Experimental Physiology

Cocaine- and amphetamine-regulated transcript (CART) peptide as an *in vivo* regulator of cardiac function in *Rana ridibunda* frog

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The aim of this study was to investigate the effect of CART peptide on cardiac performance and on the physiological signalling pathways involved using *Rana ridibunda* frog heart preparations *in vivo*. The CART peptide, when injected into the venous sinus, significantly and reproducibly increased the force of frog heart contractions by up to 33.0 ± 6.4% during the first 15 min after its application but did not influence the chronotropic activity of the frog heart. The positive inotropic effect was entirely blocked by prazosin, pertussis toxin, R_p-adenosine 3′,5′-cyclic monophosphorothioate, autosauvagine 30 or metyrapone, as well as by extirpation of the pituitary gland, functional elimination of the inter-renal glands and long-lasting starvation, and was not observed on isolated heart preparations. Propranolol and double pithing were without significant effect on this phenomenon. It was concluded that: (i) CART peptide, administered to frogs *in vivo*, increases the force of heart contractions; (ii) this effect of the peptide is exerted via activation of the hypothalamic–pituitary–inter-renal gland axis through a corticoliberin-sensitive mechanism; (iii) CART augments the pumping function of the heart via a corticosteroid-dependent potentiation of myocardial α_1-adrenoreceptors signalling; and (iv) prolonged food deprivation abolishes the positive inotropic effect of CART, suggesting the participation of endogenous CART in the physiological adaptation of the circulatory system to limitations of energy consumption.

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Cocaine- and amphetamine-regulated transcript (CART) peptide was first identified by a differential display polymerase chain reaction in rat striatum after acute *in vivo* administration of cocaine or amphetamine (Douglass *et al.* 1995). The association of the CART transcript with acute drug abuse has generated considerable scientific interest in the physiological functions of the CART system. Additionally, the locomotor effects of CART peptide administration, as well as the effects of drugs on CART peptide expression in the hypothalamus, prompted the investigation of its participation in drug-related locomotor aberrations (Kimmel *et al.* 2000; Jaworski *et al.* 2003*a*), in feeding behaviour as a satiety factor regulated by leptin (Kristensen *et al.* 1998; Lambert *et al.* 1998) and in tasks with reinforcement and reward (Jaworski *et al.* 2003*b*).

Several observations suggest that CART peptide may serve as a signalling molecule outside the nervous system, in rat gastrointestinal tract (Murphy *et al.* 2000; Ekblad *et al.* 2003) and adrenal glands (Murphy *et al.* 2000), as well as in various cell types of the endocrine pancreas (Wierup *et al.* 2004). It has been found that in two different rat models of type 2 diabetes CART peptide at low concentration is co-stored within the secretory granules of β-cells, thus suggesting cosecretion of insulin and CART peptide, as well as a role of CART peptide as a paracrine factor (Wierup *et al.* 2006).

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Expression of CART peptide was also detected in poikilothermic animals. Immunoreactivity for CART peptide was observed in brain and pituitary gland of *Xenopus laevis*, where it was dramatically reduced during starvation (Calle *et al.* 2006). Lazar *et al.* (2004) found CART peptide in frog brain. Additionally, a CART-like peptide, bombinakinin M, was isolated from toad skin. Like CART peptides, it decreased food intake in rats when applied directly to the brain ventricles (Lai *et al.* 2003).

While the physiological importance of CART peptides is being revealed over time, CART receptor protein still remains to be discovered. Little is known about the cellular signalling pathways by which CART peptide exercises its effects. Recently, Lakatos *et al.* (2005) established that CART peptide activates $G_{i/o}$-protein in mouse pituitary cell line AtT20, which strongly supports the existence of a specific G-protein-coupled receptor for CART peptides. This finding is in agreement with an earlier report for CART peptide-induced L-type Ca^{2+} channel inhibition via a pertussis toxin-blockable and membrane-delimited mechanism in hippocampal neurones (Yermolaieva *et al.* 2001). In contrast, Wierup *et al.* (2006) observed that CART peptide could potentiate cAMP-enhanced insulin secretion via a cAMP/protein kinase A-dependent pathway, suggesting the existence of one or more CART peptide receptor(s) coupled with various G-proteins.

The effects of CART peptide on the functional performance of rat heart have also been studied. It was found that intrathecally injected CART peptide potentiates the central pressor action of glutamate in a dose-dependent manner (Scruggs *et al.* 2005). Intracisternally administered CART peptide fragments increased the heart rate and blood pressure in anaesthetized adult male rats (Hwang *et al.* 2004), while their intrathecal or intravenous administration did not produce such effects (Hwang *et al.* 2004; Scruggs *et al.* 2005). These observations place CART peptides among the emerging family of endogenous ligands of recently de-orphanized G-protein-coupled receptors, all of them engaged in a complex physiological regulation of energy homeostasis and cardiovascular functions (Enomoto *et al.* 2003; Jaszberenyi *et al.* 2004; Richard & Baraboi, 2004).

While the role of CART peptides in the modulation of feeding behaviour and energy balance has been extensively explored, the mechanisms by which they exert their cardiovascular effects have been largely unknown. Therefore, the aim of our study was to investigate the *in vivo* effect of CART on cardiac performance and on the physiological signalling pathways involved. Being a small and relatively simple vertebrate, the frog provides the researcher with the advantage of studying complex hormonal signalling *in vivo* at modest expense. Since *Rana* species have been shown to express CART peptide (Lazar *et al.* 2004), we decided to conduct our experiments on *Rana ridibunda* frog heart preparations.

Methods

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Bulgarian Center for Bioethics. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the Institutional Animal Care and Use Committee, April 1997, Oakland University, MI, USA. Anaesthetics were applied according to the standards given by the guide of the Oakland University.

Frog heart preparation *in vivo*

All experiments were performed at room temperature (20–22°C). Frogs were placed in a bell jar with anaesthetic-soaked cotton (ethyl ether). Ethyl carbamate (urethane), 5% solution, 0.05 ml g^{-1}, was injected for anaesthesia in the dorsal lymph sack. Animals were killed by double pithing at the end of the experiments or were double pithed before the start of experiments when frogs with destroyed CNS were studied.

Anaesthetized frogs were fixed on their backs, and a triangular incision of the chest was made. The heart was exposed from pericardium and lifted vertically and was connected to a force transducer. Contractions were recorded and analysed on a computer using interface and TENZO1 software (Stoks, Sofia, Bulgaria).

Separate time control measurements were performed with the addition of R_p-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS), antisauvagine 30, pertussis toxin and prazosin. Time control measurements were also conducted for the experimental models, i.e. frogs without CNS, without a pituitary gland and with ligation of both main renal arteries for functional inter-renalectomy. Several treatments, such as the addition of antisauvagine 30, Rp-cAMPS or prazosin and the elimination of the pituitary gland, CNS or inter-renal glands, decreased the force of heart muscles during the first 60 min of the experiments significantly more than the observed decrease in experiments with CART only. However, there are several measurements of CART without any treatment, where the force of contraction an hour after the beginning of the experiments, i.e. at the moment of CART application, was as low as the values observed after the above-mentioned conditions. The positive inotropic effect of CART in these preparations without any treatment was as significant as that observed in preparations with a higher basal force of contraction. Therefore, we decided that the lower force of heart contraction alone is not an important factor that could significantly change CART effectiveness.

Data analysis

All data are presented as means ± S.E.M. The *n* in the text refers to the number of experiments, i.e. frog heart preparations *in vivo*. Statistical significance was determined by Student's unpaired *t* test. $P < 0.05$ was considered statistically significant.

Solutions and drugs

All substances were dissolved in 200 μl modified Ringer solution (100 mmol l^{-1} NaCl, 1.3 mmol l^{-1} KCl, 0.7 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} $NaHCO_3$) and injected into the frog venous sinus. Concentrations are given in grams per gram body weight (bw). The injections with Ringer solution were performed regularly at 15 min intervals during 105 min experiments, as presented in Fig. 1. Pertussis toxin (PTX) holotoxin was injected as inactive precursor. The CART peptide was applied 30 min after PTX application to allow the entry of the enzymatic component of the holotoxin, the PTX A protomer, into the target cells.

The sources of chemicals used were as follows: CART peptide fragment 55–102 (human), from Bachem AG, Bubendorf, Switzerland; pertussis toxin from Calbiochem, EMD Biosciences Inc., La Jolla, CA, USA; and prazosin, propranolol, antisauvagine 30 (D-Phe^{11},His^{12}-sauvagine fragment 11–40), R_p-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS), metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) and all salts from Sigma-Aldrich Inc. (St Louis, MO, USA). Metyrapone (up to 10 mg ml^{-1}) was dissolved directly in the Ringer solution.

Results

Frog heart preparations *in vivo* usually develop regular contractions, repetitive in pattern and force. That is why this preparation is widely used in various physiological and pharmacological tests, including those of newly discovered neurotransmitters and hormones. In our experimental conditions, frog heart preparations developed slightly declining contractions, preceded by an initial moderate decrease of the force lasting for half an hour (Fig. 1*A*; ♦). Thus, although the median force of the maximal contractions was suppressed after the first two injections, further repetitions of this procedure did not produce any significant effect on heart contractility (Fig. 1*A*). Therefore, we decided to inject CART peptide fragment 55–102 (CART peptide), which is widely used for physiological studies, particularly studies of the regulation of energy homeostasis and the control of appetite (Murphy, 2005), 1 h after the start of the experiment. This timing provided stable contractile activity for at least half an hour,

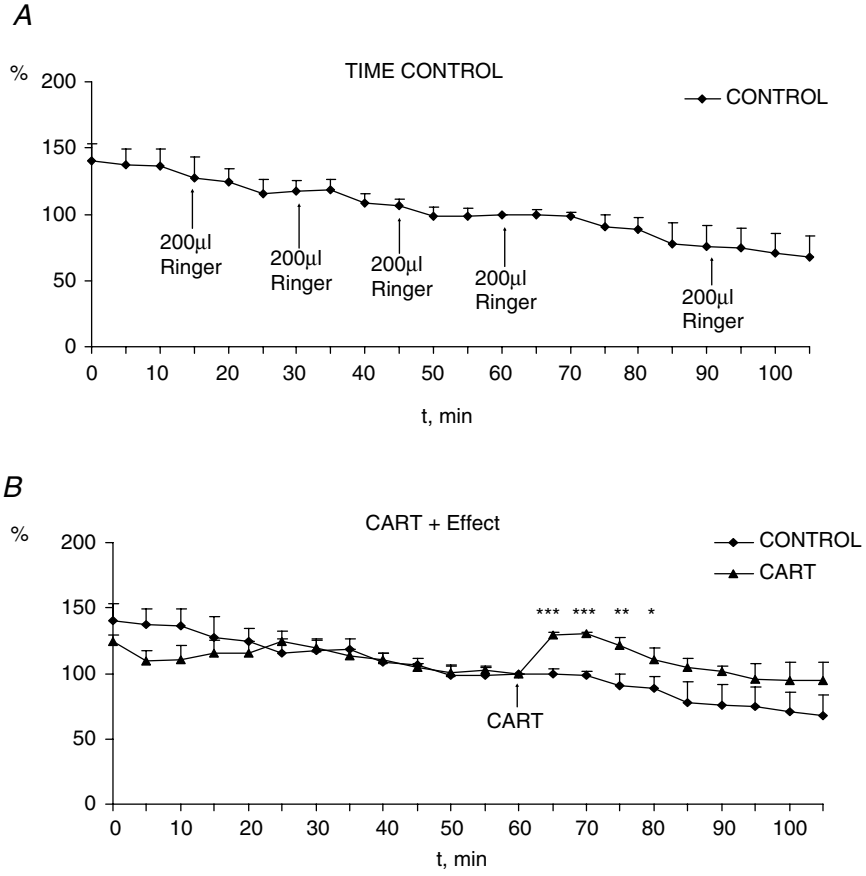

Figure 1. Effect of CART peptide on maximal force of contractions of the frog heart
A, time course of spontaneous frog heart contractions (♦), expressed as percentages of the initial contractile force measured 60 min from the start of the experiment (taken as 100%), prior to CART administration. *B*, the second curve (▲) presents the maximal force of contractions after the injection of 18 ng (g bw)$^{-1}$ CART peptide. Data are means + S.E.M. of 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control.

as well as a 30 min preconditioning interval, suitable for the application of different inhibitors, before the application of CART.

The CART was used at two concentrations, usually 18 ng (g bw)$^{-1}$ and sometimes 54 ng (g bw)$^{-1}$, applied in 200 μl Krebs solution. The weight of frog blood is about 4.6% of the body weight and the plasma is about 59% of the blood (Terentiev, 1950), which means concentrations of about 30 and 100 nM CART in the blood plasma, respectively, assuming that the whole amount of CART remains there. Further dilution of CART peptide due to extracellular fluid–blood plasma exchange could decrease these CART levels around 10 times, giving concentrations of 3 and 10 nM, respectively. These amounts of CART are, respectively, approximately two and six times higher than those reported for the hypothalamic–pituitary portal blood (Larsen *et al.* 2003) and are approximately 10 and 30 times higher than those in the general circulation (Stanley *et al.* 2004). We decided not to estimate the CART positive inotropic effect dose–response relationship and to study the effect of 18 ng CART (g bw)$^{-1}$ only, in order to have near physiological conditions. In our view, the use of concentrations 30 or more times higher than the reported ones for blood plasma levels of the systemic circulation in a CART dose–response study can induce different non-physiological responses that may significantly influence or even mask the natural signalling pathway. When injected into the venous sinus, CART peptide (18 ng (g bw)$^{-1}$) significantly and reproducibly augmented the force of the frog heart contractions by 29.9 ± 2.5% ($n = 6$) at 65 min (5 min after its application), 32.7 ± 1.5% ($n = 6$) at 70 min (10 min after its application), 33.0 ± 6.4% ($n = 6$) at 75 min (after 15 min) and 24.3 ± 9.4% ($n = 6$) at 80 min (20 min after application; Fig. 1*B*; ▲). The maximal force of contractions is expressed as a percentage of the initial contractile force measured 60 min from the start of each experiment (taken as 100%), prior to CART administration. In these conditions, CART peptide was found to produce a positive inotropic effect. This effect was statistically significant for 20 min following the injection of CART (Fig. 1*B*). The higher concentration of the peptide (54 ng (g bw)$^{-1}$) increased the force of frog heart contractions but was also without any effect on the heart rate (not shown).

The positive inotropic effect of CART peptide was significantly suppressed by 42 ng (g bw)$^{-1}$ prazosin, a selective peripheral α_1-adrenoreceptor blocker, injected with 200 μl modified Krebs solution 15 min before CART peptide (Fig. 2*A*), while 140 ng (g bw)$^{-1}$ prazosin (Fig. 2*A*) entirely abolished it. Prazosin (both concentrations) failed to affect the heart contractility (compare the data between 45 and 60 min after the beginning of the experiments presented in Fig. 2). However, the effect of CART peptide on the heart contraction force

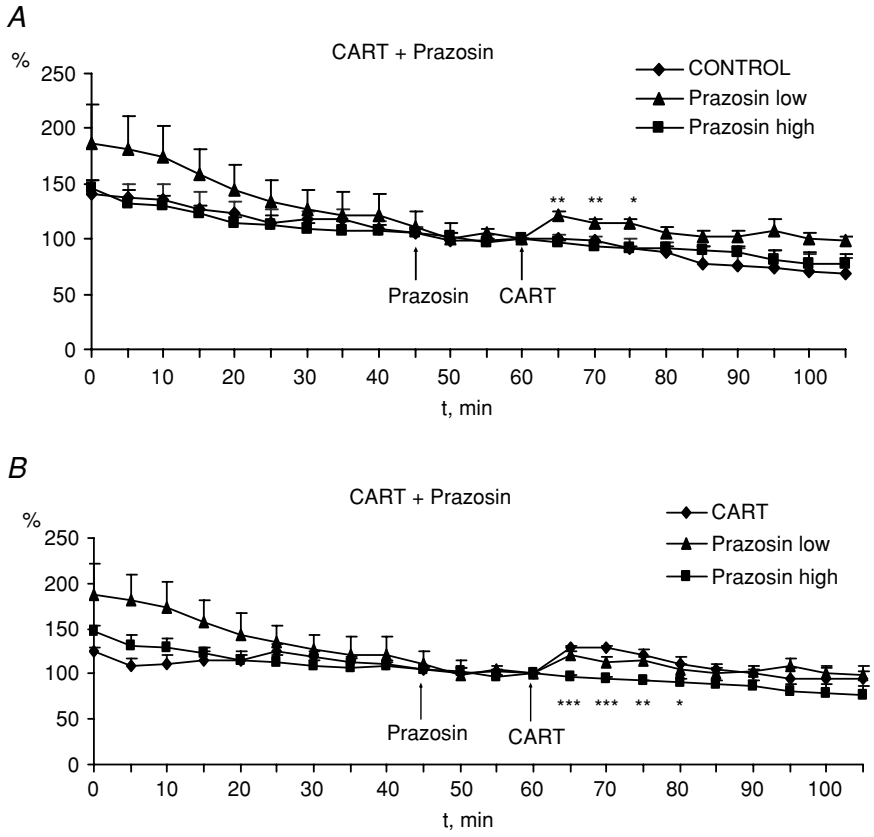

Figure 2. Effect of CART peptide on maximal force of contractions of the frog heart in the presence of prazosin
Time course and conditions of the experiments are the same as in Fig. 1. Prazosin was administered before 18 ng (g bw)$^{-1}$ CART at concentrations of 42 (*A*; ▲) or 140 ng (g bw)$^{-1}$ (*A*; ▲). Time control data (♦), as well as data showing the effect of CART peptide alone (■), are given for comparison. Data are means + S.E.M. of 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control.

was not modulated by pretreatment with 30 ng (g bw)$^{-1}$ propranolol, a non-selective β-adrenoreceptor blocker (data not shown). Therefore, it was suggested that the effect of CART on the heart was mediated by adrenaline, released as a neurotransmitter or as a hormone, via α_1-adrenergic receptors. This suggestion was further tested on double-pithed frogs. The involvement of secondary neurotransmission in the action of CART on frog heart was not studied, because CART peptide was without any effect on preparations of isolated frog hearts (data not shown).

Pithing(s) was(were) performed prior to the start of the experiments. The mechanical destruction of the spinal cord with a metal spindle had no apparent influence on either basal heart activity or on CART-induced positive inotropic effect (not shown). In these conditions, CART-induced positive inotropic action was not changed (Fig. 3*A*; contractile force augmentation was $15.0 \pm 3.2\%$ at 5 min, $25.0 \pm 3.6\%$ at 10 min, $33.0 \pm 4.7\%$ at 15 min and $24.0 \pm 2.3\%$ at 20 min after the CART peptide injection). These data suggest that the positive inotropic action of CART peptide was not relayed by central or peripheral neurotransmission, thus rendering the hypothesis of the involvement of endocrine glands more likely.

In order to test this hypothesis, we subjected the animals to hypophysectomy. The extirpation alone resulted in a larger drop of the initial values of basal heart contractions, by $140.0 \pm 35.9\%$ during the first 30 min following the operation, as estimated by time control measurements of frogs without a pituitary gland (Fig. 3*B*; ■), *versus* only a $40.0 \pm 13.2\%$ decline in the initial values in untreated controls (Fig. 1*A*). This decline was supervened by a long-lasting stabilization of the contractile force and pattern, indistinguishable from the control records. The removal of the pituitary gland almost entirely abolished the effect of CART on frog heart, suggesting the involvement of a pituitary hormone in CART peptide-induced positive inotropic effect in frogs.

Further, the participation of the pituitary gland was investigated using antisauvagine 30, a high-affinity antagonist of corticotrophin-releasing factor (CRF) receptor type 2. Antisauvagine 30 entirely inhibited the positive inotropic effect of CART when it was present in the blood plasma before the application of CART (Fig. 4; antisauvagine 30 is referred to as CRF-I). The participation of cAMP-dependent protein kinase (PKA) in CART-induced activation of the heart contractions was also studied using Rp-cAMPS, a specific membrane-permeable inhibitor of activation by cAMP of PKA I and II which is resistant to cyclic nucleotide phosphodiesterases. The Rp-cAMPS was applied 30 min before CART to allow

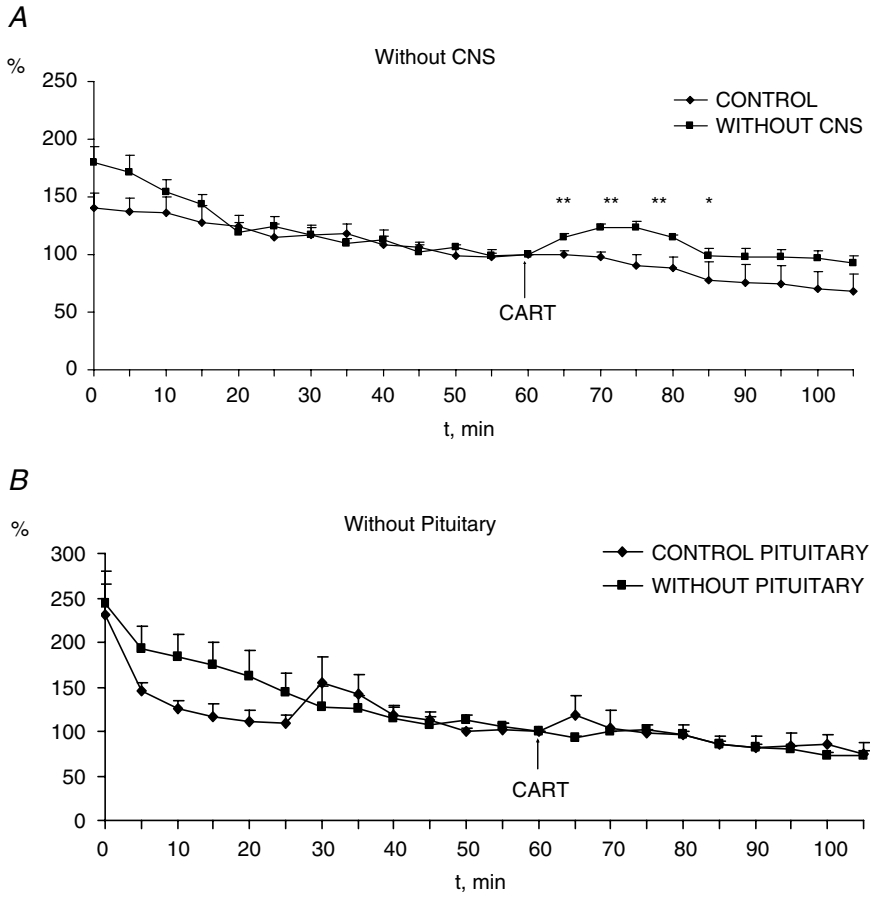

Figure 3. Effect of CART peptide on maximal force of contractions of the frog heart after mechanical destruction of CNS (pithing) or extirpation of the pituitary gland
Time course and conditions of the experiments are the same as in Fig. 1. *A*, pithing was performed prior to the start of the measurements, and the effect of 18 ng (g bw)$^{-1}$ CART on heart contractions was monitored as usual (■). Time control measurements of frog heart preparations *in vivo* with destroyed CNS are given for comparison (♦). *B*, experiments on frog heart preparations *in vivo* were performed without pituitary gland (■) and compared with time control data of frog heart preparations *in vivo* without pituitary gland (♦). Data are means + S.E.M. of 6 experiments in *A* and *B*. $^*P < 0.05$, $^{**}P < 0.01$ *versus* control.

sufficient time for it to cross the cell membranes and reach the cytoplasm (Fig. 4; Rp-cAMPS is referred to as PKA-I). The Rp-cAMPS, like the CRF receptor type 2 antagonist, entirely abolished the effect of CART application on frog heart contractions in the same experimental conditions.

Since the pituitary gland does not secrete adrenaline-related hormones, we suggested its involvement as an intermediate link between CART peptide receptors and cardiac α_1-adrenoreceptors. The anatomy of the frog does not permit surgical removal of its inter-renal glands, which renders the ligation of both main renal arteries the only means of functional inter-renalectomy. This procedure also resulted in a sharp decline of the force of cardiac contractions, which reached steady-state levels in 60 min. These changes in the heart dynamics were ascribed to the decrease in the circulating levels of adrenal hormones, an observation repetitively documented by many earlier studies (Floyer, 1951; Webb *et al.* 1965; de Champlain, 1977 for review). In these experimental conditions, the positive inotropic effect of CART was not manifested (Fig. 5), implying that the final target of the CART-activated hormonal axis was the inter-renal glands. Next, the involvement of corticosteroid hormones of the inter-renal glands in the CART effect was studied using 2 μg (g bw)$^{-1}$ metyrapone, an 11β-hydroxylase inhibitor that blocks adrenal cortisol synthesis in mammals (Fornhem *et al.* 1995). In the presence of metyrapone, CART did not influence the pumping function of frog heart (Fig. 5*B*).

The participation of G proteins in CART peptide-dependent signalling was investigated using pertussis toxin (PTX) as an irreversible inhibitor of G_i proteins (Koslow & Burns, 1992). The injection of 30 ng (g bw)$^{-1}$ PTX diluted in 200 μl Krebs solution 30 min prior to the application of CART did not affect either the force or the frequency of the frog heart contractions. Subsequent administration of CART peptide to PTX-pretreated animals had no further effect on the heart contractile activity, suggesting the participation of PTX-sensitive $G_{i/o}$ proteins in CART peptide-triggered signalling (Fig. 6).

During these studies, another interesting observation was made. In winter, when frogs usually fast for 2 months or more, the potency of CART to stimulate heart contractility is completely lost. This loss of activity is reversible upon feeding, and fades away a few days after a single meal.

Discussion

Frog heart preparations *in vivo* are widely employed in the study of direct and indirect effects of various biologically active substances on myocardial tissue. Unfortunately, selective receptors of CART peptides have not yet been identified. Therefore, given the absence of selective CART receptor inhibitors, the hypothesis of a direct effect of CART peptides on myocardium could not be ruled out completely. In accordance with this suggestion, our findings indicated that efferent signalling from spinal cord or CNS did not interfere with the positive inotropic action of CART *in vivo*. However, the latter was completely abolished by pretreatment of animals with a selective

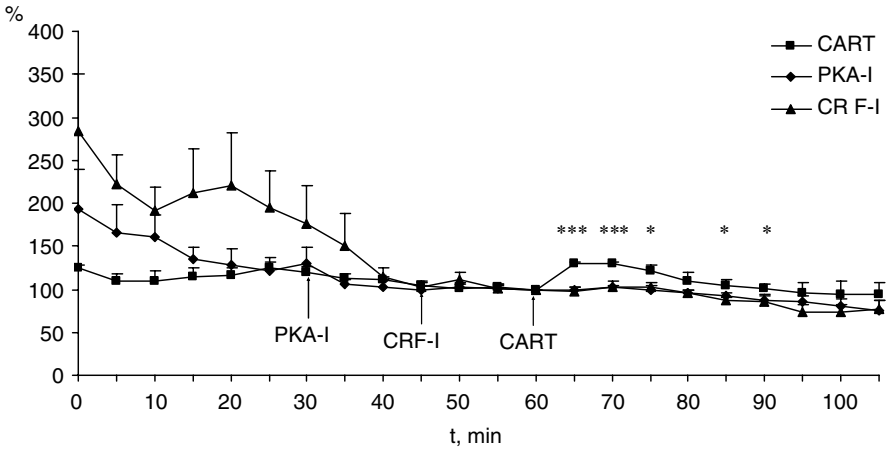

Figure 4. Effect of CART peptide on maximal force of contractions of the frog heart following administration of antisauvagine 30 or Rp-cAMPS
Time course, conditions of experiments and CART peptide concentration are the same as in Fig. 1. Antisauvagine 30 (CRF-I) was injected at 336 ng (g bw)$^{-1}$ 45 min after the start of the experiment and 15 min before administration of 18 ng (g bw)$^{-1}$ CART peptide (▲). The Rp-cAMPS (PKA-I) was injected at 135 ng (g bw)$^{-1}$ 30 min after the start of the experiment and 30 min before the administration of 18 ng (g bw)$^{-1}$ CART peptide (▲). Control data of contractions without any application of blockers and their response to the injection of 18 ng (g bw)$^{-1}$ CART peptide 60 min after the start of the experiment are given for comparison (■). Statistical significance was estimated for CART *versus* CART in the presence of Rp-cAMPS (i.e. PKA-I) and for CART *versus* CART in the presence of antisauvagine 30 (i.e. CRF-I). Data are means + S.E.M. of 5 experiments. $^*P < 0.05$, $^{***}P < 0.001$ *versus* CART peptide.

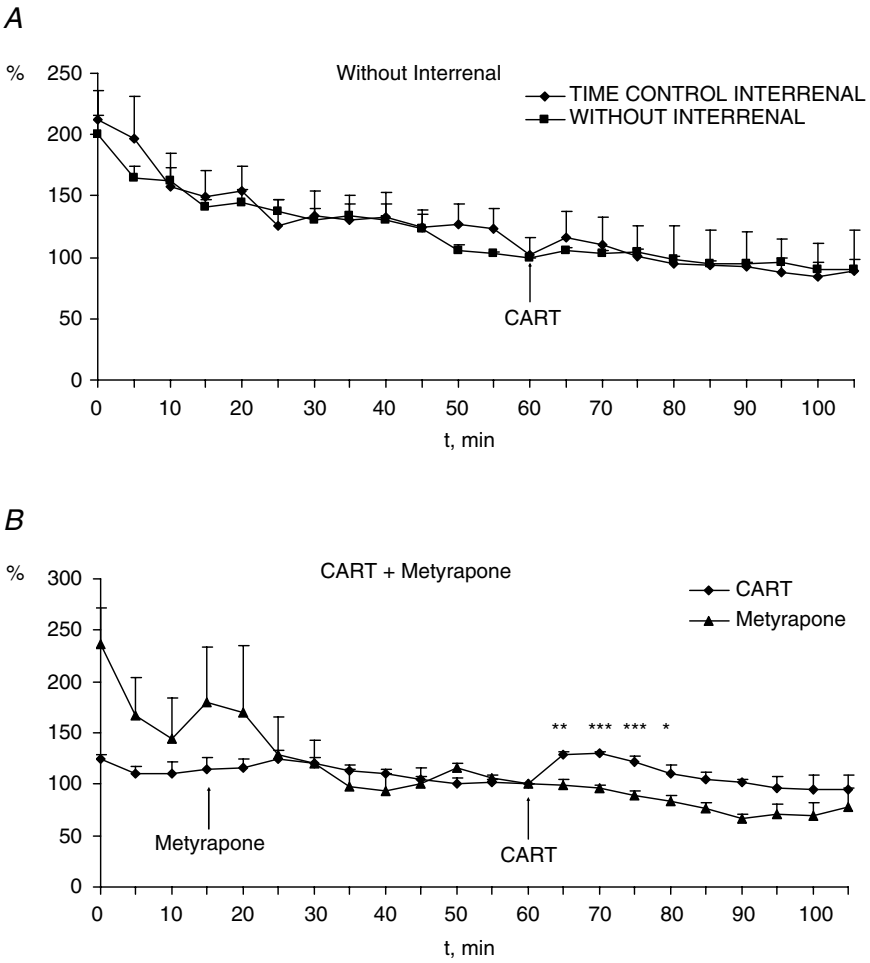

Figure 5. Effect of CART peptide on maximal force of contractions of the frog heart after functional elimination of inter-renal glands
Time course, conditions of the experiments and CART peptide concentration are the same as in Fig. 1. *A*, the inter-renal gland blood supply was interrupted by ligation of the main renal arteries just before the beginning of the experiments. Data are means ± S.E.M. of 6 experiments. Time control measurements of preparations with eliminated inter-renal glands are given for comparison (♦). *B*, metyrapone was injected at 20 μg (g bw)$^{-1}$ 15 min after the start of the experiment and 45 min before administration of 18 ng (g bw)$^{-1}$ CART peptide (▲). Control data without metyrapone before the application of CART are given for comparison (♦). Data are means + S.E.M. of 6 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control.

α_1- adrenoceptor blocker, thus suggesting the involvement of a non-neuronal source of adrenergic hormones in CART-induced augmentation of myocardial function. A first-choice candidate for this role would be the inter-renal gland, which is the most important regulator of cardiac performance in amphibians and a functional analogue of the mammalian adrenal gland. It is noteworthy that in frogs the only catecholamine produced by inter-renal glands is adrenaline and, unlike the situation in mammals, the adrenaline binds to α_1-adrenoceptors with high affinity (Lazou *et al.* 2002). Indeed, in our studies the functional removal of inter-renal glands effectively prevented the positive inotropic action of *in vivo* CART administration.

All this leads to the question of whether CART acts directly on inter-renal glands or, as in mammals, affects some other endocrine gland (Murphy *et al.* 2000; Baranowska *et al.* 2006), which in turn could stimulate the inter-renal gland. We explored the second alternative

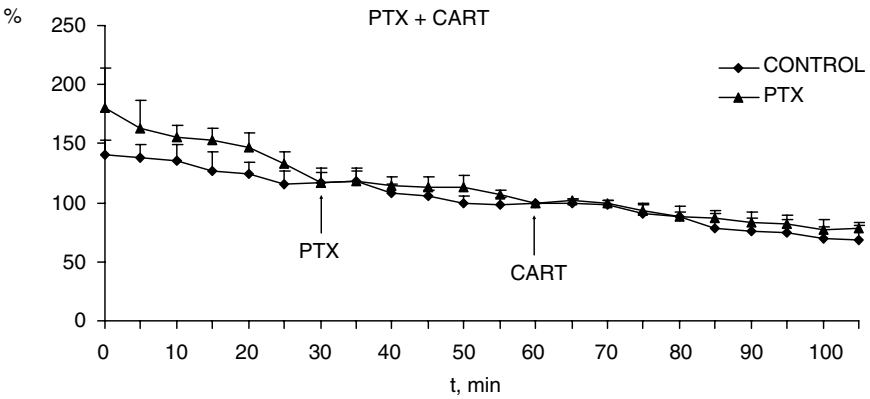

Figure 6. Effect of CART peptide on maximal force of contractions of the frog heart following administration of PTX
Time course, conditions of the experiments and CART peptide concentration are the same as in Fig. 1. The PTX was injected at 30 ng (g bw)$^{-1}$ 30 min after the start of the experiment and 30 min before administration of 18 ng (g bw)$^{-1}$ CART peptide (▲). Time control data are given for comparison (♦). Data are means + S.E.M. of 6 experiments.

by performing hypophysectomy, which also resulted in abrogation of the positive inotropic action of CART. This observation implies the participation of a pituitary hormone or hormones in CART-induced signalling *in vivo*. Since pituitary hormones are mostly trophic to other endocrine glands, very few of them are known to exert direct effects on non-endocrine targets (see Boron & Boulpaep, 2005 for details). None of them are known to be endogenous cardiac stimulants. Therefore, it is reasonable to assume that in frogs CART peptide exerts its positive inotropic effect via the hypothalamic–pituitary–inter-renal axis, as in mammals (Baranowska *et al.* 2006). The positive CART-immunostaining in the pituitary neuronal lobe of the amphibian *Xenopus laevis* (Calle *et al.* 2006) suggests a release of CART peptide into the general circulation to act on peripheral targets, as reported for mammals (Vicentic *et al.* 2004). Therefore, the possibility of a CART peptide binding to a CART-sensing molecule on the plasma membrane of inter-renal gland secretory cells cannot be completely ignored. Its physiological significance, however, remains unclear.

The data reported in this paper provide evidence that CART peptides could affect cardiac function *in vivo* by activating the hypothalamic-pituitary-inter-renal hormonal axis. Thus, the expression of a CART-sensing molecule (CART receptor has not yet been identified) in the hypothalamus and/or pituitary of frog should be considered. This can be expected, since CART was originally discovered in the hypothalamic arcuate nucleus (Kristensen *et al.* 1998) and high hypothalamic CART immunoreactivity was found in amphibians (Calle *et al.* 2006). The hypothesis for the participation of the hypothalamus and the pituitary gland in a CART-induced stimulation of frog heart function was further supported by the complete inhibition of the positive inotropic effect of CART by antisauvagine 30, a specific peptide antagonist of CRF type 2 receptors. Corticotrophin-releasing factor (corticoliberin) is a hypothalamic releasing factor that activates the synthesis of pro-opiomelanocortin in the pituitary. Hence, it is the main physiological stimulator of pituitary corticotrophin (ACTH) release that targets adrenal cortex or inter-renal glands in order to increase corticosteroid synthesis (for details see Boron & Boulpaep, 2005). Moreover, metyrapone, an 11β-hydroxylase inhibitor that blocks adrenal cortisol synthesis (Fornhem *et al.* 1995), entirely abolished the effect of CART. This result suggests the participation of enhanced corticosteroid synthesis in the mechanism of CART-induced α_1-adrenoreceptor activation and rules out the possibility of a direct increase of the blood plasma level of adrenaline in frogs due to CART.

The cAMP-dependent protein kinase (PKA) is involved in the effect of CART peptide on frog heart contractions. In the presence of PKA inhibitor, the application of CART remains physiologically silent, suggesting a main role of PKA during one or several steps of CART signalling. The most probable candidates for it are the pituitary CRF type 2 receptors, whose main signal transducer pathway involves G_s protein and adenylate cyclase activation (Liaw *et al.* 1996; Grammatopoulos *et al.* 2000). Other cAMP/PKA-sensitive steps of CART peptide effect, such as the interaction of CART with CART binding molecule followed by G_s protein activation (Wierup *et al.* 2006) or downstream signalling at the level of inter-renal glands, are also possible. However, their more detailed study requires isolated organs, tissues or cells, instead of the anaesthetized whole organisms in our experiments.

The very few reports on the coupling of CART peptide with G protein suggest the participation of $G_{i/o}$ protein in AtT20 cells (Lakatos *et al.* 2005) and hippocampal neurones (Yermolaieva *et al.* 2001), and G_s protein in rat pancreatic cells (Wierup *et al.* 2006). In our study, the action of CART on cardiac performance was averted by pretreatment with pertussis toxin, which suggests the involvement of $G_{i/o}$ protein in CART peptide signalling (Koslow & Burns, 1992). Thus, the existence of $G_{i/o}$ protein-coupled CART receptor could be hypothesized in frog hypothalamus and/or pituitary. Like other G protein-coupled receptors, it could be coupled to more than one G protein (for review see Hermans, 2003; Wettschureck & Offermanns, 2005). Unfortunately, this assumption cannot yet be verified *in vivo* owing to the lack of inhibitors selective for each known G protein.

Although our results indicate a connection between the feeding status and the myocardial effectiveness of CART peptide, they are still insufficient for identifying the primary target of CART peptide in frogs. The data from the literature, however, point to the magnocellular nucleus, which is known to play a pivotal role in the regulation of feeding behaviour. In this nucleus, an almost complete absence of CART immunoreactivity, as well as reduced amounts of two other neuropeptides (urocortin and metenkephalin) have been observed in *Xenopus laevis* after long-lasting starvation (Calle *et al.* 2006). Our results also suggest a substantial decrease in CART-sensing molecules (receptors) that can follow the above-mentioned vast decrease of hypothalamic CART peptide expression after food deprivation. Furthermore, in *Xenopus laevis*, CRF secretion modulates food intake as an anorexic factor in the absence of stress. In contrast to mammals and birds, the stress axis (hypothalamus–pituitary–inter-renal glands) is not activated during periods of food deprivation in amphibians (Crespi *et al.* 2004). Taken together, these data corroborate the implication of CART in adaptive mechanisms that tune the activity of the circulatory system to the current metabolic state of the body.

Additionally, CART was found in the rat adrenal gland (Murphy *et al.* 2000), which suggests its participation as a paracrine regulatory factor of adrenaline synthesis and release. The presented complete inhibition of CART

positive inotropic effect after the addition of an inhibitor of CRF receptor type 2 or the extirpation of the pituitary gland strongly supports the participation of the pituitary trophic hormone as a main inter-renal gland activator after CART injection. Thus, a direct effect of CART binding to CART receptors on the plasma membrane of inter-renal endocrine cells cannot be completely excluded but such a mechanism seems to be unimportant for the observed increase of the force of heart contractions in frogs.

In conclusion: (i) CART peptide, administered to frogs *in vivo*, increases the force of heart contractions; (ii) this effect of the peptide is exerted via activation of the hypothalamic-pituitary-inter-renal gland axis through a corticoliberin-sensitive mechanism; (iii) CART augments the pumping function of the heart by a corticosteroid-dependent potentiation of myocardial α_1-adrenoreceptor signalling; and (iv) prolonged food deprivation abolishes the positive inotropic effect of CART, suggesting the participation of endogenous CART in the physiological adaptation of the circulatory system to limitations of energy consumption.

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