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Anirban Roy

William F. Wonderlin

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The Permeability of the Endoplasmic Reticulum Is Dynamically Coupled to Protein Synthesis*

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Anirban Roy and William F. Wonderlin‡
From the Department of Biochemistry and Molecular Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506

Proteins synthesized by the rough endoplasmic reticulum (RER) co-translationally cross the membrane through the pore of a ribosome-bound translocon (RBT) complex. Although this pore is also permeable to small molecules, it is generally thought that barriers to their permeation prevent the cyclical process of protein translation from affecting the permeability of the RER. We tested this hypothesis by culturing Chinese hamster ovary-S cells with inhibitors of protein translation that affect the occupancy of RBTs by nascent proteins and then permeabilizing the plasma membrane and measuring the permeability of the RBT to a small molecule, 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG). The premature or normal release of nascent proteins by puromycin or pactamycin, respectively, increased the permeability of the RBT 4-MUG by 20–30%. In contrast, inhibition of elongation and the release of nascent proteins by cycloheximide did not increase the permeability, but it prevented the increase in permeability by pactamycin. We conclude that the permeability of the RER is coupled to protein translation by a simple gating mechanism whereby a nascent protein blocks the pore of an RBT during translation, but after release of the nascent protein the pore is permeable to small molecules as long as an empty ribosome remains bound to the translocon.

In eukaryotes, secretory and most integral membrane proteins are synthesized by a translationally active 80 S ribosome composed of 60 S and 40 S subunits and bound to a translocon complex, a heteromeric assembly of proteins embedded in the membrane of the rough endoplasmic reticulum (RER) (Fig. 1). Nascent proteins emerging from the exit tunnel of the 60 S subunit co-translationally cross the membrane of the RER by passing through a protein-conducting channel (PCC) in the translocon complex (1). The exit tunnel of the 60 S subunit and the pore of the PCC must be large enough to be permeated by a nascent protein chain, and this pathway is therefore large enough to be permeated by many other small molecules when a ribosome-bound translocon (RBT) is translationally inactive and empty, i.e. the pore is not occupied by a nascent protein. We previously demonstrated (2) that the permeability of RBTs to a small, polar molecule (4-methyl-umbelliferyl-α-D-glucopyranoside (4-MUG)) was increased after puromycin, a tRNA analog, prematurely terminated translation and released nascent protein chains from the PCC (path a, Fig 1). The increased permeability to 4-MUG was consistent with a previous report that RBTs incorporated into planar bilayers and opened by puromycin are permeable to ions (3), and our results were also supported by a recent report that puromycin can release calcium from the RER (4). The permeation of empty RBTs is especially significant in the context of recent studies by Nicchitta and colleagues (5, 6), who reported that approximately two-thirds of the 60 S subunits remain bound to translocons after the normal completion of protein translation, thereby constituting a large pool of persistent, translationally inactive RBT complexes. Although permeation of these empty RBTs could have very important consequences for RER-dependent signaling and the maintenance of essential gradients across the RER membrane, this pathway has received relatively little attention.

The permeation of translocons by small molecules has also been investigated by Johnson and colleagues (7, 8), who concluded that a permeability barrier is maintained at the luminal end of the translocon by the binding of BiP, a luminal chaperone protein, and that the junction between the 60 S subunit and the translocon is tightly sealed. Their conclusions have led to the broadly held view that RBTs are tightly sealed at all times, preventing the process of protein translation from influencing the permeability of the RER to small molecules. We propose, however, that the notion that the permeability of the RER is not affected by permeation of RBTs might be limited to experimental conditions in which translationally inactive, empty ribosomes have been stripped from ER microsomes by a high-salt wash. This experimental detail is important because it limits the detection of permeation to only two states of the translocon, the translationally active 80 S-bound translocon and the ribosome-free translocon (blocked and closed states, respectively, in Fig. 1). A novel feature of our current study is that we have used experimental conditions in which permeation of a third state of the translocon, translationally inactive 60 S-bound RBTs, can also be detected.

In this article we examine two models that describe the relationship between the translational activity of RBTs and the permeability of the RER to a small molecule, 4-MUG. The first model is a “static permeability” model in which RBTs are never permeable to small molecules other than nascent proteins, and the permeability of the RER is therefore independent of translational activity. The second model is a “dynamic permeability” model in which the pore of an RBT is “gated” closed when it is...
occupied by a nascent protein, but the pore of translationally inactive, 60 S-bound translocans is open and permeable to small molecules. This simple physical mechanism for coupling the permeability of the RER to protein translation predicts a dynamic relationship in which increasing the level of protein translation and the occupancy of RBTs by nascent proteins should decrease the permeability of the RER, whereas inhibiting the initiation of protein translation and decreasing the occupancy of RBTs by nascent proteins should increase the permeability of the RER.

The appropriateness of the static versus dynamic permeability models can be tested by determining the extent to which the permeability of the RER is dependent on the level of protein translation and the occupancy of RBTs by nascent proteins. The static permeability model predicts complete independence, whereas the dynamic permeability model predicts that the permeability of the RER is inversely related to the level of occupancy by nascent proteins during protein translation. The goal of the current study was to test these predictions by measuring the permeability of the RER to 4-MeG while manipulating protein translation with drugs that inhibit protein translation by different mechanisms of action. Puromycin (PUR) resembles aminocycl-tRNA, and it is a substrate for the peptidyltransferase of the 60S subunit, accepting a nascent peptide and causing the premature release of incomplete nascent proteins (9). Pactamycin (PAC) inhibits the initiation of protein translation by preventing formation of the 43S initiation complex, but it permits the normal elongation and release of full-length proteins whose synthesis was initiated prior to addition of the drug (10, 11). Cycloheximide blocks elongation and prevents the normal completion and release of nascent proteins from RBTs (12). Cycloheximide stabilizes polyribosomes and the association of 80S ribosomes with translocans, whereas PUR and PAC elicit rapid breakdown of polyribosomes, but leave the majority of translationally inactive 60S subunits bound to the RER (5). We report that the permeability of the RER was sensitive to the manipulation of protein translation and changes in the occupancy of RBTs by nascent proteins, and we conclude that the permeability of the RER is dynamically coupled to protein translation.

**EXPERIMENTAL PROCEDURES**

**Materials**—4-MeG, cycloheximide, and puromycin-HCl were from Calbiochem. Pactamycin was a generous gift from The Upjohn Co. All other reagents were from Sigma.

**Cell Culture**—CHO-S cells (Invitrogen), a cell line derived from Chinese hamster ovary K1 cells and selected for growth in suspension, was used for most experiments. A cell line derived from HEK-293 cells for growth in suspension (HEK-293-F) was also obtained from Invitrogen. HepG2 and BHK-21 cells were obtained from the ATCC (Manassas, VA). CHO-K1 cells were a gift from Ed Levitan (University of Pittsburgh). CHO-S and HEK-293-F cells were grown in serum-free medium (CHO-SFII and 293SFII medium, respectively, from Invitrogen). CHO-K1 cells were grown in Ham’s F12 medium containing 5% serum, and BHK-21 and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% serum. All cells were cultured at 37 °C, 8% CO₂.

**4MeG Assay**—The 4-MeG assay was performed as described by Heritage and Wonderlin (2). Briefly, CHO-S cells and HEK-293-F cells were typically grown to a density of 0.5–1.0 × 10⁶ cells/ml in T-75 flasks or in spinner flasks. For a 32-well assay (eight conditions in quadruplicate), 20 ml of cells was pelleted and resuspended in 20 ml of K-G buffer (140 mM potassium glutamate, 2.5 mM MgCl₂, 10 mM HEPES, pH 7.25) and gently permeabilized by N₂ cavitation (2 min, 80 p.s.i.). This procedure was modified for adherent cells. BHK-21 and CHO-K1 cells were grown in T-75 flasks, harvested by scraping, and then homogenized in K-G buffer by N₂ cavitation. HepG2 cells were grown to confluence in 48-well plates, and the medium was replaced with K-G buffer containing 0.08% digitonin (100 µg/ml) for 5 min to permeabilize the cells and then diluted 1:5 with K-G buffer. Nunc 48-well plates were loaded with 0.5 ml of sample/well, the plate with solutions was prewarmed to 35 °C, and 4-MeG (20 µM from a 20 mM stock in methanol) was added immediately before placing the plate into a CytoFluor 4000 plate reader. The fluorescence was measured for 30 min at 2-min intervals with 10 s mixing before each measurement. The center wavelengths/bandwidths of the excitation and emission filters were 360/40 and 460/40, respectively.

**Data Analysis**—In CHO-S cells, 4-MeG is activated when it enters the lumen of the RER and it is hydrolyzed by α-glucosidase II. The slope of the fluorescence versus time curve, S₀, is proportional to the rate of entry and activation of the dye at time t. The linear and exponential contributions to S₀ were fitted by Equation 1,

\[ S₀ = S₀,e^{kt} \]  

Equation 1

where \( S₀ \) is the initial slope (ΔF/min) and \( k \) is an exponential rate constant (min⁻¹). Best-fit estimates of \( S₀ \) and \( k \) for each well were obtained using the Solver nonlinear curve-fitting routine in Excel (Microsoft). All experimental conditions were assayed in parallel ± detergent (0.05% Igepal, Fig. 2A) so that the permeability to 4-MeG under a specific experimental condition could be expressed as a dimensionless fractional permeability (P),

\[ P = S₀/S₀,max \]  

Equation 2

where \( S₀,max \) is the rate of activation after the RER membrane was permeabilized by detergent and the access of 4-MeG to the α-glucosidase II was maximal. Statistical analysis was performed using JMP®in (SAS Institute). Averages are plotted ± standard error of the mean.

**Calcium Measurements**—CHO-S cells were loaded with 10 µM Fluo-3-AM (30 min, 23 °C) and then washed twice in Hanks’ balanced salt solution. Fluorescence emission at 510 nm was measured with excitation at 485 nm in a CytoFluor 4000 plate reader. Adherent BHK-21 cells grown in 10% Dulbecco’s modified Eagle’s medium (8% CO₂, 37 °C) were released by trypsinization and gentle trituration and then loaded with 10 µM Mag-Fura-2-AM (60 min, 37 °C) in Dulbecco’s modified Eagle’s medium minus serum. The cells were washed twice in Hanks’ balanced salt solution and loaded into a stirred cuvette in a SFXE Fluorolog spectrofluorometer. The ratio of fluorescence measured at 510 nm with excitation alternating between 340 and 380 nm was calculated at each time point without further calibration.

**RESULTS**

**Rationale for Testing the Permeability Models**—We have studied the permeation of RBTs in CHO-S cells by 4-methyl-umbelliferyl-α-D-glucopyranoside (4-MeG), a small, membrane-impermeant probe; it’s entry into the RER can be detected when it is hydrolyzed to a fluorescent product by luminal α-glucosidase II. We previously reported (2) that the activation of 4-MeG in intact CHO-S cells was negligible, but the selective rupture of the plasma membrane by N₂ cavitation enabled 4-MeG to access the RER, and it revealed a substantial basal permeability of the RER to 4-MeG (Pₜₐₜₜ). This permeability was further increased to a level termed \( P_{\text{PUR}} \) when nascent
measured in CHO-S cells to other cell lines, (2) the specificity of $\Delta P_{\text{PUR}}$ as a measure of the occupancy of translationally active RBTs by nascent chains, and (3) the contribution of RBT-mediated permeation to $P_{\text{basal}}$.

**Similarities in Permeability ($P$) among Different Cell Lines**—We examined the activation of 4-MeG in the absence and presence of PUR in CHO-S, CHO-K1, HEK-293-F, BHK-21, and HepG2 cells. We observed in all of these cell lines a substantial $P_{\text{basal}}$ and a similar 30–40% increase in $P$ when puromycin was added to the assay (Fig. 2B). The similarity between CHO-S and HepG2 cells is especially notable, because Seiser and Nicchitta (5) reported that approximately two-thirds of the ribosomes remain bound to the RER membrane in HepG2 cells following either the premature termination of translation by PUR or the normal completion of translation with pactamycin. We conclude from these similarities that the mechanisms that regulate the permeation of RBTs are probably also similar among these cell lines and that the high $P_{\text{basal}}$ and the $\Delta P_{\text{PUR}}$ are not unusual features of CHO-S cells. These results support our use of CHO-S cells in the present study as a model system for investigating the permeability of the RER.

**$\Delta P_{\text{PUR}}$ Is a Measure of the Pool of Translationally Active RBTs**—We previously concluded (2) that $\Delta P_{\text{PUR}}$ was produced by the release of nascent chains from translationally active RBTs on the basis of the generally recognized mechanism of action of puromycin as a tRNA analog (9), the similar sensitivity to high salt of $\Delta P_{\text{PUR}}$ and the binding of ribosomes to ER membranes (13), and the absence of $\Delta P_{\text{PUR}}$ in the presence of detergent, which demonstrated that $\Delta P_{\text{PUR}}$ required the entry of 4-MeG into a membrane-bound compartment. In the current study we wanted to use the size of $\Delta P_{\text{PUR}}$ to track changes in the size of the pool of translationally active RBTs (i.e., those RBTs occupied by nascent proteins that could be released by PUR) in response to treatments with inhibitors of protein synthesis. The puromycin reaction catalyzed by the peptidyltransferase is essential for the release of nascent proteins, and we performed the following additional experiments to provide a more rigorous test of the specific dependence of $\Delta P_{\text{PUR}}$ on the puromycin reaction.

Anisomycin is an inhibitor of the peptidyltransferase and the puromycin reaction (14). When added simultaneously with PUR, 50 $\mu$M anisomycin completely inhibited $\Delta P_{\text{PUR}}$, but it did not inhibit $\Delta P_{\text{PUR}}$ when it was added 10 min after PUR, sufficient time for the irreversible release of nascent chains by PUR (Fig. 3A). The failure of anisomycin to significantly inhibit $\Delta P_{\text{PUR}}$ when added after PUR demonstrated that the ability of anisomycin to inhibit $\Delta P_{\text{PUR}}$ when applied with PUR did not result from nonspecific effects, such as blocking the pore of empty RBTs. We conclude from the complete inhibition of $\Delta P_{\text{PUR}}$ when anisomycin was applied with PUR that the production of $\Delta P_{\text{PUR}}$ required an anisomycin-sensitive peptidyltransferase reaction.

The puromycin reaction is maximum at pH 8.5–9.5, with a $pK_a$ of 7.2–7.6 and a steep decrease in rate at neutral pH and below (15, 16). We observed that $\Delta P_{\text{PUR}}$ was inhibited when the buffer was acidified from pH 7.2 to pH 6.6 before the addition of PUR, but acidification to pH 6.6 15 min after the addition of PUR had no effect (Fig. 3B). The temporal dependence of the inhibition by low pH leads us to conclude, again, that the inhibitory effect was on the puromycin reaction and not the permeability pathway.

We conclude that the production of $\Delta P_{\text{PUR}}$ is entirely dependent on the puromycin reaction catalyzed by the peptidyltransferase center, and it is therefore a valid measure of the release of nascent proteins from translationally active RBTs. The measurement of $\Delta P_{\text{PUR}}$ provides an alternative approach for...
The Contribution of RBTs to $P_{\text{basal}}$—We have observed over a 2-year period that $P_{\text{basal}}$ can slowly change in CHO-S cells over the course of several weeks or months, with $P_{\text{basal}}$ ranging from 0.2 to 0.7. This variability led us to speculate that the entry of 4-MoG via pathways other than RBTs might contribute a labile background component to $P_{\text{basal}}$ and cause us to underestimate the importance of changes in $P_{\text{basal}}$ mediated specifically by permeation of RBTs. In the absence of drugs that can specifically inhibit the permeation of RBTs, we have estimated the component of $P_{\text{basal}}$ contributed by the permeation of RBTs, relative to the background permeability contributed by other, unidentified pathways, by examining the sensitivity of $P_{\text{basal}}$ to high salt, which has been reported to release up to 85% of the translationally inactive ribosomes from the RER membrane in rat hepatocytes (path c, Fig. 1) (13). Although high salt might also inhibit RBT-independent pathways for the entry of 4-MoG, we have demonstrated previously (2) that both the concentration of salt producing half-maximal block and the slope of the salt concentration-inhibition curve were identical for $\Delta P_{\text{PUR}}$ and the salt-sensitive component of $P_{\text{basal}}$. We surmised from the remarkably similar sensitivities to high salt that both salt-sensitive components were produced by the entry of 4-MoG through a common pathway. Cells were split into two samples, with one sample broken open in high-salt (300 mM K) K-G buffer and the other sample broken open in normal (140 mM K) K-G buffer. A plot of $P_{\text{basal}}$ measured in high-salt versus normal buffer is shown in Fig. 3C. The dotted line with unity slope indicates the relationship expected if $P_{\text{basal}}$ was not affected by high salt, and it is clear that $P_{\text{basal}}$ measured in high salt was depressed relative to $P_{\text{basal}}$ measured in normal salt, consistent with our previous report that $P_{\text{basal}}$ was sensitive to high salt (2). Most importantly, a regression line fitted to these data had a slope of 0.94 ($\pm$0.10), insignificantly different from a value of 1. We conclude from the parallel downward shift of the relationship that $P_{\text{basal}}$ was decreased on average by $\approx$0.2 in high-salt relative to normal-salt buffers regardless of the size of $P_{\text{basal}}$ in normal salt. The data in Fig. 3C also indicate that the apparent lower limit of $P_{\text{basal}}$ is 0.2, from which we can conclude that in some samples of cells 4-MoG enters the RER exclusively through empty RBTs. These data indicate that the size of the salt-sensitive permeability to 4-MoG was surprisingly constant compared with the high variability of the salt-insensitive component.

Effect of Puromycin—The primary test of the static permeability model was to determine whether pretreating cells with PUR prior to permeabilization could reversibly increase $P_{\text{basal}}$. CHO-S cells were pretreated with 200 $\mu$M PUR for 15 min to terminate translation and release nascent proteins from RBTs, after which half of the cells were permeabilized and assayed. Pretreatment with PUR significantly increased $P_{\text{basal}}$ while substantially reducing $\Delta P_{\text{PUR}}$ (Fig. 4A), as expected if all of the available nascent proteins were released from translationally active RBTs during the pretreatment period. If the increase in $P_{\text{basal}}$ resulted from the release of nascent proteins and the opening of RBTs, it should be reversed by restarting translation and increasing the occupancy of RBTs by nascent proteins. The remaining control and PUR-treated cells were pelleted, washed, and transferred to control medium for 30 min, at which time $P_{\text{basal}}$ was not significantly different from the control level (Fig. 4A), demonstrating that restarting protein synthesis could reverse the increase in $P_{\text{basal}}$.

Effect of Pactamycin—To ensure that the increase in $P_{\text{basal}}$ produced by PUR was not an artifact of the premature release of truncated proteins by PUR, we also examined pactamycin, which should release full-length proteins. A 1-h pretreatment with PAC significantly increased $P_{\text{basal}}$ and eliminated $\Delta P_{\text{PUR}}$. 

determining the size of the pool of translationally active RBTs that is much simpler than other methods (e.g., radiolabeling nascent proteins). It is also insensitive to translationally active ribosomes in the cytosol, because only the release of nascent proteins from translationally active ribosomes that are bound to the RER can increase the activation of 4-MoG. Although $\Delta P_{\text{PUR}}$ provides only a relative measure of the translational activity of RBTs, that was sufficient for our current study in which we wanted to use $\Delta P_{\text{PUR}}$ to track changes in the size of the pool of translationally active RBTs in response to inhibitors of protein synthesis.
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FIG. 4. Pretreatment with PUR, PAC, and CHX differentially affects P. Gray and black bars represent $P_{\text{basal}}$ and $P_{\text{PUR}}$, respectively. Asterisks indicate a significant ($p < 0.05$, Dunnet's test) increase in $P_{\text{basal}}$ relative to the control $P_{\text{basal}}$, which is also marked by a dotted line. A. pretreatment with PUR reversibly increased $P_{\text{basal}}$ and reduced $\Delta P_{\text{PUR}}$. Identical assays were performed with cells incubated in 200 mM PUR for 15 min (left set of bars) or following a 30-min washout (right set of bars) ($n = 6$). B. a 1-h incubation of CHO-S cells in 200 mM PAC increased $P_{\text{basal}}$, and eliminated $\Delta P_{\text{PUR}}$ when cells were broken open in 140 mM K-G buffer (left set of bars, $n = 7$), but the increase in $P_{\text{basal}}$ was absent when cells were broken open in 300 mM K-G buffer (right set of bars, $n = 4$). C. a 1-h incubation in 50 mM CHX did not increase $P_{\text{basal}}$ (left set of bars, $n = 5$), in contrast to the significant increase produced by PAC. When PAC and CHX were combined (right set of bars, $n = 4$), the inhibitory effect of CHX was dominant.

(Fig. 4B), as observed with PUR pretreatment. Similar, significant increases in $P_{\text{basal}}$ were observed following pretreatments with PAC for 5, 30, 60, or 360 min, indicating that the increase in $P_{\text{basal}}$ was both rapid and sustained (data not shown).

The increase in $P_{\text{basal}}$ was eliminated when control and PACTreated cells were permeabilized in a buffer in which the potassium glutamate concentration was increased to 300 mM (Fig. 4B), which supports a role for empty RBTs because translationally inactive ribosomes can be stripped from the RER by a high-salt buffer (13). Also, unlike PUR, the acute application of PAC to permeabilized control cells did not increase $P_{\text{basal}}$ (data not shown), which is consistent with the ability of PUR to release nascent proteins directly versus the PAC requirement that synthesis be completed and nascent proteins be released prior to permeabilization.

The similar increase in $P_{\text{basal}}$ produced by pretreating intact cells with either PAC or PUR can be accounted for by the conversion of blocked RBTs to open RBTs. The increase in $P_{\text{basal}}$ was balanced entirely by the loss of $\Delta P_{\text{PUR}}$, resulting in no net increase in $P_{\text{PUR}}$ (Fig. 4, A and B), which was expected if the increase in $P_{\text{basal}}$ depended solely on the conversion of blocked RBTs to open RBTs along path a in Fig. 1. Also, the increase in $P_{\text{basal}}$ produced by both PUR and PAC was about 70% of the $\Delta P_{\text{PUR}}$ measured in control cells (Fig. 4, A and B, where $\Delta P_{\text{PUR}}$ reflects the size of the translationally active pool in control cells), which was remarkably consistent with the previous report of a persistent binding of ~65% of 60 S subunits after the release of nascent proteins (5).

Effect of Cycloheximide—In contrast to PUR and PAC, a 1-h pretreatment with 200 mM cycloheximide (CHX) did not increase $P_{\text{basal}}$ (Fig. 4C). A similar lack of effect was observed with 5- or 360-min pretreatments (data not shown), indicating that the different effects of PAC and CHX were not limited to a specific point in time. The failure of CHX to increase $P_{\text{basal}}$ demonstrated that the increased $P_{\text{basal}}$ produced by PUR or PAC was not a nonspecific consequence of inhibiting protein translation (e.g. clearing of proteins from the lumen of the RER). The fact that PAC, but not PUR, requires elongation for the release of nascent chains led us to predict that CHX would prevent the increase in $P_{\text{basal}}$ by PAC, but CHX should have much less of an effect on the increase in $P_{\text{basal}}$ by PUR. Cotreatment with CHX completely prevented PAC from increasing $P_{\text{basal}}$ (Fig. 4C), whereas CHX decreased $\Delta P_{\text{PUR}}$ by only 20–30% (data not shown).

PUR-dependent Release of Calcium in Intact Cells—We also examined changes in the permeability of the ER to Ca$^{2+}$ to determine whether changes in the permeability to 4-MoG could be generalized to Ca$^{2+}$ and as a control to ensure that changes in the permeability of the ER could be observed in intact cells in which no cytosolic constituents that might regulate the permeability of RBTs could be lost, in contrast to the 4-MoG assay that requires permeabilization of the plasma membrane for the entry of 4-MoG. intact CHO-S cells were loaded with the Ca$^{2+}$ indicator Fluo-3 and treated in parallel with PUR and thapsigargin (TG), a selective inhibitor of the Ca-ATPase in the ER (17), with the response to TG providing a positive control for a Ca$^{2+}$ signal produced by the release of Ca$^{2+}$ from the ER. Thapsigargin (100 nM) and PUR produced similar, triphasic changes in [Ca$^{2+}$] (Fig. 5, A and B), both beginning with a transient increase. The increase in [Ca$^{2+}$] elicited by PUR was not a nonspecific effect of inhibiting protein synthesis, because it was observed in cells in which protein synthesis was already inhibited by pretreatment with CHX (Fig. 5A). We also used Mag-Fura-2 to monitor more specifically changes in the permeability of Ca$^{2+}$. BHK-21 cells were loaded with Mag-Fura-2 under conditions in which the [Ca$^{2+}$] in the lumen of the ER could be preferentially monitored (18). Although we could not detect changes in luminal [Ca$^{2+}$] with the addition of PUR to intact BHK-21 cells (data not shown), PUR significantly increased the rate of release of Ca$^{2+}$ from the ER in intact cells when re-uptake was inhibited by TG (Fig. 5C), evidence that the permeability to Ca$^{2+}$ was increased. Our observation that PUR increased the permeability of the RER to calcium in intact cells is consistent with a recent report that PUR depleted calcium in the RER of dialyzed or permeabilized cells (4), and our observations in intact cells demonstrated that changes in the permeability to 4-MoG produced by PUR did not result from the loss of potential regulatory factors following permeabilization of the plasma membrane.

DISCUSSION

We conclude from the experiments reported above that the permeability of the RER can be dynamically coupled to the level of protein translation by changes in the number of empty (i.e. open) versus blocked RBTs. Dynamic coupling could be produced by a simple mechanism requiring only the following features: (1) the pore of the PCC and the exit tunnel of the 60 S subunit are blocked by a nascent protein when the RBT is...
translocation; (2) following the normal completion of translation and the release of a nascent protein, some empty ribosomes remain persistently bound to translocons; (3) the pore of empty RBTs is permeable to small molecules; and (4) the relative distribution of blocked versus empty translocons is influenced by the level of protein translation. The apparent high barrier to the permeation of translocons by small molecules, as described by Johnson and colleagues (Ref. 7; also reviewed in Ref. 8), is probably limited to experimental conditions in which permeable, translationally inactive polysomes (e.g. Fig. 174 in Ref. 20). However, our results are more consistent with a study by Adelman et al. (13) in which ~40% of the ribosomes could be released from rat liver microsomes with salt alone, and an additional 40–45% could be released with the combination of high salt and puromycin. These authors concluded that the ribosomes released by high salt in the absence of puromycin were mostly translationally inactive, empty ribosomes, and their results would support a ratio of empty to occupied RBTs of ~1:1. Our results are also consistent with a high permeability to small solutes observed in rough microsomes prepared from rat liver without treatment with high salt (19). Resolution of this issue will require parallel measurements of permeability and ultrastructure in the same cells.

A high resting permeability to small molecules mediated by empty RBTs also raises the question as to how the RER might maintain essential gradients across its membrane or different environments (e.g. oxidizing versus reducing) in the lumen versus the cytosol. Although the Ca-ATPase in the ER membrane could maintain a calcium gradient in the presence of a leak pathway, it is less clear which transporters might maintain gradients for other molecules of interest. Of course, any speculation as to the potential effect of empty RBTs on the permeability of the RER to small molecules must be tempered by the fact that thus far we have only demonstrated permeation by 4-MoG and calcium, and further study will be required to determine the repertoire of small molecules that can permeate empty RBTs.

The macroscopic changes in $P_{\text{basal}}$ described above might be especially important during global changes in protein translation, such as when the initiation of protein synthesis is inhibited in response to cellular stress (21). In this context, the increase in $P_{\text{basal}}$ produced by PAC provides a new clue into the mechanism of stress-induced apoptosis. Many cytosolic and ER stressors inhibit the initiation of protein translation by activating the phosphorylation of the translation initiation factor eIF-2α (21), an effect mimicked by PAC. If inhibiting the initiation of translation increases the permeability of the RER, as we observed with PAC, then RER-dependent homeostatic processes might be disrupted, thereby promoting apoptosis. The

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**Fig. 5.** PUR increased the permeability to calcium. A. CHO-S cells were loaded with Fluor-3 and PUR or TG (100 nM) was added at time zero. A single representative experiment is shown with cells pretreated for 30 min with 50 μM CHX to inhibit protein synthesis prior to the addition of PUR. B, averaged responses in five experiments performed as described in A and plotted with standard error bars. For TG, the latency to the decline from the peak to the nadir was highly variable. C, decay of ER luminal Ca²⁺ in BHK-21 cells loaded with Mag-Fura-2. 100 nM TG alone or TG and 200 μM PUR were added at time zero. The decay of luminal [Ca²⁺] was fitted with a single exponential function and plotted with the fitted line on a semi-log plot. The inset shows a significant decrease (p < 0.05, t test, n = 6) in the average time constant for the decay with PUR (black) and without PUR (gray).
ability of cycloheximide to stabilize nascent chains within RBTs and prevent PAC from increasing \( P_{\text{basal}} \) might explain why CHX can also prevent stress-induced apoptosis under conditions in which protein synthesis is already inhibited, such as during ischemia/reperfusion (22); and the opposing actions of CHX and PUR on \( P_{\text{basal}} \) might account for the paradoxical, pro-apoptotic effects of PUR in situations where CHX is protective (23, 24). From a methodological perspective, experimental manipulations that stress cells and inhibit the initiation of protein synthesis (e.g. removing extracellular calcium) could have large effects on the permeability of the RER.

Dynamic fluctuations in \( P_{\text{basal}} \) might also occur at a microscopic level as protein synthesis within local regions of the RER undergoes its normal cyclical course. For example, the permeation of empty RBTs by calcium or other small molecules following the release of a completed protein could produce a diffusible signal coupled to the end of translation, perhaps playing a role in stimulating the disassembly and reassembly of a translationally active RBT with successive cycles of protein translation. Molecules entering the lumen through an empty RBT might also signal its availability for the retrograde export of misfolded proteins through the pore of the RBT (25). At a microscopic level, the opening of RBTs might produce nearly quantal changes in \( P_{\text{basal}} \) compared with the macroscopic changes in \( P_{\text{basal}} \) averaged over larger dimensions. For example, the opening of an RBT could produce a rapid and large change in the local concentrations of molecules within the lumen of the RER, given the very small volume of the lumen and the potentially large flux of molecules through an empty RBT.

In conclusion, the permeability of the RER to small molecules should be viewed as a dynamic property influenced by protein translation. We propose that dynamic coupling could occur in any cells in which empty ribosomes remain bound to translocons following the completion of translation. Recognition of the dynamic nature of this relationship provides new insight into the generation of novel signals and the potential loss of homeostatic regulation under pathophysiological conditions.

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REFERENCES

1. Beckmann, R., Spahn, C. M., Eswar, N., Helmers, J., Penczek, P. A., Sali, A., Frank, J., and Blobel, G. (2001) Cell 107, 361–372
2. Heritage, D., and Wonderlin, W. F. (2001) J. Biol. Chem. 276, 22655–22662
3. Simon, S. M., and Blobel, G. (1991) Cell 65, 371–380
4. Lemax, R. B., Camello, C., Van Copenolle, F., Petersen, O. H., and Tepikin, A. V. (2002) J. Biol. Chem. 277, 26479–26485
5. Seiser, R. M., and Nicchitta, C. V. (2000) J. Biol. Chem. 275, 33820–33827
6. Poter, M. D., and Nicchitta, C. V. (2002) J. Biol. Chem. 277, 23314–23320
7. Hamman, B. D., Hendershot, L. M., and Johnson, A. E. (1998) Cell 92, 747–758
8. Johnson, A. E., and van Waes, M. A. (1999) Annu. Rev. Cell Dev. Biol. 15, 799–842
9. Pestka, S. (1974) Methods Enzymol. 30, 261–282
10. Lodish, H. G., Housman, D., and Jacobsen, M. (1971) Biochemistry 10, 2348–2356
11. Stewart-Blair, M. L., Yanowitz, I. S., and Goldberg, I. H. (1971) Biochemistry 10, 4198–4206
12. Baliga, B. S., Pronczuk, A. W., and Munro, H. N. (1969) J. Biol. Chem. 244, 4480–4489
13. Adelman, M. R., Sabatini, D. D., and Blobel, G. (1973) J. Cell Biol. 56, 206–229
14. Ioannou, M., Coutsogeorgopoulos, C., and Synetos, D. (1998) Mol. Pharmacol. 53, 1089–1096
15. Pestka, S. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 624–628
16. Maden, B. E., and Monro, R. E. (1968) Eur. J. Biochem. 6, 309–316
17. Lytton, J., Westlin, M., and Hanley, M. R. (1991) J. Biol. Chem. 266, 17067–17071
18. Hofer, A. M., Landolfi, B., Debellis, L., Pozzan, T., and Curi, S. (1998) EMBO J. 17, 1986–1995
19. Meissner, G., and Allen, R. (1981) J. Biol. Chem. 256, 6413–6422
20. Pawson, D. W. (1981) The Cell, 2nd Ed., p. 321, W. B. Saunders Company, Philadelphia
21. Brostrom, C. O., and Brostrom, M. A. (1998) Prog. Nucleic Acids Res. Mol. Biol. 58, 79–125
22. Musat-Maruc, S., Gunter, H. E., Jugdutt, B. I., and Docherty, J. C. (1999) J. Mol. Cell Cardiol. 31, 1073–1082
23. Chow, S. C., Peters, L., and Orrenius, S. (1995) Exp. Cell Res. 216, 149–159
24. Rainey, L. D., Hendershot, L. M., and Bondurant, M. C. (1992) J. Cell. Physiol. 151, 487–496
25. Romisch, K. (1999) J. Cell Sci. 112, 4185–4191