Identification and function of type-2 and type-3 ryanodine receptors in gut epithelial cells

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Reverse transcription-PCR (RT-PCR) techniques were used to identify the expression of ryanodine receptor (RyR) isoforms in gut epithelial cells. Restriction digest and sequence analysis of the PCR product showed the presence of RyR 2 and RyR 3. [³H]Ry binding studies on a microsome preparation, in a high-salt buffer, showed specific binding with an EC₅₀ of 15 μM. In order to determine a potential functional role for these RyRs, we first characterized the response of the cells to acetylcholine. At all concentrations used acetylcholine induced sinusoidal cytosolic Ca²⁺ concentration ([Ca²⁺]_i) oscillations. In response to 10⁻⁴ M acetylcholine, levels of inositol 1,4,5-trisphosphate (InsP₃) showed a peak of six times the basal level, at 30 s after stimulation. Application of caffeine alone failed to elicit a rise in cytosolic Ca²⁺. However, caffeine (5–50 mM) did rapidly and reversibly

inhibit the acetylcholine-induced [Ca²⁺]_i oscillations. The effects of Ry were more complex. Applied alone, Ry had no effect on the [Ca²⁺]_i signal. When applied during agonist-evoked [Ca²⁺]_i oscillations, Ry (10 μM) slowly blocked the response. In the continuous presence of Ry (10 μM) a short application of acetylcholine elicited a [Ca²⁺]_i response that continued as oscillations even when the agonist was removed. The oscillations, in the presence of Ry (10 μM) but absence of agonist, were blocked either by removal of extracellular Ca²⁺ or by an application of a higher concentration of Ry (100 μM). These effects are consistent with the known use-dependence and dose-dependence for Ry action at the RyR. We conclude that the RyR 2 and RyR 3, identified by RT-PCR, play a central role in [Ca²⁺]_i oscillations in gut epithelial cells.

INTRODUCTION

Ca²⁺ signalling is a ubiquitous process in epithelial cells and is thought to be fundamental to cell functions as diverse as fluid secretion, exocytosis and cell division [1,2]. Many agonists promote a rise in cytosolic Ca²⁺ via the production of the second messenger inositol 1,4,5-trisphosphate (InsP₃), which in turn promotes the opening of InsP₃ receptors and the release of Ca²⁺ from intracellular stores. In addition to the InsP₃ receptor, it is becoming clear that the ryanodine receptor (RyR), first identified in muscle cells, plays a role in calcium signalling in some types of epithelial cell [3]. Molecular biological techniques, such as reverse transcription-PCR (RT-PCR) and RNase protection, have been used to screen whole organs for the three known isoforms of the RyR [4,5]. Whole organs such as submaxillary gland, kidney and gut, which are composed predominantly of cells of epithelial origin, have been shown to express RyR. These studies show the ubiquity of the RyR, but are of limited value because the mixed cell populations in each organ cannot allow positive identification of the cell type that carries the RyR. However, recent work on cell lines has shown RyRs in two epithelial cell types, HeLa and LLC-PK1 cells [6]. Functional effects of RyRs in epithelial cells have been found in pancreatic acinar cells [7–9], in hepatocytes [10] in transforming growth factor β (TGF-β)-treated mink lung epithelia [11] and HeLa cells [6]. However, it is only in HeLa cells (RyR 2) [6] and in TGF-β-treated mink lung epithelial cells (RyR 3) [11] where receptors have been identified.

In our study, we have used RT-PCR to identify the presence of mRNA coding for RyR 2 and RyR 3 in T84 cells. Specific [³H]ryanodine (Ry) binding was detected in T84 microsomes, indicating that the RyRs were functionally expressed. The cytosolic Ca²⁺ concentration ([Ca²⁺]_i) response of the cells to acetylcholine was assessed and InsP₃ production measured. The

effects of caffeine and Ry on the [Ca²⁺]_i oscillations were assessed. Our analysis represents the most complete study of the [Ca²⁺]_i signalling in gut epithelial cells to date, and reveals the presence of RyRs that are functionally significant in agonist-evoked [Ca²⁺]_i responses.

EXPERIMENTAL

Materials

Materials were obtained as follows: Fura-2 AM from Molecular Probes (Eugene, OR, U.S.A.), cell culture solutions from Gibco U.K. and Ry from Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture and solutions

T84 cells were grown in standard culture conditions. In brief, the culture medium was Dulbecco's modified Eagle's medium (DMEM)/HAM's F12 supplemented with 10% (v/v) fetal-calf serum (Gibco, U.K.) and penicillin/streptomycin and cells were split 1:20 once a week. For experimental use, cells were plated on to 22-mm-diam. glass coverslips and were studied after 1–3 days. The standard extracellular bathing solution contained (in mM): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 10 Hepes, pH 7.4 (NaOH). Caffeine was added to this solution with no adjustment to the osmolality.

RT-PCR

Methods were essentially as described in Bennett et al. [6]. In brief, total RNA was extracted according to Chomczynski and Saachi [12]. A sample (5 μg) of RNA was used in 15 μl of a cDNA synthesis reaction carried out using a cDNA synthesis kit (First strand cDNA synthesis, Pharmacia, Biotech) according to

Abbreviations used: Ry, ryanodine; RyR, ryanodine receptor; RT-PCR, reverse transcription-PCR; InsP₃, inositol 1,4,5-trisphosphate; [Ca²⁺]_i, cytosolic Ca²⁺ concentration; TGF-β, transforming growth factor β; DMEM, Dulbecco's modified Eagle's medium.

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the manufacturer's instructions. Two control cDNA reactions were carried out. In one we added RNA but no reverse transcriptase (called RNA control), in the other we added no RNA (called cDNA control). PCR reactions contained 2 μ l of the T84 cDNA reaction, or cDNA control reaction, or 1 μ l of mouse brain cDNA, 50 pmol of each primer, 100 μ M dNTPs, and 2.5 units of *Taq* polymerase (Boehringer Mannheim) in 10 mM Tris/HCl (pH 8.3) containing 50 mM KCl, 1.5 mM MgCl₂ in a total volume of 50 μ l. Reactions were carried out in a Techne PHC-1 programmable dry-block. Following hot start (5 min at 94 °C), the samples were subjected to 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C. This was followed by a final extension at 72 °C for 10 min. PCR was carried out using degenerate primers designed to sequences conserved among the three isoforms of RyR [6]. The forward primer was 5'-CA(C/T)(C/T)T(A/C/G/T)(C/T)T(A/C/G/T)GA(C/T)-AT(A/C/T)GCIATGGG-3' and the reverse primer was 5'-(A/G/T)(A/G)TA(A/G)TT(A/C/G/T)GCIA(A/G)(A/G)TT-(A/G)TG(C/T)-TC-3'.

Nested PCR was performed by amplifying 5 μ l of each original PCR reaction with the same forward primer and an internal degenerate downstream primer, thus ensuring only Ry-like sequences were amplified producing a band of approximately 480 bp. The downstream primer was 5'-CA(T/C)GG(T/C/G)-TT(T/C)GAAAC(A/C/T)CA(C/T)AC-3'. The resulting PCR products were analysed by agarose gel electrophoresis.

Restriction enzyme analysis of RT-PCR products

To determine which RyR isoforms were present within the PCR products, restriction enzymes were employed. *SacI* was predicted to cut RyR 1 only into fragments of approx. 280 and 200 bp. *BglII* was predicted to cut RyR 2 only into fragments of approx. 270 and 210 bp respectively. *BstEII* should cut RyR 3 only into fragments of approx. 260 and 220 bp. The results obtained by this analysis were confirmed by cloning and sequencing the PCR products.

Cloning and sequencing of RT-PCR products

PCR products were separated electrophoretically on an 1.5% agarose gel and visualized under UV light. Bands of interest were cut out and purified using the GENECLEAN II DNA Purification kit (Bio 101). The purified DNA was cloned into TA cloning vector (Invitrogen), according to the manufacturer's instructions. Positive clones were subjected to restriction enzyme analysis with *AvaII* to determine which isoforms were present and this was verified by sequencing clones manually using the Sequenase kit (version 2.0) (Amersham Corp.).

[³H]Ry binding

Microsomes were prepared from T84 cells by Turrax homogenization, on ice, in a solution containing (in mM): 340 sucrose, 5 EDTA, 20 Mops, pH 7.3, adjusted with Tris. Also included in the sucrose solution were enzyme inhibitors (Sigma) as follows: 1.6 μ g/ml pepstatin, 1.6 μ g/ml aprotinin, 1.6 μ g/ml leupeptin, 2.7 mM benzamidine, 40 μ g/ml PMSF and 0.5 mg/ml trypsin/chymotrypsin inhibitor. After centrifugation at 600 g for 2 min to remove cell debris, the supernatant was removed and spun at 36000 g for 20 min at 4 °C in a Sorvall RC-5B centrifuge (DuPont) to pellet a crude microsome fraction. This was then resuspended in sucrose solution and immediately stored at -80 °C. The concentration of microsomal protein was determined using a Bradford assay with BSA (Sigma) as a standard. [³H]Ry binding was carried out in high-salt buffer: 1.5 M KCl,

25 mM Na/Pipes, 500 nM CaCl₂, 40 μ g/ml PMSF, pH 7.4. The reaction mix contained 320 μ g of microsomal protein, 1 nM [³H]Ry (Amersham International) with a specific radioactivity of 93.0 Ci/mmol, and a range of concentrations of Ry (Sigma). The assay was carried out in triplicate and full equilibration was reached after an overnight incubation at 4 °C. The membranes were pelleted in a microfuge and, after removing the supernatant, the pellet was resuspended in 10% (w/v) SDS (Sigma) and radioactivity measured in a scintillation counter. Examples of typical count values were 24391 d.p.m. after incubation with no added Ry and 13857 d.p.m. in the presence of 250 μ M Ry. A graph was produced where the counts at each Ry concentration were expressed as a percentage of the maximum counts obtained in the absence of added Ry (see Figure 3). This plot was fitted according to the equation:

$$y = 100/(1 - [\text{Ry}]/\text{EC}_{50})$$

InsP₃ binding assay

[³²P]InsP₃ {made from [³²P]ATP (Amersham)}, unlabelled InsP₃ and binding protein (prepared from bovine adrenal microsomes) were obtained from Robin Irvine, Pete Cullen and Kathy Hinchcliffe (The Babraham Institute) who used the methods of Palmer et al. [13]. T84 cells were plated into 24-well plates (Nunc). After 3 days in culture, cells were stimulated with acetylcholine and the reaction stopped at set time points by adding ice-cold 5% perchloric acid. The supernatant was then spun for 2 min in a microfuge at 0 °C to remove any cell debris and 350 μ l of this supernatant was mixed with 35 μ l of EDTA (100 mM) and adjusted to pH 6.0 with KOH. After 1 min on ice and a 1 min spin in the microfuge the supernatant was removed and used in the binding assay. Protein content of each well was determined using a Bradford assay (approx. 200 μ g of protein per well). The binding buffer was (mM): 100 Tris, 4 EGTA, 4 EDTA, 4 mg/ml BSA (fraction V), pH 9.0. The incubation mix contained: 25 μ l of sample (or unlabelled InsP₃ standard), 25 μ l of labelled InsP₃ (20000–30000 c.p.m.), 10 μ l of binding protein, 25 μ l of binding buffer and 15 μ l of water. This was then placed on ice for 20 min, followed by an 8 min spin in a microfuge. The pellet was then washed with water and the specific radioactivity was counted. Standard curves were produced for each assay and the samples calibrated against these. All data points were carried out in quadruplicate.

Single-cell [Ca²⁺]_i measurement

Cells were loaded for 20 min at 37 °C with 1 μ M Fura-2 AM in culture medium. The cells were then washed thoroughly in extracellular solution and placed on the microscope stage. All experiments were carried out at room temperature (20 °C). Details of Fura-2 imaging are described elsewhere [9]; in brief, we used an Applied Imaging Magical machine with an extended, ISIS Photonic Sciences camera. Off-line ratio images were used to calculate cytosolic Ca²⁺ employing a calibration table drawn up from data obtained with ionomycin-permeabilized cells bathed in 10 mM Ca²⁺ (*R*_{max}) and 10 mM EGTA (*R*_{min}). The bath solution was maintained at a constant volume of around 0.3 ml by a suction pipette. Agonists were applied to the cells by a bolus application of 3 ml of solution into the bath. This allowed a rapid and complete bath solution exchange within a second.

RESULTS

Degenerate PCR primers, designed to amplify a 530 bp product from the 3' region of all three known RyRs, were used to screen cDNA prepared from total RNA isolated from gut epithelial

Figure 1 RT-PCR detection of RyR

RT-PCR was carried out as described and the resulting PCR products analysed by agarose gel electrophoresis on 1.5% gels. A 530 bp product was amplified from cDNA from T84 cells (lane 1) and mouse brain (lane 2). No product was detected in PCR reactions containing RNA control (lane 3) or the cDNA control (lane 4). Similar results were obtained on three separate occasions.

Figure 2 Restriction enzyme analysis of the RyR PCR product

The RyR PCR product from T84 cells was subjected to restriction enzyme analysis as described and the resulting fragments analysed by agarose gel electrophoresis on 1.5% gels. Lane 1 shows the undigested 480 bp product, lane 2 shows that *SacI* (RyR 1-specific) had no effect, lane 3 shows that *BglII* (RyR 2-specific) cut the PCR product, indicating it was predominantly a type-2 RyR. Lane 4 shows that *BstEII* (RyR 3-specific) had little effect on digesting the product.

cells [6]. As a positive control, the primers were used to amplify cDNA prepared from mouse brain total RNA since all three RyRs have been found in various regions of the brain [4]. A 530 bp product was amplified from both brain and T84 cDNA, whereas PCR reactions containing RNA or the cDNA reaction mix minus RNA were negative (Figure 1). The results indicate the presence of mRNA coding for RyRs in gut epithelial cells. To increase both product yield and specificity a second amplification step was carried out using a nested downstream primer, resulting in a product of 480 bp. To determine which RyR isoforms were present in this second product, we used restriction digest analysis with enzymes directed specifically against each receptor isoform (three separate PCR reactions and digests). *SacI* (RyR 1-specific) did not cut the PCR product, *BglII* (RyR 2-specific) cut the majority of the PCR product into two fragments of the expected sizes 280 and 200 bp, and *BstEII* (RyR 3-specific) appeared to have little or no effect (Figure 2). These results indicate that RyR 2 is the major RyR isoform expressed in T84 cells. The presence

Figure 3 $[^3\text{H}]\text{Ry}$ binding to T84 microsomes

The data, at each Ry concentration, are expressed as a percentage of the binding in the absence of added Ry. A total of three separate experiments were carried out. Points on the graph were derived from one or two experiments. Where appropriate the data from more than one experiment are shown as the mean $\pm$ S.E.M. The plot was approximated to a sigmoidal relationship with an EC_{50} of $15.25 \mu\text{M}$ (see the Experimental section).

of some undigested PCR product in the *BglII* digest suggested that other RyR isoform(s) may be present, although at a much lower level. In two of three experiments very faint bands of the predicted size were visible after digestion with *BstEII*, suggesting that RyR 3 may also be expressed. To verify these results, PCR products from two separate experiments were cloned and analysed by restriction digest with *AvaII* or sequencing (results not shown). Restriction digest analysis of 13 clones identified 11 RyR 2 and two RyR 3. Of these, nine clones were successfully sequenced as RyRs, seven were found to be RyR 2, and two were found to be RyR 3 by comparison with known database sequences [V. Sorrentino, G. Giannini, P. Malzac and M. G. Mattei, unpublished work; *Homo Sapiens* RyR 3 gene (partial) submitted in 1993 to the EMBL/GenBankTM/DDBJ databases under accession number X74270].

To determine whether functional RyRs were expressed in T84 cells we carried out an $[^3\text{H}]\text{Ry}$ binding assay. We incubated microsomes prepared from T84 cells in a high-salt buffer containing $[^3\text{H}]\text{Ry}$ and a range of concentrations of Ry. The data were derived from three separate experiments on two different microsome preparations. Figure 3 shows a graph of specific $[^3\text{H}]\text{Ry}$ binding, at each Ry concentration, expressed as a percentage of the binding determined in the absence of Ry. The approximate EC_{50} was determined from the fitted line and had a value of $15.25 \mu\text{M}$.

In order to determine a potential role for an RyR in $[\text{Ca}^{2+}]_i$ signalling in gut epithelial cells it was necessary to describe the agonist-evoked $[\text{Ca}^{2+}]_i$ response. In our experiments, acetylcholine reversibly elicited sinusoidal $[\text{Ca}^{2+}]_i$ oscillations across the dose-response range in T84 cells ($n = 149$ cells, Figure 4). The threshold for this response was around $1 \mu\text{M}$ and both the amplitude of the $[\text{Ca}^{2+}]_i$ oscillations increased with increased agonist concentrations up to a maximum at $100 \mu\text{M}$ acetylcholine (Figure 4B). Additional experiments on the acetylcholine-induced $[\text{Ca}^{2+}]_i$ oscillations showed that, after removal of extracellular Ca^{2+} , one or two additional $[\text{Ca}^{2+}]_i$ spikes were observed before the response was abolished ($n = 8$, results not shown). Reintroduction of extracellular Ca^{2+} rapidly restored the $[\text{Ca}^{2+}]_i$ oscillations. These results indicate that the $[\text{Ca}^{2+}]_i$ oscillations are dependent on Ca^{2+} influx, but that acutely, cytosolic Ca^{2+} spikes occur in the absence of extracellular Ca^{2+} .

It has been shown pharmacologically that the acetylcholine response of T84 cells is dependent on muscarinic M3 receptors

Figure 4 Acetylcholine-induced sinusoidal oscillations in single cells

(A) A typical example of the response of a single T84 cell to the application of 10^{-4} M acetylcholine. The Ca^{2+} response was rapid in onset, rose to a peak and then decayed to a plateau level with superimposed oscillations. The response was completely reversible on removal of agonist. (B) A dose-response curve was drawn up from the measured peak Ca^{2+} rise at each concentration of acetylcholine. The Figure shows the mean and S.E.M. and each point is derived from 4–8 cells.

coupled to phospholipase C [14]. Atropine (1 mM) abolished the acetylcholine-evoked $[\text{Ca}^{2+}]_i$ signal ($n = 4$, results not shown) and more directly we were able to measure the production of InsP_3 by a mass assay. Figure 5 summarizes our data obtained from T84 cells stimulated with $100 \mu\text{M}$ acetylcholine (maximum effective dose). A basal level of InsP_3 of 6 nM/ng of protein was measurable in unstimulated cells. The peak InsP_3 level, of six times basal concentration, occurred 30 s after the application of agonist. This was then followed by a decay to a plateau level, above the initial baseline. Prior to the peak InsP_3 response, it was noticed that the levels of InsP_3 in the cell actually dropped. In summary, we have shown that acetylcholine stimulates InsP_3 production and sinusoidal $[\text{Ca}^{2+}]_i$ oscillations in T84 cells. In the next series of experiments we set out to determine a potential role for RyRs in the agonist-evoked $[\text{Ca}^{2+}]_i$ oscillations.

Initial experiments were carried out using caffeine which is known to promote Ca^{2+} release from RyR 1 and RyR 2 [15]. When applied alone, at a range of concentrations (5–50 mM), caffeine failed to elicit any increase in cytosolic Ca^{2+} ($n = 22$, Figure 6). In fact, in many cells it was apparent that caffeine concentrations above 25 mM actually decreased the fluorescence signal. Furthermore, it was shown that caffeine applied during a response to acetylcholine rapidly and reversibly abolished the

Figure 5 Time course of the stimulated production of InsP_3 by acetylcholine

The histogram shows InsP_3 measured relative to basal levels and expressed as nM/ng of protein in T84 cells. An initial drop in levels of InsP_3 was followed by a peak at 30 s and a plateau level. The line was drawn by eye.

Figure 6 Block of the acetylcholine-induced $[\text{Ca}^{2+}]_i$ signals by caffeine

High concentrations of caffeine had no effect alone but reversibly blocked the oscillatory $[\text{Ca}^{2+}]_i$ response to acetylcholine in T84 cells.

Figure 7 Slow block of the acetylcholine-induced $[\text{Ca}^{2+}]_i$ response by Ry

Application of 10 μM Ry slowly blocked the agonist-evoked $[\text{Ca}^{2+}]_i$ signal in T84 cells. In some cells this effect was partially reversible.

$[\text{Ca}^{2+}]_i$ oscillations (Figure 6). These rapid and reversible effects of caffeine suggest that its predominant action, in these gut epithelial cells, is either to directly block the InsP_3 receptor [16] or to block the production of InsP_3 [17,18].

Figure 8 Persistent $[\text{Ca}^{2+}]_i$ oscillations induced by a short agonist application, in the presence of Ry, are dependent on extracellular Ca^{2+}

Preincubation with 10 μM Ry followed by a short application of acetylcholine in T84 cells led to persistent $[\text{Ca}^{2+}]_i$ oscillations that were abolished when extracellular Ca^{2+} was removed.

Figure 9 Persistent $[\text{Ca}^{2+}]_i$ oscillations induced by a short agonist application, in the presence of Ry, are blocked by high Ry concentrations

Persistent $[\text{Ca}^{2+}]_i$ oscillations evoked by a short application of agonist in T84 cells were blocked when the Ry concentration was increased from 10 to 100 μM .

The effect of Ry, applied alone, was also tested at concentrations from 0.1 to 10 μM . We failed to observe any change in the $[\text{Ca}^{2+}]_i$ ($n = 27$, results not shown). However, when Ry (10 μM) was applied during an agonist-evoked $[\text{Ca}^{2+}]_i$ response a slow block of the $[\text{Ca}^{2+}]_i$ oscillations was observed. Figure 7 shows two typical examples: in one cell Ry blocked the $[\text{Ca}^{2+}]_i$ oscillations completely (observed in 17/24 cells) while in the other cell a $[\text{Ca}^{2+}]_i$ spike was observed when Ry was washed off. In another experimental protocol, we applied Ry (10 μM) and then briefly applied a pulse of acetylcholine. In 16/24 cells, this evoked a $[\text{Ca}^{2+}]_i$ response in the presence of agonist that persisted as $[\text{Ca}^{2+}]_i$ oscillations, even after the agonist had been removed (Figure 8). The persistent oscillations could be blocked either by removal of extracellular Ca^{2+} (Figure 8) or by raising the Ry concentration to 100 μM (Figure 9). Taken together, these data indicate that alone Ry has very little effect, but that when Ry is combined with an agonist it leads a prolonged activation of the Ca^{2+} signal.

DISCUSSION

The results show the presence of mRNA coding for RyR 2 and RyR 3 in gut epithelial cells. We also provide evidence that these

receptors are expressed and are functionally important in the agonist-evoked $[\text{Ca}^{2+}]_i$ oscillations. The involvement of both InsP_3 and RyRs in the Ca^{2+} signalling pathway could be used to provide the cell with a mechanism to initiate a wider range of responses than would be possible with a single receptor.

Previous work using patch-clamp techniques have shown Ca^{2+} -dependent channels in the plasma membrane of many intestinal cells, the opening of which, in response to agonist, implies a cytosolic Ca^{2+} rise. Ca^{2+} -dependent Cl^- channels have been shown in HT29 cells [19], Ca^{2+} -dependent K^+ channels in HT29 cells [20], T84 cells [21], rabbit colonic epithelium [22,23], human colonic crypts [24] and Ca^{2+} -dependent cation channels in rat crypts [25]. The underlying $[\text{Ca}^{2+}]_i$ rise has been recorded directly in T84 cells in response to carbachol [26], in Intestine 407 cells in response to acetylcholine and histamine [27] and in CaCo-2 cells in response to vitamin D_3 [28]. An important observation from the work on T84 and Intestine 407 cells was that the agonists induced oscillating cytosolic Ca^{2+} signals [21,29]. The above studies, however, shed no light on the second messenger signalling pathways in the $[\text{Ca}^{2+}]_i$ response of enterocytes or on the nature of the intracellular Ca^{2+} stores. Only two studies have addressed these issues. In CaCo-2 cells, InsP_3 levels rose measurably in response to vitamin D_3 [30] and, using subtype-specific antibodies, a type-3 InsP_3 receptor has been identified in gut epithelial cells [31]. This receptor type has the lowest affinity for InsP_3 of all the known family of InsP_3 receptors [32] but we know little of its functional activation.

Our RT-PCR experiments indicate the presence of mRNA coding for the type-2 and type-3 RyRs. Although not quantitative, it appears as if the type-2 receptor mRNA is relatively more abundant than that of the type 3. Experiments with $[\text{H}^3]\text{Ry}$ binding demonstrate that the RyR is expressed in T84 cells. Under high-salt conditions (1.5 M KCl) the low-affinity binding we observed (EC_{50} 15.25 μM) is consistent with previous reports for skeletal muscle [33] and cardiac [34] RyRs. Further detailed work will be required to determine higher-affinity binding sites, perhaps under different conditions.

In our experiments, sinusoidal $[\text{Ca}^{2+}]_i$ oscillations, that is, oscillations on an elevated $[\text{Ca}^{2+}]_i$ baseline, were induced by suprathreshold concentrations of acetylcholine in T84 cells. At an acetylcholine concentration of 100 μM we detected a measurable production of InsP_3 which peaked at 30 s and fell to a steady plateau. Interestingly at 2 and 5 s intervals after agonist application we observed a decrease in the InsP_3 concentration. This decrease has not been shown before and would be difficult to detect without using a mass assay system similar to that used in our study. It could be that the 5-phosphatase that breaks down InsP_3 may be under receptor regulation, and this might provide an explanation of our results if this was activated prior to activation of the production of InsP_3 . Further work will be required to investigate this phenomenon. Our results are consistent with acetylcholine acting through an M3 receptor, promoting a production of InsP_3 and release of Ca^{2+} from intracellular Ca^{2+} stores.

We demonstrated that caffeine, applied alone, failed to elicit a $[\text{Ca}^{2+}]_i$ rise in these gut epithelial cell lines. This contrasts to the Ca^{2+} -releasing action of caffeine on muscle [35] and neurons [36], but is consistent with the lack of effect of caffeine in HeLa cells [6] and mink lung epithelial cells [11]. The RyR 3 isoform found in the latter cell type was thought to be insensitive to caffeine, but it has now been shown that in dyspedic mice (that lack RyR 1) the remaining RyR 3 in skeletal muscle is responsive to caffeine [37]. In pancreatic acinar cells, under whole-cell patch-clamp conditions, caffeine at low concentrations (less than 1 mM) can elicit $[\text{Ca}^{2+}]_i$ spikes [9,38]. However, these effects are only revealed

when the Ca^{2+} buffering of the intracellular solution is very low. Under more normal Ca^{2+} buffering conditions caffeine does not produce a rise in cytosolic Ca^{2+} . When applied against an agonist-induced $[\text{Ca}^{2+}]_i$ response caffeine is inhibitory in these gut epithelial cells. This is consistent with findings in many cell types where caffeine is thought to both block the InsP_3 receptor directly [16] and inhibit the production of InsP_3 . We conclude that caffeine is most probably acting to inhibit the acetylcholine response via an action on the InsP_3 pathway. Further experiments, using the patch-clamp technique, will be required to determine if conditions of low cytosolic Ca^{2+} buffering can reveal a direct effect of caffeine on the RyR.

The interpretation of the Ry block of the agonist-evoked $[\text{Ca}^{2+}]_i$ signal in T84 cells depends on the position of RyRs in the signal cascade that leads to oscillations. RyRs could be an essential part of the oscillator mechanism and open with each oscillatory cycle. Alternatively, RyRs could have a regulatory role and provide a leak of Ca^{2+} to feed forward on to an InsP_3 -dependent oscillator [9]. The slow block of the agonist-evoked oscillations by $10\text{ }\mu\text{M}$ Ry does not allow us to discriminate between the two hypotheses. However, the persistent $[\text{Ca}^{2+}]_i$ oscillations observed in the continued presence of Ry ($10\text{ }\mu\text{M}$), after a brief application of agonist, suggest one possible model. At this concentration of Ry we would expect binding to an open channel and promotion of a 'locked', low-conductance state [39]. Under these conditions the RyRs could not play a role as oscillators and the $[\text{Ca}^{2+}]_i$ spikes observed must be due to an oscillatory mechanism dependent on InsP_3 receptors. The data from the persistent oscillations therefore place the RyR in a regulatory role, feeding Ca^{2+} forward and sensitizing InsP_3 receptors. Despite the evidence for a regulatory role of RyRs in the persistent oscillations, it is important to understand that agonist-evoked oscillations may utilize the RyR in a different role, possibly via modulation with factors such as cADP-ribose [9].

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