Bisphenol A inhibits compound action potentials in the frog sciatic nerve in a manner independent of estrogen receptors

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ABSTRACT

Although the endocrine disruptor bisphenol A (BPA) is reported to inhibit nerve conduction, the underlying mechanisms are unclear. Therefore, in the present study, we examined the effect of BPA on compound action potentials (CAPs) recorded from the frog sciatic nerve using the air-gap method. Treatment of the sciatic nerve with BPA (0.5 mM) for 20 min reduced the peak amplitude of the CAP by approximately 60% in a partially reversible manner. The reduction in the CAP peak amplitude was concentration-dependent, with a half-maximal inhibitory concentration (IC_{50}) value of 0.31 mM. This effect of BPA was unaffected by an estrogen-receptor antagonist, 4-hydroxytamoxifen, which by itself reduced CAP peak amplitude, with an IC_{50} value of 0.26 mM (comparable to that of BPA). The natural estrogen 17β-estradiol, at the highest dissolvable concentration (0.05 mM), had an effect similar to that of BPA. The IC_{50} value of BPA was comparable to those of some local anesthetics in inhibiting frog CAPs. Our findings suggest that BPA inhibits nerve conduction in a manner independent of estrogen receptors. This action of BPA may underlie, at least in part, the neurotoxicity of the compound.

1. Introduction

Bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane], an organic synthetic compound, is easily absorbed into the body through food and drink, owing to its lipophilicity (for review see [1–3]). Because BPA has an estrogenic action, albeit comparatively weak [4–7], it perturbs normal endocrine functions (for review see [8,9]). Furthermore, there are numerous studies demonstrating that BPA acts on the nervous system (for review see [3,9,10]). For example, BPA has been shown to affect, in a sex-specific manner, the motivation to explore- and anxiety-related behavior in rats [11]. BPA also impacts the behavioral response to pain produced by the subcutaneous injection of formalin in rats [12]. Interestingly, Pandey and Deshpande [13] reported that BPA inhibits fast-conducting compound action potentials (CAPs) in the frog (Rana nigromaculata) sciatic nerve. This effect of BPA was found to be sensitive to the estrogen-receptor antagonist tamoxifen, as well as Ca^{2+}+-free and voltage-gated L-type Ca^{2+}-channel antagonists (i.e., nifedipine and diltiazem), but not T- or P-type Ca^{2+}-channel antagonist (Ni^{2+}). Accordingly, the researchers concluded that BPA modulates voltage-gated L-type Ca^{2+} channels via estrogen receptor α (ERα). However, Ca^{2+}-channel involvement appears unlikely in the frog (Rana nigromaculata) sciatic nerve, because CAPs in this preparation are completely blocked by a voltage-gated Na^{+}-channel blocker, tetrodotoxin (TTX; [14]). Therefore, the effects of BPA on nerve conduction are currently unclear.

The present study was undertaken to evaluate the effect of BPA on the TTX-sensitive CAPs recorded from the frog sciatic nerve using the air-gap method. We chose this preparation because it is about 150-fold more effective than BPA in stimulating prolactin secretion [5] and uterotrophic activity [6]. Because BPA has been reported to bind to a local anesthetic (LA) receptor site on human cardiac Na^{+} channels [22], we further examine the effects of prilocaine and pramoxine, which are amide-type and ester-type LAs, respectively [23], on frog sciatic nerve CAPs. A part

Abbreviations: BPA, bisphenol A; CAP, compound action potential; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; IC_{50}, half-maximal inhibitory concentration; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERRγ, estrogen-related receptor γ; nH, Hill coefficient; 4-OHT, 4-hydroxytamoxifen; LA, local anesthetic; TTX, tetrodotoxin

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of the present study has been reported in abstract form [24].

2. Materials and methods

This study was approved by the Animal Care and Use Committee of Saga University, and was conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used.

2.1. Preparation of frog sciatic nerves

The method used for obtaining frog sciatic nerve preparations has been described previously [14,15,25,26]. Because there appears to be no difference between females and males in the overall distribution pattern of ERα in the rodent spinal cord or dorsal root ganglion (DRG) [27,28] (data on the frog sciatic nerve are lacking), we used either sex of the frog Rana nigromaculata. In brief, frogs were decapitated and then pithed. Thereafter, the sciatic nerve (length: 3–4 cm; diameter: 0.4–1 mm) was dissected from the lumbar plexus to the knee in Ringer’s solution. The isolated sciatic nerve was carefully desheathed under a binocular microscope and then loosely placed on five platinum wires that were glued to a Lucite plate, and the two ends of the nerve were tied to the wires with threads. The plate was put on a beaker containing Ringer’s solution, immersing the sciatic nerve. Throughout the experiments, the Ringer’s solution was continuously stirred at a rate of about 350 rpm with a Teflon-covered magnetic stir bar to maintain a uniform and steady solute concentration around the sciatic nerve. The composition of the Ringer’s solution used was (mM): NaCl, 115.5; KCl, 2.0; CaCl₂, 1.8; Na₂HPO₄, 1.3; and NaH₂PO₄, 0.7 (pH = 7.0). Before the start of the experiment, the sciatic nerve was preincubated for at least 15 min with Ringer’s solution.

2.2. Recordings of CAPs from frog sciatic nerve fibers

As described previously [14,15,25,26], the Lucite plate with the platinum wires attached to the sciatic nerve was moved from the beaker containing Ringer’s solution (100 mL) to an empty beaker, and CAPs were recorded in air using a preamplifier. Two of the platinum wires were used to record CAPs, and the other two were used to stimulate the sciatic nerve. The stimulation was performed at a frequency of 1 Hz with a stimulator, using rectangular pulses of 0.1-ms duration and of the desired concentration. The CAPs obtained from four sciatic nerves were excited; see below). As done previously [14,15,25,26], the peak amplitude of the maximal CAP was measured as the difference between baseline and CAP peak level, as done previously [14,15,25,26]. The peak amplitude of the CAP depended on the strength of the stimulus given to the sciatic nerve, and the CAP peak amplitude increased with increasing stimulus strength, eventually attaining a maximal value (where all fibers contained in the nerve were excited; see below). As done previously [14,15,25,26], we analyzed the peak amplitude of the maximal CAP. When the effect of a drug on CAPs was examined, the sciatic nerve was soaked for 20 min in Ringer’s solution containing the drug (sufficient for a near-maximal effect) and then for ≤60 min in drug-free Ringer’s solution. Each sciatic nerve was used only once to examine the effect of a drug, unless otherwise mentioned, because the effects of many of the drugs were partially reversible. The conduction velocity was determined using the fifth electrode as an additional stimulation site and by measuring the distance between the stimulus artifact and the peak of the CAP. All experiments were carried out at room temperature (20–28 °C).

2.3. Drugs

The drugs used were BPA (Tokyo Chemical Industries, Co. Ltd., Tokyo, Japan), 17β-estradiol, 4-OHT (≥98% trans isomer), pramoxine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) and prilocaine hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All of the drugs (except for pramoxine and prilocaine, which were directly dissolved in Ringer’s solution) were first dissolved in dimethyl sulfoxide (DMSO) to make a stock solution, and then diluted to the desired concentrations in Ringer’s solution immediately before use, where the concentration of DMSO was less than 1%. BPA, 17β-estradiol and 4-OHT were tested at concentrations less than their highest dissolvable concentration.

2.4. Data analysis

The concentration-dependence curve for the reduction of the peak amplitude of the CAP in the sciatic nerve soaked with a drug for 20 min was analyzed using the following Hill equation:

\[ \text{CAP amplitude (% of control)} = \frac{100}{1 + \left(\frac{[\text{Drug}]}{IC_{50}}\right)^n_H} \]

where [Drug] is drug concentration, IC₅₀ is the concentration of the drug for half-maximal inhibition, and n_H is the Hill coefficient. Data were indicated as mean ± S.E.M., and statistical significance was set at p < 0.05 using paired or unpaired Student’s t-test. In all cases, n refers to the number of sciatic nerves studied. An average of the peak amplitudes of five CAPs measured during 10 min before drug application was taken as control.

3. Results

The effects of various drugs on fast-conducting CAPs were examined in a total of 82 sciatic nerves, and the CAPs had an average peak amplitude of 21.8 ± 0.2 mV (n = 82). The conduction velocity of the fibers averaged 26 ± 2 m/s (range: 16–49 m/s; n = 28). These values were comparable to those reported previously [14,26,29–35]. DMSO at 1% (the maximal concentration used in the present study) did not affect CAPs; the peak amplitude of the CAP was 106 ± 2% of control (taken as 100%; 32.2 ± 3.5 mV; n = 4) (p > 0.05), 20 min after treatment with DMSO.

3.1. Effect of BPA on frog sciatic nerve CAPs

First, we examined the effect of BPA on CAPs recorded from the frog sciatic nerve. Soaking the sciatic nerve into Ringer’s solution containing BPA at 0.5 mM (a concentration enough to inhibit frog sciatic nerve CAPs [13] and cardiac Na⁺ channels [22]) for 20 min reduced the peak amplitude of the CAP (Fig. 1A, top). Fig. 1A, bottom, shows the average time course of the change in CAP peak amplitude after incubation in the BPA (0.5 mM) solution, relative to that before application (control), obtained from four sciatic nerves. The BPA-induced reduction in CAP peak amplitude was near maximal 20 min following exposure, where the peak amplitude of the CAP was 38 ± 2% (p < 0.05) of control (22.1 ± 0.8 mV; n = 4). In nerves treated with BPA and then returned to drug-free Ringer’s solution (washout) for up to 60 min, the CAP amplitude recovered to 65 ± 6% (p < 0.05; n = 4) of the control level (Fig. 1A). Fig. 1B shows the time course of the changes in CAP peak amplitude after treating the sciatic nerve with BPA of various concentrations ranging from 0.002 mM to 0.5 mM. The rate of the reduction in CAP peak amplitude during 20 min of exposure to BPA increased with increasing concentration of the compound. The CAP amplitude reduction after 20 min of exposure increased in magnitude with increasing BPA concentration. The concentration-response curve for the BPA-induced reduction in CAP amplitude in the nerve trunks is given in
Fig. 1C. The IC₅₀ value obtained from analysis based on the Hill equation was 0.31 mM. In the following experiments, the inhibitory action of BPA on CAPs was examined at 0.2 mM, a value similar to the IC₅₀.

Fig. 1D shows the dependency of the peak amplitude of the CAP on stimulus intensity in the control and after 20 min of exposure to BPA (0.2 mM). In the absence and presence of BPA, the CAP amplitude increased with increasing stimulus intensity, eventually reaching a maximal value. The inhibitory effect of BPA was seen for CAPs evoked by the maximal stimulus strength, without an effect on the threshold to elicit the CAP. This finding was obtained in four other nerves.

To reveal whether the CAP inhibition produced by BPA is mediated by estrogen receptors, especially ERRγ, we examined the effect of 4-OHT, administered at the same concentration as BPA (0.2 mM). Pretreatment with 4-OHT for 20 min reduced the peak amplitude of the CAP. This reduction was stable within this period (Fig. 2A, top). There was no difference in the relative CAP amplitude between 4-OHT treatments of 18 and 20 min (55 ± 8 and 54 ± 8%, respectively; p > 0.05; n = 4), indicative of a stable effect of 4-OHT (0.2 mM) itself. Following this pretreatment, adding BPA (0.2 mM) to the 4-OHT-containing Ringer’s solution markedly inhibited the CAP (Fig. 2A, top). The average result obtained from four nerves is given in Fig. 2A, bottom. The peak amplitude after treatment with both 4-OHT and BPA for 20 min was reduced to 52 ± 5% (p < 0.05; n = 4) of that just before the co-treatment. The CAP peak amplitude after 20 min of co-treatment with BPA and 4-OHT was not different from that obtained with BPA alone (p > 0.05; Fig. 2B).

3.2. Effect of 4-OHT on frog sciatic nerve CAPs

Because 4-OHT alone inhibited CAPs, we examined this inhibition in great detail. 4-OHT, at a concentration of 0.1 mM, irreversibly reduced CAP peak amplitude (Fig. 3A, top). Pretreatment with 4-OHT for 20 min reduced the peak amplitude of the CAP. This reduction was stable within this period (Fig. 2A, top). There was no difference in the relative CAP amplitude between 4-OHT treatments of 18 and 20 min (55 ± 8 and 54 ± 8%, respectively; p > 0.05; n = 4), indicative of a stable effect of 4-OHT (0.2 mM) itself. Following this pretreatment, adding BPA (0.2 mM) to the 4-OHT-containing Ringer’s solution markedly inhibited the CAP (Fig. 2A, top). The average result obtained from four nerves is given in Fig. 2A, bottom. The peak amplitude after treatment with both 4-OHT and BPA for 20 min was reduced to 52 ± 5% (p < 0.05; n = 4) of that just before the co-treatment. The CAP peak amplitude after 20 min of co-treatment with BPA and 4-OHT was not different from that obtained with BPA alone (p > 0.05; Fig. 2B).

3.3. Effect of 17β-estradiol on frog sciatic nerve CAPs

Next, we compared the effect of BPA on CAP inhibition with that of
an estrogen-receptor agonist, 17β-estradiol. Similar to BPA, 17β-estradiol at a concentration of 0.05 mM (the maximally dissolvable concentration in Ringer’s solution containing 1% DMSO) inhibited CAPs slightly in a partially reversible manner (Fig. 4A, top). The reduction in the CAP peak amplitude produced by 17β-estradiol (0.05 mM) was maximal within 20 min of exposure to the drug; the amplitude was 90 ± 2% (p < 0.05) of control (21.7 ± 1.8 mV; n = 3; Fig. 4A, bottom). This percentage value was not different from that of 0.05 mM BPA (91 ± 3%; n = 5; Fig. 1C) (p > 0.05). Fig. 4B shows the effect of 17β-estradiol in a concentration range of 0.01–0.05 mM on CAPs. Although this inhibition by 17β-estradiol weakened with increasing concentration, the relative CAP amplitudes at 0.02 and 0.05 mM were not significantly different (p > 0.05). Because the concentration dependency of this effect of 17β-estradiol was very slight, we did not test a wider concentration range, although 17β-estradiol has a biphasic effect on GH cell growth in a manner dependent on the concentration tested [36].

3.4. Effects of LAs on frog sciatic nerve CAPs

BPA reportedly binds to an LA receptor site on voltage-gated Na+ channels [22]. Therefore, we compared the effect of BPA with those of two different types of LAs (prilocaine and pramoxine). Prilocaine at 1 mM reversibly reduced CAP peak amplitude (Fig. 5A, top). Fig. 5A, bottom, shows the average time course of the change in CAP peak amplitude following exposure to prilocaine (1 mM), relative to control. Prilocaine stably reduced CAP amplitude within 20 min of exposure; the peak amplitude was reduced to 68 ± 4% (n = 5; p < 0.05) of control (21.9 ± 2.8 mV). The peak amplitude of the CAP 30 min after washout was 101 ± 6% (n = 5) of control. This value was not significantly different from 100% (p > 0.05). The extent of the reduction in the CAP peak amplitude produced by prilocaine increased with increasing concentration, within a range of 0.01–5 mM (Fig. 5B; IC50 = 1.8 mM).

Compared with prilocaine, pramoxine at 0.5 mM reduced CAP peak amplitude in a partially reversible manner (Fig. 5C, top). Pramoxine reduced CAP peak amplitude 20 min after exposure to 15 ± 3% (n = 4; p < 0.05) of control (21.9 ± 2.8 mV). The peak amplitude of the CAP 60 min after washout was 83 ± 3% (n = 4; p < 0.05; Fig. 5C, bottom) of control. The magnitude of the CAP peak amplitude reduction produced by pramoxine increased with increasing concentration within a range of 0.001–1 mM (Fig. 5D; IC50 = 0.21 mM).

4. Discussion

We demonstrate that the endocrine disruptor BPA reduces the peak amplitude of fast-conducting and TTX-sensitive CAPs recorded from the
frog (Rana nigromaculata) sciatic nerve in a partially reversible manner. This action was concentration-dependent, with an IC$_{50}$ value of 0.31 mM. This inhibition by BPA was observed for CAPs evoked by a maximal stimulus strength, and BPA did not change the threshold to elicit the CAP. This effect of BPA was mimicked by a natural estrogen, 17β-estradiol; the effect of this estrogen was comparable in magnitude to that of BPA. The CAP inhibition elicited by BPA and 17β-estradiol occurred within 2 min of exposure (see Figs. 1A and 4A). Similar CAP inhibitions were produced by an amide-type LA (prilocaine) and an ester-type LA (pramoxine). Prilocaine reversibly reduced CAP peak amplitude, with an EC$_{50}$ value of 1.8 mM. In comparison, pramoxine did so with an efficacy (EC$_{50} = 0.21$ mM) higher than that of prilocaine, with a slow recovery to control level.

### 4.1. No involvement of estrogen receptors or estrogen-related receptors in the CAP inhibition produced by BPA

Because the CAP inhibition is rapid, and our preparation is the dissected sciatic nerve that lacks neuronal cell bodies, it is unlikely that the effect of BPA is mediated by nuclear estrogen receptors that affect gene transcription. Estrogen receptors are located in the plasma membrane as well as the nucleus (for review see [37]). Plasma membrane receptors are involved in many actions of BPA; for example, the modulation of the NMDA receptor (a glutamate receptor)-mediated increase in intracellular Ca$^{2+}$ concentration in rat hippocampal neurons in culture [38] and the modulation of dopamine transporter function in rat pheochromocytoma cells [39]. ERα and estrogen receptor β (ERβ) are expressed in rat DRG [27] and spinal cord ventral horn neurons [40] whose fibers are contained within the sciatic nerve. The CAP inhibition produced by BPA, therefore, may be mediated by plasma membrane estrogen receptors. However, this possibility is
Na+ channels, because tamoxifen reduces TTX-sensitive Na+-channel activity (see above). Although 4-OHT by itself inhibits CAPs, this action also involves mechanisms of bisphenol A action, Reprod. Toxicol. 24 (2007) 139–177.

There is much evidence that BPA acts on the nervous system. For example, BPA increases spontaneous motor activity along with a large reduction in tyrosine hydroxylase activity in the midbrain [48], and it produces memory impairment accompanied by a reduction in acetylcholine production in the hippocampus [49] in rodents. Synaptogenesis induced by 17β-estradiol is inhibited by BPA in the hippocampus of rodents and primates [50,51]. Additionally, BPA affects exploratory, anxiety- and pain-related behaviors [11,12]. Many of these effects of BPA might be mediated by estrogen receptors expressed in the central nervous system (for example see [40]). The inhibitory action of BPA on frog sciatic nerve CAPs was seen at concentrations higher than 0.1 mM. Völkel et al. [52] reported that 80 min after the oral administration of BPA (5 mg), the maximal plasma concentration of BPA, albeit in its glucuronide form, attains some 0.8 μM in humans. Even though this concentration is much lower than 0.1 mM, BPA is lipophilic, and therefore may accumulate in fatty tissues, such as the central nervous system, resulting in a high concentration of BPA that inhibits nerve conduction without estrogen-receptor activation.

In conclusion, our findings indicate that BPA inhibits nerve conduction independent of estrogen-receptor activation. This action of BPA might contribute to the neurotoxic effects of the compound on the central nervous system.

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