Regulation of a Ca\(^{2+}\)-sensitive Adenylly Cyclase in an Excitable Cell

ROLE OF VOLTAGE-GATED VERSUS CAPACITATIVE Ca\(^{2+}\) ENTRY

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In nonexcitable cells, we had previously established that Ca\(^{2+}\)-sensitive adenylly cyclases, whether expressed endogenously or heterologously, were regulated exclusively by capacitative Ca\(^{2+}\) entry (Fagan, K. A., Mahey, R. and Cooper, D. M. F. (1996) J. Biol. Chem. 271, 12438–12444; Fagan, K. A., Mons, N., and Cooper, D. M. F. (1998) J. Biol. Chem. 273, 9297–9305). Relatively little is known about how these enzymes are regulated by Ca\(^{2+}\) in excitable cells, where they predominate. Furthermore, no effort has been made to determine whether the prominent voltage-gated Ca\(^{2+}\) entry, which typifies excitable cells, overwhelms the effect of any capacitative Ca\(^{2+}\) entry that may occur. In the present study, we placed the Ca\(^{2+}\)-stimulable, adenylly cyclase type VIII in an adenosivirus vector to optimize its expression in the pituitary-derived GH\(_{4}\)C\(_{1}\) cell line. In these cells, a modest degree of capacitative Ca\(^{2+}\) entry could be discerned in the face of a dramatic voltage-gated Ca\(^{2+}\) entry. Nevertheless, both modes of Ca\(^{2+}\) entry were equally efficacious at stimulating adenylly cyclase. A striking release of Ca\(^{2+}\) from intracellular stores, triggered either by ionophore or thyrotrophin-releasing hormone, was incapable of stimulating the adenylly cyclase. It thus appears as though the intimate colocalization of adenylly cyclase with capacitative Ca\(^{2+}\) entry channels is an intrinsic property of these molecules, regardless of whether they are expressed in excitable or nonexcitable cells.

Ca\(^{2+}\)-sensitive adenylly cyclases provide a means of coordinating the activities of the two major signaling systems of cAMP and Ca\(^{2+}\) (1). The fact that these cyclases are regulated by Ca\(^{2+}\) entering the cells ensures an acute response of the cAMP-generating system to the elevation of [Ca\(^{2+}\)]\(_i\). In nonexcitable cells, we have previously shown a strict dependence for capacitative Ca\(^{2+}\) entry (CCE) as the mode of [Ca\(^{2+}\)]\(_i\) elevation that would regulate Ca\(^{2+}\)-sensitive adenylly cyclases, whether they were expressed endogenously or heterologously (2, 3). In particular, Ca\(^{2+}\) released from internal stores by any mechanism was unable to regulate adenylly cyclase activity (4), whereas Ca\(^{2+}\) entering via CCE modulated cAMP synthesis positively or negatively, depending on the adenylly cyclase species expressed (2, 3). These findings have now been extended to other nonexcitable cell systems (5–7). Although the endogenous Ca\(^{2+}\)-stimulable adenylly cyclase of cerebellar granule cells (8) and of hippocampal slices (9) is stimulated by Ca\(^{2+}\) influx through voltage-gated calcium channels (VGCCs), it is not known whether adenylly cyclases in excitable cells are as discriminating as those expressed in nonexcitable cells for the nature of the Ca\(^{2+}\) rise to which they respond. In the present study, the Ca\(^{2+}\)-stimulable ACVIII was placed in an adenosivirus vector to provide efficient expression in the excitable, anterior pituitary-derived tumor line, GH\(_{4}\)C\(_{1}\). GH\(_{4}\)C\(_{1}\) cells are spontaneously electrically active and express VGCCs that give rise to prominent intracellular rises in [Ca\(^{2+}\)]\(_i\); upon membrane depolarization (10). They also express TRH receptors coupled to phospholipase C that elevate [Ca\(^{2+}\)]\(_i\), both by inositol 1,4,5-trisphosphate-linked mechanisms and by modifying the activity of VGCCs (11). It seemed possible that GH\(_{4}\)C\(_{1}\) cells, like most neuronal cells, might not display prominent CCE; however, if CCE were detectable, the opportunity would be provided to determine whether any selectivity was displayed in the regulation of the adenylly cyclase for either type of [Ca\(^{2+}\)]\(_i\) rise in the same cell type.

EXPERIMENTAL PROCEDURES

Materials—Thapsigargin and forskolin were from Calbiochem. [2-\(^{32}\)P]ATP were obtained from Amersham Pharma Biotech. Fura-2/AM and pluronic acid were from Molecular Probes, Inc. (Eugene, OR). Other reagents were from Sigma.

Cells and Viruses—Viruses were constructed and propagated using HER293 cells, a human embryonic kidney cell line transformed by and expressing high levels of Ad5 E1A and E1B proteins (12). The virus used for recombination was Ad5\(_{327}\)BstIa\(_{gal}\)-gal, which encodes LacZ in place of the E1A and E1B genes and permits color screening for recombinant viruses (13). Ad5\(_{327}\)BstIa\(_{gal}\)-gal was purified after infection of HER293 cells, and thawing and pelleting of the cell debris. The supernatants were concentrated cell pellet by three cycles of rapid freezing and thawing; the cell debris was pelleted and re-extracted twice by resuspension in a small volume of phosphate-buffered saline followed by rapid freezing and thawing and pelleting of the cell debris. The supernatants were combined and banded for 50 min at 36,000 rpm using a CsCl step gradient consisting of 1 ml of 1.4 g/ml CaCl\(_2\) in phosphate-buffered saline and 1.5 ml of 1.25 g/ml CaCl\(_2\) in phosphate-buffered saline in a SW40 rotor. The virion band was collected by side puncture, diluted in 1.35 g/ml CaCl\(_2\) in phosphate-buffered saline, and rebanded for 3 h at 65,000 rpm in a VTi65 rotor. The virion band was again collected by side puncture, to prepare Ad5\(_{327}\)BstIa\(_{gal}\)-term-

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were combined and dialyzed versus 6 changes of 2 liters each of 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 0.2 mM EDTA at 4 °C. The presence of TP on the DNA greatly increases the infectivity after transfection (15), presumably due to both increased nuclear uptake facilitated by the nuclear localization signal in TP and protection of the DNA from degradation. TP was cotransfected with BstBI-for BstBI-resistant plasmids, the completion of digestion, an aliquot was digested with proteinase K before agarose gel electrophoresis. BstBI-digested Ad5dl327β-gal-TP was aliquoted and stored at −20 °C.

**Plasmid Construction**—A cDNA encoding ACVIII was cloned into pACCMV under the control of the adenovirus type 5 E1A promoter. pXC15E1A-ACVIII was further modified by insertion of a 3′-end of the ACVIII coding sequence and the sequences encoding the intron and poly(A) sites (which are contributed by the 3′-end of the EIB gene) to generate pXC15E1A-ACVIII

**Transfections**—HEK 293 cells at approximately 70% confluence were transfected using a modified calcium phosphate procedure (17) using 6 μg of plasmid DNA and approximately 0.2 μg of Ad5dl327β-gal-TP complex. Cells were incubated with the transfection solution overnight before being fed with fresh medium.

**Construction of Adenovirus Transducing Vector Encoding ACVIII**—Because cAMP is a prominent regulator of cell growth and numerous cellular processes, it would be expected that adenylyl cyclase expression would be tightly regulated and constrained to low levels (18). Thus, whereas attempts were made to construct transducing viruses placing ACVIII expression under the control of both the strong CMV promoter and weaker E1A promoters, it was expected that the use of the E1A promoter would yield a virus that directed expression at a level closer to the normal level. The use of the E1A promoter was also expected to make construction and growth of the virus easier because high-level expression of the cyclase might be expected to alter expression of a variety of genes and interfere specifically with regulation of adenovirus gene expression (e.g., Refs. 19–22). In addition to being a generally weaker promoter than the CMV promoter, the activity of the E1A promoter is inhibited by the high level of E1A protein expressed in HEK 293 cells (23 and data not shown). Attempts to introduce pXC15E1A-ACVIII into BstBI-digested Ad5dl327β-gal-TP by standard overlap recombination (13, 24) were unsuccessful. As an alternative, ligation of the modified plasmid, pXC15E1A-ACVIIIint, with the large, right arm of the chromosome-terminal complex was used. 6 μg of pXC15E1A#12-ACVIIIint was digested with BstBI to generate a ligation site for the viral arm and with XmnI, which cleaves in the β-lactamase coding sequence within the plasmid vector backbone to leave a blunt end, to inhibit recircularization of the plasmid as well as ligation to form concatamers. The restriction enzyme-digested plasmid was ligated with Ad5dl327β-gal-TP complex that had been digested with BstBI, and the mixture was used to transfect the virus. It was expected that expression of ACVIII during the transfection would be significantly reduced because of the reduction in the number of ACVIII templates that contained exon and poly(A) sequences. Furthermore, direct ligation with the large arm of Ad5dl327β-gal-TP complex was expected to efficiently introduce the E1A-ACVIII cassette into the virus. Ligation to the large arm of adenovirus restores the intron and a poly(A) site provided by the 3′-end of the EIB gene, thus ACVIII expression should be directed by the recombinant virus. Plaques were purified from the transfection stock after serial dilution in HEK 293 cells and overlaying with Noble agar-containing medium and serum. Plates were stained with neutral red and 5-bromo-4-chloro-3-indolyl-β-D-galactoside 7 days after infection, and clear plaques were picked on day 8. Plaques were grown in HEK 293 cells and tested for the presence of the ACVIII gene by polymerase chain reaction. A positive clone was grown in large stock, purified by banding consecutively on CsCl step and isopycnic gradients as indicated above, and dialyzed versus three changes of 1 liter each of 135 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, and 50% (v/v) glycerol at 4 °C. The virus particle concentration was determined by reading absorbance at 260 nm, with 1 A₅₅₀ unit considered equivalent to 10²⁷ particles. The particle/plaque-forming unit ratio was approximately 100:1 for all preparations.

**Cell Culture and Infection**—Rat anterior pituitary GH₃ cells were maintained in 13 ml of Ham’s F-10 medium (Life Technologies, Inc.) with 15% (v/v) horse serum and 2.5% fetal bovine serum (Gemini) in 75-cm² flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

![Functional expression of ACVIII in GH₃ cells.](http://www.jbc.org/)

**FIG. 1.** Functional expression of ACVIII in GH₃ cells. GH₃ cells were infected with the ACVIII/adenovirus construct (at a m.o.i. of 100) 48 h before assay. cAMP accumulation was measured in populations of GH₃ cells as described under "Experimental Procedures." A, uninfected (open bars) and ACVIII-infected GH₃ cells (right-hatched bars) were pretreated with IBMX (100 μM) and maintained in Ca²⁺-free Krebs buffer. cAMP accumulation was measured over a 1-min period starting with the addition of forskolin (10 μM) and baseline, and the presence or absence of Ca²⁺ (2 mM), as indicated. B, cAMP accumulation was measured in GH₃ cells infected with the ACVIII adenovirus as described above, in the presence of vasoactive intestinal peptide (VIP; 200 nM), KCl (20 mM), and the presence or absence of Ca²⁺ (2 mM), as indicated. Basal cAMP accumulation values were 0.3 ± 0.02 and 0.43 ± 0.01 for uninfected and infected cells, respectively. Data are representative of three similar experiments.
RESULTS

The functional expression of ACVIII was determined by biochemical assay. Because adenylyl cyclase type II, a Ca\(^{2+}\)-insensitive isoform, is the predominant mRNA in GH\(_4\)C\(_1\) cells (29), the expression of ACVIII activity could be demonstrated conclusively by an increase in cAMP accumulation in response to an elevation in [Ca\(^{2+}\)]. The most robust means of elevating [Ca\(^{2+}\)] in GH\(_4\)C\(_1\) cells is via VGCCs (30). Consequently, VGCCs were activated by membrane depolarization by increasing [K\(^{+}\)], in the presence or absence of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{o}\)). When assayed with forskolin, control cells showed no response to VGCC-mediated Ca\(^{2+}\) entry, whereas ACVIII-infected cells exhibited a robust stimulation in cAMP accumulation upon Ca\(^{2+}\) entry, yielding an approximately 3.5-fold stimulation compared with the Ca\(^{2+}\)-free condition (Fig. 1A). Vasoactive intestinal peptide stimulation of the cyclase via \(\alpha_7\) activation could also be augmented approximately 2.5-fold by VGCC-mediated Ca\(^{2+}\) entry (Fig. 1B). Therefore, the use of an adenovirus construct to express ACVIII provided a simple and efficient means of heterologously expressing this protein in this excitable cell type.

Little is known about the regulation of adenylyl cyclases by VGCC-mediated Ca\(^{2+}\) entry in excitable cells. In nonexcitable cells, we have previously established that Ca\(^{2+}\) stimulation of heterologously expressed ACVIII occurs exclusively via CCE. Therefore, we wanted to characterize more fully the ability of VGCCs to regulate ACVIII expressed heterologously in an excitable cell. The activity of VGCCs relies on the membrane potential, which is dictated, in part, by the K\(^{+}\) concentration in the medium bathing the cells. The amount of Ca\(^{2+}\) entry after membrane depolarization with varying [K\(^{+}\)], was assessed (Fig. 2A). Populations of Fura-2-loaded GH\(_4\)C\(_1\) cells were maintained in Ca\(^{2+}\)-free Krebs buffer (which contains 4.75 mM KCl). At 300 s, the [K\(^{+}\)], was increased by the addition of KCl (0–20 mM) and CaCl\(_2\) (2 mM), which resulted in a rapid, robust Ca\(^{2+}\) rise. The magnitude of the peak Ca\(^{2+}\) rise ranged from approximately 230 nM to approximately 1050 nM, depending on the [K\(^{+}\)]. The apparently equivalent Ca\(^{2+}\) rise, elicited by 10 and 20 mM KCl indicates maximal stimulation of the VGCCs by modest membrane depolarization (indeed, addition of 40 mM KCl resulted in a Ca\(^{2+}\) rise identical to that elicited by 20 mM KCl; data not shown).

The ability of the progressive Ca\(^{2+}\) rises triggered by incremental [K\(^{+}\)] increases to regulate the exogenously expressed ACVIII was next examined. GH\(_4\)C\(_1\) cells infected with the ACVIII virus were incubated in Ca\(^{2+}\)-free Krebs buffer. cAMP accumulation was measured over a 1-min period starting with the addition of forskolin and varying [KCl], and [Ca\(^{2+}\)], as indicated (Fig. 2B). In the absence of [Ca\(^{2+}\)], there was no effect of membrane depolarization on cAMP accumulation. Inclusion of [Ca\(^{2+}\)], (2 mM) increased cAMP production at all [K\(^{+}\)] values, with an approximately 2-fold stimulation in the 5 mM [K\(^{+}\)] condition and an approximately 2.8-fold stimulation for the 10 and 20 mM [K\(^{+}\)] conditions, thus. The observed “plateau” in the ability of high [K\(^{+}\)] to stimulate further Ca\(^{2+}\) entry was reflected in the regulation of ACVIII. Note that the cAMP accumulation is measured over the first minute after the addition of [Ca\(^{2+}\)], where the [Ca\(^{2+}\)], rises triggered by 10 and 20 mM [K\(^{+}\)], are very similar (cf. Fig. 2A).

The above-mentioned findings showed that a Ca\(^{2+}\)-sensitive adenylyl cyclase could be regulated by Ca\(^{2+}\) entry through VGCCs in excitable cells. Furthermore, the magnitude of the stimulation mirrored the extent of Ca\(^{2+}\) entry. We have seen similar results with CCE in nonexcitable cells. In excitable cells, the role of CCE has been explored only sparingly in the face of the much more pronounced Ca\(^{2+}\) rise generated by VGCCs. However, we wondered whether CCE was present in excitable cells, if it could also regulate ACVIII, and how this might compare with the regulation by VGCC-mediated Ca\(^{2+}\) entry. CCE is activated by depletion of intracellular Ca\(^{2+}\) stores using the sarcoplasmic/endoplasmic Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG) (31). Treatment of the cells with TG resulted in a modest [Ca\(^{2+}\)] rise (approximately 130 nM) that returned toward baseline because the cells were in Ca\(^{2+}\)-free media (Fig. 3A). Addition of [Ca\(^{2+}\)], either 0.5 or 2 mM, resulted in a rapid [Ca\(^{2+}\)] rise that reached a peak of approximately 230 or 380 nM, respectively, within the time course of the CCE measurements. Although the [Ca\(^{2+}\)] rise due to CCE was rather robust, it was considerably smaller than the [Ca\(^{2+}\)], rise generated by VGCC-mediated Ca\(^{2+}\) entry (Fig. 2B). Depolarization of the cells with KCl (10 mM) in the presence of either 0.5 or 2 mM [Ca\(^{2+}\)], resulted in peak [Ca\(^{2+}\)] rises of approximately 400 and 550 nM, respectively. Therefore, triggering of CCE by intracellular Ca\(^{2+}\) store depletion resulted in a modest [Ca\(^{2+}\)] rise compared with that arising from VGCC-mediated Ca\(^{2+}\) entry. Next, the ability of CCE and VGCC-mediated Ca\(^{2+}\) entry to stimulate ACVIII was compared. Cells maintained in Ca\(^{2+}\)-free Krebs buffer were treated with TG 4 min before the addition of varying [Ca\(^{2+}\)], (Fig. 3C). The stimulation of ACVIII
The clear demonstration of CCE in GH4C1 cells can be difficult because individual GH4C1 cells demonstrate spontaneous Ca\(^{2+}\) oscillations due to intermittent activation of VGCCs, even in “resting” conditions (10). Therefore, in a population of GH4C1 cells, considerable VGCC activity may underlie the magnitude of the Ca\(^{2+}\) entry ascribed to CCE. The extent of VGCC-mediated Ca\(^{2+}\) entry in resting conditions was explored by comparing Ca\(^{2+}\) entry in untreated and TG-treated cells. Untreated cells reveal Ca\(^{2+}\) entry due to spontaneously active VGCCs along with CCE from passive store depletion due to the cells being in Ca\(^{2+}\)-free buffer. The t-type VGCC blocker, nimodipine, can then be used to verify the contribution of the predominant t-type VGCC occurring in GH4C1 cells. Populations of Fura-2 loaded GH4C1 cells were either untreated or treated with TG 4 min before the addition of [Ca\(^{2+}\)]\(_i\) (2 mM). Prior treatment of the cells with TG may have augmented the [Ca\(^{2+}\)]\(_i\) rise, giving a peak [Ca\(^{2+}\)]\(_i\) rise of approximately 250 nM (Fig. 4A, trace a), compared with a peak [Ca\(^{2+}\)]\(_i\) rise of approximately 220 nM (Fig. 4A, trace b) in the untreated cells (to illustrate the two traces more clearly, a running average (5-s intervals) of each trace has been overlaid on the actual trace). The magnitude of the [Ca\(^{2+}\)]\(_i\) rise generated by CCE was clearly discerned in the presence of the VGCC blocker nimodipine. Addition of nimodipine (1 \(\mu\)M) along with [Ca\(^{2+}\)]\(_i\) resulted in a greatly reduced [Ca\(^{2+}\)]\(_i\) rise in the untreated cells (approximately 125 nM as compared with 220 nM without nimodipine (Fig. 4A, cf. b versus d)). The effect of nimodipine on TG-treated cells was much less drastic, decreasing the peak [Ca\(^{2+}\)]\(_i\) rise from 250 nM without nimodipine to 180 nM in the presence of the VGCC blocker (Fig. 4A, cf. a versus c). The ability of nimodipine to greatly attenuate the [Ca\(^{2+}\)]\(_i\) rise generated by Ca\(^{2+}\) addition alone illustrated the prominent, spontaneous VGCC-mediated Ca\(^{2+}\) entry occurring in these cells. Accordingly, stimulation of ACVIII by the above-mentioned conditions also revealed the presence of spontaneous VGCC activity. Addition of [Ca\(^{2+}\)]\(_i\) to GH4C1 cells maintained in Ca\(^{2+}\)-free Krebs buffer resulted in an approximately 1.5-fold stimulation of ACVIII as compared with the control, Ca\(^{2+}\)-free condition (Fig. 4A, inset, open bar versus right-hatched bar). Pretreatment of the cells with TG increased the amount of ACVIII stimulation, resulting in a 2.4-fold increase in activity (Fig. 4, inset, cross-hatched bar). In the presence of the VGCC blocker nimodipine, the ability of [Ca\(^{2+}\)]\(_i\) to the Gh4C1 cells maintained in Ca\(^{2+}\)-free Krebs buffer resulted in an approximately 1.5-fold stimulation of ACVIII as compared with the control, Ca\(^{2+}\)-free condition (Fig. 4A, inset, left-hatched bar), whereas cells pretreated with TG still showed a robust regulation of ACVIII (approximately 1.9-fold stimulation; Fig. 4A, inset, horizontal striped bar). Thus, basal VGCC activity in resting cells did contribute significantly to the stimulation of ACVIII. Conversely, there was no indication of CCE in resting, untreated cells, because the Ca\(^{2+}\) entry generated by Ca\(^{2+}\) addition was completely blocked by nimodipine.

Another method to verify the presence of CCE is to determine whether the combination of the two Ca\(^{2+}\) entry mechanisms (CCE and VGCC) yields augmented [Ca\(^{2+}\)]\(_i\), rises and/or regulation of ACVIII. Populations of GH4C1 cells were treated with TG (Fig. 4B, traces a and c), followed by depolarization with KCl (10 mM; traces a and b) and addition of [Ca\(^{2+}\)]\(_i\) (0.5 mM; note that lower [Ca\(^{2+}\)]\(_i\) values were used to prevent maximal Ca\(^{2+}\) entry with depolarization alone). TG treatment alone (trace c) resulted in a modest [Ca\(^{2+}\)]\(_i\) rise of approximately 250 nM after the addition of [Ca\(^{2+}\)]\(_i\). Activation of VGCCs with the addition of [K\(^{+}\)]\(_i\) along with [Ca\(^{2+}\)]\(_i\), resulted in a [Ca\(^{2+}\)]\(_i\) rise of approximately 400 nM (trace b). Combining the two (TG and [K\(^{+}\)]\(_i\), (trace a)) resulted in an augmented [Ca\(^{2+}\)]\(_i\) rise of approximately 510 nM. The combination of CCE and VGCC-mediated Ca\(^{2+}\) entry also resulted in increased

**Fig. 3.** Comparison of CCE and VGCC-mediated Ca\(^{2+}\) entry and their effect on ACVIII-infected GH4C1 cells. A–C, [Ca\(^{2+}\)]\(_i\), and cAMP accumulation were determined in GH4C1 cells as described under "Experimental Procedures." A, cells were maintained in nominally Ca\(^{2+}\)-free Krebs buffer containing IBMX (100 \(\mu\)M) before the addition of TG (100 nM; 60 s) followed by the addition of [Ca\(^{2+}\)]\(_i\) \(_o\) (a, 2 mM; b, 0.5 mM) at 300 s. B, cells were incubated in nominally Ca\(^{2+}\)-free Krebs buffer containing IBMX (100 \(\mu\)M) before the addition of KCl (10 mM) and [Ca\(^{2+}\)]\(_i\) \(_o\) (a, 2 mM; b, 0.5 mM) at 300 s. C, cAMP accumulation was measured in ACVIII-infected GH4C1 cells under the same conditions as described above. Cells were assayed in the presence of forskolin (100 \(\mu\)M) and [Ca\(^{2+}\)]\(_i\) \(_o\) (0.5 or 2 mM, as indicated). CCE was stimulated with the prior addition of TG (100 nM; open bars), whereas VGCC-mediated Ca\(^{2+}\) entry was triggered with the concomitant addition of KCl (10 mM; hatched bars). Data are representative of two similar experiments.

(approximately 3.5-fold) by CCE was apparent at low [Ca\(^{2+}\)]\(_i\) \(_o\) (0.5 mM). Addition of higher [Ca\(^{2+}\)]\(_i\) \(_o\) (2 mM) resulted in further stimulation of ACVIII (approximately 5.2-fold). Comparing the ability of CCE and VGCC-mediated Ca\(^{2+}\) entry to regulate ACVIII, it was apparent that at higher [Ca\(^{2+}\)]\(_i\) \(_o\) they were very similar, with a slight difference observed at lower [Ca\(^{2+}\)]\(_i\). The ability of CCE and VGCC-mediated Ca\(^{2+}\) entry to stimulate ACVIII similarly is somewhat surprising in the light of the more robust Ca\(^{2+}\) entry arising from VGCCs (cf. Fig. 3, A and B).
regulation of ACVIII (Fig. 4B, inset). GH3C1 cells that were pretreated with TG before [Ca\(^{2+}\)]\(_i\) addition showed a stimulation of ACVIII by approximately 2.4-fold as compared with control, untreated cells (Fig. 4B, inset, open bar versus right-hatched bar). Depolarization of the cells by the addition of [K\(^+\)], along with [Ca\(^{2+}\)]\(_i\), also resulted in a stimulation of the cyclase, giving an approximately 2.2-fold increase (Fig. 4B, inset, cross-hatched bar). Combination of the two modes of Ca\(^{2+}\) entry resulted in an augmented regulation of ACVIII, yielding an approximately 2.8-fold stimulation (Fig. 4B, inset, left-hatched bar). In the same experiment as Fig. 4B, the ability of VGCC-mediated Ca\(^{2+}\) entry activated by KCl addition to regulate the cyclase was also blocked by nimodipine (Fig. 4C). Furthermore, treatment of the cells with both TG and KCl in the presence of nimodipine resulted in a stimulation of ACVIII that was identical to that seen with TG treatment alone (Fig. 4C, cross-hatched versus vertical-stripped bars). Therefore, the two Ca\(^{2+}\) entry mechanisms, CCE and VGCC-mediated Ca\(^{2+}\) entry, in combination, led to an augmentation in both the [Ca\(^{2+}\)]\(_i\) rise and the magnitude of the stimulation of ACVIII produced by either Ca\(^{2+}\) entry mechanism alone, indicating they are separate Ca\(^{2+}\) entry processes.

The previous results revealed the presence of CCE with the use of nimodipine to block the spontaneously active VGCCs. It was also shown that the two Ca\(^{2+}\) entry mechanisms are quite similar in their ability to regulate ACVIII when the underlying VGCC-mediated Ca\(^{2+}\) entry is removed from the CCE (cf. Fig. 4, A versus B, cross-hatched bars). Although the effects of VGCC-mediated Ca\(^{2+}\) entry and “pure” CCE on the regulation of the cyclase are similar, the corresponding [Ca\(^{2+}\)]\(_i\) rises are quite different (Fig. 5, A and B). A robust [Ca\(^{2+}\)]\(_i\) rise was generated by VGCC-mediated Ca\(^{2+}\) entry triggered by [K\(^+\)]\(_o\), (Fig. 5A, 10 mM; trace a), reaching a peak of approximately 700 nM, whereas TG-mediated Ca\(^{2+}\) entry reached a peak of approximately 250 nM (Fig. 5B, trace a). In the presence of nimodipine (1 mM), the [Ca\(^{2+}\)]\(_i\), rise generated by depolarization was almost eliminated (Fig. 5A, trace b), dropping to approximately 120 nM, whereas TG-mediated Ca\(^{2+}\) entry was decreased to approximately 170 nM (Fig. 5B, trace b). This relatively small [Ca\(^{2+}\)]\(_i\) rise generated by TG treatment in the presence of nimodipine, “pure” CCE, could still effectively stimulate ACVIII (cf. Fig. 4C). Therefore, a [Ca\(^{2+}\)]\(_i\) rise of approximately 170 nM generated by CCE was as efficacious as the approximately 700 nM [Ca\(^{2+}\)]\(_i\) rise produced by VGCC-mediated Ca\(^{2+}\) entry in regulating ACVIII.

The observation that a modest amount of Ca\(^{2+}\) entry via CCE was able to stimulate ACVIII to a similar extent as a much more robust VGCC-mediated Ca\(^{2+}\) entry prompted us to investigate whether Ca\(^{2+}\) release from intracellular stores could also regulate the exogenously expressed ACVIII. Although TG releases intracellular Ca\(^{2+}\), the small release occurs over a prolonged period. Phospholipase C-coupled agonists produce a much more rapid and significant [Ca\(^{2+}\)]\(_i\) rise. Addition of TRH (100 nM) to populations of GH3C1 cells maintained in Ca\(^{2+}\)-free Krebs buffer resulted in a very rapid, large [Ca\(^{2+}\)]\(_i\) rise of approximately 500 nM (Fig. 6A). Intracellular Ca\(^{2+}\) can also be released by Ca\(^{2+}\) ionophores. Treatment of GH4C1 cells incubated in Ca\(^{2+}\)-free Krebs buffer with ionomycin (IM; 2 \(\mu\)M) yielded a rapid and robust [Ca\(^{2+}\)]\(_i\) rise that reached approximately 900 nM (Fig. 6B). The ability of these large [Ca\(^{2+}\)]\(_i\) rises, generated by releasing intracellular Ca\(^{2+}\) with either TRH or IM to stimulate ACVIII was explored. cAMP accumulation was measured in GH3C1 cells that were either uninfected (open bars) or infected with the ACVIII-containing virus (hatched bars) and maintained in Ca\(^{2+}\)-free Krebs buffer before the addition of forskolin along with TRH (100 nM; Fig. 6A, inset) or IM (2 \(\mu\)M; Fig. 6B, inset). Neither TRH or IM stimulated ACVIII activity as compared with the uninfected control cells. Therefore, the triggering of a large [Ca\(^{2+}\)]\(_i\) rise due to the release of Ca\(^{2+}\) from intracellular stores via either inositol 1,4,5-trisphosphate generation or ionophore-mediated release did not affect ACVIII.

**DISCUSSION**

One of the more surprising findings to emerge from the study of Ca\(^{2+}\)-sensitive adenyl cyclases—whether they are expressed heterologously or endogenously—is their strict dependence on CCE for their regulation in nonexcitable cells (2, 3). Release from internal stores (4) or Ca\(^{2+}\) entry via ionophore (2, 3) or triggered by arachidonic acid (7) is without effect. Extremely little is known about the regulation of Ca\(^{2+}\)-sensitive adenyl cyclases in excitable cells. In the present study, we have investigated the regulation of ACVIII by Ca\(^{2+}\) in an excitable cell line, rat anterior pituitary tumor-derived GH3C1 cells. The study addressed four major issues: (a) whether Ca\(^{2+}\) entry via VGCCs could regulate a Ca\(^{2+}\)-stimulable adenyl cyclase, (b) whether the modest CCE expressed in these cells could regulate the adenyl cyclase, (c) whether Ca\(^{2+}\) release from internal stores could regulate adenyl cyclase in this excitable cell line, and (d) whether the relative magnitudes of the [Ca\(^{2+}\)]\(_i\) rises generated by CCE and VGCCs could predict the degree of stimulation of the adenyl cyclase.

Does Ca\(^{2+}\) entry via VGCCs regulate a Ca\(^{2+}\)-stimulable adenyl cyclase? Ca\(^{2+}\)-sensitive adenyl cyclases are expressed mainly in excitable tissues, with the Ca\(^{2+}\)-stimulable ACI and ACVIII found exclusively in neuronal cells (32–34), whereas ACV and ACVI, the Ca\(^{2+}\)-inhibitable isofoms, predominate in cardiac tissue (35–37). Given the expression pattern of Ca\(^{2+}\)-sensitive adenyl cyclases and the prevalence of VGCCs in those tissues, we were surprised at the paucity in the literature on the ability of VGCC-mediated Ca\(^{2+}\) entry to regulate cAMP accumulation. Although there is abundant literature on the ability of the cAMP-signaling cascade to regulate L-type VGCCs (reviewed in Ref. 38), little has been done to directly address the ability of the cAMP signaling cascade to regulate t-type VGCCs (reviewed in Ref. 38), little has been done to directly address the potential and the effect of unknown Ca\(^{2+}\) entry by such channels on adenyl cyclase activity. An exception was a study showing that Ca\(^{2+}\) entry through VGCCs inhibited adenyl cyclase activity in embryonic chick ventricle myocytes (39). In the case of Ca\(^{2+}\)/calmodulin stimulation of adenyl cyclase activity, depolarization of hippocampal CA1 slices by 50 mM KCl increased cAMP accumulation, which was blocked by calmodulin antagonists (9). Furthermore, in cultured cerebellar granular cells, depolarization-induced Ca\(^{2+}\) entry stimulated cAMP accumulation, which was blocked by nimodipine (8). The current study extends these findings in showing that heterologous expression of a specific Ca\(^{2+}\)-stimulable adenyl cyclase, ACVIII, is regulated by Ca\(^{2+}\) entry through VGCCs in a clonal cell line. The potent block of this effect by nimodipine establishes that the effect is mediated by t-type VGCCs (40).

Does CCE regulate a Ca\(^{2+}\)-stimulable adenyl cyclase in an excitable cell? The potential role of CCE in excitable cells has been overshadowed by the more robust Ca\(^{2+}\) entry generated by VGCCs. Also hindering the study of CCE not only in excitable cells, but in all cell types, is the uncertainty of the molecular nature of the channels responsible for CCE. The Drosophila transient receptor potential protein, which is involved in insect phototransduction, is increasingly being viewed as a putative CCE channel (41). A family of mammalian transient receptor potential protein isoforms have been found in...
FIG. 4. Examination of the presence of CCE in GH4C1 cells. [Ca^{2+}]_{i}, and cAMP accumulation was determined in GH4C1 cells as described under "Experimental Procedures." A, [Ca^{2+}]_{i}, measurements in populations of Fura-2-loaded GH4C1 cells maintained in nominally Ca^{2+}-free Krebs buffer containing IBMX (100 μM). Cells were either untreated (b and d) or treated with TG (100 nM, 60 s; a and c) before the addition of [Ca^{2+}]_{o}, (2 mM, 300 s) in the absence or presence of nimodipine (1 μM, 300 s; c and d). Trace averages were generated by averaging 5-s, overlapping data segments and are overlaid on the actual traces. Inset, cAMP accumulation was measured in ACVIII-expressing GH4C1 cells maintained in nominally Ca^{2+}-free Krebs buffer. Control cells ( forskolin stimulation alone, open bars) were compared with untreated (right-hatched and left-hatched bars) and TG-treated cells (100 nM; cross-hatched and horizontal striped bars), in the absence or presence of nimodipine (1 μM, right-hatched and horizontal striped bars). B, populations of Fura-2-loaded GH4C1 cells were incubated in nominally Ca^{2+}-free Krebs buffer containing IBMX (100 μM) before the addition of TG (100 nM, 60 s; a and c) and/or KCl (10 mM, 300 s; a and b), along with [Ca^{2+}]_{o}, (0.5 mM, 300 s). Inset, cAMP accumulation was measured in ACVIII-expressing GH4C1 cells maintained in nominally Ca^{2+}-free Krebs buffer. Control cells (forskolin stimulation alone, open bar) were compared with untreated (right-hatched bar), KCl-treated (10 mM; cross-hatched bar), and TG/KCl-treated (left-hatched bar) cells in the presence of [Ca^{2+}]_{o}, (0.5 mM). Asterisks denote significant differences from the TG/KCl condition (left-hatched bar) as judged by Student’s t test (p < 0.005). C, cAMP accumulation was measured in ACVIII-expressing GH4C1 cells maintained in nominally Ca^{2+}-free Krebs buffer. Control cells (forskolin stimulation alone, open bar) were compared with untreated (left-hatched bar), TG-treated (100 nM; cross-hatched bar), KCl-treated (10 mM, horizontal striped bar), and TG/KCl-treated (vertical striped bar) cells in the presence of [Ca^{2+}]_{o}, (0.5 mM). Cells were assayed in the presence of nimodipine (1 μM), which was added at the beginning of the 1-min cAMP accumulation assay. The same fold-stimulation is observed in ACVIII-expressing cells after activation of CCE or VGCC-mediated Ca^{2+} entry under basal conditions, i.e. in the absence of forskolin. Data are representative of several similar experiments.
various brain regions, further suggesting a potential role for CCE in excitable cells (44, 45). One such role for CCE in excitable cells emerged from the work of Koizumi and Inoue (46), who showed in PC12 cells that caffeine, TG, and cyclopiazonic acid, all agents that release intracellular Ca\(^{2+}\) and therefore stimulate CCE, evoked dopamine release. Exocytosis has also been shown to be regulated by CCE in adrenal chromaffin cells (47). Our findings show that CCE, which gives rise to a modest increase in [Ca\(^{2+}\)], was very effective at stimulating ACVIII. Furthermore, the findings that CCE augmented the stimulation of ACVIII by VGCC-mediated Ca\(^{2+}\) entry in a manner that was insensitive to nimodipine establishes the fact that CCE can regulate the cyclase.

Does Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores regulate ACVIII in an excitable cell? We have previously shown an inability of Ca\(^{2+}\) release to regulate Ca\(^{2+}\)-sensitive adenyl cyclases in nonexcitable cells (4), and furthermore, we have shown that Ca\(^{2+}\) regulation of adenyl cyclases relies totally on Ca\(^{2+}\) entry through CCE channels (3). In the current study, releasing intracellular Ca\(^{2+}\) with either phospholipase C-coupled agonists or ionophore was without effect on ACVIII activity. Similar to our findings, dopamine release in PC12 cells was insensitive to Ca\(^{2+}\) release from intracellular stores and in fact depended on Ca\(^{2+}\) entry (46, 48). Thus we are now inclined to generalize that Ca\(^{2+}\) release from intracellular stores will not regulate Ca\(^{2+}\)-sensitive adenyl cyclases, regardless of cell type.

Does the magnitude of the [Ca\(^{2+}\)]\(_i\) rise generated by various means predict the amount of stimulation of ACVIII? We have compared the ability of three modes of raising [Ca\(^{2+}\)]\(_i\), VGCC-mediated Ca\(^{2+}\) entry, CCE, and Ca\(^{2+}\) release from internal stores, to stimulate ACVIII. The [Ca\(^{2+}\)]\(_i\) values achieved by VGCC-mediated Ca\(^{2+}\) entry and ionophore-mediated Ca\(^{2+}\) release were both substantial (peak values of approximately 700 and 900 nM, respectively), with CCE being much more modest (approximately 250 nM). However, the magnitude of these [Ca\(^{2+}\)]\(_i\) rises in no way predicts the amount of stimulation of ACVIII. The large [Ca\(^{2+}\)]\(_i\) rise promoted by Ca\(^{2+}\) release was totally without effect, whereas a similar peak [Ca\(^{2+}\)]\(_i\) rise generated by VGCC-mediated Ca\(^{2+}\) entry was very potent at stimulating ACVIII. CCE, the least effective in terms of producing a large [Ca\(^{2+}\)]\(_i\) rise, was as efficacious as VGCC-mediated Ca\(^{2+}\) entry in stimulating ACVIII.

Obviously the inability of the magnitude of different forms of [Ca\(^{2+}\)]\(_i\) rise to predict subsequent effects on ACVIII is due to the unresolved spatial information provided by Fura-2 in population measurements of [Ca\(^{2+}\)]\(_i\). One approach to addressing this issue directly would be to measure the [Ca\(^{2+}\)]\(_i\) in the vicinity of the cyclase by an adenyl cyclase/aequorin chimera (49), with the prediction that the chimera would report similar [Ca\(^{2+}\)]\(_i\) in response to VGCC and CCE and far less in response to release from stores. It is becoming increasingly obvious that

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**Fig. 5. Effect of nimodipine on TG- and VGCC-mediated Ca\(^{2+}\) entry.** [Ca\(^{2+}\)]\(_i\) was determined in GH4C1 cells as described under "Experimental Procedures." A and B, [Ca\(^{2+}\)]\(_i\) measurements were made in GH4C1 cells maintained in nominally Ca\(^{2+}\)-free Krebs buffer. A, cells were treated with KCl/[Ca\(^{2+}\)]\(_i\), (10 mM/2 mM) at 300 s in the absence (a) or presence (b) of nimodipine (1 μM, 300 s). B, cells were treated with TG (100 nM, 60 s), followed by the addition of [Ca\(^{2+}\)]\(_i\), (2 mM, 300 s) in the absence (a) or presence (b) of nimodipine (1 μM, 300 s). Data are representative of several similar experiments.

**Fig. 6. Effect of Ca\(^{2+}\) release from intracellular stores on ACVIII expressed in GH4C1 cells.** [Ca\(^{2+}\)]\(_i\) and cAMP accumulation was determined in GH4C1 cells as described under "Experimental Procedures." A and B, [Ca\(^{2+}\)]\(_i\) measurements were made in GH4C1 cells maintained in Ca\(^{2+}\)-free Krebs buffer. Ca\(^{2+}\) was released from intracellular stores using either TRH (100 nM, 60 s; A) or IM (2 μM, 60 s; B) with the resultant [Ca\(^{2+}\)]\(_i\) rises shown. Insets, cAMP accumulation was measured in either uninfected (open bars) or ACVIII-infected cells (hatched bars), as indicated. Cells in Ca\(^{2+}\)-free Krebs buffer were treated with either forskolin/TRH (10 μM/100 nM; A) or forskolin/IM (2 μM/2 μM; B).
Ca$^{2+}$ Regulation of Adenylyl Cyclase in Excitable Cells

the plasma membrane is not uniform in lipid composition or in the distribution of regulatory elements. Recent findings indicate that adenylyl cyclases must occur in cholesterol-rich domains to be susceptible to CCE in nonexcitable cells (50). In this context, it is also relevant that transient receptor potential protein 1 has recently been reported in rafts (51). It would be very interesting to determine whether the same residence in cholesterol-rich domains would apply to the adenylyl cyclase or to any of these channels in excitable cells. The nonequivalence in the ability of VGCC-mediated Ca$^{2+}$ entry and CCE to regulate ACVIII in GH$_4$C$_1$ cells may indicate that [Ca$^{2+}$] achieved by VGCC in the cytosol is underestimated (28). Consequently, the VGCC may be even more distant and in quite a different domain from the adenylyl cyclase.

It seems fair to conclude from these studies that the intimate relationship that was first demonstrated between CCE channels and adenylyl cyclase in nonexcitable cells is maintained in excitable cells, even though the adenylyl cyclase is also susceptible to CCE in nonexcitable cells (50). In these cells. Whether this association is maintained by colocalization within cholesterol-rich domains of the plasma membrane, as in nonexcitable cells (50), or by some additional process remains to be determined.

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Regulation of a Ca$^{2+}$-sensitive Adenylyl Cyclase in an Excitable Cell: ROLE OF VOLTAGE-GATED VERSUS CAPACITATIVE Ca$^{2+}$ ENTRY

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