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CHARACTERIZATION OF METACHRONAL WAVE OF BEATING CILIA ON FROG'S PALATE EPITHELIUM IN TISSUE CULTURE

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SUMMARY

1. A method is suggested to measure phase *versus* distance between beating cilia by means of a photoelectric device. A statistical method interpreting the results thus obtained is discussed.

2. It was found that: (a) an average phase exists between beating cilia, (b) despite strong fluctuations in phase on a short time scale, the average phase was kept constant over periods of 8 h, (c) the ciliary frequency and the length of the metachronal wave can be measured simultaneously.

3. The average phase differences are linearly dependent on distance.

4. The effective range of synchronization between cilia is of the order of 10 μm indicating that it occurs within one cell.

5. During the cycle of ciliary beating there are periods where coupling is stronger.

INTRODUCTION

It has been observed previously that cilia create a metachronal wave on the epithelium (Sleigh, 1974; Aiello & Sleigh, 1977; Sanderson & Sleigh, 1981; Marino & Aiello, 1982; Sanderson & Sleigh, 1984).

The quasi-periodic optical signal which describes ciliary motion (Eshel, Grossman & Priel, 1985) is defined by three parameters: frequency, amplitude and phase. The frequency and the amplitude of the signal which represent the frequency and the amplitude of ciliary motion, at least when a small number of cilia are studied (Eshel & Priel, 1986*a*), can be easily and accurately obtained with photoelectric devices (Eshel & Priel, 1986*b*) and by other techniques (Cheung & Jahn, 1976; Aiello & Sleigh, 1977). However, it is impossible to measure directly phase differences between beating cilia by a photoelectric method. This is a serious limitation of the method which does not permit characterization of the metachronal wave – one of the most interesting features of cilia. There is, nevertheless, the possibility of utilizing two methods, each of which is capable of demonstrating (among other things) the existence of the metachronal wave and of estimating its wave-length: (a) high-speed cinematography – which, in addition to estimating the wave-length, provides in-

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formation on the frequency and shape of the beating cilia. This technique has greatly contributed to the study of ciliary motion (Aiello & Sleigh, 1977; Sanderson & Sleigh, 1981; Marino & Aiello, 1982; Sanderson & Sleigh, 1984); (b) electron microscopy which has made it possible to both estimate the wave-length and to study the detailed inner structure of cilium and suggests a molecular mechanism for ciliary movement (Warner, 1974; Sanderson & Sleigh, 1981).

In this manuscript we suggest a photoelectric method which measures simultaneously the frequency of ciliary beating and the wave-length of the metachronal wave. Our method permits, moreover, measurement of the degree of synchronization between beating cilia located at two different areas. The distance between the two areas is well defined and can be varied at will. As far as we know, there is no other technique which can measure this parameter.

The method suggested here is a direct extension of our photoelectric method for measuring frequency and amplitude (Eshel *et al.* 1985). It is based on simultaneous measurement of scattered light from two points on the ciliary epithelium or its tissue culture. The distance between the two points can be varied from zero (focused on the same place) to hundreds of micrometres by steps of 0·5 μm. The two signals observed at a given distance are analysed and the parameters describing the quasi-periodic movement are calculated, so that the phase difference at this distance can be determined.

METHODS

The experiments were carried out on locally supplied frogs (*Rana ridibudna*). Ciliary tissue cultures were prepared from the palate as previously described (Eshel *et al.* 1985). Before the measurement the medium over the tissue culture was changed several times, until a total depletion of mucus was achieved. Therefore during the measurements there was no mucus over the cilia, so that the signals observed were derived directly from them.

The preparation was placed flat on the stage, cilia uppermost, with the light coming from above (100 W tungsten–halogen lamp fed from a stabilized home-made d.c. power supply) and passing through the epithelium. An inverted microscope (Olympus, IMT) was used, so that the objective beneath the specimen could be focused on the ciliated surface. Optical fibres (50 μm cross-sectional diameter) were placed in the focal plane of the oculars. One fibre was fixed exactly in the centre of the field (Gama Scientific, 700–10–36a) and the other (Gama Scientific, 700–10–62) could slide along an axis lying in the focal plane, by means of a micrometric screw. The orientation of this axis could be changed by simply rotating the eyepiece. An objective of $\times 20$ corresponding to a field diameter of 2·5 μm was used (Eshel & Priel, 1986*a*). Each fibre was connected to a separate photomuliplier (EMI, 9635B). The photomultipliers' output was further amplified (Par. 113A) and digitalized into the memory of a microcomputer (Apple, IIe) at a sampling rate of 360 Hz.

The two fibres were brought above the same location on the preparation in the following manner: first, some marking point was brought exactly below the tip of the fixed fibre, and then the movable fibre was adjusted also above the same point by means of the micrometric screw. An area of beating cilia was then brought below the fibres and final corrections made for producing on the oscilloscope screen identical time-dependent signals. On comparing visually the identity and the '0' phase between the two signals an estimated accuracy of $\pm$ 1 μm is achieved in determining the '0'.

The signals with '0' distance between the two fibres were sampled and analysed. The movable fibre was then moved along its axis (which was chosen as the one with the greatest phase gradient) in steps of 1 or 1·5 μm ($\pm$0·5 μm) and the signals again sampled, simultaneously.

The calibrations of the data acquisition system and the computer analysis were performed as described previously (Eshel *et al.* 1985).

RESULTS

The power of the suggested method can be best illustrated by representing the actual pairs of signals at various distances between them. In Fig. 1 we present 0·5 s segments of the signals at different positions, one relative to the other. When the two fibres are focused on the same point of the ciliary culture, one expects to get identical signals. However, for detecting the signals two completely separate measuring

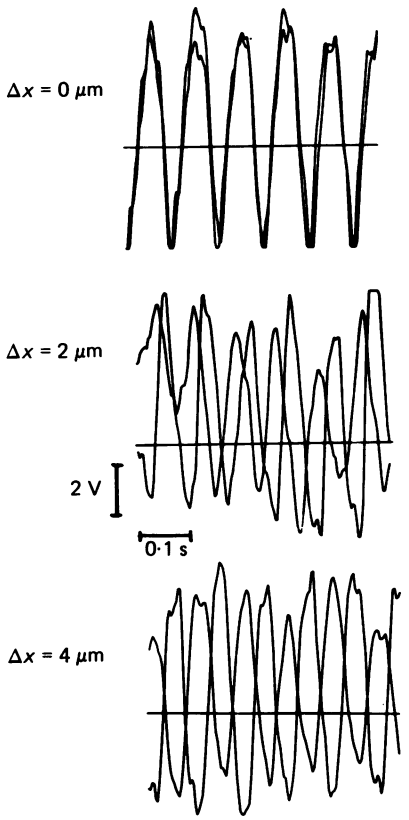

Fig. 1. 0·5 s segments of simultaneously sampled optical signals at different distances (Δx) between two fibres. The field diameter of each fibre is 2·5 μm.

systems are used, each of which may produce a different noise and small distortions. Moreover, the fibres can only be focused on the same point with an accuracy of ± 1 μm, so the overlap is not wholly perfect. The signals are therefore similar, but not identical, as they intrinsically are (Fig. 1, $\Delta x = 0$ μm). With no further calculations Fig. 1 ($\Delta x = 0$ μm) shows that the phase between the two signals is very close to zero.

When the position of the movable fibre is changed by 2 μm with respect to the fixed fibre (Fig. 1, $\Delta x = 2$ μm), the phase between the two signals changes while all the other signal parameters (frequency and amplitude) remain (visually) similar. An increase of distance up to 4 μm further heightened the phase difference between the

signals (Fig. 1, $\Delta x = 4\ \mu m$). The three signals in Fig. 1 were recorded at half-hour intervals and even after 8 h the effect was completely reversible and the average phase difference remained constant.

Careful examination of longer segments of the signals, a typical half-second specimen whereof is shown in Fig. 1, showed the existence of strong phase fluctuations between the two signals over relatively short periods of time. The calibration of the measuring system by external oscillating lamps (Eshel *et al.* 1985) reveals that each signal was stable in phase as a function of time. Recently, frequency fluctuation on a similar system was reported (Eshel *et al.* 1985). For a phase difference to be meaningful the frequencies measured at the two areas must be the same; frequency fluctuation can cause apparent phase fluctuations. However, no correlation was found between frequency fluctuations and phase fluctuations. Moreover, even when the frequency was stable and equal at the two areas, phase fluctuations occurred. Consequently, the fluctuations in phase difference between the two signals are interpreted as being an intrinsic property of ciliary beating.

The most convenient way to represent graphically the phase difference ($\Delta\phi$) between two longer signals, e.g. 40 s records composed of 14400 points each (360 Hz sampling rate), is by Lissajous figures (Burns & MacDonald, 1975). By this method, the amplitude of one signal is drawn *versus* the amplitude of the other one, at a given constant time. It may be shown analytically that:

(a) When two periodic signals are identical (with zero phase difference between them), then the resulting Lissajous figure is a straight line at 45 deg of the 'X' axis.

(b) At $0 < \Delta\phi < 90$ deg an ellipse is created by the principal axis inclined to 45 deg, the width of the ellipse being proportional to the phase difference.

(c) At $\Delta\phi = 90$ deg a circle is created.

(d) At 90 deg $< \Delta\phi <$ 180 deg an ellipse is formed as in case 'b', but with the principal axis at 135 deg with respect to the 'X' axis.

(e) At $\Delta\phi = 180$ deg a straight line inclined to 135 deg is formed.

(f) In the absence of any correlation between the two signals, the points are scattered uniformly over the whole space.

Fig. 2 shows experimental Lissajous figures at four different positions of the two fibres. Each panel is composed of 14400 experimental points of simultaneous sampling. Fig. 2*A* shows that when the two signals are sampled from the same place (within $\pm 1\ \mu m$) most of the points are located around a straight line at 45 deg (as referred to in case 'a' above), which indicates that the average phase difference between the two signals is close to zero. Fig. 2*A* also shows a distribution of points well away from the straight line indicating strong fluctuations of phase. It may also be noted that the line is asymmetric and there is a larger density of points at the upper right-hand corner of the Figure. This indicates that synchronization between cilia is asymmetric and that there are positions during the cycle of ciliary beating where the coupling is stronger.

On changing the position of the movable fibre by 1·5 μm relative to the fixed one, the beating of the cilia was scanned simultaneously from two circles with their centres 1·5 μm apart (Fig. 2*B*). The result was a relatively broad ellipse with its main axis aligned at 45 deg (as referred to in case 'b' above) which shows that the phase difference is $0 < \Delta\phi < 90$ deg. The other features mentioned for Fig. 2*A* (fluctuations, asymmetry) are also apparent in Figs. 2*B*, *C* and *D*.

An additional change of the fibre position to 3 μm with respect to the fixed fibre resulted in almost a disk (Fig. 2*C*) which indicates that the phase difference is close to 90 deg (case 'c' above). When the areas measured were 4·5 μm apart (Fig. 2*D*), an ellipse was again formed, but with its main axis at 135 deg, indicating a phase difference between the two signals of 90 deg $< \Delta\phi <$ 180 deg (case 'd' above).

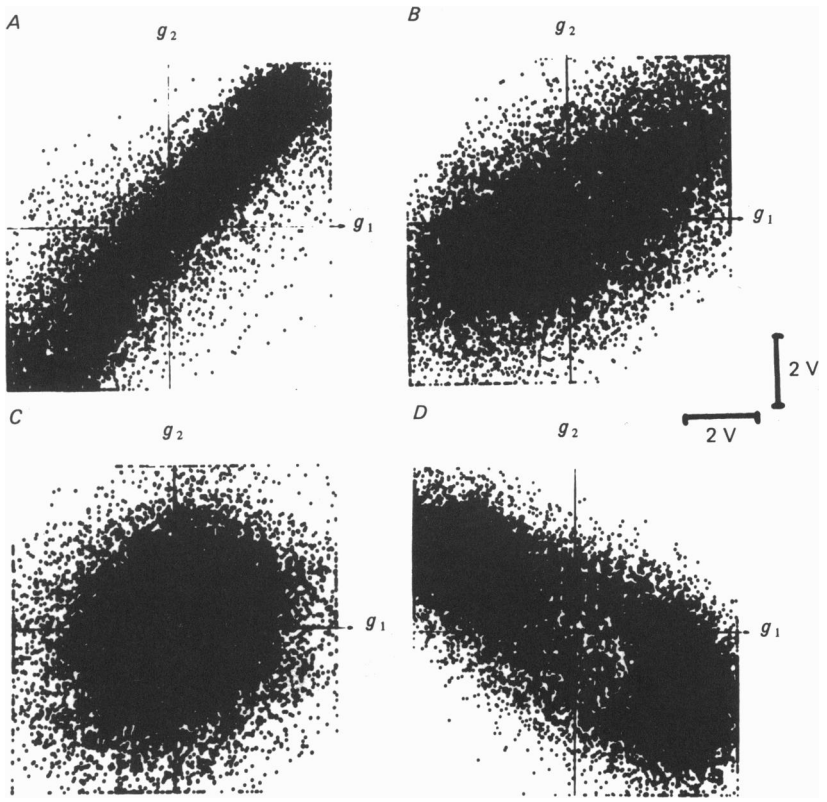

Fig. 2. Lissajous figures of simultaneously sampled optical signals. *A*, $\Delta x = 0$ μm; *B*, $\Delta x = 1{\cdot}5$ μm; *C*, $\Delta x = 3$ μm; and *D*, $\Delta x = 4{\cdot}5$ μm. Each figure composed of 14400 points (40 s of the signals).

From the illustrations it is possible to estimate the metachronal wave-length without the need to resort to calculations. From Fig. 1 it can be seen that for the particular segment that was examined a metachronal wave-length of *ca.* 10–11 μm is found. Clearly a further measurement may yield a significantly different value due to the observed fluctuations in the phase differences. For this reason when we average out over the entire signal (Fig. 2) the estimated wave-length differs from the above value of 10–11 μm and the averaged value is found to be *ca.* 13–16 μm.

DISCUSSION

Simultaneous scrutiny of ciliary movement in relatively close areas of tissue culture shows, even without any sophisticated calculations, that:

(a) The signals observed are, in principle, very similar (Fig. 1).

(b) An average phase difference, as a function of distance between the two measured areas (Fig. 1), does exist.

(c) The signals are quite deterministic; they are defined with high signal-to-noise ratio (Fig. 1).

(d) There are pronounced fluctuations in the phase difference (Fig. 2). This signifies, taking into account the deterministic behaviour of the signals, that these fluctuations are an intrinsic property of ciliary beating.

(e) The average phase difference is a reversible quantity which remains stable for long periods of time. The area was scanned back and forth for almost 8 h, with essentially the same results.

(f) The metachronal wave-length can be estimated from Lissajous figures (Fig. 2) and agrees satisfactorily with estimations by other techniques (Aiello & Sleigh, 1977).

These conclusions were arrived at by visual measurement and scanning of the signals (Figs. 1 and 2). Nevertheless, calculating the changes of phase difference with distance is far from trivial, because of fluctuations in amplitude and phase of the ciliary beating. These fluctuations dictate that a time-averaged procedure must be utilized in order to obtain valid results. We have previously shown (Eshel & Priel, 1986*a*) that by measuring over a single relatively small area, fluctuations in frequency do occur. Therefore, if frequency fluctuations in two relatively close areas are not correlated, there is no significance to phase difference. It was found that although frequency fluctuations do exist, the two signals are correlated most of the time, which means that when a frequency change takes place in one area, the same change occurs in the other over distances of at least 10 μm. This means that, from a phase-measurement point of view, the frequencies are almost constant.

According to Gorelik (1959), the average phase difference between two signals with fluctuating amplitudes and phases is given by:

$$\overline{\cos(\phi_2-\phi_1)} = \frac{\overline{a^2}-(\overline{a_1^2}+\overline{a_2^2})}{2\overline{a_1 a_2}}, \tag{1}$$

where a_1 and a_2 are the average amplitudes of the two signals respectively, a is the average amplitude of the arithmetic sum of the two signals and $\phi_2-\phi_1$ is the average phase difference between the two signals.

The analysis of Gorelik is a statistical one, and our signals are composed of 14400 experimental points which is an adequate sample for a statistical approach. Fig. 3 shows that the average phase difference is linearly related to distance, at least at intervals of $\Delta x = 10$ μm. Moreover, from the slope of the straight line it is seen that a change of one micrometre in distance changes the phase by 23 deg, corresponding to a metachronal wave-length of $15{\cdot}6 \pm 1$ μm.

The effective range of synchronization between cilia, which represents the interval in which cilia beat coherently, can be measured in two ways: (a) by the linear dependence of average phase difference *versus* distance (Fig. 3) and (b) from Lissajous figures (Fig. 2), as was already discussed above. It was found that the range of synchronization is of the order of 8–10 μm and only rarely exceeds this length. The tissue culture from the frog's palate is grown as patches (as for most other tissue cultures) which include several ciliated cells in each patch. The average dimension

of the ciliary cell is $\sim 75\ \mu m^2$ and its form is elliptical with the length of its principal axis of the order of 10 μm. This indicates that the synchronization between cilia is largely limited to the dimension of the ciliary cell. As far as we know, the mechanism of ciliary synchronization is still unproven. However, one of the suggestions is that the external liquid affords synchronization between cilia. Even if we assume that this

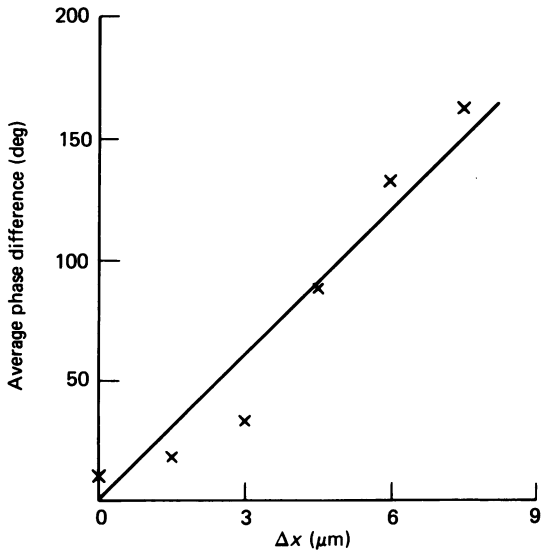

Fig. 3. Average phase difference between signals plotted against the distance between the two fibres (Δx) according to eqn. (1). Each point is calculated from 40 s of simultaneously sampled signals.

is indeed the mechanism of synchronization the hydrodynamic forces are a necessary but not sufficient condition. One further condition that needs to be fulfilled is that the cilia have to be aligned in space in a manner that in principle allows synchronization. Therefore, our findings that the range of synchronization between cilia is of the order of the length of one ciliary cell is only valid for the tissue culture. This is because the possibility exists that during the growth of the explant, cells may change their relative orientation within the patches themselves. Another point that needs to be emphasized is that our measurements are performed in a liquid of relatively low viscosity in comparison to mucus. It was shown that even under such conditions synchronization between cilia does exist; however, the phase fluctuations and the range of synchronization as well as the wave-length are influenced by the external conditions. In order to generalize our findings, additional work on tissue cultures in various media is needed as well as the study of ciliary organs of different origin. This work is now under way in our laboratory.

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REFERENCES

AIELLO, E. & SLEIGH, M. A. (1977). Ciliary function of the frog oropharyngeal epithelium. *Cell and Tissue Research* **178**, 267–278.

BURNS, D. M. & MACDONALD, S. G. G. (1975). *Physics for Biology and Pre-Medical Students*, pp. 216–219. Philippines: Addison–Wesley Publishers Ltd.

CHEUNG, A. T. & JAHN, T. L. (1976). High speed cinemicrographic studies on rabbit tracheal (ciliated) epithelia: determination of the beat pattern of tracheal cilia. *Pediatric Research* **10**, 140–144.

ESHEL, D., GROSSMAN, Y. & PRIEL, Z. (1985). Spectral characterization of ciliary beating: variations in frequency with time. *American Journal of Physiology* **249**, C161–165.

ESHEL, D. & PRIEL, Z. (1986*a*) Spectral characterization of ciliary beating: biological meaning of the spectral line-width. *Biophysical Chemistry* **23**, 261–265.

ESHEL, D. & PRIEL, Z. (1986*b*) Spectral characterization of ciliary beating: temperature dependence of spectral parameters. *Biophysical Chemistry* (in the Press).

GORELIK, G. S. (1959). *Oscillations and Waves* (in Russian), ed. RITOV, C. M., pp. 447–443. Moscow: Physical and Mathematical Press.

MARINO, M. R. & AIELLO, E. (1982). Cinemicrographic analysis of beat dynamics of human respiratory cilia. *Cell Motility*, suppl. 1, 35–39.

SANDERSON, M. J. & SLEIGH, M. A. (1981). Ciliary activity of cultured rabbit tracheal epithelium: beat pattern and metachrony. *Journal of Cell Science* **47**, 331–347.

SANDERSON, M. J. & SLEIGH, M. A. (1984). The effect of serum immunoglobins on the metachronal coordination of the lateral cilia of *Mytilus edulis*. *Cell Motility* **4**, 103–119.

SLEIGH, M. A. (1974). Metachronism of cilia of metazoa. In *Cilia and Flagella*, ed. SLEIGH, M. A., pp. 287–304. New York: Academic Press.

WARNER, F. D. (1974). The fine structure of the ciliary and flagellar axoneme. In *Cilia and Flagella*, ed. SLEIGH, M. A., pp. 11–39. New York: Academic Press.