Ca²⁺ signalling in K562 human erythroleukaemia cells: effect of dimethyl sulphoxide and role of G-proteins in thrombin- and thromboxane A₂-activated pathways

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The human leukaemic cell line K562 is a pluripotent stem cell with the potential to mature along a megakaryocytic or erythroid line. In these cells, thrombin and U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy prostaglandin F_{2 α}), a thromboxane A₂ analogue, increased intracellular Ca²⁺ in a rapid and concentration-dependent manner. The peak transient observed with both thrombin and U46619 was preserved upon stimulation in the absence of extracellular calcium and blunted with phorbol myristate acetate, suggestive of activation of phospholipase C. Short-term treatment with leupeptin abolished the calcium response to thrombin, but did not alter that to U46619. Both pertussis toxin (PT) and DMSO pretreatment inhibited thrombin- but not U46619-stimulated intracellular calcium elevation, indicating that these agonists signal through different G-proteins. Western blot analysis of crude membranes from K562 cells revealed the

presence of G₁₂ α and G₁₃ α ; the other known PT-substrates, G₁₁ α and G_o α , were not detected. Consistent with this observation, ADP-ribosylation experiments revealed the presence of two PT substrates which co-migrated with human erythrocyte G₁₂ α and G₁₃ α . An antibody raised against G_{q/11} α , a subfamily of G-protein α subunits unmodified by PT, specifically recognized 42 kDa protein(s) in K562 cells. PCR amplification of reverse-transcribed K562 RNA followed by DNA sequencing showed that these cells express messages for both G_q α and G₁₁ α . Treatment of K562 cells with DMSO reduced the levels of thrombin receptor mRNA, without simultaneous changes in the expression of G₁₂ α and G₁₃ α . We have thus identified Ca²⁺-mobilizing agonists and related G-proteins in K562 cells, together with changes induced by DMSO in this signalling pathway.

INTRODUCTION

Multipotent haemopoietic cell lines that can be induced to mature to a stable phenotype have been used as model systems for studying the regulation of gene expression during proliferation and differentiation. One such cell line, the K562 line, was established from the pleural fluid aspirate of a patient in chronic myeloid leukaemia blast crisis [1]. Phenotypically considered to be an erythroleukaemic cell type, this highly undifferentiated cell is of variable size and expresses specific markers of erythroid, myeloid and megakaryocytic lineage in keeping with its origin as the clonal expansion of a pluripotent stem cell [2]. Initially shown to differentiate to an erythroid phenotype in response to haemin and sodium butyrate, the list of inducers now includes a variety of agents such as the anti-neoplastic drugs, *cis*-diaminedichloroplatinum II, daunomycin and 1- β -D-arabinofuranosylcytosine (Ara-C) [3–5].

G-proteins are membrane-bound heterotrimeric GTPases composed of α , β and γ subunits which couple activated receptors to intracellular effectors such as adenylate cyclase, phospholipase C

(PLC), phospholipase A₂ (PLA₂), cyclic GMP phosphodiesterase, and K⁺, Na⁺ and Ca²⁺-channels (for reviews and recent methodology see articles contributed to [6] and [7] respectively). Members of the G_i subgroup of this family, composed of G₁₁, G₁₂, G₁₃, G_{o1}, G_{o2}, T₁ and T₂ are covalently modified by pertussis toxin (PT), resulting in the uncoupling of receptors from their cognate G-proteins and impairment of agonist action. In some cell types, agonist-mediated activation of PLC is PT-sensitive; this appears to reflect the interaction of $\beta\gamma$ dimers, released following coupling of the occupied receptors with G_i proteins, with the PLC- β 2 isoform (for review see [8]). On the other hand, PT-insensitive activation of PLC, as detected in most cell types, arises from the interaction of α subunits corresponding to members of the G_q class (including G_q and G₁₁₋₁₆) with PLC- β 1 and/or PLC- β 3 [8]. Early cellular events following receptor and G-protein activation, such as PLC stimulation, with calcium mobilization, induction of various protein kinases and the transcription of early growth response genes, are important, at least in some biological systems, for cellular proliferation and differentiation [9,10]. Changes in G-protein levels or activity

Abbreviations used: Ara-C, 1- β -D-arabinofuranosylcytosine; PLC, phospholipase C; PLA₂, phospholipase A₂; PT, pertussis toxin; U46619, 9,11-dideoxy-9 α ,11 α -methanoepoxy prostaglandin F_{2 α} ; IMDM, Iscove's modified Dulbecco's medium; KHH, Krebs–Henseleit–N–Hepes; [Ca²⁺]_i, intracellular calcium concentration; DPBS, Dulbecco's phosphate-buffered saline; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TxA₂, thromboxane A₂; PMA, phorbol myristate acetate; I-BOP, [1S-(1 α ,2 β (5Z),3 α -(1E,3S),4 α)-7-[3-(3-hydroxy-4-(*p*-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid.

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have been shown to accompany, or directly promote, cell differentiation, proliferation or transformation. In HL-60 myeloid cells, induction to a neutrophil-like phenotype is accompanied by down-regulation of $G_{16}\alpha$ and its corresponding mRNA [11]. Differentiation of K562 cells with haemin, Ara-C and daunomycin is accompanied by inhibition of *c-myc* proto-oncogene expression [5,12], although *c-myc* expression can be inhibited by other agents, such as DMSO, without concomitant differentiation [13]. Yet in K562 cells, very little is known about Ca^{2+} -mobilizing receptors, G-protein expression and changes in signalling during phenotypic modification. The purpose of this study was to characterize receptor-mediated, G-protein-coupled calcium signalling in K562 cells and examine the effect of DMSO on components of this pathway.

EXPERIMENTAL

Materials

U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy prostaglandin $F_{2\alpha}$) was from Cayman Chemical (Ann Arbor, MI, U.S.A.) and bovine thrombin from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.) and PT was purchased from List Biological Laboratories (Campbell, CA, U.S.A.). Leupeptin, aprotinin and PMSF were from Boehringer Mannheim (Indianapolis, IN, U.S.A.) and trypsin [treated with L-(tosylamido-2-phenyl)ethyl chloromethane ketone] from Worthington Biochemicals (Freehold, NJ, U.S.A.). [α - 32 P]dCTP (3000 Ci/mmol) and [adenylate- 32 P]NAD (300 Ci/mmol) were from ICN Biochemicals (Irvine, CA, U.S.A.). 125 I-labelled goat anti-(rabbit IgG) (10 μ Ci/mg) was from New England Nuclear (Wilmington, DE, U.S.A.). Previously characterized rabbit antisera against specific decapeptides of the G-proteins $G_{11}\alpha$ (#3646), $G_{12}\alpha$ (#1521), $G_{13}\alpha$ (#1518) and $G_{14}\alpha$ (#2919) were a generous gift from David Manning, Department of Pharmacology, University of Pennsylvania [14,15]. An antibody raised against the common C-terminal decapeptide of $G_{11/12}\alpha$ (#0946) was also provided to us by David Manning. Rabbit antibody against $G_{16}\alpha$ was obtained from Dr. K.-J. Chang, Burroughs Wellcome Co. [16]. Culture media were from Gibco BRL (Grand Island, NY, U.S.A.) and all other chemicals from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

K562 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD, U.S.A.) and were routinely maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) bovine calf serum in a 7.5% CO_2 -enriched incubator. For certain experiments, cells were treated with 1% DMSO for 3 days in serum-free IMDM supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin and 5 ng/ml sodium selenite. This treatment was typically associated with a reduction in growth rate and the emergence of small, uniform, loosely adherent cells. Cells subjected to this treatment are referred to as 'treated with DMSO in chemically defined medium' throughout the text or as 'DMSO-treated' in the Figures and titles of Figure legends.

Intracellular calcium determination

On the day of the experiment, K562 cells in suspension were centrifuged at 200 g and 22 °C and the cell pellet resuspended in Krebs-Henseleit-N-Hepes (KHH) buffer, pH 7.4, containing 1 mg/ml fatty acid-poor BSA. The suspended cells were loaded with 1 μ M fura-2 acetoxymethyl ester (fura-2) and incubated for

30 min in a 5% CO_2 incubator at 37 °C. The cells were then spun down and washed in fura-2-free KHH, incubated for a further 20 min at 37 °C, to allow for intracellular cleavage of the dye, and finally suspended in KHH buffer at a concentration of 10^6 cells per ml.

Calcium measurements were performed in a single-wavelength spectrofluorimeter (University of Pennsylvania Biomedical Instruments Group) with excitation at 339 nm and emission at 500 nm. Aliquots of the cell suspension (1.5 ml) were loaded in a quartz cuvette and the effects of a variety of agonists tested at 37 °C. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated using a standard formula and assuming an equilibrium dissociation constant of 224 nM at 37 °C for the fura-2/ Ca^{2+} interaction [17]. F_{max} was measured by adding 20 μ l of 5 mM digitonin and F_{min} by adding sequentially 50 μ l of 0.4 M EGTA and 50 μ l of 3 M Tris. To examine whether the elevation of $[Ca^{2+}]_i$ was secondary to intracellular calcium release, some experiments were performed in the absence of extracellular Ca^{2+} , or in the presence of the Ca^{2+} -channel blockers $NiCl_2$ or $LaCl_3$.

Membrane protein preparation

Control or DMSO-treated K562 cells were pelleted at 200 g and washed twice in Dulbecco's PBS (DPBS) and then resuspended in a cold solution containing 100 mM KCl, 5 mM NaCl, 3 mM $MgCl_2$, 50 mM Hepes, pH 7.4, and 1 mM dithiothreitol (DTT) (lysis buffer) and placed in a nitrogen cavitation chamber at 3105 kPa (450 p.s.i.) for 20 min. The suspension was centrifuged at 500 g and 4 °C for 10 min, to remove nuclei and unbroken cells, and the supernatant then spun at 14000 g and 4 °C for 10 min. The resulting pellet was resuspended in lysis buffer containing 1 mM EGTA and then homogenized in a Teflon-glass homogenizer with 10 strokes of the pestle. Following a further spin at 14000 g and 4 °C for 10 min, the crude membrane fractions were resuspended in 50 mM Tris/HCl, pH 7.0, at approx. 2 mg/ml and stored at -70 °C till use. For membranes prepared for immunoblotting, all solutions also contained 0.5 μ g/ml aprotinin, 0.5 μ g/ml leupeptin and 200 μ M PMSF. Protein determinations were by the Lowry method using BSA as standard.

ADP-ribosylation

ADP-ribosylation was as described by Ribeiro-Neto et al. [18] with some modifications. PT (500 μ g/ml) was activated with 50 mM DTT for 20 min at 32 °C followed by a 15-fold dilution with cold 0.025% BSA. The ADP-ribosylation mixture consisted of 20 μ l of a 0.25 μ g/ μ l suspension of membranes and 20 μ l of pre-activated and diluted PT in a final volume of 100 μ l reaction buffer that also contained 12.5 mM Tris/HCl, pH 7.4, 0.35 mM EDTA, 0.1 mM ATP, 0.1 mM GTP, 0.033% Lubrol and 5×10^7 c.p.m./ml [32 P]NAD. The mixtures were incubated at 32 °C for 40 min, the reactions were stopped by the addition of 100 μ l of cold 20% trichloroacetic acid, and then centrifuged at 1200 g for 20 min at 4 °C. The supernatants were aspirated, the pellets washed with diethyl ether and spun again at 1200 g for 30 min. The final pellets were dissolved in 62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% SDS and 0.001% Pyronin Y (modified Laemmli buffer) and resolved by SDS/PAGE on a 9% acrylamide gel with a 4-8 M urea gradient [19]. The gels were then stained with Coomassie Blue, dried and subjected to autoradiography with Kodak X-OMat XAR film at room temperature for 1-48 h.

Western blot analysis

Membrane preparations from K562 cells and rat brain myelin (positive control for $\text{G}_{11\alpha}$, G_α and G_β ; see [20]) were used. Proteins were solubilized in modified Laemmli buffer, subjected to electrophoresis on a 10% acrylamide gel, transferred at 4 °C and 40 V for 12–16 h on to 0.45 μm -pore-size nitrocellulose (Immobilon NC, Millipore) and then blocked for 2 h at 22 °C in DPBS containing 3% (w/v) BSA and 0.02% sodium azide. After incubation with the primary antiserum (anti- $\text{G}_{11\alpha}$, anti- $\text{G}_{13\alpha}$) for 2 h at 22 °C, the membrane was washed once with 150 mM NaCl, 50 mM Tris/HCl, pH 8.5, 1% Nonidet P-40, and 0.1% SDS and twice with 150 mM NaCl, 50 mM Tris/HCl, pH 8.5. Following a further blocking step, the nitrocellulose membrane was incubated with 10^6 c.p.m./ml of ^{125}I -labelled goat anti-(rabbit IgG) serum for 1 h at 22 °C, washed as before, and dried prior to autoradiography with Kodak X-OMat XAR film at –70 °C for 1–10 days. In experiments using anti- $\text{G}_{12\alpha}$, anti- $\text{G}_{q/11\alpha}$ or anti- G_α as primary antisera, the immunoblotting procedure was as described previously (blotting for mitogen-activated protein kinase in [21]). Briefly, the incubations were carried out at 20 °C in 10 mM Tris/HCl, pH 7.4, containing 0.05% Tween-20, 0.15 M NaCl and 1 mM EDTA (blotting buffer). Blocking (using 3% dried milk in blotting buffer) and incubations with solutions of primary and secondary antisera (prepared using blocking buffer as diluent) were carried out for 1 h at 20 °C. A 1:2000 dilution of horseradish peroxidase-coupled donkey anti-(rabbit IgG) was used as secondary antiserum. Blots were washed with blotting buffer before the blocking step and the incubations with primary and secondary antisera (one wash of 15 min, followed by two washes of 5 min each). Detection was carried out using an enhanced chemiluminescence kit (Amersham), following the instructions indicated by the supplier.

Isolation of RNA

Total RNA was prepared from control and DMSO-treated K562 cells by the method of Chirgwin et al. [22]. Briefly, K562 cells were washed twice in cold 0.9% NaCl and then homogenized in a 4 M guanidine thiocyanate solution (containing 40% CsCl, 0.5% sodium *N*-lauryl sarcosine, 0.7% 2-mercaptoethanol, 0.1% antifoam A and 25 mM sodium citrate, pH 7.0), by syringing the sample through a 21G needle multiple times. Samples were then cushioned on a 5.7 M CsCl/ 0.1 M EDTA, pH 7.5, solution and centrifuged in an SW50.1 rotor at 150000 g for 16 h at 20 °C. The RNA pellet was subsequently dissolved in a buffer containing 10 mM Tris/HCl, pH 7.2, 5 mM EDTA, 0.2 M NaCl and 0.5% SDS. RNA was extracted twice with chloroform/butanol (4:1, v/v) and then precipitated with ethanol in the presence of 0.3 M sodium acetate, pH 5.2.

cDNA probes

The 2.26 kb $\text{G}_{13\alpha}$ cDNA was originally isolated from a human liver cDNA library, the 1.47 kb *Eco*RI fragment of $\text{G}_{12\alpha}$ from a mouse macrophage cDNA library and the 1.3 kb G_α probe from a rat heart cDNA library (described in [23]). The full-length $\text{G}_{11\alpha}$ probe was originally cloned from a human brain cDNA library [24] and the G_β probe from a rat brain cDNA library [25]. The thrombin receptor cDNA was originally cloned from human megakaryocytic-like Dami cells [26] (a 670 bp *Sma*I fragment was used as probe). The rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was obtained from Ambion (Austin, TX, U.S.A.). All probes were labelled by random primer extension [27].

Northern analysis

RNA samples were denatured and fractionated on a 1.5% agarose, 6% formaldehyde gel and then transferred on to nylon (Biotrans, ICN) membranes. Equivalence of RNA loading was assessed by staining ribosomal RNA with Methylene Blue. The membranes were prehybridized in 50% formamide, 5× Denhardt's, 50 mM sodium phosphate (pH 6.5), 0.75 M NaCl, 75 mM sodium citrate, 0.1% SDS and 75 $\mu\text{g}/\text{ml}$ yeast tRNA at 42 °C for 4–6 h. Hybridization was in fresh prehybridization solution containing 10% dextran sulphate and (2–3) × 10^6 c.p.m./ml of random primer extended probe (10^8 – 10^9 c.p.m./ μg) at 42 °C for 16–20 h. The membranes were washed twice in 2× SSC/0.1% SDS at 22 °C (SSC: 0.15 M NaCl/0.015 M sodium citrate) and then 2–3 times in 0.1× SSC/0.1% SDS at 50 °C. Autoradiograms were generated on Kodak X-OMat XAR film at –70 °C for 1–7 days. To confirm the equivalence of RNA loading, and to correct for non-specific differences in transcription between experimental conditions, parallel lanes were probed with a GAPDH cDNA.

PCR amplification of $\text{G}_\alpha/\text{G}_{11\alpha}$ cDNA fragments from K562 cells

A degenerate primer corresponding to the common C-terminal heptapeptide of mouse G_α and $\text{G}_{11\alpha}$ was used for first-strand DNA synthesis using AMV reverse transcriptase. The reaction mixtures (50 μl) contained 50 mM Tris/HCl, pH 8.3, 10 mM MgCl_2 , 50 mM KCl, 10 mM DTT, 50 μM spermidine, 500 μM each of dNTPs, 10 μg of total RNA and 10 units of AMV reverse transcriptase (Promega). The RNA–DNA heteroduplexes were subjected to PCR amplification using degenerate primers 5'-AATGAGAA[C/T]CG[C/A]ATGGAGGA-3' (upstream) and 5'-GATGTT[T/C]TCGGT[G/A]TC[T/G]GTGGC-3' (downstream). These primers correspond to sequences NENRME and ATDTENI which are conserved in mouse G_α , mouse $\text{G}_{11\alpha}$ and in *Drosophila melanogaster* G_α (residues 244–250 and 331–337, respectively in the murine proteins; residues 238–244 and 325–331 in the *Drosophila* protein). PCR mixtures (100 μl) consisted of 5 μl of reverse transcription products, 4 μM primers, 200 μM each of dNTPs, 4.5 mM MgCl_2 and 2.5 units of *Taq* DNA polymerase; amplification involved two rounds of 30 cycles (95 °C, 1 min; 48 °C, 30 s; 72 °C, 1 min) followed by an additional cycle (48 °C, 2 min; 70 °C, 7 min). The amplified fragments were ligated into the *Eco*RV site of *p*BluescriptII SK where a T overhang had been created using *Taq* polymerase and dTTP. Positive recombinants were identified by loss of α -complementation of the β -galactosidase gene product on isopropyl β -D-thiogalactoside/5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside plates. Individual clones were sequenced in both directions by the dideoxy termination method [28].

RESULTS

A number of agonists including erythropoietin, platelet activating factor, angiotensin II, arginine vasopressin, endothelin, leukotrienes B_4 and D_4 , carbachol and noradrenaline were screened, and two agents, U46619 and thrombin, were identified for their ability to increase $[\text{Ca}^{2+}]_i$ in K562 cells. U46619, a stable analogue of thromboxane A_2 (TxA_2), increased $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner, with a threshold at 20 nM and maximum values seen at 2 μM (Figures 1a–1c). A rapid transient followed by a sustained phase of lower amplitude was evident in the presence of extracellular calcium. In the absence of extracellular calcium, the calcium transient was preserved and the amplitude relatively unchanged, implying that the initial rapid rise in $[\text{Ca}^{2+}]_i$ was secondary to intracellular release, suggestive of

Figure 1 Effect of U46619 on $[Ca^{2+}]_i$ in undifferentiated K562 cells

Fura-2 loaded K562 cells, suspended in KHH buffer, were exposed to various concentrations of U46619 and Ca^{2+} transients measured. In (d) and (e), experiments were performed in Ca^{2+} -free KHH ($[Ca^{2+}]_0 = 0$). Results are representative of at least three independent determinations assayed in duplicate. The indicated time scale is identical for all panels.

activation of PLC (Figure 1d). Addition of non-specific blockers of calcium channels, such as Ni^{2+} (1 mM with $[Ca^{2+}]_0 = 0$) and La^{3+} (10 μ M with $[Ca^{2+}]_0 = 1.25$ mM), did not abolish the early peak in $[Ca^{2+}]_i$ (Figures 1e and 1f). Pretreatment with phorbol myristate acetate (PMA), a tumour-promoting phorbol ester, at 100 nM for 1–2 min, abolished the ability of U46619 subsequently to elevate $[Ca^{2+}]_i$ (Figure 2a). Pretreatment of K562 cells for 12–16 h with 100 ng/ml of PT, which catalyses the transfer of the ADP-ribose moiety from NAD^+ to amino groups present in certain G-proteins (rendering them unresponsive to receptor

Figure 2 Effect of U46619 on $[Ca^{2+}]_i$ in undifferentiated and DMSO-treated K562 cells

Where indicated, K562 cells were pretreated with 100 ng/ml pertussis toxin (PT) for 16 h prior to experiments. Representative calcium tracings from at least three independent experiments, assayed in duplicate, are shown. Data in the bar graph represent the change in $[Ca^{2+}]_i$ following addition of U46619 (peak of transient minus basal) and are expressed as mean $\pm$ S.E.M. from 4 to 8 individual determinations. The indicated time scale is identical for all panels.

activation), did not affect the calcium response to U46619 (Figures 2b and 2d).

Bovine thrombin increased $[Ca^{2+}]_i$ in a concentration-dependent manner in undifferentiated cells, with a threshold effect at 0.01–0.05 unit/ml and maximum effect measured at 5 units/ml (Figure 3a). At relatively high concentrations, a rapid initial transient was followed by a slower sustained elevation of $[Ca^{2+}]_i$,

Figure 3 Effect of thrombin on $[Ca^{2+}]_i$ in undifferentiated and DMSO-treated K562 cells

Where indicated, K562 cells were pretreated with 100 ng/ml pertussis toxin (PT) for 16 h prior to experiments. Representative calcium tracings from two to three independent experiments, assayed in duplicate, are shown. In (a) and (b), different calcium tracings are superimposed for comparison. Experiments in Ca^{2+} -free KHH are shown as $[Ca^{2+}]_0 = 0$. The time bar denoted is identical for all panels.

Figure 4 Desensitization of agonist responses and the role of proteolytic activation on Ca^{2+} signalling in undifferentiated K562 cells

Thrombin (2.5 units/ml) and U46619 (1 μM) were used for all experiments. Where indicated, the concentrations of trypsin and leupeptin were 0.1 μM and 100 $\mu\text{g/ml}$ respectively. The indicated time scale is identical for all panels.

while at lower concentrations the rate of initial rise in $[\text{Ca}^{2+}]_i$ was lower (Figure 3a). In the absence of extracellular calcium, the peak transient was preserved with a lower amplitude, but the sustained phase was lost, suggesting that the first phase was due to intracellular release and the second due to opening of calcium channels (Figure 3b). Similar to its effects on the response to U46619, short-term treatment with 100 nM PMA inhibited the ability of thrombin to elevate $[\text{Ca}^{2+}]_i$ (Figure 3c). Cells treated with DMSO in chemically defined medium showed a dramatically inhibited calcium response to thrombin without any alteration in the response to U46619 (Figures 2c, 3d and 3e). Pretreatment of K562 cells with 100 ng/ml PT for 12–15 h substantially inhibited the effect of thrombin, while the combination of DMSO and PT treatment resulted in almost complete abolition of the thrombin response (Figure 3e); in contrast, responses to U46619 were totally preserved by both manoeuvres (Figure 2d). This indicated that thrombin, as opposed to U46619, coupled to PLC through a PT-sensitive pathway and that some component of this pathway was disrupted upon treatment with DMSO in chemically defined medium.

Addition of 1–2 μM ionomycin to undifferentiated K562 cells increased $[\text{Ca}^{2+}]_i$ from 94 ± 20 nM to 995 ± 252 nM (average $\pm$ S.E.M., $n = 3$); the maximally effective concentrations of thrombin (2–4 units/ml) mobilized approximately one-third of the intracellular Ca^{2+} available to ionomycin, from 102 ± 27 to 380 ± 62 nM (average $\pm$ S.E.M., $n = 3$). The efficacy of U46619 was similar to that of thrombin.

Both thrombin and U46619 exhibited a very rapid and complete homologous desensitization upon re-exposure to the same agonist (Figures 4c and 4d), but neither response was affected by prior stimulation with the other agent (Figures 4a and 4b). Addition of 0.1 μM trypsin (a serine protease specific for lysyl and arginyl residues at the amino side of peptide bonds) also triggered a calcium transient in these cells (Figure 4e); pretreatment with 200 $\mu\text{g/ml}$ leupeptin specifically prevented

Figure 5 ADP-ribosylation (a) and Western blotting (b and c) of membranes from undifferentiated or DMSO-treated K562 cells

(a) Membranes were prepared from K562 cells and subsequent ADP-ribosylation carried out in the presence of activated pertussis toxin and $[\text{P}^{32}]\text{NAD}$ at 32 $^{\circ}\text{C}$ for 40 min. Solubilized proteins were then analysed by SDS/PAGE on 10% acrylamide/4–8 M urea gradient gel. Each lane contained 5 μg of membrane protein. (b) and (c): K562 cell membranes (100 μg each) were solubilized and analysed by SDS/PAGE on a 10% acrylamide gel. Transferred proteins were blotted with either a 1:300 dilution of anti- $\text{G}_{13}\alpha$ serum (b) or a 1:300 dilution of anti- $\text{G}_{12}\alpha$ serum (c). For details see the Experimental section. Lane 1, control cells; lane 2, cells treated with DMSO in chemically defined medium.

the subsequent responses to thrombin (Figure 4f) and trypsin (results not shown).

To identify G-proteins that may be involved in calcium signalling in K562 cells we initially performed *in vitro* ADP-ribosylation assays using K562 cell membranes, PT and $[\text{P}^{32}]\text{NAD}$. To enhance the resolution of G-protein α subunits, the proteins were resolved on 4–8 M urea gradient gels. Both control and DMSO-treated K562 cells appear to express at least two PT-sensitive substrates with apparent molecular masses of 40 and 38 kDa (Figure 5a). The mobility of these bands was identical to that of α_{13} and α_{12} purified from human erythrocytes (results not shown). The lower apparent molecular mass on the ADP-ribosylation experiments may result from the use of urea gradient gels [19].

The identity of PT substrates in K562 cells was assessed by immunoblotting using specific anti-peptide sera. Bands specifically reacting with anti- $\text{G}_{13}\alpha$ and anti- $\text{G}_{12}\alpha$ sera were detected equally in control and DMSO-treated cells (Figures 5b and 5c respectively). These results were confirmed by total RNA using $\text{G}\alpha$ -subunit cDNAs as probes. Both control and DMSO-treated cells express similar levels of a 2.5–2.6 kb message for $\text{G}_{12}\alpha$ and a 3.6–3.7 kb message for $\text{G}_{13}\alpha$ (Figures 6a and 6b respectively). We could not detect expression of message encoding $\text{G}_{11}\alpha$ or $\text{G}_o\alpha$ subunits by Northern blot analysis using their corresponding cDNA probes. Consistent with the results of the RNA blots, no specific immunoreactivity was detected in K562 crude membranes using antisera against $\text{G}_{11}\alpha$ or $\text{G}_o\alpha$ (Figures 7a and 7b). K562 cell membranes also contain ≈ 42 kDa protein(s) reacting positively with anti- $\text{G}_{q/11}\alpha$ (an antiserum directed against the PT-insensitive $\text{G}_q\alpha$ subfamily), but no specific immunoreactivity was detected against anti- $\text{G}_x\alpha$ (Figures 7c and 7d respectively).

The antiserum against $\text{G}_{q/11}\alpha$ recognizes a common C-terminal decapeptide and hence does not differentiate between them. Using cDNAs encoding murine $\text{G}_q\alpha$ and $\text{G}_{11}\alpha$ we were unable to identify a clear transcript for either α subunit in K562 cells by Northern blot analysis. This could reflect low copy message, message instability or sequence differences between the murine probes and the mRNAs expressed in K562 cells. Therefore, the

Figure 6 Northern blotting of total cellular RNA from K562 cells

Samples (20 µg) of total RNA from control K562 cells (lanes 1) or K562 cells treated with DMSO in chemically defined medium (lanes 2) were probed with either ³²P-labelled G_{α} cDNA (a) or ³²P-labelled G_{β} cDNA (b). The equivalence of loading was controlled by probing parallel lanes with a GAPDH cDNA probe.

Figure 7 Western blot experiments using antisera against G_{α} , G_{β} , and G_{γ}

(a) K562 membranes (100 µg, lane 1) and rat brain myelin (80 µg, lane 2) were incubated with a 1:300 dilution of rabbit anti- G_{α} . (b) K562 membranes (100 µg, lane 1) and rat brain myelin (80 µg, lane 2) were incubated with a 1:400 dilution of rabbit anti- G_{β} . (c) 100 µg each of membranes from undifferentiated K562 cells (lane 1) and K562 cells treated with DMSO in chemically defined medium (lane 2) were incubated with a 1:200 dilution of rabbit anti- $G_{\gamma/11}$ serum. (d) Aliquots (100 µg each) of membranes from undifferentiated K562 cells (lane 1) and K562 cells treated with DMSO in chemically defined medium (lane 2), together with 50 µg of rat brain myelin (lane 3) were incubated with a 1:100 dilution of a polyclonal anti- G_{γ} serum. Several non-specific bands were detected when blotting K562 membranes with the anti- G_{γ} serum (d); the position corresponding to brain myelin G_{γ} is indicated by the arrowhead.

expression of message encoding G_{α} and G_{11} in K562 cells was studied by PCR amplification using degenerate primers corresponding to conserved regions in the murine and *Drosophila melanogaster* proteins. Sequencing of individual clones generated by ligation of the amplified PCR fragments (282 bp) revealed the expression of both G_{α} and G_{11} mRNA in K562 cells (nucleotide sequences were 95% and 94% similar, respectively, to the corresponding murine cDNA fragments; Figures 8a and 8b).

The expression of thrombin receptor was studied by Northern blot analysis using a human thrombin receptor probe. In three out of four experiments we observed a significant reduction of message (≈ 3.7 kb) in the K562 cells treated with DMSO in chemically defined medium (Figure 9), with the remaining blot showing only a minor decrease. These results demonstrate that there is reduced expression of the human thrombin receptor with

Figure 8 Nucleotide sequence and deduced primary structure of fragments corresponding to human G_{α} (panel A) and G_{11} (panel B)

Reverse-transcribed mRNA from K562 cells was subjected to PCR amplification using the indicated primers (see underlined sequences). Nucleotide substitutions compared with the murine cDNAs are shown. The primary structure of the fragment corresponding to human G_{α} is identical to that of the murine protein; the amino acid sequence of human G_{11} shows a single amino acid substitution compared with its murine counterpart (N residue in position 266 of murine G_{11} replaced by H, see asterisk). For details see the Experimental section.

Figure 9 Expression of thrombin receptor mRNA in undifferentiated and DMSO-treated cells

Aliquots of 20 µg of total RNA from undifferentiated K562 cells (lane 1) and K562 cells treated with DMSO in chemically defined medium (lane 2) were probed with an *Sma*I fragment (670 bp) of ³²P-labelled human thrombin cDNA probe. Equivalence of loading was controlled by probing parallel lanes with GAPDH cDNA probe (shown in lower window). For details see the Experimental section.

this treatment which correlates with the observed inhibition of the thrombin-stimulated Ca^{2+} response.

DISCUSSION

In this study we have identified ligands that elevate $[\text{Ca}^{2+}]_i$ in K562 cells, demonstrated that these ligands signal through different G-proteins and observed changes in their coupling following treatment with DMSO in chemically defined medium. This elevation of $[\text{Ca}^{2+}]_i$ appears to occur through both intracellular release and influx through an undefined Ca^{2+} channel. Given that the K562 cell line shows several megakaryocytic features, it is of interest that two potent stimulants of PLC in human platelets, U46619 and thrombin, trigger their responses in a dedifferentiated precursor cell type, though other agonists such as the leukotrienes, platelet activating factor and arginine vasopressin, which are also active in platelets, do not mobilize calcium in K562 cells. Previously, receptors for TxA_2 [29] and $\text{P}_{2\text{T}}$ purinoceptors [30,31] had been identified in K562 cells and contradictory observations were made on the effects of thrombin on $[\text{Ca}^{2+}]_i$ in this line ([30,32]; see discussion below). We observed that PMA abolished the Ca^{2+} response triggered by U46619 and thrombin in K562 cells (Figures 2 and 3), similar to what was reported with the ADP-induced $[\text{Ca}^{2+}]_i$ response in the same line [30] and with similar alterations in the response of platelets to various agonists (for discussion see [30]).

With respect to the TxA_2 receptor-driven effects, our results agree with those reported by Dorn [29] who observed that the TxA_2 mimetic I-BOP was also able to increase $[\text{Ca}^{2+}]_i$ in K562 cells. The rapid desensitization that we detected following occupancy of the TxA_2 receptor in K562 cells (Figure 4d) is in contrast with slower responses previously reported for this cell line [29], but coincides with observations made in platelets [33]. A possible reason for the above discrepancy may lie in the use of two different TxA_2 mimetics (U44069 and I-BOP) in the desensitization and assay protocols followed in the previous study on K562 cells [29].

We have observed that while U46619 and thrombin exhibit homologous agonist-induced desensitization, they do not cross-desensitize each other, an observation similar to that made in platelets [33].

The TxA_2 receptor has been cloned recently, showing features common to the members of the G-protein-coupled receptor superfamily [34,35]. In human platelets, TxA_2 receptor activation is associated with PT-insensitive G-protein-dependent stimulation of PLC [36], similar to our observation in K562 cells. Pretreatment of platelet membranes with an antibody directed against the common C-terminal decapeptide of $\text{G}_{\text{q}}\alpha$ and $\text{G}_{11}\alpha$ inhibited TxA_2 -stimulated GTPase [37]. Both reconstitution and transfection experiments have shown that the α subunits of G_{q} , G_{11} and G_{16} can activate PLC- $\beta 1$, PLC- $\beta 3$ and also PLC- $\beta 2$ (albeit less efficaciously) (for review see [8]). These data suggest that the $\text{G}_{\text{q}}\alpha$ and/or $\text{G}_{11}\alpha$ subunits expressed in K562 cells (Figures 7c and 8) represent the PT-insensitive G-proteins that couple TxA_2 receptors to activation of PLC. In this regard it has been recently pointed out that G_{q} and G_{11} are activated with similar agonist concentration-response curves and kinetics by thyrotropin, bombesin or vasopressin [38,39]. The above finding and their similar ability to stimulate PLC- β isoforms suggested the existence of functional redundancy in these two G-proteins [39].

The ability of thrombin to increase $[\text{Ca}^{2+}]_i$ in K562 cells (threshold and maximum effect at 0.01–0.05 and 5 units/ml, respectively, in our hands) agrees with the recent observation by Mari et al. [32]; these authors also reported that thrombin

increases $[\text{Ca}^{2+}]_i$ in the human leukaemic T-cell line Jurkat with EC_{50} and maximum effect at 0.3 and 1–3 units/ml respectively. Consequently, the previously reported inability of thrombin to raise $[\text{Ca}^{2+}]_i$ in fura-2-loaded K562 cells [30] may have resulted from the low concentration of agonist used in those studies (0.001 unit/ml). Thrombin is a mitogenic agent for fibroblast and vascular smooth muscle cells [40]; in platelets this compound, like U46619, initiates a cascade of biochemical events that begins with a rapid rise in $[\text{Ca}^{2+}]_i$ and culminates in aggregation and degranulation, critical steps for haemostasis and thrombosis [35,41]. The thrombin receptor, cloned from human megakaryocyte-like cells, is a member of the seven-transmembrane-domain receptor superfamily which is cleaved at its N-terminus by the agonist; this uncovers a tethered peptide agonist (SFLLRN) and results in receptor activation [42]. Trypsin can mimic the effect of thrombin-induced activation of PLC in K562, and leupeptin selectively inhibits this response, in agreement with similar observations made in platelets and megakaryocyte-like cells [26,43]. The PT-sensitive stimulation of PLC is currently considered to depend on the interaction of $\beta\gamma$ dimers generated following activation of the toxin-sensitive G-protein heterotrimers with the $\beta 2$ and/or $\beta 3$ isoforms of PLC expressed in some cell types. PT-sensitive α subunits may also activate PLC, as microinjection of activated $\text{G}_{\text{o}}\alpha$ in *Xenopus laevis* oocytes evoked Cl^- currents mediated by $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release [44]. Different combinations of $\beta\gamma$ dimers are equally efficacious in activating PLC- $\beta 2$ and PLC- $\beta 3$. The specificity of $\beta\gamma$ dimers originating from PT substrates to activate PLC- $\beta 2/\beta 3$, as opposed to those released from other heterotrimers (G_{s} , G_{q} , etc.), appears to reflect either the relative abundance of the G_i -like proteins and/or their co-localization with the relevant effectors [8]. The K562 cells express two PT-sensitive substrates that were identified as $\text{G}_{12}\alpha$ and $\text{G}_{13}\alpha$ on the basis of ADP-ribosylation pattern, Western and Northern blot analysis (Figures 5 and 6). The PT-sensitive elevation in $[\text{Ca}^{2+}]_i$ triggered by thrombin in K562 is therefore mediated by G_{12} and/or G_{13} . In this context, inactivation of the $\text{G}_{12}\alpha$ gene in embryonic stem cells did not alter PT-sensitive $\alpha_{2\text{A}}$ -adrenergic-mediated inhibition of adenylate cyclase, suggesting that either more than a G_i -like protein is normally involved in coupling a given receptor to the effector, or that down-regulation of the predominant transducer can be compensated for by a highly homologous one [45].

DMSO is a planar-polar compound which was first described to induce erythroid differentiation in Friend murine leukaemic cells and has subsequently been shown to induce differentiation and maturation in a number of other cell lines [13,46]. While DMSO has not been reported to induce obvious erythroid differentiation or haemoglobinization in K562 cells [4,47], there is some evidence that this agent modulates globin gene expression, as transient transfection of K562 cells with a human α -globin promoter coupled to the chloramphenicol acetyltransferase gene showed a 10-fold induction of reporter activity in response to DMSO [48]. Even prolonged culture of K562 cells in nutrient-deficient media can induce the differentiation of these cells to committed precursors of the erythroid series [49]. Our results show that Ca^{2+} signalling receptors and their related G-proteins are expressed at a very early stage in erythropoiesis and that DMSO treatment in chemically defined medium induced a marked resistance to thrombin stimulation within 3 days of such exposure. Plausible explanations for the loss of the thrombin response with this treatment are the down-regulation of the thrombin receptor, its associated PT-sensitive G-protein or a selective isoform of PLC (such as PLC- $\beta 2$). In this regard, changes in expression or activation of G-proteins have been

considered as cause or markers of proliferation or differentiation. However, the similar message and protein levels measured with the corresponding probes for $G_{12}\alpha$ and $G_{13}\alpha$ (Figures 5 and 6) point to differentiation-induced alterations at the level of receptor and/or effector. The results of the Northern blots using a thrombin receptor cDNA probe (Figure 9) show that reduced expression of this receptor represents at least one of the changes occurring during DMSO treatment. Future work is in progress to define changes in other elements of the Ca^{2+} signalling cascade during phenotypic transformation of K562 cells. These studies are required to obtain a complete picture of the changes associated with maturation of K562 and other leukaemic cells.

In summary, we have demonstrated that human myelogenous leukaemic K562 cells express thrombin and TxA_2 receptors which, following agonist occupancy, trigger PT-sensitive and insensitive increases in $[Ca^{2+}]_i$, respectively. We have also documented the expression of $G_{12}\alpha$, $G_{13}\alpha$, $G_q\alpha$ and $G_{11}\alpha$ in K562 cells, without finding evidence of the presence of other α subunits including $G_{11}\alpha$, $G_o\alpha$ and $G_z\alpha$. The deduced amino acid sequence corresponding to the human $G_q\alpha$ and $G_{11}\alpha$ fragments showed 100% and 98.7% similarity, respectively, to their murine counterparts [50]. DMSO-induced phenotypic alterations in K562 cells are accompanied by a significant reduction in the thrombin response without any change in that due to agonist occupancy of the TxA_2 receptor.

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