Phorbol ester inhibits myoblast fusion and activates β-adrenergic receptor coupled adenylate cyclase

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Received 10 May 1985

Primary cultures of myoblasts, derived from embryonic chick pectoral muscle, were treated with phorbol ester (TPA) for 8–96 h TPA treatment blocked the fusion of myoblasts along with the expression of the MM form of creatine kinase Interestingly, TPA treatment markedly increased the activity of β-adrenergic receptor coupled adenylate cyclase (AC) activity The study suggests that TPA treatment augments the functional interaction between a coupling N_s protein and catalytic unit of AC The likely significance of these results is briefly presented

Myoblast *Adenylate cyclase* *Phorbol ester* *Fusion* *Creatine kinase* *β-Adrenergic receptor*

1. INTRODUCTION

Phorbol esters such as 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) have been shown to be potent tumor promoters and their site of action is the cellular plasma membrane [1–3]. TPA inhibits the fusion of mononucleated myoblasts [4–6]. Precisely how TPA exerts this inhibitory action and the relation between the inhibition of fusion and a few reported biochemical changes [4–8] evoked by TPA in myoblasts are unclear. In addition to the likely involvements of membrane (phospho)lipids, glycolipids and glycoproteins [9], a limited number of studies have implicated a transient increase of cAMP in the process of myoblast fusion [10,11].

In primary cultures of myoblasts, the catalytic unit (C) of the cAMP generating enzyme, adenylate cyclase (AC) and a GTP-binding coupling protein called N_s are present prior to fusion [12] However, the stimulation of AC by β-adrenergic receptor (β-AR) agonists (isoproterenol, ISO) is not readily seen in myoblasts [12]. At the time of fusion and subsequent to the fusion of myoblasts into myotubes, there appears to be an increase in the number of β-AR which are functionally linked to the AC such that ISO increases the AC activity in the presence of GTP [11–14]. Thus, the blockade by TPA of myoblast fusion would be expected to maintain the plasma membrane bound, β-AR coupled AC system in a low activity state. As will be shown here, the reverse was observed in the 'fusion inhibited state' of myoblasts due to TPA. Also, the activation of the β-AR coupled AC system by the exposure to TPA of myoblasts seemed to result from the action of TPA on the coupling N_s protein. The likely significance of this novel and intriguing observation is discussed in terms of whether cAMP plays any role in myoblast fusion or other facets of muscle differentiation In our study the activities of creatine kinase were markedly decreased by the exposure of myoblasts to TPA and this was associated with marked decreases in the proportion of MM isozyme (a suitable marker of myoblast differentiation) and increases in the proportion of BB isozyme. The site for action of TPA in the cell membrane may be protein kinase C [15], the enzyme discovered by Nishizuka and associates [15]. In view of the postulated roles of kinase C in the actions of

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00145793/85/$3 30

various biological stimuli on cellular processes including proliferation [15, 16], it is tempting to consider that the activation of myoblast protein kinase by TPA is somehow linked to its other effects - (i) the blockade of fusion and (ii) the activation of the β-AR linked AC system This is briefly discussed as well.

2. MATERIALS AND METHODS

2.1. *Culture conditions*

Skeletal muscle cultures were prepared from myoblasts isolated from pectoral muscle of 11-day-old white Leghorn chick embryos according to [17] with the exception that 10% horse serum was used rather than fetal calf serum To determine the effect of TPA, normal culture media were replaced with TPA- (0 1 μM) containing media 8 h after plating the myoblasts. In TPA-treated cultures the media were changed at 24 h intervals thereafter to ensure the continued presence of TPA

2 2 *Measurement of CK activity and isoenzyme patterns*

Control and TPA-treated cultures were harvested after 72 h of development, sonicated in 50 mM Tris-HCl buffer, to which dithiothreitol (100 mM) was added, and then frozen at $-80°C$ until assayed. Upon thawing samples were centrifuged at $12\,800 \times g$ for 5 min and assayed for total CK activity using the Boehringer Mannheim *N*-acetylcysteine activated CK kit. Separation of the CK isoenzymes was achieved by ion-exchange chromatography using DEAE-Sephadex as described in [18]

2 3. *Adenylate cyclase assay*

Homogenate (30–50 μg protein) was incubated at 37°C for 5 min in assay mixture (0.15 ml) containing 50 mM glycylglycine (pH 7.5), 4 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP (containing 10 000 cpm as cyclic [^{3}H]AMP), ATP-regenerating system (20 mM creatine phosphate, 8 units creatine kinase), isomethylbutylxanthine and 0.4 mM [α-^{32}P]ATP. Other details were as described previously [19,20]. The concentrations of GTP, Gpp(NH)p, ISO, and forskolin are listed in table 2. Protein was estimated according to Lowry et al [21] using bovine serum albumin as the standard

3 RESULTS AND DISCUSSION

The continuous presence of TPA (0.1 μM) in primary cultures of chick myoblasts inhibited fusion As shown in fig.1, extensive myotube formation was present in control cultures by 72 h in vitro whereas only large clusters of non-fused cells were observed in TPA-treated cultures. Quantitative measurements of the fusion index showed, that after TPA treatment, the number of nuclei present in myotubes was never more than 10% of the total cells present

Several markers have been used to study the normal course of differentiation of presumptive myoblasts to myotubes in cultures of skeletal muscle CK is a dimeric enzyme that exists in two prin-

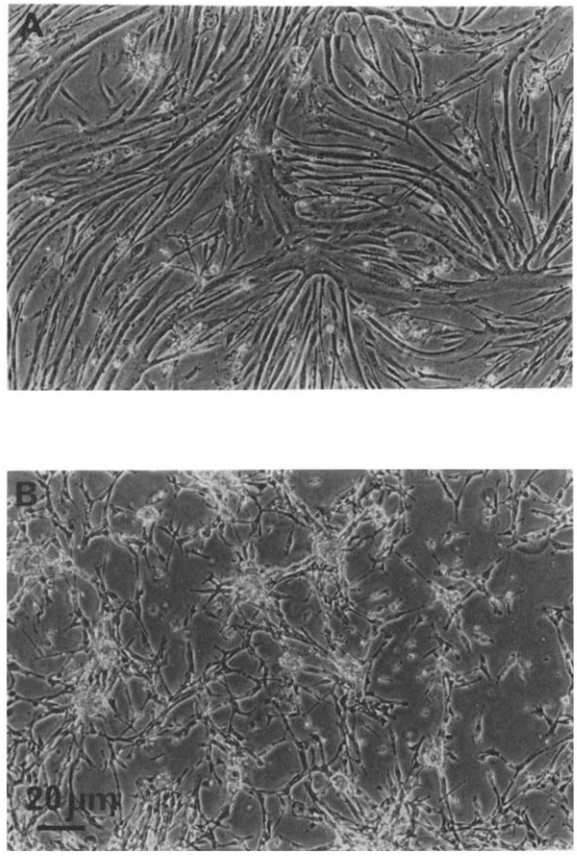

Fig 1 Photomicrograph (A) of a control muscle culture prepared from myoblasts isolated from 11-day-chick embryos and maintained in vitro for 72 h Note extensive myotube formation In (B) fusion was blocked by the presence of 0 1 μM TPA beginning 8 h after plating

cipal isoenzymic forms, the MM-CK dimer in adult skeletal muscle and the BB-CK dimer in early embryonic muscle. The heterodimer MB-CK exists transiently during early differentiation of skeletal muscle. Both total CK actvity [22] and the time course of appearance of the isoenzymes have been used as markers of differentiation [23].

When CK activity was compared in control and TPA-treated cultures harvested after 72 h of culture there was a significant deficit of CK activity in TPA-treated cultures (table 1). It should be noted that this effect of TPA on CK activity occurred in the absence of a significant effect on total protein content of the cultures (table 1). Examination of the isoenzyme profile revealed that the isoenzyme CK-MM was absent from TPA-treated cultures, the heterodimer CK-MB was decreased and the CK-BB form markedly increased (table 1). Since CK-BB is known to be the primary isoenzyme present in undifferentiated myoblasts, the present data indicate that the presence of TPA inhibits differentiation of the presumptive myoblasts. The preponderance of the CK-BB form indicates that in the presence of TPA the myoblasts continue to replicate but do not withdraw from the cell cycle to enter a differentiating state in preparation for fusion.

Table 2 summarizes the results of the effect of TPA on myoblast homogenate AC activity measured under various assay conditions. The enzyme activities determined in the presence of N_s activators like NaF or Gpp(NH)p [24] and in the presence of ISO plus GTP were significantly higher in the TPA exposed myoblast fraction. Basal activity (measured without added activators of the enzyme) was also higher. Since homogenate enzyme activity was measured, there is the likelihood of the presence of endogenous GTP in homogenate and thus basal activity may not reflect the activity of the 'true basal' state of AC. AC measured with forskolin present was also higher in the TPA-exposed myoblast preparations. Forskolin is believed to act on the catalytic unit of AC [25], although it can potentiate the effect of N_s activators on the enzyme [25]. The β-adrenergic stimulation (with GTP present) was determined in the presence of saturating concentrations of ISO. However, in preliminary experiments, we noted that TPA treatment did not alter the affinity towards this β-receptor agonist (not shown). Thus these results indicated that TPA treatment altered the 'funtional interaction' between the N_s and C subunit of AC, which seemed to be more effective in stimulating the cyclase activity. Mn^{2+} is known to increase AC activity, in fact even to a greater extent than Mg^{2+} and the evidence supports its stimulation occurring via action on the catalyst TPA treatment increased only modestly Mn^{2+}-stimulated AC (not shown). This indicates that the major site of TPA action is the N_s protein and not the C unit of AC.

The 3 alterations (inhibition of fusion and of the expression of CK-MM, and activation of AC) evoked by TPA exposure of myoblasts offer an interesting opportunity to comment on whether cAMP plays any role in the fusion of myoblasts or

Table 1

Effects of fusion-inhibiting concentration of TPA (0 1 μM) on protein content, creatine kinase activities, and creatine kinase isozymes of myoblasts in primary cultures

TPA in myoblast culture medium	Protein content (mg/dish, 6 wells)	Creatine kinase (units/mg protein)		Isozymes (%)		
		Homogenate	Supernatant	MM	MB	BB
Absent	1 07 (0.07)	4 73 (0 26)	8.45 (0 65)	28 9 (3 65)	41 0 (3.6)	30.1 (2 3)
Present	1 21 (0 12)	1 84 (0 16)[a]	2 63 (0 35)[a]	0	23 9 (5 4)	75 8 (5.4)[b]

Values in parentheses represent ± SE, $n = 8$

[a]Significantly lower ($p < 0$ 02) than control

[b]Significantly higher ($p < 0$ 02) than control

Table 2

Effect of exposure to TPA of myoblasts on adenylate cyclase activities

Assay addition	Adenylate cyclase activity (pmol cAMP/min per mg protein)	
	Control myoblasts	Treated myoblasts
None	14 ± 2	20 ± 2
Forskolin	78 ± 3	136 ± 5
NaF	109 ± 9	137 ± 6
Gpp(NH)p	56 ± 3	145 ± 7
Isoproterenol + Gpp(NH)p	60 ± 4	150 ± 7
Isoproterenol + GTP	20 ± 1	30 ± 2

Results are means ± SE, when present, NaF, Gpp(NH)p, isoproterenol and GTP were 5, 0 1, 0 05 and 0 05 mM, respectively Myoblasts were exposed for 3 days to TPA (0 1 μM) (treated myoblasts) prior to their use Control myoblasts were cultured identically but in the absence of TPA The enzyme activity was determined within 10 min of homogenization (by sonication) of myoblasts

in the post-fusion differentiation process and if there is any relation between the expression of CK isozymes and the state of differentiation The observations that a transient rise in cAMP precedes the fusion of myoblasts [10] and that prostaglandins or ISO via elevation of cAMP augment the onset of fusion [11] have been challenged [26]. Recent studies [26] in fact show that an increase in cAMP is seen reproducibly after fusion and in proliferative states We report here, that marked increases in the cAMP synthetic enzyme activity are noted when the fusion is almost completely inhibited and myoblasts are in the state of 'dedifferentiation' or proliferation It may be that the higher cAMP content in these myoblasts is attempting to 'force' them to fuse but TPA-induced blockade of the fusion cannot be overcome It is at the same time attractive to consider that higher cAMP content is in some way related to the inhibition of the expression of CK-MM forms, which admittedly is also dependent on the state of differentiation. The latter has been well-documented However this is a paradoxical situation For example, in the Ca^{2+}-induced myoblast fusion, which leads to elevation of cAMP faster than the increase in CK activity [26], it has been postulated that cAMP may be involved in the expression of the CK-MM form activity. We in fact observed the reverse, i.e. higher cAMP synthetic ability associated with the low CK activity. It was also noted that TPA exposure did not elevate cAMP phosphodiesterase (unpublished).

On the other hand, there exists a suggestion that phospholipid metabolism is critical in the overall process of myogenesis in vitro [26] and in particular the likely involvement of phosphatidylinositol has been suggested. There is growing support for the view that a major target for TPA action is protein kinase C [15]. It is thus tempting to consider that the inhibitory effect of TPA on myoblast fusion and the activation by TPA of the β-adrenergic receptor linked AC system of myoblasts are linked to the activation of protein kinase C It could be that the coupling protein N_s is a substrate for the kinase C-catalyzed phosphorylation such that the functional interaction between N_s and C is dependent on the state of N_s phosphorylation. Whether this bears any relation to the inhibitory effect of TPA on the fusion of myoblast, however, requires careful further examination.

ACKNOWLEDGEMENT

This study was supported by a grant from the Muscular Dystrophy Association of Canada

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