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# SYNERGISTIC ACTIVATION OF RAT HEPATOCYTE GLYCOGEN PHOSPHORYLASE BY A23187 AND PHORBOL ESTER

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Summary: The combination of 1.6 μM 4β phorbol, 12β myristate, 13α acetate (PMA) and 1 μM A23187 produced a five-fold greater stimulation of rat hepatocyte glycogen phosphorylase activity than was seen with PMA alone. Vasopressin activation of glycogen phosphorylase was comparable to that seen with PMA plus A23187. Glycogen phosphorylase activity due to PMA plus A23187 was increased significantly after 30 sec, maximal at 120 and sustained at elevated levels for 240 sec. In contrast, activation due to vasopressin was maximal at 30 sec followed by a decrease. The addition of PMA 5 min prior to the A23187 abolished the synergism between these two agents. These data are compatible with the hypothesis that diacylglycerol and $Ca^{2+}$ synergistically increase glycogen phosphorylase activity in rat hepatocytes.

Introduction: In platelets, addition of the tumor-promoting agent 4β phorbol, 12β myristate, 13α acetate (PMA) results in activation of protein kinase C which is phospholipid-dependent and calcium-activated (1). Diacylglycerol or PMA activate protein kinase C (2) but do not increase free cytosolic calcium in platelets (3). Slight increases in cytosolic $Ca^{2+}$ induced by an ionophore such as A23187 or ionomycin in combination with PMA markedly increased platelet secretion (3,4). Similar synergistic effects of PMA and A23187 have been seen on insulin release by rat pancreatic islets (5), aldosterone secretion by isolated adrenal glomerulosa cells (6) and DNA synthesis by lymphocytes (7). There is an emerging view that diacylglycerol and cytosolic $Ca^{2+}$ are synergistic cellular signals (1-9). Hormones that elevate cytosolic $Ca^{2+}$ concurrently increase the level of diacylglycerol by activation of phosphoinositide breakdown via

Abbreviations used: PMA for 4β phorbol-12β myristate, 13α acetate which is also known as 12-tetradecanoyl-phorbol-13acetate.

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phospholipase C (8-10). Phosphoinositide breakdown has been linked to the receptor mechanism involved in $Ca^{2+}$ mobilization (8,10).

In rat hepatocytes, vasopressin increases the phosphorylation of 10 major proteins (11,12). Seven of these proteins are also phosphorylated in response to A23187 which elevates cytosolic $Ca^{2+}$ or by glucagon which elevates cyclic AMP. Phosphorylation of three proteins is not affected by either of the above agents but is observed after incubation of hepatocytes with 1.6 μM PMA for 90-120 sec (11,12). Garrison et al. (11,12) also reported that 1.6 μM PMA had little effect on glycogen phosphorylase activity while 10 μM A23187 mimicked the activation of glycogen phosphorylase due to vasopressin. However, the present results indicate that in the presence of 1 μM A23187, PMA synergistically increases rat hepatocyte glycogen phosphorylase activity.

Methods: Hepatocytes were isolated from fed female rats (120-160 g) of the Charles River CD strain as described by Tolbert et al. (13). The hepatocytes were incubated in Krebs-Ringer bicarbonate buffer containing 20 mM glucose for 15 min prior to the experiments. At various times after the addition of agents the reactions were terminated by the addition of 100 μl of a solution (pH 7.0) containing 50 mM EDTA, 20 mM dithiothreitol, 100 mM morpholinopropane sulfonic acid and 500 mM NaF. The samples were immediately frozen in a dry ice-ethanol bath. They were homogenized at 2-5°C with a Willems polytron (Brinkmann) for 20 sec at setting 5. The homogenates were centrifuged at 300 rpm for 10 min at 2-5°C and the pellets were discarded. The supernatant contained 3 to 5 mg of protein/ml and 10 microliters were assayed for glycogen phosphorylase activity as described by Birnbaum and Fain (14). The basal glycogen phosphorylase activity ranged from 1.1-1.6 micromoles of glucose-1-P converted to glycogen per mg of protein during a 20 min assay.

The 4β phorbol, 12β myristate, 13α acetate and 4β phorbol 13α-monoacetate were obtained from Sigma Chemical Co. and the A23187 from Calbiochem Co.. Stock solutions were prepared in DMSO at a concentration of 1 and 5 mM and diluted with water. An equal volume of DMSO was added to control tubes. Ethanol was not used as a solvent since it activates glycogen phosphorylase.

Results: The data in Table 1 indicate that after a 2 min incubation, 1 μM A23187 or 1.6 μM PMA activated by 5 and 10%, respectively, glycogen phosphorylase activity in rat hepatocytes. The combination of A23187 plus PMA increased glycogen phosphorylase activity to values approximately 5-fold greater than those observed with either agent alone (Table 1).

Table 1
Stimulation of rat hepatocyte glycogen phosphorylase activity by PMA in the presence of A23187

| Additions | % increase in glycogen phosphorylase |
|---|---|
| 1 μM A23187 | 4.7±1.5* |
| 1.6 μM, 4β phorbol, 12β myristate, 13α acetate | 10.6±3.0** |
| A23187 + phorbol ester | 48.0±6.7*** |

Rat hepatocytes (5.0 x $10^6$/tube) were incubated for 15 min prior to the addition of A23187 or 4β phorbol, 12β myristate, 13α acetate (PMA). The reactions were stopped 2 min after the addition of these agents. The values are the means ± S.E. of 16 paired experimental replications.

* Significant with $p < .01$ by t test
** Significant with $p < .005$ by t test
***Significant with $p < .001$ by t test

The data in Figure 1 compare the time course for activation of glycogen phosphorylase by A23187 + PMA as compared to A23187 + vasopressin. The increase due to vasopressin was more rapid in onset followed by a decrease in activity. In contrast, the

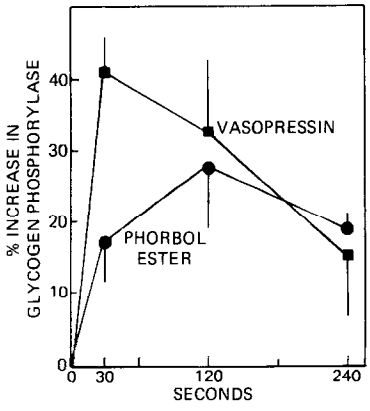


Figure 1 Time course for stimulation of glycogen phosphorylase by vasopressin and PMA.

Rat hepatocytes (5.4 x $10^6$) were incubated for 30, 120 or 240 sec in the presence of 1 μM A23187 and either 10 mU/ml of vasopressin or 1.6 μM 4β phorbol, 12β myristate, 13α acetate (PMA). The values are the mean ± S.E. of the % increases due to either agent over those due to the ionophore alone for 5 experiments. The increases in glycogen phosphorylase due to vasopressin were significant at 30 ($p < 0.001$) and 120 sec ($p < 0.02$) while those due to phorbol ester were significant at 30 ($p < 0.05$), 120 ($p < 0.025$) and 240 sec ($p < 0.001$) by paired t-test.

Table 2
Comparison of different concentrations of PMA in the presence of 0.1 or 1 μM A23187

| Additions | A23187 .1 μM | A23187 1 μM |
|---|---|---|
| | % increase in glycogen phosphorylase | |
| None | + 1.1±4.5 | + 6.0±5.8 |
| PMA .016 μM | + 3.4±6.3 | + 3.6±4.1 |
| PMA .16 μM | - 1.1±3.0 | + 8.9±1.8** |
| PMA 1.6 μM | - 0.6±4.1 | +33.8±3.7*** |
| Vasopressin 10 mU/ml | +36.3±7.8* | +38.6±9.5* |

Rat hepatocytes ($5.4 \times 10^6$) were incubated for 120 sec in the presence of 0.1 or 1 μM A23187 with the indicated concentrations of 4β phorbol, 12β myristate, 13α acetate (PMA) or vasopressin. The values are the mean ± S.E. of 6 paired replications.
* Significant with $p < 0.01$ by paired t test
** Significant with $p < 0.005$ by paired t test
***Significant with $p < 0.001$ by paired t test

effect of PMA + A23187 was maximal after 2 min and remained significantly elevated over 4 min incubation (Fig. 1).

PMA at a concentration of 0.16 μM gave an activation of glycogen phosphorylase which was 26% of that due to 1.6 μM PMA (Table 2). In both Fig. 1 and Table 2 the effects of 1.6 μM PMA + 1 μM A23187 were comparable to those produced by 10 mU/ml of vasopressin at 2 min. The data in Table 2 also indicate that 1.6 μM PMA in the presence of only 0.1 μM A23187 was ineffective as a stimulator of glycogen phosphorylase activity.

The synergistic effects of A23187 at 1 μM and PMA at 1.6 μM were not seen if the PMA was added 5 min prior to the ionophore (Table 3). However, the ability of vasopressin or forskolin to activate glycogen phosphorylase was not decreased whether PMA was added 5 min prior to or at the same time as these agents (Table 3).

The biologically inactive 4β phorbol 13α-monoacetate was tested at a concentration of 1.6 μM in the presence of 1 μM A23187 and did not increase glycogen phosphorylase or block the effect of 1.6 μM PMA. In five experiments the % increase in glycogen phosphorylase activity in the presence of 1 μM A23187 due to the inactive phorbol ester was 7% while PMA gave a 130% increase in the absence and a 169% increase in the presence of the inactive ester.

Table 3
Effect of prior incubation with PMA on the response to A23187, vasopressin and forskolin

| Additions | Without | + PMA 1.6 μM | + PMA added 5 min prior to other additions |
|---|---|---|---|
| | % increase in glycogen phosphorylase due to agents | | |
| Vasopressin 10 mU/ml | 33.1±7.1 | 42.9±14.1 | 45.5±10.0 |
| Vasopressin + A23187 1.0 μM | 34.9±6.4 | 47.8±15.0 | 54.0±12.2 |
| A23187 1.0 μM | 8.8±5.4 | 42.8±13.1 | 4.9± 4.9 |
| Forskolin 0.5 μM | 21.9±3.1 | 30.9±7.9 | 34.8± 8.1 |

Rat hepatocytes ($3.3 \times 10^6$) were incubated for 120 sec in the presence of A23187, vasopressin and forskolin. (PMA) 4β phorbol, 12β myristate, 13α acetate (1.6 μM) was either added with the agents or 5 min prior to their addition. The values are the mean ± S.E. of the % increase due to the added agents for 8 paired replications as compared to controls or PMA alone. PMA added at the same time as the other agents reduced basal phosphorylase activity by 1% while, if added 5 min prior to other agents, it reduced phosphorylase activity by 12%.

Discussion: The present data support the hypothesis that vasopressin activation of glycogen phosphorylase requires both elevation of intracellular $Ca^{2+}$ and increases in diacylglycerol derived from phosphoinositide breakdown (8-10). Vasopressin-stimulated phosphoinositide breakdown is readily detected within 7.5 sec of hormone addition to rat hepatocytes (15). The phosphorylation of soluble proteins in rat hepatocytes seen after addition of vasopressin can be reproduced by the combination of A23187 and 1.6 μM PMA (11,12). Possibly one of the unique proteins phosphorylated by protein kinase C is involved in glycogen phosphorylase activation through a direct or indirect mechanism. The hypothesis that phorbol ester action involves protein kinase C activation is supported by the recent findings that the receptors for phorbol ester co-purify with protein kinase C in soluble extracts of brain (16,17).

The concentrations of PMA which are sufficient in the presence of A23187 or some other calcium ionophore to affect lymphocyte activation (7), aldosterone secretion (6), insulin secretion (5) and platelet secretion (3,4) are 0.1, 0.005, 0.1 and 0.02 μM, respectively. These concentrations are all lower than those used in the present studies with hepatocytes where 0.16 μM PMA was ineffective. However, 1.6 μM PMA was the

concentration used in the experiments on protein phosphorylation (11,12). It is unclear why higher concentrations of PMA are required in hepatocytes, but the same concentration of an inactive phorbol ester did not affect glycogen phosphorylase activity.

If PMA was added 5 min prior to the A23187, there was no activation of glycogen phosphorylase. However, prior incubation with PMA did not reduce the response to vasopressin or forskolin (Table 3). Forskolin increases hepatocyte glycogen phosphorylase activity and cyclic AMP at concentrations comparable to those required for elevation of both parameters by isoproterenol (18). These data indicate that in rat hepatocytes synergistic activation of glycogen phosphorylase by A23187 and PMA is only seen if these agents are added at the same time.

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