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Basal and Physiological Ca^{2+} Leak from the Endoplasmic Reticulum of Pancreatic Acinar Cells

SECOND MESSENGER–ACTIVATED CHANNELS AND TRANSLOCONS*

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We have studied the Ca^{2+} leak pathways in the endoplasmic reticulum of pancreatic acinar cells by directly measuring Ca^{2+} in the endoplasmic reticulum ($[Ca^{2+}]_{ER}$). Cytosolic Ca^{2+} ($[Ca^{2+}]_C$) was clamped to the resting level by a BAPTA-Ca^{2+} mixture. Administration of cholecystokinin within the physiological concentration range caused a graded decrease of $[Ca^{2+}]_{ER}$, and the rate of Ca^{2+} release generated by 10 pM cholecystokinin is at least 3× as fast as the basal Ca^{2+} leak revealed by inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Acetylcholine also evokes a dose-dependent decrease of $[Ca^{2+}]_{ER}$, with an EC_{50} of 0.98 ± 0.06 μM. Inhibition of receptors for inositol 1,4,5-trisphosphate (IP_3) by heparin or flunarizine blocks the effect of acetylcholine but only partly blocks the effect of cholecystokinin. 8-NH_2 cyclic ADP-ribose (20 μM) inhibits the action of cholecystokinin, but not of acetylcholine. The basal Ca^{2+} leak from the endoplasmic reticulum is not blocked by antagonists of the IP_3 receptor, the ryanodine receptor, or the receptor for nicotinic acid adenine dinucleotide phosphate. However, treatment with puromycin (0.1–1 mM) to remove nascent polypeptides from ribosomes increases Ca^{2+} leak from the endoplasmic reticulum by a mechanism independent of the endoplasmic reticulum Ca^{2+} pumps and of the receptors for IP_3 or ryanodine.

Many physiological and pharmacological responses rely on the ability of intracellular messengers to generate cytosolic Ca^{2+} signals by releasing Ca^{2+} from the endoplasmic reticulum (ER)[1] (1–4). Thus, in the pancreatic acinar cell the neurotransmitter acetylcholine (ACh) stimulates the release of Ca^{2+} from the ER via inositol 1,4,5-trisphosphate (IP_3), while the hormone cholecystokinin (CCK) evokes Ca^{2+} release by a complex interaction between the messengers IP_3, cyclic ADP-ribose, and nicotinic acid adenine dinucleotide phosphate (NAADP) (5). Although it is well established that low concentrations of these agonists generate long lasting trains of different forms of cytosolic Ca^{2+} oscillation (5, 6), there are few data on the kinetics of Ca^{2+} in the ER lumen ($[Ca^{2+}]_{ER}$), particularly during the action of physiological concentrations of agonists.

After agonist-induced depletion, the Ca^{2+} content of the ER is replenished by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, but even in the resting state there is a basal leak of Ca^{2+} from the lumen of ER, which can be revealed by the inhibition of SERCA with thapsigargin or cyclopiazonic acid (CPA) (7, 8). Although the molecular nature of the basal leak is still unclear, in permeabilized hepatocytes the temperature dependence and kinetics of the leak suggest that it occurs through a channel (9). Missiaen *et al*. (10) found that in A7r5 smooth muscle cells the leak rate was fitted by a two-exponential decay.

In skeletal and cardiac muscle, sarcoplasmic reticulum basal Ca^{2+} efflux may occur through the ryanodine receptor (11, 12), and in non-muscle cells it has been suggested that basal Ca^{2+} leak from the ER reflects the flow of Ca^{2+} through the IP_3 receptor induced by the action of resting levels of IP_3 (13, 14). However in a study in baby hamster kidney fibroblasts, Hofer *et al*. (7) clearly demonstrated that the leak was not blocked by either the IP_3 receptor antagonist heparin or the ryanodine receptor antagonist ruthenium red.

In contrast to the hypothesis that the basal Ca^{2+} leak occurs through second messenger-activated Ca^{2+} channels in the ER membrane, it has been suggested that the leak could occur through the translocon pore complex in the ER membrane (8). Recent studies have suggested that the empty pore of the translocon complex is permeable to small ions and neutral molecules (15–17). Experimentally, the permeability of the translocon can be modified by puromycin (16, 17), an adenosine derivative that purges the translocon of nascent polypeptides, creating an empty pore (18, 19), and we have used this tool to investigate for the first time the permeability of the translocon pore to Ca^{2+}.

In this study we have investigated the basal and agonist-evoked pathways of Ca^{2+} leak by directly measuring the depletion of $[Ca^{2+}]_{ER}$ in isolated pancreatic acinar cells. Moreover, to avoid the profound regulatory effects of cytosolic Ca^{2+} ($[Ca^{2+}]_C$) on Ca^{2+} release channels (20), $[Ca^{2+}]$ in the solution bathing the ER was "clamped" at a quasi-resting level (~90 nM) by dialyzing the cell with a mixture of calcium and the Ca^{2+} chelator BAPTA. This provides a particularly sensitive method for studying very slow Ca^{2+} fluxes.

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[1] The abbreviations used are: ER, endoplasmic reticulum; $[Ca^{2+}]_{ER}$, intraluminal Ca^{2+}; $[Ca^{2+}]_C$, cytosolic Ca^{2+}; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetate; CCK, cholecystokinin; ACh, acetylcholine; IP_3, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; Iono, ionomycin; CPA, cyclopiazonic acid; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

Using this experimental approach we have set out to study the following: (i) the depletion of $[Ca^{2+}]_{ER}$ by physiological doses of CCK and ACh, (ii) the relative magnitudes of the basal Ca^{2+} leak and the Ca^{2+} leak stimulated by physiological doses of agonists, (iii) the contribution of IP_3 and ryanodine receptors to agonist-evoked and basal Ca^{2+} leak, and (iv) the effect on $[Ca^{2+}]_{ER}$ on the dissociation of nascent polypeptide chains from the ribosome with puromycin.

EXPERIMENTAL PROCEDURES

Cell Preparation and Solutions—Mouse pancreatic acinar cells were isolated by digestion with purified collagenase (200 units ml^{-1}, Worthington Biomedical Corp., Lakewood NJ) as described previously (8). Freshly isolated cells were incubated with 5 μM fura-2FF/AM (Teflabs, Austin TX) or 5 μM mag-fura-2/AM (Molecular Probes Europe BV, Leiden, The Netherlands) and pluronic F-127 (0.025%) for 30–45 min at 37 °C. Cells were attached to poly(L-lysine)-coated cover slips, installed in a flow chamber and placed on the stage of a Nikon Diaphot inverted fluorescence microscope (Nikon Ltd., Kingston, UK). Imaging experiments were performed at room temperature (20–22 °C). Extracellular solutions contained (in mM): NaCl, 140; KCl, 4.7; $MgCl_2$, 1.13; $CaCl_2$, 1; glucose, 10; and HEPES-NaOH, 10 (pH 7.2) and were perfused rapidly under gravity using electronic valves (Lee Products Ltd., Gerrard's Cross, Bucks, UK). Cells were alternatively illuminated by 340 and 380 nm light from a monochromator using a ×40, 1.3 NA objective lens, and emission light with a wavelength longer than 400 nm was collected. Images from a CCD camera (Photonic Sciences, Beaconsfield, UK) were digitized, averaged, and analyzed using a Quanticell 700 m imaging system from Visitech International (Sunderland, UK). In order to remove fura-2FF or mag-fura-2 from the cytosol and control the composition of the intracellular medium, imaging experiments were performed either with patch-clamped cells after dialysis of the cytosol (21) or with streptolysin O-treated cells after permeabilization of the plasma membrane. Experiments with streptolysin O-permeabilized cells allowed the composition of the solution bathing the ER to be changed rapidly (7, 22).

Patch Clamping and Dialysis of Single Acinar Cells—Single cells were dialyzed with intracellular solution using the whole-cell patch clamp configuration (21) with microelectrodes of resistance 2–5 MΩ made from borosilicate glass capillaries (GC150TF-7.5, Harvard Apparatus Ltd., Kent, UK) and an EPC-8 amplifier (Heka elektronik, Lambrecht/Pfalz, Germany). The intracellular solution contained (in mM): KCl, 120; NaCl, 20; HEPES-KOH, 10; ATP, 2; $MgCl_2$, 1.13; BAPTA, 10; $CaCl_2$, 2 (pH 7.2), so that free cytosolic $[Ca^{2+}]$ was clamped at ~90 nM (8).

Permeabilization of Acinar Cells—Cells attached to cover slips were perfused with intracellular medium containing 0.5 units/ml reduced streptolysin O (Corgenix UK, Peterborough, UK) for 30 s at room temperature (7, 22). Experiments were carried out in medium with an ionic composition identical to the patch-pipette intracellular solution.

Measurement of $[Ca^{2+}]_{ER}$—Averaged measurements of the ratio of fluorescence at 340 nm to fluorescence at 380 nm were made at 5-s intervals. In experiments with low, physiological doses of CCK, analysis was performed only in the basolateral, non-nuclear area of the acinar cell, where the density of ER is highest (23, 24); this area also has a lower density of other organelles and is less susceptible to movement artifacts. $[Ca^{2+}]_{ER}$ was estimated from ratio measurements by an *in situ* cell calibration (25) using R_{min} values obtained with 10 μM ionomycin (Iono) and 10 mM EGTA, and R_{max} values with 10 μM ionomycin and 20 mM $CaCl_2$. The K_d for fura-2FF-free acid was found to be 31.5 μM by a cell-free calibration using Ca^{2+}/EGTA buffers (Molecular Probes Europe BV).

Chemicals and Statistics—All chemicals were obtained from Sigma-Aldrich Co. Ltd. unless otherwise stated. All quantitative data refer to fluorescence ratios of fura-2FF–loaded cells and are presented as means ± S.E. of the mean. Statistical comparisons were by paired or unpaired two-tailed Student's *t* test, and analysis of sigmoid curves was performed with Microcal Origin using a Boltzmann fit.

RESULTS

Following permeabilization of the plasma membrane or whole-cell dialysis of dye-loaded pancreatic acinar cells, the ratio of fluorescence at 340 to 380 nm rose as cytosolic dye was washed out (8). In 51 fura-FF-loaded patch-clamped cells, the mean ratio in the basolateral non-nuclear area after dialysis was 0.63 ± 0.03, corresponding to a mean apparent $[Ca^{2+}]_{ER}$ of 80 μM.

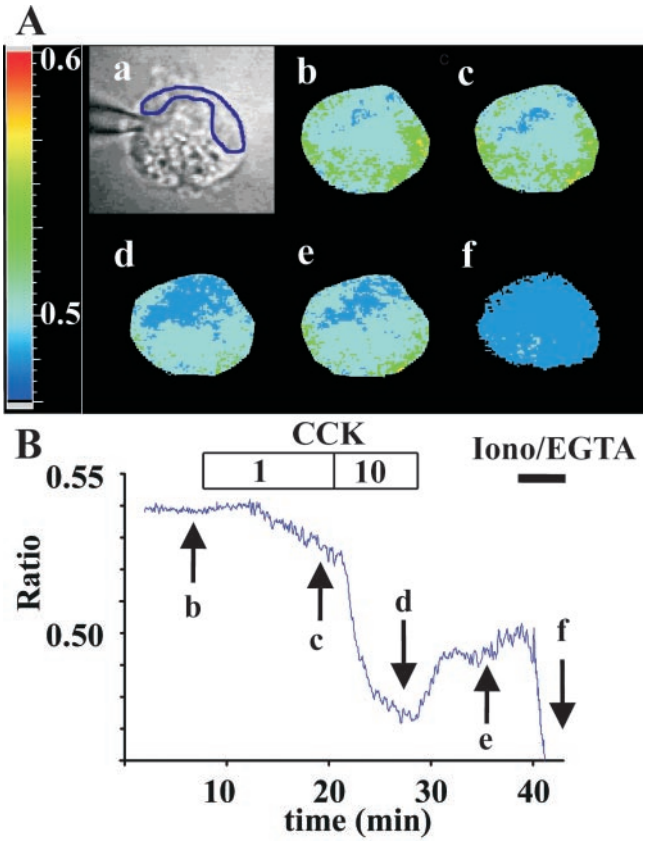

FIG. 1. **Effects of physiological doses of CCK (1 and 10 pM) on $[Ca^{2+}]_{ER}$ in a dialyzed, fura-2FF-loaded acinar cell.** *A*, montage of brightfield image (*a*) and fluorescence ratio images (*b–f*) obtained during illumination at 340 and 380 nm, with the pseudocolor ratio scale shown on the *left*. Images show the fluorescent ratio in the control state (*b*), after continuous incubation with 1 pM CCK for 11 min (*c*), after incubation with 10 pM CCK for 7 min (*d*), following washout of *CCK* (*e*), and in the presence of ionomycin (10 μM) and EGTA (10 mM) (*Iono/EGTA*) (*f*). *B*, time course of the change in ratio representing a fall in $[Ca^{2+}]_{ER}$. CCK concentration is in picomolar. Ratio measurements were made in the basolateral area indicated in the brightfield image in *a*, and *arrows* in *B* indicate the time points at which corresponding ratio images shown in *A* (*b–f*) were captured.

Physiological Concentrations of Cholecystokinin Deplete $[Ca^{2+}]_{ER}$—CCK (CCK-8, sulfated form) generated stepwise decreases in ratio, but at picomolar concentrations of the agonist the ratio reached the new steady-state level relatively slowly. At 1 pM CCK, the plasma concentration seen in the fasting state in the mouse[2] and in humans (26), there was a small but detectable decrease in ratio (Fig. 1). In 7 cells the mean decrease was 0.014 ± 0.003 (Fig. 2). However at 10 pM CCK, a concentration seen in the plasma after a meal, there was a more substantial mean decrease in ratio of 0.043 ± 0.006 (13 cells). The CCK-induced decreases in ratio were uniform throughout the basolateral non-nuclear area of the cell, where the resting ratio was highest. 10 nM CCK evoked a large average decrease in the ratio of 0.142 ± 0.005 (Fig. 2).

Depletion of $[Ca^{2+}]_{ER}$ by Different Doses of Acetylcholine—ACh (10 nM to 100 μM) caused a sharp decrease in ratio (Fig. 3) with low concentrations (10–100 nM) often being associated with a small "rebound" from the initial decrease (Fig. 3*B*). The decrease in the ratio evoked by ACh was most pronounced in the basal area of the cell and was uniform in this region. In

[2] J. F. Rehfeld, personal communication.

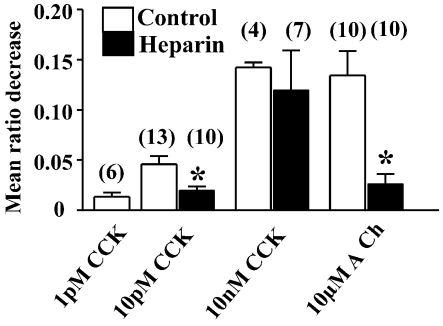

FIG. 2. **Mean decreases in ratio (± S.E.) evoked by CCK and ACh in cells dialyzed with control BAPTA/Ca^{2+} intracellular solution and in cells dialyzed with intracellular solution containing heparin.** Control data are represented by *open bars*, and the data from experiments with heparin (500 μg/ml) are shown by *solid bars*. *, statistically significant difference ($p < 0.01$) compared with control intracellular solution, which was obtained by unpaired Student's *t* test.

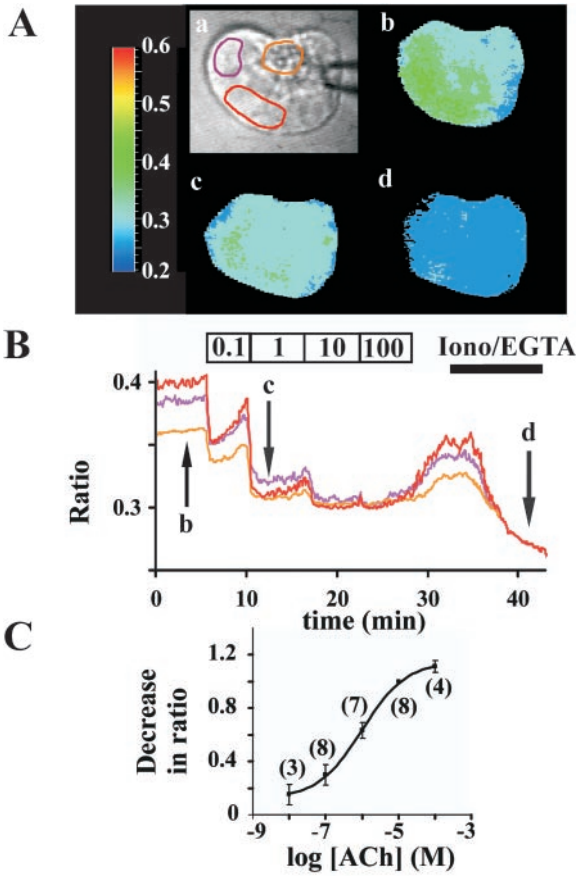

FIG. 3. **Depletion of $[Ca^{2+}]_{ER}$ by different doses of ACh in dialyzed cells.** *A*, montage of brightfield image (*a*) showing basal (*red*), lateral (*pink*), and apical (*orange*) regions of interest and (*b–d*) fluorescence ratio images obtained during illumination at 340 and 380 nm, with the pseudocolor ratio scale shown on the *left*. Ratio images show the control state (*b*), the effect of 1 μM ACh (*c*), and the effect of ionomycin (10 μM) and EGTA (10 mM) (*Iono/EGTA*) (*d*). *B*, time course of ratio changes in basal, lateral, and apical areas showing the decrease in ratio with increasing concentrations of ACh (μM) with a small "rebound" effect of 0.1 μM ACh. *Arrows* indicate the time points at which corresponding ratio images in *A* (*b–d*) were recorded. *C*, plot of decrease in mean ratio (data normalized relative to effect of 10 μM ACh) against log[ACh] (M), with half-maximal depletion at 0.98 ± 0.06 μM. Numbers in *parentheses* refer to the number of cells for each data point.

other regions of the cell including the lateral and apical regions, ratio decreases had similar time courses but slightly smaller amplitudes (Fig. 3*B*). The EC_{50} for the ACh-evoked

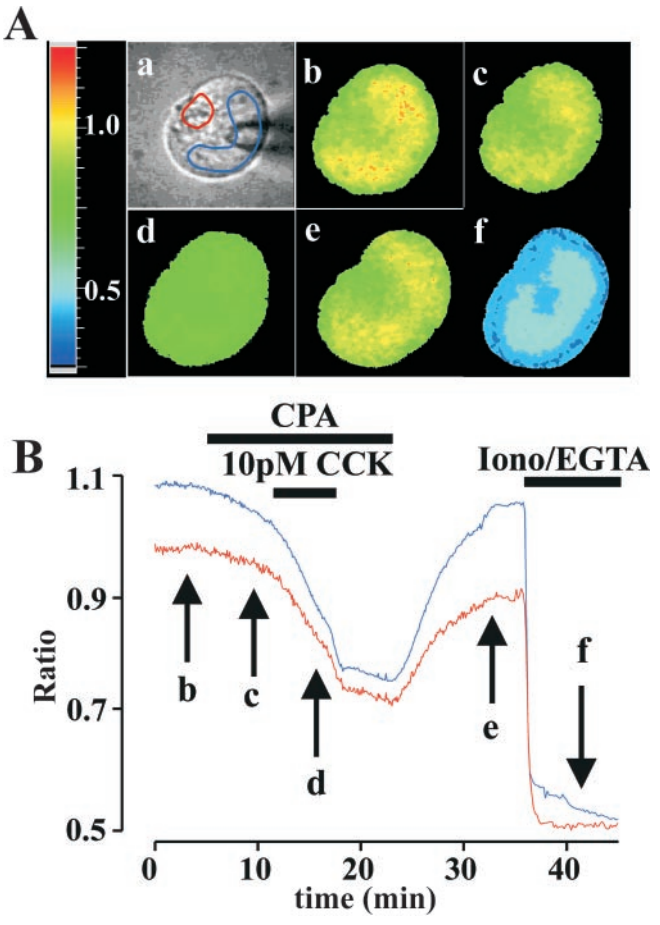

FIG. 4. **Relative magnitudes of the basal Ca^{2+} leak revealed by CPA and the Ca^{2+} leak evoked by a physiological concentration of CCK in a dialyzed acinar cell.** *A*, montage of brightfield image (*a*) showing basolateral (*blue*) and apical (*red*) regions of interest, and fluorescent ratio images (*b–f*) with the pseudocolor ratio scale shown on the *left*. Ratio images show the control state (*b*), the effect of CPA (10 μM) (*c*), the effect of CPA and 10 pM CCK together (*d*), washout (*e*), and the effect of ionomycin (10 μM) and EGTA (10 mM) (*Iono/EGTA*) (*f*). *B*, time course of ratio in the basolateral and apical regions of the cell, which is measured in the regions indicated in *A* (*a*). *Arrows* indicate the time points at which corresponding ratio images shown in *A* (*b–f*) were recorded.

decrease in ratio was 0.98 ± 0.06 μM (Fig. 3*C*). In 10 cells 10 μM ACh gave a mean decrease in ratio of 0.135 ± 0.024; this was similar in size to the decrease evoked by 10 nM CCK (Fig. 2). In the presence of a supramaximal concentration of ACh, 10 nM CCK was unable to cause further depletion of $[Ca^{2+}]_{ER}$ ($n = 5$, data not shown).

The Basal Leak Is Considerably Smaller than the Leak Evoked by 10 pM CCK—After a short lag, the application of the SERCA pump inhibitor CPA (10 μM) evoked a steady decrease in the ratio (Fig. 4). This is consistent with the unmasking of the basal leak of Ca^{2+} from intracellular stores following inhibition of Ca^{2+} uptake. Thapsigargin (2 μM) evoked a quantitatively similar depletion of $[Ca^{2+}]_{ER}$ (data not shown). The mean rate of decrease evoked by CPA in 14 cells was 0.018 ± 0.006 min^{-1}, much smaller than the rate of decrease of the ratio due to 10 μM ACh (0.15 ± 0.02 min^{-1}, $n = 8$). When 10 pM CCK was applied to cells in which the basal leak had already been revealed by CPA treatment the mean rate of Ca^{2+} loss was accelerated (Fig. 4). The decreases in the ratio evoked by CPA and CCK were greatest in the basolateral area; slightly smaller decreases with similar kinetics were seen throughout the cell (Fig. 4). In 7 cells, 10 pM CCK significantly increased ($p < 0.05$, paired Student's *t* test) the mean rate of decline evoked by CPA

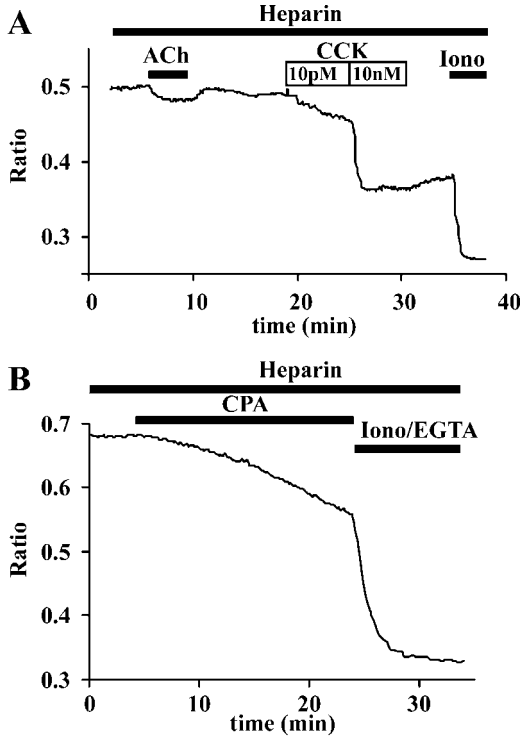

FIG. 5. **Responses of cells dialyzed with intracellular solution containing the IP_3 receptor antagonist heparin (500 μg/ml).** *A*, the response to ACh (1 μM) is inhibited, but CCK (10 pM and 10 nM) still evokes a response. *B*, CPA (10 μM) still reveals the basal leak when heparin is present in the intracellular solution.

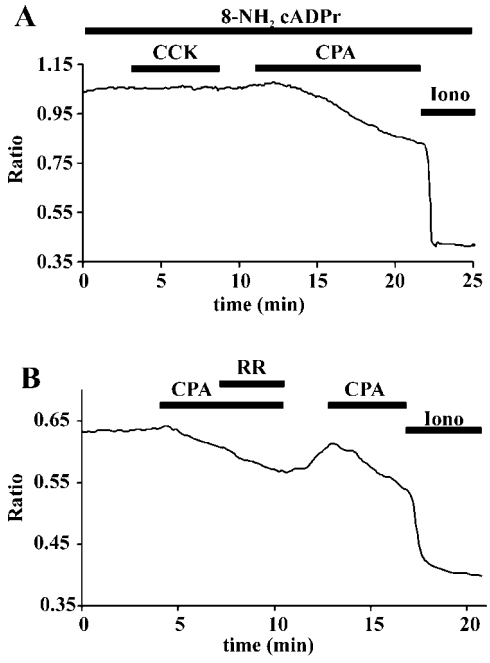

FIG. 6. **Effects of inhibitors of ryanodine receptors on the response to CCK and on the basal leak.** *A*, in a patch-clamped cell dialyzed with 8-NH_2 cyclic ADP-ribose (20 μM) the store-depleting effect of physiological dose of CCK (10 pM) is inhibited, but CPA (10 μM) is still able to reveal the basal leak. *B*, in a streptolysin O-permeabilized cell, the basal leak evoked by CPA is not affected by the ryanodine receptor blocker ruthenium red (*RR*, 10 μM). Subsequently, $[Ca^{2+}]_{ER}$ is depleted by a mixture of ionomycin (10 μM) and EGTA (10 mM) (*Iono*).

(by 265 ± 82%), whereas in 6 cells 1 pM CCK increased the CPA-evoked rate of decline by only 10 ± 20%.

IP_3 Receptor Antagonists Block the Action of ACh but Only Partially Block the Action of CCK and Do Not Block the Basal Leak—In 17 cells dialyzed with the competitive IP_3 receptor antagonist heparin (M_r 6000; 500 μg/ml) the mean resting ratio (0.59 ± 0.03) was not significantly different from cells dialyzed with control pipette solution. Heparin substantially blocked the response to ACh (Fig. 5*A*), and in 10 cells dialyzed with heparin the response to 10 μM ACh was decreased by 80% compared with control cells (Figs. 2 and 5*A*). Heparin did not completely block the effect of CCK (Fig. 5*A*). In heparin-dialyzed cells the mean response to 10 nM CCK was not different from the mean response in control cells (Figs. 2 and 5*A*); however the effect of 10 pM CCK was more than halved by heparin (Fig. 2). In heparin-dialyzed cells CPA was still able to reveal the basal leak (0.012 ± 0.005 min^{-1}, $n = 4$) and deplete $[Ca^{2+}]_{ER}$ (Fig. 5*B*). We also studied the effects of another receptor antagonist flunarizine (10 μM) on agonist-evoked and basal Ca^{2+} release. In our study this compound, which is membrane-permeant and has been previously reported to block directly the Ca^{2+} channel of the IP_3 receptor (27, 28), inhibited the effect of ACh; however CCK and CPA were still able to deplete $[Ca^{2+}]_{ER}$ in the presence of flunarizine ($n = 4$, not shown).

Ryanodine Receptor Antagonists Substantially Inhibit the Action of CCK but Not the Effect of ACh or the Basal Leak—Dialysis with 8-amino-cyclic ADP-ribose (20 μM, Molecular Probes Europe BV), a competitive inhibitor of cyclic ADP-ribose action (29, 30), had no effect on mean resting $[Ca^{2+}]_{ER}$ (mean ratio = 0.67 ± 0.08 in the presence of 8-amino-cyclic ADP-ribose, $n = 7$) or on the action of ACh, but blocked the depletion of $[Ca^{2+}]_{ER}$ by 10 pM CCK (Fig. 6*A*). In 5 cells dialyzed with this inhibitor the mean ratio decrease evoked by 10 pM CCK was reduced by 87 ± 11% ($p < 0.02$ compared with control cells). In the presence of 8-amino-cyclic ADP-ribose, CPA was still able to deplete the Ca^{2+} store at a rate similar to in control conditions (0.02 ± 0.009 min^{-1}, $n = 4$). Inhibition of CPA responses by 8-amino-cyclic ADP-ribose was not seen even when the IP_3 receptor blocker flunarizine was present as well ($n = 4$). In order to investigate further whether the basal leak could occur through the ryanodine receptor we studied the effect on the basal leak of ruthenium red, a blocker of the ryanodine receptor (20, 31). These experiments were performed on permeabilized cells, allowing relatively fast and simple changes of the solutions in contact with the ER. In 16 permeabilized cells CPA (10 μM) evoked a mean ratio decrease of 0.021 ± 0.004 min^{-1}, but ruthenium red (10 μM) had no effect on this basal leak (Fig. 6*B*). We also studied the effect of nifedipine and verapamil (both 100 μM) on the basal Ca^{2+} leak in permeabilized cells. It has been reported that in sea urchin eggs these Ca^{2+} channel blockers completely inhibit the release of stored Ca^{2+} evoked by NAADP (32), but we found that in the pancreatic acinar cell they had no effect on the basal Ca^{2+} leak evoked by CPA ($n = 8$ cells for both compounds, data not shown).

Puromycin Evokes a Decrease in $[Ca^{2+}]_{ER}$—In patch-clamped and dialyzed cells the protein synthesis inhibitor puromycin (100–500 μM) produced an initial decrease in ratio, which slowed down after a short period (Fig. 7*A*). Mean fura-2FF ratio decreases with 100 and 500 μM puromycin were 0.08 ± 0.02 and 0.167 ± 0.04, respectively (10 cells). Ratio measurements in acinar cells loaded with the low-affinity Ca^{2+} indicator mag-fura-2 and dialyzed with BAPTA/Ca^{2+} intracellular solution confirmed the ability of puromycin to deplete $[Ca^{2+}]_{ER}$ ($n = 4$; data not shown). We also studied the effects of puromycin in fura-2FF-loaded acinar cells permeabilized by streptolysin O, because this allowed faster changes of bathing solution during the experiment. Responses to puromycin were seen in permeabilized cells (Fig. 7*B*), and the mean ratio decrease evoked by 500 μM puromycin was 0.11 ± 0.02 ($n = 16$), and by 1 mM puromycin 0.14 ± 0.03 ($n = 7$). At 20 μM, puro-

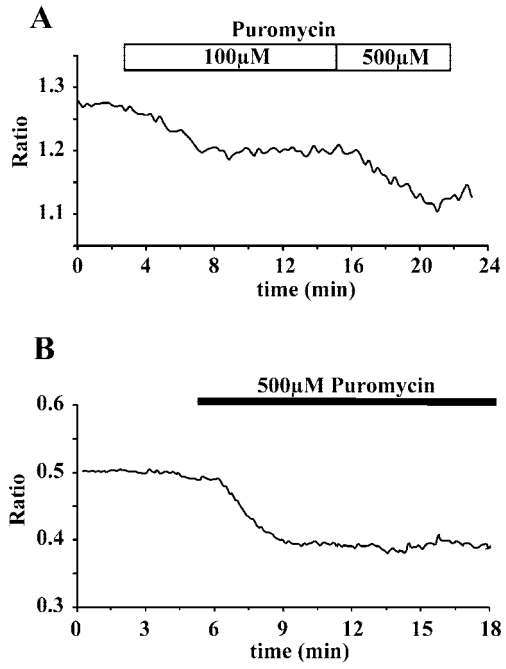

FIG. 7. **Time courses of depletion of $[Ca^{2+}]_{ER}$ by puromycin.** *A*, puromycin (100 and 500 μM) depletes $[Ca^{2+}]_{ER}$ in a dialyzed cell. *B*, 500 μM puromycin evokes a decrease in $[Ca^{2+}]_{ER}$ in a streptolysin O-permeabilized cell.

mycin had no effect ($n = 4$). To exclude the possibility that the effects of puromycin were mediated via agonist-activated Ca^{2+} release channels, we applied puromycin to permeabilized cells during blockade of IP_3 and ryanodine receptors. In the presence of the IP_3 receptor inhibitor heparin (500 μg/ml), puromycin (500 μM) decreased the mean ratio of 10 permeabilized cells by 0.091 ± 0.015 (Fig. 8*A*), and in the presence of the ryanodine receptor antagonist ruthenium red (10 μM) puromycin decreased the mean ratio of 8 permeabilized cells by 0.074 ± 0.019 (Fig. 8*B*). Neither of these values was significantly different from the effect of puromycin alone. To investigate the possibility that the depletion of $[Ca^{2+}]_{ER}$ by puromycin could be because of a decreased activity of the SERCA pump rather than an increase in Ca^{2+} permeability of the ER membrane, we studied the effect of puromycin on $[Ca^{2+}]_{ER}$ when SERCA was inhibited by CPA. Puromycin (500 μM) accelerated the Ca^{2+} leak evoked by CPA (Fig 8*C*). In 36 cells the mean Ca^{2+} leak was increased from 0.013 ± 0.002 to 0.018 ± 0.002 ($p < 0.01$, paired Student's t test).

DISCUSSION

In this study we were able to dissociate the effects of physiological doses of agonists on the initial or intrinsic Ca^{2+} efflux from the ER from any effects on Ca^{2+} signaling due to secondary Ca^{2+}-induced Ca^{2+} release. This was achieved by direct measurements of $[Ca^{2+}]_{ER}$ while $[Ca^{2+}]_C$ was clamped at a quasi-resting level with a BAPTA/Ca^{2+} mixture. In these highly sensitive experimental conditions, where feedback of Ca^{2+} on its own efflux was prevented, we were able to resolve the depletion of $[Ca^{2+}]_{ER}$ produced by the hormone CCK at 1 pM (the fasting plasma level), and at 10 pM, a level achieved in the plasma after a meal. 1 and 10 pM CCK produced ratio changes corresponding to ~10 and 32% of the decrease induced by a supramaximal dose of CCK, respectively. Our experiments indicate that SERCA pumps can balance the leak induced by all doses of CCK in the physiological range and prevent substantial depletion of the store. This is important because substantial depletion of ER can inhibit protein synthesis, facilitate protein degradation and affect protein folding (33–37).

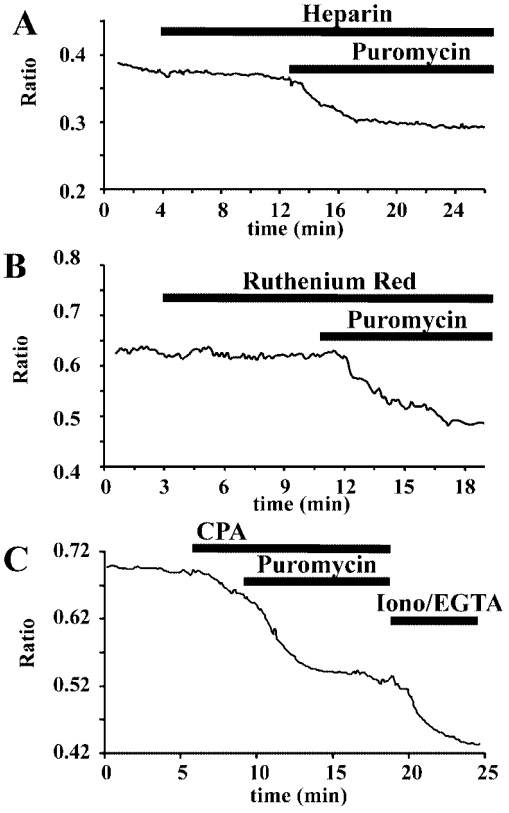

FIG. 8. The depletion of $[Ca^{2+}]_{ER}$ by puromycin (500 μM) in permeabilized cells is not affected by the IP_3 receptor antagonist heparin (500 μg/ml) (*A*) or the ryanodine receptor antagonist ruthenium red (10 μM) (*B*). *C*, in a permeabilized cell in which inhibition of the SERCA pump by CPA (10 μM) activates the basal Ca^{2+} leak, puromycin (500 μM) accelerates the depletion of $[Ca^{2+}]_{ER}$. Subsequent application of a mixture of ionomycin (10 μM) and EGTA (10 mM) (*Iono/EGTA*) further depletes the Ca^{2+} store.

A recent study by Pinton *et al*. (38) in HeLa cells has indicated that overexpression of the anti-apoptotic protein bcl-2 can decrease the Ca^{2+} content of the ER, suggesting that this protein could mediate (or regulate) Ca^{2+} leak from the ER. Moreover, this study implies a new "trophic" action of low physiological doses of Ca^{2+}-releasing hormones, *i.e.* protection of the ER from Ca^{2+} overload and consequently from apoptotic destruction of the cell.

Previous measurements of cytosolic Ca^{2+} have indicated that in intact cells physiological concentrations of CCK generate, after a delay of 1–2 min, a mixture of fast, local spikes and slow global Ca^{2+} oscillations (5, 30, 39). Our study describes an increase of the permeability of the ER that is purely due to action of the hormone (without amplification by Ca^{2+}-induced Ca^{2+} release). The mechanism by which moderate increases in Ca^{2+} efflux from the ER are converted into different forms of Ca^{2+} oscillation by other cellular mechanisms is an interesting subject for further theoretical and experimental studies. For ACh the physiological concentration range in the vicinity of the pancreatic acinus is unknown, therefore we have characterized the effects of a broad range of ACh concentration on $[Ca^{2+}]_{ER}$. The ability of low doses of ACh (10 and 100 nM) to generate a pattern of short lasting local Ca^{2+} spikes similar to that produced by IP_3 infusion into patch-clamped cells (6) has been well characterized (5, 39). Our measurements of $[Ca^{2+}]_{ER}$ suggest that 10 and 100 nM ACh cause between 13 and 25% of the maximal agonist-dependent ratio changes, respectively. The depletion of $[Ca^{2+}]_{ER}$ induced by these doses of ACh, and by physiological doses of CCK, is clearly quantitatively similar. Therefore the difference in the patterns of cytosolic Ca^{2+} re-

sponses of the two agonists cannot simply be explained by different levels of Ca^{2+} efflux from the ER.

In this study we found that the ability of ACh to induce Ca^{2+} leak from the ER was blocked by heparin, a competitive IP_3 receptor antagonist (40), and by flunarizine, which does not affect IP_3 binding to its receptor but blocks the Ca^{2+} release channel activated by IP_3 (27, 28). This strongly suggests that the primary mechanism of ACh-induced Ca^{2+} leak is mediated by IP_3 receptors.

In contrast, the role of IP_3 receptors in acinar cell Ca^{2+} signaling by CCK is more complex. Using a biochemical radioreceptor assay Matozaki *et al*. (41) showed that supraphysiological (>50 pM) concentrations of CCK generate measurable amounts of IP_3, and studies using permeabilized cells (42) and patch-clamped cells (5, 43) have reported that inhibitors of the IP_3 receptor completely block CCK-evoked $[Ca^{2+}]_C$ signals. However in another study Thorn *et al*. (39) described that when acinar cells were dialyzed with low concentrations (<250 μg/ml) of heparin, physiological doses of CCK (5–20 pM) still produced long lasting oscillations in $[Ca^{2+}]_C$ (although short, IP_3-type $[Ca^{2+}]_C$ spikes were blocked). In the present study in which "intrinsic," messenger-evoked Ca^{2+} efflux from the ER and secondary Ca^{2+}-induced Ca^{2+} release were dissociated, we found that inhibition of the IP_3 receptor caused partial inhibition of the effect of 10 pM CCK, as did inhibition of the ryanodine receptor with 8-NH_2 cyclic ADP-ribose. These data support the hypothesis that although ACh-induced efflux is mediated by IP_3, physiological concentrations of CCK stimulate efflux dependent on multiple messengers, including IP_3 and cyclic ADP-ribose. Because inhibition of IP_3 receptors has been found to block $[Ca^{2+}]_C$ oscillations evoked by the putative Ca^{2+} releasing messengers cyclic ADP-ribose and NAADP (5), this suggests that CCK-evoked $[Ca^{2+}]_C$ oscillations rely on the recruitment of IP_3 receptors to amplify the $[Ca^{2+}]_C$ signal. Our data show that the CCK-induced Ca^{2+} efflux is partially dependent on IP_3 receptors. We found that heparin did not block the depletion of $[Ca^{2+}]_{ER}$ by 10 nM CCK, suggesting that although nanomolar concentrations of CCK do generate IP_3 (41), when IP_3 action is blocked by heparin other CCK-stimulated messengers could be able to evoke a substantial release of Ca^{2+} from the ER. Our observations that in the presence of supramaximal doses of ACh, the addition of supramaximal doses of CCK is unable to deplete $[Ca^{2+}]_{ER}$ further, suggesting that although primary CCK-induced Ca^{2+} efflux does involve additional Ca^{2+}-releasing messengers, it does not involve a separate Ca^{2+} store. Qualitatively similar time courses of $[Ca^{2+}]_{ER}$ were seen within different regions of a single cell during depletion of $[Ca^{2+}]_{ER}$ with secretagogues and with CPA (Figs. 3 and 4). This supports the concept that the endoplasmic reticulum of the pancreatic acinar cell acts as a single agonist-releasable Ca^{2+} store (8, 22, 24).

Hofer *et al*. (7) reported that neither heparin nor the ryanodine receptor antagonist ruthenium red blocked the basal leak revealed by SERCA inhibition. Our study supports this finding. We showed that cells dialyzed with heparin had a similar resting $[Ca^{2+}]_{ER}$ to control cells, suggesting no substantial differences from control cells in the pump/leak relationship. Neither heparin, nor the membrane-permeant Ca^{2+} channel blocker flunarizine, which does not affect IP_3 binding to its receptor but has been reported to inhibit Ca^{2+} release by IP_3 (27, 28), blocked the basal leak evoked by CPA. Furthermore, neither of the two ryanodine receptor antagonists used (ruthenium red and 8-NH_2 cyclic ADP-ribose) nor nifedipine or verapamil, which in sea urchin eggs completely block the Ca^{2+} release evoked by NAADP (32), had any effects on the basal Ca^{2+} leak. We therefore found no evidence to suggest that in the pancreatic acinar cell the basal Ca^{2+} leak occurs through any of the Ca^{2+} release channels so far identified in the ER membrane.

In contrast it has been hypothesized that the basal Ca^{2+} leak from the ER into the cytosol may occur through the aqueous pore in the translocon of the ER membrane during the protein synthetic cycle (8). During the normal cycle of protein synthesis the permeability of the translocon is tightly controlled, possibly because of the binding of the ribosome to the cytosolic surface of the translocon pore (44). The permeability of the translocon is also regulated at the luminal side of the pore by the prominent ER chaperone BiP (45), this protein being released from the translocon shortly after the completion of ribosome-nascent chain targeting. However in the empty state, when the translocon pore is ribosome-bound but unoccupied by polypeptide, the ribosome-translocon complex seems to allow the passage of small molecules (17).

In the present study we have examined the effect of puromycin on the Ca^{2+} permeability of the ER membrane, and found a substantial puromycin-induced Ca^{2+} efflux. Puromycin is an antibiotic that selectively terminates ribosomal translation by releasing the nascent polypeptide from the protein channel of the ribosome (18, 19). Simon *et al*. (15, 16) used an electrophysiological approach to show that in pancreatic rough microsomes puromycin activates an ion-permeable pore. Interestingly, spontaneous openings of large conductance ion channels in the ER membrane, possibly representing subconductance states of the translocon channel, were reported even in the absence of puromycin (16). These spontaneous openings could be responsible for the basal Ca^{2+} leak. If such a translocon-mediated Ca^{2+} leak exists, then it should be particularly prominent in the pancreatic acinar cell with its extremely well developed rough endoplasmic ER and very high protein-synthesizing activity. Importantly, a very recent study by Potter and Nicchitta (46) has demonstrated that ribosomes maintain stable associations with translocons after the termination of protein synthesis. According to our results, in the protein-free state such endogenous ribosome-translocon complexes could serve as mediators of the basal Ca^{2+} leak from the ER.

In our study we have shown, for the first time, that removal of the polypeptide chain from the ribosome by puromycin causes a depletion of $[Ca^{2+}]_{ER}$ by a mechanism independent of IP_3 receptors and ryanodine receptors and independent of inhibition of the SERCA pump. Our study therefore provides experimental support for the hypothesis that the basal Ca^{2+} leak from the rough ER occurs through translocon pores in the ER membrane.

REFERENCES

1. Berridge, M. J. (1993) *Nature* **361,** 315–325
2. Berridge, M. J. (2001) in *Novartis Foundation Symposium* (Bock G., and Goode, J., eds) Vol. 239, pp. 52–64, John Wiley and Sons, Sussex, UK
3. Thomas, A. P., St. Bird, G., Hajnóczky, G., Robb-Gaspers, L. D., and Putney, J. W. (1996) *FASEB J.* **10,** 1505–1517
4. Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994) *Physiol. Rev.* **74,** 595–636
5. Cancela, J. M., Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (2000) *EMBO J.* **19,** 2549–2557
6. Petersen, O. H. (1992) *J. Physiol.* **448,** 1–51
7. Hofer, A. M., Curci, S., Machen, T. E., and Schulz, I. (1996) *FASEB J.* **10,** 302–308
8. Mogami, H., Tepikin, A. V., and Petersen, O. H. (1998) *EMBO J.* **17,** 435–442
9. Beecroft, M. D., and Taylor, C. W. (1998) *Biochem. J.* **334,** 431–435
10. Missiaen, L., de Smedt, H., Raeymaekers, L., Droogmans, G., van den Bosch, L., and Casteels, R. (1996) *Biochem. J.* **317,** 849–853
11. Pessah, I. N., Molinski, T. F., Mely, T. D., Wong, P., Buck, E. D., Allen, P. D., Mohr, F. C., and Mack, M. M. (1997) *Am. J. Physiol.* **272,** C601-C614
12. Overend, C. L., O'Neill, S. C., and Eisner, D. A. (1998) *J. Physiol.* **507,** 750–769
13. Smith, P. M., and Gallacher, D. V. (1994) *Biochem. J.* **299,** 37–40
14. Favre, C. J., Lew, D. P., and Krause, K.-H. (1994) *Biochem. J.* **302,** 155–162
15. Simon, S. M., Blobel, G., and Zimmerberg, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6176–6180
16. Simon, S. M., and Blobel, G. (1991) *Cell* **65,** 371–380
17. Heritage, D., and Wonderlin, W. F. (2001) *J. Biol. Chem.* **276,** 22655–22662

18. Pestka, S. (1974) *Methods Enzymol.* **30,** 261–282
19. Skogerson, L., and Moldave, K. (1968) *Arch. Biochem. Biophys.* **125,** 497–505
20. Bezprozvanny, I., Watras, J., and Ehrlich, B. E. (1991) *Nature* **351,** 751–754
21. Hammill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100
22. van de Put, F. H. M. M., and Elliott, A. C. (1996) *J. Biol. Chem.* **271,** 4999–5006
23. Bolender, R. P. (1974) *J. Cell Biol.* **61,** 269–287
24. Park, M. K., Petersen, O. H., and Tepikin, A. V. (2000) *EMBO J.* **19,** 5729–5739
25. Grynciewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
26. Ballinger, A., McLoughlin, L., Medbak, S., and Clark, M. (1995) *Clin. Sci.* **89,** 375–381
27. Seiler, S. M., Arnold, A. J., and Stanton, H. C. (1987) *Biochem. Pharmacol.* **36,** 3331–3337
28. Palade, P., Dettbarn, C., Alderson, B., and Volpe, P. (1989) *Mol. Pharmacol.* **36,** 673–680
29. Walseth, T. F., and Lee, H. C. (1993) *Biochim. Biophys. Acta* **1178,** 235–242
30. Cancela, J. M., and Petersen, O. H. (1998) *Pflügers Arch.* **435,** 746–748
31. Palade, P. (1987) *J. Biol. Chem.* **262,** 6135–6141
32. Genazzani, A. A., Empson, R. M., and Galione, A. (1996) *J. Biol. Chem.* **271,** 11599–11602
33. Stevens, F. J., and Argon, Y (1999) *Semin. Cell Dev. Biol.* **10,** 443–454
34. Wetmore, D. R., and Hardman, K. D. (1996) *Biochemistry* **35,** 6549–6558
35. Prostko, C. R., Dholakia, J. N., Bromstrom, M. A., and Bromstrom, C. O. (1995) *J. Biol. Chem.* **270,** 6211–6215
36. Bromstrom, C. O., Prostko, C. R., Kaufmann, R. J., and Bromstrom, M. A. (1996) *J. Biol. Chem.* **271,** 24995–25002
37. Jeffery, J., Kendall, J. M., and Campbell, A. K. (2000) *Biochem. Biophys. Res. Commun.* **268,** 711–715
38. Pinton, P., Ferrari, D., Rapizzi, E., di Virgilio, F. D., Pozzan, T., and Rizzuto, R. (2001) *EMBO J.* **20,** 2690–2701
39. Thorn, P., Lawrie, A. M., Smith, P. M., Gallacher, D. V., and Petersen, O. H. (1993) *Cell* **74,** 661–668
40. Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L., and Gill, D. L. (1988) *J. Biol. Chem.* **263,** 11075–11079
41. Matozaki, T., Göke, B., Tsunoda, Y., Rodriguez, M., Martinez, J., and Williams, J. A. (1990) *J. Biol. Chem.* **265,** 6247–6254
42. Tortorici, G., Zhang, B.-X., Xu, X., and Muallem, S. (1994) *J. Biol. Chem.* **269,** 29621–29628
43. Yule, D. I., Stuenkel, E., and Williams, J. A. (1996) *Am. J. Physiol.* **271,** C1285-C1294
44. Crowley, K. S., Liao, S., Worrell, V. E., Reinhart, G. D., and Johnson, A. E. (1994) *Cell* **78,** 461–471
45. Hamman, B. D., Hendershot, L. M., and Johnson, A. E. (1998) *Cell* **92,** 747–758
46. Potter, M. D., and Nicchitta C. V. (2002) *J. Biol. Chem.* **277,** 23314–23320

Basal and Physiological Ca^{2+} Leak from the Endoplasmic Reticulum of Pancreatic Acinar Cells: SECOND MESSENGER–ACTIVATED CHANNELS AND TRANSLOCONS

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