Involvement of the Cyclic AMP-Protein Kinase A Pathway in Gap Junctional Communication in Preimplantation Mouse Embryos

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In this study, we have examined the role of cAMP in gap junctional communication (GJC) in preimplantation mouse embryos. GJC was monitored by Lucifer Yellow (LY) injected into one blastomere of compacted embryos. The speed of GJC was defined as the time taken for the last blastomere of the embryo to become visibly fluorescent. The median time for 8-cell embryos (140 sec) was similar to that for 16-cell (135 sec). To determine whether cAMP and cAMP-dependent protein kinase (PKA) are involved in the regulation of GJC, the effects of PKA inhibitor (H8) and cAMP analogues (Rp-cAMP and 8-Br-cAMP) on dye transfer between blastomeres of compacted embryos were examined. Some of the embryos treated with either H8 or Rp-cAMP failed to transfer LY to all blastomeres within 10 min. In contrast, 8-Br-cAMP speeded up fluorescent dye transfer. The median time to fill all blastomeres with LY was 140 sec in untreated controls and 90 sec in siblings treated with 8-Br-cAMP. Inhibition of PKA by H8 or Rp-cAMP induced delay or arrest in embryo development after compaction, but the increase of intracellular cAMP showed no effect. These findings suggest that GJC in preimplantation mouse embryos is regulated by cAMP-PKA pathway and transient interference by PKA inhibitors induces the developmental delay beyond compaction.

In the preimplantation development of mouse embryos, two phenotypically distinct cell populations appear at the 16-cell stage: non-polarized inner cells and polarized outer cells. These two populations give rise to the inner cell mass (mainly the origin of the embryo proper) and the trophectoderm (the origin of extra-embryonic tissues), respectively (Rossant, 1986; Fleming, 1987). The first cellular event leading to the divergence between these two cell populations takes place at the 8-cell stage and is called compaction (Johnson and Maro, 1986; Gueth-Hallonet and Maro, 1992).

During compaction, morphological and physiological changes including cell flattening, positional reorganization of cellular components, and gap junction formation occur. The assembly of gap junctions in the 8-cell stage does not depend on cell flattening (Goodall, 1986; Kidder et al., 1987), but functional gap junctions are required for the maintenance of the compacted state (Buehr et al., 1987; Lee et al., 1987). In addition, perturbation of gap junctional communication (GJC) between blastomeres results in developmental abnormalities including blastomere exclusion and delays in blastulation (Bevilacqua et al., 1989; Leclerc et al.,

1994). Gap junctions are therefore essential for the embryo to develop into the blastocyst stage.

Gap junctions are cell membrane specializations made up of hexameric aggregates of one or more members of a family of proteins, the connexins. Mirror images of these connexin hexamers interpose at the cell surface to form connexin dodecamers (Good-enough et al., 1974). These proteins are seen at the cell membrane interface, where they form low-resistance channels for the exchange of ions, low molecular-weight metabolites, and possibly second messengers (Gilula et al., 1972). Intercellular coupling mediated by these channels is implicated in cell growth regulation (Zhu et al., 1992) and the spatial patterning of cell differentiation, but the nature of the signals transmitted through them and the exact roles of gap junctions in development are unclear (reviewed by Guthrie and Gilula, 1989).

The permeability of the junctional channels can be reversibly altered by a wide variety of effectors, including changes in free Ca^{2+}, voltage, cytoplasmic pH, and cyclic nucleotide (Musil et al., 1990). Cyclic AMP, one of these effectors has been shown to be important in the control of cellular proliferation (Atkinson et al., 1995) and gap junctional permeability (Saez et al., 1986; Mehta et al., 1992).

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Cyclic AMP is an important regulator of GJC in various tissues. Cyclic AMP has been shown to enhance GJC by increasing the conductance of gap junctions, by affecting the gap junctional area on the surface of cells, by phosphorylation of gap junction proteins, and by affecting gating (open versus closed state) of gap junctional channels (Grazul-Bilska et al., 1996). It has also been reported that an increase in intracellular cAMP provokes speeding of communication through gap junctions in normal embryos and prevents the lethal condition characteristic of fertilization of DDK mouse eggs by alien sperm (Leclerc et al., 1994). However, what mechanism rescues the DDK mouse phenotype is not well understood.

In the present study, we have used cAMP-dependent protein kinase (PKA) inhibitor, cAMP antagonist, and cAMP agonist to examine the relationship beween GJC and cAMP-PKA pathway. We tested whether PKA inhibitor and cAMP analogues induce the variation of GJC, and then these changes of GJC by treatment of the agents are involved in the development of mouse embryos after compaction.

Materials and Methods

Collection of preimplantation mouse embryos

ICR mice (Department of Biology, Hanyang University) were bred under 14 h light and 10 h dark cycles. ICR female mice (7 to 8 wk-old) were superovulated by intraperitoneal injection of 5 I.U. of pregnant mare's serum gonadotrophin (PMSG, Sigma) followed by human chorionic gonadotrophin (hCG, Sigma) 48 h later. Females were paired overnight with ICR males (10 to 12 wk-old) and checked for vaginal plugs next morning. In this conditions, fertilization is considered to occur 12 h post-hCG. Embryos at compacted 8-cell stage were collected in HEPES-buffered M2 medium supplemented with 4 mg/ml bovine serum albumin (M2+BSA) by flushing the oviduct, 70-72 h post-hCG (Hogan et al., 1994).

Culture of preimplantation mouse embryos with H8, Rp-cAMP, or 8-Br-cAMP

Embryos were collected in HEPES-buffered M2 medium (M2+BSA) and cultured in M16 medium (M16+BSA, Hogan et al., 1994) under mineral oil (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air in 60 mm plastic dishes (Falcon).

Embryos in each experimental group were cultured in the drop (20 μl) of M16 medium with 50 μM H8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide, Sigma) as cAMP-dependent protein kinase inhibitor, 2 mM Rp-cAMP (adenosine 3;5-monophosphothioate, cyclic (cAMP-S) Rp-isomer, Boehringer Mannheim) as cAMP-antagonist, or 1 mM 8-Br-cAMP (8-bromo-adenosine 3;5-cyclic monophosphate, Sigma) as cAMP agonist, respectively. Rp-cAMP is a cAMP analog and a competitive inhibitor for protein kinase A, which prevents

dissociation of the kinase holoenzyme into its catalytic and regulatory subunits, resulting in complete loss of phosphorylation activity (Grazul-Bilska et al., 1996). Embryos cultured in the drop of M16 medium without any agents were used as controls.

Assay of gap junctional communication

Gap junctional communication (GJC) was monitored by LY injection into one blastomere of compacted 8-cell embryos as previously described (Buehr et al., 1987; Lee et al., 1987). The speed of GJC was defined as the time taken for the last blastomere of the embryo to become visibly fluorescent. The ability of blastomeres in embryos treated with H8, Rp-cAMP, or 8-Br-cAMP to communicate through gap junctions was determined quantitatively by measuring the time taken for LY injected into one blastomere to transfer throughout the embryo and comparing it with transfer times observed in controls.

Microinjection was carried out with a micromanipulator (NT-88, Nikon-Narishige) mounted on an inverted microscope (Diaphot-200, Nikon). A fire-polished holding pipette (outer diameter: 80~100 μm; inner diameter: 20 μm) was used to hold the embryos stationary during injection. Microinjection of LY was carried out with micropipette mounted on the Eppendorf microinjector 2542. Diameter of opening of tip in injection pipette was 0.5 μm±0.2 μm. Lucifer Yellow CH, lithium salt (Molecular Probes) was used at 5% in distilled water.

Statistical analysis

The distributions of the time taken for LY transfer to all blastomeres were compared by Mann-Whitney test. The proportion of non-transferred embryos after treatment with H8 or Rp-cAMP was compared by Fisher's exact test. The proportions of compaction and blastulation in the presence of H8, Rp-cAMP or 8-Br-cAMP were compared by x^2-test. Values of $p<0.05$ were considered significant.

Results

Gap junctional communication (GJC) between blastomeres in preimplantation mouse embryos

To understand GJC between blastomeres, the time taken for LY to transfer to all blastomeres in compacted 8-cell and 16-cell embryos and difference for the time between both embryos were examined.

The times taken for the last blastomere to become visibly fluorescent after injection of LY into one blastomere of embryos at the 8-cell (n=45) and 16-cell (n=33) stage are shown in Fig. 1. The end-point of the measurement was the time when the last had just become visibly fluorescent.
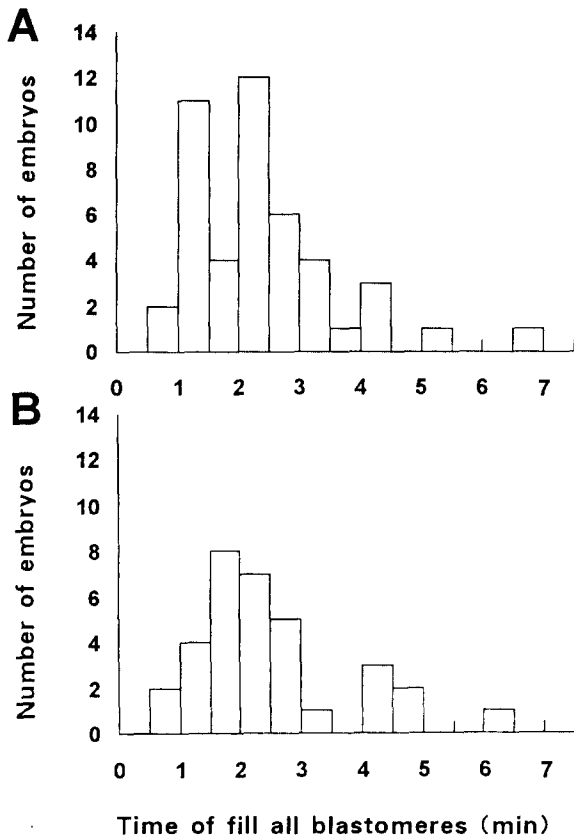
Two embryos (4%) showed transfer time of more

Fig. 1. The time taken for LY to transfer to all blastomeres after injection into one blastomere of 8-cell (A) and 16-cell (B) embryos. In each case, the ordinate gives the number of embryos and the abscissa gives the times taken for the last blastomere in the embryo to become visibly fluorescent.

than 5 min with the majority taking between 1 min and 3 min for dye to transfer throughout 8-cell embryos. Only one embryo (3%) showed the time over 5 min in 16-cell embryos. The median time of 8-cell embryos (140 sec) was very similar to that of 16-cell (135 sec). In addition, the distribution of time to fill all blastomeres of embryos at 16-cell stage embryos was not different from that observed at the 8-cell stage.

Gap junctional communication between blastomeres in embryos treated with H8, Rp-cAMP, or 8-Br-cAMP

The effects of H8 (PKA inhibitor), Rp-cAMP (cAMP antagonist), or 8-Br-cAMP (cAMP agonist) on dye transfer of compacted embryos were tested for examining whether cAMP and PKA are involved in the regulation of GJC.

The times taken for dye transfer through gap junctions in H8 treated embryos are shown in Fig. 2. When one or more blastomeres were still not visibly fluorescent 10 min after injection of LY into one blastomere, embryos were designated non-transferring. In untreated control embryos, only 1 of 23 embryos (4%) failed to transfer LY to all blastomeres within 10
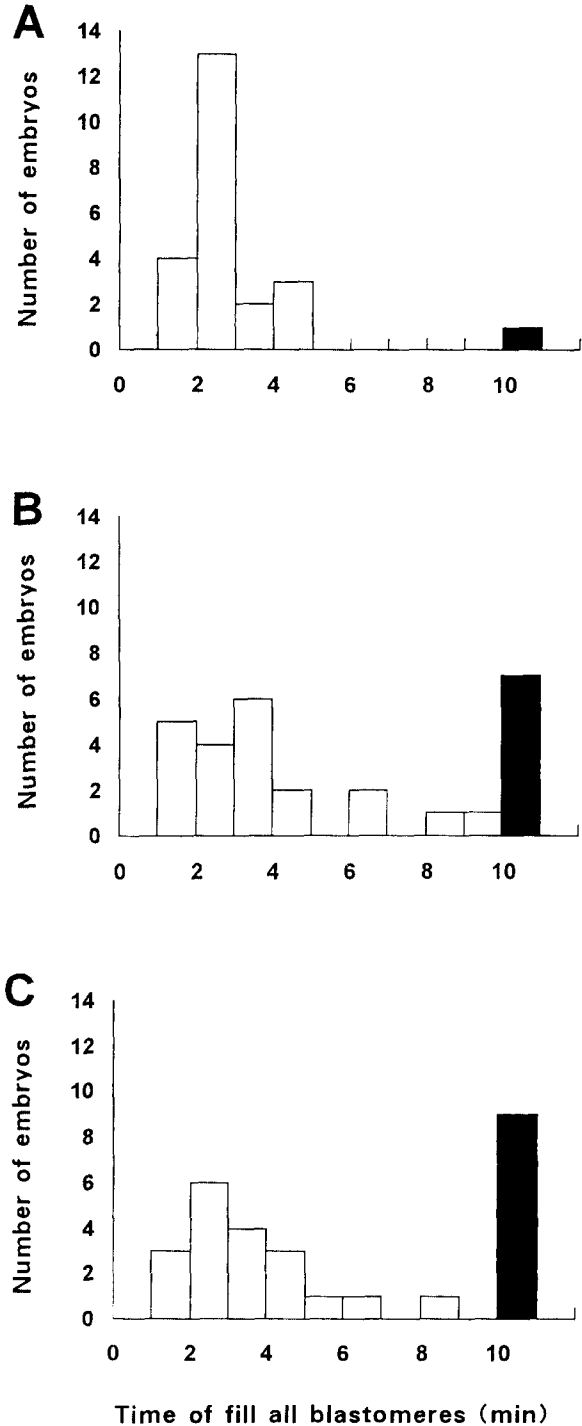
Time of fill all blastomeres (min)

Fig. 2. The time taken for LY to transfer to all blastomeres of compacted 8-cell embryos after treatment with 50 µM H8. In each case, the ordinate gives the number of embryos and the abscissa gives the time taken for the last blastomere in the embryo to become visibly fluorescent. When one or more blastomeres were still not visibly fluorescent 10 min after injection of LY into one blastomere, the embryos were designated non-transferring. All embryos taking longer than 10 min are in last filled bar. A, Control. B, H8 treatment for 30 to 60 min. C, H8 treatment for 60 to 120 min.

min (Fig. 2A). These embryos had distribution of transfer

A

B

C

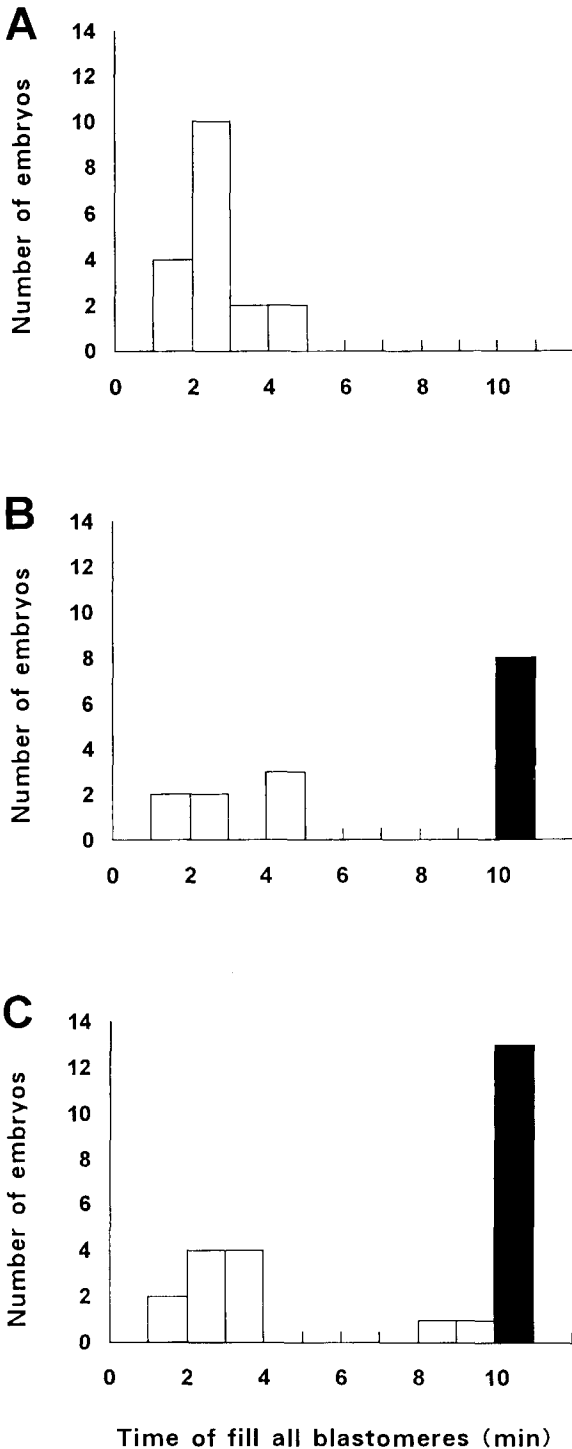**Time of fill all blastomeres (min)**

Fig. 3. The time taken for LY to transfer to all blastomeres of compacted 8-cell embryos after treatment with 2 mM Rp-cAMP. In each case, the ordinate gives the number of embryos and the abscissa gives the time taken for the last blastomere in the embryo to become visibly fluorescent. When one or more blastomeres were still not visibly fluorescent 10 min after injection of LY into one blastomere, the embryos were designated non-transferring. All embryos taking longer than 10 min are in last filled bar. A, Control. B, R$_p$-cAMP treatment for 30 to 60 min. C, R$_p$-cAMP treatment for 60 to 120 min.
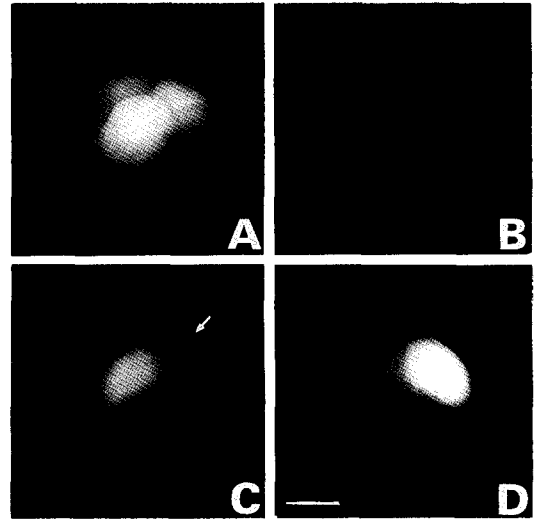

Fig. 4. The disturbance of GJC by either H8 or Rp-cAMP in compacted 8-cell embryos. A, LY is transferred to all blastomeres 4 min after injection (control). B, LY remains only in the blastomere injected even 3 min after injection (H8). C, A third blastomere (arrow) becomes fluorescent 10 min after injection (H8), D, Within 4 min, LY was transferred to all blastomeres, but this embryos show the severe non-even transfer 10 min after injection (Rp-cAMP). Scale bar=30 min.

time similar to those of the embryos in Fig. 1A. However, embryos (Fig. 2B and C) treated with H8 (50 µM) showed the different distribution of time taken for fluorescent transfer. Treatment with H8 for 30 to 60 min slowed dye transfer and a large proportion (25%) took longer than 10 min for all blastomeres to fill. However, the distributions plotted in Fig. 2A and B were not significantly different (p>0.05, Fisher's exact test). Treatment with H8 for 60 to 120 min brought significantly a large proportion (32%) of embryos into the non-transferring category (p<0.05, Fisher's exact test). Embryos treated with H8 (Fig. 2B and C) have the similar distribution of time to fill all blastomeres and embryos under transferring category have the median time slower than that of the controls. Median times of control (Fig. 2A) and both under transferring category (Fig. 2B and C) were 150 sec, 190 sec and 180 sec, respectively.

The effect of Rp-cAMP (2 mM) on GJC was similar to those of H8 (Fig. 3). Rp-cAMP brought more proportion of embryos into non-transferring category than H8. Over the half of total embryos treated with Rp-cAMP (53% and 52%) were included in the non-transferring category. Both embryos, irrespective of the time treated with Rp-cAMP, significantly increased the numbers under non-transferring category (Fig. 3B, p<0.01; Fig. 3C, p<0.01, Fisher's exact test). As shown in Fig. 4, it was found that LY transfer between blastomeres in some embryos after H8 or Rp-cAMP treatment is severely perturbed.

8-Br-cAMP (1 mM) speeded up dye transfer significantly compared to the controls (treated vs. control p<0.05, Mann-Whitney test). There was a significant
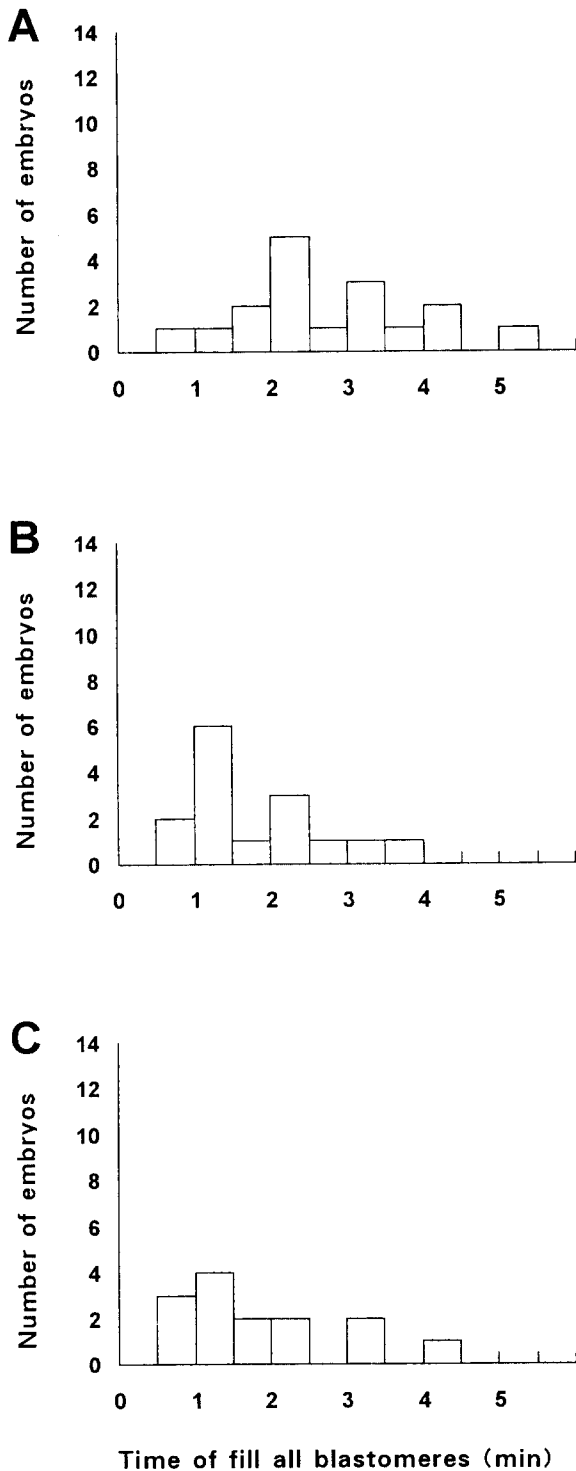
Time of fill all blastomeres (min)

Fig. 5. The time taken for LY to transfer to all blastomeres of compacted 8-cell embryos after treatment with 1 mM 8-Br-cAMP. In each case, the ordinate gives the number of embryos and the abscissa gives the times taken for the last blastomere in the embryo to become visibly fluorescent. A, Control. B, 8-Br-cAMP treatment for 30 to 60 min. C, 8-Br-cAMP treatment for 60 to 120 min.

difference between them in median time, too. The

median time was 150 sec in untreated controls, 90 sec in siblings treated with 8-Br-cAMP for 30 to 60 min and 95 sec for 60 to 120 min (Fig. 5). No embryo treated with 8-Br-cAMP took over 5 min for dye transfer through gap junctions.

Developmental consequence of changing GJC

We tested whether the change of GJC brought about by agents being involved in PKA activity was expressed in development to blastocyst after compaction. For these experiments, non-injected embryos treated for 1 up to 10 h with H8, Rp-cAMP, or 8-Br-cAMP were washed free of agents by passage through several drops of control culture medium M16 and then observed for their ability to form blastocyst.

The results are summarized in Table 1. The number of embryos that reached blastocyst stage was reduced depending on the time to exposure to H8 after 24 h culture. Developmental arrest and delay of embryos in H8 over 5 h were not recovered even after 48 h. Only one embryo that had been treated with H8 for 10 h reached the blastocyst stage and most embryos were still compacted morula at 24 h. Some embryos treated with H8 over 5 h were decompacted or abnormally developed to blastocyst stage, and then degenerated after 48 h culture. In Rp-cAMP for 10 h, the number of embryos which reached blastocyst stage was reduced significantly after 24 h culture, but this reduction induced by Rp-cAMP was inclined to overcome in 48 h culture. Thus, though blastocyst formation was delayed, transient treatment with Rp-cAMP, but not H8, did not irreversibly damage the ability of embryos to resume normal development. 8-Br-cAMP had no effect in development in all cases observed after 24 and 48 h culture. This result indicates that 8-Br-cAMP has no effect on development to blastocyst after compaction.

Discussion

We have shown that PKA inhibitor and cAMP analogues have effects on dye transfer through gap junction and development of preimplantation mouse embryos. We have demonstrated that median time of dye transfer at 8-cell stage does not differ from that observed at 16-cell stage (Fig. 1). However, Buehr et al. (1987) have reported that dye transfer of 16-cell stage embryos becomes significantly faster than that of 8-cell stage. A possible reason for this discrepancy between the current results and those reported by Buehr et al (1987) may be a difference of strain or stage in which embryos were manipulated. However, our results agree with those of a previous study (Leclerc et al., 1994) in that an increase of intracellular cAMP induced the speeding of dye transfer, indicating that cell-cell communication through gap junction is improved (Fig. 5). The increase of intracellular cAMP provoked speeding of communication through gap junctions and the effect

Table 1. Effect of H8, Rp-cAMP or 8-Br-cAMP on development of 8-cell mouse embryos to blastocyst stage

Agent	Culture time (h)	Exposure time (h)				
		0	1	3	5	10
H8 (50 μM)	24	86.3 (63/73)	74.7 (59/79)	53.4[a] (39/73)	35.8[a] (29/81)	1.7[a] (1/59)
	48	95.9 (70/73)	97.5 (77/79)	95.9 (70/73)	72.8[a] (59/81)	20.3[a] (12/59)
Rp-cAMP (2 mM)	24	56.4 (44/78)	45.8 (33/72)	46.7 (35/75)	44.4 (32/72)	28.2[a] (20/71)
	48	94.9 (74/78)	94.4 (68/72)	97.3 (73/75)	93.1 (67/72)	87.3 (62/71)
8-Br-cAMP (1 mM)	24	52.3 (34/65)	40.0 (24/60)	59.7 (35/62)	53.1 (34/64)	41.7 (25/60)
	48	96.9 (63/65)	100.0 (60/60)	96.8 (60/62)	98.4 (63/64)	93.3 (56/60)

The numbers indicate percentage of the embryos developed to blastocyst stage. The numbers in parentheses show the number of embryos developed to blastocyst stage as a fraction of the total number of embryos cultured.
[a]Values differ significantly from that of the control (p<0.01).

was too fast to be assigned plausibly to an effect on gap junction formation, suggesting that cAMP is possibly involved in posttranslational modification in mouse embryos (Leclerc et al., 1994).

A recent study has identified connexins other than connexin43 (Cx43) in preimplantation mouse embryos, suggesting that gap junctions are comprised of different connexins which prepare for differentiation after post-implantation, and that functional regulation of connexins is complicated (Davies et al., 1996). The molecular basis by which GJC is regulated is largely unknown; however, differences of phosphorylation sites among connexins may represent one such regulatory mechanism of GJC through changing connexin functions per se (Zhang and Nicholson, 1989). Previous studies have demonstrated a direct correlation between increased level of cAMP and increased GJC in various cell types (Flagg-Newton et al., 1981; Leclerc et al., 1994; Saez et al., 1986, 1990).

Musil et al. (1990) suggested that cAMP-dependent protein kinase may mediate the serine phosphorylation of Cx43, one member of the connexin family which was associated with the up-regulation of GJC in normal rat kidney cell line. Conversely, reduced junctional permeability has been associated with the tyrosine and/or serine phosphorylation of connexin proteins (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990; Berthoud et al., 1992; Moreno et al., 1992). It was also reported that 6-dimethylaminopurine, serine-threonine protein kinase inhibitor, is able to induce cell flattening and gap junction formation prematurely at the 4-cell stage in mouse embryos (Aghion et al., 1994). These studies indicate that many kinases are involved in regulating posttranslational modifications of gap junction proteins. To understand the more precise mechanism of GJC in preimplantation mouse embryos, it has to be verified whether other kinases are involved in GJC through posttranslational modification of gap junction proteins, and whether protein kinases alter the distribution of gap junctions.

We have found that cAMP is an important regulator of GJC in preimplantation mouse embryos. However, because cell-cell communication through gap junction is a complicated process connected with other factors including extracellular matrix (Musil et al., 1990; Jongen et al., 1991), many studies have to be carried out to explain the biological meaning of communication between blastomeres in preimplantation embryos. It can be postulated that Rp-cAMP might be involved in the function of compaction-associated proteins (e.g. uvomorulin), because Rp-cAMP had a stronger effect on the suppression of GJC than H8, and partially induced the loss of cell flattening or compaction when embryos was treated for over 1 h. Furthermore, Jongen et al. (1991) have supported this possibility that there is a good correlation between the level of GJC and that of immunohistochemical staining of E-cadherin, a calcium-dependent cell adhesion molecule, at cell-cell contact areas in mouse epidermal cell lines, and Duffey et al. (1981) have suggested that cAMP is implicated in regulating the permeablility of tight junctions.

The current results suggest that inhibition of the function of PKA by H8 or Rp-cAMP leads to interference of GJC. Rp-cAMP had the stronger effect on the suppression of GJC compared to H8, but both inhibitors could not inhibit dye transfer through gap junction completely. Some embryos treated with either H8 or Rp-cAMP required times for dye transfer similar to controls (Fig. 2 and Fig. 3). The difference of dye transfer among embryos treated with PKA inhibitors could be caused by different resistance of injected embryos to these inhibitors. This different response to inhibitors is similar to the finding that some embryos treated with butyrate for lowering intracellular pH do not fail to transfer longer than 10 min (Leclerc et al., 1994).

Many studies have focused on the relationship between the GJC and development beyond compaction (Buehr et al., 1987; Lee et al., 1987; Bevilacqua et al., 1989; De Sousa et al., 1993; Leclerc et al., 1994).

When compacted eight-cell embryos are injected with gap junction antisense RNA or antibody and then cultured, substantial proportion of injected embryos decompact and fail to cavitate (Lee et al., 1987; Bevilacqua et al., 1989). However, De Sousa et al. (1993) have reported that protein trafficking inhibitors, Brefeldin-A and monensin, induce decompaction of one or more blastomeres and delay blastocyst formation, but these inhibitors do not irreversibly damage the ability of embryos to resume normal development, and our study showed that Rp-cAMP, but not H8, has similar results (Table 1). Irreversible arrest and delay caused by H8 may be related to other effects of H8 including cytotoxic effects on suppressing embryonic metabolisms, but Manejwala et al. (1989) have reported that early culturing of blastocysts in H8 for 4 h does not have significantly inhibitory effects on the rate of blastocoel expansion. This difference in the effect of H8 suggests that inhibitory effect of H8 is concerned with gene expression and change of cellular components structure in compacted embryos rather than in blastocoel forming embryos because we have observed that compacted 8-cell embryos cultured in H8 over 3 h show the developmental arrest or delay. 8-Br-cAMP had no advantage in development after compaction although facilitating the speeding of gap junctional transfer and expanding the blastocoel, indicating that there seems to be no direct correlation between increase of GJC and development after compaction (Table 1). The diversity of results described above shows that communication through gap junctions is a complicated process.

Thus, we conclude that GJC in preimplantation mouse embryos is regulated by cAMP-PKA pathway, and that transient interference of gap junctional communication by PKA inhibitors induces the developmental delay beyond compaction, but increase of communication does not promote the development of preimplantation mouse embryos.

Acknowledgements

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