THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 264, No. 31, Issue of November 5, pp. 18414-18418, 1989
Printed in U.S.A.

Differential Expression of Protein Kinase C Isozymes and Erythroleukemia Cell Differentiation*

(Received for publication, May 4, 1989)

Edon Melloni‡, Sandro Pontremoli‡, Pier Luigi Viotti‡, Mauro Patrone‡, Paul A. Marks§, and Richard A. Rifkind§

From the ‡Institute of Biological Chemistry, University of Genoa, Genoa, Italy and the §DeWitt Wallace Research Laboratory, Memorial Sloan-Kettering Cancer Center and the Sloan-Kettering Division of The Graduate School of Medical Sciences, Cornell University, New York, New York 10021

Hexamethylene bisacetamide (HMBA) and other polar/apolar chemical agents are potent inducers of erythroid differentiation in murine erythroleukemia cells (MELC), as well as other transformed cell lines. Although the mechanism of action of HMBA is not yet known, evidence has been obtained that protein kinase C (PKC) plays a role in this process. In this study we provide further evidence that establishes this relationship. MELC contain two principal PKC activities, PKCβ and PKCα. MELC variants, selected for resistance to vincristine (VC), which display acceleration of their rates of induced differentiation, are enriched in PKCβ activity. When MELC are exposed to HMBA there is a fall in PKC activity, largely accounted for by a decline in PKCβ. This decline in PKC activity is faster in the VC-resistant, rapidly differentiating MELC. We previously demonstrated that VC-resistant MELC are resistant to the inhibition of differentiation by the phorbol ester, phorbol 12-myristate 13-acetate (PMA). In both VC-sensitive and -resistant MELC, PMA causes rapid membrane translocation and then a decline in PKC activity, accompanied by a generation of a Ca^{2+}- and phospholipid-independent protein kinase activity. In VC/PMA-resistant variants, this Ca^{2+}/phospholipid-independent protein kinase activity persists considerably longer than in the VC-sensitive variants. This correlates with the resistance to PMA and provides additional evidence for a role for the Ca^{2+}/phospholipid-independent protein kinase activity during induced differentiation.

The induction of differentiation in MELC,[1] by exposure to HMBA and related polar/apolar compounds (1, 2) is a multistep process leading, after a latent period of about 10–12 h, to commitment to terminal erythroid differentiation and cessation of proliferation (3–5). A number of metabolic changes occur during the latent period which precedes irreversible commitment to differentiation. Among these changes are altered membrane ion flux (6–8), cell volume (9), cyclic nucleotide concentration (10), and PKC activity (11). With respect to PKC activity, we have previously demonstrated that HMBA initiates a progressive decline in native PKC activity, accompanied by the proteolytic generation of a Ca^{2+}/PL-independent protein kinase activity which appears to be important during commitment (11). There is, in addition, HMBA-mediated modulation of expression of several nuclear protooncogenes, including c-myb, c-myc, c-fos, and p53, among which the suppression of expression of c-myb appear to be of particular importance to the commitment process (12–16). Following commitment, MELC display the morphological and molecular changes characteristic of normal erythroid differentiation, including coordinated expression of the several globin genes, the heme-synthetic enzyme pathway, the red cell-specific membrane proteins, progressive suppression of DNA and RNA synthesis, and cessation of cell division (1, 17, 18).

Recently, we have demonstrated that MELC rendered resistant to low concentrations of vincristine are 1) markedly more sensitive to induction of differentiation by low concentrations of HMBA, 2) induced to commitment without a latent period, and 3) resistant to the differentiation-inhibitory effects of the phorbol ester, PMA, and the glucocorticoid, dexamethasone (19). We have suggested that these VC-resistant MELC, which display accelerated differentiation, may prove to be constitutive for a factor (or factors) critical for early events in commitment (16, 19).

In this paper we examine the relationship between PKC activity and the inducibility of MELC. We demonstrate that in MELC, PKC exists predominantly as two isozymes, identified as PKCα and PKCβ. In all MELC variants examined, the level of activity of PKCα exceeds PKCβ. In general, the level of total PKC activity is about 25% higher in the VC-resistant cells, compared to VC-sensitive cells, and this is accounted for largely by increased PKCβ activity. We have found that cells with higher levels of PKCβ activity display faster rates of HMBA-induced differentiation. Upon exposure to HMBA, MELC lose PKC activity. Rapidly differentiating, VC-resistant lines lose their PKC activity faster, and the decline is predominantly in PKCβ activity. We also demonstrate that VC-resistant MELC are, in fact, sensitive to the inhibitory effect of PMA, but only if preincubated with the phorbol ester for sufficient time to deplete the Ca^{2+}/PL-independent protein kinase activity which is generated by exposure to PMA. Taken together, the accumulated evidence suggests an important role for PKC activity, and PKCβ activity in particular, during induction of MELC to erythroid differentiation. The data are consistent with and supportive

* This work was supported in part by Grants CA-31768 and CA-08748 from the National Cancer Institute and by grants from the Japanese Foundation for Promotion of Cancer Research, the Italian Consiglio Nazionale delle Richerche, Progetto Finalizzato Oncologia, Sottoprogetti Biologia Cellulare e Molecolare, and the Associazione Italiana per la Ricerca sul Cancro, Milano. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: MELC, murine erythroleukemia cells; HMBA, hexamethylene bisacetamide; PKC, protein kinase C; VC, vincristine; PMA, phorbol 12-myristate 13-acetate; PL, phospholipid; HA, hydroxyapatite; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of the concept that HMBA-mediated generation, by proteolytic cleavage, of an active, Ca^{2+}/PL-independent PKC fragment, perhaps derived from PKCβ, is a critical step in the induction process.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—MEL cell line DS19, and all the variant lines described in this paper which were derived from it, were maintained in α minimal essential medium containing 10% (v/v) fetal calf serum (5). Sc9 and N23 are unselected subclones of DS19. V3.17 and each of the subclones of V3.17 (subclones 14, 44, and 74) were selected for resistance to VC as previously reported (19). They are routinely maintained in 5 ng/ml of vincristine; the presence or absence of VC has no effect on their property of accelerated differentiation when exposed to HMBA. Sc9[VCR]c15 was selected from DS19 by successive single-step selections, first in 1 ng/ml of VC, then in 2 ng/ml. It is maintained in 2 ng/ml VC. R1 were derived from DS19 by selection for resistance to induction of terminal cell division and differentiation by HMBA (20). R1[VCR] was selected from R1 cells by growth in 5 ng/ml of VC and now displays the property of accelerated response to HMBA instead of the HMBA-resistance characteristic of the parent line (21). Cell line A120 was selected from Sc9 cells by stepwise growth in Adriamycin (daunarubicin, Sigma), starting at 20 ng/ml. It is maintained in 120 ng/ml Adriamycin.[2]

Cells were induced to differentiate by exposure to 5 mM HMBA and the accumulation of differentiated cells was assayed by the benzidine reaction for hemoglobin using benzidine dihydrochloride (Sigma) as previously described (22).

Hydroxyapatite (HA)-Ultrogel was purchased from LKB, and DEAE-cellulose (DE-52) from Whatman. Phosphatidylserine, 1,2-diolein, histone (type III-S), leupeptin, phenylmethylsulfonyl fluoride, PMA, ATP, HEPES, and HMBA were from Sigma. [^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Corp., and Triton X-100 from Rohm and Haas.

HA-Ultrogel Chromatography and PKC Assay—The cells (40×10^6) were suspended in 2 ml of 20 mM KPO_4 buffer, pH 7.5, containing 10% glycerol, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride, lysed by sonication at 4 °C (six bursts of 10 s each) and then centrifuged at 100,000 $\times g$ for 10 min. The supernatant (cytosol fraction) was collected and loaded onto an HA-Ultrogel column (0.7 × 20 cm) as described by Ono *et al.* (23), with the following modifications: the flow rate was 0.5 ml/min and fractions of 1 ml were collected; the column was washed with 15 ml of starting buffer followed by 30 ml of 40 mM KPO_4 buffer. A linear gradient (90 ml) of from 40 to 90 mM KPO_4, followed by 20 ml of 90 mM and 35 ml of 200 mM KPO_4 buffer were then applied to the column. PKC activity was determined on 100 μl of the eluted fractions as previously described (11).

Separation of Native PKC and Ca^{2+}/PL-independent PKC Activities by DEAE-Chromatography—The cytosol fraction from 40×10^6 cells was prepared and subjected to anion-exchange chromatography as previously described (11). PKC activity and the Ca^{2+}/PL-independent protein kinase activity were assayed using 100 μl of the eluted fractions and the amount of each enzyme activity calculated from the area under the eluted peaks. For assay of membrane-associated PKC activity, the pellet obtained after centrifugation of the lysed cells at 100,000 $\times g$ was suspended in 1 ml of a lysing buffer containing 0.2% Triton X-100, and aliquots of 10 μl were immediately used to assay PKC activity.

RESULTS

Fractionation of PKC Activity in MELC—Two distinct peaks of protein kinase activity, identified as PKC by their absolute requirement for Ca^{2+} and phospholipid (PL; phosphatidylserine and dioleoyl glyceral) for activity, were detected in MELC by HA-Ultrogel chromatography (Fig. 1*A*). The first peak (*I*) elutes at approximately 60 mM KPO_4 buffer, and represents 12–16% of the total Ca^{2+}/PL-dependent kinase activity in the N23 variant of MELC. The second peak (*II*) elutes at about 80–84 mM KPO_4 buffer and accounts for 84–88% of total activity. Employing antipeptide antibodies which specifically recognize the α, β, and τ isozymes of PKC (kindly provided by Dr. Ora M. Rosen, Memorial Sloan-Kettering Cancer Center) (24), it was demonstrated that the first peak corresponds to PKCβ activity, and the second to PKCα activity (Table I). No PKC activity reactive with anti-PKCτ antibody was detected in these MELC lines.

PKC Activity in MELC Variants with Different Rates of Induction—We have recently described the selection of a

[2] Z-X. Chen, P. A. Marks, and R. A. Rifkind, unpublished observations.

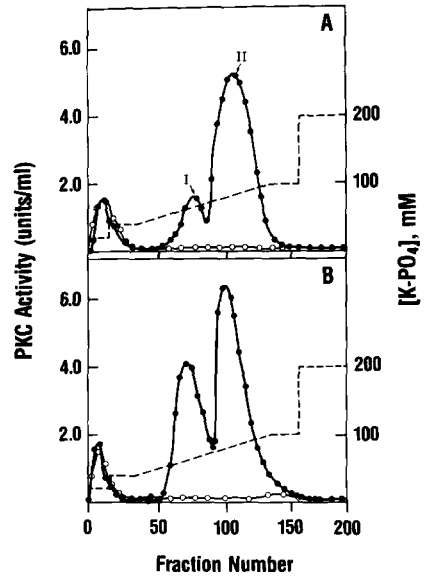

FIG. 1. **Separation of PKC isozymes by HA-Ultrogel chromatography.** The cytosol fraction from 4×10^7 cells was prepared and chromatographed on an HA-Ultrogel column, as described under "Experimental Procedures." PKC activity was assayed in the presence (●——●) or absence (○——○) of Ca^{2+} and phospholipids on 100 μl of eluted fractions. [KPO_4] buffer gradient (- - -). PKC peak I (column fractions 60–85) and PKC peak II (column fractions 100–130) were pooled separately and concentrated by ultrafiltration to an activity of about 100 units/ml (unit = nmol ^{32}P incorporated/min). The specific activities of each isozyme were, for N23 cells, about 830 units/mg protein (*peak I*) and 5000 units/mg protein (*peak II*), and for V3.17[44] cells, 4150 and 4850 units/mg protein, respectively. *A*, PKC fractionation for MELC line N23; *B*, PKC fractionation for V3.17[44] cells.

TABLE I

Identification of PKC isozymes with isozyme-specific antisera

Antiserum	Source of PKC activity[a]			
	Fraction I	Fraction II	Total cytosol	Rat brain
	m net cpm/cm nitrocellulose strip			
Anti-PKCα	1	159	171	56
Anti-PKCβ	126	14	136	154
Anti-PKCτ	1	1	2	39

[a] PKC fractions I and II, isolated from V3.17 [44] cells by HA-Ultrogel chromatography, were further purified by threonine-Sepharose (58). Each concentrated PKC fraction, as well as the unfractionated cytosol from the same cells, and an unfractionated cytosol preparation from rat brain (positive control for PKCα, -β, and -τ) were individually adsorbed in triplicate onto nitrocellulose strips (0.5 × 3.0 cm) for 1 h at room temperature. The strips were saturated with 5% bovine serum albumin in phosphate-buffered saline, cut into three 1-cm pieces, and each piece exposed to one of three antipeptide antisera, specific for PKCα, -β, and -τ. After 2 h the strips were washed in phosphate-buffered saline, exposed for 2 h to ^{125}I-anti-rabbit Ig, exhaustively washed in phosphate-buffered saline, with 0.1% Tween 20, and then bound radioactivity was determined with an Ambis β scanner and by radioautography (not shown). The numbers reported, for each antiserum applied to each source of PKC activity, are the mean counts/min/strip, less background (38–40 cpm/strip) of the triplicate preparations.

cloned variant MELC line resistant to inhibition of cell growth by low concentrations of vincristine (19). This variant, designated V3.17, is significantly more sensitive to the induction of erythroid differentiation by HMBA (and other polar/apolar inducing agents, such as dimethyl sulfoxide and HMBA analogs; data not shown), than the parental MELC cell line, DS19-Sc9, derived from Friend's original isolate, 745A (25). This variant, and a series of cloned VC-resistant MELC lines subsequently isolated, is induced to differentiate by low concentrations of HMBA, becomes committed to terminal differentiation without the 10–12-h latent period characteristic of the parental line, and accumulates differentiated (hemoglobin-containing, benzidine-reactive) erythroid cells at an accelerated rate (19). A number of clones have also been isolated which are resistant to induction by HMBA or differentiate at a distinctly lower rate (see "Experimental Procedures." The kinetics of induced differentiation of these variants and the parental line, as measured by accumulation of benzidine-reactive cells, are illustrated in Fig. 2. All of these variants grow at approximately the same rate, achieving a density of about 2–3 × 10^6 cells/ml within 48–72 h.

Each of these variant clones was examined by HA-Ultrogel chromatography for the level and distribution of PKC activity represented by each of the two isozymes characteristic of MELC (Fig. 1*B* and Table II). Although all variants demonstrate both β- and α-PKC activity, and in each, PKCα is the principal component, the fraction contributed by PKCβ differs among the several cell lines. In general, the clones least responsive to HMBA (R1, A120, and N23) display the lowest content of PKCβ, while the most sensitive lines (R1[VCR], Sc9[VCR]c15, and the V3.17 subclones, 74, 44, and 14) display the highest levels of this isozyme, compared to the parental line, Sc9.

Effect of HMBA on PKC Activity and PKC Isozymes—We have examined the kinetics of change of total cytosolic PKC activity during the induction of differentiation with HMBA of a low-response (N23) and a high-response (V3.17[44]) MELC variant (Fig. 3). PKC activity falls gradually and steadily during induction of N23 cells, reaching about 60% of initial activity by 72 h, by which time appreciable numbers of differentiated cells can be detected (by the benzidine reaction). V3.17[44] cells start, as is true for all of the VC-resistant, high-response clones, with a higher level of PKC activity, and there is an approximately 50% fall in PKC activity within 4 h of exposure to 5 mM HMBA (or 3 mM HMBA, as well, data not shown, which is sufficient for complete induction of the HMBA-sensitive VC-resistant clones (19)). By 24 h, when considerable numbers of differentiated V3.17[44] cells can be detected, PKC activity has decreased to 30% of initial activity.

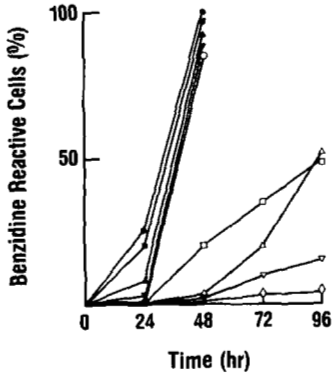

FIG. 2. **Kinetics of induced differentiation of MELC variants.** Each MELC variant was inoculated into suspension culture (see "Experimental Procedures") at 10^5 cells/ml in the presence of 5 mM HMBA. The proportion of benzidine-reactive cells was determined in each culture at the indicated intervals. ●, V3.17[14]; ■, V3.17[44]; ▲, V3.17[74]; ▼, Sc9[VCR]c15; ▽, A120; ◇, R1; □, Sc9; △, N23; ○, R1[VCR].

TABLE II
Protein kinase C activity in MELC variants

Variant	Protein kinase C activity[a]			
	PKCβ	PKCα	Total PKC	β/total
	units/10^6 cells[b]			
R1	0.39	3.60	3.99	0.10
	0.36	4.20	4.56	0.08
A120	0.24	4.68	4.92	0.05
	0.18	4.08	4.26	0.04
N23	0.69 ± 0.10	3.90 ± 0.45	4.59	0.15
SC9	0.70	3.60	4.30	0.16
	0.58	4.10	4.68	0.12
R1(VCR)	1.70	3.30	5.00	0.34
	1.60	3.95	5.55	0.29
SC9(VCR)c15	1.30	3.40	4.70	0.28
	1.38	3.95	5.33	0.26
V3.17[74]	1.50 ± 0.16	4.50 ± 0.36	6.00	0.25
V3.17[44]	2.48 ± 0.16	3.80 ± 0.30	6.28	0.39
V3.17[14]	2.23 ± 0.14	3.27 ± 0.36	5.50	0.41

[a] For each variant, PKC values were determined at least two times; for some variants three to five determinations were made, and the value given is the mean ± S.D.
[b] Unit = nmol ^{32}P incorporated/min.

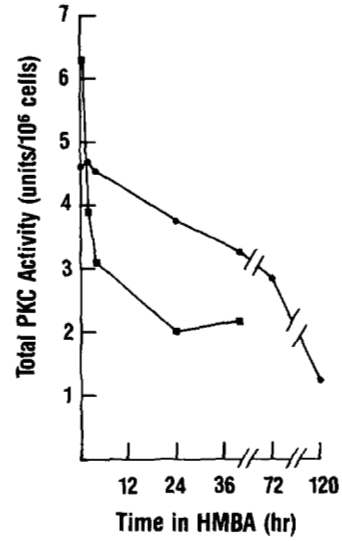

FIG. 3. **Effect of HMBA on total cytosolic PKC activity.** N23 and V3.17[44] cells were inoculated at 2 × 10^5 cells/ml in culture with 5 mM HMBA and maintained (by repeated dilution) at a cell density <9 × 10^5 cells/ml in order to obviate the suppressive effect of growth-to-plateau-density on PKC activity (11). At the indicated intervals, up to 40 h (for V3.17[44] cells, ■——■) and 120 h (for N23 cells, ●——●), 40 × 10^6 cells were harvested and total cytosolic PKC activities determined as described under "Experimental Procedures."

We have examined the changes in the β- and α-PKC isozymes during HMBA-induced differentiation of N23 and V3.17[44] cells (Fig. 4). In both cell lines the decrease in PKC activity is mainly accounted for by a decline in PKCβ. The early (<4 h) and rapid fall in PKC activity (from about 6.3 units/10^6 cells to about 3.1 units/10^6 cells; Fig. 3) in the VC-resistant cell line (V3.17[44]) is largely due to a fall in PKCβ (from 2.5 units/10^6 cells to 0.5 units/10^6 cells), which has declined to about 20% of initial activity by 3 h of exposure to HMBA (Fig. 4). In N23 cells, neither PKCβ nor total PKC

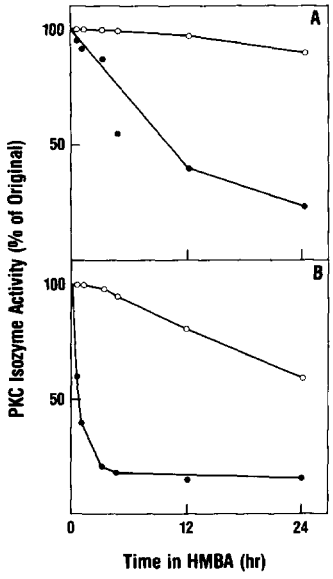

FIG. 4. **Effect of HMBA on PKCα and PKCβ activities.** N23 (*A*) and V3.17[44] (*B*) cells were cultured with 5 mM HMBA under the conditions described in Fig. 3 and assayed by HA-Ultrogel chromatography (see Fig. 1), at the indicated intervals up to 24 h, for changes in the activities of PKCα (chromatographic peak II) and PKCβ (peak I). The 100% values (activity at 0 h) were: for N23 cells, PKCβ = 0.7 and PKCα = 4.15 units/ml; for V3.17[44] cells, PKCβ = 2.5 and PKCα = 3.95 units/ml. O—O, PKCα; ●—●, PKCβ.

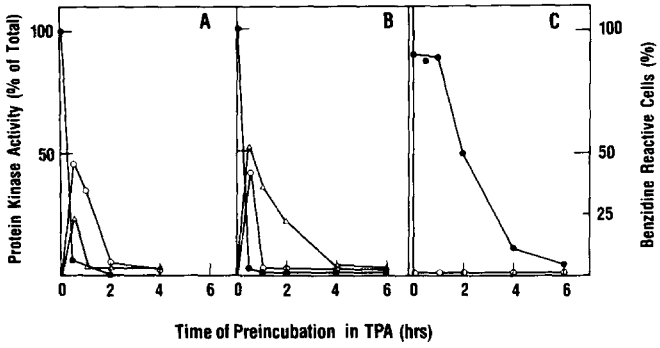

FIG. 5. **Effect of PMA on differentiation and PKC activity.** N23 and V3.17[44] cells at a density of 10^5 cells/ml were preincubated with PMA (100 ng/ml) for the periods indicated, up to 6 h, then aliquots of 4×10^7 cells (*A*, N23 cells; *B*, V3.17[44] cells) were lysed, the membrane and cytosol fractions separated (see "Experimental Procedures"), and the cytosol fractions chromatographed on a DE-52 column for separation and assay of native PKC and the Ca^{2+}/PL-independent kinase, as previously described (11). The membrane fraction was assayed for total PKC activity. ●, cytosolic PKC; O, membrane PKC; Δ, Ca^{2+}/PL-independent protein kinase. *C*, a second aliquot was inoculated into culture (at 10^5 cell/ml) with 5 mM HMBA and PMA (100 ng/ml). After 40 h (for V3.17[44] cells, ●) and 72 h (for N23 cells, O) the proportion of benzidine-reactive cells was determined.

activities decline to these levels until after 24 h of culture with HMBA. The decline in PKCα is more gradual in both cell lines, although definitely faster in V3.17 cells than in the N23 line.

Effect of PMA on Induction and PKC Activity—We have previously shown that the phorbol ester, PMA, is a powerful inhibitor of HMBA-induced MELC erythroid differentiation, acting during the latent period for commitment (1, 5, 26). PMA depletes MELC PKC activity by over 90% within 2–3 h and the suppressed inducibility by HMBA recovers in proportion to the recovery of PKC activity (11). HMBA-hypersensitive, VC-resistant MELC appear relatively insensitive to the differentiation-inhibitory effects of PMA when both agents are added simultaneously (19). We have now examined the effects of various periods of preincubation of N23 and V3.17[44] cells both on PKC activity and on induced differentiation in response to subsequent addition of HMBA (Fig. 5).

From the data in Fig. 5*C* it can be seen that N23 cells are completely inhibited by PMA, with respect to differentiation (accumulation of benzidine-reactive cells), even if PMA is added as late as simultaneously with the HMBA (0 h of preincubation). On the other hand, it requires at least 2 h of preincubation with PMA to achieve partial (50%) inhibition of induction of V3.17[44] cells, and complete inhibition requires 6 h of pre-exposure to the phorbol ester. In both cell lines cytosolic PKC is rapidly depleted, within 1 h of exposure to PMA, while a membrane-associated activity is transiently generated (within 30 min) and depleted by 1–2 h (Fig. 5, *A* and *B*). The two lines differ, however, with respect to the amount and persistence of a Ca^{2+}/PL-independent protein kinase activity generated during exposure to PMA. This activity is detected in both lines by 30 min in PMA, but reaches a higher proportion (almost 50%) of total PKC activity and persists longer (until 4 h) in V3.17 cells (Fig. 5*B*), compared to the N23 line (Fig. 5*A*), in which all of this form of protein kinase activity is gone by 1 h of exposure to the phorbol ester. Taken together, these observations raise the possibility that the relative resistance to inhibition of V3.17 cells by PMA is related to the delayed depletion of one form of kinase activity, coupled with the rapid rate of induction of these VC-resistant MELC.

DISCUSSION

The present findings provide evidence that PKC plays an important role during HMBA-mediated induction of MELC differentiation (11, 27). MELC contain, principally, two PKC isozymes, which we have identified as PKCβ and PKCα by virtue of their reactivity with specific antipeptide antibodies (24). Although PKCα predominates in all MELC variants examined, the proportion of PKCβ is distinctly higher (as is total PKC activity) in a series of cloned MELC variants which display an accelerated response to HMBA and sensitivity to lower concentrations of the inducer (19, 20), compared to the parental line and to other MELC clones selected for resistance to induction by HMBA or for their relatively slow response. The rapid fall in PKCβ activity in response to HMBA, characteristic of a hypersensitive cell line, compared to the much more gradual decline exhibited by a less responsive line, implicates a role for this isozyme during early stages of the induction process. It is now established that in virtually all cell systems total cellular PKC activity represents the aggregate activities of multiple PKC isozymes, including the products of at least three PKC genes, PKCα, -β, and -τ (28–39), and it has recently been reported that these isozymes can be differentially down- and up-regulated (24–40). A role for PKC in the regulation of cell proliferation and differentiation, both of which are elements of the response of MELC to inducing agents (1), has been suggested by reports on a variety of biological systems (28, 41–51), including hematopoietic precursors (52, 53).

We have previously demonstrated that in MELC, HMBA induces the formation of a proteolytically generated, cytosolic Ca^{2+}/PL-independent kinase activity accompanied by progressive decline in total cytosolic PKC activity (11). Although both the generation of this kinase activity and induced differentiation are inhibited by leupeptin, an inhibitor of protease

activities, such as calpain, it has not been demonstrated that this Ca^{2+}/PL-independent PK activity is a proteolytic product of native PKC (11, 54–56). Consistent, however, with the suggested importance during differentiation of inducer-activated generation of a cytosolic Ca^{2+}/PL-independent PK activity is the present observation that the kinetics of inhibition of induced differentiation by PMA appear to correlate with the rate of decline of this kinase activity. The more rapid decay of kinase activity, the more effective is PMA in inhibiting induced differentiation.

Precisely how an elevated level of PKCβ might contribute to the greater sensitivity and rapid response to inducer characteristic of the series of VC-resistant MELC variants is not known. We have speculated (16, 19) that the characteristic rapid response of VC-resistant MELC to HMBA may reflect their constitutive expression of a factor (or factors) critical and rate-limiting for an early event in the commitment process. Indeed, we have demonstrated (57) that, unlike the VC-sensitive MELC variant, Sc9, the induction of VC-resistant V3.17 cells by HMBA is not retarded by inhibition of protein synthesis with cycloheximide. It is possible that phosphorylation of a specific protein target by an activated PKC, perhaps a proteolytically generated, Ca^{2+}/PL-independent fragment of PKCβ, comprises one required, but probably not sufficient component of this complex regulatory pathway. What role is played by the more slowly responding PKCα also remains to be determined. This isozyme declines somewhat more rapidly in cells which are induced more rapidly (*i.e.* V3.17[44]), suggesting that its activation or deactivation may play some role during post-commitment differentiation, such as a role in the actual implementation of the developmental program including terminal cell division.

Acknowledgment—We are grateful to Lang Ngo for her expert technical assistance.

REFERENCES

1. Marks, P. A., Sheffery, M., and Rifkind, R. A. (1987) *Cancer Res.* **47,** 659–666
2. Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A., and Marks, P. A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 862–866
3. Gusella, J., Geller, R., Clarke, B., Weeks, V., and Housman, D. (1976) *Cell* **9,** 211–229
4. Fibach, E., Reuben, R. C., Rifkind, R. A., and Marks, P. A. (1977) *Cancer Res.* **37,** 440–444
5. Chen, Z., Banks, J., Rifkind, R. A., and Marks, P. A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 471–475
6. Mager, D., and Bernstein, A. (1978) *J. Supermol. Struct.* **8,** 431–438
7. Bernstein, A., Hunt, D. M., Crichley, V., and Mak, T. W. (1976) *Cell* **9,** 375–381
8. Bridges, K., Levenson, R., Housman, D., and Cantley, L. (1981) *J. Cell Biol.* **90,** 542–544
9. Gazitt, Y., Deitch, A. D., Marks, P. A., and Rifkind, R. A. (1987) *Exp. Cell Res.* **117,** 413–420
10. Gazitt, Y., Reuben, R. C., Deitch, A. D., Marks, P. A., and Rifkind, R. A. (1978) *Cancer Res.* **38,** 3779–3783
11. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Cakiroglu, A. G., Jackson, J. F., Rifkind, R. A., and Marks, P. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5282–5286
12. Lachman, H. M., and Skoultchi, A. I. (1984) *Nature* **310,** 592–594
13. Kirsch, I. R., Bertness, V., Silver, J., and Hollis, G. (1985) in *Leukemia: Recent Advances in Biology and Treatment* (Gale, R. P., and Golde, D. W., eds) pp. 91–98, Alan R. Liss, New York
14. Ramsay, R. G., Ikeda, K., Rifkind, R. A., and Marks, P. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6849–6853
15. Todokoro, K., and Ikawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **135,** 1112–1118
16. Marks, P. A., and Rifkind, R. A. (1989) in *Tumor Suppressor Genes* (Klein, G., ed) Marcel Dekker, Inc., in press
17. Marks, P. A., and Rifkind, R. A. (1978) *Annu. Rev. Biochem.* **47,** 419–448
18. Tsiftsoglou, A. S., and Robinson, S. H. (1985) *Int. J. Cell Cloning* **3,** 349–366
19. Melloni, E., Pontremoli, S., Damiani, G., Viotti, P., Weich, N., Rifkind, R. A., and Marks, P. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3835–3839
20. Chen, Z-X., Banks, J., Rifkind, R. A., and Marks, P. A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 471–475
21. Michaeli, J., Weich, N., Marks, P. A., and Rifkind, R. A. (1988) *Blood* **72,** 97a
22. Singer, D., Cooper, M., Maniatis, G. M., Marks, P. A., and Rifkind, R. A. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 2668–2670
23. Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K., and Nishizuka, Y. (1987) *Science* **236,** 1116–1120
24. Makowske, M., Ballester, R., Cayre, Y., and Rosen, O. M. (1988) *J. Biol. Chem.* **263,** 3402–3410
25. Friend, C., Scher, W., Holland, J., and Sato, T. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 378–382
26. Yamasaki, H., Fibach, E., Nudel, U., Weinstein, I. B., Rifkind, R. A., and Marks, P. A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 3451–3455
27. Balazovich, K. J., Portnow, D., Boxer, L. A., and Prochownik, E. V. (1987) *Biochim. Biophys. Acta* **927,** 247–255
28. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. S., and Ullrich, A. (1986) *Science* **233,** 853–859
29. Ohno, S., Kawasaki, H., Imajoh, S., and Suzuki, S. (1987) *Nature* **325,** 161–166
30. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. (1986) *Science* **233,** 859–866
31. Ono, Y., Kurokawa, T., Kawahara, U., Nishimura, O., Marumoto, R., Igarashi, K., Sugino, Y., Kikkawa, U., Ogita, K., and Nishizuka, Y. (1986) *FEBS Lett.* **203,** 111–115
32. Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Kikkawa, U., Ogita, K., Sugino, Y., and Nishizuka, Y. (1986) *FEBS Lett.* **206,** 347–352
33. Knopf, J. L., Lee, M.-H., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M., and Bell, R. M. (1986) *Cell* **46,** 491–502
34. Housey, G. M., O'Brian, C. A., Johnson, M. D., Kirschmeier, P., and Weinstein, I. B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1065–1069
35. Huang, K.-P., Nakabayashi, H., and Huang, F. L. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8535–8539
36. Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K., and Nishizuka, Y. (1987) *FEBS Lett.* **217,** 227–231
37. Pelosin, J.-M., Vilgrain, I., and Chambaz, E. M. (1987) *Biochem. Biophys. Res. Commun.* **31,** 382–391
38. Huang, F. L., Yoshida, Y., Nakabayashi, H., and Huang, K.-P. (1987) *J. Biol. Chem.* **262,** 15714–15720
39. Jaken, S., and Kiley, S. C. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4418–4422
40. Huang, F. L., Yoshida, Y., Cunha-Melo, J. R., Beaven, M. A., and Huang, K.-P. (1989) *J. Biol. Chem.* **264,** 4238–4243
41. Castagna, M., Takai, Y., Kaibuchi, L., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257,** 7847–7851
42. Miyake, R., Tanaka, Y., Tsuda, T., Kaibuchi, K., Kikkawa, U., and Nishizuka, Y. (1984) *Biochem. Biophys. Res. Commun.* **121,** 649–656
43. Couturier, A., Bazgar, S., and Castagna, M. (1984) *Biochem. Biophys. Res. Commun.* **121,** 448–455
44. Arcoleo, J. P., and Weinstein, I. B. (1985) *Carcinogenesis* **6,** 213–217
45. Hiroka, K., Hirota, T., Aguilera, G., and Catt, K. J. (1985) *J. Biol. Chem.* **260,** 3243–3246
46. Wolf, M., LeVine, H., III, May, W. S., Jr., Cuatrecases, P., and Sayoun, N. (1985) *Nature* **317,** 546–549
47. Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B., and Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 8309–8313
48. Sekar, M. C., and Hokin, L. E. (1986) *J. Membr. Biol.* **89,** 193–210
49. Ase, K., Berry, N., Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1988) *FEBS Lett.* **236,** 396–400
50. Stone, R. M., Weber, B. L., Spriggs, D. R., and Kufe, D. W. (1988) *Blood* **72,** 739–744
51. Stone, R. M., Sariban, E., Pettit, G. R., and Kufe, D. W. (1988) *Blood* **72,** 208–213
52. Katayama, N., Nishikawa, M., Minami, N., and Shirakawa, S. (1989) *Blood* **73,** 123–130
53. Faleto, D. L., Arrow, A. S., and Macara, I. G. (1985) *Cell* **43,** 315–325
54. Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T., and Nishizuka, Y. (1989) *J. Biol. Chem.* **264,** 4088–4092
55. Tapley, P. M., and Murray, W. W. (1984) *Biochem. Biophys. Res. Commun.* **122,** 158–164
56. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., and Horecker, B. L. (1986) *J. Biol. Chem.* **261,** 4101–4105
57. Michaeli, J., Richon, V. M., Jackson, J. F., Rifkind, R. A., and Marks, P. A. (1988) *Blood* **72,** 97a
58. Kikkawa, A., Go, M., Kuomoto, J., and Nishizuka, J. (1986) *Biochem. Biophys. Res. Commun.* **135,** 636–643