Bisphenol S and Bisphenol F Are Less Disruptive to Cardiac Electrophysiology, as Compared With Bisphenol A

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ABSTRACT

Bisphenol A (BPA) is a high-production volume chemical used to manufacture consumer and medical-grade plastic products. Due to its ubiquity, the general population can incur daily environmental exposure to BPA, whereas heightened exposure has been reported in intensive care patients and industrial workers. Due to health concerns, structural analogs are being explored as replacements for BPA. This study aimed to examine the direct effects of BPA on cardiac electrophysiology compared with recently developed alternatives, including BPS (bisphenol S) and BPF (bisphenol F). Whole-cell voltage-clamp recordings were performed on cell lines transfected to express the voltage-gated sodium channel (Nav1.5), L-type voltage-gated calcium channel (Cav1.2), or the rapidly activating delayed rectifier potassium channel (hERG). Cardiac electrophysiology parameters were measured using human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and intact, whole rat heart preparations. BPA was the most potent inhibitor of fast/peak (INa-P) and late (INa-L) sodium channel ($IC_{50} = 55.3, 23.6\,\mu M$, respectively), L-type calcium channel ($IC_{50} = 30.8\,\mu M$), and hERG channel current ($IC_{50} = 127\,\mu M$). Inhibitory effects on L-type calcium channels were supported by microelectrode array recordings, which revealed a shortening of the extracellular field potential (akin to QT interval). BPA and BPF exposures slowed atrioventricular (AV) conduction and increased AV node refractoriness in isolated rat heart preparations, in a dose-dependent manner (BPA: $+9.2\%$ 0.001 μM, $+95.7\%$ 100 μM; BPF: $+20.7\%$ 100 μM). BPS did not alter any of the cardiac electrophysiology parameters tested. Results of this study demonstrate that BPA and BPF exert an immediate inhibitory effect on cardiac channels, whereas BPS is markedly less potent. Additional studies are necessary to fully elucidate the safety profile of bisphenol analogs on the heart.

Key words: bisphenol; plastics; cardiac electrophysiology; comprehensive in vitro proarrhythmia assay (CiPA).

Bisphenol A (BPA) is a high-production volume chemical, with roughly 8 million metric tons used each year to manufacture polycarbonate plastics (eg, food and beverage containers, medical devices), epoxy resins (eg, aluminum can liners), and in thermal printing applications (PR newswire, 2016; Shelby, 2008). Human exposure to BPA can occur daily, and as a result, biomonitoring studies have detected BPA in 91–99% of the general population (Calafat et al., 2005; Chen et al., 2016a; Lehmler et al., 2018; Vandenberg et al., 2010, 2007). Although environmental exposure to BPA occurs at a relatively low dose (Koch and Calafat, 2009; Vandenberg et al., 2007, 2010), occupational (Bousoumah et al., 2021; Hines et al., 2018; Ribeiro et al., 2017), and clinical environments can result in exceedingly high BPA exposure (Calafat et al., 2009; Duty et al., 2013; Gaynor et al., 2019; Huygh et al., 2015; Testai et al., 2016). Indeed, BPA was detected in 60% of neonatal intensive care unit (NICU) supplies, including items used for feeding, bandages, breathing support, intravenous, and parenteral infusion (Iribarne-Durán et al., 2019). Clinical exposure can also result in heightened and/or prolonged BPA exposure in young patients, due to an underdeveloped metabolic system (Calafat et al., 2009). In the NICU setting, premature infants had urinary BPA levels that ranged from 1.6 to 946 μg/l (Calafat et al., 2009) and the degree of exposure was linked to high-intensity treatment that required multiple (plastic) medical devices (Duty et al., 2013). Furthermore, higher urinary concentrations of BPA were detected in very low birth weight infants, as compared with term infants, likely due to increased medical device usage (Strømmen et al., 2021). Adult ICU patients were found to have urinary BPA levels that ranged from 6.1 to 680 μg/l and serum levels ranged from 2.6 to 255 μg/l when undergoing extracorporeal membrane oxygenation in conjunction with continuous veno-venous hemofiltration (Huygh et al., 2015). Similarly, urinary BPA levels increased approximately 4-fold in adult cardiac surgery patients postoperatively, as compared with preoperative levels (Shang et al., 2019).

BPA exposure is concerning, particularly in sensitive patient populations, as accumulating evidence suggests that BPA exerts a negative impact on cardiovascular health (Bae et al., 2012; Bae and Hong, 2015; Han and Hong, 2016; Melzer et al., 2010, 2012). A 10-year longitudinal study found that BPA exposure was associated with a 46–49% higher hazard ratio for cardiovascular and all-cause mortality (Bao et al., 2020). Furthermore, epidemiological studies have reported associations between BPA exposure and an increased risk of myocardial infarction, hypertension, coronary and peripheral artery disease, and a decrease in heart rate variability (reviewed previously; Posnack, 2014; Ramadan et al., 2020). Experimental studies have noted that BPA exposure can antagonize ion channels, impair electrical conduction, and precipitate triggered arrhythmias (Belcher et al., 2012; Deutschmann et al., 2013; Feiteiro et al., 2018; Michaela et al., 2014; Posnack et al., 2015; Wang et al., 2011; Yan et al., 2011). In vitro studies performed in kidney, neuronal, and smooth muscle cells have shown that BPA inhibits T-type and L-type calcium channel current (Deutschmann et al., 2013; Feiteiro et al., 2018; Michaela et al., 2014). In cardiac tissue, such an alteration in calcium channel current would alter nodal cell depolarization, atrioventricular (AV) conduction, and the plateau phase of the cardiac action potential. Furthermore, BPA exposure can disrupt intracellular calcium handling, resulting in calcium leak from the sarcoplasmic reticulum and an increased propensity for triggered arrhythmias (Gao et al., 2013; Liang et al., 2014; Ramadan et al., 2018). Of interest, BPA exposure was observed to increase calcium-mediated triggered activity and ventricular arrhythmias in females (but not males) subjected to

catecholamine stress (Patel et al., 2015). Notably, such alterations in calcium handling were attenuated in an estrogen-receptor knockout model (Yan et al., 2011), suggesting that BPA-induced effects may be sex-specific. To the best of our knowledge, experimental studies examining the impacts of bisphenol chemicals using human cardiomyocyte models are lacking.

With increasing health concerns, structurally similar chemicals are being explored as replacements for BPA (Chen et al., 2016a). Two such substitutes, bisphenol S (BPS) and bisphenol F (BPF), are used to manufacture consumer products that don "BPA-free" labeling. For example, BPS is used to produce polyethersulfone plastic food containers, medical-grade products, epoxy resins, and is found in thermal printing applications (Lehmler et al., 2018; Chen et al., 2016a). Unfortunately, many of these alternative chemicals are considered "regrettable substitutions," as BPS and BPF may exert biological effects that are similar to BPA (Kojima et al., 2019; Moon, 2019; Trasande, 2017). To date, it is unclear whether BPA alternatives are less cardiotoxic, as recent work suggests that BPS and BPF may also impair cardiac function (Ferguson et al., 2019; Gao et al., 2015; Mu et al., 2019). Recent biomonitoring studies have observed an uptick in BPS and BPF exposure in the general population as BPA is phased out and replaced (Lehmler et al., 2018), highlighting the urgent need to investigate the impact of BPA analogs on cardiac physiology.

We compared the cardiac electrophysiology effects of BPA, BPS, and BPF using whole-cell voltage-clamp experiments to identify the half-maximal inhibitory concentration (IC$_{50}$) of 4 key cardiac ion channels, highlighted by the comprehensive in vitro proarrhythmia assay (CiPA) initiative (Colatsky et al., 2016; Sager et al., 2014). Follow-up studies on human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) were performed using microelectrode array (MEA) recordings. Importantly, hiPSC-CM have been widely adopted as a tool for preclinical safety testing to measure alterations in cardiac automaticity, conduction velocity, depolarization, and repolarization time (Chen et al., 2016b). To aid in the translation of our in vitro cell studies, we employed an intact, whole rat heart preparation for direct assessment of cardiac electrophysiology. We previously reported that BPA treatment prolongs AV conduction in isolated rat heart preparations during sinus rhythm (Posnack et al., 2014), accordingly, we hypothesized that inhibitory effects of bisphenol chemicals on calcium current would impair AV conduction and increase refractoriness in cardiac preparations. Furthermore, we hypothesized that BPS and/or BPF exposure would have less effect on cardiac electrophysiology due to differences in chemical structure that may increase the potency of BPA for voltage-gated channels. The latter is supported by a study by Deutschmann et al. (2013), which reported a direct interaction between BPA and the extracellular part of voltage-gated calcium channels with structure-effect analyses suggesting that its angulated orientation may promote this interaction.

MATERIALS AND METHODS

Reagents. Chemical reagents were purchased from Sigma Aldrich (St Louis, MO). BPA (CAS #80-05-7), BPS (CAS #80-09-1), and BPF (CAS #620-92-8) ($\geq$98% purity, analytical standard) stock solutions were prepared in 99+% dimethyl sulfoxide (DMSO). Working concentrations were prepared directly in iCell culture media (Fujifilm cellular dynamics; Madison, Wisconsin) for voltage-clamp recordings and MEA studies or Krebs-Henseleit (KH) crystalloid buffer (Reilly et al., 2020) for intact heart

preparations to obtain a final concentration between 0.001 and 100 μM BPA, BPS, or BPF. The range of concentrations was selected to mimic human environmental exposure (1–100 nM), maximum clinical or occupational exposure ($\leq$ 10 μM), and supraphysiological exposure levels ($>$ 10 μM) (Bousoumah et al., 2021; Ramadan et al., 2020). A wider range of doses were employed for whole-cell voltage-clamp recordings, when necessary, to pinpoint the half half-maximal inhibitory concentration (0.01–3 mM). To reduce the risk of environmental contamination, nonpolycarbonate bisphenol-free plastic was used for experiments (including Tygon tubing [Saint-Gobain in North America; Malvern, Pennsylvania], polystyrene cell culture plates [Axion biosystems; Atlanta, Georgia], and glass tissue bath [Radnoti; Covina, California]).

Whole-cell voltage-clamp recordings. Environmental chemicals can alter the cardiac action potential, electrical conduction, or elicit arrhythmias through inhibition of sodium, calcium, and potassium ion currents. To test the effects of bisphenol chemicals on cardiac currents, Nav1.5, Cav1.2, and hERG channel recordings were performed using stably transfected cell lines, as previously described (Jaimes et al., 2019). For each measurement, cells were exposed to bisphenol-containing solutions for approximately 5–10 min, until currents reached stability. For *Nav1.5 recordings*, the extracellular solution included 137 mM sodium chloride, 10 mM dextrose, 10 mM HEPES, 4 mM potassium chloride, 1 mM magnesium chloride, and 1 mM calcium chloride. The intracellular solution consisted of 120 mM cesium hydroxide, 120 mM aspartic acid, 10 mM egtazic acid, 10 mM cesium chloride, 10 mM HEPES, 5 adenosine 5′-triphosphate magnesium salt, and 0.4 mM guanosine 5′-triphosphate salt. The voltage protocol was approximately 1 sec in duration, repeated at 0.1 Hz. Sodium channel recordings were performed using human embryonic kidney cells (HEK293) transfected with Nav1.5 cDNA. Cells were repolarized from −95 to −120 mV for 200 ms, depolarized from −120 to −15 mV for 40 ms, and then further depolarized to +40 mV for 200 ms. This was followed immediately by a voltage ramp-down phase from +40 to −95 mV for 100 ms. Neurotoxin-2 (ATXII, 20 nmol/l) was included in the extracellular solution to induce Nav1.5 late current, as previously described (Mantegazza et al., 1998). Tetrodotoxin (30 μM) was applied at the end of each recording to determine the current baseline. *Cav1.2 recordings* were performed using Chinese hamster ovary cells (CHO) stably transfected with Cav1.2 cDNA. Cells were depolarized from −80 to 0 mV for 40 ms, further depolarized to +30 mV for 200 ms, followed by a voltage ramp-down phase from +30 to −80 mV for 100 ms. Recording stability was assessed by applying the voltage protocol in the control solution for 12 consecutively recorded traces with $<$ 10% difference. *hERG recordings* were performed using HEK293 cells stably transfected with hERG cDNA. The extracellular solution included 130 mM sodium chloride, 12.5 mM dextrose, 10 mM HEPES, 5 mM potassium chloride, 1 mM magnesium chloride hexahydrate, and 1 mM calcium chloride. The intracellular solution consisted of 120 potassium-gluconate, 20 mM potassium chloride, 10 mM HEPES, 5 egtazic acid, and 1.5 adenosine 5′-triphosphate magnesium salt. The voltage protocol was 5 sec in duration, repeated at 0.1 Hz. Cells were depolarized −80 to +40 mV for 500 ms, followed by a voltage ramp-down phase from +40 to −80 mV for 100 ms. An hERG potassium channel blocker (10 μM E-4031) was applied at the end of each recording to determine the baseline. Recordings were collected before and after bisphenol chemical exposure; chemical potency was calculated by dividing the steady-state current amplitude by the average amplitude from the last 5 traces measured in the control solution to calculate the fractional block. This was plotted against the bisphenol chemical concentration tested, fitted with the Hill Equation to generate a half-maximal inhibitory concentration (IC$_{50}$) and the Hill coefficient.

Human cardiomyocyte MEA recordings. hiPSC-CM (iCell cardiomyocytes[2], female donor #01434, Fujifilm cellular dynamics; Madison, Wisconsin) were plated onto fibronectin-coated MEAs at a density of 50–75 000 cells/well (24-well plate, Axion Biosystems; Atlanta, Georgia). Cells were defrosted in iCell cardiomyocyte plating media in a cell culture incubator (37°C, 5% CO$_2$) for 2 h, thereafter cells were cultured in iCell maintenance media for 4–7 days, per the manufacturer's instructions. Treatment groups included 0.01% DMSO (vehicle), 0.01–100 μM BPA, BPS, or BPF. Cells were treated for 15 min, and then extracellular field potential signals were recorded in response to external stimulation (1–2 Hz). Extracellular field potential duration (FPD) was measured and rate corrected with the Fridericia formula (FPDc). Disturbances in the recorded waveform can be used to predict the identity of ion channels impacted by chemical exposure, with FPD analogous to an in vitro QT interval that correlates with action potential duration at 50% repolarization (Asakura et al., 2015; Clements, 2016). Although hiPSC-CM can display an immature phenotype as compared with adult cardiomyocytes, recent comparative studies have demonstrated good correlation between FPDc and clinical trial QTc prolongation in response to 26 different drugs when using commercially available hiPSC-CM cell lines (iCell and Cor4.U) (Blinova et al., 2017).

Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee at Children's National Research Institute and followed the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.* Bisphenol chemicals are xenoestrogens that may cause exaggerated cardiac effects in females (Ben-Jonathan and Steinmetz, 1998; Yan et al., 2011); accordingly, experiments were performed using female Sprague Dawley rats, aged 3–4 months (strain NTac: SD, from NIH Genetic Resource stock, Taconic Biosciences; Germantown, New York). Animals were housed in conventional acrylic rat cages in the Research Animal Facility, under standard environmental conditions (12:12 h light:dark cycle, 18°C–25°C, 30%–70% humidity). Each animal served as its own control, with electrophysiology measurements collected at baseline and again after treatment.

Intact heart preparations. Animals were anesthetized with 3% isoflurane; the chest was opened, the heart was rapidly excised, and the aorta was cannulated. The isolated, intact heart was then transferred to a temperature-controlled (37°C) constant-pressure (70 mmHg) Langendorff-perfusion system. Excised hearts were perfused with a modified KH buffer bubbled with carbogen, as previously described (Jaimes et al., 2019; Swift et al., 2020). Pseudo-electrocardiograms (ECG) were recorded in lead II configuration, and biosignals were acquired in iox2 and analyzed in ecgAUTO (EMKA technologies; Sterling, Virginia). Isolated hearts remained stable with minimal fluctuations in heart rate or electrophysiology parameters following 0.01% DMSO media perfusion (vehicle; Figure 1). To account for animal variability, ECG recordings were collected during control media perfusion (15 min) and in response to bisphenol exposure (15 min). Similarly, electrophysiology measurements (see below) were measured at baseline, after 15 min chemical exposure, and again after 15 min washout with KH media. Results of 2-

dimensional hiPSC-CM studies may/may not translate to a whole heart with specialized anatomy, cell populations (atrial, nodal, ventricular), spatial tissue heterogeneity, a coordinated conduction system, and increased metabolic demand. Accordingly, an intact heart model can aid in the translational impact of our findings—and also allows for a direct comparison to the existing bisphenol chemical literature (Belcher *et al.*, 2015; Ferguson *et al.*, 2019; Gear *et al.*, 2017; Yan *et al.*, 2011).

Electrophysiology measurements. A pacing electrode was positioned on the right atrium for assessment of AV conduction time, AV node refractory period (AVNERP), and Wenckebach cycle length (WBCL) (Swift *et al.*, 2019). WBCL was defined as the shortest S1-S1 pacing interval that resulted in 1:1 AV

conduction. AVNERP was defined as the shortest S1-S2 pacing interval that resulted in 1:1 AV conduction. Electrophysiology studies were performed using a Bloom Classic electrophysiology stimulator (Fischer Medical; Wheat Ridge, Colorado) set to a pacing current $1.5\times$ the minimum pacing threshold, with a 1 ms monophasic pulse width. For each parameter, the pacing cycle length (PCL) was decremented to pinpoint the PCL before loss of capture was observed.

Statistical analysis. Results are reported as mean $\pm$ standard deviation. Data normality was assessed by Shapiro-Wilk testing (GraphPad Prism, GraphPad Software; San Diego, California). Statistical analysis was performed using one-way analysis of variance (ANOVA) for MEA recordings or 2-way analysis of variance with repeated measures (RM-ANOVA) to compare baseline versus treatment in whole heart experiments. Significance was defined by an adjusted p-value ($q < 0.1$) after correction for multiple comparisons using a 2-stage linear step-up procedure to control the false discovery rate (0.1) as described by Benjamini *et al.* 2006). Significance is denoted in the figures with an asterisk (*).

RESULTS

BPA Exerts a Greater Inhibitory Effect on Ion Channels, Compared with BPS or BPF

Whole-cell voltage-clamp recordings were performed on cells transfected with 1 of 4 cardiac ion channels, as highlighted by CiPA (Colatsky *et al.*, 2016; Sager *et al.*, 2014). Currents were evoked and recorded before and after exposure to BPA, BPS, or BPF, and a half-maximal inhibitory concentration (IC_{50}) was computed by testing the respective potency to each ion channel. Collectively, BPA had the highest affinity for each ion channel tested, compared with both BPF and BPS, and current suppression was concentration-dependent (**Figures 2 and 3**). Peak sodium current (I_{Na-P}) was suppressed with an IC_{50} of 55.3 μM BPA, 232 μM BPF, and 1090 μM for BPS; late sodium current (I_{Na-L}) was suppressed at lower doses, with a computed IC_{50} of 23.6 μM BPA, 100 μM BPF, and 369 μM BPS (**Figs. 2A and 2B**). In ventricular tissue, I_{Na-P} is responsible for action potential upstroke (phase 0), and I_{Na-L} is involved in the plateau phase (phase 2). Accordingly, inhibition of I_{Na-P} is likely to slow depolarization and electrical conduction, whereas inhibition of I_{Na-L} can shorten the action potential duration. L-type calcium channel current (I_{CaL}) was also the most sensitive to BPA exposure, with an IC_{50} of 30.8 μM, compared with 76 μM BPF and 333 μM BPS (**Figure 3A**). Calcium current (I_{CaL}) plays a prominent role in the plateau phase of the ventricular action potential and also contributes to the action potential upstroke in nodal cells. Inhibition of I_{CaL} can slow sinus rate, delay AV conduction, increase AV refractoriness, and shorten the ventricular myocyte action potential. Finally, the rapid delayed rectifier potassium current (I_{Kr}) was suppressed at higher bisphenol concentrations, with a measured IC_{50} of 127 μM BPA, 209 μM BPF, and 633 μM BPS (**Figure 3B**). Bisphenol exposure could suppress I_{Kr} and prolong cardiac repolarization (phase 3) at high concentrations.

BPA and BPF Exposures Alter the Extracellular Field Potential of Human Cardiomyocytes

To further investigate the effects of bisphenol chemicals on cardiac electrophysiology, we employed hiPSC-CM that express key cardiac ion channels (Edwards and Louch, 2017). hiPSC-CM were cultured atop microelectrodes and extracellular field
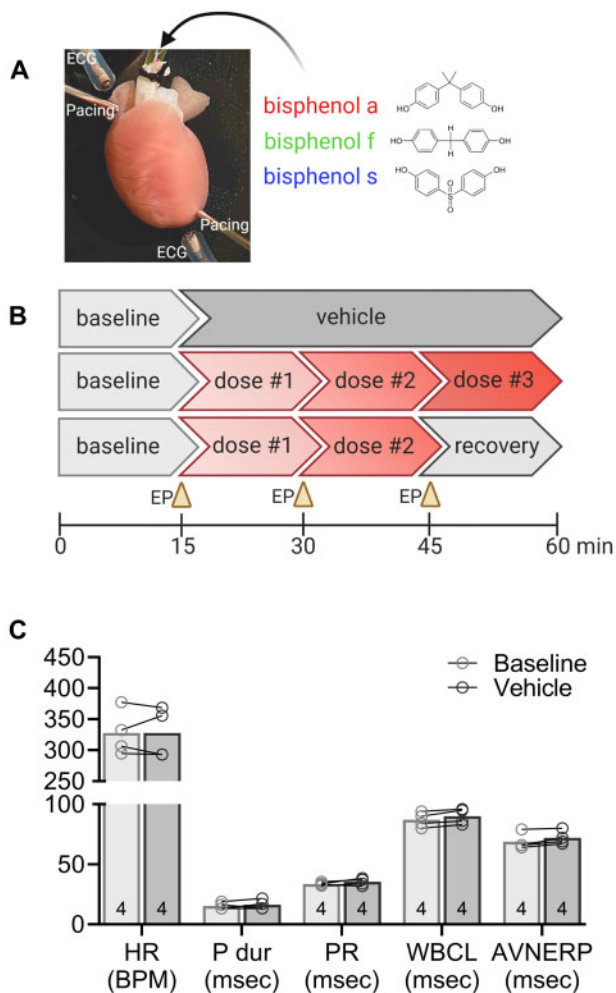

Figure 1. Experimental design and vehicle control parameters. A, Langendorff-perfused rat heart shown with pacing electrodes on the right atria and apex, and monopolar electrodes placed to record electrocardiograms. B, The schematic timeline depicts perfusion protocols used in the study, including (top) vehicle control exposure, (middle) bisphenol chemical dose response, and (bottom) bisphenol chemical dose response and subsequent recovery. C, Cardiac electrophysiology parameters are consistent over time, and similar between baseline and vehicle (0.01% DMSO) exposure. Values reported as mean $\pm$ SD. Statistical significance determined by RM-ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph ($n = 4$). Abbreviations: ECG, location of electrocardiogram electrode; EP, electrophysiology protocol; HR, heart rate; BPM, beats per minute; P dur, P wave duration; PR, PR interval; WBCL, Wenckebach cycle length; AVNERP, atrioventricular node effective refractory period; msec, milliseconds; DMSO, dimethyl sulfoxide.

Nav1.5 ($I_{Na\text{-}P}$) current
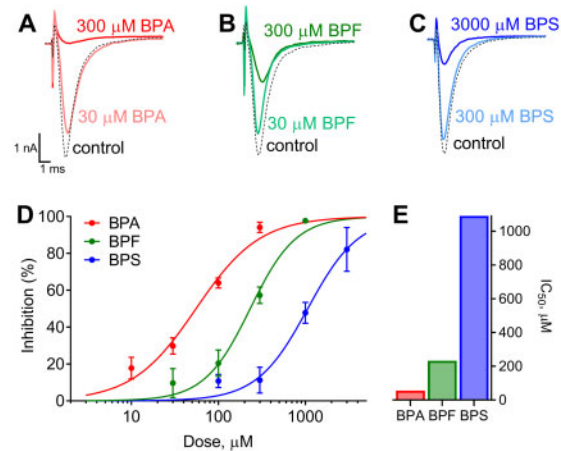
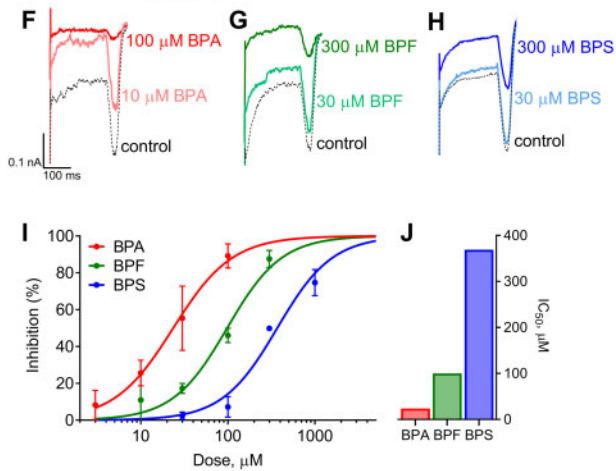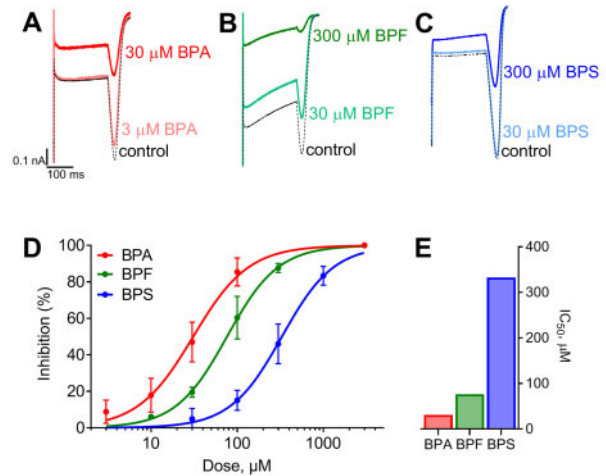## Nav1.5 ($I_{Na\text{-}L}$) current

Figure 2. Bisphenol inhibition of sodium currents. Whole-cell voltage-clamp recordings of fast/peak sodium current ($I_{Na\text{-}P}$) following acute (5–10 min) exposure to (A) BPA, (B) BPF, or (C) BPS. D, Dose-dependent inhibition of $I_{Na\text{-}P}$ (mean ± SD). E, Calculated IC_{50} values are shown. Whole-cell voltage-clamp recordings of late sodium current ($I_{Na\text{-}L}$) following exposure to (F) BPA, (G) BPF, or (H) BPS. I, Dose-dependent inhibition of $I_{Na\text{-}L}$ (mean ± SD). J, Calculated IC_{50} values are shown. $n = 4$ independent cell recordings per study. Abbreviations: BPA, bisphenol A; BPS, bisphenol S; BPF, bisphenol F.

CaV1.2 (I_{CaL}) current
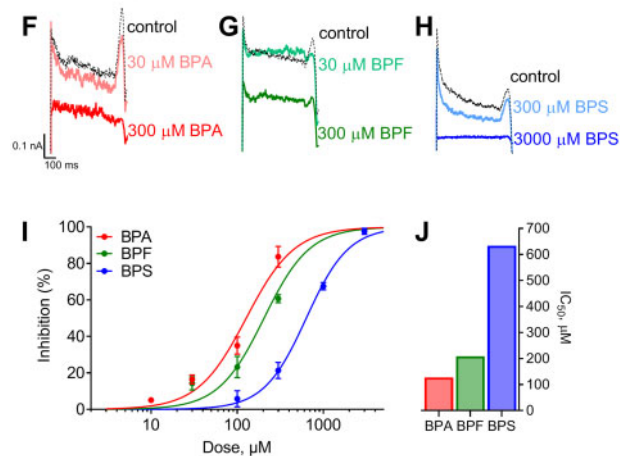

hERG (I_{Kr}) current

Figure 3. Bisphenol inhibition of calcium and potassium currents. Whole-cell voltage-clamp recordings of L-type calcium current (I_{CaL}) following acute (5–10 min) exposure to (A) BPA, (B) BPF, or (C) BPS. D, Dose-dependent inhibition of I_{CaL} (mean ± SD). E, Calculated IC_{50} values are shown. Whole-cell voltage-clamp recordings of hERG current (I_{Kr}) following exposure to (F) BPA, (G) BPF, or (H) BPS. I, Dose-dependent inhibition of I_{KrL} (mean ± SD). J, Calculated IC_{50} values are shown. $n = 4$ independent cell recordings per study.

potentials were recorded (**Figs. 4A, 4D, and 4G**), with FPD measurements analogous to an in vitro QT interval that correlates with the action potential duration (Asakura *et al.*, 2015; Clements, 2016). Acute BPA exposure resulted in a slight non-monotonic dose response (**Figure 4B**), wherein no effect on FPDc was observed at the lowest BPA dose tested (0.01 μM), and a 7.5% increase in FPDc was observed at 0.1 μM BPA ($q < 0.005$). Notably, low-dose effects have previously been reported for BPA and other endocrine-disrupting chemicals that can present with a nonmonotonic dose response (Birnbaum, 2012; Gao *et al.*, 2015; Liang *et al.*, 2014; Vandenberg, 2014). At higher BPA doses, FPDc shortened significantly compared with the vehicle (13.8% at 30 μM, 37.3% at 100 μM, $q < 0.0001$). Low-dose effects were not observed for either BPF or BPS (**Figs. 4E and 4H**). However, BPF exposure resulted in FPDc shortening at higher concentrations (3.7% at 10 μM [$q < 0.05$], 12.5% at 30 μM [$q < 0.0001$], 32.4% at 100 μM [$q < 0.0001$]). Treatment with BPS did not alter FPDc at

any of the tested concentrations. FPDc restitution curves were generated by increasing the pacing frequency (1–2 Hz). A frequency-dependent effect was not observed for BPA in hiPSC-CM, although BPF exhibited a slight reverse-use dependency with FPDc shortening more prominent at lower frequencies (**Figs. 4C and 4F**).

BPA and BPF Exposures Slow Heart Rate

To aid in the translation of our in vitro findings, we quantified the acute effects of BPA, BPF, and BPS on cardiac electrophysiology using an ex vivo intact heart preparation. Heart preparations exhibited normal sinus rhythm when perfused with control KH media (327.7 ± 36.8 BPM) and KH media supplemented with vehicle (327.6 ± 40.3 BPM; **Figure 1C**). BPA exposure resulted in a measurable decline in heart rate, which may be partly attributed to calcium channel current inhibition. Sinus
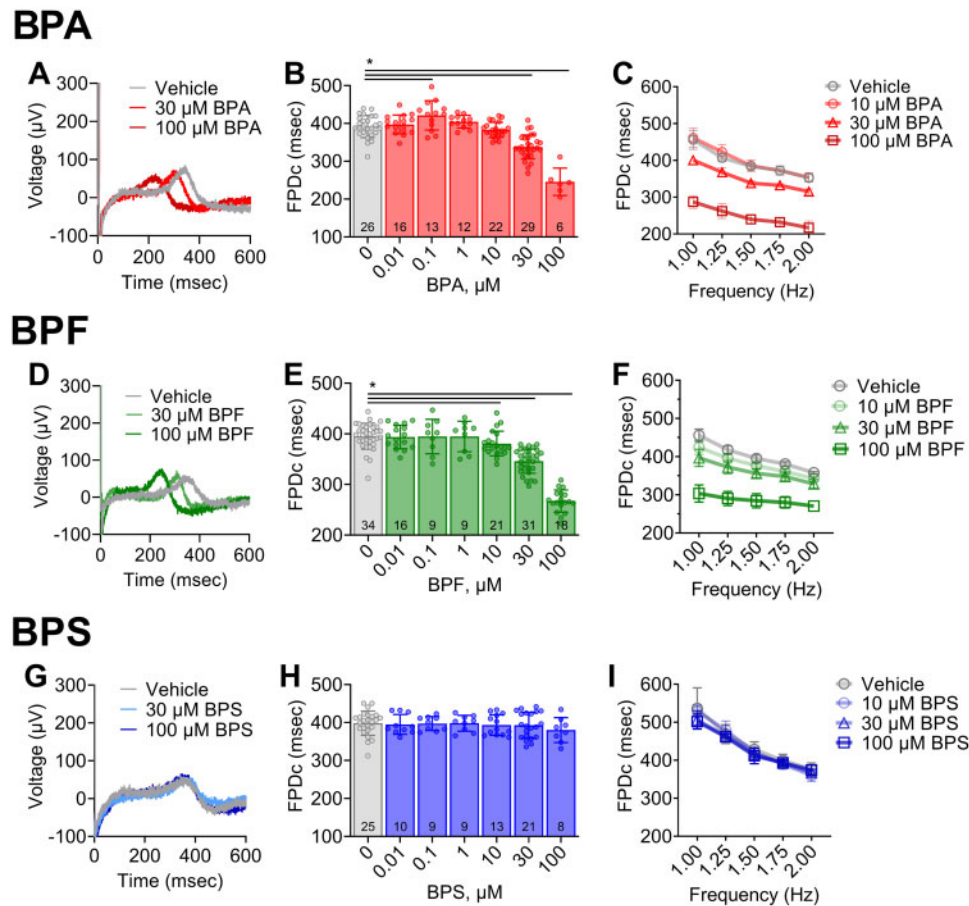
Figure 4. Bisphenol chemical effects on cardiomyocyte field potential duration. A, Representative traces of extracellular field potentials recorded from hiPSC-CM following acute (15 min) exposure to vehicle, 30, or 100 µM BPA. B, Field potential duration (corrected using Fridericia formula: "FPDc") shortens with increasing BPA exposure; single pacing frequency (1.5 Hz). C, FPDc restitution curve at multiple pacing frequencies (1–2 Hz). D, Local field potential traces following exposure to vehicle, 30, or 100 µM BPF. E, FPDc shortens with increasing BPF exposure (1.5 Hz). F, FPDc restitution curve (1–2 Hz). G, Local field potential traces following exposure 30–100 µM BPS. H, FPDc remains constant with increasing BPS exposure (1.5 Hz). I, FPDc restitution curve (1–2 Hz). Values reported as mean ± SD. *$q < 0.05$ as determined by ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph.

rate slowed by 16.6% ($q < 0.01$) and 85.4% ($q < 0.0001$) after exposure to 10 and 100 µM BPA, respectively (relative to baseline recording; Figs. 5A and 5B). This depressive effect culminated in the cessation of ventricular electrical activity in 62% of heart preparations treated with 100 µM BPA (Figure 5D). Heart rate slowing was immediate, yet reversible, as sinus rhythm recovered quickly after removal of BPA and replacement with control media perfusion. A nonmonotonic BPA dose-response relationship was not observed. Heart rate also slowed by 12.5% ($q < 0.001$) after exposure to 100 µM BPF, the only dose to significantly affect automaticity (Figure 5E). Conversely, no significant change in sinus rhythm or rate was observed after exposure to BPS at the concentrations tested (Figure 5G).

BPA and BPF Exposures Slow AV Conduction
Heart preparations exhibited stable atrial and AV conduction with control media perfusion (15.3 ± 2.8 ms P duration, 33.6 ± 1.4 ms PR interval), and during perfusion with media supplemented with vehicle (16.3 ± 4.0 msec P duration, 35.3 ± 3.1 ms PR interval). Concurrent with heart rate slowing due to BPA exposure, we also observed significant lengthening of both the P duration and PR interval. A prolonged P duration was only observed at the highest BPA dose (39.8 ± 16.6 msec at 100 µM BPA, Figure 6B), whereas the PR interval progressively lengthened

with higher BPA concentrations (Figs. 6A and 6D). BPA exposure resulted in variable degrees of AV block, ranging from 1st degree to intermittent 3rd degree AV block (Figure 6D). Notably, the acute effect of BPA on AV conduction was reversible with a rapid recovery of the PR interval time after 15-min washout (Figure 6E). Atrial pacing was implemented, and AV conduction slowing persisted at multiple PCL (Figure 6F). BPF exposure also lengthened the PR interval, albeit the effect was much less pronounced (+19.6% 100 µM BPF). There was no observable change in AV conduction following BPS exposure at the tested concentrations (Figure 6I).

BPA and BPF Exposures Increase AV Nodal Refractoriness
To further investigate slowed AV conduction in the presence of bisphenols, incremental atrial pacing was implemented to pinpoint the Wenckebach phenomenon. WBCL was comparable between control media perfusion (87 ± 6.2 msec) and during perfusion with vehicle (90 ± 6.5 msec). However, it proved to be a highly sensitive parameter for bisphenol-induced slowing of AV conduction (Figs. 7A and 7B). BPA exposure altered WBCL in a dose-dependent manner beginning at a low nanomolar concentration (+7.6% 0.01 µM BPA [$q < 0.1$]; +68% 100 µM BPA [$q < 0.0001$]), suggesting a lengthening of the relative refractory period. Pinpointing an accurate WBCL after 100 µM BPA
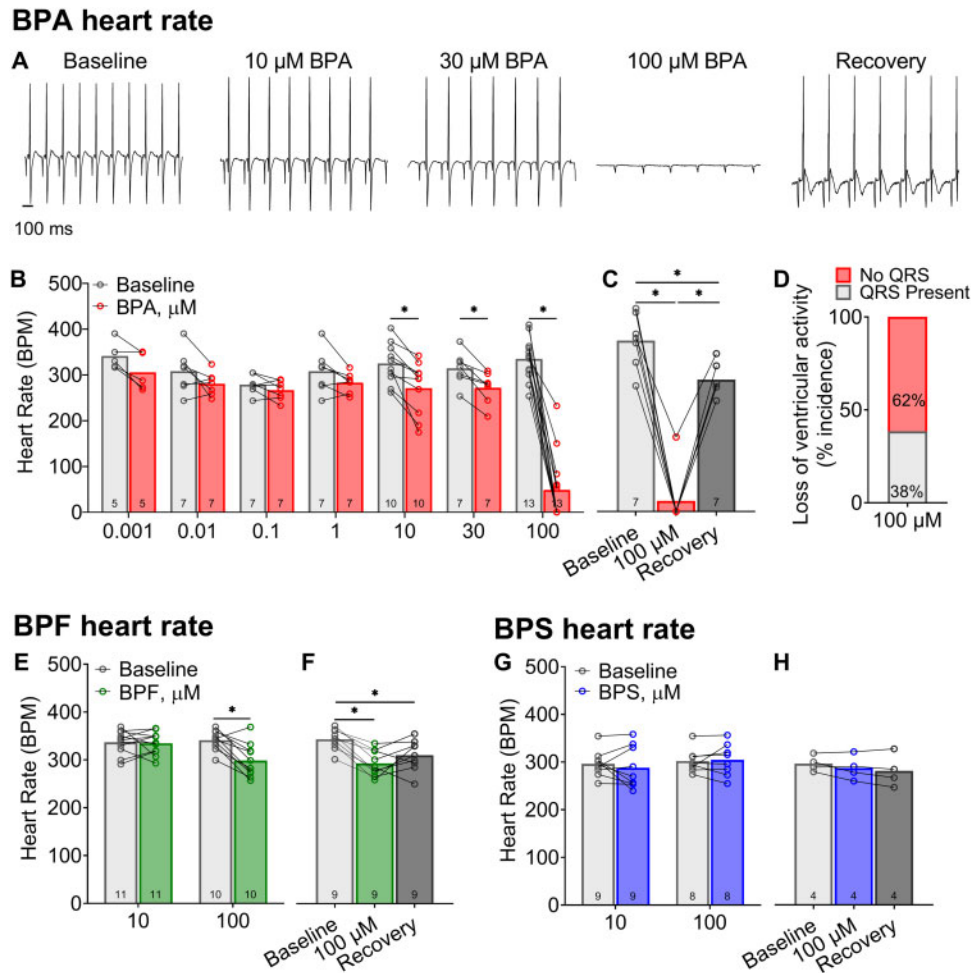
Figure 5. Bisphenol chemical effects on heart rate. A, Representative ECG recordings from Langendorff-perfused hearts at baseline, acute (15 min) exposure to BPA, or recovery (100 μM BPA exposure, followed by 15 min washout). B, BPA exposure results in sinus rate slowing, beginning at 10 μM BPA. C, Heart rate slowing after 100 μM BPA exposure largely recovers after washout (15 min). D, Heart rate measurements at high BPA doses were confounded by intermittent 3rd degree heart block, with loss of ventricular electrical activity. E, Heart rate slowing following BPF exposure occurred at the highest concentration tested (100 μM BPF). F, Heart rate slowing after 100 μM BPF recovered slightly after washout. G and H, BPS exposure had no discernable effect on heart rate. Values reported as mean ± SD. *$q < 0.05$ as determined by RM-ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph.

exposure was confounded by the loss of capture at the slowest cycle length tested (150 ms; **Figure 7C**). BPF exposure had a moderate effect on WBCL, but only at the highest concentration tested (22.5% 100 μM BPF [$q < 0.0001$]). BPS exposure did not alter WBCL at the tested concentrations, which was in agreement with PR interval measurements during sinus rhythm (**Figure 7G**). Extrastimulus pacing was also performed to measure the effective refractory period of the AV node. Similar to WBCL measurements, BPA exposure increased AV node refractoriness in a dose-dependent manner (**Figs. 8A and 8B**), beginning with modest changes at a low nanomolar concentration (+9.2% 0.001 μM BPA [$q < 0.05$]) and increasing thereafter (+95.7% 100 μM BPA [$q < 0.0001$]). BPF exposure had a moderate effect on AVNERP, but only at the highest concentration tested (20.7% 100 μM BPF [$q < 0.0001$]). BPS exposure did not alter AVNERP at the tested concentrations (**Figure 8G**).

DISCUSSION

This is the first study to compare the acute effects of BPA, BPS, and BPF on cardiac electrophysiology using both in vitro and ex vivo cardiac preparations. In the described study, we

demonstrate that BPA is the most potent inhibitor of sodium, calcium, and potassium channel currents—as compared with the chemical alternatives, BPS and BPF. In the context of industrial or clinical environments, individuals can present with urinary BPA concentrations that are exceedingly high—reaching 4–8 μM (Calafat et al., 2009; He et al., 2009; Wang et al., 2012). Accordingly, we tested a range of concentrations that encompass environmental, clinical, occupational, and supraphysiological exposures (Bousoumah et al., 2021; Ramadan et al., 2020). We found that BPA exerted the greatest effect on automaticity and AV conduction—which may be mediated by inhibitory effects on calcium current. Electrical disturbances were largely focused on nodal and AV conduction, with negligible effects on cardiac repolarization or arrhythmia susceptibility. Results of this study suggest that acute BPS exposure is less disruptive to cardiac electrophysiology, as compared with BPA or BPF. Epidemiological or clinical studies are needed to investigate an association between bisphenol chemical exposure and bradycardia or slowed AV conduction; moreover, bisphenol chemical exposure could exert an additive effect in patients with subclinical AV node abnormalities, ischemic conditions, or in patients being treated for cardiac arrhythmias (eg, beta-blocker usage).
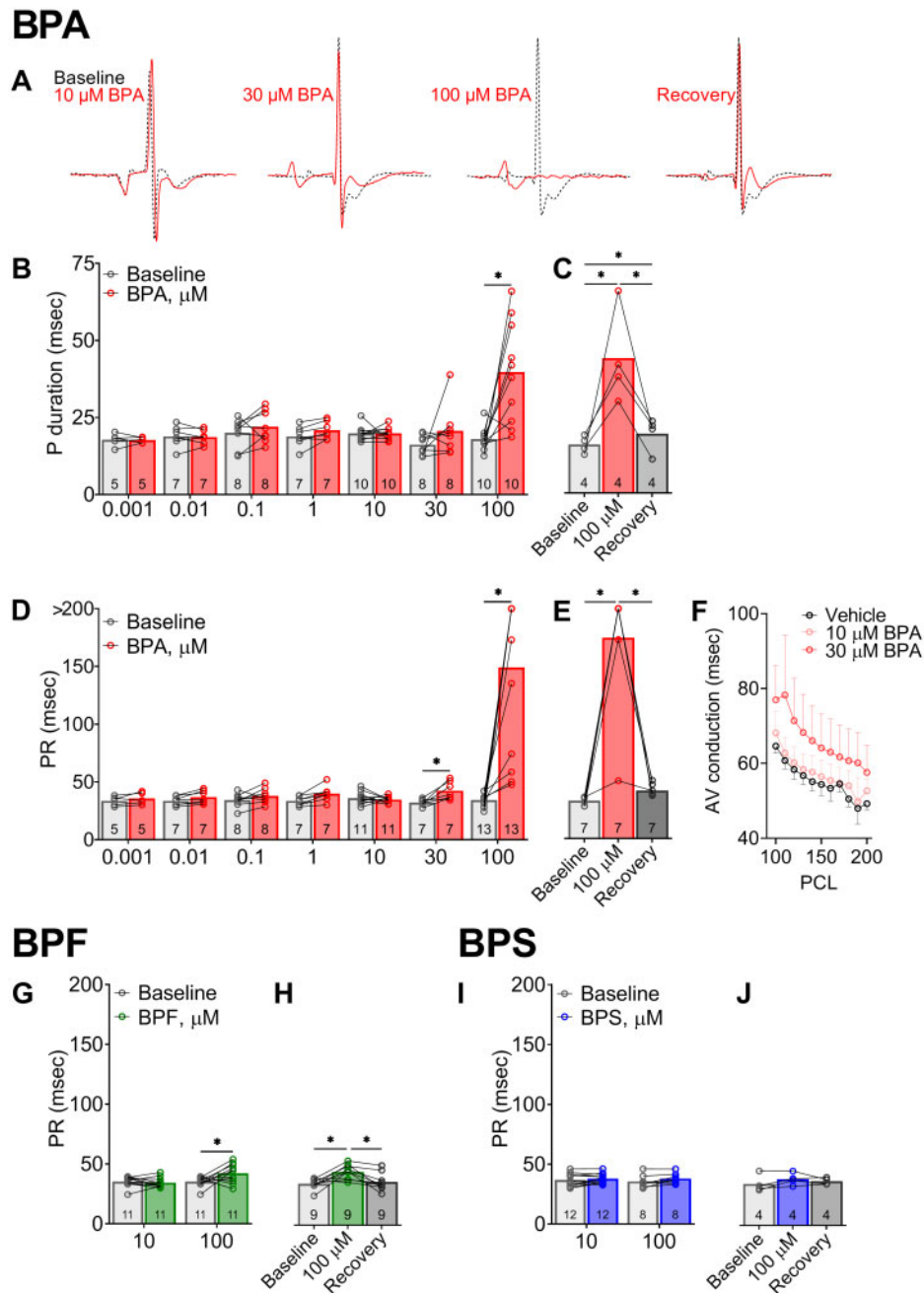
Figure 6. Bisphenol chemical effects on atrial and atrioventricular conduction during sinus rhythm. A, Representative ECG waveform from Langendorff-perfused hearts at baseline, acute BPA exposure (15 min), or recovery (100 µM BPA exposure, followed by 15 min washout). Each waveform pair recorded from the same animal, before and after exposure. B, P duration indicates longer atrial depolarization time at the highest BPA dose (100 µM). C, Slowed atrial conduction after 100 µM BPA exposure recovers after washout. D, The progressive lengthening of PR duration indicates slowed AV conduction following 30–100 µM BPA exposure, often resulting in intermittent 3rd degree heart block (denoted by data point > 200 ms). E, AV conduction slows after 100 µM BPA exposure and recovers after washout. F, AV conduction slowing persists with external pacing to correct for heart rate. G, Atrioventricular conduction slowing occurs at 100 µM BPF concentration and (H) recovers after washout. I and J, BPS exposure had no discernable effect on atrioventricular conduction time. Values reported as mean ± SD. *$q < 0.05$ as determined by RM-ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph. Abbreviation: PCL, pacing cycle length.

Additional mechanistic studies are required to fully elucidate the safety profile of bisphenol chemicals on cardiac electrical and mechanical function, and report on the chronic effects of bisphenol exposure.

Bisphenol Chemicals and Calcium Ion Homeostasis

Of the bisphenol chemicals tested in this study, BPA was the most potent inhibitor of L-type calcium channels with an $IC_{50} =$ 30.8 µM. This finding is in agreement with the literature, which reported an immediate inhibitory effect of BPA on T-type calcium channels in human embryonic kidney cells ($IC_{50} = 6$–33 µM, depending on channel subtype [Michaela et al., 2014]). Similarly, Deutschmann et al. (2013) reported that BPA rapidly and reversibly inhibited calcium current through L-, N-, P/Q-, R-, and T-type calcium channels in rat endocrine cells, dorsal root ganglion, cardiomyocytes, and transfected human embryonic
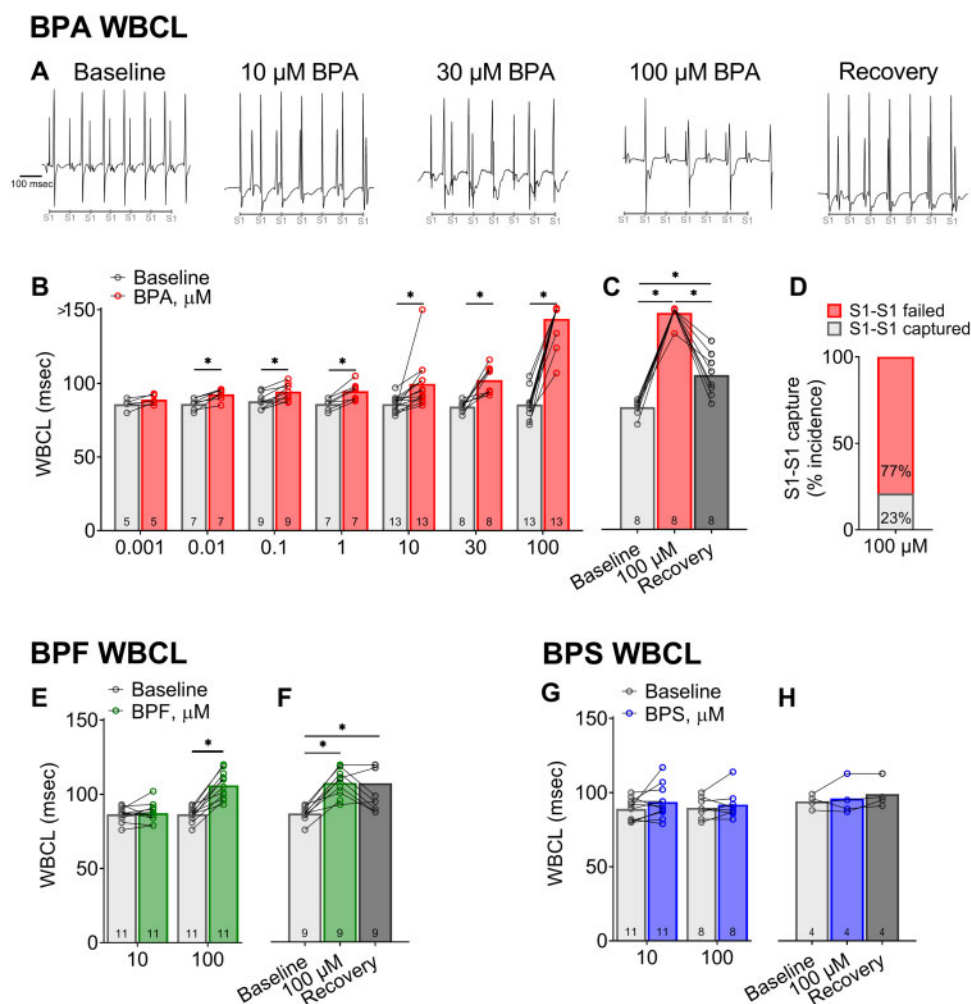
Figure 7. Bisphenol chemical effects on atrioventricular conduction in response to atrial pacing. A, Representative ECG recordings during atrial pacing show failure to capture in BPA-treated hearts, indicating slowed atrioventricular conduction. The timing of S1-S1 pulses (90 msec) is indicated below. B, Longer Wenckebach cycle length (WBCL) following exposure to BPA concentrations (0.01–100 μM), as compared with baseline. Note: Complete Heart block denoted by measurement > 150 msec (longest S1 pacing interval tested). C, Longer WBCL after 100 μM BPA exposure largely recovers after 15-min washout. D, WBCL measurements at high BPA doses were confounded by complete heart block. E, 100 μM BPF exposure results in longer WBCL. F, Moderate recovery in atrioventricular conduction after BPF washout. G and H, No change in WBCL was observed after exposure to 10–100 μM BPS. Values reported as mean ± SD. $^*q < 0.05$ as determined by RM-ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph. Abbreviation: ECG, electrocardiogram.

kidney cells (IC_{50} = 26–35 μM). Studies suggest that the inhibitory effects of bisphenol chemicals on calcium channel current are influenced by the chemical structure and bridge between the 2 phenol rings, with reduced inhibitory effects anticipated for BPS and BPF (Deutschmann et al., 2013). In cardiac tissue, calcium channels play an important role in nodal cell depolarization, AV conduction, the plateau phase of the cardiac action potential, and contractility. Indeed, recent studies have shown that BPA can alter cardiac electrophysiology, likely through a calcium-dependent mechanism. Sinus bradycardia and delayed electrical conduction have been reported after BPA exposure, using in vivo and ex vivo models (Belcher et al., 2015; Patel et al., 2015; Posnack et al., 2014; Valokola et al., 2019). Patel et al. (2015) observed conduction slowing in BPA-exposed animals subjected to catecholamine stress, although this effect was limited to females. BPA-induced heart rate slowing was also reported in in vivo studies conducted by Belcher et al. (2015), although the authors noted that this effect might be attributed to autonomic dysregulation. In addition to electrophysiology disturbances, BPA exposure has been shown to alter intracellular calcium

handling, which can increase calcium leak from the sarcoplasmic reticulum (Yan et al., 2011), increase the incidence of calcium-mediated arrhythmias, and precipitate calcium amplitude alternans (Ramadan et al., 2018). Studies suggest that intracellular calcium handling may be influenced by post-translational modifications of key calcium proteins via an estrogen-mediated mechanism (Liang et al., 2014).

Bisphenol Chemicals and Sodium Channel Current
Similar to calcium channel inhibition, we found that BPA was the most potent inhibitor of fast (INa-P) and late (INa-L) sodium channel (IC_{50} = 55.3 and 23.6 μM, respectively). This finding is in agreement with previously published studies, which reported that BPA blocks fast voltage-gated sodium channels in transfected HEK cells (IC_{50} = 25 μM; O'Reilly et al., 2012) and isolated dorsal root ganglion neurons (IC_{50} = 40 mM; Wang et al., 2011). These effects were rapid, reversible, and dose-dependent (Wang et al., 2011). Moreover, in isolated ganglion neurons, the described effects were attenuated with protein kinase A (PKA) or protein kinase C (PKC) inhibitors, suggesting an underlying
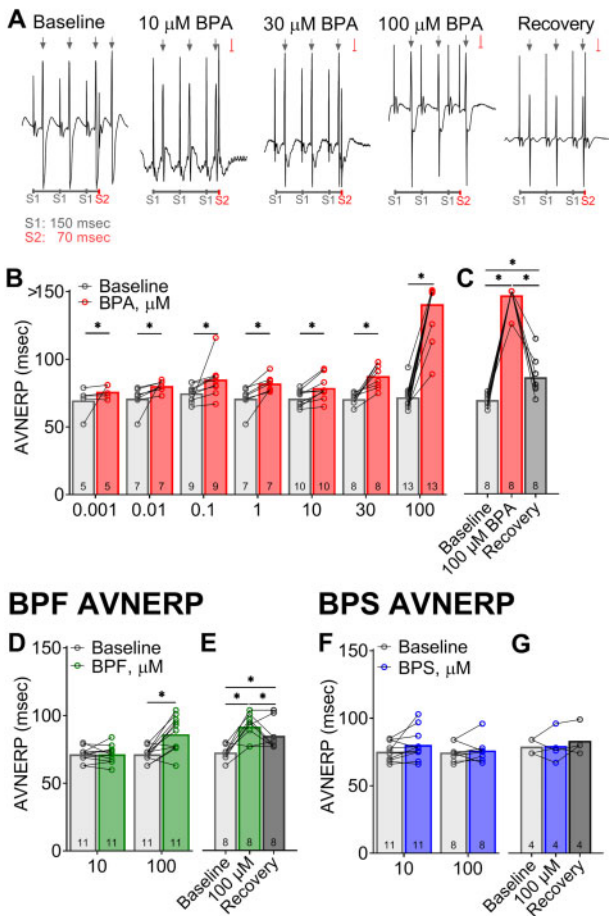
Figure 8. Bisphenol chemical effects on atrioventricular nodal refractoriness in response to atrial pacing. A, Representative ECG recordings during atrial pacing show capture (↓) and failure to capture (⊥) in response to S1-S2 pacing (150, 70 ms). B, Longer atrioventricular nodal effective refractory period (AVNERP) following exposure to BPA concentrations (1–100 μM), as compared with baseline. Note: Complete heart block denoted by measurement > 150 msec (S1 pacing interval). C, Longer AVNERP after 100 μM BPA exposure recovers after 15-min washout. D, BPF exposure results in longer AVNERP at the highest dose tested. E, Moderate recovery of AVNERP after BPF washout. F and G, No change in AVNERP was observed after exposure to 10–100 μM BPS. Values reported as mean ± SD. *$q < 0.05$ as determined by RM-ANOVA with multiple comparisons testing (0.1 FDR). Number of replicates indicated in each bar graph.

protein kinase-dependent pathway. In cardiac tissue, the fast voltage-gated sodium channel (INa-P; tetrodotoxin-sensitive) is responsible for the action potential upstroke, and blockade is likely to reduce the rate of depolarization and slow conduction velocity. Late-sodium channel current (tetrodotoxin-insensitive) is active during the action potential plateau phase, and blockade is expected to shorten repolarization time (Horváth *et al.*, 2020). This highlights the importance of performing electrophysiology studies using cardiac models (eg, human cardiomyocytes, isolated whole heart, in vivo studies) in conjunction with whole-cell recordings, given the dynamic nature of cardiac electrophysiology.

Bisphenol Chemicals and Estrogen-Receptor Signaling
Since BPA is classified as a xenoestrogen, alterations in cardiac function may also be attributed to its interaction with estrogen receptors. In the presented study, we utilized female cardiac preparations because previous reports have indicated that BPA-induced effects on calcium handling and electrical instabilities can be heightened in female animals, exacerbated in the presence of estradiol, and attenuated in estrogen-receptor-beta knockout animals (Belcher *et al.*, 2012; Yan *et al.*, 2011). Studies have shown that 17β-estradiol alone can rapidly and reversibly inhibit sodium and calcium channels in a concentration-dependent manner (Lee *et al.*, 2002; Wang *et al.*, 2013). Accordingly, the effects of BPA on cardiac electrophysiology may be mediated by both direct interaction with ion channels at the cell membrane and/or intracellular signaling pathways precipitated by estrogen-receptor binding. Although BPS and BPF have also been shown to display estrogenic activity comparable to BPA (Kojima *et al.*, 2019; Moreman *et al.*, 2017), in the presented study, we identified clear differences in the potency of BPA, BPS, and BPF as it relates to ion channel inhibition and cardiac electrophysiology parameters.

Cardiotoxic Profile of BPA Analogs
Biomonitoring studies have recently reported an uptick in BPS and BPF exposure in the general population, as manufacturers begin to phase out and replace BPA in some consumer and medical products. For example, data from the 2013–2014 National Health and Nutrition Examination Survey (NHANES) detected BPA, BPS, and BPF in 96%, 90%, and 67% of urinary samples from the general population (Lehmler *et al.*, 2018). Nevertheless, very little is known about the effects of these substitute chemicals on cardiovascular health, and whether they offer a superior safety profile. Using a zebrafish model, Qiu *et al.* (2020) reported that BPS exposure results in transcriptional changes that can increase inflammation, alter cardiac morphology, and *decrease* heart rate. In a rodent cardiac model, BPS-treatment alone was shown to *increase* heart rate, whereas the addition of catecholamine stress increased the propensity for premature ventricular contractions and calcium-mediated triggered activity (Gao *et al.*, 2015). The authors noted that the observed effect on cardiac electrophysiology was sex-specific and mediated via estrogen-receptor-beta signaling, which alters the phosphorylation status of key calcium handling proteins. Notably, the same group has reported nearly identical effects with BPA treatment, which suggests that the 2 chemicals may act via a common mechanism (Yan *et al.*, 2011). Variable results between species could be attributed to intrinsic differences in ion channel expression and/or ectothermic regulation of zebrafish. In a separate study by Ferguson *et al.* (2019), BPS or BPA-treatment rapidly reduced mechanical function in heart preparations, but slightly different post-translational modifications were observed in myofilament proteins. Investigations into the cardiac effects of BPF are even more limited, with a single report noting a decrease in the heart rate of zebrafish following BPF-exposure (Mu *et al.*, 2019). The current study was focused on cardiac electrophysiology outcomes; therefore, future work is still needed to assess the impact of BPA, BPF, and BPS on myocardial contractility.

To the best of our knowledge, our study is the first to compare the acute effects of BPA, BPS, and BPF exposure on cardiac ion channels and electrophysiology. We aimed to identify the IC_{50} concentrations for BPA, BPS, and BPF on key cardiac ion channels highlighted by the CiPA initiative—and validate the effect of those concentrations on human cardiomyocyte and intact heart preparations. Collectively, we observed that BPA exposure has a more potent effect on cardiac electrophysiology, as compared with the chemical substitutes BPF and BPS. Our results suggest that BPS may be a reasonable alternative, particularly for medical devices used to treat vulnerable patient

populations at increased risk for bisphenol chemical exposure. Nevertheless, limitations to our study should be considered. First, we included a number of models in our study, but additional mechanistic work is necessary to fully elucidate the safety profile of bisphenol chemicals—including the impact on intracellular targets, genomic and proteomic expression profiles (subacute or chronic studies), and further investigation of in vivo conditions with crosstalk between systems (autonomic regulation, metabolism, hormonal fluctuations). Second, there are notable differences in cardiac electrophysiology between species (eg, ion channel expression, sinus rate, action potential morphology), which should be noted when considering the translation of experimental studies to humans. Third, our study was focused on the acute effects of bisphenol chemicals on cardiac electrophysiology and the chronic impact of such exposures remains unclear. Finally, future work is necessary to investigate a full range of bisphenol chemical exposures beyond those reported here—as endocrine-disrupting chemicals can present with a nonmonotonic dose response (Birnbaum, 2012; Gao et al., 2015; Liang et al., 2014; Vandenberg, 2014).

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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