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The marine secondary metabolites 2,4-dibromophenol and 2,4,6-tribromophenol differentially modulate voltage dependent ion currents in neuroendocrine (PC12) cells

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Abstract

2,4-Dibromophenol (2,4-DBP) and 2,4,6-tribromophenol (2,4,6-TBP) are marine secondary metabolites, with 2,4,6-tribromophenol playing an important role as industrially produced flame retardant and pesticide. Both substances disturb cellular calcium signals in neuroendocrine cells as previously shown by Hassenklöver et al. (2006) [Hassenklöver, T., Predehl, S., Pilli, J., Ledwolorz, J., Assmann, M., Bickmeyer, U., 2006. Bromophenols, both present in marine organisms and in industrial flame retardants, disturb cellular Ca^{2+} signaling in neuroendocrine cells (PC12). Aquat. Toxicol. 76, 37–45]. We investigated calcium channel currents in detail and outward membrane currents as potential cellular targets of both bromophenols. In this electrophysiological approach, 2,4-DBP reduced voltage dependent calcium channel currents with a half-maximal concentration of 45 ± 32 μM (S.D.) and a Hill coefficient of 0.87 ± 0.49 (S.D.). 2,4,6-TBP reduced calcium channel currents with a half-maximal concentration of 28 ± 19 μM (S.D.) and a Hill coefficient of 0.79 ± 0.31 (S.D.). The major contribution to calcium channel currents was mediated by L-type (67%) and N-type channels (30%) in PC12 cells; both bromophenols modulated both current types. Whole cell outward currents, mainly carried by potassium ions, were reduced by 2,4-DBP with a half-maximal concentration of 41 ± 9 μM (S.D.) showing a Hill coefficient of 1.71 ± 0.31 (S.D.). 2,4,6-TBP showed a weak reduction of outward currents at high concentrations of 300 μM. 2,4,6-TBP selectively decreased calcium entry via calcium channels as revealed in whole cell patch clamp experiments, whereas 2,4-DBP reduced both in- and outward currents.

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1. Introduction

In natural marine environments many very complex as well as relatively simple brominated compounds are found, for example phenols with different grades of bromination. These bromophenols are natural products and can be found in bryozoans, sponges, crustaceans (Whitfield et al., 1999), polychaetes (Goerke and Weber, 1991), molluscs (Chung et al., 2003a), fish (Boyle et al., 1992) and mammals (Vetter and Janussen, 2005). In the North Sea bromophenols have been described in macroalgae, sponges and ascidians (Kotterman et al., 2003). Highest concentrations with up to 7000 ng/g dry weight were found in the common seaweed *Lobophora variegata* (Chung et al., 2003b). 2,4-Dibromophenol and 2,4,6-tribromophenol are major constituents of the total bromophenol content (Chung et al., 2003a). The primary producers of 2,4-DBP and 2,4,6-TBP seem to be macroalgae which synthesise these bromophenols as secondary metabolites (Flodin and Whitfield, 1999). Because of their lipophilicity, an accumulation in the food chain is postulated by Whitfield et al. (1998). Marine animals raised in aquaculture lack the typical marine taste, which can be restored by feeding diets containing bromophenol compounds (Ma et al., 2005). Some polychaetes, such as *Notomastus lobatus* and *Thelepus crispus* accumulate bromophenols, which change the microbial community inside their living tubes (Lincoln et al., 2005). The bromophenol 2,4,6-TBP, known from natural sources, can also be produced industrially during flame retardant production in resins and is further used in pesticides. In 2001, an amount of 9500 t of 2,4,6-TBP was compounded (IUCLID, 2003).

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Bromophenols are suspected to act as endocrine disrupters (Legler and Brouwer, 2003). While 2,4-DBP binds to the estrogen receptor (Olsen et al., 2002), 2,4,6-TBP interacts with thyroid hormone-binding transport proteins (Meerts et al., 2000). Additionally, 2,4,6-TBP seems to be neurotoxic during development (Lyubimov et al., 1998; Ríos et al., 2003) and inhibits moulting of copepods at low concentrations (Wollenberger et al., 2005). Disturbance of the endocrine system may partly be caused by an influence of bromophenols on cellular calcium signaling as shown by Hassenklöver et al. (2006) in neuroendocrine cells—particularly in reference to free calcium ions, which fulfill many cellular tasks, e.g. as second messenger and trigger of exocytosis.

Cells of the adrenal gland have been subject matter for many toxicological and pharmacological studies e.g. PCBs (Messeri et al., 1997; Westerink and Vijverberg, 2002a) heavy metals (Shafer and Atchison, 1991; Weinsberg et al., 1995; Westerink and Vijverberg, 2002b) and alkaloids (Lee and Kim, 1996; Gafni et al., 1997; Bickmeyer et al., 1998; Kim et al., 2001; Smith et al., 2002; Bickmeyer et al., 2004). 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). Because of the disturbance of intracellular calcium signals by 2,4-DBP and 2,4,6-TBP mentioned above, which is partly induced by reduction of voltage dependent calcium influx (Hassenklöver et al., 2006), we focussed on a detailed electrophysiological approach to describe concentration–effect-relationships of involved ion channel currents. Ionic membrane currents, other than calcium currents, have not been investigated so far in bromophenol toxicity, therefore we measured in- and outward currents of PC12 cells in the presence of bromophenols using the whole cell configuration of the patch clamp technique (Hamill et al., 1981).

2. Material and methods

2.1. Culture methods

PC12 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 U penicillin/streptomycin per millilitre. Cells were incubated at 37 °C, 90% humidity and 5% CO_2 and grown on collagen coated cover slips and/or in collagen coated dishes (30 mm). Culture medium was exchanged every 3 days and cells were split when necessary.

2.2. Voltage clamp experiments using the whole cell configuration of the patch clamp technique

Recordings were done using the EPC-7 patch clamp amplifier (List electronics) and analyzed with the computer program Signal 3 (CED). All experiments were carried out 1 or 2 days after plating cells in collagen coated dishes (see above). The physiological bath solution for measurement of in- and outward currents comprised: 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 30 mM glucose, 26 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonicacid (HEPES), pH was adjusted to 7.2 with NaOH. The physiological pipette solution contained: 140 mM K-gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP, 10 mM ethylenglycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraaceticacid (EGTA), 10 mM HEPES, 0.4 mM Na_2GTP, adjusted to pH 7.2 with NaOH. The bath solution to measure calcium channel currents contained: 135 mM tetraethylammonium-chloride (TEA-Cl), 10 mM HEPES, 1.2 mM $MgCl_2$, 10 mM $BaCl_2$, 2 μM tetrodotoxin (TTX) (pH was adjusted to 7.2 with TEA-OH). The pipette solution comprised: 135 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 2 mM Na_2ATP (adjusted to pH 7.2 with TEA-OH), which prevented "run down" of currents (Bickmeyer et al., 1993). Currents were recorded with patch pipettes of approximately 5 MΩ resistance. Currents evoked from a holding potential of −70 to +10 mV for 200 ms every 30 s or by increasing voltage steps (+10 mV, 100 ms, every 10 s) starting from −90 up to +60 mV for current–voltage (*I–V*) relationship. Current traces for *I–V* relationships were leak subtracted.

Series resistance voltage errors were neglected (average value was 0.8 mV, maximal 2.8 mV during calcium channel current measurements). Data were not corrected for liquid junction potentials. For statistical evaluation (half-maximal concentrations, Hill coefficient) the program Igor Pro 4 (wavemetrics) was used. Results are presented as mean ± standard deviation (S.D.), unless otherwise stated. In all figures error bars indicate standard error of the mean (S.E.M.) derived from at least three individual experiments. The mean input resistance of cells after establishment of the whole cell configuration was 690 ± 31 MΩ and cell capacitance was 6.7 ± 2.5 pF.

2.3. Substances

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

3. Results

3.1. Electrophysiological measurement of voltage-operated calcium channel currents

Calcium ion channel currents were isolated by exclusion of other ion channel currents using specific pipette and bath solutions. Barium was used as charge carrier through calcium channels (see above). Currents were elicited from a holding potential of −70 mV to various voltages.

3.1.1. 2,4-Dibromophenol

2,4-Dibromophenol dose-dependently reduced currents through voltage dependent calcium channels. Current–voltage relationships in the presence of various concentrations of 2,4-DBP showed no obvious shift in the overall shape of the relationship (Fig. 1). The half-maximal concentration was calculated from the concentration–current amplitude curve, which mirrored a concentration–effect curve ($100 - I/I_{max}\%$). The concentration–effect curve was fitted electronically by the Hill-

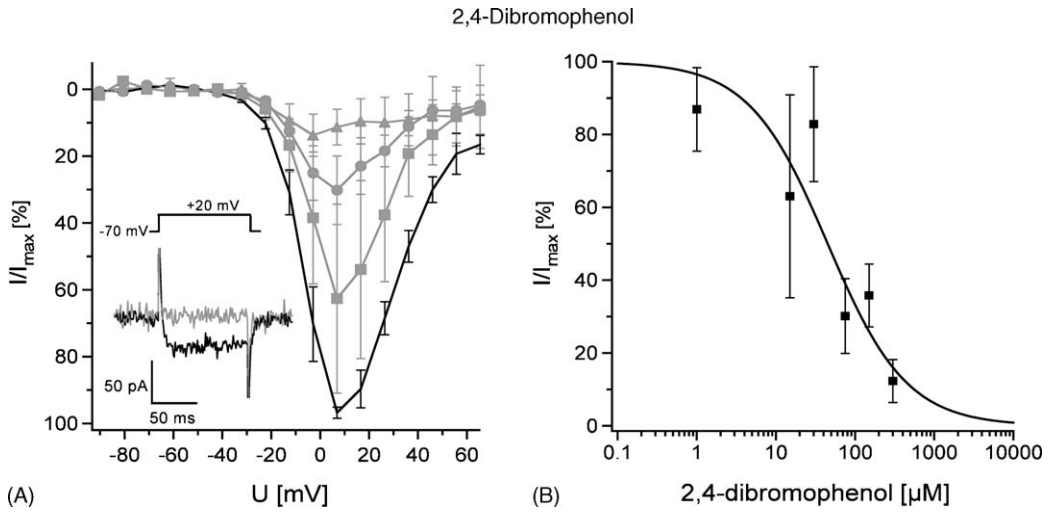

Fig. 1. (A) Current–voltage relationship (*I–V*) without (black) and with 2,4-dibromophenol (grey) in concentrations of 15 μM (■), 75 μM (●) and 300 μM (▲). Original trace of calcium channel currents before (black) and after application of 30 μM 2,4-dibromophenol (−70 to +20 mV, 100 ms). (B) Concentration–current amplitude curve of 2,4-DBP. The half-maximal concentration is 45 ± 32 μM (S.D.) and the Hill coefficient 0.87 ± 0.49 (S.D.). Error bars indicate S.E.M.

equation and showed a half-maximal value of 45 ± 32 μM and a Hill coefficient of 0.87 ± 0.49. All values for the concentration–effect curve were measured after 10 min of exposure.

3.1.2. 2,4,6-Tribromophenol

2,4,6-TBP dose-dependently reduced currents through voltage dependent calcium channels. Current–voltage relationships (*I–V*) in presence of various concentrations of 2,4,6-TBP are demonstrated in Fig. 2. The half-maximal concentration is calculated from the concentration–effect curve, fitted electronically and showed a value of 28 ± 19 μM and a Hill coefficient of 0.79 ± 0.31 ($100 - I/I_{max}$%). The duration of exposure had a strong effect on inward currents as demonstrated in Fig. 4. The values for the concentration–current amplitude curve were measured after 10 min of exposure.

The current–voltage relationship showed no obvious alteration in its shape after application of 2,4,6-TBP, but at a concentration of 15 μM, a possible shift to negative voltages can be seen, which may be due to the high variability of measured values at +10 and +20 mV (Fig. 2). To exclude the possibility that the origin of the shift is a preferential reduction of either L- or N-type currents, we looked for contribution of L- and N-type currents to calcium channel currents. N-type currents contributed 30 ± 10% and L-type 67 ± 27% to calcium channel currents. We blocked one, the L-type of the two major calcium current components, and measured the remaining N-type calcium channel currents in the presence of 2,4,6-TBP (Fig. 3). The reduction of calcium channel currents was comparable to the reduction in data obtained using untreated cells. From this data we conclude that there is no change in the contribution by L- and N-type calcium currents

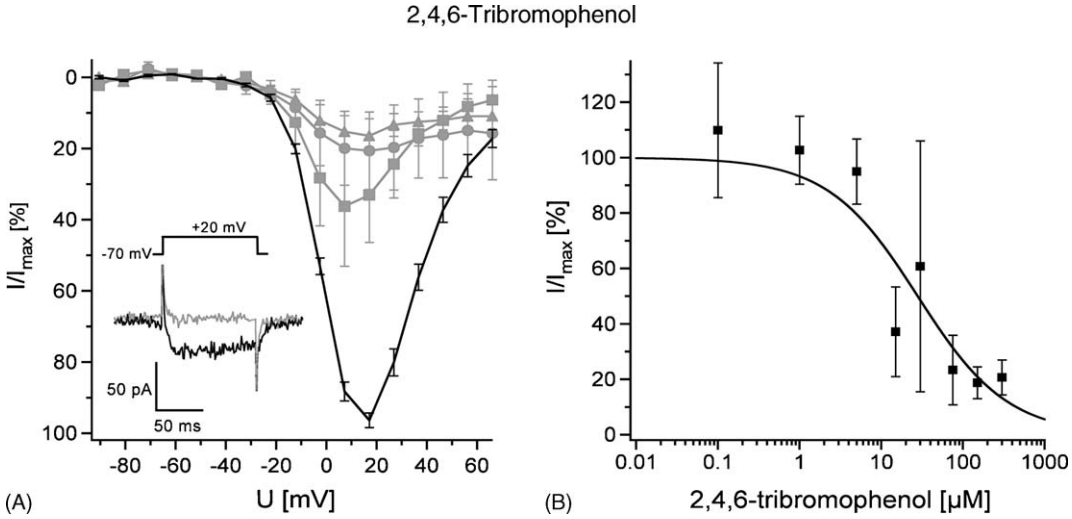

Fig. 2. (A) Current–voltage relationship (*I–V*) without (black) and with 2,4,6-tribromophenol (grey) in concentrations of 15 μM (■), 75 μM (●) and 150 μM (▲). Original trace of calcium channel currents before (black) and after application of 150 μM 2,4,6-tribromophenol (−70 to +20 mV, 100 ms). (B) Concentration–current amplitude curve of 2,4,6-TBP. The half-maximal concentration is 28 ± 19 μM (S.D.) and the Hill coefficient of 0.79 ± 0.31 (S.D.). Error bars indicate S.E.M.

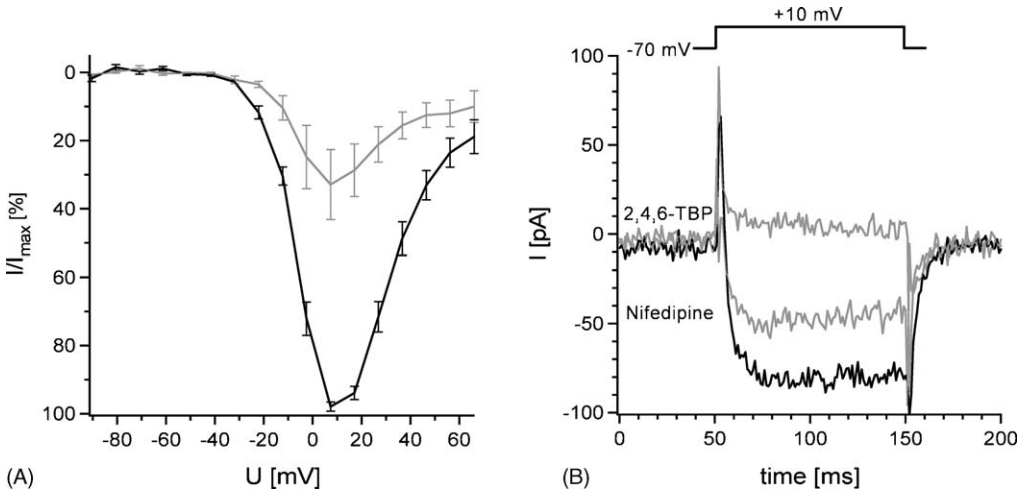

Fig. 3. (A) 5 μM nifedipine blocks 67% of inward currents (L-type) without a shift in current–voltage relationship. (B) After application of 5 μM nifedipine, remaining N-type currents are fully blocked using 300 μM 2,4,6-TBP. Error bars indicate S.E.M.

during application of 2,4,6-TBP. Both investigated bromophenols (0.1–300.0 μM) clearly showed dose-dependent reductions of inward currents through voltage-dependent calcium channels.

3.1.3. Time course of 2,4,6-TBP effects

In long-term exposures with bromophenols much lower effective concentrations can be estimated than presented in the concentration–current amplitude curves after an exposure of 10 min. Because of unstable recording conditions for experiments lasting longer than 30 min ("run down"), long term exposure was technically not possible in this electrophysiological approach. Fig. 4 demonstrates a time course of calcium channel currents after application of 2,4,6-TBP at different concentrations.

After application of low concentrations of 2,4,6-TBP, a slow decay of current amplitudes over time was visible. It, therefore, can be estimated that these concentrations affect calcium channel currents during a long-term exposure of hours or days, which is experimentally not feasible in whole cell patch clamp experiments.

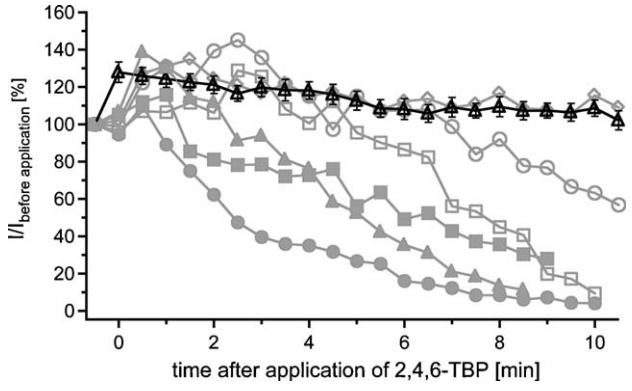

Fig. 4. Time course of calcium channel currents after application of 1 μM (◊), 15 μM (○), 30 μM (□), 75 μM (▲), 150 μM (●) and 300 μM (■) 2,4,6-TBP and only bath solution (Δ). Current amplitudes measured during voltage pulses from −70 to +10 mV (duration 200 ms, every 30 s). Error bars indicate S.E.M. of eight cells.

The current increase after application of a substance (see time course Fig. 4) is related to the perturbation of the bath solution by application of substances and has been observed for several years and may originate in mechano-sensitive, barium-permeable ion channels (Peng et al., 2005; Bickmeyer, 2005).

3.2. Current measurements with physiological solutions

The main contribution to voltage dependent ion currents comes from calcium channels and potassium channels in PC12 cells. The major components of voltage operated outward currents are potassium channels. We made no attempt to differentiate potassium channel types in the experiments, but measured all currents without addition of any blocker or pharmacological tool. Outward currents are clearly more susceptible to 2,4-DBP than to 2,4,6-TBP as indicated in Fig. 5. 2,4-DBP reduces outward currents in a dose-dependent manner. A half-maximal concentration of 41 ± 9 μM and a Hill coefficient of 1.71 ± 0.31 ($100 - I/I_{max}$%) was calculated (Fig. 5). 2,4-DBP is very effective in reducing outward currents with a lower half-maximal concentration than measured for calcium channel currents.

2,4,6-TBP shows no dose-dependent reduction of outward currents. Instead, a high selectivity for voltage operated calcium channel currents and a comparably weak effect on potassium outward currents could be shown (Fig. 6). This may be reflected in the current–voltage relationship (*I–V*) curves by a current increase after 2,4,6-TBP application, possibly origined by a reduction of inward calcium channel currents leading to a net increase of outward currents. Only at highest concentrations of 300 μM, 2,4,6-TBP revealed a visible effect on outward currents (Fig. 6).

The results indicate a high selectivity of 2,4,6-TBP affecting calcium channel currents, which in comparison to 2,4-DBP may reflect the different toxicological profiles of both substances.

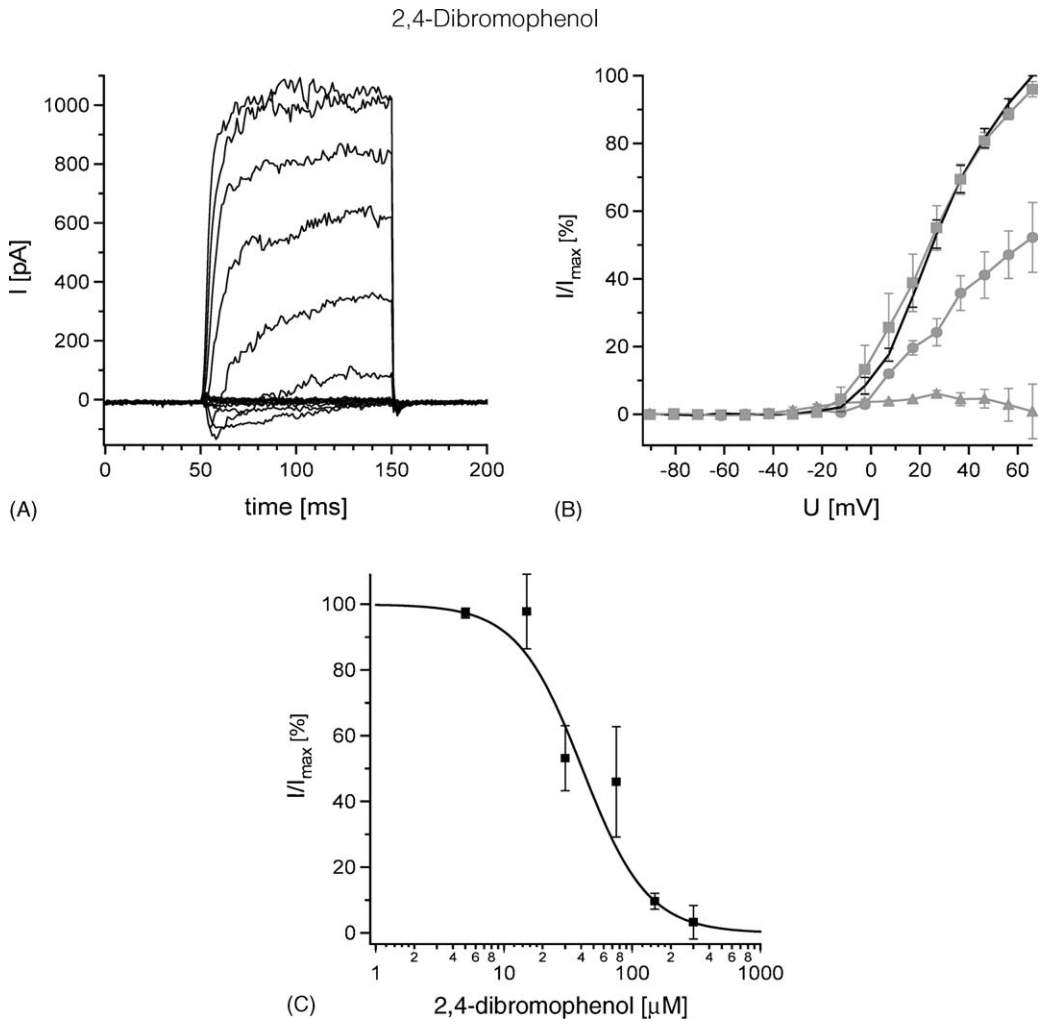

Fig. 5. In- and outward currents of PC12 cells, measured using physiological bath and pipette solutions. (A) Original current traces elicited from voltages of −70 to +60 mV. (B) Current–voltage relationship measured at the end of voltage pulses (100 ms). Control: black trace and after application of 2,4-dibromophenol 5 μM (■), 30 μM (●) and 300 μM (▲). (C) Concentration–current curve of 2,4-DBP on outward currents elicited between −70 and +50 mV. Error bars indicate S.E.M. Half-maximal concentration 41 ± 9 μM (S.D.), Hill coefficient 1.71 ± 0.31 (S.D.).

4. Discussion

2,4,6-TBP and 2,4-DBP are major compounds naturally found in marine organisms in the North Sea and elsewhere. Both compounds are found in high concentrations in, e.g., macroalgae and polychaetes (Goerke and Weber, 1991; Chung et al., 2003b). An additional impact of 2,4,6-TBP, originates from anthropogenic sources such as industrial production of flame retardants. Hassenklöver et al. (2006) suggested cellular calcium signaling as one target of bromophenol toxicity, especially because these substances are supposed to act as endocrine disruptors, and therefore the authors used neuroendocrine cells for investigations. In this study we focused on voltage operated calcium channel currents and outward potassium currents using an electrophysiological approach. As suggested by Hassenklöver et al. (2006), one target of 2,4-DBP and 2,4,6-TBP are cellular calcium signals elicited by voltage dependent calcium entry. Calcium channel currents are reduced by both substances in a dose-dependent manner with half-maximal concentrations in micromolar ranges. There is no differential effect on either N- or L-type calcium channel currents of either substance. Other voltage operated calcium channel types are present in undifferentiated PC12 cells, but contribute only marginally to calcium entry (Liu et al., 1996; Shafer and Atchison, 1991; own unpublished data). 2,4,6-TBP as the most abundant natural bromophenol, which increases intracellular calcium levels induced by a release from intracellular calcium stores (Hassenklöver et al., 2006), was most effective in reducing voltage operated calcium entry. 2,4,6-TBP showed a high selectivity to reduce calcium channel currents in comparison to potassium outward currents. Presumably this selectivity for voltage operated calcium channels separates voltage dependent calcium entry from a store operated entry, which is still visible in fluorometric measurements. 2,4-DBP reduced both voltage operated inward and outward currents in comparable concentration ranges. The relevance of clearly different cellular targets of closely related bromophenols may be reflected in a differential toxicity profile as suggested from investigations on kidneys, liver and developmental studies (Bruchajzer et al., 2002; Szymanska et al., 1995; Wollenberger et al., 2005). Even a small differential disturbance

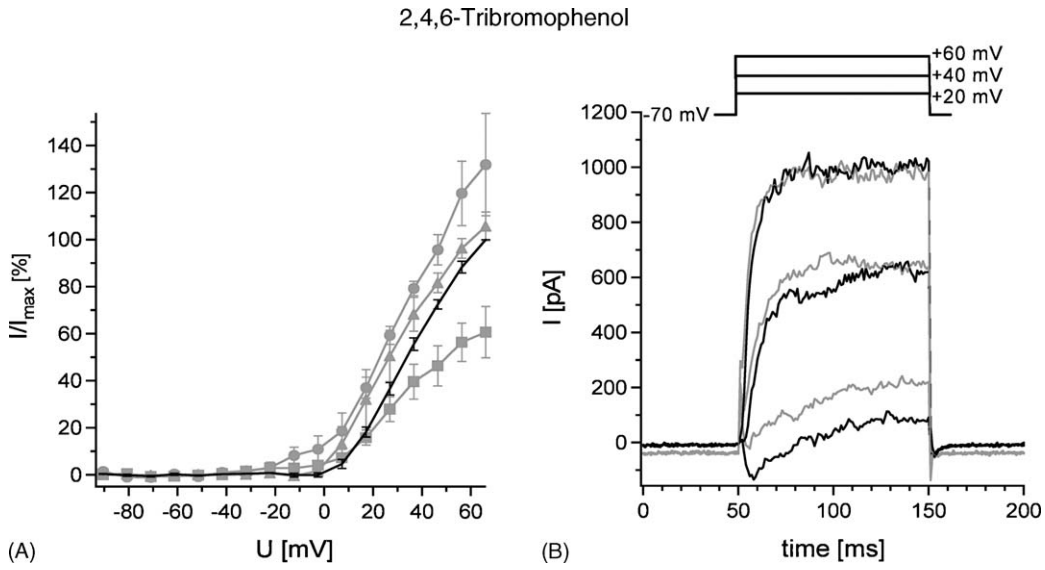

Fig. 6. (A) Current–voltage relationship of outward currents elicited from a holding potential of −70 mV (100 ms). Control: black trace; after application of 2,4,6-tribromophenol: grey trace, in concentrations of 75 μM (●), 150 μM (▲) and 300 μM (■). (B) Original current traces before (black) and after application of 75 μM 2,4,6-tribromophenol (grey) at indicated voltages.

of the cellular calcium homeostasis could possibly change the regulation of various physiological processes, including secretion of hormones. The selectivity of 2,4,6-TBP for calcium signals (reduction of calcium entry and release from intracellular stores) indicates the toxicity of this substance and therefore its impact on developmental processes, since calcium signals are crucial for hormonal and therefore developmental regulation. In addition, 2,4,6-TBP induces aromatase activity, which balances estrogen levels (Cantón et al., 2005). 2,4-DBP comparably reduces in- and outward currents; this interesting finding suggests more general target structures and target molecules for this compound and in support of this, 2,4-DBP binds to estrogen receptors as possible endocrine disruptors (Olsen et al., 2002). Some brominated natural products may interfere in membrane organization by disturbance of structurally organized membrane channels and signal pathways such as brominated molecules which show generally high lipophilicity (for review Howe et al., 2005).

Analyses of human blood samples showed that 2,4,6-TBP is taken up via seafood and directly from the environment (Thomsen et al., 2002). To which extent, however, human or wildlife tissues are exposed to bromophenol concentrations and whether these concentrations negatively affect calcium signaling remains unclear.

In marine ecosystems bromophenols may play an important role, as concentrations, e.g. in macroalgae and polychaetes are high enough to disturb calcium signaling of exposed cells and animal tissues. Disturbed endocrine systems may be a reason for the inhibition of the development of copepods with an EC_{50} of 2.5 μM by 2,4,6-TBP (Wollenberger et al., 2005).

Further, long term effects of bromophenols are an important and methodically challenging task to investigate in the future in order to assess the effects of bromophenol on calcium signaling and impairment of endocrine systems in marine animals.

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