Regulation of Phospholipase D in L6 Skeletal Muscle Myoblasts

ROLE OF PROTEIN KINASE C AND RELATIONSHIP TO PROTEIN SYNTHESIS*

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The addition of vasopressin or 12-O-tetradecanoylphorbol-13-acetate (TPA) to prelabeled L6 myoblasts elicited increases in [14C]ethanolamine release, suggesting the activation of phospholipase D activity or activities. While the effects of both agonists on intracellular release were rapid and transient, when extracellular release of [14C]ethanolamine was measured, the effect of vasopressin was again rapid and transient, whereas that of TPA was delayed but sustained. Effects of both agonists on intra- and extracellular release were inhibited by the protein kinase C (PKC) inhibitor, Ro-31-8220, and PKC down-regulation by preincubation with TPA. The formation of phosphatidylbutanol elicited by vasopressin and TPA mirrored their effects on extracellular [14C]ethanolamine release in that the former was transient, whereas the latter was sustained. Responses to both agonists were abolished by PKC down-regulation. When protein synthesis was examined, the stimulation of translation by TPA and transcription by vasopressin were inhibited by Ro-31-8220. In contrast, down-regulation of PKC inhibited the synthesis response to TPA but not vasopressin. Furthermore, following down-regulation, the effect of vasopressin was still blocked by the PKC inhibitors, Ro-31-8220 and bisindolylmaleimide. Analysis of PKC isoforms in L6 cells showed the presence of α, ε, δ, µ, τ, and ζ. Down-regulation removed both cytosolic (α) and membrane-bound (ε and δ) isoforms. Thus, the elevation of phospholipase D activity or activities induced by both TPA and vasopressin and the stimulation of translation by TPA involves PKC-α, ε, and/or δ. In contrast, the increase in transcription elicited by vasopressin involves µ, τ, and/or ζ. Hence, although phospholipase D may be linked to increases in transcription elicited by TPA, it is not involved in the stimulation of transcription by vasopressin.

Loss of skeletal muscle is an acute metabolic response to infection and neoplastic disease and results from a decrease in the rate of protein synthesis and an increase in the rate of protein degradation (e.g. see Refs. 1–3). To reverse this process, an understanding of the signaling pathways regulating protein turnover is essential.

We have used 12-O-tetradecanoylphorbol-13-acetate (TPA)1

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1The abbreviations used are: TPA, 12-O-tetradecanoylphorbol 13-acetate; PLD, phospholipase D; PA, phosphatidic acid; PtdBuOH, phosphatidylbutanol; PE, phosphatidylethanolamine; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; ANOVA, analysis of variance; bIM, bisindolylmaleimide.
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EXPERIMENTAL PROCEDURES

Materials—(2-14C)Ethanolamine (specific activity 54 Ci/mmol), [methyl-3H](choline chloride (specific activity 84 Ci/mmol), [9,10-3H]oleic acid (specific activity 10 Ci/mmol), Calbiochem (Beeston, UK). H-phospholipase (specific activity 87 Ci/mmol) and Hyperfilm MP were from Amersham International (Amersham, Bucks, UK). Phenylmethylsulfonyl fluoride, leupeptin, TPA, and vasopressin were obtained from Sigma (Poole, UK).

All materials for tissue culture were purchased from Life Technologies Ltd. (Paisley, UK), and EN3HANCE spray was from DuPont NEN (Stevenage, UK). Silica gel K6 thin layer chromatography plates were obtained from Whatman (Maidstone, UK), and scintillation fluid (Ultima Gold XR) was from Packard (Paisford, UK). Molecular weight markers for electrophoresis were from BDH (Lutterworth, UK). The PKC inhibitors, Ro-31-8220 and bisindolylmaleimide (GF 109203X), and an antibody to PKC-γ (pre pared against a peptide corresponding to residues 681–697 of human PKC-γ and which reacts with this isoform from rodent tissues) were obtained from Calbiochem (Beeston, UK). In all experiments, Ro-31-8220 and bisindolylmaleimide were used at 1 μM concentration within the range shown to inhibit effects mediated by PKC-γ (18, 19). Monoclonal antibodies to PKC-α, -β, -δ, -ε, -θ, -η, -μ, and -ζ, which react with these isoforms from rodents, were obtained from Affiniti Research Products Ltd. (Nottingham, UK) and used as per suppliers’ instructions. These antibodies have been widely used in similar studies (e.g., 20–22).

Cell Culture—In all experiments, L6 cells (PHLS, Porton Down, UK) were plated into 60-mm Petri dishes (Greiner, UK) in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 12% fetal calf serum and grown to approximately 75% confluence.

Determination of Intracellular [14C]Ethanolamine—Prior to each experiment, cells were transferred to 2 ml of serum-free DMEM containing 2-14C]ethanolamine (0.1 μCi/ml) for 24 h. At the end of this labeling period, the cells were washed and incubated in fresh DMEM containing 5 mM unlabeled ethanolamine for an additional 2 h. After a further wash with DMEM, the cells were then incubated with or without agonists in the presence of 1 mM unlabeled ethanolamine and phosphoethanolamine. At the end of the incubation, the total medium was removed from the dishes, and to 0.8 ml of this, 3 ml of ice-cold chloroform/methanol (1:2, v/v) were added. After 30 min on ice, phase separation was achieved with chloroform (1 ml) and water (1 ml). The upper (water/methanol) phase was then removed. Extracellular metabolites were separated (23) and subjected to scintillation counting. In addition, increases in [14C]ethanolamine were confirmed by thin layer chromatography (TLC) as described below for intracellular metabolites.

To analyze intracellular metabolites, following removal of the medium, each dish was washed three times with 1 ml of phosphate-buffered saline and 1 ml of ice-cold methanol was added. The cells were scraped and washed into glass tubes with a further 1 ml of methanol. Chloroform (1 ml) and 12 mM HCl (20 μl) were added to each tube. The contents were mixed vigorously and extracted on ice for 30 min. Phase separation was achieved with chloroform (1.25 ml) and water (1.25 ml). The upper phase was then removed, and the intracellular metabolites were separated (23) and subjected to scintillation counting. Increases in [14C]ethanolamine were confirmed by TLC. Briefly, samples were concentrated by rotary evaporation, resuspended in ethanol/water (1:1, v/v), dried at 60 °C under nitrogen, and separated by TLC on Silica Gel K6 plates with water/methanol/concentrated acetic acid (2:6:1, v/v/v). Fractions comigrating with ethanolamine standard (Rf 0.53) were scraped into scintillation vials and counted.

Measurement of Extracellular [3H]Choline Release and [3H]PtdBuOH Formation—Cells were plated on dishes with either [3H]choline or [3H]oleic acid, and metabolites were determined as described previously (4).

Measurement of Intracellular PKC—Cells were incubated with 1 μM TPA for 24 h prior to use. Determination of the PKC isoforms removed by this protocol was performed as described below.

Western Blot Analysis of PKC Isoforms—To obtain cytosolic and particulate fractions, L6 cells were washed three times with phosphate-buffered saline and scraped from the dish with 2 × 100 μl of Buffer A (20 mM Tris, pH 7.5, 0.25 mM sucrose, 10 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin) (24). The cells were then lysed by six passages through a 25-gauge needle, and cytosolic fractions were obtained by centrifugation at 100,000 × g for 60 min. Membrane pellets were resuspended in the same volume of Buffer A supplemented with 1% Triton X-100 and incubated on ice for 60 min with gentle agitation. The supernatant following centrifugation at 100,000 × g for 60 min was taken as the particulate fraction. To obtain total cell PKC content, 1% Triton X-100 was included in Buffer A when the cells were lysed, and the extract was incubated on ice for 60 min prior to centrifugation as above. The supernatant was taken as total cell PKC. Protein content was determined by the method of Bradford (25). Samples were stored, without boiling, at 70 °C in SDS-polyacrylamide gel electrophoresis sample buffer (5% [w/v] SDS, 13% [w/v] glycerol, 60 mM Tris-HCl, 0.2% bromphenol blue, and 5% mercaptoethanol, pH 6.8). Equal amounts (15 μg) of cytosolic or solubilized membrane protein were subjected to SDS-polyacrylamide gel electrophoresis (10% gels), and Western blot analysis was performed with a Mini Deca-Probe (26).

Measurement of Protein Synthesis—Following 24 h in serum-free DMEM, protein synthesis was measured by the addition of L-[2,6-3H]phenylalanine for the final 60 min of incubations lasting 90 min or 6 h. Subsequent treatment to determine the specific radioactivity of protein-bound phenylalanine was as described previously (27).

Data Presentation—All lipid experiments were done with n = 3 and those for protein synthesis with n = 5. Each assay was performed at least twice under identical conditions. In studies designed to assess the effect of inhibitors, experiments were combined and analyzed by an analysis of variance (ANOVA) using set as a blocking factor with the Genstat 5 version 3.1 statistics package to determine differences between control and agonist-treated samples. Then, by fitting a contrast using each experimental group, ([agonist with inhibitor] – inhibitor) – (agonist – control), it was possible to determine the effect of an inhibitor on any stimulated event. Where ANOVA showed no difference between experiments, the combined data are shown. Results are expressed as means ± S.E., and significance was assessed by Student’s t test.

RESULTS

Stimulation of [14C]Ethanolamine Release by Phorbol Esters—Incubation of L6 myoblasts with TPA stimulated the release of [14C]ethanolamine into the medium in a concentration (EC50 ~ 10 nM; Fig. 1A) and time-dependent manner. The effect of 100 nM TPA was detected after 15 min (Fig. 2A) and continued for at least 2 h; after 1 h, the release of [14C]ethanolamine had increased from 810 ± 45 to 1555 ± 90 dpm/fraction, and after 2 h, the release had increased from 1470 ± 115 to 2715 ± 160 dpm/fraction (combined data from two experiments, n = 6; p < 0.001 for both time points). The PKC inhibitor, Ro-31-8220 (1 μM) significantly inhibited (75%; p < 0.001) the effect of 100 nM TPA following a 30-min incubation (Fig. 3A), and the response was completely abolished in PKC-down-regulated cells (Fig. 4A). The inactive phorbol ester, 4a-phorbol 12,13-didecanoate (100 μM), had no effect (e.g. 525 ± 10 versus 510 ± 15 dpm/fraction, n = 3, after a 30-min incubation). TPA also stimulated a transient, concentration-dependent (EC50 ~ 10 nM; data not shown) increase in intracellular [14C]ethanolamine. This response was detected within 3 min (the earliest time point examined) and had returned to control values by 20 min (Fig. 2B). The PKC inhibitor (Fig. 3C) and PKC down-regulation (Fig. 4C) blocked intracellular [14C]ethanolamine release in response to TPA.

Stimulation of [14C]Ethanolamine Release by Vasopressin—In contrast to TPA, vasopressin stimulated a transient, concentration-dependent release of [14C]ethanolamine into the extracellular medium (EC50 ~ 5 nM; Fig. 1B) over 10–15 min, by which time both basal and vasopressin-induced tracer values had returned parallel (Fig. 2C). The PKC inhibitor (Fig. 3B) and PKC down-regulation (Fig. 4B) completely prevented the effect of 100 nM vasopressin after 15 min. Like TPA, vasopressin also elicited a transient, concentration-dependent (EC50 ~ 5 nM; data not shown) increase in intracellular [14C]ethanolamine. This response was maximal at 3–6 min and had returned to control values by 20 min (Fig. 2D). It was partially attenuated (~70%) by the PKC inhibitor (Fig. 3D) and completely inhibited by...
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Stimulation of [3H]Choline Release and [3H]PtdBuOH Formation by TPA and Vasopressin—In an earlier study, we demonstrated that vasopressin increased [3H]choline release and [3H]PtdBuOH formation in L6 cells (4) over a similar time course to that reported here for [14C]ethanolamine. Although we have previously shown that TPA also stimulates extracellular [3H]choline release in these cells in a similar manner to that reported in this study for extracellular [14C]ethanolamine (4), we had not investigated the time course of its effects on [3H]PtdBuOH formation in detail. Initially, we examined the effect of TPA using an identical methodology to that employed for vasopressin, i.e. butanol and TPA were added at time 0. Under these conditions, TPA elicited [3H]PtdBuOH production within 3 min, the earliest time point examined, and that at which increased intracellular release of [14C]ethanolamine was also detected. However, like vasopressin (4), the formation of [3H]PtdBuOH in response to TPA was transient and had reached a plateau within 15 min (data not shown). This observation appeared to be in conflict with the effect of TPA on [3H]choline (4) and extracellular [14C]ethanolamine release, which suggested that the effect of TPA on PLD was sustained. To investigate this discrepancy, we considered the possibility that with the current approach, the inability to demonstrate sustained PLD activity in response to TPA might be due to insufficient butanol at the later time points. To examine this potential explanation, we added butanol for 20 min either at time 0 with and without agonists or at increasing times after agonist treatment. Using this approach, both TPA (14,124 ± 1342, n = 3; p < 0.001) and vasopressin (10382 ± 753, n = 3; p,0.001) significantly increased [3H]PtdBuOH formation compared with control (2231 ± 345) when butanol was added at time 0. However, when butanol was added 40 min after the agonists, a different picture emerged. TPA (10,314 ± 1119, n = 3; p < 0.001), but not vasopressin (2974 ± 603, n = 3) significantly increased [3H]PtdBuOH production when compared with control (2182 ± 489, n = 3). Similar results were also obtained when butanol was added 100 min after agonists (data not shown). The data clearly demonstrate that the effect of vasopressin on [3H]PtdBuOH formation was transient, whereas that of TPA was sustained. Down-regulation prior to the addition of either agonist completely abolished effects on [3H]PtdBuOH formation in an identical manner to that observed for [14C]ethanolamine release. For example, in the presence of TPA, [3H]PtdBuOH production increased from 112 ± 7 to 1506 ± 31 dpm/10^6 total [3H]dpm (n = 6, p < 0.001) after a 30-min incubation, but no change was observed in down-regulated cells (109 ± 4 versus 108 ± 6). Similarly, for vasopressin, [3H]PtdBuOH increased from 115 ± 6 to 1045 ± 19 dpm/10^6 total [3H]dpm (n = 6, p < 0.001) after a 15-min incubation, but again, no effect was seen in down-regulated cells (109 ± 7 versus 110 ± 4). Qualitatively identical results were also obtained for [3H]choline release (data not shown).

Stimulation of Protein Synthesis by TPA and Vasopressin—We have previously demonstrated that TPA stimulates protein synthesis at 90 min but not at 6 h, whereas vasopressin has effects at 6 h but not at 90 min in L6 cells (4). The prolonged

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**Fig. 1.** Concentration-dependent stimulation of extracellular ethanolamine release from L6 myoblasts by TPA and vasopressin. [14C]Ethanolamine-prelabeled cells were treated with increasing concentrations of either 100 nM TPA (A) for 30 min or 100 nM vasopressin (B) for 15 min. The medium was removed, and extracellular metabolites were extracted and separated. Each point represents the mean ± S.E. (n = 6) from two experiments performed in triplicate. By Student’s t test, values significantly different from control are labeled as follows. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Fig. 2.** Time course of TPA- and vasopressin-induced ethanolamine release in L6 myoblasts. Cells, prelabeled with [14C]ethanolamine, were treated with either TPA (A and B) or vasopressin (C and D) for the times shown. The [14C]ethanolamine-containing metabolites released into extracellular (A and C) and intracellular (B and D) fractions were determined by extracting the medium and cells separately. Each point represents the mean ± S.E. (n = 6) from two experiments performed in triplicate. ○, control; ●, agonist (100 nM). By Student’s t test, values significantly different from control are labeled as follows. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
(24-h) incubation of L6 myoblasts with 1 μM TPA to down-regulate PKC abolished the ability of TPA to increase protein synthesis (Fig. 5A), but in complete contrast, the response to vasopressin was unaffected (Fig. 5B). TPA also failed to stimulate protein synthesis in the presence of the PKC inhibitor (Fig. 5C), and, unlike down-regulation of PKC, this inhibitor also partially attenuated (~60%) the response to vasopressin at 1 μM (Fig. 5D). To confirm the involvement of PKC in the stimulation of transcription by vasopressin, an additional inhibitor (bisindolylmaleimide; bIM) was used. Like Ro-31-8220, bIM partially blocked the effect (~50%). Vasopressin increased protein synthesis from 12.1 ± 0.3 to 15.8 ± 0.3 dpm/μg protein (n = 10 from two combined experiments, p < 0.001) in the absence of 1 μM bIM and from 10.5 ± 0.3 to 12.2 ± 0.2 dpm/μg protein (n = 10) by ANOVA; p < 0.001 versus bIM control and vasopressin alone) in its presence. Furthermore, the partial inhibition with both Ro-31-8220 and bIM was still present following the removal of specific isoforms of PKC by down-regulation (Fig. 6).

Stimulation of Protein Synthesis by Exogenous PLD—We have previously shown that exogenous PLD elicits an increase in protein synthesis at both 90 min and 6 h in these cells (4). Preincubation with 1 μM TPA had no significant effect on the ability of exogenous PLD to stimulate protein synthesis at 90 min (data not shown). In contrast, the response at 6 h was significantly attenuated by TPA preincubation. 5 units/ml PLD increased synthesis from 12.1 ± 0.2 to 14.3 ± 0.1 dpm/μg protein (n = 10, p < 0.001) in non-down-regulated cells but only from 12.0 ± 0.3 to 13.5 ± 0.1 dpm/μg of protein (n = 10, by ANOVA; significantly different from the increase observed in the absence of down-regulation, p < 0.05) in TPA-pretreated cells.

PKC Isoforms in L6 Myoblasts—Immunoblotting of total cell extracts demonstrated the presence of the following PKC isoforms (with approximate molecular masses): α (81 kDa), δ (77 kDa), μ (115 kDa), ε (88 kDa), ζ (74 kDa), and ξ (73 kDa). PKC-β, -γ, and -θ were not detected (data not shown). When extracts were fractionated, α was detected in the cytosol, whereas ε and δ were found in the membrane. In contrast, μ, ζ, and ξ were present in both cytosolic and membrane fractions (Fig. 7A). Following a 24-h incubation with 1 μM TPA, immunoblotting of total cell extracts demonstrated that PKC-α and -δ could no longer be detected, and densitometric analysis showed that the PKC-ε content had decreased by ~90%. In contrast, PKC-μ, -ζ, and -ξ appeared resistant to down-regulation under these conditions (Fig. 7B).

DISCUSSION

We demonstrate in this study that TPA and vasopressin stimulate [14C]ethanolamine release from prelabeled L6 myoblasts. These effects were elicited in the presence of a large excess of unlabeled ethanolamine and phosphoethanolamine,
increase observed with vasopressin in the absence of Ro-31-8220, p < 0.001. The vast majority of any ethanolamine taken up by the cells is likely to be unlabeled, and thus [14C]ethanolamine is likely to accumulate. When taken together with effects on [3H]PtdBuOH formation, the data imply that both TPA and vasopressin activate a basal [3H]PtdBuOH production at the end of a 2-h incubation and that for TPA was almost restored when intra- and extracellular release observed with TPA may reflect the activity of both cytosolic and membrane-bound PLD activities (e.g. see Refs. 13, 14, 28, and 29) or have measured only extracellular release (12). When intra- and extracellular release of choline has been measured, both vasopressin and TPA rapidly stimulated intracellular release. The vasopressin response had returned to control values within 10 min, and that for TPA was almost restored within 20 min (e.g. see Ref. 30). The different time course of intra- and extracellular release observed with TPA may reflect hydrolysis of PE pools in the inner and outer leaflet of the plasma membrane or in intracellular membranes. It might also involve the activity of both cytosolic and membrane-bound PLD activities (e.g. see Ref. 31 and see below). In addition to TPA and vasopressin, PE hydrolysis by PLD has also been reported to be stimulated by adenine nucleotides (28), platelet-derived growth factor and bombesin (29), lipid A (32), and endothelin (33).

Vasopressin, which generates diacylglycerol and thus stimulates PKC through inositol lipid hydrolysis (4), and TPA, activates PLD in many cell types, suggesting a role for PKC in the activation of PLD. In this and several other studies, inhibitors of PKC and down-regulation of PKC have been shown to attenuate TPA and vasopressin-stimulated PLD activity (e.g. see Refs. 34–36). Our observation that down-regulation com-

FIG. 5. Effect of protein kinase C down-regulation and the protein kinase C inhibitor, Ro-31-8220, on the stimulation of protein synthesis elicited by TPA and vasopressin in L6 myoblasts. Cells were preincubated with or without 1 μM TPA (A and B) for 24 h or with or without Ro-31-8220 (Ro, C and D) for 15 min prior to the addition of agonist. Protein synthesis was measured during the final 60 min of either a 90-min incubation with TPA (A and C) or a 6-h incubation with vasopressin (B and D) as described under "Experimental Procedures." Values for down-regulation experiments are means ± S.E. (n = 15) from three combined experiments, and those with Ro-31-8220 are means ± S.E. (n = 10) from two combined experiments. a, significantly different from control, p < 0.01; b, significantly different from control, p < 0.001; c, significantly different from control in the absence of Ro, p < 0.01; d, significantly different from control in the absence of Ro-31-8220, p < 0.001; e, by ANOVA, significantly different from the increase observed with vasopressin in the absence of Ro-31-8220, p < 0.001.

thus precluding the possibility that [14C]ethanolamine was derived from [14C]phosphoethanolamine. Furthermore, the excess unlabeled ethanolamine also explains the steady increase in extracellular [14C]ethanolamine with time in unstimulated cells. The vast majority of any ethanolamine taken up by the cells is likely to be unlabeled, and thus [14C]ethanolamine released by continuous basal PLD activity (as demonstrated by basal [3H]PtdBuOH production at the end of a 2-h incubation) is likely to accumulate.

When taken together with effects on [3H]PtdBuOH formation, the data imply that both TPA and vasopressin activate a PLD that degrades PE. Furthermore, the time- and concentration-dependent release of extracellular [14C]ethanolamine in response to both agonists mirrored their effects on extracellular [3H]choline release in these cells (4). Comparison of the time-dependent effects on [3H]PtdBuOH formation by TPA using the two different protocols suggests that some of the data in the literature and the conclusions drawn from it should be treated with caution. When sufficient butanol is present for transphosphatidylation to take place, the TPA response clearly correlates with the effect on extracellular [14C]ethanolamine release; i.e. both events are sustained for at least 2 h. Interestingly, using the modified protocol to assess [3H]PtdBuOH formation, we have found in C2C12 myoblasts that the activation of PLD by TPA continues for at least 6 h.2

TPA also stimulates ethanolamine release within 2 min in HeLa cells (12) and after a lag period (10 min) in HL-60 cells, NIH 3T3 fibroblasts, and baby hamster kidney cells (13). In contrast, as far as we are aware, this is the first demonstration that vasopressin stimulates a PLD that degrades PE in any cell type or tissue. Many of these previous studies have measured either total ethanolamine metabolites and failed to distinguish between intra- and extracellular release (e.g. see Refs. 13, 14, 28, and 29) or have measured only extracellular release (12). When intra- and extracellular release of choline has been measured, both vasopressin and TPA rapidly stimulated intracellular release. The vasopressin response had returned to control values within 10 min, and that for TPA was almost restored within 20 min (e.g. see Ref. 30). The different time course of intra- and extracellular release observed with TPA may reflect hydrolysis of PE pools in the inner and outer leaflet of the plasma membrane or in intracellular membranes. It might also involve the activity of both cytosolic and membrane-bound PLD activities (e.g. see Ref. 31 and see below). In addition to TPA and vasopressin, PE hydrolysis by PLD has also been reported to be stimulated by adenine nucleotides (28), platelet-derived growth factor and bombesin (29), lipid A (32), and endothelin (33).

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FIG. 6. Effect of the protein kinase C inhibitors, Ro-31-8220 and bisindolylmaleimide, on the stimulation of protein synthesis elicited by vasopressin following the down-regulation of specific isoforms by TPA pretreatment in L6 myoblasts. Cells were incubated with or without 1 μM TPA for 24 h and then treated with either 1 μM Ro-31-8220 (Ro) or bIM for 15 min prior to the addition of vasopressin. Protein synthesis was measured during the final 60 min of a 6-h incubation as described under "Experimental Procedures." Data are presented as means ± S.E. (n = 10) from two combined experiments. a, significantly different from control, p < 0.001; b, significantly different from down-regulated control, p < 0.001; c, significantly different from Ro-31-8220-treated control, p < 0.01; d, by ANOVA, significantly different from the increase observed with vasopressin in the absence of Ro-31-8220, p < 0.01; e, significantly different from bIM-treated control, p < 0.001; f, by ANOVA, significantly different from the increase observed with vasopressin in the absence of bIM, p < 0.001.
completely prevented TPA- and vasopressin-induced [3H]choline or [14C]ethanolamine release or [3H]PtdBuOH formation clearly implicates PKC in this event. This suggests that the inability of Ro-31-8220 to completely inhibit TPA-induced extracellular and vasopressin-induced intracellular release of [14C]ethanolamine may be due to its action as a competitive inhibitor (18). Contrastingly, in other cell types, the activation of PLD by TPA may not involve PKC. For example, PKC inhibitors failed to block the activation of PLD by TPA in lymphocytes (37) and mast cells (38).

To gain further insight into the mechanism through which PKC regulates PLD in L6 cells, we examined which isoforms are present and subject to down-regulation. As far as we are aware, this is the first attempt to study PKC isoforms in L6 cells. A previous study in skeletal muscle and L6 skeletal muscle cells demonstrated the presence of PKC-α, -δ, and -ζ, but not PKC-β and -γ. Furthermore, α and δ, but not ζ, were down-regulated by TPA in L6 cells (39). These are very similar to our findings reported in this study and in C2C12 skeletal muscle cells.3 Other work has also reported the presence of PKC-α (40, 41), -δ (41, 42), and -ε (41) in skeletal muscle.

The data showing a correlation between the disappearance of PKC-α, -δ, and -ε and the loss of PLD activity by TPA and vasopressin clearly implicate one or more of these isoforms in this response. Their down-regulation upon TPA treatment has been observed in other cell lines (e.g. see Refs. 39, 43, and 44). Furthermore, PKC-α has been shown to activate PLD in Madin Darby canine kidney cells (45) and CCL39 fibroblasts (46), while PKC-ε has been suggested to regulate PLD activity in rat mesangial cells (47). There is no evidence to date of a role for PKC-δ in the regulation of PLD activity. It is not yet clear from our data if TPA and vasopressin activate the same PKC and/or PLD. Interestingly, although PKC-α is largely cytosolic in L6 and other cell lines (e.g. see Ref. 43), we have found PKC-ε to be membrane-associated, and this is also the case to some degree in U937 cells (48), rat6 fibroblasts (49), and renal mesangial cells (50). It is intriguing to suggest that isoform and/or location-specific PKC/PLD activities may be responsible for both the rapid but transient responses and the delayed but sustained stimulation observed with TPA. Such a possibility requires further investigation.

We have previously shown in L6 myoblasts that the EC50 for vasopressin stimulation of transcription is 10-fold higher than that for [3H]PtdBuOH formation and [3H]choline release (4). Data from the current study show that this is also true for [14C]ethanolamine release. Furthermore, the maximal stimulation of protein synthesis observed with exogenous PLD at 6 h was 12%, whereas an increase of 30% or more was elicited by vasopressin (4). Thus, while data in this and our previous study (4) support a link between the activation of PLD and the stimulation of translation by TPA, it also implies that, at best, activation of PLD is only part of the mechanism by which vasopressin increases protein synthesis in these cells. It is now clear that while PKC is involved in the stimulation of both PLD and transcription by vasopressin, the down-regulation protocol makes it possible to dissociate the two events completely. Thus, different isoforms of PKC mediate vasopressin effects on PLD and protein synthesis. Consequently, it appears that the increase in transcription we have previously observed with exogenous PLD in these cells (4) must involve an alternative mechanism to that employed by vasopressin (see below). This conclusion is also supported by the finding that the stimulation of protein synthesis at 6 h by exogenous PLD, but not vasopressin, is partially attenuated by PKC down-regulation. One possible mechanism involves the generation of lysophosphatidic acid and its action through an extracellular receptor (16).

Immunoblotting of the down-regulated cells implicates PKC-μ, -ι, and/or -ζ in the stimulation of protein synthesis by vasopressin. However, all three isoforms have a high degree of sequence homology, raising the possibility of cross-reactivity between the antibodies. Studies have shown that the ζ antibody cross-reacts with ζ-4 and, since we have been unable to obtain an alternative source of ζ antibody, its presence in L6 cells remains unproven at present. The antibody to PKC-μ clearly detects a protein of 115 kDa, but it fails to recognize any bands in the 70–90-kDa range. Furthermore, the antibodies to ζ and ζ do not detect the 115-kDa protein. Thus, from the data available on the three atypical isoforms, it appears likely that only μ and ζ are present. The inability of TPA to down-regulate PKC μ is also of interest, since when human PKC-μ was propagated in the baculovirus expression system and purified to homogeneity, it displayed high affinity TPA binding (51). However, this finding differs markedly from an earlier study in which only a very weak increase in TPA binding was observed in cellular extracts from PKC-μ transfectants (52). The difference between the two observations may be due to unknown factors present in the cellular extracts that prevent TPA binding.

Although the increase in translation elicited by insulin in L6 cells was not prevented by Ro-31-8220 (53), this inhibitor (54), but not PKC down-regulation, also attenuated the stimulation of transcription by insulin, suggesting that vasopressin and insulin utilize the same subset of PKC isoforms.

3 M. G. Thompson and A. Thom, unpublished observation.

4 C. Davies, personal communication.

Fig. 7. Identification of PKC isoforms in L6 myoblasts and the effect of prolonged incubation with TPA. Cytosolic (C) and membrane (M) fractions were prepared from nontreated cells (A). Total cell extracts were prepared from cells incubated with (+) or without (−) 1 μM TPA for 24 h (B). 15 μg of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and then immunoblotted with isomor-specific antibodies to PKC-α, -ε, -δ, -μ, -ι, or -ζ. The molecular mass standards used were β-galactosidase (116,300 kDa), Phosphorylase b (97,400 kDa) and ovottransferrin (76,000–78,000 kDa). Immunoblots are from a representative experiment of three.
In many of the cell lines investigated so far, PKC-ζ seems to be present as a cytosolic enzyme (e.g. see Ref. 55). As predicted from its structure, most studies, including the work reported here, indicate that PKC-ζ is resistant to TPA-induced translocation or down-regulation (e.g. see Refs 50 and 56). In addition, both of the PKC inhibitors that partially blocked the effect of vasoressin on transcription have been shown to inhibit PKC-ζ (e.g. see Ref. 57). The observation that higher concentrations of both agents (1 μM) were required to partially inhibit PKC ζ than to completely inhibit the α, δ, and ε isoforms (57) and our finding that Ro-31-8220 and BIM still elicited a partial inhibition of the vasoressin response following down-regulation further support a role for the down-regulation-resistant isoforms such as ζ in mediating the vasoressin response. In addition, vasoressin has been shown to stimulate the translocation of PKC ζ from the cytosol to a membrane fraction in human platelets (58), and in smooth muscle, phenylephrine elicits PKC-ζ translocation to the nucleus (59), suggesting a role in events such as gene expression.

PKC-ζ has been shown to be stimulated in vitro by phosphatidylinositol 3,4,5-trisphosphate (60), the phospholipid that is thought to be the physiologically important product of phosphatidylinositol-3-kinase (61). However, the phosphatidylinositol-3-kinase inhibitor, wortmannin (62), had no effect on the ability of vasoressin to stimulate transcription in L6 cells, suggesting that phosphatidylinositol-3-kinase is not involved in mediating this response.5 PKC-ζ is also activated by arachidonic acid (63), and preliminary data show that vasoressin increases arachidonic acid release from L6 cells.5 Furthermore, we have previously shown that vasoressin stimulates mitogen-activated protein kinase in these cells (64) and mitogen-activated protein kinase is known to phosphorylate and activate cytosolic PLA2 (65), releasing arachidonic acid. Thus, it is possible that vasoressin may, at least in part, stimulate protein synthesis in L6 cells through PKC-ζ via mitogen-activated protein kinase, cytosolic PLA2, and arachidonic acid. This potential sequence of events is under further investigation.

Although PLD is clearly not involved in the stimulation of protein synthesis by vasoressin, both this agonist and TPA also reduce myofibrillar protein degradation in skeletal muscle (6, 7). It remains to be determined whether PLD plays a role in the regulation of this component of protein turnover.

REFERENCES
1. Hasselgren, P. O., Talanimi, M., James, H., and Fischer, J. E. (1986) Arch. Surg. 121, 919–923
2. Strelov, A. B. S., Fields, A. L. A., and Baracos, V. E. (1989) Am. J. Physiol. 257, C261–C269
3. Beck, S. A., Smith, K. L., and Tisdale, M. J. (1991) Cancer Res. 51, 6098–6093
4. Thompson, M. G., Mackie, S. C., Morrison, K. S., Thom, A., and Palmer, R. M. (1994) Biochem. Biophys. Res. Commun. 208, 198–204
5. Morrison, K. S., Mackie, S. C., Palmer, R. M., and Thompson, M. G. (1995) J. Cell. Physiol. 165, 273–283
6. Thompson, M. G., Palmer, R. M., Thom, A., Garden, K., Loblly, G. E., and Calder, G. (1996) Am. J. Physiol. 270, C1875–C1879
7. Goodman, M. N. (1987) Biochem. J. 247, 151–156
8. Axelrod, J. (1990) Biochim. Biophys. Acta. 1024, 188–194
9. McNally, S., Lloyd, G. S., Ramsay, R. M., Sayner, R. M., and Ramsay, M. G. (1992) Neurosci. Lett. 139, 183–187
10. Durieux, M. E., and Lynch, K. R. (1993) Trends Pharmacomol. Sci. 14, 249–254
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