Activity and Regulation of Calcium-, Phospholipid-dependent Protein Kinase in Differentiating Chick Myogenic Cells

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Abstract. The activity of calcium-, phospholipid-dependent protein kinase (PKc) was measured in (*a*) total extracts, (*b*) crude membrane, and (*c*) cytosolic fractions of chick embryo myogenic cells differentiating in culture. Total PKc activity slowly declines during the course of terminal myogenesis in contrast to the activity of cAMP-dependent protein kinase, which was also measured in the same cells. Myogenic cells at day 1 of culture possess high particulate and low soluble PKc activity. A dramatic decline of particulate PKc activity occurs during myogenic cell differentiation and is accompanied, through day 4, by a striking rise of the soluble activity. The difference in the subcellular distribution of PKc between replicating myoblasts and myotubes is confirmed by phosphorylation studies conducted in intact cells. These studies demonstrate that four polypeptides whose phosphorylation is stimulated by the tumor promoter 12-O-tetradecanoyl phorbol 13-acetate in myotubes, are spontaneously phosphorylated in control myoblasts. Phosphoinositide turnover under basal conditions in [^{3}H]inositol-labeled cells is faster in myoblasts than in myotubes, a finding that may in part explain the different distribution of PKc observed during the course of myogenic differentiation.

CALCIUM-, phospholipid-dependent protein kinase (PKc)[1] is a widely distributed enzyme that plays a central role in the regulatory system based on phosphoinositide hydrolysis (36). PKc activity has been shown to be modified in response to a variety of factors such as hormones, neurotransmitters, growth factors, and tumor promoters, and mounting evidence suggests that this enzyme mediates (many of) the cellular responses to such factors (for reviews see 6, 8). PKc, an amphitropic protein, appears to be subjected to a complex posttranslational regulation, translocating between membrane and cytoplasm according to the local concentrations of calcium, phospholipids, and diacylglycerols, and it is thought that the enzyme is physiologically active only in the membrane-associated state (5, 12, 32). Acute and transient translocation of PKc from the cytoplasmic to the membrane compartment can be induced with appropriate stimuli in different experimental systems (22, 30) and spontaneous modifications of PKc subcellular distribution have been recently described to be related to modifications of cell proliferative activity (3).

Muscle is one of the tissues in which specific roles of PKc have been identified or hypothesized on the basis of strong evidence. Several muscle proteins appear to be possible physiological substrates of PKc, including phosphorylase kinase (36, 39), phospholamban and other cardiac sarcolemma proteins (26, 33), myosin light chain (35), and subunits of the acetylcholine receptor (34). Furthermore, the dramatic effects exerted upon muscle differentiation and expression of the differentiated phenotype by the tumor promoter 12-O-tetradecanoyl phorbol 13-acetate (TPA), a powerful PKc activator, suggest an important role for this enzyme during myogenesis (15, 17, 21, 41). We have therefore analyzed the changes in PKc activity and in its subcellular distribution during myogenesis in culture. Our data demonstrate that the activity of this enzyme varies dramatically as replicating presumptive myoblasts fuse and form mature postmitotic myotubes.

1. *Abbreviations used in this paper*: EE, chick embryo extract; HS, horse serum; IP1, inositol monophosphate; PI, phosphatidylinositol; PKa, cAMP-dependent protein kinase; PKc, calcium-, phospholipid-dependent protein kinase.

Materials and Methods

Cell Cultures

Chick embryo myogenic cells were prepared and cultured in MEM (Hazleton Research Products, Denver, PA) supplemented with 10% horse serum (HS) and 3% chick embryo extract (EE), as previously described (1, 17). Cultures consisting of myogenic cells, virtually devoid of contaminating fibroblasts, were obtained using a double 10-min preplating of the embryonic muscle cell suspension (1). Furthermore, cultures to be assayed on days 4–6 were treated with cytosine arabinoside (2.5 μg/ml) from the 48th to the 24th hour preceding each experimental point (17). Myogenic cell differentiation was monitored to evaluate the percentage of nuclei present in multinucleated myotubes in Wright's stained dishes, as previously described (1).

Protein Kinase Activities

At the times indicated, the cultures were rinsed extensively with PBS, scraped, and collected by low speed centrifugation. Homogenization and protein kinase assay methods (2), mainly based on those of Kikkawa et al. (27), were slightly modified in order to assay both the PKc and the cAMP-dependent protein kinase (PKa). Cell pellets were suspended in 5–10 vol ice-cold H-buffer (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 50 mM 2-mercaptoethanol, 2 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin) and rapidly Dounce homogenized (40 strokes). An aliquot of the homogenate was diluted with 30 vol of buffer A (50 mM phosphate buffer, pH 6.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 20 mM NaF, 10 mM theophylline) and assayed for PKa activity in the presence of 1 mg/ml salmon protamine, 10 μM γ[^{32}P]ATP (sp act, 28–33 Ci/mmol, New England Nuclear, Boston, MA), 5 μCi/ml, ± 2 μM cAMP (16).

To determine PKc activity of total cell extracts (total activity), an aliquot of the homogenate was diluted with 8 vol H-buffer supplemented with 2 mM EDTA, 10 mM EGTA, and 0.5% sodium deoxycholate (final concentrations), and extracted for 30 min at 0°C with continuous stirring.

A third aliquot of the homogenate was centrifuged at 100,000 *g* for 1 h. The resulting 100,000 *g* pellet (particulate fraction) was extracted and solubilized for 30 min at 0°C with continuous stirring in H-buffer containing 2 mM EDTA, 10 mM EGTA, and 0.5% sodium deoxycholate. Before assaying the 100,000 *g* supernatant (soluble fraction) EDTA, EGTA, and deoxycholate were added to the final concentrations indicated above.

PKc activity was measured as previously described (3). The activity measured in the presence of Ca^{++} alone (without phosphatidylserine and diolein) was subtracted as nonspecific (3). Protein content of cell extracts was determined as described (7) using BSA as a standard.

Phosphorylation Studies

Myoblasts (24 h of culture) and myotubes (96 h of culture) were washed extensively with Hepes-buffered, phosphate-free saline and incubated for 45 min with the same saline supplemented with 0.5 mg/ml BSA and 2.5 mCi/ml carrier-free ^{32}P-orthophosphoric acid ($^{32}P_i$; New England Nuclear) (23). TPA (0.1 μM, 0.1% ethanol) or 0.1% ethanol were added during the last 15 min of incubation. The cultures were then transferred on ice, rapidly washed three times with ice-cold PBS, scraped in PBS containing 2 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 mM NaF, and the cells were sedimented by centrifugation. The cell pellet was dissolved in Laemmli sample buffer (31), incubated at 100°C for 3 min, and this material was subjected to SDS-PAGE and autoradiographed as previously described (10, 11), except that a minigel apparatus (Elekon Co., Chiba, Japan) was used. Molecular mass markers were obtained from Sigma Chemical Co. (St. Louis, MO). Densitometric analysis of the autoradiograms was performed using a computer-interfaced Ultroscan XL laser densitometer (LKB Produkter AB, Bromma, Sweden).

Phosphoinositides Hydrolysis and Labeling Studies

24-h mononucleated myoblasts and 96-h myotubes, growing in 60-mm dishes, were labeled with [^{3}H]inositol (sp act, 16.5 Ci/mmol; New England Nuclear), 5 μCi/ml for the last 22 h before experiments. The cultures were extensively washed (five times at 37°C over a period of 90 min) with basal medium (without HS and EE) or with complete medium (with 10% HS and 3% EE). The cultures then received 2 ml/dish of either basal or complete medium (preequilibrated with respect to temperature and CO_2) with or without 20 mM LiCl, and the incubation proceeded for the time periods indicated in Fig. 4. The incubations were arrested at the appropriate times by rapidly removing the medium, transferring the cultures at 0°C, and pipetting 1 ml of ice-cold 10% TCA into each dish. Acid extraction and ion exchange chromatography of the inositol phosphates were performed as described in a previous paper (2).

The time course of [^{3}H]inositol labeling of phosphatidylinositol (PI) was investigated incubating myoblasts and myotubes with the labeled precursor (see above) for different time periods ending at 24 and 96 h of culture. At the end of the incubation the cultures were extensively rinsed with saline, precipitated with 10% TCA, and repeatedly extracted with 5% TCA, 1 mM EDTA. The phospholipids were extracted from the acid insoluble residue as described by Creba et al. (20). The lipid extracts were applied to oxalate-impregnated silica gel G thin layer plates (Whatman Inc., Clifton, NJ) and the chromatography was developed with chloroform/methanol/4 N NH_4OH (45:35:10) (9). Standard PI, PI-4-monophosphate, and PI-4,5-bisphosphate (Sigma Chemical Co.) were applied to TLC plates along with the experimental samples. After development, the plates were air dried and exposed to iodine vapors to visualize the standards. The plates were then scraped in 0.5-cm sections and the radioactivity was measured by liquid scintilometry.

Results

Protein Kinase Activities

PKc activity was measured in total cell extracts of chick embryo myogenic cells at different days of culture. As shown in Fig. 1, total PKc activity declines as the mononucleated cells withdraw from the cell cycle and fuse to form myotubes. The specific activity of PKc at day 1, when fusion is minimal, is approximately twice that at day 6, when fusion is >80%. The decline of total PKc specific activity associated with myogenic maturation occurs at an approximately constant rate. It does not seem to be related to the rate of fusion, which is maximal during day 2 and slower at other culture times. A different protein kinase activity, PKa, follows a different pattern in the same cultures. The activity of PKa is maximal when the rate of fusion is high, declines when the rate of fusion decreases during the third day of culture, and remains approximately constant during the last three days of culture (Fig. 1).

Separate evaluation of the soluble and particulate PKc activities reveals that the decline of total PKc activity may be resolved into a more complex phenomenon, as shown in Fig. 2. In fact, in mononucleated myoblasts, the specific activity of the particulate fraction-associated PKc is ninefold higher than that of the soluble fraction-associated PKc. The specific activity of particulate PKc dramatically decreases during myogenic maturation reaching, by day 6 of culture, a level of ~5% the initial value. Conversely, soluble PKc activity shows a biphasic pattern, increasing greatly as the particulate

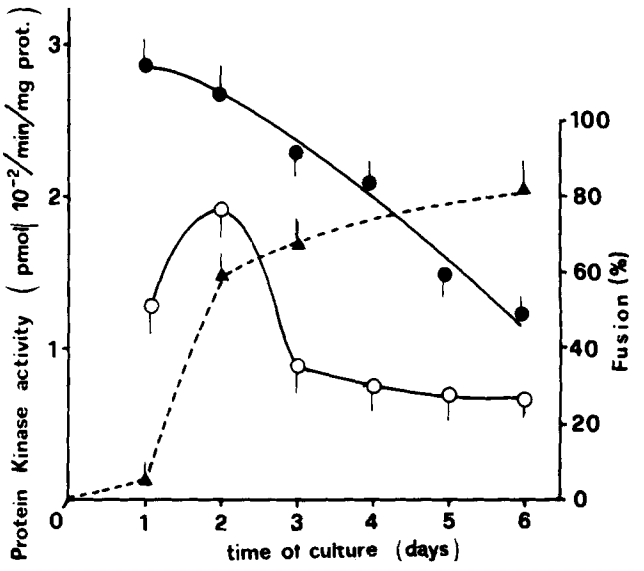

Figure 1. Protein kinase activities of total cell extracts of differentiating muscle cells. The activities of PKc and PKa and the percent of fusion were determined at the times indicated as described in Materials and Methods. (●) Diolein-stimulated PKc activity; (○) cAMP-stimulated PKa activity; (▲) percent of fusion. *prot.*, protein.

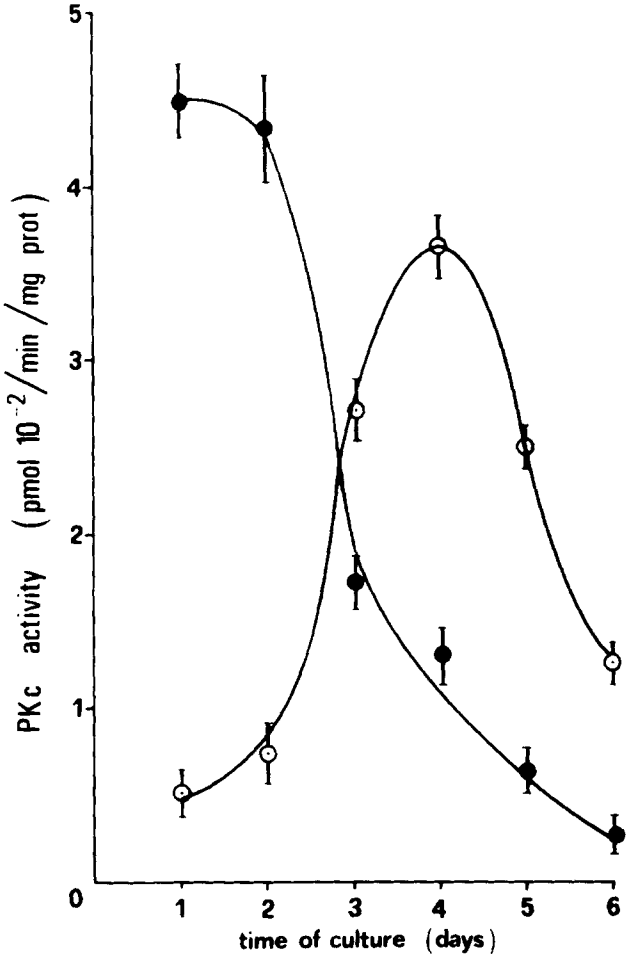

Figure 2. Subcellular distribution of PKc activity during myogenic cell differentiation. Particulate and soluble fractions were prepared as described under Materials and Methods. (●) Diolein-stimulated particulate PKc activity; (○) diolein-stimulated soluble PKc activity.

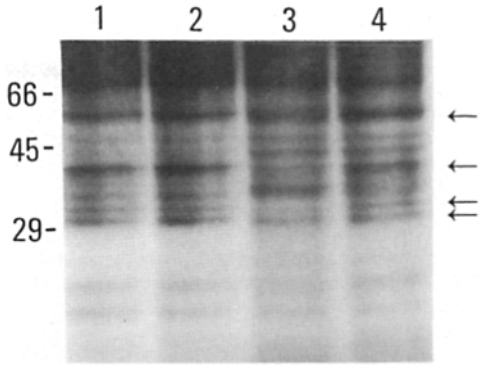

Figure 3. Effect of TPA on the phosphorylation of endogenous substrates in myogenic cells. Autoradiogram of SDS-PAGE separation of samples of control and TPA-treated 24-h myoblasts (lanes *1* and *2*) and of control and TPA-treated 96-h myotubes (lanes *3* and *4*). The numbers on the left refer to the molecular mass standards; the arrows on the right point to the position of the four polypeptides whose phosphorylation is stimulated by TPA in myotubes. Details of $^{32}P_i$ labeling, TPA treatment, SDS-PAGE, and autoradiography are in Materials and Methods.

enzyme decreases during the first 4 d of culture, but declining afterwards (Fig. 2).

Protein Phosphorylation

We analyzed the phosphorylating activity associated with PKc in intact myoblasts and myotubes. 24-h myoblasts and 96-h myotubes were labeled with $^{32}P_i$ and treated with TPA to stimulate PKc. The results (Figs. 3 and 4) indicate that the phosphorylation of four polypeptides (apparent molecular masses, 30, 32, 38, and 59 kD) is stimulated by TPA in myotubes. The same polypeptides are fully phosphorylated in control myoblasts, and TPA treatment of myoblasts does not significantly modify their level of phosphorylation. It is worth noting that TPA treatment of myotubes causes decreased phosphorylation of a polypeptide of 35 kD, a phenomenon not occurring in myoblasts.

Phosphoinositide Hydrolysis

The amount of inositol phosphates was measured in 24-h myoblasts and 96-h myotubes in order to obtain an estimate of the basal level of phosphoinositide hydrolysis in these cells. Since a labeling period longer than 24 h could not be used, the time course of [^{3}H]inositol labeling of both myoblast and myotube phosphoinositides was determined as detailed in Materials and Methods. PI labeling (the mono- and bis-phosphate derivatives of PI accounting for <8% of total phosphoinositide radioactivity throughout the experimental period) follows a pattern slightly different in myoblasts from that in myotubes, but in both cases a condition of isotopic equilibrium appears to be reached with a labeling time of 22 h (Fig. 5 *A*). Wakelam (40) has previously reported an equal equilibrium labeling time for [^{3}H]inositol-labeled phospholipids in fusion-competent myoblasts.

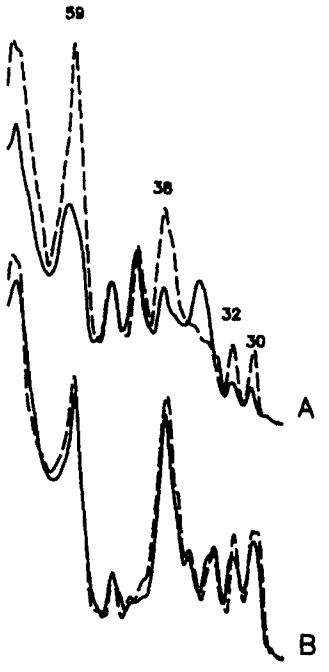

Figure 4. Densitometric scanning of the 66–29-kD region of the autoradiogram shown in Fig. 3. (*A*) 96-h myotubes; (*B*) 24-h myoblasts. (———) control cells. (– – –) TPA-treated cells. The numbers indicate the apparent molecular mass (kD) of the polypeptides whose phosphorylation is stimulated by TPA in myotubes. Each lane of the gel was scanned three times along parallel lines and the three sets of data for each lane were averaged.

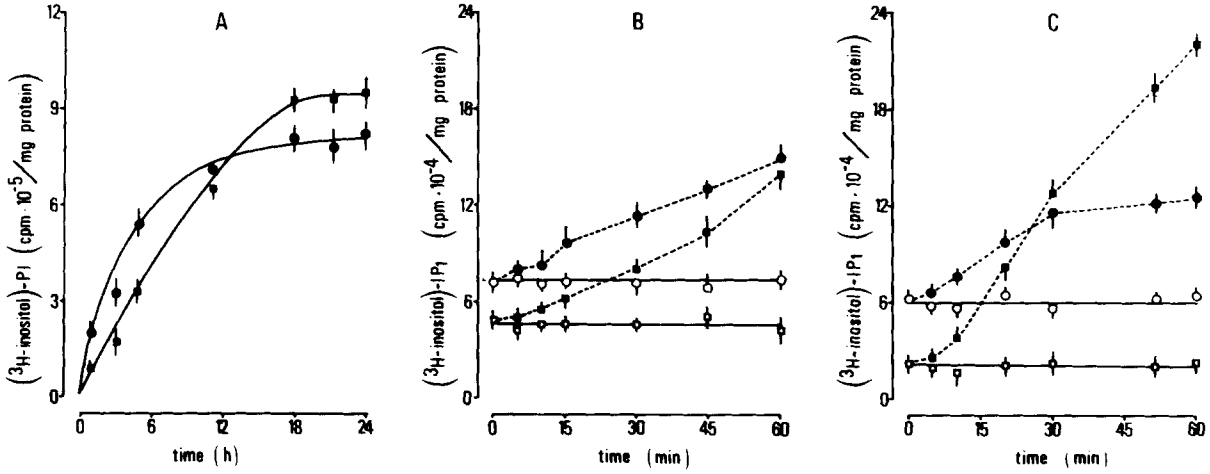

Figure 5. Phosphoinositides turnover of myogenic cells. (*A*) Time course of [^{3}H]inositol labeling of myoblast (●) and myotube (■) PI. (*B* and *C*) [^{3}H]inositol IP1 formed in myoblasts (○, ●) and myotubes (□, ■) incubated from 0 time with (solid symbols) or without (empty symbols) 20 mM LiCl. After the end of the labeling period the cells were maintained with (*B*) or without (*C*) HS and EE (see Materials and Methods).

The measurements of the basal level of phosphoinositide hydrolysis were conducted with cells maintained in the presence of HS and EE during the 90-min washing period and the incubation period (Fig. 5 *B*) as well as with cultures washed and incubated with basal medium alone (Fig. 5 *C*). In both cases, in the absence of lithium (an inhibitor of inositol monophosphate [IP1] phosphatase) the level of IP1 production is steadily higher in myoblasts than in myotubes throughout the 60-min incubation period. Also, in the presence of lithium the level of IP1 is higher in myoblasts than in myotubes during the first part of the incubation (e.g., during the first 45 min for the cells incubated in complete medium and during the first 20 min for the cells incubated in medium without HS and EE). However, the level of IP1 in myotubes approaches that of myoblasts by the end of the incubation time (Fig. 5 *B*) or overrides that of myoblasts after 30 min (Fig. 5 *C*) depending upon the presence or absence of HS and EE. At present, we are unable to explain such phenomenon. The levels of inositol bisphosphate and inositol trisphosphate (not shown for graphical clarity) represent respectively 9–13 and 1.5–3% of the values of IP1 obtained at each timepoint in the absence of lithium for both myoblasts and myotubes.

Discussion

Our data on the activity of PKc in total extracts of myogenic cells undergoing maturation in culture (Fig. 1) indicates that a relatively constant decline of total PKc activity occurs during myogenesis. No direct correlation can be established between the rate of decline of total PKc activity and the rate of fusion. Conversely, the activity of a different protein kinase (PKa; measured in the same cell preparations and thus serving as an internal control) shows a significant increase around the period of maximal rate of cell fusion, as already described by Zalin and Montague (44).

In contrast to the described pattern of total PKc activity, separate evaluation of cytosolic and particulate PKc activity of differentiating myogenic cells (Fig. 2) indicates that a dramatic decline of particulate PKc activity occurs after the burst of cell fusion and that an increase of soluble PKc activity (during the same period) is followed by a decline during days 5 and 6. The ratio of particulate to soluble PKc specific activity favors the particulate form during the first two days of culture, and is inverted between days 3 and 6, when the specific activity of soluble PKc is higher than that of the particulate fraction-associated enzyme. This result is consistent with previous data from this laboratory indicating the PKc is predominantly cytosolic in mouse cultured myotubes (19). It should be considered that, because of the particular phospholipid requirements of PKc for its activity, only the membrane-bound PKc should express the actual enzyme activity in the intact cell (14, 27, 30). On the other hand, soluble PKc activity may be regarded as a potential form of enzyme activity, which can be recruited to the membrane compartment upon appropriate stimulation of the cell (14, 22, 23, 30, 32, 38). Therefore, it seems, on the basis of our observations, that the physiological activity of PKc, as expressed by the particulate activity, undergoes a dramatic reduction along with myogenic cell maturation and/or exit from the cell cycle. Such reduction is much more pronounced than anticipated from only measuring the total activity.

We have recently shown that cell proliferation is associated with a high level of PKc, while this enzyme activity is strongly reduced in mitotically quiescent cells (3). This reduction is mainly due to a decrease of the particulate fraction-associated PKc. Since myogenic cells become irreversibly postmitotic before fusion, the observed decline of PKc activity could be related to such phenomenon. However, (*a*) a strong correlation exists between exit from the cell cycle and the terminal differentiation of myogenic cells, as well as of other types of cells; and (*b*) we have recently shown that a correlation exists between cell differentiation and decline of (particulate) PKc in cultured rat Sertoli cells in the absence of any change in the mitotic activity (24). These considerations suggest that the observed decline of (particulate) PKc activity occurring in myogenic cells may be more strictly related to myogenic cell maturation than to the exit of myoblasts from the cell cycle. In fact, our data show that the activity of particulate PKc is still high at day 2, when the

majority of myogenic cells have become postmitotic and have fused. The rapid decline of particulate PKc occurring during the 3rd and 4th day of culture may thus be related with the progressive myogenic maturation.

Another intriguing question is posed by the marked decline of particulate and the rise of soluble PKc specific activities during the first 4 d of culture (Fig. 2). It can be hypothesized that this results from detachment of PKc from the membrane compartment and its recovery in the soluble fraction of the cell. Only at later times does a decline of soluble PKc become apparent, which is a finding that may be ascribed (*a*) to a reduction in the net synthesis of the enzyme, (*b*) to specific inhibition of its activity, or (*c*) to the accumulation of contractile proteins (25, 43).

To substantiate further our findings on the differential subcellular distribution of PKc and on the basis of the preceding considerations, we analyzed the phosphorylating activity attributable to PKc in intact myoblasts and myotubes. With regard to the specific interaction between TPA and PKc, protein phosphorylation stimulated by TPA may be considered as the result of PKc activity (14, 28, 29, 36, 37). As shown in Figs. 3 and 4, TPA treatment of intact 96-h myotubes enhances the phosphorylation of four polypeptides. This suggests that (*a*) such polypeptides are substrates of PKc activity; (*b*) PKc is not maximally active in control myotubes since its activity may be increased by TPA treatment of the cells; and (*c*) those observations are consistent with the observation that PKc is predominantly cytosolic in myotubes and with the hypothesis that cytosolic PKc activity represents a "potential" form of enzyme activity. Conversely, the same four substrates of PKc phosphorylation appear to be fully phosphorylated in control myoblasts since either slight or no increase of their phosphorylation is induced by TPA; this is consistent with the observation that PKc is predominantly membrane bound ("actually" active) in myoblasts.

Several factors could be responsible for the differential distribution of PKc activity during myogenesis. Among these, a reduced calcium level, a reduction of the phosphatidylserine/phosphatidylcholine ratio in the membrane, and a reduced level of basal diacylglycerol production would constitute conditions favoring a shift of the equilibrium between membrane-associated and soluble PKc towards the soluble compartment. Previous results from our group reported elsewhere (4, 13, 18, 23) indicate that incubation of myogenic and other cell types with phosphatidylcholine-containing liposomes induces translocation of membrane-bound PKc to the soluble compartment and reduces or abolishes biological responses to TPA. Conversely, incubation of cells with phosphatidylserine-containing liposomes induces translocation of soluble PKc to the membrane compartment and enhances cell responsiveness to TPA.

We have evaluated the basal level of phosphoinositide turnover in both mononucleated myoblasts and myotubes. Since diacylglycerol is produced in equimolar amounts with inositol phosphates by the action of phospholipase C upon phosphoinositides, we measured the level of production of inositol phosphates under basal (unstimulated) conditions in both myoblasts and myotubes. It is important to note that our basal conditions refer to cells cultured in the presence of both HS and EE and therefore exposed to a variety of stimulatory agents; however, the medium composition is the same for both myoblasts and myotubes. The results obtained (Fig. 5) clearly indicate that basal hydrolysis of inositol phospholipids is substantially higher in myoblasts than in myotubes. This finding positively correlates with the observed subcellular distribution of PKc in these two types of myogenic cells. It is possible to speculate that a progressive reduction in the basal level of phosphoinositides hydrolysis, possibly accompanied by changes in the phospholipid composition of the membrane, may account for the progressive reduction of particulate PKc activity observed during myogenic cell maturation.

Rapid and specific breakdown of phosphoinositides, with production of phosphatidic acid and 1,2-diacylglycerol, has been shown by Wakelam (40–42) to accompany calcium-induced fusion of fusion-competent myoblasts. While it is difficult to compare those results with ours in terms of phosphoinositides hydrolysis (since neither the 24-h myoblasts nor the 96-h myotubes we have used are comparable with the calcium-stimulated, fusion-competent myoblasts used by Wakelam [40–42]) a causal link can be hypothesized between the fusion related burst of phosphoinositides hydrolysis and the postfusion modifications of PKc activity here described.

The developmental modifications of PKc activity reported in this paper do not contrast with recent observations from our laboratory that suggest PKc is involved in the regulation of the acetylcholine receptor in differentiated myogenic cells (2, 23), nor do they contrast with the reported specific functions of PKc in muscle (26, 33–35, 39). It is possible to speculate that PKc activity, while decreasing during myogenic maturation, specializes in terms of substrates and functions, maintaining a quantitatively reduced, but physiologically unique, role in terminally differentiating muscle cells.

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