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Effects of the carbamates fenoxycarb, propamocarb and propoxur on energy supply, glucose utilization and SH-groups in neurons

Received: 15 October 2003 / Accepted: 20 January 2004 / Published online: 19 February 2004
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Abstract Carbamates belonged to an older insecticide group, with propoxur being representative of this group. However, today carbamates with hormonal effects on insects, like fenoxycarb, or with fungicide properties, like propamocarb, are also used. The goal was a comparison of three structurally and functional different carbamates with a possibly common toxicological mechanism. Primary neuronal cell cultures of the rat are a well established model to identify neurotoxic compounds like *n*-hexane or acrylamide. In this cell culture model endpoints such as viability, energy supply, glucose consumption, glutathione (GSH) levels and cytoskeleton elements were determined. Added to cultured rat cortical neurons for 1 week, fenoxycarb, propamocarb and propoxur considerably decreased ATP levels, mitochondrial membrane potential and glucose consumption. Besides this, fenoxycarb and propamocarb had an impact on neurofilaments. After recovery for 1 week, propoxur also showed effects on neurofilaments, whereas with the other carbamates no tendency for a recovery was seen. These effects were prevented completely by pyruvate for propoxur and propamocarb, and partly so for fenoxycarb. In contrast to the main experimental design, GSH was determined after 1-h treatment with the test substances. Surprisingly, the compounds had only slight or no effect on the GSH level within this time. Further mechanistic studies indicated that carbamates primarily interacted with SH-groups, most likely by interfering with glycolysis and the construction of fibrillary proteins like neurofilaments. The prevention by pyruvate and acetylcysteine pointed to these biochemical endpoints.

Keywords Glycolysis · Cellular energy supply · Neurofilaments · Glutathione

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Introduction

Carbamates are an old group of insecticides, acting like organophosphates by inhibition of acetyl cholinesterase (AChE). The alkaloid physostigmine was the first representative of this group followed by insecticides like propoxur and others. In contrast to organophosphate insecticides, the inhibition of AChE is reversible and therefore the lethality is lower and the picture of a delayed organophosphate neuropathy is not present (Alvares 1989).

Nowadays, a several other classes of carbamates, like growth regulators (fenoxycarb, diflubenzuron) and herbicidal (metham, pebulate) or fungicidal (propamocarb) compounds are on the market. Here, two different insecticide compounds were used: propoxur (2-[1-methylethoxy]phenol methylcarbamate), a reversible inhibitor or the AChE, and fenoxycarb (2-[4-phenoxy]ethyl-ethylester carbamate), a juvenile hormone analogue (Holbrook et al. 2000; Liang and Schal 1994). As a consequence of an increased concentration of juvenile hormones in the corpora allata of adult insects, reproductive behaviour, mating efficacy and sexual receptivity decreased significantly in various insect species (Lin and Lee 1998). A third compound was chosen from the fungicide group. Propamocarb (3-[dimethylamino]propyl-propylcarbamate) is a fungicide affecting the biosynthesis of membrane constituents. As a consequence, unusual fatty acids accumulate (Burden et al. 1988; Langcake et al. 1983; Papavizas et al. 1978).

The neurotoxicity of thiocarbamates like disulfiram is well known, but some studies also pointed to a neurotoxic potential of other carbamates by influencing neurobehaviour or inducing delayed neuropathies, impairment of the electric activity of neurons, and changes in oxygen consumption and the ATPase system (Agawal et al. 1990; Babu et al. 1990; Dickoff et al. 1987; Miller 1982).

In order to further investigate these findings, biochemical endpoints were studied in vitro to elucidate

possible mechanisms for the described effects, which were not based on an inhibition of the AChE. Neuronal cells have an extensive energy demand since they have to maintain their complex architecture as well as energy-dependent ion gradients over their large cell surface (Canavagh 1984). One prominent example in this respect is 3-nitropropionic acid, an inhibitor of mitochondrial succinate dehydrogenase (Bossi et al. 1993).

This aspect was therefore addressed in various experiments using a well-established nerve cell culture system (Schmuck and Schlüter 1996; Schmuck et al. 2000). Parameters investigated were cell viability, glucose uptake, cellular ATP and reduced glutathione (GSH) levels, the potential across the inner mitochondrial membrane, and the amount of neurofilaments. For mechanistic purposes, supplementation of the cell culture medium with pyruvate, to bypass glycolysis, or with acetylcysteine, to protect SH groups, was done.

Material and methods

Chemicals

Fenoxycarb, propamocarb and propoxur were purchased from Riedl de Haen (Seelze, Germany), in a purity of 99%.

Cell culture of primary cells

The cortex was dissected from the whole brain tissue of fetal rats in the developmental stage E18–E19 and subsequently removed from the cerebral membrane. The tissues were pooled in sterile cultivation medium (Neurobasal medium; Invitrogen, Heidelberg, Germany) containing 10 ml B27 (Invitrogen). The isolation of individual cells from the cortex tissue was performed by filtration of the neuronal cells through two nylon meshes with different pore diameters (135 and 22 μm). The single cell suspension was centrifuged (500–700 g) and washed twice with culture medium. The cell pellet was then suspended in 10 ml culture medium and the cell number was counted by a cell analyzer system (Schärfe System, Reutlingen, Germany). The cells were transferred at a cell concentration of 5×10^5 to 1×10^6 cells per well to 24-well poly-D-lysine-coated cell culture plates (Biocoat; Becton and Dickinson, Heidelberg, Germany).

As neuronal cell culture generates a permanent neuronal network within 10 days, the test procedure was started at day 11. During this time, the cell composition did not change because the exclusion of serum inhibited glial growth. The cultures consisted of 90–95% neurons and 5–10% glia cells. These data were determined by immunohistochemistry (data not shown) using NSE (neuron specific enolase; Roche, Mannheim, Germany) and GFAP (glial fibrillary acid protein; Roche) antibodies. Microglia and oligodendrocytes were only seen in a minor amount (<1%) as determined by fluorescence-activated cell-sorter scan analysis using CD11/B/C antibody (1:600) (Pharmingen, Inc., San Diego, CA, USA) and the galactocerebroside antibody (Roche). These cultures are highly reproducible using the above-described, serum-free conditions. This was also documented by statistical analysis of the results.

Dosing of cell cultures

Fenoxycarb, propamocarb and propoxur were each dissolved in dimethyl sulfoxide (DMSO) and applied at 0.1, 1, 5, 10 or 50 $\mu\text{g}/\text{ml}$ to primary cortical neuronal cell cultures. DMSO concentration

did not exceed 0.1%, which induced no cytotoxicity (solvent control). The treatment period was 7 days. Evaluations were made 7 days after first dosing and after a treatment-free (recovery) period of 7 days. For mechanistic purposes, in some cases the neuronal cell culture medium was supplemented with 0.5 g/l pyruvate sodium or 0.5 g/l acetylcysteine.

Biochemical endpoints

Viability

Cells were washed twice with phosphate-buffered saline (PBS; Sigma, Deisenhofen, Germany) and subsequently incubated in a Calcein-AM/PBS solution (6 μM) (Molecular Probes, Eugene, OR, USA) for 30 min in a cell incubator. After rinsing with PBS, fluorescence was determined with a Fluostar fluorimeter (SLT, Crailsheim, Germany) at 485/530 nm.

Mitochondrial membrane potential

To determine mitochondrial membrane potential, tetramethylrhodamine was added to the culture medium at a concentration of 3.3 μM . Cells were incubated for 30 min, washed with PBS and fluorescence was determined at 550/575 nm.

Glucose consumption

Glucose was determined in cell culture medium. Glucose was determined colorimetrically in a Fluostar plus photometer (SLT) using the Trinter kit 315 (Sigma) with detection at 505 nm. Glucose consumption was estimated as the difference between glucose concentration in medium without (glucose total) and that with cells. The difference in the presence of control cells was defined as 100% uptake.

Intracellular ATP

For ATP determinations, a kit from Molecular Probes using the luciferase reaction was applied. The resultant chemiluminescence was measured in a Spectrafluor Plus luminometer (SLT).

Intracellular GSH

Intracellular GSH concentration was measured according to Mundy et al. (1997). The cells were washed with PBS and then treated with the indicated concentrations of compound for 1 h. Afterwards the cells were loaded with 5-methylchloro fluorescein diacetate (Molecular Probes) at a concentration of 20 μM in PBS for 1 h. Then, the developed fluorescence was determined in a Fluorostar spectrophotometer (SLT) at 485/530 nm.

Amount of neurofilaments

The cell culture plates were fixed in cold methanol (4°C) for 10 min and subsequently incubated for 1 h in 0.1% human albumin/PBS solution. Then, the cells were treated with a detergent (0.3% Triton X-100 in PBS) for 10 min before washing twice with PBS (plus 0.3% gelatin). The first antibody (neurofilament: mouse; Roche) was added for 2 h, and the second antibody (anti-mouse; Roche) for 1 h at 4°C. After removal of antibody 1 and 2, the plates were washed three times with PBS (plus 0.3% gelatin). The attached antibodies were exposed to peroxidase as an ABTS solution (Boehringer, Mannheim, Germany) for 30 min. The quantification of the attached antibodies was performed at 405 nm in an ELISA plate reader.

Statistical analysis

Cell culture assays were evaluated by (a) analysis of variance (ANOVA), and (b) *t*-test (Student-Newman-Keuls method) using the SigmaStat program (Jandel Scientific, Erkrath, Germany).

Results

A comparison among all three carbamates was made according to a semiquantitative ranking procedure (Table 1). For each carbamate, a slight reaction was defined as a no-observable-effect concentration (NOEC) of > 10 µg/ml and an EC₅₀ of > 50 µg/ml, a moderate or medium reaction as a NOEC of 5 µg/ml and an EC₅₀ of < 50 µg/ml, and a strong reaction as a NOEC of < 1 µg/ml and an EC₅₀ value of < 10 µg/ml. In the case where the scheme did not fit in both parameters, the EC₅₀ was ranked higher than the NOEC.

Fenoxycarb

A moderate cytotoxicity and effects on the inner mitochondrial membrane potential and the cytoskeleton were observed after treatment for 7 days, with a NOEC of 10 µg/ml and an EC₅₀ of 50 µg/ml fenoxycarb for cytotoxicity and mitochondrial membrane potential, respectively, and EC₅₀ of 10 µg/ml for the cytoskeleton effects (Fig. 1a). However, a strong reduction of the intracellular ATP level and glucose consumption was evidence that these were the most sensitive parameters, with a NOEC of 0.1 µg/ml and an EC₅₀ of 7.3 µg/ml for ATP, and a NOEC of < 0.1 µg/ml and an EC₅₀ of 9.2 µg/ml for glucose consumption. The addition of 0.5 g/l pyruvate prevented cytotoxicity and effects on mitochondrial membrane potential and the cytoskeleton completely, and on the other parameters partially (Fig. 1b).

During the recovery period fenoxycarb effects on cytotoxicity, mitochondrial membrane potential and cytoskeleton were enhanced, with NOEC levels being 5 µg/ml (Fig. 1c). The effect of intracellular ATP

concentration was nearly unchanged, but the effect on glucose consumption was increased, with a NOEC of < 0.1 µg/ml and an EC₅₀ of 0.45 µg/ml. However, similar to the situation after 7 days, pyruvate prevented completely cytotoxicity and the mitochondrial membrane potential changes, whereas effects on ATP content and glucose consumption were only partly reduced (Fig. 1d).

Propamocarb

The fungicide propamocarb had a slight cytotoxicity to cortical neurons and a moderate effect on the intracellular membrane potential, glucose consumption, ATP levels and the cytoskeleton (Fig. 2a). However, supplementation of the medium with pyruvate abolished the cytotoxicity and the effects on the cytoskeleton and reduced the effects on mitochondrial functions and the glucose consumption (Fig. 2b).

During the recovery period, effects on cytotoxicity were reduced and effects on mitochondrial functions became more obvious. The inner mitochondrial membrane potential and intracellular ATP levels were strongly reduced. Additionally, the glucose consumption was significantly reduced and neurofilaments destroyed (Fig. 2c). All of these effects were completely (cytotoxicity) or almost completely prevented by pyruvate (Fig. 2d).

Propoxur

Propoxur up to 50 µg/ml was not cytotoxic during the 7-day exposure period and had no influence on the neuronal cytoskeleton (Fig. 3a). However, propoxur showed a strong effect on energy-producing mechanisms represented by a decreased ATP production and glucose consumption, and a moderate effect mitochondrial inner membrane potential. This effect was completely reversible by a supplement of 0.5 g/l pyruvate to the cell culture medium (Fig. 3b).

Table 1 Effects of carbamates on cortical neurons in culture after a 7-day treatment period and after a recovery period of 7 days: + slight effect, NOEC (no-observable-effect concentration) > 10 µg/ml, EC₅₀ > 50 µg/ml; ++ medium/moderate effect, NOEC > 5 µg/ml, EC₅₀ < 50 µg/ml; +++ strong effect, NOEC < 1 µg/ml, EC₅₀ < 10 µg/ml; - no effect detected. EC₅₀ values are ranked higher

than NOEC. The last column summarizes the effect of supplementing the culture medium with pyruvate: ↓ reduction or reversibility of the effects, (↓) partly effective reversal with effects on glucose consumption (G), ATP or neurofilaments (N) still present

| Treatment | | Cytotoxicity | Mitochondrial functions | | Glucose consumption | Neurofilaments | Effect of pyruvate supplement |
|--------------------------------------|-------------|--------------|-------------------------|-----|---------------------|----------------|-------------------------------|
| Duration | Compound | | Rhodamine | ATP | | | |
| 7 Days exposure | Fenoxycarb | ++ | ++ | +++ | +++ | ++ | (↓) G, ATP |
| | Propamocarb | + | ++ | ++ | ++ | ++ | ↓ |
| | Propoxur | - | +++ | +++ | +++ | - | ↓ |
| 7 Days exposure + 7 days recovery | Fenoxycarb | +++ | +++ | +++ | +++ | +++ | (↓) ATP |
| | Propamocarb | + | +++ | +++ | ++ | +++ | ↓ |
| | Propoxur | + | + | +++ | +++ | ++ | (↓) G, N |

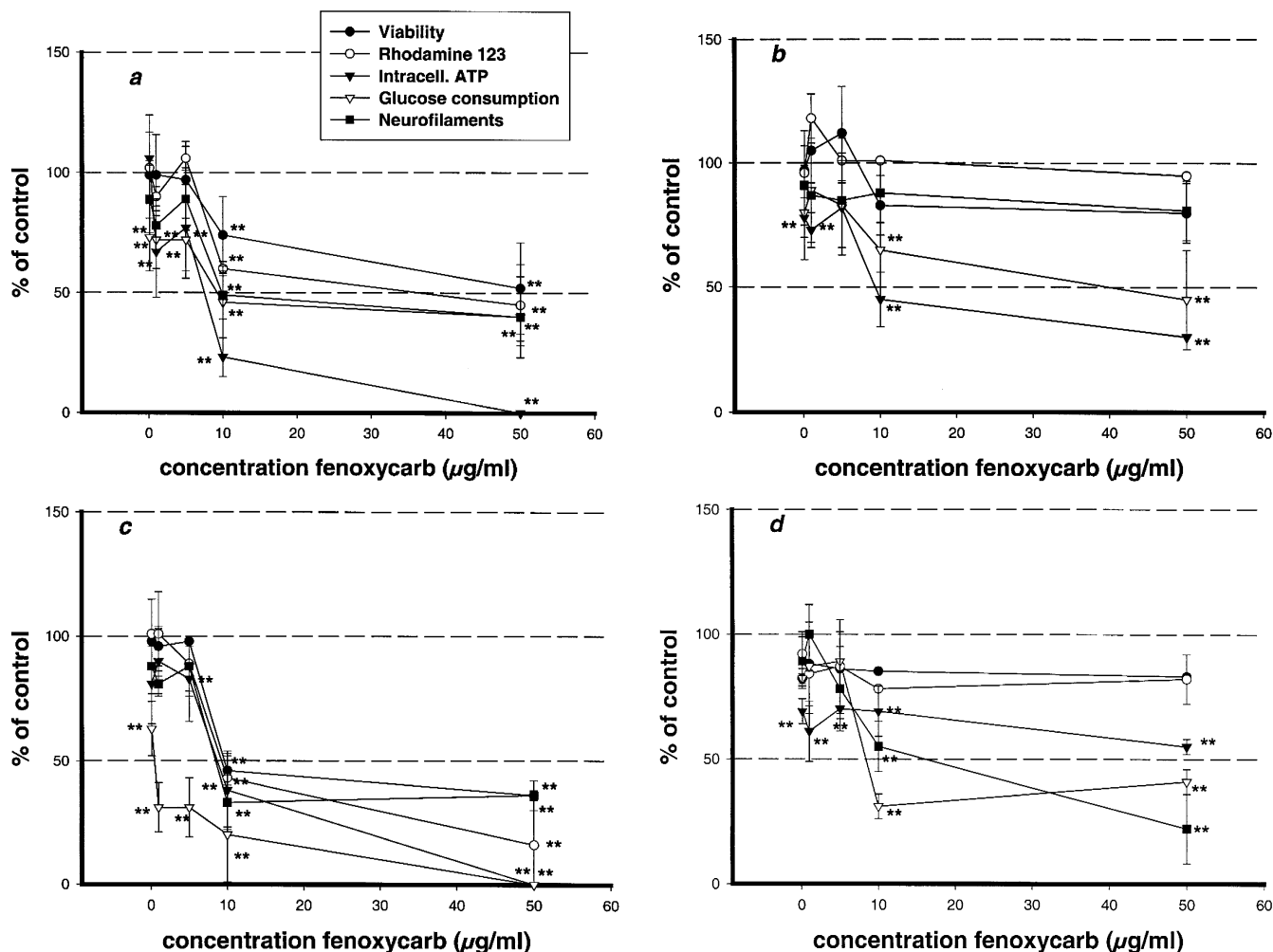


Fig. 1a–d Effects on fenoxycarb on viability, cellular-associated parameters and amount of neurofilaments. **a** Cultured neurons treated for 7 days. **b** Treatment for 7 days in cell culture medium supplemented with 0.5 g/l pyruvate. **c** Treatment for 7 days followed by a recovery period of 7 days, or **d** for 7 days followed by a recovery period of 7 days in cell culture medium supplemented with 0.5 g/l pyruvate. Mean values of three independent experiments are given. ** $P < 0.01$, indicating a significant difference from corresponding controls (one-way ANOVA followed by Student's *t*-test)

After a treatment-free period of 7 days, slight to moderate effects on cytotoxicity and cytoskeleton became obvious, while effects on mitochondrial function were decreased and glucose consumption was comparable with that during treatment (Fig. 3c). This development was only partly counteracted by pyruvate. Pyruvate was able to protect viability and mitochondrial functions but not the destruction of the cytoskeleton and the reduction of the glucose consumption (Fig. 3d).

Supplementation with acetylcysteine

The results from the three structurally different carbamates showed common effects on mitochondria and

glucose consumption, which were partly or completely inhibited by pyruvate. This excludes direct effects on mitochondrial functions comparable to those of KCN or paraquat (Schmuck et al. 2000) and pointed to indirect effects via biochemical pathways such as glycolysis. SH-groups may play a central role in this pathway as has been shown for *n*-hexane or acrylamide (Sabri et al. 1979a, 1979b).

To verify this hypothesis, mechanistic investigations were made on the intracellular GSH level and on the effect of supplementing the medium with acetylcysteine to protect SH-groups. The ATP level and glucose consumption were chosen as the most sensitive endpoints after treatment with these carbamates over 7 days. Table 2 shows clearly that acetylcysteine (0.5 g/l) strongly prevented the effects on ATP levels and on glucose consumption after treatment with all tested carbamates. However, the GSH level itself was not, or only slightly, decreased. An explanation may lay in the test procedure to assess GSH. Here, only a treatment period of up to 1 h is possible and this is obviously not sufficient for compounds like carbamates, which are possibly metabolized (Mundy et al. 1997). Therefore, a reaction of carbamates with SH-groups can clearly be shown over a treatment period of 7 days, but not after short-term treatment.

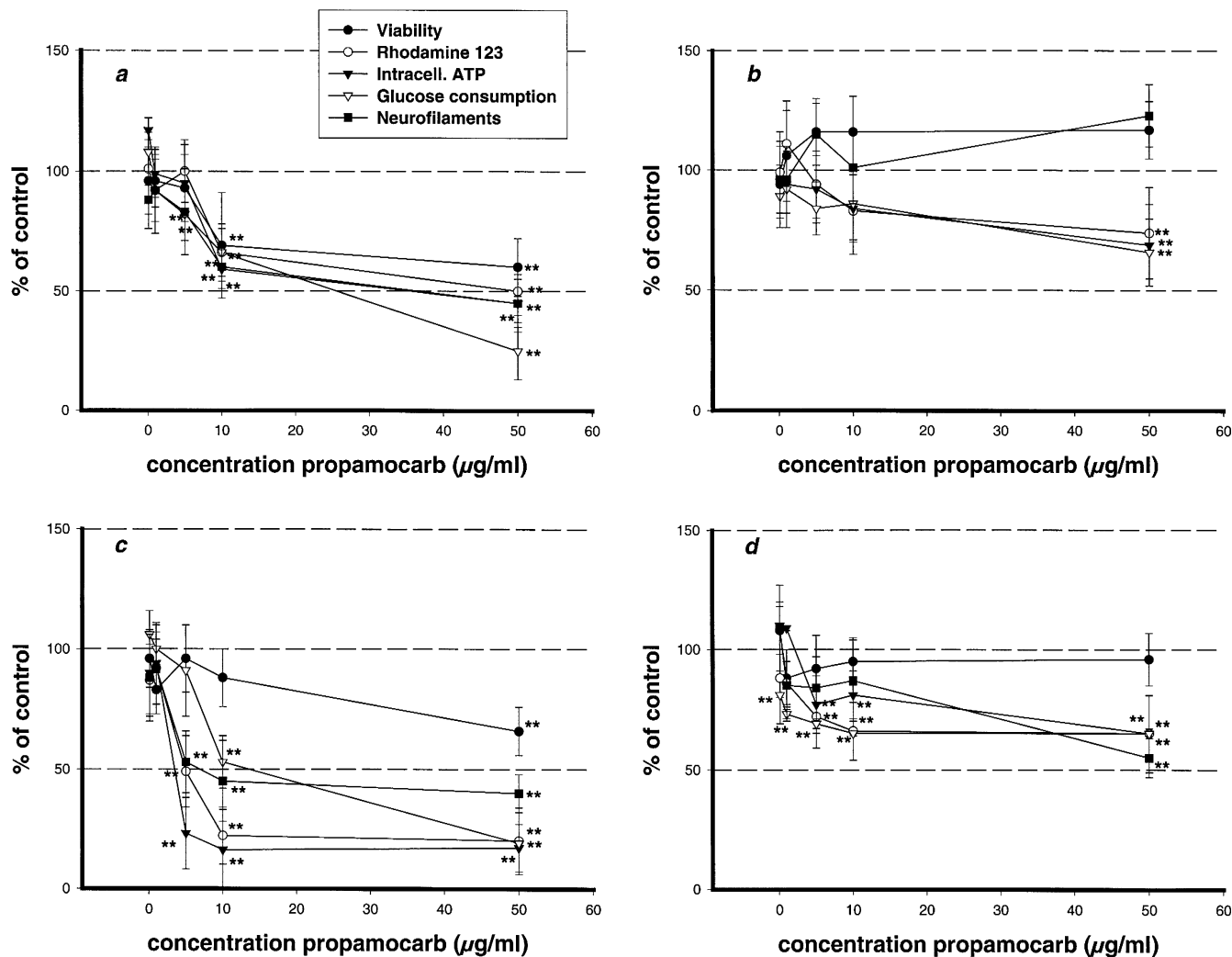


Fig. 2a–d Effects on propamocarb on viability, cellular-associated parameters and amount of neurofilaments. **a** Cultured neurons treated for 7 days. **b** Treatment for seven days in cell culture medium supplemented with 0.5 g/l pyruvate. **c** Treatment for seven days followed by a recovery period of seven days, or **d** for 7 days followed by a recovery period of 7 days in cell culture medium supplemented with 0.5 g/l pyruvate. Mean values of three independent experiments are given. ** $P < 0.01$, indicating a significant difference from corresponding controls (one-way ANOVA followed by Student's *t*-test)

Discussion

Cellular energy supply is the central motor for neuronal cells to proceed in managing all their highly specific functions, and this makes them extremely vulnerable to oxygen deprivation or cellular poisons like KCN or 3-nitropropionic acid (Schmuck et al. 2000). The power plant of the mitochondria itself is highly dependent on energy resources coming from biochemical pathways, such as glycolysis or the Krebs cycle, because neuronal cells use only the nutrient glucose for their survival. Well-known neurotoxicants like *n*-hexane, acrylamide and 3-nitropropionic acid have shown that the glycolysis

plays a central role in aggravating neuropathies (Sabri et al. 1979a, 1979b; Schmuck et al. 2000). The same effects on the energy supply were seen with disulfiram, a thiocarbamate, and carbon disulfide (Schmuck et al. 2002b).

Effects of carbamates on behaviour, drug response and the induction of polyneuropathies were not exclusively explained by the inhibition of the AChE. An influence on the neuropathy target esterase (NTE), comparable to that of organophosphates, could be excluded (Agawal et al. 1990; Dickoff et al. 1987; Miller 1982). However, biochemical work of Babu et al. (1990) showed in the brain of albino rats that propoxur affects oxygen consumption, ATPase systems and the movement of ions across ionic pumps—all energy-dependent processes.

Investigations with three structurally and functionally different carbamates—fenoxycarb and propoxur, both insecticides, and propamocarb, a fungicide—showed, in common, that basic mitochondrial functions and glucose consumption were influenced by these compounds. The effects could be counteracted by pyruvate, the end product of the glycolysis. A recovery

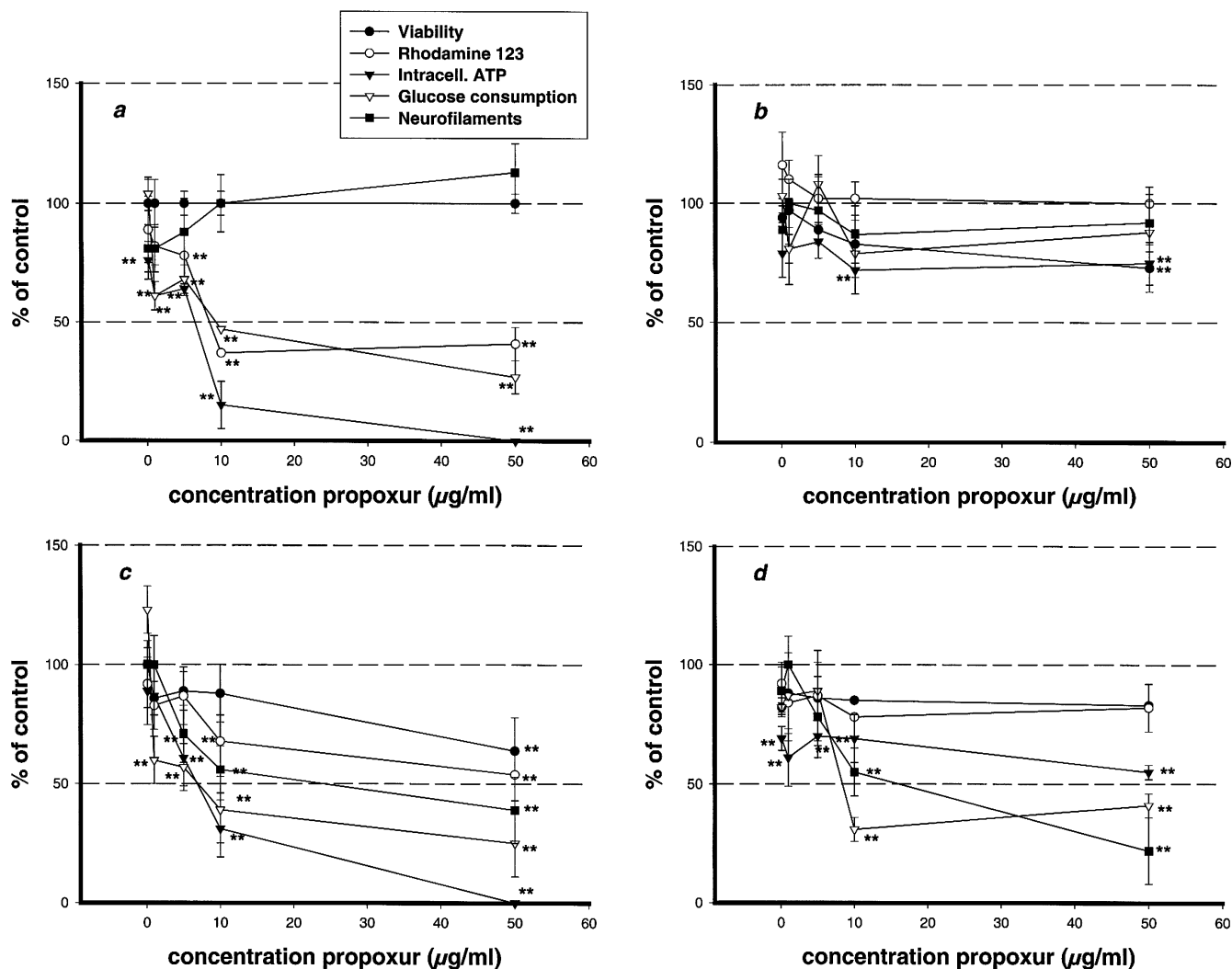


Fig. 3a–d Effects on propoxur on viability, cellular-associated parameters and amount of neurofilaments. **a** Cultured neurons treated for seven days. **b** Treatment for 7 days in cell culture medium supplemented with 0.5 g/l pyruvate **c** Treatment for 7 days followed by a recovery period of 7 days, or **d** for 7 days followed by a recovery period of 7 days in cell culture medium supplemented with 0.5 g/l pyruvate. Mean values of three independent experiments are given. ** $P < 0.01$, indicating a significant difference from corresponding controls at (one-way ANOVA followed by Student's *t*-test)

period of 7 days increased these effects and additionally increased cytotoxicity and effects on the cytoskeleton, a marker for neuropathy events. The three carbamates differ in their cytotoxicity; propoxur is non-cytotoxic up to a concentration of 50 µg/ml, whereas propamocarb and fenoxycarb were slightly to moderately cytotoxic after a 7-day treatment period. This may be the reason for their moderate effects on cellular targets like mitochondria, glucose consumption and the cytoskeleton. Propoxur showed no effects on the cytoskeleton after 7 days, in contrast to the other carbamates, and had only moderate effects after the recovery period. Therefore, these carbamates may be compared in principal to be very weak mitochondrial inhibitors, like KCN or

paraquat, or effectors on biochemical pathways, like 3-nitropropionic acid, acrylamide or *n*-hexane. However, the protection against neurotoxicity by supplementation of pyruvate to the culture medium strongly argues against a direct effect on mitochondrial respiration as the primary reason for the impaired energy supply of nerve cells. This view is further substantiated by the finding that pyruvate was unable to ameliorate effects of the respiratory chain inhibitor KCN in neurons, which are characterized by initial rapid and selective decrease of ATP levels and mitochondrial membrane potential (results not shown), but was able to abolish carbamate-induced changes (Figs. 1b,2b,3b).

However, a broad screening program with different compounds exhibiting delayed neurotoxicity in this cell culture model documents that all these chemicals can be grouped into several classes (Schmuck et al. 2000). There were compounds, such as organophosphates, IDPN (β -iminodipropionitrile), acrylamide, 2,5-hexandione or paraquat, that act directly on the cytoskeleton by different mechanisms. Others like KCN or 3-nitropropionic acid, which selectively disrupt the balance between energy supply and glycolysis, were indirectly neurotoxic

Table 2 Influence of 0.5 g/l acetylcysteine in the cell culture medium on compound-related effects of fenoxycarb, propamocarb and propoxur. Effects of glucose consumption and the intracellular ATP concentration in cortical neuronal cell cultures were deter-

| Test parameter | Fenoxycarb | | Propamocarb | | Propoxur | |
|---------------------|----------------|----|-------------|----|----------|----|
| | Acetylcysteine | | | | | |
| | - | + | - | + | - | + |
| ATP | 1 | 50 | 0.1 | 5 | 1 | 10 |
| Glucose consumption | 1 | 10 | 5 | 5 | 0.1 | 10 |
| Glutathione | 10 | 10 | 5 | 10 | 10 | 10 |

mined after a 7-day exposure period. The reduction of the glutathione (GSH) content was measured 1 h after treatment. The data are NOEC (no-observable-effect concentration, µg/ml) values

as a consequence of energy depletion. The current investigations with the three carbamates pointed to a similar mechanism. They all primarily inhibited ATP production and had moderate or strong effects on glucose consumption. This did not change after the recovery period of 7 days. This is different from the actions of KCN and 3-nitropropionic acid, where energy processes were reversible after the recovery period (Schmuck et al. 2000). These differences and the finding that pyruvate abolished nearly all toxic influences of the carbamates pointed on a mechanism in the glycolysis process.

Taken together, the observed reduction of glucose uptake and markedly decreased intracellular energy equivalents strongly suggest that carbamates inhibited glucose utilization at the level of glycolysis. The further metabolism of pyruvate via the citric acid cycle and respiratory chain seemed to be unaffected because the inner mitochondrial membrane potential is only moderately inhibited in contrast to that induced by KCN, where the breakdown of the membrane potential parallels the ATP loss. The evolving deficit of pyruvate and consequent reduction of reducing equivalents for the respiratory chain will then result in a decrease of ATP production.

A second difference from KCN and 3-nitropropionic acid is a direct effect on the cytoskeleton by fenoxycarb and propamocarb. A combination of action on both targets—energy supply and cytoskeleton—was seen with paraquat, *n*-hexane and acrylamide, for which a second mechanism may be postulated. In the case of paraquat, oxidative stress was identified (Schmuck et al. 2002a).

For *n*-hexane and acrylamide, different mechanisms were postulated starting with the production of cross-links of the neurofilaments with 2,5-hexandione, the active metabolite of *n*-hexane, or with an accumulation of the light neurofilament chains by acrylamide (Abou-Donia and Gupta 1994). A second plausible explanation for acrylamide and *n*-hexane neurotoxicity was given by Sabri and coworkers (Sabri 1994; Sabri et al. 1979a, 1979b) and Lopachin and Lehning (1997), who showed that both compounds interact with enzymes of the glycolysis carrying SH-groups at their active centre. The amelioration of carbamate-induced effects on ATP production and glucose consumption by acetylcysteine may be related to a comparable mechanism. However, a

reaction with SH-groups should be a slow process and may include metabolic degradation of the test compounds because GSH itself is not degraded within 1 h. This is the case with compounds inducing oxidative stress like paraquat and hydrogen peroxide, and therefore this alternative mechanism can also be excluded for carbamates (Röhrdanz et al. 2001; Schmuck et al. 2002b).

It was shown that the one toxic event of these tested carbamates may be an impairment of the energy state of the neurons, most likely caused by an inhibition of the glycolysis. Additionally, other cellular targets carrying SH-groups like fibrillary proteins may also be affected. However, the direct impact of these carbamates on the energy status in vivo is probably small because lesions occurred only in long-term studies using high dosages of the compounds.

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