Hormone Stimulation of Type III Adenylyl Cyclase Induces Ca^{2+} Oscillations in HEK-293 Cells*

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Various forms of cross-talk between the Ca^{2+} and cAMP signal transduction systems can occur in animal cells depending upon the types of adenylyl cyclases present. Here, we report that Ca^{2+} oscillations can be generated by hormone stimulation of type III adenylyl cyclase expressed in HEK-293 cells. These Ca^{2+} oscillations are apparently due to the unique regulatory features of type III adenylyl cyclase, which is stimulated by hormones and inhibited by elevated Ca^{2+} in vivo. Ca^{2+} oscillations were generated by glucagon, isoproterenol, or forskolin stimulation of type III adenylyl cyclase and were dependent upon the activity of cAMP- and calmodulin-dependent protein kinases. Ca^{2+} oscillations were not solely dependent upon cAMP increases since dibutyryl cAMP or (S<sub>p</sub>)cAMP did not stimulate Ca^{2+} oscillations. We hypothesize that stimulation of type III adenylyl cyclase leads to increased cAMP, activation of inositol 1,4,5-trisphosphate receptors, and elevation of intracellular Ca^{2+}. As free Ca^{2+} increases, type III adenylyl cyclase activity is attenuated by CaM kinase(s) and intracellular cAMP levels decrease. When cAMP levels drop below a threshold level, the inositol 1,4,5-trisphosphate receptor is dephosphorylated and Ca^{2+} is reesterified. This cycle is repeated if type III adenylyl cyclase is chronically exposed to an activator. This unique mechanism for generation of Ca^{2+} oscillations in cells is distinct from others documented in the literature.

In most mammalian tissues the Ca^{2+} and cAMP signal transduction systems are tightly coupled, and cross-talk between these two regulatory systems may play an important role for various physiological phenomena including synaptic plasticity (Xia et al., 1991; Choi et al., 1993b). Intracellular free Ca^{2+} (Ca_i) can affect cAMP levels by modulation of adenylyl cyclase or phosphodiesterase activities (reviewed by Choi et al. (1993a) and Beavo and Reifsnyder (1990)). On the other hand, cAMP-dependent protein kinase (PKA) or cAMP can affect Ca_i, by regulating Ca^{2+} ion channel activity (reviewed by Hell et al. (1994)). Because of the regulatory diversity of adenylyl cyclases, phosphodiesterases, and protein kinases, different patterns of cross-talk between the Ca^{2+} and cAMP regulatory systems may be established in specific cell types.

cDNA clones for eight adenylyl cyclases have been isolated, and each of these enzymes has distinct regulatory properties (Krupinski et al., 1989, 1992; Bakalyar and Reed, 1990; Feinstein et al., 1991; Gao and Gilman, 1991; Ishikawa et al., 1992; Katsumi et al., 1992; Yashihito and Cooper, 1992; Cali et al., 1994; Watson et al., 1994). Five of these enzymes; I-AC (Tang et al., 1991; Choi et al., 1992b), III-AC (Choi et al., 1992a; Wayman et al., 1994), V-AC and VI-AC (Yashihito and Cooper, 1992; Katsumi et al., 1992), and VIII-AC (Cali et al., 1994), are regulated by Ca^{2+}. I-AC and VIII-AC are stimulated by intracellular Ca^{2+} in vivo (Choi, 1992b; Cali et al., 1994), whereas V-AC and VI-AC are inhibited by Ca^{2+}.

III-AC is stimulated by Ca^{2+} and calmodulin (CaM) in isolated membranes when the enzyme is also activated by Gs (Choi et al., 1992). However, we recently discovered that Ca^{2+} inhibits hormone-stimulated III-AC activity in vivo (Wayman et al., 1995). Ca^{2+} inhibition of III-AC is not due to activation of G, or protein kinase C and is apparently mediated by one of the CaM kinases. For example, Ca^{2+} inhibition of III-AC is blocked by Mn-62, which is an inhibitor of CaM kinases. Furthermore, III-AC is inhibited by coexpression of III-AC and constitutively activated CaM kinase-II in HEK-293 cells. The CaM kinase construct used was under the control of the metallothionein promoter, which allows the induction of CaM kinase-II expression with Zn^{2+}. Ca^{2+} inhibition of III-AC in vivo provides a feedback mechanism for attenuation of hormone-stimulated adenylyl cyclase activity. Since activation of PKA can increase Ca^{2+}, and hormone stimulation of III-AC is inhibited by Ca^{2+}, one might expect Ca^{2+} oscillations to be generated by hormone stimulation of III-AC. Here, we report glucagon and isoproterenol stimulation of Ca^{2+} oscillations in HEK-293 cells expressing III-AC and the glucagon receptor.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Human embryonic kidney 293 (HEK-293) cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified 95% air, 5% CO<sub>2</sub> incubator. Cell culture materials were from Life Technologies, Inc. unless otherwise noted.

Expression of I-AC, III-AC, and the Glucagon Receptor in HEK-293 Cells—The I-AC cDNA clone was isolated from a bovine brain cDNA library as described by Xia et al. (1991). The III-AC cDNA clone in pBlueScript SK± (Bakalyar and Reed, 1990) was obtained from R. R. Reed (John Hopkins University, Baltimore, MD). The coding sequence of III-AC was ligated to CD M-8 (CD M-8(III-AC)) for expression in HEK-293 cells. Neomycin-resistant HEK-293 cells stably transfected with an expression vector CD M-8 that contained cDNA for I-AC (CD M-8(I-AC)), III-AC (CD M-8(III-AC)), or no exogenous DNA were used for this study. These clones have been characterized previously (Choi et al., 1992a, 1992b, 1993b; Wu et al., 1993) and were used for subsequent cotransfection with the rat glucagon receptor (Jelink et al., 1993). Each of these cell lines was stably transfected with either the pPCEP expression vector encoding the rat glucagon receptor (pLJ 4) or vector alone. For DNA transfections, cells were plated on 100-mm dishes at a density of 2 × 10<sup>6</sup> cells/plate, grown overnight, and transfected with the pPCEP control vector (1 µg of DNA/plate) and a hygro-
mycin resistance vector (1 μg of DNA/plate) by the Ca2+ phosphate method (Chen and Okayama, 1987). Hygromycin-resistant cells were selected in culture medium containing hygromycin B (Sigma, 460 units/ml) and 300 μg/ml G418. Hygromycin/neomycin-resistant cells were assayed for glucagon-stimulated adenyl cyclase activity by use of a cAMP accumulation assay. After selection, cells were maintained in medium containing 230 units/ml hygromycin B and 300 μg/ml G418. Multiple hygromycin/neomycin-resistant clones of each type, expressing the rat glucagon receptor (GluR) and III-AC were isolated. Cells were grown for imaging as follows. Day 1 cells were plated on poly-L-lysine- or poly-o-lysine-coated Lab-Tek four-chambered coverglass slides (60,000 cells/well) and were Ca2+ imaged on day 6.

○ cAMP Accumulation—Changes in intracellular cAMP levels were measured by determining the ratio of [3H]cAMP to total ATP, ADP, and AMP pool in [3H]adenine-loaded cells as described by Wong et al. (1991). This assay system allows rapid and extremely sensitive measurements of relative changes in intracellular CAMP levels in response to various effects. Absolute ratios for cAMP accumulation generally show some variation between experiments using different sets of cells (Federman et al., 1992; Dittman et al., 1993). It is important to emphasize, however, that relative changes in cAMP were highly consistent between experiments. Confluent cells in six-well plates were initially incubated in Dulbecco’s modified Eagle’s medium containing [3H]adenine (2.0 μCi/ml, ICN) for 16–20 h, washed once with 150 mM NaCl, and incubated at 37°C for 30 min in incubation buffer (218 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 0.5 mM EDTA, 10.0 mM glucose, 20.0 mM HEPES, pH 7.4) containing 1.0 mM IBMX, and various effectors as indicated. Reactions were terminated by aspiration, washing cells once with 150 mM NaCl, and the addition of 1.0 ml of ice-cold 5% trichloroacetic acid containing 1.0 μM cAMP. Culture dishes were maintained at 4°C for 1–4 h, and acid-soluble nucleotides were separated by ion-exchange chromatography as described (Salomon et al., 1979). Reported data are the average of triplicate determinations.

Determination of Phosphoinositide Production—Cells were plated in six-well (35 mm diameter) plates and allowed to grow until nearly confluent. Cells were then assayed for phosphoinositide turnover in response to forskolin, isoproterenol, glucagon, and carbachol by the method of Masters et al. (1985) and Subers et al. (1988). Reported data are the average of triplicate determinations.

Calcium Imaging—A 75-watt xenon lamp and a Metaltex (NC) filter wheel and shutter were separated from the Nikon microscope to prevent vibrations from affecting the optical recordings. A G. W. Ellis fiberoptic light scrambler (Technical Video Ltd.) was used to transmit the light to the microscope. Excitation, emission, and neutral density filters were from Omega Optical. The objectives used were a Nikon Fluor 20/1.3 and a Nikon Fluor 40/0.85 NA. The images were intensified with a Genesis image intensifier (Dage-MTI) and acquired with a Dage-MTI CCD-72 series camera. All image acquisition was computer-controlled with the Universal Imaging Corporation’s Image-1/FL program. Images were viewed on a Sony Trinitron color video monitor (PVM-1343MD) and printer (UP-5000) and a Javelin Electronics video gram. Images were viewed on a Sony Trinitron color video monitor controlled with the Universal Imaging Corporation’s Image-1/FL program. All image acquisition was computer-controlled with the Universal Imaging Corporation’s Image-1/FL program. Images were viewed on a Sony Trinitron color video monitor (PVM-1343MD) and printer (UP-5000) and a Javelin Electronics video gram. Images were viewed on a Sony Trinitron color video monitor controlled with the Universal Imaging Corporation’s Image-1/FL program.

RESULTS

Glucagon Stimulates Ca2+ Oscillations in HEK-293 Cells Expressing III-AC—In these experiments we used several types of transformed HEK-293 cell lines to analyze the contribution of III-AC to Ca2+ transients. Glucagon does not increase intracellular Ca2+ or cAMP in the control HEK-293 cells because they do not express glucagon receptors (Wayman et al., 1994). I-AC-expressing cells were also used as a control. I-AC is stimulated by concentrations of intracellular Ca2+ that inhibit the activity of glucagon-stimulated III-AC activity, and it is not stimulated by glucagon or β-adrenergic agonists in vivo (Wayman et al., 1994).

HEK-293 cells stably expressing the glucagon receptor (293-G), the glucagon receptor with I-AC (I-AC-G), or III-AC (III-AC-G) were treated with 100 nM glucagon, and Ca2+ imaged using Fura-2 as described under “Experimental Procedures.” Representative traces from individual cells are presented.

![FIG. 1. Glucagon stimulation of Ca2+ oscillations in HEK-293 cells expressing the glucagon receptor and III-AC.](image)

- Glucagon elicited three general types of Ca2+ response in these cells (Fig. 2, Table I). Only 7% of III-AC-G cells gave a single Ca2+ spike (Fig. 2A), 9% showed an intermediate response best described as spike-plateau (Fig. 2B), and 84% exhibited Ca2+ oscillations (Fig. 2C). In contrast, only 4% of the
control 293-G cells responded with Ca²⁺ oscillations (Table I). The initial Ca²⁺ spike in III-AC-G cells averaged 500 ± 136 nM and was followed by multiple Ca²⁺ transients (336 ± 128 nM), which continued for at least 60 min. These oscillations were at an average frequency of 4.3 peaks/15 min. In 293-G cells, the single Ca²⁺ spike averaged 332 ± 75 nM (Table I). Several different stable cell lines expressing III-AC and the glucagon receptor were examined with analogous results.

Isoproterenol Stimulates Ca²⁺ Oscillations in HEK-293 Cells Expressing III-AC—To determine if initiation of Ca²⁺ oscillations was strictly dependent on glucagon, III-AC-G cells were treated with the β-adrenergic agonist isoproterenol. HEK-293 cells express endogenous β-adrenergic receptors that are coupled to the stimulation of III-AC in vivo (Wayman et al., 1994). Incubation of HEK-293, I-AC-G (data not shown), or 293-G cells with 10 µM isoproterenol caused a single transient Ca²⁺ peak (Fig. 3A). This response was similar in amplitude and duration to that elicited by glucagon. Exposure of III-AC-G cells to isoproterenol caused Ca²⁺ oscillations that strongly resembled those induced by glucagon (Fig. 3B). Therefore, the phenomenon under consideration is not specific to glucagon stimulation of III-AC.

Forskolin Stimulates Ca²⁺ Oscillations in HEK-293 Cells Expressing III-AC—To determine if other adenyllyl cyclase activators stimulate Ca²⁺ oscillations, III-AC-G cells were treated with forskolin and Ca²⁺ imaged. Forskolin stimulates adenyllyl cyclases through direct interactions with the catalytic subunit and does not require G proteins (Seamon and Daly, 1981). Forskolin, but not its inactive analogue 1,9-dideoxy-forskolin, induced Ca²⁺ oscillations in III-AC-G cells (Fig. 4). These oscillations were of comparable amplitude and frequency to those stimulated by either glucagon or isoproterenol. Treatment of 293-G cells with forskolin caused a single Ca²⁺ peak (Fig. 7C). These results indicate that Ca²⁺ oscillations in III-AC-G cells can be initiated by several different activators of III-AC and are not dependent upon hormone stimulation of the enzyme.

Comparison of Intracellular cAMP Increases Stimulated by Hormones in III-AC-G and 293-G Cells—The data described thus far suggest that elevations in cAMP may stimulate Ca²⁺ oscillations in III-AC-G cells. The absence of hormone-stimulated Ca²⁺ oscillations in 293-G cells, which express low levels of endogenous III-AC, might be due to insufficient cAMP increases. Therefore, 293-G, I-AC-G, and III-AC-G cells were treated with 100 nM glucagon (Fig. 5A) or 10 µM isoproterenol (Fig. 5B) and intracellular cAMP accumulations were measured. Glucagon- or isoproterenol-stimulated cAMP increases were 2–3-fold greater in III-AC-G cells than in 293-G or I-AC-G cells (Fig. 5, A and B). Forskolin-stimulated increases in cAMP were also significantly greater in III-AC-G cells compared to 293-G cells (Fig. 6). These data suggest that Ca²⁺ oscillations in HEK-293 cells may require a threshold cAMP increase that is generated in III-AC-G cells, but not in 293-G or I-AC-G cells. However, other data discussed below suggest that increases in cAMP may be necessary but not sufficient for generation of Ca²⁺ oscillations.

If Ca²⁺ oscillations in HEK-293 cells require a threshold cAMP increase, then it might be possible to stimulate Ca²⁺ oscillations in 293-G cells using glucagon or forskolin in combination with cAMP phosphodiesterase inhibitors, which increase cAMP signals. The cAMP increases produced in III-AC-G cells by 100 nM glucagon or 10 µM isoproterenol are comparable to those produced in 293-G cells by a combination of IBMX and glucagon. Exposure of 293-G cells to either glucagon or forskolin in the absence of IBMX, a phosphodiesterase inhibitor, produced a single Ca²⁺ peak (Fig. 7, A and C). IBMX alone had no effect on intracellular Ca²⁺ (data not shown); however, combinations of glucagon and IBMX (Fig. 7B) or forskolin and IBMX (Fig. 7D) resulted in Ca²⁺ oscillations. The predominant form(s) of endogenous adenyllyl cyclase in HEK-293 cells is Ca²⁺-inhibitable. For example, isoproterenol stimulated adenyllyl cyclase activity in HEK-293 control cells approximately 6.0-fold, and this stimulation was inhibited 60% by increasing intracellular free Ca²⁺. These data are consistent with the proposal that a minimal cAMP increase is necessary for Ca²⁺ oscillations.

cAMP Analogues Alone Do Not Produce Ca²⁺ Oscillations in HEK-293 Cells—If Ca²⁺ oscillations are solely dependent upon minimal cAMP increases, then it should be possible to generate oscillations with high concentrations of membrane-permeable cAMP analogues such as dibutyryl cAMP or (S₃₅)CAMP. Incubation of 293-G cells with either 1 mM dibutyryl cAMP (Fig. 8A) or 400 µM (S₃₅)CAMP (Fig. 8B) resulted in a single Ca²⁺ transient. Secondary challenges with greater concentrations of cAMP analogues (e.g. 5 mM dibutyryl cAMP) caused no further increase in Ca²⁺. These levels of (S₃₅)CAMP or dibutyryl cAMP are sufficient to fully activate PKA in 293 cells (Impey et al., 1994). We conclude that elevated cAMP may be necessary but not sufficient for Ca²⁺ oscillations. Other regulatory properties of III-AC, for example its sensitivity to Ca²⁺ inhibition, may contribute to this phenomenon.

Activation of PKA Is Required for Generation of Ca²⁺ Oscillations—cAMP can regulate intracellular Ca²⁺ by several mechanisms including direct interactions with Ca²⁺ channels or indirectly by activation of PKA, which phosphorylates IP₃ receptors (Nakade et al., 1994) and ryanodine receptors channels (Suko et al., 1993; Hohenegger, 1993). To determine if PKA activation is required for generation of Ca²⁺ oscillations in III-AC-G cells, the effects of two PKA inhibitors, H-89 and (R)₂-cAMP (Rothermel et al., 1988), were examined. Preincubation of III-AC-G cells for 30 min with 20 µM H-89 did not block the initial Ca²⁺ rise stimulated by glucagon, but it did inhibit Ca²⁺ oscillations (Fig. 9A). Similarly, treatment of these cells with (R)₂-cAMP, prior to addition of isoproterenol, also blocked Ca²⁺ oscillations.
but not the initial Ca\textsuperscript{2+} response (Fig. 9B). These PKA inhibitors had no significant effect on basal or hormone-stimulated intracellular Ca\textsuperscript{2+} levels (Wayman et al., 1994). Furthermore, basal Ca\textsuperscript{2+} levels and the magnitude of the first Ca\textsuperscript{2+} spike were also unaffected by (R)\textsubscript{p}-cAMP or H-89. The inability of H-89 or (R)\textsubscript{p}-cAMP to block the initial cAMP induced Ca\textsuperscript{2+} transients may be due to incomplete inhibition PKA or to a PKA-independent mechanism for mobilization of Ca\textsuperscript{2+} by cAMP. These data indicate that PKA activity is required for both glucagon- and isoproterenol-stimulated Ca\textsuperscript{2+} oscillations.

### TABLE I

| Cells expressing type III adenyl cyclase and the glucagon receptor | Average basal Ca\textsuperscript{2+} | Cells responding | Responding cells with single spike | Responding cells oscillating | Average [Ca\textsuperscript{2+}] 1st spike | Average [Ca\textsuperscript{2+}] 2nd spike | Frequency | Cells |
|---------------------------------------------------------------|-----------------------------------|-----------------|-----------------------------------|-------------------------------|----------------------------------|----------------------------------|-----------|-------|
| HEK-293-G                                                    | 40.83 ± 11.72                     | 88.46           | 95.56                             | 4.44                          | 332 ± 75                        | 279 ± 75*                        | 2*        | 78    |
| AC-III-G                                                     | 66.86 ± 14.25                     | 80.68           | 7.04                              | 83.80                         | 500 ± 136                       | 336 ± 128                       | 4.34 ± 1.92 | 176   |

*Statistical analysis of HEK-293 cells that respond to glucagon by oscillations in intracellular calcium.

![Graph A: Intracellular Free Calcium (nM)](image1)

**Fig. 3. Isoproterenol stimulation of Ca\textsuperscript{2+} oscillations in HEK-293 cells expressing III-AC.** A, HEK-293 cells expressing the glucagon receptor (293-G) or the glucagon receptor with III-AC (III-AC-G) were treated with 10 \textmu M isoproterenol and Ca\textsuperscript{2+} imaged using Fura-2 as described under “Experimental Procedures.” Representative traces from individual cells are presented.

![Graph B: Intracellular Free Calcium (nM)](image2)

**Fig. 4. Forskolin stimulation of Ca\textsuperscript{2+} oscillations in HEK-293 cells expressing III-AC.** HEK-293 cells expressing type III adenyl cyclase (III-AC-G) were treated with 100 \textmu M forskolin and Ca\textsuperscript{2+} imaged using Fura-2 as described under “Experimental Procedures.” A representative trace from an individual cell is presented.

![Graph C: Hormone-stimulated cAMP increases in 293-G, I-AC-G, and III-AC-G cells](image3)

**Fig. 5. Hormone-stimulated cAMP increases in 293-G, I-AC-G, and III-AC-G cells.** HEK-293 cells expressing the glucagon receptor alone (293-G, ■) or the glucagon receptor with I-AC (I-AC-G, □) or III-AC (III-AC-G, ▲) were treated with 100 nM glucagon (A) or 10 \textmu M isoproterenol (B). Relative cAMP accumulations were determined as described under “Experimental Procedures.” The data are the mean ± S.D. of triplicate assays.

The CaM Kinase Inhibitor KN-62 Blocks Ca\textsuperscript{2+} Oscillations—It is our working hypothesis that CaM kinase activity may contribute to Ca\textsuperscript{2+} oscillations by inhibiting hormone or forskolin stimulation of III-AC as intracellular Ca\textsuperscript{2+} increases. III-AC is inhibited by elevations in intracellular Ca\textsuperscript{2+} in HEK-293 cells, and this inhibition is blocked by KN-62, a specific inhibitor of CaM kinases (Wayman et al., 1995). Consequently, Ca\textsuperscript{2+} oscillations may arise from a cycle that includes hormone activation of III-AC, inhibition of III-AC by CaM kinases as Ca\textsuperscript{2+} increases, and subsequent decreases in cAMP, followed by sequestration of Ca\textsuperscript{2+} and reinitiation of the cycle when Ca\textsuperscript{2+} drops. If this hypothesis is valid, then KN-62 should inhibit glucagon and forskolin stimulation of Ca\textsuperscript{2+} oscillations.

KN-62 had no effect on either basal or carbachol-stimulated intracellular free Ca\textsuperscript{2+} (data not shown). Although KN-62 did not block the initial Ca\textsuperscript{2+} peak stimulated by glucagon and
forskolin, Ca\textsuperscript{2+} oscillations were inhibited by KN-62 (Fig. 10). However, Ca\textsuperscript{2+}i did increase approximately 2-fold over the baseline and stayed at this level for at least 30 min. These data are consistent with the hypothesis that Ca\textsuperscript{2+} inhibition of III-AC, by CaM kinases, may contribute to Ca\textsuperscript{2+} oscillations. Because CaM kinases regulate a number of proteins involved in the regulation of intracellular Ca\textsuperscript{2+}, they may also be important for the resequstration of intracellular Ca\textsuperscript{2+}. For example, phospholambin (Xu et al., 1993) and a sarcoplasmic reticulum Ca\textsuperscript{2+} pump (Hawkins et al., 1994) are both phosphorylated by CaM kinases. Phosphorylation of the sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase results in a 2-fold increase in catalytic activity. Therefore, inhibition of CaM kinase activity in HEK-293 cells may inhibit the cell’s ability to return intracellular free Ca\textsuperscript{2+} to basal levels.

Ca\textsuperscript{2+} Oscillations Are Not Due to Hormone Stimulation of IP\textsubscript{3} Turnover—One of the major mechanisms for coupling of hormone receptors to mobilization of intracellular Ca\textsuperscript{2+} is through stimulation of phospholipase C and activation of the inositol trisphosphate cascade (reviewed by Berridge (1993)). Inositol 1,4,5-trisphosphate (IP\textsubscript{3}) stimulates release of Ca\textsuperscript{2+} from nonmitochondrial intracellular stores and, in some systems, activation of the IP\textsubscript{3} pathway stimulates Ca\textsuperscript{2+} oscillations (reviewed by Berridge (1990) and Fewtrell (1993)). Intracellular Ca\textsuperscript{2+} was measured and determined as described under “Experimental Procedures.” Representative traces from individual cells are presented.
phosphoinositide turnover 80%, glucagon, isoproterenol, and forskolin had no significant effect. These data suggest that Ca\textsuperscript{2+} oscillations induced by hormone or forskolin stimulation of III-AC were not due to stimulation of the IP\textsubscript{3} pathway.

The IP\textsubscript{3}-regulated Ca\textsuperscript{2+} pool is the primary source for Ca\textsuperscript{2+} oscillations. Elevations in Ca\textsuperscript{2+} can occur by several mechanisms including the opening of plasma membrane Ca\textsuperscript{2+} channels or the release of Ca\textsuperscript{2+} from intracellular stores. To identify the Ca\textsuperscript{2+} pool that contributes to glucagon-stimulated Ca\textsuperscript{2+} oscillations in III-AC-G cells, we examined the effect of glucagon and forskolin on Ca\textsuperscript{2+} oscillations in the absence of extracellular Ca\textsuperscript{2+}. Glucagon-stimulated Ca\textsuperscript{2+} oscillation in the absence of extracellular Ca\textsuperscript{2+} (Fig. 12A). The amplitude of the initial Ca\textsuperscript{2+} peak was comparable in the presence and absence of extracellular Ca\textsuperscript{2+}. However, the amplitude of subsequent Ca\textsuperscript{2+} peaks decayed relatively rapidly suggesting that internal pools were depleted. Therefore, extracellular Ca\textsuperscript{2+} is not required for the initiation of oscillations but may be required for maintenance of Ca\textsuperscript{2+} oscillations over an extended period of time.

If the primary source of Ca\textsuperscript{2+} for oscillations is an internal Ca\textsuperscript{2+} pool, then thapsigargin should inhibit glucagon-stimulated Ca\textsuperscript{2+} oscillations since this drug is an inhibitor of the intracellular sarcoendoplasmic reticulum Ca\textsuperscript{2+} ATPases (Thastrup et al., 1990; Lytton et al., 1991). Treatment of III-AC-G cells with thapsigargin in Ca\textsuperscript{2+}-free medium caused a rapid release and depletion of intracellular Ca\textsuperscript{2+} stores, and intracellular free Ca\textsuperscript{2+} returned to basal levels within 15 min (Fig. 12B). Glucagon-stimulated Ca\textsuperscript{2+} oscillations were completely blocked by pretreatment with thapsigargin, suggesting that
Ca²⁺ oscillations were dependent upon intracellular Ca²⁺ pools.

Two of the major intracellular Ca²⁺ pools are the ryanodine- and IP₃-sensitive pools, both of which are regulated by cAMP through PKA (Bird et al. 1993; Nakade et al. 1994; Yoshida et al. 1992). High concentrations of ryanodine inhibits the release of Ca²⁺ from the ryanodine-sensitive Ca²⁺ pool. Treatment of III-AC-G cells with ryanodine (1–50 μM) had no effect on glucagon-stimulated Ca²⁺ oscillations in III-AC-G cells, indicating that the ryanodine-sensitive Ca²⁺ pool does not contribute to this phenomenon (Fig. 13).

HEK-293 cells express muscarinic receptors, which are coupled to mobilization of intracellular free Ca²⁺ through the phospholipase C/IP3 pathway. The muscarinic agonist carbachol increases IP₃ turnover and intracellular Ca²⁺ in these cells. Furthermore, PKA phosphorylation of IP₃ receptors stimulates Ca²⁺ release from intracellular stores (Burgess et al. 1991; Bird et al. 1993; Joseph and Ryan 1993; Nakade et al. 1994) and could account for the cAMP-generated Ca²⁺ transients caused by forskolin, glucagon, or isoproterenol in III-AC-G cells. If the IP₃-sensitive Ca²⁺ pool contributes to the Ca²⁺ oscillations stimulated by forskolin, then pretreatment of III-AC-G cells with carbachol in the absence of extracellular Ca²⁺ should exhaust the IP₃-sensitive pool and inhibit forskolin-stimulated Ca²⁺ oscillations. Incubation of III-AC-G cells with carbachol in Ca²⁺-free media gave a single Ca²⁺ transient, and subsequent addition of forskolin did not stimulate Ca²⁺ oscillations (Fig. 14). Furthermore, pretreatment of these cells with forskolin for 5 min, in the absence of external Ca²⁺, diminished the Ca²⁺ increase caused by subsequent application of carbachol, indicating that both reagents stimulated Ca²⁺ release from a common pool. Collectively, these data suggest that the major Ca²⁺ pool contributing to glucagon and forskolin-stimulated Ca²⁺ oscillations was the IP₃-sensitive pool.

**DISCUSSION**

There is increasing interest in molecular mechanisms for generation of Ca²⁺ oscillations in non-excitable cells (reviewed by Fewtrell (1993) and Berredge (1990, 1992)). Presumably Ca²⁺ oscillations provide enhanced Ca²⁺ signals averaged over an extended period of time without the toxicity associated with persistently elevated Ca²⁺. One of the most extensively characterized mechanism for generation of Ca²⁺ oscillations is through stimulation of the phospholipase C/IP₃ pathway. For example, Bird et al. (1993) have proposed that IP₃-generated sinusoidal oscillations in intracellular Ca²⁺ require negative feedback regulation of phospholipase C by protein kinase C. In this study we describe a new mechanism for generation of Ca²⁺ oscillations that is based upon the unique regulatory features of III-AC, an enzyme that is stimulated by Gs-coupled receptors in vivo but inhibited by elevated Ca²⁺.

Forskolin, glucagon, and isoproterenol stimulated Ca²⁺ oscillations in HEK-293 cells that were stably transfected with III-AC. Control HEK-293 cells did not show Ca²⁺ oscillations unless glucagon or forskolin were applied with IBMX. Since HEK-293 cells express III-AC activity (Xia et al. 1993) and hormone stimulation of endogenous adenylyl cyclase activity is
also Ca2+-inhibitable, it seems likely that this enzyme contributes to Ca2+ oscillations in 293-G cells when endogenous cAMP phosphodiesterase activity is inhibited by IBMX. Hormone-stimulated Ca2+ oscillations in III-AC cells were dependent upon PKA activity, the IP3-sensitive Ca2+ pool, and they were inhibited by KN-62, a CaM kinase inhibitor. The low levels of cAMP analogues that were sufficient to generate single Ca2+ peaks, did not stimulate Ca2+ oscillations suggesting that elevated cAMP was necessary but not sufficient to account for Ca2+ oscillations. Glucagon, forskolin and isoproterenol did not generate Ca2+ oscillations by stimulating IP3 turnover.

What is the mechanism for hormone-stimulated Ca2+ oscillations in III-AC-G cells? Our data are most consistent with the following model (Fig. 15). When III-AC is activated by hormones, cAMP stimulates PKA, which phosphorylates and activates IP3 receptors. As intracellular Ca2+ rises, III-AC activity is attenuated by CaM kinase(s) and intracellular cAMP levels decrease because of cAMP phosphodiesterases. When cAMP inhibition of adenylyl cyclase activity can lead to Ca2+ oscillations. Data described in this report are the first evidence that Ca2+ inhibition of adenylyl cyclase activity can lead to Ca2+ oscillations in animal cells.

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