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Bromophenols, both present in marine organisms and in industrial flame retardants, disturb cellular Ca^{2+} signaling in neuroendocrine cells (PC12)

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Abstract

Bromophenols are present in polychaetes as well as in algae in marine environments including the North Sea. They are thought to cause the typical sea-like taste and flavour. The ecological function of brominated phenols is not clear yet, but they may play a role in chemical defence and deterrence [Kicklighter, C.E., Kubaneck, J., Hay, M.E., 2004. Do brominated natural products defend marine worms from consumers? Some do, most don't. Limnol. Oceanogr. 49, 430–441]. Some brominated phenols are commercially used as industrial flame retardants as, e.g., 2,4,6-tribromophenol and are suspected to disrupt the humoral system by showing tyroid hormone-like activity [Legler, I., Brouwer, A., 2003. Are brominated flame retardants endocrine disruptors? Environ. Int. 29, 879–885]. In this study 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,4-dibromophenol (2,4-DBP), 2,6-dibromophenol (2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP), all of which are present in marine organisms, were tested. Especially 2,4-DBP and 2,4,6-TBP showed a significant effect on the Ca^{2+} homeostasis in endocrine cells (PC 12). The reduction of depolarization induced Ca^{2+} elevations by 2,4-DBP and 2,4,6-TBP and the increase of intracellular Ca^{2+} by both substances, partly released from intracellular stores, may suggest a link to the disrupting effect of endocrine systems by brominated phenols. 2,4-DBP was the most potent substance we tested in respect to inhibition of voltage dependent Ca^{2+} currents as revealed in whole cell patch clamp experiments. Brominated phenols disturb cellular Ca^{2+} signaling with differential efficacy, depending on the number and position of bromine.

Keywords: Calcium signaling; Neurotoxicology; Flame retardant; Algae; 2,4-Dibromophenol; 2,4,6-Tribromophenol

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1. Introduction

Bromophenols are present in marine organisms and are thought to cause the typical sea-like taste and flavour (Kotterman et al., 2003; Chung et al., 2003a). Several bromophenols are present in marine polychaetes as well as in algae like, e.g., the brown macroalgae *Sargassum siliquastrum, Padina arborescens* and *Lobophora variegata* containing high concentrations up to 7000 ng/g (2,4-DBP 1280 ng/g; 2,4,6-TBP 5870 ng/g) (Chung et al., 2003b). Bromophenols including 2,4,6-TBP are found additionally in mussels and in blubber of mammals (Vetter and Janussen, 2005). The ecological role of brominated phenols is not clear yet. However, they may play a role in chemical defence and deterrence (Woodin et al., 1997; Kicklighter et al., 2004). Some brominated phenols, e.g., 2,4,6-TBP are also commercially used and industrially produced as flame retardants and as wood preservatives/fungicide. 2,4,6-TBP represents a high volume flame retardant with a worldwide production of 9500 t/year in 2001 (IUCLID, 2003). It is suspected to disrupt the humoral system by showing thyroid hormone like activity (Legler and Brouwer, 2003), whereas 2,4-DBP (weakly 2,4,6-TBP) binds to the human estrogen receptor (Olsen et al., 2002). In human milk 2,4,6-TBP is found in concentrations of 110 ng/g lipid (Ohta et al., 2004). 2,4,6-TBP is in addition to its synthesis generated by photolytic degradation of tetrabromobisphenol A (Eriksson and Jakobsson, 1998), which is the most abundant brominated flame retardant. Even in vineyards, 2,4,6-TBP is found in vine and cellars probably via treated wooden barrels (Chatonnet et al., 2004).

The potency of bromophenols to interact with the humoral system has led us to the question, if the naturally occurring as well as industrially produced bromophenols interact with basic signal pathways, directly linked to exocytosis and secretion of hormones. We chose endocrine cells, which are part of the nervous system but not protected by a blood brain barrier, and focussed on interactions of the brominated phenols 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP (naturally occurring and of industrial origin) with cellular Ca^{2+} signals. Ca^{2+} functions as an ubiquitous cellular second messenger mediating hormonal release. We chose the rat cell line PC12 from a phaeochromocytoma of the adrenal gland, which produces and secretes noradrenalin as well as dopamine (Greene and Tischler, 1976). Cells of the adrenal gland have been used previously for many toxicological and pharmacological studies, e.g., PCBs (Messeri et al., 1997; Westerink and Vijverberg, 2002a) heavy metals (Weinsberg et al., 1995; Westerink and Vijverberg, 2002b) and alkaloids (Lee and Kim, 1996; Gafni et al., 1997; Bickmeyer et al., 1998, 2004; Kim et al., 2001; Smith et al., 2002). The aim of the present study was to investigate the interactions of naturally as well as industrially important chemicals, which are suspected to interact with the humoral system (Legler and Brouwer, 2003), with cellular signaling. This approach may additionally in part elucidate one possible role of brominated phenols in marine organisms.

2. Materials and methods

PC 12 cells from the DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany) were kept in culture medium containing RPMI 1640, 10% fetal calf serum, 5% horse serum, and 100 units penicillin/streptomycin per ml. Cells were cultivated in an incubator at 37 °C, 90% humidity and 5% CO_2. Cells grew on collagen coated cover slips and/or in collagen coated dishes. Medium was changed every three days and the cells were subcultured when necessary.

2.1. Fluorometric measurements of intracellular Ca^{2+} levels

Cells were incubated with buffer (in mM: 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.3 NaH_2PO_4, 30 Glucose, 26 Na HEPES) containing 5 μM Fura II acetoxymethylester for 30 min at room temperature (22 ± 2 °C). The incubation buffer was removed and cells were washed for 20 min. Fluorescence of cells was monitored by an imaging system (Visitron, Puchheim, Germany) with a CCD camera mounted on an inverted microscope (Zeiss Axiovert 100). About 30 PC12 cells were simultaneously measured, separated using “the region of interest” function of the software (Metafluor, Meta Imaging Series). Fluorescence was obtained through an UV objective (Zeiss NeoFluar 20X). Data were obtained from division of two images, one obtained at 340 nm, the other at 380 nm

excitation. For determination of R_{min} and R_{max}, Fura II loaded cells were permeabilised with 10 μM ionomycin. Ca^{2+} buffer was used for determination of R_{max} and buffer without $CaCl_2$ but with additional 10 mM EGTA for determination of R_{min} in calibration experiments. In Figs. 2–4 ratio values are presented on the *y*-axis; the calcium concentration changes are indicated for a relevant area as space bar. Fluorescence ratios were converted into Ca^{2+} concentrations by the formula according to Grynkiewicz et al. (1985). All experiments were carried out at day one or two after plating of cells in collagen coated dishes (30 mm).

2.2. Experimental design

The recording chamber mounted on an inverted microscope had a volume of 2 ml and the pumping speed of the peristaltic pump was adjusted to 3 ml/min exchanging the chamber volume in less than a minute. To depolarize the cells, 80 mM KCl was used (instead of 80 mM NaCl) in the experimental buffer. The depolarization of the cellular membrane potential therefore increased gradually in less than a minute during perfusion. Cells were depolarized three times for 1 min during the course of a single experiment. Usually about 30 cells were measured simultaneously. In another set of experiments cells were exposed to Ca^{2+} free buffer ($CaCl_2$ removed and EGTA added) followed by application of bromophenols and subsequent re-supplementation of Ca^{2+} leading to store operated Ca^{2+} entry.

Results are presented as mean ± S.E.M. unless otherwise stated. Statistics (ANOVA) and calculations were performed using computer software Prism (Graphpad) and Igor (WaveMetrics).

2.3. Whole cell voltage clamp experiments

Recordings were done using the EPC-7 patch clamp amplifier (List electronics) and analyzed with the computer program Signal 2 (CED). All experiments were carried out at day one or two after plating of cells in collagen coated dishes (30 mm). The bath solution contained: 135 mM tetraethylammonium-chloride (TEA-C1), 10 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethane-sulfonicacid (HEPES), 1.2 mM $MgCl_2$, 10 mM $BaCl_2$, 2 μM tetrodotoxin (TTX) (pH was adjusted to 7.2 with TEA-OH). Currents through Ca^{2+} channels were recorded with patch pipettes of roughly 5 MΩ resistance. The pipette solution comprised of 135 mM CsCl, 10 mM HEPES, 10 mM ethyleneglycol-bis-(2-aminoethylether)-*N*,*N*,*N*′-tetraacetic acid (EGTA), 2 mM $MgCl_2$, 2 mM Na_2-ATP (adjusted to pH 7.2 with TEA-OH). Ca^{2+} channel currents were evoked from a holding potential of −70 to +10 mV for 200 ms every 30 s or by increasing voltage steps (+10 mV) starting from −90 mV up to +60 mV for current-voltage (IV) relationship.

2.4. Substances

All chemicals were obtained from Sigma–Aldrich, Merck, Fluka and Molecular Probes.

3. Results

3.1. Concentration–effect relationship of K^+ induced Ca^{2+} elevations by 2-BP, 2,6-DBP, 4-BP, 2,4-DBP and 2,4,6-TBP

From the large group of brominated phenols we chose five molecules, found in relatively high concentrations in marine organisms (Fig. 1) and looked for interactions with the cellular Ca^{2+} homeostasis. The most abundant compounds are 2,4-DBP, 2,6-DBP and 2,4,6-TBP as shown by Whitfield et al. (1999) and Kotterman et al. (2003).

Initially, the experimental series was started with 2-BP, which showed no significant effect on K^+ induced Ca^{2+} elevations in the concentrations of 30

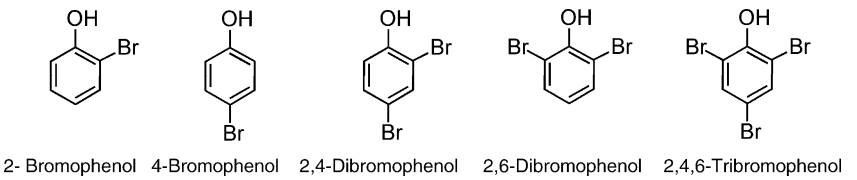


Fig. 1. Chemical structure of used compounds.

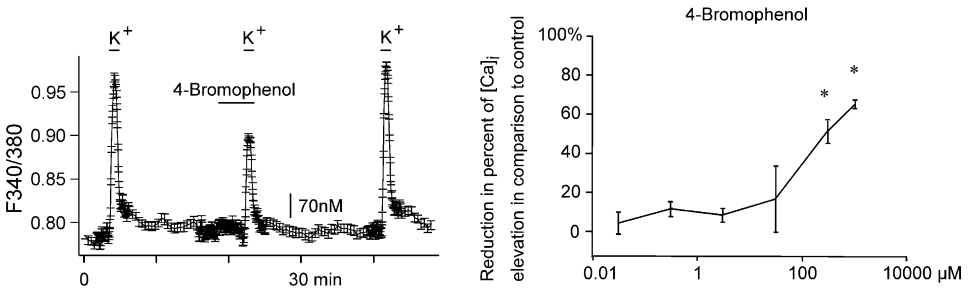


Fig. 2. (Left) Changes of the ratio F340/380 nm which represent intracellular Ca^{2+} changes following depolarization with high potassium (K^+) in the presence of 300 μM 4-BP. Averaged trace of about 30 cells measured simultaneously, space bars represent S.E.M. (Right) The dose–response relationship between 4-BP and intracellular Ca^{2+}, calculated from ≥3 independent experiments for each concentration. Asterisks indicates statistical significance ($p < 0{,}001$, ANOVA).

and 300 μM, nor did the substance itself induce any change in basal free intracellular Ca^{2+} concentration in the applied concentrations (data not shown).

2,6-DBP also showed no effect on intracellular Ca^{2+} concentration, neither during K^+ induced Ca^{2+} signals nor without at concentrations of 30 and 300 μM. An additional bromination of 2-BP in 6-position was not effective in respect to disturbance of Ca^{2+} signaling (data not shown). A bromination at the 4-position (4-BP) showed a reduction of depolarization induced Ca^{2+} elevations in concentrations of 300 μM and higher. The position of the bromine clearly affected the potency of the substance to disturb cellular Ca^{2+} signals (Fig. 2).

2,4-DBP reduced K^+ induced Ca^{2+} elevations significantly at a concentration of 30 μM ($p < 0{,}001$, ANOVA), but showed no clear effect on intracellular Ca^{2+} levels without stimulation. 300 μM eliminated K^+ induced Ca^{2+} elevations and elevated intracellular Ca^{2+} levels, before stimulation with high K^+. There was neither a significant effect of 3 μM 2,4-DBP on K^+ induced Ca^{2+} elevations nor on basal intracellular Ca^{2+} concentrations (Fig. 3). Additional bromination of 4-BP in 2-position showed a clear increase of efficacy to disturb cellular Ca^{2+} signaling in comparison to 4-BP.

The substance 2,4,6-TBP reduced K^+ induced Ca^{2+} influx in the concentration of 300 μM significantly.

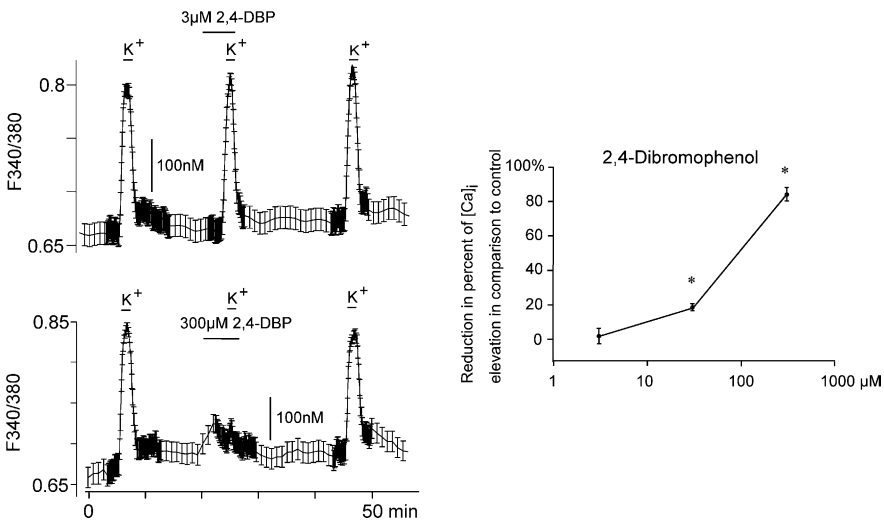


Fig. 3. (Left) Changes of the ratio F340/380 nm which represent intracellular Ca^{2+} changes following depolarization with high potassium (K^+) in the presence of 3 μM (upper drawing) and 300 μM (lower drawing) 2,4-DBP. (Right) Reduction of Ca^{2+} elevation in % of controls by 2,4-DBP. Asterisks indicate statistical significance ($p < 0{,}001$, ANOVA).

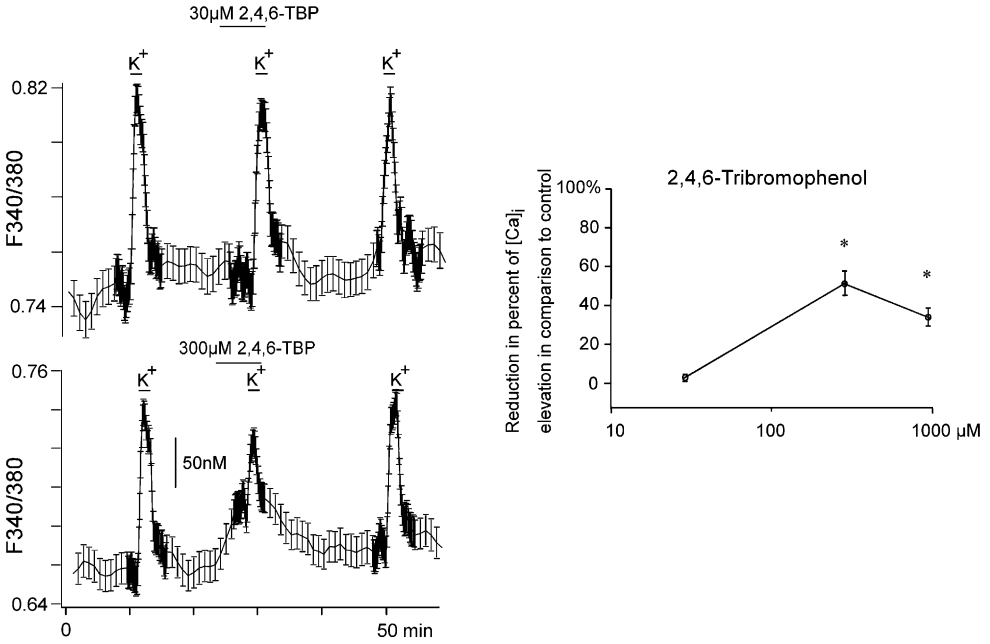


Fig. 4. (Left) Changes of the ratio F340/380 nm which represent intracellular Ca^{2+} changes following depolarization with high potassium (K^+) in the presence of 30 μM (upper drawing) and 300 μM (lower drawing) 2,4,6-TBP. (Right) Reduction of Ca^{2+} elevation in % of controls by 2,4,6-TBP. Asterisks indicate statistical significance ($p < 0{,}001$, ANOVA).

Fig. 4 demonstrates the reduction of depolarization induced Ca^{2+} elevation by 2,4,6-TBP and clearly demonstrates, that 2,4,6-TBP itself increases intracellular Ca^{2+} levels at a concentration of 300 μM.

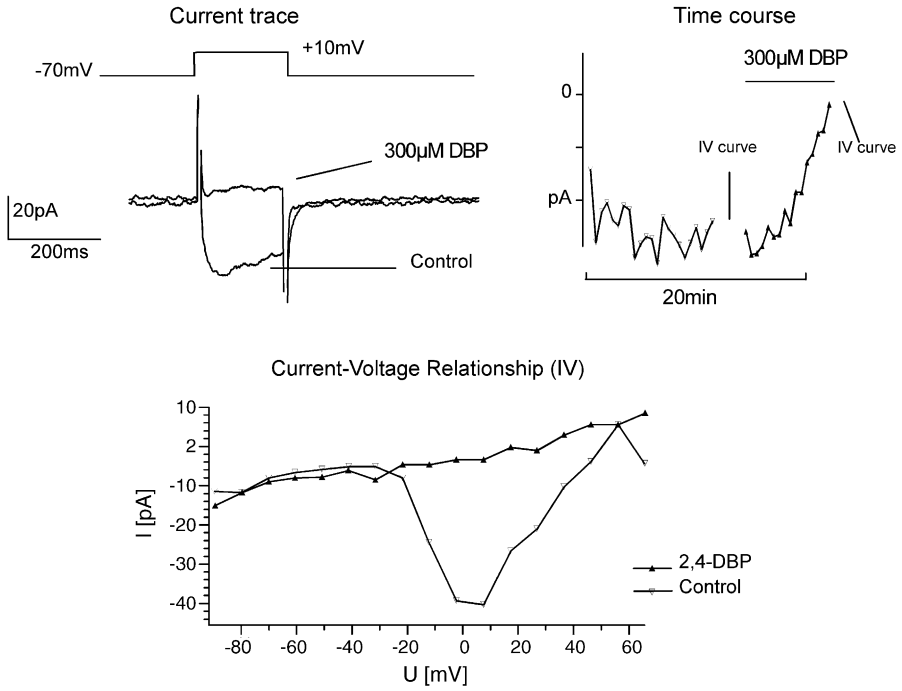
3.2. 2,4-DBP and 2,4,6-TBP increase intracellular Ca^{2+} concentrations partly by a release from intracellular stores

An elevation of intracellular Ca^{2+} levels was observed using 2,4,6-TBP and 2,4-DBP (Fig. 4) and may be caused by three possibilities: (1) The drug depolarises the cell by an unspecific membrane permeabilisation (a possible Ca^{2+} entry path). (2) The substance itself increases the open probability of voltage operated or other Ca^{2+} entry channels. (3) Intracellular calcium stores like mitochondria or the endoplasmic reticulum release Ca^{2+} into the cytosol after application of chemicals- or the reuptake into the stores is inhibited. Both mechanisms would lead to a store operated Ca^{2+} entry from the extracellular solution.

These three possibilities were investigated in the following experiments. Whole cell patch clamp experiments were performed to address (1) and (2).

Point (1): after application of the drug, no increase of a "leakage current" at potentials of −80 to −30 mV was observed. An unspecific permeabilisation of the cell membrane by 2,4-DBP could therefore be excluded. Point (2): we used a pipette solution, which prevents a "run down" of Ca^{2+} currents (Bickmeyer et al., 1993). Current through voltage operated Ca^{2+} channels was measured using Ba^{2+} as a charge carrier to increase current amplitudes and to prevent from Ca^{2+} induced inactivation of Ca^{2+} channels. Fig. 5 shows a current-voltage relationship (IV) with and without 2,4-DBP. 2,4-DBP inhibited voltage dependent Ba^{2+} currents through Ca^{2+} channels in a dose dependent manner.

After establishment of the whole cell configuration there was an increase of voltage dependent Ba^{2+} currents through Ca^{2+} channels for 3 min (−70 to +10 mV, 200 ms). This process was dependent on used pipette solution (Bickmeyer et al., 1993). Following this initial phase there was a spell of time of minimum 30 min with a stable condition. Indeed, most control experiments revealed 60 min of a "steady state" (data not shown).



2,4-Dibromophenol	Inward currents in comparsion to control (-70mV-+10mV)	
0µM	100%	
30µM	79 ± 7 %	p<0.005
300µM	9,9 ± 3,5%	p<0.001

Fig. 5. Original traces (left) of whole cell patch clamp recordings, time course (right) and current–voltage relationship (IV) (bottom) of voltage operated Ca^{2+} channel currents with and without 300 µM 2,4-DBP. Table: normalized current amplitudes (mean ± S.E.M.), elicited by voltage steps from −70 to +10 mV.

The application of 2,4-DBP was 15 min after establishment of the whole cell configuration and the time course of current amplitudes is shown in Fig. 5. 2,4-DBP inhibited inward currents maximally after about 5 min of exposure, but induced no unspecific membrane permeabilisation. This result corroborates the results of the inhibition of voltage dependent calcium entry by 2,4-DBP shown by fluorometric measurements.

To address point 3 above, we performed fluorometric measurements of intracellular Ca^{2+} levels. After removal of Ca^{2+} from the extracellular solution, a substance-induced cytosolic Ca^{2+} elevation is caused by Ca^{2+} release from intracellular stores. 2,4-DBP (300 µM) and 2,4,6-TBP (data not shown) elevated intracellular Ca^{2+} levels in the absence of extracellular Ca^{2+}. After re-supplementation of Ca^{2+} to the extracellular solution a store operated Ca^{2+} entry can be measured (Fig. 6).

This demonstrates that one reason of induced Ca^{2+} elevation by 2,4-DBP and 2,4,6-TBP is the release of Ca^{2+} from intracellular stores.

4. Discussion

Bromophenols are found in very high concentrations in sponges and algae like the brown macroalgae *S. siliquastrum* and *P. arborescens* (Chung et al., 2003b). Most but not all brominated metabolites are believed to be palatable to predators and they are not effective antibacterial agents either (Kicklighter et al., 2004). Interestingly some polychaetes (*Notomastus lobatus,*

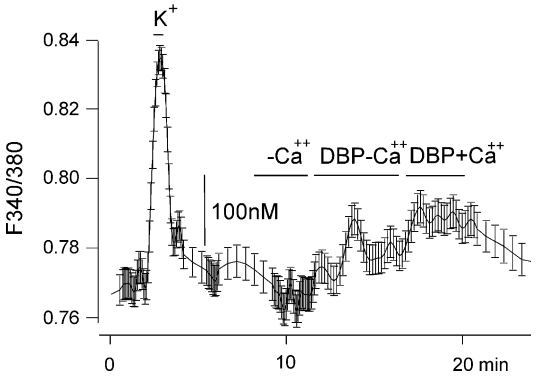


Fig. 6. Changes of the ratio F340/380 nm, which represent intracellular calcium changes following depolarization with high potassium (K^+), removal of Ca^{2+} from the extracellular ($-Ca^{++}$), addition of 300 μM 2,4-DBP to the Ca^{2+} free solution (DBP − Ca^{++}) and resupplementation of Ca^{2+} to the buffer (DBP + Ca^{2+}).

Thelepus crispus) accumulate and contaminate sediments with bromophenols, which influence benthic communities and therefore ecosystems (Lincoln et al., 2005). Some structurally identical bromophenols are used industrially as flame retardants and fungicides and are suspected to disrupt the humoral system (Olsen et al., 2002; Legler and Brouwer, 2003). For instance 2,4,6-TBP impairs cell differentiation and proliferation and induces neuronal cell differentiation (Ríos et al., 2003) in a human neuroblastoma cell line (SH-SY5Y). 2,4-DBP has been described as endocrine disruptor, which binds to the estrogen receptor (Olsen et al., 2002). We demonstrate that especially 2,4-DBP and 2,4,6-TBP show another mechanism of action, namely a disturbance of the cellular Ca^{2+} homeostasis in endocrine cells.

Secretion of hormones is controlled by Ca^{2+} influx into the cells. The reduction of depolarisation induced Ca^{2+} elevation may be a link to the disrupting effect of endocrine systems by bromophenols. Direct proof, that voltage dependent Ca^{2+} entry is inhibited by 2,4-DBP is shown in whole cell patch clamp experiments. 2,4-DBP and 2,4,6-TBP obviously not only inhibit voltage dependent Ca^{2+} entry but induce an elevation of intracellular Ca^{2+} levels in the absence of extracellular Ca^{2+}, indicating Ca^{2+} release from intracellular calcium stores. Intracellular stores like mitochondria or the endoplasmic reticulum may release Ca^{2+} into the cytosol or the reuptake into the stores is inhibited by bromophenols. Both mechanisms induce a store operated Ca^{2+} entry from the extracellular solution. Since 2,4-DBP induces intracellular Ca^{2+} release, a reduction of voltage dependent Ca^{2+} entry may partly be caused by Ca^{2+} dependent inactivation of calcium channels. Therefore we used barium ions as charge carrier in patch clamp experiments, minimizing the effect of Ca^{2+} dependent inactivation of voltage operated Ca^{2+} channels, but nevertheless, voltage operated currents were reduced by 2,4-DBP.

2,4-DBP was the most potent substance we tested. Interestingly 2-BP and 2,6-DBP were least potent. Not only the grade of bromination, but the position of the bromine substituents, especially bromination in the 4-position in conjunction with the 2-position, seemed to be responsible for the efficacy of the drug to disturb Ca^{2+} signaling. In 2,4,6-TBP, the additional bromination in the 6-position seemed to increase intracellular calcium elevations without other stimuli. 4-BP as well as 2-BP and pentabromophenol have been shown to be nephrotoxic (Lau et al., 1984; Rush et al., 1984; Bruchajzer et al., 2002; Szymanska et al., 1995) in the context of our results possibly in part by interaction with cellular calcium stores. Bradykinin, which via G-proteins increases intracellular Ca^{2+}, plays an important role in the kidneys regulating fluid and electrolyte transport (Hébert et al., 2005).

Brominated phenols are suspected to interact with the humoral system of man and mice; we show a clear disruption of cellular calcium signaling, which is the trigger for transmitter and hormonal release from glands and synapses and plays important roles in electrolyte transport in kidney. In respect to a related experimental approach using brominated pyrrole alkaloids from marine *Agelas* sponges (Bickmeyer et al., 2004; Bickmeyer, 2005) some brominated marine metabolites, depending on number and position of bromine, seem to interact with cellular Ca^{2+} signaling. The naturally produced bromophenols may serve their producers as antifeedant or allelochemicals by bad taste as well as by disturbance of developmental processes. 2,4,6-TBP inhibits the development of copepods in the low micromolar concentration range (Wollenberger et al., 2005), possibly by alterations of cellular calcium signaling. The antifeedant activity may be caused by the intensive odour, which is probably as effective in water as in air, leading to, e.g., musty and corked odours in wine (Chatonnet et al., 2004).

2,4,6-TBP is found in blubber of marine mammals (Vetter and Janussen, 2005), in human milk (Ohta et al., 2004), is produced as flame retardant as well as fungicide and is (from natural sources) found in high concentrations in marine algae (Kicklighter et al., 2004). Additionally bromophenol-rich feed can be used in aquaculture to enhance the sea-like flavour of fish and other seafood (Ma et al., 2005). The most abundant flame retardant tetrabromobisphenol A is additionally a major source of 2,4,6-TBP by photolytic degradation (Eriksson and Jakobsson, 1998). Therefore bromophenols such as 2,4,6-TBP should be in the focus of ecological as well as toxicological studies to increase awareness of their impact on human and wildlife safety.

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