IgD transduces intracellular signals more efficiently than does IgM.

DISCUSSION

Inositol phosphate isomers

Structural characterization of inositol phosphates in T5-1 cells confirmed that the putative $\text{Ins}(1,4,5)\text{P}_3$ peak was 95% authentic compound. However, the $\text{Ins}(1,3,4)\text{P}_3$ peak was shown to contain other isomers, with the authentic compound increasing from 33% in control cells to 85% in stimulated cells. We also found a small amount of $\text{Ins}(2,4,5)\text{P}_3$, probably produced during the extraction procedure from $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$. Although potentially effective in intracellular Ca^{2+} release (Wilson *et al.*, 1985), it appears in low concentrations relative to $\text{Ins}(1,4,5)\text{P}_3$, and as in other systems is probably not a significant Ca^{2+} effector.

Our estimates of resting concentrations of $\text{Ins}(1,4,5)\text{P}_3$ (approx. 750 nM for Ramos cells and 1700 nM for T5-1 cells; see Table 1) are high relative to the K_d for the $\text{Ins}(1,4,5)\text{P}_3$ receptor, which has been estimated to be between 20 and 100 nM (Supattapone *et al.*, 1988; Meyer *et al.*, 1990). Although the receptor should be saturated at the basal levels of $\text{Ins}(1,4,5)\text{P}_3$ that we have determined, we have still observed substantial increases in intracellular Ca^{2+} upon elevation of the $\text{Ins}(1,4,5)\text{P}_3$ level. Other studies (Lambert & Nahorski, 1990; Bird *et al.*, 1991) also reported high resting levels of $\text{Ins}(1,4,5)\text{P}_3$ of between 1 and 2 μM , and found that Ca^{2+} is released in the manner expected for the linear range of the receptor. It seems clear that in cells where this occurs there must be compartmentalization of endogenous $\text{Ins}(1,4,5)\text{P}_3$, so that it does not all have access to the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

As in many other mammalian cells (Shears, 1989a), the major InsP_4 isomers in T5-1 cells were $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3,4,5,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ (see also McConnell *et al.*, 1991). Of these, the most direct metabolite of $\text{Ins}(1,4,5)\text{P}_3$ is $\text{Ins}(1,3,4,5)\text{P}_4$, which may mediate regeneration of internal Ca^{2+} stores (Changya *et al.*, 1989; Irvine, 1989). The presence of two other isomers, in particular a large component of $\text{Ins}(3,4,5,6)\text{P}_4$ which is unchanged by an anti-Ig stimulus, means that equating the InsP_4 peak with $\text{Ins}(1,3,4,5)\text{P}_4$ underestimates the increase in the putative messenger compound substantially [a 2-fold increase in total InsP_4 hides a 5-fold increase in $\text{Ins}(1,3,4,5)\text{P}_4$]. $\text{Ins}(1,3,4,6)\text{P}_4$ is possibly derived from $\text{Ins}(1,3,4)\text{P}_3$, and has been linked to InsP_3 and InsP_6 production (Hughes *et al.*, 1989; Menniti *et al.*, 1990; Stephens & Irvine, 1990). In our T5-1 samples the amount of $\text{Ins}(1,3,4,6)\text{P}_4$ increased 3.5-fold upon anti-Ig stimulation. Such a marked increase in a highly phosphorylated compound seems likely to reflect a function of some importance to the cell; at present, however, there is no explanation for the substantial presence of the higher inositol phosphates in many cell types.

Detection limits of inositol phosphate assays

The high cost of [^3H]inositol constrains the ultimate sensitivity of inositol phosphate assays even when h.p.l.c. analysis is undertaken. This is a particular problem with tonsillar B cells, which incorporate relatively little [^3H]inositol due to their metabolic inertness (see above). Even in the cell lines, our radioisotope assay required at least 10^7 cells to detect significant changes in intracellular $\text{Ins}(1,4,5)\text{P}_3$ concentrations. Flow cytometric assay of Ca^{2+} , by comparison, deals easily with cellular Ca^{2+} concentrations around 30 nM using only 10^6 cells. Thus in cell lines our assay for an increase in inositol phosphates is at least 10 times less sensitive than our assays for increase in intracellular Ca^{2+} . Despite these problems, we have been able to measure changes in $\text{Ins}(1,4,5)\text{P}_3$ concentrations in lymphocytes that are significant even when they are as small as 15% of basal levels (see the Materials and methods section and Table 1).

The sensitivity of the inositol phosphate assay becomes an important issue with respect to reports that the extent of inositol lipid hydrolysis does not correlate with the degree of either Ca^{2+} signalling (Monroe *et al.*, 1989) or cell activation (Brunswick *et al.*, 1989a). In the latter studies, less than 1200 c.p.m. of radioactivity was incorporated into all inositol phosphates. Moreover, in these and other studies (Gold *et al.*, 1987; Harnett & Klaus, 1988; Rigley *et al.*, 1989), the problems inherent in analysing low levels of radioactivity were compounded by not separately analysing individual isomers of inositol phosphates, particularly the relatively minor contribution from $\text{Ins}(1,4,5)\text{P}_3$. These studies also used lithium, which in some cells (Kennedy *et al.*, 1989) can non-specifically decrease the accumulation of both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_3$. We are unaware of any previous study on lymphocytes performed in the absence of lithium where individual isomers of inositol phosphates have been analysed.

Consequences of the dose-response relationship between anti-IgM and the intracellular messengers

The experiments reported here describe the relationships between the degree of cross-linking of sIg on the human Burkitt line, Ramos, and the subsequent inositol phosphate turnover and release of intracellular Ca^{2+} . The dose of stimulating antibody was used to vary the degree of cross-linking. Our results show that the inositol phosphate turnover continues to increase over a 100-fold range of stimulating antibody (0.7–60 $\mu\text{g}/\text{ml}$; see Table 1). The Ca^{2+} response increases with respect to anti-Ig dose with a similar predictability, but can be detected at anti-Ig concentrations 5-fold lower than those giving a minimal inositol phosphate response. The relative difficulty of observing inositol phosphate turnover may account for observations of Ca^{2+} release without significant inositol phosphate production (e.g. Monroe *et al.*, 1989).

The Ca^{2+} measurements are averages for cell populations, but it has been demonstrated that individual cells in a population of anti-Ig-stimulated B cells exhibit repeated oscillations in their levels of Ca^{2+} , rather than the smooth rise that appears in population averages (Wilson *et al.*, 1987). Actual levels of Ca^{2+} in individual cells are therefore likely to reach significantly higher concentrations at the oscillation peaks than are measured in the population response. Individual cells may thus be receiving stronger stimuli than the population Ca^{2+} indicates. This suggests that the type of signalling response we see at our lowest doses of anti-Ig is more likely to be physiologically appropriate than the manifold increases in $[\text{Ca}^{2+}]_i$ and inositol phosphate production induced *in vitro* in Ramos cells with 10–60 μg of anti-IgM/ml.

The accurate translation of the degree of cross-linking of sIg into intracellular messengers may provide the mechanism for important processes in normal B cell physiology. Amongst cells with similar Ig expression, an individual B cell with higher affinity for a cross-linking antigen will command greater receptor occupancy (Mason & Williams, 1986) and therefore receive a stronger intracellular signal. If the later consequences of the Ca^{2+} signal include enhancement of cell division, then the accurate transduction of the Ca^{2+} signal can provide the basis for the clonal proliferative advantage conferred by high-affinity Ig receptors. The majority of physiological antigens do not appear to cross-link surface Ig directly, the exceptions being thymus-independent polysaccharide antigens. However, monovalent protein antigen complexes localize on follicular dendritic cells and might provide a cross-linking stimulus for antigen-specific B cells through their Ig receptors.

The different sensitivities of T5-1 cells to cross-linking IgD and IgM suggest that these molecules may be linked differently to the signal transduction machinery. The two receptors are believed to interact with the same membrane-associated proteins (Campbell

& Cambier, 1990; Hombach *et al.*, 1990; Wienands *et al.*, 1990), so differences in their signalling capability warrant further investigation.

We thank Dr. K. Bomszyk and Mr. Kevin Draves for performing the cell water determinations, and Dr. Colin Watts and Dr. David Gray for critical reading of the manuscript. The work was supported by United States Public Health Service Grants DE-08229, GM-42508 and RR00166. The Basel Institute was founded and is supported by F. Hoffmann-La Roche & Co. AG, Basel, Switzerland.

REFERENCES

- Berridge, M. J. (1989) *J. Am. Med. Assoc.* **262**, 1834–1841
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berridge, M. J., Cobbold, P. H. & Cuthbertson, K. S. (1988) *Philos. Trans. R. Soc. London B* **320**, 325–343
- Bird, G. S. J., Oliver, K. G., Horstman, D. A., Obie, J. & Putney, J. W., Jr. (1991) *Biochem. J.* **273**, 542–546
- Brunswick, M., June, C. H., Finkelman, F. D., Dintzis, H. M., Inman, J. K. & Mond, J. J. (1989a) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6724–6728
- Brunswick, M., June, J. H., Finkelman, F. D. & Mond, J. J. (1989b) *J. Immunol.* **143**, 1414–1421
- Cambier, J. C. & Ransom, J. T. (1987) *Annu. Rev. Immunol.* **5**, 175–199
- Campbell, K. S. & Cambier, J. C. (1990) *EMBO J.* **9**, 441–448
- Changya, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. & Petersen, O. H. (1989) *J. Membr. Biol.* **109**, 85–93
- Clark, E. A. & Shu, G. (1987) *J. Immunol.* **138**, 720–725
- Clark, E. A., Ledbetter, J. A., Holly, R. C., Dinndorf, P. A. & Shu, G. (1986) *Hum. Immunol.* **16**, 100–113
- Coggeshall, K. M. & Cambier, J. C. (1984) *J. Immunol.* **133**, 3382–3386
- Cooke, A. M., Nahorski, S. R. & Potter, B. V. L. (1989) *FEBS Lett.* **242**, 373–377
- Dymecki, S. M., Niederhuber, J. E. & Desiderio, S. V. (1990) *Science* **247**, 332–336
- Gold, M. R., Jakway, J. P. & DeFranco, A. L. (1987) *J. Immunol.* **139**, 3604–3613
- Gold, M. R., Law, D. A. & DeFranco, A. L. (1990) *Nature (London)* **345**, 810–813
- Harnett, M. M. & Klaus, G. G. B. (1988) *J. Immunol.* **140**, 3135–3139
- Harnett, M. M., Holman, M. J. & Klaus, G. G. B. (1989) *Eur. J. Immunol.* **19**, 1933–1939
- Hombach, J., Tsubata, T., Leclercq, L., Stappert, H. & Reth, M. (1990) *Nature (London)* **343**, 760–762
- Hughes, P. J., Hughes, A. R., Putney, J. R. & Shears, S. B. (1989) *J. Biol. Chem.* **264**, 19871–19878
- Irvine, R. F. (1989) *Biochem. Soc. Trans.* **17**, 6–9
- Kennedy, E. D., Challiss, R. A. & Nahorski, S. R. (1989) *J. Neurochem.* **53**, 1652–1655
- Kirk, C. J., Morris, A. J. & Shears, S. B. (1990) in *Peptide Hormone Action: A Practical Approach* (Siddle, K. & Hutton, J. C., eds.), pp. 151–184, IRL Press, Oxford
- Klaus, G. G. B., Bijsterbosch, M. K., O'Garra, A., Harnett, M. M. & Rigley, K. P. (1987) *Immunol. Rev.* **99**, 19–38
- Kubagawa, H., Gathings, W. E., Levitt, D., Kearney, J. F. & Cooper, J. D. (1982) *J. Clin. Immunol.* **2**, 264–270
- Lambert, D. G. & Nahorski, S. R. (1990) *Biochem. J.* **265**, 555–562
- Lane, P. J. L., Ledbetter, J. A., McConnell, F. M., Draves, K., Deans, J., Schieven, G. L. & Clark, E. A. (1990a) *J. Immunol.* **146**, 715–722
- Lane, P. J. L., McConnell, F. M., Schieven, G. L., Clark, E. A. & Ledbetter, J. A. (1990b) *J. Immunol.* **144**, 3684–3692
- Ledbetter, J. A. & Clark, E. A. (1986) *Hum. Immunol.* **15**, 30–43
- Mason, D. W. & Williams, A. F. (1986) *Handb. Exp. Immunol.* **38**, 1–17
- McConnell, F. M., Stephens, L. R. & Shears, S. B. (1991) *Biochem. J.* **280**, 323–329
- Meisenhelder, J., Suh, P.-G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57**, 1109–1122
- Menniti, F. S., Oliver, K. G., Nogimori, K., Obie, J. F., Shears, S. B. & Putney, J. W., Jr. (1990) *J. Biol. Chem.* **265**, 11167–11176
- Meyer, T., Wensel, T. & Stryer, L. (1990) *Biochemistry* **29**, 32–37
- Monroe, J. G. & Haldar, S. (1989) *Biochim. Biophys. Acta* **1013**, 273–278
- Monroe, J. G., Seyfert, V. L., Owen, C. S. & Sykes, N. (1989) *J. Exp. Med.* **169**, 1059–1070
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–697

- Rabinovitch, P. S., June, C. H., Grossman, A. & Ledbetter, J. A. (1986) *J. Immunol.* **137**, 952–961
- Rigley, K. P., Harnett, M. M. & Klaus, G. G. B. (1989) *Eur. J. Immunol.* **19**, 481–485
- Shears, S. B. (1989a) *Biochem. J.* **260**, 313–324
- Shears, S. B. (1989b) *J. Biol. Chem.* **264**, 19879–19886
- Shears, S. B., Evans, W. H., Kirk, C. J. & Michell, R. H. (1988) *Biochem. J.* **256**, 363–369
- Simon, M. I., Strathmann, M. P. & Guatam, N. (1991) *Science* **252**, 802–808
- Stanton, T. H., Maynard, M. & Bomsztyk, K. (1986) *J. Biol. Chem.* **261**, 5699–5701
- Stephens, L. R. & Irvine, R. F. (1990) *Nature (London)* **346**, 580–583
- Stephens, L. R., Hawkins, P. T. & Downes, C. P. (1989) *Biochem. J.* **262**, 727–737
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Wahl, M. I., Nishibe, S., Suh, P.-G., Rhee, S. G. & Carpenter, G. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1568–1572
- White, M. W., McConnell, F., Shu, G. L., Morris, D. R. & Clark, E. A. (1991) *J. Immunol.* **146**, 846–853
- Wienands, J., Hombach, J., Radbruch, A., Riesterer, C. & Reth, M. (1990) *EMBO J.* **9**, 449–455
- Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J. & Brown, J. E. (1985) *J. Biol. Chem.* **260**, 13496–13501
- Wilson, H. A., Greenblatt, D., Poenie, M., Finkelman, F. D. & Tsien, R. Y. (1987) *J. Exp. Med.* **166**, 601–606
- Wong, N. S., Barker, C. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1988) *Biochem. J.* **252**, 1–5
- Wreggett, K., Howe, L., Moore, J. & Irvine, R. (1987) *Biochem. J.* **245**, 933–934
- Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T. & Toyoshima, K. (1991) *Science* **251**, 192–194

Received 10 September 1991/9 December 1991; accepted 2 January 1991