Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) induced in pig kidney embryo cells a loss of rhodamine 123 staining of mitochondria in 2-3 min. Within 5 min after FCCP inoculation of cells prestained with rhodamine 123, the diffuse staining of the cytoplasm was absent. FCCP did not induce changes in the cytoplasmic microtubule complex, but induced nonrandom (preferentially perpendicular to the substrate surface) orientation of maternal centrioles. Nonrandom orientation of maternal centrioles occurred 10 min after treatment and remained for 2 hr. At 30 min after introduction of the drug, FCCP treatment increased the mean number of pericentriolar satellites on maternal centrioles and the frequency of primary cilia. The percentage of centrioles perpendicular to the substrate induced by FCCP treatment was slightly increased by disruption of microtubules and slightly diminished by disruption of microfilaments. In both cases centrioles were oriented significantly differently from random \((P < 0.01)\). These results suggest that microtubules are neither involved in the signaling pathway from plasma membrane to the centriole, nor do they anchor the centrioles perpendicular to the substrate, as proposed by Albrecht-Buehler and Bushnell (Experimental Cell Research 120, 1979). 

By using stereological analysis, it has been possible to obtain quantitative data on the number of centrosome-associated microtubules in tissue culture cells. It was shown that a variety of drugs, including 2,4-dinitrophenol (DNP), DNP with deoxy-glucose, sodium azide, and ouabain, can "activate" the centrosome, causing an increase in the number of microtubules radiating from the centrosome (Gudima et al., 1983a,b, 1986). By using stereological analysis, it has been possible to obtain quantitative data on the number of centrosome-associated microtubules in tissue culture cells. It was shown that a variety of drugs, including 2,4-dinitrophenol (DNP), DNP with deoxy-glucose, sodium azide, and ouabain, can "activate" the centrosome, causing an increase in the number of microtubules radiating from the centrosome (Gudima et al., 1983a,b, 1986).
cular to the plane of the substrate. The data suggest that this orientation is independent of the ATP level in the cell which sharply decreases under all these treatments (Spurck et al., 1986; Bershadsky and Gelfand, 1981), but could be associated with depolarization of the plasma membrane (Alieva and Vorobjev, 1995). Depolarization of the plasma membrane might be achieved by a variety of treatments. For example, ouabain is a very specific agent, but its effect depends on the cell type (Hulser et al., 1974). Protonophores like DNP, CCCP, or carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) are acting less specifically affecting all charged membranes, but more directly increasing membrane conductivity (Nicholls, 1982). Among a variety of drugs inducing loss of the membrane potential FCCP is characterized as most effective (Nicholls, 1982).

The mechanism involved in turning centrioles remains unknown. Since active centrioles in interphase cells always have microtubules associated with them, the perpendicular orientation of centrioles to the substrate is thought to be due to their anchorage in the cytoplasm with the aid of long microtubules running parallel to the surface (Albrecht-Buehler and Bushnell, 1979). This supposition appears to be supported by our evidence that all the drugs inducing nonrandom orientation of the centrioles also increase the number of microtubules radiating from the centrosome (Alieva et al., 1992; Alieva and Vorobjev, 1995).

Microfilaments were also shown to participate in centriole movement in the cytoplasm (Sherline and Mascaro, 1982; Euteneuer and Schliwa, 1985). Recently centrinactin, an actin-like protein found in the centrosome, was thought to be involved in the association between centrioles and the microfilament network (Clark and Meyer, 1992). Therefore, microfilaments may also be involved in the process which leads to the perpendicular orientation of centrioles. To gain insight into the mechanisms regulating the behavior of centrioles, we have investigated whether microtubules and microfilaments do affect the induced perpendicular orientation of centrioles. In experiments with pig kidney embryo (PK) cells, FCCP induced nonrandom (preferentially perpendicular) orientation of maternal (active) centrioles to the substrate independently of drugs known to depolymerize microtubules or microfilaments.

MATERIALS AND METHODS

Tissue culture and drug treatment. PK cells were grown on M199 tissue culture medium supplemented with 10% bovine serum and antibiotics (penicillin + streptomycin). Rhodamine staining of cultured cells was performed as described elsewhere (Alieva and Vorobjev, 1989). FCCP (20 mM) was added to the culture medium. Cells were fixed for electron microscopy 5, 10, 30 min and 2 hr after the introduction of the drug. In the second experimental series, cells were treated with 10 µg/ml nocodazole for 1 hr, or with 10 µg/ml cytochalasin B for 1 hr, prior to incubation with 20 mM FCCP for 30 min. After that time, cells were fixed for electron microscopy.

Immunofluorescence. Immunofluorescent staining of the microtubules was performed according to the standard indirect method (Bershadsky and Gelfand, 1981). Cells grown on the coverslips were lysed in the microtubule stabilizing medium, fixed with a 0.5% glutaraldehyde on phosphate-buffered saline (PBS) processed with borohydrid, and stained with primary anti-a-tubulin monoclonal antibodies (Sigma) and then with anti-mouse FITC-conjugated IgG (Sigma). Photographs were taken on an Opton Photomicroscope using Planapochromate 63/1.4 and an FITC filter set (No. 09).

Electron microscopy. For electron microscopy, cells were prepared as described elsewhere (Vorobjev and Chentsov, 1982). Cells grown on the coverslips were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.2), postfixed with 1% osmium tetroxide on the same buffer, stained with uranyl acetate, dehydrated, and embedded in Epon 812 mixture. Serial silver sections were obtained on an LKB-II ultramicrotome (LKB, Sweden). By using the tilted head of the ultramicrotome the angle of sectioning was adjusted parallel to the substrate. Actually, the section plane was inclined to the substrate at an angle less than 1.5°. Sections were mounted on single slot grids coated with Formvar film, stained with lead citrate, and then examined and photographed in HitachiHU-11B and HU-12 electron microscopes (Hitachi, Japan) operating at 75 kV. For the measurements of the centriole, microtubule negatives were taken at a magnification of x20,000.

The orientation of centrioles to the substrate was determined according to the method of Albrecht-Buehler and Bushnell (1979), which allows one to calculate the angle between the centriolar cylinder axis and the substrate by the length of projection of centriolar microtubules in the plane of the section. Histograms of the distribution of centriole projections were made as described elsewhere (Vasiliev et al., 1988). Class value (difference between minimal and maximal projection length) was taken 0.03 µm (i.e., first class, 0-0.03 µm; second, 0.03-0.06 µm, etc.). Taking the section thickness to be about 70 nm, that gives an inclination angle for the centrioles in the first class of the histogram (which are named below "perpendicular to the substrate") of >74°. The difference between treatments was compared by the x2 criterion as previously described (Vasiliev et al., 1988).

RESULTS

Light Microscopy

AT the concentration used, FCCP induced a loss of rhodamine 123 staining of mitochondria in 2-3 min (Alieva and Vorobjev, 1990). Mitochondria were neither capable of accumulating dye in the presence of FCCP nor did they retain rhodamine 123 after introduction of FCCP. In 5 min after inoculation with FCCP of the cells prestained with rhodamine 123, the diffuse staining of the cytoplasm (often observed after the action of uncouplers of oxidative phosphorylation; D. B. Zorov, personal communication) was also eliminated, demonstrating complete absence of plasma membrane potential (data not shown).

Figures la and lb show immunofluorescent staining of microtubules in normal PK cells and in PK cells after nocodazole treatment. Antibodies revealed a dense meshwork in normal cells, while 1 hr after nocodazole inoculation of the cells single microtubules remained in some of them. Typically, only weak staining of the centrosome was evident (Fig. 1b).
Electron Microscopy

The ultrastructure of centrosomes in normal and drug-treated cells was studied using a complete series of sections including both centrioles in a cell studied. To describe the centrosome several parameters were used: the presence of a primary cilium; the number of pericentriolar satellites on the active (maternal) centriole; the presence of striated rootlets; the distance between two centrioles; and the orientation of the centrioles to the substrate. The effect of FCCP on the centrosome was studied starting from 5 min after introduction of the drug.

Centrosomes in control cells. The fine structure of the centrosome in normal PK cells has been described in detail previously (Alieva and Vorobjev, 1989). A total of 1-2 pericentriolar satellites were situated on the active centriole; sometimes they were not present. One-third of the cells possessed a short primary cilium. One-quarter of the cells had striated rootlets; in 3 of the 25 cells studied by complete serial sectioning these rootlets ran from both centrioles (Table I). Only 2 of 31 active centrioles lay perpendicular to the substrate. The histogram of the distribution of active centriole inclination to the substrate was similar to the random distribution histogram (Fig. 2a) and this difference was statistically insignificant according to the $\chi^2$ criterion.

Centrosomes after FCCP treatment. At 5 min after the introduction of FCCP into the culture medium (34 cells studied) as well as after more prolonged treatment the position of the centrosome toward the nucleus and the relative of each pair of centrioles did not change compared with control cells. The mean number of satellites on the active centriole was two. Under FCCP treatment the percentage of cells with the primary cilium increased, sometimes considerably. The cilia could be relatively long—up to 1 $\mu$m (i.e., relative to the short stubby bases most usually seen in controls). The percentage of cells with striated rootlets remained close to that in the controls (Table I). The mean angle of inclination of active centrioles to the substrate slightly increased. Six of 33 active centrioles studied

<table>
<thead>
<tr>
<th>Drugs, time of treatment</th>
<th>Number of cells studied</th>
<th>Mean number of satellites (mean ± SD)</th>
<th>Percentage of the cells with primary cilium</th>
<th>Percentage of cells with striated rootlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>1.57 ± 0.15</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>FCCP, 5 min</td>
<td>34</td>
<td>2.00 ± 0.21*</td>
<td>55</td>
<td>29</td>
</tr>
<tr>
<td>FCCP, 10 min</td>
<td>32</td>
<td>2.58 ± 0.10*</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>Nocodazole, 60 min + FCCP 30 min</td>
<td>35</td>
<td>1.09 ± 0.16*</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>Cytochalasin B, 60 min + FCCP 30 min</td>
<td>38</td>
<td>1.24 ± 0.18</td>
<td>55</td>
<td>38</td>
</tr>
</tbody>
</table>

* Number of satellites significantly different from that for control cells.
FIG. 2. (a) Histograms of the distribution of length of projections of the active centrioles in PK cells after short FCCP treatment. From front to back: theoretical histogram of randomly directed centrioles (from Vasiliev et al., 1988); control cells; after 5 min FCCP treatment. (b) Histograms of the distribution of length of projections of the active centrioles in PK cells after prolonged FCCP treatment. From front to back: after 10 min FCCP treatment; after 30 min FCCP treatment; after 2 hr FCCP treatment. (c) Histograms of the distribution of length of projections of the active centrioles in PK cells after selective depolymerization of cytoskeletal elements under the 30-min action of FCCP. From front to back: after 30 min FCCP treatment; after nocodazole treatment (10 µg/ml, 1 hr) then FCCP + nocodazole treatment; after cytochalasin B treatment (10 µg/ml, 1 h) then FCCP + cytochalasin B treatment.

After 10 min of FCCP treatment, the mean number of satellites had increased significantly (Table I, \( P < 0.05 \)), and sometimes active centrioles carrying as many as five satellites were found (Fig. 3a). The number of microtubules radiating from the centrosome obviously increased (Fig. 3a). The histogram of the distribution of active centrioles differed from the control pattern. A total of 20 of the 30 centrioles studied were perpendicular to the substrate (Fig. 2b). The dramatic increase in the mean angle of inclination of active centrioles means that the majority of the centrioles were inclined at an angle of more than 38° (1-3 classes on Fig. 2b), but some still remained parallel to the substrate (last class).

By 30 min after the introduction of FCCP, the mean number of satellites on active centrioles and the percentage of cells with primary cilia had increased (Table I). Striated rootlets were seen more frequently, being present on about 1/3 of active and 1/5 nonactive centrioles. The histogram of the distribution of active centrioles was also different from the control (Figs. 2a and 2b; \( P < 0.01 \)). Half the active centrioles were oriented perpendicularly to the substrate. At this time no centrioles were inclined at an angle of <21°, i.e., active centrioles which had been parallel to the substrate also turned. A similar distribution of active centrioles persisted 2 hr after the FCCP treatment (Fig. 2b).

**Centrosomes after nocodazole and FCCP treatment.** At 30 min after the introduction of FCCP to cells preincubated for 60 min with nocodazole, the position of centrioles in relation to the nucleus had not changed. The distance between the two centrioles in each cell was <1 µm. Microtubules in the cell center were mostly absent (Fig. 3b), and occasionally one, rarely two, long microtubules were attached to the lateral surface of an active centriole or to the head of satellite. One or two satellites of characteristic shape were present on the active centriole (Fig. 3b) in most cells, or none at all. One-third of the cells studied possessed primary cilia (Table I). Striated rootlets were observed near 7 active and 6 nonactive centrioles (of the 33 cells studied). Both centrioles had striated rootlets in 1 cell.

The histogram of the distribution of projections of active centrioles after this treatment was strictly different from control (Fig. 2c; \( P < 0.01 \)). Active centrioles were predominantly perpendicular to the substrate—21 of 31 observed were inclined at an angle >74°. No centrioles were found inclined to the substrate at angles of <38° (Fig. 2c).

**Centrosomes after cytochalasin B and FCCP treatment.** In the FCCP-treated cells after preincubation with cytochalasin B (38 cells studied) the posi-
FIG. 3. (a) Ultrastructure of the centrosome in PK cell after 10 min FCCP treatment. S, pericentriolar satellite. Bar, 0.2 µm. (b) Ultrastructure of the centrosome in PK cells after nocodazole treatment (10 µg/ml, 1 hr) then FCCP + nocodazole treatment, 30 min. S, pericentriolar satellite. Bar, 0.2 µm. (c) Ultrastructure of the centrosome in PK cells after cytochalasin B treatment (10 µg/ml, 1 hr) then FCCP + cytochalasin B treatment, 30 min. S, pericentriolar satellite. Bar, 0.2 µm. (d) Pericentriolar satellites after 30 min FCCP treatment. S, pericentriolar satellite. Bar, 0.25 µm. (e) Pericentriolar satellites after nocodazole treatment (10 µg/ml, 1 hr) then FCCP + nocodazole treatment, 30 min. S, pericentriolar satellite. Bar, 0.25 µm. (f) Pericentriolar satellites after cytochalasin B treatment (10 µg/ml, 1 hr) then FCCP + cytochalasin B treatment, 30 min. S, pericentriolar satellite. Bar, 0.25 µm.
tion of the centrosome was different from that in the controls. In 16 cells the distance between maternal and daughter centrioles was >0.5 μm (Fig. 3c). The average distance was 1-2 μm, and sometimes reached 10 μm. One of the centrioles in each cell (usually the nonactive centriole) was located far away from the nucleus, near the periphery of the cell. In some cells both centrioles were distant from the nucleus. One or two satellites were present on the active centriole, but sometimes none were found. In 3 cells, the active centriole possessed three satellites. In the majority of cells pericentriolar satellites differed from those in normal or FCCP-treated cells. The filamentous basalts of satellites were thin, with striations hardly visible (Figs. 2d-2f). More than half of the cells studied possessed primary cilia (Table I). In 11 cells, active centrioles had striated rootlets, and in 5 of these cases, cilia were present. Striated rootlets were found in only 4 cells associated with nonactive centrioles.

The histogram of the distribution of active centrioles after these treatments changed relative to controls. Of the 38 active centrioles studied 12 were oriented perpendicularly to the substrate; additionally, the second and the last classes of the histogram were less well represented than those of the control cells (Fig. 2c). According to the χ² test, the orientation of the active centrioles was nonrandom, i.e., perpendicularly oriented centrioles dominated (P < 0.05). It should be mentioned that after cytochalasin B pretreatment, the percentage of active centrioles perpendicular to the substrate was less than after nocodazole pre-treatment (Fig. 2c) or treatment with FCCP alone (Fig. 2b), but for the number of centrioles studied the difference in both cases was insignificant (P > 0.2).

DISCUSSION

Our data on the action of FCCP confirm the previous observations (Alieva et al., 1992; Alieva and Vorobjev, 1995) on the "activation" of the centrosome as a microtubule-organizing center in response to treatment with uncouplers of oxidative phosphorylation. Besides increase in quantity of microtubules radiating from the centrosome, the number of pericentriolar satellites also increased significantly 30 min after introduction of FCCP. It is interesting to note that under the action of nocodazole or cytochalasin B together with FCCP, the number of pericentriolar satellites was slightly less than in control cells and significantly less than under action of FCCP alone.

The phenomenon of perpendicular orientation of centrioles to the substrate in different types of cultured cells has been described elsewhere (Albrecht-Buehler and Bushnell, 1979; Vorobjev and Chentsov, 1982; Gudima et al., 1983a,b). Reevaluation of these studies using the χ² criterion confirmed that nonrandom orientation of active centrioles in PK cells and mouse fibroblasts (Vorobjev and Chentsov, 1982; Gudima et al., 1983a,b) was statistically significant (P < 0.05; see Vasiliev et al., 1988). In PK cells, a predominantly perpendicular orientation of active centrioles to the substrate was described for the early G₁ period when cells were spreading after mitosis (Vorobjev and Chentsov, 1982). In a previous study, we have shown that the perpendicular orientation of active centrioles to the substrate surface could be induced independently of the cell cycle by depolarization of membranes, particularly of the plasma membrane (Alieva and Vorobjev, 1989). This occurred after different drug treatments, including dinitrophenol, dinitrophenol together with deoxyglucose, the calcium ionophore A23187, and ouabain.

Proceeding from the assumption that the perpendicular orientation of active centrioles to the substrate is a result of, or related to, plasma membrane depolarization (Alieva and Vorobjev, 1989, 1995), we have now studied the dynamics of centriole movement with the help of the most effective uncoupler, FCCP. The introduction of this drug led to a loss of rhodamine 123 accumulated in mitochondria in 2-3 min. In 5 min no rhodamine was retained within treated cells. Active centrioles were found to turn perpendicularly to the plane of the substrate 10 min after FCCP introduction, i.e., after mitochondria and plasma membranes had lost their membrane potential. These data suggest that the rotational movement of centrioles is somehow linked with membrane depolarization. This is also supported by the observation that the nonrandom orientation of active centrioles persists for up to 2 hr of FCCP treatment.

In all cases described above, the nonrandom orientation of centrioles was associated with an increased number of microtubules radiating from the centrosome. To determine whether microtubules are directly involved in centriole orientation, as proposed by Albrecht-Buehler and Bushnell (1979), we used nocodazole treatment prior to the introduction of FCCP. In cells pretreated with drugs selectively destroying cytoskeleton elements, FCCP treatment unexpectedly showed that the orientation of active centrioles could not be related to microtubules. Indeed, after depolymerization of microtubules with nocodazole the percentage of active centrioles perpendicular to the substrate actually increased, although the difference was not statistically significant. Thus, our data argue against the supposition of Albrecht-Buehler and Bushnell (1979) that long microtubules attached to the centriole serve as anchors and assist in the orientation of centrioles perpendicular to the substrate.

Cytochalasin B induced numerous changes in the centrosome of PK cells. Centrioles often move apart...
from each other and the satellites on the active centriole were altered. Certain centrosome microfilament relationships were observed in the studies of the mechanism of centriole splitting (Sherline and Mascardo, 1982; Euteneuer and Schliwa, 1985). In both these works, cytochalasin B was described to prevent centriole splitting induced by epidermal growth factor and tumor promoting agent (TPA). However, in our study centriole separation seemed to be stimulated by this drug. This discrepancy might be due to the different origin of the cells used. Furthermore, it is possible that the centriole separation by a distance of about 2 µm seen in our study is insignificant and was not taken into account in light microscopic studies.

It should be stressed that the nonrandom orientation of centrioles under the action of FCCP was still established in the presence of cytochalasin B. In the absence of a microfilament network, active centrioles seemed to be partially disoriented. However, the number of perpendicular centrioles was significantly higher than that expected from a random distribution. Taking into account the dramatic changes in cell morphology observed (data not shown), it is unlikely that centriolar turning in the presence of cytochalasin B can be due to the remnants of the microfilament system. We suggest that the mechanisms responsible for centriole orientation in the cytoplasm, if microfilaments are not involved, is due to some as yet undefined cytoskeletal system whose nature remains to be determined.

Summing up the data obtained in the present work and other studies (Alieva and Vorobjev, 1989, 1995), we suppose that centriole orientation is induced by the depolarization of plasma membrane, though the nonspecific effect of all the drugs used can’t be excluded. Centriole orientation occurs independently of the action of the anti-microtubule and relatively independently of the anti-microfilament drugs. This means that centrioles must be associated with some cytoskeletal structures besides microtubules and microfilaments, whose molecular basis needs to be elucidated. One of the possible components of such system associated with centrioles had been identified recently as tektins (Steffen and Linck, 1992). The relationship between membrane potential and centriole behavior partially supports the idea that centrioles may be involved in the process of the intracellular signaling pathway (Born-ens, 1979) or might operate as a cell sensor (Albrecht-Buehler, 1992), but, unlike these authors, we conclude that at least some of the centrosome-membrane relations do not include microtubules.

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REFERENCES


Alieva, I. B., and Vorobjev, I. A. (1995) Centrosome splitting (Sherline and Mascardo, 1982; Euteneuer and Schliwa, 1985). In both these works, cytochalasin B was described to prevent centriole splitting induced by epidermal growth factor and tumor promoting agent (TPA). However, in our study centriole separation seemed to be stimulated by this drug. This discrepancy might be due to the different origin of the cells used. Furthermore, it is possible that the centriole separation by a distance of about 2 µm seen in our study is insignificant and was not taken into account in light microscopic studies.

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