Increased expression of the inositol 1,4,5-trisphosphate receptor in human leukaemic (HL-60) cells differentiated with retinoic acid or dimethyl sulphoxide

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The $\text{Ins}(1,4,5)\text{P}_3$ receptor was examined in human promyelocytic leukaemic cells (HL-60) and in HL-60 cells differentiated towards granulocytes with either retinoic acid (RA) or dimethyl sulphoxide (Me_2SO). HL-60 cell membranes enriched in marker enzyme activities of the endoplasmic reticulum and the plasma membrane possess a high-affinity binding site for $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ ($K_D = 22$ nM). Electrotransfer studies indicate that $\text{Ins}(1,4,5)\text{P}_3$ binds specifically to a 260 kDa protein of HL-60 cell membranes. This $\text{Ins}(1,4,5)\text{P}_3$ -binding protein selectively binds Ca^{2+} -mobilizing inositol phosphates and other inositol phosphates which also bind to the purified InsP_3 receptor, suggesting that the $\text{Ins}(1,4,5)\text{P}_3$ -binding protein of HL-60 cell membranes is the InsP_3 receptor. When HL-60 cells are incubated with $1\text{ }\mu\text{M}$ -RA or with 1.25% Me_2SO the cells differentiate within 5–7 days into cells resembling neutrophils in both structure and function. Treated cells cease to proliferate, acquire the ability to reduce Nitro Blue Tetrazolium dye, and undergo morphological changes typical of differentiated granulocytes. Concomitant with HL-60 cell differentiation, the maximal $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in membranes increases 3–4-fold, with no change in K_D . The results suggest that there is an absolute increase in the level of the InsP_3 receptor during HL-60 cell differentiation and that the expression of this signal-transducing protein may be specifically regulated by differentiation factors.

INTRODUCTION

The HL-60 cell is a continuously proliferating tissue-culture cell which was established from the peripheral blood of a patient with acute promyelocytic leukaemia [1]. HL-60 cells resemble promyelocytes in morphology and have been particularly useful because of their capacity to differentiate in culture into any of several myelomonocytic cell types [2]. Treatment of HL-60 cells with polar-planar compounds such as dimethyl sulphoxide (Me_2SO) or with more specific agents including retinoic acid (RA) induces a myelocytic differentiation process resulting in the conversion into mature granulocytic cells. After incubation with $1\text{ }\mu\text{M}$ -RA for 5 days, the cells acquire many of the structural and functional characteristics of peripheral-blood granulocytes [3]. This chemically driven HL-60 cell differentiation process has been useful as a model system in which to study the molecular events of normal granulocytic maturation.

The phosphoinositide second-messenger pathway has been implicated in stimulus–response coupling in mature granulocytes, in peripheral-blood neutrophils and in Me_2SO -differentiated HL-60 cells [4–6]. Chemotactic peptides and other degranulating agents bind to cell-surface receptors, stimulating the activity of phosphoinositide-specific phospholipase C, with the resultant formation of the second-messenger molecules diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,4,5)\text{P}_3$ releases Ca^{2+} from specialized Ca^{2+} -sequestering organelles within cells [7,8]. Specific $\text{Ins}(1,4,5)\text{P}_3$ receptors have been demonstrated in many cell types, including neutrophils [9], and endogenously produced $\text{Ins}(1,4,5)\text{P}_3$ has been shown to bind to these receptors, causing the release of

intracellular stores of Ca^{2+} [10]. The $\text{Ins}(1,4,5)\text{P}_3$ receptor from rodent cerebellar Purkinje cells has been purified and its encoding cDNA cloned and sequenced [11–13]. The $\text{Ins}(1,4,5)\text{P}_3$ -receptor protein has characteristics of a ligand-operated ion channel, and is similar in sequence to the cloned ryanodine receptor, the Ca^{2+} channel of skeletal-muscle sarcoplasmic reticulum [14]. The identification of the $\text{Ins}(1,4,5)\text{P}_3$ receptor and similar proteins in diverse cell types, including neutrophils, neurons and skeletal-muscle cells, suggests the existence of elaborate genetic controls which regulate the expression and distribution of the $\text{Ins}(1,4,5)\text{P}_3$ receptor in these cell types.

The RA-induced HL-60 cell offers a unique system in which to study transcriptional events involved in terminal cell differentiation. The effects of RA and of other stimulating agents in triggering HL-60 cell differentiation are complex and involve a cascade of events which culminate in the expression of the granulocytic phenotype. The present data suggest that an enhanced expression of the $\text{Ins}(1,4,5)\text{P}_3$ receptor is one such event under the control of specific differentiation factors in the RA-treated HL-60 human leukaemic cell.

MATERIALS AND METHODS

Materials

$[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (17 Ci/mmol) and $\text{Ins}(1,4,5)\text{P}_3$ (148 Ci/mmol) were obtained from NEN–DuPont. Non-radiolabelled inositol phosphates were purchased from Calbiochem. $\text{Ins}(3,4,5)\text{P}_3$ was very generously given by Dr. R. F. Irvine (A.F.R.C. Research Institute, Babraham, Cambridge, U.K.). HL-60 cells

Abbreviations used: HL-60, human promyelocytic leukaemic cell; RA, retinoic acid; Me_2SO , dimethyl sulphoxide; NBT, Nitro Blue Tetrazolium; PMA, phorbol 12-myristate 13-acetate; IC_{50} , competitor concn. causing displacement of 50% of bound ligand.

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were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). All other reagents were obtained from commercial sources and were of analytical grade.

Cell culture

HL-60 cells were cultured between passages 20 and 60 in RPMI 1640 media supplemented with 5 mM-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml). Cells were grown under sterile conditions at densities of $(0.2\text{--}2.0) \times 10^6$ cells/ml in roller-culture bottles ($\frac{1}{2}$ rev./min) at 37 °C under an atmosphere of 5% CO₂ in air. To induce granulocytic differentiation, cells were seeded at low density and grown for 4–7 days in supplemented media containing 1 μ M-all-*trans*-RA or 1.25% Me₂SO. Morphologic assessment of cell differentiation was performed on cytocentrifuged cells stained with Diff-Quik (American Scientific Products).

Preparation of membranes

Control, Me₂SO-treated or RA-treated HL-60 cells at comparable cell densities were harvested by centrifugation (200 g, 10 min), resuspended ($\sim 10^6$ cells/ml) in ice-cold cavitation buffer (100 mM-KCl, 20 mM-NaCl, 1 mM-MgSO₄, 5 mM-Hepes, pH 7.2, 1 mM-dithiothreitol, 0.1 mM-phenylmethanesulphonyl fluoride and 1 μ g each of benzamidine and soybean trypsin inhibitor/ml), and lysed by N₂ cavitation [2.76 MPa (400 lb/in²) 25 min]. The cavitate was collected dropwise into EGTA (final concn. 1.25 mM) and centrifuged (1000 g, 10 min) to remove unlysed cells and nuclear debris. Membranes in the supernatant were sedimented by centrifugation (100 000 g, 30 min), resuspended at 5–10 mg of protein/ml in 50 mM-Tris (pH 8.2)/1 mM-EDTA buffer and stored at –80 °C until used (usually less than 2 weeks).

Ins(1,4,5)P₃ binding assay

[³H]Ins(1,4,5)P₃ binding to membranes was assayed by vacuum filtration. A 50 μ l portion of membranes (0.2–0.5 mg of protein) was added to 50 mM-Tris (pH 8.2)/1 mM-EDTA buffer containing approx. 6 nM-[³H]Ins(1,4,5)P₃ and in some cases non-radiolabelled Ins(1,4,5)P₃, other inositol phosphates, or heparin. For Scatchard analyses, [³H]Ins(1,4,5)P₃ was included at 3–60 nM. Mixtures of final volume 0.5 ml were incubated with shaking at 4 °C for 20 min, rapidly diluted with 4 ml of ice-cold Tris/EDTA buffer, and then immediately filtered under vacuum through pre-soaked filters (Whatman GF/B). Preliminary studies using membranes prepared from [³H]inositol-labelled cells showed that membranes are quantitatively retained (> 95%) on GF/B filters by this method. Incubation tubes and filters were quickly rinsed with an additional 4 ml of buffer. Dilution, washing and filtration took less than 5 s. Filters were air-dried, mixed with 8 ml of Ecolume scintillation cocktail (ICN Biomedicals), and counted for radioactivity in a Beckman LS 5801 scintillation counter. Non-specific binding of [³H]Ins(1,4,5)P₃ was determined in the presence of 1 μ M-Ins(1,4,5)P₃, and in most cases was less than 10% of the total binding. Radioligand metabolism during the incubation as determined by h.p.l.c. or Dowex AG 1 $\times$ 8 chromatography was consistently less than 5%. Hill plots were used to assess co-operativity of binding. K_i values for competitors were calculated from IC₅₀ values by the method of Cheng & Prusoff [15].

Ins(1,4,[³²P]5)P₃ binding to electrotransferred proteins

Binding of Ins(1,4,[³²P]5)P₃ to electrophoresed and immobilized proteins of HL-60 cell membranes was performed by a

modification of the procedure initially used to identify GTP binding to the *ras* p21 protein [16]. Briefly, membrane proteins were solubilized in SDS sample buffer, electrophoresed on SDS/10%-polyacrylamide gels, and then electrophoretically transferred to nitrocellulose filters. The filters were washed at 20 °C several times over the course of 15–20 min in 50 mM-Tris (pH 8.2)/1 mM-EDTA buffer with 0.1% Nonidet P-40. The filters were cut into strips and then incubated for 4 h at 20 °C in 1.5 ml of Tris/EDTA/0.1% Nonidet P-40 buffer with 50 nCi of Ins(1,4,[³²P]5)P₃ in the presence or absence of 10 μ M non-radiolabelled competitor. Strips were washed twice in succession with Tris/EDTA/Nonidet P40 buffer and once with Tris/EDTA alone, dried and then subjected to autoradiography. Under the experimental conditions, metabolism of Ins(1,4,[³²P]5)P₃ was less than 5%. In parallel, portions of the blots were stained for protein with Amido Black [0.1%, in 45% (v/v) methanol/10% (v/v) acetic acid] and destained with 90% methanol/2% acetic acid.

Miscellaneous methods

Nitro Blue Tetrazolium (NBT) reduction was assayed by incubating cells [$(1\text{--}2) \times 10^6$ /ml] with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) in the presence of NBT (0.5 mg/ml) at 37 °C for 30 min [17]. The percentage of cells containing blue-black formazan deposits was determined on Wright-stained cytocentrifuged preparations. Marker enzyme activities (5'-nucleotidase and glucose-6-phosphatase) were determined as described [18], except that incubations were at 30 °C for 30 min and the released P_i was determined by the method of Itaya & Ui [19]. Total cell lysozyme activity was determined as previously described [5]. Protein concentration was measured by a modified Lowry assay as described by Miller [20].

RESULTS AND DISCUSSION

[³H]Ins(1,4,5)P₃ binding to HL-60 cell membranes

The membrane fractions from control and differentiated HL-60 cells used in this study contained microsomes and plasma membranes. Marker enzyme activities of the plasma membrane (5'-nucleotidase) and microsomes (glucose-6-phosphatase) were enriched 3–4-fold in membranes from HL-60 cells and from cells treated for 5 days with 1 μ M-RA as compared with activities in the respective total cavitated-cell preparation (Table 1). Although there was a slight increase (30–50%) of enzymic activities in the

Table 1. Marker enzyme activities of control and RA-treated HL-60 cells

Enzyme activities were determined as described in the Materials and methods section. Values are expressed as nmol of released P_i/mg of protein and represent means of four (HL-60) and two (RA-treated HL-60) separate preparations.

	Enzyme activity (nmol/mg)	
	5'-Nucleotidase	Glucose-6-phosphatase
HL-60 cells		
Total cavitated-cell preparation	233	352
Membrane fraction	751	1132
RA-treated HL-60 cells		
Total cavitated-cell preparation	306	523
Membrane fraction	1011	1592

Fig. 1. Scatchard plots of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to membranes from control and RA-treated HL-60 cells

Analyses are of single experiments each representative of four or five determinations from separate membrane preparations. In these examples, performed on the same day, the calculated K_D and B_{max} values are 23 nM and 105 fmol/mg of protein for native HL-60 cell membranes and 17 nM and 358 fmol/mg of protein for RA-treated HL-60 cell membranes. Mean values ($\pm$ S.E.M.) from all determinations are 22 ± 2 nM and 102 ± 13 fmol/mg for HL-60 cell membranes and 19 ± 2 nM and 344 ± 14 fmol/mg for RA-treated HL-60 cell membranes.

Fig. 2. Competition by inositol phosphates for $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ -binding sites in HL-60 cell membrane

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding was determined in the presence of the indicated concentrations of various inositol phosphates: ∇ , $\text{Ins}(1,4,5)\text{P}_3$; $\blacksquare$, $\text{Ins}(2,4,5)\text{P}_3$; $\blacktriangle$, glycerophosphoinositol 4,5-bisphosphate [$\text{GroPIns}(4,5)\text{P}_2$]; $\square$, $\text{Ins}(3,4,5)\text{P}_3$; $\circ$, $\text{Ins}(1,3,4,5)\text{P}_4$; $\bullet$, InsP_5 ; $\triangle$, $\text{Ins}(1,3,4)\text{P}_3$; $\blacktriangledown$, $\text{Ins}(1,4)\text{P}_2$. Total $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding averaged 2700 d.p.m./mg of protein. Values are means of triplicate experiments; S.D. values (not shown) are less than 10%.

RA-treated cells compared with control HL-60 cells, membranes from both cell types showed similar enrichment (3–4-fold) of activities compared with the respective total cell cavitate.

These cell membranes also contained specific high-affinity binding sites for $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$. Binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to membranes occurred rapidly, reaching saturation at 4 °C within 20 min, and was readily reversed upon addition of excess unlabelled $\text{Ins}(1,4,5)\text{P}_3$ (results not shown). Scatchard analysis of

Fig. 3. Binding of $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ to electrotransferred HL-60 cell membrane proteins

Samples were subjected to SDS/PAGE and electrotransferred to nitrocellulose paper. Strips were incubated with $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ for 4 h in the presence or absence of 10 μM competitor. (a) Lanes 1 and 2, no competitor; lane 3, $\text{Ins}(1,4,5)\text{P}_3$. (b) Lanes 1 and 2, no competitor; lane 3, $\text{Ins}(1,4)\text{P}_2$; lane 4, $\text{Ins}(1,3,4)\text{P}_3$; lane 5, $\text{Ins}(1,4,5)\text{P}_3$; lane 6, $\text{Ins}(2,4,5)\text{P}_3$; lane 7, $\text{Ins}(1,3,4,5)\text{P}_4$; lane 8, $\text{GroPIns}(4,5)\text{P}_2$. Migration of molecular-mass standards is indicated on the left ordinate. The apparent molecular masses of the $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ -binding proteins are shown on the centre ordinate. The Figure shown is representative of two of three similar experiments.

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to HL-60 membranes indicated a single high-affinity binding site with a K_D of 22 nM and a binding-site density of 102 fmol/mg of protein (Fig. 1). The calculated Hill coefficient for $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding was 1.00 ± 0.07 ($n = 3$), suggesting a lack of binding co-operativity among sites.

Steady-state binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to HL-60 cell membranes was competitively inhibited by $\text{Ins}(1,4,5)\text{P}_3$ and other Ca^{2+} -mobilizing inositol phosphates, including $\text{Ins}(2,4,5)\text{P}_3$ and glycerophosphoinositol 4,5-bisphosphate, but not by other inositol phosphates including $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ (Fig. 2). $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding was also inhibited by $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(3,4,5)\text{P}_3$ and micromolar concentrations of InsP_5 . $\text{Ins}(1,3,4,5)\text{P}_4$ and InsP_5 lack significant Ca^{2+} -mobilizing activity, but have been reported to bind to the purified InsP_3 receptor from rat cerebellum [21]. The effects of $\text{Ins}(3,4,5)\text{P}_3$ on Ca^{2+} mobilization and on binding to the purified InsP_3 receptor are not known. The approximate K_i values for binding of inositol phosphates to the HL-60 cell membrane $\text{Ins}(1,4,5)\text{P}_3$ -binding site are: 280 nM for $\text{Ins}(2,4,5)\text{P}_3$, 400 nM for glycerophosphoinositol 4,5-bisphosphate, 400 nM for $\text{Ins}(3,4,5)\text{P}_3$, 500 nM for $\text{Ins}(1,3,4,5)\text{P}_4$ and 7 μM for InsP_5 . Heparin, an inhibitor of InsP_3 binding and InsP_3 -stimulated Ca^{2+} release in rat brain membranes and rat liver cells [11,22], also inhibited $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to HL-60 cell membranes (IC_{50} 1.4 $\mu\text{g}/\text{ml}$) (results not shown).

$\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ binding to a 260 kDa HL-60 cell membrane protein

Fig. 3(a) shows the specific binding of $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ to electrotransferred and immobilized proteins of HL-60 cell membranes. $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ bound on the nitrocellulose membrane to a protein (260 kDa), and this binding was inhibited by 10 μM non-radiolabelled $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,4,[^{32}\text{P}]5)\text{P}_3$ binding was also selectively inhibited by 10 μM - $\text{Ins}(2,4,5)\text{P}_3$, -glycerophospho-

Fig. 4. Micrographs of (a) control, (b) RA-treated and (c) Me₂SO-treated HL-60 cells

HL-60 cells or HL-60 cells treated in culture with either 1 μ M-RA (5 days) or 1.25 % Me₂SO (6 days) were cytocentrifuged and stained as described in the Materials and methods section. Bar represents 20 μ m.

inositol 4,5-bisphosphate and -Ins(1,3,4,5) P_4 , but not by equivalent concentrations of Ins(1,3,4) P_3 and Ins(1,4) P_2 (Fig. 3b). Another protein (26 kDa) also bound Ins(1,4,[³²P]5) P_3 in a specific manner; however, this binding, although not quantified, was visibly less intense than the binding to the high-molecular-mass species and appeared to be variable among preparations. The nature of this 26 kDa Ins P_3 -binding protein of HL-60 cell membranes requires investigation. The molecular mass of the Ins P_3 receptor as determined from the purified rodent brain protein [11,12] or by molecular target sizing of the rat liver and cerebellar protein [23] is 250–260 kDa.

HL-60 cell differentiation

HL-60 cells can be stimulated to differentiate in culture towards mature granulocytic-like cells by incubation with a number of agents, including RA and Me₂SO. Treatment of HL-60 cells with 1 μ M-RA for 5–7 days stimulated differentiation from a starting population of primarily promyelocytic-type cells into one dominated by granulocytic-type cells (Fig. 4). Accompanying this differentiation was a flattening of the growth curve, a lengthening of the apparent cell doubling time, and an achievement of a terminal cell density ($\sim 1.0 \times 10^6$ /ml). Moreover, if cells treated with RA were reseeded in fresh medium there was no further proliferation consistent with an irreversible cell differentiation. Coincident with this change in growth was the virtual disappearance of mitotic figures from cytocentrifuged cell preparations. After 5 days incubation with RA, most of the cells no longer resembled promyelocytes, but had morphologies typical of metamyelocytes and banded neutrophils (Fig. 4). After 7 days of incubation, a more complete differentiation could be achieved, with most of the cells resembling polymorphonuclear and segmented neutrophils (results not shown). However, after 7 days the cell density and viability were greatly decreased, and for this reason 5-day-treated cells were used routinely for the preparation of membranes and Ins P_3 -receptor binding assays. Treatment of cells for 6 days with 1.25 % Me₂SO induced a differentiation

process qualitatively similar to that with RA, with most of the cells resembling myelocytes and metamyelocytes (Fig. 4).

HL-60 cell differentiation was also assessed by the reduction of NBT dye. In mature myeloid cells and in differentiated HL-60 cells, but not in uninduced HL-60 cells, phorbol esters (including PMA) stimulate superoxide production, which is able to convert soluble NBT into insoluble blue formazan [17]. HL-60 cells treated for 6 days with 1.25 % Me₂SO or for 5 days with 1 μ M-RA were able to reduce NBT dye and develop blue-black precipitates in the presence of PMA (averaging 47 % and 82 % respectively of the total cells in the field), whereas control HL-60 cells had much decreased PMA-stimulated activity (averaging 9 %). Total cellular lysozyme activity was also increased after treatment with RA. Lysozyme, a prominent constituent of specific and azurophilic granules of mature neutrophils, was increased 3-fold after 5 days treatment of HL-60 cells with RA (results not shown).

Differentiation-induced increase in Ins(1,4,5) P_3 binding

Membranes prepared from Me₂SO- or RA-differentiated HL-60 cells exhibited significantly greater [³H]Ins(1,4,5) P_3 binding than did membranes from native HL-60 cells (Table 2). Under identical reaction conditions, specific [³H]Ins(1,4,5) P_3 binding in membranes was 2.5-fold greater in Me₂SO-treated cells and 8-fold greater in RA-treated cells as compared with control HL-60 cells. The smaller increase in [³H]Ins(1,4,5) P_3 binding after Me₂SO treatment compared with RA treatment is consistent with their relative effects on cellular differentiation (see above and Fig. 4). Morphologic differentiation in response to Me₂SO has been reported to be less complete than that in response to RA [3].

The effect of RA on the level of the Ins P_3 receptor was further investigated. Scatchard analysis of the [³H]Ins(1,4,5) P_3 -binding data showed that [³H]Ins(1,4,5) P_3 bound to a single class of sites in both native and RA-treated HL-60 cells (Fig. 1). The dissociation constants were virtually identical (22 ± 2 nM and 19 ± 2 nM); however, receptor density was markedly increased in

Table 2. [^3H]Ins(1,4,5) P_3 binding to membranes of HL-60 cells after treatment with Me_2SO or RA

Control HL-60 cells or cells treated for 6 or 5 days with 1.25% Me_2SO or 1 μM -RA, respectively, were harvested and membranes were prepared. Binding experiments with different membrane preparations were performed with equal amounts of protein and equivalent amounts and concentrations of [^3H]Ins(1,4,5) P_3 as described in the Materials and methods section. Values are means of triplicate determinations from four separate membrane preparations; s.d. values were less than 10%.

Membrane source	Specific [^3H]Ins-(1,4,5) P_3 binding (d.p.m./mg of protein)
HL-60 cells	1620
Me_2SO -treated HL-60 cells	3860
RA-treated HL-60 cells	12828

membranes from RA-treated cells compared with membranes from untreated cells, averaging 102 ± 13 (S.E.M., $n = 4$) fmol/mg of protein in HL-60 cells and 344 ± 14 (S.E.M., $n = 5$) fmol/mg of protein in RA-treated HL-60 cells. Recoveries during cavitation and membrane preparation were assessed by using [^3H]inositol-labelled cells and by measuring total [^3H]inositide levels. Although HL-60 cells incorporated twice as much radioactivity into the lipid fraction (10.4 pmol/ 10^9 cells for HL-60 cells versus 4.9 pmol/ 10^9 cells for RA-treated cells), recoveries of [^3H]inositide in the prepared membrane fractions were similar (79.2% and 76.0% respectively), and this [^3H]inositide was quantitatively retained (> 95%) by GF/B filters. These measurements allow an estimation to be made of the number of InsP_3 receptors per cell: 4400 receptors per HL-60 cell and 12400 receptors per RA-treated cell.

General conclusions

Ins(1,4,5) P_3 , the second-messenger molecule for Ca^{2+} -mobilizing hormones, binds to specific intracellular receptors in responsive cells and stimulates the rapid efflux of Ca^{2+} from specialized Ca^{2+} -handling organelles. The Ins(1,4,5) P_3 -binding site identified in HL-60 cell membranes has characteristics which suggest that it is the InsP_3 receptor. Thus a single affinity class of [^3H]Ins(1,4,5) P_3 -binding sites is found in HL-60 cell membranes, and the dissociation binding constant (22 nM) for this site is similar to that observed for InsP_3 binding in many [24–26], but not all [11,27], other membrane preparations. In support of this binding site being the authentic InsP_3 receptor, the protein expressed from cloned cDNA of the InsP_3 receptor displays a K_D of 21.7 nM [12]. Moreover, inositol phosphates which are active in Ca^{2+} -release assays [7], or which have been shown to bind to the purified InsP_3 receptor of rat brain [21], compete for binding at the Ins(1,4,5) P_3 -binding site of HL-60 cell membranes. Other inositol phosphates which are inactive in Ca^{2+} -release assays do not bind to the Ins(1,4,5) P_3 -binding site of HL-60 cell membranes. Furthermore, both the rank order potency of inositol phosphates for binding and the molecular mass (260 kDa) of the HL-60 cell Ins(1,4,5) P_3 -binding protein as determined by electrotransfer studies are equivalent to those of the purified InsP_3 receptor protein [11,12]. Thus the Ins(1,4,5) P_3 -binding protein of HL-60 cells has characteristics consistent with the contention that it is the InsP_3 receptor which regulates Ca^{2+} homeostasis in these cells.

HL-60 cells can be induced to undergo terminal differentiation into granulocytic cells by exposure to Me_2SO , RA or other

selected agents. These differentiated cells are structurally and functionally similar to peripheral-blood neutrophils in that they display enhanced chemotaxis, adherence, phagocytosis and degranulation in response to stimuli [1,2]. In the present study, it is shown that [^3H]Ins(1,4,5) P_3 binding by membrane preparations increased significantly as HL-60 cells undergo Me_2SO - or RA-driven granulocytic differentiation. Scatchard analysis indicates that the increase in binding to RA-induced cells is due not to a change in the K_D of [^3H]Ins(1,4,5) P_3 binding, but rather to an increase in the apparent B_{max} . This suggests that there is an absolute increase in the number of InsP_3 receptors in chemically differentiated HL-60 cells. The level of the InsP_3 receptor is increased 3–4-fold after 5 days of treatment with 1 μM -RA. Analysis of marker enzyme activities indicate that the apparent increase in InsP_3 binding in differentiated cells is not simply the result of a more enriched membrane fraction. The mechanism responsible for the increased InsP_3 -receptor expression in differentiated HL-60 cells is not known, although preliminary studies indicate that there is increased transcription of the InsP_3 -receptor gene.

The levels of several other signal-transduction proteins of HL-60 cells are known to increase after granulocytic differentiation. These proteins include the formyl-peptide receptor [28], the granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor [29], and the leucocyte-adherence-protein β -subunit [30]. In contrast, the level of DNA polymerase α has been shown to decline in Me_2SO -differentiated HL-60 cells [31]. The controlled expression of these and other proteins responsible for the differentiated HL-60 cell phenotype may well be the result of regulated transcriptional events. Indeed, the changes in expression of the leucocyte-adherence-protein β -subunit and DNA polymerase α have been shown to be the result of changes in mRNA transcription [30,31]. The similarity of chemically induced HL-60 cells to mature granulocytes suggests that the molecular events occurring in RA-treated HL-60 cells in tissue culture resemble the natural events of normal granulocytic maturation. This maturation process involves the regulated transcription of a discrete set of genes with the resultant expression of select proteins, including the InsP_3 receptor.

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REFERENCES

- Collins, S., Gallo, R. & Gallagher, R. (1977) *Nature* (London) **270**, 347–349
- Collins, S. J. (1987) *Blood* **70**, 1233–1244
- Breitman, T. R., Selonick, S. E. & Collins, S. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2936–2940
- Dougherty, R. W., Godfrey, P. P., Hoyle, P. C., Putney, J. W., Jr. & Freer, R. J. (1984) *Biochem. J.* **222**, 307–314
- Bradford, P. G. & Rubin, R. P. (1985) *Mol. Pharmacol.* **27**, 74–78
- Cockcroft, S. (1986) *Recept. Biochem. Methodol.* **7**, 287–310
- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **312**, 315–321
- Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Lew, D. P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1091–1095
- Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W., Jr. (1986) *Nature* (London) **319**, 514–516
- Bradford, P. G. & Rubin, R. P. (1986) *J. Biol. Chem.* **261**, 15644–15647
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) *Nature* (London) **342**, 32–38
- Mignery, G. A., Sudhof, T. C., Takei, K. & De Camilli, P. (1989) *Nature* (London) **342**, 192–195

14. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. & Numa, S. (1989) *Nature (London)* **339**, 439–445
15. Cheng, Y. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108
16. McGrath, J. P., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1984) *Nature (London)* **310**, 644–649
17. Collins, S. J., Ruscetti, F. W., Gallagher, R. E. & Gallo, R. C. (1979) *J. Exp. Med.* **149**, 969–974
18. Aronson, N. N., Jr. & Touster, O. (1974) *Methods Enzymol.* **31**, 90–102
19. Itaya, K. & Ui, M. (1966) *Clin. Chim. Acta* **14**, 361–366
20. Miller, G. L. (1959) *Anal. Chem.* **31**, 964
21. Ferris, C. D., Haganir, R. L., Supattapone, S. & Snyder, S. H. (1989) *Nature (London)* **342**, 87–89
22. Hill, T. D., Berggren, P. & Boynton, A. L. (1987) *Biochem. Biophys. Res. Commun.* **149**, 897–901
23. Nunn, D. L., Potter, B. V. & Taylor, C. W. (1990) *Biochem. J.* **265**, 393–398
24. Lo, W. W. Y. & Hughes, J. (1987) *Neurosci. Lett.* **81**, 331–334
25. Worley, P. F., Barban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136
26. Rossier, M. F., Capponi, A. M. & Vallotton, M. B. (1989) *J. Biol. Chem.* **264**, 14078–14084
27. Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) *J. Biol. Chem.* **263**, 4541–4548
28. Nidel, J., Kahane, I., Lachman, L. & Cuatrecasas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1000–1004
29. Smithgall, T. E., Yu, G. & Glazer, R. I. (1988) *J. Biol. Chem.* **263**, 15050–15055
30. Hickstein, D. D., Hickey, M. J. & Collins, S. J. (1988) *J. Biol. Chem.* **263**, 13863–13867
31. Geis, A. M., Wahl, A. F. & Wang, T. S.-F. (1989) *J. Cell. Biochem. Suppl.* **13D**, 154

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