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ALTERATION OF CYCLIC NUCLEOTIDE LEVELS IN PHORBOL 12-MYRISTATE 13-ACETATE TREATED MYOBLASTS

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SUMMARY: The tumor promoter phorbol 12-myristate 13-acetate alters the levels of both cGMP and cAMP in chick myoblasts. There is a transient 7-fold increase in cellular cGMP 40-60 sec after promoter addition. The changes in cAMP are slower with a 50% decrease at 30 min followed by a 3.5-fold increase at 3 h. The relationship between these cyclic nucleotide changes and the previously reported stimulation of hexose transport by the promoter [Fed. Proc. 37, 1817 (1978)] was investigated. Addition of 8-bromo cGMP, 8-bromo cAMP or phosphodiesterase inhibitors had no effect on hexose transport while LiCl (an inhibitor of adenyl cyclase) was stimulatory. The tumor promoter and LiCl, however, appear to stimulate hexose transport by different mechanisms.

The potent tumor promoter, phorbol 12-myristate 13-acetate, induces many transformation-like responses in a variety of cell types. In cultured myogenic cells, such responses include the inhibition of differentiation (1,2), and the stimulation of both hexose transport (2,3) and plasminogen activator protein synthesis (4). Alterations in cyclic nucleotide levels have been reported to influence a variety of cellular properties associated with transformation (5). It has been reported that exogenous manipulation of cyclic nucleotide levels can alter cellular responses to PMA[2] in some instances (6,7). In addition, PMA has been shown to alter cGMP levels in mouse 3T3 cells (8) and platelets (8) and cAMP metabolism in mouse skin (9). Accordingly, we have investigated the effect of PMA on cyclic nucleotide levels in cultured myogenic cells and have investigated the relationship of the changes which were found to the PMA-induced stimulation of hexose transport.

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[2] Abbreviations: PMA, phorbol 12-myristate 13-acetate; cAMP, 3',5'-cyclic adenosine monophosphate; cGMP, 3',5'-cyclic guanosine monophosphate.

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MATERIALS AND METHODS

PMA, cyclic nucleotide analogues and all inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-2-Deoxy-D-glucose (8.26Ci/mmole) and radioimmunoassay kits for cyclic nucleotides were purchased from New England Nuclear (Boston, MA). Myoblast cell cultures were prepared from breast muscle of 12-day white leghorn chick embryos essentially as described previously (10), except that 2.5 ng/ml collagenase (Worthington Type III) was used in place of trypsin. Cells (1.5 x 10^6/60 mm dish) were grown in Ca^{++}-free DMEM containing 10% fetal bovine serum (treated with Chelex-100 resin) and 1% 11-day chick embryo extract. The final concentration of Ca^{++} in the medium was adjusted to 0.1mM, which permits the normal differentiation of mononucleated myoblasts but prevents myotube formation (11). After 30-40 hours, the cells were fed with fresh medium containing 10^{-5}M cytosine arabinoside to kill any proliferating cells. Three-day cultures were used for all experiments.

Assay of cyclic nucleotides - The culture medium was rapidly removed from the culture dish and ice-cold 5% $HClO_4$ was added immediately. After scraping and centrifugation (1000 xg, 5 min), duplicate portions of the supernatant fraction were neutralized with an equal volume of cold 1M $KHCO_3$ and assayed for cAMP and/or cGMP using the acetylation protocol supplied with the radioimmunoassay kits. The standard error for duplicate determinations was < ± 5% for both assays.

Assay of hexose transport - Cell monolayers were washed 3x at 37° with Dulbecco's phosphate buffered saline (PBS), pH 7.4 (0.1mM $CaCl_2$, 2.6mM KCl, 1.5mM KH_2PO_4, 0.5mM $MgCl_2$, 138mM NaCl, 8.1mM Na_2HPO_4), and then incubated for 10 min at 37° with 2 µCi/ml of [^{3}H]-2-deoxy-D-glucose in PBS. The cells were then washed 3x with ice-cold PBS, solubilized in 1% sodium dodecyl sulfate and the amount of label per mg total protein determined. Uptake of [^{3}H]-deoxyglucose was linear for at least 15 min using this assay. All data shown are the means of transport assays in duplicate culture dishes (S.E. < ± 5%).

RESULTS

Cyclic nucleotide changes induced by PMA - Cyclic GMP levels in myoblasts increased 3.5-fold within 15 sec and 6.5-fold within 20-40 sec after addition of 20 ng/ml PMA (Figure 1). Within one minute, the level of cGMP decreased to 2.5-fold over that in untreated control cells (Figure 1) after which no further alterations were found between 5 min and 5 h (not shown). A similar though smaller response occurred with 2 ng/ml PMA and the levels returned to those of control cells within one minute.

No changes in cAMP levels were detected during the first two minutes after PMA addition. Assays at longer times, however, revealed a 50% decrease in cAMP levels at 30-40 min followed by a rise to 320% of the control at 60-90 min after PMA addition (Figure 2). Thereafter, cAMP levels decreased slowly, reaching control values at 5 h after PMA addition.

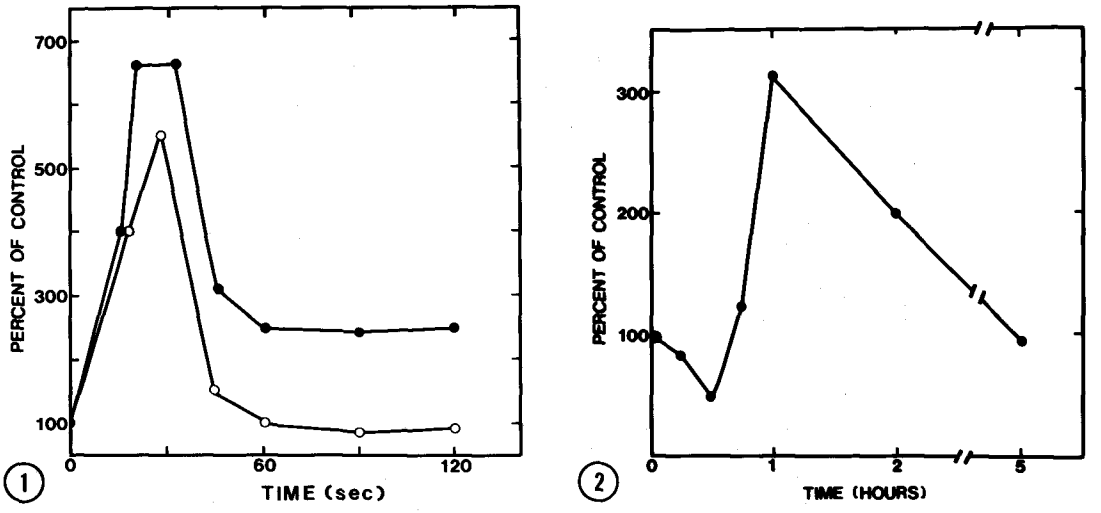

Fig. 1: Effect of PMA on cGMP levels in myoblasts. Cells were treated with 20 (●) or 2 (o) ng/ml PMA for the indicated times and cGMP was assayed in duplicate as described in Materials and Methods. Basal cGMP was 0.17 pmoles/ 60mM dish.

Fig. 2: Effect of PMA on cAMP levels in myoblasts. Cells were treated with 20 ng/ml PMA for the indicated times and cAMP was assayed in duplicate as described in Materials and Methods. Basal cAMP was 58 pmoles/dish.

Relationship of cyclic nucleotide changes to the stimulation of hexose transport - It had been found previously that hexose transport is stimulated in myoblasts treated for 5 h with PMA (2). To determine if any of the cyclic nucleotide alterations were related to the subsequent changes in hexose transport, various analogues of cAMP and cGMP and other agents known to alter cellular cyclic nucleotide levels were tested for their ability to stimulate hexose transport. Under conditions where PMA stimulated hexose transport 59%, no stimulation was observed after 5 h of treatment with 8-Bromo cAMP, 8-Bromo cGMP, aminophylline or theophylline (Table I). Further, the addition of 8-Bromo cAMP or aminophylline to PMA-treated cells had no effect on the stimulation of hexose transport (Table I). These results suggested that neither the increased cGMP nor cAMP levels in PMA-treated cells was directly related to the stimulation of hexose transport.

To determine if the initial decrease in cAMP levels could be a causal factor in hexose transport stimulation, cells were treated with LiCl, which has

TABLE I

EFFECT OF CYCLIC NUCLEOTIDE ANALOGUES, PHOSPHODIESTERASE INHIBITORS AND PMA ON HEXOSE TRANSPORT

Addition	Percent of Control
None	100
8-Bromo cAMP (4mM)	98
8-Bromo cGMP (1mM)	104
Aminophylline (1mM)	110
Theophylline (5mM)	108
PMA (20 ng/ml)	159
PMA + 8-Bromo cAMP	162
PMA + Aminophylline	161

Myoblasts (66 h post-plating) were incubated for 5 h in the presence of the indicated agent after which hexose transport was measured. Values shown are the mean of duplicate culture dishes corrected for small variations in total protein content of each dish. Mean cpm/dish in the control was 21100 for 10 min uptake. The concentration of aminophylline employed was shown to increase the intracellular cAMP concentration 5-fold.

been shown to inhibit adenyl cyclase in several tissues (12). Treatment of myoblasts with various concentrations of LiCl for 6 h caused a concentration-dependent increase in hexose transport which was maximal (20-28%) at 10mM salt and half-maximal at 1-3mM salt. The time course of the response is shown in Figure 3, where the response was half-maximal at approximately 2 h and was essentially maximal by 6 h. This is in marked contrast to the time course for

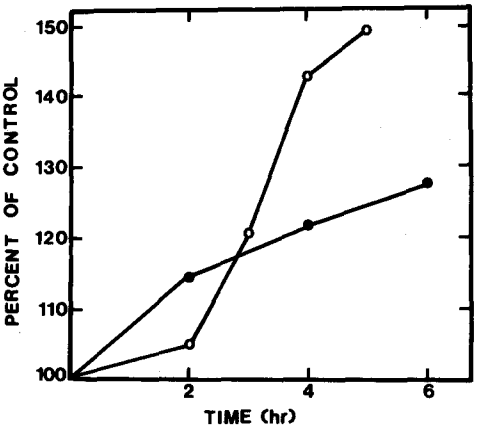

Fig. 3: Stimulation of hexose transport by PMA and LiCl. Myoblasts were treated with 20 ng/ml PMA (o) or 10mM LiCl (•) for the indicated times and [^{3}H]-2-deoxyglucose uptake was assayed in duplicate cultures as described in Materials and Methods. The mean cpm/dish in the control (0.2% acetone vehicle only) was 22,100 for 10 min uptake.

TABLE II

EFFECTS OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON THE STIMULATION OF HEXOSE TRANSPORT BY PMA AND LiCl

Treatment	Percent of Control
PMA (20 ng/ml)	131 ± 2
LiCl (10mM)	127 ± 1
PMA + Cycloheximide[a]	101 ± 3[b]
LiCl + Cycloheximide[a]	113 ± 4[b]
PMA + Actinomycin D[c]	98 ± 2[d]
LiCl + Actinomycin D[c]	119 ± 1[d]
PMA + LiCl	150 ± 5

Myoblasts were treated for 5 hours with the indicated agents, after which hexose transport was measured in duplicate culture dishes. Transport in control dishes was 19000 cpm/dish for 10 min uptake. Values are mean ± S.E.

a 1 ug/ml added 1 h prior to addition of PMA or LiCl; incorporation of [^{3}H]-leucine was inhibited by approximately 90% during the 5 h incubation

b compared with cycloheximide alone

c 0.1 ug/ml added 1 h prior to addition of PMA or LiCl; incorporation of [^{3}H]-uridine was inhibited by > 99% during the 5 h incubation

d compared with actinomycin D alone

the stimulation of hexose transport by PMA, which exhibited a 1-2 h lag before any increase became evident.

The delay in the PMA response has been shown to be due to a requirement for RNA and protein synthesis (3). The requirement for RNA and protein synthesis was therefore investigated in the LiCl response. The data in Table II demonstrate that in contrast to the complete block of the PMA response, actinomycin D and cycloheximide blocked the LiCl response only 30% and 45%, respectively. These data suggest that the LiCl response occurs by a mechanism different from that of the PMA response. In further support of this conclusion, it was found that treatment of cells with both PMA and LiCl at maximally effective doses resulted in an additive stimulation of hexose transport (Table II).

DISCUSSION

The transient rapid rise of cGMP levels in PMA-treated myoblasts is similar to that seen in PMA-treated 3T3 cells (8) and platelets (8) or in

mitogen-treated lymphocytes (13). It has been proposed (13) that, at least in the case of lymphocytes, the rise in cGMP is required for the initiation of proliferation from the G_0 stage of the cell cycle and that this rise might trigger the subsequent decline in cAMP levels which is generally associated with proliferation. In differentiated muscle cells, however, PMA is not mitogenic (1,2) despite the rise in cGMP and the subsequent transient decrease in cAMP. These data and the dramatic alteration in gene expression in PMA-treated differentiated myogenic cells (2-4) are consistent with the model for lymphocyte regulation proposed by Whitfield (14) in which the rise in cGMP is associated with progression of the cell from the G_0 to the G_1 state and this progression is necessary but not sufficient for subsequent proliferation.

The stimulation of hexose transport by PMA does not appear to be the consequence of the cyclic nucleotide alterations, since exogenous cyclic nucleotide analogues and phosphodiesterase inhibitors had no significant effect either on hexose transport itself or on the PMA-induced stimulation of hexose transport (Table I). Although it is technically difficult to mimic the endogenous levels or transience of the cGMP rise or the cAMP fall by exogenous manipulation, these findings are in agreement with those reported for the PMA stimulation of plasminogen activator protein synthesis in chick myotubes (4) and of ornithine decarboxylase synthesis in mouse skin (15).

LiCl, which probably acts to decrease cAMP levels (12), stimulated hexose transport in myoblasts, but appears to do so by a mechanism different from that of PMA, since the Li^+ stimulation, unlike PMA stimulation, was not blocked completely by inhibitors of RNA and protein synthesis (Table II). In addition, Li^+ was able to further stimulate hexose transport in cells that were stimulated with a maximally effective concentration of PMA, suggesting that they do not operate via a common mechanism.

It appears that PMA responses in myogenic cells proceed along several divergent pathways which may branch from some primary rapid event(s) following PMA interaction with the cell surface. We have found that in addition to its

effects on cyclic nucleotide levels, PMA rapidly alters Ca^{++} influx rate (16) and glycerophospholipid metabolism in differentiated myoblasts (submitted). The elucidation of the relationship among all of these early responses and the subsequent alteration in gene expression should provide further insight into tumor promoter action.

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