Effect of Taxol on Secretory Cells: Functional, Morphological, and Electrophysiological Correlates

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ABSTRACT The effect of 0.5–1.0 μM taxol, a potent promoter of microtubule polymerization in vitro, was studied on the secretory activity of chromaffin cells of the adrenal medulla. Taxol was found to have a dual effect: the long-term effect (after a 1-h incubation) of taxol was to induce almost complete inhibition of catecholamine release, whereas after a short incubation (10 min) a massive, nicotine-independent release of catecholamine was produced. From results obtained using the patch-clamp technique to study the Ca+²-dependent K+ channels (IC channels), it was possible to conclude that taxol probably provokes an augmentation of free [Ca++], in the cytoplasm, values increasing from 10⁻⁶ M at rest to several 10⁻⁷ M. The increased spontaneous release of stored neurohormones and the increased frequency of opening of IC channels occur simultaneously and could both originate from a rise of [Ca⁺⁺] upon taxol addition. Immunofluorescence and ultrastructural studies showed that 13-h taxol treatment of chromaffin cells led to a different distribution of secretory organelles, and also to microtubule reorganization. In treated cells, microtubules were found to form bundles beneath the cell membrane and, at the ultrastructural level, to be packed along the cell axis. It is concluded that in addition to its action on microtubules, the antitumor drug taxol has side effects on the cell secretory activity, one of them being to modify free [Ca⁺⁺].

Taxol, a diterpenoid isolated from Taxus brevifolia, has been shown to be a potent mitotic spindle poison and for that reason is an antitumour agent. Taxol inhibits division of exponentially growing HeLa cells (38), blocks the replication of Trypanosoma cruzi (10), inhibits the migration behavior of mammalian fibroblast cells (31, 39), and affects the morphology of PC₁₀ cells (14) and dorsal root ganglion and spinal cord neurons in cultures (30, 32). The effects of taxol appear to be related to the tubulin-microtubule system (15, 19, 27, 38, 40). Taxol has the unusual capacity to promote assembly of microtubules in vitro, stabilizes microtubules against depolymerizing effects of low temperatures, and binds specifically to cellular microtubules. Taxol is able to alter the original network of microtubules and to form bundles (4, 14, 15, 18, 30).

While various studies have described the biochemical modifications of tubulin produced by taxol (27, 38, 40), only one short report has dealt with its pharmacological action on cellular activity. In 1982, Howell et al. (22) described an inhibitory effect of taxol on glucose-stimulated insulin secretion of pancreatic cells. This present report concerns the effects of taxol on mammalian chromaffin cells in culture and emphasizes short- and long-term effects of taxol on their secretory activity. Extensive studies on the biochemistry, the ultrastructure, and the ionic controls of catecholamine secretion from these paraneuronal cells of the adrenal gland have been described (13). In addition, a role for microtubules in such a secretory process has been suggested (8, 42). In the present study, the short- and long-term effects of taxol on catecholamine release were investigated; the effect of taxol on the ionic permeability of chromaffin cell membrane was recorded and taxol-treated chromaffin cells were examined at the ultrastructural level. Using these three approaches, it is suggested that beside the likely involvement of microtubules in secretory processes, taxol also affects the cytoplasmic calcium pool.

MATERIALS AND METHODS

Release Experiments from Chromaffin Cells in Culture: The procedure for culturing chromaffin cells isolated from bovine adrenal medullae was as previously described (7). Briefly, bovine adrenal glands were retrogradely
perfused for 30 min with Ca**+-free Locke's solution buffered with 5 mM HEPES (pH 7.4) at 37°C. Glands were then perfused with Ca**+-free Locke's solution containing 0.05% collagenase (Warthington Biochemical Corp., Freehold, NJ), 140 U/ml, 0.01% DNase (Serva Feinbiochemica, Heidelberg, FRG; 200 U/mg) and 0.3% bovine serum albumin (BSA) (Serva Feinbiochemica). Dissociated cells were then filtered through nylon mesh and purified on 47.5% self-generating Percoll gradients. Chromaffin cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and containing 5.10^{-3} M cytosine arabinoside. They were plated at 5 x 10^{3} cells/well in 24 multiple-well Costar plates (Costar, Data Packaging Corp., Cambridge, MA), and incubated at 37°C in a water-saturated, 5% CO_{2}/95% air atmosphere.

For release experiments, chromaffin cells were loaded with (7-)H noradrenaline (Amersham France SA, Les Ulis, France; 16.5 Ci/mmol). Cells were incubated for 60 min with 125 nM noradrenaline and 10^{-3} M ascorbic acid (23). In our conditions, the amount of radioactive noradrenaline taken up by cells was 13 ± 5% of total exogenous radioactivity (factors known to affect noradrenaline uptake are cell membrane integrity, cell attachment, age of the culture... [see reference 3]). Cells were washed first with DMEM, and then with Locke's solution containing 2.2 mM Ca**++ at room temperature. Washing intervals were 10 min each. Cells were then stimulated with 10 μM nicotine for 10 min at room temperature and released radioactivity was determined in scintillation vials containing 10 ml of Rotisint 22 (Rotth OHG, Karlsruhe, FRG) in an SL-4000 InterTechnique Scintillation Counter. Remaining cells were collected and radioactivity was determined.

In experiments using microtubule-interacting agents, drugs were added at the concentration indicated in the text. Taxol was dissolved in dimethylsulfoxide (DMSO); stock solution was 10^{-3} M. Podophyllotoxin was stored in ethanol at 10^{-3} M.

Recording Conditions for Electrophysiology: Chromaffin cells were grown as above, but plated on 35-mm diameter Petri dishes coated with rat tail collagen. Optimal recording requirements were met by cells which adhered tightly to the bottom of the dish, and especially with cells displaying a fusiform shape. Such a conformation was usually attained during the first few days after plating (1-4 d).

Most records were obtained using the patch-clamp technique (20, 28) in the cell-attached configuration. In this protocol, the cell remains intact, so there is no alteration of its protoplasm (organelles and cytoplasmic components) and it maintains a resting potential Em close to -60 mV (potentials are measured under the grounded bath as a reference). Once a tight seal between cell and electrode is realized, the current crossing the patch of membrane is measured under voltage-clamp conditions. The potential difference across the patch of membrane under the pipette (patch potential) can be established by changing the potential of the pipette with respect to ground. The recording apparatus used was an EPC 5 (List Electronic, Germany) equipped with a 10 GΩ feedback resistor. The electric characteristics of the whole system allow current recordings in the pA range.

Bath medium contained (in mM) NaCl (130), KCl (5), MgCl_{2} (2), CaCl_{2} (2), and HEPES/Tris (pH 7.4) (5). The pipette was filled either with solution A or by an isotonic KCl solution (solution B). Solution A was composed of (in mM) NaCl (130), HEPES/NaOH (pH 7.2) (10), KCl (5), MgCl_{2} (2) (pCa 8). Solution B was composed of (in mM) Cl_{2} (130) and HEPES/KOH (pH 7.2) (10), and MgCl_{2} (2) (pCa 8). For Ca**+-free medium in the pipette, pCa was fixed at around 8 using an EGTA buffer composed of (in mM) EGTA (11), and CaCl_{2} (1) (EGTA/KOH in high K**+ medium, or EGTA/NaOH in high Na**+ medium) (see reference 12). To prepare Ca**+-free extracellular medium, no calcium was added but the solution was supplemented with 10 mM Mg^{2+} to minimize shift of the surface potential. No correction for liquid junction potentials was made; they never exceeded 5 mV under the conditions used.

1-μM taxol solutions were prepared either by adding 10 μl of a 10-fold diluted stock solution (taxol 10^{-3} M in DMSO) to 1 ml bath medium, or directly superfused at this final concentration.

Fluorescence and Electron Microscopy: Secretory granules were visualized by use of anti-chromogranin A antibody raised in rabbits (5) and fluorescein-conjugated goat anti-rabbit immunoglobulins. Chromaffin cells were fixed with 4% paraformaldehyde, and sequentially treated with acetone 50%, 100%, and 50% (21). The distribution of microtubules was followed using antitubulin antibody (7).

For electron microscopy, cultures were fixed in situ with buffered glutaraldehyde (1% glutaraldehyde in 0.1 M sodium phosphate containing KCl [50 mM], MgCl_{2} [5 mM] [pH 7.0]) to which guanosine triphosphate, 1 mM final concentration, and EGTA (2 mM) had been added. The addition of guanosine triphosphate is considered to help the preservation of microtubules. Preliminary experiments in which tannic acid was added to the fixative which also helps their preservation did not result in significant improvement: when used with the saponin to permeabilize cell membranes and permit penetration of tannic acid into the cells, morphological preservation of the cells was worse. Cells were postfixed (% h) in 1% cacodylate-buffered osmium tetroxide, dehydrated, removed intact from the plastic Petri dishes, and flat-embedded in Spurr resin (26). Thin sections, counterstained with uranyl-acetate and lead citrate, were viewed in a Philips 300 electron microscope.

Materials: Taxol was obtained from the National Institute of Cancer (Dr. M. Saffi, Bethesda, Maryland). Nordic Immunological Laboratories (Tilburg, The Netherlands) was the source for conjugated-goat anti-rabbit immunoglobulins.

RESULTS
Effect of Taxol on Catecholamine Secretion from Cultured Chromaffin Cells
In culture, chromaffin cells from adult bovine adrenal medulla maintain their capacity to synthesize, store, secrete, and take up catecholamines. As shown in Fig. 1, the nicotine-induced release of catecholamines is linear with time up to 10 min, and is maximal at a nicotine concentration of 10 μM. When incubated in the presence of the microtubule-affecting agents colchicine, podophyllotoxin, vinblastine, and taxol, the secretory activity of chromaffin cells was modified. However,
when these drugs were added during uptake of exogenous noradrenaline, no evident effect on the uptake mechanism was observed (Table I). As shown in Table II, taxol is a powerful inhibitor of the release. Almost complete inhibition was achieved at a low taxol concentration of 0.5–1.0 µM, the concentrations which were found to give maximal inhibition under these experimental conditions. When cells were preincubated first with 0.5 µM taxol for 2 h or 16 h before noradrenaline uptake, release was inhibited by 38 and 20%, respectively. This indicates a slow decrease of the taxol effect with time. Vinblastine, podophyllotoxin, and colchicine had lesser effects.

The effect of taxol on the release of catecholamines after short-term preincubation was also studied (Table III). Cells were incubated with 0.5 µM taxol during the last 10 min washing period before nicotine stimulation, and the radioactivity released was measured before and after nicotine stimulation. The radioactivity released after taxol incubation was found to be increased by 40–100%. In cases of maximal taxol-induced release, the nicotine-induced noradrenaline liberation was decreased. In contrast, when taxol-induced release was minimal, there was a slight stimulation of nicotine-induced catecholamine release.

In summary, according to the experiment conditions used (short- or long-term incubation), taxol was shown to have opposite effects. The reasons for the variability of the short term effect of taxol on different cultures were sought by studying ionic movements underlying exocytosis using electrophysiological techniques.

**Electrophysiological Data**

The most obvious explanation for an increased release of catecholamines is that taxol induces an increase of intracellular Ca++. To approach the problem, we studied another phenomenon related to [Ca++] that of the opening of membrane channels with a high K+ ion conductance. Initially, the existence of such channels, e.g., the Ca++-dependent K+ channels or Kc channels, has been shown on patches excised from embryonic rat skeletal muscles grown in culture (9), chromaffin cells (28), and neuronal cells (1). Subsequent reports show them to be present in a large variety of cells (29). In vertebrate-excitable cells, these channels share a number of properties. Most importantly, they show a high conductance (100 pS in normal conditions) and allow an almost specific flow of K+ ions. This is demonstrated by the reversal potential of the responses which is always close to the equilibrium potential.

### Table I

| Drug       | Concentration | Uptake |
|------------|---------------|--------|
| Control    | —             | 11.2   |
| Taxol      | 0.1           | 8.8    |
|            | 0.2           | 11.2   |
|            | 0.5           | 12.5   |
|            | 1.0           | 11.8   |
|            | 10.0          | 9.6    |
| Colchicine | 5             | 11.7   |
|            | 50            | 12.5   |
|            | 100           | 13.5   |
| Podophyllotoxin | 5   | 13.0   |
|            | 50            | 10.5   |
| Vinblastine| 5             | 11.2   |
|            | 10            | 11.0   |

* 5 x 10⁵ cells/well were incubated at 37°C for 60 min with [3H] noradrenaline (125 nM; 6 x 10⁵ cpm) in the presence of drugs at the indicated concentrations. Cells were washed and stored radioactivity was determined after precipitation with 0.4 N perchloric acid. Uptake is expressed as percentage of total exogenous radioactive noradrenaline, as determined on three wells of three cell preparations (taxol), and two cell preparations (podophyllotoxin, colchicine), and a single cell preparation (vinblastine).

### Table II

| Experiment | Spontaneous release | Nicotine-induced release |
|------------|---------------------|--------------------------|
| A*         | +41                 | +25                      |
| Control    | 0                   | 0                        |
| B*         | +100                | -45                      |
| Control    | 0                   | 0                        |
| C*         | +100                | -58                      |
| Control    | 0                   | 0                        |

* The effect of taxol was followed on three different chromaffin cell preparations (A, B, and C). Experimental conditions were identical; cells were incubated in the presence of radioactive noradrenaline (see Table II) in taxol-free DMEM, washed, and during last 10 min washing 0.5 µM taxol was present in the Ca++-supplemented Locke's solution. Cell medium was collected and counted radioactivity gave the spontaneous release. Induced release was provoked with 10 µM nicotine.

Data are expressed as per cent of release in control cells (0), (+) and (−) meaning, respectively, stimulation and inhibition. Each value is the average of three determinations; values given are ±5%.

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potentially dependent on both voltage and intracellular Ca\(^{++}\) concentration. So under normal conditions ([Ca\(^{++}\)]\(_i\) below 10\(^{-8}\) M), the channels open at a threshold potential of +50 mV on the cytoplasmic side.

When using the cell-attached patch configuration, which has the advantage of keeping the cells intact, the main effect of taxol was to induce an increase in the probability of opening of these Ca\(^{++}\)-dependent channels.

We assumed that a change in the average number of open channels was more likely to be directly related to a change in [Ca\(^{++}\)], rather than to a change in the voltage-dependence of these channels. [Ca\(^{++}\)] was estimated from previous data obtained on chromaffin plasma membranes (28) where its effects on the Ic channel activity had been measured. From this analysis, the increase of the frequency of channel opening produced by 1 \(\mu\)M taxol was tentatively interpreted in terms of an increase in [Ca\(^{++}\)], from \(\sim 10^{-8}\) M to the order of \(10^{-7}\) M.

Figs. 2 and 3 show that certain channels with a unique large conductance (100 pS) can be demonstrated on cell-attached patches when voltage clamped at +50 mV and above. These channels were identified as Ic channels, the population of large K channels recently shown to be dependent on [Ca\(^{++}\)], (see references 1 and 28). Moreover, their opening is known to be independent of Ca\(^{++}\) in the extracellular medium. In control experiments, these currents were recorded with an electrode filled either with normal Ringer's solution or with a solution buffered at pH 8, and found to be evoked in the same voltage range.

This high threshold for evoking an Ic activity was used to assess the physiological state of the cells in a single dish. A culture dish was discarded at the start of an experiment if the first three patches obtained in normal Ringer's solution did not display this characteristic. If this criteria was met, 1 \(\mu\)M taxol was then added to the bath and cells studied over the following hour, since in control experiments the initial physiological conditions have been shown to be maintained over this period.

The most characteristic effect of taxol was to alter the voltage-dependence of the frequency of opening of the Ic channels. As illustrated in Fig. 2, after a 7-min superfusion of taxol at 1 \(\mu\)M (a taxol concentration identical to the one used in the release experiments), Ic channels were frequently observed at voltages as low as −20 mV (see also Fig. 3). This effect was not due to the 120 \(\mu\)M of DMSO, which was used as a solvent for taxol, as DMSO per se had no effect on the opening of these channels (Fig. 4).

During long-term recordings, the opening frequency of Ic channels seen on cell-attached patches varied spontaneously. One might wonder whether such fluctuations are related to slight mechanical disturbances around the patch, causing a transient local entry of external Ca\(^{++}\), a phenomenon possibly increased by taxol application. To circumvent this problem, experiments were performed in Ca\(^{++}\)-free medium, i.e., a medium with no Ca\(^{++}\) added but supplemented with 10 mM Mg\(^{2+}\) to minimize the shifts in surface potentials on Ca\(^{++}\) withdrawal. In fact, this manipulation of the Ca\(^{++}\) gradient did not alter the main effect of taxol which was observed in 2 mM Ca\(^{++}\) medium and in Ca\(^{++}\)-free medium as well (see Table IV). That the described effect was a proper effect of taxol and not a spontaneous long-term increase in Ic activity was assessed by two distinct sets of observations summarized in Table IV: (a) on three cells, the effect of taxol was shown to develop after a 5–10-min latency, and (b) no cell (in a same culture dish) ever displayed any sign of recovery in the hour after taxol application. On the average, 1 \(\mu\)M taxol shifted the threshold potential of Ic channels from 55.7 \(\pm\) 14.5 mV (n = 14, \(\pm\)SD) to −20.0 \(\pm\) 28.5 mV (n = 15, \(\pm\)SD, p < 0.001, unpaired t test).

The probability of opening of Ic channels is known to depend both on the membrane potential and the internal Ca\(^{++}\) concentration. Using a different patch-clamp recording technique, that of the outside-out configuration, the intracellular medium can be buffered slightly and the entry of calcium prevented by perfusing with a Ca\(^{++}\)-free medium. With such methods, the calcium concentration on both sides of the membrane can be imposed and the cytoplasm side is free of organelles. Under such conditions, [Ca\(^{++}\)], is constant and no effect of taxol on channel opening is observed (Fig. 5). We therefore assume that the effect of taxol is mediated through an increased [Ca\(^{++}\)], though whether or not this drug is acting directly on the system responsible for the spontaneous fluctuations in Ic channel activity is not known.

A higher intracellular calcium concentration has also been shown to lengthen the individual open time of the Ic channels (see reference 9, which refers to myotube plasma membrane). Data presented here (Fig. 3 C) show that taxol also induces this effect: the increase in the time the channels were open was due both to an increase of the frequency of opening and in the average duration of the individual events.

According to Marty (28), the probability of opening was shown to increase by a factor of almost 4 at +60 mV and a factor of 40 at 0 mV when changing cytoplasmic-free calcium concentration from \(10^{-8}\) M to \(2.10^{-7}\) M on the same patch of
chromaffin plasma membrane. To estimate the effective Ca\(^{++}\) concentration under the conditions described here, we calculated the probability of opening by measuring the percent of time the channels spent in the open state and dividing it by the number of channels present in the patch. The latter was estimated to be approximately equal to the number of superimposed events that can be obtained with high Ca\(^{++}\) cytoplasmic concentration, i.e. during an application of 10 \(\mu\)M nicotine which provokes maximal release (Fig. 1). It is estimated that 3–5 channels were present in a single patch. In keeping with the value of four channels, the probability of opening at +60 mV changed from about 5 \(\times\) 10\(^{-3}\) in normal conditions to 5 \(\times\) 10\(^{-2}\) after addition of 1 \(\mu\)M taxol; this suggests that taxol induces an increase of intracellular Ca\(^{++}\).

![Diagram of ion-channel activity](image)

**Figure 2** Effect of superfusion of taxol on Ic channel activity. (A) In control conditions (calcium-free medium), Ic channels are only evoked by steps to +60 mV or above. (B) After superfusion with 1 \(\mu\)M taxol for 7 min, a consistent change of threshold is observed on the same cell: Ic channels are present at -20 mV. Recording conditions (as summarized on the schema): in normal Ringer’s solution (5 mM KCl), the cell resting potential \(E_m\) is \(-60\) mV. A patch of membrane still attached to the cells is electrically isolated from the bath by the edges of the tip of an electrode. The pipette potential is displaced by a 325-ms step, so as to bring the patch potential from -20 to the indicated value, +100 mV for instance. Some voltage jumps have elicited upward deflections of large amplitude (outward currents according to our convention). The corresponding channels were identified as the large Ca\(^{++}\)-dependent K\(^+\) channels (Ic channels). Since the pipette solution contained a 5-mM KCl solution (solution A), potassium equilibrium potential was below -60 mV at the level of the patch. A step to +100 mV introduces -160 mV driving force on K\(^+\) ions, so Ic channel conductance: 80 pS. Traces were obtained from a single patch in these conditions; 10-s intervals between each of the four successive recordings at an indicated voltage. i, inside; o, outside; p, pipette.
FIGURE 3 Continuous recordings of Ic activity after incubation with taxol. (A) Recordings obtained in cell-attached conditions from two distinct patches: one in normal conditions (top trace), the other after 10-min addition of 1 μM taxol in the bath (bottom trace). Experiments were performed in the absence of Ca++ in the bath and in the recording pipette. pCa was fixed at 8. Potential of each patch (as given below the traces) was stepped from -60 to +120 mV. Bath medium and solution A to fill this pipette were prepared as given in Materials and Methods. (B) The reversal potential of Ic channel currents is estimated in two distinct conditions after addition of taxol. Filled circles refer to a recording obtained using an isotonic KCl electrode (solution B); currents reversed then close to 0 mV (E_K). Filled triangles correspond to an experiment performed with a Ringer-filled electrode (solution A, 5 mM KCl). Ic channel currents reversed below -60 mV. (C) Quantitative analysis of the above experiments. The frequency of opening (open symbols) and percent of time open (filled symbols) are plotted versus potentials in normal conditions (squares) and after addition of taxol (circles). Both parameters were measured by hand on recordings taken at 100 mm/s on a Gould 4000 pen-writer. For each point, 50–100 events were sampled over 5–10 s. The time the channels spent open is the sum of the duration of the individual events, and the mean duration of a single event was calculated as a ratio of this time open (in percent) and the frequency.
concentration from the order of $10^{-8}$ M to the order of $10^{-7}$ M.

The normal release of catecholamines induced by acetylcholine derived from splanchnic nerve terminals is preceded by an entry of $\text{Ca}^{++}$ both through nicotinic receptors and voltage-dependent channels (37, 41) activated by the depolarizing effect of acetylcholine. Release experiments following short-term taxol incubation showed an unusual response to nicotine stimulation. The effect of nicotine on the release would be amplified if one effect of taxol was to shift the activation curve of the voltage-dependent calcium current to lower potentials. No obvious displacement of the amplitude versus voltage curves of either the $\text{Na}^{+}$ or the $\text{Ca}^{++}$ currents was observed, other than a slight shift of 5 mV towards more hyperpolarized potentials seen in two cells 10 min after starting the taxol superfusion (Fig. 6). Thus, the increased release of catecholamines after simultaneous application of nicotine and taxol can also be tentatively explained as the result of an effect of taxol on intracellular calcium stores, although the shift of the activation curve may indicate a slight effect of taxol on the surface charges. An attempt was made to study long-term taxol-treated chromaffin cells with the patch clamp technique. However, after more than 1 h of taxol treatment, cells were not robust enough to support patch clamp conditions. Immunofluorescence and ultrastructural approaches were thus used to study this long-term effect.

**Immunofluorescence**

In intact chromaffin cells, granules are distributed throughout the cytoplasm, and were seen to accumulate in the endings of neurites (Fig. 7 a). When treated with taxol, neurites were no longer fluorescent, thus indicating an absence of granules, while the cell body was strongly fluorescent (Fig. 7 b). Longer treatment led to a polar segregation of granules in the cell body (Fig. 7, c and d). In untreated chromaffin cells, microtubules formed a typical, dense network extending in neurite-like structures (Fig. 7 e). When treated with DMSO, no alteration of immunofluorescence staining was noticeable. Incubation of cells in the presence of 1 $\mu$M taxol for 13 h resulted in the modification of microtubular arrangement. They were found to pack together forming bundles abundant in regions close to the plasma membrane (Fig. 7, f-h).

**Ultrastructure**

Chromaffin cells cultured on collagen-coated glass or plastic coverslips showed many features typical of their in vivo counterparts. Cells had abundant cytoplasm with the normal complement of mitochondria, endoplasmic reticulum, and secretary granules. However, cells frequently formed clusters from which extended neurite-like cell processes. In cells from control, DMSO-treated, and taxol/DMSO-treated cultures, the presence of empty membrane-bound vacuoles was noted, a feature normally absent in chromaffin cells in the adrenal medulla. Such vacuoles were seen more frequently in DMSO-treated cultures while in taxol/DMSO-treated cells "myelin figures" (tightly packed whorls of membranes) were also occasionally seen. Even in the same culture, the general appearance of taxol/DMSO-treated cells varied widely: in some cells, the cytoplasm was more "aqueous" than in others, an
DMSO for 13 h, the most evident difference in the ultrastructural appearance concerned the microtubule number which was observed. In contrast, in cultures treated with taxol, no significant increase in the number of microtubules was observed between DMSO-treated and control cultures. In general, in DMSO-treated chromaffin cells, microtubules were isolated structures, and not structures organized in bundles. As in control cells, microtubules were sometimes aligned in the subplasma membrane region (Fig. 8). There was an indication that the length of microtubules observed in such sections was slightly longer than in controls which may result from the fact that on the whole they tended to be more rectilinear.

In chromaffin cells in culture treated for 1 h with 1 μM taxol, a significant increase in the number of microtubules was observed. In contrast, in cultures treated with taxol/DMSO for 13 h, the most evident difference in the ultrastructural appearance concerned the microtubule number which was considerably greater in most cells. No great differences with regard to size and number of microtubules were observed between DMSO-treated and control cultures. In general, in DMSO-treated chromaffin cells, microtubules were isolated structures, and not structures organized in bundles. As in control cells, microtubules were sometimes aligned in the subplasma membrane region (Fig. 8). There was an indication that the length of microtubules observed in such sections was slightly longer than in controls which may result from the fact that on the whole they tended to be more rectilinear.

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**DISCUSSION**

We performed the experiments reported in this paper to study the role of microtubules during release of stored material from secretory cells. Microtubule-affecting drugs, such as colchicine, have been reported to partially inhibit the release of catecholamines from chromaffin cells of the adrenal medulla (8). However, the inhibition was incomplete and an effect on membrane integrity could not be excluded (44). In contrast to studying a microtubule-disrupting drug such as colchicine, this report describes the effect of taxol, which induces microtubule polymerization and stabilization (15, 19, 27, 38, 40). Surprisingly, depending on incubation conditions, taxol was found to either inhibit release of catecholamines after long-term incubation, or to provoke so-called spontaneous release after short-term incubation. These opposite effects are likely to be a consequence of different modes of action. Taxol is known to promote polymerization of microtubules and to stabilize them in vitro; it also induces a vast increase in the number of microtubules in functional chondroblasts (43), in fibroblasts (15, 31, 39), in neurones of fetal mouse dorsal-root ganglion and spinal-cord cultures (30, 32) and in PC12 cells (14). In other morphological studies, taxol was found to provoke more profound disturbances of microtubular arrangement: the increase in number of microtubules was frequently greater than that observed in this study and unusual associations with endoplasmic reticulum and even the cell nucleus have been reported (32, 39, 43). However, in all such studies, either the concentration of taxol or the time of treatment were much greater than those used here. Cells have
FIGURE 7 Immunofluorescent labeling of 7-d-old chromaffin cell cultures. Cells were treated with antichromogranin A antibody (a–d) and antitubulin antibody (e–h). Untreated cells are shown in a and e; cells treated with 1 μM taxol for 13 h are shown in b, f, and g, and for 16 h in c, d, and h. Arrows in b indicate unlabeled neurites and arrowheads point to neurite endings. Arrows in f–h show microtubular arrangements beneath the plasma membrane. × 550.

generally been treated with concentrations of 1 μM–10 μM taxol for as long as 6 d. Chromaffin cells in culture did not survive such treatment: after 24 h in the presence of 1 μM taxol, cell mortality was high and surviving cells were too fragile to support cell clamping conditions. However, after shorter treatment periods of 13–16 h, a significant accumulation of microtubules was observed in chromaffin cells at the ultrastructural level. No particular relationship was noted between microtubules and endoplasmic reticulum cisternae as has been reported in neurones (30, 32) and fibroblasts (31, 39) in culture. Taxol-induced microtubules were found (a) free in the cytoplasm, or (b) forming oriented bundles in certain cell areas, which were either just beneath the plasma membrane or free in the cytoplasm giving the cell a polarity.

The massive increase of cytoplasmic microtubules was observed after 13–16 h of incubation with taxol, when inhibition of catecholamine release was only 20%. In contrast, a nearly complete inhibition of catecholamine release was observed in cells treated for 60 min with taxol; at that time, cytoplasmic microtubule numbers were not modified. So there is a time lag between maximal effects of taxol on secretory activity and microtubule organization. On pancreatic B-cells, Howell et al. (22) have shown that exposure of isolated rat islets of Langerhans to 10 μM taxol for 30 min results in marked inhibition of glucose-stimulated insulin secretion. Ultrastructural studies showed that 90-min treatments with taxol did not affect the structure of pancreatic cells, except for an indication of increased numbers of cytoplasmic microtubules. In this work, there were no data concerning the effect of longer incubation times on release and cell microtubules, but it is likely that the situation is similar to that of chromaffin cells, pancreatic B-cells and chromaffin cells both being paraneuronal cells (17). After overnight treatment with taxol, it is reasonable to assume that the consequent increase in cellular
FIGURES 8 and 9  (Figure 8) Culture treated with DMSO (120 μM) 13 h before fixation. The distribution of microtubules (arrows) resembles that of control cultures. × 19,600. (Figure 9) Culture treated with 1 μM taxol, 13 h before fixation. Note the abundant microtubules (arrows). In this elongated cell, long bundles are aligned along the cell axis. Chromaffin granules are excluded from regions of high density of microtubules. × 32,300.
levels of microtubules provokes a decrease of cytoplasmic fluidity. Virtually nothing is known concerning the relationship between microtubule density, cytoplasmic fluidity, and secretory organelle movement. From recent experiments, it was shown that tubulin subunits are able to bind to chromaffin granule membrane (11), though it is not clear if microtubules themselves have a similar capacity. Thus, in taxol-treated cells, granule movement towards the plasma membrane could be partially inhibited. This rationale, however, is not fully satisfactory since in chromaffin cells taxol-induced inhibition of catecholamine release was not coincident with the increase of microtubule numbers. Therefore, in addition to its property of altering microtubules, taxol probably has side effects which also affect secretory activity of cells and which remain to be elucidated.

The very rapid effect of taxol on the secretory activity of chromaffin cells during short-term incubation is one of these unexpected side effects. The increased basal release observed on short-term application of taxol may be mediated through an alteration of the excitability properties of the plasma membrane. A non-specific effect on surface charges is not expected of this neutral molecule. But suggestions of a role of microtubules in the maintenance of the membrane excitability repeatedly appear in the literature, based on reports of a tendency towards membrane stabilization on disruption of the microtubules with colchicine, and in contrast towards an increased excitability on their assembly (16, 35). Thus, after colchicine-induced breakdown, Na⁺ excitability is restored after addition of microtubular proteins and a 260,000-mol-wt protein (34). The low concentrations of taxol used here do not have any such effects on either the Na⁺ or Ca²⁺ permeabilities. In contrast, Ic channels were shown to have a consistently higher probability of opening in the presence of taxol. An explanation could be that some calcium-dependent step shows a modified sensitivity to calcium. But since taxol had no effect in conditions where [Ca²⁺] was imposed on both sides of an isolated patch any such calcium-dependent molecule is unlikely to be localized in the cell membrane. An alternate hypothesis is that taxol induces an actual increase of [Ca²⁺]. If such a [Ca²⁺] increase was to be the sole explanation, we estimate that taxol could induce a maximal 10-fold rise in [Ca²⁺] from 10⁻⁶ M at rest (but see also reference 25) to several 10⁻⁶ M. This rise in [Ca²⁺] could explain the observed spontaneous release; since taxol itself did not provoke catecholamine release from isolated secretory organelles (data not shown), the increased catecholamine release induced on short-term application of taxol is likely to be due to exocytosis. Recent investigations have shown that during exocytosis, a series of events are calcium-dependent (see reference 6 for review), and any alterations of their sensitivity to calcium or any changes of [Ca²⁺], are followed by modifications of catecholamine secretion (24).

The present data pose as yet an unanswered question: where is the [Ca²⁺], coming from? It is possible that Ca²⁺ is released directly by taxol from an internal store such as the protein-bound Ca²⁺. Alternatively, taxol may modify the activity of storage-sites such as mitochondria or reticulum (36). Spontaneous fluctuations of [Ca²⁺] have been assumed to occur in cultured neurons (2, 33). Indeed, another possibility is that taxol acts by blocking the plasma Ca²⁺ pump and as a result provokes a rise in free cytoplasmic calcium concentrations.

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Note Added in Proof: Since this paper was submitted, a report (McKay, D. B., and A. S. Schneider, 1984, J. Pharmacol. Exptl. Therap., 231:102-108) on the effect of taxol on Ca²⁺ uptake and catecholamine secretion in adrenal chromaffin cells has appeared which suggests that taxol may, in addition, block cholinergic receptors.

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Note: The text continues with a list of references, which are not transcribed here.
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