Changes in calcium influx affect the differentiation of murine erythroleukaemia cells

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As indicated by direct evidence, obtained by altering the cell-membrane permeability for Ca^{2+} in murine erythroleukaemia (MEL) cells, calpain is the triggering factor which connects fluctuations of the intracellular Ca^{2+} concentrations to the decay of protein kinase C (PKC), as well as to the kinetics of cell differentiation induced by hexamethylenebisacetamide. Cell exposure to verapamil caused a profound decrease in the rate of PKC down-regulation and a slower initial rate of accumulation of mature erythroid cells, whereas addition of the Ca^{2+} ionophore A23187 produced opposite effects. The high susceptibility of

PKC- δ to calpain degradation, at concentrations of Ca^{2+} much lower than those required for degradation of the other PKC isoforms, may be explained by the finding that this kinase isoform is predominantly associated with the cell membrane. The different cellular localizations, as well as the different susceptibilities to calpain digestion, further support the hypothesis that in MEL cells the various PKC isoforms play distinct biological functions that are critical for the maintenance of the undifferentiated state of the cell and for its commitment to terminal erythroid differentiation.

INTRODUCTION

Identical types of protein kinase C (PKC) isoforms were found to be present in two murine erythroleukaemia (MEL) cell clones characterized by a rapid (C44) or by a slow (N23) rate of hexamethylenebisacetamide (HMBA)-induced differentiation [1]. The level as well as the rate of down-regulation, however, were characteristic of each kinase form, and correlated functionally with the constitutive properties of each phenotypic cell variant [1]. The presence of high levels of PKC- δ was found to be associated with the undifferentiated phenotype and to be inversely correlated with the rate of HMBA-induced differentiation [2]. It has also been found that fluctuation in the intracellular concentration of Ca^{2+} represents the initial signal in the activation of those biochemical events that are sequentially and specifically induced by activation of the Ca^{2+} -dependent neutral proteinase calpain, and that are capable of affecting the rate of MEL-cell differentiation [3,4].

By means of a direct intracellular inhibition of calpain activity, obtained through the introduction of an anti-calpain antibody or of calpastatin into permeabilized MEL cells, we have recently demonstrated that the proteinase promotes down-regulation of PKC isoenzymes, a process that occurs in the course of MEL-cell differentiation (B. Sparatore, unpublished work). Thus, on the basis of a well-documented correlation between the rate of PKC decay and the rate of accumulation of benzidine-reactive cells, a rate-limiting role of calpain in the early stages of differentiation has been suggested. Further support for this conclusion was provided by the finding that, in the fast-differentiating MEL cell line C44, calpain is subject to a more rapid activation, due to the constitutive absence of the natural calpain inhibitor, calpastatin, in these cells [2], a condition that favours an accelerated down-regulation of PKC isoforms and a correlated rapid rate of differentiation.

The present study was carried out to explore: (1) the effects of fluctuations of the intracellular concentration of Ca^{2+} on the kinetics of PKC decay and of the induced MEL-cell differentiation; (2) the conditions required for translocation to, and

association with, MEL-cell membranes, and hence activation, of those PKC forms (δ, α, α_1), known to be involved in the differentiation process; (3) the rate and extent of degradation by calpain of the three kinase isoforms, as an index of the extent of their activation. Experimental evidence is provided suggesting mechanism(s) by which a PKC- δ -related signal is modulated by a constitutive PKC- δ down-regulation in the untreated undifferentiated MEL-cell variants investigated.

MATERIALS AND METHODS

Cell culture

MEL cell clones N23 and V3.17.44 (named C44 in this paper) were obtained and cultured as previously described [5]. Cells ($10^5/\text{ml}$) were induced to differentiate by addition of 5 mM HMBA to the cell culture. At different times of incubation, samples of the cell culture were collected and the proportion of differentiated cells was evaluated by staining with benzidine [6].

Cell permeabilization

MEL cells were permeabilized as previously specified [7]. Monoclonal anti-calpain antibody 56.3 [8] was added to the permeabilized cells at a final concentration of 30 $\mu\text{g}/\text{ml}$; finally the cells were incubated and induced with HMBA as described previously [9].

Immunoblot analysis of PKC isoenzymes

MEL cells were collected by centrifugation, and samples were subjected to SDS/PAGE [10], followed by immunoblot on to pure nitrocellulose membranes (Bio-Rad) by using an anti-PKC- α or anti-PKC- δ anti-peptide antibody as primary antibody (Gibco BRL). The presence and amount of specific immunoreactive material were evaluated as described previously [1,11].

Preparation of cell fractions

MEL cells (10^7 cells) were collected and suspended in 1 ml of 10 mM HEPES, pH 7.5, containing 0.25 M sucrose, 10 mM 2-mercaptoethanol and 1 mM EDTA. The cell suspension was sonicated (6×10 s at 0°C) and centrifuged at $100\,000\text{ g}$ for 15 min. The precipitate (cell membrane fraction) was suspended in 1 ml of 10 mM HEPES, pH 7.5, containing 0.1 mM EDTA (buffer A) and centrifuged at $100\,000\text{ g}$ for 15 min. This washing step was repeated three times. Finally the cell membrane fraction was suspended in 0.5 ml of buffer A. This cell membrane preparation did not contain detectable amounts of PKC- α , - α_1 or - δ , as demonstrated by SDS/PAGE followed by immunoblot analysis.

Purification and assay of calpain

Calpain was purified from 5×10^8 C44 MEL cells, grown to a cell density of 10^6 cells/ml, and assayed by the procedures described for the human erythrocyte enzyme [12]. One unit of calpain activity is defined as the amount that causes the release of 1 nmol of free amino groups/min under the specified conditions [13].

Measurement of $^{45}\text{Ca}^{2+}$ exchange

N23 MEL cells (5×10^6 cells/ml) were suspended in 2 ml of a balanced salt solution (BSS buffer) (135 mM NaCl, 4.5 mM KCl, 5.6 mM glucose, 0.5 mM MgCl_2 and 10 mM HEPES, pH 7.4) containing 1 mM Ca^{2+} (final concn.). At zero time $5\text{ }\mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$ /ml and verapamil, HMBA or Ca^{2+} ionophore A23187 were added, as specified in the Figure legends. At the indicated times, samples (100 μl) of the cell suspension were collected and centrifuged through a layer of silicone oil to measure cell-associated radioactivity [14].

RESULTS

Effects of changes in the intracellular concentration of Ca^{2+} on the HMBA-induced down-regulation of PKC isoenzymes in MEL cells

In order to establish whether modifications of membrane permeability for Ca^{2+} ions affected the kinetics of MEL-cell differentiation, verapamil, a Ca^{2+} -channel blocker, and the Ca^{2+} ionophore A23187 were selected to explore the effects of decreased or increased exchange of Ca^{2+} between the extra- and intra-cellular compartments on the rate of decay of single PKC isoforms. N23 MEL cells were incubated with these two compounds in the presence of $^{45}\text{Ca}^{2+}$, and the exchange of this isotope was monitored as a function of time. As shown in Figure 1(a), ionophore A23187 at a concentration of 5 nM, which is not toxic for cells, increases the exchange of $^{45}\text{Ca}^{2+}$ over the basal rate, whereas verapamil, at a concentration of $5\text{ }\mu\text{M}$, decreases the exchange of $^{45}\text{Ca}^{2+}$ significantly below the basal rate. Identical results were obtained with MEL cells incubated in the simultaneous presence of 5 mM HMBA. Comparable findings were obtained by conducting the same experiments with C44 cells (results not shown).

The same experimental conditions were used to study the effect of the induced modifications of Ca^{2+} permeability on the responses to HMBA of two MEL cell clones, characterized by a rapid (C44) or by a low (N23) rate of differentiation.

As shown in Figures 2 and 3, in both cell lines verapamil promoted a significant decrease in the rate and in the extent of HMBA-induced decay of both PKC- α (Figures 2a and 3a) and PKC- α_1 (Figures 2b and 3b), resulting in 85 % inhibition even

Figure 1 Ca^{2+} exchange in MEL cells exposed to ionophore A23187 and verapamil

(a) N23 MEL cells were equilibrated in BSS buffer as reported in the Materials and methods section for 30 min at 20°C . $^{45}\text{CaCl}_2$ ($5\text{ }\mu\text{Ci/ml}$) was added in the absence (●) or in the presence of 5 nM A23187 (□) or $5\text{ }\mu\text{M}$ verapamil (△). At the indicated times, samples (0.1 ml) were collected and radioactivity associated with cells was counted as described in the Materials and methods section. (b) Cells were incubated as in (a), except that 5 mM HMBA was present in the mixtures together with the additions specified in (a).

after 24 h, a time at which in control cells the down-regulation process is completed. Down-regulation of PKC- δ (Figure 2c) does not occur under these conditions, being on the contrary replaced by a significant increase in its intracellular level. In N23 cells (Figure 2c) the increase is 1.5-fold, whereas in C44 cells (Figure 3c) the increase is 3.1-fold.

In order to test the effects of an opposite type of alteration of the membrane permeability for Ca^{2+} ions, the two MEL cell lines were exposed to the Ca^{2+} ionophore A23187. As shown in Figures 2 and 3, the presence of the ionophore promotes a large increase in the rate and in the extent of the decay of the three PKC isoenzymes, accompanied by a decrease in the time required to reach completion of the overall process. In the MEL cell clone N23, down-regulation of PKC- α (Figure 2a) and PKC- α_1 (Figure 2b) is increased 1.6-fold, whereas the decay of PKC- δ (Figure 2c) is increased approx. 2-fold. Also in C44 cells, subjected to the same experimental conditions, down-regulation of PKC- α (Figure 3a) and PKC- α_1 (Figure 3b) is increased approx. 2.0-fold, and that of PKC- δ (Figure 3c) 1.6-fold. The effects of these compounds are dose-dependent (results not shown); the amount used in these experiments has been selected on the basis of its maximal efficiency without negative effects on cell viability.

Effect of changes in the intracellular concentration of Ca^{2+} on the rate of HMBA-induced differentiation of MEL cells

We have now evaluated the effects of verapamil or Ca^{2+} ionophore on the HMBA-induced terminal erythroid differentiation of rapidly (C44) or slowly (N23) responding MEL cell lines. As shown in Figure 4, the addition of verapamil together with HMBA promotes a profound inhibition of the rate of accumulation of benzidine-reactive cells. In N23 MEL cells (Figure 4a) the inhibition results in a longer latent period, before the onset of differentiation, which then proceeds at a constant but significantly lower rate. Thus, after 120 h of incubation with the drug, a time at which control cells have undergone complete differentiation, only 18 % of the cell population has reached the

Figure 2 Changes in the levels of PKC isoenzymes during differentiation of N23 MEL cells treated with Ca^{2+} ionophore or verapamil

N23 MEL cells ($10^5/\text{ml}$) were induced to differentiation by addition of 5 mM HMBA in the absence (●) or in the presence of 5 nM A23187 (□) or 5 μM verapamil (Δ). At the indicated times, portions of the cell culture were subjected to SDS/PAGE and immunoblotting using anti-PKC- α or anti-PKC- δ anti-peptide antibody as primary antibody, as reported in the Materials and methods section: (a) PKC- α ; (b) PKC- α_1 ; (c) PKC- δ . The area of each peak, obtained by densitometric scanning of the immunoblots, was normalized on the basis of the number of cells used and is expressed as arbitrary units (area of the peak/ 10^6 cells).

Figure 3 Changes in the level of PKC isoenzymes during differentiation of C44 MEL cells treated with Ca^{2+} ionophore A23187 or verapamil

C44 MEL cells ($10^5/\text{ml}$) were induced to differentiate by addition of 5 mM HMBA in the absence (●) or in the presence of 5 nM Ca^{2+} ionophore A23187 (□) or 5 μM verapamil (Δ). At the indicated times, portions of the cell culture were subjected to SDS/PAGE and immunoblotting using anti-PKC- α or anti-PKC- δ anti-peptide antibody as primary antibody: (a) PKC- α ; (b) PKC- α_1 ; (c) PKC- δ . The amount of each PKC isoenzyme is reported as arbitrary units calculated as in the legend to Figure 2.

terminal erythroid stage. In C44 cells (Figure 4b) a similar effect was observed; in fact, whereas in control cells terminal erythroid differentiation is completed in approx. 50 h, in cells exposed to verapamil only 13% of the cell population has reached the differentiated state at the same time. On the contrary, after incubation with HMBA and Ca^{2+} ionophore, N23 cells displayed a considerably shorter latent period, followed by a 4-fold increase in the rate of accumulation of benzidine-reactive cells (Figure 4a). In C44 cells, addition of the ionophore together with HMBA produced almost identical effects, as indicated by the shorter latent period and by a 2-fold increase in the rate of differentiation (Figure 4b). Thus the presence of the ionophore promotes in both cell lines the achievement of the fully differentiated state in times of incubation much shorter than those required by control cells.

Taken together, these results strongly suggest that modifications in the membrane permeability for Ca^{2+} ions affect, in an ordered and correlated sequence, calpain activation and expression of activity, down-regulation of PKC and the rate at which MEL cells reach the terminal erythroid differentiated state. Additional and direct demonstration of this hypothesis was provided by introducing into C44 cells, before exposure to the Ca^{2+} ionophore and to HMBA, an anti-calpain antibody selected for its full inhibitory capacity on calpain activity. The kinetics of MEL-cell induced differentiation was then evaluated. As shown in Figure 5, the introduction of the antibody into both N23 (Figure 5a) and C44 (Figure 5b) cells not only abolished the effects produced by the ionophore, but also promoted a longer latent period and a decrease in the initial rate of differentiation, especially in the slowly responding clone. Furthermore, in both

Figure 4 Kinetics of differentiation of MEL cells induced in the presence of Ca^{2+} ionophore A23187 or verapamil

N23 (a) or C44 (b) MEL cells were induced as described in the legend to Figure 2. At the indicated times, the proportion of benzidine-reactive cells was evaluated as reported in the Materials and methods section. The symbols refer to the presence of Ca^{2+} ionophore or verapamil as in the legends to Figures 2 and 3.

Figure 5 Differentiation of MEL cells loaded with Ca^{2+} and anti-calpain antibody

MEL cells ($3 \times 10^6/\text{ml}$) were permeabilized as specified in the Materials and methods section in the absence (□, ●) or in the presence of $30 \mu\text{g}/\text{ml}$ monoclonal anti-calpain antibody 56.3 (◇, ○). The cells were then induced by addition of 5 mM HMBA in the absence (●, ○) or in the presence of 5 nM Ca^{2+} ionophore A23187 (□, ◇). At the indicated times the proportion of benzidine-reactive cells was evaluated: (a) N23 cells; (b) C44 cells.

cell clones in the presence of the antibody, the rate of differentiation becomes much slower than that observed in control cells treated with HMBA alone.

Correlation between the levels of PKC- δ in MEL cells and the onset of differentiation

Previous experiments have demonstrated the existence of an inverse correlation between the level of PKC- δ and the rate of MEL cell differentiation [2]. To support this conclusion further, C44 cells were exposed to verapamil for 48 h, in order to induce a large increase in PKC- δ (see Figure 2c). Verapamil was then

Figure 6 Effect of preincubation with verapamil on the kinetics of C44 MEL-cell differentiation

C44 cells (10^5 cells/ml) were cultured in the absence (●) or in the presence of $5 \mu\text{M}$ verapamil (○). After 48 h the cells were collected by centrifugation, diluted in fresh culture medium without verapamil and induced by addition of 5 mM HMBA, at a cell density of $10^5/\text{ml}$. At the indicated times the proportion of benzidine-reactive cells was determined.

washed out and HMBA was added. Under these conditions, cells displayed a significantly longer latent period, shifted from 20 to more than 30 h, before the onset of differentiation (Figure 6), followed by a normal rate of accumulation of benzidine-reactive cells. The fact that the washing out has no effect on the delay of the onset of differentiation (cf. Figures 6 and 4b) indicates that this effect is not dependent on the actual concentration of verapamil, potentially retained on the cell surface.

Relationship between membrane binding properties and degradation by calpain of MEL-cell PKC isoenzymes

Previous reports have indicated that activation of PKC occurs after its binding to plasma membranes [15,16], a site at which also calpain becomes active [17,18] and thereby able to promote degradation of the kinase through a proteolytic process. In order to explore the mechanisms responsible for the modulation of the levels of PKC isoforms in untreated and HMBA-induced MEL cells, a first group of experiments was performed to verify the conditions required for the binding of PKC- α , - α_1 and - δ to isolated plasma membranes. Binding was evaluated as a function of increasing concentrations of Ca^{2+} , known to be the metal ion driving the interaction of both PKC and calpain with cell membranes. As shown in Figure 7, maximal binding of PKC- δ to isolated MEL-cell plasma membranes was found to occur in the complete absence of Ca^{2+} . On the contrary, binding of PKC- α and - α_1 was detectable at concentrations of Ca^{2+} above $2\text{--}3 \mu\text{M}$, reaching half-maximal binding between 5 and $7 \mu\text{M}$.

These results indicate that, unlike c-PKC isoenzymes, binding of PKC- δ to the surface of the cell membrane appears to be largely unrelated to a translocation process induced by a rise in Ca^{2+} concentration, and strongly suggest that PKC- δ is predominantly present in a membrane-associated form in resting as well as in stimulated cells. This is supported by the findings that PKC- δ is recovered in a particulate form, even when cells are lysed in EDTA, and can be solubilized only after repeated and extensive washing of the membranes (results not shown).

To explore whether this high affinity between a kinase isoform and MEL-cell membranes could be responsible for the different susceptibilities for the down-regulation process, PKC- α , - α_1 and - δ were mixed with purified plasma membranes in the presence of

Figure 7 Binding of PKC isoenzymes to MEL-cell membrane fraction

N23 cytosolic and membrane fractions were prepared as reported in the Materials and methods section. Membranes from 10^5 cells were then incubated in a total volume of 1 ml with the cytosolic fraction derived from 5×10^5 cells, in the presence of the indicated amounts of Ca^{2+} (final concentration calculated as reported in [28]). After 5 min at 20 °C, the incubation mixture was centrifuged for 5 min at 200 000 *g* and the precipitate subjected to SDS/PAGE and immunoblotting for PKC- α ($\blacktriangle$), - α_1 ($\bullet$) and - δ ($\blacksquare$) as reported in the Materials and methods section. The total amount of each PKC isoenzyme originally present in the cytosolic fraction was also determined by SDS/PAGE followed by immunoblotting.

Figure 8 Susceptibility of membrane-bound PKC isoenzymes to degradation by calpain

N23-cell membranes were prepared and incubated with the cytosolic fraction as in the legend to Figure 7, in the presence of 10 μM Ca^{2+} . The cell membrane fraction containing PKC isoenzymes was isolated by centrifugation, and purified calpain (10 units) and the indicated amounts of Ca^{2+} (final concentration calculated as described in [28]) were added to a final volume of 0.1 ml. After 5 min at 20 °C the reaction was stopped by addition of 1 mM EDTA. SDS/PAGE and immunoblot analysis were carried out as described in the Materials and methods section: $\blacktriangle$, PKC- α ; $\bullet$, PKC- α_1 ; $\blacksquare$, PKC- δ .

calpain and of increasing concentrations of Ca^{2+} . As shown in Figure 8, degradation of PKC- δ largely precedes that of PKC- α and - α_1 , becoming appreciable even at concentrations of Ca^{2+} of the order of 1–2 μM and reaching half-maximal decay at approx. 5 μM . On the contrary, degradation of PKC- α and - α_1 is detectable at 5–10 μM and reaches the half-maximal level at 40 μM Ca^{2+} .

These results further support the idea that changes in intracellular concentration of Ca^{2+} and the related activation of calpain represent crucial and primary events that control the

down-regulation of PKC isoforms and the kinetics of MEL-cell differentiation.

DISCUSSION

The role of individual PKC isoenzymes in the regulation of cell differentiation [19–25] and, more specifically, the well-documented existence of a correlation between the initial levels and the rate of selective down-regulation of single PKC isoforms and the kinetics of MEL-cell induced differentiation [1] are in strong support of different biological functions of the various PKC isoenzymes [26]. Calpain has been identified as the proteinase that catalyses a selective decay of the PKC isoforms [1].

In the present paper, we provide evidence indicating that fluctuations in the Ca^{2+} concentration represent the signal that triggers activation of calpain and thus initiates an ordered sequence of biochemical events that are critically correlated to the kinetics of cell differentiation. With respect to the specific biological function of single PKC isoforms, data are presented that confirm the existence of a direct correlation between high intracellular concentrations of PKC- δ and slow response of the erythroleukaemia cell to HMBA-induced differentiation. Moreover, PKC- δ has been found to be present in untreated cells in a tight association with MEL-cell membranes and to re-associate rapidly in reconstituted systems in the absence of Ca^{2+} . We tentatively speculate that this constitutive association of PKC- δ with the MEL-cell membrane, as also observed in other cell types [25,27], suggests an activated state of this kinase and therefore the continuous delivery of a signal that appears to be critically related to the characteristics of the undifferentiated phenotype. As a result of this cellular localization, PKC- δ is also subjected, unlike other PKCs, to a continuous down-regulation in MEL cells. The rate of this decay is much more rapid in C44 cells, due to the absence of calpastatin, a condition that favours a more rapid activation of calpain. This conclusion is supported by the finding of a higher increase in these cells in the level of PKC- δ after addition of verapamil, resulting from a decreased rate of down-regulation. On the contrary, the levels of c-PKC isoenzymes (α and α_1) are not affected by verapamil, and their translocation–activation and down-regulation are promoted by HMBA and appear to be directly correlated with an acceleration of the rate of MEL-cell induction to terminal erythroid differentiation [1].

The results reported here represent an additional and final demonstration of the central role of PKC isoforms in signal activation of pathways that are essential for MEL-cell differentiation. It is shown here that the function of each PKC isoform is expressed through a co-ordinated mechanism, mediated by Ca^{2+} , and including selectivity in the translocation and activation at the membrane level of each kinase isoform, as well as a continuous modulation by calpain of the amounts of the active kinase molecules.

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