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Selective Enhancement of L-Type Calcium Currents by Corticotropin in Acutely Isolated Rat Amygdala Neurons

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ABSTRACT

The modulation of voltage-dependent calcium currents (I_{Ca}) by corticotropin was studied in acutely dissociated rat amygdala neurons using whole-cell, patch-clamp recording techniques. Application of corticotropin$_{1-24}$ or corticotropin$_{4-10}$ increased I_{Ca} in a concentration-dependent manner, with half-maximal effective concentrations of 65 and 176 nM and maximal increases of ~75% and ~50%, respectively. Nimodipine (1 μM) reduced the I_{Ca} by ~30%. Subsequent application of corticotropin in the presence of nimodipine failed to produce an enhancement of I_{Ca}, suggesting that corticotropin acts selectively on L-type channels. In addition, corticotropin-mediated enhancement of I_{Ca} after exposure to ω-conotoxin-GVIA and ω-agatoxin-IV was not significantly different from that observed in the control neurons, ruling out the involvement of N- and P/Q-type channels. The effect of corticotropin was mimicked by forskolin and (S_p)-cyclic adenosine 3′,5′-monophosphothioate [(S_p)-cAMPS] and was significantly enhanced in the presence of phosphodiesterase or protein phosphatase inhibitors. On the other hand, the effect of corticotropin was markedly reduced in neurons intracellularly dialyzed with (R_p)-cAMPS, a regulatory site antagonist of cAMP-dependent protein kinase (PKA) or by extracellular perfusion of KT 5720, a catalytic site antagonist of PKA. Taken together, these results show for the first time that corticotropin enhances voltage-dependent Ca^{2+} currents in brain neurons and that this increase is mediated through L-type channels and involves a cAMP-dependent mechanism.

During stress or psychiatric disturbance, corticotropin, a 39-amino-acid polypeptide, is released from the anterior pituitary in response to corticoliberin stimulation. Corticotropin then acts on zona fasciculata cells of the adrenal cortex to activate steroidogenesis and stimulate cortisol secretion (Bondy, 1985). In addition to its hypothalamus-pituitary-adrenal (HPA) axis-activating property, corticotropin has been implicated in many neural and behavioral functions (Hol et al., 1995; van Rijzingen et al., 1996). Corticotropin originates from the precursor molecule pro-opiomelanocortin (POMC). POMC is synthesized mainly in the pituitary and to a lesser degree in the neurons of the hypothalamus, the nucleus tractus solitarius and the amygdala (de Wied and Jolles, 1982). After intraventricular administration of corticotropin analog, corticotropin-radiolabeled cells were observed in the amygdala (Rees et al., 1980). Behavioral studies demonstrated that intra-amygdala administration of corticotropin analog counteracted changes in social behavior induced by isolation, an effect comparable with that produced by subcutaneous injection (Hol and Spruijt, 1992), suggesting that the amygdala is a possible site of action.

The amygdala plays a key role in the modulation of endocrine function, visceral effector mechanisms, and complex patterns of integrated behavior such as defense, aggregation, epilepsy, learning, and memory (Gallagher et al., 1981; Racine, 1981). A primary model for studying the neural substrates of emotional memory related to the function of the amygdala is Pavlovian fear conditioning (Davis et al., 1994; LeDoux, 1994). During fear conditioning, sensory inputs from the auditory thalamus and cortex reaches the lateral and basolateral amygdala, which in turn project to central nucleus. The central nucleus of the amygdala projects to the several brain areas involved in autonomic and endocrine regulation, such as the paraventricular nucleus of the hypothalamus, which links the amygdala directly to the HPA axis (Pitkanen et al., 1997; Maren, 1999). It has been shown that corticoliberin causes a pronounced facilitation of acoustic startle and an enhancement of Ca^{2+} currents in the amygdala neurons (Liang et al., 1992; Yu and Shinnick-Gallagher,

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ABBREVIATIONS: HPA, hypothalamus-pituitary-adrenal; POMC, pro-opiomelanocortin; VDCCs, voltage-dependent Ca^{2+} channels; PIPES, 1,4-piperazine(ethanesulfonic acid; TEA, tetraethylammonium hydrochloride; ω-CgTX, ω-conotoxin-GVIA; ω-AgTX, ω-agatoxin-IV; TTX, tetrodotoxin; I-V, current-voltage; PKA, cAMP-dependent protein kinase; cAMPS, cyclic adenosine 3′,5′-monophosphothioate; IBMX, isobutylmethylxanthine; PP, protein phosphatase; MC, melanocortin; L-LTP, long-lasting potentiation.

1997). Therefore, we sought to determine whether corticotropin exerts similar action on brain cells.

Transmitter and hormone release by many secretory cells is tightly coupled to depolarization-dependent Ca^{2+} entry. In pituitary corticotrophs, corticoliberin increases cytosolic Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs), and this Ca^{2+} entry is responsible for the corticoliberin-mediated corticotropin release from those cells (Guerineau et al., 1991). In adrenocortical cells, corticotropin inhibited a noninactivating K^+ current leading to depolarization-dependent Ca^{2+} entry and cortisol secretion (Capponi et al., 1984; Enyeart et al., 1996). Cortisol secretion induced by corticotropin could be inhibited by antagonists of T-type Ca^{2+} channels, the primary Ca^{2+} channel subtype expressed by these cells (Enyeart et al., 1993). These results suggest that VDCCs are the targets modulated by corticotropin, leading to hormone release. In the present study, we analyze the action of corticotropin$_{1-24}$ and corticotropin$_{4-10}$ on the VDCCs in isolated amygdala neurons using patch-clamp recording in a whole-cell configuration.

Materials and Methods

Cell Preparation. The techniques used to prepare brain slices and cell dissociation were similar to those described previously (Wang et al., 1996). In brief, 10- to 18-day-old, male Sprague-Dawley rats were decapitated and transverse slices (500 μm) were cut from tissue block of the brain using a Vibroslice (Campden Instruments, Silbey, UK). Lateral and basolateral amygdala regions were visualized under a stereomicroscope and dissected from the brain slices with a scalpel. The pieces of brain tissue containing the amygdala were transferred to 10 ml of PIPES saline solution in a spinner flask. The temperature was maintained at 32 ± 1°C and 2–3 mg of protease XIV (Sigma, St. Louis, MO) was added. The amygdalar minislices were stirred gently at a rate sufficient to prevent them from settling, and the solution was bubbled continuously with 95% O_2/5% CO_2. The PIPES saline solution contained 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM PIPES, pH 7.0.

One hour later, minislices were washed with 10 ml of PIPES saline solution (three times) and transferred for storage to a holding chamber filled with continuously bubbled PIPES saline solution at room temperature. When needed, a slice was transferred to 1 ml of external solution and the neurons were dissociated by trituration using a fire-polished Pasteur pipette with a ~1-mm tip diameter. The suspension of dissociated amygdalar neurons then was transferred to a 0.5- to 1-ml recording chamber mounted on an Olympus IMT-2 inverted microscope (Olympus, Tokyo, Japan). Neurons were allowed to settle on poly-L-lysine-coated coverslips.

Electrophysiological Recordings. Whole-cell Ca^{2+} currents were recorded using a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) and filtered at 10 kHz. Patch pipettes were pulled from borosilicate glass and fire polished with a resistance of 2 to 5 MΩ. The external solution contained 3 mM $CaCl_2$, 10 mM glucose, 120 mM tetraethylammonium hydrochloride (TEA), 5 mM 4-aminopyridine, and 10 mM PIPES; 2 μM tetrodotoxin was always added. Pipette solution contained 2 mM $MgCl_2$, 10 mM PIPES, 10 mM glucose, 20 mM TEA, 100 mM Cs methanesulfonate, 10 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 20 mM phosphocreatine, and 0.2 mM leupeptin. All the experiments were performed at room temperature (22 to 25°C). After establishing a gigaseal, the membrane underlying the pipette was ruptured by a gentle suction to obtain whole-cell recording. Experimental tests were initiated when the size of I_{Ca} became stable, which usually took ~5 min. When studying the effect of corticotropin on the voltage-dependent, steady-state activation, we used the voltage ramps and Ba^{2+} as the charge carrier (3 mM $CaCl_2$ was replaced by 5 mM $BaCl_2$ in the external solution).

Online and offline data acquisition and analysis were accomplished using a DigiData 1200 interface (Axon Instruments) between a Axopatch 200B preamplifier and a PC 486 computer using pClamp 6.0 software program. Current traces (not including those of the current-voltage curves) shown in the figures were the averaged responses of two identical voltage steps elicited consecutively at 7-s intervals. Statistical significance was determined at the level of $p \leq 0.05$ using paired or unpaired Student's t tests. All data were expressed as mean ± S.E.M.

Drugs. Corticotropin and its analogs were obtained from Sigma. Nimodipine and ω-CgTX were obtained from Research Biochemicals (Natick, MA). ω-AgTX and tetrodotoxin (TTX) were obtained from Calbiochem (La Jolla, CA). Corticotropin and neurotoxins were dissolved in water and were aliquoted and frozen. Each of the stocks was diluted to the appropriate concentrations in the external solution immediately before the experiment. Nimodipine was dissolved in dimethyl sulfoxide and protected from light. Final dilution of nimodipine resulted in dimethyl sulfoxide concentration of 0.1%, which, when delivered alone, had no effect on calcium currents.

Results

These data were obtained from ≥100 neurons acutely dissociated from rat lateral and basolateral areas. From various cell shapes obtained after trituration, we selected only medium- or small-size neurons with a pyramidal or stellate morphology. No obvious differences in properties of these two cell types were observed, and the results were pooled. Under our recording conditions, low voltage-activated Ca^{2+} current was rarely seen.

Effect of Corticotropin. Whereas corticotropin$_{1-39}$ is the naturally occurring neuropeptide processed from POMC, corticotropin$_{1-24}$ has the full biological activity of the 39-amino acid molecule, possessing both corticotropic and neurotropic characteristics (Hol et al., 1995). Small corticotropin fragments, especially the heptapeptide sequence corticotropin$_{4-10}$ common to corticotropin and melanotropin, have potent neurotropic and behavioral effects and evidence has accumulated to show that they are also physiologically produced. In the present study, we mainly applied these two peptides (1–24 and 4–10) to investigate their actions on the VDCC. When elicited by 200-ms step depolarization from a holding potential of −70 mV, a whole-cell I_{Ca} was normally activated around −40 mV, peaked at −10 mV and reversed between +30 to +40 mV (Wang et al., 1996; Huang et al., 1998). Figure 1A illustrates examples of the effects of corticotropin on I_{Ca}. Application of 1 μM corticotropin$_{4-10}$ or corticotropin$_{1-24}$ increased the amplitude of I_{Ca}. The ability of corticotropin analogs to potentiate I_{Ca} was plotted as a function of concentrations tested (Fig. 1B). Mean values were calculated from six applications at the concentrations tested. The mean experimental data was fitted with a sigmoid curve (r^2 = 0.964 for corticotropin$_{1-24}$ and 0.989 for corticotropin$_{4-10}$). The half-maximal effective concentrations for corticotropin$_{1-24}$ and corticotropin$_{4-10}$ were estimated to be 65 and 176 nM, respectively.

The increase in I_{Ca} occurred within 1 min of application and this effect was persistent after the washout of corticotropin within the recording period of experiment. To see whether the lack of reversibility is caused by the washout of some cytoplasmic constituent during whole-cell dialysis or a slow washout of corticotropin, sharp electrode recordings

from basolateral amygdala neurons were made in brain slices treated with TTX (1 μM) and TEA (10 mM). Previous work has shown that somatic Ca^{2+}-dependent action potential could be evoked under this condition (Wang et al. 1997). Figure 1C shows that, in artificial cerebrospinal fluid containing TTX and TEA, depolarizing current step command (300 ms, 0.8 nA) evoked a high threshold Ca^{2+}-spike. Superfusion of corticotropin$_{1-24}$ (1 μM) to the slice prolonged the duration of Ca^{2+}-dependent action in a reversible manner. The durations measured at half-peak amplitude were 35.0 $\pm$ 2.5 ms in control, 54.3 $\pm$ 4.2 ms in the presence of corticotropin ($n = 6, p < 0.01$ paired t test) and 39.5 $\pm$ 2.7 ms 40 min after the washout of drug.

The voltage-dependent effect of corticotropin$_{1-24}$ was analyzed in current-voltage (I-V) relationships in the presence and absence of 1 μM corticotropin$_{1-24}$. As shown in Fig. 2A, corticotropin increased I_{Ca} to a greater extent at negative potentials (≤ -10 mV) than at more depolarized membrane potentials (0$-$ +40 mV) and the increase was not accompanied by a shift in the current-voltage relationship (peak I_{Ca} voltages were -9 ± 1 mV in control and -8 ± 1 mV in corticotropin, $n = 6$). The I-V relationship was also studied with voltage ramps using 5 mM Ba^{2+} as the charge carrier (Fig. 2B). Currents were evoked by voltage ramps from -70 to +50 mV (0.33 mV/ms); consistent with the above observation, corticotropin$_{1-24}$ did not shift the I-V relationship ($n = 5$).

The possibility that corticotropin$_{1-24}$ may affect the voltage dependence of steady-state inactivation was examined. In this series of experiments, I_{Ca} was elicited by step commands to 0 mV from holding potentials ranging from -80 mV to 0 mV. The inactivation curve was fitted with the following Boltzmann equation: $I / I_{max} = 1/\{1 + \exp[(V - V_{1/2} / k)]\}$, where $V_{1/2}$ is the potential at which $I / I_{max} = 0.5$, and k is the slope factor of the curve. As shown in Fig. 3, corticotropin$_{1-24}$ did not shift the voltage-dependent steady-state inactivation. The values for $V_{1/2}$ and k in control and corticotropin were -21.2 ± 1.9 mV and -19.2 ± 1.4 mV and -20.8 ± 2.0 mV and -16.6 ± 1.5 mV ($n = 4$), respectively. Taken together, these data suggest that corticotropin-induced enhancement of I_{Ca} is not caused by a shift in the voltage dependence of steady-state activation or inactivation.

We also investigated the effect of a C-terminal fragment of corticotropin, corticotropin$_{18-39}$ (CLIP), on the I_{Ca}. Corticotropin$_{18-39}$ also enhanced I_{Ca}, with a less maximal effect of ~30% (Fig. 1B). If corticotropin$_{18-39}$ and corticotropin$_{4-10}$ were exerting their effects via the same receptor, then saturating concentration of corticotropin$_{4-10}$ should occlude the modulation produced by corticotropin$_{18-39}$. Indeed, this is the case. As shown in Fig. 4A, application of corticotropin$_{18-39}$ (1 μM) increased the I_{Ca} by 33.1 $\pm$ 3.2% ($n = 6$), but subsequent addition of corticotropin$_{4-10}$ (1 μM) in the presence of corticotropin$_{18-39}$ only increased the I_{Ca} by 3.3 $\pm$ 3.5% ($n = 6$), which was significantly less than that produced by corticotropin$_{4-10}$ alone (51.3 $\pm$ 1.1%, $n = 6$, $p < 0.01$). We also performed the reverse experiments in which corticotropin$_{4-10}$ was applied before the addition of corticotropin$_{18-39}$. Shown in Fig. 4B is a time course of these experiments in which peak current evoked by a voltage step to -10 mV was plotted as a function of time. Representative current traces were shown in the figure inset. Initially, application of corticotropin$_{4-10}$ (1 μM) increased the I_{Ca} by 44.7 $\pm$ 2.4% ($n = 6$). Subsequently, corticotropin$_{18-39}$ (1 μM) had little or no effect (2.0 $\pm$ 1.8%, $n = 6$, $p > 0.1$).

Types of Ca^{2+} Channels Modulated. Rat amygdala neurons express several pharmacologically distinct subtypes of high-threshold Ca^{2+} channels (Foehring and Scroggs, 1994; Yu and Shinnick-Gallagher, 1997). To determine which Ca^{2+} channel subtypes were modulated, corticotropin was applied after selective block of populations of Ca^{2+} channels. Figure 5A shows that application of nimodipine (1 μM), a selective L-type Ca^{2+} channel blocker, reduced the whole-cell I_{Ca} by 29.3 $\pm$ 1.8% ($n = 6$). However, subsequent application of corticotropin$_{1-24}$ (1 μM) in the presence of nimodipine failed to cause an enhancement (1.8 $\pm$ 1.4%, $n = 6$). If corticotropin acts selectively on the L-type channels, then nimodipine should be able to abolish the component of corticotropin-mediated enhancement. Figure 5B shows that ap-

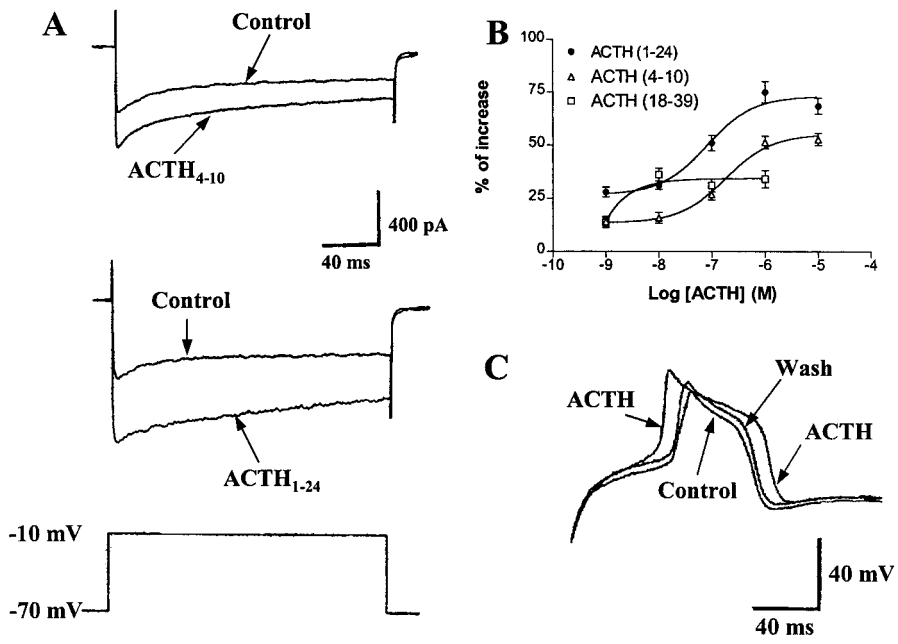

Fig. 1. corticotropin increases I_{Ca} in rat amygdala neurons. A, I_{Ca} was elicited by 200-ms step command from a holding potential of -70 mV to -10 mV. Application of corticotropin$_{4-10}$ (1 μM) or corticotropin$_{1-24}$ (1 μM) increased the I_{CA}. ACTH, corticotropin. B, concentration-dependent enhancement of I_{Ca} by corticotropin. Data were expressed as mean $\pm$ S.E.M. Points on the curve were obtained from six applications on different neurons for each concentration. C, sharp-electrode, current clamp recording from a basolateral amygdala neuron in the slice preparation. The slice was bathed in 1 μM TTX and 10 mM TEA containing solution to allow the expression Ca^{2+}-dependent action potential. Superfusion of corticotropin$_{1-24}$ (1 μM) prolonged the spike duration that reversed with 40 min of washing. ACTH, corticotropin.

plication of corticotropin$_{1-24}$ (1 μM) increased the I_{Ca} to 170.8 ± 3.6% (n = 6) of control; subsequent addition of nimodipine (1 μM) reduced the I_{Ca} to 69.4 ± 4.0% (n = 6) of the initial control, a value similar to that without corticotropin treatment (70.2 ± 2.6%, n = 6, $p > 0.1$). These results suggest that corticotropin selectively acts on the L-type Ca^{2+} channels.

To further exclude the involvement of N- and P/Q-type Ca^{2+} channels in the action of corticotropin, we applied submaximal concentrations of ω-CgTX and ω-AgTX simultaneously. Application of ω-CgTX (100 nM) plus ω-AgTX (200 nM) reduced the whole-cell I_{Ca} to 70.2 ± 2.6% (n = 6) of control. As shown in Fig. 6, in the presence of ω-CgTX + ω-AgTX, corticotropin$_{4-10}$ still increased I_{Ca} to 114.3 ± 3.6% (n = 6) of the initial control, suggesting that the effect of corticotropin is not influenced by ω-CgTX + ω-AgTX. Taken together, these data suggest that the primary effect of corticotropin on the I_{Ca} is to enhance dihydropyridine-sensitive L-type currents.

Involvement of PKA Pathway. Because corticotropin receptor is known to be coupled positively to adenylyl cyclase (Florijn et al., 1993), experiments were performed to determine whether the cAMP-dependent protein kinase (PKA) was involved in mediating the I_{Ca} potentiation. Forskolin (25 μM), an adenylyl cyclase activator, mimicked the effect of corticotropin, increasing the I_{Ca} by 38.4 ± 5.0% (n = 6). Similarly, application of membrane-permeant analog of cAMP, (S_p)-cAMPS (50 μM), caused a 62.6 ± 2.7% (n = 6) enhancement (Fig. 7A). Consistent with these results, phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) also enhanced the effect of subsaturating concentration of corticotropin$_{1-24}$. As depicted in Fig. 7A, corticotropin$_{1-24}$ (10 nM) alone increased the I_{Ca} by 31.3 ± 3.8% (n = 6). However, in the presence of IBMX (50 μM), the same concentration of corticotropin$_{1-24}$ increased the I_{Ca} by 53.3 ± 6.4% (n = 8). The difference is statistically significant ($p < 0.05$).

(R_p)-cAMPS, a PKA regulatory site antagonist, was dissolved into the pipette solution (50 μM). Figure 7B shows that inclusion of intracellular (R_p)-cAMPS markedly reduced I_{Ca} potentiation in response to corticotropin$_{4-10}$ (1 μM); corticotropin increased the I_{Ca} by only 13.3 ± 1.8% (n = 9, $p <$ 0.01). In another three experiments, cells were preincubated with (R_p)-cAMPS (100 μM) for at least 2 h before whole-cell recordings were made with normal pipette solution. Under this condition, the effect of corticotropin was also markedly reduced, causing an increase of only 9.8 ± 2.1% (n = 3). Similar result was obtained when neurons were pretreated with KT 5720 (1 μM), a PKA catalytic site antagonist. Corticotropin$_{4-10}$ (1 μM) increased the I_{Ca} by only 11.4 ± 1.4% (n = 7) in KT 5720-treated neurons compared with 51.3 ± 1.1% (n = 6, $p < 0.01$) without KT 5720 treatment (Fig. 7B). Thus, the action of corticotropin is reduced by the treatments that inhibit PKA.

To investigate whether an ongoing phosphorylation-dephosphorylation process mediated by PKA and a serine-threonine phosphatase is involved in corticotropin action on the Ca^{2+} currents, we studied the role of phosphatase in the

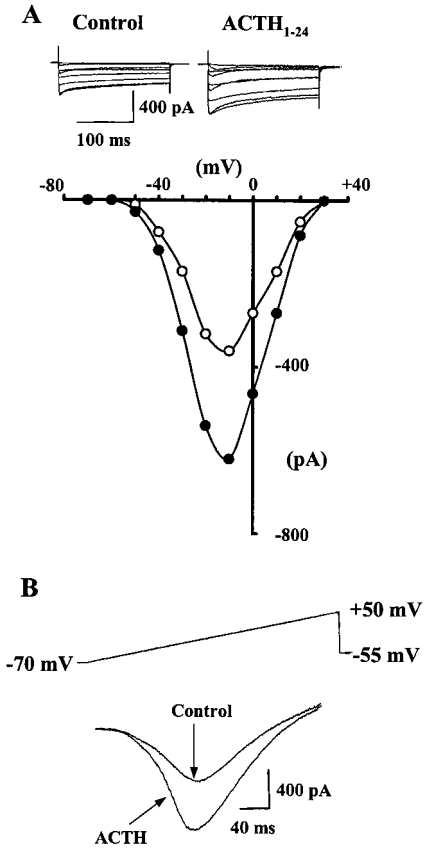

Fig. 2. Effect of corticotropin on current-voltage relationship in amygdala neurons. A, a family of current traces elicited by depolarizing steps (10-mV increments) from a holding potential of −70 mV in the absence and presence of 1 μM corticotropin$_{1-24}$. The current-voltage plot was constructed by measuring the peak amplitude of I_{Ca} before and after application of corticotropin$_{1-24}$. ACTH, corticotropin. B, effect of corticotropin$_{1-24}$ on the I_{Ca} with a voltage ramp (0.33 mV/ms) using 5 mM Ba^{2+} as the charge carrier. ACTH, corticotropin.

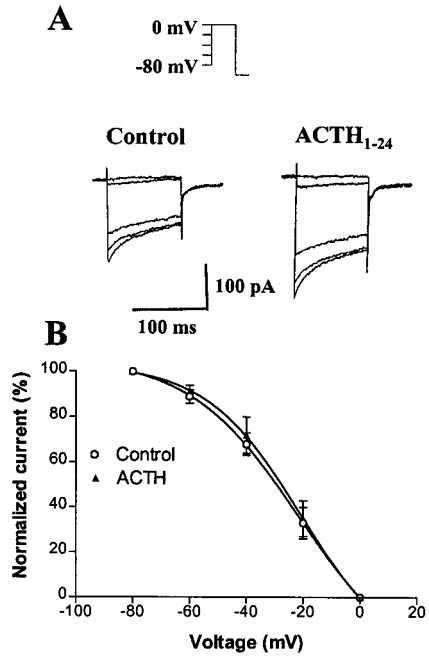

Fig. 3. Corticotropin does not affect steady-state inactivation. A, effect of corticotropin$_{1-24}$ (1 μM) on the I_{Ca} values, which were elicited by step commands to 0 mV from holding potentials ranging from −80 to 0 mV. ACTH, corticotropin. B, steady-state inactivation of I_{Ca}. Solid lines are the best fit of Boltzmann equation: $I / I_{max} = 1 / \{1 + \exp[(V - V_{1/2}/k)]\}$, where $V_{1/2}$ is the potential at which $I / I_{max} = 0.5$, and k is the slope factor of the curve. ACTH, corticotropin.

corticotropin enhancement of I_{Ca}. In these experiments, the membrane permeant inhibitor of protein phosphatase 1 (PP1) and PP2A, okadaic acid, was applied. As illustrated in Fig. 7A, in the presence of okadaic acid (1 μM), corticotropin$_{1-24}$ (10 nM) increased the I_{Ca} from 31.3 ± 3.8% to 60.9 ± 3.8% ($n = 6$, $p < 0.05$).

Discussion

The present results show for the first time that corticotropin enhances voltage-dependent Ca^{2+} currents in brain neurons and that this increase is mediated through dihydropyridine-sensitive L-type channels. The effect was concentration-dependent, with estimated EC_{50} values of 65 and 176 nM and a maximal increase of 50 to 75% for corticotropin$_{1-24}$ and corticotropin$_{4-10}$, respectively. The mimicry of corticotropin effect by active analogs suggests a receptor-mediated mechanism. The effect of corticotropin probably involves a cAMP- and protein kinase-dependent mechanism because the increase was mimicked by forskolin and (S_p)-cAMPS and was markedly reduced in neurons intracellularly dialyzed with (R_p)-cAMPS or extracellular perfusion of KT 5720. Moreover, inhibition of the cAMP-degradative enzyme phosphodiesterase potentiated the corticotropin-induced enhancement of I_{Ca}. The enhancement of corticotropin-mediated modulation by inhibition of PP1/PP2A with okadaic acid further supports the hypothesis that protein phosphorylation was involved.

Enhancement of Ca^{2+} Currents by Corticotropin in the Amygdala Neurons. Corticotropin and corticotropin-like peptides originate from the precursor molecule POMC. POMC is processed by tissue-specific enzymes to yield small peptides, such as corticotropin, α-melanotropin, β-melanotropin, γ-melanotropin, and endorphins, collectively termed melanocortins (MC). Five MC receptors have been identified so far, of which MC3, MC4, and MC5 were located in the brain (Hol et al., 1995; van Rijzingen et al., 1996). Detailed neuroanatomical mapping by in situ hybridization demonstrated that MC3 and MC4 were expressed in the hypothalamus and limbic system including the amygdala. MC3 differs from MC4 in that it specifically recognizes peptides with the corticotropin$_{4-10}$ core sequence. All peptides with the corticotropin$_{4-10}$ core are approximately equipotent for MC3 receptor; on the other hand, corticotropin$_{4-10}$ is 100 times less potent than corticotropin$_{1-24}$ in activating the

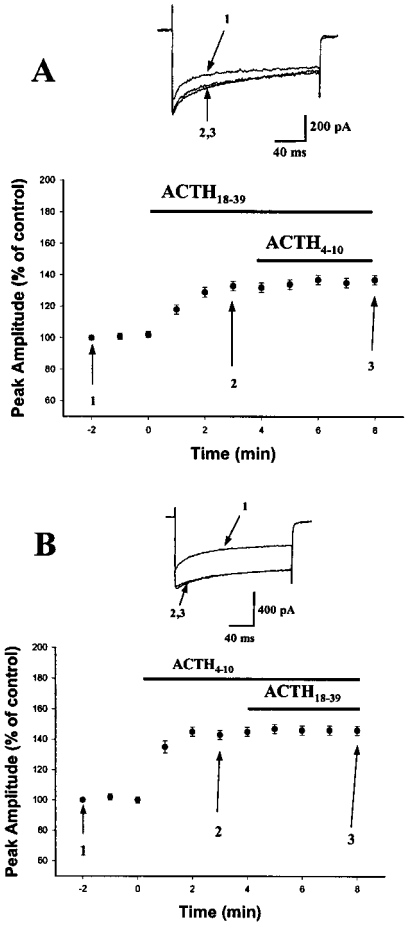

Fig. 4. Enhancement produced by corticotropin$_{18-39}$ and corticotropin$_{4-10}$ can mutually occlude each other. A, application of corticotropin$_{18-39}$ increased the I_{Ca} by 33.1 ± 3.2%, but largely reduced the effect of corticotropin$_{4-10}$. ACTH, corticotropin. B, on the other hand, corticotropin$_{4-10}$ increased the I_{Ca} by 44.7 ± 2.4% and occluded the effect of corticotropin$_{18-39}$. ACTH, corticotropin.

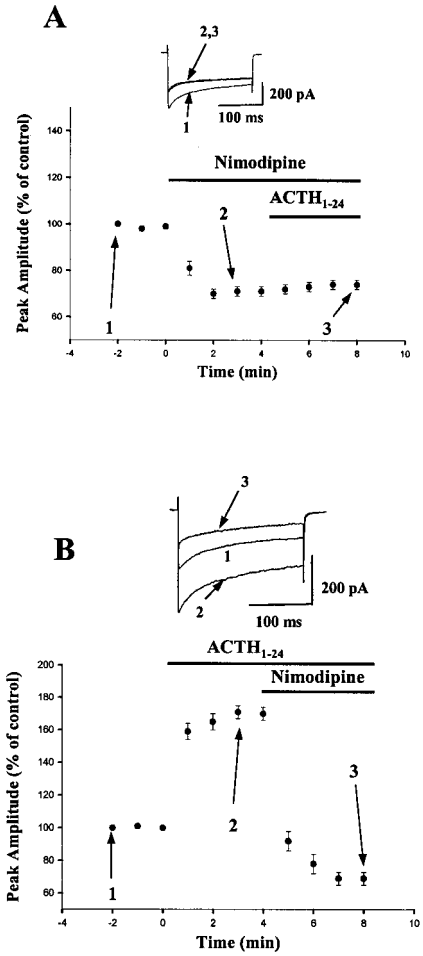

Fig. 5. Corticotropin acts selectively on the L-type calcium currents. The percentage change of peak I_{Ca} was plotted as a function of time. Bars denote the periods of delivery of corticotropin$_{1-24}$ (1 μM) or nimodipine (1 μM). A, as expected, nimodipine decreased I_{Ca}, and subsequent application of corticotropin$_{4-10}$ in the presence of nimodipine failed to produce an enhancement of I_{Ca}. *Inset* shows the current traces taken at the time point as indicated. ACTH, corticotropin. B, application of corticotropin$_{1-24}$ increased the I_{Ca} to 171 ± 4% ($n = 6$) of control, subsequent addition of nimodipine reduced the I_{Ca} to 69 ± 4% ($n = 6$) of the initial control. ACTH, corticotropin.

MC4 receptor (Gantz et al., 1993). Thus, although it is now impossible to distinguish between the MC receptor subtypes without developing specific antagonists, based on ED_{50} values, our data are more likely to be consistent with mediation through an MC3 receptor subtype.

In brain tissues, the concentrations of corticotropin$_{1-24}$ needed to elicit a half-maximal response ranged from 10 to 100 nM (Hol et al., 1995), which is comparable with the results reported herein but is ~1000-fold higher than those in endocrine cells (Enyeart et al., 1996). The difference is probably caused by the differential expression of subtype receptors in different tissues. In situ hybridization analysis revealed that adrenal cortex expressed MC2 receptors (Mountjoy et al., 1992).

The increase in I_{Ca} could be caused by a shift of the current-voltage relationship in the negative direction by corticotropin, which would cause more channels to open at negative potentials, thereby enhancing the I_{Ca}. Alternatively, corticotropin could increase I_{Ca} by shifting voltage-dependent steady state inactivation to a more depolarized level. In the present study, neither the peak of the current-voltage relationship nor the voltage-dependent steady-state inactivation were affected by corticotropin. These results suggest that corticotropin-induced enhancement of I_{Ca} was not caused by a shift in the voltage dependence of steady-state activation or inactivation. Further study with single-channel recordings is needed to determine whether corticotropin-induced increase in I_{Ca} results from an opening of more calcium channels and/or an increase in single-channel conductance.

L-Type Ca^{2+} Channel Is the Target of Modulation. At least four different high-threshold, voltage-dependent Ca^{2+} currents (L-, N-, P/Q-, and R types) have been identified in the amygdala neurons based on pharmacological criteria (Foehring and Scroggs, 1994; Yu and Shinnick-Gallagher, 1997; Huang et al., 1998). In the present study, sequential application of nimodipine and corticotropin revealed that the effect of corticotropin on I_{Ca} was completely blocked by nimodipine. Conversely, pretreatment of neurons with corticotropin did not affect the inhibitory effect of nimodipine; nimodipine not only completely abolished the component of corticotropin-mediated enhancement but also reduced the I_{Ca} to ~69% of the initial control, a value not significantly different from that without corticotropin pretreatment. In contrast to the clear participation of L-type channels, blockade of N-type Ca^{2+} channels with ω-CgTX and P/Q-type channels with ω-AgTX did not reduce the corticotropin response. Thus, corticotropin acts selectively on the dihydropyridine-sensitive L-type Ca^{2+} channels. Possible modulation of Ca^{2+} channels by corticotropin has been reported in several systems. For instances, in a rat model of volume-controlled hemorrhagic shock, corticotropin produced a sustained restoration of arterial pressure, pulse pressure and respiratory function and these effects were inhibited by N- and L-type Ca^{2+} channel blockers (Guarini et al., 1993). Intracerebroventricular administration of corticotropin induced a peculiar symptomatology characterized by recurrent episodes of stretching, yawning, and penile erection that could be blocked by ω-CgTX, implying the involvement of N-type Ca^{2+} channels (Argiolas et al., 1990).

PKA-Mediated Modulation of L-type Currents. The MC receptors belong to the superfamily of G protein-coupled

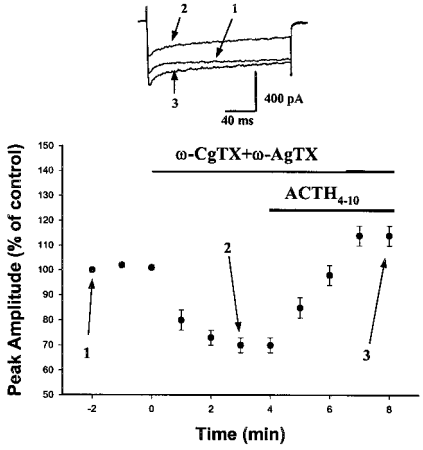

Fig. 6. corticotropin still increases the I_{Ca} in the presence of N- and P/Q-type Ca^{2+} channel blockers. The percentage change of peak I_{Ca} was plotted as a function of time. Bars denote the periods of delivery of corticotropin$_{4-10}$ (1 μM) or ω-CgTX + ω-AgTX. Application of ω-CgTX (100 nM) plus ω-AgTX (200 nM) decreased I_{Ca} without affecting the corticotropin-induced I_{Ca} enhancement. ACTH, corticotropin.

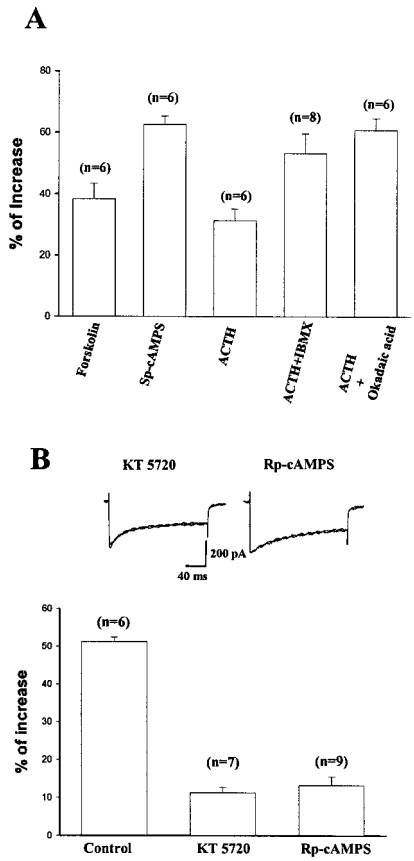

Fig. 7. Corticotropin-induced enhancement of I_{Ca} is mediated by a PKA signaling cascade. A, forskolin (25 μM) or cAMP analog [(S_p)-cAMPS, 50 μM] mimics the corticotropin modulation of I_{Ca}. In addition, IBMX (50 μM) or protein phosphatase inhibitor okadaic acid (1 μM) potentiate the modulation. ACTH, corticotropin. B, effect of corticotropin$_{4-10}$ (1 μM) on the I_{Ca} in neurons intracellularly dialyzed with (R_p)-cAMPS (50 μM) or pretreated with KT 5720 (1 μM). The bars represent the enhancement of I_{Ca} (mean ± S.E.M.) by corticotropin$_{4-10}$ in control, in neurons intracellularly dialyzed with (R_p)-cAMPS or pretreatment with KT 5720. ACTH, corticotropin.

receptors and are positively coupled to adenylyl cyclase. Early studies in adrenal cortex showed that increasing intracellular cAMP mimicked the stimulation of corticosteroid production, indicating that there was a direct functional coupling between the corticotropin receptor, cAMP production, and corticosteroid synthesis (Halkerston, 1975). In brain tissues (striatal and septal slices), an increase in cAMP after corticotropin treatment has also been observed (Wiegant et al., 1979; Florijn et al., 1993). In the present study, we have demonstrated that forskolin, but not 1,9-dideoxyforskolin, mimics the effect of corticotropin. Similarly, activation of PKA by (S_p)-cAMPS results in a potentiation. The effect of subsaturating concentration of corticotropin$_{1-24}$ is significantly enhanced in the presence of phosphodiesterase inhibitor or protein phosphatase inhibitor. Furthermore, modulation of I_{Ca} by corticotropin was blocked by (R_p)-cAMPS and KT 5720, the respective PKA regulatory and catalytic site antagonists. These results suggest that PKA mediates the enhancement of I_{Ca} by corticotropin in the amygdala neurons.

It is well established that stimulation of adenylyl cyclase or PKA leads to phosphorylation of cardiac L-type Ca^{2+} channels, resulting in an increase in contractility and heart rate. However, the selective potentiation of L-type Ca^{2+} currents in central neurons by neuromodulators is limited in the literature. In neostriatal neurons recorded in the presence of phosphatase inhibitors, dihydropyridine-sensitive L-type currents were enhanced by dopamine D_1 agonists or cAMP analogs (Surmeier et al., 1995). An enhancement of L-type currents by β-adrenergic agonists and cAMP analogs has been reported in hippocampal neurons (Gray and Johnston, 1987; Kavalali et al., 1997).

Functional Implications. In central neurons, L-type Ca^{2+} channel plays a critical role in the synthesis of new proteins important for the expression of long-lasting potentiation (L-LTP) of synaptic transmission and long-term memory. The generation of L-LTP requires a sufficiently large influx of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels and de novo RNA transcription and protein synthesis. L-LTP and L-LTP-associated increases in cAMP response element-mediated gene expression are attenuated by L-type Ca^{2+} channel blockers (Impey et al., 1996). Up-modulation of I_{Ca} reported here implicates that corticotropin may influence behaviors in mammals. Indeed, this hypothesis was supported by the behavioral studies showing that corticotropin enhanced the acquisition and retention of passive avoidance as well as delayed extinction of pole-jumping avoidance (Greven and de Wied, 1977; Fekete and de Wied, 1982). Furthermore, deficits in spatial orientation induced by an acute administration of *N*-methyl-D-aspartate receptor antagonist could be counteracted by corticotropin analog (Spruijt et al., 1994).

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