Type I Adenylyl Cyclase Functions as a Coincidence Detector for Control of Cyclic AMP Response Element-Mediated Transcription: Synergistic Regulation of Transcription by Ca^{2+} and Isoproterenol

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Studies carried out with mammals and invertebrates suggest that Ca^{2+} -sensitive adenylyl cyclases may be important for neuroplasticity. Long-term potentiation in the hippocampus requires increases in intracellular Ca^{2+} which are accompanied by elevated cyclic AMP (cAMP). Furthermore, activation of cAMP-dependent protein kinase is required for the late stage of long-term potentiation in the CA1 region of the hippocampus, which is also sensitive to inhibitors of transcription. Therefore, some forms of synaptic plasticity may require coordinate regulation of transcription by Ca^{2+} and cAMP. In this study, we demonstrate that the expression of type I adenylyl cyclase in HEK-293 cells allows Ca^{2+} to stimulate reporter gene activity mediated through the cAMP response element. Furthermore, simultaneous activation by Ca^{2+} and isoproterenol caused synergistic stimulation of transcription in HEK-293 cells and cultured neurons. We propose that Ca^{2+} and neurotransmitter stimulation of type I adenylyl cyclase may play a role in synaptic plasticity by generating optimal cAMP signals for regulation of transcription.

Mammalian adenylyl cyclases are regulated by neurotransmitter and hormone receptors coupled to G proteins (52) and also by intracellular free Ca^{2+} (16). Protein phosphorylations catalyzed by the cyclic AMP (cAMP)-dependent protein kinase (PKA) regulate several aspects of neuronal function, including ion channel activity, gene expression, and neurotransmitter synthesis and release (47, 48). Furthermore, cAMP has been implicated in the regulation of synaptic plasticity and may play an important role in mechanisms underlying learning and memory (19, 35, 71), including long-term potentiation (LTP) (7, 8, 23, 25, 33, 58).

Although some forms of LTP may decay over several hours, long-lasting LTP (L-LTP), which is produced by multiple trains of high-frequency stimulation, can last hours or even days (46) and is sensitive to inhibitors of transcription and translation (24, 28, 39, 46, 57). For example, L-LTP in the CA1 region of the hippocampus is blocked by inhibitors of transcription (23, 25), and both cAMP (56) and activation of PKA can induce L-LTP in area CA1 (23). Various forms of LTP in the hippocampus require entry of Ca^{2+} through N-methyl-D-aspartate receptors or voltage-gated Ca^{2+} channels (36, 48), and an increase in intracellular Ca^{2+} can induce LTP (45). Activation of N-methyl-D-aspartate receptors and LTP increases cAMP in area CA1 (10, 11). Furthermore, LTP in the dentate gyrus has also been reported to increase cAMP (59). These data suggest that Ca^{2+} -sensitive adenylyl cyclases may be stimulated when Ca^{2+} channels are activated during LTP. Increased cAMP generated during L-LTP may contribute to LTP by stimulating transcription of specific genes through CRE, the cAMP response element (27, 32).

Several distinct adenylyl cyclases have been cloned and characterized (13, 16); however, only the type I (14, 40, 61) and III adenylyl cyclases (I AC and III AC, respectively) (15) have

been reported to be stimulated by Ca^{2+} and calmodulin (CaM). I AC is directly activated by Ca^{2+} and CaM in vivo (14, 44, 68). However, III AC is stimulated by CaM and Ca^{2+} in membrane preparations only when the enzyme is activated by other effectors such as forskolin or GppNHp (15).

The physiological and biochemical functions of I AC are of particular interest to neurobiologists because the enzyme is neurospecific (69) and is expressed in those areas of the brain implicated in neuroplasticity (71). Furthermore, the *Drosophila* learning mutant bearing the mutation *rutabaga*, which is deficient in associative learning, lacks Ca^{2+} -stimulated adenylyl cyclase activity (20, 21, 42, 43), and the gene for the enzyme maps to the *rut* locus (41). Feaney (22) has shown that Ca^{2+} responsiveness of adenylyl cyclase is the critical defect in *rutabaga* flies. In *rutabaga* larvae, voltage clamp analysis of neuromuscular transmission indicated deficient synaptic facilitation and posttetanic potentiation (73). Furthermore, disruption of the gene for I AC in transgenic mice leads to deficiencies in spatial learning measured by the Morris water task (67). The marine snail *Aplysia californica* also contains Ca^{2+} -sensitive adenylyl cyclase activity that may play a role in association between conditioned and unconditioned stimuli (1, 30, 64). However, the *A. californica* Ca^{2+} adenylyl cyclase(s) has not been cloned, and its relationship to the mammalian enzymes is unknown.

We have proposed that synergistic stimulation of I AC by Ca^{2+} and neurotransmitters may play an important role in synaptic plasticity and that the enzyme may facilitate coupling of intracellular Ca^{2+} to stimulation of transcription mediated through cAMP response elements such as CRE (16, 71). The objective of this study was to determine if the expression of I AC in cultured cells allows intracellular Ca^{2+} to control transcription through the CRE enhancer element. The data presented in this study illustrate that synergistic regulation of CRE-mediated transcription by Ca^{2+} and isoproterenol occurs in neurons and nonneuronal cells expressing I AC.

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MATERIALS AND METHODS

Plasmid and DNA subcloning. The I AC cDNA clone was isolated from a bovine brain cDNA library as described by Xia et al. (72). The III AC cDNA clone (5) was obtained from R. R. Reed (The Johns Hopkins University, Baltimore, Md.). FR-I AC, a mutant of I AC lacking CaM and Ca^{2+} sensitivity, was prepared as previously described by Wu et al. (68). DNA fragments were isolated and ligated into the *Hind*III and *Not*I sites in pCEP4 (Invitrogen) by using standard techniques (2, 52). The CRE-*lacZ* reporter construct contained six repeats of a CRE consensus enhancer (TGACGTCA) and a minimal Rous sarcoma virus promoter that drives *lacZ* expression. The dominant negative CREB mutant KCREB in the Rc/RSV vector (Invitrogen) was provided by R. H. Goodman.

Cell culture. Human embryonic kidney HEK-293 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum in a humidified 95% air–5% CO_2 incubator. Primary cortical neurons were maintained in DMEM or NeuroBasal (Gibco-BRL) with B27 supplement (Gibco-BRL). Primary cerebellar neurons were maintained in DMEM supplemented with 5% fetal bovine serum. Specific regions of the brain were removed by dissection and minced in Earle's balanced salt solution plus 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7). The neurons were then incubated in 1 mg of activated papain (Worthington Biochemicals) per ml at 37°C for 20 min. Ovomucoid trypsin inhibitor at 10 mg/ml (Worthington Biochemicals) was added, and neurons were incubated for 10 min at 37°C. The neurons were then dissociated via gentle passage through a 2-ml serological pipette for up to 50 repetitions. Large fragments were gravity precipitated, and the supernatant was carefully removed and plated at a density of approximately 10^6 cells per 35-mm-diameter well in Opti-MEM (Gibco-BRL). After 12 to 18 h, Opti-MEM was removed and the appropriate maintenance medium was added. With these techniques, primary cerebellar cultures were composed of at least 90% neurons, of which 95% were cerebellar granule cells. Primary cortical cultures typically contained 70 to 90% neurons.

Generation of stably transfected HEK-293 cells. HEK-293 cells were stably transfected with the G418-resistant (G418^r) CRE-*lacZ* reporter construct and an expression vector, pCDM8, that contained cDNA for I AC, III AC, or the parent plasmid. Briefly, cells were plated in 100-mm-diameter dishes at a density of 2.5×10^6 cells per plate, grown overnight, and transfected with the pCDM8 vector (10 μg of DNA per plate) and CRE-*lacZ* (1 μg DNA per plate) by the calcium phosphate method (9). Polyclonal stables were made with I AC or FR-I AC in pCEP4, which carries hygromycin resistance, and CRE-*lacZ*. Transfections were carried by using 10 μg of each vector. Transfected cells were incubated in a 37°C incubator with 3% CO_2 overnight, washed, and further incubated in DMEM–10% bovine calf serum (BCS) for 40 to 48 h at 10% CO_2 . G418^r cells were selected in culture medium containing 500 μg of G418 (Gibco-BRL) per ml. G418^r, hygromycin-resistant cells were selected in culture medium containing 350 μg of hygromycin per ml and 250 μg of G418 per ml.

Transient transfections. Cells were plated in 100-mm-diameter dishes at a density of 2.5×10^6 cells per plate and grown overnight at 37°C in a 10% CO_2 incubator. The cells were transfected with 20 μg of DNA by the calcium phosphate method (9).

Determination of cAMP accumulation. Changes in intracellular cAMP levels were measured by determining the ratio of [^3H]cAMP to a total ATP and ADP pool in [^3H]adenine-

loaded cells as described by Wong et al. (68). Confluent cells in six-well plates were initially incubated in DMEM containing [^3H]adenine (2.0 $\mu\text{Ci}/\text{ml}$; ICN) for 16 to 20 h, washed once with 150 mM NaCl, and incubated at 37°C for 30 min in incubation buffer (118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 0.5 mM EDTA, 10.0 mM glucose, 20.0 mM HEPES [pH 7.4]) containing 1.0 mM 3-isobutyl-1-methylxanthine (IBMX) and various effectors as indicated. Reactions were terminated by aspiration, washing cells once with 150 mM NaCl, and addition of 1.0 ml of ice-cold 5% trichloroacetic acid containing 1.0 mM cAMP. Culture dishes were maintained at 4°C for 1 to 4 h, and acid-soluble nucleotides were separated by ion-exchange chromatography as described previously (15).

Primary neuron transfections. Cells were plated at approximately 10^6 cells per well of a six-well plate. The transfections were performed after 3 to 4 days in culture. Briefly, 16 μl of Lipofectamine (Gibco-BRL), 2 μg of CRE-*lacZ* reporter construct, and 1 μg of the simian virus 40-chloramphenicol acetyltransferase (CAT) vector pCAT (Promega) were mixed in 200 μl of Opti-MEM (Gibco-BRL). The mixture was allowed to form DNA-lipid complexes for 15 to 20 min and added to one well of cells along with 800 μl of Opti-MEM. Following incubation for 5 h at 37°C, growth medium was substituted, and incubation was continued for another 48 to 72 h before the mixture was assayed for β -galactosidase (β -Gal) activity.

Determination of β -Gal activity in HEK-293 cells and neuron primary cultures. HEK-293 cells were plated in six-well plates and grown until confluent. The medium was removed, and the cells were washed once with phosphate-buffered saline. Various effectors, including agents which stimulate cAMP levels, were then added to cells in serum-free DMEM and incubated at 37°C for 30 min. The effector was removed, and DMEM-BCS was added. The cells were then incubated for 2.5 h, rinsed, and lysed in 0.5 ml of 0.5% Nonidet P-40. The assay was then carried out by adding 3.5 mM *o*-nitrophenyl- β -D-galactopyranoside (ONPG), a chromogenic substrate for β -Gal, and the mixture was incubated at 37°C for 30 min. The assay was stopped by heating at 100°C for 2 min, the mixture was centrifuged at 15,000 rpm for 30 min, and the supernatant was collected. The optical density at 420 nm of the supernatant was measured, and protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical). The enzyme activity was calculated by using an extinction coefficient for ONPG determined under these experimental conditions. Enzyme activity is expressed as micromoles of ONPG hydrolyzed per minute per milligram.

Approximately 10^6 neurons were plated in each well of a six-well plate. The neurons were then incubated in serum-free DMEM with effectors for 45 min. This medium was changed to neuronal maintenance medium, and the neurons were incubated at 37°C for 5 to 6 h. The neurons were lysed in 0.5% Nonidet P-40 with three freeze-thaw cycles. The β -Gal assay was carried out as described above with the exception that the reaction was allowed to proceed 2 to 4 h. Activities were normalized for protein determined by the bicinchoninic acid method (Pierce Chemical). Transfection efficiency was normalized by performing a CAT assay of the pCAT control vector, using the Promega CAT assay kit.

RESULTS

I AC confers Ca^{2+} regulation of CRE-*lacZ* in 293 cells. HEK-293 cells which were stably transfected with either I AC or III AC and a CRE-*lacZ* reporter construct were used to

FIG. 1. Calcium ionophore-stimulated CRE-mediated transcription in cells expressing I AC. (A) Stably transfected HEK-293 cells were incubated with effectors in serum-free medium for 30 min and in growth medium alone without effectors for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. Cells expressing the CRE- β -Gal reporter without I-AC and cells containing the CRE- β -gal reporter and I-AC are designated CRE and I AC/CRE, respectively. Cells expressing the CRE- β -Gal reporter and III AC are designated III AC/CRE. (B) Cells were pretreated with 20 μ M H89 for 15 min before presentation of A23187. The cells were then treated as for panel A. (C) Cells containing the CRE- β -gal reporter with a mutant I-AC that lacks CaM/Ca²⁺ sensitivity are designated FR-I AC/CRE. All cell lines used were polyclonal. When present, the A23187 concentration was 10 μ M; 2 mM CaCl₂ was present in all experiments. Error bars are standard deviations for triplicate determinations.

monitor Ca²⁺ regulation of transcription mediated through the CRE enhancer element. These cell lines are designated I AC/CRE and III AC/CRE, respectively. Control cells that contained only CRE-*lacZ* are designated CRE cells and express low levels of endogenous III AC (70) but not I AC (69). The induction of β -Gal transcription was monitored by measuring β -Gal activity in cell extracts after treatment with agents that affect intracellular Ca²⁺ and/or adenylyl cyclase activity.

In initial experiments, intracellular Ca²⁺ was elevated by using the Ca²⁺ ionophore A23187 and 2 mM CaCl₂ (Fig. 1). Treatment of control cells lacking I AC with A23187 did not stimulate expression of β -Gal, consistent with other studies which have shown no stimulation of CRE-mediated transcription by intracellular Ca²⁺ in various cultured cell lines (32, 65).

In I AC/CRE cell lines, A23187 increased β -Gal activity 2.5 $\pm$ 0.4-fold in 10 independent experiments using three different monoclonal and two different polyclonal cell lines expressing I AC (Fig. 1A). Although the absolute values for β -Gal expression generated by A23187 treatment varied in different I AC/CRE cell lines, A23187 stimulation of β -Gal expression was consistently obtained with all I AC/CRE cell lines examined. In contrast, III AC/CRE cell lines showed no increased β -Gal activity in response to A23187, consistent with previous data indicating that III AC is not stimulated by Ca²⁺ in the absence of other effectors (15). These data suggested that Ca²⁺ may couple to CRE-mediated transcription by Ca²⁺ or CaM stimulation of I AC.

If Ca²⁺ stimulation of β -Gal transcription in type I/CRE cells was due to Ca²⁺-induced elevation of cAMP levels and activation of PKA, then H89, a specific PKA inhibitor (12), should antagonize this phenomenon. H89 did in fact block the Ca²⁺-stimulated increase in β -Gal expression seen in I AC/CRE cells, suggesting that PKA activation was the primary mechanism for increased transcription of β -Gal (Fig. 1B).

To confirm that Ca²⁺ coupling to CRE-mediated transcription was due to Ca²⁺ stimulation of I AC, the experiments described above were carried out with a point mutant of I AC that is not stimulated by Ca²⁺ and CaM (68). This mutant, FR-I AC, contains an Arg in place of Phe-503 within the CaM binding domain of the enzyme. FR-I AC has full catalytic activity but is not stimulated by Ca²⁺ and CaM in vitro or in vivo. In contrast to I AC/CRE cells, FR-I AC/CRE cells did not show ionophore stimulation of β -Gal expression (Fig. 1C). These data confirmed that the coupling of Ca²⁺ to CRE-controlled transcription was due to Ca²⁺ and CaM stimulation of I AC and was not attributable to other Ca²⁺ regulatory mechanisms such as activation of CaM kinases.

It was of interest to determine if intracellular Ca²⁺ signals generated from physiologically relevant agonists would also couple to stimulation of CRE-mediated transcription through activation of I AC. The HEK-293 cells used in this study contain muscarinic receptors that couple to increases in intracellular Ca²⁺ when the cells are treated with the muscarinic agonist carbachol (14). Carbachol stimulated expression of β -Gal in I AC/CRE cell lines but not III AC/CRE or CRE cell lines (Fig. 2). Half-maximal stimulation occurred at approximately 0.3 mM carbachol in cells expressing I AC. Furthermore, the muscarinic antagonist atropine completely blocked the carbachol-stimulated β -galactosidase induction (Fig. 3A). The Ca²⁺ chelator 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl)-ester (BAPTA-AM) and H89 also inhibited carbachol stimulation of transcription, demonstrating a requirement for intracellular free Ca²⁺ and PKA activation (Fig. 3B and C). Calphostin C, a protein kinase C inhibitor (38), and KN-62, an inhibitor of CaM kinases II and IV (62), had no effect on carbachol or A23187 stimulation of CRE-mediated transcription (data not shown).

Synergistic stimulation of I-AC activity in vivo by Ca²⁺ and isoproterenol. We examined β -adrenergic receptor stimulation of I AC activity in HEK-293 cells because β -adrenergic agonists have been shown to potentiate LTP in various regions of the brain (34, 61). Furthermore, it was of interest to determine if combinations of Ca²⁺ and β -adrenergic agonists would produce synergistic stimulation of intracellular cAMP or transcription mediated through CRE. Several laboratories have reported synergistic activation of CRE-mediated transcription via pairing of Ca²⁺ and cAMP signals (37, 53, 54, 63).

Although I AC can be stimulated by addition of activated recombinant Gs α to membranes containing the enzyme (60), the enzyme is not stimulated by Gs-coupled receptors in

FIG. 2. Carbachol dose-response curve for CRE-mediated transcription in cells containing I AC. Stably transfected HEK-293 cells were incubated with effectors in serum-free medium for 30 min and in growth medium alone for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. CRE, cells expressing the CRE- β -Gal reporter without I-AC; III AC/CRE, cells expressing the CRE- β -Gal reporter with III AC; I AC/CRE, cells expressing the CRE- β -Gal reporter with I AC. Error bars are standard deviations for triplicate determinations.

HEK-293 cells unless it is also activated by intracellular Ca^{2+} (Table 1). HEK-293 cells express β -adrenergic receptors which couple to stimulation of endogenous adenylyl cyclase activity. Treatment of I AC cells with isoproterenol resulted in stimulation of cAMP levels, which was attributable to isoproterenol stimulation of endogenous adenylyl cyclase activity present in HEK-293 cells (Table 1). Sensitivity of the I-AC activity to isoproterenol was greatly potentiated when I AC was also activated by carbachol, which elevates intracellular Ca^{2+} , and the combination of isoproterenol and carbachol synergistically stimulated I AC. In the presence of carbachol, isoproterenol stimulated the enzyme 23-fold. Endogenous adenylyl cyclase activity in HEK-293 cells did not show synergistic activation by intracellular Ca^{2+} and isoproterenol. These data indicate that I AC generates a maximal cAMP signal when simultaneously stimulated by CaM- and Gs-coupled receptors and may therefore function as a coincidence detector (6) for integration of Ca^{2+} and neurotransmitter signals.

Synergistic stimulation of transcription by Ca^{2+} and isoproterenol. The influence of isoproterenol on CRE-mediated transcription in I AC/CRE cells was examined in the presence or absence of Ca^{2+} ionophore. In the absence of A23187, isoproterenol had only a minor influence on β -Gal expression, with maximum increases of approximately 50%, which is attributable to stimulation of endogenous adenylyl cyclase activity by isoproterenol (Fig. 4). However, combinations of isoproterenol and Ca^{2+} gave synergistic stimulation of β -Gal expression, with half-maximal stimulation at 250 nM isoproterenol. In the presence of Ca^{2+} , isoproterenol stimulated β -Gal expression 3.5 ± 0.5 -fold in 10 independent experiments using three different monoclonal and one polyclonal I AC/CRE cell line. The β -Gal expression levels generated by combinations of Ca^{2+} and isoproterenol were consistently greater than that seen with 100 μM forskolin, a reagent commonly used to maximize cAMP levels in cultured cells (data not shown). Presumably, stimulation of β -Gal expression by Ca^{2+} and isoproterenol was greater than additive because of synergistic increases in intracellular cAMP (Table 1).

FIG. 3. Inhibition of carbachol stimulation of CRE-mediated transcription by atropine, BAPTA-AM, and H89. (A) Stably transfected HEK-293 cells were incubated with effectors in serum-free medium for 30 min and in growth medium for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. CRE, cells expressing the CRE- β -Gal reporter without I-AC; I AC/CRE, cells expressing the CRE- β -Gal reporter with I AC. Cells were pretreated with 1 μM atropine for 15 min before addition of carbachol (carb.). (B) Cells were pretreated with 32 μM BAPTA-AM for 15 min before addition of carbachol. Cells were treated as for panel A. (C) Cells were pretreated with 20 μM H89 for 5 min before addition of carbachol. Cells were treated and assayed as for panel A. When present, the carbachol concentration was 1 mM. Error bars are standard deviations for triplicate determinations.

The CRE control cells have a basal level of *lacZ* induction that is presumably caused by endogenous levels of cAMP. When this basal level of induction is removed by subtraction, the I AC/CRE data more closely reflect the contribution of I AC (Fig. 5A). Furthermore, the apparent stimulation is increased to 5- to 7-fold for Ca^{2+} stimulation and 12- to 14-fold for Ca^{2+} and isoproterenol stimulation, which is fairly close to the fold stimulation under similar conditions in the cAMP accumulation assay (Table 1). It is also interesting that when serum-starved cells are used, the basal level of CRE-*lacZ* induction is reduced and similar results are obtained (data not shown).

The β -adrenergic antagonist propranolol inhibited synergistic stimulation of transcription by isoproterenol and Ca^{2+} in I AC/CRE cells (Fig. 5A). Although control CRE cells responded to isoproterenol, A23187 was without effect, and combinations of A23187 and isoproterenol were not synergistic.

TABLE 1. Ca^{2+} and β -adrenergic stimulation synergistically activate type I adenylyl cyclase in whole cells

Treatment ^a	Intracellular cAMP [(cAMP/ATP + ADP + AMP) $\times$ 100]			Stimulation ^c (fold)
	-I AC	+I AC	+I AC, -I AC ^b	
No addition	0.06 $\pm$ 0.01	0.12 $\pm$ 0.01	0.06 $\pm$ 0.01	1
Isoproterenol	0.35 $\pm$ 0.002	0.40 $\pm$ 0.05	0.05 $\pm$ 0.01	1
Carbachol	0.07 $\pm$ 0.002	0.70 $\pm$ 0.05	0.63 $\pm$ 0.06	10.5
Carbachol + isoproterenol	0.25 $\pm$ 0.005	1.65 $\pm$ 0.10	1.40 $\pm$ 0.10	23

^a HEK-293 cells expressing I AC (+I AC) or cells of the same cell line without I AC (-I AC) were treated with reagents for 30 min in the presence of 1.0 mM IBMX as indicated and then assayed for cAMP accumulation as described in Materials and Methods. When present: isoproterenol, 1.0 μ M; carbachol, 1 mM. Error is standard deviation for triplicate determinations.

^b cAMP ratios determined in non-I AC-expressing cells were subtracted from those in I AC-expressing cells to obtain the contribution from I AC alone.

^c Stimulation of corrected I AC activities relative to basal activity.

Synergistic stimulation of β -Gal expression by Ca^{2+} and isoproterenol was not observed with FR-I AC/CRE cells, illustrating that CaM stimulation of I AC was required for this phenomenon (Fig. 5B). Combinations of carbachol and isoproterenol also produced synergistic stimulation of transcription, which was shown to be dependent on Ca^{2+} by attenuation with the calcium chelator BAPTA-AM (Fig. 5C).

The stimulation of β -Gal transcription by Ca^{2+} and isoproterenol may be due to synergistic increases in cAMP or to Ca^{2+} stimulation of CaM-sensitive kinases. The selective PKA inhibitors H89 and adenosine-3',5'-cyclic phosphorothiolate-Rp (Rp-cAMP) (51) blocked increased β -Gal expression stimulated by isoproterenol or A23187, whereas KN-62, a CaM kinase inhibitor, did not significantly inhibit A23187 stimulation of β -Gal transcription or synergism observed between Ca^{2+} and the β -adrenergic agonist (Table 2). Calmidazolium, a competitive CaM antagonist, blocked the synergism seen between isoproterenol and A23187. Collectively, these data indicate that I AC can integrate Ca^{2+} and receptor-mediated

FIG. 4. Synergistic stimulation of CRE-mediated transcription in I AC/CRE cells by Ca^{2+} and isoproterenol. HEK-293 cells stably expressing I AC were incubated with effectors in serum-free medium for 30 min and in growth medium alone for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. When present, A23187 was at 10 μ M with 2 mM CaCl_2 . Error bars are standard deviations for triplicate determinations.

FIG. 5. Calcium and β -adrenergic stimulation synergistically induce CRE-mediated transcription via I AC. (A) Stably transfected HEK-293 cells were incubated with effectors in serum-free medium for 30 min and in growth medium alone for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. Endogenous β -Gal activity was subtracted. I AC/CRE, cells expressing the CRE- β -Gal reporter with I AC. A23187 was at 5 μ M, isoproterenol (iso) was at 10 μ M, and propranolol (prop) was at 10 μ M. AI, the combination of 5 μ M A23187 and 10 μ M isoproterenol. (B) FR-I AC/CRE, cells expressing the CRE- β -Gal reporter with FR-I AC, a mutant I AC lacking CaM sensitivity. A23187 was at 5 μ M, and isoproterenol was at 1 μ M. (C) Cells were pretreated with 32 μ M BAPTA-AM for 15 min. When present, carbachol was at 1 mM, A23187 was at 5 μ M, and isoproterenol was at 10 μ M. CI, carbachol plus A23187 at the same concentrations used individually. Error bars are standard deviations for triplicate determinations.

signals in vivo to produce optimal cAMP signals for control of transcription through the CRE enhancer element.

Attenuation of CRE-mediated transcription in HEK-293 cells by a dominant negative CREB mutant. HEK-293 cells may contain *trans*-acting factors other than the CREB family of transcription factors which may mediate cAMP-dependent induction of CRE-*lacZ* and have a role in the CRE-mediated synergistic induction of transcription. We explored this issue by using the dominant negative CREB mutant (KCREB) developed in Goodman's laboratory (65). When the CRE cell line was transiently transfected with KCREB, the cells showed

TABLE 2. Inhibition of CRE-mediated transcription in I-AC/CRE cells by protein kinase inhibitors

Treatment ^a	β -Gal activity ^b (μ mol of ONPG hydrolyzed/min/mg)
No addition	0.5 $\pm$ 0.1
Isoproterenol	0.7 $\pm$ 0.1
A23187 + Ca ²⁺	1.1 $\pm$ 0.2
A23187 + Ca ²⁺ + isoproterenol	2.3 $\pm$ 0.2
A23187 + Ca ²⁺ + isoproterenol + calmidazolium	0.9 $\pm$ 0.1
A23187 + Ca ²⁺ + isoproterenol + H89	0.6 $\pm$ 0.1
A23187 + Ca ²⁺ + isoproterenol + Rp-cAMP	0.7 $\pm$ 0.1
A23187 + Ca ²⁺ + isoproterenol + KN-62	2.0 $\pm$ 0.2

^a I-AC/CRE cells were treated with reagents for 30 min as indicated and then assayed for β -Gal activity as described in Materials and Methods. When present: isoproterenol, 1.0 μ M; A23187, 5 μ M; Ca²⁺, 2 mM; calmidazolium, 10 μ M; KN-62, 10 μ M; Rp-cAMP, 50 μ M; H89, 20 μ M. Error is standard deviation.

isoproterenol stimulation of *lacZ* similar to that of CRE cells (data not shown). CRE cells transiently transfected with I AC and KCREB showed approximately 60% inhibition of Ca²⁺-mediated *lacZ* induction relative to CRE cells transiently transfected with I AC (Fig. 6). Furthermore, the synergism between isoproterenol and Ca²⁺ was completely abolished in cells transfected with KCREB and I AC. These data suggest that a member of the CREB family of transcription factors is required for synergistic activation of CRE-mediated transcription via I AC.

Synergistic amplification of CRE-*lacZ* transcription in primary neurons. To address the physiological significance of the data described above, it was of interest to determine if CRE-mediated transcription in cultured neurons could be activated synergistically by intracellular Ca²⁺ and β -adrenergic agonists. This question was addressed by transiently expressing the CRE- β -Gal reporter in primary cultures of neocortex (postnatal days 1 to 2), hippocampus (postnatal days 1 to 2), and cerebellum (postnatal days 7 to 8), all of which express I AC activity (71). A23187 or isoproterenol alone increased β -Gal expression in cultured cortical and cerebellar neurons, and the combination of the two was greater than additive (Fig. 6 and 7A). Similar synergism was observed with cultured neurons from the hippocampus (data not shown). The selective PKA inhibitors H89 and Rp-cAMP both blocked isoproterenol- and A23187-mediated synergism, indicating a primary role for PKA. Furthermore, calmidazolium attenuated isoproterenol and A23187 synergistic production of β -Gal expression in cortical and cerebellar cultures. Calphostin C, a protein kinase C inhibitor, did not have a statistically significant effect on β -Gal expression stimulated by a combination of A23187 and isoproterenol (Fig. 7A).

It is interesting that high concentrations of KN-62 partially antagonized the synergism seen with A23187 and isoproterenol in cerebellar neurons, suggesting that there may be some contribution from Ca²⁺ activation of a CaM kinase in these cells (Fig. 8A). Nevertheless, there was synergistic stimulation of transcription even in the presence of KN-62, suggesting that Ca²⁺ and isoproterenol stimulation of adenylyl cyclase activity and activation of PKA is a major pathway operative for regulation of CRE-mediated transcription in these cells. This conclusion is also supported by the finding that PKA inhibitors completely abolished the increases in β -Gal expression obtained with combinations of A23187 and isoproterenol. Synergistic stimulation of CRE-mediated transcription in cortical and cerebellar neurons was also observed with isoproterenol and KCl, presumably because of intracellular Ca²⁺ increases

FIG. 6. A dominant negative CREB mutant blocks synergistic stimulation of CRE-mediated transcription. CRE cells were transiently transfected with the following ratios of DNA: for KCREB/I AC-CRE cells, 3 $\times$ KCREB, 1 $\times$ I AC/pCEP4, and 0.5 $\times$ pCAT; for I AC-CRE cells, 3 $\times$ pCDM8, 1 $\times$ I AC/pCEP4, and 0.5 $\times$ pCAT. The transient transfections were carried out as described in Materials and Methods. The cells were incubated with effectors in serum-free medium for 30 min and in growth medium alone for 2.5 h and then assayed for β -Gal activity as described in Materials and Methods. When present, isoproterenol (iso) was at 1 μ M and A23187 was at 10 μ M. The data were normalized for transfection efficiency by determining CAT activity. Error bars are standard deviations for triplicate determinations.

produced by membrane depolarization and opening of voltage-sensitive Ca²⁺ channels (Fig. 7B and 8B). Furthermore, the synergism obtained with combinations of 60 mM KCl and 10 μ M isoproterenol was blocked by H89 (Fig. 8B). In accord with the work of Bading et al. (4), we found that KCl alone induced CRE-mediated transcription in primary rat cerebellar and cortical cultures. Taken collectively, these experiments show that Ca²⁺ and β -adrenergic stimulation can result in synergistic induction of cAMP-mediated transcription in neurons. Furthermore, we propose that a Ca²⁺- and CaM-sensitive adenylyl cyclase may provide a molecular mechanism for this synergism by integration of Gs and Ca²⁺ signals.

DISCUSSION

I AC is directly stimulated by CaM and Ca²⁺ in vitro and in vivo but is insensitive to β -adrenergic receptor stimulation in HEK-293 cells unless the enzyme is also activated by Ca²⁺ and CaM. Presumably, conformational changes caused by binding of CaM to the catalytic subunit of the enzyme enhance interactions with activated Gs. Synergistic stimulation of I AC by Ca²⁺ and β -adrenergic receptors is clearly distinct from that reported for *Aplysia* adenylyl cyclase activity, which showed synergistic activation only upon the ordered addition of Ca²⁺ and serotonin (72). The synergism described in this study was seen in steady-state assays and was quantitatively much greater than that reported for *Aplysia* adenylyl cyclase.

Although the molecular role of I AC in neuroplasticity has not been defined, we have proposed that integration of Ca²⁺ and neurotransmitter signals by this enzyme may be important for long-term adaptive changes in neurons, perhaps by facilitating synergistic regulation of cAMP-mediated transcription through multiple signaling pathways (16, 17, 70). Because cAMP regulation of transcription may be important for some forms of LTP in vertebrates (23, 25), we designed experiments to determine if Ca²⁺ can induce CRE-mediated transcription by stimulation of I AC. Our data indicate that Ca²⁺ can activate CRE-mediated transcription in cells containing I AC

FIG. 7. Calcium and isoproterenol synergistically induce CRE-mediated transcription in cortical neurons. (A) Primary cortical neurons from postnatal day 1 rats were cultured and transfected with CRE-lacZ on days 4 to 5 as described in Materials and Methods. Two days after transfection, the neurons were treated with agonists for 45 min in serum-free medium and then incubated for 5 to 6 h in growth medium. The β -Gal assay was carried out as described in Materials and Methods. Neurons were pretreated with inhibitors for 15 min. Rp-cAMP pretreatment was for 60 min. Rp-cAMP was at 50 μ M, H89 was at 20 μ M, calmidazolium was at 20 μ M, KN-62 was at 10 μ M, and calphostin C was at 0.5 μ M. Inhibitors did not affect basal activity by more than 25%. Isoproterenol was at 10 μ M, and A23187 was at 5 μ M. (B) Experimental conditions were as for panel A. Isoproterenol was at 10 μ M, and KCl was at 60 mM. iso, isoproterenol; AI, combination of A23187 and isoproterenol; KI, combination of KCl and isoproterenol; Rp, Rp-cAMP; Calm, calmidazolium; Calph., calphostin C. Error bars are standard deviations for triplicate determinations.

but not in the same cell line lacking this enzyme. Furthermore, simultaneous stimulation with a Gs-coupled receptor and Ca^{2+} produced synergistic activation of transcription in HEK-293 cells expressing I AC.

Several lines of evidence indicate that Ca^{2+} coupling of CRE-stimulated transcription in I AC/CRE cells was due to CaM stimulation of I AC and activation of PKA. First, this phenomenon was not observed in control cells lacking I AC, in cells containing a mutant I AC that is insensitive to CaM and Ca^{2+} , or in cells expressing III AC, an adenylyl cyclase that is not directly stimulated by Ca^{2+} and CaM. The observation that III AC did not confer responsiveness to Ca^{2+} also argues against the possibility that elevation of basal cAMP indirectly promoted Ca^{2+} sensitivity to the CRE. Furthermore, Ca^{2+} stimulation of β -Gal expression was blocked by inhibitors of PKA but not by other protein kinase inhibitors.

Ca^{2+} and isoproterenol also synergistically stimulated CRE-mediated transcription in cultured cortical and cerebellar neurons. Stimulation of CRE-mediated transcription in neu-

FIG. 8. Calcium and isoproterenol synergistically induce CRE-mediated transcription in cerebellar neurons. (A) Lipofectamine (Gibco-BRL) was used to transfect primary cerebellar neurons with the CRE-lacZ reporter construct. The neurons were from 8-day rat pups and were 7 to 8 days in culture at the time of the assay. They were treated with agonists for 45 min in serum-free medium and then incubated for 5 to 6 h in growth medium. The β -Gal assay was carried out as described in Materials and Methods. Neurons were pretreated with inhibitors for 15 min. Rp-cAMP pretreatment was for 60 min. Rp-cAMP was at 50 μ M, H89 was at 20 μ M, calmidazolium was at 20 μ M, and KN-62 was at 10 μ M. Inhibitors did not affect basal activity by more than 25%. Isoproterenol was at 10 μ M, and A23187 was at 5 μ M. (B) Experimental conditions were as reported for panel A. Isoproterenol was at 10 μ M, and KCl was at 60 mM. iso, isoproterenol; AI, combination of A23187 and isoproterenol; KI, combination of KCl and isoproterenol; Rp, Rp-cAMP; Calm, calmidazolium. Error bars are standard deviations for triplicate determinations.

rons by Ca^{2+} and isoproterenol was insensitive to calphostin C but inhibited by the PKA inhibitors H89 and Rp-cAMP, indicating that the major pathway for this phenomenon required activation of PKA. Furthermore, the attenuation of β -adrenergic and Ca^{2+} -mediated synergism in neurons by the CaM inhibitor calmidazolium suggests a possible role for a Ca^{2+} - and CaM-sensitive adenylyl cyclase. This mechanism for conferring Ca^{2+} inducibility to the CRE provides an alternative and unique pathway distinct from mechanisms involving activation of CaM kinases, which undoubtedly contribute to Ca^{2+} stimulation of CRE-mediated transcription in some cells (18, 26, 53–55). The relative contributions of these two pathways in different populations of neurons will depend upon a number of factors, including the distribution of I AC and CaM kinases in different areas of the brain. For example, the data reported in Fig. 8A suggested that Ca^{2+} stimulation of adenylyl cyclase and CaM kinase activities may both contribute to CRE-mediated transcription in primary neuron cultures from the cerebellum.

Our data indicate that the maximum expression of β -Gal arose when the optimal cAMP signal was generated by synergistic stimulation of I AC. The quantitative relationships between intracellular cAMP and CRE-mediated transcription

have been very difficult to establish under physiologically relevant conditions because of the low sensitivity of cAMP assays. Under most circumstances, increases in intracellular cAMP in response to adenylyl cyclase activators cannot be detected by conventional cAMP assays unless cyclic nucleotide phosphodiesterase inhibitors are included (14). In this study, the detection of CRE-mediated transcription in I AC-containing cells was monitored without the inclusion of phosphodiesterase inhibitors or artificial stimulators of adenylyl cyclase such as forskolin. Without phosphodiesterase inhibitors, Ca^{2+} -stimulated increases in cAMP levels in HEK-293 cells or neurons were undetectable. Clearly, however, the increases in Ca^{2+} -stimulated transcription were mediated by cAMP because of the requirement for CaM-sensitive I AC activity and the sensitivity to PKA inhibitors. Thus, under physiological conditions, cAMP levels in neurons and other cells are rapidly attenuated by phosphodiesterases.

Stimulation of transcription by cAMP, which requires the nuclear translocation of PKA (49), may require higher or more persistent cAMP signals than other cAMP-regulated events, particularly in neurons. For example, CREB phosphorylation by PKA is relatively slow compared with other PKA-regulated processes, probably because CREB is not very abundant; it is a relatively poor substrate for PKA, and nuclear translocation of PKA is required (29). Furthermore, stimulation of PKA nuclear translocation in *Aplysia* neurons (3) and serotonin stimulation of transcription through CRE (34) are relatively slow processes that required multiple doses of serotonin for adenylyl cyclase activation. Robust cAMP signals may be required for transcriptional control in neurons because a cAMP gradient must be established from the nerve termini to the cell body. The novel function of I AC for neuroplasticity may therefore be to integrate Ca^{2+} and neurotransmitter signals to generate optimal signals for cAMP-mediated transcription. Our data indicate that the enzyme can indeed function as a coincidence detector for transcriptional control and provides one mechanism that may explain the role of this enzyme for long-term synaptic changes in neurons.

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