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Effect of Calcium on the Pellet Height Response of Tetrahymena Cilia

J. J. Blum and Alvernon Hayes

Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

The pellet height response (a measure of the increase in height of the pellet of cilia obtained by brief centrifugation in the presence of ATP as compared to the absence of ATP) of Tetrahymena cilia prepared by deciliation in the presence of $Ca^{2+}$is sensitive to the concentration of free Ca^{2+} during the pellet height assay. The magnitude of the increase in pellet height and the sharpness of the pellet boundary both increase markedly with increasing $[Ca^{2+}]$. The half-maximal effect is attained at a free $[Ca^{2+}]$ of about 1.5×10^{-7} M. The pellet height assay thus measures a Ca^{2+}-sensitive component of the ciliary motile system. The possibility that this is the Ca^{2+}-sensitive orientation system is discussed.

Key words: cilia, Ca^{2+}-sensitivity, N-ethylmaleimide

When glycerol-extracted cilia of Tetrahymena are centrifuged at low speed in the presence of ATP, the height of the pellet of cilia is severalfold larger than the height of a control centrifuged in the absence of ATP (1). The increase in pellet height is in part due to a swelling of the cilia and thus the sedimentation of a larger fraction of the cilia, and in part to a decrease in pellet density. Studies on the effects of thiourea, nucleotide specificity, and NiCl demonstrated that the pellet height response was a measure of some aspect of ciliary motility (2), but no insight has been forthcoming as to what aspect of the motile system was involved. A requirement for an unmodified SH group on the ciliary dynein was indicated by the finding that NEM, at concentrations which caused a slight enhancement of ATPase activity, inhibited the pellet height response (3). A requirement for Ca^{2+} or Mg^{2+} was also established with the finding that glycerol-treated cilia suspended in 20 mM imidazole, 0.1 mM EDTA, pH 7.5, had no pellet height response and no ATPase activity in the absence of Mg^{2+} or of Ca^{2+} (4). If Mg^{2+} was added, the ATPase activity and the pellet height response increased in parallel, attaining their maximum at about 1.0 mM Mg^{2+}. Very low concentrations of Ca^{2+}, however, caused a marked increase in pellet height response, but larger concentrations were required before there was an appreciable increase in ATPase activity (4). Except for this early indication of an important difference

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Abbreviations: EDTA – ethylenediaminetetraacetic acid; EGTA – [ethylenebis (oxyethylenenitrilo)]-tetraacetic acid; NEM – N-ethylmaleimide; PPDM – N,N′-p-phenylenedimaleimide; DTT – dithiothreitol.

between the effects of Ca^{2+} and Mg^{2+} on the pellet height response, there have been no further studies on the effect of Ca^{2+} on the pellet height response, and, in particular, the effect of Ca^{2+} in the presence of Mg^{2+} had not been investigated. In this paper we report that very low concentrations of Ca^{2+} alter the hydrodynamic properties of cilia as measured by the size of the pellet formed on brief centrifugation in the presence of ATP.

MATERIALS AND METHODS

In earlier studies cilia were prepared by suspending the Tetrahymena at −20°C in 5 volumes of 70% glycerol, containing 2.5 mM $MgSO_4$, 50 mM KCl, and 20 mM thioglycollate, and shearing the cilia off the cells by shaking on a Vortex mixer (1). In the present studies, the cells were suspended at 0°C in a buffer containing 25 mM Tris, 25 mM acetate, 0.1% (wt/vol) EDTA and 15% (wt/vol) glycerol, pH 7.5, and, after preliminary swirling, 25 mM Ca^{2+} was added and the cilia detached by further swirling at 0°C, as described in detail elsewhere (5). Before use, the cilia were stirred at 0°C for 5 min in 20 ml of 0.1% (wt/vol) Triton X-100 in IM 7.5 buffer (20 mM imidazole, 2.5 mM $MgSO_4$, pH 7.5). The demembranated axonemes were washed twice in this buffer (12,000 × g for 10 min each time) and resuspended in IMG buffer (20 mM imidazole, 2.5 mM $MgSO_4$, 0.1 mM EGTA pH 7.5). Axonemes were always prepared from storage at −20°C in IM 8.3 buffer containing 60% (vol/vol) glycerol just before use. All components for the pellet height assays and for ATPase assays (NEM, ATP, DTT, and $CaCl_2$) were dissolved in IMG buffer.

Pellet height assays were performed as follows: 0.3 ml of cilia were added to 0.6 ml of the desired buffer at 25°C and 0.05 ml of NEM or of buffer was added and a timer started. At desired intervals, 0.10 ml of 10 mM DTT was added, and a sample of 0.1 ml removed and added to 0.8 ml of the desired buffer at 0°C and kept at 0°C for about 3 min before assay of its ATPase activity. To the remaining 0.95 ml of cilia, 0.05 ml of 10 mM ATP was added, and the contents of each tube transferred to a Wintrobe Hematocrit tube. Centrifugation [in a bench top clinical centrifuge, as described earlier (1)] was begun within ~ 110–130 sec after the addition of ATP to the first sample, and continued for the desired time (see below). The height of the pellet and of the meniscus in each tube was then recorded; pellet heights were corrected to a meniscus height of 10.0 cm. The sharpness of the boundary depends on the duration of centrifugation (1) as well as on the presence of Ca^{2+} (as reported in this paper). An increase of centrifugation time (e.g., from 2.5 to 3.5 min) can cause a fairly sharp boundary to be formed for some preparations even in the absence of Ca^{2+}, and conversely a very brief centrifugation (e.g., 1.5 min) will yield a fuzzy boundary even in the presence of Ca^{2+}. For all the preparations used in this paper, centrifugation time was 3.5 min (plus about 1.1 min for the centrifuge to stop). Use of the term "fuzzy" to describe a boundary indicates that there is not a sharp demarcation between the pellet of cilia and a clear fluid above it. Instead, the boundary is indistinct because the fluid immediately above it is very opalescent (due to the presence of many cilia). The opalescence decreases with increasing height above the more densely packed pellet and the fluid becomes clear well below the meniscus.

ATPase activity was measured by adding 0.1 ml of 10 mM ATP in IMG buffer to the 0.9-ml samples after briefly warming them to 25°C. Assays were run for 10 min and stopped by addition of 0.5 ml of 10% (wt/vol) trichloroacetic acid. Orthophosphate was measured as described elsewhere (6).

Computations for the amount of free Ca^{2+} in the various solutions were made with the aid of a slightly modified computer program (7) that takes into account the binding of

both Ca^{2+} and Mg^{2+} to ATP as well as to EGTA (Kim and Padilla, submitted for publication). We are grateful to Dr. Y.S. Kim for performing these computations and for measuring the amount of Ca^{2+} in our buffers.

Protein was measured by the method of Lowry et al. (8) using bovine serum albumin as a standard.

RESULTS

In previous studies using cilia prepared by shearing in the absence of added Ca^{2+}, we generally obtained pellet height responses of two to fourfold, although occasionally larger responses were obtained. Cilia prepared by the present method generally gave a much larger response to ATP. In the absence of ATP, most of the cilia remained in suspension, the opacity decreasing with height above a very small pellet. In the presence of ATP a much larger pellet was obtained but contrary to our earlier findings, the pellet boundary was often diffuse and therefore difficult to quantitate. It was also observed that, contrary to our earlier findings (3), preincubation with NEM caused an increase in pellet height. It occurred to us that the change in both qualitative appearance and response to NEM might be due to the presence of EGTA (i.e., the absence of Ca^{2+}) in the present experiments (in addition to any changes caused by the different method of deciliation) and experiments were set up to test for a possible effect of Ca^{2+} on the pellet height response.

Figure 1 shows the results obtained when cilia were incubated with NEM at 25°C for times up to 7.2 min in the absence of Ca^{2+} ($[Ca^{2+}] < 10^{-9}$M) or in the presence of 6.0×10^{-5} M Ca^{2+}. The pellet height in the absence of ATP was very small in both the presence and absence of Ca^{2+}, and was not altered by NEM. In the absence of Ca^{2+}, the boundary of the pellet was "fuzzy"; in the presence of Ca^{2+}, the boundary was sharp. Upon the addition of ATP, the pellet height increased about 10- 15-fold for the cilia without Ca^{2+}. Preincubation with 26 μM NEM caused a small but definite increase in pellet height response, although quantitation of the effect was difficult because in the absence of Ca^{2+} the pellet boundary was indistinct. In the presence of Ca^{2+}, the pellet height increased over 30-fold, and the boundary between the pellet and the clear fluid above was sharp. If the cilia were preincubated with 26 μM NEM, there was only an inhibition of the pellet height response, confirming our previous observations (made without added Ca^{2+} but in the absence of any chelating agent) that NEM inhibits the pellet height response. The upper part of Fig. 1 shows that about the same amount of enhancement of axonemal ATPase activity was obtained whether the cilia were preincubated with NEM in the presence or absence of Ca^{2+}. In the experiments shown in Fig. 1 the ATPase activities were assayed at free Ca^{2+} concentrations comparable to those at which the pellet height assays were performed. As can be seen in Fig. 2, however, there was practically no effect of Ca^{2+} concentration in the range studied here on axonemal ATPase activity whether or not the cilia were preincubated with NEM.

To determine the amount of free Ca^{2+} required for this change in magnitude and quality of the pellet height response, pellet height assays were performed at known concentrations of free Ca^{2+}. Figure 2 shows that at a free Ca^{2+} of $\sim 1.5 \times 10^{-7}$M the response was about midway between the small fuzzy pellet formed in the absence of Ca^{2+} and the larger and sharply delineated pellet formed in the presence of ample Ca^{2+}. Figure 2 also shows that if the cilia were preincubated for 2.5 min with 26 μM NEM, which caused about a 25% enhancement of ATPase activity, there was a slight increase in pellet height in the absence of Ca^{2+} but an inhibition of the pellet height response in the

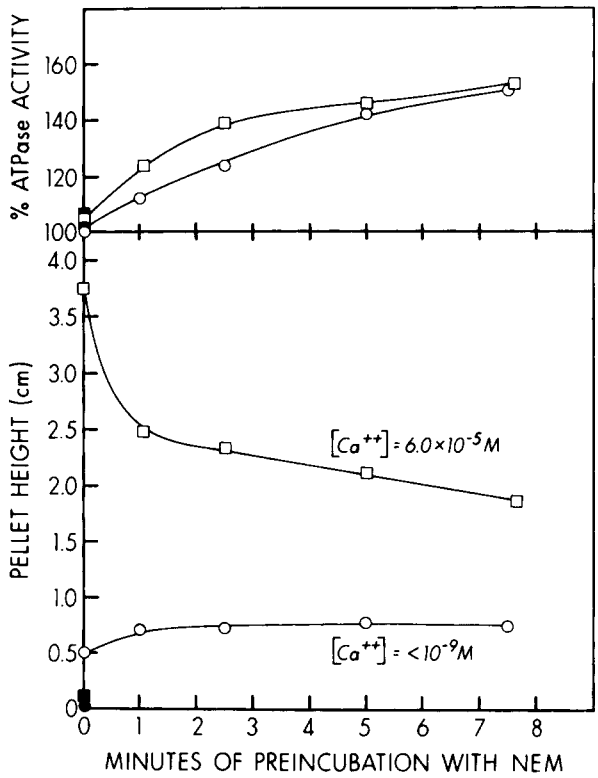

Fig. 1. Effect of preincubation with NEM on pellet height response and ATPase activity of Tetrahymena cilia in the presence and absence of Ca^{2+}. Cilia were preincubated at 25°C for the times shown on the abscissa with 26μM NEM in IMG buffer with or without added Ca^{2+}, as shown. At the indicated time, 0.10-ml samples were removed for assay of ATPase activity, as described in Materials and Methods. The free Ca^{2+} concentration during the ATPase assay of cilia that had been preincubated in the presence or absence of Ca^{2+} was 4.5×10^{-5} M and 1.1×10^{-8} M, respectively. After removal of the samples for assay of ATPase activity, 0.05 ml of 10 mM ATP (open symbols) or 0.05 ml of IMG buffer was added (closed symbols) and the pellet height assay performed as described in Materials and Methods. Protein concentration during preincubation was 1.72 mg/ml; during the pellet height assay, 1.48 mg/ml; during the ATPase assay, 0.172 mg/ml. ATPase activity of 100% corresponds to 166 nmoles/min-mg.

presence of Ca^{2+}. The ATPase activity was practically independent of the free Ca^{2+} concentration during the pellet height assay and was enhanced as expected by preincubation with NEM.

It should be noted that in previous experiments much higher concentrations of NEM were used and a correspondingly larger inhibition of the pellet height response was obtained (3). In experiments with cilia preincubated with 66 μM NEM at 25°C in the absence of Ca^{2+} the pellet height response increased with time of preincubation for the first few minutes. By 9 min there was no net change in pellet height and at longer times a decrease in pellet height response compared to controls was observed.

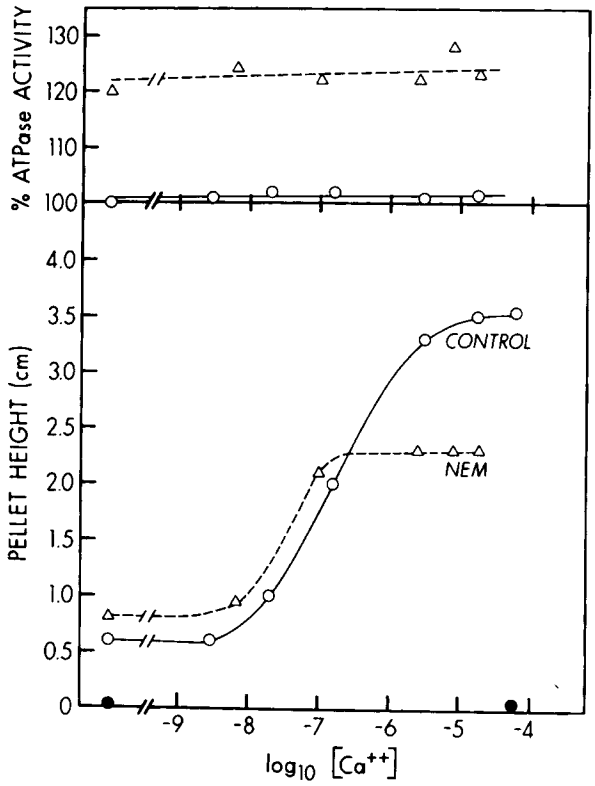

Fig. 2. Effect of Ca^{2+} on the pellet height response of Tetrahymena cilia. Cilia from the same preparation as used in the experiment shown in Fig. 1. were exposed to varying concentrations of free Ca^{2+} as shown on the abscissa in the presence of NEM (○—○) or after preincubation with 26 μM NEM (△- - -△) for 2.5 min at 25°C. ATPase activity and pellet height response were measured as described in the legend to Fig. 1. The free Ca^{2+} concentration during the ATPase assay ranged from 3.5 to 4.1 × 10^{-5} M. Protein concentrations are as listed in the legend to Fig. 1. ATPase activity of 100% corresponds to 179 nmoles/min-mg.

DISCUSSION

Our results show that low concentrations of Ca^{2+} strongly influence the sedimentation properties of demembranated axonemes in the presence of Mg^{2+} and ATP. Under these conditions Ca^{2+} has very little effect on axonemal ATPase activity. Although it is well established that cilia can beat normally at free Ca^{2+} concentrations below 10^{-9}M (9), it has become clear in recent years that Ca^{2+} nevertheless plays an important role in several aspects of ciliary and flagellar beating. Naitoh and Eckert (10) have reviewed much of the literature on the effect of Ca^{2+} on ciliary motility, and suggest that there are at least 2 kinds of motile components in the protozoan cilium, one a Mg^{2+}-dependent system concerned with cyclic bending, the other a Ca^{2+}-sensitive system which governs the orientation of the effective stroke. The Ca^{2+}-sensitive system appears to be localized on

the cilium proper, since deciliation causes loss of the Ca^{2+}-dependent responses in Paramecium (11, 12). A similar role for Ca^{2+} is indicated by studies on glycerol-extracted models of the flagellate Crithidia oncepelti, which in the presence of free [Ca^{2+}] propagate waves from base to tip but which when the free [Ca^{2+}] is reduced to below about 10^{-7}M initiate waves at the tip, as in vivo (13). In addition to playing a key role in determining the orientation of cilia in protozoa (10, 14, 15) and the arrest response of gill cilia of Mytilus (16), Ca^{2+} is also required for the chemotactic turning response of fern spermatozoids (17). Of particular interest is the report (18) that sea urchin sperm extracted in the presence of 5 mM Ca^{2+} have nearly symmetrical flagellar beat patterns when reactivated in Mg-ATP solutions containing $\sim 10^{-9}$ M Ca^{2+} but asymmetrical beat patterns when Ca^{2+} is present. In our present method of preparing cilia, the cilia were detached by swirling in the presence of a high concentration of Ca^{2+}, and then resuspended in a solution where free [Ca^{2+}] was $< 10^{-9}$ M. In the presence of 2.5 mM Mg^{2+}, addition of ATP then causes an $\sim$ 10-fold increase in pellet height. If now sufficient Ca^{2+} ($> 10^{-6}$ M) is also present, there is a further increase of two to fourfold in the height of the pellet. This Ca^{2+}-sensitive component of the pellet height response implies a change in the hydrodynamic properties of the cilia which further enhances their sedimentation rate in a weak centrifugal field. The increase in sharpness of the pellet boundary may also indicate a change in the way the cilia pack. In so far as this Ca^{2+}-sensitive component of the pellet height response is indeed separate from the dynein-powered sliding system, a possible candidate for the Ca^{2+}-sensitive elements is the radial link system, which appears to be responsible for converting active interdoublet sliding into local bending (19). If Ca^{2+} increased the number of attached radial links this could increase the bending resistance and hence decrease the density of the pellet formed during low speed centrifugation. Whatever the mechanism of this Ca^{2+}-sensitive change in the sedimentation of cilia during brief low speed centrifugation, it is not accompanied by an appreciable change of total axonemal ATPase activity.

In earlier experiments with cilia made according to the present procedure and reacted with very low concentrations of PPDM for 18 h at 0°C there was little if any inhibition of the pellet height response (assayed in the absence of Ca^{2+}), but enhancement of the axonemal ATPase activity did occur (5). Conversely, in the present experiments, preincubation with 26 μM NEM for 1 min at 25°C caused an appreciable change in pellet height response (an increase if [Ca^{2+}] $< 10^{-9}$, a decrease if [Ca^{2+}] $> 10^{-6}$ M) but only a slight enhancement of total ATPase activity. Since under these conditions, both NEM and PPDM caused an enhancement of the ATPase activity of solubilized 30S dynein (3, 5, 20) and since the Ca^{2+}-sensitive component of the pellet height response appears not to assay the properties of the dynein arms, it appears unlikely that the SH group responsible for enhancement of dynein ATPase activity is the same as the SH group(s) responsible for inhibition (or, in the absence of Ca^{2+}, enhancement) of the pellet height response. At present we are uncertain as to the significance of the increase in pellet height caused by NEM in the absence of free Ca^{2+}. Because of the increased sedimentability of the cilia in the presence of Ca^{2+}, small changes in ciliary swelling could lead to an appreciable increment in pellet height which might not be of particular importance, but it is also possible that the NEM-caused enhancement of the pellet height response in the absence of Ca^{2+} may reflect an interesting aspect of axonemal motility.

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