The nucleus of an inositol lipid cycle (6) and the Ca\(^{2+}\) receptors on the nuclear membrane (4, 5) and the presence of inositol 1,4,5-trisphosphate such as mitosis, apoptosis, and gene transcription (reviewed in Ref. 3) is suggested by the presence of inositol 1,4,5-trisphosphate that [Ca\(^{2+}\)]\(_{n}\) may be independently regulated.

The results reported in this study address the controversial issue that nuclear free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{n}\)) may be regulated independently of cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{c}\)). We have measured [Ca\(^{2+}\)]\(_{n}\) and [Ca\(^{2+}\)]\(_{c}\), with recombinant aequorin targeted to the nucleus and cytosol in HeLa cells. We found that histamine, ATP, and ionomycin increased [Ca\(^{2+}\)]\(_{n}\), quantitatively more than [Ca\(^{2+}\)]\(_{c}\), although the time course of these changes was similar. The difference between [Ca\(^{2+}\)]\(_{n}\) and [Ca\(^{2+}\)]\(_{c}\) depended on the stimulus, and the relative difference between [Ca\(^{2+}\)]\(_{n}\) and [Ca\(^{2+}\)]\(_{c}\) was less with ionomycin than with histamine or ATP. After depletion of the internal Ca\(^{2+}\) store, restoration of extracellular Ca\(^{2+}\) resulted in only increased [Ca\(^{2+}\)]\(_{c}\), without a significant increase in [Ca\(^{2+}\)]\(_{n}\). Treatment with cyclopiazonic acid resulted in a delayed increase in [Ca\(^{2+}\)]\(_{c}\) compared to [Ca\(^{2+}\)]\(_{n}\). These differences in both timing and magnitude of nuclear Ca\(^{2+}\) signals confirm that the cell can limit or delay increases in nuclear free Ca\(^{2+}\). Taken with the fact that an inositol phosphate signaling system resides in the nucleus and its envelope, our data support the hypothesis that [Ca\(^{2+}\)]\(_{n}\) may be independently regulated.

Cytosolic Ca\(^{2+}\) is a pivotal regulator of many cytosolic functions (1, 2). However, the role of Ca\(^{2+}\) in the nucleus is more controversial. The involvement of nuclear Ca\(^{2+}\) in processes such as mitosis, apoptosis, and gene transcription (reviewed in Ref. 3) is suggested by the presence of inositol 1,4,5-trisphosphate (InsP\(_3\)) receptors and inositol 1,3,4,5-tetrakisphosphate receptors on the nuclear membrane (4, 5) and the presence in the nucleus of an inositol lipid cycle (6) and the Ca\(^{2+}\) binding proteins, calmodulin, calreticulin and calpain (7).

It is widely assumed that the nuclear pore forms a large channel for the free diffusion of ions and macromolecules (8). However, electrophysiological data from patch clamping the nuclear envelope revealed that the nuclear pore is not freely permeable to all ions (9, 10) and that transport across the pore may be regulated by the perinuclear Ca\(^{2+}\) store (11) and/or ATP (12). In addition, there was no diffusion of fura-2 (13) or InsP\(_3\) (14) into the cytoplasm after they had been injected into oocyte nuclei. The combined evidence from the literature therefore strongly suggests that the nucleus is capable of independently regulating the transport of ions such as Ca\(^{2+}\).

Previous reports comparing [Ca\(^{2+}\)]\(_{n}\) and [Ca\(^{2+}\)]\(_{c}\), using Ca\(^{2+}\)-sensitive fluorescent dyes have been divided on whether [Ca\(^{2+}\)]\(_{n}\) is independently controlled (14–20). The controversy has arisen primarily from problems with dye compartmentalization and with alterations in Ca\(^{2+}\) sensitivity of the dye by protein binding (16). Reported differences in nuclear and cytosolic fluorescence have therefore not been universally accepted as evidence for differential regulation of [Ca\(^{2+}\)]\(_{n}\) and [Ca\(^{2+}\)]\(_{c}\) (15–17). Despite this controversy most authors support the hypothesis that changes in [Ca\(^{2+}\)]\(_{n}\) regulate nuclear events (15–20).

The Ca\(^{2+}\)-sensitive photoprotein aequorin can be localized to specific organelles within the cell by the addition of protein targeting sequences. It is therefore ideally suited to monitoring Ca\(^{2+}\) within subcellular compartments, as unlike the fluorescent indicator dyes it is insensitive to pH or protein binding (21). This strategy has been successfully used to target aequorin to the mitochondria (22), nucleus (23, 24), and endoplasmic reticulum (25, 26) of live cells. We have previously demonstrated efficient targeting of apoaequorin to the nucleus by addition of the nuclear structural protein, nucleoplasmin, to the N terminus (24). The N-terminal addition of luciferase to apoaequorin targets aequorin to the cytosol and prevents passive diffusion of the 22-kDa apoaequorin into the nucleus (24). The luciferase aequorin is also serendipitously a more stable cytosolic apoaequorin variant (27). cDNAs encoding luciferase-aequorin and nucleoplasmin-aequorin have been inserted into replication-deficient adenovirus (RAd) vectors, which allow efficient expression of the aequorin chimeras in a wide range of cell types (28). A major advantage of this method of gene transfer is that approximately 100% of the cells express recombinant protein, compared with 10–20% achieved by transfection (29), and it therefore removes the need to create stable cell lines expressing the photoprotein.

We have used the targeted Ca\(^{2+}\) indicator proteins to demonstrate that increases in [Ca\(^{2+}\)]\(_{n}\) are not reflected by an equivalent increase in [Ca\(^{2+}\)]\(_{c}\) in HeLa cells stimulated by agonist, Ca\(^{2+}\) ionophore, and the endoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor, CPA. Our results provide evidence consistent with the independent regulation of [Ca\(^{2+}\)]\(_{n}\).

**EXPERIMENTAL PROCEDURES**

**Materials**—Coelenterazine, zero Ca\(^{2+}\) calibration buffer, and Ca\(^{2+}\)/EGTA calibration buffers were purchased from Molecular Probes (Eugene, Oregon). Tissue culture reagents, ionomycin, and CPA were from Sigma (UK), and all other chemicals were of analytical reagent grade and from either Sigma or Fisons (UK).

**Immunolocalization**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml amphotericin, and 10%
fetal calf serum. The RAd vectors, luciferase-aequorin (RAdLA) and nucleoplasmin-aequorin (RAdNPA) previously developed (28) were used to infect HeLa cells at a multiplicity of infection of 100 plaque-forming units/cell. Cells were trypsinized 24 h postinfection with the appropriate RAd vector, resuspended on glass coverslips, and examined by indirect immunofluorescence as described for COS7 cells (28).

In Vitro Ca\textsuperscript{2+} Calibration of Recombinant Aequorin Variants—HeLa cells were infected with RAdLA, RAdNPA, and a RAd vector-expressing recombinant untargeted aequorin, RAdCA (28), as described above. Two days postinfection cells were washed twice with phosphate-buffered saline, scraped from the culture plates, and resuspended in lysis buffer (0.5 mM EDTA, 5 mM β-mercaptoethanol, 20 mM Tris HCl, pH 7.4). Harvested cells were subjected to three freeze-thaw cycles and centrifuged at 12,000 rpm for 5 min at room temperature. The supernatants were spin dialyzed for 1 h at 4°C with three exchanges of zero Ca\textsuperscript{2+} buffer (120 mM KCl, 1 mM MgSO\textsubscript{4}, 10 mM EGTA, 10 mM MOPS pH 7.1 at 37°C). The aequorin variants were reconstituted with 2 μM coelenterazine in zero Ca\textsuperscript{2+} buffer for 3 h on ice, and light production was measured in triplicate in Ca\textsuperscript{2+}/EGTA buffers with free Ca\textsuperscript{2+} concentrations ranging from 11.6 nM to 39.8 μM as described previously (25). Rate constants were then calculated from fractional luminescence (30).

Intracellular Ca\textsuperscript{2+} Measurements—HeLa cells were washed, detached with trypsin, and reseeded on glass coverslips 24 h after infection with the appropriate RAd vector. Following attachment, cells were incubated in medium supplemented with 5 mM sodium butyrate for 16–18 h in order to enhance expression. Recombinant aequorin was reconstituted with 2 μM coelenterazine in culture medium at 37°C for a minimum of 3 h prior to each experiment. Coverslips were loaded onto a heated chamber (37°C) and perfused with modified Krebs-Ringer Hepes buffer (containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl\textsubscript{2}, 5.5 mM glucose, 25 mM Hepes, pH 7.4, at 37°C). For experiments performed in the absence of extracellular Ca\textsuperscript{2+}, 1 mM EGTA was substituted for 1.3 mM CaCl\textsubscript{2} in the Krebs-Ringer Hepes buffer. The unconsumed aequorin was determined at the end of each experiment by exposing cells to 5 mM CaCl\textsubscript{2} in water, which causes the cells to lyse by exposure to the hypotonic solution. Cells were imaged for chemiluminescence via direct contact of the coverslip with a fiberoptic array, onto a three-stage image-intensified photon counting CCD camera. Data were analyzed using both supplied (Photek Ltd.) and custom (C. M. Rembold) software.

RESULTS

Intracellular Localization of Aequorin Variants—cDNAs encoding recombinant aequorin, luciferase-aequorin, and nucleoplasmin-aequorin were inserted into a RAd vectors which has previously been shown to infect HeLa cells efficiently (28). Indirect immunofluorescence of luciferase-aequorin (Fig. 1A) and nucleoplasmin-aequorin (Fig. 1B) in HeLa cells clearly demonstrates localization of nucleoplasmin-aequorin to the nucleus and of luciferase-aequorin to the cytosol. The level of expression in cells was variable and the proportion of cells which were immunopositive was >95%.

In Vitro Calibration of Recombinant Aequorin Variants—Calibration of the Ca\textsuperscript{2+} response in vitro was performed on expressed aequorin variants extracted from RAd vector-infected HeLa cells (Fig. 1C). In order to transform the bioluminescence data into [Ca\textsuperscript{2+}], the rate constants were determined for each variant at [Ca\textsuperscript{2+}] between 11.6 nM and 39.8 μM. The rate constants, k, were determined for each [Ca\textsuperscript{2+}] either from the slope of the line when log(counts/s) was plotted versus time or counts/s/total remaining counts (21, 25). Calibration of the recombinant aequorins (Fig. 1C) demonstrated that the addition of nucleoplasmin or luciferase to the N terminus of aequorin did not affect the Ca\textsuperscript{2+} dependent light emission in vitro when compared to unmodified recombinant aequorin.

Comparison of [Ca\textsuperscript{2+}], and [Ca\textsuperscript{2+}], in HeLa Cells Stimulated with ATP, Histamine, or CPA—HeLa cells expressing either luciferase-aequorin or nucleoplasmin-aequorin were imaged with a three microchannel plate intensified photon counting CCD camera directly with contact with a fiberoptic. The resolution allowed stimulated luminescence from individual cells to be monitored. Cells which contained large amounts of aequorin after pharmacological manipulation but before lysis were chosen for analysis. For accurate measurement of resting [Ca\textsuperscript{2+}], the bioluminescent signal from 60–100 cells on each coverslip...
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We compared the Ca\(^{2+}\) signal in response to ATP or histamine stimulation in cells expressing either nucleoplasmin-aequorin or luciferase-aequorin. Basal [Ca\(^{2+}\)] was similar in the nucleus and cytosol, with a mean value of approximately 100 nM. Stimulation with 1 \(\mu\)M ATP in the presence of extracellular Ca\(^{2+}\) resulted in similar increases in both [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) (Fig. 2A). Subsequent stimulation with a higher ATP concentration (10 and 100 \(\mu\)M) increased [Ca\(^{2+}\)] more than [Ca\(^{2+}\)]\(_n\). Increases in [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) in response to 100 \(\mu\)M histamine occurred at the same rate initially (Fig. 2B); however, the increase in [Ca\(^{2+}\)]\(_n\) stopped prior to the increase in [Ca\(^{2+}\)]\(_c\), resulting in higher sustained [Ca\(^{2+}\)]\(_n\), than [Ca\(^{2+}\)]\(_c\).

A similar pattern was observed with 100 \(\mu\)M ATP stimulation in the absence of extracellular Ca\(^{2+}\). Initially the increases in [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) were similar but were followed by a larger increase in [Ca\(^{2+}\)]\(_c\) than [Ca\(^{2+}\)]\(_n\), and the nuclear Ca\(^{2+}\) signal returned to baseline more rapidly (Fig. 2C). Subsequent stimulation with 100 \(\mu\)M histamine resulted in no change in [Ca\(^{2+}\)]\(_n\), or [Ca\(^{2+}\)]\(_c\), consistent with depletion of the InsP\(_3\)-responsive Ca\(^{2+}\) store. Restoration of extracellular Ca\(^{2+}\) after 450 s (Fig. 2C) induced a clear increase in [Ca\(^{2+}\)]\(_c\) but no significant change in [Ca\(^{2+}\)]\(_n\).

Treatment with CPA (10 \(\mu\)M) induced a large increase in [Ca\(^{2+}\)]\(_n\) that clearly preceded any increase in [Ca\(^{2+}\)]\(_c\) (Fig. 2D) by 50–60 s. The overall increase in [Ca\(^{2+}\)]\(_n\) was slower, reached a lower peak [Ca\(^{2+}\)]\(_c\), and was still increasing when the [Ca\(^{2+}\)]\(_c\) was already declining. Subsequent stimulation with 100 \(\mu\)M histamine increased [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) with a similar time course but with the maximal increase in [Ca\(^{2+}\)]\(_n\) again greater than [Ca\(^{2+}\)]\(_c\).

Ionomycin-induced Changes in [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\).—In both the presence (Fig. 3A) and absence (Fig. 3B) of extracellular Ca\(^{2+}\), 2 \(\mu\)M ionomycin induced rapid increases in [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\). The response in the presence of extracellular Ca\(^{2+}\) was larger than the response in its absence, consistent with ionomycin-induced release of intracellular Ca\(^{2+}\) stores in both and induction of a calcium release-activated current (I\(\text{CRAc}\)) in the former. The sustained increase in [Ca\(^{2+}\)]\(_n\) was significantly larger than the increase in [Ca\(^{2+}\)]\(_c\) (Fig. 3A). The relative difference between [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) was less than that observed with histamine or ATP stimulation and is demonstrated by an xy comparison of [Ca\(^{2+}\)]\(_n\) and [Ca\(^{2+}\)]\(_c\) (Fig. 3C), which shows that the slope of the relation observed with ionomycin in the presence of extracellular Ca\(^{2+}\) was significantly greater than that observed with either ATP or histamine.

**DISCUSSION**

The results reported here clearly show that HeLa cells can prevent [Ca\(^{2+}\)]\(_n\) rising to the same level as that in the cytosol. The magnitude of this effect depended on the conditions presented to these cells. When the source of the cytosolic Ca\(^{2+}\) signal was entirely extracellular, there was no detectable rise in nuclear free Ca\(^{2+}\) (Fig. 2C), which demonstrates that [Ca\(^{2+}\)]\(_n\) does not necessarily increase when a Ca\(^{2+}\) signal is generated in the cytosol. These results have important implications for the role of Ca\(^{2+}\) as a specific regulator of nuclear events through Ca\(^{2+}\)-binding proteins, such as calmodulin, calreticulin, and calpain.

Evidence suggesting a role for free Ca\(^{2+}\) in the nucleus has centered on the existence of a mechanism for generating and
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FIG. 3. Comparison of [Ca\(^{2+}\)]\(_{\text{n}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\) in Hela cells stimulated with ionomycin. Cells expressing either nuclear or cytosolic luciferase-aequorin were perfused with medium containing 2 \(\mu\)M ionomycin: A, in the presence of extracellular Ca\(^{2+}\) and B, in the absence of extracellular Ca\(^{2+}\). C, the relation between the mean nuclear and cytosolic signals from HeLa cells stimulated with 1, 10, and 100 \(\mu\)M ATP (data from Fig. 2A), 100 \(\mu\)M histamine (data from Fig. 2B), and 2 \(\mu\)M ionomycin (data from Fig. 3A). Linear regression shows the slope of the ionomycin-treated cells (short dashed line) was significantly higher than the slope of from the ATP- or histamine-treated cells (long dashed lines). The solid line is the line of identity.

responding to Ca\(^{2+}\) signals in the nucleus (3–7). The fluorescent Ca\(^{2+}\) indicator dyes have been extensively used to study nucleocyttoplasmic Ca\(^{2+}\) gradients at rest and following stimulation in a wide variety of cell types, often with conflicting results (14–20,32–34). A major problem with the dyes is in situ calibration. This is because they load differently into various intracellular organelles where the local environment is critical for indicator calibration (16,17). Unlike these previous studies, our results are not dependent on calibration methods. Addition of proteins or peptides to the amino terminus of aequorin has been shown to be an efficient means of targeting the protein to a variety of subcellular organelles without affecting the specific activity of the photoprotein (24), unlike changes to the C terminus (25). Nuclear targeting of aequorin did not alter its Ca\(^{2+}\) sensitivity in vitro (Fig. 1C), and aequorin calibration is independent of indicator concentration since estimating the rate constant is a ratiometric method. In addition, calibration of the nuclear targeted aequorin in situ demonstrated by Brini et al. (35) showed no difference from the in vitro calibration. However, we are not able to exclude the possibility that the differences in [Ca\(^{2+}\)]\(_{\text{n}}\) reported here were influenced by focal changes in cytosolic Ca\(^{2+}\). The kinetics of aequorin mean that high local changes in [Ca\(^{2+}\)] could affect the precise estimation of the mean [Ca\(^{2+}\)] (36). Although our data cannot rule out the existence of local elevations in [Ca\(^{2+}\)]\(_{\text{n}}\), the major differences in timing and magnitude of Ca\(^{2+}\) signals reported here cannot be explained entirely by such a mechanism.

The dose-response relationship for ATP and nuclear free Ca\(^{2+}\) showed that the lower nuclear Ca\(^{2+}\) relative to cytosolic was much more obvious at high doses of agonist (Fig. 2, A and B). Stimulation of HeLa cells by agonist (ATP or histamine) always increased both [Ca\(^{2+}\)]\(_{\text{n}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\). When the source of cytosolic Ca\(^{2+}\) signal was entirely through the plasma membrane there was no detectable rise in [Ca\(^{2+}\)]\(_{\text{n}}\), which is consistent with the importance of Ca\(^{2+}\) release from internal stores as the mechanism regulating [Ca\(^{2+}\)]\(_{\text{n}}\). The delay in increase and the slower rate of rise of [Ca\(^{2+}\)]\(_{\text{n}}\) when endoplasmic reticulum Ca\(^{2+}\) was released by the sarco, endoplasmic reticulum calcium ATPase inhibitor CPA (Fig. 2D), is also consistent with this hypothesis. The question therefore arises as to whether these agonist-induced differences in [Ca\(^{2+}\)]\(_{\text{n}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\) are a result of differences in the amount of Ca\(^{2+}\) released into these two compartments from the internal Ca\(^{2+}\) store or differences in Ca\(^{2+}\) buffering capacity of the relevant compartment. A direct route for Ca\(^{2+}\) into the nucleus from the ER is supported by the presence of InsP\(_{3}\) receptors on the inner membrane of the nuclear envelope (37) and the reports that InsP\(_{3}\) stimulated release of Ca\(^{2+}\) directly into the nucleus from the nuclear envelope (14,38). These conclusions are also supported by a report that depolarization-induced increases in [Ca\(^{2+}\)]\(_{\text{n}}\) were attenuated in the nucleus of neuroblastsoma cells (18). Although our results showing attenuation of the nuclear Ca\(^{2+}\) signal could be explained by mechanisms in the cytoplasm preventing Ca\(^{2+}\) reaching the nuclear membrane, the possibility also exists that the nuclear pore may have some selectivity against Ca\(^{2+}\), in spite of its large size observed microscopically. It is now well established that the nuclear pore is not freely permeable to other small molecules and ions (9,10,13,14). The correlation plot between [Ca\(^{2+}\)]\(_{\text{n}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\), show that

(36)
control of [Ca\(^{2+}\)]\(_n\) must involve at least two different mechanisms, direct release of Ca\(^{2+}\) into the nucleus and control of Ca\(^{2+}\) movement from the cytosol into the nucleus either through the buffering of Ca\(^{2+}\) by the cytoplasm, which would prevent a Ca\(^{2+}\) signal generated at the plasma membrane reaching the nuclear envelope, or regulation across the nuclear membrane. Independent regulation does require that the nucleus reaches the nuclear envelope, or regulation across the nuclear membrane.

Independent control of nuclear free Ca\(^{2+}\) mechanisms, direct release of Ca\(^{2+}\) through the buffering of Ca\(^{2+}\) ions to the nucleus generates a nuclear Ca\(^{2+}\) signal (14) clearly supports this hypothesis. Although nuclear Ca\(^{2+}\) has been extensively studied there is currently no clear consensus on its regulation and role in nuclear events. Individual variation between cell types and the signaling reported in the literature (13–19, 32–35) has been extensively studied; there is evidence in the literature to support this (18, 24, 40) and a possible intranuclear Ca\(^{2+}\) store (41). The presence of InsP\(_3\) receptors on the inner nuclear membrane (37), of nuclear InsP\(_3\) generating enzymes in the nucleus (6), the impermeability of the nuclear envelope to InsP\(_3\) (14), and the finding that injection of InsP\(_3\) into the nucleus generates a nuclear Ca\(^{2+}\) signal (14) clearly supports this hypothesis.

Although nuclear Ca\(^{2+}\) has been extensively studied there is currently no clear consensus on its regulation and role in nuclear events. Individual variation between cell types and the mechanisms regulating [Ca\(^{2+}\)]\(_n\) may explain the differences in nuclear Ca\(^{2+}\) signaling reported in the literature (13–19, 32–34). Our results highlight the value of using targeted indicators in intact cells to monitor changes in organelle [Ca\(^{2+}\)]. They also highlight the need to search for the molecular basis of independent control of nuclear free Ca\(^{2+}\), and whether this is due to a Ca\(^{2+}\) gradient involving the buffering of cytosolic Ca\(^{2+}\) signals before they reach the nuclear envelope or a gated mechanism involving the nuclear envelope.

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