

Regulation of Ca^{2+} -Sensitive Adenylyl Cyclase in Gonadotropin-Releasing Hormone Neurons

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In immortalized GnRH neurons, cAMP production is elevated by increased extracellular Ca^{2+} and the Ca^{2+} channel agonist, BK-8644, and is diminished by low extracellular Ca^{2+} and treatment with nifedipine, consistent with the expression of adenylyl cyclase type I (AC I). Potassium-induced depolarization of GT1-7 neurons causes a dose-dependent monotonic increase in $[\text{Ca}^{2+}]_i$ and elicits a bell-shaped cAMP response. The inhibitory phase of the cAMP response is prevented by pertussis toxin (PTX), consistent with the activation of G_i -related proteins during depolarization. Agonist activation of the endogenous GnRH receptor in GT1-7 neurons also elicits a bell-shaped change in cAMP production. The inhibitory action of high GnRH concentrations is prevented by PTX, indicating coupling of the GnRH receptors to G_i -related proteins. The stimulation of cAMP production by activation of endogenous LH receptors is enhanced by low (nanomolar) concentrations of GnRH but is abolished by micromolar concentrations of GnRH, again in a PTX-sensitive manner. These findings indicate that GnRH neuronal cAMP production is maintained by Ca^{2+} entry through voltage-sensitive calcium channels, leading to activation of Ca^{2+} -stimulated AC I. Furthermore, the Ca^{2+} influx-dependent activation of AC I acts in conjunction with AC-regulatory G proteins to determine basal and agonist-stimulated levels of cAMP production. (Molecular Endocrinology 15: 429-440, 2001)

INTRODUCTION

Mammalian sexual development and reproductive function are controlled by the episodic stimulation of pituitary gonadotropin secretion by the hypothalamic decapeptide, GnRH. GnRH is produced by a small

number (~1,500) of specialized neurosecretory cells in the hypothalamus (1-4). The secretory activity of normal and immortalized GnRH neurons (GT1-7 neurons) is dependent on the depolarization-induced entry of extracellular calcium that results from their spontaneous firing of calcium-dependent action potentials (5-7). GnRH secretion from hypothalamic neurons and GT-1 cells is also stimulated by receptor-mediated increases in intracellular cAMP, and by treatment with forskolin and cAMP analogs (8-11).

Immortalized GnRH neurons (GT1-7 neurons) express several G protein-coupled receptors (GPCRs), including those for GnRH and LH/human (h) CG (9, 12, 13) as well as β_1 adrenergic (10), D1 dopaminergic (14), muscarinic (15, 16), cholinergic (4, 17, 18), and serotonergic receptors (19). Agonist activation of specific GPCRs and dissociation of their cognate G proteins release α - and $\beta\gamma$ -subunits that regulate phospholipase C- β , adenylyl cyclase (AC), and ion channels, which in turn control the intracellular levels of inositol phosphates, calcium, cAMP, and other second messengers (20, 21). The regulation of AC in hypothalamic neurons by changes in cytosolic $[\text{Ca}^{2+}]_i$ is an important step in the integration of the actions of the two major second messengers, cAMP and Ca^{2+} .

Agonist-induced activation of phospholipase C (PLC) is the major signal transduction pathway in cells that express GnRH receptors, and the consequent Ca^{2+} mobilization and activation of protein kinase C (PKC) by GnRH are key elements in the hypothalamic control of gonadotropin hormone secretion by the pituitary gland (22). Activation of the GnRH receptors expressed in GT1-7 neurons also increases PLC and phospholipase D activity and stimulates voltage-gated Ca^{2+} entry (23). The ability of elevated intracellular cAMP levels to increase GnRH release in GT-1 cells has also implicated receptors that are positively coupled to AC in the signaling cascade that regulates GnRH release (10). Recent studies in GT-1 cells have suggested that the cAMP signaling pathway is an essential component of the mechanism responsible for the pulsatile release of GnRH from hypothalamic neurons (11).

In the present study, the regulation of Ca^{2+} -dependent AC was investigated in cultured rat hypothalamic cells and immortalized murine GnRH neurons (GT1-7 neurons). The role of $[\text{Ca}^{2+}]_i$ in AC regulation was analyzed during receptor-dependent and -independent changes in Ca^{2+} influx and mobilization. Receptor-independent increases in $[\text{Ca}^{2+}]_i$ were elicited by high extracellular $[\text{Ca}^{2+}]_e$; the calcium channel agonist BK-8644; K^+ -induced depolarization; and treatment with ionomycin. Conversely, $[\text{Ca}^{2+}]_i$ levels were decreased by treatment with the calcium channel blocker nifedipine and incubation in low extracellular $[\text{Ca}^{2+}]_e$. In addition, the consequences of agonist stimulation of the Ca^{2+} -mobilizing GnRH receptor, and associated changes in cAMP production, were investigated in low and high extracellular $[\text{Ca}^{2+}]_e$ and in the presence of Ca^{2+} channel agonist and antagonist analogs. The control of cAMP production was also analyzed during activation of LH/hCG receptors expressed in GT1-7 neurons. Those studies have demonstrated that the prevailing level of $[\text{Ca}^{2+}]_i$ is a critical determinant of AC activity and cAMP production in GnRH neuronal cells.

RESULTS

Dependence of Basal cAMP Production on Ca^{2+} Influx

In GT1-7 neurons, treatment with the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 1 mM), caused a 5-fold increase in cAMP production (2.1 ± 0.3 pmol/ml vs. 13.7 ± 2.7 pg/ml). This was accompanied by increased GnRH release (23.8 ± 3.4 pg/ml vs. 36.4 ± 4.5 pg/ml; $P < 0.01$; $n = 6$) and had no significant effect on $[\text{Ca}^{2+}]_i$ (187 ± 23 nM control vs. 215 ± 37 nM IBMX; $n = 8$). The intracellular cAMP concentration in the presence of IBMX reached a maximum during the first 60 min and declined thereafter. In contrast, cAMP release into the incubation medium showed a slower and prolonged rise between 30 and 240 min (Fig. 1, A and B). The rate of cAMP production by cultured GT1-7 neurons was highly sensitive to the changes in extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$). Increases in $[\text{Ca}^{2+}]_e$ caused concentration-dependent elevations of both $[\text{Ca}^{2+}]_i$ and cAMP production, with EC_{50} values of 1.3 mM and 2.2 mM, respectively (Fig. 1, C and D). Concentration-dependent increases in cAMP production were also observed when cells were incubated with increased $[\text{Ca}^{2+}]_e$ in the absence of IBMX (Fig. 1E). AC activity, in membrane preparations from GT1-7 neurons, was also stimulated by Ca^{2+} in a concentration-dependent manner, reaching a maximum at 10 μM Ca^{2+} (Fig. 1F).

The stimulatory action of extracellular Ca^{2+} on cAMP production was highly dependent on Ca^{2+} entry through L-type Ca^{2+} channels. The dihydropyridine Ca^{2+} channel agonist, BK-8644, caused time- and dose-dependent increases in cAMP production, with an EC_{50} of 9.6 nM (Fig. 2, A and B). cAMP production

was significantly increased (12.7 ± 2.1 to 16.8 ± 1.6 pmol/ml; $P < 0.05$; $n = 4$) after 5 min of incubation with 1 μM BK-8644 and was maximal after 15 min. The stimulatory effect of BK-8644 on cAMP production was abolished by incubation in low $[\text{Ca}^{2+}]_e$ (Fig. 2B). Conversely, L-type channel blockade by nifedipine caused a dose-dependent decrease in cAMP production with an IC_{50} of 6.7 nM (Fig. 2D), and this was also maximal after 15 min (Fig. 2C). A significant decrease in cAMP production (12.7 ± 2.1 pmol/ml, control vs. 8.6 ± 1.2 pmol/ml; $P < 0.05$; $n = 4$) was observed after 5 min of incubation with 1 μM nifedipine.

In contrast to the stimulatory effect of increased Ca^{2+} influx on cAMP production, ionomycin-induced $[\text{Ca}^{2+}]_i$ increases in cells bathed in Ca^{2+} -free medium did not lead to increased cAMP formation. The 4-fold elevation of $[\text{Ca}^{2+}]_i$ induced by 400 nM ionomycin (Fig. 3A), and the 5-fold increase induced by 1 μM ionomycin (Fig. 3B) had no effect on cAMP production (Fig. 3, C and D). The $[\text{Ca}^{2+}]_i$ level in cells treated with 1 μM ionomycin was comparable to that of $[\text{Ca}^{2+}]_i$ in cells bathed in 1.5 mM $[\text{Ca}^{2+}]_e$, yet cAMP production remained low despite the 5-fold increase in $[\text{Ca}^{2+}]_i$ during Ca^{2+} mobilization from intracellular stores.

Depolarization of GT1-7 neurons by increased extracellular $[\text{K}^+]$ caused a progressive, concentration-dependent increase in $[\text{Ca}^{2+}]_i$ (Fig. 4A). This was associated with a bell-shaped cAMP response (Fig. 4B) in which the maximal level was elicited at 35 mM KCl, a concentration that caused only a half-maximal increase (462 nM) in $[\text{Ca}^{2+}]_i$. Higher KCl concentrations caused further increases in $[\text{Ca}^{2+}]_i$ of up to 920 nM and were associated with a progressive and marked decrease in cAMP production almost to the baseline level. As expected, the stimulatory effect of K^+ on cAMP production was abolished by incubation in 100 nM extracellular Ca^{2+} (Fig. 4B). There was also a major decrease in the baseline cAMP level below that observed at 1.5 mM Ca^{2+} .

The inhibitory effect of 55 mM K^+ on cAMP production was prevented by pretreatment of GT1-7 neurons with 100 ng/ml pertussis toxin (PTX), indicating that activation of G_i or G_o was responsible for the inhibition of cAMP production (Fig. 4C). In this experiment, PTX treatment significantly increased both the stimulated cAMP response to 35 mM K^+ and the inhibited response of 50 mM K^+ , suggesting that G_i -mediated impairment of AC activity increased progressively with increasing degrees of depolarization.

Effects of GnRH Receptor Activation on cAMP Production

In addition to stimulating the inositol phosphate/ Ca^{2+} -signaling pathway via $G_{q/11}$, activation of the GnRH receptor has been shown to increase cAMP production (24-26). The action of GnRH on cAMP production in GT1-7 neurons was biphasic and dose-dependent. GnRH concentrations of up to 10 nM caused progressive increases in $[\text{Ca}^{2+}]_i$ and cAMP production (Fig. 5,

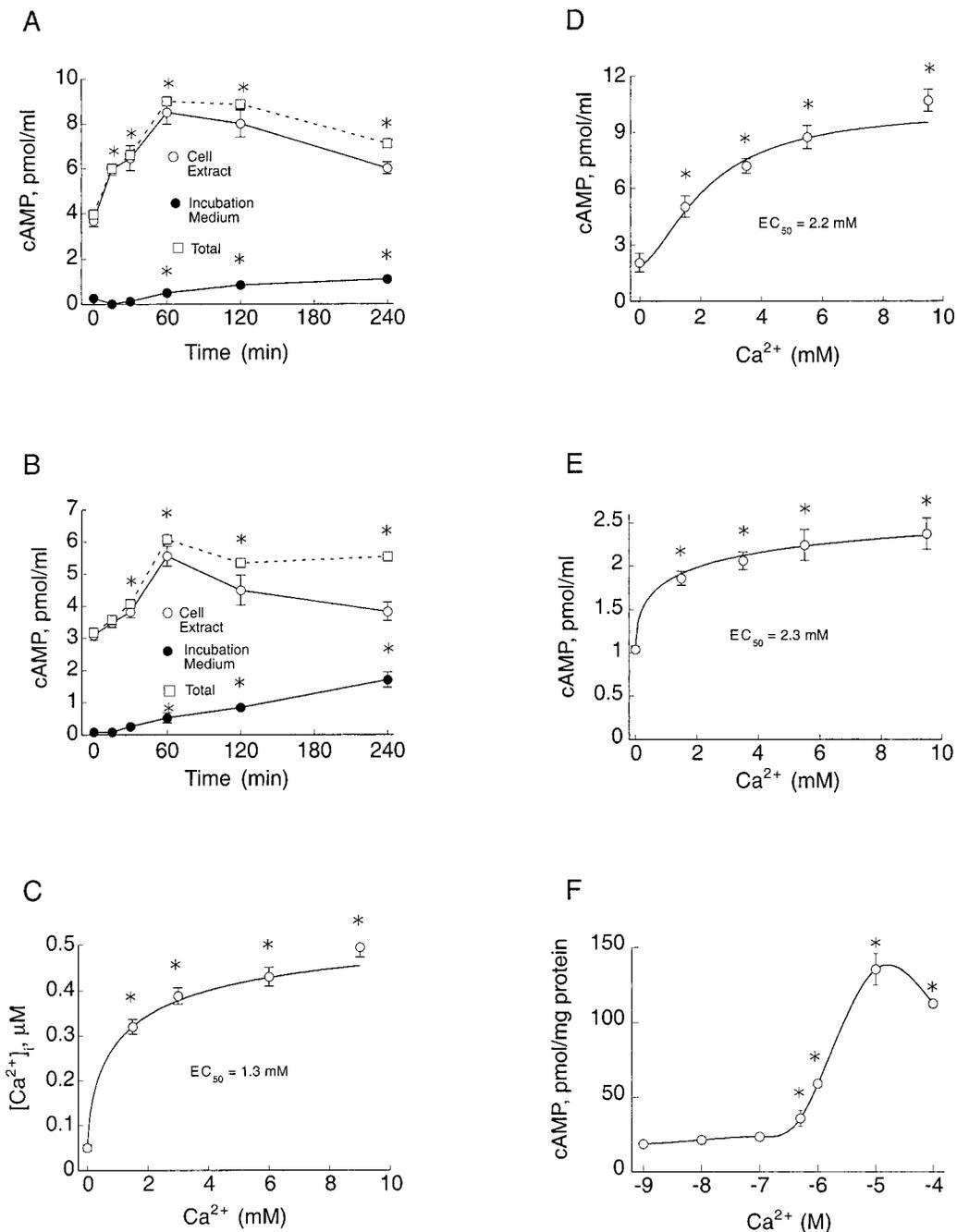


Fig. 1. Extracellular Calcium-Regulated cAMP Production in GnRH Neurons

A and B, Time course of basal cAMP production in hypothalamic cells (A) and GT1-7 neurons (B). C, Extracellular $[\text{Ca}^{2+}]_i$ dependence of $[\text{Ca}^{2+}]_i$ in GT1-7 neurons. D, Extracellular $[\text{Ca}^{2+}]$ -dependence of cAMP production in GT1-7 neurons. E, Extracellular $[\text{Ca}^{2+}]$ -dependence of cAMP production in the absence of IBMX. F, $[\text{Ca}^{2+}]_i$ -dependence of AC activity assayed by cAMP production in GT1-7 membrane preparations. Data are means \pm SE of three independent experiments. Asterisks indicate significant differences ($P < 0.05$) compared with basal cAMP production and Ca^{2+} free-medium.

A and B). Membrane-associated G_s α -subunit immunoreactivity decreased during treatment with low nanomolar GnRH concentrations but was unchanged after 30 min treatment with $1 \mu\text{M}$ GnRH or with a GnRH antagonist analog (Fig. 5C). However, higher concentrations of GnRH that continued to increase $[\text{Ca}^{2+}]_i$, caused a progressive decrease in cAMP production.

This inhibitory action of GnRH on cAMP formation was prevented by treatment with PTX. The bell-shaped dose-response curve and the reversal of the inhibitory actions of high GnRH concentrations on cAMP production by PTX are again consistent with the activation of G_i -related proteins (Fig. 5D). Both the stimulatory action of low nanomolar GnRH concentrations on

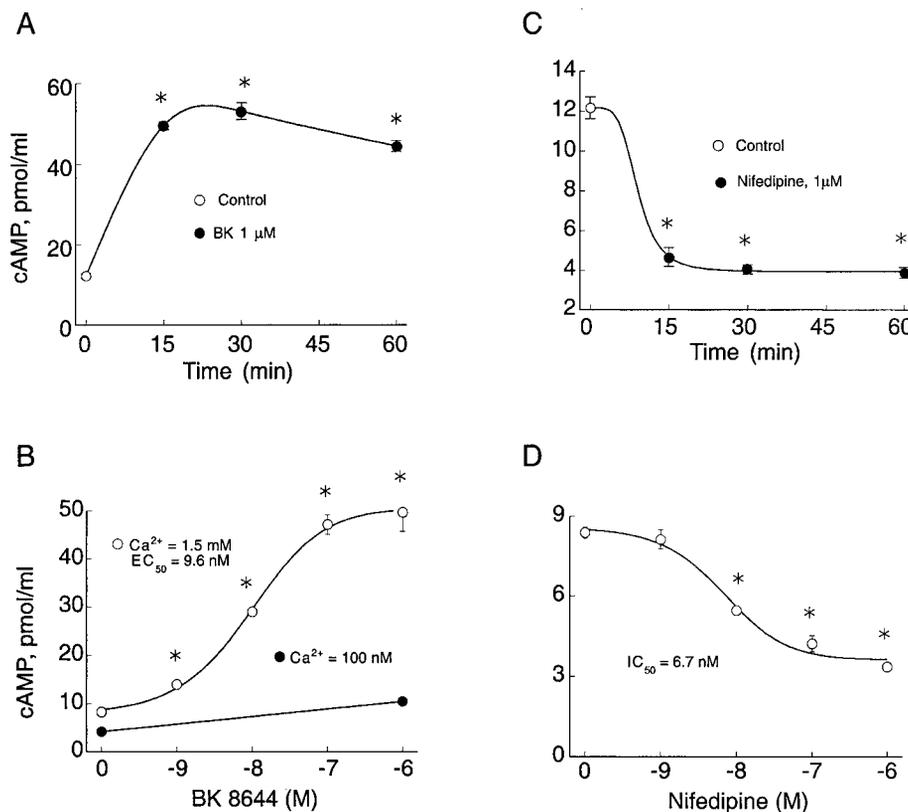


Fig. 2. Effects of Dihydropyridines on cAMP Production in GT1-7 Neurons

A and B, Time- and dose-dependent stimulation of cAMP production by BK-8644. C and D, Time- and dose-dependent inhibition of cAMP production by nifedipine. Data are means ± SE of three independent experiments. Asterisks indicate significant differences ($P < 0.05$) compared with the basal cAMP production.

cAMP production and the inhibitory action of micromolar GnRH concentrations were accentuated by increases in $[Ca^{2+}]_e$ (Fig. 6A). Conversely, inhibition of Ca^{2+} influx by nifedipine converted the biphasic cAMP response to GnRH into a small monotonic increase (Fig. 6B). Furthermore, the stimulatory action of BK-8644 was enhanced by nanomolar GnRH concentrations (Fig. 7A) but was abolished by micromolar concentrations of GnRH (Fig. 7B).

Effects of LH Receptor Activation on cAMP Production

Exposure of GT1-7 neurons to increasing concentrations of hCG for 30 min caused dose-dependent increases in cAMP production, with EC_{50} of 0.3 nM. Both basal and hCG-stimulated cAMP production were markedly reduced in Ca^{2+} -free medium (Fig. 8A) and by treatment with 1 μM nifedipine (not shown). Treatment with hCG also decreased G_s α-subunit immunoreactivity, consistent with coupling of the activated LH/hCG receptor to the AC-stimulatory G protein (Fig. 8B). The reduction of cAMP production by inhibition of Ca^{2+} entry, with no change in EC_{50} , indicates that Ca^{2+} influx through voltage-sensitive calcium channels (VSCC) is necessary to maintain hCG-induced

activation of AC (Fig. 8A). The maximal stimulatory effect of 2 nM hCG was potentiated by increasing $[Ca^{2+}]_e$, with a similar EC_{50} for both control and hCG-treated cells (Fig. 8C). The stimulatory action of hCG was biphasically regulated by concomitant activation of GnRH receptors, with enhancement at low nanomolar GnRH concentrations, and inhibition at high nanomolar and micromolar concentrations of GnRH (Fig. 8D). In the presence of 1.5 mM $[Ca^{2+}]_e$, treatment with K^+ (35 mM) or hCG (2 nM) increased cAMP production from 8.8 ± 0.3 pmol/ml to 35.9 ± 1.3 pmol/ml; $n = 4$; $P < 0.001$ for K^+ , and from 8.8 ± 0.3 pmol/ml to 16.5 ± 0.9 pmol/ml; $n = 4$; $P < 0.01$ for hCG. No additive effect on cAMP production (33.2 ± 1.9 pmol/ml; $n = 4$) was observed during concomitant treatment with 35 mM K^+ and 0.5 nM hCG. There was also no additivity between 35 mM K^+ and 0.5 nM hCG in Ca^{2+} -free medium, since the stimulatory action of K^+ was abolished.

AC Isoforms in GT1-7 Neurons

Western blot analysis of membrane preparations from GT1-7 neurons with specific antiserum for AC I revealed that this Ca^{2+} -stimulated and brain-specific enzyme is expressed in GT1-7 neurons (Fig. 9A). AC

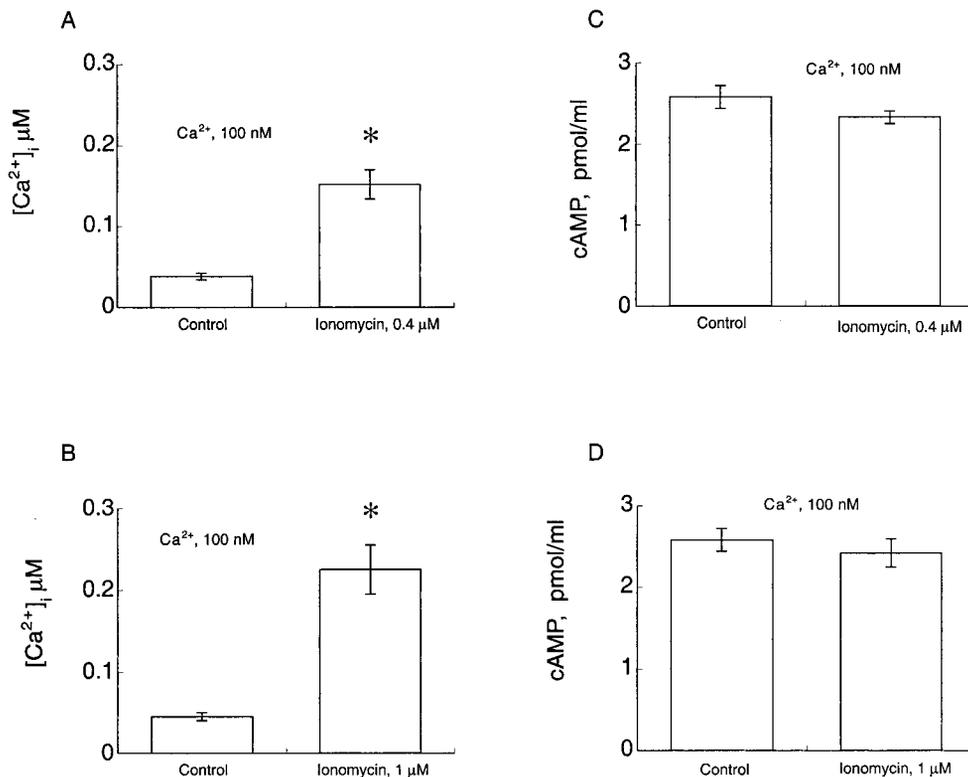


Fig. 3. Effects of Ionomycin on $[\text{Ca}^{2+}]_i$ and cAMP Production in GT1-7 Neurons

A and B, $[\text{Ca}^{2+}]_i$ responses of GT1-7 neurons treated with ionomycin (400 nM and 1 μM) in Ca^{2+} -free medium. C and D, cAMP production during ionomycin-induced increases in $[\text{Ca}^{2+}]_i$. Data are means \pm SE of three independent experiments. Asterisks indicate significant differences ($P < 0.01$) compared with the basal $[\text{Ca}^{2+}]_i$ level.

III, which was initially cloned as an AC restricted to the olfactory neuroepithelium, was also detected in GT1-7 neurons by Western blotting with specific antiserum (Fig. 9B). AC V, which is abundant in the brain and heart tissue, and AC VI, abundant in peripheral tissue and low in the brain, are structurally related and are inhibited by Ca^{2+} . One or both of these isoforms are also expressed in GT1-7 neurons as revealed by blotting with an antiserum that cross-reacts with both enzymes (Fig. 9C). The specificity of immunoreactivity for each specific AC was confirmed by preadsorption of primary antibody with the relevant antigenic peptide (shown as controls in Fig. 9, A-C).

DISCUSSION

The episodic mode of GnRH release from perfused hypothalamic cells and immortalized GnRH neurons is highly $[\text{Ca}^{2+}]_o$ dependent, suggesting that GnRH secretion is controlled by Ca^{2+} entry through plasma membrane Ca^{2+} channels (27, 28). Normal GnRH neurons and immortalized GnRH neurons express numerous plasma-membrane channels, including tetrodotoxin-sensitive Na^+ channels, low-voltage-activated Ca^{2+} channels, dihydropyridine-sensitive Ca^{2+} channels, inward-rectifying K^+ channels, several types of outward K^+ channels, the BK subtype of Ca^{2+} -sensi-

tive K^+ channels (I_{K-Ca}), and apamin-sensitive I_{K-Ca} channels (SK channels) (6, 29-33). An analysis of the relationship between electrical membrane activity and Ca^{2+} influx in differentiated GT1-7 neurons revealed that most cells exhibit spontaneous, extracellular Ca^{2+} -dependent action potentials (5, 6). In spontaneously active GT1-7 neurons firing regular action potentials, each spike generates a significant increase in $[\text{Ca}^{2+}]_i$. Such $[\text{Ca}^{2+}]_i$ increases are consistently larger in cells exhibiting broad endocrine-like action potentials than in those exhibiting sharp neuronal-like action potentials (5).

The present studies have demonstrated that Ca^{2+} entry through VSCC and the consequent changes in $[\text{Ca}^{2+}]_i$ levels also regulate AC activity and cAMP production. Under basal conditions, Ca^{2+} channel blockade by nifedipine, or incubation in Ca^{2+} -free medium, inhibits cAMP production in a time- and concentration-dependent manner. These observations are consistent with the expression of Ca^{2+} -stimulated ACs (34-40) in GnRH cells and other neurons. Such treatment also abolished pulsatile neuropeptide secretion (27, 28), inhibited spontaneous electrical activity, and reduced $[\text{Ca}^{2+}]_i$ levels (5). Thus, Ca^{2+} coordinately regulates spontaneous electrical activity and cAMP production, and its removal or reduction leads to abolition of pulsatile GnRH secretion.

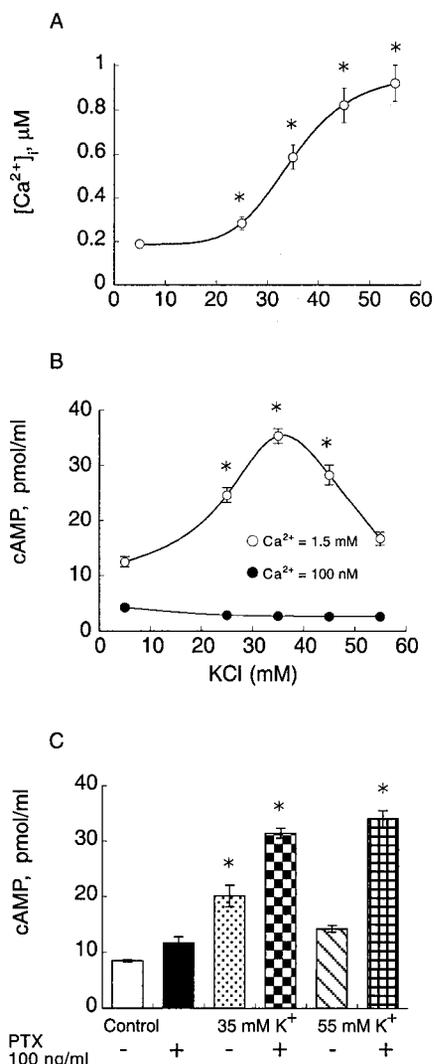


Fig. 4. Effects of Depolarization-Induced Calcium Entry on cAMP Production in GT1-7 Neurons

A, Elevation of $[Ca^{2+}]_i$ during progressive depolarization of GT1-7 neurons by increased extracellular K^+ . B, The concomitant biphasic change in cAMP production, with stimulation at K^+ concentrations up to 35 mM and progressive inhibition at higher concentrations. C, Effects of PTX on the inhibition of cAMP production by high KCl concentrations on cAMP production. Data are means \pm SE of three independent experiments. Asterisks indicate significant differences ($P < 0.01$) compared with the basal cAMP production.

GT1-7 neurons express type I AC, and increased cAMP production was observed when $[Ca^{2+}]_i$ was increased as a consequence of Ca^{2+} channel activation by BK-8644. The stimulatory action of Ca^{2+} was evident in the $[Ca^{2+}]_i$ range from 100 nM to 500 nM, levels that are produced in these excitable cells by increases in $[Ca^{2+}]_e$. These observations indicate that physiological elevations in $[Ca^{2+}]_i$ secondary to Ca^{2+} entry can regulate cAMP production. In contrast, mobilization of $[Ca^{2+}]_i$ from intracellular stores by ionomycin in the absence of extracellular $[Ca^{2+}]$ did not increase cAMP production.

The finding that only Ca^{2+} entry can stimulate cAMP production indicated that AC may be functionally co-localized with the Ca^{2+} entry channels in GT1-7 neurons, as in other neurons (41-43). AC V and AC VI are also expressed in GnRH neurons, but their role in the Ca^{2+} -dependent regulation of cAMP production appears to be minor since inhibition of Ca^{2+} entry was not accompanied by increased cAMP production.

In cerebellar granule cells, K^+ -induced depolarization caused initial stimulation and subsequent inhibition of cAMP production in the absence of extracellular Ca^{2+} . This biphasic response was attributed to progressive increases in the extracellular $K^+ : Na^+$ ratio in the absence of extracellular Ca^{2+} . Despite the lack of direct evidence for the modulation of cerebellar granule cell AC by membrane potential, the results demonstrated that membrane depolarization-induced influx of Na^+ , as well as Ca^{2+} , can elevate cAMP levels (44). In rat brainstem synaptoneurosome, membrane depolarization has been found to activate PTX-sensitive G proteins (45). This action is mediated by the voltage-gated sodium channel (VGSC), and its gating properties determine the activation of PTX-sensitive G proteins (46). In addition, in depolarized brainstem and cortical synaptoneurosome, the VGSC α -subunit was most efficiently cross-linked with G_o -proteins. These findings suggest that interactions between the VGSC α -subunit and G_o -proteins occur during membrane depolarization (47).

In GnRH neurons, depolarization by progressive elevation of $[K^+]_e$ elicited a bell-shaped cAMP response, with initial stimulation and subsequent inhibition. Stimulation of cAMP production was evident for K^+ concentrations from 5 mM to 35 mM K^+ and was correlated with increased $[Ca^{2+}]_i$, presumably reflecting activation of the Ca^{2+} -stimulated type I AC expressed in GnRH neurons. The stimulatory effect of increased K^+ was completely abolished in Ca^{2+} -free medium, indicating that Ca^{2+} entry is necessary for activation of the endogenous Ca^{2+} -stimulated AC in GnRH neurons. Also, the lack of synergistic stimulation of cAMP production by K^+ -induced depolarization and activation of the G_s -coupled LH receptors in GnRH neurons indicates the absence of voltage-sensitive AC (48). In contrast, it appears that membrane depolarization of GnRH neurons is linked to activation of $G_{i/o}$ inhibitory proteins, since PTX pretreatment prevented K^+ -induced inhibition of cAMP production. The PTX sensitivity of the inhibitory action of high K^+ concentrations in GnRH neurons resembles that observed in brain synaptoneurosome during membrane depolarization (47).

Activation of GnRH receptors expressed in GnRH neurons leads to a rapid and concentration-dependent increase in $[Ca^{2+}]_i$ that consist of an initial spike phase, driven by inositol trisphosphate-induced Ca^{2+} mobilization, and a sustained Ca^{2+} response that depends on Ca^{2+} entry through VSCC (12). Agonist activation of the GnRH receptors expressed in GnRH-producing neurons also modulated cAMP production.

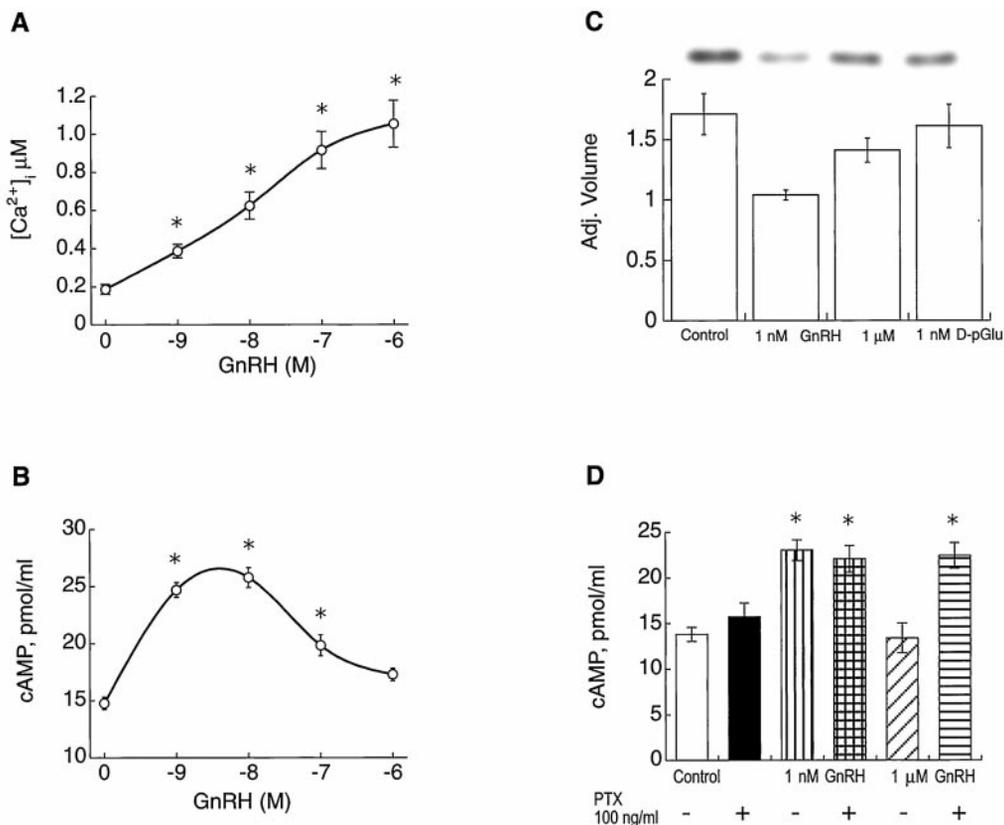


Fig. 5. GnRH-Stimulated Ca^{2+} and cAMP Responses in GT1-7 Neurons

A and B, Dose-dependent actions of GnRH on $[\text{Ca}^{2+}]_i$ and cAMP production. C, Effects of GnRH on membrane-associated G_s α -subunit immunoreactivity. D, Effects of PTX on GnRH-induced changes in cAMP production. Data are means \pm SE of three independent experiments. Asterisks indicate significant differences ($P < 0.01$) compared with the basal $[\text{Ca}^{2+}]_i$ or cAMP production.

Treatment with increasing GnRH concentrations caused a dose-dependent and biphasic change in cAMP production. The stimulation of cAMP production by low nanomolar GnRH concentrations was inhibited by the Ca^{2+} channel blocker, nifedipine, and was not observed in Ca^{2+} -free medium. In contrast, increased $[\text{Ca}^{2+}]_e$, and the Ca^{2+} channel agonist BK-8644, potentiated the stimulatory actions of low nanomolar GnRH concentrations. These findings indicate that the stimulatory actions of GnRH on cAMP production are mediated in part by GnRH-induced Ca^{2+} entry through ion channels and activation of Ca^{2+} -stimulated AC. The concentration of GnRH that stimulates cAMP production was associated with a decrease in membrane-bound $G_{\alpha s}$ immunoreactivity, consistent with G_s dissociation and subunit redistribution during GnRH receptor activation (15, 49–51). In COS-7 cells transiently expressing the GnRH receptor, agonist-stimulated cAMP production is dependent on specific residues in the first intracellular loop that are not essential for activation of the phosphoinositide signaling pathway (25). This suggests that the GnRH receptor in GnRH neurons can regulate cAMP production by coupling to G_s , as well as through activation of Ca^{2+} -stimulated AC.

The inhibition of cAMP production by high nanomolar and micromolar GnRH concentrations and its reversal by PTX indicate that the GnRH receptors expressed in GnRH neurons are also coupled to inhibitory $G_{i/o}$ proteins. GnRH receptors have also been reported to couple to G_i in human reproductive tract tumors (52–54). The inhibition of cAMP production by high GnRH concentrations was prevented by nifedipine-induced blockade of VSCC and was potentiated by increased $[\text{Ca}^{2+}]_e$. These findings indicate that high GnRH concentrations activate PTX-sensitive G protein(s) that in turn inhibit types I, V, and VI ACs, causing a decrease in cAMP production (55, 56). High concentrations of GnRH appear to activate G_i to an extent that abrogates the stimulatory action of G_s and Ca^{2+} entry on cAMP production.

Activation of the endogenous LH receptor expressed in GnRH neurons (9, 57) causes a dose-dependent increase in cAMP production. The stimulatory action of LH/hCG on cAMP production is highly Ca^{2+} -dependent and is significantly reduced in Ca^{2+} -free medium and by treatment with nifedipine. In contrast, increased $[\text{Ca}^{2+}]_e$ and treatment with BK-8644 significantly increased cAMP production. These results suggest that Ca^{2+} entry through VSCC acts as a

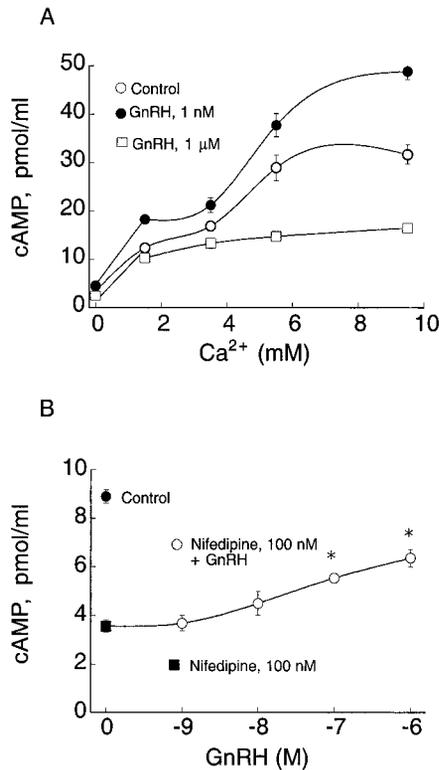


Fig. 6. Effects of Extracellular [Ca²⁺] and Nifedipine on GnRH-Induced cAMP Production in GT1-7 Neurons

A, Extracellular [Ca²⁺] dependence of the stimulatory and inhibitory actions of nanomolar and micromolar GnRH concentrations, respectively, on cAMP production. B, Inhibition by nifedipine of basal cAMP production and the biphasic GnRH-induced cAMP response. Data are means ± SE of three independent experiments. Asterisks indicate significant differences (*P* < 0.01) compared with the basal cAMP production.

conditioning factor to increase the catalytic activity of G_{sα}-activated AC in GnRH neurons. Thus, VSCC-dependent Ca²⁺ influx in spontaneously active GnRH neurons provides a pathway that links Ca²⁺ and the cAMP signaling system triggered by activation of G_s-coupled LH receptors. The stimulatory action of hCG is dually regulated by concomitant activation of GnRH receptors. The synergistic effect of hCG and low nanomolar GnRH concentrations in GnRH neurons suggests that convergent signaling from LH and GnRH receptors acts cooperatively on G_s to stimulate AC activity. In contrast, higher degrees of agonist activation of the GnRH receptor attenuate the stimulatory action of hCG by activation of PTX-sensitive G_{i/o} proteins.

These observations indicate that the firing of spontaneous action potentials in GnRH neurons causes increased Ca²⁺ influx and activation of Ca²⁺-stimulated AC to maintain basal cAMP production. In addition, convergent signaling from LH/hCG and GnRH receptors via G_s acts in conjunction with Ca²⁺ influx to stimulate cAMP production. The ability of micromolar GnRH concentrations to terminate hCG-stimulated

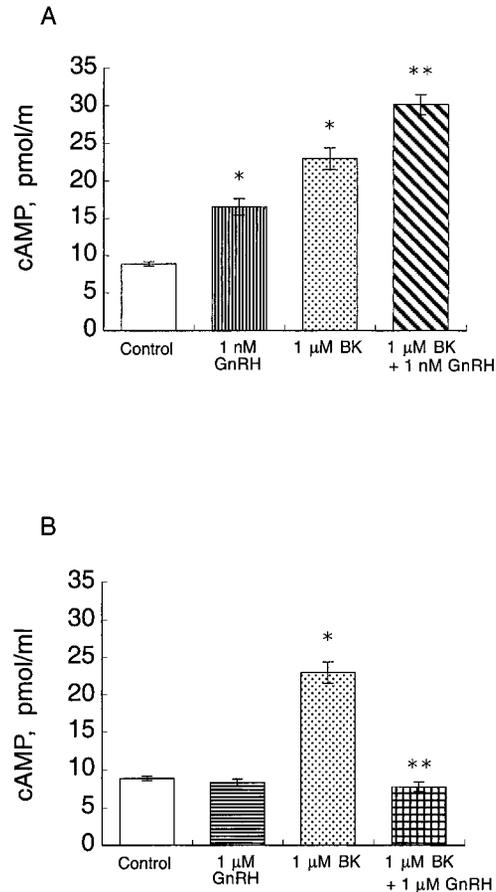


Fig. 7. Effects of BK-8644 on GnRH-Induced cAMP Production in GT1-7 Neurons

A, Enhancement of the maximal stimulatory action of BK 8644 on cAMP production by concomitant treatment with 1 nM GnRH. B, Inhibition of the maximum stimulatory action of BK 8644 by concomitant treatment with 1 μM GnRH. Data are means ± SE of three independent experiments. Asterisks indicate significant differences (*P* < 0.01) compared with the basal cAMP production. Double asterisks indicate significant differences (*P* < 0.05) compared with 1 μM BK 8644.

cAMP production in a PTX-sensitive manner indicates that the GnRH receptor also couples to G_{i/o} proteins and inhibits G_s-activated AC and cAMP production.

It is likely that this mechanism is mediated by AC I, which is stimulated by Ca²⁺ entry and is also regulated by receptor-dependent activation of G_s and G_i. Our findings suggest that changes in GnRH neuronal cAMP production are largely secondary to alterations in cell excitability and Ca²⁺ influx. This process, in conjunction with autocrine inhibitory actions of GnRH itself on cAMP production, could contribute to the pulsatile neuropeptide secretion that is characteristic of GnRH neuronal function *in vivo* and *in vitro*.

The nature of the latter process has not yet been clarified, but may involve more than one mechanism to provide redundancy to this essential component of the hypothalamic control of pituitary secretion. For example, Vitalis *et al.* (11) have proposed that phosphory-

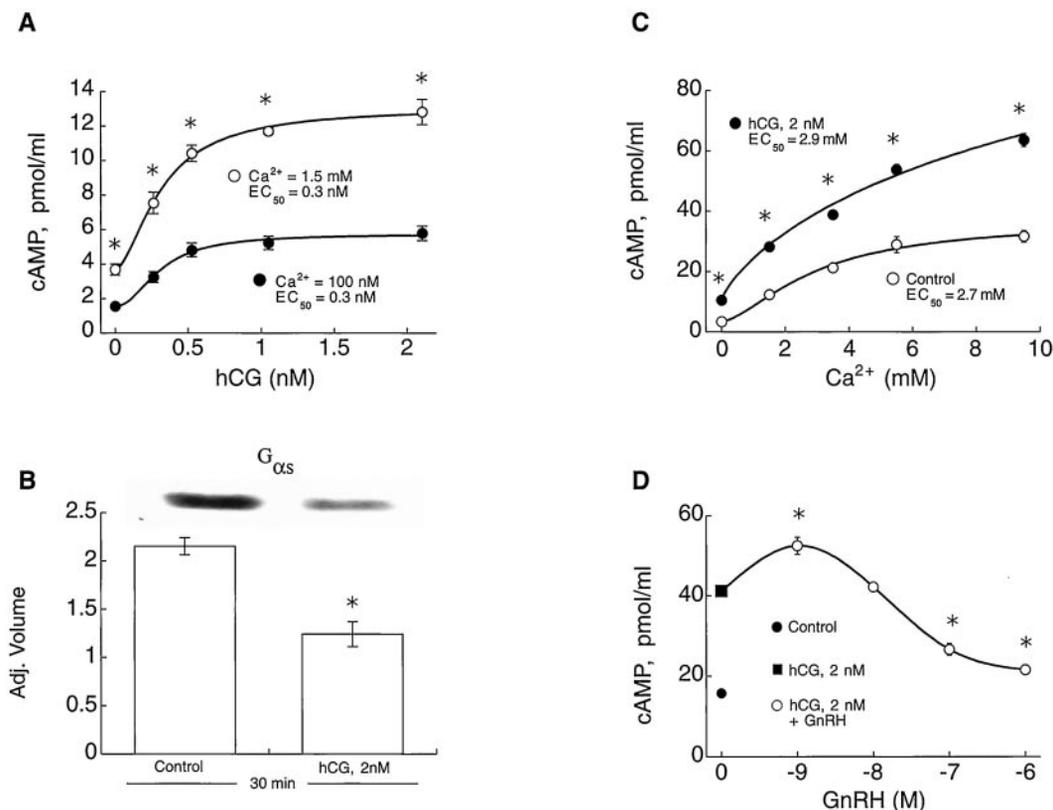


Fig. 8. Regulation of cAMP Production in GnRH Neurons by hCG and GnRH

A, hCG-stimulated cAMP production of cells incubated in normal and low extracellular [Ca²⁺]_e. B, hCG-induced reduction of immunoreactive G_s α-subunit in GT1-7 cell membranes. C, Potentiation of the maximal hCG-stimulated cAMP production by increased [Ca²⁺]_e. D, Biphasic action of GnRH receptor activation on hCG-induced cAMP production. Data are means ± SE of three independent experiments. Asterisks indicate significant differences ($P < 0.01$) compared with the basal cAMP production.

lation and inhibition of AC V and/or AC VI by protein kinase A (PKA) cause oscillations in cAMP production that in turn activate cyclic nucleotide-gated cation channels, which increase cell excitability and promote GnRH secretion. Also, Sakakibara *et al.* (58) have proposed another negative feedback limb that is dependent on the ability of cAMP-induced activation of PKA to inhibit phosphodiesterase activity in GT1 cells. The extent to which each of the currently proposed mechanisms contributes to the operation of the GnRH pulse generator, and the degree to which this depends on the prevailing steroid milieu and other factors, has yet to be determined.

MATERIALS AND METHODS

Tissue and Cell Culture

Hypothalamic tissue was removed from fetuses of 17-day pregnant Sprague Dawley rats (Taconic Farms, Inc., Germantown, NY). The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. After dissection, hypothalami were placed in ice-cold dissociation buffer containing 137 mM

NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 26 mM HEPES, and 100 mg/liter gentamicin, pH 7.4. The tissues were washed and then incubated in a sterile flask with dissociation buffer supplemented with 0.2% collagenase, 0.4% BSA, 0.2% glucose, and 0.05% DNase I. After 60 min incubation in a 37°C water bath with shaking at 60 cycles/min, the tissue was gently triturated by repeated aspiration into a smooth-tipped Pasteur pipette. Incubation was continued for another 30 min, after which the tissue was again dispersed. The cell suspension was passed through sterile mesh (200 μm) into a 50-ml tube, sedimented by centrifugation for 10 min at 200 × *g*, and washed once in dissociation buffer and once in culture medium consisting of 500 ml DMEM containing 0.584 g/liter L-glutamate and 4.5 g/liter glucose, mixed with 500 ml F-12 medium containing 0.146 g/liter L-glutamine, 1.8 g/liter glucose, 100 μg/ml gentamicin, 2.5 g/liter sodium bicarbonate, and 10% heat-inactivated FCS. Each dispersed hypothalamus yielded about 1.5 × 10⁶ cells. Immortalized GnRH neurons (GT1-7 neurons) obtained from Dr. R. I. Weiner (University of California at San Francisco) (59) were cultured under the same conditions as primary hypothalamic cells.

Measurement of cAMP Production

For studies on cAMP release, GnRH-producing cells were stimulated in serum-free medium (1:1 DMEM/F-12) containing 0.1% BSA, 30 mg/liter bacitracin, and 1 mM IBMX. For measurement of cAMP production in GT1-7 cell membrane preparations, cells were washed twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupepe-

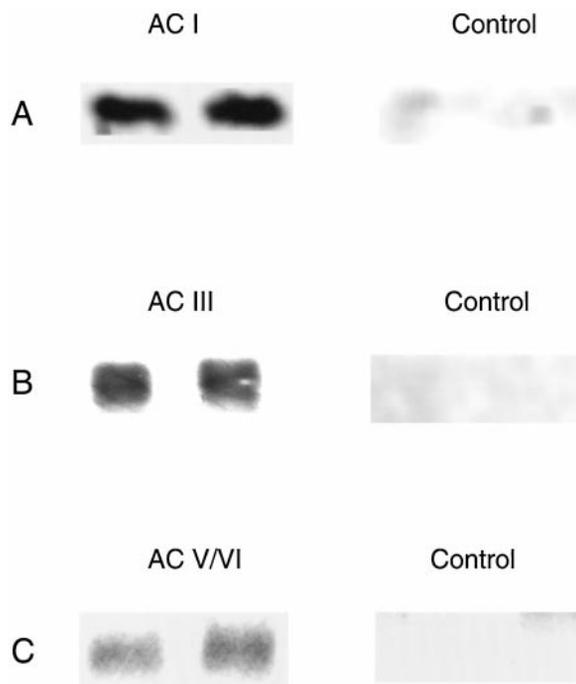


Fig. 9. Immunoblot Analysis of Ca^{2+} -Dependent AC Isoforms in GT1-7 Neurons

Solubilized GT1-7 membrane preparations were analyzed by SDS-PAGE and Western blotted with specific antibodies to AC I (A), AC II (B), and AC V/VI (C). In control samples the specificity of each antibody was determined by preadsorption with the corresponding blocking peptide.

tin, pH 7.4), removed from the plates by scraping, and lysed by freeze thawing. After centrifugation at $12,000 \times g$ for 15 min at 4 C, the pellet was resuspended in TE buffer and stored at -70 C until assayed. Protein contents were measured by the BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Aliquots (25 μg) of membrane proteins were incubated with 20 mM Tris/HCl, 10 mM MgCl_2 , 100 μM ATP, 20 mM creatine phosphate, 10 U/ml creatine phosphokinase, selected concentrations of Ca^{2+} , at 32 C for 5 min. RIA of cAMP was performed as previously described (60), using a specific cAMP antiserum at a titer of 1:5,000. This assay showed no cross-reaction with cGMP, 2',3'-cAMP, ADP, GDP, CTP, or IBMX. cAMP production is expressed as picomoles/mg protein/5 min.

Measurement of Calcium Ion Concentration

For single-cell $[\text{Ca}^{2+}]_i$ measurements, cultures were incubated at 37 C for 60 min with 2 μM fura-2/AM in phenol red-free DMEM. The cells were subsequently washed with Krebs-Ringer solution containing 1.2 mM Ca^{2+} and kept for at least 30 min in this medium before measurements. All experiments were performed in cells bathed in Krebs-Ringer solution containing 1.2 mM Ca^{2+} at room temperature. Coverslips with cells were mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a $40\times$ oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. All $[\text{Ca}^{2+}]_i$ values were derived from a standard curve that was constructed by addition of known concentrations of Ca^{2+} to 2 μM fura-2.

Immunoblot Analysis of Membrane-Associated G Proteins and AC Isoforms

For immunoblot analysis, cells were washed twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), scraped from the plates, and lysed by freeze thawing. After centrifugation at $12,000 \times g$ for 15 min at 4 C, the pellets were resuspended in TE buffer and stored at -70 C. Protein contents were measured by the Pierce Chemical Co. BCA protein assay kit. SDS-gel electrophoresis was performed on 8% acrylamide gels, followed by blotting with polyvinylidene difluoride (PVDF) membrane of 0.45- μm pore size. The blots were incubated with first antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or first antibody preadsorbed with the corresponding peptide antigen (1:1000 + 10 μg peptide), followed by peroxidase-coupled goat-antirabbit IgG (H+L), and visualized by chemiluminescence (Life Technologies, Inc., Gaithersburg, MD). The immunoreactive bands were analyzed as three-dimensional digitized images using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc. Hercules, CA). The optical density (OD) of images is expressed as volume (OD \times area) adjusted for the background, which gives arbitrary units of adjusted volume (Adj. Volume).

Materials

Western blotting reagents and ECL were obtained from Amersham Pharmacia Biotech, Arlington Heights, IL; collagenase (149 U/mg) was from Worthington Biochemical Corp., Freehold, NJ; DNase I, trypsin, bacitracin, IBMX, CTP, GDP, guanosine 3',5'-cyclic monophosphate (cGMP), adenosine 2',3'-cyclic monophosphate (2',3'-cAMP), and BSA were from Sigma (St. Louis, MO); GnRH was from Peninsula Laboratories, Inc. (Belmont, CA); ^{125}I -cAMP was from Covance Laboratories, Inc. (Vienna, VA); protein assay kits were from Pierce Chemical Co., and Membrane Immobilon-P was from Millipore Corp. (Bedford, MA). Peroxidase-coupled goat-antirabbit IgG (L+H), FBS, and DMEM/F12 1:1 powder were from Life Technologies, Inc.. Antibodies to AC I, AC III, and AC V/VI, were purchased from Santa Cruz Biotechnology, Inc., as well as the corresponding peptide antigens. Antibodies to $\text{G}_{\alpha q/11}$, $\text{G}_{\alpha s}$, $\text{G}_{\alpha i1}$, $\text{G}_{\alpha i2}$, and $\text{G}_{\alpha i3}$ were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), as well as the corresponding standard peptides. Other reagents, if not specified, were obtained from Sigma.

Data Analysis

All results are expressed as mean \pm SEM, and statistical comparisons were performed using the Kruskal-Wallis test followed by the Mann-Whitney *U* test. A difference between groups was considered to be significant if the *P* value obtained from the Mann-Whitney *U* test was less than 0.05. The calculations were performed on a Macintosh power PC using the Statistica/Mac 4.1 (StatSoft, Tulsa, OK).

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