Direct observation of hexokinase translocation in stimulated macrophages

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1. Fluorescence imaging of antibodies was used to show that phorbol 12-myristate 13-acetate (PMA) induces a 4-fold increase in the amount of hexokinase relative to the control in the cortical shell of rat peritoneal macrophage cytosol adjacent to the plasma membrane, and a corresponding depletion in the amount of hexokinase in the central core of the cytosol. However, there was no significant PMA-dependent change in the distribution of glucose-6-phosphate dehydrogenase. 2. Cytochalasin D, an inhibitor of actin microfilament polymerization, prevented the PMA-induced hexokinase translocation and also reduced the PMA-dependent increases in 2-deoxy-D-glucose transport and glucose-dependent PMA-stimulated superoxide production. 3. PMA caused a contraction of the width of the cortical F-actin

zone. Cytochalasin D caused some dispersal of F-actin within the cell, increasing the density of F-actin within the central cytosolic core and causing aggregation of the F-actin within the cortex. These data are consistent with the view that PMA induces attachment of hexokinase to microfilaments within the cortical zone adjacent to the cell membrane of macrophages, and cytochalasin D prevents this attachment. This is the first direct demonstration of the translocation of hexokinase to the plasma membrane in activated cells, and supports the view that enhanced hexokinase activity in the cortical region of the cytosol is an important early component of the macrophage activation process.

INTRODUCTION

Macrophages, together with the other phagocytic cells of the immune system, have an important role in the destruction of pathogens, producing cytotoxic proteins and superoxide radicals when activated by a variety of agents. In addition to their bactericidal function, macrophage products cause host tissue damage following their inappropriate release in many disease conditions.

One of the characteristics of macrophage and neutrophil activation is an increase in oxygen utilization by up to 50-fold above resting levels, known as the respiratory burst [1]. This oxygen is used for the production of superoxide radicals by membrane-bound NADPH oxidase and is dependent on the supply of NADPH produced from a concomitant increase in hexose monophosphate shunt (HMPS) activity [2]. The production of NADPH is in turn directly dependent on the supply of glucose to the HMPS, and it has been shown that macrophages are unable to produce superoxide radicals in the absence of external glucose [3,4].

Following activation of macrophages by phorbol myristate acetate (PMA), there is an increased net rate of sugar transport across the plasma membrane. It has been suggested that this increase is due to loose coupling of the sugar transport process to hexokinase activity at the endofacial surface of the glucose transporter [3,5]. This model proposes that, upon activation of the macrophage, hexokinase is translocated from a cytosolic location to the area adjacent to the plasma membrane, i.e. in the vicinity of the endofacial surface of the glucose transporter.

It has been reported that hexokinase interacts with erythrocyte glucose transporters *in vitro* [6]. Hexokinase was shown to bind to a cytoplasmic domain of the transporter and this binding was specific for the enzyme, sensitive to ATP concentrations and required the regulatory domain of the enzyme to be intact. However, there is no direct evidence to support the suggestion

that hexokinase is translocated to the endofacial surface of the glucose transporter.

The association of hexokinase with mitochondria has been extensively studied in a number of cell types [7,8], and is of great metabolic importance to the cell [9]. In brain cells most of the total hexokinase activity can be found in the mitochondrial fraction [10,11], and in malignant tumour cells the increased association of the enzyme with the mitochondria has been suggested to be the cause of the very high rate of metabolism in these cells [12–15]. However, the distribution of hexokinase is not static and can be altered according to the metabolic state of the cell [10,11,16].

It has been reported that glycolytic enzymes are aggregated into multienzyme complexes [17] and that these complexes may confer kinetic advantages, allowing for rapid reaction times and channelling of substrates [18,19]. The binding of glycolytic enzymes to cytoskeletal structures may be important in this respect [20,21] and may also facilitate the translocation of enzymes to different parts of the cell in response to changes in metabolic demands. The glycolytic enzyme aldolase has recently been shown to be associated with the actin cytoskeleton in a reversible and dynamic process which is modulated by glycolytic inhibitors and also by cytochalasin D, an inhibitor of actin microfilament polymerization [22].

The object of the present investigation was to provide direct evidence in support of the translocation of hexokinase to the cell surface in relation to activation and cytoskeletal structures by using quantitative epifluorescence microscopy.

MATERIALS AND METHODS

Sugar-dependent superoxide production

Macrophages isolated by peritoneal lavage from three or four rats, as previously described [3], were washed and diluted to a cell concentration of approx. 1×10^6 /ml in Hepes-buffered saline

Abbreviations used: HMPS, hexose monophosphate shunt; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; G-6-PD, glucose-6-phosphate dehydrogenase; 2-dGlc, 2-deoxy-D-glucose.

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(Hepes, 5 mM; NaCl, 140 mM; KCl, 5 mM; MgCl_2 , 1.2 mM), pH 7.3, at 37 °C. Cells were pre-incubated for 3 h at 37 °C in the presence or absence of cytochalasin D (5 μM) (Sigma Chemical Co.), and superoxide production was measured by the reduction of 20 μM cytochrome *c* (from horse heart; Sigma) over 20 min in various concentrations of glucose and with PMA (Sigma) at 40 nM [3].

2-Deoxy-D-glucose (2-dGlc) transport studies

Macrophages were isolated, incubated for 3 h at 37 °C in the presence or absence of cytochalasin D (5 μM), and then the uptake of various concentrations of 2-deoxy-D-[2,6- ^3H]glucose was measured for 10 min in either the presence or the absence of 40 nM PMA [3].

Fluorescence microscopy

Rat peritoneal macrophages were isolated as described above, and the cells were then washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing glucose (5.5 mM; ICN-Flow Laboratories) at a cell concentration of $> 1 \times 10^7/\text{ml}$. The concentrated suspension was then placed on coverslips and the cells were allowed to attach for 1 h at 37 °C in humidified CO_2/air (1:19). After washing with PBS to remove any non-adherent cells, DMEM was again applied together with the relevant treatment. PMA (40 nM) was added alone (for 30 min at 37 °C) or after pre-incubation with cytochalasin D (5 μM) for 3 h at 37 °C. Cells were then pre-fixed for 5 min at 37 °C in 0.25% paraformaldehyde in PBS, rinsed in PBS and permeabilized for 15 min in 0.1% Triton X-100 in PBS at room temperature. Fixation for 5 min in paraformaldehyde was repeated, and residual free aldehyde autofluorescence was decreased by several washes in PBS, before the addition of antibody.

Coverslips were treated either with rabbit anti-hexokinase antibody [23,24] or with rabbit anti-[glucose-6-phosphate dehydrogenase (G-6-PD)] antibody for 45 min at 37 °C. The cells were washed in PBS and exposed to a second antibody, raised against rabbit IgG (Sigma), which had been affinity-purified and labelled with tetramethylrhodamine-5- (and 6-) isothiocyanate (TRITC). After 1 h, these cells were washed extensively overnight at 4 °C, after which the coverslips were mounted on glass slides in glycerol/PBS (1:1, v/v).

Staining of F-actin

Coverslips of permeabilized cells were bathed in PBS containing BODIPY phalloidin 581/591 (Molecular Probes, Eugene, OR, U.S.A.), a specific stain for F-actin. One unit per coverslip was applied for 30 min, and then excess stain was removed by repeated washing in PBS.

Microscopy

The cells were examined using a Leitz Dialux 20 microscope with epifluorescence illumination via an N-2 filter block. Fluorescence was imaged with a Silicon-Intensified Target (SIT) camera (Javelin), digitized and averaged using a Hamamatsu C1966 image processor. Frames were stored and analysed by a micro-computer (Acorn Archimedes, Cambridge, U.K.).

Analysis of enzyme translocation

The data analysis was automated using a computer program which executed the following procedures on the pseudocolour images of cell fluorescence obtained from 16 video-frame averages. A best-fit ellipse was drawn around the perimeter of each

Figure 1 Diagrammatic representation of the numerical analysis of the translocation of G-6-PD and hexokinase in macrophages

A best-fit ellipse was drawn around the circumference of each cell and then 20 concentric ellipses were drawn within it (three are shown here). The total pixel intensity in each annulus was measured (see the Materials and methods section). The sums of the products of the vertical distances (Δy_n) between each of the annuli at 20 equally spaced positions from 0 to 1 along the *x* axis and the relative densities of antibody within each annulus (ρ_x ; see the Materials and methods section) were obtained as follows:

$$L_{x0} = \{(r20 - r19) \cdot \rho_{20} \dots (r3 - r2) \cdot \rho_c + (r2 - r1) \cdot \rho_b + (r1 - 0) \cdot \rho_a\} \cdot S$$

Values at all vertices between the 20 equally spaced vertical projections along the *x* axis were obtained and plotted against the radial position *x* (from 0 to 1). The scale factor *S* was obtained by first matching the simulated data to the observed cytochalasin D distributions $L_{x(0-1)}$ (where the density of enzyme ρ is uniformly distributed). The exponential distributions ρ_x for control and PMA data were obtained from the observed exponential functions of the ratios of the fluorescence intensity $L_{x,\text{control}}/L_{x,\text{cyto.D}}$ and $L_{x,\text{PMA}}/L_{x,\text{PMA+cyto.D}}$ (Figure 5).

cell on the video-screen, then a series of 20 concentric ellipses with principal radii decrementing in steps of 5% were drawn within the cell perimeter and the total pixel intensity within each ellipse was obtained (Figure 1). The successive differences in total pixel intensity between the neighbouring integrands give the fluorescence intensity within each annulus, and the average pixel intensity within each annulus was obtained by dividing each annular integral of pixel intensity by the number of pixels within the annulus. The average pixel fluorescence at each of the 20 points along the radius is plotted as a percentage of the total sum. This procedure normalizes the elliptical cell shape to that of a circle with a radius of unit length and also the total fluorescence intensities between sets of cells.

Data analysis of radial distribution of fluorescence within cells

Cytochalasin D-treated cells

The radial distribution of fluorescence intensity of the anti-hexokinase antibody in cells exposed to cytochalasin D was plotted and fitted to the circular equation $L_x = S \sqrt{1 - x^2}$. This is the equation of a circle with a normalized radius of 1, where L_x is the fluorescence intensity at any radial position *x* along the horizontal axis between 0 (the centre of the circle) and 1 (the perimeter), and *S* is a scale factor relating the average percentage fluorescence intensity to the position *x* along the radius. The best-fit line through the cytochalasin D data had a highly significant goodness-of-fit to the observed set of 20 intensities [$\Sigma \chi^2 = 0.019$ (d.f. = 17; $P > 0.99$)]. Each data point was the average from approx. 250 cells from five separate experiments, i.e. 50 cells per experiment. The S.E.M.s were obtained from the entire cell population.

The analysis is consistent with the view that anti-hexokinase antibody in cytochalasin D-treated cells is uniformly distributed

Figure 2 Diagrammatic representation of the two-compartment model for protein distribution

The cell is assumed to consist of an inner core and an outer cortex of cytosol. Hexokinase, G-6-PD and actin are distributed uniformly (but at different concentrations) within the core and cortex. The distributions of fluorescent materials between cortex and core are estimated by obtaining a best fit of the simulated distributions with the observed radial distribution of fluorescence by varying the following parameters: m , the marginal position between core and cortex, which is any distance between the centre (0) and the plasma membrane (1); ϵ , the ellipsoidicity factor (e.g. $\epsilon = 1 = \text{circle}$); ρ , the density of material within the core relative to the cortex, where $\rho_{\text{core}} + \rho_{\text{cortex}} = 1$; and S , the scale factor. Thus $S \cdot [(y_{r2} - y_{r1}) \cdot \rho_{\text{cortex}} + y_{r1} \cdot \rho_{\text{core}}]$ is the simulated percentage distribution of fluorescence at any radial position x between 0 and 1.

throughout the entire cytosol of a normalized spherical cell. The distributions of hexokinase within control and PMA-treated cells differed significantly from those in cytochalasin D-treated cells. The attenuation of the fluorescence signal on moving from the centre to the periphery of the cell is consistent with the decrease in light path length on moving from the central to the peripheral segments of the cell.

Control and PMA-treated cells (exponential fit)

The relative radial density distributions (ρ_r) of anti-hexokinase antibody in control and PMA-treated cells were obtained from the ratios of the observed radial fluorescence distributions of $L_{x, \text{control}}/L_{x, \text{cyto.D}}$ and $L_{x, \text{PMA}}/L_{x, \text{PMA+cyto.D}}$. The observed ratio distributions were fitted to the following exponential function: $a + b \cdot \exp(cx)$, where x is any distance along the x axis of the quadrant between 0 and 1. The errors of the distribution ratios $L_{x, \text{control}}/L_{x, \text{cyto.D}}$ and $L_{x, \text{PMA}}/L_{x, \text{PMA+cyto.D}}$ were obtained by Gaussian summation of the errors of the data points. The fits of the density distributions of control and PMA to the exponential functions indicated that the distributions of hexokinase within control and PMA-treated cells were not uniform, but there was an increase in density towards the cell cortex. The increase in cortical density was greater in the case of PMA-treated cells than in control cells ($P < 0.001$).

The estimates of average radial distributions of hexokinase in control and PMA-treated cells are shown as the continuous lines through the data points of observed radial fluorescence distributions in control and PMA-treated cells. The lines are obtained from the sum of the products of the relative densities of hexokinase in each annulus of the cytosol at a radial distance x from the cell centre (see above) and annular thickness Δy_{rx} at any position x between the centre and periphery of the spherical cell, i.e. from the product $\Sigma \rho_r \times \Sigma S \Delta y_{r1}$, where S is the scale factor used to match the data with the fluorescence distribution in

cytochalasin D-treated cells and $\Sigma \Delta y_{rx}$ is sum of the matrix of the vertical distances between the 20 successive annuli spaced at intervals of 0.05 along the radius R between 0 and 1. S is an identical scale to that used with cytochalasin D (Figure 1).

Two-compartment fit for hexokinase and actin

F-actin is non-uniformly distributed within the cytosol even after exposure to cytochalasin D, and thus the relative density function, ρ_r , could not be used to monitor changes in F-actin distribution. A simpler two-compartment model of protein distribution within the cell was therefore adopted, in which the cell is assumed to consist of an inner core surrounded by an outer cortex of cytosol (Figure 2). Hexokinase, G-6-PD and actin are assumed to be distributed in both compartments at different uniform densities. The margin dividing the core from the cortical compartment can be assigned any distance m along the principal radius between the centre (0) and the periphery (1). The density of material within the cortex (ρ) is expressed as a relative density with respect to the core; hence cortical density is equal to $1 - \rho_{\text{core}}$. The cells can vary in shape from an oblate to a prolate ellipse, controlled by the ellipsoidicity function ϵ . The equation describing the relationship between the vertical height y_{xcr} at any position x of the cell cortex between the centre (0) and surface of the cell (1) is:

$$y_{xcr} = \epsilon \sqrt{1 - x^2}$$

The relationship between the vertical height y_{xcr} at any position x between the centre (0) and margin (m) between the core and cortex is:

$$y_{xcr} = \epsilon \sqrt{m^2 - x^2}$$

Thus the fluorescence distribution at any position x can be described as:

$$S \times [y_{x, \text{cortex}} (1 - \rho) - y_{x, \text{core}} \rho]$$

Thus the enzyme distribution is determined by four independent parameters: S , the scale factor; ϵ , the ellipsoidicity factor; m , the position of the margin between core and cortex; and ρ , the density of material within the core relative to the cortex.

The theoretical distributions were fitted to the observed data by independent variation of ϵ , ρ , m and S using the least-squares parameter-fitting routine Microsoft Excel Solver, available as a macro in the Microsoft Excel 4 spreadsheet, which minimizes the $\Sigma \chi^2$ between the observed and theoretical distributions. The errors of the parameters are estimated from the parameter changes required to double the minimum $\Sigma \chi^2$.

Individual cell fluorescence intensity ratio

The fluorescence intensity ratio for the anti-hexokinase or anti-G-6-PD antibody from each cell was obtained from the average fluorescence intensity in the outer 70–90%/inner 0–20% of the radial distribution, and values were plotted as a frequency histogram. A high ratio means that the enzyme is distributed more peripherally than in cells having a low ratio. The best-fit Gaussian distributions to the frequency–distribution-ratio histograms of the data are shown in Figure 6. This method measures enzyme distribution in an individual cell and shows the range of the data better than the lumped radial distribution. However, the radial distribution data (see Figures 7 and 8) show the overall shape of the enzyme distribution within the cell.

RESULTS

Effects of PMA and cytochalasin D on 2-dGlc transport and superoxide production

Following activation of rat peritoneal macrophages by PMA

Figure 3 Effects of PMA and cytochalasin D on glucose-dependent superoxide production

Results are expressed as superoxide dismutase-inhibitable superoxide production ($\mu\text{mol} \cdot 5 \text{ min}^{-1} \cdot \text{ml of cell water}^{-1}$), with the zero-sugar value subtracted and plotted by a non-linear least-squares fit to the Michaelis–Menten equation (KaleidaGraph 2.1; Abelbeck Software). The mean cell volume of the macrophages was 330 fl, and all superoxide production rates are normalized to 3×10^9 cells, or approx. 1 ml of cell water. Cytochalasin D significantly decreased the V_{max} for PMA-dependent superoxide radical production from 17.35 ± 1.31 (PMA) to 10.13 ± 0.27 (PMA + cytochalasin D) $\mu\text{mol} \cdot 5 \text{ min}^{-1} \cdot \text{ml of cell water}^{-1}$ ($P < 0.001$; $n = 7$), without significantly changing the K_m (PMA, $0.43 \pm 0.10 \text{ mM}$; PMA + cytochalasin D, $0.38 \pm 0.04 \text{ mM}$). ■, PMA; ○, PMA + cytochalasin D.

Table 1 Effects of PMA and cytochalasin D on net 2-dGlc uptake

Phloretin/HgCl₂-inhibitable uptakes (means $\pm$ S.E.M.) were fitted by non-linear least-squares regression to the Michaelis–Menten equation (KaleidaGraph 2.1; Abelbeck Software). The K_m (mM) and V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$) values obtained were compared by Student's t tests; * $P < 0.02$ for test versus control value; n = number of separate determinations.

	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$)	K_m (mM)	n
Control	1.03 ± 0.05	0.75 ± 0.12	8
PMA (40 nM)	1.11 ± 0.01	$0.42 \pm 0.01^*$	8
PMA + cytochalasin D (5 μM)	1.12 ± 0.04	0.69 ± 0.08	8
Cytochalasin D (5 μM)	0.97 ± 0.11	0.71 ± 0.18	6

(40 nM), there was an increase in glucose-dependent superoxide production (Figure 3) and a decrease in the K_m of net 2-dGlc uptake (Table 1). Cytochalasin D (5 μM) inhibited PMA-induced glucose-dependent superoxide production by reducing the V_{max} of the process without altering the K_m (i.e. non-competitive inhibition). In contrast, cytochalasin D significantly increased the K_m of PMA-induced net 2-dGlc uptake without altering the V_{max} (i.e. competitive inhibition).

Figure 4 Photomicrographs of anti-hexokinase-antibody-labelled cells

Rat peritoneal macrophages were isolated, treated with anti-hexokinase antibody and photographs taken of the fluorescent images. (a) Fluorescent antibody distribution in control cells; (b) cells after exposure to PMA (40 nM) for 30 min (scale bar = 10 μm); (c) fluorescent image of cells treated with cytochalasin D (5 μM) for 3 h; (d) image from cells treated for 3 h with cytochalasin D and then exposed to PMA for 30 min.

Figure 5 Average relative fluorescence intensities and fluorescence ratios of control, PMA- and cytochalasin D-treated cells

Shown are the average relative fluorescence intensities (upper three scattergraphs) of control, PMA- and cytochalasin D-treated cells, and the fluorescence ratios, i.e. the ratio of relative fluorescence intensity (control)/relative fluorescence intensity (cytochalasin D) and fluorescence intensity (PMA)/relative fluorescence intensity (PMA + cytochalasin D) against radial position (lower two lines). The radial position 0 refers to the centre of the cell, and 1 refers to the plasma membrane. The means of 50–100 cells of each condition are shown. The estimates of hexokinase radial distributions in control and PMA-treated cells were calculated from the product of the radial densities and the radial distribution function L_r obtained from the cytochalasin D data (see the Materials and methods section), and were found to be good fits to the predicted values. For control, $\Sigma\chi^2 = 0.108$, $P > 0.99$; for PMA, $\Sigma\chi^2 = 0.125$, $P > 0.99$, i.e. both distributions of hexokinase are consistent with exponential increases in hexokinase density towards the cell surface. The concentrations of hexokinase in the most peripheral 10% of the cell radius was increased above that found in cells treated with cytochalasin D by $23 \pm 3\%$ in control cells ($P < 0.001$) and by $79 \pm 10\%$ in PMA-treated cells, ($P < 0.001$ versus cyto. D; also $P < 0.001$ versus control cells).

Figure 6 Frequency distribution histogram of the intensity ratio of the relative radial fluorescence in individual cells treated with anti-hexokinase antibody

The intensity ratio from each cell was obtained from the average fluorescence intensity in the outer 70–90%/inner 0–20% of the radial distribution and plotted as a frequency histogram. The lines are the best-fit Gaussian distributions to the data shown in Figure 5. The control cells have a mean ratio of 0.941 ± 0.032 ($n = 55$), whereas PMA-stimulated cells have a mean ratio of 1.30 ± 0.037 ($n = 90$; $P < 0.001$), indicating translocation of the fluorescence to the cell periphery. Cytochalasin D reduced the radial intensity ratio of anti-hexokinase antibody distribution in both control and PMA-stimulated cells, to 0.787 ± 0.032 ($n = 90$) and 0.793 ± 0.035 ($n = 95$; $P < 0.001$) respectively. These findings are consistent with a cytochalasin D-dependent increase in hexokinase translocation in PMA-stimulated macrophages. ●, Control; ■, PMA; ○, cytochalasin D; □, PMA + cytochalasin D.

Figure 7 Radial distribution of anti-G-6-PD antibody

Results are expressed as means ($n = 120$ per condition). PMA (40 nM) and cytochalasin D (5 μ M) had no significant effect on G-6-PD enzyme distribution. ○, Control; □, PMA; ●, cytochalasin D. The radial position 0 refers to the centre of the cell, and 1 refers to the plasma membrane.

Figure 8 Ratio of radial distributions of anti-hexokinase to anti-G-6-PD antibodies in control and PMA-treated cells

Results are means $\pm$ S.E.M.; $n = 120$ cells from each condition, i.e. 480 cells were analysed. The radial position 0 refers to the centre of the cell, and 1 refers to the plasma membrane. PMA causes a significant depletion of hexokinase compared with its effect on G-6-PD in the core region of the cell and a significant enhancement of hexokinase in the cytosolic shell adjacent to the cell membrane. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for PMA versus control; all other data points were not significantly different. ●, Control; ○, PMA.

Effects of PMA and cytochalasin D on macrophage cell diameter

Cytochalasin D significantly reduced the mean cell diameter of the macrophages from $12.28 \pm 0.23 \mu\text{m}$ (control, $n = 40$) to $10.92 \pm 0.08 \mu\text{m}$ ($n = 37$; $P < 0.00015$ by Student's t test). The cytochalasin D-induced decrease in cell diameter is probably due to cell rounding, related to the breakdown of microfilaments. PMA increased the mean cell diameter to $13.86 \pm 0.18 \mu\text{m}$ ($n = 44$; $P < 0.0001$), and there was no significant difference

Figure 9 Photomicrographs of BODIPY-phalloidin-stained macrophages

Following treatment with or without PMA (40 nM; 30 min) and cytochalasin D (5 μ M; 3 h), permeabilized cells were bathed for 30 min in PBS containing 1 unit per coverslip BODIPY-phalloidin 581/591. (a) Control cells; (b) fluorescence distribution after treatment with PMA (40 nM); (c) fluorescent image of cytochalasin D-treated cells (5 μ M for 3 h); (d) image from macrophages treated with cytochalasin D for 3 h and then exposed to PMA for 30 min. Scale bar = 10 μ m.

between cells treated with PMA plus cytochalasin D and cells exposed to cytochalasin D alone. It can be concluded that cytochalasin D causes shrinkage of the cell cross-sectional area by 28 %, whereas PMA causes an increase of the cross-sectional area by 27 %.

Effects of PMA and cytochalasin D on the distribution of the anti-hexokinase antibody in macrophages

Figure 4 shows photomicrographs of epifluorescence images of anti-hexokinase antibody in control, PMA- (40 nM), cytochalasin D- (5 μ M) and PMA+cytochalasin D-treated macrophages. In PMA-treated cells, the majority of fluorescent antibody was distributed around the perimeter of the cell, with the cell centre apparently clear of fluorescence. In control cells, the antibody was seen to be more homogeneously distributed, and in cytochalasin D-treated cells the antibody was apparently completely uniformly distributed throughout the cell. In cells treated with anti-G-6-PD antibody, the antibody was uniformly distributed within all cells under all conditions tested (results not shown).

PMA stimulation altered the hexokinase distribution within the cytosol (Figure 5). There was a steep exponential increase in the hexokinase density in the outer 10 % of the cytosolic cortex. This was observed as a 79 ± 10 % increase in the density of anti-hexokinase antibody in the peripheral 10 % of cell radius, as calculated from the difference in the exponential regression lines of the data of the lower two lines in Figure 5 ($P < 0.001$; PMA

versus cyto. D). Cytochalasin D (5 μ M) treatment for 3 h prevented the PMA-induced translocation of hexokinase to the plasma membrane (Figures 4d, 5 and 6). The distribution of anti-hexokinase antibody in cytochalasin D-treated cells was consistent with a uniform distribution throughout a spherical cell (Figure 4b). In control cells there was a small increase (23 ± 3 %; $P < 0.001$) in the observed density of hexokinase within the cell cortex in comparison with cytochalasin D-treated cells (Figures 5 and 6). This is equivalent to a 12 ± 2 % increase in the amount of hexokinase in the cortical shell compared with cytochalasin D-treated cells. The increase in hexokinase in the peripheral cortex of PMA-treated cells was 49 ± 6 % greater than that seen in cytochalasin D-treated cells. This 4-fold enhancement in comparison with control cells was highly significant ($P < 0.001$).

The values of the coefficients for the best-fit exponential regression lines of the relative density of anti-hexokinase antibody for control/cytochalasin D-treated cells were: $a = 0.83 \pm 0.23$; $b = 0.063 \pm 0.018$ and $c = 1.94 \pm 0.24$ ($r = 0.992$; $P < 0.001$), and the equivalent coefficients for the relative radial density functions in PMA-treated cells versus cytochalasin D-plus-PMA-treated cells were $a = 0.77 \pm 0.018$; $b = 0.022 \pm 0.006$ and $c = 4.02 \pm 0.31$ ($r = 0.992$; b and c in control versus PMA-treated, $P < 0.05$ and $P < 0.001$ respectively). In PMA-treated cells there was also a matching decrease in the amount of antibody within the central region of the cell. The density of anti-hexokinase antibody within the cell core was approx. 80 % of that observed in cells treated with cytochalasin D ($P < 0.01$) (Figure 5). Hence PMA treatment

Figure 10 Effects of PMA and cytochalasin D on the radial distribution of fluorescent F-actin in macrophages

Cells were treated as described in the legend to Figure 9, then the fluorescent images were processed and lines fitted by least-squares analysis using the two-compartment model (see the Materials and methods section). PMA (40 nM) induced a narrowing of the peripheral F-actin layer ($P < 0.05$) and accumulation of actin in the cell core from zero (control) to $12 \pm 2\%$ of total. Cytochalasin D increased actin in the central core from zero to $16 \pm 2\%$. The best fits for the parameters were: cytochalasin D, $m = 0.855 \pm 0.012$; $\rho = 0.056 \pm 0.03$ ($P < 0.01$); $S = 18.46 \pm 1.3$ and $\epsilon = 1.19 \pm 0.08$; PMA, $m = 0.876 \pm 0.007$ ($P < 0.05$); $\rho = 0.040 \pm 0.016$ ($P < 0.01$); $S = 20.22 \pm 0.9$ and $\epsilon = 1.26 \pm 0.06$; control, $m = 0.822 \pm 0.002$; $\rho = 0$; $S = 17.85 \pm 2.1$ and $\epsilon = 1.11 \pm 0.12$. $\circ$, Control; $\bullet$, PMA; $\square$, cytochalasin D. Theoretical actin distributions: —, control; ---, PMA; ----, cytochalasin D. The radial position 0 refers to the centre of the cell, and 1 refers to the plasma membrane.

caused no overall change in the total amount of anti-hexokinase antibody within the cell.

Fluorescence ratios within individual cells

The changes in hexokinase distribution were also illustrated by the changes in the ratio of antibody observed within the outer 70–90% of the cell radius compared with that in the inner 0–20% within the same cell: the higher the ratio, the greater the relative amount of peripherally bound enzyme (Figure 6).

The anti-G-6-PD antibody was homogeneously distributed within the cytosol (Figure 7). There was no significant alteration from the control state in the distribution of G-6-PD antibody following activation by PMA.

Comparison of anti-hexokinase and anti-G-6-PD antibody distributions

A comparison of the relative distributions of anti-hexokinase and anti-G-6-PD indicates no difference in the radial distribution of antibodies in control cells. However, in the peripheral 20% of PMA-treated cells, the ratio of anti-hexokinase to anti-G-6-PD antibody fluorescence was between 1.45 ± 0.15 and 1.65 ± 0.20 ($P < 0.001$; PMA versus control), and in the central region of the cell the ratio fell to 0.90 ± 0.10 ($P < 0.05$) (Figure 8). This indicates that PMA alters the distribution of hexokinase, but not of G-6-PD.

Effects of PMA and cytochalasin D on F-actin distribution

Macrophages stained with fluorescent BODIPY-phalloidin for F-actin are shown in Figure 9. Under all conditions, the majority of the actin was found within the peripheral cortex of the cell. PMA caused a slight narrowing of the peripheral F-actin layer and also some accumulation of actin near the cell core. In the cytochalasin D-treated cells there was an increased amount of F-actin within the cell core; however, the main effect of cytochalasin D was to produce punctate aggregates of F-actin within the cell cortex which were not found in either control or PMA-treated cells.

The radial distributions of F-actin in control, PMA and cytochalasin D-treated cells are shown in Figure 10. The lines through the data points are the least-square fits using the two-compartment fit (see the Materials and methods section). Comparison of control and PMA-treated cells indicated that there was narrowing of the peripheral zone from $2.22 \pm 0.24 \mu\text{m}$ in control cells to $1.66 \pm 0.14 \mu\text{m}$ in PMA-treated cells ($P < 0.05$), despite the significant increase in the cross-sectional area of the PMA-treated cells. In control cells all of the actin lay within the cortex, whereas in PMA-treated and cytochalasin D-treated cells, $12 \pm 2\%$ and $16 \pm 2\%$ respectively was present within the cell core. Thus it is evident that exposure to either PMA or cytochalasin D leads to a small amount of F-actin redistribution within the cell.

DISCUSSION

Our results indicate that, in PMA-stimulated rat peritoneal macrophages, there is a significant translocation of hexokinase from the cell cortex to the area adjacent to the plasma membrane (Figure 5). The PMA-induced translocation is relatively specific and cannot be ascribed simply to alterations resulting from any change in cell shape, since the ratio of the radial distributions of anti-hexokinase antibody to anti-G-6-PD antibody (Figure 8) grows exponentially towards the cell cortex as would be predicted on the basis that hexokinase (but not G-6-PD) is translocated to the cell periphery following exposure to PMA.

The data shown here are the first direct demonstration of the translocation of hexokinase to the plasma membrane in activated cells. However, translocation of other enzymes to the plasma membrane upon activation has been reported previously. In Swiss 3T3 fibroblasts, PMA treatment induced a partial redistribution of diacylglycerol kinase from the cytosol to the plasma membrane [25], while protein kinase C has been shown to be translocated to the plasma membrane in response to a number of stimuli in many cell types [26–29]. In activated human neutrophils, proteins associated with NADPH oxidase are translocated to the plasma membrane [30], and these results suggest that alteration of enzyme activity at the endofacial surface is a means of promoting large changes in transport and metabolism in response to activation.

Hexokinase is found in a number of isoforms, some of which have a hydrophobic N_2 -terminus that may be important for the binding of the enzyme to mitochondrial membranes [31]. This hydrophobic tail region is a post-translational modification of the enzyme that results in different kinetic and regulatory properties, and it may also be crucial in the proposed binding of cytosolic hexokinase to the plasma membrane.

Cytochalasin D is a fungal metabolite which destabilizes actin filaments, but unlike cytochalasin B it does not bind to the sugar transporter [32]. Despite the absence of a direct interaction of cytochalasin D with the sugar transporter, it inhibited both PMA-induced 2-dGlc transport and glucose-dependent superoxide production (Figure 3 and Table 1). These data indicate that

disturbance of cytosolic microfilaments in macrophages by cytochalasin D inhibits PMA-induced hexokinase translocation (Figure 5) and so decreases net 2-dGlc uptake and glucose-dependent superoxide production.

The distribution of the glycolytic enzyme aldolase has also been shown to be altered by treatment with cytochalasin D [22]. Using fluorescent techniques in living Swiss 3T3 cells, it was shown that aldolase is attached to F-actin in the perinuclear region of the cell and that cytochalasin D treatment leads to detachment of aldolase from F-actin within 10 min.

Cytochalasin D shows non-competitive inhibition of glucose-dependent superoxide production in macrophages and competitive inhibition of PMA-dependent net 2-dGlc uptake (Figure 3 and Table 1). PMA and dexamethasone have been reported to show similar non-competitive and competitive effects on sugar transport and superoxide production in macrophages, and these observations can be explained in terms of the differences in metabolism of the two sugars [33]. Accumulation of free 2-dGlc within the macrophage cytosol leads to saturation of hexokinase, and hence the effects of PMA and cytochalasin D, which alter the coupling between hexokinase and the transporter, are only observed at low concentrations of 2-dGlc, where it is accumulated to sub-saturating levels. However, with glucose, which is completely metabolized by macrophages, PMA (which induces coupling between hexokinase activity and sugar transport) increases superoxide production at all glucose concentrations. Cytochalasin D, which uncouples hexokinase and sugar transport, decreases superoxide production at all glucose concentrations.

Although there is some controversy about the precise relationship between protein kinase C activation and the interaction of F-actin with the cytoskeleton, it is clear that activation of phagocytic cells by PMA results in changes in F-actin density and accumulation within the cell cortex, along with altered cell motility and ruffling of the plasma membrane [34–38]. PMA-activated actin polymerization is inhibited by inhibitors of protein kinase C, e.g. staurosporine [37], which have also been shown to inhibit free radical production in neutrophils [38].

Although the data support the suggestion that F-actin and hexokinase are associated in some way in rat peritoneal macrophages, the distributions of the two differ in several ways. Firstly, nearly all of the F-actin is present within the cytosolic cortex, whereas hexokinase is much more homogeneously distributed throughout the cytosol. Secondly, PMA induces a large increase in the fraction of hexokinase bound to the peripheral cortex, but causes little change in the amount of F-actin in this region. The PMA-induced change in F-actin is seen as a decrease in the width of the peripheral zone of F-actin, perhaps corresponding to the membrane ruffling process [34–38]. Additionally, membrane ruffling is consistent with the observed PMA-induced increase in cell diameter reported here. Thirdly, cytochalasin D has a small effect on F-actin distribution, resulting in an increase in the amount of F-actin present within the cell core, whereas the augmented amount of hexokinase attached to the cell cortex is completely detached following exposure to cytochalasin D.

These data are consistent with the view that PMA leads to

attachment of hexokinase to F-actin within the cortical zone of the cytosol and that this linkage can be broken following exposure to cytochalasin D. The observations that the distributions of the hexokinase and F-actin differ in some respects, together with the absence of an effect of PMA on the cellular distribution of G-6-PD, support the idea that the translocation of hexokinase is a specific process which is an important early event in the activation of macrophages.

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