Long-Lasting Effects of a PEGylated Mutant Cocaine Esterase (CocE) on the Reinforcing and Discriminative Stimulus Effects of Cocaine in Rats

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Recent mutagenesis studies have identified a mutant G4C/S10C/T172R/G173Q cocaine esterase (CCRQ CocE) with an *in vitro* duration of action of >40 days. Although the *in vivo* duration of CCRQ CocE's action was <24 h, modification of this enzyme with polyethylene glycol (PEG) polymers resulted in a CocE (PEG-CCRQ CocE) capable of preventing cocaine-induced lethality for up to 72 h. The current studies were aimed at providing a detailed characterization of the effectiveness, selectivity, and duration of PEG-CCRQ CocE's actions in cocaine self-administration and discrimination assays in rats. Pretreatment with PEG-CCRQ CocE produced dose-dependent rightward shifts in the dose–response curves for cocaine self-administration and discrimination, with the highest dose of PEG-CCRQ CocE capable of producing an initial shift of cocaine's reinforcing and interoceptive effects of >30-fold to the right, with significant inhibition of these effects observed for up to 72 h. Although PEG-CCRQ CocE also produced slight reductions in the rates of methylphenidate- and food-reinforced responding, these effects were short-lived, lasting <24 h. Finally, when taken together with the finding that PEG-CCRQ CocE failed to alter the cocaine-like interoceptive effects of either methylphenidate or d-amphetamine, these results suggest that PEG-CCRQ CocE possesses a high degree of pharmacologic specificity for cocaine and a prolonged *in vivo* duration of action. In conclusion, these studies provide strong evidence to support the further development of long-lasting, highly efficient CocEs, such as PEG-CCRQ CocE, as a potential therapeutic option for the treatment of cocaine abuse in humans.
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INTRODUCTION

Cocaine abuse remains a significant worldwide public health problem, with an estimated 20 million individuals using cocaine during the 2009 calendar year (UNODC, 2010). Despite longstanding efforts to identify small molecules capable of selectively inhibiting the reinforcing effects of cocaine (eg, Dackis and O'Brien, 2003; Grabowski *et al*, 2004; Mello, 1990; Platt *et al*, 2002; Roberts and Brebner, 2000; Tanda *et al*, 2009; Vocci *et al*, 2005), there are currently no approved pharmacotherapies for the treatment of cocaine abuse. Recently, significant efforts have been made toward developing protein-based pharmacotherapies as an alternative approach to treating cocaine abuse with the goal of reducing the amount of cocaine that

is able to reach its central sites of action by altering the pharmacodynamic and/or pharmacokinetic properties of cocaine. Cocaine-specific antibodies achieve this by sequestering cocaine in the periphery (eg, Fox *et al*, 1996; Martell *et al*, 2005), whereas cocaine-specific enzymes and catalytic antibodies reduce the central effects of cocaine by enhancing the metabolism and clearance of cocaine, thus reducing the circulating concentrations of cocaine within both the central and peripheral nervous systems (eg, Landry and Yang, 1997; Turner *et al*, 2002).

Although cocaine-specific antibodies have been shown to reduce the reinforcing and/or subjective effects of cocaine in both preclinical (Carrera *et al*, 2000; Fox *et al*, 1996; Kantak *et al*, 2000), and clinical studies (Haney *et al*, 2010; Martell *et al*, 2005), many of these studies also suggest that the protective effects of cocaine-specific antibodies are easily surmounted by increasing the rate and/or amount of cocaine intake. One approach to circumvent these stoichiometric limitations has been to develop catalytic antibodies, such as mAb 15A10, which not only sequester cocaine in the periphery, but also catalyze its breakdown (Landry *et al*, 1993). Although this approach has been shown to selectively decrease cocaine self-administration in rats (Baird *et al*,

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2000; Mets *et al*, 1998), its utility has been limited by the relatively poor efficiency with which mAb 15A10 metabolizes cocaine (Kcat/Km ∼26-fold lower than butyrylcholinesterase (BChE)). Nevertheless, together with the demonstration that exogenous BChE is capable of reducing the toxic effects of cocaine (Lynch *et al*, 1997), these experiments provided the theoretical basis for the development of highly efficient cocaine-specific enzymes as pharmacotherapies for cocaine toxicity and abuse (eg, Zhan *et al*, 2003).

Cocaine is naturally metabolized by BChE to the inactive metabolites ecgonine methyl ester and benzoic acid, with elimination half-lives for cocaine of ∼26 min in rats and ∼45 min in non-human and human primates (Carmona *et al*, 2005; Mello *et al*, 2002; Mendelson *et al*, 1999). In an attempt to improve upon BChE's catalytic efficiency, Zhan and colleagues have used site-directed mutagenesis to create mutant BChEs capable of hydrolyzing cocaine ∼450–2000 times more efficiently than native BChE (Pan *et al*, 2005; Zheng *et al*, 2008). These mutant BChEs have shown promising results when evaluated in rodents, effectively reducing the cardiovascular, lethal, and response-reinstating effects of cocaine (Brimijoin *et al*, 2008; Xue *et al*, 2010; Zheng *et al*, 2008). In addition, an albumin-fused mutant BChE (Albu-CocH) has been shown to decrease progressive ratio responding for cocaine (Carroll *et al*, 2011), suggesting that it is also capable of reducing the reinforcing effectiveness of cocaine.

In addition to mutant BChEs, a highly efficient (Kcat/Km ∼800-fold greater than BChE; Larsen *et al*, 2002; Turner *et al*, 2002), bacterial cocaine esterase (CocE) has been isolated from a strain of *Rhodococcus* growing in the soil around coca plants (Bresler *et al*, 2000) and extensively evaluated as a possible treatment for cocaine toxicity and abuse. Although the wild-type (wt) form of CocE is capable of dose dependently protecting mice and rats against the cardiovascular, convulsant, and lethal effects of cocaine, it is rapidly inactivated at body temperature resulting in an *in vivo* half-life of ∼15 min (Cooper *et al*, 2006; Jutkiewicz *et al*, 2009; Ko *et al*, 2007, 2009; Wood *et al*, 2010). Site-directed mutagenesis studies aimed at improving the thermostability of CocE have identified an equally efficient mutant CocE (T172R/G173Q CocE, RQ CocE, DM CocE) with an *in vivo* half-life of ∼4.5 h in mice (Gao *et al*, 2009; Narasimhan *et al*, 2010), and a high degree of pharmacologic specificity (Brim *et al*, 2011). In addition to dose dependently inhibiting the cardiovascular, convulsant, lethal, and reinforcing effects of cocaine in rats (Collins *et al*, 2009, 2011b), DM CocE has also been shown to effectively reverse the cardiovascular effects associated with large intravenous (IV) doses of cocaine in rhesus monkeys (Collins *et al*, 2011a). While these findings suggest that DM CocE should provide an effective treatment option for acute cocaine toxicity in humans, its *in vivo* duration of action is likely too short to provide a realistic option for the treatment of cocaine abuse.

Recently, substitution of cysteine residues at the Gly4 and Ser10 positions of RQ CocE has been shown to result in a mutant G4C/S10C/T172R/G173Q CocE (CCRQ CocE) with an *in vitro* half-life of >40 days at 37 °C. In rodents, however, the duration of CCRQ CocE's actions was <24 h. Modification of biologic entities with polyethylene glycol

(PEG) moieties has been shown to lengthen *in vivo* residence times by imparting stealth properties (Caliceti and Veronese, 2003). The addition of two 40 kDa branched PEG moieties (one per monomer) to CCRQ CocE (PEG-CCRQ CocE) dramatically increased its *in vivo* duration of action in rodents, with a 32.0-mg/kg dose of PEG-CCRQ CocE capable of reducing the reinforcing effects of 0.1 mg/kg/inj cocaine for up to 96 h, and the lethal effects of 180.0 mg/kg cocaine for up to 72 h (Narasimhan *et al*, 2011). Briefly, the current studies were aimed at extending these findings by providing a detailed characterization of the effectiveness, selectivity, and duration of PEG-CCRQ CocE's actions in cocaine self-administration and discrimination assays in rats. Accordingly, the effects of three doses of PEG-CCRQ CocE were evaluated against at least a 100-fold range of cocaine doses in self-administration and drug discrimination procedures, with the daily assessment of cocaine dose–response curves used to determine the *in vivo* duration of PEG-CCRQ CocE's actions. In addition, methylphenidate (self-administration and drug discrimination) and d-amphetamine (drug discrimination only) were also used to evaluate PEG-CCRQ CocE's pharmacologic selectivity, whereas rates of food-reinforced responding were used to evaluate the behavioral selectivity of PEG-CCRQ CocE's effects.

METHODS

Subjects

Male Sprague-Dawley rats (325–350 g) were obtained from Harlan (Indianapolis, IN) and maintained in a temperature and humidity controlled environment, on a 12-h dark/light cycle with lights on at 0700 h. All rats were singly housed with free access to tap water and ∼20 g of food per day. All experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals, and performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Surgery

Rats were prepared with chronic indwelling catheters in the left femoral vein under ketamine:xylazine (90 : 10 mg/kg; intraperitoneal (IP)) anesthesia. Catheters were passed under the skin and attached to stainless steel tubing that exited through a metal tether button that was sutured to the muscle between the scapulae. A 5–7-day recovery period was provided before experimentation. Catheters were flushed daily with 0.2 ml of heparinized saline (100 U/ml) before the start, and after the completion of operant sessions.

Apparatus

All experimental sessions were conducted in operant conditioning chambers (30.5 cm W × 24 cm D × 21 cm H; Med Associates, St Albans, VT) placed inside sound attenuating cubicles. For self-administration studies, each chamber was equipped with two nosepoke devices (ENV-114BM; Med Associates) positioned 6 cm above the stainless

steel grid floor and illuminated with a yellow stimulus light. Each chamber was also equipped a red, yellow, and green LED stimulus complex located above each nosepoke, and a white house light located on the top of the opposite wall. A syringe driver (PMH-100-20; Med Associates) delivered solutions through Tygon tubing connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA) and spring tether, which was held in place by a counterbalanced arm. For discrimination and sucrose reinforcement studies, chambers were equipped with two retractable levers, located 6 cm above the floor and on either side of a pellet dispenser (ENV-203M; Med Associates) which delivered 45 mg sucrose pellets (Bio-Serv, Frenchtown, NJ) into an external pellet cup. In these chambers, a LED stimulus complex was located above each of the levers as well as the pellet cup.

Self-Administration Studies

Acquisition of responding for IV drugs. Rats were initially trained to nosepoke for injections of either 0.32 mg/kg cocaine or 0.1 mg/kg methylphenidate ($n = 6$/drug) during daily, 60 min sessions under a fixed ratio (FR) 1 schedule of reinforcement. Illumination of the yellow stimulus light inside the left nosepoke signaled drug availability, with subsequent responses on this nosepoke resulting in drug injection (320 µl/kg delivered over ∼1 s). Injections were accompanied by the illumination of the red, yellow, and green LEDs above the left nosepoke, and followed by a 5-s time out (TO) during which the house light was illuminated and responses were recorded but had no scheduled consequence. Ratio requirements were gradually increased (1, 2, 3, and 5) until rats were responding under an FR5TO5 schedule of reinforcement. Upon stabilization of responding, defined as three consecutive sessions with less than a 20% variation and no increasing or decreasing trend in responding, rats were transferred to a multi-component, 5-dose procedure. Under this procedure, increasing doses of cocaine (0, 0.032, 0.1, 0.32, and 1.0 mg/kg/inj) or methylphenidate (0, 0.01, 0.032, 0.1, and 0.32 mg/kg/inj) were available during five sequential 25 min components (eg, Barrett *et al*, 2004; Hiranita *et al*, 2009). Components were initiated by the non-contingent delivery of the unit dose of the drug available for responding, and separated by a 2-min inter-component interval (ICI) during which time the house light was illuminated and responding had no scheduled consequence. During this phase of the experiment, the concentrations of cocaine (1.0 mg/ml) and methylphenidate (0.32 mg/ml) in the syringe were constant, with the different unit doses delivered by altering the injection duration (adjusted for body weight). For example, the volumes and injection durations for a 320-g rat were as follows: 0.0 mg/kg/inj = 0 µl over 0 s (stimuli only); 0.032 mg/kg/inj = 10 µl over 0.1 s; 0.1 mg/kg/inj = 32 µl over 0.3 s; 0.32 mg/kg/inj = 100 µl over 1 s; and 1.0 mg/kg/inj = 320 µl over 3 s. Rats were maintained on this schedule for the remainder of the experiment, with stability defined as three consecutive sessions with <20% difference in responding during each of the five components.

Effects of PEG-CCRQ CocE on the IV self-administration of cocaine and methylphenidate. Upon the acquisition of stable responding for cocaine or methylphenidate under the

5-dose schedule, rats were exposed to a single session saline substitution to determine the effects of removing the reinforcer on the maintenance of responding. Maintenance doses of cocaine (0.032–1.0 mg/kg/inj), or methylphenidate (0.01–0.32 mg/kg/inj) were reintroduced on the following day. In order to evaluate the capacity of PEG-CCRQ CocE to shift the dose–response curve for cocaine-maintained responding, pretreatments with PEG-CCRQ CocE were administered before sessions in which a 3.2-mg/ml dose of cocaine was loaded into the syringe, resulting in larger unit doses of cocaine being available for responding (0.1–3.2 mg/kg/inj). This range of cocaine doses was available during the session that occurred 0 and 24 h after pretreatment with 3.2 mg/kg PEG-CCRQ CocE, 0, 24, and 48 h after pretreatment with 10.0 PEG-CCRQ CocE, and 0, 24, 48, and 72 h after pretreatment with 32.0 mg/kg PEG-CCRQ CocE. Subsequently, the syringe was filled with 1.0 mg/ml cocaine, and rats were once again allowed to respond for the maintenance doses of cocaine (0.032–1.0 mg/kg/inj). All rats received all doses of PEG-CCRQ CocE, with the doses presented in random order, and separated by at least 7 days to allow for drug washout and the reacquisition of stable responding under maintenance conditions.

The effects of PEG-CCRQ CocE (10.0 and 32.0 mg/kg; IV) on responding maintained by methylphenidate were also evaluated, with pretreatments administered before sessions in which the maintenance doses of methylphenidate (0.01–0.32 mg/kg/inj) were available for responding. The order of PEG-CCRQ CocE doses was counterbalanced across rats and separated by at least seven sessions to allow for drug washout and the reacquisition of stable responding under the maintenance conditions.

Acquisition of responding for sucrose pellets. Rats were initially trained to respond for 45 mg sucrose pellets during daily, 60 min sessions under an FR1TO5 schedule of reinforcement identical to that described for IV self-administration, and the ratio requirements were gradually increased until rats were stably responding under an FR5TO5. Subsequently, sucrose pellets were made available under an FR5TO5 schedule of reinforcement during five sequential 25 min components, each separated by a 2-min ICI signaled by illumination of the house light. Food pellets were unavailable during the first component; instead, ratio completion resulted in the stimuli that normally accompanied pellet delivery and a 5-s TO. Subsequent components were signaled by the non-contingent delivery of a sucrose pellet, with an additional 29 pellets available for responding. After all 29 pellets for an individual component were earned, the house light was illuminated and rats spent the remainder of the 25-min component in TO. The next component began after the TO and 2 min ICI had elapsed.

Effects of PEG-CCRQ CocE on responding for sucrose pellets. Upon stabilization of responding under maintenance conditions, rats were pretreated PEG-CCRQ CocE (10.0 and 32.0 mg/kg; IV) or phosphate-buffered saline (PBS; vehicle) immediately before the start of sessions. These sessions were identical to maintenance sessions with the exception that rats were briefly removed from the chamber during the 2-min ICI and injected with saline (IP). In order to confirm PEG-CCRQ CocE's activity, a second

test session was conducted 48 h later during which cumulative doses of cocaine (1.0, 3.2, 10.0, and 32.0 mg/kg; IP) were administered during the 2-min ICI before components 1 through 4, respectively. No injections were given before the start of the extinction (EXT) component.

Drug Discrimination Studies

Discrimination training. Rats were initially trained to respond under an FR10TO5 for sucrose pellets on both the right and left levers during alternating components (ie, 10 pellets on the right and 10 pellets on the left) with a maximum of 40 pellets per session. Once rats consistently earned all 40 pellets, they were trained to discriminate 10.0 mg/kg; IP cocaine from saline using a single-alternation, two-lever discrimination procedure, with the position (right/left) of the cocaine-appropriate lever counterbalanced across rats. Following cocaine or saline administration, rats were placed into an operant chamber, illuminated by the house light, for a 10-min pretreatment period. The start of the session was signaled by the EXT of the house light, insertion of the levers, and the illumination of the yellow LEDs above each of the levers. Ratio completion (FR10) on the injection-appropriate lever resulted in sucrose pellet delivery followed by a 5-s TO during which the yellow LEDs above the levers were extinguished and the red, yellow, and green LEDs above the pellet tray were illuminated. Responding on the alternate lever was recorded, but had no scheduled consequence. Sessions terminated after 10 reinforcers or 10 min had elapsed, whichever came first, at which point the levers were retracted, all LEDs were extinguished, and the house light was illuminated. Upon acquisition of the discrimination, defined as three consecutive sessions with >80% injection-appropriate responding during the first ratio and entire session, the number of components was gradually increased to 5, with each component identical to the initial discrimination training session. Daily sessions comprising 1–5 components (randomized across days), with each component preceded by the administration of either saline or 10.0 mg/kg; IP cocaine. Although saline could be administered before any component, the administration of cocaine always occurred before the terminal component of the session. Thus, if cocaine was administered before the first component, the session was one component long. Training continued until rats were allocating at least 80% of their responding on the injection appropriate during the first ratio of each component, as well as for components as a whole.

Discrimination testing. Once rats met training criteria, two types of test were performed: substitution tests and pretreatment tests. During test sessions, completion of a ratio on either lever resulted in the delivery of a sucrose pellet, and the resetting of the ratio requirements on both levers. Sessions comprising 3–5 components, and terminated after rats allocated at least 80% of their total responding on the cocaine-appropriate lever, or if rats failed to complete a ratio on either lever during the component, which ever occurred first.

Substitution tests. The following drugs were evaluated using a cumulative dosing procedure: cocaine (0.32, 1.0, 3.2,

and 10.0 mg/kg; IP), methylphenidate (0.32, 1.0, 3.2, 10.0, and 18.0 mg/kg; IP), d-amphetamine (0.032, 0.1, 0.32, and 1.0 mg/kg; IP), and ketamine (1.0, 3.2, 10.0, 18.0, and 32.0 mg/kg; IP). Most drugs were administered as 10 min pretreatments.

Effects of PEG-CCRQ CocE on the discriminative stimulus effects of cocaine and cocaine-like drugs. Pretreatments with PEG-CCRQ CocE (3.2, 10.0, and 32.0 mg/kg; IV) or PBS occurred 1 min before the first injection of the session, with cumulative doses of 0.32, 1.0, 3.2, and 10.0 mg/kg; IP cocaine tested in PBS-treated rats, and the cumulative doses of 10.0, 32.0, 56.0, 100.0, and 180.0 mg/kg; IP cocaine evaluated after pretreatment with PEG-CCRQ CocE. These doses of cocaine were evaluated daily until rats allocated at least 80% of their responding on the cocaine-appropriate lever following the 10.0-mg/kg training dose of cocaine. Test sessions were identical to those described above, with the exception that >80% cocaine-appropriate responding or failure to complete a ratio would terminate sessions after any of the five components (ie, sessions comprising 1–5 components).

A second set of pretreatment tests were conducted to evaluate the effects of a 32.0-mg/kg; IV dose of PEG-CCRQ CocE against methylphenidate, d-amphetamine, and cocaine over 3 successive days. Substitution tests with methylphenidate (0.32, 1.0, 3.2, 10.0, and 18.0 mg/kg; IP) and d-amphetamine (0.032, 0.1, 0.32, and 1.0 mg/kg; IP) were initiated either 1 min or 24 h after PEG-CCRQ CocE administration, with the order counterbalanced across rats. Cocaine (0.32, 1.0, 3.2, 10.0, and 18.0 mg/kg; IP) was always administered during the third session, 48 h after PEG-CCRQ CocE administration.

Drugs

Cocaine, methylphenidate, and d-amphetamine were obtained from the National Institute on Drug Abuse (Bethesda, MD). Ketamine was purchased from Henry Schein (Denver, PA). All drugs were dissolved in physiologic saline, and administered IV for self-administration studies, and IP for drug discrimination studies. PEG-CCRQ CocE was prepared and purified as previously described (Narasimhan et al, 2011), and administered IV on a mg/kg basis in a volume of no >10 ml/kg immediately before the beginning of operant sessions.
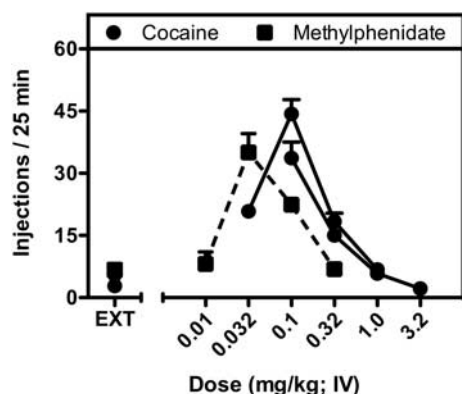
Data analysis

For self-administration studies, data are presented as the mean ± the standard error of the mean (SEM), $n = 6$, number of injections earned (IV drug self-administration), or ratios completed (food reinforcement) per 25 min component (GraphPad Prism; GraphPad Software, San Diego, CA). For food reinforcement studies, data are also presented as the mean ± SEM of the overall rate of responding (responses per second) during the active portion of each component. Significant effects of PEG-CCRQ CocE on the number of injections earned, ratios completed, and rates of responding were determined by two-way ANOVA with *post hoc* Bonferroni tests (GraphPad

Prism). For drug discrimination studies, data are presented as the mean ± SEM, $n = 5$, of the percent of overall responding that occurred on the cocaine-appropriate lever, and the overall response rate (responses per second) during the active portion of each component. Linear regression analyses (GraphPad Prism) were used to determine if there was a significant correlation between dose and percent cocaine-appropriated responding, with a full generalization to the discriminative stimulus effects of cocaine defined as a significant non-zero slope and at least 80% of the overall responding occurring on the cocaine-appropriate lever during any component. Dose ratios (dose required to produce 50% maximal effect (ED_{50}) PEG-CCRQ/ED_{50} PBS) were determined for each rat, with the average (95% confidence interval (CI)) dose ratio used to describe the degree to which PEG-CCRQ CocE shifted the dose–response curves for substitute drugs, with a significant shift defined as a 95% CI not inclusive of 1.0.

RESULTS

Baseline dose–response curves for responding maintained by increasing doses of cocaine (0.032–1.0 mg/kg/inj or 0.1–3.2 mg/kg/inj) and methylphenidate (0.01–0.32 mg/kg/inj) are shown in Figure 1. Both drugs maintained responding in a dose-dependent manner (cocaine 0.0–1.0 mg/kg/inj: $F_{(4, 20)} = 82.3$; $p < 0.001$; cocaine 0.0–3.2 mg/kg/inj: $F_{(4, 20)} = 56.0$; $p < 0.001$; methylphenidate: $F_{(4, 20)} = 31.0$; $p < 0.001$) with inverted U-shaped dose–response curves, and peak levels of responding observed at doses of 0.032 and 0.1 mg/kg/inj for methylphenidate and cocaine, respectively. Importantly, there were no significant differences in the numbers of cocaine injections (0.1, 0.32, or 1.0 mg/kg/inj) earned when the maintenance range of cocaine doses (0.032–1.0 mg/kg/inj), or a higher range of cocaine doses (0.1–3.2 mg/kg/inj) were available for injection. In addition, although responding

occurred at significantly lower rates when ratio completion resulted in saline injections ($F_{(1, 25)} = 35.7$; $p < 0.001$), baseline levels of responding were recovered during the first session after the reintroduction of cocaine (Figure 2; top left panel).

When administered immediately before the start of an operant session in which rats had access to cocaine injections, pretreatment with PEG-CCRQ CocE dose dependently ($F_{(3, 75)} = 4.8$; $p < 0.001$) affected the capacity of cocaine to maintain responding, with doses of 3.2 and 10.0 mg/kg PEG-CCRQ CocE shifting the dose–response curve for IV cocaine self-administration ~10-fold and 30-fold to the right, respectively (Figure 2). Pretreatment with 32.0 mg/kg; IV PEG-CCRQ CocE resulted in cocaine maintaining saline-like levels of responding, despite being available at unit doses as large 3.2 mg/kg/inj (Figure 2). In addition to the dose-dependent nature of PEG-CCRQ CocE's effects on cocaine-reinforced responding, the duration of these inhibitory effects was also dependent on the dose of PEG-CCRQ CocE, with larger doses producing a longer lasting inhibition. For instance, although the dose–response curve began to move back to the left as early as 24 h after PEG-CCRQ CocE, baseline-like rates of cocaine-
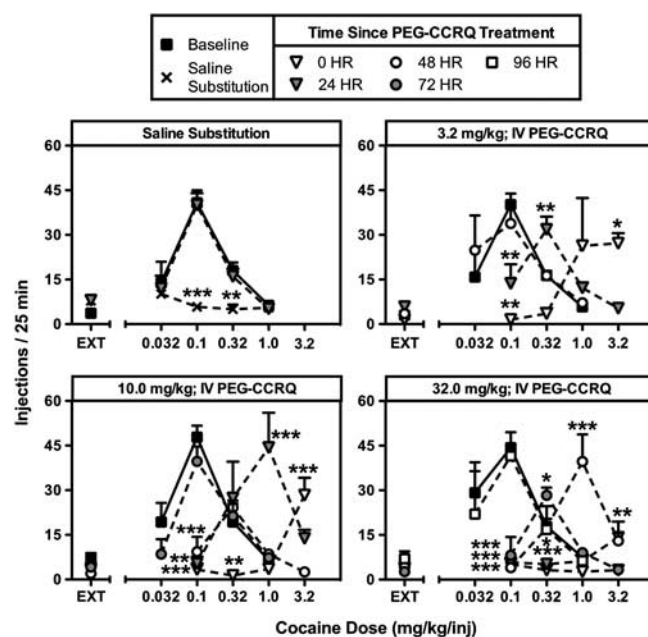
Figure 2 Time course of the effects of PEG-CCRQ CocE on FR5TO5 responding maintained by cocaine (0–3.2 mg/kg; IV) during five sequential 25 min components. Responding for cocaine (filled squares; 0.0–1.0 mg/kg/inj) during sessions immediately preceding treatment were used for baseline. Upper left panel: Responding during the substitution of saline for cocaine (X), and upon reintroduction of cocaine 24 h later (shaded triangles). Data represent the mean ± the standard error of the mean (SEM) number of cocaine injections ($n = 6$) earned during each 25 min component immediately after (open triangles), 24 h after (shaded triangles), 48 h after (open circles), 72 h after (shaded circles), and 96 h after (open squares) the administration of 3.2 mg/kg; IV (upper right panel), 10.0 mg/kg; IV (lower left panel), or 32.0 mg/kg; IV PEG-CCRQ CocE (lower right panel). Points above extinction (EXT) refer to the number of completed ratios that resulted in presentation of stimuli associated with injections, but no injection. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ represent points that were significantly different than baseline (0.0–1.0 or 0.0–3.2 mg/kg/inj cocaine) as determined by two-way, repeated measures ANOVA with post hoc Bonferroni tests.

Figure 1 Dose–response curves for responding maintained during five sequential 25 min components in which increasing doses of cocaine (filled circles) or methylphenidate (filled squares) were available for injection under an FR5TO5 schedule of reinforcement. Cocaine was available over one of two dose ranges (0.0, 0.032, 0.1, 0.32, and 1.0 mg/kg/inj or 0.0, 0.1, 0.32, 1.0, and 3.2 mg/kg/inj), and methylphenidate was available over one range of doses (0.0, 0.01, 0.032, 0.1, and 0.32 mg/kg/inj). Data represent the mean ± the standard error of the mean (SEM) number of injections ($n = 6$) earned during each 25 min component. Points above extinction (EXT) refer to the number of completed ratios that resulted in presentation of stimuli associated with injections, but no injection.

maintained responding were not observed until 48 h after 3.2 mg/kg PEG-CCRQ CocE, 72 h after 10.0 mg/kg PEG-CCRQ CocE, and 96 h after pretreatment with the 32.0 mg/kg dose of PEG-CCRQ CocE.

As was observed when cocaine served as the reinforcer, substituting saline for methylphenidate resulted in a significant reduction in responding $(F(1, 25) = 31.9; p < 0.001)$, with significantly fewer injections earned during the third and fourth components $(p < 0.001$ for both), and baseline levels of responding recovered during the first session that methylphenidate was made available for injection (Figure 3; top panel). Unlike with cocaine,

the 10-mg/kg dose of PEG-CCRQ CocE failed to alter responding maintained by methylphenidate; however, a significant disruption $(F(1, 25) = 9.0; p < 0.01)$ of methylphenidate-reinforced responding was observed following the 32.0-mg/kg dose of PEG-CCRQ CocE. Despite this apparent interaction between PEG-CCRQ CocE and methylphenidate, these effects were only observed at the 0.032-mg/kg/inj dose of methylphenidate, and only at the 0-h time point, with baseline levels of responding observed for all doses of methylphenidate 24 h later (Figure 3).

The effects of PEG-CCRQ CocE on high rates of food-reinforced responding are shown in Figure 4. Unlike the relatively restricted suppression of methylphenidate-reinforced responding, PEG-CCRQ CocE produced a slight but significant suppression of the rates of responding across the session as a whole during the 0-h time point $(F(2, 50) = 5.5; p < 0.01)$, however, *post hoc* tests failed to identify significant difference between the rates of responding for the PBS- and PEG-CCRQ CocE-treated groups during any of the five components. These effects were also short-lived, with baseline-like rates of responding observed 24 h later (data not shown). There were no significant effects of PEG-CCRQ CocE on the number of food pellets earned at either the 0- or 24-h time points. When evaluated 48 h after PBS treatment, cocaine produced a dose-dependent suppression of both the rates of responding $(F(4, 20) = 9.4; p < 0.001)$ and the total numbers of pellets earned per component $(F(4, 20) = 5.1; p < 0.01)$. These effects of cocaine were prevented in a dose-dependent manner by pretreatment with PEG-CCRQ CocE (rate of responding: $F(2, 50) = 11.6; p < 0.001$; pellets earned: $F(2, 50) = 9.5; p < 0.001$), despite the fact that it was administered 48 h before cocaine (Figure 4; right panels).

In order to further evaluate the selectivity and duration of PEG-CCRQ CocE's effects, a group of rats were trained to discriminate cocaine (10.0 mg/kg; IP) from saline using a two-lever drug discrimination task. As shown in Figure 5, cocaine produced dose-dependent increases in the selection of the cocaine-associated lever, with $96.9 \pm 1.2\%$ of responding occurring on the cocaine-appropriate lever following a cumulative dose of 10.0 mg/kg; IP cocaine. Similar dose-dependent increases in cocaine-appropriate responding were also observed when rats were pretreated with methylphenidate (maximum $88.1 \pm 7.1\%$) and d-amphetamine (maximum $93.6 \pm 4.6\%$), with a full generalization to
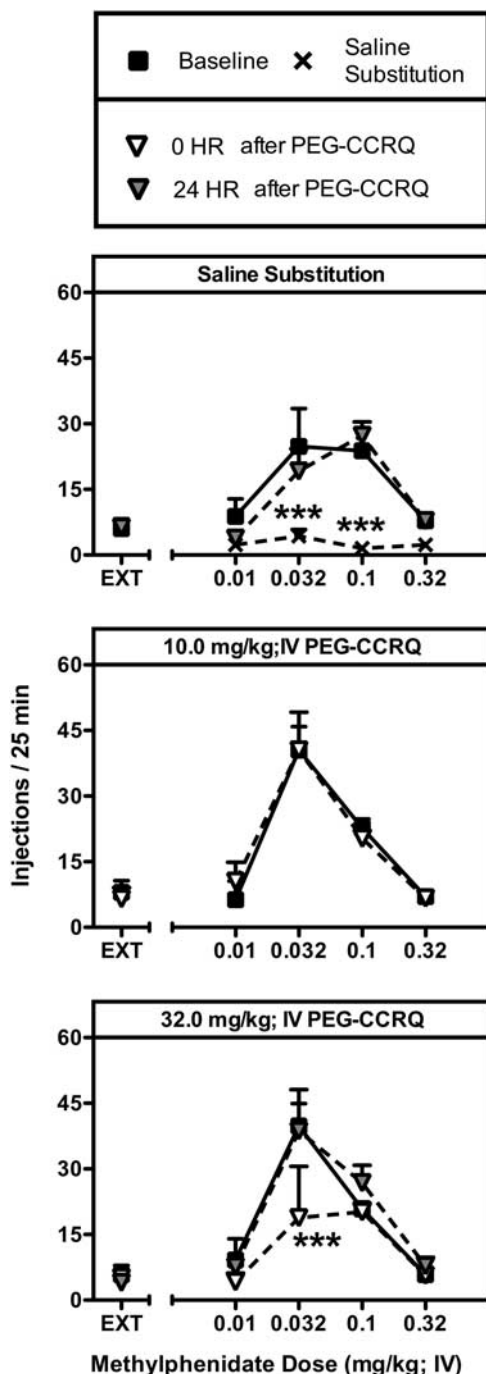
Figure 3 Time course of the effects of PEG-CCRQ CocE on FR5TO5 responding maintained by methylphenidate (0.0–0.32 mg/kg/inj) during five sequential 25 min components. Responding for methylphenidate (filled squares; 0.0–0.32 mg/kg/inj) during sessions immediately preceding treatment were used for baseline. Upper panel: Responding during the substitution of saline for methylphenidate (X), and upon reintroduction of methylphenidate 24 h later (shaded triangles). Data represent the mean ± the standard error of the mean (SEM) number of methylphenidate injections ($n = 6$) earned during each 25 min component (middle panel) immediately after (open triangles), and 24 h after (shaded triangles) the administration of 10.0 mg/kg; IV (middle panel) 32.0 mg/kg; IV PEG-CCRQ CocE (lower panel). Points above extinction (EXT) refer to the number of completed ratios that resulted in presentation of stimuli associated with injections, but no injection. ***$p < 0.001$ represents points that were significantly different than baseline (0.0–32 mg/kg/inj methylphenidate) as determined by two-way, repeated measures ANOVA with *post hoc* Bonferroni tests.
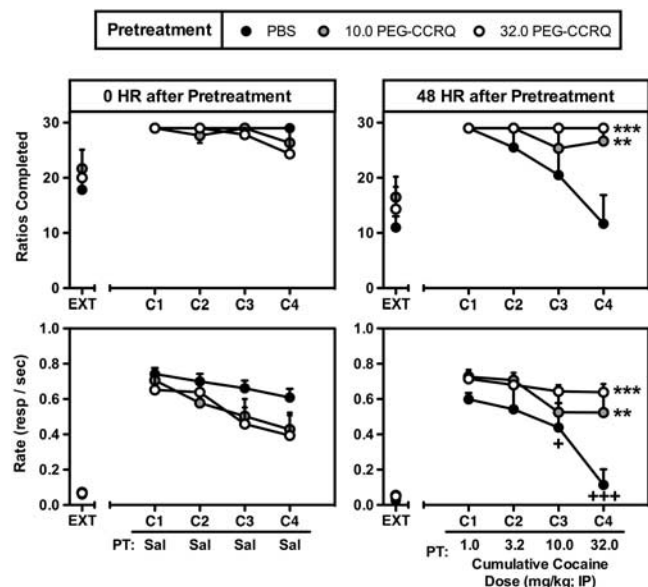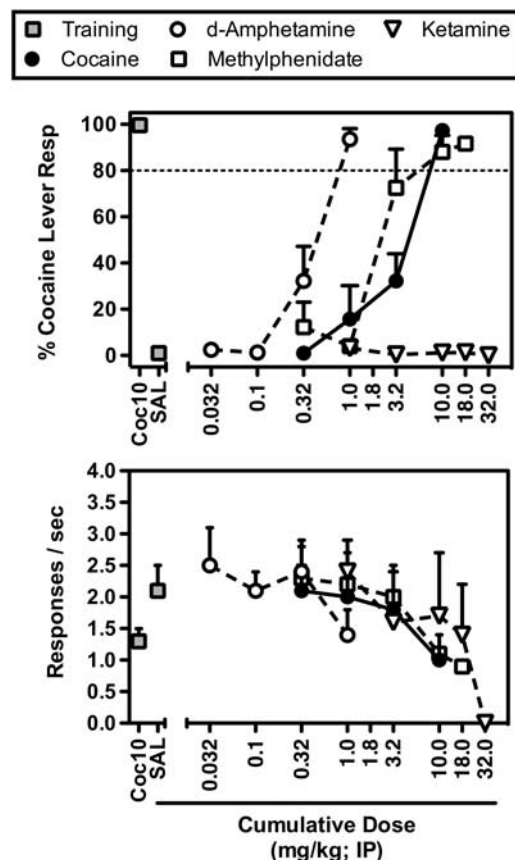
Figure 4 Effects of pretreatment with PEG-CCRQ CocE on FR5TO5 responding maintained by sucrose pellets. Number of ratios completed (upper panels) and rate of responding (responses per second; lower panels) during sessions that were preceded by the administration of PBS (filled circles), 10.0 mg/kg; IV (shaded circles), or 32.0 mg/kg; IV PEG-CCRQ CocE (open circles). Left panels represent responding that occurred immediately after PBS or PEG-CCRQ CocE administration, with saline injections delivered before components 1 through 4. Right panels represent responding during sessions that occurred 48 h after PBS or PEG-CCRQ CocE administration, with cumulative doses of cocaine (1.0, 3.2, 10.0, and 32.0 mg/kg; IP) administered before components 1 through 4. Data represent the mean ± the standard error of the mean (SEM) number of ratios completed or responses per second ($n = 6$) during each 25 min component. Points above extinction (EXT) refer responding that resulted in presentation of stimuli associated with pellet delivery, but no pellet. $**p < 0.01$; $***p < 0.001$; $+p < 0.05$; $+++p < 0.001$. Significant difference in the number of ratios completed, or rate of responding between PEG-CCRQ CocE-treated rats and PBS-treated rats receiving cocaine (*), and PBS-treated rats not receiving cocaine (ie, 24 h prior) (+) were determined by two-way, repeated measures ANOVA with post hoc Bonferroni tests.

Figure 5 Effects of cocaine, methylphenidate, d-amphetamine, and ketamine in PBS-treated rats trained to discriminate 10.0 mg/kg; IP cocaine from saline. Upper panel: Dose–response curves for the percent of responding that occurred on the cocaine-appropriate lever during cumulative dose substitution tests. Lower panel: Dose–response curves for the overall rate of responding during cumulative dose substitution tests. Data represent the mean ± the standard error of the mean (SEM) for each dosing condition ($n = 5$). Shaded squares depict the effects of saline or 10.0 mg/kg cocaine on responding during training session. The dashed line at 80% represents the cutoff for full substitution.

the discriminative stimulus effects of cocaine at cumulative doses of 10.0 and 1.0 mg/kg; IP, respectively. Unlike methylphenidate and d-amphetamine, pretreatment with ketamine failed to increase selection of the cocaine-appropriate lever (maximum $3.2 \pm 1.8\%$), with responding occurring almost exclusively on the saline-appropriate lever over the entire range of cumulative doses tested. The relative potencies (ED_{50}) of these compounds to produce cocaine-appropriate responding are shown in Table 1.

Similar to the effects of PEG-CCRQ on cocaine self-administration, pretreatment with PEG-CCRQ CocE produced rightward shifts in the dose–response curve for cocaine-induced increases in cocaine-appropriate responding (Figure 6). Although high cumulative doses of cocaine (180.0 mg/kg; IP) were capable of producing slight increases (>20%) in cocaine-appropriate responding during sessions immediately after pretreatment with lower doses of PEG-CCRQ CocE (3.2 or 10.0 mg/kg; IV), these effects were due to one rat in particular who showed higher proportions of cocaine-appropriate responding (66–78%) following the 180.0-mg/kg; IP dose of cocaine at the 0-h time point.

Pretreatment with 32.0 mg/kg; IV PEG-CCRQ CocE resulted in nearly exclusive responding on the saline-appropriate lever at all doses of cocaine tested, including the cumulative dose of 180.0 mg/kg; IP cocaine. The duration of CocE's effects against the discriminative stimulus effects of cocaine was also dependent upon the dose of PEG-CCRQ CocE administered, with the training dose of 10.0 mg/kg; IP cocaine producing >80% cocaine-appropriate responding in the majority of rats 48 h after treatment with 3.2 mg/kg or 10.0 mg/kg PEG-CCRQ CocE, and 96 h after treatment with 32.0 mg/kg PEG-CCRQ CocE. In addition to altering cocaine's discriminative stimulus effects, pretreatment with PEG-CCRQ CocE produced a similar inhibition of cocaine's rate suppressant effects, although increasing doses of cocaine more easily surmounted these protective effects of PEG-CCRQ CocE. Nevertheless, cumulative cocaine doses as large 180.0 mg/kg; IP failed to completely suppress responding at any time during the first 48 h after 32.0 mg/kg; IV PEG-CCRQ CocE.

As shown in Figure 7, the capacity of PEG-CCRQ CocE to alter the discriminative stimulus effects of psychostimulants

Table 1 Relative Potency of Substitute Drugs to Produce a Cocaine-Like Interoceptive Effect Following Pretreatment with PBS or 32.0 mg/kg PEG-CCRQ CocE

Drug	PBS treatment		32.0 mg/kg; IV PEG-CCRQ CocE treatment			
	ED_{50}[a]	(95% CI)	ED_{50}	(95% CI)	Dose ratio	(95% CI)
Cocaine	4.8	(3.7–6.2)	n.s.		n.a.	
Methylphenidate	4.9	(2.2–7.9)	6.1	(3.8–9.3)	1.1	(0.1–2.0)
d-Amphetamine	0.6	(0.5–0.9)	0.6	(0.4–0.9)	1.0	(0.3–1.6)
Ketamine	n.s.		n.d.			

Abbreviations: n.a., not applicable; n.d., not determined; n.s., slope not significant different than zero based on linear regression analysis.
[a]Dose that produced 50% cocaine-appropriate responding based on linear regression analysis.
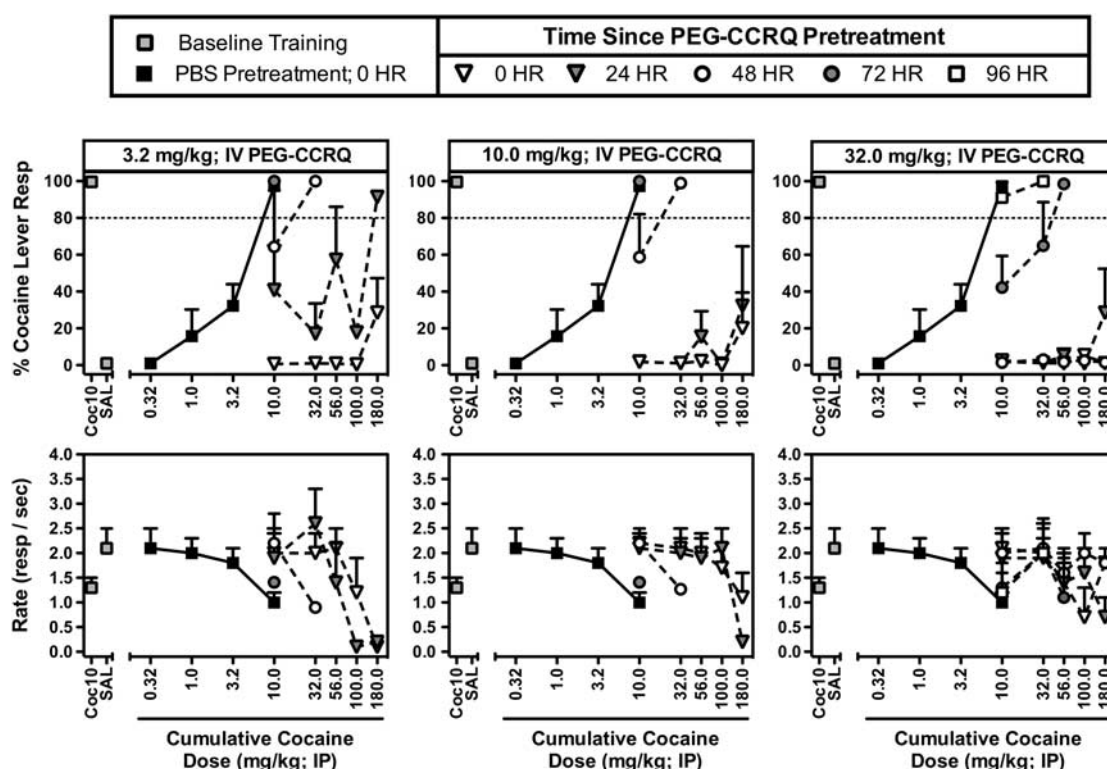
Figure 6 Time course of the effects of pretreatment with PEG-CCRQ CocE in rats trained to discriminate 10.0 mg/kg IP cocaine from saline. Effects of PEG-CCRQ (3.2, 10.0, and 32.0 mg/kg; IV) on the percent of responding that occurred on the cocaine-appropriate lever (upper panels), and the overall rate of responding (lower panels) immediately after (open triangles), 24 h after (shaded triangles), 48 h after (open circles), 72 h after (shaded circles), and 96 h after (open circles) PEG-CCRQ CocE administration. Data represent the mean ± the standard error of the mean (SEM) for each dosing condition ($n = 5$). Filled squares depict the effects of saline or 10.0 mg/kg cocaine on responding during training session. The dashed line at 80% represents the cutoff for full substitution.

was limited to cocaine, as there were no significant differences between the generalization dose–response curves obtained for methylphenidate or d-amphetamine following PBS pretreatments and those obtained 0–24 h after the administration of 32.0 mg/kg PEG-CCRQ CocE (Table 1). Despite this inability of PEG-CCRQ CocE to alter the discriminative stimulus effects of methylphenidate or d-amphetamine, cumulative doses of cocaine as large as 18.0 mg/kg resulted in nearly exclusive responding on the saline-appropriate lever when it was evaluated 48 h after the administration of 32.0 mg/kg PEG-CCRQ CocE. Although slight increases in cocaine-appropriate responding were observed at the 10.0 and 18.0 mg/kg doses of cocaine, these effects represent increased levels of cocaine-appropriate

responding in a single rat (different rat for 10.0 and 18.0 mg/kg doses), rather than a systematic increase in cocaine-appropriate responding. The ED_{50} values for methylphenidate, d-amphetamine, and cocaine observed following PEG-CCRQ pretreatment, as well as the fold shift from PBS produced by PEG-CCRQ CocE for each of these drugs are shown in Table 1.

DISCUSSION

We have recently described mutations resulting in a highly thermostable bacterial CocE (CCRQ CocE), as well as a PEGylated form of this enzyme (PEG-CCRQ CocE) capable
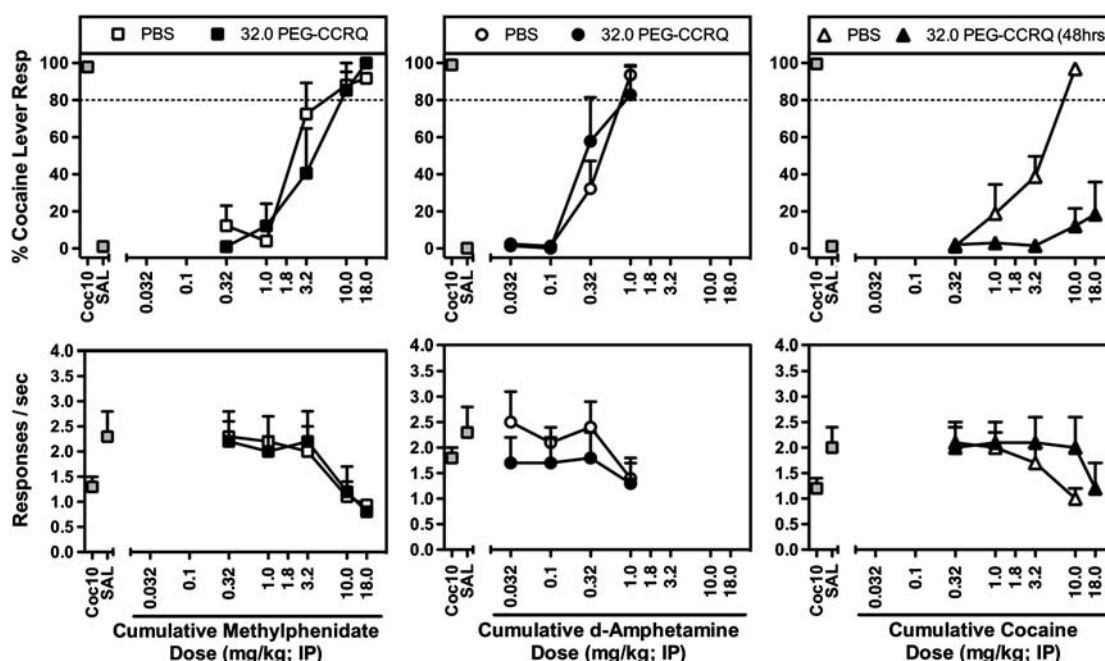
Figure 7 Effects of 32.0 mg/kg; IV PEG-CCRQ CocE on the cocaine-like interoceptive effects of methylphenidate, d-amphetamine, and cocaine. Percent of responding that occurred on the cocaine-appropriate lever (upper panels), and overall rate of responding (lower panels) following the cumulative doses of methylphenidate (left; 0.32–18.0 mg/kg; IP), d-amphetamine (middle; 0.032–1.0 mg/kg; IP), and cocaine (right; 0.32–18.0 mg/kg; IP). Dose–response curves for methylphenidate and d-amphetamine were generated 0 and 24 h after PEG-CCRQ CocE (counterbalanced), whereas the dose–response curve for cocaine was generated 48 h after PEG-CCRQ CocE. Data represent the mean ± the standard error of the mean (SEM) for each end point ($n = 5$). Filled squares depict the effects of saline or 10.0 mg/kg cocaine on responding during training session. The dashed line at 80% represents the cutoff for full substitution.

of producing a long-lasting protection against cocaine's effects in mice, rats, and rhesus monkeys (Narasimhan et al, 2011). The current studies were aimed at extending these findings by providing a thorough evaluation of the capacity of PEG-CCRQ CocE to reduce the reinforcing and discriminative stimulus effects of cocaine in rats, and there were four main findings. First, pretreatment with PEG-CCRQ CocE produced a dose-dependent antagonism of IV cocaine self-administration, with a > 30-fold rightward shift in the reinforcing effects of cocaine observed immediately after the administration of a 32.0-mg/kg dose of PEG-CCRQ CocE. Second, PEG-CCRQ CocE produced a similar dose-dependent antagonism of the discriminative stimulus effects of cocaine, with a 32.0-mg/kg dose of PEG-CCRQ CocE shifting cocaine's dose–response curve at least 25-fold to the right. Third, in addition to producing greater shifts in cocaine's effects, the duration of PEG-CCRQ CocE's effects was also dose dependent, with the 32.0-mg/kg dose producing at least a three-fold shift in the discriminative stimulus and reinforcing effects of cocaine for at least 72 h. And fourth, pretreatment with PEG-CCRQ CocE had little to no effect on the reinforcing or discriminative stimulus effect of methylphenidate and d-amphetamine, suggesting that it possesses a high degree of specificity for cocaine, and thus cocaine-related behaviors. When taken together, these studies demonstrate the capacity of PEG-CCRQ CocE to provide a long-lasting antagonism of cocaine's discriminative stimulus and reinforcing effects, and provide strong evidence to support the further development of highly efficient cocaine-hydrolyzing enzymes with extended durations of action, such as PEG-CCRQ CocE, as candidate medications for the treatment of cocaine abuse in humans.

In an extension of previous findings with DM CocE (T172R/G173Q CocE or RQ CocE; Collins et al, 2009, 2011a, b; Gao et al, 2009; Narasimhan et al, 2010), the current studies evaluated impact of introducing two cysteine residues at the dimer interface, in combination with the PEGylation of this crosslinked CocE dimer (PEG-CCRQ CocE; Narasimhan et al, 2011) on duration and effectiveness of CocE to alter the behavioral effects of cocaine. Importantly, just as these changes did not alter the enzymatic activity of PEG-CCRQ CocE in vitro (Narasimhan et al, 2011), the DM (RQ) and PEG-CCRQ forms of CocE appeared to be equally effective at eliminating cocaine in vivo as both enzymes produced at least 10-fold rightward shifts in the reinforcing effects of cocaine when comparable doses of ~3 mg/kg were administered immediately before the start of cocaine self-administration sessions (Collins et al, 2009). Nevertheless, just as the prolonged duration of PEG-CCRQ CocE's protective effects differentiated it from DM CocE in rodent models of acute cocaine toxicity (Collins et al, 2011b; Narasimhan et al, 2011) differences between these enzymes began to emerge when PEG-CCRQ CocE was evaluated against cocaine self-administration using a multiple dose procedure that allowed for the longitudinal evaluation of an animal's sensitivity to the reinforcing effects of cocaine. Unlike the effects of ~3 mg/kg DM CocE, which had completely dissipated by 24 h (Collins et al, 2009), pretreatment with 3.2 mg/kg PEG-CCRQ CocE produced a longer lasting change in cocaine self-administration with a three-fold rightward shift in the dose–response curve observed 24 h later, and baseline-like rates of responding not observed until 48 h after administration. Interestingly, in addition to producing larger

rightward shifts in the reinforcing effects of cocaine (30-fold or greater), the duration of PEG-CCRQ CocE's effects also increased with dose. Similar to the increased duration of protection reported for PEG-CCRQ CocE against the lethal effects of cocaine (Narasimhan et al, 2011), larger doses of PEG-CCRQ CocE not only produced larger rightward shifts in the reinforcing effects of cocaine (30-fold or greater), but these effects were also longer lasting, with baseline-like rates of cocaine self-administration gradually returning over 72–96 h after the administration of higher doses of PEG-CCRQ CocE.

In contrast to the ~30-fold rightward shift in the reinforcing effects cocaine, there were no significant effects of 10.0 mg/kg PEG-CCRQ CocE on methylphenidate-reinforced responding, and only modest decreases in responding maintained by an intermediate dose of 0.032 mg/kg/inj methylphenidate following pretreatment with the 32.0-mg/kg dose of PEG-CCRQ CocE. Importantly, however, unlike the prolonged inhibition observed with cocaine self-administration, the inhibition of methylphenidate-maintained responding by PEG-CCRQ CocE was short-lived, with baseline rates of responding returning within 24 h. A similarly short-lived inhibition of the rate of food-reinforced responding was also observed following pretreatment with this high dose of PEG-CCRQ CocE, however, it is important to note that the number of food pellets earned was unaffected. Although these findings suggest that PEG-CCRQ CocE can produce a mild, non-specific disruption of behavior when administered at high doses, these effects were relatively short-lived, and only observed with relatively high rates of responding suggesting that they resulted from the effects of the bolus administration of a large quantity of bacterially derived protein (eg, LPS-induced sickness-like behavior), rather than an enzymatic inhibition of the reinforcing effects of methylphenidate or food, per se. Nevertheless, it will be important to continue to evaluate the behavioral and pharmacologic specificity of PEG-CCRQ, and other CocEs in future studies.

Further evidence for PEG-CCRQ CocE's prolonged duration of action and high degree of pharmacologic specificity was provided by the results of studies in which rats were trained to discriminate the interoceptive effects of cocaine from saline using a multiple component, cumulative dose procedure (eg, Barrett et al, 2005; Ukai and Holtzman, 1988; Woolfolk and Holtzman, 1997), such that an animal's sensitivity to the discriminative effects of cocaine could be evaluated longitudinally after treatment with CocE. Just as with cocaine self-administration, PEG-CCRQ CocE produced a dose-dependent rightward shift in the cumulative dose–response curve for selection of the cocaine-appropriate lever. Even though slight differences in the effectiveness were observed during the initial time point following pretreatment with doses of PEG-CCRQ CocE ranging from 3.2 to 32.0 mg/kg, each of these doses resulted in the majority of rats responding on the saline-appropriate lever following tests with cumulative doses of cocaine as large as 180.0 mg/kg. Not only do these results suggest that PEG-CCRQ CocE is capable of shifting the discriminative stimulus effects of cumulative cocaine doses at least 56-fold to the right, but they also suggests that the rats are unable to discriminate an otherwise LD_{100} dose of cocaine for up to 48 h after administration of 32.0 mg/kg PEG-CCRQ CocE.

Although the use of drug discrimination and self-administration assays provided a consistent profile of PEG-CCRQ CocE's activity with respect to the duration and magnitude of its effects against cocaine, the evaluation of PEG-CCRQ CocE against other cocaine-like drugs in the drug discrimination assay provided much stronger evidence in support of PEG-CCRQ CocE's pharmacologic selectivity. Unlike the decrease in responding maintained by an intermediate dose of methylphenidate that was observed following pretreatment with 32.0 mg/kg PEG-CCRQ CocE in the self-administration studies, this high dose of PEG-CCRQ CocE failed to alter the doses at which either methylphenidate or d-amphetamine substituted for the discriminative stimulus effects of cocaine. Thus, even though self-administration and food reinforcement assays suggested that PEG-CCRQ CocE may produce a mild, non-selective, disruption of behavior, the absence of any non-specific effects of PEG-CCRQ CocE in the drug discrimination assay demonstrates the importance of utilizing rate-independent assays for the evaluation of pharmacologic selectivity.

Previous studies with mutant bacterial CocEs (Brim et al, 2010; Collins et al, 2009) or mutant human BChEs (Carroll et al, 2011) have demonstrated the capacity of highly efficient cocaine-specific hydrolase to inhibit the reinforcing effects of cocaine; however, in each of these studies the inhibitory effects of the enzymes were short-lived, lasting from minutes to hours. The results of the current studies with PEG-CCRQ CocE not only confirm this effect of cocaine-specific esterases, but they also provide convergent evidence that PEG-CCRQ CocE represents a significant improvement over previously described mutant bacterial and human cocaine hydrolases with respect to both the magnitude and duration of its antagonism of the reinforcing effects of cocaine. In addition to producing an initial shift of the reinforcing and discriminative stimulus effects of cocaine of >30-fold to the right, larger doses of PEG-CCRQ CocE retained their capacity to significantly alter these abuse-related effects of cocaine for up to 72 h after treatment. Although some of this improvement can undoubtedly be attributed to the incorporation of the thermostabilizing cysteine residues at the dimer interface, previous reports suggest that the PEGylation of CCRQ CocE also had a significant role in increasing the in vivo duration of CCRQ CocE's action while also decreasing its immunogenic potential in rodents (Narasimhan et al, 2011). Not only are these effects consistent with the PEGylation of other biologic entities (Caliceti and Veronese, 2003), but they also suggest that optimizing the location and number of PEGylation sites may result in even longer lasting CocEs. Thus, when taken together with the findings of Narasimhan et al (2011) these findings suggest that PEGylated forms of highly efficient, cocaine-specific esterases, such as PEG-CCRQ CocE may provide a safe, effective, and long-lasting therapeutic option for the treatment of cocaine abuse in humans.

DISCLOSURE

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