Bisphenol A Inhibits Voltage-Activated Ca\(^{2+}\) Channels in Vitro: Mechanisms and Structural Requirements

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ABSTRACT

Bisphenol A (BPA), a high volume production chemical compound attracts growing attention as a health-relevant xenobiotic in humans. It can directly bind to hormone receptors, enzymes, and ion channels to become biologically active. In this study we show that BPA acts as a potent blocker of voltage-activated Ca\(^{2+}\) channels. We determined the mechanisms of block and the structural elements of BPA essential for its action. Macroscopic Ba\(^{2+}\)/Ca\(^{2+}\) currents through native L-, N-, P/Q-, T-type Ca\(^{2+}\) channels in rat endocrine GH3 cells, mouse dorsal root ganglion neurons or cardiac myocytes, and recombinant human R-type Ca\(^{2+}\) channels expressed in human embryonic kidney (HEK) 293 cells were rapidly and reversibly inhibited by BPA with similar potency (EC\(_{50}\) values: 26–35 \(\mu\)M). Pharmacological and biophysical analysis of R-type Ca\(^{2+}\) channels revealed that BPA interacts with the extracellular part of the channel protein. Its action does not require intracellular signaling pathways, is neither voltage- nor use-dependent, and does not affect channel gating. This indicates that BPA interacts with the channel in its resting state by directly binding to an external site outside the pore-forming region. Structure-effect analyses of various phenolic and bisphenolic compounds revealed that 1) a double-alkylated (R-C(CH\(_3\))\(_2\)-R, R-C(CH\(_3\))(CH\(_2\)CH\(_3\))-R), or double-trifluoromethylated sp\(^3\)-hybridized carbon atom between the two aromatic rings and 2) the two aromatic moieties in angulated orientation are optimal for BPA’s effectivness. Since BPA highly pollutes the environment and is incorporated into the human organism, our data may provide a basis for future studies relevant for human health and development.

Introduction

Bisphenol A (BPA) is a chemical that is extensively used (>3.8 million tons/yr worldwide) to produce polycarbonate plastic and epoxy resins. Both synthetics are manufactured into highly diverse mass products such as optical media (CD and DVD), protective coatings inside metal food containers, baby bottles, thermal paper, composites, and sealants in dentistry and medical tubing (for review see Vandenberg et al., 2007; Dekant and Volkel, 2008). BPA is an environmental pollutant that is incorporated into living organisms (for review see von Goetz et al., 2010). Common routes of BPA exposure in humans are oral intake, respiration, and dermal absorption (Braunrath, 2005; Biedermann et al., 2010; Loganathan and Kannan, 2011). Detectable levels of BPA were found in over 90% of people living in industrialized countries due most likely to chronic exposure (Calafat et al., 2005; Calafat et al., 2008; see also He et al., 2009). BPA’s adverse effects on human health and the ecosphere are being increasingly recognized (for review see Chapin et al., 2008).

Numerous studies have shown that BPA influences a wide range of physiologic functions (for review see Rubin, 2011). It is very well documented that BPA and various related compounds bind to hormone receptors and influence multiple endocrine pathways (Matsushima et al., 2008; Okada et al., 2008; Swedenborg et al., 2009; Riu et al., 2011; Soriano et al., 2012). Beyond this, recent studies provide evidence that BPA can directly interact with biologically active proteins such as enzymes and ion channels (Hiroi et al., 2006; Asano et al., 2010; Hashimoto et al., 2012; O’Reilly et al., 2012; Pandey and Deshpande, 2012). Protein disulfide isomerase (PDI) has been isolated as a binding protein of BPA in the rat brain. BPA binds to different domains of protein disulfide isomerase with K\(_D\) values in the range of 10\(^{-6}\) to 10\(^{-4}\) molar (Hashimoto et al., 2012). A rapid and reversible increase of large conductance Ca\(^{2+}\)/voltage-sensitive K\(^+\) (Maxi-K) channel activity by BPA (10–100 \(\mu\)M) has been shown in human and canine coronary smooth muscle cells as well as in AD-293-cells expressing the recombinant form of the channel (Asano...
et al., 2010). Furthermore, inhibitory effects of BPA in the micromolar range have been reported for mouse neuronal (Wang et al., 2011) and human cardiac Na⁺ channels (O'Reilly et al., 2012). In the latter case it has been shown that BPA directly binds to the channel protein at the local anesthetic receptor site.

In the present study we have characterized the interaction of BPA and several of its related compounds with voltage-activated Ca²⁺ channels. This family of channels can be divided into subtypes that are expressed in different cells of the body (for review see Catterall et al., 2005). The different types of voltage-activated Ca²⁺ channels play key roles in various physiologic and pathophysiological processes such as excitation-contraction coupling, synaptic transmission, hormone release, gene expression, and cell death and differentiation (for review see Catterall, 2011).

We sought to elucidate the mechanisms underlying BPA’s interaction with voltage-activated Ca²⁺ channels expressed in GH3 pituitary tumor cells, mouse DRG neurons, mouse cardiac myocytes, and with voltage-activated recombinant human R-type Ca²⁺ channels stably expressed in human embryonic kidney (HEK) 293 cells. The blocking mechanism of BPA was analyzed in detail in recombinant human R-type Ca²⁺ channels. We aimed at characterizing state-dependence of the block and the site where BPA binds to the channel protein. Furthermore, we intended to determine which structural features of the BPA molecule are basic for its ability to block Ca²⁺ channels. For that purpose we compared the degree of Ca²⁺ channel block induced by BPA-related compounds to that of BPA.

Material and Methods

Cells and Cell Culture. Endocrine rat pituitary tumor cells (GH3 line; DSMZ, Braunschweig, Germany) were grown on 100 mm culture dishes (Becton Labware, Franklin Lakes, NJ) in Ham F10 medium supplemented with 5% horse serum, 1% penicillin-streptomycin, and 2 mM glutamine. Two to seven days before electrophysiological recordings, cells were resuspended in culture medium and cells were plated on poly-L-lysine coated glass coverslips and kept in humidified atmosphere (37°C).

Cells were used for electrophysiological recordings within the following 12 hours. All experiments were conducted in accordance with the guidelines of the Animal Care Committee of the University of Bonn.

Cardiac myocytes were isolated from male or female (ratio ~1:1) mice (C57BL/6) as described previously (Linz and Meyer, 2000). Briefly, hearts were removed and cardiopleged in cold Tyrode solution containing 135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 0.2 mM CaCl₂, 130 mM K-glutamate. For digestion 200 IU/ml trypsin type I (Sigma) and 0.4 mg/ml collagenase type II (Sigma-Aldrich) were added. The ventricles were cut down, chunked and allowed to settle in oxygenated Tyrode solution containing 135 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM HEPES, 1.8 mM CaCl₂, 1 mM BSA, and 16.6 mg/l trypsin inhibitor. The solution was filtered through a 125-μm nylon mesh, centrifuged briefly, and the pellet was resuspended in fresh Tyrode solution. Cells were plated on poly-L-lysine–coated glass coverslips and recordings were performed 1–8 hours after plating.

HEK293 cells stably expressing human α₁B and β₃ Ca²⁺ channel-subunits were kindly provided by T. Schneider, University of Cologne (Nakashima et al., 1998). Cells were maintained in DMEM supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) (Invitrogen, Darmstadt, Germany), and 10% fetal bovine serum. Geneticin (0.5 mg/ml) and hygromycin (0.2 mg/ml) were used for selection of α₁B- and β₃-subunit expression, respectively. Cells were plated on poly-L-lysine–coated glass coverslips and used within 24–48 hours after plating for recordings.

Drugs. Electrophysiological Recordings. Ca²⁺ and/or Ba²⁺ currents were recorded in the whole-cell configuration of the patch-clamp technique using an EPC9 patch-clamp amplifier and the PULSE software (HEKA Electronic, Lambrecht, Germany). Data were filtered and digitized at 3 and 10 kHz, respectively, and stored on hard disk. For recordings of tail currents the sampling rate was 100 kHz. In ventricular cardiac myocytes Ca₉, 1.2 L-type Ca²⁺ channels constitute the major pathway for high voltage–activated (HVA) Ca²⁺ currents (Bers and Perez-Reyes, 1999). Ba²⁺ (2 mM) currents through N-type Ca²⁺ channels were elicited by voltage ramps (200-millisecond) from −50 mV to −70 mV. In mouse DRG neurons Ca₉ currents through T-type Ca²⁺ channels were evoked by depolarizations (100-millisecond) from −70 mV to −20 mV using CaCl₂ (10 mM) as charge carrier. Currents through N-type Ca²⁺ channels were elicited by voltage ramps (100-millisecond) from −70 mV to +50 mV using BaCl₂ (1 mM) as charge carrier. Currents were recorded in the presence of nifedipine (10 μM) and α,β-methylene-ATP (0.2 mM) to eliminate contribution from L- and P/Q-type Ca²⁺ channels, respectively. The internal solution of the recording electrodes contained: 160 mM cesium-aspartate, 10 mM EGTA, 2 mM MgATP, 20 mM phosphocreatine, 0.2 mM Na₂GTP, 20 mM HEPES (pH 7.3, 290–300 mOsmol/l). Filled electrodes had resistances between 1.5 and 4 MΩ. The extracellular solution contained: 160 mM TEA-Br, 3 mM KCl, BaCl₂ or CaCl₂, 1 mM NaHCO₃, 1 mM MgCl₂, 10 mM HEPES, and 4 mM glucose (pH 7.4, 300–310 mOsmol/l). The final concentrations of Ba²⁺ and Ca²⁺ are indicated throughout the text. All recordings were performed at room temperature (20–23°C).

The recording chamber was continuously perfused at a rate of 1 ml/min. The bath volume was exchanged every 20 seconds. Drugs were applied in external solution using a fast-pressure–application system (DAD-VM Superfusion System;ALA Scientific Instruments, Farmingdale, NY). The tip of the application pipette (diameter 100 μm) was positioned within 100 μm off the cells and solution exchange was obtained within ~20 milliseconds.

Drugs. Bisphenol A and the related compounds were purchased from Alfa Aesar (Karlsruhe, Germany) or Sigma-Aldrich (Taufkirchen, Germany). Stock solutions of the drugs were prepared in ethanol at a concentration of 200 mM and stored at room temperature. All toxins were freshly prepared from stock solution. The highest final concentration of the solvent ethanol was 0.15% which did not affect...
voltage-activated Ca\(^{2+}\) channels. All other chemicals were obtained from Sigma-Aldrich.

**Data Analysis and Statistics.** The analysis of the whole-cell recordings was carried out online using PulseFit (HEKA, Germany) or IGOR software (Wavemetrics, Lake Oswego, OR). Data fitting and statistical analysis were performed using PRISM 5.0 (GraphPad Software Inc., San Diego, CA). EC\(_{50}\) values and Hill slopes were determined by fitting data points to a logistic function. Charge-voltage relationships were fitted by an equation in the form \(y = \frac{G_{\text{max}}\cdot(\text{V}_{\text{test}} - \text{V}_{\text{rev}})}{(1 + \exp(\text{V}_{1/2} - \text{V}_{\text{test}})/k)}\), with \(G_{\text{max}}\) maximum conductance, \(\text{V}_{1/2}\) half-maximal activation, \(\text{V}_{\text{test}}\) test potential, \(\text{V}_{\text{rev}}\) reversal potential, and \(k\) slope factor. Data for \(m_\alpha\) and \(h_\alpha\) were fitted to a Boltzmann function \(Y = (\text{Min} + (\text{Max} - \text{Min})/(1 + \exp((\text{V}_{0.5} - \text{V}_{\text{test}})/k)))\). Time course for recovery from inactivation was

**Fig. 1.** BPA inhibits HVA whole-cell Ba\(^{2+}\) currents in GH3 cells. (A) Concentration-dependent inhibition of Ba\(^{2+}\) (5 mM) currents by BPA. Currents were elicited by depolarizing steps (50-millisecond) to 0 mV at 0.1 Hz. Increasing concentrations of BPA (as indicated) were successively applied for 40 seconds. Note that the current recovered completely upon washout of 100 \(\mu\)M BPA. (B) Time-course of current inhibition by BPA (100 \(\mu\)M). Currents were evoked by brief depolarizing steps (15-millisecond) to 0 mV at 1 Hz. Current amplitudes were measured 10 milliseconds after beginning of the depolarization and current inhibition (%) was plotted versus time. Application of BPA is indicated by the horizontal bar. (C) Charge-voltage relationship (C-V) for total Ba\(^{2+}\) currents for control (circles) and in the presence of 70 \(\mu\)M BPA (squares; \(n = 3\)). Data points represent the charge transferred for each current pulse (100-millisecond). Solid lines represent fit of data points to the equation given in Materials and Methods. BPA did not significantly affect values for half-maximum activation (\(m_\alpha\); control: \(-10.3 \pm 0.9\) mV, BPA: \(-4.9 \pm 2.7\) mV; \(P > 0.05\)). Inset shows fractional block for the data from Fig. 1C. (D1) Summary of pharmacological dissection of HVA Ba\(^{2+}\) currents using \(\omega\)-conotoxin GVIA (1.5 \(\mu\)M), \(\omega\)-conotoxin MVIIC (1.5 \(\mu\)M), nifedipine (10 \(\mu\)M) or nifedipine plus \(\omega\)-conotoxin MVIIC. Current inhibition of normalized peak currents is shown in percent (\(n\) in parentheses). (D2) Contribution of the different Ca\(^{2+}\) channel types to the total Ba\(^{2+}\) current. (E1) Normalized peak current inhibition (%) of total Ba\(^{2+}\) current by BPA (70 \(\mu\)M). (E2) Inhibition of pharmacologically dissected Ba\(^{2+}\) current fractions by BPA (70 \(\mu\)M; \(n\) in parentheses; drug concentrations as in D1). (F) CER for BPA-mediated inhibition of total (filled symbols) or P/Q- (+R-) type HVA currents (open symbols). CERs were obtained by plotting normalized peak current inhibition (%) against BPA concentration. Data points were fitted to a logistic function yielding an EC\(_{50}\) of 28 \(\pm\) 1.1 and 32 \(\pm\) 1.9 \(\mu\)M for total and P/Q- (+R-) type HVA currents, respectively (\(n = 3\); see Table 1).

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fitted by a double exponential function. The data in the manuscript are presented as mean ± standard error unless stated otherwise. Statistical analysis was performed using Students t test or one-way analysis of variance and Tukey’s post-test. Differences with P value <0.05 were regarded as significant and levels of significance are indicated by asterisks (***, P<0.001, ** P<0.01, * P<0.05).

Results

Inhibition of High Voltage-Activated Ca\(^{2+}\) Channels by BPA in GH\(_3\) Cells. GH\(_3\) cells used in our experiments express essentially HVA Ca\(^{2+}\) channels. BPA was applied extracellularly to single cells at micromolar concentrations using a fast application system (see Materials and Methods). Fig. 1A shows the effect of BPA on Ba\(^{2+}\) currents (5 mM) elicited by depolarizing voltage steps (50-millisecond) to 0 mV at 0.1 Hz for three different concentrations. Each concentration (starting with 10 \(\mu\)M) was applied for 40 seconds during which maximal inhibition was achieved. The current inhibition was almost fully reversible and currents recovered completely within about 1 minute upon washout of BPA. Similar results were obtained with Ca\(^{2+}\) (10 mM) as charge carrier. Note that internal perfusion with BPA even at high concentrations (100 \(\mu\)M) was ineffective (unpublished data).

Fig. 1B illustrates the time course of Ca\(^{2+}\) channel block by 100 \(\mu\)M BPA and of its washout when brief depolarizing steps (15-millisecond) to 0 mV were applied at 1 Hz. The finding that the amount of block by BPA did not vary with different stimulation frequencies (0.1 and 1 Hz) implies that the block is not use-dependent (for a more detailed analysis see R-type Ca\(^{2+}\) channels below).

In further experiments the effect of BPA (70 \(\mu\)M) was studied at different depolarizing voltages. The charge transferred by an individual Ba\(^{2+}\) current was determined from the area under the current curves. Fig. 1C shows the charge-voltage relationship obtained in the presence and absence of BPA. While charge transfer was significantly reduced there was no significant shift in the charge-voltage curve in the presence of BPA. The mean value of the block at different membrane potentials was calculated from corresponding data points and plotted against voltage (fractional block, see inset). The degree of block did not vary significantly within the voltage range tested (P = 0.92; n = 3).

To analyze the blocking action of BPA in more detail total currents were pharmacologically dissected using nifedipine and/or \(\omega\)-conotoxins. As illustrated in Fig. 1D1 the application of \(\omega\)-conotoxin GVIA (1.5 \(\mu\)M) was ineffective, indicating that N-type channels did not contribute to the total current.
Application of nifedipine (10 μM), which blocks L-type Ca\(^{2+}\) channels, resulted in a 52 ± 3.9% \((n = 6)\) reduction of the total current. Block of P/Q-type Ca\(^{2+}\) channels by \(\omega\)-conotoxin MVIIC (1.5 μM) reduced the total current by 30 ± 0.9% \((n = 7)\). A small current fraction (probably R-type) of 17 ± 2.0% \((n = 3)\) of the total current was resistant to both nifedipine and \(\omega\)-conotoxin MVIIC. Figure 1D2 illustrates the contribution of the different HVA Ca\(^{2+}\) channel types to the total current.

In the presence of BPA (70 μM) the total Ba\(^{2+}\) current was inhibited by 74.2 ± 2.6% \((n = 7\); Fig. 1E1). Figure 1E2 shows that inhibition of the current components remaining after nifedipine and/or \(\omega\)-conotoxin MVIIC application did not significantly differ from inhibition of the total current by BPA \((P > 0.7\) between the four columns). These findings indicate that BPA does not discriminate between the different HVA Ca\(^{2+}\) channel types and blocks L-, P/Q-, and probably R-type Ca\(^{2+}\) channels to the same extent.

Figure 1F illustrates the concentration-effect relationship for total Ba\(^{2+}\) currents and currents in the presence of nifedipine. The potency of BPA to inhibit currents is similar for both curves (see Table 1). This supports our findings that BPA does not distinguish between the different HVA channel types.

**Inhibition of High and Low Voltage–Activated Ca\(^{2+}\) Channels by BPA in DRG Neurons.** In DRG neurons N-type Ca\(^{2+}\) channels are predominant (Fig. 2A2), as indicated by the finding that 78 ± 0.6% \((n = 3)\) of the total Ba\(^{2+}\) currents were blocked by the application of \(\omega\)-conotoxin GVIA (500 nM) (Fig. 2A1). Application of nifedipine (10 μM) reduced the current by 26 ± 0.9%. Application of agatoxin-TK (200 nM), a P/Q-type Ca\(^{2+}\)-channel blocker, was ineffective. A small current fraction (~6%) was resistant to the blockers applied and was presumably through R-type Ca\(^{2+}\) channels (Fig. 2A2). Dose-dependent inhibition of N- (+ R-) type channels by BPA was studied in the presence of nifedipine and agatoxin TK (Fig. 2B). The EC\(_{50}\) of 35 ± 1.3 μM was comparable to the EC\(_{50}\) values obtained for other HVA Ca\(^{2+}\)–channel types in GH3 cells. In DRG neurons the effect of BPA was also investigated on low voltage–activated (T-type) Ca\(^{2+}\) channels. BPA also reduced this type of Ca\(^{2+}\) channel with a potency comparable to that obtained for HVA Ca\(^{2+}\) channel types (Fig. 2C; Table 1).

**Inhibition of L-Type Ca\(^{2+}\) Channels by BPA in Cardiac Myocytes.** In ventricular cardiac myocytes HVA Ca\(^{2+}\) currents are through Cav 1.2 L-type Ca\(^{2+}\) channels (Bers and Perez-Reyes, 1999). BPA inhibited these Ca\(^{2+}\) channels in a concentration-dependent manner with an EC\(_{50}\) of 35 ± 1.3 μM (Fig. 2D). As in endocrine and neuronal cells, the current fraction (~6%) was resistant to the blockers applied and was presumably through R-type Ca\(^{2+}\) channels (Fig. 2A2).

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**Table 1: Summary of the EC\(_{50}\) values of CERs**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ca(^{2+}) Channel</th>
<th>EC(_{50}) ± S.E.M.</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH3 cells</td>
<td>Total currents</td>
<td>26 ± 1.12</td>
<td>1.05</td>
</tr>
<tr>
<td>GH3 cells</td>
<td>P/Q- (+R-) type</td>
<td>32 ± 1.9</td>
<td>1.18</td>
</tr>
<tr>
<td>Cardiac myocytes</td>
<td>L-type</td>
<td>35 ± 1.3</td>
<td>1.39</td>
</tr>
<tr>
<td>DRG neurons</td>
<td>N- (+ R-) type</td>
<td>35 ± 1.3</td>
<td>1.12</td>
</tr>
<tr>
<td>DRG neurons</td>
<td>T-type</td>
<td>26 ± 1.12</td>
<td>0.84</td>
</tr>
<tr>
<td>HEK cells (HP(^{–})–70 mV)</td>
<td>R-type Ca(_{1.2})</td>
<td>26 ± 1.04</td>
<td>1.19</td>
</tr>
<tr>
<td>HEK cells (HP(^{–})–100 mV)</td>
<td>R-type Ca(_{1.2})</td>
<td>32 ± 1.03</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* Holding potential.

\(^{1}\) P = 0.97 for all CERs; \(n = 3–8\) for each concentration.

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**Fig. 3.** BPA inhibits R-type Ca\(^{2+}\) channels expressed in HEK293 cells. (A) Ba\(^{2+}\) (15 mM) currents elicited by depolarizing steps (100-millisecond) to the potentials indicated are shown for control (left panel), in the presence of BPA (35 μM; middle panel), and upon washout (recovery) (right panel). (B) Charge-voltage relationship (CVR) for total Ba\(^{2+}\) currents. Data points represent the charge transferred by current pulses at different depolarizing voltages for control \((n = 4\); circles) and in the presence of 35 μM BPA \((n = 4\); squares). (C) CER for inhibition of Ba\(^{2+}\) currents by BPA is not dependent on resting membrane potential. Currents were evoked from a membrane potential of either −70 mV (filled circles) or −100 mV (open circles). Normalized peak current inhibition (%) was plotted against BPA concentration and data points were fitted to a logistic function (at −70 mV: EC\(_{50}\) 88 ± 1.04 μM, Hill slope = 1.19 ± 0.06; at −100 mV: EC\(_{50}\) 32 ± 1.03 μM, Hill slope = 1.16 ± 0.06; \(n = 3–9\)).
time course of block and of its washout was fast and occurred within seconds. Together these findings suggest that BPA inhibits the different classes of Ca2+ channels found in different tissues with similar potency (for summary see Table 1).

Biophysical and Pharmacological Characterization of BPA’s Action on Human R-Type Ca2+ Channels Expressed in HEK293 Cells. A detailed analysis of the blocking mechanisms of BPA was performed on human R-type Ca2+ channels in HEK293 cells stably expressing α1E and β3 Ca2+-channel-subunits. Figure 3A shows a family of Ba2+(15 mM) currents elicited by depolarizing steps (100-millisecond) to voltages between −10 and +40 mV. BPA (35 μM; middle panel) reversibly inhibited currents and nearly full recovery from current inhibition was observed within 2 minutes upon washout (Fig. 3A, right panel). Small reductions in current amplitudes after washout were most likely due to current rundown during internal perfusion. In some cells full recovery could be obtained. To test for use-dependence of the block, depolarizing steps (15-millisecond) to 0 mV were applied at 0.1, 1, and 10 Hz. The amount of block obtained by BPA (35 μM) at each frequency did not vary significantly (P = 0.88; unpublished data; see also Fig. 1, A and B). This was also the case when the frequency was changed to 10 Hz after block had reached its equilibrium at 0.1 or 1 Hz.

Figure 3B illustrates the charge transferred by Ba2+ ions through R-type Ca2+ channels at different depolarizing voltages for control and in the presence of 35 μM BPA (C-V relationship). Inhibition of Ba2+ currents through R-type Ca2+ channels by BPA was concentration-dependent and occurred with an EC50 of 26 ± 1 μM (Fig. 3C).

To test whether binding of BPA is state-dependent, holding potential was shifted from −70 mV to −100 mV where almost all of the channels are in the resting state (Fig. 4A). As we did not observe any significant change in potency (Fig. 3C; EC50 32 ± 1 μM; P = 0.66), this finding strongly suggests that BPA binds to the channel in its resting state.

As illustrated in Fig. 3A current activation and inactivation kinetics were hardly affected by BPA. In further experiments we determined steady state activation and inactivation under control conditions and in the presence of 35 μM BPA (Fig. 4A) and found that neither the steady state activation nor the inactivation curves were significantly affected by BPA. Taken together these findings provide additional evidence that BPA binds to and stabilizes the resting state of the channel. Further support comes from the observation that neither recovery from inactivation (Fig. 4B; P = 0.824) nor deactivation kinetics (Fig. 4C; P = 0.73) were significantly changed by BPA.

To exert its action BPA has to be present for a certain time period (preapplication time) before current activation. When preapplied for 100 milliseconds BPA (70 μM) started to become effective and approached its maximal inhibitory effect after 10 seconds of preapplication (Fig. 5A). When block was brought to equilibrium by prolonged preapplication (3 minutes) of BPA (35 μM) before a long-lasting depolarizing step (1-second) was applied, we observed only a reduction in current amplitude and no change in the rate of current decay (Fig. 5, C and D). These findings demonstrate that BPA is not blocking open channels. Further support for these results came from the observation that BPA did not affect either current amplitude or current kinetics when applied during depolarizing pulses with channels in the open state (Fig. 5B).
Even concentrations as high as 300 μM did not produce any current inhibition.

To test for a possible involvement of intracellular signaling pathways in BPA’s action, modulators of G proteins and protein kinases A and C were applied (Fig. 6). Irreversible activation or inhibition of G proteins by adding guanosine 5’-O-(3-thiotriphosphate) (20 μM) or GDPbS (1 mM) to the internal perfusion solution had no significant effect on the blocking action of BPA. Neither was BPA’s action affected by the inhibition of protein kinases A and C by H-89 (10 μM) and GÖ-6983 (10 μM), respectively. These modulators were applied extracellularly for up to 10 minutes. It is noteworthy that GÖ-6983 itself inhibited Ca\(^{2+}\) channels to about 77.6 ± 1.8% (n = 3) after 4–5 minute. Similar effects of GÖ-6983 have been previously described for L-type Ca\(^{2+}\) channels (Welling et al., 2005). BPA was always applied after the inhibitory effect of GÖ-6983 had reached its maximum.

Several reports have suggested or demonstrated that hormones are able to modulate Ca\(^{2+}\) channels by direct interaction (for review see Boonyaratanakornkit and Edwards, 2007). In particular 17β-estradiol has been shown to rapidly and reversibly reduce cardiovascular L-type Ca\(^{2+}\) channels at micromolar concentrations (Jiang et al., 1992; Nakajima et al., 1995; Meyer et al., 1998). Since we found that BPA inhibited L-type Ca\(^{2+}\) channels in cardiomyocytes (Fig. 2D) we wondered if it shares a common binding site on Ca\(^{2+}\) channels with E\(_2\). Indeed E\(_2\) (100 μM) also reduced R-type Ca\(^{2+}\) channels rapidly and reversibly by 40.7 ± 2.3% (Fig. 7A).

At this concentration the E\(_2\)-mediated inhibition was near saturation (Fig. 7B) but was significantly smaller than that observed with BPA (more than 80%) at the same concentration (Fig. 7, A and B). Similar results were obtained with total currents in GH\(_3\) cells (unpublished data).

Fig. 5. BPA’s action requires binding to the resting state of the R-type Ca\(^{2+}\) channel. When BPA (70 μM) was applied before the onset of a depolarizing step (100-millisecond) to 0 mV (preapplication) its effectiveness increased with preapplication time. (A) Bars represent normalized peak current amplitude inhibition (%) after different preapplication times. After 10 seconds, inhibition was almost complete and did not increase significantly with longer preapplication times (P > 0.05 for 10 seconds versus 20 seconds and 10 seconds versus 40 seconds, n = 3–4). (B) BPA was completely ineffective when applied after the onset of the depolarizing step. Representative current traces shown for control and coapplication of BPA. Application time is indicated by the horizontal bar. (C) Current traces were elicited by 1-second depolarizing steps to −10 mV and were recorded before (control) and 3 minutes after incubation with BPA (35 μM). No channel openings were elicited during the incubation period. Note that the block by BPA was fully developed after incubation and that Ca\(^{2+}\) channel kinetics were not altered. Current decay was fitted to a double exponential function. The time constants for control and BPA were τ\(_1\): 63 ± 9 milliseconds and 43 ± 3.6 milliseconds, τ\(_2\): 299 ± 58 milliseconds and 261 ± 41 milliseconds, respectively (τ\(_1\); P = 0.8; τ\(_2\); P = 0.6; n = 5). (D) Normalized traces from (C). ***P < 0.001, *P < 0.05

Fig. 6. BPA’s action on R-type Ca\(^{2+}\) channels in HEK293 cells is independent of intracellular signaling pathways. The modulators of intracellular signaling pathways GTP\(_g\)S [guanosine 5’-O-(3-thiotriphosphate)] (20 μM), GDPbS (1 mM) (G proteins), GÖ-6983 (10 μM) (protein kinase C inhibitor) and H-89 (10 μM) (protein kinase A inhibitor) had no effect on BPA (70 μM) control-induced Ba\(^{2+}\) current inhibition. G protein modulators were added to the internal solution and allowed to diffuse into the cell for 10 minutes before current recording started. Protein kinase C and protein kinase A inhibitors were applied via bath for up to 10 minutes. Currents were elicited by voltage ramps ranging from −70 mV to +50 mV for 50 milliseconds every 15 seconds. (P > 0.35; n = 3–10)
In addition, the double-methylated sp³-hybridized carbon atom present in BPA and TBBA, which bridges the two phenol rings, is optimal for the ability of the compounds to inhibit Ca²⁺ channels. In fact, successive removal of the methyl groups revealed less effective bisphenols (BPE, BPF). Remarkably, BPAF, where the methyl groups are replaced with trifluoromethyl moieties was twice as potent as BPA (EC₅₀ = 13 ± 1.15 μM, data not shown). BPAB, which has a bulky bridging structure, (R-C(CH₃)(CH₂CH₃)-R), was almost equally effective compared with BPA. Furthermore, sp³-bridging of this carbon atom or introduction of different bridging structures led to bisphenols that were ineffective (4'-HBP, BPS). It is interesting to note, that biphenol [(1,1'-biphenyl)-4,4'-diol] did not show any activity, which again confirms that a sp³-hybridized carbon atom bridging the aromatic rings is essential for the blocking effect on Ca²⁺ channels.

In summary, our biophysical and pharmacological analysis in case of a common binding site one would expect that under these conditions the combination of the two compounds is less effective than BPA alone. This, however, was not observed (Fig. 7, A and B). On the contrary, the combination of the two compounds was slightly but significantly more effective (BPA, 81.5 ± 1.3%, n = 14; BPA + E₂, 85.6 ± 1.3%, n = 12; P < 0.05).

**Effect of BPA and Related Compounds on Ca²⁺ Channels in GH3 Cells.** To study the structure-activity relationship of bisphenol A and related substances, compounds with (1) different bridging structures between the two phenolic rings (e.g., BPAF, BPF, 4,4'-HBP), (2) different aromatic substitution patterns (4'-CP, 2,2'-DPP, TMBPA), and (3) different sterically demanding structure moieties (4'-TBP, 4'-TAP, BPM, BPP) were investigated in GH3 cells (Fig. 8). Table 2 summarizes the effect of the various phenolic and bisphenolic compounds on HVA Ba²⁺ currents in GH3 cells in relation to BPA.

TBBA, one of the most abundantly produced halogenated flame retardants, effectively inhibited Ba²⁺ currents at 100 μM concentration. As shown in Fig. 8A, TBBA was slightly but significantly less effective in blocking Ca²⁺ channels than BPA (Table 2). In contrast, BPS, another high production monomer, had no significant effect at this high concentration (Table 2).

Comparing the effects of BPA, 4'-CP, 2,2'-DPP, TBBA, and TMBPA, the aromatic substitution pattern strongly influences the ability to inhibit Ca²⁺ channels. The chemical structure of BPA is composed of two methyl groups and two phenol moieties on the central sp³-hybridized carbon atom. 4'-CP, which lacks one phenol-hydroxyl group, was about half as active as BPA. 2,2'-DPP, lacking both of the phenol-hydroxyl groups, was ineffective (≤20%). Furthermore, four aromatic methyl groups at meta-position (TMBPA) decrease the activity, whereas TBBA with four bromines as substituents was almost as active as BPA. Obviously, an aromatic substituent like bromine with a positive mesomeric effect and a predominant negative inductive effect supports the ability of meta-substituted compound to inhibit Ca²⁺ channels by reducing the electron density of π-systems.

In this study we present evidence that BPA interacts with voltage-activated Ca²⁺ channels as inhibitory ligand. BPA’s efficacy as inhibitor is comparable to that of polyvalent cations such as cadmium, cobalt, and manganese (Carbone and Swandulla, 1989). Pharmacological experiments with specific organic blockers (nifedepine and v-conotoxins) for the different Ca²⁺ channel types showed that BPA affects all subtypes studied here (L-, N-, P/Q-, R-, T-type) to the same extent. Detailed analysis of biophysical properties on human R-type channels revealed that channel kinetics (activation, inactivation, and deactivation) and steady-state characteristics (activation and inactivation) were not significantly altered by BPA.

In summary, our biophysical and pharmacological analysis strongly suggests that BPA exerts its action by binding to the channels in their resting state. This is supported by the findings that 1) the amount of block is independent of the
frequency and duration of current activation, 2) the current kinetics are not altered, 3) there is no shift in the steady state inactivation curve. Furthermore, our findings suggest that the binding site is located at the extracellular part of the pore-forming subunit. BPA as a highly lipophilic substance might reach a binding site at the transmembranal part of the channel protein. However, when applied intracellularly BPA was ineffective.

Direct evidence that BPA interacts with a specific binding site comes from the analysis of structurally related phenol and bisphenol derivatives. To be effective the molecules have to meet certain structural requirements. From the binding motive of a double-methylated or double-trifluoromethylated sp³-hybridized carbon atom flanked by two phenol moieties in angulated orientation, one can strongly assume a specific binding site at the various Ca²⁺ channels that interacts with effective compounds. The ability of BPA and related compounds to specifically interact with proteins has already been demonstrated for certain hormone receptors (Matsushima et al., 2007). Similar findings were described for BPA estrogen-related receptor-γ (ERR-γ) interactions. Whereas BPA (IC₅₀ = 9.78 ± 0.87 nM) showed a high binding affinity to ERR-γ, BPF was approx. (IC₅₀ = 131 ± 17.9 nM) 16-fold less potent, which clearly demonstrated the importance of the double-methylated central carbon atom for ERR-γ interactions. (Matsushima et al., 2008; Okada et al., 2008). In BPAF the two methyl groups are replaced by two electron-rich trifluoromethyl moieties, which reduced the binding affinity to the ERR-γ considerably by a factor of 35. In contrast, the inhibitory effect on Ca²⁺ channels was more pronounced with BPAF compared with BPA. Furthermore, 2,2'-DPP lacking both of the phenol-hydroxyl groups did not show any ERR-γ

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**Fig. 8.** Structural requirements of BPA for inhibition of HVA Ca²⁺ channels in GH3 cells. Comparison of BPA to various bisphenolic (A) and phenolic (B) compounds. Ba²⁺ currents were elicited by applying voltage ramps (100-millisecond) from −70 mV to +50 mV. Compounds (100 μM) were applied 15 seconds before the onset of the voltage ramps and washed out immediately after return to the holding potential. Onset of the block was fast and recovery was complete for all compounds applied except for BPM and BPP. Bars illustrate normalized peak current amplitude reduction (%) induced by the various compounds (systematic names for compounds and statistical values are given in Table 2).
binding compared with 4'-CP, which was as active as BPA. Although there are some matches in the compound’s activity profiles for ERR-γ and Ca2+ channels, we do not assume that the two binding sites for BPA possess major structural similarities on the two proteins.

Since BPA and several of its related compounds are environmental pollutants that can be incorporated into the human organism, exposure to these chemicals may cause serious health problems. Urinary levels of BPA have recently been associated with chronic diseases, including heart disease, diabetes, as well as neurobehavioral changes in toddlers (Lang et al., 2008; Melzer et al., 2010; Sathyarayanan et al., 2011).

The Environmental Protection Agency considers a safe level of exposure to be 50 μg/kg of body weight per day (U.S. EPA, 2010). Recent experiments with primates, however, indicate that human exposure may be much higher than previously assumed (Taylor et al., 2011). Under certain conditions exposure to BPA may exceed putative safe levels. This could be the case in manufacturing facilities. Indeed recent studies have shown that workers in epoxy resin factories can have approximately 1000 times higher urinary BPA concentrations compared with control groups (He et al., 2009; Melzer et al., 2010; Wang et al., 2012). The highest levels measured were almost up to 10 μM (Wang et al., 2012), which is within the effective concentration range of Ca2+ channels block by BPA. High exposure to BPA has also been reported for patients, particularly premature infants undergoing intensive medical care treatment (Calafat et al., 2009).

In general the incorporation of the lipid-soluble compound BPA in certain body compartments is not very well studied and it is conceivable that BPA could accumulate to micromolar concentrations in the human body (see above). This may apply also to highly lipophilic, halogenated BPA derivatives such as tetrabromobisphenol A (TBBA) one of today’s most abundantly used brominated flame retardants. Indeed, it has been shown that in mice fed with 100 μg TBBA/kg body weight TBBA accumulates over-proportionally in the striatum compared with other brain regions (Nakajima et al., 2009).

The toxicity of BPA has been evaluated with respect to development, reproduction, and cancer (see, e.g., Xu et al., 2010; Fernandez et al., 2012). In this context, a recent Food and Drug Administration update points to “some concern about the potential effect (of BPA) on the brain, behavior and prostate gland in fetuses, infants, and young children” (for review see Wolstenholme et al., 2011). The BPA concentration administered in toxicological studies is expected to result in micromolar concentrations in different compartments of the body and should critically depend on BPA metabolism. Exact values in humans, however, are currently not available.

Given the fact that BPA and its related compounds are ubiquitously contaminating our environment, we expect that further studies will surface that medical diseases such as cardiovascular, respiratory, and metabolically caused disorders can at least partly be attributed to the effect of BPA on voltage-activated Ca2+ channels. Our findings that certain BPA-related compounds are less or even non-effective on voltage-activated Ca2+ channels may provide a key to finding molecules that could substitute for BPA in large-scale plastic production.

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Authorship Contributions

Participated in research design: Swandulla, Hans, Meyer. Conducted experiments: Deutschmann, Swandulla, Hans. Performed data analysis: Deutschmann, Hans, Swandulla, Häberlein, Meyer. Wrote or contributed to the writing of the manuscript: Swandulla, Hans, Häberlein, Meyer, Deutschmann.

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