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Effects of La^{3+} on Surface Charges, Dielectrophoresis, and Electrofusion of Barley Protoplasts[1]

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SHUNNOSUKE ABE* AND JUNKO TAKEDA
Institute of Agricultural Environment Control, College of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790, Japan (S.A.); *and Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kitashirakawa Oiwake-chou, Sakyo-ku, Kyoto 606, Japan* (J.T.)

ABSTRACT

When dielectrophoresis and electrofusion of barley (*Hordeum vulgare* var Moor) leaf protoplasts were assayed in the presence of 0.1 to 1 millimolar lanthanum ion (La^{3+}) in the basal medium (0.7 molar mannitol, 1 millimolar piperazine-*N*,*N*-bis[2-ethanesulfonic acid]-Na [pH 6.7], 0.1 millimolar $CaCl_2$), dielectrophoresis and induction of electrofusion were strongly inhibited. The latter remained inhibited and the former recovered by about 60% after washing the La^{3+}-treated protoplasts without EDTA. These inhibitions were almost completely abolished by washing the La^{3+}-treated protoplasts with 1 millimolar EDTA. Inductively coupled plasma atomic emission spectroscopic analysis revealed that protoplasts retained a considerable amount of La^{3+} after washing without EDTA and released most of the bound La^{3+} by washing with 1 millimolar EDTA. This tightly bound La^{3+} seemed responsible for the inhibition of electrofusion and dielectrophoresis that was observed in the La^{3+}-treated protoplasts after washing. ζ-potentials of protoplasts were -39.0 ± 3.2 millivolts, -16.7 ± 2.6 millivolts, and virtually zero in media containing 0, 0.1, and 0.3 millimolar La^{3+} ($I = 7.2$ millimolar), respectively, and had a positive value ($+14.2 \pm 2.2$ millivolts) in the presence of 1 millimolar La^{3+}. These effects of La^{3+} on ζ-potentials were easily abolished by washing without EDTA. This indicates that charged species located at the surface of plasma membrane of protoplasts cannot account for the sites at which La^{3+} exerts its inhibition of dielectrophoresis and electrofusion. In contrast, the promotion of spherical fusion and the reduction of broken fusion products observed in the presence of La^{3+} were almost completely abolished by washing without EDTA. Our results also indicate that the initial induction and development of electrofusion can be studied independently.

In our earliest study of electrofusion (19) and in related studies (1–3), the importance of calcium ions and a change of the membrane state into a fusible state in induction of electrofusion have been pointed out. We used Ca^{2+} in order to stabilize and enhance electrofusion. The effectiveness of Ca^{2+} on electrofusion was recently confirmed with plant (7, 14, 17, 20, 21) and animal cells (15). On the other hand, a low fusion efficiency, compared with a weakly conductive pure osmoticum medium, has been reported because addition of Ca salts increases conductivity of media (25). In mesophyll protoplasts from peas, Ca^{2+} reduces the threshold for electrofusion (7). In protoplasts from tobacco leaf cells and cultured potato cells, fusion frequencies are increased by the addition of Ca^{2+} (20). An absolute requirement of Ca^{2+} in electrofusion is reported with animal cells (15). In some cases, Mg^{2+} ions also facilitate electrofusion, in addition to or instead of Ca^{2+} (25). These stimulative effects of Ca^{2+} on electrofusion do not seem to be adequately explained by the reduction of the fluidity of the lipid domain of the cytoplasmic membrane. Indeed, detectable changes of fluidity have not always been recorded with less than 10 mM Ca^{2+} (14) except with pea mesophyll protoplasts (7), although electrofusion is stimulated below this level of Ca^{2+}. Phase separation within the lipid domain might explain the observed phenomena (15), but this has not been substantiated.

In a previous study (2), we defined three components of electrofusion—initial induction, development, and recovery—and showed that these processes could be studied independently. Above all, the initial induction is the most important because factors necessary for undergoing subsequent cell fusion are set during the initial induction by an electrical stimulus and, as a result, the cytoplasmic membrane and the associated structures are turned into a fusible state which is, in turn, returned to a resting state by the recovery process. This fusible state may also develop to maturity some time after the pulsation. We also found that a surface Ca^{2+} antagonist, La^{3+},[2] strongly inhibited dielectrophoresis and the initial induction of fusion while it promoted the development of fusion and reduced broken fusion products. These effects of La^{3+} may be important for understanding of a possible role of Ca^{2+} in electrofusion. Ln^{3+} are known to antagonize Ca^{2+} ions at membrane sites and can thus be used as a potential tool for studying the role of Ca^{2+} (10, 23). Since the surface charges of protoplasts are believed to be important in cell fusion (13), a direct effect of La^{3+} on the surface charge of protoplasts may be crucial in the inhibition of electrofusion. In the present study, we investigated the effect of La^{3+} on dielectrophoresis, electrofusion, and surface charges in further detail. Comparisons of other trivalent cations with La^{3+} were also investigated to distinguish the specific effects of La^{3+} from the nonspecific effects of surface charges of protoplasts, conductivity of media, and the valency of La^{3+}.

MATERIALS AND METHODS

Plant Materials and Isolation of Protoplasts. Barley seeds (*Hordeum vulgare* L. var Moor) were sown in vermiculite after being soaked in distilled water overnight and were placed in a growth cabinet (Koitotron KG-306) at 21°C day (16 h/114 μE $m^{-1}s^{-1}$) and night for 7 to 9 d. A Hyponex solution (2%) was used for watering. Protoplasts were isolated from the primary leaves of the plants as previously described (2) with a modification adapted

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[2] Abbreviations: Ln^{3+}, lanthanide ion; Pipes, piperazine-*N*,*N*-bis(2-ethanesulfonic acid); $HACo^{3+}$, hexaaminecobalt(III); Spd, spermidine; Spd^{3+}, fully protonated spermidine; FA ratio, fusion frequency to alignment ratio; ICP, inductively coupled plasma atomic emission spectroscopy.

for a large scale isolation. The leaves were floated on an enzyme solution containing 1% Cellulase Onozuka (Kinki Yakult Co. Ltd.) and 0.7 M mannitol at pH 5.7 for 3 to 4 h at 21°C after removing the lower epidermis. Protoplasts were collected by centrifugation for 3 min at 110*g* in a 50 ml tube, after filtration through Miracloth, and resuspended in the basal medium composed of 0.7 M mannitol, 0.1 mM $CaCl_2$, and 1 mM Pipes-Na at pH 6.7. The suspension was centrifuged in the same condition, and the pelleted protoplasts were resuspended in the basal medium (50 ml per tube) and again were centrifuged in the same conditions. This washing procedure was repeated once more, and the pelleted protoplasts were resuspended in the basal medium. The protoplast density was adjusted to 1.3 to 1.5×10^6 cells/ml.

Fusion Assay. Of the suspended protoplasts, 200 μl were mixed with an equal volume of the basal medium, with or without chemicals to be tested, and were incubated for an appropriate period. After the incubation, small aliquots of protoplasts were subjected to electrofusion at 21°C, using an electric cell fusion processor, SCF-1 (Sankei Company Limited, Japan) with an electrofusion chamber having a pair of parallel Pt-plate-electrodes under microscopic observation (Nikon TMD-Mi: Nihon Kogaku Kogyo Co, Ltd/Inohara Firm). The gap between the electrodes was 450 μm and the length was 5 mm. The AC field and DC pulse strengths for the standard assay for electrofusion were 90 V/cm and 1.33 kV/cm with 82 kHz frequency and 100 μs duration, respectively. We prefer to use a simple term 'DC pulse' for an electrical impulse which is applied to protoplasts in order to induce cell fusion. Terms 'permeabilization,' 'breakdown pulses,' etc., which apparently assume particular events, are not appropriate here because their role in electrofusion is not fully understood yet. The AC field was applied for 10 s before application of the DC pulse. Fusion was scored 5 min after the application of the DC pulse. Cells (150–200) were generally counted for every fusion assay, and the assay was replicated until sufficient counts were accumulated. All protoplasts and fusion events in the gap were counted.

The fusion frequency, broken rate, and spherical rate were calculated as follows. Fusion frequency, (No. of total fusion products)/(No. of total fusion products + No. of unfused protoplasts); broken fusion rate, (No. of broken fusion products)/(No. of total fusion products); spherical fusion rate, (No. of spherical fusion products)/(No. of total fusion products); No. of total fusion products, (No. of unbroken fusion products + No. of broken fusion products). Alignment of protoplasts, as a measure of dielectrophoresis, was scored immediately after application of the AC field. Alignment was calculated by dividing numbers of protoplasts aligned by numbers of protoplasts examined.

$LaCl_3(6\ H_2O)$ and $HACo^{3+}Cl_3$ were purchased from Wako Pure Chemicals Industries, Ltd. As the free base of spermidine is strongly basic (pH > 10) and cations are no longer trivalent at such high pH, spermidine trihydrochloride from Sigma Chemical Co. (pH about 7.5 at 100 mM) was used for the source of Spd^{3+} (a protonated form). With La^{3+}, plastic or quartz wares were used because La^{3+} tends to adsorb on glass surfaces (8).

Washing Experiments with Chemical-Treated Protoplasts. After incubation of protoplasts in media containing chemicals to be tested in a microfuge tube, protoplasts were collected on the bottom of the tube by centrifugation and the supernatant was discarded. After the removal of the incubation medium, washing was performed as follows. The pelleted protoplasts were carefully resuspended in washing medium (400 μl) that was added to them in several aliquots. The suspension was then centrifuged in the same conditions, and the supernatant was removed. This washing procedure was repeated twice, and the washed protoplasts were resuspended in a desired medium. For the EDTA wash, the washed protoplasts were resuspended and incubated in a medium (400 μl) with 1 mM EDTA (0.7 M mannitol, 1 mM Pipes-Na, 0.1 mM $CaCl_2$, 1 mM EDTA-2 Na [pH 6.7]) for 10 min. Protoplasts were collected by centrifugation, and the supernatant was removed. These protoplasts were then washed twice with the basal medium as above to remove EDTA. As protoplasts treated with inhibitors often became fragile, they were centrifuged carefully (20–40*g* for 3 min). In the case of La^{3+}, excess centrifugation, 110*g* for example, rendered protoplasts tightly packed, and considerable disruption of protoplasts occurred on resuspending.

Analysis of La^{3+}. Amounts of La^{3+} bound to protoplasts and eluted in washing media were determined using ICP which provides a sensitive assay for La with easy pretreatment of samples. An ICP instrument (model 750N from Nippon Jarrel-Ash Co.Ltd. Kyoto, Japan) was used with a conventional cross-flow type nebulizer. The instrumentation and operating conditions have been described (16). The protocol of washing experiments described above was followed except that 10 ml aliquots of protoplast suspensions and washing media were used. Washing media removed from the protoplasts (10 ml) and the pelleted protoplasts, were digested with concentrated nitric acid and perchloric acid (analytical grade). The digestion was continued by adding aliquots of nitric acid and perchloric acid under extensive heating until no organic substances remained. These digested samples were concentrated by continuous heating, filled up to 10 ml with distilled water, and subjected to analysis of La using ICP. The results were presented as mol per protoplast.

RESULTS

Reversibility of the effects of La^{3+} on Dielectrophoresis and Electrofusion. When protoplasts which had been incubated in media containing La^{3+} were washed with and then assayed in the basal medium as in the control, fusion frequency recovered only partially (Table I). Alignment of protoplasts treated with 0.1 and 1 mM La^{3+} recovered 60 to 70% of the control value after the wash. Rotation of protoplasts by an AC field was also inhibited in the presence of La^{3+} and partially recovered after the wash. FA ratios were 0.35 and 0.14 for the washed protoplasts which had been treated with 0.1 and 1 mM La^{3+}, respectively, and 0.67 for the control. This indicates that induction of fusion remained inhibited. In the presence of 0.1 mM La^{3+}, the FA ratio was 0.36 and close to that after washing. Therefore, a recovery of fusion frequency after washing the La^{3+}-treated protoplasts is attributable to a recovery of dielectrophoresis. In the presence of 1 mM La^{3+}, dielectrophoresis of protoplasts was inhibited almost completely, while tight clusters of several protoplasts increased as reported earlier (2). Fusion efficiency was very low even after prolonged incubation which caused a considerable increase of aggregation (>50%). The number of these strongly aggregated protoplasts was more than that of protoplasts aligned by an AC field by manyfold. Thus, we could not accurately describe the degree of induction of fusion using FA ratios in the presence of 1 mM La^{3+}. On the other hand, these inhibitions of dielectrophoresis and electrofusion, and induction of the strong aggregation were almost completely abolished by washing the La^{3+}-treated protoplasts for 10 min with an EDTA containing (1 mM) basal medium. Washing protoplasts with EDTA caused an increase of fusion frequency, while washing without EDTA did not. In contrast, a promotion of the development of fusion with a marked decrease of broken fusion products was observed in the presence of 0.1 and 1 mM La^{3+} and was abolished by washing without EDTA.

Inhibition of electrofusion by La^{3+} was observed almost instantaneously within a minimum time resolution (90 s) and reached a maximum within 10 to 15 min. Promotion of spherical fusion and reduction of broken fusion products in the presence of La^{3+} were observed almost immediately after the incubation.

Amount of La^{3+} Bound to Protoplasts. As the above results

Table I. *Effects of Washing on Dielectrophoresis and Electrofusion of La^{3+}-Treated Protoplasts*

Fusion of protoplasts that had been incubated in 0.1 or 1 mM La^{3} for 15 min was assayed in the presence (presence) or absence (washed) of La^{3+}. Washing was performed with (EDTA washed) or without 1 mM EDTA. Control represents an assay with protoplasts without La^{3+} treatment. Alignment was determined after dielectrophoresis at 90 V/cm for 10 s. Values are presented as a percentage with SD.

Treatment	No. of Cells	Fusion Frequency	Broken Rate	Spherical Rate	Alignment
			%		
Control	477	32.7 ± 2.1	3.2 ± 1.4	35 ± 4	49 ± 5
Control, washed	619	31.5 ± 1.9	3.6 ± 1.3	37 ± 3	46 ± 6
Control, no AC	369	3.0 ± 0.9	ND	ND	0 ± 0
0.1 mM La^{3+}, presence	429	7.9 ± 1.3	0.0 ± 0	79 ± 7	22 ± 9
0.1 mM La^{3+}, washed	463	11.4 ± 1.5	8.0 ± 3.7	16 ± 5	33 ± 7
1.0 mM La^{3+}, presence	772	1.7 ± 0.5	0.0 ± 0	77 ± 12	ND
1.0 mM La^{3+}, washed	568	4.0 ± 0.8	4.3 ± 4.2	22 ± 9	29 ± 6
1.0 mM La^{3+}, EDTA washed	719	30.7 ± 1.7	4.1 ± 1.3	47 ± 3	47 ± 5
Control, EDTA washed	581	36.8 ± 2.0	6.5 ± 1.7	46 ± 3	54 ± 8

suggested a strong binding of La^{3+} to the cytoplasmic membrane of protoplasts, La^{3+} content of La^{3+}-treated protoplasts was analyzed by ICP (Table II). When protoplasts were incubated with 0.1 and 1 mM La^{3+} for 15 min and then washed without EDTA, a considerable amount of La^{3+} was left absorbed in protoplasts. When these protoplasts were washed in 1 mM EDTA for 10 min, the majority (about 80%) of the bound La^{3+} was eluted in the washing medium. In contrast, when protoplasts treated with 0.1 and 1 mM La^{3+} were washed without EDTA, only 5 to 16% of the bound La^{3+} was eluted in the washing medium. A leakage of cell contents by the washing procedure was monitored by simultaneous measurements of lanthanum and cellular potassium and phosphorus, and found to be small (data not shown).

Comparisons of Effects between La^{3+} and Other Trivalent Cations and Effects of Conductivity of Media on Electrofusion. In Table III, the effects of La^{3+} are compared with those of $HACo^{3+}$ (18). NaCl was also used to compare the effects of conductivity of media on electrofusion. These ions were added to the basal medium. It is noted that $HACo^{3+}$ promoted the development of fusion with a reduction of broken fusion products at 0.3 to 1 mM and that the effects of $HACo^{3+}$ were mostly abolished by washing. Spd^{3+} exerted a similar effect but was less effective (data not shown). This resembled the promotive effects of La^{3+}. $HACo^{3+}$ inhibited fusion, but less effectively than La^{3+}, while conductivity in $HACo^{3+}$ media was higher than that of La^{3+} media. When 3.3 mM NaCl was used to give approximately the same conductivity as 1 mM La^{3+} medium, fusion frequency was 10 times higher. By adding 6 mM NaCl, conductivity of the medium was approximately doubled, but fusion frequency was still higher than in 1 mM La^{3+}. Fusion frequency in 1 mM $HACo^{3+}$, while its conductivity was only 13% higher than 3.3 mM NaCl medium, was one-half of that in 3.3 mM NaCl medium. In addition, electrofusion was still induced in the presence of 100 mM Na^{+} (conductivity 10 ms/cm) when a high power equipment (SCF-2K, Sankei Co. Ltd.) was used (data not shown).

Effects of $HACo^{3+}$, Spd^{3+}, and Na^{+} on induction of electrofusion and dielectrophoresis are shown in Table IV. The FA ratio in $HACo^{3+}$, Spd^{3+}, and Na^{+} was not different from that of the control (0.71). K^{+} had a similar effect as Na^{+} (data not shown). Although previous workers (20) reported that Spd enhances induction of electrofusion drastically, we could not observe such an effect with barley protoplasts. Conductivity of the 1 mM Spd^{3+} medium was 358 μs/cm. Results in Table III and IV show that the effects of ions on electrofusion were much more dependent on the cationic species than on their conductivities.

Effects of La^{3+} and Other Trivalent Cations on ζ-Potentials of Protoplasts. Because trivalent cations are expected to interact strongly with the negatively charged surface of the cytoplasmic membrane and thereby modify dielectrophoresis and electrofusion, we examined the effects of La^{3+}, $HACo^{3+}$, and Spd^{3+} on ζ-potentials of protoplast surfaces using the Brigg's cell (Table V). Trichloride salts of these cations were added to the basal medium and the pH values of the media containing them were 6.7 ± 0.2. The pH value should not be higher than this value otherwise La^{3+} becomes unstable (8). The ionic strength of these salts at 1 mM was calculated as 6 mM and that of the basal medium was close to 1.2 mM. ζ-Potentials of protoplasts were virtually zero at 0.3 mM La^{3+}, and reversed to positive values above 0.3 mM La^{3+} (also Fig. 1), when the ionic strength of the media was kept constant at 7.2 mM by adding NaCl. ζ-Potentials of protoplasts was approximately exponential against the bulk concentrations of La^{3+}. $HACo^{3+}$ and Spd^{3+} also neutralized the negative charges of protoplast surface, but less effectively than La^{3+}. Na^{+} and K^{+} had the same effect on ζ-potentials and were the least effective among the cations examined. ζ-Potentials of protoplasts in 1 mM La^{3+} increased slightly by lowering the pH values to 3.6, and fell to zero in the control. Such low pHs also inhibited dielectrophoresis and electrofusion irreversibly but did not induce strong aggregation of protoplasts.

Table II. *Analysis of Bound La^{3+} to Protoplasts Using ICP*

Protoplasts treated with 0.1 or 1 mM La^{3+} for 15 min were assayed for La^{3+} content after washing with or without incubation in 1 mM EDTA media (1 mM Na_2EDTA, 0.7 M mannitol, 1 mM Pipes-Na, 0.1 mM $CaCl_2$). Density of protoplasts was 1.3 × 10^6/ml, and values are expressed as fmole per a protoplast with SD. The average diameter of protoplasts were 32.5 ± 5.6 μm.

	0.1 mM La^{3+} Treatment			1 mM La^{3+} Treatment		
	Supernatant[a]	Cells[b]	Total	Supernatant[a]	Cells[b]	Total
			fmol			
La^{3+}-treated						
Without EDTA wash	2.1 ± 0.1	11.1 ± 0.5	13.2 ± 0.6	7.2 ± 0.2	136 ± 5	143 ± 5
With EDTA wash	12.9 ± 0.3	2.0 ± 0.1	14.9 ± 0.4	118 ± 3	15.5 ± 0.5	134 ± 4

[a] Analysis of washing medium after protoplasts were pelleted down by centrifugation. [b] Analysis of the pelleted protoplasts.

Table III. *Effects of La^{3+} and $HACo^{3+}$ With or Without Washing*

Fusion parameters of protoplasts treated with $HACo^{3+}$ and La^{3+} were determined with (washed) or without (presence) washing and compared with conductivities of media. Results with NaCl were also compared. Values are presented with SD.

Treatment	No. of Cells	Fusion Frequency	Broken Rate	Spherical Rate	Conductivity
			%		μs/cm
Control	496	30.0 ± 2.0	6.1 ± 2.0	35 ± 4	96
$HACo^{3+}$					
0.3 mM, presence	439	13.4 ± 1.7	1.7 ± 1.7	97 ± 3	192
1 mM, presence	306	7.8 ± 1.5	0.0 ± 0	96 ± 5	406
1 mM, washed	475	20.4 ± 1.8	5.2 ± 2.2	40 ± 5	96
$LaCL_3$					
1 mM, presence	525	2.1 ± 0.6	0.0 ± 0	73 ± 13	345
1 mM, washed	426	2.8 ± 0.8	6.7 ± 1.8	33 ± 14	96
1 mM, EDTA washed	607	28.7 ± 1.8	6.9 ± 1.9	40 ± 4	96
NaCl					
3.3 mM	590	20.3 ± 1.7	6.7 ± 2.3	13 ± 3	360
6 mM	461	10.8 ± 1.4	6.0 ± 3.4	16 ± 6	610

Table IV. *Effects of Na^+, $HACo^{3+}$, and Spd^{3+} on Alignment and Fusion Frequency*

Fusion frequency and alignment of protoplasts were assayed in the presence of none (basal medium), 1 mM $HACo^{3+}$, 1 mM Spd^{3+}, and 6 mM Na^+. These cations were added to the basal medium as chlorides, and the pHs were 6.7 ± 0.2. Alignment was determined after dielectrophoresis at 90 V/cm for 10 s. Values are expressed as a percentage with SD.

Treatment	Fusion Frequency	Alignment	FA Ratio
	%		
Basal medium	34.4 ± 2.4	48 ± 7	0.71
1 mM $HACo^{3+}$	9.8 ± 1.3	12 ± 4	0.78
1 mM Spd^{3+}	11.6 ± 1.4	16 ± 4	0.73
6 mM Na^+	18.9 ± 1.7	30 ± 5	0.63

Table V. *Effect of Cations on ζ-Potentials of Protoplasts*

Treatment	ζ-Potential
	mV
Normal pH, pH 6.7 (buffered)[a]	
Basal medium (I = 1.2 mM)	−48.3 ± 3.2
Basal medium, EDTA washed	−48.8 ± 3.2
1 mM La-treated, washed	−43.6 ± 3.6
1 mM $LaCl_3$, presence (I = 7.2 mM)	+14.4 ± 2.4
1 mM $HACo\text{-}Cl_3$	−3.8 ± 0.9
1 mM $Spd\text{-}Cl_3$	−6.5 ± 0.6
6 mM NaCl	−38.9 ± 2.9
6 mM KCl	−38.6 ± 2.9
Low pH, pH 3.6 (not buffered)[b]	
6 mM NaCl	−2.1 ± 2.6
1 mM $LaCl_3$	+22.9 ± 2.5

[a] Salts were added to the basal medium (0.7 M mannitol, 1 mM Pipes-Na, 0.1 mM $CaCl_2$). [b] Salts were added to an unbuffered medium (0.7 M mannitol, 0.1 mM $CaCl_2$), and pH was adjusted by HCl (ionic strength, close to 7.2). Z. potentials of protoplasts in the presence (presence or no remarks) or in the absence (washed) of La^{3+}, $HACo^{3+}$, Spd^{3+}, Na^+, and K^+ are presented with SD. I, ionic strength.

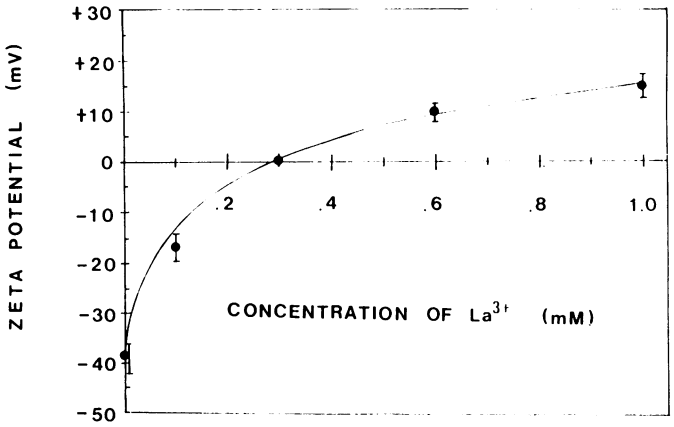

FIG. 1. Concentration effect of La^{3+} on ζ-potentials of protoplasts. ζ-Potentials of protoplasts (vertical axis), which had been determined in media containing 0.7 M mannitol, 1 mM Na-Pipes (pH 6.7), 0.1 mM $CaCl_2$, and various concentrations of $LaCl_3$ by using the Brigg's apparatus for electrophoresis, keeping the ionic strength of media constant at 6 mM plus ionic strength of the basal medium by adding NaCl, are plotted against the concentration of La^{3+}. The ionic strength of the basal medium (0.7 M mannitol, 1 mM Na-Pipes, 0.1 mM $CaCl_2$) at pH 6.7 was approximately 1.2 mM and was kept constant by using the same stock solutions throughout the experiments. Each point includes 40 to 60 determinations. Vertical bars beside data points represent the standard deviations.

DISCUSSION

In the present study, we confirmed our previous observation (2) that, in the presence of La^{3+}, dielectrophoresis and the initial induction of electrofusion are inhibited while spherulation of fusion products (development of electrofusion) is promoted. However, the conductivity of the media and surface charges of the protoplasts did not seem to be major factors of electrofusion in the present study, although they have been shown to affect electrofusion (6, 13). The observation that washing restored the ζ-potentials of La^{3+}-treated protoplasts (Table V) but did not release the inhibition of the initial induction is also consistent with this conclusion. ζ-Potentials of protoplasts were slightly increased further by lowering the pH to 3.6 in the presence of 1

mM La^{3+}, while the same reduction of pH values caused a major increase of ζ-potentials to virtually zero in the absence of La^{3+} (Table V). This might indicate that a H^+-binding site is a major source of the reversible site for La^{3+}. Trivalent cations markedly reduced alignment of protoplasts as a consequence of inhibition of dielectrophoresis. Increase of conductivity and the ionic strength of the media, and specific interactions of ions with protoplast surfaces may be responsible for the reductions of dielectrophoresis (16).

The inhibition of the initial induction of fusion was specific for La^{3+} and not reversible by washing without EDTA. Recovery of fusion frequency of the La^{3+}-treated protoplasts was almost complete by washing with EDTA. We also found analytically that a considerable amount of La^{3+} was found to be absorbed by protoplasts after a short exposure to La^{3+} and that the majority of the absorbed La^{3+} in protoplasts was removed by a brief incubation with EDTA. Thus, we conclude that the inhibitory effect of La^{3+} on the initial induction of fusion is exerted by La^{3+} ions strongly bound to protoplasts and that this bound La^{3+} also caused the inhibition of dielectrophoresis in the washed, La^{3+}-treated protoplasts. This bound La^{3+} must exist in an easily accessible portion from the outside, perhaps around the cytoplasmic membrane. Requirement of a short incubation time for exerting the inhibition and no major loss of cellular phosphorus and potassium accompanied by the wash are also consistent with this. This bound La^{3+} may cause an alteration of membrane structures or functions to modify dielectrophoresis and electrofusion. Neutralization or reversal of surface charges which are thought to be important for cell fusion (13) cannot account for the inhibition, because the surface charges of La-treated protoplasts were no longer affected, while the inhibition remained after washing without EDTA. In contrast, Hagiwara and Takahashi (9) reported with lobster axon membranes that the binding of La^{3+} was irreversible and affected the surface charges even after washing.

Since the average diameter of protoplasts was 32.5 ± 5.6 μm, the amount of La^{3+} bound to protoplasts treated with 0.1 and 1 mM La^{3+} after washing without EDTA is 0.39 and 3.6 nmol/cm^2, respectively. This calculation assumes that the La^{3+} which is released by EDTA is located at the surface of the protoplasts. An estimation of the surface charge density (approximately 3700 esu/cm^2 at $I = 1.2$ mM) is 13 pmol/cm^2. In this situation, a drastic decrease of the surface negative charges or a reversal of the sign of the charges should be observed because the bound La^{3+} ions overwhelm the negative charges of protoplasts. However, although there were striking effects on surface charges of protoplasts in the presence of La^{3+}, we failed to find such changes of ζ-potentials of La^{3+}-treated protoplasts washed without EDTA. This indicates that the negatively charged species at the membrane surface of protoplasts do not account for the sites for the EDTA-washable La^{3+}. Another candidate for the site of La^{3+}-binding is the undissociated group at near neutral pH. La^{3+} can thus be replaced with undissociated protons or other cations as observed in chelators. In this type of binding, La^{3+} should be replaced with exactly the same numbers of charges to keep the net surface charges unchanged. Existence of La^{3+} binding sites behind the layer of the surface charges is also a possibility.

Ln^{3+} ions are known to antagonize Ca^{2+} and bind strongly to Ca^{2+} sites. Ca^{2+} channels and Ca^{2+}-binding molecules may provide such sites for Ln^{3+} (8, 10, 23). The binding sites for La^{3+} to antagonize Ca^{2+} are generally located on the superficial regions of cells (8, 23). If displacement of Ca^{2+} in the membrane is the event in the initial induction, this tightly bound La^{3+} may not be easily displaced by an electrical stimulus from the site and inhibit the initial induction. Other alterations of membrane function and structure related to fusion also may be exerted by antagonizing Ca^{2+} in the membrane structure. Alternatively, it is possible that La^{3+} ions bind to various molecules without antagonizing Ca^{2+}, since Ln^{3+} ions are also known to bind strongly to various ligands in biological molecules such as phosphate moieties, aromatic rings, and carboxyl groups (11). Since La^{3+} ions bind to protoplast moieties very strongly (4), phospholipids may also provide sites for La^{3+} binding. If this is the case, the binding of La^{3+} should be on the inner surface of the bilayer because binding of La^{3+} to the outer surface may accompany significant alterations of the net surface charges. Integrated components in the membrane structure such as proteins (11) are also candidates for the sites. As cytoskeletal elements may exist in the cytoplasmic membrane (5), they might provide the sites with or without antagonizing Ca^{2+} (11, 23). La^{3+} ions can bridge between or within molecules and cause aggregation (4, 8, 11, 18), thereby hindering a change of the cytoplasmic membrane into the fusible state. Increase of rigidity or reduction of fluidity of the cytoplasmic membrane seems too simple an explanation for the present results. The inhibition of fusion and also the promotion of the development of fusion, implying an ability for the membrane to be reorganized, were observed in the presence of La^{3+}. The formation of a mechanically rigid structure in the cytoplasmic membrane will cause inhibitions of both induction and development of electrofusion.

Promotion of the development of fusion and protection of protoplasts from being disintegrated by pulsation in the presence of La^{3+} were readily abolished by washing without EDTA. A promotion of the recovery process also seems likely. Such effects were also observed with $HACo^{3+}$ and Spd^{3+}, and therefore did not seem specific for La^{3+}, while the inhibition of the initial induction of fusion was specific for La^{3+} and not reversible without EDTA. This indicates that the inhibitory effect on the initial induction and the promotive effect on the development of fusion are exerted by different mechanisms. These promotive effects might arise from a modification of the cytoskeleton, since Ln^{3+} (23) and other multivalent cations (21) are reported to interact with it, and effects similar to those of cytochalasin B have been reported (2). The possibility that these trivalent cations are taken up during electroporation and exert their promotive effects internally cannot be excluded.

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