Inhibition of Mammalian RNA Synthesis by the Cytoplasmic Ca^{2+} Buffer BAPTA. Analyses of [^{3}H]Uridine Incorporation and Stress-Dependent Transcription†

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ABSTRACT: To determine whether oscillations of cytoplasmic [Ca^{2+}] might be involved in transcription regulated by the unfolded protein response (UPR), dermal fibroblasts were loaded with the widely used Ca^{2+} buffer BAPTA, which is expected to dampen cytoplasmic [Ca^{2+}] changes without affecting resting [Ca^{2+}]. BAPTA inhibited UPR-dependent transcription of the GRP78/BiP and EDEM genes. However, BAPTA also blocked cytoplasmic stress-dependent (UPR-independent) transcription of the HSP70 gene. These results led to the unexpected demonstration that BAPTA was a general inhibitor of cellular RNA synthesis in dermal fibroblasts. BAPTA is delivered to the cytoplasm as the acetoxymethyl (AM) ester BAPTA/AM, but released AM groups, as well as formaldehyde generated from AM breakdown, were ruled out as causes of RNA synthesis inhibition. BAPTA inhibited RNA synthesis in all mammalian cell types tested except CHO-K1. GRP78/BiP RNA induction in CHO-K1 cells was not blocked by BAPTA. Thus, there does not appear to be a critical requirement for cytoplasmic [Ca^{2+}] changes in CHO-K1 UPR-dependent transcription. However, general inhibition of RNA synthesis by the [Ca^{2+}] buffer BAPTA was unanticipated. This might possibly reflect a fortuitous interaction of BAPTA with the RNA synthesis machinery or a requirement for [Ca^{2+}] changes.

Interference with protein folding and processing within the lumenal space of the endoplasmic reticulum (ER) in mammalian cells causes ER stress and triggers signaling events collectively known as the unfolded protein response (UPR). The UPR, in turn, acts to return the status of the ER to normal (*1*). Two UPR-specific signaling pathways, involving the ER transmembrane protein families ATF6 α/β and Ire1p α/β, generate transcription factors that target ER stress-responsive genes encoding ER chaperones, folding enzymes, and quality control factors. In response to ER stress, the ATF6 proteins translocate to the Golgi apparatus and undergo regulated intramembrane proteolysis catalyzed by the S1P/S2P protease system, resulting in soluble cytoplasmic fragments that enter the nucleus and activate transcription of target genes with ERSE promoter elements, such as GRP78/BiP (*1*). ER stress also causes dimerization of Ire1p proteins, resulting in autophosphorylation by their kinase domains and activation of their endoribonuclease domains located in the cytoplasm. The endoribonuclease substrate is the mRNA encoded by the XBP1 gene, whose translation product is a weakly functioning transcription factor. The activated endoribonuclease domain of Ire1p initiates excision of a 26-nt intervening sequence from XBP1, resulting in a frame shift. The resulting spliced form of XBP1 mRNA encodes a highly active transcription factor that targets genes controlled by the UPRE promoter element, such as EDEM, as well as genes driven by ERSE elements (*1*).

Primary dermal fibroblasts exhibit quantifiable and concentration-dependent UPRs with the ER stress agents dithiothreitol (DTT),[1] thapsigargin (TG), azetidine-2-carboxylic acid (AZC), and tunicamycin (TN), and this cell type was used to demonstrate differences in various aspects of the UPR (increased transcription of GRP78/BiP, translation attenuation, cell death, and extension of lipid-linked oligosaccharides) to different UPR inducers (*2*). Subsequently, quantitative analysis of ATF6 and Ire1p-XBP1 activation showed that the magnitudes of these signaling events caused by different stress inducers did not correlate with the extent of transcription of the GRP78/BiP and EDEM (*3*). This suggested that other factors contributed greatly to stress-dependent transcription.

Ca^{2+} is involved in many physiological processes, including those relevant to stress responses (*4*). As revealed by application of TG (*2*), an inhibitor of the SERCA pump, loss of ER Ca^{2+} can cause ER stress. However, there is no clear evidence that loss of Ca^{2+} is necessary for ER stress responses. Similarly, the potential role of cytoplasmic Ca^{2+} in the UPR is unknown. Since the active forms of the ATF6 and XBP1 transcription factors are formed in the cytoplasmic compartment, the possibility was considered that cytoplasmic Ca^{2+} might account for previously reported modulation of UPR-dependent transcription (*3*). To dampen Ca^{2+} oscilla-

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[1] Abbreviations: ADC, anthracene-1,5-dicarboxylic acid; ADC/AM, ADC diacetoxymethyl ester; AZC, azetidine-2-carboxylic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA/AM, BAPTA tetrakis(acetoxymethyl) ester; [Ca^{2+}]$_i$, internal (cytoplasmic) Ca^{2+} concentration; DIA, diamide; DTT, dithiothreitol; TG, thapsigargin; TN, tunicamycin.

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tions, without affecting resting Ca^{2+}, we employed the commonly used approach of loading cells with the Ca^{2+} buffer BAPTA. We found that BAPTA strongly attenuated UPR transcriptional responses in dermal fibroblasts. Unexpectedly, this result was traced to a general inhibition of RNA synthesis by BAPTA. This indicates that BAPTA may have an inhibitory interaction with a component of the RNA synthesis machinery or that cytoplasmic Ca^{2+} changes may be involved in RNA synthesis.

MATERIALS AND METHODS

Reagents. Formaldehyde (F1268), BAPTA/AM (A1076), Pluronic F-127 (P24443-250G), actinomycin D (A9415), uridine (U3750), L-azetidine-2-carboxylic acid (AZC) (A0760), thapsigargin (TG, T9033), dithiothreitol (DTT, D9779), diamide (D3648), and tunicamycin (TN, T7765) were from Sigma. ADC/AM (A6871) was from Molecular Probes. [5-^{3}H]Uridine (17.7 Ci/mmol) was from Sigma, and [^{32}P]-dCTP (3000 Ci/mmol) was from Amersham Biosciences.

Cell Culture. Dermal fibroblasts (ATCC CRL-1904) were grown in RPMI 1640 medium as described (*2*).

ER and Cytoplasmic Stress Treatments. ER stress was induced with 100 nM TG for 30 min, 0.4 mM DTT for 20 min, 5 μg/mL TN for 60 min, or 40 mM AZC for 60 min as described earlier (*2*). Cytoplasmic stress was induced with AZC as well as with 0.5 mM diamide [DIA (*5*, *6*)] for 20 min.

RT-PCR Analysis of XBP1 Splicing. XBP1 activation was monitored by analyzing the amounts of PCR fragments representing the unspliced and spliced forms of XBP1 message (*3*).

Northern Blot Analysis of mRNAs Encoding GRP78/BiP, EDEM, and HSP70. Total RNA was harvested, fractionated by formaldehyde−agarose gel electrophoresis, transferred to nylon membrane, and probed with ^{32}P probes for the indicated human RNAs as described (*2*, *6*). Signals were measured with a phosphorimager (Fuji) and normalized to signals for actin (*2*).

Loading of Cell Cultures with BAPTA/AM, Formaldehyde, and ADC/AM. BAPTA/AM (20−50 mM) and ADC/AM (25 mM) were dissolved in DMSO and stored at −20 °C. Pluronic F-127 solution (20% w/v in DMSO) was mixed with pure DMSO and/or one of the AM ester solutions for a concentration of 10% Pluronic. These mixtures were then added to culture media buffered to pH 7.2 with Na-HEPES and containing 10% serum to include 0.2−2% DMSO, 0.02−0.2% Pluronic (these two concentrations were the same for all cultures compared in the same experiment), and the desired amount of AM ester. Formaldehyde was handled in an analogous manner. The treated media were used to incubate cells (typically 30 min). After being washed twice with warm PBS, cells were allowed to recover (typically for 30 min) in the same medium but without AM esters, formaldehyde, Pluronic, or DMSO.

Alkaline Hydrolysis of ADC/AM. The procedure was adapted from the Molecular Probes Inc. bulletin entitled *Acetoxymethyl (AM) and Acetate Esters*. Fifty microliters of 0.8 mM ADC/AM in DMSO was mixed with 50 μL of methanol and either 25 μL of 2 M KOH or 25 μL of water. After 1 h at room temperature the pH was adjusted to approximately 7 with concentrated HCl. The sample was mixed with 200 μL of water and 800 μL of water-saturated butanol, vortexed vigorously for 10 s, and partitioned in a microcentrifuge at 13000 rpm for 15 min. Each layer was collected and adjusted to 1 mL with water (lower) or butanol (upper) as needed. The ADC (lower) or ADC/AM (upper) in each phase was estimated by absorbance at 365 nm.

Measurement of RNA Synthesis. Incorporation of [^{3}H]-uridine into total RNA was based upon established protocols (*7*, *8*). After treatments with potential RNA synthesis inhibitors as indicated, cells (approximately 4×10^5 in 60 mm wells) were washed twice with warm PBS, allowed to recover for at least 30 min in medium without inhibitors, and then labeled for 10 min after addition of 1 μCi of [^{3}H]-uridine/mL. Cells were washed three times with ice-cold PBS and lysed with 1 mL of lysis buffer (PBS containing 1% SDS and 10 mM Na_3EDTA). Ice-cold 100% TCA solution was added to lysates to a final concentration of 5% (v/v). Cell lysates were vortexed immediately and kept on ice for 30 min, and precipitates were collected by vacuum-assisted filtration on glass microfiber filter disks (Whatman, 2.4 cm). Tubes were rinsed once with 1 mL of cold PBS containing 5% TCA, which was applied to the filter disk. The filter disks were washed three times with 10 mL of ice-cold PBS containing 5% TCA, once with 10 mL of ice-cold water containing 5% TCA, and twice with 10 mL of an ethanol−ether (1:1 v/v) solution prewarmed to 37 °C. Filters were allowed to dry in air, and radioactivity was determined by immersing filters in 6 mL of scintillation fluid (Liquiscint, no. LS-121, National Diagnostics) followed by liquid scintillation counting. Radioactivity was normalized to protein in the lysates, which was determined with the Pierce BCA protein assay kit against bovine serum albumin.

RESULTS

Loading the Cytoplasm with the Ca^{2+} Chelator BAPTA Inhibits UPR-Induced Transcription of Stress-Dependent Genes but Not XBP1 Splicing. Cells were loaded with the Ca^{2+} buffer BAPTA for 30 min and then allowed to recover for 30 min in medium containing a normal Ca^{2+} concentration before application of stress. In this manner, BAPTA is not expected to strongly alter resting $[Ca^{2+}]_i$ but to clamp $[Ca^{2+}]_i$ at resting levels. BAPTA loading does not appear to prevent replenishment of ER Ca^{2+} stores (*9*). To load BAPTA into cultured cells, it is applied as BAPTA/AM, a membrane-permeable acetoxymethyl (AM) ester (*10*, *11*). BAPTA/AM is unable to chelate Ca^{2+}, but cleavage of BAPTA/AM by cytoplasmic esterases releases and activates free BAPTA, which is membrane impermeable and is trapped in the cytoplasm.

Splicing of XBP1 by Ire1p and proteolytic activation of ATF6 were monitored (*3*). As shown in Figure 1, panel a, and as reported earlier for primary neuronal cells (*12*), BAPTA/AM treatment itself resulted in appreciable splicing of XBP1. The basis for this property of BAPTA/AM is not known. BAPTA/AM had no appreciable effect on XBP1 splicing induced by 100 nM TG or 2 mM DTT, but as shown earlier (*3*) the degree of splicing by these agents was near the maximum attainable. In preliminary experiments (data not shown) BAPTA/AM alone had no discernible effect on ATF6 cleavage, but activation of ATF6 by ER stress was inhibited by BAPTA/AM, suggesting that BAPTA interfered

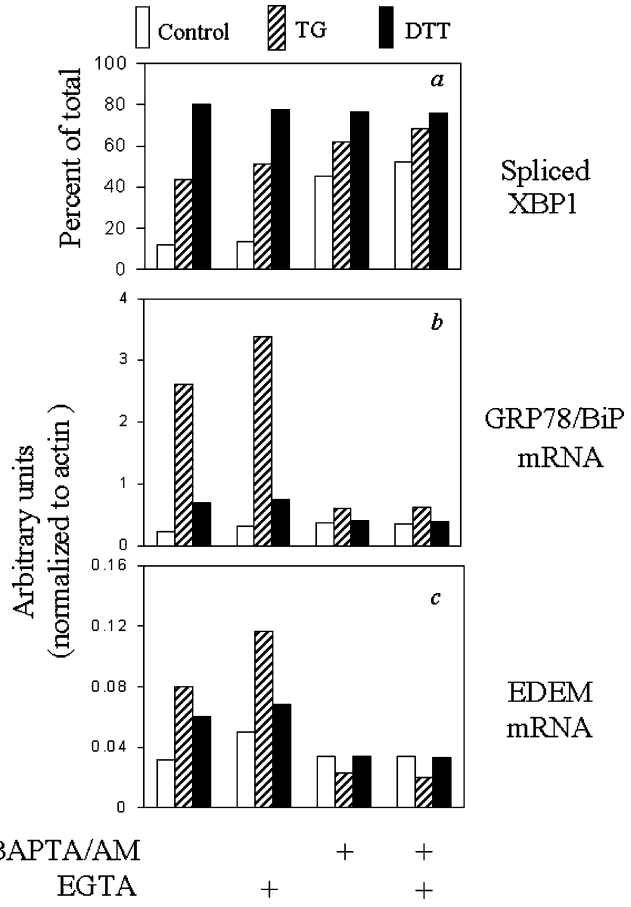

FIGURE 1: Inhibition of GRP78/BiP and EDEM gene expression, but not XBP1 splicing, by BAPTA/AM. Dermal fibroblasts were treated for a 35 min period with or without 2.5 mM EGTA in the medium in the absence or presence of 50 μM BAPTA/AM for the final 30 min, as indicated. This was followed by a 30 min period without BAPTA/AM or EGTA. After this 65 min loading protocol, cells were left unstressed for 30 min (white bars), stressed with 100 nM TG for 30 min (crosshatched bars), or left unstressed for 10 min followed by stress with 2 mM DTT for 20 min (black bars). RNA was then harvested to determine percentage splicing of XBP1 (panel a). Alternatively, 5 h after the beginning of the stress treatments RNA was harvested to determine the fold increases of GRP78/BiP (panel b) or EDEM (panel c) mRNA.

with either the initial activation of ATF6, its transport to the Golgi apparatus, or proteolytic cleavage. Thus, BAPTA promoted XBP1 splicing but inhibited ATF6 cleavage.

The EDEM gene has a UPRE promoter element responsive to XBP1, while the GRP78/BiP gene has an ERSE promoter element responsive to both XBP1 and ATF6 (*1*, *13*). Thus, BAPTA might be expected to stimulate transcription of both EDEM and GRP78/BiP. However, BAPTA-induced XBP1 splicing was accompanied by little or no increase of EDEM mRNA and perhaps a slight increase for GRP78/BiP mRNA (Figure 1, panels b and c). Curiously, BAPTA strongly inhibited TG, DTT (Figure 1, panels b and c), TN, and AZC (Figure 2, panels a, b, d, and e) dependent transcription of the GRP78/BiP and EDEM genes, even though there was abundant splicing of XBP1. This suggested that a step after splicing of XBP1 was sensitive to BAPTA.

The effective concentration range of BAPTA/AM for inhibition of GRP78/BiP transcription (12−50 μM; data not shown) was typical for this reagent compared with other studies (*12*, *14*, *15*). Similar effects on XBP1 splicing, and GRP78/BiP and EDEM transcription, were observed in the absence or presence of sufficient EGTA in the medium to chelate all available extracellular Ca^{2+} during BAPTA/AM loading (Figure 1). Since external Ca^{2+} was restored during and after UPR induction, these findings suggest that clamping $[Ca^{2+}]_i$ at resting levels during ER stress may have prevented GRP78/BiP and EDEM transcription.

The Effect of BAPTA/AM on Stress-Dependent Transcription Was Not Mimicked with Formaldehyde. Other than chelation of Ca^{2+} (*11*), we are unaware of any significant interactions of BAPTA with cellular components. However, the AM groups released from BAPTA/AM spontaneously break down to acetic acid and formaldehyde (*10*). Since the resulting formaldehyde concentrations are expected to be well below those needed to cause tissue fixation (for example, approximately 0.2 mM formaldehyde results from 0.05 mM BAPTA/AM, compared with 330 mM formaldehyde in 1% fixative solutions) and formaldehyde is highly membrane permeable, it is generally held that any formaldehyde resulting from breakdown of the AM group rapidly diffuses out of the cell and does not significantly interfere with cellular function (*10*). However, formaldehyde delivered by 0.05 mM Benz2/AM esters into erythrocytes strongly inhibited ATP production (*16*). On a molar basis, the Benz2/AM ester used was approximately 4-fold as potent as pure formaldehyde, consistent with the release of four AM groups/mol of Benz2/AM ester.

Since formaldehyde readily reacts with amino groups of proteins, it is a potential cause of misfolding and other protein defects, and side effects of the AM ester were a particular concern. The possibility that formaldehyde derived from BAPTA/AM might have interfered with UPR-dependent transcription of GRP78/BiP and EDEM was addressed in two ways. First, the ability of formaldehyde to mimic the effects of BAPTA/AM was tested. Second, the AM ester of 1,5-dicarboxylic anthracene (ADC) (*17*), i.e., ADC/AM, which should have similar membrane permeability and esterase sensitivity as BAPTA/AM, was used. ADC does not significantly bind Ca^{2+} or any other physiological ion.

The effects of formaldehyde on stress-dependent transcription of GRP78/BiP and EDEM are presented in Figure 2. As shown above, 50 μM BAPTA/AM strongly suppressed UPR-dependent transcription of GRP78/BiP and EDEM (panels d and e). As pointed out above (Figure 1), a slight increase of GRP78/BiP mRNA was detected with BAPTA/AM treatment alone (Figure 2, panel d) and was repeatable. In contrast, 200 μM formaldehyde had no appreciable effect on TG-dependent GRP78/BiP or EDEM transcription (panels g and h), indicating that the formaldehyde potentially released from BAPTA/AM was inconsequential. Compared with BAPTA/AM, a 20-fold higher (in terms of AM group equivalents) concentration of formaldehyde (4 mM) did block UPR-dependent transcription (panels j and k). However, as presented below, 50 μM BAPTA/AM did not produce an equivalent amount of formaldehyde (Table 1).

No single concentration of formaldehyde replicated the effects of 50 μM BAPTA/AM, which caused mild GRP78/BiP mRNA transcription by itself, suppressed the robust effect of AZC on GRP78/BiP mRNA (Figure 3, panel a), and caused 48% of XBP1 mRNA to be spliced (panel b). High concentrations of formaldehyde did not appreciably increase GRP78/BiP transcription, although 4 mM formal-

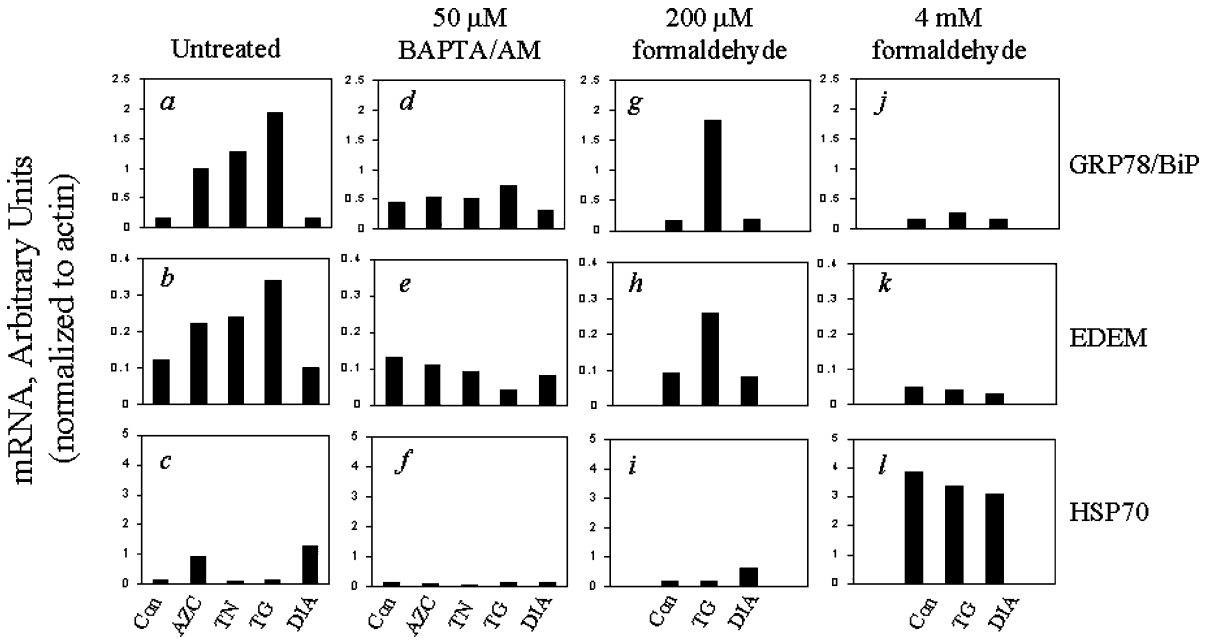

FIGURE 2: Effects of BAPTA/AM on target gene activation by stress is not replicated with formaldehyde. Dermal fibroblasts were treated with medium containing 0.02% Pluronic F-127 alone (panels a−c), 50 μM BAPTA/AM (panels d−f), 200 μM formaldehyde (panels g−i), or 4 mM formaldehyde (panels j−l). After a 30 min recovery period, cells were left unstressed (Con) or stressed with 40 mM AZC for 60 min, 5 μg/mL TN for 60 min, 100 nM TG for 30 min, or 0.5 mM DIA for 20 min. Five hours after the beginning of the stress treatments RNA was harvested, and the amounts of mRNAs for GRP78/BiP (panels a, d, g, and j), EDEM (b, e, h, and k), and HSP70 (c, f, i, and l) were determined after normalization to actin mRNA.

Table 1: Summary of Effects of AM Esters and Formaldehyde on Stress-Dependent mRNA Synthesis

criterion	50 μM BAPTA/AM	50 μM ADC/AM	200 μM formaldehyde	4 mM formaldehyde
stimulation of XBP1 splicing	Y	N	N	Y
stimulation of GRP78/BiP mRNA synthesis	Y	N	N	N[a]
stimulation of EDEM mRNA synthesis	Y	Y	Y	N[a]
inhibition of ER stress-dependent mRNA synthesis (GRP78/BiP; EDEM)	Y	N	N	Y
stimulation of HSP70 mRNA synthesis	N	N	N	Y
inhibition of cytoplasmic stress-dependent mRNA synthesis (HSP70)	Y	N	N	N[b]
inhibition of total RNA synthesis	Y	N	N	Y

[a] Any ability to stimulate is most likely masked by inhibition. [b] Ability to detect inhibition possibly obscured by strong stimulatory effect.

dehyde caused 17% XBP-1 splicing (panel b) and 1 mM formaldehyde strongly suppressed GRP78/BiP transcription induced by AZC (panel a). Formaldehyde of different sources (Sigma, Sargent-Welch, and Scientific Products) and different lengths of storage at room temperature (from recently purchased to 25 years at room temperature) had equivalent suppression of AZC-induced GRP78/BiP transcription (data not shown). Thus, suppression was likely due to formaldehyde itself rather than a byproduct formed during storage.

The Effects of BAPTA/AM Are Not Replicated with ADC/AM. To independently evaluate the potential contribution of the AM groups released from BAPTA/AM, we tested an irrelevant ester, ADC/AM. The only known relevant activity of ADC is binding to Cs^+ (*17*). Yet, ADC/AM would be expected to introduce formaldehyde into cells in a manner similar to BAPTA/AM. Since the AM groups are subject to hydrolysis during storage and dissolution, the intactness of ADC/AM stocks (to assess the portion that might have broken down to ADC) was verified by demonstrating increased polarity after deliberate conversion to ADC by alkaline hydrolysis and partitioning between water and butanol. A total of $4 \pm 2\%$ ($n = 4$) of the material partitioned into water without hydrolysis, compared with $84 \pm 1\%$ ($n = 5$) after hydrolysis, indicating the ADC/AM was largely intact in our stock solutions.

As shown in Figure 4, BAPTA/AM strongly suppressed AZC-induced transcription of GRP78/BiP and EDEM (panels a and b), but ADC/AM (panels d and e) had no effect, arguing against inhibition of transcription by released formaldehyde. ADC/AM also failed to cause XBP1 splicing (Figure 3, panel b). Slight increases in the levels of EDEM mRNA (but not GRP78/BiP mRNA) were noticed with 6.25−50 μM ADC/AM alone (Figure 4, panel e) and 0.2−1 mM formaldehyde alone (data not shown). Therefore, it appears that we detected a mild effect of formaldehyde generated by AM esters on EDEM mRNA. With 6.25 μM BAPTA/AM a similar effect on basal EDEM was detected (Figure 4, panel b), with little or no effect on GRP78/BiP (panel a), consistent with a possible effect of formaldehyde. However, this increase was erased at higher concentrations, presumably due to the suppressive effect of BAPTA/AM on stress-induced transcription.

BAPTA/AM Suppresses Cytoplasmic Stress-Dependent Transcription of HSP70. Although the activity of BAPTA/AM was not due to released AM groups, it remained to be determined whether BAPTA selectively affected UPR-

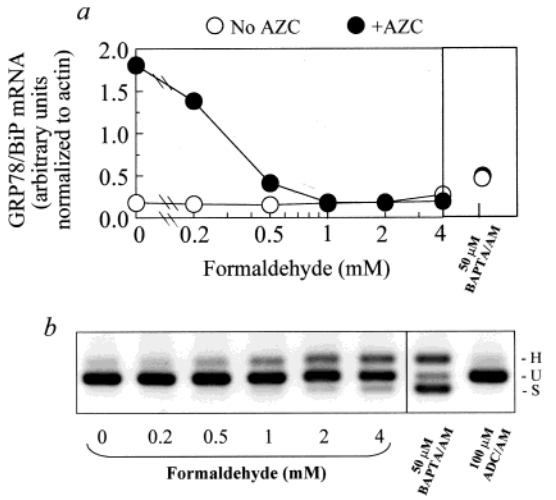

FIGURE 3: Differential effects of BAPTA/AM, ADC/AM, and formaldehyde on GRP78/BiP mRNA transcription and XBP1 splicing. Cells were treated with BAPTA/AM, formaldehyde, and AZC as described for Figure 2 but with 0.08% Pluronic F-127. ADC/AM (100 μM) was loaded exactly as done for BAPTA/AM. RNA was harvested, and GPR78/BiP mRNA (panel a) and XBP1 splicing (panel b) were assessed as for Figure 2. H, U, and S refer to the hybrid, unspliced, and spliced XBP1 PCR products (*3*).

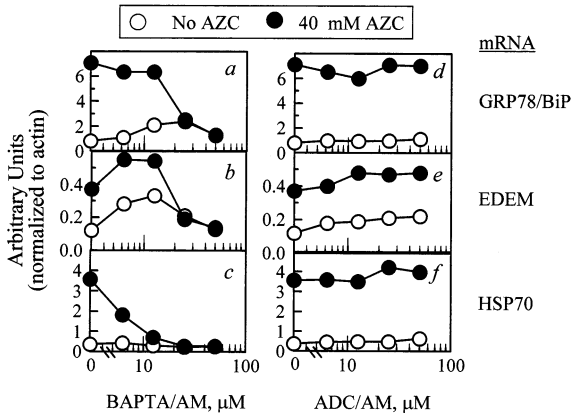

FIGURE 4: Inhibition of stress-induced gene expression by BAPTA/AM is not mimicked by ADC/AM. Cells were loaded with different concentrations of BAPTA/AM (panels a−c) or ADC/AM (panels d−f), and mRNAs for GRP78/BiP (panels a and d), EDEM (panels b and e), or HSP70 (panels c and f) were determined with (black symbols) or without (white symbols) AZC-induced stress as for Figure 2.

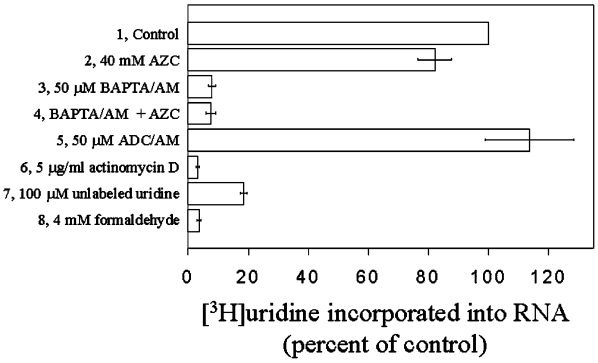

FIGURE 5: BAPTA/AM is a general inhibitor of RNA synthesis. As indicated, cells were left untreated (bars 1, 6, and 7), stressed with 40 mM AZC (bars 2 and 4), loaded with 50 μM BAPTA/AM (bars 3 and 4), loaded with 50 μM ADC/AM (bar 5), or treated with 4 mM formaldehyde (bar 8) as described for Figure 2. Two hours and 20 min after completion of loading, some cells were incubated for 10 min with 5 μg/mL actinomycin D (bar 6). Two hours and 30 min after loading, all cells were incubated for 10 min with [^{3}H]uridine in the absence (bars 1−6 and 8) or presence of 5 μg/mL actinomycin D (bar 6) or 100 μM unlabeled uridine (bar 7).

dependent transcription. HSP70 mRNA is induced by cytoplasmic stress in a manner unrelated to the UPR (*6*). HSP70 mRNA was examined after treatment with diamide, a sulfhydryl oxidant (*5*) that causes robust cytoplasmic stress but no ER stress, and after treatment with AZC, which causes cytoplasmic stress in addition to ER stress (*6*). Unexpectedly, 50 μM BAPTA/AM strongly inhibited the HSP70 responses of diamide and AZC (Figure 2, panels c and f). These results were not obtained with ADC/AM (Figure 4, panels c and f) or 0.2 mM formaldehyde (Figure 2, panel i). Formaldehyde (4 mM) (panel l) itself caused cytoplasmic stress, resulting in robust stimulation of HSP70 transcription, but 50 μM BAPTA/AM itself (panel f) did not. Thus, 50 μM BAPTA/AM did not generate an amount of cellular formaldehyde equivalent to that resulting from exogenous 4 mM formaldehyde. Even though these two treatments inhibited UPR-dependent transcription (Figure 2, panels d, e, j, and k), they did so for different reasons.

BAPTA/AM Is a General Inhibitor of RNA Synthesis in Many Mammalian Cell Types. Since UPR-dependent transcription and cytoplasmic stress-dependent transcription are regulated by different mechanisms, but both responses require RNA synthesis, the previous results raised the possibility that BAPTA/AM might be a general inhibitor of RNA synthesis. Cellular RNA was labeled with 1 μCi/mL [^{3}H]uridine for 10 min after various treatments. As shown in Figure 5, incorporation of label into a cold TCA-insoluble fraction was specific for RNA (bar 1) because it was inhibited greater than 95% by 5 μg/mL actinomycin D (bar 6) and was diminished by 80% with 0.1 mM unlabeled uridine (bar 7).

Little or no effect on RNA synthesis was seen with 50 μM ADC/AM (bar 5), 0.2 mM formaldehyde (not shown), or 40 mM AZC (bar 2). Formaldehyde (4 mM) inhibited RNA synthesis by 95% (bar 8). This was somewhat unexpected since 4 mM formaldehyde also strongly induced HSP70 transcription (Figure 2, panel l). It is possible that the inhibitory effect of 4 mM formaldehyde was offset by a strong cytoplasmic stress response or that the formaldehyde selectively inhibited transport or utilization of the [^{3}H]uridine while failing to inhibit RNA synthesis from endogenous NTPs. The basis for this apparent discrepancy was not determined.

Surprisingly, 50 μM BAPTA/AM inhibited RNA synthesis by 90% by itself (bar 3) or in combination with 40 mM AZC (bar 4). In these experiments [^{3}H]RNA synthesis was measured 2.5 h after BAPTA loading, whereas a 5.5 h delay was used to measure stress-induced mRNA synthesis (Figures 1−4). RNA synthesis was inhibited about 90% for the first 4 h after completion of BAPTA loading and was still strongly inhibited at 5 h (Figure 6, panel a). Twenty minutes of loading (panel b) and 25 μM BAPTA/AM (panel c) were sufficient to inhibit RNA synthesis by 90%. BAPTA/AM (6−12 μM; that is, 24−48 μM AM) was sufficient to inhibit RNA synthesis by half. Similar inhibition required 500 μM formaldehyde (panel d). Although ADC/AM contains two AM groups while BAPTA/AM has four, inhibition of RNA synthesis was negligible with 200 μM ADC/AM (panel e).

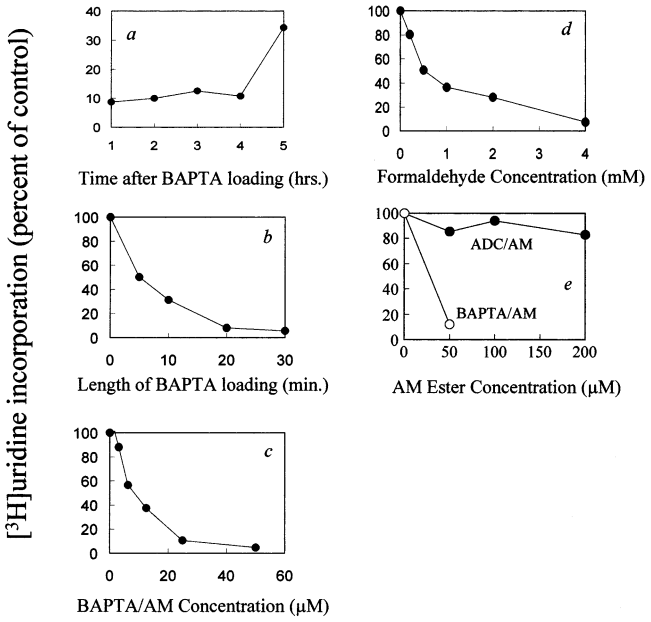

FIGURE 6: Variables affecting inhibition of RNA synthesis by BAPTA/AM. Treatments were as described for Figure 2, and RNA synthesis was measured as for Figure 5. The standard condition was loading of 50 μM BAPTA/AM for 30 min and recovery for 2 h without BAPTA/AM prior to measurement of RNA synthesis. The variables tested were length of recovery period (panel a), length of the loading period (panel b), concentration of BAPTA/AM (panel c), replacement of BAPTA/AM with formaldehyde (panel d), and replacement of BAPTA/AM (white symbols) with ADC/AM (black symbols) (panel e).

In addition to dermal fibroblasts (approximately 90% inhibition), other cell types were also sensitive to inhibition of RNA synthesis by 50 μM BAPTA/AM: MDBK, 70% inhibition; mouse embryonic fibroblasts, RAW 246.7, and HeLa, 50% inhibition; COS-1, 30% inhibition. Higher concentrations of BAPTA/AM did not appreciably increase inhibition. We determined that the saturation point for BAPTA/AM in RPMI 1640 medium with 10% FCS was approximately 20 μM, suggesting that inhibition of RNA synthesis might be limited by the availability of soluble BAPTA/AM. Consistent with this idea, we tested the aforementioned cells by loading for 90 min instead of 30 min, and in all cases RNA synthesis inhibition by 50 μM BAPTA/AM was reproducibly increased (data not shown). In all cell types tested, RNA synthesis was inhibited by at least 95% by 5 μg/mL actinomycin D.

BAPTA Does Not Inhibit Total or UPR-Dependent RNA Synthesis in CHO-K1 Cells. Curiously, even though they were fully sensitive to actinomycin D, CHO-K1 cells did not respond to BAPTA/AM as did the other cells. BAPTA/AM up to 200 μM did not inhibit RNA synthesis. BAPTA/AM (12−25 μM) actually increased incorporation of [^{3}H]-uridine by about 50%, and longer times for loading of 50 μM BAPTA/AM also increased [^{3}H]uridine incorporation (data not shown). Although the basis for these unusual observations was not determined, these data showed that CHO-K1 cells could be successfully loaded with BAPTA but without inhibiting RNA synthesis. This offered an opportunity to determine whether BAPTA loading affected UPR-dependent transcription, the original goal of the study. Loading with 50 μM BAPTA/AM itself had no appreciable effect on GRP78/BiP mRNA expression in CHO-K1 cells (1.1 ± 0.06-fold compared with untreated controls). ER stress with 40 mM AZC had a significant effect on GRP78/BiP mRNA (2.5 ± 0.09-fold), though less robust than that typically observed in dermal fibroblasts. Interestingly, GRP78/BiP mRNA still increased substantially (2.2 ± 0.04-fold) when the AZC treatment was preceded by BAPTA loading. Thus, BAPTA/AM did not appear to block UPR-dependent transcription in CHO-K1 cells.

DISCUSSION

In an effort to determine whether changes in $[Ca^{2+}]_i$ might be important for the UPR, the Ca^{2+} buffering reagent BAPTA was unexpectedly found to prevent stress-dependent transcription of GRP78/BiP, EDEM, and HSP70 by acting as a general inhibitor of RNA synthesis. BAPTA also activated Ire1p-mediated splicing of XBP1 mRNA, as reported previously (*12*). Neither stimulation of XBP1 splicing nor inhibition of RNA synthesis by BAPTA could be attributed to the released AM ester group or formaldehyde resulting from its breakdown.

To our knowledge general inhibition of RNA synthesis by BAPTA, or a possible role for $[Ca^{2+}]_i$ changes in RNA synthesis, has not been reported earlier. However, there have been indications that BAPTA can interfere with UPR-dependent transcription. In one case, the TG-dependent increase in transcription of GRP78/BiP was fully eliminated. However, this involved an extensive 9 h treatment with BAPTA/AM with no recovery period (*14*). In another study, expression of a reporter gene containing the promoter of the calreticulin gene, which is known to respond to ER stress, was inhibited by about 50% after a 30 min loading period with BAPTA/AM followed by recovery for 16 h (*15*). In both studies, interpretation of the effect on the stress response was complicated by the use of TG as the stress inducer. TG blocks the SERCA protein, which pumps Ca^{2+} from the cytoplasm into the ER, and thus plays a critical role in cellular Ca^{2+} homeostasis at rest and after stimulation. In other words, in these studies BAPTA might have altered the ability of TG to deplete ER Ca^{2+} [especially since the pool of BAPTA-bound Ca^{2+}, by itself, can be adequate to replenish the ER with Ca^{2+} (*9*)], in which case any inhibition of transcription would be secondary to attenuation of ER stress. ER stress agents that do not directly affect Ca^{2+} homeostasis were not evaluated, and the possible effects of the AM ester were not considered. In the present study BAPTA blocked the GRP78/BiP and EDEM transcriptional responses of DTT, TN, and AZC (agents that are not known to directly affect Ca^{2+} homeostasis), in addition to TG, and the potential artifact of AM-derived formaldehyde was addressed with formaldehyde itself and an irrelevant AM ester, ADC/AM.

These data suggest the possibility that $[Ca^{2+}]_i$ changes may be involved in RNA synthesis. As examples, this could involve maintenance of NTP pools, formation of transcription complexes, or catalysis by RNA polymerases. Consistent with this, 12−50 μM BAPTA/AM, which was necessary for RNA synthesis inhibition, is also the range needed for effective Ca^{2+} buffering. However, at this time the possibility cannot be excluded that BAPTA might fortuitously interact with a required enzyme or cofactor, independent of its chelation properties. As examples, it is plausible that BAPTA

might interfere with RNA polymerase or an enzyme involved in synthesis of ribonucleotide triphosphates. If so, BAPTA may prove to be a useful reagent for transcriptional analysis.

In summary, BAPTA is a potent inhibitor of cellular RNA synthesis in many mammalian cell types, suggesting a potential role for $[Ca^{2+}]_i$ changes in transcription or a novel inhibitory interaction. This is not due to generation of formaldehyde from the AM ester. However, CHO-K1 RNA synthesis was not sensitive to BAPTA, and in these cells there was no indication that UPR-dependent RNA synthesis involved $[Ca^{2+}]_i$ changes.

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