Activation of corticotropin-releasing factor receptor 1 selectively inhibits Ca\textsubscript{\textsc{v}}3.2 T-type calcium channels

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Activation of CRFR1 selectively inhibits CaV3.2

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Number of text pages: 41 (including title, running title and footnote pages)

Figures: 8

Number of references: 39

Abstract: 223

Introduction: 654

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ABBREVIATIONS:
UCN, urocortin; CRF, corticotrophin-releasing factor receptor; PKA, protein kinase A; PKC, protein kinase C; Gβγ, G-protein βγ subunit; PLC, phospholipase C; TK, tyrosine kinases; CaMKII, Ca2+/calmodulin-dependent protein kinase II; GDP-β-S, guanosine-5′-O-(2-thiodiphosphate); GTP-γ-S, guanosine-5′-O-(3-thiotriphosphate); MAS-GRK3, G-protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; PI3K, phosphatidylinositol 3-kinase; RGS2, regulator of G-protein signaling 2
Abstract

The corticotropin-releasing factor (CRF) peptides, CRF and urocortins 1-3, are crucial regulators of mammalian stress and inflammatory responses and are also implicated in disorders such as anxiety, depression and drug addiction. There is considerable interest in the physiological mechanisms by which CRF receptors mediate their widespread effects and here we report that the native CRF receptor 1 (CRFR1) endogenous to the HEK293 cells can functionally couple to mammalian CaV3.2 T-type calcium channels. Activation of CRFR1 by either CRF or urocortin 1 (UCN) reversibly inhibits CaV3.2 currents (IC50 ~30 nM) but does not affect CaV3.1 or CaV3.3 channels. Blockade of CRFR1 by the antagonist, astressin, abolished the inhibition of CaV3.2 channels. The CRFR1-dependent inhibition of CaV3.2 channels was independent of the activities of phospholipase C (PLC), tyrosine kinases (TK), Ca2+/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and other kinase pathways, but was dependent upon a cholera toxin-sensitive G-protein mediated mechanism relying upon G-protein βγ subunits (Gβγ). The inhibition of CaV3.2 channels via the activation of CRFR1 was due to a hyperpolarized shift in their steady-state inactivation and was reversible upon washout of the agonists. Given that UCN affect multiple aspects of cardiac and neuronal physiology and that CaV3.2 channels are widespread throughout the cardiovascular and nervous systems, the results point to a novel and functionally relevant CRFR1-CaV3.2 T-type calcium channel signaling pathway.
Introduction

The corticotropin-releasing factor (CRF) family, consisting of CRF, urocortin 1 (UCN), UCN2 and UCN3, are critical regulators of stress and inflammatory responses and have been variously associated with being cardioprotective and contributing towards alcohol and drug dependencies (Bale and Vale, 2004; Reul and Holsboer, 2002, Gravanis et al., 2005, Bruijnzeel et al., 2005). The two major receptors for CRF and UCNs, CRF receptor 1 (CRFR1) and CRFR2, have been identified as G-protein coupled receptors (GPCR) that can mediate responses via activation of the protein kinase signaling pathways (Gravanis et al., 2005, Bruijnzeel et al., 2005). CRF has a higher affinity for CRFR1 than for CRFR2, UCN shows high affinity for both CRFR1 and CRFR2, while UCN2 and UCN3 are selective for CRFR2 (Bale and Vale, 2004). The CRFR1 is expressed primarily in the brain and pituitary, and activation of CRFR1 exerts numerous central and peripheral effects associated with pathological diseases (Dautzenberg and Hauger, 2002). Within the hypothalamus-pituitary-axis, CRF and CRF-related peptides such as UCN activate CRFR1 receptors to regulate pituitary function in response to stress (Bale and Vale, 2004; Reul and Holsboer, 2002). In addition, overactive CRFR1 receptors in extra-hypothalamic circuits have been implicated in affective disorders (Reul and Holsboer, 2002).

Low voltage-activated calcium channels play critical roles in thalamocortical processes in both the awake and sleep states, in pacemaking activity, action potential burst firing (Steriade and Llinas, 1988), pain transmission and hormone secretion (Hildebrand and Snutch, 2006; Perez-Reyes, 2003). In mammals, three $\alpha_1$-subunit genes have been described
that encode distinct low voltage-activated calcium channels (T-type) with unique biophysical and pharmacological properties: \( \text{Ca}_{V}3.1 \) (\( \alpha_{1G} \)), \( \text{Ca}_{V}3.2 \) (\( \alpha_{1H} \)), and \( \text{Ca}_{V}3.3 \) (\( \alpha_{1I} \)) (Perez-Reyes, 2003). Previous reports have shown that \( \text{Ca}_{V}3.1 \), \( \text{Ca}_{V}3.2 \), and \( \text{Ca}_{V}3.3 \) are differentially and widely expressed in brain and various peripheral tissues (Cribbs et al., 1998; McKay et al., 1998; Molineux et al., 2006). Altered T-type calcium channel activity has been implicated in cardiac hypertrophy (Nuss and Houser, 1993), generalized epilepsies (Nelson et al., 2006), and both acute and chronic pain signaling (Bourinet et al., 2005). While the electrophysiological properties of \( \text{Ca}_{V}3 \) T-type channels are primarily regulated by dynamic changes in membrane potential, their functional properties can also be modulated by the actions of hormones or neurotransmitters acting via GPCR that trigger downstream transduction pathways (Welsby et al., 2003; Kim et al., 2006; Wolfe et al., 2003; Chemin et al., 2006).

UCN, an endogenous agonist for CRF receptors, has been shown to either attenuate or stimulate low threshold calcium currents depending on the type of native cells examined (Tao et al., 2005; Kim et al., 2007; Lee et al., 1997). Various T-type calcium channel subtypes are expressed in these cells (Jagannathan et al., 2002), however, due to the lack of discriminatory antagonists, the selective inhibitory effect of CRFR1 activation on each of the three \( \text{Ca}_{V}3 \) channels - \( \text{Ca}_{V}3.1 \), \( \text{Ca}_{V}3.2 \) and \( \text{Ca}_{V}3.3 \) - could not be investigated pharmacologically in these cells. As CRFR1, \( \text{Ca}_{V}3 \) and various calcium channels are localized to peripheral and central neuron regions including the amygdala, hippocampus, hypothalamus and pituitary (Chalmers et al., 1995; Talley et al., 1999), it is of interest to ask
by what mechanism does the activation of the CRFR1 underline the inhibition of CaV3 channels and to ask whether the CaV3 calcium channels are specifically inhibited.

In the present study, we report that activation of CRFR1 endogenous to the HEK293 cells selectively inhibited CaV3.2 calcium channels while CaV3.1 and CaV3.3 channels were not affected. Besides, the mechanism for the inhibition was mediated via a cholera-toxin sensitive, G_{iγ}-dependent pathway. Importantly, the decrease in CaV3.2 currents was due to a novel mechanism involving the hyperpolarized shift in the steady-state inactivation property of the channels and the effect is reversible upon wash-out of the CRFR1 agonists. As UCN affect multiple aspects of cardiac and neuronal physiology and as CaV3.2 channels are expressed widely throughout the cardiovascular and nervous systems, our results point to a novel and functionally relevant CRFR1-calcium channel signaling pathway.
Materials and Methods

Cell culture and transient transfection protocols

HEK293 cells were maintained in DMEM + 10% FBS. Transient transfection was performed using the standard calcium phosphate transfection method with a DNA mix containing 1:9 ratios (by weight) of GFP plasmid and constructs encoding for rat or human Cav3.1, Cav3.2, and Cav3.3 isoforms. The full-length rat (McRory et al., 2001) and human (kindly provided by David Parker, Neuromed Pharmaceuticals, Inc) Cav3.1, Cav3.2, and Cav3.3 α1-subunits were cloned into the pcDNA3 vector (Invitrogen). RGS2 and MAS-GRK3 constructs (both kindly provided by Dr. Brett Adams, Utah State University, USA) were subcloned into pEGFP-C2 and pIRES vectors, respectively (Clontech, USA).

Reverse transcription-PCR

Total RNA was extracted from HEK293 cells using the RNeasy kit (QIAGEN). Reverse transcription was carried out with SuperScript™ II (Invitrogen). Negative control (reactions without reverse transcriptase) was carried out in all RT-PCRs to exclude contamination. The sequences of the primers employed in this study are summarized in Table 1 (supplemental data). Hypothalamus and left cardiac atrium were used as positive controls for the expression of CRFR2α and CRFR2β, respectively. The expression of β-actin mRNA was examined as an internal control. The PCR protocol includes a denaturation step at 95 °C for 2 min, and the denaturation, annealing, and elongation cycle was carried out at 94 °C for 30 sec, at 65 °C (CRFR1, R2α, and R2β) or 60 °C (β-actin) for 20 sec, and at 72 °C for 1 min. PCR was carried out for 35 cycles (CRFR1, R2α, R2β), or 25 cycles (β-actin), respectively.
PCR analysis was repeated at least twice with the same samples to confirm reproducibility of the results.

**Western blotting**

HEK293 cells were lysed for 1 h in PBST buffer containing 1% Triton X-100, 10 µg/mL aprotinin and 1 mm PMSF in phosphate-buffered saline (PBS). Following centrifugation at 40,000 rpm for 30 min at 4°C, 30 µg of soluble protein was separated in 10% SDS polyacrylamide gel. The proteins were then transferred electrophoretically onto polyvinylidene difluoride membrane (Bio-Rad) using a semi-dry transfer system (Bio-Rad) with methanol omitted from the transfer buffer. For immunolabelling experiments, membranes were first incubated with 5% non-fat milk in TBST (20mM Tris pH 7.6, 137mM NaCl and 0.05% Tween 20) for 1 h at room temperature. The membranes were then incubated with diluted primary antibody anti-CRFR1 (1:500 dilution, V-14, Santa Cruz, CA) or anti-CRFR2 (1:500 dilution, C-15, Santa Cruz, CA) at 4°C overnight. After 5 washes with TBST, membranes were incubated for 2 h with 2000-fold diluted rabbit anti-goat secondary antibody (Sigma). After another 5 washes with TBST, the specific binding of the primary antibody was detected with SuperSignal West Pico chemiluminescent substrate (Pierce). The intensities of immunoreactive staining were measured using a scanning densitometer (Scion Image, Scion Corp., Frederick, MD).

**Immunohistochemical localization of CRFR1**

HEK293 cells were grown on polylysine coated sterile coverslips, and fixed in PBS containing 4% sucrose and 4% paraformaldehyde for 20 min at 4°C. The fixed cells were
washed three times with PBS before permeabilization in PBS containing 0.1% Triton X-100 for 5 min. Blockade was then carried out with 4% horse serum in PBS for 1 h. This was followed by incubation in primary antibody (goat anti-CRFR1, 1:500, Santa Cruz, CA) for 1 h at 25°C. After washing three times with PBS, Alexa Fluor 488 Chicken Anti-goat IgG (green) (Invitrogen, USA) was applied to the samples at a dilution of 1:500. The immunolabelled cells were visualized using a laser-scanning confocal microscope (Fluoview BX61, Olympus). Negative controls, omitting each primary antibody, were performed in each case and no significant immunolabellings were observed (data not shown).

**Measurement of cyclic AMP (cAMP)**

To determine intracellular cAMP levels, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, was added to each well 30 min before the addition of CRF, UCN, or astressin to prevent breakdown of accumulated cAMP. After incubation with CRF or UCN for 10 min, cells were immediately immersed in 0.25 ml of 0.1M HCl to stop the reaction. For experiments using antagonist, the astressin was applied for 30 min before the 10-min incubation as described above. Cells were collected and then centrifuged at 3000 rpm for 15 min at room temperature. The intracellular cAMP content was determined from the supernatant using the direct cAMP enzyme immunoassay kit in accordance with the manufacturer's high-sensitivity acetylation protocol (Sigma, USA).

**Cloning of rat CRFR1 receptor**

Rat CRFR1 mRNA was isolated from rat (Wistar) brain and RT-PCR was performed
using CRFR1 forward (5-ATGGGACGGCGCCGCAGCTCCGGCTCG-3) and reverse (5-TCACACTGCTGTGGACTGCTTGATGC-3) primers. The PCR product was cloned into pGEM-T Easy (Promega, USA) vector and the identity of the rat CRFR1 was confirmed by DNA sequencing. The full length rat CRFR1 digested with restriction enzyme SpeI and NotI was subcloned into MCS B site of the pIRES vector (Clontech, USA), while mCherry (gift from Dr. Roger Y. Tsien, University of California, San Diego, USA) was cloned into the MCS A site using the XhoI and EcoRI restriction enzyme sites.

Electrophysiological recordings and data Analysis

Whole-cell currents were recorded at room temperature. Extracellular solution contained (in mM) 140 tetraethylammonium methanesulfonate, 10 HEPES, 5 BaCl₂ (pH to 7.3 with CsOH). Patch pipettes (World Precision Instruments) have a resistance of 2-3 MΩ when filled with an internal solution (in mM): 138 Cs-MeSO₃, 5 CsCl, 0.5 EGTA, 1 MgCl₂, 4 MgATP and 10 HEPES (pH 7.3, adjusted with CsOH). Complete replacement of external solution 2ml/min in the chamber was achieved within 2-3 minutes. Whole-cell Ba²⁺ currents were recorded using a MultiClamp 700B amplifier (Axon Instruments, Foster City, CA), controlled and monitored with a PC running p-CLAMP software version 9.3 (Axon Instruments). Series resistance was typically <5 MΩ and was electronically compensated by at least 70%. Data were low pass-filtered at 2 kHz using the built-in Bessel filter of the amplifier, and subtraction of capacitance and leakage current was carried out on-line using the P/4 protocol. Student’s t-tests were used to compare the different values, and were considered significant at P<0.05. All data are expressed as means±S.E.M., and GraphPad
Prism software was used for data plotting. Concentration–response curves were fitted by sigmoidal Hill equation $I/I_{\text{control}}=1/(1+10^{\log{IC_{50}-X}}n_H)$, where $X$ is the decadic logarithm of the concentration used, $IC_{50}$ is the concentration at which the half-maximum effect occurs, and $n_H$ is the Hill coefficient. The I-V curves were fitted by $I_{Ba}=G_{\text{max}}(V-E_{\text{rev}})/(1+\exp((V-V_{1/2})/k_{I-V}))$. Activation data were fitted by: $G/G_{\text{max}}=F_{\text{low}}/[1+\exp((V_{1/2,\text{low}}-V)/k_{\text{low}})]+(1-F_{\text{low}}/[1+\exp((V_{1/2,\text{high}}-V)/k_{\text{high}}))$, where $V_{1/2\text{act}}$ is the potential for half-activation calculated from dual Boltzmann functions when $G=0.5G_{\text{max}}$. Steady-state inactivation data were fitted by a Boltzmann function of the form: $III/I_{\text{max}}= (A1-A2)/[1+\exp((V-V_{1/2\text{inact}})/k_{\text{inact}})]+A2$. Recovery of currents from inactivation was tested using a double-pulse protocol. The peak current from the second test pulse was always normalized to the first 1-sec pulse, and the plot of normalized current versus repolarization time was fitted with a double exponential equation: $Y=Y_{\text{min}}+A_1\times[1-\exp(-t/\tau_f)]+A_2\times[1-\exp(-t/\tau_s)]$, where $Y$ is the fraction of recovery, $A_1$ and $A_2$ are the maximum values of the fast and slow component, and $\tau_f$ and $\tau_s$ are the time constants, respectively.

Pharmacological agents

All drugs were obtained from SIGMA (USA), unless otherwise indicated. Stock solutions of UCN, CRF, PKI 5-24, chelerythrine chloride, PTX, CTX and astressin were prepared in distilled deionized water. Stock solutions of GF-109203X, H-89, wortmannin, genistein, KN-93 and U-73122 were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the bath solution is expected to be less than 0.01%, and had no

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functional effects on the T-type calcium currents. The QEHA peptide (QEHAQEPERQYMHIGTMVEFAYALVGK) and SKEE peptide (SKEEKSDKERWQHL ADLADFALAMKDT) were synthesized by GenScript Corporation (NJ, USA). The peptides were purified by HPLC (>95%) and the identities verified by mass spectrometry.

Results

HEK 293 cells endogenously express functional CRF receptor 1 (CRFR1)

Previous reports have shown that HEK 293 cells endogenously express CRF receptors (Dautzenberg et al., 2000). In order to confirm both the receptor subtype and the ability of endogenous receptors to transduce downstream signals after binding CRF or UCN, we characterized HEK 293 cells by RT-PCR, western blot and immunohistochemistry analyses.

RT-PCR analysis demonstrated that CRFR1 mRNA was expressed in HEK 293 cells, but that neither CRFR2α nor CRFR2β mRNA could be detected (Fig. 1A). As controls for the RT-PCR, CRFR2α and CRFR2β transcripts could be clearly amplified from mRNAs isolated from mouse hypothalamus and left atrium respectively (Fig. 1A). All RT-PCR reactions were able to amplify β-actin while the negative controls performed in parallel in the absence of reverse transcriptase enzyme in the RT reaction showed no product. Western blot analysis of HEK 293 cell protein lysates using anti-CRFR1 antibody revealed that CRFR1 was expressed at the predicted size of ~55 kDa while CRFR2 was not detected by anti-CRFR2 antibody (Fig. 1B). As a positive control, mouse hypothalamus which is known to express both CRFR1 and CRFR2 showed prominent bands of ~55 kDa (CRFR1) and ~65kDa (CRFR2) and both HEK 293 cell and hypothalamus lysates produced robust
staining for β-actin (Fig. 1B). As CRF receptors belong to the family of seven transmembrane GPCRs (Markovic et al., 2006) and would be expected to be localized to the plasma membrane, we examined the distribution of CRFR1 on HEK 293 cells by confocal microscopy. Fig. 1C clearly shows the membrane localization of CRFR1. To test the functionality of the endogenous CRFR1 in HEK 293 cells, we measured the levels of cAMP produced from CRFR1 activation by either CRF or UCN, both of which are known to activate adenylate cyclase (Markovic et al., 2006). Upon either CRF (0.01 µM) or UCN (0.01 µM) application to HEK 293 cells, cAMP production was stimulated ~7-fold over basal levels (Fig. 1D). In the presence of the CRFR1 antagonist, astressin (1 µM), the stimulation of cAMP accumulation by UCN or CRF was completely abrogated (Fig. 1D). Taken together, the results show that the HEK 293 cells endogenously expressed fully functional CRFR1 and could therefore be used to investigate the mechanisms by which Cav3 T-type calcium channels might be affected.

**CRFR1 selectively inhibits Cav3.2 T-type calcium channels**

The binding affinities of CRF and UCN peptides to CRFR1 are roughly similar (Dautzenberg et al., 2000) and we decided to first investigate the activation of CRFR1 by UCN on Cav3 T-type calcium channels. Fig. 1 showed that rat Cav3.2 channels were selectively inhibited by application of 0.1 µM UCN while the rat Cav3.1 and Cav3.3 T-type calcium channels were not affected. A similar selective inhibition of Cav3.2 channels was observed at all test potentials (Fig. 2, A-C, right panels). Overall, application of 0.1 µM UCN significantly reduced Cav3.2 currents by ~31% (I/I_{control}=0.70±0.05, n=15, P<0.05).
while neither Cav3.1 nor Cav3.3 currents were significantly affected (Cav3.1: \( I/I_{\text{control}} = 0.98 \pm 0.03, n=13, P>0.05 \); Cav3.3: \( I/I_{\text{control}} = 0.96 \pm 0.04, n=6, P>0.05 \)). After washout, Cav3.2 channel currents returned to baseline levels within 5 min (Fig. 2F), indicating the effect of UCN on Cav3.2 currents was not due to rundown. In order to determine whether the observed selective inhibition of Cav3 was species-specific, we further investigated the effects of 0.1 µM UCN on the three human brain Cav3 T-type calcium channel isoforms expressed in HEK293 cells. Fig. 1E shows a similar degree of UCN-mediated inhibition (~37%) of human Cav3.2 currents (\( I/I_{\text{control}} = 0.63 \pm 0.06, n=7, P<0.01 \)), with no significant effects on either Cav3.1 (\( I/I_{\text{control}} = 0.97 \pm 0.03, n=9, P>0.05 \)) or Cav3.3 (\( I/I_{\text{control}} = 0.98 \pm 0.07, n=7, P>0.05 \)) currents. Electrophysiological recordings for the UCN-mediated inhibition of human Cav3 T-type calcium channels and the time course of the inhibition are shown (supplemental data, Fig. 8).

**CRFR1 activation affects Cav3.2 channels by shifting steady-state inactivation properties**

Prior to investigating the mechanism for the selective inhibition of Cav3.2 calcium channels via UCN activation of CRFR1, we examined: 1) whether the response is dosage-dependent, and 2) whether channel electrophysiological properties were affected. To address the first issue, we performed a UCN dose-response study for the inhibition of Cav3.2 currents. At a holding potential of -110 mV, application of UCN at 0.01, 0.1, 1, and 10 µM increasingly inhibited Cav3.2 currents by 15% (\( I/I_{\text{control}} = 0.85 \pm 0.03, n=8, P<0.05 \)), 31% (\( I/I_{\text{control}} = 0.70 \pm 0.05, n=15, P<0.05 \)), 41% (\( I/I_{\text{control}} = 0.59 \pm 0.04, n=9, P<0.01 \)), and 43%
The maximum inhibition reduced the original peak current by ~43% with a calculated IC$_{50}$=30.41 nM and a Hill coefficient=0.98. We next evaluated the inhibition of UCN at a more physiological holding potential of -80 mV and found that the Ca$_{V3.2}$ channels were even more sensitive to inhibition by UCN with an ~4-fold decrease in the IC$_{50}$ to 6.93 nM (Hill coefficient of 0.97; Fig. 3B). To address the second point, we investigated whether the steady-state activation and inactivation properties of Ca$_{V3.2}$ channels were affected by bath application of 0.1 µM UCN. We observed a slight but statistically insignificant shift of 2 mV in the hyperpolarizing direction of the activation potential (V$_{1/2}$ from -41.8±2.6 to -43.7±1.3 mV, and k value from 5.9±0.3 to 6.0±0.6, n=19, P>0.05) (Fig. 3C). In contrast, 0.1 µM UCN significantly shifted the steady-state inactivation potentials of Ca$_{V3.2}$ calcium channels in the hyperpolarizing direction by ~13 mV (V$_{1/2}$ from -66.7±0.6 to -79.5±0.7 mV, and k value from 4.9±0.6 to 7.2±0.7, n=17, P<0.05) (Fig. 3D). In addition, UCN at 30 nM (~IC$_{50}$ at holding potential of -110 mV) shifted the steady-state inactivation potentials of Ca$_{V3.2}$ calcium channels in the hyperpolarizing direction by an expected smaller potential of ~8 mV (V$_{1/2}$: -75.3±0.7 mV, and k value: 6.6±0.8, n=6, P<0.05 vs. control) (Fig. 3E) suggesting a dose-dependence in the shift of the steady-state inactivation upon activation of CRFR1 by UCN. Furthermore, after UCN (0.1 µM) washout, the steady-state inactivation reversed to values similar to controls prior to UCN application (V$_{1/2}$: -68.4±0.7, k: 5.2±0.6, n=8, P<0.05). This result suggests that the reduction in Ca$_{V3.2}$ currents observed upon application of UCN could be the result of increased channels remaining in the inactivated
state. We further determined whether activation of CRFR1 will affect Ca\textsubscript{v}3.2 recovery from inactivation. A typical two-pulse protocol was used with a prepulse of 1-s duration (Fig. 3F). We observed a slight but statistically insignificant effect of UCN (0.1 μM) (p>0.05) on the fast and slow components of recovery from inactivation (Control: $\tau_{\text{fast}} = 110\pm19$ ms, n=9, $\tau_{\text{slow}} = 916\pm113$ ms, n=9; 0.1 μM UCN: $\tau_{\text{fast}} = 103\pm17$ ms, n=9, $\tau_{\text{slow}} = 684\pm72$ ms, n=9) (Fig. 3F), which suggested that the UCN blockade purely shifted the steady-state of inactivation potentials to the left and did not affect the rate of recovery. Interestingly, however, there appeared to be some facilitation of the Ca\textsubscript{v}3.2 channels under both conditions.

**UCN and CRF similarly inhibit Ca\textsubscript{v}3.2 currents by activation of CRFR1**

In order to confirm that the UCN-mediated response occurred via CRFR1, we examined its effects on Cav3.2 currents in the presence or absence of the CRFR antagonist astressin. Pre-incubation with 1 μM astressin blocked the UCN-induced inhibition ($I/I_{\text{control}}=0.95\pm0.06$, n=6, p<0.05; Fig. 4A) indicating that the inhibition of Ca\textsubscript{v}3.2 channels is CRFR1-dependent. Importantly, CRF peptide activation of CRFR1 also showed robust inhibition (by ~28%) of the Ca\textsubscript{v}3.2 currents ($I/I_{\text{control}}=0.72\pm0.04$, n=6, P<0.05; Fig. 4B) and was also CRFR1-dependent as demonstrated by the abrogation of inhibition after pre-incubation with 1 μM astressin.

**Ca\textsubscript{v}3.2 channel inhibition can be enhanced by exogenous CRFR1 expression**

We next explored whether augmentation of CRFR1 levels through the over-expression of cloned CRFR1 in HEK 293 cells could affect the degree of inhibition of Cav3.2 channels mediated by UCN or CRF. We first cloned and then characterized the rat CRFR1 in HEK
293 cells. Western blot analysis of CRFR1 proteins isolated from mouse hypothalamus and CRFR1-transfected HEK293 lysates showed a predominant ~55 kDa band stained by anti-CRFR1 (Fig. 5A). The total amount of CRFR1 protein expressed in the HEK 293 cells after transient transfection with cloned CRFR1 showed a 3-4 fold increase over the endogenous level, after normalization with the β-actin expression level (Fig. 5A and 1B).

Application of UCN (0.01 µM) or CRF (0.01 µM) on HEK 293 cells transfected with the cloned CRFR1 stimulated cAMP production by about 80-fold over basal levels (82.91±6.40 for CRF and 79.29±6.37 for UCN). This represented a ~10-fold increase in cAMP compared to that for non-transfected HEK 293 cells (Fig. 5B and 1D). In the presence of the CRFR1 antagonist, astressin (1 µM), the level of cAMP production after application of either UCN or CRF was not significantly different from basal level exhibited by control HEK 293 cells (Fig. 5B).

We also examined whether the level of CaV3.2 channel inhibition might be increased in the CRFR1-transfected HEK 293 cells. Fig. 5C shows that upon application of either 0.1 µM UCN or CRF that CaV3.2 currents were significantly reduced by 46.3% (I/I_{control}=0.54±0.04, n=6, P<0.05) and 42.5% (I/I_{control}=0.57±0.04, n=6, P<0.05), respectively. These values represent a ~50% increase in the degree of inhibition of CaV3.2 currents compared to that for non-CRFR1-transfected HEK 293 cells. Dose-response analysis of UCN inhibition in CRFR1 transfected HEK 293 cells showed an enhanced affinity by ~6-fold (IC_{50}=5.07 nM and a Hill coefficient=0.96) (Fig. 5C, lower panel). Again, pre-treatment of the cells with astressin (1 µM) abrogated the inhibitory effects of UCN and CRF on CaV3.2 currents.
Overall, the apparent non-linearity of the relationship between the level of CRFR1 protein, the production of cAMP and the degree of Cav3.2 inhibition may reflect the fact that cAMP/PKA-dependent intracellular signaling factors required to affect inhibition of Cav3.2 channels might be limiting. Alternatively, it may indicate that a signaling mechanism distinct from the well described CRFR1-mediated cAMP/PKA pathway is involved in the inhibition of Cav3.2 T-type channels (see below).

**Involvement of G-proteins in CRFR1-mediated Cav3.2 calcium channel inhibition**

To address whether G-proteins are directly involved in the CRFR1-dependent inhibition of Cav3.2 calcium channels, we dialyzed into cells guanosine-5’-O-(2-thiodiphosphate) (GDP-β-S, 1 mM), a non-hydrolysable GDP analog. It shows that GDP-β-S completely abolished the inhibition of Cav3.2 currents by 0.1 µM UCN (Fig. 6A, left panel; I/Icontrol=0.97±0.03, n=6). In contrast, the nonhydrolyzable GTP analog guanosine-5’-O-(3-thiotriphosphate) (GTP-γ-S, 100 µM) did not prevent the UCN-mediated inhibition of Cav3.2 currents (I/Icontrol=0.58±0.03, n=6, p<0.05) and that the inhibition remained for up to 10 min after the washout of UCN. For control cells not dialyzed with intracellular GTP-γ-S, the inhibition by UCN was reversed and the currents returned to baseline levels within 5 min after the washout of UCN (Fig. 6B). These results indicated that G-proteins are involved in the CRFR1-mediated inhibition of Cav3.2 currents. We further determine that which isoform of Ga was involved in this inhibition. To investigate whether the UCN-mediated response occurred via GaS, we examined its effects on Cav3.2 currents...
in the presence of cholera toxin (CTX), which could catalyze ADP-ribosylation of $\alpha_S$. Pretreatment of Cav3.2 expressing cells with CTX (500 ng/ml for 24h), abolished the UCN-induced inhibition ($I/I_{\text{control}}=0.95\pm0.06$, n=6, Fig. 6C), indicating that the inhibition of Cav3.2 channels is $\alpha_S$ dependent. In addition, after pretreatment of the cells with pertussis toxin (PTX, 200 ng/ml for 24h), which catalyzes the ADP-ribosylation of $\alpha_{i/o}$, UCN still mediated robust inhibition of the Cav3.2 currents ($I/I_{\text{control}}=0.67\pm0.07$, n=5, Fig. 6C). The Cav3.2 currents inhibition induced by CRFR1 activation was sensitive to CTX but not PTX, implicating $\alpha_S$, instead of $\alpha_{i/o}$, was involved. As CTX activated $\alpha_S$, we also examined whether CTX would by itself trigger a shift in half inactivation potential as compared to control cells. In CTX treated cells, we observed a significant shift of $\sim$10 mV in the hyperpolarizing direction of the inactivation potential (66.7±0.6 to -76.8±0.9 mV, and k value from 4.9±0.6 to 6.5±0.8, n=6, P<0.05) (Fig. 6D). To further test whether UCN-mediated Cav3.2 currents inhibition proceeds through a $\alpha_q/11$-mediated pathway, Cav3.2 currents inhibition was examined in the presence of RGS2 (0.1 $\mu$g/$\mu$l), which could selectively bind $\alpha_q/11$-GTP (Kammermeier et al., 1999). In RGS2 expressing cells, UCN still inhibited Cav3.2 currents by $\sim$29% ($I/I_{\text{control}}=0.70\pm0.02$, n=5, Fig. 6C), which is not significantly different compared with the RGS2 non-transfected cells. These results suggested that $\alpha_S$, not $\alpha_{i/o}$ and $\alpha_q/11$, is involved in the UCN-induced Cav3.2 channels inhibition.

**UCN inhibits Cav3.2 channels independent of PLC and downstream protein kinases**

Previous reports have shown that cardiovascular and neuronal protections by UCN are
mediated via activation of either cAMP/PKA or PKC (Markovic et al., 2006). As it has also been reported that T-type calcium channels can be regulated by serine-threonine kinases, tyrosine kinases (TK) and PLC pathways (Chemin et al., 2006), we investigated whether the inhibitory effects of CRFR1 are mediated by these known pathways. After pretreatment of CaV3.2 expressing cells with the selective PKC inhibitors, GF109203X (1 µM) or chelerythrine chloride (1 µM), UCN (0.1 µM) still mediated robust inhibition of the CaV3.2 currents ($I/I_{\text{control}}=0.70\pm0.04$, $n=6$, $p<0.05$ vs. control and $I/I_{\text{control}}=0.70\pm0.08$, $n=5$, $p<0.05$ vs. control, respectively; Fig. 7A, G). Similarly, pretreatment with selective PLC inhibitors U-73122 (3 µM) (Suh et al., 2002) or ET-18-OCH3 (ET, 10 µM), did not prevent the inhibition of CaV3.2 currents by UCN ($I/I_{\text{control}}=0.67\pm0.03$, $n=5$, $p<0.05$ vs. control and $I/I_{\text{control}}=0.68\pm0.05$, $n=5$, $p<0.05$ vs. control, respectively; Fig. 7B, G). The degree of inhibition by UCN in the presence or absence of PKC inhibitors (GF109203X or chelerythrine chloride) and PLC inhibitors (U-73122 or ET-18-OCH3) were not significantly different. After pretreatment with genistein (100 µM), a broad-spectrum inhibitor of TK, and after current amplitudes had stabilized, application of UCN (0.1 µM) inhibited the $I_{Ba}$ by ~27% ($I/I_{\text{control}}=0.73\pm0.06$, $n=5$, Fig. 7C) and which was not different from the inhibition obtained in the absence of genistein. Previous reports also showed that CaMKII and phosphatidylinositol 3-kinase (PI3K) were involved in G-protein-mediated regulation of T-type calcium channels (Chemin et al., 2006). Here, pre-incubation with wortmannin (1 µM), a specific PI3K inhibitor, or KN-93 (0.5 µM), a selective CaMKII inhibitor, failed to prevent the UCN-induced inhibition of CaV3.2 channel currents ($I/I_{\text{control}}=0.69\pm0.08$, $n=5$,
p<0.05 vs. control; Fig. 7D; I/I_{control}=0.65±0.06, n=5, p<0.05 vs. control; Fig. 7E), indicating that the PI3K and CaMKII pathways were not involved. As activation of CRFR1 increases cAMP accumulation and PKA activity (Dautzenberg et al., 2002), blocking PKA activity might be expected to block the effect of UCN-mediated inhibition. To test this possibility we dialyzed the cells with a pipette solution containing PKI 5-24 (1 µM) and found no effect on the CaV3.2 currents over 5 min. After this pretreatment of PKI 5-24 for 5 min, application of 0.1 µM UCN still reduced current amplitudes by 30% (I/I_{control}=0.70±0.05, n=6, p<0.05 vs. control, Fig. 7F) similar to that for currents recorded in the absence of PKI 5-24. Similar inhibition by UCN on CaV3.2 currents with H-89 (0.5 µM) (~29%, I/I_{control}=0.71±0.07, p<0.05 vs. control; n=5) was also obtained (Fig. 7G). Taken together, these results indicate that the inhibition of CaV3.2 currents mediated by CRFR1 is not likely to be mediated via PLC or downstream PKA, PKC, CaMKII, PI3K, or TK signaling pathways.

**UCN inhibits CaV3.2 T-type calcium channels via G-protein βγ subunits (G_{βγ})**

We further tested whether the UCN-induced inhibition of CaV3.2 currents might be mediated via G_{βγ} subunits by introducing into the recording pipette a synthetic peptide, QEHA, (encoding residues 956 to 982 of AC II) which competitively binds G_{βγ} and blocks G_{βγ}-mediated signaling (Chemin et al., 2006). As a control, we used a synthetic SKEE peptide, representing the cognate region of AC III, that does not bind G_{βγ}. Our results showed that intracellular pipette application of QEHA (200 µM) suppressed the effects of 0.1 µM UCN (I/I_{control}=0.97±0.03, n=6 for -UCN; and 0.95±0.04, n=9 for +UCN; Fig. 8A, B). Furthermore, the intracellular application of the QEHA peptide also eliminated the
hyperpolarizing shift in the steady-state inactivation potential normally elicited by UCN (V\(_{1/2}\): -69.6±0.7 mV for control, and V\(_{1/2}\): -67.8±0.9 mV for UCN+QEHA, n=5, P>0.05; Fig. 8E). In contrast, intracellular application of the SKEE peptide (200 µM) did not significantly affect the UCN-induced inhibition of Ca\(_{V}3.2\) currents (I/I\(_{control}\)=0.97±0.02, n=6 for -UCN; and 0.69±0.04, n=7 for +UCN; Fig. 8C, D). Beside intracellular application of QEHA, a membrane-associating C-terminal construct of a G-protein-coupled receptor kinase that sequesters G\(_{b\gamma}\) (MAS-GRK3) was transfected into HEK293 cells to further determine whether G\(_{b\gamma}\) is involved in the inhibition of Ca\(_{V}3.2\) currents. When MAS-GRK3 was co-expressed in Ca\(_{V}3.2\)-expressing HEK293 cells, the robust inhibition by UCN was almost completely eliminated (I/I\(_{control}\)=0.92±0.09, n=5; Fig. 8G). These results demonstrate that Ca\(_{V}3.2\) channel inhibition is mediated via a G\(_{b\gamma}\)-dependent mechanism upon the activation of the CRFR1.

G-protein dependent inhibition of calcium channels has been previously demonstrated to require the G\(_{b2}\) subunit, while the requirement for a specific G\(_{\gamma2}\) subunit is less stringent (Wolfe et al., 2003). To support our results that the inhibition of Ca\(_{V}3.2\) channels induced by CRFR1 activation was via G\(_{b\gamma}\), we investigated whether G\(_{b2}\) or G\(_{\gamma2}\) subunit was endogenously expressed in HEK293 cells. We found by RT-PCR that G\(_{b2}\) and G\(_{\gamma2}\) transcripts are endogenously expressed in HEK293 cells (Fig. 8F, left panel). Endogenous G\(_{b2}\) protein was also detected by anti-G\(_{b2}\) in HEK293 cells (Fig. 8F, right panel). Taken together, these results strongly suggest that native CRFR1 receptors couple via an endogenous G\(_{b\gamma}\)-dependent signaling pathway to mediate the selective inhibition of Ca\(_{V}3.2\) channels.
among the T-type subfamily of calcium channels. This pathway represents a novel CRFR1-coupled mechanism distinct from the previously described PLC and downstream kinase signaling pathways.

Discussion

Corticotropin-releasing factor (CRF) peptides mediate multiple physiological responses including those involved in mammalian responses to stress and inflammation. There is also growing evidence that CRF peptides contribute towards human disorders such as anxiety, depression, and alcohol and drug addictions (Bale and Vale, 2004; Reul and Holsboer, 2002, Gravanis et al., 2005, Bruijnzeel et al., 2005). Several CRF receptor subtypes are known to be expressed and there is considerable interest in determining both the downstream effectors and mechanisms by which these GPCR mediate their widespread physiological functions. In the present paper we demonstrate that activation of CRFR1 receptors by either CRF or UCN robustly inhibits CaV3.2 channels via a GDP–dependent mechanism with no effect on either CaV3.1 or CaV3.3 T-type calcium channels. We also find that the CRFR1-mediated inhibition of CaV3.2 channels is state-dependent, likely occurring through stabilizing the channels in the inactivated state, and the inhibition is reversible after washout of UCN.

HEK293 cells are an appropriate expression system as these cells endogenously and exclusively express the corticotropin-releasing factor receptor 1, the CRFR1 subtype (Dautzenberg et al., 2000) (Fig. 1). Both the activation of endogenous and over-expressed cloned CRFR1 receptors produced robust and selective inhibition of cloned rat and human brain CaV3.2 T-type calcium channels. CRF and UCN have both been shown to bind CRFR1
receptors with similar affinities (Dautzenberg et al., 2000) and we found that CaV3.2 channel inhibition was also not ligand-dependent as both CRF and UCN elicited comparable levels of T-type calcium channels inhibition.

The G-protein dependent inhibition of T-type calcium channels has been previously demonstrated to require the Gβ2 subunit, while the requirement for a specific Gγ subunit is less stringent (Wolfe et al., 2003). These reports have found that inhibition mediated by G-protein decreases the probability of opening of the CaV3.2 calcium channels, while activation and inactivation properties are unaltered. Interestingly, in our present findings, the activation of CRFR1 receptors by either CRF or UCN produced a distinct and significant hyperpolarizing shift in the steady-state inactivation potentials while activation potentials remained unaltered. Unlike the activation of the dopamine receptor 1 which requires both cAMP and G-protein to act in combination to inhibit T-type calcium currents (Drolet et al., 1997), a similar requirement was not evident for the CRFR1-mediated inhibition of the Cav3.2 channels. Evaluating the role of cAMP in CRFR1-mediated inhibition of Cav3.2 channels, application of PKI 5-24, a potent and selective peptide inhibitor of cAMP-dependent protein kinase (PKA), failed to diminish the inhibitory effects of CRFR1 activation indicating that PKA is not directly involved in Cav3.2 inhibition.

Previous reports have shown that T-type calcium currents can also be modulated by the PKC signaling pathway. In one instance, examining GH3 cells showed that native T-type calcium currents are inhibited by OAG (a diacylglycerol, DAG analogue) (Herrington et al., 1992). Similar results were reported for native T-type currents in chick DRG neurons.
although in these neurons the direct inhibition was independent of PKC activation (Hockberger et al., 1989). Here, GF109203X, a selective PKC inhibitor, failed to prevent the inhibition by UCN indicating that PKC is also not involved in Cav3.2 channel inhibition. Previous reports have suggested that phospholipase C (PLC) activation could also be part of the CRFR1-signaling pathway (Radulovic et al., 2003). PLC is a critical component of the phosphoinositol pathway that generates two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), and which are thought to be involved in some types of T-type calcium channel regulation (Chemin et al., 2006). We found that U-73122, a phospholipase C inhibitor, was unable to abrogate the inhibition of Cav3.2 induced by the UCN-mediated activation of CRFR1 receptor, ruling out the possible involvement of the PLC pathway. Furthermore, the inhibition of Cav3.2 channels via the CRFR1 receptor is independent of the activation of tyrosine kinases, CaMKII, and PI3K, through which T-type calcium channels could be regulated. The explanation for the differences remains to be determined but it might be attributed to the diversity of UCN activities on cells or that the A-kinase-anchoring-protein deficient HEK293 cells may not be capable of supporting robust ion channel phosphorylation. In addition, it has been reported that kinase modulation of T-type calcium channels has been shown to be temperature-dependent in which phosphorylation was observed at 37°C but was less obvious when the experiments were performed at room temperature (Chemin et al., 2007). These factors may be involved in CRFR1-mediated Cav3.2 regulations and need to be further examined.

Native and cloned T-type calcium channel properties have been reported to be regulated
by cAMP-dependent PKA, PKC, PKG, calmodulin-dependent protein kinase II and tyrosine kinases through various G-protein coupled receptors (Kim et al., 2006; Herrington et al., 1992). We find that Gβ2 and Gγ2 transcripts and proteins are endogenously expressed in HEK293 cells which suggest the potential involvement of Gβ2 in the selective inhibition of Cav3.2 channels by activation of the CRFR1 receptor. However, other possible subtypes of G-protein βγ subunits could not be excluded, and need to be further investigated. Previous reports have shown that activation of dopamine D1 and D2 receptors could mediate the T-type calcium channels inhibition (Wolfe et al., 2003; Chemin et al., 2006). Nevertheless, the inhibitory mechanism of Cav3.2 channels mediated by CRFR1 receptor is distinctly different from the activation of the dopamine receptors (Wolfe et al., 2003). Our data suggested that the CRFR1-mediated selective inhibition of Cav3.2 was through the stabilization of the channels in their inactivated state, while activation of D1 receptors reduces the open probability of the Cav3.2 channels, and the voltage-dependent steady-state inactivation of the channels remains unchanged. Evidences to suggest our conclusion are: (1) robust hyperpolarized shift of voltage-dependent steady-state inactivation; (2) reversal of the hyperpolarized shift of inactivation potentials after introduction of the QEHA competitor peptide or the washout of UCN from the bath solution; and (3) a reduction in the concentration of UCN to trigger inhibition of Cav3.2 channels when cells are held at the more depolarized membrane potential of -80 mV compared to that for -110 mV. However, a comparison of the rat Cav3.2 (AF290213) used in this report with the human Cav3.2 channel used by the Barrett’s group (AF290213) did not show obvious differences in the
choice of alternatively spliced exons. But, the two clones contained differences in amino acid residues that code for the II-III linker. Whether these species differences in the cytoplasmic regions of the channels, including the II-III linker, are important to account for the differences in mechanisms for inhibition by CRFR1 and dopamine receptors require future work. Besides, we used a 15-second protocol that is different from the 6-second voltage prepulses used by the Barrett’s group (Wolfe et al., 2003). This difference in experimental protocol may be relevant as it has been demonstrated that a shorter duration of the prepulse resulted in a depolarizing shift in the steady-state inactivation in the \(G_{\beta\gamma}\)-mediated inhibition of \(CaV2.2\) channels (McDavid et al., 2006). Nonetheless, besides the observed hyperpolarized shift in steady-state inactivation, we cannot exclude the possibility that the probability of opening (\(P_o\)) of the \(CaV3.2\) channels may also be reduced upon activation of CRFR1 receptors by UCN. There is a possibility that both mechanisms act as a unified pathway to inhibit the \(CaV3.2\) currents upon activation of CRFR1 receptors by UCN.

In the present study, we found that G-protein mediated \(CaV3.2\) channels inhibition was reversible, which is consistent with the previous reports about \(CaV2\) channels. However, our result showed that the kinetics of \(CaV3.2\) channels inhibition induced by CRFR1 activation appeared to be slow, whereas \(G_{\beta\gamma}\)-inhibition of \(CaV2\) channels was very fast (De Waard et al., 1997). The explanation for the differences remains to be determined but it might be attributed to channel internalization during the slowly-developing process or to the diversity of UCN activities on cells. In addition, while \(G_{\beta\gamma}\) subunits inhibit high voltage-gated \(CaV2\)
calcium channels via binding to the I-II linker region, they distinctly bind to the II-III linker of Cav3.2 channels (Ikeda, 1996; Zamponi and Snutch, 1998). The binding of the Gβγ subunits to Cav2.2 channels has been reported to alter the voltage-dependent activation or inactivation properties of the channels, depending upon the frequency of the action potential-like waveforms (APWs) examined (Zamponi and Snutch, 1998). Using low frequency APWs, Cav2.2 N-type channels display a depolarizing shift in the steady-state inactivation potentials when the conditioning pulse is applied for 3 sec (McDavid et al., 2006). Given that the Gβγ effects on Cav2.2 N-type channels can vary depending upon the nature of the stimulus, future studies will be required to dissect the differences in the effects on steady-state inactivation properties of Cav3.2 channels observed after activation of CRFR1. Recent studies by Iftinca and colleagues have identified a depolarizing shift on Cav3.2, but not Cav3.1 and Cav3.3, inactivation properties in response to Rho-associated kinase (ROCK) activation via the endogenous ligand lysophosphatidic acid (LPA) (Iftinca et al., 2007). Like Gβγ regulation on Cav3.2 channels, this mechanism involved the II-III loop and it is tempting to speculate the existence of cross-talk between these two regulatory pathways.

In conclusion, the present study provides evidence that activation of CRFR1 results in the selective inhibition of Cav3.2 T-type calcium currents while Cav3.1 and Cav3.3 were not affected. The mechanism for the inhibition was via a cholera-toxin sensitive, Gβγ-mediated signaling pathway that resulted in a hyperpolarized shift in the steady-state inactivation property of the Cav3.2 calcium channels. Importantly, the effect is reversible.
upon wash-out of the CRFR1 receptor agonists such as CRF or UCN. As UCN affect multiple aspects of cardiac and neuronal physiology and as Ca\textsubscript{V}3.2 channels are expressed widely throughout the cardiovascular and nervous systems, our results point to a novel and functionally relevant CRFR1-calcium channel signaling pathway. In future experiments, it will be interesting to determine whether selective inhibition of Ca\textsubscript{V}3.2 calcium channels via activation of CRFR1 receptors may directly play a role in stress response, affective disorders or in cardiovascular pathophysiology.

**Acknowledgements**

We thank Dr. Brett Adams for the kind gifts of MAS-GRK3ct and EGFP-RGS2 and Dr. David Parker for the human Cav3.1, Cav3.2 and Cav3.3 T-type calcium channels. We also thank Tan Fong Yong and Dejie Yu for excellent technical assistance.
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**Footnotes**

T.W.S is supported by grants from the Biomedical Research Council, (BMRC) Singapore. T.P.S. is supported by an operating grant from the Canadian Institutes for Health Research and a Canada Research Chair.
Legends for Figures

**Fig.1. CRFR1 is endogenously expressed in HEK 293 cells.** A) Detection of CRFR1, CRFR2α and CRFR2β in HEK 293 cells. Total RNA from mouse hypothalamus or left atrium was used as positive controls for CRFR2α and CRFR2β respectively. Negative control indicates RT-PCR reaction without the addition of RT. B) Western blot analysis of CRFR1 (upper panel), CRFR2 (middle panel) and β-actin (lower panel) of HEK 293 membrane protein extracts. Mouse hypothalamus expressing both CRFR1 (~55 kDa) and CRFR2 (~65 kDa) is used as positive control, while β-actin expression controls for loading. Quantifications of band intensities are measures of relative expression levels. C) Membrane expression of CRFR1 determined by confocal microscopy. Merged picture (upper panel) of bright field (lower panel, left) and fluorescent signals of CRFR1 (lower panel, right). Scale bar, 20 µm. Proteins were immunolabelled by a monoclonal goat anti-CRFR1 antibody (green, Alexa). D) Application of UCN (0.01 µM) and CRF (0.01 µM) stimulated robust cAMP production, but not in the presence of antagonist, astressin (1 µM). The results are representative of three independent experiments. *P<0.05 vs. control.

**Fig.2. Effects of UCN-mediated CRFR1 activation on cloned T-type Cav3 calcium channels.** A-C) Lefts panels, representative traces showing the effect of 0.1 µM UCN (black triangles) on Ba^{2+} currents (5 mM) elicited by a -30 mV test pulse. Right panels, current-voltage (I-V) profiles (evoked by a series of depolarizing pulses from a holding potential of -110 mV to test potentials between -90 and 0 mV, in 10-mV increments) obtained for cloned rat Cav3.1 (A, n=13), Cav3.2 (B, n=15) and Cav3.3 subunits (C, n=6). D)
Effect of 0.1 µM UCN on I\textsubscript{Ba} flowing through cloned rat Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2 and Ca\textsubscript{v}3.3 channels elicited by a -30 mV test pulse.

E) Effect of 0.1 µM of UCN on I\textsubscript{Ba} flowing through cloned human Ca\textsubscript{v}3.1 (n=9), Ca\textsubscript{v}3.2 (n=7) and Ca\textsubscript{v}3.3 (n=7) channels elicited by a -30 mV test pulse.

F) Exemplary traces (left panel) and time course (right panel) of Ca\textsubscript{v}3.2 currents obtained before (control), during, and after (washout) the application of 0.1 µM UCN. After washout, Ca\textsubscript{v}3.2 currents recorded in control cells returned to original amplitude.

**Fig. 3. Dose-response and effects of UCN on Ca\textsubscript{v}3.2 channel biophysical properties.**

A,B) Concentration-dependent effects of UCN on Ca\textsubscript{v}3.2 currents. Ca\textsubscript{v}3.2 currents were evoked by a 100 ms depolarizing step to -30 mV from a holding potential of -110 mV (A) and -80 mV (B). Dose-response curves were established by fitting the normalized currents with a sigmoidal Hill equation where \( \frac{I}{I_{\text{control}}} = \frac{1}{1 + 10^{(\log I_{50} - X)}} \frac{h}{n} \) (left panel, see Supplementary data). Exemplary traces in the absence and presence of UCN on evoked Ca\textsubscript{v}3.2 currents are shown in the right panel.

C) Effects of UCN (0.1 µM) on the steady-state activation curve of Ca\textsubscript{v}3.2. Exemplary tail currents evoked by repolarizations to -100 mV after depolarizing test pulses at -90, -60, 0, or 20 mV (upper panel) and normalized steady-state activation curves (lower panel) in the absence (■) and presence (▲) of UCN.

D) Effect of UCN (0.1 µM) on the steady-state inactivation curve of Ca\textsubscript{v}3.2. Typical current traces after 15-s conditioning depolarizing pulses evoked at -120, -90, -60, or -30 mV (upper panel) and normalized steady-state inactivation curves (lower panel) in the absence (■) and
presence (▲) of UCN, and washout (O). Steady-state inactivation curves were obtained by evoking CaV3.2 currents with a test depolarization to -30 mV applied at the end of 15-s conditioning pulses ranging from -120 to -30 mV. E) Effect of UCN (30 nM) on the steady-state inactivation of CaV3.2 (n=6). F) Effect of UCN (0.1 µM) on the recovery from inactivation of CaV3.2 channels. Double-pulse protocol started from a holding potential of -110 mV, with a 1 second depolarizing prepulse to -30 mV, following by a variable length (from 10 ms to 5.12 s) of repolarization to -110 mV, and finally a second 50 ms depolarizing pulse to -30 mV. Sweeps were separated by 20 seconds to allow for complete channel recovery. Fraction recovery was calculated as the ratio of the peak current measured during the second test pulse divided by the peak current measured during the prepulse.

**Fig.4. UCN and CRF inhibit CaV3.2 currents via activation of CRFR1.** CaV3.2 currents were evoked by a 100 ms depolarizing step to -30 mV from a holding potential of -110 mV. Cells were incubated with 1 µM astressin for 30 min before application of UCN or CRF. A) Exemplary traces (left panel) and pooled data (right panel) show the effects of UCN (0.1 µM) on CaV3.2 currents in the presence of astressin (1 µM). B) Exemplary traces (upper panel) and pooled data (lower panel) show the effects of CRF (0.1 µM) on CaV3.2 currents in the absence (left) and presence (right) of astressin.

**Fig.5. Effects of over-expression of CRFR1 on CaV3.2 channels.** A) Western blot analysis showed robust expression of cloned CRFR1 transfected in HEK 293 cells. Anti-CRFR1 immunolabelled mouse hypothalamus (left lane, positive control) and transfected HEK293 cells (right lane) showing the ~55kDa CRFR1, with β-actin as control.
(42 kDa, lower panel). B) Stimulation of cAMP in CRFR1-transfected HEK 293 cells by CRF (0.01 µM) (n=6), UCN (0.01 µM) (n=6) in the presence or absence of astressin (1µM) (n=6). The results are from three independent experiments done in duplicates; *P<0.05 vs. control. C, D) Effects of UCN (C) (n=6) and CRF (D) (n=6) on CaV3.2 currents in HEK 293 cells co-transfected with CaV3.2 and CRFR1 cDNA. Exemplary traces show the effects of UCN (0.1 µM) or CRF (0.1 µM) on CaV3.2 currents in the absence (upper panel, left) (n=6) and presence (upper panel, right) (n=6) of astressin (1 µM). Pooled data is shown as mean±S.E.M. for control and drug treated cells (lower panel, left). Dose-response curves were fitted by a sigmoidal Hill equation. CaV3.2 currents were evoked by a 100 ms depolarizing step to -30 mV from a holding potential of -110 mV. Astressin alone did not affect the amplitudes of CaV3.2 currents (C, lower panel, n=6), *P<0.05 vs. control.

**Fig.6. G-protein mediated inhibition of CaV3.2 channels by UCN.** A) Exemplary traces of CaV3.2 currents before (-UCN) and during 0.1 µM UCN (+UCN) application in the presence of GDP-β-S (1 mM, left panel). Pooled data shows the mean current amplitude ±S.E.M. of CaV3.2 currents (n=6). B) Time course of UCN inhibition on CaV3.2 currents in absence and presence of GTP-γ-S. Exemplary traces of CaV3.2 currents obtained in presence (left panel) of GTP-γ-S, before, during and after washout of the application of 0.1 µM UCN. Application of UCN (dark bar, lower) was for 5 min prior to wash-out for the next 10 min (light bar). Both control cells (■) and cells recorded with GTP-γ-S (0.1 mM) in the patch pipette (▲) show current inhibition as evaluated at time=0 min. After washout, Cav3.2 currents recorded in control cells, in the absence of GTP-γ-S, returned to original amplitude.
while GTP-γ-S-treated cells show a continuous inhibition for up to 10 min. Numbers in parentheses indicate number of cells tested. C) Effects of UCN on CaV3.2 currents in PTX (200 ng/ml for 24h, n=5) or CTX (500 ng/ml for 24h, n=5) pretreated cells, and RGS2 expressing HEK293 cells (0.1 µg/µl, n=5). CaV3.2 currents (A-C) were evoked by a 100 ms depolarizing step to -30 mV from a holding potential of -110 mV. D) Effect of CTX (500 ng/ml) on the steady-state inactivation curve of CaV3.2. Steady-state inactivation curves in the absence (■) or presence (▲) of CTX were obtained by evoking CaV3.2 currents with a test depolarization to -30 mV applied at the end of 15-s conditioning pulses ranging from -120 to -30 mV.

Fig.7. UCN inhibits CaV3.2 channels independently of PKC, PKA, TK, PI3K, CaMKII, and PLC (A-G). CaV3.2 currents were evoked by a 100 ms depolarizing step to -30 mV from a holding potential of -110 mV. Cells were incubated with GF109203X (1 µM) for 30 min (A, n=6), U-73122 (3 µM) for 30 min (B, n=5), genistein (100 µM) for 30 min (C, n=5), wortmannin (1 µM) (D, n=5), and KN-93 (0.5 µM) (E, n=5), or intracellular applied PKI 5-24 (1 µM) (F, n=6). Pretreatment of cells with H-89 (0.5 µM, n=5), ET-18-OCH3 (ET, 10 µM, n=5), and chelertrine chloride (1 µM, n=5) were shown (G). The amplitude of the peak CaV3.2 current in the presence of UCN is normalized to the peak current recorded before drug application. Exemplary traces (A-F, top panels) and pooled data (A-F, bottom panels) show CaV3.2 currents in the absence (-UCN) or presence (+UCN) of UCN (0.1 µM). *P<0.05 vs. control.

Fig.8. Evidence that Gβγ mediates CRFR1-dependent inhibition of CaV3.2 currents. A)
Synthetic QEHA peptide (200 µM) prevented current inhibition by 0.1 µM UCN perfused continuously as indicated by arrow. Inset: without UCN (■); UCN (0.1 µM) (▲). B) Pooled data indicates QEHA peptide abolition of UCN-induced inhibition of CaV3.2 currents in the absence (-UCN, n=6) or presence of UCN (0.1 µM) (+UCN, n=9). C) Control SKEE peptide (200 µM) did not prevent UCN-induced inhibition. Inset: without UCN (■); +UCN (0.1 µM) (▲). D) Pooled data indicates SKEE peptide effect on UCN-induced inhibition of CaV3.2 currents in the absence (-UCN, n=6) and presence of UCN (0.1 µM) (+UCN, n=7); *P<0.05 vs. control. E) Steady-state inactivation curve of QEHA peptide in the absence (■) (n=5) and presence (0.1 µM, n=5) (▲) of UCN. F) Left panel, PCR products of Gβ2 (1023 bp) and Gγ2 (216 bp) amplified from HEK293 mRNA. Negative controls: without adding RT (-RT) or template (water). Right panel, Gβ2 (upper panel) was expressed in HEK293 cells and rat brain, with β-actin as control (lower panel). G) Effects of UCN on CaV3.2 currents in MAS-GRK3 (0.01 µg/µl) co-expressed HEK293 cells (n=5).
Figure 4

A

+Astressin
+UCN
-UCN

200 pA
25 ms

I / Icontrol

B

-Astressin

CRF 0.1μM

Control

200 pA
25 ms

I / Icontrol

-UCN

+UCN

Control

CRF 0.1μM CRF+astressin

*
Figure 5

A

B

C

D

Molecular Pharmacology Fast Forward. Published on February 21, 2008 as DOI: 10.1124/mol.107.043612
Figure 7

A-C: Graphs showing the effect of different compounds on current amplitude. The y-axis represents the current normalized to control, and the x-axis shows the concentration of the compounds. 

D-F: Additional graphs showing the effect of different compounds on current amplitude. The y-axis represents the current normalized to control, and the x-axis shows the concentration of the compounds. 

G: A graph showing the effect of UCN, H-89, ET, and Chelerytrine on current amplitude. The y-axis represents the current normalized to control, and the x-axis shows the concentration of the compounds.