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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Increases Cardiac Myocyte Intracellular Calcium and Progressively Impairs Ventricular Contractile Responses to Isoproterenol and to Calcium in Chick Embryo Hearts

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SUMMARY

Binding by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the Ah receptor leads to transcriptional activation of several genes and a toxicity syndrome that includes tumor promotion, wasting, hormonal and immune system dysfunction, and death. Recent findings indicate that TCDD may also affect cardiac function. Here, we used the chick embryo, a TCDD-sensitive species, to further characterize the effects of TCDD on ventricular muscle contraction and on cardiac myocyte $[Ca^{2+}]_i$ assessed with fura 2. The results show that TCDD causes an evolving sequence of contractile defects, independent of changes in diet, first impairing cAMP-modulated contraction (after 48 hr) and later (by seven days) decreasing responses to $[Ca^{2+}]_o$. Phenobarbital, even at high doses, failed to affect the inotropic response to isoproterenol, supporting the specificity of the ventricular contractile effects of TCDD. TCDD treatment also depressed inotropic responses to theophylline and forskolin, indicating that it has a post-β-adrenergic receptor effect on cAMP action. In contrast to its depression of responses to β-adrenergic stimuli and to $[Ca^{2+}]_o$, TCDD did not affect initial tensions of ventricular muscle stimulated at 1 Hz or the force-frequency response up to 1 Hz, indicating that TCDD-treated ventricles can respond normally at slow rates of stimulation. TCDD treatment depressed lusitropic (relaxation) responses to isoproterenol and to increasing $[Ca^{2+}]_o$ indicating that it impairs the ability of the sarcoplasmic reticulum to sequester Ca^{2+}. Fura 2-based measurements showed that $[Ca^{2+}]_i$ was nearly doubled after TCDD treatment. The increase in $[Ca^{2+}]_i$ is consistent with the decrease in the contractile response to $[Ca^{2+}]_o$, amelioration of the response to isoproterenol by subphysiologic concentrations of $[Ca^{2+}]_o$, and intermittent lack of response to electrical stimulation in high K^+ observed in ventricles from TCDD-treated embryos. TCDD treatment also depressed the initial increase in $[Ca^{2+}]_i$ by isoproterenol, consistent with the decreased contractile response to isoproterenol. The findings show that TCDD causes well defined, progressive impairment of avian ventricular responses to inotropic stimuli, providing new evidence that the heart is a target of TCDD action and that TCDD disturbs intracellular calcium processing.

The toxicity syndrome caused by TCDD, PCBs, and chemically related polyhalogenated aromatic hydrocarbons includes weight loss, involution of the thymus gland, edema, keratinocyte proliferation, tumor promotion, and increased mortality (1). Characteristically, animals die suddenly after a period of decreased food intake, weight loss, and inanition (2), but the proximate cause of death is not known. Although the heart has not been considered a major target of TCDD action, recent findings indicate that PCBs and TCDD can affect cardiac function (3–6).

We previously reported that TCDD treatment diminished papillary muscle contractile reponsiveness to β-adrenergic stimuli in guinea pigs, a species highly sensitive to TCDD toxicity (5). TCDD had other effects, including impairment of the inotropic response to increasing $[Ca^{2+}]_o$, indicating that it also increased myocardial cell $[Ca^{2+}]_i$. Here, we have used the chick embryo, another species sensitive to TCDD toxicity, to demonstrate that TCDD causes contractile changes independently of the decreased food intake associated with the wasting syndrome, to further characterize the ventricular effects of

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ABBREVIATIONS: TCDD, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; SR, sarcoplasmic reticulum; $[Ca^{2+}]_i$, intracellular calcium; $[Ca^{2+}]_o$, extracellular calcium; Ca^{2+}, calcium; K^+, potassium; Na^+, sodium; standard Tyrode's, 149.3 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.4 mM $H_2PO_4^-$, 11.9 mM HCO_3^-, and 10 mM glucose, pH 7.4; ED_{50}, median effective dose; HEPES buffer, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM glucose, and 10 mM HEPES, pH 7.4; T½R, time for the tension to fall to half peak height; R_{min}, fluorescence ratio in the absence of calcium; R_{max}, fluorescence ratio in the presence of saturating calcium.

TCDD, and to examine its effects on $[Ca^{2+}]_i$ by fura 2-based measurements in cardiac myocytes.

We report here that TCDD progressively impairs avian ventricular contractile responses, first affecting inotropic responses to β-adrenergic stimuli and later to $[Ca^{2+}]_o$. TCDD also depressed lusitropic responses to isoproterenol and to increasing $[Ca^{2+}]_o$ indicating that it impairs the ability of the SR to sequester $[Ca^{2+}]_i$. We show further that TCDD causes postreceptor impairment of cAMP function, and that phenobarbital (which, like TCDD, is a cytochrome P-450 inducer, but which acts independently of the Ah receptor) did not affect ventricular contractile function, even at high doses, supporting TCDD's specificity. Finally, we report that TCDD increases cardiac myocyte $[Ca^{2+}]_i$ as previously predicted (5), and also suppresses the further rise in $[Ca^{2+}]_i$ in response to isoproterenol in cultured myocardial cells. The findings establish the cross-species cardiotoxicity of TCDD and show that the depression of inotropic responsiveness of TCDD is associated with impairment of cardiac cell calcium processing.

Experimental Procedures

Materials. TCDD was a gift of the Monsanto Co. (St. Louis, MO). Sources of other chemicals were: isoproterenol ((−)-isoproterenol (+)-bitartrate salt), collagenase type I, trypsin, 4,6-diamidino-2-phenylindole, ionomycin, *N*-methyl-D-glucamine, HEPES, EGTA, and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO); forskolin (Coleus forskolii) from Calbiochem-Behring Diagnostic, (San Diego, CA); dioxane (1,4-dioxane) from Fisher Scientific (Pittsburgh, PA); fura 2-AM from Molecular Probes (Eugene OR); and Eagle's Basal Medium and additives from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). The monoclonal anti-chick heart myosin antibody MF20 and 4,6-diamidino-2-phenylindole were gifts from Dr. David Bader (Cornell University Medical College, New York, NY).

Chick embryos: source and treatment. Fertilized White Leghorn eggs were obtained from Shamrock Farms (North Brunswick, NJ) or Burr Farms, Inc. (Hampton, CT). The eggs were incubated at 37° and 70% relative humidity. TCDD in 0.01 ml dioxane or 0.01 ml dioxane alone was administered to embryos by injection through a hole in the shell into the fluids surrounding the embryo.

Physiologic experiments. Hearts from 18- to 20-day-old chick embryos were removed, kept in standard Tyrode's, and aerated with 95% O_2 and 5% CO_2. The right ventricular wall was suspended vertically in a double-walled tissue chamber containing 2.5 ml of the aerated standard Tyrode's maintained at 30°. One end of the preparation was fixed to the floor of the bath with a stimulating electrode. The other end was connected by a nylon thread to an isometric force displacement transducer (Grass Co., Quincy, MA, model FTO3C). The contractile force was recorded on a polygraph (Grass Co., Quincy, MA, model 7D). Unless otherwise indicated, the preparations were paced at a frequency of 1 Hz using square wave pulses (duration, 5 milliseconds; voltage, 1.5 times the threshold) delivered by two coiled platinum electrodes connected to a stimulator (Grass Co., model S88) via a stimulus-isolation unit (Grass Co., model SIU5). The resting tension was adjusted to 0.5 g. Ventricular muscles were equilibrated routinely under these conditions for 40 min before experimental procedures were started. The tension developed at the end of this equilibration period is referred to as "initial tension".

Isoproterenol was dissolved in standard Tyrode's containing ascorbic acid at 1 mg/ml, forskolin in ethanol/water (95:5, v/v), and theophylline in water. Further dilutions were in standard Tyrode's. Contractile responses to changes in Ca^{2+} were examined between 1.8 mM and 7.7 mM Ca^{2+} by adding calcium chloride to the bathing medium in increments of 0.8 to 0.9 mM. Dose-response curves were obtained by measuring the peak developed tension in response to cumulative addition of drugs to the organ bath. Drug concentrations were increased after the response to the previous concentration had reached a plateau. Except for the experiments shown in Fig. 7, a separate heart was used for each dose-response curve. ED_{50} values were determined from dose-response curves by inspection.

T½R was measured from high-speed recordings before and after various inotropic interventions. The values for three successive contractions were averaged for each determination.

To examine the effects of TCDD when contraction was dependent on L-type Ca^{2+} channel function, standard Tyrode's was replaced with Tyrode's containing 22 mM K^+ after equilibration, to partially depolarize the ventricles. Osmolarity was maintained by lowering the Na^+ concentration. Under these conditions, the fast Na^+ channels are voltage-inactivated and contractions are governed by L-type Ca^{2+} channel activity (7). Stimulation frequency was reduced to 0.2 Hz, intensity was increased 2-fold, and pulse duration was held at 5 milliseconds. After 10 min in high-K^+ Tyrode's, the capacity of isoproterenol (10^{-7} to 10^{-6} M) to restore contractions was examined.

The force-frequency response, the ventricular contractile response to increasing stimulus frequency, was measured after equilibration for 40 min at 1 Hz in standard Tyrode's, by stepwise stimulation of the muscle at frequencies between 0.1 and 1 Hz. The frequency was increased each time the developed tension reached a plateau.

Primary cultures of cardiac myocytes. Cardiac myocytes were cultured from hearts of 15-day-old embryos treated five days before dissection with 1 nmol of TCDD in 0.01 ml of dioxane or with 0.01 ml of dioxane alone. Myocytes were isolated essentially as described by De Haan (8). Hearts (6 to 8 for each treatment group in each experiment) were removed under sterile conditions, razor-cut into small pieces and washed twice with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (140 mM NaCl, 5.5 mM D-glucose, 5.5 mM KCl, 0.4 mM KH_2PO_4, and 1 mM Na_2HPO_4). The fragments were pipetted up and down in the same solution containing 0.0125% (w/v) of trypsin and 0.05% (w/v) of collagenase at 37° for 3 min. The dissociated cell suspension was placed in 10 ml of the cold Ca^{2+}- and Mg^{2+}-free Hank's solution containing 1% (w/v) bovine serum albumin to inhibit trypsinization and then centrifuged at 300 × *g* for 5 min at 4°. The pellet was reserved and the supernatant was discarded. Residual undigested heart fragments were subjected again to enzymic digestion (total of four cycles). The combined pellets were resuspended in Ham's F-12 medium without insulin (9) and containing 10% (v/v) fetal bovine serum (culture medium) and placed in a 10-cm Petri dish for about 10 min to allow fibroblasts to separate and attach to the plastic. The myocyte-enriched supernatant was decanted. Myocyte contents of enriched preparations were determined immunohistochemically. Cells were fixed in ice-cold methanol on a glass slide and reacted for successive 30-min periods at room temperature, first with MF20 (a monoclonal antibody that binds to an epitope on chick embryo cardiac myosin heavy chains (10)) in 1 M potassium phosphate, pH 7.0, and then, in the dark, after washing in the same buffer, with goat anti-mouse IgG linked to tetramethylrhodamine isothiocyanate. Nuclei were stained for 10 min with the fluorescent dye, 4,6-diamidino-2-phenylindole. The number of MF20-positive cells and total cell nuclei were counted using a Zeiss fluorescence microscope. Myocytes were found to comprise 85% or more of the cells in the final preparations.

Cell viability was evaluated by trypan blue exclusion and regularly found to exceed 95%. Cells were counted in a Neubauer chamber and diluted to 5×10^6 cells per ml. Aliquots of 75 μl were plated on 7 × 22 mm glass coverslips (SLM Instruments, Urbana, IL) that had been coated previously with 1% (w/v) gelatin and placed in 35-mm Petri dishes (two per dish). After incubation for 2 hr in 5% CO_2 in air at 37°, 2 ml of culture medium were added and then replaced with fresh medium after further incubation for 24 hr. Confluent monolayers developed by 36 hr.

Measurement of $[Ca^{2+}]_i$. After 48 hr, the culture medium was removed and replaced with 1.5 ml of a solution containing HEPES buffer, to which 3 μl of a 5 mM solution of the acetoxymethyl ester of fura 2 (fura 2-AM) (11) in Me_2SO was added (final concentrations: 10

μM fura 2-AM, 0.2% (v/v) Me_2SO). After incubation for 90 min at 37°, the cells were washed twice with HEPES buffer without the fura 2-AM and the cells were further incubated for 45 min to permit intracellular deesterification of fura 2-AM.

Fluorescence was measured at 37° in a thermostatically controlled DMX-1000 spectrofluorometer (SLM Instruments, Urbana IL). Coverslips were placed in a variable angle coverslip holder with the surface of the coverslips containing the cells facing the excitation and emission beams. The angle between the cover slip and the emission beam was held at 27°, a setting found in preliminary experiments to minimize background autofluorescence (fluorescence in unloaded cells). Fluorescence was monitored at an emission wavelength of 510 nm with excitation wavelengths alternating between 340 and 380 nm. The two rapidly alternating emission signals were discriminated electronically and ratios were derived by the software. Solutions were added or removed from the cuvette through a tube connected to a hole in the cap of the coverslip holder.

After washing cells twice with HEPES buffer, cell function was routinely tested by examining the increase in $[Ca^{2+}]_i$ in HEPES buffer, in which sodium was replaced with 140 mM *N*-methyl-D-glucamine (12). Coverslips containing cells that failed to increase $[Ca^{2+}]_i$ in response to zero sodium were discarded. The effect of isoproterenol on $[Ca^{2+}]_i$ was examined by replacing the HEPES buffer with a solution of the same composition containing 1 μM isoproterenol. After measuring $[Ca^{2+}]_i$ and the response to isoproterenol, calibration was performed by measuring the R_{min} in Ca^{2+}-free HEPES buffer containing 10 mM EGTA and 5 μM ionomycin. R_{max} was obtained by adding HEPES buffer containing 1.8 mM Ca^{2+} and 5 μM ionomycin. $[Ca^{2+}]_i$ was derived from the 340 nm/380 nm ratios and the R_{min} and R_{max} values were derived by the SLM8000 software according to the equation of Grynkiewicz *et al.* (11), using a K_d of 224 nM. A coverslip in each group was subjected to all of the same procedures (except for the fura 2-AM loading) and was used to measure autofluorescence, which was automatically subtracted by the program.

Statistical evaluations. Group means were compared by Student's *t* tests where appropriate. Dose-response curves were evaluated by two-way analyses of variance. Post-hoc comparisons were made with *t* tests using the error term from the antecedent analysis of variance. Differences were considered significant at $p < 0.05$. Levels of statistical significance for data shown in figures are mainly given only in the figure legends.

Results

Initial tensions and responses to isoproterenol in untreated or solvent-treated chick embryos. Initial tensions and responses to inotropic stimuli did not differ for untreated or solvent-treated controls. Unless otherwise indicated, controls received the solvent dioxane. Mean initial tensions for ventricular preparations from 18- to 20-day-old embryos did not differ significantly from each other. However, responses to isoproterenol were higher in 18- than in 19- or 20-day-old embryos ($p = 0.0001$). Isoproterenol, at 3×10^{-7} to 10^{-6} M, elicited maximal increases over initial tensions (mean maximal % increase ± standard error) of 169 ± 14% ($n = 14$), 134 ± 23% ($n = 17$), and 116 ± 51% ($n = 5$) for 18-, 19-, and 20-day-old embryos, respectively. The findings are consistent with a reported decline in cardiac sensitivity to isoproterenol in chick embryos between 16 and 21 days old (13). Contractile responses to other stimuli (i.e., forskolin, theophylline, and calcium) did not differ significantly for 18- to 20-day-old embryos.

Effect of TCDD treatment on the inotropic response to isoproterenol. TCDD decreased inotropic responses to isoproterenol as a function of dose and exposure time. Fig. 1 shows data for 19-day-old embryos. TCDD exposure for 48 hr decreased mean maximal responses to isoproterenol by 28% at 1 nmol per egg and 53% at 5 nmol per egg (*top panel*); exposure for 7 days decreased responses by 65% at 1 and 5 nmol per egg (*bottom panel*). TCDD also depressed ventricular responses to isoproterenol in 18- and 20-day-old embryos. After 7 days in the 18-day-old embryos, TCDD depressed the inotropic response to isoproterenol by 20% at 1 nmol ($p = 0.0018$), and by 55% at 5 nmol ($p < 0.0001$), $n = 6$ for each group. In the 20-day-old embryos tested at 1 nmol, TCDD depressed the inotropic response to isoproterenol by 70%, essentially the same effect as in the 19-day-old embryos ($n = 5$ for controls, $n = 6$ for TCDD ($p < 0.0001$)). In contrast to its depression of contraction stimulated by isoproterenol, TCDD did not affect ventricular initial tensions either at 48 hr or 7 days of treatment, even at 5 nmol per egg. (Values for initial tensions are given in the figure legends.)

Fig. 1 also shows that TCDD shifted the dose-response curves

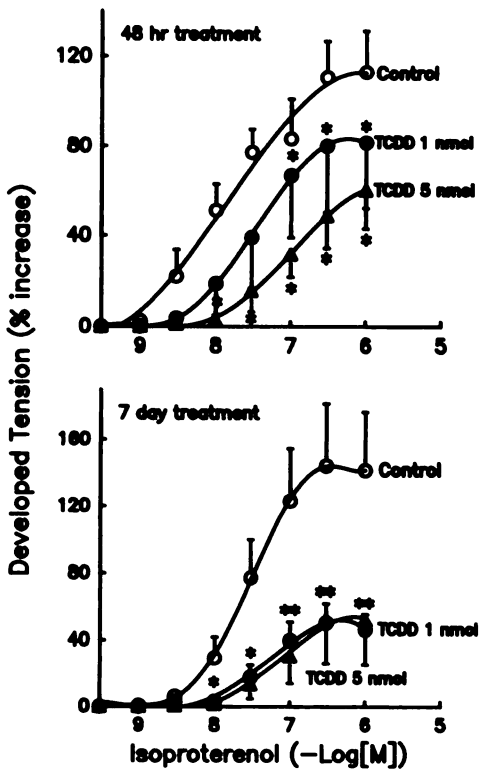

Fig. 1. Effect of TCDD treatment on inotropic responses to isoproterenol in right ventricles from hearts of 19-day-old embryos at 48 hr (*top panel*) and 7 days (*bottom panel*) after treatment. Controls (○), TCDD, 1 nmol (●), and 5 nmol (▲). TCDD was administered in 0.01 ml dioxane and controls received 0.01 ml of dioxane for data shown in this and all other figures. Contractility was measured in response to increasing concentrations of isoproterenol added to the bathing medium as described in Experimental Procedures. Results are expressed as the percent increase in tension developed over the initial tension before addition of isoproterenol. *Error bars* here and in all other figures represent 1 SE. For the 48-hr experiments, mean initial tensions (mg ± standard error) were 258 ± 43 for controls ($n = 7$), 219 ± 39 for TCDD at 1 nmol per egg ($n = 5$), and 176 ± 25 for TCDD at 5 nmol ($n = 4$). For the 7-day experiments, mean initial tensions were 162 ± 20 for controls ($n = 10$), 199 ± 49 for TCDD at 1 nmol ($n = 6$), and 180 ± 53 at 5 nmol ($n = 7$). Mean initial tensions among treatment groups in this and in other experiments did not differ significantly. Analysis of variance for TCDD versus control: $p < 0.01$ for 1 nmol at 48 hr, $p \leq 0.0001$ for all other treatments. Post-hoc analysis for TCDD versus control: *, $p < 0.05$; **, $p < 0.01$ (significance values were the same for 1 and 5 nmol after 7 days of treatment).

for isoproterenol to the right, increasing the ED_{50} for isoproterenol. Thus, TCDD treatment decreased ventricular sensitivity to isoproterenol.

Effects of TCDD on the lusitropic response to isoproterenol (T½R). Isoproterenol elicited statistically significant decreases in mean relaxation times (T½R) in 19-day-old embryos treated with dioxane alone but did not decrease the relaxation times in the ventricles from embryos treated with TCDD (Fig. 2). Thus, TCDD inhibited isoproterenol's acceleration of relaxation.

Effects of TCDD on inotropic responses to theophylline and forskolin. Theophylline increases the force of contraction by depressing phosphodiesterase, thus increasing basal levels of cAMP. Forskolin increases contraction independently of the β-adrenergic receptor by activating adenylate cyclase directly. Fig. 3 shows that theophylline and forskolin increased the force of contraction in control and TCDD-treated ventricles but that the responses to both agents were significantly lower after TCDD treatment.

Effects of TCDD on responses in high K^+. To determine whether TCDD impaired contraction under conditions dependent only on slow Ca^{2+} channel function, contractile responses were examined in high K^+. Partial depolarization of ventricles by high (22 mM) K^+ terminates ventricular responsiveness to paced stimulation because the higher resting potential prevents the influx of sodium that normally initiates the action potential (7). Under these conditions contraction can normally be restored by isoproterenol or other agents that enhance Ca^{2+} channel opening. All of the control and TCDD-treated ventricular preparations became nonresponsive to paced stimulation in 22 mM K^+. An example of representative responses for ventricular preparations from a control and a TCDD-treated embryo (1 nmol for 7 days) is shown in Fig. 4. Isoproterenol restored contractile responsiveness in all of the controls and in 11 of 13 preparations from TCDD-treated embryos. The force of contraction at each concentration of isoproterenol was weaker (example shown in Fig. 4C), and the T½ for relaxation

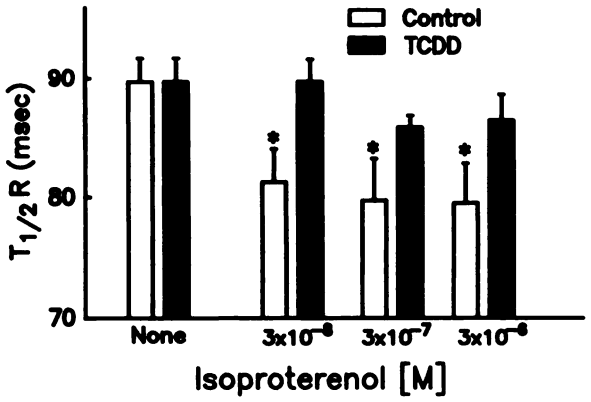

Fig. 2. Effect of TCDD on relaxation (T½R) of ventricles after stimulation with isoproterenol. The T½R was examined in ventricles of 19-day-old chick embryos 7 days after treatment with TCDD at 1 nmol per egg (*solid bars*) and in controls (*clear bars*); $n = 8$ for both groups. Concentrations of isoproterenol added to the bathing medium are shown on the *abscissa*. Results are expressed as the time for the maximum developed tension to fall to half the peak height as determined from high speed recordings as described in Experimental Procedures. Mean T½R (msec ± standard error) before adding isoproterenol was 90 ± 2 for controls and 90 ± 2 for the TCDD-treated ventricles; $p = 1.0$ (*, significantly shorter than without isoproterenol, $p < 0.05$).

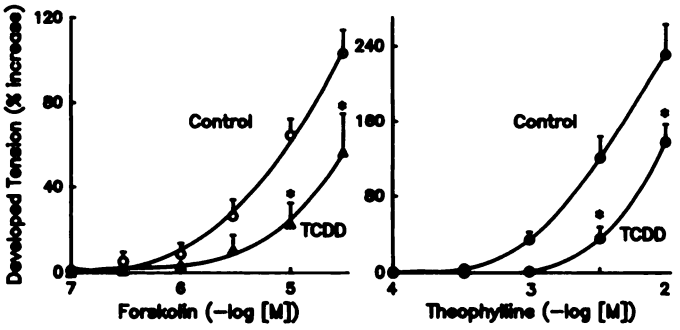

Fig. 3. Effect of TCDD on the positive inotropic effects of forskolin (*left panel*) and theophylline (*right panel*). Contraction was measured in ventricular preparations from 18- or 19-day-old embryos, 7 days after treatment with TCDD. Increasing concentrations of forskolin or theophylline were added to the bathing medium as described in Experimental Procedures. Results are expressed as the percent increase in tension developed over the initial tension before adding forskolin or theophylline. Controls (○); TCDD, 1 nmol per egg, (●); 5 nmol per egg (▲). For the experiments with forskolin, mean initial tensions (mg ± standard error) were 221 ± 20 for controls ($n = 8$) and 219 ± 24 for the TCDD-treated ventricles ($n = 6$); $p = 0.96$. In the experiments with theophylline, mean initial tensions were 208 ± 27 for controls ($n = 10$) and 227 ± 16 for the TCDD-treated ventricles ($n = 10$); $p = 0.55$. Analysis of variance for TCDD-treated versus control ventricles: $p < 0.0001$ for forskolin and $p < 0.001$ for theophylline. Post-hoc analysis for TCDD versus control: *, $p < 0.001$.

was longer, for ventricles from TCDD-treated embryos. At 3×10^{-6} M isoproterenol, for example, mean developed tensions ± standard error (inotropic effect) were 104 ± 10 mg, $n = 13$, for controls and 64 ± 15 mg, $n = 11$, for ventricles from the TCDD-treated embryos ($p < 0.05$). Mean relaxation times (T½R) ± standard error were 73 ± 4 msec, $n = 13$, for controls and 100 ± 9 msec, $n = 9$, for the TCDD-treated preparations ($p < 0.02$). Thus, TCDD inhibited the inotropic and lusitropic effects of isoproterenol in high K^+ as in standard Tyrode's.

In addition, more isoproterenol was needed to restore contractile activity in the ventricles from TCDD-treated embryos in high K^+. Thus, at 10^{-7} M isoproterenol contractions were restored in 92% of controls and in 23% of the TCDD-treated ventricles; at 3×10^{-7} M isoproterenol contractions were restored in all of the controls and 77% of the TCDD-treated ventricles, and at 10^{-6} M isoproterenol contractions were restored in 85% of the TCDD-treated ventricles. Also, although all of the control preparations contracted in response to each electrical pulse in the presence of isoproterenol, seven of the 11 TCDD-treated ventricles that had responded to isoproterenol in high K^+ were intermittently refractory to electrical stimulation (Fig. 4C).

$[Ca^{2+}]_o$ modulation of the effect of TCDD on the response to isoproterenol. The effect of the calcium concentration of the medium on TCDD's impairment of the inotropic response to isoproterenol was examined by comparing responses to isoproterenol in medium containing 0.9 mM and 1.8 mM $[Ca^{2+}]_o$. TCDD at 1 nmol per egg for 7 days decreased the maximum response to isoproterenol by 33% in 0.9 mM $[Ca^{2+}]_o$ versus 65% in 1.8 mM $[Ca^{2+}]_o$ (Fig. 1). Lowering $[Ca^{2+}]_o$ to subphysiologic concentrations therefore ameliorated the impairment by TCDD of isoproterenol's stimulation of contraction.

Effect of TCDD on contractile responses to $[Ca^{2+}]_o$. TCDD treatment for 48 hr did not significantly affect inotropic responsiveness to $[Ca^{2+}]_o$, an agent that acts independently of

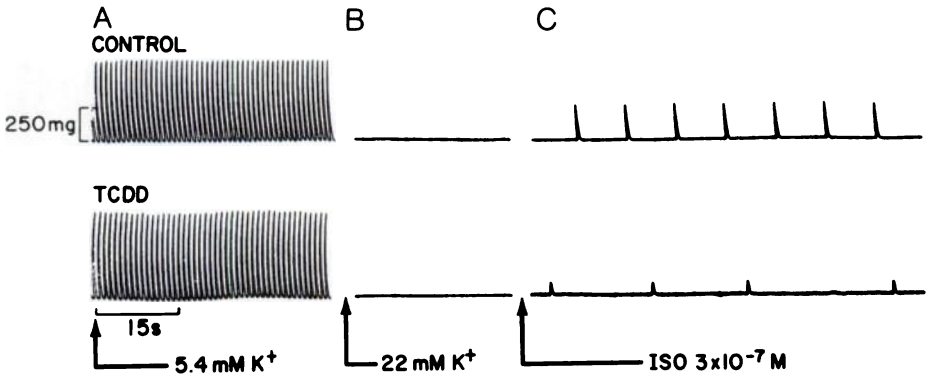

Fig. 4. Representative recordings showing contraction of ventricles from control and TCDD-treated embryos in normal Tyrode's and high K^+. The *bottom panel* shows tracings from a ventricular strip from a 19-day-old embryo treated for 7 days with TCDD at 1 nmol per egg in 0.01 ml dioxane. The *top panel* shows tracings from a control treated with dioxane alone. The scale for top and bottom panels is the same. A, Preparations were equilibrated in standard Tyrode's (5.4 mM K^+) for 40 min with pacing at 1 Hz. B, The bathing medium was changed to another containing 22 mM K^+ to partially depolarize the ventricles and render them unexcitable. Stimulus frequency was reduced to 0.2 Hz and intensity was increased 2-fold. C, Isoproterenol (3×10^{-7} M) was added to the bathing medium to restore contractile responsiveness. Stimulation parameters were the same as for B. The *bottom tracing* shows intermittent nonresponsiveness to paced stimulation in the TCDD-treated ventricular preparation.

cAMP (Fig. 5, *top panel*). The inotropic response to $[Ca^{2+}]_o$, however, seemed to begin to level off at about 6.1 mM $[Ca^{2+}]_o$ in the TCDD-treated ventricles but continued to increase in the controls, even at 7.7 mM $[Ca^{2+}]_o$, the highest concentration tested. However, after 7 days, TCDD decreased the inotropic response to $[Ca^{2+}]_o$ by 30% and 50% at 1 nmol and 5 nmol, respectively (significantly more inhibitory at 5 nmol than at 1 nmol ($p = 0.0014$)) (Fig. 5, *bottom panel*). TCDD also significantly prolonged the T½ for ventricular muscle relaxation in response to $[Ca^{2+}]_o$ (Fig. 6).

The impression that TCDD was relatively sparing of the response to calcium when it severely affected the response to isoproterenol was further examined by constructing dose-response curves to $[Ca^{2+}]_o$ and then to isoproterenol in the same preparations (Fig. 7). In this experimental subset, TCDD at 1 nmol for 7 days decreased the response to $[Ca^{2+}]_o$ by a mean of 12% (not statistically significant) and the subsequent response to isoproterenol by a mean of 70% (about the same as found for ventricles not previously exposed to increasing $[Ca^{2+}]_o$) (Fig. 1). These findings showed that within the same preparations, TCDD affected the response to isoproterenol substantially more severely than the response to calcium.

Force-frequency relationship. Gradually increasing the stimulus frequency from 0.1 to 1 Hz (force-frequency response) produced positive staircase responses in both control and TCDD-treated ventricles. This response was unaffected by TCDD treatment at up to 5 nmol per egg for 7 days (Fig. 8).

Effect of phenobarbital on contractile responses to isoproterenol. The inotropic and lusitropic responses to isoproterenol were examined in ventricular preparations of 18- and 19-day-old chick embryos treated for 7 days with phenobarbital at 3, 10, or 30 mg per egg. Phenobarbital, like TCDD, induces hepatic cytochrome P-450 but increases different isoforms, and does so independently of the Ah receptor in chick embryo as in rodent liver (14). Phenobarbital at up to 30 mg per egg (half of a typical dose for a 70-kg man), a dose that maximally induces P-450 in chick embryo liver, had no significant effect on the inotropic or lusitropic responses to isoproterenol.

Effect of TCDD on $[Ca^{2+}]_i$ in cardiac myocytes. The ability of cardiac myocytes to increase $[Ca^{2+}]_i$ when the bathing medium was replaced with a sodium-free solution was routinely measured to insure that a representative cardiac cell function, Na^+-Ca^{2+} exchange, was operative (15) and that the cells had loaded with fura 2. With zero sodium there was a rapid and marked increase in $[Ca^{2+}]_i$, to a mean level of 804 nM ± 78 (standard error), $n = 15$, in the control cells. We did not find any consistent difference in the degree of this response for control and TCDD-treated cells.

Representative tracings for measurement of $[Ca^{2+}]_i$ in cardiac myocytes of control (*lower tracing*) and TCDD-treated embryos (*upper tracing*) and responses to isoproterenol are shown superimposed in Fig. 9 (*top panel*). The *top panel* shows first that $[Ca^{2+}]_i$ was higher in the cells from TCDD-treated embryos. The increase in $[Ca^{2+}]_i$ was highly reproducible as it was observed in each of 10 independent experiments including a group of preliminary experiments in which the effect of TCDD treatment *in ovo* on $[Ca^{2+}]_i$ in cells cultured from 7- and 10-day-old embryos was examined. The *bottom panel* of Fig. 9 shows mean values ± standard error for $[Ca^{2+}]_i$ derived from four independent experiments in myocytes from 15-day-old embryos treated with 1 nmol TCDD for 5 days, or with dioxane alone. Mean $[Ca^{2+}]_i$ ± standard error was 103 ± 11 nM for myocytes from control embryos and 190 ± 25 nM for myocytes cultured from TCDD-treated embryos ($p < 0.01$). Thus TCDD treatment *in ovo* at 1 nmol for 5 days approximately doubled cardiac myocyte $[Ca^{2+}]_i$.

Effect of TCDD on the increase in $[Ca^{2+}]_i$ in response to isoproterenol. The *top panel* of Fig. 9 also shows that isoproterenol gradually increased $[Ca^{2+}]_i$ in cells cultured from both control and TCDD-treated embryos. However, the rate of increase was suppressed in the cells from the TCDD-treated embryos. Fig. 10 shows that most of the difference in the responses of cells from control and TCDD-treated embryos occurred during the first minute after exposure to isoproterenol, when mean $[Ca^{2+}]_i$ increased by 19.2 nM for control cells and 5.8 nM for the TCDD-treated cells ($p < 0.01$, 70% depression by TCDD). Thus, TCDD markedly attenuated the initial increase in $[Ca^{2+}]_i$ in response to isoproterenol. Later, the differences became non significant. At 5 min, for example, the rate of increase was 8.3 nM per min for the controls and 7.3 nM per min for the TCDD-treated embryos.

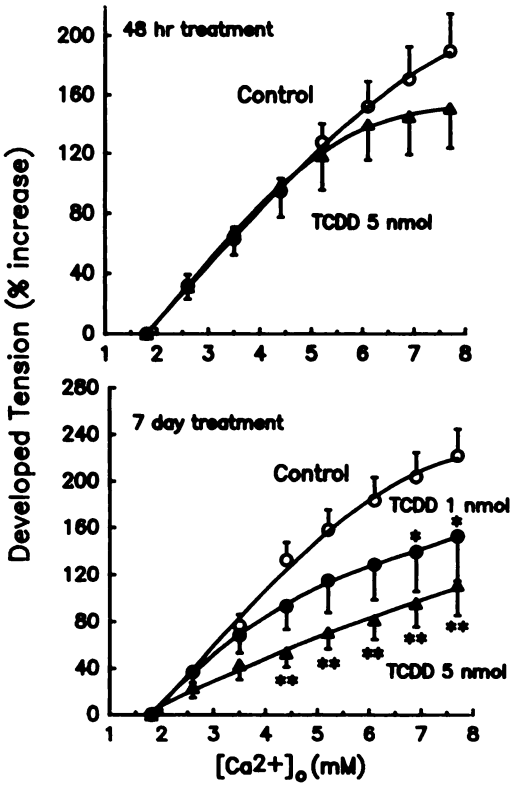

Fig. 5. Effect of TCDD treatment on the positive inotropic effect of calcium 48 hr (*top panel*) and 7 days (*bottom panel*) after treatment. Increasing concentrations of $[Ca^{2+}]_o$ were added to the bathing medium and the force of contraction was measured as described in Experimental Procedures. Results are expressed as the percent increase in tension developed over the initial tension before addition of $[Ca^{2+}]_o$. Controls (○), TCDD at 1 nmol per egg (●), and 5 nmol per egg (▲). For the experiments at 48 hr after treatment with TCDD, mean initial tensions (mg ± standard error) were 159 ± 21 for controls (n = 6) and 226 ± 34 for the TCDD-treated ventricles (n = 7); p = 0.13. Analysis of variance for TCDD-treated versus control: p = 0.30. For the experiments 7 days after treatment with TCDD, mean initial tensions ± standard error were 211 ± 28 for controls (n = 15), 305 ± 41 for TCDD 1 nmol (n = 6) (p = 0.09 for TCDD-treated versus control), and 204 ± 16 for TCDD 5 nmol (n = 11) (p = 0.88 for TCDD-treated versus control). Analysis of variance for TCDD-treated versus control: p = 0.001 at 1 nmol and $p < 0.0001$ at 5 nmol. Post-hoc analysis for TCDD-treated versus control: *, $p < 0.05$; **, $p < 0.01$.

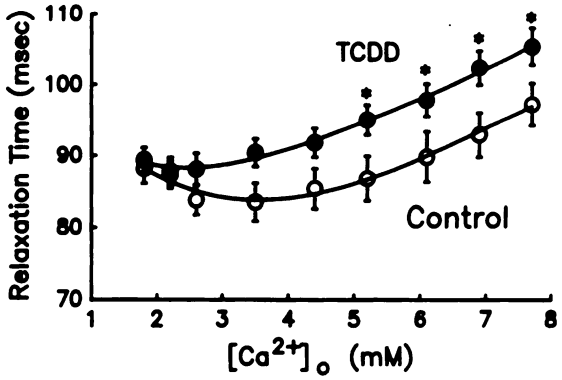

Fig. 6. Prolongation of relaxation in ventricles from TCDD-treated embryos in response to addition of calcium to the bathing medium. The T½R was examined in ventricles of 19-day-old chick embryos 7 days after treatment with TCDD at 1 nmol per egg (●), n = 13, and in controls (○), n = 11. The concentrations of $[Ca^{2+}]_o$ in the bathing medium are shown on the *abscissa*. Results are expressed as the time for the maximum developed tension to fall to half the peak height as determined from high speed recordings as described in Experimental Procedures. The mean T½R (msec ± standard error) in 1.8 mM $[Ca^{2+}]_o$ was 88 ± 2 for controls and 90 ± 2 for the TCDD-treated; p = 0.64. Analysis of variance for TCDD-treated versus control: $p < 0.001$. Post-hoc analyses for TCDD-treated versus control: *, $p < 0.05$.

Discussion

These results show that TCDD treatment causes contractile defects in avian ventricular muscle independently of changes in diet. They also show that the effects of TCDD are progressive, initially affecting cAMP-modulated contraction and only later decreasing responses to $[Ca^{2+}]_o$. Further, TCDD impairs ventricular relaxation as well as contraction, and does so both in response to isoproterenol and to $[Ca^{2+}]_o$, indicating that it affects the ability of the SR to sequester Ca^{2+}. The results also provide experimental verification of our hypothesis that TCDD increases cardiac myocyte $[Ca^{2+}]_i$. This finding, as discussed further below, is consistent with the decrease in the contractile response to $[Ca^{2+}]_o$, amelioration of the response to isoproterenol by subphysiologic concentrations of $[Ca^{2+}]_o$, and intermittent lack of response to electrical stimulation in high K^+, observed in ventricles from TCDD-treated chick embryos. The results show also that TCDD depresses the initial increase in cardiac cell $[Ca^{2+}]_i$ by isoproterenol. As changes in $[Ca^{2+}]_i$ have been temporally (16) and quantitatively (17) related to the positive contractile responses to isoproterenol, the depression of the increase in $[Ca^{2+}]_i$ by isoproterenol is consistent with the decreased contractile response to isoproterenol.

TCDD impairment of β-adrenergic-stimulated contraction is evidenced by its depression of the inotropic and lusitropic effects of isoproterenol, the need for more isoproterenol to restore contractility in high K^+, the depressed inotropic and lusitropic responses to isoproterenol once contractile activity was restored in high K^+, and the depressed inotropic effects of theophylline and forskolin. The latter show that TCDD has a postreceptor action. Moreover, the similar degrees to which TCDD suppressed responses to isoproterenol and forskolin (70% and 65%, respectively, at 5 nmol per egg for 7 days) indicates that its postreceptor effect predominates. This could involve one or more of the following: function or synthesis of G proteins or adenylate cyclase; cAMP-mediated phosphorylation of phospholamban in the SR, of calcium channels in the sarcolemma, or of contractile proteins; or the synthesis or function of SR Ca^{2+} ATPase.

The postreceptor actions of TCDD could be primary or secondary to a receptor effect because postreceptor defects can follow receptor desensitization by high concentrations of catecholamines in cardiac cells and failing myocardium (18, 19). TCDD has been found to down regulate other receptors, including the epidermal growth factor (20), estrogen (21), and glucocorticoid (22) receptors, although different mechanisms may be involved in each case. However, cAMP measured by radioimmunoassay in hearts from control and TCDD-treated embryos did not differ significantly, and isoproterenol doubled cAMP levels in both conditions (data not shown). Although these results indicate that TCDD does not have marked effects on receptor-mediated cAMP synthesis, they do not exclude some receptor effects because small changes in cAMP in subcellular compartments may not be reflected in changes in the whole organ (23).

The depression by TCDD of β-adrenergic stimulation of ventricular contraction in chick embryos and guinea pigs (5),

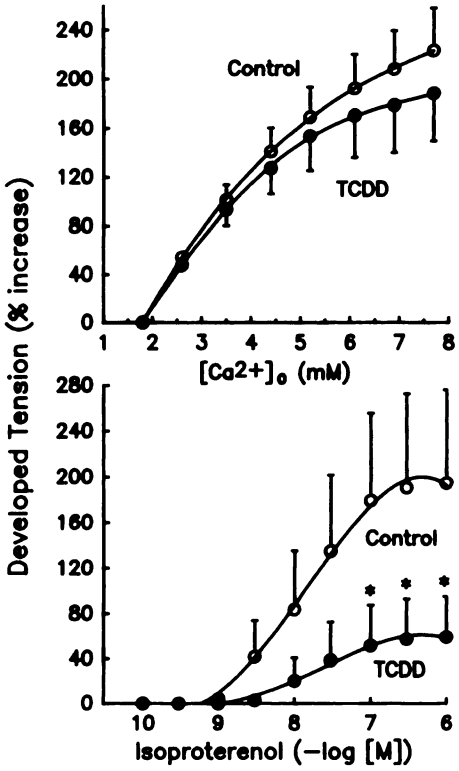

Fig. 7. Comparison of the effects of TCDD on the inotropic effects of calcium and isoproterenol in the same ventricular preparations. Right ventricles were removed from 19-day-old embryos 7 days after treatment with TCDD at 1 nmol per egg (●) and controls (○). Contractile responses were measured first in response to increasing concentrations of $[Ca^{2+}]_o$ added to the bathing medium, as described in Experimental Procedures. The preparations were then washed, reequilibrated in standard Tyrode's, and dose-response curves for isoproterenol were examined. Results are expressed as the percent increase in tension developed over the initial tension before addition of $[Ca^{2+}]_o$ (*top panel*) or isoproterenol (*bottom panel*), each considered as 100% ($n = 7$ for both control and TCDD-treated preparations). Mean initial tensions (mg ± standard error) before adding $[Ca^{2+}]_o$ were 296 ± 33 for controls and 304 ± 27 for TCDD-treated; $p = 0.86$. Mean initial tensions ± standard error before adding isoproterenol were 176 ± 14 for control and 199 ± 26 for the TCDD-treated; $p = 0.44$. Analysis of variance for TCDD-treated versus control for the response to $[Ca^{2+}]_o$, $p = 0.18$; for the response to isoproterenol, $p < 0.01$. Post-hoc analysis for TCDD-treated versus control: *, $p < 0.05$.

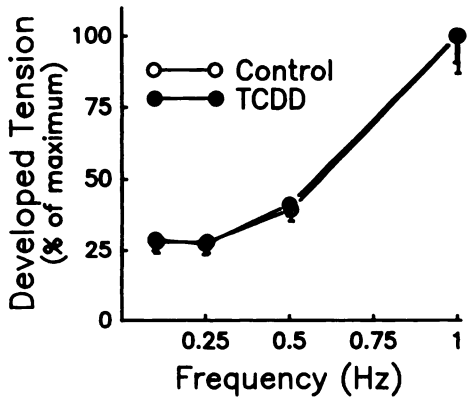

Fig. 8. Effect of TCDD on the force-frequency response. Contraction of ventricles from 18- or 19-day-old embryos treated with TCDD at 5 nmol per egg (●) for 7 days and controls (○) was examined at increasing frequencies of stimulation from 0.1 to 1 Hz. Stimulation frequency was successively increased after the developed tension at the previous frequency had reached a plateau. Developed tension is expressed as percent of the tension at 1 Hz. The variance for the 100% value at 1 Hz is derived from the variance for the developed tensions. Mean initial tensions (mg ± standard error) after equilibration at 1 Hz before initiating the experiment were 210 ± 13 for controls ($n = 19$) and 250 ± 16 for the TCDD-treated ($n = 15$); $p = 0.062$. TCDD did not significantly affect the force-frequency response.

two different species sensitive to other aspects of TCDD toxicity, indicates that TCDD is cardiotoxic across species. Thus, cardiotoxicity can be included in the TCDD toxicity syndrome. Our findings are consistent with reports of Hermansky *et al.* (6) of decreased chronotropic responses to isoproterenol in TCDD-treated rats *in vivo* and of Brewster *et al.* (4) of decreased atrial sensitivity to isoproterenol in TCDD-treated guinea pigs. Brewster *et al.* also concluded that cardiac failure could be a contributing cause of death in TCDD toxicity. The contrasting findings of Kelling *et al.* (24) of augmented inotropic and chronotropic responses to isoproterenol in atria from TCDD-treated rats could reflect differences in dose or exposure time or species differences in atrial responses.

Depression of cAMP-regulated processes may extend to other toxic effects of TCDD. Thus, TCDD has been reported to inhibit cAMP-mediated stimulation by adrenocorticotropic hormone of cortisol synthesis (25), an effect that could underly its depression of steroid hormone levels. Inhibition by TCDD of rat liver phosphoenolpyruvate carboxykinase (26), a cAMP-dependent enzyme that controls the rate of gluconeogenesis, has been implicated in the wasting syndrome. It is interesting to speculate further that depressed cAMP-mediated glucose production *per se* could contribute both to the cardiac toxicity of TCDD and the wasting syndrome. Forcing the chick embryo to rely on carbohydrates for fuel by administration of β-amino-γ-trimethylammonium Glutyrate (DL-aminocarnitine) to inhibit fatty acid oxidation presented a lethal stress to TCDD-treated chick embryos (27). The heart relies mainly on fats for fuel but increases glucose utilization when glucose is increased by a high carbohydrate diet or by stress-induced catecholamine release. Impaired ability of the heart to use glucose when needed could lead to an energy deficit and a decreased ability to respond to inotropic stresses; this has been suggested to occur in failing hearts (28).

There are several similarities between ventricular contractile defects caused by TCDD and changes in human failing myocardium in clinical and experimental heart failure (29, 30). In both conditions, there is worsening impairment of inotropic and lusitropic responsiveness to isoproterenol and abnormal calcium handling, although initial tensions of ventricles paced at 1 Hz are unaffected even with severe and prolonged dysfunction. However, there are some differences. Failing hearts show myocardial hypertrophy; this has not been reported after TCDD treatment, nor did we find any increase in heart weight for TCDD-treated chick embryos (data not shown). Moreover, even when isoproterenol responsiveness is severely depressed in failing myocardium, $[Ca^{2+}]_i$ is not reported to be increased. Decreased β-adrenergic responsiveness superimposed on increased $[Ca^{2+}]_i$ could hasten deterioration of cardiac function and favor development of calcium overload, a potentially lethal condition (31, 32). The development of a decreased response to $[Ca^{2+}]_o$ may herald the development of calcium overload. It may be noteworthy in this regard that the avian ventricular response to $[Ca^{2+}]_o$ was not affected at 48 hr after TCDD

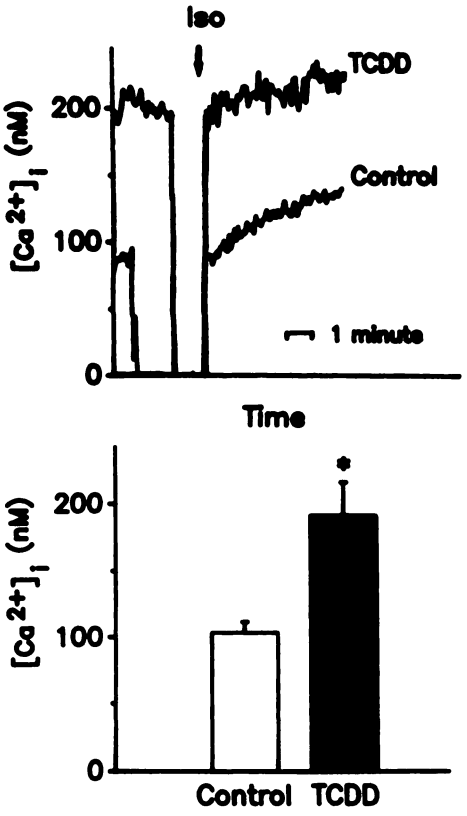

Fig. 9. Effect of TCDD treatment *in ovo* on $[Ca^{2+}]_i$ of cultured cardiac myocytes and on the response to isoproterenol. *Top panel*, representative tracings for coverslips containing cardiac myocytes cultured from 15-day-old embryos treated for 5 days with 0.01 ml dioxane alone or 1 nmol TCDD. After loading with fura 2-AM, coverslips were placed in the fluorimeter and were equilibrated in HEPES-buffered medium for 3 min at 37°. Fluorescence was monitored at 340 and 380 nm (excitation). Readings were taken for 1 min to obtain $[Ca^{2+}]_i$. The HEPES-buffer was then replaced with fresh buffer containing 1 μM of isoproterenol (*at arrow*). At the end of the experiment, $[Ca^{2+}]_i$ was calibrated as described in Experimental Procedures and the *tracing* shown was derived by the software according to the formula of Grynkiewicz *et al.* (11). *Bottom panel*, mean values ± standard error for $[Ca^{2+}]_i$ derived from measurements on four independent cell culture preparations (four different egg cohorts), each on at least three coverslips (*, significantly different from controls, $p < 0.01$).

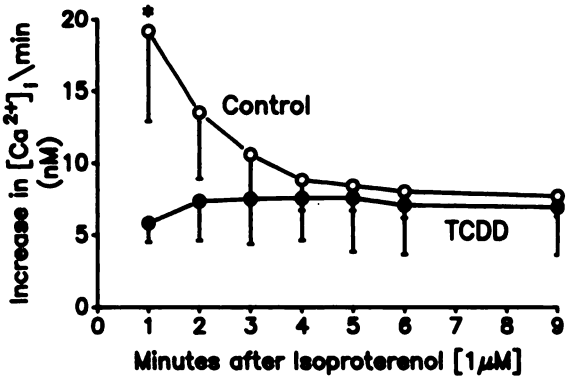

Fig. 10. Inhibition by TCDD of the rate of increase in $[Ca^{2+}]_i$ in response to isoproterenol in cardiac myocytes. Cells from control and TCDD-treated embryos were cultured, and $[Ca^{2+}]_i$ was measured in response to isoproterenol as described in Experimental Procedures and in the legend to Fig. 9. Results are shown for measurements derived from three independent cell culture preparations, each on at least three coverslips. Analysis of variance for TCDD-treated vesus control: $p = 0.01$. Post-hoc analysis for TCDD-treated versus control: *, $p < 0.01$.

treatment when there was no increase in mortality, but was significantly depressed after 7 days of exposure when mortality was increased. (The incidence of mortality at 7 days, examined in a subset of 269 eggs included in these experiments, was 10% for controls, 29% at 1 nmol TCDD per egg, and 47% at 5 nmol per egg.)

These findings are consistent with other evidence for increases in $[Ca^{2+}]_i$ by TCDD. A transient increase in Ca^{45} influx was observed minutes after adding TCDD to cultured mouse hepatoma cells (33). More persistent increases in $[Ca^{2+}]_i$ have been found in rat thymocytes exposed to TCDD (34) and in rat liver several days after TCDD treatment (35). In our experiments, the effect of TCDD on $[Ca^{2+}]_i$ in cardiac myocytes was clearly persistent; it was found in cardiac myocytes removed from embryos treated *in ovo* and cultured for 48 hr. The finding indicates that TCDD increases the set point at which $[Ca^{2+}]_i$ is maintained.

Increased cardiac myocyte $[Ca^{2+}]_i$ is consistent with several observed effects of TCDD on ventricular contraction that are not readily explained by depression of β-adrenergic responsiveness alone. The response to increasing $[Ca^{2+}]_o$, for example, would be expected to be depressed in cells in which $[Ca^{2+}]_i$ is elevated because levels of $[Ca^{2+}]_i$ that exceed the handling capacity of the SR and diminish L-type channel conductance (36) would be reached at lower concentrations of $[Ca^{2+}]_o$. For like reasons, increased $[Ca^{2+}]_i$ is consistent with the amelioration of TCDD's depression of the response to isoproterenol when $[Ca^{2+}]_o$ was lowered from 1.8 to 0.9 mM. Further, the intermittent lack of contraction in response to successive voltage stimuli in high K^+ indicates that ventricular myocardium from TCDD-treated embryos has a prolonged refractory period. The length of the refractory period can reflect the amount of $[Ca^{2+}]_i$ needed to be removed during relaxation and would be expected to be longer in cells with increased $[Ca^{2+}]_i$.

Even if TCDD depresses cAMP-dependent SR function as our findings indicate, it is not clear why normal levels of $[Ca^{2+}]_i$ should not be maintained if the cAMP-independent mechanisms regulating Ca^{2+} efflux through the sarcolemma, Na^+-Ca^{2+} exchange and the Ca^{2+} pump (37), are functioning normally. Thus, TCDD may increase $[Ca^{2+}]_i$ by a mechanism independent of its effects on β-adrenergic function. Thapsigargin, which inhibits Ca^{2+} uptake by the SR, may present an analogous situation in this respect because an effect on the sarcolemma has had to be invoked to explain the increase by thapsigargin in $[Ca^{2+}]_i$ in cardiac cells (38).

Among the many mechanisms by which TCDD could increase $[Ca^{2+}]_i$, inhibition of Na^+ or Ca^{2+} ATPases or changes in lipid composition may merit consideration first because these are related to known actions of TCDD. Thus, TCDD has been reported to inhibit Na^+ and Ca^{2+} ATPases in liver membranes (39). Inhibition of Na^+ ATPase in heart could increase $[Ca^{2+}]_i$ by increasing $[Na^+]_i$ and secondarily increasing Ca^{2+} influx by reverse Na^+-Ca^{2+} exchange (37, 40); this would be consistent with inferences that TCDD increases Ca^{2+} influx in hepatoma cells (33) and thymocytes (34). Depression of the synthesis or function of Ca^{2+}ATPases could decrease Ca^{2+} efflux through the sarcolemma and impair Ca^{2+} uptake by the SR (41) and thus could provide a single explanation for the decrease in β-adrenergic responsiveness and the increase in $[Ca^{2+}]_i$ by TCDD.

Changes by TCDD in cardiac membrane lipid composition by arachidonic acid, or by epoxide or monohydroxylated metabolites of arachidonic acid generated by TCDD-induced cytochrome P-450 (14, 42), for example, could affect ion passage and electrical responsiveness, increasing $[Ca^{2+}]_i$ and altering cardiac contractile responses (43, 44). P-450-generated arachidonic acid products have been found in plasma (45). Therefore, arachidonic acid metabolites generated by liver or kidney could potentially reach the heart through the circulation.

The known biochemical and toxic effects of TCDD require binding by TCDD to the cytosolic Ah receptor, a ligand-activated transcription factor (1, 46). The effects of TCDD on ventricular contraction are consistent with an Ah receptor-mediated action because they were elicited by treatment of chick embryos with TCDD and with toxic PCBs that bind to the Ah receptor (3), but not by agents that are not Ah receptor ligands, such as non-toxic PCBs (3) or phenobarbital. We do not yet know whether the increase by TCDD in $[Ca^{2+}]_i$ is also consistent with an Ah receptor-mediated mechanism.

The failure of TCDD to affect initial tensions or the force frequency response up to 1 Hz, even after treatment for 7 days at 5 nmol per egg, as was found in papillary muscles from TCDD-treated guinea pigs (5), indicates that differences in function of TCDD-treated and untreated ventricles are not apparent under basal conditions. Thus, SR from TCDD-treated embryos seems to be able to take up and release the Ca^{2+} needed to sustain contraction at slow rates of stimulation. The ventricular effects of TCDD may become functionally significant at heart rates too rapid for the SR to fill with Ca^{2+} at each cycle. This could normally occur with catecholamine stimulation and perhaps even under basal conditions in birds and small mammals *in vivo*. Thus, TCDD may diminish ability to handle normal cardiac stresses. It is interesting to speculate that the cardiac effects of TCDD could have contributed to its high toxicity for birds and small mammals seen after environmental TCDD exposures, such as in the industrial accident at Seveso, Italy (47).

In summary, we have shown that TCDD has well defined, progressive effects, independent of changes in diet, on avian ventricular responses to inotropic stimuli. These effects are reflected in complementary changes at the cellular level: an increase in $[Ca^{2+}]_i$ and suppression of the increase in $[Ca^{2+}]_i$ by isoproterenol. Our findings provide new evidence that the heart is a target of TCDD action and that TCDD disturbs intracellular calcium processing. Understanding the basic mechanisms involved may provide insights into the toxicity and lethality of TCDD and related polyhalogenated aromatic hydrocarbons, and into the processes by which the cell normally maintains calcium homeostasis.

Acknowledgments

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