promoting the intersectional hybridization of poplars, must prove a boon to breeders. Large numbers of hybrid progeny can be obtained, much greater than with recognition pollen, so that selections for cloning may be made from a much wider choice. This is very desirable since the percentage of valuable types produced by these wider crosses will always be smaller than for compatible crosses.

Summary. Pretreatment of stigmas of poplar flowers with hexane or ether promoted hybridization between normally incompatible black and white poplar species. Success rates, as measured by setting of fertile hybrid seed, exceeded 95% of those resulting normally from compatible crosses. Results suggest the existence of some incompatibility factor, possibly of a lipoid nature, located on the stigmatic surfaces.

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A23187 and Red Cells: Changes in Deformability, K^+, Mg^{2+}, Ca^{2+} and ATP

Incorporation of calcium into the interior of the red cell markedly reduces the deformability of the cell membrane[1,2]. Because calcium is efficiently pumped out of the normal red cell[3], experimental incorporation of calcium has required depletion of cellular ATP to allow leakage[1], or hemolysis and resealing[4]. The ionophore A23187 transports divalent cations across cell membranes[5–7], and we have used this ionophore to introduce calcium into red cells containing normal amounts of hemoglobin and ATP in order to investigate deformability in a system as intact as possible.

Materials and methods. Human blood was collected in ACD solution and immediately washed 3 times with Na-*tris* (135 m*M* NaCl; 4.5 m*M* KCl; 15 m*M* choline; 10 m*M* *tris*-HCl, pH 7.3) or K-*tris* (140 m*M* KCl; 15 m*M* NaCl; 10 m*M* *tris*-HCl, pH 7.3) buffer, supplemented with 1m*M* EDTA in the first wash to remove exogenous calcium[8]. Stock solutions (1 *M*) of the above monovalent salts were treated with Chelex resin (Dow) to remove Ca^{2+} and Mg^{2+}. Cells were resuspended to 10% hematocrit in the washing buffer and the divalent cation transport antibiotic A23187 (at 6 mg/ml in 75% ethanol: 25% DMSO) was added to make 0.1% of the volume for a final antibiotic concentration of 10 μm. (A23187 was a gift of Dr. R. L. HAMMILL of Eli Lilly and Co.[9].) After 30 min equilibration at room temperature divalent cations were added and incubated with the cells for 30 min.

Uptake of Ca^{2+} and Mg^{2+} was measured using atomic absorption spectroscopy. Intracellular Na and K contents were measured by flame photometry on cells washed 1 to 3 times in isotonic $MgCl_2$.

The deformability of the cells was measured by filtration through polycarbonate filters with 2.5 μm pores (Nuclepore, General Electric Corp), at 10 mm H_2O pressure, or by measurement of the pressure required to pull red cells through micropipettes as described previously[1]. ATP content was measured by a luciferin-luciferase method[10].

Results. The minimal solution concentration of A23187 required to produce the effects described below was 1 λ of stock A23187 solution per ml of red cells at 10% hematocrit. Upon addition of A23187, cells suspended in Na-*tris* medium shrank by 25%, while intracellular K^+ fell from 95 to 50 m*M* and intracellular Na^+ rose from 9.3 to 12–16 m*M* (Table I). The rapid K^+ efflux has been reported by others[6].

Addition of calcium to A23187-treated cells altered the mean cell volume (MCV) in proportion to the calcium added. The apparent shrinkage caused by 5 μ*M* Ca^{2+} correlates with increased filterability of these cells, but higher concentrations of calcium caused filterability to decrease markedly despite shrinkage. Addition of magnesium caused a similar loss of MCV but filterabtyili was unchanged. Thus MCV and K^+ content appear to be most strongly influenced by A23187, but cell filterability appears to be regulated primarily by calcium.

Deformability of cells was also measured by use of a micropipette technique[1] (Table II). Addition of A23187 alone slightly increased the pressure required to aspirate the cells, and also changed the morphology. Addition of calcium caused a marked increase in Pt for A23187 cells.

Since loss of intracellular ATP can cause loss of deformability, the ATP levels of cells containing A23187 and A23187 plus calcium were measured. The results, shown in Table III, indicate that addition of A23187 caused accelerated depletion of ATP relative to controls. However, addition of calcium did not produce further loss of ATP. Depletion of ATP thus appears to be caused by the A23187 itself. The mechanism of this rapid loss is not readily explained.

Table I. Changes in filterability, mean cell volume and monovalent cation content

Addition	Filterability (%)	MCV (μ³)	K_i	Na_i
None	59	91	95	9
A23187 only	59	73	50	13
A23187 + 5 μ*M* $CaCl_2$	71	62	42	—
A23187 + 50 μ*M* $CaCl_2$	46	67	33	16
A23187 + 500 μ*M* $CaCl_2$	10	72	35	—
A23187 + 50 μ*M* $MgCl_2$	61	68	37	19

Washed red cells at 10% hematocrit in Na-*tris* buffer were incubated 30 min with A23187 and an additional 30 min with divalent cations. Controls were incubated in parallel. Filterability was measured as described. Mean cell volume (MCV) in cubic microns; intracellular K and Na, in mmole/l packed cells. Concentrations of divalent cations are the amounts added to the cell suspension.

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Table II. Pressure required to aspirate A23187-treated cells

Addition	No. cells	Pt, mm $H_2O \pm$ SD	Cell morphology
None	22	6.6 ± 2.4	100% disc
$CaCl_2$ (500 μM)	10	9.5 ± 4.0	100% disc
A23187	31	17.5 ± 13.1	90% crenated disc, rest misc. forms
A23187 + 500 μM $CaCl_2$	33	67.4 ± 37.8	90% smooth sphere, 10% crenated spheres
	(9)	(22.0 ± 5.9)	(crenated spheres)
	(24)	(84.2 ± 37.2)	(smooth spheres)

Cells in Na-*tris* were treated as described in Table I, and aspirated into a 2.9 μm micropipette. The pressure required to pull the cells into the pipette was recorded. Standard deviations are calculated. For cells with both A23187 and calcium, distinct differences in pressure were seen for crenated spheres versus spheres, and these cells are tabulated separately.

The accumulation of calcium and magnesium by red cells in the presence of A23187 was determined and the results are shown in Table IV. Concentration dependence was slight, indicating that intracellular sites were not saturated even at a final intracellular concentration of 4 mM. In the absence of added magnesium, intracellular magnesium (2.5 mM) leaked out. Addition of 0.5 mM $MgCl_2$ to the cell suspension resulted in magnesium accumulation. These values for Ca^{2+} and Mg^{2+} distribution were attained by 30 min and were stable for several hours. Since loss of filterability occurs in a range of calcium concentrations in which percent calcium accumulation varies only slightly, titration of the affinity of the sites responsible for rigidity with extracellular EGTA was not attempted.

Table III. ATP content of A23187-treated cells

Addition	ATP level, mM/l packed cells		
	Na-*tris* (2 h)	Na-*tris* (8 h)	K-*tris* (2 h)
None	0.88	0.58	0.81
A23187	0.60	0.15	0.58
A23187 + 50 μM $CaCl_2$	0.58	0.09	0.67
A23187 + 50 μM $MgCl_2$	0.60	0.09	0.64

Cells incubated as described in Table I were assayed for ATP by the method of ALEDORT et al.[10], using luciferin-luciferase. ATP was measured either at 2 h or at 8 h after addition of A23187 to cells, or of ethanol: DMSO to control cells. Data at 2 h correspond to data for filterability, K and Na content, and pipette aspiration, all of which were determined at about 2 h also.

Table IV. Calcium and magnesium uptake in presence of A23187

Medium [$CaCl_2$] (μM)	% Ca uptake		% Mg uptake	
	− Mg	+ Mg	− Mg	+ Mg
500	83	79	71	50
50	83	80	—	55
5	88	81	—	—
0	(100)	(100)	60	50

Red cells at 10% hematocrit in Na-*tris* were treated with A23187 and calcium and magnesium were added to the suspension to obtain solution concentrations of calcium as shown. Magnesium was present either as the intrinsic Mg^{2+} of the cells, about 250 μM in the entire suspension (− Mg) or an additional 500 μM of $MgCl_2$ was added to the suspension (+ Mg). After incubation for 1 h, cation contents of whole suspensions and of supernatants were measured, and hematocrits were taken. After correction for cell volume in the suspension, the percent of each cation in the cells was calculated.

Discussion. The above results confirm earlier observations[1,2,5] that incorporation of calcium into the interior of the red cell causes the membrane to become more rigid. Confirmation of previously reported effects with A23187 indicate that loss of intracellular components is not responsible for the observed loss of deformability with calcium. In addition, it is confirmed, in intact cells, that even large increases in intracellular magnesium do not cause rigidity.

The rapid loss of potassium in the presence of A23187 may be a manifestation of the well-known increase in K^+ efflux caused by intracellular Ca^{2+}[11–14]. It has been shown by REED[6] that Mg-EGTA prevents this K^+ efflux. Other workers have found that total intracellular levels of 80 to 100 μM Ca are required to produce this effect[15,16] and that the intracellular ionized Ca^{2+} level must be about 1 μM[16]. The total level of Ca^{2+} in our system, in the absence of added $CaCl_2$, is about 1 μM. Depletion of intracellular Ca can also cause K^+ efflux[17] but this effect is accompanied by an increase in Na^+ permeability, which we did not observe. Hence the K^+ permeability change is most likely to involve efficient redistribution of intracellular Ca^{2+} to critical sites, as observed for Mg^{2+}[5] in mitochondria.

The great disparity in the levels of calcium required for K^+ efflux and loss of cellular deformability indicates that the mechanism is probably different, and hence the red cell fibrillar network[1,2] is unlikely to be involved in the K^+ efflux.

Summary. A23187 allows red cells to accumulate Ca^{2+} and become rigid. Excess Mg^{2+} does not make cells rigid. A23187 also causes K^+ efflux, even the absence of added calcium.

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