Corticotropin-Releasing Factor Receptors Couple to Multiple G-Proteins to Activate Diverse Intracellular Signaling Pathways in Mouse Hippocampus: Role in Neuronal Excitability and Associative Learning

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Corticotropin-releasing factor (CRF) exerts a key neuroregulatory control on stress responses in various regions of the mammalian brain, including the hippocampus. Using hippocampal slices, extracts, and whole animals, we investigated the effects of human/rat CRF (h/rCRF) on hippocampal neuronal excitability and hippocampus-dependent learning in two mouse inbred strains, BALB/c and C57BL/6N. Intracellular recordings from slices revealed that application of h/rCRF increased the neuronal activity in both mouse inbred strains. Inhibition of protein kinase C (PKC) by bisindolylmaleimide I (BIS-I) prevented the h/rCRF effect only in hippocampal slices from BALB/c mice but not in slices from C57BL/6N mice. Inhibition of cAMP-dependent protein kinase (PKA) by H-89 abolished the h/rCRF effect in slices from C57BL/6N mice, with no effect in slices from BALB/c mice. Accordingly, h/rCRF elevated PKA activity in hippocampal slices from C57BL/6N mice but increased only PKC activity in the hippocampus of BALB/c mice. These differences in h/rCRF signal transduction were also observed in hippocampal membrane suspensions from both mouse strains. In BALB/c mice, hippocampal CRF receptors coupled to $G_{q/11}$ during stimulation by h/rCRF, whereas they coupled to G_s, $G_{q/11}$, and G_i in C57BL/6N mice. As expected on the basis of the slice experiments, h/rCRF improved context-dependent fear conditioning of BALB/c mice in behavioral experiments, and BIS-I prevented this effect. However, although h/rCRF increased neuronal spiking in slices from C57BL/6N mice, it did not enhance conditioned fear. These results indicate that the CRF system activates different intracellular signaling pathways in mouse hippocampus and may have distinct effects on associative learning depending on the mouse strain investigated.

Key words: **neuronal excitability; h/rCRF; PKC; PKA; classical fear conditioning; G-protein; mouse; hippocampus**

Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide that has been implicated in both physiological and behavioral responses to stress (Spiess et al., 1981; Vale et al., 1981). During exposure to stress, CRF can be secreted directly from nerve terminals located in the hippocampus. Specifically, numerous, large CRF-immunoreactive neurons have been found in the hippocampal CA1 and CA3 region (Swanson et al., 1983; Merchenthaler, 1984). Previous studies have shown the modulation of hippocampus-dependent learning and memory by CRF. Human/rat CRF (h/rCRF) injected directly into the dentate gyrus consistently enhanced memory retention in rats in a one-way passive avoidance task (Lee et al., 1993). Injection of h/rCRF into the dorsal hippocampus shortly before the training enhanced context- and tone-dependent fear conditioning in BALB/c mice through CRF receptor 1 (CRFR1) (Radulovic et al., 1999). In addition to the effects on hippocampal learning tasks, CRF exerts a profound action on hippocampal neuronal activity. Recent studies have demonstrated that h/rCRF produces a long-lasting enhancement of synaptic efficacy in the rat hippocampus *in vivo* (Wang et al., 1998, 2000). h/rCRF reversibly increases the spiking of rat hippocampal pyramidal cells (Aldenhoff et al., 1983) and enhances the amplitude of CA1 population spikes evoked by stimulation of the Schaffer collateral pathway (Hollrigel et al., 1998). We showed recently that application of h/rCRF facilitates the induction and stability of long-term potentiation (LTP) under defined stimulation conditions in area CA1 of mouse hippocampal slices (Blank et al., 2002).

To examine the signal transduction pathways of h/rCRF in mouse hippocampus, we studied the G-protein and second-messenger activation after CRF receptor stimulation in hippocampi of two mouse inbred strains, C57BL/6N and BALB/c. We chose these two inbred strains because C57BL/6 and BALB/c mice have repeatedly been found to differ strongly in several behavioral responses (Oliverio et al., 1973; Peeler and Nowakowski, 1987; Beuzen and Belzung, 1995) and in neurodevelopmental and neurochemical parameters (Nowakowski, 1984). For example, BALB/c mice exhibit stronger anxiety-like responses in the light–dark choice test (Beuzen and Belzung, 1995), in the

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open-field paradigm (Oliverio et al., 1973), and in a runway traversal locomotor activity test (Peeler and Nowakowski, 1987). The impact of h/rCRF on neuronal excitability of CA1 pyramidal cells was investigated in hippocampal slices from both mouse inbred strains. Finally, we investigated the effect of h/rCRF on hippocampus-dependent learning in C57BL/6N and BALB/c mice.

Materials and Methods

Animals. Experiments were performed with male BALB/c and C57BL/6N mice (Charles River, Sultzfeld, Germany) 9–12 weeks old. The mice were housed individually and maintained on a 12 hr light/dark cycle (lights on at 7:00 A.M.) with access to food and water *ad libitum*. All experimental procedures were in accordance with the European Council Directive (86/609/EEC) and the Animal Section Law under the supervision of the District Government of Braunschweig (Lower Saxony, Germany).

Electrophysiology. Mice were briefly anesthetized with isoflurane and then decapitated. In <1 min, the skull was opened, and the brain was removed and transferred to ice-cold artificial CSF (aCSF) solution of the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 1.5 $MgSO_4$, 2 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose, pH 7.4 (equilibrated with 95% O_2–5% CO_2). Hippocampi were dissected from the chilled brain hemispheres on ice. Transverse hippocampal slices (400 μm) were obtained on a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK) and kept submerged (minimum of 1 hr at room temperature before recordings) in aCSF.

Conventional intracellular recording techniques were used, with glass microelectrodes filled with 3 M potassium acetate. Microelectrodes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) on a horizontal electrode puller (Zeitz-Instrumente, Augsburg, Germany). The microelectrode tip resistances ranged from 60 to 100 MΩ for recordings from mouse hippocampal neurons. Intracellular signals were recorded with a single-electrode voltage-clamp amplifier (SEC-05L; NPI Electronics, Tamm, Germany), which performed current-clamp measurements at high switching frequencies in the range of 25–30 kHz. Bridge balance was monitored throughout the experiment and adjusted as required. Traces were stored on a computer using Pulse 7.4 software (Heka, Lambrecht, Germany) for offline analysis. For intracellular recordings, only neurons were included that exhibited overshooting action potentials, stable membrane potentials of at least −60 mV, and input resistances of ≤35 MΩ. Input resistance was determined by measuring the voltage deflection at the end of a 100 msec hyperpolarization current step (−0.2 nA). Depolarizing current pulses of 3–5 msec duration were injected through the recording electrode to elicit single action potentials. Spike frequency adaptation was investigated by injecting each cell with a series of 600 msec depolarizing current pulses (0.2–1 nA; increment, 100 pA). To compare neuronal responses, the membrane potential of each cell was manually clamped to −65 mV by discontinuous current injection. In all electrophysiological experiments, *n* values represent the number of slices.

Drugs. h/rCRF (Rühmann et al., 1996) and [Glu^{11,16}] astressin (Eckart et al., 2001) were synthesized in our laboratory as described. H-89 and bisindolylmaleimide I (BIS-I) were obtained from Calbiochem (San Diego, CA). Phorbol 12,13-dibutyrate (PDBu) and 4α-phorbol were both purchased from Sigma (St. Louis, MO).

Drug treatment. [Glu^{11,16}] astressin was dissolved in aCSF to a final concentration of 280 μM. h/rCRF stock solutions were prepared in 10 mM acetic acid. For cannula injections, dilutions in aCSF to a final concentration of 400 ng/μl were prepared immediately before the experiments. The final pH of the peptide solution was 7.4. BIS-I was stored as 1 mM stock solution in dimethylsulfoxide (DMSO). For injection, the solution was diluted with aCSF to a final concentration of 0.4 nmol/μl. PDBu and 4α-phorbol were both dissolved in DMSO to 5 μg/μl. For injection, the solutions were diluted with aCSF to a final concentration of 10 ng/μl.

Cannulation. Double guide cannulas (C235; Plastics One, Roanoke, VA) were implanted using a stereotactic holder during anesthesia with 1.2% avertin (0.02 ml/g, i.p.) under aseptic conditions as described previously (Stiedl et al., 2000; Blank et al., 2002). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull of the mouse with dental cement. The cannulas were placed into both lateral brain ventricles, with anteroposterior (AP) coordinates zeroed at bregma AP 0 mm, lateral 1 mm, and depth 3 mm or directed toward both dorsal hippocampi, AP −1.5 mm, lateral 1 mm, and depth 2 mm (Franklin and Paxinos, 1997). The animals were allowed to recover for 4–5 d before the experiments started. On the day of the experiment, bilateral injections were performed using an infusion pump (CMA/100; CMA Microdialysis, Solna, Sweden) at a constant rate of 0.33 μl/min (final volume, 0.25 μl per side). Cannula placement was verified *post hoc* in all mice by injection of methylene blue. For electrophysiological experiments, double guide cannula placement was verified by unilateral methylene blue injection.

Fear conditioning. The fear conditioning experiments were performed as described previously (Stiedl et al., 2000; Blank et al., 2002) using a computer-controlled fear conditioning system (TSE, Bad Homburg, Germany). Fear conditioning was performed in a Plexiglas cage (36 × 21 × 20 cm) within a fear conditioning box constantly illuminated (12 V, 10 W halogen lamp, 100–500 lux). In the conditioning box, a high-frequency loudspeaker (KT-25-DT; Conrad, Hirschau, Germany) provided constant background noise [white noise, 68 dB sound pressure level (SPL)]. The training (conditioning) consisted of a single trial. The mouse was exposed to the conditioning context (180 sec) followed by a tone (30 sec, 10 kHz, 75 dB SPL, pulsed 5 Hz). After termination of the tone, a foot shock (0.7 mA, 2 sec, constant current) was delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 sec after shock termination to avoid an aversive association with the handling procedure. Under these conditions, the context served as background stimulus. Background contextual fear conditioning but not foreground contextual fear conditioning, in which the tone is omitted during training, has been shown to involve the hippocampus (Phillips and LeDoux, 1994). Memory tests were performed 24 hr after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 sec without tone or shock presentation (with background noise). Freezing, defined as lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice (Blanchard and Blanchard, 1969; Bolles and Riley, 1973; Fanselow and Bolles, 1979) by a time-sampling procedure every 10 sec throughout the memory test. In addition, activity-derived measures (inactivity, mean activity, and exploratory area) were recorded by a photobeam system (10 Hz detection rate).

Protein kinase A and protein kinase C assays. cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) activities were assayed using the PepTag Assay for nonradioactive detection of PKC or PKA (Promega, Madison, WI) on the basis of the phosphorylation of fluorescent-tagged PKC- or PKA-specific peptides. After incubation in either aCSF or 250 nM h/rCRF for 30 min, hippocampal slices were placed in ice-cold homogenization buffer [20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 48 mM mercaptoethanol, 0.32 M sucrose, and freshly added protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany)]. The tissue was homogenized with a Teflon-plastic homogenizer and centrifuged at 100,000 × *g* for 30 min in a Beckman Instruments (Fullerton, CA) XL-80 ultracentrifuge. The resulting supernatant contained the PKA preparation. The pellet was rehomogenized in homogenization buffer and sonicated (four times for 15 sec), incubated for 30 min with Triton X-100 (0.2%), and centrifuged at 100,000 × *g* for 30 min. The supernatant contained the membrane-bound PKC preparation, which was used for the PKC assay. Protein concentrations were determined with the Bradford assay (Bio-Rad, Munich, Germany). The assay was performed as described by the manufacturer. An aliquot of the PKA preparation was incubated for 30 min at 30°C in PepTag PKA 5× reaction buffer (in mM: 100 Tris-HCl, pH 7.4, 50 $MgCl_2$, and 5 ATP) and 0.4 μg/μl of the PKA-specific peptide substrate PepTag A1 (L-R-R-A-S-L-G; Kemptide). The same procedure was used for the PKC preparations that were incubated in PepTag PKC reaction buffer (in mM: 100 HEPES, pH 7.4, 6.5 $CaCl_2$, 5 DTT, 50 $MgCl_2$, and 5 ATP) containing 0.4 μg/μl of the PKC-specific peptide substrate PepTag C1 (P-L-S-R-T-L-S-V-A-A-K). The reaction was stopped by heating to 95°C for 10 min. Phosphorylation of the PKA- and PKC-specific substrates was used to measure

kinase activity. Phosphorylated and unphosphorylated PepTag peptides were separated on a 0.8% agarose gel by electrophoresis. The gel was photographed with a transilluminator, and bands indicating substrate phosphorylated by PKA or PKC were quantified by densitometry (WinCam 2.2; Cybertech, Berlin, Germany). For the PKA and PKC assays, 4.5 and 6.5 μg of protein, respectively, were applied.

Western blotting. Hippocampi of C57BL/6N or BALB/c mice were dissected out and homogenized in TBS (10 mM Tris, pH 7.6, and 150 mM NaCl), 10% sucrose, and a protease inhibitor cocktail tablet (Boehringer Mannheim). The homogenate was centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was removed, and the membrane pellet was resuspended in a second identical wash step and centrifuged again at 20,000 × *g* for 30 min at 4°C. The supernatant was removed, and the membrane pellet was resuspended in TBS, 1 mM EDTA, and 1% sodium cholate and incubated for 60 min with constant mixing at 4°C. By centrifugation at 155,000 × *g* for 60 min (4°C), the supernatant containing soluble membrane proteins was obtained. Protein concentrations were determined with a Bradford assay (Bio-Rad). Equal amounts of protein for each group were separated on a 10% SDS gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA) using a semidry transfer apparatus. The blot was probed using an anti-$G_{q/11}\alpha$ subunit antibody (1:4000; Calbiochem), an anti-$G_s\alpha$ subunit antibody (1:1000; NEN, Boston, MA), or an antibody directed against $G\alpha_{i\text{-}1,2,3}$-protein (1:200; Calbiochem). These antibodies were detected by secondary antibodies conjugated to alkaline phosphatase. CDP-*Star* (Tropix, Bedford, MA) was used as a chemiluminescence substrate. During dephosphorylation, the substrate decomposed, producing a prolonged emission of light that was imaged on photographic film (Fuji Super RX; Fujifilm, Tokyo, Japan). The relative density of the bands was measured by densitometry using the software WinCam 2.2 for Windows.

Preparation of hippocampal membranes. Membranes were prepared as described previously (Grammatopoulos et al., 2001). Hippocampi of C57BL/6N or BALB/c mice were homogenized in Dulbecco's PBS (extraction buffer) containing 10 mM $MgCl_2$, 2 mM EGTA, 1.5 gm/l bovine serum albumin (BSA) (w/v), 0.15 mM bacitracin, and 1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.2, at 22°C. The homogenate was centrifuged at 1500 × *g* for 30 min at 4°C. The pellet was discarded, and the supernatant was spun at 45,000 × *g* for 60 min at 4°C. Using the homogenizer, the final pellet was resuspended in 10 ml of the described extraction buffer. The protein concentration of the membrane suspension was determined using the bicinchoninic acid method (Smith et al., 1985) with BSA as a standard.

Synthesis of ^{32}P-GTP-γ-azidoanilide and photolabeling of Gα subunits. ^{32}P-GTP-γ-azidoanilide (^{32}P-GTP-AA) was synthesized as described previously (Schwindinger et al., 1998). Mouse hippocampal membranes were incubated in a darkroom with or without h/rCRF (100 nM) for 5 min at 30°C before the addition of 5 μCi of ^{32}P-GTP-AA in 120 μl of 50 mM HEPES buffer, pH 7.4, containing 30 mM KCl, 10 mM $MgCl_2$, 1 mM benzamidine, 5 μM GDP, and 0.1 mM EDTA. After incubation for 3 min at 30°C, membranes were collected by centrifugation and resuspended in 100 μl of the above buffer containing 2 mM glutathione, placed on ice, and exposed to UV light (254 nm) at a distance of 5 cm for 5 min.

G-protein immunoprecipitation. ^{32}P-GTP-AA-labeled G-proteins were precipitated by centrifugation and solubilized in 120 μl of 2% SDS. Then, 360 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 1% (v/v) Triton X-100, 1% (v/v) deoxycholate, 0.5% (w/v) SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, and 10 μg/ml aprotinin was added, and insoluble material was removed by centrifugation. Solubilized membranes were divided into 100 μl aliquots, and each aliquot was incubated with 10 μl of undiluted G-protein antiserum at 4°C. Subsequently, 50 μl of protein A-Sepharose beads (10% w/v in the above buffer) was added, and the incubation was continued at 4°C overnight. The beads were collected by centrifugation, washed twice, and dried under vacuum. The immune complexes were dissociated from protein A by reconstitution in Laemmli's buffer (100 μl) and boiling in a water bath for 5 min. Samples were subjected to gel electrophoresis. The gels were stained with Coomassie blue, dried, and exposed to Fuji x-ray film at −70°C for 2–5 d. The relative density of the bands was measured by optical density scanning using the software Scion Image-β 3b for Windows (Scion, Frederick, MD).

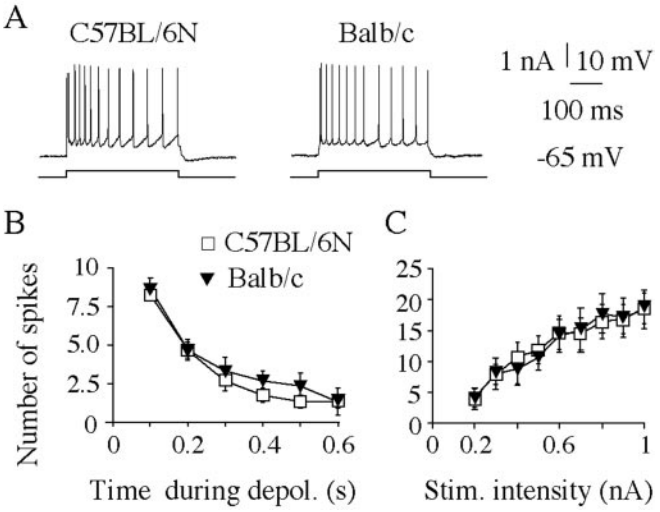

Figure 1. *A*, Representative intracellular recordings from CA1 pyramidal neurons in hippocampal slices from C57BL/6N mice and BALB/c mice showing responses to 600 msec depolarizing current pulses. *B*, Number of spikes elicited in 100 msec fragments during a single depolarizing (*depol.*) current pulse (600 msec, 1 nA). *C*, Plot of the number of spikes elicited by a 600 msec depolarizing pulse versus stimulus (*stim.*) intensity.

Statistics. Statistical comparisons were made by using Student's *t* test and ANOVA. Data were expressed as mean ± SEM. Significance was determined at the level of $p < 0.05$.

Results

In hippocampal slices from both C57BL/6N and BALB/c mice, stable intracellular recordings were obtained from CA1 pyramidal neurons. The resting membrane potentials of pyramidal neurons from C57BL/6N mice (−68.4 ± 0.9 mV; $n = 38$) and BALB/c mice (−69.6 ± 1.2 mV; $n = 33$) did not differ significantly, nor did the membrane input resistance of CA1 cells of C57BL/6N mice (56.5 ± 3.5 MΩ; $n = 38$) and BALB/c mice (58.7 ± 3 MΩ; $n = 33$) differ significantly from each other. Likewise, there were no significant differences between the spike amplitudes, with values of 63.4 ± 1.2 mV ($n = 19$) found for C57BL/6N mice and 62.2 ± 1.3 mV ($n = 13$) for BALB/c mice.

When mouse CA1 pyramidal cells of either strain were excited by prolonged depolarizing current pulses, they responded with prolonged spiking (Fig. 1*A*). The discharge rate was highest at the beginning of the current pulse (1 nA) and declined to a steady rate during the course of the depolarizing pulse (Fig. 1*B*). Increasing stimulus intensities elicited enhanced neuronal spiking. In response to strong depolarizing current pulses (1 nA, 600 msec), C57BL/6N and BALB/c mouse pyramidal cells fired 18.7 ± 2.5 ($n = 12$) and 18.6 ± 7.3 ($n = 7$) spikes, respectively (Fig. 1*C*).

h/rCRF was applied to mouse hippocampal slices to investigate the effects on the neuronal spiking behavior. The number of spikes elicited by a 600 msec depolarizing current pulse was increased by 88 ± 24% ($n = 7$; $p < 0.05$) in C57BL/6N (data not shown) mice and by 87 ± 39% ($n = 8$; $p < 0.05$) in BALB/c mice after addition of 250 nM h/rCRF (Fig. 2*A*). After 30 min of washing in aCSF, spiking was still elevated by 85 ± 21% ($n = 7$; $p < 0.05$) in C57BL/6N (data not shown) mice and by 86 ± 35% ($n = 8$; $p < 0.05$) in pyramidal cells from BALB/c mice (Fig. 2*A*). Within 90 min, the firing rate returned to control values and was no longer significantly different from the firing rate before h/rCRF application in C57BL/6N mice (2 ± 6%; $n = 7$) and

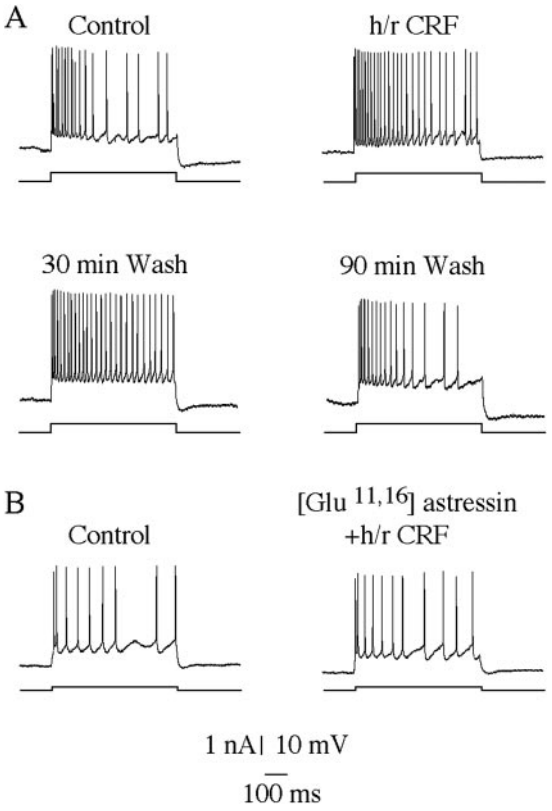

Figure 2. Effect of h/rCRF on neuronal spiking of BALB/c mouse CA1 pyramidal cells elicited by 600 msec depolarizing current pulses. *A*, Traces were sampled before, during, and 30 and 90 min after h/rCRF (250 nM, 10 min) application. *B*, Recordings were made before and 20 min after coapplication of h/rCRF (250 nM, 10 min) and [Glu ^{11,16}] astressin (1 μM) over a period of 10 min. Pulse intensity was kept constant during each experiment; holding potential, −65 mV.

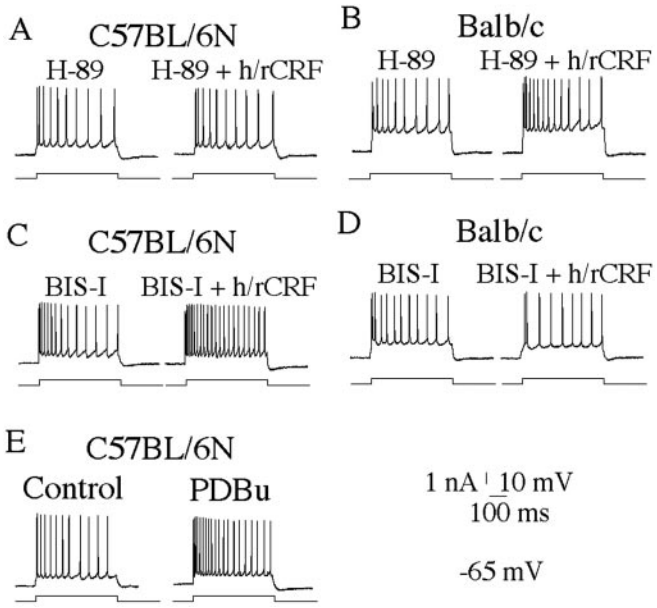

Figure 3. Effect of the PKC inhibitor BIS-I and of the PKA inhibitor H-89 on h/rCRF-mediated modulation of excitability. Representative recordings in CA1 pyramidal cells from C57BL/6N (*A*, *C*) and BALB/c (*B*, *D*) mice showing the effect of 250 nM h/rCRF applied over a period of 20 min after preincubation with BIS-I (1.2 μM, 1 hr) or H-89 (10 μM, 3 hr). *E*, Spiking behavior of CA1 pyramidal cells from C57BL/6N before and during bath application of PDBu (100 nM). Pulse intensity was kept constant during each experiment.

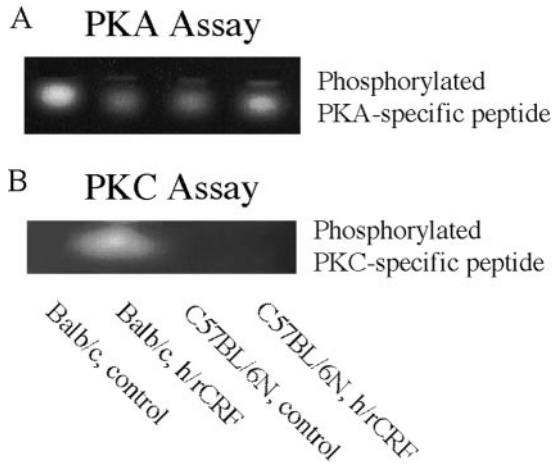

Figure 4. PKA and PKC activity in hippocampal slices of C57BL/6N and BALB/c mice. Hippocampal slices were incubated in either 250 nM h/rCRF (30 min) or aCSF (30 min, as control). Partially purified homogenates of these slices ($n = 11$) from six animals were tested for the ability to phosphorylate a PKA-specific (L-R-R-A-S-L-G; Kemptide) (*A*) or a PKC-specific (P-L-S-R-T-L-S-V-A-A-K) (*B*) peptidic substrate in a nonradioactive assay. Identical amounts of protein were used for each sample.

BALB/c mice (4 ± 7%; $n = 8$) (Fig. 2*A*). In CA1 hippocampal neurons from both mouse strains, the h/rCRF effect was antagonized by the CRF receptor antagonist [Glu ^{11,16}] astressin (Fig. 2*B*).

In subsequent experiments, we investigated the underlying second-messenger pathways activated by h/rCRF to increase the neuronal excitability in mouse hippocampus. When slices were preincubated with the selective and cell-permeable PKA inhibitor H-89, the firing rate of hippocampal neurons from C57BL/6N mice was not significantly enhanced by h/rCRF (7 ± 5%; $n = 6$; p = NS) (Fig. 3*A*). In contrast, after the H-89 treatment, h/rCRF still enhanced the neuronal activity of hippocampal neurons from BALB/c mice by 55 ± 10% ($n = 5$; $p < 0.05$) (Fig. 3*B*). When hippocampal slices from BALB/c mice were preincubated with BIS-I, a highly selective cell-permeable PKC inhibitor, subsequent h/rCRF application did not significantly increase the neuronal firing rate (4 ± 2%; $n = 5$; p = NS) (Fig. 3*D*). In contrast, after BIS-I treatment, h/rCRF application still enhanced neuronal spiking in CA1 cells from C57BL/6N mice by 52 ± 9% ($n = 6$; $p < 0.05$) (Fig. 3*C*). In these mice, bath application of the potent PKC activator PDBu increased the spiking behavior of hippocampal neurons by 79 ± 24% ($n = 5$; $p < 0.05$) (Fig. 3*E*).

Under basal conditions, PKA activity, as measured by the phosphorylated state of a PKA-specific target peptide, was lower in hippocampal brain slices from C57BL/6N mice than in hippocampal brain slices from BALB/c mice. After h/rCRF treatment, PKA activity in hippocampal slices from C57BL/6N mice was increased, whereas it was decreased in hippocampal slices from BALB/c mice compared with the corresponding PKA activities in control slices (Fig. 4*A*). Because membrane translocation of PKC is considered to be an indicator of PKC activation (Kraft and Anderson, 1983), we assayed PKC activity in the membrane-bound fraction of hippocampal slice homogenates. After h/rCRF incubation of slices, PKC activity was apparent only in hippocampal slices of BALB/c mice (Fig. 4*B*), with no detectable PKC activity in hippocampal slices of C57BL/6N mice. The observed differences in the activation of second-messenger pathways after h/rCRF application can be attributed to variations in the abundance of G-proteins. However, using immunoblots, we

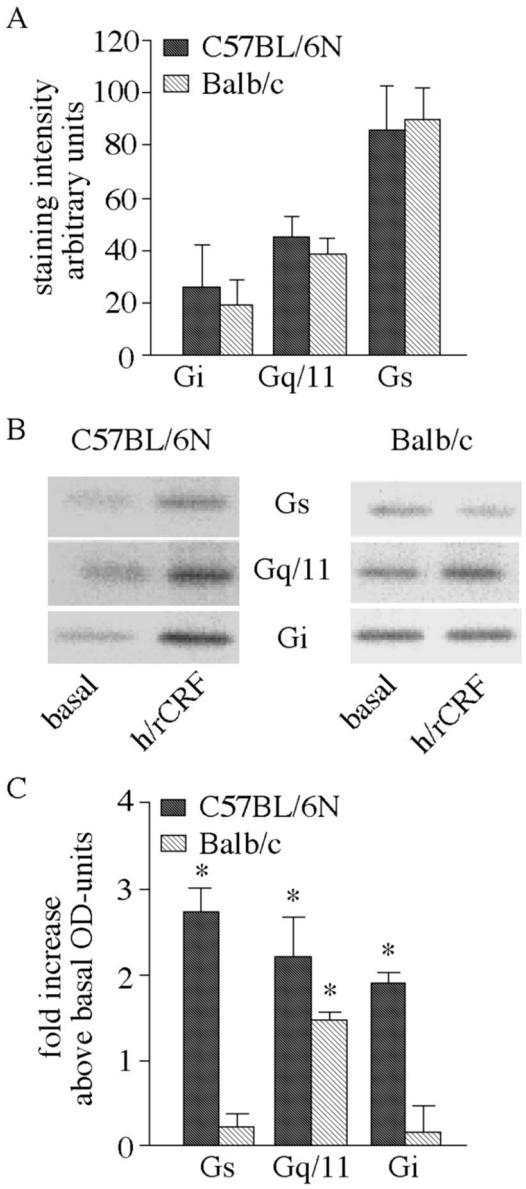

Figure 5. h/rCRF-induced activation of G_s-, G_I-, and $G_{q/11}$-proteins. *A*, Basal levels of G_s, G_i, and $G_{q/11}$ in hippocampal membrane fractions from C57BL/6N and BALB/c mice. The bar graph summarizes Western blot data (mean ± SEM) of three independent experiments each with five animals per mouse strain. *B*, Autoradiograph of h/rCRF-induced photolabeling of Gα subunit subtypes from hippocampal membranes of C57BL/6N ($n = 30$) and BALB/c ($n = 30$) mice. Membranes were incubated with ^{32}P-GTP-AA in the presence and absence of h/rCRF (100 nM), followed by UV cross-linking and immunoprecipitation of the Gα subunit subtypes using specific antibodies. Proteins were resolved by SDS-PAGE, followed by autoradiographic visualization. *C*, Bar graph summarizing autoradiograph data. *$p < 0.05$ indicates statistically significant differences.

did not observe any significant differences in the abundance of G_s-, G_I-, and $G_{q/11}$-proteins (Fig. 5*A*). In subsequent experiments, we analyzed CRF receptor-mediated activation of G-proteins in hippocampal membrane suspensions. After h/rCRF application, the nonhydrolyzable GTP analog ^{32}P-GTP-AA binds to the GTP-binding site of activated G-protein α-chains and forms a stable complex, which can be identified with specific G_α antibodies (Offermanns et al., 1991). Thus, specific activation of individual G-proteins can be demonstrated. In hippocampal membranes of C57BL/6N mice, h/rCRF induced activation of G_s, G_i, and $G_{q/11}$ with an order of potency $G_s > G_{q/11} > G_i$, whereas in hippocampal membranes of BALB/c mice, only stimulation of $G_{q/11}$ was detectable after h/rCRF treatment (Fig. 5*B*,*C*).

To further delineate the impact of the observed different h/rCRF-mediated signaling pathways on learning and memory, mice were subjected to contextual fear conditioning, a hippocampus-dependent associative learning paradigm (Kim and Fanselow, 1992; Phillips and LeDoux, 1992, 1994). When BALB/c mice received a bilateral h/rCRF injection intracerebroventricularly ($n = 7$) (Fig. 6*A*) and were trained 2 hr after the injection, contextual fear was significantly enhanced compared with naive ($p < 0.05$; $n = 9$) (Fig. 6*A*) and vehicle-treated ($p < 0.01$; $n = 30$) animals (Fig. 6*A*). This h/rCRF effect was prevented by either [Glu^{11,16}] astressin ($n = 7$) or BIS-I ($n = 7$). Both compounds had no effect when applied alone (Fig. 6*A*). To exclude the possibility that h/rCRF was acting via a brain structure that has projections to the hippocampus, h/rCRF and BIS-I were administered locally into the dorsal hippocampus. Contextual fear was also significantly elevated when h/rCRF was injected intrahippocampally ($p < 0.05$; $n = 6$) (Fig. 6*A*). BIS-I had no effect when administered intrahippocampally alone ($n = 5$) but abolished the h/rCRF-mediated enhancement of conditioned fear ($n = 6$; p = NS) (Fig. 6*A*). In C57BL/6N mice, freezing was not significantly changed when h/rCRF was injected 2 hr ($n = 15$; p = NS) (Fig. 6*B*) before the training session. However, injection of PDBu 2 hr before the training ($n = 9$) significantly enhanced contextual fear compared with the contextual fear of naive ($p < 0.05$; $n = 9$) (Fig. 6*B*) and vehicle-treated ($p < 0.05$; $n = 27$) animals (Fig. 6*B*). There was no significant change of contextual fear after injection of the inactive isomer 4α-phorbol (p = NS; $n = 4$) (Fig. 6*B*).

Discussion

In this study, we provide evidence that signal processing of h/rCRF in mouse hippocampus was mediated through two different signal transduction pathways. Slice experiments revealed that h/rCRF increased CA1 hippocampal neuronal activity via PKC in the hippocampus of BALB/c mice and via PKA in the hippocampus of C57BL/6N mice. Hippocampus-dependent learning evaluated by context-dependent fear conditioning was improved only in BALB/c mice after h/rCRF injection but not in C57BL/6N mice. Western blots from mouse hippocampal membrane proteins showed identical amounts of the relevant G-protein subunits in both mouse strains. However, application of h/rCRF induced activation of $G_{q/11}$ in the hippocampus of BALB/c mice and G_s, $G_{q/11}$, and G_i in the hippocampus of C57BL/6N mice. h/rCRF increased neuronal excitability in the hippocampus of both mouse strains but improved fear conditioning only in BALB/c and not in C57BL/6N mice. Thus, it might be concluded that the h/rCRF-induced increase in neuronal activity is not sufficient to enhance fear conditioning but that the stimulation of specific intracellular signaling cascades is also required. In support of this hypothesis, we observed recently that inhibition of hippocampal Ca^{2+}/calmodulin-dependent kinase II (CaMKII) prevents stress-mediated facilitation of fear conditioning with no effect on primed hippocampal LTP (Blank et al., 2002). This observation implies that facilitation of neuronal activity was necessary along with activation of CaMKII to enhance fear conditioning.

In mouse hippocampus, CRFR1 was reported to be the predominant CRF receptor subtype (Van Pett et al., 2000). However, we cannot conclude whether the differences in G-protein activation result from the different coupling of a single receptor sub-

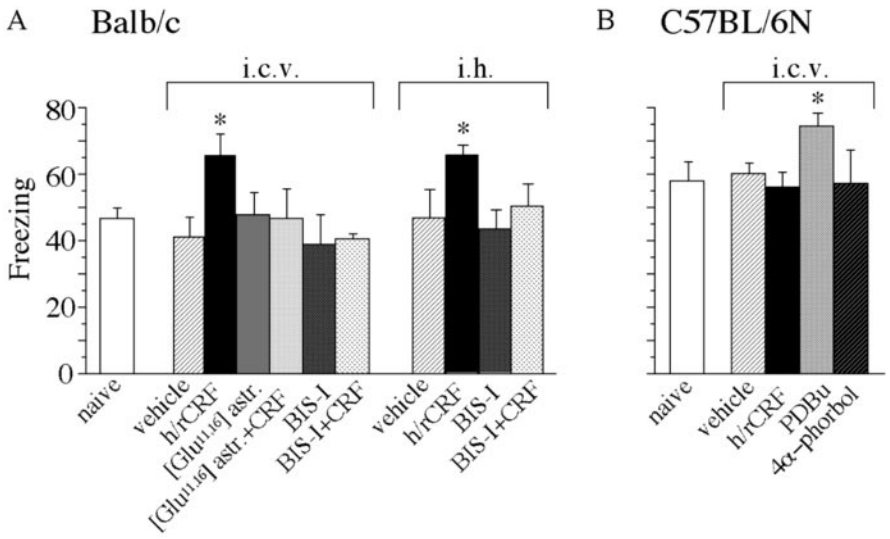

Figure 6. Effect of h/rCRF on context-dependent fear conditioning of BALB/c (*A*) and C57BL/6N (*B*) mice injected with aCSF, h/rCRF, [Glu ^{11,16}] astressin, PDBu, or 4α-phorbol 2 hr before the training as indicated. For combined treatment, [Glu ^{11,16}] astressin and BIS-I were given 15 min before h/rCRF application. Freezing was measured in the retention test performed 24 hr after training. Injections were performed intracerebroventricularly (*i.c.v.*) or intrahippocampally (*i.h.*) as indicated. *$p < 0.05$ indicates statistically significant differences versus vehicle-injected animals and naive animals.

type or the different coupling in combination with differences in the distribution profile of CRF receptor subtypes in the hippocampus of both mouse strains.

All of the known effects of CRF in the rat hippocampus involve receptor-coupled activation of G_s and adenylate cyclase and an increase in cellular levels of cAMP (Chen et al., 1986; Battaglia et al., 1987; Pihoker et al., 1992; Haug and Storm, 2000). This is in agreement with the activation of G_s in hippocampi of C57BL/6N mice. However, it was reported that h/rCRF also activates the phospholipase C (PLC)–PKC-pathway in rat Leydig cells (Ulisse et al., 1990), in cultured rat astrocytes (Takuma et al., 1994), in rat cerebellum (Miyata et al., 1999), and in rat cerebral cortex (Grammatopoulos et al., 2001). In addition, Malenka et al. (1986) reported that activation of PKC markedly reduces accommodation of neuronal spiking in rat hippocampal pyramidal cells. Both aspects together are in agreement with our conclusion that, in BALB/c mice, $G_{q/11}$-dependent PKC activation mediated the h/rCRF-induced increase of neuronal activity. Surprisingly, PKA activity was reduced in hippocampal slices from BALB/c mice during application of h/rCRF. This effect might be initiated by $G_{q/11}$ stimulation, which has been shown to be associated with an increase of the abundance of G-protein $\beta\gamma$ subunits. These subunits inhibit type I adenylyl cyclase and thereby decrease PKA activity (Taussig et al., 1993; Chen et al., 1997). Activation of $G_{q/11}$, G_s, and G_i, as observed in hippocampi of C57BL/6N mice, synergistically stimulates adenylyl cyclase type 2 (Lustig et al., 1993), thus also increasing the cAMP formation. In the membrane-bound fraction of hippocampal slice homogenates prepared from C57BL/6N mice, no PKC activity was detected after h/rCRF application. We did not detect any significant contribution of PKC to the h/rCRF-induced increase in neuronal spiking behavior of CA1 pyramidal cells in C57BL/6N mice. However, the treatment of hippocampal slices from C57BL/6N mice with BIS-I and the H-89 treatment of slices from BALB/c mice showed the tendency to reduce the spiking rate compared with controls. This observation suggests that, in both mouse strains, neuronal activity is sensitive to changes in PKA and PKC activity.

Our observation that only PKA was activated in hippocampal slices of C57BL/6N mice during application of h/rCRF might be because receptors with dual signaling properties often stimulate different pathways with different efficacies. A_3 adenosine receptors, for example, interact with G_i-proteins and, to a lesser extent, with $G_{q/11}$-proteins in CHO cells (Palmer et al., 1995). These receptors were shown to inhibit adenylyl cyclase in all cell types tested, whereas stimulation of PLC was cell type dependent. Although activated CRF receptors coupled to $G_{q/11}$ in hippocampal membranes of C57BL/6N mice, h/rCRF neither activated PKC in hippocampal slices nor enhanced the conditioned fear response. This result is surprising because, in experiments using PDBu, we demonstrated that hippocampal neuronal excitability and conditioned fear of C57BL/6N mice was enhanced by activation of PKC. In contrast, h/rCRF also stimulated $G_{q/11}$ in hippocampal membranes of BALB/c mice and improved hippocampus-dependent learning via activation of PKC in this mouse strain. Similar results were reported by Fordyce et al. (1985), who found that stimulation of hippocampal PKC activity enhances contextual learning, as determined by the fear conditioning task in DBA mice. In the hippocampus of C57BL/6J mice, a PKA-dependent period for contextual memory consolidation develops between 1 and 3 hr after training (Bourtchouladze et al., 1998). Considering the activation of the PKA system in the hippocampus of C57BL/6N mice during h/rCRF application, it is surprising that h/rCRF did not facilitate contextual fear conditioning in C57BL/6N mice. In a recent study, the crucial temporal relationship between PKA inhibition and training necessary to produce impairment of the consolidation of fear memory was demonstrated (Bourtchouladze et al., 1998). A narrow time window exists for PKA inhibition before the training. When mice are treated with PKA inhibitor 20–30 min before contextual conditioning, they show dramatic amnesia. However, inhibition of PKA 3 hr before training does not affect retention 24 hr after training. Thus, in the present study, h/rCRF might have had no effect on long-term contextual memory in C57BL/6N mice because PKA was not activated within the decisive time window.

To summarize, we demonstrated that h/rCRF activated at least two different signaling cascades in mouse hippocampus, the PLC–PKC pathway (via interaction with $G_{q/11}$) and the cAMP–PKA pathway (via interaction with G_s, $G_{q/11}$, and G_i). Future experiments will have to determine whether hippocampal CRF receptors can switch their coupling between different G-protein subunits triggered by the activation of specific signaling events such as protein phosphorylation (Lawler et al., 2001). Alternatively, the observed multisignaling activity of h/rCRF might be caused by the activation of different types of CRF receptors coupling to G_s and to $G_{q/11}$ to initiate independent activation of adenylyl cyclase and PLC. These findings suggest a possible intermediary role for differential CRF receptor coupling in determining distinct endocrine and behavioral stress responses. In support of this hypothesis, both mouse strains are differentially responsive to neurogenic, psychogenic, and systemic stress, with a greater stress reactivity and adrenal glucocorticoid release in

BALB/cByJ mice than in C57BL/6ByJ mice (Anisman et al., 2001). Our results add to the existing data showing that the genetic background can affect the behavioral phenotypes of genetically modified mice generated for elucidating the molecular basis of learning and memory (McNamara et al., 1998; Dobkin et al., 2000; Dockstader and van der Kooy, 2001). In view of the contribution of the hippocampus to numerous forms of learning (for review, see Kesner et al., 2000; Kim and Baxter, 2001; Maren, 2001) and the fact that h/rCRF represents an early signal in the neuroendocrine response to stress (Koob and Bloom, 1985), our present findings may represent an important step toward understanding the cellular and molecular processes underlying interstrain variability concerning the impact of stress on learning and memory (Brush et al., 1988; Francis et al., 1995; Palmer and Prinz, 1999).

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