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# Phorbol 12-myristate 13-acetate, ionomycin or ouabain, and raised extracellular magnesium induce proliferation of chicken heart mesenchymal cells

(diacylglycerol/kinase C/calcium/mitogenesis/*onc* genes)

SAMUEL D. BALK, ANDREA MORISI, AND HARRIET S. GUNTHER

Department of Pathology and Cancer Research Institute, New England Deaconess Hospital, Boston, MA 02215; and Department of Pathology, Harvard Medical School, Boston, MA 02115

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ABSTRACT **Cultured chicken heart mesenchymal cells are proliferatively quiescent at low densities in medium containing plasma at 10%. Mitogenic hormones like epidermal growth factor and insulin-like growth factors cause these cells to proliferate very actively, as does infection with avian sarcoma viruses, erythroblastosis virus, or myelocytomatosis virus. We have found that the combination of phorbol 12-myristate 13-acetate (PMA), ionomycin or ouabain, and raised extracellular magnesium, likewise, causes these cells to proliferate very actively. Although these agents have no significant effect when acting singly, the combination of PMA at 100 ng/ml and 0.5 μM ionomycin induces a 6-fold increase in cell number at 4 days, and the combination of PMA, ionomycin, and 5.6 mM magnesium induces 12-fold multiplication. Likewise, PMA plus 1 μM ouabain induces 3-fold multiplication, whereas the combination of PMA, ouabain, and magnesium induces 6-fold multiplication. The tumor promoter PMA, like diacylglycerol released by breakdown of plasma membrane phosphatidylinositol diphosphate, is known to activate the serine- and threonine-specific intracellular enzyme kinase C. The divalent cation ionophore ionomycin is known to carry calcium into cells down an electrochemical gradient, and the $Na^+,K^+$-ATPase inhibitor ouabain appears to elevate intracellular calcium by means of a sodium-mediated exchange mechanism. Magnesium, like calcium, is known to enter cells passively down an electrochemical gradient and to be involved in the regulation of many key intracellular reactions. Our findings with PMA, ionotropes, and magnesium support a hypothesis that diacylglycerol-mediated activation of kinase C plus cellular divalent cation influx and/or mobilization, caused by the action of mitogenic hormones or the protein products of *onc* genes, are key events in the initiation of cell replication.**

Cell replication is initiated when mitogenic hormones bind to their receptors or, it would appear, when the protein products of viral or cellular *onc* genes themselves activate critical steps in the mitogenic hormone–hormone receptor cascade (1). Cell replication induced by exogenous mitogenic hormones represents appropriate ("normal") proliferation, whereas replication induced by the protein products of *onc* genes represents the autonomous or mitogenic hormone-independent proliferation that characterizes neoplasia (2). Examples of the latter include proliferation resulting from the production of a platelet-derived growth factor-like protein by the v-*sis* gene of simian sarcoma virus (3) and proliferation apparently induced by the truncated, constitutively active epidermal growth factor (EGF) receptor, or closely related protein, encoded by the *erb*B gene of avian erythroblastosis virus (4). Therefore, both appropriate and autonomous initiation of cell replication appear to involve the same complex of intracellular messengers and messenger-activated effectors.

A considerable amount of evidence suggests that breakdown of plasma membrane phosphatidylinositol 4,5-diphosphate, with release of diacylglycerol as an intracellular messenger, is a key event that follows the binding of many hormones to their receptors (5, 6). Diacylglycerol, in turn, appears to activate the intracellular enzyme kinase C, a phospholipid and calcium-dependent enzyme that phosphorylates proteins on serine and threonine residues and is believed to be involved in the activation of many critical metabolic processes (6). Current evidence indicates that the tumor promoter phorbol 12-myristate 13-acetate (PMA) serves as a surrogate for diacylglycerol by activating kinase C in the presence of a physiological intracellular concentration of calcium (6). Additional current evidence indicates that $p60^{src}$, the kinase encoded by the *src* gene of Rous sarcoma virus (RSV), is capable of phosphorylating phosphatidylinositol to phosphatidylinositol 4,5-diphosphate, a prerequisite for the generation of diacylglycerol (7). The kinase $p60^{src}$ is known to phosphorylate intracellular proteins on tyrosine residues as do the kinase activities associated with many hormone receptors. The finding that $p60^{src}$ is capable of phosphorylating phosphatidylinositol to phosphatidylinositol diphosphate raises the possibility that this *onc* gene product functions by increasing the generation of diacylglycerol and the activity of kinase C as well as by tyrosine-specific protein phosphorylation (7).

In 1971, one of us (S.D.B.) discovered that chicken pectoral muscle fibroblasts reversibly ceased to proliferate in plasma-containing culture medium of radically reduced calcium concentration, while the proliferation of RSV-infected pectoral muscle fibroblasts was essentially unaffected (8, 9). This observation, which has been confirmed in a number of systems (10), formed the basis for a hypothesis that intracellular calcium was involved in the initiation of cell replication and that a failure of intracellular calcium homeostasis contributes to the autonomous proliferation that characterizes the neoplastic state. Other investigators then demonstrated that lowered culture medium magnesium caused reversible arrest of proliferation of chicken embryo fibroblasts and hypothesized that this ion, which, like calcium, tends to enter cells passively down an electrochemical gradient and regulates the activities of many key intracellular reactions, is involved in mitogenesis (11). We subsequently demonstrated that lowering of extracellular magnesium, like calcium, selectively inhibits the proliferation of normal, as compared to RSV-infected, chicken pectoral muscle fibroblasts (12). When both calcium and magnesium were reduced in plasma-

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; RSV, Rous sarcoma virus; EGF, epidermal growth factor.

containing medium, more modest reductions were required to arrest the proliferation of normal pectoral fibroblasts than were required when the respective ions were reduced singly; the proliferation of RSV-infected fibroblasts was, again, essentially unaffected. We hypothesized, accordingly, that both divalent ions are involved in initiation of cell replication and that failure of intracellular divalent cation homeostasis contributes to the autonomous proliferation of neoplastic cells (12).

We have subsequently developed a culture system in which chicken heart mesenchymal cells are proliferatively quiescent, at low culture densities, in plasma-containing medium of *physiological* ion concentrations (13–15). Unlike chicken pectoral muscle fibroblasts and chicken embryo fibroblasts (13), the proliferative quiescence of chicken heart mesenchymal cells does not require that culture medium divalent cation concentrations be lowered, thereby eliminating potential artifactual and nonphysiological effects like increased cell membrane permeability due to decreased extracellular calcium concentration. The proliferative quiescence of low-density chicken heart mesenchymal cells in plasma-containing medium is, likewise, not based on the use of nonphysiological culture conditions like serum starvation or density-dependent inhibition (16). Chicken heart mesenchymal cells proliferate very actively when exposed to insulin-like growth factors and EGF or brain fibroblast growth factor (17) or when infected with avian sarcoma viruses, avian erythroblastosis virus or avian myelocytomatosis virus (18).

Proliferative quiescence under physiological culture conditions makes chicken heart mesenchymal cells well suited for test of intracellular messengers that are hypothesized to initiate cell replication in response to mitogenic hormones or to the protein products of viral *onc* genes. The divalent cation-lowering experiments that have been done with fibroblasts (8–12) have been liable to indirect effects of ion reduction and would not detect the existence of a diacylglycerol messenger. Chicken heart mesenchymal cells, on the other hand, are proliferatively quiescent without manipulation of culture conditions and so will replicate only when stimulated with all, or at least most, of the required complex of intracellular messengers or their surrogates. We report here the initiation of replication of chicken heart mesenchymal cells by the combination of the diacylglycerol surrogate PMA, the ionotropes ionomycin or ouabain, and hyperphysiological extracellular magnesium.

## MATERIALS AND METHODS

Our methods and synthetic medium for culturing chicken heart mesenchymal cells have been published (13–15, 17, 18). Primary cultures were prepared by using synthetic medium with heparinized, heat-inactivated rooster plasma at 5%. Ventricles from the hearts of two 8- to 12-week-old SPF-Cofal-negative cockerels (SPAFAS, Norwich, CT) were enzymatically dissociated (0.1% trypsin/0.05% collagenase). The suspension of cells and tissue debris obtained by the enzymatic dissociation was centrifuged and the pellet was resuspended in plasma-containing medium and used to seed 60-mm Falcon tissue culture dishes. After a 3-hour attachment period in the incubator, the cultures were washed four times with a physiological electrolyte solution and changed to fresh, plasma-containing medium. During the 3-hour attachment period, chicken heart mesenchymal cells attach to the culture dish, whereas myocytes, capillary segments, and debris do not. Therefore, after washing there remains an essentially pure population of chicken heart mesenchymal cells. A few dishes from each set of primary cultures were inoculated with RSV (Schmidt–Ruppin strain) on the day following their preparation.

All cultures were incubated in a Napco model 6300 incubator at 42°C in a humidified 95% air/5% $CO_2$ atmosphere. During the first 3 days after preparation of primary cultures, EGF at 100 ng/ml and insulin at 1 $\mu$g/ml were included in the plasma-containing medium. On the third day after preparation, primary cultures were changed to plasma-containing medium without added mitogenic hormones. Four days after preparation, the primary cultures of normal and RSV-infected chicken heart mesenchymal cells were subcultured for experiments, again by using medium containing plasma at 5%, into replicate secondary 35-mm Falcon culture dishes.

Heparinized, heat-inactivated rooster plasma was prepared by collecting rooster blood in 10-ml siliconized tubes, each containing 0.8 ml of 25 mM EGTA and 0.1 ml of heparin at 0.25 mg/ml. By this modified method, rooster plasma, after heat inactivation and stoichiometric recalcification, contained heparin at a concentration of 5 $\mu$g/ml.

Experiments were begun on the day following subculture. All experimental culture media contained heparinized, heat-defibrinogenated rooster plasma at 10%. Two milliliters of experimental culture medium was used per 35-mm dish; media were changed on days 2 and 3. Cells were counted with a Coulter electronic cell counter. Cell proliferation was determined over 4-day periods. This period was chosen because RSV-infected cells, the maximal proliferation standard for these experiments, reach saturation density during this time.

PMA (Sigma) was diluted into experimental medium from a 200 $\mu$g/ml stock in dimethyl sulfoxide ($Me_2SO$) to yield our highest experimental concentration of 200 ng/ml as was ionomycin (Squibb) from a 1 mM $Me_2SO$ stock to yield our highest experimental concentration of 1 $\mu$M. The solvent $Me_2SO$, at a dilution of 1:500 (0.2%, vol/vol), was without effect on chicken heart mesenchymal cells. Ouabain (Sigma) was diluted from a 2 mM stock in water to yield our highest experimental concentration of 2 $\mu$M. Receptor-grade EGF was purchased from Collaborative Research (Waltham, MA), and crystalline bovine insulin was purchased from Sigma. Synthetic medium in which 106 mM NaCl was isoosmotically replaced with 71 mM $MgCl_2$ was used for the preparation of experimental media of hyperphysiological magnesium concentrations: magnesium-enriched (71 mM) synthetic medium was combined with standard synthetic medium (mg, 0.68 mM) and plasma (Mg, 1.0 mM) to yield experimental media of desired magnesium concentrations. Identical experimental results were obtained when $MgCl_2$ was isoosmotically substituted for NaCl, as above, or when $Mg^{2+}$ was simply added from a 1 M $MgCl_2$ stock.

## RESULTS

Fig. 1 represents the results of a single experiment that examines the effects of PMA, ionomycin or ouabain, and raised extracellular magnesium on the proliferative behavior of low-density chicken heart mesenchymal cells in medium containing plasma at 10%.

PMA, ionomycin, ouabain, or raised extracellular magnesium, acting singly, induced no significant proliferative activity. The combination of PMA at 100 ng/ml (162 nM) and ionomycin at 0.5 $\mu$M, their optimally effective concentrations, induced a 6-fold increase in cell number after 4 days of incubation. The combination of PMA, ionomycin, and 5.6 mM extracellular magnesium induced a 12-fold increase in cell number. Magnesium at 5.6 mM represents eight times the physiological extracellular magnesium concentration (0.7 mM). We have determined (data not shown) that magnesium is optimally active at this level.

The combination of PMA at 100 ng/ml and ouabain at 1 $\mu$M induced a 3-fold increase in cell number after 4 days of

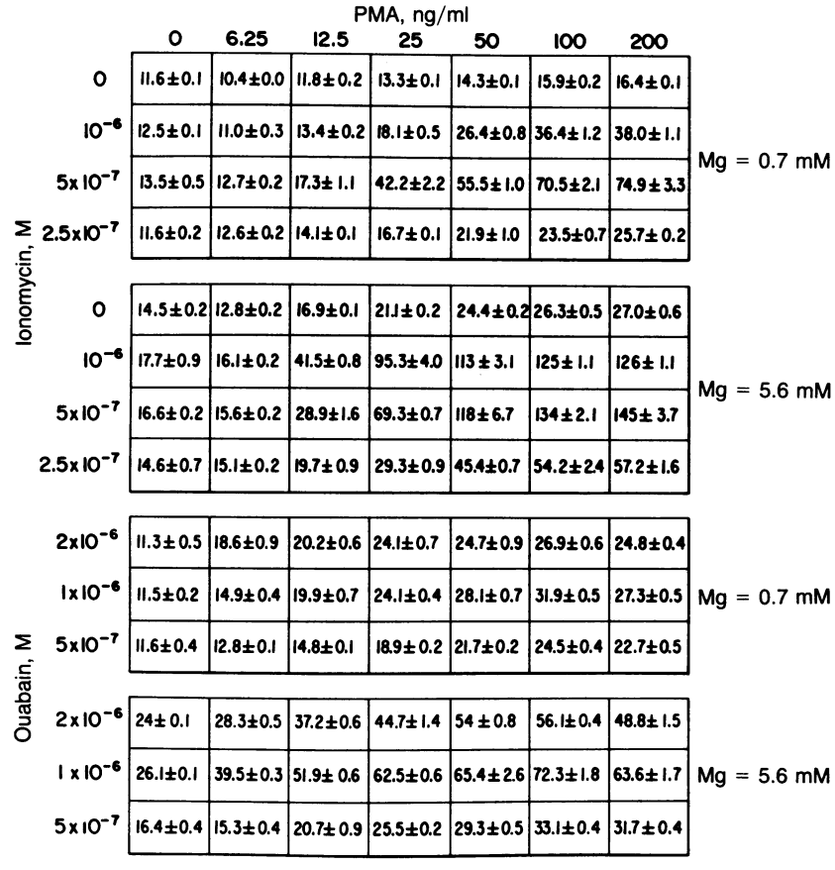


FIG. 1. A single experiment examining the effects of PMA, ionomycin or ouabain, and raised extracellular magnesium on the proliferative behavior of low-density chicken heart mesenchymal cells in medium containing chicken plasma at 10%. Each datum represents the mean number of cells ± SEM × $10^{-4}$, from three replicate 35-mm secondary experimental culture dishes counted with a Coulter electronic cell counter after 4 days of incubation. At the beginning of the experiment (day 0), there were 8.4 ± 0.2 × $10^4$ cells per dish. After 4 days of incubation in the presence of EGF at 1 μg/ml there were 99.3 ± 2.0 × $10^4$ cells per dish. Insulin at 10 μg/ml yielded 24.3 ± 0.4 × $10^4$ and EGF plus insulin yielded 371 ± 5.4 × $10^4$ cells per dish. RSV-infected chicken heart mesenchymal cells increased from 2.9 ± 0.1 × $10^4$ per dish on day 0 to 546 ± 20 × $10^4$ per dish on day 4. Culture media were changed on the second and third days of incubation. Preliminary titrations of PMA vs. ionomycin or ouabain vs. culture medium magnesium indicated that magnesium is optimally stimulatory at 5.6 mM, eight times the physiological extracellular concentration. This experiment was repeated in excess of three times.

incubation, whereas the combination of PMA, ouabain, and raised extracellular magnesium induced a 6-fold increase.

EGF at 1 μg/ml induced a 9-fold multiplication of chicken heart mesenchymal cells during 4 days of incubation, and insulin at 10 μg/ml, a somatomedin surrogate, induced a 2-fold increase. EGF and insulin together induced a 32-fold increase. Chicken heart mesenchymal cells that had been infected with RSV in primary culture increased 188-fold during 4 days of secondary culture in plasma-containing medium in the absence of added mitogenic hormones. (Experimental cultures of RSV-infected cells had been seeded at one-third the density of normal cells and so could proliferate more extensively before attaining saturation density.) Cells that had been induced to proliferate with PMA, ionomycin or ouabain, and raised extracellular magnesium, like hormone-stimulated cells or RSV-infected cells, manifested exponential proliferation kinetics during these experiments.

PMA became toxic to chicken heart mesenchymal cells at 1 μg/ml. Other β-phorbol tumor promoters were considerably less active than PMA in acting with ionotropes (ionomycin or ouabain) to induce cell proliferation. β-Phorbol didecanoate, for example, had activity comparable to PMA at 100 ng/ml (162 nM) only at a concentration of 1100 ng/ml (1620 nM). α-Phorbol esters had no proliferation-inducing activity. Ionomycin became toxic at concentrations in excess of 2 μM, ouabain at concentrations in excess of 4 μM, and extracellular magnesium at concentrations in excess of 22 mM. The combination of ionomycin and ouabain was less active in inducing cell replication than ionomycin alone. A23187, a standard divalent cation ionophore, was toxic to chicken heart mesenchymal cells at 10 μM, whereas lower concentrations failed to act in concert with PMA and raised extracellular magnesium in the induction of cell replication. The sodium ionophore monensin was toxic at 1 μM and failed to manifest replication-inducing activity at lower concentrations.

## DISCUSSION

The phorbol ester PMA appears to act as a surrogate for diacylglycerol in the activation of the intracellular serine- and threonine-specific enzyme kinase C (6). The divalent cation ionophore ionomycin promotes the entry of calcium and magnesium into cells down their electrochemical gradients (19). The cardiac glycoside ouabain inhibits the plasma membrane $Na^+,K^+$-ATPase, raises intracellular $Na^+$, and, in so doing, raises intracellular $Ca^{2+}$ by means of the $Na^+/Ca^{2+}$ exchange mechanism or by displacement of bound calcium (20). Because, unlike the calcium gradient, the electrochemical gradient favoring the movement of magnesium into cells is not steep (21), elevation of extracellular magnesium can be expected to have a significant effect in

promoting influx of this ion. (The extracellular concentrations of ionized calcium and magnesium are both ≈1 mM; intracellular ionized magnesium is ≈0.1 mM, whereas intracellular ionized calcium is ≈0.1 μM.) The electrochemical gradient for calcium is so steep that elevation of extracellular calcium would probably have little effect on net movement of that ion into cells. In addition, culture medium calcium cannot be raised above physiological levels without causing the formation of calcium phosphate precipitates.

Our observation that the combination of PMA, ionomycin or ouabain, and raised extracellular magnesium induces proliferation of chicken heart mesenchymal cells supports a hypothesis that diacylglycerol and divalent cations are important intracellular messengers in the initiation of cell replication. The proliferation-inducing effect of ionomycin, whose dominant effect would be exerted on the more steeply poised calcium movement as well as the activity of ouabain, which elevates intracellular $Ca^{2+}$ without elevating intracellular $Mg^{2+}$, speaks for the importance of $Ca^{2+}$ in the initiation of cell replication. Elevated intracellular magnesium, presumably a consequence of elevation of extracellular magnesium, may contribute to initiation of cell replication by virtue of the effect of this ion on many key metabolic activities. Alternatively, elevated intracellular magnesium may displace bound intracellular calcium and so act indirectly by means of that ion (21).

The putative intracellular messenger diacylglycerol, as noted in the Introduction, appears to be generated by hormone-induced breakdown of phosphatidylinositol diphosphate (5). EGF, for example, appears to enhance phosphatidylinositol turnover in A-431 cells (22), and the transforming protein $p60^{src}$ of RSV, again as noted earlier, appears to phosphorylate phosphatidylinositol to polyphosphoinositol, a prerequisite for generation of diacylglycerol (7).

Four hypotheses are extant concerning the mechanism of hormone-induced calcium influx or mobilization: (*i*) that inositol 1,4,5-triphosphate, the second product of hydrolysis of phosphatidylinositol diphosphate, mobilizes intracellular calcium (5); (*ii*) that phosphatidic acid formed by phosphorylation of diacylglycerol mobilizes intracellular calcium (23); (*iii*) that binding of hormones directly activates calcium channels in the plasma membrane (24); (*iv*) that polyamines, synthesized as a result of hormone binding, stimulate calcium influx or intracellular calcium mobilization (25). Little is known about the mechanisms that regulate intracellular magnesium concentration.

A23187, a standard divalent cation ionophore, has been shown to be comitogenic with PMA for lymphocytes (26). We have found that A23187, unlike ionomycin, does not act with PMA and raised extracellular magnesium to induce replication of chicken heart mesenchymal cells. The probable explanation for this difference in effect is that only one molecule of ionomycin is required to bind and carry a molecule of $Ca^{2+}$, whereas two molecules of A23187 are required (19). This difference makes ionomycin a more effective mobile carrier for calcium than A23187. Although the combination of PMA, ionomycin, and raised extracellular magnesium induces chicken heart mesenchymal cells to proliferate at a rate comparable to that induced by EGF (a 9-fold increase in 4 days), this rate does not equal the rate of proliferation induced by EGF plus hyperphysiological insulin (32-fold increase) or by RSV infection (188-fold increase). It is possible that the use of divalent cation ionophores that are still more-effective mobile calcium carriers than ionomycin may induce proliferation of chicken heart mesenchymal cells at rates comparable to our maximal proliferation standards.

The resumption of proliferative activity that follows relief of fibroblasts from serum starvation has been reported by others to be associated with sodium influx and proton efflux that leads to intracellular alkalinization. Recently published studies suggest that this $Na^+/H^+$ exchange may be triggered by increased intracellular calcium activity (27).

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