Morphological Effects of Protein Kinase C Activators in Mouse Late Morulae

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Abstract: *The effects of activators of protein kinase C (PKC) on the development of mouse late morulae were examined by morphological techniques. Late morulae were cultured with low levels of the PKC activators phorbol 12-myristate 13-acetate (PMA; 0.01-1 nM) and 1-oleoyl 2-acetyl-sn-glecerol (OAG; 0.1-10 μM) for 24 h, their blastulation ratios were determined and morphological changes in intracellular distribution of actin filaments were examined. PMA treatment induced the decompaction of morulae and delayed blastocoel formation. OAG treatment induced delayed embryonic development. Both PMA and OAG treatment induced reorganization of actin filaments in late morulae. It is assumed that PKC activation induces reversible arrest of embryonic morphogenesis in mouse late morulae.*
Key words: *Protein kinase C activators, Mouse morulae, Decompaction, Blastocoel formation, Actin filament.*

Compaction is a morphogenetic process consisting of a major rearrangement of cell structure and intercellular interactions which occurs in mouse embryos at the 8-cell stage and is a prerequisite for morula to blastocyst transition. Initially rounded blastomeres adhereto and flatten onto each other [1]. Compaction is mediated by the Ca^{2+}-dependent cell-cell adhesion molecule E-cadherin requiring extracellular Ca^{2+} at millimolar concentrations [2]. Accompanying these changes in surface properties, each blastomere becomes polarized along the radial axis determined by the position of cell-cell contacts [3]. Compaction requires the polarized distribution of cytoplasmic microfilaments, i.e. actin filaments, and is reversible by disruption of microfilaments [4–7]. It has been reported that application of activators of protein kinase C (PKC) to the culture medium can cause compaction-like adhesion of blastomeres in 2-cell, 4-cell and early 8-cell mouse embryos [8, 9], and that microfilaments are involved in the adhesion of cells induced by PKC activators [9, 10]. Up to the early 8-cell stage, interphase cells showed limited disruption and loss of microvilli when exposed to the PKC activator phorbol 12-myristate 13-acetate (PMA), and foci of polymerized actin filaments remain visible in the cortex of embryos [10], but many microvilli are conserved and few polymerized actin filaments remain in the cytocortex in compacted 8-cell embryos cultured with PMA.

In the present study we morphologically assessed the effects of low levels of PKC activators on the development of mouse late morulae. Mouse embryos were cultured with PMA and 1-oleoyl 2-acetyl-sn-glecerol (OAG) for 24 h from the late morula stage, and then the blastulation rate and changes in actin filament distribution in the embryonic cells were morphologically determined.

Materials and Methods

Preparation of culture medium: PMA (Sigma Chemical Co., St. Louis, MO, USA) dissolved in ethanol (EtOH; Wako Pure Chemical Co., Osaka, Japan) at a concentration of 1 mM and OAG (Sigma) dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan) at concentration of 10 mM were diluted in embryo culture medium to the desired concentrations before use. In vehicle only control cultures, embryos were exposed to 0.1% (v/v) EtOH and 0.1% DMSO (v/v), respectively. Non-treatment controls were cultured without any vehicle.

Recovery of embryos: Female and male ICR mice purchased from Clea Japan (Tokyo, Japan) were housed in group cages under conditions of controlled temperature (22 ± 2°C, RH 55 ± 5%) and lighting (12L/12D).

Received: December 10, 1996
Accepted: February 7, 1997
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They were fed a standard laboratory pellet diet (CE-2, Clea Japan) and water *ad libitum*. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985). Female mice were induced to superovulate by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG; 7.5 IU/mouse, Teikokuzoki Co., Tokyo, Japan) followed by intraperitoneal injection of human chorionic gonadotropin (hCG; 7.5 IU/mouse, Sankyo Co., Tokyo, Japan) after 48 h. Females were mated with male mice overnight and checked for vaginal plugs the following morning. Late morulae were recovered at 77 h post-hCG administration by flushing the uterus with HEPES buffered medium-2 containing 4 mg/ml bovine serum albumin (BSA; Sigma) (M-2 + BSA). Recovered embryos were cultured in a drop (0.3 ml) of medium-16 containing 4 mg/ml BSA (M-16 + BSA) on culture dishes (Falcon 3912, Oxnard, CA) for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

Treatment of embryos: After preincubation, embryos (10 embryos/drop) were incubated with 0 (EtOH vehicle control), 0.01, 0.1 or 1.0 nM PMA or with 0 (DMSO vehicle control), 0.1, 1.0, or 10 μM OAG for 24 h. The blastocoel formation rate was assessed under a phase contrast microscope (Olympus, Tokyo, Japan) as percentages of morulae with a blastocoel after 0, 1, 4, 8, 12, 16, 20 and 24 h of incubation. The experiments were repeated three times.

Immunohistochemistry: Embryos were incubated with PKC activators as described above and fixed in 3.5% paraformaldehyde (Wako) in phosphate buffered saline (PBS, pH 7.4) at room temperature (RT; 23 ± 2°C) for 20 min. After washing with PBS, the embryos were incubated with rabbit anti-actin antibody (Sigma) diluted 1:50 with PBS containing 0.5% BSA and 0.01% NaN_3 at 4°C for 18 h. They were rinsed with PBS containing 0.25% Tween-20 (Sigma) (PBS-Tw), and then treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H and L chains) antibody (1/400 dilution; Sigma) at RT for 90 min. The embryos were washed gently 5 times for 15 min with PBS-Tw, and were then placed in the center of vaseline spots on a slide. A cover slip was then carefully placed on the vaseline spots and pressed down gently to anchor the embryos between the cover slip and the slide glass. The embryos were examined under a fluorescent microscope (BH2-RFC, Olympus, Tokyo, Japan) and with a confocal laser scanning microscope (LSM 410, Carl Zeiss, Oberkochen, Germany).

Statistical analysis: χ^2 analysis for blastocoel formation and Wilcoxon's signed rank test for morphological estimation were carried out with the Statview IV program in a Macintosh computer. Differences at a probability of $P<0.05$ were considered significant. All data are shown as the means ± SD.

Results

Effects of PKC activators on blastocoel formation: The time courses of blastocoel formation rate in morulae are shown in Fig. 1A and B. In non-treated and vehicle control groups, blastocoel formation began within 1 h. Blastocoel formation also began within 4 h in 0.01 and 0.1 nM PMA-treated groups (Fig. 1A) and in all OAG-treated groups (Fig. 1B).

After 4 h of incubation, vehicle control embryos had already formed cavities (Fig. 2A), but 1.0 nM PMA-

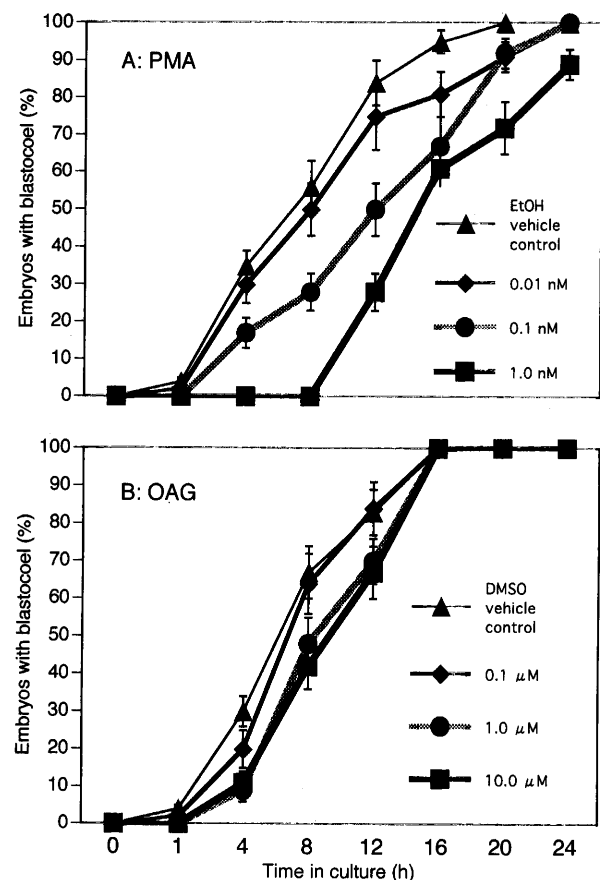

Fig. 1. Time courses of blastocoel formation in embryos treated with (A) PMA and (B) OAG. Late morulae were obtained 77 h after hCG administration and were cultured as described in experimental procedures.

treated embryos (Fig. 2B) were partially decompacted and 10 μM OAG-treated embryos (Fig. 2C) remained compacted. After 8 h of exposure, vehicle control embryos (Fig. 2D) increased their blastocoel volume, but 1.0 nM PMA-treated embryos (Fig. 2E) were partially decompacted and 10 μM OAG-treated embryos (Fig. 2F) remained compacted. In the 1.0 nM PMA-treated group, delayed blastocoel formation was shown in all

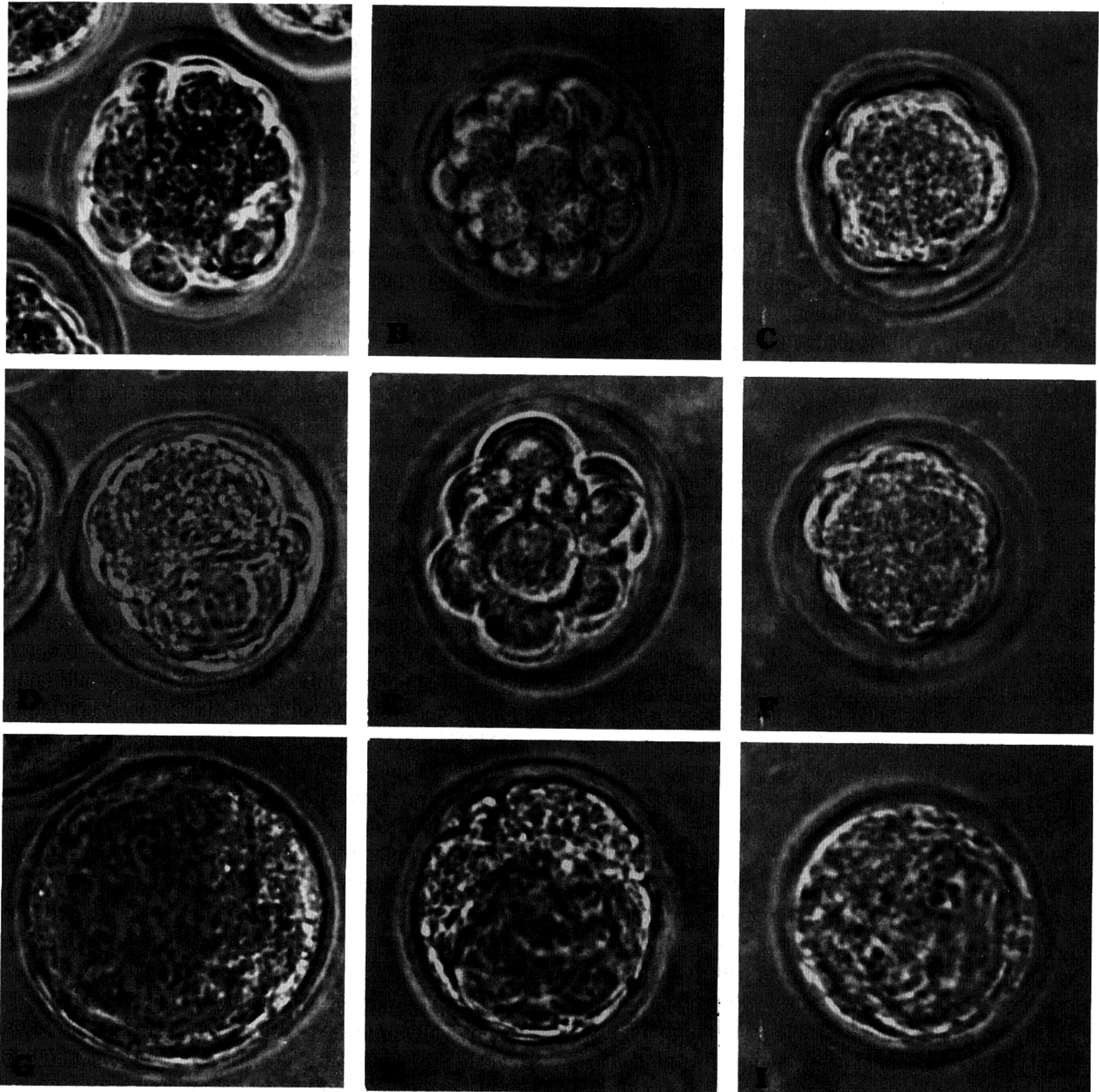

Fig. 2. Effects of PMA and OAG on blastulation. Late morulae were cultured in control media (A, D, G), 1.0 nM PMA (B, E, H) or 10 μM OAG (C, F, I). After 4 h incubation, control embryos (A) had already formed cavities (arrowed), whereas PMA-treated embryos (B) were partially decompacted and OAG-treated embryos (C) remained compacted. After 8 h of exposure, control embryos (D) showed an increase in blastocoel (arrowed) volume, while PMA-treated embryos (E) were partially decompacted and OAG-treated embryos (F) remained compacted. After 24 h of treatment, all embryos except for some in the 1 nM PMA group formed blastocysts. Control embryos (G) formed fully expanded blastocysts, while PMA-treated embryos (H) had an irregular appearance and OAG-treated embryos (I) were not expanded. Original magnification, × 400.

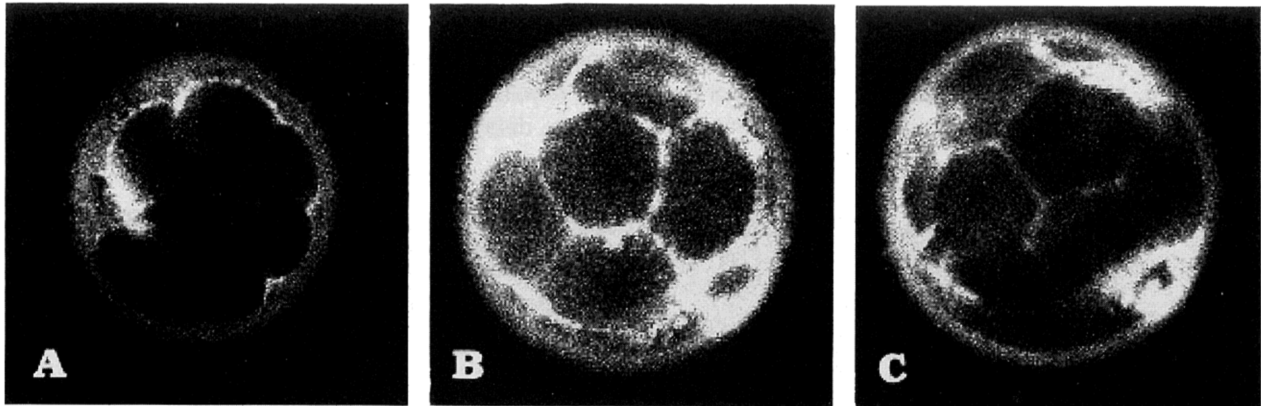

Fig. 3. Composite confocal images of mouse embryos. The embryos were stained with rabbit anti-actin-antibody and FITC-conjugated goat anti-rabbit IgG to show the distribution actin filaments. The embryos were optically sectioned at 2 μm and nine serial images were generated under a confocal microscope. In vehicle control embryos, anti-actin-antibody-positive filaments were abundant in the peripheral cytoplasm of outer flattened blastomeres facing the zona pellucida (A). In contrast, in 1.0 nM PMA- (B) and 10 μM OAG (C)-treated embryos, many actin fibers were observed in regions of cell-cell adhesion. Original magnification, × 400.

embryos by 8 h. The embryos incubated with 1 and 10 μM OAG showed delayed blastocoel formation. At the end of the experimental period (after 24 h of treatment), all embryos in vehicle (Fig. 2G) and 10 μM OAG-treated groups (Fig. 2I), but not in the 1 nM PMA-treated group (Fig. 2H), developed to blastocysts. In the 1 nM PMA-treated group, most of the embryos had an irregular appearance, had degenerating cells and were not expanded. In 0.1, 1.0 and 10 μM OAG-treated groups, all embryos contained blastocoels by 16 h, but most of the embryos incubated with 10 μM OAG were not expanded by 24 h.

Localization of actin filaments: In non-treated and vehicle controls, all embryos were still tightly compacted after 1 h incubation. In contrast, all embryos incubated with 1.0 nM PMA for 1 h were completely decompacted. Embryos treated with 10 μM OAG for 1 h were partially decompacted. In non-treated (data not shown) and vehicle control embryos, anti-actin-antibody-positive filaments were abundant in the peripheral cytoplasm of outer flattened blastomeres facing the zona pellucida (Fig. 3A). In PMA- and OAG-treated embryos, many actin fibers were observed at regions of cell-cell adhesion (Fig. 3B and C, respectively).

Discussion

It has been reported that activation of PKC induces premature compaction in 2-cell and 4-cell stage mouse embryos [8, 11].The responses of embryos to different PKC activators are varied. PMA induces compaction, and then causes a prolonged decompaction phase. OAG induces only the compaction phase [8]. PMA is a stronger PKC activator than OAG because the former is not metabolized within cells and continuously activates PKC. Such chronic activation of PKC may lead to the reduction of PKC production. Since OAG, a diacylglycerol, is rapidly metabolized in the cells, this reagent causes transient activation of PKC [12].

In the present study, late morulae treated with PMA and OAG showed delayed blastocoel formation. After 1 h incubation, 1 nM PMA-treated embryos were completely decompacted, and the decompaction phase continued for 8 h after the beginning of treatment. When treated with 1 and 10 μM OAG, blastocoel formation of embryos was slightly delayed, similarly to that in PMA-treated embryos, but decompaction was not observed in OAG-treated embryos. Decompaction induced by PMA treatment is considered to be the result of the reduction of PKC production [9]. The delayed blastocoel formation induced by OAG is considered not to depend on the reduction of PKC production but on the transient activation of PKC. Blastocoel formation and expansion may be caused by activation of Na^+/K^+-ATPase [13] and adenylate cyclase [14]. PKC activators decrease Na^+/K^+-ATPase [15] and adenylate cyclase activities [16]. Our results are in agreement with these reports.

PMA has a variety of effects on the actin filament organization in various cells in culture [17–19], unfertil-

ized ova [20], and preimplantation mouse embryos [10]. In mouse embryos, the effects of PMA on the cytoskeleton depend on the developmental stage. PMA does not affect actin organization up to the early 8-cell stage. When late 8-cell embryos with compaction were exposed to culture medium containing PMA, most microvilli were lost and few polymerized actin filaments remained in the cytocortex [10]. In the present study, however, we observed thick ribbon-like actin filaments at the periphery of the cytoplasm in regions of cell-cell adhesion after exposures to PMA and OAG. These differences in the effects of PMA and OAG might be dependent on the embryonic developmental stage, i.e. differences between early and late morulae, respectively. In conclusion, the present findings demonstrated that PKC activation induces reversible arrest in embryonic morphogenesis in mouse late morulae.

Acknowledgments

This work was supported by a Grant-in-Aid to N.M., and H.M. from the Ministry of Education, Science and Culture of Japan, by a Grant to N.M. from the Inamori Foundation, and by a Grant to N.M. from the Itoh Memorial Foundation.

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