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Rapid, microtubule-dependent fluctuations of the cell margin

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SUMMARY

Using data automatically acquired by microinterferometry from large numbers of chick fibroblasts, we have detected rapid microfluctuations in the rates of protrusion and retraction of the cell margin which were strongly suppressed by colcemid, nocodazole and taxol. Fluctuations in the rate of retraction of the margin were about twice as powerful as fluctuations in the rate of protrusion. High-frequency fluctuations were also apparent in the cell track and in measures of cell spreading, shape and speed. These rapid fluctuations were also all suppressed by colcemid and nocodazole, sometimes by doses insufficient to disrupt the majority of microtubules. Taxol on the other hand did not suppress fluctuations in direction of the cell track nor fluctuations in the spreading of the cells but it was very effective at suppressing variations in protrusion and retraction and in cell speed and shape.

We discovered that much slower, larger-scale variations in protrusion, retraction, spreading, shape and speed resulted from the accumulation of these rapid, microtubule-dependent fluctuations of the cell margin. These large-scale variations in cell behaviour were also suppressed by the same drug treatments that were effective in suppressing the corresponding high-frequency fluctuations. We speculate that a function of microtubules is to enhance the fibroblast's responses to its environment by causing microfluctuations of the cell's margin which give rise to large-scale variations in cell behaviour.

Key words: Colcemid, Nocodazole, Taxol, Dynamic instability, Interference microscopy

INTRODUCTION

It has long been suspected that small, localised protrusions and retractions of the cell margin might be coupled to the local activities of microtubules. Couchman et al. (1985) observed that mitochondria tend to be good markers for microtubules in phase contrast microscopy of the leading lamellae of fibroblasts; they found that the movement of a single mitochondrion towards the leading edge under the influence of azide would always give rise to a small localised protrusion at the cell margin. More recently, direct observations of fluorescently labelled microtubules in the living growth cones of cultured *Xenopus* neurons have revealed that the reorientation of microtubules may predetermine growth cone turning (Tanaka and Kirschner, 1991, 1995). Although the advance of the growth cone and the extension of microtubules are not strictly coordinated, there does appear to be a closer coupling when the microtubules are splayed out rather than looped back on themselves (Tanaka and Kirschner, 1991). Furthermore, one effect of disrupting microtubules in the leading lamella of fibroblasts is to reduce the protrusion and retraction activity of the leading edge (Bershadsky et al., 1991). These observations and many more suggest that, although microtubules do not drive the protrusion of the leading edge, they can locally modulate and direct protrusion and may even exert this effect over some distance (Rosania and Swanson, 1996).

We undertook this study primarily to discover which aspects of the protrusion/retraction activity of the cell margin are dependent on the integrity of the microtubule system. Using the recently developed method of phase-shifting microinterferometry (Dunn and Zicha, 1993; Zicha and Dunn, 1995) we were able to acquire large quantities of data revealing rapid microfluctuations as well as slower, larger scale excursions of the cell margin. Parallel measurements were made of changes in the spreading, shape and translocation of the cells. We have previously shown how the translocation of the cell is determined by the areas and relative positions of the regions of protrusion and retraction of the cell margin (Dunn et al., 1997). Liao et al. (1995) have proposed that low concentrations of nocodazole and taxol can interfere with fibroblast locomotion by inhibiting the dynamic cycling of microtubules. Here we have sought to trace any such effects on cell translocation and shape back to the microtubule-dependent activity of the cell margin.

MATERIALS AND METHODS

Cell culture – secondary chick heart fibroblasts

Explants from E7 chick embryo heart ventricles were set up in flasks of Medium 199 with Hanks' buffered salts (Sigma-Aldrich Company Ltd, Poole UK) supplemented with 10% heat inactivated foetal bovine serum (Life Technologies Ltd, Paisley, UK), 5% EE_{50} embryo extract (Imperial Laboratories (Europe) Ltd, Andover, UK), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma-Aldrich) and 2 mM

L-glutamine (ICN Biomedicals Ltd, Thame, UK). The cultures were left for 2 days at 37°C in a humidified incubator in 2% CO_2, after which they were washed twice in calcium/magnesium free PBS (Imperial Laboratories) and briefly treated with 0.05% trypsin/Versene (Life Technologies Ltd) until the cells had rounded up. The fibroblasts were then suspended in medium and seeded onto acid-washed coverslips after allowing the explants to settle out.

Each filming chamber consisted of a 1 mm thick glass microscope slide with a central 15 mm diameter hole covered by cementing a 38 mm × 38 mm No. 2 coverslip to one face of the slide using a u.v.-curing glass cement (Southern Watch and Clock Supplies Ltd, Orpington, UK). After thorough washing and soaking in several changes of serum-containing medium, each chamber was filled with test medium consisting of the above culture medium with or without the addition of one of the test agents. Colcemid (demecolcine, Sigma-Aldrich cat. ref. D-6279) was used at a final concentration of 0.1, 0.25, 0.5 or 1.0 μg ml^{-1}; nocodazole (Sigma-Aldrich cat. ref. M-1404) at 100, 200 or 500 nM and taxol (Paclitaxel, Sigma-Aldrich cat. ref. T-7402) at 25, 50 or 100 nM. Data from the two lowest doses of colcemid were pooled for statistical analysis, to give a sample size comparable with the others, and we have referred to this group as having a concentration of 0.2 μg ml^{-1} in the plot labels. A coverslip seeded with cells as above, and incubated for 2-3 hours, was then inverted onto the chamber and sealed in position using a hot wax mixture (1:1:1, paraffin:Vaseline:beeswax). A small bubble of air was left in each chamber to allow CO_2 equilibration with the Hanks' salts. The cultures were then ready for interference microscopy or fluorescent staining.

Fluorescent staining for tubulin

After three hours of incubation at 37ºC, coverslips were removed from the filming chambers, washed in Hanks' BSS, and fixed by immersing in methanol at –70ºC for one minute followed by six dips into acetone at –70ºC. Once dry, the fixed cultures were incubated at 4ºC overnight with the anti α-tubulin antibody DM1A (a gift from Dr P. A. M. Eagles) diluted 1:500 in 2% BSA/PBS. The cultures were washed in PBS and incubated with Texas Red-conjugated rabbit anti-mouse antibody (Amersham International plc, Bucks., UK) diluted 1:25 in 2% BSA/PBS for 2 hours then washed with PBS and mounted on a slide with a PVA/DABCO mixture (consisting of 5 mM Tris-HCl, pH 8.5, made up in dH_2O and 33% v/v glycerol to which 15% w/v polyvinyl alcohol (Mw 30-70 000, Sigma-Aldrich) and 2.5% w/v DABCO (diazabicyclo[2.2.2]octane, Sigma-Aldrich) had been added which acted as an anti-photobleaching agent. The cultures were viewed using a Leica TCS NT laser scanning confocal system fitted to a Leica DMRBE microscope using a ×63 (NA 1.32) objective.

Digitally recorded interference microscopy with automatic phase-shifting (DRIMAPS)

Recording of cell behaviour was started as soon as possible after applying the chosen treatment regime, usually within 5 minutes of assembling the chamber, and the exact time of application was written in the file header. Cell behaviour was observed in monochromatic illumination (546 nm) using a Horn interference microscope fitted with a ×20 twin interference objective (Leitz, Wetzlar, Germany) and adapted for automatic phase-shifting (Dunn and Zicha, 1997). This was kept at 37°C in a temperature-controlled warm room. Interference images were acquired by a TM-765e CCD camera (Pulnix Europe Ltd, Basingstoke, UK) and a Matrox Magic image acquisition board (Matrox UK Ltd Swindon, UK). A sequence of eight interference images captured at video rate during 0.32 seconds, while phase-shifting under computer control, was used to calculate each phase-shifted interference (PSI) image. Time-lapse sequences of PSI images were gathered at a frame interval of one minute. An automatic shutter blocked the illumination of the cells between frames during which time each PSI image was calculated and stored on hard disk.

After recording a total of 1,200 frames over a 20 hour period, the PSI image sequence was processed to compensate for drift in the microscope settings and to remove any residual distortion of the wavefront introduced by the microscope optics (Zicha and Dunn, 1995). In the resulting PSI images the brightness is linearly related to the optical path difference (OPD) introduced by the cells and, since the OPD is closely related to the quantity of non-aqueous material in the light path, each image can be interpreted directly and automatically as the distribution of dry matter in the cells. Next each cell in the image sequence was tracked manually to identify the cell in successive frames (Zicha and Dunn, 1995). The stored tracking coordinates were then used by a computer to find each identified cell in each image and to calculate its total dry mass, spread area, the coordinates of its mass centroid and area centroid and several parameters describing its shape and mass distribution. Parameters describing protrusion and retraction were obtained by comparing images separated by a fixed time lag (Zicha and Dunn, 1995), see Results for more details.

The data obtained by the DRIMAPS system were analysed in *Mathematica*® (Wolfram Research Inc., Champaign, Illinois, USA). All data from cells in contact with each other or with the frame edge were automatically removed from further analysis. The remaining data from each isolated cell consisted of one or more continuous sequences or runs of varying length sampled at 1 minute intervals. Only runs of length at least 60 were used in the following spectral analysis and for the analysis of variance these were partitioned into runs of 60, rejecting any remainder, to give 1 hour runs. The total number of 1 hour runs for each treatment were: control, 511; colcemid, 529; nocodazole, 464; taxol, 367.

Statistical methods

In analysing the effects of the different treatments on a parameter's values, we found that it was critically important to take account of random variations between cultures and between cells within a culture; simply taking means and standard errors within each treatment category could yield very misleading significance levels. The statistical model required for analysing the sources of variation in such nested data structures is known as a hierarchical Analysis of Variance (ANOVA). The critical null hypothesis to be tested is usually that the treatments do not introduce a significant difference in the parameter's values in addition to the random variations between cultures, between cells and within cells. The standard hierarchical ANOVA requires balanced data (equal numbers of elements at each level) but cell-behavioural data are rarely balanced and the number of recorded cells within each culture can vary widely. Fortunately, several methods have recently been developed for treating unbalanced data and we have elected to use the method-of-moments for estimating the variance components at each level and the Satterthwaite approximation to test the significance of the additional variance due to each level (Milliken and Johnson, 1992). This yields F-ratios which can have non-integer degrees of freedom and the significances of these were tested using the FRatioPValue routine in *Mathematica*®.

For each parameter, we applied nine pairwise ANOVAs to the 1 hour runs to determine whether any of the three colcemid, three nocodazole and three taxol treatments differed significantly from the control. The resultant probability values were given a star-rating as follows: ***≡$<P\leq 0.001$; **≡$0.001<P\leq 0.01$; *≡$0.01<P\leq 0.05$. In occasional cases where the distributions of the data deviated badly from normality, we achieved much closer approximations to normal distributions by applying a log transform before performing the ANOVA. The results of the tests revealed that generally there were highly significant variations between cells, and occasionally between cultures, which would often have led to false indications of significant effects in the absence of a fully nested test. For the same reason, the use of error bars in plotting the data is inappropriate and we have instead indicated the distribution of the data using a dark grey bar to show the range of values lying within the inner two quartiles (50% of the data) and two light grey bars extending from this to the first and ninth deciles so that the combined bars cover 80% of the data. The

reason for choosing these values is that, if the data are normally distributed, then the median value shown by a dot should bisect the dark grey bar and each half should be approximately equal in length to the light grey bars.

For analysing the variation with time of each parameter, and how this was affected by the treatments, we used spectral analysis routines from the Time Series Pack issued as a supplement to the *Mathematica*® package. Each power spectrum reveals the strength of the variations in the data as a function of the frequency of oscillation. These spectra were derived from sample auto-covariances obtained from the long runs of data up to a lag of 50 minutes as described in the handbook for the Time Series Pack. The auto-covariances for all runs of one treatment were pooled by weighting according to run length and the smoothed power spectrum was obtained using the Tukey-Hanning lag window of width 50. Further details are given in Results.

RESULTS

Microtubule disruption

Fig. 1 shows six scanning confocal images of fibroblasts stained with the fluorescent anti-tubulin. Most of the tubulin appears to be polymerised in the untreated fibroblast (Fig. 1A) and in fibroblasts treated for 3 hours with 100 nm nocodazole (Fig. 1D) or with 50 nm taxol (Fig. 1F). In contrast, fibroblasts treated for 3 hours with 0.1 μg ml^{-1} colcemid (Fig. 1B), with 0.5 μg ml^{-1} colcemid (Fig. 1C) or with 500 nm nocodazole (Fig. 1E) all show some degree of depolymerisation resulting in a diffuse or punctuate cytoplasmic staining. In these cases, the background of depolymerised tubulin generally obscures intact microtubules except for those overlapping the nucleus but a few intact microtubules can usually be seen radiating from the centrosome when this is visible. We made no attempt to quantify the dose dependency of microtubule disruption by colcemid or nocodazole but it became clear in a survey that some intact microtubules persisted in the majority if not in all the cells at all the concentrations studied.

Protrusion-retraction analysis

In order to obtain a quantitative description of the fluctuations of the cell margin, we considered the regions of protrusion and retraction as defined by Dunn et al. (1997). Protrusion is defined for the time interval Δt as the region of the substratum which was not covered by the cell at time t but was covered at time $t + \Delta t$. Conversely, retraction is the region which was covered at time t but not at time $t + \Delta t$. The upper part of Fig. 2 shows a small portion of two PSI images illustrating the change in mass distribution of an isolated chick fibroblast during a 5 minute interval. Each pixel in these images was approximately 0.5 μm^2 in area and was considered to belong to the cell if it contained at least 12 fg of dry matter which was the threshold sensitivity of the system. The two regions covered by the cell were then used to obtain the regions of protrusion (grey) and retraction (black).

If protrusion and retraction are sampled over an interval of Δt = 5 minutes as in Fig. 2C, the typical picture that emerges

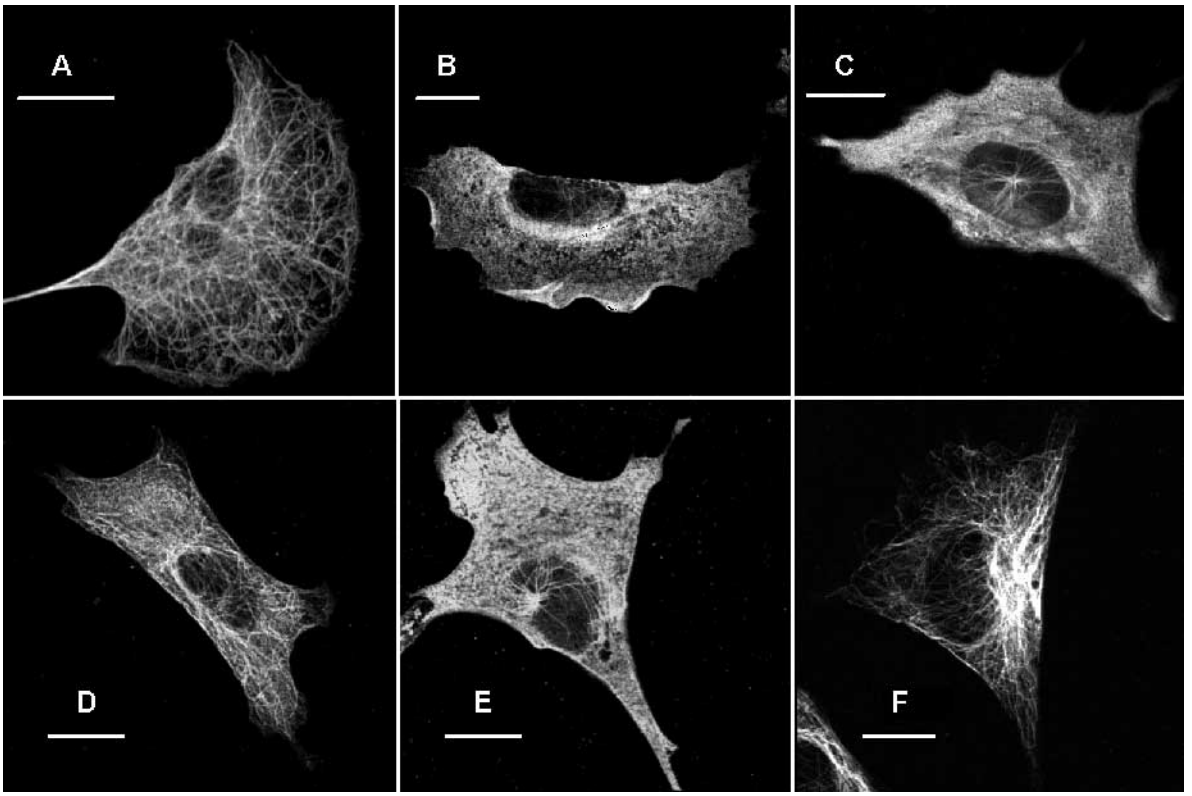

Fig. 1. Scanning confocal images of secondary E7 chick heart fibroblast immunolabelled for α-tubulin (see Materials and Methods). Bars, 10 μm. (A) Untreated cell. (B) Cell after 3 hours exposure to 0.1 μg ml^{-1} colcemid. (C) Cell after 3 hours exposure to 0.5 μg ml^{-1} colcemid. (D) Cell after 3 hours exposure to 100 nM nocodazole. (E) Cell after 3 hours exposure to 500 nM nocodazole. (F) Cell after 3 hours exposure to 50 nM taxol.

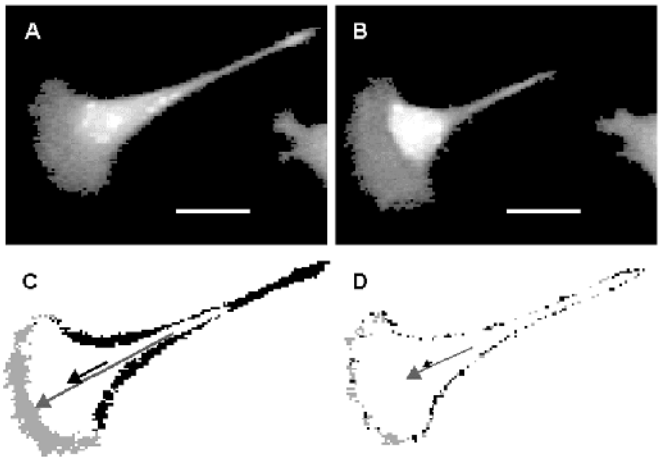

Fig. 2. (A,B) Portions of two time-lapse PSI images recorded 5 minutes apart. Bar 20, μm. (C) Regions of protrusion (grey) and retraction (black) extracted from the two upper images. (D) The same regions extracted from two intermediate PSI images recorded 1 minute apart.

is of a broad band of protrusion at the leading edge of the cell and a retracting margin in the tail region. In other words protrusion and retraction each tend to form a contiguous region and these tend to lie at opposite ends of the cell. On the other hand, when the sampling interval is reduced to 1 minute, the sites of protrusion and retraction tend to break up into many small regions interspersed along the active edges of the cell margin as in Fig. 2D. This indicates that protrusion and retraction in these fibroblasts are not smooth continuous processes but tend to be noisy when viewed with sufficient time resolution. This paper is concerned with the nature of this noise, its dependence on microtubules and its consequences for the long term motile behaviour of the cell.

We first present the effects of microtubule stabilising and destabilising drugs on the mean levels of protrusion and retraction. Measures of the total areas of the regions of protrusion and retraction were sampled at a time interval of Δt = 1 minute and then means were taken over the 1 hour runs. The results of the ANOVA tests revealed that the areas of protrusion were generally reduced by treatment with all three drugs although this reduction was not significant at the lower concentrations in the cases of colcemid and nocodazole (Fig. 3). Taxol was significantly effective at all three concentrations. The mean levels and dose-response curves for the areas of retraction were practically identical to those for protrusion, with exactly the same pattern of significance levels, and so the retraction results are not shown. This close matching of the mean levels of protrusion and retraction over 1 hour periods is to be expected when the cell is maintaining a fairly constant spread area (Dunn and Zicha, 1995). However, we next looked at their short-term variations which turned out to be quite different.

Variations in the rates of protrusion and retraction were examined using spectral analysis of runs of data of length at least 60 minutes. Fig. 4 shows the power spectrum of the 1 minute area of protrusion (Fig. 4A) and of the 1 minute area of retraction (Fig. 4B) for the pooled untreated data (black line) and for the various drug treatments (grey lines). The highest frequency theoretically detectable in the data is half the sampling frequency and the horizontal frequency axis thus

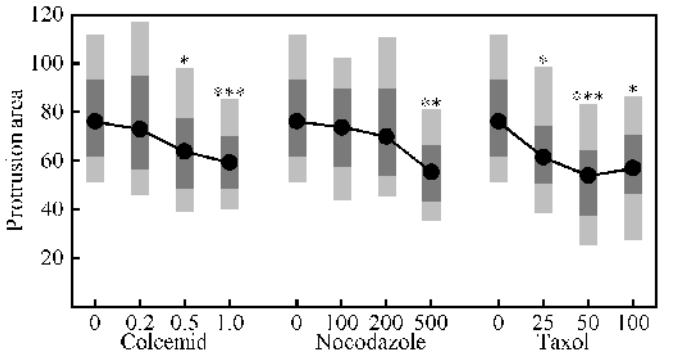

Fig. 3. The 1 minute area of protrusion in μm^2 plotted against the dose of colcemid in $\mu g\ ml^{-1}$, the dose of nocodazole in nM and the dose of taxol in nM. Means were taken over 1 hour intervals and the distributions of these means are represented by the dots (median values), the dark grey bars spanning 50% of the values and the light grey bars spanning 80% of the values (see Materials and Methods). The data were then nested according to cells and to cultures and the star rating in each plot indicates the results of nested, unbalanced ANOVA tests (see Materials and Methods).

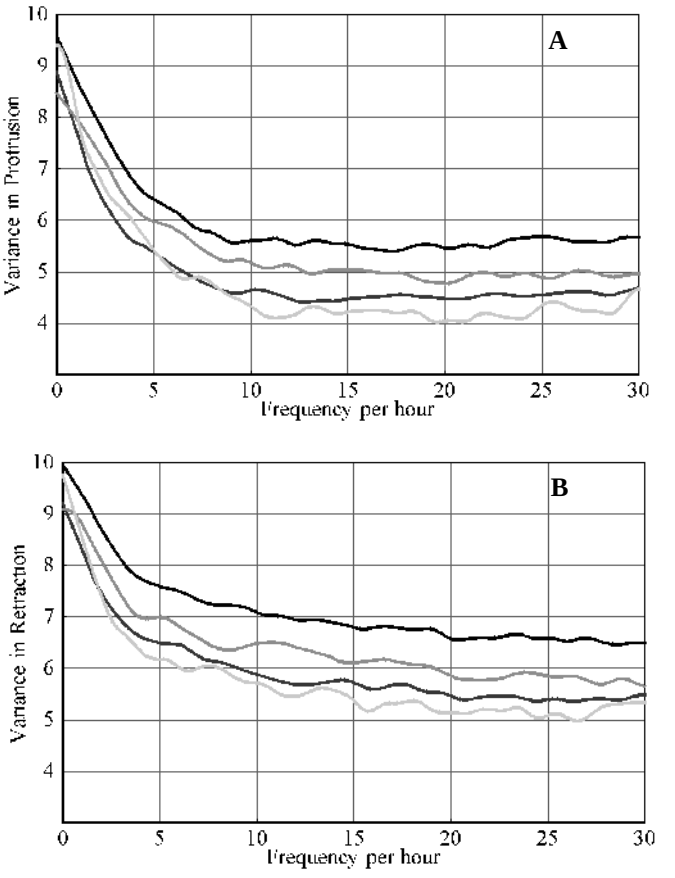

Fig. 4. (A) Power spectrum of the 1 minute protrusion areas for untreated cells (black line), for the pooled colcemid-treated cells (dark grey line), for the pooled taxol-treated cells (mid grey) and for cells treated with 500 nM nocodazole (light grey). (B) The same for the 1 minute retraction areas. The horizontal axis in each plot is the frequency in $hour^{-1}$ and the vertical axis is the $\log_2$ of the variance in the data (the power) attributed to each frequency.

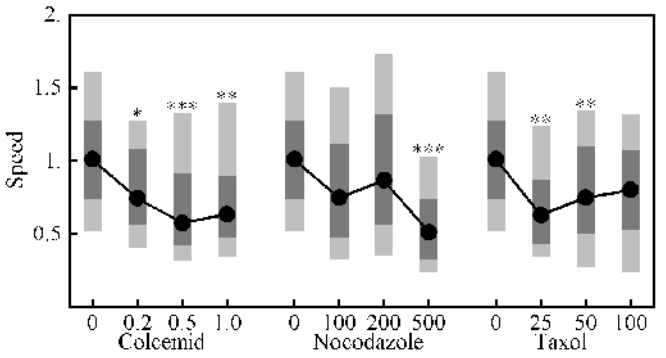

Fig. 5. Cell speed measured as the magnitude in μm of displacement of the area centroid during a 1 minute interval. Means were taken over 1 hour intervals and further details are given in the legend to Fig. 3.

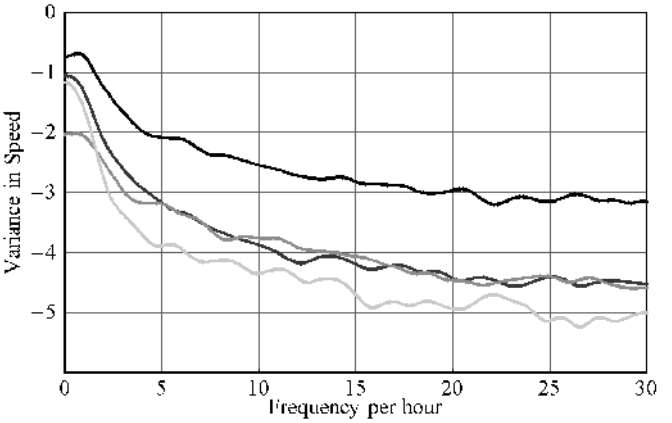

Fig. 6. Power spectrum of the cell speed measured as described in the legend to Fig. 5 for untreated cells (black line), for the pooled colcemid-treated cells (dark grey line), for the pooled taxol-treated cells (mid grey) and for cells treated with 500 nM nocodazole (light grey). Other details as in Fig. 4.

covers the full range of 0 to 30 cycles per hour. The vertical axis is labelled with a $\log_2$ scale indicating how much of the variance in the data is due to oscillations with the corresponding frequency. Thus an increase of 1 on the vertical axis corresponds to a doubling of the power of the oscillations.

In each plot, the power falls off rapidly and levels out to a plateau as the frequency increases. Concentrating first on the untreated data in each plot, the low-frequency fluctuations in protrusion (Fig. 4A) were around 16 times more powerful than the high-frequency fluctuations (4 doublings on the $\log_2$ scale) and around 10 times more powerful in the case of retraction (Fig. 4B). It is clear that the high-frequency fluctuations in retraction were about twice as powerful as those for protrusion. As with the mean areas of protrusion and retraction, the effects of the drugs were dose-dependent and we have represented in the plot only those concentrations which were significantly effective at reducing the mean areas of protrusion and retraction. It can be seen that the two microtubule disrupting drugs, colcemid (dark grey) and nocodazole (light grey) were very effective in reducing the power of the fluctuations of both protrusion and retraction to less than half their control levels. Taxol (mid grey) appears not to have been quite so effective. It is particularly interesting that the suppression of the fluctuations by all three drugs spans the whole frequency spectrum.

We conclude that protrusion is a much smoother process than retraction: in untreated cells, the high-frequency fluctuations in protrusion have only half the power of those of retraction. All three drugs suppress not only these high-frequency fluctuations but equally suppress the much more powerful, lower frequency variations in the rates of protrusion and retraction.

Cell translocation

We have shown previously that the translocation of the cell, as measured by the displacement of its area centroid, is directly dependent on the areas of protrusion and retraction, relative to the total cell area, and on the distance separating the regions of protrusion and retraction (Dunn et al., 1997). The three drugs might therefore be expected to reduce the speed of cell translocation in the same way as they reduced the mean levels of protrusion and retraction. This is what we found. Fig. 5 shows cell speed expressed as the 1 minute displacement of the area centroid and the effects of the three drugs were generally to suppress cell speed from about 1.0 μm minute^{-1} to around half this value. However, the dose dependency of this speed suppression is not a close match to that of the suppression of protrusion and retraction in Fig. 3 and we conclude that the drugs must therefore have some effect on the relative disposition of protrusion and retraction as well as on their areas. We did not analyse this in further detail.

Variations in the speed of the area centroid were examined using spectral analysis (Fig. 6) as with protrusion and retraction. In this case the slower variations in speed of the untreated cells (black line) were only some 10 times more powerful than the high frequency fluctuations. Again, all three drugs suppressed the variations in speed across the whole spectrum. The response to the drugs was again dose-dependent and only those concentrations that were significantly effective at reducing mean speed were included in the spectral analysis.

The high frequency noise that we detected in the speed of the untreated cells is only one aspect of the fluctuations in cell translocation. When examining a sample of the tracks traced out by their area centroids, we noticed that these tended to be noisy in comparison with the tracks simultaneously traced out by the mass centroids of the same cells and that this noise appeared to be suppressed by colcemid (Fig. 7). In other words,

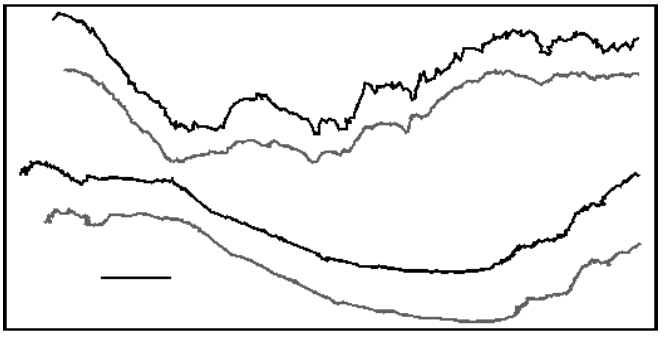

Fig. 7. The upper black line is the track of the area centroid of an untreated fibroblast recorded for 222 minutes and the lower black line is the same for a cell treated with 1.0 μg ml^{-1} colcemid and recorded for 280 minutes. The grey lines shown below each of these tracks are the corresponding tracks of the mass centroids. Bar, 10 μm.

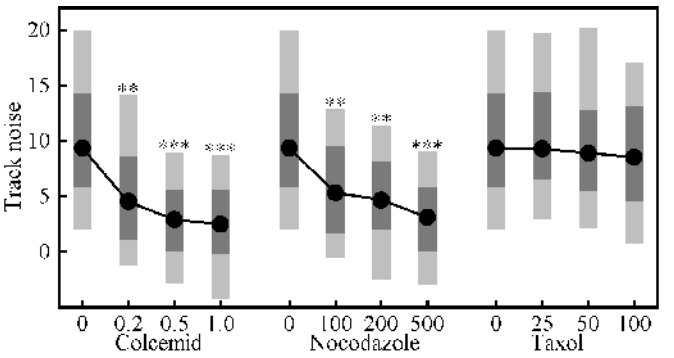

Fig. 8. The track noise measured as the difference in length in μm between the 1 hour track of the area centroid and the 1 hour track of the mass centroid. Further details are as in Fig. 3.

the area centroid of untreated cells shows rapid fluctuations in direction in comparison with the mass centroid. This would be expected if the cell were rapidly protruding and withdrawing thin lamellipodia since their low mass density would have little effect on the position of the mass centroid relative to the effect on the area centroid. We quantified this track noise by simply measuring over each 1 hour period the total length of the area centroid track minus the total length of the mass centroid track. In Fig. 8 it can be seen that this measure was generally positive and was strongly suppressed by all concentrations of the two microtubule-disrupting agents, colcemid and nocodazole, from a median value of about 9.5 μm to less than 3.0 μm at the highest concentrations, a threefold decrease. In contrast, the microtubule-stabilising agent taxol had no significant effect on track noise. The samples in Fig. 7 were chosen as typical cases and the track noise for the untreated cell was 11.14 μm compared with 2.75 μm for the colcemid-treated cell.

Cell spreading and shape

In ANOVA tests the mean spread area of the cells was not significantly affected by any of the three drugs at any concentration. As we reported previously for primary chick fibroblasts,

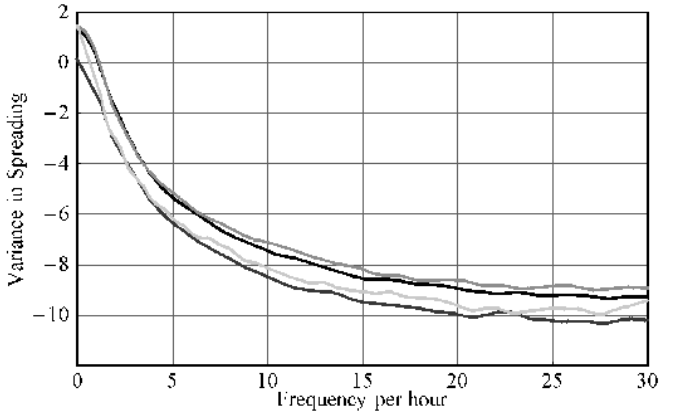

Fig. 9. Power spectrum of cell spreading measured as the area-to-mass ratio in $\mu m^2\ pg^{-1}$ for untreated cells (black line), for the pooled colcemid-treated cells (dark grey line), for the pooled taxol-treated cells (mid grey) and for cells treated with 500 nM nocodazole (light grey). Other details as in Fig. 4.

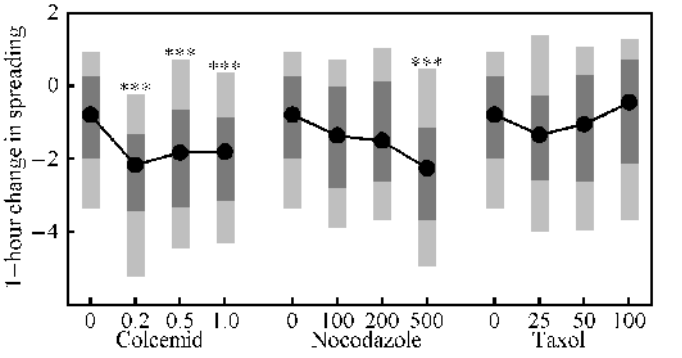

Fig. 10. The absolute change in the area-to-mass ratio during one hour (log_2 scale). Other details as in Fig. 3.

these secondary fibroblasts showed a distinct upper limit on the range of spread areas which appeared to be linearly related to mass and which was around 7.2 $\mu m^2\ pg^{-1}$ as with the 7-day primary cells (Dunn and Zicha, 1995). Thus the spread areas of the cells tended to increase as they grew in mass during the cell cycle. In order to remove this upward trend, which would have invalidated the spectral analysis, we measured cell spreading as the area-to-mass ratio. Repeating the ANOVA tests for this new measure of spreading gave the same result as previously: no effect by any drug at any concentration.

The spectral analysis of cell spreading is presented in Fig. 9. The spectrum falls steeply with increasing frequency and shows that low-frequency variations in cell spreading were around 1,000 times more powerful than the high-frequency fluctuations (10 doublings on the log_2 scale). It can be seen that the high-frequency variations in spreading were suppressed by all concentrations of colcemid (dark grey line: pooled colcemid data) and by 500 nm nocodazole (light grey) but not by taxol (mid grey). The suppression by colcemid and nocodazole appears to span the whole spectrum, as with the suppression of variations in cell speed, and here we confirmed this directly by examining the long-term variation in spreading in ANOVA tests. These variations were measured as the absolute difference between the area-to-mass ratios at the beginning and end of each 1 hour run of data and then logarithms (base 2) were taken to normalise the distributions. Fig. 10 shows the results of the ANOVA tests and it can be seen that all concentrations of colcemid and the highest concentration of nocodazole suppressed the 1 hour variation in spreading to about half of the untreated level. This corresponds very well with the suppres-

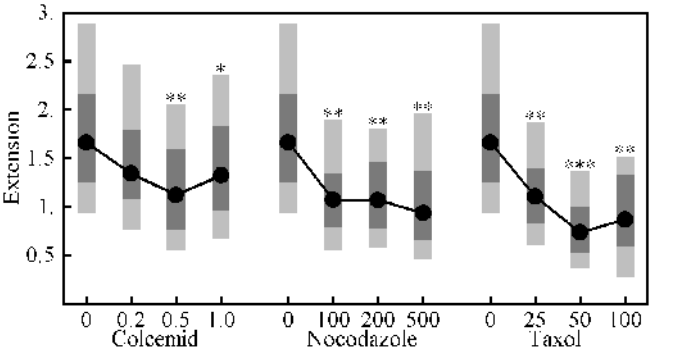

Fig. 11. The effect of the three drugs on the extension of cell shape.

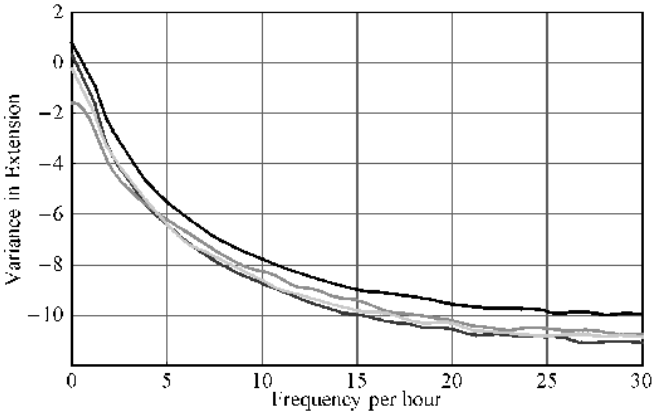

Fig. 12. Power spectrum of fluctuations in the extension of cell shape for untreated cells (black line), for the pooled colcemid-treated cells (dark grey line), for the pooled taxol-treated cells (mid grey) and for the pooled nocodazole-treated cells (light grey).

sion of the high-frequency fluctuations in spreading revealed by the spectral analysis. In each case the same concentrations of the same drugs were effective and the level of suppression was around 1.0 on the $\log_2$ scale. Also there is a suggestion that taxol increases variations in spreading in both cases but this was not significant in the ANOVA tests.

Cell shape was measured using the shape index called extension which is based on 2nd-order geometrical moments and can be thought of as a measure of the rotational inertia of the shape (Dunn and Brown, 1986, 1990). This takes the minimum value of zero only if the cell shape is circular and increases as the shape becomes less compact. The mean cell extension was significantly depressed by colcemid treatment at the two highest doses and by nocodazole and taxol treatment at all doses with taxol being particularly effective (Fig. 11). In spectral analysis, all three drugs were effective at reducing high frequency variations in cell extension to about half the untreated levels and again their effect extended across the whole spectrum (Fig. 12). Again we used ANOVA tests to confirm that the long term variations were significantly suppressed, in this case by all three drugs, but the strong suppression of the mean level of extension by all three drugs suggests a trivial explanation of this suppression of its variation.

DISCUSSION

It has long been known that drugs that affect the state of polymerisation of cytoplasmic microtubules can reduce the speed of cell translocation (Vasiliev et al., 1970) and it has more recently been observed that the rates of protrusion and retraction of the cell margin in human fibroblasts are reduced by colcemid treatment (Bershadsky et al., 1991). Here we have found that colcemid, nocodazole and taxol all suppressed the mean areas of protrusion and retraction and the mean cell speed. There are good theoretical reasons for believing that these responses are directly connected since there is an exact and simple relationship between the areas and disposition of the regions of protrusion and retraction and the displacement of the cell's area centroid (Dunn et al., 1997). This view is supported by the dose-dependency of the effects which, although they do not match exactly, show that the concentrations of colcemid and taxol that we used were generally effective in each case whereas only the highest dose of nocodazole had significant effects.

The spectral analysis of the 1 minute protrusion and retraction revealed fluctuations in their areas that were similarly sensitive to treatment with the three drugs. The power of the fluctuations declined steadily from a maximum at the lowest frequencies to a plateau at frequencies greater than about 10 hour^{-1}, indicating that the high-frequency fluctuations were random noise. The noise associated with retraction was about twice as powerful as that associated with protrusion. Colcemid and the highest dose of nocodazole suppressed the power of all these fluctuations to less than half the untreated levels whereas taxol was not quite so effective. In all cases, the suppression extended uniformly across the whole spectrum so that slower, larger-scale variations were equally suppressed.

Protrusion and retraction, when sampled over intervals as short as one minute, tend to occur as small regions interspersed along the active cell edge as shown in Fig. 2D. These small regions tend not to persist from one time interval to the next since, when sampled over longer intervals as in Fig. 2C, protrusion and retraction have each coalesced into larger regions at opposite ends of the cell. We propose that it is the rapid appearance and disappearance of these small regions that is responsible for the noise detected in the spectral analysis. The suppression of this noise by the three drugs indicates that the appearance and disappearance of these small regions is dependent on the activities of microtubules.

If the areas and relative positions of the regions of protrusion and retraction tended to remain constant then the cell would glide smoothly with constant speed and direction. Variations in the speed and direction of cell translocation must therefore arise from variations in the areas and positions of protrusion and retraction. It is not surprising, therefore, that the spectrum of cell speed also reveals a high level of noise that is effectively suppressed by the three drugs. Again the lowest two doses of nocodazole were only marginally effective, and were excluded from the plot, but the highest dose of nocodazole and the pooled doses of colcemid and taxol were all highly effective and reduced the noise level to almost one quarter of its untreated level.

Variations in the direction of cell translocation showed a very different response to treatment by the three drugs. Again it was clear that there were high-frequency variations in untreated cells as revealed by the noise or small wiggles on the tracks of the area centroid. Again it was apparent that the variations were caused by fluctuations in protrusion and retraction since the tracks of the mass centroid showed much less noise. This would be expected if the noise were due to the appearance and disappearance of small protrusions since these would have a very low mass density in comparison with the bulk of the cell. Unlike the speed noise, the track noise was strongly suppressed by low doses of nocodazole and not significantly affected by taxol, although both were suppressed by the highest nocodazole dose and by all doses of colcemid. It is clear, therefore, that this noise depends on a very different aspect of the variations in protrusion and retraction. Perhaps it depends not so much on how the areas of protrusion and retraction are fluctuating but on how their relative positions are fluctuating. The fact that only colcemid and nocodazole are effective suggests that the suppression of this noise is due to microtubule

disruption but then the two lowest doses of nocodazole are probably insufficient to cause much disruption to microtubules (Liao et al., 1995). They are, however, sufficient to interfere with the dynamic cycling of microtubules but then so are the concentrations of taxol that we used (Liao et al., 1995). We conclude that the aspect of microtubule activity that generates rapid variations in cell direction may be different from that which generates rapid fluctuations in cell speed.

The mean spread area of the cells was unaffected by all three drugs but we have noticed previously that cells treated with colcemid often tend to maintain a much more constant spread area than untreated cells (Dunn et al., 1997). A change in cell area is due to a difference between the areas of protrusion and retraction; therefore fluctuations in spreading are not caused directly by fluctuations in protrusion and retraction but by a failure of these fluctuations to match each other. The spectral analysis of spreading (measured as area-to-mass ratio) revealed a high-frequency noise that was suppressed by colcemid and by the highest dose of nocodazole but not by taxol. This suggests yet a third pattern of response to the drugs and in this case it may be the disruption of microtubules that causes the effect. It can also be seen that the high-frequency fluctuations in spreading were very small; some 1,000 times less than the large scale changes that occurred over longer periods. The spectral analysis revealed that these large scale changes were equally suppressed by the same drug treatments and this was confirmed by direct measurement and ANOVA tests of the mean 1 hour changes in spreading which were reduced by colcemid and nocodazole to one third of their untreated levels. In the case of spreading, therefore, we have found a property of the cells that is affected only in its fluctuations and not in its mean values.

Finally, the mean extension of cell shape was strongly reduced by all three drugs including the lowest concentrations of nocodazole. Since the extension of cell shape is simply the sum of the two components elongation and dispersion, this observation is consistent with that of Middleton et al. (1988) who found that both components of the shape of secondary chick heart fibroblasts were reduced by colcemid and nocodazole. The reduction of extension means that the treated cells were assuming a more rounded compact appearance but it does not mean that the cells were rounding up as from lack of adhesion because the level of spreading was unaffected. As with the other parameters, we found rapid fluctuations in cell shape that were also suppressed by the drugs although taxol, which was most effective at reducing extension, was least effective at reducing its rapid fluctuations. We also found that large scale changes in cell shape were significantly reduced by all three drugs but this is hardly surprising since the reduced mean levels of extension means that there was simply less room for large scale changes to occur. Nevertheless, it may be that the primary effect of microtubules on cell shape is to increase its variation, as with cell spreading, and the raising of the mean level of extension is a secondary consequence of this.

In conclusion, we have found rapid fluctuations of the cell margin that lead to large-scale, long-term changes in cell motile behaviour. All these fluctuations were significantly suppressed by at least some of the three drugs that we used and we infer that all are dependent on microtubules. The large scale disruption of microtubules by colcemid and the highest dose of nocodazole was effective in all cases. It is likely that the functional link between microtubules and the activity of the cell margin is complex and that some activities of the margin can be suppressed by altering the state of dynamic cycling of the microtubules which all three drugs are known to do even at low concentrations. Other aspects of marginal activity may require the interaction of microtubules with motor proteins or simply the presence of microtubules as mechanical elements that may entrain or direct the flow of material towards the cell margin. A possible adaptive advantage of this constant, microtubule-dependent vibration of the cell margin may be to increase the sensitivity of the cell to its environment. In all cases we have found that the rapid microvariations of the cell margin tend to accumulate to produce slower, larger scale changes. Any external influence which biases the microvariations could thus result in a large-scale response. This might therefore explain why the sensitivity of cells to ultrafine surface topography, for example, is considerably reduced by the action of colcemid (Wójciak-Stothard et al., 1995).

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