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# RAPID ALTERATION IN $Ca^{++}$ CONTENT AND FLUXES IN PHORBOL 12-MYRISTATE 13-ACETATE TREATED MYOBLASTS

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SUMMARY: The tumor promoter phorbol 12-myristate 13-acetate rapidly induces alterations in both $Ca^{++}$ content and transport in cultured differentiated chick myoblasts. At 4 ng/ml (6nM), the promoter caused a 25 ± 12% decrease in total intracellular $Ca^{++}$ within 5 h after its addition. Measurement of $^{45}Ca^{++}$ transport at this time revealed a 15 ± 6% decrease in the rate constants for both efflux and influx. Values of $t_{1/2}$ for the cytosolic $Ca^{++}$ pool in control and treated cells were 9.1 and 10.7 min, respectively, for efflux and 8.6 and 10.4 min, respectively, for influx. $Ca^{++}$ influx was decreased maximally within 90 sec after promoter addition. No effect was observed on $^{86}Rb^{+}$ uptake or intracellular concentration at equilibrium. The $Ca^{++}$ response is among the most rapid yet reported and may play a primary role in altering cellular metabolism.

Phorbol 12-myristate 13-acetate, a potent tumor promoter induces the expression of transformation-like properties in a variety of normal cells in vitro. In cultured myogenic cells, such transformation-like responses include the reversible inhibition of myogenic differentiation from progenitor cells (1-3), reversible inhibition of $Ca^{++}$-dependent multinucleated myotube formation from differentiated mononucleated myoblasts (1,2), and other alterations in gene expression (2-4). The mechanism by which PMA[1] at very low concentrations ($10^{-8}$-$10^{-9}$M), causes these multiple cellular responses remains unknown. Although the mechanism of PMA action is unknown, it appears likely that PMA binds to the cell surface and initiates one or a small number of primary responses which give rise to multiple secondary responses. In view of the diverse roles of ions as regulators of cellular metabolism, we have initiated an investigation of the effect of PMA on $Ca^{++}$ and $K^{+}$ ($Rb^{+}$) content and fluxes

[1]ABBREVIATIONS: PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium

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in non-proliferative differentiated myoblasts. We report here that PMA causes no change in $K^+$ fluxes or concentration but causes a decrease in $Ca^{++}$ efflux, influx, and intracellular content and that the decreased influx of $Ca^{++}$ is evident within the first minute of exposure to PMA. These data suggest that altered $Ca^{++}$ flux could be a primary event in PMA action in these cells.

## MATERIALS AND METHODS

PMA was obtained from Sigma Chemical Co. (St. Louis, Mo.). $^{45}Ca^{++}$ (932 mCi/mmole) and $^{86}Rb^+$ (611mCi/mmole) 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 (5), except that 2.5 ng/ml collagenase (Worthington Type III) was used in place of trypsin. Cells were plated at a density of 1.5 x $10^6$/dish on collagen-coated 25 $cm^2$ tissue culture flasks (Falcon) or 60 mm tissue culture dishes (Corning) 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 (6). Cultures were maintained at 37° in 10% $CO_2$-90% air. 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.

Measurement of relative $Ca^{++}$ levels - myoblasts were grown for 20 h in the presence of 3.3 µCi/ml of $^{45}Ca^{++}$ and were then treated with PMA dissolved in acetone (0.2% final concentration of solvent) or acetone alone. Total $^{45}Ca^{++}$ in the cells was determined by removing the labeled medium, washing the monolayers in phosphate-free balanced salt solution (7) containing either 1mM $LaCl_3$ or 2mM $CaCl_2$, and solubilizing the washed cells in 1% sodium dodecyl-sulfate for protein and radioactivity measurements. Protein was determined by the method of Lowry, et. al. (8) and radioactivity was measured by liquid scintillation counting with a carbon-14 window. Relative $^{86}Rb^+$ levels were measured by an identical procedure.

$Ca^{++}$Efflux Rates - Cells were pre-loaded for 20 h with 10-20 µCi/ml of $^{45}Ca^{++}$ and then treated for 5 h on day 3 with PMA or acetone. The monolayers were washed rapidly at 37° with phosphate-buffered saline, pH 7.4, containing 0.1 mM $Ca^{++}$ and then incubated at 37° in 3 ml fresh bicarbonate-free HEPES-buffered complete medium (0.1 mM $Ca^{++}$, 20 mM HEPES buffer, pH 7.4). Aliquots were withdrawn at appropriate intervals. At the end of the efflux period, the cells were solubilized and protein and residual radioactivity were determined. Efflux rate constants were calculated (9) from the slope x(-1) given by linear regression analysis of a plot of ln $(1-R_t/R_\infty)$ vs time, where $R_t$ is the amount of $^{45}Ca^{++}$ in the efflux medium at time t and $R_\infty$ is the total amount of $^{45}Ca^{++}$ in the medium at equilibrium (45-100 min).

$Ca^{++}$ Influx Rates - Cell monolayers in culture flasks were washed rapidly at 37° with phosphate-buffered saline (pH 7.4, 0.1 mM $Ca^{++}$) and then incubated at 37° in 2 ml fresh bicarbonate-free HEPES-buffered complete medium containing 20 mM HEPES buffer, pH 7.4, 0.1 mM $Ca^{++}$ and 7-20 µCi/ml $^{45}Ca^{++}$. At the appropriate time, the radioactive medium was removed by rapid aspiration and the cells were washed for 10 sec at 37° with a continuous stream of phosphate-free balanced salt solution containing 2mM $CaCl_2$. The cells were then solubilized for protein and radioactivity determinations. Influx rate constants were obtained in a manner identical to that used for efflux rate constants, except that $R_t$ and $R_\infty$ refer to $^{45}Ca^{++}$/mg protein in the cells at time t and at equilibrium (60 or 120 min), respectively.

$^{86}Rb^{+}$ Influx Rates - Cultures were incubated for 10 min in complete medium containing 2 μCi/ml (3.3 μM) $^{86}Rb^{+}$, after which they were washed and solubilized. Uptake was linear for at least 20 min.

## RESULTS

As shown in Table I, treatment with PMA for 5 h caused a dose-dependent decrease in the amount of cell-associated $^{45}Ca^{++}$. The decrease was found to be maximal at approximately 4 ng/ml PMA and one-half maximal at approximately 1-2 ng/ml PMA. In 14 other experiments in which myoblasts were treated with 40 ng/ml PMA for 5 h, the mean decrease in cell-associated $^{45}Ca^{++}$ was 24.5 ± 12.3% S.D. (significant at the $P < .001$ level using the paired Student's t-test). A similar decrease (not shown) was found at 2.5 h after PMA addition, suggesting a rapid onset for the alteration. Washing the cells with 2mM $LaCl_3$ instead of 2mM $CaCl_2$ prior to harvest and counting gave essentially similar results. Since $La^{+++}$ would be expected to remove any $Ca^{++}$ bound to the cell's external surface, these results suggest that PMA lowers the total intracellular $Ca^{++}$ content of treated myoblasts. Since this decreased content could have arisen from a PMA-induced decrease in cell volume with no change in $Ca^{++}$ concentration, relative cell volume was ascertained in PMA-treated and control myoblasts equilibrated either with $[^{3}H]$-$H_2O$ or $[^{3}H]$-3-O-methylglucose. These experiments revealed a variable decrease in cell volume which did not exceed 10%, even at 40 ng/ml PMA. It thus appears that PMA

TABLE I

EFFECT OF PMA CONCENTRATION ON RELATIVE $Ca^{++}$ CONTENT OF MYOBLASTS

| PMA Concentration (ng/ml) | $^{45}Ca^{++}$ Content (cpm/mg protein) | Percent of Control |
|---|---|---|
| 0 | 66432 ± 72 | 100.0 ± 0.1 |
| 1 | 66130 ± 1041 | 99.5 ± 1.6 |
| 2 | 60370 ± 3452 | 90.0 ± 5.2 |
| 4 | 56942 ± 694 | 85.7 ± 1.0 |
| 40 | 57400 ± 1429 | 86.4 ± 2.1 |

Myoblasts (50 h post-plating) were incubated for 16 h with 5 μCi/ml $^{45}Ca^{++}$ and then treated for 5 h with the indicated concentration of PMA. Calcium contents were determined as described in Materials and Methods. Each value is the mean ± S.D. for duplicate determinations.

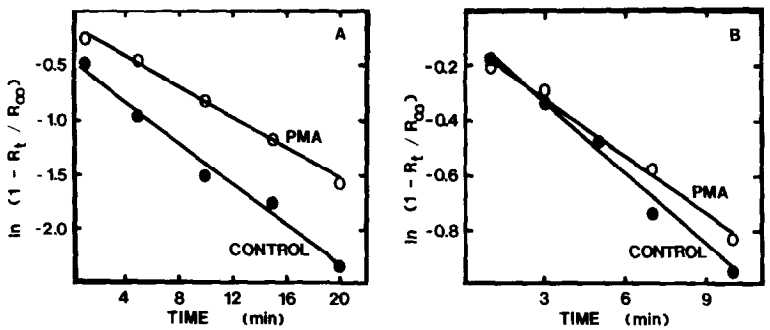


Fig. 1: Effect of PMA on $Ca^{++}$ efflux and influx. A. Efflux: Myoblasts (50 h post-plating) were incubated with 16.7 μCi/ml $^{45}Ca^{++}$ for 20 h and then treated with 40 ng/ml PMA or 0.2% acetone for 5 h. $^{45}Ca^{++}$ efflux was measured in duplicate as described in Methods. The calculated efflux rate constants are .0699 $min^{-1}$ and .0913 $min^{-1}$ for PMA-treated and control cells, respectively. B. Influx: Three-day myoblasts were treated for 5 h with 40 ng/ml PMA, after which influx of $^{45}Ca^{++}$ was measured in duplicate as described in Methods. The calculated influx rate constants are .0698 $min^{-1}$ and .0884 $min^{-1}$ for PMA-treated and control cells, respectively.

causes a decrease in $Ca^{++}$ concentration within one or more intracellular compartments. Similar experiments with $^{86}Rb^{+}$ revealed no changes (not shown).

To determine the effect of PMA on $Ca^{++}$ transport across the myoblast plasma membrane, cells were treated with 40 ng/ml PMA for 5 h and the rates of both $^{45}Ca^{++}$ efflux and influx were measured. The efflux and influx rate constants,which are proportional to the slopes x(-1) of the lines shown in Figure 1, were decreased in PMA-treated vs control myoblasts. In five separate experiments, each done in duplicate, the mean ratio of the slopes given by the efflux rate data for PMA-treated vs control cells was 0.850 ± 0.064 S.D. (statistically significant at $P < .05$ by paired Student's t-test). The mean values of $t_{1/2}$ for $Ca^{++}$ efflux from control and PMA-treated cells were 9.1 and 10.7 min respectively. Similarly, in four separate experiments, the mean ratio (PMA/control) of the slopes given by the influx rate data was 0.850 ± 0.055 S.D. ($P < .05$). The mean values of $t_{1/2}$ for control and PMA-treated cells were 8.6 min and 10.4 min, respectively. Since the outward and inward $Ca^{++}$ fluxes are proportional to the respective rate constants and the cytosolic $Ca^{++}$ concentration (9), and since the intracellular $Ca^{++}$ concentration decreases 10-20% after 5 h of exposure to PMA, it is calculated

TABLE II

EFFECT OF PMA CONCENTRATION ON $Ca^{++}$ UPTAKE

| PMA Concentration | $^{45}Ca^{++}$ Uptake (cpm/mg protein) | Percent of Control |
|---|---|---|
| 0.0 | 16969 ± 539(3) | 100 |
| 0.2 | 17815 ± 105(3) | 105 |
| 1.0 | 15728 ± 210(3) | 92.7 |
| 4.0 | 12965 ± 1002(4) | 76.4 |
| 20.0 | 12683 ± 494(4) | 74.7 |

Three-day myoblasts were washed continuously for 10 sec with phosphate-buffered saline (0.1mM $Ca^{++}$) and then incubated for 1 min at 37° in HEPES-buffered medium containing 6.7 μCi/ml $^{45}Ca^{++}$ and the indicated concentration of PMA. The cells were washed and solubilized as described in Materials and Methods. Each value is the mean ± S.D. for n = 3 or 4 (n in parentheses).

that PMA causes a 25-30% decrease in bidirectional $Ca^{++}$ fluxes in PMA-treated myoblasts.

To determine the time of onset of $Ca^{++}$ flux changes, $Ca^{++}$ influx was measured at very short times after PMA addition. In four separate experiments, each with quadruplicate measurements, $^{45}Ca^{++}$ uptake was measured during the initial 90 sec of myoblast exposure to 20 ng/ml PMA. The mean uptake (cpm/mg protein) decreased 18.9 ± 5.4% (S.D.) in PMA treated myoblasts relative to controls (significant at the $P < .01$ level by paired Student's t-test). In one experiment, uptake was reduced 13% during the initial 45 sec of PMA treatment. The dose-response for the diminution of $Ca^{++}$ influx during the first minute of PMA treatment is shown in Table II. The maximum response was obtained with 4 ng/ml PMA with a very sharp response curve between 1 and 4 ng/ml similar to the dose response shown in Table I.

$^{86}Rb^{+}$ influx was measured at various times from 0 min to 5 h after PMA addition. No changes were observed at any of these times.

## DISCUSSION

To our knowledge, this is the first report of a PMA-induced alteration in cellular $Ca^{++}$ movements or concentrations. The decrease in $Ca^{++}$ influx which occurs within the first minute after PMA addition to myoblasts is among

the most rapid cellular responses to this tumor promoter so far reported for any cell type. The concentration dependence of this response (Table II) is essentially identical to all of the other myoblast responses to PMA which have been examined (1-3), and is consistent with a possible primary role of this response in PMA action in these cells. No alterations were found in $K^+$ transport or intracellular concentration (measured with $^{86}Rb^+$) or in amino acid transport (unpublished observations). Hexose transport likewise was not altered at early times, although, as reported earlier, it increased as a secondary response which was maximal at 5 h (2,4). Thus, the $Ca^{++}$ response appears to be a specific alteration, rather than a perturbation of membrane transport systems, in general.

One of the earliest membrane changes which occurs during the mitogenic stimulation of quiescent cells by serum (10) or PMA (11) is an increase in $Rb^+$ flux. The absence of a change in $Rb^+$ transport in non-proliferative myoblasts, in which PMA is not a mitogen (1 and unpublished observations), is in accord with previous work (reviewed in 12) which suggests that a change in the activity of the $Na^+$, $K^+$ pump is required for a mitogenic response to occur.

It is widely held that $Ca^{++}$ is a key intracellular regulatory messenger. We have demonstrated that PMA rapidly alters cyclic nucleotide and polyphosphoinositide metabolism (submitted for publication) in myoblasts, both of which are thought to be regulated by $Ca^{++}$ (13,14). The present data suggest that these two responses, and perhaps some or all of the other pleiotropic responses to the tumor promoter, may result from an initial alteration in $Ca^{++}$ fluxes.

Whitfield, et. al. (15) suggested that an alteration in $Ca^{++}$ fluxes might be a primary event in the mitogenic responses of lymphocytes to PMA. The present results with myoblasts are in accord with this suggestion, although the decreased influx in myoblasts is opposite to their prediction of increased influx in PMA-treated lymphoblasts. It would appear that different cell types may respond differently with respect to $Ca^{++}$ movements following PMA treatment. Indeed, preliminary studies with PMA-treated multinucleated

myotubes indicate that intracellular $Ca^{++}$ concentration increases significantly in this cell type in response to PMA treatment. Further work with both myoblasts and myotubes will be required to more fully understand the relationship between the $Ca^{++}$ responses in these cells and the many other alterations in cellular properties which have been described.

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