oocytes with a protruding membrane were transferred into Hepes-buffered TCM-199 with the osmolarity adjusted to 300 mOsm by adding sucrose supplemented with 0.5 μg/ml demecolcine, 7.5 μg/ml cytochalasin B, and 10% FCS and then the protrusion was removed by aspiration with a 15-μm inner diameter glass pipette [19]. A single donor cell was inserted into the perivitelline space of each enucleated oocyte using the same glass pipette. Cell-oocyte complexes were transferred to mPZM-3 and kept in a CO_2 incubator adjusted to 5% CO_2 in air at 38.5 C until fusion.

The chamber for fusion was a 60-mm dish filled with 7 ml of the sorbitol medium with or without 0.1 mM $Ca(CH_3COO)_2$. Two stainless-steel wires (100-μm diameter) were used as electrodes and they were attached to micromanipulators. The single cell-oocyte complex was sandwiched between the electrodes and oriented with the contact surface between the cytoplast and the donor cell perpendicular to the electrodes. Membrane fusion was induced by applying a single direct-current pulse of 25 V for a duration of 20 μsec with a prepulse of alternating-current field of 5 V, 1 MHz for 2 sec using an LF 101 Fusion Machine (Nepa Gene Co., Chiba, Japan). Following the fusion pulse, the complexes were cultured for a period of 2 h in 100 μl of mPZM-3 supplemented with 2.2 μg/ml cytochalasin B. Fusion was determined by microscopic examination at 1 h after applying the pulse. At 2 h after culture, some fused embryos were activated using the same methods as those for oocytes in the sorbitol medium supplemented with 0.1 mM $Ca(CH_3COO)_2$ and treated with cytochalasin B for 2 h. The culture and evaluation methods of embryos were also the same as those described above.

Statistical analysis

All percentage data were subjected to an arcsin transformation in each replicate.

The transformed values and the numbers of cells in blastocysts were analyzed using one-way (experiment 3) or two-way (experiments 1, 2, and 4) ANOVA followed by Fisher's protected least significant difference test. A probability of $P<0.05$ was considered statistically significant.

Results

Experiment 1

The percentages of activated oocytes (85.5-92.9%) were not affected by the concentrations of calcium in the activation medium and the presence or absence of treatment with cytochalasin B (Table 1). However, the second polar body extrusion rate (13.7%) of activated oocytes after applying electric pulses in the presence of 0.1 mM calcium and treatment with cytochalasin B was significantly ($P<0.05$) lower than those (51.9-65.3%) of activated oocytes that were not treated with cytochalasin B regardless of calcium concentrations in the activation medium.

Experiment 2

There were no significant differences in the percentages of cleaved oocytes (63.5-85.3%) among different treatment groups (Table 2). However, the blastocyst formation rate (38.8%) of oocytes activated in the presence of 0.1 mM calcium and treated with cytochalasin B was significantly ($P<0.05$) higher than those (14.3-16.7%) of oocytes in other treatment groups. No significant differences in the mean numbers of cells (52.2-63.8 cells) in the blastocysts were observed among different treatment groups.

Experiment 3

The percentage of cleaved oocytes (76.8%) at 0.1 mM was significantly ($P<0.05$) higher than that (52.0%) at 0 mM, although the value was not different from those (56.0-69.6%) at 0.01-0.05 and 0.5-1.0 mM (Table 3). More oocytes (28.6%) developed to the blastocyst stage at 0.1 mM ($P<0.05$), although the value was not different from that (23.0%) at 0.5 mM. There were no significant differences in the mean numbers of

cells (42.3-53.6 cells) in the blastocysts among different concentrations of calcium.

Experiment 4

The percentages of fused embryos (62.7-77.3%) were not affected by the presence or absence of calcium in the fusion medium and activation treatment (Table 4). The cleavage rate (33.3%) of embryos that were fused in the absence of calcium without activation was significantly ($P<0.05$) lower than those (67.4-73.5%) of embryos in other treatment groups. More embryos (28.9%) developed to the blastocyst stage (Fig. 1) when they were fused in the absence of calcium and activated ($P<0.05$). There were no significant differences in the mean numbers of cells (48.4-61.0 cells) in the blastocysts (Fig. 2) among different treatment groups.

Discussion

The results of the present study show that concentrations of calcium in fusion and activation media affect the *in vitro* developmental competence of pig parthenogenetic oocytes and cloned embryos derived from miniature pig somatic cells. The optimal concentration of calcium in an activation medium for the development of pig oocytes is 0.1 mM under our experimental conditions. The highest blastocyst formation rate of miniature pig cloned embryos is obtained when donor cells and recipient oocytes are fused in the absence of calcium and the fused embryos are activated in the presence of 0.1 mM calcium.

Our results are inconsistent with those of the previous report [14] in which it was indicated that an activation medium with 1.0 mM calcium is more favorable than that with 0.1 mM calcium for the development of pig parthenogenetic oocytes. This contradiction may be attributed to the presence or absence of cytochalasin B treatment after activation. Oocytes were not treated with cytochalasin B after activation in the previous report [14] in which 46.7% of oocytes activated in the presence of 1.0 mM calcium were apparently diploid having two pronuclei, whereas 90.0% of oocytes activated in the presence of 0.1 mM calcium were apparently haploid extruding one pronucleus as a polar body. This difference might be responsible for the different developmental potential after activation because the developmental capacity of activated diploid oocytes is known to be superior to that of haploid oocytes in the mouse [20]. In contrast, oocytes were treated with cytochalasin B after activation to prevent extrusion of polar bodies in the present study in which 69.8-86.3% of oocytes were apparently diploid regardless of calcium concentrations in the activation medium. It is suggested that stimulation by 0.1 mM calcium is sufficient to induce activation of porcine oocytes because most oocytes had pronuclei after applying electric pulses in the

presence of 0.1 mM calcium in both the previous report [14] and present study. Additional stimulation by higher concentrations of calcium would be required to form diploid oocytes by preventing extrusion of polar bodies after activation. When extrusion of polar bodies is prevented by cytochalasin B, however, excessive stimulation by higher concentrations of calcium may impair the development of porcine parthenogenetic oocytes. In addition, it is possible that differences in oocyte quality, activation media or equipments for applying electric pulses between the previous report [14] and present study brought about the contradiction because there were no significant differences in the rates of diploid oocytes and blastocyst formation between different concentrations of calcium (0.1 and 1.0 mM) even when the oocytes were not treated with cytochalasin B after activation in the present study.

The results of the present study demonstrated that removing calcium from a fusion medium improves the development of embryos reconstituted with miniature pig somatic cells when they are activated in the presence of 0.1 mM calcium after fusion. This improvement may be attributed to increasing MPF activity in recipient oocytes after fusion. Because MPF activity during oocyte maturation is maximal at metaphase of both the first and second meiotic divisions, MPF activity remains high in oocytes arrested at metaphase II [21]. Upon fertilization or activation, however, MPF activity declines rapidly [22,23]. When donor bovine somatic cells, regardless of cell cycle stage, are transferred into preactivated recipient oocytes, development of the resulting embryo is limited because all embryos arrest at the 8-cell stage [16]. Because activation of the embryonic genome of bovine embryos occurs between the 8- and 16-cell stages [24], preactivated recipient oocytes may not reprogram somatic cell nuclei. In addition, cloned mouse embryos reconstructed by injection of somatic cell nuclei into zygotes after removal of both pronuclei exhibited abnormal chromosomes and did not develop

into blastocysts [15]. In contrast, when nuclei of differentiated cells are transferred into unactivated recipients having high MPF activity, reconstructed embryos develop into offspring in many species [2,25-30]. These data suggest that high levels of MPF activity are required for reprogramming a donor nucleus from a differentiated cell. In the present study, when cloned embryos were not activated after fusion, only 2.7% of them fused in the absence of calcium developed to the blastocyst stage and the blastocyst formation rate was enhanced by addition of calcium into the fusion medium. These results consist with those of the previous report [7] in which 69% of porcine fetal fibroblast nuclear transfer embryos that were fused in the presence of calcium and not receiving additional activation stimulation cleaved compared with only 10% of their counterparts fused under calcium-free conditions. The results suggest that it is difficult to induce concurrent activation of porcine recipient oocytes by electric pulses for fusion in the absence of calcium but the concurrent activation occurs when donor cells and recipient oocytes were fused in the presence of calcium. Therefore, donor nuclei would be exposed to high MPF activity for 2 h after fusion in the absence of calcium, which would improve the development into blastocysts of cloned embryos in the present study.

In conclusion, we have shown that the optimal concentration of calcium in an activation medium for the development of pig parthenogenetic oocytes is 0.1 mM when they are treated with cytochalasin B after activation. In addition, it has been indicated that removing calcium from a fusion medium improves the development of cloned embryos derived from miniature pig somatic cells when they are activated in the presence of 0.1 mM calcium after fusion.

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Figure Legends

Fig. 1. Blastocysts developed from cloned embryos produced by fusing miniature pig somatic cells and enucleated oocytes in the absence of calcium and activating in the presence of 0.1 mM calcium. Magnification 238×.

Fig. 2. Nuclei stained with Hoechst 33342 of a blastocyst developed from cloned embryos produced by fusing miniature pig somatic cells and enucleated oocytes in the absence of calcium and activating in the presence of 0.1 mM calcium. Magnification 300×.

Table 1. Activation of porcine oocytes applied electric pulses in the presence of 0.1 or 1.0 mM calcium with or without cytochalasin B treatment[a]

Calcium concentration (mM)	Cytochalasin B treatment	No. of oocytes examined	No. (%)[b] of oocytes activated	No. (%)[c] of activated oocytes with[d]			
				1PB			2PB
				1PN	2PN	Total	1PN
0.1	−	55	49 (89.1)	3 (6.1)	14 (28.6)	17 (34.7)[e]	32 (65.3)[e]
	+	59	51 (86.4)	5 (9.8)	39 (76.5)	44 (86.3)[f]	7 (13.7)[f]
1.0	−	56	52 (92.9)	4 (7.7)	21 (40.4)	25 (48.1)[e]	27 (51.9)[e]
	+	62	52 (85.5)	9 (17.0)	28 (52.8)	37 (69.8)[ef]	16 (28.3)[ef]

[a]Experiments were repeated five times.

[b]Percentage per oocytes examined.

[c]Percentage per oocytes activated.

[d]PB, polar body; PN, pronucleus.

[e,f]Values with different superscripts within each column are significantly different ($P<0.05$).

Table 2. Development of porcine oocytes applied electric pulses in the presence of 0.1 or 1.0 mM calcium with or without cytochalasin B treatment[a]

Calcium concentration (mM)	Cytochalasin B treatment	No. of oocytes cultured	No. (%)[b] of oocytes developed to ≥2-Cell (2)[c]	No. (%)[b] of oocytes developed to Blastocyst (7)[c]	Mean no. ± SEM of cells in blastocysts
0.1	−	136	108 (79.4)	22 (16.2)[d]	53.9 ± 6.1
	+	116	99 (85.3)	45 (38.8)[e]	53.5 ± 4.6
1.0	−	96	61 (63.5)	16 (16.7)[d]	63.8 ± 5.0
	+	91	58 (63.7)	13 (14.3)[d]	52.2 ± 5.2

[a]Experiments were repeated five times.

[b]Percentage per oocytes cultured.

[c]Numbers in parentheses indicate the time of examination (days of culture).

[d,e]Values with different superscripts are significantly different ($P<0.05$).

Table 3. Development of porcine oocytes applied electric pulses in the presence of calcium at various concentrations[a]

Calcium concentration (mM)	No. of oocytes cultured	No. (%)[b] of oocytes developed to ≥2-Cell (2)[c]	No. (%)[b] of oocytes developed to Blastocyst (7)[c]	Mean no. ± SEM of cells in blastocysts
0	127	66 (52.0)[d]	14 (11.0)[d]	52.5 ± 5.5
0.01	175	98 (56.0)[de]	21 (12.0)[d]	45.8 ± 3.2
0.05	175	116 (66.3)[de]	32 (18.3)[de]	42.3 ± 2.4
0.1	112	86 (76.8)[e]	32 (28.6)[f]	49.7 ± 2.8
0.5	100	62 (62.0)[de]	23 (23.0)[ef]	53.6 ± 3.4
1.0	112	78 (69.6)[de]	18 (16.1)[de]	47.9 ± 6.3

[a]Experiments were repeated five times.

[b]Percentage per oocytes cultured.

[c]Numbers in parentheses indicate the time of examination (days of culture).

[d,e,f]Values with different superscripts within each column are significantly different ($P<0.05$).

Table 4. Development of miniature pig somatic cell nuclear transfer embryos fused in the presence or absence of calcium with or without activation

Calcium	Activation treatment	No. of trials	No. of complexes treated	No. (%)[a] of complexes fused	No. (%)[b] of embryos developed to ≥2-Cell (2)[c]	No. (%)[b] of embryos developed to Blastocyst (7)[c]	Mean no. ± SEM of cells in blastocysts
−	−	4	95	75 (77.3)	25 (33.3)[d]	2 (2.7)[d]	61.0 ± 11.4
+	−	4	142	89 (62.7)	60 (67.4)[e]	8 (9.0)[e]	51.0 ± 12.0
−	+	4	117	83 (70.9)	61 (73.5)[e]	24 (28.9)[f]	49.5 ± 2.7
+	+	5	187	133 (71.1)	94 (70.7)[e]	22 (16.5)[g]	48.4 ± 2.9

[a]Percentage per complexes treated.

[b]Percentage per complexes fused.

[c]Numbers in parentheses indicate the time of examination (days of culture).

[d,e,f,g]Values with different superscripts within each column are significantly different ($P<0.05$).