Mitotic aberrations induced by carbaryl reflect tyrosine kinase inhibition with coincident up-regulation of serine/threonine protein phosphatase activity: implications for coordination of karyokinesis and cytokinesis

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The insecticide carbaryl and its metabolite 1-naphthol cause partial uncoupling of karyokinesis and cytokinesis in V79 Chinese hamster fibroblasts; karyokinesis is blocked in metaphase, the microtubules of the spindle depolymerize and the chromosomes and spindle remnants become displaced to the periphery of the cell. A high frequency of these disturbed cells elongate and a smaller fraction initiate a cleavage furrow. Here, we attempt to determine the potential targets for carbaryl and 1-naphthol in cytokinesis-specific signalling, led by the fact that the potential protein phosphatase inhibitor 1-naphthyl phosphate was previously identified in treated cells. We found that the typical cytological pattern induced by carbaryl and 1-naphthol could be obtained with tyrphostins, specific tyrosine kinase inhibitors, indicating that the carbaryl-induced effects could be due to tyrosine kinase inhibition. This was confirmed by tyrosine kinase assays showing that carbaryl, 1-naphthol and 2-naphthol were equally efficient at inhibiting tyrosine kinase activity as tyrphostin B44(−). As tyrosine kinases can act as regulatory factors in determining dephosphorylation rates, the activities of type-1 (PP1) and type-2A (PP2A) serine/threonine protein phosphatases were also determined. There was a clear up-regulation of the overall PP1/PP2A activities in cells treated with carbaryl, 1-naphthol or tyrphostin B44(−). This stimulation was shown to be indirect because these compounds had no effect on the activity of purified human PP1 in the test tube. 2-Naphthol, which has been found to be less efficient with regard to displacement of chromatin, did not cause up-regulation, but a significant decrease in PP1/PP2A activity. We suggest that a net decrease in tyrosine kinase activity in combination with a net increase in PP1/PP2A activity is a precondition for cell elongation and cytokinesis in mammalian cells and that the corresponding enzymes are targets in the network of activities serving to coordinate karyokinesis and cytokinesis.

Introduction

In mammalian cells karyokinesis and cytokinesis are normally perfectly coordinated, making it difficult to separate biochemical steps necessary for chromosome segregation and cytokinesis, respectively. Although a number of players have been identified (Norbury and Nurse, 1992; Glotzer, 1997), including integrating factors (Rappaport, 1991; Mackay et al., 1998), the primary signals for coordination of the two processes have remained poorly known. However, the fortuitous combination of the compound carbaryl and a clone of the V79 cell line (Önfelt and Klasterska, 1983; Soderpalm-Berndes and Onfelt, 1988) appears to provide a possibility to get closer to an understanding of how this coordination is organized.

Carbaryl is an insecticide which acts primarily by inhibiting acetylcholinesterase by carbamoylation of a serine hydroxyl group at the active site (International Programme on Chemical Safety, 1994). The compound also has other biochemical effects, as it reacts with reduced glutathione and probably directly with other accessible protein hydroxyl and sulphhydryl groups (Hinderer and Menzer, 1976; Miller et al., 1979; Önfelt 1983) or indirectly through reactions involving the glutathione conjugate (Baille and Slatter, 1991). During carbamoylation the aromatic part of carbaryl will be released in the form of 1-naphthol, which is an uncoupler of oxidative phosphorylation. Carbaryl causes a reversible block in metaphase, spindle microtubule depolymerization, displacement of chromosomes to the periphery of the cell and, concomitant with this, a remarkable progression of the cytoplasmic changes typical of anaphase and telophase (Önfelt and Klasterska, 1983; Soderpalm-Berndes and Onfelt, 1988; Renglin et al., 1998). An immediate block in metaphase is seen with most compounds at some concentration (Önfelt, 1986) and, consequently, there is a vast number of routes leading into a state eliciting the spindle assembly check-point (Jordan et al., 1992; Sorger et al., 1997). While carbaryl (at 0.4 mM) blocks several other types of cells in metaphase (unpublished results), the concomitant changes in shape were found to be most frequent and reproducible in the V79 cells used here. The typical changes in cell shape can occur with a few other compounds, but are not as frequent and reproducible as with carbaryl (unpublished results). Therefore, V79 cells are most probably stably modified in some respect resulting in a propensity to uncouple cytokinesis from karyokinesis when blocked in metaphase in a certain fashion. Further, it seems likely that carbaryl selectively elicits reactions serving to stimulate progression to cytokinesis, possibly involved also in the coordination of karyokinesis and cytokinesis. 

An investigation of the mechanism of action of carbaryl at first appears to be aggravated by a bewildering number of possible reactions. However, the current knowledge of the effects of carbaryl presents us with some details that may be helpful when the mechanisms behind the above-mentioned mitotic effects are being elucidated. (i) At 0.4 mM 1-naphthol induces the same cytological pattern as carbaryl, without any decrease in free sulphydryl groups (Soderpalm-Berndes and Önfelt, 1988); (ii) carbaryl at 0.4 mM causes less uncoupling of oxidative phosphorylation but, with respect to mitotic aberrations, shows remarkable similarities to the effects of 1-naphthol (Soderpalm-Berndes and Önfelt, 1988); (iii) the structural isomer 2-naphthol, also an efficient uncoupler of oxidative phosphorylation, blocks mitotic cells in metaphase,
but is considerably less efficient with regard to displacement of chromosomes and precocious cell elongation (Renglin et al., 1998); (iv) all three compounds cause a decrease in protein phosphorylation and the phosphorylated product, 1-naphthyl phosphate, can be identified in cells treated with 1-naphthol (Renglin et al., 1998). Thus, we tentatively concluded that 1-naphthol is phosphorylated by some kinase(s) and, hypothetically, carbarly would act by carbamoylating the enzyme(s) in question. Because of the aromatic part of the compounds we speculated that both carbarly and 1-naphthol may inhibit a tyrosine kinase(s) not as easily inhibited by 2-naphthol.

Here we have tested this hypothesis in a comparative study of all three agents and a set of tyrophostins, which are benzylidine malononitrile derivatives designed to inhibit protein tyrosine kinase activity with weak or no effects on serine/threonine kinases (Yaish et al., 1988; Levitzki 1992; cf. Nowak et al., 1997). Our study shows that tyrophostins, carbarly and 1-naphthol all induce the same aberrations in mitosis. At corresponding concentrations, these compounds induce an efficient inhibition of tyrosine kinase activity concurrently with up-regulation of the activity of protein phosphatase 1 (PP1) and/or 2A (PP2A). These effects suggest that the mitotic aberrations induced by carbarly could be related to a targeted modulation of reversible phosphorylation involved in the regulation of mitosis.

Materials and methods

Chemicals

Carbarly was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). The ATP measurements were performed with a Bio Orbit ATP monitoring kit (Turku, Finland). Cell culture media, the Protein Phosphatase and the Protein Tyrosine Kinase Assay Systems were purchased from Gibco BRL (Grand Island, NY). Tyrophostins were bought from Calbiochem (La Jolla, CA), Tyrosine Kinase Assay Systems were purchased from Gibco BRL (Grand Island, NY). Cell culture media, the Protein Phosphatase and the Protein Tyrosine Kinase Assay Systems were purchased from Gibco BRL (Grand Island, NY).

Cell culture

V79 Chinese hamster cells were seeded in flasks or on plastic coverslips in Petri dishes and incubated in Eagle's minimum essential medium supplemented with 1.8 mM L-glutamine, 90 U/ml penicillin, 90 µg/ml streptomycin, 45 µg/ml kanamycin and 10% heat-inactivated fetal calf serum, in 5% CO₂ at 37°C before use in experiments.

The V79 cells used here carry the same mutations in p53 (C.Arnaudeau, personal communication) as reported by Chaung et al. (1997).

Mouse fibroblasts (IVS) overexpressing the oncogenic p60src protein tyrosine kinase were seeded on Petri dishes in Dulbecco’s modified Eagles medium containing 5% fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 200 µg/ml G418 at 37°C with 5% CO₂ before use in experiments. All materials were from Gibco BRL.

Treatment

Medium was removed and the V79 cells were rinsed in 2×5 ml Hank’s balanced salt solution (HBSS) at 37°C before treatment with compounds or vehicle alone. Carbarly, 1-naphthol, 2-naphthol and tyrophostins were all dissolved in acetone giving a final concentration of 0.2% acetone in HBSS. Okadaic acid was dissolved in dimethylsulphoxide (DMSO) giving a final concentration of 0.2% DMSO in HBSS. Controls were treated with the same amount of vehicle alone in HBSS.

Mitosis: Giemsa staining

Cultures on coverslips were incubated for 30 min except for the time course experiments where parallel cultures, and also controls, were fixed every 5 min up to 30 min. Fixation was carried out in methanol:glacial acetic acid (3:1), and the slides were stained for 4 min in 4% Gurr’s Giemsa and coded before scoring. All experiments were repeated once or twice.

Recovery

Cells treated for 30 min were rapidly rinsed with 2×5 ml HBSS and fixed after 0, 5, 10, 20 and 30 min of incubation in medium. Each compound, time and concentration was run in duplicate with their own controls.

Scoring

All slides were coded and analysed by the same person; 20 mitotic cells/ slide were scored and classified in accordance with a previous protocol (Renglin et al., 1998), but with some of the classes summarised here: (1) round cells with the chromosomes in a metaphase-like arrangement in the middle; (2) round cells with the metaphase-like plate located at the periphery of the cell; (3) elongated cells with metaphase-like chromosomes at one pole; (4) elongated cells with metaphase-like chromosomes at one pole and with signs of a symmetrically placed cleavage furrow; (5) cells apparently cleaved so that one tetraploid cell and one cytoplasm had been formed; (6) anaphases; (7) telophases (Figure 1a–c).

Immunofluorescence labelling

Cells grown on coverslips and treated as described above were fixed with 0.5% glutaraldehyde in PHEM (10 mM EGTA, 60 mM PIPES, 25 mM HEPES, pH 6.9) with 1% TritonX-100 for 10 min, followed by NaBH₄ (1 mg/ml) for 2×5 min, with rinses in PHEM between and followed by immunolabelling of tubulin; the cells were incubated with mouse anti-β-tubulin antibody (Amersham) for 20 h at 4°C, washed in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.3) and then incubated with biotinylated rabbit anti-mouse antibody (Zymed, San Francisco, CA) for 60 min at 37°C. After washing in PBS the cells were incubated with FITC– streptavidin for 30 min at 37°C. Chromosomes were counterstained with propidium iodide before the coverslips were mounted in glycerol and 0.2 M Tris, pH 8.5, with DABCO as antioxidant.

ATP measurements

Cultures (5×10⁵ cells/flask, 25 cm²) were incubated for 24 h before the cells were preincubated for 30 min in Krebs Ringer solution supplemented with glucose or deoxyglucose, before addition of compound and incubation for 30 min. After removal of treatment solution and rinsing with 0.9% NaCl (0°C), extraction was carried out with 0.5 ml of 10% trichloroacetic acid for 30 min at 0°C. The ATP levels were measured according to instructions in the kit and values were related to the protein content, which was measured according to Lowry (Lowry et al., 1951) with bovine serum albumin as protein standard. All compounds were tested in four or five experiments.

Protein phosphorylation: ⁴²P-labelling

An estimate of the overall protein phosphorylation was obtained by ⁴²P-labelling; cultures (5×10⁵ cells/flask, 25 cm²) were incubated for 24 h and then preincubated for 30 min in 4 ml of Dulbecco’s phosphate-free modified Eagles medium. [³²P]orthophosphoric acid (specific activity 10.00 mCi/ml, carrier free) was added to a final concentration of 0.126 mCi/ml and incubated for 15 min. The cells were treated with the respective compounds for 30 min, rinsed with ice-cold 0.9% NaCl and then the cells were scrapped off and washed three times in cold 10% trichloroacetic acid by centrifugation. The final pellets were suspended in cold TBS (10 mM Tris and 150 mM NaCl) and the ⁴²P content was counted with a Beckman scintillation chamber. Aliquots were set aside for determination of protein content later on. Within an experiment, each treatment group was run in duplicate and five experiments were performed.

Serine/threonine phosphate assay

Cultures (1.5×10⁵ cells/flask, 80 cm²) were set up and incubated for 48 h before rinsing and treatment for 30 min in HBSS. Quickly rinsed cultures were then put on ice and scraped off in a lysis buffer consisting of 20 mM HEPES, pH 7.4, 1 mM MgCl₂, 30 mM β-mercaptoethanol, 10% glycerol, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride and 10 µg/ml antipain, leupeptin and pepstatin. The enzyme activities in the extracts were measured by a commercial protein phosphate assay from Gibco BRL according to the manufacturer’s instructions. Briefly, ³²P-labelled phosphorylase a is used as substrate and the reactions start when the substrate is added to the cell extracts. The reaction was allowed to proceed for 10 min at 30°C and stopped by the addition of 20% trichloroacetic acid. The samples were placed on ice for 10 min and then centrifuged at 10 000 g for 3 min. An aliquot of the clear supernatant was taken to determine the amount of radioactivity released in the assay as ³²P. The protein phosphate activity was calculated as nmoi P_i released/min/ml (for details on the assay procedures see, for example, MacKintosh, 1993, and references therein). Two or three experiments were performed with each compound and with samples run in duplicate or triplicate.

By using the protein phosphate assay described above, the effects of the chemicals alone on the activity of a purified human type 1 serine/threonine protein phosphatase (M₅, 37 kDa) expressed in Escherichia coli (Alessi et al., 1993) were also studied.

Effects on tyrosine kinase activity

The src-transformed IVS cell line has a high constitutive tyrosine kinase activity (Chen et al., 1992). In order to further stimulate mitogenic tyrosine kinase
activity, subconfluent IV5 cells (1.4 × 10⁶/Petri dish), grown in medium with 0.5% fetal calf serum for 30 h, were fed with medium containing 5% serum for 15 or 30 min. Then medium was removed and the cells were rinsed with ice-cold PBS, scraped off the dishes with a rubber policeman into 200 ml lysis buffer (50 mM HEPES, pH 7.4, 50 mM β-glycerophosphate, 25 mM NaF, 0.1% Triton X-100, 150 mM NaCl, 20 mM EGTA, 15 mM MgCl₂, 1 mM diethioctetil, 25 µg/ml leupeptin and 25 µg/ml aprotinin), homogenized 20 times with a 1 ml pipette and put on ice for 20 min. After centrifugation at 4000 g for 2 min the supernatant was divided into aliquots and frozen.

Cell extracts melted on ice were diluted 10 times. The tyrosine kinase assay (Gibco BRL) was performed according to the manufacturer’s instructions with the modification that the compound to be tested was added. The target peptide (RR–SRC) mimics the immediate surroundings of and the pp60v–src tyrosine residue phosphorylated by EGF. The amount of [γ-³²P]ATP in each sample was 0.08 mCi/ml. Carbaryl, 1-naphthol, 2-naphthol and tyrphostin B44(–) were applied in 10 µl acetone to give the same final concentrations as used with intact V79 cells. Each control was run in triplicate with acetone instead of compound. Three experiments were performed with each compound.

### Results

#### Cytology

Carbaryl, 1-naphthol and 2-naphthol were all used at 0.4 mM, while the tyrphostins were tested at concentrations in the range 0.5–25 µM according to suggestions by the manufacturer. The different B-tyrphostins in particular were found to induce displacement of chromosomes (Figure 1a–c), but precocious cell elongation was less frequent than with 1-naphthol and carbaryl (Table I). All in all the B-type tyrphostins were more efficient than 2-naphthol (Table I). After 30 min treatment there were virtually no anaphases or telophases with 1-naphthol, carbaryl or tyrphostin B44(–).

Tyrphostin B44(–) at 10 µM was selected for further investigation, first verifying by immunofluorescent staining that there was depolymerization of spindle microtubules (Figure 2). The temporal aspects of induction of aberrant cells and reversion of effect after rinsing, were similar to those with 1-

<table>
<thead>
<tr>
<th>Tyrophostin</th>
<th>Concentration tested (µM)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Types 2–5 (%) ± SE</td>
<td>Types 3–5 (%) ± SE</td>
</tr>
<tr>
<td>A25 3,4,5-Trihydroxy-benzylidene-malonitrile</td>
<td>0.5</td>
<td>23 ± 5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25 ± 2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>B42 N-Benzyl-3,4-dihydroxyBCAα</td>
<td>0.5</td>
<td>43 ± 5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40 ± 3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>B44(–) (R)-N-(1-Methylbenzyl)-3,4-dihydroxy-BCAα</td>
<td>0.5</td>
<td>72 ± 6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>B44(–) (R)-N-(3-Phenylpropyl)-3,4-dihydroxy-BCAα</td>
<td>0.5</td>
<td>38 ± 13</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>57 ± 6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>B46 N-(3-Phenylpropyl)-3,4-dihydroxy-BCAα</td>
<td>0.5</td>
<td>41 ± 11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>68 ± 10</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>B48 N-Phenyl-3,4-dihydroxyBCAα</td>
<td>0.5</td>
<td>31 ± 5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35 ± 8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>400</td>
<td>80 ± 12</td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>400</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>400</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>Control</td>
<td>50</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

The table shows the concentrations tested and the average frequencies (%) of mitotic cells having the metaphase plate displaced to the periphery (types 2–5) and the frequency of those showing an elongated shape similar to normal anaphase or telophase (types 3–5). Descriptions are given in Materials and Methods and Figure 1. n = 4.

αBCA, benzylidene cyanoacetamide.
Recovery of V79 cells treated with 20 nM okadaic acid for 60 min

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th>c-mitosis (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid (20 nM)</td>
<td>20</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>61.8 ± 12.8</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>67.0 ± 3.5</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>4.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>3.5 ± 0</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>2.8 ± 0.3</td>
</tr>
</tbody>
</table>

Although c-mitosis were seen after 60 min, none of the scoring parameters 1–5 used for scoring of the typical appearances induced by carbaryl could be detected. \( n = 2 \).

<table>
<thead>
<tr>
<th>Table III.</th>
<th>Response of V79 cells treated with 20 nM okadaic acid for 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Deoxyglucose (nmol ATP/mg prot ± SE)</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Carbyl</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>DNP</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>Tyrphostin</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>Control</td>
<td>7.4 ± 1.1</td>
</tr>
</tbody>
</table>

ATP was measured after treatment in the presence of deoxyglucose to depress glycolysis and measure oxidative phosphorylation and under normal conditions, i.e. in the presence of glucose. Averages and standard errors were based on values from 4–5 cultures/compound. Crude measurements of protein phosphorylation were made by incorporation of \( ^{32}P \) and the values are expressed as a per cent of controls. Averages and standard errors were based on 3 cultures/compound (Fig. 3).

Measurements of ATP and protein bound \( ^{32}P \)

V79 cells were treated with the chemicals in Krebs Ringer solution supplemented with glucose or deoxyglucose in order to investigate their action on oxidative phosphorylation (Table IV). There were significantly lower amounts of ATP in cells supplemented with deoxyglucose and treated with 1-naphthol, 2-naphthol or the conventional uncoupler of oxidative phosphorylation 2,4-dinitrophenol, compared with controls (\( P < 0.005 \)). In the presence of deoxyglucose instead of glucose the decreases with 10 \( \mu \)M tyrphostin B44(–) and carbaryl were not significant, but the \( P \) value for carbaryl treatment was low (\( P = 0.1 \)).

The total amount of ATP measured in the presence of glucose suggested compensation through glycolysis (Table IV). Tyrphostin had no significant effect on ATP levels.

\( ^{32}P \) bound to protein decreased by 10 ± 3.9% (x ± SE) during treatment with 10 \( \mu \)M tyrphostin B44(–) and by 29 ± 3.1% with 1 mM 2,4-dinitrophenol. Previously observed decreases for carbaryl, 1-naphthol and 2-naphthol were 47 ± 5.8, 42 ± 4.9 and 31 ± 5.3%, respectively (Renglin et al., 1998). These relative decreases appeared to correlate well with the total relative amount of ATP (linear regression, \( P = 0.008 \), \( r = 0.965, n = 5 \)) after treatment (Figure 3).

Effects on tyrosine kinase activity in extracts from IV5 cells

Tyrphostin B44(–), carbaryl, 1-naphthol and 2-naphthol were applied at the concentrations used in cell culture experiments. They were all found to decrease the \( \textit{in vitro} \) tyrosine kinase activity.
Carbaryl-induced mitotic aberrations

Fig. 3. Relationship between ATP levels and $^{32}$P incorporation. Values in Table IV were plotted to show that the average level of protein phosphorylation correlated with a decrease in ATP with the compounds applied: carbaryl, 1-napthol and 2-napthol (0.4 mM), tyrphostin B44(–) (10 $\mu$M) and 2,4-dinitrophenol (1 mM).

Table V. Effects of 1-naphthol, 2-naphthol, carbaryl and tyrphostin B44(–) on tyrosine kinase activity in extracts from serum-stimulated mouse IV5 fibroblasts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tyrosine kinase activity (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol (0.4 mM)</td>
<td>61.1 ± 2.2</td>
</tr>
<tr>
<td>2-Naphthol (0.4 mM)</td>
<td>62.1 ± 3.1</td>
</tr>
<tr>
<td>Carbaryl (0.4 mM)</td>
<td>68.9 ± 9.5</td>
</tr>
<tr>
<td>Tyrphostin B44(–) (10 $\mu$M)</td>
<td>53.2 ± 9.6</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 3.8</td>
</tr>
</tbody>
</table>

The target peptide mimics the immediate surroundings of and the pp60$^{v–src}$ tyrosine residue phosphorylated by EGF. Averages and standard errors were based on values from 4–8 samples/compound and are expressed as a percent of controls.

activity by ~50% compared with controls ($P < 0.005$ in all cases; Table V).

Protein phosphatase assay: chemical effects on isolated enzyme

Carbaryl, 1-naphthol and 1-naphthyl phosphate were tested for their inhibitory effects on purified human type 1 serine/threonine phosphatase ($M_r$ 37 kDa) at concentrations ranging from 0.01 nM to 1 mM. Microcystin-LR, the positive control (1 $\mu$M) in the in vitro assay, inhibited the enzyme when added (not shown). 1-Naphthol and carbaryl showed only slight effects at the highest concentrations (1 mM), while 1-naphthyl phosphate showed very low or no effect in the assay (data not shown).

Effects on cellular serine/threonine phosphatase activity

Phosphatase activity in extracts from treated V79 cells was measured and expressed as a percentage of the corresponding controls (Figure 4). After 5 min treatment with 0.4 mM 1-naphthol, 0.4 mM carbaryl or 10 $\mu$M tyrphostin B44(–) the phosphatase activity was significantly increased ($P < 0.01$). Cells that had been treated with 0.4 mM 2-naphthol or 20 nM okadaic acid showed lower activities than controls ($P = 0.01$). The positive control, microcystin-LR, inhibited phosphatase activity very effectively.

Discussion

The present study was carried out to elucidate the possibility that modifications of protein kinase and or phosphatase activities cause the characteristic mitotic disturbances induced by the insecticide carbaryl and its metabolite 1-naphthol in V79 Chinese hamster cells. Both compounds cause spindle microtubule depolymerization and a block in metaphase, but blocked cells elongate and a fraction may even show a furrow or cleave (Soderpalm-Berndes and Onfelt, 1988; Renglin et al., 1998). The block in metaphase seems to occur before activation of the anaphase promoting complex (Cohen-Fix and Hall, 1997) because chromatid separation cannot be observed in blocked cells. However, cells in anaphase at the start of treatment decondense chromosomes and progress into G1 within minutes even when full chromosome segregation is more or less hampered (Onfelt, unpublished observations of live cells).

We discuss below the finding that carbaryl and 1-naphthol may exert their particular effect on mitosis of V79 cells through inhibition of some tyrosine kinase, in conjunction with an increase in serine/threonine phosphatase activity.

Tyrosine kinase inhibition mimics the effects of carbaryl and 1-naphthol

1-Naphthyl phosphate was earlier isolated from cells treated with 1-naphthol, suggesting that 1-naphthol may function as a substrate for a kinase(s) and, presumably, then cause a reduction in phosphorylation of endogenous substrates. Due
to the aromatic part of carbaryl and 1-naphthol, it seemed appropriate to test protein tyrosine kinase inhibitors.

We applied eight different tyrphostins, which have been designed to selectively inhibit protein tyrosine kinase activities (Yaish et al., 1988; Levitzki, 1992). Effects on serine/threonine kinases, which most probably are indirect, are known to occur though (Nowak et al., 1997). All eight tyrphostins caused mitotic aberrations similar to those observed with 1-naphthol and carbaryl (Table I). Tyrphostin B44(−) was selected for further tests because its effects showed clear resemblances to the effects of 1-naphthol and carbaryl, with about the same frequency of displacement of chromosomes but a lower frequency of elongation (Table I). The selectivity of tyrphostins toward tyrosine kinase activity, in combination with the tyrosine kinase inhibiting activity of carbaryl and 1-naphthol, strongly suggest that tyrosine kinase inhibition is an important primary event behind the observed mitotic aberrations. Yet, 0.4 mM 2-naphthol was found to inhibit tyrosine kinase activity in vitro to the same extent as carbaryl, 1-naphthol and tyrphostin B44(−), so the cytological effects observed cannot be due solely to this factor. The target peptide in the assay mimics the part of pp60−src containing the tyrosine residue phosphorylated by EGF (peptide 3 in Pike et al., 1982), but the above-mentioned compounds may be small enough to target a broad range of tyrosine kinases. It cannot be excluded that 2-naphthol and its phosphorylated derivative, 2-naphthyl phosphate, together give a different activity profile with regard to cellular tyrosine phosphorylation state (see below) and thereby optimal concentration of 2-naphthol, which is different from that of 1-naphthol. Preliminary results with higher concentrations of 2-naphthol suggest that this hypothesis is reasonable; 0.5 mM induces the typical aberrations in ~50% of blocked cells (not shown).

Up-regulation of phosphatase activity

It is known that there are tyrosine kinases that are able to regulate PP2A phosphatase activity (Chen et al., 1992). The assay as applied here did not discriminate between PP1 and PP2A activities but clearly demonstrated increased activity in V79 cells treated with 1-naphthol, carbaryl and tyrphostin B44(−), while decreased activity was observed with 2-naphthol and okadaic acid. Hence, the treatments that gave almost identical results with regard to displacement of chromatin and were efficient with regard to precocious elongation in V79 cells also caused the same degree of up-regulation. We suggest therefore that both the increase in phosphatase activity and the cytological effects with 1-naphthol, carbaryl and tyrphostin B44(−) could be due to tyrosine kinase inhibition.

It is to be noted though that the increased PP1/PP2A activity did not reduce 32P-labelling by >10% with tyrphostin B44(−), which may agree with the usually small amount of tyrosine to be phosphorylated, but also suggests a certain selectivity or restriction to cell cycle stage of the enhanced protein phosphatase activity.

The more prominent decreases in overall 32P-labelling with 1-naphthol and carbaryl treatment, although to some extent explained by a general decrease in ATP (Figure 3), suggests that carbaryl and 1-naphthol are far less selective than tyrphostin B44(−). It remains to be tested if this suggested difference in effects on protein phosphorylation contain the source of the more effective elongation among aberrant cells with carbaryl and 1-naphthol treatment. It also remains to be tested if the formed 1-naphthyl phosphate reduces protein tyrosine phosphatase activity and if carbaryl can inhibit such enzymes by carbamoylation; both compounds hamper progression from G2 to mitosis, a process requiring an active dual tyrosine/serine phosphatase cdc25 (Strausfeld et al., 1994). It is of interest considering the difference between 1-naphthol and 2-naphthol that 2-naphthyl phosphate has been found to be a more swiftly transformed substrate than 1-naphthyl phosphate for mammalian protein tyrosine phosphatase, PTP 1 (Zhang, 1995). In any case, the observed increase in PP1/PP2A activity concomitant with induction of a morphology typical of anaphase and telophase is in line with the finding that PP1 activity is required for the cell to exit mitosis (Fernandez et al., 1992).

Comments on control experiments

The measurements presented here confirm that uncoupling of oxidative phosphorylation does not cause the high frequencies of displacement of chromosomes and elongation of V79 cells blocked in metaphase; tyrphostin B44(−) did not affect ATP levels under any conditions, while 2-naphthol, an efficient uncoupler of oxidative phosphorylation, was found to be inefficient with regard to the cytological effects studied (Table I; Renglin et al., 1998), as was the conventional uncoupler 2,4-dinitrophenol (Soderpalm-Berndes and Onfelt, 1988).

1-Naphthyl phosphate, which is formed in V79 cells treated with 1-naphthol, is claimed to be a broad phosphatase inhibitor (Li, 1984; Pondaven and Meijer, 1986), while our test did not show any effect of 1-naphthyl phosphate on isolated human PP1 activity in vitro. Yet, both 1-naphthyl phosphate and the established serine/threonine protein phosphatase (PP1/PP2A) inhibitor okadaic acid have been found to hamper chromatid separation and to cause asynchronous commitment in Tradescantia stamen hair cells (Wolniak and Larsen, 1992). Okadaic acid also affected chromatid separation in PtK2 cells, without interfering with spindle microtubules or their attachment to kinetochores (Larsen and Wolniak, 1993).

Here, we tested okadaic acid because charged 1-naphthyl phosphate is not likely to be taken up by the cells. All in all our observations on the action of okadaic acid appear in full accordance with those already presented for HeLa cells (Ghosh and Paweletz, 1992; Ghosh et al., 1996). Similar to V79 cells, okadaic acid had very little effect initially in HeLa cells, but within 1 h c-mitotic configurations appeared (with 12 nM). Impaired sister chromatid separation was indicated by the appearance of diplochromosomes in 20% of the mitotic cells after 24 h recovery (Ghosh and Paweletz, 1992). Further, stimulation of premature entrance into mitosis from G2 by okadaic acid could be demonstrated (Ghosh et al., 1996; see also Ajiro et al., 1996). We observed c-mitotic V79 cells with scattered chromosomes characteristic of spindle disruption, but this effect was dependent on time, not concentration, and therefore we assume that a mitotic population treated in G2 was incapable of forming a spindle. It is most important though that none of the protocols applied by us resulted in elongation and furrowing in V79 cells.

As mentioned above, none of 1-naphthyl phosphate, 1-naphthol or carbaryl had any effect on isolated human type 1 serine/threonine phosphatase in the test tube. This shows that PP1/PP2A are not likely targets for carbaryl and 1-naphthol.

Summary

It is suggested that carbaryl, 1-naphthol and tyrphostin B44(−) inhibit some tyrosine kinase activity, the down-regulation of which is connected with enhanced serine/
threonine phosphatase activity. This combination of effects seems important for induction of precocious cell elongation and cytokinesis in metaphase, but yet another factor is involved, as shown by the lower frequencies of elongated cells with tyrphostin compared with carbaryl/1-naphthol. Among different types of cells, tested in the same protocol, V79 cells show a particular propensity for the progression of the change of shape despite the block in metaphase with these compounds. Therefore, some separate function communicating the stage of the chromosome cycle may be constitutionally relaxed, just as the spindle assembly/ chromosome attachment-checkpoint is found to be endogenously in these cells (Onfelt et al. 1993). The putative tyrosine kinase would be a possible target for interaction with that unknown function and thus may play a role in the coordination of karyokinesis and cytokinesis.

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