Mobilization of intracellular Ca^{2+} by adenine nucleotides in human T-leukaemia cells: evidence for ADP-specific and P_{2y}-purinergic receptors

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The expression of purinergic receptors on human T-cells was investigated and the receptors were shown to be functionally coupled to intracellular signals in two out of eight T-leukaemia cell-lines. Addition of adenine nucleotides resulted in mobilization of intracellular Ca^{2+} in HPB-ALL cells and a cell line (CB1) recently isolated from a patient with T-acute lymphoblastic leukaemia. Of a range of nucleotides tested only ADP and ATP elevated intracellular levels of Ca^{2+}, with ADP being the more potent agonist. Ca^{2+} mobilization by ATP was accompanied by increased inositol phosphate production and was blocked by the purinergic receptor antagonist, Reactive Blue 2, indicating that ATP was interacting with a P_{2y} receptor. Intracellular Ca^{2+} release triggered by ADP was independent of both inositol phosphate production and protein tyrosine phosphorylation. Expression of the transmembrane phosphotyrosine phosphatase, CD45, had no effect on ADP-stimulated Ca^{2+} mobilization. Our results show that functional P_{2y} receptors can be expressed on T-cells, and also identify a novel T-cell ADP receptor. Signals mediated by these purinergic receptors could play important roles in modulating T-cell function.

INTRODUCTION

Adenine nucleotides interact with any of four purinergic receptors defined by the selectivity of the receptors for different analogues of ATP (Gordon, 1986). P_{2x} receptors couple directly to cation channels, P_{2y} receptors are linked to Ca^{2+} mobilization by activation of phospholipase C and release of total inositol phosphate (InsP_t), whereas P_{2z} receptors are stimulated by ATP in its dissociated form ATP^{4-}, producing permeabilization of the plasma membrane (Gordon, 1986). P_{2t} receptors are expressed on platelets and the principal agonist is ADP (Fisher et al., 1985). Apart from distinguishing certain purinergic receptors by their sensitivity to different ATP analogues, selective antagonists have also been utilized to discriminate between receptor sub-types. Reactive Blue 2 (Cibacron Blue 3GA) antagonizes P_{2y} effects (Burnstock and Warland, 1987; Inoue et al., 1991) whereas Brilliant Blue G is an antagonist of P_{2z} receptors (Soltoff et al., 1989).

Mitogen-stimulated proliferation of a human mixed lymphocyte population and human natural killer cell activity have previously been shown to be inhibited by ATP (Fishman et al., 1980; Schmidt et al., 1984). In both of these cases the purinergic receptor sub-type was not known. Whereas ATP stimulated DNA synthesis and increased the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse thymocytes (Ikehara et al., 1981; El-Moatassim et al., 1987) higher concentrations of ADP were necessary to stimulate DNA synthesis, and ADP was a less effective agonist than ATP at mobilizing $[Ca^{2+}]_i$ (El-Moatassim et al., 1987).

The receptors via which ADP and ATP mediated their effects in thymocytes were suggested to be P_2-purinergic (El-Moatassim et al., 1987) although the mechanism of coupling to Ca^{2+} mobilization was not determined. In platelets (Fisher et al., 1985) and brain capillary endothelial cells (Frelin et al., 1993) ADP mobilized $[Ca^{2+}]_i$ in the apparent absence of InsP_t, though in hepatocytes and aortic endothelial cells ADP increased $[Ca^{2+}]_i$ via release of Ins(1,4,5)P_3, an effect mediated via P_{2y} receptors (Charest et al., 1985; Pirotton et al., 1987).

We have investigated the presence of purinergic receptors in eight human T-leukaemia cell lines and identified adenine nucleotide receptors which couple to rapid mobilization of $[Ca^{2+}]_i$ in HPB-ALL T-cells and in a recently isolated T-cell line (CB1) derived from a patient with T-acute lymphoblastic leukaemia (Biffen et al., 1994). Ca^{2+} mobilization was induced by ATP, probably via a P_{2y} receptor which couples to InsP_t generation. We also describe a novel ADP receptor which has not previously been reported in human T-cells. This ADP receptor mediates mobilization of $[Ca^{2+}]_i$independently of both protein tyrosine phosphorylation and InsP_t production.

MATERIALS AND METHODS

Cells

We are grateful to V. Broadbent and S. O'Connor (Addenbrookes Hospital, Cambridge, U.K.) for supplying us with samples from patients with T-acute lymphoblastic leukaemia. A bone-marrow sample (CB1) was maintained in culture and established as a cell line (Biffen et al., 1994), whereas cells from a peripheral blood sample (CB3) were used on the day of receipt. $CD45^-$ and $CD45^+$ HPB-ALL cells are described in (Shiroo et al., 1992) whereas the NY subclone of HPB-ALL cells was provided by P. Beverley (I.C.R.F. Human Tumour Immunology Group, London, U.K.). T-ALL cell lines SUP-T1, SUP-T11 and SUP-T13 were generously given by S. Smith (University of Chicago Medical Centre, Chicago, U.S.A.) and the immunophenotype of the cells is given in Smith et al. (1988, 1989a). Cells were maintained in RPMI 1640 supplemented with fetal-calf serum (FCS) at 37 °C in air/CO_2 (95/5, v/v). Identification of cell-surface-marker expression was by fluorescence-activated cell sorter (FACS) analysis using a FACS Star flow cytometer (Becton Dickinson).

Abbreviations used: ADP[S], adenosine 5′-[β-thio]diphosphate; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; $CD45^-$, CD45-depleted cells; $CD45^+$, CD45-expressing cells; FACS, fluorescence-activated cell sorter; FCS, fetal-calf serum; HBSS, Hanks' Balanced Salts Solution; InsP_t, total InsP; mAb, monoclonal antibody; PdBu, phorbol 12,13-dibutyrate; PGE_2, prostaglandin E_2; PKC, protein kinase C; PtdOH, phosphatidic acid; TCR, T-cell antigen receptor.

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Chemicals and reagents

Fura-2/AM was from Calbiochem Novabiochem, Nottingham, U.K. Hanks' Balanced Salts Solution (HBSS) was from Gibco, Paisley, Scotland, U.K. and purinergic agonists and antagonists, phorbol 12,13-dibutyrate (PdBu), prostaglandin E_2 (PGE_2), together with other chemicals used, were of the highest purity grade obtainable from Sigma, Poole, Dorset, U.K. The purified OKT3 monoclonal antibody (mAb) (IgG2a) against the CD3 antigen, comprising the γ, δ and ϵ polypeptides of the T-cell antigen receptor (TCR), was a kind gift from CILAG, Paris, France; the OKT8 mAb (IgG2a) against the CD8 co-receptor of the TCR was purified from the hybridoma supernatant by affinity chromatography on Protein A–Sepharose (Pharmacia, Uppsala, Sweden).

Calcium determinations

Cells at a density of 1×10^6/ml in 2% FCS/RPMI were loaded with 1 μM Fura-2/AM at 37 °C for 20 min, recovered by centrifugation and resuspended in Hepes-buffered HBSS, pH 7.3, supplemented with 2% FCS and 0.1 mM sulfinpyrazone. Assays were performed on 4×10^6 cells in a Perkin–Elmer LS5B luminescence spectrophotometer using Fura-2 software provided by Perkin–Elmer. $[Ca^{2+}]_i$ values were calculated and plotted using Lotus software.

Protein tyrosine phosphorylation

Cells (5×10^6) were incubated in Hepes-buffered HBSS and reactions were terminated by addition of SDS-sample buffer and heated at 100 °C for 10 min. Details of the Western-blotting procedure and identification of phosphotyrosine-containing proteins using specific anti-phosphotyrosine sera are detailed in Biffen et al. (1993).

Inositol phosphate production

Cells (5×10^5/ml) were incubated overnight in inositol-free medium before adding [^{3}H]*myo*-inositol (37 kBq/ml) and incubating the cells for a further 48 h. Cells were harvested, washed and resuspended in Hepes-buffered HBSS, pH 7.3. After a 15 min pre-incubation LiCl (10 mM) was added and after a further 5 min incubations were started by addition of agonists. Termination of the reaction, extraction and separation of InsP_t are detailed in Biffen et al. (1993).

DNA synthesis

Cells (1×10^5) were incubated in microtitre plate wells with agonists for 24 h then [^{3}H]thymidine (18 kBq) was added per well and the incubations continued overnight. The cells were lysed under vacuum in a cell-harvester (Titertek) and the DNA collected on glass-fibre filters, dried and the radioactivity associated with each filter quantified by liquid-scintillation counting.

RESULTS

ADP and ATP increase $[Ca^{2+}]_i$ in CB1 cells

T-cells derived from the bone-marrow of a patient with T-acute lymphoblastic leukaemia were maintained in culture and established as a cell line (called CB1). The phenotype of CB1 cells was $CD2^{hi}$, $CD3^{lo}$, $CD4^{hi}$, $CD8^{hi}$, as determined by flow cytometry and the cells were a mixed population of CD45-negative and CD45-positive cells (Biffen et al., 1994). These were separated with magnetic beads coated with CD45-specific mAb into two distinct sub-clones, hereafter referred to as $CD45^-$ ($CD45^-$ depleted) and $CD45^+$ ($CD45^+$ expressing). The level of expression of other antigens on the two sub-clones was the same as the parent cells above.

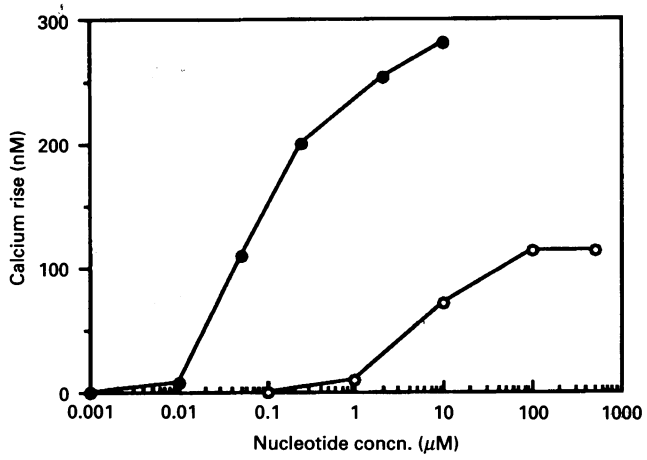

Figure 1 Dose-dependent mobilization of $[Ca^{2+}]_i$ by ADP and ATP in $CD45^-$ CB1 cells

Fura-2-loaded $CD45^-$ CB1 cells were incubated at 37 °C for 1 min before addition of ADP (●) or ATP (○) at the concentrations indicated. The rise in $[Ca^{2+}]_i$ was calculated by subtracting the basal level from the maximally elevated level for each concentration of nucleotide. The data are representative of three separate experiments.

Signal transduction via the TCR has been shown to be uncoupled in $CD45^-$ cells (Koretzky et al., 1991; Shiroo et al., 1992). This is associated with decreased activity of receptor-associated tyrosine kinases such as $p59^{fyn}$ (Shiroo et al., 1992) and $p56^{lck}$ (Biffen et al., 1994) probably as the result of tyrosine phosphorylation of the regulatory C-terminal tyrosine residue (Hurley et al., 1993). As signal transduction via the TCR is blocked in $CD45^-$ cells we used the $CD45^-$ CB1 cells to determine whether all receptor-mediated signalling is uncoupled in these cells. Addition of adenine nucleotides to Fura-2-loaded $CD45^-$ CB1 cells resulted in a dose-dependent increase in $[Ca^{2+}]_i$ (Figure 1). The cells were more sensitive to addition of ADP (half-maximal stimulation at 0.09 μM) compared with ATP (half-maximal stimulation at 6 μM). The magnitude of the Ca^{2+} rise following addition of 10 μM ADP was nearly 3-fold greater compared with stimulation with a maximal dose of ATP.

To determine whether CD45 expression did have any effect on the ability of adenine nucleotides to mobilize $[Ca^{2+}]_i$, $CD45^+$ CB1 cells were assayed for their ability to mobilize $[Ca^{2+}]_i$ following addition of ADP (Table 1). It was apparent that expression of CD45 did not alter the response of CB1 cells to ADP, although CD45 was necessary for CD3-antigen-stimulated Ca^{2+} mobilization.

Expression of ADP receptors on T-cells

A number of T-cell lines or cells from patients with T-cell leukaemia were tested for expression of ADP-sensitive Ca^{2+} mobilization (Table 1). As a comparison CD3-mAb-stimulated Ca^{2+} mobilization was also assessed. There was no correlation between CD3-induced Ca^{2+} mobilization and the ability of ADP to elevate $[Ca^{2+}]_i$. This is consistent with ADP coupling to second messenger production via a mechanism distinct from that employed by the TCR. Furthermore, ADP-dependent Ca^{2+} release was equivalent in $CD45^-$ cells compared with $CD45^+$ cells, whereas TCR-mediated signalling was reduced or absent in

Table 1 Expression of ADP receptors on T-leukaemia cells

Fura-2-loaded cells were stimulated with either ADP (10 μM) or OKT3 (1 μM) and the stimulated rise in $[Ca^{2+}]_i$ above basal levels determined. The stimulated Ca^{2+} rises were as follows: −, no rise; +, 10–50 nM; ++, 50–150 nM; +++, 150–300 nM; ++++, above 300 nM. The data are representative of three separate experiments.

	Ca^{2+} rise stimulated	
Cell line	ADP (10 μM)	OKT3 (1 μg/ml)
CB1 $CD45^-$	+++	−
CB1 $CD45^+$	+++	+
CB3	−	++
HPB-ALL $CD45^-$	+++	+
HPB-ALL $CD45^+$	+++	+++
NY-HPB-ALL	−	++++
Jurkat	−	++++
SUP-T1	−	−
SUP-T11	−	++
SUP-T13	−	++++

Table 2 Effect of selective purinergic inhibitors on Ca^{2+} mobilization

Fura-2-loaded CB1 $CD45^-$ cells were incubated at 37 °C with the purinergic antagonist for 1 min before the cells were challenged with agonist. The rise in $[Ca^{2+}]_i$ above basal levels is shown below. The data are representative of two separate experiments.

	Ca^{2+} rise (nM) stimulated	
Inhibitor added	ADP (0.2 μM)	ATP (10 μM)
None	248	67
Reactive Blue (2.5 μM)	314	76
Brilliant Blue G (20 μM)	257	76

$CD45^-$ cells. SUPT-1 cells did not respond to CD3 cross-linking (Table 1) as CD3 antigen is expressed at a very low level on these cells (results not shown). Of all the cells tested, only CB1 and HPB-ALL cells expressed functionally active ADP receptors.

Characterization of the adenine nucleotide receptors

To determine the structural requirements for agonists and thereby further characterize the receptor type, the ability of various nucleotides to mobilize Ca^{2+} was assessed. ATP (10 μM) stimulated a sub-maximal increase in $[Ca^{2+}]_i$ (Figure 1) and this concentration was chosen to test other nucleotide trisphosphates to establish rank orders of potency for the agonists. Substitution of a methylene bridge in the polyphosphate chain of ATP produces an analogue which is a less potent P_{2y} agonist than ATP (Gordon, 1986; Burnstock and Warland, 1987). In an experiment using CB1 cells in which 10 μM ADP and adenosine 5′-[β-thio]diphosphate (ADP[S]) stimulated increases of 245 and 208 nM Ca^{2+} respectively, neither α,β- nor β,γ-methylene-ATP elicited a rise in $[Ca^{2+}]_i$, consistent with the receptor being of the P_{2y} sub-type. 3′-*O*-(4-Benzoyl)benzoyl-ATP is a selective agonist of P_{2z} receptors (El-Moatassim and Dubyak, 1992) and this also had no effect on Ca^{2+} mobilization. Similarly UTP, which has been shown to increase $[Ca^{2+}]_i$ in some cells via an unclassified nucleotide receptor (Frelin et al., 1993), also failed to raise Ca^{2+} levels. The specificity of the receptor for ADP was confirmed by showing that only ADP or ADP[S] stimulated a rise in $[Ca^{2+}]_i$, whereas CDP, GDP, IDP or TDP did not. When AMP or adenosine were added at 10 μM no increase in Ca^{2+} was observed,

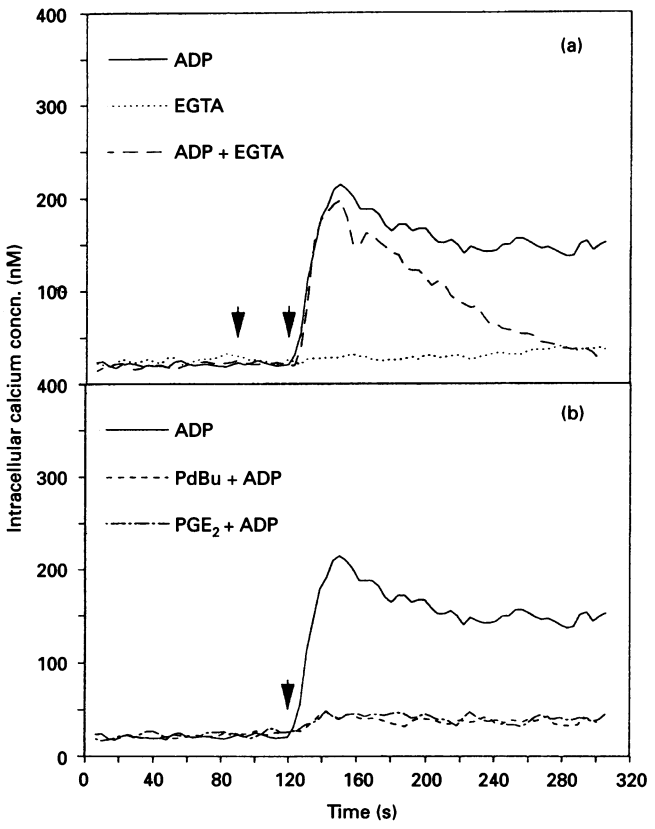

Figure 2 The ADP receptor couples to release of $[Ca^{2+}]_i$ and is regulated by activation of protein kinases A and C

Experiments were conducted on Fura-2-loaded $CD45^-$ CB1 cells. (**a**) Cells were incubated for 90 s at 37 °C before addition of EGTA (2.5 mM) (first arrow) followed 30 s later by addition of ADP (200 nM) (second arrow). (**b**) PdBu (100 ng/ml) or PGE_2 (2.5 μM) were added at t = 0 followed 2 min later by addition of ADP. The data are representative of three separate experiments.

showing that the effect of ADP and ATP was not dependent on subsequent dephosphorylation to AMP or adenosine. The ability of ADP[S], a non-hydrolysable analogue, to increase Ca^{2+} provided further evidence that the active agonist species was ADP and dephosphorylation to AMP or adenosine was not required. In a separate experiment all of the above reagents were added at 50 μM, but in each case similarly elicited no change in $[Ca^{2+}]_i$.

The effects of selective inhibitors of P_{2y} and P_{2z} receptors were tested (Table 2). Reactive Blue 2, a P_{2y} antagonist (Burnstock and Warland, 1987; Inoue et al., 1991), increased rather than reduced the ADP induced rise in $[Ca^{2+}]_i$. However, it was not possible to use higher concentrations of Reactive Blue 2 as concentrations greater than 2.5 μM resulted in significant quenching of the Fura-2 signal. Similar observations have been reported by Soltoff et al. (1989). No such quenching problem was observed with Brilliant Blue G (20 μM), a P_{2z} antagonist (Soltoff et al., 1989), and at this concentration it inhibited neither the ADP- nor the ATP-induced Ca^{2+} increases (Table 2). A concentration of 10 μM Brilliant Blue G has been shown to completely inhibit Ca^{2+} entry stimulated by 300 μM ATP in rat parotid acinar cells (Soltoff et al., 1989). Therefore, the data are not compatible with ADP mediating its effects via P_{2z} or P_{2y} receptors.

Table 3 Receptor-coupled inositol phosphate generation in CD45$^+$ CB1 cells

Cells prelabelled with [^{3}H]*myo*-inositol were incubated in Hepes-buffered HBSS in the presence of 10 mM LiCl. Cells were stimulated with the shown ligands for 10 min before extraction and quantification of InsP_t as described in the Materials and methods section. mAbs to CD3 (OKT3) and CD8 (OKT8) were cross-linked by addition of sheep anti-mouse F(ab')$_2$ fragments (5 μg/ml). Results show the level of InsP_t following the 10 min incubation and are the mean ± S.D. of incubations performed in triplicate. n.d. denotes that the value was not determined.

	Radiolabel in total InsP_t (d.p.m.)	
Addition	Control	Reactive Blue (100 μM)
None	1176 ± 66	901 ± 112
ATP (100 μM)	1515 ± 134	933 ± 12
OKT3 (1 μg/ml) + OKT8 (2 μg/ml)	1716 ± 149	1399 ± 37
ADP (0.2 μM)	1232 ± 17	n.d.

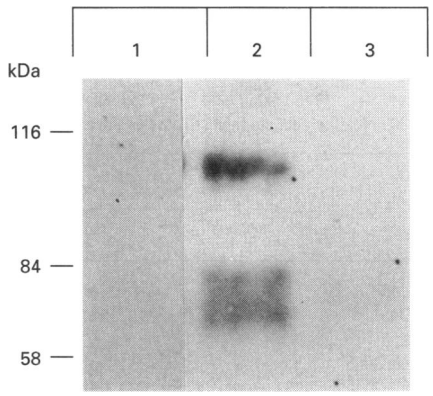

Figure 3 ADP does not induce protein tyrosine phosphorylation in CD45$^+$ CB1 cells

Cells (5×10^6) were stimulated with: (lane 1) no addition; (lane 2) 1 μg/ml OKT3 and 2 μg/ml OKT8 for 1 min followed by 5 μg/ml sheep anti-[mouse F(ab)$_2$ IgG fragment]; or (lane 3) 10 μM ADP. All incubations were terminated after 3 min incubation at 37 °C by addition of SDS-sample solubilization buffer. Detection of phosphotyrosine by specific antisera is described in the Materials and methods section. The same result was obtained in two separate experiments.

Mechanisms underlying Ca^{2+} mobilization by ADP

The source of the rise in $[Ca^{2+}]_i$ mediated by addition of ADP could result from the release of intracellular Ca^{2+} pools, stimulated Ca^{2+} influx, or both. To discriminate between these two events, CB1 cells were incubated in medium containing EGTA to chelate extracellular Ca^{2+}. Under these conditions ADP induced a rapid rise in Ca^{2+} which returned to basal levels within 3 min (Figure 2a). The magnitude of the Ca^{2+} increase was similar to that induced by addition of ADP in normal medium and showed that ADP releases Ca^{2+} initially from an intracellular pool and Ca^{2+} influx is necessary to maintain the elevated Ca^{2+} level.

The finding that ADP releases Ca^{2+} from an intracellular pool is evidence that ADP interacts with a specific receptor at the plasma membrane and couples to second messenger production. Activation of protein kinase C (PKC) or protein kinase A inhibits TCR-mediated Ca^{2+} mobilization in some T-cells (Ward and Cantrell, 1990; Park et al., 1992). To determine whether activation of these kinases might modulate ADP-receptor signalling CB1 cells were treated for 2 min with either PdBu, a potent PKC agonist, or PGE_2, an agonist which increases cyclic AMP in T-cells (Holter et al., 1991). Mobilization of Ca^{2+} was abolished by both treatments, indicating that either the receptor or the coupling process is regulated by serine/threonine phosphorylation by protein kinases A and C (Figure 2b).

ADP does not couple to inositol phosphate production nor to protein tyrosine phosphorylation

As ADP releases Ca^{2+} from an intracellular pool it was possible that ADP coupled via a receptor to production of Ins(1,4,5)P_3 which mobilizes $[Ca^{2+}]_i$ (Berridge and Irvine, 1989). CB1 CD45$^+$ cells were prelabelled with [^{3}H]*myo*-inositol and stimulated with ADP, ATP or by cross-linking CD3 and CD8 (Table 3). Addition of 200 nM ADP did not significantly increase InsP_t levels (Student's paired t test, $P > 0.1$) even though this concentration of ADP stimulated Ca^{2+} levels by 200 nM (Figure 2a). Cross-linking CD3 with CD8 increases tyrosine phosphorylation of phospholipase Cγ1 in T-cells (Shiroo et al., 1992) and raised $[Ca^{2+}]_i$ levels by 100 nM in CB1 CD45$^+$ cells (Biffen et al., 1994) as well as stimulating InsP_t production by 46% (Table 3). This demonstrated that receptor-linked InsP_t production was functionally coupled in these cells and could be measured. CD45$^+$ CB1 cells were used in these experiments as a positive control since receptor-stimulated InsP_t production is severely reduced in CD45$^-$ cells (Koretzky et al., 1991; Shiroo et al., 1992). However, similar results for ADP- and ATP-stimulated InsP_t production were observed in CD45$^-$ cells (results not shown), further showing that CD45 expression is not essential to maintain coupling of the ADP and ATP receptors, in contrast with signalling mediated by the TCR. Addition of 100 μM ATP, which raises Ca^{2+} by 100 nM (Figure 1), stimulated InsP_t production by 29% (Table 3). Therefore, ADP is most effective at raising $[Ca^{2+}]_i$ in CB1 cells but the least effective at stimulating InsP_t production.

The data obtained from Ca^{2+} assays suggested that ATP may be mediating its effects via P_{2y} receptors. This was again investigated using Reactive Blue 2 as a selective P_{2y}-receptor antagonist, although for InsP_t production it was possible to add equimolar concentrations of the antagonist which had not been possible with Fura-2-loaded cells (cf. Table 2). Table 3 shows that addition of Reactive Blue 2 alone resulted in a decrease in the level of InsP_t. Addition of ATP in the presence of Reactive Blue 2 did not stimulate the production of InsP_t above that observed with Reactive Blue 2 alone. There was a possibility that Reactive Blue 2 suppresses all stimulated InsP_t production. However, this was not the case as Reactive Blue 2 had no effect on InsP_t production in CD45$^+$CB1 cells triggered by cross-linking CD3 and CD8. The CD3 × CD8-stimulated increase in InsP_t production was 540 d.p.m. and 498 d.p.m. in the absence and presence of Reactive Blue 2 respectively. The results clearly showed that Reactive Blue 2 selectively inhibited InsP_t production stimulated by ATP in CB1 cells, providing further evidence that ATP mediates its effect via a P_{2y} receptor.

As TCR-coupling to intracellular second messenger pathways is mediated by a rise in protein tyrosine phosphorylation, the ability of ADP to induce tyrosine phosphorylation was assessed. CD3–CD8 cross-linking induced a rise in protein tyrosine phosphorylation of three proteins of molecular masses 107 kDa, 78 kDa and 69 kDa in CD45$^+$ CB1 cells as shown in Figure 3. However, ADP addition did not increase the level of tyrosine phosphorylation of these proteins (Figure 3) nor that of any protein with a molecular mass of less than 58 kDa (results not

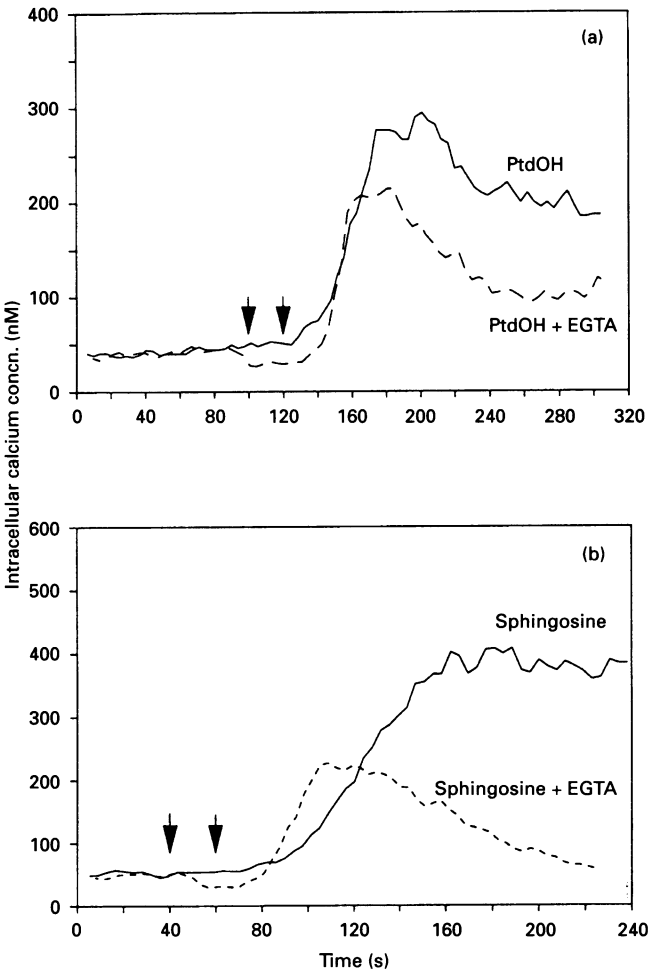

Figure 4 Mobilization of Ca^{2+} in CD45$^-$ CB1 cells by sphingosine and PtdOH

Fura-2-loaded CD45$^-$ CB1 cells were incubated with (**a**) PtdOH (10 μg/ml) or (**b**) sphingosine (50 μM). Agonist additions are shown by the second arrow at 120 s whereas the first arrow denotes addition of EGTA (2.5 mM) at 90 s. The data are representative of three separate experiments.

shown). Therefore ADP appears to couple to Ca^{2+} mobilization by a tyrosine kinase- and InsP_t- independent process.

Possible mediators of Ca^{2+} mobilization by ADP

As ADP at low concentrations (200 nM) mobilizes $[Ca^{2+}]_i$ in the absence of InsP_t production, potential mediators for this Ca^{2+} release were investigated. Sphingosine (10–100 μM) has been shown to raise Ca^{2+} levels in pancreatic acinar cells (Yule et al., 1993) whereas in Jurkat T-cells phosphatidic acid (PtdOH) has been demonstrated to release intracellular Ca^{2+} (Stewart et al., 1991). In both these cases Ca^{2+} mobilization occurred in the absence of InsP_t production. In CB1 cells addition of PtdOH or sphingosine (Figures 4a and 4b) raised $[Ca^{2+}]_i$ levels, although there was a short time lag consistent with the compounds being incorporated into the cells and further metabolized to form potential second messengers. When extracellular Ca^{2+} was chelated with EGTA, addition of either PtdOH or sphingosine stimulated a smaller increase in $[Ca^{2+}]_i$ compared with cells in normal Ca^{2+} medium. These results suggest that there are alternative mechanisms for release of $[Ca^{2+}]_i$ in CB1 cells.

Effect of stimulation of ADP receptors on cell proliferation

In mouse thymocytes addition of ATP or ADP stimulates DNA synthesis (Ikehara et al., 1981; El-Moatassim et al., 1987). Both CB1 and HPB-ALL cells are $CD4^+CD8^+$ and therefore have the phenotype of early thymocytes. In thymocytes, elevation of $[Ca^{2+}]_i$ has been associated with induction of apoptosis (Smith et al., 1989b). We were interested to observe whether addition of ADP and the subsequent elevation of $[Ca^{2+}]_i$ would have any effect on apoptosis or cell proliferation. Cells were incubated for 2 days with ADP (10 μM) before [^{3}H]thymidine was added and incorporation into DNA measured after a further overnight incubation. Addition of ADP did not affect incorporation of [^{3}H]thymidine into DNA (40973 $\pm$ 3654 d.p.m. for control and 40714 $\pm$ 2121 d.p.m. for incubations with ADP; mean $\pm$ S.D. for triplicate incubations), and neither was [^{3}H]thymidine incorporation affected by pretreating the cells with ionomycin (0.1 μg/ml) (result not shown), further demonstrating that elevated $[Ca^{2+}]_i$ did not affect cell proliferation. It was anticipated that a decrease in the rate of cell proliferation might indicate that apoptotic mechanisms had been activated. As neither ADP nor ionomycin pretreatment affected the rate of cell proliferation it appeared that apoptosis was not being induced. This was confirmed by observing Acridine Orange-stained cells by fluorescence microscopy, a method which enables the number of cells with condensed chromatin, a measure of apoptosis, to be assessed (Gregory et al., 1991). In both control and ADP-treated cells the number of Acridine Orange-stained condensed nuclei was less than 5%, indicating that, unlike freshly isolated thymocytes, CB1 cells do not apoptose following a Ca^{2+} signal.

DISCUSSION

The data show that of eight T-leukaemia cell lines investigated two responded to addition of adenine nucleotides by mobilizing $[Ca^{2+}]_i$. The effect is likely to be receptor mediated for a number of reasons: (i) of a range of nucleotides tested only ADP and ATP were agonists; (ii) the effect was not observed in all T-cell types (Table 1); (iii) ADP was active in the submicromolar range and the effect was dose-dependent (Figure 1); (iv) an intracellular pool of Ca^{2+} was released showing that ADP was acting at the plasma membrane by coupling to a transmembrane signalling process (Figure 2a); (v) phosphorylation of proteins following activation of protein kinases A and C inhibited the effect of ADP (Figure 2b); and (vi) the effect of ATP was blocked by the antagonist Reactive Blue 2 (Table 3). As the data suggest that ADP and ATP mediate their effects via different receptors, the evidence for each receptor will be discussed separately.

ATP receptor

ATP has been shown to mediate its effects via a number of purinergic-receptor subtypes (Gordon, 1986). In the CB1 cells used in this series of experiments the effect of ATP is probably mediated via interaction with a P_{2y} receptor, since the potent P_{2x}-receptor agonist α,β-methylene-ATP and the P_{2z}-receptor agonist 3′-*O*-(4-benzoyl)benzoyl-ATP had no effect on Ca^{2+} levels, and Brilliant Blue G, a P_{2z}-receptor antagonist (Soltoff et al., 1989), did not block the effect of ATP on Ca^{2+} mobilization (Table 2).

P_{2y} receptors couple to the breakdown of phosphatidylinositol-4,5-bisphosphate (Pirotton et al., 1987) and generation of InsP_t (Charest et al., 1985). We demonstrated that ATP stimulated the production of InsP_t (Table 3) and this was blocked by the P_{2y}-receptor antagonist Reactive Blue 2 (100 μM) (Burnstock and Warland, 1987). Recently a mouse P_2 receptor was cloned and both ATP and UTP were found to be agonists (Lustig et al., 1993). Such a receptor is unlikely to be expressed in CB1 cells as UTP had no effect on Ca^{2+} mobilization.

ADP receptor

Unlike ATP, ADP does not appear to mediate its effects via a P_{2y} receptor. Although ADP stimulated Ca^{2+}-mobilization, this did not involve stimulated InsP_t production (Table 3). Brilliant Blue G did not block Ca^{2+} release by ADP, indicating that P_{2z} receptors were not involved (Table 2). As ADP does not mediate its effect via either a P_{2y} or P_{2z} receptor, and the cells do not express a functional P_{2x} receptor, it is therefore likely that CB1 cells express an ADP-specific receptor. This is borne out by the sensitivity of the cells to low concentrations of ADP. 50 nM ADP stimulated a rise in $[Ca^{2+}]_i$ equivalent to that mobilized by 100 μM ATP (Figure 1) or by cross-linking CD3 and CD8 in $CD45^+$ CB1 cells (result not shown). ADP receptors similarly couple to Ca^{2+} mobilization in platelets (Sage and Rink, 1986) and brain capillary endothelial cells (Frelin et al., 1993) in the absence of InsP_t production (Fisher et al., 1985; Frelin et al., 1993).

In platelets ADP induces the rapid tyrosine phosphorylation of several proteins which have been implicated in the regulation of Ca^{2+} entry across the plasma membrane (Sargeant et al., 1993). However, we have no evidence to suggest that the ADP receptor in CB1 cells couples to protein tyrosine phosphorylation (Figure 3). Triggering the TCR induces increases in protein tyrosine phosphorylation but this is not observed following ADP addition. TCR signalling is dependent upon CD45 expression (Koretzky et al., 1991; Shiroo et al., 1992) and the ability of ADP to stimulate Ca^{2+} mobilization in $CD45^-$ HPB-ALL and CB1 subclones is further evidence for non-involvement of tyrosine kinases in the action of ADP and demonstrates that CD45 is unnecessary for ADP-receptor actions.

Agents which raise cyclic AMP block ADP-induced shape changes in platelets (Macfarlane and Mills, 1975). Similarly addition of PGE_2, which has been shown to elevate cyclic AMP levels in T-cells (Holter et al., 1991), completely blocked ADP-stimulated Ca^{2+} mobilization in CB1 cells (Figure 2b). Activation of PKC had the same effect (Figure 2b). It is likely that the ADP receptor is a substrate for protein kinases A and C, although it is also possible that one or more components in the signal transduction mechanism could also be the target.

The adenine nucleotide receptors that we have described are clearly of potential importance in T-cell function. In transformed cells we have not observed any effect of ADP on cell proliferation or apoptosis. However, assuming that ADP receptors are expressed on a sub-set of thymocytes (possibly depending on their activation and/or differentiation state) a rise in Ca^{2+} may be expected to induce apoptosis. Expression of the ADP receptors on mature lymphocytes could provide a novel pathway for modulating proliferative responses. It will be interesting to investigate these receptors in thymocytes and peripheral lymphocytes to determine whether addition of ADP has any effect on cell proliferation or cell death.

In conclusion we have shown that certain T-leukaemia cells express an ADP receptor and a P_{2y} purinergic receptor which couple to mobilization of $[Ca^{2+}]_i$ independently of expression of the CD45 phosphotyrosine phosphatase. To the best of our knowledge this is the first report of an ADP receptor on human T-cells.

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