UNIVERSIDADE ESTADUAL DE CAMPINAS



IRAÍDES NUNES DOS SANTOS

# RECEPTORES DE GLICOCORTICÓIDES E SUBTIPOS DE ADRENOCEPTORES BETA EM ÁTRIO DIREITO DE RATOS SUBMETIDOS A ESTRESSE



**Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Funcional e Molecular - Área de Fisiologia**

**Orientação**

**Prof. Dra. Regina Célia Spadari-Bratfisch**

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**BANCA EXAMINADORA:**

**Profa. Dra. REGINA CÉLIA SPADARI-BRATFISCH (orientadora)**

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PRÓPRIA RAZÃO DESCONHECE...

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**SUMÁRIO**

# RESUMO

Átrios direitos de ratos machos e de fêmeas sacrificadas em diestro submetidos a estresse por choques nas patas apresentam supersensibilidade a agonistas não-seletivos e seletivos $\beta_2$ acompanhada por aumento na expressão da proteína do receptor $\beta_2$. Este tecido também apresenta subsensibilidade a agonistas seletivos $\beta_1$, sem alteração na expressão da proteína do receptor $\beta_1$. Estes resultados sugerem que a supersensibilidade pode ser devida a aumento na expressão do subtipo $\beta_2$ mas que a subsensibilidade à noradrenalina demonstrada em experimentos farmacológicos, deve-se, provavelmente, a alterações na sinalização intracelular deste receptor e/ou nos processos de recaptação e de metabolização dos agonistas e não a alterações no próprio receptor. Não houve alteração na resposta cronotrópica ao CGP12177 em átrio direito de ratos, machos ou fêmeas, submetidos a estresse por choques nas patas, indicando que o receptor que medeia esta resposta é mais resistente ao estresse do que as isoformas clássicas $\beta_1$ e $\beta_2$, e não é influenciado pelas fases do ciclo estral.

Em átrio direito de ratos submetidos à desnervação sinoaórtica houve dicotomia na sensibilidade deste tecido aos agonistas clássicos noradrenalina, isoprenalina e CGP12177, sugerindo que o sítio receptor que responde ao CGP12177 se comporta de modo independente da isoforma clássica de adrenoceptor $\beta_1$, embora nossos dados não permitam esclarecer se esta resposta é mediada por um novo subtipo de adrenoceptor, denominado $\beta_4$, ou a uma nova isoforma do receptor $\beta_1$, com baixa afinidade pelos agonistas convencionais.

Ratos e ratas sacrificadas em diestro ou estro e submetidos a estresse por choques nas patas apresentaram aumento de cerca de duas vezes na concentração sérica de corticosterona. Nenhuma alteração foi observada na expressão das proteínas dos receptores de glicocorticóides em átrios direitos destes mesmos animais, comparados com ratos controle.

Nossos resultados permitem concluir que em átrios direitos de ratos submetidos a estresse por choque nas patas ocorre aumento da expressão do adrenoceptor $\beta_2$ e que este resulta em aumento da sensibilidade a agonistas não-seletivos para os subtipos de adrenoceptores $\beta$ e para agonistas seletivos para o subtipo $\beta_2$, o que confere importância fisiológica ao processo. Permitem concluir também que a isoforma clássica do adrenoceptor $\beta_1$ apresenta comportamento independente da isoforma não-convencional, uma vez que a resposta mediada por adrenoceptores $\beta_1$ sofre dessensibilização, mas a expressão dos adrenoceptores $\beta_1$, assim como a resposta a agonistas não convencionais permanece inalterada. A expressão de receptores para glicocorticóides também não se apresentou alterada, embora a concentração de corticosterona estivesse aumentada em ratos submetidos a estresse por choque nas patas.

## ABSTRACT

Right atria of male or female rats sacrificed at diestrus and submitted to foot shock stress show supersensitivity to non-selective and selective $\beta_2$-adrenoceptor agonists. This supersensitivity was due to increased $\beta_2$-adrenoceptor protein level expression. This tissue also showed subsensitivity to non selective $\beta_1$-adrenoceptor agonist, but no changes in $\beta_1$-adrenoceptor protein level was observed, suggesting that the subsensitivity showed in the phamacological approches is probably due to changes in intracelullar sinalying rather than in alterations at the receptor level. The chronotropic response to (±)-CGP12177 was not changed in right atria from male or female rats sacrificed at estrus or diestrus, indicating that the response is more resistant to stress than the response to conventional agonists, and it is not influenced by the estrous cycle phase. Additionally we observed that in right atria from sinoaortic denervated rats there was a dichotomy between sensitivity to the classical agonists and CGP12177. Our data do not clarify whether the chronotropic response to (±)-CGP12177 was mediated by the putative $\beta_4$-adrenoceptor or by a $\beta_1$-adrenoceptor isoform, with low affinity for the conventional agonists. Moreover, our data show that this receptor shows a behavior independent from the $\beta_1$-adrenoceptor classical isoform. Male or female rats sacrificed at diestrus or at estrus and submitted to foot shock stress showed an increase of around 2-fold in the serum corticosterone level. No alteration was observed in the glucocorticoid receptor protein expression in right atria of stressed compared to control rats.

We conclude that in right atria from rats submitted to foot shock stress the $\beta_2$-adrenoceptor enhanced expression results in increased sensitivity to β-adrenoceptor non-selective

agonists and to $\beta_2$-adrenoceptor selective agonists, that confers physiological importance to this process. We also conclude that the $\beta_1$-adrenoceptor classical isoform shows a behavior independent from the non-conventional isoform, because the response to agonists mediated by $\beta_1$-adrenoceptor was desensitizated but the response to the non conventional agonist was not altered. The expression of glucocorticoid receptor was not altered by foot shock stress, although the corticosterone levels increased in rats submitted to foot shock stress. Moreover, the adaptive mechanisms triggered by stress were not dependent on gender but they are influenced by the estrous cycle.

# III. INTRODUÇÃO

A presença de agentes estressores externos e/ou internos pode ameaçar a integridade ou o equilíbrio do organismo. Frente a esta situação, o organismo produz uma série de alterações metabólicas e fisiológicas que visa manter a sua homeostase e que foi denominada por SELYE (1936) de Síndrome Geral da Adaptação. Esta resposta está intimamente relacionada com a ativação do sistema nervoso simpático-medula da adrenal, que culmina com a liberação das catecolaminas (OSTMAN-SMITH, 1979; NATELSON *et al.*, 1981; AXELROD & REISINE, 1984; NATELSON *et al.*, 1988) e com a ativação do eixo hipotálamo-hipófise-córtex da adrenal, que resulta na liberação dos glicocorticóides (AXELROD & REISINE, 1984). Os glicocorticóides e as catecolaminas possuem um papel fundamental em regular as atividades fisiológicas normais e em manter a homeostase frente a situações de estresse. A inter-relação entre estes dois sistemas é de grande importância fisiológica. Em geral, os hormônios esteróides induzem o refinamento ou a regulação dos processos mediados pelas catecolaminas.

O sistema periférico responsivo às catecolaminas é principalmente caracterizado por cinco subtipos de receptores de superfície celular ($\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ e $\beta_3$). Estes receptores são codificados por genes específicos (EMORINE, 1989) e estão acoplados a diferentes sistemas efetores. A resposta celular às catecolaminas depende do subtipo e da proporção de cada subtipo de receptor adrenérgico presente em uma determinada célula. Esta proporção pode ser controlada por fatores externos como temperatura, ou internos como a ação de hormônios tireoideanos, estrogênios ou glicocorticóides (WILLIANS *et al.*, 1977; FRASER & VENTER, 1980).

No tecido cardíaco, a resposta cronotrópica às catecolaminas é mediada pelos adrenoceptores $\beta_1$ e $\beta_2$. A contribuição relativa de cada um destes subtipos de adrenoceptores $\beta$ para a modulação da função cardíaca varia entre as espécies, não apenas do ponto de vista quantitativo, mas também qualitativo (MOLENAAR *et al.*, 1997). Além dos adrenoceptores $\beta_1$ e $\beta_2$, alguns autores propuseram que, no coração de várias espécies (KAUMANN, 1989; KAUMANN, 1997; SARSERO *et al.*, 1998) haveria um terceiro subtipo de adrenoceptor $\beta$ com propriedade cardioestimulante. Este receptor seria farmacologicamente distinto dos subtipos $\beta_1$, $\beta_2$ e $\beta_3$, sendo, por isso, referido como adrenoceptor $\beta_4$ atípico. O suposto adrenoceptor $\beta_4$ apresenta afinidade por agonistas de adrenoceptores $\beta_3$ e pelo agonista parcial não convencional CGP12177A (KAUMANN, 1997). KAUMANN & LYNHAM (1997) e KAUMANN (1997) demostraram que o receptor $\beta_4$ (assim como as isoformas clássicas $\beta_1$ e $\beta_2$) se acopla ao sistema proteína G-adenilil-ciclase e tem a capacidade de aumentar os níveis intracelulares de AMP cíclico. Apesar das evidências que indicam que este receptor possui um papel funcional no coração, as opiniões a respeito de sua existência são controversas, pois além deste receptor não ter sido clonado, vários autores propuseram que o efeito cardioestimulante do CGP12177 poderia ser mediado por uma isoforma do receptor $\beta_1$ com baixa afinidade pelos agonistas convencionais (PAK & FISHMANN, 1996; KONKAR *et al.*, 2000; KOMPA & SUMMERS, 1999, KAUMANN *et al.*, 2001), e não por um novo subtipo de adrenoceptor $\beta$, como proposto inicialmente por KAUMANN e seus colaboradores.

A ativação do sistema cardiovascular é condição *sine qua non* para a sobrevivência diante de um estressor. Neste sentido, a interação entre os glicocorticóides e as catecolaminas é de grande importância fisiológica. Glicocorticóides exercem efeitos

permissivos sobre a ação das catecolaminas no sistema cardiovascular, por meio de vários mecanismos. Estes hormônios induzem a expressão da fenilalanina-N-metiltransferase, enzima limitante na síntese de adrenalina (KENNEDY & ZIEGLER, 1991; WURTMAN & AXELROAD, 1996) e prolongam a ação das catecolaminas nas sinapses, inibindo a recaptação das catecolaminas e diminuindo a concentração periférica das enzimas catecol-orto-metiltransferase e monoamino oxidase (DAILELY & WESTFALL, 1978; GIBSON, 1981), aumentando assim a reatividade do sistema cardiovascular à ativação simpática. Adicionalmente, tem-se verificado que os glicocorticóides regulam a expressão e/ou a densidade dos adrenoceptores β em vários tecidos, incluindo células do ducto deferente de hamster (HADCOCK & MALBON, 1988), tecido adiposo humano (BRÖNNEGAR *et al.*, 1995) pulmão de ratos (MANO *et al.*, 1979; MAK *et al.*, 1995), células de músculo liso arterial humano (JAZAYERI & MEYER, 1988). Em geral, observa-se que os glicocorticóides aumentam a expressão dos adrenoceptores $\beta_2$ e diminuem ou não alteram a expressão dos adrenoceptores $\beta_1$ (FÈVE *et al.*, 1990; KIELY *et al.*, 1994). Glicocorticóides também atuam aumentando o acoplamento dos adrenoceptores β com o seu sistema efetor (MARONE *et al.*, 1980; DAVIES *et al.*, 1981). Embora na literatura sejam abundantes os dados que indicam a relação entre glicocorticóides e modulação de receptores β, estudos como estes são escassos em tecido cardíaco.

Receptores de glicocorticóides foram detectados em coração de humanos e de ratos (FUNDER *et al.*, 1973; LOMBES *et al.*, 1992). No tecido cardíaco os glicocorticóides regulam a expressão de vários genes (LINDPAINTENER *et al.*, 1990; DELLA *et al.*, 1995), afetam a contratilidade (LEUNG & MUNK, 1975) o peso do coração (HICKS *et al.*, 1982) e participam dos processos de hipertrofia cardíaca

(McDERMOTT *et al.*, 1989). SATO *et al.* (1996) sugeriram uma possível ligação cruzada entre os glicocorticóides e o segundo mensageiro AMPc no estímulo ao crescimento de cardiomiócitos.

TUNER & MOSES (1986) verificaram que há diferença na concentração de receptores de glicocorticóides cardíacos, relacionada ao sexo. Fêmeas têm maior concentração de receptores citosólicos em miócitos atriais do que em miócitos ventriculares, enquanto em machos a proporção é semelhante nas duas câmaras. Em fêmeas, o tratamento com dexametasona não produz diminuição significativa nos receptores para glicocorticóides atriais, mas reduz a concentração de receptores ventriculares para 65% do controle. Isto sugere que receptores para glicocorticóides cardíacos, em fêmeas, podem estar menos sujeitos à "down-regulation" pelos altos níveis de glicocorticóides circulantes do que em machos.

Vários autores demonstraram o efeito do estresse e, conseqüentemente, das altas concentrações plasmáticas de glicocorticóides e de catecolaminas sobre a resposta mediada por β adrenoceptores em átrio direito de ratos. BASSANI & De MORAES (1988) demonstraram que o estresse por choques nas patas pode induzir aumento da resposta cronotrópica positiva a agonistas seletivos de adrenoceptores $\beta_2$. Posteriormente, NOURANI *et al.* (1992) demonstraram o envolvimento dos glicocorticóides neste processo ao verificarem que o tratamento de ratos com antagonista de receptores para glicocorticóidedes, abole a supersensibilidade verificada para agonistas de adrenoceptores $\beta_2$. Em átrio direito de ratas sacrificadas durante o diestro, MARCONDES *et al.* (1996), verificaram que estresse por choques nas patas induziu subsensibilidade da resposta cronotrópica eliciada pela noradrenalina. VANDERLEI *et al.* (1996), ampliaram estes

achados ao verificarem que parte desta subsensibilidade era gerada por aumento na eficiência dos mecanismos de recaptação neuronal da noradrenalina. Adicionalmente, estes autores verificaram que o tecido que apresentava subsensibilidade para noradrenalina, desenvolvia supersensibilidade ao efeito cronotrópico da adrenalina, sugerindo, que assim como em átrio direito de ratos machos, o protocolo de estresse utilizado, induziu uma população heterogênea de receptores $\beta_1/\beta_2$-adrenérgicos, no tecido atrial de fêmeas sacrificadas em diestro. Posteriormente, SPADARI-BRATFISCH *et al.* (1999) demonstraram que átrios direitos de ratas estressadas e sacrificadas em diestro responderam a concentrações nanomolares de TA2005 (um agonista seletivo para receptores $\beta_2$), e que esta resposta foi abolida pelo ICI118551 (antagonista de receptores $\beta_2$). Estes dados, permitiram a estes autores, não só confirmarem a participação do receptor $\beta_2$ na resposta cronotrópica, como também quantificarem esta participação em 10%. É interessante ressaltar, que nenhuma das alterações mencionadas acima, foram obervadas em átrio direito de ratas sacrificadas durante o estro, o que indica que a sensibilidade do tecido atrial de fêmeas às catecolaminas, depende das alterações hormonais que ocorrem durante o ciclo estral.

A hipertensão neurogênica induzida por desnervação sinoaórtica em ratos, apresenta algumas características que permitem classificá-la como modelo de estresse. Os ratos submetidos a este procedimento cirúrgico apresentam aumento da atividade simpática (ALEXANDER *et al.*, 1980) e concentrações plasmáticas elevadas de corticosterona. Nos estágios iniciais do processo, os ratos apresentam taquicardia e elevação da pressão arterial. Após um período de cerca de 15 dias, estes animais apresentam diminuição na freqüência cardíaca (BRODY *et al.*, 1983, VASSALO *et al.*, 1991, TRAPANI *et al.*, 1986, ALPER *et al.*, 1987, MAUAD *et al.*,

1992) enquanto a hipertensão apresenta labilidade e pode persistir por alguns meses (ALEXANDER *et al.*, 1980, KRIEGER *et al.*, 1980, VASQUEZ & KRIEGER, 1980. ZANESCO *et al.* (1997) demonstraram que em átrio direitos de ratos submetidos à desnervação sinoaórtica, ocorre diminuição da sensibilidade à noradrenalina que é causada por diminuição dos sítios de ligação dos adrenoceptores $\beta_1$. Diferentemente do que ocorre em átrio direito de ratos submetidos ao estresse por choques nas patas, esta subsensibilidade não é acompanhada por alterações na resposta mediada por adrenoceptores $\beta_2$.

Diante dos dados da literatura que indicam que o estresse e consequentemente elevados níves de catecolaminas e glicocorticóides, alteram a sensibilidade do tecido atrial de ratos machos e fêmeas e diante dos trabalhos que indicam que há um terceiro tipo de receptor β-adrenérgico, com atividade cardioestimulante, o objetivo geral do nosso trabalho foi analizar se a resposta cronotrópica do CGP12177, é alterada pelo estresse (por choques nas patas ou desnervação sinoaórtica). Adicionalmente, nós também analizamos o nível das proteínas dos receptores de glicocorticóides e de adrenoceptores β, em átrio direito de ratos submetidos a estresse.

Para a apresentação desta tese, os resultados obtidos foram organizados em capítulos que correspondem aos manuscritos gerados durante o seu desenvolvimento, os quais passamos a apresentar.

## IV. OBJETIVOS GERAIS

Nossos objetivos foram verificar se:

1- o estresse por choques nas patas ou desnervação sinoaórtica altera a resposta mediada pelo CGP12177 em átrio direito de ratos;

2- a resposta cronotrópica ao CGP12177 é alterada pelo ciclo estral e pelo estresse;

3- estresse por choques nas patas modula a expressão das proteínas dos receptores de glicocorticóides e dos subtipos de adrenoceptores β em átrio direito de ratos machos e de fêmeas.

4- a expressão destes receptores é influenciada pelo ciclo estral.

# V. MANUSCRITOS

# CHRONOTROPIC RESPONSE TO (±)-CGP12177 IN RIGHT ATRIA FROM STRESSED RATS

**Santos, I.N. & Spadari-Bratfisch, R. C.**

Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil

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## Abstract

Foot-shock stress changes the sensitivity of the rat right atria to $\beta_1$- and $\beta_2$-adrenoceptor agonists. In this study, we investigated whether the same stress protocol also changes the atrial sensitivity to the non-conventional agonist, (±)-CGP12177. Concentration-response curves to (±)-CGP12177, a $\beta_1$- and $\beta_2$-adrenoceptor antagonist with agonist properties at the putative $\beta_4$-adrenoceptors, were obtained in the absence and in the presence of propranolol (200 nM or 2 μM), CGP20712A 10 nM plus ICI118,551 50 nM, or CGP20712A (1 μM or 3 μM), in right atria from rats submitted to three daily foot-shock sessions (120 mA pulses of 1.0 s duration applied at random intervals of 5-25 s over 30 min) and sacrificed after the third session. The $pD_2$ for (±)-CGP12177 was not influenced by foot-shock stress. The stimulant effect of (±)-CGP12177 was resistant to the blockade by 200 nM and 2 μM (±)-propranolol, or to the combined blockade by CGP20712A and ICI118,551. However in right atria from stressed rats with the former dose of propranolol the concentration-response curve to the agonist was shifted 2.0-fold to the right. CGP20712A shifted the concentration-response curve to (±)-CGP12177 to the right by 4.6 (1μM) and 19-fold (3 μM) in atria from control rats, and by 2.2-fold (1 μM) and 43-fold (3 μM) atria from in stressed rats. Maximum response to CGP12177 was not affected by propranolol or CGP20712A in concentrations ranging from 0.1 nM to 10 μM. These results show that the chronotropic effect of (±)-CGP12177 is mediated by atypical $\beta_4$-adrenoceptors, and that, in constrast to $\beta_1$-and/or $\beta_2$-adrenoceptors, this receptor is resistant to the effects of foot-shock stress, suggesting that the putative $\beta_4$- adrenoceptor

is a receptor different from a low affinity state $\beta_1$-adrenoceptor, as previously proposed, unless both proposed isoforms of $\beta_1$-adrenoceptor show independent stress-induced behavior.

**Key words:** putative $\beta_4$-adrenoceptor, low affinity $\beta_1$-adrenoceptor isoform, stress, right atria, chronotropic response.

## Introduction

We have previously shown that right atria from stressed rats have lower sensitivity to the chronotropic effects of noradrenaline and a decreased affinity for $\beta_1$-AR selective antagonists. This subsensitivity was accompanied by an increase in the sensitivity to non-selective β-AR agonists (Bassani and De Moraes 1987; Marcondes et al. 1996; Vanderlei et al. 1996). Moreover, right atria from stressed but not from control rats responded to nanomolar concentrations of TA2005, a $\beta_2$-AR selective agonist, and these responses were abolished by ICI118,551 (50 nM). Based on these data we suggested that foot-shock induces a $\beta_2$-AR subtype mediated response in rat right atria (Spadari-Bratfisch et al. 1999).

Evidence for a "putative fourth β-adrenoceptor" in cardiac tissues was first presented when it was observed that some compounds traditionally classified as $\beta_1$ and $\beta_2$-adrenoceptor antagonists caused cardiostimulant effects at much higher concentrations than those causing antagonism (Kaumann 1973, 1989). Kaumann (1989) proposed that these compounds, termed "non-conventional agonists", may mediate their effects through a β-adrenoceptor (β-AR) distinct from $\beta_1$ ($\beta_1$-AR) and $\beta_2$ ($\beta_2$-AR)-adrenoceptors. Afterwards this subtype, known as the "putative $\beta_4$-adrenoceptor" ($\beta_4$-AR), has been demonstrated in heart tissues from cats, rats (Kaumann 1973; Malinowska and Schicker 1996, Kaumann and Molenaar 1996; Kaumann and Lynham 1997), guinea-pigs and mice (Kaumann 1989, 1996), ferrets and humans (Kaumann 1989, 1996, 1997; Kauman and Molenaar 1997; Molenaar et al. 1997). The cardiostimulant effect of (-)-CGP12177A, a non-conventional agonist, is unaffected by the $\beta_1$ and $\beta_2$-adrenoceptor antagonist (-)-propranolol (200 nM) but is blocked with moderate affinity by (-)-bupranolol and CGP20712A (Kaumann and Molenaar 1996, 1997). A phosphodiesterase inhibitor, IBMX,

potentiated the effect of (-)-CGP12177 in rat right and left atria (Kaumann and Lynham 1997), suggesting $G_s$-protein coupling of the putative $\beta_4$-AR to adenylate cyclase. (-)-[$H^3$]-CGP12177 has been also used as a radioligand for putative $\beta_4$-AR in rat atrium (Sarsero et al. 1998).

Despite the evidence from functional and binding studies indicating the existence of putative $\beta_4$-AR, it seems that the responses originally attributed to putative $\beta_4$-AR may also be produced by $\beta_1$-AR. In hamster fibroblasts (CHW) expressing the human $\beta_1$-AR, (-)-CGP12177A acted as a low potency agonist. Based on an experimental model of failing heart produced by myocardial infarction, Kompa and Summers (1999) proposed that the properties attributed to the putative $\beta_4$-AR may be explained by an interaction of the agonist with a low affinity state of the $\beta_1$-AR or that $\beta_1$-AR and the putative $\beta_4$-AR use the same signalling pathway. Lowe et al. (1999) also argued that the putative $\beta_4$-AR could correspond to a low affinity state of $\beta_1$-AR. Since a $\beta_4$-AR gene has not yet been identified, the controversy is still open.

In this study, we examined the chronotropic response to (±)-CGP12177A in right atria from rats to determine whether the response mediated by the "putative" $\beta_4$-AR was modified after the same stress protocol known to affect the sensitivity to noradrenaline, isoprenaline and TA20005 (Spadari-Bratfisch et al. 1999).

## Material and methods

### Animals

Male Wistar rats (*Rattus norvergicus*) 250 to 350 g, were housed in standard cages in a temperature-controlled room (22°C), with a 12 h light / dark cycle with the lights on at 6:30 a.m. Standard laboratory chow and tap water were available *ad libitum*.

During the experiments, the animals were cared for in accordance with the principles reported by Olfert and Cross (1993) and the experimental protocols were approved by the animal care committee (CEEA), of the Institute of Biology, UNICAMP.

### Stress protocol

Rats individually underwent three daily sessions of unsignaled, inescapable foot-shocks. The rats were placed in a plexiglass chamber (26 cm long x 21 cm wide x 26 cm high) provided with a grid floor consisting of stainless steel rods (0.3 cm in diameter and spaced 1.0 cm apart). During the 30 min foot-shock sessions, which occurred between 7:30 a.m. and 11:00 a.m., the shocks were delivered by a constant current source controlled by a microprocessor-based instrument constructed at the Center for Biomedical Engineering, UNICAMP. Each rat received 120 foot-shocks with a current intensity of 1.0 mA and a duration of 1.0 s at random intervals of 5-25 s (mean interval, 15 s). The shocks were scrambled as described by Hoffman and Fleshler (1962). After the foot-shock session, the rats were returned to their cages or were sacrificed.

This stress protocol modifies the catecholamine sensitivity of right atria from male rats (Bassani and De Moraes 1987, 1988) and female rats in diestrus (Vanderlei et al. 1996; Marcondes et al. 1996, Spadari-Bratfisch et al. 1999).

**Organ-bath studies**

Immediately after the last foot-shock session, the rats were sacrificed by a blow to the back of the head and exsanguinated. The hearts were immediately removed and the right atria isolated and suspended in 20 ml organ baths containing Krebs-Henseleit solution of the following composition (in mM): NaCl 115.0; KCl 4.6; $CaCl_2.2H_2O$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4.7H_2O$ 2.5; $NaHCO_3$ 25.0; glucose 11.0 and ascorbic acid 0.1. The solution was warmed (36.5 ± 0.1°C) and gassed continuously with 95% $O_2$-5% $CO_2$ (pH 7.2-7.4). The atria were attached to isometric force transducers (Narco F-60, Narco Biosystems) under a resting tension of 5 mN. The tissues were allowed to stabilize for 1 h during which the bathing medium was changed at 15 min intervals.

A complete cumulative concentration-response curve (Van Rossun 1963) to (±)- CGP12177A was obtained by stepwise increases in the concentration (0.5 log unit), in the absence of any antagonist. After this curve the preparation was washed with Krebs-Henseleit solution to remove the agonist and to allow recovery of the initial beating rate. Antagonist was then added and left in contact with the tissue for 2h before another concentration-response curve was obtained, using the same agonist, in the presence of antagonist. Experiments to control for the desensitization caused by obtaining two concentration-response curves to the agonist in the same tissue were also done. In this case, two concentration-response curves to the agonist were obtained in the absence of antagonist (Figure 1). CGP20712A (3 μM) was used to antagonize the effect of (±)-CGP12177A mediated by $\beta_4$-AR (Kaumann and Molenaar 1996). Propranolol (200 nM or 2 μM) or CGP20712A (10 nM) plus ICI118,551 (50 nM) were used to antagonize $\beta_1$- and $\beta_2$-AR mediated responses and to guarantee that the observed effect of the agonist resulted from its

interaction with $\beta_4$-AR (Kaumann 1996; Kaumann and Molenaar 1996; Kompa and Summers 1999). A maximum response was reached when a 0.5 log unit increase in the agonist concentration produced no additional increase in atrial beating frequency. The experiments ended with the administration of a saturating concentration of (-)-isoprenaline (400 μM).

Concentration-response curves were also obtained for the effect of an antagonist (propranolol or CGP20712A) on the beating rate of atria which have been previously stimulated by a saturating concentration (10 μM ) of CGP12177.

**Analysis of the concentration-response curves**

Changes in the sensitivity to the agonist were evaluated by determining the concentration which produced a response that was 50% of the maximum response ($EC_{50}$). This calculation was done using the software Graph Pad Prism (GraphPad Software, San Diego, CA). The data are presented as mean negative logarithm of the $EC_{50}$ ($pD_2$) ± s.e.m.

The concentrations of agonist producing a half-maximal response in the absence [A] and presence [A'] of antagonist were estimated (Arunlakshana and Schild 1959) as follows:

$$\log (CR - 1) = n \log [B] - \log K_B$$

where CR is [A'] / [A], n is the slope, [B] is the concentration of the antagonist, and -log $K_B$ is the antagonist dissociation constant. The apparent molar equilibrium dissociation constant for the interaction of the antagonist with the receptor, $K_B$, was determined using the equation:

$$K_B = [B] / \log (CR - 1).$$

The dissociation constants are given as $pK_B$ values, i.e. -log $K_B$.

### In vitro pretreatment

Concentration-response curves were obtained after incubating isolated right atria with phenoxybenzamine (10 $\mu$M) for 15 min to block $\alpha$-adrenoceptors (Besse and Furchgott 1976), extraneuronal uptake (Iversen et al. 1972) and muscarinic receptors (Furchgott and Bursztyn 1967). This period was followed by 45 min of thorough washing. After recovery of the spontaneous rate, corticosterone (30 $\mu$M) and desipramine (0.1 $\mu$M) were added and maintained in the organ-bath throughout the experiment to inhibit extraneuronal uptake (Iversen and Salt 1970) and neuronal uptake (Salt 1972), respectively.

### Statistical analysis

Statistical differences were assessed by Student's *t*-test for unpaired samples or ANOVA plus Tukey test (Zar 1984). Differences were considered significant at $p < 0.05$.

## Results

(±)-CGP12177A produced a concentration-dependent, positive chronotropic effect in right atria from control or stressed rats, both of which had similar spontaneous beating frequencies and maximum responses (Table 1). The $pD_2$ value of (±)-CGP12177 in right atria was not modified by stress. The maximum response to (±)-CGP12177A compared to (-) isoprenaline was around 65%. Since in most groups two concentration-response curves were obtained in the same tissue, in the absence and presence of antagonist, a control experiment to check for tissue desensitization to the agonist was done (Figure 1). Initial beating rate, $pD_2$ and maximum response to (±)-CGP12177A were not significantly different comparing the first and the second concentration-response curves, either in control or stressed rat right atria.

In right atria from control rats, the concentration-response curve to (±)-CGP12177 was not affected by 200 nM or 2 μM (±)-propranolol (Figure 2A), although the higher concentration of propranolol produced a 13% depression on the spontaneous atria beating rate. In contrast, in right atria from stressed rats, 200 nM (±)-propranolol significantly shifted the concentration-response curve to (±)-CGP12177A to the right by 2.0-fold (Figure 2B, Table 2). The $pK_B$ estimated for (±)-propranolol in right atria from stressed rats was 7.10 ± 0.10.

The incubation of the tissues with CGP20712A (10 nM) plus ICI118,551 (50 nM) caused a statistically significant ($p<0.05$, Student's *t* test) depression in the initial beating rate of atria from control (257 ± 7 to 208 ± 8 beats/min) and stressed ( 275 ± 12 to 210 ± 13 beats/min) rats. However, (±)-CGP12177A potency ( 7.09 ± 0.20, control; 6.98 ± 0.21, stressed) and efficacy (80 ± 17, control; 75 ±7 beats/min, stressed) were not altered by the presence of the $\beta_1$- and $\beta_2$-adrenoceptor antagonists (Figure 3; $p>0.05$, Student's *t* test).

In atria from control (Figure 4A) and stressed (Figure 4B) rats, the concentration-response curves to (±)-CGP12177A in the presence of 1 μM CGP20712A were shifted to the right 3.7-fold and 2.6-fold, respectively (Table 3). This concentration produced a 16% depression on the spontaneous beating rate in right atria from foot-shock stressed rats. CGP20712A (3 μM) decreased the spontaneous beating frequency by around 18% in right atria from control and foot-shock stressed rats, without modifying the maximum responses to the agonist. The concentration-response curves to (±)-CGP12177A were shifted to the right by around 33-fold in atria from control rats, but only 23-fold in atria from stressed rats ($p<0.05$; Figure 3, Table 3).

The concentration-response curves for the antagonism of the effect of (±)-GCP12177 by increasing concentrations of propranolol or CGP20712A are presented in Figure 5. The response to the agonist was not altered by any of the antagonists in a concentration range from 0.1 nM to 10 μM, both in control and foot-shock stressed rats right atria.

## Discussion

Foot-shock stress induces supersensitivity in the chronotropic response mediated by $\beta_2$-adrenoceptors and subsensitivity in the chronotropic response mediated by $\beta_1$-adrenoceptors in right atria from male rats (Bassani and De Moraes 1988) and female rats sacrificed during diestrus (Marcondes et al. 1996; Vanderlei et al. 1996; Spadari-Bratfisch et al. 1999). The results obtained here show that the same stress protocol did not change the sensitivity of right atria from rats to the chronotropic effect of (±)-CGP12177, suggesting that the "putative $\beta_4$-AR" is unaffected by the same stress protocol that changes the sensitivity to classic $\beta_1$ and $\beta_2$- selective agonists.

The hypothesis proposed by Kompa and Summers (1999) that the response to (-)-CGP12177 in cardiac tissue is mediated by a low affinity isoform of $\beta_1$-AR was based on data showing that changes in sensitivity of both $\beta_1$- and putative $\beta_4$-AR mediated responses occur exactly in parallel, as does the resensitization induced by prior administration of pertussis toxin. By using the same rational, then the sensitivity to that non-conventional agonist should also be altered in this stress model, since the response to classic $\beta_1$-AR agonists was modified (Vanderlei et al. 1996). However, our data do not agree with this hypothesis but rather support the proposal that the receptors mediating the chronotropic response to (±)-CGP12177 represent a class of receptors distinct from $\beta_1$, and are most probably the putative $\beta_4$-adrenoceptor proposed by Kaumann (1989, 1996), unless one assumes that changes in sensitivity could occur independently for each receptor isoform.

The chronotropic effect of (±)-CGP12177 in right atria from control male rats was resistant to blockade by propranolol (200 nM and 2 μM), and to the combined blockade by CGP20712A (10 nM) and ICI1187,551 (50 nM), but was blocked by CGP20712A (1μM or 3μM), which confirms that the response results from the interaction of the agonist with the "putative $\beta_4$-AR", rather than with $\beta_1$-AR or $\beta_2$-AR (Kaumann and Molenaar 1996). Although the $pK_B$ of CGP20712A was higher than that reported by Kaumann and Molenaar (1996; $pK_B$= 6.4 using 3 μM of antagonist), it was similar to the results reported by Kompa and Summers (1999; $pK_B$= 7.1 using 1 μM CGP20712A). Different results were obtained in right atria from foot-shock stressed rats, in which 200 nM (±)-propranolol caused a significant rightward shift in the concentration-response curve to (±)-CGP12177A and allowed the estimation of a $pK_B$ value for propranolol (7.10 ± 0.1). This value was higher than that reported for the interaction of propranolol with the "putative" $\beta_4$-AR ($pK_B$ < 5.7; Kaumann and Molenaar 1996) but much lower than the $pK_B$ for propranolol at $\beta_1$-AR and $\beta_2$-AR ($pK_B$ = 8.5 and 8.9, respectively; Gille et al. 1985). On the other hand they were similar to the value obtained by Kompa and Summers (1999) in right atria from rats with myocardial infarction ($pK_B$ = 6.8 ± 0.1). The combined blockade of $\beta_1$-AR by CGP20712A (10 nM) and $\beta_2$-AR by ICI1187,551 (50 nM), did not affect the concentration-response curve to (±)-CGP12177, confirming that the response is not mediated by those two adrenoceptor subtypes.

Both concentrations of CGP20712A (1μM and 3μM) produced rightward shifts in the concentration-response curves to (±)-CGP12177 in atria from control and foot-shock stressed rats. The estimated $pK_B$ for CGP20712A in right atria from control rats were similar to those reported by Kaumann and Molenaar (1997) in this same tissue (6.4) and by Kompa and Summers

(1999) in right atria from rats with heart failure (7.1 ± 0.2). Additionally confirming these data, the response to a saturating concentration of the agonist was not altered by concentrations of propranolol or CGP20712A, in a range from 0.1 nM to 10 μM.

In those experiments using antagonists there was a depression in the spontaneous beating rate of atria. However, this effect did not altered the $pD_2$ and maximum response to the agonist, since in atria from control rats data are similar to some previously reported by others (Kaumann and Molenaar 1996, Kompa and Summers 1999).

Thus, in contrast to the heart failure model (Kompa and Summers, 1999), in this model of stress the alterations in right atrial sensitivity to $\beta_1$-AR and $\beta_2$-AR agonists and antagonists did not parallel those on the sensitivity of the "putative" $\beta_4$-AR and suggest that these receptors are in fact different from a low affinity state of $\beta_1$-AR, as proposed by those authors.

**Table 1**

Spontaneous beating rates (BR), $pD_2$ values and maximum responses to (±)-CGP12177 (MR) in right atria from control and foot-shock stressed rats.

| Group | BR[a] (beats/min) | $pD_2$ | MR (beats/min) | Isoprenaline MR (beats/min) |
|---|---|---|---|---|
| *Control* | 265 ± 8[a] | 7.00 ± 0.07 | 73 ± 8 | 112 ± 11 |
| | (11)[b] | (11) | (11) | (11) |
| *Foot-shock* | 269 ± 7 | 6.90 ± 0.07 | 66 ± 7 | 102 ± 11 |
| | (13) | (13) | (13) | (11) |

[a]Means ± s.e.m.; [b]Number of experiments.

**Table 2**

Spontaneous beating rates (BR), $pD_2$ values for (±)-CGP12177 in the presence of 200 nM propranolol and the $pK_B$ values for this antagonist in right atria from control and foot-shock stressed rats.

| Group | BR[a] (beats/min) | $pD_2$[a] | $pK_B$[a] | CR[a,b] |
|---|---|---|---|---|
| *Control* | 280 ± 6 (3)[c] | 6.86 ± 0.20 (3)[a] | - | 1.4 |
| *Foot-shock* | 259 ± 11 (7) | 6.61 ± 0.14* (7) | 7.10 ± 0.10 (5) | 2.0 (7) |

[a]Means ± s.e.m; [b]Ratio between $EC_{50}$ values for (±)-CGP12177 in the presence and absence (Table 1) of antagonist; [c] Number of experiments. * significantly different from CGP12177 $pD_2$ value in the absence of antagonist, in right atria from stressed rat (Table 1).

**Table 3**

Spontaneous beating rate (BR), $pD_2$ values for (±)-CGP12177 in the presence of CGP20712A and the estimated $pK_B$ for this antagonist in right atria from control and foot-shock stressed rats.

| Group | n[a] | BR | (±)-CGP12177 $pD_2$ | CGP20712A $pK_B$[b] | CR[b,c] |
|---|---|---|---|---|---|
| **CGP20712A (1 μM)** | | | | | |
| *Control* | 04 | 240 ± 6 | 6.43 ± 0.10 | 6.40 ± 0.20 | 3.7 |
| *Foot-shock* | 04 | 230 ± 16 | 6.49 ± 0.10 | 6.20 ± 0.10 | 2.6 |
| **CGP20712A (3 μM)** | | | | | |
| *Control* | 07 | 223 ± 8 | 5.48 ± 0.07 | 7.00 ± 0.10* | 33 |
| *Foot-shock* | 06 | 215 ± 6 | 5.54 ± 0.08 | 6.90 ± 0.10* | 23 |

[a]Number of experiments. [b]Mean ± s.e.m. [c]Ratio between (±)-CGP12177 EC50 values in the presence and absence of the antagonist. *$p<0.05$ compared to 1 μM CGP20712A group (Anova and Tukey test).

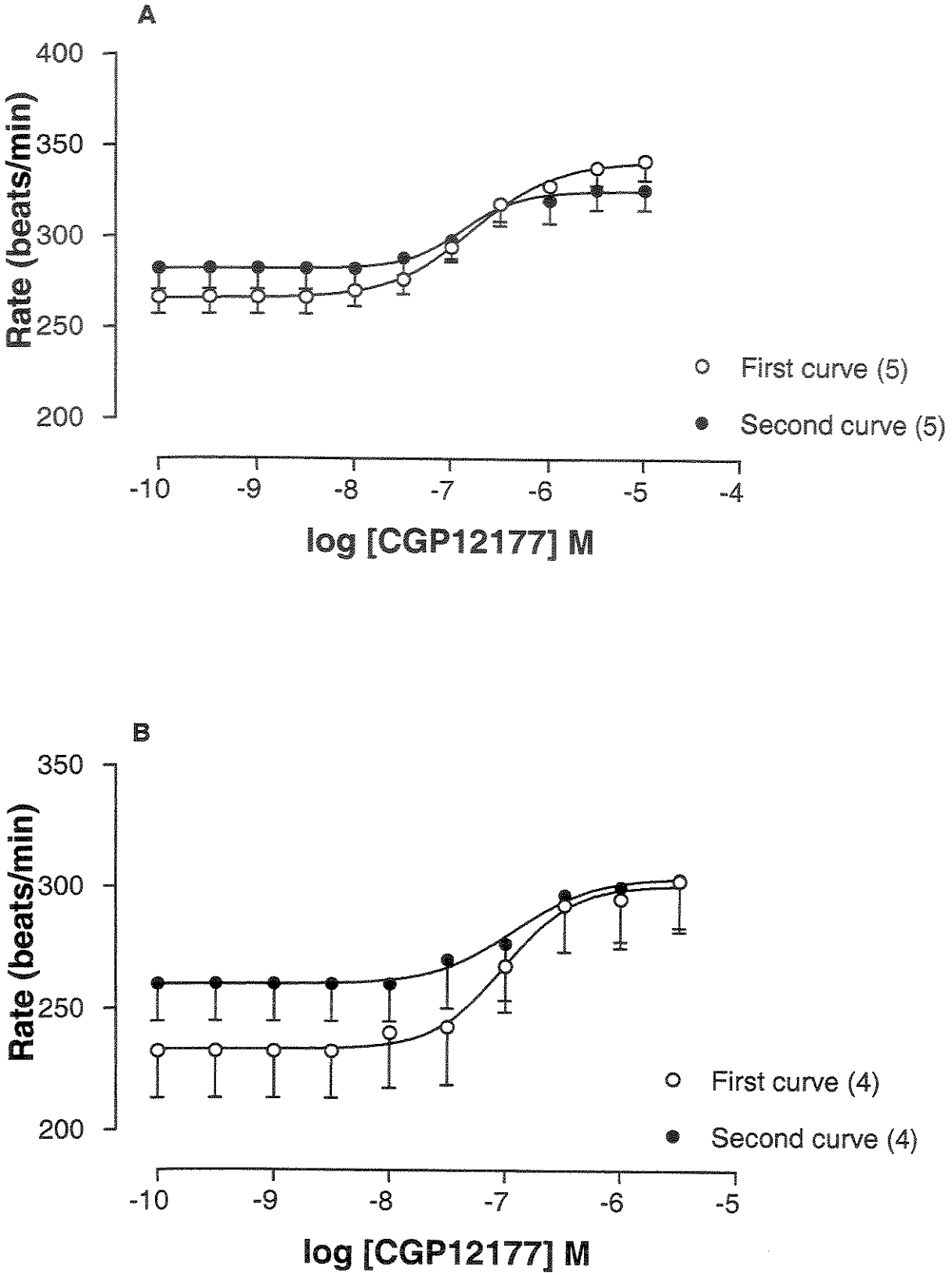


**Figure 1.** First (○) and second (●) concentration-response curves to (±)-CGP12177A obtained in the same right atrial preparation from control (**A**) and stressed (**B**) rats. The points are the mean ± s.e.m. of the number of experiments in parantheses.

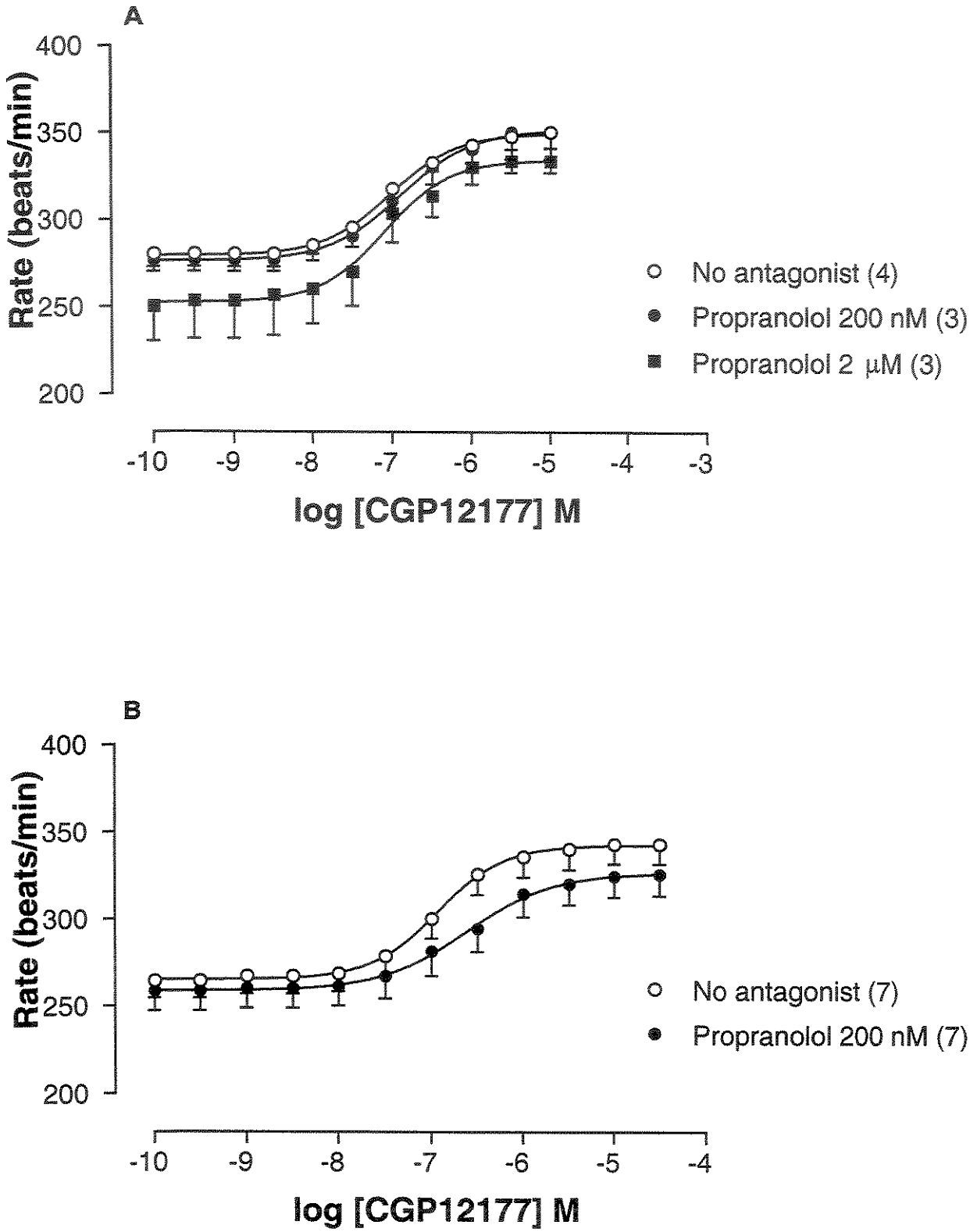


**Figure 2.** Dose-response curves to (±)-CGP12177A in right atria from control (**A**) and stressed rats (**B**) in the absence (○) or presence of 200 nM (●) and 2 uM. (■) propranolol.

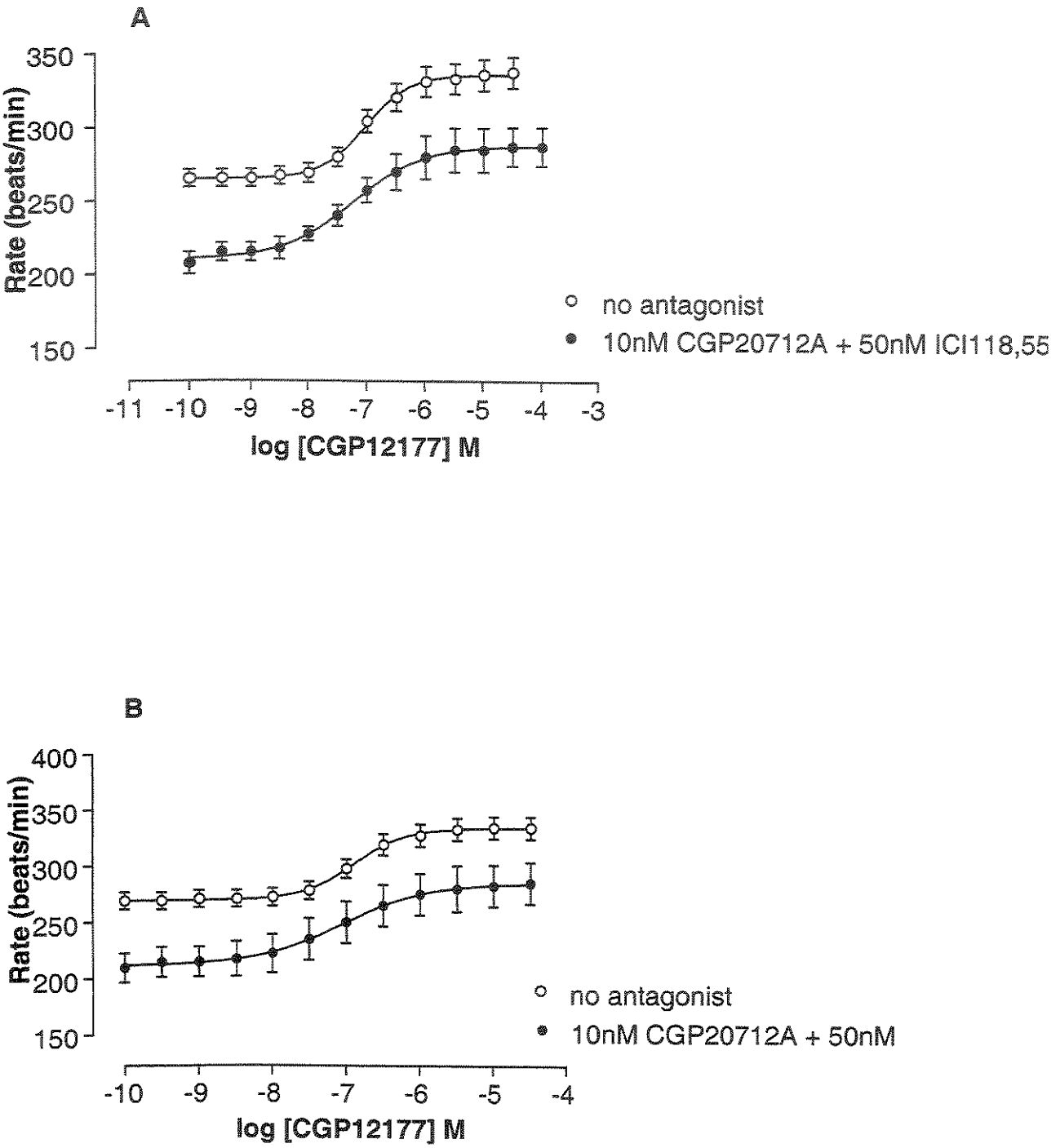


**Figure 3**. Dose-response curves to (±)-CGP12177A in right atria from control (**A**) and stressed rats (**B**) in the absence (○) or presence of (●) 50nM ICI118,551 plus 10nM CGP20712A.

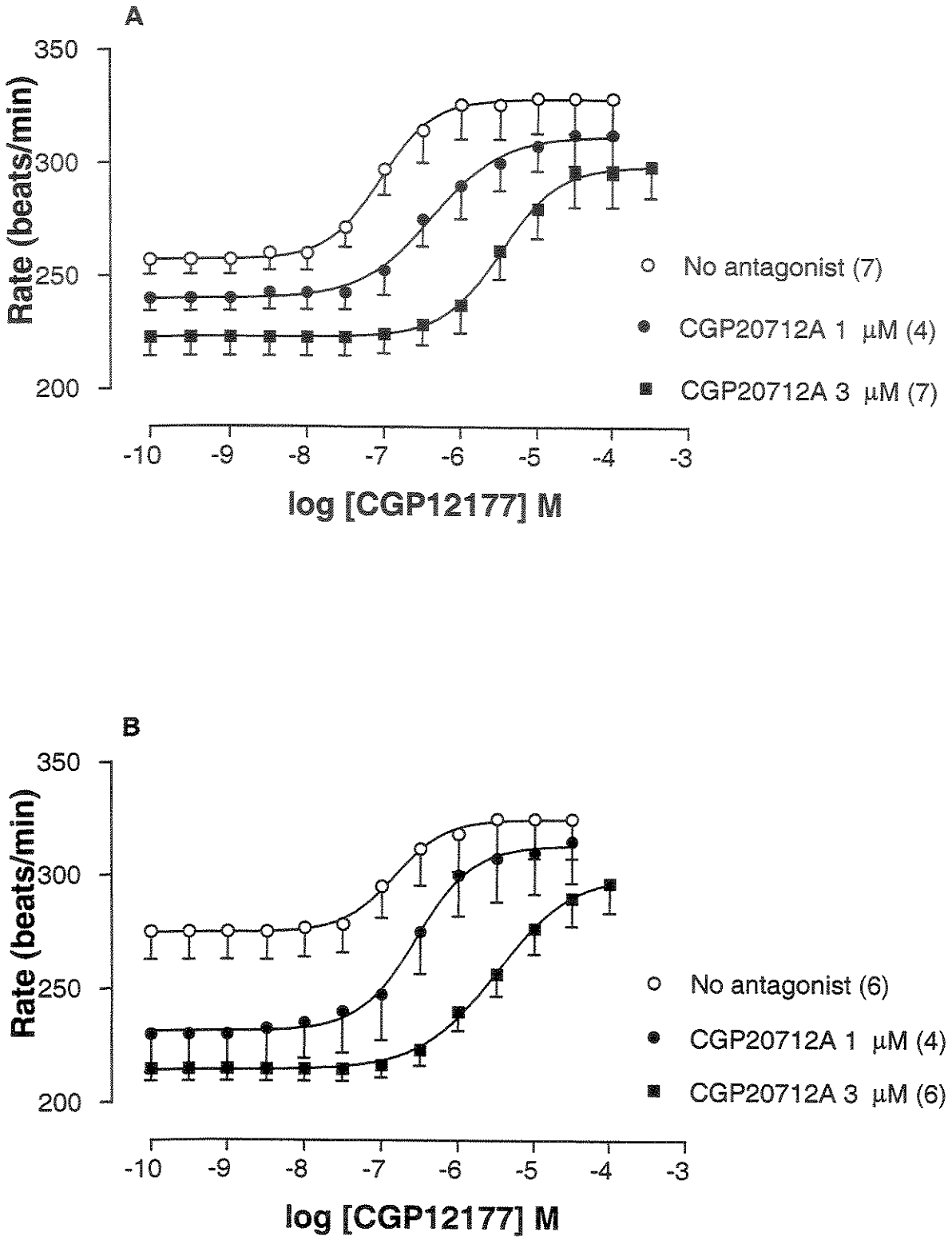


**Figure 4**. Dose-response curves to (±)-CGP12177A in right atria from control (A) and stressed rats (B) in the absence (○) or presence of (●) 1 μM and (■) 3 μM CGP20712A.

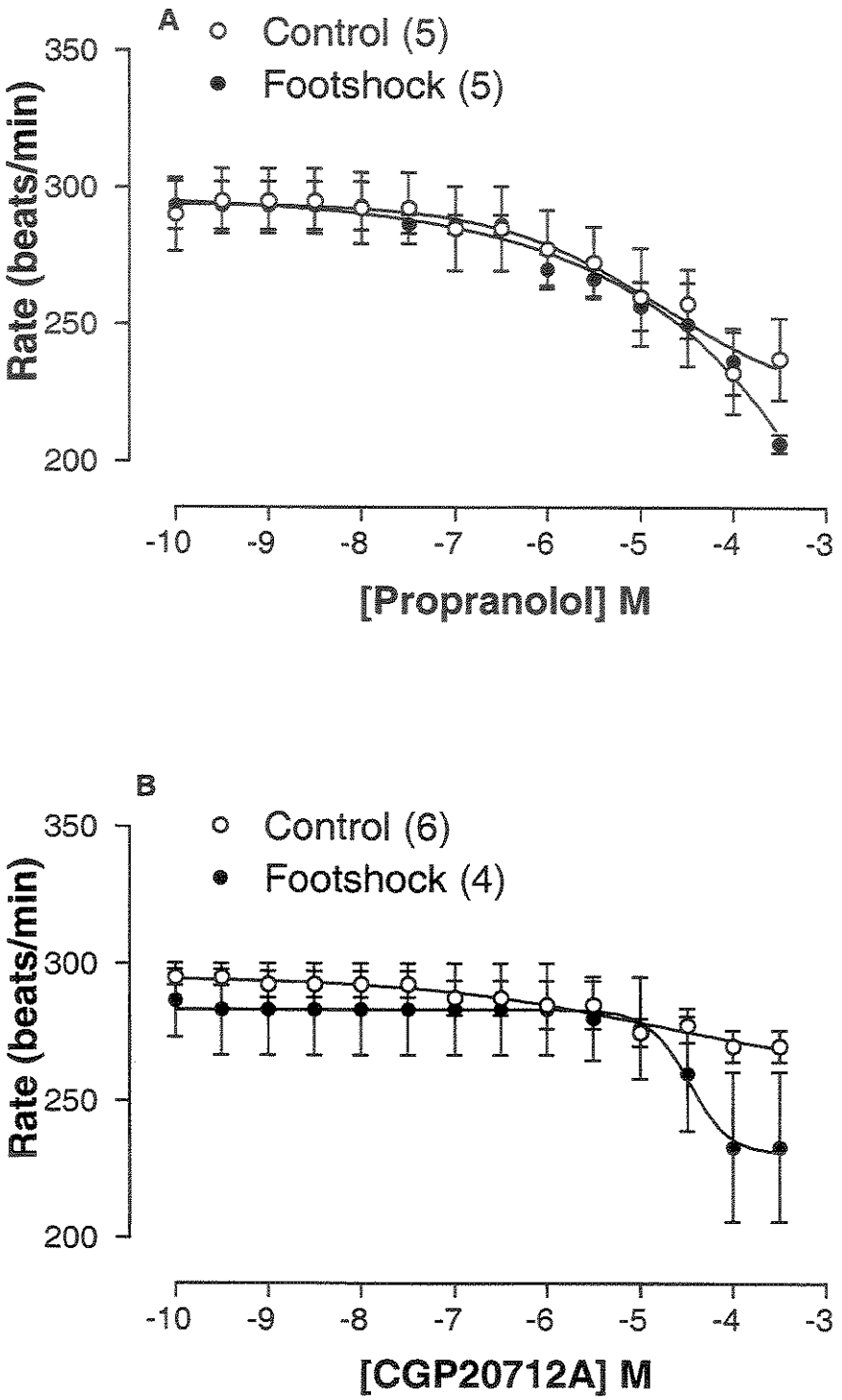


**Figure 5**. Negative chronotropic effects of $\beta_2$-AR-selective antagonist ICI118,551 plus $\beta_1$-AR-selective antagonist CGP20712A in right atria from control (**A**) and stressed (**B**), in the presence of 100μM (±)-CGP12177.

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# RESPONSE TO (±)-CGP12177 IN RIGHT ATRIA FROM FEMALE STRESSED RATS

Santos, I.N. & Spadari-Bratfisch, R. C.

Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil

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**Running title**: female rats and atria sensitivity to CGP12177.

**Abstract**

We investigated whether foot-shock stress causes any change in the chronotropic response to CGP12177 of right atria from female rats and whether these alterations are dependent on the gender and the estrus cycle phases. Concentration-response curves to (±)-CGP12177 were obtained in right atria from foot-shock stressed rats sacrificed at estrus or diestrus. The results have shown that in right atria from female rats CGP12177 $pD_2$ value was not altered by stress or the estrus cycle, but values were lower than those obtained in right atria from male rats. 200nM propranolol shifted to the right the dose-response curve to CGP12177 by around 6-fold in atria from control or foot-shock stressed female rats sacrificed at estrus and 13-fold in right atria from diestrus control female rats. On the other hand right atria from foot-shock stressed rats sacrificed at diestrus were resistant to the propranolol blockade. 3 μM CGP20712A shifted to the right the dose-response curve to CGP12177 by around 30-fold in right atria from estrus or control diestrus rats and 17-fold in right atria from diestrus foot-shock stressed rats. The CGP20712A $pK_B$ value in right atria from diestrus foot-shock stressed rats was lower than control diestrus as well as control and foot-shock stressed rats sacrificed at estrus. Therefore, our results have shown that the CGP12177 potency is lower in right atria from female rats than in atria from male rats, and that it is not altered by the estous cycle or foot-shock stress. However, during diestrus the affinity for the antagonists propranolol and CGP20712A was lower in right atria from foot-shock stressed rats, than control. Those results suggest that the β-adrenoceptor site activated by CGP12177 show different behaviours according to the estrous cycle.

## Introduction

It is well described that the chronotropic effect of catecholanines in right atria is mainly mediated by $\beta_1$-AR (Bryan et al. 1981, O'Donnell and Wanstall 1985, Juberg et al. 1985). Althoug radioligand binding detected $\beta_2$-AR in atrium from control rats (Minneman et al. 1979; Juberg et al., 1985), it seems not to participate of the chronotropic response to basal concentration of norepinephrine and epinephrine (Kaumann, 1986). In right atria from male and diestrus female rats submitted to foot-shock stress, $\beta_2$-AR plays a role to isoprenaline (Bassani and De Moraes 1988; Vanderlei et al. 1996) and TA2005 (Spadari-Bratfisch et al. 1999) whereas the response to rorepinephrine was desensitized.

Additionally to $\beta_1$- and $\beta_2$-AR, it has been suggested the participation of a third cardiostimulant β-adrenoceptor in the heart of rats and several other species (Kaumann, 1973; Malinowska and Schicker 1996, Kaumann and Molenaar 1996, Kaumann and Lynhan, 1997, Kaumann 1989, 1996), including human's heart (Kaumann, 1989, 1996, 1997; Kaumann and Molenaar 1997; Molenaar et al. 1997). This putative fourth β-AR was proposed to be activated by the non-conventional partial agonist, (-)-CGP12177, which elicited cardiostimulation by coupling to Gs-protein and adenylate cyclase (Kaumann and Lynham 1997) in doses higher than those needed to block the classical $\beta_1$-and $\beta_2$-AR (Kaumann, 1973, 1989). The cardiostimulant effect of (-)-CGP12177 was resistant to the blockade by propranolol, a $\beta_1$-and $\beta_2$-AR antagonist, or SR59230A, a $\beta_3$-AR antagonist (Kaumann and Molenaar, 1996), but it was blocked with moderated affinity by (-)-bupranolol and CGP20712A (Kaumann and Molenaar, 1996, 1997). In rat atrium (-)-CGP12177 was used as a radioligand for the putative new β-adrenoceptor subtype (Sarsero et al., 1998).

In spite of evidence from functional and binding studies, the hypothesis of the existence of a putative $\beta_4$-AR was challenged when it was demonstrated that the responses to non-conventional agonists might be produced by $\beta_1$-AR, because in hamster fibroblasts (CHW) expressing the human $\beta_1$-AR, (-)-CGP12177A behaved as an agonist with potency similar to that seen for the putative $\beta_4$-AR (Pak and Fishman, 1996). Additionally, it was demonstrated that desensitization and resensitization of $\beta_1$ and putative $\beta_4$-AR mediated responses occur in parallel in a rat model of cardiac failure (Kompa and Summers, 1999). Cells expressing rat or human $\beta_1$-AR were stimulated by high concentration CGP12177 or LY362884 (Konkar et al. 2000). Finally, the hypothesis of the existence of a fourth β-AR was abandoned when Kaumann et al (2001) demonstrated that the cardiostimulant effect of (-)-CGP12177 was absent in $\beta_1/\beta_2$-adrenoceptor double knockout mice. This result reinforced the hypothesis that the agonistic effects of (-)-CGP12177 might be produced through its interaction with an allosteric site at the $\beta_1$-AR. However, the density and affinity of the binding sites for (-)-[$^3$H]CGP12177, formerly attributed to the putative $\beta_4$-AR (Sarsero et al., 1999), and found in the ventricles of wild-type mice, remained in the ventricles from $\beta_1/\beta_2$-double knockout mice (Kaumann et al., 2001).

Recently, we showed (Santos and Spadari-Bratfisch, 2001) that in right atria from stressed male rats, the cardiostimulant effect of CGP12177 was not altered, whereas the classical $\beta_1$-AR isoform mediated response to noradrenaline was is desensitized. That was an indication of independent behaviors of those two ligand sites of the $\beta_1$-AR.

In this present study, we used right atria from stressed male or female rats (a model where we also observed changes in the classical $\beta_1/\beta_2$-AR) to examine if the foot-shock stress causes any

change in the chronotropic response to CGP12177 and whether this alteration is dependent of the estrous cycle or the gender.

## Material and methods

### Animals

Female (200 to 300g) or male (250 –350g) Wistar rats (*Rattus norvergicus*), were housed in standard cages in a temperature-controlled room (22°C), with a 12 h light / dark cycle with the lights on at 6:30 a.m. Standard laboratory chow and tap water were available *ad libitum*.

The estrous cycle phases were determined by vaginal smear taken every morning at 7:30 a.m., and rats presenting at least two 4-day regular cycles (proestrus, estrus, metaestrus, diestrus) were used.

During the experiments, the animals were cared for in accordance with the principles reported by Olfert and Cross (1993). The experimental protocols were approved by the animal care committee (CEEA), of the Institute of Biology, UNICAMP.

### Stress protocol

Female rats with regular cycles were individually underwent three sessions of unsignaled, inescapable foot-shock at estrus, metestrus and diestrus, or at diestrus, proestrus and estrus.

The rats were placed in a Plexiglas chamber (26 cm long x 21 cm wide x 26 cm high) provided with a grid floor consisting of stainless steel rods (0.3 cm in diameter and spaced 1.0 cm apart). During the 30 min foot-shock sessions, which occurred between 7:30 a.m. and 11:00 a.m., the shocks were delivered by a constant current source controlled by a microprocessor-based instrument constructed at the Center for Biomedical Engineering, UNICAMP. Each rat received 120 foot-shocks whit a current intensity of 1.0 mA and duration of 1.0 s at random intervals of 5-

25 s (mean interval, 15 s). The shock was scrambled by the mechanism developed at Center for Biomedical Engineering, UNICAMP. After the foot-shock session, the rats were returned to their cages or were sacrificed.

This stress protocol modifies the catecholamine sensitivity of right atria from male rats (Bassani and De Moraes, 1987; 1988) and female rats in diestrus (Marcondes et al., 1996; Vanderlei et al., 1996; Spadari-Bratfisch et al., 1999).

**Organ-bath studies**

Immediately after the last foot-shock session, the rats were sacrificed by a blow to the back of the head and exsanguined. The hearts were immediately removed and the right atria isolated and suspended in 20 ml organ baths containing Krebs-Henseleit solution of the following composition (in mM): NaCl 115.0; KCl 4.6; $CaCl_2.2H_2O$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4.7H_2O$ 2.5; $NaHCO_3$ 25.0; glucose 11.0 and ascorbic acid 0.1. The solution was warmed (36.5 ± 0.1°C) and gassed continuously with 95% $O_2$-5% $CO_2$ (pH 7.2-7.4). The atria were attached to isometric force transducers (Narco F-60, Narco Biosystems) under a resting tension of 5 mN. The tissues were allowed to stabilize for 1 h during which the bathing medium was changed at 15 min intervals.

A complete cumulative concentration-response curve (Van Rossun, 1963) to (±)- CGP12177A was obtained by stepwise increases in the concentration (0.5 log unit), in the absence of any antagonist. After this curve the preparation was washed with Krebs-Henseleit solution to remove the agonist and to allow recovery of the initial beating rate. Antagonist was then added and left in contact with the tissue for 2h before another concentration-response curve was obtained, using the same agonist, in the presence of the antagonist. Experiments to control for the desensitization

caused by obtaining two concentration-response curves to the agonist in the same tissue were also done. In this case, two concentration-response curves to the agonist were obtained in the absence of antagonist, as previously reported (Santos and Spadari-Bratfisch, 2001). CGP20712A (3 μM) was used to antagonize the effect of (±)-CGP12177A mediated by the newly proposed β-AR isoform or subtype (Kaumann and Molenaar, 1996). Propranolol (200 nM) was used to antagonize $\beta_1$- and $\beta_2$-AR mediated responses and to guarantee that the observed effect of the agonist resulted from its interaction with newly proposed β-AR isoform or subtype (Kaumann, 1996; Kaumann and Molenaar, 1996). A maximum response was reached when a 0.5 log unit increase in the agonist concentration produced no additional increase in the atrium beating frequency. The experiments ended with the administration of a saturating concentration of (-)-isoprenaline (400 μM).

**Analysis of the concentration-response curves**

Changes in the sensitivity to the agonist were evaluated by determining the concentration which produced a response that was 50% of the maximum response ($EC_{50}$). This calculation was done using the software Graph Pad Prism (GraphPad Software, San Diego, CA). The data are presented as mean negative logarithm of the $EC_{50}$ ($pD_2$) ± s.e.m.

The concentrations of agonist producing a half-maximal response in the absence [A] and presence [A'] of antagonist were estimated (Arunlakshana and Schild, 1959) as follows:

$$\log (CR - 1) = n \log [B] - \log K_B$$

where, CR is [A'] / [A], n is the slope, [B] is the concentration of the antagonist, and -log $K_B$ is the dissociation constant. The apparent molar equilibrium dissociation constant for the interaction of the antagonist with the receptor, $K_B$, was determined using the equation:

$$K_B = [B] / \log (CR - 1).$$

The dissociation constants are given as $pK_B$ values, i.e. -log $K_B$.

**In vitro pretreatment**

Concentration-response curves were obtained after incubating isolated right atria with phenoxybenzamine (10 $\mu$M) for 15 min to block α-adrenoceptors (Besse and Furchgott, 1976), extraneuronal uptake (Iversen et al., 1972) and muscarinic receptors (Furchgott and Bursztyn, 1967). This period was followed by 45 min of thorough washing. After recovery of the spontaneous rate, corticosterone (30 μM) and desipramine (0.1 μM) were added and maintained in the organ-bath throughout the experiment to inhibit extraneuronal uptake (Iversen and Salt, 1970) and neuronal uptake (Salt, 1972), respectively.

**Statistical analysis**

Statistical differences were assessed by the Student *t*-test for unpaired samples or Analysis of variance (ANOVA) plus Tukey test (Zar, 1984). Differences were considered significant at $p < 0.05$.

## Results

(±)-CGP12177A produced a positive, concentration-dependent chronotropic effect in right atria from control or stressed female rats. The atria spontaneous beating frequencies and the Hill slopes of (±)-CGP12177 concentration-response curves were similar among groups as well as the maximum responses to the (±)-CGP12177A, which were around 65% of the maximum response to isoprenaline (101 beats/min) in all groups (Table 1).

In right atria from female rats the $pD_2$ value of (±)-CGP12177 was independent of the estrus cycle phase and it was not modified after stress. However the potency of (±)-CGP12177 was lower in right atria from control or stressed female rats compared to control male rats, by around 2.0-and 3.0-fold in right atria from estrus (control and foot-shock, respectively) or diestrus female rats (Table 1; Figure 1).

In right atria from control and stressed rats sacrificed during estrus, 200nM propranolol shifted to the right the CGP12177 concentration-response curves by 7.0- and 5.1-fold respectively (Figure 2A and 2B). In right atria from control diestrus rats, propranolol caused a 13-fold shift to the right in the dose-response curve to CGP12177 (Figure 2C). The estimated propranolol $pK_B$ values were 7.40 ± 0.11 and 7.37 ± 0.04 in right atria from control or stressed rats sacrificed at estrus, and 7.70 ± 0.11 in right atria from control rats sacrificed during diestrus. Right atria from stressed female rats sacrificed at diestrus were not blocked by 200 nM propranolol so that it was not possible to estimate any propranolol $pK_B$ value in this group (Figure 2D; Table 2), as expected to occur since the response to CGP12177 as known as resistant to the propranolol blockade (Kaumann and Molennar, 1996).

Concentration-response curves to (±)-CGP12177A obtained in the presence of 3$\mu$M of CGP20712A were shifted to the right by around 30-fold in right atria from both control and stressed rats sacrificed during estrus or control rats sacrificed at diestrus (Figure 3A to 3C). Right atria from stressed rats sacrificed at diestrus were more resistant to the blockade by CGP20712A (Table 3; Figure 3D) and the antagonist $pK_B$ value (6.68 ± 0.1) was lower than that obtained in the control group (7.00 ±0.03; Table 3; $p< 0.05$; Student *t* test).

## Discussion

It has been demonstrated that foot-shock stress induced supersensitivity of the chronotropic response mediated by $\beta_2$-adrenoceptors and subsensitivity of the chronotropic response mediated by $\beta_1$-adrenoceptors in right atria from male (Bassani and De Moraes 1987, 1988) and female rats sacrificed during diestrus (Marcondes et al., 1996; Vanderlei et al., 1996; Spadari-Bratfisch et al., 1999), but not at estrus. Recently, we demonstrated that the same stress protocol did not change the chronotropic response to (±)-CGP12177 in right atria from male rats (Santos and Spadari-Bratfisch, 2001) and that this response is resistant to the propranolol blockade. These data suggested that the response to the non-conventional agonist is mediated by a receptor site which is not affected by the foot-shock stress, differently from the classical $\beta_1$-adrenoceptor, because both sites, the classical and the low affinity one, show independent behavior.

Our present data show that as well as in right atria from male rats, foot-shock stress do not change the sensitivity to (±)-CGP12177 in right atria from female rats. Nevertheless, right atria from control female rats are less sensitive to the (±)-CGP12177 chronotropic effect compared to atria from male rats and the dose-response curves to this non-conventional β-adrenoceptor agonist are shifted to the right by propranolol and CGP20712A. Both antagonists $pK_B$ values estimated in those groups (Table 2 and 3, respectively) are not compatible with the $pK_B$ value proposed for the interaction of propranolol or CGP20712A with the "putative" $\beta_4$-AR site (propranolol $pK_B < 5.7$ and CGP20712A $pK_B = 6.4$; Kaumann and Molenaar, 1996), neither for the interaction of these antagonists with the classical $\beta_1$-adrenoceptor isoform (propranolol $pK_B = 8.5$, Gille et al., 1985; CGP20712A $pK_B = 9.6$, Lemoine and Kaumann, 1991). On the other

hand, the $pK_B$ values that we estimated for these antagonists interaction with the adrenoceptor population of right atria from female rats are similar to those proposed by Kompa and Summers (1999) for the interaction with a low affinity $\beta_1$-AR isoform (propranolol $pK_B$ = 6.8 ± 0.1; CGP20712A $pK_B$= 7.1). Our data also agree with those reported by Pak and Fishman (1996) who showed that in CHW cells expressing human $\beta_1$-AR, CGP20712 antagonized the response to CGP12177 with an affinity 100 fold lower than that exhibited at $\beta_1$-AR.

Interestingly, in right atria from foot-shock stressed diestrus female rats propranolol was unable to antagonize the effect of CGP12177. Moreover, the shift induced by CGP20712A in the concentration-response curve to CGP12177 was lower in this group than it was in the other ones (Table 3). Unpublished data obtained in our laboratory showed that in right atria from rats sacrificed at diestrus, foot-shock stress decreased the affinity to CGP20712A ($pK_B$= 8.91 and 8.61, control and foot-shock, respectively; $p<0.05$) and metoprolol ($pK_B$= 8.41 and 8.02, control and foot-shock, respectively; $p<0.05$) by the $\beta_1$-AR classical isoform. Taken together those data show that the decreased affinity of the atypical $\beta_1$-AR isoform to propranolol and CGP20712A occurs in parallel with decreased affinity of the classical $\beta_1$-AR isoform.

Many other authors using tissues affected by pathological conditions (Kompa and Summer, 1999) or cell culture (Pak and Fishman, 1996; Konkar et al., 1999; Kaumann et al., 2001) showed consistent evidences that CGP12177 cardiostimulant effect is mediated by an atypical $\beta_1$-AR isoform. We show in this work, a physiological condition in which the response mediated by the low affinity $\beta_1$-AR isoform can be altered. Probably the changing hormonal profile during the estrous cycle in female rats or in those pathological conditions modulates the stability of this atypical low affinity site located at the $\beta_1$-AR.

We conclude that, the classical $\beta_1$-AR and the low affinity site located at the $\beta_1$-AR are independently regulated. Our data also show that the affinity of the atypical site located at the $\beta_1$-AR to antagonists depends on estrous cycle.

This feature of this β-AR site would open a new perspective suggesting that the β-AR could oscillate between the classical β-AR profile and the newly proposed low affinity β-AR site so that it would be possible to be found in several intermediate affinity states among those two states previously proposed.

**Table 1**

Spontaneous beating rates (BR), (±)-CGP12177 $pD_2$ values, maximum responses (MR) and concentration-effect curves Hill Slope obtained in right atria from control and foot-shock stressed male or female rats.

| Group | n[a] | BR[b] (beats/min) | $pD_2$[b] | MR[b] (beats/min) | Hill Slope[b] |
|---|---|---|---|---|---|
| **MALE** | | | | | |
| Control | 7 | 260 ± 8[a] | 7.10 ± 0.05 | 71 ± 11 | 1.33 ± 0.10 |
| **ESTRUS** | | | | | |
| Control | 12 | 247 ± 7 | 6.81 ± 0.08* | 73 ± 4 | 1.24± 0.1 |
| Foot-shock | 7 | 255 ± 10 | 6.66 ± 0.05* | 63 ± 8 | 1.27 ± 0.09 |
| **DIESTRUS** | | | | | |
| Control | 11 | 264 ± 9 | 6.81 ± 0.09* | 71 ± 5 | 1.02 ± 0.09 |
| Foot-shock | 8 | 275 ± 10 | 6.61 ± 0.09* | 61 ± 4 | 1.25 ± 0.17 |

[a]Number of experiments. [b]Means ± S.E.M. *$p<0.05$ compared with (±)-CGP12177A $pD_2$ value of in atria from control male rat (Anova and Tukey test, $p<0.05$).

**Table 2**

(±)-CGP12177 $pD_2$ values in presence of 200 nM propranolol and the $pK_B$ values for this β-antagonist in right atria from control and foot-shock stressed female rats.

| Group | n[a] | BR[b] (beats/min) | $pD_2$[b] | CR[b,c] | $pK_B$[b] |
|---|---|---|---|---|---|
| **ESTRUS** | | | | | |
| Control | 5 | 178 ± 10 | 6.00 ± 0.09* | 7.0 ± 1.1 | 7.40 ± 0.11 |
| Foot-shock | 3 | 230 ± 6.0 | 5.96 ± 0.03* | 5.1 ± 0.4 | 7.37 ± 0.04 |
| **DIESTRUS** | | | | | |
| Control | 5 | 182 ± 12 | 5.75 ± 0.10* | 13 ± 2 | 7.70 ± 0.11 |
| Foot-shock | 3 | 215 ± 23 | 6.42 ± 0.15 | 1.8 ± 1.0 | - |

[a]Number of experiments. [b]Mean ± S.E.M. [c]Ratio between $EC_{50}$ values for (±)-CGP12177 in the presence and absence of the antagonist (table 1). *$p<0.05$ compared with (±)-CGP12177 $pD_2$ values in table 1 (Student *t*-test).

**Table 3**

(±)-CGP12177 $pD_2$ values obtained in the presence of 3μM CGP20712A and the $pK_B$ values of this β-AR antagonist in right atria from control and foot-shock stressed female rats.

| Group | n[a] | BR[b] | $pD_2$[b] | CR[b,c] | $pK_B$[b] |
|---|---|---|---|---|---|
| **ESTRUS** | | | | | |
| Control | 7 | 207 ± 7 | 5.34 ± 0.07* | 31 ± 5 | 6.98 ± 0.07 |
| Foot-shock | 8 | 214 ± 12 | 5.30 ± 0.10* | 29 ± 8 | 6.88 ± 0.09 |
| **DIESTRUS** | | | | | |
| Control | 6 | 233 ± 8 | 5.30 ± 0.04* | 33 ± 2.2 | 7.00 ± 0.03 |
| Foot-shock | 4 | 253 ± 19 | 5.49 ± 0.10* | 17 ± 3.0$^{+}$ | 6.68 ± 0.1$^{+}$ |

[a]Number of experiments. [b]Values are mean ± s.e.m. [c]Ratio between EC50 values for (±)-CGP12177 in the presence and absence of the antagonist (Table 1). * $p<0.05$ compared with (±)-CGP12177 $pD_2$ values in table 1 (Student *t*-test). $^{+}p< 0.05$ compared with other groups.

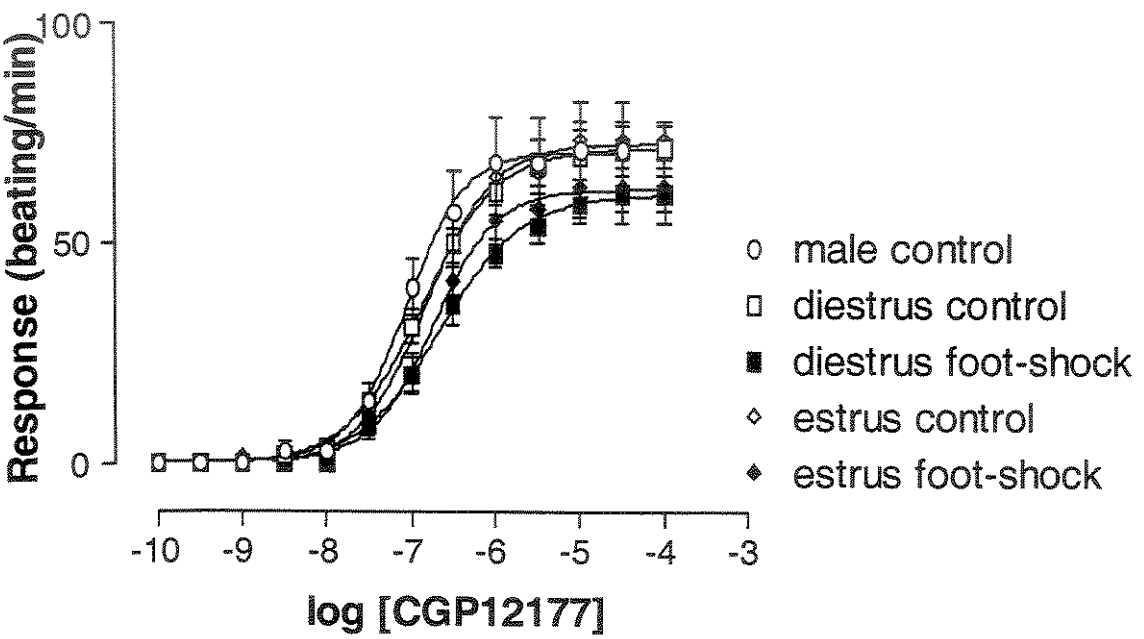


**Figure 1.** Dose-response curves to (±)-CGP12177A in right atria isolated from control male (O), control diestrus female (□), foot-shock diestrus female (■), control estrus female (◇) and foot-shock estrus female (◆).

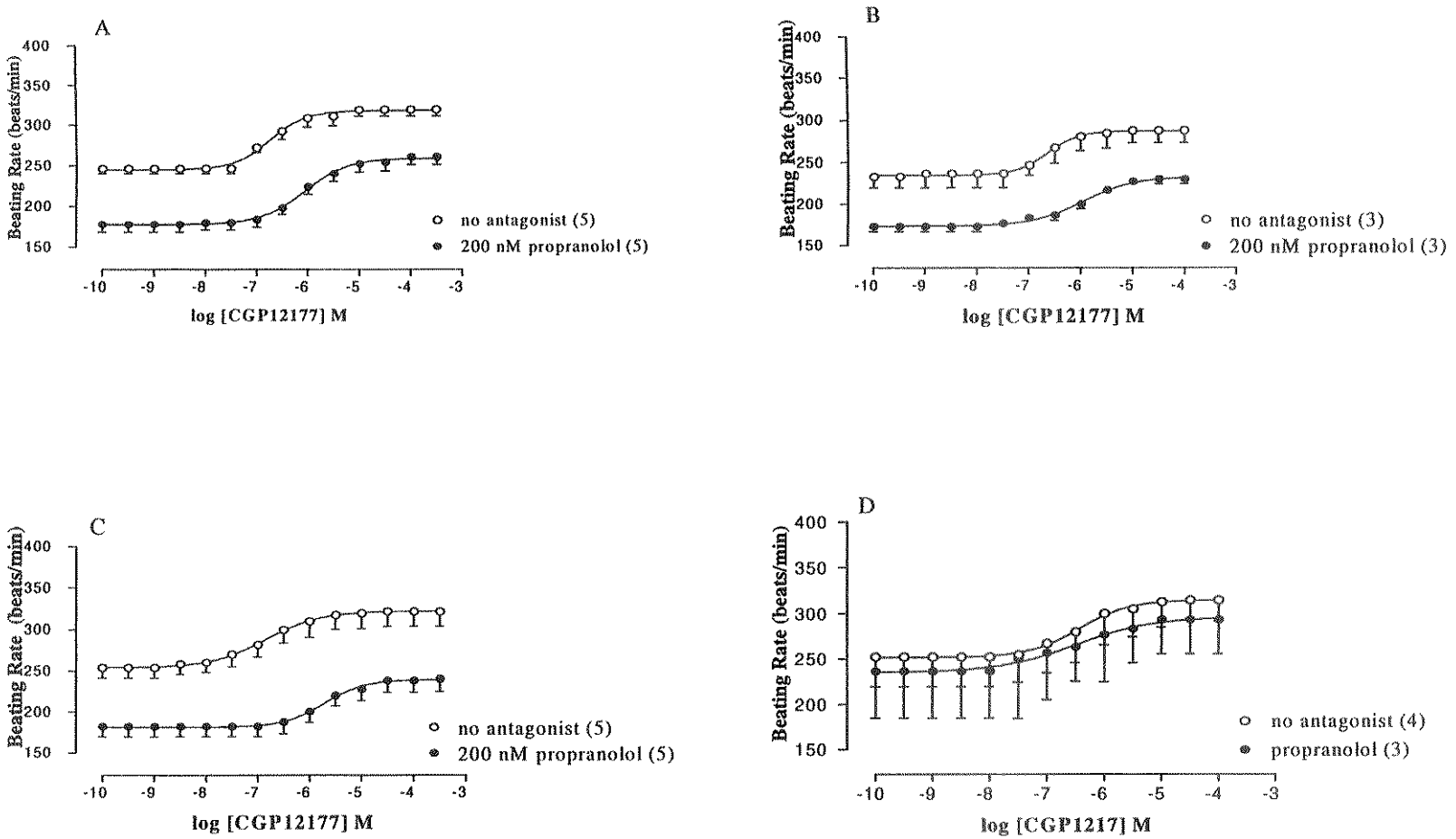


**Figure 2.** Dose-response curves to (±)-CGP12177A in right atria isolated from control (**A**) or foot-shock stressed rats sacrificed at estrus (**B**), control (**C**) or foot-shock stressed rats sacrificed at diestrus (**D**), in the absence (O) or the presence of 200nM propranolol (●).

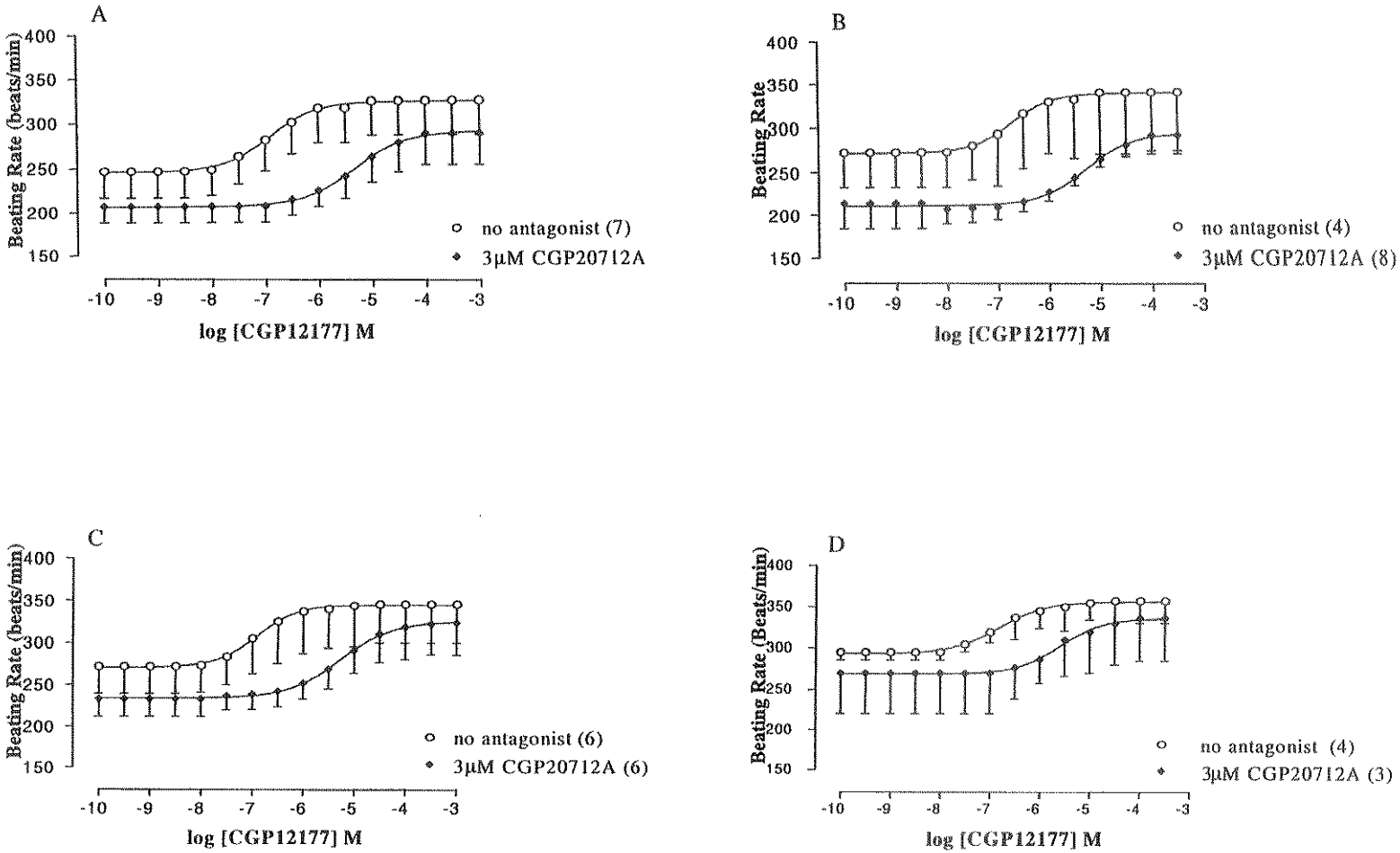


**Figure 3.** Dose response curves to (±)-CGP12177A in right atria isolated from control (**A**) or foot-shock stressed rats sacrificed at estrus (**B**), control (**C**) or foot-shock stressed rats sacrificed at diestrus (D), in the absence (O) or the presence of 3$\mu$M CGP20712A (◆).

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# CHRONOTROPIC RESPONSE TO (±)-CGP12177 IN RIGHT ATRIA OF SINOAORTIC DENERVATED RATS

**Santos, I.N.; Sumitani M.; Krieger, M. H. & Spadari-Bratfisch, R. C.**

Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil

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**Running title**: SAD and atrial sensitivity to CGP12177

## Abstract

The existence of a fourth subtype of β-adrenoceptor (β-AR) has been proposed and it still is a matter of controversy. In sinoaortic denervated (SAD) rats we demonstrated that right atria shows subsensitivity to $\beta_1$-agonists. Now, we investigated whether the cardiostimulant effect of (±)-CGP12177 (proposed to be mediated by $\beta_4$-AR or atypical states of $\beta_1$-AR) was altered after SAD. Right atria isolated from rats 48h after SAD surgery were subsensitive to isoprenaline, norepinephrine and (±)-CGP12177. In right atria from 48h SAD the concentration-response curve to the (±)-CGP12177 was shifted to the right by 200nM propranolol (7.4-fold; $pK_B$=7.50 ± 0.05) or 3 μM CGP20712A (4.6-fold; $pK_B$= 5.97 ± 0.11). In right atria from control rats, the curve to (±)-CGP12177 was resistant to the blockade by (±)-propranolol, or to blockade by 10nM CGP20712A plus 50nM ICI118,551. In right atria isolated from rats one week after SAD, there was a dichotomy between sensitivity to agonists. The tissue was subsensitivity to isoprenaline and norepinephine but not to (±)-CGP12177A. The effect of the CGP12177 was not affected by propranolol. These results suggest that shortly after SAD, an adaptive response involving all β-AR subtypes is triggered, causing subsensitivity to agonists. Nevertheless, one week after SAD, the putative $\beta_4$-AR subtype and/or the low-affinity $\beta_1$-AR isoform recovers sensitivity, whereas the classical $\beta_1$ and $\beta_2$-AR subtypes mediated responses were still subsensitive. Again, as it was demonstrated in right atria from stressed rats, the classical $\beta_1$-AR and the site activated by (±)-CGP12177 showed independent behaviour.

## Introduction

Cardiac tissues contain $\beta_1$- and $\beta_2$-adrenoceptors (Carlson et al., 1972). In the rat the chronotropic and inotropic responses to neurally released and circulating norepinephrine are mediated by the $\beta_1$-adrenoceptor ($\beta_1$-AR) subtype (Juberg et al., 1985). However, it has been proposed that the chronotropic response to high concentrations of epinephrine could be mediated also by $\beta_2$-adrenoceptors ($\beta_2$-AR; Kaumann, 1986).

Kaumann (1989) proposed that some compounds, termed "non-conventional agonists", may mediate their effects in cardiac tissue through a β-adrenoceptor (β-AR) distinct from $\beta_1$-AR and $\beta_2$-AR, and suggested that it should be named "putative $\beta_4$-adrenoceptor" ($\beta_4$-AR). The cardiostimulant effect of (-)-CGP12177A, a non-conventional agonist, is unaffected by the $\beta_1$ and $\beta_2$-AR antagonist (-)-propranolol (200 nM) but is blocked with moderate affinity by (-)-bupranolol and CGP20712A (Kaumann and Molenaar, 1996, 1997). A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), potentiated the effect of (-)-CGP12177 in rat right and left atria (Kaumann and Lynham, 1997), suggesting $G_s$-protein coupling of the putative $\beta_4$-AR to adenylate cyclase. (-)-[$H^3$]-CGP12177 has been also used as a radioligand for putative $\beta_4$-AR in rat atrium (Sarsero et al., 1998).

Despite the evidence from functional and binding studies indicating the existence of putative $\beta_4$-AR, it was demonstrated that the responses originally attributed to putative $\beta_4$-AR may also be produced by $\beta_1$-AR. In hamster fibroblasts (CHW) expressing the human $\beta_1$-AR, (-)-CGP12177A acted as a low potency agonist. Based on an experimental model of failing heart produced by myocardial infarction, Kompa and Summers (1999) proposed that the properties attributed to the putative $\beta_4$-AR may be explained by an interaction of the agonist with a low

affinity state of the $\beta_1$-AR or that $\beta_1$-AR and the putative $\beta_4$-AR use the same signalling pathway. Lowe et al. (1999) also argued that the putative $\beta_4$-AR could correspond to a low affinity state of $\beta_1$-AR. Since a $\beta_4$-AR gene has not yet been identified and that a cardiac response to CGP12177 could not be elicited in $\beta_1/\beta_2$-AR double knockout mice (Kaumann et al., 2001) the response to CGP12177 seems to be definitively attributed to a low affinity site located at the $\beta_1$-AR.

We have previously shown that right atria from stressed rats have lower sensitivity to the chronotropic effects of norepinephrine and a decreased affinity for $\beta_1$-AR selective antagonists. This subsensitivity was accompanied by an increase in the sensitivity to non-selective $\beta$-AR agonists (Bassani and De Moraes, 1987; Marcondes et al., 1996; Vanderlei et al., 1996). Moreover, right atria from stressed but not from control rats responded to nanomolar concentrations of TA2005, a $\beta_2$-AR selective agonist, and these responses were abolished by ICI118,551(50 nM). Based on these data we suggested that foot-shock stress induces a $\beta_2$-AR subtype mediated response in rat right atria simultaneously with a decrease in the response mediated by $\beta_1$-AR (Spadari-Bratfisch et al., 1999). In this stress model, the response to CGP12177 was not modified suggesting that the response is mediated by a different receptor from a low affinity state of $\beta_1$-AR, as previously proposed, unless both proposed isoforms of $\beta_1$-AR show independent behavior (Santos and Spadari-Bratfisch, 2001).

Sinoaortic denervation (SAD) disrupts baroceptor-mediated regulation of heart rate and blood pressure (Krieger, 1964), causing tachycardia and labile hypertension (Vasquez and Krieger, 1980). Although blood pressure lability persists for months, tachycardia is transient and usually returns to near control rates within 2 weeks after SAD. During this period, right atria are subsensitive to $\beta$-AR agonists due to a down regulation of $\beta_1$-AR (Zanesco et al., 1997).

In this study, we examined the chronotropic response to (±)-CGP12177A in right atria from SAD rats to determine whether the response to this non-conventional agonist was modified in parallel to the $\beta_1$-AR down-regulation.

## Methods

### Animals

Male Wistar rats (*Rattus norvergicus*) 300 to 350 g, were housed in standard cages in a temperature-controlled room (22°C), with a 12 h light / dark cycle with the lights on at 6:30 a.m. Standard laboratory chow and tap water were available *ad libitum*.

During the experiments, the animals were cared for in accordance with the principles reported by Olfert and Cross (1993) and the experimental protocols were approved by the animal care committee (CEEA), of the Institute of Biology, UNICAMP.

### Surgical procedures

All surgical procedures were performed under aseptic conditions and were carried out under anesthesia produced by ketamine, 50mg/Kg, and xylazine, 5mg/Kg, i.m. Bilateral SAD was performed as described by Krieger (1964). Briefly, after the induction of anesthesia, the external and internal branches of the carotid arteries were exposed. The vagas nerve, the sympathetic trunk and surrounding connective tissue were gently dissected away from the vessels, the superior laryngeal nerve was resected and a section of the sympathetic trunk removed. Sham surgery consisted of the same proceduresused to expose and free the arteries, but without denervation.

After SAD or sham surgery, catheters containing sterile saline were placed in the left femoral vein and artery for the subsequent administration of drugs and recording blood pressure and heart rate. The vein and artery were catheterized using sterile PE-50 and PE-10 tubing, respectively.

Catheters were exteriorized at the dorsal neck region. Following surgery, all animals were treated with benzathine penicillin, 100,000 U, i.m., to minimize infections.

Twenty-four hours before the sacrifice, the efficacy of baroceptor deafferentation was evaluated. The animals received an intravenous injection of phenylephrine (1,5 – 3.0 ug/Kg) to elicit at least a 50 mmHg increase in arterial pressure. Animals were considered to have an adequate denervation of baroreceptors if the subsenquent maximal decrease in heart rate was less than 30 beats/mim. The bradycardia typically observed in sham or controls was 60 – 120 beats/mim after the same dose of phenylephrine.

**Organ-bath studies**

The rats were killed by a blow to the back of the head and exsanguinated. The hearts were immediately removed and the right atria isolated and suspended in 20 ml organ baths containing Krebs-Henseleit solution of the following composition (in mM): NaCl 115.0; KCl 4.6; $CaCl_2.2H_2O$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4.7H_2O$ 2.5; $NaHCO_3$ 25.0; glucose 11.0 and ascorbic acid 0.1. The solution was warmed (36.5 ± 0.1°C) and gassed continuously with 95% $O_2$-5% $CO_2$ (pH 7.2-7.4). The atria were attached to isometric force transducers (Narco F-60, Narco Biosystems, Houston, Texas, USA) under a resting tension of 5 mN. The tissues were allowed to stabilize for 1 h during which the bathing medium was changed at 15 min intervals.

Complete cumulative concentration-response curves (Van Rossum, 1963) to isoprenaline, norepinephrine and (±)- CGP12177A were obtained by stepwise increases in the concentration (0.5 log unit), in the absence of any antagonist. After this curve the preparation was washed with Krebs-Henseleit solution to remove the agonist and to allow recovery of the initial beating rate. Antagonist was then added and left in contact with the tissue for 2h before another

concentration-response curve was obtained, using the same agonist, in the presence of antagonist. CGP20712A (1 and 3 μM) was used to antagonize the effect of (±)-CGP12177A mediated by $\beta_4$-AR (Kaumann and Molenaar, 1996). Propranolol (200 nM) or CGP20712A (10 nM) plus ICI118,551 (50 nM) were used to antagonize $\beta_1$- and $\beta_2$-AR mediated responses and to guaranteethat the observed effect of the agonist resulted from its interaction with $\beta_4$-AR (Kaumann, 1996; Kaumann and Molenaar, 1996; Kompa and Summers, 1999). A maximum response was reached when a 0.5 log unit increase in the agonist concentration produced no additional increase in atrial beating frequency. The experiments ended with the administration of a saturating concentration of (-)-isoprenaline (400 μM).

**Analysis of the concentration-response curves**

Changes in the sensitivity to the agonist were evaluated by determining the concentration which produced a response that was 50% of the maximum response ($EC_{50}$). This calculation was done using the software Graph Pad Prism (GraphPad Software, San Diego, CA). The data are presented as mean negative logarithm of the $EC_{50}$ ($pD_2$) ± s.e.m.

The concentrations of agonist producing a half-maximal response in the absence [A] and presence [A'] of antagonist were estimated (Arunlakshana and Schild, 1959) as follows:

$$\log (CR - 1) = n \log [B] - \log K_B$$

where CR is [A'] / [A], n is the slope, [B] is the concentration of the antagonist, and -log $K_B$ is the antagonist dissociation constant. The apparent molar equilibrium dissociation constant for the interaction of the antagonist with the receptor, $K_B$, was determined using the equation:

$$K_B = [B] / \log (CR - 1).$$

The dissociation constants are given as $pK_B$ values, i.e. -log $K_B$.

**In vitro pretreatment**

Concentration-response curves were obtained after incubating isolated right atria with phenoxybenzamine (10 $\mu$M) for 15 min to block $\alpha$-adrenoceptors (Besse and Furchgott, 1976), extraneuronal uptake (Iversen et al., 1972) and muscarinic receptors (Furchgott and Bursztyn, 1967). This period was followed by 45 min of thorough washing. After recovery of the spontaneous rate, corticosterone (30 $\mu$M) and desipramine (0.1 $\mu$M) were added and maintained in the organ-bath throughout the experiment to inhibit extraneuronal uptake (Iversen and Salt, 1970) and neuronal uptake (Salt, 1972), respectively.

**Statistical analysis**

Statistical differences were assessed by Student's *t*-test for unpaired samples or ANOVA plus Tukey test (Zar, 1984). Differences were considered significant at $p < 0.05$.

## Results

The concentration response parameters for isoprenaline, norepinephrine and CGP12177 in isolated right atria obtained from control rats and from rats that have been submitted to SAD surgery are summarized in Table 1. The spontaneous beating frequency in right atria from 48h SAD rats was not significantly different from those seen in right atria from control (Table 1), but right atria of 1 week SAD rats exhibited a decrease in spontaneous beating rate ($p<0.05$), as previously reported (Zanesco et al., 1997). Right atria from rats submitted to sham-surgery (both 48 h or 7 days after surgery) presented $pD_2$ values of isoprenaline (8.77 ± 0.16; 8.72 ± 0.10, respectively), norepinephrine (7.28 ± 0.09; 7.42 ± 0.08, respectively) and (±)-CGP12177 (6.85 ± 0.10) similar to control (Table 1). Isoprenaline and norepinephrine potencies, but not efficacies, were also significantly lower in 48h and 1week SAD rats right atria when compared to control (Table 1).

The concentration-response curves to (-)-isoprenaline and (-)-norepinephrine are shown in Figures 1A and 1B. The Hill slope of isoprenaline concentration-response curves was not significantly altered in 48h or 1 week SAD rats right atria (Table 1). However, there was an increase in the Hill slope of the norepinephrine concentration-response curve in right atria from 48 h SAD rats. (±)-CGP12177 produced a positive, concentration-dependent chronotropic effect in right atria from control rats (Figure 1C), with a maximum response around 53% of the maximum response to isoprenaline or norepinephrine. (Table 1). The efficacy of (±)-CGP12177A as well as the Hill slopes of the respective concentration-response curves were lower in right atria isolated from rats sacrificed 48h after SAD compared to control, sham or 1 week SAD rats (Table 2; Figure 1C). The potency of (±)-CGP12177A was lower in right atria from rats sacrificed 48h after SAD compared to control or sham groups.

The concentration-response curves to (±)-CGP12177 were resistant to the blockade by 200 nM propranolol in right atria from control (Figure 2A, Table 2) and 1 week SAD rats (Figure 2C, Table 2). Propranolol did not cause any change in the spontaneous beating rate of right atria from control or 1 week SAD rats (Table 2 versus Table 1), but decreased in 33% ($p < 0.05$) the spontaneous beating rate of right atria from 48h SAD rats. This caused a steeper (Hill slope: 1.82 ± 0.22) concentration-response curve to (±)-CGP12177 in this group, when compared with the curve obtained in the absence of this antagonist (Hill slope: 0.66 ± 0.06). Concentration-response curve to (±)-CGP12177 was 7.41-fold shifted to the right by 200nM propranolol (Table 2; Figure 2B). Concentration response curves to (±)-CGP12177A were also obtained in the presence of 10 nM CGP20712A plus 50 nM ICI118,551. In these concentrations the compounds antagonize $\beta_1$- and $\beta_2$-AR, respectively (Dooley et al., 1986; O'Donnell and Wanstall, 1980). The right atria from control or 48h SAD rats showed a decrease of 17% ($p < 0.05$) in spontaneous beating rate (Table 2 versus Table 1), and the concentration-response curves to (±)-CGP12177 were resistant to the blockade by CGP20712A plus ICI118,551 (Figure 3). The concentration-effect curves for the antagonism of the (±)-CGP12177 chronotropic effect by CGP20712A are shown in Figure 4. The spontaneous beating rates of right atria from control or 1 week SAD rats were not altered by the antagonist dose of 1μM (Table 3 versus Table 1). However 3μM CGP20712A significantly decreased ($p<0.05$) the spontaneous beating rate in 14% and 22%, respectively, and caused a significantly dose-dependent shift to the right in the dose-response curve to CGP12177 (Figure 4). Because the basal beating rate was lower and the maximum response to the agonist was not altered the dose-response curves to (±)-CGP12177 were steeper in the presence of propranolol, CGP20712A plus ICI118,551 or 3μM CGP20712A (Table 2 and 3, respectively).

**Discussion**

Our results confirm that right atria isolated from rats sacrificed 1 week after sinoaortic denervation were subsensitive to the chronotropic effects of isoprenaline or norepinephrine. This subsensitivity was previously atributed to $\beta_1$-AR down-regulation (Zanesco et al., 1997). Our results have also shown that the sensitivity to (-)-CGP12177 was not altered in right atria from 1 week SAD rats. The chronotropic response to CGP12177 has been proposed to be mediated by the putative $\beta_4$-AR (Kaumann and Molenaar, 1996; 1997). These authors have described that the response mediated by putative $\beta_4$-AR is resistant to the blockade by the classical $\beta_1$-and $\beta_2$-AR antagonist, propranolol; and it is blocked by the $\beta_1$-AR antagonist CGP20712A and by bupranolol (in a concentration range that these compounds interacts with the putative $\beta_4$-AR). In spite of evidences from functional and binding studies pointing to the existence of putative $\beta_4$-AR there are also evidences that the responses to non-conventional agonists might be produced by $\beta_1$-AR (Pak and Fishman, 1996; Konkar et al., 2000). The response to CGP12177 in an experimental model of failing heart produced by myocardial infarction, had been proposed to be in fact mediated by a low affinity site located on the $\beta_1$-AR (Kompa and Summers, 1999). More recently, Kaumann et al (2001) demonstrated that the cardiostimulant effect of (-)-CGP12177 was absent in $\beta_1/\beta_2$-adrenoceptor double knockout mice. This result reinforced the hypothesis that the agonistic effects of (-)-CGP12177 might be produced through its interaction with an allosteric site at the $\beta_1$-AR. However, the density and affinity of the binding sites for (-)-[$^3$H]CGP12177, formerly attributed to the putative $\beta_4$-AR (Sarsero et al., 1999), and found in the ventricles of wild-type mice, remained in the ventricles from $\beta_1/\beta_2$-double knockout mice (Kaumann et al., 2001).

Our present results have shown that the interaction of CGP12177 with the β-AR population in right atria from control or 1 week SAD rats was resistant to the propranolol blockade, a behaviour which is consistent with the properties attributed to the putative $\beta_4$-AR subtype (Kaumann and Molenaar, 1996; 1997). Moreover, in this model, the dichotomy between sensitivity to isoprenaline and norepinephrine versus CGP12177 suggest that those responses are mediated by two different adrenoceptor populations, or that both, the high and the low affinity sites at the $\beta_1$-AR show different behaviour. Similar results have been shown in right atria isolated from stressed rats (Santos and Spadari-Bratfisch, 2001).

However, in right atria isolated from 48h sinoaortic denervated rats the picture seems to be quite different, since tissue is subsensitivity to the full agonists isoprenaline and norepinephrine, and also to the non-conventional partial agonist (±)-CGP12177. Moreover, 200 nM (±)-propranolol caused a significant rightward shift in the concentration-response curve to (±)-CGP12177, which allowed the estimation of a $pK_B$ value for propranolol (7.50 ± 0.05). This value was higher than that reported for the interaction of propranolol with the putative $\beta_4$-AR ($pK_B < 5.7$; Kaumann and Molenaar, 1996) and it was also higher than the value obtained by Kompa and Summers (1999) in right atria from rats with myocardial infarction (6.8 ± 0.1). On the other hand the propranolol $pK_B$ value we obtained was between the $pK_B$ value for propranolol interacting with the classical $\beta_1$-AR isoform ($pK_B = 8.5$; Gille et al., 1985) and at the $\beta_1$-AR novel state proposed by Konkar et al (2000, $pK_B = 7.2$ ) in CHO cells expressing only $\beta_1$-AR.

Analysis of the concentration-effect curves can be done taking into account not only an estimation of the location parameter and upper asymptote to describe potency and intrinsic activity, respectively, but also the estimates of the steepness of the concentration-response curves, which can also provide critical information for quantitative interpretation of the drug

response interaction (Van der Graaf and Schoemaker, 1999). The efficacy of the partial agonist (±)-CGP12177 as well as the slope of the dose-response curve in right atria from rats sacrificed 48h after SAD were lower compared to control or 1 week SAD rats. The Hill slope of the (±)-CGP12177 concentration-response curve is also lower in right atria from 48h SAD rats, which is consistent with agonist profile that interact with two receptor sites which present different coupling efficiency (Kenakin, 1999). The antagonist-induced curve steepening is predict by two-receptor/transducer models (Kenakin, 1993; Van der Graaf et al., 1999), as we have shown to occur in the concentration-response curves to CGP12177 obtained in the presence of propranolol or CGP20712A (Table 4). Parallel to that, in right atria from 48h SAD rats, CGP20712A pKB value decreased compared to control, which suggests that this antagonist is blocking more than one receptor site.

Taken together, our results suggest that shortly after SAD, when catecholamines levels are high (Alexander et al., 1980) an adaptive response is triggered involving all β-AR subtypes and causing subsensitivity to agonists. At this time, CGP12177 seems to interact with two receptor sites. Nevertheless, one week after SAD, when the right atria intrinsic beating rate is lower than control, $\beta_4$-AR and/or low affinity site of $\beta_1$-AR recover whereas the classical $\beta_1$-AR mediated responses are still subsensitive. Again, as it was demonstrated in right atria from stressed rats (Santos and Spadari-Bratfisch, 2001) the classical $\beta_1$-AR and the site activated by CGP12177 showed independent behaviour.

**Table 1**

Spontaneous beating rate (BR), isoprenaline, norepinephrine and (±)-CGP12177 $pD_2$ values, maximum response (MR) and concentration-effect curves Hill slope obtained in right atria from control and sinoaortic denervated (SAD) rats.

| | n[a] | BR[b] | $pD_2$[b] | MR[b] | Hill Slope[b] |
|---|---|---|---|---|---|
| **ISOPRENALINE** | | | | | |
| *CONTROL* | 4 | 283 ± 8 | 8.75 ± 0.08 | 140 ± 21 | 0.92 ± 0.07 |
| *48h SAD* | 3 | 267 ± 9 | 8.44 ± 0.03* | 100 ± 10 | 1.24 ± 0.14 |
| *1week SAD* | 5 | 232 ± 7*$^{+}$ | 8.21 ± 0.09* | 146 ± 11 | 1.06± 0.06 |
| **NOREPINEPHRINE** | | | | | |
| *CONTROL* | 7 | 290 ± 6 | 7.56 ± 0.13 | 146 ± 10 | 0.79 ± 0.11 |
| *48h SAD* | 4 | 270 ± 11 | 7.05 ± 0.04* | 105 ± 19 | 1.18 ± 0.08*$^{\#}$ |
| *1week SAD* | 5 | 220 ± 6*$^{+}$ | 7.11 ± 0.08* | 150 ± 8 | 0.70 ± 0.06 |
| **CGP12177** | | | | | |
| *CONTROL* | 7 | 260 ± 8 | 7.10 ± 0.10 | 71 ± 11 | 1.31 ± 0.09 |
| *48 h SAD* | 6 | 235 ± 9 | 6.45 ± 0.07* | 35 ± 5$^{*}$ | 0.66 ± 0.06* |
| *1 week SAD* | 6 | 210± 11$^{+}$ | 6.95 ± 0.08 | 55 ± 8 | 1.34 ± 0.24 |

[a]Number of experiments. [b]Mean ± S.E.M. *$p<0.05$ compared to control. $^{+}p,0.05$ compared to 48 h SAD group. $^{\#}p<0.05$ compared to 1 week sad group (Anova plus Tukey test).

**Table 2**

(±)-CGP12177 $pD_2$ values obtained in the presence of 200 nM propranolol or 10 nM CGP20712A plus 50 nM ICI118,551 in right atria from control and sinoaortic denervated rats.

| | n[a] | BR[b] (beats/min) | $pD_2$[b] | Hill Slope[b] |
|---|---|---|---|---|
| **PROPRANOLOL** | | | | |
| *CONTROL* | 3 | 280 ± 6 | 6.89 ± 0.05 | 0.98 ± 0.05 |
| *48h SAD* | 4 | 158 ± 5[#] | 5.58 ± 0.04*[+] | 1.82 ± 0.22[#] |
| *1week SAD* | 4 | 175 ± 10 | 7.25 ± 0.10* | 1.17 ± 0.34 |
| **CGP20712A+ICI118,551** | | | | |
| *CONTROL* | 4 | 208 ± 8[#] | 7.10 ± 0.2 | 0.73 ± 0.06 |
| *48h SAD* | 4 | 200 ± 4[#] | 7.47 ± 0.1 | 1.98 ± 0.23* |

[a]Number of experiments. [b]Mean ± S.E.M. [#]$p<0.05$ compared to CGP12177 concentration-response curve in the absence of antagonist (Table 1; Student *t*-test). *$p<0.05$ compared to control. [+]$p<0.05$ compared to 1week SAD group (Anova plus Tukey test).

**Table 3**

(±)-CGP12177 $pD_2$ value in the presence of CGP20712A and $pK_B$ values for this β-adrenoceptor antagonist in right atria from control and sinoaortic denervated rats.

| | n[a] | BR[b] (beats/min) | $pD_2$[b] | CR[b,c] | $pK_B$[b] | Hill coeficient[b] |
|---|---|---|---|---|---|---|
| **1 μM CGP20712A** | | | | | | |
| *CONTROL* | 4 | 243 ± 8 | 6.39 ± 0.11[#] | 5.3 ± 1.3 | 6.63 ± 0.14 | 1.01 ± 0.11 |
| *48h SAD* | 6 | 217 ± 10 | 6.24 ± 0.09 | 2.0 ± 0.3[*] | 5.97 ± 0.11[*] | 1.08 ± 0.06 |
| *1week SAD* | 3 | 193 ± 19 | 5.93 ± 0.10[#] | 10.0 ± 4.0 | 6.97 ± 0.16 | 0.96 ± 0.20 |
| **3 μM CGP20712A** | | | | | | |
| *CONTROL* | 7 | 223 ± 8[#] | 5.48 ± 0.07[#] | 43 ± 4.8 | 7.10 ± 0.07 | 1.22 ± 0.08 |
| *48h SAD* | 3 | 203 ± 3 | 5.42 ± 0.03[#] | 9.1 ± 0.5[*] | 6.48± 0.03[*] | 3.68 ± 0.04[*] |
| *1week SAD* | 4 | 163 ± 10[#] | 5.55 ± 0.20[#] | 23 ± 9.0 | 6.90 ± 0.20 | 0.90 ± 0.03 |

[a]Number of experiments. [b]Mean ± S.E.M. [c]Ratio between $EC_{50}$ values for (±)-CGP12177 in the presence and absence (Table 1) of the antagonist. [#]$p<0.05$ compared to CGP12177 concentration-response curve in the absence of antagonist (Table 1; Student *t*-test). [*]$p<0.05$ compared with control or SAD 1 week group (Anova plus Tukey test).

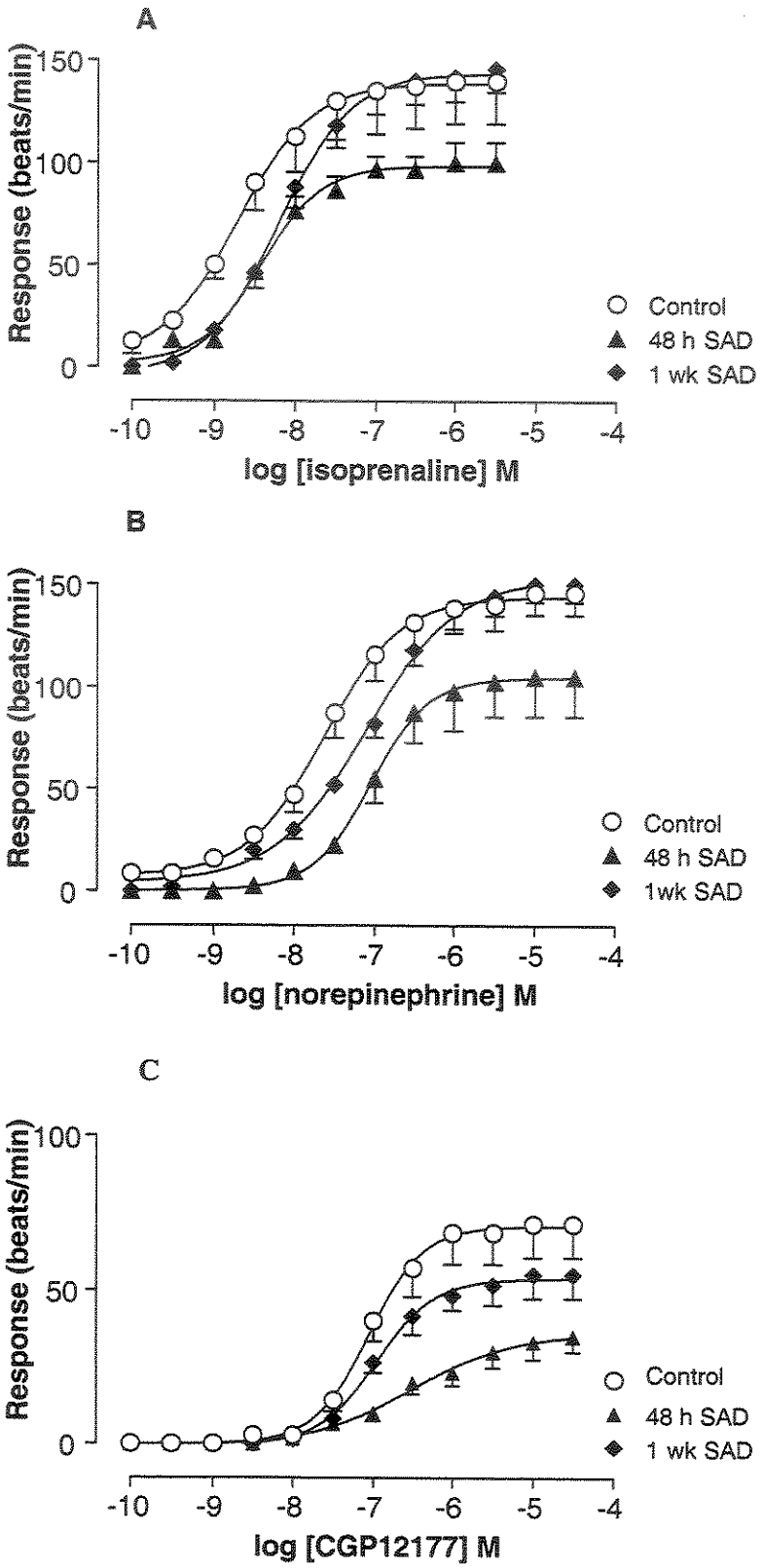
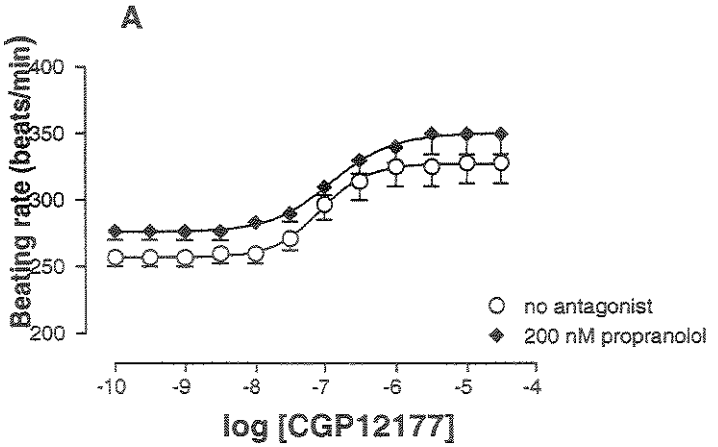
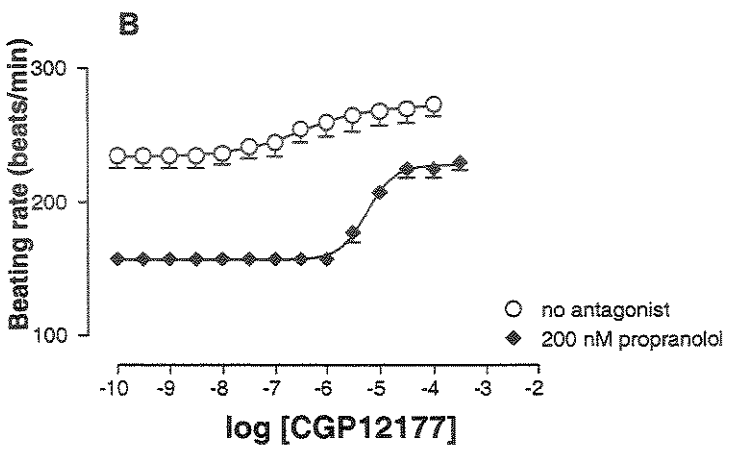



**Figure 1.** Concentration-response curves to isoprenaline (A), norepinephrine (B) and (±)-CGP12177A (C), in right atria isolated from control rats (○) and 48 h (▲) or 1 week after sinoaortic denervation (◆).



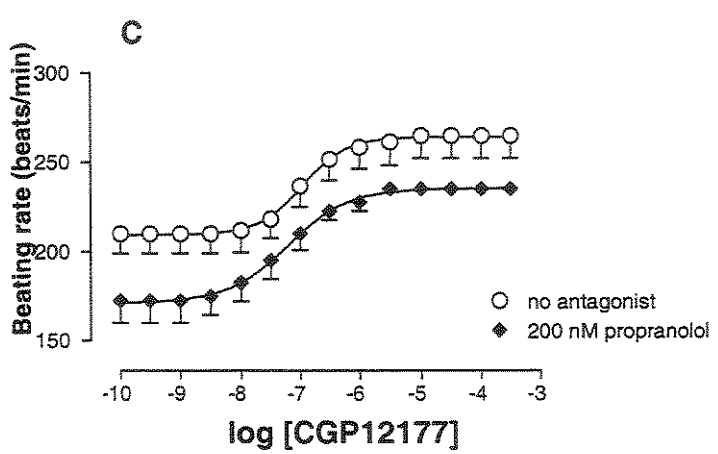




**Figure 2.** Concentration -response curves to (±)-CGP12177A in right atria isolated from control (A), 48 h SAD (B) and 1 week SAD rats (C), in the absence (○) or presence of 200 nM propranolol (◆).

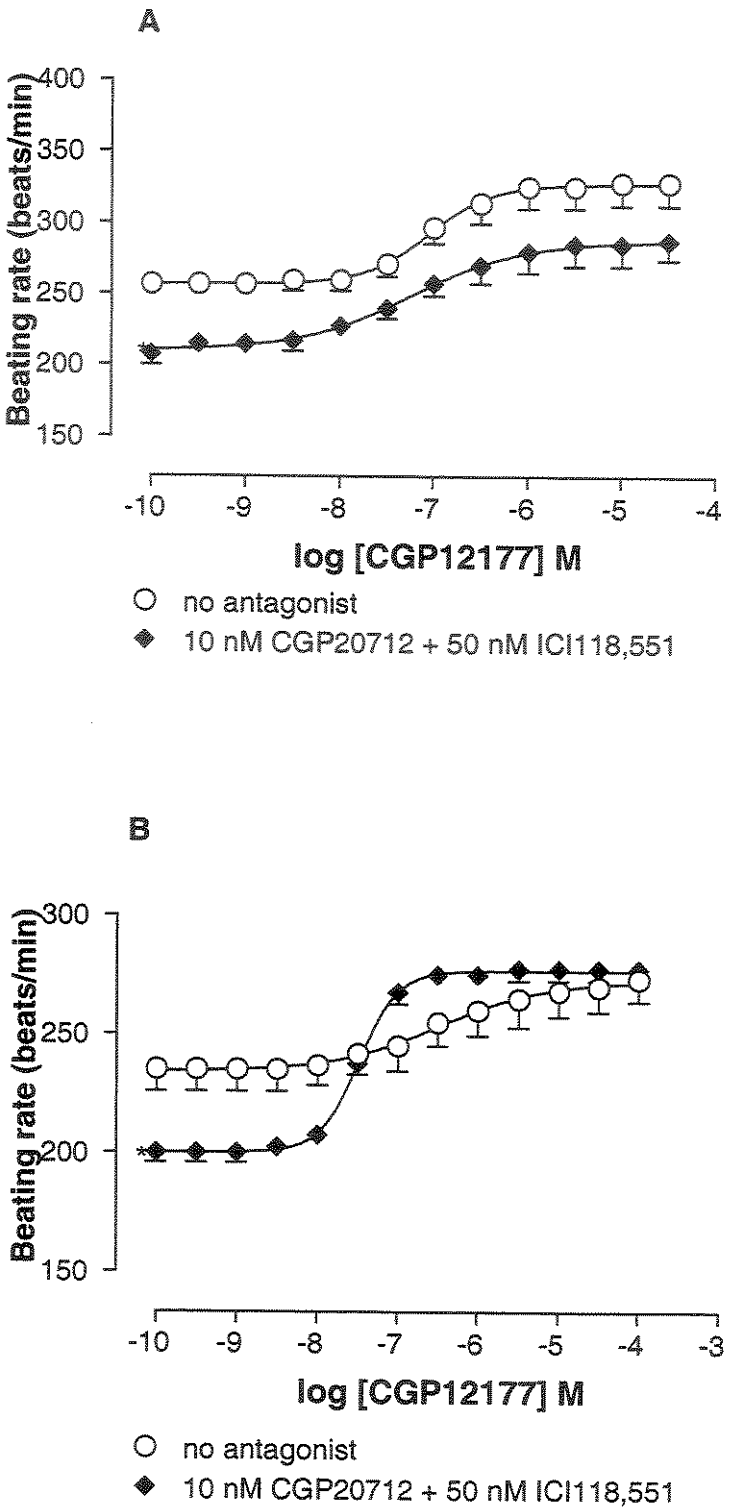



**Figure 3**. Concentration-response curves to (±)-CGP12177A in right atria isolated from control (A), 48 h SAD (B) in the absence (O) or presence of (◆) 10 nM CGP20712 + 50nM ICI118,551.

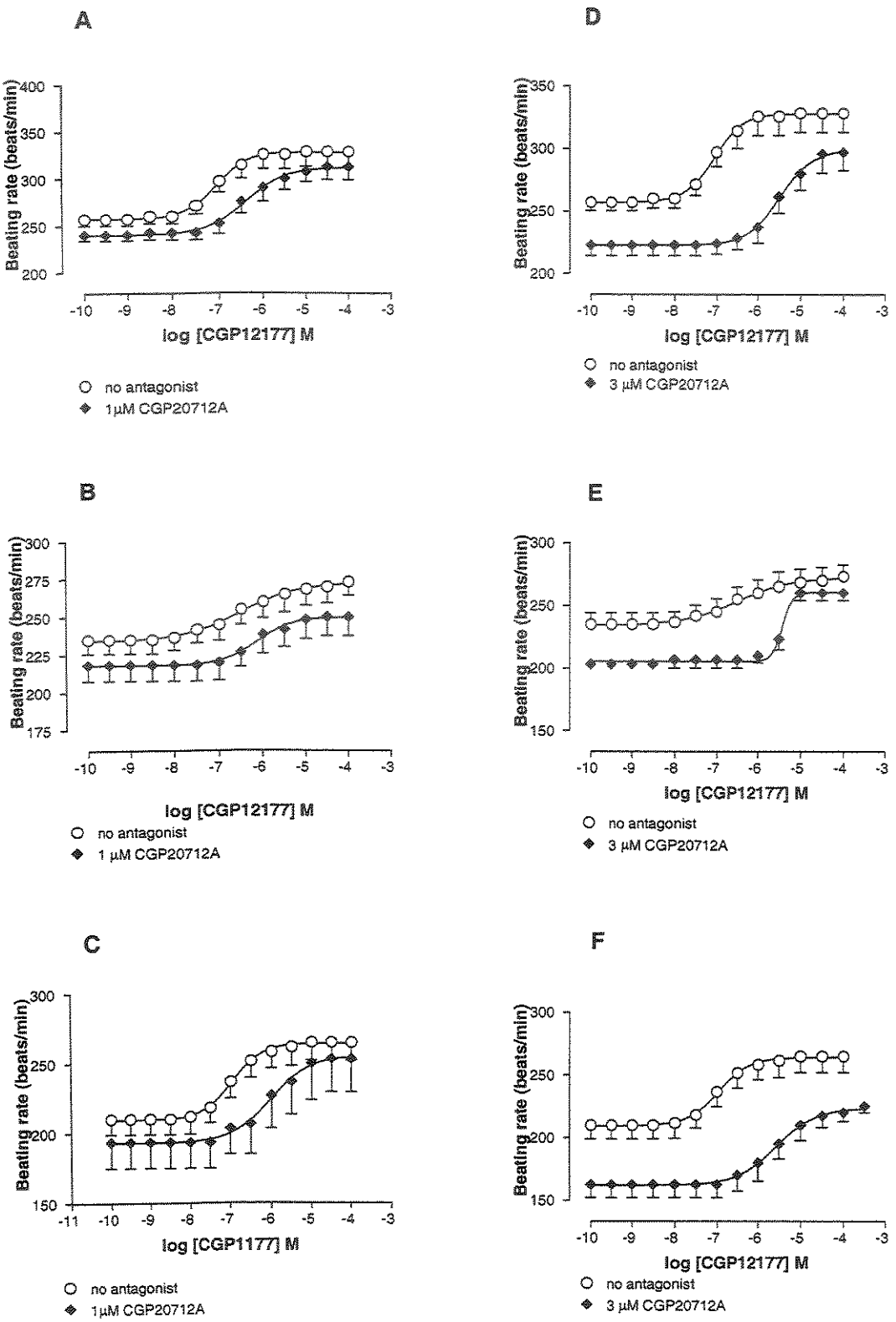



**Figure 4**. Concentration-response curves to (±)-CGP12177A in right atria isolated from control (A and D), 48 h SAD (B and E) and 1 week SAD rats (C and F), in the absence (○) or presence of (◆)1$\mu$M or 3$\mu$M CGP20712

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# EFFECT OF FOOT-SHOCK STRESS ON GLUCOCORTICOID RECEPTOR AND β-ADRENOCEPTOR PROTEINS IN RIGHT ATRIA FROM RATS

**Santos, I. N.[1]; Souza, E. P. M.[1], Grassi-Kassisse, D. M.[1], Marcondes, F.K[2].; Sonir, A.[3]; Castro, M.[3], & Spadari-Bratfisch, R. C.[1]**

[1] Laboratory of stress study (Labeest), Department of Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

[2] Department of Physiological Science, Dental School of Piracicaba, State University of Campinas (UNICAMP), Piracicaba, SP, Brazil.

[3] Department of Physiology, Faculty of Medical Sciences of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil.

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**Running title**: glucocorticoid receptor and β-adrenoceptor protein levels in atria from stressed rats.

**Key words:** atria, stress, β-adrenoceptor, corticosterone, glucocorticoid receptor

**Abstract**

The stress reaction is characterized by increased serum catecholamines and glucocorticoids levels. The responses to catecholamines are mediated by heptahelical G-protein coupled receptors known as β-adrenergic receptors (βAR) whereas glucocorticoids signals by binding to intracelular receptors (GR). Right atria from rats submitted to foot-shock stress show supersensitivity to isoprenaline (non-selective $\beta_1/\beta_2$AR agonist) and TA2005 (selective $\beta_2$AR agonist) together with subsensitivity to noradrenaline (selective $\beta_1$AR agonist). Stressed female rats sacrificed during estrus do not show any of these above described cardiac stress effects. The alterations on atria sensitivity to catecholamines were dependent on the stress-induced increase in the plasma corticosterone level. In the present work we verify if the GR as well as the $\beta_1$ and $\beta_2$-ARs expression was altered in right atria from male or female rats submitted to foot-shock stress. Our data showed that serum corticosterone levels increased after foot-shock stress and returned to basal levels 60 min after the end of the stress session, the estrous cycle related corticosterone cycling is not affected by foot-shock stress as well as the GR and $\beta_1$AR expression in right atria. Additionally, data also showed that in right atria from foot-shock stressed male rats and diestrus female rats $\beta_2$AR protein level increased. The $\beta_2$AR protein expression was not altered in right atria from female rats sacrificed at estrus probably because it was already high in right atria from control rats. We concluded that the glucocorticoid receptor or $\beta_1$-AR were not down-regulated in this stress paradigm so that the subsensitivity to noradrenaline previously observed was not due to alterations on the $\beta_1$AR protein expression. However, the supersensitivity to non-selective agonists might be due to an up-regulation of the $\beta_2$AR. Those alterations are not dependent on the gender but they are influenced by the estrous

cycle phase and confer a cardio protection against the damage induced by chronic or repeated $\beta_1$AR activation during stress and the estrous cycle.

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## Introduction

The stress reaction is orchestrated by the catecholamines and the glucocorticoids that were designed the stress hormones (Axelrod & Reisine, 1984).

The physiological responses to catecholamines are mediated by heptahelical G-protein coupled receptors known as β-adrenergic receptors ($\beta_1$AR, $\beta_2$AR and $\beta_3$AR). The cardiac tissue is a major target of these hormones since both $\beta_1$ and $\beta_2$ARs are expressed in the heart, and play critical roles in regulating cardiac function. Until recently a question was made why two adrenoceptors subtypes if both use the same signaling way. However, evidence now available suggests that in contrast to $\beta_1$ARs, the $\beta_2$ARs undergo sequential coupling to both Gs and Gi, resulting in a biphasic effect on mice cardiomyocytes contraction rate with an initial small increase followed by a sustained decrease (Xiang et al, 2002). It has also been reported that Gs activity may be involved in myocyte apoptosis (Zhu et al., 2001; Xiao et al., 2001). Therefore, while $\beta_2$AR/Gi coupling only generates a small negative effect, if any, on heart rate (Devic et al., 2001), its main function may be to activate the anti-apoptosis pathway to protect against the damage induced by chronic $\beta_1$ARs activation during periods of prolonged stress (Xiang et al, 2002).

In rat right atria the $\beta_1$AR subtype is about 67-83%, whereas the $\beta_2$AR subtype corresponds to 30 - 17% of the βARs population (Minneman et al., 1979; Juberg et al., 1985) but only $\beta_1$ARs seem to be coupled to the control of cardiac chronotropism under normal conditions, although a $\beta_2$AR mediated chronotropic response to high concentrations of adrenaline has been demonstrated (Kaumann, 1986).

We have previously reported that the right atria from rats submitted to foot-shock stress were supersensitive to isoprenaline and TA2005, a selective $\beta_2$AR agonist (Voss et al., 1994). This supersensitivity was abolished by the $\beta_2$AR selective antagonist, ICI118,551, suggesting that the response mediated by $\beta_2$AR increased after foot-shock stress (Vanderlei et al., 1996, Spadari-Bratfisch et al., 1999; Santos & Spadari-Bratfisch, 2001). In the same tissue, there was a decrease in the response to norepinephrine mediated by the $\beta_1$AR subtype (Vanderlei et al., 1996). These stress-induced alterations in the sensitivity to catecholamines were dependent on the presence of high plasma corticosterone levels (Nourani et al., 1992) and the estrous cycle phases (Vanderlei et al., 1996). If confirmed that stress induced a change in the ratio between $\beta_1$AR and $\beta_2$AR it may be an evidence of an adaptive response being triggered which confers a protective effect to the cardiac tissue.

During stress the high levels of glucocorticoids binds to a low affinity high capacity glucocorticoid receptor (Reul et al., 1985). In its unliganded form, glucocorticoid receptors are located in the cytoplasm, associated with the heat shock proteins. When the glucocorticoid hormone binds to the receptor, the specific heat shock proteins dissociate and the glucocorticoid receptor-hormone complex enters the nucleus and modulates some responsive genes transcription (Picard et al., 1990; Pratt 1990). GR have been identified in human and rodent heart (Funder et al., 1973; Agarval and Philippe, 1979; Lombes et al., 1992) where glucocorticoids regulate the expression of a subset of steroid-responsive genes (Lindpaintner et al., 1990; Della et al., 1995) including the $\beta_1$ and the $\beta_2$ARs (Hadcock and Malbon, 1988; Fève et al., 1990; Kiely et al., 1994; Brönnegard et al., 1995), and increase the coupling of β-AR with their effector systems (Davies and Lefkowitz, 1984), affect cardiac contractility (Leung and Munck, 1975) and cardiac weight (Hicks et al., 1982).

Changes in the glucocorticoid receptor have been described in many tissues from rats submitted to several stress protocols causing long-term elevation in the circulating levels of corticosterone (Cure, 1989; Kant et al., 1987; Katz et al., 1981;Ottenweller et al, 1989; Scribner et al., 1991). Alexandrova & Farkas (1992) showed that acute or repeated stress causes GR down-regulation whereas repeated immobilization stress increases total cytosolic GR in rat liver (Al-Mohaisen et al., 2000). On the other hand, five days immobilization stress had no effect on the corticosteroid receptors in the hippocampus, circulating lymphocytes or pituitary (Lowy 1991). The corticosteroid receptor binding was not altered in whole-cell GR immunoblot from adrenalectomized or adrenal-intact rats submitted to inescapable tail shocks (Deak et al., 1999). Although many studies reported the effect of stress on GR regulation, there are few studies that examine the effect of stress on cardiac GR regulation. Ho-Kim et al. (1983) showed that stress by exposure to ether do not change the number of glucocorticoid receptor binding sites in the heart. In this present study we examined the impact of the foot-shock stress on serum corticosterone levels as well as on $\beta_1$AR, $\beta_2$AR and GR protein levels in right atria from male and female rats sacrificed in two different phases of the estrous cycle.

## Material and methods

Male and female Wistar rats (*Rattus norvergicus*) 250 to 350 g were housed in standard cages in a temperature controlled room (22°C), with a 12 h light / dark cycle, with the lights on at 6:30 a.m. Standard laboratory chow and tap water were available *ad libitum*.

During the experiments, the animals were cared for in accordance with the principles reported by Olfert et al. (1993) and the experimental protocols were approved by institutional comitte for ethics in animal experimentation (COBEA), of the Institute of Biology, UNICAMP.

### Stress protocol

Rats individually underwent one daily session of unsignaled, inescapable foot-shocks, repeated in three consecutive days. Female rats with regular cycles individually underwent the foot-shock sessions at estrus, metestrus and diestrus, or at diestrus, proestrus and estrus. The rats were placed in a Plexiglass chamber (26 cm long x 21 cm wide x 26 cm high) provided with a grid floor consisting of stainless steel rods (0.3 cm in diameter and spaced 1.0 cm apart). During the 30 min foot-shock sessions, which occurred between 7:30 a.m. and 11:00 a.m., the shocks were delivered by a constant current source controlled by a microprocessor-based instrument constructed at the Center for Biomedical Engineering, UNICAMP. Each rat received 120 foot-shocks with a current intensity of 1.0 mA and duration of 1.0 s at random intervals of 5-25 s (mean interval 15 s). The shocks were scrambled by a mechanism developed at Center for Biomedical Engineering, UNICAMP. After the foot-shock session, the rats were returned to their cages or were sacrificed.

**Tissue preparing**

Right atria were isolated and maintained at the temperature of -70°C until experiment. The tissues were homogenized in TAPS buffer with sucrose (0,25 M) in combination with proteases inhibitors (2mg/ml aprotinin, 34 mg/ml phenylmethyl-sulfonyl fluoride, 5mg/ml leupeptin), in a cell destroyer Branson Sonifier 185 (Danbury, CT, USA) operated at the maximum velocity. Protein content was measured with a standard Micro BCA Reagent (Pierce, Illinois, USA).

Samples were mixed with Laemmeli's sample buffer and dithiothreitol (1:1) and heated in a water bath at 65°C for 4 min. An aliquot of each sample (50µg of protein) was loaded onto an 8% gel using the Laemmli method (Laemmli, 1970). Electrophoresis was done at 125 V, using a Mini-Protean II slab gel apparatus. Immunoblotting was carried out by transferring proteins from the slab gels to nitrocellulose membranes using an electrophoretic transfer apparatus (Mini Transblot, Bio-Rad, Hercules, CA, USA), at 80 V, for 2h at 4°C. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (1 M Tris, 5 M NaCl, Tween 20) for 1 hour in a shaker at room temperature. After blocking, glucocorticoid receptor (GR M-20, Santa Cruz Biotechnology, Inc., CA, USA), $\beta_1$ (V-19, Santa Cruz Biotechnology, Inc.) or $\beta_2$-AR (M-20, Santa Cruz Biotechnology, Inc.) polyclonal antibody were added at 1:100 dilution and blots were further incubated at 4°C overnight. Following this step, the membranes were washed in sample buffer followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (Dako a/s, Denmark), diluted 1:2000 for 1h at room temperature. The membranes were incubated with the ECL chemiluminescence substrate solution (Amersham Pharmacia Biotech., England) for 1 min and then exposed to Amersham X-ray film for 15 - 30 s. The optical density of GR

(approximately 95 KDa) or β-AR (approximately 47 KDa) reactive bands visible on X-ray film was analysed using the Gel-Pro analyser program.

**Hormonal Assay**

After the last foot shock session, rats were sacrificed by a blow to the back of the head followed by the section of the cervical vessels and the blood was collected from the trunk. In the female rats blood was collected from control animals between 7:30 -11:00 a.m. In the stressed female rats blood was collected immediately, 1 h and 6 h after foot shock sessions.

Following collection, blood was allowed to coagulate at room temperature. Serum was frozen until the serum levels of corticosterone were measured by radioimmunoassay (commercial kit, ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA).

**Statistical analysis**

Statistical differences were assessed by Student's *t*-test for unpaired samples or ANOVA plus Tukey test (Zar, 1984). Differences were considered significant at $p < 0.05$.

## Results

### Effect of foot-shock stress on the expression of β-AR or GR proteins

Western blot analysis demonstrated that no significant differences occurred in GR content in right atria from male (Figure 1A) or female rats sacrificed at diestrus (Figure 1B) or estrus (Figure 1C) following exposure to foot-shock stress. The same stress protocol increased by 4- and 6-fold the $\beta_2$AR protein level in right atria from male (Figure 2A) or female rats sacrificed at diestrus (Figure 2 B), whereas no significant change was observed in the $\beta_2$AR protein level in right atria from estrus female rats (Figure 2C). However, in right atria from control female rats sacrificed at estrus, $\beta_2$ARs were already expressed at the same level observed in right aria from foot-shock stressed female rats (Figure 2C). No alteration was observed in the $\beta_1$AR protein level in right atria from male (Figure 3A), diestrus (Figure 3B) or estrus (Figure 3C) female rats.

### Effect of foot-shock stress on serum corticosterone level

After foot-shock stress there was an increase of 2-fold in the serum corticosterone level of male ($p<0.05$, Student *t*-test, Figure 4A) or female rats sacrificed at diestrus or estrus ($p<0.05$, ANOVA plus Tukey test Figure 4B).

Figure 5, reproduced from Verago et al. (2001), shows that in stressed male rats there was a trend towards an increase in the levels of corticosterone after stress on days 1, 2 and 3. However, ANOVA for repeated measures followed by the Tukey test showed that only on days 2 and 3 the plasma corticosterone levels after foot-shock stress were significantly higher than those before foot-shock. Plasma corticosterone levels on day 3 were also higher than the levels on days 0 and 1 ($p<0.05$) indicating that there was no adaptation to the foot-shock stress with repetition. However, 24h after each foot-shock session serum corticosterone levels have returned to the

basal levels. Figure 6A and B, shows that, in fact, as early as 60 min after the foot-shock session the serum corticosterone level had already decreased toward basal levels and that those levels remained low until the next day, before the next foot-shock session. This hormonal profile is not dependent of the estrous cycle phase and do not interfere with the serum corticosterone levels alterations observed along the normal estrous cycle since the peak at the afternoon of proestrus remained unaltered in stressed female rats (Figure 6B).

## Legends to the figures

**Figure 1** - Western blot of glucocorticoid receptor in right atria from control and stressed male (A) and female rats. In the stressed rats tissue was collected immediately after the last foot-shock session, which was applied during diestrus (B) or estrus (C) in the female animals. The plots on the right side represent the mean ± sem of experiments represented by the figure on the left side.

**Figure 2** - Western blot of $\beta_2$-adrenoceptor in right atria from control and stressed male (A) and female rats. In the stressed rats tissue was collected immediately after the last foot-shock session, which was applied during diestrus (B) or estrus (C) in the female animals. The plots on the right side represent the mean ± sem of experiments represented by the figure on the left side. * significantly different from control ($p<0.05$, Student *t* test).

**Figure 3** - Western blot of $\beta_1$-adrenoceptor in right atria from control and stressed male (A) and female rats. In the stressed rats tissue was collected immediately after the last foot-shock session, which was applied during diestrus (B) or estrus (C) in the female animals. The plots on the right side represent the mean ± sem of experiments represented by the figure on the left side.

**Figure 4** - Serum corticosterone levels of control and stressed male (A) and female (B) rats. Stressed rats were sacrificed after three foot-shock sessions, applied in three consecutive days. Blood was collected immediately after sacrifice, between 7:30 and11:00 a.m. * Significantly different from control ($p<0.05$, Student *t*-test).

**Figure 5** – Plasma corticosterone levels of rats before and after 30 min of foot-shock stress. On day 0, animals of both groups were individually placed in the foot shock cage but did not receive foot shocks. On days 1, 2 and 3, rats in the stressed group received 120 shocks (1.0 mA, 1.0 s, intervals of 5-25 s between shocks) over a 30-min period. Rats from the control group remained in the foot shock cage but did not receive foot shock. The columns represent the means ± SEM for 5 experiments. * $P< 0.05$ compared to the day 0 and 1. + $P<0.05$ compared to the stressed group before the session on day 2 (ANOVA plus Tukey test). This figure has been reproduced from Verago et al. (2001) with the permission of the Brazilian Journal of Biological and Medical Research.

**Figure 6** - Serum corticosterone levels of control and stressed female rats. In the stressed rats blood was collected immediately, 1 h and 6 h after the foot shock stress, which was applied during estrus, metaestrus and diestrus (A) or diestrus, proestrus and estrus (B). * Significantly different from control ($p<0.05$, ANOVA plus Tukey test).

Figure 1

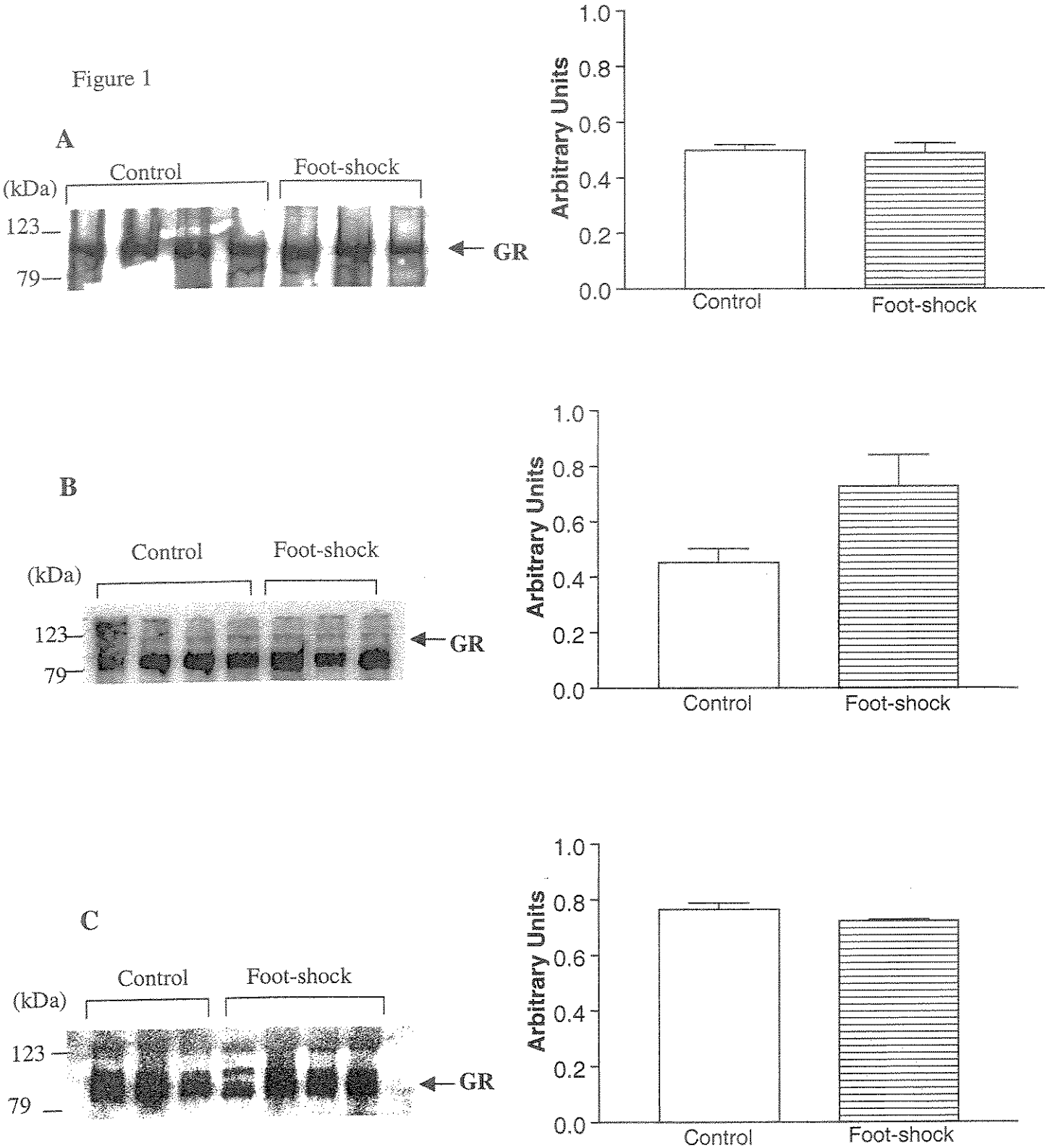
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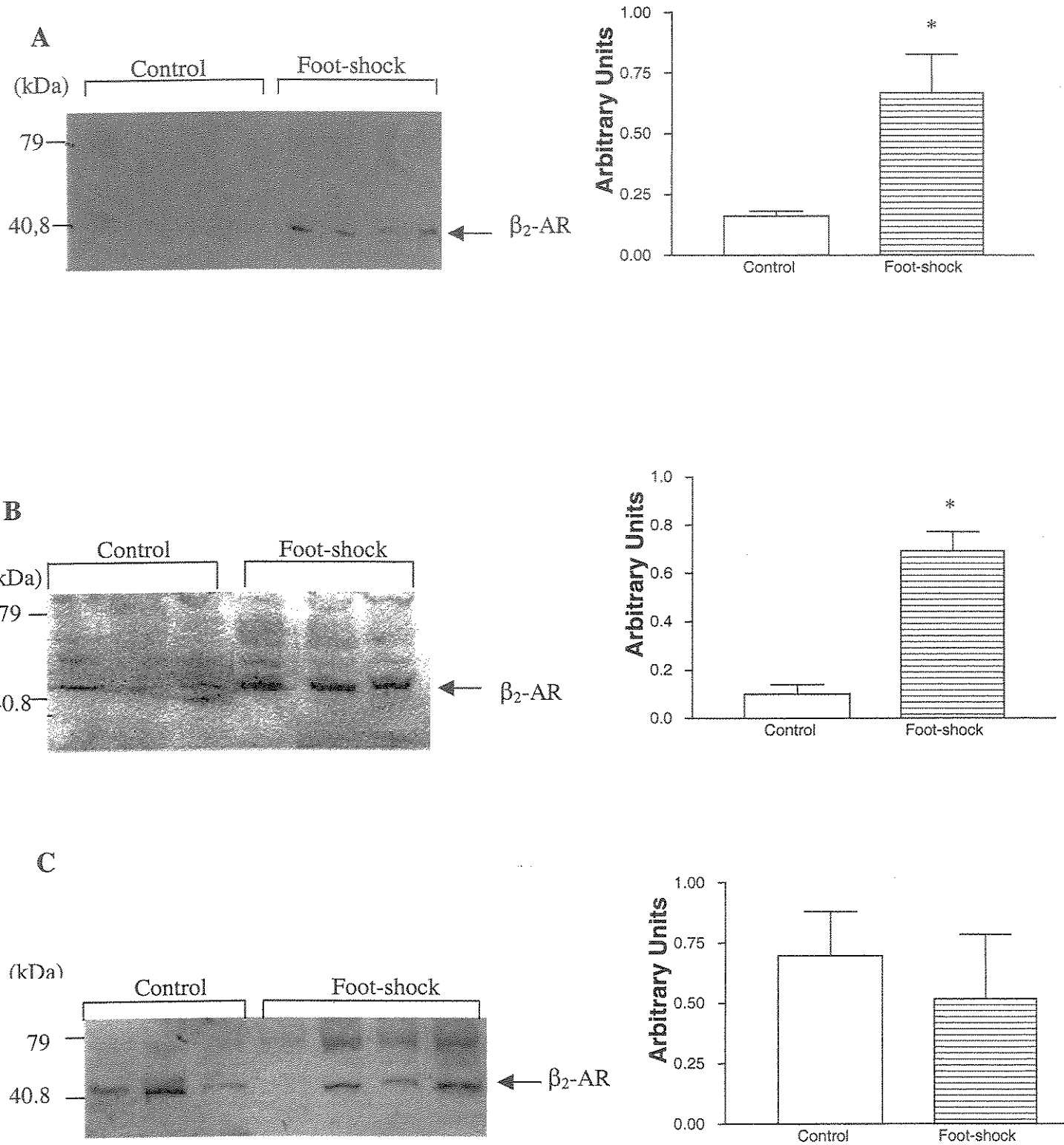
Figure 2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Arbitrary Units
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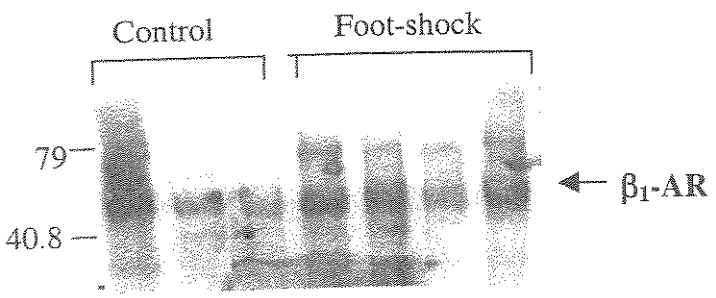
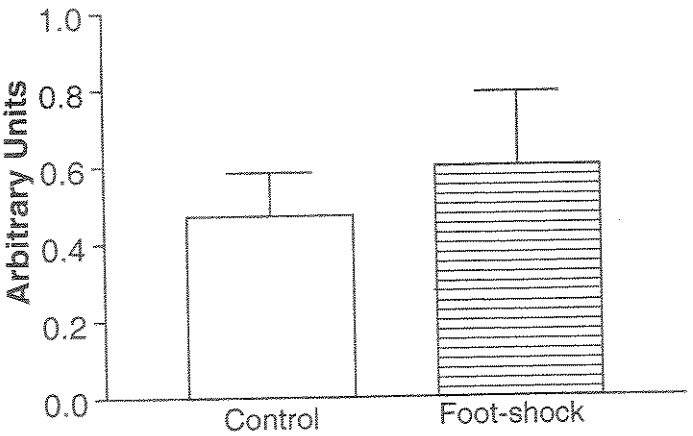
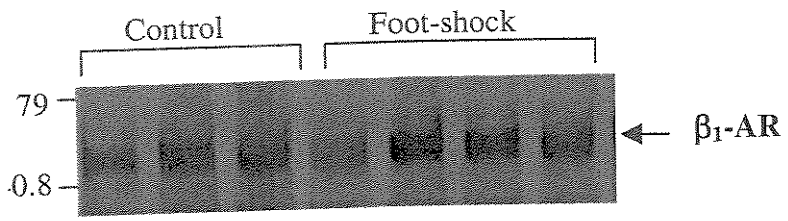
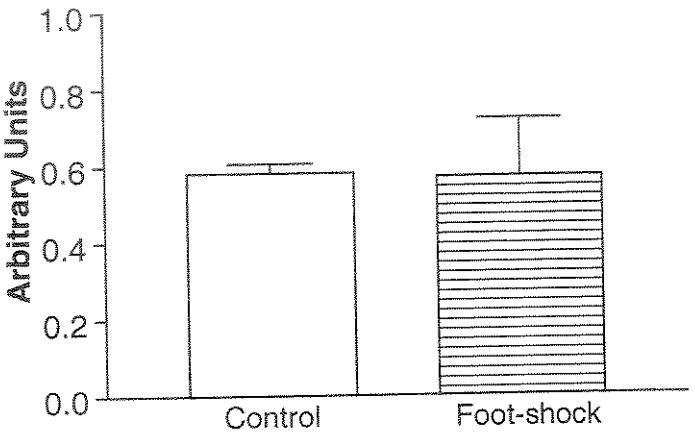
0.75
0.50
0.25
0.00
*
Control
Foot-shock
B
Control
Foot-shock
(kDa)
79
40.8
β2-AR
Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Foot-shock
C
(kDa)
Control
Foot-shock
79
40.8
β2-AR
Arbitrary Units
1.00
0.75
0.50
0.25
0.00
Control
Foot-shock

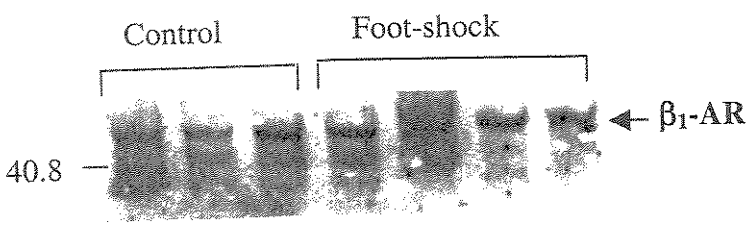
Figure 3


Control
Foot-shock
79
40.8
β1-AR


Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
Control
Foot-shock


Control
Foot-shock
79
0.8
β1-AR


Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
Control
Foot-shock


Control
Foot-shock
40.8
β1-AR

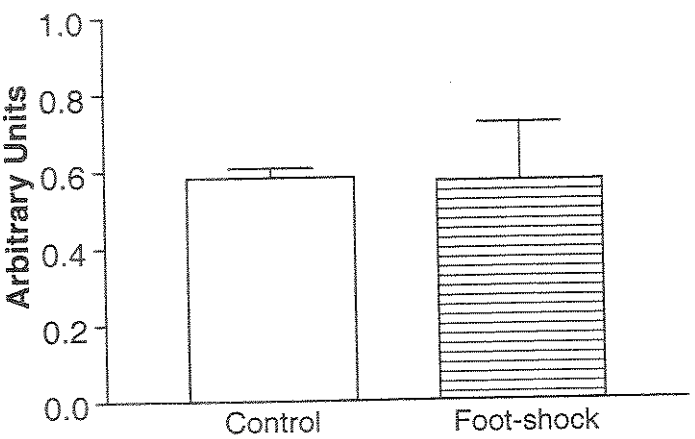
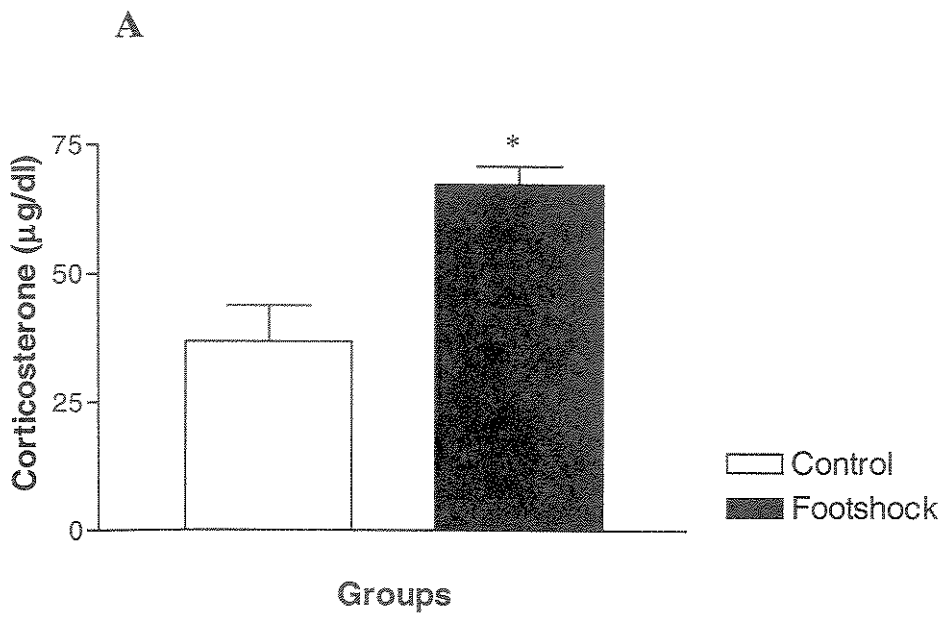

Arbitrary Units
1.0
0.8
0.6
0.4
0.2
0.0
Control
Foot-shock

Figure 4


A
75
50
25
0
Corticosterone (µg/dl)
*
Control
Footshock
Groups

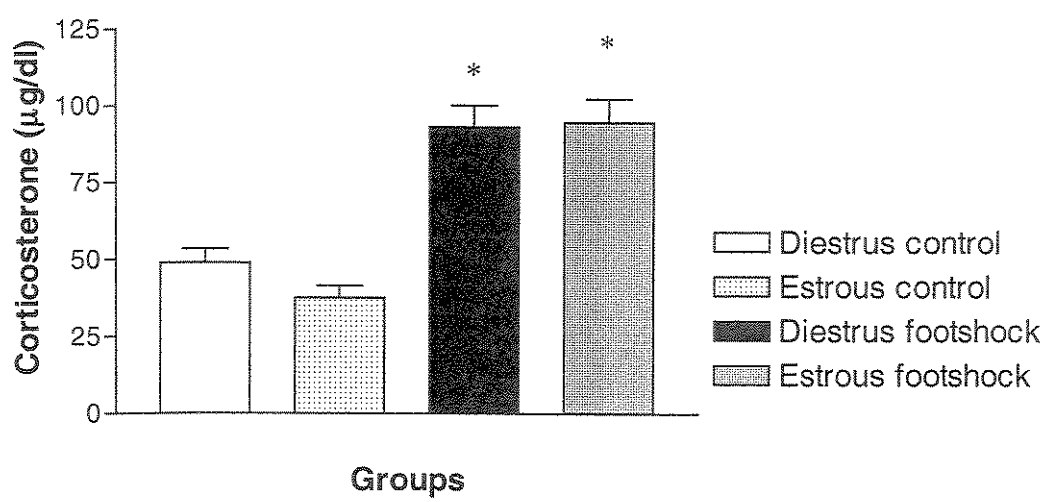

125
100
75
50
25
0
Corticosterone (µg/dl)
*
*
Diestrus control
Estrous control
Diestrus footshock
Estrous footshock
Groups

Figure 5

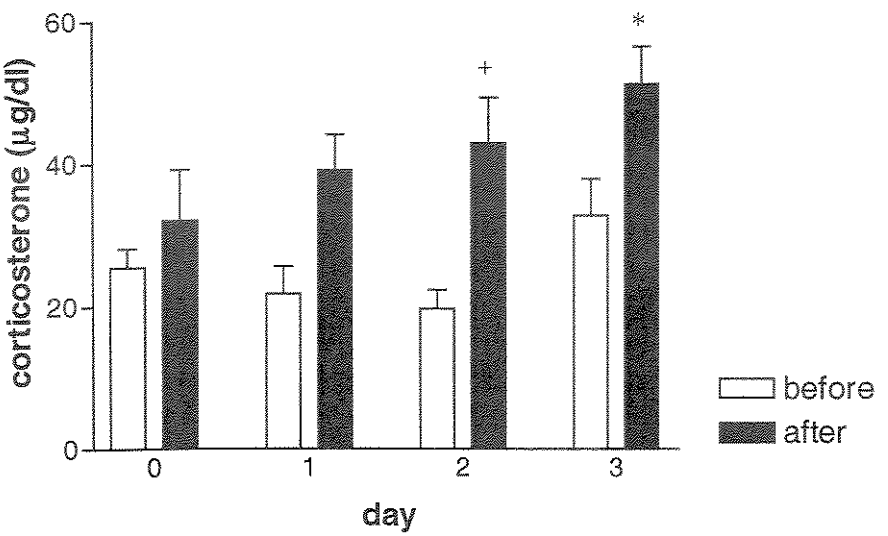
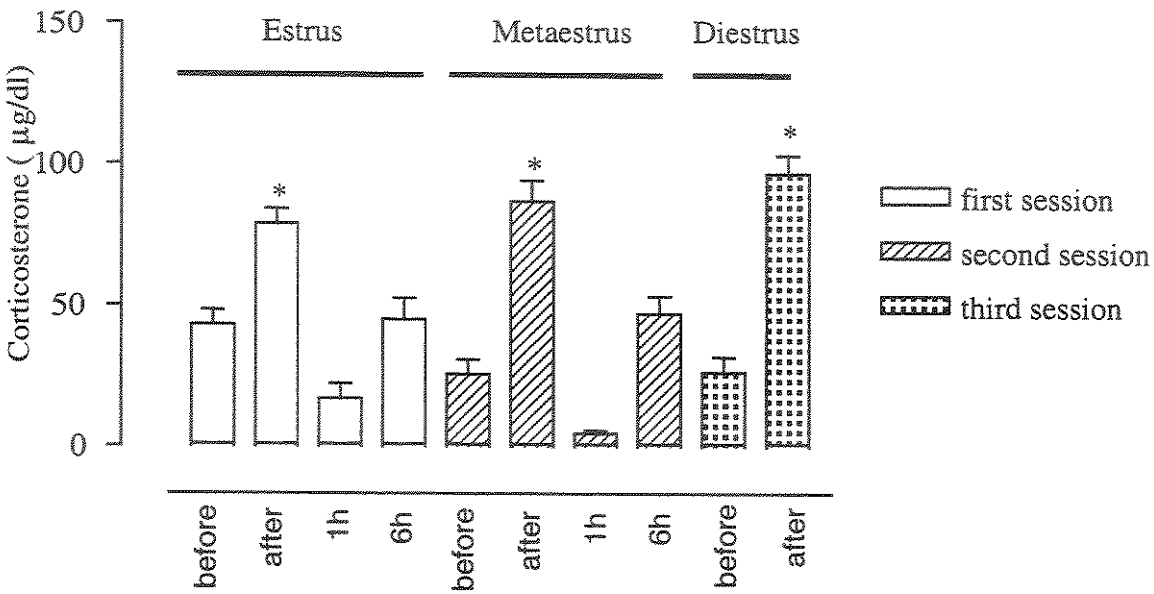
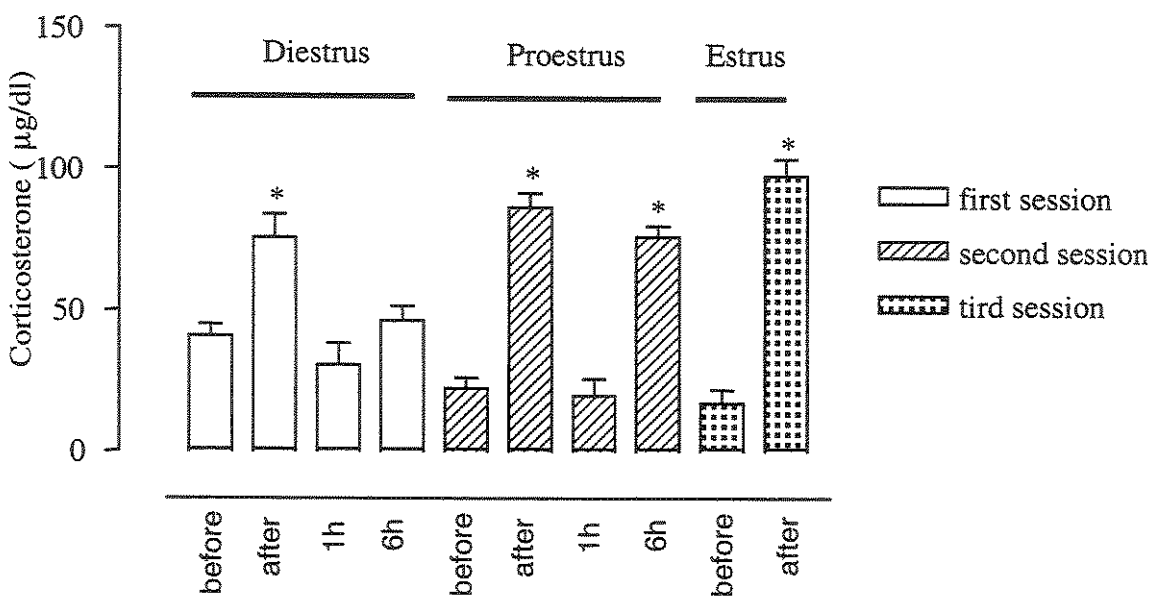


Figure 6


150
100
50
0
Corticosterone ( μg/dl)
Estrus
Metaestrus
Diestrus
*
*
*
before
after
1h
6h
before
after
1h
6h
before
after
first session
second session
third session


150
100
50
0
Corticosterone ( μg/dl)
Diestrus
Proestrus
Estrus
*
*
*
*
before
after
1h
6h
before
after
1h
6h
before
after
first session
second session
tird session

## Discussion

Our data have shown that in right atria from male or female rats submitted to foot-shock stress there was no alteration in the GR protein density, although serum corticosterone levels were 2-fold increased immediately after the stress session. In this same tissue, the $\beta_1$AR protein also remained unchanged whereas the $\beta_2$AR protein expression increased in right atria from male and female rats sacrificed at diestrus but not from those rats sacrificed at estrus.

The available data regarding GR regulation remain controversial and inconclusive. Adrenalectomy increases the cytosolic GR number, and the percentage of GR translocated from cytosol to nuclei (McEwen et al., 1992; Alexandrova and Farkas, 1992; Al-Mohaisen, et al., 2000), while large doses of glucocorticoids reduce cytosolic GR (Alexandrova and Farkas, 1992). Changes in the GR are generally reported in tissues from rats submitted to treatments causing long-term elevation or reduction in the circulating levels of corticosterone (Katz et al., 1981; Kant et al., 1987; Cure 1989; Ottenweller et al., 1989; Scribner et al., 1991). In our experimental conditions the peak serum corticosterone level induced by foot-shock stress dissipates around 60 min following the stress. Thus, although the peak concentrations of serum corticosterone induced by foot-shock stress were similar to those reported after exposition to other stressors, which are known to down-regulate the GR (Deak et al., 1999b), the duration of the stress response after this foot-shock stress protocol might not be enough to down-regulate the GR protein (Alexandrova and Farkas, 1992). Some other investigators have reported no changes in GR with stress. Svec et al. (1989) detected no down-regulation of the hepatic GR in rodents after 3 days of exposure to several stressors. In addition Lowy (1991) found no changes in GR in various tissues of rats after repeated immobilization stress for 5 days. Our results are in accordance with those findings.

Anyway, it is possible that the high serum corticosterone levels acting throughout normal GR density were able to induce $\beta_2$AR up-regulation. It had been previously reported that glucocorticoids modulate βAR expression in many tissues. DDT1MF-2 cells of hamster vas deferens treated with glucocorticoids increased the $\beta_2$AR mRNA and density (Hadcock and Malbon, 1988). The $\beta_2$AR mRNA concentration in the lung from adrenalectomized rats was lower than control. Those animals treatment with dexametasone restored the $\beta_2$AR mRNA levels. On other hand, glucorticoid treatment decreased $\beta_1$AR in adipocytes (Fève et al, 1990). Exposure of C6 glioma cells to dexametasone for 48h, but not for 24h, changed the $\beta_1/\beta_2$AR ratio from 80:20 to 50:50, without modifying the whole content of these receptors (Kiely et al., 1994).

We have previously demonstrated that the response to non-selective (Bassani and De Moraes, 1998) and selective $\beta_2$AR agonists (Spadari-Bratfisch et al., 1999) increases in right atria from male or female rats sacrificed at diestrus but no alteration was observed when the stressed female rats were sacrificed during estrus (Marcondes et al., 1996; Vanderlei et al., 1996). Thus, the present results confirm that the stress-induced right atria supersensitivity to $\beta_2$AR agonists previously demonstrated in right atria from rats is associated with an increase in the $\beta_2$AR protein level.

Whereas the $\beta_2$AR protein increases in right atria from stressed rats sacrificed during diestrus, there was no change during estrus, probably because during estrus $\beta_2$AR are already highly expressed as shown in Figure 2C. We have previously proposed that the increase in the serum corticosterone level induced by stress was a condition for triggering the alterations in the right atria sensitivity to βAR agonists, because those sensitivity alterations were not demonstrated in adrenalectomized rats or in rats treated with RU-38486, a GR antagonist (Nourani et al., 1992).

Accordingly, the present results showed that during the oestrus cycle the peak of corticosterone in the afternoon of the proestrus observed in the female rat (Figure 6B) might be the trigger for the increased $\beta_2$AR expression in right atria from female rats during estrus. Thus, what protection could a high expression of $\beta_2$AR confers to the cardiac tissue that is present during estrus in control female rats and in rats submitted to foot-shock stress?

$\beta_2$AR has been shown to associate with scaffolding proteins that connect $\beta_2$AR to PKA, PKC, PP2A, and/or L-type $Ca^{++}$ channels (Davare et al., 2001; Cong et al., 2001; Shih et al., 1999); Fraser et al., 2000). Those receptors can also be recruited into the signalling complexes and can interact with the $Na^+/H^+$ exchanger regulatory factor (NHERF, Hall et al., 1998). This interaction is critical for the receptor recycling after sequestration from the cell surface and for the $\beta_2$AR coupling to Gi (Xiang et al., 2002). Moreover, the $\beta_2$ARs undergo sequential coupling to Gs and Gi (Xiang et al., 2002). It has been reported that Gs activity may be involved in myocyte apoptosis, whereas activated Gi confers a protective effect against myocyte apoptosis (Zhu et al., 2001; Xiao 2001). While $\beta_2$AR-Gi coupling only generates a small negative effect on myocytes beating rate (Devic et al., 2001) and a no detectable effect on rats heart rate (Spadari-Bratfisch et al., 1999), its main function may be to activate the anti-apoptosis pathway to protect against the damage induced by chronic or repeated $\beta_1$AR activation during stress. Indeed, it has been demonstrated that moderate overexpression of $\beta_2$AR in the heart of transgenic mice leads to an enhanced cardiac contractility without developing cardiomyopathy, suggesting that $\beta_2$AR can provide contractile support without significant cardiotoxic consequence (Dorn et al., 1999).

The $\beta_1$AR lacks the domain needed for those interactions that cause receptor sequestration (Xiang et al., 2002). So, this adrenoceptor subtype is expected to be retained at the cell surface and does not interact with Gi what may be essential for its physiologic function which is

mediated only by Gs (Xiang et al., 2002). Consistent with those evidences, our present data showed no alteration in the $\beta_1$AR protein expression in right atria from foot-shock stressed rats. Zhang et al. (2000) have also shown that in cardiomyocytes where a moderate overexpression of $\beta_2$AR has been induced, the response mediated by $\beta_1$AR was desensitized. This is similar to what happens in the right atria from foot-shock stressed rats where an increase in the $\beta_2$AR expression which contributes with 10% of the total chronotropic response to non-selective agonists (Spadari-Bratfisch et al., 1999) is accompanied by a desensitization in the response to norepinephrine which is not caused by a decreased $\beta_1$AR expression.

The dual regulation mechanism mediated by both $\beta_1$ and $\beta_2$ARs in cardiac tissue represents a sophisticated mechanism critical for the ability of the hearts to respond to, but not be damaged by catecholamines during stress. The results presented here, demonstrate for the first time, this mechanism operating in a physiological condition in a wild type animal rather than cultured myocytes or genetically modified mice. Moreover, the regulation of the proportion of subtypes expressed in cardiac cells during stress gives them an additional protective feature. Our data also have shown that this adaptive process is operating during the oestrus cycle in female rats. The factors controlling these mechanisms are to be elucidated.

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## VI. CONCLUSÕES

1- Nossos dados demonstram que o receptor que medeia a resposta cronotrópica eliciada pelo CGP12177 é mais resistente ao estresse por choques nas patas que as isoformas clássicas dos adrenoceptores $\beta_1$ e $\beta_2$ e que, diferentemente destes dois subtipos, o sítio receptor ativado pelo CGP12177 não é alterado pelas variações hormonais que ocorrem durante o ciclo estral.

2- Em átrio direito de ratos, isolados 48 h após a desnervação sinoaórtica, observamos subsensibilidade à isoprenalina, noradrenalina e CGP12177. No entanto, sete dias após esta cirurgia, o sítio receptor que medeia a resposta cronotrópica eliciada pelo CGP12177 já havia recuperado sua sensibilidade, enquanto a isoforma clássica do receptor $\beta_1$ permanecia subsensível à noradrenalina, graças a um processo de *down-regulation*. Esses dados sugerem que a isoforma clássica do receptor $\beta_1$ e o sítio receptor ativado pelo CGP12177 são diferentemente regulados.

3- Estresse por choques nas patas promoveu aumento nas concentrações séricas de corticosterona em ratos machos e fêmeas. A concentração de proteínas do receptor de glicocorticóide, detectado pela técnica de *Western Blot*, não foi alterada em átrio direito de ratos machos ou de fêmeas, sugerindo que o pico de secreção da corticosterona, não foi suficiente para induzir alterações na proteína do referido receptor.

4- A expressão das proteínas dos adrenoceptores $\beta_1$ não se altera com o estresse nem com as variações hormonais que ocorrem durante as fases do ciclo estral. Estes dados indicam que a subsensibilidade aos agonistas $\beta_1$ adrenérgicos, verificada em experimentos farmacológicos, não é desencadeada por alterações na densidade de adrenoceptores e, provavelmente, se deve a outros mecanismos de sinalização intracelular deste receptor.

5- A supersensibilidade para agonistas não seletivos $\beta_1/\beta_2$, observada em experimentos farmacológicos, em átrio direito de ratos machos ou de fêmeas sacrificadas em diestro, está associada ao aumento da expressão das proteínas dos adrenoceptores $\beta_2$. Provavelmente, o ambiente hormonal ao qual ratas estão expostas durante o estro, evita alterações semelhantes na expressão dos adrenoceptores $\beta_2$, o que adiciona complexidade na interação dos fatores que regulam a resposta tecidual às catecolaminas.

## VII. REFERÊNCIAS BIBLIOGRÁFICAS REFERENTES À INTRODUÇÃO


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