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Corticotropin-Releasing Factor Increases Dihydropyridine- and Neurotoxin-Resistant Calcium Currents in Neurons of the Central Amygdala

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ABSTRACT

Corticotropin-releasing factor (CRF) is an important mediator of stress responses in the brain, and CRF receptors and CRF-containing neurons and terminals are located within the central nucleus of the amygdala (CeA). CeA neurons possess multiple types of Ca^{++} channels, including L, N and Q types and a current resistant to saturating concentrations of dihydropyridine and neurotoxin antagonists. In this study, we used whole-cell patch-clamp techniques to study the effects of CRF on whole-cell Ca^{++} current (I_{Ca}) in acutely dissociated CeA neurons and determine components of the current affected. CRF (1–400 nM) increased the peak of the I_{Ca} in ≈50% of the CeA neurons recorded. In the remaining neurons, CRF had little effect. The CRF-induced increase in the I_{Ca} was concentration dependent and the estimated EC_{50} value was 14.9 nM. CRF (50 nM) increased the peak I_{Ca} by 25 ± 5% (n = 9). CRF produced an increase in both the transient and the steady state current but did not shift the peak of the current-voltage relationship. CRF did not affect the voltage dependence of activation and inactivation, and the CRF effect on I_{Ca}s was not significantly different when the neuron was held at −80 or −40 mV. The competitive CRF receptor antagonist (α-helical CRF_{9-41}, 3 μM) blocked the CRF-induced increase in I_{Ca}, suggesting that the effect of CRF is receptor mediated. CRF (50 nM) enhanced the I_{Ca} (20 ± 3%) in the presence of saturating concentrations of the L-type blocker nimodipine and neurotoxin N- and Q-type blockers ω-conotoxin GVIA and ω-conotoxin MVIIC. We conclude that CRF increased, through a receptor mechanism, dihydropyridine- and neurotoxin-resistant current(s) in CeA neurons.

CRF, a 41-amino acid polypeptide, plays a major role in the coordination of endocrine, autonomic and behavioral responses to stressful stimuli. In addition to activation of the HPA axis (Aguilera *et al.*, 1992; Axelrod and Reisine, 1984; Hauger *et al.*, 1988), CRF regulates responses to stress through activation of extrahypothalamic brain regions. Anatomically, CRF, CRF receptors, CRF-containing neurons and terminals are widely present in brain regions other than the HPA axis (De Souza *et al.*, 1985; Palkovits *et al.*, 1985; Sakanaka *et al.*, 1986; Sawchenko and Swanson, 1985; Swanson *et al.*, 1983), including the bed nucleus of the stria terminalis, septum and the medial, basolateral and central amygdala. Behavioral and autonomic effects of CRF administered centrally are also mediated through extrahypothalamic brain areas and occur in the presence of dexamethasone, a treatment that blocks HPA activation. In addition, CRF, applied *in vivo* or *in vitro*, can directly alter neuronal behaviors in extrahypothalamic brain regions. For example, CRF excites neurons of the cortex (Eberly *et al.*, 1983), hippocampus (Aldenhoff *et al.*, 1983) and the locus ceruleus (Valentino *et al.*, 1983; Valentino and Foote, 1988), whereas neurons of the thalamus and lateral septal areas are inhibited (Eberly *et al.*, 1983).

The CeA, a prominent nuclear complex within the corpus amygdaloideum, is thought to be one of the key extrahypothalamic regions in responses to stress involving CRF. Anatomically, CeA contains a relatively high amount of CRF and high density of CRF-immunoreactive (CRF-ir) neurons and fibers, as well as a moderate concentration of CRF receptors (Cassell and Gray, 1989; Cummings *et al.*, 1983; De Souza *et al.*, 1985; Grigoriadis and De Souza, 1992; Imaki *et al.*, 1991; Olschowka *et al.*, 1982; Palkovits *et al.*, 1985; Swanson *et al.*, 1983; Uryu *et al.*, 1992). The CRF-ir neurons in the CeA have efferent projections to nuclei involved in central control of autonomic activity and stress (see Gray, 1989, 1990) such as the parabrachial nucleus (Moga and Gray, 1985; Sakanaka *et al.*, 1986), the dorsal vagal complex (Veening *et al.*, 1984) and

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ABBREVIATIONS: CRF, corticotropin-releasing factor; CeA, central nucleus of the amygdala; NIM, nimodipine; DHP, dihydropyridine; CRF-ir, corticotropin-releasing factor immunoreactive; GABA, γ-aminobutyric acid; ACTH, adrenocorticotropin; HPA, hypothalamic-pituitary-adrenal; TEA, tetraethylammonium chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid TTX, tetrodotoxin; 4-AP, 4-aminopyridine; Aga IVA, ω-agatoxin IVA; HVA, high voltage activated calcium current.

the midbrain central gray. Furthermore, a calcium-dependent release of CRF has been measured in fetal and adult rat amygdala (Smith *et al.*, 1986; Takuma *et al.*, 1994) and CRF release in the CeA is increased in cocaine-treated rats (Richter *et al.*, 1995). Electrophysiologically, CRF inhibits the slow-afterhyperpolarizing potential, hyperpolarizes the membrane and broadens the duration of Ca^{++} spikes in amygdala neurons *in vitro* (Rainnie *et al.*, 1992). Behaviorally, CRF microinfused into the CeA nucleus causes tachycardia (Wiersma *et al.*, 1993), whereas infusion of CRF receptor antagonist α-helical CRF_{9-41} into the CeA attenuates stress-induced freezing behavior (Swiergiel *et al.*, 1993) and reverses anxiogenic-like effects of ethanol withdrawal (Rassnick *et al.,* 1993). Furthermore, during restraint stress, CRF mRNA content in amygdala is increased (Kalin *et al.*, 1994).

In extrahypothalamic brain areas, CRF stimulates the release of neurotransmitters, including, GABA, dynorphin and methionine-enkephalin (Sirinathsinghji *et al.*, 1989; Sirinathsinghji and Heavens, 1989), as well as dynorphin A (Song and Takemori, 1992). In the HPA axis, CRF increases cytosolic calcium in rat and human pituitary corticotrophs through voltage-gated calcium channels, and this calcium entry is responsible for the CRF-dependent ACTH release from those cells (Guerineau *et al.*, 1991). L- and P-type calcium channel antagonists (Kuryshev *et al.*, 1996) can block this increase in corticotrophs. These data suggest that CRF may affect voltage-activated Ca^{++} channels, leading to neurotransmitter release, but the mechanism underlying these actions of CRF on brain neurons is not known.

Amygdala neurons possess multiple types of I_{Ca}s (Foehring and Scroggs, 1994; Kaneda and Akaike, 1989), and we have described four different types of I_{Ca} in isolated CeA neurons (Yu and Shinnick-Gallagher, 1994a, 1997). This study focuses on the membrane mechanism of action of CRF acting on receptors in neurons intimately involved in integrating the autonomic, neuroendocrine and behavioral responses to stressful stimuli. The purpose of the present study was to analyze in isolated CeA neurons the actions of CRF on the I_{Ca} using whole-cell patch-clamp recording technique and pharmacological agents and neurotoxins. A portion of these results has been published in abstract form (Yu and Shinnick-Gallagher, 1994a).

Methods

Cell preparation. The techniques used to prepare 300- to 330-μm brain slices and cell dissociation were similar to those described previously (Yu and Shinnick-Gallagher, 1994b, 1997). Pregnant female Sprague-Dawley rats at 16 to 18 days of gestation were purchased from Harlan (Houston, TX). Pups of either sex ranging from 8 to 18 days were decapitated, and the brains were rapidly removed and cooled in dissecting solution (0–5°C), bubbled continuously with 100% O_2. The dissecting solution contained 120 mM NaCl, 10 mM KCl, 2 mM KH_2PO_4, 1 mM $CaCl_2{\cdot}2H_2O$, 6 mM $MgSO_4{\cdot}7H_2O$, 10 mM D-glucose and 10 mM piperazine-N,N′-bis-[2-ethanesulfonic acid]; the pH was adjusted to 7.40 with 10 N NaOH. The osmolarity of the dissecting solution was measured using 5100 Vapor Pressure Osmometer (Wescor) and adjusted to 300 to 310 mOsM with sucrose in every experiment. In cold solution, the brain was transversely cut posterior to the first branch and anterior to the last branch of the superior cerebral vein. The resulting block of brain tissue was hemisected. Three or four serial coronal slices per hemisphere were obtained using a Vibroslice (Campden) and incubated in a beaker containing oxygenated dissecting solution at room temperature for 20 to 30 minutes. The slices were then transferred to an oxygenated enzyme solution preheated to 34.5°C and incubated for 12 to 15 min. The enzyme solution contained a mixture of 20 to 30 mg/15 ml of pronase E (Type XXV; Sigma, St. Louis, MO) and 18 to 20 mg/15 ml of trypsin (Sigma). The slices were washed twice after enzyme treatment and stored in an oxygenated dissecting solution at room temperature for ≈10 min. The CeA area was visualized under a stereomicroscope and dissected from the brain slices with a scalpel. The pieces of brain tissue containing the CeA were triturated gently with a series of flame-polished Pasteur pipettes of decreasing diameter to dissociate mechanically the individual neurons. The suspended cells were pipetted into the recording chamber that was mounted on the stage of a Nikon inverted microscope (Nikon Diaphot).

Electrophysiological recordings. The whole-cell patch-clamp methodology (Hamill *et al.*, 1981) was used for recording I_{Ca}s. Patch electrodes were made from Corning 7052 glass (1.5-mm outer diameter, Garner Glass, Claremont, CA), pulled using a Flaming-Brown micropipet puller (model P-97, Sutter Instruments, Novato, CA) or a laser puller (model P-2000, Sutter Instruments, Novato, CA) and polished using a Narishige microforge (model MF-9). The patch electrodes were coated with 20% Sylgard before polishing and had resistances of 3 to 6 MΩ when filled with an internal solution of the following composition (in mM): 90 Cs acetate (or 100 CsF), 18 TEA, 18 HEPES, 9 BAPTA, 9 D-glucose, 5 MgATP, 0.2 NaGTP and 0.1 leupeptin. The internal fluoride solution was used in a few preliminary experiments. Data were pooled because (1) dialysis of fluoride into dissociated amygdala neurons (Kaneda and Akaike, 1989) occurs in 10 to 20 min, a time frame longer than the protocols used in the present experiments, and (2) neither the I_{Ca} or the effect of CRF on I_{Ca} recorded with acetate or fluoride was different (see Joels and Karst, 1995). The pH of the internal solution was adjusted from 7.1 to 7.2 with 1 N CsOH at room temperature and had a final osmolarity of 270 to 280 mOsM. The external solution consisted of the following: 120 mM TEA-Cl, 3 mM $CaCl_2$, 10 mM HEPES, 10 mM CsCl, 5 mM 4-AP, 10 mM glucose and 2 μM TTX. The pH of the external solution was titrated to ≈7.4 with 1 N HCl or 1 N CsOH, and the osmolarity was adjusted to 320 ± 5 mOsM with sucrose. All the experiments were performed at room temperature (22–25°C). Both the internal and external solutions were designed to suppress pharmacologically the interfering sodium and potassium currents. The I_{Ca}s recorded in this study are thought to be in the relative physiological range because the extracellular Ca^{++} concentration used (3 mM) is close to the "normal" condition (2.5 mM; Allen *et al.*, 1993). The reference electrode was filled with the same solution as the internal recording solution; junction potentials calculated according to the method of Neher (1992) ranged from 2 to 4 mV with electrode tip diameters of 1.0 to 1.5 μm.

After establishing a gigaseal, the membrane underlying the pipette was ruptured by a gentle suction to obtain whole-cell recording. The recordings were considered acceptable if neurons displayed robust inward sodium currents in the dissecting solution. An Axoclamp-2A was used for whole-cell recording in the continuous single-electrode voltage-clamp mode. The capacitance neutralization, gain and phase controls were adjusted to produce optimal clamp efficiency. Under these conditions, a clamp gain of 8 to 10 nA/mV could be obtained. Unless otherwise noted, cells were clamped at −70 mV (in some cells, −80 mV) or −40 mV. A holding potential of −70 mV is close to the resting membrane potential of these cells recorded in slice preparation (−67 mV; Rainnie *et al.*, 1992; Schiess *et al.*, 1993), a condition permitting the I_{Ca} recorded to be within a physiological range for activation and inactivation. The series resistance (R_s) was calculated according to the equation $R_s = V/I_t$ and determined by fitting the decay of the whole cell current to the capacitative transient in response to a voltage step (V) to obtain the value of I_t when t (time) = 0. The value of R_s was ≈5 to10 MΩ; series resistance was

not compensated. Currents were usually <1 nA, suggesting that the series resistance error was not significant in the present study.

Online and offline data acquisition and analysis were accomplished using an DigiData 1200 interface (Axon Instruments, Foster City, CA) between a Axoclamp-2A preamplifier and a Gateway 2000 486/33C computer using pClamp 6.0 software programs (Axon Instruments). Analog signals were also stored as hard copy on a Gould (model 3400; Cleveland, OH) chart recorder for further analysis. Signals were filtered at 1 or 3 kHz before digitizing using a built-in filter on the recording amplifier. Capacitance and leak currents were estimated as the current evoked by depolarizing voltage commands in the presence of $CdCl_2$ (200 μM) and were digitally subtracted for the analyses. The reduction and enhancement of the I_{Ca} by blocking agents and CRF, respectively, were expressed as a percentage of the control whole-cell I_{Ca} obtained in normal recording solution. Current traces (not including those of the current-voltage curves) shown in the figures are the averaged responses of two or three identical voltage steps elicited consecutively at 7-sec intervals. Statistical significance was determined at the level of $P \leq .05$ using paired or unpaired Student's t tests and Mann-Whitney rank-sum test. All data are expressed as mean ± S.E.M.

The I_{Ca} generally runs down slowly with time, although the ATP and GTP are present in the pipette (Eliot and Johnston, 1994; Mintz, 1994; Mintz, *et al.* 1992a; Mynlieff and Bean, 1992; Randall and Tsien, 1995). The percent increase in I_{Ca} recorded in the presence of CRF was calculated as $[I_{Ca}\ (CRF) - I_{Ca}\ (control)]/I_{Ca}\ (control) \times 100\%$. In the majority of CeA neurons, the I_{Ca} showed a slow, progressive rundown. As shown (see fig. 3; control or no effect group), the I_{Ca} decayed ≈8% for the first 2 to 3 min, after which the current stabilized with a rate of decay ≈4% to 5%/min thereafter. No correction was made for the rundown of the I_{Ca} except in data used for the concentration-response curve, in which the percent increase in I_{Ca} (x) induced by CRF (at different concentrations) after 1 min of application was divided by the relative amplitude of the control I_{Ca} (0.9157) at 1 min (see fig. 3); the value of the increase induced by CRF at each concentration was calculated as $x/0.9157$. The concentration-response curve was plotted using Inplot Graph Pad (San Diego, CA) software.

Drug application. CRF (human and rat; Peninsula Laboratories, Belmont, CA), α-helical CRF_{9-41} (Bachem, Torrance, CA), ω-conotoxin GVIA (RBI, Natick, MA), Aga IVA (Alamone Labs, Jerusalem, Israel) and ω-conotoxin MVIIC (Bachem) were prepared as concentrated stock solution in distilled water and stored in aliquots at −20°C. The stock solutions of drugs were diluted with the external solution immediately before each experiment. Nimodipine and (±)-BAY K 8644 (RBI) were prepared as concentrated stock solutions in 90% dimethylsulfoxide and protected from light. TTX was obtained from Sigma.

All drugs were applied using a bath microsuperfusion technique similar to the "concentration-clamp" described by Akaike *et al.* (1986). After formation of a gigaohm seal and subsequent whole-cell recording, the cell was lifted from the bottom of the chamber and then inserted into an acetate tube (volume of ≈80 μl) through an access hole in which the Sylgard that coated the electrode formed a tight fit around the circumference of the access opening. One end of the tube was connected to a small chamber into which test solutions were introduced. The other end of the tube was connected *via* tubing to a solenoid valve. When the valve was opened, the solution moved through the tube by gravity; due to the small volume exchanged, fast bath applications of agonist were obtained.

Results

CRF increased the I_{Ca} in CeA neurons in a concentration-dependent manner. These data were obtained from ≥120 neurons acutely dissociated from rat CeA. We previously described the I_{Ca} in CeA neurons (Yu and Shinnick-Gallagher, 1994a, 1997). The HVA I_{Ca} in CeA neurons is composed of at least four pharmacologically distinct components. We found that the N-type, ω-conotoxin GVIA (1 μM)-sensitive current accounted for 30% of the total I_{Ca}; the Q-type current defined as the current sensitive to ω-conotoxin MVIIC (250 nM) or ω-agatoxin IVA (Aga IVA; 1 μM) amounted to 13% to 18% of I_{ca}; the L-type, NIM-sensitive current represented 22% of the total I_{Ca}; and a resistant current, a non-L-, N- and Q-type current, comprised 37% to 53% of the total I_{Ca}. (Yu and Shinnick-Gallagher, 1997). Figure 1 illustrates an example of the effects of sequential applications of calcium channel blockers on I_{Ca} evoked by voltage-step commands to +10 or 0 mV from a holding potential of −70 mV (fig. 1A, left & 1B) or −40 mV (fig. 1A, right), respectively. The antagonists block similar proportions of total current whether held at −70 or −40 mV. L-type currents are found in only 70% of CeA neurons, and a P-type current blocked by low nanomolar concentrations of ω-agatoxin (Mintz *et al.*, 1992a, 1992b) is not recorded in CeA neurons (Yu and Shinnick-Gallagher, 1997). In our previous study (Yu and Shinnick-Gallagher, 1997), we defined the resistant current as the current remaining in ω-conotoxin MVIIC (250 nM), NIM (5 μM) and ω-conotoxin GVIA (1 μM) or Aga IVA (1 μM); furthermore, increasing the concentrations of NIM (10 μM) and neurotoxins ω-conotoxin GVIA (2 μM) and ω-conotoxin MVIIC (500 nM) resulted in a similar percentage (36%) of resistant current recorded. We (Yu and Shinnick-Gallagher, 1997) have also shown that low-threshold T-type currents are not recorded from the holding potentials of −70 and −40 mV used in the present study. Our previous findings (Yu and Shinnick-Gallagher, 1997) are in agreement with other studies on unidentified (Kaneda and

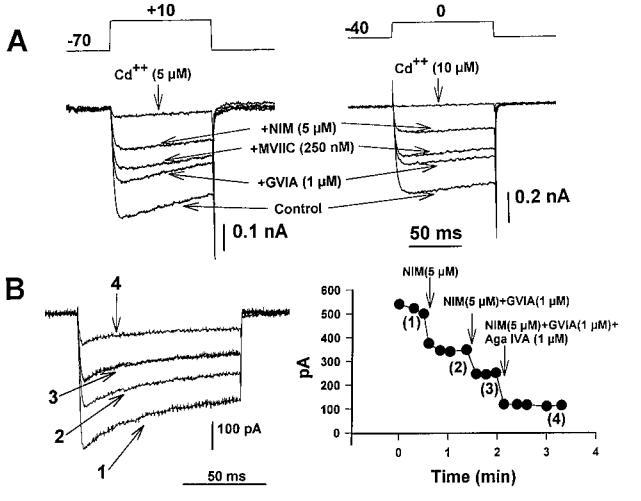

Fig. 1. Components of I_{Ca} in CeA neurons. A, Left, I_{Ca} elicited using 100-ms depolarizing steps from −70 to +10 mV. The combination of CgTx GVIA (1 μM) plus CgTx MVIIC (250 nM) plus NIM (5 μM) reduced I_{Ca} by 64% in this neuron; the resistant current represented 36% of the total I_{Ca}. Cd^{++} (5 μM) reduced the resistant current. A, Right, I_{Ca} elicited using 100-ms depolarizing steps from −40 to 0 mV in another neuron. The addition of CgTx GVIA (1 μM) plus CgTx MVIIC (250 nM) plus NIM (5 μM) sequentially reduced the I_{Ca} by 69% and revealed a resistant current comprising 31% of the total I_{Ca}. Cd^{++} (10 μM) completely blocked the resistant current. Currents recorded in a high concentration of Cd^{++} (200 μM) were leak subtracted. B, Time course of NIM and neurotoxin block. Left, I_{Ca} elicited in response to a voltage step from −70 to +10 mV. I_{Ca} recorded at times indicated (right). In this neuron, NIM blocked 32% of I_{Ca}, ω-conotoxin GVIA blocked 23% and Aga IVA blocked 21%. In this neuron, the resistant current amounted to 23% of the total I_{Ca}.

Akaike, 1989) and pyramidal (Foehring and Scroggs, 1994) neurons in the amygdaloid complex.

The application of CRF (1–400 nM) increased the I_{Ca} elicited by 100-ms step commands from −70 mV to 0 or +10 mV in ≈52% of the neurons recorded (65 of 124 neurons tested; fig. 2). This enhancement of I_{Ca} was not accompanied by a change in leak current. In the remaining half of the neurons, CRF had no effect. The CRF-induced increase in I_{Ca} was observed when the cell was held at either −70 (or −80 mV) or −40 mV (fig. 2, A and B, and see fig. 4, A and B).

The CRF-sensitive component showed a slight inactivation during 100-ms step command in neurons held at −70 and −40 mV (fig. 2). CRF (100 nM) enhanced the peak I_{Ca} by 28 ± 7% (n = 19) and 26 ± 5% (n = 6) when neurons were depolarized from −80 or −40 mV, respectively ($P \geq .05$). These results suggest that CRF increased HVA I_{Ca}s because at a holding potential of −40 mV, LVA channels are completely inactivated (Fox *et al.*, 1987; Nowycky *et al.*, 1985; Tsien *et al.*, 1988).

The increase in I_{Ca} occurred within 1 min of application of CRF (100 nM; fig. 3). However, I_{Ca} itself ran down within that time period in the absence of an ATP-regenerating system (n = 9; fig. 3). A comparison of I_{Ca} with respect to time in control (n = 8) and in CRF-treated neurons indicated that the CRF effect was sustained and not reversible within the recording period of the experiment (n = 9; fig. 3). Neurons not responding to CRF (n = 9; 100 nM) showed a rundown similar to that recorded in the absence of the peptide ($P \geq .05$ at each point; fig. 3), even at higher concentrations (400 nM, $P \geq .05$, n = 5). These data suggest that CRF did not produce an inhibitory action on I_{Ca} in CeA neurons. The effects of CRF in CeA but not basolateral amygdala neurons recorded in slice preparations were also difficult to reverse (Rainnie *et al.*, 1992).

We analyzed the effect of CRF on the current-voltage relationship for the I_{Ca} (fig. 4). CRF increased I_{Ca} to a greater extent at more depolarized membrane potentials (−10 to +40 mV) than at more negative potentials (<−20 mV). Analysis of the CRF current-voltage relationship obtained by subtraction (fig. 4Ac, Bc) showed that the current(s) sensitive to CRF began to activate around −10 mV and reached maximal values between +10 and +20 mV. The greatest enhancement of I_{Ca} produced by CRF occurred at +11 ± 3 mV (n = 7) which is close to the maximum value of I_{Ca} in the current-voltage relationship termed the peak I_{Ca} (fig. 4, Ab and Bb). The CRF-induced increase in the peak I_{Ca} current was not accompanied by a shift in the current-voltage relationship in seven of eight neurons (peak I_{Ca} voltage in control, +11 ± 3 mV; peak I_{Ca} voltage in CRF, +11 ± 3 mV; n = 7).

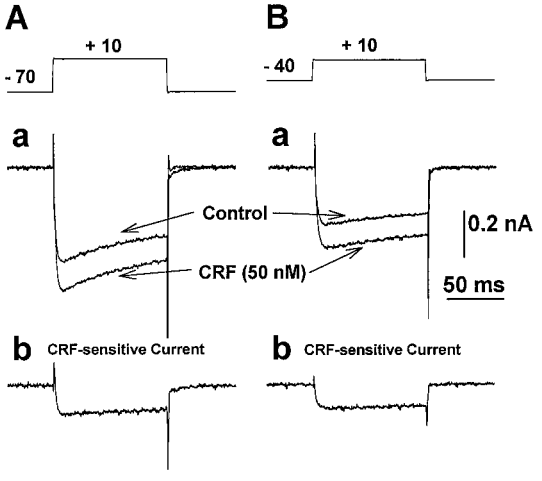

Fig. 2. CRF increased the peak I_{Ca} in rat CeA neurons. Aa, CRF (50 nM) increased the peak I_{Ca} by 28% in this neuron, which was held at −70 mV and stepped to +10 mV. Ab, CRF-sensitive current was obtained by digitally subtracting the current recorded in control from that measured in CRF in the neuron shown in Aa. Ba, CRF (50 nM) increased the peak I_{Ca} by 40% when the neuron in A was stepped from −40 to +10 mV. Bb, Current enhanced by CRF at a holding potential of −40 mV. Leak currents were subtracted.

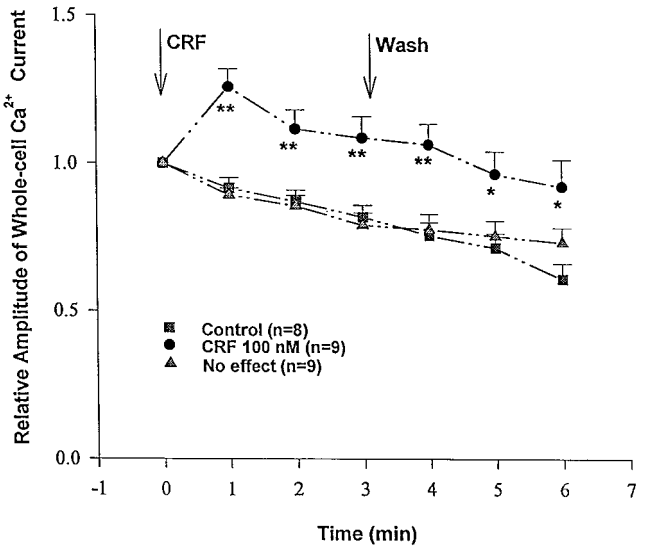

Fig. 3. Time course of CRF action on the I_{Ca}. The effect of CRF was measured per min after the initial application. I_{Ca} was normalized to the peak current in control. Control recordings were averaged for 1 min, and CRF subsequently was added. Cells, which were treated but did not respond to CRF, were labeled "no effect." The run down of the peak whole-cell I_{Ca} occurs in these experiments because there was no ATP-regenerating system added to the solution in the electrode. Peak I_{Ca} was elicited from −80 to 0 mV or +10 mV using 100-ms voltage step commands. n = number of neurons tested.

We further tested whether the CRF effect on the current was mediated by shifts in voltage-dependent steady-state activation (fig. 5). The activation curve could be fitted with the following Boltzmann equation: $I_{step}/I_{max} = 1/\{1 + \exp[(V - V_{½})/k]\}$, where $V_{½}$ is the potential at which $I_{step}/I_{max} = 0.5$, and k is the slope factor of the curve. The values for $V_{½}$ (I_{step}/I_{max}) and k in control and CRF were −10.5 and −11 mV and −6.8 and −7.3 mV, respectively. We also analyzed the effect of CRF on the voltage dependence of steady-state inactivation (fig. 6). In this series of experiments, I_{Ca} was elicited by the same voltage commands from two different holding potentials in the presence and absence of CRF (fig. 6A). If CRF increased the I_{Ca} by shifting voltage-dependent steady-state inactivation to a more depolarized level, we would expect to see a larger effect on the I_{Ca} evoked from a more positive holding potential. The ratio of currents elicited by step commands to +20 mV from holding potentials of −100 mV (I_{Max}) and −40 mV (I_{Test}) was determined in four neurons. The ratio of I_{Test}/I_{Max} was not significantly different in the absence (46 ± 3%; fig. 6B1) or presence (50 ± 4%; fig. 6B2) of CRF. These data suggest that the CRF-induced enhancement of I_{Ca} was not due to a shift in the voltage dependence of steady-state activation or inactivation.

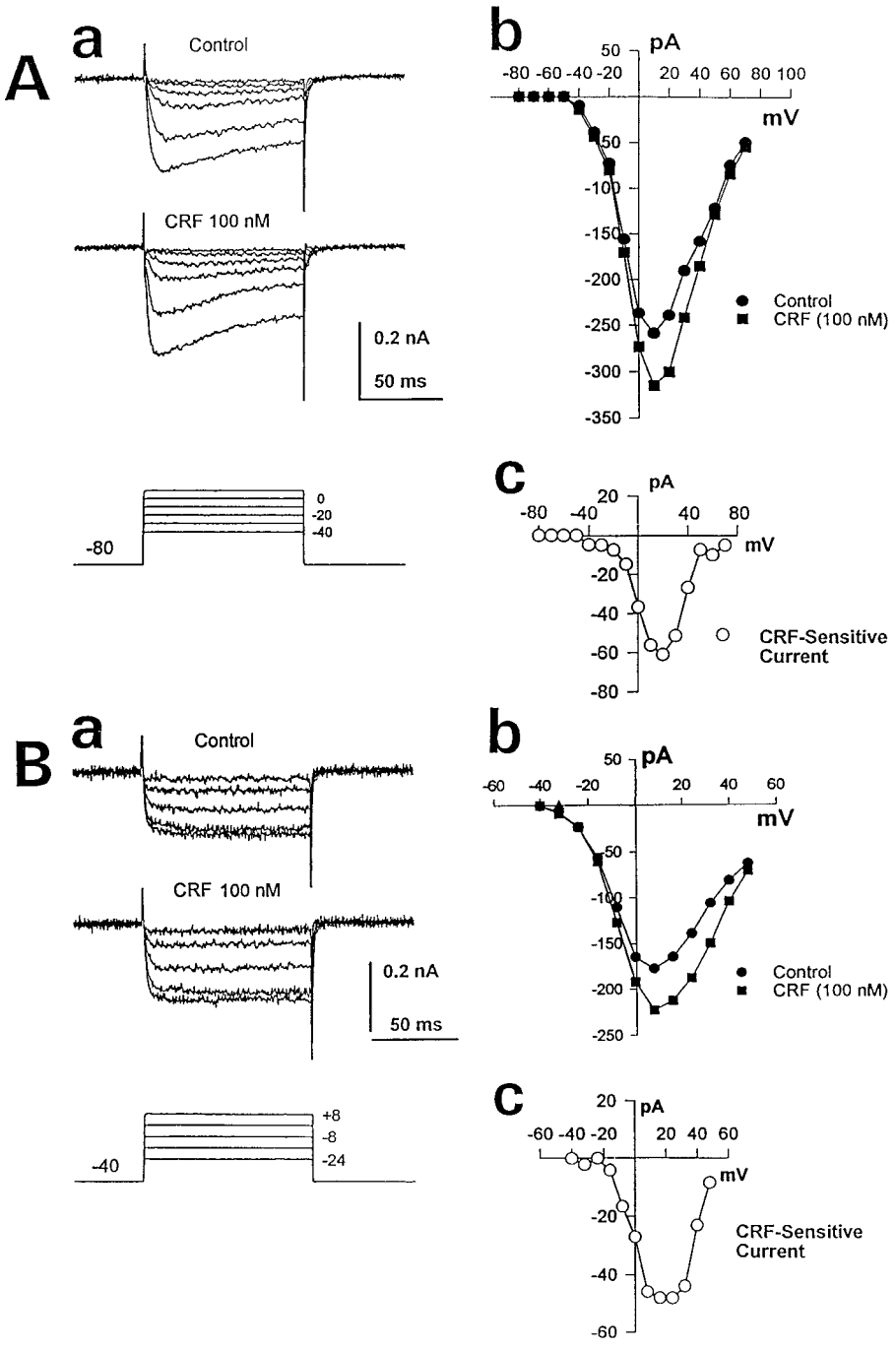

Fig. 4. Effect of CRF on current-voltage relationships in CeA neurons. The current-voltage plot was constructed by measuring the magnitude of the peak I_{Ca} recorded before (control) and after application of CRF (100 nM) as well as during wash as a function of command potential. Currents were evoked using 100-ms depolarizing steps from −80 to +70 mV in 10-mV increments. A, V_h = −80 mV; CRF (100 nM) increased the maximal I_{Ca} (at +10 mV) by 22% in this cell. a, Original current traces recorded in control and CRF. b, Current-voltage plot of data obtained in a. c, CRF-sensitive current obtained by subtracting current in control from that recorded in CRF. The I_{Ca} was maximally enhanced by CRF (100 nM) at +20 mV. B, V_h = −40 mV; CRF (100 nM) increased the maximal I_{Ca} by 26% in another neuron held at −40 mV. Currents were evoked using 100-ms depolarizing steps from −40 to +50 mV in 8-mV increments. a, Current traces recorded in control and CRF. b, Current-voltage relationship in control and in the presence of CRF (100 nM) in same neuron as a. c, CRF-sensitive current obtained by digitally subtracting the current in control from that recorded in CRF in a neuron held at −40 mV. Leak currents in both A and B recorded in the presence of 200 μM Cd^{++} were subtracted.

The effect of CRF on the I_{Ca} was concentration dependent with an estimated EC_{50} value of 14.9 nM (fig. 7). In these experiments, the rundown of the I_{Ca} was corrected with respect to time (see Methods). CRF (400 nM) increased the peak I_{Ca} by 31 ± 6% (n = 6) above control value.

The CRF receptor antagonist α-helical CRF_{9-41} blocked the CRF-induced increase in I_{Ca}. We analyzed the effect of CRF antagonist α-helical CRF_{9-41} to examine whether a direct receptor mechanism mediated the CRF effect on the I_{Ca}. α-Helical CRF_{9-41}, an amino-terminal-shortened analog of CRF and a synthetic competitive CRF antagonist (Rivier *et al.*, 1984), can block many effects of CRF, including CRF-induced ACTH secretion in rat anterior pituitary (Rivier *et al.*, 1984), CRF stimulation of neurotransmitter release (Sirinathsinghji and Heavens, 1989; Song and Takemori, 1992) and behavioral changes induced by stress and CRF (Boadle-Biber *et al.*, 1993; Kiang, 1994; Menzaghi *et al.*, 1994; Swiergiel *et al.*, 1993). We first applied α-helical CRF_{9-41} (3 μM) and subsequently added CRF (50 nM) to the α-helical CRF_{9-41} (3 μM)-containing solution. In 8 of 15 neurons tested, α-helical CRF_{9-41} (3 μM) induced a slight but insignificant increase in the I_{Ca} (7 ± 2%), a possible partial agonist action. A partial agonist action for α-helical CRF_{9-41} has been reported previously in the amygdala (Rainnie *et al.*, 1992) and other brain regions (Menzaghi *et al.*, 1994). CRF (50 nM) applied in the presence of α-helical CRF_{9-41} (3 μM) resulted in only a 4.5 ± 1.5% increase in I_{Ca} (fig. 8) in 6 of 15 neurons, whereas the remaining cells (9 of

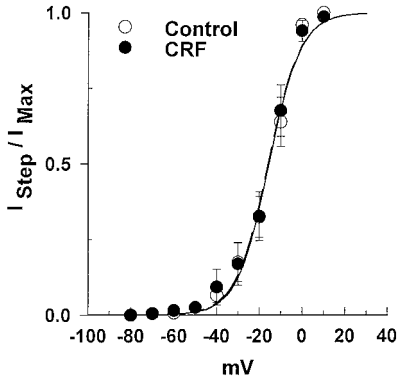

Fig. 5. Effect of CRF on voltage-dependent steady-state activation. Currents were measured at various test potentials (−70 to +20 mV) from a holding potential of −80 mV. I_{step}/I_{max} was calculated as described in text. Curve fitting with a Boltzmann equation yielded a $V_{½}$ of −10.5 mV in control and −11 mV in CRF; k values were −6.8 and −7.3 mV, respectively. Each point represents the mean ± S.E.M. of 4 cells. Step commands were 100 ms.

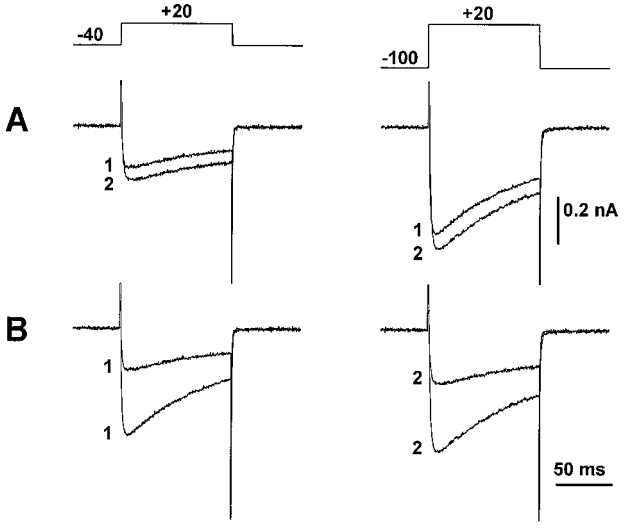

Fig. 6. CRF does not affect steady-state inactivation. A and B were obtained from the same neuron before (trace 1) and after (trace 2) a 1-min application of CRF (100 nM). A, Effect of CRF (trace 2) on control currents (trace 1) elicited with 100-ms step commands from either −100 mV (I_{Max}) or −40 mV (I_{Test}) to +20 mV. B, Ratio of I_{Test}/I_{Max} in the absence (trace 1 in A, 39%, B1) and presence (trace 2 in A, 43%, B2) of CRF was similar. Leak currents were subtracted.

15) showed no response. This change in I_{Ca} (4.5 ± 1.5%, n = 6) was significantly less than that induced by CRF alone (50 nM, 25 ± 5%, n = 9, P ≤ .05, unpaired t test). In addition, when the CRF-responding and nonresponding neurons were considered together, as shown in fig. 8B, the percent change in I_{Ca} in CRF alone (50 nM) was significantly different (P ≤ .01, two-tailed Mann-Whitney rank-sum test) from that recorded in the presence of CRF (50 nM) plus CRF antagonist. These data suggest that the CRF-induced increase in the I_{Ca} is mediated by direct CRF receptor activation.

CRF increased the resistant component of the I_{Ca}. We next tested the effect of CRF on DHP- and neurotoxin-sensitive and -resistant components of I_{Ca}. We recently described the presence of the multiple types of the I_{Ca}s in acutely dissociated CeA neurons and found four components of HVA I_{Ca} s (Yu and Shinnick-Gallagher, 1994a, 1997): (1) NIM-sensitive, L-type (22%), (2) ω-conotoxin GVIA-sensitive,

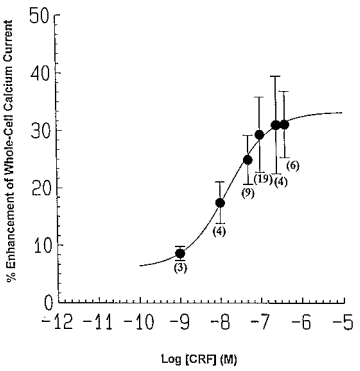

Fig. 7. CRF increased I_{Ca} in a concentration-dependent manner in rat CeA neurons. Concentrations of CRF tested ranged from 1 to 400 nM; the estimated EC_{50} value was 14.9 nM. Currents were elicited in neurons by 100-ms voltage commands from −80 to +10 mV. Currents were leak subtracted, and the value of I_{Ca} was corrected for decay of the I_{Ca} (see Methods). Numbers in parentheses are the numbers of neurons tested. Data are expressed as mean ± S.E.M. Hill coefficient = .82.

N-type (30%), (3) a Q-type (13–18%) current sensitive to ω-conotoxin MVIIC or Aga IVA and (4) resistant current recorded in the presence of NIM (5 μM), ω-conotoxin GVIA (1 μM) and ω-conotoxin MVIIC (250 nM) or Aga IVA (1 μM), which amounted to 37% to 53% (mean, 49%) of the I_{Ca} in CeA neurons. We examined the effect of CRF on the resistant I_{Ca} in the presence of all the pharmacological blocking agents. In these experiments, the I_{Ca} was elicited by voltage commands to 0 mV from holding potentials of −70 mV in cells pretreated with NIM (5 μM), ω-conotoxin GVIA (1 μM) and ω-conotoxin MVIIC (250 nM). Under these conditions, CRF (50 nM) still increased the remaining current by 31% (fig. 9). The CRF-sensitive current obtained in the presence of NIM, ω-conotoxin GVIA and ω-conotoxin MVIIC was similar with respect to that recorded in the absence of the antagonists (fig. 2, Ab, and fig. 8, Ab). Plotting the resistant current *vs.* time (fig. 9) showed that CRF increased the resistant current maximally at 1 min after application and then followed a time course of action of CRF similar to that observed with the whole-cell I_{Ca} (fig. 3). In a total of 7 neurons tested, CRF (50 nM) increased the resistant I_{Ca} by 20 ± 3%. The CRF (50 nM)-induced increase in resistant current recorded in the presence of the three calcium channel blockers was similar to that obtained in the absence of the antagonists (25 ± 6%, n = 7; P ≥ .05). The estimated total current in the absence of the antagonists 1 to 6 min after the addition of CRF (96, 82, 79, 77, 68 and 64 pA, respectively) was not significantly greater than the CRF current (52 pA) recorded at 1 min in the presence of the antagonists. However, because the effect of CRF is numerically larger in the absence of the antagonists, it is possible that CRF may have some effect on component(s) of the DHP- and neurotoxin-sensitive current not detected under the present experimental conditions. Altogether, these data suggest that the primary effect of CRF on the I_{Ca} in CeA neurons is to enhance a DHP- and neurotoxin-resistant current.

Discussion

The present results show for the first time that CRF enhances HVA I_{Ca}s in brain neurons and that this increase is mediated through DHP- and neurotoxin-resistant components in CeA neurons. The CRF enhancement of I_{Ca} was concentration dependent with an estimated EC_{50} value of

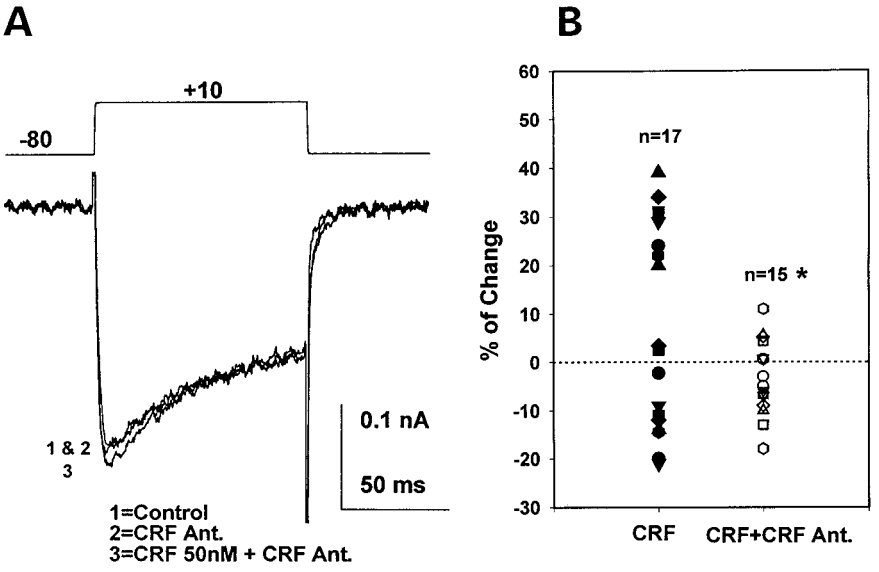

Fig. 8. α-Helical CRF_{9-41}, a competitive CRF receptor antagonist, blocks the CRF-induced increase in the I_{Ca} in CeA neurons. A, Recording of I_{Ca} in a single neuron. I_{Ca} was elicited in neurons by voltage commands from −80 to +10 mV as control (trace 1); the addition of α-helical CRF_{9-41} (3 μM) had no effect on I_{Ca} (trace 2). The subsequent addition of CRF (50 nM) in the presence of α-helical CRF_{9-41} caused only a 5.6% increase of the whole-cell current (trace 3). B, Graph of the percentage change in I_{Ca} in neurons treated with CRF alone (50 nM) and CRF (50 nM) in the presence of α-helical CRF_{9-41} (3 μM). The effect of CRF (50 nM) in each control CeA neuron is indicated by the solid symbols (n = 17). The effect of CRF (50 nM) in each neuron recorded in the presence of α-helical CRF_{9-41} (3 μM) is indicated by the open symbols (n = 15). In the presence of the antagonist, CRF (50 nM) produced a 4.5 ± 1.5% (n = 6) increase in the I_{Ca}, a change significantly ($P \leq .05$, two-tail unpaired t test) less than that CRF (50 nM) induced alone (25 ± 5%, n = 9). The difference between the two populations of neurons is significant ($P \leq .01$, two-tailed Mann-Whitney rank-sum test).

14.9 nM. The effect of CRF was maximal at the peak of the current-voltage relationship and not mediated through alteration of the voltage dependence of steady-state activation or inactivation. These data also provide evidence that the effect of CRF on the I_{Ca} is through a receptor-mediated mechanism.

The finding here that CRF increased the I_{Ca} is consistent with the following studies implicating a calcium mechanism for CRF action: (1) CRF stimulates the release of neurotransmitters in central nervous system, including ACTH and β-endorphin from the anterior pituitary (Axelrod and Reisine, 1984; Vale *et al.*, 1981); GABA, dynorphin and methionine-enkephalin from rat neostriatum and globus pallidus (Sirinathsinghji *et al.*, 1989; Sirinathsinghji and Heavens, 1989); and dynorphin A from mouse spinal cord (Song and Takemori, 1992). (2) CRF increases the duration of a Ca^{++} action potential in amygdala neurons (Rainnie *et al.*, 1992). (3) CRF increases cytosolic calcium of rat and human pituitary corticotrophs (Guerineau *et al.*, 1991). (4) CRF enhances Ca^{++} entry into human epidermoid A-431 cells and rat astrocytes (Kiang, 1994; Takuma *et al.*, 1994), an action blocked by L- and P-type calcium channel blockers in rat corticotropes (Kuryshev *et al.*, 1996).

The pharmacology of CRF receptor-mediated enhancement of I_{Ca} is in good agreement with that observed for native CRF receptors in the brain and cloned CRF receptors expressed in COS cells. In CeA neurons, the I_{Ca} was quite sensitive to CRF (EC_{50} = 14.9 nM), suggesting the CRF receptors in the CeA neurons might bind CRF with high affinity, as reported in basolateral amygdala neurons (EC_{50} = 40 nM; Rainnie *et al.*, 1992) and other brain regions such as frontoparietal cortex (EC_{50} = 36 nM; Battaglia *et al.*, 1987) and retina (EC_{50} = 20–30 nM; Olianas *et al.*, 1993). Taken together, the data obtained in the present study indicate that CRF-induced increase in I_{Ca} occurs through activation of CRF receptors.

There are two subtypes of CRF receptors, CRF_1 and CRF_2, the latter of which has two splice variants: $CRF_{2\alpha}$, found primarily in brain, and $CRF_{2\beta}$, localized in non-neuronal brain cells and in the periphery (Lovenberg *et al.*, 1995). Rat/human CRF has an EC_{50} value of 20 nM on adenylate cyclase activity in $CRF_{2\alpha}$ Ltk^- transiently transfected cells but an EC_{50} value of 4 nM in CRF_1 Ltk^- stably transfected cells (Chalmers *et al.*, 1996). K_i values for binding of CRF to CHO cells expressing CRF_1 *vs.* $CRF_{2\alpha}$ were ≈1 and ≈13 nM, respectively (Donaldson *et al.*, 1996). Although it is not possible to distinguish between CRF receptor subtypes without the use of specific antagonists, the EC_{50} value for CRF in the present study suggests that based on previous studies in transfected cells, the CRF effect on I_{Ca} in CeA neurons is more likely to be consistent with mediation through a CRF_2 receptor subtype.

The CRF-induced increase in I_{Ca} could be due to several mechanisms. A shift of the current-voltage relationship in the negative direction would cause more channels to open at negative potentials, thereby enhancing the I_{Ca}, but CRF did not shift the peak of the current-voltage relationship and voltage dependence of steady-state activation, suggesting that the CRF-induced increase in I_{Ca} may not be due to a change in the voltage dependence of channel activation. Furthermore, the ratio of I_{Test}/I_{Max} recorded at different holding potentials in the absence and presence of CRF was not statistically different, suggesting the CRF-induced increase in I_{Ca} was not mediated by a shift in the voltage dependence of steady-state inactivation over this range. It is possible that small changes in individual current components could be obscured in measurements of the voltage-dependent properties of the whole-cell current. However, these changes would have to be relatively small to not be detected in the present experiments. Further studies with single-channel recordings are required to determine definitively whether CRF enhances the I_{Ca} by increasing the single-channel conductance and/or increasing the channel open time.

Other neuropeptides increase HVA I_{Ca}s; these include luteinizing hormone-release hormone in pituitary cells (Anwyl, 1991; for a review; see Rosenthal *et al.*, 1987), angiotensin II in nodose neurons (Bacal and Kunze, 1994) and CGRP in nodose neurons (Wiley *et al.*, 1992). In these studies, the luteinizing hormone-release hormone- and CGRP-induced enhancement of I_{Ca}s is pertussis toxin sensitive, whereas the angiotensin II effect is insensitive and neither angiotensin II nor CGRP shift the current-voltage relationship. The CGRP-induced increase in HVA I_{Ca} is not caused by a change in voltage dependence of channel activation or steady-state in-

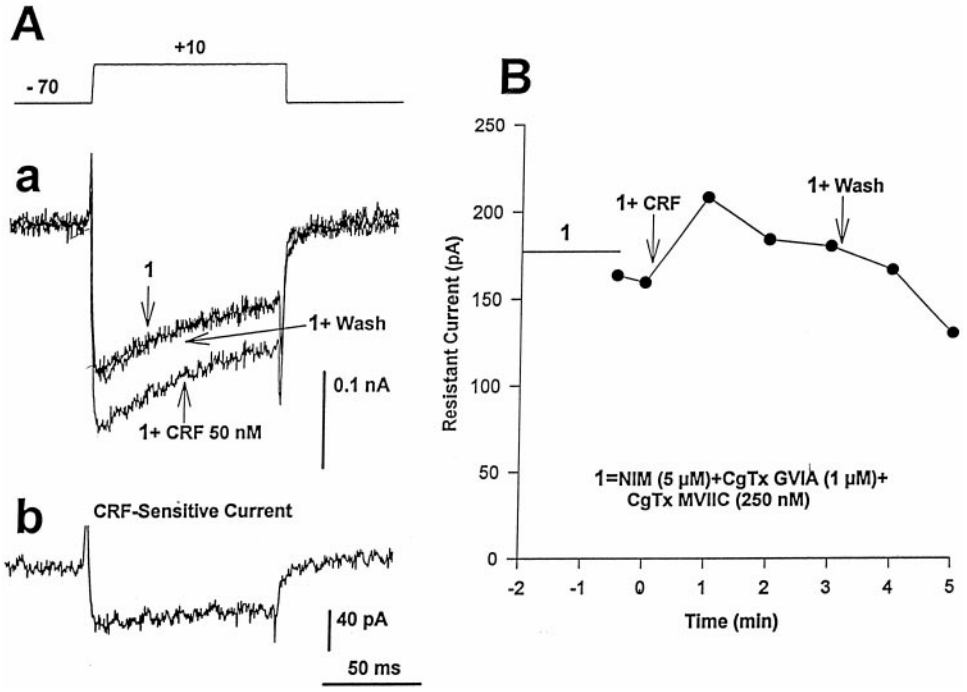

Fig. 9. CRF increased the NIM- and neurotoxin-resistant component of I_{Ca}. Data depicted in A and B were recorded from the same neuron. Aa, CRF (50 nM) increased the I_{Ca} by 31% after pretreating the cell with CgTx GVIA (1 μM) plus CgTx MVIIC (250 nM) plus NIM (5 μM) for 2 min. Ab, CRF-sensitive current was computed by subtracting the current in control from that in the presence of CRF. B, Time course of CRF action on the resistant I_{Ca} recorded from the same neuron as A. The holding potential was −70 mV, and the command potential was +10 mV.

activation but rather by an increase in maximal calcium conductance, probably by opening more channels (Wiley *et al.*, 1992). The CRF-induced increase in the I_{Ca} in this study is similar to that of previous studies of CGRP because CRF did not shift voltage dependence of activation or inactivation of I_{Ca}, suggesting that the CRF effect, like CGRP, may also be due to an increase in maximal conductance resulting from an opening of more calcium channels in the presence of the peptide.

We found that CRF enhanced the DHP- and neurotoxin-resistant component of the HVA I_{Ca} in CeA neurons. Our data showed that CRF increased an HVA rather than an LVA I_{Ca} because the percent increase in currents elicited from −40 mV (26%) and −80 mV (28%) were not different and because LVA currents were not recorded under the experimental conditions. CeA neurons express multiple types of HVA Ca^{++} channels (Yu and Shinnick-Gallagher, 1994a, 1997), namely, L, Q and N types as well as a resistant current accounting for the largest portion of the I_{Ca}. CRF increased the resistant I_{Ca}. The percentage increase in the whole-cell I_{Ca} (25%) induced by CRF (50 nM) was similar to that observed for the resistant current at equal molar concentrations of the peptide (20%). There is a possibility, however, that CRF may have an effect on some components of the DHP- and neurotoxin-sensitive currents not detected under our experimental conditions because the numerical values of the CRF currents and the percentage increase of I_{Ca} induced by CRF are larger in the absence than in the presence of the antagonists.

The DHP- and neurotoxin-resistant current in CeA neurons has some electrophysiological and pharmacological characteristics in common with cloned doe-1 and *alpha*-1E channels and native R-type I_{Ca}s but remains unclassified (Yu and Shinnick-Gallagher, 1997). Our findings are consistent with CRF receptors being linked to resistant channels, perhaps of a doe-1, *alpha*-1E or R type.

The source of CRF terminals in the CeA originates intrinsically from CRF-containing cell bodies located in this nucleus and extrinsically *via* projections from other brain regions such as lateral hypothalamus, dorsal raphe and the medial geniculate complex (Imaki *et al.*, 1991; Uryu *et al.*, 1992). CRF terminals from projection neurons form synapses with non-CRF-containing neurons more frequently than with CRF-containing neurons (Imaki *et al.*, 1991; Uryu *et al.*, 1992). Furthermore, calcium-dependent CRF release evoked by a high concentration of potassium has been demonstrated in the amygdaloid complex (Smith *et al.*, 1986; Takuma *et al.*, 1994). These data along with our findings here suggest that CRF released locally at synapses may enhance the DHP- and neurotoxin-resistant I_{Ca}s in predominantly non-CRF-containing neurons.

The CeA, as the major intra-amygdala target and output structure of the amygdaloid complex (Krettek and Price, 1978; McDonald, 1991, 1992; Stefanacci *et al.*, 1992) projects to the nuclei involved in central control of autonomic activity and stress. The anatomic and pharmacological importance of the neuronal circuitry involving the CeA suggests that alteration in interneuronal communication within the CeA nucleus by a CRF-induced enhancement of I_{Ca}s may be functionally relevant in regulating the autonomic, behavioral and endocrine responses to stress.

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