RDX Binds to the GABA<sub>A</sub> Receptor–Convulsant Site and Blocks GABA<sub>A</sub> Receptor–Mediated Currents in the Amygdala: A Mechanism for RDX-Induced Seizures

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BACKGROUND: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a high-energy, trinitrated cyclic compound that has been used worldwide since World War II as an explosive in both military and civilian applications. RDX can be released in the environment by way of waste streams generated during the manufacture, use, and disposal of RDX-containing munitions and can leach into groundwater from unexploded munitions found on training ranges. For >60 years, it has been known that exposure to high doses of RDX causes generalized seizures, but the mechanism has remained unknown.

OBJECTIVE: We investigated the mechanism by which RDX induces seizures.

METHODS AND RESULTS: By screening the affinity of RDX for a number of neurotransmitter receptors, we found that RDX binds exclusively to the picrotoxin convulsant site of the γ-aminobutyric acid type A (GABA<sub>A</sub>) ionophore. Whole-cell in vitro recordings in the rat basolateral amygdala (BLA) showed that RDX reduces the frequency and amplitude of spontaneous GABA<sub>A</sub> receptor–mediated inhibitory postsynaptic currents and the amplitude of GABA-evoked postsynaptic currents. In extracellular field recordings from the BLA, RDX induced prolonged, seizure-like neuronal discharges.

CONCLUSIONS: These results suggest that binding to the GABA<sub>A</sub> receptor convulsant site is the primary mechanism of seizure induction by RDX and that reduction of GABAergic inhibitory transmission in the amygdala is involved in the generation of RDX-induced seizures. Knowledge of the molecular site and the mechanism of RDX action with respect to seizure induction can guide therapeutic strategies, allow more accurate development of safe thresholds for exposures, and help prevent the development of new explosives or other munitions that could pose similar health risks.


Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; royal demolition explosive) is a high-energy cyclic compound developed before World War II and has a history of worldwide use in explosive mixtures and formulations, such as C-4 (McLellan et al. 1992; Yinon 1990). RDX can be released into the environment by way of waste streams generated during the manufacture, use, and disposal of the pure product or RDX-containing munitions (McLellan et al. 1992). Another possible route of exposure is unexploded munitions found on training ranges, with the accompanying risk of groundwater leaching [Agency for Toxic Substances and Disease Registry (ATSDR) 2010]. The risk of inadvertent release to groundwater or soil has resulted in U.S. Environmental Protection Agency (EPA) regulations governing human oral exposure. Based on more recent studies (Crouse et al. 2006; Gust et al. 2009; Kasuske et al. 2009; Kucukardali et al. 2003), the U.S. EPA is currently reassessing RDX information available in the Integrated Risk Information System database (U.S. EPA 1993).

For >60 years it has been known that human exposure to high doses of RDX causes headache, dizziness, vomiting, and confusion, followed by tonic-clonic seizures (Barsotti and Croft 1949; Kasuske et al. 2009; Kucukardali et al. 2003). Although initial isolated reports were of accidental exposure during manufacture or other contamination (ATSDR 2010; U.S. EPA 1993), case reports peaked during the Vietnam War, when RDX was inadvertently ingested by soldiers using it to ignite cooking fires, or intentionally taken as an illicit intoxicant (Stone et al. 1969); such case reports still appear (Kasuske et al. 2009). Intense seizures and status epilepticus after experimental RDX exposure have also been observed in a wide range of species, from lizards and birds to nonhuman primates (Burdette et al. 1988; Crouse et al. 2006; Gust et al. 2009; Martin and Hart 1974; Quinn et al. 2009; Schneider et al. 1977; Von Oettingen et al. 1949). It has been speculated that the limbic plays a primary role in RDX-induced seizures (Burdette et al. 1988), but the cellular mechanism of seizure induction has been unknown. Here, we present evidence that RDX binds at the convulsant site of the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor (Benarroch 2007; Johnston 2005; Kalueff 2007), causing reduction of GABA<sub>A</sub> receptor–mediated synaptic transmission and induction of epileptiform activity, as we demonstrate in the amygdala, a seizure-prone structure of the limbic system (Aroniadou-Anderjaska et al. 2007, 2008).

Materials and Methods

RDX. We obtained RDX from the Department of the Navy (Naval Ordnance and Security Activity, Indian Head, MD); HPLC analysis showed it to be >99.5% pure, with the remainder being water. For animal experiments, RDX was dissolved in 1% methylcellulose and 0.2% Tween-80 and administered to rats by oral gavage at the dose of 75 mg/kg, a dose that consistently induces seizures within 30 min after administration (McCain W., personal communication). Control rats received a vehicle solution that consisted of 1% methylcellulose and 0.2% Tween-80 without RDX; vehicle was also administered by oral gavage. For in vitro studies, RDX was dissolved in dimethyl sulfoxide (DMSO) at a 100× concentration to minimize the percentage of DMSO in the final dilution.

In vivo electrophysiological experiments. Male Sprague-Dawley rats were individually housed in an environmentally controlled room (20–23°C; 12-h light/dark cycle, with lights on 0600 hours), with food and water available ad libitum. The rats weighed 150–250 g at the beginning of the experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the U.S. Army Center for
homogenates using the 96-well microplate method of Padilla et al. (1998). A 2% (wt/wt) homogenate was made in 0.1 M Na-phosphate buffer (pH 8.0) plus 1% Triton, using a 20-sec burst (on ice) of a Polytron AT 10/35 (Brinkman Instruments, Rexdale, Ontario, Canada). Total cholinesterase [brain cholinesterase is 95% AChE (Padilla et al. 1998)] was determined at 37°C by measuring the change in absorbance at 412 nm over 5 in in a 96-well plate reader (Bio-Tek Synergy HT, Winsoski, VT) using acetylthioleucine iodide as substrate and 5,5-dithio-bis[2-nitrobenzoic acid] as the colorimetric indicator. We generated a glutathione sulhydryl standard curve for conversion of absorbance units into nanomoles of sulphydryl groups. AChE enzymatic activity in the brain samples was then calculated as micromoles of substrate hydrolyzed per minute per gram wet weight (ww) (Padilla et al. 1998). Blood and brain samples were assayed for RDX content by the U.S. Army Public Health Command Directorate of Laboratory Sciences using gas chromatography with electron capture detection (GC-ECD) (Bishop et al. 2003).

Receptor binding assays. Neurotransmitter receptor binding assays for RDX were performed by Ricerca Biosciences (Concord, OH). The specific assays and catalog numbers are listed in Table 1. Initially, RDX was tested at a single concentration of 33 µM. Routine screening for receptor affinity is usually done with a compound concentration of 10 µM (Ricerca Biosciences). To better ensure the probability of a “hit” with RDX, the initial test of RDX was performed using 33 µM, a half-log higher concentration. Subsequently, a complete dose response of RDX was tested in the [35S]-rabiltybicyclophosphorothionate ([35S]-TBPS) convulsant site assay (catalog no. 226830) using picrotoxin as standard; this assay is based on the method of Malsay (1993). The maximal inhibitory concentrations (IC50) were determined by a nonlinear least-squares regression analysis using MathIQ (ID Business Solutions Ltd., Guildford, Surrey, UK). The inhibition constant (Ki) values were calculated using the equation of Cheng and Prusoff (1973), the observed IC50 of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the dissociation constant (Kd) of the ligand (obtained experimentally at Ricerca Biosciences).

In vitro extracellular and whole-cell patch-clamp recordings. Acute amygdala slices were prepared from male Sprague–Dawley rats (200–250 g body weight) as described previously (Aroniadou-Anderjaska et al. 2001; Braga et al. 2003). The artificial cerebrospinal fluid (ACSF) consisted of 125 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 25 mM NaHCO3, 1.25 mM NaH2PO4, and 11 mM glucose, bubbled with 95% O2/5%CO2 to maintain a pH of 7.4. For extracellular field potential recordings, slices were transferred to an interface-type recording chamber maintained at 32°C, where they were perfused with ACSF at 0.7–1 mL/min. Spontaneous activity was recorded in the basolateral amygdala (BLA) in the gap-free mode (continuous recordings), while evoked field potentials were sampled by delivering single stimulation pulses to the external capsule at 30-sec intervals. Recording glass pipettes were filled with ACSF and had a resistance of approximately 5 MΩ. Bipolar stimulating electrodes were constructed from twisted stainless steel wire, 50 µm in diameter. Spontaneous and evoked analog signals were digitized using pClamp10 software (Molecular Devices, Division of MDS Analytical Technologies, Sunnyvale, CA).

For whole-cell recordings, slices were transferred to a submersion-type recording chamber (0.7 mL volume), where they were continuously perfused with oxygenated ACSF at a rate of 3–5 mL/min. The techniques employed were similar to those described previously (Braga et al. 2003; Pidoplichko and Dani 2005). All experiments were performed at 32–33°C. We visualized neurons under infrared light using Nomarski optics of an upright microscope (Zeiss Axioskop 2; Carl Zeiss MicroImaging, Thornwood, NY) equipped with a CCD-100 camera (Dage-MTI, Michigan City, IN). Tight-seal (> 1 GΩ) whole-cell recordings were obtained from the cell body of pyramidal-shaped neurons in the BLA region. Access resistance (5–24 MΩ) was regularly monitored during recordings, and cells were rejected if the resistance changed by > 15% during the experiment. Patch electrodes were fabricated from borosilicate glass and had a resistance from 3 to 4 MΩ when filled with solution A (60 mM CsCl, 60 mM KCl, 10 mM EGTA, 10 mM HEPES, 5 mM MgATP, 0.3 mM NaGTP, pH 7.2; 280–290 mOsm/kg) or with solution B (135 mM Cs-glucosone, 10 mM MgCl2, 0.1 mM CaCl2, 1 mM EGTA, 10 mM HEPES, 2 mM Na-ATP, 0.2 mM NaGTP, pH 7.2; 280–290 mOsm/kg). Solution B (high Cs+) stabilizes cell leakage (allowing prolonged periods of recording) but depresses the hyperpolarization-activated current (Ih), whereas solution A (lower Cs+) still keeps the leakage low without affecting the Ih current. Neurons were voltage-clamped at +40 mV (intrapiptide solution A) or at ~70 mV (intrapiptide solution B) holding potential, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA).

We performed pressure application of GABA with the help of the push–pull experimental arrangement (Pidoplichko and
Effects of RDX on brain AChE.

Organophosphorus pesticides and nerve agents are potent inhibitors of peripheral and central nervous system AChE. Exposure to these agents causes excessive salivation and lacrimation in addition to seizure induction (Shih et al. 2003). Because of anecdotal reports of increased salivation and/or lacrimation associated with RDX intoxication (Burkett et al. 1988; Crouse et al. 2006; Schneider et al. 1977), we speculated that RDX-induced seizures might involve a similar inhibition of brain AChE. To examine this possibility, we administered RDX (75 mg/kg) or vehicle solution to rats (n = 6/group). Seizures appeared 10–21 min after dosing, but we did not observe increased salivation or lacrimation. At the onset of seizures, the rats were euthanized, and samples of the frontal lobe and blood were collected for measurements of AChE activity and content of RDX. AChE activity in the vehicle- and RDX-treated groups was identical (6.6 ± 0.4 µmol/min/g ww). Thus, seizure induction by RDX does not involve inhibition of AChE.

Blood and brain concentrations of RDX during seizures. Analysis of the blood and frontal cortex samples taken at the onset of RDX-induced seizures indicated a direct correlation of blood to brain concentration of RDX (Figure 2A); the correlation coefficient (CC) was 0.81. This result indicates that RDX readily enters the brain in direct proportion to the level of RDX in the blood after intestinal absorption. The correlation of brain RDX concentration with time to seizure onset after the oral gavage dose is presented in Figure 2B (CC = −0.61). These data indicate that the higher the brain concentration of RDX, the shorter the time interval between RDX administration and seizure onset.

RDX binds to the convulsant site of the GABA_A receptor. To determine the binding sites of RDX in the brain that may be involved in seizure induction, we screened a battery of neurotransmitter receptors for affinity to RDX. The receptors assayed included those implicated as targets of known convulsants, such as the glutamate family of receptors, nicotinic and muscarinic acetylcholine receptors, the glycine receptor, the family of GABA_A receptor ligand sites, the batrachotoxin site of the sodium channel (site 2) (Catterall et al. 1981), and several others (Table 1). Out of this comprehensive list, the only binding site for which RDX had significant affinity was the TBPS/picrotoxin/butyrylcholoethersulphonate (TBOB) convulsant site of the GABA_A receptor (Kalinec 2007; Maksay 1993). RDX inhibited [35S]-TBPS and [35S]-TBOB binding by > 70% at the screening RDX concentration of 33 µM. In Figure 3, the full dose–response [35S]-TBPS binding curve is shown for both RDX and picrotoxin, which...
was used as a positive control; RDX has an apparent $K_i$ of $21.1 \pm 2.1 \mu M$, compared with picrotoxin, which has an apparent $K_i$ of $0.20 \pm 0.042 \mu M$.

**RDX reduces GABA$_A$ currents and induces seizure-like activity in the BLA in vitro.** Having found that RDX binds to the convulsant site of the GABA$_A$ receptor, we next examined the functional consequences by determining the effect of RDX on GABA$_A$ receptor–mediated currents in the basolateral nucleus of the amygdala (BLA). The amygdala plays a central role in seizure generation, with the BLA being the most important nucleus involved in this role (Aroniadou-Anderjaska et al. 2007, 2008; Mohapel et al. 1996; White and Price 1993), and previous studies have suggested that the amygdala is a target for RDX (Burds et al. 1988; Levine et al. 1990; Macphail et al. 1985). In *in vitro* brain slices, we identified recorded neurons in the BLA as principal cells on the basis of their pyramidal shape and the recorded neurons in the BLA as principal cells (Materials and Methods) or at action-potential–dependent spontaneous inhibitory post synaptic currents (sIPSCs), from $21 \pm 3$ events/sec in control conditions to $8 \pm 2$ events/sec and $31 \pm 4$ pA in the presence of RDX ($n = 4$, $p < 0.05$). Examples are shown in Figure 4A and B, and the group results are shown in Figure 4C and D. sIPSCs recovered only partially after 12-to 15-min washout of RDX. The recovered currents were completely blocked by the GABA$_A$ receptor antagonist bicuculline (20 µM; Figure 4B).

To directly demonstrate the blockade of GABA currents by RDX, we examined the effect of RDX on the currents induced by local pressure application of GABA. Pressure-applied GABA (200 µM) for 500 msec to pyramidal-shaped BLA neurons, in the presence of CNQX (10 µM), AP-5 (50 µM), and SCH50911 (20 µM), elicited currents that were significantly reduced by 40 µM RDX added to the bath (37 ± 3% reduction, mean ± SE; $n = 6$; Figure 5). Current amplitudes recovered only partially after the wash-out of RDX.

To determine the impact of the reduction of sIPSCs by RDX on the overall activity of the BLA neuronal network, we applied RDX (100 µM) to the slice medium while recording extracellular field spontaneous activity. RDX induced prolonged, seizure-like neuronal discharges in the BLA within 15–25 min after exposure ($n = 4$; Figure 6). We applied single-pulse stimuli every 30 sec to sample the evoked field potential responses. Seizure-like activity was triggered by each stimulus pulse but was also present when stimulation was
turned off (Figure 6B,C). The effect of RDX was not reversible at least after 40 min of RDX washout.

**Discussion**

RDX has been used extensively as an explosive in both military and civilian applications. The health hazards from RDX exposure have been known since the 1940s, with brain seizures and status epilepticus as one of the major symptoms of RDX intoxication seen in humans (Barsotti and Crofti 1949; Stone et al. 1969) and rats (Burstone et al. 1988; Macphail et al. 1985; Schneider et al. 1978; Von Oettingen et al. 1949). However, until the present day there has been no information regarding the mechanisms by which RDX induces seizures. The major finding of the present study is that RDX binds to the picrotoxin convulsant site of the GABA_A receptor and inhibits GABA_A receptor-mediated synaptic transmission; this is likely to be the primary mechanism by which RDX induces brain seizures.

**RDX-induced seizures.** Previous reports have described seizures in humans after accidental acute consumption of RDX (Hollander and Colbach 1969; Kasuake et al. 2009; Stone et al. 1969; Woody et al. 1986). Animal studies have also demonstrated the induction of seizures after RDX administration (Merou et al. 1988; Crouse et al. 2006; Gust et al. 2009; Macphail et al. 1985; Martin and Hart 1974; Schneider et al. 1977; Von Oettingen et al. 1949). Because RDX causes rats to convulse within seconds after intravenous injection, Schneider et al. (1977) concluded that convulsions are caused by the parent compound rather than a neurotoxic metabolite of RDX. The present study supports this view because the brain concentration of RDX correlated positively with the time to seizure initiation and because RDX reduced GABAergic transmission and induced epileptiform activity in isolated brain slices. In addition, the present findings indicate that RDX is rapidly absorbed after oral administration and readily crosses the blood–brain barrier. A brain level of 8 µg/g ww was the lowest level we observed in the RDX-treated rats at the time of seizure onset, implying that this brain level is adequate for seizure initiation. The variability in blood-brain RDX concentrations among animals is probably related to differential absorption of the water-insoluble RDX in the methylcellulose suspension.

Burstone et al. (1988) suggested that the limbic system is significantly involved in RDX-induced seizures, based on the observation that amygdala kindling was accelerated in rats that received a relatively low daily dose of RDX. Here, we show that RDX reduces GABA_A receptor–mediated inhibitory synaptic transmission in the rat BLA, producing hyperexcitability and the appearance of seizure-like neuronal discharges *in vitro*. Consistent with the view that the amygdala is a primary target of RDX, and the well-known role of the amygdala in startle responses (Davis et al. 2008) and aggressive behavior (Makropoulou et al. 2008), rats that are administered RDX and do not reach the threshold for seizures display an increased acoustic startle response (Macphail et al. 1985), as well as an overall hyperreactivity and increased fighting (Levine et al. 1990).

**Mechanisms of RDX seizure induction.**

Many convulants induce seizures by reducing GABAergic inhibition, either by competitively antagonizing GABA or by directly blocking chloride influx through the GABA_A channel (Kaluff et al. 2007; Rowlett et al. 2005). The “cage convulsants,” compounds with cyclical structure, bind at a convulsant binding pocket inside the GABA_A ionophore at different—and possibly overlapping—positions. This “binding pocket” of the GABA_A channel is also referred to as the picrotoxin convulsant site (Ito and Ho 1994; Kaluff 2007; Makas and van Rijn 1993). We found that RDX, a cyclic nitramine, binds to the GABA_A receptor convulsant site but does not bind to any of the other neurotransmitter or neuromodulator receptors we studied. RDX displaced TBPS, a non-competitive (for GABA) ionophore blocker, which acts at the picrotoxin convulsant site (Ito and Ho 1994; Kaluff 2007; Makas and van Rijn 1993). RDX also displaced TBOB, which appears to bind at the same site as TBPS, and with kinetics similar to those of TBPS (Makas and van Rijn 1993). Thus, RDX binds inside the chloride ionophore at a convulsant site overlapping with TBPS. The potency of RDX at the GABA_A convulsant site (K_i of 21.1 µM) is similar to pentylene tetrazol, a convulsant commonly used in animal models of seizure and epilepsy that is also known to bind at the picrotoxin convulsant site (Coulter et al. 1990).

We tested the functional implications of RDX binding to the picrotoxin convulsant site in the amygdala, a limbic structure that plays a central role in the generation and propagation of seizures (Araniadou-Anderjaska et al. 2008) and is a primary target for neurotoxins, including warfarine neurotoxins such as nerve agents (Apland et al. 2009; Aroniadou-Anderjaska et al. 2009; Shih et al. 2003). We found that RDX significantly reduced the frequency and amplitude of action-potential–dependent iPSCs in the BLA, the amygdala nucleus that plays the primary role.

**Figure 5.** RDX reduced GABA-evoked currents in the BLA. Whole-cell recordings from principal neurons in the BLA were obtained in the presence of CNQX, AP-5, and SCH50911. (A) RDX (40 µM) reduced the outward postsynaptic current elicited by 500-msec pressure application of 200 µM GABA onto a pyramidal BLA neuron (V_h = +40 mV). (B) RDX (40 µM) reduced the inward postsynaptic current elicited by 500-msec pressure application of 200 µM GABA onto a pyramidal BLA neuron (V_h = −70 mV). In both A and B, each current trace is the average of four current recordings, and the black bar indicates the period of GABA application. After recording control current traces, RDX was applied to the bath; 4 min later, several GABA-evoked currents were recorded. Current amplitudes recovered partially after the washout of RDX. (C) Group data (mean ± SE; absolute current amplitude from both V_hold = +40 mV and V_hold = −70 mV experiments; n = 6) showing the effect of RDX on the amplitude of GABA-evoked currents. *p < 0.05.
in seizure generation (Aroniadou-Anderjaska et al. 2007, 2008; Mohapel et al. 1996; White and Price 1993). Consistent with the results from the binding studies described here, RDX reduced dIPSCs by a postsynaptic action on GABA<sub>A</sub> receptors. This is supported by the significant reduction of the postsynaptic currents evoked by locally applied GABA in the presence of RDX. The prolonged, seizure-like neuronal discharges recorded in the BLA in the presence of RDX were probably a result of the reduced inhibitory tone (reduced dIPSCs) throughout the BLA network because of the blockade of the GABA<sub>A</sub> channels by RDX. In a recent study in the northern bobwhite quail, Gust et al. (2009) found many molecular alterations in the brain after terminal RDX-induced seizures, including alterations in the expression of genes involved in the regulation of neuronal excitability.

RDX did not affect the activity of brain or peripheral blood AChE. The reported increased salivation and lacrimation after prolonged, low-dose RDX intoxication (Burdette et al. 1988; Crouse et al. 2006; Schneider et al. 1977) may be mediated by the effect of RDX at the GABA<sub>A</sub> receptor, similar to the effects of type II pyrethroid esters (Ecbichon 2001).

**Conclusions**

In the present study, we found that RDX induces seizures by binding to the picrotoxin convulsant site of the GABA<sub>A</sub> ionophore, thereby reducing GABAergic inhibitory transmission. We studied the effect of RDX on GABAergic synaptic transmission in the basolateral nucleus of the amygdala. Along with previous studies (Burdette et al. 1988; Levine et al. 1990; Macphail et al. 1985), the present study suggests that the amygdala is involved in the seizureogenic effects of RDX; however, other brain regions may also be significantly involved. In addition, although we performed RDX binding studies for a number of neurotransmitter and neuromodulator receptors as well as sodium channels, the list certainly is not exhaustive; RDX might also bind to other channels that affect neuronal excitability, which we did not test in the present study. The mechanism of RDX seizure induction revealed in the present study in rats is probably similar in humans, because the binding characteristics of TBPS to the GABA<sub>A</sub> receptor are similar in rat and human brain (Attack et al. 2007; Cole et al. 1984). Knowing that RDX induces seizures by binding to the GABA<sub>A</sub> ionophore can guide treatment efforts in cases of RDX overexposure and contribute to the development of drugs that will prevent the onset of seizures without producing sedation or other undesirable effects. Furthermore, with other potential munitions in development by the military, knowledge of the molecular site and the mechanism of RDX action with respect to seizure induction can help prevent the development of explosives or other munitions that could pose similar health risks.

**References**


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 figure. 6. RDX induced seizure-like neuronal discharges in the BLA in vitro. Spontaneous field activity was recorded extracellularly from the BLA, in the gap-free mode, in amygdala slices. Single-pulse stimulation was applied at 30-sec intervals (regularly spaced vertical lines are the stimulus artifacts). (A) No spontaneous activity was present in control conditions (before application of RDX). (B) Bath application of RDX (100 µM) induced seizure-like discharges, which were triggered by the stimulus pulse, but were also present when stimulation was turned off. (C) The effects of RDX were not reversed after 30 min of wash.


NOTE: In Figure 2 of the paper by Williams et al. [Environ Health Perspect 119:357–363 (2011)], the units for “Brain RDX concentration” (y-axis) should have been micrograms per gram wet weight (µg/g ww) instead of micrograms per milligram wet weight (µg/mg ww).

The authors regret the error.

The corrected text is presented in the PDF version of this article.