REACTIVATION OF ETHANOL–CALCIUM–ISOLATED CILIA FROM *TETRAHYMENA PYRIFORMIS*

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Cilia isolated from *Tetrahymena* after ciliary shedding is induced by the addition of $CaCl_2$ or other salts to cells suspended in an ethanol solution (4, 6, 11) have been studied chemically and enzymatically (4, 6, 11, 12, 13), but until now have never been shown to be capable of motility in the presence of ATP. In contrast to this, Gibbons (7) has demonstrated the reactivation of glycerol-isolated cilia by techniques similar to those developed for grasshopper sperm tails (8) and *Polytoma* flagella (2). However, the glycerol technique is much less satisfactory than the ethanol-calcium method for isolating pure cilia.

This paper describes the reactivation, which can be obtained with ATP or ADP, of cilia isolated by an ethanol-calcium method (6), followed by glycerination or other treatments.

MATERIALS AND METHODS

Tetrahymena pyriformis W was grown in 8-liter aerated cultures of 1% Difco Proteose-Peptone, 0.25% Difco Yeast Extract, with Dow Corning Antifoam A Spray added to control foaming. After 2½ days, the cells were harvested in a Lourdes continuous flow centrifuge at 300 *g*. The cilia were isolated by an ethanol-calcium procedure similar to Gibbons' modification of Watson's technique (6, 11).

The isolated cilia, precipitated at 15,000 *g*, were cooled to about 0°C and combined with five volumes (unless otherwise stated) of extraction solution at 0°C. Care was taken to prevent the extraction solution from getting too cold, as this caused the cilia to freeze before extraction was accomplished and to subsequently fragment. The total elapsed time from the first addition of ethanol to addition of the final extraction solution was usually 1½ hr. The resulting cilia suspension was reactivated after 30 min by dilution with two parts of ATP solution at 19°C. Activity was studied from 30 min up to 3 hr after addition of extraction solution, during which time the cilia could be reactivated when added to an ATP solution. Movement was measured microscopically by counting the beat frequency with the aid of a stopwatch.

With the exception of the experiments on the effect of temperature, cilia were observed in a room maintained at 19°C. One drop of cilia suspension was added to two drops of reactivation solution, mixed rapidly with a glass rod, and a small drop of the mixture was immediately placed on a glass slide and observed by phase-contrast microscopy at 1000 magnification. A 40-ml container of water placed over the 6-volt light source in the Zeiss microscope base served as a heat filter. By using a glass slide with a built-in thermocouple, the author found that this filtered illumination did not raise the temperature of the slide more than a fraction of a degree over that produced by dark-field illumination at lower magnification.

The apparatus used to regulate and determine temperature in the studies on the effects of temperature is described by Holwill and Silvester (9).

All the extraction solutions used, with the exception of Tris-EDTA and TMSK, contained, in addition to the extracting reagent, 50 mM KCl, 2.5 mM $MgSO_4$, and 20 mM Tris-thioglycollate buffer pH 8.3 at 0°C. The Tris-EDTA solution is identical to Gibbons' Tris-EDTA solution for membrane disruption of the ethanol-isolated cilia, containing 0.1 mM EDTA and 1 mM Tris-thioglycollate buffer, pH 8.3 at 0°C. TMSK solution, designed by Gibbons for maintaining ciliary shape, contains 25 mM KCl, 2.5 mM $MgSO_4$, 200 mM sucrose, and 30 mM Tris-thioglycollate buffer, pH 8.3 at 0°C (6).

The reactivation solutions contained, in addition to ATP or ADP, 50 mM KCl, 2.5 mM $MgSO_4$, and 20 mM imidazole-thioglycollate buffer, pH 6.8 at room temperature. The ATP concentration in the reactivation solution was 0.5 mM unless otherwise stated.

RESULTS AND DISCUSSION

When the ethanol-calcium–isolated cilia are diluted directly into a reactivation solution with 0.5 mM ATP, the membrane appears to swell

and the cilia curl up or, alternatively, fragment. The cilia are not motile, but if they are observed immediately after being rapidly diluted, an occasional cilium can be seen to beat for a few seconds before it too is inactivated.

When the ethanol-calcium–isolated cilia are diluted with five volumes of extraction solution containing 70% glycerol, as used directly by Gibbons to isolate cilia (7), and subsequently diluted into a reactivation solution with 0.5 mM ATP, they retain their shape and do not appear to swell. Initially about 50%, and occasionally as high as 80%, of the cilia are motile, but this fraction decreases rapidly, about one-half of the motile cilia stopping within the 1st min.

The motility of these cilia preparations appears to be more sensitive to temperature variation than that of the glycerol-isolated cilia (7). Motility was maximal at 16°C, and slightly less at temperatures up to 20°C, but fell off sharply outside of this range, with only occasional movement at 24° or 12°C.

The amount of dilution into the glycerol solution also appears to be critical. If the dilution with extraction solution is increased from 5:1 to 20:1, only about one-half as much motility is subsequently observed, and higher dilutions cause an even greater diminution of motility. Gibbons has reported an adverse effect on the motility of his glycerin-isolated cilia upon dilution into a large volume of reactivation solution (7). On the other hand, washing the cilia in TMSK solution and reprecipitating them before extraction did not noticeably alter the results.

Addition of 3% bovine serum albumin to the reactivation solutions caused an increase (greater than 20%) in the motility observed after extraction at a 1:5 dilution in glycerol, but did not improve the reduced motility observed after extraction at a 1:20 dilution, nor did it improve motility when included in the extraction solution. The beneficial effect of albumin, and of the polyvinylpyrrolidone used in other reactivation systems (1, 3) may be partly an osmotic effect. A rough calculation suggests that the readily soluble matrix protein within the cilium, about 25% of the total protein (6), represents an approximately 2% solution of protein within the cilium. The parameters for ciliary size here are considered to be the same as those for *Paramecium* cilia (10), and the protein/cilium is derived from the data of Watson and Hopkins (11). Alternatively, the main effect of albumin or polyvinylpyrrolidone may be simply to reduce the sticking of the cilia to the glass slide and cover glass.

All preparations were improved to some degree by the use of imidazole-thioglycollate buffer rather than imidazole-HCl. In some preparations which showed extremely poor motility due to high temperature or other unknown reasons, thioglycollate increased motility up to 400%, and even the most motile cilia preparations showed about a 15% improvement.

As the ATP concentration was increased, the frequency of wave propagation increased from about 2 beats/sec at 0.1 mM ATP to a maximum of 5–6 beats/sec at 0.5 mM. Further increase in ATP concentration caused a decrease in beat frequency and a corresponding decrease in the number of motile cilia, so that considerably less movement was seen by 2 mM ATP. The adverse effect of increasing ATP concentration on the number of motile cilia was not significantly affected by increasing the Mg^{++} concentration. Approximately the same results were obtained when the experiment was repeated with glycerol-isolated cilia.

Beat frequency at low ATP concentrations could generally be improved by 1 or 2 beats/sec by increasing the Mg^{++} concentration to about 5 mM in the reactivation solution. The addition of Ca^{++} between 0.01 mM and 0.1 mM caused no increase in the per cent of motile cilia, but the beat frequency increased slightly and the duration of motility improved significantly. At concentrations of 10 mM Ca^{++} or Mg^{++}, all movement stopped. This inhibitory effect of high Ca^{++} and Mg^{++} has been observed routinely in similar systems (2, 7, 8).

Cilia could also be reactivated in ADP. At a concentration of 0.1 mM, which was sufficient for some motility in ATP, no movement occurred with ADP. At 0.3 mM, about half as many cilia beat in ADP as in an equivalent ATP concentration, and then only after a 20- to 30-sec lag period. This is comparable to the effect of ADP on *Polytoma* flagella (2), and probably indicates the conversion of ADP to ATP by an adenylate kinase, the presence of which has been indicated in *Tetrahymena* by Culbertson (5). Movement at 0.3 mM ADP is slow, about 1 beat/sec, with higher amplitude waves than in ATP. At higher concentrations of ADP, the per cent motility increased, and between 2 and 3 mM ADP (the highest value used) it was significantly greater than for equivalent concentrations of ATP and

at least as great as at optimal ATP. The frequency of wave propagation was equivalent to that for high ATP concentrations and the lag period had decreased slightly.

The swelling and inactivation of ethanol-calcium–isolated cilia when diluted directly into reactivation solution suggest that an intact semipermeable ciliary membrane is still present, preventing the entry of externally applied ATP. The effect of a number of reagents on membrane permeability to ATP has been studied under the same conditions as were used for glycerol treatment and ATP reactivation. A continued absence of motility after a treatment concomitant with the maintenance of ciliary shape in nonisotonic solution may indicate large-scale membrane destruction leading to a loss of matrix protein, solubilization of some ciliary component necessary for motility, or inhibition of the ATPase or some other enzyme.

Reactivation was not found after use of TMSK as the extraction solution, although shape was maintained, possibly due to isotonicity of the solution. In Tris-EDTA solution, which Gibbons has used to extract proteins from ethanol-calcium–isolated cilia (6), most of the cilia became swollen and curled. Less than 1% of the cilia showed subsequent motility in reactivation solutions, but these moved rapidly, at 5–10 beats/sec, for 5 min or more.

Digitonin has been used for making reactivatable models of mammalian spermatozoa (1). Treatment of ethanol-calcium–isolated cilia with extraction solutions containing 0.5% digitonin and 60% sucrose led to about 40% reactivation; with 0.05% digitonin in 60% sucrose, about 20% reactivation was observed. In both cases, there was a noticeable lag period, up to half a minute, before full motility was observed in the reactivation solution. Less than 1% reactivation occurred following extraction with 60% sucrose without digitonin, or with 0.05% digitonin in TMSK solution. Cilia treated with digitonin alone were never reactivatable.

Substitution of 70% ethylene glycol for glycerol in the extraction solution led to about 50% motility. In this case also, there was a noticeable lag before full motility was obtained. The duration of movement was about three times as long as after glycerol extraction, but few cilia could still be reactivated after 3 hr in the ethylene glycol. Although the cilia maintained their shape after extraction with solutions containing 65% ethanol, no motility was observed. This may have been due to the inhibitory effect of ethanol on ciliary ATPase (unpublished results).

In both the digitonin-sucrose and ethylene glycol treatments, movement in ADP was sparse and slow at all concentrations studied.

SUMMARY

A number of methods are discussed for reactivation of the motility of *Tetrahymena* cilia isolated by ethanol-calcium treatment of the cells. Some characteristics of the reactivation treatments and motility of the cilia are described.

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